EFFECT OF MORPHINE ON THE STIMULI-INDUCED CALCIUM UPTAKE INTO SYNAPTOSOMES ISOLATED FROM MORPHINE-TOLERANT RATS

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Accepted March 24, 1982

Abstract—Effects of morphine on calcium uptake into synaptosomes isolated from acutely or chronically morphine-tolerant rat brain was studied. Addition of morphine inhibited the depolarization-stimulated uptake of calcium without affecting uptake under nondepolarizing conditions. This inhibition was prevented by simultaneous addition of naloxone with morphine before calcium uptake was initiated. Acute tolerance to morphine increased depolarization-stimulated synaptosomal calcium uptake. On the other hand, chronic exposure of rats to morphine to elicit tolerance to and physical dependence on morphine did not influence synaptosomal calcium uptake. However, these preparations apparently lost the ability of in vitro morphine-inhibition of calcium uptake into the synaptosomes. Our results suggested that adaptive changes of synaptosomal calcium uptake produced by exposure to morphine may be involved in tolerance and physical dependence development, but influence of morphine on calcium uptake by the synaptosomes isolated from the rats acutely tolerance to morphine was differed from that of chronic tolerant rats.

Recent reports have suggested that the concentration of free calcium plays an important role in the tolerance to and physical dependence on opiates. Guerrero-Munoz et al. (1, 2) and Ross (3) reported that subcutaneous implantation of a morphine pellet or subcutaneous injection of morphine for 8 hr for 3 days in mice to induce tolerance and physical dependence resulted in a selective increase of calcium uptake into the synaptosomes, the pinched-off nerve terminals isolated from brain homogenates. Furthermore, this increment was antagonized by pretreatment with naloxone (1, 2). Since an influx of calcium is required for release of neurotransmitters (4) and morphine has been shown to inhibit the release of neurotransmitters (5, 6), these studies suggest the plausible mechanisms for tolerance to and physical dependence on morphine. As most of earlier studies described above on calcium uptake by synaptosomes isolated from the morphine-tolerant animals have been performed at a relatively short time exposure to morphine and represent an acute tolerant stage, it is interesting to test the synaptosomal calcium uptake at a different stage of morphine tolerance.

In this study, we isolated synaptosomes from the brain of rats acutely or chronically tolerant to morphine and used these preparations to evaluate the effect of short or long time exposure to morphine on the depolarization-stimulated uptake of calcium.

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MATERIALS AND METHODS

Tolerance

**Acute tolerance:** Male Wistar strain rats (60 to 100 g in body weight) were subcutaneously injected with 15 mg/kg of morphine every 4 hr for 3 days. Antinociceptive activity of morphine was measured after each injection with a Randall-Selitto apparatus (Ugo Basile) (7). The maximal pressure measured was 250 g. Rats were killed and used for synaptosomal calcium uptake at 30 min after the final administration of morphine.

**Chronic tolerance to and physical dependence on morphine:** The rats (initial body weights of 80-100 g) were made dependent on morphine by a daily subcutaneous injection of morphine. The amount of morphine administered was increased during a period of 4 weeks until a daily dose of 80 mg/kg (40 mg/kg twice a day at 10 a.m. and 6 p.m.) was reached: the first week, 20 mg/day; the second week, 40 mg/day; the third week, 60 mg/day and the fourth week, 80 mg/day (8, 9). After the physical dependence on morphine was confirmed, the animals were still administered 40 mg/kg of morphine at 10 a.m. and 6 p.m., and the animals were killed to obtain synaptosomes at 30 min after the final administration of morphine.

Preparation of synaptosomes

Synaptosomes were isolated by the method of Cotman and Matthews (10). Briefly, whole brains (except the cerebellum) were homogenated using a glass-Teflon homogenizer at the lowest possible speed using eight up and down strokes. Centrifugation procedures were identical to those described by Cotman and Matthews (10). The final synaptosomal pellet was resuspended in incubation medium to give protein concentrations of approximately 0.5 to 1.0 mg/ml which were determined by the method of Lowry et al. (11) using bovine serum albumin as the standard, and the suspensions were kept on ice prior to the incubation.

Calcium uptake procedures

The accumulation of $^{45}$Ca$^{2+}$ into the synaptosomes was studied as described by Blaustein (12). The synaptosomes were preincubated in a physiological salt solution which contained 132 mM NaCl, 5 mM KCl, 1.2 mM Na$_2$HPO$_4$, 1.3 mM MgCl$_2$, 1.2 mM CaCl$_2$, 10 mM glucose, and 20 mM Tris, buffered to pH 7.4 at 25°C by titration with maleic acid for 15 min at 30°C. When in vitro effects of drugs were examined, drugs were added at the beginning of the preincubation period. For the depolarized samples, $^{45}$Ca$^{2+}$-loading was initiated by the addition of an equal volume of depolarizing media (137 mM KCl, 1.2 mM Na$_2$HPO$_4$, 1.3 mM MgCl$_2$, 1.2 mM CaCl$_2$, 10 mM glucose, and 20 mM Tris, buffered to pH 7.4 at 25°C by titration with maleic acid or 132 mM NaCl, 5 mM KCl, 1.2 mM Na$_2$HPO$_4$, 1.3 mM MgCl$_2$, 1.2 mM CaCl$_2$, 60 µg/ml of veratrine, 10 mM glucose, and 20 mM Tris, buffered to pH 7.4 at 25°C by titration with maleic acid) containing $^{45}$Ca$^{2+}$ (specific activity 1 mCi of $^{45}$Ca$^{2+}$/m mol of $^{40}$Ca$^{2+}$). The mixture was then incubated for 3 min at 30°C and terminated by adding an equal volume of ice-cold stopping solution (120 mM NaCl, 5 mM KCl, and 30 mM ethyleneglycol-bis (β-aminoethyl ether)-N,N′-tetraacetic acid (GEDTA), titrated to pH 7.4 at 25°C with Tris-base). Nondepolarized samples were handled in the same manner except that after the 15 min preincubation period, an equal volume of incubation medium (5 mM KCl) containing $^{45}$Ca$^{2+}$ (1 mCi of $^{45}$Ca$^{2+}$/m mol of $^{40}$Ca$^{2+}$) was added. Each sample was immediately filtered through a Whatmann glass fiber filter (GF-C) prewashed with ice-cold washing solution containing 132 mM choline-Cl, 5 mM KCl, 1.2 mM Na$_2$HPO$_4$, 1.3 mM MgCl$_2$, 1.2 mM CaCl$_2$, 10 mM glucose, and 20 mM Tris, buffered to pH 7.4 at 25°C by titration with maleic acid, and the filters were
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 with a ALOKA LSC-900 liquid scintillation counter. Net influx of calcium across the synaptosomal membrane was calculated by subtracting the values obtained in the nondepolarized (5 mM KCl) synaptosomes from the values in the high KCl- or veratrine-depolarized synaptosomes. These differences are expressed as JK (potassium dependent changes) or JVer. (veratrine dependent changes), which means the amount of calcium that traverses the plasma membrane.

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

Drugs used: Morphine hydrochloride and naloxone hydrochloride were purchased from Sankyo Co., Japan. Veratrine from Merck, $^{45}$CaCl$_2$ (specific activity: 16 mCi/mg Ca) from New England Nuclear, and ethyleneglycol-bis (β-aminoethylether)-N,N’-tetraacetic acid (GEDTA) from Wako-Junyaku Co., Japan. Other chemicals used were of analytical grade. Drugs were dissolved in double-distilled and deionized water.

RESULTS

Development of acute tolerance to morphine: The antinociceptive action of morphine was gradually reduced when morphine (15 mg/kg, s.c.) was repeatedly administered every 4 hr. The antinociceptive activity of morphine in animals treated with morphine disappeared after the 13th administration of morphine (Fig. 1).

Development of chronic tolerance to morphine: The results on loss in body weight during withdrawal of morphine are shown in Fig. 2. Decrease in body weight of the rats repeatedly treated with morphine was 16.2 ±1.0 g (mean±S.E.) after the injection of saline instead of morphine. When doses of morphine were decreased on the last day, decrease in body weight of the morphine-dependent rats were found to be dose-dependent, while an increase of body weight was observed with 80 mg/kg of morphine.

![Graphs showing development of tolerance in antinociceptive activity.](image_url)

Fig. 1. Development of tolerance in antinociceptive activity. Each point represents a mean±S.E. of 8 animals. 1st, 2nd, 4th, 7th, 10th, and 13th: the 1st, 2nd, 4th, 7th, 10th, and 13th administration of morphine, ○: saline (control), □: morphine (15 mg/kg, s.c.).
a day, the mean±S.E. being 2.0±2.5 g. The body weights of 6 rats untreated with morphine increased when saline was injected.

Time course of the uptake of calcium into the synaptosomes: Figure 3 shows the uptake of calcium into the depolarized and nondepolarized synaptosomes as a function of time. The uptake of calcium reached a plateau level within 3 min. Figure 3 also shows the uptake of calcium into the synaptosomes as a function of different concentrations of veratrine. Calcium was taken up by veratrine-stimulated synaptosomes in a concentration-dependent manner. From these results, an incubation time of 3 min was used in the following experiments.

Effects of morphine on synaptosomal calcium uptake: The changes in levels of synaptosomal calcium uptake stimulated by high KCl or veratrine following in vitro additions of opiates are shown in Tables 1

![Fig. 2.](image)

**Fig. 2.** Loss in body weight during withdrawal of morphine in morphine dependent rats. ○: subcutaneous injection of saline to the untreated rats, ●: saline (s.c.) (withdrawal), □: morphine 40 mg/kg (s.c.) twice a day, ▽: morphine 20 mg/kg (s.c.) twice a day, △: morphine 10 mg/kg (s.c.) twice a day, at 24 hr: readministration of morphine (40 mg/kg, s.c.). Each point represents a mean±S.E. of 6 rats. *, **: significantly different from the value of the rats treated with morphine 40 mg/kg (s.c.) at P<0.05 and P<0.01, respectively.

![Fig. 3.](image)

**Fig. 3.** Calcium accumulation as a function of different incubation times in depolarized and nondepolarized synaptosomes. Each point represents a mean±S.E. of 4 experiments. ●: nondepolarized synaptosomes, ○: depolarized (71 mM K+) synaptosomes, □: depolarized (30 µg/ml veratrine) synaptosomes, ▽: depolarized (10 µg/ml veratrine) synaptosomes, △: depolarized (3 µg/ml veratrine) synaptosomes. *: significantly different from the nondepolarized synaptosomes at P<0.01.

| Treatment          | Na+5 mM K⁺ | Veratrine | ∆Ver. |
|--------------------|------------|-----------|-------|
| Control            | 7.22±0.12  | 11.12±0.13| 3.90±0.18 |
| Naloxone 10⁻⁶ M    | 7.39±0.13  | 10.77±0.21| 3.38±0.25 |
| Morphine 10⁻⁶ M    | 7.48±0.21  | 10.21±0.14*| 2.73±0.25*|
| Naloxone 10⁻⁶ M    | 7.18±0.47  | 11.36±0.25| 4.18±0.53 |
| Morphine 10⁻⁶ M    | 7.18±0.47  | 11.36±0.25| 4.18±0.53 |

Table 1. Effect of opiates on veratrine-stimulated calcium uptake in the synaptosomes isolated from naive rats

Each value represents the mean±S.E. of 5 experiments. Veratrine dependent changes, ∆Ver., were calculated as the difference between Ca uptake from veratrine media and 5 mM K⁺ media. *, significantly different from the control value at P<0.01.
and 2. In both KCl- and veratrine-stimulated synaptosomal calcium uptakes, significant inhibition could be induced by 10^-6 M morphine (P<0.05 and P<0.01, respectively). Morphine also significantly reduced depolarization-dependent changes which are represented by JK and JVer. The inhibition of depolarization-stimulated uptake by morphine was prevented by the addition of naloxone (10^-6 M) with morphine before calcium uptake was initiated, but 10^-6 M naloxone alone had no influences on the basal and depolarization-stimulated calcium uptakes.

Calcium uptake into the synaptosomes from rats acutely tolerant to morphine: Table 3 shows the effect of morphine on calcium uptake by synaptosomes isolated from rats acutely tolerant to morphine. Both KCl- and veratrine-stimulated synaptosomal calcium uptakes were significantly increased in acute morphine tolerant animals as compared to naive animals (P<0.01), while basal uptake was not influenced. Therefore, depolarization-dependent changes significantly increased. These results suggested that acute tolerance to morphine in rats produced an increase in the calcium content of synaptosomes. These results were in good agreement with those of Ross (3).

Calcium uptake into the synaptosomes of rats chronically tolerant to morphine: To clarify the relationship between the synaptosomal calcium uptake and development of morphine dependence, we studied the uptake of calcium into the synaptosomes isolated from the brains of rats chronically tolerant to morphine. As can be seen in Table 4, chronically morphinized animals did not show significant changes in calcium uptake compared to controls.

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**Table 2. Effect of opiates on potassium-stimulated calcium uptake in the synaptosomes isolated from naive rats**

| Treatment          | Na+5 mM K+   | 71 mM K+   | JK          |
|--------------------|--------------|------------|-------------|
| Control            | 4.05±0.14    | 13.65±0.50 | 9.60±0.54   |
| Naloxone 10^-6 M   | 4.49±0.17    | 13.39±0.36 | 8.90±0.40   |
| Morphine 10^-6 M   | 3.99±0.34    | 12.03±0.19*| 8.04±0.39*  |
| Naloxone 10^-6 M+  | 4.28±0.24    | 14.31±0.28 | 10.03±0.36  |
| Morphine 10^-6 M   |              |            |             |

Each value represents the mean±S.E. of 5 experiments. KCl dependent changes, JK, were calculated as the difference between Ca uptake from high KCl media and 5 mM K+ media. *, significantly different from the control value at P<0.05.

**Table 3. Stimuli-induced calcium uptake in the synaptosomes isolated from acutely tolerant rat brain**

| Ca uptake (pmoles/g protein) | Control | Morphine-tolerance |
|------------------------------|---------|--------------------|
| Na+5 mM K+                   | 6.78±0.11 | 6.48±0.23          |
| Veratrine                    | 10.71±0.22| 12.10±0.22*        |
| JVer.                        | 3.93±0.25 | 5.62±0.32*         |
| Na+5 mM K+                   | 5.50±0.24 | 5.22±0.20          |
| 71 mM K+                     | 14.32±0.34| 16.22±0.38*        |
| JK                           | 8.82±0.42 | 11.00±0.42*        |

Each value represents the mean±S.E. of 5 to 8 experiments. *, significantly different from the control value at P<0.01. See the legends for Tables 1 and 2.
Table 4. Stimuli-induced calcium uptake in the synaptosomes isolated from chronically tolerant rat brain

| Ca uptake (μmoles/g protein) | Control       | Morphine-tolerance |
|-----------------------------|---------------|-------------------|
| Na+5 mM K+                  | 4.94±0.19     | 4.96±0.25         |
| Veratrine                   | 8.45±0.31     | 7.89±0.10         |
| JVer.                       | 3.51±0.36     | 2.93±0.27         |
| Na+5 mM K+                  | 6.27±0.65     | 7.08±0.60         |
| 71 mM K+                    | 16.59±0.73    | 16.96±0.23        |
| JK                          | 10.32±0.98    | 9.88±0.83         |

Each value represents the mean±S.E. of 5 to 7 experiments. See the legends for Tables 1 and 2.

Table 5. Effect of morphine on stimuli-induced calcium uptake in the synaptosomes isolated from chronically tolerant rat brain

| Ca uptake (μmoles/g protein) | Control       | Morphine (10^-6 M) |
|-----------------------------|---------------|-------------------|
| Na+5 mM K+                  | 6.18±0.22     | 5.91±0.28         |
| Veratrine                   | 9.14±0.34     | 9.29±0.20         |
| JVer.                       | 2.98±0.40     | 3.38±0.34         |
| Na+5 mM K+                  | 5.19±0.20     | 5.51±0.24         |
| 71 mM K+                    | 15.64±0.19    | 15.98±0.26        |
| JK                          | 10.45±0.28    | 10.47±0.35        |

Each value represents the mean±S.E. of 5 to 6 experiments. See the legends for Tables 1 and 2.

not have significant changes in the depolarization-stimulated and basal calcium uptakes into the synaptosomes as compared to the naive animals.

Table 5 shows the data for in vitro addition of morphine on calcium uptake into the synaptosomes isolated from chronically morphinized rat brain. No significant difference was found between any of the treatment groups as compared with the control groups. These results were different from the in vitro effects of morphine on synaptosomal calcium uptake isolated from naive rats as shown in Tables 1 and 2.

DISCUSSION

Recent findings have suggested that calcium ions play an important role in the production of analgesia and tolerance by opiates (13–15). Since most of earlier studies on synaptosomal calcium uptake isolated from the morphine-tolerant animals have been performed at a relatively short time exposure to opiates, it is interesting to test the synaptosomal calcium uptake at a different stage of morphine tolerance.

In this study, in vitro addition of morphine inhibited depolarization-stimulated calcium uptake by the synaptosomes isolated from naive rats, and this inhibition was prevented by the addition of naloxone with morphine before calcium uptake was initiated. These results suggested that a part of the inhibition by morphine on the synaptosomal calcium uptake was mediated through the opiate receptors. It is likely that only depolarization-stimulated calcium uptake is involved in presynaptic actions of morphine since the present results show that the ability for calcium uptake by the nondepolarized synaptosomes is not altered by morphine. This result was in good agreement with a recent
report (16) which showed that opiates decreased the release of neurotransmitters from brain slices subsequent to KCl-induced depolarization, but did not inhibit neurotransmitter release induced by A 23187, a calcium ionophore that causes transport of calcium across membranes through depolarization independent processes (17, 18). Chronic exposure of rats to morphine to elicit tolerance to and physical dependence on morphine did not influence the synapticosomal calcium uptake. However, these preparations apparently lost the ability for in vitro morphine-inhibition of calcium uptake into the synaptosomes. The present study also suggests that in order to demonstrate chronic tolerance at the membrane level subsequent to chronic morphine administration in vivo, synaptosomes from the animals chronically tolerant to morphine must be challenged with a dose of morphine added in vitro. There is no significant difference in calcium uptakes into the synaptosomes isolated from naive and chronically tolerant groups. The effects of chronic exposure to morphine on synapticosomal calcium uptake are strikingly similar to the effects of chronic exposure to barbiturates (19, 20), chlorpromazine (21), and ethanol (22). On the other hand, acute tolerance to morphine increased synapticosomal calcium uptake compared to that by naive rats. The present results are in good agreement with those of Guerrero-Munoz et al. (1, 2) and Ross (3). It was suggested from the results described above that adaptive changes of synapticosomal calcium uptake produced by exposure to morphine may be involved in tolerance and physical dependence development.

Since it is known that calcium is so intimately coupled with the exocytotic release of neurotransmitters (4) and that morphine has been shown to influence the release of neurotransmitters (5, 6), these results suggest that the analgesia by morphine and development of tolerance and physical dependence to morphine are at least partially due to an alteration of calcium influx. However, since the influence of morphine on calcium uptake by the synaptosomes isolated from the rats acutely tolerant to morphine was differed from the chronically tolerant rats, it is suggested that the studies of tolerance to and physical dependence on morphine would be more useful if done at different stages of tolerance.

Acknowledgement: We thank Miss Kumi-ko Suzuki for excellent technical assistance.

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