Destabilization of actin filaments as a requirement for the secretion of catecholamines from permeabilized chromaffin cells

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In the search for a functional role of cytoskeletal proteins in the mechanism(s) of stimulus-secretion coupling, we have previously demonstrated that the actomyosin system might be involved in the transport of cations across the plasma membrane of bovine adrenal chromaffin cells [(1986) J. Biol. Chem. 261, 5745–5750]. To establish whether actin and myosin might also be involved in later stages of the cellular response, we have examined the possible effects of various actin-specific reagents on the calcium-mediated secretion of catecholamines from digitonin-permeabilized cells. F-Actin-destabilizing agents, such as cytochalasin D or DNase 1, were found to promote Ca^{2+}-stimulated (as well as basal) secretion. By contrast, stabilizers, like phalloidin, produced the opposite effect. It is concluded that stimulus-secretion coupling in chromaffin cells might require the reorganization of actin for modulating both ion transport across the plasma membrane and exocytotic secretion per se.

Actin *Chromaffin cell* *Cytochalasin* *DNase 1* *Secretion*

1. INTRODUCTION

Reorganization of cytoskeletal protein assemblies following stimulation of the plasma membrane by a variety of ligands appears to be a common event [1], suggesting that reassembly plays a fundamental role in the transmission of signals from the external 'world' to the cellular machinery. In particular, following receptor-mediated stimulation of some secretory cells, such as basophils and chromaffin cells, the redistribution of microfilaments [2] and of microfilament-associated proteins [3] has been observed visually. It makes sense to believe that prevention of any such rearrangement should lead to inhibition of subsequent steps in the cellular response to stimulation. We have recently reported that stimulation by ACh of bovine adrenal medullary chromaffin cells will lead to an increase in the total cytosolic actin content, accompanied by a decrease in the G-actin pool [4]. We also introduced into these cells, via liposomes, a variety of actin-specific macromolecules, which are believed to affect the state of assembly of actin and/or its attachment to membranes. The F-actin-depolymerizing agent DNase 1 [5] was found to cause electrical depolarization at the plasma membrane and an elevation of both Ca^{2+} uptake and CA secretion [6,7]. Qualitatively similar effects were observed with rabbit skeletal HMM [4,7–9], which is capable of bundling actin filaments [10] or might, similarly to HMM subfragment-1 [11], cause

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Abbreviations: ACh, acetylcholine; DBH, dopamine-β-hydroxylase; CA, catecholamine; CD, cytochalasin D; HMM, heavy meromyosin; NEM-HMM, *N*-ethylmaleimide-poisoned HMM; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); Rh-HMM, rhodamine isothiocyanate-labelled HMM

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detachment of microfilaments from plasma membranes. Both stimulation and secretion are thus affected by the reorganization of microfilaments. To determine whether changes in the assembly of the actomyosin system would directly affect the secretion of CA without modulating at the same time signal transduction at the plasma membrane level, we have permeabilized the cells with digitonin [12,13]. In doing so we by-passed the plasma membrane barrier and thus all related signalling events. Addition of micromolar concentrations of Ca^{2+} to such permeabilized cells has been shown to stimulate exocytotic CA secretion [12,13]. Since the actin-specific reagents employed in this study, including macromolecules, e.g. HMM, are able to enter the cells freely, any effect exerted by these reagents on CA secretion should then be directly associated with the functional role(s) played by the microfilaments and/or changes in their organization in the secretory event per se.

2. MATERIALS AND METHODS

2.1. *Cells*

Bovine adrenal glands were obtained several minutes after slaughter from a local slaughterhouse, and transported to the laboratory on ice. Cells were prepared from the medulla essentially as described by Schneider et al. [14]. In short, dissociation from the tissue was carried out by successive incubations in buffer I (118 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.7 mM KCl and 10 mM D-glucose adjusted to pH 7.2 and oxygenated with 95%/5% O_2/CO_2) and 0.2% collagenase (Worthington). Following the final washing in buffer II (buffer I + 2.2 mM $CaCl_2$ and 0.5% BSA (Sigma, fraction V)), the 3rd–5th fractions were pooled together. Viability was >95%, as demonstrated by eosin red exclusion. The cells were resuspended in Dulbecco's modified Eagle's medium (DMEM from Biofluids, Rockville, MD), supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and cytosine arabinoside (10 μg/ml), to inhibit bacterial contamination and to prevent the culture from being overgrown by other cells in the preparation. Cells were plated in 24-well Costar plates at an initial density of approx. 300000 cells/well. The medium was changed after 3 days, and again the day before the experiments, usually 5–7 days after plating.

2.2. *Digitonin treatment*

Prior to each experiment, plated cells were washed briefly with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline and then preincubated for 20 min with 10 μM digitonin in a buffer composed of 140 mM Na glutamate, 5 mM $MgSO_4$, 5 mM MgATP, 5 mM glucose, 20 mM Pipes, pH 6.8, 0.5 mM ascorbic acid, 5 mM EGTA and the drug or protein being utilized [1,15]. Following preincubation, the medium was removed (minimal secretion had occurred, not exceeding 5% of the total CA contents), and replaced by fresh buffer (without digitonin) for another 15 min. At the beginning of this second incubation period 5 mM $CaCl_2$ was added to one half of the samples yielding a free $[Ca^{2+}]$ of 20 μM. This is called Ca^{2+}-stimulated secretion. In presenting the data, CA release in the presence of the various reagents is normalized to the amount released upon Ca^{2+} stimulation of control cells (representing on average 10% of the total CA contents). All experiments were carried out at least three times in triplicate.

2.3. *Catecholamines*

Following termination of the experiments, an aliquot (150 μl) of the medium in each well was aspirated and clarified by centrifugation in an Eppendorf microfuge. 100 μl of the supernatant was removed and added to 100 μl of 2% acetic acid. Catecholamines were then assayed fluorimetrically in a Perkin-Elmer 650-40 spectrofluorimeter using a modification of the trihydroxyindole method [15].

2.4. *Microfilament-specific proteins and drugs*

HMM was prepared according to Weeds and Pope [16]. Actin-activated HMM–Mg^{2+}-ATPase activity was measured by titration with 0.01 N NaOH to maintain a constant pH (7.4), using a Radiometer (Copenhagen) pH-stat. Actin was prepared according to Spudich [17]. HMM was poisoned with NEM as described by Meeusen and Cande [18]. HMM was labelled with rhodamine isothiocyanate (Sigma, grade 1) as described by us for actin [19]. Non-reacted rhodamine was re-

moved by gel filtration in a PDP 10 column (Pharmacia). Rhodamine phalloidin was purchased from Molecular Probes (Junction City, OR). Cytochalasin D, DNase 1 and phalloidin were Sigma products.

2.5. *Fluorescence microscopy*

For microscopic visualization, cells were plated in plastic 2-well tissue culture chambers/microscope slides (Miles), rather than in Costar plates but otherwise treated identically as described above. During the permeabilization procedure, cells were incubated with either Rh-HMM (0.1 mg/ml) or with rhodamine-phalloidin (5 U/ml) and (to identify positively chromaffin cells) with goat anti-rat DBH (kindly supplied by Dr Pat Fleming, Georgetown University). For further processing, the cells were fixed after 20 min in 2% formaldehyde, washed several times in phosphate-buffered saline and counterstained for DBH with fluorescein-labelled rabbit anti-goat IgG (Sigma). For microscopic observations the tissue culture chamber/slides were mounted on a Zeiss IM35 inverted microscope equipped with the appropriate filter combination for rhodamine and fluorescein, respectively. Micrographs were taken with automatic exposure settings.

3. RESULTS AND DISCUSSION

In contrast to the permeabilization of cells by exposure to high electric fields [20], permeabilization with digitonin will result in permanent pores in the plasma membrane [12,13], thus providing for free solute exchange to and from the cytoplasm [15]. In addition, access to the cytoplasm in electrically permeabilized cells is limited to compounds with a molecular mass < 1000 Da [20]. The accessibility of the cytoplasm of detergent-permeabilized chromaffin cells to externally supplied macromolecules was verified using Rh-HMM, as well as rhodamine phalloidin. Uptake of the fluorescent (macro)molecules into the cells and their binding to F-actin could be followed directly under the microscope during the permeabilization period, and occurred usually within 3–5 min. However, more detailed studies showed that rhodamine phalloidin and Rh-HMM were being taken up primarily by the adrenomedullary endothelial cells [21], rather than by chromaffin cells. Cultured chromaffin cells, positively identified by staining for DBH (fig.1A), revealed a distinct lack of actin stress fibres; however, an enhanced fluorecent rim could be observed at the cell periphery (fig.1B), presumably corresponding to the cortical web of actin filaments underlying the plasma membranes. A similar appearance could also be seen upon staining the cells with Rh-HMM (not shown). This lack of cytoplasmic stress fibres in chromaffin cells at the level of resolution by fluorescence light microscopy is in sharp contrast to the appearance of adrenal medullary endothelial cells, which were

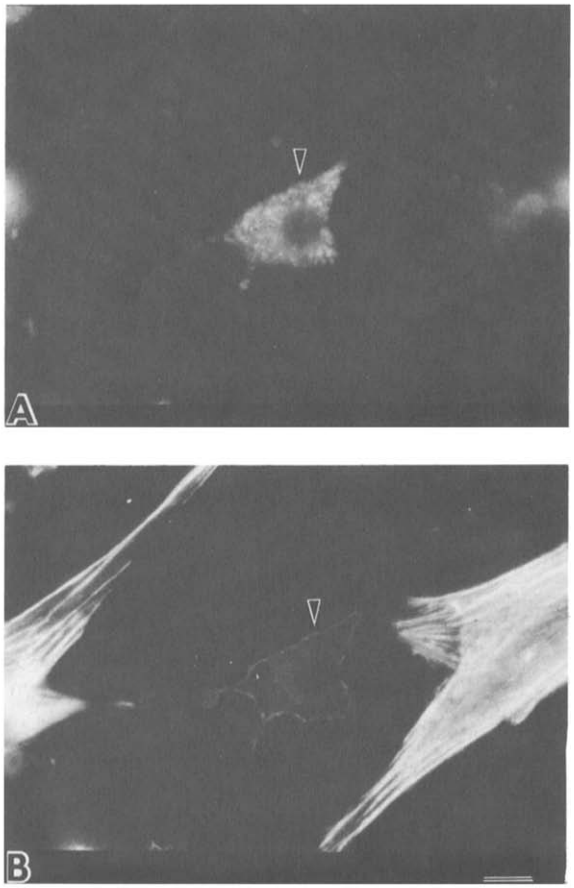

Fig.1. Fluorescence micrographs of cultured bovine adrenal medullary cells double-stained for DBH (A) and F-actin (B). Chromaffin cells, which are DBH positive, are nearly completely devoid of cytosolic actin stress fibres, except for a distinct rim underneath the plasma membranes; by contrast, the endothelial cells are DBH negative, but reveal an elaborate network of microfilaments. Bar: 10 μm.

also present in the culture [21], but which were anti-DBH negative. The endothelial cells revealed a highly organized network of stress fibres which was also susceptible to staining with Rh-HMM (Lelkes, in preparation).

The F-actin-destabilizing agents CD and DNase 1 were found to enhance significantly both basal and Ca^{2+}-stimulated secretion in a dose-dependent fashion (fig.2). At the highest doses employed (10 μM CD and 1 mg/ml DNase 1), the Ca^{2+}-evoked release was enhanced by 60 and 30%, respectively. This interrelationship between secretion and destabilization of actin filaments is reminiscent of our previous observation that stimulation of intact cells by ACh [4] or by depolarizing concentrations of K^+ [22] is accompanied by a transient augmentation of the cytosolic actin content. This increase is probably due to a stimulation-induced breakdown of F-actin originally attached to insoluble matrices such as the plasma membrane or the storage granules [23]. Such detachment might also occur in intact cells that had been fused with liposomes containing DNase 1. These cells have been shown to exhibit depolarization of the plasma membrane concomitant with enhanced Ca^{2+} uptake and augmented CA secretion [6,7], just like ACh-stimulated cells, suggesting the involvement of microfilaments in ion transport across the plasma membrane during the stimulation-secretion sequence.

On the other hand, the F-actin-stabilizing agent phalloidin, which can shift the G-F equilibrium in favor of F-actin [24], caused a significant (up to 40%) reduction in both the basal and the Ca^{2+}-induced CA secretion from digitonin-permeabilized cells (fig.3). A similar, even more pronounced decrease in the CA secretion was obtained, following preincubation of the cells with G-actin, which polymerizes under the conditions employed. It has been shown that the complex formed between F-actin and NEM-HMM, in contrast to acto-HMM, is not dissociated by ATP [18]. HMM [10], and similarly NEM-HMM (unpublished), can cross-link neighbouring thin filaments in skeletal muscle myofibrils from which myosin had been removed. NEM-HMM is therefore supposed to be able to induce the formation of microfilament bundles, and this presumably stabilizes the filaments against disintegration. Thus, the effect of NEM-HMM on

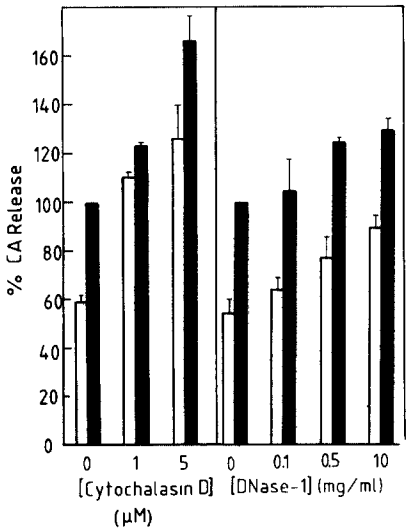

Fig.2. Effects of cytochalasin D and DNase 1 on catecholamine release from digitonin-permeabilized chromaffin cells. Shaded bars, Ca^{2+}-stimulated release; open bars, basal release. Catecholamine release is normalized to that obtained from control cells stimulated with 20 μM Ca^{2+} in the absence of the drugs.

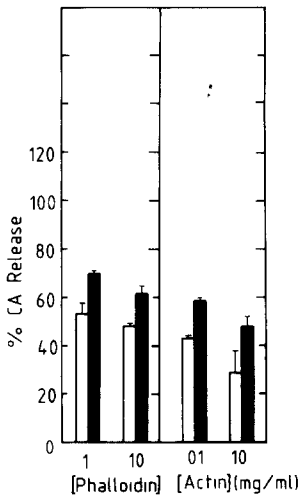

Fig.3. Effects of phalloidin and actin on CA release from permeabilized cells. The data are normalized to Ca^{2+}-stimulated catecholamine release from permeabilized control cells. Shaded and open bars represent Ca^{2+}-stimulated and basal release, respectively.

chromaffin cells is expected to be similar to that of phalloidin, or opposite to that induced by CD or DNase 1. As seen in fig.4, this is actually the case for Ca^{2+}-stimulated cells, when using sufficiently high concentrations of NEM-HMM. NEM-HMM also exerted a similar inhibitory effect on secretion when incorporated into intact cells, and caused hyperpolarization rather than depolarization of the plasma membrane [9]. By contrast, HMM had no effect on Ca^{2+}-stimulated release from permeabilized cells and, at a concentration of 1 mg/ml, had only a moderately suppressing effect on the basal secretion. This might be due to the hydrolysis of MgATP, which is also necessary for secretion [20].

We have recently shown [9] that introduction of HMM into intact cells gave effects qualitatively quite similar to those obtained with DNase 1, i.e. depolarization and an increase in secretion associated with enhanced influx of Ca^{2+} as well as Na^{+}. Modulation of secretion from intact cells by agents such as DNase 1, HMM or NEM-HMM could be attributed, at least partly, to the changes observed in ion fluxes across the plasma membrane. However, from the present set of experiments, it appears that DNase 1, CD, phalloidin, actin, NEM-HMM, and probably also HMM, can control secretory processes by directly affecting events which are distal to and/or independent of stimulus-mediated ion movements.

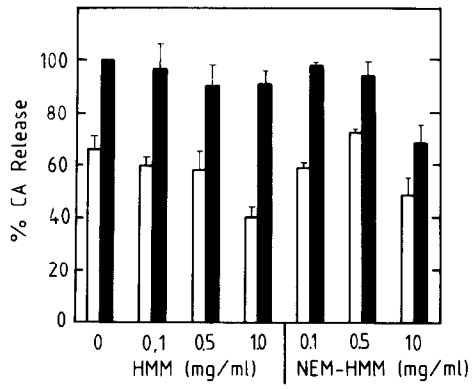

Fig.4. CA release by permeabilized cells in the presence of HMM and NEM-HMM. Shaded and open bars represent Ca^{2+}-stimulated and basal release, respectively. The data are normalized to the Ca^{2+}-stimulated catecholamine release from control, permeabilized cells.

In analyzing our results, we have hitherto been concerned merely with effects of the reagents employed on actin in its polymerized state, i.e. its possible transformation into monomeric (and also, in the case of CD, into oligomeric) actin, its detachment from membranes, or its cross-linking. However, non-stimulated (resting) cells generally contain large amounts of monomeric actin in complex with proteins such as profilin [25]. It has been shown that this G-actin pool can undergo polymerization following stimulation [26]. DNase 1 could inhibit this polymerization by strongly binding to G-actin and/or by displacing some actin-binding proteins, e.g. profilin. It is feasible to believe that the stimulatory effect of DNase 1 on CA secretion, observed in both intact and digitonin-permeabilized cells, could be caused by such an inhibition of actin polymerization and/or indirectly by displacement of proteins. The latter might in turn, by binding to the plasma membrane [26] and/or to cytosolic organelles (e.g. the chromaffin granules), affect the charge and the hydration layer of these membranes and thus control exocytosis.

Attention has recently been drawn to the possibility that the lipid composition of the plasma membrane may affect the state of assembly of actin [26,27]. It is quite feasible that the reverse may also occur, namely that attachment, detachment and changes in the mode of assembly of membrane-bound microfilaments could modify a host of membrane properties including the density and distribution of electrical charges and/or the hydration of the plasma and granule membranes. It is safe to assume that changes in the mechanochemical reactivity of membrane-associated microfilaments might modulate local cytoplasmic streaming and its associated electrokinetic effects in the vicinity of the plasma membrane. Such dynamic modifications may regulate a variety of intrinsic membrane properties, e.g. enzyme activities and transmembranal ion transport. In particular, the dynamics of microfilament assembly may directly and/or indirectly influence the fusion competence of biological membranes [4,8,9].

Our data, obtained in digitonin-permeabilized cells, to a certain extent do not agree with those

from similar experiments carried out with electrically permeabilized chromaffin cells. Knight and Baker [20] have reported that phalloidin as well as cytochalasins B and D were without effect on the Ca^{2+}-dependent release of CA from 'leaky' bovine adrenal medullary cells. We note that this is not the only discrepancy between the results obtained with digitonin- and electrically permeabilized cells [12,13]. Knight and Baker argue that their failure to affect Ca^{2+}-stimulated secretion in leaky cells by drugs specifically directed against microfilaments provides strong evidence against a direct involvement of actin in exocytosis [20]. However, as we have demonstrated here and elsewhere [4,8,9], these results and conclusions can at most concern CA secretion from leaky cells only and not the global events termed stimulus-secretion coupling. The fact that the electrically permeabilized cells did not respond to actin-specific reagents raises the possibility that the reagents could not find any suitable actin with which to interact, e.g. G-actin for polymerization by phalloidin, and F-actin to be stabilized by phalloidin or broken by the cytochalasins. Thus, it was recently demonstrated that electric field pulses can induce changes in the state of cellular actin, including an increase in the content of a fraction which does not interact with DNase 1 unless treated with guanidine hydrochloride [28]. The very treatment of cells with digitonin may also, in principle, affect the assembly and binding to membranes of microfilaments as well as the G:F ratio in the cytosol. Digitonin renders membranes permeable by preferentially interacting with cholesterol [12], which is present in both the granule and plasma membranes of chromaffin cells. Cholesterol is known to affect strongly the microviscosity of membranes [29]. The interaction of digitonin with cholesterol may induce a large enough change in membrane composition and structure so as to affect the assembly, binding and distribution of the membrane-associated actin. Thus the treatment of cells with digitonin may not only cause pore formation but also affect cytoskeletal organization, at least indirectly via its association with the plasma membranes. Just as actin (at least partly, in its non-polymerized form) could enter the permeabilized cells from the medium, so should intracellular actin be capable of leaving the cells during their pretreatment with digitonin and the application of CD or DNase 1. However, the very fact that these and other reagents influenced secretion suggests that at least part of the actin must have been retained by the cells.

In conclusion, microfilaments seem to play more than one role in the secretory process by chromaffin cells: they seem to be involved both in stimulus-induced transmembranal ion movement and in subsequent events related to exocytotic secretion. This might also be true for secretory processes in other cells and, more generally, for stimulus-response coupling induced by hormones, neurotransmitters, growth factors, mitogens and tumor promoters.

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