J. Membrane Biol. 96, 193–198 (1987)

The Journal of
Membrane Biology

ADH and Phorbol Ester Increase Immunolabeling of the Toad Bladder Apical Membrane by Antibodies Made to Granules

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Summary. Polyclonal antibodies were raised to isolated toad bladder granules. On immunoblots, the anti-granule antiserum specifically stained components of isolated granules. Immunocytochemically, the anti-granule antiserum labeled the apical surface of the bladder. Immunolabeling increased at the apical surface when the bladder was exposed to antidiuretic hormone (ADH) serosally or phorbol ester (PMA) mucosally—conditions which stimulate apical granule exocytosis. The increase in granule epitopes on the apical surface was sixfold greater than the net increase in surface area.

Key Words antidiuretic hormone · exocytosis · hydroosmosis · phorbol myristate acetate (PMA) · transport

Introduction

Antidiuretic hormone, ADH, induced hydroosmosis is accompanied by an increase in the apical surface area of the toad urinary bladder as measured morphometrically and electrically [4, 9, 16, 18]. It is presumed that this addition does not represent more of the pre-existing membrane but rather addition of membrane domains which contain the effector units of the hormone-induced apical permeability changes (11).

Exocytosis of apical electron-dense granules and tubules containing intramembranous particle aggregates is a likely source of the additional membrane [4, 12, 15]. We have recently isolated the electron-dense, membrane-bounded granules from the toad urinary bladder [7] and now report that we have raised, in rabbits, polyclonal antibodies to the granules which have been evaluated in immunoblots and by immunocytochemistry. We present data showing that material which reacts with the anti-granule antibody is increased on the apical surface after stimulation by serosal ADH or mucosal phorbol ester, phorbol myristate acetate (PMA), consistent with apical granule exocytosis previously reported [12, 13]. There is a sixfold enrichment in immunolabeling of granule epitopes over the increase in surface area, reinforcing the idea that the material added contains specialized domains with a greater than proportional representation of epitopes which resided previously in the intracellular granules.

Materials and Methods

Materials

4β-Phorbol-12-myristate 13-acetate (PMA), dimethylsulfoxide (DMSO), 3,3′diaminobenzidine tetrachloride (DAB), 4-chloro-1-naphthol, bovine serum albumin (BSA), non-ionic detergent (Nonidet NP-40), HEPES and Tris were purchased from Sigma Chemical (St. Louis, MO); antidiuretic hormone (ADH, pitressin) from Parke, Davis (Morris Plains, NJ) and goat anti-rabbit IgG-horseradish peroxidase from Kirkegaard & Perry Laboratories (Gaithersburg, MD).

Protein A-gold made according to the method of Frens [2] was provided by Dr. Tulio Farragiana.

HEPES-saline consisted of 110 mM NaCl, 10 mM HEPES, 3 mM $KHCO_3$, 1 mM Na_2HPO_4, 5 mM dextrose, 1 mM $CaCl_2$, 1 mM $MgCl_2$ (pH 7.6), 230 mosmol/kg. Phosphate buffered saline (PBS) was 70 mM NaCl, 5 mM Na_2HPO_3, pH 7.2.

Antibody Production

Toad bladder epithelial cells were homogenized and their granules isolated using Percoll gradients [7]. Approximately 1 mg of granule protein was extracted from the bladders of 40 toads. Polyclonal antibodies to the granules were produced in rabbits. The first injection consisted of 1 ml of granule fraction (0.5 mg/ml protein), mixed with 1 ml of pertussis vaccine in complete Freund's adjuvant. The second injection, was made 10 days later with 0.5 mg granule protein in Freund's adjuvant. Ten days after the second injection, the production of specific antiserum was assayed on immunoblots of SDS polyacrylamide gels of granules and mitochondria. If the test was negative, a third injection was administered. When the antibodies specifically reacted to immunoblots of granules, 20 ml of serum was collected. Control,

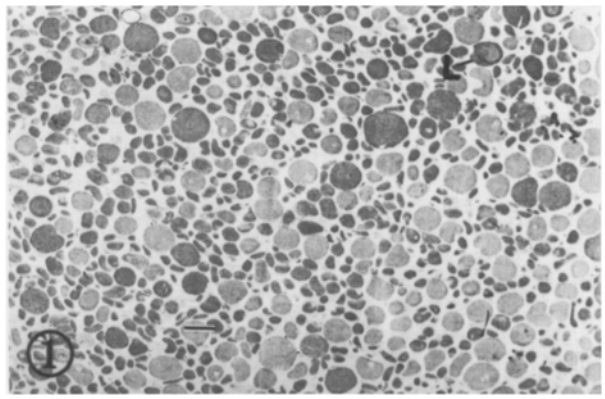

Fig. 1. Low magnification electron micrograph of the immunogen, the isolated granules of the toad urinary bladder, isolated as described in [7]. All the organelles in the field are granules except for a few secondary lysosomes (*L*, residual bodies) identified cytochemically in other sections. Although the granule's boundary membrane is not distinguishable because of the electron-dense contents, one can often discern vesicular or tubular membranes associated with the granules. The thin section was stained with uranyl acetate and lead citrate. Bar = 1 μm. ×4,000

preimmune serum was collected prior to the first injection of immunogen.

To remove high molecular weight IgG complexes, the serum was centrifuged at 100,000 × *g* for 30 min with the brake off and the supernatant was collected and stored in small aliquots at −70°C until use.

Immunoblot

Polyacrilamide gel electrophoresis (PAGE) of SDS-solubilized organelle fractions was performed [6] on 7.5% acrylamide slabs (mini-gels, 8 × 10 cm) with 3% sieving gels using 1 μg of protein per lane (Mighty Small Slab Gel Electrophesis Unit, Hoefer Scientific Instruments, San Francisco, CA). Gels (1.5 ml thick) were done in pairs and one gel was stained with silver [14] and the contents of the second gel were electrophoretically transferred to nitrocellulose paper, 0.45 μm pore size (200 mA for 1.5 hr) [19]. The nitrocellulose was cut into strips, and nonspecific binding was blocked in Tris-buffered saline with 3% BSA for 1 hr at 37°C. The strips were placed in sealed plastic bags with polyclonal antigranule rabbit antisera diluted 1 : 2000 with Tris-buffered saline with 1% BSA (TBS-BSA) on a shaker for 1 hr at room temperature. A TBS rinse was followed by two rinses of TBS with 0.05% NP-40 (detergent) and one TBS rinse. The strips were then exposed to affinity purified goat anti-rabbit IgG-HRP diluted 1 : 2000 with TBS-BSA for 1½-2 hr at room temperature with shaking. The strips were washed as above in TBS and in TBS-NP-40 and incubated in 1 mg/ml imidazole, 1 μl/ml 30% H_2O_2 and 1 mg/ml DAB or 0.6 mg/ml 4-chloro-1-naphthol, pH 7.4, until color developed, usually less than 1 min for DAB and 5 min for 4-chloro-1-naphthol. After air drying, the strips were photographed using ASA 50 Polaroid type 55 film.

Toad Bladder Preparation

Each bladder of three well-hydrated toads was subdivided into four pieces. Each piece was stretched [1], mucosal side out, on a 1-cm diameter Lucite ring and held in place by slipping an O-ring over the bladder and Lucite ring. The bladder rings were suspended in aerated HEPES-buffered saline for 30–60 min. For incubation, the bladder-ring was placed on a drop of "mucosal" solution and the ring concavity contained the serosal solution. Each of the bladder rings from a single bladder was incubated for 10 min either with (i) 50 mU pitressin (ADH) in the serosal solution or (ii) 10^{-6} M PMA in the mucosal solution or (iii) and (iv) HEPES-saline on both mucosal and serosal sides [unstimulated bladder (iii) and immunocytochemical control (iv)] [8].

Fixation

After the 10-min incubation, each ring was immersed in 1% *p*-formaldehyde, in 0.1 M cacodylate buffer, pH 7.2, for 15 min followed by several rinses in PBS (phosphate buffered saline, 5 mM phosphate, 110 mM NaCl, pH 7.2) for a total of 20 min at room temperature followed by immunocytochemical incubation as described below.

After immunocytochemical incubations, the bladder pieces were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, rinsed briefly in 0.1 M cacodylate and post-fixed in reduced osmium tetroxide [5] for 1 hr and then dehydrated and embedded in Epon. Thin sections, whose identity was coded, were examined in a JEOL 100B electron microscope with and without prior uranyl acetate and lead citrate staining [17, 21].

Immunocytochemistry

An initial exposure of the bladders to 0.5% BSA in PBS (PBS-BSA) blocks sites which might bind antibodies nonspecifically. After 10 min at 37°C in PBS-BSA, bladder rings i, ii, and iii were floated on a drop of antigranule antiserum, diluted 1 : 100 with PBS-BSA while bladder ring iv, which was the immunocytochemical control, was floated on a drop of preimmune serum, diluted 1 : 100 with PBS-BSA. After 1 hr at room temperature, the tissues were rinsed with agitation in three changes in PBS-BSA. All of the bladder rings were exposed for 1 hr at room temperature to protein A-gold (IgG detector molecule) diluted 1 : 10 with PBS-BSA. This is equivalent to indirect cytochemistry in that the site of the antigranule antibody binding is inferred from the position of the protein A-gold bound to it. The gold is electron dense and we used 15 nm diameter colloidal gold. The bladder rings were rinsed three times in PBS-BSA and then fixed for electron microscopy as described above.

Morphometry

To quantitate changes in the apical surface area and the expression of granule epitopes on the apical surface we used standard morphometric techniques relating linear dimensions to surface areas [4, 9]. Briefly, a ratio of the mean apical plasma membrane length/mean lateral length (tight junction-to-tight junction distance) was determined for each experimental condition. The plasma membrane length *including microvilli* of the apical surface membrane of at least ten cells per bladder was measured by planimetry. The lateral length was similarly measured by following the cells' surface but *excluding the microvilli*. We have previously determined that the lateral length is not altered by ADH [4].

The number of gold particles identifying binding sites of antigranule antibody were counted and expressed per plasma membrane length as a measure of the proportion of apical mem-

brane derived from granule exocytosis. The mean and standard error for the number of binding sites per plasma membrane length was determined for each condition in a minimum of 17 micrographs containing the apical regions of at least 30 cells.

Results

Assay of Antibodies to Isolated Granules

The isolated granule fraction was used as an immunogen in rabbits. The granules constitute one of the three organelle fractions separated by intrinsic density on Percoll gradients [7]; the other fractions were surface membranes and mitochondria. Lysosomes were not separated as a unique fraction but were present in small numbers in both the granule and the mitochondria fractions. The purity of the granule fraction as determined by electron microscopy is seen in Fig. 1, and its typical silver-staining pattern in SDS-PAGE is seen in Fig. 2, lane *2*. SDS-polyacrylamide gels of the granules and mitochondria have typical silver-staining patterns seen in Fig. 2 (lanes *1* and *2*), and we used immuno(Western)blots (lanes *3* and *4*) of these fractions to assay the specificity of the antisera. On the granule gel (lane *2*), the prominent bands are at 14, 33, 43, 52–56, 62, 67–72, 72–77, 104, 106, 116, 138 and 151 kD. Granule components are probably high in glycoproteins as indicated by their diffuse bands and requirement for silver stain rather than Coomassie blue. (Our current procedure, in which we isolate the fractions in the presence of EGTA and a single proteolysis inhibitor, phenylmethylsulfonyl fluoride, results in slightly different molecular weights than in our previous report [7] where we used several proteolysis inhibitors. The use of multiple inhibitors was discontinued because this combination was found to inhibit several transport functions; this combination of inhibitors was not found to be essential to maintain the larger molecular weight components (*manuscript in preparation*).)

The granule fraction proved to be highly immunogenic in rabbits. Several polyclonal rabbit antisera were raised to isolated granules. In this paper we present data on one antiserum, A-19, which had the ability to stain several components of the granule fraction on nitrocellulose immunoblots from SDS-PAGE (Fig. 2): 14, 50, 55, 68–74, 100, 120 and 150 kD (lane *4*); immunoblots of comparable fractions of mitochondria do not stain (lane *3*). The granule fractions used for electrophoresis and immunochemistry were from different preparations than those used as immunogens. A-19's staining pattern was consistent for five preparations of isolated granules whose immunoblots were evaluated with A-19. With 1 : 2000 dilutions of A-19 we always get staining of 68–74, 100 and 150 kD bands at minimum.

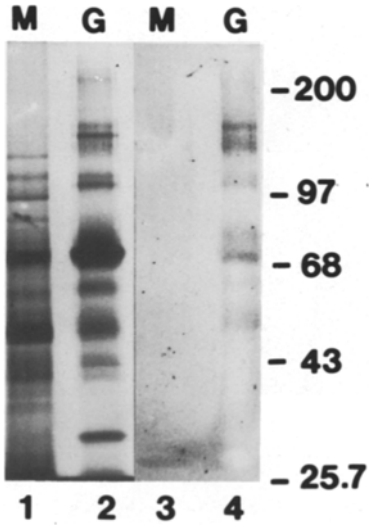

Fig. 2. SDS-PAGE (*1* and *2*) and immunoblot (*3* and *4*) of two fractions: mitochondria (*M*) and granules (*G*), isolated from the epithelium of the toad urinary bladder using Percoll gradients [7]. PAGE was silver stained to visualize protein bands. Each lane had 1 μg of protein. The immunoblot was stained with a 1 : 2000 dilution of A-19 (antigranule serum)/PBS followed by visualization of goat antirabbit IgG-HRP by 4-chloro-1-naphthol. In the granule fraction, which has 12 bands on this silver-stained gel (*2*), approximately half of its immunoblot bands react with A-19 antibody (*4*) while the mitochondrial fraction (PAGE, silver-stained, *1*) has little if any staining with the antigranule antibody (*3*). Migration positions of molecular weight standards are marked on the right, and are myosin, phosphorylase B, bovine serum albumin, ovalbumin, and α-chymotrypsinogen (200, 97, 68, 43 and 25.7 kD)

Immunostaining of the Apical Surface

Antibodies raised to isolated granules label the apical surface of the granule-rich cells (Fig. 3). The epitopes identified by the presence of protein A-gold remain with the bladder's surface after extensive washing both prior to the application of antibody and prior to the addition of protein A-gold. The gold particles are found associated with the membrane as well as on the closely associated glycocalyx. On occasion one detects an apparent clustering of gold on microvilli tips in ADH- and PMA-treated bladders which could result from antigen clustering or from the antibody being excluded by charge or size from the membrane regions at the base of the microvilli. Substituting preimmune rabbit serum for antigranule serum followed by protein A-gold does not result in the localization of gold at the apical surface (Fig. 4); thus the localization with A-19 is not a general, nonspecific binding of rabbit serum to the toad bladder surface.

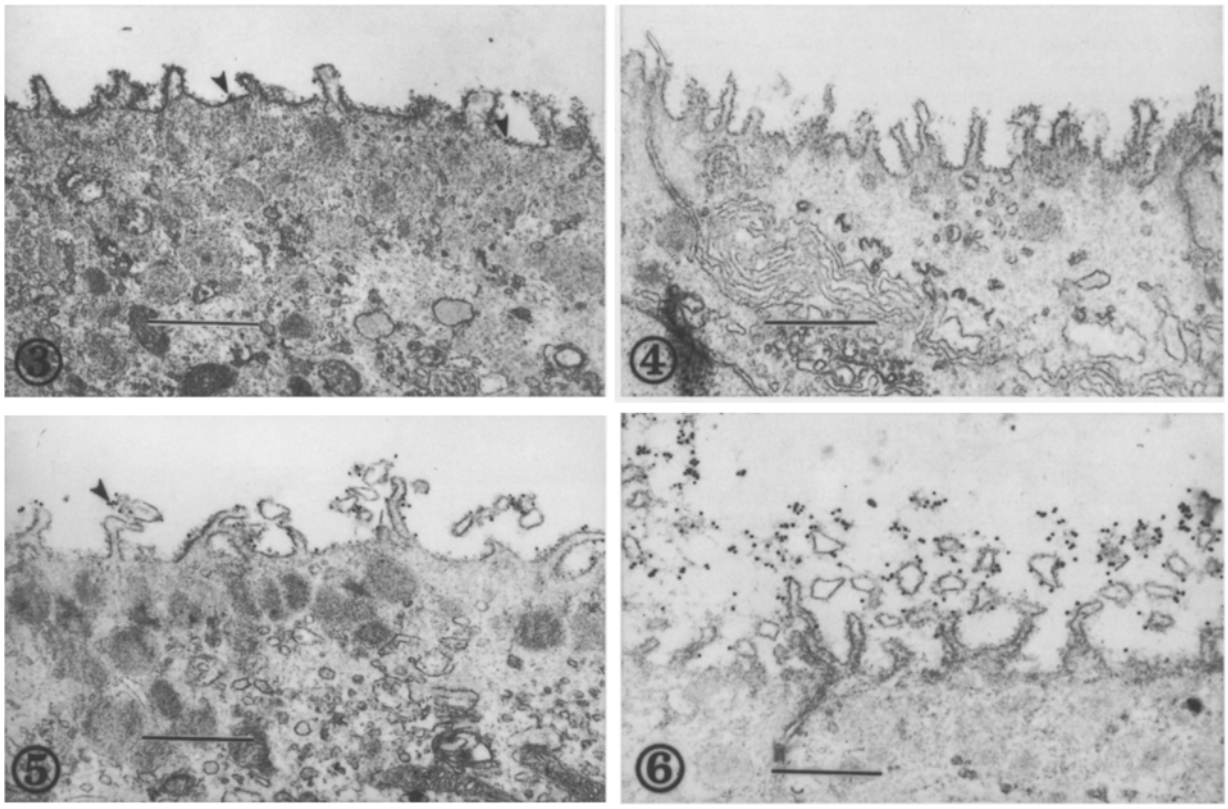

Fig. 3. Electron micrograph of the apical region of a toad bladder granule-rich cell showing pre-embedding immunocytochemical localization of granule-derived antigen on the apical surface. Colloidal gold particles are in the region immediately adjacent to the membrane (arrows) in this bladder which was a physiological control, i.e., not challenged with either ADH or PMA in vitro. Bar = 1 μm. ×15,000

Fig. 4. Electron micrograph of the apical region of granule-rich cells of a nonstimulated bladder to which has been applied "preimmune" rabbit antiserum and protein A-gold. No gold particles were associated with the apical surface as compared to the bladders which have had the antigranule antiserum and protein A-gold (Figs. 3, 5, and 6). Bar = 1 μm. ×15,000

Fig. 5. Increased immunolabeling by antigranule antibody on the apical surface after ADH stimulation of the bladder was detected by protein A-gold. The microvilli tips are seen in longitudinal and cross-section and often have gold particles in clusters at the microvillus tips (arrow). The gold particles are more numerous than in the unstimulated bladder (*cf.* Fig. 3). Bar = 1 μm. ×15,000

Fig. 6. Electron micrograph of the apical region in which mucosal PMA-induced granule exocytosis is accompanied by a great increase in surface area and in surface-associated granule epitopes on the reticulated apical borders of two adjacent granule-rich type cells. The antigranule antiserum followed by protein A-gold were applied prior to embedding as above; the gold particles were seen often in groups in PMA-treated bladders. Bar = 1 μm. ×15,000

When the bladder had been stimulated with ADH prior to the application of antigranule antibody there was an increase in the number of gold particles/μm of apical plasma membrane length (Fig. 5). In ADH-treated bladders the number of gold particles on the apical surface/μm apical plasma membrane length is more than double that seen on unstimulated bladder (Table). The increased number of gold particles are found as individual particles or in clusters (Fig. 5, arrow). Similarly, bladders treated with mucosal PMA, which induces a massive exocytosis [13], have more than fourfold the number of gold particles/μm apical plasma membrane length associated with their greatly elongated microvilli (Fig. 6, Table) and these are often in groupings of five to seven particles. When we calculated the ratio of the measured apical plasma membrane length/lateral length, we confirmed that both ADH and PMA increase the apical surface area (Table, lines 1 and 2). To evaluate whether the increase in gold labeling represented a specific increase in granule-derived material, we divided the *increase* in antigranule antibody surface label by the *increase* in surface area: gran-

Table. Specific increase in antigranule antibody labeling of the apical surface induced by ADH and PMA

	Unstimulated (control)	ADH	PMA
Apical surface area plasma membrane length lateral length	2.52 ± 0.18	2.98 ± 0.14	4.03 ± 0.26
Percent of control	100%	118%	160%
Granule-derived epitopes (number of colloidal gold particles per μm plasma membrane length)	0.77 ± 0.16	1.67 ± 0.41	3.51 ± 0.26
Percent of control	100%	216%	455%
Enrichment of granule-derived membrane-associated material	—	6.4-fold	5.9-fold

ule-derived epitopes were found to be enriched sixfold on the apical surface of the ADH- and PMA-treated bladders (Table, line 5).

Discussion

Antidiuretic hormone increases the antigranule immunolabeling of the bladder's apical surface. Since this antibody is made to isolated granules, this finding provides immunocytochemical support for previous evidence that ADH induces exocytosis of the granules concomittant with hydroosmotic permeability at the apical surface. The previous studies were based on exocytic images, linear measurement and histochemistry of the apical surface in ADH-treated and control hemibladders [4, 9, 12]. In those studies, 30 min ADH treatment resulted in a 25% increase in surface area comparable to the present finding (after 10 min ADH) of 18% increase in surface area. This increase is accompanied by a 116% increase in granule epitopes on the apical surface.

Similarly, PMA-initiated massive granule exocytosis [13] is corroborated in these studies: the 60% increase in surface area represented in elongated microvilli in the PMA-treated bladders is accompanied by a 355% increase in immunolabeling by the antigranule antibody. It is of interest, however, that the proportional increase in granule epitopes over the control hemibladders is comparable, sixfold, for both ADH and PMA.

At this time, we cannot associate the surface staining with an individual peptide or component nor discriminate between granule membrane and contents; the polyclonal nature of the antigranule antibodies is seen in the staining of immunoblots. We can say, however, that the epitopes identified by the immunostaining are likely to originate in the interior of the granules and the membrane facing it, which, as a result of exocytosis, become continuous with the exterior of the apical surface. Less likely, but not excluded, is the possibility that these epitopes pre-exist in the apical membrane and are unmasked as the result of ADH.

These immunocytochemically localized epitopes apparently stay on the surface even during the extensive washing prior to immunocytochemical labeling and could be useful as an apical label. In contrast, we found that, although enzymatically labeled plasma membrane components (e.g. borohydride-labeled glycoprotein) provide a consistent plasma membrane marker in other tissues [3], labeled plasma membrane glycoproteins were shed by toad bladder apical surface during preparative washes [10]. Similarly, recent studies using antibody made to a denatured 70-kD toad bladder peripheral membrane peptide report that its release into the mucosal medium was initiated by mucosal PMA (but not serosal ADH) [20]. It is of interest that a 70-kD peptide is seen in gels of granule *content* but absent from gels of granule membrane (*manuscript in preparation*).

The subcellular localization of the epitopes which bind antigranule antibody in permeabilized and frozen sections will provide information of the relatedness of the membranes known to cycle through the apical surface coincident with the ADH-induced permeability increase and decrease. Among other uses, the antibodies have potential for immunoisolation of apical membrane vesicles from an homogenate of toad bladder epithelial cells [10].

We appreciate the generous advice and help of Drs. V. Sapirstein, R. Kulikowski, T. Farragiana and T.A. Krulwich. This work was supported by NIH grant AM25110 to S.K.M.

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Received 19 June 1986; revised 5 January 1987