Role of Intracellular Calcium in Modification of Mu and Delta Opioid Receptor-Mediated Antinociception by Diabetes in Mice

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ABSTRACT

We examined the effects of calcium modulators on mu and delta opioid receptor agonist-induced antinociception in both diabetic and nondiabetic mice. In nondiabetic mice, intracerebroventricular (i.c.v.) pretreatment with calcium and thapsigargin, which increase intracellular calcium, reduced [D-Ala²,N-MePhe⁶,Gly-ol⁵]-enkephalin (DAMGO)-induced antinociception by shifting its dose-response curve to the right. However, in diabetic mice, such as EGTA and thapsigargin did not affect DAMGO-induced antinociception. In contrast i.c.v. administration of agents that decrease intracellular calcium, such as EGTA and ryanodine, enhanced DAMGO-induced antinociception in both diabetic and nondiabetic mice. In contrast with DAMGO i.c.v. pretreatment with calcium and thapsigargin enhanced (−)-TAN67-induced antinociception in nondiabetic mice by shifting its dose-response curve to the left. However, (−)-TAN67-induced antinociception in diabetic mice was not affected by pretreatment with calcium or thapsigargin. Moreover i.c.v. pretreatment with EGTA, but not with ryanodine, reduced (−)-TAN67-induced antinociception in nondiabetic mice. In diabetic mice i.c.v. pretreatment with both EGTA and ryanodine reduced (−)-TAN67-induced antinociception. These results suggest that cytosolic calcium has different effects on mu and delta opioid receptor agonist-induced antinociception. Further, these results suggest that the modification of mu and delta opioid receptor agonist-induced antinociception by diabetes in mice may be due to increased levels of intracellular calcium.

There is considerable evidence of a close relationship between opioid antinociception and Ca²⁺ levels within the central nervous system. Agents that increase cytosolic Ca²⁺ in neurons and synaptosomes block opioid antinociception when injected i.c.v. Hano et al. (1964) reported that intracisternal administration of Ca²⁺ antagonizes the antinociceptive effect of morphine, a prototype mu opioid receptor agonist. The ionophores X-537A and A23187, which increase intracellular calcium, reduced [D-Ala²,N-MePhe⁶,Gly-ol⁵]-enkephalin (DAMGO)-induced antinociception by shifting its dose-response curve to the right. However, in diabetic mice i.c.v. pretreatment with calcium and thapsigargin did not affect DAMGO-induced antinociception. In contrast i.c.v. administration of agents that decrease intracellular calcium, such as EGTA and ryanodine, enhanced DAMGO-induced antinociception in both diabetic and nondiabetic mice. In contrast with DAMGO i.c.v. pretreatment with calcium and thapsigargin enhanced (−)-TAN67-induced antinociception in nondiabetic mice by shifting its dose-response curve to the left. However, (−)-TAN67-induced antinociception in diabetic mice was not affected by pretreatment with calcium or thapsigargin. Moreover i.c.v. pretreatment with EGTA, but not with ryanodine, reduced (−)-TAN67-induced antinociception in nondiabetic mice. In diabetic mice i.c.v. pretreatment with both EGTA and ryanodine reduced (−)-TAN67-induced antinociception. These results suggest that cytosolic calcium has different effects on mu and delta opioid receptor agonist-induced antinociception. Further, these results suggest that the modification of mu and delta opioid receptor agonist-induced antinociception by diabetes in mice may be due to increased levels of intracellular calcium.

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1 This study was carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted by the Committee on Care and Use of Laboratory Animals of Hoshi University, which is accredited by the Ministry of Education, Science, Sports and Culture.

ABBREVIATIONS: i.t., intrathecal; i.c.v., intracerebroventricular; EGTA, ethylene glycol bis(β-aminoethyl ether)N,N′-tetraacetic acid; DAMGO, [D-Ala²,N-MePhe⁶,Gly-ol⁵]-enkephalin; DPDPE, [d-Pen²,d-Pen⁵]enkephalin; PKC, protein kinase C; PKA, protein kinase A; STZ, streptozotocin; %MPE, percentage of maximum possible effect; IP₃, inositol 1,4,5-triphosphate; (−)-TAN67, (−)-2-methyl-4α-(3-hydroxyphenyl)-1,2,3,4,4a,5,12,12α-octahydroquinolino[2,3-g]isoquinoline; DMSO, dimethylsulfoxide; [Ca²⁺], intracellular calcium concentration; PAG, periaqueductal gray.
Therefore, we suggested that diabetic mice are selectively hyporesponsive to supraspinal mu opioid receptors agonists and hyperresponsive to supraspinal delta opioid receptors agonists (Kamei et al., 1994a, b). Recently, we reported that the reduction of mu opioid receptor-mediated antinociception in diabetic mice may be in part due to the enhancement of protein kinase C activity (Ohsawa and Kamei, 1997). However, the detailed mechanisms that are responsible for this hyporesponsiveness to supraspinal mu receptor-mediated antinociception and hyperresponsiveness to supraspinal delta opioid-mediated antinociception in diabetic mice are unclear.

Considerable evidence suggests 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$^{2+}$] 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 mu and delta opioid receptor-mediated antinociception by diabetes. Thus, to test this hypothesis, we examined the effect of intracellular calcium modulators on the change in mu and delta opioid receptor agonist-induced antinociception in diabetic and nondiabetic mice.

Materials and Methods

**Animals.** Male ICR mice (Tokyo Laboratory Animals Science, Tokyo, Japan), weighing ~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 ± 1°C with a 12-hr light/dark cycle. Animals were rendered diabetic by an injection of STZ (200 mg/kg i.v.) prepared in 0.1 N citrate buffer at pH 4.5. Age-matched nondiabetic 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 to 4 sec. To obtain the same magnitude of antinociceptive potency, cutoff latencies of 10 and 30 sec were used for (-)-TAN67 and DAMGO, respectively. Animals that did not respond within the cutoff time were removed and assigned a score equivalent to the cutoff time. The percent maximum possible effect (%MPE) was calculated for each animal as %MPE = 100 × (postdrug latency – predrug latency)/(cutoff time – predrug latency)

**Intracerebroventricular injection.** The i.c.v. administration was performed following the method described by Haley and McCor- mick (1957) using a 50-μl Hamilton syringe. The injection site was 1.5 mm from the midline, 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 following drugs were used: STZ (Sigma Chemical, St. Louis, MO), DAMGO (Peninsula Laboratories, San Carlos, CA), thapsigargin (Research Biochemical International, Natick, MA), ry- anodine (Calbiochem-Novabiochem, San Diego, CA), EGTA (Sigma Chemical) and (-)-TAN67. (-)-TAN67 was synthesized by Dr. Na- gase (Toray Laboratory, Kamakura, Japan). Thapsigargin was dis- solved 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 hr 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 nondiabetic 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 nondiabetic 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 nondiabetic mice (fig. 1A). As shown in figure 1B, DAMGO given i.c.v. at doses of 3 to 10 ng caused a dose-dependent inhibition of the tail-flick response in nondiabetic mice. The following drugs were used: STZ (Sigma Chemical, St. Louis, MO), DAMGO (Peninsula Laboratories, San Carlos, CA), thapsigargin (Research Biochemical International, Natick, MA), ryanodine (Calbiochem-Novabiochem, San Diego, CA), EGTA (Sigma Chemical) 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 hr 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).

**Fig. 1.** A. Dose-response effect of i.c.v. pretreatment with CaCl$_2$ (100–300 nmol, hatched column) on DAMGO (10 or 30 nmol i.c.v.)-induced antinociception in diabetic and nondiabetic mice. B. Effect of i.c.v. pretreatment with CaCl$_2$ (300 nmol, closed symbol) and its vehicle (open symbol) on the dose-response curve for DAMGO-induced antinociception in diabetic (diamond) and nondiabetic mice (circle). Nondiabetic mice injected with DAMGO received either saline (○) or CaCl$_2$ (300 nmol, ●). Diabetic mice injected with DAMGO received either saline (◇) or CaCl$_2$ (300 nmol, ●). CaCl$_2$ 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 to 15 mice in each group. *, P < .05 compared with respective nondiabetic mice. #, P < .05 compared with the saline (open column)-pretreated group.
The potency ratio (95% CL) of DAMGO-induced antinociception was shifted to the left. The potency ratio (95% CL) of DAMGO-induced antinociception in calcium-treated nondiabetic mice vs. saline-treated nondiabetic 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 CaCl₂ (100–300 nmol; fig. 1A). Moreover, CaCl₂ (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 vs. that in saline-treated diabetic mice was 1.2 (1.0–1.3) (fig. 1B). The i.c.v. pretreatment with CaCl₂ (300 nmol) by itself had no effect on the baseline tail-flick latencies in diabetic (mean tail-flick latencies of 2.59 ± 0.17 sec, n = 10 for before CaCl₂ treatment; 2.60 ± 0.12 sec, n = 10 for after CaCl₂ treatment) and nondiabetic mice (mean tail-flick latencies of 2.73 ± 0.14 sec, n = 10 for before CaCl₂ treatment; 2.70 ± 0.15 sec, n = 10 for after CaCl₂ treatment). Furthermore, CaCl₂ (100–300 nmol i.c.v.) did not produce apparent behavioral changes, such as convulsion and hyperlocomotion, in diabetic and nondiabetic mice.

EGTA injected i.c.v. (1–60 nmol) significantly enhanced the antinociceptive effect of DAMGO (5.6 ng i.c.v.) in nondiabetic mice (fig. 2A). Furthermore i.c.v. pretreatment with EGTA (30 nmol) enhanced the inhibition of the tail-flick response induced by i.c.v. DAMGO in nondiabetic 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 nondiabetic mice vs. that in saline-treated nondiabetic 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. 2A). However, significant potentiation of DAMGO-induced antinociception was observed in diabetic mice at a higher dose of EGTA (30 and 60 nmol; fig. 2A). EGTA (30 nmol i.c.v.) increased the potency of DAMGO-induced antinociception in diabetic mice; the dose-response curve for DAMGO-induced antinociception in diabetic (di-amond) and nondiabetic mice (circle). Nondiabetic mice injected with DAMGO received either saline (○) or EGTA (30 nmol, ●). Diabetic mice injected with DAMGO received either saline (△) or EGTA (30 nmol, ◆). 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 to 15 mice in each group. *, P < .05 compared with respective nondiabetic mice. #, P < .05 compared with the saline (SA, open column)-pretreated group.

Fig. 2. 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 nondiabetic mice. B, Effect of i.c.v. pretreatment with ETGA (30 nmol, closed symbol) and saline (open symbol) on the dose-response curve for DAMGO-induced antinociception in diabetic (di-amond) and nondiabetic mice (circle). Nondiabetic mice injected with EGTA 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 to 15 mice in each group. *, P < .05 compared with respective nondiabetic mice.

Effects of i.c.v. CaCl₂ and EGTA on (−)-TAN67-induced antinociception in diabetic and nondiabetic mice. In contrast with DAMGO, as shown in fig. 3A i.c.v. pretreatment with CaCl₂ (300 nmol) enhanced the inhibition of the tail-flick response induced by i.c.v. (−)-TAN67 in nondiabetic 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 nondiabetic mice vs. saline-treated nondiabetic mice was 3.6 (3.1–4.2). However, in diabetic mice i.c.v. pretreatment with CaCl₂ (300 nmol) did not affect (−)-TAN67-induced antinociception (fig. 3A). The potency ratio (95% CL) of (−)-TAN67-induced antinociception in calcium-treated diabetic mice vs. saline-treated diabetic mice was 1.2 (0.8–1.8). As shown in figure 3B i.c.v. pretreatment with EGTA (10 nmol) attenuated the inhibition of the tail-flick response induced by i.c.v. (−)-TAN67 in nondiabetic 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 nondiabetic mice vs. saline-treated nondiabetic 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 (−)-TAN67-induced antinociception was shifted to the right (fig. 3B). The potency ratio (95% CL) of (−)-TAN67-induced antinociception in EGTA-treated diabetic mice vs. saline-treated diabetic mice was 7.7 (5.8–10.4).

Effects of thapsigargin and ryanodine on DAMGO-induced antinociception in diabetic and nondiabetic 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 nondiabetic mice (fig. 4A). As shown...
latencies of 2.71 sec for before thapsigargin treatment; 2.83 ± 0.11 sec, 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 nondiabetic mice, while it has been reported that thapsigargin potently affect the intracellular calcium level (Take-mura et al., 1991; Premack et al., 1994). Ryanaidine injected i.c.v. (0.3–3 nmol i.c.v.) significantly and dose-dependently enhanced the antinociceptive effect of DAMGO (5.6 ng i.c.v.) in nondiabetic mice (fig. 5A). Furthermore, ryanodine (3 nmol i.c.v.) significantly enhanced the potency of DAMGO in diabetic mice; the dose-response curve for DAMGO-induced antinociception as shifted to the left (fig. 5B). The potency ratio (95% CL) of DAMGO-induced antinociception in ryanaidine-treated nondiabetic mice vs. saline-treated nondiabetic 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. 5A). 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. 5B). The potency ratio (95% CL) of DAMGO-induced antinociception in ryanodine-treated diabetic mice vs. saline-treated diabetic mice was 4.4 (4.0–4.8). The i.c.v. pretreatment with ryanodine (3 nmol) by itself did not affect the tail-flick latencies in diabetic (mean tail-

Fig. 3. A, The effect of i.c.v. pretreatment with CaCl$_2$ (300 nmol, closed symbol) and saline (○) on the dose-response curve for (−)-TAN67-induced antinociception in diabetic (diamond) and nondiabetic (circle) mice. Nondiabetic mice injected with (−)-TAN67 received either saline (○) or CaCl$_2$ (300 nmol, ●). Diabetic mice injected with (−)-TAN67 received either saline (○) or CaCl$_2$ (300 nmol, ●). CaCl$_2$ 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 to 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 nondiabetic (circle) mice. Nondiabetic mice injected with (−)-TAN67 received either saline (○) or EGTA (10 nmol, ●). Diabetic mice injected with (−)-TAN67 received either saline (○) or EGTA (10 nmol, ●). 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 to 15 mice in each group.

in figure 4B, i.c.v. pretreatment with thapsigargin (3 nmol) attenuated the inhibition of the tail-flick response induced by i.c.v. DAMGO in nondiabetic 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 nondiabetic mice vs. vehicle-treated nondiabetic 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. 4A). The i.c.v. pretreatment with thapsigargin did not affect the potency of DAMGO in diabetic mice (fig. 4B). The potency ratio (95% CL) of the antinociceptive effect of DAMGO in thapsigargin-treated diabetic mice vs. vehicle-treated diabetic mice was 1.0 (1.0–1.1). The 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 sec, n = 8 for before thapsigargin treatment; 2.72 ± 0.16 sec, n = 8) and nondiabetic mice (mean tail-flick latencies of 2.71 ± 0.22 sec, n = 9 for before thapsigargin treatment; 2.83 ± 0.11 sec, 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 nondiabetic mice, while it has been reported that thapsigargin potently affect the intracellular calcium level (Take-mura et al., 1991; Premack et al., 1994). Ryanaidine injected i.c.v. (0.3–3 nmol i.c.v.) significantly and dose-dependently enhanced the antinociceptive effect of DAMGO (5.6 ng i.c.v.) in nondiabetic mice (fig. 5A). Furthermore, ryanodine (3 nmol i.c.v.) significantly enhanced the potency of DAMGO in diabetic mice; the dose-response curve for DAMGO-induced antinociception as shifted to the left (fig. 5B). The potency ratio (95% CL) of DAMGO-induced antinociception in ryanaidine-treated nondiabetic mice vs. saline-treated nondiabetic 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. 5A). 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. 5B). The potency ratio (95% CL) of DAMGO-induced antinociception in ryanodine-treated diabetic mice vs. saline-treated diabetic mice was 4.4 (4.0–4.8). The i.c.v. pretreatment with ryanodine (3 nmol) by itself did not affect the tail-flick latencies in diabetic (mean tail-

Fig. 4. 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 nondiabetic 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 nondiabetic mice (circle). Nondiabetic mice injected with DAMGO received either vehicle (20% DMSO, ○) or thapsigargin (3 nmol, ●). Diabetic mice injected with DAMGO received either vehicle (20% DMSO, ○) or thapsigargin (3 nmol, ●). 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 to 15 mice in each group. *P < 0.05 compared with respective nondiabetic mice. #, P < 0.05 compared with the vehicle (open column)-pretreated group.
Effects of thapsigargin and ryanodine on \((\text{-})\)-TAN67-induced antinociception in diabetic and nondiabetic mice. As shown in figure 6A, i.c.v. pretreatment with thapsigargin (3 nmol) potentiated the inhibition of the tail-flick response induced by i.c.v.-administered \((\text{-})\)-TAN67 in nondiabetic mice; the dose-response curve for \((\text{-})\)-TAN67-induced antinociception was markedly shifted to the left. The potency ratio (95% CL) of \((\text{-})\)-TAN67-induced antinociception in thapsigargin-treated nondiabetic mice vs. vehicle-treated nondiabetic mice was 3.1 (2.5–3.9). In diabetic mice, i.c.v. pretreatment with thapsigargin (3 nmol) did not affect \((\text{-})\)-TAN67-induced antinociception (fig. 6A). The potency ratio (95% CL) of \((\text{-})\)-TAN67-induced antinociception in ryanodine-treated nondiabetic mice vs. vehicle-treated nondiabetic mice was 1.4 (0.7–1.4). In diabetic mice, however, ryanodine (3 nmol i.c.v.) attenuated \((\text{-})\)-TAN67-induced antinociception; the dose-response curve for \((\text{-})\)-TAN67-induced antinociception was markedly shifted to the right (fig. 6B). The potency ratio (95% CL) of \((\text{-})\)-TAN67-induced antinociception in ryanodine-treated diabetic mice vs. saline-treated diabetic mice was 4.0 (2.7–6.2).

Discussion

The i.c.v. administration of CaCl2, which has been reported to increase the intracellular concentration of calcium, attenuated the antinociceptive effect of DAMGO, an mu opioid receptor agonist in nondiabetic 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 nondiabetic mice. This observation is consistent with a previous report that calcium an-
agonized morphine-induced antinociception while EGTA potentiated morphine-induced antinociception (Harris et al., 1975). Thus, these results and present results indicate that mu opioid receptor agonist-induced antinociception is reduced by an increase in intracellular Ca\(^{2+}\) levels. In the present study, we observed that pretreatment with thapsigargin reduced DAMGO-induced antinociception in nondiabetic 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 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\(^{2+}\)-selective, nonvoltage-activated membrane current (Takeamura 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 mu opioid receptor agonist-induced antinociception.

In contrast to DAMGO, we observed that calcium injected i.c.v. enhanced the antinociceptive effect of (−)-TAN67, a selective delta-1 opioid receptor agonist (Kamei et al., 1997), in nondiabetic mice. Moreover i.c.v. EGTA blocked (−)-TAN67-induced antinociception in nondiabetic mice. Bharucha and Zhao (1996) reported that competitive and noncompetitive antagonists of the N-methyl-D-aspartate receptor antagonize the analgesic action of delta-1 opioid receptor agonists. Furthermore, recent studies have reported that calcium channel blockers attenuate the antinociception induced by delta and kappa but not mu opioid receptor agonists (Spampinato et al., 1994). These results and those of the present study suggest that delta 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 mu and delta opioid receptor-induced antinociception. Furthermore, in the present study, we observed that (−)-TAN67-induced antinociception in nondiabetic 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 delta 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 mu and delta opioid receptor agonist-induced antinociception. On the other hand, (−)-TAN67-induced antinociception in nondiabetic mice was not affected by pretreatment with ryanodine, which decrease cytosolic calcium levels. It is not clear why ryanodine does not affect (−)-TAN67-induced antinociception in nondiabetic. It has not been shown that the mu and delta 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 mu opioid receptor expressing neurons show the expected changes in calcium levels in response to ryanodine, while neurons expressing delta opioid receptor are not directly affected by ryanodine. However, Miyamae et al. (1993) reported that a cloned delta opioid receptor expressed in Xenopus oocytes can mediate agonist activation of phospholipase C. It has recently been reported that delta 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 suggest that the activation of delta 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 that underlie this differential modulation of the mu and delta 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 mu opioid receptor agonists is mediated by the activation of ATP-gated potassium channels, whereas that induced by delta opioid receptor agonists is mediated by the activation of calcium-gated potassium channels. Therefore, it is possible that delta opioid receptor-mediated antinociception may be mediated through the enhancement of intracellular calcium levels. It is likely that the differential modulation of mu and delta opioid receptor agonist-induced antinociception by intracellular calcium may be due to the different mechanisms of mu and delta opioid receptor-mediated signal transduction. On the other hand, recent study has demonstrated a differential distribution of mu and delta receptors in the rat brain. The mu opioid receptors were detected in some cortical and thalamic nuclei, including the prefrontal region, which involved in the central pain-inhibiting system, and delta opioid receptors in cortex and limbic structures (Goodman et al., 1980). Thus, it is possible that there are several supraspinal sites at which the mu and delta opioid receptor agonists can induce antinociception. Furthermore, it has been reported that antinociception is produced by microinjection of morphine, a mu opioid receptor agonist, into a variety of brain sites including the PAG, locus ceruleus, mesencephalic reticular formation and structures within the rostral ventromedial medulla (Jensen and Yaksh, 1986). In contrast to mu opioid receptor agonists, the brain sites which mediate the antinociception induced by delta opioid receptor agonists have yet to be identified. Microinjection of DPDPE into either the PAG or the locus ceruleus 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 mu and
delta opioid receptor agonist-induced antinociception by intracellular calcium may reflect the differences in the primary sites of action of mu and delta opioid receptor agonists.

The antinociceptive effect of DAMGO in diabetic mice is less than that in nondiabetic mice. We observed that agents that increase intracellular calcium (e.g., Ca\(^{2+}\) and thapsigargin) did not affect DAMGO-induced antinociception in diabetic mice. Moreover, agents that reduce intracellular calcium levels (e.g., 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 nondiabetic 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 nondiabetic 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; Kostyk et al., 1995). These results suggest that the diabetic state may affect [Ca\(^{2+}\)], in neurons and various tissues. Thus, these results and the present data strongly suggest that the enhancement of delta opioid receptor agonist-antinociception in diabetic mice may be due to increased [Ca\(^{2+}\)]. 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 because 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, we observed that the antinociception induced by (-)-TAN67 in diabetic mice, but not in nondiabetic 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 nondiabetic 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 mu and delta opioid receptor agonists are modulated differently by intracellular calcium. Furthermore, changes of mu and delta opioid receptor agonist-antinociception in diabetic mice may be due to excessive intracellular calcium overload caused by the dysfunction of calcium store function.

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Ohsawa et al. (1994) Activation of Ca\(^{2+}\) current in diabetic aorta because 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, we observed that the antinociception induced by (-)-TAN67 in diabetic mice, but not in nondiabetic 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 nondiabetic 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 mu and delta opioid receptor agonists are modulated differently by intracellular calcium. Furthermore, changes of mu and delta opioid receptor agonist-antinociception in diabetic mice may be due to excessive intracellular calcium overload caused by the dysfunction of calcium store function.
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