Effects of Morphine, Clonidine and Papaverine on Synaptosomal $^{45}\text{Ca}^{2+}$ Uptake and Antinociceptive Action in Rats

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Abstract—We studied the relationship between the inhibition of extracellular $^{45}\text{Ca}^{2+}$ uptake into synaptosomes and the antinociceptive action induced by morphine, clonidine and papaverine in rats. The antinociceptive action induced by clonidine was as potent as that by morphine, but that by papaverine was less potent than those by morphine and clonidine. Antinociceptive action by morphine was considerably potentiated by the simultaneous administration of clonidine. However, the antinociceptive actions induced by morphine and clonidine were found to be mediated through different receptor mechanisms. Although the pretreatment by papaverine blocked the morphine-induced antinociception, the inhibition induced by papaverine was not found to be mediated through the opiate receptor because papaverine did not displace $[^3\text{H}]$-dihydromorphine binding to the membrane fraction from rat brain. Papaverine also inhibited the antinociceptive action induced by clonidine. Morphine inhibited the veratrine-stimulated synaptosomal $^{45}\text{Ca}^{2+}$ uptake by a naloxone-reversible process. Papaverine also strongly inhibited the veratrine-stimulated synaptosomal $^{45}\text{Ca}^{2+}$ uptake, while clonidine had virtually no effect. The inhibition of synaptosomal $^{45}\text{Ca}^{2+}$ uptake induced by morphine was not increased by simultaneous addition of clonidine. The strong inhibitions of synaptosomal $^{45}\text{Ca}^{2+}$ uptake were still observed by simultaneous addition of papaverine with morphine and clonidine. These results suggest that it is difficult to account for the drugs-induced antinociception by the inhibition of extracellular $\text{Ca}^{2+}$ influx into the synaptosomes other than the inhibition of extracellular $^{45}\text{Ca}^{2+}$ uptake into the synaptosomes.

It is generally accepted that the movement of $\text{Ca}^{2+}$ across neural membranes is an important step leading to the release of neurotransmitters (1), and many of the central nervous acting drugs affect the movement of $\text{Ca}^{2+}$ into nerve endings isolated from brain homogenate (synaptosomes) (2–4). The antinociceptive effect of morphine is also considered to involve such a mechanism, that is, inhibition of extracellular $\text{Ca}^{2+}$ influx into synaptosomes (5) and that of release of neurotransmitters (6).

In our previous results, however, we demonstrated that pretreatment by papaverine blocked the antinociceptive effect induced by morphine, while in vitro simultaneous addition of papaverine and morphine possessed a potent inhibitory effect on $^{45}\text{Ca}^{2+}$ uptake into synaptosomes isolated from rat brain (7). These results seem to contradict the relationship between the inhibition of synaptosomal $^{45}\text{Ca}^{2+}$ uptake and morphine-antinociception.

Though the inhibition of synaptosomal $^{45}\text{Ca}^{2+}$ uptake by morphine and the antinociceptive effect induced by morphine are mediated through the opiate receptor (8–10), it is considered that papaverine inhibits drug-induced antinociception without the

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mediation of the opiate receptor. Clonidine, an \( \alpha_2 \)-adrenoeceptor agonist, was known to produce an antinociceptive effect in animals (11, 12), and the antinociceptive effect induced by clonidine was not mediated through the opiate receptor (12). Therefore, we studied whether or not papaverine also inhibited the antinociceptive effect induced by clonidine. Furthermore, we investigated the effects of morphine, clonidine and papaverine on synaptosomal \(^{45}\)Ca\(^{2+}\) uptake.

Materials and Methods

Assessment of antinociceptive activity:
Male Wistar strain rats, 70 to 130 g, were used. Antinociceptive activities of drugs were measured after each subcutaneous injection of drugs with a Randall-Selitto apparatus (Ugo Basile) (13). The maximal pressure measured was 250 g.

\(^{45}\)Ca\(^{2+}\) uptake: The synaptosomal fraction of the whole brain (except the cerebellum) homogenate of male Wistar strain rats (weighing 200 to 250 g) was prepared by the method of Cotman and Matthews (14). The uptake of \(^{45}\)Ca\(^{2+}\) into synaptosomes was examined as described by Blaustein (15) and described previously (9, 10). A 0.5 ml aliquot of the synaptosomal preparation in a physiological salt solution (132 mM NaCl, 5 mM KCl, 1.2 mM CaCl\(_2\), 1.2 mM NaH\(_2\)PO\(_4\), 1.3 mM MgCl\(_2\), 10 mM glucose, 20 mM Tris base, pH adjusted to 7.4 with maleic acid) was preincubated for 15 min at 30°C. Each drug was added at the beginning of the preincubation period. The uptake of \(^{45}\)Ca\(^{2+}\) into synaptosomes was started by addition of 0.5 ml of the above physiological salt solution containing \(^{45}\)Ca\(^{2+}\) (specific activity: 1 mCi/m mol) and \(60 \mu g/ml\) of veratrine. The mixture was then incubated for 3 min at 30°C and terminated by adding 1 ml of ice-cold stopping solution (120 mM NaCl, 5 mM KCl, 30 mM ethyleneglycol-bis-(\(\beta\)-aminoethyl-ether)-N,N'-tetraacetic acid (EGTA), pH adjusted to 7.4 with Tris base). The mixture was then incubated for 3 min at 30°C and terminated by adding 1 ml of ice-cold washing solution (132 mM choline-Cl, 5 mM KCl, 1.2 mM CaCl\(_2\), 1.2 mM NaH\(_2\)PO\(_4\), 1.3 mM MgCl\(_2\), 10 mM glucose, 20 mM Tris base, pH adjusted to 7.4 with maleic acid). Each filter was washed twice with 3 ml of ice-cold washing solution and placed in a scintillation vial with a toluene scintillator. Radioactivities of samples were counted using an ALOKA LSC-900 liquid scintillation counter. The values were corrected for the \(^{45}\)Ca\(^{2+}\) remaining on the filters in the absence of the preparations.

Binding assay: The crude mitochondrial fraction from rat brain (except the cerebellum) was suspended in 5 vol. (original wet weight) of 50 mM Tris/HCl buffer, pH 7.4, and centrifuged at 17,000x g for 10 min. The tight, brownish pellet (which contains most of mitochondria) was discarded, while the top, loose pellet, was separated and resuspended in 50 mM Tris/HCl buffer, and recentrifuged at 17,000x g for 10 min. The pellet was resuspended in 50 mM Tris/HCl buffer containing 100 mM NaCl and incubated at 0°C for 60 min to facilitate dissociation of endogenous inhibitors of ligand binding (16). NaCl was removed from the membrane fraction by three further centrifugations and resuspended in 50 mM Tris/HCl buffer, and then it was stored at -20°C until use.

The measurement of labeled ligand binding in rat brain membranes was performed using a filtration method. Potencies of competing ligands were determined by co-incubating of unlabeled ligands with 1 nM \([3H]\)-dihydro-morphine (DHM). Nonspecific binding was determined in the presence of 1 \(\mu M\) naloxone. The binding reaction was performed at 25°C for 60 min and stopped by rapid filtration through a Whatman glass fiber filter (GF/C). Filters were washed twice with 5 ml of ice-cold 50 mM Tris/HCl buffer and placed in a scintillation vial with a toluene scintillator. Radioactivities of samples were counted using an ALOKA LSC-900 liquid scintillation counter.

The protein concentration was determined by the method of Lowry et al. (17) using bovine serum albumin (F-V) as the standard. Statistical significance was evaluated by Student's t-test.

Drugs used: Morphine hydrochloride and naloxone hydrochloride (Sankyo Co., Japan), clonidine hydrochloride (Tokyo-Kasei), papaverine hydrochloride and EGTA (ethyl-
energyglycol-bis-(β-aminoethyl ether)-N,N'-tetraacetic acid (Wako-Junyaku), phentolamine methanesulfonate (Takeda-Yakuin) and $^{45}\text{CaCl}_2$ (specific activity: 16 mCi/mg) and $[^3\text{H}]$-dihydromorphine (specific activity: 70.7 Ci/m mol) (New England Nuclear). Other chemicals used were of analytical grade. Drugs were dissolved in double-distilled and deionized water.

Results

Antinociceptive action: The antinociceptive effects of morphine, clonidine and papaverine were increased in a dose-dependent manner, which was estimated at the maximal effective time (30 min) after subcutaneous injection of drugs (Fig. 1). The antinociceptive activity of clonidine in rats was as potent as that of morphine, but papaverine was less potent than morphine and clonidine. When morphine (2.5 mg/kg, s.c.) or clonidine (2.5 mg/kg, s.c.) was simultaneously administered with naloxone (1 mg/kg, s.c.), an opiate antagonist, the antinociceptive action induced by morphine was prevented by naloxone, but that by clonidine was not (Fig. 2). On the other hand, in the case of simultaneous administration of morphine (2.5 mg/kg, s.c.) or clonidine (2.5 mg/kg, s.c.) with phentolamine (2.5 mg/kg, s.c.), an α-adrenoceptor antagonist, the antinociceptive action induced by clonidine was prevented by phentolamine, but that by morphine was not (Fig. 3). Administration of naloxone (1 mg/kg, s.c.) or phentolamine (2.5 mg/kg, s.c.) alone was without any antinociceptive action (Figs. 2 and 3). When morphine (1.0 mg/kg, s.c.) was simultaneously administered with clonidine (1.0 mg/kg, s.c.), the antinociceptive action in rats induced by both the drugs was greatly potentiated, though the antinociceptive action of morphine or clonidine alone was weak at

![Fig. 1. Dose response curves for the antinociceptive actions of morphine, clonidine and papaverine. Ordinate: antinociceptive effect is shown as the ratio of threshold pressure (g) 30 min before and after injection. Abscissa: log dose (mg/kg, s.c.). ● morphine, ○ clonidine, ■ papaverine. Each point represents a mean with S.E. of 8 animals.](image)

![Fig. 2. Effects of naloxone on the antinociceptive activities of morphine and clonidine in rats. Each bar represents a mean with S.E. of 8 animals. Antinociceptive effect is shown as the ratio of threshold pressure (g) 30 min before and after injection. Control: saline, s.c.; Naloxone: 1 mg/kg, s.c.; Morphine: 2.5 mg/kg, s.c.; and Clonidine: 2.5 mg/kg, s.c. *: Significantly different from the control at P<0.01. #: Significantly different from the morphine treated group at P<0.01.](image)

![Fig. 3. Effect of phentolamine on the antinociceptive activities of morphine and clonidine in rats. Each bar represents a mean with S.E. of 9 animals. Antinociceptive effect is shown as the ratio of threshold pressure (g) 30 min before and after injection. Control: saline, s.c.; Phentolamine: 2.5 mg/kg, s.c.; Clonidine: 2.5 mg/kg, s.c.; and Morphine: 2.5 mg/kg, s.c. *: Significantly different from the control at P<0.01. #: Significantly different from the clonidine treated group at P<0.01.](image)
this dose (Fig. 4). We previously reported that the maximal effective time of the antinociceptive action induced by papaverine was 30 min, and this effect reduced to about control level at 60 min after papaverine injection (7). Therefore, we examined the effect of papaverine on the antinociceptive action induced by morphine or clonidine when these drugs were administered 30 min after pretreatment of the rats with papaverine. Figure 5 shows that the antinociceptive action induced by morphine and clonidine is strongly prevented by the pretreatment by papaverine.

**Synaptosomal 45Ca2+ uptake:** When the crude mitochondrial fraction (P2-fraction) was separated into the myelin, synaptosomal and mitochondrial fractions using discontinuous Ficoll gradient centrifugation, most of the material exhibiting veratrine-stimulated 45Ca2+ uptake was found in the synaptosomal fraction (Fig. 6-A). When 10^-6 g/ml of tetrodotoxin was present in the incubation medium, the stimulatory effect of veratrine on 45Ca2+ uptake into the synaptosomes was completely prevented, while high K+ stimulated (71 mM K+) 45Ca2+ uptake was unaffected (Fig. 6-B).

Morphine (10^-6 M) slightly but significantly inhibited the veratrine-stimulated 45Ca2+ uptake into various fractions from rat brain homogenate (A) and 45Ca2+ uptake into synaptosomes in the presence and absence of tetrodotoxin (10^-6 g/ml). (A) Open bars show 45Ca2+ uptake in the presence of veratrine and closed bars that in the absence of veratrine. Each bar represents a mean with S.E. of 5 experiments. *: Significantly different from the control at P<0.01. **: Significantly different from the morphine and clonidine treated groups at P<0.05 and P<0.01, respectively.

![Fig. 4. Simultaneous administration of clonidine and morphine on the antinociceptive activity in rats.](image)

![Fig. 5. Effect of papaverine on antinociceptive actions induced by morphine and clonidine in rats.](image)

![Fig. 6. 45Ca2+ uptake into various fractions from rat brain homogenate (A) and 45Ca2+ uptake into synaptosomes in the presence and absence of tetrodotoxin (10^-6 g/ml).](image)
synaptosomal $^{45}\text{Ca}^{2+}$ uptake, while $10^{-6}$ M morphine did not affect the $^{45}\text{Ca}^{2+}$ uptake into the synaptosomes in the absence of veratrine (basal uptake). Papaverine at $10^{-4}$ M strongly inhibited the veratrine-stimulated synaptosomal $^{45}\text{Ca}^{2+}$ uptake and slightly but significantly inhibited the basal uptake. Clonidine at $10^{-6}$ M and $10^{-6}$ M naloxone did not affect the basal and veratrine-stimulated synaptosomal $^{45}\text{Ca}^{2+}$ uptakes. The inhibition of veratrine-stimulated synaptosomal $^{45}\text{Ca}^{2+}$ uptake by morphine was prevented by the addition of naloxone in the incubation medium. Although clonidine potentiated morphine-antinociception, the inhibition of veratrine-stimulated $^{45}\text{Ca}^{2+}$ uptake into synaptosomes induced by morphine was not affected by simultaneous addition of clonidine. Furthermore, $^{45}\text{Ca}^{2+}$ uptake into the synaptosomes in the presence of papaverine and morphine or clonidine was still strongly inhibited, though papaverine blocked the antinociceptive action induced by morphine or clonidine. However, the inhibition induced by both morphine and papaverine was not increased more than that by papaverine alone (Table 1).

**Table 1. Combined effects of morphine, clonidine, naloxone and papaverine on synaptosomal $^{45}\text{Ca}^{2+}$ uptake in the presence and absence of veratrine**

|                | $-\text{Veratrine}$ | $+\text{Veratrine}$ | $\Delta \text{Veratrine}$ |
|----------------|----------------------|----------------------|--------------------------|
| Control        | $5.19\pm0.16$        | $9.04\pm0.17$        | $3.85\pm0.23$            |
| Morphine, $10^{-6}$ M | $5.17\pm0.14$        | $8.23\pm0.21^*$      | $3.06\pm0.25^*$          |
| Naloxone, $10^{-6}$ M | $5.45\pm0.20$        | $9.16\pm0.13$        | $3.71\pm0.26$            |
| Papaverine, $10^{-4}$ M | $4.60\pm0.11^*$      | $6.56\pm0.16^{**}$   | $1.96\pm0.19^{**}$       |
| Clonidine, $10^{-6}$ M | $5.54\pm0.14$        | $9.15\pm0.26$        | $3.61\pm0.30$            |
| Morphine, $10^{-6}$ M + | $5.38\pm0.21$        | $9.11\pm0.27$        | $3.73\pm0.34$            |
| Naloxone, $10^{-6}$ M | $4.60\pm0.15^*$      | $7.03\pm0.27^{**}$   | $2.43\pm0.31^*$          |
| Morphine, $10^{-6}$ M + | $5.23\pm0.10$        | $8.32\pm0.18^*$      | $3.09\pm0.21^*$          |
| Papaverine, $10^{-4}$ M | $5.73\pm0.19^*$      | $6.83\pm0.20^{**}$   | $2.26\pm0.28^{**}$       |

Veratrine dependent changes, $\Delta$ Veratrine, were calculated as the difference between $^{45}\text{Ca}^{2+}$ uptake from the media in the presence and absence of veratrine. Each value represents a mean with S.E. of 6 experiments. *: **: Significantly different from the corresponding control at $P<0.05$ and $P<0.01$, respectively.

**Fig. 7. Displacement of specific $[3\text{H}]-\text{DHM}$ binding by naloxone (○), morphine (●), clonidine (□) and papaverine (△). Ordinate: specific binding of $[3\text{H}]-\text{DHM}$ (%). Abscissa: concentration of drugs ($-\log \text{M}$). Each point represents a mean with S.E. of 4 experiments.**

**Binding assay:** The binding of $[3\text{H}]-\text{DHM}$ to the membrane fraction from rat brain was displaced by the addition of nonradioactive opiate ligands. Morphine, an opiate agonist, inhibited 50% of the $[3\text{H}]-\text{DHM}$ binding at a concentration of $10^{-8}$ M. Naloxone, an opiate antagonist, inhibited 50% of the
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[3H]-DHM binding at a concentration of 5x10^{-9} M. On the other hand, the inhibitory effects of papaverine and clonidine on [3H]-DHM binding were very weak, if any (Fig. 7).

Discussion

It has been suggested that a certain relationship may exist between the inhibition of stimuli-induced synaptosomal 45Ca^{2+} uptake and the antinociceptive action of morphine (5). These actions were mediated through the opiate receptor because naloxone reversed both actions (8–10). However, these relationships seemed to contradict our previous results because the pretreatment by papaverine inhibited the morphine-induced antinociception notwithstanding that the strong inhibition of synaptosomal 45Ca^{2+} uptake was still observed by in vitro simultaneous addition of papaverine with morphine (7). Therefore, we studied whether or not the inhibition of morphine-induced antinociception by papaverine was mediated through the opiate receptor.

In this paper, naloxone, an antagonist of the opiate receptor, prevented the antinociceptive action induced by morphine, but not that by clonidine. On the other hand, phentolamine, a potent α-adrenoceptor antagonist, prevented the antinociceptive action induced by clonidine, but not that by morphine. These results suggested that the antinociceptive actions induced by morphine and clonidine were mediated through different receptor mechanisms and further confirmed that the binding of [3H]-DHM to the membrane fraction from rat brain was not displaced by the addition of clonidine. These observations are supported by the results of Lipman and Spencer who reported that naloxone did not inhibit the antinociceptive action induced by clonidine in mice (12). The pretreatment with papaverine blocked the morphine-induced antinociception. This inhibition by papaverine was not mediated through the opiate receptor because papaverine did not displace the [3H]-DHM binding to the membrane fraction from rat brain. Papaverine also prevented the antinociceptive action induced by clonidine. For the inhibition of papaverine on the morphine- and clonidine-induced antinociception, papaverine was administered 30 min before the drug was given because the antinociceptive action induced by papaverine alone reduced to the control level at the assessment time. On the other hand, the antinociceptive action induced by morphine was significantly potentiated by the simultaneous administration of clonidine.

As shown in Table 1, morphine inhibited the veratrine-stimulated synaptosomal 45Ca^{2+} uptake by a naloxone-reversible process. This result is in good agreement with our previous findings (9, 10). Papaverine also strongly inhibited the veratrine-stimulated synaptosomal 45Ca^{2+} uptake. Although the activity of morphine on the inhibition of synaptosomal 45Ca^{2+} uptake was much less than that of papaverine, morphine possessed a potent antinociceptive action as compared with papaverine. This discrepancy may be due to the sites of action of the drugs. The inhibition of synaptosomal 45Ca^{2+} uptake by morphine was mediated through the opiate receptor, and the opiate receptor is localized in a certain region of the brain (18). Our synaptosomal preparations are heterogeneous because they contain nerve endings from diverse origins. On the other hand, papaverine is considered to inhibit the synaptosomal 45Ca^{2+} uptake non-specifically as reported in smooth muscle (19). Papaverine and morphine possessed the antinociceptive action and inhibited the veratrine-stimulated synaptosomal 45Ca^{2+} uptake; these results, at least partly, support the hypothesis that the antinociceptive action induced by morphine and papaverine may be attributable to its ability to affect the extracellular Ca^{2+} influx into the nerve endings. However, clonidine had virtually no effect on the synaptosomal 45Ca^{2+} uptake, and the inhibition of synaptosomal 45Ca^{2+} uptake induced by morphine was not increased by simultaneous addition of clonidine. Although the antinociceptive action induced by simultaneous administration of morphine and clonidine was greatly potentiated, further studies are needed to clarify the differences in the mode of action of these drugs.
and clonidine. These results suggest that it may be difficult to account for the drug-induced antinociception by the inhibition of extracellular Ca\(^{2+}\) influx into the nerve endings only and that other mechanisms may be involved in the drug-induced antinociception.

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