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Immunocytochemical demonstration of calmodulin in cells secreting by exocytosis

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Summary. Calmodulin is a regulator of several calcium-dependent cellular processes. It has been suggested that it plays a role in the mechanism of secretion. Employing an indirect immunoperoxidase technique at the light microscope level, this study demonstrates the presence of calmodulin in several exocytotic cells (mast cells, thyroid follicular cells, neurohypophyseal neurosecretory terminals, pancreatic β-cells and pancreatic acinus cells) in rat and man. The positive staining reaction for calmodulin was granular and at least in the case of rat mast cells it appeared to be associated with the granule membrane.
Key words. Immunocytochemistry; calmodulin; secretory granules.

Many intracellular processes, including stimulus-secretion coupling, are known to be calcium-dependent. One of the modulators of these processes is probably the troponin C-related protein, calmodulin (148 amino acid residues, mol.wt around 17,000) which appears to be ubiquitous in eukaryotic cells[1,2]. Calmodulin could play a role in stimulus-secretion coupling at several steps, as the protein is involved in cyclic AMP metabolism, arachidonic acid metabolism, activation of the actomyosin system, control of microtubule assembly and calcium-magnesium ATP-ase activity[3,4].

In indirect pharmacological studies and using radioimmunoassays and phosphodiesterase activation assays, the presence of calmodulin has been demonstrated in exocytotic cells: mast cells[4,5], neurohypophyseal neurosecretory terminals[6–9], follicular cells of the thyroid[10,11], pancreatic β-cells[12,13] and acinus cells of the exocrine pancreas[14].

In the present study, the indirect immunoperoxidase technique was employed to investigate the subcellular distribution of calmodulin in the above-mentioned cells.

Material and methods. Reagents and buffers. Calmodulin was purified from bovine brain according to Watterson et al.[15]. Polyclonal rabbit antiserum to calmodulin was prepared according to Slaninova and Thorn[9]. The antibody showed no cross-reactivity with troponin. The buffer used for the radioimmunoassay contained 1 mM EGTA. Measurements of calmodulin concentration by means of radioimmunoassay and by the phosphodiesterase activation method gave the same results in rat brain homogenates, bovine neurosecretomes and neurosecretory granules respectively. Normal goat serum, biotinylated goat antirabbit serum and avidin peroxidase were purchased from Vector. A Tris/HCl buffer, 50 mM, pH 7.4, containing 0.5 M NaCl and 0.01% of the detergent Nonidet P-40 was used for washing. Reagents were diluted in phosphate buffered saline containing 1% bovine serum albumin.

Tissue. Rat mast cells were obtained by peritoneal and thoracic cavity lavage, purified by Ficoll (Pharmacia) density gradient separation, washed and suspended in a Tris/HCl buffer[16]. Smears were prepared following resuspension in plasma. The smears were air-dried and fixed in methanol for 30 min.

Specimens from rat and human neurohypophysis, thyroid and pancreas were formalin fixed, paraffin-embedded and cut in serial sections. Mast cells were found in the loose connective tissue of these specimens.

All the paraffin-embedded sections were deparaffinized prior to immunostaining.

Immunostaining procedure. All steps apart from the primary antibody incubation were carried out at room temperature. The slides were treated with methanolic hydrogen peroxide 0.5% for 15 min followed by normal goat serum 1:50 for 15 min to block endogenous peroxidase activity and reduce background staining. They were incubated at 4 °C with anti-calmodulin 1:1000 for 72 h, with biotinylated goat antirabbit serum 1:200 for 35 min and with avidin peroxidase 1:400 for 50 min. Calcium concentrations were controlled using defined amounts of EGTA and calcium cloride in the incubation buffers. Staining was achieved by incubating the slides for 15 min with 0.5 mg diaminobenzidine per ml Tris/HCl buffer, with the addition of hydrogen peroxide for the last 8 min. Finally, the slides were dehydrated and mounted in DPX.

Control trials were performed employing non-immunized rabbit serum 1:1000 in place of anti-calmodulin. Moreover, the experiments were carried out following absorption of antibody with purified calmodulin.

Results. Immunoperoxidase staining for calmodulin revealed a characteristic positive staining reaction in all exocytotic cells investigated including pancreatic β-cells and acinus cells, different cell types in the adenohypophysis, nonterminal neurosecretory dilatations in the neurohypophysis, thyroid follicular cells and mast cells in both species.

The huge accumulations of granules, 0.1–0.2 µm wide, along the nerve fibers in the neurohypophysis corresponding to the nonterminal dilatations and known as Herring bodies exhibited a strongly positive granular staining reaction for calmodulin (fig. 1A). Due to the small size of the granules it was not possible to locate the specific site of reactivity to granule membrane and/or granule interior and it was not possible to exclude a diffuse cytoplasmic reactivity. Similar considerations apply to the detection of the positive staining reaction in thyroid follicular cells (fig. 1B) and human mast cells.

Smears of large rat mast cells with large granules (more than 0.5 µm in diameter) were stained with toluidine blue at pH 5. The interior of the mast cell granules exhibited a strong metachromasia (fig. 2A). Following immunoperoxidase staining for calmodulin the rims of the individual granules exhibited a distinct staining reaction (figs 2B and C).

The immunohistochemical staining pattern of exocytotic cells was similar in spite of different preparative methods and all appropriate controls were negative. Variations in calcium concentrations from 4 mM Ca^{++} to 25 mM EGTA did not influence the results.

Discussion. Secretory cells are characterized by an ability to transport macromolecules across their plasma membrane. This process involves sequential formation and fusion of membrane-bound granules or vesicles. Secretion by exocytosis is considered to be a localized response to a regional increase in cytoplasmic calcium levels. This causes local structural changes in the adjacent plasma membrane and probably involves a calcium-binding protein such as calmodulin[17].

Studies using immunological and non-immunological secretory agents combined with calmodulin antagonists such as phenothiazines and the smooth muscle relaxant W-7 indicate that exocytosis is a calcium-calmodulin regulated process[4]. Furthermore, studies of human basophilic granulocytes have provided indirect pharmacological evidence that histamine release in these cells is associated with calmodulin or calmodulin-dependent enzymes[5].

The mast cell provides a well-documented model of exocytosis. Utilizing a specific antibody against calmodulin in the indirect immunoperoxidase technique we have demonstrated the presence of this protein in association with the secretory granule membranes of the rat mast cell.

It would appear likely that a large part of the calmodulin found in resting cells with normal levels of free calcium in their cyto-

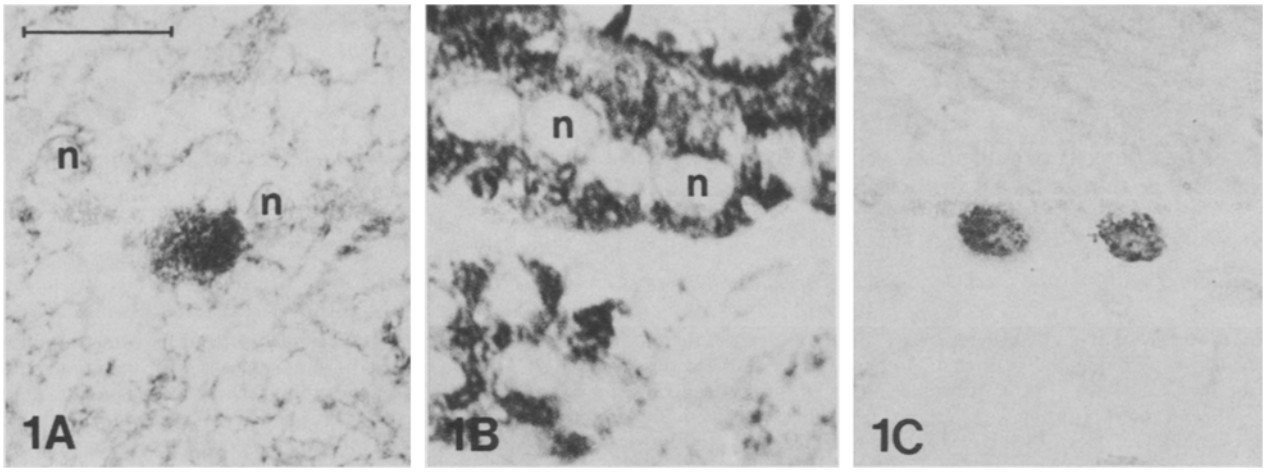

Figure 1. A Herring body (nonterminal dilatation) from rat neurohypophysis *(A)*, follicular cells from human thyroid *(B)* and two mast cells from human dermis *(C)* show a positive staining reaction for calmodulin. Note the granularity of the reaction product. n = unstained nuclei of pituicytes *(A)* and of follicular cells *(B)*. × 950.

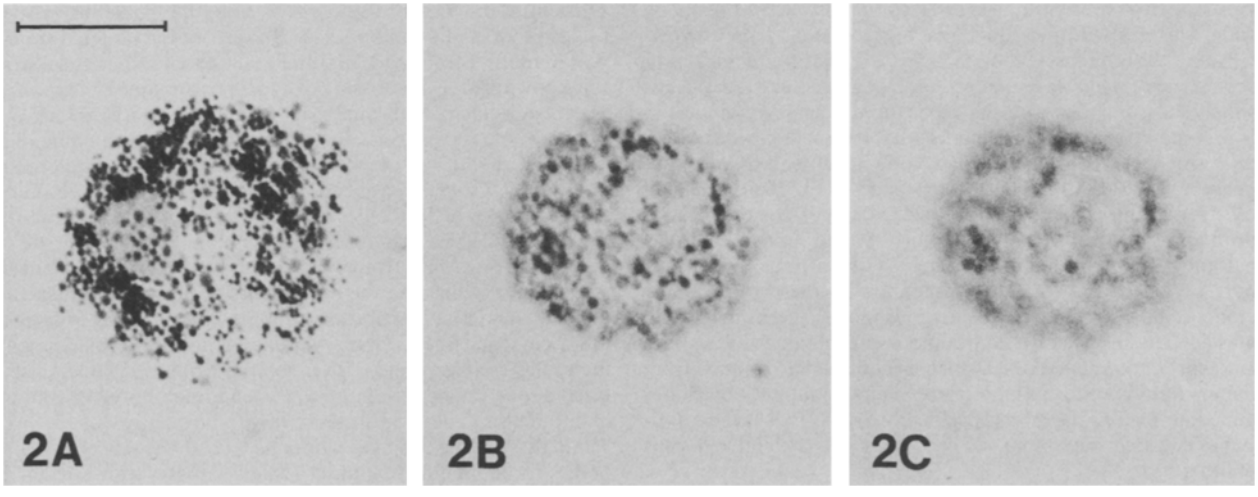

Figure 2. Smears of large rat mast cells stained with toluidine blue at pH 5 *(A)* and for calmodulin *(B* and *C)*. The metachromagia in *A* is confined to the interior of the large granules, whereas a distinct positive staining reaction is associated with the membranes of the granules. The same mast cell is shown in *B* and *C* in different focus planes. × 950.

plasm would not be bound to calcium[18]. It is not clear to what extent the immunostaining procedure interferes with the binding of calcium to calmodulin or with the subcellular distribution of calcium. Binding of radiolabeled calmodulin to the cytoplasmic surface of neurosecretory granules has, however, been shown to occur both in the presence and in the absence of calcium[8].
The subcellular, granular distribution of calmodulin in the cells investigated in the present study is compatible with the involvement of calmodulin in exocytosis.

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Selective killing of smooth muscle cells in culture by the ricin A-chain conjugated with monoclonal antibodies to a cell surface antigen via a dextran bridge

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Summary. Monoclonal antibodies to a surface antigen of the modulated smooth muscle cells originally isolated from the rat aorta media were conjugated with ricin A-chain via an oxidized dextran bridge. The interaction of cultured cells with the conjugates obtained and with control substances was monitored following incorporation of ^{14}C-leucine radioactivity. It was found that ^{14}C-leucine incorporation was suppressed by 80–90% at a conjugate concentration of 10^{-6}–10^{-7} M. Antigen-negative cells (line IAR; rat hepatocytes) were insensitive to the conjugate at any concentration used. Control use of purified ricin A-chain, native or oxidized dextran, specific and nonspecific IgG did not affect normal ^{14}C-leucine incorporation. The data obtained may be useful for designing targeted drug transport systems and for selective screening of modulated smooth cells in vascular pathology models in vivo.
Key words. Smooth muscle cells; monoclonal antibodies; ricin A-chain; drug transport-system; dextran bridge.

It is known at present that in different disease states (including widely-distributed ones such as atherosclerosis and hypertension) smooth muscle cells participate in the pathological process; some of them leave the resting state and enter the cell cycle, which ends in either true cell replication or endoreplication[1,2]. It was shown in cell culture models that only cells whose phenotype is modified into the 'synthetic' one can enter a proliferative phase[3,4]. The discovery of markers specific for the modulated cells is very important for the localization of these cells in a normal vessel, for the investigation of their role in vascular pathology and for the construction of systems for targeted transport of biologically active compounds to these cells. Using model cellular cultures with analogous modifications of smooth muscle cells monoclonal antibodies were obtained to surface antigens of modulated cells, which were originally isolated from the media of rat aorta. One of the monoclonal antibodies obtained, termed L_1, interacts specifically only with a surface antigen of cultured smooth muscle cells and does not bind with cells of other types[5,6].
At present antibodies to cell surface antigens are widely used for immunotoxin synthesis and selective action on the antigen-bearing cells[7].

The aim of the present work was to study the possibility of the use of monoclonal antibodies L_1 as vectors for targeted transport of model compounds to the modulated smooth muscle cells. It is known that direct binding of a toxin or another biologically-active substance to an antibody sometimes noticeably affects antibody specific properties or does not permit the binding of a sufficient quantity of active molecules to each immunoglobulin molecule. To solve the problem drugs (for example, antibiotics) can be coupled to antibodies via a polymeric bridge, particularly via an activated dextran macromolecule[8,9].
In our case dextran activated by periodate oxidation was used as a polymeric bridge and the A-chain of the plant toxin ricin was used as a model active substance. In principle, instead of one or a few ricin A-chains a larger number of low-molecular-weight molecules can be bound with a dextran bridge, capable of targeted action on smooth muscle cells entering into proliferation in different pathological states.
The activity of the conjugates obtained, i.e. the ability of L_1-antibodies to be modified, without loss of activity, with a reactive polymer capable of binding biologically active compounds, was observed by following the selective killing of antigen-positive smooth muscle cells in vitro.