Regulation of Ca\textsuperscript{2+} sensitization by PKC and rho proteins in ovine cerebral arteries: effects of artery size and age

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Akopov, Sergey E., Lubo Zhang, and William J. Pearce. Regulation of Ca\textsuperscript{2+} sensitization by PKC and rho proteins in ovine cerebral arteries: effects of artery size and age. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H930–H939, 1998.—G protein-regulated Ca\textsuperscript{2+} sensitivity of vascular contractile proteins plays an important role in cerebrovascular reactivity. The present study examines the intracellular mechanisms that govern G protein-regulated Ca\textsuperscript{2+} sensitivity in cerebral arteries of different size and age. We studied β-escin-permeabilized segments of common carotid, basilar, and middle cerebral arteries from nonpregnant adult and near-term fetal sheep. Activation of protein kinase C (PKC) by (-)-indolactam V or a phorbol ester produced receptor-independent increases in Ca\textsuperscript{2+} sensitivity. Such increases were more marked in immature arteries and were inversely correlated with artery size in both mature and immature arteries. However, inhibitors of PKC did not significantly affect increases in Ca\textsuperscript{2+} sensitivity in responses to either serotonin (5-hydroxytryptamine, 5-HT) or guanosine 5’-O-(3-thiotriphosphate) (GTP\textsuperscript{γ}S). Alternatively, deactivation of rho p21, a small G protein associated with Rho kinase, by exotoxin C3 fully prevented increases in Ca\textsuperscript{2+} sensitivity in responses to 5-HT or GTP\textsuperscript{γ}S in both adult and fetal arteries of all types. Neither inhibitors of PKC nor exotoxin C3 altered baseline Ca\textsuperscript{2+} sensitivity. We conclude that patterns of receptor- and/or G protein-mediated modulation of Ca\textsuperscript{2+} sensitivity are dependent on an intracellular pathway that involves activation of small G proteins and Rho kinase. In contrast, PKC has little, if any, role in agonist-induced Ca\textsuperscript{2+} sensitization under the present experimental conditions.

calcium sensitivity; G proteins; rho p21; maturation; serotonin; sheep

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PKC- and/or rho p21-dependent pathways of G protein-induced Ca\(^{2+}\) sensitization influence cerebrovascular reactivity in the ovine cerebral circulation. From our previous data demonstrating that Ca\(^{2+}\) sensitization may be modulated by age (2), a second goal of these studies was to evaluate the effects of maturation on the mechanisms by which G proteins alter cerebrovascular Ca\(^{2+}\) sensitivity.

**METHODS**

All protocols and procedures used in these studies were reviewed and approved by the Animal Research Committee of Loma Linda University.

**General Preparation**

Common carotid (Com), basilar (Bas), and middle cerebral (MCA) arteries were obtained from young adult sheep (18–24 mo old) and near-term (≈140 days of gestation) fetuses. Segments of cranial arteries were withdrawn and placed in a Krebs solution containing (in mM) 122 NaCl, 25.6 NaHCO\(_3\), 5.56 dextrose, 5.17 KCl, 2.49 MgSO\(_4\), 1.60 CaCl\(_2\), 0.114 ascorbic acid, and 0.027 EGTA, which was continuously bubbled with 95% O\(_2\)-5% CO\(_2\). Each artery segment was cleaned of adhering tissues, cut into a segment ≈2–3 mm long, mounted on wires, and suspended between a force transducer and a post attached to a micrometer. Measurements of vessel contractility were performed at optimal stretch as previously described (7). To avoid any possible endothelium-mediated effects, we removed the endothelium by rotating each arterial segment around the mounting wires several times to gently scrape the entire luminal surface. After equilibration at optimal baseline stretch, the artery segments were incubated in a relaxing solution that contained (in mM) 5 EGTA, 5 ATP, 110 potassium acetate, 6 magnesium acetate, 1 dithiothreitol, 0.01 leupeptin, and 20 imidazole, at pH 6.8 (titrated with KOH). Chemical skinning was achieved by treating with β-escin (100 µM for Bas and MCA, 150 µM for Com) at 25°C for 20 min. Permeabilization procedures, Ca\(^{2+}\) buffer preparations, and verification of permeabilization in cerebral arteries of different size and age have previously been described in detail and shown to be optimum for each artery type used in this study (1, 2). All measurements of Ca\(^{2+}\) sensitivity were performed in the presence of calmodulin (1 µM) after irreversible depletion of internal Ca\(^{2+}\) stores in permeabilized preparations by incubation with 10 µM A-23187 for 15 min. All materials used for permeabilization were obtained from Sigma Chemical (St. Louis, MO).

**Experimental Protocols**

Three major protocols were used in the present study.

**Protocol A.** Effects of PKC activation on Ca\(^{2+}\) sensitivity. Segments of MCA, Bas, and Com were first contracted by exposure to 120 mM K\(^+\) to obtain a maximal contraction under intact conditions and then permeabilized. Contractile responses of permeabilized Ca\(^{2+}\)-depleted artery rings were recorded during sequential administrations of Ca\(^{2+}\) buffer solutions with increasing free Ca\(^{2+}\) concentrations between 0.01 and 10 µM. After completion of the Ca\(^{2+}\) dose-response protocol, the arteries were returned to relaxing solution. To test the effects of the selective PKC activator, (−)-indolactam V (Sigma), on Ca\(^{2+}\) sensitivity, the arteries were exposed to a submaximal concentration of free Ca\(^{2+}\), which in accordance with previous measurements of Ca\(^{2+}\)-dependent force was approximately the EC\(_{30}\). Once the contractile responses to this concentration of Ca\(^{2+}\) had stabilized, graded concentrations of indolactam V (0.03–10 µM) were added. The arteries were then returned to relaxing solution, treated with indolactam V at its EC\(_{50}\), and exposed once again to graded concentrations of Ca\(^{2+}\) (0.01 and 10 µM). Finally, the arteries were returned to relaxing solution, contracted by exposure to a submaximal concentration of free Ca\(^{2+}\), and then treated with 3 µM of (−)-indolactam V shown to be biologically inactive with respect to PKC activation (13). In some experiments, the arteries precontracted with a submaximal concentration of free Ca\(^{2+}\) were additionally exposed to a phorbol ester, phorbol 12,13-dibutyrate (PDBu, 1 µM; Sigma).

**Protocol B.** Effects of PKC inhibitors on 5-HT- and guanosine 5′-O-(3-thiotriphosphate)-induced increases in Ca\(^{2+}\) sensitivity. Paired segments of MCA, Bas, and Com were mounted and studied in parallel. First, the segments were exposed to 120 mM K\(^+\) to obtain a maximal contraction under intact conditions. The segments were then permeabilized, and one member of each pair served as a control while the other was exposed to an inhibitor of PKC. All subsequent measurements were performed on the second segment. Two PKC inhibitors were tested, 1-((5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7, Sigma) and calphostin (Calbiochem, La Jolla, CA). Dose-finding experiments were conducted to determine the optimal concentration of these agents (see RESULTS). Because calphostin is light sensitive, its solutions were made in the dark and constantly protected from light. After addition of calphostin to the bath, the latter was illuminated by visible light (a 100-W incandescent bulb) because calphostin inhibits PKC only in the presence of light, which is needed for formation of free radicals and subsequent site-specific oxidative modification of PKC (11).

Both treated and control segments were exposed to graded concentrations of free Ca\(^{2+}\) (0.01–10 µM) to obtain Ca\(^{2+}\)-dose-response curves characterizing baseline Ca\(^{2+}\) sensitivity. Then, to test the effects of 5-HT on Ca\(^{2+}\) sensitivity, the arteries were exposed to a submaximal concentration of free Ca\(^{2+}\), which in accordance with our previous measurements of Ca\(^{2+}\)-dependent force was approximately the EC\(_{30}\). Once the contractile responses to this concentration of Ca\(^{2+}\) had stabilized, 10 µM 5-HT was added and contractile responses were again recorded. The arteries were then returned to relaxing solution, precontracted by the EC\(_{30}\) of Ca\(^{2+}\), and exposed to 1 µM of (−)-indolactam V. Finally, the arteries were once again returned to relaxing solution, contracted by exposure to a submaximal concentration of free Ca\(^{2+}\), and then treated with 100 µM guanosine 5′-O-(3-thiotriphosphate) (GTPγS) to activate all G proteins regardless of receptor occupation. We have previously shown that the concentrations of 5-HT and GTPγS used are maximal in these preparations (1, 2).

**Protocol C.** Effects of exotoxin C3 on 5-HT- and GTPγS-induced increases in Ca\(^{2+}\) sensitivity. Protocol C was similar to protocol B except that, instead of PKC inhibitors, the treated segments were treated with exotoxin C3. This treatment was applied in a relaxing solution containing 1 µg/ml exotoxin C3 (Calbiochem, La Jolla, CA), 10 µM NAD, and 50 µM GTP for 25 min at 28°C, and these substances were then washed out three times with normal relaxing solution. As shown previously, this procedure provides ADP ribosylation of rho p21 proteins, resulting in their full inactivation (9, 19, 24).

**Data Analysis and Statistics**

All values are given as means ± SE. In all cases, n refers to the number of animals studied. Unless indicated otherwise,
RESULTS

A total of 197 artery preparations were obtained from 25 young adult sheep and 21 near-term fetuses.

General Characteristics

Values of absolute tension produced by 120 mM K+ averaged 5.7 ± 0.4, 3.6 ± 0.4, and 1.9 ± 0.3 g for adult Com, Bas, and MCA segments, respectively. Corresponding values in fetal arteries averaged 4.4 ± 0.3, 2.2 ± 0.2, and 1.1 ± 0.1 g. For all arteries studied, the constrictor effect of K+ disappeared after permeabilization. Conversely, the addition of 10 µM Ca2+ before permeabilization had no effect on contractile tension in any artery type, whereas after β-escin treatment, Ca2+ induced sustained contractions whose magnitudes varied in relation to β-escin concentration. As previously described, the ratio of Ca2+-induced contraction after permeabilization to K+-induced contraction before permeabilization was calculated as a criterion of full permeabilization (2). Across all adult artery segments used in these studies, maximal levels of contractile tensions induced by 10 µM Ca2+ after permeabilization averaged 125 ± 6, 133 ± 5, and 131 ± 7% of the corresponding maximal tensions induced before permeabilization by 120 mM K+ for Com, Bas, and MCA, respectively. Across all fetal artery segments used, the corresponding values averaged 123 ± 4, 127 ± 6, and 123 ± 4%, respectively.

All permeabilized arterial preparations responded to graded concentrations of Ca2+ in a dose-dependent manner. Analysis of the pCa-force relations demonstrated that they were generally left shifted in Bas relative to Com segments and in MCA relative to Bas segments in both adult and fetal arteries. Correspondingly, Ca2+-pD2 values were lower for Com (5.97 ± 0.03, n = 17) than for Bas (6.43 ± 0.04, n = 20, P < 0.0001) or MCA (6.74 ± 0.05, n = 14, P < 0.0001) in adult arteries. In fetal arteries, corresponding values averaged 6.26 ± 0.04 (n = 14), 6.65 ± 0.05 (n = 16), and 6.78 ± 0.06 (n = 12) for Com, Bas, and MCA, respectively. Differences between Com and intracerebral arteries were statistically significant (P < 0.001).

To avoid complications created by vessel rundown during our experimental measurements, we repeatedly challenged the permeabilized artery preparations with a submaximal concentration of Ca2+ and monitored the resulting development of contractile force. In preparations in which force production in response to consecutive administrations of a given level of Ca2+ began to decrease, that particular segment was excluded from further study. In addition, this approach enabled repeated measurements of Ca2+-sensitization at similar levels of tension produced by submaximal Ca2+ concentrations (2) and thereby standardized the initial level of contractile tension. This standardization facilitated direct comparisons within and between artery types regarding the effects of variable agonists on Ca2+ sensitivity. In adult arteries, the estimated EC50 of Ca2+ produced contractile tensions of 26.5 ± 1.6, 27.5 ± 1.7, and 31.4 ± 3.5% of maximal contractile tensions in Com, Bas, and MCA segments, respectively. These values did not differ significantly from one another or from those obtained from corresponding fetal arteries, which averaged 26.7 ± 1.4, 27.0 ± 1.3, and 25.5 ± 1.3% of maximal tensions, respectively. Addition of 5-HT (10 µM) or GTPγS (100 µM) produced sustained increases in force in both adult and fetal arteries. In adult arteries, the 5-HT-induced increases averaged 54.1 ± 8.3 (n = 11), 60.9 ± 2.9 (n = 14), and 57.5 ± 5.2% (n = 10) above initial Ca2+-induced tension for Com, Bas, and MCA, respectively. These values were significantly (P < 0.01) less than corresponding values in fetal arteries, which averaged 86.5 ± 7.8 (n = 10), 96.9 ± 4.5 (n = 12), and 107.7 ± 8.6% (n = 8). Similarly, effects of GTPγS were significantly (P < 0.01) greater in fetal than adult arteries. In Com, Bas, and MCA, GTPγS increased force an average of 57.5 ± 8.1 (n = 11), 73.9 ± 6.7 (n = 14), and 65.6 ± 4.5% (n = 10) in adult arteries and 104.5 ± 4.8 (n = 10), 107.3 ± 6.5 (n = 12), and 101.7 ± 6.4% (n = 8) in fetal arteries.

Effects of PKC Activation on Ca2+ Sensitivity

When incubated in relaxing solution containing 5 mM EGTA and no added Ca2+, no contractile response was produced in any of the preparations by PDBu, (-)-indolactam V, or (+)-indolactam V. In contrast, in both adult and fetal vessel preparations precontracted with submaximal Ca2+ concentrations, PDBu and (-)-indolactam V increased force significantly, whereas (+)-indolactam V remained inactive. Curves representing these responses are shown for Bas segments in Fig. 1. Further analysis focused on the effects of (-)-indolactam V, which is a highly sensitive activator of PKC with clear dose-dependent characteristics of contractile effects (12, 13, 26).

Under our conditions, (-)-indolactam V produced dose-dependent increases in force in the presence of submaximal concentrations of Ca2+ in both adult and fetal arterial preparations (Fig. 2). Maximal contractions were observed at (-)-indolactam V concentrations of 1 µM, and further increases in concentration did not further increase arterial tone. Maximal effects of (-)-indolactam V in adult arteries averaged 56.5 ± 5.6 (n = 12), 88.5 ± 4.5 (n = 13), and 109.4 ± 7.2% (n = 9) of initial Ca2+-induced tone for Com, Bas, and MCA, respectively. Corresponding values in the fetal arteries averaged 136.7 ± 10.7 (n = 9), 128.5 ± 8.3 (n = 11), and 176.8 ± 9.5% (n = 9). Two-way ANOVA revealed that the maximal (-)-indolactam V-produced force was significantly greater (P < 0.01) in fetal compared with...
adult arteries across all artery types. For between-artery comparisons, no significant differences in maximal effect between Com and Bas segments were observed in either the fetus or adult, although in the adult, the values tended to be greater in Bas than in Com. In both the fetus and adult, the maximal magnitude of (-)-indolactam V-produced force was significantly greater in MCA segments than in either Bas or Com segments. With regard to the dose-related effects of (-)-indolactam V, the magnitude of (-)-indolactam V-induced force was greater in fetal than adult arteries at all concentrations tested (Fig. 2). However, pD2 values for (-)-indolactam V-induced contractions were similar in all arteries independent of age or artery type (Fig. 2).

To evaluate the effects of PKC activation on pCa-force relations, we compared dose-dependent contractile effects of graded concentrations of Ca2+ in the absence and presence of (-)-indolactam V at a concentration of 0.1 µM (EC50). Figure 3 shows that, in the presence of (-)-indolactam V, the pCa-force curves were shifted to the left across all adult and fetal arteries studied, with statistically significant increases in all corresponding pD2 values.

**Effects of PKC Inhibitors on Ca2+ Sensitivity**

Analysis of the effects of H-7 on increases in force produced by (-)-indolactam V (1 µM) revealed that, at concentrations of >0.1 µM, H-7 fully eliminated (-)-indolactam V-induced contractile responses in both adult and fetal arteries (Fig. 4). Control measurements further revealed that, at concentrations of 1 µM, H-7 also fully prevented contractile effects of 1 µM PDBu (not shown).

At 1 µM, H-7 did not influence pCa-force relations in either adult or fetal arteries. In adult arteries, pD2 values for Ca2+ averaged 5.95 ± 0.03, 6.46 ± 0.03, and 6.76 ± 0.06 for Com (n = 4), Bas (n = 5), and MCA (n = 3), respectively, in the absence of H-7. In the presence of H-7, these values averaged 5.96 ± 0.05, 6.43 ± 0.03, and 6.76 ± 0.08. Corresponding values in fetal arteries averaged 6.40 ± 0.08, 6.82 ± 0.09, and 6.99 ± 0.09 in the absence of H-7 and 6.36 ± 0.07, 6.81 ± 0.09, and 7.04 ± 0.11 in the presence of H-7 for Com (n = 3), Bas (n = 5), and MCA (n = 3), respectively. Furthermore, H-7 (1 µM) did not affect the increases in force produced by 5-HT or GTPγS in arterial preparations precontracted with submaximal concentrations of Ca2+ (Fig. 5). However, at higher concentrations (10 µM), H-7 moderately attenuated these effects in both adult and fetal arteries (Fig. 5).

Calphostin reliably prevented the responses to 1 µM (-)-indolactam V in both adult and fetal arteries at a concentration of 0.1 µM. In adult control arteries, (-)-indolactam V produced increases in force averaging 53.5 ± 9.9, 75.3 ± 3.8, and 136.9 ± 1.9% for Com, Bas,
and MCA, respectively, whereas in the same arterial preparations treated with 0.1 μM calphostin, responses to (-)-indolactam V averaged 2.5 ± 1.7, 0.8 ± 0.8, and 1.6 ± 1.5%. Similarly, in control fetal arteries, (-)-indolactam V increased tensions by 136.7 ± 29.3, 112.4 ± 5.3, and 209.0 ± 26.5% for Com, Bas, and MCA, respectively, whereas in calphostin-treated arteries, its effect averaged 0.1 ± 3.7, 2.3 ± 1.9, and 0.0 ± 1.5%. At higher concentrations, however, calphostin diminished the amplitudes of Ca²⁺ contractions (not shown), and it was therefore not appropriate to use this agent at concentrations of >0.1 μM. Like H-7, calphostin (0.1 μM) did not alter either pCa-force relations or 5-HT and GTPγS effects on arteries precontracted with submaximal Ca²⁺ concentrations (Fig. 6). An absence of such effects was observed both in adult and fetal arteries of all types (Fig. 6).

Fig. 2. Effects of (-)-indolactam V on Ca²⁺-induced force in β-escin-permeabilized ovine cranial arteries (Com, common carotid artery; MCA, middle cerebral artery). Segments of cranial arteries from adults (solid lines) and fetuses (dashed lines) were permeabilized with β-escin and then contracted with a submaximal concentration of Ca²⁺ (−EC30). When contractile responses had stabilized, cumulatively increasing concentrations of (-)-indolactam V (abscissa) were then added and percent increases in tension were recorded (ordinate). Corresponding pD2 values for (-)-indolactam V-force relations are presented on right. All values are given as means ± SE for n = 4–6 in all groups. *Significant differences between corresponding adult and fetal arteries.

Effects of Exotoxin C3 on Ca²⁺ Sensitivity

Exotoxin C3 (1 μg/ml) did not alter pCa-force relations in any of the arteries studied (Fig. 7). However, at the same concentration, exotoxin C3 markedly reduced increases in force produced by 5-HT or GTPγS. Their effects virtually disappeared in both adult and fetal arteries after treatment with exotoxin C3 (Fig. 7). Interestingly, the effects of (-)-indolactam V and PDBu (1 μM) were not altered by exotoxin C3 in either adult or fetal arteries (Fig. 8).

DISCUSSION

Recent studies clearly establish Ca²⁺ sensitivity as a variable, biochemically regulated determinant of vascular reactivity in many vascular beds (21, 22, 33), including the cerebral circulation (1, 2, 26). Thus regulation of cytosolic Ca²⁺ concentration is only one component of the pharmacomechanical coupling that triggers and maintains contraction of smooth muscle cells in response to receptor activation. In addition, agonist-induced Ca²⁺ sensitization is responsible for a significant portion of the increase in contractile tone resulting from activation of adrenergic, serotonergic, thromboxane, and endothelin receptors. The diversity of receptor types that involve Ca²⁺ sensitization suggests a universal mechanism coupling receptor activation to contraction.

Despite clear indications that activation of G proteins is a first step in Ca²⁺ sensitization, the subsequent events coupling this activation to Ca²⁺ sensitivities remain uncertain (see Fig. 9). Given that PKC activators and inhibitors can modulate agonist-induced Ca²⁺ sensitization (21, 22), some authors have suggested that agonist-induced G protein-dependent generation of diacylglycerol (18) or arachidonic acid release (27) could activate PKC, which in turn could phosphorylate MLC and/or actin-regulating proteins, such as calponin or caldesmon, thereby influencing thin filament-dependent regulation of Ca²⁺ sensitivities (21, 37). Alternatively, the rho family of small G proteins may also couple G protein activation to Ca²⁺ sensitization, as suggested by work with the exotoxin C3 from C. botulinum, which ADP ribosylates only rho p21 and is therefore a highly selective tool in arterial preparations (9, 34). In both permeabilized and nonpermeabilized smooth muscle preparations, inactivation of rho p21 depresses GTPγS-induced Ca²⁺ sensitization (8–10, 14, 17, 19, 20, 24, 25). Under resting conditions, rho p21 is largely bound to the cytoplasmic protein GDI but on activation is translocated to the sarcolemma (8, 10), after which it activates Rho kinase and in turn inhibits MLC phosphatase (MLCP) and thereby influences MLC phosphorylation (3, 17, 20). Although it remains uncertain how activation of Rho kinase near the sarcolemma interacts with MLC phosphatase located near the myofilaments, it appears likely that some agonists may activate rho p21 and thereby activate Rho kinase, leading to phosphorylation and inactivation of MLCP (see Fig. 9).

To date, most studies of agonist-induced Ca²⁺ sensitization have focused on adult smooth muscle preparations and have not explored how this mechanism varies among arteries of different size and age. Because differences in artery size and age are associated with substantial differences in reactivity to many agonists (7, 28, 36), the present study has examined for the first
time how these differences relate to differences in PKC- and rho p21-mediated signaling. We examined cerebral arteries permeabilized with β-escin, an agent that preserves receptor function and intracellular structure while enabling investigation of the intracellular mechanisms governing Ca^{2+} sensitivity (9, 12–14, 16–18, 20–22). A key advantage of this approach was that possible effects of agonists on transmembrane Ca^{2+} fluxes or intracellular Ca^{2+} release (11, 12) were totally eliminated. Consistent with our previous observations, the present study revealed that baseline Ca^{2+} sensitivity, as characterized by pD2 values for Ca^{2+} in the absence of receptor and/or G protein stimulation, was greater in small than in large arteries but was significantly affected by age only in the common carotid. Conversely, 5-HT and GTPγS increased Ca^{2+} sensitivity much more in immature than in mature arteries, and this effect was independent of artery size. Together, these results emphasize that regulation of Ca^{2+} sensitivity is modulated by both age and artery type, at least in ovine cerebral arteries.

To examine the mechanisms coupling receptor occupation to Ca^{2+} sensitization, we first studied the role of PKC. PKC activation by either PDBu or (-)-indolactam V produced sustained increases in Ca^{2+} sensitivity (Figs. 1–3). The specificity of these effects was confirmed by the observations that 1) the inactive stereoisomer (+)-indolactam V had no effect on Ca^{2+} sensitivity, 2) both PDBu and (-)-indolactam V had no effect on vascular tone in the absence of Ca^{2+}, and 3) the contractile effects of both activators were completely prevented by the PKC inhibitors H-7 and calphostin. These findings strongly corroborate previously published observations suggesting that PKC can modulate Ca^{2+} sensitivity and sensitize contractile proteins to Ca^{2+} (12, 13, 16, 22, 31).

Although previous studies have established that PKC can modulate Ca^{2+} sensitivity, the present studies extend these observations for the first time to arteries of different size and age (Fig. 2) and demonstrate that PKC activation produced greater increases in Ca^{2+} sensitivity in intracerebral arteries than in the com-
mon carotid. This difference could potentially compensate for the relatively small contribution of Ca\(^{2+}\) from the sarcoplasmic reticulum characteristic of small, compared with large, arteries (35). Independent of artery type or size, PKC activation also increased Ca\(^{2+}\) sensitivity more in immature than in mature arteries. Combined with the observation that receptor agonists increase Ca\(^{2+}\) sensitivity more in immature than in mature arteries (2) and the finding that PKC activity may be greater in immature than in mature arteries (5), the present observations suggest that upregulation of the PKC-dependent pathway of Ca\(^{2+}\) sensitization may contribute to age-related differences in cerebrovascular reactivity. Alternatively, it remains possible that sensitivity of the contractile apparatus to modification by PKC is greater in immature than mature arteries, but this appears unlikely because the pD\(_2\) for (–)-indolactam V did not vary with either artery type or age (Fig. 2). This latter finding also argues against the possibility that age-related differences in agonist-induced sensitization are attributable to differences in PKC isoform; different isoforms might be expected to exhibit different sensitivities to various activators of PKC.

The observation that concentrations of the PKC inhibitors H-7 and calphostin which prevented the effects of (–)-indolactam V and PDBu (Figs. 5 and 6) had little effect on 5-HT- and GTP\(\gamma\)S-induced Ca\(^{2+}\) sensitization suggests that PKC activation can induce Ca\(^{2+}\) sensitization but is not involved in receptor- or G protein-induced Ca\(^{2+}\) sensitization under our experimental conditions. Although these results contradict reports that PKC inhibitors, such as staurosporine, H-7, and Ro-31–8220, can attenuate Ca\(^{2+}\) sensitization responses to \(\alpha\)-agonists, 5-HT or GTP\(\gamma\)S (21–23, 27, 30, 32), many of the PKC inhibitors used are nonspecific and inhibit PKC at its ATP binding site, a region with a high degree of sequence homology in most kinases (6). Such inhibitors may also inhibit other kinases, including cGMP kinase, MLC kinase (MLCK), or Rho kinase, which also are involved in regulation of Ca\(^{2+}\) sensitivity (16). Evidence of poor selectivity of PKC inhibitors was demonstrated by our findings that H-7 could inhibit 5-HT- and GTP\(\gamma\)S-induced contractions only at concentrations 10 times higher than needed to eliminate effects of (–)-indolactam V or PDBu (Fig. 5). In contrast, the more selective PKC inhibitor calphostin did not modify 5-HT- or GTP\(\gamma\)S-induced Ca\(^{2+}\) sensitization even at its highest concentration (Fig. 6), which was more than adequate to block the effects of (–)-indolactam V. Similarly, other authors (9, 16, 38) who have used the highly specific inhibitor of PKC, PKC-(19–36), also did not observe any modification of agonist- or GTP\(\gamma\)S-induced Ca\(^{2+}\) sensitization in permeabilized arteries. The observation that concentrations of the PKC inhibitors H-7 and calphostin which prevented the effects of (–)-indolactam V and PDBu (Figs. 5 and 6) had little effect on 5-HT- and GTP\(\gamma\)S-induced Ca\(^{2+}\) sensitization suggests that PKC activation can induce Ca\(^{2+}\) sensitization but is not involved in receptor- or G protein-induced Ca\(^{2+}\) sensitization under our experi-
abilized smooth muscle cells. PKC downregulation by prolonged exposure to a phorbol ester also failed to block agonist-induced Ca\textsuperscript{2+} sensitization (15). Together with previous work the present results suggest that PKC has little, if any, role in agonist- or G protein-induced Ca\textsuperscript{2+} sensitization in permeabilized ovine cerebral arteries. At least in permeabilized preparations, it appears that PKC activation is uncoupled from either the sarcolemmal receptors or their associated G proteins governing Ca\textsuperscript{2+} sensitization. It remains possible that permeabilization with \(\beta\)-escin may alter signal transduction by allowing leakage of unidentified but functionally important constituents, such as diacylglycerol (9), from skinned smooth muscle (16), suggesting that it may be worthwhile to repeat the present experiments with \(\alpha\)-toxin, which generally produces smaller pores and is associated with less possible leakage of cytosolic constituents. In light of these considerations, it appears imperative that the role of PKC in agonist-induced Ca\textsuperscript{2+} sensitization be further investigated in intact arterial preparations.

Although the present data suggest a limited role for PKC in Ca\textsuperscript{2+} sensitization, our experiments with exotoxin C3 strongly suggest that activation of rho p21 is involved in coupling receptor and G protein activation to contraction in cerebrovascular smooth muscle under our conditions. As mentioned above, exotoxin C3 ribosylates rho p21 and thereby inactivates the rho-dependent pathway of Ca\textsuperscript{2+} sensitization. Although the extent of ribosylation of Rho was not directly quantitated in our experiments, the specificity of C3 has previously been demonstrated in many preparations (9, 14, 17, 19, 20, 24, 25). More importantly, the ability of C3 to dramatically inhibit the Ca\textsuperscript{2+} sensitization induced by maximally effective concentrations of either 5-HT or GTP\(\gamma\)S in all artery types in both fetuses and adults (Fig. 7) was consistent with previous reports and suggests that ribosylation of Rho was in all likelihood complete. In contrast, exotoxin C3 had no effect on baseline Ca\textsuperscript{2+} sensitivity (Fig. 7). This dichotomy of effects of exotoxin C3 on baseline and agonist-induced Ca\textsuperscript{2+} sensitization reinforces the view that regulation of these two general aspects of Ca\textsuperscript{2+} sensitivity are quite independent of one another and that small G proteins are critically important in agonist-induced Ca\textsuperscript{2+} sensitization, regardless of age or artery type.

Overall, our data support the concept that agonist-induced Ca\textsuperscript{2+} sensitization in smooth muscle is primar-
ily mediated by small G proteins including rho p21, whereas PKC activation plays perhaps only a minor role (3, 8, 9, 16, 25, 38). Although some authors have suggested that PKC may mediate activation of small G proteins by agonists or GTP\(\gamma\)S (19), the present study argues against this possibility because 1) PKC inhibitors did not modify Ca\(^{2+}\) sensitization induced by 5-HT or GTP\(\gamma\)S and 2) exotoxin C3 did not affect Ca\(^{2+}\) sensitization induced by the PKC activators (\(-\))indolactam V and PDBu. On the basis of these findings, we agree with suggestions that the Rho-dependent pathway for Ca\(^{2+}\) sensitization is in general PKC independent in both permeabilized and nonpermeabilized preparations (3, 8, 9, 25). Certainly, further investigations are needed to better clarify the potential interactions between PKC and Rho, particularly in intact nonpermeabilized smooth muscle preparations from different vascular beds and species.

Together with our previous findings (2), the present findings suggest two distinct patterns of regulation of Ca\(^{2+}\) sensitivity in ovine cerebral arteries of different size and age. First, baseline Ca\(^{2+}\) sensitivity varies in relation to artery size and age, is greater in immature than in mature arteries, and is also greater in small than in large arteries. Because neither PKC inhibitors nor exotoxin C3 affect basal Ca\(^{2+}\)-force relations, their regulation appears not to be associated with variations in the major pathways of pharmacomechanical coupling but instead to more likely reflect developmental variations in the contractile apparatus including possible differences in calmodulin content and MLCK and MLCP activities, for example. In the second main pattern of Ca\(^{2+}\) sensitivity regulation, agonists and GTP\(\gamma\)S dramatically enhance Ca\(^{2+}\) sensitivity, and the magnitude of this effect is greater in immature than mature arteries and is relatively independent of artery type or size. Overall, the data suggest that developmental variations in Ca\(^{2+}\) sensitization reflect underlying variations in organization of the receptor-rho p21-Rho kinase-MLC pathway of intracellular signaling. Thus maturation may have important effects on the content and/or activity of rho p21-Rho kinase and/or on the sensitivity of MLCP to these influences. Although speculative, this hypothesis may serve as a basis for planning further investigations of molecular mechanisms governing physiological variations of cerebrovascular reactivity.

Fig. 8. Representative traces of responses to PKC activators in presence and absence of exotoxin C3. After permeabilization with \(\beta\)-escin, 1 segment of each artery pair was treated with 1 \(\mu\)g/ml exotoxin C3, and other segment served as control. After this treatment, intracellular Ca\(^{2+}\) stores were irreversibly depleted with A-23187, after which artery segments were exposed to a submaximal concentration of Ca\(^{2+}\) (EC\(_{50}\)). When resulting contraction had stabilized, segments were exposed to PDBu or (\(-\))indolactam V (indo). Tracings shown are from 1 adult Bas and 1 fetal Bas segment each and are generally representative of tracings obtained in all arteries.

Fig. 9. Current concepts regarding intracellular signaling pathways governing Ca\(^{2+}\) sensitization in vascular smooth muscle. In this summary of potential mechanisms involved in coupling receptor occupation to Ca\(^{2+}\) sensitization, agonist interaction leads to 2nd messenger production as determined by receptor affinity, density, and gain (mass of 2nd messenger produced for each ligand-receptor interaction). This ligand-receptor interaction may lead to activation of PKC via liberation of diacylglycerol (DAG), which in turn can act on either thin filaments or possibly on myosin light chain (MLC) phosphatase (MLCP). Alternatively, receptor occupation may stimulate activation of rho p21 via an unknown mechanism, which then activates Rho kinase and can phosphorylate and inactivate MLCP, thereby leading to an apparent increase in Ca\(^{2+}\) sensitivity. PLC\(_{\beta}\), phospholipase C\(_{\beta}\); MLCK, MLC kinase. See text for details and references.
REFERENCES


