Purification and characterization of protein kinase C from rabbit iris smooth muscle

Myosin light-chain phosphorylation *in vitro* and in intact muscle

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Protein kinase C of rabbit iris smooth muscle was purified by the sequential use of three chromatographic steps, i.e. anion-exchange (DEAE-cellulose), gel filtration (Sephadex G-150) and substrate affinity (protamine-agarose), and its properties were investigated by using as substrate myosin light-chain protein (MLC) isolated from the same tissue. The enzyme appeared as a single band on SDS/polyacrylamide-gel electrophoresis, with a molecular mass of approx. 80 kDa. Histone H-1 and iris muscle MLC, but not rabbit skeletal-muscle MLC, were effective substrates for the enzyme, with apparent K_m values of 3.0 and 16.6 μM respectively. The enzyme, with MLC as substrate, had the following characteristics. (a) Its activity was dependent on Ca^{2+} and phosphatidylserine (PS). In the presence of Ca^{2+} and PS, diolein and phorbol dibutyrate (PDBu) increased its activity by 61 and 65 % respectively. Half-maximal activation of the enzyme (K_a) occurred at 10 μM free Ca^{2+} , and in the presence of diolein and PDBu the apparent K_a for Ca^{2+} was decreased to 3 μM and 2 μM respectively. (b) Studies on the relative potency of various cofactors in activating the enzyme revealed that PS, phorbol myristate acetate and 1-stearoyl-2-arachidonylglycerol were the most potent of the phospholipids, phorbol esters and diacylglycerols respectively. (c) H-7, a protein kinase C inhibitor, inhibited MLC phosphorylation in a dose-dependent manner, with 50 % inhibition at 10 μM . (d) Addition of carbamoylcholine (for 1 min) or PDBu (for 25 min) to iris sphincter muscle prelabelled with [^{32}P]P_i specifically increased MLC phosphorylation, and only the stimulatory effect of the muscarinic agonist was blocked by atropine. The data provide additional support for a role for protein kinase C in the contractile response of the iris smooth muscle.

INTRODUCTION

There is now general agreement that an elevation of the intracellular Ca^{2+} concentration mediates the action of a number of hormones, neurotransmitters and other pharmacological agents. In smooth muscle, as in other tissues, one way by which these agents increase intracellular Ca^{2+} and induce contraction involves formation of inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DG) from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) (for reviews see [1–7]). However, although receptor-mediated breakdown of PIP_2 was first reported in the iris smooth muscle more than a decade ago [8], our knowledge of the role of the polyphosphoinositides and their derived second messengers in receptor function in smooth muscle is still incomplete. The contraction of smooth muscle in response to various agonists is usually composed of two phases: a fast ‘phasic’ component, followed by a slower, more sustained, ‘tonic’ component. There is now good experimental evidence which suggests that synergism between IP_3 and DG could play an important role in regulating smooth-muscle contraction (see [5] and [7] and references therein). According to this, the agonist-induced hydrolysis of PIP_2 may lead to the following events: (a) release of IP_3 , followed by Ca^{2+} mobilization

from the sarcoplasmic reticulum, an increase in cytosolic free Ca^{2+} , activation of myosin light-chain kinase to phosphorylate the myosin light-chain protein (MLC) and consequently lead to phasic contraction; (b) formation of DG, followed by activation of protein kinase C (PKC), phosphorylation of MLC, and consequently lead to tonic contraction. In support of these concepts, in vascular smooth muscle IP_3 (0.5–30 μM) caused Ca^{2+} release and tension development in rabbit main-pulmonary-artery muscle permeabilized with saponin [9], which has also been shown in other types of smooth muscle (for review see [5]). In addition, synergism between Ca^{2+} and activators of PKC, such as phorbol esters, has been shown to mediate contraction in various types of smooth muscle [10–14], and furthermore phosphorylation of MLC by PKC has been demonstrated [15–17].

Although there has been a considerable amount of effort directed towards understanding the role of the DG arm of the polyphosphoinositide response in smooth-muscle contraction, there has been very little work on the purification and characterization of PKC from this tissue. In the past 2 years we have reported that in the iris smooth muscle contractions by carbamoylcholine (CCh) indicated close correlations, using different concentrations of CCh, atropine and pirenzepine antagonism, time course, temperature and Ca^{2+} , between muscarinic

Abbreviations used: PIP_2 , phosphatidylinositol 4,5-bisphosphate; IP_3 , inositol 1,4,5-trisphosphate; DG, 1,2-diacylglycerol; PS, phosphatidylserine; MLC, myosin light chain; PDBu, phorbol 12,13-dibutyrate; CCh, carbamoylcholine; PKC, protein kinase C.

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acetylcholine-receptor occupancy, PIP_2 hydrolysis, MLC phosphorylation and contraction [18,19]. In addition, we have shown that in this muscle the phorbol ester phorbol 12,13-dibutyrate (PDBu), which mimics the action of DG, induced MLC phosphorylation and contraction in a dose- and time-dependent manner [14]. In the present study we report on the purification and characterization of PKC from the iris smooth muscle, with as substrate MLC prepared from the same tissue, and furthermore we show that both CCh and PDBu specifically increase MLC phosphorylation in the intact muscle.

MATERIALS AND METHODS

Animals

Albino rabbit eyes and bovine eyes were obtained from local slaughterhouses. Eyes were enucleated immediately after the animals were killed, and were transported to the laboratory packed in ice.

Purification of protein kinase C and MLC from iris muscle

PKC was purified from rabbit iris by the sequential use of three chromatographic steps: anion-exchange (DEAE-cellulose), gel filtration (Sephadex G-150) and substrate affinity (protamine-agarose). DEAE-cellulose and Sephadex G-150 chromatographies were based on the methods of Kikkawa *et al.* [20] and protamine-agarose chromatography was based on the procedures of Walton *et al.* [21] and Wooten *et al.* [22].

The phosphorylatable MLC from bovine iris was prepared by the procedure used for purification of turkey gizzard MLC by Perrie & Perry [23] and Adelstein & Klee [24].

Protein kinase C assay

PKC was assayed by a minor modification of the method of Takai *et al.* [25]. The enzyme activity was measured by incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into iris MLC or lysine-rich calf thymus histone (H-1). Unless otherwise stated, the standard reaction mixture (total vol. 100 μl) contained 25 mM-Tris/HCl (pH 7.4), 1 mM-EGTA, 10 mM- MgCl_2 , 400 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ $[(4\text{--}6) \times 10^5$ c.p.m./nmol], 60 μg of MLC or 50 μg of histone H-1, 10 μg of phosphatidylserine (PS), 1 μg of diolein, 100 μM free CaCl_2 , and 0.5–2.0 μg of enzyme protein. The reaction was carried out for 3 min at 37 °C, and stopped by the addition of 1 ml of 25% (w/v) trichloroacetic acid. The acid-precipitable protein was collected on a Gelman GA6-S filter, and the radioactivity of the filter was determined by counting in a liquid-scintillation counter. Free Ca^{2+} concentration in the reaction mixture was maintained with Ca^{2+} /EGTA (1 mM) buffers as described by Godt & Lindley [26]. Incorporation of ^{32}P was linear during the time of incubation, and the enzyme activity was proportional to the protein concentration. PKC activity was expressed as the activity (pmol of ^{32}P transferred/min per mg of protein) in the presence of Ca^{2+} , PS and diolein minus that of the basal activity (1 mM-EGTA).

Incubation and measurement of the effect of CCh and PDBu on ^{32}P incorporation into MLC of intact iris muscle

Rabbit iris sphincter was isolated and incubated in a modified Krebs–Ringer bicarbonate buffer (pH 7.4)

containing 50 μCi of ^{32}P P_i /ml at 37 °C for 90 min as described previously [18]. The pH of the buffer was maintained at 7.4 with O_2/CO_2 (97:3). After the 90 min preincubation, a period sufficient for equilibration of ^{32}P with intracellular ATP, CCh or phorbol esters were added and incubation was continued for various time intervals. In experiments where atropine was used, the tissue was incubated with the drug for 5 min before addition of the agonist. The reaction was stopped by immersion of the tissue in a methanol/solid- CO_2 slurry at -80 °C. The tissue was then homogenized in ice-cold 5% trichloroacetic acid. The acid-insoluble material was solubilized in 200 μl of SDS sample buffer by boiling for 30 min in sealed tubes. Samples containing 125 μg of protein were separated by SDS/polyacrylamide-gel electrophoresis [27]. Gels were stained with Coomassie Blue and dried on to filter paper. Results were analysed by autoradiography on Kodak XAR-5 film. For quantification, the band corresponding to MLC was excised from the dried gel and counted for radioactivity in a Beckman liquid-scintillation counter.

Other methods

Protein was measured by the method of Bradford [28], with bovine serum albumin as standard. SDS/polyacrylamide-gel electrophoresis was performed on 10% or 12%-acrylamide as described by Laemmli [27].

Chemicals

Phorbol esters were purchased from LC Services Corp. (Woburn, MA, U.S.A.). Phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.). ^{32}P P_i (carrier free) was purchased from ICN (Irvine, CA, U.S.A.) and the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ preparation kit was from Promega Biotec (Madison, WI, U.S.A.). H-7 was obtained from Seikagaku America (St. Petersburg, FL, U.S.A.). DEAE-cellulose (DE-52) was purchased from Whatman (Clifton, NJ, U.S.A.). Sephadex G-150, protamine-agarose, lysine-rich histone H-1 (calf thymus type III-S), skeletal-muscle MLC, skeletal-muscle myosin and the diacylglycerols were obtained from Sigma (St. Louis, MO, U.S.A.).

RESULTS

Purification of protein kinase C from the iris muscle

Sequential chromatography by anion-exchange (DEAE), gel filtration (Sephadex G-150) and substrate affinity (protamine-agarose) resulted in an approx. 250–300-fold purification of PKC from rabbit iris muscle. Table 1 summarizes the results obtained in a typical run using this purification procedure. The specific activity of PKC in the crude extract was 1.23 nmol/min per mg, and after the three chromatographic steps it was 322, or a 262-fold purification. The yield from 715 mg of protein in the crude extract, corresponding to about 45 g of irises, was approx. 400 μg , representing an activity yield of 15%, which is comparable with yields described for the purification of the enzyme from brain by using protamine-agarose columns [21,22].

On SDS/polyacrylamide-gel electrophoresis the purified enzyme exhibited a single, broad and diffuse Coomassie Blue band of approx. 80 kDa (Fig. 1a). No other major contaminating proteins were detected by Coomassie Blue staining. Further autoradiography of a sample of purified PKC autophosphorylated in the

Table 1. Purification of protein kinase C from rabbit iris smooth muscle

The chromatographic purification steps and the method of assay for PKC, with histone H-1 (type III-S) as substrate, were as described in the Materials and methods section. Enzyme activity was calculated as the difference between activities obtained in the presence of PS/DG/Ca²⁺ and basal activity (with 1 mM-EGTA present). In this experiment the wet weight of irises was 45 g.

Purification step	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg of protein)	Yield (%)	Purification (fold)
Crude extract (100 000 g supernatant)	715	880	1.23	100	1
DEAE-cellulose	46	681	14.8	77	12
Sephadex G-150	9.5	508	53.5	58	43
Protamine-agarose	0.4	129	322	15	262

Fig. 1. SDS/polyacrylamide-gel electrophoresis patterns at different stages of purification of iris muscle PKC and (b) representative autoradiogram of autophosphorylated PKC

(a) Coomassie Blue stain of proteins from chromatographies of: 100 000 g (crude extract), 70 μ g (lane 1); DEAE-cellulose, 52 μ g (lane 2); Sephadex G-150, 25 μ g (lane 3); protamine-agarose, 5 μ g (lane 4). Molecular masses (kDa) are shown of standard proteins: myosin, phosphorylase b, bovine serum albumin, ovalbumin, α -chymotrypsinogen, β -lactoglobulin. (b) PKC was incubated with [γ -³²P]ATP in the absence (lane 1) or the presence (lane 2) of 100 μ M-Ca²⁺, 100 μ g of PS/ml and 10 μ g of DG/ml for 3 min at 37 °C, and then run on gel electrophoresis as described in the Materials and methods section. The gel chromatogram was exposed for autoradiography for 3 days.

presence of [γ -³²P]ATP revealed the presence of a doublet protein in the 80 kDa region. It is likely that the broad and diffuse Coomassie Blue band present in Fig. 1(a), lane 4, contains some minor proteins which are poorly

resolved from each other when analysed by Coomassie Blue staining; however, because of possible differences in their specific radioactivities these PKC species are resolved with autoradiography (Fig. 1b, lane 1). Coomassie Blue staining of certain proteins is not a reliable marker of their relative abundance, since different proteins incorporate the dye to different extents. Therefore, although the band in Fig. 1(a), lane 4, appears minor by Coomassie Blue staining, it is in fact highly phosphorylatable, indicating that it is not a minor species of PKC. Furthermore, phosphorylation of proteins is known to retard their mobility on SDS/polyacrylamide-gel electrophoresis.

Characterization of protein kinase C

In the present study MLC was prepared from the iris muscle as described by Perrie & Perry [23] and Adelstein & Klee [24] and, as shown in Fig. 2(a), the protein migrated in SDS/polyacrylamide-gel electrophoresis as two bands, of 20 kDa and 18.5 kDa. Studies on the effects of the PKC cofactors PS, DG and Ca²⁺ on iris MLC phosphorylation indicate that all three are required for maximal activation of the enzyme (Fig. 2b). The increases in PKC activity by Ca²⁺, PS, Ca²⁺ + PS and Ca²⁺ + PS + DG were 27, 153, 868 and 1110 % of the control (lane 1) respectively. Thus iris muscle PKC is dependent on Ca²⁺, PS and DG to activate the transfer of phosphate from ATP to MLC.

In preliminary studies we investigated the effect of enzyme concentration and time course of MLC phosphorylation. Under the standard assay conditions, MLC phosphorylation by PKC was a linear function of the amount of enzyme added up to 0.5 μ g. Further, at optimum concentrations of MLC (30 μ M) and enzyme (0.5 μ g), the rate of substrate phosphorylation was linear up to 10 min and then levelled off with time of incubation. The time course of phosphorylation of MLC revealed that at 90 min (37 °C) PKC can incorporate about 1.9 mol of phosphate/mol of 20 kDa light chain.

The rates of phosphorylation of MLC and histone H-1 by PKC were studied over a wide range of substrate concentrations. The activity of the enzyme increased as a function of substrate concentration and reached a maximum at about 30 μ M-MLC and 5 μ M-histone. Lineweaver-Burk analysis of the data gave an apparent K_m for PKC of 16.6 μ M and a V_{max} of 313 nmol/min per

Fig. 2. Coomassie-Blue-stained gel (a) and autoradiogram (b) showing Ca²⁺- and phospholipid-stimulated phosphorylation of iris muscle MLC by PKC

The reaction mixture (final volume 100 μ l) contained 25 mM-Tris/HCl buffer (pH 7.4), 1 mM-EGTA, 10 mM-MgCl₂, 400 μ M-[γ -³²P]ATP [(4–6) $\times$ 10⁵ c.p.m./nmol], 30 μ M iris MLC, 2.0 μ g of enzyme protein, and PKC activators (PS, 10 μ g; diolein, 1 μ g; Ca²⁺, 100 μ M) as indicated. Incubation was carried out at 37 °C for 3 min and terminated by the addition of 2 $\times$ concentrated Laemmli SDS-sample buffer (v/v), and boiled for 3 min. Samples were subjected to SDS/polyacrylamide (12 %)-gel electrophoresis. The gel chromatogram was exposed for autoradiography for 8 h. ³²P radioactivity (c.p.m.) in MLC bands was: lane 1, 17265; lane 2, 21990; lane 3, 43706; lane 4, 167174; lane 5, 208309.

mg of protein with MLC as substrate and a K_m of 3 μ M and a V_{max} of 385 nmol/min per mg of protein with histone H-1 as substrate. The K_m of PKC for ATP as a phosphate donor was 9.4 μ M. These data indicate that histone H-1 is a better substrate for iris muscle PKC than is isolated iris MLC (3.0 versus 16.6 μ M).

Substrate specificity of protein kinase C

In addition to iris muscle MLC and histone H-1, MLC from turkey gizzard smooth muscle and MLC and myosin from rabbit skeletal muscle were also investigated as substrates (Table 2). With equal amounts (30 μ g) of each protein substrate, PKC activity was 100 % for histone H-1, 50 % for gizzard MLC, 41 % for iris muscle MLC, 2 % for skeletal-muscle MLC and 1.5 % for rabbit skeletal myosin. These findings indicate that histone H-1, as was reported in other tissues [25,29], is by far the most effective substrate for iris muscle PKC; however, more significantly, these studies demonstrate that only smooth-muscle MLC serves as a substrate for the enzyme.

Activation of protein kinase C by PS, DG and PDBu as a function of free Ca²⁺ concentration

These studies on the effects of activators and inhibitors

Table 2. Activity of iris muscle protein kinase C with smooth- and skeletal-muscle MLC proteins and histone H-1

The enzyme was assayed as described in the Materials and methods section, and the substrate concentration was 30 μ g/assay. The values are means $\pm$ S.E.M. for three separate experiments, each run in duplicate.

Protein substrate	PKC activity (nmol/min per mg of protein)	Relative activity
Histone H-1	266 $\pm$ 9.0	1.00
MLC (turkey gizzard smooth muscle)*	133 $\pm$ 2.0	0.50
MLC (bovine iris smooth muscle)	110 $\pm$ 2.2	0.41
MLC (rabbit skeletal muscle)	5.6 $\pm$ 0.5	0.02
Myosin (rabbit skeletal muscle)	3.5 $\pm$ 0.3	0.015

* A gift from Dr. James R. Sellers (NIH, Bethesda, MD).

Fig. 3. Protein kinase C activation by PS, DG and PDBu as a function of free Ca²⁺ concentration

Phosphorylation of iris muscle MLC (30 μ M), in the absence or presence of PS (50 μ g/ml), DG (10 μ g/ml) and 100 nM-PDBu, was conducted as described in the Materials and methods section. The free Ca²⁺ concentrations were calculated as described by Godt & Lindley [26], by using 1 mM-EGTA buffer. Data are means $\pm$ S.E.M. for three separate experiments.

were designed to characterize the regulation of the iris muscle PKC with the MLC purified from the same tissue as substrate. As with PKC from other tissues [29–31], PS alone, Ca²⁺ alone, or DG alone did not significantly activate the enzyme to phosphorylate MLC (results not shown). Only high concentrations of PS (> 20 μ g/ml) caused minimal activation of the enzyme (results not shown). As shown in Fig. 3, in the absence of the phospholipid Ca²⁺ had no effect on the enzyme activity. However, in the presence of 50 μ g of PS/ml, Ca²⁺ increased PKC activity by 3-fold, with half-maximal activation occurring at approx. 10 μ M free Ca²⁺ and maximal activation occurring at approx. 50 μ M-Ca²⁺ (Fig.

Table 3. Effects of major phospholipids, diacylglycerols and phorbol esters on protein kinase C activity

The enzyme was assayed as described in the Materials and methods section with 30 μM iris muscle MLC as substrate. Specificity of phospholipids was determined by adding 100 μg of each phospholipid to a 0.1 ml incubation mixture containing 10 μg of DG/ml and 100 μM - Ca^{2+} (control). Specificity of diacylglycerols and phorbol esters was determined by adding 10 μg of each diacylglycerol/ml or 100 nM of each phorbol ester to a 0.1 ml incubation mixture containing 50 μg of PS/ml and 100 μM - Ca^{2+} (control). Data are means $\pm$ S.E.M. for three separate experiments performed in duplicate.

Addition	MLC phosphorylation (nmol of P incorporated/min per mg of protein)	Stimulation (fold)
Control (DG + Ca^{2+})	26.3 $\pm$ 3.0	—
Phosphatidylserine (PS)	262.7 $\pm$ 10.6	10
Phosphatidic acid	217 $\pm$ 6.0	8
Phosphatidylinositol	198 $\pm$ 12.0	7.6
4,5-bisphosphate (PIP_2)		
Phosphatidylinositol	191 $\pm$ 18.0	7.3
Phosphatidylglycerol	97 $\pm$ 4.5	3.7
Phosphatidylethanolamine	71.4 $\pm$ 1.3	2.7
Phosphatidylcholine	41.3 $\pm$ 6.3	1.6
Control (PS, Ca^{2+})	105 $\pm$ 8.0	—
1-Stearoyl-2-arachidonyl-glycerol	268 $\pm$ 25.0	2.6
1,2-Dioleoylglycerol	229 $\pm$ 23.0	2.2
1,2-Dioctanoylglycerol	201 $\pm$ 24.0	1.9
1-Oleoyl-2-acetyl-glycerol	177 $\pm$ 20.0	1.7
Phorbol 12-myristate 13-acetate	252 $\pm$ 18.5	2.4
Phorbol 12,13-dibutyrate (PDBu)	216 $\pm$ 19.0	2.1
Phorbol 12,13-diacetate	167 $\pm$ 16.0	1.6
4 α -Phorbol	116 $\pm$ 7.0	1.1

3). At 10 μM free Ca^{2+} and in the presence of PS the rate of ^{32}P incorporation from [γ - ^{32}P]ATP into iris muscle MLC was about 126 nmol/min per mg of protein, and this increased by 61 and 65% on addition of DG and PDBu respectively. Moreover, addition of DG or PDBu to the reaction mixture decreased the Ca^{2+} requirement for the enzyme, causing a leftward shift of the Ca^{2+} dose-response curve (Fig. 3). Thus, in the presence of DG, half-maximal enzyme activation occurred at approx. 3 μM free Ca^{2+} , or more than a 50% decrease in K_a for the cation (10 μM versus 3 μM). Similarly, in the presence of PDBu half-maximal enzyme activation occurred at about 2 μM free Ca^{2+} .

Phospholipid specificity of protein kinase C

To determine the specificity of the phospholipid requirement for PKC activation and iris MLC phosphorylation, the effects of other phospholipids were investigated (Table 3). PS was the most effective phospholipid, when assayed under standard assay conditions, increasing PKC activity by 10-fold. This was followed by phosphatidic acid (8-fold), PIP_2 (7.6-fold), phosphatidylinositol (7.3-fold) and phosphatidylglycerol (3.7-fold). Phosphatidylethanolamine and phosphatidyl-

choline were least effective in stimulating the enzyme activity. These data demonstrate a requirement for a negatively charged phospholipid for optimal phosphorylation of MLC by PKC.

Effects of diacylglycerols and phorbol esters on protein kinase C activity

Iris muscle MLC (30 μM) phosphorylation by PKC was stimulated by the addition of diolein and PDBu under standard assay conditions, in a concentration-dependent manner, with half-maximally effective concentrations of 5 $\mu\text{g}/\text{ml}$ and 10 nM respectively (results not shown). When diolein and PDBu were replaced by other diacylglycerols and phorbol esters, the results obtained are shown in Table 3. 1-Stearoyl-2-arachidonyl-glycerol was the most active diacylglycerol, stimulating PKC activity by 2.6-fold. This was followed by 1,2-dioleoylglycerol (2.2-fold), 1,2-dioctanoylglycerol (1.9-fold) and 1-oleoyl-2-acetyl-glycerol (1.7-fold). Of the phorbol esters investigated, phorbol myristate acetate was the most potent activator, with a stimulatory effect of 2.4-fold (Table 3). This was followed by PDBu (2.1-fold) and phorbol 12,13-diacetate (1.6-fold). The inactive stereoisomer 4 α -phorbol was ineffective in activating PKC.

Effect of protein kinase C inhibitor H-7 on MLC phosphorylation

H-7 has previously been described as a potent and specific inhibitor of PKC [32]. H-7 inhibited iris muscle MLC phosphorylation by PKC in a dose-dependent manner, with 50% inhibition at 10 μM .

Effects of CCh, atropine and PDBu on MLC phosphorylation in the intact iris sphincter muscle

Addition of the muscarinic agonist CCh to iris sphincter muscle pre-labelled with ^{32}P consistently increased phosphorylation of the 20 kDa light chain of myosin by about 35–40% (average of 11 experiments) of that of the control within 1 min (Fig. 4). The radioactivity of other phosphoproteins remained relatively unaffected. Similarly, addition of PDBu also increased phosphorylation of the 20 kDa MLC by about 25–30% (average of 11 experiments), but, in contrast with CCh, the muscle was exposed to the phorbol ester for 25 min. Atropine alone had no effect on MLC phosphorylation. However, the CCh-stimulated phosphorylation of MLC was blocked by the muscarinic antagonist, demonstrating that the CCh action on MLC phosphorylation is mediated through muscarinic cholinergic receptors. In contrast, the PDBu-stimulated phosphorylation of MLC was insensitive to atropine, supporting the view that these DG analogues do by-pass the receptor-mediated events and directly activate PKC.

DISCUSSION

In the present work, PKC was purified from rabbit iris smooth muscle by a three-step chromatographic procedure, namely anion-exchange (DEAE-cellulose), gel filtration (Sephadex G-150) and substrate affinity (protamine-agarose), resulting in a 262-fold purification and 15% yield of the enzyme (Table 1). SDS/polyacrylamide-gel electrophoresis of the enzyme revealed a broad band

Fig. 4. Typical autoradiogram, after protein separation by SDS/polyacrylamide-gel electrophoresis, showing the effects of CCh, atropine and PDBu on protein phosphorylation in the iris intact sphincter muscle

The tissues were first incubated for 90 min in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 50 μ Ci of [32 P] P_i . Atropine (0.1 μ M) was added 5 min before addition of CCh (50 μ M) or PDBu (200 nM), and the incubation continued for 1 min with CCh and for 25 min with PDBu. Incubations were terminated and subjected to SDS/polyacrylamide (10%) gel electrophoresis as described in the Materials and methods section. 32 P radioactivity in MLC bands was (c.p.m./mg of total tissue protein): lane 1, 7448; lane 2, 10256; lane 3, 7336; lane 4, 7624; lane 5, 9536; lane 6, 9480.

with a molecular mass of 80 kDa (Fig. 1a). When the enzyme was autophosphorylated in the presence of [γ - 32 P]ATP and the PKC activators, a doublet protein was observed in the 80 kDa region (Fig. 1b). A similar doublet protein was reported for rat brain PKC, and this was attributed to the presence of two distinct isoforms of the enzyme [33]. Recent molecular studies on PKC in rat and bovine brain indicate the presence of at least three isoforms of the enzyme [34,35]. It is possible that the doublet protein observed here represents different isoforms of the iris muscle PKC.

The findings presented here on characterization of iris muscle PKC show that the enzyme is activated by Ca^{2+} , DG and PS. When added individually these agents have little effect on MLC phosphorylation (Fig. 2b). The Ca^{2+} concentrations required for maximal activation of the enzyme are dependent on the presence of PS, and can be decreased by either diolein or PDBu (Fig. 3). Half-maximal activation of the enzyme (K_a) occurred at 10 μ M free Ca^{2+} in the presence of PS. However, in the presence of DG and PDBu the apparent K_a for Ca^{2+} was decreased to 3 μ M and 2 μ M respectively.

The findings on the relative potencies of various phospholipids, diacylglycerols and phorbol esters (Table 3) in activating iris muscle PKC are in general agreement with those reported in other tissues [36]. PS was the most active of the phospholipids tested, although other acidic phospholipids were also potent activators of iris muscle PKC. It is not clear yet which of these phospholipids are

essential in the activation of PKC *in vivo*. Our data indicate a requirement for a negatively charged head group, and this is in accord with the enzyme-activation model of Bell [37]. According to this, four negatively charged PS molecules bind a single Ca^{2+} ion to form a Ca^{2+} -PS complex on the cytoplasmic leaflet of the membrane bilayer. PKC binds to this complex and is activated only after the binding of DG. If we assume that the function of the phospholipid is to bind Ca^{2+} and to anchor this Ca^{2+} -phospholipid complex in the membrane, then other anionic phospholipids, such as phosphatidic acid and the phosphoinositides, could function as potential regulators of PKC activity. The ability of DG to activate PKC by increasing its affinity for Ca^{2+} has been shown to be dependent on the structural specificity of the fatty acyl moieties esterified to the diacylglycerol [38,39]. To determine the specificity of the fatty acyl moieties, the effects of various diacylglycerols and phorbol esters on MLC phosphorylation by PKC were investigated. The results show that under our standard assay conditions 1-stearoyl-2-arachidonoylglycerol was the most potent activator. This is the major DG formed from receptor-stimulated PIP_2 hydrolysis (for review see [5]), and the finding that this species of DG is the most potent activator of PKC is in accord with the hypothesis that PIP_2 and its derived second messengers are involved in smooth-muscle contraction. Of the phorbol esters tested, phorbol myristate acetate was the most active, followed by PDBu and phorbol diacetate. As expected, the inactive stereoisomer 4 α -phorbol was ineffective in activating the enzyme. Most studies on MLC phosphorylation in smooth muscle have emphasized the role of MLC kinase, and therefore little is known about the physiological occurrence of PKC phosphorylation of MLC. Recently, it has been reported that phorbol esters induce a slowly developing, yet sustained, contractile response, but the precise extent of MLC phosphorylation during these responses remains controversial [5,7]. The present findings of specific increases in MLC phosphorylation in the iris sphincter by CCh and PDBu, coupled with our previous observations on the close relationships between muscarinic-receptor occupancy, IP_3 and DG formation, MLC phosphorylation and contraction in this tissue [19,40], lend further support to the hypothesis that agonist-stimulated PIP_2 hydrolysis couples activated Ca^{2+} -mobilizing receptors to smooth-muscle contraction. IP_3 , through intracellular Ca^{2+} mobilization, and MLC-kinase-dependent site-specific phosphorylation of MLC may serve to initiate contraction, whereas DG, acting through PKC and its site-specific phosphorylation of MLC, may serve to sustain the tonic phase of the contractile response. The data in the present paper on the purification and characterization of PKC enzyme from the iris muscle, with iris muscle MLC as substrate, together with the findings on the effects of various activators and inhibitors on MLC phosphorylation *in vitro* and in the intact muscle provide additional support for a role for PKC in the iris muscle contractile response.

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