PKC-induced ERK1/2 interactions and downstream effectors in ovine cerebral arteries

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Zhao, Yu, Lubo Zhang, and Lawrence D. Longo. PKC-induced ERK1/2 interactions and downstream effectors in ovine cerebral arteries. Am J Physiol Regul Integr Comp Physiol 289: R164–R171, 2005; doi:10.1152/ajpregu.00847.2004.—Both protein kinase C (PKC) and extracellular signal-regulated kinases (ERK1/2) are involved in mediating vascular smooth muscle contraction. We tested the hypotheses that in addition to PKC activation of ERK1/2, by negative feedback ERKs modulate PKC-induced contraction, and that their interactions modulate both thick and thin myofilament pathways. In ovine middle cerebral arteries (MCA), we measured isometric tension and intracellular free calcium concentration ([Ca2+]i) responses to PKC stimulation [phorbol 12,13-dibutyrate (PDBu), 3 × 10−6 M] in the absence or presence of ERK1/2 inhibition (U-0126, 10−5 M). After PDBu ± ERK1/2 inhibition, we also examined by Western immunoblot the levels of total and phosphorylated ERK1/2, myosin light chain20 (MLC20), and CPI-17. PDBu induced significant increase in tension in the absence of increased [Ca2+]i. PDBu also increased phosphorylated ERK1/2 levels, a response blocked by U-0126. In turn, U-0126 augmented PDBu-induced contractions. PDBu also was associated with significant increases in phosphorylated caldesmonSer789 and MLC20 levels, each of which peaked at 5 to 10 min. PDBu also increased phosphorylated CPI-17 levels, which peaked at 2 to 3 min. Rho kinase inhibition (Y-27632, 3 × 10−7 M) did not alter PDBu-induced contraction. These results support the idea that PKC activation can increase CPI-17 phosphorylation to decrease myosin light chain phosphatase activity. In turn, this increases MLC20 phosphorylation in the thick filament pathway and increases Ca2+ sensitivity. In addition, ERK1/2-dependent phosphorylation of caldesmonSer789 was not necessary for PDBu-induced contraction and appears not to be involved in the reversal of caldesmon’s inhibitory effect on actin-myosin ATPase.

vascular smooth muscle; U-0126; caldesmon; CPI-17; myosin light chain 20

A major contractile pathway of vascular smooth muscle cells (SMCs) is via agonist-mediated G protein and phospholipase C-β (PLC) activation, Ca2+ release, and Ca2+/calmodulin-mediated phosphorylation of the 20-kDa regulatory myosin light chain 20 (MLC20), which, in concert with the myosin heavy chain and actin, effects contraction. This Ca2+-dependent, thick filament regulatory pathway, with its dependence on the balance of activities of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP), has been well described (16, 19, 21, 39, 44). Recently, we and colleagues (33) reported that activation of protein kinase C (PKC) also induces robust contractile responses in ovine cerebral arteries, a response not associated with an increase in intracellular Ca2+ concentration ([Ca2+]i) and that these responses differ significantly as a function of developmental age. Nonetheless, the mechanism of these responses was unclear (33). Other reports have demonstrated PKC-mediated Ca2+-independent vascular contraction (18, 27, 53, 54), and PKC has been shown to modulate myogenic tone (12). During the past decade, accumulating evidence has suggested that components of the mitogen-activated protein kinase (MAPK) cascade may be involved in vascular contraction via an increase in sensitivity of the contractile machinery to Ca2+ (1, 9, 11, 16, 22, 53, 55).

Key components of this MAPK cascade include the extracellular signal-regulated kinase 1 and 2 (ERK1/2) subfamily, the activation of which is dependent on dual phosphorylation of a tyrosine (Try185) and a threonine (Thr187) residue (2, 3). Although the relationship of ERK1/2 activation to cell proliferation and differentiation is well established (5, 17), relatively less is known of the effects of ERKs in vascular smooth muscle contraction and relaxation and of the mechanism(s) by which this is effected (1, 9, 20, 22, 49). In many cell types, PKC activation is associated with phosphorylation of ERKs (25, 34). Several workers have suggested that ERKs modulate vascular SMC contraction via caldesmon phosphorylation and the thin filament (actin) pathway (1, 16, 36). Our recent studies have indicated that ERK-mediated contraction also involves the Ca2+-dependent pathway (52, 55).

Knowledge of specific ERK1/2 activation patterns and their downstream effectors may provide insight into the role of this pathway in regulating cerebrovascular reactivity. From a clinical perspective, the vulnerability of cerebral vessels to dysregulation with altered cerebral blood flow may result, in part, from dysfunction of the agonist-induced signal transduction cascade. During the past decade, work in our laboratory has explored a number of aspects of pharmacomechanical coupling in the cerebral vasculature, particularly in regard to changes with developmental maturation (for instance, see Refs. 26, 28, 29, 30, 32, 33, 55). Although providing useful insights, clearly, the mechanistic basis of interactions of receptors, second messengers, and downstream effectors is much more complex than may first appear.

In the present study, we tested the hypotheses that in cerebral arteries, PKC modulates contraction via both thick filament and thin filament regulatory pathways. We examined the extent to which activation of PKC by phorbol 12,13-dibutyrate (PDBu) leads to ERK1/2 phosphorylation and, in turn, modulates PDBu-induced contraction via phosphorylation of the downstream effector caldesmonSer789. In addition, we tested the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
the hypothesis that PDBu-induced contraction is mediated through MLC20 phosphorylation.

METHODS

Tissue preparation. For these studies, we used cerebral arteries from nonpregnant adult sheep (≤2 yr) obtained from Nebecker Ranch (Lancaster, CA). The ewes were anesthetized and killed with 100 mg/kg intravenous pentobarbital sodium, after which we obtained main branch anterior, middle, and posterior cerebral arteries (MBC), or in the case of tension and [Ca2+]i measurements, the main branch middle cerebral artery (MCA). We have shown that this method of death has no significant effect on vessel reactivity compared with the use of other anesthetic agents (31, 32). All surgical and experimental procedures were performed within the regulations of the Animal Welfare Act. The National Institutes of Health’s Guide for the Care and Use of Laboratory Animals was strictly adhered to, as were “The Guiding Principles in the Care and Use of Animals” approved by the Council of the American Physiological Society, and this work was approved by the Animal Care and Use Committee of Loma Linda University. Studies were performed in isolated vessels cleaned of adipose and connective tissue, as previously described (32). We used the vessels immediately for the experiments. In arteries used for response to PKC agonists or antagonists and/or ERK1/2 inhibitors, we added the agent 30 min before administration of agonist/antagonist. Unless otherwise noted, all chemicals were obtained from Sigma Chemical (St. Louis, MO).

Measurement of isometric tension. As noted, we isolated and removed MCA without stretching from adult sheep. Eight artery segments were obtained from each animal, one for each of eight vessel baths. We cannulated 4-mm segments of each vessel ring with tungsten mounting wires. To avoid the complications of endothelium-baths. We cannulated 4-mm segments of each vessel ring with segments were obtained from each animal, one for each of eight vessel removed MCA without stretching from adult sheep. Eight artery

Biotechnology, Santa Cruz, CA), and the protein band was detected on Hyperfilm (Amersham Life Science, Arlington Heights, IL). To maintain immunoblotting labeling conditions constant, we used the same titer of polyclonal ERK1/2 antibodies and protein concentration in all samples. We chose the antibody titers and protein concentrations on the basis of experiments determining optimal conditions on the linear portion of the titration curve (55). We used α-actin as an internal control for uniform protein loading (56).

For the cytosolic proteins caldesmon and CPI-17, the methods for immunoblot were similar to those for ERK1/2 except that we used a 7.5% gel (Bio-Rad) for caldesmon and a 10–20% gradient gel (Bio-Rad) for CPI-17, with appropriate antibodies (Santa Cruz Biotechnology).

For MLC20 immunoblots, tissues were frozen in tissue freezing buffer [5% trichloracetic acid, 10 mM dithiothreitol (DTT), 5 mM sodium fluoride (NaF), and 95% acetonitrile] on dry ice. The tissues were then brought to room temperature in washing buffer (10 mM DTT, 100% acetonitrile, and 5 mM NaF) and washed three times with washing buffer. Protein was extracted (0.05 g wet wet/ml) in extraction buffer (8.0 M urea, 20 mM Tris base, 23 mM glycine, 10 mM DTT, 10 mM EGTA, 10% glycerol, 0.05% bromphenol blue, and 5 mM NaF, pH 8.6) at room temperature for 90 min. Next, 20 μl of each sample were loaded on a urea gel and electrophoresed at 12 mA for 3 h. Proteins were transferred to a nitrocellulose membrane and subjected to immunoblotting with specific MLC20 antibody (1:200; Sigma). Anti-mouse IgM conjugated with horseradish peroxidase was used as a secondary antibody (1:2,000; Santa Cruz Biotechnology). Bands were detected with enhanced chemiluminescence using a ChemiImager (Alpha-Innotech, San Leandro, CA). We calculated moles of phosphate per mole of MLC20 by dividing the density of the phosphorylated band by the sum of the density of the phosphorylated and the nonphosphorylated band.

Protein determination. Soluble protein concentration was measured using the method of Bradford (4), as modified by Read and Northcote (42), using BSA (BDH Chemicals, Poole, UK) as standard.

Simultaneous measurement of [Ca2+]i and tension in cerebral arteries. Investigators in our laboratory have published this method in several reports (28, 29, 30, 33, 55). Briefly, MCA was sectioned into 2-mm rings, mounted on platinum wires, and attached to a strain gauge in a bath mounted on a Jasco CAF-110 intracellular Ca2+ analyzer (Easton, MD). After equilibration, loading with fura-2 AM, and washing, we measured simultaneously fura-2 fluorescence and force. Vessels were illuminated alternatively at 340 and 380 nm, with fluorescence emission measured at 510 nm. We recorded intensity at each excitation wavelength, as well as the fluorescence ratio (R340/380). When fura-2 is present only as free acid, there is an inverse change in fluorescence at 340- and 380-nm excitation. We also measured maximum R340/380 (Rmax) and Rmin and used these to normalize changes in fluorescence ratios from different preparations. Before treatment with PDBu with or without U-0126, we measured the K+ -induced contraction and increase in [Ca2+]i as a positive control.

Data analysis. We tested the null hypothesis by using one-way ANOVA. We used the Student-Newman-Keuls post hoc test to determine differences between groups. We used Student’s t-test to compare two groups. We analyzed concentration-response curves by using computer-assisted nonlinear regression to fit the data with the use of GraphPad Prism (GraphPad Software, San Diego, CA). The null hypothesis was rejected at P < 0.05. For each study, the n value equaled the number of animals from which we obtained cerebral arteries.

RESULTS

Effect of ERK1/2 inhibition on PDBu-induced contraction. To more completely understand PKC-ERK interactions in mediating agonist-induced contraction, we examined several
aspects of PDBu-induced (3 x 10^{-6} M) increases in tension in the absence or presence of the ERK inhibitor U-0126 (10^{-5} M). As shown in Fig. 1A, PDBu-induced MCA tension increased significantly over a period of 3 to 10 min, in a manner similar to that which we and colleagues previously reported (33). In the presence of ERK inhibition by U-0126, maximal tension increased ~25% over that in response to PDBu alone. The increases in tension in response to PDBu in the absence or presence of U-0126 were maintained for up to 30 min. Figure 1, insert, summarizes the maximum contractile responses for PDBu alone and in the presence of U-0126 (P < 0.05). Previously, we have reported that PDBu-induced contraction in adult sheep was not associated with [Ca^{2+}], change. As shown in Fig. 1B, importantly, PDBu-induced increase in tension with U-0126 also was not accompanied by an increase in [Ca^{2+}]. A similar increase in tension but not in [Ca^{2+}], was shown by using another PKC activator (−)-indolactam V (10^{-6} M) (data not shown).

To further explore the role of PKC in modulating ERK-induced responses, we examined the time course of total and phosphorylated ERK1/2 protein levels after administration of PDBu (3 x 10^{-6} M). As shown in Fig. 2A, this resulted in a twofold increase in phosphorylated p44 and p42 ERK levels that peaked at ~5 min, whereas total ERK1/2 remained constant. Figure 2B shows the densitometric analysis of phosphorylated p44 and p42 under these conditions, normalized to the total ERK1/2 protein levels. At 5 and 10 min, U-0126 (10^{-5} M) completely blocked this increase (n = 4 each; P < 0.05).

**PDBu-induced phosphorylation of caldesmon**. To further explore the mechanisms of PKC in non-Ca^{2+}-dependent contraction via thin filament regulation, we measured the extent to which PKC activation by PDBu increased phosphorylation of caldesmon. As shown in Fig. 3A, PDBu increased caldesmon phosphorylation in a time-dependent manner. The maximum phosphorylation occurred at 5 min with an ~1.7-fold increase compared with the control value. This response was blocked by U-0126 (10^{-5} M) at a concentration that had no effect on the control level. Figure 3B shows the mean values for these responses in relation to basal levels (P < 0.05).

**PDBu-induced MLC20 phosphorylation.** To further explore the mechanisms of PKC in Ca^{2+}-dependent contraction via thick filament regulation, we measured the extent to which PDBu (3 x 10^{-6} M) increased the phosphorylation of MLC20. As shown in Fig. 4A, Western immunoblot density of phosphorylated MLC20 levels increased ~1.5-fold by 1 min and then continued to increase to 2-fold greater than the control level at 5–10 min. Figure 4B shows the time course of PDBu-induced MLC20 phosphorylation in relation to total MLC20.
phosphorylated and nonphosphorylated MLC20 levels under and MLCK, we used Western immunoblot to examine both several conditions. As shown in Fig. 5, the presence of the Rho-kinase inhibitor Y-27632 (3×10^{-5} M) induced contraction. Y-27632 concentration was, indeed, effective for Rho-kinase inhibition, as demonstrated by its significant blockade of phenylephrine (10^{-5} M)-induced contraction.

**PDBu-induced CPI-17 phosphorylation.** To further examine the mechanism of PKC-mediated contraction, we measured total and phosphorylated CPI-17 levels in response to PDBu (3×10^{-6} M). As shown in Fig. 7A, Western immunoblotting with PDBu increased phosphorylated CPI-17 levels ~1.7-fold by 2 min, and this level of activation was maintained until 10 min, after which it decreased slightly (not significant). Figure 7B presents the mean densitometric values of the phosphorylated CPI-17 response to PDBu (n = 4; P < 0.05). Importantly, ERK1/2 inhibition (U-0126, 10^{-5} M) failed to block the PDBu-induced increase in phosphorylated CPI-17 levels (Fig. 7C), again suggesting that, in this regard, PKC acts independently of ERK1/2 activation.

**DISCUSSION**

In vascular smooth muscle, contraction is mediated by both thick and thin filament regulatory pathways. MLC20 is phosphorylated on the Ser19 residue by MLCK and dephosphorylated by MLCP. In general terms, the Ca^{2+}-dependent pathway operates via MLCK, and increased myofilament Ca^{2+} sensitivity may occur by inhibition of MLCP. We and others have shown that both PKC (18, 33, 40) and ERKs (52, 55) operate by a non-Ca^{2+}-dependent mechanism. PKC is an important mediator of myogenic tone (12), and PDBu is a tool commonly used to study PKC-mediated responses (16, 18, 33). Some of our previous studies have raised important questions in regard to PKC- and ERK-mediated pathways in cerebral vascular contraction. Thus, in the present study, we tested the hypothesis that in these arteries, ERK1/2 not only are activated by PKC but, in turn, modulate PKC-mediated contraction.

**Role of RhoA/Rho-kinase activation.** To test the hypothesis that RhoA/Rho-kinase are key effectors of PDBu-induced contraction, we measured vascular tension in the absence and presence of the Rho-kinase inhibitor Y-27632 (3×10^{-7} M). As shown in Fig. 6A, PDBu (3×10^{-6} M) induced a maximum increase in tension, but this was not altered in the presence of Y-27632. As a positive control, and as shown in Fig. 6B, this Y-27632 concentration was, indeed, effective for Rho-kinase inhibition, as demonstrated by its significant blockade of phenylephrine (10^{-5} M)-induced contraction.

**Fig. 3.** PDBu-induced phosphorylation of caldesmon in cerebral arteries. A: Western immunoblot of p-caldesmon (CaDSer789) at 0, 1, 2, 4, 5, 10, 15, 20, and 30 min in response to PDBu (3×10^{-6} M). B: Western immunoblot of p-CaDSer789 at 0, 1, 2, 4, 5, 10, 15, 20, and 30 min in response to the ERK1/2 inhibitor U-0126 (10^{-5} M) and PDBu (3×10^{-6} M). C: densitometric analysis of p-CaDSer789 normalized to the density of the total CaD for each respective lane (n = 4 each). *P < 0.05 compared with control.

Figure 4B also shows that inhibition of ERK1/2 by U-0126 had no significant effect on PDBu-induced MLC20 phosphorylation. Values are those from densitometric analysis of phosphorylated MLC20 levels (n = 5 each; P < 0.05).

One might argue that PDBu-induced MLC20 phosphorylation, and thus contraction, occurs as a result of MLCK activation. To examine this possibility, we repeated this study in the presence of the MLCK inhibitor ML-7. As shown in Fig. 5A, PDBu-induced MCA tension was not significantly decreased in the presence of ML-7 (2×10^{-5} M). In contrast, as a positive control, and as shown in Fig. 5B, ML-7 dramatically blocked 95 ± 5% of phenylephrine (10^{-5} M)-induced MCA contraction (n = 4; P < 0.01). To further test the relationship of PKC and MLCK, we used Western immunoblot to examine both phosphorylated and nonphosphorylated MLC20 levels under several conditions. As shown in Fig. 5C, 5 min after administration of PDBu, the ratio of phosphorylated to total MLC20 had doubled. This PDBu-induced increase in phosphorylated MLC20 was not altered significantly in the presence of ML-7. In addition, ERK1/2 inhibition by U-0126 (10^{-5} M) showed no significant effect on the time course of MLC20 phosphorylation following PDBu (3×10^{-6} M) treatment. Figure 5D presents the densitometric analysis of these data (n = 4; P < 0.05).

**Role of RhoA/Rho-kinase activation.** To test the hypothesis that RhoA/Rho-kinase are key effectors of PDBu-induced contraction, we measured vascular tension in the absence and presence of the Rho-kinase inhibitor Y-27632 (3×10^{-7} M). As shown in Fig. 6A, PDBu (3×10^{-6} M) induced a maximum increase in tension, but this was not altered in the presence of Y-27632. As a positive control, and as shown in Fig. 6B, this Y-27632 concentration was, indeed, effective for Rho-kinase inhibition, as demonstrated by its significant blockade of phenylephrine (10^{-5} M)-induced contraction.

**Fig. 4.** Time course of PDBu-induced activation of myosin light chain 20 (MLC20) in cerebral arteries. A: Western immunoblot of nonphosphorylated (non-p) and p-MLC20 at 1, 2, 5, 10, 15, and 30 min. By 2 min the phosphorylated MLC20 levels increased significantly, continuing to increase to 10 min, after which they remained stable. B: time course of p-MLC20 normalized to the ratio of p-MLC20 to t-MLC20 for each respective lane (n = 4). *P < 0.05 compared with control.
In a previous study (55), we showed in MCA that inhibition of ERK1/2 activity by U-0126 (10⁻⁵ M) abolished any increase in [Ca²⁺]i while only slightly decreasing the phenylephrine-induced tension. This response pattern (increase in tension with no change in [Ca²⁺]i) was similar to that which we had demonstrated following PKC stimulation by PDBu (10⁻⁸ to 10⁻⁴ M) (33). [We also demonstrated that in the presence of PKC inhibition by staurosporine (3 x 10⁻⁸ M), the U-0126-mediated failure of [Ca²⁺]i to increase in response to norepinephrine was relieved (55).] These findings thus raised several questions in regard to the role of ERK1/2 in PKC-mediated contraction.

Role of ERKs in arterial contraction. Traditionally, the MAPK/ERK pathway has been viewed in terms of receptor

Fig. 5. PDBu-induced contraction and MLC20 phosphorylation were not altered by myosin light chain kinase (MLCK) inhibition. Maximum tension induced by PDBu (3 x 10⁻⁶ M) or phenylephrine (Phe; 10⁻⁵ M) is shown in the absence or presence of the MLCK inhibitor ML-7 (2 x 10⁻⁵ M). A: ML-7 did not significantly decrease PDBu-induced contraction. B: in contrast, ML-7 blocked 95 ± 11% of Phe-induced contraction (n = 4). †P < 0.01. C: Western immunoblot of non-p- and p-MLC20 at 5 min after administration of PDBu in the absence or presence of ML-7 (2 x 10⁻⁵ M) or U0126 (10⁻⁵ M). Cont, control. D: densitometric analysis of p-MLC20 normalized to the ratio of p-MLC20 to t-MLC20 for the several treatment conditions (n = 4). *P < 0.05 compared with control.

Fig. 6. Lack of effect of PDBu on Rho-kinase activation. Maximum tension induced by PDBu (3 x 10⁻⁶ M) or Phe (10⁻⁵ M) is shown in the absence or presence of the Rho-kinase inhibitor Y-27632 (3 x 10⁻⁷ M). A: Y-27632 did not significantly decrease PDBu-induced contraction. B: in contrast, Y-27632 blocked 62 ± 13% of Phe-induced contraction compared with that in response to Phe alone (n = 4). *P < 0.05 compared with control.
Several studies have demonstrated that MAPK/ERK phosphorylation plays a role in contraction of rabbit femoral artery (41), sheep uterine artery (53), pig carotid artery (1, 20), ferret aorta (9, 22), rat aorta, mesenteric, and tail arteries (49), rat mesenteric artery smooth muscle cells (45), and bovine carotid artery (10). Nonetheless, a study in pig carotid artery (15) and in rabbit portal vein and femoral artery (37) denied such a role. One report demonstrated that pharmacological inhibition of ERK1/2 phosphorylation abolished the cerebral artery tone developed in response to increased intraluminal pressure (24). Recently, we reported in ovine cerebral arteries (55) that α1-adrenergically induced (phenylephrine, 10^{-5} M) contraction was associated with ERK phosphorylation. In these arteries, administration of the relatively selective MEK inhibitor U-0126 (10^{-5} M; a dose somewhat lower than the concentration required for 50% inhibition) slightly decreased phenylephrine-induced tension but totally eliminated the [Ca^{2+}]_i increase, thus augmenting Ca^{2+} sensitivity (55).

PKC and vascular contraction. The past decade has seen significant progress in elucidating the role of the PKC family of kinases in vascular contractility. Current evidence supports the idea of PKC being an important mediator of myogenic tone (12, 16, 33) and of isoform-selective activity, with individual isoforms mediating specific aspects of SMC contraction (6, 35). Thus delineation of isoform-selective downstream effectors and the full characterization of the enzymes and associated molecules should provide insight into mechanisms and pathogenesis of vascular disease and be of value in the development of pharmacological/genetic approaches for effective prevention and/or treatment (46).

Interaction of PKC and ERKs. As shown in the present study, and as our group has reported previously (33), in ovine cerebral arteries, activation of PKC by PDBu resulted in a significant increase in tension with no increase in [Ca^{2+}]_i. Unique in this study was the significant further increase in tension in the presence of the ERK inhibitor U-0126. This finding strongly supports the idea that under normal circumstances, ERKs feed back to inhibit PKC-mediated contraction. In our previous report, we observed that in the adult arteries, PDBu resulted in a significant decrease in sensitivity to noradrenaline (NE), whereas PKC inhibition (staurosporine) resulted in a marked increase in NE sensitivity. This would be expected because of PKC’s negative feedback on α1-adrenergically mediated Ins(1,4,5)P_3 generation and downstream effectors (33). As noted above, in that study we were unable to explain the paradox of a robust PKC-induced increase in tension despite no increase in [Ca^{2+}]_i and evidence that this increased Ca^{2+} sensitivity was unchanged when extracellular [Ca^{2+}] was zero (33). In light of evidence from the present...
studies of significant inhibition by ERKs on PKC, it may be that further PKC inhibition can only attenuate adrenergically mediated contraction further. This idea is supported by the previous study in which PKC inhibition by staurosporine blocked the augmentation of phenylephrine-induced contraction produced by U-0126 (33). A further point is that PKC-induced ERK1/2 phosphorylation did not occur before the maximum PDBu-induced contraction. Thus ERK1/2 activation may well not be the direct downstream effector for PKC-mediated contraction.

PDBu-induced phosphorylation of caldesmon. As noted above, in addition to its influence on ERK activation in the contraction pathway that does not require an increase in [Ca^{2+}], PKC has been demonstrated to phosphorylate the thin filament-associated protein caldesmon (23, 36, 50, 52). PKC-mediated phosphorylation of caldesmon also significantly decreases its ability to inhibit actin-myosin ATPase (46, 47). Although the major site of ERK-dependent phosphorylation in caldesmon is at Ser^{789}, PKC may phosphorylate other sites. In the present study, we have demonstrated that PKC activation by PDBu phosphorylates both ERK and caldesmon^{Ser^{789}}, U-0126 inhibition of ERK phosphorylation blocked caldesmon^{Ser^{789}} phosphorylation and yet increased PDBu-induced contraction. This suggests that caldesmon^{Ser^{789}} phosphorylation was not directly involved in releasing the inhibitory effect on actin-myosin ATPase. In fact, PKC-specific phosphorylation of caldesmon^{Ser^{789}} may inhibit other PKC-mediated phosphorylation sites on caldesmon. This finding agrees with a recent report from the uterine arteries of pregnant sheep (53).

PDBu-induced phosphorylation of MLC_{20}. The present finding in cerebral arteries that PDBu increased MLC_{20} phosphorylation in the absence of changes in [Ca^{2+}], suggests an increase in Ca^{2+} sensitivity of MLC_{20} phosphorylation. As has been demonstrated, MLCP plays a key role in the regulation of Ca^{2+} sensitivity of MLC_{20} phosphorylation in vascular smooth muscle (44). Several agonists increase Ca^{2+} sensitivity of MLC_{20} phosphorylation by inhibiting MLCP activity. A major protein in the regulation of MLCP activity is CPI-17, which when phosphorylated is a potent inhibitor of the catalytic subunit of MLCP, thus inhibiting MLCP activity and increasing Ca^{2+} sensitivity of MLC_{20} phosphorylation. In the present study, PDBu significantly increased CPI-17 phosphorylation. In part, this increased Ca^{2+} sensitivity also is associated with an increase in MLC_{20} phosphorylation (44) in response to G protein-coupled receptor activation through Rho A/Rho-kinase (13, 14). In turn, Rho A/Rho-kinase may act by phosphorylating CPI-17 (43) to promote contraction (8, 51). Nonetheless, in the present study, PDBu-induced contraction was unaltered by the Rho-kinase inhibitor Y-27632. This suggests that PKC, acting directly, can phosphorylate CPI-17 in the absence of activation of either RhoA/Rho-kinase or ERK1/2. In addition, PDBu-induced MLC_{20} phosphorylation was not blocked by the MLCK inhibitor ML-7, indicating that there may be direct PKC phosphorylation sites on MLC_{20} (Fig. 5C).

Figure 8 presents a scheme for the mechanisms suggested by the present study. Activation of PKC leads to the phosphorylation of ERK1/2, which, in turn, appears to have negative feedback on PKC-mediated responses. PKC also phosphorylates CPI-17, which, by inhibiting MLCP, increases myofilament Ca^{2+} sensitivity and promotes MLC_{20} phosphorylation and contraction. In contrast, by phosphorylating caldesmon^{Ser^{789}}, ERKs may inhibit other caldesmon phosphorylation sites mediated by PKC and attenuate actin-myosin interaction, thereby inhibiting PDBu-induced contraction. Overall, it would appear that in cerebral arteries there exists a fine balance of several elements in the regulation of myofilament Ca^{2+} sensitivity and vascular tone. These include the balance between thick and thin filament regulatory pathways and the balance between MLCP activity in determining the degree of MLC_{20} phosphorylation.

Perspective

The present study supports the hypothesis that PKC phosphorylates and thereby activates ERK1/2 via the MAPK cascade. In addition, by negative feedback, the ERKs appear to inhibit PKC-mediated contraction. Our additional goal was to help sort out the extent to which PKC and ERKs activate the downstream effectors caldesmon, CPI-17, and MLC_{20}. The present studies support the hypothesis that ERK1/2 phosphorylates caldesmon^{Ser^{789}} and thus attenuates the thin filament regulatory pathway. In addition, by phosphorylating CPI-17, which interacts with the catalytic subunit of MLCP to inhibit its activity, PKC acts to maintain or increase phosphorylated MLC_{20}. Taken as a whole, the data in this study emphasize that PKC and the ERKs may operate in a “yin and yang” fashion to modulate thick and thin filament regulation of cerebrovascular tone. PKC thus plays a key role, acting through CPI-17 to increase myofilament Ca^{2+} sensitivity and alternatively acting through the ERKs and caldesmon to decrease contraction. Nonetheless, many details of the virtual spider’s web of interacting regulatory mechanisms remain an enigma. A challenge in the coming years is to elucidate details of the role of these elements in determining myofilament Ca^{2+} sensitivity.

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