Kinase activation and smooth muscle contraction in the presence and absence of calcium

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Purpose: **The intracellular signalling mechanisms that modulate the sustained vascular smooth muscle contractions that occur with vasospasm are not well understood. The purpose of this investigation was to examine cell signalling mechanisms that account for sustained vascular smooth muscle contraction, independent of increases in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$).**

Methods: **Fresh bovine carotid artery smooth muscles contractile responses were examined in a muscle bath. $[Ca^{2+}]_i$ was depleted by use of the extracellular Ca^{2+} chelator, ethylene glycol-bis(β-aminoethylether) N, N, N′, N′-tetraacetic acid and the intracellular chelator, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′,-tetraacetic acid.**

Results: **In Ca^{2+}-free conditions, depolarizing the membrane with high extracellular KCl failed to elicit a contraction. In addition, in Ca^{2+}-free conditions the ($[Ca^{2+}]_i$) was less than 10 nmol/L as determined with the Ca^{2+}-indicator, Fura 2. The protein kinase C (PKC) activator, phorbol 12, 13-dibutyrate (PDBu), induced slowly developing sustained contractions in bovine carotid artery smooth muscle, and the magnitude of the contractile response to PDBu (10 nmol/L to 10 μmol/L) was the same in the presence and absence of Ca^{2+}. PDBu induced contractions in Ca^{2+}-free conditions were not inhibited by the myosin light chain kinase inhibitor, ML-9 (50 μmol/L), but were inhibited by the PKC inhibitor, staurosporine (50 nmol/L).**

Conclusions: **These data suggest that vascular smooth muscle contractions can occur under conditions where the $[Ca^{2+}]_i$ is low and fixed and that these contractions may be mediated by PKC. (J Vasc Surg 1995;22:37-44.)**

Vascular smooth muscle is unique in that sustained contractions can be maintained with minimal energy expenditure. Sustained vascular smooth muscle contraction is important for maintaining vasomotor tone. However, sustained vascular smooth muscle contraction may be deleterious in pathologic vasospasm. Vasospasm contributes to mesenteric ischemia, myocardial infarction, and stroke associated with subarachnoid hemorrhage and often complicates peripheral arterial reconstructions.

The *initiation* of contraction in vascular smooth muscle is believed to arise from an increase in intracellular Ca^{2+} with activation of the Ca^{2+}-calmodulin-dependent enzyme, myosin light chain kinase (MLCK) that in turn phosphorylates 20 kd myosin light chains.[1] However, considerable controversy exists regarding the mechanisms involved in the maintenance of vascular smooth muscle contraction. This controversy centers on data that show that *sustained* vascular smooth muscle contractions can be maintained independent of changes in intracellular calcium concentration ($[Ca^{2+}]_i$) and the extent of myosin light chain phosphorylation.[2-4] To account for these data, the "latch bridge hypothesis" has been proposed that suggests that dephosphorylation of myosin generates a slowly cycling cross-bridge, the latch bridge, which is responsible for force maintenance.[5] Alternatively, a number of investigators have

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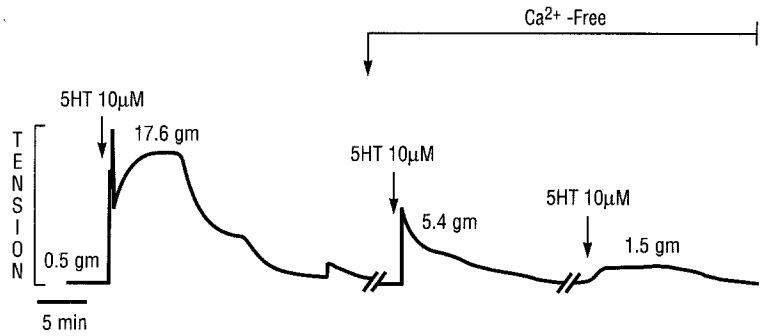

Fig. 1. Contractile response to repeated additions of serotonin in Ca^{2+}-free conditions. This is representative tracing of response of BCASM strips to repeated additions of serotonin (5-HT, 10 μmol/L, at 30 minute intervals) after equilibration in Ca^{2+}-free (+4 mmol/L EGTA and 0.1 mmol/L BAPTA) buffer. Contractile response progressively diminished, suggesting that intracellular Ca^{2+} stores were eventually depleted. After 3 to 5 additions of 5-HT contractile response was less than 5% of response in presence of Ca^{2+}.

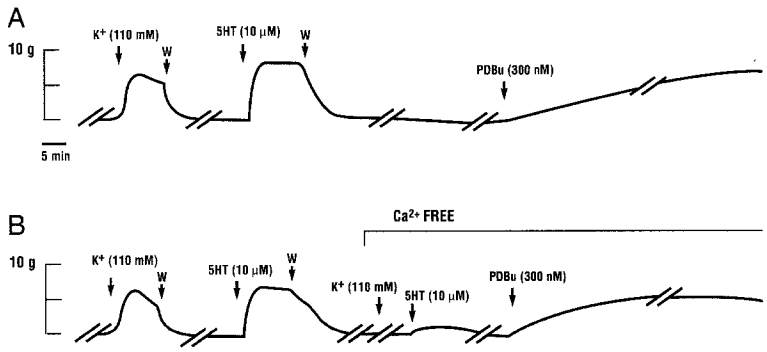

Fig. 2. Contractile responses of BCASM induced by PDBu in presence and absence of Ca^{2+}. BCASM strips were equilibrated in normal bicarbonate buffer for 60 minutes. Baseline contractile responses to 110 mmol/L KCL and serotonin (5HT, 10 μmol/L) were determined in Ca^{2+} containing buffer. Some muscle strips were then incubated in Ca^{2+}-free (+4 mmol/L EGTA and 0.1 mmol/L BAPTA) buffer for 60 minutes and then treated with PDBu (300 nmol/L) until contraction reached plateau. PDBu elicited slowly developing, sustained contractions of similar magnitude in presence **(A)** and absence **(B)** of Ca^{2+}. These are actual tracings that are representative of at least 8 separate experiments.

proposed that activation of other kinase cascades may be important for the sustained phase of vascular smooth muscle contraction.[6-8]

A significant body of data supports a role for protein kinase C (PKC) in sustained vascular smooth muscle contraction.[6,7,9] In particular, most vasoconstricting peptides interact with receptors linked to the phosphoinositide system.[10-12] Phosphoinositide hydrolysis leads to the production of inositol trisphosphate and diacylglycerol. Although inositol triphosphate liberates Ca^{2+} from intracellular stores, the major function of diacylglycerol is activation of PKC.[13] Phorbol esters, which directly activate PKC,[14] induce contraction in numerous vascular smooth muscles.[15-17] PKC translocation is temporally associated with agonist-induced vascular smooth muscle contraction.[18] In addition, similar late-phase phosphoproteins are phosphorylated and dephosphorylated in agonist-induced contractions and in phorbol ester–induced contractions.[19]

The hypothesis of this investigation is that increases in $[Ca^{2+}]_i$ and activation of MLCK are important for the *initiation* of vascular smooth muscle contraction but that other kinases such as PKC are important for the *sustained* phase of vascular smooth muscle contraction. The purpose of this investigation was to produce experimental conditions under which Ca^{2+}-dependent signalling mechanisms were not operative. Under these conditions, agonists that increase intracellular Ca^{2+} do not elicit smooth

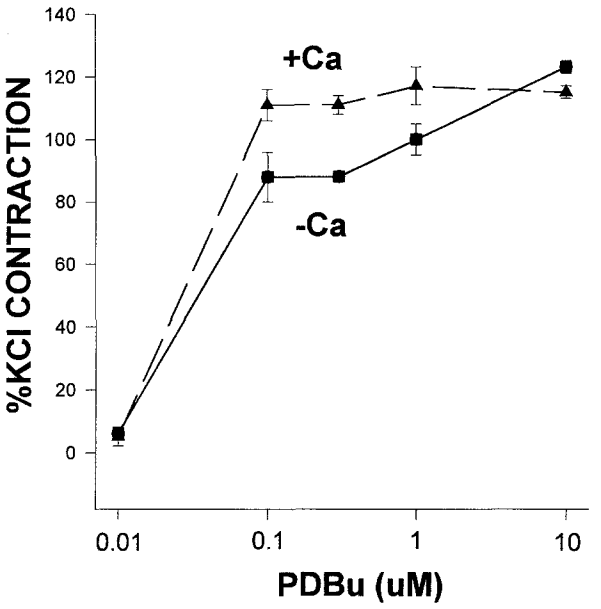

Fig. 3. Dose responses of BCASM to PDBu in presence and absence of Ca^{2+}. After baseline responses to KCl (110 mmol/L) were determined, strips were placed in Ca^{2+}-containing or Ca^{2+}-free (+4 mmol/L EGTA and 0.1 mmol/L BAPTA) buffer for 60 minutes and then treated with PDBu at doses ranging from 10 nmol/L to 10 μmol/L (n = 5 for each dose of PDBu). Responses are displayed as percent of initial KCl contraction in Ca^{2+}-containing buffer. There was no significant difference in magnitude of contractile response in Ca^{2+}-containing *(triangles)* or Ca^{2+}-free *(squares)* conditions to doses of PDBu ranging from 10 nmol/L to 10 μmol/L.

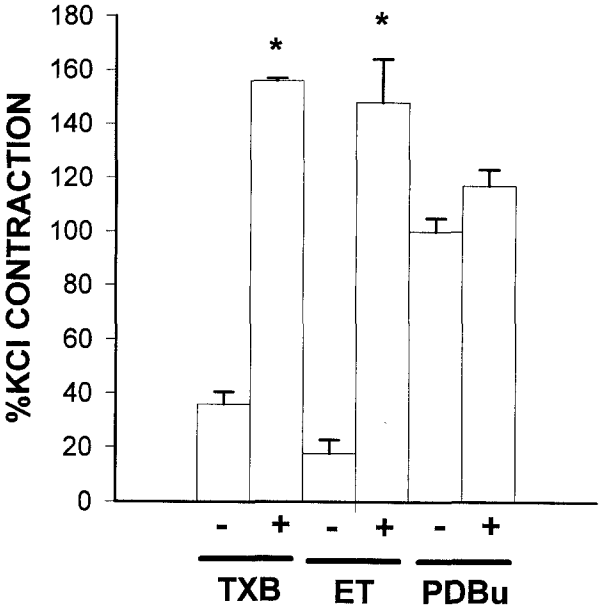

Fig. 4. Contractile responses of BCASM to agonists in absence of Ca^{2+}. Contractile response of BCASM to doses of stable thromboxane agonist U46619 *(TXB,* 100 μmol/L) and endothelin-1 *(ET,* 1 μmol/L), which cause maximal contractions, were significantly attenuated in absence *(minus sign)* of Ca^{2+} ($p < 0.05, n = 4$) compared with Ca^{2+}-containing conditions *(plus sign)*. There was no significant difference in magnitude of contraction to PDBu (1 μmol/L) in presence or absence of Ca^{2+}.

muscle contractions, whereas the phorbol ester, phorbol 12, 13-dibutyrate (PDBu), elicits slowly developing sustained contractions of vascular smooth muscle. This model suggests that Ca^{2+}-independent smooth muscle contractions that are operative through PKC activation.

MATERIAL AND METHODS

Chemicals and reagents. ML-9, KN-62, and phorbol esters were obtained from LC laboratories (Woburn, Mass.), endothelin-1 and staurosporine from Calbiochem (LaJolla, Calif.), the thromoxane analogue U46619 from Cayman Chemical (Ann Arbor, Mich.), Fura-2 from Molecular Probes (Eugene, Ore.), and aequorin from Friday Harbor Photoproteins (Friday Harbor, Wash.).

Smooth muscle contractions. Bovine calf carotid arteries were obtained at a local abattoir and dissected free of adventitial tissue. The arteries were opened longitudinally, and the endothelium was denuded with gentle rubbing of the intimal surface. Transverse strips, 1 to 2 mm in diameter, were cut, and a loop of surgical suture was tied to each end of the strip. The strips were mounted in an automated tissue bath system (Buxco Electronics, Troy, N.Y.). The strips were equilibrated in Krebs bicarbonate solution (NaCl 120 mmol/L, KCl 4.7 mmol/L, $MgSO_4$ 1.0 mmol/L, NaH_2PO_4 1.0 mmol/L, glucose 10 mmol/L, $CaCl_2$ 1.5 mmol/L, and Na_2HCO_3 25 mmol/L) at 37° C and gassed with 95% O_2 and 5% CO_2 for 1 hour. Initial responses to high extracellular KCl (110 mmol/L with equimolar replacement of NaCl) were determined, and the data are reported as a percentage of this initial response. Subsequently, the muscles were equilibrated in Ca^{2+}-free conditions containing 4 mmol/L ethylene glycol-bis(β-aminoethylether) N, N, N′, N′-tetraacetic acid (EGTA) and 0.1 mmol/L 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA/acetyl methyl ester) (an intracellular calcium chelator), for 1 hour.[20] Depletion of extracellular Ca^{2+} was confirmed by repeating the high extracellular KCl contraction. High KCl depolarizes the muscle membrane, allowing extracellular Ca^{2+} to enter the cell.

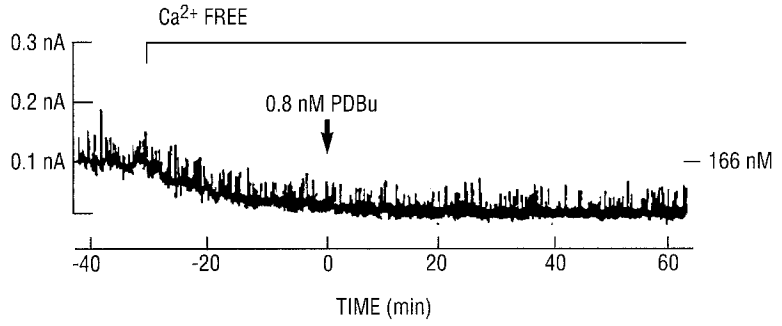

Fig. 5. Intracellular Ca^{2+} concentrations in Ca^{2+}-free conditions with photoprobe aequorin. Aequorin-loaded bovine carotid artery strips were equilibrated in Krebs Ringers bicarbonate solution containing 1.5 mmol/L Ca^{2+}. After 30 minutes strips were placed in Ca^{2+}-free, 4 mmol/L EGTA containing buffer for 60 minutes. Phorbol ester (PDBu) was then added. Intracellular $[Ca^{2+}]_i$ progressively decreased during equilibration in Ca^{2+}-free conditions and did not change with addition of phorbol ester. Calibrated values of $[Ca^{2+}]_i$ are indicated in legend on right.

Intracellular Ca^{2+} stores were considered depleted when 5-hydroxytryptamine (5-HT) failed to elicit a response. Agonists were added directly to the muscle baths in the appropriate dilutions, and the contractions are reported as a percentage of the initial KCl contraction.

Intracellular Ca^{2+} determinations. Intracellular $[Ca^{2+}]_i$ was determined by use of aequorin and Fura-2 as Ca^{2+}-indicators.[21,22] Strips of bovine carotid artery were loaded with aequorin by use of a method that uses permeablizing solutions. The strips were placed sequentially into four solutions for 30 minutes each: solution 1: 10 mmol/L EGTA, 5 mmol/L Na_2ATP, 120 mmol/L KCl, 2 mmol/L $MgCl_2$ 20 mmol/L TES; solution 2: 0.1 mmol/L EGTA 5 mmol/L Na_2ATP, 120 mmol/L KCl, 2 mmol/L Cl_2, 20 mmol/L N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid, aequorin 0.2 mg/ml; solution 3: 0.1 mmol/L EGTA, 5 mmol/L Na_2ATP, 120 mmol/L KCl, 2 mmol/L $MgCl_2$, 20 mmol/L TES; solution 4: 5 mmol/L ATP, 120 mmol/L NaCl, 3.5 mmol/L KCl, 1.5 mmol/L dextrose, 15.5 mmol/L $NaHCO_3$, 10 mmol/L $MgCl_2$, 1.4 mmol/L NaH_2PO_4. Aequorin signals and isometric tensions were measured simultaneously as test substances were added to the reservoir by perifusion. Light output was detected with a photomultiplier tube interfaced with a computer.

For Fura-2 determinations, smooth muscle cells were isolated with enzymatic digestion. Smooth muscle strips were incubated in Hepes buffer containing 3 mg/ml collagenase, 0.7 mg/ml elastase, 1 mg/ml hyaluronidase, 2 mg/ml ATP, 0.1 mg/ml deoxyribonuclease, 1 mg/ml trypsin inhibitor, 15 mg/ml bovine serum albumin, 0.1 mmol/L Ca^{2+} for 30 minutes at 37° C. The smooth muscle cells were pelleted with centrifugation and washed three times in buffer. Dispersed smooth muscle cells were loaded with the Ca^{2+} indicator Fura-2 (1 μmol/L) for 45" at 37° C and placed in a cuvette in a dual wavelength spectrophotometer (PTI, South Brunswick, N.J.) and fluorescence measured by use of excitation wavelengths of 340/380 nmol/L and emission wavelengths of 510 nmol/L. The intracellular Ca^{2+} concentrations were calculated by use of the equation $[Ca^{2+}]_i = k_d \times (R - R_{min}/R_{max} - R)$ where k_d = 224 nmol/L, R = the observed fluorescence, R_{max} = maximal Ca^{2+} concentration (that occurs with lysis of the cells), and R_{min} = minimal Ca^{2+} concentration (in the presence of 4 mmol/L EGTA).

Statistical analysis. Statistical analysis was performed with use of a one-way analysis of variance (ANOVA), and differences were considered significant when the *p* value was less than 0.05.

RESULTS

Contractile responses of carotid artery smooth muscle in the presence and absence of Ca^{2+}. Carotid smooth muscle strips incubated in a Ca^{2+}-free buffer containing 4 mmol/L EGTA and 0.1 mmol/L BAPTA for 60 minutes did not contract on addition of high extracellular KCl. Repeated additions of 10 μmol/L serotonin (5-HT) resulted in progressive decrease in the contractile response, suggesting that intracellular stores of Ca^{2+} were progressively depleted (Fig. 1). Stimulation with PDBu in Ca^{2+}-containing, or Ca^{2+}-free conditions, resulted in a slowly developing sustained contraction (Fig. 2). The tension reached a plateau by 60

minutes. The magnitude of the PDBu-induced contraction, over a range of 10 nmol/L to 10 μmol/L, was similar in the presence and absence of Ca^{2+} (Fig. 3). The magnitude of contractile responses to the thromboxane agonist U-46610 (100 μmol/L) and endothelin-1 (1 μmol/L) were significantly greater in the presence of Ca^{2+} compared to Ca^{2+}-free conditions (Fig. 4).

Effect of Ca^{2+}-free conditions on free cytosolic Ca^{2+} concentrations. The effect of Ca^{2+}-free conditions and of the addition of PDBu on intracellular Ca^{2+} concentrations ($[Ca^{2+}]_c$) was determined by use of aequorin as a Ca^{2+} indicator. When extracellular Ca^{2+} is removed, the $[Ca^{2+}]_c$ in bovine carotid artery smooth muscle (BCASM) decreases (Fig. 5). The subsequent addition of 0.8 μmol/L PDBu resulted in no change in $[Ca^{2+}]_c$.

The intracellular Ca^{2+} concentrations under Ca^{2+}-free conditions were less than 10 nmol/L as determined with the Ca^{2+} indicator, Fura-2. These Ca^{2+} concentrations did not change with the addition of 5-HT (1.0 or 10 μmol/L), suggesting that intracellular Ca^{2+} stores were significantly depleted.

The effect of protein kinase inhibitors on Ca^{2+}-free phorbol ester induced contractions. To determine which kinases are operative in sustained vascular smooth muscle contraction under Ca^{2+}-free conditions, the specific protein kinase inhibitors, ML-9 (an inhibitor of MLCK) and staurosporine (an inhibitor of PKC) were used.[23,24] Preincubation with ML-9 (50 μmol/L) significantly inhibited KCl (110 mmol/L) and 5-HT (10 μmol/L) contractions in Ca^{2+}-containing buffer, yet there was minimal effect on a Ca^{2+}-free PDBu (300 nmol/L) contractions (Fig. 6). Preincubation with staurosporine (100 nmol/L) did not significantly inhibit KCl (110 mmol/L) or 5-HT (10 μmol/L) contractions in Ca^{2+}-containing buffer (Fig. 7). However, staurosporine (50 nmol/L) significantly inhibited Ca^{2+}-free PDBu (300 nmol/L) contractions.

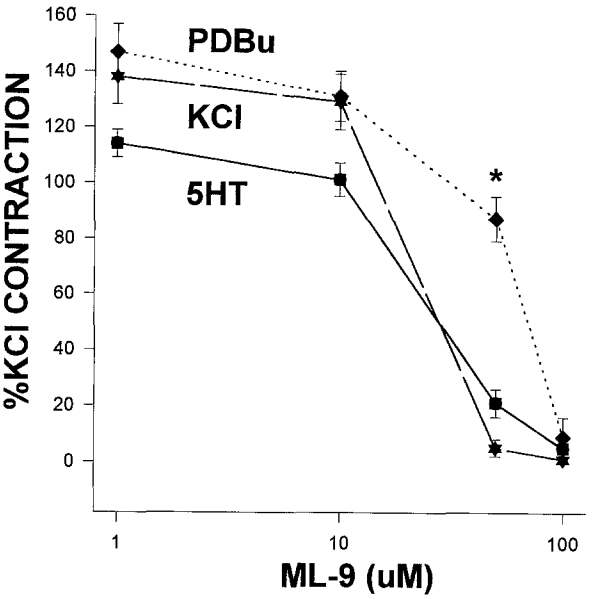

Fig. 6. The effect of ML-9, MLCK inhibitor, on Ca^{2+}-free PDBu contractions. BCASM strips were equilibrated in normal bicarbonate buffer. After baseline responses to KCl (110 mmol/L) were determined, some strips were placed in Ca^{2+}-free (+4 mmol/L EGTA and 0.1 mmol/L BAPTA) buffer for 60 minutes. Strips were preincubated with ML-9 (10 to 100 μmol/L) for 30 minutes and then KCL (110 mmol/L in Ca^{2+}-containing buffer, *closed stars*), 5-HT (10 μmol/L in Ca^{2+}-containing buffer, *closed squares*), or PDBu (300 nmol/L in Ca^{2+}-free buffer, *closed diamonds*) were added. ML-9 (50 μmol/L) significantly *(asterisk)* inhibited KCl (110 mmol/L) and 5-HT (10 μmol/L) contractions in presence of Ca^{2+} yet did not significantly inhibit PDBu (300 nmol/L) contractions in Ca^{2+}-free conditions ($p < 0.05$ one-way ANOVA, $n = 4$ for each dose of ML-9).

DISCUSSION

Vascular smooth muscle is unique in that sustained contractions can be maintained with minimal energy use.[6] This tonic contractile state may account for pathologic vasospastic states. Although Ca^{2+}-dependent activation of MLCK has been implicated in the rapid initiation of vascular smooth muscle contraction,[1] less is known about the cellular signalling events that modulate the sustained phase of contraction that occurs in vasospasm. PKC activation has been implicated in activating a kinase cascade that leads to a tonic contractile state.[6-8] Phorbol esters pharmacologically activate PKC and have been shown to induce vascular smooth muscle contraction.[15,16]

In this study, a model of Ca^{2+}-independent vascular smooth muscle contraction was developed. The phorbol ester, PDBu, induced contraction in bovine carotid artery smooth muscle (BCASM) in the absence of extracellular Ca^{2+} and under conditions where the intracellular Ca^{2+} concentration was at a low and fixed level (Figs. 1 to 3). Under these Ca^{2+}-free conditions, high extracellular KCl did not induce a contraction. The contraction elicited by repeated additions of the agonist serotonin progressively diminished to less than 5% of that which occurred when Ca^{2+} was present (Fig. 1). Normal resting intracellular Ca^{2+} concentrations in vascular smooth muscle are 150 to 200 nmol/L.[23] Under the Ca^{2+}-free conditions used in this study, the intracel-

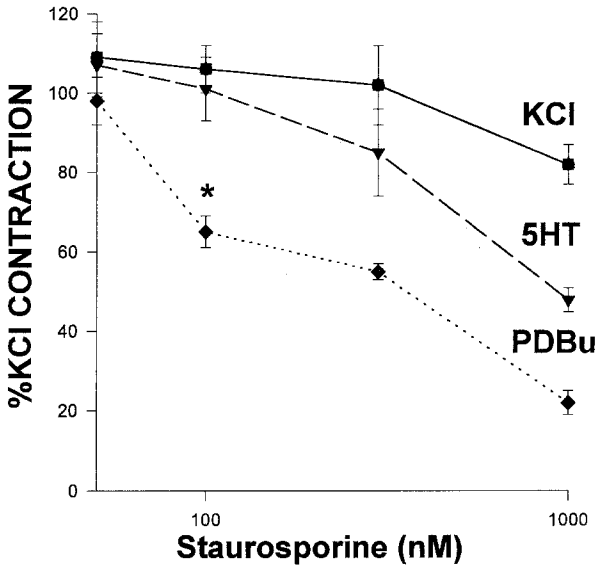

Fig. 7. Effect of staurosporine, PKC inhibitor on Ca^{2+}-free PDBu contractions. BCASM strips were equilibrated in normal bicarbonate buffer. After baseline responses to KCl (110 mmol/L) were determined, some strips were placed in Ca^{2+}-free (+4 mmol/L EGTA and 0.1 mmol/L BAPTA) buffer for 60 minutes. Strips were preincubated with staurosporine (10 to 1000 nmol/L) for 30 minutes and then KCL (110 mmol/L in Ca^{2+}-containing buffer, *closed squares*), 5-HT (10 μmol/L in Ca^{2+}-containing buffer, *closed triangles*), or PDBu (300 nmol/L in Ca^{2+}-free buffer, *closed diamonds*) were added. Staurosporine (100 nmol/L) significantly *(asterisk)* inhibited PDBu contractions in Ca^{2+}-free conditions to greater extent than KCl or 5-HT contractions in presence of Ca^{2+} ($p < 0.05$ one-way ANOVA, $n = 4$ for each dose of staurosporine).

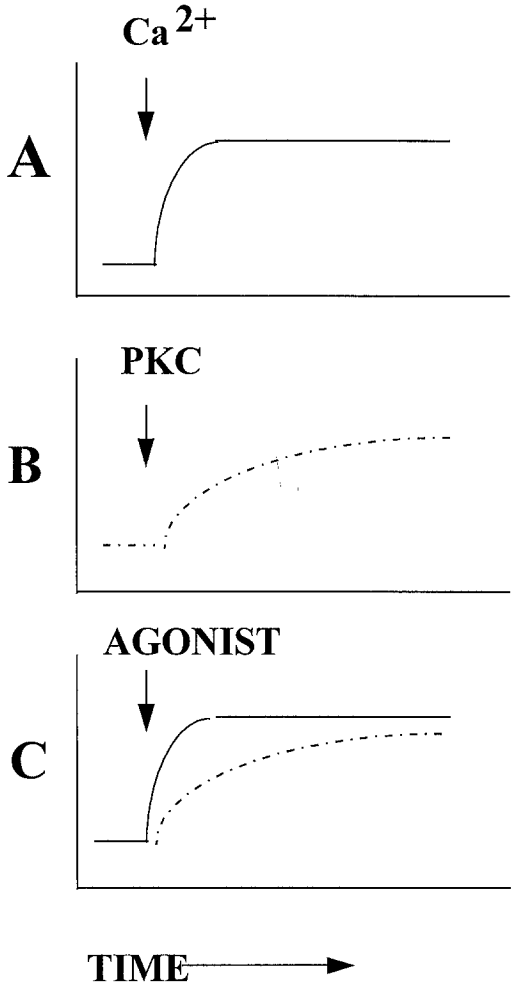

Fig. 8. Model of signalling mechanisms in VSM contraction. Rapid rise in intracellular Ca^{2+}, that occurs when depolarization of smooth muscle membrane with high extracellular KCl leads to rapid initiation of VSM contraction **(A)** *(solid line)*. Phorbol esters, PKC activators, induce slowly developing sustained contractions in presence or absence of Ca^{2+} **(B)** *(broken line)*. Composite of increase in intracellular Ca^{2+} and PKC activation may account for initial and sustained aspects of agonist induced contractions **(C)**.

lular Ca^{2+} concentration was less than 10 nmol/L and did not change with serotonin or PDBu stimulation (Fig. 4).

Activation of PKC and not MLCK leads to Ca^{2+}-free PDBu-induced contractions in that Ca^{2+}-free contractions were not inhibited by low concentrations (50 μmol/L) of the MLCK inhibitor ML-9, but Ca^{2+}-dependent contractions induced by high extracellular KCl and 5-HT were inhibited by ML-9 (Fig. 5). The ED_{50} of ML-9 for MLCK in vitro is 4 μmol/L and for PKC 54 μmol/L.[25] Thus, at higher concentrations of ML-9 (100 μmol/L), the inhibitor is probably producing complete inhibition of KCl, 5-HT, and PDBu contractions through inhibition of both MLCK and PKC. The PKC inhibitor, staurosporine (100 nmol/L), inhibits Ca^{2+}-free PDBu contractions to a greater extent than Ca^{2+}-dependent KCl or 5-HT–induced contractions (Fig. 6). The ED_{50} of staurosporine for PKC in vitro is 7 nmol/L and for MLCK 13 nmol/L.[26] The relatively high doses of staurosporine required to inhibit muscle contractions in these experiments may be due to poor penetration of the inhibitor into the muscle strips. The partial inhibition of KCl and 5-HT contractions may be due to overlap between PKC and MLCK inhibition.

Phorbol ester induced contractions in Ca^{2+}-free conditions may be due to the activation of Ca^{2+}-independent isoforms of PKC. Vascular smooth

muscle contains the Ca^{2+}-dependent α-isoform and the calcium-independent ε-isoform.[27] Alternatively, phorbol esters may be overcoming the Ca^{2+} requirement of PKC for activation.[28] Further work is needed to determine which isoforms of PKC are operative in both physiologic and pharmacologic contractions of vascular smooth muscle.

Ca^{2+}-independent contractions were elicited in BCASM by the physiologically relevant thromboxane analogue (U46619) and endothelin (Fig. 4). The magnitude of these contractions were significantly less than the magnitude of contractions in the presence of Ca^{2+}. This is similar to what has been described for Ca^{2+}-free endothelin-induced contractions in porcine coronary arteries and prostaglandin ($PGF_{2\alpha}$)-induced contractions single permeabilized ferret aorta cells.[24,29] Endothelin-induced, Ca^{2+}-free contractions are inhibited by the PKC inhibitor H-7,[24] and the $PGF_{2\alpha}$-induced Ca^{2+}-independent contractions were inhibited by staurosporine,[29] suggesting at least a partial role for PKC in agonist-induced Ca^{2+}-independent smooth muscle contraction.

It has been suggested that PKC modulates sustained contraction through the activation of a kinase cascade that involves mitogen-activated protein kinase, which leads to caldesmon phosphorylation.[30] Caldesmon is a thin filament regulatory protein in smooth muscle that inhibits actinomyosin ATPase. This inhibition is reversed by the phosphorylation of caldesmon.[31] Thus it is possible that activation of "Ca^{2+}-independent" PKC pathways may lead to sustained vascular smooth muscle contraction through the activation of a kinase cascade.

Activation of PKC has been implicated in the pathologic vasospasm that accompanies cerebral vasospasm.[32,33] In an animal model of subarachnoid hemorrhage–induced vasospasm, the PKC-mediated vascular smooth muscle contractions occurred in Ca^{2+}-free media.[34] The possible role of Ca^{2+}-independent PKC activation in other acute vasospastic states has yet to be determined.

These data support a model of vascular smooth muscle contraction where the initial rapid phase of contraction that occurs with depolarization of the membrane with KCl is caused by Ca^{2+}-dependent, MLCK-mediated mechanisms (Fig. 8). However, the slowly developing tonic phase of vascular smooth muscle contraction can be pharmacologically induced with the PKC activators phorbol esters under circumstances where the extracellular and intracellular Ca^{2+} concentrations are low and fixed. Thus the sustained phase of vascular smooth muscle contraction may be mediated by a kinase cascade that involves activation by PKC. This model of phorbol ester–induced Ca^{2+}-free vascular smooth contraction will be useful in further elucidating the subsequent intracellular signalling events that lead to tonic vascular smooth muscle contraction or vasospasm.

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