EFFECTS OF MORPHINE, CODEINE AND CODEINE-EPOXIDE ON CALCIUM UPTAKE INTO THE SYNAPTOSOMES ISOLATED FROM NAIVE AND TOLERANT RATS

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Abstract—We studied the effects of morphine, codeine and codeine-7,8-oxide (codeine-epoxide) on the stimuli-induced $^{45}$Ca$^{2+}$ uptake into the synaptosomes isolated from naive and tolerant rats and clarified the relationship between pharmacological responses of opiates and synaptosomal $^{45}$Ca$^{2+}$ uptake. In vitro additions of morphine and codeine-epoxide inhibited the synaptosomal $^{45}$Ca$^{2+}$ uptakes induced by two stimuli, that is, high KCl and veratrine in a concentration-dependent manner; and the inhibitions could be reversed by naloxone. However, the inhibitory action of codeine was less than that of morphine and codeine-epoxide. Since the potency ratios of the anti-nociceptive action of opiates are higher in the order of morphine $>$ codeine-epoxide $>$ codeine, the inhibitory effect of opiates on synaptosomal $^{45}$Ca$^{2+}$ uptake may partly relate to their antinociceptive action. On the other hand, opiates significantly increased synaptosomal $^{45}$Ca$^{2+}$ uptake when animals were rendered tolerant to their antinociceptive action, and data showed that the elevation of stimuli-induced $^{45}$Ca$^{2+}$ uptake into the synaptosomes isolated from tolerant animals may reflect the degree of antinociceptive tolerance. Our results support the hypothesis that some of the pharmacological effects of opiates may be attributable to its ability to affect calcium accumulation in synaptosomes.

Recent findings suggest that calcium has been implicated in the antinociceptive action of opiate drugs in the central nervous system (1-4). Furthermore, morphine has been reported to inhibit the calcium uptake into the synaptosomes by a naloxone-reversible process, while tolerance to morphine resulted in an elevation of synaptosomal calcium uptake (5-7). Since an influx of calcium is required for release of neurotransmitters (8) and since opiate drugs have been shown to inhibit the release of neurotransmitters (9, 10), these results suggested that a certain relationship may exist between pharmacological responses of opiate drugs and synaptosomal calcium uptake. Since it has been reported that the antinociceptive action of codeine-7,8-oxide (codeine-epoxide), a new metabolite of codeine (11), was about 2 times as potent as codeine and tolerance developed more slowly than that of codeine (12), it was of interest to examine whether or not these differences reflect synaptosomal calcium uptake. In this paper, we examined the effects of morphine, codeine and codeine-epoxide on stimuli-induced calcium uptake into the synaptosomes isolated from naive and tolerant rats and did studies to clarify the relationship...
relationship between pharmacological responses of opiate drugs and synaptosomal calcium uptake.

MATERIALS AND METHODS

Antinociceptive activity: The antinociceptive responses of morphine, codeine and codeine-epoxide were determined by the method of pressure stimuli on the rat hindpaw using the Randall-Selitto apparatus (Ugo Vasile) (13). Groups of 8 male Wistar strain rats weighing 80 to 100 g were used. A control response was determined for each animal before treatment, and test thresholds were assessed at 30 min after drug injection. Thirty min after drug injection was chosen because our previous data (12) had shown that the antinociceptive responses at 30 min after drug dosing correlated with the antinociceptive index (data not shown). The maximal pressure measured was 250 g.

Tolerance: Animals in the tolerant groups received 10 mg/kg, s.c., of morphine or 20 mg/kg, s.c., of codeine every 4 hr for three days. These drug doses were chosen because our previous study (12) had shown that the antinociceptive potency ratio of morphine, codeine-epoxide and codeine was about 4:2:1. The antinociceptive activities of drugs were measured by the method of pressure stimuli on the rat hindpaw at 30, 60, 90 and 120 min after drug injection. The antinociceptive index (A.I.) was calculated as follows: A.I. = sum of threshold (g) obtained at 30, 60, 90 and 120 min after injection/ [threshold(g) before injection] × 4. After the tolerance was confirmed on the third day, animals were killed and used for synaptosomal calcium uptake at 30 min after the final administration of drugs.

Calcium uptake: Synaptosomes were prepared from brain homogenates using discontinuous Ficoll gradient centrifugation according to a modification of the procedure of Cotman and Matthews (14). The final synaptosomal pellet was resuspended in incubation medium to give a protein concentration of approximately 0.5 to 1.0 mg/ml as determined by the method of Lowry et al. (15) and kept on ice prior to the incubation. The uptake of $^{45}$Ca$^{2+}$ into the synaptosomes was studied as described by Blaustein (16). Briefly, the synaptosomes were first preincubated in a physiological salt solution (132 mM NaCl, 5 mM KCl, 1.2 mM NaH$_2$PO$_4$, 1.3 mM MgCl$_2$, 1.2 mM CaCl$_2$, 10 mM glucose and 20 mM Tris/maleic acid buffer, pH 7.4) for 15 min at 30°C. The uptake of $^{45}$Ca$^{2+}$ into the synaptosomes was initiated by the addition of an equal volume of depolarizing media (137 mM KCl, 1.2 mM NaH$_2$PO$_4$, 1.3 mM MgCl$_2$, 1.2 mM CaCl$_2$, 10 mM glucose and 20 mM Tris/maleic acid buffer, pH 7.4 or 132 mM NaCl, 5 mM KCl, 1.2 mM NaH$_2$PO$_4$, 1.3 mM MgCl$_2$, 1.2 mM CaCl$_2$, 60 μg/ml of veratrine, 10 mM glucose and 20 mM Tris/maleic acid buffer, pH 7.4) containing $^{45}$Ca$^{2+}$ (specific activity 1 mCi of $^{45}$Ca$^{2+}$/m mol). The mixture was then incubated for 3 min at 30°C, and the reaction was terminated by adding an equal volume of ice-cold GEDTA stopping solution (120 mM NaCl, 5 mM KCl and 30 mM GEDTA, titrated to pH 7.4 with Tris-base). The 3 min $^{45}$Ca$^{2+}$ loading period was chosen because our preliminary study had shown that the peak influx was reached at this time (17). Nondepolarized samples were handled in the same manner except that after 15 min preincubation period, an equal volume of incubation medium (5 mM KCl) containing $^{45}$Ca$^{2+}$ (specific activity 1 mCi/m mol) was added. When in vitro effects of drugs were examined, drugs were added at the beginning of the preincubation period. Each sample was immediately filtered by passing it through a Whatmann glass fiber filter (GF-C) prewashed with ice-cold washing solution (132 mM choline-Cl, 5 mM KCl, 1.2 mM NaH$_2$PO$_4$, 1.3 mM MgCl$_2$, 1.2 mM CaCl$_2$, 10 mM...
glucose and 20 mM Tris/maleic acid buffer, pH 7.4) and washed twice with 3 ml of ice-cold washing solution. The filters were then brought to complete dryness under an infrared lamp. They were then placed directly into vials with a toluene scintillator and counted using an ASOKA LSC-900 liquid scintillation counter. The values were corrected for the $^{45}$Ca$^{2+}$ remaining on the filters in the absence of the synaptosomal fraction.

Statistical significance was evaluated by the Student's t-test.

Drugs used: The following were purchased from commercial sources: codeine phosphate, morphine hydrochloride and naloxone hydrochloride from the Sankyo Co., Japan; Tris (Tris (hydroxymethyl) aminomethane) from the Sigma Chemical Co.; GEDTA (ethylene-glycol-bis-(β-aminoethylether)-N,N-tetraacetic acid) from the Wako Junyaku Co., Japan; and Ficoll 400 from Pharmacia Fine Chemicals. $^{45}$CaCl$_2$ (specific activity: 16 mCi/mg Ca) was obtained from New England Nuclear. Veratrine was from Merck. Codeine-epoxide was synthesized according to the method of Uba et al. (11). Other chemicals used were analytical grade. Drugs were dissolved in double-distilled and deionized water.

RESULTS

Antinociceptive activity: In this experiment, morphine, codeine and codeine-epoxide were administered in equipotent doses to produce an antinociceptive response (morphine 2.5 mg/kg, s.c.; codeine 10 mg/kg, s.c.; and codeine-epoxide 5 mg/kg, s.c.). Antinociceptive responses of morphine, codeine and codeine-epoxide were abolished by naloxone (1 mg/kg, s.c.). Administration of naloxone (1 mg/kg, s.c.) alone was without any antinociceptive effect (Fig. 1).

Effects of morphine, codeine and codeine-epoxide on the stimuli-induced synaptosomal

![Antinociceptive activity](chart)

Fig. 1. Inhibitory effect of naloxone on opiate drug-induced antinociceptive activity in rats. Each bar represents the mean±S.E. of 8 animals. Control: saline, s.c.; Naloxone: 1 mg/kg, s.c.; and Codeine-epoxide: 5 mg/kg s.c. Open bars show the values of antinociceptive activity before drug dosing. Closed bars show the values of antinociceptive activity at 30 min after drug dosing. Naloxone was administered at the same time of drug dosing. *P<0.01: significantly different from the control at 30 min after saline dosing.

![Effect of morphine](chart)

Fig. 2. Effect of morphine on stimuli-induced synaptosomal $^{45}$Ca$^{2+}$ uptake. Each point represents the mean±S.E. of 8 experiments. ■: non-depolarized (5 mM KCl) synaptosomes ($Y=-0.041X+8.705$, $r=-0.114$, $P>0.1$), ○: depolarized (30 µg/ml of veratrine) synaptosomes ($Y=-0.366X+10.376$, $r=-0.7549$, $P<0.01$) and ◦: depolarized (71 mM KCl) synaptosomes ($Y=-0.67X+15.253$, $r=-0.7771$, $P<0.01$). *P<0.05, **P<0.01: significantly different from the control (without addition of morphine).
45Ca2+ uptake: Effects of morphine, codeine and codeine-epoxide on the stimuli-induced synaptosomal 45Ca2+ uptake are shown in Figs. 2, 3 and 4, respectively. As can be seen from the control values (without addition of drugs), depolarization of synaptosomes by the elevated concentration of potassium or addition of veratrine increased the uptake of 45Ca2+ more than about 2.5-fold and 1.5-fold, respectively. The basal uptakes (5 mM KCl) were not significantly influenced by various concentrations (10^-8 M to 10^-5 M) of drugs. However, stimuli-induced 45Ca2+ uptakes were inhibited by morphine and codeine-epoxide in a concentration-dependent manner (see Fig. 2 and Fig. 4 legends), while codeine was without effect on the stimuli-induced 45Ca2+ uptakes except for 10^-5 M codeine on the potassium-stimulated 45Ca2+ uptake (Fig. 3). The inhibitions of veratrine-stimulated synaptosomal 45Ca2+ uptake by morphine (10^-6 M) and codeine-epoxide (10^-6 M) were prevented by the addition of naloxone (10^-6 M), but 10^-6 M naloxone alone and 10^-6 M of codeine alone had no influence (Fig. 5).

Antinociceptive responses to morphine, codeine and codeine-epoxide in tolerant rats: Tolerance development to morphine, codeine and codeine-epoxide were measured by the reduction of antinociceptive responses of rats when morphine (10 mg/kg, s.c.), codeine (40 mg/kg, s.c.) and codeine-epoxide (20 mg/kg, s.c.) at their equipotent doses were repeatedly administered every 4 hr. The antinociceptive responses of morphine, codeine and codeine-epoxide were gradually reduced. Tolerance to morphine develops...
Fig. 5. Combined effects of morphine, codeine and codeine-epoxide with naloxone on veratrine-induced synaptosomal $^{45}\text{Ca}^2+$ uptake. Each bar represents the mean±S.E. of 5 experiments. Open bars show the nondepolarized (5 mM KCI) uptake. Closed bars show the depolarized uptake. Drugs (10$^{-6}$ M) were added at the beginning of the preincubation period. *P<0.05, **P<0.01: significantly different from the depolarized control.

Fig. 5.

Fig. 6. Development of tolerance in the antinociceptive activity. Each point represents the mean±S.E. of 6 animals. : Control (saline, s.c.). : Morphine (10 mg/kg, s.c.), : Codeine (40 mg/kg, s.c.) and : Codeine-epoxide (20 mg/kg, s.c.). *P<0.01: significantly different from the corresponding control.

Fig. 7. Stimuli-induced $^{45}\text{Ca}^2+$ uptake into the synaptosomes isolated from tolerant rat brain. Each bar represents the mean±S.E. of 6 experiments. Open bars show the nondepolarized (5 mM KCI) uptake. Closed bars show the depolarized (30 µg/ml of veratrine) uptake. Dotted bars show the depolarized (71 mM KCI) uptake. Morphine (10 mg/kg), codeine (40 mg/kg) and codeine-epoxide (20 mg/kg) were subcutaneously administered every 4 hours for 3 days. *P<0.01: significantly different from each depolarized control. ++P<0.05, +++P<0.01: significantly different from each depolarized morphine-treated uptake.

Fig. 7.

most rapidly among the three drugs, and it develops most slowly in the rats treated with codeine-epoxide (Fig. 6).

Synaptosomal $^{45}\text{Ca}^2+$ uptake in the tolerant rats: In this experiment, we studied the stimuli-induced calcium uptake into the synaptosomes isolated from tolerant rat brains. Figure 7 shows that continuous treatments of morphine, codeine and codeine-epoxide produce a significant increase in the stimuli-induced synaptosomal $^{45}\text{Ca}^2+$ uptakes, except for codeine-epoxide on the potassium-stimulated uptake, while basal uptakes (5 mM KCI) were not influenced. Animals treated with morphine showed the most elevation of the stimuli-induced synaptosomal $^{45}\text{Ca}^2+$ uptake among the three drugs, and significantly higher in the animals treated with codeine or codeine-epoxide on the potassium-stimulated uptake (P<0.01). Moreover, with codeine-epoxide, the trend for increase in stimuli-induced synaptosomal $^{45}\text{Ca}$ uptake was less than that with codeine.

**DISCUSSION**

Codeine-epoxide was recently identified as a new metabolite of codeine (11), and the antinociceptive activity of codeine-epoxide was weaker than that of morphine, but more effective than its parent compound (12).
Furthermore, Takayanagi et al. (12) reported that tolerance developed more slowly in the rats treated with codeine-epoxide than with codeine.

In this study, we also found that the antinociceptive activity of codeine-epoxide was abolished with naloxone, suggesting that the antinociceptive activity of codeine-epoxide was also mediated through the opiate receptors. Furthermore, the degree for tolerance development of opiate drugs used was higher in the order of morphine > codeine > codeine-epoxide, and these results were also supported by the findings of Takayanagi et al. (18) who found that codeine-epoxide showed a trend of decrease in dependence liability.

Recent findings have suggested a role for calcium in the production of analgesia and tolerance by opiates (1-4). Furthermore, Guerrero-Munoz et al. (6, 7) and Ross (5) indicated by biochemical studies that the acute or in vitro administration of morphine inhibited synaptosomal $^{45}$Ca$^{2+}$ uptake; and chronic administration of morphine, when animals were rendered to tolerance/dependence on morphine, resulted in an elevation of synaptosomal $^{45}$Ca uptake. In this study, we used the $^{45}$Ca uptake into the synaptosomes by two stimuli, high KCI and veratrine, and clarified the relationship between the relative potency of the antinociceptive response or tolerance to opiates and synaptosomal $^{45}$Ca uptake. Both high KCI and veratrum alkaloid were shown to depolarize synaptosomes (19, 20), and the depolarization was associated with an increased uptake of $^{45}$Ca$^{2+}$ in the synaptosomes and an elevated synaptosomal calcium level (16). Furthermore, the depolarization was related to the release of neurotransmitters (16, 21, 22).

Our results showed that morphine and codeine-epoxide significantly inhibited the synaptosomal $^{45}$Ca uptake by the two stimuli in a concentration-dependent manner, and the inhibitions were naloxone reversible processes. However, the inhibitory action of codeine was less effective than those of morphine and codeine-epoxide. The inhibitions of $^{45}$Ca$^{2+}$ uptake by opiates were reproducible, although the amount of $^{45}$Ca$^{2+}$ taken up by the synaptosomes varied from one experiment to another. These discrepancies are probably related to the viability of the intra-synaptosomal mitochondria because Åkerman and Nicholls suggested that intrasynaptosomal mitochondria respond to an increase in $^{45}$Ca$^{2+}$ uptake across the plasma membrane upon depolarization (23).

On the other hand, opiates significantly increased synaptosomal $^{45}$Ca uptake in the tolerant animals. Data showed that the degree for the elevation of stimuli-induced synaptosomal $^{45}$Ca$^{2+}$ uptakes was higher in the order of morphine > codeine > codeine-epoxide. These effects may reflect the development of antinociceptive tolerance to drugs; however, it remains obscure whether or not the elevation of synaptosomal $^{45}$Ca$^{2+}$ uptake is a primary or a secondary action for the development of antinociceptive tolerance to drugs.

It has been postulated that an influx of calcium ions into the nerve endings is required for the release of neurotransmitters (8). Moreover, in vitro addition of opiates significantly depressed potassium-evoked noradrenaline release from brain slices (9, 10), and addition of naloxone significantly enhanced noradrenaline release from the slices of morphine-dependent rat brains (10). Therefore, based on these studies, a more likely mechanism for presynaptic actions is that opiates inhibit transmitter release by reducing the availability of calcium to the stimulus-release coupling mechanism to cause the antinociceptive response; and an elevation of synaptosomal calcium in tolerant animals causes an increase in neurotransmitter...
release to oppose the inhibitory effect of opiates. These results parallel the findings in the peripheral tissues. For instance, Huidobro-Toro et al. (24) suggested that calcium antagonized the inhibitory response of opiates in the isolated preparation of guinea-pig ileum which seemed to be a suitable in vitro model to assess the opiate action (25), and the response of ileum strips to normorphine in animals rendered tolerance/dependence on morphine was also reduced by calcium. Illes et al. (26) also showed that the opiate depressed the stimulated release of the excitatory transmitter by a reduction in the supply of calcium ions to the stimulus-release coupling mechanisms in the mouse vas deferens.

Our data partly support the hypothesis that some of the pharmacological effects of opiates may be attributable to its ability to reduce calcium accumulation by synaptosomes, and tolerance to this effect of opiates on 45Ca2+ uptake develops within the same time period as the antinociceptive tolerance to opiates. These effects may relate to the degree of the pharmacological actions of opiates. However, since in vitro addition of codeine (10^-6 M) had virtually no effect on the synaptosomal 45Ca2+ uptake, it is likely that the site of the acute opiate actions for the inhibitory effect on synaptosomal 45Ca2+ uptake is not affected by the development of tolerance, and other mechanisms also may exist for the antinociceptive action of opiates. Further studies are required.

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