Characterization of two forms of protein kinase C α, with different substrate specificities, from skeletal muscle

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We have investigated protein kinase C (PKC) in skeletal muscle cytosol and demonstrated the presence of two major activities. These did not correspond to different PKC isoenzymes but seemed to represent two species of PKC α as deduced by: elution during hydroxyapatite chromatography at KH_2PO_4 concentrations expected of PKC α; detection of the two species by three specific but unrelated anti-(PKC α) antibodies; immunodepletion of both activities with anti-(PKC α) antibody; and demonstration of identical requirements of both Ca^{2+} ions and lipid for activation. These species, termed PKC α1 and PKC α2, phosphorylated the modified conventional PKC pseudosubstrate peptide (19–31, Ser-25) equally well. Importantly, however, the activities differed in that PKC α1 phosphorylated histone IIIS, and also peptides derived from the EGF receptor and glycogen synthase, to a much greater extent than did PKC α2. Similarly, incubation of crude muscle extracts with either PKC α1 or α2 gave rise to different protein phosphorylation patterns. The involvement of proteolysis, dephosphorylation or oxidative modification in the interconversion of PKC α1 and PKC α2 during preparation was ruled out. Although some PKC-binding proteins were detected in overlay assays, their presence did not explain the anomalous PKC α2 activity. The results suggest that a modification of PKC α *in situ* limits its substrate specificity, and indicate an additional level of control of the kinase that may be a site for modulation of PKC-mediated signal transduction.

INTRODUCTION

The protein kinase C (PKC) family consists of at least nine isoenzymes, which are dependent on certain activators: the conventional PKC isoenzymes α, β and γ, requiring Ca^{2+} ions, diacylglycerol and phospholipid [1]; the novel PKC forms (δ, ϵ, η and θ), which are independent of Ca^{2+} but still require lipid activators; and the atypical PKCs (ζ and ι), which are also insensitive to diacylglycerol [1,2]. The PKC family is involved in many signal transduction pathways, and because the isoenzymes show different tissue distributions and substrate specificities as well as activator requirements, it is likely that they perform different functions. PKC-mediated events include cell proliferation and differentiation, hormone secretion and gene expression [3].

Whereas activation of the conventional PKCs requires elevation of intracellular Ca^{2+} and diacylglycerol, attenuation of kinase activity can involve processes other than the removal of these activators. A number of such processes have been described. First, chronic activation of PKC can lead to increased proteolysis and hence down-regulation of the kinase, in some cases involving the temporary generation of M-kinase, the activator-independent catalytic domain [4–6]. A second form of regulation involves the phosphorylation state of PKC. Studies have indicated that PKC dephosphorylated at certain sites is inactive, as is nascent, unphosphorylated PKC [7–11]. A further process is the modification of the thiol/disulphide status of PKC, which has been studied after treatment of intact cells [12,13], tissue homogenates [14] or purified PKC [15] with various oxidizing agents such as quinones, periodate or H_2O_2. Depending on the conditions employed and hence the region of the PKC molecule affected, oxidative modification of PKC can have several effects, including alteration of its chromatographic properties, generation of an activator-independent form or even complete inactivation [15]. Lastly, other proteins that might be involved in the regulation of PKC activity have been described, including inhibitors [16–18] and the receptors for activated C-kinase, cytoskeletal proteins that are thought to be involved in the subcellular localization of kinase activity [19]. Although the physiological effects of these proteins are unclear, it is likely that their functions are also regulated in response to specific signals, giving rise to another level of control of the PKC response.

PKC activity might therefore be affected not only after direct activation of the kinase, as with proteolysis, but also by signalling pathways that are PKC-independent. During characterization of the soluble PKC isoenzymes present in rat skeletal muscle, we have detected two forms of PKC α with different chromatographic properties as well as distinct substrate specificities. Our findings are best explained by the modification in intact muscle of a proportion of the total cytosolic PKC α, resulting in decreased activity towards specific substrates, whereas phosphorylation of others is unaffected.

EXPERIMENTAL

Materials

Hydroxyapatite (DNA-grade Biogel HTP) was from Bio-Rad Laboratories (Sydney, N.S.W., Australia). Peptides derived from the EGF receptor (650–657), glycogen synthase (1–12) and the PKC β pseudosubstrate site (19–31, Ser-25) (mPSP) were from Auspep (Parkville, Vic., Australia). Rabbit anti-peptide antibodies against PKCs α, δ, ϵ and ζ were from Gibco BRL, Life Technologies (Mulgrave, Vic., Australia). Rabbit anti-peptide antibody against PKC θ was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Mouse monoclonal antibodies MC-1a, -2a and -3a against rabbit brain PKC γ, β and α respectively were from Seikagaku Corporation (Tokyo, Japan). Mouse monoclonal antibody against rat brain PKC α was from Trans-

Abbreviations used: DOG, 1,2-dioctanoyl-*sn*-glycerol; DTT, dithiothreitol; mPSP, modified PKC β pseudosubstrate peptide (19–31, Ser-25); PKC, protein kinase C; PS, L-α-phosphatidyl-L-serine.

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duction Laboratories (Lexington, KY, U.S.A.). Other biochemicals were from Sigma Chemical Co., Boehringer Mannheim or BDH Laboratory Supplies.

Tissue extraction

Soleus, red gastrocnemius and red quadriceps muscles (1.5–2.0 g) were taken from male Wistar rats (300–350 g) immediately after being killed and extracted in 8 ml of ice-cold homogenizing buffer [20 mM Mops (pH 7.5)/250 mM mannitol/1.2 mM EGTA/1 mM dithiothreitol (DTT)/2 mM PMSF/200 μg/ml leupeptin/2 mM benzamidine] with a Janke and Kunkel Ultra-Turrax tissue homogenizer at setting 7 for two 15 s intervals. This and all subsequent procedures were performed at 4 °C. The extract was centrifuged at 100000 ***g*** for 45 min, and the supernatant was termed the cytosolic fraction. The 100000 ***g*** pellet was washed once by resuspension in homogenizing buffer and recentrifugation, and protein was solubilized by resuspension in 8 ml of 20 mM Mops (pH 7.5)/0.5% (w/v) decanoyl-*N*-methylglucamide/2 mM EDTA/5 mM EGTA/1 mM DTT/2 mM PMSF/200 μg/ml leupeptin/2 mM benzamidine. After being left for 1 h this extract was recentrifuged as above and the supernatant was retained as the solubilized particulate fraction.

Hydroxyapatite chromatography

A cytosolic fraction, equivalent to 0.6 g of wet tissue (i.e. 2–3 ml), was passed through a 0.45 μm filter and applied to a 7.5 ml hydroxyapatite column (0.8 cm × 15 cm) equilibrated with either 20 or 80 mM KH_2PO_4 as specified, pH 7.5, containing 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 20 μg/ml leupeptin and 2 mM benzamidine. The column was washed with 25 ml of the same buffer, and protein eluted in 60 fractions of 3.75 ml each, with a linear gradient (confirmed by conductivity measurements) from 20 to 180 mM or 80 to 140 mM KH_2PO_4, at 0.2 ml/min.

PKC assay

Column fraction samples (10 μl) were assayed as previously described [2], with either 5 μM mPSP/0.5 mg/ml histone IIIS/0.5 mg/ml myelin basic protein/30 μM glycogen synthase peptide (1–12) or 10 μM EGF receptor peptide (650–657) as substrate, with combinations of 1 mM $CaCl_2$, 125 μg/ml L-α-phosphatidyl-L-serine (PS) and 2.5 μg/ml 1,2-dioctanoyl-*sn*-glycerol (DOG) as stated. The ATP concentration was decreased to 10 μM to determine the sensitivity of PKC fractions to the specific inhibitor GF-109203X (bisindolylmaleimide I), which is competitive with ATP. In this study, PKC activity is defined as that stimulated in the presence of lipid activators, above that seen with Ca^{2+} alone.

Immunoblotting

Protein was precipitated by the addition of 1 ml of ice-cold acetone and 10 μl of 2.5 mg/ml BSA to 250 μl of each column fraction, and incubation at −70 °C for 1 h. The samples were centrifuged for 10 min at 20000 ***g*** at room temperature; the protein pellets were heated to 95 °C for 2 min after addition of 30 μl of water and 10 μl of Laemmli sample buffer, and subjected to SDS/PAGE and immunoblotting with a chemiluminescence detection system (NEN–DuPont). Primary antibodies used were rabbit anti-peptide antibodies against PKC isoenzymes α, δ, ϵ or ζ (5 μg/ml) or PKC isoenzyme θ (1 μg/ml), mouse monoclonal antibody raised towards an 18 kDa expressed fragment of PKC α (1 μg/ml), or mouse monoclonal antibodies directed against rabbit PKC α, β or γ (1, 1 or 2 μg/ml respectively). The specificities of the polyclonal antibodies were confirmed by including the peptides against which they were raised during the primary incubations to block specific binding (results not shown). As reported by others [20,21], the rabbit anti-peptide antibody raised against the C-terminus of PKC ζ gave two bands, of molecular masses 82 and 70 kDa, sensitive to competition with peptide. Only the lower band corresponds to PKC ζ.

Immunodepletion

Samples of peak PKC α fractions from hydroxyapatite chromatography (200 μl) were incubated on ice after the addition of 15 μl of a 0.25 mg/ml solution of Transduction Laboratories anti-(PKC α) mouse monoclonal antibody or vehicle [20 mM Na_2HPO_4 (pH 7.5)/50% (v/v) glycerol/150 mM NaCl/1.5 mM NaN_3/1 mg/ml BSA]. After 3 h (PKC α2) or 20 h (PKC α1), goat anti-(mouse IgG)–Sepharose conjugate was added to all incubations, followed by gentle rocking for 1 h at 4 °C. Incubations were then centrifuged for 30 s at 20000 ***g*** at room temperature and the resulting supernatants were assayed for PKC activity.

Phosphorylation of muscle proteins *in vitro*

Incubations were performed at 30 °C for between 1 and 20 min as indicated, in a volume of 90 μl, containing 10 μl of hydroxyapatite peak fraction, 10 μl of skeletal muscle solubilized particulate fraction, 20 mM Mops, pH 7.5, 0.04% (v/v) Triton X-100, 1 mM $CaCl_2$, 125 μg/ml PS, 2.5 μg/ml DOG, 120 nM cAMP-dependent protein kinase inhibitor peptide, 100 μM [γ-^{32}P]ATP (7.5–10 μCi per incubation; 1500–2000 c.p.m./pmol) and 5 mM $Mg(CH_2CO_2)_2$. The PKC pseudosubstrate inhibitor peptide (19–31) was present at 100 μM where specified. Reactions were terminated by the addition of Laemmli sample buffer and samples were subjected to SDS/PAGE and autoradiography.

Purification of PKC α

The partial purification of PKC α was adapted from the method of House et al. [22]. Briefly, 10 rat brains were extracted in the presence of 1 mM $CaCl_2$ and centrifuged at 40000 ***g***, and the pellet was washed twice by resuspension in buffer containing only 0.1 mM $CaCl_2$ and recentrifugation. Finally the pellet was resuspended in buffer containing 2 mM EDTA and 2 mM EGTA instead of $CaCl_2$ and again centrifuged. PKC α in the supernatant was further purified by DE52 and hydroxyapatite chromatography. Immunoblotting indicated that this preparation was highly enriched in PKC α.

Overlay assay

Cytosolic and hydroxyapatite fractions were assayed for PKC-binding proteins by using an overlay assay modified from Chapline et al. [23]. Samples were subjected to SDS/PAGE followed by electroblotting as described above, except that ovalbumin was used as carrier protein in acetone precipitates, and electroblotting was performed in the absence of methanol. The membranes were preincubated for 16 h at 4 °C in three changes of Tris-buffered saline containing 0.05% (v/v) Tween-20, 10 mg/ml BSA and 1 mM DTT and then incubated with partly purified PKC α for 2 h at room temperature in a total volume of 2 ml, containing 50 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 5 mM $Mg(CH_2CO_2)_2$, 0.25 mM EDTA, 1 mM $CaCl_2$, 125 μg/ml PS, 2.5 μg/ml DOG, 10 mg/ml BSA, 2 mM DTT, 20 μg/ml leupeptin and 1 mM PMSF. Membranes were then washed five times with PBS containing 1 mM $CaCl_2$, 20 μg/ml PS and 0.4 μg/ml DOG for 5 min, once with 2.5% (v/v)

formaldehyde in PBS for 40 min, once with 10 % (w/v) glycine for 20 min and twice in Tris-buffered saline containing 0.05 % (v/v) Tween-20. Bound PKC α was then detected as described for immunoblotting, with the use of the Transduction Laboratories monoclonal antibody.

General methods

SDS/PAGE was performed as described by Laemmli [24]. Protein in gels was electroblotted in 25 mM Tris (pH 8.3)/192 mM glycine/20 % (v/v) methanol unless specified. Densitometry was performed with a Medical Dynamics Personal Densitometer SI and results analysed with IP Lab Gel software (Signal Analytics, Vienna, VA, U.S.A.).

RESULTS

To characterize the complement of PKC isoenzymes in rat skeletal muscle, cytosolic fractions from muscle, and also brain for comparison, were applied directly to a hydroxyapatite column and eluted with a linear 20–180 mM KH_2PO_4 gradient. Fractions were assayed for PKC activity with mPSP and histone IIIS in the presence of Ca^{2+}, PS and DOG to derive the profiles shown in Figure 1. The brain profile exhibits major activity in the region of the gradient (fractions 24–34 in Figure 1a) previously shown to yield PKC β and γ [25], whereas a second peak of activity was observed to elute in the region of the gradient corresponding to PKC α (fractions 38–44 in Figure 1a). This contrasts markedly with the muscle profile, which displayed only a very minor but reproducible activity eluting in the PKC β/γ region (fractions 30–34 in Figure 1b). Most noticeably the two major peaks of PKC activity in muscle were observed to elute in the PKC α region (fractions 38–55 in Figure 1b). The second of these (fractions 43–55), which exhibited a trailing shoulder, was virtually absent from the brain profile. Activity in the general PKC α region, measured in the presence of Ca^{2+} but in the absence of PS and DOG, was very low for each substrate (see Table 2 for basal activities in peak fractions), indicating that any contribution from lipid-independent protein kinases was small. Importantly, although each peak in the brain profile was apparent with either mPSP or histone IIIS as substrate, the second major peak in the muscle profile was only poorly detected with histone IIIS.

To confirm the identity of the muscle PKC peaks shown in Figure 1b, protein in the column fractions from a similar separation was subjected to immunoblotting with isoenzyme-specific antibodies raised against a 14-residue peptide derived from the rat PKC α sequence (Gibco). PKC α was detected in the fractions of both major activities and seemed to be most concentrated in peak fractions (Figure 1c, top panel). Complementary results were obtained in further experiments, where activity profiles were again similar to those in Figure 1b, with the use of two alternative anti-(PKC α) antibodies generated towards purified rabbit brain PKC α (Seikagaku) or an 18 kDa fragment of PKC α (Transduction Laboratories). Although immunoblots with these antibodies suggested that hydroxyapatite fractions corresponding to the second major muscle PKC activity peak contained less PKC α protein than was indicated by the activity measurements (Figures 1b and 1c), this can be explained by the greater sensitivity of the second peak to proteolysis (demonstrated in Figure 3), together with the delay between PKC activity assay and immunodetection, during which active fractions were identified and concentrated.

The presence of other PKC isoenzymes was also examined by immunoblotting. PKC β was detected only in fractions corresponding to the early minor peak of activity (Figure 1c). PKC

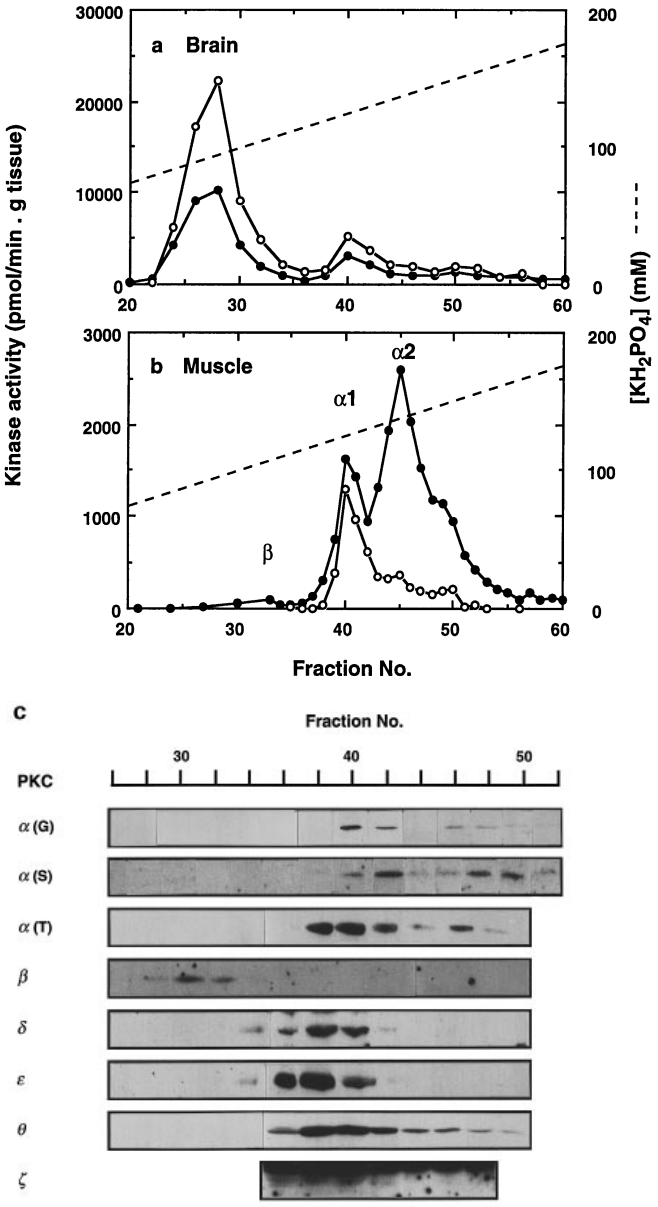

Figure 1 PKC profiles after hydroxyapatite chromatography of brain and skeletal muscle cytosolic fractions

Hydroxyapatite chromatography was performed with either brain (**a**) or skeletal muscle (**b**) cytosol as detailed in the Experimental section, and fractions were assayed for PKC activity in the presence of Ca^{2+}, PS and DOG, with mPSP (●) or histone IIIS (○) as substrate. Results shown in (**a**) and (**b**) are typical of 2 and 23 chromatographic separations respectively. (**c**) Protein in hydroxyapatite column fractions from three similar experiments to that shown in (**b**) was subjected to SDS/PAGE and immunoblotting as detailed in the Experimental section, with Transduction Laboratories monoclonal antibodies specific for PKC α (T), Seikagaku monoclonal antibodies specific for PKC α (S), and PKC β, Gibco polyclonal antibodies raised against PKC α (G), δ, ε, or ζ, or Santa Cruz polyclonal antibodies specific for PKC θ, as indicated. The three PKC α immunoblots were of separate chromatographic separations and all other immunoblots were of the same separation as the PKC α (T) immunoblot. Exposure times for chemiluminescent detection were between 1 and 10 min, except for the PKC ζ immunoblot, which required 1 h.

γ, which in brain extracts is eluted before PKC β [25], was not detected in the muscle profile. It therefore seems that PKC α is the major conventional PKC activity present in red skeletal muscle cytosol but exists in two discrete forms: the first, here termed PKC α1, exhibiting the expected substrate specificity;

Table 1 Immunodepletion of PKC α activity in peak fractions

Peak fractions of PKC α1 and α2 activity, prepared as in Figure 1b, were subjected to immunodepletion by using a monoclonal antibody against PKC α, then assayed with the substrates indicated as detailed in the Experimental section. Results are given as means ± S.E.M. for three independent experiments each assayed in triplicate, and are corrected for activity observed in the absence of PS and DOG.

		PKC activity (fmol/min)		
Peak	Substrate	Control	Immunodepleted	Immunodepletion (%)
α1	mPSP	215 ± 25	52 ± 22	78 ± 4
α1	Histone IIIS	231 ± 16	48 ± 10	82 ± 5
α2	mPSP	364 ± 25	91 ± 17	75 ± 3
α2	Histone IIIS	55 ± 14	9 ± 4	85 ± 3

Table 2 Activator dependence of PKC α1 and PKC α2

Peak hydroxyapatite chromatographic fractions containing either PKC α1 or PKC α2 activity were assayed with mPSP as substrate as detailed in the Experimental section, with the indicated combinations of EGTA, Ca^{2+}, PS and DOG. Results are shown as means ± S.E.M. for three independent experiments each performed in triplicate.

	PKC activity (pmol/min per g of tissue)	
Additions	PKC α1	PKC α2
EGTA	117 ± 27	195 ± 26
Ca^{2+}	49 ± 25	66 ± 23
EGTA + PS	110 ± 18	200 ± 28
EGTA + DOG + PS	165 ± 42	252 ± 45
Ca^{2+} + DOG + PS	788 ± 176	1069 ± 57

Table 3 Substrate specificities of PKC α1 and α2

Chromatography fractions, prepared as in Figure 1b, were assayed with the substrates indicated as detailed in the Experimental section. Results are means ± S.E.M. for the peak PKC α1 and PKC α2 fractions, from at least three observations made with different preparations.

	PKC activity (pmol/min per g of tissue)		
Substrate	α1	α2	α1/α2
Pseudosubstrate peptide (Ser-25)	1310 ± 111	1834 ± 92	0.7
Myelin basic protein	550 ± 118	393 ± 105	1.4
EGF receptor peptide	314 ± 52	157 ± 13	2.0
Histone IIIS	1126 ± 197	223 ± 26	5.1
Glycogen synthase peptide	1415 ± 144	249 ± 39	5.7

and the second, termed PKC α2, displaying novel characteristics. The presence of two peaks was highly reproducible, with PKC α1 averaging 36 ± 2 % (mean ± S.E.M.) of the activity of PKC α2 towards mPSP in six different muscle profiles obtained with this gradient. The novel PKCs δ, ε and θ, and the atypical PKC ζ, were also located by immunoblotting (Figure 1c). Although some of these partly co-eluted with PKC α1, the activity measured in these fractions is largely Ca^{2+}-dependent (see Table 2), making it unlikely that the Ca^{2+}-independent PKCs contributed significantly to total activity as measured under our assay conditions.

Further evidence that PKC α is responsible for both of the major muscle PKC activities was obtained by immunodepletion studies. Approx. 80 % of the PKC activity in the PKC α1 peak fraction could be abolished by incubation with an anti-(PKC α) antibody, as measured in activity assays with either mPSP or histone IIIS (Table 1). Immunodepletion of the PKC α2 peak fraction led to a similar loss of PKC activity, although activity measured with histone IIIS was extremely low even in control incubations when compared with that seen with mPSP (Table 1), as expected from the muscle activity profile in Figure 1b. These results demonstrate that at least a major proportion of the PKC activity in both PKC α1 and PKC α2 fractions is indeed due to PKC α. Importantly, the fact that the two activities were decreased to similar extents, towards both mPSP and histone IIIS, suggests that they are unlikely to have arisen from a combination of different PKC isoenzymes.

The activator requirements of the two major PKC activities were determined with mPSP as substrate. These experiments showed clearly that Ca^{2+}, in addition to PS and DOG, was necessary to achieve full activation of both peaks (Table 2). In fact the activator requirements seemed identical, supporting the hypothesis that the same PKC isoenzyme is responsible for both peaks. Because no increase in the background kinase activity was observed in the presence of lipid activators alone, it is unlikely that any Ca^{2+}-independent PKC isoenzymes were responsible for either of these PKC peaks, particularly as our assay conditions were not optimized for the detection of novel or atypical PKC isoenzymes. In addition, Ca^{2+} alone led to inhibition of the background activity seen with EGTA, which argues against the involvement of a Ca^{2+}-dependent, but lipid-independent, protein kinase.

Because the two PKC α peaks differed in their ability to phosphorylate histone IIIS (Figure 1b), further assays were undertaken to characterize the different substrate specificities of PKC α1 and α2 (Table 3). Substrates included myelin basic protein as well as the peptides derived from the PKC phosphorylation sites of the EGF receptor and glycogen synthase. When the ratios of phosphorylation rates by the two activities were compared, a range was observed from mPSP, which was a better substrate for PKC α2, to glycogen synthase peptide, which was a far better substrate for PKC α1 (Table 3). The preferences are not related to the size of the substrate, as protein and peptides are found throughout the range.

To determine whether PKC α1 and PKC α2 also phosphorylated endogenous skeletal muscle proteins to different extents, hydroxyapatite fractions were incubated for between 1 and 20 min with a solubilized particulate fraction from muscle. After SDS/PAGE and autoradiography of the incubations, a number of phosphoproteins were detected (Figure 2a). Some of these have previously been observed when endogenous PKC was activated in isolated triad preparations from rabbit skeletal muscle [26]. Whereas a 43 kDa protein was more highly phosphorylated in incubations with PKC α1 than with PKC α2, a 20 kDa protein exhibited greater selectivity for PKC α2 (Figure 2a). These differences are most clearly seen in incubations performed for 10 min, beyond which the effects of phosphatases and substrate depletion presumably play a greater role. In similar experiments, incubations were performed for 10 min in the absence or presence of the PKC pseudosubstrate inhibitor peptide, which demonstrated that these two proteins were specific PKC substrates in that their phosphorylation was abolished by the inhibitor. Each lane was analysed by densitometry and the profiles from incubations performed in the presence of the PKC inhibitor were subtracted from those performed in its absence, to determine the specific contribution of PKC activity to the phosphorylation of these proteins (Figure 2b). This showed that when PKC α1 and PKC α2 activity to the 20 kDa protein was normalized, the 43 kDa protein was phosphorylated 2.5-fold

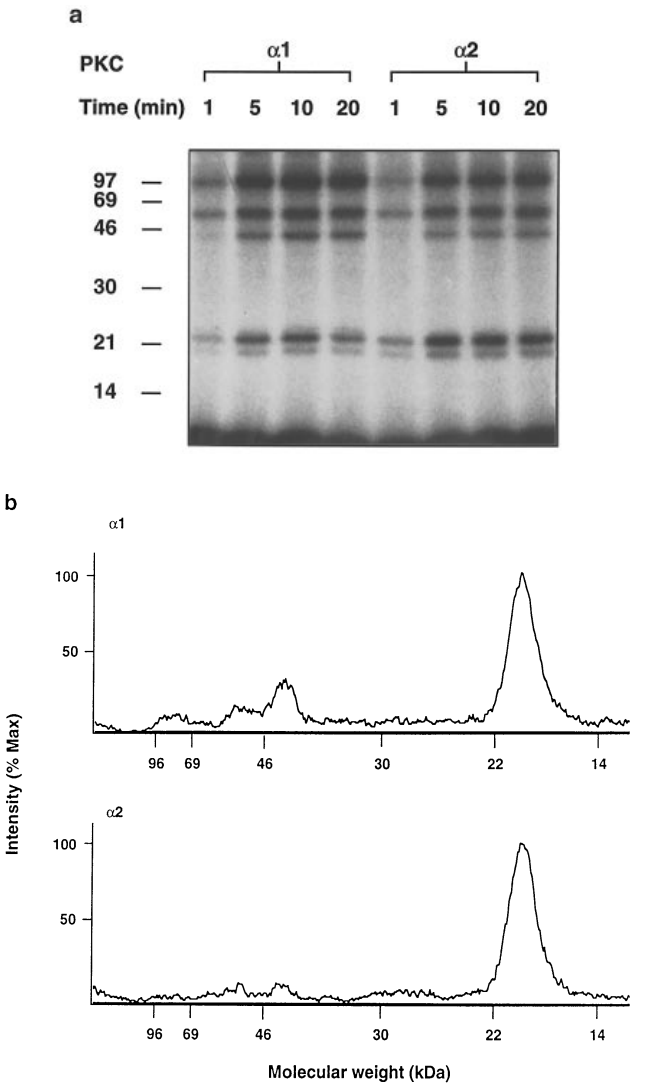

Figure 2 Phosphorylation of skeletal muscle particulate proteins by PKC α1 and PKC α2

(**a**) Peak hydroxyapatite chromatography fractions containing PKC α1 or PKC α2 activity were incubated with a solubilized particulate fraction from skeletal muscle together with PKC assay reagents, including Ca^{2+} and lipid activators, for the times indicated. For further details see the Experimental section. Incubations were subjected to SDS/PAGE and autoradiography. The positions of protein markers, with molecular masses given in kDa, are indicated at the left. Results are from a single experiment representative of three. (**b**) An autoradiograph from a similar experiment, in which incubations were performed for 10 min in the absence and presence of 100 μM pseudosubstrate inhibitor peptide, was analysed by densitometry, as described in the text, to give the profiles of PKC-dependent phosphorylation shown.

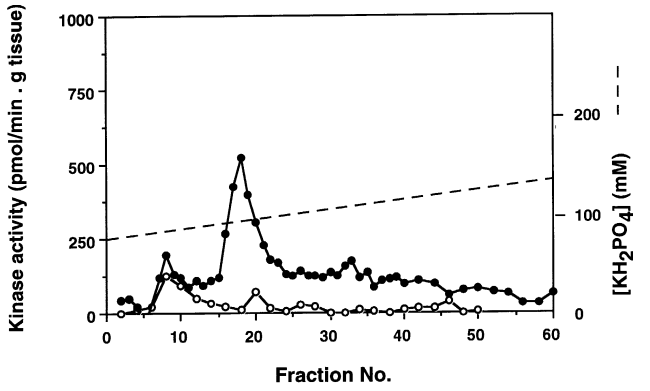

Figure 3 Elution of PKC from hydroxyapatite after preparation under conditions leading to increased protease activity

Muscle cytosol was incubated for 10 min at 30 °C in the presence of 2 mM $CaCl_2$, 125 μg/ml PS and 2.5 μg/ml DOG, before chromatography in the presence of protease inhibitors. Assays were performed with Ca^{2+} but in the presence (●) or absence (○) of PS and DOG, with mPSP as substrate. The results shown are typical of three similar experiments.

more in the presence of PKC α1. These results demonstrate that the different substrate specificities of the two PKC forms seen with exogenous substrates reflect their activities towards endogenous muscle proteins and are therefore likely to be physiologically important characteristics.

The differences between the PKC α activities were found to extend to their sensitivities to a synthetic inhibitor. PKC α2 was more susceptible to inhibition by submaximal doses of chelerythrine chloride in three independent experiments with mPSP as substrate (results not shown). The IC_{50} values were 107 ± 12 and 21 ± 5 μM for PKC α1 and PKC α2 respectively, as determined from dose–response curves. The fact that this inhibitor shows competitive kinetics for protein substrate [27] again underlines the differing substrate interactions of the two PKC α species. In contrast, no difference was seen in the effects of GF-109203X (bisindolylmaleimide I) on the two forms, with IC_{50} values of 36 ± 5 and 24 ± 6 nM for PKC α1 and PKC α2 respectively, similar to that previously reported for PKC α [28]. This inhibitor shows competitive binding kinetics for ATP [28], suggesting that interaction with ATP does not differ between the PKC α activities.

The next experiments were undertaken to elucidate the mechanism responsible for the generation of the different PKC α activities. To determine whether proteolysis was involved, muscle cytosol that had been prepared in the absence of leupeptin, PMSF and benzamidine was applied to hydroxyapatite and eluted with a shallow gradient in a buffer also lacking these protease inhibitors. Activity assays indicated that although the overall recovery of PKC was decreased by 31% in comparison with that seen in Figure 1b, the ratio of PKC α1 to PKC α2 activity was unchanged (PKC α1 being 36% and 40% of PKC α2 in two experiments; results not shown). A further investigation of the effects of proteolysis involved the incubation of muscle cytosol for 10 min at 30 °C in the presence of $CaCl_2$, PS and DOG, before hydroxyapatite chromatography (Figure 3). This gave an altered elution profile: PKC α1 activity was again diminished, PKC α2 activity was almost absent and an early, previously unseen peak was now observed that was lipid-independent (fractions 6–10 in Figure 3). Because these incubation conditions were conducive to proteolysis of PKC, especially by calpain [4,29], the early peak of activity is likely to represent the activator-independent catalytic domain of PKC α, also known as M-kinase [4].

Although an association of PKC α with calpain in skeletal muscle has been reported [30], we were unable to detect endogenous calpain activity in either PKC α1 or PKC α2 hydroxyapatite fractions (C. Schmitz-Peiffer, C. L. Browne and T. J. Biden, unpublished work). In addition, immunoblotting with PKC α-specific antibodies did not detect any faster-migrating PKC species (see, for example, Figure 5). Taken together, these results suggest that PKC α2 is not a proteolytic product of PKC α but is in fact more sensitive to proteolysis than PKC α1.

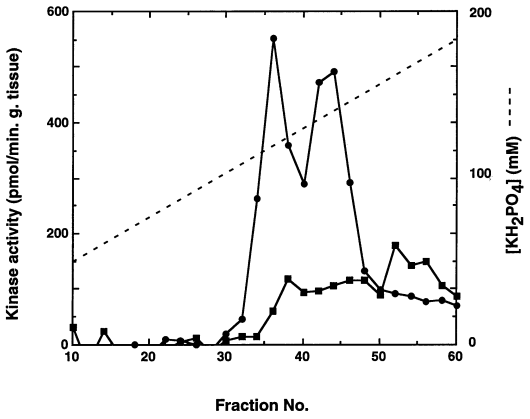

Figure 4 The effects of incubation of muscle cytosol with H_2O_2 before hydroxyapatite chromatography

A skeletal muscle cytosolic fraction was incubated at 4 °C for 20 h in the absence (●) or presence (■) of 100 mM H_2O_2 and 25 mM $FeCl_2$, before hydroxyapatite chromatography as in Figure 1b. Fractions were assayed for PKC in the presence of Ca^{2+} and lipid activators, with mPSP as a substrate. Results shown are typical of four similar experiments.

However, we cannot exclude the possibility that under the incubation conditions used to generate the profile shown in Figure 3, residual cytosolic ATP might enable PKC autophosphorylation to occur, possibly leading to increased proteolysis of either PKC α species [31]. In this case, transient conversion of one species to the other by concurrent minor proteolysis might be hard to detect.

We also investigated whether the two activities of PKC α might represent differently phosphorylated forms of the same protein, as it is possible that an altered phosphorylation state could affect both elution from hydroxyapatite and substrate recognition without altering electrophoretic mobility. Immunoblotting had revealed that the isoenzyme had a consistent apparent molecular mass of 82 kDa on SDS/PAGE of column fractions from each peak (Figure 1c). This demonstrated the absence of the unphosphorylated nascent 74 kDa form of PKC α, previously reported to be inactive in assays employing histone as a substrate [7,8]. Furthermore muscle extracted in the presence of the protein phosphatase inhibitors 50 mM NaF and 40 mM β-glycerophosphate gave a similar activity profile to that shown in Figure 1b (results not shown). The total PKC activity measured in these peaks was increased 1.4-fold, which is consistent with earlier reports that PKC α must be phosphorylated to be active [7,8]. However, peaks α1 and α2 were again evident, eluting at similar KH_2PO_4 concentrations as previously. The ratio of activities in the peaks assayed with mPSP was also preserved, PKC α1 again being 35 ± 4 % of PKC α2 in three experiments. Thus although the presence or absence of phosphatase inhibitors did alter total PKC activity, it did not selectively influence either PKC α1 or PKC α2. This argues against the generation of either of the two activities by dephosphorylation during preparation.

In a recent study, Allen et al. [21] observed a second PKC α activity (termed PKC α') after hydroxyapatite chromatography of purified rat brain PKC. PKC α' also phosphorylated histone IIIS poorly but, in contrast with the activator dependency of PKC α2 reported here, displayed a high degree of Ca^{2+}- and lipid-independence. These authors showed that PKC α' was probably generated during preparation, by an oxidative modification of PKC α, a process previously found to activate the kinase [15], and that its formation could be prevented by using peroxide-free Triton X-100 and an antioxidant during purification. To determine whether PKC α2 was derived from PKC α1 by oxidative modification, experiments were performed with anti-oxidants and oxidizing agents. After increasing the concentration of DTT in the extraction buffer to 50 mM, the activity of PKC α1 remained at 41 % of that of PKC α2 (results not shown), similar to that seen in Figure 1b and therefore demonstrating that PKC α2 was not generated by oxidative modification during extraction. Conversely, experiments were performed with muscle cytosol that had been prepared in the absence of DTT and incubated on ice overnight in the absence or presence of 100 mM H_2O_2 and 25 μM $FeCl_2$ before hydroxyapatite chromatography (Figure 4). The resultant decrease in PKC α2 activity relative to PKC α1 activity seen in the control incubation (compare with Figure 1b) is likely to be a consequence of the higher sensitivity of PKC α2 to proteolysis during the overnight incubation. However, after treatment with H_2O_2 a decrease in both PKC α1 and PKC α2 peaks was observed, together with an increase in a third peak eluting after PKC α2. This peak again phosphorylated histone IIIS poorly and also exhibited a low level of lipid-activator-independent activity (10 % of total activity; results not shown). Thus although PKC α2 itself is probably not derived by oxidative modification during extraction, an activity displaying some but not all of the features of PKC α2 can be generated by subsequent oxidation of the cytosolic fraction. This further activity is probably more closely related to PKC α' and might correspond to the trailing shoulder of PKC α2 seen in Figure 1b.

Another mechanism by which the chromatographic and kinetic properties of PKC α could be altered, but not its electrophoretic mobility under denaturing conditions, is by association of the kinase with a regulatory factor. The possibility that PKC α2, which displayed anomalous substrate specificity, was associated with a lipid was investigated by incubation of the cytosolic extract with 1 % (v/v) Tween-80 on ice for 30 min. The extract was then loaded on a hydroxyapatite column equilibrated with detergent-containing buffer, washed with the same buffer and eluted with a phosphate gradient in the absence of detergent. The PKC activity profiles observed when assaying fractions with mPSP and histone IIIS as substrates were still similar to those shown in Figure 1b, with PKC α1 being 39 % and 44 % of PKC α2 in two experiments. A similar procedure was employed in further attempts to dissociate PKC α2 from a putative binding factor, by using 1 M NaCl in place of Tween-80, again with no effect on the activity profiles (PKC α1 being 39 % of PKC α2).

PKC α overlay assays were employed to determine the presence of a binding protein that might selectively inhibit the activity of PKC α2 in a substrate-dependent fashion. Samples of muscle cytosol and hydroxyapatite peak fractions were electroblotted after SDS/PAGE, and membranes were preincubated under renaturing conditions before incubation with partly purified PKC α and subsequent immunodetection. In this way both the PKC α in the original sample and also bound PKC α were observed, the latter indicating the presence of PKC α-binding proteins. A number of such proteins were detected in the cytosol, ranging from 30 to 140 kDa (Figure 5). However, only a 69 kDa PKC α-binding protein was found in the hydroxyapatite fractions as well as in the cytosol, and although abundant in the PKC α1 peak fraction it was barely detected in the PKC α2 peak fraction. These results demonstrate that PKC α2 substrate specificity is not explained by association of PKC α with a binding protein. The sequential elution of PKC α1 and PKC α2 during hydroxyapatite chromatography is probably not explained by the association of PKC α1 with the 69 kDa binding protein because further overlay assays suggested that these did not co-elute

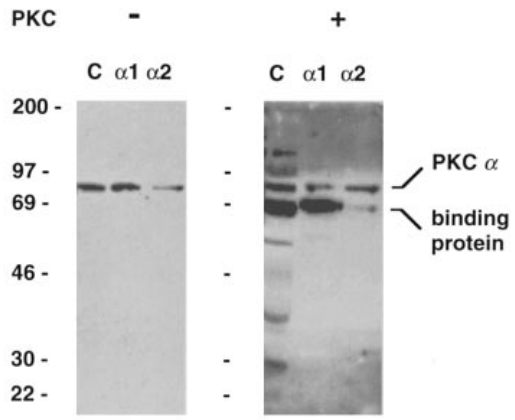

Figure 5 PKC overlay assays of skeletal muscle cytosol and hydroxyapatite chromatography fractions

Muscle cytosol (C), and PKC α1 and PKC α2 peak fractions, prepared as in Figure 1b, were subjected to SDS/PAGE and electroblotting, followed by PKC α overlay assay in the absence (−) and presence (+) of partly purified PKC α, as described in the Experimental section. The positions of protein markers, with molecular masses given in kDa, are indicated at the left. Results are typical of five similar overlay assays.

precisely and that both Ca^{2+} and phospholipid are required for the interaction of the proteins (C. Schmitz-Peiffer, C. L. Browne and T. J. Biden, unpublished work).

DISCUSSION

Characterization of PKC isoenzymes in rat skeletal muscle

Although several earlier studies have identified various PKC isoenzymes in muscle, results have differed and there has been little attempt to correlate expression with activity. Immunohistochemical analysis of human skeletal muscle detected PKC α, PKC βI and βII [32], whereas PKC β was not detected in rat skeletal muscle cells [33]. PKC α, PKC β, PKC ε and PKC θ were detected by immunoblotting in one recent study [34], and PKC α, PKC δ and PKC ζ in another [35]. In two studies involving Northern analyses, mRNA for PKC β was poorly detected in skeletal muscle extracts: the major isoenzymes observed were PKC α and PKC θ [36] and PKC α, PKC ε and PKC θ [34]. These results are consistent with our finding that PKC α is the most abundant conventional PKC activity in muscle cytosol, and that PKC β activity is relatively scarce, whereas our results from immunoblotting of chromatographic fractions support the presence of all of the above isoenzymes. A study of PKC activity in soleus muscle of Zucker rats [37] described PKC βI and βII activities and a single form of PKC α. Differences from our observations can be explained by the use of detergent during muscle extraction and the use of histone IIIS alone during the assay of hydroxyapatite fractions. Although the results shown here are derived from a mixture of three red skeletal muscle groups, we obtained similar hydroxyapatite profiles in experiments using a single red muscle, soleus, and also white quadriceps (C. Schmitz-Peiffer, C. L. Browne and T. J. Biden, unpublished work).

Hydroxyapatite chromatography, either of crude extracts or of partly purified PKC, has been widely used to examine PKC activity. Kosaka et al. [38] used this technique to investigate PKC isoenzymes in several rat tissues, and in each case PKC α, detected with histone I as substrate, was eluted as a single peak. However, in several other reports, for example of rat retina [39], bovine neutrophils [40], PC12h cells [41] and EL4 thymoma cells [42], PKC activity, eluting in the region of the gradient expected to yield PKC α, gave an indication of two chromatographically separable forms of this isoenzyme. It is therefore probable that the present findings are applicable to tissues other than skeletal muscle, even though we found little indication of PKC α2-like activity in rat brain (Figure 1a). Because PKC α differed between skeletal muscle and brain in this way, despite identical preparation, and because of the highly reproducible ratio of the two activities, we conclude that PKC α2 is unlikely to have been generated during tissue extraction but already exists in intact muscle. The presence of two forms of PKC α might have been overlooked in other studies, especially when only histone was employed as substrate to generate activity profiles [37], or if chromatographic separation was suboptimal.

The major PKC activities observed after chromatography of muscle cytosol corresponded immunologically to PKC α, whereas the minor PKC activity corresponded to PKC β. In addition to these conventional PKC isoenzymes, we detected the novel PKC species δ, ε and θ, as well as the atypical PKC ζ. The elution of these isoenzymes was in agreement with previous studies of other tissues [39,43]. The two major muscle PKC activities therefore seem to represent different forms of PKC α on the basis of several criteria: elution from hydroxyapatite (Figure 1b) in the region of the KH_2PO_4 gradient expected of PKC α [25]; detection of protein in immunoblots by three unrelated but PKC α-specific antibodies (Figure 1c); immunodepletion of activity towards both histone IIIS and mPSP with PKC α-specific antibody; and finally the exhibition of identical requirements for both Ca^{2+} and lipid activators when phosphorylating mPSP (Table 2).

Although PKC α is responsible for both peaks of activity, the kinase in the second peak seems to have undergone some form of modification, making it less able to phosphorylate certain substrates such as histone IIIS. The effects of the two differently acting synthetic PKC inhibitors were consistent with a difference in the interaction of the PKC forms with protein or peptide substrates, but not with ATP. This altered specificity was observed not only in assays with synthetic peptides and exogenous proteins (Table 3), but also in incubations with endogenous skeletal muscle proteins (Figure 2). The latter finding underscores the possible physiological relevance of the presence of PKC α2. If PKC α2 activity is derived from PKC α1, this could represent an additional means of regulating the kinase in skeletal muscle, beyond the elevation of activators.

Derivation of PKC α2

Our studies aimed at clarifying the presence of the two PKC α species have been constrained by the difficulties in purifying PKC α2, apparently because of its increased susceptibility to proteolytic degradation. However, we have shown that proteolysis is unlikely to account for the generation of PKC α2 because preparation or incubation of skeletal muscle cytosol under conditions promoting proteolysis of PKC (Figure 3) did not simply convert one form into the other, and immunoblotting demonstrated that they exhibited identical molecular masses (Figures 1c and 5). Hence neither M-kinase nor a novel proteolytic form of PKC α can explain either of the PKC α activities described here.

It has been well established that PKC α exists in different phosphorylation states [9–11], and our experiments with phosphatase inhibitors confirmed that preventing dephosphorylation during preparation can influence overall activity. In contrast, the inhibitors did not affect the ratio of the two activities, indicating

that neither was a result of phosphatase activity after tissue extraction. We cannot, however, exclude the possibility that some difference in phosphorylation occurring in intact muscle might underlie interconversion of the two species of PKC α. An example might be tyrosine phosphorylation, as recently described for controlling the substrate specificity of PKC δ [44]. However, we were unable to detect tyrosine-phosphorylated protein in hydroxyapatite fractions containing either PKC α1 or PKC α2 by using two different anti-phosphotyrosine antibodies in immunoblots (results not shown). Moreover, PKCs α1 and α2 showed no differences in migration on SDS/PAGE (Figure 1c), nor in pI as determined by isoelectric focusing (results not shown). This suggests that any differences in phosphorylation that might be present between the two species would need to be rather subtle.

Our investigation into the role of oxidative modification in the generation of two PKC α forms indicated that PKC α2 was not produced by this means during muscle extraction and was not identical with the previously described PKC α' [21]. However, treatment of muscle cytosol with H_2O_2 increased a minor peak of PKC α2-like activity, which might be more closely related to PKC α' (Figure 4). Modification of different regions of the PKC molecule by oxidation has been reported to lead to different effects in terms of regulation by activators and also on catalytic activity; more than one form of oxidatively modified PKC, displaying different chromatographic properties and levels of activator dependence, has been described [15]. In addition to altered chromatographic elution, PKC α2 shares a further property with previously described oxidatively modified PKC in that it exhibits an increased sensitivity to proteolysis [15]. Thus PKC α2 could be a form of oxidatively modified PKC α that exists in intact muscle but is difficult to generate *in vitro* with oxidizing agents. This manner of regulating PKC has been described under certain conditions in studies with hepatocytes [13], hippocampus [14], C6 glioma cells [15] and B16 melanoma cells [45].

In summary, the findings of the present study give an indication of the PKC α activity existing in intact muscle. The generation of a form with substrate-delimited catalytic activity might be important in the selective phosphorylation of PKC α substrates in this tissue. Such a system might also occur in certain other cell types, although the PKC α activity in brain was found to be predominantly of the fully active form. Although proteolysis and phosphorylation are probably not involved, oxidative modification remains a candidate for the mechanism of conversion of PKC α1 into the more limited PKC α2. We have also found evidence that activated PKC α binds to a 69 kDa protein, which may be a physiologically important event in skeletal muscle.

This work was supported by an N.H.M.R.C. block grant to the Garvan Institute of Medical Research.

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Received 25 March 1996/22 July 1996; accepted 30 July 1996