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PROTEIN PHOSPHORYLATION AND THE EXOCYTOSIS-LIKE INTERACTION BETWEEN ISOLATED ADRENAL MEDULLARY PLASMA MEMBRANES AND CHROMAFFIN GRANULES

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The interaction between isolated adrenal medullary plasma membranes and chromaffin granules has been proposed as a cell-free model for exocytosis. Phosphorylation experiments showed that isolated chromaffin granules as well as isolated plasma membranes contain protein kinases and phosphate accepting membranous proteins. Upon joint incubation however, the chromaffin granule-located proteins are preferentially phosphorylated. β-γ-methylene-ATP, a non-hydrolysable analogue, was able to reduce both the plasma membrane-induced release of the soluble chromaffin granular content and the phosphate incorporation into the protein fraction. The results of these experiments on a cell-free model system fit in the hypothesis originating from work on several types of intact cells that the exocytotic event is linked with protein phosphorylation.

Chromaffin granules (CG) release their soluble content upon incubation with isolated adrenal medullary plasma membranes (PM) (1, 2). The features of this temperature-, pH- and Ca^{2+}-dependent interaction closely resemble some essential properties of exocytosis, a process in which secretory vesicles release their content into the extracellular space upon fusion with the plasma membrane. The interaction can be blocked by addition of EGTA and is stimulated when $[Ca^{2+}]$ exceeds 2×10^{-7}M with a half maximal response around 10^{-6}M and the maximal response at 10^{-5}M Ca^{2+} (3). Comparison of the density gradient distribution of interacted CG and PM with the distribution of non-interacted material in self-generating Percoll gradients revealed that the loss of each granule's content is an "all-or-none" phenomenon rather than a partial release. Because of these and other resemblances, the PM-induced release of the CG content has been proposed as a putative cell-free model for exocytosis in which the intracellular sites of control would be accessible to manipulation.

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Recently an enhanced phosphorylation of membranous protein has been correlated with the secretory activity in several cell-types. Catecholamine secretion from cultured adrenal medullary cells stimulated by different secretagogues was shown to be accompanied by enhanced phosphorylation (4). Similar observations were made in other neuronal (5, 6) and non-neuronal (7) secretory cells. Since protein phosphorylation could be a necessary step between Ca^{2+}-influx and the exocytotic fusion it was the purpose of this study to investigate whether this phenomenon also occurs in the cell-free CG-PM system. The plasma membrane barrier can also be passed in "leaky" cells, another model for exocytosis (for a review see 8). With this technique, which involves the permeabilization of the plasma membrane with intense electric fields, it has been demonstrated that adrenal medullary cells require ATP for their Ca^{2+}-dependent catecholamine release (9). The presence of Ca^{2+}-dependent protein kinases and phosphate accepting proteins on chromaffin granule-ghosts has been demonstrated (10). Because ATP may exert this function acting as a phosphate donor for membranous protein phosphorylation we also looked at a possible requirement for ATP in the cell-free CG-PM system. Since the soluble CG matrix contains a considerable amount of ATP (for a review see 11) the ATP concentration of the incubation medium before interaction is usually situated between 5×10^{-6}M and 10^{-5}M due to a small but inevitable fraction of broken granules. This concentration can rise to 5×10^{-5}M or more during the interaction (2); therefore we used β-γ-methylene-ATP, a non-hydrolysable analogue, to compete with the endogenous ATP (12).

METHODS

Bovine adrenal PM and CG were isolated essentially as described by (13). The isolated CG and PM were resuspended in 0.3M sucrose (10 mM Tris-HCl, pH 7.4) and the CG catecholamine and PM protein concentrations were determined. After dilution of the CG and PM to a concentration adequate for the incubation experiments the PM suspension or and equivalent blank was preincubated for 5 min at 37°C and the CG suspension equilibrated at room temperature. Then CG were added to the PM suspension and the incubation mixture shaken for a few seconds. After 1 min incubation at 37°C this mixture was placed in iced water and 3 volumes of ice-cold buffered 0.3M sucrose were added to stop the temperature-dependent interaction. The incubated samples were then centrifuged at 15,000 x g for 20 min in a refrigerated centrifuge to pellet the intact CG (MSE HiSpin 21 with 24 x 14 rotor). By this procedure granule-bound catecholamines (or other markers of the soluble CG content) were separated from free catecholamines. The supernatants were kept for assay. The resulting data were corrected for the catecholamines or soluble protein already set free before incubation (see 1).

For the ^{32}P incorporation experiments the incubation method was modified as follows : 60 µCi (γ-^{32}P) ATP (3000 Ci/mmol, Amersham

International) were added to each of the preincubating PM suspensions 1 min before the actual incubation with CG. The total incubation volume was 1000 µl. The incubation was stopped with 9 volumes of 10 mM Tris-HCl buffer (pH 7.4) in order to lyse the vesicles. The membrane fraction was recovered by centrifugation at 200,000 x g for 60 min (Beckman L8-55 with SW41 rotor). The resulting pellets were resuspended in 100 µl of a solubilizing solution containing 10 mM Tris-HCl (pH 6.8), 2.5 % SDS (w/v) and 5 % β-mercaptoethanol (v/v). After being boiled for 10 min the samples were centrifuged in an Eppendorf table-top centrifuge for 10 min. 20 µl of each of the resulting supernatants (containing solubilized membrane proteins) were applied on 12.5 % polyacrylamide gels with 4 % stacking gels. SDS-gelelectrophoresis was performed by the method of (14) in a vertical slab gel electrophoresis apparatus (Pharmacia Fine Chemicals GE-4). The electrode buffer (pH 8.3) contained 0.05 M Tris-HCl, 0.384M glycine and 0.1 % SDS. The samples were electrophoresed together with molecular weight standards (Pharmacia Fine Chemicals) for 6 h at 150V and stained with 0.1 % Coomassie brilliant blue R250. Dried gels were placed in contact with Kodak XAR-5 film for 3 days at -70°C. The autoradiographs were scanned in a Perkin-Elmer densitometer by the Research and Development department of AGFA-Gevaert, Mortsel.

In another series of experiments 50 µl of each of the solubilized membrane protein containing samples were run through an HPLC-gel-filtration column (Spherogel-TSK 3000 SW, 7.5 x 600 mm) at 1 ml/min with 0.1M phosphate buffer (pH 7.0, 0.1 % SDS) (6000A pump and U6K injector, Waters Ass.; UV-50 detector, Varian). In this step proteins were separated from smaller molecules like ATP. The fractions containing proteins larger than 15,000 dalton (determined by molecular weight markers) were pooled and counted for ^{32}P-activity in a liquid scintillation counter (Packard A460 with Pico-Fluor 30 scintillator). The ^{32}P-incorporation into the membranous protein fraction could be measured quantitatively this way.

The catecholamine concentrations were measured by the colorimetric method of (15) and by the HPLC method with amperometric detection described by (2).

The protein concentrations were determined according to the methods of (16) and (17) using bovine serum albumin for a standard.

It should be remarked that no additional Ca^{2+} was required since the concentration of endogenous Ca^{2+} from broken granules exceeds the treshold concentration for interaction between CG and PM.

RESULTS AND DISCUSSION

Addition of (γ-^{32}P)ATP to the incubation medium revealed that several membranous proteins of CG and PM become phosphorylated under the conditions used for interaction experiments (Figure 1). CG and PM both have their own protein kinases and phosphate accepting proteins as can be seen on the densitometric tracings of CG and PM incubated separately. The major CG-located phosphorylated protein seems to have a molecular weight of about 45kD. The two most important phosphorylated proteins on the PM have an apparent molecular weight of approximately 20 and 60kD. When CG and PM are incubated together the CG-located proteins are preferentially phosphorylated. Only the 60kD-PM protein can still be detected on the autoradiographs. The latter protein band most

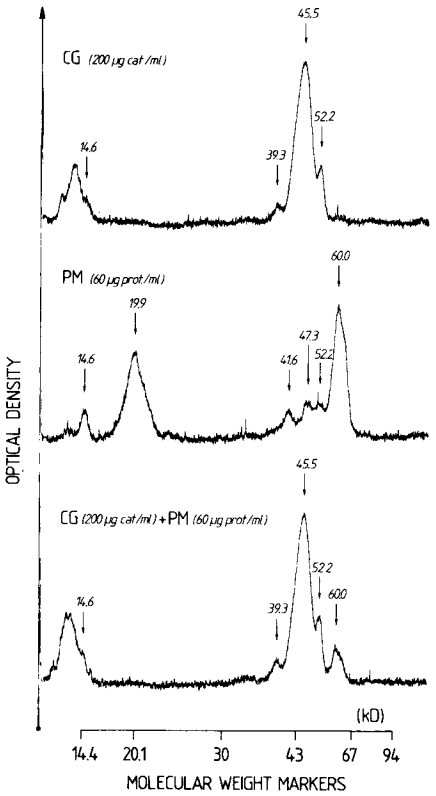

Figure 1 - Membranous phosphate accepting proteins of the chromaffin granule-plasma membrane system and its components. Chromaffin granules and plasma membranes were incubated for 1 min, apart and together, in 1 ml 0.3M sucrose (10 mM Tris-HCl, pH 7.4) containing 150 µCi γ-^{32}P-ATP. After this incubation and hypotonic lysis the membrane fraction was recuperated by high-speed centrifugation. The solubilized membranous proteins were subjected to SDS polyacrylamide gel electrophoresis in a 12.5 % gel. Densitometric scans of the autoradiograph from the dried gel are presented with the estimated molecular weight of the phosphate accepting proteins.

probably contains two different polypeptides in close approximation (observation from other experiments) and its phosphorylation seems to be Ca^{2+}-dependent. Addition of 1 mM EGTA to the incubation mixture with CG and PM resulted in a 70 % decrease in phosphorylation of the 60kD band compared to a control without EGTA (figure not shown). On the other hand the 14.6kD band and the lighter material near the front (which might be polyphosphoinositides, 18) showed a 6-fold increase in phosphorylation in the presence of 1 mM EGTA.

β-γ-methylene-ATP provokes a decreased ^{32}P-incorporation into the membranous protein fraction of the CG-PM system (figure 2) and it has an inhibitory effect on the PM-induced release of the CG content (figure 3). At high concentrations the non-hydrolysable ATP analogue also caused effects which were not interaction-specific as demonstrated by an

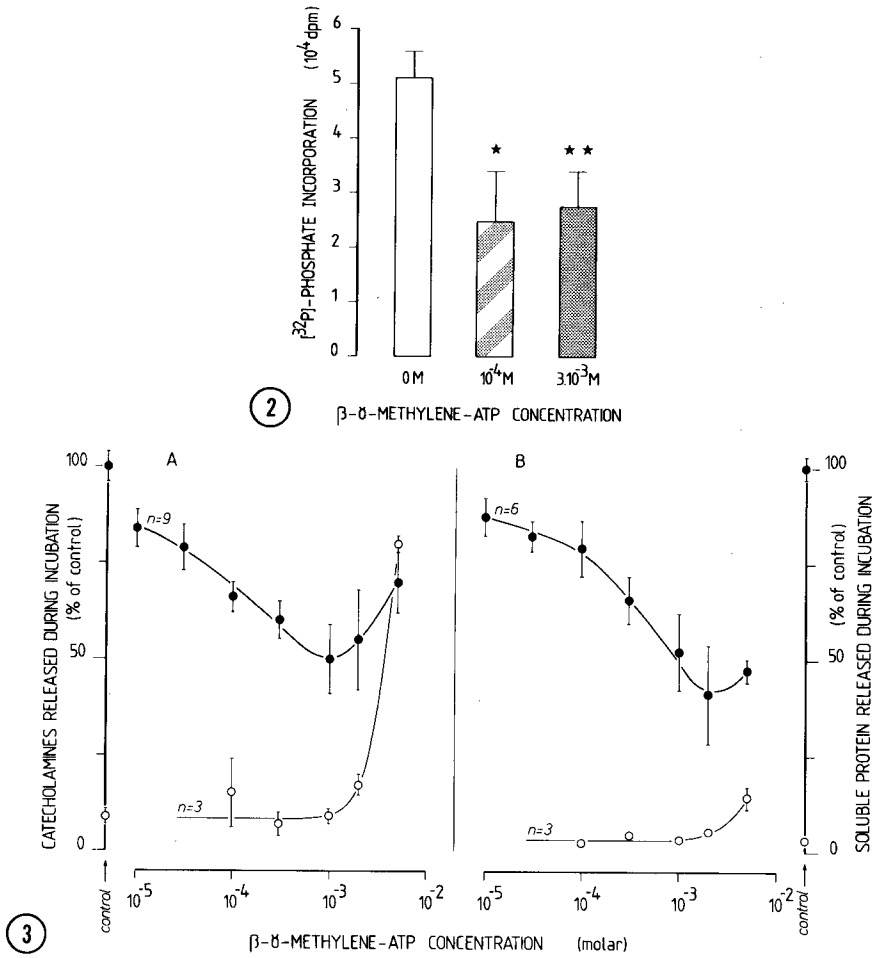

Figure 2 - The effect of a non-hydrolysable ATP-analogue on the phosphorylation of the membranous fraction of the chromaffin granule-plasma membrane system. Chromaffin granules were incubated with plasma membranes as in Figure 1. After solubilisation the proteins from the membranous fraction were separated from smaller molecules using HPLC-gel filtration. The ^{32}P-incorporation was measured in a scintillation counter. Results are presented with their standard deviation (n = 3; * : $P < 0.05$; ** : $P < 0.01$; Student's t-test).

Figure 3 - The effect of a non-hydrolysable ATP-analogue on the plasma membrane-induced release of the chromaffin granular content. Chromaffin granules (200 µg cat./ml) were incubated for 1 min at 37°C without (open symbols) and with (closed symbols) plasma membranes (50 µg prot./ml) in 400 µl 0.3M sucrose (10mM Tris-HCl, pH 7.4). Immediately after this incubation the released granular content markers like catecholamines (A) and soluble protein (B) were separated from those still present in the chromaffin granules by centrifugation. In the control situation (no ATP analogue added) about 30 % of the catecholamines present in the CG were released during incubation with PM. The results are presented with their s.e.m. Correction for catecholamines or soluble protein already free before the incubation has been made.

increased release of soluble material from CG incubated without PM. Up to 1 mM, however, it inhibits the CG-PM interaction without affecting CG incubated alone. These observations suggest that hydrolysis of ATP is

necessary for the membranous protein phosphorylation as well as for the exocytosis-like interaction in the CG-PM system. (9) also suggested that the hydrolysis of ATP is necessary for exocytosis in bovine adrenal medullary cells. This assumption was based on their experiments with "leaky" cells, a model which showed a Ca^{2+}-dependency remarkably similar to the one of the cell-free CG-PM interaction (19). The CG-PM system contains at least one other important ATP hydrolysing enzyme : the CG-located Mg^{2+}-ATPase responsible for the energetisation of the catecholamine uptake. This enzyme can be inhibited with N-ethylmaleimide (20), a drug which does not interfere with the PM-induced release of the CG content (up to 10^{-4}M NEM, unpublished results), and is therefore not a likely mediator between ATP and the exocytosis-like event. If protein phosphorylation proves to be a prerequisite for the interaction between CG and PM the main role of ATP might be that as a substrate for protein kinases.

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