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ON THE SPECIFICITY OF VERAPAMIL AS A CALCIUM CHANNEL-BLOCKER

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Abstract—The stimulated uptake of $^{45}Ca^{2+}$ into incubated cerebrocortical synaptosomes caused by veratrine (75 μM) was blocked by low concentrations of verapamil (0.5–30 μM) which did not prevent or reduce depolarization as judged by efflux of potassium (K^+). However, verapamil did not prevent amino acid neutrotransmitter release at these low concentrations and this is discussed in terms of mobilization of internal calcium (Ca^{2+}) stores.

At higher concentrations (30–200 μM) verapamil appeared to act additionally at sodium (Na^+) channels since both depolarization-induced K^+ efflux and neurotransmitter release were reduced or prevented. When K^+, at a high concentration (56 mM), was used as the depolarizing agent, both $^{45}Ca^{2+}$ influx and neurotransmitter release were prevented by verapamil across a wide concentration range (0.5–200 μM). The data are discussed in terms of the specificity of action of verapamil on Ca^{2+} channels.

Calcium enters neurones and muscle cells through voltage-sensitive calcium (Ca^{2+}) channels. These channels are also sensitive to a range of calcium antagonists including verapamil, a phenylalkylamine, and its methoxy derivative, D-600 [1–3]. At low concentrations verapamil appears to block "slow" Ca^{2+} channels [4, 5]. It will prevent calcium entering through cardiac and smooth muscle sarcolemma [6, 7], which accounts for its peripheral and coronary vasodilation effects. However, at higher micromolar concentrations there is evidence that verapamil has a more complex action, including an apparent blockade of sodium (Na^+) channels [8, 9]. Existing evidence for this non-specific action of verapamil at higher concentrations in neural tissue remains circumstantial and is complicated by the lack of a consistent Ca^{2+}-dependence in the transmitter-releasing properties of veratrine [9]. Here we present further evidence for an action of verapamil at higher concentrations on sodium-channels based on a comparison of its effects on the enhanced $^{45}Ca^{2+}$ entry and on amino acid neurotransmitter release evoked by high potassium (K^+) concentrations and veratrine.

MATERIALS AND METHODS

Preparation of synaptosomes. Whole rat cerebral cortex was removed from female Sprague–Dawley rats after exsanguination and placed in ice-cold 0.32 M sucrose. Synaptosomes were prepared by a modified version [10] of the method of Gray and Whittaker [11].

Incubation procedure. Pellets of synaptosomal fractions were resuspended in mammalian Krebs–Ringer solution of composition (mM); NaCl, 138; KCl, 5; $MgSO_4$, 1; Tris–HCl 20; glucose, 10; pH 7.4 to give a protein concentration of 2 mg/ml. The synaptosomes were incubated for 15 min at 37° in a shaking water bath. Appropriate additions were then made; verapamil at the relevant concentration was added, followed by the depolarizing agent after 5 min. Incubation was continued for a further 10 min before sedimentation of the synaptosomes in a bench-top ultracentrifuge at 15,600 *g* for 3 min. The supernatants were retained for amino acid analysis. The pellets were digested with NaOH and samples taken for protein determination and K^+ content as described previously [12].

$^{45}Ca^+$ uptake studies. Incubation was carried out as described above. $^{45}CaCl_2$ (1.2 mM final concentration; sp. act. 0.8 mCi/mmoles) was added together with the depolarizing agent and incubation was continued for 10 min. The suspensions were decanted into polypropylene tubes containing ice-cold EGTA solution of composition (mM): NaCl, 120; KCl, 5; EGTA, 30; $MgCl_2$, 1.2; Tris–HCl 20; NaH_2PO_4, 1.2; pH 7.4 to give a final EGTA concentration of 6 mM. The synaptosomal suspensions were sedimented and the supernatants retained for amino acid analysis. The pellets were then washed twice with 1 ml of K^+-free Na medium of composition (mM); NaCl, 137; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; Tris–HCl 20; glucose, 10; pH 7.4 and once with a similar medium but containing Ca^+ (1.2 mM) and no glucose. The pellets were digested with NaOH and 1.0 ml. aliquots were taken for radioactive counting in a Packard Tricarb scintillation counter. Aliquots were also taken for protein and potassium content determinations.

Amino acid analysis. The supernatants were acidified with 20% formic acid containing an appropriate amount of norleucine and immediately frozen in solid CO_2/propanol. The supernatants were taken to dryness, re-extracted with methanol, freeze-dried and dissolved in 0.025 M HCl. Amino acids were measured with a Chromaspek J1 80 autoanalyser as described previously in detail [12].

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Protein determination. This was by the method of Lowry *et al.* [13] using bovine serum albumin as a standard.

Potassium determination. Potassium was measured by flame photometry as described previously [12].

Sources of materials. Verapamil was kindly donated as a gift by Abbott Laboratories Ltd., Queensborough, Kent, U.K. Veratrine hydrochloride was obtained from Sigma Chemical Company St. Louis, MO.

RESULTS

Verapamil action: veratrine as depolarizing agent

As reported previously [9], verapamil at concentrations above 30 μM prevented the loss of K^+ from synaptosomes induced by veratrine at 25–75 μM. It also reduced the evoked release of neuroactive amino acids (glutamate, aspartate and GABA) and prevented the associated enhancement of $^{45}Ca^{2+}$ entry caused by this depolarizing agent (Table 1). The results for glutamate only are reported in Table 1 but aspartate and GABA were equally affected, with no action being observed in two other non-transmitter amino acids monitored (i.e. threonine and leucine). Verapamil added alone had no action on any of these parameters. This selective release of neurotransmitters and the loss of K^+ induced by veratrine has previously been shown in many studies to be prevented by low concentrations of tetrodotoxin (e.g. 1 μM;), indicating that it is due to depolarization of neurone-derived structures (synaptosomes) with associated activation of voltage-sensitive Na^+ channels.

However, at relatively low concentrations (0.5–30 μM), verapamil blocked $^{45}Ca^{2+}$ entry without preventing release of either K^+ or amino acids (Table 1). Thus its action on evoked amino acid neurotransmitter release was dissociated from its action on $^{45}Ca^{2+}$ fluxes into synaptosomes. In this low concentration range, therefore, the veratrine-induced release of amino acids was not calcium-dependent, whilst at higher verapamil levels (30–50 μM) it appeared to be so dependent.

Verapamil action: potassium as depolarizing agent

When added at levels above 25 mM potassium evokes a calcium-dependent release of neuroactive amino acids from synaptosomes [14]. This response at 56 mM is seen in Table 2. Potassium at this concentration also stimulated $^{45}Ca^{2+}$ uptake by synaptosomes by approximately 73%. Concentrations of verapamil at 0.5 μM and above prevented both the K^+ evoked release of amino acids and the K^+-evoked entry of $^{45}Ca^{2+}$ (Table 2).

DISCUSSION

Many previous studies have shown that Ca^{2+} uptake into incubated synaptosomes occurs through voltage-sensitive calcium channels. Thus depolarizing agents enhance $^{45}Ca^{2+}$ uptake and provide a test system for the potency and mode of action of Ca^{2+}-channel blocking agents. In the present study the Ca^{2+} antagonist, verapamil, was found to have a bimodal action depending on the concentration employed.

Effects of low concentrations of verapamil

At low levels (0.5–30 μM) verapamil prevented the $^{45}Ca^{2+}$ uptake induced by veratrine or by high K^+ concentrations without diminishing the membrane depolarization as judged by efflux of K^+ (Table 1). In this low concentration range the specific release of neuroactive amino acid induced by high K^+ concentrations, but not that induced by veratrine, was also blocked. The dose–response relationships for these two parameters (i.e. a sharp change at 0.5 μM verapamil) were very similar (Table 2) and indicate the Ca^{2+}-dependent nature of this high K^+-induced neurotransmitter release from synaptosomes.

Thus verapamil at low concentrations (< 30 μM) effectively blocks the entry of $^{45}Ca^{2+}$ into synaptosomes but does not prevent neurotransmitter

Table 1. Effects of verapamil on the responses due to veratrine stimulation of synaptosomes

	^{45}Ca uptake (nmoles/mg protein)	K^+ content (μequiv/mg protein)	Glutamate release (nmoles/100 mg protein)
Control	3.73 ± 0.12	29.9 ± 1.0	218 ± 41
Veratrine alone (75 μM)	8.11 ± 0.17	17.0 ± 0.6	1976 ± 116
Verapamil alone (200 μM)	3.82 ± 0.14	29.0 ± 3.0	236 ± 33
Veratrine (75 μM) plus verapamil at the folowing concentrations:			
0.01 μM	7.95 ± 0.12	nm	1899 ± 87
0.10 μM	8.07 ± 0.15	18.0 ± 3.0	1983 ± 134
0.50 μM	3.69 ± 0.14	nm	1936 ± 98
5.0 μM	3.75 ± 0.14	nm	1955 ± 108
10.0 μM	nm	17.7 ± 0.5	nm
30.0 μM	3.77 ± 0.11	24.0 ± 0.7	1704 ± 85
75 μM	3.69 ± 0.13	30.0 ± 2.0	1355 ± 79
100	3.65 ± 0.14	26.2 ± 0.5	964 ± 63
150 μM	3.77 ± 0.11	nm	591 ± 47
200 μM	3.77 ± 0.11	29.6 ± 0.8	380 ± 62
N	12	18	10

Synaptosomes were incubated as described in Methods. Results are mean ±S.E.M. for the number of observations indicated by N. Each group involved at least three synaptosome preparations. n.m. = not measured.

Table 2. Effects of verapamil on responses due to the potassium stimulation of synaptosomes

	$^{45}Ca^{2+}$ uptake (nmoles/mg protein)	Glutamate release (nmoles/100 mg protein)
Control	3.68 ± 0.07	196 ± 36
Potassium alone (56 mM)	6.37 ± 0.13	617 ± 57
Potassium (56 mM) plus Verapamil at the following concentrations:		
0.01 μM	6.51 ± 0.12	nm
0.05 μM	nm	590 ± 46
0.10 μM	6.40 ± 0.14	nm
0.50 μM	3.73 ± 0.14	284 ± 32
1.0 μM	nm	261 ± 48
5.0 μM	3.78 ± 0.13	nm
10.0 μM	3.66 ± 0.15	257 ± 36
30.0 μM	3.63 ± 0.12	234 ± 26
200.0 μM	3.73 ± 0.10	287 ± 54
N		10

Synaptosomes were incubated as described in Methods. Potassium (56 mM final concentration) was added as KCl.

The data are mean ±S.E.M. for the number of observations indicated by N.

At least three synaptosome preparations were employed. n.m. = not measured.

release. This lack of effect of low concentrations of verapamil on the amino acid release induced by veratrine, could be explained if the rapid and large Na^+ entry induced by veratrine allowed internal Na^+ levels to rise to a level which released Ca^{2+} from internal stores (e.g. mitochondria and endoplasmic reticulum) by an exchange process. Good evidence exists in suport of this [15, 16]. The many conflicting reports on whether veratrine-induced neurotransmitter release is Ca^{2+}-dependent could be explained by this phenomenon of Ca^{2+} release from endogenous stores (see ref. 9). Similar considerations apply to other agents which activate sodium channels such as Tityustoxin, a Scorpion venom toxin [17, 18], which causes substantial and rapid entry of Na^+.

Effects of high concentrations of verapamil

At concentrations above 30 μM, verapamil blocked not only $^{45}Ca^{2+}$ uptake induced by veratrine but also amino acid and K^+ release (Table 1). The latter indicates that depolarization is being prevented by the Ca^{2+}-channel blocking agent. This is supported by the inhibition of the respiratory response to added veratrine by this concentration range of the drug [9]. The most likely mode of action of verapamil under these conditions (i.e. >30 μM) is through blockade of active Na^+ channels. Evidence for an action of verapamil and related Ca^{2+}-channel inhibitors on Na^+ channels is now quite substantial [8, 19, 20] and contrasts with the more specific actions of the dihydropyridine class of calcium antagonists (e.g. Nifedipine and Nitrendipine) [3]. The Na^+-channel interaction of verapamil may be due to the (+) enantiomer which appears to have a substantial blocking action on the fast Na^+ channel of cardiac muscle. The (−) enantiomer principally affects the slow Ca^{2+} channel [4, 5, 21].

Kohlhardt *et al.* [22] found that 8.8 mM Ca^{2+} would reverse the inhibitory action of 5 μM verapamil on Ca^{2+} conductance across myocardial fibres indicating that the drug is interacting with a Ca^{2+}-binding site. In this respect it is surprising that verapamil and its methoxy derivative gallopamil (D-600) are thought to act on the inside of cardiac membranes after entering the cell in the unchanged form [3]. Thus these agents block Ca^{2+} only after the calcium channels are open and the degree of blockade increases with the frequency of stimulation and membrane depolarization. It is noteable, therefore, that it is a consistent finding that these drugs have no detectable action on Ca^{2+} fluxes in control unstimulated synaptosomes (Tables 1 and 2).

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