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Involvement of Intracellular Calcium in Morphine Tolerance in Mice

FORREST L. SMITH, DANIEL S. DOMBROWSKI AND WILLIAM L. DEWEY

Department of Pharmacology and Toxicology, Medical College of Virginia of Virginia Commonwealth University, Richmond, VA 23298-0613

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SMITH, F. L., D. S. DOMBROWSKI AND W. L. DEWEY. *Involvement of intracellular calcium in morphine tolerance in mice.* PHARMACOL BIOCHEM BEHAV **62**(2) 381–388, 1999.—Opioid analgesic tolerance is associated with a disruption in Ca^{++} homeostasis. Drugs reducing Ca^{++} influx can prevent and reverse tolerance. The hypothesis was tested that both Ca^{++} influx and mobilization from intracellular pools maintains the expression of morphine tolerance. Ca^{++} modulating drugs were injected ICV at doses not affecting morphine's potency in placebo pellet-implanted mice, in order to determine whether tolerance would be reversed in morphine pellet-implanted mice. The Ca^{++} chelator EGTA significantly reversed tolerance. The Ca^{++} channel antagonists nifedipine and omega-conotoxin GVIA also reversed tolerance. The role of intracellular Ca^{++} was investigated using the membrane permeable intracellular Ca^{++} chelator EGTA-AM. EGTA-AM reversed tolerance at lower morphine doses, but not at higher morphine doses. Thus, mobilization of intracellular Ca^{++} contributes to the expression of tolerance. Finally, 1,4-dihydropyridine–sensitive Ca^{++} channels are known to stimulate Ca^{++}-induced Ca^{++} release (CICR) from Ca^{++}/caffeine-sensitive microsomal pools possessing ryanodine receptors. We examined whether blocking Ca^{++} mobilization from these pools with ryanodine would reverse morphine tolerance. Ryanodine's effects were similar to EGTA-AM. Tolerance was reversed at lower morphine doses, but not at higher doses. Thus, morphine tolerance appears to be associated with increases in Ca^{++} influx and mobilization from Ca^{++}/caffeine-sensitive pools. © 1999 Elsevier Science Inc.

Morphine tolerance Intracellular calcium Analgesia

ABUNDANT evidence indicates a close relationship between opioid antinociception and Ca^{++} levels within the central nervous system. Agents that increase cytosolic Ca^{++} in intact cultured neurons and synaptosomes isolated from the CNS, also block opioid antinociception when injected intracerebroventicularly (ICV). Over 30 years ago, Hano et al. (25) reported that intracisternal administration of Ca^{++} ions antagonize morphine antinociception. The ionophores X-537A and A23187, which facilitate Ca^{++} uptake by cells, also block opioid antinociception (26,57). Because the activity of ionophores is largely that of increasing intracellular Ca^{++} (41), it was postulated that Ca^{++} alters intracellular events to antagonize the antinociceptive effects of opioids [for review, see (14)]. Conversely, Ca^{++} chelators (i.e., EGTA, EDTA), or Ca^{++} channel antagonists of the verapamil, diltiazem, and dihydropyridine type potentiate antinociception in animals (6,7, 13,18,20,29,30,50). In humans, Ca^{++} channel antagonists enhance opioid analgesia in cancer patients and surgical patients without concomitant respiratory depression (11,13,40,49) or enhancement of the rewarding properties of opioids (56).

Numerous reports indicate that opioid tolerance is associated with an alteration in Ca^{++} homeostasis. Not only is synaptosomal $^{45}Ca^{++}$ uptake increased (15), but basal free-intracellular Ca^{++} concentrations are higher in the brain and spinal cord (28,58,59). In morphine tolerance, 1,4-dihydropyridine Ca^{++} channel density is increased (42,43,48). Not surprisingly, Ca^{++} channel antagonists have been shown to prevent the development of opioid tolerance (22), reverse tolerance (1,18, 22), and attenuate the signs of physical dependence in animals (1,4,10,43,52). Tolerance has also been reversed in humans with nimodipine (49).

Previous studies have demonstrated a clear role for extracellular Ca^{++} influx through voltage-sensitive Ca^{++} channels in opioid tolerance. Yet less is known about the potential contribution of mobilization of Ca^{++} from intracellular pools to opioid tolerance. Therefore, the hypothesis was tested that

Requests for reprints should be addressed to Forrest L. Smith, Ph.D., Department of Pharmacology and Toxicology, Medical College of Virginia, P.O. Box 980613, Richmond, VA 23298-0613.

the expression of morphine tolerance is related to alterations in Ca^{++} influx and mobilization from intracellular pools. Our results indicate that extracellular Ca^{++} influx through voltage-sensitive channels, and Ca^{++} mobilization from Ca^{++}/caffeine-sensitive microsomal pools, contribute to the expression of morphine tolerance.

METHOD

Methods of Handling Mice

Male Swiss–Webster mice (Harlan Laboratories, Indianapolis, IN), weighing 25–30 g, were housed six or eight to a cage in animal care quarters maintained at 22 ± 2°C on a 12 L:12 D cycle. Food and water were available ad lib. The mice were brought to a test from (22 ± 2°C, 12-h light–dark cycle), marked for identification and allowed 24-h to recover from transport and handling. All procedures were approved by the Institutional Animal Care and Use Committee at the Medical College of Virginia.

Surgical Implantation of Pellets

Mice were anesthetized with ether before depilation of the hair around the base of the neck. The skin was cleansed with 10% providone iodine (General Medical Corp., Prichard, WV) and rinsed with alcohol before making a 1-cm transverse incision at the base of the neck. The underlying subcutaneous space toward the dorsal flanks was opened using a sterile glass rod. Maintenance of a stringent aseptic surgical field minimized any potential contamination of the pellet, incision, and subcutaneous space. A placebo pellet or a 75-mg morphine pellet was inserted in the space before closing the site with Vetbond Tissue Adhesive (3M Animal Care Products, St. Paul, MN), and again applying providone iodine to the surface. The animals were allowed to recover in their home cages, were they remained throughout the experiment.

Intracerebroventricular Injections

Intraventricular injections were performed as described by Pedigo et al. (39). Mice were lightly anesthetized with ether and a transverse incision was made in the scalp. A free-hand 5-μl injection of the drug or the vehicle was made into the lateral ventricle. The extensive experience of this laboratory has made it possible to inject drugs with a greater than 95% accuracy.

The Tail-Flick Test

The tail-flick test used to assess for antinociception in mice was developed by D'Amour and Smith (19) and modified by Dewey et al. (21). Before injections the baseline (control) latency for each mouse was determined. Only mice with a control reaction time from 2- to 4-s were used. The test latency after drug treatment was assessed at the appropriate time, and a 10-s maximum cutoff time was used to prevent damage to the tail. Antinociception was quantified according to the method of Harris and Pierson (27) as the percentage of maximum possible effect (% MPE), which was calculated as: $\%MPE = [(test - control)/(10 - control)] \times 100$. Percent MPE was calculated for each mouse using at least six mice per dose.

Experimental Design

Agents that affect intracellular Ca^{++} were tested for their ability to reverse morphine tolerance. Baseline tail-flick latencies were obtained before administration of morphine SC and vehicle or Ca^{++} modulating drug ICV. Test latencies were measured 30 min after morphine administration, although pretreatment times with ICV drugs varied, as indicated in the Results section. The peak time effect of ICV drugs was based on an earlier study (54), and confirmed in morphine pellet-implanted animals. Because the overall hypothesis was that Ca^{++} modulating drugs would reverse tolerance, dose and time-course experiments were first conducted in tolerant animals. Using a dose of morphine SC calculated to elicit 20% MPE, increasing doses of Ca^{++} modulating agent were injected ICV in different groups of tolerant mice. Initial dose was based on previous studies, or in the case of no previous reports, a 1- or 10-μg dose was injected. Doses of Ca^{++} modulating agent were adjusted to elicit a maximum reversal of tolerance (i.e., an increase from 20 %MPE to 80–100 %MPE). Following this, the time Ca^{++} modulating agent was injected was varied in several groups to estimate the time of maximum Ca^{++} modulating effect. Based on these parameters, placebo pellet-implanted mice received morphine calculated to elicit 20 %MPE and the Ca^{++} modulating agent. If the agent enhanced antinociception in placebo pellet-implanted mice, the dose was reduced until it had no effect, and was then retested in morphine pellet-implanted mice. Thus, Ca^{++} modulating agent doses were adjusted to reverse morphine tolerance without affecting placebo pellet-implanted mice. For the sake of brevity, data on Ca^{++} modulating agent dose and time-course studies are not shown. Morphine dose–response curves were generated for calculation of ED_{50} values and 95% confidence limits according to a modification of Procedures 5 and 8 by Tallarida and Murray (55). Tests for parallelism were conducted before calculation of potency ratio values and 95% confidence limits using the method of Colquhoun (17).

Swiss–Webster mice were selected because of their high susceptibility to morphine tolerance compared to other mouse strains (46). The large degree of tolerance, as indicated by a reduction in potency, provided enough separation between dose–response curves to determine statistically whether a Ca^{++} modulating drug could reverse tolerance.

Drugs and Chemicals

The 75-mg morphine pellets were obtained from the National Institute on Drug Abuse. Ethylene glycol-bis(β-Aminoethyl ether)-*N,N,N′*,N′-tetraacetic acid tetrasodium (EGTA) (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water. Ryanodine >98% purity and omega conotoxin GVIA (Research Biochemical International, Natick, MA) were dissolved in distilled water, which also served as a vehicle control. Nifedipine (RBI, Natick, MA), BAPTA-AM [1,2-*bis*-(*o*-Amino-5′,5′-debromophenoxy)ethane-*N,N,N′*,N′-tetraacetic acid tetra(acetoxymethyl) ester], and EGTA-AM [ethylene glycol-bis(β-Aminoethyl ether)-*N,N,N′*,N′-tetraacetic acid tetra(acetoxymethyl) ester] (Calbiochem-Novabiochem Int., La Jolla, CA) were dissolved in 2:1:17 dimethylsulfoxide: emulphor (Rhone-Poulenc Roreer, Ft. Washington, PA):distilled water. Morphine sulfate was dissolved in pyrogen-free isotonic saline (Baxter Healthcare Corp., Deerfield, IL).

RESULTS

Role of Extracellular Ca^{++} in Morphine Tolerance

The hypothesis was tested that the influx of extracellular Ca^{++} participates in the expression of morphine tolerance. For these experiments, mice were surgically implanted with either a placebo pellet or a 75-mg morphine pellet. Seventy-two hours later the mice were injected with morphine SC for

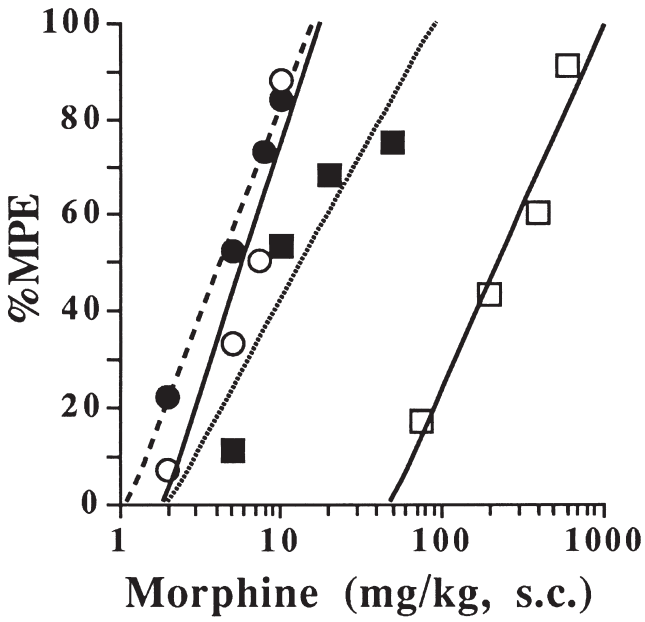

FIG. 1. Chelation of extracellular Ca^{++} with EGTA reverses morphine tolerance. Baseline tail-flick latencies were obtained before injecting mice with morphine SC. Twenty-five minutes later the mice received either vehicle (5 μl, ICV) or EGTA (10.7 nmol, ICV), and were then tested 5 min later. Placebo-pelleted mice received either vehicle (○) or EGTA (●), while morphine-pelleted mice received either vehicle (□) or EGTA (■).

generation of dose–response curves. The tolerance observed is consistent with the first report characterizing morphine pellets in mice (38), and the high susceptibility of Swiss–Webster mice to tolerance compared to other strains (46). The animals in this study were also pretreated with either vehicle or Ca^{++} modulating drug injected ICV. Doses of the modulating drug were adjusted to have no effect in placebo pellet-implanted mice, so that its effect in tolerant mice could be assessed. Selection of timing and dose of Ca^{++} modulating drug were based on a previous study (54) or were tested as detailed in the Method section. For this experiment, placebo and morphine-tolerant mice received either vehicle ICV or the Ca^{++} chelator EGTA ICV. Placebo and morphine pellet-implanted mice injected with saline SC and EGTA ICV responded with less than 6 %MPE. As seen in Fig. 1 and Table 1, morphine pellet-implanted mice injected with vehicle ICV were significantly tolerant to morphine relative to ICV vehicle-injected placebo mice. EGTA-injected ICV did not affect morphine's potency in placebo pellet-implanted mice, but almost completely reversed tolerance in morphine pellet-implanted mice. Thus, extracellular Ca^{++} plays a role in the expression of morphine tolerance.

Other experiments were conducted to test the hypothesis that Ca^{++} influx through voltage-sensitive Ca^{++} channels participates in morphine tolerance. Placebo and morphine pellet-implanted mice injected with saline SC and the 1,4-dihydropyridine Ca^{++} channel antagonist nifedipine ICV responded with less than 15 %MPE. Nifedipine injected ICV had no influence in placebo pellet-implanted mice administered morphine (Fig. 2A, Table 2). However, this same nifedipine dose significantly reversed tolerance in morphine pellet-implanted mice. Tolerance was only partially reversed, because the ED_{50} values for the morphine pellet-implanted mice remained significantly higher than the placebo pellet-implanted mice. In other experiments ω-CgTx GVIA was tested, which is a marine snail neurotoxin peptide first isolated from *Conus geographus.* ω-CgTx GVIA antagonizes Ca^{++} channels involved with synaptic transmission (33,34). Placebo and morphine pellet-implanted mice injected with saline SC and ω-CgTx GVIA ICV responded with less than 14 %MPE. Pretreatment with ω-CgTx GVIA significantly reversed tolerance in the morphine pellet-implanted mice (Fig. 2B, Table 3). Similar to nifedipine, tolerance was partially reversed, suggesting that both channel types contribute to tolerance through Ca^{++} influx.

Role of Intracellular Ca^{++} Mobilization in Morphine Tolerance

Because Ca^{++} influx leads to higher intracellular Ca^{++} levels, experiments were conducted to determine whether chelation of intracellular Ca^{++} would reverse morphine tolerance. The hydrolysis of the acetoxymethyl ester groups on membrane permeable BAPTA-AM by intracellular esterase leaves

TABLE 1

ROLE OF EXTRACELLULAR CALCIUM IN MORPHINE TOLERANCE IN MICE

Group	Treatment ICV	ED_{50} mg/kg (95% C.L.)	Potency Ratio (95% C.L.)
Placebo-P	Veh	5.9 (4.6 to 7.6)	—
Placebo-P	EGTA	4.6 (3.5 to 6.2)	vs. Pbo+Veh 0.8 (0.5 to 1.1)
Morphine-P	Veh	224.7 (164.0 to 306.2)*	vs. Pbo+Veh 37.6 (24.8 to 55.5)*
Morphine-P	EGTA	13.2 (8.6 to 20.3)†‡	vs. Pbo+EGTA 3.0 (1.7 to 5.2)† vs. MP+Veh 16.9 (9.5 to 29.1)‡

Seventy-two hours after surgical implantation of placebo or 75 mg morphine pellets, mice received morphine SC and were tested 30 min later in the tail-flick test. Twenty-five minutes after morphine administration, vehicle, or EGTA (10.7 nmol) was injected ICV.

*Significantly different from Placebo-P/Veh group.

†Significantly different from Placebo-P/EGTA group.

‡Significantly different from Morphine-P/Veh group.

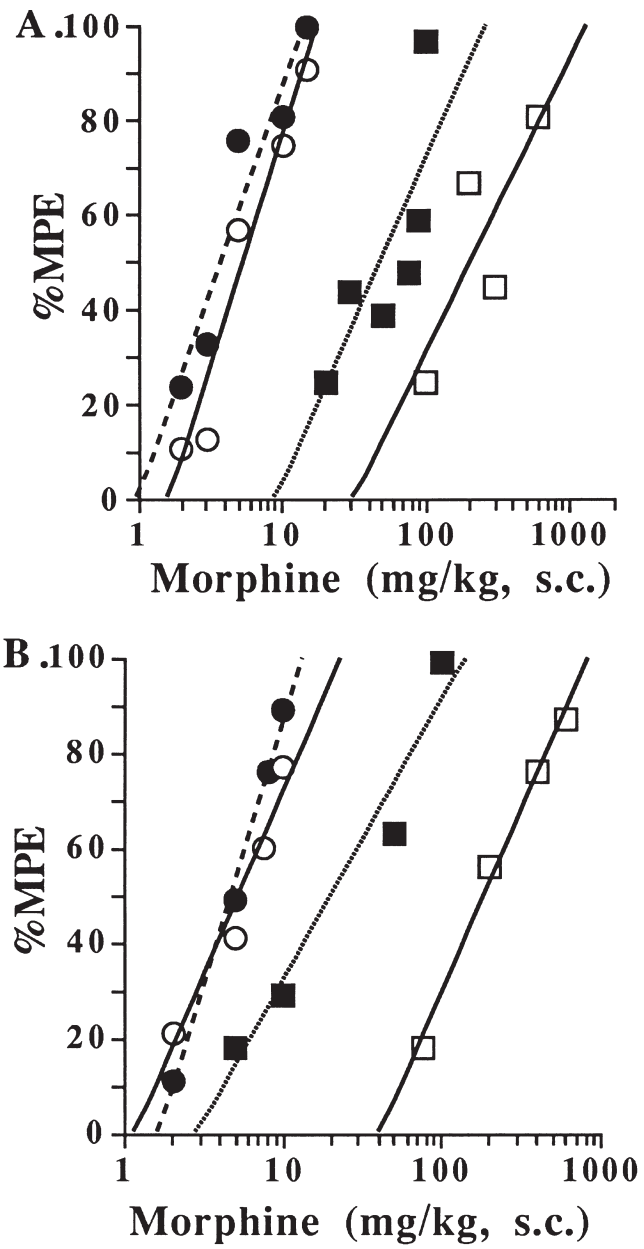

FIG. 2. (A) Antagonism of dihydropyridine-sensitive Ca^{++} channels partly reverses morphine tolerance. Baseline tail-flick latencies were obtained before injecting mice with nifedipine (14.4 nmol, ICV). Ten minutes later the mice received morphine SC, and were then tested 30 min later. Placebo-pelleted mice received either vehicle (○) or nifedipine (●), while morphine-pelleted mice received either vehicle (□) or nifedipine (■). (B) Antagonism of ω-conotoxin GVIA-sensitive Ca^{++} channels partly reverses morphine tolerance. Baseline tail-flick latencies were obtained before injecting mice with morphine SC. Ten minutes later the mice received ω-CgTx (1.7 pmol, ICV), and were then tested 20 min later. Placebo-pelleted mice received either vehicle (○) or ω-CgTx (●), while morphine-pelleted mice received either vehicle (□) or ω-CgTx (■).

the BAPTA free-acid Ca^{++} chelator trapped inside the cell (9). However, BAPA-AM partially reversed morphine tolerance by shifting the curve 2.3-fold to the left, although the shift was not significant (Fig. 3A). BAPTA-AM was soluble in the 2:1:17 DMSO:emulphor:distilled water vehicle. However, the mixing of BAPTA-AM with the CSF may have reduced its solubility and limited its access to intracellular sites. Alternatively, the more soluble EGTA-AM was tested. Placebo and morphine pellet-implanted mice injected with saline SC and EGTA-AM ICV responded with less than 16 %MPE. EGTA-AM elicited a complex response, making it difficult to analyze in the absence of a complete linear relationship between dose and response (Fig. 3B). Tolerance was reversed at lower morphine doses, but not at higher morphine doses. This biphasic effect indicates that intracellular Ca^{++} may play a complex role in the expression of tolerance.

Role of Ca^{++}/Caffeine-Sensitive Ca^{++} Pools in Morphine Tolerance

The hypothesis was tested that Ca^{++} mobilization from Ca^{++}/caffeine-sensitive microsomal pools participates in morphine tolerance. Experiments were conducted to determine whether preventing Ca^{++} mobilization from these pools would reverse morphine tolerance. In vitro studies reveal that the plant alkaloid ryanodine (from *Ryania speciosa vahl.*) binds ryanodine receptors and blocks Ca^{++} mobilization from neuronal Ca^{++}/caffeine-sensitive microsomal pools (36,47). We previously reported that ryanodine reduced the ability of ICV Ca^{++} to antagonize morphine antinociception, indicating that Ca^{++} injected ICV mobilized Ca^{++} from Ca^{++}/caffeine-sensitive pools (54). Therefore, experiments were conducted to examine the role of this pool in morphine tolerance. Placebo and morphine pellet-implanted mice injected with saline SC and ryanodine ICV responded with less than 16 %MPE. Ryanodine had no effect in placebo pellet-implanted mice injected with morphine (Fig. 4). However, ryanodine elicited a complex response in morphine tolerant mice that was very similar to EGTA-AM. Tolerance was reversed at low morphine doses but not at higher morphine doses. These results indicate that the Ca^{++}/caffeine-sensitive CA^{++} pool participates in the expression of morphine tolerance.

DISCUSSION

Role of Ca^{++} in Morphine Tolerance

Several lines of evidence indicate that opioid tolerance leads to alterations in Ca^{++} homeostasis. 1,4-Dihydropyridine Ca^{++} channel binding density is increased in morphine tolerance (42,43,48). Tolerance causes an increase in $^{45}Ca^{++}$ uptake by synaptosomes (15), higher brain, and spinal cord basal intracellular free Ca^{++} levels as measured by fura-2 (58), and higher Ca^{++} levels in synaptosomes and tissue from selected brain regions (28,32,59). However, it is unknown whether higher intracellular Ca^{++} levels are static or are sustained by continuous influx that maintain tolerance. Based on these reports, the hypothesis was tested that chelation of extracellular Ca^{++} with EGTA would reverse morphine tolerance. The 10.7 nmol EGTA dose injected ICV in placebo pellet-implanted mice was 50 to 100 times lower than doses reported to enhance acute opioid antinociception (7). Yet EGTA almost completely reversed morphine tolerance, indicating that sustained Ca^{++} influx plays a role in maintaining tolerance.

At this time, it is unknown in which brain regions Ca^{++} homeostasis may be disrupted. The periaqueductal gray (PAG), lining the aqueduct between the third and forth ventricle, is readily accessible to drugs injected into the lateral ventricles of the brain. This region, besides possessing μ-opioid receptors and receiving β-endorphin-containing terminals from cell

TABLE 2

ROLE OF DIHYDROPYRIDINE-SENSITIVE CALCIUM CHANNELS IN MORPHINE TOLERANCE IN MICE

Group	Treatment ICV	ED_{50} mg/kg (95% C.L.)	Potency Ratio (95% C.L.)
Placebo-P	Veh	5.5 (4.3 to 7.0)	—
Placebo-P	Nifedipine	3.8 (2.9 to 5.0)	vs. Pbo + Veh 0.7 (0.4 to 1.0)
Morphine-P	Veh	210.7 (132.0 to 336.2)*	vs.Pbo + Veh 39.2 (24.7 to 61.1)*
Morphine-P	Nifedipine	50.9 (35.7 to 72.7)†‡	vs.Pbo + Nif 14.1 (9.2 to 22.8)†
			vs. MP + Veh 4.2 (2.2 to 7.9)‡

Seventy-two hours after surgical implantation of placebo or 75 mg morphine pellets, mice received vehicle or nifedipine (14.4 nmol) ICV 10 min before morphine. Thirty minutes after SC morphine administration antinociception was measured in the tail-flick test.

*Significantly different from Placebo-P/Veh group.

†Significantly different from Placebo-P/Nifedipine group.

‡Significantly different from Morphine-P/Veh group.

bodies in the arcuate nucleus, plays an important role in modulating the activity of bulbospinal monoaminergic antinociceptive systems (3,45). μ-Opioids injected directly into the PAG elicits not only dose-dependent antinociception, but significantly reduces the levels of intraneuronal Ca^{++} (60). Alternatively, Ca^{++} injected directly into the PAG blocks the antinociceptive effects of μ-opioids injected parenterally or directly into the PAG (24). Because the PAG is readily accessible to the Ca^{++} modulating drugs, it is tempting to speculate that this region may be affected in morphine tolerance. However, our model of injecting Ca^{++} modulating drugs ICV and morphine SC has several limitations. The expression of morphine tolerance also involves the spinal cord and peripheral sites. Thus, Ca^{++} modulating drugs injected ICV may reverse tolerance in brain regions but not in other areas. It may be shown that disruptions in Ca^{++} homeostasis in the spinal cord and peripheral sites also contribute to morphine tolerance.

Role of Ca^{++} Channels in Morphine Tolerance

Opioid tolerance-induced disruptions in Ca^{++} homeostasis may involve Ca^{++} channels. As mentioned earlier, opioid tolerance results in an increase in 1,4-dihydropyridine binding sites. In addition, Ca^{++} channel antagonists can prevent the development of opioid tolerance (22), reverse tolerance (1,18,22), and attenuate the signs of physical dependence in animals (1,4,10,43,52). ω-CgTx GVIA also reduced opioid withdrawal in rats (5). Remarkably, in cancer patients, nimodipine reversed tolerance, enabling an average 44% reduction in daily morphine intake (49). In our hands, both nifedipine and ω-CgTx GVIA only partially reversed morphine tolerance. This is consistent with the partial reversal of sufentanil tolerance reported with nimodipine (22). Whether tolerance should have been completely reversed by the individual Ca^{++} channel blockers is difficult to say. In two other studies, the use of a single opioid dose make it difficult to interpret the extent of reversal (1,18). Furthermore, the different physiochemical properties of each drug could limit the access of drug to critical sites. Therefore, a conservative qualitative interpretation is that both 1,4-dihydropyridine-sensitive and ω-CgTx GVIA-sensitive Ca^{++} channels play a role in morphine tolerance.

Role of Intracellular Ca^{++} Mobilization in Morphine Tolerance

Because evidence indicated that extracellular Ca^{++} influx might be higher in morphine-tolerant mice, the hypothesis was tested that chelation of intracellular Ca^{++} with BAPTA-

TABLE 3

ROLE OF ω-CONOTOXIN GVIA-SENSITIVE CALCIUM CHANNELS IN MORPHINE TOLERANCE IN MICE

Group	Treatment ICV	ED_{50} mg/kg (95% C.L.)	Potency Ratio (95% C.L.)
Placebo-P	Veh	5.8 (4.2 to 8.0)	—
Placebo-P	ω-CgTx	4.7 (3.7 to 6.0)	vs. Pbo + Veh 0.8 (0.5 to 1.2)
Morphine-P	Veh	184.5 (127.5 to 267.0)*	vs. Pbo + Veh 32.4 (16.8 to 57.0)*
Morphine-P	ωCgTx	22.0 (14.7 to 32.8)†‡	vs. Pbo + CgTx 5.1 (3.0 to 8.7)†
			vs. MP + Veh 7.7 (4.1 to 14.1)‡

Seventy-two hours after surgical implantation of placebo or 75mg morphine pellets, mice received morphine SC and were tested in the tail-flick test 30 min later. Vehicle or omega Conotoxin GVIA (ω-CgTx) (1.7 pmol) was administered ICV 10 min after morphine.

*Significantly different from Placebo-P/Veh group.

†Significantly different from Placebo-P/ ω-CgTx group.

‡Significantly different from Morphine-P/Veh group.

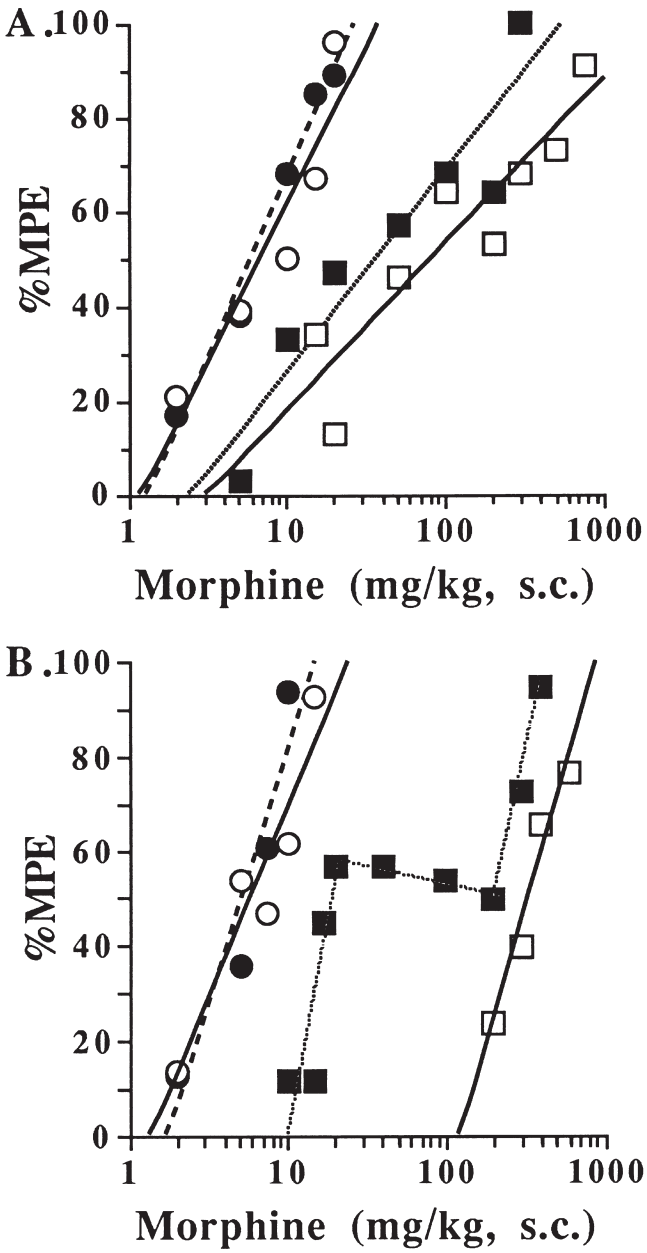

FIG. 3. The intracellular Ca^{++} chelator BAPTA-AM fails to reverse morphine tolerance. Baseline tail-flick latencies were obtained before injecting mice with morphine SC. Twenty minutes later the mice received either vehicle or BAPTA-AM (13.1 nmol, ICV) and were then tested 10 min later. Placebo-pelleted mice received either vehicle (○) or BAPTA-AM (●), while morphine-pelleted mice received either vehicle (□) or BAPTA-AM (■). The intracellular Ca^{++} chelator EGTA-AM partially reverses morphine tolerance. Baseline tail-flick latencies were obtained before injecting mice with morphine SC. Twenty-five minutes later the mice received either vehicle or EGTA-AM (75 nmol, ICV), and were then tested 5 min later. Placebo-pelleted mice received either vehicle (○) or EGTA-AM (●), while morphine-pelleted mice received either vehicle (□) or EGTA-AM (■).

AM would reverse tolerance. However, BAPTA-AM only partially reversed tolerance. BAPTA-AM was soluble in the 2:1:7 emulphor:DMSO:water vehicle, which we used in another study to dissolve several water-insoluble drugs (54).

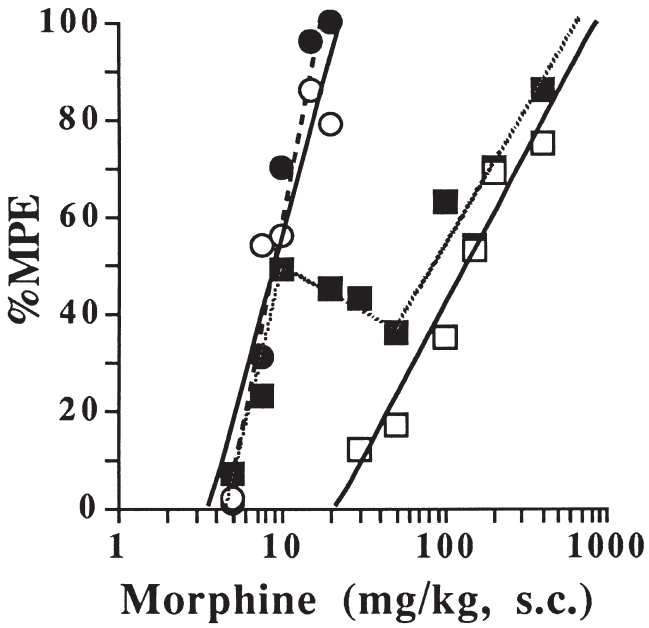

FIG 4. Antagonism of Ca^{++}-induced Ca^{++} release with ryanodine partially reverses morphine tolerance. Baseline tail-flick latencies were obtained before injecting mice with morphine sc. Twenty minutes later the mice received either vehicle or ryanodine (10.1 nmol, ICV), and were then tested 10 min later. Placebo-pelleted mice received either vehicle (○) or ryanodine (●), while morphine-pelleted mice received either vehicle (□) or ryanodine (■).

However, BAPTA-AM as well as other acetoxymethyl-containing Ca^{++} chelators are notoriously insoluble in many vehicle solutions, and BAPTA-AM may have had limited access to the sites of action. To our knowledge, this is the first reported attempt at injecting BAPTA-AM in vivo. It is notable that EGTA-AM partially reversed morphine tolerance. The complex curve could reflect the physiochemical properties of a sparingly soluble drug in CSF. However, the similarity to the ryanodine curve suggests that EGTA-AM could have affected the Ca^{++}/caffeine-sensitive Ca^{++} pools.

Role of Ca^{++}/Caffeine-Sensitive Ca^{++} Pools in Morphine Tolerance

Within neurons, homeostatic mechanisms that tightly control cytosolic free Ca^{++} levels are important in regulating many functions, such as membrane excitability and second-messenger pathways involving adenylyl cyclase and the phosphatidylinositol system (16,31). The pools that sequester and mobilize Ca^{++} allow neurons to tightly regulate the levels of cytosolic free Ca^{++}. Neurons have two separate intracellular Ca^{++} mobilization pathways. One pool mobilizes Ca^{++} from the endoplasmic reticulum following inositol-1,4,5-trisphosphate receptor stimulation. Another pool, the Ca^{++}/caffeine-sensitive pool, possesses a Ca^{++} channel closely resembling the ryanodine receptor purified from skeletal muscle that mobilizes Ca^{++} channel upon stimulation by intracellular Ca^{++} influx or caffeine (2,36). Three ryanodine receptor isoforms are expressed in rodent brain (ryanodine receptor-1, -2, and -3). In vitro studies indicate that ryanodine receptors participate in physiological processes throughout the CNS (35,44, 51,53). Within neurons, this pool is involved with the phenom-

enon of Ca^{++}-induced Ca^{++} release (CICR), in which Ca^{++} entry through 1,4-dihydropyridine-sensitive Ca^{++} channels amplifies its signal by stimulating ryanodine receptors to mobilize pooled Ca^{++} (23). In fact, Ca^{++} mobilized from these pools acts as a positive feedback to trigger more release by activating adjacent ryanodine receptors [for review, see (8)]. In neurons, low concentrations of the plant alkaloid ryanodine slightly stimulates Ca^{++} mobilization, whereas 1 μM ryanodine blocks Ca^{++} release by either preventing channel opening or by stabilizing an open subconductance state (12,37,47).

If morphine tolerance resulted from Ca^{++} mobilization from Ca^{++}/caffeine microsomal pools, we hypothesized that ryanodine injected ICV would reverse tolerance by blocking release from these pools. The results indicate that ryanodine partially reversed tolerance. However, the nature of the response to ryanodine is difficult to interpret, and may reflect the complex phenomenon of CICR. The dose–response curve could be interpreted as biphasic, with tolerance reversed at low morphine doses, reaching a plateau at intermediate doses, with a loss of reversal at higher morphine doses. Thus, if tolerance involves greater Ca^{++} influx through 1,4-dihydropyridine channels, the data indicates that tolerance at low morphine doses involves CICR. At higher morphine doses, other disruptions in Ca^{++} homeostasis may be revealed. For example, the IP_3-sensitive pool may contribute to tolerance, although this cannot be examined in the absence of selective membrane-permeable IP_3 receptor antagonists.

SUMMARY

The evidence is clear that disruptions in intracellular Ca^{++} contributes to the expression of antinociceptive tolerance. Our results indicate that extracellular Ca^{++} influx through voltage-sensitive Ca^{++} channels, and mobilization from Ca^{++}/caffeine-sensitive pools are involved with morphine tolerance. These findings agree with reports that Ca^{++} channel antagonists reverse tolerance, and supports the potential widespread use of Ca^{++} channel antagonists to prevent or reverse tolerance, and to decrease the signs of dependence during opioid withdrawal in humans.

ACKNOWLEDGEMENTS

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