Modifications of μ-opioid receptor agonist-induced several pharmacological actions by diabetes in mice: possible involvement of protein kinase C and intracellular calcium

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Major drugs used in this study

Morphine	prototype μ agonist
DAMGO	μ agonist
Naloxone	preferring μ antagonist
β-FNA	μ (μ_1, μ_2) antagonist
Naloxonazine	μ (μ_1) antagonist
TAN-67	non-peptidic δ (δ_1) agonist
BNTX	δ (δ_1) antagonist
NTB	δ (δ_2) antagonist
Calphostin C	protein kinase C inhibitor
KT5720	protein kinase A inhibitor
PDBu	protein kinase C activator
Thapsigargin	Ca^{2+}-ATPase inhibitor
Ryanodine	ryanodine receptor antagonist
Methamphetamine	dopamine releaser
Cocaine	dopamine uptake inhibitor
Quinpirole	dopamine (D_2/D_3) agonist
7-OH-DPAT	dopamine D_3 agonist

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General Introduction

Diabetes mellitus is currently a complicated disease. There are 132 million patients with diabetes mellitus in the world: especially in Japan, there are more than seven million diabetic patients. This disease exhibits persistent elevations of glucose levels in serum or blood. The elevation of glucose levels induces a bewildering list of changes in vascular or neuronal cells. The major diabetic complications make diabetes more convoluted. There are three major diabetic complications, i.e., diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy. Although many studies have been carried out on diabetic complications, the detailed mechanisms and etiology underlying diabetic complications, especially diabetic neuropathies, are unclear at this time.

Diabetic neuropathy is the most convoluted complication. Diabetic gastropathy, ulcers, diarrhea, and bladder dysfunction are the major peripheral neuropathies. Peripheral neuropathies have been the primary neuroscience focus of diabetes research. In contrast to the periphery, the brain is not usually thought to be a target of chronic diabetic complications. However, the impact of diabetes mellitus on the central nervous system has gained attention recently (Mooradian, 1988; Ryan, 1988). Chronic diabetes mellitus afflicts the central nervous system in several ways. Diabetes increases the risk of stroke and the extent of stroke-related damage, over-medication with insulin or oral agents can permanently damage the brain, and diabetes may increase the prevalence of seizure disorders. Diabetes changes brain transport, blood flow and metabolism, and these changes may produce a chronic encephalopathy (see McCall, 1992 for a review). Furthermore, it is well known that diabetes or hyperglycemia influences the sensitivity of laboratory animals to various pharmacological agents. An increased sensitivity of hyperglycemic or diabetic animals to barbiturates (Lamson et al., 1951; Strother et al., 1971; Ackerman and Leibman, 1975; Ackerman 1976; Strother, 1979) and a decreased sensitivity to D-amphetamine (Fernando and Cuzon 1974; Marshall et al., 1976, 1978; Marshall, 1978a,b), *p*-chloroamphetamine (MacKenzie and Trulson, 1978), and carbon tetrachloride (Hanazono et al., 1975a, 1975b) have been demonstrated. Recently, we have reported that the pharmacological sensitivity to ethanol in diabetic mice is less than that in non-diabetic mice (Ohsawa and Kamei, 1997). Although many investigators have shown the differential sensitivity to various centrally acting drugs in diabetic mice, the detailed mechanisms underling this differential sensitivity to these drugs have not been clearly defined.

Simon and Dewey (1981) reported that mice and rats with streptozotocin-induced

diabetes and spontaneously diabetic mice are significantly less sensitive than non-diabetic mice to the antinociceptive effect of morphine. Furthermore, Kamei et al. (1992a) also demonstrated that the antinociceptive effect of intracerebroventricularly administered morphine and [D-Ala^2, N-MePhe^4, Gly-ol^5]enkephalin (DAMGO) is smaller in diabetic mice than in non-diabetic mice. On the other hand, there were no significant differences between the antinociceptive potencies of these μ-opioid receptor agonists between diabetic and non-diabetic mice when they were administered intrathecally (Kamei et al., 1994a). These results indicated that the reduction in the antinociceptive potency of μ-opioid receptor agonists in diabetic mice is due to the dysfunction of supraspinal μ-opioid receptors. However, there is little information available regarding the mechanism responsible for these changes.

Several studies have indicated that the binding and metabolism of neurotransmitters within the brain, especially monoamines, are abnormal in diabetes mellitus. Lozovsky et al. (1981) reported that the number of dopamine receptors, measured as 3H-spiperone binding, is increased by 30-50% in alloxan- or streptozotocin-induced diabetic rats. Kamei et al. (1994d) indicated that the dopamine turnover ratio in the limbic forebrain is increased in diabetic mice compared to non-diabetic mice. Thus, it has been suggested that diabetic mice have increased limbic forebrain dopaminergic neurotransmission (Kamei et al., 1994d). Regional noradrenergic neurotransmission is also altered in diabetes. Trulson and Himmel (1983) showed that the forebrain noradrenaline (NA) content increases in diabetes. 3-Methoxy-4-hydroxyphenylglycol (MHPG), a major metabolite of NA, decreases in diabetes suggesting that diabetes decreases forebrain noradrenergic neurotransmission.

Opioids, the prototype of which is morphine, are the most potent available analgesic compounds. Opiates act by mimicking endogenous opioid peptides and specifically activate membrane receptors in the nervous system. The receptors, named opioid receptors, were first discovered in 1973 with the demonstration of stereospecific and saturable binding of radiolabeled opiates to brain membrane preparations (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). Martin and his colleagues (Gilbert and Martin, 1976; Martin et al., 1976) performed pharmacological studies in chronic spinal dogs and found that morphine and several of its analogs differed in their pharmacological profiles. The study of the pharmacological activity of a wide variety of alkaloid compounds and the discovery of enkephalins (Hughes et al., 1975) led to the classification of opioid binding sites into three classes referred to as μ, δ and κ opioid receptors (Goldstein and Naidu, 1989). Autoradiography showed a distinct distribution for each

receptor class in the brain (Mansour et al., 1988) and evidence has accumulated for a differential role of the three classes of receptors in pain modulation (Dickenson, 1991). Specific μ-, δ- and κ-opioid receptor agonists have also been associated with variable acute liability and distinct mood-altering, autonomic and neuroendocrine effects which have been shown to be mediated by each receptor type (Millan, 1990). Recently, Evans et al. (1992) and Kieffer et al. (1992) independently reported cloning of δ-opioid receptor from neuroblastoma x glioma NG-108-15 hybrid cells using an heterologous expression cloning strategy and ligand-binding activity to monitor the expression of receptors. Following the identification of the δ-opioid receptor, μ-(Chen et al., 1993) and κ-opioid receptor (Yasuda et al., 1993) cDNA were also cloned. Molecular analysis of these three opioid receptors indicated that they conform to the structural motif of the guanine-nucleotide-binding regulatory protein (G-protein) coupled receptor superfamily, including the conserved seven hydrophobic domains.

Morphine, the prototypic μ-opioid receptor agonist, produces analgesia, drowsiness, changes in mood, respiratory depression, decreased gastrointestinal motility, nausea, vomiting, and alternations of the endocrine and autonomic nervous systems. Morphine is used primarily for the treatment of pain. Some of the central nervous system mechanisms that reduce the perception of pain also produce a state of well being or euphoria. Morphine also is taken outside of medical channels for the purpose of obtaining these effects on mood. This potential for abuse has generated much research on separating the mechanism of analgesia from that of euphoria in the hope of eventually developing a potent analgesic that dose not produce euphoria. Although this research has led to advances in understanding the physiology of pain, the standard medications for severe pain remain the derivatives of the opium poppy and synthetic drugs that activate the same receptors. Furthermore, patients who have received opioids develop tolerance routinely, and if the medication is stopped abruptly, they will show the signs of an opioid withdrawal syndrome, the evidence for physical dependence. Although morphine is one of the most potent analgesics, the use of morphine and related drugs is limited clinically by these problems.

The physiological mechanisms implicated in the development of opioid dependence and the expression of withdrawal symptoms consist of adaptive changes that include the processes of homologous regulation, affecting the endogenous opioid system, and heterologous regulation that affects other neurotransmitter systems. Numerous non-opioid neurotransmitters have been proposed to participate in this heterologous regulation. Many of the pharmacological and biochemical studies have been focused on the central noradrenergic system, which seems to have an important role in the expression of the somatic signs of opioid withdrawal. This involvement

is supported by several lines of evidence based on the biochemical changes reported in noradrenergic transmission during opiate dependence and withdrawal, and on the pharmacological responses induced after opiate withdrawal by the administration of adrenergic compounds. Much of the evidence for the noradrenergic involvement in opioid dependence has been derived from studies of the locus coeruleus (LC). The LC is the largest cluster of noradrenergic neurons in the brain (Dahlstrom and Fuxe, 1964; Foote et al., 1983). The firing rate of noradrenergic neurons in the LC increases during naloxone-precipitated withdrawal from morphine dependence (Aghajanian, 1978). Treatment with DSP-4, which destroys the NA-containing neurons, prior to induction of dependence attenuates any withdrawal signs precipitated by systemic administration of naloxone in morphine-dependent mice (Funada et al., 1993). Furthermore, an enhancement in the brain content of MHPG, a principal metabolite of NA, was found in several brain regions innervated by the LC during morphine abstinence withdrawal behavior (Sesak et al., 1983). This increase in the levels of the NA metabolite was also confirmed in the frontal cortex of mice (Funada et al., 1993). The physiological activation of LC neurons follows a time course that closely parallels the expression of morphine abstinence withdrawal behaviors (Rasmussen and Aghajanian, 1989). The recent development of in vivo microdialysis techniques in freely moving rats allows the measurement of the extracellular concentrations of neurotransmitters in discrete areas of the central nervous system, which is a direct reflection of the balance between synaptic release and uptake/clearance of these transmitters. Using this technique, it has been reported that the extraneuronal NA concentration was enhanced immediately after the expression of naloxone-precipitated morphine withdrawal symptoms, reaching the maximal levels within 30 min after naloxone and remaining elevated for over 90 min. The symptomatology of withdrawal paralleled these changes in the cortical output of NA (Rossetti et al., 1993), which is compatible with the previous idea that some of the behavioral signs of abstinence may be mediated by an increased activity of the noradrenergic system (Redmond, 1987). Thus, many investigators have suggested that the central noradrenergic system plays a significant role in morphine withdrawal.

Another problem with the use of morphine and other opioid drugs is their potent reinforcing properties. Opioids and other drugs of abuse exert marked effects on mood and motivation (Belluzzi and Stein, 1977; Stein and Belluzzi, 1978; Dum and Herz, 1987). Animals will readily self-administer drugs either intravenously or orally, and drugs that are self-administered by animals correspond well with those of high abuse potential in humans. Self-

administration and intracranial self-stimulation -- obviously associated with feelings of pleasure -- have been widely used to determine the motivational effects of drugs of abuse (Wise and Bozarth, 1982; Bozarth, 1987), These operant techniques measure the primary reinforcing processes using specific behavioral tasks such as lever-pressing. In the behavioral experiments, place conditioning, a technique that measures the secondary reinforcing effects of drugs, assessed the drug-induced motivational effects. In this procedure, the association that develops between the presentation of a drug and a previously neutral stimulus (e.g., differently colored compartments of a shuttle box) is evaluated. The results obtained with this paradigm for rewarding drugs are largely identical to those obtained with the self-administration technique (Spyraki, 1988; Carr et al., 1989). Besides the evaluation of rewarding properties, the place conditioning procedure also allows the detection of aversive (negatively reinforcing) properties of drugs, which is of particular importance when investigating the effects of opioids.

Two major dopamine systems originate in the ventral midbrain: the nigrostriatal dopamine system and the mesolimbic dopamine system. The mesolimbic dopamine system has been implicated in drug reinforcement. The cell bodies of this system originate in the ventral tegmental area (VTA), originally described as the A10 group of catecholamine-containing neurons (Dahlstrom and Fuxe, 1964), and project to the forebrain, largely the nucleus accumbens, olfactory tubercle, frontal cortex, amygdala, and septal area (Koob, 1992). Opioids can increase dopamine release in the nucleus accumbens as measured by in vivo microdialysis in awake, freely moving animals (Di Chiara and Imperato, 1988). Microinjection of opioids into the VTA, a region containing the cell bodies of origin of the mesolimbic dopamine system, lowers the reward thresholds for brain stimulation and produces robust place preferences (Di Chiara and North, 1992). Furthermore, it has been shown that the place preferences produced by opioids appear to have a major dopaminergic component (Spyraki et al., 1983; Shippenberg et al., 1993). Other evidence supporting the role of dopamine in opioid reinforcement comes from lesion studies. Dopaminergic lesions of the nucleus accumbens have been shown to antagonize morphine (Kelsey et al., 1989) and heroin (Spyraki et al., 1983) induced place preference. Similarly, destruction of the ascending dopaminergic pathways ipsilateral to the injection site of DAMGO blocked DAMGO-induced place conditioning when dopamine levels were reduced by more than 90% (Phillips et al., 1983). Thus, mesolimbic dopaminergic neurons may be involved in the reinforcing effect of opioids.

μ-, δ- And κ-opioid receptors inhibit neurotransmission, and this is generally accepted as

the mechanism underlying analgesia. The inhibition of neurotransmission is caused by coordinated changes at the cellular level (Smart et al., 1994). The opioid receptors, as well as many other hormone, lymphokine, neurotransmitter, and neuromodulator receptors, signal to specific intracellular effectors through G-proteins (Gillman, 1987; Birnbaumer, 1990). The family of G-protein coupled receptors share a number of structural features, including seven conserved hydrophobic domains that form the membrane spanning regions. Through G-protein coupling to specific second messenger systems, all three classes of opioid receptors elicit cellular responses such as inhibition of adenylyl cyclase, increased potassium conductance, and inactivation of calcium channels (Simonds et al., 1985; Childers, 1991). These intracellular changes may underlie the antinociceptive action of opioids.

Repeated or sustained exposure to opioids leads to a reduction in their potencies for evoking pharmacological effects, especially those causing analgesia. This phenomenon is termed tolerance. Chronic exposure to morphine stimulates cytosolic protein kinase C (PKC) activity in rat brain (Narita et al., 1994a), as well as increasing membrane-bound PKC activity in rat brain homogenates (Narita et al., 1994b) and dorsal root ganglion (DRG) neurons from laminae I and II of the spinal cord (Mao et al., 1995; Mayer et al., 1995). This increase in PKC activity parallels the development of tolerance to opioid-induced antinociception in vivo (Narita et al., 1994a, 1994b; Mao et al., 1995; Mayer et al., 1995). Furthermore, inhibition of PKC with H-7, calphostin C or GM1 ganglioside prevents the development of tolerance, while inhibition of protein kinase A (PKA) with KT5720 dose not (Narita et al., 1994c; Narita et al., 1995; Mao et al., 1994). Thus, chronic opioid-induced activation of PKC may play a role in the development of tolerance by causing coordinated changes in the cellular mechanisms that mediate antinociception.

Chronic opioid exposure induced several intracellular changes. These intracellular changes may be involved in the development and expression of opioid physical dependence. Upon chronic opioid treatment, LC neurons developed tolerance to the acute inhibitory actions of opioids, as neuronal firing rates recovered toward pretreatment levels (Aghajanian, 1978; Andrade et al., 1983; Christie et al., 1987). The neurons also become dependent on opioids after chronic exposure, as indicated by the fact that abrupt cessation of opiate treatment, for example, by administration of an opioid receptor antagonist, leads to an elevation in the firing rates in the LC several-fold above the pretreatment level (Aghajanian, 1978; Rasmussen et al., 1990). The rebound of adenylyl cyclase activity during withdrawal was first demonstrated by Sharma et al. (1975), and has long been considered as a candidate for a role in generating opioid withdrawal.

Type VIII adenylyl cyclase mRNA in the LC was selectively increased after chronic morphine treatment in the LC, and the time course of these changes in adenylyl cyclase mRNA was related to the incidence of jumping behavior during the withdrawal syndrome (Matsuoka et al., 1994). Another argument in favor of the participation of this intracellular signal transduction pathway is the strong attenuation in the expression of the somatic symptoms of morphine withdrawal that results from the inhibition of protein kinase activities in the LC (Maldonado et al., 1995). Protein kinases belonging to the family of the serine/threonine kinases seem to be selectively implicated since this alleviator response was induced by the local administration of the serine/threonine kinase inhibitor H-7, but not after the microinjection of the tyrosine kinase inhibitor KB23 (Maldonado et al., 1995). The results obtained in the LC were correlated with the behavioral manifestations of the somatic symptoms of abstinence, and suggest that many intracellular changes throughout the brain produced by opioid withdrawal may be involved in the activation of noradrenergic neuron in the LC.

A role for Ca^{2+} in the action of opioids has long been advocated based initially on pharmacological evidence. Previous findings may provide an explanation for some of these effects and implicate Ca^{2+} more definitively in the action of opioids (Hano et al., 1967). Two acute effects of opioids commonly observed are a reduction in neurotransmitter release and inhibition of neuronal electrical activity. The electrophysiological evidence indicates that opioids decrease Ca^{2+} influx by depression of voltage-sensitive Ca^{2+} channels (Mudge et al., 1979) and membrane hyperpolarization of cell bodies by increased K^{+} conductance (North and Williams, 1983). An another electrical finding that may be very important is that opioids also prolong Ca^{2+}-dependent after hyperpolarization. These electrical findings may be related to Ca^{2+} disposition and may be explained by an opioid-induced decrease in intracellular Ca^{2+} binding. Early pharmacological evidence pointed the way and provided strong circumstantial evidence to implicate Ca^{2+} in the action of opioids. For instance, manipulating cellular calcium can alter many of the observed effects of opioids. Invariably, maneuvers that tend to elevate neuronal Ca^{2+}, either with Ca^{2+} itself or with Ca^{2+} ionophores, reduce opioid actions. On the other hand, procedures that lower cytosolic Ca^{2+}, such as reductions in the extracellular Ca^{2+} concentration, removal of extracellular Ca^{2+} with chelating agents, or blocking of Ca^{2+} entry with La^{3+}, all enhance the action of opioids (Chapman and Way, 1980; Schmidt and Way, 1980).

The increase in Ca^{2+} accumulation requires the concomitant presence of the opioids and this may explain the development of physical dependence. When opioids are discontinued or

treatment with an antagonist removes the agonist, the high synaptosomal Ca^{2+} content in the absence of the agonist produces greatly increased neurotransmitter release. This neuronal hyperexcitability then gives rise to withdrawal signs and symptoms. The abstinence syndrome can be attenuated by reducing intracellular Ca^{2+}. Furthermore, La^{3+} administration reduces abrupt or naloxone-induced withdrawal jumping in mice (Chapman and Way, 1980). The mechanisms underlying the enhancement of intracellular calcium by chronic opioid exposure are physiological counter-adaptation. Opioid could cause a transient displacement of Ca^{2+} from its intracellular binding sites to effect increased K^+ conductance, membrane hyperpolarization, decreased neuronal firing, and neurotransmitter release. The Ca^{2+} released by opioids might act to reduce further Ca^{2+} entry, or the Ca^{2+} channel itself may be blocked by opioids, resulting in a fall in neuronal Ca^{2+}. However, this lowering sets in to motion other homeostatic processes within the cell, which become increasingly manifest as opioid administration continues. In particular, the counter-adaptive effect occurs at the intracellular binding sites. The displacement of Ca^{2+} from its binding sites in the presence of the opioids becomes more difficult so that a higher dose of the opioid is required before an acute response can be elicited (tolerance). However, the counter-adaptive effect of retained Ca^{2+} within the cell also increases, but requires the presence of the opioid (dependence). Removal of the opioid by discontinuing its administration or by administering an antagonist results in a rise in intracellular Ca^{2+}, higher excitability, and increased neurotransmitter release (abstinence syndrome; Chapman and Way, 1980).

Excessive glucose can also be transported intracellularly, mainly by the glucose transporter GLUT-1, and metabolized to change the redox potential, increase sorbitol production via aldose reductase, or alter signal transduction pathways, such as the activation of diacylglycerol (DAG) and PKC levels (Kaiser et al., 1993; Greene et al., 1987; Williamson et al., 1993; King et al., 1997; De Rubertis and Craben, 1994; Sharma and Ziyadeh, 1995; Baynes, 1991). It is possible and, in fact, likely that the common pathway by which all of the intra- and extracellular changes induced by hyperglycemia are mediating their adverse effects is the alteration of various signal transduction pathways. Increases in total DAG content have been demonstrated in a variety of tissues associated with diabetic vascular complications, including the retina (Shiba et al., 1993), aorta, heart (Inoguchi et al., 1992), and renal glomeruli (Craven et al., 1990; Ishii et al., 1996) from animal models of diabetes and patients. Furthermore, in the sciatic nerve of diabetic animals, the activity of PKC is increased. This activation of PKC causes higher phosphorylation of Na^+-K^+-ATPase, which results in a decrease in its activity (Hermenegildo et al., 1993). In an electrophysiological study, it has been shown that the injection of PKC inhibitors,

such as staurosporine and PKC (19-31), reduced the hyperexcitability of C-fibers in streptozotocin-induced diabetic rats (Ahlgren and Levine, 1994). Thus, it seems likely that several diabetic neuropathies may be caused by the activation of intracellular messengers, especially PKC.

Numerous investigators have indicated that chronic diabetes mellitus is associated with pronounced changes in cellular calcium homeostasis, which in turn lead to substantial complications in most system functions. In vascular smooth muscle cells, a definite increase of Ca^{2+} influx through voltage-operated sarcolemma Ca^{2+} channels and decrease in the productivity of the Ca-ATPase pump in diabetic animal lead to enhanced vascular smooth muscle contractions (Sowers, 1990). In peripheral nerves of diabetic rats, mitochondrial and axoplasmic calcium levels were indeed found to be increased with electron-probe X-ray microanalysis (Lowery et al., 1990). Moreover, voltage-dependent calcium currents through L-, and N-type channels are enhanced in DRG neurons in BB/Wor rats in vivo (Hall et al., 1995). The impairment of the activity of the Na^{+}/Ca^{2+} exchanger (Greene et al., 1984) and Ca^{2+}-ATPase (Janicki et al., 1994) causes a net calcium overload. Furthermore, in sensory neurons, depolarization-induced Ca^{2+} transients in small DRG neurons became substantially prolonged during streptozotocin-induced and spontaneously occurring diabetes (Kostyuk et al., 1995). The increased residual Ca^{2+} in synaptic terminals is very substantial for the potentiation of synaptic transmission (Zucker, 1989). As altered calcium homeostasis that results in elevation of $[Ca^{2+}]_i$ may contribute to neuronal cell injury and death (Nichotera et al., 1992), enhanced calcium levels may contribute to the impairment of neuronal function in diabetes mellitus.

Aims and Scope

The purpose of this research is to investigate the mechanisms underlying dysfunction of the central nervous system in diabetic mice and painful diabetic neuropathies. In my experimental approach, behavioral and neurochemical analyses are employed.

The specific aims of the proposed research

In Chapter 1

First, I shall attempt to determine the dysfunction of μ–opioid receptors in diabetic mice. The μ–opioid receptor agonist inductions of several pharmacological actions, such as the Straub tail reaction and antinociception, in diabetic mice were examined. In addition, to define the role of the protein kinase (PK) C in the dysfunction of μ–opioid receptors in diabetic mice, I have examined the influence of central administration of PKC and PKA inhibitors on the μ–opioid receptor agonist-induced pharmacological actions in diabetic mice. Furthermore, to distinguish between the differential modulation of μ– and δ–opioid receptor agonist-induced antinociception in diabetic mice and the changes of intracellular calcium levels, the influence of central administration of intracellular calcium modulators on the μ– and δ–opioid receptor agonist-induced antinociception in diabetic mice were examined.

In Chapter 2

In order to determine the effect of the dysfunction of μ–opioid receptors in diabetic mice on the expression of morphine withdrawal, I have investigated the naloxone-induced withdrawal signs in morphine-dependent diabetic mice. Furthermore, to evaluate the role of the protein kinase (PK) C in the changes of morphine withdrawal in diabetic mice, I have examined the influence of central administration of PKC and PKA inhibitors on the expression of naloxone-induced withdrawal jumping and naloxone-induced enhancement of noradrenaline turnover in morphine-dependent diabetic mice. Moreover, to demonstrate the role of the intracellular calcium in the central dysfunction in diabetic mice, the influence of supraspinal and spinal injections of intracellular calcium modulators on the expression of morphine withdrawal and naloxone-induced enhancement of noradrenaline turnover in morphine-dependent diabetic mice were also investigated.

In Chapter 3

In order to evaluate the rewarding effect of several addictive drugs in diabetic mice, I have determined the morphine-, methamphetamine- and cocaine-induced place preferences in diabetic mice.

Chapter 1

Modification of μ-opioid receptor-mediated pharmacological action by diabetes in mice: possible involvement of protein kinase C and intracellular calcium

Introduction

It has been reported that the antinociceptive potency of morphine is decreased in several rodent models of hyperglycemia, including a spontaneously diabetic strain mice and streptozotocin-induced diabetes, an animal model of type I diabetes (Simon and Dewey, 1981). Kamei et al. (1994a) previously reported that the antinociceptive effects of i.c.v., but not i.t., administration of μ-opioid receptor agonists, such as morphine and [D-Ala^2, NMePhe^4, Gly-ol^5]enkephalin (DAMGO), in non-diabetic mice were significantly less than those in diabetic mice. In contrast with these μ-opioid receptor agonists, Kamei et al. (1994b, 1995) recently reported that the antinociceptive effect of i.c.v. administration of δ-opioid receptor agonists, such as [D-Pen^2, D-Pen^5]enkephalin (DPDPE) and (±)-TAN67 (Suzuki et al, 1996), in diabetic mice were markedly greater than those in non-diabetic mice. Therefore, they suggested that diabetic mice are selectively hypo-responsive to supraspinal μ-opioid receptors agonists and hyper-responsive to supraspinal δ-opioid receptors agonists (Kamei et al., 1994a, 1994b). However, the detailed mechanisms that are responsible for this hypo-responsiveness to supraspinal μ-receptor-mediated antinociception and hyper-responsiveness to supraspinal δ-opioid-mediated antinociception in diabetic mice are unclear.

Various studies have demonstrated the existence of two μ-opioid receptor subtypes, which have been referred to as μ_1-opioid and μ_2-opioid receptors (Heyman et al., 1988; Kamei et al., 1993b, 1993c; Pasternak et al., 1980; Pasternak and Wood, 1986; Paul et al., 1989; Wolozin and Pasternak, 1981). In vitro studies have indicated that naloxonazine selectively blocks a high-affinity morphine binding, which has been ascribed to μ_1 receptors (Hahn et al., 1982). Other studies using naloxonazine have implicated μ_1-opioid receptors in several μ-opioid receptor-mediated actions, such as supraspinal antinociception, feeding and prolactin release, but not in others, such as spinal antinociception, antitussive action and physical dependence (Heyman et al., 1988; Kamei et al., 1993b, 1993c; Pasternak et al., 1980; Pasternak and Wood, 1986; Paul et al., 1989; Wolozin and Pasternak, 1981).

Morphine contracts the sacrococcygenus muscle in mice, which results in erection of the tail (Straub tail reaction) (Bilby et al., 1960). Previous studies have suggested that the morphine-induced Straub tail reaction is evoked through the activation of μ-opioid receptors (Kameyama and Ukai, 1979; Narita et al., 1993). However, there is little information available regarding the involvement of μ-opioid receptor subtypes in the morphine-induced Straub tail reaction. Recently, Murray and Cowan (1990) suggested that δ-opioid receptors are not

involved in the opioid agonist-induced Straub tail reaction. Thus, the morphine-induced Straub tail reaction is useful model which can serve to examine the effect of diabetes on μ-opioid receptor functions, without the influence of δ-opioid receptor functions. Thus, in Experiment 1-1, I investigated the influence of naloxonazine, a selective μ_1-opioid receptor antagonist, on morphine-induced Straub tail reaction to determine the involvement of the μ-opioid receptor subtypes in the morphine-induced Straub tail reaction. In addition, I investigated the influence of diabetes on the morphine-induced Straub tail reaction to clarify the hypothesis that diabetic mice are selectively hypo-responsive to μ_1-opioid receptor-mediated pharmacological action, but not to that which is mediated by μ_2-opioid receptors.

Three opioid receptors, μ-, δ-, and κ-opioid receptors, have recently been cloned (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Yasuda et al., 1993; Liang et al., 1995). These three opioid receptors contain several potential phosphorylation sites in the first and third loops and the C-terminus of intracellular domains (Miotto et al., 1995). It has been suggested that phosphorylation of these three opioid receptors is involved in desensitization. There is accumulating evidence that the activation of protein kinase C (PKC) regulates several cellular functions through the phosphorylation of proteins, including some receptors, whose function is then down-regulated or up-regulated (Moran and Dascal, 1989). Activation of PKC by treatment with phorbol ester potentiates the desensitization of the μ-opioid receptor-induced K^+ current (Chen and Yu, 1997). Furthermore, we recently reported that activation of PKC by phorbol 12,13-dibutyrate leads to the desensitization of μ-opioid receptor-mediated antinociception (Narita et al., 1997). These results suggest that PKC may be involved in the desensitization of μ-opioid receptor-mediated pharmacological action in mice.

Many investigators have reported that hyperglycemia or elevated glucose levels can increase diacylglycerol (DAG) levels and activate PKC in vascular tissues, cardiac tissues, or cultured cells (Craven and DeRubertis 1989; King et al. 1990; Tanaka et al. 1991; Inoguchi et al. 1992). Activation of the DAG-PKC cellular signal pathway is linked to vasculature dysfunction in diabetes (Craven and DeRubertis 1989; Wolf et al. 1990; Shiba et al. 1993). Furthermore, Ahlgen and Levine (1994) reported that both the mechanical behavioral hyperalgesia and C-fiber hyperexcitability in response to mechanical stimuli seen in streptozotocin-induced diabetic rats are reduced by agents that inhibit PKC. This result suggests that increased PKC activity might alter the excitability of primary afferent nociceptors. It is possible that PKC may be involved in the desensitization of μ-opioid

receptor-mediated pharmacological actions in the mice. Thus, in Experiment 1-2, I examined the effects of a PKC activator and inhibitor on the i.c.v. morphine-induced Straub tail reaction in diabetic and non-diabetic mice. Furthermore, in Experiment 1-3, the role of PKC in the attenuation of the antinociception induced by DAMGO in diabetic mice was examined.

There is considerable evidence of a close relationship between opioid antinociception and Ca^{2+} levels within the central nervous system. Agents that increase cytosolic Ca^{2+} in neurons and synaptosomes block opioid antinociception when injected i.c.v. Hano et al. (1964) reported that intracisternal administration of Ca^{2+} antagonizes the antinociceptive effect of morphine, a prototype μ-opioid receptor agonist. The ionophores X-537A and A23187, which facilitate Ca^{2+} uptake by cells, also block morphine-induced antinociception. (Harris et al., 1975; Vocci et al., 1980). Since ionophores act mainly by increasing intracellular Ca^{2+}, it has been postulated that Ca^{2+} alters intracellular events to antagonize the antinociceptive effects of morphine (Chapman and Way, 1980). Conversely, Ca^{2+} chelators (i.e., ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid (EGTA)) or Ca^{2+} channel antagonists of the verapamil, diltiazem and dihydropyridine types potentiate opioid antinociception (Ben-Sreti et al., 1983; Hoffmeister and Tettenborn, 1986).

Considerable evidences suggest that calcium signaling is abnormal in cardiac myocytes (Nobe et al., 1990), vascular smooth muscle (Kamata et al., 1988) and other tissues (Levy et al., 1994) from diabetic animals. A recent study has shown that verapamil has a beneficial effect on the cardiac function of diabetic rats without affecting glucose metabolism or insulin secretion (Afzal et al., 1988). It has been suggested that chronic excessive intracellular calcium overload might induce cardiac dysfunction in chronic diabetes (Heyliger et al., 1987; Nishio et al., 1990). Moreover, it has been suggested that the diabetic state may change $[Ca^{2+}]_i$ in neuron and various tissues (Lowery et al., 1990; Hall et al., 1995; Kostyuk et al., 1995). It is possible that increased cytosolic calcium may play an important role in the modification of μ- and δ-opioid receptor-mediated antinociception by diabetes. Thus, to test this hypothesis, in Experiment 1-4, I examined the effect of intracellular calcium modulators on the change in μ- and δ-opioid receptor agonist-induced antinociception in diabetic and non-diabetic mice.

Experiment 1-1: Effects of diabetes on the morphine-induced Straub tail reaction in mice.

Materials and Methods

Animals

Male ICR mice (Tokyo Animal Laboratories Inc., Tokyo, Japan), weighing about 20 g at the beginning of the experiments, were used. They had free access to food and water in an animal room which was maintained at 22 ± 2 °C with a 12-h light-dark cycle. Animals were rendered diabetic by an injection of streptozotocin (200 mg/kg, i.v.) prepared in 0.1 N citrate buffer at pH 4.5. Age-matched non-diabetic mice were injected with vehicle alone. The experiments were conducted two weeks after injection of streptozotocin or vehicle. Mice with serum glucose levels above 400 mg/dl were considered diabetic.

Procedure

The Straub tail reaction was graded using a minor modification of the numerical scores of Kameyama et al. (1978) as follows; 0 = 0°, 0.5 = 1-30°, 1 = 31-45°, 1.5 = 46-60°, 2.0 = 61-90°, 2.5 = more than 90°. The angle was measured above the horizontal plane of the table. Morphine was injected subcutaneously (s.c.). The Straub tail reaction was observed 20 min after s.c. administration of morphine. Motor coordination in mice was measured using rotarod performance apparatus (3 cm in diameter, 3.25 rpm; Natsume Co., Tokyo, Japan).

Drugs

Morphine hydrochloride was obtained from Sankyo Co. (Tokyo, Japan). β-Funaltrexamine and naloxonazine were synthesized by Dr. Nagase (Toray Industries, Inc., Kamakura, Japan). β-Funaltrexamine (20 mg/kg, s.c.) and naloxonazine (35 mg/kg, s.c.) were given to mice 24 h prior to morphine treatment. The dose and schedule for β-funaltrexamine and naloxonazine treatment in this study were determined according to previous report (Kamei et al., 1992b, 1993a). Drugs were dissolved in 0.9 % saline solution.

Statistical analysis

Data are shown as the mean ± S.E. One-way ANOVA followed by Dunnett's multiple comparison test was used for the statistical evaluation.

Results

The pre-drug Straub tail reaction score is zero in both non-diabetic and diabetic mice. As shown in Fig. 1-1, s.c. administration of morphine, at doses of 3 - 10 mg/kg, dose-dependently increased the Straub tail reaction score in both non-diabetic and diabetic mice. Mice with diabetes showed significantly less sensitivity in the morphine-induced Straub tail reaction. Indeed, there was a significant difference in the intensity of the Straub tail reaction induced by 3, 5.6 and 10 mg/kg of morphine between non-diabetic mice and diabetic mice (Fig. 1-1). When the mice were pretreated with naloxonazine, the Straub tail reaction induced by morphine (10 mg/kg, s.c.) in both non-diabetic and diabetic mice was significantly reduced (Table 1-1). Furthermore, the Straub tail reaction induced by morphine (10 mg/kg, s.c.) was also antagonized by pretreatment with β-funaltrexamine in both non-diabetic and diabetic mice (Table 1-1).

On the other hand, there was no significant difference in the duration of rotarod performance between diabetic (114.7 ± 25.5 s, n=6) and non-diabetic (110.0 ± 16.4 s, n=7) mice.

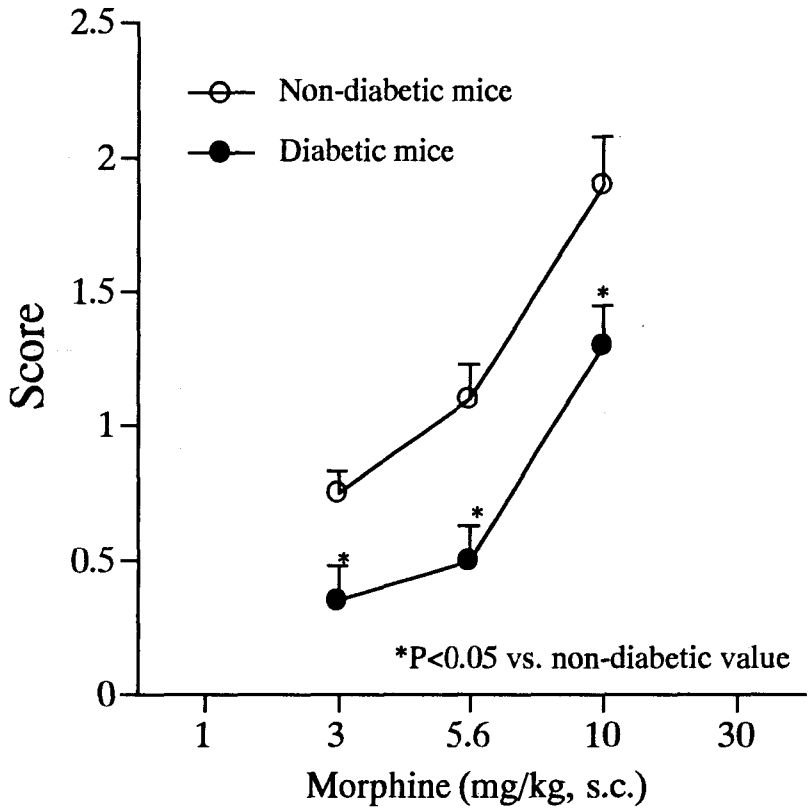

Figure 1-1. Dose-response curves for the morphine-induced Straub tail reaction in diabetic (open circle) and diabetic (closed circle) mice. The Straub tail reaction was graded using numerical scores, and was observed 20 min after s.c. administration of morphine. *P<0.05 vs. non-diabetic mice.

Table 1

Antagonism of the s.c. morphine-induced Straub tail reaction by μ-opioid receptor antagonists in non-diabetic and diabetic mice.

Treatments	Tail reaction scores	
	Non-diabetic mice	Diabetic mice
Morphine	1.90 ± 0.18	1.30 ± 0.15#
Morphine+β-funaltrexamine	0.90 ± 0.16**	0.75 ± 0.15*
Morphine+naloxonazine	0.85 ± 0.13**	0.70 ± 0.13**

The Straub tail reaction was observed 20 min after the s.c. administration of morphine (10 mg/kg). Each group consisted of 10 mice. Mice were treated with β-funaltrexamine (20 mg/kg, s.c.) or naloxonazine (35 mg/kg, s.c.) 24 h before testing.

#P<0.05 vs. non-diabetic mice. *P<0.05 and **P<0.01 vs. morphine alone.

Experiment 1-2: Possible involvement of protein kinase C in the attenuation of the morphine-induced Straub tail reaction in diabetic mice

Materials and Methods

Animals

Male ICR mice (Tokyo Laboratory Animals Science Co., Tokyo), weighing about 20 g at the beginning of the experiments, were used. The mice were housed under the condition as described in Chapter 1-1. Animals were rendered diabetic by an injection of streptozotocin (200 mg/kg, i.v.) prepared in 0.1 N citrate buffer at pH 4.5. Age-matched non-diabetic mice were injected with vehicle alone. The experiments were conducted 2 weeks after injection of streptozotocin or vehicle. Mice with serum glucose levels above 400 mg/dl were considered diabetic.

Measurement of the Straub tail reaction

The Straub tail reaction was graded using a minor modification of the numerical scoring system as described in Chapter 1-1. The angle was measured above the horizontal plane of the table.

Intracerebroventricular injection

Intracerebroventricular (i.c.v.) administration was performed following the method described by Haley and McCormick (1957) using a 50-μl Hamilton syringe. The injection site was 1.5 mm from the mid line, 0 mm from the bregma and 3.0 mm from the surface of the skull. Injection volumes for i.c.v. administration were 5 μl.

Drugs

The drugs used were streptozotocin (Sigma Chemical Co., St. Louis, MO) and morphine hydrochloride (Sankyo Co., Tokyo, Japan). Calphostin C and phorbol-12,13-dibutyrate (PDBu) were purchased from Calbiochem-Novabiochem International (San Diego, CA), and were injected 1h before the i.c.v. injection of morphine. The dose and schedule for

calphostin C and PDBu in this study were determined as described previously (Narita et al. 1997).

Data analysis

The data are expressed as means ± S.E. The statistical significance of differences between groups was assessed with an analysis of variance (ANOVA) followed by the Bonferroni test. The potency ratio for non-diabetic mice and diabetic mice was calculated using Program 11 of the Pharmacological Calculation system of Tallarida and Murray (1987)

Results

Effects of a protein kinase C activator, phorbol 12,13-dibutyrate (PDBu), on the morphine-induced Straub tail reaction in diabetic and non-diabetic mice.

As shown in Fig. 1-2A, i.c.v. administration of morphine, 15 μg, induced a Straub tail reaction in both diabetic and non-diabetic mice. Mice with diabetes showed significantly less sensitivity in the i.c.v. morphine-induced Straub tail reaction. I.c.v. pretreatment with PDBu (10 and 50 pmol) 60 min prior to an i.c.v. challenge with morphine (15 μg) attenuated the morphine-induced Straub tail reaction in non-diabetic mice. In diabetic mice, however, PDBu had no significant effect on the morphine (15 μg)-induced Straub tail reaction (Fig. 1-2A). As shown in Fig. 1B, i.c.v. pretreatment with calphostin C (10 pmol) 60 min prior to an i.c.v. injection of morphine did not affect the morphine-induced Straub tail reaction in non-diabetic mice. In diabetic mice, i.c.v. pretreatment with calphostin C, at doses of 3 and 10 pmol, progressively enhanced the morphine-induced Straub tail reaction (Fig 1-2B).

The i.c.v.-administered morphine-induced Straub tail reaction in diabetic mice was less than that in non-diabetic mice, as evidenced by a 2.3-fold rightward shift in the dose-response curve (Fig. 1-3). As shown in Fig. 1-3, i.c.v. pretreatment with a protein kinase C (PKC) activator, PDBu, at a dose of 50 pmol attenuated the i.c.v. morphine-induced Straub tail reaction in non-diabetic mice; the dose response curve for the morphine-induced Straub tail reaction was markedly shifted to right by 2.1-fold. The potency ratio (95% CL) of the morphine-induced Straub tail reaction in PDBu-treated non-diabetic mice versus that in vehicle-treated non-diabetic mice was 2.1 (1.4 - 4.3) (Fig. 1-3). In contrast, the i.c.v. morphine-induced Straub tail reaction in diabetic mice was not affected by i.c.v. pretreatment with PDBu (Fig. 1-3). I.c.v. pretreatment with a PKC inhibitor, calphostin C, did not affect the morphine-induced Straub tail reaction in non-diabetic mice (Fig. 1-3), but enhanced in diabetic mice. The dose-response curve for the morphine-induced Straub tail reaction in latter mice was markedly shifted to the left by 2.8-fold. The potency ratio (95 % CL) of the morphine-induced Straub tail reaction in calphostin C-treated diabetic mice versus that in

vehicle-treated diabetic mice was 2.8 (2.3 - 3.5) (Fig. 1-3). There was no significant difference in the potency of the morphine-induced Straub tail reaction between calphostin C-treated diabetic mice and naive non-diabetic mice. The potency ratio (95 % CL) of the morphine-induced Straub tail reaction in calphostin C-treated diabetic mice versus that in naive non-diabetic mice was 1.0 (0.98 - 1.07).

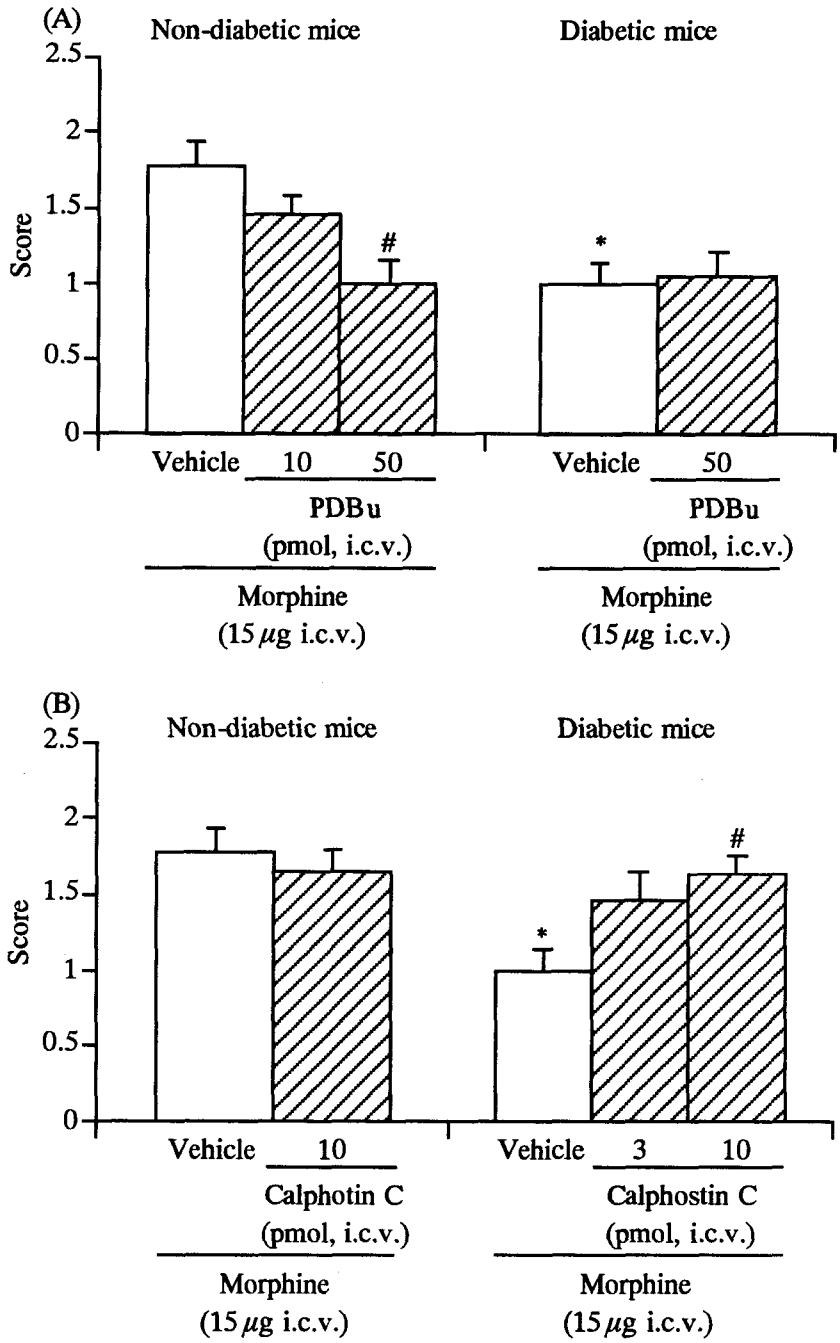

Figure 1-2. Effect of i.c.v. pretreatment with phorbol 12,13-dibutyrate (PDBu; A) and calphostin C (B) on the i.c.v. morphine-induced Straub tail reaction. PDBu (10 and 50 pmol) or calphostin C (1 or 3 pmol) was injected i.c.v. 60 min before administration of morphine (15 ml, i.c.v.). The Straub tail reaction was graded using numerical scores, and was observed 20 min after the i.c.v. administration of morphine. Each column represents the mean with S.E. for 9-15 mice in each group. *P<0.05 vs. non-diabetic mice. #P<0.05 vs. respective vehicle treated group.

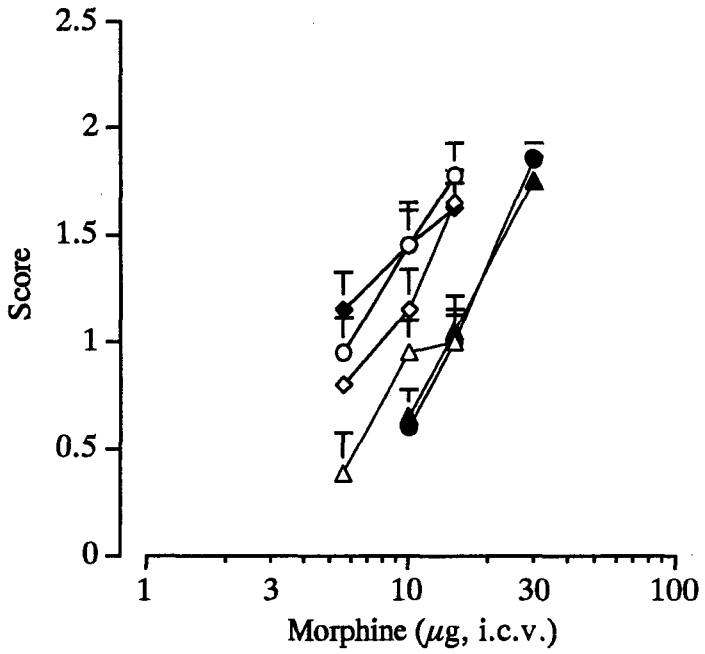

Figure 1-3. Effects of i.c.v. pretreatment with phorbol 12,13-dibutyrate (diamond) and calphostin C (triangle) on the dose-response curve for the i.c.v. morphine-induced Straub tail reaction in diabetic (closed symbol) and non-diabetic mice (open symbol). Phorbol 12,13-dibutyrate (50 pmol) or calphostin C (3 pmol) was injected i.c.v. 60 min before the administration of morphine. The Straub tail reaction was graded using numerical scores, and was observed 20 min after the i.c.v. administration of morphine. Each column represents the mean with S.E. for 9-15 mice in each group. *P<0.05 vs. non-diabetic mice. #P<0.05 vs. respective vehicle treated group.

Experiment 1-3: Possible involvement of protein kinase C in the attenuation of DAMGO-induced antinociception in diabetic mice.

Materials and Methods

Animals

Male ICR mice (Tokyo Laboratory Animals Science Co., Tokyo), weighing about 20 g at the beginning of the experiments, were used. The mice were housed under the condition as described in Chapter 1-1. Animals were rendered diabetic by an injection of streptozotocin (200 mg/kg, i.v.) prepared in 0.1 N citrate buffer at pH 4.5. Age-matched non-diabetic mice were injected with vehicle alone. The experiments were conducted 2 weeks after injection of streptozotocin or vehicle. Mice with serum glucose levels above 400 mg/dl were considered diabetic.

Antinociceptive assay.

The antinociceptive response was evaluated by recording the latency in the tail-flick test using radiant heat as a stimulus. The intensity of the thermal stimulus was adjusted so that the animal flicked its tail in 2-4 s. A cut-off latency of 30 s was used to prevent injury to the tail. Animals which did not respond within 30 s were removed and assigned a score of 30 s. The percent maximum possible effect (%MPE) was calculated for each animal as %MPE = 100 x (post drug latency - pre drug latency)/(30 - pre drug latency).

Intracerebroventricular injection

Intracerebroventricular (i.c.v.) administration was performed following the method described in Chapter 1-2.

Drugs

The following drugs were used: streptozotocin (Sigma Chemical Co., St. Louis, MO, USA), [D-Ala^2, N-MePhe^4, Gly-ol^5] enkephalin (DAMGO; Peninsula Laboratories, Inc., San Carlos, CA, USA), phorbol 12,13-dibutyrate (PDBu; Calbiochem-Novabiochem International, San Diego, CA, USA) and calphostin C (Calbiochem-Novabiochem International, San Diego, CA, USA). PDBu and calphostin C were dissolved in ethanol 0.1% in saline (0.9% NaCl solution). DAMGO was dissolved in saline. The doses of opioid agonist, PDBu and

calphostin C in this study were determined as described previously (Narita et al. 1997).

Data analysis

The data are expressed as means ± S.E. The statistical significance of differences between groups was assessed with Student's t test (comparison of two groups) or an analysis of variance (ANOVA) followed by the Bonferroni test (comparisons among multiple groups). The potency ratio for non-diabetic mice and diabetic mice was calculated using Program 11 of the Pharmacological Calculations system of Tallarida and Murray (1987).

Results

Effect of i.c.v. pretreatment with phorbol 12,13-dibutyrate (PDBu) on the antinociceptive effect induced by i.c.v.-administered DAMGO in diabetic and non-diabetic mice.

As shown in Fig. 1-4, i.c.v. administration of DAMGO (10 ng) produced an average %MPE of 84.2 ± 9.3 % and 33.3 ± 8.4 % in non-diabetic and diabetic mice, respectively. Diabetic mice were significantly less sensitive to i.c.v. DAMGO than non-diabetic mice, as assessed by the tail-flick test. Pretreatment with PDBu (50 pmol, i.c.v.) 60 min prior to an i.c.v. challenge with DAMGO attenuated the antinociceptive effect of DAMGO (10 ng) in non-diabetic mice. The attenuation of DAMGO-induced antinociception in non-diabetic mice was reversed by concomitant pretreatment with calphostin C (3 pmol, i.c.v.) In diabetic mice, however, PDBu (50 and 100 pmol) had no significant effect on the antinociceptive effect of DAMGO (10 ng).

Effects of i.c.v. pretreatment with calphostin C on DAMGO-induced antinociception in diabetic and non-diabetic mice.

As shown in Fig. 1-5A, pretreatment with calphostin C, at a dose of 3 pmol, i.c.v., did not affect DAMGO (10 ng, i.c.v.)-induced antinociception in non-diabetic mice. Furthermore, calphostin C, at a dose of 3 pmol, also had no effect on the antinociception induced by a lower dose (3 ng, i.c.v.) of DAMGO-induced antinociception. However, pretreatment with a higher dose (10 pmol, i.c.v.) of calphostin C for 60 and 120 min significantly increased DAMGO (3 ng, i.c.v.)-induced antinociception in non-diabetic mice (Fig. 1-6).

In diabetic mice, pretreatment with calphostin C (3 pmol, i.c.v.) for 60, 120 and 240 min, but not for 30 min, progressively increased DAMGO-induced antinociception. DAMGO produced dose-dependent antinociception at 5.6-30 ng i.c.v. in diabetic mice and 3-10 ng i.c.v. in non-diabetic mice. The antinociceptive potency of i.c.v. DAMGO in diabetic mice was less than that in non-diabetic mice, as evidenced by a 2.3-fold rightward shift in the dose-response curve for DAMGO-induced antinociception (Fig. 1-5B). Figure 1-5B shows that pretreatment with calphostin C (3 pmol, i.c.v.) for 60 min can prevent the rightward shift in the DAMGO dose-response curve, which is indicative of a decreased potency of DAMGO in diabetic mice. However, pretreatment with calphostin C (3 pmol) for 60 min had no effect on the dose-

response curve for DAMGO-induced antinociception in non-diabetic mice.

The effects of various doses of calphostin C on the antinociceptive effect of DAMGO in both diabetic and non-diabetic mice are shown in Fig. 1-7. Pretreatment with calphostin C for 60 min, at doses of 1 to 20 pmol, did not have any effect on the antinociception induced by DAMGO (10 ng). In diabetic mice, pretreatment with calphostin C dose-dependently increased the antinociceptive effect of DAMGO (10 ng).

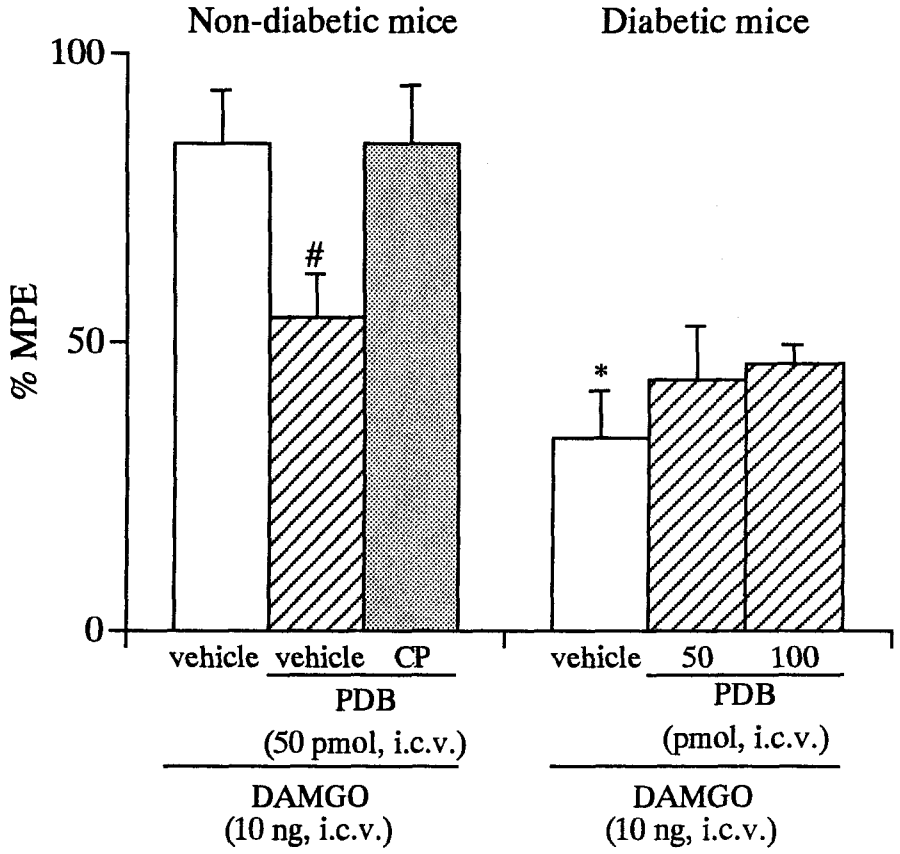

Figure 1-4. Effect of i.c.v. pretreatment with phorbol 12,13-dibutyrate (PDBu) on DAMGO-induced antinociception in diabetic and non-diabetic mice. PDBu (50 or 100 pmol) alone or a combination of PDBu (50 pmol) and calphostin C (CP; 3 pmol) was injected 60 min before the administration of DAMGO (10 ng, i.c.v.). Mice were tested 10 min after the injection of DAMGO in the tail-flick test. Each column represents the mean with S.E. for 10 mice in each group. #P<0.05 compared with the vehicle-pretreated group. *P<0.05 compared with respective non-diabetic mice.

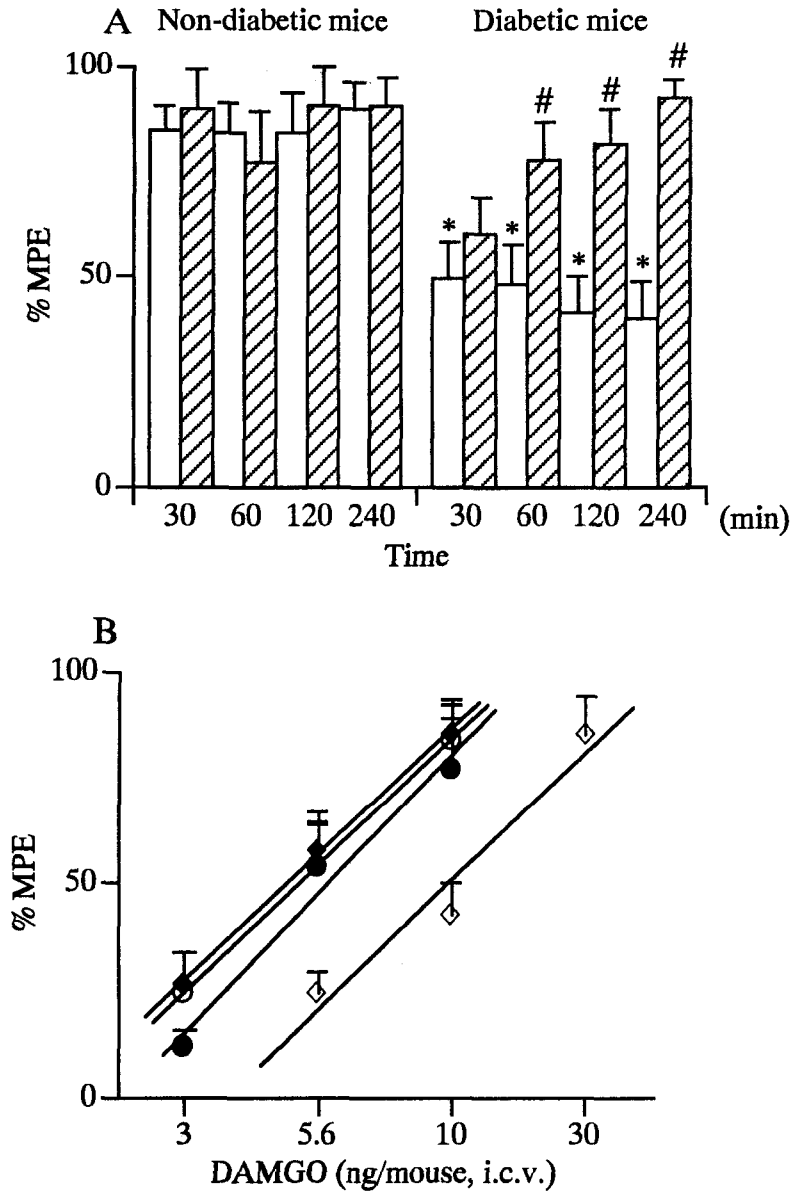

Figure 1-5. (A) effect of calphostin C (3 pmol, hatched column) on DAMGO-induced antinociception after different pretreatment times in diabetic and non-diabetic mice. (B) effect of i.c.v. pretreatment with calphostin C (3 pmol, closed symbol) and its vehicle (open symbol) on the dose-response curves for DAMGO-induced antinociception in diabetic (diamond) and non-diabetic (circle) mice. Calphostin C (3 pmol) was injected i.c.v. 30, 60, 120 and 240 min (A) or 60 min (B) before the administration of DAMGO (10 ng, i.c.v.). Mice were tested 10 min after the injection of DAMGO in the tail flick test. Each column represents the mean with S.E. for 10 mice in each group. #P<0.05 compared with the vehicle-pretreated group (open column). *P<0.05 compared with respective non-diabetic mice.

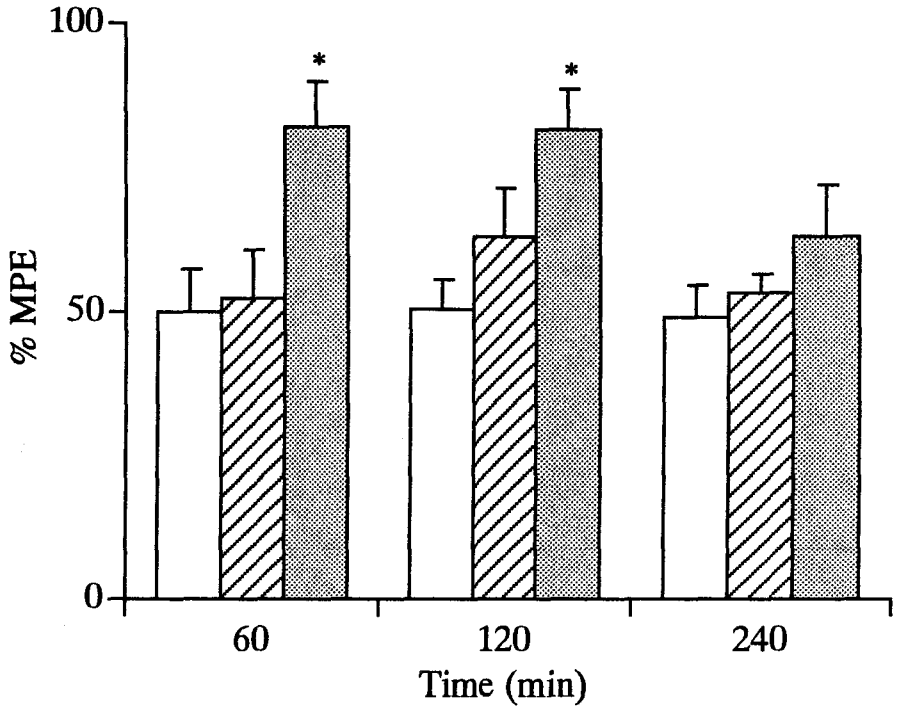

Figure 1-6. The effect of calphostin C on DAMGO-induced antinociception in non-diabetic mice after different pretreatment times. Calphostin C (3 pmol, hatched column; 10 pmol, closed dotted column) was injected i.c.v. 60, 120 and 240 min before the administration of DAMGO (5.6 ng, i.c.v.). Mice were tested 10 min after the injection of DAMGO in the tail-flick test. Each column represents the mean with S.E. for 10 mice in each group. *P<0.05 compared with the vehicle-pretreated group (open column).

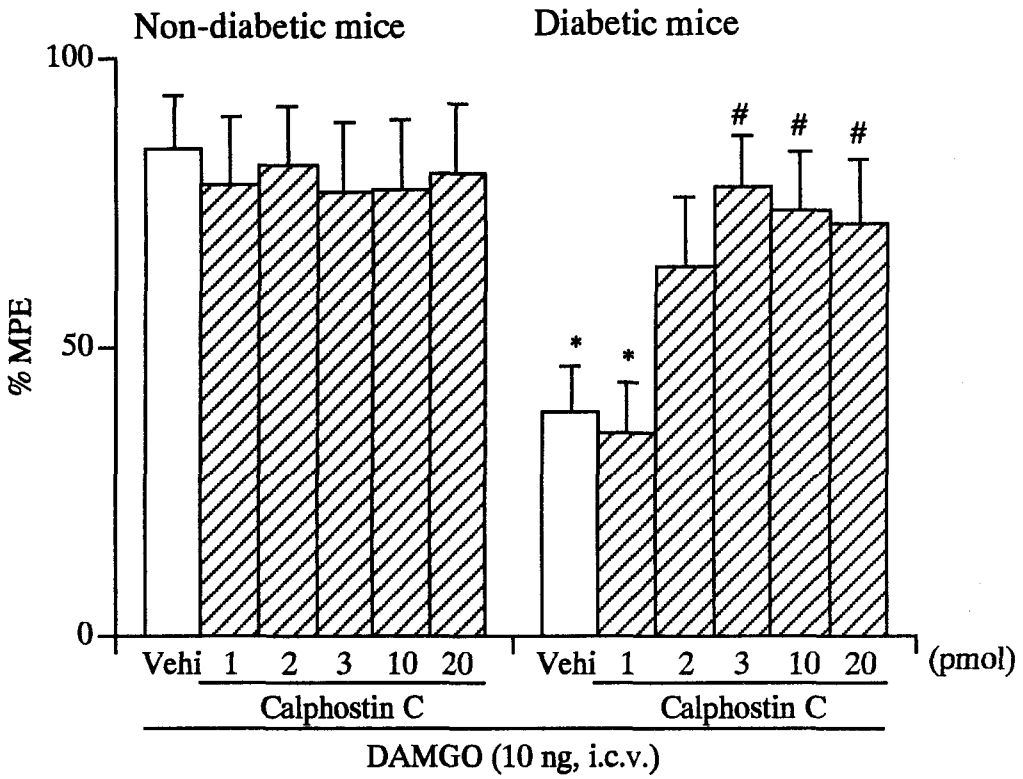

Figure 1-7. Dose-response effect of i.c.v. pretreatment with calphostin C on DAMGO-induced antinociception in diabetic and non-diabetic mice. Calphostin C and vehicle (Vehi) were injected i.c.v. 60 min before the administration of DAMGO. Mice were tested 10 min after the injection of DAMGO in the tail-flick test. Each column represents the mean with S.E. for 10 mice in each group. #P<0.05 compared with the vehicle-pretreated group. *P<0.05 compared with respective non-diabetic mice.

Experiment 1-4: Role of intracellular calcium in modification of μ- and δ-opioid receptor-mediated antinociception by diabetes in mice.

Materials and Methods

Animals

Male ICR mice (Tokyo Laboratory Animals Science Co., Tokyo), weighing about 20 g at the beginning of the experiments, were used. The mice were housed under the condition as described in Chapter 1-1. Animals were rendered diabetic by an injection of streptozotocin (200 mg/kg, i.v.) prepared in 0.1 N citrate buffer at pH 4.5. Age-matched non-diabetic mice were injected with vehicle alone. The experiments were conducted two weeks after injection of streptozotocin or vehicle. Mice with serum glucose levels above 400 mg/dl were considered diabetic.

Antinociceptive assay

The antinociceptive response was evaluated by recording the latency in the tail-flick test using radiant heat as a stimulus. The intensity of the thermal stimulus was adjusted so that the animal flicked its tail in 2-4 s. To obtain the same magnitude of antinociceptive potency, cut-off latencies of 10 and 30 sec were used for (-)-TAN67 and DAMGO, respectively. Animals which did not respond within the cut-off time were removed and assigned a score equivalent to the cut-off time. The percent maximum possible effect (%MPE) was calculated for each animal as %MPE = 100 × (post-drug latency – pre-drug latency)/(cut-off time – pre-drug latency)

Intracerebroventricular injection

Intracerebroventricular (i.c.v.) administration was performed following the method described in Chapter 1-2.

Drugs

The following drugs were used: streptozotocin (Sigma Chemical Co., St. Louis, Mo, USA), [D-Ala^2, N-MePhe^4, Gly-ol^5]enkephalin (DAMGO; Peninsula Laboratories, Inc., San Carlos, CA, USA), thapsigargin (Research Biochemical International, Natic, MA), ryanodine

(Calbiochem-Novabiochem, San Diego, CA), EGTA (Sigma Chemical Co., St. Louis, Mo, USA) and (-)-TAN67. (-)-TAN67 was synthesized by Dr. Nagase (Toray Laboratory, Kamakura, Japan). Thapsigargin was dissolved in 20% DMSO in saline (0.9% sodium chloride solution). DAMGO, (-)-TAN67, EGTA, $CaCl_2$ and ryanodine were dissolved in physiological saline. Thapsigargin was injected 1 h before the injection of agonists. Ryanodine and $CaCl_2$ were injected 10 min before the injection of agonists. EGTA was injected 15 min before the administration of agonists. The dose and schedule for each opioid agonist, EGTA, $CaCl_2$, ryanodine and thapsigargin in this study were determined as described previously (Smith and Stevens, 1995; Kamei et al., 1997)

Data analysis

The data are expressed as means ± S.E. The statistical significance of differences between groups was assessed with an analysis of variance (ANOVA) followed by the Bonferroni test. The potency ratio for non-diabetic mice and diabetic mice was calculated using Program 11 of the Pharmacological Calculation system of Tallarida and Murray (1987)

Results

Effects of i.c.v. $CaCl_2$ and EGTA on DAMGO-induced antinociception in diabetic and non-diabetic mice

$CaCl_2$ injected i.c.v. (100-300 nmol) significantly and dose-dependently reduced the antinociceptive effect of DAMGO (10 ng, i.c.v.) in non-diabetic mice (Fig. 1-8A). As shown in Fig. 1-8B, DAMGO given i.c.v. at doses of 3-10 ng caused a dose-dependent inhibition of the tail-flick response in non-diabetic mice. I.c.v. pretreatment with $CaCl_2$ (300 nmol) attenuated this inhibition of the tail-flick response induced by i.c.v. DAMGO in non-diabetic mice; the dose-response curve for DAMGO-induced antinociception was shifted to the left. The potency ratio (95% confidence limits (CL)) of DAMGO-induced antinociception in calcium-treated non-diabetic mice versus saline-treated non-diabetic mice was 2.2 (2.0-2.5). On the other hand, in diabetic mice, DAMGO (30 ng, i.c.v.)-induced antinociception was not reduced by i.c.v. pretreatment with $CaCl_2$ (100-300 nmol; Fig. 1-8A). Moreover, $CaCl_2$ (300 nmol, i.c.v.) did not affect the potency of DAMGO in diabetic mice. The potency ratio (95% CL) of antinociceptive effect of DAMGO in calcium-treated diabetic mice versus that in saline-treated diabetic mice was 1.2 (1.0-1.3) (Fig. 1-8B). I.c.v. pretreatment with $CaCl_2$ (300 nmol) by itself had no effect on the baseline tail-flick latencies in diabetic (mean tail-flick latencies of 2.59 ± 0.17 s, n=10 for before $CaCl_2$ treatment; 2.60 ± 0.12 s, n=10 for after $CaCl_2$ treatment) and non-diabetic mice (mean tail-flick latencies of 2.73 ± 0.14 s, n=10 for before $CaCl_2$ treatment; 2.70 ± 0.15 s, n=10 for after $CaCl_2$ treatment). Furthermore, $CaCl_2$ (100-300 nmol, i.c.v.) did not produce apparent behavioral changes, such as convulsion and hyperlocomotion, in diabetic and non-diabetic mice.

EGTA injected i.c.v. (1-60 nmol) significantly enhanced the antinociceptive effect of DAMGO (5.6 ng, i.c.v.) in non-diabetic mice (Fig. 1-9A). Furthermore, i.c.v. pretreatment with EGTA (30 nmol) enhanced the inhibition of the tail-flick response induced by i.c.v. DAMGO in non-diabetic mice; the dose-response curve for DAMGO-induced antinociception was shifted to the left. The potency ratio (95% CL) of the DAMGO-induced antinociception in EGTA-treated non-diabetic mice versus that in saline-treated non-diabetic mice was 2.5(1.7-3.7). Furthermore, in diabetic mice, DAMGO (10 ng, i.c.v.)-induced antinociception was potentiated by i.c.v. pretreatment with EGTA (1-60 nmol; Fig. 1-9A). However, significant potentiation of DAMGO-induced antinociception was observed in diabetic mice at

a higher dose of EGTA (30 and 60 nmol; Fig. 1-9A). EGTA (30 nmol, i.c.v.) increased the potency of DAMGO in diabetic mice; the dose-response curve for DAMGO-induced antinociception was shifted to the left. The potency ratio (95% CL) of the antinociceptive effect of DAMGO in EGTA-treated diabetic mice versus that in saline-treated diabetic mice was 2.4 (2.3-2.5) (Fig. 1-9B). Moreover, i.c.v. pretreatment with EGTA (60 nmol) by itself had no effect on the tail-flick latencies in diabetic (mean tail-flick latencies of 2.62 ± 0.13 s, n=10 for before EGTA treatment; 2.56 ± 0.14 s, n=10 for after EGTA treatment) and non-diabetic mice (mean tail-flick latencies of 2.59 ± 0.21 s, n=10 for before EGTA treatment; 2.64 ± 0.20 s, n=10 for after EGTA treatment). Furthermore, EGTA (1-60 nmol, i.c.v.) did not affect general behavior in diabetic and non-diabetic mice.

Effects of i.c.v. $CaCl_2$ and EGTA on (-)-TAN67-induced antinociception in diabetic and non-diabetic mice

In contrast with DAMGO, as shown in Fig. 1-10A, i.c.v. pretreatment with $CaCl_2$ (300 nmol) enhanced the inhibition of the tail-flick response induced by i.c.v. (-)-TAN67 in non-diabetic mice; the dose-response curve for (-)-TAN67-induced antinociception was markedly shifted to the left. The potency ratio (95% CL) of (-)-TAN67-induced antinociception in calcium-treated non-diabetic mice versus saline-treated non-diabetic mice was 3.6 (3.1-4.2). However, in diabetic mice, i.c.v. pretreatment with $CaCl_2$ (300 nmol) did not affect (-)-TAN67-induced antinociception (Fig. 1-10A). The potency ratio (95% CL) of (-)-TAN67-induced antinociception in calcium-treated diabetic mice versus saline-treated diabetic mice was 1.2 (0.8-1.8). As shown in Fig. 3B, i.c.v. pretreatment with EGTA (10 nmol) attenuated the inhibition of the tail-flick response induced by i.c.v. (-)-TAN67 in non-diabetic mice; the dose-response curve for (-)-TAN67-induced antinociception was markedly shifted to the right. The potency ratio (95% CL) of (-)-TAN67-induced antinociception in EGTA-treated non-diabetic mice versus saline-treated non-diabetic mice was 4.1 (2.3-9.1). In diabetic mice, i.c.v. pretreatment with EGTA (10 nmol) attenuated (-)-TAN67-induced antinociception; the dose-response curve for (-)-TAN-67-induced antinociception was shifted to the right (Fig. 1-10B). The potency ratio (95% CL) of (-)-TAN67-induced antinociception in EGTA-treated diabetic mice versus saline-treated diabetic mice was 7.7 (5.8-10.4).

Effects of thapsigargin and ryanodine on DAMGO-induced antinociception in diabetic and non-diabetic mice

Thapsigargin injected i.c.v. (0.3-3 nmol) significantly and dose-dependently reduced the antinociceptive effect of DAMGO (10 ng, i.c.v.) in non-diabetic mice (Fig 1-11A). As shown in Fig. 1-11B, i.c.v. pretreatment with thapsigargin (3 nmol) attenuated the inhibition of the tail-flick response induced by i.c.v. DAMGO in non-diabetic mice; the dose-response curve for DAMGO-induced antinociception was markedly shifted to the right. The potency ratio (95% CL) of the antinociceptive effect of DAMGO in thapsigargin-treated non-diabetic mice versus vehicle–treated non-diabetic mice was 3.0 (2.5-3.8). However, in diabetic mice, DAMGO (30 ng, i.c.v.)-induced antinociception was not affected by i.c.v. pretreatment with thapsigargin (3 nmol; Fig. 1-11A). I.c.v. pretreatment with thapsigargin did not affect the potency of DAMGO in diabetic mice (Fig. 1-11B). The potency ratio (95% CL) of the antinociceptive effect of DAMGO in thapsigargin-treated diabetic mice versus vehicle-treated diabetic mice was 1.0 (1.0-1.1). I.c.v. pretreatment with thapsigargin (3 nmol) by itself, had no effect on the tail-flick latencies in diabetic (tail-flick latencies of 2.72 ± 0.16 s, n=8 for before thapsigargin treatment; 2.72 ± 0.16 s, n=8) and non-diabetic mice (mean tail-flick latencies of 2.71 ± 0.22 s, n=9 for before thapsigargin treatment; 2.83 ± 0.11 s, n=9 for after thapsigargin treatment). Furthermore, thapsigargin (0.3-3.0 nmol, i.c.v.) did not produce any apparent behavioral change in diabetic and non-diabetic mice, while it has been reported that thapsigargin potently affect the intracellular calcium level (Takemura et al., 1991; Premack et al., 1994).

Ryanodine injected i.c.v. (0.3-3 nmol) significantly and dose-dependently enhanced the antinociceptive effect of DAMGO (5.6 ng, i.c.v.) in non-diabetic mice (Fig. 1-12A). Furthermore, ryanodine (3 nmol, i.c.v.) significantly enhanced the potency of DAMGO in non-diabetic mice; the dose-response curve for DAMGO-induced antinociception as shifted to the left (Fig. 1-12B). The potency ratio (95% CL) of DAMGO-induced antinociception in ryanodine-treated non-diabetic mice versus saline-treated non-diabetic mice was 2.2 (1.9-2.6). In diabetic mice, DAMGO (10 ng, i.c.v.)-induced antinociception was also dose-dependently enhanced by pretreatment with ryanodine (0.3-3 nmol; Fig. 1-12A). Moreover, ryanodine (3 nmol, i.c.v.) enhanced the potency of DAMGO in diabetic mice; the dose-response curve for DAMGO-induced antinociception was markedly shifted to the left (Fig. 1-12B). The potency ratio (95% CL) of DAMGO-induced antinociception in ryanodine-treated diabetic mice versus saline-treated diabetic mice was 4.4 (4.0-4.8). I.c.v. pretreatment with ryanodine (3

nmol) by itself did not affect the tail-flick latencies in diabetic (mean tail-flick latencies of 2.78 ± 0.18 s, n=10 for before ryanodine treatment; 2.53 ± 0.12 s, n=10 for after ryanodine treatment) and non-diabetic mice (mean tail-flick latencies of 2.36 ± 0.11 s, n=10 for before ryanodine treatment; 2.31 ± 0.07 s, n=10 for after ryanodine treatment). Furthermore, ryanodine (0.3-3 nmol) did not affect the general behavior in diabetic and non-diabetic mice.

Effects of thapsigargin and ryanodine on (-)-TAN67-induced antinociception in diabetic and non-diabetic mice

As shown in Fig. 1-13A, i.c.v. pretreatment with thapsigargin (3 nmol) potentiated the inhibition of the tail-flick response induced by i.c.v.-administered (-)-TAN67 in non-diabetic mice; the dose-response curve for (-)-TAN67-induced antinociception was markedly shifted to the left. The potency ratio (95% CL) of (-)-TAN67-induced antinociception in thapsigargin-treated non-diabetic mice versus vehicle-treated non-diabetic mice was 3.1 (2.5-3.9). In diabetic mice, i.c.v. pretreatment with thapsigargin (3 nmol) did not affect (-)-TAN67-induced antinociception (Fig. 1-13A). The potency ratio (95% CL) of (-)-TAN67-induced antinociception in thapsigargin-treated diabetic mice versus vehicle-treated diabetic mice was 1.4 (0.8-2.6). Ryanodine (3 nmol, i.c.v.) did not affect the potency of (-)-TAN67 in non-diabetic mice (Fig. 1-13B). The potency ratio (95% CL) of (-)-TAN67-induced antinociception in ryanodine-treated non-diabetic mice versus saline-treated non-diabetic mice was 1.0 (0.7-1.4). In diabetic mice, however, ryanodine (3 nmol, i.c.v.) attenuated (-)-TAN67-induced antinociception; the dose-response curve for (-)-TAN67-induced antinociception was markedly shifted to the right (Fig. 1-13B). The potency ratio (95% CL) of (-)-TAN67-induced antinociception in ryanodine-treated diabetic mice versus saline-treated diabetic mice was 4.0 (2.7-6.2).

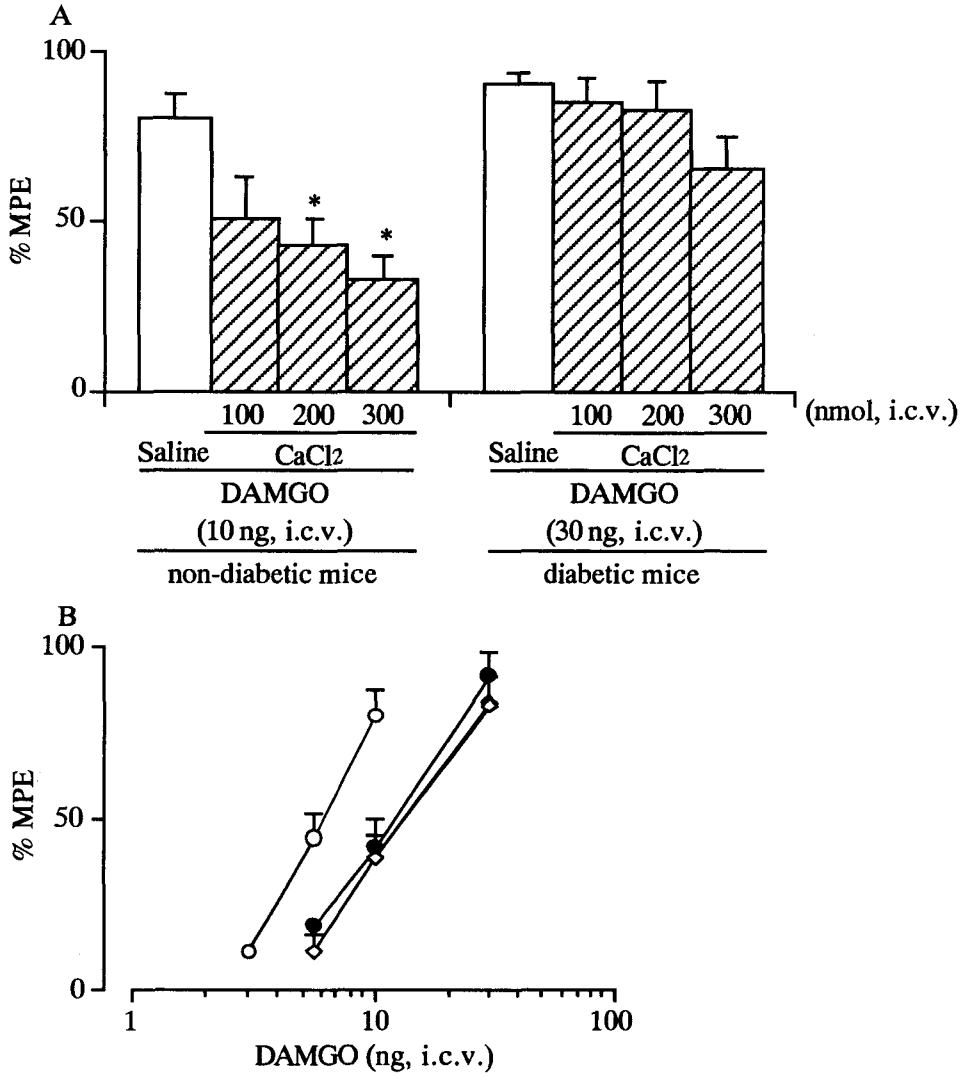

Figure 1-7. (A) dose-response effect of i.c.v. pretreatment with CaCl2 (100 and 300 nmol, hatched column) on DAMGO (10 or 30 nmol, i.c.v.)-induced antinociception in diabetic and non-diabetic mice. (B) effect of i.c.v. pretreatment with CaCl2 (300 nmol, closed symbol) and its vehicle (open symbol) on the dose-response curve for DAMGO-induced antinociception in diabetic (diamond) and non-diabetic mice (circle). Non-diabetic mice injected with DAMGO received either saline (open circles) or CaCl2 (300 nmol, closed circles). Diabetic mice injected with DAMGO received either saline (open diamonds) or CaCl2 (300 nmol, closed diamonds). CaCl2 was injected 10 min before the administration of DAMGO. Mice were tested 10 min after the injection of DAMGO in the tail-flick test. Each column and point represents the mean with S.E. for 10-15 mice in each group. *P<0.05 compared with respective non-diabetic mice. #P<0.05 compared with the saline (open column)-pretreated group.

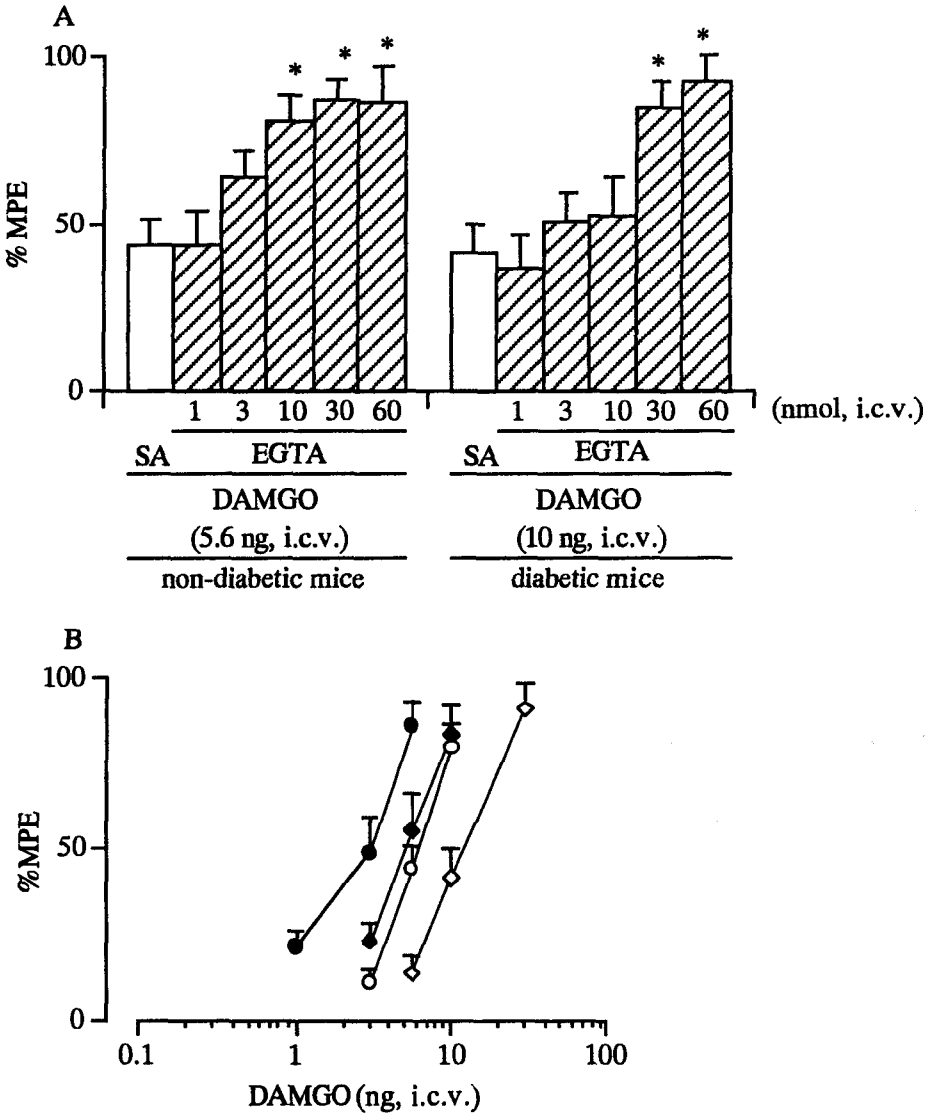

Figure 1-8. (A) dose-response effect of i.c.v. pretreatment with EGTA (1-60 nmol, hatched column) on DAMGO (10 or 5.6 nmol, i.c.v.)-induced antinociception in diabetic and non-diabetic mice. (B) effect of i.c.v. pretreatment with EGTA (30 nmol, closed symbol) and saline (open symbol) on the dose-response curve for DAMGO-induced antinociception in diabetic (diamond) and non-diabetic mice (circle). Non-diabetic mice injected with DAMGO received either saline (open circles) or EGTA (30 nmol, closed circles). Diabetic mice injected with DAMGO received either saline (open diamonds) or EGTA (30 nmol, closed diamonds). EGTA was injected 15 min before the administration of DAMGO. Mice were tested 10 min after the injection of DAMGO in the tail-flick test. Each column and point represents the mean with S.E. for 10-15 mice in each group. *P<0.05 compared with respective non-diabetic mice. #P<0.05 compared with the saline (SA, open column)-pretreated group.

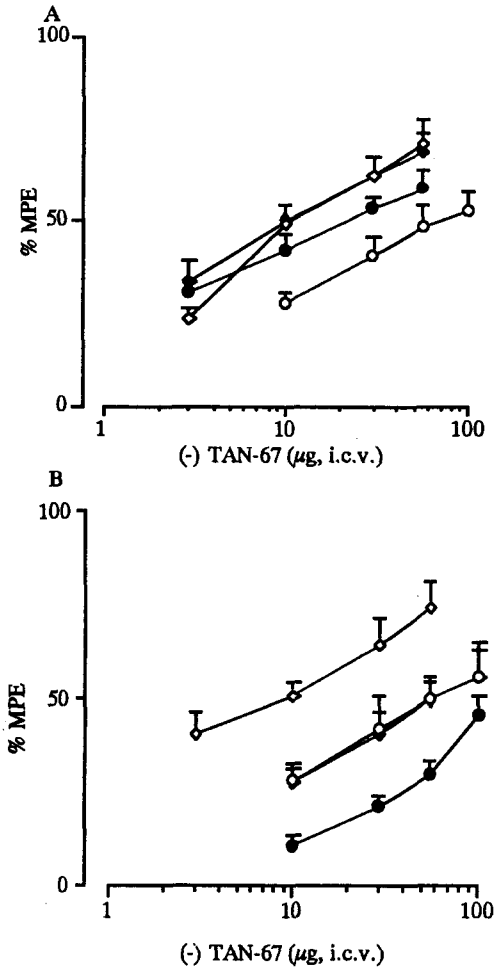

Figure 1-9. (A) the effect of i.c.v. pretreatment with CaCl2 (300 nmol, closed symbol) and saline (open circle) on the dose-response curve for (-)-TAN67-induced antinociception in diabetic (diamond) and non-diabetic (circle) mice. Non-diabetic mice injected with (-)-TAN67 received either saline (open circles) or CaCl2 (300 nmol, closed circles). Diabetic mice injected with (-)-TAN67 received either saline (open diamonds) or CaCl2 (300 nmol, closed diamonds). CaCl2 was injected 10 min before the administration of (-)-TAN67. Mice were tested 30 min after the injection of (-)-TAN67 in the tail-flick test. Each point represents the mean with S.E. for 10-15 mice in each group. (B) the effect of i.c.v. pretreatment with EGTA (10 nmol, closed symbol) and saline (open symbol) on the dose-response curve for (-)-TAN67 in diabetic (diamond) and non-diabetic (circle) mice. Non-diabetic mice injected with (-)-TAN67 received either saline (open circles) or EGTA (10 nmol, closed circles). Diabetic mice injected with (-)-TAN67 received either saline (open diamonds) or EGTA (10 nmol, closed diamonds). EGTA was injected 15 min before the administration of (-)-TAN67. Mice were tested 30 min after the injection of (-)-TAN67 in the tail-flick test. Each point represents the mean with S.E. for 10-15 mice in each group.

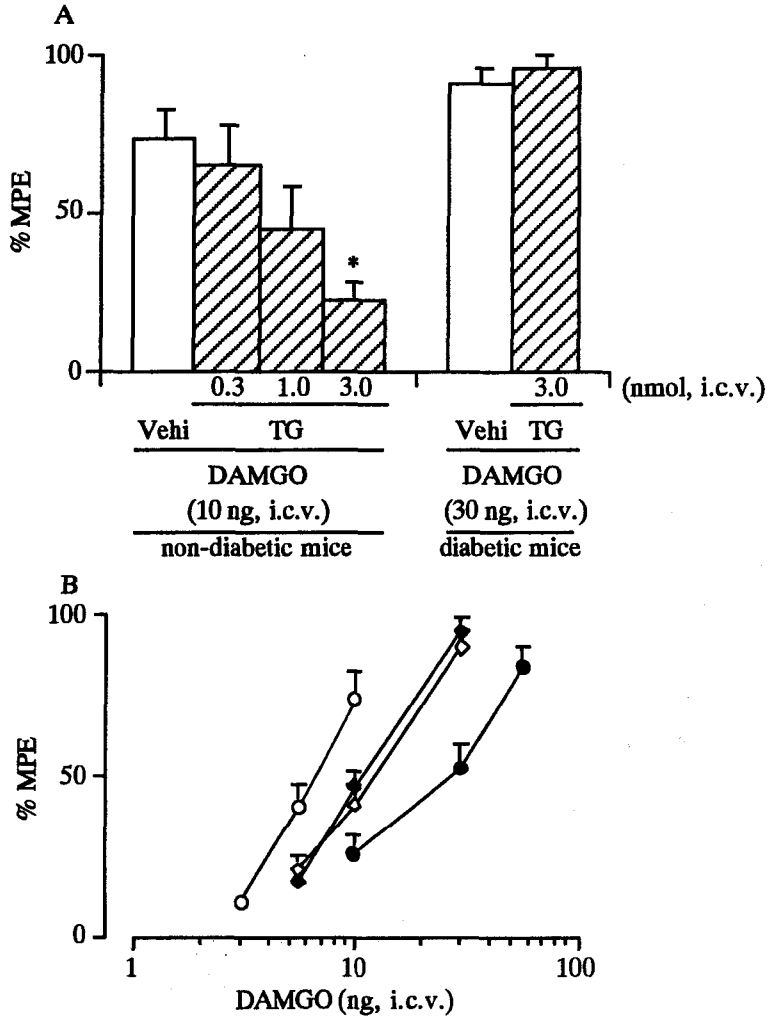

Figure 1-10. (A) dose-response effect of i.c.v. pretreatment with thapsigargin (hatched column) on DAMGO (30 or 10 nmol, i.c.v.)-induced antinociception in diabetic and non-diabetic mice. (B) effect of i.c.v. pretreatment with thapsigargin (3 nmol, closed symbol) and its vehicle (open symbol) on the dose-response curve for DAMGO-induced antinociception in diabetic (diamond) and non-diabetic mice (circle). Non-diabetic mice injected with DAMGO received either vehicle (20% DMSO, open circles) or thapsigargin (3 nmol, closed circles). Diabetic mice injected with DAMGO received either vehicle (20% DMSO, open diamonds) or thapsigargin (3 nmol, closed diamonds). Thapsigargin was injected 60 min before the administration of DAMGO. Mice were tested 10 min after the injection of DAMGO in the tail-flick test. Each column and point represents the mean with S.E. for 10-15 mice in each group. *P<0.05 compared with respective non-diabetic mice. #P<0.05 compared with the vehicle (open column)-pretreated group.

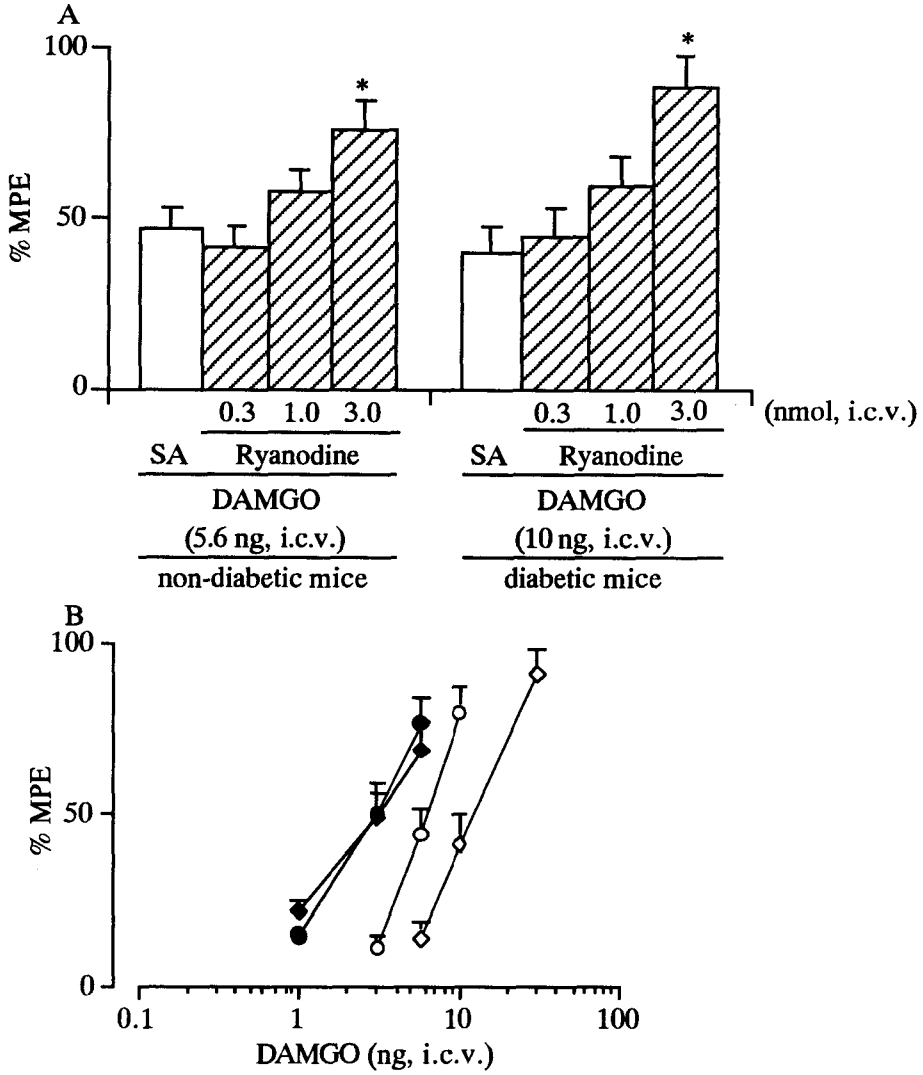

Figure 1-11. (A) dose-response effect of i.c.v. pretreatment with ryanodine (hatched column) on DAMGO (10 or 5.6 nmol, i.c.v.)-induced antinociception in diabetic and non-diabetic mice. (B) effect of i.c.v. pretreatment with ryanodine (3 nmol, closed symbol) and its vehicle (open symbol) on the dose-response curve for DAMGO-induced antinociception in diabetic (diamond) and non-diabetic (circle) mice. Non-diabetic mice injected with DAMGO received either saline (open circles) or ryanodine (3 nmol, closed circles). Diabetic mice injected with DAMGO received either saline (open diamonds) or ryanodine (3 nmol, closed diamonds). Ryanodine was injected 10 min before the administration of DAMGO. Mice were tested 10 min after the injection of DAMGO in the tail-flick test. Each point represents the mean with S.E. for 10-15 mice in each group.

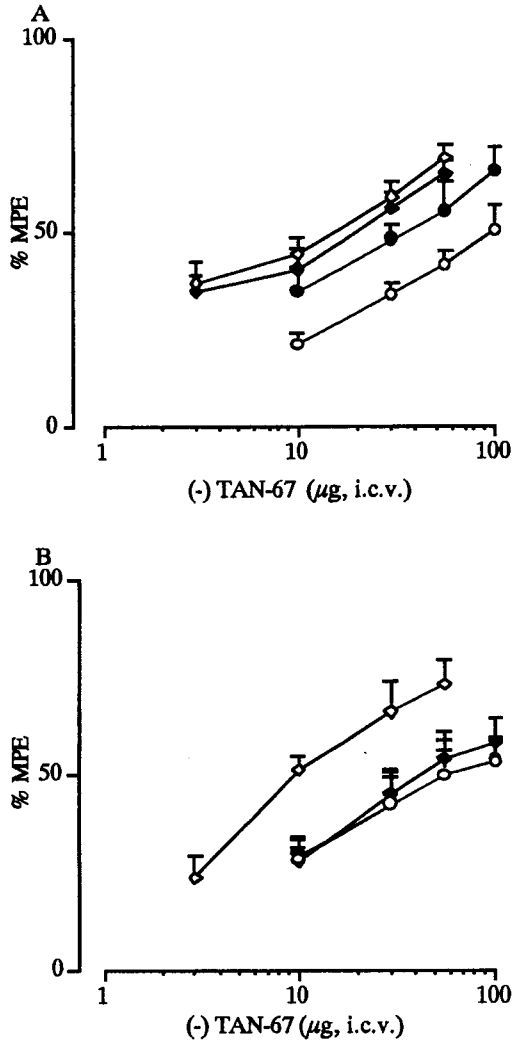

Figure 1-12. (A) the effects of i.c.v. pretreatment with thapsigargin (3 nmol, closed circle) and its vehicle (open symbol) on the dose-response curve for (-)-TAN67-induced antinociception in diabetic (diamond) and non-diabetic (circle) mice. Non-diabetic mice injected with (-)-TAN67 received either vehicle (20% DMSO, open circles) or thapsigargin (3 nmol, closed circles). Diabetic mice injected with (-)-TAN67 received either vehicle (20% DMSO, open diamonds) or thapsigargin (3 nmol, closed diamonds). Thapsigargin was injected 60 and min before the administration of (-)-TAN67. Mice were tested 30 min after the injection of (-)-TAN67 in the tail-flick test. Each point represents the mean with S.E. for 10-15 mice in each group. (B) the effects of i.c.v. pretreatment with ryanodine (closed symbol) and saline (open symbol) on the dose-response curve for (-)-TAN67-induced antinociception in diabetic (diamond) and non-diabetic (circle) mice. Non-diabetic mice injected with (-)-TAN67 received either saline (open circles) or ryanodine (3 nmol, closed circles). Diabetic mice injected with (-)-TAN67 received either saline (open diamonds) or ryanodine (3 nmol, closed diamonds). Ryanodine was injected 10 min before the administration of (-)-TAN67. Mice were tested 30 min after the injection of (-)-TAN67 in the tail-flick test. Each point represents the mean with S.E. for 10-15 mice in each group.

Discussion

The results of "Experiment 1-1" clearly indicated that the Straub tail reaction induced by s.c. administration of morphine is significantly antagonized by β-funaltrexamine, which suggests that μ-opioid receptors may play an important role in the morphine-induced Straub tail reaction. These results are consistent with previous reports of Kameyama and Ukai (1979) and Narita et al. (1993). In the present study, I also observed that the Straub tail reaction induced by s.c. administration of morphine is significantly less in diabetic mice than in non-diabetic mice. This result supports the previous suggestion that mice with diabetes are selectively hypo-responsive to μ-opioid receptor-mediated pharmacological actions (Kamei et al., 1992a, 1992b). There was no significant difference in the pre-drug Straub tail reaction score between diabetic and non-diabetic mice. Furthermore, motor incoordination was not observed in diabetic mice. These results further support the conclusion that the hypo-responsive to morphine-induced Straub tail reaction in diabetic mice may be due to the dysfunction of μ-opioid receptors, but not of motor coordination.

It has been proposed that μ_1-opioid receptors, but not μ_2-opioid receptors, mediate supraspinal antinociception, since Heyman et al. (1988) and Paul et al. (1989) reported that naloxonazine, a selective μ_1-opioid receptor antagonist, selectively attenuates i.c.v. administered DAMGO-induced antinociception, but not intrathecally administered DAMGO-induced antinociception. In this regard, Kamei et al. (1992b, 1994a) recently demonstrated that the antinociceptive effect of i.c.v. morphine in diabetic mice was significantly less than that in non-diabetic mice. Furthermore, the antinociceptive effect of i.c.v. morphine was significantly reduced in both diabetic and non-diabetic mice following pretreatment with naloxonazine (Kamei et al., 1994a). However, there were no significant differences in the antinociceptive effects of i.t.-administered morphine in diabetic and non-diabetic mice (Kamei et al., 1992b, 1994a). Moreover, naloxonazine had no significant effect on the antinociceptive effect of i.t. morphine in either diabetic or non-diabetic mice (1994b). The sensitivities of the antinociceptive effects of i.c.v. and i.t. morphine to naloxonazine agree with the suggestions of other investigators (Heyman et al., 1988; Paul et al., 1989) that μ_1-opioid receptors play a major role in supraspinal antinociception, while μ_2-opioid receptors play a major role in spinal antinociception. Based on these results, Kamei et al. (1994a) previously concluded that mice with diabetes are selectively hypo-responsive to μ_1-opioid receptor-mediated pharmacological action, but not to that which is mediated by μ_2-opioid

receptors. This hypothesis is further supported by the present results that 1) the Straub tail reaction induced by s.c. administration of morphine is significantly less in diabetic mice than in non-diabetic mice, and 2) naloxonazine, a selective μ_1-opioid receptor antagonist, significantly reduced the Straub tail reaction induced by s.c. administration of morphine in both non-diabetic and diabetic mice, indicating that μ_1-opioid receptors play a major role in the morphine-induced Straub tail reaction.

Although Murray and Cowan (1990) suggested that supraspinal δ-opioid receptors are not involved in the opioid agonist-induced Straub tail reaction, the role of spinal δ-opioid receptors in opioid-induced Straub tail reaction is unclear. Narita et al. (1993) suggested that κ-opioid receptors are not involved in opioid-induced Straub tail reaction. Furthermore, Kamei et al. (1992b) also suggested that there was no significant change in the function of spinal δ-opioid receptors between diabetic and non-diabetic mice. It has been shown that the morphine-induced Straub tail reaction is produced at the level not only of the supraspinal site but also spinal site. In the present study, β-funaltrexamine and naloxonazine only partially antagonized the morphine-induced Straub tail reaction in both diabetic and non-diabetic mice. Although the detail mechanisms of this partial antagonism are unclear, it is possible that spinal δ-opioid receptors might be involved in the morphine-induced Straub tail reaction. Further studies are needed to resolve this problem.

There have been several suggestions regarding the possible functions of protein kinase C (PKC), including involvement in secretion and exocytosis, modulation of ion conductance, regulation of receptor interaction with components of the signal transduction apparatus, smooth muscle contraction, gene expression and cell proliferation (Nishizuka, 1988). PKC regulates several cellular functions through the phosphorylation of proteins, including some receptors. The results of "Experiment 1-2" demonstrated that the i.c.v. morphine-induced Straub tail-reaction in non-diabetic mice is attenuated by i.c.v. pretreatment with PDBu (50 pmol), which stimulates PKC. Many investigators have proposed that the phosphorylation of receptors by PKC may be a possible mechanism for the development of desensitization (Shearman et al., 1989). Activation of PKC by phorbol ester attenuates the opioid-induced inhibition of adenylyl cyclase activity in neuroblastoma × glioma NG108-15 hybrid cells (Louie et al., 1990). Furthermore, activation of PKC by phorbol ester potentiates the desensitization of μ-opioid receptor-induced K^+ current (Chen and Yu, 1994; Zhang et al., 1996). We previously indicated that i.c.v. pretreatment with PDBu attenuates DAMGO- and morphine-induced antinociception (Narita et al., 1997). Thus, these previous results and the

present data suggest that the activation of PKC by phorbol ester attenuates the pharmacological action of μ-opioid receptor agonists.

In contrast, PDBu had no significant effect on the morphine-induced Straub tail reaction in diabetic mice. Furthermore, i.c.v. pretreatment with calphostin C (10 pmol), which had no significant effect on the morphine-induced Straub tail reaction in non-diabetic mice, significantly and dose-dependently reversed the attenuation of the morphine-induced Straub tail reaction in diabetic mice. Indeed, there was no significant difference in the potency of the morphine-induced Straub tail reaction between calphostin C-treated diabetic mice and naive non-diabetic mice. Calphostin C inhibits the binding of diacylglycerol to the regulatory domain of PKC (Kobayashi et al., 1989). Thus, it is likely that the attenuation of several of morphine's pharmacological actions, i.e. antinociception and the Straub tail reaction, in diabetic mice may be due, in part, to the increased phosphorylation of μ-opioid receptors by the activation of PKC.

The morphine-induced the Straub tail reaction involves both central and peripheral components of the nervous system. The results from "Experiment 1-1" indicated that the systemic morphine-induced Straub tail reaction in non-diabetic mice is greater than that in diabetic mice. In Experiment 1-2, the i.c.v. morphine-induced Straub tail reaction in non-diabetic mice was greater than that in diabetic mice, as with the systemic administration of morphine. Many investigators have indicated that the central administration of morphine induces the Straub tail reaction in mice (Narita et al., 1994; Nath et al., 1994). Diabetes mellitus causes various complications, including dysfunction of skeletal muscles and peripheral nerves (Pain and Garlick, 1974). It has been reported that the activation of PKC induces the dysfunction of skeletal muscle in streptozotocin-induced diabetic mice (Nojima et al., 1995). Thus, it is possible that the dysfunction of peripheral neurons and/or muscle causes the attenuation of the systemic morphine-induced Straub tail reaction in diabetic mice. However, the i.c.v. administration of morphine induced the Straub tail reaction in both diabetic and non-diabetic mice. Furthermore, i.c.v. pretreatment with calphostin C reverses the attenuation of the i.c.v. morphine-induced Straub tail reaction in diabetic mice. These results indicate that the attenuation of the morphine-induced Straub tail reaction in diabetic mice may be due to the desensitization of supraspinal μ-opioid receptors, but not the dysfunction of peripheral nerves and muscles, by the activation of PKC.

The results from "Experiment 1-3" demonstrated that i.c.v. pretreatment with PDBu (50 pmol) attenuated the inhibition in the tail-flick test induced by i.c.v. DAMGO in non-

diabetic mice. Furthermore, the attenuation of i.c.v. DAMGO-induced antinociception by PDBu was reversed by concomitant i.c.v. pretreatment with calphostin C, a selective PKC inhibitor. These results are consistent with our previous observation and support the suggestion that the attenuation by PDBu of μ-opioid receptor-mediated antinociception is specifically mediated by the activation of PKC (Narita et al., 1997).

In contrast, PDBu, by itself, had no significant effect on DAMGO-induced antinociception in diabetic mice. Furthermore, pretreatment with calphostin C (3 pmol, i.c.v.), which had no significant effect on DAMGO-induced antinociception in non-diabetic mice, significantly and dose-dependently increased DAMGO-induced antinociception in diabetic mice. Indeed, there was no significant difference in the potency of DAMGO-induced antinociception between calphostin C-pretreated diabetic mice and naive non-diabetic mice. In the present study, moreover, I found that pretreatment with a higher dose of calphostin C (10 pmol) potentiated low-dose (3 ng, i.c.v.) DAMGO-induced antinociception in non-diabetic mice. As noted above, calphostin C specifically inhibits the binding of diacylglycerol to the regulatory domain of PKC (Kobayashi et al., 1989), and therefore is a more selective inhibitor than staurosporine or 1-(5-isoquinolinesulfonyl)-2-methylpiperazine hydrochloride (H-7), which interact with the ATP-binding site of PKC that shares substantial homology with other protein kinases. The phosphorylation of receptors by PKC has been proposed to be a possible mechanism for the development of tolerance or desensitization (Shearman et al., 1989). According to recent cloning studies, several potential phosphorylation sites by protein kinases are present in cloned opioid receptors, including μ-opioid receptors (Miotto et al., 1995). Previously, we reported that i.c.v. pretreatment with PDBu produced a calphostin C-sensitive attenuation of DAMGO-induced antinociception (Narita et al., 1997). These results suggest that μ-opioid receptors can be phosphorylated by the activation of PKC, and this receptor phosphorylation by PKC leads to desensitization of μ-opioid receptor-mediated responses. Several studies have suggested that PKC systems may be up-regulated in diabetes (Craven and DeRubertis, 1989; Wolf et al., 1990; Shiba et al., 1993). We recently reported that at least 30 to 60 min of pretreatment is required for PDBu to desensitize μ-opioid receptor-mediated responses (Narita et al., 1997). In the present study, at least 60 min of pretreatment was required for calphostin C to increase DAMGO-induced antinociception in diabetic mice. Thus, it is possible that the attenuation of the antinociceptive effect of DAMGO in diabetic mice may be due, in part, to the increased phosphorylation of μ-opioid receptors by the activation of PKC.

In "Experiment 1-4", i.c.v. administration of $CaCl_2$ which has been reported to increase the intracellular concentration of calcium, attenuated the antinociceptive effect of DAMGO, an μ-opioid receptor agonist in non-diabetic mice. Moreover, i.c.v. administration of EGTA, which has been reported to reduce the intracellular concentration of calcium, enhanced the antinociceptive effect of DAMGO in non-diabetic mice. This observation is consistent with a previous report that calcium antagonized morphine-induced antinociception while EGTA potentiated morphine-induced antinociception (Harris et al, 1975). Thus, these results and present results indicate that μ-opioid receptor agonist-induced antinociception is reduced by an increase in intracellular Ca^{2+} levels. In the present study, I observed that pretreatment with thapsigargin reduced DAMGO-induced antinociception in non-diabetic mice. This result is consistent with a previous finding that i.c.v. pretreatment with thapsigargin reduced the antinociceptive effect of morphine (Smith and Stevens, 1995). It has been reported that thapsigargin selectively inhibits Ca^{2+} uptake into the inositol 1,4,5-trisphosphate (IP_3)-sensitive microsomal Ca^{2+} pool by inhibiting ATP/Mg^{2+}-dependent ATPase (Bian et al., 1991). The subsequent depletion of this pool activates a low-conductance, Ca^{2+}-selective, non-voltage activated membrane current (Takemura et al., 1991; Premack et al., 1994). Thus, the increase in cytosolic Ca^{2+} caused by thapsigargin blocks the antinociceptive effect of DAMGO. Furthermore, pretreatment with ryanodine potentiates the antinociceptive effect of DAMGO. It has been reported that ryanodine blocks Ca^{2+} release from Ca^{2+}/caffeine-sensitive microsomal pools, which is involved in the phenomenon of Ca^{2+}-induced Ca^{2+} release (McPherson et al., 1991). It has been reported that ryanodine blocks Ca^{2+} release and accumulation by either preventing the opening of ryanodine channels or stabilizing an open subconductance state (McPherson et al., 1991). Furthermore, it has been reported that ryanodine reduces the rate at which $[Ca^{2+}]_i$ increases with Ca^{2+} entry (Friel and Tsien, 1992). Thus, it is possible that the potentiation of DAMGO-induced antinociception caused by ryanodine may be due to a decrease in $[Ca^{2+}]_i$. Therefore, the present results suggest that an increase in cytosolic Ca^{2+} levels antagonize μ-opioid receptor agonist-induced antinociception.

In contrast to DAMGO, I observed that calcium injected i.c.v. enhanced the antinociceptive effect of (-)-TAN67, a selective δ_1-opioid receptor agonist (Kamei et al., 1997), in non-diabetic mice. Moreover, i.c.v. EGTA blocked (-)-TAN67-induced antinociception in non-diabetic mice. Bhargava and Zhao (1996) reported that competitive and noncompetitive antagonists of the *N*-methyl-*D*-aspartate (NMDA) receptor antagonize the

analgesic action of δ_1-opioid receptor agonists. Furthermore, recent studies have reported that calcium channel blockers attenuate the antinociception induced by δ and κ but not μ-opioid receptor agonists (Spampinato et al., 1994). These results and those of the present study suggest that δ-opioid receptor agonist-induced antinociception is potentiated by an increase in intracellular Ca^{2+} levels. Thus, the present results suggest that cytosolic calcium differentially modulates the μ- and δ-opioid receptor-induced antinociception. Furthermore, in the present study, I observed that (-)-TAN67-induced antinociception in non-diabetic mice is potentiated by i.c.v. pretreatment with thapsigargin. As mentioned above, thapsigargin causes the increase in cytosolic calcium levels. Therefore, it is possible that δ-opioid receptor agonist-mediated antinociception is potentiated by the increase in cytosolic calcium levels. Thus, the present results suggest that cytosolic calcium differentially modulates the μ- and δ-opioid receptor agonist-induced antinociception. On the other hand, (-)-TAN67-induced antinociception in non-diabetic mice was not affected by pretreatment with ryanodine, which decreased cytosolic calcium levels. It is not clear why ryanodine does not affect (-)-TAN67-induced antinociception in non-diabetic. It has not been shown that the μ and δ opioid receptors regulating antinociception are always expressed on the same neuron, or even in the same pain-regulating neural pathway. Thus, it is possible that μ-opioid receptor expressing neurons show the expected changes in calcium levels in response to ryanodine, while neurons expressing δ-opioid receptor are not directly affected by ryanodine. However, Miyamae et al. (1993) reported that a cloned δ-opioid receptor expressed in *Xenopus oocytes* can mediate agonist activation of phospholipase C. It has recently been reported that δ-opioid receptor-mediated increases in intracellular $[Ca^{2+}]_i$ result from IP_3-induced Ca^{2+} release from intracellular stores (Smart and Lambert, 1996a). It is suggested that the activation of δ-opioid receptor enhances $[Ca^{2+}]_i$ presumably *via* a phospholipase C mechanism (Connor et al., 1994). Thus, it is possible that the lack of an effect by ryanodine on (-)-TAN67-induced antinociception may be due to the differences between ryanodine receptor- and IP_3 receptor-mediated intracellular calcium release.

The detailed mechanisms which underlie this differential modulation of the μ- and δ-opioid receptor agonist-induced antinociception by intracellular calcium are unclear. Welch and Dale Dunlow (1993) reported that the antinociception produced by intrathecal injection of morphine was partially blocked by glyburide, an ATP-gated potassium channel blocker, but not apamin, a calcium-gated potassium channel blocker, whereas that produced by DPDPE was completely reduced by apamin. These results suggest that the antinociception induced by

μ-opioid receptor agonists is mediated by the activation of ATP-gated potassium channels, whereas that induced by δ-opioid receptor agonists is mediated by the activation of calcium-gated potassium channels. Therefore, it is possible that δ-opioid receptor-mediated antinociception may be mediated through the enhancement of intracellular calcium levels. It is likely that the differential modulation of μ- and δ-opioid receptor agonist-induced antinociception by intracellular calcium may be due to the different mechanisms of μ- and δ-opioid receptor-mediated signal transduction. On the other hand, recent study has demonstrated a differential distribution of μ and δ receptors in the rat brain. The μ opioid receptors were detected in some cortical and thalamic nuclei, including the pretectal region, which involved in the central pain-inhibiting system, and δ opioid receptors in cortex and limbic structures (Goodman et al, 1980). Thus, it is possible that there are several supraspinal sites at which the μ- and δ- opioid receptor agonists can induce antinociception. Furthermore, it has been reported that antinociception is produced by microinjection of morphine, a μ-opioid receptor agonist, into a variety of brain sites including the periaqueductal gray (PAG), locus coeruleus, mesencephalic reticular formation and structures within the rostral ventromedial medulla (Jensen and Yaksh, 1986). In contrast to μ-opioid receptor agonists, the brain sites which mediate the antinociception induced by δ-opioid receptor agonists have yet to be identified. Microinjection of DPDPE into either the PAG or the locus coeruleus did not produce antinociception (Bodnar et al., 1988). Thus, it is possible that DAMGO and (-)TAN67 dose not act on the same brain region to produce antinociception. Therefore, it seems likely that differential modulation of μ- and δ-opioid receptor agonist-induced antinociception by intracellular calcium may reflect the differences in the primary sites of action of μ- and δ-opioid receptor agonists.

The antinociceptive effect of DAMGO in diabetic mice is less than that in non-diabetic mice. I observed that agents which increase intracellular calcium, i.e. Ca^{2+} and thapsigargin, did not affect DAMGO-induced antinociception in diabetic mice. Moreover, agents which reduce intracellular calcium levels, i.e. EGTA and ryanodine, significantly potentiated the antinociceptive effect of DAMGO in diabetic mice. However, the effective dose of EGTA for the potentiation of DAMGO-induced antinociception in diabetic mice is greater than that in non-diabetic mice. Thus, it is likely that the attenuation of DAMGO-induced antinociception in diabetic mice may be due to enhanced intracellular calcium levels. The antinociceptive effect of (-)-TAN67 in diabetic mice is greater than that in non-diabetic mice. Moreover, EGTA blocks the antinociceptive effect of (-)-TAN67 in diabetic mice. On

the other hand, calcium does not affect (-)-TAN67-induced antinociception in diabetic mice. These results suggest that the enhancement of (-)-TAN67-induced antinociception in diabetic mice may be due in part to the enhancement of the Ca^{2+} level. It has been reported that chronic excessive intracellular calcium overload might induce cardiac dysfunction in chronic diabetes (Heyliger et al., 1987; Nishio et al., 1990). In peripheral nerves of diabetic rats, mitochondrial and axoplasmic calcium levels were found to be increased by electron-probe X-ray microanalysis (Lowery et al., 1990). Moreover, voltage-dependent calcium currents through L- and N-channels are enhanced in dorsal root ganglion neurons of BB/Wor rats and diabetic mice in vivo (Hall et al., 1995; Kostyuk et al., 1995). These results suggest that the diabetic state may affect $[Ca^{2+}]_i$ in neurons and various tissues. Thus, these results and the present data strongly suggest that the enhancement of δ-opioid receptor agonist-induced antinociception in diabetic mice may be due to increased $[Ca^{2+}]_i$. Furthermore, it has been suggested that the ability of caffeine, a ryanodine receptor agonist, to mobilize Ca^{2+} from intracellular stores is impaired in the diabetic aorta, since caffeine-induced contraction is significantly reduced in diabetic aorta compared with that in control aorta. Moreover, it has been reported that the activity of Ca^{2+}-ATPase is impaired in the diabetic rat (Janicki et al, 1994). In the present study, I observed that the antinociception induced by (-)-TAN67 in diabetic mice, but not in non-diabetic mice, was reduced by pretreatment with ryanodine. Furthermore, i.c.v. pretreatment with thapsigargin, which inhibits Ca^{2+}-ATPase, affected both DAMGO- and (-)-TAN67-induced antinociception in non-diabetic mice, but not in diabetic mice. Therefore, these results strongly suggest that diabetic state may alter intracellular calcium store function. It is possible that the modification of DAMGO- and (-)-TAN67-induced antinociception by diabetes may be due to excessive intracellular calcium overload following changes in calcium store function. In conclusion, the antinociceptive effects of μ- and δ-opioid receptor agonists are modulated differently by intracellular calcium. Furthermore, changes in of μ- and δ-opioid receptor agonist-induced antinociception in diabetic mice may be due to excessive intracellular calcium overload caused by the dysfunction of calcium store function.

Chapter 2

Modification of naloxone-precipitated withdrawal syndrome by diabetes in mice: possible involvement of protein kinase C and intracellular calcium

Introduction

Chronic administration of morphine produces physical dependence, which is exhibited by various specific and vegetative signs after withdrawal of morphine or administration of an opioid antagonist (Bläsic et al., 1973; Wei et al., 1973). The degree of physical dependence on morphine may be assessed by the intensity of the withdrawal signs, or by the amount of naloxone required to precipitate an effect of a given intensity (Way et al., 1968). Morphine withdrawal signs are characterized by the expression of jumping, ptosis, "wet dog" shakes and diarrhea (Way et al., 1968; Way et al., 1969).

Suzuki et al. (1992) previously suggested that μ-opioid receptor subtypes may be differentially modulate some naloxone-precipitated withdrawal signs in morphine-dependent mice, since morphine-dependent CXBK mice, which are known to be selectively deficient in μ_1-opioid receptors, possess a differential sensitivity to naloxone challenge with regard to body weight loss, diarrhea and ptosis and jumping and body shakes (Suzuki et al., 1992a). Furthermore, Kamei et al. (1994a) proposed that mice with diabetes are selectively hyporesponsive to activation of μ_1-opioid receptors, but are normally responsive to activation of μ_2-opioid receptors. Thus, it is possible that μ_1-opioid receptor-mediated naloxone-precipitated withdrawal signs may be selectively reduced in morphine-dependent diabetic mice, as in CXBK mice.

Thus, in Experiment 2-1, I compared the development of morphine dependence in diabetic and non-diabetic mice to clarify our hypothesis that diabetic mice are selectively hyporesponsive to μ_1-opioid receptor-mediated pharmacological action, but not to that mediated by μ_2-opioid receptors.

The effects of opioids on gastrointestinal motor function have been attributed both to action within the central nervous system and to direct action on peripheral receptors within the enteric nervous system (Heyman et al., 1988; Porreca and Burks, 1983; Porreca et al, 1984; Tavani et al, 1980). Pick et al (1991) and Heyman et al (1988) suggested that the antitransit properties of μ-opioid receptor agonists, such as morphine and [D-Ala^2, N-methyl-Phe^4, Gly^5-ol]enkephalin (DAMGO), are mediated mainly by naloxonazine-insensitive (μ_2) opioid receptors, since the gastrointestinal antitransit effect of μ-opioid receptor agonists is antagonized by pretreatment with β-funaltrexamine, but not with naloxonazine. On the other hand, naloxonazine antagonizes μ-opioid receptor-mediated supraspinal antinociception, implying that μ_1-opioid receptors mediate supraspinal antinociception (Heyman et al, 1988;

Paul et al., 1989). We previously reported that the antinociceptive effects of i.c.v., but not i.t., administration of μ-opioid receptor agonists in non-diabetic mice were markedly greater than those in diabetic mice (Kamei et al, 1992a, 1992b, 1994a). Furthermore, in Chapter 1, I indicated that the morphine-induced Straub tail reaction, which is mediated mainly by μ_1-opioid receptors, was greater in non-diabetic mice than in diabetic mice. Therefore, Kamei et al. (1994a) suggested that diabetic mice are selectively hyporesponsive to μ_1-opioid receptor-mediated pharmacological action, but not to that which is mediated by μ_2-opioid receptors. In Experiment 2-2, to examine the hypothesis, I investigated the influence of diabetes on the inhibition of gastrointestinal transit by the s.c. administration of morphine in mice.

The central noradrenergic system has been hypothesized to play an important role in the development of physical dependence on μ-opioid agonists and in the expression of their withdrawal signs (Redmond and Krysatal, 1984). It has been reported that the firing rate of noradrenergic neurons in locus ceruleus (LC), which is a cluster of noradrenaline (NA)-containing cell bodies in the brain, increases during naloxone-precipitated withdrawal from morphine dependence (Aghajanian, 1978). Furthermore, in a biochemical study, the level of 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), the major metabolite of NA, in the cerebral cortex which projects from the LC, increased following naloxone injection in morphine-dependent rats (Crawley et al., 1979). These biochemical changes and various withdrawal signs were blocked by clonidine, an α_2-agonist (Crawley et al., 1979; Tseng et al., 1975; Laverty and Roth, 1980). Thus, these reports suggest that the central noradrenergic system may play a significant role in morphine withdrawal.

The aim of Experiment 2-3 was to investigate the role of noradrenergic systems in the modulation of naloxone-precipitated morphine withdrawal by diabetes. The effects of diabetes on naloxone-precipitated jumping and naloxone-induced changes in NA turnover were evaluated in morphine-dependent mice.

The cellular and molecular bases of opiate addiction remain largely unknown (Cox, 1990; Nestler, 1992). The sensitization of the adenylate cyclase/ cyclic AMP/ cyclic AMP-dependent protein kinase (PKA) transduction system has been demonstrated to be robust and to show a consistent compensatory response to the sustained inhibition of opioid receptors. It has been reported that the acute administration of opiate receptor agonists induces the inhibition of adenylate cyclase through guanine nucleotide-binding Gi/o proteins and decreases the basal production of cyclic AMP (Nestler et al., 1989). In contrast, chronic treatment with these drugs results in up-regulation of this transduction system. Indeed, it has

been reported that chronic treatment with morphine increases both basal and stimulated production of cyclic AMP (Sharma et al., 1975, García-Sevilla et al., 1987) and specific G protein and their messenger (m)RNAs (Nestler et al., 1993; Escribá et al., 1994; Parolaro et al., 1993). Moreover, chronic treatment with morphine also enhances the activity of PKA (Nestler et al., 1988, Terwilliger et al., 1991) and increases the abundance of adenylate cyclase mRNA (Matsuoka et al., 1994). Therefore, up-regulation of the adenylate cyclase/ cyclic AMP transduction system is currently the best-characterized potential mechanism for opiate tolerance and dependence (Nestler, 1992; Nestler et al., 1989; Collier and Roy, 1974; Collier, 1980).

Several recent studies have indicated that protein kinase C (PKC) is involved in opiate tolerance and dependence (Mayer et al., 1995; Narita et al., 1994; Smart et al., 1996b). Pretreatment with an inhibitor of serine/threonine kinase (H7) inhibited the development of tolerance to supraspinal antinociception induced by opiates in rats (Narita et al., 1994). Recently, Maldonado et al., (1995) reported that the behavioral expression of naloxone-precipitated withdrawal syndromes was strongly attenuated by the administration of H7 into the locus coeruleus or into the periaqueductal gray matter. It has also been reported that i.c.v. pretreatment with H7 reduced naloxone-precipitated jumping in acutely morphine-dependent mice (Bilsky et al., 1996). Furthermore, a marked decrease (50 %) in the immunoreactivity of PKC-α and β isoforms (cytosolic and membrane-bound isoenzymes) has recently been found in postmortem brains of heroin addicts and in brains of morphine-dependent rats (Busques et al., 1995). Moreover, it has been reported that both PKA and PKC were markedly activated in chronically morphine-treated rats (Makimura et al., 1997). These findings suggest that the PKC system may also play a major role in opiate addiction.

Many investigators have reported that hyperglycemia or elevated glucose levels can increase diacylglycerol (DAG) levels and activate PKC in vascular tissue, cardiac tissues or cultured cells (Craven and De Rubertis, 1989; King et al., 1990; Inoguchi et al., 1992; Tanaka et al., 1991). Activation of the DAG-PKC cellular signal pathway is linked to vasculature dysfunction in diabetes (Craven and De Rubertis, 1989; Wolf et al., 1990; Shiba et al., 1993). Furthermore, in Chapter 1, I indicated that calphostin C, a protein kinase C inhibitor, reverses the attenuation of DAMGO-induced antinociception in diabetic mice to the level in non-diabetic mice. In contrast to PKC, it has been reported that the hepatic PKA activity measured both in the absence and presence of cyclic AMP is decreased in streptozotocin-induced diabetic animals (Khandelwal et al., 1977; Davies et al., 1995). Decreased PKA activity is

caused by decreased phosphorylation of the cyclic AMP response element binding protein in the diabetic rat (Davies et al., 1995). The induction of physical dependence on morphine was significantly decreased in genetically diabetic mice (Shook et al., 1986). Although the mechanisms of this modulation of the naloxone-precipitated jumping in the morphine-dependent mice by diabetes are not yet clear, it is possible that functional changes in protein kinase activity may be involved.

Therefore, the primary aim of Experiment 2-4 was to investigate the involvement of PKC and PKA in the modulation of naloxone-precipitated morphine withdrawal in mice. Furthermore, I examined the involvement of PKC and PKA on the naloxone-induced enhancement of NA turnover in the frontal cortex of morphine-dependent mice. Moreover, to evaluate the modification of naloxone-precipitated jumping in morphine-dependent mice by diabetes, the effect of protein kinase inhibitors on the expression of naloxone-precipitated morphine withdrawal and naloxone-induced enhancement of NA turnover in the frontal cortex were evaluated in morphine-dependent diabetic and non-diabetic mice.

Many acute opiate effects can be partially explained by the inhibition of Ca^{2+} ion flux in neuronal cells (Guerrero-Muñoz et al., 1979) and by a reduction in synaptosomal Ca^{2+} content (Ross et al., 1977). It has been reported that intracisternal administration of Ca^{2+} and Ca^{2+} ionophores antagonizes the antinociceptive effect of morphine, a prototype μ-opioid receptor agonist (Chapman et al., 1980; Hano et al., 1964). Conversely, Ca^{2+} chelators (i.e., ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid (EGTA)) or Ca^{2+} channel antagonists of the verapamil, diltiazem and dihydropyridine types potentiate opioid antinociception (Ben-Sreti et al., 1983; Hoffmeister and Tettenborn, 1986). Ca^{2+} seems to play a basic role not only in the acute effects of opiates but also in the development of tolerance and physical dependence. Chronic morphine administration has been shown to produce, as a contra-adaptive reaction, an increase in Ca^{2+} uptake in various brain preparations, resulting in an increase of vesicular Ca^{2+} content (Yamamoto et al., 1978). Furthermore, it is reported that N- and L-type Ca^{2+} channel blockers prevent naloxone-precipitated withdrawal syndromes in morphine-dependent mice and rats (Barrios and Baeyens, 1991; Basilico et al., 1992; Bourreau et al., 1996). In contrast to Ca^{2+} channel blockers, L-type Ca^{2+} channel stimulators increased naloxone-precipitated withdrawal signs in acute morphine-dependent mice (Barrios and Baeyens, 1991). Thus, it is possible that influx of calcium through the L- and N-type Ca^{2+} channels plays an important role in the expression of naloxone-precipitated withdrawal signs in morphine-dependent animals.

Previously, it has been reported that metabolic glutamate receptor antagonist attenuates naloxone-precipitated withdrawal signs in rats given chronic subcutaneous morphine (Fundytus and Coderre, 1994). Activation of metabolic glutamate receptors stimulates phospholipase C which hydrolyzes phosphatidyl inostol to produce diacylglycerol and inositol-1,4,5-trisphosphate (IP_3) (Schoepp and Conn, 1993). IP_3 promotes the release of Ca^{2+} from internal stores on the endoplasmic reticulum. Furthermore, it has been reported that during morphine withdrawal, phosphatidyl inositol turnover has been found be greatly enhanced (Pellegrini-Giampietro et al., 1988). Thus, these reports suggest that enhancement of the release of Ca^{2+} from internal stores may be involved in the expression of naloxone-precipitated withdrawal signs in morphine-dependent animals.

It has been reported that veratrine, which contains a Na^+ channel activator, veratridine, could release NA from brain synaptosomes even in a Ca^{2+} free medium, possibly as a result of mobilization of intracellular stores (Schoepp and Conn, 1993). Furthermore, ryanodine induced a modest decrease in NA overflow, whereas cyclopiazonic acid, an inhibitor of sacroplasmic reticulum Ca^{2+}-ATPase, slightly increased NA overflow in rat vas deferens (Bourreau, 1996). These results suggest that internal Ca^{2+} stores could participate in the process of NA release. Thus, it is possible that the naloxone-induced enhancement of NA turnover in the frontal cortex modulated by enhancement of intracellular $[Ca^{2+}]_i$ followed by the release of Ca^{2+} from intracellular Ca^{2+} stores.

Considerable evidences suggest that calcium signaling is abnormal in cardiac myocytes (Nobe et al., 1990), vascular smooth muscle (Kamata et al., 1988) and other tissues (Levy et al., 1994) from diabetic animals. A recent study has shown that verapamil has a beneficial effect on the cardiac function of diabetic rats without affecting glucose metabolism or insulin secretion (Afzal et al., 1988). It has been suggested that chronic excessive intracellular calcium overload might induce cardiac dysfunction in chronic diabetes (Heyliger et al., 1987; Nishio et al., 1990). Moreover, it has been suggested that the diabetic state may change $[Ca^{2+}]_i$ in neuron and various tissues (Hall et al., 1995; Kostyuk et al., 1995; Lowery et al., 1990). Furthermore, in Chapter 1-4, changes in μ- and δ-opioid receptor agonist-induced antinociception in diabetic mice may be due to excessive intracellular calcium overload caused by the dysfunction of calcium store function. Therefore, it is possible that the dysfunction of calcium store function in diabetic mice may affect the naloxone-precipitated withdrawal jumping in morphine-dependent diabetic mice. Thus, the primary aim of Experiment 2-5 was to investigate the involvement of intracellular calcium in the modulation

of naloxone-precipitated morphine withdrawal in mice. Furthermore, I examined the role of intracellular calcium on the naloxone-induced enhancement of NA turnover in the frontal cortex of morphine-dependent mice. Moreover, to evaluate the modification of naloxone-precipitated jumping in morphine-dependent mice by diabetes, the effects of intracellular calcium modulators on the expression of naloxone-precipitated morphine withdrawal and naloxone-induced enhancement of NA turnover in the frontal cortex were evaluated in morphine-dependent diabetic and non-diabetic mice.

Experiment 2-1: Modification of development of morphine dependence by diabetes

Materials and Methods

Animals

Male ICR mice (Tokyo Laboratory Animals Science Co., Tokyo), weighing about 20 g at the beginning of the experiments, were used. The mice were housed under the condition as described in Chapter 1-1. Animals were rendered diabetic by an injection of streptozotocin (200 mg/kg, i.v.) prepared in 0.1 N citrate buffer at pH 4.5. Age-matched non-diabetic mice were injected with vehicle alone. The experiments were conducted two weeks after injection of streptozotocin or vehicle. Mice with serum glucose levels above 400 mg/dl were considered diabetic.

Chronic administration of morphine

Morphine was injected s.c. daily at 9 a.m. and 7 p.m. According to the schedule described by Maldonado et al. (1989), the morphine dose was increased progressively from 8 to 45 mg/kg over a period of 5 days. The doses of morphine (mg/kg) injected at 9 a.m. and 7 p.m. were: 1st day (8, 15), 2nd day (20, 25), 3rd day (30, 35), 4th day (40, 45) and 5th day (45 at 9 a.m. only), respectively.

Morphine withdrawal

Withdrawal signs were precipitated by injecting naloxone (0.3, 1, 3 and 10 mg/kg, s.c. in a volume of 10 ml/kg) 2 h after the final morphine administration. After the naloxone challenge, mice were immediately placed on a circular platform (30 cm diameter x 70 cm height). Naloxone-precipitated withdrawal signs were recorded for 60 min according to our previous papers (Suzuki et al., 1984, 1992a, 1992b). The number of mice which expressed withdrawal jumping was counted for 15 min after the naloxone injection. The numbers of mice which expressed other withdrawal signs, such as ptosis, diarrhea, and body shakes, within 60 min after naloxone injection were also recorded. Body weight was measured before and 15, 30, 45 and 60 min after naloxone injection.

Drugs

The drugs used in the present study were streptozotocin (Sigma Chemical Co., St.

Louis, MO, USA), morphine hydrochloride (Sankyo Co., Tokyo Japan) and naloxone hydrochloride (Research Biochemicals Inc., Wayland, MA, USA). All drugs were dissolved in saline solution.

Statistical analysis

Differences in weight loss were analyzed using one-way analysis of variance followed by Dunnett's multiple comparison test. The ED50 values, the ED50 ratio and their 95 % confidence intervals for naloxone-precipitated withdrawal signs were computed according to Litchfield and Wilcoxon (1949) using Program 47 of the Pharmacological Calculations system of Tallarida and Murray (1987).

Results

Naloxone-precipitated withdrawal signs in morphine dependent diabetic mice.

Naloxone-precipitated jumping tended to increase in a dose-related manner in morphine-dependent non-diabetic and diabetic mice (Fig. 2-1). However, diabetes reduced the number of animals respond to naloxone-precipitated jumping to half.

Naloxone-precipitated body shakes also tended to increase in a dose-related manner in morphine-dependent non-diabetic and diabetic mice (Fig. 2-2). Diabetes produced a rightward shift of the dose response curve of the naloxone-precipitated body shakes in morphine-dependent mice. Thus, diabetes reduced the number of animals respond to naloxone-precipitated body shakes to about half.

There was no significant difference in the incidence of naloxone-induced diarrhea between morphine-dependent diabetic mice and morphine-dependent non-diabetic mice (Fig. 2-3A). The loss of body weight induced by naloxone increased in a dose-related manner in morphine-dependent non-diabetic and diabetic mice. However, the naloxone-induced body weight loss in diabetic mice was significantly greater than that in non-diabetic mice 60 min after administration of naloxone (Fig. 2-4). Furthermore, the loss of body weight 60 min after administration of naloxone (3 mg/kg, s.c.) in chronically saline-treated (morphine-non-dependent) diabetic mice (7.9 ± 1.1 %, n=10) was also significantly ($P<0.01$) greater than that in chronically saline-treated (morphine-non-dependent) non-diabetic mice (2.4 ± 0.2 %, n=10).

The incidence of ptosis induced by naloxone in morphine-dependent diabetic mice was similar to that in morphine-dependent non-diabetic mice (Fig. 2-5B).

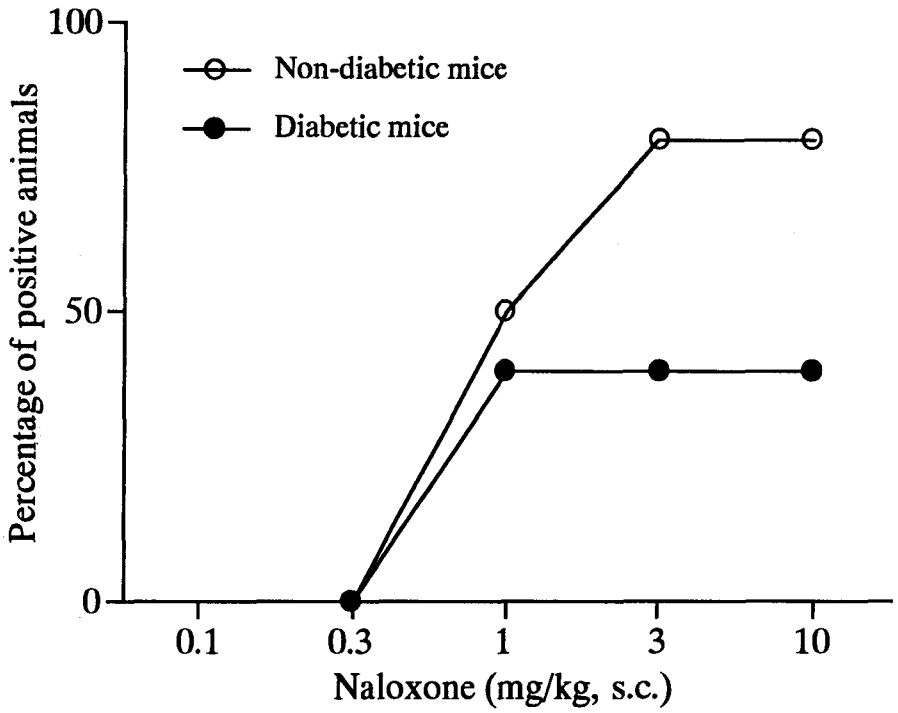

Figure 2-1. Naloxone-precipitated jumping in morphine-dependent diabetic and non-diabetic mice. Diabetic and non-diabetic mice were treated with morphine (8 - 45 mg/kg, s.c.) twice a day for 5 days. Withdrawal jumping was observed for 15 min after administration of naloxone (s.c.). Each point represents the number of animals responding within 10 animals.

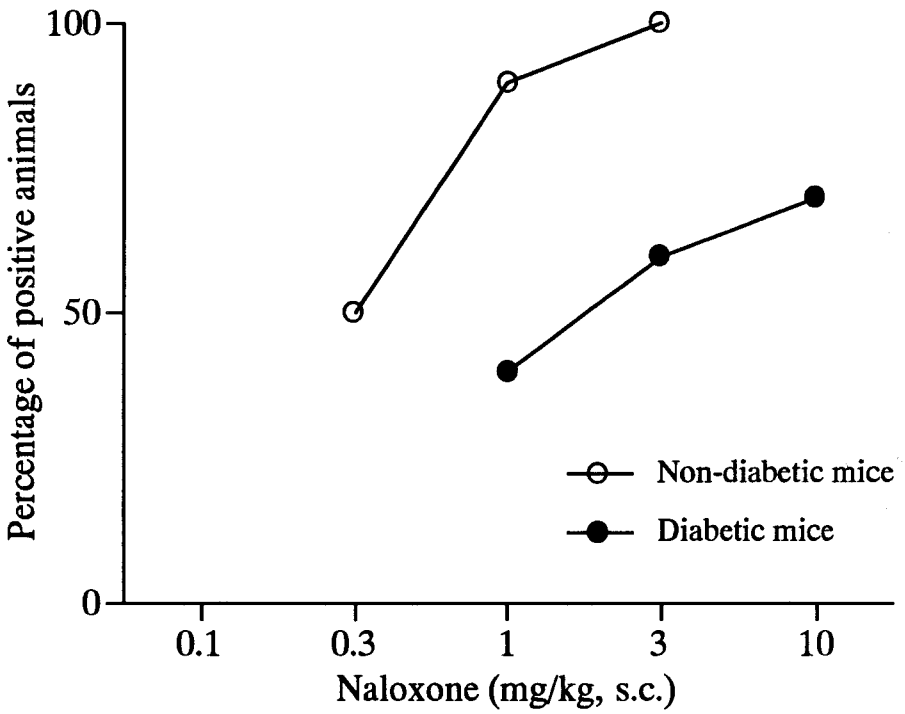

Figure 2-2. Naloxone-precipitated body shakes in morphine-dependent diabetic and non-diabetic mice. Diabetic and non-diabetic mice were treated with morphine (8 - 45 mg/kg, s.c.) twice a day for 5 days. Withdrawal body shakes was observed for 60 min after administration of naloxone (s.c.). Each point represents the number of animals responding within 10 animals.

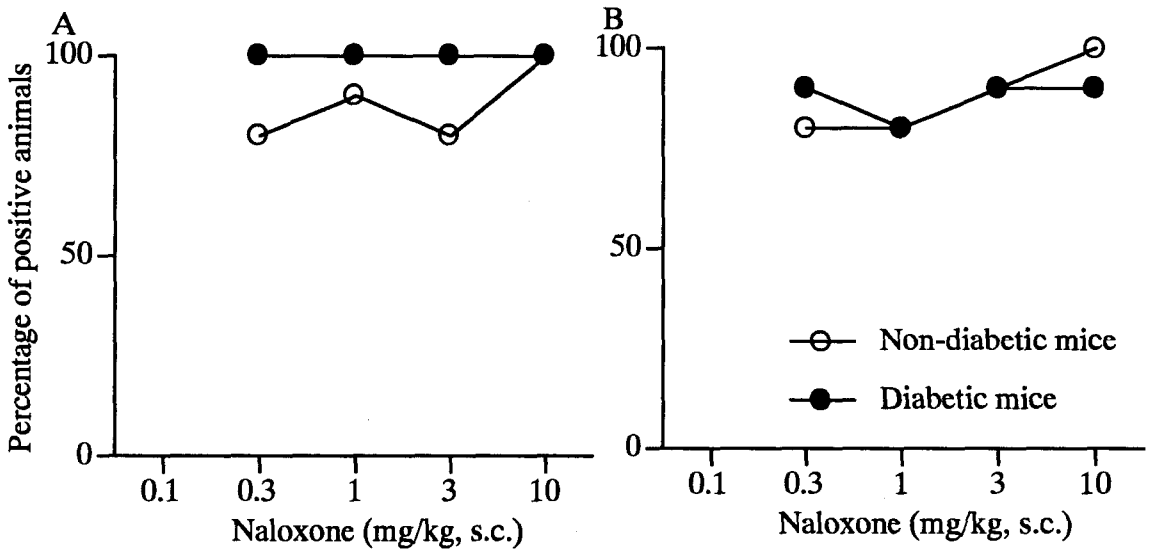

Figure 2-3. Naloxone-precipitated diarrhea (A) and ptosis (B) in morphine-dependent diabetic and non-diabetic mice. Diabetic and non-diabetic mice were treated with morphine (8 - 45 mg/kg, s.c.) twice a day for 5 days. Withdrawal signs were observed for 60 min after administration of naloxone (s.c.). Each point represents the number of animals responding within 10 animals.

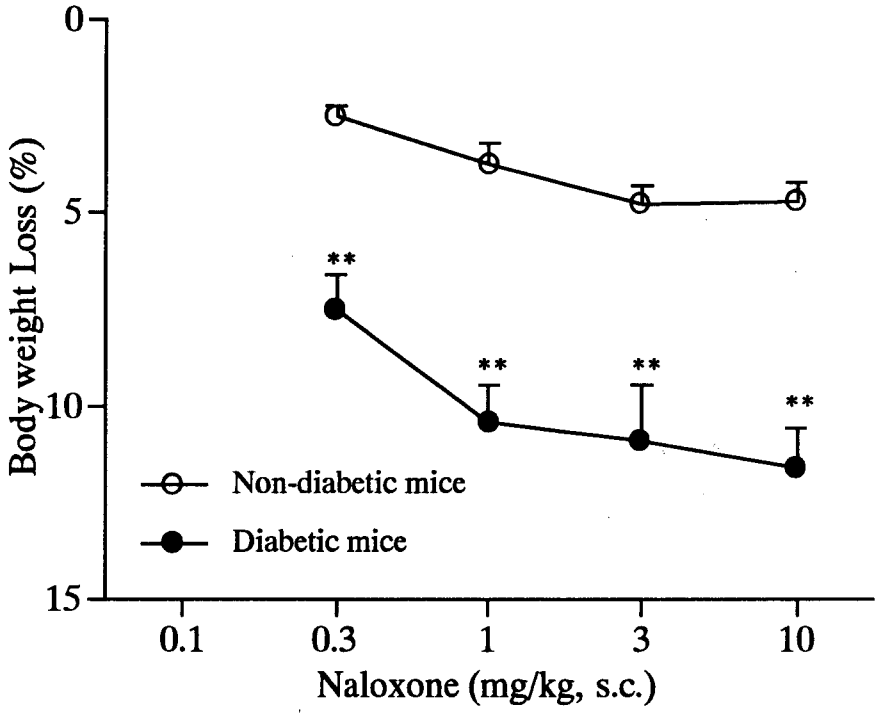

Figure 2-4. Body weight loss 60 min after naloxone challenge in morphine-dependent diabetic and non-diabetic mice. Diabetic and non-diabetic mice were treated with morphine (8 - 45 mg/kg, s.c.) twice a day for 5 days. Withdrawal was precipitated by naloxone (s.c.). Each point represents the number of animals responding within 10 animals.

Experiment 2-2: Effect of diabetes on the morphine-induced inhibition of gastrointestinal transit.

Materials and Methods

Animals

Male ICR mice (Tokyo Laboratory Animals Science Co., Tokyo), weighing about 20 g at the beginning of the experiments, were used. The mice were housed under the condition as described in Chapter 1-1. Animals were rendered diabetic by an injection of streptozotocin (200 mg/kg, i.v.) prepared in 0.1 N citrate buffer at pH 4.5. Age-matched non-diabetic mice were injected with the vehicle alone. The experiments were conducted 2 weeks after injection of streptozotocin or vehicle. Mice with serum glucose levels above 400 mg/dl were considered diabetic.

Gastrointestinal transit

Mice were fasted for 12 h before the experiments. Thirty min after s.c. administration of morphine, or saline, a 5% aqueous suspension of charcoal in 10% gum arabic solution was administered p.o. at a volume of 0.1 ml/mouse. Thirty minutes after charcoal administration, the animal was killed by cervical dislocation and its small intestine was removed. The small intestine was placed on a ruled template and both the length from the pylorus to the cecum and the distance traveled by the charcoal were measured. The % transit was calculated as: 100 x (length from the pylorus to the cecum - distance traveled by the charcoal)/length from the pylorus to the cecum. To calculate the antitransit effect of morphine, % transit for each animal was compared with the mean % transit in the saline-treated group according to the formula: percent inhibition = 100 x (mean % transit of saline-treated mice - % transit of morphine-treated mouse)/mean % transit of saline-treated control mice.

Data analysis

Data are expressed as the mean ± S.E. Statistical significance of differences was assessed with a one-way analysis of variance (ANOVA) with repeated measures followed by a Dunnett's test. A level of probability of 0.05 or less was accepted as significant. The ED50 values and their 95% confidence intervals (95% CI) for the antitransit effect of morphine were computed according to Litchfield and Wilcoxon (1949) using Program 47 of the

Pharmacological Calculations system of Tallarida and Murray (1987).

Drugs

Streptozotocin was purchased from Sigma Chemical Co., St. Louis, MO, USA. Morphine hydrochloride was purchased from Sankyo, Co., Tokyo, Japan. β-Funaltrexamine and naloxonazine were synthesized by Dr. Nagase (Toray Industries, Kamakura, Japan). All drugs were dissolved in 0.9% saline solution. β-Funaltrexamine (40 mg/kg) and naloxonazine (35 mg/kg) were injected s.c. 24 hr before testing.

Results

Subcutaneous injection of morphine (1-10 mg/kg) dose-dependently inhibited gastrointestinal transit in both non-diabetic and diabetic mice (Fig 2-6). The ED50 values (95% CI) for the antitransit effect of morphine were 1.3 (1.0 - 1.8) mg/kg in non-diabetic mice and 2.4 (0.9 - 6.0) mg/kg in diabetic mice. There was no significant difference in the antitransit properties of morphine between non-diabetic and diabetic mice.

When the mice were pretreated with β-funaltrexamine (40 mg/kg, s.c.), the antitransit effects of morphine (10 mg/kg, s.c.) were significantly reduced in both non-diabetic (Fig. 2-7A) and diabetic (Fig. 2-7B) mice. In contrast, pretreatment with the selective μ_1-opioid receptor antagonist naloxonazine (35 mg/kg, s.c.) 24 hr before testing failed to antagonize the morphine (10 mg/kg, s.c.)-induced inhibition of gastrointestinal transit in both non-diabetic and diabetic mice.

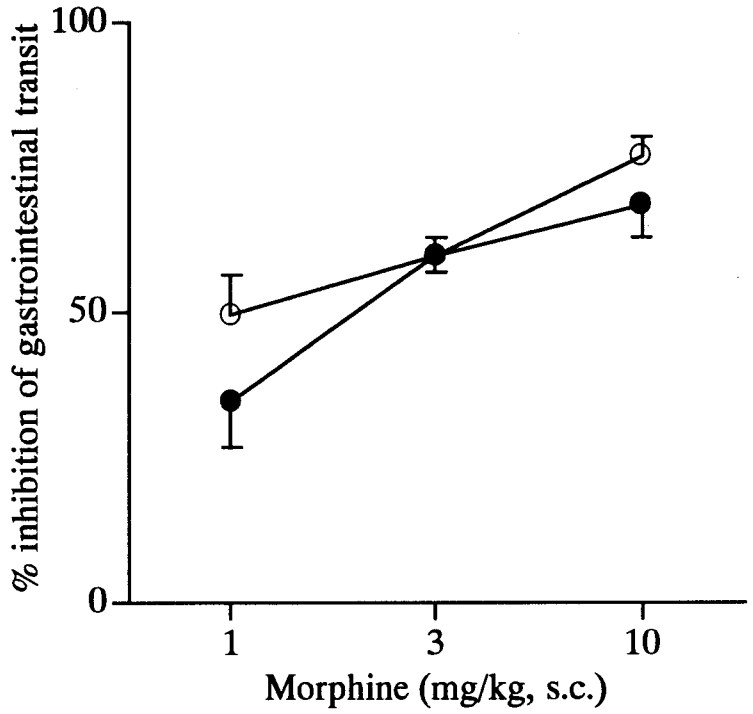

Figure 2-5. Dose-response curves for the antigastrointestinal transit effect of morphine in non-diabetic (open circles) and diabetic (closed circles) mice. Each point represents the mean with S.E. from 8 animals.

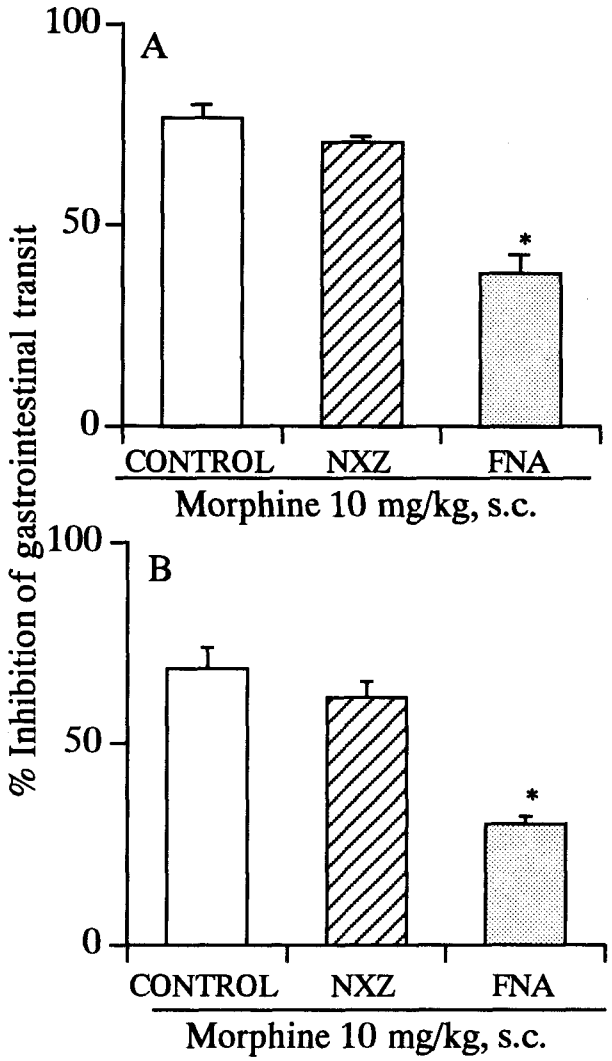

Figure 2-6. Effects of β-funaltrexamine (FNA) and naloxonazine (NXZ) on the antitransit effects of morphine (10 mg/kg, s.c.) in both non-diabetic (A) and diabetic (B) mice. Mice were treated with FNA (40 mg/kg, s.c.) or NXZ (35 mg/kg, s.c.) 24 h before testing. Each column represents the mean with S.E. from 8 animals. *P<0.01 vs. morphine alone.

Experiment 2-3: Role of noradrenergic functions in the modification of naloxone-precipitated withdrawal jumping in morphine-dependent mice by diabetes

Materials and Methods

Animals

Male ICR mice (Tokyo Laboratory Animals Science Co., Tokyo), weighing about 20 g at the beginning of the experiments, were used. The mice were housed under the condition as described in Chapter 1-1. Animals were rendered diabetic by an injection of streptozotocin (200 mg/kg, i.v.) prepared in 0.1 N citrate buffer at pH 4.5. Age-matched non-diabetic mice were injected with vehicle alone. The experiments were conducted 2 weeks after injection of streptozotocin or vehicle. Mice with serum glucose levels above 400 mg/dl were considered diabetic.

Chronic administration of morphine

Morphine was injected s.c. daily at 9 A.M. and 7 P.M. According to the schedule described in Chapter 2-1, the morphine dose was increased progressively from 8 to 45 mg/kg over a period of 5 days.

Morphine withdrawal

Withdrawal signs were precipitated by injecting naloxone (0.3 mg/kg, s.c. in a volume of 10 ml/kg) 2 hr after the final morphine administration. After the naloxone challenge, mice were immediately placed in an acrylic cylinder (30 cm high, 20 cm in diameter). The number of jumps during each 5-min period was counted for 15 min after naloxone injection.

Neurochemical analysis

The concentrations of noradrenaline (NA) and 3-methoxy-4-hydroxyphenyleneglycol (MHPG) were estimated using high-performance liquid chromatography (HPLC) with electrochemical detection (ECD), as previously described (12). Non-dependent (chronically saline-treated) mice (diabetic and non-diabetic) and morphine-dependent mice (diabetic and non-diabetic) were sacrificed 5, 10 and 15 min after naloxone (0.3 mg/kg, s.c.) or saline (10 ml/kg, s.c.) injection, and then immersed in a dry ice-ethanol solution. The brain was quickly

removed and the cerebral cortex was dissected on an ice-cold glass plate. The HPLC system consisted of a delivery pump (EP-300, Eicom Co., Japan), an analytical column (EICOMPAC, MA-50DS, Eicom Co., Japan) and a guard column (Eicom, Japan). The electrochemical detector (EC-100, Eicom, Japan) included a graphite electrode (WE-3G, Eicom, Japan).

Drugs

The drugs used were streptozotocin (Sigma Chemical Co., St. Louis, MO), morphine hydrochloride (Sankyo Co., Tokyo, Japan) and naloxone hydrochloride (Sigma Chemical Co., St. Louis, MO). All drugs were dissolved in 0.9% saline solution.

Statistical analysis

Differences in the number of jumps and NA turnover were analyzed using a one-way analysis of variance followed by Dunnett's multiple comparison test. NA turnover was determined as the NA ratio: NA ratio = MHPG (ng/g of wet tissue)/NA (ng/g of wet tissue).

Results

Naloxone-precipitated withdrawal jumping in morphine-dependent diabetic mice

The time-course of naloxone-precipitated jumping in morphine-dependent diabetic and non-diabetic mice is shown in Figure 2-8. Subcutaneous injection of naloxone (0.3 mg/kg) invariably provoked withdrawal jumping within 2 to 15 min in morphine-dependent non-diabetic mice and reached its maximum within 5 min after naloxone injection. In morphine-dependent diabetic mice, s.c. injection of naloxone (0.3 mg/kg) also provoked withdrawal jumping within 15 min. However, the number of naloxone-precipitated withdrawal jumps within 5 min after naloxone challenge was significantly less in morphine-dependent diabetic mice than in morphine-dependent non-diabetic mice (Fig. 2-8).

Effects of diabetes on naloxone-induced changes in the level of NA turnover in the frontal cortex

The level of NA turnover in the frontal cortex was significantly lower in diabetic mice than in non-diabetic mice (Fig. 2-9A, B, C). Chronic treatment with morphine did not significantly modify the level of NA turnover in either non-diabetic or diabetic mice. Furthermore, the level of NA turnover did not significantly change after saline challenge in either morphine-dependent non-diabetic or morphine-dependent diabetic mice. As shown in Figure 2-9A and B, the level of NA turnover in frontal cortex in morphine-dependent non-diabetic mice was significantly increased 5 and 10 min after naloxone (0.3 mg/kg, s.c.) injection, compared with that in the saline-challenged morphine-dependent group. Although the level of NA turnover in frontal cortex in morphine-dependent diabetic mice was significantly increased 10 min after naloxone challenge, a naloxone-induced increased in the NA turnover was not observed 5 min after naloxone challenge (Fig. 2-9 A, B).

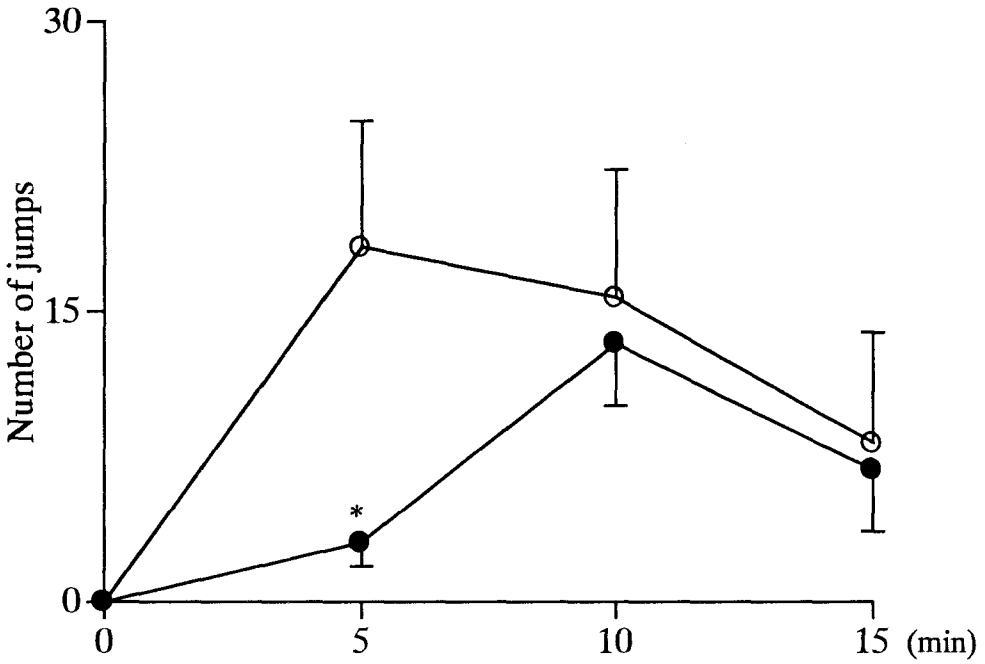

Figure 2-8. Time course of naloxone precipitated jumping in morphine-dependent diabetic (closed circle) and non-diabetic (open circle) mice. Withdrawal was precipitated by naloxone (0.3 mg/kg, s.c.), and the number of jumps during each 5-min period was counted for 15 min after naloxone injection. Each point represents the mean with S.E. of 10 mice. *P<0.05 vs. non-diabetic mice

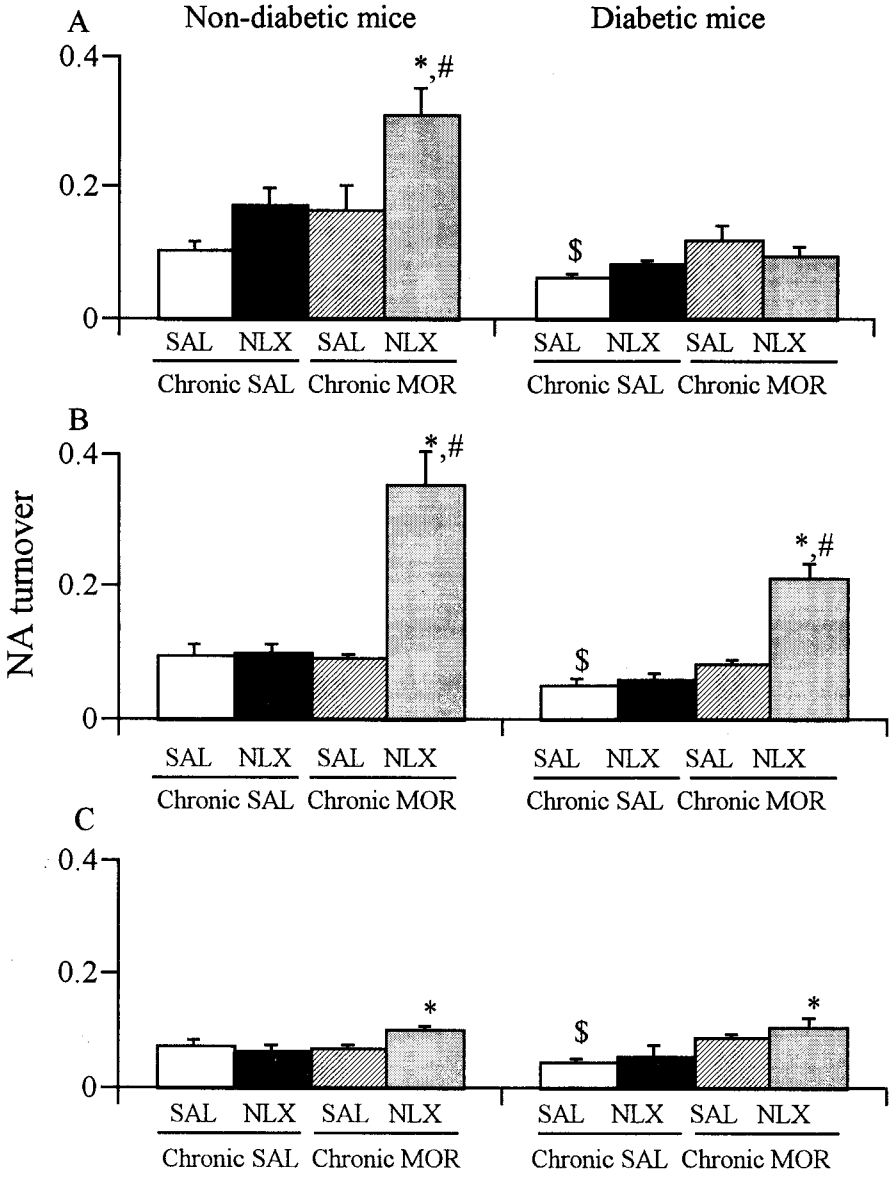

Figure 2-9. Naloxone-precipitated changes in the rate of noradrenaline turnover (NA ratio) in the cortex of diabetic and non-diabetic mice after chronic morphine (MOR) treatment. Mice were sacrificed 5 (A), 10 (B) and 15 (C) min after s.c. injection of naloxone (NLX) or saline (SAL). Each group consisted 6 mice. NA turnover was determined as the NA ratio. NA ratio = MHPG/NA. $ $P<0.05$ vs. respective non-diabetic mice. * $P<0.05$ vs. respective saline (SAL)-challenged chronic saline (SAL)-treated group. # $P<0.05$ vs. respective saline (SAL)-challenged chronic morphine-treated group.

Experiment 2-4: Modification of the expression of naloxone-precipitated withdrawal signs in morphine-dependent mice by diabetes: possible involvement of protein kinase C.

Materials and Methods

Animals

Male ICR mice (Tokyo Laboratory Animals Science Co., Tokyo), weighing about 20 g at the beginning of the experiments, were used. The mice were housed under the condition as described in Chapter 1-1. Animals were rendered diabetic by an injection of streptozotocin (200 mg/kg, i.v.) prepared in 0.1 N citrate buffer at pH 4.5. Age-matched non-diabetic mice were injected with vehicle alone. The experiments were conducted 2 weeks after the injection of streptozotocin or vehicle. Mice with serum glucose levels above 400 mg/dl were considered diabetic.

Chronic administration of morphine

Morphine was injected s.c. daily at 9 A.M. and 7 P.M. According to the schedule described in Experiment 2-1, the morphine dose was increased progressively from 8 to 45 mg/kg over a period of 5 days.

Morphine withdrawal

Withdrawal signs were precipitated by injecting naloxone (0.3 mg/kg, s.c. in a volume of 10 ml/kg) 2 h after the final morphine administration. After the naloxone challenge, mice were immediately placed in acrylic cylinder (30 cm high, 12 cm in diameter). The number of jumps during a 5-min period was counted for 30 min after naloxone injection.

Neurochemical analysis

The concentrations of noradrenaline (NA) and 3-methoxy-4-hydroxyphenyleneglycol (MHPG) were estimated using high-performance liquid chromatography (HPLC) with electrochemical detection (ECD), as previously described (34). Morphine-dependent diabetic and non-diabetic mice were sacrificed 5 min after naloxone (0.3 mg/kg, s.c.) or saline (10 ml/kg, s.c.) injection, then immersed in a dry ice-cold solution. The brain was quickly removed and the cerebral cortex was dissected on an ice-cold glass plate. The HPLC system

consisted of a delivery pump (EP-300, Eicom Co., Japan), an analytical column (EICOMPAC MA-50DS, Eicom Co., Japan) and a guard column (Eicom, Co., Japan). The electrochemical detector (EC-100, Eicom Co., Japan) included a graphite electrode (WE-3G, Eicom Co., Japan).

Intracerebroventricular injection

Intracerebroventricular (i.c.v.) administration was performed following the method described in Chapter 1-2.

Drugs

The drugs used were streptozotocin (Sigma Chemical Co., St. Louis, MO), morphine hydrochloride (Sankyo Co., Tokyo, Japan), and naloxone hydrochloride (Sigma Chemical Co., St. Louis, MO). Calphostin C, phorbol-12,13-dibutyrate (PDBu) and (8R,9S,11S)-(-)-9-hydroxy-9-n-hexyloxy-carbonyl-8-methyl-2,3,9,20-tetrahydro-8-,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo[a,g]cycloocta[cde]-trinden-1-one (KT5720) were purchased from Calbiochem-Novabiochem International (San Diego, CA). Calphostin C and KT-5720 were injected 1hr before the injection of naloxone. PDBu was injected 1hr before the last injection of morphine. The dose and schedule for calphostin C, PDBu and KT-5720 in this study were determined as described previously (Narita et al., 1997).

Data analysis

The data are expressed as means ± S.E. The statistical significance of differences between groups was assessed with Student's t-test (comparison of two groups) or an analysis of variance (ANOVA) followed by Dunnet's test (comparison among multiple groups). NA turnover was determined as the NA ratio: NA ratio=MHPG (ng/g of wet tissue) / NA (ng/g of wet tissue).

Results

Influence of a protein kinase C (PKC) inhibitor, calphostin C, on naloxone-precipitated withdrawal jumping

The effect of calphostin C, a PKC inhibitor, on the number of naloxone-precipitated withdrawal jumps in morphine-dependent diabetic and non-diabetic mice is shown in fig. 2-10. Naloxone-precipitated withdrawal jumps were markedly attenuated by the i.c.v. administration of calphostin C (20 pmol) in morphine-dependent non-diabetic mice. However, naloxone-precipitated withdrawal jumping in morphine-dependent diabetic mice was increased by i.c.v. pretreatment with calphostin C (20 pmol). As shown in fig. 2-11, the number of naloxone-precipitated withdrawal jumps within 5 min after naloxone challenge was dose-dependently attenuated by i.c.v. pretreatment with calphostin C (3-20 pmol) in morphine-dependent non-diabetic mice. However, the number of naloxone-precipitated withdrawal jumps in morphine-dependent diabetic mice was dose-dependently enhanced by i.c.v. pretreatment with calphostin C (10 and 20 pmol).

Influence of a cyclic AMP-dependent protein kinase (PKA) inhibitor, KT-5720 on naloxone-precipitated withdrawal jumping

The effect of KT-5720, a PKA inhibitor, on the number of naloxone-precipitated withdrawal jumps in morphine-dependent diabetic and non-diabetic mice is shown in fig. 2-12. In morphine-dependent non-diabetic mice, naloxone-precipitated withdrawal jumping within 5 and 10 min after naloxone challenge was attenuated by i.c.v. pretreatment with KT-5720. However, i.c.v. pretreatment with KT-5720 did not affect naloxone-precipitated withdrawal jumping in morphine-dependent diabetic mice. As shown in fig. 2-13, the number of naloxone-precipitated withdrawal jumps within 5 min after naloxone challenge in morphine-dependent non-diabetic mice, but not in morphine-dependent diabetic mice, was dose-dependently attenuated by i.c.v. pretreatment with KT5720 (10 and 20 pmol).

Influence of a PKC activator, phorbol-12,13-dibutyrate (PDBu), on the expression of naloxone-precipitated withdrawal jumping in morphine-dependent diabetic and non-diabetic mice

The effect of phorbol-12,13-dibutyrate (PDBu), a PKC activator, on the number of

naloxone-precipitated withdrawal jumps in morphine-dependent diabetic and non-diabetic mice is shown in fig. 2-14. In morphine-dependent non-diabetic mice, but not in morphine-dependent diabetic mice, naloxone-precipitated withdrawal jumping within 5 min after naloxone challenge was attenuated by i.c.v. pretreatment with PDBu (10 pmol). As shown in fig. 2-15, i.c.v. pretreatment with PDBu (3 and 10 pmol) 3 h before naloxone injection dose-dependently attenuated naloxone-precipitated withdrawal jumping within 5 min after naloxone injection. However, i.c.v. pretreatment with PDBu (3 and 10 pmol) had no significant effect on naloxone-precipitated withdrawal jumping within 5 min after naloxone injection in morphine-dependent diabetic mice.

Influences of protein kinase C and A modulator on naloxone-induced changes in the level of NA turnover in the frontal cortex

The level of NA turnover in the frontal cortex was significantly lower in morphine-dependent diabetic mice than in morphine-dependent non-diabetic mice (Fig. 2-16A and B). The level of NA turnover in frontal cortex in morphine-dependent non-diabetic mice was significantly increased 5 min after naloxone challenge, compared with that in the saline-challenged morphine-dependent non-diabetic mice (Fig 2-16A and B). However, a naloxone-induced increase in the NA turnover was not observed 5 min after naloxone challenge in morphine-dependent diabetic mice (Fig. 2-16A and B). As shown in fig. 2-16A, a naloxone-induced increased in the NA turnover was attenuated by pretreatment with calphostin C (20 pmol, i.c.v.) and KT5720 (20 pmol, i.c.v.) in morphine-dependent non-diabetic mice (Fig. 2-16A). In morphine-dependent diabetic mice, i.c.v. pretreatment with neither calphostin C nor KT5720 affected the NA turnover in naloxone-challenged morphine-dependent diabetic mice (Fig. 2-16A). I.c.v. pretreatment with PDBu (10 pmol) 1 h before last injection of morphine attenuated a naloxone-induced increase in NA turnover in the frontal cortex in morphine-dependent non-diabetic mice (Fig. 2-16B). However, i.c.v. pretreatment with PDBu (10 pmol) had no significant effect on the level of NA turnover in the frontal cortex in naloxone-challenged morphine-dependent diabetic mice (Fig 2-16B).

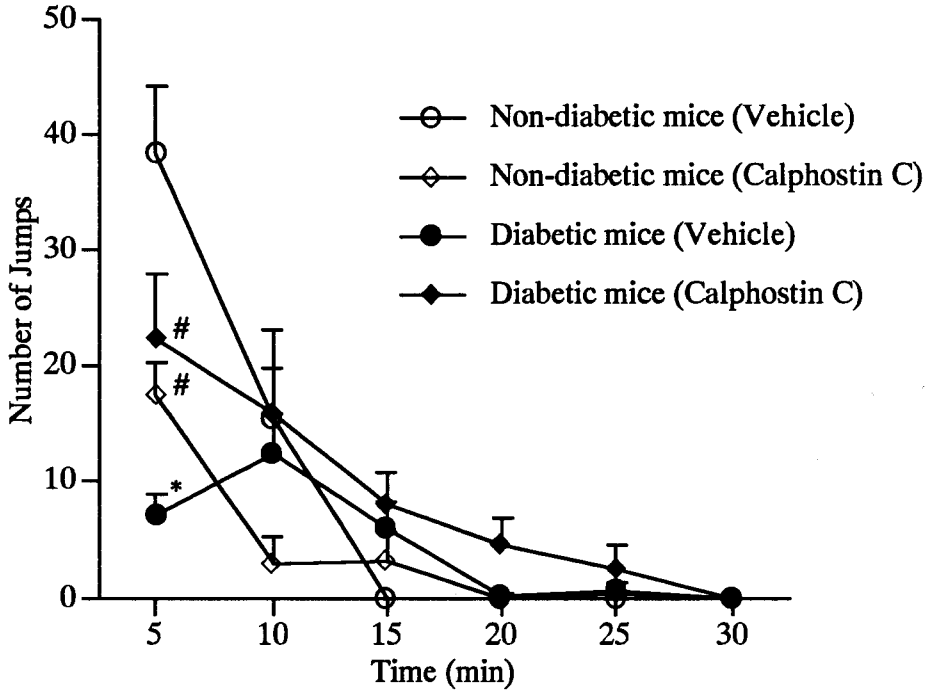

Figure 2-9. The effect of a protein kinase C inhibitor, calphostin C, on the time course of the number of naloxone-precipitated withdrawal jumps in morphine-dependent diabetic and non-diabetic mice. Calphostin C (20 pmol) and vehicle were injected i.c.v. 1h before the administration of naloxone (0.3 mg/kg, s.c.). Each point represents the mean ± S.E. of 9-10 mice. *P<0.05 versus non-diabetic mice. #P<0.05 versus the respective vehicle-treated groups.

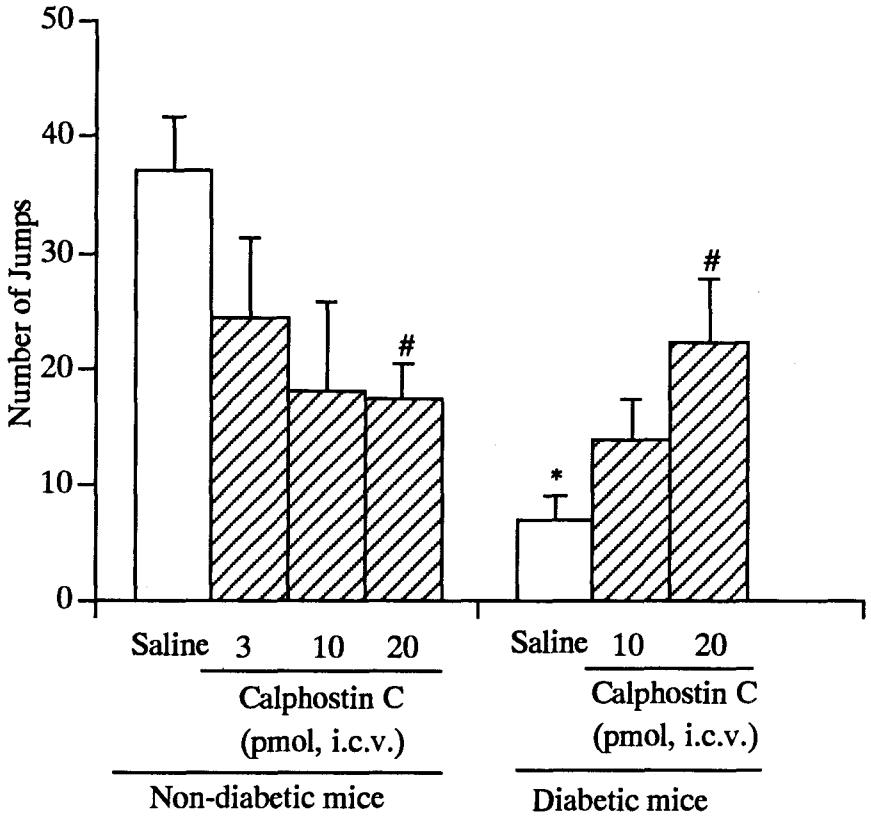

Figure 2-10. The dose-related effect of a protein kinase C inhibitor, calphostin C, on the number of naloxone-precipitated withdrawal jumps within 5 min after naloxone challenge in morphine-dependent diabetic and non-diabetic mice. Calphostin C (3 pmol, 10 pmol and 20 pmol) and vehicle were injected i.c.v. 1h before the administration of naloxone (0.3 mg/kg, s.c.). Each point represents the mean ± S.E. of 9-10 mice. *P<0.05 versus non-diabetic mice. #P<0.05 versus the respective vehicle-treated groups.

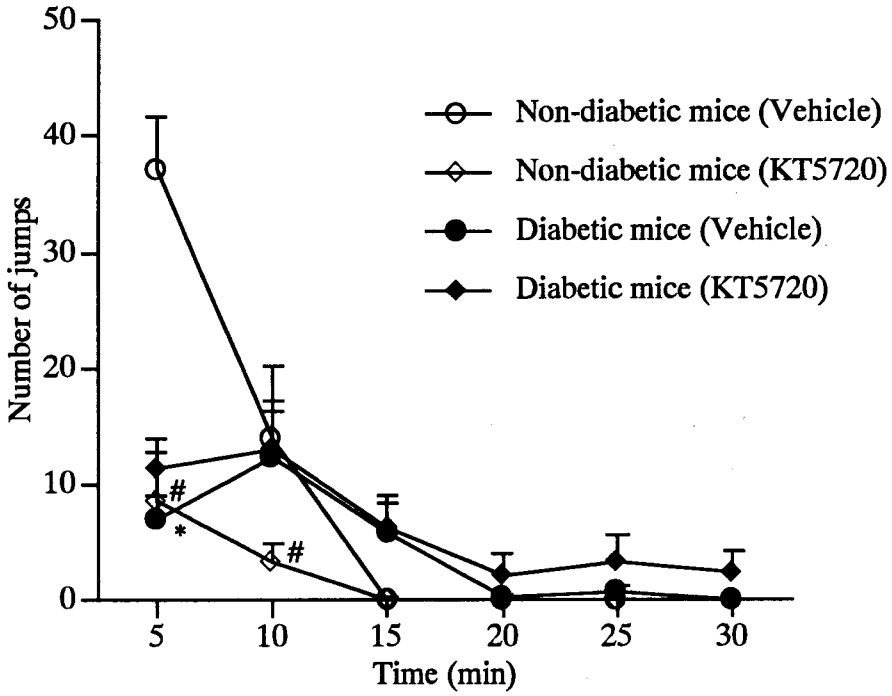

Figure 2-11. The effect of a protein kinase A inhibitor, KT-5720, on the time course of the number of naloxone-precipitated withdrawal jumps in morphine-dependent diabetic and non-diabetic mice. KT-5720 (20 pmol) and vehicle were injected i.c.v. 1h before the administration of naloxone (0.3 mg/kg, s.c.). Each point represents the mean ± S.E. of 10-12 mice. *P<0.05 versus non-diabetic mice. #P<0.05 versus the respective vehicle-treated groups.

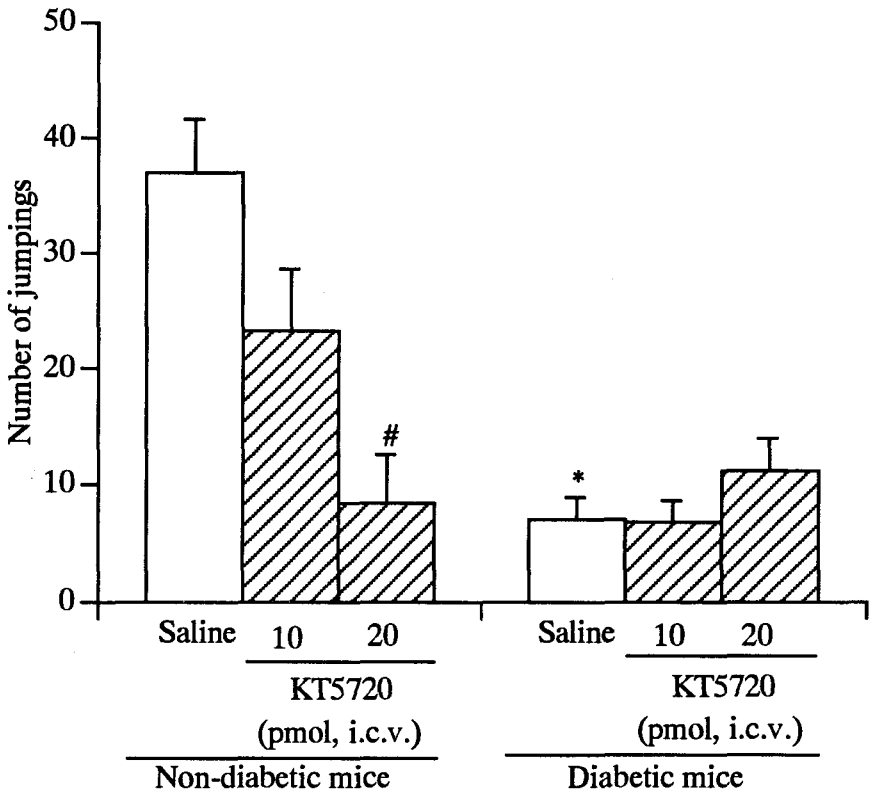

Figure 2-12. The effect of a protein kinase A inhibitor, KT-5720, on the number of naloxone-precipitated withdrawal jumps in morphine-dependent diabetic and non-diabetic mice. KT-5720 (10 and 20 pmol) and vehicle were injected i.c.v. 1h before the administration of naloxone (0.3 mg/kg, s.c.). Each point represents the mean ± S.E. of 10-12 mice. *P<0.05 versus non-diabetic mice. #P<0.05 versus the respective vehicle-treated groups.

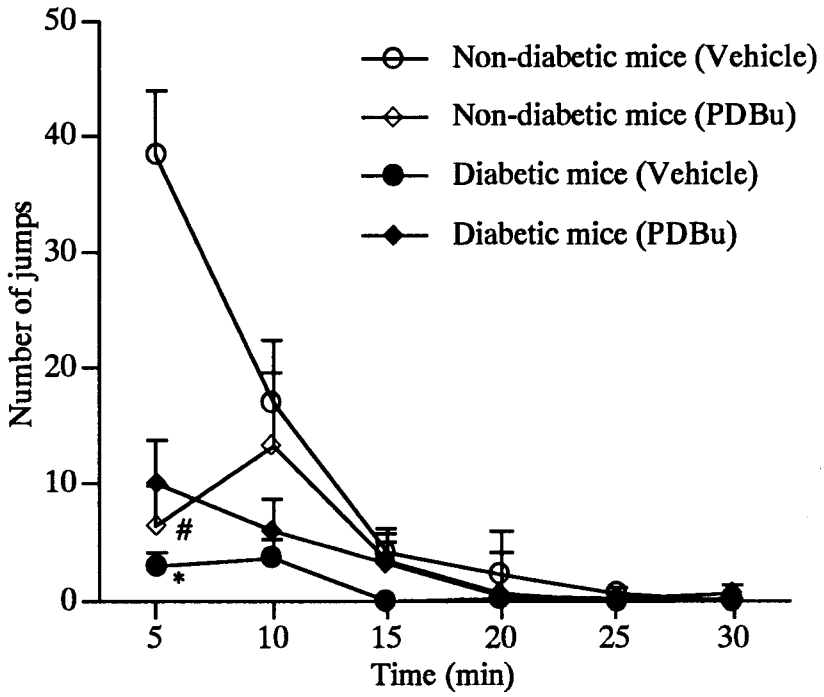

Figure 2-13. The effect of a protein kinase C activator, phorbol-12,13-dibutyrate (PDBu), on the time course of the number of naloxone-precipitated withdrawal jumps in morphine-dependent diabetic and non-diabetic mice. PDBu (10 pmol) and vehicle were injected i.c.v. 1 h before the last injection of morphine (45 mg/kg, s.c.). Each point represents the mean ± S.E. of 10 mice. *P<0.05 versus non-diabetic mice. #P<0.05 versus the respective vehicle-treated groups.

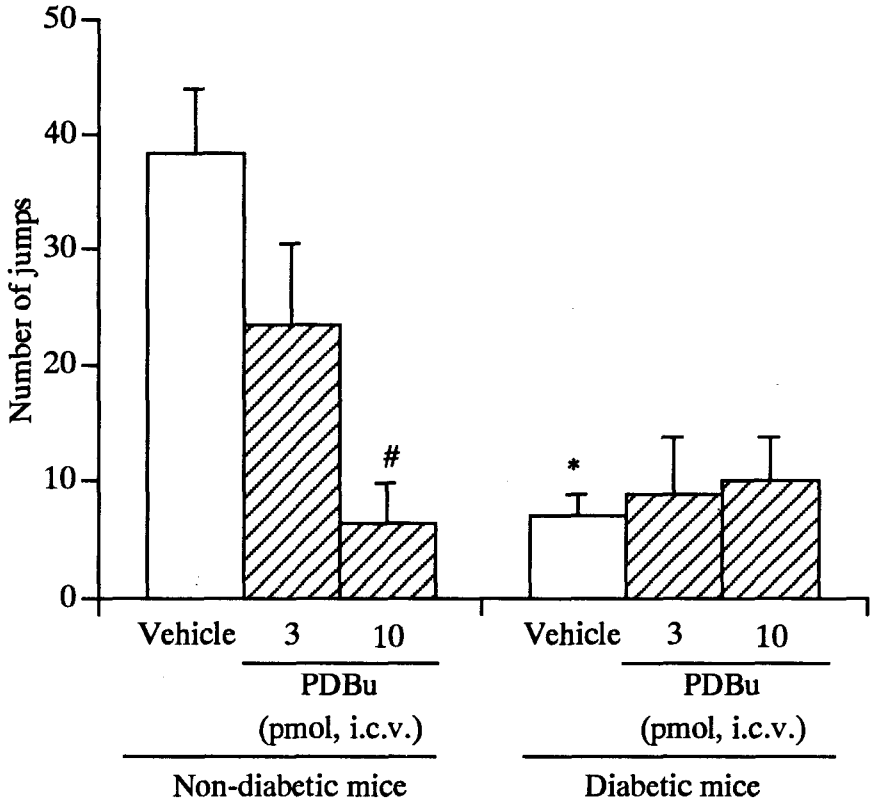

Figure 2-14. The dose-related effect of a protein kinase C activator, phorbol-12,13-dibutyrate (PDBu), on the number of naloxone-precipitated withdrawal jumps within 5 min after naloxone challenge in morphine-dependent diabetic and non-diabetic mice. PDBu (3 pmol and 10 pmol) and vehicle were injected i.c.v. 1 h before the last injection of morphine (45 mg/kg, s.c.). Each point represents the mean ± S.E. of 10 mice. *P<0.05 versus non-diabetic mice. #P<0.05 versus the respective vehicle-treated groups.

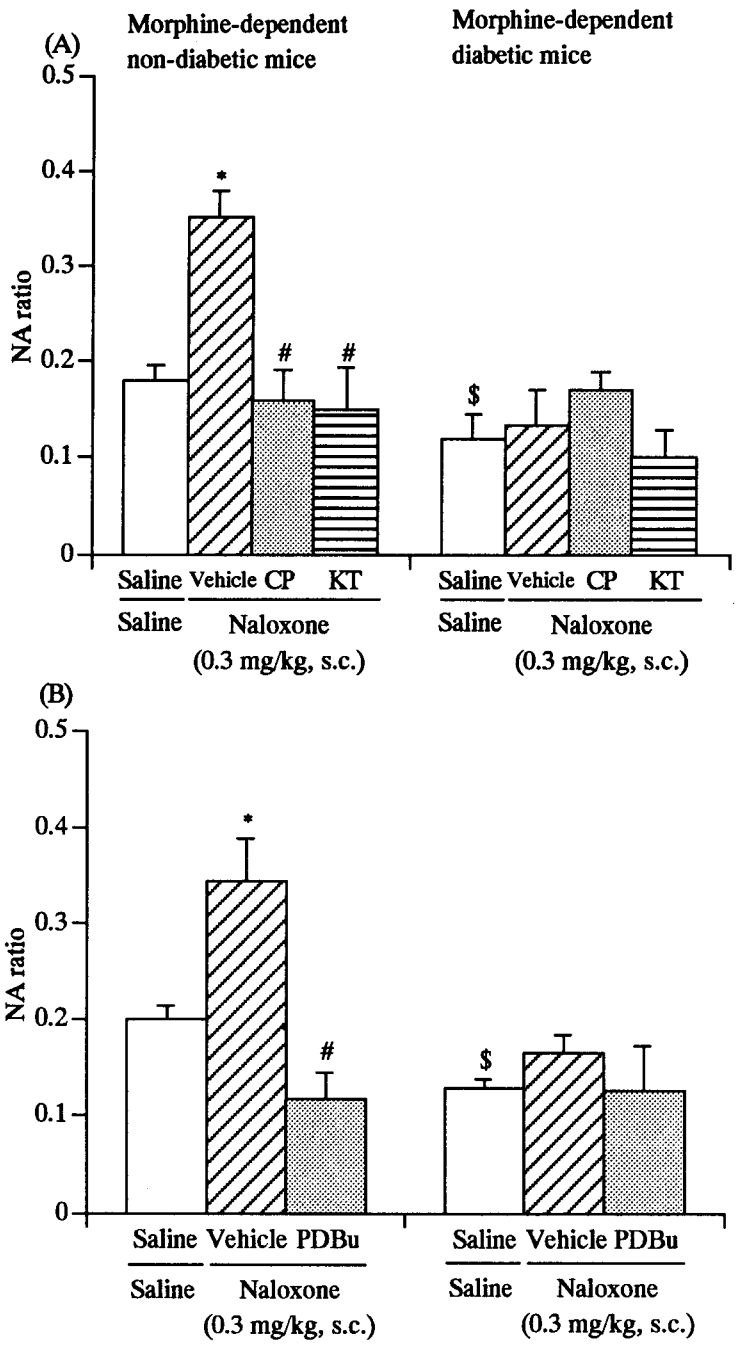

Figure 2-15. The effects of protein kinase C and A inhibitor (A) and protein kinase C activator (B) on a naloxone-induced increase of NA turnover in frontal cortex in morphine-dependent diabetic and non-diabetic mice. Calphostin C (CP; 20 pmol, ic.v.) and KT5720 (KT; 20 pmol, i.c.v.) and vehicle were injected 1 h before the last injection of naloxone (0.3 mg/kg, s.c.) (A). PDBu (10 pmol) and vehicle were injected i.c.v. 1 h before the last injection of morphine (45 mg/kg, s.c.). Each column represents the mean with S.E. of 5-10 mice. *P<0.05 versus saline-challenged morphine-dependent non-diabetic mice. #P<0.05 versus naloxone challenged chronic morphine-treated group. $P<0.05 versus respective non-diabetic mice.

Experiment 2-5: Role of intracellular calcium on the modulation of naloxone-precipitated withdrawal jumping in morphine-dependent mice by diabetes.

Materials and Methods

Animals

Male ICR mice (Tokyo Laboratory Animals Science Co., Tokyo), weighing about 20 g at the beginning of the experiments, were used. The mice were housed under the condition as described in Chapter 1-1. Animals were rendered diabetic by an injection of streptozotocin (200 mg/kg, i.v.) prepared in 0.1 N citrate buffer at pH 4.5. Age-matched non-diabetic mice were injected with vehicle alone. The experiments were conducted 2 weeks after the injection of streptozotocin or vehicle. Mice with serum glucose levels above 400 mg/dl were considered diabetic.

Chronic administration of morphine

Morphine was injected s.c. daily at 9 A.M. and 7 P.M. According to the schedule described in Experiment 2-1, the morphine dose was increased progressively from 8 to 45 mg/kg over a period of 5 days.

Morphine withdrawal

Withdrawal signs were precipitated by injecting naloxone (0.3 mg/kg, s.c. in a volume of 10 ml/kg) 2 h after the final morphine administration. After the naloxone challenge, mice were immediately placed in acrylic cylinder (30 cm high, 12 cm in diameter). The number of jumps during a 5-min period was counted for 30 min after naloxone injection.

Neurochemical analysis

The concentrations of noradrenaline (NA) and 3-methoxy-4-hydroxyphenyleneglycol (MHPG) were estimated using high-performance liquid chromatography (HPLC) with electrochemical detection (ECD), as previously described (34). Morphine-dependent diabetic and non-diabetic mice were sacrificed 5 min after naloxone (0.3 mg/kg, s.c.) or saline (10 ml/kg, s.c.) injection, then immersed in a dry ice-cold solution. The brain was quickly removed and the cerebral cortex was dissected on an ice-cold glass plate. The HPLC system

consisted of a delivery pump (EP-300, Eicom Co., Japan), an analytical column (EICOMPAC MA-50DS, Eicom Co., Japan) and a guard column (Eicom, Co., Japan). The electrochemical detector (EC-100, Eicom Co., Japan) included a graphite electrode (WE-3G, Eicom Co., Japan).

Intracerebroventricular injection

Intracerebroventricular (i.c.v.) administration was performed following the method described in Experiment 1-2.

Drugs

The drugs used were streptozotocin (Sigma Chemical Co., St. Louis, MO), morphine hydrochloride (Sankyo Co., Tokyo, Japan), and naloxone hydrochloride (Sigma Chemical Co., St. Louis, MO). Ryanodine was purchased from Calbiochem-Novabiochem International (San Diego, CA). Thapsigargin was purchased from Research Biochemical International (Natic, MA). Ryanodine was injected 15 min before the injection of naloxone. Thapsigargin was injected 1h before the injection of naloxone. The dose and schedule for ryanodine and thapsigargin in this study were determined as described previously (Smith and Stevens, 1995).

Data analysis

The data are expressed as means ± S.E. The statistical significance of differences between groups was assessed with Student's t-test (comparison of two groups) or an analysis of variance (ANOVA) followed by Dunnet's test (comparison among multiple groups). NA turnover was determined as the NA ratio: NA ratio = MHPG (ng/g of wet tissue) / NA (ng/g of wet tissue).

Results

Influence of a ryanodine on naloxone-precipitated withdrawal jumping

Subcutaneous injection of naloxone (0.3 mg/kg) invariably provoked withdrawal jumping within 2 to 15 min in morphine-dependent non-diabetic mice and reached its maximum within 5 min after naloxone injection (Fig. 2-17A, 2-18A). In morphine-dependent diabetic mice, s.c. injection of naloxone (0.3 mg/kg) also provoked withdrawal jumping within 15 min. However, the number of naloxone-precipitated withdrawal jumps within 5 min after naloxone challenge was significantly less in morphine-dependent diabetic mice than in morphine-dependent non-diabetic mice (Fig. 2-18A, 1-18B). The effects of ryanodine on the number of naloxone-precipitated withdrawal jumps in morphine-dependent diabetic and non-diabetic mice are shown in fig. 2-17. Naloxone-precipitated withdrawal jumping was markedly attenuated by the i.c.v. administration of ryanodine (1.0 nmol) in morphine-dependent non-diabetic mice. However, naloxone-precipitated withdrawal jumping in morphine-dependent diabetic mice was not affected by i.c.v. pretreatment with ryanodine (1.0 nmol). As shown in fig. 2-17B, the number of naloxone-precipitated withdrawal jumps within 5 min after naloxone challenge was dose-dependently attenuated by i.c.v. pretreatment with ryanodine (0.3 and 1.0 nmol) in morphine-dependent non-diabetic mice. However, the number of naloxone-precipitated withdrawal jumps in morphine-dependent diabetic mice was not affected by i.c.v. pretreatment with ryanodine (0.3 and 1.0 nmol).

Influence of thapsigargin on naloxone-precipitated withdrawal jumping

The effect of thapsigargin on the number of naloxone-precipitated withdrawal jumps in morphine-dependent diabetic and non-diabetic mice is shown in fig. 2-18. In morphine-dependent non-diabetic mice, but not in morphine-dependent diabetic mice, naloxone-precipitated withdrawal jumping within 5 min after naloxone challenge was attenuated by i.c.v. pretreatment with thapsigargin (1.0 nmol). As shown in fig. 2-18B, i.c.v. pretreatment with thapsigargin (0.3 and 1.0 nmol) 1 h before naloxone injection dose-dependently increased naloxone-precipitated withdrawal jumps within 5 min after naloxone injection. However, i.c.v. pretreatment with thapsigargin (0.3 and 1.0 nmol) had no significant effect on the number of naloxone-precipitated withdrawal jumping within 5 min after naloxone

injection in morphine-dependent diabetic mice.

Influences of ryanodine and thapsigargin on naloxone-induced changes in the level of NA turnover in the frontal cortex

The level of NA turnover in the frontal cortex was significantly lower in morphine-dependent diabetic mice than in morphine-dependent non-diabetic mice (Fig. 2-19A and B). The level of NA turnover in the frontal cortex in morphine-dependent non-diabetic mice was significantly increased 5 min after naloxone challenge, compared with that in the saline-challenged morphine-dependent non-diabetic mice (Fig 2-19A and B). However, a naloxone-induced increase in the NA turnover was not observed 5 min after naloxone challenge in morphine-dependent diabetic mice (Fig. 2-19A and B). As shown in fig. 2-19A, a naloxone-induced increase in the NA turnover was attenuated by pretreatment with ryanodine (1.0 nmol, i.c.v.) in morphine-dependent non-diabetic mice (Fig. 2-19A). I.c.v. pretreatment with ryanodine did not affect the NA turnover in naloxone-challenged morphine-dependent diabetic mice (Fig. 2-19A). I.c.v. pretreatment with thapsigargin (1.0 nmol) 1 h before the injection of naloxone increased a naloxone-induced increase in NA turnover in the frontal cortex in morphine-dependent non-diabetic mice (Fig. 2-19B). However, this enhancement of the naloxone-induced increase in NA turnover was not statistically significant.

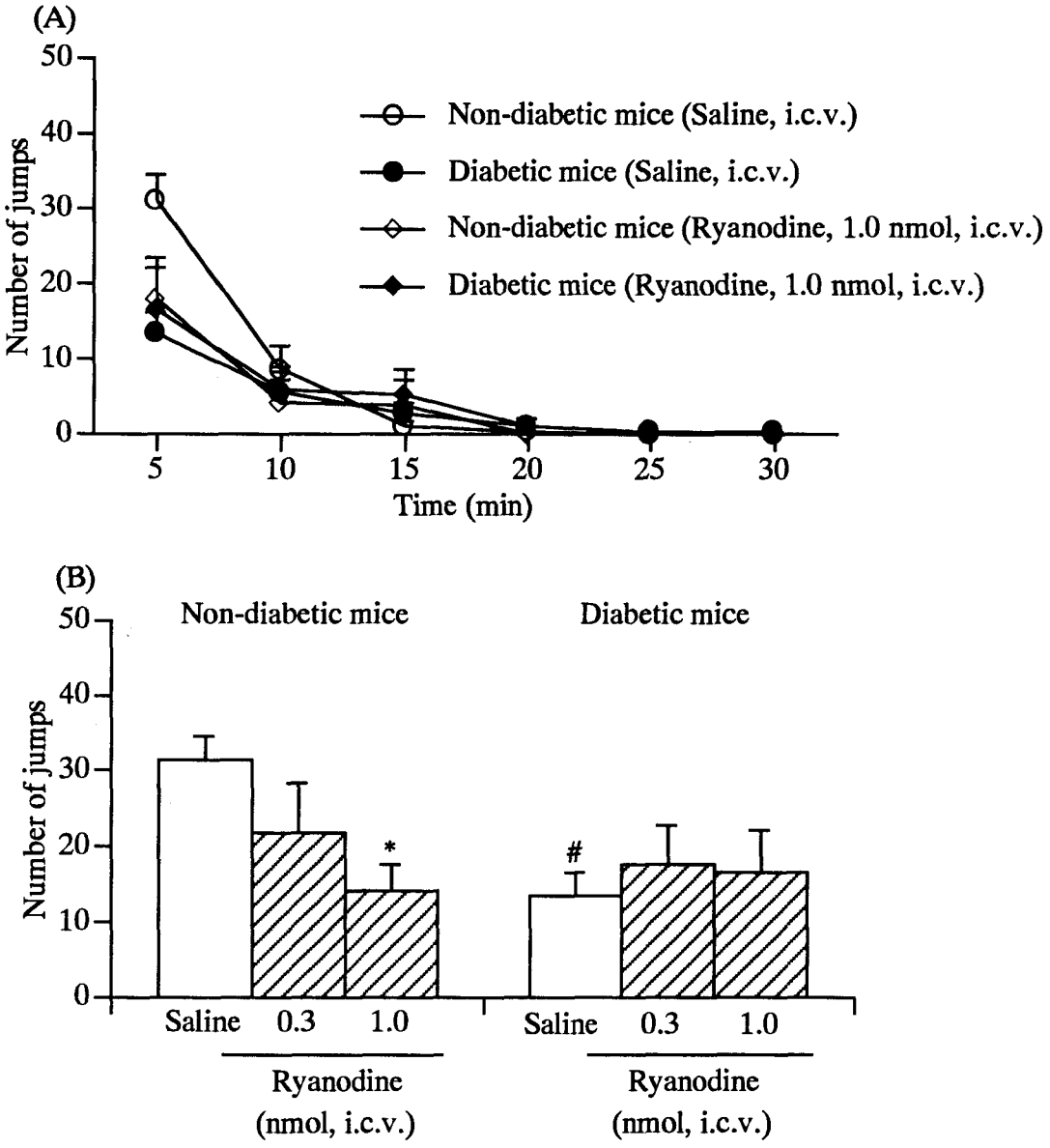

Figure 2-16. (A) The effect of ryanodine on the time course of the number of naloxone-precipitated withdrawal jumps in morphine-dependent diabetic and non-diabetic mice. (B) The dose-related effect of ryanodine (0.3 and 1.0 nmol) on the number of naloxone-precipitated withdrawal jumps within 5 min after naloxone challenge in morphine-dependent diabetic and non-diabetic mice. Ryanodine (0.3 or 1.0 nmol) and saline were injected 15 min before the administration of naloxone (0.3 mg/kg, s.c.). Each point or column represents the mean ± S.E. of 9-10 mice. *$P<0.05$ versus non-diabetic mice. #$P<0.05$ versus the respective saline-treated groups.

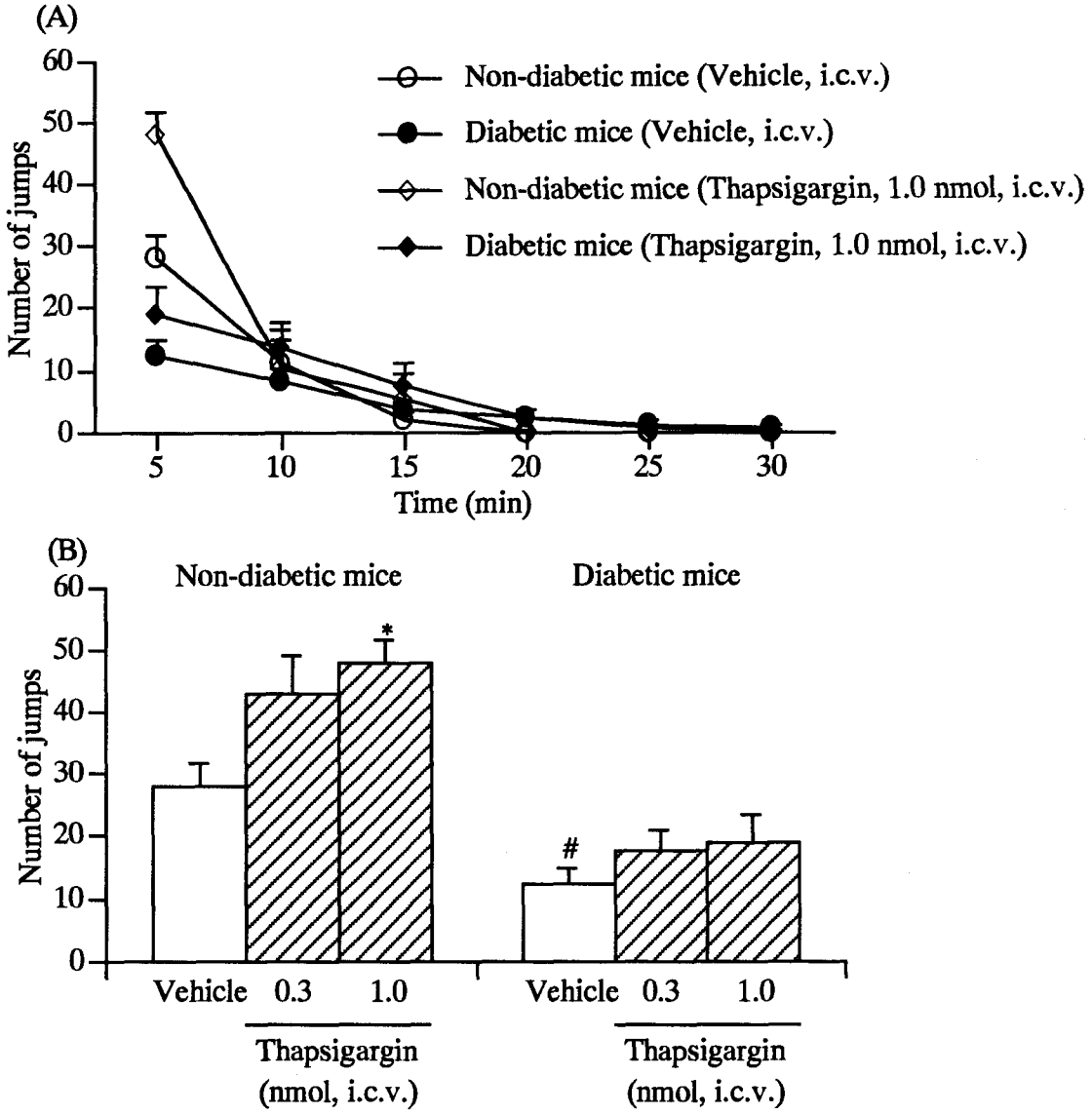

Figure 2-17. (A) The effect of thapsigargin on the time course of the number of naloxone-precipitated withdrawal jumps in morphine-dependent diabetic and non-diabetic mice. (B) The dose-related effect of thapsigargin on the number of naloxone-precipitated withdrawal jumps within 5 min after naloxone challenge in morphine-dependent diabetic and non-diabetic mice. Thapsigargin (0.3 and 1.0 nmol) and vehicle were injected i.c.v. 1h before the administration of naloxone (0.3 mg/kg, s.c.). Each point or column represents the mean ± S.E. of 9-10 mice. *P<0.05 versus non-diabetic mice. #P<0.05 versus the respective vehicle-treated groups.

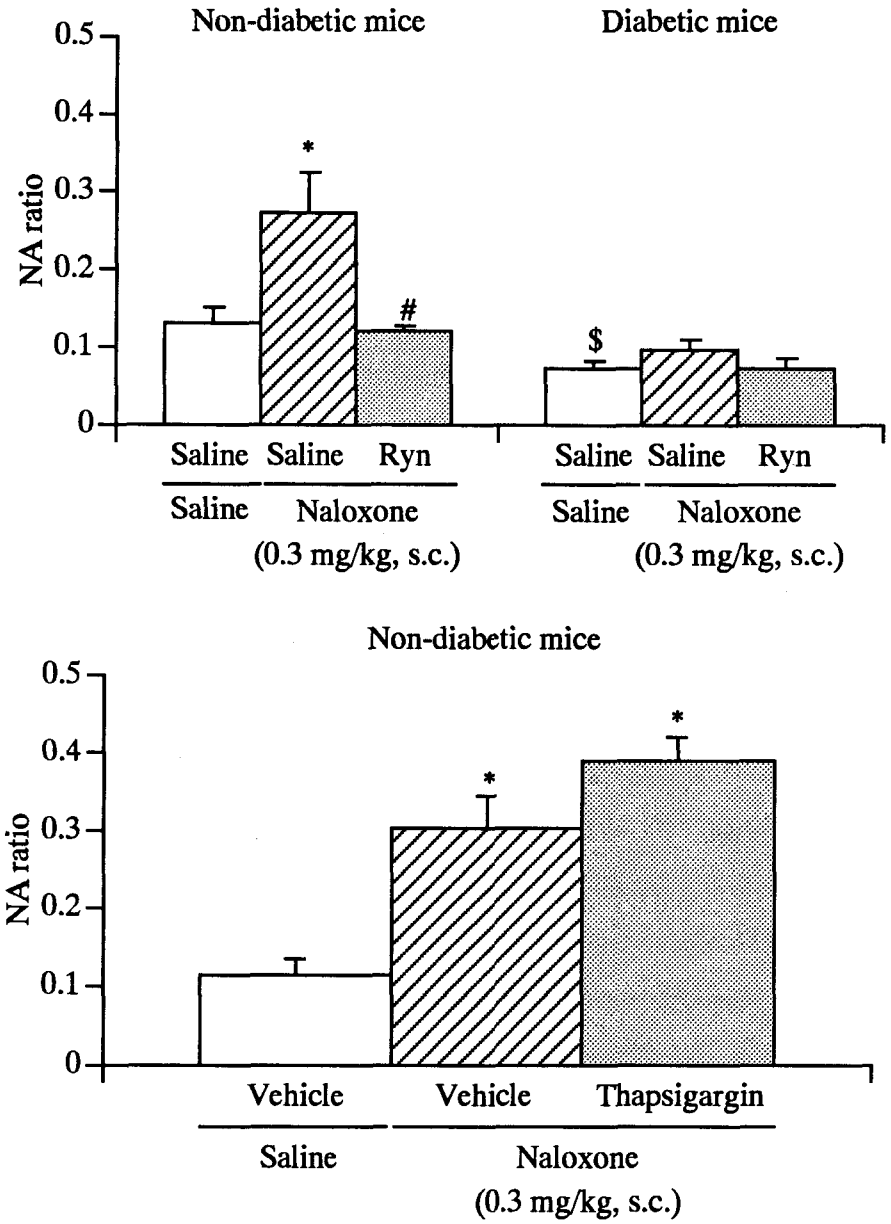

Figure 2-18. The effects of ryanodine (A) and thapsigargin (B) on naloxone-induced increase of NA turnover in the frontal cortex in morphine-dependent diabetic and non-diabetic mice. Ryanodine (Ryn; 1.0 nmol) and saline were injected 15 min before the administration of naloxone (0.3 mg/kg, s.c.) (A). Thapsigargin (1.0 nmol) and vehicle were injected i.c.v. 1 h before the administration of naloxone (0.3 mg/kg, s.c.). Each column represents the mean with S.E. of 4-5 mice. *P<0.05 versus saline-challenged morphine-dependent non-diabetic mice. #P<0.05 versus naloxone challenged chronic morphine-treated group. $P<0.05 versus respective non-diabetic mice.

Discussion

The results of "Experiment 2-1" show that morphine-dependent diabetic mice exhibit a differential sensitivity to naloxone challenge with regard to body weight loss, and jumping and body shakes. Indeed, the naloxone-induced loss of body weight is much greater in diabetic mice than in non-diabetic mice, whereas naloxone-induced jumping and body shakes are relatively less in diabetic mice. Suzuki et al. (1992a) previously demonstrated that naloxone-precipitated jumping and body shakes are much less in CXBK mice than in C57BL/6 mice. However, they found no significant difference in naloxone-precipitated diarrhea, body weight loss and ptosis between CXBK and C57BL/6 mice (Suzuki et al., 1992a). Moskowitz and Goodman (1985) found that the CXBK mouse is deficient in μ-opioid receptors, whereas the δ-opioid receptor population is less consistently altered. Furthermore, they demonstrated that the CXBK mouse was particularly deficient in μ_1-opioid receptor binding (Moskowitz and Goodman, 1985). Based on these results, Suzuki et al. (1992a) previously suggested that naloxone-precipitated jumping and body shakes in morphine-dependent mice may be mediated by μ_1-opioid receptors, but that diarrhea, body weight loss and ptosis appeared to be mediated by μ_2- and/or δ-opioid receptors. Furthermore, Kamei et al. (1994a) proposed that the diabetic mouse is selectively deficient in μ_1-opioid receptors, but not in μ_2-opioid receptors. Thus, the present and previous findings support the hypothesis that naloxone-precipitated jumping and body shakes in morphine-dependent mice may be mediated by μ_1-opioid receptors.

Cowan et al. (1988) reported that rats treated with μ- but not δ-opioid agonists showed ptosis after naloxone injection. Moreover, Suzuki et al. (1992a) reported that there was no difference in the incidence of naloxone-precipitated ptosis between morphine-dependent CXBK and C57BL/6 mice. Thus, they have concluded that naloxone-precipitated ptosis may be mediated by μ_2-, but not μ_1- or δ-opioid receptors. In the present study, naloxone-precipitated ptosis in diabetic mice was not different from that in non-diabetic mice, suggesting that μ_2-opioid receptor-mediated function in the development of physical dependence on morphine is not altered in diabetic mice, as compared to that in non-diabetic mice.

In the present study, although the incidence of naloxone-precipitated diarrhea in morphine-dependent diabetic mice was similar to that in morphine-dependent non-diabetic mice, the amount of naloxone-precipitated body weight loss in morphine-dependent diabetic

mice was significantly greater than that in morphine-dependent non-diabetic mice. This result is not consistent with previous result using CXBK mice (Suzuki et al., 1992a). The amount of naloxone-precipitated body weight loss in morphine-dependent CXBK mice was similar to that in morphine-dependent C57BL/6 mice, as was the incidence of naloxone-precipitated diarrhea (Suzuki et al., 1992a). Heyman et al. (1988) reported that morphine and DAMGO mediated their antitransit effects at a naloxonazine-insensitive site, referred to as μ_2-opioid receptor. Based on these results, we suggested that naloxone-precipitated diarrhea and body weight loss appeared to be mediated by μ_2- and/or δ-opioid receptors (Suzuki et al., 1992a). As mentioned above, it is suggested that μ_2-opioid receptor-mediated function in the development of physical dependence on morphine is not altered in diabetic mice, as compared to that in non-diabetic mice. Furthermore, Kamei et al. (1992a, 1994b) previously suggested that supraspinal δ-opioid receptors are up-regulated in diabetic mice, since the antinociceptive effect of i.c.v. [D-Pen^{2,5}]enkephalin (DPDPE), a selective δ-opioid receptor agonist, in diabetic mice was significantly greater than that in non-diabetic mice. Moreover, Miyamoto et al. (1993a, 1993b) recently suggested that δ-opioid receptors were involved in the development of morphine dependence. Thus, naloxone-precipitated body weight loss may be mediated by δ-opioid receptors. However, it is well-known that naloxone-precipitated body weight loss in morphine-dependent animals may be primarily due to naloxone-precipitated diarrhea (Goode, 1971). It has been assumed that the diarrhea which results from morphine withdrawal is caused by an increase in gastrointestinal motility (Kaymakcalan and Temelli, 1964; Collier et al., 1972; Brown et al., 1988). In this regard, accumulating evidence suggests that autonomic neuropathy is a common complication in diabetic patients. In fact, it has been reported that diarrhea is common in patients with diabetes mellitus, suggesting that diabetes is associated with dysfunction of the autonomic nervous system of the gut (Hosking et al., 1978). In the present study, furthermore, I observed that the amount of naloxone-precipitated body weight loss in chronically saline-treated (non-dependent) diabetic mice was also significantly greater than that in chronically saline-treated (non-dependent) non-diabetic mice. Furthermore, in Chapter 2-2, I demonstrated that there was no significant difference between the ED50 values of the naloxonazine-insensitive antitransit effect of morphine in non-diabetic and diabetic mice. Namely, diabetes failed to alter the μ_2-opioid receptor-mediated antitransit effect of morphine. Thus, it is also possible that the potent naloxone-precipitated body weight loss in morphine-dependent diabetic mice, as compared to that in morphine-dependent non-diabetic mice, may be due to the intensity of the increase in gastrointestinal motility caused by

dysfunction of the autonomic nervous system of the gut.

In conclusion, the present results suggest that μ_1-opioid receptor-mediated function in the development of physical dependence on morphine in diabetic mice is significantly less than that in non-diabetic mice, and support our hypothesis that mice with diabetes are selectively hypo-responsive to μ_1-opioid receptor-mediated pharmacological action, but not to that which is mediated by μ_2-opioid receptors.

In Experiment 2-2, I indicated that the antitransit effect of s.c. morphine is significantly antagonized by pretreatment with β-funaltrexamine, but not by pretreatment with naloxonazine. These results are consistent with the findings of Heyman et al. (1988) and Pick et al. (1991), and suggest that μ_2-opioid receptors may play an important role in the antitransit effect of morphine.

The results of Experiment 2-2 also demonstrated that the dose-dependent antitransit effect of morphine can be observed not only in non-diabetic, but also in diabetic mice. Furthermore, there was no significant difference between the ED50 values of the antitransit effect of morphine in non-diabetic and diabetic mice. The antitransit effect of s.c. morphine in diabetic mice is also significantly antagonized by pretreatment with β-funaltrexamine, but not by pretreatment with naloxonazine. Moreover, there is no significant difference in the effect of β-funaltrexamine on morphine-induced antitransit effect between diabetic and non-diabetic mice. Kamei et al. (1992 a, b, 1994a) previously reported that diabetic mice are selectively hyporesponsive to μ_1-opioid receptor-mediated pharmacological actions, i.e. supraspinal antinociception, but not to those mediated by μ_2-opioid receptors, i.e. spinal antinociception (Kamei et al, 1994a) and the antitussive effect (Kamei et al, 1994c). This hypothesis is further supported by the present results.

In the present study, the antitransit effect of morphine was not completely antagonized by pretreatment with β-funaltrexamine. In this regard, Porreca and Burks (1983) suggested that δ-opioid receptors may mediate the antigastrointestinal transit effects of opioids at the spinal cord level, since i.t. administration of δ-opioid receptor agonists such as D-Ala^2-D-Leu^5-enkephalin, D-Ser^2-Leu-enkephalin-(Thr^6) and D-Pen^2-L-Cys^5-enkephalin, consistently inhibited gastrointestinal transit. These results suggest that the antitransit effects of opioids are mediated by both μ- and δ-opioid receptors. Kamei et al. (1992b) previously reported that there was no significant difference in the supraspinal and spinal δ-opioid receptor-mediated antinociceptive effects between non-diabetic and diabetic mice. Thus, it seems likely that spinal δ-opioid receptors may have an important role in the antitransit effect

of morphine in both non-diabetic and diabetic mice. This possibility appears to provide a plausible explanation for the partial antagonism of β-funaltrexamine on the antitransit effect of morphine.

In conclusion, the results from Experiment 2-2 demonstrated that diabetes failed to alter the μ_2-opioid receptor-mediated antitransit effect of morphine. This conclusion supports the hypothesis that diabetic mice are selectively hyporesponsive to μ_1-opioid receptor-mediated pharmacological action, but not to that which is mediated by μ_2-opioid receptors.

In Experiment 2-3, I indicated that morphine-dependent diabetic mice jump less within 5 min after naloxone challenge than morphine-dependent non-diabetic mice. This result supports the previous suggestion that morphine-dependent diabetic mice exhibit a less sensitivity to naloxone challenge with regard to withdrawal jumping.

In the present study, I observed a significant increase in NA turnover in the frontal cortex in morphine-dependent non-diabetic mice 5 min after naloxone challenge, when the incidence of withdrawal jumping was at its peak. The central noradrenergic system has been hypothesized to be involved in morphine dependence and withdrawal (Redmond and Krysatal, 1984). In a microinjection study using naltrexone, it was shown that the LC region is the most sensitive site for the expression of morphine withdrawal signs (Maldonado et al., 1992). It has been reported that the firing rate of noradrenergic neurons in the LC, which is a cluster of NA-containing cell bodies in the brain, increases during naloxone-precipitated withdrawal from morphine (Aghajanian, 1978). Furthermore, it has been reported that treatment with DSP-4, a noradrenergic neurotoxin that selectively damages the NA-containing nerve endings that project from the LC, before the naloxone challenge suppresses the expression of morphine withdrawal signs, including jumping and "wet dog" shakes (Funada et al., 1994). Moreover, in a biochemical study, the level of 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), the major metabolite of NA, in the cerebral cortex which projects from LC, increased following naloxone challenge in morphine-dependent rats and mice (Crawley et al., 1979; Funada et al., 1994). These reports and the present study strongly suggest that LC noradrenergic neurons are involved in morphine dependence and/or withdrawal.

In the present study, I found that NA turnover in the frontal cortex was significantly lower in chronically saline-treated (non-dependent) diabetic mice than in chronically saline-treated (non-dependent) non-diabetic mice. Thus, it is likely that noradrenergic neurotransmission in the frontal cortex, especially in the LC, may be reduced in diabetic mice, compared with that in non-diabetic mice. Furthermore, the results of our experiment suggest

an association between noradrenergic neurotransmission in the frontal cortex and the incidence of naloxone-precipitated withdrawal jumping. Indeed, NA turnover in the frontal cortex had not changed at 5 min after naloxone challenge in morphine-dependent diabetic mice, although the incidence of naloxone-induced jumping was significantly lower in morphine-dependent mice than in morphine-dependent non-diabetic mice at this point. In contrast, the NA turnover in frontal cortex was significantly increased at 10 min after naloxone challenge in morphine-dependent mice, when the incidence of naloxone-induced jumping was increased to the levels that were observed in morphine-dependent non-diabetic mice.

In conclusion, the findings of Experiment 2-3 raise possibility that the reduction in noradrenergic neurotransmission in the LC noradrenergic system may be associated with the reduction in the incidence of naloxone-precipitated withdrawal jumping at 5 min after naloxone challenge in morphine-dependent diabetic mice, as compared with that in non-diabetic mice.

In Experiment 2-4, I found that the i.c.v. administration of a PKC inhibitor, calphostin C, attenuated the expression of naloxone-precipitated withdrawal jumping in morphine-dependent non-diabetic mice. Moreover, the expression of naloxone-precipitated withdrawal jumping was also attenuated by i.c.v. pretreatment with the PKA inhibitor KT-5720 in morphine-dependent non-diabetic mice. Furthermore, naloxone-induced increase in NA turnover in the frontal cortex in morphine-dependent non-diabetic mice is attenuated by i.c.v. pretreatment with either calphostin C and KT5720 1 hr before naloxone challenge. Many investigators indicated that chronic morphine administration increases levels of adenylyl cyclase and PKA in the LC (Nestler et al., 1992; Duman e al., 1988). These adaptations have been shown to increase the electrical excitability of LC neurons (Alreja and Aghajanian, 1991; Kogan et al., 1992; Shiekhattar and Aston-Jonse, 1993) and appear to contribute to activation of the LC seen on precipitation of withdrawal (Kogan et al., 1992; Rasmmussen et al., 1990). Thus, it is also possible that the attenuation of naloxone-induced increase in the level of NA turnover in the frontal cortex in morphine-dependent non-diabetic mice is produced by the inhibition of PKA by KT5720 in LC. Furthermore, several recent studies have indicated that PKC activity is involved in opiate tolerance and dependence (Collier. 1980; Mayer et al., 1995). It was reported that chronic treatment with morphine induced a modest, naloxone-sensitive, increase in cytosolic, but not membrane, PKC activity in rat brain (pons/medulla but not cortex and midbrain regions)(Narita et al., 1994). Furthermore, H-7

have been inhibited the withdrawal signs and the increase in glutamate levels, which induce NA release in the frontal cortex, in the LC of opioid-dependent animal (Tokuyama et al., 1997). Thus, it is possible that the activation of PKC, as well as PKA, in the LC may be modulate naloxone-induced increase of NA turnover in the frontal cortex and the naloxone-precipitated withdrawal jumping in morphine-dependent rodents.

In the present study, i.c.v. pretreatment with PDBu 1 hr before the last injection of morphine attenuated increase in NA turnover in the frontal cortex and the naloxone-precipitated withdrawal jumping in morphine-dependent non-diabetic mice. The mechanisms which underlie the attenuation of naloxone-induced increase in NA turnover in the frontal cortex and the expression of naloxone-precipitated withdrawal jumping in morphine-dependent non-diabetic mice by PKC activator are unclear. It was reported that the abundance of μ-opioid receptors was not significantly altered in morphine-dependent human and rats (García-Sevilla et al., 1997). The phosphorylation of receptors by PKC has been proposed to be a possible mechanism for the development of desensitization (Shearman et al., 1989). It is reported that activation of PKC by phorbol ester potentiates the desensitization of μ-opioid receptor induced K^+ current (Chen and Yu, 1994). We recently reported that the activation of PKC by PDBu leads to the desensitization of μ-opioid receptor-mediated antinociception (Narita et al., 1997). In view of these results, present data suggests that attenuation of naloxone-induced increase in NA turnover in the frontal cortex and the expression of naloxone-precipitated withdrawal jumping by the activation of PKC may be due to the desensitization of μ-opioid receptor. However, it was reported that long term treatment with phorbol ester has desensitized or downregulated PKC in mouse neuroblastoma N1E-15 cells, when the cells were treated with 100 nM PDBu for 24 hr (Lai and El-Fakahany, 1988). In the present study, the effect of PDBu on naloxone-precipitated withdrawal jumping in morphine dependent non-diabetic mice exerts the same effect as the PKC inhibitor. Therefore, it is also possible that the effect of PDBu on naloxone-induced increase in NA turnover in the frontal cortex and naloxone-precipitated withdrawal jumping in morphine-dependent non-diabetic mice may be due to the desensitization or down-regulation of PKC. Because desensitization of PKC by phorbol ester requires a long-term treatment, it is unlikely that the desensitization of PKC by phorbol ester produce the attenuation of naloxone-induced increase in NA turnover in the frontal cortex and naloxone-precipitated withdrawal jumping in morphine-dependent non-diabetic mice. Therefore, it is most likely that the attenuation of naloxone-induced increase in NA turnover in the frontal cortex and naloxone-precipitated withdrawal

jumping in morphine-dependent non-diabetic mice may be due to the desensitization of μ-opioid receptors induced by activation of PKC.

In Experiment 2-4, I observed that morphine-dependent diabetic mice jump less within 5 min after naloxone challenge than morphine-dependent non-diabetic mice. Furthermore, NA turnover in the frontal cortex was not affected by naloxone challenge. Moreover, the expression of naloxone-precipitated withdrawal jumping in morphine-dependent diabetic mice was not attenuated by i.c.v. pretreatment with the protein kinase A inhibitor KT-5720. There was a significant increase in the number of naloxone-precipitated withdrawal jumps within 5 min after naloxone challenge in morphine-dependent diabetic mice by i.c.v. pretreatment with calphostin C. Furthermore, I observed that pretreatment with a PKC activator, phorbol-12,13-dibutyrate (PDBu), 1 h before morphine injection attenuated the naloxone-precipitated withdrawal jumping in morphine-dependent non-diabetic mice, but not in morphine-dependent diabetic mice. This result suggests that the activation of PKC by PDBu before morphine injection inhibits the expression of naloxone-precipitated withdrawal jumping in morphine-dependent non-diabetic mice. Thus, it is likely that the attenuation of naloxone-precipitated withdrawal jumping in morphine-dependent diabetic mice may be due to the increased activity of PKC, since i.c.v. pretreatment with calphostin C increased, while i.c.v. pretreatment with PDBu did not affect, the number of naloxone-precipitated withdrawal jumps in morphine-dependent diabetic mice. In the present study, pretreatment with KT5720 did not affect the NA turnover in naloxone-challenged morphine-dependent diabetic mice. This result is consisted with behavioral data. Although pretreatment with calphostin C increased the naloxone-precipitated withdrawal jumping in morphine-dependent diabetic mice, pretreatment with calphostin C did not affect the NA turnover in naloxone-challenged morphine-dependent diabetic mice. This result contravenes the hypothesis that naloxone-precipitated withdrawal jumping is produced by the increase of NA turnover in the frontal cortex. There is accumulating evidence that the activation of PKC regulates several cellular functions through the phosphorylation of proteins, including some receptors, whose function is then down-regulated (Moran and Dascal, 1989). It has been reported that the activation of PKC by phorbol esters induces to decrease the affinity and/or responsiveness of α- and β-adrenergic receptors (Corvera and Garcia-Sainz, 1984; Sibley et al., 1984). Many investigators have reported that hyperglycemia or elevated glucose levels can increase diacylglycerol (DAG) levels and activate PKC in vascular tissue, cardiac tissues or cultured cells (Craven and De Rubertis, 1989; King et al., 1990; Inoguchi et al., 1992; Tanaka et al.,

1991). Furthermore, in Chapter 1, I indicated that calphostin C, a protein kinase C inhibitor, reverses the attenuation of DAMGO-induced antinociception in diabetic mice to the level in non-diabetic mice. Thus, it is expected that activation of PKC in diabetic mice may induce the phosphorylation of several proteins, including some receptors, in the brain. In view of these data and present data, it is possible that the enhancement of naloxone-precipitated withdrawal jumping in calphostin C-treated morphine-dependent diabetic mice may be result from the inhibition of phosphorylation of several receptors and proteins, including α- and β-adrenergic receptors, but not from the enhancement of NA release, induced by PKC. However, further detail studies are necessary before this possibility can be established with greater certainty.

In conclusion, the results of Experiment 2-4 indicate that both the brain PKC and PKA systems may play a major role in morphine dependence. Elucidating the possible in vivo cross-communication between these regulatory enzymes may be important for a better understanding of the cellular and molecular bases of morphine addiction. Moreover, the functional up-regulation in intracellular PKC systems may be partly associated with the reduction in the incidence of naloxone-precipitated withdrawal jumping 5 min after naloxone challenge in morphine-dependent diabetic mice.

In Experiment 2-5, pretreatment with ryanodine attenuated naloxone-precipitated withdrawal jumping in morphine-dependent non-diabetic mice. Furthermore, naloxone-induced enhancement of noradrenaline (NA) turnover in morphine-dependent non-diabetic mice was also attenuated by pretreatment with ryanodine. It has been reported that ryanodine blocks the Ca^{2+}/caffeine sensitive microsomal pools, which are involved in the phenomenon of Ca^{2+}-induced Ca^{2+} release (McPherson et al., 1991). Furthermore, it has been reported that ryanodine reduces the rate at which $[Ca^{2+}]_i$ increases with Ca^{2+} entry (Friel and Tsien, 1992). Furthermore, it is reported that ryanodine reduces the NA overflow (Bourreau, 1996). Thus, it is likely that the attenuation of naloxone-precipitated withdrawal jumping and NA turnover in morphine-dependent non-diabetic mice caused by ryanodine may be due to a decrease in $[Ca^{2+}]_i$. In contrast to ryanodine, thapsigargin enhanced the naloxone-precipitated withdrawal jumping in morphine-dependent non-diabetic mice. Moreover, thapsigargin also increased naloxone-induced enhancement of NA turnover in morphine-dependent non-diabetic mice. Thapsigargin selectively inhibits Ca^{2+} uptake into the IP_3-sensitive microsomal Ca^{2+} pool by inhibiting ATP/Mg-dependent ATPase (Bian et al., 1991). The subsequent depletion of this pool activates a low-conductance, Ca^{2+}-sensitive, non-voltage activated membrane current (Premack et al., 1994; Takemura et al., 1991). Thus, it is possible that thapsigargin increases

$[Ca^{2+}]_i$. It has been reported that cyclopiazonic acid, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase inhibitor, enhances the NA overflow in rat vas defences (Bourreau, 1996). Thus, it is possible that NA release in neurons may modulate the intracellular $[Ca^{2+}]_i$. Therefore, thapsigargin-induced enhancement of naloxone-precipitated withdrawal jumping and naloxone-induced increase in NA turnover in morphine-dependent non-diabetic mice may be due to the increase in $[Ca^{2+}]_i$.

Many investigators have indicated that the blockade of L- and N-type calcium channels inhibits the expression of naloxone-precipitated withdrawal signs in morphine-dependent mice and rats (Basilico et al., 1992; Bongianni et al., 1986; Tokuyama et al., 1995). In contrast to calcium channel blocker, L-type calcium channel opener, Bay K 8644, increases the severity of naloxone-precipitated withdrawal in acute morphine-dependent rats (Barrios and Baeyens, 1991). Furthermore, it has been reported that naloxone-induced enhancement of NA metabolism is blocked by L-type calcium channel blockers, nimodipin and verapamil (Bongianni et al., 1986). Hence, influx of Ca^{2+} into the cytoplasm through the calcium channels may be involved in the expression of naloxone-precipitated withdrawal signs and naloxone-induced activation of NA neurons in morphine-dependent animals. Intracellular calcium modulators used in this study have the same properties as calcium channel blocker or activator, since ryanodine suppresses and thapsigargin increases the naloxone-induced withdrawal jumping in morphine-dependent non-diabetic mice. However, Fundytus and Coderre (1994) reported that a single injection of thapsigargin prior to the precipitation of withdrawal failed to increase the severity of abstinence symptoms in morphine-dependent rats. This result contradicted our present data. In the study of Fundtus and Coderre (1994), thapsigargin was injected 10 min prior to naloxone injection, whereas in the present study, thapsigargin was injected 60 min before injection of naloxone. Furthermore, the estimation of naloxone-precipitated withdrawal in the present study also differed from Fundytus and Coderre (1994). Thus, differences in the results between our data and the results of Fundytus and Coderre (1994) may be due to different experimental conditions.

In Experiment 2-5, I observed that morphine-dependent diabetic mice jump less within 5 min after naloxone challenge than morphine-dependent non-diabetic mice. Furthermore, NA turnover in the frontal cortex was not affected by naloxone challenge. Moreover, the expression of naloxone-precipitated withdrawal jumping in morphine-dependent diabetic mice was not affected by pretreatment with either thapsigargin or ryanodine. It has been reported that chronic excessive intracellular calcium overload might

induce cardiac dysfunction in chronic diabetes (Heyliger et al., 1987; Nishio et al., 1990). In peripheral nerves of diabetic rats, mitochondrial and axoplasmic calcium levels were found to be increased by electron-probe X-ray microanalysis (Lowery et al., 1990). Moreover, voltage-dependent calcium currents through L- and N-channels are enhanced in dorsal root ganglion neurons of BB/Wor rats and diabetic mice in vivo (Hall et al., 1995; Kostyuk et al., 1995). These results suggest that the diabetic state may affect calcium homeostasis in neurons and various tissues. Furthermore, it has been suggested that the ability of caffeine, a ryanodine receptor agonist, to mobilize Ca^{2+} from intracellular stores is impaired in the diabetic aorta, since caffeine-induced contraction is significantly reduced in the diabetic aorta compared with that in the control aorta. Moreover, it has been reported that the activity of Ca^{2+}-ATPase is impaired in the diabetic rat (Janicki et al., 1994). Therefore, it is possible that diabetes is an anomalous intracellular calcium homeostasis. In the present study, i.c.v. pretreatment with thapsigargin and ryanodine did not affect the naloxone-precipitated withdrawal jumping in morphine-dependent diabetic mice. Thus, it is possible that the attenuation of naloxone-precipitated withdrawal jumping in morphine-dependent diabetic mice may be due to anomalous intracellular calcium homeostasis followed by changes in calcium store function.

In conclusion, the results of Chapter 2 suggested that the attenuation of naloxone-precipitated withdrawal jumping in morphine-dependent diabetic mice may be due to the dysfunction of central noradrenergic systems, followed by the activation of PKC and increase in $[Ca^{2+}]_i$.

Chapter 3

Modification of reinforcing effect of several addictive drugs by diabetes in mice

Introduction

The conditioned place preference paradigm is reliable technique for measuring the reinforcing properties of drugs, and is particularly useful for evaluating the reinforcing effects of addictive drugs, such as morphine, methamphetamine and cocaine. The conditioned place preference paradigm was recently used to demonstrate that morphine has a strong conditioning effect in mice as well as rats (Suzuki et al., 1993). Moreover, an additional important feature of the conditioned place preference paradigm is that both agonist and antagonists can be evaluated without any complicating behavioral motor effects (Hoffman, 1989).

The reinforcing effects of opioids, such as morphine and heroin, have been demonstrated using self-administration and conditioned place preference procedure in rodents and other mammalians. The place conditioning study in rats and mice provided evidence that motivational effects of μ- and δ-opioid agonists result from the activation of μ- and δ-opioid receptors in the central neuron systems, respectively (Shippenberg et al., 1987; Suzuki et al., 1991, 1993, 1994). Such findings suggest that the activation of supraspinal μ- and/or δ-opioid receptors is required for the expression of the reinforcing effect of opioids. Suzuki et al. (1993) previously demonstrated that systemic administered morphine produced a place preference in μ_1-opioid receptor-deficient CXBK mice. Furthermore, the morphine-induced place preference in mice was not blocked by pretreatment naloxonazine, a selective μ_1-opioid receptor antagonist. Based on these results, Suzuki et al. (1993) suggested that morphine produces its motivational effects via naloxonazine-insensitive μ-opioid receptors; namely μ_2-opioid receptors. Furthermore, they suggested that δ_1- and δ_2-opioid receptors may be involved in the modulation of the reinforcing effect of morphine, since the morphine-induced place preference is blocked by pretreated with naltrindole, a selective δ-opioid receptor antagonist, 7-benzylidenenaltrexone, a selective δ_1-opioid receptor antagonist, and naltriben, a selective δ_2-opioid receptor antagonist (Suzuki et al., 1994).

It has been reported that the antinociceptive potency, but not maximal effect, of morphine was decreased in several rodent models of hyperglycemia, including a spontaneously diabetic strain mice, streptozotocin-induced diabetes, a model animal of type I diabetes (Simon and Dewey, 1981). In Chapter 2, I indicated that the induction of physical dependence on morphine was also significantly decreased in streptozotocin-induced diabetic mice. In a clinical study, Morley et al. (1984) showed a significant decreased pain tolerance in

diabetic patients and in normal fasted subjects and suggested that the painful neuropathy experienced by some diabetic patients might involve an interaction of glucose with the action of endogenous opioid peptides.

Recently, Kamei et al. (1994a) demonstrated that the potency of naloxonazine-sensitive pharmacological actions of μ-opioid receptor agonists, i.e. supraspinal antinociception, Straub tail reaction and locomotor-enhancing effect, in diabetic mice were markedly reduced as compared with in non-diabetic mice. Furthermore, in Chapter 2, I indicated that μ_1-opioid receptor-mediated naloxone-precipitated signs of withdrawal from physical dependence on morphine in diabetic mice are significantly less than those in non-diabetic mice. In contrast, there was no significant difference in naloxonazine-insensitive pharmacological actions of μ-opioid receptor agonist, i.e. spinal antinociception and antitussive effect, between diabetic mice and non-diabetic mice (Kamei et al., 1993a, b, 1994a). Therefore, we proposed that mice with diabetes are selectively hyporesponsive to activation of μ_1-opioid receptors, but are normally responsive to μ_2-opioid receptors. On the other hand, Kamei et al. (1994b) previously reported that the 7-benzylidenenaltrexone-sensitive antinociceptive effect of [D-Pen^{2,5}]enkephalin (DPDPE) was significantly greater in diabetic mice than in non-diabetic mice, whereas there was no significant difference in the naltriben-sensitive antinociceptive effect of [D-Ala^2]deltorphineII between diabetic and non-diabetic mice. These findings suggested that mice with diabetes are selectively hyperresponsive to activation of δ_1-opioid receptors, but are normally responsive to δ_2-opioid receptors. Therefore, functional abnormalities in μ- and δ-opioid receptor functions of diabetic animals may alter the reinforcing effect of morphine.

Thus, in Experiment 3-1, I compared the morphine-induced place preference in diabetic and non-diabetic mice to clarify the hypothesis that functional abnormalities in μ- and δ-opioid receptor functions of diabetic animals may alter the reinforcing effect of morphine.

It has been reported that the psychostimulant-induced place preference is abolished by pretreatment with dopamine receptor antagonists. Indeed, amphetamine-induced place preference was antagonized by pretreatment with haloperidol, a dopamine receptor antagonist (Spyraki et al., 1982), or 7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SCH23390), a selective dopamine D_1 receptor antagonist (Leone and Di Chiara, 1987). Furthermore, microinjection of (+)-amphetamine into the nucleus accumbens resulted in a place preference (Carr and White, 1986). Moreover, amphetamine increases extracellular

dopamine in various terminal dopamine areas, as estimated by brain microdialysis studies in free-moving rats (Di Chiara and Imperato, 1988). Based on these results, Di Chiara (1995) proposed that enhanced dopamine release from the nucleus accumbens may play a critical role in the acquisition and expression of psychic dependence on drugs of abuse.

Dopamine D_3 receptors are part of the dopamine D_2-like receptor family (Sokoloff et al. 1990; Seeman and Van Tol 1994). Dopamine D_3 receptors are found mainly in limbic regions of the brain, and are involved in cognition, emotion and endocrine functions (Léveque et al., 1992). The dopamine receptor agonist 7-hydroxy-*N,N*-di-n-propyl-2-aminotetralin (7-OH-DPAT) reportedly has a 50- to 100-hold higher affinity for dopamine D_3 receptors than dopamine D_2 receptors in genetically transfected cells (Lévesque et al. 1992). Thus, 7-OH-DPAT has been used to investigate possible dopamine D_3 receptor-mediated functions. Recent evidence has suggested that dopamine D_3 receptors may modulate the reinforcing effects of cocaine and morphine. Since the self-administration of cocaine in rats was decreased by co-administration of 7-OH-DPAT, Caine and Koob (1993) suggested that preferential stimulation of dopamine D_3 receptors by a low dose of 7-OH-DPAT may modulate the reinforcing properties of cocaine. Furthermore, Suzuki et al. (1995) reported that morphine-induced hyperlocomotion was attenuated by pretreatment with 7-OH-DPAT. They also indicated that the morphine-induced increase in the dopamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the limbic forebrain (nucleus accumbens and olfactory tubercle) was attenuated by 7-OH-DPAT. These results suggest that the activation of the presynaptic dopamine D_3 receptors in the mesolimbic dopamine system may attenuate the expression of morphine-induced hyperlocomotion. Moreover, it has been reported that the acquisition and expression of the morphine-induced place preference are antagonized by pretreatment with 7-OH-DPAT (Fonseca et al., 1995). These results suggest that the activation of dopamine D_3 receptors attenuates the rewarding properties of opioids.

It is reported that spontaneous locomotor activity in diabetic mice was significantly greater than that in non-diabetic mice (Kamei et al. 1994d). Furthermore, haloperidol and SCH23390, a selective dopamine D_1 receptor antagonist, significantly reduced spontaneous locomotor activity in diabetic mice, but not in non-diabetic mice (Kamei et al. 1994d). Moreover, dopamine turnover (DOPAC+HVA/dopamine) in the limbic forebrain in diabetic mice was significantly higher than that in non-diabetic mice (Kamei et al. 1994d). These results led to propose the possibility that neurotransmission in mesolimbic dopamine systems may be enhanced, rather than reduced, in diabetic mice relative to that in non-diabetic mice

(Kamei et al., 1994d). Thus, it is possible that methamphetamine-induced place preference may be greater in diabetic mice than in non-diabetic mice.

Thus, in Experiment 3-2, I compared methamphetamine-induced place preference in diabetic and non-diabetic mice to clarify the hypothesis that functional abnormalities in mesolimbic dopamine systems, especially dopamine D_3 receptor function, in diabetic mice may alter the reinforcing effect of methamphetamine.

Although the reinforcing and stimulant properties of cocaine and related drugs have been shown to correlate with their binding properties at the dopamine transporter (Bergman et al., 1989; Ritz et al., 1987), it is possible that the cocaine-induced place preference may be changed in diabetic mice. The aim of Experiment 3-3 was to compared the cocaine-induced place preference in diabetic and non-diabetic to clarify the hypothesis that functional abnormalities in mesolimbic dopamine systems in diabetic mice may alter the reinforcing effect of cocaine.

Experiment 3-1: Modification of morphine-induced place preference by diabetes

Materials and Methods

Animals

Male ICR mice (Tokyo Laboratory Animals Science Co., Tokyo), weighing about 20 g at the beginning of the experiments, were used. The mice were housed under the condition as described in Chapter 1-1. Animals were rendered diabetic by an injection of streptozotocin (200 mg/kg, i.v.) prepared in 0.1 N citrate buffer at pH 4.5. Age-matched non-diabetic mice were injected with vehicle alone. The experiments were conducted 2 weeks after injection of streptozotocin or vehicle. Mice serum glucose levels above 400 mg/dl were considered diabetic.

Place conditioning

Place conditioning was conducted as previously described using a minor modification of biased procedure (Suzuki et al., 1991) according to the method of Bardo et al. (1984). The apparatus used was a shuttle box (15 x 30 x 15 cm) which was divided into two compartments of equal size. One compartment was white with a textured floor and the other was black with a smooth floor. For conditioning, mice were immediately confined to the white compartment after drug injections and to the black compartment after saline injections. Saline conditioning was conducted before confinement to both compartment. In addition, some group of mice was immediately confined to the black compartment after morphine injection and to the white compartment after saline injections. Conditioning sessions (3 for drug: 3 for vehicle) were conducted for a 60-min period once a day. The order of the 6 conditioning sessions was conducted alternating. On day 7, tests of conditioning were performed as follows: the partition separating the two compartments was raised 7 cm above the floor, and a neutral platform was inserted along the seam separating the compartments. Mice were not given any injections before test session. The time spent in each compartment during a 900-s session was then measured by a infrared beam sensor (KN-80, Natume, Tokyo, Japan) in a blinded fashion. The position of the mouse was defined by the position of its body. All sessions were conducted under conditions of dim illumination (40 lux) and white masking

noise. Mice exhibited no preference for either of the place states under these conditions.

Drugs

Streptozotocin was purchased from Sigma Chemical Co., St. Louis, MO, USA. Morphine hydrochloride was purchased from Sankyo, Co., Tokyo, Japan. β-Funaltrexamine, naloxonazine, 7-benzylidenenaltrexone, naltriben and 2-methyl-4aa-(3-hydroxyphenyl)-1,2,3,4,4a,5,12,12aa-octahydroquinolino[2,3,3-g]isoquinoline (TAN-67) were synthesized by Dr. Nagase (Toray Industries, Kamakura, Japan). All drugs were dissolved in 0.9% saline solution. β-Funaltrexamine (10 and 20 mg/kg, s.c.) and naloxonazine (35 mg/kg, s.c.) were injected 24h before injection of morphine. 7-Benzylidenenaltrexone (0.3 and 0.7 mg/kg, s.c.) was injected 30 min before injection of morphine. Naltriben (0.1 and 0.5 mg/kg, s.c.) was injected 30 min before injection of morphine. The dose and schedule for each opioid receptor antagonist in this study were determined as described previously (Suzuki et al., 1993, 1994). Each antagonist was injected before each morphine injection. Glucose was injected 30 min before injection of morphine.

Data analysis

Conditioning scores represent the time spent in the drug-paired place minus the time spent in the vehicle-paired place and are expressed as mean ± S.E.. Statistical analysis of difference between groups was assessed with Wilcoxon rank-sum test (comparison of two groups) or one-way analysis of variance (ANOVA) followed by Kruskal-Wallis test (comparison between multiple groups). The potency ratio of the morphine-induced place preference between non-diabetic mice and diabetic mice was calculated using Program 11 of the Pharmacological Calculations systems of Tallarida and Murray (1987).

Results

Effects of diabetes on morphine-induced place preference

As shown in Fig. 3-1, none of the mice receiving saline in conditioning sessions exhibited a significant preference for either compartment of the test box. Testing of experimentally saline-treated mice on the test session revealed that neither non-diabetic nor diabetic mice showed a significant preference for one side of the test box over the other. The place conditioning produced by morphine is shown in Fig. 3-1. In non-diabetic mice, morphine, at dose ranges from 3 to 30 mg/kg, s.c., caused a dose-related preference for the drug-associated place, and significant conditioning was observed at doses of 5, 10 and 30 mg/kg. In diabetic mice, morphine (1 mg/kg) induced a slight place preference, although this effect was not statistically significant. Significant conditioning was observed at doses of 3, 5 and 10 mg/kg in diabetic mice. At these doses, all diabetic mice exhibited preference for the drug-associated place. On the other hand, when morphine (5 mg/kg, s.c.) was paired with black compartment, morphine also caused a significant place preference in both diabetic (mean conditioning score of 178.9 $\pm$ 67.9 s, n=7) and non-diabetic mice (mean conditioning score of 117.2 $\pm$ 34.6 s, n=8). Pretreatment with glucose (30 mmol/kg, i.p.) increased the serum glucose levels of non-diabetic mice (432.3 $\pm$ 11.3 mg/dl, n=8) to the level of diabetic mice. However, morphine-induced place preference in non-diabetic mice was not affected by pretreatment with glucose. There was no significant difference in the morphine-induced place preference between glucose-treated non-diabetic (139.6 $\pm$ 38.1 s, n=8) and glucose-untreated non-diabetic mice (112.9 $\pm$ 36.4 s, n=10).

Influence of μ-opioid receptor antagonists pretreatment upon the morphine-induced place preference

β-Funaltrexamine (10 and 20 mg/kg, s.c.), a selective μ-opioid receptor antagonist, caused aversion for the drug-associated place, and significant conditioning aversion was observed at dose of 20 mg/kg in non-diabetic mice (Fig. 3-2). However, β-funaltrexamine did not cause neither a place aversion nor place preference in diabetic mice. Moreover, naloxonazine (35 mg/kg, s.c.), a selective μ_1-opioid receptor antagonist, caused a significant place aversion in non-diabetic mice, but not in diabetic mice.

The effects of pretreatment with β-funaltrexamine on the place preference produced by morphine in both diabetic and non-diabetic mice are shown in Fig. 3-3. After pretreatment with saline, morphine (5 mg/kg) produced a significant preference for drug-paired place in both non-diabetic mice and diabetic mice. Thus, pretreatment with saline did not alter this effect of morphine. However, the morphine-induced place preference was significantly antagonized by pretreatment with β-funaltrexamine in both non-diabetic and diabetic mice. The mean conditioning score of morphine (5 mg/kg) in both non-diabetic and diabetic mice pretreated with β-funaltrexamine at doses of 10 mg/kg and 20 mg/kg were significantly lower than those in saline-pretreated non-diabetic and diabetic mice, respectively. Furthermore, morphine (3 mg/kg)-induced place preference in diabetic mice was also antagonized by pretreatment with β-funaltrexamine. On the other hand, as shown in Fig. 3-4, pretreatment with naloxonazine did not modify the morphine (5 mg/kg)-induced place preference in non-diabetic mice. The mean conditioning score did not significantly differ from that in mice that had been pretreated with saline. Furthermore, naloxonazine also had no effect on morphine-induced place preference in diabetic mice. Indeed, there was no significant difference in the mean conditioning score of morphine (3 and 5 mg/kg) between saline-pretreated diabetic mice and naloxonazine-treated diabetic mice. Thus, pretreatment with naloxonazine did not modify the appetitive effect of morphine in either diabetic or non-diabetic mice.

Influence of δ_1- and δ_2-opioid receptor antagonists pretreatment upon morphine-induced place preference

7-Benzylidenenaltrexone (0.3 and 0.7 mg/kg, s.c.), a selective δ_1-opioid receptor antagonist, alone caused neither place preference nor place aversion in both non-diabetic and diabetic mice. Furthermore, naltriben (0.1 and 0.5 mg/kg, s.c.), a selective δ_2-opioid receptor antagonist, alone also did not cause either a place preference or a place preference. The effects of 7-benzylidenenaltrexone and naltriben on the morphine-induced place preference are shown in Fig. 3-5 and 3-6. Morphine-induced place preference was antagonized by pretreatment with naltriben in a dose-related manner in both diabetic and non-diabetic mice. Indeed, naltriben, at a dose of 0.5 mg/kg, significantly abolished the morphine (5 mg/kg)-induced place preference. In diabetic mice, naltriben, at a dose of 0.5 mg/kg significantly antagonized the morphine (3 mg/kg)-induced place preference. Moreover, naltriben, at a dose of 0.5 mg/kg, significantly suppressed the morphine (5 mg/kg)-induced place preference in

diabetic mice. On the other hand, 7-benzylidenenaltrexone, at a dose of 0.7 mg/kg, significantly abolished the morphine (5 mg/kg)-induced place preference in non-diabetic mice. Moreover, 7-benzylidenenaltrexone, at a dose of 0.7 mg/kg, also abolished the morphine (10 mg/kg)-induced place preference in non-diabetic mice. In diabetic mice, although the lower dose (3 mg/kg) of morphine-induced place preference was significantly suppressed by pretreatment with 7-benzylidenenaltrexone, at a dose of 0.7 mg/kg, the higher dose of morphine (5 mg/kg)-induced place preference was not antagonized by pretreatment with 7-benzylidenenaltrexone.

As shown in Fig. 3-3 and 3-6, the morphine (5 mg/kg)-induced place preference in diabetic mice was significantly but not completely suppressed by pretreatment with β-funaltrexamine, and was not affected by 7-benzylidenenaltrexone. However, when β-funaltrexamine (10 mg/kg) and 7-benzylidenenaltrexone (0.7 mg/kg) was co-pretreated with morphine, the morphine (5 mg/kg)-induced place preference in diabetic mice was completely abolished (morphine alone, 199.4 ± 42.0 s, n=8; morphine with β-funaltrexamine, 71.2 ± 44.9 s, n=8; morphine with β-funaltrexamine and 7-benzylidenenaltrexone, 19.1 ± 52.1 s, n=8).

TAN-67-induced place preference in diabetic mice

The place conditioning produced by TAN-67, a non-peptidic selective δ-opioid receptor agonist, is shown in Fig. 3-7. In non-diabetic mice, TAN-67, at doses of 10, 30 and 56 mg/kg, s.c., cause neither a place preference nor a place aversion for the drug-associated place. However, in diabetic mice, TAN-67 caused a dose-dependent place preference. TAN-67, at a dose of 10 mg/kg, induced a slight place preference, although this effect was not statistically significant. Significant conditioning was observed at a dose of 30 mg/kg.

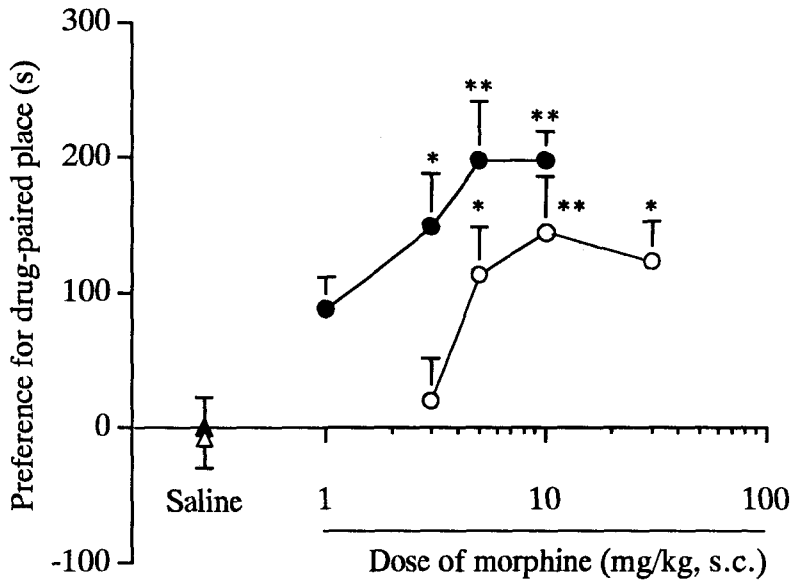

Figure 3-1. Place conditioning produced by morphine (circle) in non-diabetic (open symbol) and diabetic (closed symbol) mice. Ordinate: mean difference (s) between times spent on drug- and saline-paired sides of test box. Each point represents the mean ± S.E. of 10-16 mice. The asterisk denotes significant preference conditioning (Wilcoxon test: *P<0.05, **P<0.01 vs. respective saline alone (triangle)).

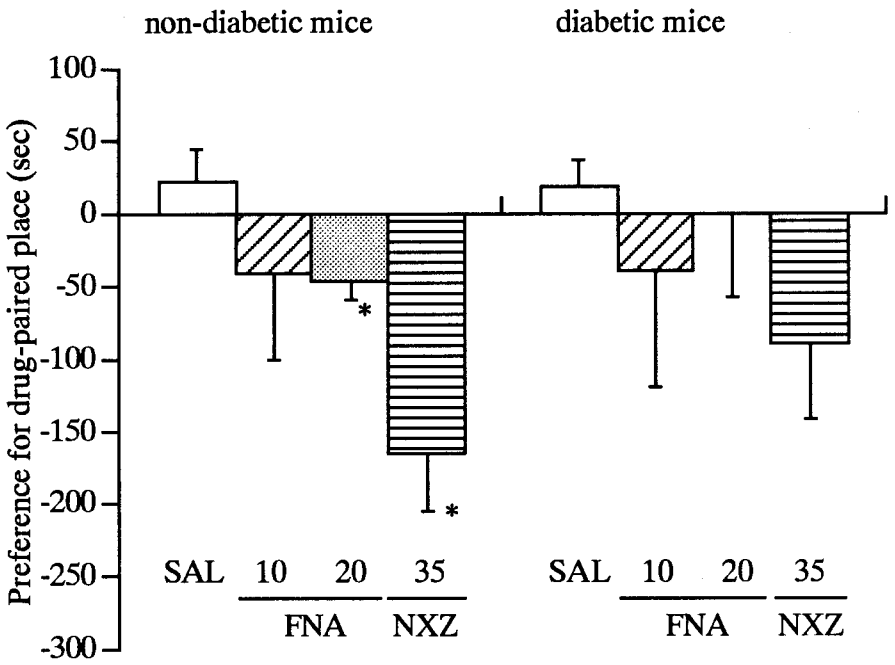

Figure 3-2. Place conditioning produced by β-funaltrexamine (FNA, 10 and 20 mg/kg, s.c.) and naloxonazine (NXZ, 35 mg/kg, s.c.) in non-diabetic and diabetic mice. Each column represents the mean ± S.E. of 7-12 mice. The asterisk denotes significant place aversion conditioning (Wilcoxon test: *P<0.05 vs. saline (SAL) alone).

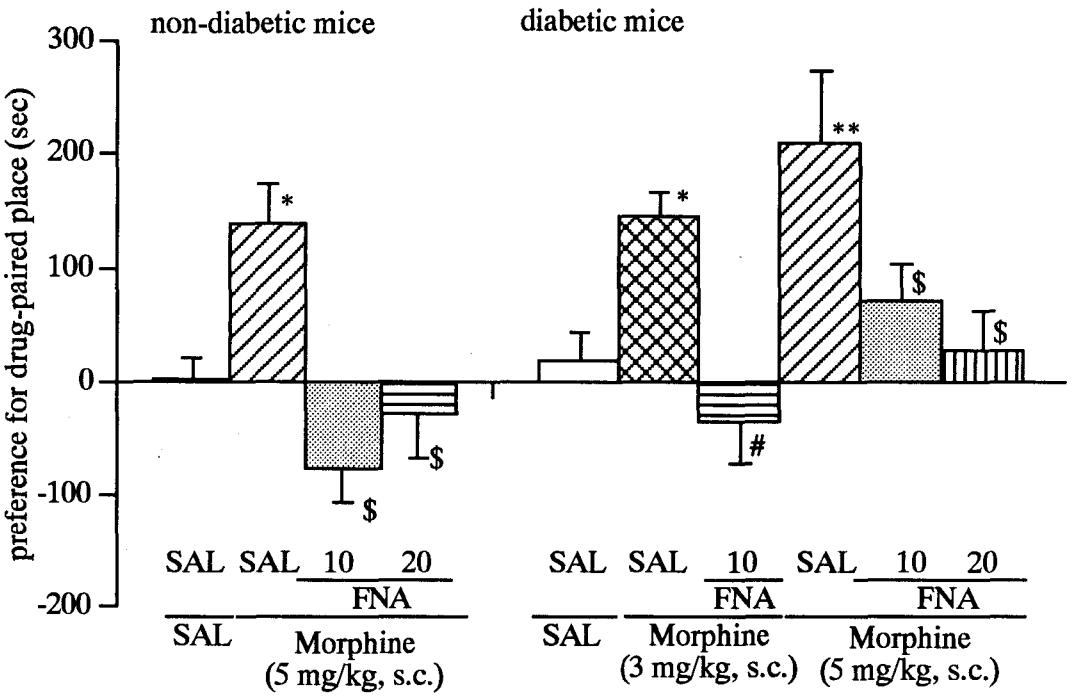

Figure 3-3. Effect of β-funaltrexamine (FNA, 10 and 20 mg/kg, s.c.) on morphine (3 or 5 mg/kg, s.c.)-induced conditioned place preference in non-diabetic and diabetic mice. Each column represents the mean ± S.E. of 7-13 mice. The asterisk denotes significant preference conditioning (Wilcoxon test: *P<0.05, **P<0.01 vs. saline (SAL) alone). The sharp denotes significant difference from morphine alone (Wilcoxon test: #P<0.05). Significant difference from respective morphine (5 mg/kg) alone (Kruskal-Wallis test followed by Wilcoxon test: $P<0.05).

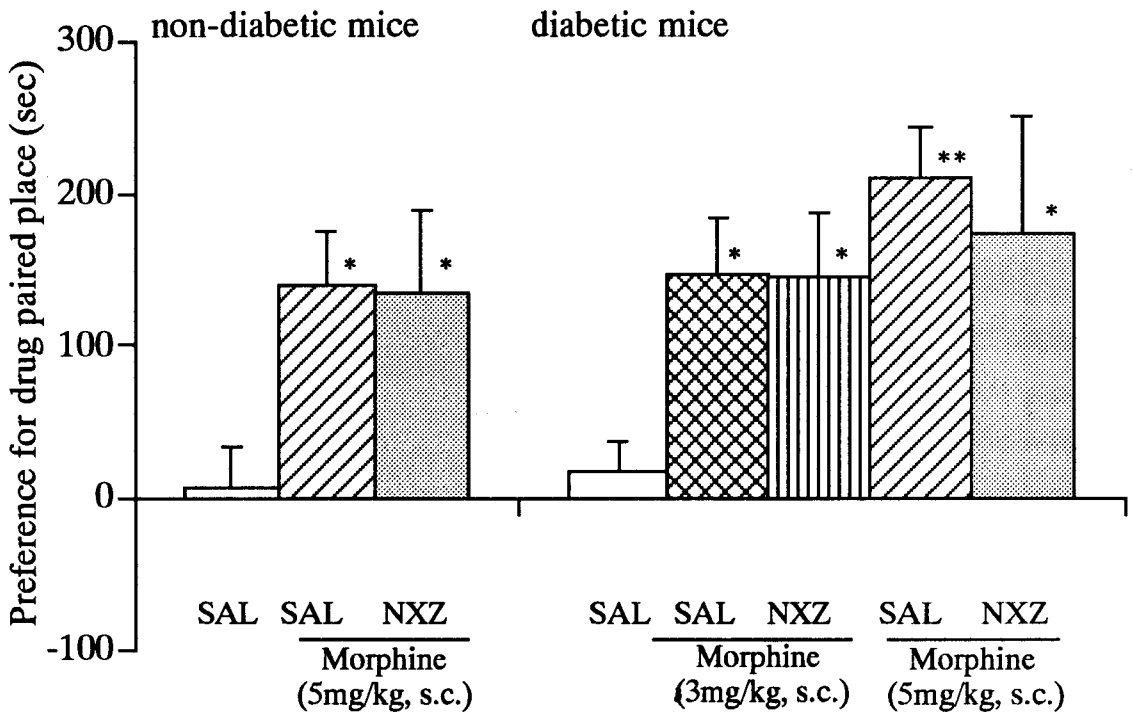

Figure 3-4. Effect of naloxonazine (NXZ, 35 mg/kg, s.c.) on morphine (3 or 5 mg/kg, s.c.)-induced conditioned place preference in non-diabetic and diabetic mice. Each column represents the mean ± S.E. of 7-12 mice. The asterisk denotes significant preference conditioning (Wilcoxon test: *P<0.05, **P<0.01 vs. saline (SAL) alone).

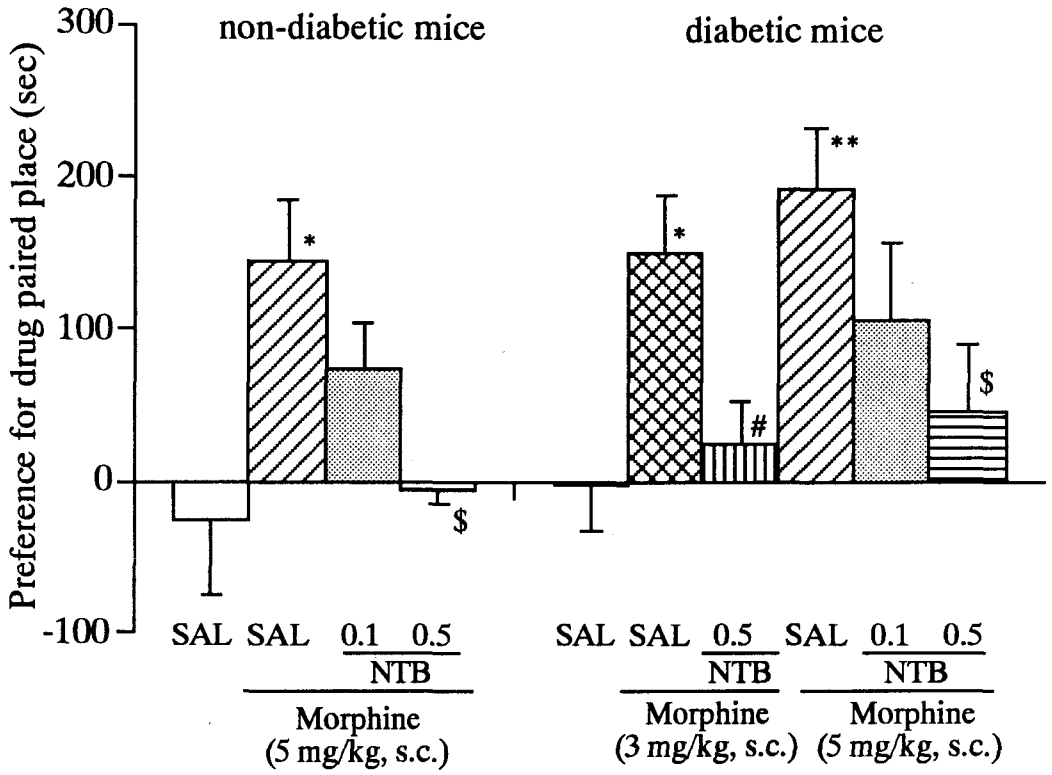

Figure 3-5. Effects of naltriben (NTB, 0.1 and 0.5 mg/kg, s.c.) on morphine (3 or 5 mg/kg, s.c.)-induced conditioned place preference in non-diabetic and diabetic mice. Each column represents the mean ± S.E. of 7-14 mice. The asterisk denotes significant preference conditioning (Wilcoxon test: *P<0.05, **P<0.01 vs. saline (SAL) alone). The sharp denotes significant difference from morphine alone (Wilcoxon test: #P<0.05). Significant difference from respective morphine (5 mg/kg) alone (Kruskal-Wallis test followed by Wilcoxon test: $P<0.05).

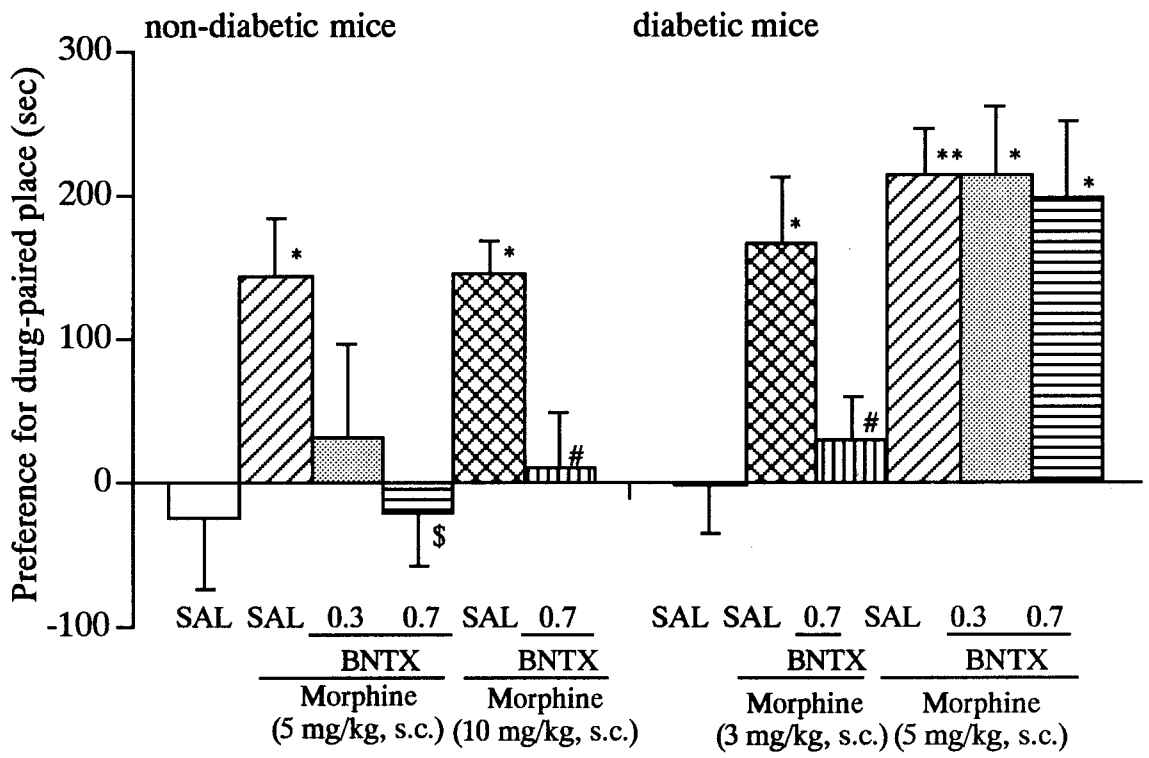

Figure 3-6. Effect of 7-benzylidenenaltrexone (BNTX, 0.3 and 0.7 mg/kg, s.c.) on morphine (3 or 5 mg/kg, s.c.)-induced conditioned place preference in non-diabetic and diabetic mice. Each column represents the mean ± S.E. of 7-13 mice. The asterisk denotes significant preference conditioning (Wilcoxon test: *P<0.05, **P<0.01 vs. saline (SAL) alone). The sharp denotes significant difference from morphine alone (Wilcoxon test: #P<0.05). Significant difference from respective morphine (5 mg/kg) alone (Kruskal-Wallis test followed by Wilcoxon test: $P<0.05).

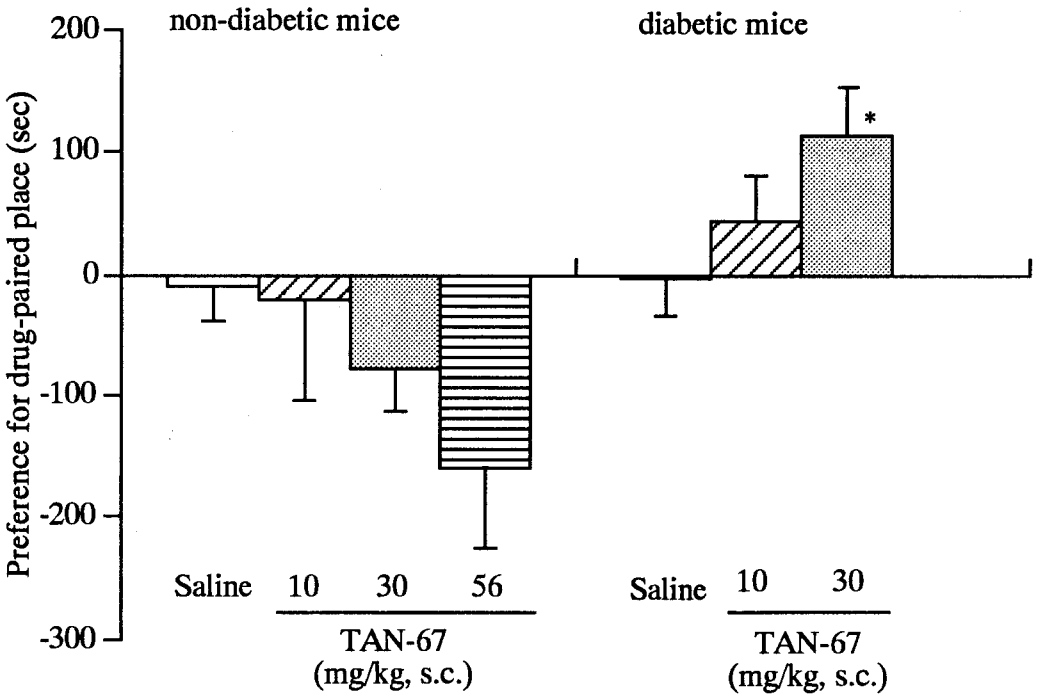

Figure 3-7. Place conditioning produced by TAN-67 (10 and 30 mg/kg, s.c.) in non-diabetic and diabetic mice. Ordinate: mean difference (s) between times spent on drug- and saline- paired sides of the test box. Each column represents the mean ± SE of 7-16 mice. The asterisk denotes significant preference conditioning (Wilcoxon test: *P<0.05 vs. saline alone).

Experiment 3-2: Effects of diabetes on methamphetamine-induced place preference in mice.

Materials and Methods

Animals

Male ICR mice (Tokyo Laboratory Animals Science Co., Tokyo), weighing about 20 g at the beginning of the experiments, were used. The mice were housed under the condition as described in Chapter 1-1. Animals were rendered diabetic by an injection of streptozotocin (200 mg/kg, i.v.) prepared in 0.1 N citrate buffer at pH 4.5. Age-matched non-diabetic mice were injected with vehicle alone. The experiments were conducted 2 weeks after injection of streptozotocin or vehicle. Mice with serum glucose levels above 400 mg/dl were considered diabetic.

Place conditioning

Place conditioning was conducted as previously described using a minor modification of an unbiased procedure (Suzuki et al., 1990). The apparatus used was a shuttle box (15 x 30 x 15 cm: w x l x h) which was divided into two compartments of equal size. One compartment was white with a textured floor and the other was black with a smooth floor. For conditioning, mice were confined to one compartment after drug injections and to the other compartment after saline injections. Conditioning session consisted of 6 alternate day injections of drug or vehicle (saline). Immediately following drug injection, mice were confined to one compartment. Following vehicle injections they were confined to the other compartment. Treatment compartment and the presentation order of drug and vehicle were counterbalanced for each drug dose. Conditioning sessions were 60 min in duration. On day 7, tests of conditioning were performed as follows: the partition separating the two compartments was raised to 7 cm above the floor, and a neutral platform was inserted along the seam separating the compartments. The time spent in each compartment during a 900-sec session was then measured in a blinded fashion by an infrared beam sensor (KN-80, Natume, Tokyo, Japan). The position of the mouse was defined by the position of its body. All sessions were conducted under conditions of dim illumination and masking white noise.

Drugs

Streptozotocin was purchased from Sigma Chemical Co., St. Louis, MO, USA. Methamphetamine hydrochloride was purchased from Dainippon Seiyaku, Co., Tokyo, Japan. 7-OH-DPAT {(±)-7-hydroxy-*N*,*N*-di-n-propyl-2-aminotetralin} and quinpirole (trans-(-)-4aR-4a,5,6,7,8,8a,9-octahydro-5-propyl-1H-pyrazolo[3,4-g]quinoline) were purchased from Research Biochemicals, Inc., Natick, MA, USA). Streptozotocin was dissolved in 0.1 N citrate buffer. Other drugs were dissolved in sterile 0.9 % NaCl solution. 7-OH-DPAT was injected s.c. at 10 min before methamphetamine injection. Quinpirole was injected i.c.v. 10 min before methamphetamine administration. Intracerebroventricular (i.c.v.) administration (5 μl/mouse) was performed according to the method described in Experiment 1-2 using a 50 μl Hamilton syringe. Each antagonist was injected before each conditioning session for methamphetamine. The dose, route and schedule for quinpirole and 7-OH-DPAT in this study were determined as described previously (Kamei et al. 1996a; Funada et al., 1995).

Data analysis

Conditioning scores represent the time spent in the drug-paired place minus the time spent in the vehicle-paired place and are expressed as mean ± S.E. Dose-response curves were analyzed using a one-way random factorial analysis of variance and linear regression analysis. The Wilcoxon test was used to determine whether individual doses produced a significant conditioning (*$P<0.05$).

Results

Effects of diabetes on methamphetamine-induced place preference

As shown in Fig. 3-8, none of the mice that received saline in conditioning sessions exhibited a significant preference for either compartment of the test box. The mean conditioning scores were -2.0 ± 33.8 s (n=8) for non-diabetic mice and -1.3 ± 47.5 s (n=8) for diabetic mice. The place conditioning produced by methamphetamine is also shown in Fig. 3-8. In non-diabetic mice, methamphetamine, at dose ranges from 0.3 to 3 mg/kg, s.c., caused a dose-related preference for the drug-associated place, and significant conditioning was observed at doses of 1 and 3 mg/kg. In diabetic mice, methamphetamine (0.1 mg/kg) produced a slight place preference, but this effect was not statistically significant. Significant conditioning was observed at doses of 0.3 mg/kg and 1 mg/kg in diabetic mice. At these doses, all of the diabetic mice exhibited preference for the drug-associated place.

Effect of quinpirole on methamphetamine-induced place preference

The effect of pretreatment with quinpirole, a dopamine D_2/D_3 receptor agonist, on the place preference produced by methamphetamine is shown in Fig. 3-9. Methamphetamine (3 mg/kg for non-diabetic mice and 0.3 mg/kg for diabetic mice) following i.c.v. pretreatment with saline produced a significant preference for the drug-paired place in both non-diabetic mice (163.6 ± 51.6 s, n=8) and diabetic mice (166.1 ± 53.5 s, n=8). However, methamphetamine-induced place preference was significantly antagonized by i.c.v. pretreatment with quinpirole (5 nmol) in both non-diabetic and diabetic mice. The mean conditioning score for methamphetamine following pretreatment with quinpirole in non-diabetic and diabetic mice was -12.9 ± 31.6 s (n=8) and 17.8 ± 48.0 s (n=12), respectively.

Effect of 7-OH-DPAT on methamphetamine-induced place preference

The effect of 7-OH-DPAT, a selective dopamine D_3 receptor agonist, on methamphetamine-induced place preference is shown in Fig. 3-10. Methamphetamine (3 mg/kg for non-diabetic mice and 0.3 mg/kg for diabetic mice) following s.c. pretreatment with saline produced a significant preference for the drug-paired place in both non-diabetic mice (176.5 ± 45.0 s, n=8) and diabetic mice (163.6 ± 37.5 s, n=8). This methamphetamine-induced place preference was significantly antagonized by pretreatment with 7-OH-DPAT (0.1 mg/kg) in non-diabetic mice. The mean conditioning score for methamphetamine

following pretreatment with 7-OH-DPAT (-31.1 ± 40.0 s, n=8) was significantly ($P<0.05$) lower than that in saline-pretreated non-diabetic mice. On the other hand, a significant methamphetamine-induced place preference was not observed in 7-OH-DPAT (0.1 mg/kg)-pretreated diabetic mice. However, a statistically significant difference in methamphetamine-induced place preference was not observed between saline-pretreated diabetic mice and 7-OH-DPAT-pretreated diabetic mice.

7-OH-DPAT-induced place conditioning in diabetic mice

The place conditioning produced by 7-OH-DPAT, a dopamine D_3 receptor agonist, is shown in Fig. 3-11. In non-diabetic mice, 7-OH-DPAT produced a significant aversion for the drug-associated place. 7-OH-DPAT, at doses of 1 and 3 mg/kg, induced a significant place aversion (mean conditioning scores of -101.4 ± 58.9 s, n=8 for 1.0 mg/kg, s.c. and -188.3 ± 64.7 s, n=8 for 3.0 mg/kg, s.c.). However, in diabetic mice, 7-OH-DPAT produced neither a preference nor aversion for the drug-associated place (mean conditioning scores of -5.0 ± 58.2 s, n=8 for 1.0 mg/kg, s.c. and -59.8 ± 33.0 s, n=8 for 3.0 mg/kg, s.c.).

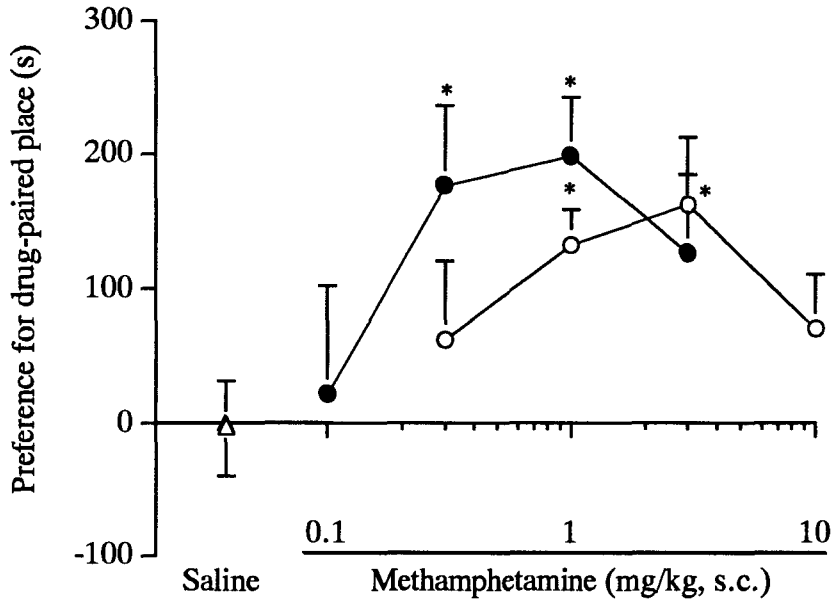

Figure 3-8. Place conditioning produced by methamphetamine in diabetic (closed symbol) and non-diabetic (open symbol) mice. Each point represents the mean conditioning score ± S.E. of 8-12 mice. The asterisks denote significant preference conditioning (Wilcoxon test: *P<0.05 vs. respective saline alone (triangle)).

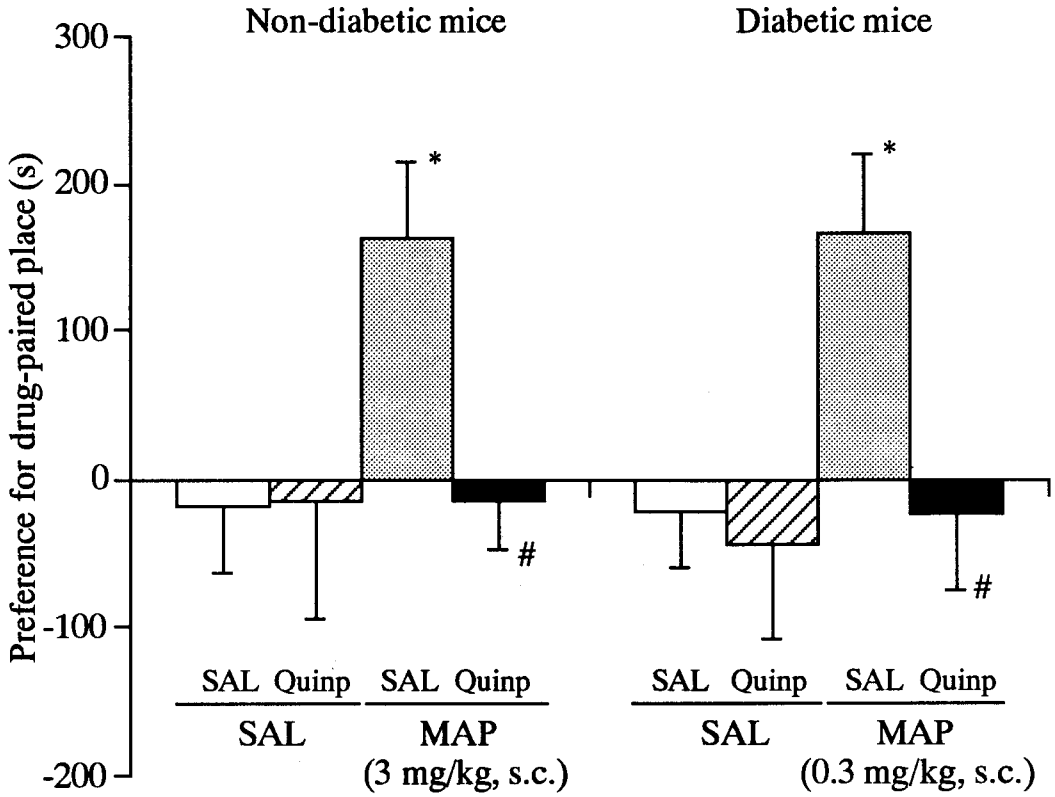

Figure 3-9. Effect of quinpirole (Quinp; 5 nmol, i.c.v.) on methamphetamine (MAP)-induced place preference in diabetic and non-diabetic mice. Each column represents the mean conditioning score ± S.E. of 8-12 mice. The asterisks denote significant preference conditioning (Wilcoxon test: *P<0.05 vs. saline (SAL) alone). The sharp denotes a significant difference from methamphetamine alone (Wilcoxon test: #P<0.05).

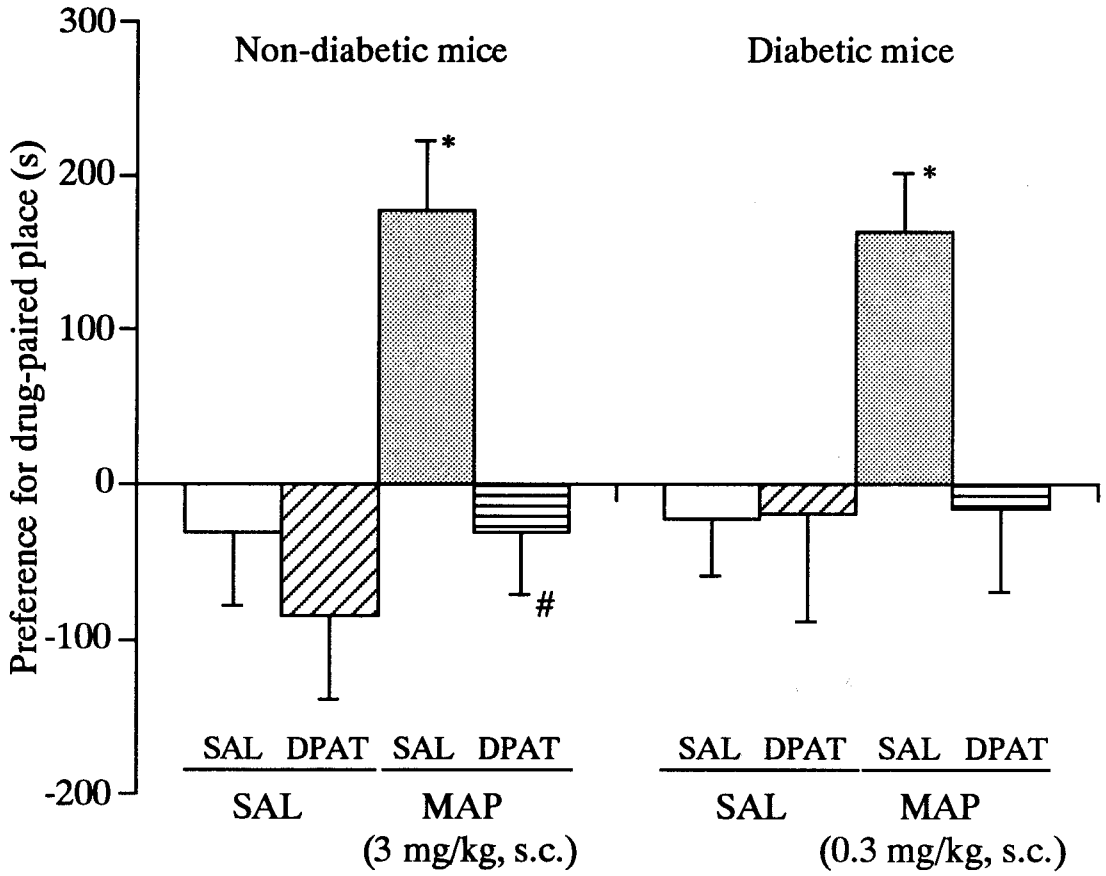

Figure 3-10. Effect of 7-OH-DPAT (DPAT; 0.1 mg/kg, s.c.) on methamphetamine (MAP)-induced place preference in diabetic and non-diabetic mice. Each column represents the mean conditioning score ± S.E. of 8-12 mice. The asterisks denote significant preference conditioning (Wilcoxon test: *P<0.05 vs. saline (SAL) alone). The sharp denotes a significant difference from methamphetamine alone (Wilcoxon test: #P<0.05).

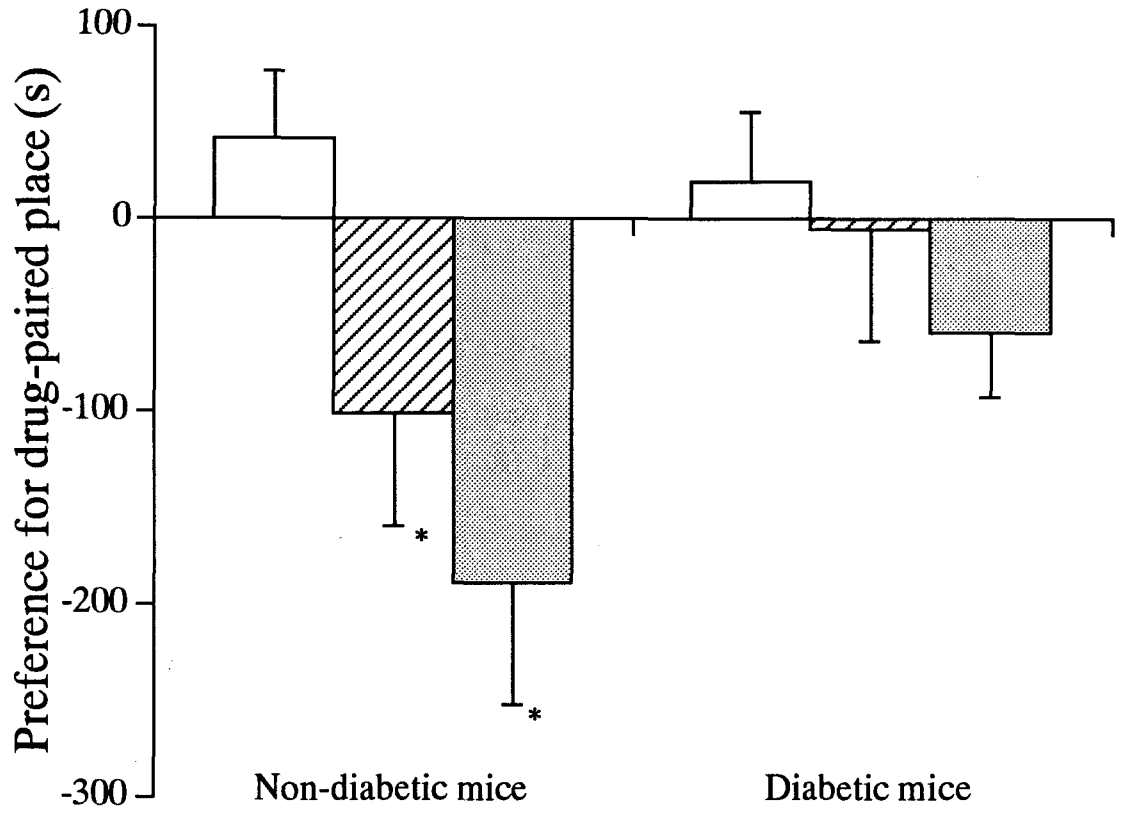

Figure 3-11. Place conditioning produced by 7-OH-DPAT (1 mg/kg, s.c., hatched column; 3 mg/kg, s.c., dotted column) in diabetic and non-diabetic mice. Each column represents the mean conditioning score ± S.E. of 8-12 mice. The asterisks denote significant aversion conditioning (Wilcoxon test: *P<0.05 vs. saline alone (open column).

Experiment 3-3: Effects of streptozotocin-induced diabetes on place conditioning action of cocaine in mice

Materials and Methods

Animals

Male ICR mice (Tokyo Laboratory Animals Science Co., Tokyo), weighing about 20 g at the beginning of the experiments, were used. The mice were housed under the condition as described in Chapter 1-1. Animals were rendered diabetic by an injection of streptozotocin (200 mg/kg, i.v.) prepared in 0.1 N citrate buffer at pH 4.5. Age-matched non-diabetic mice were injected with vehicle alone. The experiments were conducted 2 weeks after injection of streptozotocin or vehicle. Mice with serum glucose levels above 400 mg/dl were considered diabetic.

Place conditioning

Place conditioning was conducted as previously described using a minor modification of an unbiased procedure as described in Experiment 3-2. The apparatus used was a shuttle box (15 x 30 x 15 cm: w x l x h) which was divided into two compartments of equal size. One compartment was white with a textured floor and the other was black with a smooth floor. For conditioning, mice were confined to one compartment after drug injections and to the other compartment after saline injections. Conditioning session consisted of 6 alternate day injection of drug or vehicle (saline). Immediately, following drug injection, mice were confined to one compartment. Following vehicle injection they were confined to the other compartment. Treatment compartment and the presentation order of drugs vehicle were counterbalanced for each drug dose. Conditioning sessions were 60 min in duration. On day 7, tests of conditioning were performed as follows: the partition separating the two compartments was raised to 7 cm above the floor, and a neutral platform was inserted along the seam separating the compartments. The time spent in each compartment during a 900-s session was then measured in a blinded fashion by an infrared beam sensor (KN-80, Natume, Tokyo, Japan). The position of the mouse was defined by the position of its body. All sessions were conducted under conditions of lim illumination and masking white noise.

Drugs

Streptozotocin was purchased from Sigma (St. Louis, MO, USA.). Cocaine hydrochloride was purchased from Takeda Pharmaceutical Industries, Inc. (Ohsaka, Japan). Streptozotocin was dissolved in 0.1 N citrate buffer. Cocaine hydrochloride was dissolved in sterile 0.9 % NaCl solution.

Data analysis

Conditioning scores represent the time spent in the drug-paired place minus the time spent in the saline-paired place and are expressed as mean ± S.E. Dose-response curve was analyzed using a one-way random factorial analysis of variance and liner regression analysis. The Wilcoxon test was used to determine whether individual doses produced a significant conditioning (*<0.05).

Results

As shown in Fig. 3-12, none of the mice receiving that received saline in conditioning sessions exhibited a significant preference for either compartment of the test box. The mean conditioning scores were -2.0 ± 33.8 s (n=8) for non-diabetic mice and -1.3 ± 47.5 s (n=8) for diabetic mice. The place conditioning produced by cocaine is shown in Fig. 3-12. In non-diabetic mice, cocaine, at dose ranges from 1 to 30 mg/kg, s.c., caused a dose-related preference for the drug-associated place, and significant conditioning was observed at doses of 10 and 30 mg/kg. In diabetic mice, cocaine (10 mg/kg) caused a slight place preference, but this effect was not statistically significant. Significant conditioning was observed at doses of 30 mg/kg in diabetic mice. At this dose, all diabetic mice exhibited preference for the drug-associated place. However, there was no significant difference in the cocaine-induced place preference between diabetic and non-diabetic mice.

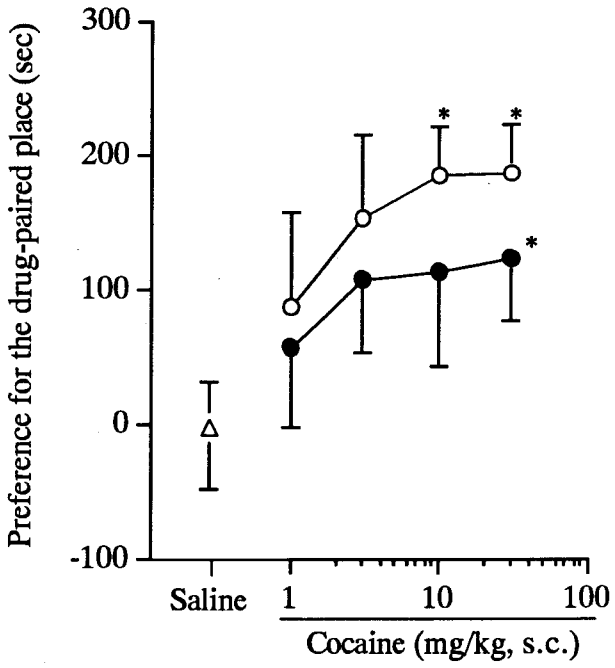

Figure 3-12. Place conditioning produced by cocaine in diabetic (closed circle) and non-diabetic (open circle) mice. Each point represents the mean conditioning score ± S.E. of 8-12 mice. The asterisks denote significant preference conditioning (Wilcoxon test: *P<0.05 vs. respective saline alone (triangle)).

Discussion

The results of Experiment 3-1 demonstrated that morphine produced dose-related conditioned place preference in both diabetic and non-diabetic mice. Furthermore, the morphine-induced place preference was significantly antagonized by pretreatment with the β-funaltrexamine, a selective μ-opioid receptor antagonist, but not naloxonazine, a selective μ_1-opioid receptor antagonist in both diabetic and non-diabetic mice. Similarly, Suzuki et al. (1993) demonstrated that the morphine-induced place preference was not suppressed by pretreatment with naloxonazine, whereas β-funaltrexamine completely suppressed morphine-induced place preference. Furthermore, they reported that morphine produced a β-funaltrexamine-sensitive place preference in μ_1-opioid receptor deficient CXBK mice. Therefore, the present results along with the findings of Suzuki et al. (1993) strongly support the hypothesis that the morphine-induced place preference may be mediated by naloxonazine-insensitive μ-opioid receptors (namely, μ_2-opioid receptors).

In the present study, place aversion was observed following s.c. administration of naloxonazine and β-funaltrexamine in non-diabetic mice, but not in diabetic mice. It has been shown that opioid receptor antagonists, such as naloxone and naltrexone, produce a conditioned place aversion in rats (Bechara and van der Kooy, 1985; Mucha and Herz, 1984) and mice (Mucha and Walker, 1987). Recent evidence suggests that the aversive properties of naloxone primarily reflect an antagonism of the activity of central β-endorphin-containing neurons in mediobasal arcute hypothalamus (Mucha et al., 1984). In receptor binding studies, it has been reported that naloxonazine inhibits [^{3}H]β-endorphin binding, suggesting that [^{3}H]β-endorphin might label μ_1 sites (Houghten et al., 1984). Furthermore, it should be noted that a reasonable naloxonazine dose to obtain a μ_1 selectivity in mice would be 35 mg/kg, s.c. (Ling et al., 1986). In the present study, naloxonazine (35 mg/kg, s.c.) produced a conditioned place aversion, suggesting that selective blockade of μ_1-opioid receptors results in aversive motivational states. Possibly, β-endorphinergic pathway would be involved in the aversive properties of naloxonazine. Nevertheless, the morphine-induced place preference was not blocked by pretreatment with naloxonazine in both non-diabetic and diabetic mice. These findings also strongly support the possibility that the motivational effect of morphine may be mediated by μ_2-opioid receptors. Recently, Kamei et al. (1994a) proposed that mice with diabetes are selectively hyporesponsive to activation of μ_1-opioid receptors, but are normally responsive to μ_2-opioid receptors, since naloxonazine-sensitive, but not naloxonazine-

insensitive pharmacological actions of μ-opioid receptor agonists in diabetic mice were markedly reduced as compared with in non-diabetic mice. This possibility might be supported by the present findings that naloxonazine and β-funaltrexamine did not produce place aversion in diabetic mice.

The morphine (5 mg/kg)-induced place preference in non-diabetic mice was antagonized by pretreatment with either 7-benzylidenenaltrexone, a selective δ_1-opioid receptor or naltriben, a selective δ_2-opioid receptor antagonist. These results are also consistent with previous findings that δ_1- and δ_2-opioid receptors may be involved in the modulation of the reinforcing effect of morphine (Suzuki et al., 1994). The morphine-induced place preference in diabetic mice was suppressed by pretreatment with naltriben. Furthermore, the lower dose (3 mg/kg) of morphine-induced place preference in diabetic mice was significantly antagonized by pretreatment with 7-benzylidenenaltrexone. These results indicate that the motivational effect of morphine in diabetic mice is also modulated by the δ_1- and δ_2-opioid receptor. In the present study, the morphine (5 mg/kg)-induced place preference in diabetic mice was significantly but not completely antagonized by pretreatment with β-funaltrexamine and was not affected by 7-benzylidenenaltrexone. However, when mice were pretreated with both β-funaltrexamine and 7-benzylidenenaltrexone, the morphine (5 mg/kg)-induced place preference in diabetic mice was completely abolished. It is not clear whether the lack of the effect of 7-benzylidenenaltrexone on morphine-induced place preference in diabetic mice may be caused by the increasing the dose of morphine overcoming the 7-benzylidenenaltrexone antagonism or the up-regulation of δ_1-opioid receptor-mediated functions. In this regard, Kamei et al. (1994b) previously demonstrated that the antinociceptive effect of DPDPE was significantly greater in diabetic mice than in non-diabetic mice, whereas there was no significant difference in the antinociceptive effect of [D-Ala^2]deltorphin II between diabetic and non-diabetic mice. Furthermore, pretreatment with 7-benzylidenenaltrexone, but not naltriben, significantly antagonized the antinociceptive effect of DPDPE. In contrast, the antinociceptive effect of [D-Ala^2]deltorphin II was antagonized by naltriben, but not by 7-benzylidenenaltrexone (Kamei et al., 1994b).

Recently, Kamei et al. (1995) demonstrated TAN-67, a novel non-peptidic δ-opioid receptor agonist, produced a marked and dose-dependent 7-benzylidenenaltrexone-sensitive, but not naltriben-sensitive, antinociceptive effect in both non-diabetic and diabetic mice. Furthermore, the antinociceptive effect of TAN-67 is significantly greater in diabetic mice than that in non-diabetic mice (Kamei et al., 1995a). Based on these results, it is suggested

that mice with diabetes are selectively hyperresponsive to δ_1-opioid receptor-mediated antinociception, but are normally responsive to activation of δ_2-opioid receptors. In the present study, I demonstrated that TAN-67 produced significant place preference in diabetic mice, but not in non-diabetic mice. This result suggests that the motivational effect mediated by δ_1-opioid receptors, which is silent in non-diabetic mice, fulfill its function in diabetic mice. On the other hand, higher dose of morphine (10 mg/kg)-induced place preference in non-diabetic mice was significantly antagonized by 7-benzylidenenaltrexone. Thus, it seems likely that the lack of the effect of 7-benzylidenenaltrexone on high dose of morphine (5 mg/kg)-induced place preference in diabetic mice might be due to the up-regulation of δ-opioid receptor-mediated functions.

In the present study, I also demonstrated that the maximal conditioning of morphine in diabetic mice was relatively higher than that in non-diabetic mice. Furthermore, the mean conditioning score of morphine, at a dose of 3 mg/kg, was significantly greater in diabetic mice than that in non-diabetic mice. However, when glucose was administered to non-diabetic mice to raise their serum glucose levels, morphine-induced place preference in non-diabetic mice was not changed. This result suggests that the enhancement of morphine-induced place preference in diabetic mice was not due to their higher serum glucose levels. In this regard, a conditioned reinforcing effect of morphine has been observed following its injection into nucleus accumbens (Koob and Goeders, 1989; Olds, 1982). Furthermore, it is reported that morphine binds with relatively high affinity to δ-opioid receptors (Magnan et al., 1982), and δ- rather than μ-opioid receptors are predominate in this region (Mansour et al., 1988). Therefore, the reinforcing effects of intra-nucleus accumbens morphine may reflect an interaction with δ- rather than μ-opioid receptors. Thus, it is possible that the enhancement of morphine-induced place preference in diabetic mice as compared to non-diabetic mice may be due to the up-regulation of δ_1-opioid receptor-mediated.

Many studies have indicated that morphine increases the activity of mesolimbic and nigrostriatal dopamine-containing neurons (Gysling et al., 1983; Matthews et al., 1984). Furthermore, it has been reported that acute morphine treatment increases dopamine release and metabolism in the caudate putamen and the nucleus accumbens (Di Chiara and Imperato, 1988; Wood et al., 1982). There is evidence that the motivational effect of morphine is mediated by dopamine neurons in these regions (Botarth and Wise, 1981; Phillips and LePiane, 1980; Spyraki et al., 1983; Van der Kooy et al., 1982). Locomotor activity in the experimental animals has been shown to be closely related to the activity of the mesolimbic

dopaminergic system. Kamei et al. (1995b) recently demonstrated that spontaneous locomotor activity in diabetic mice was significantly greater than that in non-diabetic mice. This enhanced spontaneous locomotor activity in diabetic mice was reduced by pretreatment with either SCH23390, a selective dopamine D1 receptor antagonist, 7-benzylidenenaltrexone or naltrindole (Kamei et al., 1994d). Furthermore, Kamei et al. (1994d) also demonstrated that the dopamine turnover in limbic forebrain (including nucleus accumbens and olfactory tubercle) is increased in diabetic mice as compared to non-diabetic mice. On the other hand, morphine-induced increase in dopamine turnover in the mouse limbic forebrain was significantly suppressed by naltrindole (Naritra et al., 1993). Based on these results, Kamei et al. (1994d) previously suggested that the enhanced spontaneous locomotor activity in diabetic mice may be result from increased dopamine release in mesolimbic dopamine systems, which might be due to the up-regulation of δ-opioid receptor-mediated functions. Therefore, the increased dopamine neurotransmission which related to the up-regulation of δ-opioid receptor-mediated functions may account for the enhancement of morphine-induced appetitive effect in diabetic mice.

In conclusion, the results from Experiment 3-1 support the hypothesis that the morphine-induced place preference may be mainly mediated through the activation of the μ_2-opioid receptor, and suggest that the enhancement of the morphine-induced place preference in diabetic may be due to the up-regulation of δ_1-opioid receptor-mediated functions.

In Experiment 3-2, methamphetamine produced a dose-dependent and significant place preference in both diabetic and non-diabetic mice. This methamphetamine-induced place preference was attenuated by pretreatment with quinpirole in both diabetic and non-diabetic mice. White and Wang (1984) reported that somatodendric dopamine-autoreceptors, which regulate the impulse flow of most mesolimbic dopamine neurons in the ventral tegmental area, exhibit the pharmacological characteristics of dopamine D_2 receptors. On the other hand, quinpirole possesses a high affinity for the recently described dopamine D_3 receptors. Sokoloff et al. (1990) reported that quinpirole has an approximately 100-fold higher affinity for the dopamine D_3 receptor than for the dopamine D_2 receptor. Tang et al. (1994) reported that quinpirole inhibits dopamine release through the activation of dopamine D_2 and dopamine D_3 receptors. Therefore, the present results suggest that the attenuation of methamphetamine-induced place preference by quinpirole may be mediated by a reduction in dopamine transmission through the activation of dopamine autoreceptors. In this regard, we also demonstrated that methamphetamine-induced place preference was attenuated by

pretreatment with 7-OH-DPAT in both diabetic and non-diabetic mice. Lévesque et al. (1992) reported that the selectivity of 7-OH-DPAT for dopamine D_3 receptors is >100-, >1000- and >10000-fold greater than that for dopamine D_2, D_4 and D_1 receptors, respectively, suggesting that 7-OH-DPAT is a highly selective agonist for dopamine D_3 receptors. Furthermore, several microdialysis and brain-slice studies have indicated that 7-OH-DPAT reduces dopamine release in the nucleus accumbens and striatum (Damsma et al., 1993; Timmerman et al., 1991; Yamada et al., 1994) Therefore, dopamine D_3 receptors may play a role in controlling dopamine release or synthesis as an autoreceptor in dopamine neuronal terminals in the limbic area (Gobert et al. 1995). Thus, present data strongly suggest that the reduction of the mesolimbic dopamine system activity can reduce the rewarding effect of methamphetamine.

The nucleus accumbens is an important site for the mediation of the reinforcing properties of drug of abuse. Furthermore, it has been reported that the dopamine-containing neurons of the ventral tegmentum and their tracts that innervate the limbic and frontal cortex are required for the acute reinforcing actions of cocaine and D-amphetamine (Roberts and Koob, 1982; Yokel and Wise, 1975; 1976). Although Spyraki et al. (1982) failed to observe an overall significant attenuation of amphetamine-induced place preference in rats with 6-hydroxydopamine lesions of the nucleus accumbens, there was a significant correlation between dopamine levels in the nucleus accumbens and the magnitude of the place preference. Furthermore, dopamine depletion in the nucleus accumbens varied between 60 and 90% of that in the controls, and the importance of a severe depletion of dopamine in attenuating a place-preference effect has been demonstrated. Moreover, it has been reported that the microinjection of (+)-amphetamine into the nucleus accumbens resulted in a place preference (Carr and White, 1986). These reports suggest that the nucleus accumbens is an important substrate in the psychostimulant-induced place preference. Locomotor activity in the experimental animals has been shown to be closely related to the activity of the mesolimbic dopaminergic system. It is recently demonstrated that spontaneous locomotor activity in diabetic mice was significantly greater than that in non-diabetic mice. Furthermore, Kamei et al. (1994d) also demonstrated that dopamine turnover (DOPAC+HVA/dopamine) in the limbic forebrain is significantly greater in diabetic mice than that in non-diabetic mice. Moreover, it is reported that increased spontaneous locomotor activity in diabetic mice was attenuated by pretreatment with 7-OH-DPAT (Kamei and Saitoh, 1996a). Based on these results, we suggested that the enhanced spontaneous locomotor activity in diabetic mice may result from increased dopamine release in mesolimbic dopamine systems, which might be due

to the down-regulation of presynaptic dopamine D_3 receptor-mediated functions (Kamei and Saitoh, 1996a). In the present study, we also demonstrated that 7-OH-DPAT, at doses of 1 and 3 mg/kg, produced dose-dependent place aversion in non-diabetic mice. However, 7-OH-DPAT produced neither place preference or place aversion in diabetic mice. It has been reported that CGS10746B (5-(4-methyl-1-piperazinyl)imidazo[2,1-b][1,3,5]-benzothiadiazepine), an inhibitor of dopamine release, produced a place aversion (Schechter and Meehan 1994). Furthermore, as mentioned above, 7-OH-DPAT inhibits dopamine release from the nucleus accumbens, a major terminal area of the mesolimbic dopaminergic system, through the activation of dopamine D_3 receptors (Damsma et al. 1993; Timmerman et al. 1991; Yamada et al. 1994; Gobert et al. 1995). These results suggest that the activation of dopamine D_3 receptors in the mesolimbic dopamine system may produce place aversion. Thus, it is possible that the reduction in place aversion caused by 7-OH-DPAT in diabetic mice may be related to the dysfunction of dopamine D_3 receptors. Furthermore, the present findings also support the previous hypothesis (Kamei and Saitoh, 1996a) that diabetic mice are hyporesponsive to dopamine D_3 receptor-mediated modulation of dopamine release in the limbic area. Therefore, the increased dopamine neurotransmission which is associated with the down-regulation of presynaptic dopamine D_3 receptor-mediated functions may be account for the enhancement of methamphetamine's reinforcing effect in diabetic mice. The mechanisms which lead to this dysfunction of dopamine D_3 receptors in diabetic mice are unclear. It was previously suggested that some factor(s) derived from spleen cells may play an important direct or indirect role in the alternation of dopamine receptor functions (Kamei and Saitoh, 1996b). It is possible that these factor(s) in diabetic mice and the dysfunction of dopamine D_3 receptors may somehow be related.

In Experiment 3-3, I demonstrated that although statistically significant difference in cocaine-induced place preference between diabetic and non-diabetic mice was not observed, cocaine preferentially produced place preference in non-diabetic mice as compared to those in diabetic mice. In Experiment 3-2, I demonstrated that methamphetamine, an enhancer of dopamine release, preferentially produced place preference in diabetic mice as compared to those in non-diabetic mice. Furthermore, it has been shown that cocaine had no significant effect on locomotor activity in diabetic mice, whereas methamphetamine produced a significant locomotor-enhancing effect in diabetic mice (Saitoh et al., 1995). Based on the results of this study and reports from Saitoh et al. (1995), it is possible that the pharmacological actions of cocaine in diabetic mice were less than those in non-diabetic mice.

In Experiment 3-2, the methamphetamine-induced place preference in both diabetic and non-diabetic mice was significantly antagonized by pretreatment with not only quinpirole, a dopamine D2/D3 receptor agonist, but also 7-OH-DPAT, a selective dopamine D3 receptor agonist. Moreover, 7-OH-DPAT produced significant place aversion in non-diabetic mice, but not in diabetic mice. Based on these results, I suggested that the increased dopamine neurotransmission which associated with the down-regulation of presynaptic dopamine D3 receptor-mediated function may account for the enhancement of methamphetamin's reinforcing effect in diabetic mice. In contrast with the reinforcing effects of methamphetamine, it has been reported that the reinforcing and stimulant properties of cocaine was shown to correlate with their binding properties at the dopamine transporter (Bergman et al., 1989; Ritz et al., 1987). Furthermore, mice lacking the dopamine transporter are indifferent to the stimulant effects of cocaine (Giros et al., 1996). Figlewicz et al. (1994) demonstrated that direct administration of insulin into the CNS resulted in significantly increased the dopamine transporter mRNA levels relative to vehicle-treated controls. In addition reports indicated that the dopamine transporter mRNA expression in ventral tegmental area/substantia nigra compacta and ventral medial bundle decreased in experimentally-induced diabetic rats (Figlewicz et al., 1996; Petrisic et al., 1997). Thus, these results and the present data suggest that changes in the dopamine transporter regulation in the dopamine neurons of the mesolimbic/nigrostriatal pathway and the reduction of cocaine's reinforcing effect in diabetic mice may some how be related. However, further studies are necessary before this issue can be resolved with greater certainty. In conclusion, cocaine-induced place preference was attenuated in diabetic mice as compared to those in non-diabetic mice. This might be due to in part by the alteration in the dopamine transporter function.

Conclusions

The above findings lead to the following conclusions:

(1) Activation of protein kinase C (PKC) plays the critical role in the attenuation of μ–opioid receptor agonist-induced pharmacological action in diabetic mice. Furthermore, intracellular Ca^{2+} differentially modulates the μ– and δ–opioid receptor-mediated antinociception. Finally, changes in μ– and δ–opioid receptor agonist-induced antinociception in diabetic mice may be due to intracellular calcium overload caused by the dysfunction of the calcium storage function (Fig. A).

(2) The findings of this study demonstrated that pretreatment with PKC and PKA inhibitors suppress naloxone-precipitated withdrawal jumping in morphine-dependent non-diabetic mice, and inhibit the naloxone-precipitated increase in cortical NA turnover. Moreover, increased intracellular calcium augmented naloxone-precipitated withdrawal jumping and the turnover rate of NA in the frontal cortex in morphine-dependent non-diabetic mice. Naloxone-precipitated withdrawal jumping was significantly less in morphine-dependent diabetic mice than in morphine-dependent non-diabetic mice. The reduction of naloxone-precipitated withdrawal jumping in morphine-dependent diabetic mice may be due to the dysfunction of central noradrenergic neurons and/or central noradrenergic receptor function. Furthermore, the mechanisms underling the reduction of naloxone-precipitated withdrawal jumping in morphine-dependent diabetic mice may be due to the activation of PKC activity and the dysfunction of intracellular calcium storage (Fig. B).

(3) The reinforcing effect of morphine and methamphetamine in diabetic mice is greater than that in non-diabetic mice. The enhancement of the reinforcing effect of morphine in diabetic mice may be due to the up-regulation of μ_1-opioid receptor-mediated functions. Furthermore, the enhancement of methamphetamine-induced place preference in diabetic mice may be due to dysfunction of the presynaptic dopamine D_3 receptors. On the other hand, the reinforcing property of cocaine in diabetic mice was less than that in non-diabetic mice. The reduction of the reinforcing property of cocaine in diabetic mice might be caused by the dysfunction of the dopamine-transporter, which may be the cause for the enhancement of dopaminergic transmission in the nucleus accumbens. I have hypothesized that the differential sensitivity

needed to produce the reinforcing effect of morphine, methamphetamine and cocaine in diabetic mice may result from the dysfunction of central dopaminergic systems.

From the above findings, I conclude that the etiology of several central diabetic neuropathies may be due to the abnormality of the intracellular events, especially activation of PKC and dysfunction of intracellular calcium storage. The reductions of μ–opioid receptor-mediated pharmacological action and naloxone-precipitated withdrawal jumping in diabetic mice may be caused by both the activation of PKC and the increase in the $[Ca^{2+}]_i$. Furthermore, the differential sensitivity of μ– and δ–opioid receptor agonist-induced antinociception in diabetic mice may be due to the increase in the $[Ca^{2+}]_i$.

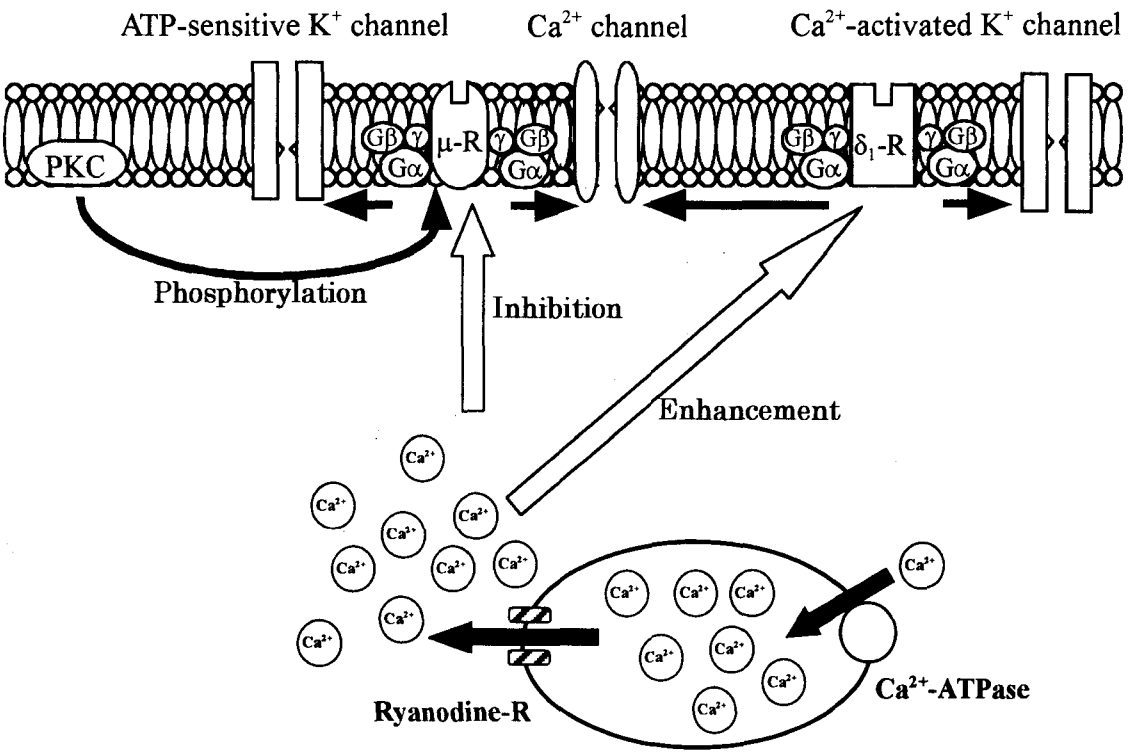

Fig. A. Activation of PKC may desensitize the μ-opioid receptor function. The activation of PKC in diabetic mice may desensitize the μ-opioid receptor function. Furthermore, μ-opioid receptor mediated antinociception may antagonize by the increase in $[Ca^{2+}]_i$, whereas δ-opioid receptor-mediated antinociception may enhance.

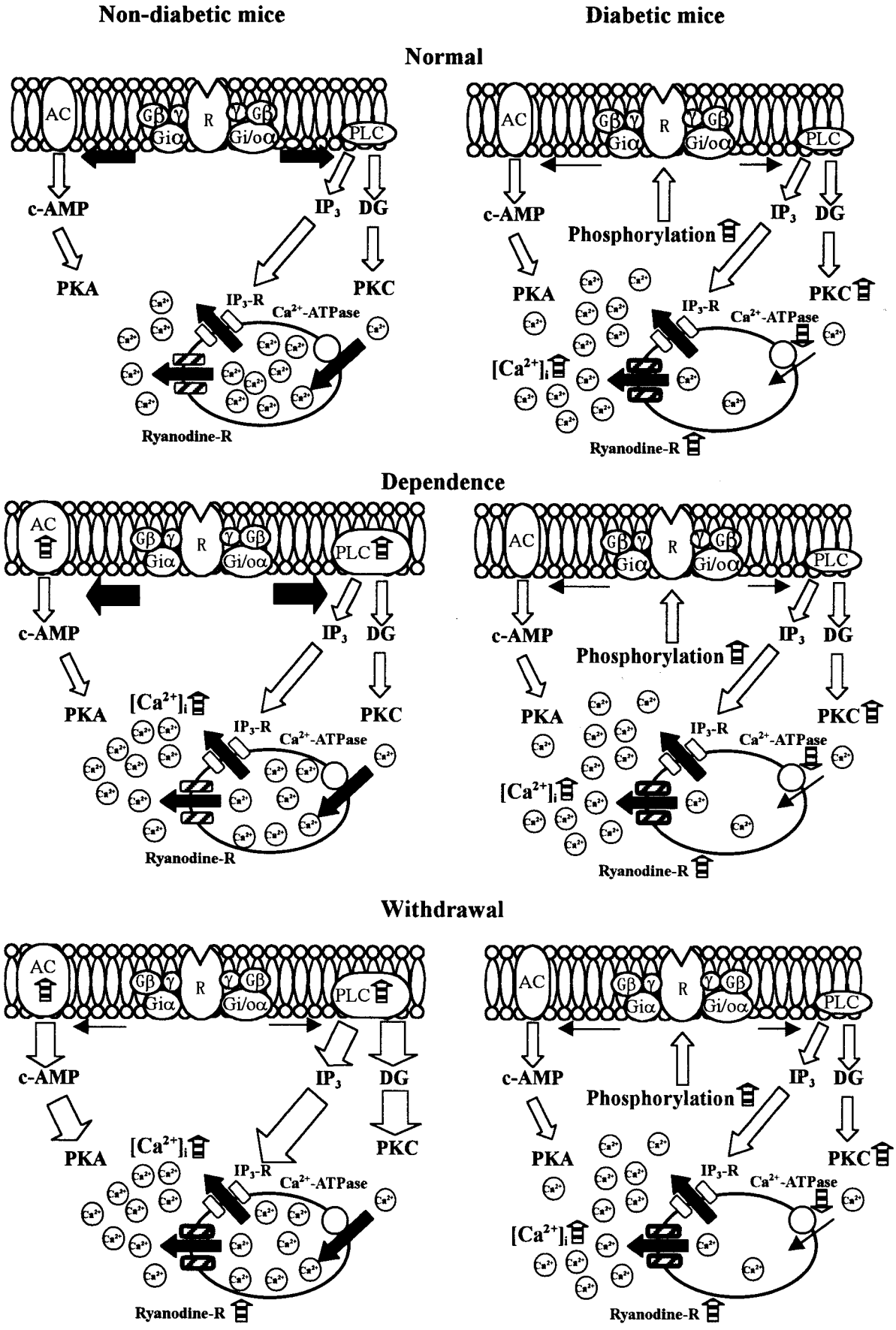
Non-diabetic mice
Diabetic mice
Normal
AC
Gβ
γ
R
Giα
Gi/oα
PLC
c-AMP
IP3
DG
PKA
PKC
IP3-R
Ca2+-ATPase
Ryanodine-R
Phosphorylation
[Ca2+]i
Dependence
Withdrawal

Fig. B. Schematic illustration of diagram of the hypothesized response of identified neuronal element during normal physiological activity, morphine tolerance and dependence in non-diabetic and diabetic mice. Note that the magnitudes of the responses depicted are illustrative only. PLC= phospholipase C, AC= adenylate cyclase, G= G-protein, PKC= protein kinase C, PKA= protein kinase A, ➡ = inhibition, ⇨ = activation, ⇧ = increase, ⇩ = decrease. In non-diabetic mice, chronic treatment with morphine increases the level of G-protein, adenylate cyclase and phospholipase C (tolerant/dependent sate). These changes mediated via persistent activation of opioid receptors. In opiate dependent-state, the combined presence of the morphine and up-regulated intracellular pathways would return excitability of the neuron to normal state. Removal of the morphine would leave the up-regulated intracellular pathways unopposed, leading to withdrawal activation of the neurons (withdrawal state). In diabetic mice, μ-opioid receptor-mediated function may be desensitized by the phosphorylation via activated PKC. It is possible that μ-opioid receptor-mediated inhibition of intracellular pathways in diabetic mice may be weaker than that in non-diabetic mice. Consequently, increase of the level of intracellular substrates in diabetic mice by chronic treatment with morphine may be less than that in non-diabetic mice. Furthermore, the increase of $[Ca^{2+}]_i$ in diabetic mice reduce the adaptive change induced by naloxone. Therefore, activation of the neurons induced by withdrawal in diabetic mice may be less than those in non-diabetic mice.

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