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Dihydropyridine-sensitive Ca^{2+} influx modulated by stretch in A7r5 vascular smooth muscle cells

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Abstract

We examined $^{45}Ca^{2+}$ influx in A7r5 vascular smooth muscle cells under cyclical stretch and static conditions and compared the results obtained at resting membrane potential (2.5 mM $[K^+]_o$, $E_m = -58$ mV according to uptake of [^{3}H]tetraphenylphosphonium) with those under depolarizing conditions (70 mM $[K^+]_o$, $E_m = -27$ mV). Application of 10% average strain (24% maximum) in cycles of 3 s on, 3 s off at resting E_m caused a 5-fold increase in Ca^{2+} influx rate to a level similar to depolarized cells and depolarized, stretched cells. 1 μM (+)-isradipine blocked 90% of the stretch- or depolarization-activated Ca^{2+} uptake. When the cells were stretched under Na^+-free conditions, a reduction, not activation, of Ca^{2+} influx rate occurred. Our results suggest that stretching of cultured aortic vascular smooth muscle cells enhances Ca^{2+} uptake through a voltage-dependent, dihydropyridine-sensitive Ca^{2+} entry pathway, whose activation by stretch is dependent upon extracellular Na^+.

Keywords: Stretch-activated channel; Na^+ dependence; Ca^{2+} uptake; Membrane potential; Ca^{2+} channel, L-type

1. Introduction

Vascular smooth muscle is a mechanosensitive tissue which responds to applied stretch in at least three ways: (1) elevation of contractile tone (the myogenic response); (2) alteration of cell proliferation rate; and (3) regulation of cell volume. Many of the cellular events by which a stretch stimulus is transduced to these responses in the vasculature remain to be elucidated. One hypothesis is that stretch activates ion channels present in vascular smooth muscle cell membranes (Meininger and Davis, 1992). According to this hypothesis, stretch opens channels that allow Na^+ and Ca^{2+} ions to enter the cell, which in turn depolarizes the cell and activates voltage-gated Ca^{2+} channels and, in contractile cells, elevates tone. An important issue is whether the bulk of Ca^{2+} entering stretched vascular smooth muscle cells enters via the stretch-activated channels themselves, or via voltage-activated channels, secondary to depolarization caused by stretch-activated channels (mechano-electrical coupling). In vascular smooth muscle cells of the synthetic (noncontractile) phenotype, influx of Ca^{2+} could alter the rate of cell proliferation, since Ca^{2+} channel blockers can inhibit DNA synthesis and proliferation (Sperti and Colucci, 1991). Stretch-activated channels in vascular smooth muscle cells have been linked to cell proliferation (Yang et al., 1993) and cell volume regulation (Bulow and Johansson, 1994).

Both single-channel and whole-cell patch-clamp techniques have demonstrated the presence of stretch-activated channels in vascular smooth muscle (Kirber et al., 1989, 1992; Dopico et al., 1994; Bevan et al., 1990; Davis et al., 1992b), urinary bladder smooth muscle cells (Wellner and Isenberg, 1994), endothelial cells (Lansman et al., 1987), mesangial cells (Craelius et al., 1989), and other cells (Sachs, 1991; French, 1992). Stretch-activated channels are typically nonselective cation channels permeable to Na^+, K^+, and Ca^{2+}, though the cation selectivity varies with cell type.

The purpose of the present study was to determine the effect of cyclical stretch on $^{45}Ca^{2+}$ entry in A7r5 cells, which are a model for the synthetic phenotype of vascular smooth muscle cells (Kimes and Brandt, 1976). We have previously reported that L-type Ca^{2+} channels in this cell line are modulated as a function of cell

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proliferation (Ruiz-Velasco et al., 1994a), probably via changes in the resting membrane potential. This is the first study to examine the effects of stretch on voltage-dependent $^{45}Ca^{2+}$ entry in vascular smooth muscle cells under conditions that simulate the dynamic conditions of mechanical load in vivo. Portions of these data have been presented elsewhere (Ruiz-Velasco et al., 1994b).

2. Materials and methods

2.1. Growth conditions

A7r5 cells, obtained from the American Type Culture Collection (Bethesda, MD), were grown in 75 cm^2 flasks in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (GIBCO), 50 μg/ml streptomycin (Mediatech, Washington, D.C.), and 50 units/ml penicillin (Mediatech) as described (Ruiz-Velasco et al., 1994a). The culture medium was replaced every 48 h. For $^{45}Ca^{2+}$ uptake determinations the cells were seeded at a density of 5000/cm^2 in 25 mm wells containing a type I collagen-coated silastic substratum (Flexcell International Corp., McKeesport, PA). All assays were performed at room temperature (20–22°C) with 10-day old cultures, whereby confluency had been reached at approximately 5 days after seeding.

2.2. $^{45}Ca^{2+}$ uptake measurements

Prior to each assay the cells were rinsed twice with 1 ml/well of Hepes Buffered Saline Solution (HBSS) containing in mM: 127.5 NaCl, 2.5 KCl, 1.5 $MgCl_2$, 10 glucose, and 25 Na-Hepes (25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, titrated with NaOH), pH 7.4. The HBSS was removed and the cells were preincubated for 10 min in 0.5 ml/well of Low K^+ Ca^{2+} Uptake Solution (LKUS) containing in mM: 65 NaCl, 2.5 KCl, 67.5 *N*-methyl-D-glucamine Cl (NMGCl), 1 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 10 Na-Hepes, and 0.2 mg/ml bovine serum albumin, pH 7.4. Following the preincubation period the solution was aspirated and 0.5 ml of either LKUS or High K^+ Uptake Solution (HKUS; same as LKUS but with 70 mM KCl and no NMGCl) containing $^{45}CaCl_2$ (2 μCi/ml; New England Nuclear, Wilmington, DE) was added to each well. In some assays 1 μM of the dihydropyridine Ca^{2+} channel blocker (+)-isradipine (Sandoz Research Institute, East Hanover, NJ) was added to the preincubation and incubation solutions. Throughout the preincubation and incubation periods the plates were mounted in the Flexercell Strain Unit (Model FX2000, Flexcell Int. Corp.) and the silastic surface was stretched and relaxed in a 6 s cycle (3 s stretched, 3 s relaxed) using a vacuum level that achieved 10% average and 24% maximum surface elongation (Banes et al., 1990). Static experiments utilized culture plates equipped with an identical substratum but made with an inflexible polystyrene bottom, and were not mounted in the instrument during the assay. After each respective elapsed time, the cells were washed 4 times with 1 ml/well of an ice-cold Wash Solution (in mM: 65 NaCl, 2.5 KCl, 1 $MgCl_2$, 67.5 NMGCl, 5 glucose, 10 Na-Hepes, 0.2 EGTA, and 0.2 mg/ml bovine serum albumin, pH 7.4). The cells from each well were then dissolved in 0.5 ml of HBSS +0.1% Triton-X-100 overnight at 22°C, which was transferred to vials together with 0.5 ml H_2O wash and 5 ml scintillation fluid, and counted.

Table 1
Effect of stretch on initial rate and 10 min Ca^{2+} uptake in A7r5 cells

$[K^+]_o$ (mM)	$[Na^+]_o$ (mM)	Isradipine (1 μM)	Ca^{2+} uptake	Condition	
				Nonstretched	Stretched
2.5	70	−	Initial rate [a]	0.14	0.76
		−	10 min uptake [b]	0.72 ± 0.49 (12)	2.76 ± 1.08 (12)
		+	Initial rate [a]	0.092	0.082
		+	10 min uptake [b]	1.28 ± 0.53 (6)	0.63 ± 0.20 (6)
2.5	0	−	Initial rate [a]	0.07	0.007
		−	10 min uptake [b]	0.40 ± 0.26 (6)	0.05 ± 0.13 (6)
70	70	−	Initial rate [a]	0.44	0.63
		−	10 min uptake [b]	2.38 ± 0.80 (12)	2.76 ± 0.51 (12)
		+	Initial rate [a]	0.09	0.068
		+	10 min uptake [b]	1.25 ± 0.31 (6)	1.08 ± 0.81 (6)

[a] Ca^{2+} uptake rates are expressed as nmol Ca^{2+}/(10^6 cells × min). The initial rate was determined from the derivative of the exponential uptake curve at $t = 0$, as described in Materials and methods section 2.3. Each rate is a single value determined from the uptake curve fit to the entire data set for that condition. [b] Ca^{2+} uptake at 10 min is expressed as nmol $Ca^{2+}/10^6$ cells. To determine these values, the background Ca^{2+} binding (parameter *C* in the uptake equation in section 2.3.) from the best fit of all the data for that experimental condition was subtracted from each 10 min Ca^{2+} uptake determination, and the average taken. Values reported are means ± S.E.M. (*n* determinations).

Cells grown in parallel in a 6-well plate were used to determine cell number at the time of assay. Each well was incubated for 5 min at 37°C in a 0.05% trypsin, 0.53 mM EDTA-4Na solution. The cells were then centrifuged at $150 \times g$ for 10 min and the pellet was resuspended in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum. The number of cells/well was determined using a hemacytometer.

2.3. Analysis of Ca^{2+} uptake

The $^{45}Ca^{2+}$ uptake curves were fit using the following single exponential equation:

$$Ca^{2+} \text{ uptake (nmol } Ca^{2+}/10^6 \text{ cells)} = A(1 - \exp^{(-Bt)}) + C$$

where A is the steady-state Ca^{2+} level (nmol $Ca^{2+}/10^6$ cells), B is the rate constant (1/min), t is time since the $^{45}Ca^{2+}$ addition (min), and C is the nonspecifically bound $^{45}Ca^{2+}$ (nmol $^{45}Ca^{2+}/10^6$ cells). The influx rate (nmol $Ca^{2+}/(10^6$cells $\times$ min)) was obtained from the first derivative of the uptake curve at $t = 0$, which is $A \times B$. Uptake curves which showed no evidence of saturation within 30 min, e.g. dihydropyridine-inhibited Ca^{2+} uptake, were fit by linear regression. The results at each time point are expressed as means ± S.E.M. For curve fitting, the mean values from all the experiments for a given condition were used rather than fitting each experiment individually, because this yielded consistently better fits (correlation coefficients > 0.92), although it precluded statistical analysis. Following the initial fit to all the data, individual values less than the nonspecific binding level (fitting parameter C) were discarded (0–4 points per data set of 12), and the data refit for some conditions. In order to make statistical comparisons between different experimental conditions we used all the Ca^{2+} uptake values at 10 min and Student's t-test for paired data (Tables 1 and 2). Data analysis and curve fitting were performed using either Prizm (GraphPad, San Diego, CA) or Quattro Pro for Windows (Borland, Scotts Valley, CA).

2.4. Membrane potential measurement

Membrane potential (E_m) of A7r5 cells under static and stretched conditions was measured from the equilibrium distribution of [^{3}H]tetraphenylphosphonium (TPP^+), a membrane permeant lipophilic cation which accumulates intracellularly as a function of membrane potential (Milligan and Strange, 1994; Morel and Godfraind, 1994). Prior to each assay the cells were rinsed once with HBSS and preincubated for 10 min in 0.5 ml/well of LKUS, at which time the cells to be stretched were mounted in the Flexercell Strain Unit and subjected to the same stretch regimen described above. The cells under static conditions were not mounted in the unit. Following the preincubation period the LKUS was aspirated and 0.5 ml of either LKUS, HKUS, or a solution containing 135 mM K^+ (same as HKUS but with 135 mM KCl and no NaCl) was added to each of the corresponding wells and incubated for 30 min (sufficient for equilibration). All three incubation solutions contained [^{3}H]TPPBr (0.20 μCi/ml, New England Nuclear) and 10 mM unlabeled TPPBr. Following the incubation period the cells were rinsed twice with ice-cold HBSS (1 ml/well), then dissolved in 0.5 ml of HBSS + 0.1% Triton-X-100 overnight and counted in a scintillation counter. The intracellular concentration of TPP^+ was determined by using the following equation:

$$[TPP^+]_i = ((TPP^+_{total} - TPP^+_{135K^+})/V_i) + [TPP^+]_o$$

where TPP^+_{total} is the TPP^+ uptake at either 2.5 or 70 mM K^+, $TPP^+_{135K^+}$ is the TPP^+ uptake at 135 mM K^+ (assumed to set $E_m = 0$), V_i is the intracellular volume, and $[TPP^+]_o$ is the extracellular TPP^+ concentration (10 mM). The units for TPP^+ uptake and V_i were nmol $TPP^+/10^6$ cells and ml/10^6 cells, respectively. The V_i of stretched and nonstretched cells was determined as described by Kletzien et al. (1975) in parallel experiments. Briefly, the cells were rinsed with HBSS and incubated for 30 min in 0.5 ml of LKUS containing 3-O-methyl[^{14}C]-D-glucose (0.2 μCi/ml, New England Nuclear). For this assay, the LKUS was glucose-free to allow unhindered uptake of 10 mM 3-O-methylglucose (3-O-MG). 2 mM pyruvate, which enters on a different carrier and therefore does not interfere with 3-O-MG uptake, was supplied to maintain cellular ATP levels. Following the incubation period, the cells were rinsed twice in ice-cold HBSS (glucose-free; same as HBSS but with 2 mM pyruvic acid and 1 mM phloretin). Phloretin, an inhibitor of sugar transport, was used in order to trap 3-O-MG that had been taken up by the cells. The cells were then dissolved and their radioac-

Table 2
Statistical comparison of stretch-modulated Ca^{2+} influx in A7r5 cells

Comparison [a]		P value [b]	Significance [c]
LKNS vs.	LKST	0.04	Significant
	HKNS	0.02	Significant
LKNS ($-Na^+$) vs.	LKST ($-Na^+$)	0.34	N.S.
LKST vs.	HKNS	0.71	N.S.
	HKST	0.99	N.S.
	LKST + isradipine	0.05	Significant
HKNS vs.	HKST	0.40	N.S.
	HKNS + isradipine	0.05	Significant
HKST vs.	HKST + isradipine	0.04	Significant

[a] Abbreviations: LKNS, 2.5 mM $[K^+]_o$ nonstretched; LKST, 2.5 mM $[K^+]_o$ stretched; HKNS, 70 mM $[K^+]_o$ nonstretched; HKST, 70 mM $[K^+]_o$ stretched. [b] Determined by Student's t-test for paired data. [c] Values of $P > 0.05$ were considered not significant (N.S.).

tivity counted as described above. Since TPP^+ distributes across cell membranes electrophoretically, E_m was calculated from the Nernst equation:

$$E_m = 58 \log[TPP^+]_o/[TPP^+]_i$$

3. Results

The time course of $^{45}Ca^{2+}$ uptake in A7r5 cells was measured under several conditions, including resting, K^+-depolarized, and stretched. These assays were carried out in solutions where $[Na^+]_o$ was held constant in order to minimize the effect of ion substitution during 70 mM $[K^+]_o$ depolarization on Na^+-dependent transport mechanisms. Therefore, we chose to vary $[NMG^+]_o$ when the cells were depolarized. For resting conditions, 2.5 mM $[K^+]_o$ was used because it gave more consistent stretch-dependent stimulation of Ca^{2+} uptake than the more physiological value of 5 mM.

Fig. 1 shows Ca^{2+} uptake in static vs. stretched 10-day postconfluent A7r5 cell cultures under basal conditions (2.5 mM $[K^+]_o$) in the absence and presence (1 μM) of the dihydropyridine Ca^{2+} channel blocker (+)-isradipine. Ca^{2+} uptake was greater in cells that were stretched at each time point measured, unless the blocker was present. The influx rate was approximately 5-fold higher for the stretched cells (0.76 vs. 0.14 nmol $Ca^{2+}/(10^6$cells $\times$ min)), and the 10-min Ca^{2+} uptake value was increased 4-fold by stretch (2.76 vs. 0.72 nmol $Ca^{2+}/10^6$ cells, Table 1; significant at $P < 0.05$, Table 2). Exposure of the cells to (+)-isradipine caused

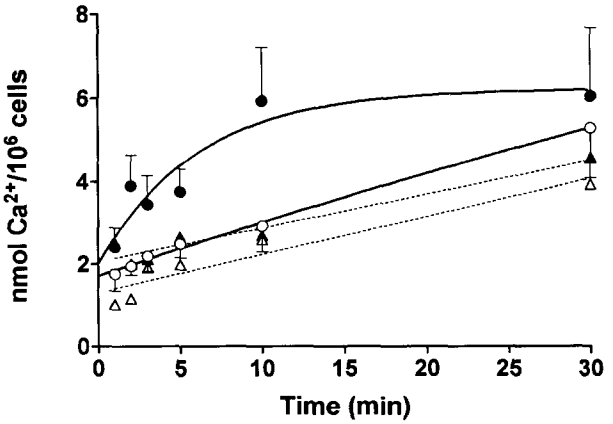

Fig. 1. Effect of stretch on $^{45}Ca^{2+}$ uptake in A7r5 vascular smooth muscle cells under low K^+ conditions (2.5 mM) at 20–22°C. The cells were preincubated for 10 min in LKUS in the presence (△,▲) or absence (○,●) of 1 μM (+)-isradipine. Following the preincubation period, LKUS containing $^{45}CaCl_2$ (2 μCi/ml) with or without 1 μM (+)-isradipine was added to each well. Cell monolayers were either stretched rhythmically (3 s on, 3 s off; filled symbols) or remained unstretched (empty symbols). Values are expressed as means ± S.E. nmol $Ca^{2+}/10^6$ cells, $n = 8$–9 determinations for ○,● and $n = 5$–6 determinations for △,▲.

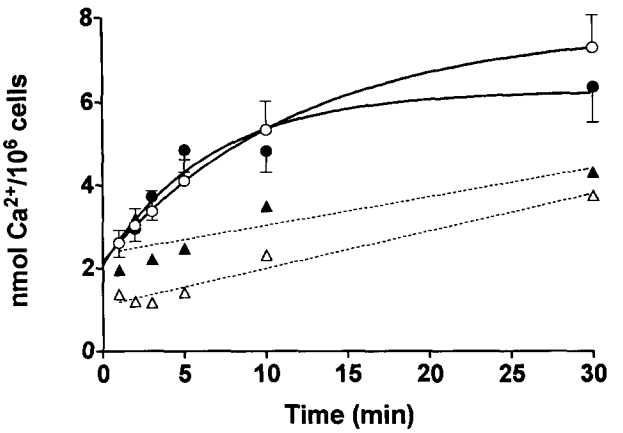

Fig. 2. Effect of stretch on $^{45}Ca^{2+}$ uptake in A7r5 vascular smooth muscle cells under depolarizing conditions (70 mM K^+) at 20–22°C. The cells were preincubated for 10 min in LKUS in the presence (△,▲) or absence (○,●) of 1 μM (+)-isradipine. Following the preincubation period, HKUS containing $^{45}CaCl_2$ (2 μCi/ml) with or without 1 μM (+)-isradipine was added to each well. Cell monolayers were either stretched rhythmically (3 s on, 3 s off; filled symbols) or remained unstretched (empty symbols). Values are expressed as means ± S.E. nmol $Ca^{2+}/10^6$ cells, $n = 8$–12 determinations for ○,● and $n = 5$–12 determinations for △,▲.

nearly complete (89%) inhibition of stretch-activated Ca^{2+} uptake, with little effect under nonstretched conditions (initial rate 0.082 nmol $Ca^{2+}/(10^6$cells $\times$ min) stretched and 0.092 nonstretched; 10-min uptake 0.63 stretched vs. 1.28 nmol $Ca^{2+}/10^6$ cells nonstretched, Fig. 1, Table 1). Comparison of the 10-min Ca^{2+} uptake values showed 78% inhibition by isradipine under stretched conditions, which was significant at $P = 0.05$.

When the cells were depolarized with 70 mM $[K^+]_o$ (Fig. 2), Ca^{2+} uptake by stretched cells was similar to that from unstretched cells, and similar to that in stretched cells at 2.5 mM $[K^+]_o$ (cf. Figs. 1 and 2, Table 1). The addition of 1 μM (+)-isradipine inhibited both the stretch-induced (89%) and nonstretched, K^+-stimulated (80%) Ca^{2+} entry rates.

We next wanted to determine if the stretch-induced Ca^{2+} entry observed under resting conditions was dependent on extracellular Na^+, since previous reports have shown that some stretch-activated channels are Na^+-permeant (Meininger and Davis, 1992; Wellner and Isenberg, 1993) and flow-induced constriction in blood vessels is dependent on $[Na^+]_o$ (McDonald et al., 1994). Under nonstretched conditions, replacing Na^+ with NMG^+ caused a slight reduction in both the Ca^{2+} influx rate (0.14 (Na^+) vs. 0.07 (NMG^+) nmol $Ca^{2+}/(10^6$cells $\times$ min)) and 10-min uptake value (0.64 (Na^+) vs. 0.40 (NMG^+) nmol $Ca^{2+}/10^6$ cells, cf. Figs. 1 and 3, Table 1). On the other hand, when the cells were stretched, Ca^{2+} entry was markedly reduced in the absence of extracellular Na^+ (0.007 nmol $Ca^{2+}/(10^6$cells $\times$ min), Fig. 3; 10-min uptake 0.05 nmol $Ca^{2+}/10^6$ cells). This is in contrast to the 5-fold in-

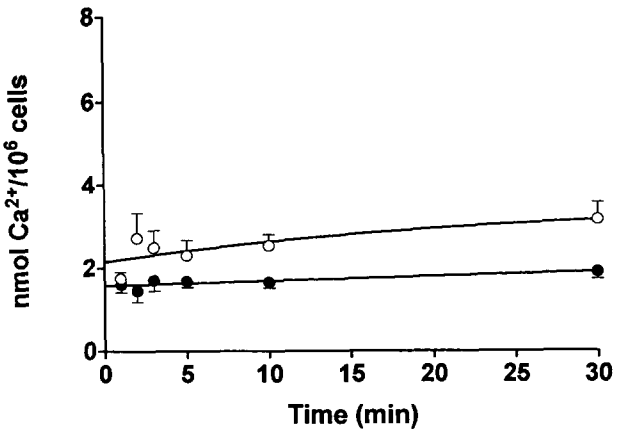

Fig. 3. Effect of stretch on $^{45}Ca^{2+}$ uptake in A7r5 vascular smooth muscle cells under low K^+ (2.5 mM), Na^+-free conditions at 20–22°C. The cells were preincubated for 10 min in Na^+-free LKUS (Na^+ replaced with equimolar NMG^+). Following the preincubation period, Na^+-free LKUS containing $^{45}CaCl_2$ (2 μCi/ml) was added to each well. Cell monolayers were either stretched rhythmically (3 s on, 3 s off; filled symbols) or remained unstretched (empty symbols). Values are expressed as means ± S.E. nmol $Ca^{2+}/10^6$ cells, $n = 5$–6 determinations.

crease in Ca^{2+} uptake by stretch in the presence of extracellular Na^+, described above.

Table 2 contains a number of statistical comparisons worth noting. Significant alteration of Ca^{2+} influx was obtained for activation by either stretch or 70 mM $[K^+]_o$ depolarization, as well as block of these effects, applied separately or combined, by (+)-isradipine. The stretch-induced reduction of Ca^{2+} entry in the absence of Na^+ was not statistically significant.

Finally, we attempted to determine whether the increase in Ca^{2+} influx rates observed under high K^+

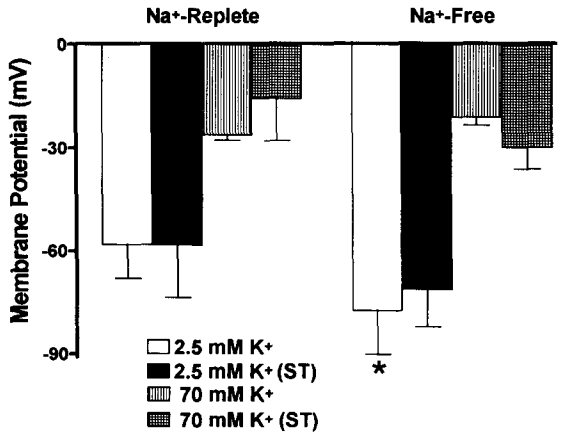

Fig. 4. Effect of 70 mM $[K^+]_o$, stretch, and Na^+ removal on E_m in A7r5 cells. E_m was determined by $[^3H]TPP^+$ equilibration for 30 min under the conditions indicated. Where indicated (ST) the cells were stretched rhythmically (3 s on, 3 s off). The results are expressed as means ± S.E.M. for three experiments, each performed in triplicate. $^*P < 0.05$ for Na^+-free vs. Na^+-replete 2.5 mM $[K^+]_o$ conditions.

or stretch conditions was due to membrane depolarization. We measured E_m from the equilibrium distribution of the lipophilic cation TPP^+. Because a 30-min incubation period was required for TPP^+ equilibration, these values reflect the average E_m over the 30-min measurement period. Therefore, rapid or transient changes in E_m cannot be detected by this method. Fig. 4 shows that under static conditions the cells depolarize when transferred from 2.5 (−58 mV) to 70 mM $[K^+]_o$ (−27 mV). However, when the cells were stretched rhythmically for 30 min, no significant change was observed in the time-averaged E_m. Similar results were obtained in Na^+-free solutions, although a small but significant hyperpolarization was observed for the static 2.5 mM $[K^+]_o$ condition.

4. Discussion

This study has demonstrated that postconfluent A7r5 vascular smooth muscle cells exhibit dihydropyridine-sensitive Ca^{2+} uptake which is activated by rhythmic stretch conditions. Both the similarity in magnitude compared to depolarization-induced Ca^{2+} entry and its dependence on extracellular Na^+ suggest a mechano-electrical transduction pathway leading to a rise of cytosolic Ca^{2+} in these cells. Since A7r5 cells are noncontractile, the possible relevance of this mechanism to the myogenic response in vascular smooth muscle cells is unclear. Conceivably, the stretch-activated Ca^{2+} entry mechanism might be important for regulating cell volume or proliferation of dedifferentiated vascular smooth muscle cells.

Both the stretch- and depolarization-induced Ca^{2+} entry were markedly blocked by the dihydropyridine (+)-isradipine. The block of stretch-induced contraction by a Ca^{2+} channel blocker has been reported in feline pulmonary arteries by Kulik and coworkers (Kulik et al., 1988), who found that 10 μM diltiazem abolished the myogenic response in those vessels. However, others have reported that depolarization- and stretch-induced Ca^{2+} entry occur via distinct pathways due to the weak effect of Ca^{2+} channel blockers on stretch-activated Ca^{2+} entry in many systems (Winquist and Baskin, 1983; Laher et al., 1988; Xiao and Bevan, 1994; Hwa and Bevan, 1986; Bevan et al., 1990; Bulow and Johansson, 1991; Davis et al., 1992b; Bialecki et al., 1992). These differences might be explained by the distinct methodologies employed in each study as well as smooth muscle cell heterogeneity.

We employed a dynamic regimen of 24% maximal and 10% mean surface elongation, interspersed with periods of relaxation, to mimic conditions encountered during the cardiac cycle. This is the first report in which $^{45}Ca^{2+}$ influx has been studied in smooth muscle cells that are subjected to continuous, dynamic stretch

and relaxation conditions. It is in contrast to other studies in which the stretch stimulus is applied once and maintained (Bialecki et al., 1992; Bulow and Johansson, 1991; Davis et al., 1992b; Hwa and Bevan, 1986; Kulik et al., 1988; Laher et al., 1988; Winquist and Baskin, 1983; Xiao and Bevan, 1994; Wellner and Isenberg, 1993, 1994), applied in a graded manner (Bulow and Johansson, 1991), or where the stimulus consists of periodically increasing or decreasing vessel luminal pressure (Bevan et al., 1990; Davis and Sikes, 1990). One study that employed both single and dynamic stretch conditions observed that 3 nM felodipine inhibited dynamic stretch-induced tone more completely than single stretch-induced tone (Bulow and Johansson, 1991). This is consistent with the nearly complete block of cyclical stretch-induced Ca^{2+} influx in our study. A possible explanation for the failure of Ca^{2+} channel blockers to inhibit single stretch-induced tone is that under these conditions Ca^{2+} channels are subject to voltage-dependent inactivation. The work of Davis and Sikes (1990) has demonstrated that both rate-dependent and rate-independent stretch-sensitive components probably contribute to the regulation of myogenic tone.

A drawback in the study of stretch-activated channels is the fact that specific blockers for these channels are presently unknown (French, 1992; Sigurdson et al., 1992). This makes it difficult to ascertain whether stretch-induced Ca^{2+} influx occurs through stretch-activated or voltage-gated Ca^{2+} channels. Nonetheless, we have observed that in A7r5 cells (+)-isradipine blocked both stretch- and voltage-gated Ca^{2+} entry with equal efficacy, suggesting that only a single Ca^{2+} entry pathway, the L-type Ca^{2+} channel, is involved. To our knowledge there is no evidence that would suggest that dihydropyridines can block Ca^{2+} influx through a stretch-activated channel. Patch clamp studies of stretch-activated nonselective cation channels have shown them to be insensitive to 500 nM (Bear, 1990) or 10 μM (Davis et al., 1992a) nifedipine. One nonselective channel in cultured heart cells was blocked by 10 μM diltiazem, however (Ruknudin et al., 1993). In A7r5 cells, since stretch plus high K^+-induced depolarization produces no further stimulation of Ca^{2+} uptake beyond either stretch or high K^+ alone, it appears that most stretch-activated Ca^{2+} entry occurs through L-type Ca^{2+} channels, and the role of stretch-activated channels is limited to providing membrane depolarization. This is further supported by the dependence of stretch-activated Ca^{2+} uptake on extracellular Na^+. Our Na^+ removal experiment is also inconsistent with direct activation of the L-type Ca^{2+} channel by membrane stretch, an hypothesis supported by Langton (1993) but disfavored by Davis et al. (1992a).

While extracellular Ca^{2+} has been shown to be important in the myogenic contractile response (Meininger and Davis, 1992; Sigurdson et al., 1992), the role of extracellular Na^+ has not been clearly defined. We found that when Na^+ ions were replaced by NMG^+, stretch-induced Ca^{2+} entry was abolished. Other laboratories have reported that myogenic tone is decreased when extracellular Na^+ is removed. Bevan and colleagues have shown that in addition to decreasing myogenic tone, the removal of Na^+ caused a significant reduction in flow-induced contractions in rabbit ear arteries (Bevan and Joyce, 1992, 1993). Unlike our observations, Bialecki et al. found that for cultured pulmonary smooth muscle, Na^+ removal had no effect on stretch-induced Ca^{2+} uptake (Bialecki et al., 1992). A possible explanation is that stretch-activated channels in those cells might conduct Ca^{2+} preferentially over Na^+. Patch-clamp studies have shown stretch-activated channels to be of variable cation selectivity, with some permeable only to Ca^{2+} and Na^+ (Lansman et al., 1987; Wellner and Isenberg, 1993) and others permeable to Ca^{2+}, Na^+, and K^+ ions (Davis et al., 1992b; Kirber et al., 1988), or somewhat selective for K^+ over Na^+ (Ruknudin et al., 1993). In A7r5 cells our results suggest that stretch-activated channels carry far less Ca^{2+} current than L-type Ca^{2+} channels under dynamic stretch conditions. Wellner and Isenberg (1993) estimated that, in guinea pig urinary bladder myocytes, although the influx of Ca^{2+} through stretch-activated nonselective cation channels is small compared to peak current through L-type Ca^{2+} channels, in chronically depolarized cells the steady state Ca^{2+} current is about equal through both pathways. Thus, we might expect increasing reliance on dihydropyridine-sensitive channels for Ca^{2+} influx in dynamically stretched compared to chronically stretched cells, since under dynamic conditions less voltage-dependent inactivation would be expected.

The Na^+ dependence of stretch-activated Ca^{2+} entry leads us to believe that stretch-activated channels exert a membrane-depolarizing influence in A7r5 cells. We therefore tried to determine whether cyclical application of 10% average strain leads to a change of E_m using uptake of the lipophilic cation tetraphenyl phosphonium (Morel and Godfraind, 1994). However, we were unable to observe significant alteration of E_m by application of stretch (Fig. 4). Since the tetraphenyl phosphonium distribution assay registers the time-averaged membrane potential during a 30-min equilibration period, the lack of observable alteration of E_m implies that any stretch-induced depolarization must be transient in nature, or compensated by a separate hyperpolarization phase during the stretch-relaxation cycle. It is conceivable that, although a stretch-activated channel could adequately depolarize the membrane to activate L-type Ca^{2+} channels, it might inactivate so rapidly as to have little effect on the time-averaged E_m. Indeed, Hamill and McBride (1992) have studied

the kinetics of stretch-sensitive channels in oocytes using the pressure-clamp technique, and found that these channels can rapidly adapt to a single aplication of suction such that the open state probability decreases sharply. Stretch-activated channels can then reactivate as they are stimulated again by suction on the pipet.

We find it likely that cyclical stretch and relaxation of A7r5 cells transiently and repeatedly depolarizes the cells via Na^+ entry, thereby intermittently activating voltage-gated Ca^{2+} entry. The inhibition of Ca^{2+} uptake by stretch in the absence of Na^+, however, points toward a second, inhibitory mechanism. Activation of K^+ efflux by stretch, with resultant hyperpolarization, could explain these results. The presence of stretch-activated Ca^{2+}-dependent K^+ channels has been shown in patch-clamp studies of rabbit pulmonary (Kirber et al., 1992), superior mesenteric (Dopico et al., 1994), and cerebral arteries (Brayden and Nelson, 1992) and their function is believed to be involved in a feedback pathway which regulates membrane depolarization and vasoconstriction. Coactivation of depolarizing and hyperpolarizing channels could provide vascular smooth muscle cells with a negative feedback mechanism which allows them to limit the amount of Ca^{2+} entry in response to dynamic changes in stretch.

Knowledge of the pathways involved in the response to stretch will prove critical for developing possible therapeutic interventions. Our study would suggest that dihydropyridines might be effective in blocking stretch-induced effects in vascular smooth muscle cells.

We conclude that A7r5 cells possess a dihydropyridine-sensitive, stretch-modulated Ca^{2+} influx pathway. The Na^+ dependence of this mechanism suggests that L-type Ca^{2+} channels are activated in these cells by Na^+ entry through stretch-activated channels, which results in transient cell depolarization.

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