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Identification of Exo2 as the catalytic subunit of protein kinase A reveals a role for cyclic AMP in Ca^{2+}-dependent exocytosis in chromaffin cells

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Digitonin-permeabilized chromaffin cells secrete catecholamines by exocytosis in response to micromolar Ca^{2+} concentrations, but lose the ability to secrete in response to Ca^{2+} as the cells lose soluble proteins through the plasma membrane pores. We have previously shown [Morgan and Burgoyne (1992) *Nature*, 355, 833–836] that cytosol can retard this loss of secretory competence and that two distinct stimulatory activities (Exo1 and Exo2) are present in cytosol. Here we report that Exo2 behaved as a single peak of activity through purification on hydroxyapatite, ammonium sulfate precipitation and gel filtration and the activity correlated with a single polypeptide of ~44 kDa on SDS gels. Protein sequencing of this band revealed it to be the catalytic subunit of cyclic AMP-dependent protein kinase (PKA). Both cyclic AMP and the commercially available catalytic subunit of PKA stimulated exocytosis in a dose-dependent manner which was absolutely dependent on the presence of micromolar Ca^{2+}. These data show that PKA (Exo2) regulates Ca^{2+}-dependent exocytosis in bovine adrenal chromaffin cells.

Key words: adrenal chromaffin cells/cyclic AMP/exocytosis/protein kinase A/secretion

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 well understood, the proteins which Ca^{2+} acts upon to bring about the exocytotic fusion of granule and plasma membranes have remained elusive. Recent work has, however, identified a number of candidate proteins (Burgoyne and Morgan, 1993). In permeabilized chromaffin cells (i.e. cells whose plasma membranes have been selectively porated) exocytosis can be directly triggered simply by raising Ca^{2+} to micromolar levels in the presence of MgATP (Baker and Knight, 1978). However, 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. Following this work, a requirement for cytosolic proteins in regulated exocytosis has been demonstrated in GH3 cells (Martin and Walent, 1989), PC12 cells (Lomneth *et al.*, 1991), mast cells (Koffer and Gomperts, 1989) and brain synaptosomes (Kish and Ueda, 1991). Although the work of Sarafian *et al.* (1987) did not identify the proteins in the leaked fraction responsible for the maintenance of secretory responsiveness, the run-down/reconstitution assay provides an opportunity to identify these proteins systematically, in an analogous manner to the spectacularly successful *in vitro* reconstitution studies of the early secretory pathway (see Rothman and Orci, 1992 for review).

By using the run-down/reconstitution approach, several soluble proteins (cytosolic and extrinsic membrane proteins) have been identified which regulate exocytosis in neuroendocrine cells, including annexin II (Ali *et al.*, 1989; Ali and Burgoyne, 1990; Sarafian *et al.*, 1991), 14-3-3 proteins (Exo1; Morgan and Burgoyne, 1992a,b; Wu *et al.*, 1992), p145 (Nishizaki *et al.*, 1992; Walent *et al.*, 1992) and several unpurified factors (Hay and Martin, 1992; Morgan and Burgoyne, 1992a). One such unpurified factor was termed Exo2 and was one of two stimulatory activities found on anion exchange fractionation of adrenal medullary or brain cytosol, the other being Exo1 (14-3-3 proteins; Morgan and Burgoyne, 1992a). Crude Exo2 generally had a less potent stimulatory effect on exocytosis than Exo1 and the effect of combining the two factors was additive (Morgan and Burgoyne, 1992a). The present paper details the purification and identification of Exo2 from brain cytosol as well as characterizing the effect of the protein on Ca^{2+}-dependent exocytosis in bovine adrenal chromaffin cells.

Results

We have previously shown (Morgan and Burgoyne, 1992a) that fractionation of sheep brain cytosol using Q-Sepharose revealed two stimulatory activities (Exo1 and Exo2) with an inhibitory activity sandwiched between. In nine separate Q-Sepharose fractionations, Exo1 activity was detected in all nine, Exo2 activity was detected in six and the inhibitory activity was found in seven. This did not indicate variability in the presence of Exo2, however, since when inhibitory fractions were subjected to gel filtration chromatography on Sephacryl S-100, the inhibitory activity was lost and a stimulatory Exo2 activity revealed (unpublished observations). From comparison with molecular weight standards run on the same column, Exo2 had an approximate molecular mass of 44 kDa (Morgan and Burgoyne, 1992a). Thus, it appears that the similar elution characteristics of

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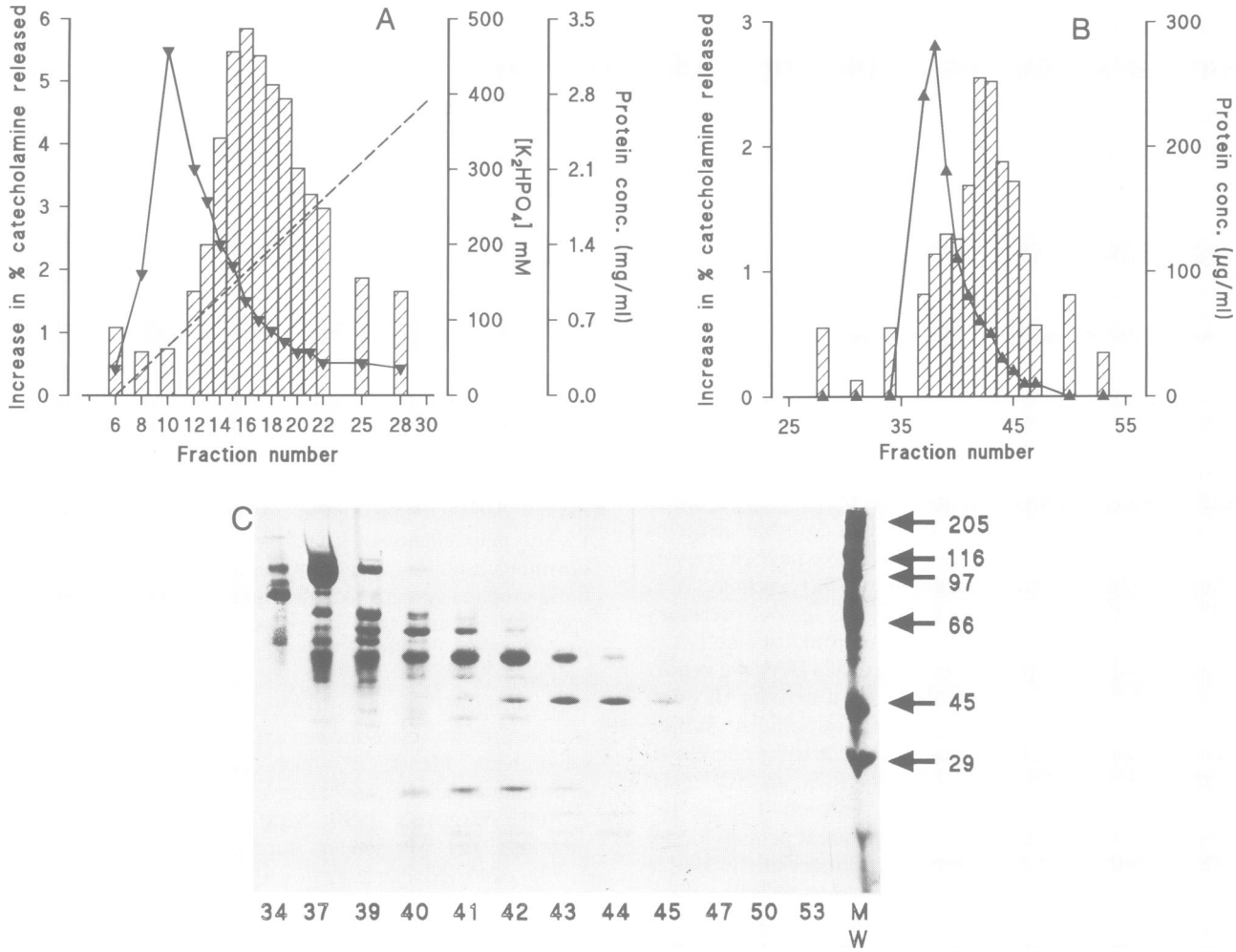

Fig. 1. Hydroxyapatite and Superdex 200 chromatography of Exo2. **(A)** Q-Sepharose fractions containing Exo2 activity were loaded on to a hydroxyapatite column and bound proteins eluted with a 0–500 mM potassium phosphate gradient. **(B)** Hydroxyapatite fractions 15–19 were precipitated with 45–65% ammonium sulfate, resuspended and loaded on to a Superdex 200 column and eluted proteins collected. The dotted line shows the phosphate gradient applied to the hydroxyapatite column and the solid lines show the protein concentrations of the eluted fractions. The hatched bars show the increase in 10 μM Ca^{2+}-induced secretion above controls as a percentage of catecholamine released. The standard protocol was used. Data shown are means (n = 2). **(C)** Shows a 10% SDS gel of the fractions eluted from the Superdex 200 column.

Exo2 and the inhibitory activity on Q-Sepharose can obscure the true efficacy of these two factors in the run-down/reconstitution assay. For this reason, initial characterization of Exo2 was carried out using material which had been partially purified from sheep brain cytosol using Q-Sepharose and Sephacryl S-100. Since catecholamine release from permeabilized chromaffin cells is Ca^{2+}-dependent, the effect of such partially purified Exo2 over a range of calculated free Ca^{2+} concentrations was investigated. Exo2 was found to be ineffective until the free Ca^{2+} concentration exceeded 300 nM and the stimulatory effect was maximal at 10 μM Ca^{2+}—the optimum for control secretion (data not shown). Furthermore, the action of Exo2 was completely abolished by the potent and specific blocker of exocytosis, tetanus toxin (A.Morgan, U.Weller, J.O.Dolly and R.D.Burgoyne, unpublished observations). Taken together, these findings provide compelling evidence that Exo2 enhances catecholamine release from run-down chromaffin cells by a bona fide action on the exocytotic process rather than some non-specific effect on secretory granule integrity.

For the purification of Exo2, chromatography on Q-Sepharose was used as a starting point (Morgan and Burgoyne, 1992a). Fractions 35–40 were routinely taken as containing Exo2 and applied to a hydroxyapatite column. Bound proteins were eluted with an increasing phosphate gradient. Eluted proteins were tested for activity in the run-down/reconstitution assay (Figure 1A). No activity was detected in the unadsorbed fraction, which contained around half of the loaded protein. It can be seen that a single stimulatory activity eluted over a range of fractions (peak activity in fractions 15–19) corresponding to 150–220 mM potassium phosphate. The stimulatory activity was precipitated at between 45 and 65% saturation with ammonium sulfate and the precipitate resuspended in a small volume and applied to a Superdex 200 gel filtration column. Eluted fractions were again tested for activity in the run-down/reconstitution assay and visualized on SDS gels (Figure 1B and C). The activity eluted as a single peak and from comparison with molecular weight standards run on the same column in identical conditions, Exo2 had an apparent molecular mass of 44 kDa. The stimulatory activity spread over fractions 41–45, with peak activity present in fractions 42 and 43. From the SDS gel, it can be seen that

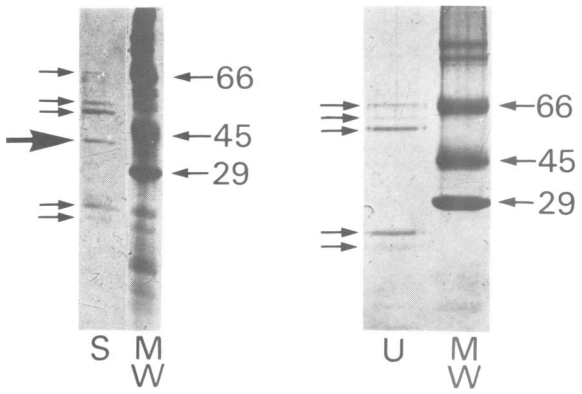

Fig. 2. Phenyl-Sepharose selectively removes a 44 kDa polypeptide. Superdex 200 fractions containing Exo2 activity (pooled fractions 42–45) were loaded on to a phenyl-Sepharose column and unadsorbed proteins collected. The left panel shows a 10% SDS gel of the Superdex fraction (S) and the right panel shows a 10% SDS gel of the unadsorbed material (U), both are aligned next to molecular weight markers (MW). The positions of the unbound polypeptides are denoted by faint arrows and the position of the selectively retained polypeptide is marked in the Superdex fraction by a bold arrow.

peptide 1:	T	L	G	T	G	S	F	G	R
PKA (C):	T	L	G	T	G	S	F	G	R
	48								56

peptide 2:	V	T	E	T	G	N	H	Y	A	M
PKA (C):	H	M	E	T	G	N	H	Y	A	M
	62									71

Fig. 3. Sequence analysis of tryptic peptides of Exo2. The putative Exo2 polypeptide was excised from SDS gels after purification to the Superdex stage, digested with trypsin and the resultant peptides separated by reverse phase HPLC. Two randomly selected peptides were sequenced using an Applied Biosystems 471A gas-phase sequencer with on-line PTH-amino acid analyser. The sequences were compared with those in the SWISS-PROT database and found to have homology only with the catalytic subunit of PKA [PKA(C)]. The sequences of the peptides are shown for comparison with the sequence of PKA(C). Homologous amino acids are boxed. The positions of residues in PKA(C) are indicated beneath. The peptide 1 sequence is present in α and β isoforms of PKA(C) and the peptide 2 sequence is compared with the α isoform. Of 19 residues identified from Exo2, 17 are identical to residues in PKA(C), and the peptides would be preceded by lysines, as expected for tryptic fragments. The two amino acid differences are likely to be sequencing errors.

the only major polypeptide which has a similar distribution to that of the activity is a band which comigrates with the 45 kDa molecular weight marker.

A further observation provided evidence that Exo2 was indeed this 44 kDa polypeptide. Exo2 purified to the Superdex stage was applied to a phenyl-Sepharose column and bound proteins eluted with a decreasing salt gradient. No Exo2 activity could be detected in the run-down/reconstitution assay from either the eluted fractions or the unadsorbed material, despite activity being detectable in the loaded material, indicating that Exo2 had bound to the column but had not eluted. Figure 2 shows SDS gel tracks of the material loaded on to this column and the unadsorbed flow-through

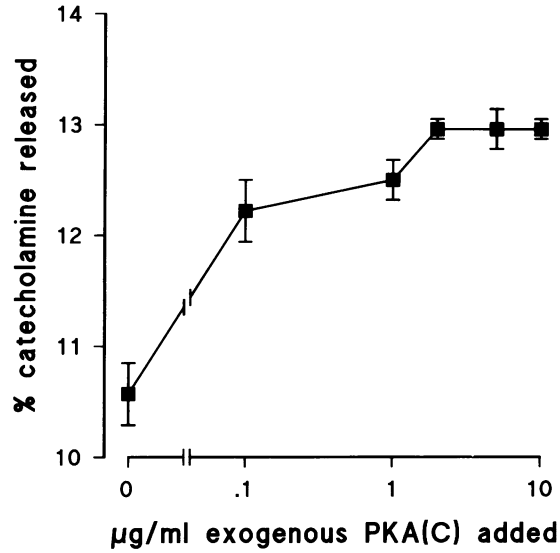

Fig. 4. Dose dependency of the stimulatory effect of PKA(C). Cells were pre-permeabilized for 10 min and then incubated for a further 15 min with buffer A containing 1 mM DTT and 0.02% NaN_3 and the indicated concentrations of PKA(C) prior to stimulation in buffer A containing 10 μM Ca^{2+}. Data shown are means ± SE (n = 4) and are expressed as a percentage of total cellular catecholamine.

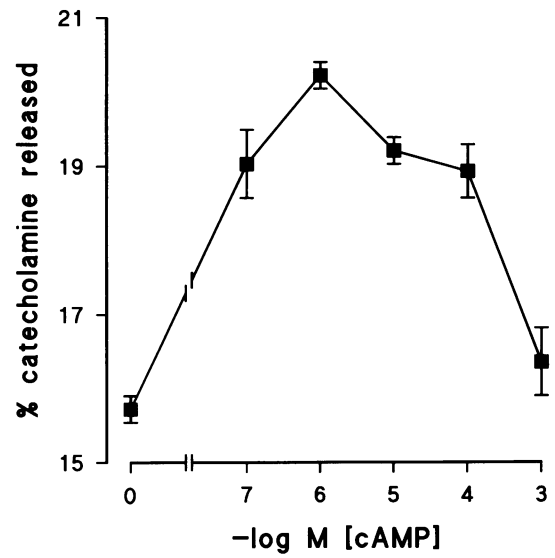

Fig. 5. Dose dependency of the stimulatory effect of cAMP. Cells were pre-permeabilized for 10 min and then incubated for a further 15 min with buffer A containing 1 mM DTT and 0.02% NaN_3 and the indicated concentrations of cAMP prior to stimulation in buffer A containing 10 μM Ca^{2+} and the indicated concentrations of cAMP. Data shown are means ± SE (n = 4) and are expressed as a percentage of total cellular catecholamine.

fraction. Although the individual polypeptides have run slightly differently on the two gels, it can nevertheless be seen that the unadsorbed fraction contains all of the polypeptides present in the parent fraction other than the band of ~44 kDa. The finding that depletion of the 44 kDa polypeptide removes activity strongly suggests that Exo2 activity resides in this polypeptide.

In order to identify the putative Exo2 polypeptide, the 44 kDa band was cut out of SDS gels, digested *in situ* with trypsin and the resultant extracted peptides separated by

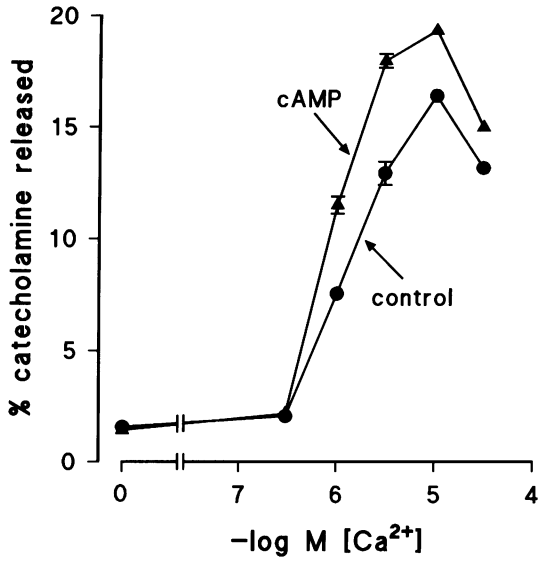

Fig. 6. Ca^{2+} dependency of the stimulatory effect of cAMP. Cells were pre-permeabilized for 10 min and then incubated for a further 15 min with buffer A containing 1 mM DTT and 0.02% NaN_3 in the presence or absence of 5 μM cAMP prior to stimulation in buffer A containing the indicated free Ca^{2+} concentrations in the presence or absence of 5 μM cAMP. Data shown are means ± SE (n = 4) and are expressed as a percentage of total cellular catecholamine. In some cases the SE was smaller than the symbols.

reverse phase HPLC. Two randomly selected peptides were then sequenced (Figure 3). It can be seen that the tryptic peptides exhibited a high degree of homology with the published sequence of the bovine catalytic subunit of cyclic AMP-dependent protein kinase [PKA(C); Shoji *et al.*, 1983], suggesting that this is the identity of Exo2. If this is true, the stimulatory action of Exo2 on exocytosis from run-down cells should be mimicked by the commercially available catalytic subunit of the kinase. It can be seen from Figure 4 that PKA(C) stimulated secretion in a dose-dependent manner. The additional presence of PKA(C) in the stimulation phase (stage 3) as well as the preincubation phase (stage 2) did not result in any further stimulation. It was also found that activation of endogenous PKA by cAMP enhanced secretion (Figure 5), but that whereas the effect of PKA(C) plateaued at around 2 μg/ml, the effect of cAMP was biphasic, peaking at around 1 μM and returning to control levels at 1 mM. The dose of PKA(C) required for a maximal effect was lower than for partially purified Exo2, probably due to loss of activity during purification. As was demonstrated for Exo2, the effect of cAMP on secretion was Ca^{2+}-dependent (Figure 6) as was the effect of commercially available PKA(C) (data not shown).

Discussion

We have previously shown (Morgan and Burgoyne, 1992a) that cytosol contains three factors capable of affecting secretion from run-down chromaffin cells, two stimulatory (Exo1 and Exo2) and one inhibitory. Exo1 activity was always detected in Q-Sepharose fractionations whereas Exo2 activity was observed less often, and in fractionations where both were detected, Exo1 was usually more active in the run-down/reconstitution assay. These findings implied a less important role for Exo2 in exocytosis than Exo1. However, the observation that inhibitory fractions contain Exo2 activity (indicating a considerable overlap in the elution properties of the two factors) suggests that the poor efficacy of Exo2 relative to Exo1 is at least in part due to contaminating inhibitory material in the Exo2 fraction. Thus, Exo2 may play a greater role in the regulation of exocytosis than was initially assumed. As with Exo1 (Morgan and Burgoyne, 1992a; Morgan *et al.*, 1993), the stimulatory action of Exo2 on secretion was absolutely Ca^{2+}-dependent and was abolished by co-incubation with the light chain of tetanus toxin. These observations strongly suggested that the stimulation of catecholamine release due to Exo2 was due to a bona fide effect on the exocytotic process.

Exo2 was taken through a four-step purification protocol. On the final step, Superdex gel filtration, Exo2 activity correlated with a polypeptide of ~44 kDa. Furthermore, application of partially purified Exo2 to a phenyl-Sepharose column resulted in the abolition of stimulatory activity and the selective removal of the 44 kDa polypeptide, suggesting that this hydrophobic protein represented Exo2. Protein sequencing of tryptic fragments of the 44 kDa polypeptide revealed strong homology with the catalytic subunit of cyclic AMP-dependent protein kinase (PKA). The molecular mass of the catalytic subunit of PKA including two phosphate groups and an *N*-myristoyl group is 40 862 (Shoji *et al.*, 1983), which is in close agreement with the estimated molecular mass of Exo2. The presence of an N-terminal fatty acyl group would be expected to increase the hydrophobicity of the protein and so is consistent with the tight binding of Exo2 to the hydrophobic interaction column, phenyl-Sepharose. The identification of Exo2 as the catalytic subunit of PKA was confirmed by the demonstration that commercially avaliable PKA(C) was stimulatory in the run-down/reconstitution assay.

The potential role of PKA in the exocytotic mechanism has been explored by the use of cAMP or its analogues in permeabilized cells. cAMP elicits Ca^{2+}-independent secretion in saponin-permeabilized parotid acini (Takuma and Ichida, 1991) and staphylococcal α-toxin-permeabilized pituitary cells (Macrae *et al.*, 1990), and potentiates Ca^{2+}-dependent secretion in electropermeabilized RINm5F cells (Vallar *et al.*, 1987) and streptolysin-O-permeabilized pancreatic acinar cells (O'Sullivan and Jamieson, 1992; Stecher *et al.*, 1992), suggesting a role for PKA in the control of exocytosis in these cell types. Using streptolysin-O-permeabilized pancreatic acini, O'Sullivan and Jamieson (1992) found that the cAMP enhancement of Ca^{2+}-dependent secretion was mediated by PKA since it could be abolished by treatment of the cells with a peptide derived from the heat stable inhibitor of PKA, whereas equipotent PMA enhancement was unaffected, as was control secretion. This suggests that PKA regulates, but is not itself essential for, exocytosis in pancreatic acinar cells.

Studies into the role of PKA in secretion from chromaffin cells have focused mainly on intact cells and the results have often appeared contradictory. Baker *et al.* (1985) reported that elevation of cAMP levels with high doses of the adenylate cyclase activator forskolin inhibited nicotine- but not KCl-induced secretion without affecting Ca^{2+} entry. Elevation of cAMP inhibits nicotine-induced depolymerization of the cortical actin network (Cheek and Burgoyne, 1987) and so this may explain the inhibitory effect of cAMP observed. In contrast, Morita *et al.* (1987a) found that low doses of forskolin enhanced nicotine-, KCl- and ionomycin-induced secretion. These apparently contradictory results are

probably due to the differences in the concentration of forskolin used, since it has been shown that acetylcholine-induced secretion is enhanced at low doses but inhibited at high doses of the drug (Morita *et al.*, 1987a). Morita *et al.* (1987b, 1991) found that forskolin and 8-bromo-cAMP enhanced Ca^{2+} entry due to acetylcholine and KCl and so proposed that this was the mechanism by which cAMP potentiates secretion. However, it is difficult to see how this could explain the potentiation of Ca^{2+}-ionophore-induced secretion seen by two groups (Morita *et al.*, 1987a; Marriot *et al.*, 1988). A more likely explanation would appear to be a direct action of cAMP on the exocytotic process downstream of Ca^{2+} entry.

Although little work has been published on the effect of cAMP on permeabilized chromaffin cells, the available data are no less contradictory. Baker and Knight (1981) found that 1 mM cAMP had no effect on secretion from electropermeabilized cells, whereas Bittner *et al.* (1986) found that 100 μM cAMP produced a modest potentiation of Ca^{2+}-dependent secretion in digitonin-permeabilized cells, and Sontag *et al.* (1991) found that forskolin potently enhanced Ca^{2+}-induced secretion in a dose-dependent manner (with 10 μM forskolin approximately doubling secretion). The reason for these discrepancies is likely to be the biphasic dose−response relationship for cAMP seen in this study. The observation that exogenous PKA(C) produces a similar enhancement of secretion suggests that the effect of cAMP is mediated by the kinase. Taken together, these results suggest that the well recognized role of PKA in stimulating exocytosis from other cell types can now be extended to chromaffin cells. Since cAMP is generated in intact chromaffin cells by a Ca^{2+}-dependent mechanism (Keogh and Marley, 1991) in response to many secretagogues (Marley *et al.*, 1991; Anderson *et al.*, 1992), activation of PKA is likely to be important in the amplification of Ca^{2+}-dependent exocytosis in chromaffin cells.

Materials and methods

Materials

High purity digitonin and hydroxyapatite were obtained from Novabiochem (Nottingham, UK). Fetal calf serum and Dulbecco's modified Eagle's medium with 25 mM HEPES were obtained from Gibco (Paisley, UK). All other chemicals, including PKA(C), were from Sigma (Poole, UK).

Isolation and culture of chromaffin cells

Chromaffin cells were isolated from bovine adrenal medullae by enzymic digestion as described by Greenberg and 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/ml gentamicin, 10 μM cytosine arabinofuranoside, 2.5 μg/ml amphotericin B, 25 U/ml penicillin, 25 μg/ml streptomycin), plated in 24 well trays at a density of one million 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 standard protocol was in three stages. (i) Cells were permeabilized with 300 μl of buffer A (139 mM potassium glutamate, 2 mM ATP, 2 mM $MgCl_2$, 5 mM EGTA, 20 mM PIPES at pH 6.5) containing 20 μM digitonin for 10 min. (ii) Cells were incubated with 200 μl of purified protein fractions [or dialysis buffer (buffer A containing 1 mM DTT and 0.02% NaN_3) for controls] for 15 min. (iii) Cells were stimulated with 300 μl of buffer A containing 10 μM free Ca^{2+} for 20 min. After stage (iii), the buffer was removed, centrifuged at 16 000 *g* for 2 min and aliquots were taken for assay of released catecholamine.

Assay of released catecholamines was performed using a fluorimetric method (von Euler and Floding, 1955). 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.

Purification of Exo2

Sheep brain cytosol was prepared as previously described (Morgan and Burgoyne, 1992a) except that the initial homogenization buffer contained 0.1 mM 3,4-dichloroisocoumarin and 0.1 mM *N*-[*N*-(L-3-*trans* carboxyrane-2-carbonyl)-L-leucyl]-agmatine as proteolytic inhibitors. Cytosol was applied to a 50 ml Q-Sepharose column (Pharmacia) which had been pre-equilibrated with buffer B (10 mM imidazole, 1 mM EGTA, 1 mM DTT, 1 mM NaN_3 at pH 7.3). Bound proteins were eluted with an increasing salt gradient from 0 to 500 mM NaCl over 240 ml at 2 ml/min and 4 ml fractions were collected. Fractions 35−40 (corresponding to 160−210 mM NaCl) were routinely taken as containing Exo2 and were pooled, dialysed overnight against buffer C (10 mM potassium phosphate, 1 mM DTT, 0.02% NaN_3 at pH 7.7) and millipore filtered (0.2 μm) before being applied to a hydroxyapatite column (1.4 × 9 cm) which had been pre-equilibrated in buffer C. Bound proteins were eluted with an increasing phosphate gradient from buffer C to buffer D (500 mM potassium phosphate, 1 mM DTT, 0.02% NaN_3 at pH 7.85) over 96 ml at 1 ml/min and 3 ml fractions were collected. Fractions 15−19 (corresponding to 150−220 mM potassium phosphate) contained the peak Exo2 activity and were pooled, and ammonium sulfate was added to 45% saturation on ice while stirring for 30 min. This material was then centrifuged at 13 000 *g* for 5 min. The supernatant was then made up to 65% saturation with ammonium sulfate and centrifuged as before. The pellets were then resuspended in 600 μl of buffer A containing 1 mM DTT and 0.02% NaN_3 and this material was loaded on to a 120 ml Superdex 200 column (Pharmacia) in the same buffer at 2 ml/min and 2 ml fractions were collected.

Attempts were made to purify Exo2 using hydrophobic interaction chromatography on phenyl-Sepharose. Here, material which had been purified to the Superdex stage (pooled fractions 42−45) was dialysed overnight against buffer E (300 mM NaCl, 2 mM EGTA, 1 mM DTT, 25 mM Tris at pH 7.5) and applied to a 22 ml phenyl-Sepharose column (Pharmacia) which had been pre-equilibrated in the same buffer. Bound proteins were eluted with a decreasing salt gradient/increasing ethylene glycol gradient from buffer E to buffer F (25 mM NaCl, 2 mM EGTA, 1 mM DTT, 60% ethylene glycol, 25 mM Tris at pH 7.5) over 96 ml at 1 ml/min and 3 ml fractions were collected.

All purifications were carried out at room temperature using a Pharmacia FPLC system. All fractions were dialysed overnight against buffer A containing 1 mM DTT and 0.02% NaN_3 before use in secretion experiments. Protein concentrations were estimated using the method of Bradford (1976). SDS−PAGE was performed using a Bio-Rad mini Protean II according to the manufacturer's instructions.

Protein sequencing

Prior to sequencing, the putative Exo2 polypeptide was excised from SDS gels of fractions purified as above and proteolytically digested by the method of Rosenfeld *et al.* (1992). Briefly, the gel band was washed for 40 min in 50% acetonitrile, dried for 10 min and then rehydrated overnight at 37°C in a trypsin solution (Sigma type IX). The digest was then separated by reverse phase HPLC using a 0−60% acetonitrile gradient in 0.05% trifluoroacetic acid and the resulting peptides concentrated before sequencing using an Applied Biosystems 471A gas-phase sequencer with on-line PTH-amino acid analyser. Derived sequences were compared for homology with sequences in the SWISS-PROT database.

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