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Calmodulin inhibits the protein kinase C-catalysed phosphorylation of an endogenous protein in A10 smooth-muscle cells

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The protein kinase C (PKC) activator phorbol 12,13-dibutyrate stimulated the phosphorylation of a 75 kDa protein (p75) in intact cultured A10 smooth-muscle cells and sonicated cell preparations; p75 was the only major substrate for endogenous PKC in sonicated A10 cells. The Ca^{2+}-dependent phosphorylation of p75 *in vitro* was dramatically decreased in PKC-down-regulated A10 cells; however, p75 from identical sonicated cell preparations was still phosphorylated by an exogenous aortic PKC preparation. Calmodulin inhibited the phosphorylation of p75 by PKC, but not the phosphorylation of other PKC substrates (platelet P47 protein and histone). The addition of calmodulin after the phosphorylation reaction was started prevented further phosphorylation, but did not decrease the extent of phosphorylation of p75 that was reached before the addition of calmodulin. The inhibition of p75 phosphorylation was concentration-dependent, with IC_{50} values (concn. giving 50% inhibition) ranging from less than 0.5 to 10 μg of calmodulin/ml, and was Ca^{2+}-dependent, requiring a free Ca^{2+} concentration of 10 μM or greater. These results suggest that the inhibition of the PKC-catalysed phosphorylation of p75 by calmodulin may be due to its interaction with the substrate, rather than a direct inhibitory effect on the enzyme, and that this inhibition could be regulated by intracellular Ca^{2+} concentration. Therefore, p75 may be a physiological link between the PKC and Ca^{2+}/calmodulin pathways.

INTRODUCTION

The protein kinase C (PKC) and Ca^{2+}/calmodulin pathways are among the most important mechanisms by which cells regulate their functions. In smooth-muscle cells, PKC can either act synergistically with Ca^{2+} or exert negative feedback control over cell responses induced by receptor-mediated Ca^{2+} mobilization [1–5]. However, the molecular basis that underlies the regulation of PKC-mediated substrate phosphorylation by Ca^{2+} has not been elucidated. For instance, although PKC is dependent for its activity on phospholipids and Ca^{2+}, diacylglycerol alone can activate the enzyme without an increase in intracellular Ca^{2+} concentrations [1,2]. Also, little is known about the substrates of PKC *in vivo*. A specific endogenous substrate for PKC, an 80–87 kDa protein referred to as MARCKS, has been identified in a wide range of cells and tissues [6,7], although its presence in vascular smooth-muscle cells has not been documented. This acidic and heat-stable protein [7] was shown to be myristoylated in murine macrophages [8] and rich in alanine [9], and thus has been designated as myristoylated alanine-rich C-kinase substrate (MARCKS). MARCKS is a calmodulin-binding protein, but phosphorylation of MARCKS by PKC decreased calmodulin binding [10]. On the other hand, there are reports that calmodulin can inhibit the phosphorylation of MARCKS by PKC [11], although it was not clear whether this inhibition was due to the inhibition of PKC directly, or, as might be predicted from the calmodulin-binding data [10], due to the direct interaction between calmodulin and MARCKS. We report here that in A10 smooth-muscle cells calmodulin specifically inhibited the PKC-catalysed phosphorylation of a 75 kDa protein (p75) which shares several unique properties with MARCKS. The characteristics of the inhibition suggest that the inhibition may be due to the direct interaction between calmodulin and p75, and that the inhibition may be of physiological significance.

MATERIALS AND METHODS

Materials

Calmodulin from bovine brain was generously provided by Dr. R. Sharma (Department of Medical Biochemistry, University of Calgary), purified as described in [12]. [γ-^{32}P]ATP (4500 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA, U.S.A.). [^{32}P]P_i was from Amersham Canada Ltd. (Oakville, Ont., Canada). A10 smooth-muscle cells derived from rat thoracic aorta were obtained from the American Type Culture Collection, Rockville, MD, U.S.A. (CRL 1476). Culture medium, fetal-calf serum, antibiotics and tissue-culture vessels (Nunc) were from Gibco (Burlington, Ont., Canada). Phosphatidylserine (PS; bovine brain) and 1,2-dioctanoylglycerol were from Serdary Research Laboratories (London, Ont., Canada). Histone (III-S), phenylmethanesulphonyl fluoride, pepstatin A, leupeptin, phorbol 12,13-dibutyrate (PDBu) and soybean trypsin inhibitor were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PKC (19–36) inhibitory peptide (PKC_{19-36}) was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.). The dye reagent for protein determination was purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.). General laboratory reagents were of analytical grade or better.

Purification of aortic PKC, platelet P47 protein and rat brain MARCKS protein

PKC was partially purified from bovine aorta by chromatography on DEAE-Sephacel and phenyl-Sepharose, as previously

Abbreviations used: PS, phosphatidylserine; PDBu, phorbol 12,13-dibutyrate; p75, 75000 Da protein; PKC, Ca^{2+}- and phospholipid-dependent protein kinase C ('C-kinase'); P47, 47000 Da platelet protein; MARCKS, myristoylated alanine-rich C-kinase substrate; PKC_{19-36}, protein kinase C (19–36) inhibitory peptide.

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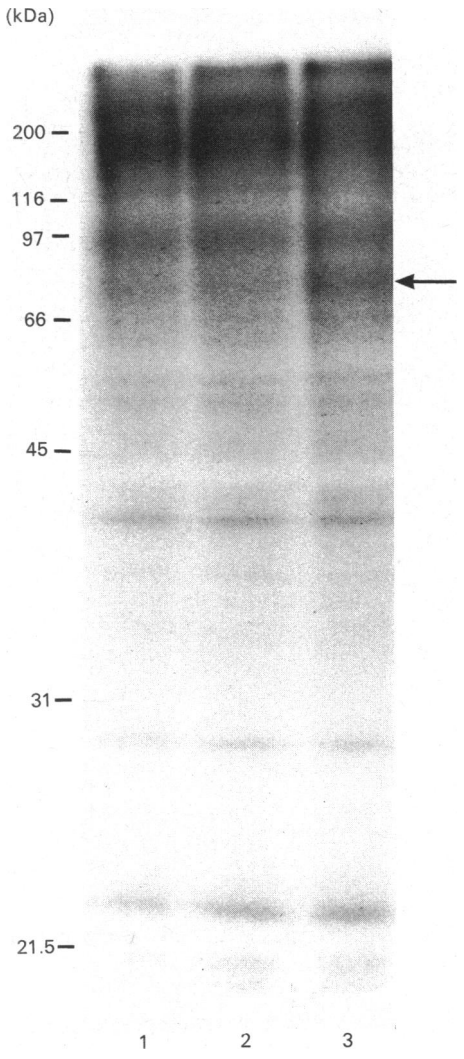

Fig. 1. Phosphorylation in intact cultured A10 cells

Cells were equilibrated with [^{32}P]P_i as described in the Materials and methods section. Lane 1, control; lanes 2 and 3, cells were treated for 1 min at 37 °C with either solvent alone (dimethyl sulphoxide, lane 2) or 1 μM-PDBu (lane 3). Monolayers were then solubilized and analysed by SDS/PAGE as outlined in the Materials and methods section. The positions of molecular-mass (kDa) marker proteins are shown on the left; the position of p75 is indicated by the arrow.

described [13,14]. This preparation was devoid of any other protein kinase activity. Platelet P47 protein was purified as reported previously [13]. Rat brain MARCKS protein was partially purified by extraction of the brain in 2.5% (w/v) $HClO_4$ and precipitation of MARCKS from the 10000 *g* supernatant with 55% saturation of $(NH_4)_2SO_4$ [15].

A10 cell culture and down-regulation of PKC

A10 cells were cultured as described previously [16]. A10 cells for experimental use were cultured in Dulbecco's modified Eagle's medium containing 25 mM-glucose, 0.01 mM-pyruvate, 2 mM-butyrate, 100 units of penicillin/ml, 100 μg of streptomycin/ml and 5% (v/v) fetal-calf serum, and grown to confluence (7 days; about 50 μg of cell protein/dish). About 18 h before each experiment, the cells were re-fed with the same medium, except that the concentration of fetal-calf serum was decreased to 0.2%. For down-regulation of PKC [16], 1 μM-PDBu was added together with the low-serum medium; an appropriate amount of the dimethyl sulphoxide solvent (final conc. 0.1%) was added to control dishes.

^{32}P labelling of intact A10 cells

The method for this was adapted from methods reported by others [17,18]. A10 cells in low-serum (0.2%) medium were washed twice with minimum essential medium containing 0.25 mM-P_i and 20 mM-Hepes, pH 7.4, and were then labelled by a 2 h incubation with 125 μCi of [^{32}P]P_i/ml. PDBu (1 μM) or dimethyl sulphoxide (0.1%) was then added to the medium; after 1 min, the medium was removed by aspiration. A10 cells were solubilized in SDS electrophoresis sample buffer [19] after being washed three times with ice-cold phosphate-buffered saline (137 mM-NaCl, 2.7 mM-KCl, 0.9 mM-$CaCl_2$, 0.5 mM-$MgCl_2$, 1.5 mM-KH_2PO_4 and 8 mM-Na_2HPO_4, pH 7.4).

Phosphorylation of sonicated A10 cells and brain MARCKS protein by PKC

Cultured A10 cells were washed twice with phosphate-buffered saline, scraped into homogenizing buffer (0.25 M-sucrose, 0.1 mM-EDTA, 1 mM-dithiothreitol, 20 mM-Tris/HCl, pH 7.5, 0.5 mM-phenylmethanesulphonyl fluoride and 1 mg each of leupeptin, pepstatin A and soybean trypsin inhibitor/l), and homogenized by sonication (4 × 30 s; 75 W, BraunSonic 1510 sonicator). The protein concentration of the sonicated cells was adjusted to either 0.5 or 2 mg/ml.

Phosphorylation of the sonicated A10 cells by PKC was performed by two methods. In one method, the sonicated cells were first boiled for 10 min to inactivate all endogenous protein kinases, phosphatases and ATPases, and then were subjected to phosphorylation by purified aortic PKC [13]. The reaction medium contained 1 μg of PKC, 10 or 125 μg of heat-treated cell protein, 20 mM-Pipes/HCl (pH 6.5), 5 mM-$MgCl_2$, 0.5 mM-$CaCl_2$, 10 μM-[γ-^{32}P]ATP (4 Ci/mmol) and PS (40 μg/ml) in a total volume of 125 μl. The reaction was started by adding [γ-^{32}P]ATP, and was stopped by adding 125 μl of SDS electrophoresis sample buffer after incubation for 15 min at 30 °C. The samples were boiled for 5 min before being subjected to SDS/12%-polyacrylamide-gel electrophoresis [19].

In the second method, the sonicated cells were not heat-treated; the incubation medium was same as above, with the omission of purified aortic PKC. When PDBu was used to stimulate endogenous PKC activity, 1 μM-PDBu and 1 mM-EGTA were added, and $CaCl_2$ and phosphatidylserine were omitted. Protein phosphatase inhibitors (10 mM- or 100 mM-β-glycerophosphate, 10 mM-NaF, 7 mg of *p*-nitrophenyl phosphate/ml and 50 μM-Na_3VO_4) were added to some experiments, without obvious differences in the observed phosphorylation levels. Routinely, the reaction was stopped by adding SDS electrophoresis sample buffer, but in some experiments the reaction was stopped by boiling the reaction medium for 5 min. The medium was then centrifuged at 16000 *g* for 15 min in an Eppendorf centrifuge, and the supernatant was subjected to SDS/PAGE.

Conditions for phosphorylation of brain MARCKS (10 μg/ml) were same as those for phosphorylation of boiled sonicated A10-cell preparations.

Other methods

Autoradiography was done with Kodak X-Omat AR diagnostic film in Kodak X-Omatic cassettes with intensifying screens. In some experiments, autoradiograms were scanned on a LKB 2202 Ultroscan laser densitometer, and the areas under the p75 phosphoprotein peak were calculated. Alternatively, the areas of gels corresponding to the p75 phosphoprotein band on

autoradiograms were cut out, and the radioactivity was measured by liquid-scintillation counting. Radioactivity in a portion of gel with same area excised immediately above the cut band was also measured as a control. Protein concentrations in sonicated A10 cells were determined by the Coomassie Blue spectrophotometric assay [20], with BSA as a standard.

RESULTS

In intact A10 cells, a short-term (1 min) incubation with PDBu, a PKC activator [1], stimulated the phosphorylation of only one prominent protein band (Fig. 1; location indicated by the arrow). The apparent molecular mass of this protein was 75 ± 2 kDa (mean $\pm$ S.E.M. of 5 determinations); consequently this protein is designated as the 75 kDa protein (p75) in this paper. Rat brain MARCKS protein was electrophoretically distinct from p75, which migrated faster than MARCKS under identical conditions; MARCKS exhibited an apparent molecular mass of 83.5 ± 0.5 kDa ($n = 4$) in our gel system (Fig. 2, lane 3). The phosphoprotein (p75) band always appeared broad and fuzzy (Fig. 1, lane 3). 1,2-Dioctanoylglycerol, a cell-permeant diacylglycerol analogue that also activates PKC in intact cells [1], also induced the phosphorylation of same 75 kDa protein band (results not shown).

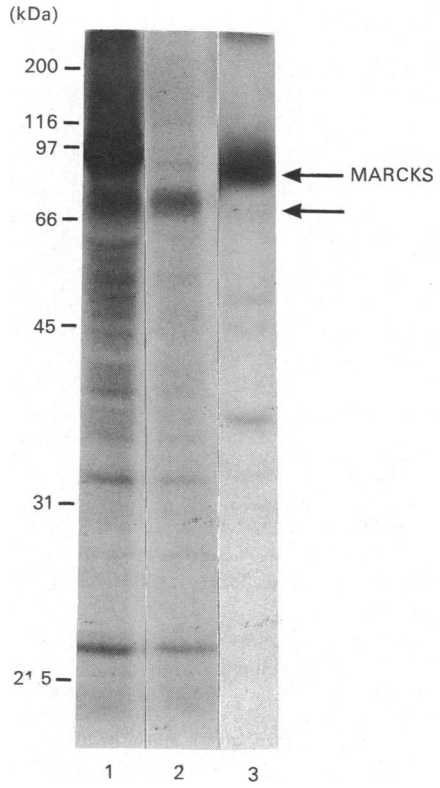

Fig. 2. Phosphorylation of sonicated A10-cell preparations and rat brain MARCKS

Sonicated A10 cells were phosphorylated by endogenous PKC in the presence of 1 mM-EGTA plus 1 μM-PDBu (lanes 1 and 2). The phosphorylation reaction was stopped by boiling the samples for 5 min. SDS electrophoresis sample buffer was then added either to the samples without prior centrifugation (lane 1) or to the supernatant after centrifuging the samples at 16000 *g* for 15 min at room temperature (lane 2). The position of p75 is indicated by the arrow; positions of molecular-mass (kDa) marker proteins are shown on the left. In lane 3, the partially purified rat brain MARCKS protein (10 μg/ml) was phosphorylated by purified aortic PKC, as described in the Materials and methods section.

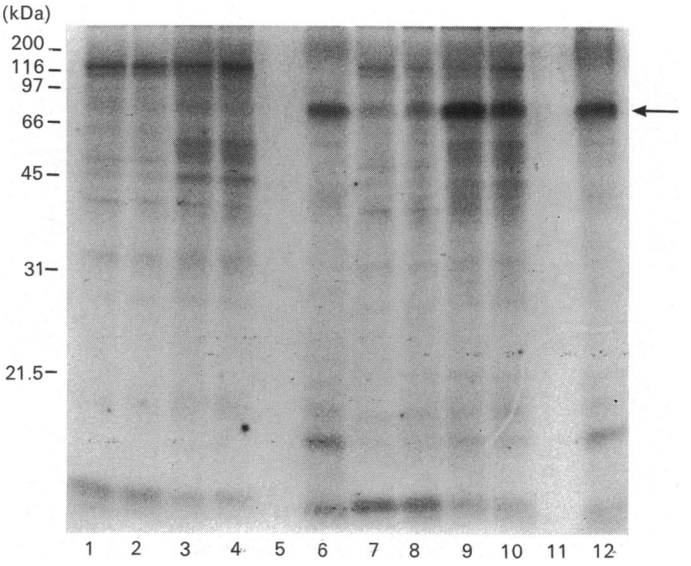

Fig. 3. Phosphorylation of sonicated cell preparations from control and PKC-down-regulated A10 cells

A10 cells were preincubated with either 1 μM-PDBu (PKC-down-regulated cells; lanes 1–6) or solvent alone (dimethyl sulphoxide, control cells; lanes 7–12) for 18 h. The cells were then sonicated and phosphorylated under the following conditions: lanes 1 and 7, phosphorylation in the presence of 1 mM-EGTA; lanes 2 and 8, 1 mM-EGTA plus 1 μM-PDBu; lanes 3 and 9, 0.5 mM-Ca^{2+}; lanes 4 and 10, 0.5 mM-Ca^{2+} plus PS (40 μg/ml). Some sonicated A10 cells (samples 5, 6, 11 and 12) were boiled as described in the Materials and methods section before phosphorylation under the following conditions: lanes 5 and 11, 0.5 mM-Ca^{2+} plus 40 μg of PS/ml; lanes 6 and 12, 0.5 mM-Ca^{2+}, 40 μg of PS/ml and 1 μg of purified PKC. The position of p75 is indicated by the arrow. The positions of molecular-mass (kDa) marker proteins are shown on the left.

In sonicated A10 cells, p75 was also the only major protein for which phosphorylation was clearly increased by the addition of PDBu in the presence of EGTA (Fig. 3, lane 8), presumably owing to the action of endogenous PKC. Addition of Ca^{2+} (Fig. 3, lane 9) strikingly increased the phosphorylation of p75. Addition of PS did not increase further the Ca^{2+}-dependent phosphorylation of p75 (Fig. 3; cf. lanes 9 and 10), perhaps because there was enough phospholipid in the sonicated cell preparation to support PKC activity. Phosphorylated p75 was found in the supernatant when the phosphorylated sonicated cells were boiled and centrifuged before adding SDS sample buffer (Fig. 2, lanes 1 and 2), indicating that this protein was heat-stable [7]. In intact A10 cells wherein PKC was down-regulated, PDBu was no longer able to stimulate p75 phosphorylation (results not shown), nor was there a stimulation of p75 phosphorylation in sonicated cells by either PDBu or Ca^{2+} (Fig. 3, lanes 2 and 3). When sonicated cells were heat-treated, the phosphorylation of p75 became completely dependent on the addition of exogenous PKC (Fig. 3, lanes 11 and 12). In heat-treated sonicated preparations of PKC-down-regulated A10 cells, exogenous PKC still phosphorylated p75 (Fig. 3, lane 6) to a level comparable with that of p75 where PKC was added to heat-treated sonicated control A10 cells (Fig. 3, lane 12). Furthermore, the peptide PKC_{19-36} (100 μg/ml), a specific PKC inhibitor [21], completely blocked the phosphorylation of p75 induced by PDBu or Ca^{2+} in sonicated cells (results not shown).

Calmodulin (50 μg/ml) blocked the Ca^{2+}-stimulated phosphorylation of p75 by endogenous PKC (Fig. 4, cf. lanes 3 and 6), but, unlike PKC_{19-36}, had no effect on PDBu-induced phosphorylation of p75 in the absence of Ca^{2+} (Fig. 4, cf. lanes

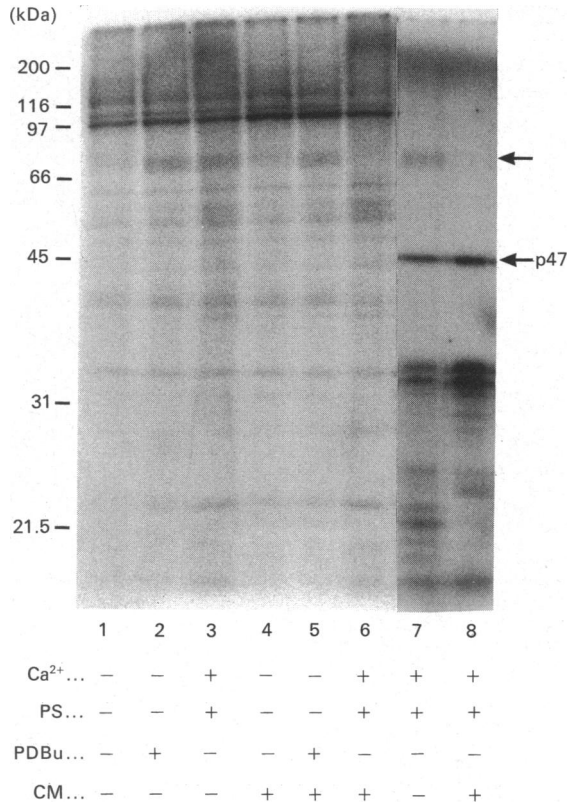

Fig. 4. Inhibition of p75 phosphorylation in sonicated A10-cell preparations by calmodulin

Sonicated A10-cell preparations either were untreated (lanes 1–6) or were boiled (lanes 7 and 8) before phosphorylation. Phosphorylation was performed as described in the Materials and methods section, under the following conditions: lanes 1 and 4, 1 mM-EGTA; lanes 2 and 5, 1 mM-EGTA plus 1 μM-PDBu; lanes 3 and 6, 0.5 mM-Ca^{2+} plus PS (40 μg/ml); lanes 7 and 8, 0.5 mM-Ca^{2+}, PS (40 μg/ml) and 1 μg of purified PKC. In addition, calmodulin (CM; 50 μg/ml) was added to lanes 4–6 and 8. A10-cell protein concentration was 80 μg/ml. In lanes 7 and 8, boiled sonicated A10 cells were supplemented by addition of 150 μg of platelet P47 protein/ml and 80 μg of histone III-S/ml (the phosphoprotein bands of 32 kDa, 33 kDa and lower). The positions of P47 and p75 are indicated by the arrows; the positions of molecular-mass (kDa) marker proteins are shown on the left.

2 and 5). Calmodulin also inhibited the phosphorylation of p75 in heat-treated sonicated A10 cells by exogenous PKC in the presence of Ca^{2+} (Fig. 4, lanes 7 and 8). However, phosphorylation of other PKC substrates, platelet P47 protein and histone, which were added to the sonicated cell preparation before the phosphorylation reaction, was not significantly inhibited by calmodulin.

Phosphorylation of p75 in sonicated A10 cells by endogenous PKC was time-dependent during 15 min of incubation. The addition of calmodulin at zero time almost totally inhibited the phosphorylation of p75 in the subsequent 15 min incubation. The addition of calmodulin after 5 or 10 min of incubation, followed by continuation of the incubation for a total of 15 min, resulted in phosphorylation of p75 to levels that were equivalent to the extent of phosphorylation observed after incubation for 5 or 10 min respectively (Fig. 5).

At a low A10-cell protein concentration (80 μg of protein/ml), calmodulin completely inhibited p75 phosphorylation at a calmodulin concentration of 5 μg/ml, with an IC_{50} (concn. giving 50% inhibition) of less than 0.5 μg/ml. When the A10-cell

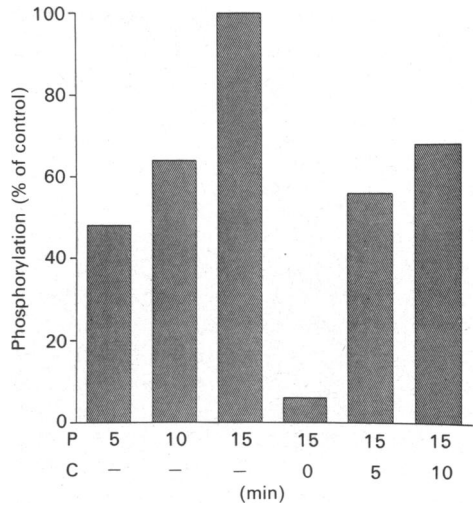

Fig. 5. Effect of calmodulin on the time course for the phosphorylation of p75 by PKC and its inhibition by calmodulin

Sonicated A10-cell preparations (80 μg of cell protein/ml) were phosphorylated by endogenous PKC during incubations with 0.5 mM-Ca^{2+} plus PS (40 μg/ml). Line P indicates the total time (min) of phosphorylation. Autoradiograms were scanned on a densitometer, and the area of the p75 phosphoprotein peak was calculated; the phosphorylation was expressed as a percentage of the 15 min value (100%). Calmodulin (5 μg/ml) was omitted (–), or was added at zero time (0) or after the phosphorylation incubation had proceeded for 5 or 10 min (line C).

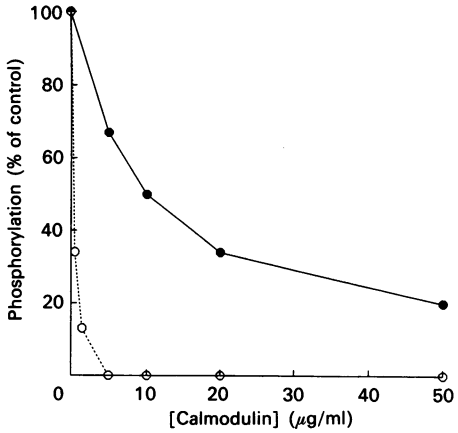

Fig. 6. Concentration-dependence for the inhibition of p75 phosphorylation by calmodulin

Sonicated A10-cell preparations containing either 80 μg of cell protein/ml (○) or 1 mg of cell protein/ml (●) were phosphorylated with endogenous PKC by incubation with 0.5 mM-Ca^{2+} plus PS (40 μg/ml) and the indicated concentrations of calmodulin. The autoradiograms were scanned with a densitometer. The integrated peak areas representing the p75 phosphoprotein were determined and expressed as a percentage of the peak area observed in the same area of the gel in the absence of calmodulin (control).

protein concentration was increased to 1 mg/ml, calmodulin inhibited the phosphorylation of p75 by about 80% at 50 μg/ml, with an IC_{50} of 10 μg/ml (Fig. 6). Unlike brain tissue, the endogenous calmodulin content in A10 cells was quite low, less than 10% of the minimum concentration (0.5 μg/ml) of exogenous calmodulin added to the phosphorylation reaction, as judged by the Coomassie Blue staining of electrophoresis gels of sonicated cells compared to added calmodulin.

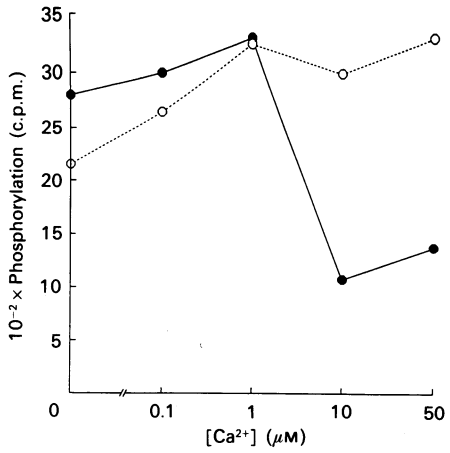

Fig. 7. Ca^{2+}-dependence for the inhibition of p75 phosphorylation by calmodulin

Sonicated A10-cell preparations were phosphorylated with endogenous PKC by incubation with 1 μM-PDBu in the absence (○) or presence (●) of 50 μg of calmodulin/ml and at the indicated free Ca^{2+} concentrations, obtained with the use of Ca^{2+}–EGTA buffers [13]. The portions of the gels corresponding to the 75 kDa phosphoprotein band on the autoradiograms were excised, and radioactivity was measured by scintillation counting. Background was subtracted by measuring radioactivity in an identical gel sample excised immediately above the phosphorylated p75 band. The A10-cell protein concentration was 1 mg/ml.

The PDBu-induced phosphorylation of p75 in sonicated A10 cells could be observed in the absence of Ca^{2+} (EGTA buffer), and was only modestly stimulated by the addition of Ca^{2+} (Fig. 7). Calmodulin did not inhibit p75 phosphorylation in the absence of Ca^{2+}, or at Ca^{2+} concentrations of 0.1 and 1 μM; full inhibitory effects were observed at 10 μM Ca^{2+} concentration (Fig. 7).

DISCUSSION

The following reasons indicate that the phosphorylation both *in vitro* (sonicated cells preparations) and in intact cells of a 75 kDa protein (p75) in cultured A10 cells was catalysed by PKC. (i) The PKC activator PDBu stimulated phosphorylation of p75 in intact A10 cells (Fig. 1). (ii) Ca^{2+}, or PDBu in the absence of Ca^{2+}, stimulated the phosphorylation of p75 in sonicated cells (Fig. 3). (iii) The phosphorylation of p75 induced by PDBu or Ca^{2+} in sonicated cells was eliminated (Fig. 3) if endogenous PKC was down-regulated [16]. (iv) Purified exogenous PKC phosphorylated p75 in heat-treated sonicated cells from both control and PKC down-regulated cells (Fig. 3).

The p75 protein, the only prominent PKC substrate in A10 cells, was heat-stable and migrated as a broad band at a variable rate in SDS electrophoresis gels. These unique properties of p75 in A10 cells are similar to those of MARCKS (80–87 kDa protein) identified in other tissues [6,7,9,10]. To our knowledge, this is the first time that a MARCKS-like protein has been detected in vascular smooth-muscle tissue; further work appears warranted to determine the precise structure of p75 and its relationship to MARCKS.

Calmodulin was a potent inhibitor of the phosphorylation of p75 by both endogenous and exogenous PKC (Fig. 4). The calmodulin inhibition of p75 phosphorylation could not be due to a calmodulin-dependent protein phosphatase, since neither heat-treatment of sonicated A10-cell preparations (Fig. 4) nor phosphatase inhibitors blocked the inhibition of phosphorylation. Furthermore, the time course of phosphorylation of p75 and its inhibition by calmodulin (Fig. 5) showed that calmodulin did not decrease the extent of phosphorylation that was reached before the addition of calmodulin. The phosphorylation of other PKC substrates (platelet P47 protein and histone) was not inhibited by calmodulin (Fig. 4). This specificity for calmodulin inhibition of p75 phosphorylation in sonicated A10 cells suggests that PKC itself was not inhibited directly; rather, the inhibition may be due to an interaction of calmodulin with the p75 substrate. In an earlier paper, calmodulin was reported to inhibit the PKC-catalysed phosphorylation of MARCKS in brain tissue as well as the phosphorylation of other PKC substrates, including histone [11]. This lack of substrate specificity for calmodulin inhibition led these authors to suggest that calmodulin inhibited PKC directly, but the possibility that calmodulin interacted directly with the substrate proteins was not excluded. This discrepancy between previous observations [11] and our own results (Fig. 4) may be due to the different species and tissue origins of PKC and its endogenous substrate. Calmodulin has also been observed to inhibit the PKC-catalysed phosphorylation of another calmodulin-binding protein, calcineurin, but to have no effect on the phosphorylation of a synthetic polypeptide substrate, syntide [22].

The conditions that affected the inhibition by calmodulin of PKC-catalysed phosphorylation of p75 were also investigated. IC_{50} values for calmodulin inhibition of p75 phosphorylation ranged from less than 0.5 μg of calmodulin/ml at a low A10-cell protein concentration to 10 μg/ml at a higher cell protein concentration (Fig. 6), suggesting a high affinity of calmodulin for p75. Experiments with purified p75 from A10 cells will, however, be required to determine the binding affinity for calmodulin and the effect of PKC-catalysed phosphorylation on calmodulin binding to p75. Calmodulin had no inhibitory effect at Ca^{2+} concentrations below 1 μM (Fig. 7), but was fully effective at Ca^{2+} concentrations above 10 μM. These Ca^{2+} concentrations are within the known intracellular changes in Ca^{2+} concentrations during the course of agonist-triggered cell activation, suggesting that this inhibition may be of physiological importance. We therefore suggest that p75 may play a role in the control of smooth-muscle function by providing a link with Ca^{2+}/calmodulin- and PKC-regulated pathways.

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