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The Effects of Some Organic "Calcium Antagonists" on Calcium Influx in Presynaptic Nerve Terminals

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SUMMARY

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The actions of the organic "Ca antagonists" verapamil and D-600 were tested on pinched-off presynaptic nerve terminals (synaptosomes) from rat brain, and on the frog neuromuscular junction. ^{45}Ca uptake was measured in control media, and in depolarizing media containing either 75 mM potassium or veratridine, an alkaloid that opens sodium channels. The extra uptake induced by depolarizing media appears to be mediated by voltage-sensitive Ca channels. Synaptosome depolarization was indirectly determined with the voltage-sensitive fluorescent dye, di-pentyl oxacarbocyanine. Verapamil or D-600 (100 μM) inhibited the K^+-induced ^{45}Ca uptake by about two thirds, but had no effect on the K^+-induced synaptosome depolarization; this inhibition of Ca uptake is, presumably, due to block of Ca-channels. Veratridine-induced ^{45}Ca influx was more than 80% inhibited by verapamil or D-600 (100 μM), and veratridine-induced depolarization was almost completely blocked. These observations indicate that Na channels as well as Ca channels are inhibited by verapamil and D-600. Recordings of miniature end-plate potentials were used to evaluate the actions of verapamil and D-600 at the frog neuromuscular junction, after miniature end-plate potential frequency had been made sensitive to changes in the bathing Ca concentration by raising the external K^+. Miniature end-plate potential frequency was not affected by verapamil (40-50 μM) or D-600 (10 μM) but was significantly reduced by Mn^{2+} (0.2 mM), a known blocker of Ca channels. Although verapamil and D-600 appear to be very potent antagonists of Ca currents in heart and smooth muscle, we conclude that Ca channels in vertebrate neurons are much less sensitive to these drugs.

INTRODUCTION

Calcium influx in many types of cells is mediated by voltage-sensitive Ca channels. These channels have certain shared properties (1): for example, they are not blocked by TTX[1] but are susceptible to blockade by polyvalent cations such as Mn^{2+}, Co^{2+} and La^{3+}. The Ca channels in some types of cells also share a marked sensitivity to several organic "Ca antagonists" (2) such as verapamil (Isoptin, Iproveratril), D-600 (a methoxy derivative of verapamil) and nifedipine (Adalat, Bay-A1040). Although the plasma membranes of presynaptic nerve terminals appear to contain voltage-sensitive Ca channels (3-5) there is indirect evidence that these channels may be relatively insensitive to the organic "Ca antagonists" (6-8).

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[1] The abbreviations used are: TTX, tetrodotoxin; CC_5, 3,3'-dipentyl 2,2'-oxacarbocyanine; MEPP, miniature end-plate potentials.

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Pinched-off presynaptic terminals (synaptosomes) retain many of the functional properties of intact presynaptic nerve endings (9, 10). Entry of Ca into synaptosomes occurs via voltage-dependent Ca channels (5). In the study reported here, we used the synaptosome preparation to show directly that high concentrations of organic "Ca antagonists" are required to inhibit Ca uptake by nerve terminals. We also confirmed previous findings (8) that indicate that Ca channels in the presynaptic terminals of the frog neuromuscular junction are insensitive to these drugs. Our observations indicate that Ca channels from different types of cells may be pharmacologically distinct. A preliminary report of some of these findings has been communicated to the Society for Neuroscience (11).

MATERIALS AND METHODS

Preparation of synaptosomes. Synaptosomes were prepared from rat brains by the method of Hajos (12). The nerve terminal-enriched material in the 0.8 M sucrose fraction from the sucrose gradient was equilibrated with physiological saline by the addition of 2.5 volumes of ice-cold standard (Na + 5K) salt solution. This solution contained (mM): NaCl, 145; KCl, 5; $MgCl_2$, 1.3; $CaCl_2$, 1.2; NaH_2PO_4, 2.4; HEPES 20, buffered to pH 7.5 at 25° with Tris base. The diluted synaptosomes were centrifuged at 4° for 10 min at 10,000 × *g* and the pellet was resuspended in cold Na + 5 K solution. Suspensions were preincubated for about 25 min at 30° (with or without drug) before initiating test uptakes of ^{45}Ca.

Measurement of ^{45}Ca influx. Aliquots of the warmed synaptosome suspension were added to incubation solutions containing ^{45}Ca and other substances (detailed below). Calcium uptake into synaptosomes was measured in both standard (Na + 5K) salt solution and "stimulating" salt solutions. Stimulating salt solutions contained either 75 mM potassium (High K solution) substituted for an equimolar concentration of sodium, or veratridine (30–80 μM) in Na + 5K solution. After test incubations lasting 10–120 seconds, ^{45}Ca uptake was stopped by diluting the incubation media (0.4 ml volume) with 4.5 ml of ice-cold Na + 5K solution. The diluted suspensions were immediately filtered by suction through Whatman glass fiber filters (GF/C, 2.4 cm diameter). The filters were rapidly washed with two 4.5 ml aliquots of ice-cold Na + 5K. The filters were then suspended in vials containing 10 ml of scintillation cocktail (containing Omnifluor (New England Nuclear), toluene and Triton X-100; cf ref. 13) and the ^{45}Ca content of the material trapped on the filters was determined by liquid scintillation spectrometry. Protein concentrations were determined by the method of Lowry *et al.* (14). The extra uptake of ^{45}Ca (K-stimulated = ΔK, or veratridine stimulated = ΔVer) was calculated as the difference between the uptake from Na + 5K and from the appropriate depolarizing solution. In each experiment Ca uptake determinations were made on 3–5 replicate samples, as indicated in the tables and figure legends. Standard errors for mean stimulated uptake (SEΔ) were calculated as:

$$SE\Delta = \sqrt{[SE(Na + 5K)]^2 + [SE\ (Stim)]^2}$$

where SE (Na + 5K) = standard error of the mean calcium uptake from Na + 5K and SE (Stim) = standard error of the mean uptake from High K or veratridine containing solutions.

Fluorescence measurements. The voltage-sensitive fluorescent dye CC_5 was used to follow changes in membrane potential (15). Aliquots of synaptosomes containing about 0.5 mg of protein, with or without drugs, were added to Na + 5K, or to High K solutions containing CC_5 at a concentration of 2.5 μM. The synaptosome suspension was illuminated with light at a wavelength of 448 nM and fluorescent emission was measured at 511 nm. Further details are given in reference 15.

Electrophysiological recordings at the frog neuromuscular junction. Experiments were performed at room temperature (20°–24°) on the sartorius nerve-muscle preparation of the frog *Rana pipiens*. The preparation was pinned on Sylgard, set in a small petri dish. The usual bathing solution was frog Ringer's of the following composition (mM): NaCl, 116; KCl, 2; $MgCl_2$, 2; $CaCl_2$, 2; and Tris-maleate 5, brought to pH

7.4 with NaOH. Microelectrodes filled with 3 M KCl (10–20 M) were used to record MEPPs from synaptic areas. Signals were amplified via a high impedance electrometer (WP Instruments), and displayed on a Tektronix storage oscilloscope. Signals were measured directly from the oscilloscope screen, or were photographed with cine camera and measured from film.

The muscles were depolarized by slowly perfusing Ringer's solution containing an additional 10 mM K (12 mM K total) into the chamber, until the resting potential stabilized (usually at around −50 mV). Measurements were only made from cells in which the smallest MEPPs discernible in High K were at least two times larger than the baseline noise. At least 50 MEPPs were counted for each determination of MEPP frequency.

Drugs. Verapamil and D-600 were the gifts of Knoll Pharmaceutical Co. Nifedipine was the gift of Delbay Pharmaceuticals. Veratridine was purchased from Aldrich Chemical Co.

RESULTS

Verapamil, D-600 and nifedipine are ineffective blockers of K-stimulated Ca uptake by synaptosomes. K-stimulated Ca uptake is mediated by voltage-sensitive Ca channels in synaptosomes (5). This uptake, which is blocked by Mg^{2+}, Mn^{2+} and La^{3+}, appears to trigger the release of neurotransmitters (5). A verapamil concentration of approximately 100 μmoles/l was required to block this uptake by two-thirds (Fig. 1, lower curve). This concentration is about 25 times greater than that needed to achieve a similar reduction of the inward Ca current in mammalian cardiac fibers (16). D-600 was no more effective than verapamil in reducing ^{45}Ca influx into synaptosomes (Table 1, Exp. A, B_2) although it is much more potent than verapamil in its action on cardiac (16) and smooth muscle (2). Neither D-600 nor verapamil affected significantly the ^{45}Ca uptake from control (Na + 5K) media.

Whereas in cardiac muscle the effects of these drugs develop slowly, over a period of 40 to 80 min (17), we found that the effects of both drugs on synaptosomes did not depend on the duration of the preincubation period with the drugs (Table 1, Exp. A). Also, as illustrated by experiment B in Table 1, drug effectiveness did not depend on the duration of the test-incubation period, when this was varied between 10 and 120 sec. Furthermore, the percent inhibition was either not changed, or even slightly reduced, when the external Ca concentration was lowered (Table 1, Exp. B). The latter finding suggests that D-600 is not a competitive antagonist of Ca influx.

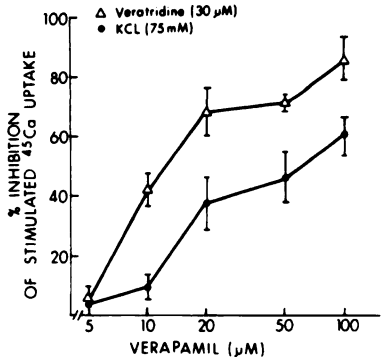

FIG. 1. *Effect of verapamil on ^{45}Ca uptake by synaptosomes*

The lower curve (●) shows the inhibitory effect of the drug on Ca uptake stimulated by incubating the synaptosomes with 30 μM veratridine. The upper curve (Δ) was obtained when uptake was stimulated by high external potassium (75 mM). Points in the lower curve represent the means ± SEM from 3–4 experiments, while points in the upper curve represent the means and the range from two experiments. In each experiment Ca uptake determinations were made on triplicate samples. Test incubations with ^{45}Ca lasted two minutes. All solutions contained 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$. Ca uptake from control solutions (Na + 5K ± verapamil) averaged 0.67 ± 0.08 nmoles/mg protein per 2 min. High-K stimulated Ca uptake 3–4 fold, while veratridine stimulated Ca uptake 2–3 fold in the absence of verapamil.

Block of ^{45}Ca uptake by verapamil did not appear to depend on the extent of synaptosome depolarization (i.e., on the concentration of K in the incubation solution). As indicated by the data in Fig. 2, the ratio of K-stimulated Ca uptake in the presence of verapamil (80 μM) to K-stimulated uptake in the absence of drug remained constant whether the test incubation media contained 20, 30, 50, or 75 mM KCl.

A third organic "Ca antagonist," nifedi-

TABLE 1

Effect of organic "Ca antagonists" on K-stimulated Ca uptake by synaptosomes

Synaptosomes were preincubated with or without drug in Na + 5 K solution, and then incubated with or without drug, with ^{45}Ca, in 5 mM or 75 mM K solution (See Fig. 1 legend). Stimulated uptake, ΔK, was then calculated as the difference between Ca uptake from 75 mM K media and 5 mM K media. Percent inhibition was calculated as:

$$\text{Percent inhibition} = \left[1 - \frac{\Delta K\ (\text{Drug})}{\Delta K\ (\text{Control})}\right] \times 100\%$$

Average uptake values were obtained from triplicate samplings.

Experiment	$[Ca^{2+}]$	Preincubation	Incubation	Ca^{2+} Uptake (nmoles/mg protein)			Inhibition
				Na + 5 mM K	75 mM K	ΔK	
	(mM)	*(min)*	*(sec)*				*(%)*
A Control	1.2	2	120	1.16 ± 0.03	3.50 ± 0.35	2.34 ± 0.35	
VPL 50 μM		2		1.15 ± 0.23	2.36 ± 0.19	1.21 ± 0.30	48
Control		45		1.00 ± 0.08	3.21 ± 0.29	2.21 ± 0.30	
VPL 50 μM		45		0.86 ± 0.15	1.87 ± 0.39	1.01 ± 0.42	54
B_1 Control	0.02	30	10	0.08 ± 0.01	0.38 ± 0.05	0.30 ± 0.05	
D-600 50 μM			10	0.07 ± 0.01	0.26 ± 0.02	0.19 ± 0.02	37
Control			120	0.18 ± 0.01	0.61 ± 0.04	0.43 ± 0.05	
D-600 50 μM			120	0.15 ± 0.01	0.45 ± 0.06	0.30 ± 0.07	30
B_2 Control	1.2	30	10	1.01 ± 0.07	4.59 ± 0.14	3.58 ± 0.18	
D-600 50 μM			10	1.12 ± 0.02	2.93 ± 0.32	1.81 ± 0.31	48
Control			120	2.28 ± 0.16	8.33 ± 0.92	6.05 ± 0.91	
D-600 50 μM			120	1.92 ± 0.15	5.26 ± 0.95	3.34 ± 0.96	45
C Control	0.06	30	15	0.38 ± 0.03	1.55 ± 0.06	1.17 ± 0.07	
Nifedipine 30 μM				0.33 ± 0.08	1.45 ± 0.07	1.12 ± 0.11	4

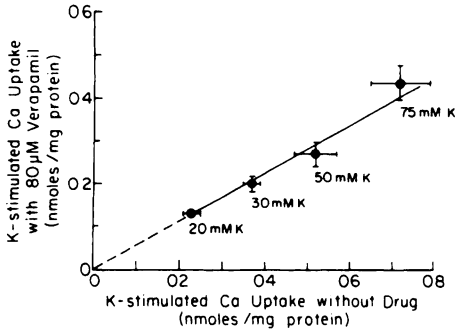

FIG. 2. *The effect of varying potassium concentrations on the inhibition of K-stimulated calcium uptake by verapamil*

The ordinate indicates K-stimulated Ca uptake in the presence of 80 μM verapamil; the abscissa indicates uptake in the absence of verapamil. The line was drawn by eye. The number beside each data point is the concentration of potassium (mM) in the external medium; all solutions contained 20 μM Ca and 1 mM Mg. Error bars indicate the standard errors of the means for quadruplicate or quintuplicate determinations. Incubation in the presence of ^{45}Ca was carried out for 10 seconds at 30° in this experiment.

pine, which in heart and smooth muscle (2) is more potent than D-600, did not block stimulated ^{45}Ca uptake into synaptosomes at concentrations of up to 30 μM (Table 1, Exp. C). In sum, our results indicate that the Ca channels in synaptosomes are relatively insensitive to the organic "Ca antagonists" verapamil, D-600, and nifedipine.

Verapamil and D-600 do not reduce K-stimulated transmitter release at the frog neuromuscular junction. In the frog neuromuscular junction, MEPP frequency is very sensitive to changes in the extracellular Ca when the muscle fibers are depolarized by potassium (18). Presumably, voltage-sensitive Ca channels are opened by depolarization (3), permitting Ca to enter the terminals and enhance spontaneous transmitter release (19). We attempted to modify this frequency increase by inhibiting Ca entry with the organic "Ca antagonists," verapamil and D-600, and also with Mn^{2+}, a known blocker of Ca channels (1). As can be seen in Fig. 3, when the potassium

concentration was increased from 2 to 12 mM, with 2 mM $CaCl_2$ present in the external solution, there was a significant increase in the MEPP frequency. Mn^{2+} reduced the MEPP frequency increase by more than 50%. Neither 50 μM verapamil (Fig. 3) nor 10 μM D-600 (data not shown) reduced MEPP frequency significantly in depolarized preparations (8 cells). Higher concentrations of verapamil were not tested in depolarized preparations because they reduced MEPP amplitude and increased the resting MEPP frequency when added to the normal (2 mM K) Ringer's solution. Our observations on the effects of the organic "Ca antagonists" at the neuromuscular junction are similar to those of Gotgilf and Magazanik (8); these findings further support the conclusion that the Ca channels in presynaptic nerve terminals are not as sensitive to the organic "Ca antagonists" as are the Ca channels in myocardial cells.

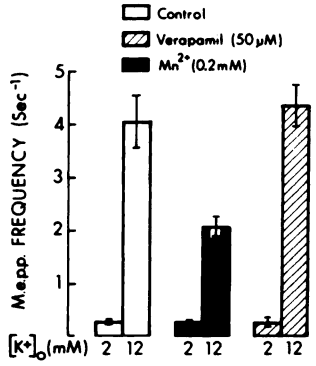

FIG. 3. *The effects of manganese and verapamil on MEPP frequency in depolarized frog neuromuscular junctions*

After each depolarization period with 12 mM K Ringer's solution, the preparation was rinsed with control Ringer's solution [containing (mM): NaCl, 116; KCl, 2; $MgCl_2$, 1.35; $CaCl_2$, 2; and Tris-maleate, 5; brought to pH 7.4 with NaOH] until MEPP frequency returned to within 25% of control value. All data shown in this figure are from a single cell; the bars, from left to right, indicate the order in which the recordings were made. Resting potential was −83 mV in 2 mM K, and −55 mV in 12 mM K solution. Error bars indicate ± SEM. In this and all other experiments where MEPP frequency was measured, the smallest MEPPs discernible in high potassium were at least 2 times larger than the baseline noise. Similar results were obtained with verapamil in 5 other cells, and with manganese in two of these cells.

Verapamil and D-600 block Na channels in synaptosomes. There have been many reports suggesting that high concentrations (200–500 μM) of the organic "Ca antagonists" suppress Na conductance changes in the squid axon (20), and reduce the action potential amplitude in frog sciatic nerve (21) and skeletal muscle (7). In experiments employing the voltage-sensitive fluorescent dye, CC_5, to monitor membrane potential changes (15), we obtained evidence that the organic "Ca antagonists" also block Na channels in synaptosomes.

When dye-treated synaptosomes are depolarized by either veratridine, an alkaloid that opens Na channels (22), or by high concentrations of K, there is an increase in CC_5 fluorescence, and this increase appears to be directly related to the membrane potential (15). The veratridine-induced, but not the K-induced, fluorescence change is blocked by tetrodotoxin, indicating that the former effect is due to the opening of Na channels. As can be seen in Fig. 4, both verapamil and D-600 depressed the veratridine-induced fluorescence increase. In control experiments (not shown) we observed that neither verapamil nor D-600 had any effect on the fluorescence of nondepolarized synaptosomes incubated in Na + 5K solution; also neither drug affected the fluorescence change induced by K-depolarization. Furthermore, block of the veratridine-induced fluorescence by verapamil was independent of the concentration of veratridine used, over a veratridine concentration range of 10–500 μM; thus verapamil does not appear to inhibit fluorescence by simply interfering with the binding of veratridine to synaptosomes.

The results described above suggest that the organic "Ca antagonists" block Na channels at concentrations similar to those required for Ca-channel blockade in synaptosomes. If this were true, one would expect veratridine-induced ^{45}Ca uptake to be inhibited more effectively than K-stimulated ^{45}Ca uptake by the organic "Ca antagonists." Indeed, this is shown in Fig. 1, where the inhibition of veratridine-stimulated (upper curve) and potassium-stimulated (lower curve) Ca uptakes are com-

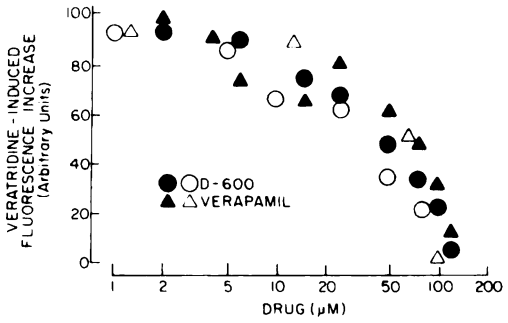

FIG. 4. *Suppression, by verapamil and D-600, of veratridine-induced CC_5 fluorescence changes in synaptosomes*

Results from two experiments are shown (filled versus open symbols). Veratridine-induced fluorescence increase was measured as the increment in synaptosome fluorescence (ΔF) due to the addition of 80 μM veratridine, in the presence [ΔF (Drug)] or absence [ΔF (Control)] of verapamil or D-600, and expressed as:

$$\text{Veratridine-induced fluorescence increase} = \frac{\Delta F\ (\text{Drug})}{\Delta F\ (\text{Control})} \times 100.$$

pared as a function of verapamil concentration. Whereas verapamil inhibits K-stimulated uptake only by blocking the voltage-sensitive Ca channels, it inhibits veratridine-stimulated uptake both by directly blocking the Ca channels, and (as indicated in Fig. 4) by reducing the depolarization (a function of the Na permeability) which opens the Ca channels. It should be noted that in this type of experiment, all of the veratridine-stimulated Ca uptake appears to be mediated by Ca channels, since it is effectively blocked by La^{3+} (data not shown).

DISCUSSION

Pinched-off presynaptic nerve endings retain many of the metabolic, osmotic, and transport properties of intact neural tissue (cf. 9, 10). Previous studies have shown that Ca uptake into synaptosomes is mediated by voltage-sensitive Ca channels, similar to the Ca channels observed in many types of excitable cells (5). We have taken advantage of the synaptosome preparation to examine the effectiveness of the organic "Ca antagonists" in blocking Ca influx into neuronal tissue.

The organic "Ca antagonists" have been found to inhibit a wide variety of membrane-related processes, including muscle end-plate sensitivity to acetylcholine (23), K-conductance (7, 24–26) and Na-stimulated Mg efflux (27). There are also indications that Na channels may be blocked by these drugs (7, 8, 20, 21) and that neuronal Ca channels may be relatively insensitive to them (6–8). Our results provide direct evidence that the organic "Ca antagonists" do not *selectively* block Ca influx into synaptosomes. Rather, these agents, at concentrations of 50–100 μM, seem to inhibit voltage-dependent Na as well as Ca channels. Thus, ionic currents blocked by high concentration of these drugs cannot be equated with "Ca currents." This contrasts with the identification of all TTX-blocked currents as "Na currents" (28).

There are reports that even in the heart, the action of the racemic (±) mixtures of the organic "Ca antagonists" may not be limited to the Ca channels (17, 24, 25). Nevertheless, marked inhibition of the Ca conductance in cardiac muscle is observed with concentrations of these drugs on the order of 10^{-6} to 10^{-5} M (2, 24, 25); in vascular smooth muscle, concentrations below 10^{-7} M may be effective (6). This contrasts with the much higher concentrations (10^{-5}–10^{-3} M) of the "Ca antagonists" needed to block, to a comparable degree, Ca uptake by synaptosomes, and Ca-currents (20) and Ca-dependent membrane processes in neural tissues (6–8). This suggests that neuronal Ca channels may be pharmacologically different from the Ca channels in cardiac and smooth muscle, with respect to receptor sites for "Ca antagonists," just as neuronal Na channels may be pharmacologically different from cardiac Na channels with respect to sensitivity to TTX (29). The differential sensitivity of the Ca channels in nerve and muscle to the organic "Ca antagonists" may have an interesting pharmacological consequence: it may allow verapamil and D-600 to inhibit Ca-dependent processes in myocardial or vascular tissue at doses having virtually no effects on neuronal activity. This may partially explain the usefulness of the organic "Ca-antagonists" as target-specific agents in the treat-

ment of arterial hypertension, hyperkinetic heart function and coronary artery disease (2).

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