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Interaction between protein kinase C and Exo1 (14-3-3 protein) and its relevance to exocytosis in permeabilized adrenal chromaffin cells

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The roles of protein kinase C (PKC) and Exo1 in exocytosis from digitonin-permeabilized adrenal chromaffin cells were explored by using exogenous purified proteins in a run-down/reconstitution system. The stimulatory action of Exo1 on exocytosis from run-down cells was found to be completely dependent on the continuous presence of exogenous MgATP, suggesting that it acts on the slow phase of exocytosis [Holz, Bittner, Peppers, Senter & Eberhard (1989), J. Biol. Chem. **264**, 5412–5419]. Partially purified rat brain PKC was found to be able to stimulate Ca^{2+}-dependent exocytosis from run-down cells in a dose-dependent manner. This effect was indeed due to PKC and not a contaminant in the PKC fraction, since the PKC activator phorbol 12-myristate 13-acetate (PMA), under conditions in which control secretion was not affected, potentiated the effect of the exogenous PKC in stimulating secretion. Furthermore, although either PKC or Exo1 alone could stimulate exocytosis from run-down cells, the effect of combining the fractions was synergistic, as had previously been observed using PMA treatment combined with Exo1 incubation [Morgan & Burgoyne (1992) Nature (London) **355**, 833–836]. The observed synergy between PKC and Exo1 was not due to PKC-mediated phosphorylation of Exo1, and Exo1 was found not to affect PKC activity in enzyme assays. We conclude that PKC and Exo1 act synergistically in the slow phase of Ca^{2+}-dependent exocytosis from adrenal chromaffin cells. Furthermore, PKC does not directly affect Exo1, but rather enhances the activity of Exo1 by a putative phosphorylation of another, unidentified, component of the exocytotic machinery which facilitates the action of Exo1 in exocytosis.

INTRODUCTION

Ca^{2+} has long been recognized as the major signal leading to exocytosis in many cell types, including adrenal chromaffin cells (Burgoyne, 1991). Although the nature of the Ca^{2+} signal responsible for exocytotic secretion in the chromaffin cell is now fairly well understood, the factors which Ca^{2+} acts upon to bring about the fusion of granule and plasma membranes have remained elusive, due mainly to the lack of an *in vitro* exocytosis system. In the absence of such a system, the best approach appears to be the permeabilized cell technique pioneered by Baker & Knight (1981) whereby lesions are created in the plasma membrane, allowing exocytosis to be triggered merely by raising Ca^{2+} to micromolar levels in the presence of exogenous MgATP.

One drawback of certain permeabilizing agents, such as digitonin and streptolysin O, is that the lesions created in the plasma membrane are so large as to allow the exit (and entry) of cytosolic proteins. This leakage of cytosolic proteins results in a run-down of the ability of the cell to secrete in response to Ca^{2+}. Ironically, this apparent drawback has great potential for identifying those proteins involved in the exocytotic process. It has been shown by Sarafian *et al.* (1987) that reintroduction of the proteins which leak from digitonin-permeabilized chromaffin cells can reconstitute secretion from such run-down cells, indicating that cytosolic proteins are essential for exocytosis. In the search for the identity of the essential cytosolic proteins, the run-down/reconstitution system was used to implicate a role for calpactin (annexin II) in exocytosis (Ali *et al.*, 1989; Ali & Burgoyne, 1990; Burgoyne & Morgan, 1990), and this was subsequently confirmed by Sarafian *et al.* (1991). More recently we have identified two cytosolic proteins, Exo1 and Exo2, which stimulate secretion from run-down cells (Morgan & Burgoyne, 1992).

In addition to annexin II, Exo1 and Exo2, protein kinase C (PKC) has been suggested by several groups to be involved in Ca^{2+}-dependent exocytosis from adrenal chromaffin cells (Knight & Baker, 1983; Pocotte *et al.*, 1985; Burgoyne *et al.*, 1988). Although the importance of PKC in the exocytotic process has been controversial, most workers now agree that the enzyme performs a modulatory role rather than being itself essential for exocytosis (Burgoyne, 1991). PKC leaks from digitonin-permeabilized cells, but this leakage can be prevented by prior treatment of the cells with phorbol 12-myristate 13-acetate (PMA), which causes the enzyme to translocate from the cytosol to membranes (Terbush & Holz, 1986). We have shown that such treatment with PMA potentiates the action of Exo1 in the run-down/reconstitution system, presumably due to retention of endogenous PKC (Morgan & Burgoyne, 1992). However, PMA treatment prior to stimulation will, in addition to preventing PKC leakage, cause enzyme activation and hence phosphorylation of key substrates. Significantly, it has been claimed that PMA pretreatment of digitonin-permeabilized chromaffin cells also prevents the leakage of the putative regulator of exocytosis, annexin II, presumably through phosphorylation of its *N*-terminal region (Sarafian *et al.*, 1991). Clearly, then, PMA treatment is an unsuitable approach with which to investigate the relationship between PKC and Exo1 in exocytosis, since the observed potentiation may result from the retention/activation of proteins other than PKC. In the present paper, this problem is obviated by using exogenous PKC along with Exo1 in the run-down/reconstitution system. Furthermore, the biochemical interaction of PKC and Exo1 is explored using *in vitro* phosphorylation studies.

MATERIALS AND METHODS

Materials

High-purity digitonin was obtained from Novabiochem (Nottingham, U.K.). Fetal calf serum and Dulbecco's modified Eagle's medium with 25 mM-Hepes were obtained from Gibco

Abbreviations used: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; DTT, dithiothreitol; KCIP, PKC inhibiting protein.
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(Paisley, Scotland, U.K.). [γ-^{32}P]ATP (3000 Ci/mmol), Hyperfilm MP and PKC enzyme assay kit were obtained from Amersham (Aylesbury, Bucks., U.K.). All other chemicals were from Sigma (Poole, Dorset, U.K.).

Isolation and culture of chromaffin cells

Chromaffin cells were isolated from bovine adrenal medullae by enzymic digestion as described by Greenberg & Zinder (1982), with modifications (Burgoyne *et al.*, 1988). Cells were washed in Ca^{2+}-free Krebs–Ringer buffer (consisting of 145 mM-NaCl, 5 mM-KCl, 1.3 mM-$MgCl_2$, 1.2 mM-NaH_2PO_4, 10 mM-glucose and 20 mM-Hepes at pH 7.4), resuspended in culture medium (Dulbecco's modified Eagle's medium with 25 mM-Hepes, 10% fetal calf serum, 8 μM-fluorodeoxyuridine, 50 μg of gentamycin/ml, 10 μM-cytosine arabinofuranoside, 2.5 μg of amphotericin B/ml, 25 units of penicillin/ml and 25 μg of streptomycin/ml), plated in 24-well trays at a density of 1×10^6 cells per well, and maintained in culture for 3–7 days before use.

Cell permeabilization and assay of catecholamine secretion

After initial washing of cells in Ca^{2+}-free Krebs–Ringer buffer, the protocol was in three stages. (1) Cells were permeabilized with 300 μl of buffer A (139 mM-potassium glutamate, 2 mM-ATP, 2 mM-$MgCl_2$, 5 mM-EGTA and 20 mM-Pipes at pH 6.5) containing 20 μM-digitonin for 10 min. (2) Cells were incubated with 200 μl of purified protein fractions {or dialysis buffer [buffer A containing 1 mM-dithiothreitol (DTT) and 0.02% NaN_3] for controls} for 15 min. (3) Cells were stimulated with 300 μl of buffer A containing 10 μM free Ca^{2+} for 20 min. After stage 3, the buffer was removed, centrifuged at 16000 *g* for 2 min and aliquots taken for assay of released catecholamine.

Assay of released catecholamines was performed using a fluorimetric method (von Euler & Floding, 1959). The total catecholamine content of the cells was determined after release of catecholamines with 1% Triton X-100. Catecholamine secretion was calculated as a percentage of total cellular catecholamine. All experiments were performed at room temperature.

Protein purification

Purification of Exo1 was as described previously (Morgan & Burgoyne, 1992), except that 0.1 mM-3,4-dichloroisocoumarin and 0.1 mM-*N*-[*N*-(L-3-transcarboxyrane-2-carbonyl)-L-leucyl]-agmatine were included in the initial homogenization buffer to inhibit proteolytic activity.

PKC was prepared from rat brain as follows, based on the method of Walsh *et al.* (1984). Five rat brains were homogenized in 20 ml of PKC buffer (1 mM-EDTA, 1 mM-EGTA, 1 mM-DTT, 0.02% NaN_3 and 20 mM-Tris at pH 7.5) containing 0.1 mM-3,4-dichloroisocoumarin and 0.1 mM-*N*-[*N*-(L-3-transcarboxyrane-2-carbonyl)-L-leucyl]agmatine, and centrifuged at 100000 *g* for 60 min. The supernatant was removed, dialysed overnight versus 1 litre of PKC buffer, and Millipore-filtered (1.2 μm pore size). $CaCl_2$ and $MgCl_2$ were now added to the supernatant to a final concentration of 2 mM each, and the material was loaded on to a 22 ml phenyl-Sepharose column (Pharmacia) pre-equilibrated with PKC buffer containing 2 mM-$CaCl_2$ and 2 mM-$MgCl_2$ (pH 7.5). The column was then flushed sequentially with PKC buffer containing 2 mM-$CaCl_2$, 2 mM-$MgCl_2$ and 1 M-NaCl (pH 7.5); PKC buffer containing 2 mM-$CaCl_2$ and 2 mM-$MgCl_2$ (pH 7.5); and finally with PKC buffer alone. The material eluted in the final step with PKC buffer was designated the PKC fraction (specific activity 55–520 nmol of phosphate transferred/min per mg of protein) and used in the experiments described. In some experiments, the PKC used was purified further by application to a 1 ml Mono Q f.p.l.c. column (Pharmacia) pre-equilibrated in PKC buffer and subsequent gradient elution with 0–500 mM-NaCl in PKC buffer over 48 ml.

Both Exo1 and PKC were dialysed against buffer A containing 1 mM-DTT and 0.02% NaN_3 prior to use in permeabilized cell secretion experiments, and against PKC buffer for phosphorylation experiments and PKC assays. Protein concentrations were determined by the method of Bradford (1976).

Phosphorylation studies

Partially purified PKC (67 μg/ml; specific activity 55 nmol of phosphate transferred/min per mg of protein) was incubated in the presence or absence of 25 μg of pure Exo1/ml in the following reaction mixture at room temperature: 15 mM-magnesium acetate, 50 μM-ATP (containing 100 μCi of [^{32}P]ATP/ml), 1 mM-calcium acetate, 66 mM-L-α-phosphatidyl-L-serine, 2 μg of PMA/ml, 2.5 mM-DTT, 0.05% NaN_3 and 50 mM-Tris at pH 7.5. After 30 min the reaction was stopped by the addition of SDS dissociation buffer (1.25% SDS, 2 mM-EDTA, 10% sucrose, 1% 2-mercaptoethanol, 10% glycerol and 125 mM-Tris at pH 6.8) followed by immediate boiling. Samples were then run on one-dimensional SDS/polyacrylamide gels, stained with Coomassie Blue, dried and exposed to Hyperfilm MP at −70 °C for 6 days.

PKC assay

Purified PKC was assayed for activity using a PKC enzyme assay kit (Amersham, code RPN77) according to the manufacturer's instructions. The enzyme assay is based upon the PKC-catalysed transfer of the γ-phosphate group of ATP to a threonine residue in a substrate peptide specific for PKC. Partially purified PKC (2.2 μg/ml; specific activity 520 nmol of phosphate transferred/min per mg of protein) and 280 μg of pure Exo1/ml were used in each sample.

Statistical analyses

Values for statistical significance, where quoted, were derived using Student's *t* test.

RESULTS

We previously showed that the ability of Exo1 to stimulate Ca^{2+}-dependent exocytosis was enhanced considerably by prior treatment of cells with PMA during permeabilization (Morgan & Burgoyne, 1992). In addition, the level of stimulation by Exo1 under control conditions (i.e. no PMA treatment) was partially inhibited (by 33%) by 20 μM-staurosporine (A. Morgan & R. D. Burgoyne, unpublished work). We set out, therefore, to investigate the interaction between PKC and Exo1. First, in order to further characterize the role of Exo1 in Ca^{2+}-dependent exocytosis, the requirement for MgATP by Exo1 in reconstituting secretion from run-down cells was examined (Fig. 1). Control secretion was dependent on the presence of MgATP as previously reported (Baker & Knight, 1981), and the activity of Exo1 was also MgATP-dependent. It should be noted that although control secretion could be significantly enhanced by preincubation of cells with MgATP in stage 2 prior to challenge with 10 μM-Ca^{2+} in the absence of MgATP, Exo1 was unable to enhance this response. Indeed, only when MgATP was present throughout the experiment did Exo1 stimulate exocytosis. This would be consistent with a requirement for protein phosphorylation during the stimulation period (stage 3) for Exo1 action.

Since PKC has been implicated in Ca^{2+}-dependent exocytosis by the use of activators (Knight & Baker, 1983; Lee & Holz, 1986) and inhibitors (Burgoyne *et al.*, 1988; Knight *et al.*, 1988) of the endogenous enzyme, the ability of exogenous purified PKC to stimulate exocytosis in the run-down/reconstitution

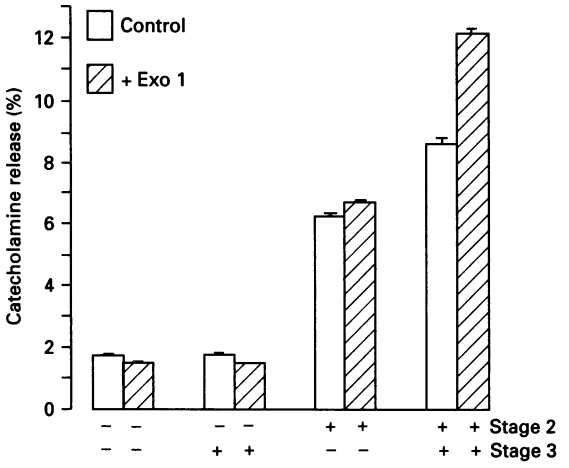

Fig. 1. Exo1 activity is MgATP-dependent

Cells were permeabilized in stage 1 in the presence of 2 mM-MgATP as in the standard protocol. In stage 2, cells were incubated with 3.5 mg of crude Exo1/ml, or dialysis buffer for controls, in the presence (+) or absence (−) of 2 mM-MgATP. In stage 3, cells were stimulated to secrete by challenge with 10 μM-Ca^{2+} in the presence (+) or absence (−) of 2 mM-MgATP. Exo1 used here differs from that in all other described experiments in that the protein was only purified to the Q-Sepharose stage. Catecholamine release is shown as a percentage of total catecholamine; results are expressed as means ± S.E.M. ($n = 4$).

Table 1. PMA enhances the stimulatory effect of PKC on Ca^{2+}-dependent exocytosis

Cells were permeabilized and incubated in the presence or absence of 200 μg of PKC/ml in stage 2 as in the standard protocol. Where used, PMA (200 nM) was present in stage 3 alone. The data show release induced by 10 μM-Ca^{2+} and the results are expressed as means ± S.E.M. ($n = 4$). *$P < 0.02$ compared with control; †$P < 0.002$ compared with PMA control. Values in parentheses indicate the percentage increase due to PKC treatment.

	Catecholamine release (%)	
	Control	+PMA
Control	13.4 ± 0.1	13.6 ± 0.4
+PKC	14.2 ± 0.2* (0.8)	16.3 ± 0.3† (2.7)

Table 2. The stimulatory effects of PKC and Exo1 are synergistic

Cells were permeabilized and incubated in the presence of 200 μg of PKC/ml or 50 μg of Exo1/ml (or both together) in stage 2 as in the standard protocol and stimulated with 10 μM-Ca^{2+} in stage 3. Results are expressed as means ± S.E.M. ($n = 4$). *$P < 0.02$; **$P < 0.02$; ***$P < 0.001$ compared with control levels.

	Catecholamine release (%)	Increase over control (%)
Control	13.4 ± 0.1	0
+PKC	14.2 ± 0.2*	0.8
+Exo1	14.4 ± 0.2**	1.0
+PKC/Exo1	17.0 ± 0.4***	3.6

system was investigated. Treatment with the PKC activator PMA in stage 3 alone had no effect on control secretion (Table 1). This is presumably due to leakage of endogenous PKC from the permeabilized cells during stages 1 and 2 (Terbush & Holz,

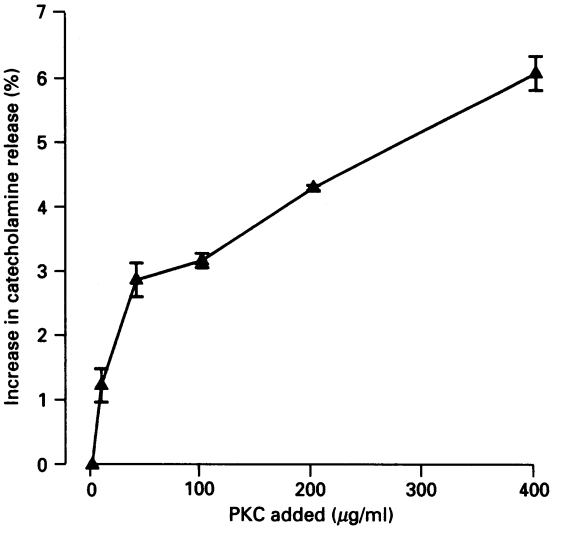

Fig. 2. PKC stimulates Ca^{2+}-dependent exocytosis in a dose-dependent manner

Cells were permeabilized and incubated in the presence of the indicated concentrations of PKC as in the standard protocol. In stage 3, cells were challenged with 10 μM-Ca^{2+} in the presence of PMA (200 nM). Results are shown as the increase in the percentage of total catecholamine released and are expressed as means ± S.E.M. ($n = 4$).

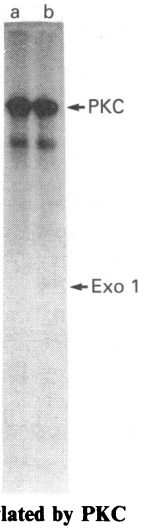

Fig. 3. Exo1 is poorly phosphorylated by PKC

Partially purified PKC (67 μg/ml; specific activity 55 nmol of phosphate transferred/min per mg of protein) without (lane a) or with (lane b) 25 μg of pure Exo1/ml was incubated in the presence of [^{32}P]ATP as described in the Materials and methods section, run on SDS/PAGE and labelled polypeptides detected by autoradiography. The positions of autophosphorylated PKC and Exo1 are shown.

1986). However, incubation of cells with exogenous PKC resulted in a small, but reproducible, increase in secretion and this effect, unlike the control situation, was potentiated by PMA treatment, implying that the agent responsible was indeed PKC. Furthermore, the ability of exogenous PKC to stimulate secretion from run-down cells in the presence of PMA was dose-dependent (Fig. 2). In addition, the effect of combining exogenous PKC and Exo1 was investigated (Table 2). Under these conditions (without PMA treatment) Exo1 is relatively ineffective (Morgan & Burgoyne, 1992), and Exo1 was used at a concentration below the EC_{50} in order to look for any synergism. It was found that,

Table 3. Exo1 does not affect PKC activity

Kinase activity was assayed by monitoring ^{32}P incorporation into a synthetic peptide substrate specific for PKC as described in the Materials and methods section. All samples (except for background) contained 2.2 μg of PKC/ml (specific activity 520 nmol of phosphate transferred/min per mg of protein) and 280 μg of Exo1/ml (or dialysis buffer for controls). Treatments were as follows: background, PKC replaced by dialysis buffer; Ca^{2+}/phospholipid (PL)-independent, calcium acetate replaced by equimolar EGTA and phosphatidylserine and PMA are replaced by 50 mM-Tris; Ca^{2+}/phospholipid (PL)-dependent, standard assay mixture containing calcium acetate, phosphatidylserine and PMA. Results are expressed as means of duplicate incubations. Similar results were found with two preparations of both PKC and Exo1.

	^{32}P incorporation (pmol/min per mg)	
Treatment	Control	+Exo1
Background	5.9	6.5
Ca^{2+}/PL-independent	20.9	21.7
Ca^{2+}/PL-dependent	95.6	99.7

as expected under these conditions, either PKC or Exo1 could stimulate only a low level of secretion from run-down cells. However, when both protein fractions were present together, their effects were synergistic. This effect was also seen with PKC that had been further purified on f.p.l.c. Mono Q ion-exchange columns to remove contaminating calmodulin (results not shown).

In order to see whether Exo1 is a PKC substrate, Exo1 and PKC were incubated together with [^{32}P]ATP and the incorporation of ^{32}P into protein was monitored by SDS/PAGE and subsequent autoradiography (Fig. 3). It can be seen that, although there was a slight incorporation of radiolabel into Exo1, the effect was tiny in comparison with PKC autophosphorylation, indicating that Exo1 is a poor substrate for PKC. Moreover, it is apparent from Fig. 3 that the presence of Exo1 had no effect on PKC autophosphorylation, and hence presumably on PKC enzyme activity. Exo1 is closely related to a family of proteins believed to be PKC inhibitors (Morgan & Burgoyne, 1992), and so any possible inhibitory action was also tested by assaying PKC activity using a synthetic peptide substrate in the presence or absence of Exo1 (Table 3). The concentration of Exo1 used (280 μg/ml) was greater than that required for maximal inhibition of PKC by PKC-inhibiting protein (KCIP) (100 μg/ml). Exo1 was without kinase activity itself, and did not inhibit Ca^{2+}/phospholipid-independent kinase activity or Ca^{2+}/phospholipid-dependent PKC activity. A lack of effect of Exo1 on PKC activity was seen with two separate preparations of both proteins.

DISCUSSION

The classic work of Baker & Knight (1981) established that catecholamine release from permeabilized chromaffin cells can be induced by micromolar Ca^{2+} concentrations and is absolutely dependent on exogenous MgATP. More recently, however, this assertion has been challenged by Holz and his co-workers (Holz *et al.*, 1989), who have argued that there are two components in exocytosis: an initial, fast, wave of exocytosis which, although primed by MgATP, does not require exogenous MgATP to proceed; and a second, slower, phase which requires exogenous MgATP for fusion to occur. We have shown here that Exo1 is unable to stimulate the MgATP-independent exocytosis of 'primed' vesicles (i.e. in cells incubated with MgATP prior to challenge without MgATP), and instead requires the continuous presence of MgATP for its effect. If one accepts Holz's hypothesis, then Exo1 must act in the second, slower, phase of exocytosis which requires exogenous MgATP. Nevertheless, the demonstration that the activity of Exo1 is completely dependent on exogenous MgATP provides additional evidence that the protein acts on the exocytotic process rather than having some non-specific effect, such as granule lysis.

Introduction of exogenous partially purified rat brain PKC into permeabilized pituitary cells (Naor *et al.*, 1989) and PC12 cells (Ben-Shlomo *et al.*, 1991) which had been depleted of endogenous PKC by chronic exposure to phorbol esters has previously been shown to reconstitute phorbol ester-stimulated exocytosis. We show here for the first time that the introduction of PKC into run-down chromaffin cells, which lose endogenous PKC in the absence of phorbol esters (Terbush & Holz, 1986), enhances Ca^{2+}-dependent exocytosis in a dose-dependent manner. Furthermore, this effect of PKC is synergistic when combined with exogenous Exo1, thus indicating that the previous observation of synergy between PMA treatment and Exo1 incubation (Morgan & Burgoyne, 1992) was indeed attributable to PKC retention and not due to the prior phosphorylation and retention of key substrates such as annexin II.

Since PKC could clearly increase the activity of Exo1 in the run-down/reconstitution system, an obvious possibility was that this was achieved by a PKC-mediated phosphorylation of Exo1. Indeed, PKC-mediated phosphorylation of annexin II has been shown to increase the activity of that protein in the run-down/reconstitution system (Sarafian *et al.*, 1991). However, phosphorylation studies revealed Exo1 to be an extremely poor substrate for PKC-mediated phosphorylation *in vitro*, and hence Exo1 is unlikely to be a physiological substrate for PKC. Thus it seems likely that PKC acts on some other component of the exocytotic process, either upstream or downstream of Exo1 action, and in so doing facilitates the involvement of Exo1 in the exocytotic mechanism. Indeed, it may be that Exo1 and PKC both act to regulate the activity of a single protein (the fusogen?). The inability of PKC and Exo1 to completely re-activate secretion to levels seen in non-run-down cells may be due to the leakage or inactivation of such a target protein.

Exo1 has been shown from protein sequencing to be a member of the 14-3-3/KCIP family of proteins (Morgan & Burgoyne, 1992), bearing most similarity to the KCIPs. The KCIPs are so named because they are PKC inhibiting proteins (Toker *et al.*, 1990; Aitken *et al.*, 1990), and so it was a surprise to find that Exo1, even though used at higher concentrations than those required for maximal PKC inhibition by KCIP, had no inhibitory effect on PKC in two enzyme assays. This may be due to the fact that our Exo1 preparation contains few of the gene products with KCIP activity, or the inhibitory activity could be overcome by the PMA in our assay (Toker *et al.*, 1990).

Bringing the data together, it appears that Exo1 acts on the second, slower, MgATP-dependent phase of exocytosis and that its activity is greatly enhanced by the presence of PKC, although this is not due to a direct effect of PKC on Exo1 or vice versa. Although the sites of action of PKC and Exo1 in the exocytotic mechanism are not yet clear, it is possible that their involvement in the slower phase of exocytosis is related to the involvement of the cortical cytoskeleton. Re-organization of the cytoskeleton has been shown to be necessary, but not in itself sufficient, for a full exocytotic response in chromaffin cells (Cheek & Burgoyne, 1987; Aunis & Bader, 1988). Activation of PKC (a MgATP-requiring protein) by PMA causes re-organization of the cytoskeleton (Burgoyne *et al.*, 1989). Thus it may be that the slow MgATP-dependent phase of exocytosis involves PKC-mediated re-organization of the cytoskeleton, which then allows granules to approach the plasma membrane in readiness for exocytotic

fusion, which is in turn modulated by Exo1 and other proteins. Nevertheless, it is clear that further biochemical studies of the interactions between the putative cytosolic regulators of exocytosis (PKC, calpactin, Exo1 and Exo2) and other cytosolic, granule membrane and plasma membrane proteins are required to assemble a more detailed picture of the exocytotic machinery.

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