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Modulation of Free Intracellular Calcium Levels [(Ca^{++})i] in Brain and Spinal Cord of Morphine-Tolerant Rats and Mice

SANDRA P. WELCH[1] AND PAMELA P. BASS

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

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WELCH, S. P. AND P. P. BASS. *Modulation of free intracellular calcium levels [(Ca^{++})i] in brain and spinal cord of morphine-tolerant rats and mice.* PHARMACOL BIOCHEM BEHAV **51**(1) 57–63, 1995.—Evaluation of the effects of tolerance to morphine in vivo on free intracellular calcium levels [(Ca^{++})i] in whole brain synaptosomes from rats and synaptosomes from brain regions and spinal cords of both rats and mice indicated that in whole brain and spinal cord synaptosomes, [Ca^{++}]i levels are significantly higher in morphine-tolerant mice. Although levels do not differ between nontolerant and tolerant preparations from the rat spinal cord, [Ca^{++}]i brain levels are significantly higher in morphine-tolerant rats. Evaluation of three brain regions from both nontolerant and tolerant rats and two brain regions from mice indicated no significant differences in basal [Ca^{++}]i, with the exception that lower basal levels of calcium were observed in the midbrain of the tolerant rat. In vivo, mice tolerant to morphine (SC) were cross tolerant to DAMGO (IT and ICV). Cross tolerance between morphine and DAMGO was observed in vitro. No cross-tolerance was observed between morphine (SC) and the δ-agonist, DPDPE (IT or ICV), or the κ-agonist, U50,488H (U50, IT or ICV) in vivo. Similarly, no cross tolerance was observed between morphine (SC) and DPDPE or U50 in vitro. These data indicate that tolerance in vivo induced a change in synaptosomal calcium modulation such that tolerance could be observed in vitro. Thus, a membrane component may be altered by the development of tolerance to morphine leading to an alteration in [Ca^{++}]i in both the brain and spinal cord.

Fura2/AM Intracellular calcium Opioid tolerance DPDPE Morphine U50,488H Brain Spinal cord Synaptosomes

ABUNDANT evidence indicates that a close relationship exists between opiate analgesia and Ca^{++} levels within the CNS. Using measures of free intracellular calcium ([Ca^{++}]i), acutely administered morphine in vitro blocks calcium entry to synaptosomes (40,59,60). Similarly, other investigators have demonstrated that acute opioid exposure leads to reductions in $^{45}Ca^{++}$ uptake (6,23,48,47). The blockade of calcium entry into neurons in response to acutely administered opiates has been proposed to lead to alterations in neurotransmission by: a) inhibiting or enhancing the activity of ATPase-modulated ion transport (13,28,30,49); b) inhibiting Ca^{++}-dependent protein kinases, which can alter phosphorylation of many cell proteins including calcium channel proteins (9,10); c) decreasing the firing of neurons (64); and d) decreasing the Ca^{++} available for the release of neurotransmitters (25). Increasing cytosolic Ca^{++} blocks acute opioid analgesia [for review, see (7,24)]. Acutely administered morphine also inhibits adenylyl cyclase activity in many brain regions via activation of pertussis toxin-sensitive inhibitory $G_{i/o}$ proteins [for review, see (11)]. Decreases in neuronal cAMP reduce the activation of cAMP-dependent protein kinase, and subsequently reduce phosphorylation of important modulatory proteins (15,36), among which are calcium channel proteins, which leads to a decrease in calcium entry. Decreased calcium entry to neurons due to the interaction of μ- and δ-opiate receptor-selective ligands with their respective receptors and the subsequently increased potassium efflux and hyperpolarization of neurons has been reported (1,37,38,54,62,63). Recently, κ-opioids have also been shown to produce hyperpolarization via potassium efflux (19).

The development of tolerance to opiates has been postulated to occur when certain neuronal, physiologic, and cellular

[1] Requests for reprints should be addressed to Sandra P. Welch, Box 613, MCV Station, Richmond, VA 23298-0613.

processes are activated as the body attempts to compensate for the chronic presence of drug. Numerous compensatory mechanisms have been demonstrated to accompany the development of tolerance and addiction, among which are increased [Ca^{++}]i (accompanied by decreases in potassium efflux), increased adenylate cyclase activation, changes in G-protein composition, induction of early response genes, and alterations in the activation of endogenous modulators and "anti-opioid" peptides. Most of these adaptations of the neuron involve mechanisms that lead to modulation of intracellular calcium possible via the alteration of G-proteins [for a review of mechanisms of tolerance associated with the nucleus accumbens, see (35)]. Thus, opioid tolerance appears to involve an alteration in the mechanisms that regulate cellular Ca^{++} homeostasis. Increased [Ca^{++}]i levels, which have been shown in synaptosomes from mouse brain, have been hypothesized to represent a homeostatic change that counters the acute effects of morphine on [Ca^{++}]i (59). The present study was designed to evaluate the effects of tolerance to morphine in vivo on [Ca^{++}]i in whole brain synaptosomes from another species—the rat—and synaptosomes from brain regions in both the rat and the mouse. In addition, we evaluated differences in [Ca^{++}]i between synaptosomes from spinal cords of morphine-tolerant rats and mice. We have previously demonstrated that a change in calcium homeostasis occurs in whole mouse brain in response to chronic morphine, and that such a change is stable enough to be detected as an increase in [Ca^{++}]i in synaptosomes (59). We tested the hypothesis that tolerance to morphine in vivo would result in specific neuronal alterations resulting in cross tolerance in vitro to μ-opioids, but not δ- or κ-opioids.

MATERIALS AND METHODS

Synaptosomal Preparation

Synaptosomes were prepared from mouse or rat brain using subcellular fractionation techniques described by McGovern et al. (34). Male Swiss-Webster mice (25–30 g, Harlan Laboratories, Frederick, MD) or male Sprague-Dawley rats (200–250 g, Harlan Laboratories) were decapitated, and either the whole brain or the spinal cord was removed. For the study of brain regions the brain was dissected and the medulla, midbrain, and corpus striatum were removed. Tissue was homogenized in 0.32 M sucrose in Kreb's buffer (pH 7.4–7.5) on ice (1 g tissue/20 ml sucrose). The Kreb's buffer was composed of (mM): NaCl, 120; KCl, 5; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; $CaCl_2$, 1; glucose, 10, $NaHCO_3$, 20, and was brought to pH 7.5 with 1 N HCl. The homogenate was centrifuged at 1000 × *g* for 10 min. The supernatant was removed and centrifuged at 18,000 × *g* for 20 min. The P2 pellet, containing synaptosomes in a crude mitochondrial pellet, was used to obtain adequate protein for the assays involving the spinal cord. For studies of the whole brain and brain regions the resultant pellet (P2 fraction) was resuspended in 16% Ficoll (16% wt./vol. Type 400 dialyzed Ficoll; Sigma), which had been prepared in 0.32 M sucrose. A 7.5% Ficoll suspension was layered by hand over the 16% Ficoll/pellet suspension to form a discontinous gradient. The discontinous gradient formed was centrifuged for 1 h at 40,000 × *g*. The synaptosomes banded at the 16%/7.5% Ficoll interface were removed by aspiration and washed with a 1 : 1 sucrose (0.32 M)/Kreb's buffer solution. The synaptosomes were centrifuged for 5 min at 18,000 × *g* and washed again in 1 : 2 sucrose/Kreb's buffer solution, and again centrifuged for 5 min at 18,000 × *g*. The synaptosomes (pellet) were resuspended in Kreb's buffer for the measurement of intracellular Ca^{++}. The protein concentration was 1 mg/ml, as determined by the Lowry method (31). The Ficoll and buffers were prepared fresh before each assay and kept on ice. Synaptosomes were kept on ice until they were used.

Measurement of [Ca^{++}]i

Synaptosomes were loaded with 5 μM fura2/AM (Molecular Probes, Inc.) dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the tube was 1%. Synaptosomes were incubated for 45 min at 35°C in a shaking water bath. At the end of the 45 min incubation, the synaptosomal suspension was washed three times in Kreb's buffer and centrifuged following each wash at 18,000 × *g* for 20 min. The SPEX Cation Measurement System (Model CM-3/System; SPEX Industries, Inc., Edison, NJ) was used to measure the free calcium within the synaptosomes. The fura-2 ester is hydrolyzed within the cell or synaptosome and the free fura-2 then binds Ca^{++}. Bound fura-2 is excited by light at 340 nm. The ratio of excitation intensities of fura-2 at both intensities (340/380) is used to calculate intracellular Ca^{++} concentrations (20). To correct for free fura-2, or that associated with extracellular structures, the fura-2 signal in the presence of a 100-fold excess of EGTA (prepared in 3 M Tris, pH 8.3) was determined to give a background value of relative fluorescence (or F_{min}). The maximal fluorescence (F_{max}) was determined when fura-2 was maximally bound to Ca^{++} following lysis of the synaptosomes with 0.1% Triton X-100 and addition of a saturating concentration of Ca^{++}. The F_{max} and F_{min} were determined for each synaptosomal preparation according to the method of Komulainen and Bondy (29). In addition, a separate P_2 crude synaptosomal preparation was incubated with 1% DMSO for 45 min before the preparation of pure synaptosomes using the Ficoll gradient. These synaptosomes that were not fura-2 loaded were used for the quantification of autofluorescence. The autofluorescence of all drugs was also examined in these unloaded synaptosomes. Correction of the signals at 340 and 380 nm for autofluorescence preceded determination of the 340/380 ratio. From the ratio the free calcium concentration was determined using the method of Grynkiewicz and Iverson (20) using 224 nm at the K_d for fura-2 and the following equation where F = the fluorescence of the sample, and S_{f2} and S_{b2} denote the fluorescence at 380 nm in calcium-free and saturating calcium conditions, respectively:

$$[Ca^{++}]_i = K_d \times \frac{(F - F_{min})}{(F_{max} - F)} \times \frac{S_{f2}}{S_{b2}}$$

Each experiment was run in duplicate and repeated using three to six separate sets of synaptosomes.

Synaptosomes were incubated at 37°C for 2 min in a quartz cuvette, with constant stirring, in the sample chamber of the Spex microfluorometer to allow equilibration to the temperature before monitoring basal levels of [Ca^{++}]i. Drugs or vehicle (20 μl addition) were incubated with the synaptosomes for 3 min before KCl-induced stimulation of [Ca^{++}]i by addition of 20 μl KCl such that the final concentration in the cuvette was 50 mM.

Tolerance Induction

Mice were implanted subcutaneously (SC) under the loose skin of the back with either a 75-mg morphine pellet or placebo pellet according to the method of Way et al. (57). Mice have been shown to be rendered tolerant to morphine in 72 h

TABLE 1

[Ca^{++}]i IN NONTOLERANT AND TOLERANT MOUSE AND RAT SYNAPTOSOMES

	Baseline [Ca^{++}]i (nM)	% KCl-Induced Increase in [Ca^{++}]i
Whole Brain		
Non-Tolerant mice	328 ± 20 (a)	70 ± 5 (a)
Tolerant mice	468 ± 22* (a)	62 ± 9 (a)
Non-Tolerant rats	514 ± 44	56 ± 11
Tolerant rats	715 ± 33**	49 ± 10
Spinal Cord		
Non-Tolerant mice	613 ± 41	12 ± 2
Tolerant mice	880 ± 69*	14 ± 5
Non-Tolerant rats	275 ± 15	17 ± 2
Tolerant rats	195 ± 16	17 ± 3

Basal [Ca^{++}]i, KCl (50 mM)-stimulated [Ca^{++}]i were determined in each preparation. The mean [Ca^{++}]i the standard error of the mean was determined using duplicate samples from 5 separate preparations of synaptosomes (mice) and 3 separate preparations (rat) and compared statistically via the Dunnett's *t*-test. *$p < 0.05$ from group labelled "Non-Tolerant mice"; **$p < 0.05$ from group labelled "Non-Tolerant rats"; (a) = data taken from Welch and Olson (58) and presented for purposes of comparison.

using this method. Rats were rendered tolerant by the implantation SC of an ALZET minipump (60 mg/kg per day delivery). The mice and rats were tested for tolerance to a 10-mg/kg challenge injection of morphine (SC) using the tail-flick test. Some mice were assessed for cross tolerance to morphine 3 μg/mouse, intrathecally (IT). Mice were rendered tolerant to morphine after a 3-day (72 h) exposure to the morphine pellets. Rats were rendered tolerant after a 7-day exposure to the ALZET pumps. Tolerance was assessed as the lack of antinociceptive effect following the morphine challenge (%MPE < 10%) in the morphine-tolerant mice or rats, whereas morphine produced > 80% MPE in the placebo mice or rats. Morphine-tolerant and nontolerant mice were tested for cross tolerance to the κ-agonist, U50,488H [50 μg/mouse, administered IT or intracerebroventricularly (ICV)], the δ-agonist DPDPE [(D-$Pen^{2,5}$)-enkephalin, 3 μg/mouse, IT or ICV] or the μ-agonist DAMGO [(D-Ala^2-,*N*-Me-Phe^4,Gly-ol^5) enkephalin, 1 μg/mouse, IT, or 0.5 μg/mouse, ICV]. The doses of the drugs to be tested represent those that produced at least 80% maximum possible effect (%MPE) in naive mice in our laboratory on the day of testing (which corresponds to previously published studies from our laboratory (32,58,61). The exception is U50,488H, which was only a partial agonist when administered ICV. The morphine pellets and the ALZET pumps remained in place at sacrifice of the animals for the preparation of synaptosomes.

IT and ICV Injections

Intrathecal injections were performed following the protocol of Hylden and Wilcox (26). Unanesthetized mice were injected between the L5 or L6 area of the spinal cord with a 30-ga, 0.5-inch needle. ICV injections were performed using the method of Pedigo et al. (43). Injection volumes of 5 μl were administered.

Tail-Flick Test

The tail-flick procedure used was that of Harris and Pierson (22). Control reaction times of 2–4 s and a cutoff time of 10 s were employed. Antinociception was quantified as the %MPE as developed by Harris and Pierson (22) using the following formula: %MPE = 100 × (test − control)/(10 − control). Percent MPE was calculated for each mouse using at least six mice per dose. Using the %MPE for each mouse, the mean effect and standard error of the mean (SEM) were calculated for each dose. Morphine (SC) was injected 20 min before testing; all other drugs were administered 10 min before testing. The time points for testing represent the time of peak antinociception produced by the drugs as previously determined in our laboratory in numerous studies (32,58,61).

RESULTS

The results presented in Table 1 indicate that in whole brain and spinal cord synaptosomes, [Ca^{++}]i levels were significantly higher in morphine-tolerant mice, whereas levels did not differ significantly between nontolerant and tolerant preparations from the rat spinal cord. In addition, although basal whole brain levels of [Ca^{++}]i were higher in rats, the percent rise in [Ca^{++}]i in response to KCl in brain did not differ between rats and mice. A direct quantitative comparison of the percent rise is not possible because the mice and the rats were not tested at the same time. However, we observed that the percent rise in [Ca^{++}]i in response to KCl did not differ significantly across experiments. The KCl-induced rise in [Ca^{++}]i in the spinal cord was significantly lower than that observed in the brains of either mice or rats. We also evaluated changes in [Ca^{++}]i induced by shorter exposure to morphine in mice. Acute administration of 10 mg/kg morphine or vehicle 20 min before sacrifice of the mice resulted in no significant differences in whole brain synaptosomal levels of [Ca^{++}]i in vitro between vehicle- and morphine-treated groups (358 ± 35 vs. 390 ± 20 nM [Ca^{++}]i for $n = 3$ separate preparations,

TABLE 2

[Ca^{++}]I IN NONTOLERANT AND TOLERANT MOUSE AND RAT SYNAPTOSOMES FROM BRAIN REGIONS

	Basal [Ca^{++}]i (nM)	% KCl-Induced Increase in [Ca^{++}]i
Midbrain		
Non-Tolerant mice	163 ± 26	31 ± 6
Tolerant mice	184 ± 18	23 ± 5
Non-Tolerant rats	716 ± 84	40 ± 17
Tolerant rats	590 ± 26**	62 ± 5
Corpus Striatum		
Non-Tolerant mice	NT	
Tolerant mice	NT	
Non-Tolerant rats	539 ± 57	34 ± 1
Tolerant rats	476 ± 32	79 ± 30**
Medulla		
Non-Tolerant mice	284 ± 26	24 ± 3
Tolerant mice	221 ± 41	43 ± 19
Non-Tolerant rats	486 ± 38	44 ± 10
Tolerant rats	490 ± 18	39 ± 15

Basal [Ca^{++}]i, KCl (50 mM)-stimulated [Ca^{++}]i were determined in each preparation. The mean [Ca^{++}]i the standard error of the mean was determined using duplicate samples from 5 separate preparations of synaptosomes (mice) and 3 separate preparations (rat) and compared statistically via the Dunnett's *t*-test. **$p < 0.05$ from group labelled "Non-Tolerant rats"; NT = not tested.

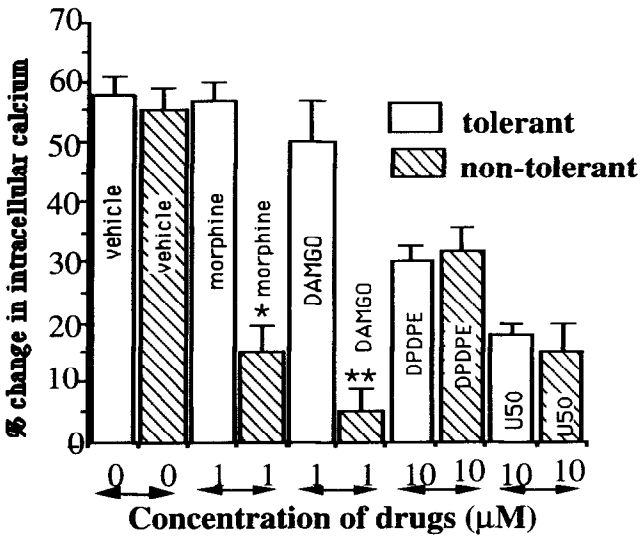

FIG. 1. Cross tolerance of DAMGO in vitro to in-vivo morphine. Synaptosomes were prepared and loaded with fura-2/AM as described in Methods from morphine-tolerant or nontolerant mouse whole brain. Synaptosomes were incubated for 3 min with either distilled water vehicle, morphine (1 μM), DAMGO (1 μM), DPDPE (10 μM), or U50,488H (U50; 10 μM) before stimulation with 50 mM KCl. The average percent change (increase) in $[Ca^{++}]i$ over basal $[Ca^{++}]i$ was calculated for three to five separate preparations of synaptosomes. $*p < 0.05$ from nontolerant synaptosomes; $**p < 0.01$ from nontolerant synaptosomes.

respectively). Because such studies yielded negative data, the studies were not repeated in the rat.

Evaluation of three brain regions from both nontolerant and tolerant rats and two brain regions from mice indicated no significant differences in basal $[Ca^{++}]i$ (Table 2) with the exception that lower basal levels of calcium were observed in the midbrain of the tolerant rat. In addition, a significantly higher KCl-induced increase in calcium was observed in the striatum from morphine-tolerant rats [79% increase vs. 34% control increase in $(Ca^{++})i$]. The levels of basal calcium were higher in the mouse spinal cord than in the mouse brain. We found that the preparation of pure synaptosomes from the mouse spinal cord required large numbers of mice to obtain adequate tissue. We therefore modified our procedure such that a crude synaptosomal preparation (P2 pellet) was substituted for use with mouse spinal cords, which may account for the higher basal calcium levels. We were unable to evaluate the corpus striatum in the mouse because of the highly variable rise in $[Ca^{++}]i$ in response to KCl in the tissue. Synaptosomes from the corpus striatum also exhibited highly variable basal levels of $[Ca^{++}]i$ and were determined to have > 25% leakage of the dye. We believe the variability was due to a lack of tissue leading to poor loading of fura-2/AM, poor hydrolysis of the dye, and dye leakage. The assay required at least 1 mg/ml protein per sample. An inordinately large number of mice would have to have been sacrificed to obtain adequate tissue. Thus, our attempt to use less tissue resulted in the variability described earlier.

Because tolerance to morphine appeared to be accompanied by an increase in basal levels of $[Ca^{++}]i$ in the brain, we examined the specificity of this effect for morphine by performing cross-tolerance experiments in vitro. The results of those experiments are shown in Fig. 1. Morphine significantly blocked KCl-induced rises in the synaptosomes from the nontolerant mice, but failed to block KCl-induced rises in the synaptosomes from morphine-tolerant mice. In vivo, mice tolerant to morphine (SC) were cross-tolerant to DAMGO (IT and ICV) (Fig. 3). Cross-tolerance between morphine and DAMGO was observed in vitro. That is, like morphine, DAMGO blocked KCl-induced rises in $[Ca^{++}]i$ in the nontolerant preparation, but not in the tolerant preparation. No cross tolerance was observed between morphine (SC) and the δ-agonist DPDPE (IT or ICV), or the κ-agonist U50,488H (U50, IT or ICV) in vivo (Table 3). Similarly, no cross tolerance was observed between morphine (SC) and DPDPE or U50 in vitro.

DISCUSSION

These data indicate that tolerance in vivo induced a change in synaptosomal calcium modulation such that tolerance could be observed in vitro. In addition, the change in the synaptosomes induced by morphine tolerance in vivo is specific for μ-agonists. Our data indicate that a membrane component may be altered by the development of tolerance to morphine, leading to an alteration in $[Ca^{++}]i$ not only in the brain as we had previously reported (59), but also in the spinal cord. Several possibilities exist as to the membrane component altered in the development of tolerance leading to a higher set point for intracellular calcium levels. Chronic exposure to opioids results in neuronal tolerance to the acute inhibitory effects of opiates on adenylyl cyclase in affected regions (36,52). Most regions show no alteration in response to chronic morphine

TABLE 3

EVALUATION OF CROSS-TOLERANCE TO MORPHINE IN VIVO IN MICE

Drug	% MPE Placebo mice	% MPE Morphine-tolerant mice
Morphine		
10 mg/kg, SC	96 ± 2 (n = 30)	5 ± 3 (n = 30)**
Morphine		
3 μg/mouse, i.t.	85 ± 9 (n = 24)	15 ± 5 (n = 24)**
DAMGO		
1 μg/mouse, i.t.	88 ± 8 (n = 12)	10 ± 5 (n = 12)**
0.5 μg/mouse, icv.	78 ± 6 (n = 6)	2 ± 5 (n = 6)
DPDPE		
3 μg/mouse, i.t.	95 ± 2 (n = 12)	85 ± 7 (n = 12)
3 μg/mouse, icv.	81 ± 9 (n = 6)	74 ± 5 (n = 6)
U50, 488H		
50 μg/mouse, i.t.	78 ± 5 (n = 12)	70 ± 3 (n = 12)
50 μg/mouse, icv.	48 ± 9 (n = 6)	55 ± 5 (n = 6)

Mice were rendered tolerant to morphine after a 3 day (72 hour) exposure to the morphine pellets. Separate groups of mice were also implanted with placebo pellets. Tolerance was assessed using the tail-flick test for antinociception. Morphine-tolerant and nontolerant (placebo-pelleted) mice were tested for cross-tolerance to the kappa agonist, U50,488H, the delta agonist, DPDPE, or the mu agonist, DAMGO (all administered i.t. and icv.). The doses of the drugs represent doses which produced at least 80% maximum possible effect (% MPE) in naive mice in our laboratory on the day of testing with the exception of U50,488H which was a partial agonist when administered icv. The average % MPE ± Standard error of the mean and the number (n) of mice per group tested are listed.

$**p < 0.01$ from the respective "placebo" group.

except the locus coeruleus, nucleus accumbens, amygdala, and dorsal root ganglia (52). In sensitive regions, tolerance affects levels of G_{ia} and G_{oa} (36), adenylyl cyclase activity (52), and the activity of cAMP-dependent protein kinase (52), which in turn phosphorylates (and activates) calcium channels, IP_3 receptors, and potassium channels, among other important cell proteins (55). Other compensatory mechanisms include an impairment of opioid receptors to stimulate $G_{i/o}$ proteins (56), an alteration in stimulation of GTPase activity (2), and an increase in "L-type" calcium channel number (39,44,45,50). In addition, the chronic administration of morphine has been shown to increase the number of "N-type" calcium channels in mouse brain (50). Thus, increased calcium channel binding has been documented to accompany opioid tolerance. Whether μ-, δ-, and δ-opioids produce similar or dissimilar effects on calcium channel number or function has not been clarified. Based on recent work measuring mRNAs for various ion channels in response to chronic morphine and the δ-opioid DADL, in cells in culture, dissimilarities may exist in the alterations of ion channels by opioid receptor subtypes (33). Such dissimilarities in channels could explain the lack of cross tolerance we observed between μ- and δ- or δ-opioid in vitro.

The effects of the chronic administration of μ-, δ-, and κ-opioids on potassium channel number or function have not been extensively studied. Mackler and Eberwine (33) showed that the mRNA for certain potassium channels is decreased by morphine, but not by DADL. Chronic morphine has been shown to decrease potassium efflux in rats (8). We have shown that there is no cross tolerance of morphine to potassium channel openers. However, the potassium channel blockers apamin and glyburide precipitate a withdrawal syndrome in morphine-tolerant mice (58). Thus, interactions between opiates and potassium channels are anatomically and functionally possible in the regulation of tolerance. A likely candidate for the point of interaction of these two classes of drugs lies in the modulation of intracellular calcium, as potassium channel blockers increase intracellular calcium (14,16,17,21,42,46). Thus, the differences on basal levels of $[Ca^{++}]i$ could be due to a down-regulation of potassium channels in the synaptosomal membrane and a resultant decrease in potassium efflux.

We also hypothesize that higher basal $[Ca^{++}]i$ levels observed with chronic morphine exposure may be due, in part, to an alteration in microsomal calcium sequestration and release. The role of opiate tolerance on microsomal activity has not been determined. Opioid withdrawal has been shown to be accompanied by increased formation of IP_3 in brain (18). Microsomal pools (along with mitochondrial pools) play a major role in sequestering Ca^{++} following nerve activity. Activation of phospholipase C from cell surface receptor stimulation leads to the breakdown of cell membrane phosphatidylinositol 4,5-bisphosphate, and the production of inositol 1,4,5-trisphosphate (IP_3). The IP_3-sensitive pool accounts for a large portion of the total intracellular stores of Ca^{++} [for reviews, see (3,5)]. The theory of capacitative Ca^{++} entry involves the interaction between cell surface inositol tetrakisphosphate (IP_4) receptors and refilling of IP_3-sensitive microsomal pools during stimulation (27,51). Neurons also possess Ca^{++}/caffeine-sensitive pools, which are stimulated by caffeine and blocked by ryanodine (4,12,41,53). Increases in the membrane receptors for IP_3 or ryanodine on the endoplasmic reticulum (ER) could lead to an increased release of calcium from the ER and contribute to the higher basal $[Ca^{++}]i$ in the synaptosomes. Because synaptosomes are largely devoid of ER, such an explanation for higher basal $[Ca^{++}]i$ seems unlikely. However, increased numbers of plasma membrane-associated IP_4 receptors could lead to increased calcium entry. In addition, another possibility for an increase in $[Ca^{++}]i$ could be that sodium–calcium exchange has been altered by the chronic administration of opioids.

Thus, chronic exposure to morphine induces a μ-opioid-selective change in synaptosomes from both brain and spinal cord, resulting in increased levels of $[Ca^{++}]i$, which is hypothesized to counter the acute effects of morphine on $[Ca^{++}]i$ and result in both the production of tolerance and a lack of cross tolerance between classes of opioid receptors. Several membrane-associated points for modulation of calcium exist, although the exact nature of the morphine-induced change remains to be determined. The data indicate that changes in $[Ca^{++}]i$ in the medulla, corpus striatum, and midbrain do not account for the overall increases in $[Ca^{++}]i$. Thus, it is likely that one or more discrete areas of the brain are altered by tolerance to morphine and account for the changes in $[Ca^{++}]i$ observed.

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REFERENCES

1. Aronsen, J. K. Potassium channels in nervous tissue. Biochem. Pharmacol. 43:11–14; 1992.
2. Barchfeld, C. C.; Maasen, F. A.; Medzihradsky, F. Receptor regulated interactions of opiated with PGE-induced adenylate cyclase in rats. Life Sci. 31:1661–1665; 1982.
3. Berridge, M. J. Inositol triphosphate and calcium signalling. Nature 361:315–325; 1993.
4. Bezprozvanny, I.; Watras, J.; Ehrlich, B. E. Bell-shaped Ca^{++}-response curves of Ins (1,4,5)P3- and Ca^{++}-gated channels from endoplasmic reticulum of cerebellum. Nature 351:751–754; 1991.
5. Blaustein, M. P. Ca^{++} transport and buffering in neurons. Trends Neurosci. Sci. 11:438–443; 1988.
6. Cardenas, H. L.; Ross, D. H. Morphine-induced Ca^{++} depletion in descrete regions of rat brain. J. Neurochem. 24:487–493; 1975.
7. Chapman, D. B.; Way E. L. Metal ion interactions with opiates. Ann. Rev. Pharmacol. Tox. 20:553–579; 1980.
8. Christie, M. J.; Williams, J. T.; North, R. A. Mol. Pharmacol. 32:633–638; 1987.
9. Clouet, D. H.; Williams, N. The role of calmodulin in opioid-induced changes in the phosphorylation of rat striatal synaptic membrane proteins. Neurochem. Res. 7:1135–1148; 1982.
10. Clouet, D. H.; Williams, N.; Yonehara, N. In a calmodulin-opioid peptide related to the mechanism of opioid action? Life Sci. 33:727–730; 1983.
11. Collin, E.; Cesselin, F. Neurobiological mechanisms of opioid tolerance and dependence. Clin. Neuropharmacol. 14:465–488; 1991.
12. Dehlinger-Kremer, M.; Zeuzem, S.; Schulz, I. Interaction of caffeine-, IP3- and vanadate-sensitive pools in acinar cells of the exocrine pancreas. J. Membrane Biol. 119:85–100; 1991.
13. Desaiah, D.; Ho, I. K. Effect of morphine on mouse brain ATPase activities. Biochem. Pharmacol. 26:89–92; 1977.
14. DeWeille, J. R.; Lazdunski, M. Regulation of the ATP-sensitive potassium channel. In: Narahashi, T., ed. Ion channels. New York: Plenum; 1990:205–222.
15. Flemming, L. M.; Ponjee, G.; Childers, S. R. Inhibition of pro-

tein phosphorylation by opioid-inhibited adenylyl cyclase in rat brain membranes. J. Pharmacol. Exp. Ther. 260:1416–1424; 1992.
16. Fosset, M.; DeWeille, J. R.; Green, R. D.; Schmid-Antomarchi, H.; Lazdunski, M. Antidiabetic sulfonylureas control action potential properties in heart cells via high affinity receptors that are linked to ATP-dependent potassium channels. J. Biol. Chem. 263:7933–7936; 1988.
17. Gaines, K. L.; Hamilton, S.; Boyd, A. E. Characterization of the sulfonylurea receptor on beta cell membranes. J. Biol. Chem. 263:2589–2592; 1988.
18. Giampietro, D. E. P.; Ruggiero, M.; Giannelli, S.; Chiarugi, V. P.; Moroni, F. Morphine withdrawal in vitro: Potentiation of agonist-dependent polyphosphoinositide breakdown. Eur. J. Pharmacol. 149:297–306; 1988.
19. Grundt, T. J.; Williams, J. T. κ-Opioid receptors also increase potassium conductance. Proc. Natl. Acad. Sci. USA 90:11429–11423; 1993.
20. Grynkiewicz, G.; Iverson, L. L. A new generation of Ca^{++} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260:3440–3450; 1985.
21. Hara, N.; Ichinose, M.; Sawada, M.; Maeno, T. Activation of calcium-dependent potassium conductance by adenaline in mouse peritoneal macrophages. Pflugers Arch. Eur. J. Physiol. 419:371–379; 1991.
22. Harris, L. S.; Pierson, A. K. Some narcotic antagonists in the benzomorphan series. J. Pharmacol. Exp. Ther. 143:141–148; 1964.
23. Harris, R. A.; Yamamoto, H.; Loh, H. H.; Way, E. L. Alterations in brain Ca^{++} localization during the development of morphine tolerance and dependence. In: Kosterlitz, H. W., ed. Opiates and endogenous opioid peptides. New York: Elsevier; 1976: 361–368.
24. Hoffmeister, F.; Tettenborn, D. Ca^{++} agonists and antagonists of the dihydropyridine type: Antinociceptive effects, interference with opiate-mu-receptor agonists and neuropharmacological actions in rodents. Psychopharmacology 90:299–307; 1986.
25. Hughes, J.; Kosterlitz, H. W.; Leslie, F. M. Effect of morphine on adrenergic transmission in the mouse vas deferens. Assessment of agonist and antagonist potencies of narcotic analgesics. Br. J. Pharmacol. 53:371–381; 1975.
26. Hylden, J. L. K.; Wilcox, G. L. Pharmacological characterization of substance P-induced nociception in mice: Modulation by opioid and noradrenergic agonists at the spinal level. Eur. J. Pharmacol. 67:313–404; 1983.
27. Irving, A. J.; Collingridge, G. L.; Schofield, J. G. Interactions between Ca^{++} mobilizing mechanisms in cultured rat cerebellar granule cells. J. Physiol. 456:667–680; 1992.
28. Jhamandas, K.; Sawynok, J.; Sutak, M. Antagonism of morphine action on brain acetylcholine release by methylxanthines and Ca^{++}. Eur. J. Pharmacol. 49:309–312; 1978.
29. Komulainen, H.; Bondy, S. C. The estimation of free calcium within synaptosomes and mitochondria with fura-2: Comparison to QUIN-2. Neurochem. Int. 10:55–64; 1987.
30. Laurian, L.; Oberman, Z.; Hoerer, E.; Graf, E. Ansiserotonergic inhibition of calcitonin-induced increase of beta-endorphin, ACTH, and cortisol secretion. J. Neural Transm. 73:167–176; 1988.
31. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:270–275; 1951.
32. Lux, F.; Welch, S. P.; Brase, D. A.; Dewey, W. L. Evidence for supraspinal mediation of intrathecal calcium-induced antinociception in mice: Modulation by intracerebroventricular naloxone, calcium and verapamil, and comparison with systemic morphine. J. Pharmacol. Exp. Ther. 246:500–507, 1988.
33. Mackler, S. A.; Eberwine, J. H. Cellular adaptation to opiates alters ion-channel mRNA levels. Proc. Natl. Acad. Sci. USA 91: 385–389; 1994.
34. McGovern, S.; Maguire, M. E.; Gurd, R. S.; Mahler, H. R.; Moore, W. J. Separation of adrenergic and cholinergic synaptosomes from immature rat brain. FEBS Lett. 31:193–198; 1973.
35. Nestler, E. J. Molecular mechanisms of drug addiction in the mesolimbic dopamine pathway. Neurosciences 5:369–376; 1993.
36. Nestler, E. J.; Erdos, J. J.; Terwilliger, R. Z.; Duman, R. S.; Tallman, J. F. Regulation of G-proteins by chronic morphine treatment in the rat locus coeruleus. Brain Res. 476:230–239; 1989.
37. North, R. A. Opioid actions on membrane ion channels. In: Herz, A.; Simon, E.; Akil, H., eds. Handbook of experimental pharmacology. Amsterdam: Springer, 1992.
38. North, R. A.; Williams, J. T.; Surprenant, A.; Christie, M. J. μ and delta receptors belong to a family of receptors that are coupled to potassium channels. Proc. Natl. Acad. Sci. USA 84:5487–5491; 1987.
39. Ohnishi, T.; Saito, K.; Maeda, S.; Matsumoto, K.; Sakuda, M.; Inoki, R. Intracerebroventricular treatment of mice with pertussis toxin induces hyperalgesia and enhances [^{3}H]-nitrendipine binding to synaptic membranes: Similarity with morphine tolerance. Naunyn-Schmiedeberg's Arch. Pharmacol. 341:123–127; 1990.
40. Olson, K. G.; Welch, S. P. The effects of Dynorphin A (1–13) and U50,488H on free intracellular calcium in guinea pig cerebellum. Life Sci. 48:575–581; 1991.
41. Palade, P.; Dettbarn, C.; Alderson, B.; Volpe, P. Pharmacologic differentiation between inositol 1,4,5-trisphosphate induced Ca^{++} release and Ca^{++} or caffeine induced Ca^{++} release from intracellular membrane systems. Mol. Pharmacol. 36:673–680; 1989.
42. Panten, U.; Burgfeld, J.; Goerke, F.; Rennicke, M.; Schwanstecher, M.; Wallasch, A.; Zinkler, B. J.; Lenzen, S. Control of insulin secretion by sulfonylureas, meglitinide and diazoxide in relation to their binding to the sulfonylurea receptor in pancreatic islets. Biochem. Pharmacol. 38:1217–1229; 1989.
43. Pedigo, N. W.; Dewey, W. L.; Harris, L. S. Determination and characterization of the antinociceptive activity of intraventricularly administered acetylcholine in mice. J. Pharmacol. Exp. Ther. 193:845–852; 1975.
44. Ramkumar, V.; El-Fakahamy, E. E. Increase in [^{3}H]-nitrendipine binding sites in the brain in morphine-tolerant mice. Eur. J. Pharmacol. 102:371–372; 1984.
45. Ramkumar, V.; El-Fakahamy, E. E. Prolonged morphine treatment increases rat brain dihydropyridine binding sites: Possible involvement in development of morphine dependence. Eur. J. Pharmacol. 146:73–83; 1988.
46. Reiser, G.; Hamprecht, B. Bradykinin causes a transient rise in intracellular calcium activity in cultured neural cells. Pflugers Arch. Eur. J. Physiol. 405:260–264; 1985.
47. Ross, D. H.; Lynne, S. C.; Cardenas, H. L. Selective control of Ca^{++} ions by naloxone. Life Sci. 18:789–796; 1976.
48. Ross, D. H.; Medina, M. A.; Cardenas, H. L. Morphine and ethanol: Selective depletion of regional brain Ca^{++}. Science 186: 63–64;1974.
49. Sanfacon, G.; Houde-DePuis, M.; Vanier, R.; LaBreque, G. Calcium-induced modifications of inhibition of acetylcholine release by morphine. J. Neurochem. 28:881–884; 1977.
50. Suematsu, M.; Ohnishi, T.; Shinno, E.; Maeda, S.; Matsumoto, K.; Sakuda, M.; Saito, K. Effect of prolonged administration of clonidine on ^{3}H-PN200–110 and ^{125}I-ω-conotoxin binding in mouse brain. Neurosci. Lett. 163:193–196; 1993.
51. Takemura, H.; Ohshika, H.; Yokosawa, N.; Oguma, K; Thastrup, O. The thapsigargin-sensitive intracellular Ca^{++} pool is more important in plasma membrane Ca^{++} entry than the IP3-sensitive intracellular pool in neuronal cell lines. Biochem. Biophys. Res. Commun. 180:1518–1526; 1991.
52. Terwilliger, R. Z; Johnson, D. B.; Sevarino, K. A.; Crain, S. M.; Nestler, E. J. A general role for adaptations in G-proteins and the cAMP system in mediating the chronic actions of morphine and cocaine on neuronal function. Brain Res. 548:100–110; 1991.
53. Thayer S. A.; Perney, T. M.; Miller, R. J. Regulation of Ca^{++} homeostasis in sensory neurons by bradykinin. J. Neurosci. 8: 4089–4097; 1988.
54. Triggle, D. J. Potassium channels and potassium channel modulators. Neurotransmissions 6:1–5; 1990.

55. Tsunoda, Y. Receptor-operated calcium signaling and crosstalk in stimulus secretion coupling. Biochim. Biophys. Acta 1154:105–156; 1993.
56. Vachon, L.; Costa, C.; Herz, A. Desensitization of opioid stimulated GTPase in neuroblastoma × glioma hybrid cells. Biochem. Biophys. Res. Commun. 128:1342–1349; 1985.
57. Way, E. L.; Loh, H. H.; Shen, F. H. Simultaneous quantitative assessment of morphine tolerance and physical dependence. J. Pharmacol. Exp. Ther. 167:1–8; 1969.
58. Welch, S. P.; Dunlow, L. D. Antinociceptive activity of intrathecally administered potassium channel openers and opioid agonists: A common mechanism of action? J. Pharmacol. Exp. Ther. 267:390–399; 1993.
59. Welch, S. P.; Olson, K. G. Opiate tolerance-induced modulation of free intracellular Ca^{++} in synaptosomes. Life Sci. 48:1853–1861; 1991.
60. Welch, S. P.; Olson, K. G. Salmon calcitonin-induced modulation of free intracellular calcium. Physiol. Biochem. Behav. 39: 641–648; 1991.
61. Welch, S. P.; Singha, A. K.; Dewey, W. L. Modulation of the antinociception produced by intrathecal morphine, calcium, A23187, U50,488H, DAMGO, and DPDPE by intrathecally administered calcitonin gene-related peptide (CGRP) in mice. J. Pharmacol. Exp. Ther. 251:1–8, 1989.
62. Werz, M. A.; Macdonald, R. L. Dynorphin and neoendorphin peptides decrease dorsal root ganglion neuron calcium-dependent action potential duration. J. Pharmacol. Exp. Ther. 234:49–56; 1985.
63. Williams, J. T.; Eagan, T. M.; North, R. A. Enkephalin opens potassium channels in mammalian central neurons. Nature 299: 74–77; 1982.
64. Zieglgansberger, W.; Fry, J. P. Opioid actions on mammalian spinal nerons. In: Herz, A., ed. Developments in opiate research. New York: Marcel Dekker; 1978:192–199.