EFFECT OF 1,1-DIPHENYL-3-PIPERIDINOBUTANOL HYDROCHLORIDE (ASPAMINOL) ON THE STIMULI-INDUCED CALCIUM UPTAKE IN RAT BRAIN SYNAPTOSOMES

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Abstract—Effects of 1,1-diphenyl-3-piperidinobutanol hydrochloride (Aspaminol), a nonspecific smooth muscle relaxant, on the uptake of calcium in the synaptosomes of rat brain were studied, using two stimuli i.e. high KCl and ATP. We also studied the effect of Aspaminol on the analgesic response of rat hindpaw using the Randall-Sellito test. The addition of Aspaminol inhibited the stimuli-induced calcium uptake in a concentration dependent manner. Aspaminol inhibited the high KCl induced calcium uptake competitively against the external calcium concentration. These findings suggest that Aspaminol may act on the surface of the synaptosomal membranes. In case of ATP stimulated calcium uptake, Aspaminol reduced the rate of uptake and maximal level of the synaptosomal calcium, and did not appear to have any effect on the rate of calcium release. These findings suggest that the inhibitory effect of Aspaminol can be mainly attributed to inhibition of the calcium influx, but not to acceleration of calcium efflux. In the five weeks old rats, Aspaminol did not influence the responses in the paw pinch test, but, in the weanling rats (about three weeks old), Aspaminol had a slight effect on the responses in the paw pinch test. This discrepancy may be due to difficulties in passing through the blood-brain barrier. Our findings suggest that a certain relationship may exist between the analgesic effect and the calcium uptake in the synaptosomes.

The concentration of free calcium outside and within neurons plays an important role in the functioning of the nervous systems. The release of neurotransmitters at the nerve endings is dependent on calcium, and appears to be triggered by a rise in the cytoplasmic calcium level (1).

Recently, numerous studies have shown that synaptosomes (pinched-off nerve terminals isolated from brain homogenate) resemble intact tissue in transport (2, 3) and

metabolic processes (4, 5), and thus synaptosomes should afford an excellent model system for examining calcium disposition at the nerve endings.

Numerous investigations have indicated a role for calcium in the production of analgesia by opiates. Morphine-induced analgesia was inhibited by the administration of calcium (6) and enhanced by a calcium ionophore, X537A (7). Furthermore, a specific inhibition by opiates of calcium uptake in the synaptosomes has been described (8–10). Moreover, it has been suggested that

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morphine blocks the release of neurotransmitters by decreasing calcium influxes. These findings indicate that a close relationship may exist between the analgesic effect and calcium movement.

Nachshen and Braustein (11) and Ichida et al. (12) showed that verapamil and D 600 inhibited the calcium uptake in the synaptosomes from rat brain. Verapamil and D 600 also inhibited the calcium uptake in smooth muscle (13) and in microsomal fractions (14). Takayanagi et al. found that 1,1-diphenyl-3-piperidinobutanol hydrochloride (Aspaminol), a nonspecific smooth muscle relaxant, also inhibited the calcium uptake in smooth muscle (15) and in microsomal fractions (14, 16).

We attempted to determine whether or not Aspaminol inhibits the stimuli-induced calcium uptake in the synaptosomes and whether or not this drug possesses the analgesic responses.

MATERIALS AND METHODS

Preparations of synaptosomes: Synaptosomes were prepared from brain homogenates of male Wistar strain rats weighing 150 to 250 g, using a discontinuous Ficoll gradient centrifugation, according to a modification of the procedure of Cotman and Matthews (17). Homogenization of brains (except the cerebellum) was carried out in 9 volumes of ice-cold 0.32 M sucrose, 5 mM N-2-hydroxyethyl piperazine-N'-2 ethanesulfonic acid (HEPES) buffer, brought to pH 7.4 at 25°C by titration with Tris base, in a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 1,000×g for 10 min. The supernatant was centrifuged at 17,000×g for 10 min. The resultant pellet was resuspended in fresh 0.32 M sucrose-5 mM HEPES buffer (half to the volume decanted) and again centrifuged at 17,000×g for 10 min. The pellet from this spin (crude mitochondrial fraction) was

resuspended in 0.32 M sucrose-5 mM HEPES buffer and layered on top of a discontinuous gradient composed of 15 ml of 7.5% (w/v) Ficoll on top of 15 ml of 12% (w/v) Ficoll, both in 0.32 M sucrose, and the preparation centrifuged at 65,000×g for 50 min in a Hitachi ultracentrifuge with a swing bucket rotor, RPS 27-2. After centrifugation, the material between the 7.5% and 12% Ficoll interface (Synaptosomal fraction) was harvested with a Pasteur pipette and, in case of potassium stimulated $^{45}\text{Ca}^{2+}$ uptake, was diluted with about 8 volumes of ice-cold Ca^{2+} -free physiological salt solution containing 132 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4 , 1.3 mM MgCl_2 , 10 mM glucose and 20 mM Tris buffered to pH 7.4 at 25°C by titration with maleic acid, or, in case of ATP stimulated $^{45}\text{Ca}^{2+}$ uptake, diluted with about 2 volumes of ice-cold 0.32 M sucrose. The dilution was made by progressive addition of aliquots of the ice-cold Ca^{2+} -free physiological salt solution or 0.32 M sucrose over a 15 min period. The diluted suspension with Ca^{2+} -free physiological salt solution was then centrifuged at 9,000×g for 10 min, and with 0.32 M sucrose was then centrifuged at 100,000×g for 30 min. The resultant pellet was resuspended in each ice-cold preincubation medium in a glass homogenizer with a Teflon pestle at lowest speed, and kept on ice prior to the incubation, which was always carried out the same day. All procedures were carried out below 4°C. These synaptosomal suspensions were used for determination of $^{45}\text{Ca}^{2+}$ uptake, as induced by two stimuli.

Incubation procedures and $^{45}\text{Ca}^{2+}$ uptake: The first estimation of $^{45}\text{Ca}^{2+}$ uptake in the synaptosomes with high KCl was done by the method of Blaustein (18). The synaptosomes were first equilibrated in a physiological salt solution containing 132 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_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. The uptake of $^{45}\text{Ca}^{2+}$ in the synaptosomes was initiated when 0.6 ml of suspension was added to 0.6 ml of pre-warmed high KCl solution containing 137 mM KCl, 1.2 mM NaH_2PO_4 , 1.3 mM MgCl_2 , 1.2 mM CaCl_2 including $^{45}\text{Ca}^{2+}$, 10 mM glucose and 20 mM Tris buffered to pH 7.4 at 25°C by titration with maleic acid. Unless otherwise indicated, the final solution had 66 mM NaCl, 71 mM KCl, 1.2 mM NaH_2PO_4 , 1.3 mM MgCl_2 , 1.2 mM CaCl_2 (specific activity 2 mCi/m mol), 10 mM glucose and 20 mM Tris. The mixture was incubated for 2 min at 30°C, unless otherwise indicated. When the effect of Aspaminol was examined, the drug was added at the beginning of the preincubation period. This experimental incubation was terminated by adding 1.2 ml of ice-cold stopping solution containing 30 mM ethyleneglycol-bis-(β -amino-ethylether)-N,N'-tetraacetic acid (GEDTA), 120 mM NaCl and 5 mM KCl titrated to pH 7.4 at 25°C with Tris base. One ml of mixture was immediately filtered by passing through a Millipore filter (HA, 0.45 μm) prewashed with ice-cold washing solution containing 132 mM choline-Cl, 5 mM KCl, 1.2 mM NaH_2PO_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 washed twice with one ml of ice-cold washing solution.

The second estimation of $^{45}\text{Ca}^{2+}$ uptake in the synaptosomes with ATP was done by the method of Guerrero-Munoz et al. (8). The synaptosomes were preincubated in 0.32 M sucrose for 5 min at 30°C. The uptake of $^{45}\text{Ca}^{2+}$ in the synaptosomes was initiated when 0.6 ml of suspension was added to 0.6 ml of prewarmed stimulator solution containing 0.1 mM CaCl_2 including $^{45}\text{Ca}^{2+}$ (specific activity 2 mCi/m mol),

3 mM MgCl_2 , 3 mM ATP disodium salt and 50 mM Tris buffered to pH 7.4 at 25°C by titration with maleic acid. At fixed intervals, $^{45}\text{Ca}^{2+}$ uptake was terminated by adding 1.2 ml of ice-cold washing solution containing 100 mM NaCl, 0.1 mM CaCl_2 , 3 mM MgCl_2 and 50 mM Tris buffered to pH 7.4 at 25°C by titration with maleic acid, and one ml of synaptosomal suspension was immediately passed through a Millipore filter (HA, 0.45 μm) prewashed with ice-cold washing solution. Each filter was washed twice with one ml of the same solution. The drug was added at the beginning of the preincubation period. The experimental incubation carried out at 30°C.

To measure the retention of previously accumulated $^{45}\text{Ca}^{2+}$, 0.5 ml of the synaptosomes were preincubated in 0.32 M sucrose for 5 min at 30°C, followed by a 10 min incubation in the presence of 0.5 ml of stimulator solution (final one ml). These synaptosomes were then diluted with 10 volumes of pre-warmed washing solution and again re-incubated at 30°C. After fixed time intervals between 0 to 10 min, two ml of the synaptosomal suspension was immediately filtered and washed twice with one ml of ice-cold washing solution. When the effect of Aspaminol was examined, the drug was added at the beginning of the re-incubation period.

The radioactive Ca^{2+} retained was estimated by a liquid scintillation spectrometer, after the filters had been dissolved in a toluene scintillator. The values were corrected for the $^{45}\text{Ca}^{2+}$ remaining on the filters in the absence of the synaptosomal fraction. $^{45}\text{Ca}^{2+}$ uptake was estimated from the radioactivity of the filter. Protein was determined by the method of Lowry et al. (19), using bovine serum albumin as the standard. The amount of protein contained in the incubation mixture was 300 to 600 $\mu\text{g/ml}$.

Assessment of analgesia: Male Wistar strain rats, weighing 30 to 40 g and 70 to 90 g, were used. Subcutaneous doses of Aspaminol (100 mg/kg and 500 mg/kg) or morphine (2.5 mg/kg) were administered, and assessed using the Randall Sellito test (20).

In the Randall Sellito test (Analgesy-meter, Ugo Basile) a force increasing at a constant rate is applied force to the rat hindpaw. The applied force is continuously monitored by an indicator moving along a linear scale calibrated in grams. The application of force is stopped when rats start to struggle to a noticeable degree.

Drugs used: Aspaminol (1,1-diphenyl-3-piperidinobutanol hydrochloride; Kowa), morphine hydrochloride (Sankyo), Tris (Tris (hydroxymethyl) aminomethane and ATP disodium salt; Sigma), HEPES (N-2-hydroxyethyl-piperazine-N'-2 ethanesulfonic acid; Nakarai), GEDTA (ethyleneglycol-bis-(β -amino-ethylether)-N,N'-tetraacetic acid; Wako-Junyaku), Ficoll 400 (Pharmacia Fine Chemicals) and $^{45}\text{CaCl}_2$ (specific activity: 16 mCi/mg; New England Nuclear). Other chemicals used were of analytical grade. Drugs were dissolved in double-distilled and deionized water.

RESULTS

Effect of Aspaminol on potassium stimulated calcium uptake in the synaptosomes: Figure 1 shows the uptake of calcium in the depolarized (71 mM KCl) and polarized (5 mM KCl) synaptosomes, as a function of time. The uptake of calcium increased linearly for approximately the first minute of incubation in the polarized synaptosomes, and reached a plateau level within 2 min. In the presence of 71 mM KCl, both the rate of uptake and plateau value were increased. Potassium stimulated calcium uptake, which was calculated as difference between the uptakes in 71 mM KCl and 5 mM

KCl at the plateau level, was about 6 $\mu\text{mol/g}$ protein. This value was in good agreement with that reported by other investigators (12,

Fig. 1. Calcium accumulation as a function of different incubation times for depolarized and polarized synaptosomes. Each point represents mean $\pm$ S.E. of 3 experiments. Synaptosomes were equilibrated in the physiological salt solution for 15 min at 30°C. The incubation was initiated by addition of high KCl (137 mM) or physiological salt solution containing $^{45}\text{Ca}^{2+}$, and terminated by addition of ice-cold GEDTA stopping solution and rapid filtration after fixed intervals. ●; depolarized synaptosomes, ○; polarized synaptosomes. *; significantly different from the polarized synaptosomes at $P < 0.05$.

Fig. 2. Calcium accumulation as a function of different concentration of external potassium. Each point represents mean $\pm$ S.E. of 3 experiments. Synaptosomes were equilibrated in the physiological salt solution for 15 min at 30°C. The incubation was initiated by addition of different concentration of high KCl solution containing $^{45}\text{Ca}^{2+}$, and terminated by addition of ice-cold GEDTA stopping solution and rapid filtration after 2 min.

21). Figure 2 shows the calcium uptake in the synaptosomes as a function of different concentrations of external potassium. An increase of external potassium stimulated the calcium uptake in the synaptosomes up to 71 mM KCl. This results was in good agreement with that reported by Blaustein (18). From these results, an incubation time of 2 min and a concentration of 71 mM KCl were used in subsequent studies on potassium stimulated calcium uptake in the synaptosomes.

Figure 3 shows the effect of Aspaminol on potassium stimulated calcium uptake in the synaptosomes. The calcium uptake in 5 mM KCl medium was not influenced significantly by various concentrations (10^{-6} to 10^{-4} M) of Aspaminol (Fig. 3, lower curve), but potassium stimulated calcium uptake was inhibited by Aspaminol in a concentration dependent manner (Fig. 3, upper curve).

Since Aspaminol inhibits the calcium uptake in the microsomal fractions from smooth muscle and this inhibition is abolished by the increase of calcium concentration in the reaction mixture (16), it was of interest to determine whether or not Aspaminol is acting as a competitive or noncompetitive inhibitor in calcium uptake. Therefore, we tested the inhibitory effect of Aspaminol on

calcium uptake at different external concentrations of calcium. Table 1 shows that Aspaminol (10^{-4} M) inhibited the potassium

Fig. 3. Effect of various concentration of Aspaminol on potassium stimulated calcium uptake in the synaptosomes. Each point represents mean $\pm$ S.E. of 5 experiments. Synaptosomes were equilibrated in the physiological salt solution in the presence of different concentration of Aspaminol for 15 min at 30°C. The incubation was initiated by addition of high KCl (137 mM) or physiological salt solution containing $^{45}\text{Ca}^{2+}$ in the presence of various concentration of Aspaminol, and terminated by addition of ice-cold GEDTA stopping solution and rapid filtration after 2 min. ●; depolarized synaptosomes, ○; polarized synaptosomes. *, ** significantly different from the control at $P < 0.05$ and $P < 0.01$, respectively.

Table 1. Effect of Aspaminol on potassium stimulated calcium uptake at different external calcium concentration

CaCl ₂ (Conc.)	⁴⁵ Ca Uptake (μmol/g protein)	
	Control	Aspaminol (10^{-4} M)
0.12 mM	2.44 $\pm$ 0.19	1.60 $\pm$ 0.07*
0.36 mM	5.20 $\pm$ 0.30	3.51 $\pm$ 0.21*
1.2 mM	10.67 $\pm$ 0.76	6.30 $\pm$ 0.32*
3.6 mM	15.97 $\pm$ 0.56	13.40 $\pm$ 1.04
12.0 mM	20.94 $\pm$ 3.23	18.93 $\pm$ 0.80

Each value represents mean $\pm$ S.E. of 5 experiments. Synaptosomes were equilibrated in the physiological salt solution containing various concentration of CaCl₂ in the presence and absence of 10^{-4} M Aspaminol for 15 min at 30°C. The incubation was initiated by addition of high KCl (137 mM) salt solution containing various concentration of CaCl₂ in the presence and absence of 10^{-4} M Aspaminol, and terminated by addition of ice-cold GEDTA stopping solution and rapid filtration after 2 min. * significantly different from the control at $P < 0.01$.

stimulated calcium uptake in the low external calcium up to 1.2 mM, but this inhibitory effect was abolished by high external calcium concentrations (3.6 mM to 12 mM). Figure 4 shows the Lineweaver-Burk plots for the above data. The reciprocal plots gave hyperbolic curves, but, in case of higher concentrations of calcium, it was apparent that the lines extrapolated to the same calcium concentration, indicating that Aspaminol inhibited the calcium uptake competitively.

Fig. 4. (A); Lineweaver-Burk plots of Aspaminol actions on potassium stimulated calcium uptake at different external calcium concentrations, (B); same plots at high concentration of external calcium. $\circ$; Aspaminol (10^{-4} M), $\bullet$; control.

Effect of Aspaminol on ATP stimulated calcium uptake in the synaptosomes: Table 2 shows that ATP stimulated calcium uptake was extremely low in the absence of ATP and/or Mg^{2+} . Figure 5 shows the calcium uptake data obtained with synaptosomes preincubated with various concentrations of

Fig. 5. Effect of various concentration of Aspaminol on ATP stimulated calcium uptake in the synaptosomes. Each point represents mean $\pm$ S.E. of 4 experiments. Synaptosomes were suspended with 0.32 M sucrose and preincubated in the presence of various concentration of Aspaminol for 5 min at 30°C . The incubation was initiated by addition of uptake solution containing $^{45}\text{Ca}^{2+}$ in the presence of various concentrations of Aspaminol, and terminated by rapid filtration after fixed intervals. $\circ$; control, $\bullet$; 10^{-6} M Aspaminol, $\odot$; 10^{-5} M Aspaminol, $\circ$; 10^{-4} M Aspaminol, $\times$; ATP omitted. * significantly different from the control at $P < 0.01$.

Table 2. Effects of components of reaction mixture on ATP stimulated calcium uptake in the synaptosomes

Addition	^{45}Ca Uptake ($\mu\text{mol/g protein}$)
Complete	19.70 ± 1.78
—ATP, — Mg^{2+}	$4.68 \pm 0.38^*$
—ATP	$3.82 \pm 0.21^*$
— Mg^{2+}	$5.11 \pm 0.57^*$

Each value represents mean $\pm$ S.E. of 4 to 5 experiments. Synaptosomes were suspended with 0.32 M sucrose and preincubated for 2 min at 30°C . The incubation was initiated by addition of uptake solution containing $^{45}\text{Ca}^{2+}$ in the presence of 3 mM ATP and/or 3 mM $MgCl_2$. The incubation terminated by rapid filtration after 10 min. * significantly different from the complete group at $P < 0.01$.

Fig. 6. Time course of calcium retention by synaptosomes. Each point represents mean $\pm$ S.E. of 4 experiments. Synaptosomes were preincubated for 5 min at 30°C, and then loaded with uptake solution for 10 min at 30°C. The incubation was initiated by addition of 10 volume of washing solution containing 100 mM NaCl, 3 mM MgCl₂, 0.1 mM CaCl₂ and 50 mM Tris-maleic acid buffer, and terminated by rapid filtration after fixed intervals. ○; control, ●; Aspaminol (10^{-4} M).

Aspaminol (10^{-6} to 10^{-4} M). ATP stimulated calcium uptake in the synaptosomes showed a rapid phase and followed by a slower phase which reached a plateau level in about 20 min. Aspaminol significantly reduced the rate of uptake and maximal level of synaptosomal calcium, in a concentration dependent manner.

The effect of Aspaminol (10^{-4} M) on the time-dependent release of $^{45}\text{Ca}^{2+}$ from $^{45}\text{Ca}^{2+}$ -loaded synaptosomes is shown in Fig. 6. From 2 to 10 min, Aspaminol did not appear to have any effect on the rate of $^{45}\text{Ca}^{2+}$ release. These results suggest that the inhibitory effect of Aspaminol is mainly due to inhibition of calcium influx, but not to acceleration of calcium efflux.

Assessment of analgesia: In case of 5 weeks old rats, morphine (2.5 mg/kg s.c.) had an analgesic effect and mean threshold force to elicit struggle of the paw was 160 ± 24 g (mean $\pm$ S.E.) at 30 min after administration. This value was significantly higher than the control value (92 ± 11 g) at the same time after administration, but Aspaminol (500 mg/kg s.c.) did not have an analgesic

Fig. 7. Effects of Aspaminol and morphine on paw-pinch test in rats. Each point represents mean $\pm$ S.E. of 5 to 6 rats. [A]; weanling rats, [B]; 5 weeks old rats, ○; control (saline s.c.), ●; morphine (2.5 mg/kg s.c.), ●; Aspaminol (500 mg/kg s.c.), ○; Aspaminol (100 mg/kg s.c.). *, ** significantly different from the control at $P < 0.05$ and $P < 0.01$, respectively.

effect at each time after administration (Fig. 7-B). On the other hand, combined administration of Aspaminol (500 mg/kg s.c.) with morphine (2.5 mg/kg s.c.) potentiated the analgesic effect of morphine (data not shown). In case of weanling rats (about 3 weeks old), morphine (2.5 mg/kg s.c.) and Aspaminol (500 mg/kg s.c. and 100 mg/kg s.c.) also induced an analgesic effect. The peak value of threshold force after administration of morphine was 250 g at 30 min, and that of Aspaminol (500 mg/kg s.c. and 100 mg/kg s.c.) were 230 ± 20 g at 30 min and 184 ± 28 g at 60 min, respectively. These values were significantly higher than the corresponding control values (Fig. 7-A).

DISCUSSION

Verapamil and D 600 which have been shown to prevent the influx of calcium in the smooth muscle (13) and its microsomal fractions (14) inhibited the potassium stimulated calcium uptake in rat brain synaptosomes (11-12). Aspaminol (1,1-

diphenyl-3-piperidinobutanol hydrochloride), a nonspecific smooth muscle relaxant, also inhibited the calcium uptake in the smooth muscle (15) and its microsomal fractions (14, 16). Therefore, we attempted to determine whether or not Aspaminol also would inhibit the calcium uptake in rat brain synaptosomes. For this purpose, we assured the extent of calcium uptake in the synaptosomes using high KCl and ATP.

Our results showed that Aspaminol clearly inhibited the synaptosomal calcium uptake by two stimuli. The inhibition was observed during the initial rate as well as in the maximal calcium uptake. The concentration of Aspaminol (10^{-4} M) required to block the uptake by about two-thirds in the maximal calcium uptake by two stimuli. Since the inhibitions of the various stimuli-induced calcium uptake were shown to same degree, Aspaminol also would act on the surfaces of the synaptosomal membranes as well as that of smooth muscle (16) and also inhibited the high KCl induced calcium uptake in the synaptosomes competitively against the extracellular calcium concentration.

The recent findings showed that morphine blocked calcium uptake in the synaptosomes (8–10). Because calcium is so intimately coupled with exocytotic release of neurotransmitters (22), a process as stimulus-secretion coupling, these findings suggest that morphine may inhibit calcium-mediated "stimulus-secretion coupling" events to cause analgesic response. To make this hypothesis clear, we carried out the paw pinch test in the rats treated with Aspaminol because this drug markedly inhibited the calcium uptake in the synaptosomes. In case of weanling rats, Aspaminol slightly but significantly affected the responses in the paw pinch test. Furthermore, we tested the combined administration of Aspaminol with morphine. Aspaminol clearly potentiated the analgesic effect of morphine. These results suggest

that a certain relationship may exist between the analgesic effect and calcium movement on the nerve endings. But Aspaminol has virtually no effects on the analgesic responses in adult rats. This discrepancy is still obscure. One of possible mechanisms is considered. Since most of Aspaminol molecules exist as ion forms in the physiological pH (pKa of Aspaminol: 8.62), these molecules may be difficult to pass through the blood-brain barrier and can not reach a sufficient concentration for the analgesic responses in the rat brain, though further study is required.

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