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DIHYDROPYRIDINE SENSITIVE CALCIUM CHANNELS IN A SMOOTH MUSCLE CELL LINE[1]

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The pharmacological properties of voltage sensitive calcium channels (VSCC) were examined in a rat aortic smooth muscle cell line (A10). The inorganic VSCC blockers Co^{2+} and Cd^{2+} blocked $^{45}Ca^{2+}$ uptake into these cells in both 5 mM K^+ and 50 mM K^+ (depolarizing) conditions. The organic VSCC antagonists nitrendipine, nimodipine, D-600 and diltiazem also blocked $^{45}Ca^{2+}$ uptake at low concentrations. The relative potencies of blockade were similar to those found in intact vascular smooth muscle. The VSCC "agonist" BAY K8644 enhanced $^{45}Ca^{2+}$ uptake and this effect could be reversed by nitrendipine. These results indicate that A10 cells possess VSCC and that these VSCC behave similarly to those in authentic smooth muscle.

Voltage-sensitive calcium channels (VSCCs) play an important role in excitation-contraction coupling in smooth muscles. Depolarization of smooth muscle leads to the opening of VSCCs allowing entry of calcium from the extracellular fluid. This influx of calcium initiates smooth muscle contraction (1).

Various substances have been found to block VSCCs (and hence contraction) in smooth muscles. Inorganic cations such as Co^{2+} and Cd^{2+} appear to block by competing with Ca^{2+} for entry through the channel (2,3). Organic VSCC antagonists block by binding to various sites on the channel and subsequently block calcium entry

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Abbreviations: VSCC, voltage-sensitive calcium channel; Hepes, N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid; EGTA, Ethyleneglycol bis (β-aminoethyl ether)-N,N,N',N',-tetraacetic acid.

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following alterations in channel gating (4-6). Examples of such organic compounds are the dihydropyridines such as nifedipine, the phenylalkylamines verapamil and D-600, and the benzothiazepine diltiazem. These compounds are used clinically to treat angina and hypertension (for reviews see 7,8,9).

More recently, a novel dihydropyridine "agonist", BAY K8644, has been developed which increases the time that VSCCs remain open (10) and consequently enhances contraction of smooth and cardiac muscles (11,12).

In the present study, we examine the influence of calcium agonist and antagonist drugs on $^{45}Ca^{2+}$ uptake in a rat aortic smooth muscle derived cell line, A10 (13). We demonstrate that this cell line contains VSCCs that behave like those known to exist in authentic smooth muscle.

MATERIALS AND METHODS

Drugs. Diltiazem was obtained from Marion Laboratories (Kansas City, MO). D-600 was from Knoll AG (Ludwigshafen, FRG). Nitrendipine, the two stereoisomers of nimodipine and BAY K8644 were kindly provided by Miles Laboratories (New Haven, CT). All other drugs and chemicals were from commercial sources.

Cell Culture. Rat aortic smooth muscle cells, A10, were routinely grown on 100 mm plastic tissue culture dishes in Eagle's Minimal Essential Medium supplemented with 10% fetal bovine serum (KC Biologicals) and 2 mM glutamine (KC Biologicals) in 10% CO_2. Cells were split by treating with 0.25% trypsin (GIBCO) and were subcultured onto 60 mm dishes (Falcon) for $^{45}Ca^{2+}$ flux assays. Cells were used for assays when 80-90% confluent.

$^{45}Ca^{2+}$ Uptake Assays. Assays were conducted in a shaking water bath adapted to hold 60 mm plates at 37°C (14). Assays were begun by aspiration of the growing medium and addition of a Hepes-buffered assay medium (135.7 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 2.62 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.81 mM $MgSO_4$, 5.6 mM glucose, 20 mM Hepes and 1 μCi/ml $^{45}CaCl_2$ (Amersham) pH 7.4). When drugs were used, they were added 5 min before and were present throughout the assay. Cells were depolarized using a 50 mM K^+ medium (composition: same as above except 91.5 mM NaCl, 50 mM KCl). Assays were terminated at the appropriate time by aspiration of the medium and four rapid rinses (total time approx. 15 sec) in an ice cold choline chloride (175 mM) and EGTA (2 mM) solution. Plates were allowed to air dry and were extracted with 5 ml of 0.2% sodium dodecyl sulfate. Samples were taken for estimation of $^{45}Ca^{2+}$ taken up and for a fluorimetric

protein assay (15) using bovine serum albumin as standard. Results were calculated as cpm $^{45}Ca^{2+}$ taken up per mg protein.

RESULTS

We examined $^{45}Ca^{2+}$ fluxes in cultured A10 cells. Fig. 1 illustrates some typical uptake curves. $^{45}Ca^{2+}$ uptake was more rapid and reached greater levels in 50 mM K^+ (depolarizing conditions) than in 5 mM K^+ (resting conditions). The magnitude of this increase varied somewhat from culture to culture ranging from a stimulation of about 30% (Fig. 1) to greater than 150% (Fig. 2). In each case, however, organic antagonists of voltage sensitive calcium channels such as nitrendipine reversed the effects of 50 mM K^+ and depressed $^{45}Ca^{2+}$ uptake to well below resting (5 mM K^+) levels (Figs. 1 and 2). Tetrodotoxin (5×10^{-6} M) had no effect on $^{45}Ca^{2+}$ uptake in 5 or 50 mM K^+. The most likely explanation for such observations is that A10 cells contain VSCCs a portion of which are open in 5 mM K^+ due to spontaneous activity in these cells (13). Thus, $^{45}Ca^{2+}$ uptake even in 5 mM K^+ is at least partially due to influx through VSCC. Any VSCC not open at 5 mM K^+ are opened when a further depolarizing stimulus (50 mM K^+) is used.

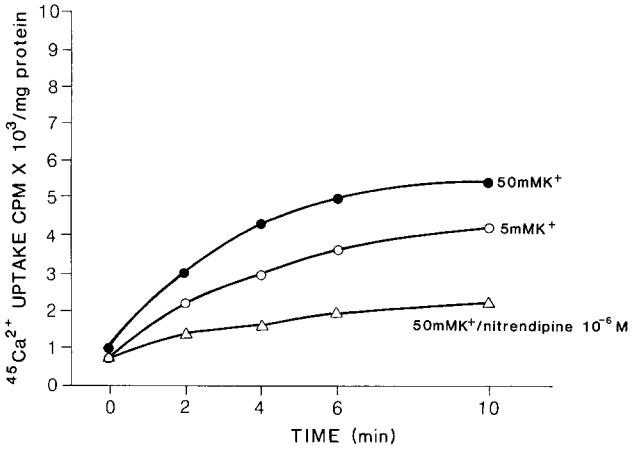

FIG. 1. Curves for $^{45}Ca^{2+}$ uptake into A10 cells. Each point is the mean of duplicate determinations. The experiment has been repeated 8 times with similar results.

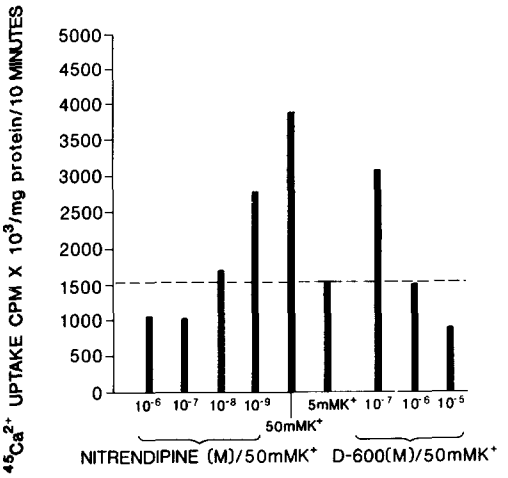

FIG. 2. Effects of the organic calcium channel antagonists nitrendipine and D-600 on $^{45}Ca^{2+}$ uptake into A10 cells. Dotted line shows level of uptake in 5 mM K^+. (n=4).

The hypothesis that $^{45}Ca^{2+}$ uptake in 5 mM K^+ is partially via VSCC is further supported by examining the effects of the inorganic VSCC antagonists Co^{2+} and Cd^{2+} (Fig. 3). It can be seen that both of these agents reduce $^{45}Ca^{2+}$ uptake in 5 mM K^+ to about the same extent as nitrendipine (10^{-6} M). Co^{2+} and Cd^{2+}

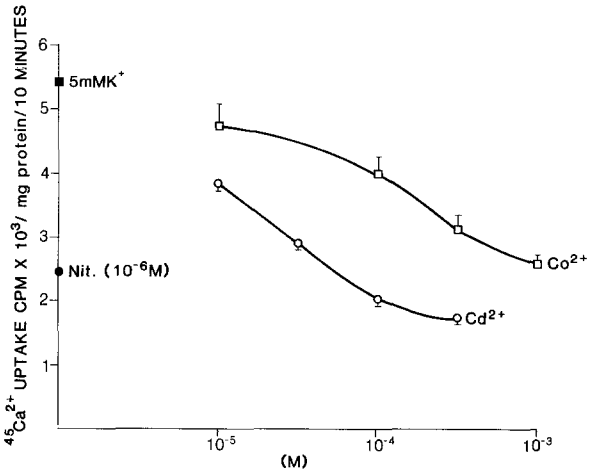

FIG. 3. Effects of the inorganic calcium channel blockers Cd^{2+} and Co^{2+} on $^{45}Ca^{2+}$ uptake into A10 cells. Incubation media contained 5 mM K^+. (■) Uptake in the presence of no blocker, 5 mM K^+. (●) Uptake in the presence of nitrendipine (10^{-6} M). Points are means ± (S.E.M.) of quadruplicate determinations.

also reversed the increase in $^{45}Ca^{2+}$ uptake observed in 50 mM K^+.

These results indicate that A10 cells possess VSCC that are sensitive to organic and inorganic VSCC blockers as with authentic smooth muscle. This hypothesis is further confirmed by observing the effects of a variety of organic VSCC blockers. It can be seen (Figs. 2 and 4) that in addition to nitrendipine, nimodipine, D-600 and diltiazem also inhibited $^{45}Ca^{2+}$ uptake. These effects occurred at very low concentrations. Moreover, the effects of nimodipine exhibited the correct stereospecificity. Nitrendipine was the most potent blocker (IC_{50} = 1.1 nM), the IC_{50}'s for D-600 and for diltiazem were 0.15 μm and 1.2 μM respectively. The relative potencies for these organic VSCC antagonists agree well with previously reported results for the inhibition of depolarization induced smooth muscle contraction and [^{3}H]-nitrendipine binding (16).

It has recently been shown that the novel dihydropyridine BAY K8644 increases $^{45}Ca^{2+}$ uptake via VSCC in cultured NG108-15 cells (17). Indeed this agent contracts smooth muscle and has a positive inotropic effect (11,12). Electrophysiological data has

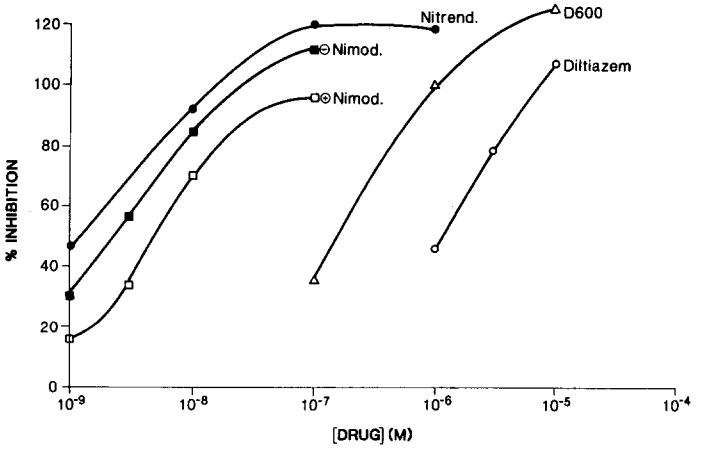

FIG. 4 Inhibition curves for the blockade of $^{45}Ca^{2+}$ uptake into A10 cells. Incubations contained 50 mM K^+. Inhibition > 100% indicates blockade of $^{45}Ca^{2+}$ uptake below that obtained in 5 mM K^+. Points are means of quadruplicate determinations.

TABLE I

Effects of BAY K8644 on $^{45}Ca^{2+}$ Uptake in A10 Cells

	$^{45}Ca^{2+}$ uptake cpm/mg protein/15 minutes
5 mM K^+	3165
50 mM K^+	4180
50 mM K^+ + Nitrendipine (10^{-6} M)	2020
50 mM K^+ + BAY K8644 (10^{-6} M)	5932
5 mM K^+ + BAY K8644 (10^{-6} M)	4630
50 mM K^+ + BAY K8644 (10^{-6} M) + Nitrendipine (10^{-6} M)	1990

Results are means of duplicate cultures. The experiment was repeated three times with similar results.

illustrated the ability of this compound to stabilize VSCC in a state where they open for increased periods of time allowing increased Ca^{2+} entry (10). In the case of NG108-15 cells, the effects of BAY K8644 on $^{45}Ca^{2+}$ uptake are only manifest in the presence of 50 mM K^+ and are not observed at 5 mM K^+. However, in the present case, BAY K8644 stimulated $^{45}Ca^{2+}$ uptake in both 50 mM K^+ and 5 mM K^+ (Table I), further indicating that with A10 cells, some VSCCs are open even in 5 mM K^+. The effects of BAY K8644 were completely reversed by 10^{-6} M nitrendipine.

DISCUSSION

A10 cells were originally derived from the thoracic aorta of embryonic rats. These cells have several ultrastructural features that are reminiscent of smooth muscle. Moreover, their content of creatine phosphokinase is also typical of muscle. A10 cells fire spontaneous action potentials and neighboring cells are electronically tightly coupled to one another (13). It should be noted that in rabbit aorta at any rate, cells are also found

to be electrically coupled; however, spontaneous action potentials are not seen (18). Thus, A10 cells do not exactly mimic the properties of authentic aortic smooth muscle in all respects.

The data presented here illustrates that A10 cells do possess one of the key properties of smooth muscle, that is the presence of dihydropyridine sensitive calcium channels. $^{45}Ca^{2+}$ uptake into A10 cells could be increased by a depolarizing stimulus (50 mM K^+). The degree to which this stimulation occurred differed somewhat from culture to culture. This may reflect the degree of spontaneous activity found with cells at different stages of growth.

It is clear that both the 50 mM K^+-induced $^{45}Ca^{2+}$ uptake and a considerable portion of the uptake seen in 5 mM K^+ is blocked by both organic and inorganic calcium channel blockers. The pharmacological specificity of this blockade is typical of drug interaction with voltage sensitive calcium channels. Indeed the block seen with nimodipine exhibited the correct stereospecificity. In addition, the potency of the dihydropyridine calcium channel blockers is typical of their effects in smooth muscle as opposed to heart for example, where they are considerably less potent (19,20). The current view as to the mode of action of dihydropyridine blockers is that these compounds bind to and stabilize an inactivated non-conducting conformation of the channel (21,22,10). Presumably under the experimental conditions used here, dihydropyridines can bind to inactivated channels that are generated during spontaneous cellular activity. Other non-dihydropyridine calcium channel blockers such as D-600 have a similar mode of action (23) and again they are potent blockers of calcium channels in smooth muscle.

The recently described dihydropyridine BAY K8644 contracts smooth muscle (11) and increases calcium currents in a variety of

cells (17,24,10). This appears to be due to stabilization of a conformation of the calcium channel which remains open for longer periods of time (10). In several of these situations, the effects of BAY K8644 are only manifest in the presence of a depolarizing stimulus, e.g. a high K^+ concentration (17,11,12). In the present case, however, BAY K8644 was able to enhance $^{45}Ca^{2+}$ uptake even in 5 mM K^+. This presumably reflects the opening of calcium channels in these cells during spontaneous activity.

Clearly therefore, A10 cells contain voltage sensitive calcium channels which behave like those in authentic smooth muscle by several pharmacological criteria. These cells should provide a useful model for the further analysis of calcium channel/drug interactions in smooth muscle.

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