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A Synthetic Pseudosubstrate Peptide of Protein Kinase C Inhibits the Phorbol-12,13-dibutyrate Effect on Permeabilized Coronary Artery Smooth Muscle

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SUMMARY

We have studied the effects of a synthetic peptide that is based on the autoinhibitory domain of protein kinase C on tension development in detergent-permeabilized coronary artery smooth muscle. This peptide inhibited two forms of protein kinase C that were isolated from the coronary artery media layer by hydroxylapatite chromatography, with apparent K_i values in the 5–8 μM range. Contractions induced by calcium in the permeabilized arteries were not affected by the peptide (30 μM). Potentiation of calcium-induced contractions by 1 μM phorbol-12,13-dibutyrate was partially inhibited by 10 μM peptide and was completely abolished by 30 μM peptide. These results indicate that phorbol-12,13-dibutyrate potentiates calcium-induced contractions of permeabilized coronary arteries by activation of protein kinase C, but activated protein kinase C is not a requirement for the induction of contractions by calcium alone.

Phorbol esters, which are putative activators of the calcium- and phospholipid-dependent PKC, have been reported to induce contractions in vascular smooth muscle preparations (1, 2). In an effort to understand the mechanism by which phorbol esters induce these contractions, a model system of permeabilized smooth muscle strips has been employed in this study. This model has proven to be a useful system in which to study the effects of one tumor-promoting phorbol ester, PDBu. Several laboratories, including ours, have reported that PDBu, although having no effect on the permeabilized smooth muscle strips in the presence of subthreshold calcium concentrations (below 100 nM), potentiates the contractions induced by higher, but submaximal, concentrations of calcium (200–800 nM) (3, 4). Thus, in these systems, PDBu, and presumably activated PKC, increases the sensitivity of the contractile apparatus to calcium.

There is widespread use of phorbol esters to induce the activation of PKC in intact cells. Phorbol esters are thought to act by substituting for the presumed endogenous PKC activator, DAG (5). However, it is difficult to accept the assumption that the active phorbol esters interact solely with PKC. Indeed, there are reports that indicate that phorbol esters and DAG do not always have the same effect in a given cell (e.g., Ref. 6). In addition, PKC is probably not the only target of DAG within the cell. For example, it has been recently reported that both DAG and phorbol esters cause the translocation and activation of cytidylyltransferase in GH3 cells, apparently via a PKC-independent mechanism (7). Because it has been proposed that PKC may be involved in the sustained contraction of smooth muscle (8), it is important to determine whether phorbol esters are acting in whole, in part, or at all through PKC activation to induce smooth muscle contraction.

Several compounds (e.g., H-7, polymyxin B, staurosporine, and sphingosine) have been reported to be inhibitors of PKC activity (9–12). Interpretation of data based on the use of many of these inhibitors is complicated by the relatively low selectivity of these compounds for PKC. One possibility for highly selective inhibition of PKC arises from the report of House and Kemp (13), in which they describe an autoinhibitory domain found within the PKC sequence. Synthetic analogues of this sequence are potent inhibitors of PKC activity *in vitro* and have been used in attempts to delineate the role of PKC in various cell systems (e.g., Refs. 14 and 15). It has been pointed

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ABBREVIATIONS: PKC, protein kinase C; PDBu, phorbol-12,13-dibutyrate; DAG, diacylglycerol; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; MLCK, myosin light chain kinase; PKC-I, RFARKGALRQKNVY; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; C-fragment, *N*-bromosuccinimide-generated carboxyl terminal fragment of histone III-S.

out, however, that many protein kinases share some of the same determinants for substrate recognition (16). Indeed, the PKC "pseudosubstrate" peptides do, at higher concentrations, inhibit MLCK and the calcium/calmodulin-dependent protein kinase II activities in *in vitro* assays (16). Nevertheless, at lower concentrations, these peptides appear to be some of the most selective inhibitors available. We have used a synthetic peptide based on residues 19–31 of PKC (PKC-I) in an attempt to determine whether the potentiation by PDBu of calcium-induced contractions in detergent-permeabilized artery strips is due to activation of PKC.

Materials and Methods

PDBu was obtained from Sigma and dissolved in dimethyl sulfoxide (Fisher) to give a stock solution of 4 mM. PKC-I was synthesized by Peninsula Laboratories, Inc. The P-81 ion exchange chromatography paper used in the PKC assay was from Whatman, and Universol from ICN Biomedicals was used as scintillant. [γ-^{32}P]ATP (3000 Ci/mmol) was from New England Nuclear. Phosphatidylserine and DAG were from Avanti Polar Lipids, Inc. Biogel P-2 and hydroxylapatite were both from Bio-Rad. Triton X-100 was purchased from Research Products, International. Leupeptin was obtained from Peptides International. All other reagents were purchased from Sigma and Fisher.

Isolation of PKC. The media layer (19 g) was removed from the right circumflex coronary artery of approximately 100 porcine hearts and was frozen in liquid nitrogen. The frozen smooth muscle was reduced to powder by percussion in a stainless steel mortar (17) and then homogenized on ice in buffer A (10 mM Tris, 10 mM EGTA, 5 mM EDTA, 2 μg/ml aprotinin, 4 μg/ml leupeptin, 4 μg/ml soybean trypsin inhibitor, 1 mM dithiothreitol, pH 7.5). After separation of the soluble fraction by centrifugation at 40,000 × *g* for 20 min, the pellet was suspended in buffer B (buffer A containing 0.3% Triton X-100) and incubated on ice, with occasional resuspension, for 30 min. After centrifugation as before, the two supernatant fractions were pooled and loaded on a DEAE-cellulose column (2.1 × 23 cm). The column was washed with buffer C (20 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, pH 7.5) until the A_{280} of the effluent was less than 0.10 (approximately 400 ml), and then PKC was eluted with a linear (500-ml) NaCl gradient (0–400 mM) in buffer C. The fractions containing PKC activity were pooled (50 ml), concentrated by reverse dialysis in polyethylene glycol 8000 to about 34 ml, and then dialyzed against buffer C to remove polyethylene glycol and salts. After centrifugation at 40,000 × *g* for 10 min, the supernatant fraction was loaded on a hydroxylapatite column (0.6 × 0.9 cm). The column was washed with 40 mM KH_2PO_4, 2 mM EDTA (pH 7.0), until the A_{280} of the eluent was less than 0.10 (approximately 60 ml). PKC activity was eluted with a linear gradient (400 ml) of 40–300 mM KH_2PO_4 in 2 mM EDTA (pH 7.0). Each peak of PKC activity was pooled and stored at −70° in 17% glycerol, 0.06% Triton X-100.

PKC assay. PKC activity was assayed in a reaction mixture containing 25 mM Tris, pH 7.5, 10 mM magnesium acetate, 0.35 mM $CaCl_2$, 0.83 mg/ml bovine serum albumin, 40 μg/ml phosphatidylserine, 5 μg/ml DAG, 80 μM ATP, 1,500,000 cpm of [γ-^{32}P]ATP, 0.3 mg/ml C-fragment (18), and appropriate concentrations of PKC-I, in a total volume of 60 μl. PKC activity was defined as the activity observed in the presence of phosphatidylserine and DAG minus the activity obtained in the absence of these two lipids. The C-fragment was prepared according to the procedure reported by Sherod *et al.* (19). Phosphatidylserine and DAG were prepared together by evaporation of the organic solvents under nitrogen and sonication in 1 mM Tris (pH 7.0). The kinase reactions were initiated by the addition of 7.5 μl of appropriately diluted enzyme. The assay tubes were incubated for 4 min at 30°, and the reaction was terminated by application of 40 μl of the reaction mixture to phosphocellulose paper (4 × 1.5 cm). The papers were washed for a total of 15 min in several changes of 75 mM phosphoric acid and finally for 5 min in absolute ethanol. The papers were dried, and the radioactivity was measured in approximately 5 ml of scintillant. Product accumulation was linear with reaction time to at least 4 min and, in the presence of added bovine serum albumin, was linear with added enzyme protein.

PKC-I purification. PKC-I was obtained from Peninsula Laboratories, Inc. Before use in any experiment, PKC-I was desalted on a Biogel P2 column (0.8 × 13 cm), using 50 mM ammonium formate (pH 9.0). The fractions were pooled and lyophilized to remove the ammonium formate. In all experiments reported here, lyophilized 50 mM ammonium formate was added to control tissues. The lyophilized ammonium formate was shown to have no effect on the calcium concentration responses of the tissues.

Artery strip preparations. The preparation of detergent-permeabilized media layers of coronary arteries has been described (4). Briefly, porcine coronary arteries were cut open longitudinally and gently rubbed with cotton gauze to remove the endothelium. The media layer was then dissected from the adventitia. Transverse strips of the isolated media layer were mounted in a tissue bath (1.5 ml) between a stationary hook and a force transducer. The strips were permeabilized by incubation with a buffer containing 20 mM imidazole, 50 mM potassium acetate, 5 mM EGTA, 50 mM sucrose, 0.5% Triton X-100, 0.5 mM dithiothreitol, and 0.5 mM leupeptin (pH 7.0), for 60 min at room temperature. The strips were washed once with a buffer containing 20 mM imidazole, 50 mM potassium acetate, 5 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM leupeptin, 10 mM magnesium acetate, 6 mM ATP, and 6 mM KH_2PO_4 (pH 7.0) and then twice with the same buffer containing 0.5 mM EGTA. Once the tissues achieved a stable baseline force, they were contracted by changes in the free calcium concentrations. Free calcium concentrations were calculated using a BASIC translation of the computer program of Perrin and Sayce (20). The stability constants, corrected for pH, were given by Sillen and Martell (21). Statistical analyses were performed using the Student's *t* test.

Results

Isolation of PKC from coronary arteries. The PKC activity was extracted from the media layer of porcine coronary arteries by using detergents and was isolated by DEAE-cellulose chromatography, essentially as described by Woodgett and Hunter (22). The enzyme eluted as a single peak between 62 mM and 95 mM NaCl. Others have reported and we have observed in this system that recovery of PKC activity from DEAE chromatography is greater than 100% (23). In our hands, the crude detergent extract from approximately 20 g of coronary artery media layer contained 18.4 nmol/min of phospholipid-dependent activity. After DEAE-cellulose chromatography, the pooled fractions contained 41.7 nmol/min of activity, a 226% recovery.

Hydroxylapatite chromatography resolved two peaks of phospholipid-dependent activity from the dialyzed fraction. One peak, which eluted at 104 mM KH_2PO_4 (peak II), contained the bulk of the recovered activity (5.7 nmol/min). Although several smaller apparent peaks were observed, they were not characterized further, due to their low kinase activity. Total recovery from the hydroxylapatite column was only 18%.

Kinetic analysis of inhibition by PKC-I. Inhibition of phospholipid-dependent activity of the two major peaks resolved by hydroxylapatite chromatography was studied with respect to varying C-fragment concentrations, under assay conditions in which maximal phospholipid-dependent activity was observed. Although these studies were done to determine appropriate PKC-I concentrations for use in the tissue studies, we also noted that PKC-I did not appear to inhibit either hydroxylapatite fraction in a competitive manner with respect to the C-fragment as substrate. These data are in contrast to

those reported by House and Kemp (13). Several differences in experimental procedures could explain the apparent discrepancy, including the use of different substrates, slightly different peptides, and different enzyme sources. The inhibition in our system appeared to be noncompetitive. Apparent K_i values were calculated from the slope replot of the Dixon plot (24) and were found to be 4.9 μM for peak I and 8.8 μM for peak II (data not shown).

Effect of PKC-I on detergent-permeabilized coronary arteries. A synthetic peptide similar to PKC-I (PKC 19–36) can, depending on the substrate used, inhibit MLCK (16). Because MLCK is thought to be responsible for the calcium-induced contractions in permeabilized smooth muscle, we tested the effects of PKC-I (30 μM) on the calcium concentration-response curve in the absence of PDBu. Fig. 1 shows that PKC-I alone had no effect on the calcium-induced contractions. These data indicate that, under these conditions, PKC-I does not inhibit MLCK and that PKC may not be involved in the calcium-induced contractions.

In the presence of 1 μM PDBu, permeabilized coronary arteries contracted with a greater force in the presence of increasing calcium concentrations, as compared with tissues treated identically in the absence of PDBu (Fig. 2), as has been reported previously (4). Treatment of the tissues with 30 μM PKC-I and 1 μM PDBu resulted in a complete inhibition of the PDBu potentiation (Fig. 2). The inhibition was dose dependent, inasmuch as a lower concentration of PKC-I (10 μM) resulted in approximately 60% inhibition of the PDBu potentiation at pCa 6.7 (data not shown).

We were concerned that PKC-I might in some way be interacting with PDBu extracellularly, thereby affecting the ability of the phorbol ester to exert its actions on the smooth muscle cells. We, therefore, tested the effect of PKC-I on PDBu-induced contractions of coronary artery media that had not been subjected to detergent. In this experiment, PKC-I should not have access to the interior of the cells, and, therefore, any extracellular inactivation of PDBu would result in a reduction of PDBu-induced contractions. Incubation of the intact tissues with 1 μM PDBu alone resulted in a maximal contraction that was 107 ± 16% that of the maximum contraction elicited by 144 mM KCl. When 10 μM PKC-I was included in the buffer, the maximal contraction was 111 ± 17% that of the KCl controls, indicating that PKC-I had no effect on the ability of PDBu to induce contraction in an intact media layer (data not shown).

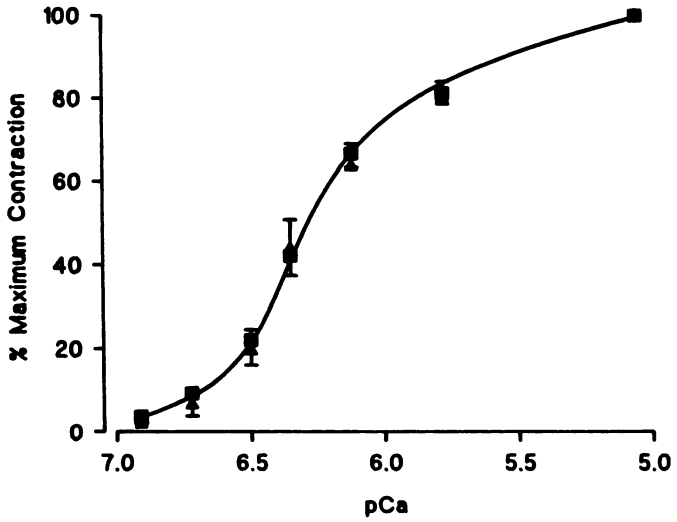

Fig. 1. Effect of 30 μM PKC-I on calcium-induced contractions of permeabilized coronary artery media layer. Tissues in low calcium buffer (pCa > 8) were treated with either 30 μM PKC-I (▲) or lyophilized ammonium formate as a control (■). The tissues were then caused to contract by the addition of $CaCl_2$ to give increasingly higher free calcium concentrations. Error bars indicate standard errors, where $n = 8$ for each curve. Contractions are expressed as a percentage of the maximum force generated at pCa 5.06. There was no statistically significant difference in the force generated at any calcium concentration.

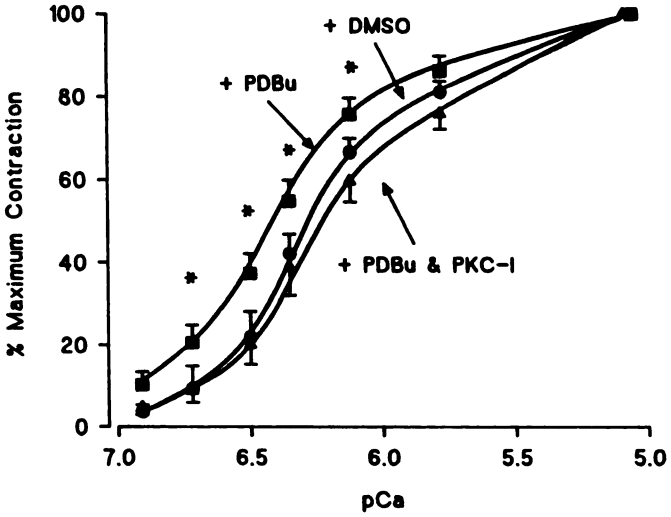

Fig. 2. Effect of 30 μM PKC-I on the PDBu potentiation of calcium-induced contractions. Tissues in low calcium buffer (pCa > 8) were treated for 10 min with 1 μM PDBu and either 30 μM PKC-I (▲) or lyophilized ammonium formate (■). Control tissues (●) were treated with 0.1% dimethyl sulfoxide ($n = 7$). The tissues were then exposed to progressively higher free calcium concentrations by addition of $CaCl_2$. There was no statistically significant difference between the contractions generated at high calcium (pCa 5.06). The mean values ($n = 8$) are expressed as a percentage of the contraction obtained at pCa 5.06. *, Statistically significant difference between contractions in the presence of PDBu alone and contractions in the presence of PDBu plus PKC-I or in the presence of dimethyl sulfoxide alone at a given pCa ($p < 0.05$).

Discussion

The synthetic peptide we have used is based on residues 19–31 of PKC, with a tyrosine introduced on the carboxyl terminal for future radiolabeling purposes. Because of this alteration, it was necessary to establish that this peptide (PKC-I) retained its ability to inhibit PKC activity. We chose to study the effects of PKC-I on PKC fractions from coronary arteries that were isolated by hydroxylapatite chromatography, to determine whether PKC-I had any different effects on the two fractions. Although the two peaks of PKC studied were not rigorously identified, comparison of the elution profile with those reported by others indicates that the two peaks may represent the α and β forms of the enzyme (25). Because the total recovered PKC activity represented only about 20% of the activity applied to the hydroxylapatite column, we cannot rule out the possibility that other forms of PKC exist in the smooth muscle cells.

Smith *et al.* (16) have reported that a peptide similar to PKC-I inhibits calcium/calmodulin-dependent protein kinase II and MLCK activities *in vitro*, in the presence of appropriate peptide substrates, with IC_{50} values of 30 and 35 μM, respectively. In order to completely inhibit the PDBu potentiation in the pres-

ent study, it was necessary to use 30 μM PKC-I, which could have been a high enough concentration to inhibit MLCK activity. If PKC-I did inhibit MLCK in our system, there should have been an effect on the calcium concentration-response curve, because MLCK activation is presumed to be the mechanism by which calcium induces contractions in permeabilized arteries. The lack of effect of PKC-I on calcium-induced contractions indicates that, at this concentration in this system, PKC-I may be relatively selective for PKC. Furthermore, these data imply that the PKC activation is not a component of the mechanism of calcium-induced contractions in permeabilized coronary arteries.

A concentration of PKC-I (10 μM) that approximates the apparent K_i value resulted in partial inhibition of the PDBu potentiation of calcium-induced contractions, and complete inhibition was observed with 30 μM PKC-I. A previous report from this laboratory has indicated that polymyxin B can also inhibit PDBu potentiation (4). It is thought that polymyxin B inhibits PKC by a different mechanism than does PKC-I (10). Because the PDBu effect on permeabilized arteries can be inhibited by two compounds that inhibit PKC by different mechanisms and because the inhibition by PKC-I is dose dependent, it seems highly probable that PDBu potentiates calcium-induced contractions solely through PKC activation.

Caution must be used in translating the role of PKC in permeabilized tissues to that in intact arteries. However, these data do lend some assurance that activation of PKC may be responsible for the PDBu-induced contractions of intact tissues. To determine more directly the role of PKC in intact tissues, we are currently investigating methods of introducing PKC-I into the cells that do not involve irreversible permeabilization.

In summary, we have shown that a synthetic peptide based on a pseudosubstrate domain of the PKC enzyme inhibits PKC activity isolated from vascular smooth muscle and that this peptide inhibits PDBu-induced potentiation of calcium-induced contractions in permeabilized smooth muscle strips without altering the calcium-induced contractions in the absence of PDBu.

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