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Regulation of calcium uptake in synaptosomes from rat brain by DL-2-amino-5-phosphonovaleric acid

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DL-2-Amino-5-phosphonovaleric acid stimulated calcium influx into synaptosomes isolated from rat brain. The increase in Ca^{2+} permeability of the synaptosomal membranes induced by this amino acid is not markedly dependent on the membrane potential or Na^+ concentration. It is postulated that 2-amino-5-phosphonovaleric acid is an agonist for a receptor(s) which regulates intraneuronal free calcium concentrations by modulating a selective calcium channel. The observed stimulation of calcium uptake may provide an assay system for purification of the endogenous ligand for this receptor and for characterization of its physiological role in neuronal function.

Ca^{2+} uptake; DL-2-Amino-5-phosphonovalerate; Synaptosome; Receptor

1. INTRODUCTION

Neurons have very active transport systems for maintaining their intracellular ionic composition and can undergo very large and rapid changes in the ionic permeability of their plasma membranes. The existence of special mechanisms for calcium permeability in excitable cells has been appreciated for almost 30 years [1–3]. At least two different mechanisms appear to be responsible for active extrusion of Ca^{2+} across the neuronal membranes: the ATP-driven calcium transporter and the Na^+/Ca^{2+} exchanger (review [4]). Return of Ca^{2+} into the interior of neurons is largely by diffusion through Ca^{2+}-selective ion channels [1,2,5–8].

At least four different types of calcium channels have been proposed or observed, of which the most important appear to be voltage-sensitive channels and receptor-modulated channels (see above reviews). Although voltage-dependent calcium channels in neuronal membranes are well described, calcium movements through receptor-mediated channels are much less well characterized. Much remains to be done before an understanding of their properties is achieved [9]. Receptor-operated calcium channels have been proposed to account for the fact that many neurotransmitter ligands activated Ca^{2+} influx in the absence of changes in membrane potential [10–13].

We have previously observed that the potent neurotoxin kainate [14] stimulated uptake of calcium by synaptosomes isolated from rat brain. Here, we provide evidence that the potent and relatively specific antagonist of the NMDA receptors [15–18] DL-2-amino-5-phosphonovalerate (APVA), at millimolar concentrations, also stimulates Ca^{2+} influx into synaptosomes from rat brain. This increase in Ca^{2+} permeability of synaptosomal membranes appears to occur via receptor-modulated calcium channels which are different from voltage-dependent Ca^{2+} channels.

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2. MATERIALS AND METHODS

Male Sprague-Dawley rats (180–220 g) were used throughout the experiments. Crude synaptosomal preparations (P_2 fraction) were prepared from homogenates of brain cortex by centrifugation for 10 min at 20000 × *g* of the initial low-speed supernatant (3 min at 1000 × *g*). The purified synaptosomal fraction was isolated according to Booth and Clark [19]. The final synaptosomal pellets were suspended at approx. 1 mg protein/ml in modified Krebs-Henseleit saline (140 mM NaCl, 3 mM KCl, 5 mM $NaHCO_3$, 1.3 mM $MgSO_4$, 1 mM Tris-phosphate, 10 mM Tris-Hepes, pH 7.4, containing 10 mM glucose and 0.1 mM $CaCl_2$). In experiments involving low sodium levels (<1 mM), the Na^+ salts were replaced by equal concentrations of choline salts.

2.1. *Measurements of calcium uptake*

For measurements of $^{45}Ca^{2+}$ uptake, 400-μl aliquots of the resuspended P_2 synaptosomal fraction were incubated for 10 min at 30°C. Uptake of $^{45}Ca^{2+}$ was then initiated by rapid addition of 400 μl Krebs-Henseleit medium containing $^{45}Ca^{2+}$ (1.25 mM final concentration, 1 μCi/ml). At appropriate times (1, 2, 3, 5, 10, 20 s), uptake of $^{45}Ca^{2+}$ was terminated by addition of 5 ml ice-cold EGTA 'stopping solution' containing 140 mM NaCl, 3 mM KCl, 5 mM $NaHCO_3$, 1 mM Tris-phosphate, 3 mM EGTA and 10 mM Tris-Hepes, pH 7.4.

Each sample was immediately filtered through a prewashed glass-fiber filter using a Millipore vacuum filtration apparatus. The filters were rapidly washed twice with 5-ml aliquots of the cold incubation medium, dried in air, and the radioactivity measured in a Searle Delta 300 liquid scintillation counter using an Amersham aqueous scintillant (ACS II). To measure the effect of amino acid on the uptake of $^{45}Ca^{2+}$, the assay solution contained APVA at final concentrations from 0.5 to 10 mM (see table and figure legends for details): the amino acid was neutralized to pH 7.4 by NaOH or Tris base. In experiments testing the efficacy of the voltage-dependent channel blockers, the assay media also contained cobalt chloride (3 mM), lanthanum chloride (6 μM), quinacrine (100 μM), verapamil (50 μM) or ruthenium red (5 or 50 μM).

2.2. *Measurements of membrane potential*

The transmembrane electrical potential was estimated from the equilibrium distribution of [^{14}C]triphenylmethylphosphonium (TPMP; final total concentration 10 μM < 0.44 μCi/ml) plus 1 μM tetraphenylboron. The synaptosomes were incubated with TPMP and tetraphenylboron, with and without amino acid, for 20 min at 30°C. Aliquots (300 μl) were withdrawn and the synaptosomes centrifuged through a layer of silicone oil. The radioactivity was then measured in both the pellet and supernatant fractions. The membrane potentials were calculated using the Nernst equation.

2.3. *Measurements of oxygen consumption*

Synaptosomes were incubated for 5 min at 25°C in a shaking water bath, transferred to a thermostatted (25°C) chamber containing a Clark-type oxygen electrode and, after a steady rate of oxygen consumption was established, the amino acid was added.

2.4. *Protein determination*

Protein concentrations were determined by the method of Lowry et al. [20] with bovine serum albumin as the standard.

2.5. *Statistical analysis*

All values are presented as means ± SE. Statistical significance was determined using the paired *t*-test applied to the raw data. Measurements in the presence of amino acid were compared to those in the absence of amino acid (control) in the same experiment. The term 'independent experiments' refers to experiments carried out on different days and using different solutions of amino acid.

3. RESULTS

3.1. *Effect of APVA on $^{45}Ca^{2+}$ uptake by synaptosomes*

The dependence of the measured $^{45}Ca^{2+}$ uptake on concentration of APVA is shown in fig.1. The $^{45}Ca^{2+}$ uptake increased with increasing APVA concentration, becoming statistically significant at 0.5 mM ($P < 0.05$). Half-maximal effect occurred at approx. 1 mM amino acid. The $^{45}Ca^{2+}$ uptake induced by addition of 10 mM APVA was 80%

above control values. This effect was specific for APVA since addition of 10 mM concentrations of D-glutamate, L-glutamate, D-aspartate, L-aspartate, or L-cysteate instead of APVA had no effect on $^{45}Ca^{2+}$ uptake by the synaptosomes. Similarly, no inhibition of the APVA-induced $^{45}Ca^{2+}$ uptake was observed when these compounds were added either before or at the same time as the APVA (not shown).

3.2. *Time dependence of the effect of APVA on $^{45}Ca^{2+}$ uptake by synaptosomes*

Uptake of $^{45}Ca^{2+}$ by the synaptosomal fraction was measured in the presence and absence of 5 mM APVA (fig.2A). The presence of APVA resulted in an increase in the uptake of $^{45}Ca^{2+}$, which became statistically significant ($P < 0.05$) by 2 s and was significant at all of the longer times. Fig.2B illustrates the difference between the uptake of $^{45}Ca^{2+}$ in the presence and absence of APVA. As shown in this figure, the uptake of $^{45}Ca^{2+}$ induced by APVA occurred with a half-time of approx. 3 s.

3.3. *Distribution of APVA-induced Ca^{2+} permeability changes in different anatomical regions of the brain*

The effect of the amino acid on the uptake of $^{45}Ca^{2+}$ was measured using crude synaptosomal fractions prepared from five different anatomical regions of the brain: cortex, striatum, hippocampus, midbrain and cerebellum (table 1). APVA stimulated the uptake of ^{45}Ca most strongly in synaptosomes from the cortex, while smaller, but still statistically significant, stimulations were observed in synaptosomes from the striatum, hippocampus and cerebellum. Only in synaptosomes from midbrain was there no significant APVA-induced increase in calcium uptake.

3.4. *The effect of calcium channel blockers and ruthenium red on the increased $^{45}Ca^{2+}$ uptake induced by APVA*

The increase in $^{45}Ca^{2+}$ uptake induced by APVA was measured in the presence and absence of 3 mM $CoCl_2$, 6 μM $LaCl_3$, 50 μM verapamil, 100 μM quinacrine, 100 μM nifedipine or 50 μM ruthenium red (table 2). None of these agents, except $LaCl_3$, significantly inhibited the increase in $^{45}Ca^{2+}$ uptake induced by APVA. Lanthanum

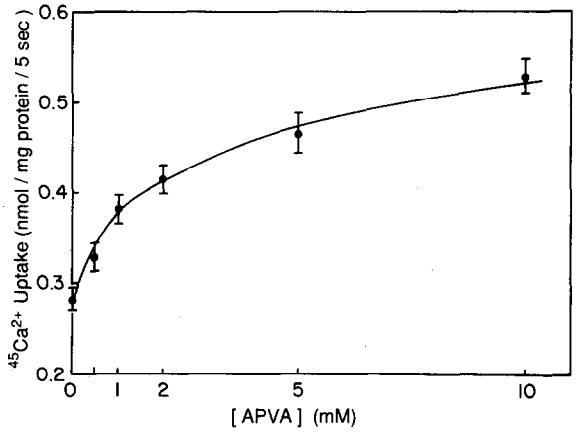

Fig.1. $^{45}Ca^{2+}$ uptake in 5 s as a function of APVA concentration. Synaptosomes were incubated for 10 min at 30°C in Krebs-Henseleit medium containing 3 mM K^+ and 0.1 mM Ca^{2+}. Aliquots (400 μl) were added to equal volumes of incubation medium containing $^{45}Ca^{2+}$ (final concentration 1.25 mM) to initiate $^{45}Ca^{2+}$ uptake. Uptake was terminated after 5 s by injection of 5 ml ice-cold stopping solution and rapid filtration. The values are the means ± SE for 4 experiments.

decreased the effect of APVA addition by about 40%.

3.5. *Effect of Na^+ concentration on the increase in $^{45}Ca^{2+}$ uptake induced by APVA*

The importance of Na^+ to the APVA-induced $^{45}Ca^{2+}$ uptake in synaptosomes was determined by comparing the uptake measured in media which contained normal Na^+ concentrations (140 mM) to that measured in media with very low Na^+ concentrations (< 1 mM). As shown in table 3, the amino acid stimulated uptake of $^{45}Ca^{2+}$ nearly as much when the synaptosomes were suspended in media with low Na^+ concentrations as when they were suspended in media with normal Na^+ concentrations. The difference was not statistically significant indicating little or no dependence on Na^+ concentration.

3.6. *Effect of the APVA on the synaptosomal membrane potential and respiratory rate*

The effect of addition of amino acid on the transmembrane electrical potential of synaptosomes was estimated from its effect on the equilibrium distribution of [^{14}C]TPMP. After 20 min incubation with 0 (control) and 5 mM

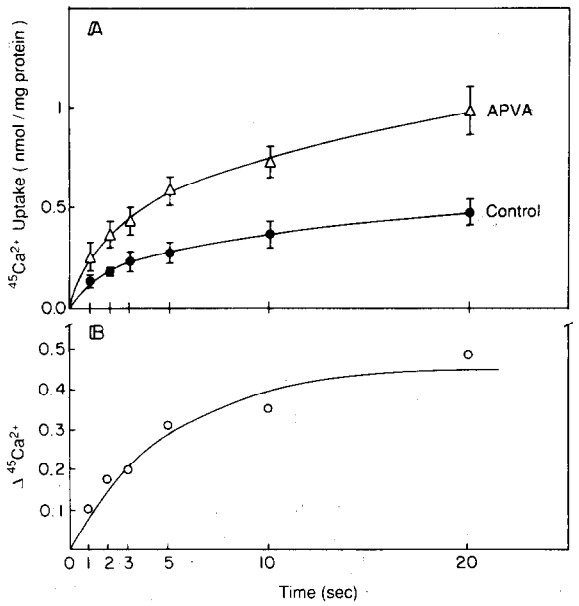

Fig.2. (A,B) Effect of APVA on uptake of $^{45}Ca^{2+}$ into synaptosomes. The uptake of $^{45}Ca^{2+}$ was initiated by media containing either 0 mM (control) or 10 mM APVA as described in the legend to fig.1. Each point in panel A is the mean ± SE for 4 experiments except for the 1 s point which was for 3 experiments. The uptake in the presence of APVA was greater than that in the control sample at each time point longer than 1 s ($P < 0.05$, paired t-test applied to the raw data). The points in panel B are the calcium uptake in the presence of amino acid minus that in its absence.

Table 1

Effect of DL-2-amino-5-phosphonovalerate on uptake of $^{45}Ca^{2+}$ by synaptosomes from different regions of the brain

Region of brain	Uptake of calcium (% control)	P value
Cortex	161 ± 8	<0.005
Striatum	140 ± 5	<0.01
Hippocampus	137 ± 6	<0.05
Midbrain	114 ± 11	n.s.
Cerebellum	128 ± 6	<0.05

The uptake of $^{45}Ca^{2+}$ in 5 s was measured as described in section 2. The concentration of APVA was 5 mM. The results are expressed as percent of the uptake in the absence of amino acid (mean ± SE for 4 experiments)

Table 2

Effect of APVA on $^{45}Ca^{2+}$ uptake by synaptosomes in the presence of calcium channel blockers

Additions	Uptake of $^{45}Ca^{2+}$ (nmol/mg protein per 5 s)		
	Control	APVA	Uptake
None	0.29 ± 0.05	0.53 ± 0.07	0.24
Quinacrine (100 μM)	0.24 ± 0.07	0.51 ± 0.06	0.27
$CoCl_2$ (3 mM)	0.20 ± 0.04	0.49 ± 0.05	0.29
$LaCl_3$ (6 μM)	0.15 ± 0.04	0.26 ± 0.05	0.11
Verapamil (50 μM)	0.26 ± 0.03	0.50 ± 0.03	0.24
Nifedipine (100 μM)	0.28 ± 0.045	0.49 ± 0.04	0.21
Ruthenium red (50 μM)	0.23 ± 0.02	0.46 ± 0.05	0.23

The uptake of $^{45}Ca^{2+}$ was initiated by diluting the synaptosomal fraction (P_2) with an equal volume of Krebs-Henseleit medium (3 mM K^+) containing $^{45}Ca^{2+}$ and the indicated amount of channel blocker in the absence (control) or presence (APVA) of 10 mM APVA. $^{45}Ca^{2+}$ uptake was measured for the first 5 s after dilution and the results are presented as means ± SE for 4 experiments

APVA, the calculated membrane potentials (uncorrected for binding of TPMP) were 83.6 ± 1.6 mV in control and 78.4 ± 3.5 mV in the presence of the amino acid (means ± SE for three experiments). This difference was not statistically significant.

Table 3

The effect of Na^+ concentration on the uptake of $^{45}Ca^{2+}$ induced by APVA

	nmol/mg protein per 5 s	
	140 mM Na^+	0 mM Na^+
Control	0.34 ± 0.01	0.31 ± 0.02
APVA	0.68 ± 0.03	0.54 ± 0.07

The uptake of $^{45}Ca^{2+}$ was measured in regular Krebs-Henseleit medium (140 mM Na^+) in a medium in which choline was substituted for Na^+ (0 mM Na^+) as described in section 2. All values are expressed as means ± SE for 4 independent experiments. APVA was added to a final concentration of 10 mM

The respiratory rates of synaptosomes were measured between 1 and 3 min after addition of APVA. The amino acid increased the respiratory rate by about 25% ($P < 0.01$). The control respiratory rate was 1.79 ± 0.2 nmol O_2/min per mg protein (means ± SE for 3 experiments).

4. DISCUSSION

The pharmacological classification of excitatory amino acid receptors indicates that several types of receptors exist in the vertebrate central nervous system. One of them is preferentially activated by *N*-methyl-D-aspartate and this effect is blocked by APVA [15–18]. Current data indicate, however, that the effects of APVA cannot be fully explained by its ability to block some *N*-methyl-D-aspartate receptors [21–23]. Electrophysiological experiments suggest that 100 μM concentrations are sufficient to block the *N*-methyl-D-aspartate receptors but at higher concentrations (100–500 μM, the maximum tested) other effects occurred which could be explained by the presence of receptors activated by APVA [21,22]. Our results ([23]; present paper) are consistent with the presence of APVA-activated receptors which increase the permeability of synaptosomal membrane to calcium with half-maximal effects at approx. 1 mM.

4.1. *Is Na^+/Ca^{2+} exchange responsible for APVA-induced $^{45}Ca^{2+}$ uptake?*

The results presented in table 3 show that lowering the concentration of Na^+ in the incubation medium to less than 1 mM had no significant effect on the increase of uptake of Ca^{2+} induced by this amino acid. This indicates that the observed effect is independent of Na^+ concentration and therefore apparently does not involve Na^+/Ca^{2+} exchange.

4.2. *Does APVA activate voltage-dependent calcium channels?*

The voltage-dependent calcium channels in neuronal membranes can be blocked by many metal ions, including Mn^{2+}, Ni^{2+}, Co^{2+}, Cd^{2+}, La^{3+} and at least partially by several organic Ca^{2+}-channel blockers, including verapamil and nifedipine (see, for example [2,6–8,24]). In addition, quinacrine [25] and ruthenium red [26–28] have been reported to block selectively voltage-dependent calcium channels. Previous experiments showed that $CoCl_2$, verapamil and quinacrine, at the concentrations used in these experiments, inhibited the depolarization-dependent $^{45}Ca^{2+}$ uptake of synaptosomes by more than 80% [14]). This is in marked contrast to the fact that these agents show little or no effect on the stimulation of calcium uptake by APVA. Although La^{3+} inhibited the increased $^{45}Ca^{2+}$ uptake, lanthanum ions are known to bind tightly to biological membranes and to have nonspecific effects on many membrane-associated functions. Thus, from these data it does not appear that the voltage-dependent calcium channels are responsible for the APVA-induced calcium movement.

Ruthenium red has also been reported to be an inhibitor of voltage-dependent calcium channels [26–28] but is generally used to inhibit both passive and active calcium uptake into isolated mitochondria [29–31]. The lack of effect of ruthenium red on the increase in Ca^{2+} permeability of the synaptosomal membrane in the presence of APVA not only supports the view that voltage-dependent calcium channels were not involved, but also provides evidence that uptake of calcium by free mitochondria does not contribute significantly to the measured uptake of $^{45}Ca^{2+}$.

4.3. *APVA appears to act as an agonist for a receptor which modulates calcium-selective channels*

The increase in Ca^{2+} permeability of the plasma membrane of synaptosomes by APVA is consistent with its activating receptor-modulated calcium channels: (i) The effect is saturated by increasing amino acid concentrations. (ii) There is a very high specificity for the structure of the amino acid; close structural analogues have no comparable effect nor do they inhibit the effect of APVA. (iii) There is biological specificity, i.e. the amount of stimulation of calcium uptake is much greater in synaptosomes from some anatomical regions of the brain than from other anatomical regions. (iv) The uptake of calcium through receptor-modulated calcium channels is generally not as sensitive to the blocking agents used as are the voltage-dependent channels. On the other hand, APVA is not a natural constituent of the central nervous system. We suggest that it is acting as a

nonphysiological agonist for a receptor involved in regulation of the metabolism of specific neurons by modulating the calcium levels in the neurons. It is interesting to note that calcium uptake can be used as an assay method for the characterization and purification of the endogenous ligand(s) for this receptor.

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