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Protein kinase C translocation in intact vascular smooth muscle strips

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Using intact muscle strips from the bovine carotid artery, the time course of translocation of protein kinase C (PKC) from the cytosol to the membrane fraction was measured in response to various agonists that induce contractile responses. PKC activity was assessed by Ca^{2+}/phospholipid-dependent phosphorylation of histone. Exposure of the muscle strips to phorbol ester (12-deoxyphorbol 13-isobutyrate) induced a rapid and sustained translocation of PKC from the cytosol to the membrane fraction, and a slowly developing but sustained contractile response. Histamine induced a comparable initial translocation of PKC to the membrane which then decreased somewhat to a stable plateau significantly above basal values. Histamine also led to a rapid and sustained increase in tension. Angiotensin II, which caused a rapid but transient contraction, induced a rapid initial translocation of PKC to the membrane. The membrane-associated PKC then declined to a stable plateau significantly lower than that seen after a histamine-induced response, and only slightly above the basal value. Endothelin, which induced a sustained contraction, caused a sustained translocation of PKC from the cytosol to the membrane. In contrast, although exposure to 35 mM-KCl induced a rapid and sustained contraction, it caused only a transient translocation of PKC; the membrane-associated PKC returned to its basal value within 20 min. These results demonstrate that PKC in intact smooth muscle can be rapidly translocated to the membrane and remains membrane-bound during sustained phorbol ester- or agonist-induced contractions, but that such a sustained translocation of PKC does not occur during prolonged stimulation with KCl.

INTRODUCTION

The Ca^{2+}/phospholipid-dependent protein kinase, protein kinase C (PKC), is widely distributed in tissues and organs [1]. Over the last few years the enzyme has been implicated in the regulation of an increasing number of cellular processes [1–4]. In vascular smooth muscle, PKC is present in relatively high concentrations [4], suggesting that it also plays an important role in the control of smooth muscle function. A unique feature of PKC regulation is the spatial translocation of the enzyme. Under basal conditions PKC is thought to be located mainly in the cytosol, but after hormonal stimulation PKC is rapidly translocated to the membrane where it associates with membrane phospholipids [4]. The activation of the enzyme is often closely linked to changes in phosphoinositide (PI) metabolism [3]. When an appropriate agonist activates a specific receptor, an immediate receptor-linked event is the activation of a specific phospholipase C that catalyses the rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to two intracellular messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) [2]. It has been shown that IP_3 triggers the release of Ca^{2+} from intracellular stores in smooth muscle [5]. Likewise, it has been demonstrated that appropriate agonists induce sustained increases in the DAG content of cultured smooth muscle cells [6] and intact tracheal [7] and carotid artery [8] smooth muscle.

Many smooth muscle agonists such as angiotensin II, histamine and α_1-adrenergic agents act through receptors coupled to the PI system [2,8,9], thereby inducing an increase in cytosolic free Ca^{2+} and the generation of DAG. The time courses of the generation of these two messengers are different. The increase in cytosolic free Ca^{2+} is only transient, and the Ca^{2+} concentration returns to near-basal levels within several minutes [9,10]. On the other hand, it has been shown that the increase in DAG concentration is sustained [6–8]. The only well-characterized effect of DAG is the activation of PKC. Hence it seems probable that PKC is also activated during the sustained phase of smooth muscle contraction. Support for this assumption comes from contraction studied with phorbol esters, activators of PKC [11]. Exposure of smooth muscle strips to phorbol ester leads to a slowly developing contraction which lasts for several hours [12–14]. It has also been shown that phorbol esters induce a sustained contraction in chemically skinned vascular smooth muscle when the Ca^{2+} concentration is maintained at 100 nM [15]. These results demonstrate that pharmacological activation of PKC can induce a slowly developing contractile response in vascular smooth muscle.

That a contraction induced by phorbol ester is similar to the sustained phase of an agonist-mediated contraction has been demonstrated by the analysis of the protein phosphorylation events induced either by phorbol esters or by agonists. By two-dimensional gel electrophoresis, the phosphoproteins from smooth muscle can be resolved into approx. 40 phosphoproteins [8,16]. The phosphorylation pattern of these proteins shows distinct temporal changes after stimulation with an agonist. For example, stimulation of bovine carotid artery with histamine or tracheal smooth muscle with carbachol leads initially to the phosphorylation of the 20 kDa myosin light chain and two other low-molecular-mass (18 kDa) proteins. Later, during the sustained contraction, the intermediate filament protein desmin, as well as caldesmon and a number of low-molecular-mass cytosolic proteins, become phosphorylated, but the extent of myosin light chain phosphorylation declines. These 'late-phase' proteins seen

Abbreviations used: DBP, 12-deoxyphorbol 13-isobutyrate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-triphosphate; PI, phosphoinositide; DAG, diacylglycerol; PKC, protein kinase C; PSS, physiological salt solution.

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during an agonist-mediated contraction are identical with the phosphoproteins observed during phorbol-ester-induced contraction [8,16]. The phosphorylation of these proteins appears to occur as a direct or indirect consequence of the activation of PKC [17].

The temporal change in the pattern of phosphoproteins and the distinct effects of phorbol esters on smooth muscle contraction [12–14] have led to the proposal that the initial and sustained phases of smooth muscle contraction are mediated by different cellular and molecular events [17]. In this model, the initial phase is characterized by an increase in the cytosolic Ca^{2+} concentration, the activation of the calmodulin-dependent myosin-light-chain kinase, the phosphorylation of the myosin light chains, an interaction of actin and myosin, and hence contraction. This initial phase of tension development is followed by a phase of tension maintenance. During this latter phase, it has been proposed that the activation of membrane-associated PKC by altered Ca^{2+} influx leads directly or indirectly to the phosphorylation of a number of cytosolic proteins as well as fibrillar proteins such as desmin and caldesmon [8,16,18]. It is postulated that these late-phase phosphoproteins are involved in tension maintenance [17,19]. In view of the recent studies by Marston and colleagues [20] showing that caldesmon interacts with actin, and those of Adam *et al.* [18] showing agonist- and phorbol ester-dependent phosphorylation of caldesmon, it is possible that the phosphorylation of caldesmon may play a role in maintaining smooth muscle contraction. A prediction of this two-phase model is that a sustained translocation of PKC should be observed during the sustained phase of agonist-induced vascular smooth muscle contractions.

To test this postulate, PKC translocation was examined in bovine carotid artery strips in response to phorbol ester, KCl-induced depolarization and several specific hormonal agonists including histamine, endothelin and angiotensin II. Our results show a sustained translocation of PKC in response to phorbol ester, histamine and endothelin, but a transient translocation in response to KCl and angiotensin II. The data indicate that the sustained contractile response seen in vascular smooth muscle after hormonal stimulation is associated with a sustained translocation of PKC.

MATERIALS AND METHODS

Materials

Phorbol ester DPB (12-deoxyphorbol 13-isobutyrate), histones (type III-S), DEAE-cellulose and histamine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), angiotensin II from Calbiochem–Behring (San Diego, CA, U.S.A.), and endothelin from Peptides International (Louisville, KY, U.S.A.). [^{32}P]ATP was obtained from Amersham (Arlington Heights, IL, U.S.A.). 1,2-Diolein and phosphatidylserine were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.).

Preparation of bovine carotid artery strips

Calf carotid arteries were obtained from a local abattoir and transported at 4 °C in a physiological salt solution (PSS) of the following composition: 140 mM-NaCl, 4.7 mM-KCl, 1.0 mM-$MgSO_4$, 1.0 mM-NaH_2PO_4, 1.5 mM-$CaCl_2$, 0.03 mM-EDTA, 0.5 μM ascorbic acid, 10 mM-glucose and 10 mM-Hepes (pH 7.4). The buffer was gassed with 100% O_2. Carotid arteries were cleared of surrounding connective tissues and the adventitia were removed with fine forceps. The endothelium was removed by rubbing the muscle strips with a cotton-tipped wooden stick. Transverse strips of approx. 1 mm width were prepared for both contraction and translocation experiments.

Measurement of isometric tension

Each carotid artery strip was mounted in a 2 ml static muscle chamber and equilibrated in PSS containing 10 μM-phentolamine at 37 °C and gassed with 100% O_2. Tissues were allowed to equilibrate for 90 min before experiments were begun. Tension was measured isometrically with Grass FT-03 force-displacement transducers (Grass Instrument Co., Quincy, MA, U.S.A.) and was displayed on a Gould 2400 S recorder (Gould Inc., Cleveland, OH, U.S.A.) with built-in preamplifiers.

Measurement of PKC translocation

Strips of carotid artery smooth muscle were weighed and incubated for at least 120 min in PSS under 100% O_2 at 37 °C [7]. The strips were then exposed to agonists. At appropriate time points, 1 g of smooth muscle strips was rapidly put on ice, washed in ice-cold homogenization buffer (20 mM-Tris/HCl, pH 7.5, 250 mM-sucrose, 3 mM-EGTA, 10 mM-mercaptoethanol, 1 mM-phenylmethanesulphonyl fluoride and 50 μM-leupeptin) and immediately homogenized using a Polytron apparatus. The homogenate was then spun at 100000 ***g*** for 60 min and the supernatant was used as the cytosolic fraction. The pellet was resuspended in buffer containing 1.0% Triton X-100 and shaken at 4 °C for 30 min. The homogenate was then diluted with buffer to a final concentration of 0.5% Triton X-100 and centrifuged at 100000 ***g*** for another 60 min. The supernatant was used as the PKC-containing fraction of the original membrane pellet. Both PKC-containing fractions then underwent DEAE-cellulose chromatography to remove substances interfering with the PKC assay [21]. The fractions were applied to 1 ml DEAE-cellulose columns equilibrated in buffer A (20 mM-Tris/HCl, pH 7.5, 0.5 mM-EGTA, 0.5 mM-EDTA, 50 mM-mercaptoethanol, 0.025% Triton X-100 and 10% glycerol). The columns were then washed with 3 vol. of buffer A containing 20 mM-NaCl. PKC activity was then eluted with buffer A containing 400 mM-NaCl.

PKC assay

Kinase activity was assessed by Ca^{2+}/phospholipid-dependent phosphorylation of histone 1, as described previously [22]. An aliquot (10 μl) was assayed in buffer containing 20 mM-Tris/HCl, 1 mM-EGTA, 1.2 mM-$CaCl_2$ (free Ca^{2+} 200 μM), 20 μg of phosphatidylserine/ml, 2 μg of diolein/ml and 200 μg of histone/ml in a total volume of 0.1 ml. Phosphatidylserine and diolein, dissolved in chloroform and dried under N_2, were suspended by sonication in water. Samples were prewarmed at 30 °C for 1 min, and [^{32}P]ATP (2–5 μCi/μmol) was added to a final concentration of 25 μM to start the reaction. After 10 min at 30 °C, the reaction was stopped with 0.8 ml of ice-cold 20% trichloroacetic acid. Samples were filtered on 0.45 μm Millipore filters and were washed with 10 ml of 5% trichloroacetic acid. Filters were counted for radioactivity in a scintillation counter. Activity in the presence of Ca^{2+} alone was subtracted from that obtained with Ca^{2+} plus diolein and phosphatidylserine. The PKC activity was expressed as nmol of phosphorus transferred to histone per 10 min per gram of original vascular tissue.

The results reported for the translocation experiments (means ± S.D.) represent duplicate measurements made on three to five separate occasions. The n value given in each Figure represents the number of separate experiments, in each of which duplicate measurements were obtained. For statistical analysis, changes from baseline to experimental conditions were compared using the paired Student's t test. A change was considered significant when the P value was <0.05.

RESULTS

Contractile responses of bovine carotid artery induced by phorbol ester DPB, histamine, angiotensin II, endothelin and 35 mM-KCl

As a guide to the experiments measuring the translocation of PKC, the time courses of the contractile response of bovine carotid artery strips to the vasoactive hormones histamine, angiotensin II and endothelin, as well as to phorbol ester and KCl, were characterized (Fig. 1). Exposure of the muscle strips to 0.8 μM-DPB caused a slowly developing contraction that reached a plateau after 20–40 min and was then sustained for several hours. The maximum tension achieved with 0.8 μM-DPB was equivalent to that induced by 10 μM-histamine. Stimulation of the muscle with histamine (10 μM) induced a rapid contractile response reaching a sustained plateau within 4–6 min. This plateau was stable for at least 60 min. Angiotensin II (100 nM) induced the same initial rapid increase in tension as induced by histamine, but the angiotensin-induced contraction was transient, and tension returned to the basal level within 20 min. The recently discovered vasoactive hormone endothelin (100 nM) induced a slowly developing but sustained contraction. The plateau of the endothelin-induced contraction was reached after 20 min. The maximum tension induced by endothelin was 80% of the histamine-induced contraction. Addition of 35 mM-KCl to the muscle caused a rapid rise in tension, with a sustained plateau which was approx. 50% of the histamine-induced sustained response.

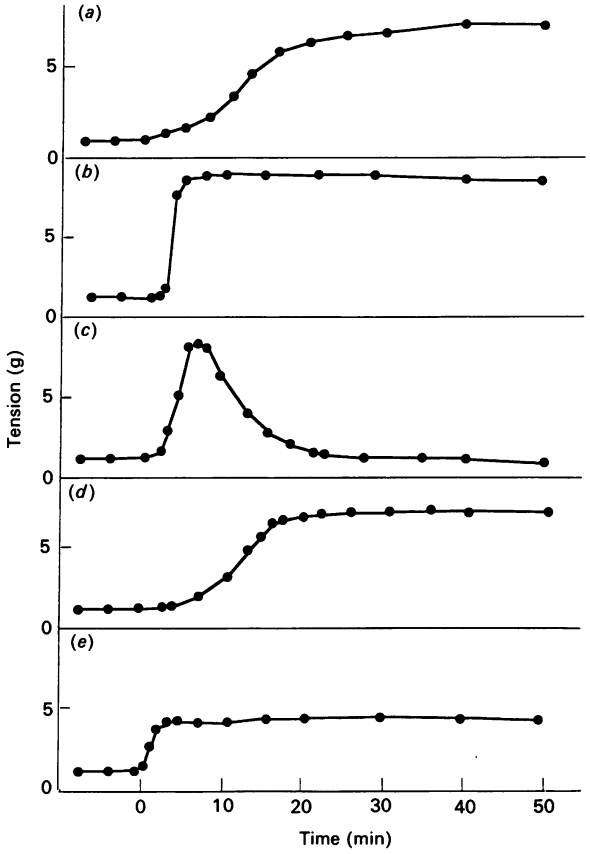

Fig. 1. Agonist-induced contractions of bovine carotid artery

Contractile effects of phorbol ester, histamine, angiotensin II, endothelin and K^+ on smooth muscle strips from bovine carotid artery. (*a*) DPB (800 nM) induced a slowly developing contraction which reached a sustained plateau after 20–40 min. (*b*) Histamine (10 μM) induced a rapid contraction followed by a stable sustained contractile response. (*c*) Angiotensin II (100 nM) led to a rapid contraction which declined thereafter and returned to basal tension after 20 min. (*d*) Endothelin (100 nM) induced a slowly developing contraction which reached a sustained plateau within 20 min. (*e*) Exposure to 35 mM-KCl induced a rapid contraction which was sustained at a tension considerably lower than that observed during a histamine- or endothelin-induced contractile response. Tracings shown are representative of 3–6 experiments. Agonists were added at zero time.

PKC translocation induced by DPB

PKC was measured by the traditional method of Ca^{2+}-dependent phosphatidylserine/diolein-stimulated phosphorylation of histone [1,21]. Recovery of PKC activity was quite consistent in different experiments. In all of our experiments, 20–30% of the measured PKC activity was membrane-bound under basal conditions (see Figs. 2–4, 6 and 7). The mean basal value of membrane-bound PKC ranged from 0.7 to 1.2 nmol of P/10 min per g wet wt. in various experiments, and the cytosolic activity ranged from 2.7 to 3.3 nmol of P/10 min per g wet wt. This constitutively membrane-bound PKC was not decreased by prolonged incubation of the muscle strips before the experiments and seemed therefore not to be due to activation of PKC during the preparation of the muscle strips. Furthermore, the total PKC activity (membrane plus cytosol) was constant throughout the period of exposure to PKC.

The first studies of PKC translocation were performed with the phorbol ester DPB. After exposure of muscle strips to 0.8 μM-DPB there was a rapid increase of PKC activity in the membrane fraction and a concomitant decrease of PKC activity in the cytosol (Fig. 2). By 5–10 min the PKC activity in the membrane had reached its maximum, and it then stayed elevated. The cytosolic PKC activity fell to a very low value and remained decreased during this same period. The total PKC activity (cytosol plus membrane) measured in serial samples of tissue did not change appreciably in response to DPB (4.1–4.8 nmol of P/10 min per g wet wt.) during the course of the experiments. The translocation of PKC activity induced by DPB (Fig. 2) occurred more rapidly than the development of contractile tension in response to this agonist (Fig. 1).

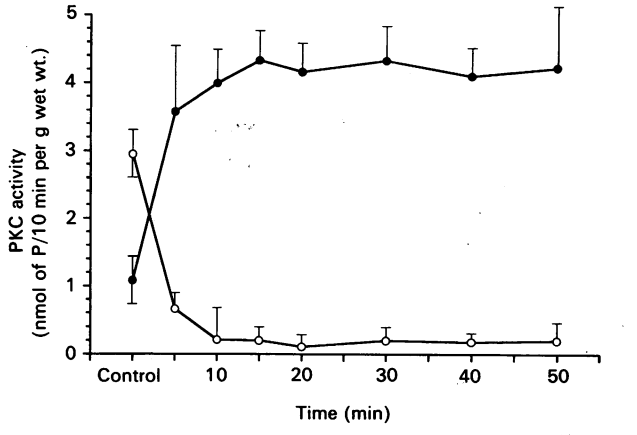

Fig. 2. Phorbol ester-induced PKC translocation

The effect of DPB (800 nM) on the distribution of PKC between the cytosolic and membrane fractions of bovine carotid artery is shown at the indicated times after addition. 'Control' represents the basal values obtained with untreated muscle. The phorbol ester induced a rapid increase of PKC activity in the membrane fraction (●) which was sustained during the experiment. The PKC activity in the cytosol (○) decreased correspondingly and disappeared almost completely (means ± S.D., $n = 4$).

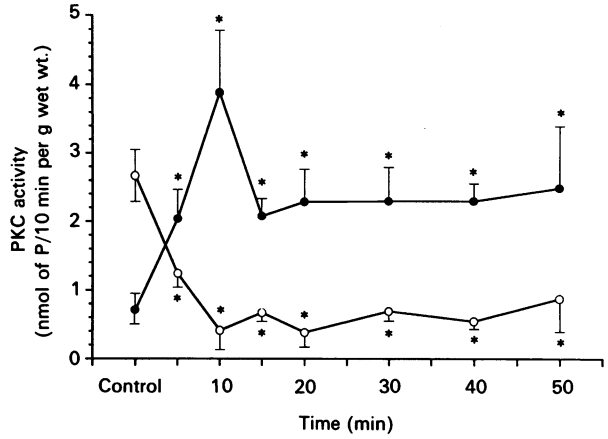

Fig. 3. Histamine-induced PKC translocation

The effect of histamine (10 μM) on the distribution of PKC between the cytosolic and membrane fractions of bovine carotid artery is shown. Histamine led to an increase in the PKC content of the membrane fraction (●). This initial increase declined to a stable plateau during which the membrane-associated PKC remained significantly elevated. The cytosolic PKC activity (○) decreased and remained low during the course of the experiment [$*P < 0.05$ compared with control (basal) values; means ± S.D., $n = 5$].

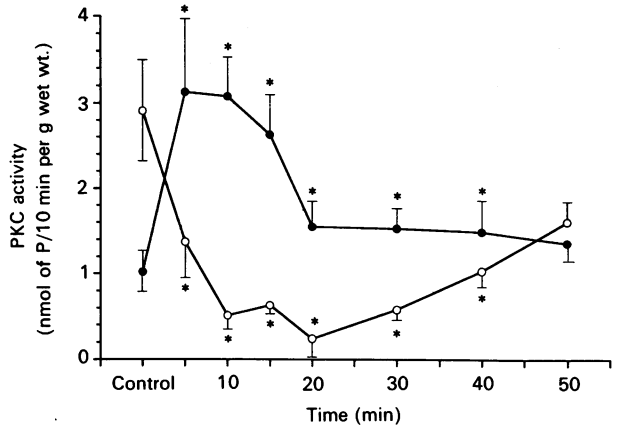

Fig. 4. Angiotensin II-induced PKC translocation

The effect of angiotensin II (100 nM) on the distribution of PKC between the cytosolic and membrane fractions of bovine carotid artery is shown. Angiotensin II induced a rapid increase in membrane-bound PKC activity (●) followed by a decrease to a plateau only slightly above basal values within 20 min. The cytosolic PKC activity (○) decreased initially. After 20 min there was a slowly increasing PKC activity in the cytosol, which had reached 60% of the basal value at the end of the experiment ($*P < 0.05$ compared with control values; means ± S.D., $n = 4$).

Effect of histamine on PKC translocation

Stimulation of carotid artery strips with histamine induced an initial rapid translocation of PKC (Fig. 3) with a time course somewhat slower than that seen with DPB (Fig. 2). The maximum PKC activity in the membrane fraction was achieved after 10 min (Fig. 3). The absolute amount of the PKC activity in the membrane fraction at this time point was 90% of the PKC activity seen after DPB treatment. However, after its initial peak, the PKC activity in the membrane of the histamine-treated muscle declined by about 50% to reach a stable plateau which was significantly above baseline ($P < 0.05$) at the time of the sustained phase of contraction (approximately half of the value observed in DPB-treated muscle). This response suggested a biphasic pattern of PKC translocation with histamine. The cytosolic PKC activity decreased rapidly and then remained at low levels during the period of exposure to histamine. Total PKC activity (cytosol plus membrane) did not change appreciably during the course of histamine action.

Effect of angiotensin II on PKC translocation

After stimulation with angiotensin II there was a rapid translocation of PKC from the cytosol to the membrane (Fig. 4). The height of the initial peak was about 80% of that observed in histamine-treated muscle. However, by 20 min of exposure, membrane-associated PKC had declined to approx. 60% of that seen during the protracted contraction of histamine-treated muscle. Angiotensin II-contracted muscle strips also relaxed at this time. In other words, in the histamine-treated muscle during the sustained response phase, the membrane-associated PKC increased from a basal value of 0.7 to a value of 2.3 nmol of P/10 min per g wet wt. (change of 1.6), whereas in the angiotensin II-treated muscle after the transient contraction, membrane-bound PKC increased from 1.0 to 1.5 nmol of P/10 min per g wet wt. (change of 0.5). A statistical comparison between the angiotension II- and the histamine-induced changes during the late phase showed a significant difference ($P < 0.05$) between the amounts of PKC that were membrane-associated in histamine- and angiotensin II-treated muscle.

Although in the angiotensin II-treated muscle the cytosolic PKC activity decreased initially, after 30 min there was a slow and steady increase in cytosolic PKC activity which reached 60% of the control value by 50 min (Fig. 4). Consequently there was a consistent decrease in total activity at 20 min (2.0 ± 0.4 nmol of P/10 min per g wet wt. at 20 min versus 3.9 ± 0.5 nmol/10 min per g at time 0) but this rose to near basal value by 50 min (3.6 ± 0.5 nmol/10 min per g) because of a progressive increase in the cytosolic PKC activity. With other contractile stimuli, the total PKC activity remained constant throughout the time course of the experiments (Fig. 5).

Effect of endothelin on PKC translocation

In contrast with angiotensin II, the endothelin-induced translocation of PKC from the cytosol to the membrane occurred slowly (Fig. 6). The maximal membrane-associated activity during endothelin action was approx. 70% of that seen with DPB (Fig. 2) and similar to that seen during the sustained phase of histamine action (Fig. 3). The decrease in cytosolic PKC activity was sustained during endothelin action. The total PKC activity did not change appreciably during the time course of endothelin action.

The contractile response to endothelin paralleled the changes in membrane-bound PKC activity. After exposure to endothelin, there was a slowly developing contraction which reached a maximum at 20 min; this was followed by a sustained contraction which lasted for several hours (Fig. 1).

Effect of 35 mM-KCl on PKC translocation

The effect of 35 mM-KCl on the time course of PKC translocation was different from that of all other agonists. Initially, there was a translocation of PKC which peaked at 10 min (Fig. 7). Thereafter, the membrane-bound PKC activity decreased, and returned to the basal level by 20 min. The cytosolic PKC activity was also initially decreased, but started rising again at 15–20 min, as the amount of membrane-bound PKC decreased. By 30 min the translocation was fully reversed and the PKC distribution was the same as that seen in the basal state.

Translocation of PKC in response to KCl occurred at the time of the initial contractile response, as was observed with

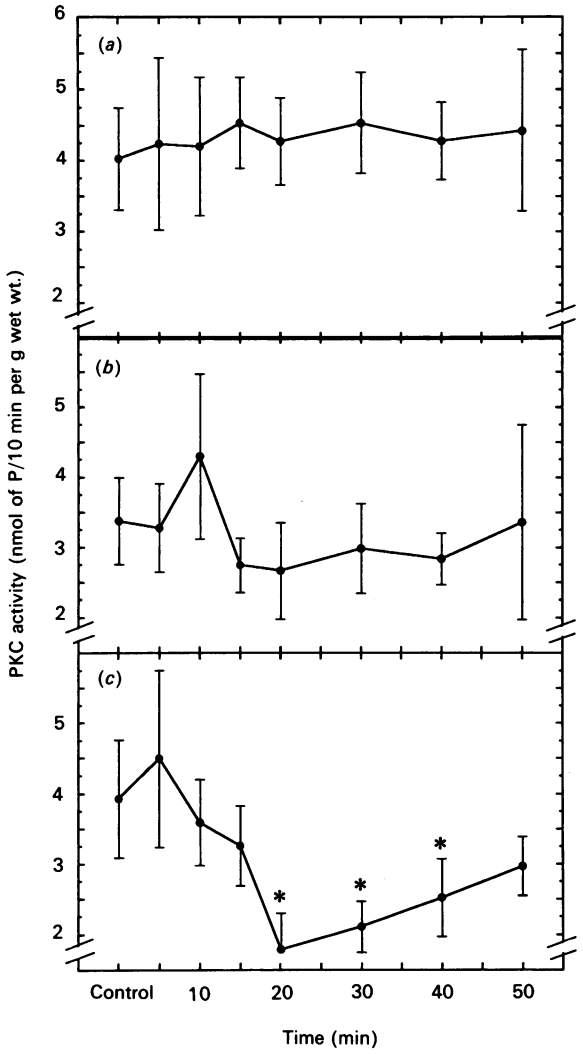

Fig. 5. Total PKC activity in membrane and cytosol fractions

The three panels show the total activities of PKC measured in carotid artery throughout the time course of experiments with DPB (800 nM, *a*), histamine (10 μM, *b*) and angiotensin II (100 nM, *c*). The plotted values were obtained by addition of the values (mean ± S.D.) of membranes to those of cytosol in each of Figs. 2, 3, and 4 respectively (*$P < 0.05$ compared with control values).

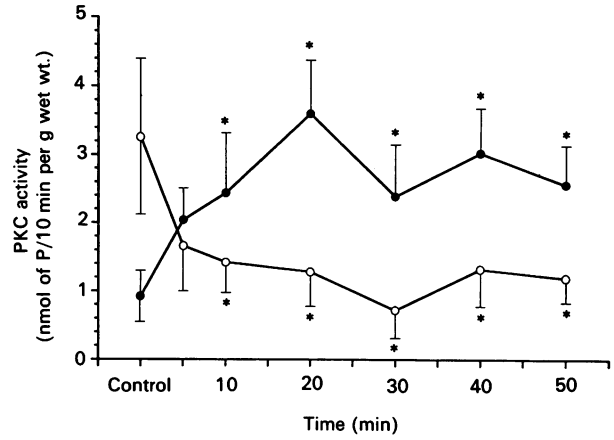

Fig. 6. Endothelin-induced PKC translocation

The effect of endothelin (100 nM) on the distribution of PKC is shown. After exposure to endothelin, the PKC activity in the membrane fraction of the muscle increased slowly and reached a peak at 20 min (●). The membrane-associated PKC activity remained elevated thereafter. In the cytosol, the PKC activity also decreased (○) (*$P < 0.05$ compared with control values; means ± S.D., $n = 3$).

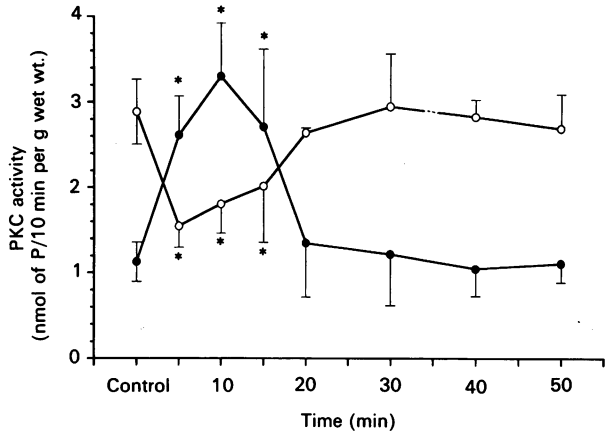

Fig. 7. KCl-induced PKC translocation

The effect of 35 mM-KCl on the distribution of PKC between the membrane (●) and cytosol (○) in bovine carotid artery is shown. KCl induced an increase in membrane-bound PKC activity, peaking at 10 min. The PKC activity then decreased and returned to basal values within 20 min. The cytosolic PKC activity showed an inverse pattern, with an initial decrease and a return to basal values after 20 min. (*$P < 0.05$ compared with control values; means ± S.D., $n = 3$).

endothelin, angiotensin II and histamine. However, in the case of KCl, there was a sustained contractile response but no significant increase in membrane-associated PKC activity during this phase of the response.

DISCUSSION

Measurements of PKC translocation in intact smooth muscle have not been published previously. The results of this study show that in vascular smooth muscle, association of PKC with the membrane occurs whether phorbol ester, histamine, angiotensin II, endothelin or KCl is the agonist (Figs. 2–4, 6 and 7). The data further demonstrate that this translocation of PKC can be sustained. In addition, our results also show that there are marked differences in the time courses and extents of PKC translocation in response to the different contractile stimuli.

One difficulty in directly correlating changes in PKC distribution with changes in tension is the fact that the contraction and translocation experiments were done under different experimental conditions: tension measurements were performed isometrically and translocation experiments isotonically. Because of the amount of tissue needed for determination of PKC activity, it was not possible to mount the multiple muscle strips isometrically for subsequent freeze-clamping at particular time points. Accordingly, our results argue that agonists which induce smooth muscle contraction also cause PKC translocation, but it is not yet possible to specifically correlate these PKC changes with changes in isometric tension. However, in protein phosphorylation experiments measuring the time course of changes in phosphorylation, comparable results have been obtained in freeze-clamped isometric muscle [18,23] and in cold-acetone-clamped isotonic muscle [8,16]. These data argue that the biochemical basis of muscle contraction is probably similar under the two conditions.

The amount of PKC activity associated with the membrane

fraction was consistently in the range of 20–30% (Figs. 2–4, 6 and 7). Although this value is higher than that reported in some studies in isolated cells [2,3,24,25], it is similar to that described in a number of other studies [26–29]. Furthermore, using a different method, i.e. immunoblotting of PKC in tissue fractions, we (J. I. Smallwood & H. Rasmussen, unpublished work) have found similar basal values in cultured mesengial cells and in cytoplasts prepared from human neutrophils. Three possible explanations for these basal membrane-associated PKC values are that: (*a*) there is PKC associated with the plasma membrane of relaxed smooth muscle cells; (*b*) PKC translocation is induced during preparation of the muscle strips; or (*c*) some cells escape homogenization and in this way contribute to the PKC measured in the pelleted 'membrane fraction'. The second possibility seems unlikely in view of the fact that prolonged incubation of the strips without agonist does not lower the basal membrane-associated PKC value. The third possibility may occur. However, the values obtained throughout this study were highly reproducible in experiments done on different days with muscle strips obtained from different animals. Any artifact of incomplete homogenization must therefore be consistent throughout all experiments. Hence it would not affect measurements of agonist-induced increases in membrane-bound PKC, but only reduce its relative magnitude.

Treatment of muscle strips with phorbol ester led to a rapid and nearly complete translocation of PKC from the cytosol to the membrane fraction (Fig. 2). This rapid and protracted redistribution of PKC in response to phorbol esters has been described in a variety of cell types [24–27,30,31].

Histamine and angiotensin II induced a similar rapid translocation of PKC (Figs 3 and 4). Both agonists caused the appearance of peak activity of membrane-bound PKC by 10 min, followed by a decline thereafter to a stable plateau. However, during this second phase there was a significant difference between the amount of membrane-associated PKC in histamine-treated as compared with angiotensin II-treated muscle. Although the height of the initial peak was comparable between the two agonists, the increment in membrane-associated PKC after 30 min of angiotensin II exposure was only 30% of the increment (above basal) seen in histamine-treated muscle. This difference in the amount of translocated PKC seen during the sustained phase of histamine-treated and angiotensin II-treated muscle may reflect the fact that histamine induces a sustained contraction but angiotensin II induces a transient one (Fig. 1). Histamine induces a sustained increase in the DAG content of the muscle, whereas angiotensin II does not [8,32]. Likewise, it is apparent that histamine induces a sustained increase in Ca^{2+} influx rate but angiotensin II does not [33]. Thus the failure of angiotensin II to induce a sustained contractile response, even though it activates PI turnover, is probably based on a failure to cause a sustained increase either in the DAG content of the membrane and/or in the Ca^{2+} influx rate. As a consequence of the first of these failures, PKC translocation would not be well sustained, and as a consequence of the second, the small incremental amount of PKC which does remain membrane-associated during the sustained phase of angiotensin II action (Fig. 4) would probably not be activated. That this small increment in membrane-associated PKC activity can be activated by increased Ca^{2+} influx is suggested by the fact that addition of the Ca^{2+} channel agonist BAY K 8644 to muscle strips which have been exposed to 0.1 μM-angiotensin II for 20 min (the time by which the muscle strips have returned to a basal tension; see Fig. 1) leads to a near-maximal contractile response even though addition of BAY K 8644 to naive strips, not previously exposed to angiotensin II, has no effect on muscle tension (Y. Takuwa & H. Rasmussen, unpublished work).

An additional feature of the angiotensin II-induced PKC translocation is the loss and subsequent recovery of total PKC activity. Since the results with histamine and phorbol ester were quite reproducible in showing no significant change in total PKC activity (Fig. 5), and the four different experiments with angiotensin II were reproducible in showing a significant loss of total activity at 20 min (Fig. 4), it seems quite likely that these results do not represent an experimental artifact. With angiotensin II, between 10 and 20 min there was a significant loss of membrane-associated PKC without a comparable rise in cytosolic PKC, whereas in the case of K^+-stimulated muscle over the same time period, as membrane-associated PKC falls, cytosolic PKC rises (Fig. 7). An explanation for this difference will require further studies. However, it may be due either to a loss and subsequent resynthesis of PKC after AII treatment or to a transient endogenous inhibition of PKC that affects its measured maximal activity *in vitro*.

The results of the studies with high K^+ (Fig. 7) support previous observations in other tissues [34–37] that an increase in intracellular Ca^{2+} concentration *per se* is able to induce the translocation of PKC to the membrane. The available evidence suggests that 35 mM-K^+ causes a marked increase in the Ca^{2+} influx rate without activating PI hydrolysis [7,33], yet this concentration of K^+ induces a rapid translocation of PKC to the membrane. However, in contradistinction to all the other agonists, this PKC translocation is completely reversed within 20 min, even though the K^+-induced increase in contraction (Fig. 1) is sustained. Also in contrast with other agonists, high K^+ causes a sustained increase in the extent of myosin light chain phosphorylation [8,23]. What is not yet clear is whether or not this K^+-initiated Ca^{2+}-dependent translocation of PKC to the membrane leads to endogenous activation of the PKC in the absence of an increase in the DAG content of the membrane. The fact that treatment with high K^+ causes no increase in the extent of phosphorylation of any of the late-phase proteins characteristic of phorbol ester-induced activation of PKC [8] argues that the K^+-initiated translocation of PKC may not lead to its activation. However, this conclusion is at variance with conclusions reached concerning the Ca^{2+}-dependent PKC translocation observed in some other tissues and cells [34,37]. Hence it remains possible that PKC may also participate in the initiation of a K^+-induced contraction.

In summary, we have shown that PKC is translocated from cytosol to membrane fractions in agonist-treated intact vascular smooth muscle strips, and that this translocation can be sustained for at least 50 min. The temporal pattern of the PKC translocation depends on the agonist used. The phorbol ester-induced translocation, which is independent of changes in intracelullar Ca^{2+}, and the KCl-induced translocation, which is totally dependent on increased cytosolic Ca^{2+}, define two extremes of this response. In the case of the physiological agonists (histamine, angiotensin II and endothelin), the temporal pattern of PKC translocation seems to reflect the respective activation of the Ca^{2+} transient, the sustained influx of Ca^{2+} and the sustained generation of DAG. These findings support but do not establish the hypothesis that PKC plays a role in mediating the sustained phase of agonist-induced contraction of vascular smooth muscle [17].

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