J. Anat. (1993) 183, pp. 309–314, with 3 figures Printed in Great Britain

Exocytosis in adrenal chromaffin cells

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(*Accepted 1 March 1993*)

ABSTRACT

Recent advances have led to an increased understanding of the Ca^{2+}-signalling pathway leading to exocytosis in bovine adrenal chromaffin cells. Video-imaging studies have allowed the temporal and spatial aspects of the Ca^{2+} signal to be investigated in detail. Ca^{2+} entry at the plasma membrane appears to be crucial for the activation of exocytosis. Ca^{2+} can enter through the nicotinic channel or characterised voltage-activated channels, or through other poorly defined pathways due to a variety of agonists. Emptying of internal Ca^{2+} stores is sufficient to activate a Ca^{2+} entry pathway. The elevation of cytosolic Ca^{2+} concentration leads to a reorganisation of the cortical actin network and to the triggering of exocytosis. Studies on permeabilised chromaffin cells have resulted in the identification of some of the proteins that control Ca^{2+}-dependent exocytosis. These include the peripheral plasma membrane protein annexin II and the cytosolic proteins, protein kinase C and 14-3-3 proteins (Exo1).

INTRODUCTION

Secretion of catecholamines and other components of the chromaffin granule is triggered by a rise in the concentration of intracellular free calcium ($[Ca^{2+}]_i$) (Knight et al. 1989*b*; Burgoyne 1991). A variety of agonists can raise $[Ca^{2+}]_i$ in chromaffin cells by activating either Ca^{2+} entry or Ca^{2+} mobilisation from internal stores. Exocytosis is only stimulated, however, by agonists that activate Ca^{2+} entry through plasma membrane Ca^{2+} channels. The movement of chromaffin granules to the plasma membrane and their subsequent exocytotic fusion occurs following disassembly of a cortical actin network (Cheek & Burgoyne, 1991). The proteins that then act to transduce the rise in $[Ca^{2+}]_i$ into membrane fusion have not yet been fully characterised. Even though exocytosis is being studied using many cell types, relatively little is known about possible components of the exocytotic machinery. Adrenal chromaffin cells provide a useful system for the investigation of all aspects of the control of exocytosis (Burgoyne, 1991) and in this article we concentrate on recent advances in the study of Ca^{2+}-signalling in bovine chromaffin cells and on molecular aspects of the exocytotic process from studies on these cells.

CALCIUM SIGNALLING IN CHROMAFFIN CELLS

A rise in $[Ca^{2+}]_i$ has been shown to be a sufficient signal to trigger exocytosis in chromaffin cells (Knight & Baker, 1982) and the use of intracellular Ca^{2+} indicators has allowed detailed analysis of the Ca^{2+} signals generated by various agonists. In particular, video-imaging techniques have allowed determination of the spatial aspects of the Ca^{2+} signals (Burgoyne et al. 1989*a*; Cheek et al. 1989*a*, *b*, 1990, 1991*b*; O'Sullivan et al. 1989). From these studies it appears that chromaffin cells possess at least 2 mobilisable Ca^{2+} pools, one selectively sensitive to IP_3 and the other to caffeine and ryanodine (Burgoyne et al. 1989*a*; Cheek et al. 1990). The distinction between these 2 pools has been confirmed in experiments with permeabilised cells which allow direct access to the intracellular storage organelles (Cheek et al. 1991*a*; Robinson & Burgoyne, 1991*b*; Stauderman et al. 1991). Release of Ca^{2+} from these internal stores appears to be ineffective in stimulating catecholamine release and, for all agonists tested, catecholamine release from bovine chromaffin cells is absolutely dependent on the presence of external Ca^{2+} (O'Sullivan & Burgoyne, 1989). Therefore, Ca^{2+} entry across the plasma membrane to raise subplasma-

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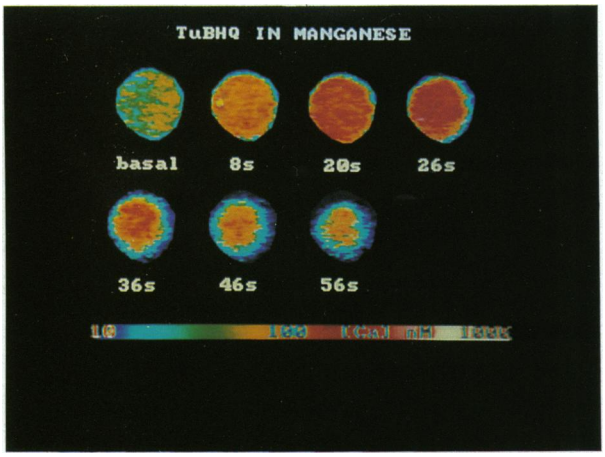

Fig. 1. Effect of the Ca^{2+}-ATPase inhibitor tuBHQ on Mn^{2+} entry into chromaffin cells. Following addition of 25 μM tuBHQ the initial response is a rise in $[Ca^{2+}]_i$ due to Ca^{2+} release from stores and after around 20 s the response becomes dominated by fluorescence quenching due to Mn^{2+} entry and the apparent size of the cell decreases as a consequence. In this experiment the cells were stimulated in 1 mM Mn^{2+} but no external Ca^{2+}.

lemmal $[Ca^{2+}]_i$ seems to be essential for exocytosis in the bovine adrenal chromaffin cell (Cheek et al. 1989*a*, *b*; Kim & Westhead, 1989; O'Sullivan et al. 1989; Stauderman et al. 1990).

Activation of nicotinic acetylcholine receptors is the most effective way of stimulating secretion from bovine chromaffin cells and secretion occurs as result of Ca^{2+} entry through voltage-dependent Ca^{2+} channels and the nicotinic receptor channel. Other agonists, such as the IP_3-mobilising agonists angiotensin II and histamine, for example, are able to stimulate catecholamine release without apparently depolarising the cells and opening voltage-dependent channels. The Ca^{2+} entry pathways opened by these agonists are still to be defined (Stauderman et al. 1990; Cheek et al. 1991*b*). It appears that Ca^{2+} mobilisation from internal stores can activate the opening of entry pathways. The Ca^{2+}-ATPase inhibitors thapsigargin and tuBHQ release Ca^{2+} from stores (Robinson & Burgoyne, 1991*a*) and stimulate Mn^{2+} entry (Fig. 1) across the plasma membrane in chromaffin cells (Robinson et al. 1992). It is not yet known whether IP_3-generating agonists such as angiotensin II and histamine stimulate Ca^{2+} entry as a consequence of store emptying being linked to the opening of plasma membrane channels (Putney, 1986) or through more direct receptor-mediated control of plasma membrane Ca^{2+} channels. Further work is required to identify and characterise the channels involved in chromaffin and other cell types.

Within chromaffin cell populations angiotensin II raises $[Ca^{2+}]_i$ in only a proportion of the cells, and in only a further subset of these is exocytosis activated (Cheek et al. 1989*a*). Ca^{2+} mobilisation from stores (Cheek et al. 1989*a*) and divalent cation entry in response to angiotensin II are both polarised (Cheek et al. 1991*b*) and, in addition, exocytosis is also polarised (Cheek et al. 1989*a*). In contrast, histamine raises $[Ca^{2+}]_i$ in almost all cells within the population due to release from internal stores (Cheek et al. unpublished observations; Stauderman et al. 1990; Stauderman & Murawsky, 1991). Divalent cation entry, monitored using Mn^{2+} as a Ca^{2+} surrogate, can be detected in response to histamine. This entry is not polarised but occurs over the whole cell. Nevertheless when exocytosis in response to histamine was monitored by following exposure on the cell surface of the granule membrane protein dopamine-β-hydroxylase (Phillips et al. 1983; Patzak et al. 1984), it was found that histamine induced exocytosis over the whole cell but only in a subpopulation of chromaffin cells (Pender & Burgoyne, 1992). In contrast, nicotine induces exocytosis in up to 98% of the cells in the population. It may be that exocytosis only occurs in those cells in which histamine is able to activate Ca^{2+} entry in addition to Ca^{2+} mobilisation.

Despite extensive work on the characterisation of calcium signals in chromaffin cells and other cell types, the exact concentration of free calcium required at subplasmalemmal sites to activate exocytosis is unknown. Knowledge of this would be useful in assessing the significance of any potential Ca^{2+}-binding proteins in exocytosis. The rise in $[Ca^{2+}]_i$ immediately beneath the plasma membrane that is necessary to activate exocytosis would occur initially in a region too small to be resolved by current videoimaging techniques, which are only able to resolve Ca^{2+} signals spread over around 1 μm. Theoretical calculations have suggested that $[Ca^{2+}]_i$ could rise to 10–100 μM close to the plasma membrane after the opening of Ca^{2+} channels (Simon & Llinas, 1985; Artalejo et al. 1987; Smith & Augustine, 1988). It seems likely that the $[Ca^{2+}]_i$ required for maximal secretion from permeabilised chromaffin cells (10 μM) is a reasonable estimate of the $[Ca^{2+}]_i$ required at the plasma membrane exocytotic sites.

Control of the actin cytoskeleton and exocytosis in chromaffin cells

Following activation of chromaffin cells and the elevation of $[Ca^{2+}]_i$ one of the early events that occurs is disassembly of the cortical actin network (Cheek & Burgoyne, 1986, 1987). It seems likely that the access of secretory granules to the plasma membrane is restricted by the subplasmalemmal actin network.

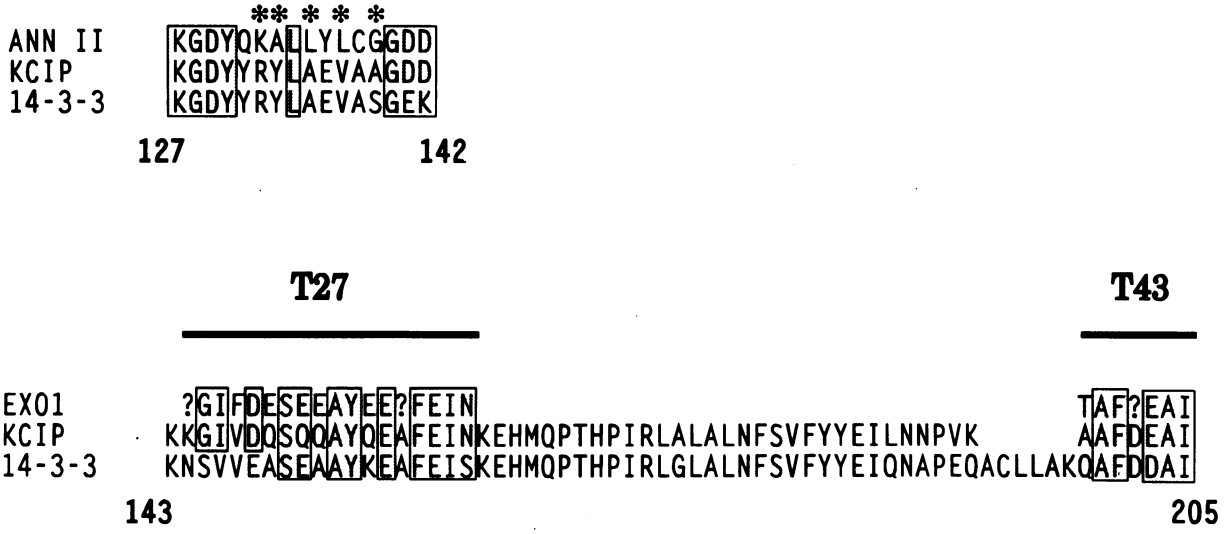

Fig. 2. Comparison of sequence data for Exo1, annexin II, 14-3-3 protein and KCIP. Part of the published data for the sequence of the eta isoform of the 14-3-3 (Ichimura et al. 1988) and KCIP, the zeta isoform (Toker et al. 1990) is shown in comparison with sequences from 2 tryptic peptides (T27 and T43) derived from Exo1 (Morgan & Burgoyne, 1992*a*). Also shown at the top is the conserved domain with homology to the C-terminus of the annexins. Identical residues are boxed and conserved substitutions shown by an asterisk.

Visualisation of this network allowed the demonstration that it is rapidly and reversibly disassembled following stimulation (Cheek & Burgoyne, 1986) and this may therefore be an important aspect of the control of exocytosis by agonists. A variety of stimuli that activate exocytosis have now been shown to result in actin reorganisation in chromaffin cells (Burgoyne et al. 1989*b*; Vitale et al. 1991) and in several other secretory cell types including mast cells (Koffer et al. 1990) and parotid acinar cells (Perrin et al. 1992). It seems unlikely that removal of this cytoskeletal barrier is sufficient to allow exocytosis to proceed since actin disassembly occurs after phorbol ester treatment without marked exocytosis (Burgoyne et al. 1989*b*) and so specific docking and fusion events may also be controlled by Ca^{2+}.

Mechanisms of Ca^{2+}-dependent exocytosis in chromaffin cells

The major area remaining to be resolved in the study of exocytosis in chromaffin cells is the characterisation of the proteins that act as Ca^{2+} receptors and form the fusion machinery in exocytosis. Candidates that have previously been suggested from pharmacological experiments include calmodulin and protein kinase C (PKC). The use of permeabilised chromaffin cells has allowed detailed analysis of the requirements for Ca^{2+} and nucleotides (Knight & Baker, 1982; Dunn & Holz, 1983; Knight et al. 1989*b*; Holz et al. 1992) and more recently cytosolic proteins. Calmodulin is not required for Ca^{2+}-dependent exocytosis in permeabilised chromaffin cells (Ahnert-Hilger et al. 1989) and PKC plays an important but purely modulatory role (Burgoyne et al. 1988; Knight et al. 1989*a*; Morgan & Burgoyne, 1990; Terbush & Holz, 1990). Members of the annexin family of calcium and phospholipid-binding proteins have been implicated in exocytosis due to their ability to aggregate and fuse chromaffin granules (Drust & Creutz, 1988). The first of the annexins shown to have this property was synexin (annexin VII). The aggregation of granules by synexin requires a rather high Ca^{2+} concentration (Creutz et al. 1978) but, as noted above, the exact magnitude of the rise in $[Ca^{2+}]_i$ in intact cells is unknown but could reach 100 μM beneath the plasma membrane. A role for synexin cannot be ruled out (Pollard et al. 1988) but a direct functional test of this protein in exocytosis has yet to be done. In contrast, direct evidence for an important role for the proteins annexin II (calpactin), Exo1 and Exo2 has come from recent studies on digitonin-permeabilised chromaffin cells.

Following digitonin-permeabilisation, exocytosis can be directly activated in chromaffin cells by introduction of micromolar calcium (Dunn & Holz, 1983). The responsiveness of the cells runs down over time after permeabilisation as the cells leak cytosolic and extractable soluble proteins (Sarafian et al. 1987; Ali et al. 1989). Amongst the leaked proteins is annexin II, previously implicated in exocytosis due to its ability to fuse chromaffin granules in vitro at micromolar Ca^{2+} concentrations (Drust & Creutz, 1988). We found that reintroduction of annexin II, purified from bovine lung, maintained the responsiveness of chromaffin cells to Ca^{2+} after perme-

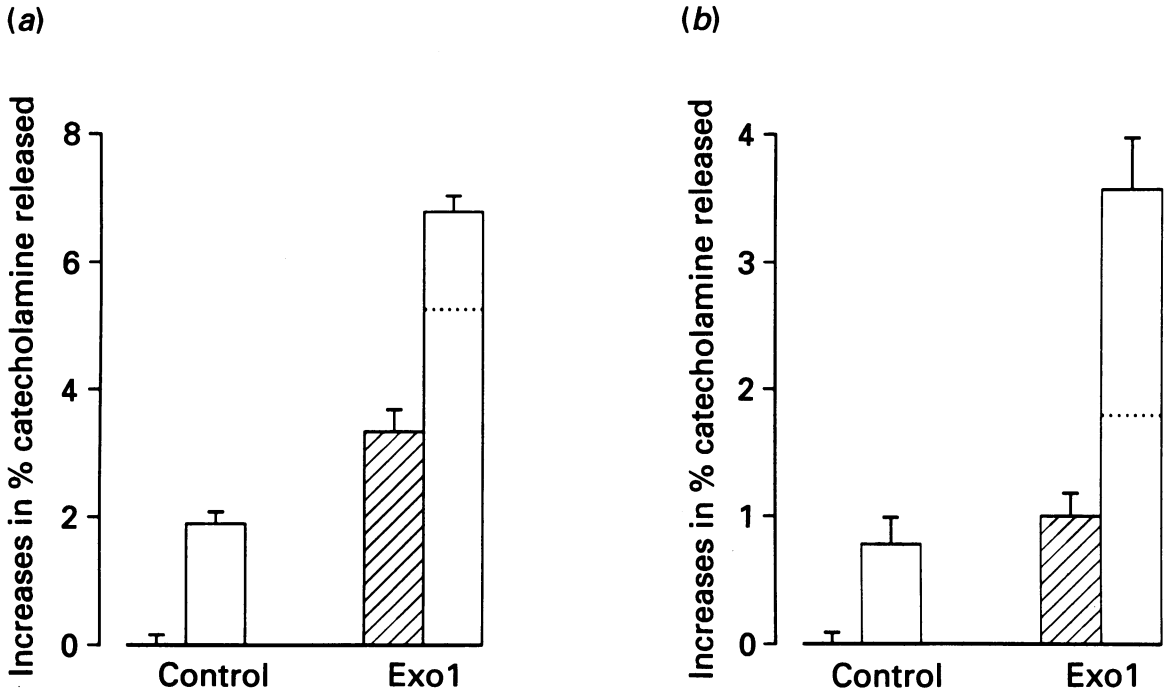

Fig. 3. Stimulatory effect of purified Exo1 on Ca^{2+}-dependent exocytosis and modulation by phorbol ester or protein kinase C. Chromaffin cells were permeabilised for 10 min, incubated with or without Exo1 for 15 min, and then stimulated with 10 μM Ca^{2+}. (a) Cells were permeabilised in the presence (□) or absence (▨) of 200 nM PMA as indicated. Exo1 concentration was 1 mg/ml (Morgan & Burgoyne, 1992*a*). (b) In the second incubation step Exo1 (50 μg/ml) partially purified protein kinase C (PKC, 200 μg/ml) or both were included (Morgan & Burgoyne, 1992*b*); ▨, control; □, +PKC. In this experiment Exo1 was used at a concentration well below its EC_{50}. Exo1 was purified to homogeneity as described by Morgan and Burgoyne (1992*a*). The dashed line in the column on the right indicates the expected value if the 2 treatments were additive. Note that PMA and Exo1 are synergistic even at supramaximal levels of Exo1.

abilisation (Ali et al. 1989; Ali & Burgoyne, 1990). In addition, a synthetic consensus peptide based on the most conserved region of the annexin family partially inhibited Ca^{2+}-dependent exocytosis (Ali et al. 1989) and an antiserum directed against the unique N-terminus of annexin II increased Ca^{2+}-dependent exocytosis (Burgoyne & Morgan, 1990). These findings together suggest that annexin II is involved in Ca^{2+}-dependent exocytosis in chromaffin cells. Ultrastructural studies have suggested that annexin II may form cross-links between granule and plasma membranes in activated cells (Nakata et al. 1990). This cross-linking could precede membrane fusion.

In order to examine whether other proteins are able to maintain or reactivate responsiveness to Ca^{2+}, the permeabilised cell/run-down system was used to screen cytosolic extracts. Adrenal medullary and brain cytosols were run on the anion exchanger Q-sepharose, to which annexin II will not bind, and 3 activities were bound and eluted. Two (Exo1 and Exo2) stimulated Ca^{2+}-dependent exocytosis and the third was inhibitory (Morgan & Burgoyne, 1992*a*). Little is known as yet about the inhibitory activity except that it inhibits control exocytosis and that stimulated by Exo1 or Exo2. Exo1 has been purified to homogeneity and consists of a family of approximately 30 kDa polypeptides. Sequence analysis of tryptic peptides (Morgan & Burgoyne, 1992*a*) revealed that the Exo1 polypeptides (Fig. 2) were related to the previously identified 14-3-3/KCIP family of proteins (Ichimura et al. 1988; Aitken et al. 1990; Toker et al. 1990). The ability of Exo1 to stimulate exocytosis shows the same Ca^{2+} dependency as controls, requires MgATP and is inhibited by tetanus toxin. In addition, the effect of Exo1 (Fig. 3) is potentiated by treatment of cells with PMA to activate and prevent leakage of PKC (Morgan & Burgoyne, 1992*a*) or by cointroduction of PKC (Morgan & Burgoyne, 1992*b*). PKC alone had only a small stimulatory effect on exocytosis. Exo1 does not appear to be a good substrate for PKC and so it is likely that the effect of PKC phosphorylation is mediated by an alternative substrate that may interact with Exo1 in exocytosis (Morgan & Burgoyne, 1992*b*).

Exo2 can be removed by chromatography from the inhibitory activity that runs similarly on ion-exchange. On gel filtration, for example, Exo2 is resolved as a single peak with an apparent molecular weight of 44 kDa (Morgan & Burgoyne, 1992*a*) and has a high specific activity indicating it to be potentially more potent in the stimulation of exocytosis than Exo1 following removal of the inhibitory compound. The

stimulatory effect of Exo2 also shows the same Ca^{2+} dependency as seen in control cells.

The finding that Exo1 belongs to the 14-3-3/KCIP family of proteins points to a potentially significant sequence homology between these proteins and the annexins. The 14-3-3/KCIP proteins possess a conserved domain (Fig. 2) very similar to the conserved C-terminus of the annexins. This domain could be of importance in exocytosis since it is possessed by the only 2 characterised families of proteins that stimulate exocytosis in permeabilised chromaffin cells. It may be that this domain is involved in protein–protein interactions necessary for exocytosis.

Considerable further work will be needed to purify and characterise the various soluble and membrane proteins involved in Ca^{2+}-dependent exocytosis and to determine their mode of action. However, we have begun to identify some of the possible protein components of the machinery responsible for exocytosis in adrenal chromaffin cells and this approach should allow further progress towards understanding the protein chemistry of exocytosis.

ACKNOWLEDGEMENTS

This work was supported by grants from the Medical Research Council, the Wellcome Trust and the AFRC.

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