Vol. 130, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1985 Pages 447-453

A smooth muscle cell line suitable for the study of voltage sensitive calcium channels

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Received June 7, 1985

A cell line originating from the fetal rat aorta has been studied with respect to $^{45}Ca^{2+}$ uptake. Kinetic experiments showed an initial rapid uptake followed by a slow linear phase; both the initial rate and the maximum uptake were increased in the presence of 55 mM potassium chloride. The calcium channel antagonists, darodipine (PY 108-068) and verapamil, inhibited both the basal and the potassium chloride stimulated uptake. Neither tetrodotoxin nor furosemide affected either basal or depolarisation induced $^{45}Ca^{2+}$ uptake. Blockade of the Na^+/K^+ ATPase by ouabain and of the Ca^{2+} ATPase by vanadate caused a net increase in cellular $^{45}Ca^{2+}$ accumulation.

Vascular smooth muscle cells are the principal cells responsible for regulation of vascular tone. An augmentation of intracellular calcium causes activation of the myosin ATPase and contraction. One of the major pathways leading to enhanced levels of cytoplasmic calcium is the voltage sensitive calcium channel (VSCC) which is present in excitable cells (1). Mechanisms controlling the gating of this channel can be studied directly by electrophysiological means, especially by the patch clamp technique (2,3).

Measurement of $^{45}Ca^{2+}$ uptake and content provides quantitative insight into the modulation of intracellular calcium. For smooth muscle, this has been extensively studied with tissue preparations such as rabbit aortic rings and taenia coli (4,5). However, measurements in whole tissue are complicated by the presence of extracellular matrix and of other cell types. The advantage of

0006-291X/85 $1.50

cultured cells is that a relatively homogeneous population can be studied directly.

This study describes a stable smooth muscle cell line. This cell line was derived from the thoracic aorta of the fetal rat and has many of the characteristics of smooth muscle cells (6). Ultrastructural analysis indicates the presence of a well developed rough endoplasmic reticulum and dispersed filaments. A muscle type creatine phosphokinase isoenzyme is also present (6). A recent report provides evidence for the existence of a $Na^+/K^+/Cl^-$ cotransport system which is insensitive to ouabain and amiloride but sensitive to the loop diuretic bumetanide (7).

The effect of calcium channel blockers on basal and KCl induced $^{45}Ca^{2+}$ uptake was examined. Furthermore, the effect of inhibiting the Na^+/K^+ and the Ca^{2+} ATPase on cellular calcium accumulation has also been studied.

METHODS

Cell culture. The A_7r_5 cell line was obtained from the American Type Tissue Culture Collection. Cells were plated at a density of 7.000 cells/well (16 mm diameter, Nunc) in Dulbecco modified Eagles medium containing 7.5% fetal calf serum and were kept at 37°C in a humidified atmosphere under 5% CO_2/95% O_2. At confluency (after 4-5 days) each well contained a monolayer of 30.000 to 50.000 cells (0.06 to 0.10 mg of protein). Cell quality was checked by phase contrast microscopy and for the absence of mycoplasm (Mycotrim TC, Hana Biologics). About 15 passages of a single batch of cells were possible without morphological changes. Hepes buffered physiological salt solution (HBPSS) consisted of: NaCl 145 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 0.1 mM, glucose 10 mM, Hepes 5 mM, pH 7.4. The depolarising buffer was HBPSS but with 95 mM NaCl and 55 mM KCl. Darodipine (8) was prepared by Sandoz. (±)Verapamil was a gift of Knoll.

Calcium uptake. The experiments were carried out on confluent monolayers of cells (5-8 days in culture) at 37°C. Cells were preincubated for 15 min with HBPSS containing no $CaCl_2$ but 0.1 mM EGTA to remove extracellular Ca^{2+} (9). Uptake measurements were performed in HBPSS or 55 mM KCl buffer (0.2 ml per well) containing 0.25 µCi of $^{45}CaCl_2$. The cells were washed 4 times with 1 ml of ice-cold HBPSS and were solubilized with 0.5 ml of 0.1% sodium dodecyl sulfate. Radioactivity was measured by scintillation counting and protein was determined with bovine serum albumin as a standard (10).

RESULTS

The time course of accumulation of Ca^{2+} by the cells was followed under various conditions. With the present experimental protocol differences in cellular Ca^{2+} content between control and stimulated values were seen after a few seconds. Under resting conditions, an initial rapid increase of $^{45}Ca^{2+}$ uptake was observed followed by a slower linear phase (Fig. 1). Exposure to 55 mM KCl led to an increase in the rate of initial uptake and to a 2-3 fold increase in the maximal accumulation. If cells were not pretreated with EGTA, a gain in $^{45}Ca^{2+}$ content (difference between control and 55 mM KCl) was observed only after 2 to 4 minutes. The dihydropyridine darodipine (1 μM) blocked both basal and depolarisation induced increase in Ca^{2+} content; only a very slow linear rise in content was observed, similar to the one seen under control conditions (Fig. 1).

Various experimental protocols were used in initial studies to optimise the signal to noise ratios in order to quantitatively evaluate the potencies of organic calcium channel blockers. The

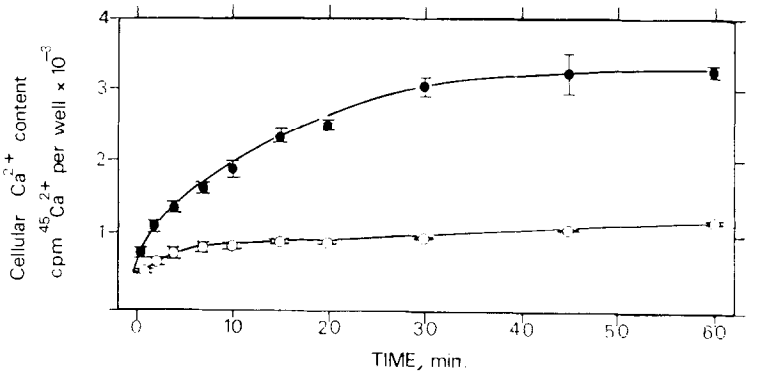

Figure 1: Time course of cellular $^{45}Ca^{2+}$ accumulation. Monolayers of cells were incubated in either HBPSS (-o-) or depolarising buffer, 55 mM KCl (-●-) containing 0.1 mM Ca^{2+} and $^{45}Ca^{2+}$ as tracer for the indicated periods of time. Results are expressed as mean ± sem cpm per well, n = 4.

Table 1

Stimulation and inhibition of $^{45}Ca^{2+}$ uptake by smooth muscle cells: effects of various channel and ATPase blockers. Cellular $^{45}Ca^{2+}$ accumulation was measured at steady state (30 min) as described in Methods. The results shown are the means ± sem of 3 independent experiments each with n = 4.

Inhibitor	5 mM KCl	55 mM KCl
	nmol Ca^{2+} per mg protein	
Control	4.2 ± 0.24	9.7 ± 0.44
Darodipine (10^{-6}M)	1.4 ± 0.11	1.5 ± 0.26
Lanthanum chloride(10^{-3}M)	0.2 ± 0.03	0.3 ± 0.04
Tetrodotoxin (10^{-6}M)	4.1 ± 0.33	10.6 ± 0.35
Furosemide (10^{-6}M)	4.7 ± 0.38	10.0 ± 0.15
Sodium vanadate (10^{-3}M)	9.0 ± 0.31	9.8 ± 0.34
Ouabain (10^{-3}M)	6.9 ± 0.27	10.7 ± 0.38

protocol utilising an EGTA preincubation followed by $^{45}Ca^{2+}$ uptake measurement in 0.1 mM Ca^{2+} (HBPSS) yielded a signal to noise ratio of about 6:1 for blockers (Table 1). Fig. 2 shows the

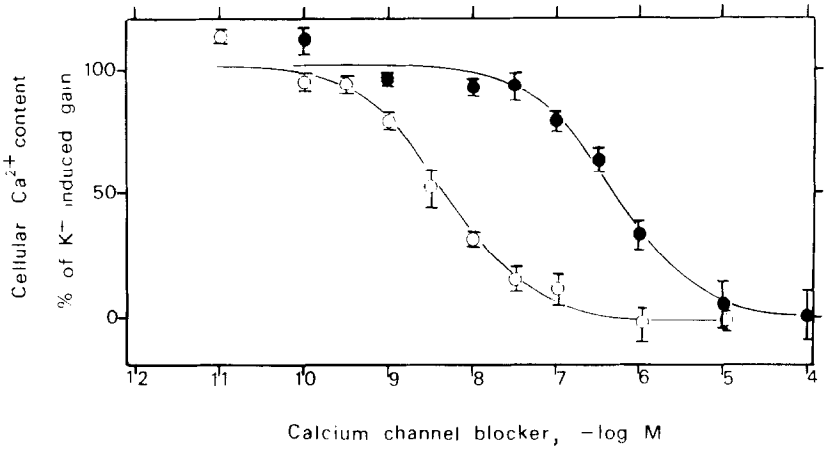

Figure 2: Effect of the calcium channel blockers verapamil (—●—) and darodipine (—○—) on cellular calcium accumulation. Cells were treated for 30 min with 55 mM KCl in HBPSS in the presence of antagonists. 100 % uptake represents the value obtained with 55 mM KCl alone and 0% uptake is the value obtained at maximal inhibition as defined with 1 µM darodipine (see Table 1). Results are expressed as mean ± sem, n = 4.

concentration-response curves for inhibition of KCl induced $^{45}Ca^{2+}$ uptake by verapamil and by the dihydropyridine darodipine. The calcium channel blockers showed an about 100-fold difference in activity and a similar extent of maximal inhibition; if added simultaneously at maximally inhibiting concentrations, no additive inhibitory effect was observed.

Table 1 shows the effect of various channel and ATPase blockers on cellular Ca^{2+} accumulation. Darodipine reduced basal and stimulated content to the same level; lanthanum further reduced this to a level significantly below that of the organic channel blockers. Tetrodotoxin, which blocks potential sensitive Na^+ channels or furosemide, a blocker of the $Na^+/K^+/Cl^-$ cotransport system did not affect the basal or KCl stimulated Ca^{2+} content of these cells. Inhibition of ATPases by vanadate led to a net increase in Ca^{2+} content. Blockade of the Na^+/K^+ ATPase by ouabain increased the basal Ca^{2+} content of unstimulated cells. In the depolarising buffer neither ouabain nor vanadate further increased cellular Ca^{2+} content.

DISCUSSION

Smooth muscle cells are known to contain several types of ionic transport systems responsible for maintaining the intracellular Ca^{2+} concentration. The functioning of these can be studied indirectly by the use of various pharmacological tools.

In this report $^{45}Ca^{2+}$ uptake and its regulation in monolayers of a smooth muscle cell line have been studied. The results shown in Fig. 1 indicate that Ca^{2+} influx into A_7r_5 cells was enhanced by KCl induced depolarisation. Blockade of the calcium channel by the dihydropyridine darodipine or by verapamil significantly reduced influx due to depolarisation (Fig. 2, Table 1). Basal

influx was also blocked by calcium antagonists (Table 1). This could be due to the resting membrane potential, or, more probably be related to the fact that these cells possess spontaneous electrical activity (6).

The pIC_{50} values (the negative logarithm of the IC_{50}) obtained for darodipine (8.5) and verapamil (6.4) were in good agreement with the pD'_2 values for darodipine (8.4) and verapamil (6.4) obtained in the KCl depolarised rabbit aorta (11). Neither tetrodotoxin, a blocker of the sodium channel, nor furosemide, an inhibitor of the $Na^+/K^+/Cl^-$ transport system (7), had any effect on $^{45}Ca^{2+}$ uptake. This indicates that Ca^{2+} does not enter to a significant degree through any of these systems.

Treatment with vanadate, which is a general ATPase inhibitor (12), resulted in an increase in calcium content similar to the increase observed in the presence of 55 mM KCl (Table 1). Inhibition of the Na^+/K^+ ATPase by ouabain increased Ca^{2+} content to a lesser extent. Either of these effects could be due to a decrease in membrane potential caused by blockade of the respective electrogenic ion pump (12,13).

Regulation of $^{45}Ca^{2+}$ uptake by modulating the VSCC has been studied in numerous cell types: heart, neuroblastoma-glioma, pheochromocytoma, and GH_3 cells (9,14-18). With regard to cultured smooth muscle cells, one very recent report has demonstrated the presence of VSCC's in another smooth muscle cell line, A_{10} (19). In agreement with our results on A_7r_5 cells, this line also showed a relatively high basal $^{45}Ca^{2+}$ uptake which was inhibited by calcium channel blockers. The A_7r_5 cells however, which have been in culture for over one year, show a faster growth rate.

The purpose of this communication is to demonstrate that a permanent aortic smooth muscle cell line possesses VSCC's and that these can be modulated by pharmacological tools. KCl depolarisation resulted in an increase in $^{45}Ca^{2+}$ uptake by the cells which was blocked by calcium antagonists. The inhibitory potency of darodipine and verapamil were comparable in these cells and in rabbit aortic rings. Alteration of the cellular Ca^{2+} content was also achieved by inhibition of the Na^{+}/K^{+} and the Ca^{2+} ATPase, presumably through a decrease in membrane potential. This vascular smooth muscle line can be considered as a convenient model for the direct study of the pharmacology of the voltage sensitive calcium channel.

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