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ANTI-SYNTAXIN ANTIBODIES INHIBIT CALCIUM-DEPENDENT CATECHOLAMINE SECRETION FROM PERMEABILIZED CHROMAFFIN CELLS

L.M. Gutierrez[1], J.L. Quintanar[1], S.Viniegra[1], E. Salinas[1], F. Moya[2] and J.A. Reig[1*]

[1]Departamento de Neuroquímica y
[2]Departamento de Genética y Microbiología
Facultad de Medicina e Instituto de Neurociencias
Universidad de Alicante. Ap. 374 Alicante 03080, Spain

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Adrenomedullary chromaffin cells release catecholamines in response to the intracellular calcium rise upon stimulation by different secretagogues. The presence of syntaxin 1, a protein presumably involved in docking of synaptic vesicles to presynaptic membranes, has been investigated in chromaffin cells. The study using two different monoclonal antibodies shows that syntaxin 1 is present in the chromaffin cell membrane fraction. Functional experiments demonstrate that anti-syntaxin antibodies inhibit calcium-dependent secretion in permeabilized cells. These results suggest that syntaxin 1 is an important component of the secretory machinery in chromaffin cells.

The exocytotic process in the synaptic terminal and other secretory systems as chromaffin cells, requires calcium entry that triggers the release of the vesicle content by a mechanism that involves the fusion of both the vesicle and the cell membrane (1,2). Recently, several proteins have been described forming a complex that establishes the docking and secretory machinery of the vesicles (3-6). These proteins include synaptotagmin and synaptobrevin/VAMP (vesicle-associated membrane protein) in the vesicle membrane and syntaxin and SNAP-25 in the presynaptic membrane (6). The presence of several of these proteins as syntaxin and the mammalian homologue of yeast Sec1 (7), synaptotagmin/p65 (8), and SNAP-25 (9) have been recently demonstrated in chromaffin cells (10). Syntaxin 1 is member of a large family of vesicular transport receptors displaying a broad tissue distribution (11).

(*) Corresponding author: J.A. Reig. Dpt. Neuroquímica, Facultad de Medicina, Universidad de Alicante. Ap 374 Alicante 03080 . SPAIN.
FAX:SPAIN 6-565 8557.

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In the present work we confirm the presence of syntaxin 1 in the chromaffin cell using both, biochemical and immunocytochemical approaches and for the first time, we demonstrate in digitonin-permeabilized chromaffin cells, that monoclonal antibodies against this protein, inhibit calcium-dependent catecholamine secretion in a doses dependent manner. The results suggest a role for syntaxin 1 in regulated secretion of chromaffin cells.

MATERIALS AND METHODS

Materials: Monoclonal anti-syntaxin (clone HPC-1), anti-mouse IgG peroxidase conjugate and mouse IgG were from Sigma. The mouse monoclonal anti-syntaxin antibody used in immunoblotting test (Mab 44D5) was isolated in a screening of neurospecific antigens expressed in *Drosophila* CNS (12). [^{3}H]noradrenaline was from Du Pont. Electrophoresis reagents were from BioRad. Immobilon PVDF (polyvinylidene difluoride) membranes were from Millipore. All other reagents were of analytical grade from Sigma.

Cell cultures and subcellular preparations: Chromaffin cells were prepared from bovine adrenal glands by collagenase digestion as previously described(13). Chromaffin cell membranes were prepared from 5x10^6 cultured cells at a density of 525,000 cells/cm^2. Cells were sonicated twice in a 50 mM Tris/ClH pH 7.2 buffer, containing 1mM PMSF (phenylmethyl sulphonyl fluoride),1mM EGTA, during 5 sec using a Sonic dismembrator at 30% maximal power (Artek System Corp.). The suspension was finally centrifuged for 30 min at 150,000 x g in a Beckman TL100. Proteins in the sediment were analyzed by 1D-PAGE. Adrenomedullary enriched plasma membrane and the cytosolic fraction were obtained according to the method of Meyer and Burger (14) using a discontinuous sucrose gradient as previously described (15). Synaptosomes from rat brain cortex were prepared in ficoll gradients according to the method of Cotman(16).

Cell permeabilization and quantification of catecholamine release: Secreted [^{3}H]noradrenaline was determined in digitonin-permeabilized cells as previously described (13) with the following modifications. During the permeabilization period cells were incubated in the absence or presence of the different immunoglobulins. After this treatment media were discarded and cells treated for 10 additional min with the same media lacking digitonin, in the presence of EGTA 5mM (basal secretion) or a 10µM buffered Ca^{2+} concentration (stimulated secretion) containing or not the different antibodies assayed. Media were collected and centrifuged at 5,000 rpm for 3 min. Released catecholamines as well as the total cell content were determined by liquid scintillation spectrometry. Net secretion is the difference between stimulated and basal release.

Electrophoresis, electroblotting and immunodetection: Were performed as previously described (17). 10% PAGE was carried out according to the method of Laemmli (18). Proteins from PAGE gels were transferred onto PVDF membranes according to the procedure of Towbin (19).

Immunocytological labeling: Chromaffin cells 50,000 cells/cm^2 were incubated with the monoclonal anti-syntaxin antibody (clon HPC-1; Sigma) for 24 h and cells were processed as previously described (20). Cells were visualized and photographed using a Olympus PM-10AD coupled to a Olympus microscope.

RESULTS

Identification of syntaxin-1 in chromaffin cells

In order to investigate the possible presence of syntaxin 1 in chromaffin cells, different samples were run on 1D-PAGE and tested using the monoclonal antibody Mab 44D5, this antibody has been shown to recognize *Drosophila* and rat syntaxin 1 (12). As it is shown in Fig. **1**. Syntaxin 1 is present in the fraction containing total chromaffin proteins (Fig.**1**,2) and is enriched in a rat brain synaptosomal fraction (Fig. **1**,3). Additionally, labeling is also positive in a plasma membrane enriched preparation from adrenal medulla (Fig. **1**,6) and a microsomal preparation from 48h cultured chromaffin cells (Fig.**1**,1). Syntaxin 1 was absent in the adrenal medulla cytosolic fraction (Fig **1**,5) and in a 100,000 x g liver membrane microsomal preparation used as control (Fig **1**,4). Similar results were obtained using the anti-syntaxin antibody (clon HPC-1) (data not shown). This experiment shows that syntaxin 1 is present in a membrane enriched fraction of adrenal medullary tissue, and confirms, as it has been demonstrated previously in culture chromaffin cells (7,9), the presence of syntaxin 1 in these cells, although in a lower relative amount than in synaptic terminals.

In order to confirm the presence of syntaxin 1 in chromaffin cells we investigated the presence of syntaxin 1 using an immunocytochemical approach. Cultured chromaffin cells were incubated with the anti-syntaxin antibody (clon HPC-1) as indicated in Methods. Fig.**2** clearly shows that cultured chromaffin cells were labeled by the anti-syntaxin antibody, as compared with the absence of label in control condition in which mouse immunoglobulins were used.

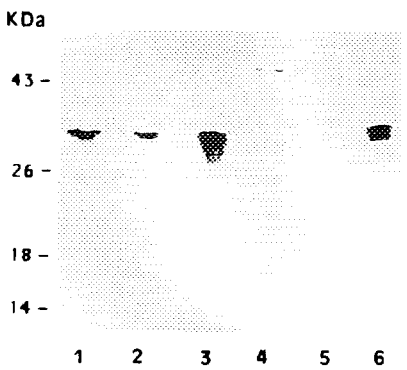

Figure 1. Western blot analysis to detect the presence of syntaxin 1

Different preparations containing approximately 100 µg protein from: chromaffin cell microsomal fraction (1), total chromaffin cell proteins (2), rat brain synaptosomes (3), rat liver microsomal fraction (4), bovine cytosolic adrenal fraction (5) and an enriched bovine adrenomedullary plasma membrane fraction (6) were analyzed by 10% PAGE, electroblotted onto a PVDF membrane and probed with anti-syntaxin antibody Mab44D5 (1/50) as described in Methods.

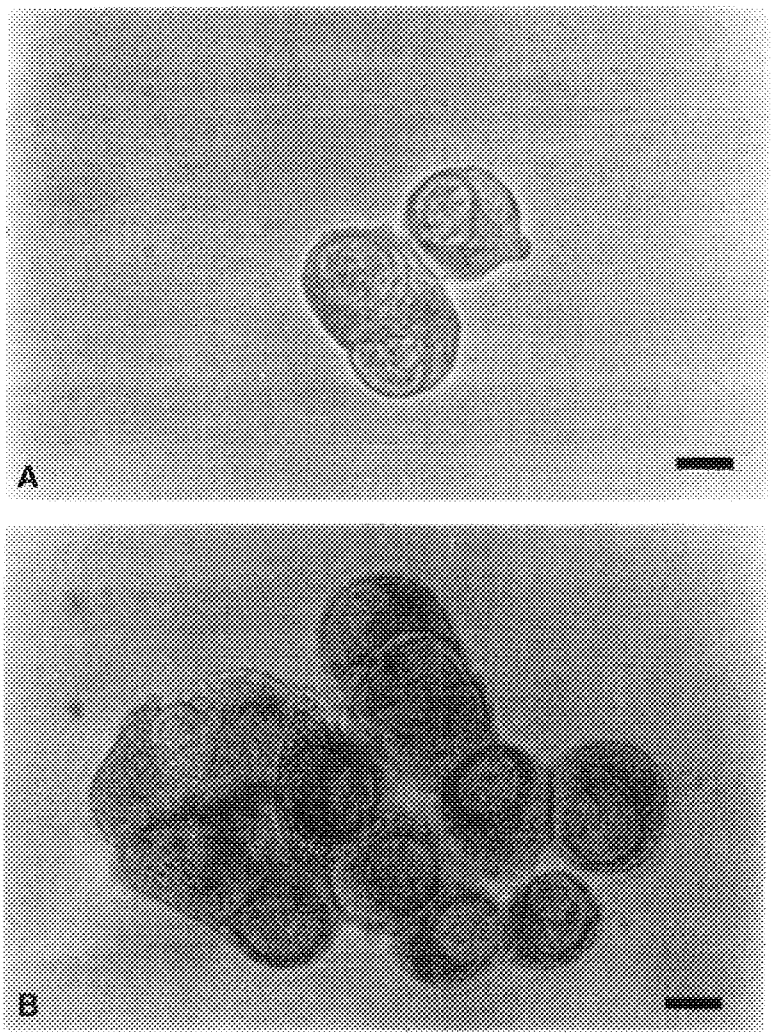

Figure 2. Immunostaining of cultured chromaffin cell using anti-syntaxin
Chromaffin cells were incubated with the anti-syntaxin antibody (clon HPC-1; Sigma) for 24h and color reaction was developed using a peroxidase-labeled secondary antibody as indicated in Methods. **A.** Control cells incubated with normal mouse immunoglobulins. **B.** Cells incubated with the anti-syntaxin antibody. The scale bar represents 10 μm.

Inhibitory effect of anti-syntaxin on catecholamine secretion

Cultured chromaffin cells were permeabilized in the presence or absence of the anti-syntaxin antibody (clon HPC-1), for the indicated time periods. Medium was then removed and catecholamine secretion was measured in the absence or presence of 10μM Ca^{2+} as indicated in Methods. Fig. **3**,A shows that the anti-syntaxin antibody provoked a time dependent decrease of net catecholamine secretion. Inhibition was also dependent on the

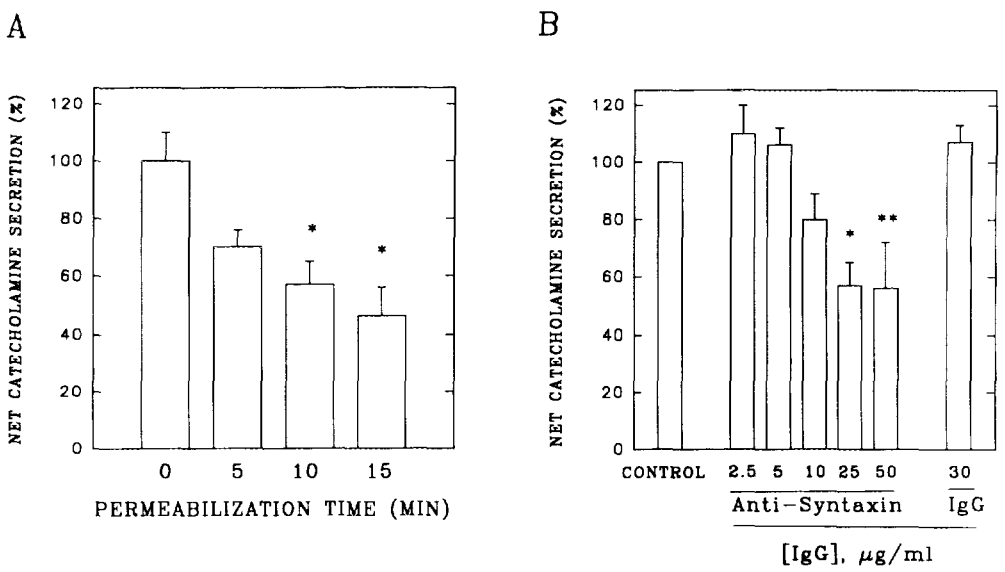

Figure 3. Effect of the anti-syntaxin antibody on catecholamine secretion in digitonin-permeabilized chromaffin cells

A. Time course effect: Anti-syntaxin antibody (clon HPC-1; 25μg ml^{-1}) was incubated for the indicated times in the presence of the digitonin-permeabilization solution as indicated in Methods, then medium was removed and secretion was induced for 10 min in the presence of 5mM EGTA or 10 μM Ca^{2+} both in absence or presence of the antibody. **B.** Dose dependent effect: Different concentrations of anti-syntaxin antibody (clon HPC-1) were incubated for 10 min in the permeabilization solution and catecholamine were measured as indicated in the former experiment. Normal mouse IgG were also tested in the same conditions as a control at the indicated concentration. Student's *t* test was used as an indication of statistical significance. Data that differ significantly from control conditions are indicated by asterisks: *p<0.01; **p<0.02.

immunoglobulin concentration reaching a 50% inhibitory effect at 25 $\mu g ml^{-1}$ (Fig. **3**,B). Basal catecholamine release was not affected by the presence of the antibody. To demonstrate the specificity of the inhibition of the anti-syntaxin antibody, mouse immunoglobulins were used as control. Concentrations as high as 30 μg ml^{-1}, had no effect on Ca^{2+}-dependent catecholamine release (Fig. **3**,B). These experiments suggest that syntaxin 1 is participating in the regulated secretory pathway of chromaffin cells.

DISCUSSION

The results of this study shows that syntaxin 1 is a protein that is present in the chromaffin cell. For the first time, using an immunocytochemical technique we demonstrate the presence of label in these

cells, however this data can not distinguish the localization of the protein. Moreover blotting experiments clearly show that syntaxin 1 is present in a plasma membrane enriched fraction of adrenal medulla and being also associated to the microsomal fraction of cromaffin cells suggesting that is clearly a membrane associated protein. At the same time we have demonstrated that syntaxin 1 is implicated in the calcium-dependent secretory pathway. The inhibition using this antibody reached 50% ($p<0.01$) of the net calcium-dependent catecholamine secretion and basal secretion was unaffected by the antibody. These results demonstrate that syntaxin 1 is a crucial element of the Ca^{2+}-dependent secretory machinery in chromaffin cells, most probably participating in a complex as it has been previously proposed (5,9). There are several posible reasons that could explain that the inhibition was not complete. The antibody could be affecting the docking of a population of granules that is not yet in the membrane, resulting in a partial inhibition, granules already docked to the membrane and possibly forming an active complex could not be affected.
This work supports the concept that molecular events involved in regulated exocytosis are similar independently of the type of vesicle involved in the process. Differences concerning the time needed for maximal secretion between the synaptic cleft and endocrine cells apparently could be caused by the high concentration of vesicles accumulated and docked at the active zones of the synaptic terminals.

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