Volume 238, number 1, 151–155 FEB 06359 September 1988

A major role for protein kinase C in calcium-activated exocytosis in permeabilised adrenal chromaffin cells

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Received 16 August 1988

The role of endogenously activated protein kinase C in calcium-activated exocytosis was examined in digitonin-permeabilised bovine adrenal chromaffin cells. Protein kinase C activity was reduced by down-regulation following long-term treatment with PMA or by using the inhibitor sphingosine. Both treatments resulted in a substantial reduction in catecholamine secretion elicited by micromolar calcium, indicating that endogenous activation of protein kinase C is a major requirement for calcium-activated exocytosis in chromaffin cells.

Exocytosis; Secretion; Chromaffin cell; Protein kinase C; Ca^{2+}; Catecholamine

1. INTRODUCTION

Adrenal chromaffin cells have been widely used in the study of the mechanisms controlling exocytosis. In these cells an elevation of cytosolic free calcium plays a major role in triggering secretion following stimulation with cholinergic agonists and other secretagogues [1,2]. In permeabilised chromaffin cells, elevation of cytosolic calcium to micromolar levels is sufficient to trigger exocytosis [3,4]. The importance of protein kinase C in calcium-activated exocytosis in chromaffin cells is not clear. Protein kinase C is translocated to the plasma membrane following stimulation of intact cells with nicotinic agonists or introduction of calcium into permeabilised cells [5] and this results in increased protein phosphorylation [6]. In permeabilised cells activation of protein kinase C using phorbol esters produces a small potentiation of calcium-activated secretion by increasing the calcium affinity of the process [7–9]. These results suggest a possible modulatory role for protein kinase C in an exocytotic pathway subserved by other calcium-dependent mechanisms. However, these experiments do not directly address the question of the role of endogenous activation of protein kinase C in calcium-activated exocytosis. We have depleted chromaffin cells of protein kinase C activity by using two independent approaches: down-regulation of protein kinase C following long-term exposure to phorbol ester; treatment with the protein kinase C inhibitor sphingosine. The results show that endogenous activation of protein kinase C must play a major role in calcium-activated exocytosis in chromaffin cells.

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2. MATERIALS AND METHODS

Bovine adrenal chromaffin cells were isolated by the method of Greenberg and Zinder [10] except that the initial injections of the glands were with 0.1% protease type XIV (Sigma) instead of collagenase. Chromaffin cells were purified by differential plating [11], seeded at 10^6 per well in 24-well trays and maintained in culture for 2–7 days in a medium consisting of Dulbecco's modified Eagles medium with 25 mM Hepes, 10% fetal calf serum, 8 μM fluorodeoxyuridine, 50 μg/ml gentamycin, 10 μM cytosine arabinoside, 2.5 μg/ml fungizone, 25 U/ml penicillin and 25 μg/ml streptomycin. Phorbol esters and sphingosine were prepared as stock solutions in DMSO.

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00145793/88/$3.50

For down-regulation of protein kinase C, cells were incubated for 24 h at 37°C in the presence of phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12,13-didecanoate (4αP) or an equivalent concentration of DMSO. Sphingosine-treated cells were preincubated with the indicated concentration of sphingosine or with DMSO in culture medium for 2 h at 37°C. Determination of catecholamine secretion and cytosolic free calcium concentration using fura2 was as described previously except that cultured cells were used and gently detached for monitoring cytosolic calcium in cells in suspension [12]. For cell permeabilisation, cells were washed twice in a calcium-free Krebs-Ringer buffer (containing in mM: 145 NaCl, 5 KCl, 1.3 $MgCl_2$, 1.2 NaH_2PO_4, 10 glucose, 20 Hepes, pH 7.4; buffer A) and permeabilised by addition of 20 μM digitonin in 139 mM potassium glutamate, 2 mM ATP, 2 mM $MgCl_2$, 5 mM EGTA, 20 mM Pipes with appropriate amounts of $CaCl_2$ to give the calculated free calcium concentration indicated at pH 6.5. In experiments with spingosine, permeabilisation buffers also contained 3.5 mg/ml fatty-acid-free BSA and the appropriate concentration of sphingosine or DMSO. Catecholamine released after 20 min was determined and expressed as percentage of total catecholamine content. For determination of binding of [^{3}H]phorbol dibutyrate ([^{3}H]PdBu, spec. act. 14.3 Ci/mmol) the cells were washed with Krebs-Ringer buffer containing 3 mM $CaCl_2$ (buffer B) and incubated for 30 min at room temperature with 500 μl per well of buffer B containing 18 nM [^{3}H]PdBu. The cell monolayers were washed once in ice-cold buffer B, left on ice for 5 min, solubilised in 0.1 M NaOH and aliquots taken for scintillation counting and protein determination. Non-specific binding was defined as that in the presence of 2 μM PMA and results shown are levels of specific binding in cpm bound per well.

3. RESULTS

3.1. *Effect of down-regulation of protein kinase C on exocytosis*

In many cell types chronic exposure to active phorbol esters results in down-regulation of protein kinase C with a loss of enzyme activity, and the protein itself as detected by immunoprecipitation and binding of [^{3}H]PdBu [13–15]. We first examined the effect of long-term (24 h) treatment with 250 nM PMA on secretion from intact cells. In these experiments control cells were treated with the phorbol ester 4αP which does not activate protein kinase C. Following treatment with PMA, secretion in response to nicotine and 55 mM K^+ was inhibited by 63 ± 4.7% (n = 4) and 66 ± 2.2% (n = 4), respectively. However, this effect may be partially due to effects on calcium entry, since the rise in cytosolic calcium due to nitocine and 55 mM K^+ was reduced by 85.8 ± 9.3% (n = 5) and 47 ± 10.9% (n = 4), respectively.

In order to examine directly the effects of down-regulation on calcium-activated exocytosis, we made use of digitonin-permeabilised cells [4]. In these cells exocytosis was maximal at 10 μM free calcium and declined at higher levels of free calcium. Therefore, we examined the effects of treatment with phorbol esters on secretion elicited by calcium up to 10 μM. As shown in fig.1, treatment with 250 nM PMA for 24 h resulted in a marked inhibition of calcium-activated secretion from permeabilised cells in comparison with control cells treated with the inactive phorbol ester 4αP. From experiments carried out on 4 separate cell preparations the range of extent of inhibition at 10 μM free calcium was 42–70%.

The concentration dependency of protein kinase C down-regulation detected using binding of [^{3}H]PdBu and the inhibition of exocytosis was compared (fig.2a,b). Treatment with 10 nM PMA was sufficient to produce a substantial down-regulation of protein kinase C as shown by the loss of binding of [^{3}H]PdBu. Treatment with higher concentrations of PMA was required to inhibit substantially exocytosis. Protein kinase C was not completely down-regulated by treatment with PMA; even at high PMA concentrations a small component of [^{3}H]PdBu binding remained. These results show that protein kinase C must be in ex-

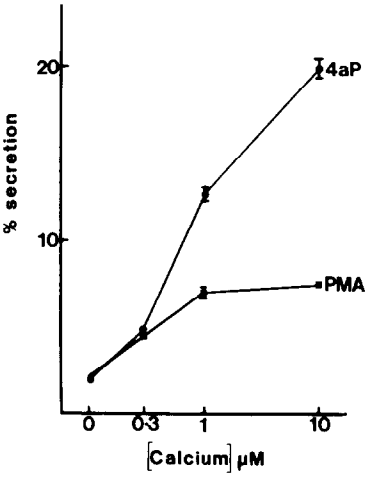

Fig.1. Effect of long-term treatment with PMA on secretion from digitonin-permeabilised chromaffin cells. Cells were treated with 250 nM PMA or 4αP for 24 h and permeabilised with digitonin in the presence of the indicated free calcium concentration. The percentage of catecholamine released over 20 min is expressed as percentage of total cellular content and is the mean ± SE of three determinations.

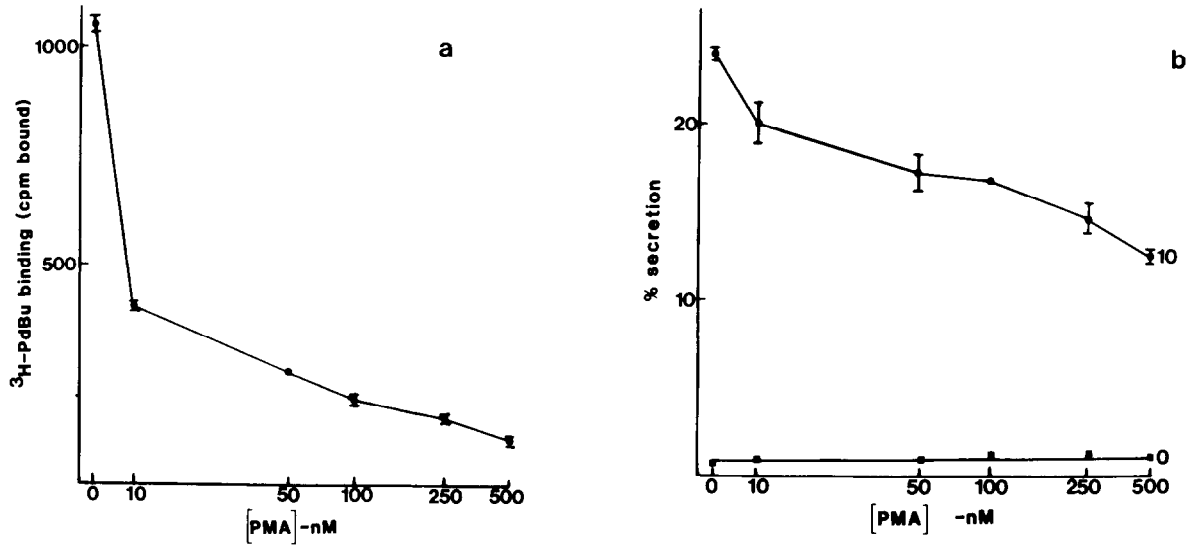

Fig.2. Concentration dependency of protein kinase C down-regulation and inhibition of exocytosis. Cells were pretreated with the indicated concentrations of PMA or with DMSO (controls) for 24 h. (a) Specific binding of ^{3}H-PdBu was determined as indicated in section 2. Control binding represents 2.74 pmol bound/mg protein. (b) After treatment with PMA, cells were permeabilised with digitonin in the absence of added calcium (0) or in the presence of 10 μM free calcium (10). Data show the percentage catecholamine released over 20 min and are means ± SE of four determinations.

cess, since a large reduction following treatment with 10 nM PMA had only a small effect on secretion. Secondly, secretion was not completely inhibited by down-regulation either due to the low level of protein kinase C remaining after PMA treatment or to protein kinase C-independent secretion.

3.2. *Effect of the protein kinase C inhibitor sphingosine on exocytosis*

In order to confirm the specificity of the effect of PMA treatment on exocytosis in permeabilised cells the effect of the protein kinase C inhibitor sphingosine [16] was examined. Treatment of cells with 100 μM sphingosine (fig.3) resulted in a marked inhibition of calcium-activated secretion from permeabilised cells. In five separate experiments the extent of inhibition of secretion by 100 μM sphingosine was in the range 73–93%. The concentration dependency of the inhibition of secretion by sphingosine is shown in fig.4. A component of secretion remained even at the highest sphingosine concentration tested. Due to the poor solubility of sphingosine in aqueous buffers higher concentrations were not tested.

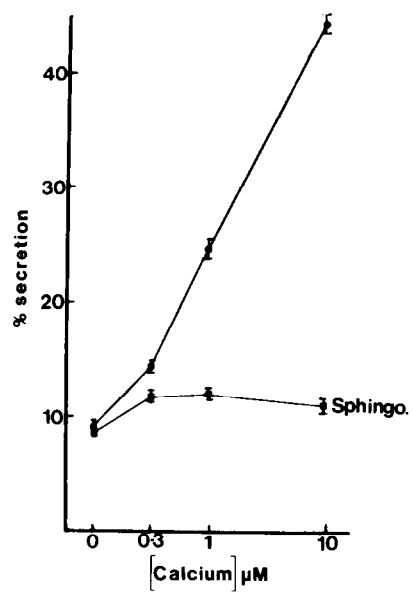

Fig.3. Effect of sphingosine on secretion from digitonin-permeabilised cells. Cells were incubated with DMSO (controls) or 100 μM sphingosine and then permeabilised in the presence of the indicated free calcium concentration. The percentage of total catecholamine released over 20 min is shown as the mean ± SE of six determinations.

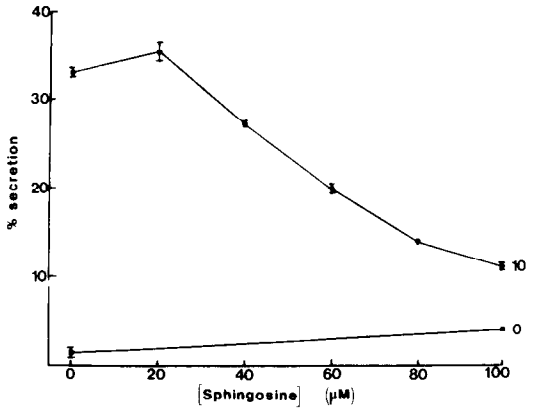

Fig.4. Concentration dependency of inhibition of secretion from permeabilised cells by sphingosine. Cells were pretreated with the indicated concentration of sphingosine and permeabilised in the presence of 0 or 10 μM free calcium with the appropriate sphingosine concentration. The percentage of total catecholamine released over 20 min is shown as the mean ± SE of six determinations.

4. DISCUSSION

Previous studies of protein kinase C and secretion from chromaffin cells have relied on the use of exogenous activation of protein kinase C by phorbol esters. In intact cells, phorbol esters stimulated no secretion [7,17] or a low level of secretion [8,18]. In permeabilised chromaffin cells, phorbol esters potentiated calcium-activated secretion [7–9]. Protein kinase C is activated following nicotinic stimulation of intact cells and during calcium-activated secretion from permeabilised cells [5,6] but the role of endogenous activation of the enzyme has not previously been directly examined.

The present results, from experiments in which protein kinase C was down-regulated by long-term treatment with PMA, showed that under these conditions nicotine and high K^+-induced secretion from intact cells is impaired. This inhibition may be due to an effect on calcium entry, since the rise in cytosolic calcium was also inhibited. Therefore, we by-passed calcium entry by examining the direct effect of down-regulation on calcium-activated exocytosis in permeabilised cells. The use of digitonin-permeabilised cells has shown that calcium-activated exocytosis is substantially inhibited in down-regulated cells. The specificity of this effect was apparent from the lack of effect of the inactive phorbol ester 4αP which was used as a control in many of the experiments. A further demonstration of the specificity of the effect of PMA pretreatment was the finding that the protein kinase C inhibitor sphingosine produced essentially the same inhibition of calcium-activated exocytosis. The extent of the inhibition of secretion was greater with sphingosine than with down-regulation. Nevertheless, a small component of secretion remained. The difference in the magnitude of the uninhibited component of exocytosis following the two treatments may result from the residual level of protein kinase C remaining after PMA treatment. These results show that endogenous activation of protein kinase C must play a major role in calcium-activated secretion with a small protein kinase C-independent component possibly also being present. The major role of protein kinase C in chromaffin cells contrasts with the situation in rat islets where down-regulation of protein kinase C had no effect on the calcium activation of insulin secretion [18].

Acknowledgements: This work was supported by a project grant from the MRC and A.O'S. was supported by an MRC Research Studentship.

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