Characterization of 14-3-3 proteins in adrenal chromaffin cells and demonstration of isoform-specific phospholipid binding

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Isoform-specific antisera were used to examine which 14-3-3 isoforms were present in bovine adrenal chromaffin cells. The η, τ and σ isoforms were not detectable, and the ϵ isoform was present at only low levels. 14-3-3 isoforms were readily detected with antisera against the β, γ and ζ isoforms. The latter isoforms were found to leak from digitonin-permeabilized chromaffin cells, as expected for cytosolic proteins, but a proportion of each isoform was retained. In subcellular fractionation studies isoforms recognized by the β and ζ antisera were found in the cytosol and Triton-insoluble cytoskeletal fractions, while the γ isoform was found in cytosol and also in microsomal and chromaffin granule membrane fractions. The γ 14-3-3 protein associated with granule membranes was partially removed by a high-salt/carbonate wash, and the membranes could bind further γ from cytosol or from a purified brain 14-3-3 protein mixture. The binding of γ 14-3-3 was not Ca^{2+}-dependent, nor was it affected by phorbol ester, GTP analogues or cyclic AMP. Using pure phospholipid vesicles it was found that γ and also ϵ 14-3-3 proteins bound directly to phospholipids. Little binding of brain β, η or ζ to phospholipid vesicles was detected. Brain 14-3-3 proteins were also able to aggregate phospholipid vesicles. Recombinant 14-3-3 isoforms (τ and the *Xenopus* protein) were able to stimulate Ca^{2+}-dependent exocytosis in digitonin-permeabilized chromaffin cells. The *Xenopus* proteins lacks part of the extreme N-terminus, indicating that this domain is not essential for function in exocytosis.

INTRODUCTION

Recent work has identified a widespread family of abundant soluble proteins known as the 14-3-3 proteins [1]. As well as several mammalian members of this family [2–12], 14-3-3 proteins from yeast [13], plants [14,15], *Drosophila* [16,17] and *Xenopus* [18] have been cloned and sequenced. Seven or eight mammalian isoforms can be detected following separation of purified brain 14-3-3 proteins by h.p.l.c. [2]. Five isoforms (β, γ, ϵ, ζ and η) are encoded by distinct genes and two (α and δ) are probably post-translationally modified forms of β and ζ respectively [19]. The sequences of two other mammalian 14-3-3 protein isoforms are known, the T-cell isoform (τ) [20] and an epithelial-cell-specific isoform (μ) [12,21]. In their native state the 14-3-3 proteins exist as dimers.

The exact cellular functions of the 14-3-3 proteins are unclear. They have been implicated in several regulatory mechanisms, including activation of tyrosine and tryptophan hydroxylases [22], regulation of protein kinase C [3,6,8] and activation of the *Pseudomonas aeruginosa* toxin exoenzyme S [11]. One isoform was suggested to have phospholipase A_2 activity [9], although other studies have failed to detect phospholipase A_2 activity in purified brain 14-3-3 preparations [23,24].

In our attempts to identify proteins that regulate Ca^{2+}-dependent exocytosis, we purified an activity from sheep brain cytosol that stimulated exocytosis in digitonin-permeabilized adrenal chromaffin cells following run-down of secretory activity due to leakage of cytosolic components [25,26]. The purified activity, which we termed Exo1, was found to have sequence identity to the known 14-3-3 protein sequences [25] and seemed likely to comprise the brain 14-3-3 isoforms [23,27]. We have used isoform-specific antisera generated using synthetic peptides [19] to examine which 14-3-3 isoforms are present in adrenal chromaffin cells and their intracellular distribution. In addition, we show that recombinant 14-3-3 proteins are effective in stimulating Ca^{2+}-dependent exocytosis in permeabilized chromaffin cells.

MATERIALS AND METHODS

Antisera

Antisera against the N-terminal sequences of sheep 14-3-3 isoforms were prepared and characterized previously [19]. The peptides used for each isoform were as follows: Ac.MDKSELVQKAC (β), Ac.VDREQLVQKAC (γ), Ac.MDKNELVQKAC (ζ), Ac.MDDREDLVYQAK (ϵ), Ac.GDREQLLQRAR (η) and Ac.MEKTELIQKAC (τ). The antisera against the β and ζ isoforms showed a low level of cross-reactivity with the other isoforms listed, but recognized the α and δ isoforms, which are post-translationally modified forms of β and ζ respectively [19]. The remaining antisera did not show any cross-reactivity with other isoforms. A further antiserum against a σ isoform was generated and was found to be isoform-specific (H. Martin and A. Aitken, unpublished work).

Purification of sheep brain 14-3-3 proteins

Our published protocol for the purification of Exo1 was used for the purification of 14-3-3 proteins from sheep brain cytosol by ion exchange, hydrophobic interaction and Mono Q f.p.l.c. chromatography followed by gel filtration [25].

Preparation of subcellular fractions

Bovine adrenal medullae were homogenized in 0.3 M sucrose, 1 mM EGTA, 5 mM Hepes, pH 7.3, and centrifuged at 800 *g* for

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15 min. The supernatant was further centrifuged at 17000 *g* for 20 min at 4 °C. The supernatant of this centrifugation was centrifuged at 100000 *g* for 60 min at 4 °C to provide the cytosol (100000 *g* supernatant) and microsomal (100000 *g* pellet) fractions. The pellet from the 17000 *g* centrifugation contained mitochondria which were washed off the top of the pellet, as well as chromaffin granules. The crude chromaffin granule pellet was resuspended in homogenization buffer, overlayed on 1.7 M sucrose, 1 mM EGTA, 1 mM $MgSO_4$, 5 mM Hepes, pH 7.3, and centrifuged at 100000 *g* for 60 min at 4 °C. The granule pellet was resuspended in 1 mM $MgSO_4$, 20 mM Hepes, pH 7.3, and lysed by freezing and thawing. The chromaffin granule membranes were pelleted by centrifugation at 100000 *g* for 60 min, and both granule and microsomal membranes were given three further washes by centrifugation. To prepare high-salt/carbonate-washed granule membranes, the membranes were taken after the first wash and extracted sequentially in 30 min incubations with 1 M KCl, 1 mM $MgSO_4$, 20 mM Hepes, pH 7.3, followed by incubation of the pelleted membranes in 110 mM Na_2CO_3, pH 11.5, and centrifuged at 100000 *g* for 60 min.

Binding of 14-3-3 proteins to chromaffin granule membranes

Control granule membranes or high-salt/carbonate-washed membranes (50 μg of protein) were incubated in 139 mM potassium glutamate, 2 mM ATP, 2 mM $MgCl_2$, 5 mM EGTA, 20 mM Pipes, pH 6.5 (total volume 140 μl), with adrenal medullary cytosol (250 μg of protein) or sheep brain 14-3-3 protein (125 μg of protein) at room temperature for 60 min. In some cases the free Ca^{2+} concentration was raised to 10 μM by addition of $CaCl_2$; other additions were made as specified in the text. The membranes were pelleted by centrifugation at 13000 *g* for 15 min and solubilized for SDS/PAGE in 160 μl of SDS-dissociation buffer; 20 μl per track was separated and probed by immunoblotting.

Binding of 14-3-3 proteins to phospholipid vesicles

Phospholipid vesicles were prepared as described by Blackwood and Ernst [28]. Phospholipids were purchased from Sigma and dried from chloroform under N_2. A 4 mg sample of phospholipid was hydrated with 0.5 ml of 100 mM KCl, 1 mM EGTA, 50 mM Hepes, pH 7.0, vortexed and sonicated twice. Pelletable vesicles were prepared after three rounds of centrifugation at 13000 *g* for 10 min in a Microfuge and pellets were resuspended in 250 μl of buffer. A 20 μl aliquot of phospholipid vesicles was mixed with 1.5 μg of brain 14-3-3 proteins in buffer (final volume 40 μl), incubated at room temperature for 10 min, centrifuged at 13000 *g* for 15 min and washed twice; pellets were analysed by SDS/PAGE and immunoblotting. For light-microscopical examination, 20 μl of phospholipid vesicles was incubated with 1.5 μg of 14-3-3 proteins or BSA for 30 min and then examined by phase-contrast microscopy.

SDS/PAGE and immunoblotting

Protein samples were separated on 12.5 % SDS/polyacrylamide gels and transferred by transverse electrophoresis to nitrocellulose. Fidelity of transfer was confirmed by Ponceau S staining of proteins on the nitrocellulose. The nitrocellulose was washed with PBS, blocked by incubation with 3 % non-fat skimmed milk for 45 min and incubated with anti-14-3-3 antisera (1:1000, except antisera for ϵ and η, which were used at 1:500) in PBS for 1 h at room temperature. After washing, the nitrocellulose was incubated with anti-rabbit IgG conjugated to horseradish peroxidase (Sigma) (1:400) in 3 % milk and 0.5 % Tween-20 in PBS for 60 min, washed in Tween/PBS and washed four times in PBS. Antibody binding was detected using the enhanced chemiluminescence system (Amersham) according to the manufacturer's instructions.

Preparation of recombinant 14-3-3 proteins

The T-cell (τ) isoform was expressed in *Escherichia coli* from the vector pKK233-2 (Pharmacia) and purified by hydrophobic interaction and anion-exchange chromotagraphy [3]. The *Xenopus* 14-3-3 protein [18] was expressed in the vector pQE32 (QA express) with a His_6 tag that allowed purification from the *E. coli* extract by metal-chelation affinity chromatography on Ni–agarose. The purity of the proteins was confirmed by SDS/PAGE.

Assay of secretion from permeabilized adrenal chromaffin cells

In order to assess the effect of 14-3-3 proteins on Ca^{2+}-dependent exocytosis, chromaffin cell cultures prepared as previously described [26,29] were permeabilized using the digitonin-permeabilization method [30,31]. The cells were incubated with 20 μM digitonin in 139 mM potassium glutamate, 2 mM ATP, 2 mM $MgCl_2$, 5 mM EGTA, 20 mM Hepes, pH 6.5, for 10 min, and then incubated in buffer with 1 mM dithiothreitol but without digitonin for 15 min in the presence or absence of added 14-3-3 proteins. The cells were then challenged with fresh buffer with no added Ca^{2+} or with $CaCl_2$ added to give to 10 μM free Ca^{2+} [25,26]. After 15 min the buffer was removed and centrifuged at 13000 *g* for 2 min, and aliquots were assayed for released catecholamine using a fluorimetric assay. Total cellular catecholamine was determined after lysis with 1 % Triton X-100. Data on catecholamine release were expressed as a percentage of total cellular catecholamine. These procedures were all carried out at room temperature (20–24 °C).

Assay of 14-3-3 protein leakage from permeabilized chromaffin cells and preparation of Triton-insoluble cytoskeletons

Chromaffin cells were permeabilized with 20 μM digitonin as indicated above in a single 25 min incubation. At the end of this time the cell supernatant and cell residue were taken for analysis by SDS/PAGE and immunoblotting. The cell residue was solubilized in 100 μl of SDS-dissociation buffer. The supernatant proteins were precipitated with methanol at −20 °C and the precipitate was solubilized in 100 μl of SDS-dissociation buffer. The Triton-insoluble cytoskeleton of cultured chromaffin cells was prepared by extraction of cells in culture with 1 % Triton X-100, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 2 mM phenylmethanesulphonyl fluoride, 10 mM Tris/HCl, pH 7.0, for 5 min on ice and solubilization of the residue with 100 μl of SDS-dissociation buffer [32]. In all cases, 20 μl of sample per track was analysed by SDS/PAGE and immunoblotting.

RESULTS

14-3-3 protein isoforms in Exo1 and in chromaffin cells

It seemed likely that the activity (Exo1) that we previously purified from sheep brain cytosol based on its ability to stimulate Ca^{2+}-dependent exocytosis in adrenal chromaffin cells [25,26] contained a mixture of the brain 14-3-3 isoforms. This was assessed using anti-peptide antisera against peptides corresponding to the N-termini of the β (beta), γ (gamma), ϵ (epsilon), η (eta), ζ (zeta), τ (tau; T-cell) and σ (sigma; epithelial) gene

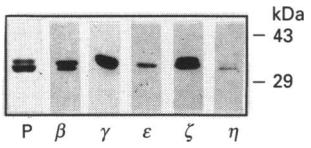

Figure 1 Characterization of 14-3-3 protein isoforms in Exo1 (brain 14-3-3 protein fraction)

Purified Exo1 was immunoblotted with the indicated anti-14-3-3 antisera (5 μg of Exo1 per lane). P indicates protein staining (Coomassie Blue) of 14-3-3 proteins, and lanes β–η are immunoblots with isoform-specific antisera. The positions of molecular mass markers are shown on the right.

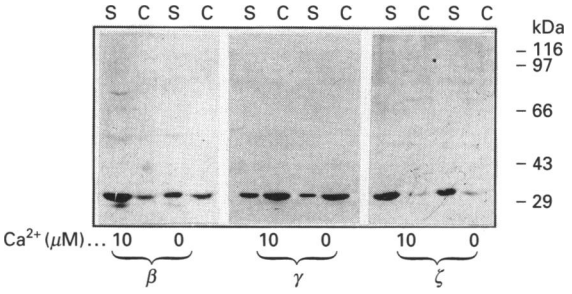

Figure 2 Characterization of 14-3-3 protein isoforms in adrenal chromaffin cells and their leakage following digitonin-permeabilization

Chromaffin cells in culture (10^6 cells per sample) were permeabilized with digitonin for 25 min in 0 or 10 μM Ca^{2+}. Cell supernatant (S) and cell residue (C) fractions were prepared in equivalent volumes of SDS-dissociation buffer at 20 μl per track and separated by SDS/PAGE, and samples were analysed by immunoblotting with anti-β, -γ and -ζ isoform-specific antisera. The positions of molecular mass markers are shown on the right. The antisera showed a high level of specificity and detected 30 kDa polypeptides in both cell residue and leaked supernatant fractions.

products [19]. The β, γ, ϵ, η and ζ isoforms were readily detectable in our purified Exo1 (Figure 1), which we will refer to henceforth as brain 14-3-3 proteins. The τ and σ isoforms were not detected in immunoblotting on the purified protein mixture, although low levels of τ isoform have been detected in brain [19].

In order to determine which 14-3-3 isoforms are expressed by adrenal chromaffin cells, the specific antisera were used in immunoblotting on samples from chromaffin cell cultures. Since exogenous 14-3-3 proteins (Exo1 [24]) are able to stimulate Ca^{2+}-dependent exocytosis in permeabilized chromaffin cells after cytosolic protein leakage and secretory run-down, it seemed probable that these soluble proteins would leak after digitonin-permeabilization. The η, τ and σ isoforms were not detectable in chromaffin cells; the ϵ isoform was present, but only at the limit of detectability, and was not studied in further detail. In contrast, the anti-β, -γ and -ζ antisera readily detected 30 kDa polypeptides in chromaffin cells (Figure 2). These isoforms were detectable in the cell residue 25 min after permeabilization, but substantial amounts were found to have leaked into the cell supernatant. The extent of retention of the isoforms varied between cell batches. It should be noted that since the anti-β and anti-ζ sera each show some cross-reactivity with the other isoform, we cannot say with certainty whether one or both of these isoforms is present in chromaffin cells.

Subcellular distribution of 14-3-3 proteins in chromaffin cells

The finding that a variable proportion of the 14-3-3 proteins had

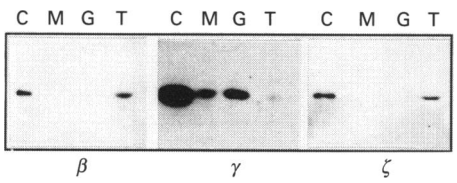

Figure 3 Distribution of 14-3-3 protein isoforms in subcellular fractions

Cytosol (C), microsomal membranes (M) and chromaffin granule membranes (G) were prepared from adrenal medulla, and a Triton-insoluble cytoskeletal fraction (T) was prepared from cultured chromaffin cells. The fractions were run at equivalent protein concentrations (0.5 mg/ml), loaded on gels at 20 μl per track and probed using the anti-β, -γ and -ζ isoform-specific antisera.

not leaked 25 min after permeabilization suggested that some interaction made by these proteins prevented them from behaving as purely cytosolic proteins and leaking completely. For this reason the subcellular distribution of 14-3-3 isoforms between cytosol, microsomal membranes, chromaffin granule membranes and the Triton-insoluble cytoskeleton was examined. Polypeptides labelled by anti-β and ζ were detected in the cytosolic fraction and in the Triton-insoluble cytoskeleton. In contrast, anti-γ detected a polypeptide in microsomal and granule membranes and γ was also present at low levels in the cytoskeletal fractions (Figure 3), suggesting isoform specificity of the subcellular distribution of the 14-3-3 proteins and a selective ability of the γ isoform to associate with membranes.

Binding of γ 14-3-3 to chromaffin granule membranes

The association of the γ isoform with chromaffin granule membranes was analysed in more detail. Granule membranes washed with high salt and carbonate to remove extrinsic membrane proteins showed a reduction in the amount of associated γ (Figure 4a). Both control and washed membranes were able to recruit further γ 14-3-3 following incubation with cytosol (Figure 4a). More γ 14-3-3 was apparently bound to washed compared with control membranes. Since the samples were run at equivalent protein concentrations, this may simply reflect a higher lipid concentration in the washed membrane fractions after removal of extrinsic proteins since, as we show below, γ 14-3-3 is able to bind to phospholipid vesicles. We examined whether the additional binding of γ 14-3-3 from cytosol was affected by a variety of factors that are known to regulate exocytosis in chromaffin cells. As shown in Figure 4(b), the extent of γ 14-3-3 binding was Ca^{2+}-independent and was unaffected by two non-hydrolysable GTP analogues, activation of protein kinase C with phorbol 12-myristate 13-acetate or addition of cyclic AMP. These binding experiments were carried out using a cytosol fraction in order to maximize the chances of detecting any factors that regulate binding. Other cytosolic proteins were not required for γ 14-3-3 binding to granule membranes, since it also bound in incubations using purified brain 14-3-3 proteins (Figure 4c).

Binding of 14-3-3 proteins to phospholipid vesicles

The presence of γ 14-3-3 on purified granule membranes and on microsomal membranes, and the ability of additional γ 14-3-3 to bind to high-salt/carbonate-washed granule membranes, suggested that this isoform interacted either with integral protein components of the membranes or with phospholipid. We used a variety of affinity-purification approaches with 14-3-3–Sepharose

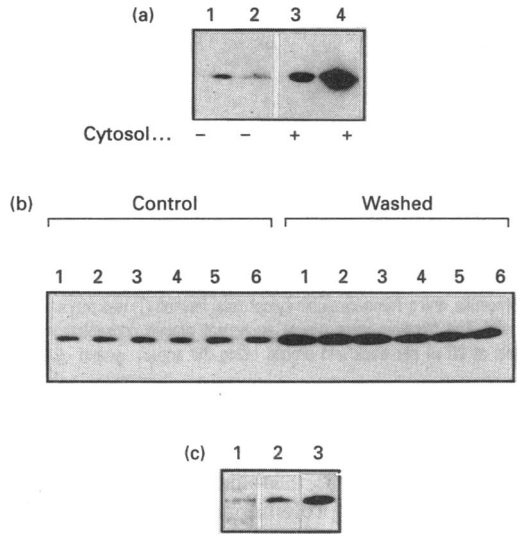

Figure 4 Characterization of γ 14-3-3 protein binding to chromaffin granule membranes

(**a**) Control (lanes 1 and 3) and high-salt/carbonate-washed (lanes 2 and 4) granule membranes were probed with anti-γ specific antiserum after the membranes had been incubated in the absence or presence of cytosol in Ca^{2+}-free buffer. (**b**) Control and high-salt/carbonate-washed granule membranes were incubated with cytosol in 0 Ca^{2+} (lanes 1), or 10 μM Ca^{2+} (2), or with 100 μM guanosine 5′-[γ-thio]triphosphate (3), 100 μM guanosine 5′-[βγ-imido]triphosphate (4), 200 nM phorbol 12-myristate 13-acetate (5) or 1 μM cyclic AMP (6) and then the membranes were separated by SDS/PAGE and probed with γ-specific antiserum. (**c**) High-salt/carbonate-washed granule membranes were incubated alone (lane 1) or with cytosol (lane 2) or brain 14-3-3 proteins (lane 3) and then the membranes were probed with γ-specific antiserum. Protein concentrations and gel loadings were as described in the Materials and methods section.

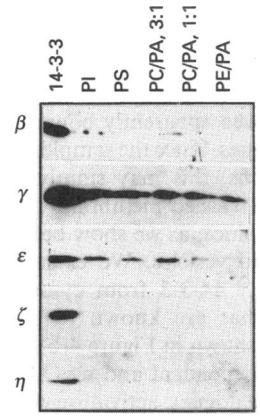

Figure 5 Binding of 14-3-3 protein isoforms to phospholipid vesicles

Brain 14-3-3 proteins (1.5 μg) were incubated with phospholipid vesicles (20 μl) consisting of phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine plus phosphatidic acid (PC/PA at 3:1 and 1:1) and phosphatidylethanolamine plus phosphatidic acid (PE/PA at 1:1). After washing, the vesicles were pelleted and resuspended in 100 μl of SDS-dissociation buffer; 20 μl of this sample and a sample of 14-3-3 proteins, adjusted to be equivalent to the protein concentration in the incubation, were separated by SDS/PAGE and probed with isoform-specific antisera.

and chemical cross-linking with cytosol fractions and with intact or solubilized membrane fractions in attempts to detect specific protein–protein interactions. The only protein that we detected to bind significantly to 14-3-3 proteins using these approaches

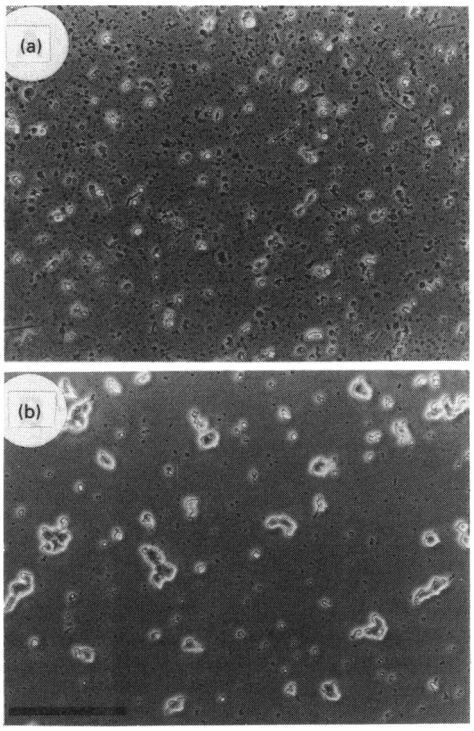

Figure 6 Aggregation of phospholipid vesicles by brain 14-3-3 proteins

Phospholipid vesicles (20 μl) formed from phosphatidylinositol were incubated alone (**a**) or with 1.5 μg of brain 14-3-3 proteins (**b**) for 30 min and then examined by phase-contrast light microscopy. Considerable aggregation of vesicles by 14-3-3 proteins had occurred over this time. The scale bar represents 100 μm.

was γ 14-3-3 itself. We therefore explored the possibility that γ 14-3-3 interacts with phospholipid.

Phospholipid vesicles were prepared with a range of phospholipids and incubated with brain 14-3-3 proteins, and bound proteins were assayed by immunoblotting of the pelleted vesicles. The β, ζ and η isoforms showed little binding to phospholipid vesicles, although low levels of binding were detected to phosphatidylcholine/phosphatidic acid (3:1, w/w) vesicles (Figure 5). In contrast, γ 14-3-3 bound efficiently to all vesicles tested. The extent of binding of γ 14-3-3 was in the range of 8–35% of total γ in the incubation, with the highest binding to phosphatidylinositol and the lowest to phosphatidylethanolamine/phosphatidic acid vesicles. In other experiments γ 14-3-3 was found to bind equally well to phosphatidylinositol, phosphatidylinositol phosphate and phosphatidylinositol bisphosphate (results not shown). Furthermore, unbound γ 14-3-3 could bind to phospholipid following a further addition of phospholipid vesicles, ruling out selective binding of a fraction of the γ isoform. These data emphasize isoform differences in the properties of the 14-3-3 proteins and indicate that the γ isoform is a phospholipid-binding protein. Binding to the same range of phospholipid vesicles was also detected for the ε isoform (Figure 5), indicating that this isoform is also a phospholipid-binding protein.

The ability of 14-3-3 proteins to bind to phospholipid vesicles was confirmed by an assay for vesicle aggregation. Light-microscopical examination indicated that incubation of phospholipid vesicles with brain 14-3-3 proteins induced their aggregation (Figure 6). In control incubations in which BSA replaced 14-3-3 proteins, no aggregation was observed (results not shown).

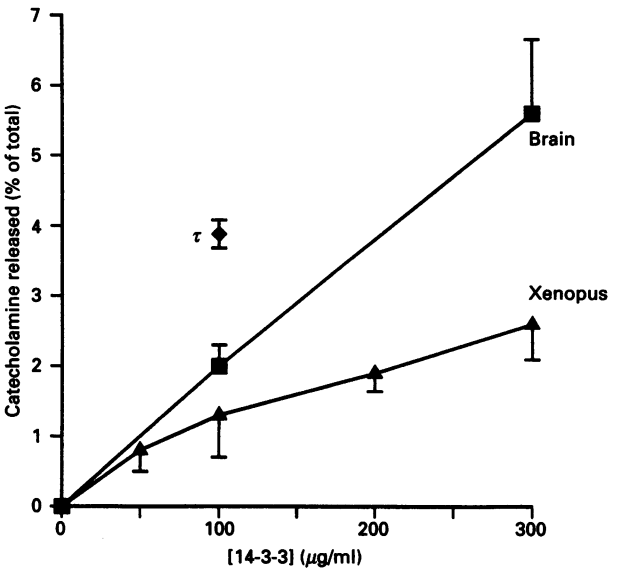

Figure 7 Effect of recombinant 14-3-3 protein isoforms on Ca^{2+}-dependent exocytosis in permeabilized adrenal chromaffin cells

Chromaffin cells were permeabilized with digitonin for 10 min, incubated in step 2 with brain 14-3-3 proteins, recombinant mammalian τ 14-3-3 or recombinant *Xenopus* 14-3-3 for 15 min, and then challenged with 0 or 10 μM Ca^{2+}. The extent of Ca^{2+}-dependent catecholamine release was expressed as a percentage of total cellular catecholamine ($n = 4$), and control values were subtracted. The recombinant proteins were expressed and purified as described in the Materials and methods section.

Effect of recombinant τ and *Xenopus* 14-3-3 proteins on Ca^{2+}-dependent exocytosis in adrenal chromaffin cells

We have demonstrated that purified brain 14-3-3 proteins (Exo1 [25,26]) stimulated Ca^{2+}-dependent exocytosis in digitonin-permeabilized chromaffin cells and that this effect was specific to a small number of identified proteins [25,33,34]. Since the active factor consisted of a number of 14-3-3 isoforms we examined whether individual purified recombinant proteins were also functional. 14-3-3 proteins are dimers in their native state and the use of recombinant proteins allows assessment of whether a homodimer is sufficient for activity. The recombinant τ and *Xenopus* isoforms were both active in stimulating Ca^{2+}-dependent exocytosis (Figure 7), although the *Xenopus* isoform was less effective than the brain 14-3-3 proteins. The *Xenopus* protein was expressed from a clone that lacked part of the N-terminus and begins at a residue equivalent to residue 11 of the mammalian β 14-3-3 protein. Therefore the extreme N-terminus of the protein is not essential for its ability to stimulate exocytosis. In control experiments a recombinant cytoplasmic fragment of the granule protein synaptotagmin was ineffective in stimulating secretion at concentrations up to 400 μg/ml (D. Roth, B. Tugal, J. Haywood, D. Apps and R. D. Burgoyne, unpublished work).

DISCUSSION

Exogenous brain 14-3-3 proteins stimulate Ca^{2+}-dependent exocytosis in digitonin-permeabilized adrenal chromaffin cells following cytosolic protein leakage and secretory run-down [25–27]. The mechanism by which these proteins regulate exocytosis is not known. Here we have characterized the 14-3-3 protein isoforms present in chromaffin cells and their distribution in subcellular fractions, and demonstrated that recombinant 14-3-3 protein isoforms are effective in stimulating Ca^{2+}-dependent secretion.

Chromaffin cells do not possess detectable levels of the η, τ and σ 14-3-3 isoforms. The ϵ isoform was at the limits of detectability and was not examined in detail. Chromaffin cell 14-3-3 proteins were detected with anti-peptide antisera that recognize the β, γ and ζ isoforms [19]. The anti-γ antiserum does not recognize any other known isoforms, but the anti-β and -ζ antisera do show a low level of cross-reactivity with the other isoform [19]. Hence we cannot say for certain whether both β and ζ isoforms are present in chromaffin cells. The mRNA for the β isoform has been detected in adrenal medulla [5] and so it seems that this isoform at least is expressed in chromaffin cells. It should be noted, however, that in experiments with brain 14-3-3 proteins, which contain both β and ζ isoforms, no differences were detected in their behaviour and so the uncertainty about the presence of the ζ isoform does not affect the central conclusions of the present paper.

Relatively little is known about the subcellular localization of the 14-3-3 proteins. While isoforms have been detected that are associated with the Golgi [12], the 14-3-3 proteins generally behave as soluble cytosolic proteins. We expected, therefore, that they would leak substantially from chromaffin cells following digitonin-permeabilization, thereby explaining the observation of a stimulatory effect of the exogenous proteins [25–27]. Substantial leakage was in fact detected with the isoform-specific antisera, but after a 25 min permeabilization period some retained 14-3-3 proteins were detected in the cells. The retention of a proportion of the 14-3-3 protein suggested that not all of the proteins were cytosolic. From an examination of subcellular fractions it was clear that anti-β and -ζ recognized proteins in the Triton-insoluble cytoskeleton and that the γ isoform was also associated with both microsomal and chromaffin granule membrane fractions. The γ isoform was probably associated as an extrinsic membrane protein since it could be partially removed by a high-salt/carbonate wash and additional γ could be bound from cytosol or purified 14-3-3 proteins. The binding of γ to chromaffin granule membranes was not Ca^{2+}-dependent, nor was it affected by other agents that regulate exocytosis in chromaffin cells, namely phorbol ester, non-hydrolysable GTP analogues or cyclic AMP [34,35]. We were unable to detect any specific protein binding partners for γ 14-3-3 apart from γ itself, and so the possibility that the γ isoform interacted with phospholipid was examined by incubation of brain 14-3-3 proteins with phospholipid vesicles. The results from these experiments showed that the γ and ϵ isoforms, but not the β, ζ or η isoforms, bind efficiently to a range of phospholipids. These findings can therefore explain the isoform-specific distribution of 14-3-3 proteins in subcellular fractions from adrenal medulla. Comparison of the sequences of the 14-3-3 protein isoforms does not provide any clues to the basis for the differing abilities of the isoforms to bind to phospholipids. We cannot rule out the possibility that β, ζ and η isoforms may bind to phospholipids under other conditions.

The functional significance for exocytosis of the interaction of 14-3-3 isoforms with phospholipids or with the cytoskeleton is not yet clear. The 14-3-3 proteins could stimulate secretion either by a direct effect on membrane fusion or by increasing the disassembly of cortical actin, which appears to be required for further mobilization of secretory granules [35,36]. It is possible that the 14-3-3 isoforms associated with the cytoskeleton could regulate actin disassembly during Ca^{2+}-dependent secretion. We have been unable, however, to detect any effect of exogenous 14-3-3 proteins on actin organization in permeabilized chromaffin

cells using rhodamine–phalloidin staining (D. Roth et al., unpublished work). Membrane fusion in exocytosis will involve proteins able to modify or reorganize lipids within the plasma and granule membranes [35]. The γ and ϵ 14-3-3 isoforms bind to phospholipids and aggregate phospholipid vesicles, suggesting that they could play some role in membrane events during exocytosis.

Two recombinant 14-3-3 isoforms, the τ and *Xenopus* proteins, were able to stimulate Ca^{2+}-dependent exocytosis in permeabilized chromaffin cells. These findings indicate that homodimers are functional in this assay. In addition, since the *Xenopus* protein begins at around residue 11 of the mammalian protein, we can conclude that the extreme N-terminus of the 14-3-3 proteins, which is one of the most variable domains [1], is not essential for their function in exocytosis. Obviously, neither the τ isoform nor the *Xenopus* protein is expressed in chromaffin cells, but the significance of the functional data using these recombinant proteins is that they indicate a lack of absolute isoform specificity in the stimulation of secretion. As other recombinant isoforms become available it will be important to carry out a full analysis of the ability of the various isoforms to stimulate secretion. In addition, the ability of recombinant proteins to function in the exocytosis assay will now allow more detailed analysis of which domains of the protein are necessary for the stimulation of exocytosis [37] and for lipid binding, and of the relationship between these two functional aspects of the 14-3-3 proteins.

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