Separate upstream and convergent downstream pathways of G-protein- and phorbol ester-mediated Ca^{2+} sensitization of myosin light chain phosphorylation in smooth muscle

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The effect of phorbol ester-induced down-regulation of protein kinase C (PKC) on diacylglycerol (sn-1,2-dioctanoylglycerol, diC_8)- and G-protein-coupled Ca^{2+} sensitization and on the relationship between phosphorylation of the regulatory myosin light chains (MLC_{20}) and force during Ca^{2+} sensitization were investigated in rabbit portal vein (PV), femoral artery (FA) and ileum smooth muscle. The effects of phorbol dibutyrate (PDBu), guanosine 5'-[γ -thio]triphosphate (GTP[S]) and agonists on the membrane versus cytosolic distribution of PKC isoenzymes were also determined. Down-regulation of PKC abolished Ca^{2+} sensitization of force and the accompanying increases in MLC_{20} phosphorylation induced by PDBu, as well as Ca^{2+} sensitization of force by diC_8 , but not that by GTP[S], aluminum fluoride

(AlF_4^-) or agonists (phenylephrine, endothelin or carbachol). Down-regulation also inhibited the PDBu-, but not the GTP[S]-induced increase in force under Ca^{2+} -free conditions. In ileum, PDBu translocated PKCs α , β_1 , β_2 , ϵ and θ to the membrane fraction, and GTP[S] caused a small translocation of PKC- ϵ . Carbachol- and GTP[S]-induced Ca^{2+} sensitization remained unaffected in down-regulated ileum in which no cytosolic PKC- ϵ was detectable. We conclude that, although both phorbol ester-induced and G-protein-coupled Ca^{2+} sensitization of force are mediated by increased MLC_{20} phosphorylation, it is likely that PKCs α , β_1 , β_2 , ϵ and θ do not play an essential role in, although they may contribute to, the G-protein-coupled mechanism.

INTRODUCTION

The normal trigger of contraction in smooth muscle is a rise in free cytosolic calcium concentration, but agonists can induce disproportionately more force at a given level of $[\text{Ca}^{2+}]$ than depolarization with high K^+ , and such agonists, as well as guanosine 5'-[γ -thio]triphosphate (GTP[S]), can increase force in permeabilized smooth muscles in which $[\text{Ca}^{2+}]$ is clamped at constant levels (Ca^{2+} sensitization, reviewed in [1]). Three lines of evidence have supported a role for protein kinase C (PKC) in agonist-induced, G-protein-coupled, Ca^{2+} sensitization. First, certain agonists can activate PKC and translocate some of its isoforms to the membrane [2–6]. Secondly, activators of PKC, phorbol esters and diacylglycerols (DAGs), induce Ca^{2+} sensitization [7–9]. The third line of evidence, based on the effect of PKC inhibitors on agonist-induced Ca^{2+} sensitization, is ambiguous, given the uncertain specificity of the inhibitors (e.g. [10]). Studies using PKC inhibitor peptides have both implicated [11,12] and rejected [13,14] a role for PKC in agonist-induced Ca^{2+} sensitization. According to recent reports down-regulation of total PKC activity does not abolish agonist-induced Ca^{2+} sensitization [15,16], but these studies did not identify the specific PKC isoforms down-regulated. Therefore, while recognizing that the conflicting conclusions about the role of PKC in Ca^{2+} sensitization may reflect differences between the receptor signalling cascades of different smooth muscles, we sought more direct information, obtainable with isoform-specific antibodies, about the role of PKC isoforms in agonist-induced, G-protein-coupled

Ca^{2+} sensitization. We also wished to determine whether down-regulation of Ca^{2+} -sensitization of force was caused by down-regulation of phosphorylation of the 20 kDa myosin light chain (MLC_{20}), the primary mechanism responsible for activation of actomyosin ATPase (see reviews in [1,17]), or, albeit less likely, from dissociation between MLC_{20} phosphorylation and force.

We have previously observed that down-regulation of PKCs α , β_1 , β_2 , θ and ϵ abolished phorbol ester-induced Ca^{2+} sensitization in tonic (femoral artery; FA) and phasic [portal vein (PV) and ileum] rabbit smooth muscles (P. E. Jensen, L. A. Walker, M. C. Gong, J. J. Sando, A. V. Somlyo, A. P. Somlyo, unpublished work). In the present study we assessed the effect of down-regulation of identified PKC isoforms on agonist-, DAG-, AlF_4^- and G-protein-mediated Ca^{2+} sensitization of MLC_{20} -phosphorylation and contraction.

MATERIALS AND METHODS

Tissue

Male New Zealand White rabbits (2.5–3 kg) were killed with halothane and exsanguinated in accordance with institutional policies and Public Health Service guidelines regarding humane use of laboratory animals. The PV was removed, cleaned of connective tissue, and cut into longitudinal strips. The longitudinal muscle layer of ileum was peeled off and cut into strips. Femoral arteries were dissected from connective tissue, opened longitudinally, the circular muscle was cut into strips and the

Abbreviations used: GTP[S]; guanosine 5'-[γ -thio]triphosphate; PKC, protein kinase C; PV, portal vein; FA, femoral artery; PDBu, phorbol dibutyrate; PMA, phorbol myristate acetate; PVDF, poly(vinylidene difluoride); ECL, enhanced chemiluminescence; AEBSEF, 4-(2-aminoethyl)benzenesulphonyl fluoride; AlF_4^- ; aluminium fluoride; DAG, diacylglycerol; diC_8 , sn-1,2-octanoylglycerol; MLC_{20} , 20 kDa myosin light chain.

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adventitia was removed. All muscle strips were 100–300 μm wide and 3–4 mm long.

Protocols for down-regulation

Muscle strips were incubated at 37 °C in Hepes-buffered Krebs solution containing phorbol myristate acetate (PMA, 3 μM), phorbol dibutyrate (PDBu, 10 μM), bryostatin-1 (10 μM) or bryostatin-2 (10 μM) for 48–96 h. Control strips were incubated under similar conditions in solutions containing only the vehicle (1 % DMSO). All solutions used to incubate tissues contained 1 % penicillin/streptomycin.

Western blotting

Rabbit polyclonal antibodies to PKC β_1 , β_2 , ϵ and θ were obtained from Santa Cruz (Santa Cruz, CA, U.S.A.) and used at a dilution of 1:400 in PBS. A monoclonal antibody specific for PKC α was a generous gift from Dr. Peter Parker (Protein Phosphorylation Laboratory, Imperial Cancer Research Fund, London, U.K.). Electrophoresis was performed on SDS/10 % polyacrylamide gels, unless otherwise noted, and proteins were transferred (30 V, 4 °C, overnight) on to poly(vinylidene difluoride) (PVDF) membranes. The membranes were exposed to primary antibodies (3–5 h) followed by secondary antibodies (1 h) and developed using enhanced chemiluminescence (ECL; Amersham).

Force measurements of Ca^{2+} sensitization and permeabilization with *Staphylococcus aureus* α -toxin

The isometric force developed by the small muscle strips mounted on a bubble plate (each bubble was approximately 270 μl [18]) was measured at 25 °C using a force transducer (AE 801; Horten, Norway). The muscle strips were stretched to $1.3 \times$ rest length. The details of the protocols for permeabilization, detection of Ca^{2+} sensitization and the solutions used have been published [19–21]. Briefly, following the down-regulation protocol carried out on intact muscles, the strips were incubated in normal relaxing solution (Ca^{2+} -free, containing 1 mM EGTA) for 5 min, the cell membranes were permeabilized with 17.5 $\mu\text{g}/\text{ml}$ *Staphylococcus aureus* α -toxin (List Biological, Campbell, CA, U.S.A.), and A23187 (10 μM) was added to deplete Ca^{2+} stores. Following a submaximal Ca^{2+} -induced contraction, Ca^{2+} sensitization was induced by addition of agonist. Maximal force, used to normalize the contractile response, was determined by exposure to pCa 4.5 (in PV and ileum) or to pCa 5.0 (in FA) solution. Buffered calcium solutions contained 10 mM EGTA, thus the Ca^{2+} concentrations were 'clamped' during exposure to the Ca^{2+} -sensitizing agents.

Separation of membrane and cytosolic fractions

To assess the effects of Ca^{2+} and PDBu on the distribution of PKC isoforms, muscle strips were homogenized in either buffer A ($-\text{Ca}^{2+}$): 1 mM EGTA, 10 mM NaCl, 25 mM Tris/HCl, pH 7.5, or buffer B ($+\text{Ca}^{2+}$): 3 mM CaCl_2 , 10 mM NaCl, 25 mM Tris/HCl, in the presence or absence of 1 μM PDBu. Both buffers also contained 10 μM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF) and 100 $\mu\text{g}/\text{ml}$ leupeptin. The homogenates were centrifuged at 100 000 g for 30 min at 4 °C to generate the cytosolic fraction. Membrane proteins were extracted for 30 min from the pellet with the respective buffers containing 1 % Triton X-100, and clarified (600 g , 5 min, 4 °C).

The distribution of PKC isoforms was also determined in α -toxin-permeabilized muscle stimulated with PDBu, GTP[S] or

carbachol at pCa 6.0; strips were frozen in liquid nitrogen, and homogenized at 4 °C, followed by membrane and cytosol fractionation (see procedure above). The effects of PDBu, GTP[S] and carbachol on the distribution of PKCs were determined in triplicate and expressed as the ratio of Western blots measured by densitometry of membrane and cytosol fractions.

Myosin light chain phosphorylation

Muscle strips for measurements of MLC₂₀ phosphorylation were frozen in liquid nitrogen-cooled freon either at steady-state force induced by submaximal Ca^{2+} or at maximal force induced by Ca^{2+} sensitization. Tissues were stored at -80 °C until processing. The frozen samples were freeze-substituted in 10 % trichloroacetic acid in acetone, subjected to two-dimensional gel electrophoresis and stained with colloidal gold [22]. MLC₂₀ phosphorylation was quantified by densitometry using a Bio-Rad GS-670 imaging densitometer.

Statistics

Statistical comparisons were made using paired or non-paired, two-tailed, Student's t -test. P values of < 0.05 were considered to be significant. For multiple comparisons analysis of variance (ANOVA) tests were made, and if significant, Bonferroni t -tests were made. Data are expressed as means $\pm$ S.E.M.

RESULTS

Down-regulation of PKC isoforms

Chronic treatment of intact rabbit FA (96 h), PV (48 h) and ileum (72 h) with phorbol esters and bryostatins caused down-regulation of PKCs α , β_1 , β_2 , ϵ and θ . PKC- η was detected only in PV and was not down-regulated (P. E. Jensen, L. A. Walker, M. C. Gong, J. J. Sando, A. V. Somlyo, A. P. Somlyo, unpublished work). Examples of down-regulation (PMA, 3 μM) of PKCs α , β_1 , ϵ and θ in FA are shown in Figure 1. PKC- θ was not detectable after treatment with PMA; α and β_1 were almost completely down-regulated, and only long exposures of ECL revealed faint bands of these isoforms. The fastest migrating band (~ 86 kDa) of ϵ was completely down-regulated and the two more slowly migrating bands were also significantly down-

Figure 1 Down-regulation of PKC isoforms in FA

The content of four of the isoenzymes of PKC detected in FA (α , β_1 , θ and ϵ) was determined on Western blots of homogenates of down-regulated (3 μM PMA, 96 h, D) and timed control FA (C). The arrows on the left indicate the position of an 80 kDa molecular mass marker, and only the regions of interest, around 80 kDa, are shown.

Figure 2 Effect of down-regulation of PKCs on agonist-, PDBu- and GTP[S]-induced Ca²⁺ sensitization

A representative trace from PV is shown in (A), and averaged results from similar experiments in PV (B), FA (C) and ileum (D) are shown below. The concentrations of agents used were: phenylephrine (PE), 100 μ M; endothelin (ET), 0.1 μ M; carbachol (carb), 100 μ M; PDBu, 1 μ M; GTP[S] (GTP_γS), 100 μ M. Columns represent net force increase induced by agonists relative to maximal Ca²⁺-induced contraction. Submaximally activating [Ca²⁺] were: ileum, pCa 6.0; PV and FA, pCa 6.7; maximally activating [Ca²⁺] were: ileum and PV, pCa 4.5; FA, pCa 5.0. Statistical significance: * P < 0.05; ** P < 0.01; *** P < 0.0001; numbers in parentheses represent the number of experiments.

regulated to ~30% of control (P. E. Jensen, L. A. Walker, M. C. Gong, J. J. Sando, A. V. Somlyo, A. P. Somlyo, unpublished work).

Effects of down-regulation of PKCs on Ca²⁺ sensitization induced by agonists and GTP[S]

The effect of down-regulation of PKCs on Ca²⁺ sensitization of force was evaluated in α -toxin-permeabilized smooth muscles. Representative traces of the contractile responses of down-regulated PV (Figure 2A) show that phenylephrine (100 μ M with 10 μ M GTP) and GTP[S] (100 μ M) elicited Ca²⁺ sensitization, but PDBu (1 μ M) had no effect, in contrast to its pronounced Ca²⁺-sensitizing effect on timed controls (results not shown). The results of similar evaluation of Ca²⁺ sensitization of different smooth muscles by PDBu (1 μ M), GTP[S] (100 μ M) and by several agonists [phenylephrine (100 μ M) and endothelin (0.1 μ M) on PV and FA; carbachol (100 μ M) on ileum] are summarized in Figures 2(B), 2(C) and 2(D). Down-regulation of PKC with PMA or bryostatin-2 did not inhibit the Ca²⁺ sensitization induced by phenylephrine (P = 0.5), endothelin (P = 0.79) or GTP[S] (P = 0.37, ANOVA) in PV (Figure 2B), although it completely abolished this effect of PDBu [15]. In ileum (Figure 2D), neither the Ca²⁺ sensitization induced by carbachol (P = 0.24) or GTP[S] (P = 0.14) nor the response to pCa 6.0 (P = 0.07) were significantly affected by chronic PMA treatment that abolished the response to PDBu.

Only in FA (Figure 2C) did the sensitization induced by phenylephrine (P = 0.0029), endothelin (P = 0.0178) and GTP[S] (P < 0.0001) appear to be significantly inhibited by chronic (96 h) PMA treatment. However, this may have been the result of residual Ca²⁺ sensitization by PMA persisting even after incubation for 96 h: the response to pCa 6.7 was significantly higher in treated strips than in time-matched controls ($12.1 \pm 1.5\%$ versus $1.4 \pm 0.3\%$, P < 0.0001). The sum of the enhancement of the response to pCa 6.7 plus the Ca²⁺ sensitization induced by the agonist on top of the (already sensitized) response to pCa 6.7 was not significantly different in down-regulated compared with timed control muscle. Thus the extent of Ca²⁺ sensitization may have been limited by a 'ceiling' of the response attainable.

Effects of down-regulation of PKCs on Ca²⁺ sensitization by diC₈ (dioctanoylglycerol) and AIF₄⁻ in FA

DAGs released from membrane lipids are thought to be the physiological messengers that activate PKC in response to agonists (see reviews in [8,9,23–25]). AIF₄⁻, another Ca²⁺-sensitizing agent [26,27], activates heterotrimeric G-proteins (G_{q,11}) that can activate phospholipase C to release DAG but is reported not to affect monomeric G-proteins [28]. Therefore we evaluated the effect of down-regulation of PKCs on Ca²⁺ sensitization by DAG, diC₈ and AIF₄⁻. Down-regulation of PKCs completely abolished the Ca²⁺ sensitization induced by diC₈, whereas it had no effect on that induced by AIF₄⁻ (P = 0.37): the diC₈-induced Ca²⁺ sensitization was 21.1 ± 0.9 (n = 3) in controls versus -3.8 ± 1.2 (n = 3, P = 0.003) after down-regulation, whereas the Ca²⁺ sensitization induced by AIF₄⁻ was 59.4 ± 5.0 (n = 4) in controls versus 54.0 ± 2.9 (n = 3, P = 0.37) after down-regulation.

Effects of down-regulation of PKCs in FA on contractions induced by GTP[S] and PDBu in Ca²⁺-free solution

In α -toxin-permeabilized FA both PDBu (1 μ M; P. E. Jensen, L. A. Walker, M. C. Gong, J. J. Sando, A. V. Somlyo, A. P. Somlyo, unpublished work) and GTP[S] [22] can cause (very slow) contraction even in Ca²⁺-free solution containing 10 mM EGTA; the contraction induced by PDBu reaches ~44% of maximal

Figure 3 Effect of Ca^{2+} -sensitizing agents on MLC_{20} phosphorylation in α -toxin-permeabilized control and down-regulated (PMA $3 \mu\text{M}$, 96 h) FA

Concentrations of the Ca^{2+} -sensitizing agents were: PDBu, $1 \mu\text{M}$; GTP[S] (GTP γ S), $100 \mu\text{M}$; phenylephrine (PE), $100 \mu\text{M}$; and endothelin (ET), $0.1 \mu\text{M}$. Statistical significance: * $P < 0.05$, ** $P < 0.01$ numbers in parentheses represent the number of experiments.

Ca^{2+} -induced force in 16 h, and this effect of PDBu is also abolished by down-regulation of PKCs. In contrast, down-regulation (with $3 \mu\text{M}$ PMA, 96 h) of PKCs had no effect ($P = 0.84$) on the contraction induced by GTP[S] in Ca^{2+} -free solution (containing 10 mM EGTA) which was $5.9 \pm 1.7\%$ ($n = 5$, $P = 0.03$) versus $5.4 \pm 1.5\%$ ($n = 4$, $P = 0.04$) in timed controls.

The effect of down-regulation of PKCs on MLC_{20} phosphorylation during Ca^{2+} sensitization

Phosphorylation of the regulatory light chains of myosin (MLC_{20} phosphorylation) is the key event in the activation of both Ca^{2+} -induced contractions and Ca^{2+} sensitization in smooth muscle (reviewed in [1, 17]). However, it has also been suggested [7, 29–31] that phorbol esters can cause force development independently of MLC_{20} phosphorylation. Therefore, we re-evaluated, in FA and PV, the effect of down-regulation of PKCs on the relationship between Ca^{2+} sensitization of MLC_{20} phosphorylation and force. In FA (Figure 3), neither the level of MLC_{20} phosphorylation ($P = 0.20$) at pCa 6.7 (at steady-state force) nor the increase in MLC_{20} phosphorylation induced by endothelin ($P = 0.56$) or AIF_4^- ($P = 0.92$) was significantly affected by down-regulation of PKCs, although the GTP[S]-induced increase in MLC_{20} phosphorylation was slightly inhibited ($P = 0.05$). In contrast, the PDBu-induced increase in MLC_{20} phosphorylation, present in controls, was absent in down-regulated FA.

In PV, PDBu caused an $18 \pm 4.2\%$ ($n = 4$, $P = 0.02$) increase in MLC_{20} phosphorylation in timed controls, but had no effect after down-regulation of PKCs ($-0.7 \pm 1.7\%$, $n = 3$, $P = 0.72$). GTP[S] increased MLC_{20} phosphorylation by $18 \pm 5.5\%$ ($n = 4$, $P = 0.04$) in timed controls and $12 \pm 2.1\%$ ($n = 3$, $P = 0.03$) in down-regulated muscles, but these differences were not significant ($P = 0.42$).

As noted above, in α -toxin-permeabilized FA, PDBu can induce force in the absence of Ca^{2+} . Therefore, we also measured the levels of MLC_{20} phosphorylation at maximal force (after ~ 16 h; P. E. Jensen, L. A. Walker, M. C. Gong, J. J. Sando, A. V. Somlyo, A. P. Somlyo, unpublished work). PDBu significantly ($P = 0.001$) increased MLC_{20} phosphorylation to $49.5 \pm 2.4\%$ ($n = 3$) compared with the level ($6.1 \pm 4.5\%$; $n = 3$) in timed controls.

Figure 4 Effect of Ca^{2+} -sensitizing agents on the distribution of PKC isoenzymes between cytosol and membrane in ileum smooth muscle

The muscles were either relaxed (pCa > 8), stimulated with pCa 4.5 or 6.0, or stimulated with pCa 6.0 plus PDBu ($1 \mu\text{M}$), GTP[S] (GTP γ S) ($100 \mu\text{M}$) or carbachol ($100 \mu\text{M}$). For each stimulatory state a membrane (M) and a cytosolic (C) fraction were obtained and analysed for the content of PKCs α , β_1 , β_2 , ϵ and θ . The arrowhead on the left indicates the position of an 80 kDa molecular mass marker, and only the regions of interest, around 80 kDa, are shown.

The effects of Ca^{2+} , PDBu, GTP[S] and carbachol on the distribution of PKC isoforms between membrane and cytosol in ileum

It is generally believed that PKCs are activated as a result of their translocation to the cell membrane and association with membrane lipids, and such translocation is often used as an index of PKC activation. We evaluated the effect of PDBu, carbachol and GTP[S] on the distribution of PKC isoforms between membranes and cytoplasm in α -toxin-permeabilized ileum smooth muscle. Ileum was used for these experiments, because relatively large quantities of tissues were needed for fractionation. The distribution and translocation of PKCs were evaluated in six different states: three in the absence of added agonist at different levels of $[\text{Ca}^{2+}]$: low (pCa > 8), maximally stimulating (pCa 4.5, frozen ~ 2 min after adding Ca^{2+} , when force was maximal), or submaximally activating (pCa 6.0, frozen 10 to 15 min after adding Ca^{2+} when force has reached steady state). In the other three groups, the effect of Ca^{2+} sensitization was evaluated by first submaximally contracting the strips with pCa 6.0 for 10 min, followed by addition of either PDBu ($1 \mu\text{M}$), GTP[S] ($100 \mu\text{M}$) or carbachol ($100 \mu\text{M}$). The strips were frozen when the responses were maximal (approx. 15 min after addition of drugs).

Representative Western blots showing the distribution of PKCs α , β_1 , β_2 , ϵ and θ are illustrated in Figure 4. PKCs α , β_1 and β_2 were primarily cytosolic at pCa 8, and were translocated to the membrane fraction by pCa 4.5 or PDBu. At pCa 6, GTP[S] and carbachol had no effect on the distribution of PKCs α , β_1 , β_2 , or θ , and GTP[S] caused a slight translocation of PKC- ϵ to the membrane fraction. PKC- θ was also primarily cytosolic, and only pCa 4.5 and PDBu affected its distribution. At pCa ≥ 8 , PKCs β_1 and θ were detected only in the cytosolic fraction; traces of α and β_2 could also be detected in the membrane fraction that contained a significant fraction ($67 \pm 8.0\%$, $n = 3$) of the ~ 92 and ~ 98 kDa band (measured together because they were not well separated by densitometry) of PKC- ϵ . Raising $[\text{Ca}^{2+}]$ from

Table 1 Summary of the effect of calcium, carbachol, GTP[S] and PDBu on the distribution of PKC- ϵ in ileum

The summarized ($n = 3$) effects of various stimulations on the membrane/cytosol ratio of ~ 92 and ~ 98 kDa PKC- ϵ bands are shown. Under resting conditions (pCa > 8), most protein was found in the cytosol; GTP[S] (100 μ M, 15 min) and PDBu (1 μ M, 15 min) significantly increased the ratio, indicating translocation of cytoplasmic protein to the membrane fraction. pCa > 8 , pCa 4.5 and pCa 6.0 were not significantly different nor was pCa 6.0 versus carbachol (100 μ M, 15 min).

	pCa > 8	pCa 4.5	pCa 6.0	Carbachol	GTP[S]	PDBu
Ratio of membrane/cytosol	0.7 ± 0.08	1.9 ± 0.95	1.3 ± 0.16	2.3 ± 0.45	$4.1 \pm 0.10^{**}$	$14 \pm 1.7^*$

Statistical significance: * $P < 0.01$ and ** $P < 0.001$ compared with the ratio in pCa 6.0 solution.

Figure 5 The membrane (M) and cytosolic (C) contents of PKC- ϵ in down-regulated (3 μ M PMA, 48 h) PV

Western blots of PKC- ϵ in down-regulated PV are compared with a timed control. Molecular mass markers are shown on left, and only the regions of interest, around 80 kDa, are shown.

pCa ≥ 8 to pCa 6.0 increased the PKC- α and - β content of the membrane fraction, and pCa 4.5 caused further translocation of PKC- α to the membrane. Surprisingly, raising $[Ca^{2+}]$ to pCa 4.5 caused the disappearance of PKC- θ and of the faster migrating (86 kDa) band of PKC- ϵ (Figure 4), perhaps due to their loss to a compartment removed by the low-speed (600 g) centrifugation used to clear the homogenates of nuclei and other large particles. If, instead of freezing the muscle at the maximal force induced by pCa 4.5, the solution was changed to pCa > 8 , and the muscle was allowed to relax for ~ 20 min, the level of the ~ 86 kDa PKC- ϵ band returned to control levels (results not shown). Thus exposure to pCa 4.5 caused a reversible disappearance of the ~ 86 kDa band of PKC- ϵ . The slower migrating bands of this isoform also reversibly translocated to the membrane. The distribution of the ~ 92 and ~ 98 kDa bands of PKC- ϵ bands between the membrane and cytosolic fractions is summarized in Table 1. The ratio of membrane/cytosol content was significantly higher during Ca^{2+} sensitization induced by GTP[S] (100 μ M, $P = 0.0001$) and PDBu (1 μ M PDBu, $P = 0.002$), compared with the ratio during stimulation with pCa 6.0. Thus, during Ca^{2+} sensitization induced by GTP[S] there was a small, but significant, translocation of the ~ 92 and ~ 98 kDa bands of PKC- ϵ to the membrane. To evaluate whether this small translocation is required for Ca^{2+} sensitization, the distribution of the PKC- ϵ remaining after down-regulation with PMA (3 μ M, 48 h) was determined and compared with timed controls. Figure 5 illustrates the distribution in PV, and identical results were obtained in FA and ileum. Only trace amounts of the slower-migrating bands of PKC- ϵ , associated with the membrane fraction, were present in down-regulated smooth muscle. There were no detectable bands in the cytosolic fractions, even after very long ECL exposure of the Western blots. Thus, chronic treatment with PMA removes all the cytosolic, and most of the membrane-associated, PKC- ϵ , without affecting GTP[S]-induced Ca^{2+} sensitization.

DISCUSSION

Our exploration of the mechanisms and relationship between G-protein-coupled and phorbol ester-induced Ca^{2+} sensitization of force suggests that the initial processes involved in the two types of Ca^{2+} sensitization are largely, though not completely, independent, whereas their final downstream effects converge on the inhibition of dephosphorylation of MLC₂₀.

The additive Ca^{2+} -sensitizing effect of supramaximal concentrations of PDBu and GTP[S] provided the first indication that these two agents may act through separate mechanisms [32], without, however, ruling out the possibility that these differences were only quantitative. The possibility of qualitative differences was considerably strengthened by the demonstration that chronic treatment with PMA, sufficient to abolish PKC activity, also abolished in permeabilized smooth muscle the potentiation of Ca^{2+} -induced contraction by PDBu, but not that by prostaglandin $F_{2\alpha}$ [15]. Down-regulation also had no effect on the response of intact (non-permeabilized) rat aorta to prostaglandin $F_{2\alpha}$, noradrenaline or endothelin-1, but the undetermined variations in intracellular $[Ca^{2+}]$ in intact muscle complicate the interpretation of these and similar [16] studies. In agreement with the previous results [15], we show that down-regulation of PKCs in two different smooth muscles (rabbit PV and ileum) had no effect on Ca^{2+} sensitization of force by three different agonists: phenylephrine, endothelin and carbachol. We also show that down-regulation of PKCs did not inhibit the G-protein-mediated Ca^{2+} -sensitizing effect of AlF_4^- [26,27], although it slightly inhibited in FA the Ca^{2+} sensitization of force and MLC₂₀ phosphorylation by GTP[S] (see below). The parallel inhibition of the Ca^{2+} -sensitizing effect of both PDBu and diC₈ by down-regulation of PKCs is consistent with these agents acting through a common, PKC-mediated mechanism. The complete or nearly complete absence of PKCs α , β_1 , β_2 , ϵ and θ found in down-regulated smooth muscles having intact or near intact G-protein-coupled responses strongly suggests that none of these isoforms is required for agonist- or GTP[S]-induced Ca^{2+} sensitization. In addition, we can also exclude a general role in this process for PKC- η , because we detected that isoform only in PV, but not in the other smooth muscles (P. E. Jensen, L. A. Walker, M. C. Gong, J. J. Sando, A. V. Somlyo, A. P. Somlyo, unpublished work) that can be equally well Ca^{2+} -sensitized. Our results do not exclude a, probably minor, contribution by a DAG-induced, PKC-mediated effect on Ca^{2+} sensitization by agonists, and the small, but significant, reduction of GTP[S]-induced Ca^{2+} sensitization of MLC₂₀ phosphorylation in FA by down-regulation provides evidence for such a mechanism. The Ca^{2+} sensitization of force by agonists (phenylephrine and endothelin) also appeared to be reduced in down-regulated FA (Figure 2C), but the residual Ca^{2+} sensitization (increased response to pCa 6.7) due to chronic treatment with PMA precluded conclusive evaluation of this finding (see the Results section). Although we did not detect

significant agonist-induced translocation of PKCs to the membrane fraction (Figure 4), fluorescent probes have indicated translocation of PKC to the membrane during the early phase of stimulation with phenylephrine [5,33]. The absence of detectable translocation of PKCs by carbachol during the steady state of contraction (present study) was possibly due to the transient nature of such translocation [2,34]. The translocation of PKC (Figure 4) by GTP[S] (at pCa 6.0) that is presumably mediated by released DAG is consistent with the notion that activation of conventional and novel PKC(s) can contribute to, although does not fully account for, G-protein-coupled Ca^{2+} sensitization.

The mechanism of Ca^{2+} sensitization of force by phorbol esters as well as by GTP[S] and agonists appears to be mediated by increased phosphorylation of MLC_{20} . This conclusion is strengthened by the present results, showing that down-regulation of PKCs inhibits both the increase in MLC_{20} phosphorylation and force induced by PDBu. Recent studies show that phorbol esters increase MLC_{20} phosphorylation at the same sites (Thr-18, Ser-19) that are phosphorylated by myosin light chain kinase (MLCK [35,36]). These activating MLCK sites are not phosphorylated by PKC, whereas phosphorylation of the PKC sites *in vitro* inhibits smooth muscle ATPase activity [37,38]. Therefore, it is clear that the Ca^{2+} -sensitizing MLC_{20} phosphorylation induced by the phorbol ester–PKC-mediated pathway is through an indirect mechanism.

We had suggested that inhibition of the protein phosphatase that dephosphorylates MLC_{20} is the major mechanism of Ca^{2+} sensitization of force in smooth muscle [39], and have demonstrated such inhibition by agonists and GTP[S] [40] and by a putative endogenous messenger, arachidonic acid [41]. We had also suggested that PKC could inhibit smooth-muscle myosin phosphatase by activating a myosin phosphatase inhibitor and/or through direct, inhibitory phosphorylation of the smooth-muscle myosin phosphatase [39]. Subsequently, the major smooth-muscle myosin phosphatase has been identified as a trimeric protein consisting of a catalytic, type 1 protein phosphatase subunit, a regulatory M110 subunit and a 20 kDa subunit of unknown function [42–45], and more recent observations show that the enhancement of MLC_{20} phosphorylation by phorbol esters is consistent with inhibition of MLC_{20} dephosphorylation by PKC [36,46]. Earlier studies that did not show increases in MLC_{20} phosphorylation during phorbol ester-induced contraction in smooth muscle may have reflected the well-known time dependence of the force/ MLC_{20} phosphorylation relationship of smooth muscle: MLC_{20} phosphorylation declines while force is maintained over long periods (latch; reviewed in [1,47]). The rather high force/ MLC_{20} phosphorylation ratio in smooth muscles activated with phorbol esters [36] or arachidonic acid [41], however, implicates other contributory mechanisms of long-term force maintenance. The high affinity of myosin for MgADP and the slow-off rate of ADP from force-generating cross-bridges in tonic smooth muscles contribute to force maintenance following dephosphorylation of MLC_{20} to baseline levels [48], and it is possible that agents causing maintained force at low levels of MLC_{20} phosphorylation increase the accumulation and/or slow the off-rate of ADP from cross-bridges.

In the absence of Ca^{2+} (pCa ≥ 8) PDBu also increased MLC_{20} phosphorylation (present study) as well as force (P. E. Jensen, L. A. Walker, M. C. Gong, J. J. Sando, A. V. Somlyo, A. P. Somlyo, unpublished work). Such contractions are considered to be due to the activity of a Ca^{2+} -independent kinase that can phosphorylate MLC_{20} when unopposed by (inhibited) myosin phosphatase activity [22,49]. This activity can be unmasked by phosphatase inhibitors that act directly (e.g. microcystin, okadaic acid, tautomycin), rather than through kinase C ([49] and refs.

therein) and, therefore, it should not be ascribed to the activity of a Ca^{2+} -independent PKC. The unimpaired Ca^{2+} sensitization of force by GTP[S] in the absence of Ca^{2+} in down-regulated FA (present study) in which the effect of PDBu was completely inhibited (P. E. Jensen, L. A. Walker, M. C. Gong, J. J. Sando, A. V. Somlyo, A. P. Somlyo, unpublished work) suggests that none of the hitherto identified (and down-regulated) PKC isoforms is required for this Ca^{2+} -independent effect mediated by G-protein(s). Finally, if a PKC plays a major, rather than auxiliary, role in agonist-induced, G-protein-coupled inhibition of smooth muscles of myosin phosphatase, then our results suggest that such a kinase is a member of the 'atypical' family: Ca^{2+} -independent and not activated by phorbol esters.

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