

Stimulation of catecholamine secretion from adrenal chromaffin cells by 14–3–3 proteins is due to reorganisation of the cortical actin network

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Abstract Catecholamine release from digitonin-permeabilized adrenal chromaffin cells is increased by exogenous 14–3–3 proteins. In order to determine how 14–3–3 proteins stimulate exocytosis their effect on the cortical actin network was examined. Increased amounts of β and γ isoforms of 14–3–3 proteins were associated with the Triton-insoluble cytoskeleton of chromaffin cells following incubation with exogenous 14–3–3 proteins. The stimulation of catecholamine release by 14–3–3 proteins was abolished by prior incubation with the actin filament stabilising drug phalloidin. Rhodamine phalloidin staining showed that the cortical actin network was disassembled and actin reorganised into intracellular foci following treatment with 14–3–3 proteins. These data suggest that 14–3–3 proteins enhance catecholamine release in permeabilized chromaffin cells by reorganisation of the cortical actin barrier to allow increased availability of secretory vesicles for exocytosis.

Key words: Exocytosis; 14–3–3 Protein; Secretion; Calcium; Chromaffin cell

1. Introduction

The release of stored catecholamines from adrenal chromaffin cells occurs by Ca^{2+} -dependent exocytosis. This involves multiple stages including secretory vesicle recruitment, docking at the plasma membrane and finally membrane fusion [1,2]. Kinetically distinct stages can be detected in patch-clamp capacitance analyses which have been attributed to rapid exocytosis of a 'release-ready' pool of vesicles near the plasma membrane and a slower release phase following recruitment from a reserve pool [3,4]. Studies on permeabilized chromaffin cells have also delineated two stages which are an ATP-dependent priming reaction followed by Ca^{2+} -triggered and ATP-independent fusion [5,6]. The ATP-dependent reaction presumably reflects secretory vesicle recruitment and is likely to involve preparation of the docking/fusion machinery. In addition, the cortical actin cytoskeleton forms a barrier to exocytosis for the majority of secretory vesicles in chromaffin cells since they are excluded from the cell periphery [7–14]. The cortical actin network disassembles transiently during cell stimulation [7–12] and this may form part of the priming reaction since cortical actin disassembly is not sufficient to allow exocytosis to occur [8,9] but results in an increase in the rate and extent of exocytosis in a subsequent stimulation [12].

The use of permeabilized chromaffin cells which lose much of their complement of cytosolic proteins by leakage through

the plasma membrane pores [15] has provided an assay for the identification of soluble proteins that act in one of other stage of the exocytotic process. These include annexin II [16], calmodulin [17,18] 14–3–3 proteins [19–21], α -SNAP [22] protein kinase C [23] and protein kinase A [24]. Studies on permeabilized PC12 cells have also identified cytosolic proteins that stimulate exocytosis and shown that one of these, p145 [25], acts in the triggering reaction while a series of proteins involved in the pathway leading to synthesis of phosphatidylinositol (4,5) biphosphate act in the ATP-dependent priming reaction [26–28].

14–3–3 Proteins stimulate exocytosis from permeabilized chromaffin cells in a Ca^{2+} -dependent manner and this stimulation is enhanced by activation of protein kinase C [19,23]. The 14–3–3 proteins are a ubiquitous family of homologous proteins which have been suggested to regulate numerous cellular processes [29]. They associate with protein kinase C and have recently been found to interact with several other protein kinases including raf, a key component of the ras/MAP kinase signalling pathway [30,31]. The mode of action of 14–3–3 proteins on exocytosis is unknown but they could potentially stimulate exocytosis in chromaffin cells by regulation of a kinase pathway or by some alternative mechanism. Recently we have shown that 14–3–3 proteins act in the ATP-dependent priming reaction [18]. They do not act by increasing levels of phosphatidylinositol (4,5) biphosphate [18] which has previously been shown to be one biochemical aspect of priming [26–28]. In this paper we have examined the possibility that 14–3–3 proteins lead to increased priming and stimulate secretion by an effect on the cortical actin network. The data presented here are consistent with a role for 14–3–3 proteins in chromaffin cells in removal of the cortical actin barrier to exocytosis.

2. Materials and methods

2.1. Materials

Anti-peptide antisera against N-terminal sequences of β and γ 14–3–3 proteins were a gift from Dr. Alastair Aitken (National Institute for Medical Research, London, UK). 14–3–3 Proteins were purified from sheep brain cytosol as previously described [19]. High purity digitonin was obtained from Novabiochem (Nottingham, United Kingdom). Immunological and Enhanced Chemiluminescence (ECL) reagents were from Amersham (High Wycombe, United Kingdom). All other reagents were of analytical grade from Sigma (Poole, United Kingdom).

2.2. Chromaffin cell cultures and cell permeabilization

Bovine adrenal medullae were dissociated by enzyme digestion as described previously and grown in culture in 24 well trays [32] for 3–6 days. The cells were washed in 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 pH 7.4 and permeabilized by incubation for 6 mins in 20 μM digitonin in KGEP buffer (139 mM potassium glutamate, 20 mM PIPES and 5 mM EGTA pH 6.5) with 2 mM MgATP. For rhodamine-phalloidin staining, the cells were grown on glass coverslips in 24 well trays at a density of 500,000 per well for 3 days.

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2.3. Extraction of the Triton-insoluble cytoskeleton and immunoblotting

Chromaffin cells were permeabilized with digitonin for 6 min and incubated in KGEP with or without added phorbol 12-myristate 13-acetate (PMA) (100 nM) or MgATP (2 mM) for 45 min and then incubated in the same buffers with or without 14–3–3 proteins (200 μ g/ml) for a further 2 min. The buffer was removed and the cells extracted by addition of ice-cold 1% Triton X-100 in 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 10 mM Tris-HCl pH 7.0 and kept on ice for 5 min [9]. The extraction buffer was removed and the Triton insoluble cytoskeletal residue solubilised with SDS-dissociation buffer [9] and samples from 3 wells were pooled (200 μ l in total). Aliquots of the pooled samples (20 μ l per lane) were separated by SDS-polyacrylamide gel electrophoresis on 10% gels, transferred to nitrocellulose and probed with rabbit antisera against β or γ 14–3–3 [33] at 1:1000 dilution using an ECL detection system [20]. ECL films were scanned and bands quantified using ImageQuant software (Molecular Dynamics).

2.4. Catecholamine secretion from permeabilized cells

Chromaffin cells were permeabilized for 6 min and then incubated in KGEP with 2 mM MgATP and 100 nM PMA for 45 min with or without 25 nM phalloidin and then incubated for 2 min with 200 μ g/ml 14–3–3 proteins in KGEP with 2 mM MgATP followed by a final stimulation incubation in KGEP containing 10 μ M free Ca^{2+} for 20 min. The extent of release of endogenous catecholamines during the final incubation was determined using a fluorometric assay [34]. In order to examine the effects of cytochalasin D on secretion the cells were permeabilised, incubated in KGEP for 45 min and then incubated in an additional 1 min step with 2.5 μ M cytochalasin D before incubation with or without 14–3–3 proteins for 2 min also in the presence of 2.5 μ M cytochalasin D immediately prior to the final stimulation incubation in KGEP plus 10 μ M Ca^{2+} for 20 min. Catecholamine release was calculated as a percentage of control release and expressed as mean \pm S.E.M.

2.5. Rhodamine-phalloidin staining of permeabilized chromaffin cells

Chromaffin cells were permeabilized for 6 min and then incubated in KGEP with or without 2 mM MgATP or 100 nM PMA for 45 min. The cells were then incubated in the same buffers with 2.5 μ M cytochalasin D for 1 min followed by the same buffers with or without 200 μ g/ml 14–3–3 proteins. After 2 min the cells were fixed in KGEP containing 2% formaldehyde for 2 h at room temperature. The cells were washed and incubated for 30 min in Tris buffered saline (TBS: 130 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 10 mM Tris HCl, pH 7.5) containing 0.3% BSA and 0.1% Triton X-100. Filamentous actin was then stained by incubation with 25 nM rhodamine-phalloidin in TBS for 15 min [7]. Fluorescence staining was examined using a conventional fluorescence microscope and using confocal laser scanning microscopy.

3. Results

3.1. Binding of exogenous 14–3–3 proteins to the Triton-insoluble cytoskeleton in permeabilized chromaffin cells

Previous findings had shown that the β and ζ isoform of 14–3–3 proteins were distributed between cytosol and the Triton-insoluble cytoskeleton [20]. In contrast, γ 14–3–3 though present in cytosol was present at only low levels in the Triton-insoluble cytoskeletal fraction and a major proportion of this isoform was membrane-associated due to its ability to bind phospholipids [20]. The ability of 14–3–3 isoforms to bind to phospholipids was not related to their stimulation of catecholamine release from chromaffin cells since two isoforms which stimulated release, the *Xenopus* and mammalian τ isoforms [20], did not bind to phospholipid vesicles (Roth and Burgoyne, unpublished observations). We, therefore, examined the possibility that the stimulation of the exocytotic pathway was related to the interaction of 14–3–3 isoforms with the cytoskeleton.

The ability of exogenous β and γ 14–3–3 isoforms to associate with the cytoskeleton in permeabilized cells was first exam-

ined. Following permeabilization and incubation for a further 45 min chromaffin cells were incubated with a mixture of brain 14–3–3 proteins, the cells extracted with Triton X-100 and the Triton-insoluble cytoskeleton probed by immunoblotting with antibodies against β and γ 14–3–3 isoforms. Incubation with exogenous 14–3–3 proteins led to increased association of both β and the γ isoforms with the Triton-insoluble cytoskeleton. The increase in cytoskeleton-associated 14–3–3 protein isoforms ranged from 1.6 to 3.4 fold and was not reproducibly affected by the activation of protein kinase C by the phorbol ester PMA or by the presence or absence of MgATP (Fig. 1).

3.2. Effects of cytoskeletal drugs on the stimulation of catecholamine release by 14–3–3 proteins

If 14–3–3 proteins acted to increase exocytotic catecholamine release by reorganising the cortical actin cytoskeleton to allow increased granule recruitment, then its effect should be modified by phalloidin which stabilizes actin filaments. In these experiments, the assay was based on the ability of a prior incubation with 14–3–3 in the presence of MgATP to rapidly prime the cells and increase catecholamine release in a subsequent stimulation step with 10 μ M Ca^{2+} in the absence of MgATP [18]. Prior incubation with 25 nM phalloidin had no effect on control secretion but prevented the stimulation of catecholamine release by 14–3–3 proteins and indeed resulted in 14–3–3 proteins producing a small inhibition of release (Fig. 2). These data are consistent with 14–3–3 proteins priming the exocytotic mechanism by an effect on the cortical actin barrier.

The effect of cytochalasin D was also examined. This drug blocks assembly of actin filaments but it had no detectable effect on the actin cytoskeleton in chromaffin cells in incubations for up to 30 min (data not shown). It was, however, included with 14–3–3 proteins since it would be expected to prevent actin reassembly if this was brought about by 14–3–3 proteins. Cytochalasin D (2.5 μ M) did not, however, modify control secretion or the stimulation by 14–3–3 proteins (Fig. 3).

3.3. Reorganisation of the cortical actin network by 14–3–3 proteins

In light of the findings with cytoskeletal drugs the effect of

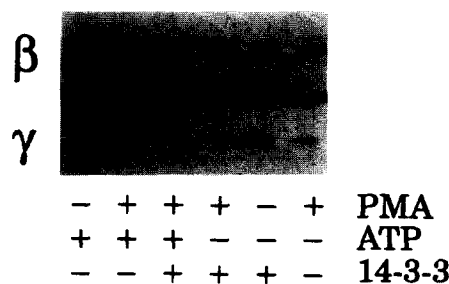


Fig. 1. Association of exogenous β and γ 14–3–3 proteins with the Triton-insoluble cytoskeleton of permeabilized chromaffin cells. Chromaffin cells were permeabilized with digitonin, incubated with KGEP with or without PMA (100 nM) or MgATP (2 mM) as indicated for 45 min and then incubated in the same buffers with or without 14–3–3 proteins (200 μ g/ml) for 2 min. The cells were extracted with 1% Triton X-100 and insoluble cytoskeletal material separated by SDS-PAGE and probed with antibodies against 14–3–3 β and γ isoforms. The levels of 14–3–3 isoforms after incubation with exogenous 14–3–3 proteins were quantified by densitometry and expressed as a percentage of control for incubations with both PMA and ATP (β , 156%; γ , 253%), with PMA alone (β , 211%; γ , 223%) and without PMA or ATP (β , 171%; γ , 347%).

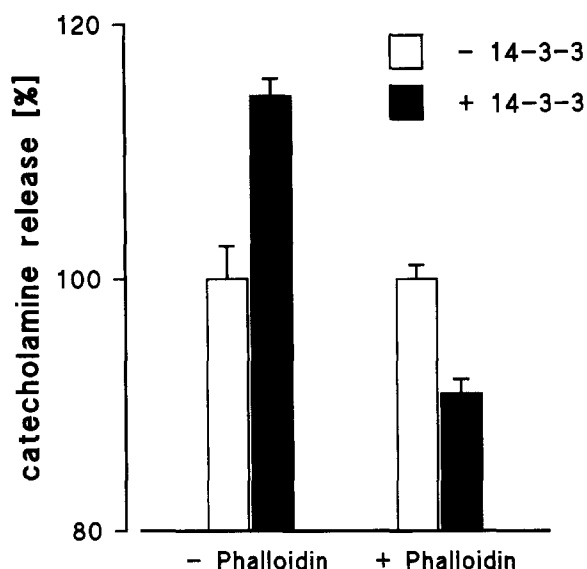


Fig. 2. Effect of phalloidin on catecholamine release stimulated by 14-3-3 proteins. Chromaffin cells were permeabilized, incubated in KGEP with 2 mM MgATP and 100 nM PMA with or without 25 nM phalloidin for 45 min, incubated for 2 min with or without 200 μ g/ml 14-3-3 proteins and then catecholamine release was assayed over 20 min in KGEP with 10 μ M free Ca^{2+} . The data are expressed as mean percentage \pm S.E.M. of control release ($n = 3$ wells). Control release was $12.3 \pm 0.4\%$ of total cellular catecholamine.

14-3-3 proteins on the cortical actin network in permeabilized chromaffin cells was examined. Changes in cortical actin in intact cells are rapid and transient [7] and the effect of 14-3-3 proteins on catecholamine release are manifest within 1–2 min [18]. Cytochalasin D had no effect on control or 14-3-3 stimulated secretion and so it was included in experiments on actin organisation so that actin reassembly would be inhibited and changes in actin essentially frozen. Staining of chromaffin cells with rhodamine-phalloidin revealed that essentially all cells possessed distinct cortical actin staining as previously shown [7,11,14]. This was unaffected by the presence of cytochalasin D in these experiments. Treatment with PMA alone (Fig. 4b) had no discernible effect on cortical actin but incubation with 14-3-3 proteins led to a disappearance of the cortical actin ring and appearance of intracellular foci of staining (Fig. 4c). PMA treatment potentiates the effect of 14-3-3 proteins on secretion and the actin changes were more marked in cells treated with both PMA and 14-3-3 proteins (Fig. 4e,f). The ability of 14-3-3 proteins to prime secretion requires the presence of MgATP and so the effects of MgATP on actin reorganisation due to 14-3-3 proteins was examined. No changes in actin organisation due to 14-3-3 proteins were discernable in the absence of MgATP (Fig. 4d). Similar results were seen in experiments on four separate batches of chromaffin cells. The changes in the organisation of actin filaments were also confirmed by confocal laser scanning microscopy.

4. Discussion

It has been previously established that addition of 14-3-3 proteins to permeabilised adrenal chromaffin cells stimulates Ca^{2+} -dependent catecholamine release [19–21,23] and does so

by an action in the ATP-dependent priming reaction [18]. 14-3-3 proteins have had many suggested functions but a common aspect is an interaction with protein kinases [29–31]. The effect of 14-3-3 proteins on catecholamine release was potentiated by activation of protein kinase C but could not be explained on the basis of a direct regulation of protein kinase C activity [23]. We now show that 14-3-3 proteins stimulate catecholamine release by reorganisation of the cortical actin cytoskeleton that normally acts as a barrier to exocytosis of the majority for secretory vesicles and which is transiently disassembled following stimulation in intact [7,11,12] or permeabilized [9] chromaffin cells. This conclusion is based on three findings: (i) exogenous β and γ 14-3-3 proteins became associated with the Triton-insoluble cytoskeleton in permeabilized cells; (ii) the stimulation of catecholamine release by 14-3-3 proteins in an assay for ATP-dependent priming was prevented by prior treatment with the actin filament stabilizing drug phalloidin; (iii) it was directly demonstrated by rhodamine-phalloidin staining that the 14-3-3 proteins lead to a reorganisation of the actin cytoskeleton including the disappearance of the cortical actin network and that this was enhanced by prior treatment with PMA and required the presence of MgATP as did the effect of 14-3-3 proteins on catecholamine release.

The stimulation of catecholamine release by 14-3-3 proteins is MgATP dependent [18]. We found that 14-3-3 proteins only lead to discernable effects on the actin cytoskeleton in the presence of MgATP but the binding of the β and γ isoforms to the Triton-insoluble cytoskeleton was not MgATP dependent and indeed there is no evidence that the 14-3-3 proteins are themselves ATP-binding proteins. Activation of protein kinase C by PMA enhances both catecholamine release [19,23] and the

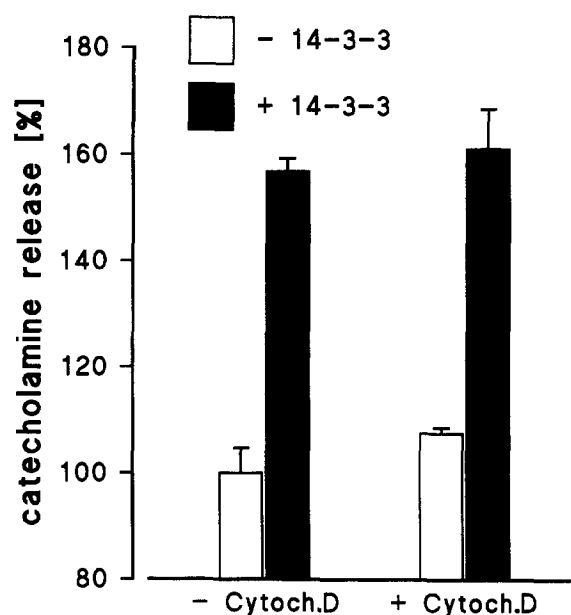


Fig. 3. Effect of cytochalasin D on catecholamine release stimulated by 14-3-3 proteins. Chromaffin cells were permeabilized, incubated in KGEP with 2 mM MgATP and 100 nM PMA for 45 min, incubated with 2.5 μ M cytochalasin D for 1 min followed by 200 μ g/ml 14-3-3 proteins for 2 min. Catecholamine release was then assayed over 20 min in KGEP with 10 μ M free Ca^{2+} . The data are expressed as mean percentage \pm S.E.M. of control release ($n = 3$ wells). Control release was $9.42 \pm 0.5\%$ of total cellular catecholamine.

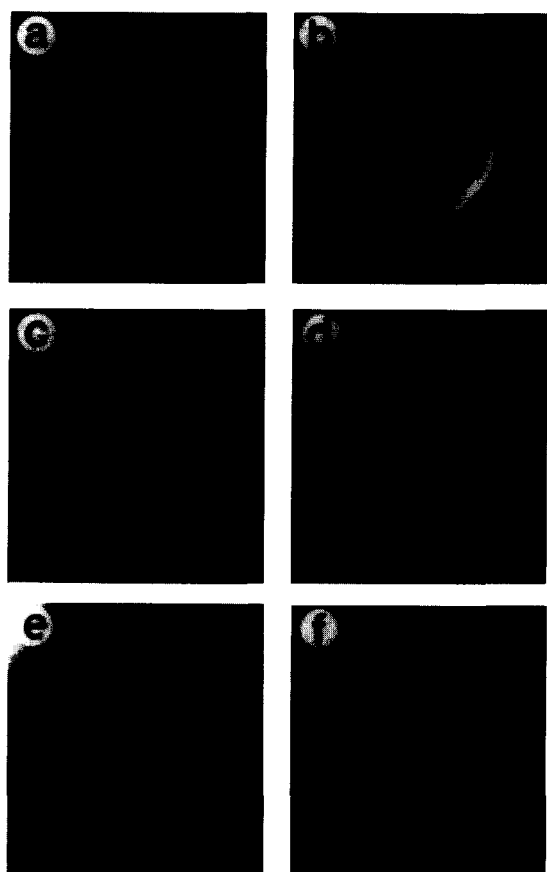


Fig. 4. Effect of 14–3–3 proteins on actin organization in chromaffin cells. Chromaffin cells were permeabilized, incubated in KGEP with or without MgATP or 100 nM PMA for 45 min and incubated with 2.5 μ M cytochalasin D for 1 min. The cells were then incubated in the same buffers with or without 200 μ g/ml 14–3–3 proteins for 2 min, fixed and stained for polymerized actin with rhodamine-phalloidin. Control cells with MgATP (a), cells with MgATP and PMA (b) cells with MgATP and 14–3–3 proteins (c), cells with PMA and 14–3–3 proteins (d), cells with MgATP, PMA and 14–3–3 proteins (e,f).

actin reorganisation. No effect was seen of PMA treatment alone on the cortical actin network in this study. It has previously been shown that phorbol ester treatment leads to rapid and reversible disassembly of cortical actin in intact chromaffin cells [9,12] and in newly permeabilized chromaffin cells in the presence of 0.3 μ M free Ca^{2+} [9]. In the present study the Ca^{2+} was clamped at low levels throughout the incubations in buffers containing 5 mM EGTA and no added Ca^{2+} and any PMA effect on cortical actin would have been reversed in the long permeabilization time used here explaining the lack of observed effect of PMA treatment alone. The stimulation of catecholamine release by 14–3–3 proteins is Ca^{2+} -dependent [19] but the actin reorganisation occurs in the absence of Ca^{2+} . Thus, it is likely that increased Ca^{2+} concentration is essential to trigger exocytosis of the recruited vesicles and that removal of the actin barrier is not sufficient to allow exocytosis to occur [8,9,12].

When the permeabilized chromaffin cells were pretreated with a low concentration of phalloidin, control catecholamine release after the prolonged permeabilization period used here was unaffected though others have found inhibitory effects using higher concentrations of phalloidin in freshly permeabilized cells [35]. Nevertheless, phalloidin prevented the stimula-

tion of catecholamine release by exogenous 14–3–3 proteins and under these conditions a small inhibitory effect of 14–3–3 proteins was apparent instead. This is consistent with earlier data showing that when cells were stimulated with 10 μ M Ca^{2+} in the presence of 14–3–3 proteins but in the absence of MgATP (a triggering reaction) the ATP-dependent stimulation by 14–3–3 proteins was lost and an inhibitory effect was observed [18].

In conclusion, we have demonstrated that exogenous 14–3–3 proteins lead to reorganisation of the actin cytoskeleton in permeabilized chromaffin cells in a manner closely paralleling their effect on catecholamine release and that the stimulatory effect on release is abolished by treatment with the actin stabilizing drug phalloidin. It seems likely, therefore, that the ability of 14–3–3 proteins to act in a priming reaction to increase exocytosis is due to removal of the cortical actin barrier to allow access of increased numbers of secretory vesicles to exocytotic sites on the plasma membrane.

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