RESEARCH COMMUNICATION

Anti-(14-3-3 protein) antibody inhibits stimulation of noradrenaline (norepinephrine) secretion by chromaffin-cell cytosolic proteins

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Incubation of digitonin-permeabilized bovine chromaffin cells in the absence of Ca^{2+} results in a loss of both cytosolic proteins and Ca^{2+} -dependent secretion. Addition of these leaked proteins prevents this loss of secretory activity. We have purified a protein from an extract of bovine adrenal medulla which can partially prevent this loss of Ca^{2+} -dependent secretion. Antibody against this protein inhibited the ability of leaked chromaffin-cell proteins to prevent the loss of Ca^{2+} -dependent secretion. Sequence analysis showed it to have sequence identity with bovine brain 14-3-3 protein. These results demonstrate that 14-3-3 protein makes a significant contribution to the ability of leaked chromaffin-cell proteins to maintain secretory activity.

INTRODUCTION

Noradrenaline (norepinephrine; NA) secretion by digitonin-permeabilized chromaffin cells is both ATP- and Ca^{2+} -dependent [1–3]. Incubation of digitonin-permeabilized chromaffin cells in the absence of Ca^{2+} results in a progressive loss of proteins and secretory activity [4]. When the proteins which leak from the digitonin-permeabilized cells are collected, concentrated and added back to the incubation buffer, this loss of Ca^{2+} -dependent secretion is largely prevented, indicating that cytosolic proteins are involved in the secretory response [4,5]. Ali *et al.* [6] and Sarafian *et al.* [7] reported that calpactin (annexin II) can prevent this loss of Ca^{2+} -dependent secretion. However, we found that leaked chromaffin-cell proteins depleted of calpactin were as effective as control proteins in preventing the loss of secretory activity [5]. Thus cytosolic proteins other than calpactin appear to be responsible for preventing this loss of secretory activity.

As only a very small quantity of proteins can be collected from digitonin-permeabilized cells, we used an extract of bovine adrenal medulla as a starting material for the isolation of cytosolic proteins involved in Ca^{2+} -dependent secretion. Here we report the isolation of one such protein and show that this protein diffuses out of digitonin-permeabilized chromaffin cells. Affinity-purified antibody against this protein partially inhibited the ability of leaked chromaffin-cell proteins to prevent the loss of secretory activity. Re-addition of the purified protein reversed this inhibition. Sequence analysis showed it to be very similar to, if not identical with, bovine brain 14-3-3 protein. After this work was completed, Morgan & Burgoyne [8] reported the identification of two cytoplasmic proteins, Exo 1 and Exo 2, in extracts of sheep brains which can partially re-activate secretion in permeabilized bovine chromaffin cells. Amino acid sequences of two peptides from Exo 1 suggest that it may be a form of sheep brain 14-3-3 protein [8].

MATERIALS AND METHODS

Chromaffin cells were isolated from bovine adrenal glands and cultured on 48-well plates [9]. Cytosolic proteins which leak from digitonin-permeabilized chromaffin cells were prepared as described by Sarafian *et al.* [4].

Adrenal medullae were homogenized on ice in 2 vol. of 150 mM-NaCl/5 mM-EGTA/20 mM-Hepes (pH 7.4)/1 mM-dithiothreitol/leupeptin (10 $\mu\text{g}/\text{ml}$)/0.2 mM-phenylmethanesulphonyl fluoride. The homogenate was centrifuged for 1.5 h at 100 000 *g*. When used in secretion assays, this supernatant (called the 'adrenal-medulla extract') was dialysed overnight at 4°C against 70 mM-potassium glutamate/2 mM- MgCl_2 /1 mM-EGTA/10 mM-Pipes (pH 6.6)/0.5 mM-dithiothreitol. All protein fractions were dialysed against this buffer before use in the secretion assay. When used for protein fractionation, the adrenal-medulla extract was applied directly to an S-300 (Pharmacia)

Fig. 1. Purification of bovine adrenal 14-3-3 protein

Protein fractions at each step during the purification were subjected to SDS/PAGE. A, molecular-mass standards; B, adrenal-medulla extract; C, pooled fractions from the S-300 column; D, proteins eluted from a Mono Q column by a step from 0.3 to 0.5 M-NaCl; E, 14-3-3 protein after elution from a Mono Q column with a 0.2–0.5 M-NaCl gradient; F, 14-3-3 protein after eluted from an S-200 column.

Abbreviation used: NA, noradrenaline (norepinephrine).

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column equilibrated with 20 mM-Tris (pH 7.5)/0.1 M-NaCl/1 mM-EGTA. Adrenal 14-3-3 protein was purified from this extract as outlined in Fig. 1.

Antibody against adrenal-medulla 14-3-3 protein was raised in rabbits and purified on a column containing bovine adrenal 14-3-3 protein coupled to Sepharose [5]. The protocol used for immunization was as follows: 0.5 ml of adrenal 14-3-3 protein (1 mg/ml) was emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously in 10 sites along the back of a rabbit. After 28 days, 0.5 ml of 14-3-3 protein was emulsified with an equal volume of incomplete Freund's adjuvant and injected subcutaneously in multiple sites. The rabbit was boosted on a monthly basis and bled 1 week later. About 30 μ g of anti-(14-3-3 protein) antibody could be isolated from 1 ml of serum after three injections of antigen.

The protocol used for measuring [3 H]NA secretion is similar to that described previously [1,3,9]. After labelling with [3 H]NA, the cells were permeabilized at room temperature in 125 μ l of 139 mM-potassium glutamate/2 mM-MgCl₂/2 mM-MgATP/5 mM-EGTA/20 mM-Pipes, pH 6.6 (KG-buffer), containing 15 μ M-digitonin. After 6 min, the permeabilization buffer was removed, and the cells were incubated for 30 min in KG-buffer containing different protein fractions or BSA. After this incubation, [3 H]NA secretion was measured in KG-buffer with or without added CaCl₂. The calculated free Ca²⁺ concentration was 10 μ M [10]. After 15 min the release medium was removed and placed on ice until centrifugation at 12000 *g* for 4 min. The supernatant from this centrifugation was removed and counted for radioactivity. The cells attached to the wells were solubilized with 125 μ l of 5 mM-Pipes/1 % Triton X-100, combined with the pellet from the above centrifugation, and counted for radioactivity. [3 H]NA release is expressed as a percentage calculated by multiplying the amount of [3 H]NA in the supernatant by 100 and dividing by the total [3 H]NA recovered. Three wells were used for all determinations, and release values are reported as the mean $\pm$ S.D.

RESULTS AND DISCUSSION

Restoration of secretion by adrenal-medulla extract

Incubation of digitonin-permeabilized chromaffin cells results in a progressive loss of Ca²⁺-dependent secretion and a release of cytosolic proteins [4,5]. As shown in Table 1, a cytosolic extract prepared from bovine adrenal medullae was almost as effective as the leaked proteins in preventing the loss of Ca²⁺-dependent secretion. As incubation in the absence of any added protein frequently resulted in an increase in basal release, control incubations contained BSA. BSA at 0.2 mg/ml was enough to prevent this increase.

Digestion of the adrenal-medulla extract with trypsin or treatment with *N*-ethylmaleimide destroyed its ability to prevent the loss of secretory activity. The increase in NA release caused by the addition of this extract was ATP-dependent. The Ca²⁺-dependence of secretion from cells incubated for 30 min with the adrenal extract was very similar to that of control cells immediately after permeabilization (results not shown). These experiments indicate that this extract is suitable starting material for the isolation of soluble proteins involved in the secretory response.

Restoration of secretion requires more than one cytosolic protein

The adrenal-medulla extract was first fractionated on an S-300 column. Those fractions which could partially prevent the loss of Ca²⁺-dependent secretion (latter third of the protein peak) were pooled and fractionated on a Mono Q (Pharmacia) column using

steps of 0.1, 0.2, 0.3 and 0.5 M-NaCl. Proteins eluted in 0.1 M-NaCl and those eluted in 0.5 M-NaCl consistently increased Ca²⁺-dependent secretion from the permeabilized cells (Table 2). They had no effect on secretion in the absence of Ca²⁺. The observation that proteins eluted by 0.1 M-NaCl as well as those eluted by 0.5 M-NaCl could partially prevent the loss of Ca²⁺-dependent secretion suggested that at least two different cytosolic proteins are involved in Ca²⁺-dependent secretion. As shown in Table 2, permeabilized cells incubated in a mixture of proteins eluted by 0.1 M- and 0.5 M-NaCl gave more Ca²⁺-dependent secretion than did cells incubated only with proteins eluted by 0.1 M-NaCl or only with proteins eluted by 0.5 M-NaCl. This increase in secretory activity appears to result from mixing several different proteins, as doubling the concentration of either fraction by itself did not cause any significant increase in activity.

Table 1. Effect of adrenal-medulla extract and chromaffin-cell cytosolic proteins on NA release

(a) The cells were permeabilized by a 6 min incubation in 15 μ M-digitonin. This permeabilization solution was removed, and the amount of [3 H]NA secretion was measured in the presence or absence of Ca²⁺. (b) The cells were permeabilized by a 6 min incubation in 15 μ M-digitonin and then incubated in the absence of Ca²⁺ for 30 min in the presence of the proteins indicated. The amount of [3 H]NA released was measured 15 min after the addition of release medium. Leaked proteins are those that diffuse out of digitonin-permeabilized chromaffin cells.

Conditions	NA released (%)		
	No Ca ²⁺	10 μ M-Ca ²⁺	Ca ²⁺ -dependent
(a) Release measured immediately after permeabilization	3.7 $\pm$ 0.5	20.6 $\pm$ 0.7	16.9
(b) Release measured after a 30 min incubation in:			
BSA (3.0 mg/ml)	3.6 $\pm$ 0.4	9.2 $\pm$ 0.6	5.6
Leaked proteins (3.0 mg/ml)	3.9 $\pm$ 0.4	17.9 $\pm$ 0.3	14
Adrenal-medulla extract (4.0 mg/ml)	3.2 $\pm$ 0.3	17.3 $\pm$ 0.7	14.1

Table 2. Effect of protein fractions from Mono Q column on NA release

The cells were permeabilized by a 6 min incubation in 15 μ M-digitonin and then incubated in the absence of Ca²⁺ for 30 min in the presence of the proteins indicated. These incubation solutions were removed, and the amount of [3 H]NA secretion was measured in the presence of 10 μ M-Ca²⁺. The amount of [3 H]NA released was measured 15 min after the addition of release media. After fractionation on an S-300 column, the adrenal-medulla extract was fractionated on a Mono Q column using a steps of 0.1, 0.2, 0.3 and 0.5 M-NaCl. Fractions eluted by 0.1 M-NaCl and those eluted by 0.5 M-NaCl were pooled separately and concentrated.

Proteins present during 30 min incubation	Concn. (mg/ml)	NA released (%)	
		In 10 μ M-Ca ²⁺	Increase above control
BSA (control)	2.0	6.7 $\pm$ 0.2	0
Proteins eluted by:			
0.1 M-NaCl	1.25	9.1 $\pm$ 0.5	2.4
	2.50	9.5 $\pm$ 0.6	2.8
0.5 M-NaCl	0.40	9.1 $\pm$ 0.2	2.4
	0.80	9.1 $\pm$ 0.1	2.4
0.1 M-NaCl plus those eluted by 0.5 M-NaCl	1.25, 0.40	13.7 $\pm$ 0.4	7.0

Fig. 2. Detection of 14-3-3 protein in leaked chromaffin-cell proteins

Samples were resolved by SDS/PAGE and analysed by immunoblotting with affinity-purified anti-(14-3-3 protein) antibody (0.4 $\mu\text{g}/\text{ml}$). Immunoreactive bands were revealed with goat anti-rabbit IgG antibodies coupled to horseradish peroxidase (Bio-Rad Laboratories). A, 30 μg of bovine brain extract; B, 30 μg of proteins which leaked from digitonin-permeabilized cells; C, 30 μg of pooled S-300 fractions; D, 10 μg of proteins eluted from a Mono Q column by a step from 0.3 to 0.5 M-NaCl; E, 6 μg of proteins after elution from a Mono Q column with a 0.2–0.5 M-NaCl gradient; F, 1 μg of protein after S-200 column.

Table 3. Effect of adrenal 14-3-3 protein on NA release

The cells were permeabilized by a 6 min incubation in 15 μM -digitonin and then incubated in the absence of Ca^{2+} for 30 min in the presence of the proteins indicated. These incubation solutions were removed, and the amount of [^3H]NA secretion was measured in the presence or absence of Ca^{2+} and the proteins as indicated. The amount of [^3H]NA released was measured 15 min after the addition of release media.

Protein present during:		NA released (%)	
Preincubation	Release	No Ca^{2+}	10 μM - Ca^{2+}
BSA*	BSA*	2.4 $\pm$ 0.3	6.5 $\pm$ 0.2
14-3-3 protein†	BSA*	Not determined	8.7 $\pm$ 0.6
BSA*	14-3-3 protein†	2.1 $\pm$ 0.5	8.0 $\pm$ 0.6
14-3-3 protein†	14-3-3 protein†	2.3 $\pm$ 0.3	10.1 $\pm$ 0.7

* 0.25 mg of BSA/ml.

† 0.1 mg of adrenal 14-3-3 protein/ml and 0.15 mg of BSA/ml.

Isolation of one of the proteins involved in this restoration

The fraction which was eluted from the Mono Q column by 0.5 M-NaCl was chosen for additional fractionation as it contained fewer proteins than did the 0.1 M-NaCl fraction. The most abundant proteins in this high-salt fraction had molecular masses between 27 and 30 kDa on SDS/PAGE, a doublet at about 27 kDa and a single band at 30 kDa (Fig. 1, lane D). These three polypeptides were co-eluted from an S-200 column with an apparent molecular mass of about 60 kDa. Chromatography on a variety of other resins did not separate these three polypeptides.

This triplet of polypeptides (molecular masses of 27–30 kDa) was purified by sequential chromatography on an S-300 column, two different Mono Q columns and an S-200 column (Fig. 1). All three polypeptides appeared to have blocked N-termini. This

mixture of polypeptides was digested with trypsin, and the resulting peptide were fractionated by h.p.l.c. Three of these peptides were sequenced using an Applied Biosystems gas-phase sequencer. These three peptides were nearly identical with peptides found in bovine brain 14-3-3 protein [11]. These sequences are EKIEAELQD, which is identical with residues 87–95 of the β -chain of brain 14-3-3 protein, TAFDEAIAELDT, which is identical with residues 199–211 of the β -chain of brain 14-3-3 protein, and AAFDDAIAELDTL, which, with the exception of the first alanine is identical with residues 199–212 of the γ -chain of brain 14-3-3 protein. On SDS/PAGE, brain 14-3-3 protein gives three polypeptides with molecular masses of 27–30 kDa. When resolved by reverse-phase chromatography [11], it separates into seven polypeptides (α – η). Under non-denaturing conditions, brain 14-3-3 is a dimer [12,13]. Thus it appears that we isolated adrenal 14-3-3 protein.

An immunoblot with affinity-purified antibody against adrenal 14-3-3 protein showed that 14-3-3 protein was present in the proteins which leaked from digitonin-permeabilized chromaffin cells (Fig. 2, lane B). About 2–3% of the total leaked proteins was 14-3-3 protein. Antibody against adrenal 14-3-3 protein reacted with 14-3-3 protein in bovine brain extract (Fig. 2, lane A) and with purified bovine brain 14-3-3 protein. In addition to reacting with 14-3-3 protein, the affinity-purified antibody also reacted with proteins with molecular masses of about 70 and 60 kDa. These proteins were present in both the leaked proteins and the adrenal-medulla extract, but they were not detected in a bovine brain extract. Fractionation on an S-300 column separated 14-3-3 protein from these proteins (Fig. 2, lane C).

Effect of purified adrenal 14-3-3 protein on secretion

Addition of adrenal 14-3-3 protein to both the incubation and release media caused about a 90% increase in Ca^{2+} -dependent secretion. (Ca^{2+} -dependent secretion from control cells was 4.1% [^3H]NA released, and Ca^{2+} -dependent secretion from cells in which 14-3-3 protein was added to both the incubation and release media was 7.8% [^3H]NA released.) Addition of 14-3-3 protein to only the incubation media or to only the release media also increased Ca^{2+} -dependent secretion. These increases were about one-half that obtained when 14-3-3 protein was added to both the incubation and release media. The experiment shown in Table 3 was performed using 0.1 mg of adrenal 14-3-3 protein/ml. Increasing the 14-3-3 protein concentration to 0.2 mg/ml did not cause any additional increase in secretion. The increase in Ca^{2+} -dependent secretion caused by adrenal 14-3-3 protein was less than that obtained with the whole tissue extract. However, if as suggested by the mixing experiment shown in Table 2, several cytosolic proteins are involved in the secretory response, one would not expect the addition of a single protein to completely restore secretion.

Inhibition using anti-(14-3-3 protein) antibody

Calpactin can also partially prevent the loss of Ca^{2+} -dependent secretion from digitonin-permeabilized chromaffin cells [5–7]. However, calpactin is not required to prevent this loss, as leaked chromaffin-cell proteins depleted of calpactin are as effective as control proteins in preventing the loss of secretory activity [5,8]. We used anti-(14-3-3 protein) antibody to examine the contribution that 14-3-3 protein makes to the ability of leaked chromaffin-cell proteins to prevent the loss of Ca^{2+} -dependent secretion. Proteins which leak from digitonin-permeabilized cells rather than the adrenal extract were used for these experiments, since the leaked proteins should more accurately reflect the chromaffin-cell cytosolic proteins. Anti-(14-3-3 protein) antibody columns were used to try to deplete leaked chromaffin-cell proteins of 14-3-3 protein. However, these columns only removed

Table 4. Effect of anti-(adrenal 14-3-3 protein) antibody on NA release

Digitonin-permeabilized cells were incubated in KG-buffer for 30 min in the presence of the proteins indicated. These incubation solutions were removed, and [3 H]NA secretion was measured in the presence or absence of Ca^{2+} . BSA was added to all of the samples such that the total protein concentration in all of the 30 min incubations was 3.2 mg/ml. Abbreviations: CP, proteins (2.5 mg/ml) which leaked from digitonin-permeabilized chromaffin cells; Ab, affinity-purified anti-(adrenal 14-3-3 protein) antibody (0.3 mg/ml); 14-3-3, purified protein from adrenal medulla (0.4 mg/ml); NA released (% above control), [3 H]NA released in 10 μM - Ca^{2+} from cells incubated in the proteins indicated minus that released from cells incubated in BSA.

Addition	NA released (% above control)
None (control)	0
CP	7.7 $\pm$ 0.3
CP + Ab	4.7 $\pm$ 0.1
CP + Ab + 14-3-3	8.2 $\pm$ 0.7

25–50 % of the 14-3-3 protein. As an alternative approach, the leaked proteins were mixed with the purified antibody and incubated overnight at 4°C before being added to the permeabilized cells. As Table 4 shows, the addition of anti-(14-3-3 protein) antibody decreased the ability of leaked proteins to prevent the loss of secretory activity. The increase in Ca^{2+} -dependent NA release caused by the addition of leaked proteins incubated with anti-(14-3-3 protein) antibody was about half that obtained with control leaked proteins. Similar results were obtained using three different preparations of leaked proteins. Addition of excess adrenal 14-3-3 protein to leaked proteins which had been preincubated overnight with the antibody reversed the inhibitory effect of the antibody (Table 4). Since purified adrenal 14-3-3 protein does not contain the 70 and 60 kDa proteins (Fig. 2, lane F), inhibition by this antibody appears to result from its binding to 14-3-3 protein. Anti-(14-3-3 protein) antibody also caused a similar decrease in the ability of a bovine brain extract to prevent the loss of the secretory activity. The brain extract contained 14-3-3 protein, but not the 70 and 60 kDa proteins (Fig. 2, lane A).

Ca^{2+} -dependent secretion from permeabilized cells incubated with anti-(14-3-3 protein) antibody and no added leaked proteins was the same as that from cells incubated with BSA (results not shown).

Using a similar approach, Morgan & Burgoyne [8] isolated a protein, Exo 1, from sheep brain which restores secretory activity to permeabilized bovine chromaffin cells. They reported the sequence of two tryptic peptides of Exo 1 which show a high degree of sequence identity with bovine brain 14-3-3 protein and suggested that Exo 1 might be a form of sheep brain 14-3-3 protein [8]. The amount of restoration they obtained with Exo 1 protein was about twice that we obtained with bovine adrenal 14-3-3 protein. This difference probably results from their treating the cells with a phorbol ester before permeabilization with digitonin. They found that this pretreatment "markedly potentiated" the response of the permeabilized cells to sheep brain Exo 1.

The inability of anti-(14-3-3 protein) antibody to completely block the restoration of secretion by leaked proteins and the additive effects of proteins eluted by 0.1 and 0.5 M-NaCl from the Mono Q column suggest that more than one cytosolic protein is involved in Ca^{2+} -dependent secretion. Morgan & Burgoyne [8] reported the partial purification of a second cytosolic protein, Exo 2, which can also partially restore Ca^{2+} -dependent secretion. Exo 1 and Exo 2 were said to have additive effects on secretion.

Experiments by Sarafian *et al.* [7] also suggest that more than one cytosolic protein is involved in secretion.

Although the results presented here and those of Morgan & Burgoyne [8] suggest that 14-3-3 protein is involved in Ca^{2+} -dependent secretion in permeabilized chromaffin cells, its precise role is not known. Although 14-3-3 protein has been shown to activate both tyrosine hydroxylase and tryptophan hydroxylase [14], it is unclear how stimulation of these hydroxylases could account for an increase in Ca^{2+} -dependent [3 H]NA release. Also, the presence of 14-3-3 protein in tissues which do not contain detectable amounts of either tyrosine or tryptophan hydroxylases suggests that it has some more general function [15–17]. An inhibitor of protein kinase C isolated from sheep brain also appears to be a form of 14-3-3 protein [18]. It is unlikely that the effect of adrenal 14-3-3 protein on secretion in permeabilized chromaffin cells results from its inhibiting protein kinase C, since other inhibitors of protein kinase C either decrease, or have no effect on, Ca^{2+} -dependent secretion in these cells, and activation of protein kinase C enhances Ca^{2+} -dependent secretion [19–26]. We have made preliminary attempts to identify what other proteins or structures might interact with 14-3-3 protein. Two obvious possibilities, secretory vesicles and F-actin, do not appear to bind adrenal 14-3-3 protein (results not shown).

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