Possible role of atypical protein kinase C activated by arachidonic acid in Ca^{2+} sensitization of rabbit smooth muscle

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1. Diacylglycerol (DAG; 10 μM), an activator of conventional and novel protein kinases C (cPKCs and nPKCs), induced Ca^{2+} sensitization of force in isolated intact and α-toxin-permeabilized femoral artery (FA) and portal vein (PV), and increased the phosphorylation of myosin light chain (MLC_{20}) at the same peptides phosphorylated by myosin light chain kinase.

2. Ca^{2+} sensitization by DAG was specifically inhibited by a pseudosubstrate peptide inhibitor of cPKCs ($PKC\alpha_{22-30}$ peptide; 50 μM). Similarly, GF 109203X (600 nM), an inhibitor of cPKCs and nPKCs, completely abolished Ca^{2+} sensitization by phorbol 12,13-dibutyrate (PDBu; 1 μM). In contrast, Ca^{2+} sensitization induced by the α_1-adrenergic agonist phenylephrine (100 μM) was not inhibited by these inhibitors of cPKCs and nPKCs.

3. A pseudosubstrate peptide inhibitor of the atypical PKCs (aPKCs) $PKC\zeta_{116-124}$ (50 μM) significantly (about 50%) inhibited the Ca^{2+} sensitization of force and MLC_{20} phosphorylation induced by 100 μM phenylephrine and by 300 μM arachidonic acid, but not that by DAG (10 μM) or PDBu (1 μM).

4. A phospholipase A_2 (PLA_2) inhibitor, ONO-RS-082 (10 μM), abolished the release of arachidonic acid and partially (by 40%) inhibited the Ca^{2+} sensitization induced by phenylephrine in FA smooth muscle. This effect was not additive to the inhibition observed with the aPKC inhibitor peptide, suggesting that arachidonic acid and aPKCs exert their effects via the same pathway, probably through activation of aPKC(s) by arachidonic acid.

5. Western blot analysis with antibodies to aPKCs revealed aPKCs ζ, λ (or ι) and an unidentified 64 kDa protein. The distribution (cytosolic and particulate) of these proteins was not affected by PDBu (1 μM).

6. Our results are consistent with a significant role for atypical (or related) PKCs through a PLA_2–arachidonic acid–aPKC pathway in agonist-induced Ca^{2+} sensitization, in parallel with a similar, but minor role of the DAG–cPKC cascade. The inability of the combination of the two (aPKC and cPKC) inhibitors to completely eliminate Ca^{2+} sensitization also suggests the presence of a third, still unidentified, pathway of this mechanism.

Phosphorylation of serine 19 of the 20 kDa regulatory light chain (MLC_{20}) of myosin by a Ca^{2+}–calmodulin-dependent myosin light chain kinase (MLCK) is the primary determinant of force development by smooth muscle (see reviews by Hartshorne, 1987; Somlyo & Somlyo, 1994). However, excitatory agonists and guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) that activate trimeric G-proteins can also increase MLC_{20} phosphorylation and force at constant [Ca^{2+}] ('Ca^{2+} sensitization') through G-protein-coupled inhibition of the smooth muscle myosin phosphatase (SMPP-1M) that dephosphorylates MLC_{20} (Somlyo *et al.* 1989; Kitazawa, Masuo & Somlyo, 1991*b*). Because excitatory agonists which activate phospholipase C (PLC) release diacylglycerol (DAG), an activator of protein kinases C (PKCs), and because phorbol esters that activate PKCs also cause Ca^{2+} sensitization of force, it has been suggested that PKCs play a role in Ca^{2+} sensitization (see reviews by Lee & Severson, 1994; Walsh, Andrea, Clément-Chomienne, Collins & Morgan, 1994). PKCs represent a family of various lipid-dependent serine/threonine kinases that can be divided into three categories, depending on their structure and regulation (Lee & Severson, 1994; Newton, 1995). Conventional PKCs (cPKCs; α, β and γ) require, in addition to phosphatidylserine, Ca^{2+} and DAG or

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a phorbol ester for maximal activity, whereas novel PKCs (nPKCs; δ, ϵ, η and θ) are Ca^{2+} independent, requiring only DAG or phorbol ester for activity. Activation of cPKCs and nPKCs is frequently, but not invariably, associated with their translocation from the cytosol to the membrane (Kraft & Anderson, 1983) and prolonged activation of these isoforms by phorbol esters causes their downregulation. Atypical PKCs (aPKCs; ζ, λ and ι) do not require and are not activated by Ca^{2+} or DAG. Their regulation is not well understood, although PKCζ is activated by arachidonic acid and other unsaturated fatty acids (Nakanishi & Exton, 1992). PKCs α, β_1/β_2, δ, ϵ, η, θ and ζ have been detected previously in smooth muscle (Donnelly, Yang, Omary, Azhar & Black, 1995; Clément-Chomienne & Walsh, 1996; Ohanian, Ohanian, Shaw, Scarth, Parker & Heagerty, 1996).

The involvement of PKCs in Ca^{2+} sensitization has been challenged by recent studies showing that the isoforms (cPKCs and nPKCs) activated by phorbol esters play only minor roles in G-protein-coupled Ca^{2+} sensitization (Hori, Sato, Miyamoto, Ozaki & Karaki, 1993; Itoh, Suzuki & Watanabe, 1994; Fujita, Takeuchi, Nakajima, Nishio & Hata, 1995; Jensen, Gong, Somlyo & Somlyo, 1996). This conclusion is based on the failure of interventions that inhibit Ca^{2+} sensitization by phorbol esters, such as downregulation of PKCs (Hori *et al.* 1993; Jensen *et al.* 1996) and inhibition of cPKCs with pseudosubstrate peptide inhibitors (Itoh *et al.* 1994; Fujita *et al.* 1995; but cf. Brozovich, 1995), to inhibit agonist- and/or GTPγS-induced Ca^{2+} sensitization. These experiments did not exclude a possible role for aPKCs in G-protein-coupled Ca^{2+} sensitization, because they are neither activated by DAG nor downregulated by phorbol esters; such a role, however, remained feasible in view of the Ca^{2+}-sensitizing effect of arachidonic acid. This fatty acid, while a direct inhibitor of SMPP-1M (Gong *et al.* 1992; Gailly *et al.* 1996), can also activate both aPKCs (Nakanishi & Exton, 1992) and an unidentified kinase associated with SMPP-1M (Ichikawa, Ito & Hartshorne, 1996*b*). Furthermore, phenylephrine increases the release of arachidonic acid in femoral artery (FA) smooth muscle, with this increase preceding force development (Gong, Kinter, Somlyo & Somlyo, 1995). Therefore, arachidonic acid may act through a dual mechanism, direct (dissociation of the phosphatase holoenzyme) and indirect (aPKC mediated), to inhibit SMPP-1M and so cause Ca^{2+} sensitization (Somlyo & Somlyo, 1994; Gong *et al.* 1995).

In order to determine whether aPKCs (PKCs ζ, λ or ι) contribute to G-protein-coupled Ca^{2+} sensitization and to examine the possible role of arachidonic acid in this process, we determined the effects of (relatively) selective PKC pseudosubstrate peptide inhibitors and of a phospholipase A_2 (PLA_2) inhibitor on agonist-, GTPγS- and arachidonic acid-induced increases in force and MLC_{20} phosphorylation at constant [Ca^{2+}]. The peptide selected to inhibit aPKCs was designed to mimic the pseudosubstrate sequence (116–124) of PKCζ that is also present in PKCλ and PKCι, but is absent from cPKCs and nPKCs (Dominguez *et al.* 1992; Akimoto *et al.* 1994). Our results are consistent with a significant role for aPKCs in G-protein-coupled inhibition of MLC_{20} dephosphorylation.

METHODS

Tissue preparation and isometric force measurement

Femoral artery, portal vein (PV) and ileum were removed from 2–3 kg rabbits anaesthetized with halothane and killed by rapid exsanguination through the carotid artery, as approved by the Animal Research Committee of the University of Virginia. Small longitudinal strips (50 μm thick, 150 μm wide, 3 mm long) of FA and PV were dissected and stretched to 1·3 times resting length. Isometric tension was measured with a force transducer (Akers AE 801; AME, Horten, Norway) in a well on a 'bubble' plate. Muscles were permeabilized at 22–24 °C, by using either *Staphylococcus aureus* α-toxin (17·5 μg ml^{-1} for 30 min; List Biological Laboratories, Campbell, CA, USA) or β-escin (50 μM for 30 min; Sigma, St Louis, MO, USA), according to previously described protocols (Kitazawa, Kobayashi, Horiuti, Somlyo & Somlyo, 1989). These treatments allowed the intracellular penetration of molecules of up to 1 kDa (α-toxin) and more than 100 kDa (β-escin). Ca^{2+} stores were depleted by the addition of 10 μM A23187 (Calbiochem, La Jolla, CA, USA) for 10 min. The strips were incubated in Ca^{2+}-buffered solutions (pCa > 8 to 4·5) containing 30 mM Pipes, 5 mM Mg-ATP, 10 mM creatine phosphate, $MgCl_2$ calculated to have 2 mM Mg^{2+}, 10 mM total EGTA and Ca-EGTA/EGTA calculated for the desired pCa (ionic strength adjusted to 0·2 M with potassium methanesulphonate; Kitazawa *et al.* 1991*b*). Calmodulin (0·5 μM; Sigma) was added to the Ca^{2+}-containing solution for experiments employing β-escin permeabilization. All the experiments were performed at 22–24 °C. Supramaximal concentrations of agonists (phenylephrine, 100 μM; GTPγS, 300 μM; arachidonic acid, 300 μM) were used to produce a large concentration gradient to reduce diffusional delays. The EC_{50} values for phenylephrine, GTPγS and arachidonic acid are ~2, ~0·1 and 65 μM, respectively (Kitazawa *et al.* 1991*a*; Gong *et al.* 1992).

Measurement of MLC_{20} phosphorylation

FA strips rapidly frozen in Freon 22 cooled with liquid nitrogen were cut away from the transducer hooks and freeze-substituted in acetone containing 10% trichloroacetic acid and subjected to two-dimensional isoelectric focusing and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) to separate the phosphorylated and unphosphorylated forms of MLC_{20} (Kitazawa, Gaylinn, Denney & Somlyo, 1991*a*). Proteins were then transferred onto poly(vinylidene difluoride) (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA, USA), stained with colloidal gold (Amersham Corp., Arlington Heights, IL, USA), and MLC_{20} phosphorylation was quantified by densitometry with a Bio-Rad GS-670 imaging densitometer, and expressed as a percentage of total (unphosphorylated plus phosphorylated) MLC_{20} (Kitazawa *et al.* 1991*a*).

Phosphopeptide analysis

Phosphopeptide analysis was performed according to Masuo, Reardon, Ikebe & Kitazawa (1994). Briefly, α-toxin-permeabilized FA strips (five strips per experiment) were incubated for 10 min in a pCa 6·7 solution containing 0·5 mM [γ-^{32}P]ATP (1000 c.p.m. pmol^{-1}) and 10 μM 1,2-*sn*-dioctanoylglycerol (DiC_8) or 1 μM phorbol 12,13-dibutyrate (PDBu) or 300 μM GTPγS. In the case of DiC_8, a

pre-incubation in the absence of Ca^{2+} and [γ-^{32}P]ATP was performed to allow the diffusion of DiC_8 in the preparation. The reaction was stopped by addition of trichloroacetic acid to a final concentration of 5%. The muscles were homogenized in a sample buffer containing 10 mM Tris–HCl (pH 6·8), 1% SDS, 1% β-mercaptoethanol, 20% glycerol and 0·014% Bromophenol Blue and subjected to SDS–PAGE (15% gel). After 2 h autoradiography, MLC_{20} bands were cut and digested with L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma). The solutions containing the peptides were subjected to C18 reverse-phase HPLC using a linear gradient to 5% CH_3CN over 30 min and to 100% over 60 min in the presence of 0·1% trifluoroacetic acid at a flow rate of 0·8 ml min^{-1}.

As a control, myosin (100 pmol), purified from pig bladder, was incubated for 30 min in the presence of 0·5 mM [γ-^{32}P]ATP (1000 c.p.m. $pmol^{-1}$), 1 mM Ca^{2+}, 10 mM Hepes (pH 7·5), 5 mM $MgCl_2$, 1 mM diothiothreitol (DTT), 1 mM 4-(2-aminoethyl)benzene-sulphonylfluoride HCl (AEBSF), 0·1 mM leupeptin and either 4 μM calmodulin and 40 μg ml^{-1} MLCK or 100 μg ml^{-1} phosphatidyl-serine and 40 μg ml^{-1} cPKC. Protein was then precipitated with trichloroacetic acid and treated as above.

Western blots

The following antibodies were used to detect aPKCs: ζSC, rabbit polyclonal antibody generated to epitope 573–592 of rat PKCζ (Santa Cruz Biotechnology, Santa Cruz, CA, USA); ζTL, monoclonal antibody generated to epitope 394–590 of human PKCζ (Transduction Laboratories, Lexington, KY, USA); λ and ι, monoclonal antibodies generated to sequence 397–558 of PKCλ (mouse) and 404–587 of PKCι (human). The ζSC antibody is less specific and cross reacts with cPKCs (Allen, Andrea & Walsh, 1994), whereas the ζTL antibody shows no such cross-reactivity, but is less suitable for immunoprecipitation.

Sample proteins were separated on 10% SDS–polyacrylamide gels and transferred (100 V, 4 °C, 2 h) onto PVDF membranes. After blocking non-specific protein binding sites with 5% fat-free milk (dry milk dissolved in phosphate-buffered saline (PBS (mM): NaCl, 137·4; KCl, 2·7; $KHPO_4$, 1·76; KH_2PO_4, 10; pH 7·4) for 2 h, membranes were incubated with the primary antibody (diluted 1 : 500 in PBS) for 3 h and then with the secondary antibody, horseradish peroxidase-conjugated IgG (Amersham, Arlington Heights, IL, USA), used at a dilution of 1 : 5000, for 1 h. Membranes were washed in PBS 3 times for 5 min between each step. Blots were developed by enhanced chemiluminescence (ECL; Amersham).

Immunoprecipitation

Ileum muscle sheets were homogenized in a buffer containing 1% Triton X-100 (v/v), 150 mM NaCl, 10 mM Tris (pH 7·4), 1 mM EDTA, 1 mM EGTA, 0·2 mM AEBSF and 0·5% NP-40 (v/v) and centrifuged for 15 min at 15000 *g* (4 °C). ζSC antibody (25 μg) was added to 500 μg protein in the supernatant diluted in 0·5 ml of the same buffer. After 1 h incubation at 4 °C, 25 μl Protein A–agarose was added to the extract. After a further incubation at 4 °C for 1 h, the agarose beads were washed 3 times with the same buffer, and the pellet was resuspended in 30 μl sample buffer (see above) and processed for Western blotting.

Preparation of cytosolic and particulate fractions

Muscles were homogenized in a buffer containing 20 mM Tris (pH 7·5), 5 mM $MgCl_2$, 2 mM EDTA, 1 mM DTT, 1 mM AEBSF, 100 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin and 100 μg ml^{-1} soybean trypsin inhibitor. Homogenates were then centrifuged at 350000 *g* at 4 °C for 30 min. The pellets were suspended in the same buffer containing 1% Triton X-100 and again centrifuged at 350000 *g* to obtain Triton X-100-soluble and -insoluble fractions.

Fractions were dissolved in sample buffer (see above). In some experiments, Triton X-100-insoluble pellets were treated with 1 mM Mg-ATP or 150 mM $MgCl_2$ to dissociate the myosin phosphatase SMPP-1M from myosin (Ichikawa, Hirano, Ito, Tanaka, Nakano & Hartshorne, 1996*a*); this was checked by using an antibody to the regulatory subunit M_{110} of the phosphatase (kindly provided by Dr D. Hartshorne, University of Arizona, USA).

Arachidonic acid measurements

Arachidonic acid was measured according to a protocol published previously (Gong *et al.* 1995). Briefly, femoral arteries were labelled with [^{3}H]arachidonic acid (218 Ci $mmol^{-1}$; Du Pont NEN) in Hepes-buffered Krebs solution (mM: NaCl, 137·4; KCl, 5·9; $CaCl_2$, 1·2; $MgCl_2$, 1·2; Hepes, 11·6; glucose, 11·5; pH 7·3; 2 μCi ml^{-1}) overnight at 37 °C. After being washed 3 times for 10 min in Krebs solution containing 0·2% fatty acid-free bovine serum albumin, muscles were incubated for 30 min in the presence of the PLA_2 inhibitor, 2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid (ONO-RS-082; 10 μM), or 1% DMSO (vehicle for controls). The muscle was then stimulated with 100 μM phenylephrine and the reaction was stopped after 5 min by addition of 2 ml of methanol chilled on dry ice, containing 0·25% butylated hydroxytoluene. Lipids were extracted twice with chloroform and the samples were spotted onto preadsorbent zones of channelled silica gel G thin-layer chromatography (TLC) plates (20 cm × 20 cm; Analtech, Newark, DE, USA), which had been activated for 1 h at 110 °C. Plates were developed for 30 min in light petroleum–diethyl ether–acetic acid (70 : 30 : 1, by volume) and dried in air. The radioactivity of the chromatogram was quantified with a Berthold linear analyser 284 (Nashua, NH, USA). The arachidonic acid peak was quantified relative to total counts incorporated into lipids.

Chemicals

3-[1-(3-(Dimethylamino)propyl]-1H-indol-3-yl]-4-(1H-indol-3-yl]-1H-pyrrolyl-2,5-dione (GF 109203X), a PKC inhibitor (with rank order of potency $\alpha > \beta_1 > \epsilon > \delta > \zeta$), 1,6-bis(cyclohexyloimino-carbonylamino)-hexane (RHC-80267), a DAG lipase inhibitor, and ONO-RS-082, a PLA_2 inhibitor, were purchased from Biomol (Plymouth Meeting, PA, USA). PKC inhibitor peptide ($PKC\alpha_{19-36}$), 1-(5-chloronaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine, an MLCK inhibitor (ML-9), PDBu and GTPγS were from Calbiochem; neomycin sulphate, a PLC inhibitor, GTP and phenyl-ephrine were from Sigma; and DiC_8 was from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). GF 109203X, RHC-80267, ONO-RS-082, ML-9 and DiC_8 were dissolved (concentrated 100 times) in DMSO and 1% DMSO was therefore used in control experiments. Other drugs and peptides (see below) were dissolved in water.

Two peptides consisting of the pseudosubstrate sequence of cPKCs α and β (sequence 22–30: RKGALRQKN, designated $PKC\alpha_{22-30}$) and aPKCs (sequence 116–124: RRGARRWRK, designated $PKC\zeta_{116-124}$), and a peptide consisting of a scrambled sequence of this latter (KARRRWRGR) were obtained from Bio-Synthesis, Inc. (Lewisville, TX, USA); $PKC\zeta_{116-124}$ is conserved between different species (mouse, rat and human) and between different aPKCs (ζ, sequence 116–124; mouse λ and human ι, sequence 117–125; see Fig. 1).

Bovine brain cPKCs and aPKCζ were from Calbiochem and Pan Vera (Madison, WI, USA), respectively. Myosin was prepared according to Persechini & Hartshorne (1983).

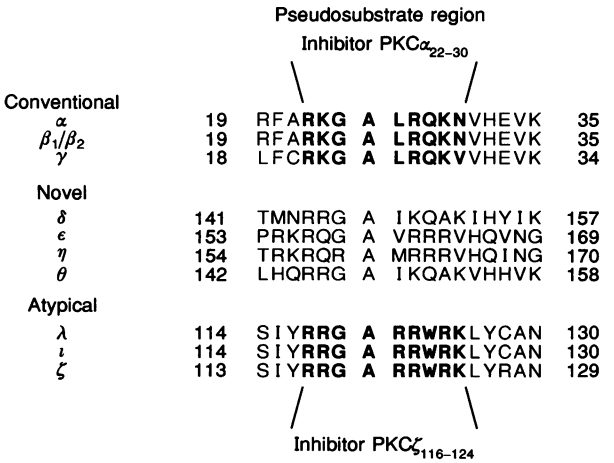

Figure 1

Pseudosubstrate sequences of different PKC isoforms (Akimoto *et al.* 1994; and references therein). The sequences used as inhibitor peptides are indicated in bold.

Statistics

Results are given as means ± S.E.M. Statistical comparisons were made using Student's t test. P values $< 0{\cdot}05$ were considered significant. Bonferroni/Dunn test (analysis of variance) was used for multiple comparisons.

RESULTS

DAG-induced sensitization in FA smooth muscle

We evaluated the Ca^{2+}-sensitizing effect of a short fatty acid chain DAG (DiC_8) on both intact and α-toxin-permeabilized FA smooth muscle. DiC_8 (10 μM) sensitized the force produced by intact FA strips in response to depolarization with high-K^+ solutions (Fig. 2*A*) and shifted the force–$[Ca^{2+}]$ curve of α-toxin-permeabilized FA strips to the left, decreasing the EC_{50} for Ca^{2+} from 363 ± 16 nM (control) to 108 ± 5 nM without affecting the maximal force developed at pCa 5·0 (Fig. 2*B*). In α-toxin-permeabilized muscles contracted at pCa 6·7 (developing 18 ± 5% of the maximal pCa 5·0-induced contraction; $n = 10$), 10 μM DiC_8 as well as 1 μM PDBu elicited a significant ($P < 0{\cdot}001$) additional increase in force (Ca^{2+} sensitization) of 63 ± 8% ($n = 5$) and 56 ± 5% ($n = 5$) of the maximal force, respectively. The effect of DiC_8 was dose dependent ($EC_{50} = 2{\cdot}4 \pm 0{\cdot}6$ μM).

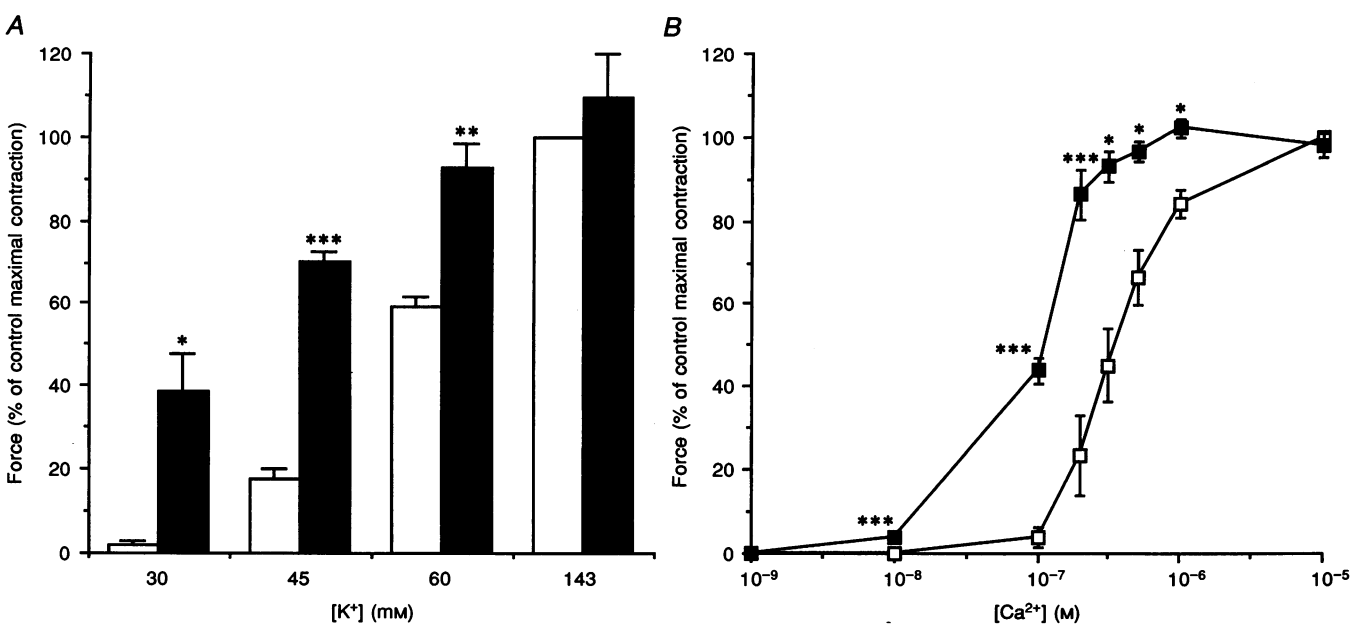

Figure 2. Potentiation of depolarization-induced contraction of intact and Ca^{2+} sensitization of permeabilized smooth muscle by DiC_8

A, intact FA strips were pre-incubated with 10 μM DiC_8 (■) or with 1% DMSO (diluent control; □) and stimulated by different high-$[K^+]_o$-containing solutions (30–143 mM). *B*, α-toxin-permeabilized FA strips were incubated with cumulatively increased $[Ca^{2+}]$ in the presence (■) or absence (□) of 10 μM DiC_8. Results (means ± S.E.M., $n = 3$–5) are expressed as a percentage of maximal control contraction induced by 143 mM K^+ (*A*) or pCa 5 (*B*). * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$.

Table 1. Effects of PKCα_{22-30} and PKC$\zeta_{116-124}$ peptides on PDBu- and agonist-induced MLC_{20} phosphorylation

	Phosphorylation (%)	
	GTP–phenylephrine	PDBu
Control	40 ± 3·2 (9)	53 ± 5·7 (7)
PKCα_{22-30}	44 ± 2 (9)	32 ± 2 (8)†
PKC$\zeta_{116-124}$	27 ± 3·6 (9)*	42 ± 6·5 (9)

β-Escin-permeabilized FA strips incubated for 30 min with 50 μM PKCα_{22-30} or PKC$\zeta_{116-124}$ peptides were rapidly frozen after 5 min contraction at pCa 6·6, followed by an additional 5 min exposure to GTP–phenylephrine or PDBu. Phosphorylated MLC_{20} is expressed as a percentage of total MLC_{20} (mean values ± S.E.M. (n)). * Significantly different from two other groups (control and PKCα_{22-30}; $P < 0·05$). † Significantly different from the control group ($P < 0·05$).

To verify the mechanism of DAG-induced sensitization, we also measured the level of MLC_{20} phosphorylation under conditions similar to those used for force measurements. α-Toxin-permeabilized FA strips were pre-incubated with 10 μM DiC_8 in Ca^{2+}-free (10 mM EGTA-containing) solution for 30 min to allow diffusion of the lipid, stimulated at pCa 6·7 for 2–30 min and rapidly frozen for phosphorylation measurements (see Methods). Phosphorylation of MLC_{20} (Fig. 3) preceded the development of force (data not shown), reached a maximum after 5 min stimulation, and was significantly increased in the presence of DiC_8.

In vitro, PKC phosphorylates MLC_{20} on Ser 1, Ser 2 and Thr 9 and decreases the actin-activated ATPase activity of myosin (Ikebe, Hartshorne & Elzinga, 1987), whereas MLCK phosphorylates Ser 19 and Thr 18 and activates the ATPase activity of myosin. We therefore performed phosphopeptide analyses (see Methods) to determine whether the PKC or the MLCK sites were phosphorylated during Ca^{2+} sensitization induced by DiC_8. α-Toxin-permeabilized FA strips were stimulated for 10 min at pCa 6·7 in the presence of 300 μM GTPγS, 10 μM DiC_8 or 1 μM PDBu. As a control, myosin was phosphorylated *in vitro* either by PKC or by MLCK. Peptides phosphorylated *in vitro* by PKC eluted at less than 5% CH_3CN, whereas peptides phosphorylated by MLCK eluted at around 60% CH_3CN. Results presented in Fig. 4 show that, *in situ*, both PDBu and DiC_8 induced phosphorylation of MLC_{20} on MLCK sites and not on PKC sites. Identical results were obtained when α-toxin-permeabilized FA strips were sensitized with 300 μM GTPγS (data not shown).

Because the level of MLC_{20} phosphorylation represents a balance between the activities of MLCK and SMPP-1M, we sought to determine which one of these enzymes was affected by DiC_8 sensitization by measuring the rate of relaxation upon removal of Ca^{2+} (with 10 mM EGTA) in the presence of ML-9, an MLCK inhibitor (Kitazawa *et al.* 1991*b*; Gong *et al.* 1992). After 30 min pre-incubation with 10 μM DiC_8 in Ca^{2+}-free solution, permeabilized muscles

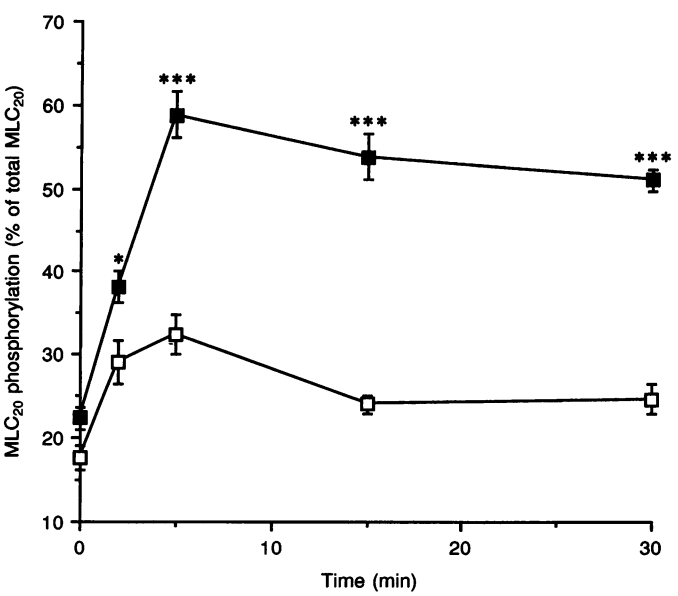

Figure 3. DiC_8-induced increase in Ca^{2+}-induced MLC_{20} phosphorylation

α-Toxin-permeabilized rabbit FA strips were incubated with 10 μM DiC_8 (■) or 1 % DMSO (diluent control; □) for 30 min in Ca^{2+}-free, 10 mM EGTA-containing solution (time point 0), and then stimulated at pCa 6·7 and frozen at the designated time points for MLC_{20} phoshorylation measurements. Phosphorylation measurements (means ± S.E.M., $n = 4–7$ for each time point) are expressed relative to total (phosphorylated and unphosphorylated) MLC_{20}. * $P < 0·05$; *** $P < 0·001$.

were maximally contracted with pCa 5 solution and transferred to a Ca^{2+}-free solution containing 100 μM ML-9. The presence of DiC_8 increased the half-time of relaxation ($t_{1/2}$) from 2·4 ± 0·29 to 4·6 ± 0·26 min ($P < 0·001$), suggesting that DiC_8 inhibits dephosphorylation of MLC_{20}.

Role of cPKCs and nPKCs in Ca^{2+} sensitization

DAG is known to activate cPKCs and nPKCs. We used two isoform-specific PKC inhibitors to assess the relative contributions of these PKCs to agonist-induced Ca^{2+} sensitization.

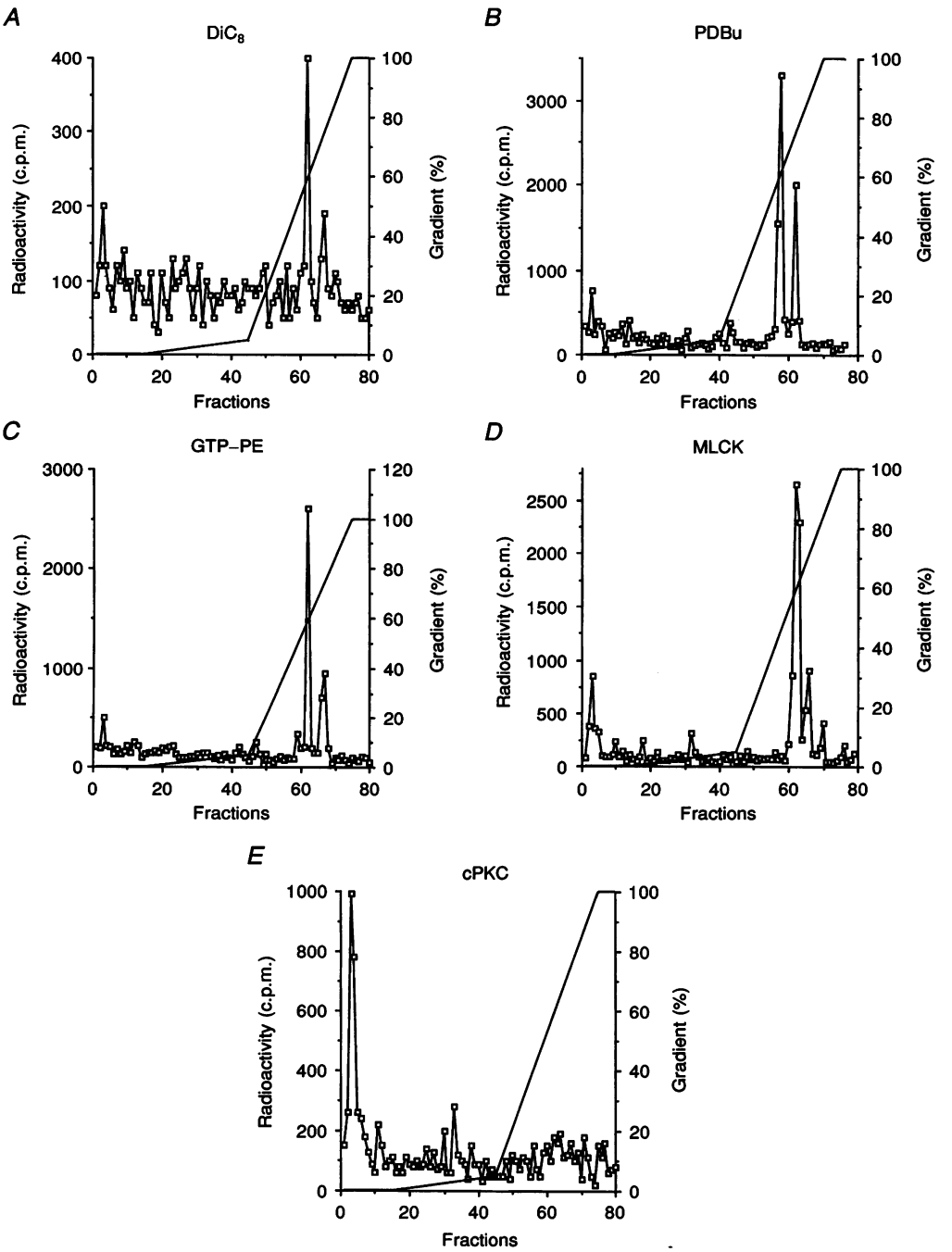

Figure 4. Phosphopeptide analysis of sites phosphorylated on MLC_{20}

FA strips permeabilized with α-toxin were stimulated at pCa 6·7 by 10 μM DiC_8 (*A*), 1 μM PDBu (*B*) or 10 μM GTP plus 100 μM phenylephrine (GTP–PE; *C*). Purified myosin was phosphorylated by MLCK (*D*) and cPKC (*E*). Note that only the peptides obtained from isolated myosin and phosphorylated with cPKC elute below 5% CH_3CN, whereas the peptides isolated from smooth muscle, whether stimulated with DiC_8, PDBu or phenylephrine, elute in the same region as the peptides obtained from myosin phosphorylated by MLCK (see Results).

The PKC inhibitory peptide $PKC\alpha_{19-36}$ is identical to a region in the PKCs α and β pseudosubstrate sequences (House & Kemp, 1987). After incubating β-escin-permeabilized FA muscles for 30 min in the presence of 20 μM $PKC\alpha_{19-36}$ peptide (in Ca^{2+}-free solution), DiC_8-induced sensitization of force (tested at pCa 6·6) was decreased from $36 \pm 4{\cdot}2\%$ ($n = 4$) of the maximal contraction to $17 \pm 2{\cdot}4\%$ ($n = 3$; $P < 0{\cdot}05$). In contrast, GTP–phenylephrine-induced sensitization (10 μM GTP, 100 μM phenylephrine) was not affected ($31 \pm 4{\cdot}6\%$ ($n = 7$) *vs.* $30 \pm 2{\cdot}4\%$ ($n = 3$)) by $PKC\alpha_{19-36}$. Similar effects were obtained with 50 μM of a shorter pseudosubstrate peptide of cPKC ($PKC\alpha_{22-30}$, see below and Fig. 8). These effects on force were accompanied by similar selective inhibition of the increase in MLC_{20} phosphorylation induced by PDBu but not by phenylephrine (Table 1). Because there was no detectable difference between the effects of DiC_8 and PDBu on Ca^{2+} sensitization of force or on the level and the site of MLC_{20} phosphorylation (Masuo *et al.* 1994; Itoh *et al.* 1994; present study, Table 1) and their inhibition by $PKC\alpha_{22-30}$, in subsequent experiments we used PDBu to activate cPKCs and nPKCs, to facilitate comparison with studies of the effects of phorbol esters in the literature.

GF 109203X inhibits PKCs α, β_1, δ, ϵ and ζ with the following EC_{50} values, respectively: 8·4, 18, 210, 132 and 5800 nM (Toullec *et al.* 1991; Martiny-Baron *et al.* 1993). At the very low concentration reported to inhibit only cPKCs α and β (60 nM), GF 109203X diminished PDBu-induced sensitization by $82 \pm 2\%$ ($n = 3$; Fig. 5, same protocol as before). At higher concentrations (up to 600 nM of GF 109203X, $n = 3$), the response to PDBu was completely abolished whereas GTP–phenylephrine-induced sensitization was not affected, suggesting that neither cPKCs nor nPKCs (such as PKCϵ) are necessary for agonist-induced sensitization. Very high concentrations of the drug, known to inhibit atypical PKCs (e.g. PKCζ), also affect other kinases such as MLCK (Toullec *et al.* 1991).

Identification and distribution of aPKCs

The effects of peptide inhibitors of aPKC and of arachidonic acid (see below) were compatible with the participation of an aPKC in G-protein coupled Ca^{2+} sensitization. Therefore, we identified the immunochemically detectable atypical isoforms in PV, FA and ileum smooth muscles. Their distribution between the cytosolic, Triton X-100-soluble and Triton X-100-insoluble fractions was studied in ileum. Using different antibodies against PKCs ζ, λ and ι, we detected by Western blot analysis four different bands of, respectively, 88, 80, 70 and 64 kDa (Fig. 6*A*).

The 88 kDa band was detected only by the ζSC antibody in the whole extract and the cytosol. This antibody was generated against a C-terminal epitope of the protein and cross reacts with PKCs α and β (Allen *et al.* 1994). The 88 kDa band is also downregulated by prolonged treatment with PDBu (P. E. Jensen, L. A. Walker, M. C. Gong, P. Gailly, J. J. Sando, A. V. Somlyo & A. P. Somlyo, unpublished results) and, therefore, is probably a cPKC that cross reacts with ζSC.

The 80 kDa band was detected by each of the four antibodies to aPKCs and co-migrated with PKCζ that was detected at a much higher concentration in rat lung by the

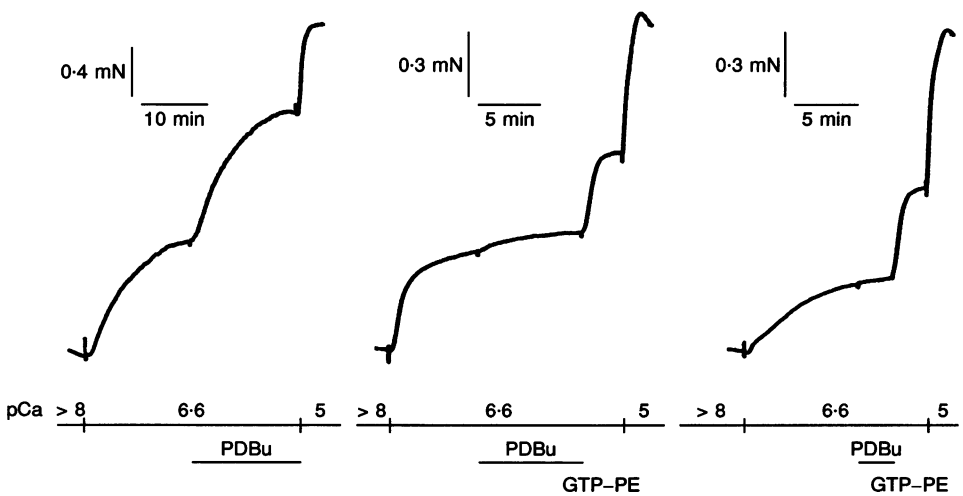

Figure 5. The effect of GF 109203X, an inhibitor of cPKCs and nPKCs, on PDBu-induced, and its lack of effect on GTP–phenylephrine-induced Ca^{2+} sensitization of force

β-Escin-permeabilized FA strips were incubated in the absence (left panel) or in the presence of 60 nM (middle panel) or 600 nM (right panel) GF 109203X, and PDBu (1 μM) and GTP–phenylephrine (GTP–PE; 10 μM GTP, 100 μM phenylephrine)-induced sensitizations were evaluated at pCa 6·6. Note that GF 109203X inhibits Ca^{2+} sensitization of force by PDBu, but not that by GTP–phenylephrine. Note also the slower rate of pCa 6·6-induced force development at 600 nM GF 109203X, suggestive of a partial inhibition of MLCK by high concentrations of the drug. Traces are representative of at least 3 experiments.

same four antibodies (Fig. 6*B*). The protein could be specifically separated from the three other bands by immunoprecipitation with ζSC antibody (Fig. 6*A*, lanes 5 and 6), suggesting that this antibody has a greater specificity for the 80 kDa protein than for the other three. We therefore consider the 80 kDa band to be the authentic PKCζ, as is also indicated by its known abundance in lung (Walsh *et al.* 1994).

The 70 kDa band was recognized (Fig. 6*A*) by λ antibody (and by ι antibody (not shown) that has the same specificity,

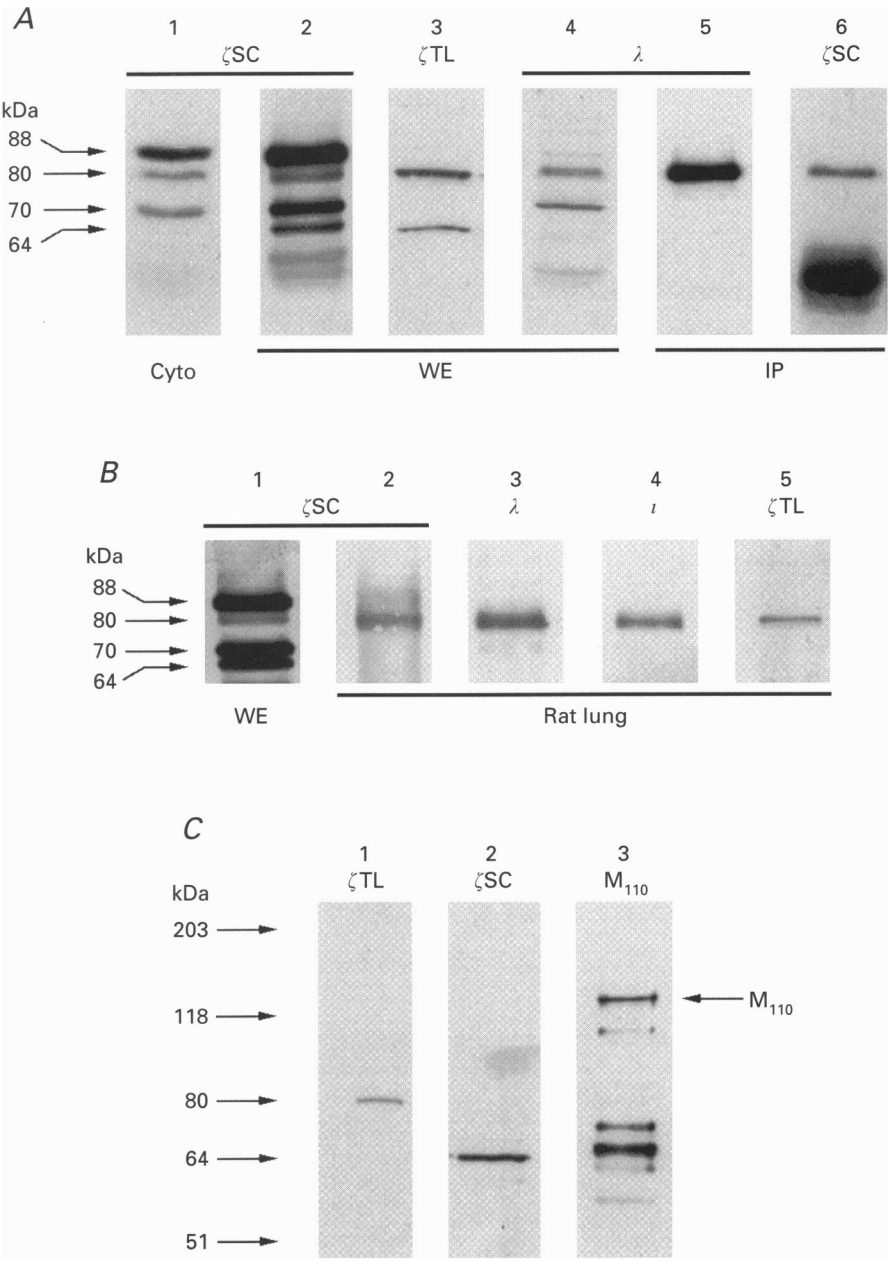

Figure 6. Identification and distribution of aPKCs

Western blots (25 to 200 μg protein per lane, detection with specific antibodies as indicated above each lane). *A*, ileum extracts: lane 1, cytosol (Cyto); lanes 2, 3 and 4, whole extracts (WE). The extracts shown in lanes 5 and 6 (IP) were immunoprecipitated with ζSC antibody but probed with λ (lane 5) or ζSC (lane 6; the band at about 50 kDa in lane 6 represents the IgG used for the immunoprecipitation). *B*, lane 1: FA whole extract; lanes 2–5: rat lung whole extracts. *C*, Western blots of proteins extracted with 150 mM $MgCl_2$ from the Triton X-100-insoluble fraction of ileum extracts. The 64 kDa band is not detected by ζTL nor the 80 kDa band by ζSC, probably due to different sensitivities of these two antibodies. Note the usual degradation of the M_{110} subunit of the phosphatase (migrating, when intact, at 130 kDa in SDS–PAGE) to its proteolytic fragments (58–72 kDa; Alessi, MacDougall, Sola, Ikebe & Cohen, 1992). Results are representative of 3–6 experiments.

since PKCs λ and ι are the same protein in, respectively, mouse and human), and co-migrated with the human PKCλ, and therefore, we consider it to be PKCλ. Not surprisingly, this protein was also recognized by ζSC antibody, as PKCs λ and ζ have very similar C-termini, but not by ζTL antibody generated to a large (22·5 kDa) region of PKCζ (see Methods). This latter observation also rules out the possibility that this band is a degradation product of PKCζ (80 kDa band).

Finally, the 64 kDa band reacted with ζSC and ζTL antibodies (Fig. 6*A*, lanes 2 and 3) but not (or very weakly) with λ and ι antibodies. This band has not been clearly identified, but could represent a new or modified atypical PKC or a related protein. ζSC antibody seemed to be more sensitive than the ζTL antibody for the detection of the 64 kDa band, but less sensitive for the detection of the 80 kDa band (Fig. 6*A*, compare lanes 2 and 3, see also Fig. 6*C*).

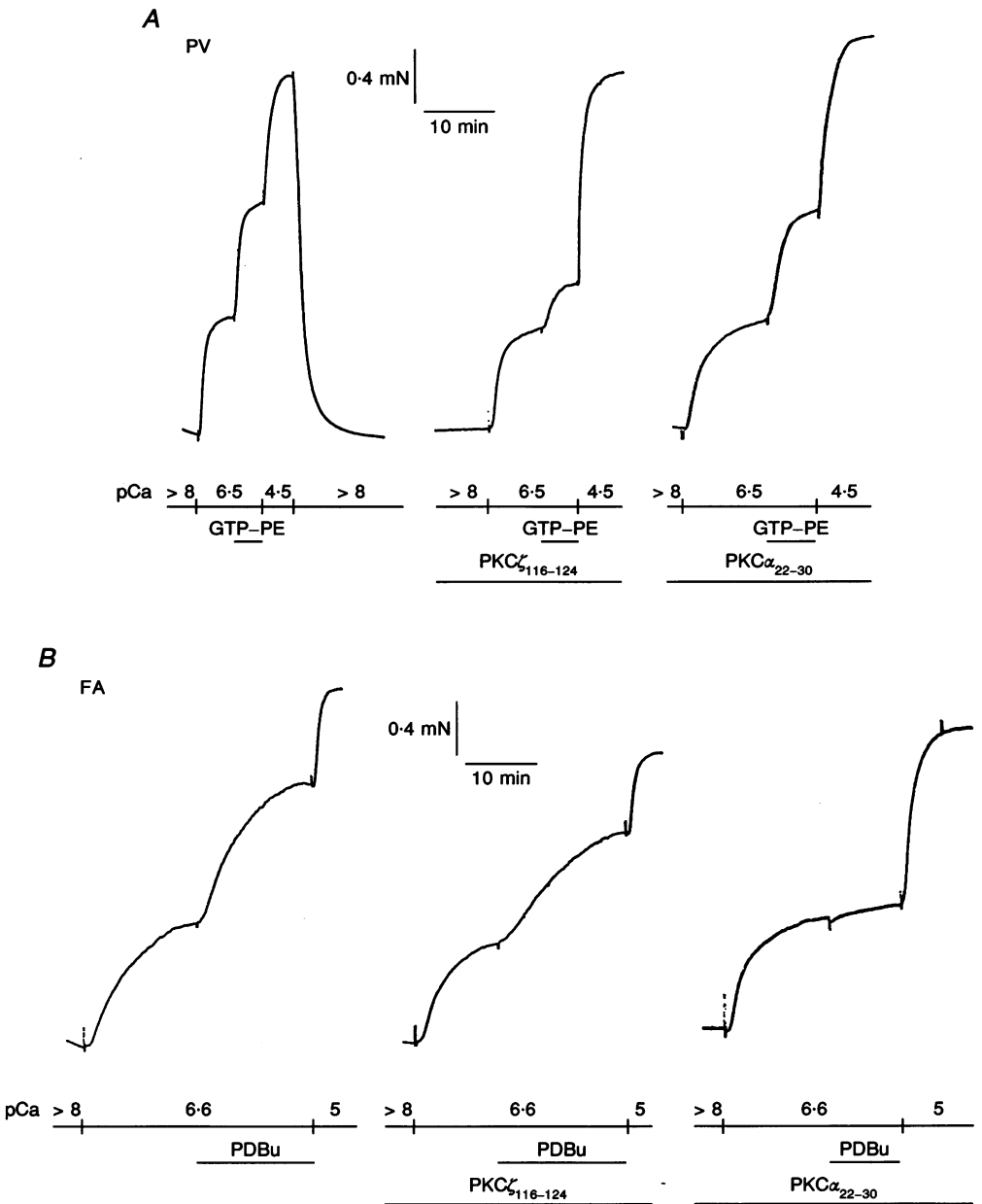

Figure 7. Effects of PKC$\zeta_{116-124}$ and PKCα_{22-30} peptides on force sensitization

β-Escin-permeabilized FA and PV strips were pre-incubated for 30 min with 50 μM PKC$\zeta_{116-124}$ or PKCα_{22-30} peptides in Ca^{2+}-free solution. Thereafter, muscles were transferred to a pCa 6·6 (FA; *B*) or pCa 6·5 (PV; *A*) solution and stimulated by GTP–phenylephrine (GTP–PE; 10 μM GTP, 100 μM phenylephrine) or 1 μM PDBu. GTP–phenylephrine-induced sensitization is illustrated in PV (*A*) and PDBu-induced sensitization in FA (*B*). Traces are representative of 5 experiments.

To summarize, the 88 kDa band detected with the ζSC antibody is a conventional isoform; the 80 and 70 kDa bands are probably PKCs ζ and λ, respectively, and the 64 kDa band has not been identified. No difference in isoform composition was observed between PV, FA and ileum (compare Fig. 6*A*, lane 2 with Fig. 6*B*, lane 1).

Activation of cPKCs and nPKCs is frequently associated with their translocation from the cytosol to the cell membrane (Kraft & Anderson, 1983). Therefore, we determined the distribution of these proteins under resting conditions (intact muscle relaxed in the absence of external Ca^{2+}) and following stimulation with PDBu or GTPγS. In unstimulated tissues, PKCλ was found exclusively in the cytosol (supernatant obtained after centrifugation of the whole extract at 350 000 *g*; see Methods), whereas PKCζ and the unidentified 64 kDa band were found both in the cytosol (about 70 and 35%, respectively) and in the Triton X-100-insoluble fraction (only traces in the Triton X-100-soluble fraction). The particulate-associated, Triton X-100-insoluble component of both these kinases could be partially removed (about 50%) by treatment with 1 mM Mg-ATP or 150 mM $MgCl_2$ (Fig. 6*C*). This treatment also removed the smooth muscle myosin phosphatase SMPP-1M (Ichikawa *et al.* 1996*a*), suggesting a possible association between these kinases and SMPP-1M. The translocation of PKC to the membrane was investigated using Triton X-100 extraction in α-toxin-permeabilized ileum, under resting conditions (Ca^{2+} free), after contraction for 10 min in pCa 6·0 solution and after sensitization (at pCa 6·0) with 1 μM PDBu or 300 μM GTPγS for 15 min. None of the atypical PKCs detected were translocated to the Triton X-100-soluble fraction. As a control, the 88 kDa band (corresponding to cPKCs) was translocated after stimulation with PDBu (from 20% in the Triton X-100-soluble fraction at pCa 6·0 to 70%) and, to a lesser extent, with GTPγS (45%, data not shown). These results confirm that the three lower bands, identified as aPKCs, are not sensitive to PDBu (Clément-Chomienne & Walsh, 1996).

Possible involvement of aPKCs in Ca^{2+} sensitization: the effect of peptide inhibitors

To determine the possible involvement of aPKCs in agonist-induced sensitization, we compared the effects of two peptides $PKC\alpha_{22-30}$ and $PKC\zeta_{116-124}$ that are identical to the pseudosubstrate sequences of, respectively, PKCs α and β and PKCs λ, ι and ζ (Fig. 1). Dominguez *et al.* (1992) previously used this strategy and showed that these peptides specifically inhibited, respectively, phorbol ester-sensitive (c and n) PKCs and PKCζ. We also determined, in isolated purified enzyme preparations, the specificity of inhibition of the activity of purified aPKCζ by the $PKC\zeta_{116-124}$ peptide and found its IC_{50} value to be ~50 nM, whereas under identical conditions the IC_{50} for the $PKC\alpha_{22-30}$ peptide was ~5 μM, i.e. an approximately 100-fold selectivity.

FA strips were permeabilized with β-escin in pCa 6·0 solution for 30 min in the presence of 50 μM of either peptide. After relaxation in Ca^{2+}-free solution, muscles were incubated for 5 min in pCa 6·6 solution and then stimulated by either 1 μM PDBu or 10 μM GTP plus 100 μM phenylephrine. $PKC\alpha_{22-30}$ peptide inhibited the DiC_8- and PDBu-induced Ca^{2+} sensitization of both force (by approximately 50%) and MLC_{20} phosphorylation (by about 40%; Figs 7 and 8; Table 1). As noted above, this peptide did not inhibit agonist (GTP–phenylephrine)-induced Ca^{2+} sensitization. Conversely, 50 μM $PKC\zeta_{116-124}$ peptide significantly inhibited the GTP–phenylephrine-induced, but not the PDBu-induced, force sensitization (Figs 7 and 8) and MLC_{20} phosphorylation (Table 1; the small inhibition of PDBu-induced MLC_{20} phosphorylation by this peptide was not statistically significant). A peptide concentration of 30–50 μM had a maximal effect, with no further inhibition observed at

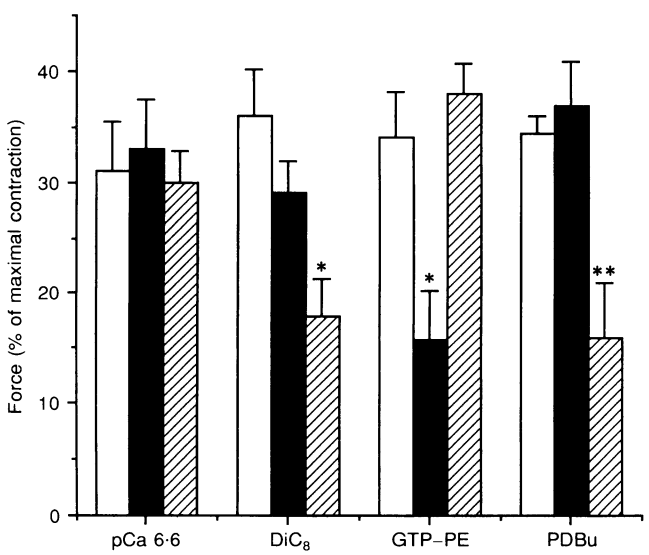

Figure 8. Effects of $PKC\zeta_{116-124}$ and $PKC\alpha_{22-30}$ peptides on force sensitization

Summary of the results illustrated in Fig. 7. GTP–phenylephrine (GTP–PE)-, DiC_8- and PDBu-induced sensitization were studied in FA muscles incubated in the absence (□) or in the presence of 50 μM $PKC\zeta_{116-124}$ (■) or $PKC\alpha_{22-30}$ peptides (▨). The sensitization by these agents was normalized to the maximal contraction (pCa 5·0) and expressed as the force developed *in addition* to the force developed and maintained at pCa 6·6. Results are means ± S.E.M., $n = 5$. * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$.

higher concentrations (up to 150 μM, although at this concentration we observed a small sensitizing effect, possibly due to Ca^{2+} contamination of the peptide stock solution).

A scrambled sequence of the PKC$\zeta_{116-124}$ peptide (50 μM) did not affect the sensitization induced by phenylephrine.

Possible role of arachidonic acid-activated aPKC pathway in agonist-induced sensitization

To explore the mechanism of arachidonic acid production elicited by phenylephrine and its possible role in Ca^{2+} sensitization, we determined, in FA smooth muscle, the effect of ONO-RS-082, an inhibitor of PLA_2 (Billah & Anthes, 1990), on the release of arachidonic acid and Ca^{2+} sensitization (see Introduction) induced by phenylephrine. Intact FA arteries labelled with [^{3}H]arachidonic acid were incubated for 30 min with 10 μM ONO-RS-082 or 1% DMSO (vehicle) as a control. Muscles were then stimulated with 100 μM phenylephrine for 5 min, and the release of arachidonic acid measured by TLC (see Methods). ONO-RS-082 (10 μM) almost completely inhibited the release of arachidonic acid induced by phenylephrine, suggesting that PLA_2 is largely responsible for this effect (Fig. 9*A*).

In α-toxin- and β-escin-permeabilized FA strips, 10 μM ONO-RS-082 inhibited the sensitization induced by GTP–phenylephrine by about 50% (10 μM GTP, 100 μM phenylephrine), but not by GTP alone (Fig. 9*B*), without affecting pCa 6·7-induced contraction or PDBu-induced Ca^{2+} sensitization. Consistently, in intact FA strips, 10 μM ONO-RS-082 also inhibited by about 40% the phenylephrine (50 μM)-induced contraction, but did not affect depolarization-induced contraction (data not shown), consistent with previous reports of the involvement of PLA_2 in Ca^{2+} sensitization (Parsons, Sumner & Garland, 1996). Neomycin (10–30 μM), a PLC inhibitor, and RHC-80267 (50 μM), a DAG lipase inhibitor, did not affect agonist-induced Ca^{2+} sensitization. A combination of 10 μM ONO-RS-082 and 50 μM PKC$\zeta_{116-124}$ peptide did not have an additive effect on GTP–phenylephrine-induced sensitization (data not shown).

The effect of PKC inhibitors on arachidonic acid-induced Ca^{2+} sensitization

Although we have shown previously that arachidonic acid directly inhibits SMPP-1M *in vitro* (Gong *et al.* 1992), the present study and the reported activation by arachidonic acid of aPKCs (Nakanishi & Exton, 1992) and of an unidentified kinase associated with SMPP-1M (Ichikawa *et al.* 1996*b*) suggested an additional, aPKC-mediated, mechanism of arachidonic acid-induced Ca^{2+} sensitization. Arachidonic acid-induced Ca^{2+} sensitization was almost completely abolished by PKC$\zeta_{116-124}$ peptide (50 μM) but not by PKCα_{22-30} peptide (50 μM; Fig. 10*A* and *B*). GTPγS still induced sensitization in the presence of PKC$\zeta_{116-124}$ peptide (Fig. 10*A*) although the response was slower than in its absence.

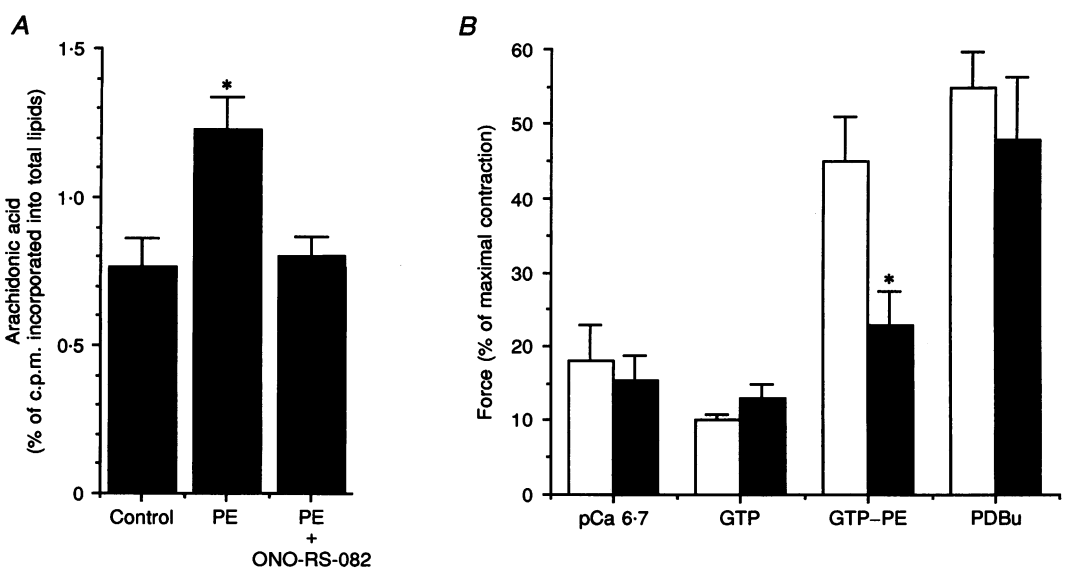

Figure 9. The effect of an inhibitor of PLA_2 on arachidonic acid release and force sensitization

A, arachidonic acid was measured in intact FA either under control conditions or after stimulation for 5 min with 100 μM phenylephrine (PE) in the presence or absence of 10 μM ONO-RS-082. The amount of released arachidonic acid is expressed relative to the amount of [^{3}H]arachidonic acid incorporated into total lipids (means ± S.E.M., $n = 8$–12). *B*, GTP- (10 μM), GTP–phenylephrine- (10 μM GTP, 100 μM phenylephrine) and PDBu (1 μM)-induced sensitizations were evaluated at pCa 6·7 in β-escin-permeabilized FA, in the presence (■) or absence (□) of 10 μM ONO-RS-082. The sensitization is expressed as the force developed in addition to the response at pCa 6·7. Force is normalized to maximal pCa 5·0-induced contraction (means ± S.E.M., $n = 4$–10). * $P < 0{\cdot}05$.

DISCUSSION

The major findings of this study: (1) suggest that an atypical PKC or related kinase that can be activated by arachidonic acid and inhibited by an aPKC peptide inhibitor plays a significant role in agonist-induced, G-protein-coupled Ca^{2+} sensitization, and (2) provide further evidence that Ca^{2+} sensitization by activation of conventional and/or novel PKCs by DAG is related to increased phosphorylation of the regulatory light chain at the MLCK phosphorylation sites and that these PKCs play only a minor role in agonist-induced sensitization. We also found evidence suggestive of a third, as yet unidentified, pathway of Ca^{2+} sensitization.

DiC_8, a synthetic analogue of the physiological PKC activator DAG, and the phorbol ester PDBu had qualitatively and quantitatively similar sensitizing effects on force developed by permeabilized smooth muscles. The increased phosphorylation on the MLCK site of MLC_{20} associated with the increase in force elicited by phorbol esters, DAG or GTPγS at constant $[Ca^{2+}]$ suggest a common pathway of sensitization through inhibition of SMPP-1M (Fig. 4, present study;

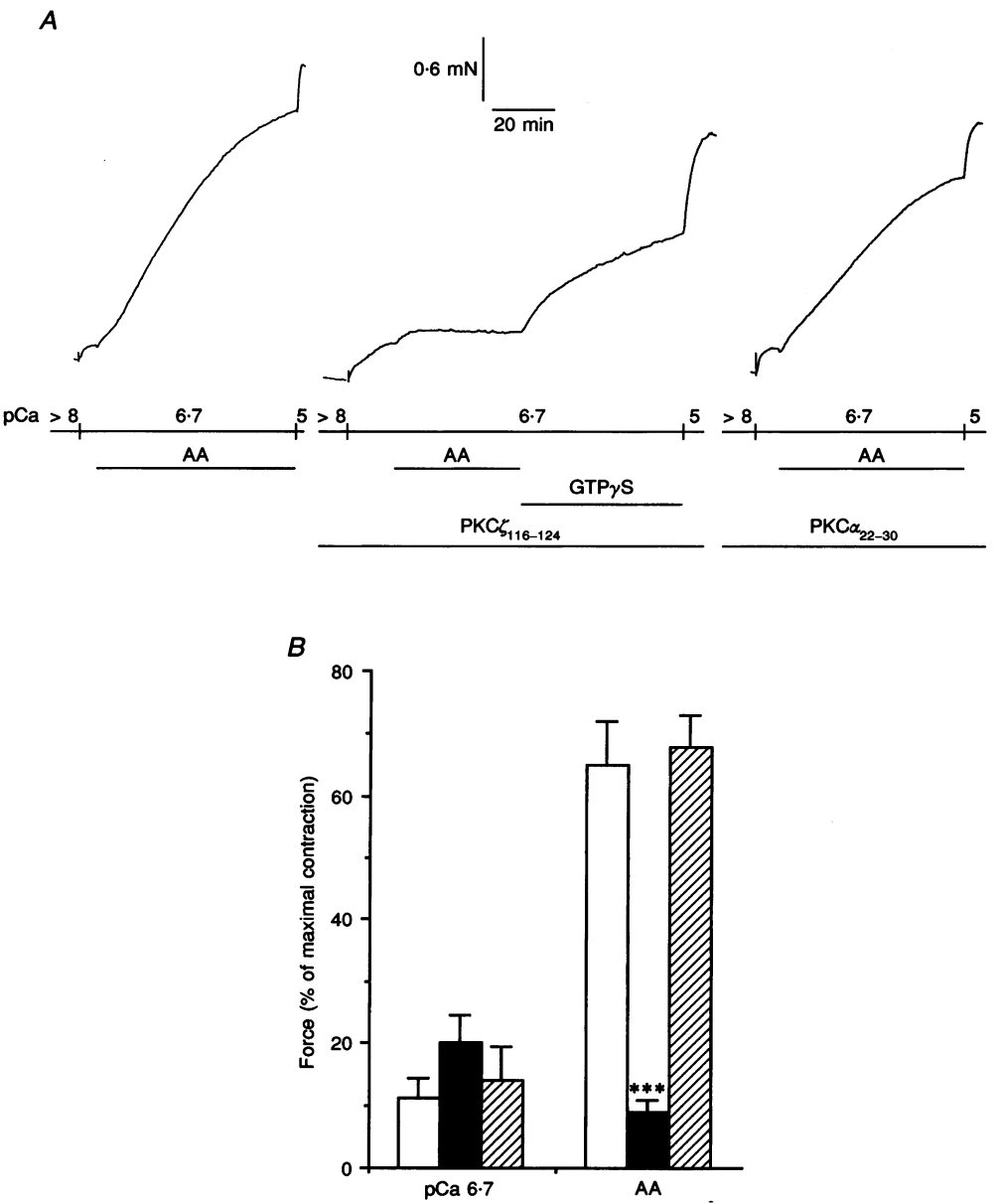

Figure 10. The effect of PKC peptide inhibitors on arachidonic acid-induced Ca^{2+} sensitization
Arachidonic acid (AA; 300 μM)-induced sensitization was evaluated in β-escin-permeabilized FA strips at pCa 6·7 (same protocol as before, see Fig. 7). *A*, examples of force traces: left trace, control; middle trace, in the presence of $PKC\zeta_{116-124}$ peptide; right trace, $PKC\alpha_{22-30}$ peptide. The effect of GTPγS (300 μM) is shown in the middle trace. *B*, results (means ± S.E.M., $n = 4$) are expressed relative to the maximal force. □, control; ■, 50 μM $PKC\alpha_{116-124}$; ▨, 50 μM $PKC\alpha_{22-30}$. *** $P < 0{\cdot}001$.

Kitazawa *et al.* 1991b; Itoh *et al.* 1994; Masuo *et al.* 1994). The mechanism of inhibition may be, as suggested previously (Somlyo *et al.* 1989; Somlyo & Somlyo, 1994), indirect, secondary to activation of an SMPP-1M inhibitor, or direct, through phosphorylation of an SMPP-1M subunit (Ichikawa *et al.* 1996b). The mechanisms of sensitization induced by activators of cPKCs and nPKCs thus seem to converge on the same target as agonist- and GTPγS-induced sensitization: inhibition of dephosphorylation of MLC_{20}. Our results, although confirming that cPKCs and/or nPKCs can induce Ca^{2+} sensitization, are consistent with the conclusion that these PKCs play only a relatively minor role in the G-protein-coupled inhibition of MLC_{20} dephosphorylation. Inter-ventions that inhibit PDBu-induced sensitization, such as downregulation of cPKCs and nPKCs (Hori *et al.* 1993; Jensen *et al.* 1996), have little or no effect on agonist- and GTPγS-induced Ca^{2+} sensitization, indicating that PDBu-sensitive isoforms of PKC are not required for these mechanisms. GF 103209X, at a concentration reported to inhibit both cPKCs and nPKCs, completely abolished PDBu-induced Ca^{2+} sensitization, without inhibiting GTP–phenylephine-induced sensitization. The incomplete inhibition by PKCα_{22-30} or PKCα_{19-36} peptides of the PDBu- and DiC_8-induced responses might be due to the fact that these peptides correspond to the pseudosubstrate sequence of cPKCs and, therefore, may not inhibit nPKCs. The failure of PKCα_{19-36} and PKCα_{22-30} to inhibit phenylephrine-induced Ca^{2+}-sensitization is consistent with earlier studies (Itoh *et al.* 1994; Fujita *et al.* 1995; and see Introduction), and may reflect the transience and/or low level of DAG produced by permeabilized smooth muscles upon stimulation by agonists. The potentiation by DAG on the K^+ contractures of intact (non-permeabilized) smooth muscle (present study) suggests that prolonged elevation of DAG can Ca^{2+} sensitize intact smooth muscle through stimulation of cPKCs and/or nPKCs. It remains to be determined whether such high levels of DAG are ever generated under physiological conditions and whether the relative contribution of these PKCs to Ca^{2+} sensitization is agonist- and/or tissue-dependent.

Phorbol ester-induced Ca^{2+} sensitization in the absence of increased MLC_{20} phosphorylation has also been reported and ascribed to the action of PKCs on thin filament-associated proteins (reviewed by Walsh *et al.* 1994), but our results provide no evidence for or bearing on such a mechanism.

We have immunochemically identified, in addition to the known isoform PKCζ (Clément-Chomienne & Walsh, 1996), aPKCλ (equivalent to ι) in smooth muscle. Neither they nor the unidentified 64 kDa band that reacted with the PKCζ antibody were translocated by PDBu to the membrane, further confirming that they are not members of the cPKC or nPKC families. A pseudosubstrate peptide inhibitor specific to aPKCs inhibited both G-protein-coupled and arachidonic acid-induced Ca^{2+} sensitization of force and MLC_{20} phosphorylation without inhibiting the effect of PDBu. Considering also that arachidonic acid is known to activate aPKCs (Nakanishi & Exton, 1992), our findings suggest that either or both of the aPKCs and the unidentified 64 kDa protein detected by the aPKC antibody may be involved in G-protein-coupled Ca^{2+} sensitization. We cannot exclude the possibility that other kinases that may also be inhibited by the PKC$\zeta_{116-124}$ peptide, such as Rho-kinase (Leung, Manser, Tant & Lim, 1995), PKN (Mukai & Ono, 1994) or a kinase associated with the regulatory subunit of SMPP-1M and activated by arachidonic acid (Ichikawa *et al.* 1996*b*) contribute to Ca^{2+} sensitization. Two of these kinases, the Rho-kinase (Matsui *et al.* 1996) and the SMPP-1M-associated kinase (Ichikawa *et al.* 1996*b*) can phosphorylate M_{110} (although at a different site(s)) and inhibit phosphatase activity. It is not known whether any of these enzymes are inhibited by PKC$\zeta_{116-124}$, but it is interesting that inhibition of SMPP-1M by arachidonic acid requires the C-terminus containing the phosphorylation sites of the M_{110} (regulatory) subunit (Gailly *et al.* 1996). It is possible that the 64 kDa protein (present study) is the SMPP-1M-associated kinase.

Phenylephrine-induced arachidonic acid release and Ca^{2+} sensitization were both inhibited by a PLA_2 inhibitor (Fig. 9), consistent with a role of arachidonic acid in Ca^{2+} sensitization (Gong *et al.* 1992, 1995), whereas the inhibition of the effect of arachidonic acid by an aPKC pseudosubstrate inhibitor (PKC$\zeta_{116-124}$) implicates a downstream role for aPKCs (Fig. 10). The effects of the PLA_2 inhibitor and the PKC$\zeta_{116-124}$ peptide were not additive, also suggesting that arachidonic acid and aPKCs act on the same pathway. Thus, although arachidonic acid can directly inhibit SMPP-1M in solution by dissociating the regulatory subunit from the catalytic subunit (Gong *et al.* 1992), its action on smooth muscle may involve dual mechanisms, both direct and indirect (aPKC mediated). In contrast to the effect of PKC$\zeta_{116-124}$, pseudosubstrate inhibitors of cPKCs (PKC_{19-36}, PKC_{22-30}) that block phorbol ester-induced Ca^{2+} sensitization did not inhibit the action of arachidonic acid (Gong *et al.* 1992; present study), further supporting the existence of at least two PKC-mediated independent pathways.

In conclusion, our studies suggest that atypical (and/or related) PKCs play the major role in G-protein-coupled Ca^{2+} sensitization in smooth muscle. The failure of the combination of two types of pseudosubstrate inhibitors to completely block the process implicates a third, as yet unidentified, pathway. This conclusion is also supported by the fact that the inhibitor of PLA_2, although completely abolishing the increase in arachidonic acid, inhibited only partially the phenylephrine-induced sensitization. The existence in smooth muscle of pharmacomechanical coupling mechanisms that can modify the level of force at constant $[Ca^{2+}]$ was predicted some time ago (Somlyo & Somlyo, 1968), but the complexities of the parallel and convergent pathways involved and their molecular mechanisms are only now being clarified.

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