RELATIONSHIP BETWEEN SYNAPTOSOMAL CALCIUM UPTAKE AND ANTINOCICEPTIVE ACTION OF MORPHINE

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Abstract—We studied the relationship between inhibition of $^{45}\text{Ca}^{2+}$ uptake and the antinociceptive action induced by morphine and effects of papaverine and 1,1-diphenyl-3-piperidinobutanol hydrochloride (Aspaminol) on both these actions of morphine. Addition of these drugs in the incubation medium significantly inhibited glutamate-stimulated synaptosomal $^{45}\text{Ca}^{2+}$ uptake. The inhibition curves of morphine and Aspaminol on glutamate-stimulated synaptosomal $^{45}\text{Ca}^{2+}$ uptake were linear, but that of papaverine was not. The inhibition of morphine on synaptosomal $^{45}\text{Ca}^{2+}$ uptake was reversed by addition of naloxone. The inhibition of synaptosomal $^{45}\text{Ca}^{2+}$ uptake induced by morphine was increased by the simultaneous addition of Aspaminol, but the inhibition induced by both morphine and papaverine was not increased to more than that by papaverine alone. Since morphine-antinociception was potentiated by Aspaminol and blocked by papaverine, these results support that the inhibition of synaptosomal calcium uptake plays an important role in the production of morphine-antinociception. However, since the inhibition of synaptosomal $^{45}\text{Ca}^{2+}$ uptake by morphine was less than that by both Aspaminol and papaverine, and papaverine blocked morphine-antinociception, notwithstanding that $10^{-4}$ M of papaverine alone completely inhibited glutamate-stimulated $^{45}\text{Ca}^{2+}$ uptake into synaptosomes, it may be difficult to account for the antinociceptive action of morphine by the inhibition of $^{45}\text{Ca}^{2+}$ uptake only.

Since influx of calcium ions into nerve endings is coupled with exocytotic release of neurotransmitters (1), movement of calcium ions outside and within neurones is an important determinant in the functioning of nervous systems. It has been reported that some of the central nervous acting drugs such as barbiturates (2, 3), chlorpromazine (4) and phenytoin (5) block stimuli-induced $^{45}\text{Ca}^{2+}$ uptake into nerve endings (synaptosomes). Morphine also inhibited synaptosomal $^{45}\text{Ca}^{2+}$ uptake (6) and release of neurotransmitter (7), but findings of Leslie et al. (8) and our previous results (9) indicated that the inhibitory effect of syn-

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plasma membrane, intrasynaptosomal mitochondria and synaptic vesicle which assumed a putative calcium pool. In this study, we investigated whether or not papaverine affects synaptosomal $^{45}\text{Ca}^{2+}$ uptake. Furthermore, we studied the relationship between inhibition of $^{45}\text{Ca}^{2+}$ uptake and antinociceptive action induced by morphine and effects of papaverine and Aspaminol on both these actions of morphine.

**Materials and Methods**

$^{45}\text{Ca}^{2+}$ uptake: Whole brains (except the cerebellum) of male Wistar strain rats (weighing 200 to 250 g) were homogenized in ice-cold 0.32M sucrose-5 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES) buffer, pH 7.4, at 25°C; and homogenates were fractionated into myelin, synaptosomes and mitochondria by discontinuous Ficoll gradient centrifugation according to the method of Cotman and Matthews (17). Each fraction was diluted with ice-cold Ca$^{2+}$-free physiological salt solution containing 132 mM NaCl, 5 mM KCl, 1.2 mM NaH$_2$PO$_4$, 1.3 mM MgCl$_2$, 10 mM glucose and 20 mM Tris/maleic acid buffer, pH 7.4, at 25°C and centrifuged at 12,000xg for 10 min at 0°C. The pellets were resuspended in Ca$^{2+}$-free physiological salt solution to give protein concentrations of about 0.5 to 1.0 mg/ml which were determined by the method of Lowry et al. (18) using a bovine serum albumin as the standard, and these suspensions were kept on ice prior to the incubation.

The uptake of $^{45}\text{Ca}^{2+}$ into the preparations was studied as described by a modification of the method of Tan et al. (19). The preparations were first preincubated in Ca$^{2+}$-free physiological salt solution for 5 min at 30°C. The uptake of $^{45}\text{Ca}^{2+}$ into the preparations was initiated by addition of an equal volume of stimulator solution containing 132 mM NaCl, 5 mM KCl, 1.2 mM NaH$_2$PO$_4$, 1.3 mM MgCl$_2$, 0.4 mM CaCl$_2$ including $^{45}\text{Ca}^{2+}$ (specific activity 1 mCi/m mol), 1 mM L-glutamate, 10 mM glucose and 20 mM Tris/maleic acid buffer, pH 7.4, at 25°C. Basal uptake was handled in the same manner except that after the 5 min preincubation period, an equal volume of incubation medium containing 0.4 mM CaCl$_2$ including $^{45}\text{Ca}^{2+}$ was added. At fixed time intervals, $^{45}\text{Ca}^{2+}$ uptake was terminated by adding an equal volume of ice-cold stopping solution which consisted of 10 mM ethyleneglycol-bis-(8-amino-ethylether)-N,N’-tetraacetic acid (EGDTA), 120 mM NaCl and 5 mM KCl, titrated to pH 7.4 at 25°C with Tris base. The drugs were added at the beginning of the preincubation period. The experimental incubation was carried out at 30°C. Each sample was immediately filtered by passing it through a Whatman glass fiber filter (GF-C) prewashed with ice-cold washing solution containing 132 mM choline chloride, 5 mM KCl, 1.2 mM NaH$_2$PO$_4$, 1.3 mM MgCl$_2$, 0.2 mM CaCl$_2$, 10 mM glucose and 20 mM Tris maleic acid buffer, pH 7.4, at 25°C, 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 ALOKA LSC-900 liquid scintillation counter. The values were corrected for the $^{45}\text{Ca}^{2+}$ remaining on the filters in the absence of the preparations.

Assessment of antinociceptive activity: Male Wistar strain rats, weighing 90 to 120 g, were used. Antinociceptive activities of drugs were measured after each subcutaneous injection of drugs with a Randall-Selitto apparatus (Ugo Basile) (20). The maximal pressure measured was 250 g.

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

**Drugs used:** Morphine hydrochloride and naloxone hydrochloride were purchased from...
Sankyo. Aspaminol (1,1-diphenyl-3-piperidinobutanol hydrochloride) was from Kowa. Papaverine hydrochloride, L-glutamate and GEDTA (ethyleneeglycol bis-(β-aminoethylether)-N,N'-tetraacetic acid) was from Wako-Junyaku. Ficoll 400 was from Pharmacia Fine Chemicals, and $^{45}$CaCl$_2$ (specific activity 16 mCi/mg) obtained from New England Nuclear. Other chemicals used were of analytical grade. Drugs were dissolved in double-distilled and deionized water.

Results

$^{45}$Ca$^{2+}$ uptake: We compared the effect of glutamate on $^{45}$Ca$^{2+}$ uptake into the P$_2$ fraction and myelin, synaptosomal and mitochondrial fractions which were subfractionated from the P$_2$ fraction using discontinuous Ficoll gradient centrifugation. Figure 1A shows that $^{45}$Ca$^{2+}$ uptake into P$_2$, synaptosomal and mitochondrial fractions are significantly increased by addition of glutamate in the incubation medium as compared to that in the absence of glutamate, while that into the myelin fraction was not. Because mitochondria accumulated $^{45}$Ca$^{2+}$, we further studied effects of sodium azide, a potent mitochondrial inhibitor, on glutamate-stimulated $^{45}$Ca$^{2+}$ uptakes into synaptosomal and mitochondrial fractions in the following experiments: Only 24.6% of the glutamate-stimulated $^{45}$Ca$^{2+}$ uptake into synaptosomes could be blocked by sodium azide, but that of mitochondria was markedly depressed (70.1%) (Fig. 1B); These results were in good agreement with that reported by Delgado-Escueta et al. (21). Figure 2 shows the effect of morphine on glutamate-stimulated synaptosomal $^{45}$Ca$^{2+}$ uptake as a function of time. The synaptosomal $^{45}$Ca$^{2+}$ uptake was rapid within the first 5 min of exposure to glutamate and appeared to approach a plateau phase at 15 min. Morphine (10$^{-6}$M)
significantly inhibited the glutamate-stimulated $^{45}$Ca$^{2+}$ uptake into synaptosomes, while morphine (10$^{-6}$ M) did not affect the synaptosomal $^{45}$Ca$^{2+}$ uptake in the absence of glutamate (basal uptake). On the other hand, papaverine (10$^{-4}$ M) and Aspaminol (10$^{-4}$ M) significantly inhibited the $^{45}$Ca$^{2+}$ uptake into synaptosomes in the presence of glutamate. Both these drugs also significantly inhibited the basal uptake, but their inhibitory effects were less than in the presence of glutamate (Fig. 3). Morphine slightly but significantly inhibited glutamate-stimulated synaptosomal $^{45}$Ca$^{2+}$ uptake in a concentration-dependent manner ($Y = -0.1079X + 5.8656$, $r = -0.5383$, $P < 0.01$) while basal uptake was not significantly inhibited by various concentrations (10$^{-8}$ M to 10$^{-5}$ M) of morphine (Fig. 4A). Papaverine (10$^{-6}$ M to 10$^{-4}$ M) significantly inhibited glutamate-stimulated synaptosomal $^{45}$Ca$^{2+}$ uptake, and its concentration-dependent curve was not straightened. Papaverine at 10$^{-4}$ M also inhibited basal uptake (Fig. 4B). Aspaminol (10$^{-6}$ M to 10$^{-4}$ M) significantly inhibited glutamate-stimulated synaptosomal $^{45}$Ca$^{2+}$ uptake in a concentration-dependent manner ($Y = -1.2225X - 1.6142$, $r = -0.9280$, $P < 0.01$), and basal uptake was less but significantly inhibited by 10$^{-6}$ M to 10$^{-4}$ M Aspaminol (Fig. 4C). The inhibition of glutamate-stimulated synaptosomal $^{45}$Ca$^{2+}$ uptake by 10$^{-6}$ M morphine was prevented by addition of 10$^{-6}$ M naloxone with morphine before calcium uptake was initiated, while glutamate-stimulated $^{45}$Ca$^{2+}$ uptake was not influenced by 10$^{-6}$ M naloxone alone (Fig. 5).

Although Aspaminol ($5 \times 10^{-5}$ M) and papaverine ($3 \times 10^{-5}$ M) inhibited the basal and glutamate-stimulated $^{45}$Ca$^{2+}$ uptakes

![Fig. 3. Effects of papaverine and Aspaminol on synaptosomal $^{45}$Ca$^{2+}$ uptake as a function of different incubation time. Each point represents the mean with S.E. of 5 experiments. Open marks: in the absence of glutamate. Closed marks: in the presence of glutamate. $\bigcirc$: control. $\triangle$, $\Delta$: 10$^{-4}$ M Aspaminol. $\square$, $\blacksquare$: 10$^{-4}$ M papaverine. *: significantly different from the corresponding control in the presence of glutamate at $P < 0.01$. **: significantly different from the corresponding control at $P < 0.05$ and $P < 0.01$, respectively.]

![Fig. 4. Effects of various concentrations of morphine (A), papaverine (B) and Aspaminol (C) on synaptosomal $^{45}$Ca$^{2+}$ uptake. Each point represents the mean with S.E. of 5 to 6 experiments. Open marks: in the absence of glutamate. Closed marks: in the presence of glutamate. *: significantly different from the corresponding control in the presence of glutamate at $P < 0.01$. **: significantly different from the corresponding control at $P < 0.05$ and $P < 0.01$, respectively.]

![Fig. 5. Effects of morphine and naloxone on the glutamate-stimulated synaptosomal $^{45}$Ca$^{2+}$ uptake. Each bar represents the mean with S.E. of 5 experiments. *: significantly different from the control at $P < 0.01$.]
Table 1. Combined effects of morphine and papaverine or Aspaminol on synaptosomal \(^{45}\)Ca\(^{2+}\) uptake in the presence (+glutamate) and absence (-glutamate) of 0.5 mM glutamate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(-)glutamate</th>
<th>(+)glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.08±0.05</td>
<td>7.53±0.10</td>
</tr>
<tr>
<td>Morphine, 10(^{-6}) M</td>
<td>3.10±0.10</td>
<td>6.92±0.08*</td>
</tr>
<tr>
<td>Aspaminol, 5×10(^{-6}) M</td>
<td>2.37±0.04*</td>
<td>5.34±0.08*</td>
</tr>
<tr>
<td>Papaverine, 3×10(^{-5}) M</td>
<td>2.60±0.05*</td>
<td>5.63±0.09*</td>
</tr>
<tr>
<td>Aspaminol 5×10(^{-5}) M</td>
<td>2.34±0.10*</td>
<td>4.49±0.01*</td>
</tr>
<tr>
<td>Morphine, 10(^{-6}) M</td>
<td>2.67±0.10*</td>
<td>5.56±0.09*</td>
</tr>
<tr>
<td>Papaverine, 3×10(^{-5}) M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morphine, 10(^{-6}) M</td>
<td></td>
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</tbody>
</table>

Each value represents the mean with S.E. of 5 experiments. Drugs were added at the beginning of the preincubation period. \*: significantly different from the control at P<0.01. \#: significantly different from the Aspaminol alone at P<0.01.

into synaptosomes and morphine (10\(^{-6}\) M) inhibited the glutamate-stimulated synaptosomal \(^{45}\)Ca\(^{2+}\) uptake, the inhibitions of synaptosomal \(^{45}\)Ca\(^{2+}\) uptake induced by morphine (10\(^{-6}\) M) was increased by addition of Aspaminol (5×10\(^{-6}\) M) but not by papaverine (3×10\(^{-5}\) M) (Table 1).

Assessment of antinociceptive activity: The maximum effective time of antinociceptive activity was 30 min for morphine (2.5 mg/kg, s.c.) and papaverine (100 mg/kg, s.c.). With the combined administration of morphine (2.5 mg/kg, s.c.) and papaverine (100 mg/kg, s.c.) at the same time, the maximum effective time was also 30 min, and the antinociceptive activity induced by both the drugs was smaller than that by morphine alone (Fig. 6). Therefore, we tested the inhibitory effect of papaverine on morphine-antinociception when morphine (2.5 mg/kg, s.c.) was administered 30 min after pretreatment of the rats with papaverine (100 mg/kg, s.c.). Figure 7 shows that the antinociceptive activity of morphine is strongly prevented by pretreatment with papaverine. The antinociceptive activity of morphine (2.5 mg/kg, s.c.) was also prevented by a 15 min pretreatment with naloxone (1 mg/kg, s.c.), an opiate antagonist, but the antinociceptive activity of the rats by injection of vehicle was not affected by the pretreatment with papaverine and naloxone. On the other hand, when morphine (2.5 mg/kg, s.c.) was administered with Aspaminol (100 mg/kg, s.c.) simultaneously, the antinociceptive action by morphine was greatly potentiated and prolonged, while Aspaminol (100 mg/kg,
Fig. 7. Effects of papaverine and naloxone on the antinociceptive activity of morphine in rats. Each bar represents the mean with S.E. of 8 animals. Measurements were made at 30 min after morphine (2.5 mg/kg, s.c.) or saline injection. Papaverine (100 mg/kg, s.c.) was administered 30 min before drugs dosing. Naloxone (1 mg/kg, s.c.) was administered 15 min before drugs dosing. *: significantly different from the control at $P<0.01$. +, ++: significantly different from the morphine treated group at $P<0.05$ and $P<0.01$, respectively.

Discussion

Several investigators have suggested that inhibition of the influx of calcium ions into the nerve endings plays an important role for the antinociceptive action of morphine (6), and this action was a naloxone reversible processes (6). Our results also showed that morphine inhibited glutamate-stimulated synaptosomal $^{45}$Ca$^{2+}$ uptake in a concentration-dependent manner, but not basal uptake. This inhibition was abolished by addition of naloxone. The inhibitory action of morphine on glutamate-stimulated synaptosomal $^{45}$Ca$^{2+}$ uptake was in agreement with a previous report using depolarizing agents (9). Aspaminol also inhibited glutamate-stimulated synaptosomal $^{45}$Ca$^{2+}$ uptake in a concentration-dependent manner. The inhibition of $^{45}$Ca$^{2+}$ uptake was similar to that of ATP and high KCl stimulated synaptosomal $^{45}$Ca$^{2+}$ uptake as reported previously (13), but the effect of Aspaminol on basal uptake was different from that in the previous report (13). This discrepancy may be mainly due to a difference in conditions for preincubation with Aspaminol. Papaverine also reduced glutamate-stimulated synaptosomal $^{45}$Ca$^{2+}$ uptake. Several investigators suggested that Ca$^{2+}$ transport across the synaptosomal membranes was affected by any change in Ca$^{2+}$-concentration which was controlled by intrasynaptosomal mitochondria (22). Some authors reported that glutamate increased $^{45}$Ca$^{2+}$ uptake into synaptic plasma membranes and synaptosomes in the presence of mitochondrial poisons (21). In these experiments, the effects of drugs on glutamate-stimulated synaptosomal $^{45}$Ca$^{2+}$ uptake were evaluated in the absence of mitochondrial poisons. Therefore, glutamate may act on both plasma membranes and intrasynaptosomal mitochondria. Takagi et al. (23) suggested that weak bases such as papaverine (pKa 5.95) are expected to penetrate into cell membranes at physiological pH and act on intracellular organelles. Papaverine blocks the mitochondrial respiratory chain between NAD and cytochrome b.

Table 8. Effect of Aspaminol on antinociceptive activity of morphine in rats. Each point represents the mean with S.E. of 8 animals. •: control; O: morphine, 2.5 mg/kg, s.c.; △: Aspaminol, 100 mg/kg, s.c.; □: morphine, 2.5 mg/kg, s.c. +Aspaminol, 100 mg/kg, s.c. *: significantly different from the corresponding control at $P<0.01$. +: significantly different from the corresponding morphine treated group at $P<0.01$. 

s.c.) alone had no effect (Fig. 8).
(16). On the other hand, Takayanagi et al. (14) reported that papaverine also inhibited calcium uptake into the plasma membrane-derived vesicles. Therefore, papaverine may act on both synaptosomal plasma membranes and intrasynaptosomal mitochondria. On the other hand, since most of the Aspaminol molecules exist as ion forms at physiological pH (pKa of Aspaminol: 8.62), it may be difficult for these molecules to penetrate into cell membranes and act on the cell surface. As the inhibition of morphine on synaptosomal $^{45}$Ca$^{2+}$ uptake was reversed by addition of naloxone, morphine acts on cell membranes mediated through opiate receptors which are localized on the cell surface (24). Therefore, the inhibition curves of morphine and Aspaminol on synaptosomal $^{45}$Ca$^{2+}$ uptake may be linear, but that of papaverine is not. The inhibition of synaptosomal $^{45}$Ca$^{2+}$ uptake induced by morphine was increased by simultaneous addition of Aspaminol, but the inhibition induced by both morphine and papaverine was not increased more than that by papaverine alone. These results may be related to the effects of Aspaminol and papaverine on the antinociceptive action of morphine. While Aspaminol increased morphine-antinociception, the antinociceptive activity of morphine was not influenced by simultaneous administration of papaverine, but was blocked by the 30 min pretreatment with papaverine. It may be difficult for Aspaminol to pass through the blood-brain barrier because most of the Aspaminol molecules exist as ion forms at physiological pH, but a small quantity of non-ionized Aspaminol molecules also exist at physiological pH. Harris et al. (25) suggested that the antinociceptive effect of morphine was potentiated by intracerebroventricular injection of EGTA, a calcium chelator, and antagonized by simultaneous intracerebroventricular injection of X537A, a calcium ionophore, and a low dose of calcium, although neither X537A nor this dose of calcium alone blocked the morphine-antinociception. Therefore, we considered that a minor change of calcium movement into the nerve endings showed a further extent of morphine-antinociception and that Aspaminol may have potentiated the morphine-antinociception by minor inhibition of calcium uptake into synaptosomes, although a sufficient concentration of Aspaminol in the rat brain to induce central actions was not obtained by subcutaneous administration. These results support previous findings that the inhibition of synaptosomal calcium uptake plays an important role for the production of morphine-antinociception. Papaverine blocked morphine-antinociception, notwithstanding that $10^{-4}$ M papaverine completely inhibited glutamate-stimulated $^{45}$Ca$^{2+}$ uptake into synaptosomes. Therefore, it may be difficult to account for the antinociceptive action of morphine by the inhibition of $^{45}$Ca$^{2+}$ uptake only. An intracerebral or intravenous administration of cyclic AMP to mice antagonized the antinociception of morphine (26). It is well known that papaverine is a potent inhibitor of phosphodiesterase (15) and that Aspaminol is found to have little inhibitory action on the phosphodiesterase activity in smooth muscle (27). Inhibition of morphine-antinociception by papaverine may be due to elevation of the cyclic AMP level which is caused by inhibition of phosphodiesterase.

References

3) Leslie, S.W., Friedman, M.B., Wilcox, R.E. and Elrod, S.V.: Acute and chronic effects of barbiturates on depolarization-induced calcium influx