CALCIUM-DEPENDENT IN VITRO INTERACTION BETWEEN BOVINE ADRENAL MEDULLARY CELL MEMBRANES AND CHROMAFFIN GRANULES AS A MODEL FOR EXOCYTOSIS

Frank KONINGS and Werner DE POTTER

Division of Pharmacology, Department of Medicine, Universitaire Instelling Antwerpen, 2610 Wilrijk, Belgium

Received 30 January 1981

1. Introduction

The adrenal medullary cell secretes catecholamines by exocytosis [3,8], a process in which secretory vesicles release their contents into the extracellular space upon fusion with the cell membrane. A similar system has been discovered for several endocrine, exocrine and neuronal cell types [3,11,17,20,22]. An in vitro interaction between secretory vesicles and their plasma membrane, followed by release of their soluble vesicular contents, has already been described for neurohypophyseal vesicles [12] and insulin granules [6]. These release systems show a definite Ca^{2+} dependence. Ca^{2+} also plays a critical part in the adrenal medullary stimulus–secretion coupling; cell membrane depolarisation causes an influx of Ca^{2+} which initiate the exocytosis of chromaffin granules [9]. Developing a cell-free model would be useful to investigate the events which occur between Ca^{2+} influx and exocytosis. [6] and [15] report, respectively, catecholamine release and fusion when adrenal medullary cell membranes are incubated with chromaffin granules. Here, we report that this interaction results in the release of other soluble granular components such as dopamine-β-hydroxylase (DBH, EC 1.14.17.1) and ATP together with catecholamines. This release is caused by μM Ca^{2+} levels and can be blocked by the addition of EGTA to the incubation medium. These data suggest that the in vitro interaction between chromaffin granules and their plasma membranes could be a simple model for exocytosis.

2. Materials and methods

Bovine adrenal glands were obtained from the local slaughterhouse and placed in ice-cold 0.3 M sucrose within 30 min of death of the animals. The medullae were separated from the cortices and homogenized with a Duall 25 homogenizer in 0.3 M sucrose (10 mM Tris–HCl (pH 7.4), 6 ml/g tissue) after a short prehomogenization with an Ultra Turrax apparatus. Chromaffin granules (CG) and plasma membranes (PM) were isolated essentially as in [18] but in the first Percoll removing centrifugation step buffered 0.3 M sucrose containing 5 mM EGTA was used. This step was performed at 15 000 × *g* for CG and at 30 000 × *g* for PM-rich material for 20 min.

The isolated CG and PM were resuspended in buffered 0.3 M sucrose and the CG catecholamine and PM protein concentrations were determined. The CG and PM suspensions were then diluted to the standard concentrations adequate for the incubation experiments. The actual incubations were performed in a warm-water bath at 37°C. After a 5 min preincubation of the PM suspension or an equivalent blank, the CG suspension was added and the incubation mixture shaken for a few seconds. To stop this incubation the mixture was placed in ice-water and 3 vol. ice-cold buffered 0.3 M sucrose were added. The incubated samples were then centrifuged at 15 000 × *g* for 20 min in a cooled centrifuge to pellet the intact CG. By this procedure granule-bound catecholamines were separated from free catecholamines. The supernatants were kept for assay.

Catecholamine concentration was measured by the colorimetric method in [24] with the exception that acetate buffer was replaced by a citrate–phosphate buffer [21].

Proteins were determined as in [16] using bovine serum albumin as a standard.

DBH activity was determined as in [5] with minor

modifications [7]. One unit (U) of activity represents the formation of 1 nmol octopamine from tyramine per hour at 37°C.

ATP was measured by the luciferin–luciferase method [23] using Sigma FLE firefly extract.

The catecholamines already released before incubation due to leakage and granule damage caused by resuspending the pellet, were determined by keeping a fraction of the CG suspension at 0°C during incubation of the other samples. The 'cold' sample was then centrifuged along with the incubated samples and the supernatant assayed for catecholamines. This value was subtracted from the concentration of the supernatant of an incubated sample to find the concentration of catecholamines released in that sample during the actual incubation. These results were graphically plotted as 'catecholamines released'; the supernatant concentration of the cold blank represented 0% and the total catecholamine concentration, 100%. The concentration or activity of the ATP and DBH released during incubation was calculated in the same way. In our experiments the fraction of catecholamines released before incubation was ~15% of the total catecholamine content of the CG suspension.

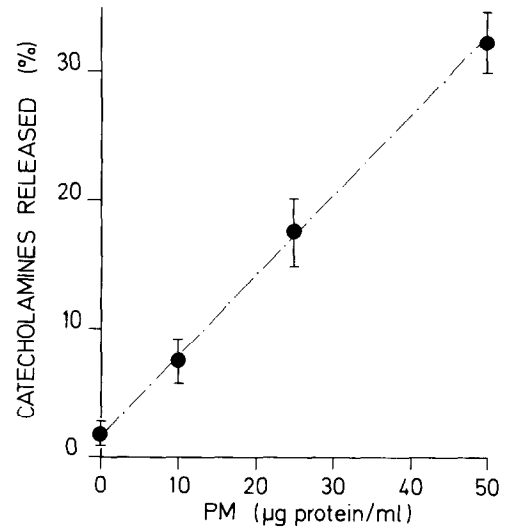


Fig.1. The effect of plasma membranes (PM) on catecholamine release from chromaffin granules (200 µg cat./ml) after 1 min of incubation at 37°C (in 700 µl total vol.). The incubation medium contained 0.3 M sucrose, 10 mM Tris–HCl (pH 7.4) and 1 µM $CaCl_2$. The results are presented with their standard deviation (n = 3); correction for the catecholamines already free before the incubation has been made.

3. Results

A small fraction of the catecholamines (±1.5%) was released during incubation of CG alone. Fig.1 shows that after joint incubation of CG with PM this release was much more important (up to 30% or more of the releasable catecholamines). The release was proportional to the amount of PM in the incubation medium and was insignificant at 0°C (not shown). The contribution of catecholamines adsorbed to PM was negligible (<1% of the total catecholamine content).

The PM-induced catecholamine release was complete in <2 min of joint CG-PM incubation (fig.2). This release was not due to a sudden increase in catecholamine-specific permeability of the granule membrane since there was a simultaneous increase of non-granule-bound ATP and DBH. The relatively high basal DBH activity was caused by membrane-bound DBH of lysed granules which stayed in the supernatant after the pelleting of the intact CG.

There was no release of the soluble granular content upon interaction between PM and CG in the absence of free Ca^{2+}. This is illustrated in fig.3. Addition of 1 mM EGTA to an incubation mixture with CG and PM resulted in a catecholamine release which was not significantly different from the basal release from CG incubated without PM.

4. Discussion

Perfused adrenal glands release their soluble vesicular components such as catecholamines, ATP and DBH, simultaneously. The ratio of these released compounds is the same as their ratio in the granule matrix (review [13]). This supports the hypothesis that chromaffin cells release their catecholamines by exocytosis, a mechanism by which the soluble vesicular content is discharged into the extracellular compartment. The analogous simultaneous release of catecholamines, ATP and DBH in the in vitro CG–PM system suggests that also under those circumstances the whole granular content is extruded. The ability of EGTA to block this process demonstrates the Ca^{2+} dependence, another property of exocytosis in chromaffin cells [10]. A resting cell has $<10^{-7}$ M intracel-

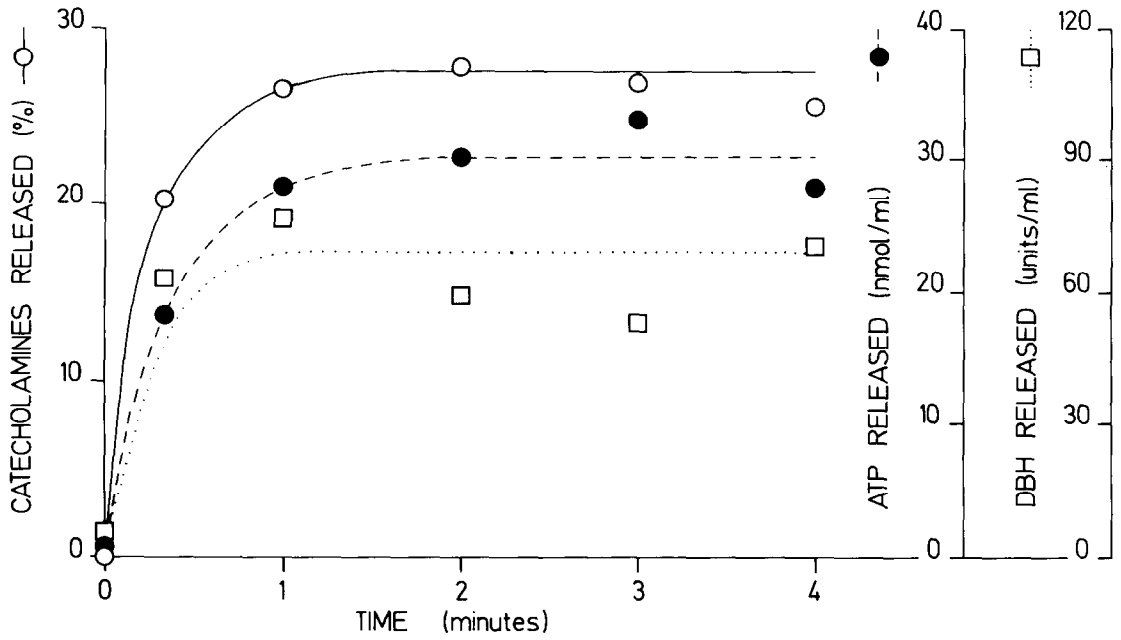
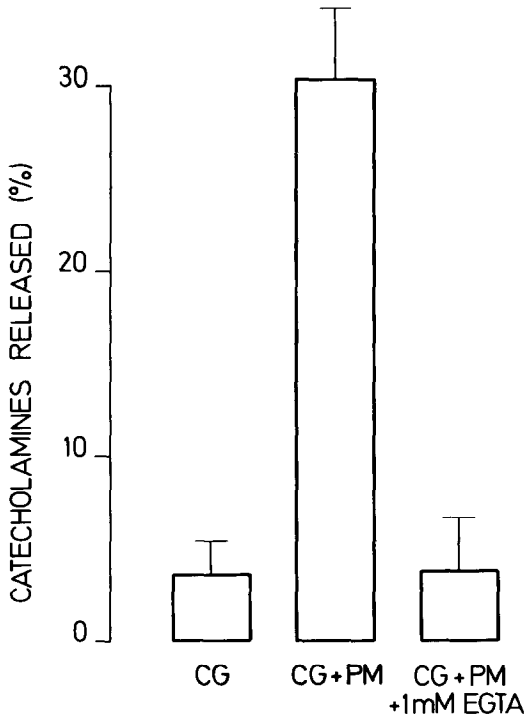


Fig.2. Time course of plasma membrane-induced release of soluble chromaffin granular components. Chromaffin granules (200 μg cat./ml) were incubated together with plasma membranes (50 μg prot./ml) in 0.3 M sucrose (10 mM Tris–HCl (pH 7.4); 1 μM $CaCl_2$) at 37°C in 700 μl total vol.). Each point represents the average result of 2 expt. A correction for catecholamines, DBH and ATP which were not granule-bound before incubation (34 μg, 97 units and 7 nmol/ml, respectively) has been made.



lular Ca^{2+} [1] but this increases to 10^{-6}–10^{-5} M upon depolarization [4]. In our experiments Ca^{2+} was ~3 × 10^{-6} M (10^{-6} M due to the $CaCl_2$ added to the incubation medium and ±2 × 10^{-6} M from lysed CG); this is within the concentration range in which exocytosis is initiated [2]. Exocytosis implies a fusion of the granular membrane with the plasma membrane followed by fission of the fused membranes. In this way, the granular material can be released into the extracellular medium without prior contact with the

Fig.3. The effect of EGTA on the plasma membrane-induced catecholamine release from chromaffin granules. Chromaffin granules (200 μg cat./ml) were incubated for 1 min at 37°C without plasma membranes (first bar), with plasma membranes (50 μg prot./ml, second bar) and with plasma membranes (50 μg prot./ml) and 1 mM EGTA (third bar) (in 700 μl total vol.). The incubation medium contained 0.3 M sucrose, 10 mM Tris–HCl (pH 7.4) and 1 μM $CaCl_2$. The results are presented with their standard deviations (n = 3). Correction for the catecholamines already released before the incubation has been made.

cytoplasm [20]. When purified zymogen granules [19] or vasopressin granules [12] are incubated with, respectively, pancreas or hypophyseal cell membranes, a similar fusion takes place if Ca^{2+} in the incubation medium reaches μM levels. [15] reported the fusion of CG with PM, although Ca^{2+} dependence was not demonstrated. These data together with the results obtained with the system described here suggest that the increase in concentration of free catecholamines can be used as an index for CG and PM fusion and for the subsequent release of soluble granular material. For this reason, the in vitro interaction between CG and PM could provide a potential model system which could, because of its simplicity, offer a better insight into the molecular mechanism of exocytosis.

Acknowledgements

F. K. is a grantee of the Belgium National Fund for Scientific Research (NFWO).

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