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# Calpactin-depleted cytosolic proteins restore $Ca^{2+}$-dependent secretion to digitonin-permeabilized bovine chromaffin cells

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Incubation of digitonin-permeabilized bovine chromaffin cells results in a loss of $Ca^{2+}$-dependent catecholamine secretion. The addition of cytosolic proteins prevents this loss of secretory activity. It has been proposed that calpactin might be the protein which is responsible for preventing this loss of activity. The experiments described in this paper show that cytosolic proteins which have been depleted of calpactin are as effective as control cytosolic proteins in preventing the loss of $Ca^{2+}$-dependent secretion. Thus, a cytosolic protein(s) other than calpactin appears to be responsible for preventing this loss of secretory activity.

Secretion; Calpactin; $Ca^{2+}$; Chromaffin cell; Catecholamine

## 1. INTRODUCTION

Digitonin-permeabilized bovine chromaffin cells are widely used as a model system to study the regulation of secretion [1–5]. Secretion of catecholamines by these digitonin-permeabilized cells is both ATP- and $Ca^{2+}$-dependent. Incubation of the permeabilized cells results in a decrease in the level of $Ca^{2+}$-dependent secretion and a release of cytosolic proteins [4]. When the proteins released during this incubation are collected, concentrated, and added back to the incubation buffer, the loss of secretory activity is prevented indicating that cytosolic proteins are involved in the secretory response [4]. Ali et al. [5] reported that incubation of permeabilized cells with calpactin or calpactin heavy chain (p36) prevented the loss of secretory activity and suggested that calpactin or a closely related protein might be the $Ca^{2+}$ receptor which regulates secretion. Calpactin is a $Ca^{2+}$-dependent phospholipid-binding and actin-binding protein (reviewed in [6,7]) which aggregates chromaffin secretory vesicles at physiological $Ca^{2+}$ concentrations [8]. However, the addition of calpactin to the incubation only resulted in an increase in secretion measured in 10 $\mu$M $Ca^{2+}$; secretion in sub-saturating $Ca^{2+}$, 0.3–3 $\mu$M, was not affected by the addition of calpactin [5]. This lack of stimulation at sub-saturating $Ca^{2+}$ suggests that $Ca^{2+}$ binding to calpactin may not cause secretion, but rather that it has some modulatory role.

We have used an anti-calpactin antibody column to remove calpactin from the proteins which leak out of the digitonin-permeabilized chromaffin cells. These calpactin-depleted proteins were used to determine whether calpactin is responsible for preventing the loss of secretory activity.

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*Abbreviations:* EGTA, ethylenebis(oxyethylenenitrilo)-tetraacetic acid; NE, norepinephrine; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); KG-buffer, potassium/glutamate/MgATP/EGTA/PIPES solution

## 2. MATERIALS AND METHODS

Chromaffin cells were isolated from bovine adrenal glands as described by Greenberg and Zinder [9], purified on self-generating Percoll gradients [10], and plated on 48-well dishes at a density of 4 × $10^5$ cells/cm$^2$ [1]. The protocol used for measuring norepinephrine (NE) secretion was similar to that of other investigators [1–5]. After labeling with [$^3$H]NE, the cells were permeabilized in 125 $\mu$l of 139 mM potassium glutamate, 2 mM $MgCl_2$, 2 mM MgATP, 5 mM EGTA, and 20 mM PIPES, pH 6.6 (KG-buffer) containing 20 $\mu$M digitonin. After 10 min, the permeabilization buffer was removed and the cells incubated for 15 min in KG-buffer containing either released cytosolic proteins or an equivalent concentration of bovine serum albumin. After this incubation, secretion was measured in KG-buffer with and without $CaCl_2$. Free $Ca^{2+}$ concentrations were calculated according to Fabiato and Fabiato [11]. After 15 min, the release medium was removed and placed on ice until centrifugation at 12000 × *g* for 4 min. The supernatant from this centrifugation was removed and counted. The cells attached to the wells were solubilized with 1% Triton X-100, combined with the pellet from the above centrifugation and counted. [$^3$H]NE release is expressed as a percentage of the total [$^3$H]NE recovered.

Cytosolic proteins which leak from digitonin-permeabilized chromaffin cells, referred to as released cytosolic proteins, were prepared as described by Sarafian et al. [4]. Calpactin was purified from bovine lung [12]. Antibodies against calpactin were raised in rabbits and purified on a column containing calpactin coupled to Sepharose (Pharmacia). The purified antibodies (6 mg) were coupled to 3 ml CNBr-activated Sepharose according to the manufacturer's

instructions. This anti-calpactin antibody column was used to remove calpactin from the released cytosolic proteins. Released cytosolic proteins (3 ml of 2 mg/ml) were loaded onto a 3 ml anti-calpactin antibody column equilibrated in 140 mM NaCl and 50 mM sodium phosphate, pH 7.0. The proteins which did not bind to this column were collected, concentrated to 2 mg/ml, and dialyzed against KG-buffer.

## 3. RESULTS AND DISCUSSION

Incubation of digitonin-permeabilized chromaffin cells for 15 min is known to result in a partial loss of $Ca^{2+}$-dependent NE secretion [4,5]. The addition of released cytosolic proteins to the incubation solution prevents this loss of secretory activity [4]. As shown in Fig. 1, the addition of released cytosolic proteins resulted in an increase in secretion measured at both saturating and subsaturating $Ca^{2+}$ concentrations. In contrast, Ali et al. [5] reported that the addition of calpactin alone to the incubation solution only results in an increase in secretion measured in 10 $\mu$M $Ca^{2+}$; there is no increase in secretion measured in subsaturating (0.3–3 $\mu$M) $Ca^{2+}$ and only a very slight increase when secretion was measured in 30 $\mu$M $Ca^{2+}$. Sarafian et al. [4] measured the effect of released cytosolic proteins only on secretion in the presence of saturating $Ca^{2+}$. The difference between the addition of calpactin alone and the addition of released cytosolic proteins suggests that some protein other than calpactin is responsible for preventing the loss of secretory activity.

As shown in Fig. 2, lane A, calpactin or p36 is one of the proteins which leaks from digitonin-permeabilized chromaffin cells. The minor band below the calpactin heavy chain or p36 in the released proteins is probably calpactin II (p35) [6,7,12]. The released cytosolic proteins were passed through an anti-calpactin antibody column. After passage through this immunoaffinity column, there was no detectable calpactin heavy chain or p36 left in the released proteins, Fig. 2, lane B. Experiments with dilutions of control released proteins and with purified calpactin showed that as little as 2.5% residual calpactin would have been detected by this immunoblot and that 2 mg/ml calpactin-depleted released proteins contained less than 0.5 $\mu$g/ml calpactin.

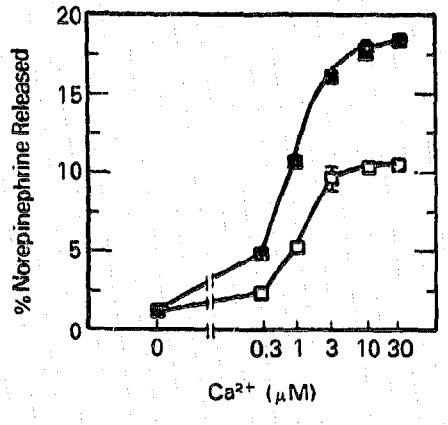


Fig. 1. Effect of released proteins on $Ca^{2+}$-dependent NE secretion. Permeabilized cells were incubated for 15 min in KG-buffer containing either 2 mg/ml bovine serum albumin (□), or 2 mg/ml released proteins (■). These incubation buffers were removed and then [$^3$H]NE secretion was measured in KG-buffer containing varying $Ca^{2+}$ concentrations.

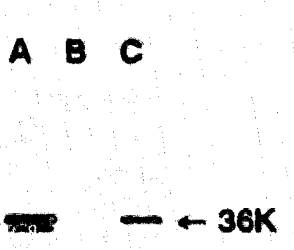


Fig. 2. Detection of calpactin in released proteins by immunoblotting. Samples were resolved on SDS-PAGE and analyzed by immunoblotting with affinity-purified anti-calpactin antibodies. Immunoreactive bands were visualized using goat anti-rabbit IgG antibodies coupled to horse radish peroxidase (Bio-Rad Laboratories). Lane A, 40 $\mu$g released proteins; lane B, 40 $\mu$g calpactin-depleted released proteins; lane C, 50 ng bovine lung calpactin.

Table I compares the ability of control released cytosolic proteins and calpactin-depleted released cytosolic proteins to prevent the loss of secretory activity. The addition of 2 mg/ml calpactin-depleted released proteins was as effective as 2 mg/ml control released proteins in preventing the loss of secretory activity. The protein (or proteins) responsible for preventing the loss of secretory activity is not present in large excess as 1 mg/ml control released proteins was less effective than 2 mg/ml control released proteins in preventing the loss

Table I

Comparison of the ability of released proteins, calpactin-depleted released proteins, and calpactin to prevent the loss of $Ca^{2+}$-dependent secretion

| Proteins present during the 15 min incubation | $Ca^{2+}$-dependent secretion (% NE released) |
|---|---|
| 2 mg/ml bovine serum albumin | 7.0 ± 0.4 |
| 1 mg/ml released proteins | 11.8 ± 0.3 |
| 2 mg/ml released proteins | 16.5 ± 2.5 |
| 2 mg/ml calpactin-depleted released proteins | 15.3 ± 0.3 |
| 10 $\mu$g/ml calpactin | 8.8 ± 0.2 |
| 17 $\mu$g/ml calpactin | 9.6 ± 0.4 |
| 17 $\mu$g/ml bovine serum albumin | 6.8 ± 0.4 |

$Ca^{2+}$-dependent secretion is: [$^3$H]NE released in 10 $\mu$M $Ca^{2+}$, minus that released in the absence of $Ca^{2+}$. $Ca^{2+}$-dependent secretion was determined after the permeabilized cells were incubated for 15 min in KG-buffer containing the proteins given above. There were no significant differences in the amounts of [$^3$H]NE released in the absence of $Ca^{2+}$. Immediately after permeabilization, $Ca^{2+}$-dependent NE secretion was 18.5 ± 0.7%.

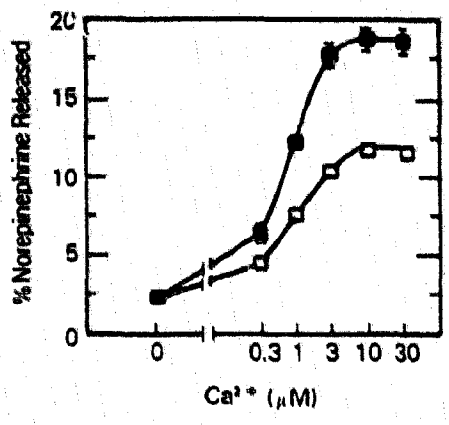


Fig. 3. Effect of calpactin-depleted released proteins on $Ca^{2+}$-dependent NE secretion. Permeabilized cells were incubated for 15 min in KG-buffer containing either 2 mg/ml bovine serum albumin (□), or 2 mg/ml calpactin-depleted released proteins (■). These incubation buffers were removed and then [$^3$H]NE secretion was measured in KG-buffer containing varying $Ca^{2+}$ concentrations.

of secretory activity. A similar dependence on the concentration of released cytosolic proteins was reported by Sarafian et al. [4]. Thus, the ability of the calpactin-depleted proteins to prevent the loss of secretory activity does not appear to result from the presence of a small amount of calpactin which was not removed by the anti-calpactin antibody column. As observed with control released proteins, the addition of calpactin-depleted released proteins resulted in an increase in secretion at both saturating and subsaturating $Ca^{2+}$ concentrations, Fig. 3.

Because there was no obvious difference in the abilities of control released proteins and calpactin-depleted released proteins to prevent the loss of secretory activity, the effect of incubation in calpactin was reexamined. Permeabilized cells incubated in 10 and 17 μg/ml calpactin gave more $Ca^{2+}$-dependent secretion than did cells incubated in bovine serum albumin, Table I, but much less than that from cells incubated in 2 mg/ml calpactin-depleted released proteins which contained less than 0.5 μg/ml calpactin. Increasing calpactin or p36 in the incubation to 80 μg/ml did not cause any additional increase in $Ca^{2+}$-dependent secretion. The results with calpactin shown in Table I are for secretion in 10 μM $Ca^{2+}$, but similar results were obtained when secretion was measured in 1, 5, 10, 30 and 100 μM $Ca^{2+}$. It is unclear why we obtained much less stimulation by calpactin than reported by Ali et al. [5], however, one possible explanation is that we were unable to reproduce the precise $Ca^{2+}$ concentration at which they obtained a large stimulation.

The results presented here indicate that a protein(s) present in the released cytosolic proteins other than calpactin is responsible for preventing the loss of secretory activity. However, calpactin may play a role in the secretory response as after the permeabilized cells have been incubated for 30 min, approximately half the calpactin is still present in the cells (data not shown).

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