Myogenic contraction by modulation of voltage-dependent calcium currents in isolated rat cerebral arteries

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1. Tissue blood flow and blood pressure are regulated by the spontaneous, myogenic, contraction developed by resistance arteries. However, the cellular mechanisms underlying myogenic contraction are not understood. In this study, the mechanisms of myogenic contraction in cerebral resistance arteries were investigated.

2. The vasoconstriction observed in response to increased pressure in cerebral resistance arteries (myogenic reactivity) was dependent on Ca^{2+} entry through voltage-dependent Ca^{2+} channels, since it was abolished by Ca^{2+} removal and by dihydropyridine antagonists of voltage-dependent Ca^{2+} channels.

3. Myogenic reactivity persisted in a high-K^+ saline, with reduced Ca^{2+}, where membrane potential is presumed to be clamped. Therefore, membrane depolarization alone does not fully account for the increased voltage-dependent Ca^{2+} channel opening.

4. Voltage-dependent Ca^{2+} currents in single smooth muscle cells isolated from the resistance artery were substantially increased by applying positive pressure to the patch electrode evoking membrane stretch.

5. Myogenic reactivity remained unaffected by ryanodine and therefore was independent of internal ryanodine-sensitive Ca^{2+} stores.

6. The myofilament Ca^{2+} sensitivity was not increased by elevated pressure in α-toxin-permeabilized arteries. However, pharmacological activation of protein kinase C or G proteins did increase the myofilament Ca^{2+} sensitivity.

7. Myogenic contraction over the pressure range 30–70 mmHg could be accounted for by an increase in $[Ca^{2+}]_i$ from 100 to 200 nM.

8. It is concluded that modest increases in $[Ca^{2+}]_i$ within the range 100–200 nM can account for that myogenic contraction, and that stretch-evoked modulation of Ca^{2+} currents may contribute to the myogenic response.

Resistance arteries contract when blood pressure increases and dilate when pressure decreases. This behaviour is termed myogenic contraction and it plays an important role in the maintenance of constant blood flow, regardless of changes in blood pressure. Despite its physiological importance, the cellular mechanisms underlying myogenic contraction are not yet understood. Of two dominant hypotheses, the most widely held is that stretch increases the smooth muscle cytosolic calcium concentration ($[Ca^{2+}]_i$) by depolarization and thereby increases opening of voltage-dependent Ca^{2+} channels. Studies correlating pressure-evoked depolarization and contraction led to this proposal, but a causal link between the two variables has not been established (Harder, Gilbert & Lombard, 1987; Bradyen & Wellman, 1989). An alternative hypothesis, based on indirect evidence, is that myogenic contraction is independent of increased $[Ca^{2+}]_i$, and relies rather on a stretch-induced activation of protein kinase C, causing an increased Ca^{2+} sensitivity of the myofilaments (e.g. Laporte, Haeberle & Laher, 1994). The purpose of this study was to determine if these mechanisms contribute to myogenic contraction in rat cerebral arteries. Preliminary results have already been presented (McCarron, Crichton, Langton, McKenzie & Smith, 1995).

METHODS

Male Wistar–Kyoto rats were killed by sodium pentobarbitone overdose (150 mg kg^{-1}, I.P.). The brain was rapidly removed and placed in physiological saline solution (PSS) containing (mM): 119 NaCl, 24 $NaHCO_3$, 4·7 KCl, 1·18 KH_2PO_4, 1·17 $MgSO_4.7H_2O$, 1·6 $CaCl_2$ and 5·5 glucose. Second-order branches of the posterior cerebral artery were dissected, transferred to a chamber and cannulated. The arteries were superfused with PSS at 37 °C

bubbled with 95% O_2 and 5% CO_2 at ~100 ml min^{-1}. Lumen diameter was measured with an electronic video dimension analyser (Living Systems Instrumentation, Burlington, VT, USA). Diameter and pressure were recorded on a chart recorder and also stored on computer for later analysis. During an initial equilibration period, the artery pressure was cycled three times between 10 and 100 mmHg to decrease mechanical hysteresis (each cycle taking about 3 min). At the end of the third cycle, the artery was equilibrated at 60 mmHg. On equilibration, the artery developed myogenic tone and luminal pressure was altered as indicated in the text. Ca^{2+}-free PSS was identical to PSS but without Ca^{2+}, and it contained 1 mM EGTA. Free [Ca^{2+}] was estimated to be less than 10 nM. The composition of the high-K^+ PSS was (mM): 100 KCl, 24 $KHCO_3$, 1·17 $MgSO_4.7H_2O$, 1·18 KH_2PO_4, 1 EGTA and 5·5 glucose; it was bubbled with 95% O_2 and 5% CO_2. [Ca^{2+}] in this solution (with no added Ca^{2+}) should be less than 10 nM.

Permeabilized arteries

Arteries were permeabilized using the α-toxin from *Staphylococcus aureus* (2 mg ml^{-1}) at 60 mmHg; thereafter pressure was altered as indicated. The composition of the bathing solution was (mM): 10 EGTA, 120 potassium methyl sulphonate, 5 Na_2ATP, 15 sodium creatine phosphate and 6 $MgCl_2$. Ca^{2+} was varied between 1 nM and 100 μM (pH 7·2) and the saline was maintained at 37 °C. Experiments on Ca^{2+} sensitivity of tension production require a high EGTA concentration (10 mM) to clamp [Ca^{2+}] throughout the preparation (Crichton & Smith, 1990). Changes of [Ca^{2+}] next to the contractile proteins are achieved by altering Ca^{2+} in the EGTA-buffered saline bathing the outside of the vessel. Luminal saline was identical to the externally applied saline, except that the [EGTA] was 0·1 mM. This allowed the saline bathing the outside of the vessel to dictate the [Ca^{2+}] within the wall and lumen of the vessel.

Cell isolation

The artery was placed in 1 or 2 ml of cold enzyme cocktail containing collagenase (725 units ml^{-1}, Sigma Type IA), elastase (0·5 mg ml^{-1}, Sigma Type IIA), and bovine albumin (2·5 mg ml^{-1}, Sigma 7906) in isolation buffer (containing (mM): 120 NaCl, 5 KCl, 0·16 $CaCl_2$, 2 $MgCl_2$, 15 $NaHCO_3$, 0·5 NaH_2PO_4, 10 taurine, 10 glucose, 10 Hepes and 0·05 Phenol Red; pH 7·3 with NaOH before adding $NaHCO_3$). This was kept at 4 °C to depress enzyme activity and to allow the enzymes to diffuse into the extracellular matrix of the tissue. After 3 h at 4 °C, the enzyme cocktail was warmed to 35 °C for 10 min in a shaking water bath to activate the enzymes. The tissue was then removed into fresh isolation buffer and triturated, at first gently, until isolated cells were seen. Cells were stored in isolation buffer at 4 °C and used within 8 h.

Current recordings

Patch pipettes were pulled from 1·5 mm o.d. borosilicate capillaries, fire polished and coated with beeswax. Their resistance was in the range 4–7 MΩ when filled with electrolyte. Seals formed by application of negative pressure were of the order of 10 GΩ. Whole-cell access was achieved by brief suction. Whole-cell currents were amplified with an Axopatch 200 amplifier (Axon Instruments). Series resistance (R_s) was measured from the capacity transients elicited by a 10 mV hyperpolarization. R_s was 9·3 ± 0·3 MΩ ($n = 10$). The largest inward current measured in this study was −300 pA, corresponding to a voltage error due to R_s of < 3 mV, and so compensation for R_s was not routinely performed. All voltages have been corrected for a measured liquid junction potential of −5 mV between the pipette and bath solution. The experimental cell was perfused with bathing solutions containing (mM): 130 NaCl, 5·4 KCl, 10 glucose and 10 Hepes; pH 7·4 with NaOH and either 10 mM $BaCl_2$–0·1 mM EGTA or 10 mM $CaCl_2$–0 EGTA. These solutions perfused the cells from a puffer pipette system (see Langton, 1993). The experimental chamber was perfused at a rate of 0·5 ml min^{-1} with (mM): 130 NaCl, 5·4 KCl, 1·8 mM Ca^{2+}, 10 glucose and 10 Hepes; pH 7·4 (with NaOH) throughout the experiment to reduce exposure of other cells to different experimental solutions. The pipette solution had the following composition (mM): 130 CsCl, 1 $MgCl_2$, 5 EGTA, 5 Na_2ATP, 0·5 GTP and 10 Hepes; pH 7·2 with CsCl. Current recordings were low-pass filtered at 1–2 kHz (−3 dB, 8-pole Bessel) and digitized at 4–10 kHz.

Analysis

The strength of the myogenic response following a rapid pressure increment from 30 to 70 mmHg was estimated by using two reference points (Fig. 1). Firstly, the diameter in the steady state following the pressure increase was compared, as a percentage, with the diameter before the pressure increase. Secondly, the steady-state diameter after the pressure increase was also compared with the diameter just after the pressure increment. Following the rapid pressure increment, the diameter transiently approached the passive diameter of the artery. However, the initial diameter increase did not usually reach the full passive diameter of the artery, since the myogenic response rapidly reversed the distension. That peak diameter provided the reference point to determine whether any contraction occurs following the pressure increase. Summarized data are presented as means ± S.E.M. and the statistical test was Student's paired t test with $P < 0·05$ considered significant.

Chemicals

Crude α-toxin was a gift from Professor Freer, Department of Bacteriology, University of Glasgow. The dihydropyridine antagonist (−)202-791 was a gift from Sandoz; the protein kinase C inhibitor R0318425 was a gift from Roche; indolactam was obtained from Sigma; and ryanodine was obtained from Calbiochem–Novabiochem Ltd.

RESULTS

Clear evidence of myogenic contraction was demonstrated in pressurized posterior cerebral arteries when pressure was increased from 30 to 70 mmHg (Fig. 2*A*; standard pressure step). Diameter initially increased when pressure was increased to 70 mmHg, but then decreased as the artery contracted. Increasing pressure to 70 mmHg caused an average initial distension to 125 ± 2% of the diameter at 30 mmHg, but the artery then contracted to 99 ± 2% of the diameter at 30 mmHg ($n = 9$). Dihydropyridine antagonists of voltage-dependent Ca^{2+} channels or the absence of external Ca^{2+} (Fig. 2*A*) abolished the myogenic contraction. The standard pressure step, in the virtual absence of extracellular Ca^{2+} (< 10 nM), increased diameter to 118 ± 3% of the diameter at 30 mmHg, no subsequent contraction was observed and the diameter remained at 119 ± 2% ($n = 6$). Similarly, nimodipine (100 nM), a dihydropyridine Ca^{2+} channel antagonist, abolished myogenic contraction. In these experiments the initial diameter at 70 mmHg was 117 ± 2% of the diameter at 30 mmHg, and remained at 116 ± 2% ($n = 3$) for the duration of the pressure step. (−)202-791 (1 μM), another dihydropyridine antagonist of voltage-dependent Ca^{2+} channels, also abolished myogenic contraction. In this series of experiments the

initial diameter at 70 mmHg was 117 ± 2% of that at 30 mmHg, contracting back to 98 ± 4%. In the presence of (−)202-791, the initial diameter at 70 mmHg was 113 ± 2%, remaining at 114 ± 2% of the diameter at 30 mmHg ($n = 4$). Figure 2*B* shows the mean steady-state diameter over the pressure range 0–80 mmHg under control conditions ($n = 13$), in the presence of nimodipine ($n = 3$) or (−)202-791 ($n = 4$) and in the absence of extracellular Ca^{2+} ($n = 13$). Between 0 and 30 mmHg, there was little difference in the responses to changes in pressure, but above 30 mmHg active myogenic contraction under control conditions maintained a smaller diameter than that seen in either the absence of extracellular Ca^{2+} or the presence of either nimodipine or (−)202-791. These results confirm that myogenic reactivity requires Ca^{2+} entry through dihydropyridine-sensitive voltage-dependent Ca^{2+} channels (e.g. Haws, Gourley & Heistad, 1983).

Since the most widely held explanation for myogenic reactivity is that pressure or stretch depolarizes the smooth muscle, leading to increased $[Ca^{2+}]_i$ (e.g. Harder *et al.* 1987; Bradyen & Wellman, 1989), we examined whether depolarization is necessary for myogenic reactivity. Like other muscle and neuronal preparations, the membrane potential of smooth muscle closely follows the K^+ equilibrium potential above an external K^+ concentration of 30 mM (Casteels, Kitamura, Kuriyama & Suzuki, 1977; Droogmans, Raeymaekers & Casteels, 1977). Cells bathed in a high-K^+ saline are depolarized and the K^+ conductance dominates to such an extent that the membrane potential is clamped. In the high-K^+ PSS the artery membrane potential will be depolarized to approximately −3 mV, assuming an internal K^+ concentration of 140 mM. Myogenic reactivity was compared both when membrane potential was free to change (in normal physiological saline) and when membrane potential was clamped in the high-K^+ physiological saline. The extracellular Ca^{2+} concentration in the high-K^+ PSS was adjusted in each experiment (mean value 60 ± 20 μM) to ensure the resting diameter was close to that observed in normal PSS. Thus in these experiments, after carrying out the control pressure step the PSS was switched to Ca^{2+}-free PSS and the artery fully dilated. The Ca^{2+}-free PSS was then exchanged with a high-K^+-containing physiological saline (120 mM K^+) with no added Ca^{2+}. Thereafter Ca^{2+} was added from a 1 M standard to increase tone to approximate that developed by the artery at the same pressure.

Under these conditions, the standard pressure step increased diameter initially to 128 ± 3%, but the artery then contracted to 114 ± 3% (Fig. 2*C*). This significant reduction in diameter ($P < 0{\cdot}05$; $n = 8$) was about 50% of the myogenic contraction observed in the same arteries, and was also abolished by nimodipine (100 nM; $n = 2$). Thus substantial reactivity remained despite a decreased driving

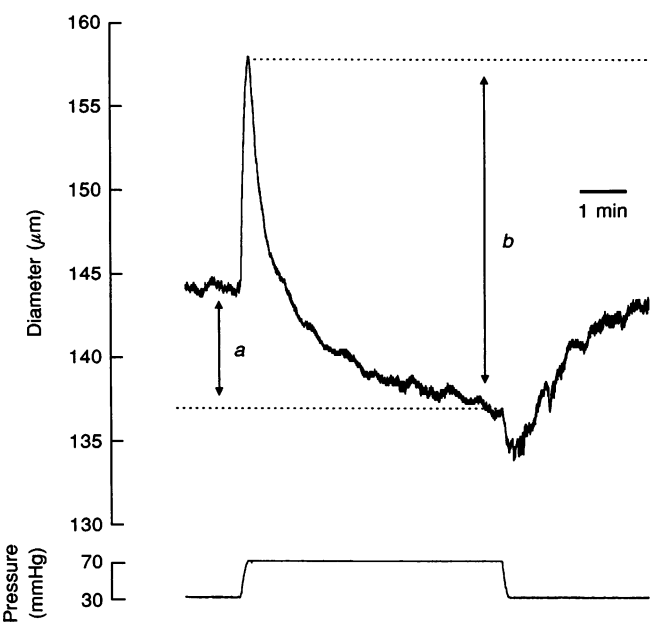

Figure 1. Illustration of a myogenic response and the reference points for summarizing the response

After a rapid pressure increment there was a passive distension of the artery followed by a constriction of the artery. Two reference points were used to characterize the response. First, the steady-state diameter after the pressure increase was compared with the diameter before the pressure increase (*a*) and expressed as a percentage. Second, the steady-state diameter after the pressure increase was also compared with the peak diameter recorded during the passive distension of the artery just after the pressure increment (*b*), and expressed as a percentage. That diameter provided the reference point to determine whether any contraction occurs following the pressure increase.

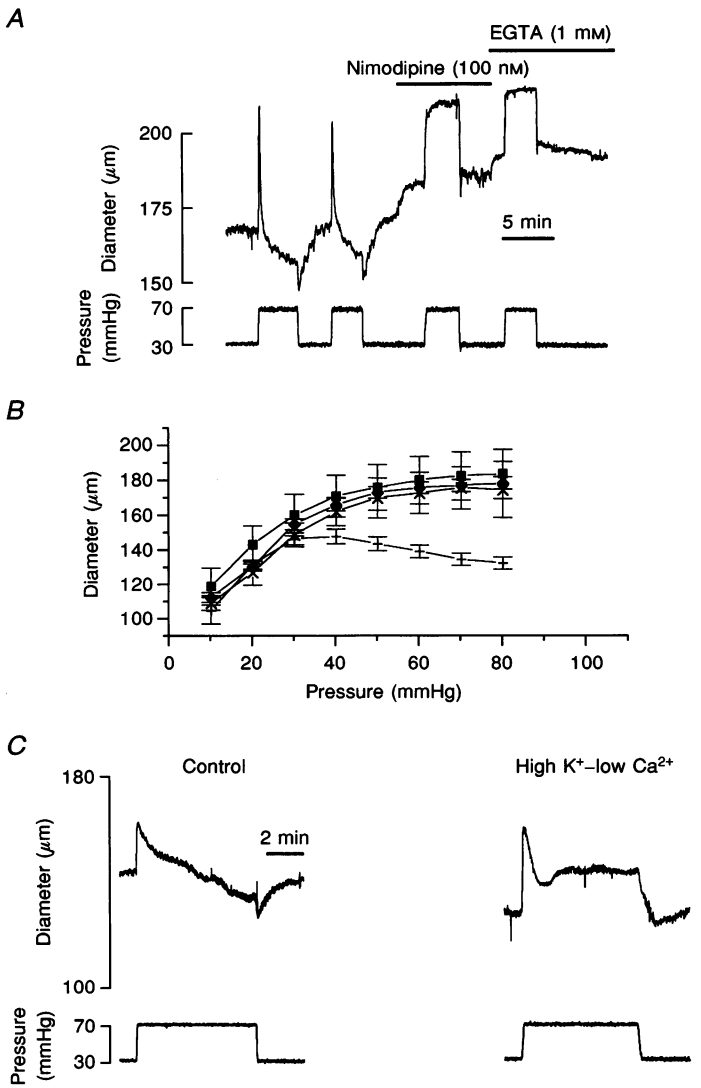

Figure 2. Role of extracellular Ca^{2+} and changes in membrane potential in myogenic reactivity

A, reproducible myogenic contractions of an intact artery (upper trace) when pressure was increased from 30 to 70 mmHg, and their abolition by nimodipine (100 nM) or the absence of extracellular Ca^{2+} with EGTA. During the equilibration period the arteries developed myogenic tone. The rapid increase in pressure from 30 to 70 mmHg increased diameter to 170 ± 9 from 136 ± 7 μm; the artery contracted back to 135 ± 7 μm ($n = 9$). In the presence of nimodipine (100 nM) the same pressure step increased the diameter to 173 ± 4 from 148 ± 4 μm; the artery did not contract and the diameter remained at 172 ± 4 μm ($n = 3$). Similarly, in the virtual absence of extracellular Ca^{2+} the standard pressure step increased the artery diameter to 181 ± 3 from 153 ± 3 μm, and the artery diameter remained at 182 ± 2 μm ($n = 6$). *B*, summarized steady-state pressure–diameter relationships obtained in PSS alone (+; $n = 13$), with nimodipine (×; 100 nM; $n = 3$) or with (−)202-791 (■; 1 μM; $n = 4$), and without Ca^{2+} (◆, Ca^{2+}-free PSS; $n = 13$). Above about 30 mmHg, a negative slope on the pressure–diameter relationship develops only in PSS. *C*, myogenic reactivity observed after a 30 to 70 mmHg pressure step (left-hand panel) is also present in a high-K^+ PSS with a reduced extracellular [Ca^{2+}] (right-hand panel). During the 20 min gap in the trace the Ca^{2+} concentration in the perfusate was first reduced from 1·6 mM to less than 10 nM. Thereafter the high-K^+–low-Ca^{2+} PSS was perfused and finally Ca^{2+} was added from a 1 M standard to increase tone to approximate that developed by the artery at the same pressure. The final free extracellular Ca^{2+} concentration in the high-K^+ PSS was 60 ± 20 μM. In the high-K^+ PSS the artery membrane potential will be depolarized to approximately −3 mV, assuming an internal K^+ concentration of 140 mM. On average, the standard pressure increased the diameter to 165 ± 9 from 129 ± 8 μm and the artery contracted back to 147 ± 7 μm ($n = 8$).

force on Ca^{2+}, as a result of the reduced extracellular Ca^{2+} concentration and the depolarization. These experiments demonstrate that changes in membrane potential are insufficient to account for myogenic reactivity. Hence pressure or stretch must alter smooth muscle tone by either modulating myofilament Ca^{2+} sensitivity, by altering Ca^{2+} removal from the cytosol or by increasing Ca^{2+} influx independently of membrane potential changes.

To distinguish between these possibilities, arteries were first chemically permeabilized using α-toxin to allow myogenic reactivity to be examined under conditions of controlled $[Ca^{2+}]_i$. Arteries were permeabilized in low $[Ca^{2+}]_o$ (1 nM) and, as shown in Fig. 3*A*, when $[Ca^{2+}]_o$ was subsequently increased to 200 nM the artery contracted. Under these $[Ca^{2+}]_i$-clamped conditions, increasing the pressure from 30 to 70 mmHg distended the artery, but unlike the intact

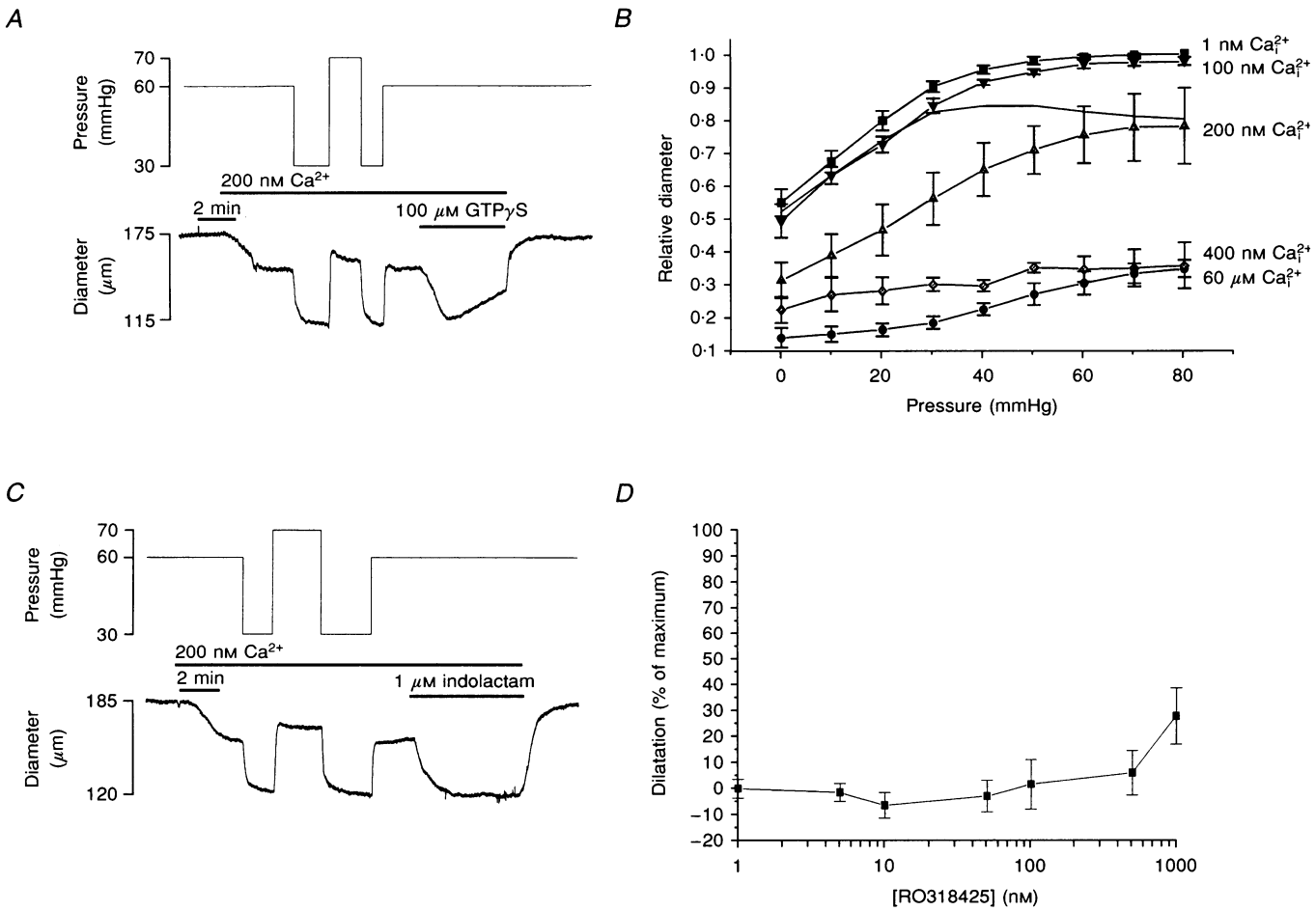

Figure 3. Stretch-evoked alteration in myofilament Ca^{2+} sensitivity

A, recording of a permeabilized artery held at 200 nM Ca^{2+} before and after the standard pressure increment from 30 to 70 mmHg. The upper panel illustrates the pressure protocol while the lower panel shows the resistance artery diameter. The artery was permeabilized with α-toxin at 60 mmHg and after increasing $[Ca^{2+}]_i$ to 200 nM, pressure was decreased to 30 mmHg and the standard pressure step applied. Under these $[Ca^{2+}]_i$-clamped conditions, the standard pressure step distended the artery, but there was no subsequent contraction. Pressure was returned to 60 mmHg and on introduction of GTPγS (100 μM) to the bathing solution the permeabilized artery contracted. *B*, steady-state pressure–diameter relationships over a range of $[Ca^{2+}]_i$. There is no evidence for a negative slope in the pressure–diameter relationship at any $[Ca^{2+}]_i$. The line without error bars illustrates the average myogenic response obtained from the intact arteries. *C*, the upper panel again illustrates the pressure protocol and the lower panel shows diameter. The artery was held at 200 nM Ca^{2+}. Although the standard pressure step did not evoke a myogenic response, protein kinase C activation with indolactam (1 μM) evoked contraction ($n = 4$). *D*, the protein kinase C inhibitor RO318425 had little effect on steady-state myogenic tone up to a concentration of 1 μM, well beyond the IC_{50} of the inhibitor for protein kinase, but similar to its IC_{50} for myosin light chain kinase. The percentage dilatation is the change in tone before the addition of RO318425 (0%) and when maximally relaxed (100%) ($n = 5$).

artery, there was no subsequent contraction. In a number of experiments, when pressure was increased from 30 to 70 mmHg, the artery diameter increased to 123 ± 5% ($n = 6$) of the diameter at 30 mmHg, with no subsequent contraction (121 ± 6%). However, activation of G proteins evoked a further contraction in the permeabilized artery (Fig. 3*A*). GTPγS (100 μM) produced an average reduction in diameter from 142 ± 15 to 121 ± 23 μm ($n = 3$).

To determine the effect of pressure on Ca^{2+}-activated force, pressure–diameter relationships were established for the

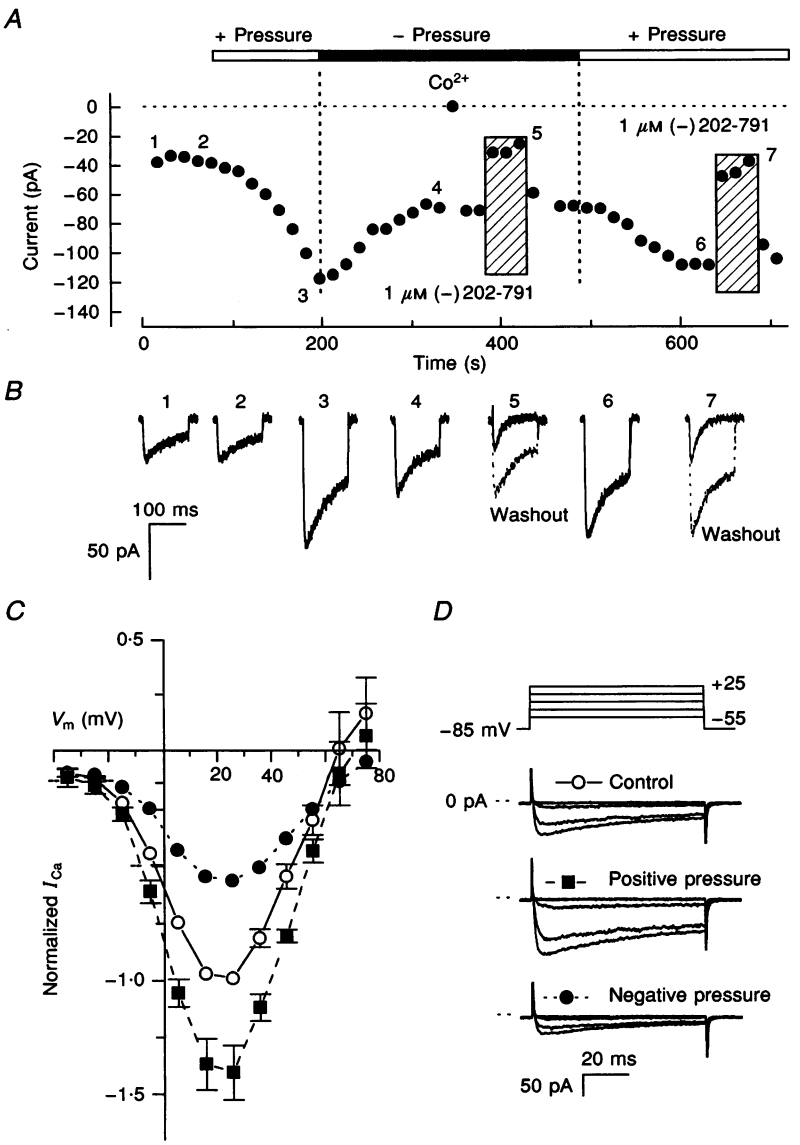

Figure 4. Stretch-evoked increases in Ca^{2+} current

A, peak inward currents recorded from a single smooth muscle cell during 200 ms depolarizations from a holding potential of −85 to +5 mV. Prior to the application of positive pressure, the peak current obtained with each depolarization was about −40 pA. However, after membrane stretch by application of positive pressure (+ Pressure) to the patch pipette, the inward current substantially increased to approximately −125 pA. The effect was reversible on application of negative pressure (– Pressure). The current was potently blocked by cobalt (1 mM) and the dihydropyridine antagonist (–)202-791 (1 μM). *B*, examples of the inward current at the time points indicated are shown. Similar results were obtained in nine other cells. The small residual current in the presence of the dihydropyridine antagonist is consistent with the voltage-dependent nature of the dihydropyridine block of Ca^{2+} current (Langton, 1993). Charge carrier was Ba^{2+} (10 mM). *C*, current–voltage relationships (I_{Ca}, calcium current; V_m, membrane potential) from three cells under control conditions (○) and after positive (■) and negative pressure (●). The Ca^{2+} current has been normalized to the peak Ca^{2+} current occurring during the control current–voltage relationship. *D*, examples of the Ca^{2+} currents are illustrated. The charge carrier was Ca^{2+} (10 mM). The current–voltage relationship has been corrected for a junction potential of −5 mV, but leak current has not been subtracted.

chemically permeabilized artery over a range of $[Ca^{2+}]_i$. Results of such experiments are summarized in Fig. 3*B*. There was no evidence of myogenic contraction ($n = 4$). For comparison, Fig. 3*B* also shows the myogenic response displayed by the intact arteries over the same range of pressures (line without error bars). Indeed, this plot provides a method of estimating the changes in $[Ca^{2+}]_i$ required to explain myogenic reactivity. An increase of $[Ca^{2+}]_i$ from 100 to 200 nM was sufficient to account for the vasoconstriction seen when pressure is increased from 30 to 70 mmHg. Thus increased pressure did not alter the myofilament Ca^{2+} sensitivity.

Activation of protein kinase C activity may mediate changes in Ca^{2+} sensitivity to produce myogenic tone (e.g. Laporte *et al.* 1994). In the present experiments no pressure-dependent increase in the myofilament Ca^{2+} sensitivity could be found. The apparent lack of effect might occur because protein kinase C was not involved in myogenic tone (i.e. it is not activated) or because protein kinase C cannot alter Ca^{2+}-activated force in this preparation. Therefore, we sought to activate protein kinase C pharmacologically using indolactam (1 μM). Indolactam increased Ca^{2+}-activated force (Fig. 3*C*), decreasing diameter from 174 ± 10 to 112 ± 17 μm ($n = 4$). These data demonstrate that protein kinase C activation altered the myofilament Ca^{2+} sensitivity, although increased pressure did not.

Although protein kinase C did not mediate stretch-induced changes in myofilament Ca^{2+} sensitivity, the kinase might still play some role if it altered $[Ca^{2+}]_i$. To test this hypothesis we used the protein kinase C antagonist RO318425 (IC_{50} 8 nM; Bradshaw, Hill, Nixon & Wilkinson, 1993) on the intact preparation. Even with 100 nM RO318425, myogenic reactivity was not impaired. Following the standard pressure step from 30 to 70 mmHg, artery diameter was 97 ± 3% of that at 30 mmHg and not different to the response observed in the same arteries in the absence of RO318425 (97 ± 4%; $n = 3$). Figure 3*D* shows the cumulative concentration-dependent response to RO318425 and illustrates that the protein kinase C inhibitor had little effect up to 1 μM. At 1 μM there was a small dilatation consistent with known inhibitory effects of the compound on myosin light chain kinase (IC_{50} 3·7 μM; $n = 5$; Bradshaw *et al.* 1993).

Myogenic reactivity is independent of protein kinase C activity but may be accounted for, at least in part, by Ca^{2+} release from the sarcoplasmic reticulum (Tanaka, Hata, Ishiro, Ishii & Nakayama, 1994). Ryanodine was used to test this possibility. Ryanodine (1 μM; at 60 mmHg) produced a small contraction; diameter was reduced from 111 ± 23 to 97 ± 21 μm ($n = 3$). This was a sustained contraction and may reflect an increased influx of Ca^{2+}, possibly as a result of store depletion, or inhibition of a Ca^{2+} removal pathway involving the sarcoplasmic reticulum and Na^+–Ca^{2+} exchange or removal of a store-dependent hyperpolarization (Moore *et al.* 1993; Randriamampita & Tsien, 1993; Nelson *et al.* 1995). In the controls, after the standard pressure step to 70 mmHg, the artery contracted to a steady-state diameter, 96 ± 4% of that at 30 mmHg ($n = 3$). After the same pressure step in the presence of 1 μM ryanodine, the artery contracted back to 97 ± 4% of the diameter at 30 mmHg ($n = 3$). Thus it seems that Ca^{2+} release from the sarcoplasmic reticulum did not play a role in either augmenting or limiting myogenic reactivity.

Since myogenic reactivity is blocked by inhibitors of voltage-dependent Ca^{2+} channels, modulation of these channels by pressure or stretch, independent of alterations in membrane potential, could play a role. To provide direct evidence that stretch-modulated voltage-dependent Ca^{2+} channels exist in these resistance arteries, voltage-dependent Ca^{2+} currents were measured using tight-seal whole-cell recording on single enzymatically dispersed smooth muscle cells from the resistance arteries. Figure 4*A* and *B* shows voltage-gated inward currents through dihydropyridine-sensitive channels before and after applying positive pressure to the patch pipette to stretch the cell membrane. Positive pressure produces a substantially increased voltage-dependent inward current and the effect was reversed by negative pressure. The inward current was sensitive to both cobalt and the dihydropyridine antagonist (−)202-791. Similar results were noted in five cells with 10 mM Ba^{2+} as the charge carrier and in seven cells with 10 mM as the Ca^{2+} charge carrier. In order to assess whether the pressure changes shifted the current–voltage relationship, inward currents were recorded over a wide range of test potentials from a holding potential of −85 mV, with 10 mM Ca^{2+} as the charge carrier (Fig. 4*C*). Positive pressure substantially increased and negative pressure decreased the Ca^{2+} current, although the voltage dependence remained unchanged. Examples of the Ca^{2+} current are illustrated in Fig. 4*D*.

DISCUSSION

The results described above demonstrate that myogenic contraction in posterior cerebral arteries was dependent on Ca^{2+} entry through dihydropyridine-sensitive Ca^{2+} channels. Positive pressure applied to the patch pipette, evoking membrane stretch, increased the magnitude of the Ca^{2+} current. Furthermore, myogenic contraction was independent of a change in myofilament Ca^{2+} sensitivity or release of $[Ca^{2+}]$ from a ryanodine-sensitive internal store.

The role of voltage-dependent Ca^{2+} channels in myogenic tone is debated based largely on the results obtained with dihydropyridines. Dihydropyridines are considered to be highly selective antagonists of voltage-dependent Ca^{2+} channels. However, there is wide variation in the reported sensitivity of myogenic tone to the dihydropyridine blockers of voltage-dependent Ca^{2+} channels. In many arterial preparations myogenic tone is abolished by dihydropyridine antagonists, but in some arteries myogenic tone is reported to be insensitive to dihydropyridines (see Meininger & Davies, 1992). This variation probably occurs for a variety

of reasons, e.g. block by dihydropyridines is voltage dependent. The more depolarized the smooth muscle membrane, the more effective these blockers will be (Bean, 1984). Thus if myogenic tone developed in some arteries at more negative membrane potentials, then block with these compounds would be reduced. Furthermore, the sensitivity to dihydropyridine antagonists may be modulated (Meisheri, Sage & Cipkus-Dubry, 1990).

Thus in the cerebral artery preparation used in the present study, activation of voltage-dependent Ca^{2+} channels was essential for myogenic contraction. Voltage-dependent Ca^{2+} channels respond to depolarization with an increased open state probability and, indeed, depolarization has been reported to accompany myogenic contraction (e.g. Harder *et al.* 1987; Bradyen & Wellman, 1989). For instance, Harder *et al.* (1987) found that increasing pressure from 20 to 120 mmHg depolarized canine renal arteries from around −60 mV to about −40 mV. Despite the correlative evidence, a causal link between depolarization and myogenic contraction is absent. The present results indicate clearly that myogenic contraction still occurred in a high-K^+ saline when, presumably, the membrane potential was clamped. That myogenic contraction remained despite a reduced driving force on Ca^{2+} (due to the depolarized membrane potential and reduced external Ca^{2+} concentration) suggests depolarization alone cannot account for myogenic contraction. In single smooth muscle cells isolated from the cerebral resistance arteries, membrane stretch (caused by application of positive pressure to the patch pipette) increased dihydropyridine-sensitive Ca^{2+} currents (see also Langton, 1993). Collectively these results suggest that stretch-evoked myogenic contraction may be mediated, at least in part, by increased voltage-dependent Ca^{2+} currents, independent of membrane potential changes.

The stress applied to the membrane may not be equivalent to the deformation of the smooth muscle cells produced by increasing intraluminal pressure in the intact vessel. However, at present there is no method that could mimick precisely, on isolated smooth muscle cells, the forces acting on the cells in the wall of the artery. An alternative method used in some studies has been to apply longitudinal stretch to the smooth muscle cells (e.g. Davis, Donovitz & Hood, 1992). However, it is important to note that intact arteries mounted on a wire myograph and stretched, presumably pulling the smooth muscle cells alone their longitudinal axis, rarely develop myogenic contraction.

The link between physical forces applied to the cell and voltage-dependent Ca^{2+} currents could be a direct effect on the cell membrane or, alternatively, physical forces may be transmitted to the channel through second messenger systems. Biochemical pathways evoked by cell stretch are wide ranging. Second messenger pathways such as tyrosine kinase, $p21^{ras}$, mitogen-activated protein kinases (MAP), S6 peptide (RRLSSLRA) kinase, protein kinase C, phospholipase C and phospholipase D have all been reported to have been activated in cardiac myocytes (e.g. Sadoshima & Izumo, 1993; Yamazaki *et al.* 1993). Interestingly, the increased S6 peptide kinase activity in rat glomerular mesangial cells, noted after stretching, was abolished by either the inhibition or downregulation of protein kinase C activity (Homma, Akai, Burns & Harris, 1992). Similarly, while mechanical stretching increased mRNA levels of c-*fos* in cardiac myocytes, this expression was markedly attenuated by downregulation of protein kinase C (Yazaki *et al.* 1993).

Given the apparent effects of mechanical loading of muscle in kinase activation, particularly protein kinase C, it is not altogether surprising that activation of protein kinase C has been suggested to play an important role in myogenic contraction. This hypothesis follows from work of Laher and colleagues who investigated the role of protein kinase C in myogenic reactivity in arteries and veins using both activators and inhibitors of protein kinase C (e.g. Hill, Falcone & Meininger, 1990; Osol, Laher & Cipolla, 1991; Henrion & Laher, 1993). Thus activation of protein kinase C enhanced myogenic tone while inhibition of protein kinase C inhibited stretched-induced tone. The hypothesis was further developed to suggest that a change in the Ca^{2+} sensitivity of the contractile proteins accounts for the changed tension in a myogenic response (Laporte *et al.* 1994). The data presented here do not support the idea of an increase in myofilament Ca^{2+} sensitivity with increased pressure. Furthermore, RO318425, a selective protein kinase C inhibitor, did not alter myogenic reactivity. RO318425 has a much greater separation in its IC_{50} for protein kinase C and myosin light chain kinase (8 nM *vs.* 3·7 μM) than other kinase inhibitors that have been used, such as H-7 and staurosporine. However, since RO318425 will inhibit several isoforms of protein kinase C, possibly with antagonistic roles in the modulation of contraction, protein kinase C may yet prove to play some role in myogenic contraction. Previous findings in larger arteries, however, also suggest little role of protein kinase C in stretch-evoked contraction. Barany, Rokolya & Barany (1990) noted that phorbol dibutyrate evoked a protein kinase C-dependent phosphorylation of arterial smooth muscle myosin light chains. However, artery stretch evoked myosin light chain phosphorylation exclusively through myosin light chain kinase. Similarly, while one or more of the steps between membrane depolarization and Ca^{2+}-dependent myosin phosphorylation has been reported as length dependent, the relationship between Ca^{2+} and myosin phosphorylation was not length dependent (Hai, 1991).

Finally the results presented suggest no role for an internal ryanodine-sensitive Ca^{2+} store in myogenic contraction. Ca^{2+} entry through voltage-dependent Ca^{2+} channels may trigger an additional release of Ca^{2+} from internal stores (calcium-induced calcium release, CICR) to contribute to the myogenic response. In the present study, however, ryanodine, an agent that disables CICR, did not alter the myogenic response.

In summary, the results presented suggest that myogenic contraction is dependent completely on Ca^{2+} entry through voltage-dependent Ca^{2+} channels. In addition, the voltage-dependent Ca^{2+} current can be increased by membrane stretch. It is possible, therefore, that stretch modulation of the voltage-dependent Ca^{2+} current plays a role in myogenic contraction.

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