

# Role of Osmotic Forces in Exocytosis: Studies of ADH-induced Fusion in Toad Urinary Bladder

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**ABSTRACT** Antidiuretic hormone (ADH) treatment of toad urinary bladder activates an exocytotic-like process by which intramembrane particle aggregates are transferred from membranes of elongated cytoplasmic tubules to the luminal-facing plasma membrane. We find that the number of these ADH-induced fusion events, and the number of aggregates appearing in the luminal membrane, are reduced when the luminal bathing medium is made hyperosmotic. As an apparent consequence of the inhibition of their fusion with the luminal membrane, the elongated cytoplasmic tubules become enormously swollen into large, rounded vesicles. These results are consistent with the view that osmotic forces are essential to the basic mechanism of exocytosis.

Exocytosis is a fundamental cellular process that involves the establishment of continuity between the plasma membrane and the membranes of cytoplasmic structures (membrane fusion). Extrapolating from their results on fusion of phospholipid vesicles with planar phospholipid bilayer membranes, Cohen et al. (5, 23) have suggested that, in biological systems, exocytosis depends critically upon osmotic water entry into cytoplasmic vesicles which are in contact with the plasma membrane. This results in vesicle swelling; the consequent rupture of vesicular and plasma membranes in the region of contact leads to fusion. Pollard and co-workers (1, 14, 16) have also proposed that osmotic swelling of cytoplasmic secretory granules is crucial to exocytotic release of their contents. Observations of vesicular swelling concomitant with exocytosis (e.g., reference 17; and W. W. Douglas, unpublished lecture quoted in reference 16) further support these ideas, but there has been no direct demonstration of the effect of transmembrane osmotic gradients on fusion until now.

Such a demonstration is difficult to make because alteration in the osmolality of the medium surrounding most cells causes them to shrink or swell rapidly so as to maintain their isosmotic status. Changes in fusion rates subsequent to modification of cell volume cannot be attributed to osmotic pressure differences across the plasma membrane, nor even simply to the resulting shift in cytosol osmolality, because this in turn could initiate a cascade of other events affecting fusion rates (e.g., shift in free  $\text{Ca}^{++}$  level, change in cyclic adenosine 5'-monophosphate (cAMP) concentration, or modification of metabolic rate). In some epithelia, however, it is possible to alter extracellular osmolality without changing cell volume. Specifically, alterations in the osmolality of the medium bathing the less permeable of the two faces of a "tight" epithelium will cause only

minimal shrinkage or swelling of cells. Thus, it is possible to change the osmolality of the mucosal (luminal) bathing solution of toad urinary bladder without significantly changing cell volume (2, 15) and thereby establish stable differences in osmolality across the luminal plasma membrane.

Addition of antidiuretic hormone (ADH) to the serosal medium of toad urinary bladder causes cAMP-mediated permeability changes in the luminal membrane of the predominant, granular-type epithelial cells (4, 7, 18). Specifically associated with the water permeability increase is the appearance in the luminal membrane of organized structures, each consisting of aggregated intramembrane particles in linear arrays (3, 11, 12). These structures have been termed "aggregates." In unstimulated bladders, aggregates are found in the membranes of elongated, tubular cytoplasmic structures (10, 19). In response to ADH treatment, these aggregate-carrying membrane structures fuse with the luminal membrane, and aggregates are translocated within the membrane matrix to areas that are away from fusion sites (13, 20). Fusion events appear in freeze-fracture replicas as round-to-oval, ice-filled invaginations, with a diameter of  $\sim 0.13 \mu\text{m}$  (13). The process by which the elongated structures bearing aggregates fuse with the luminal membrane exemplifies exocytosis, although it is not secretion in the usual sense. Our experiments were designed to investigate the role of osmotic forces in exocytosis. They demonstrate that the number of fusion events associated with ADH treatment of toad bladder diminishes when the mucosal bathing medium of the bladder is made hyperosmotic.

## MATERIALS AND METHODS

We mounted paired urinary hemibladders from eight Dominican toads (*Bufo marinus*) as sacs on the ends of glass cannulae. One bladder of each pair served

as "control." Both were filled to capacity with Ringer solution (111 mM NaCl, 3.5 mM KCl, 2.5 mM NaHCO<sub>3</sub>, 1.0 mM CaCl<sub>2</sub>, pH 7.6–8.2; 220 mosmol) and suspended for 30 min in an aerated Ringer bath at room temperature. At the end of this period, transbladder electrical potential was measured, and if a value <20 mV was obtained for either bladder of a pair, the experiment was terminated. Otherwise, the mucosal bathing medium of both experimental and control bladders was replaced with Ringer solution diluted 1:5 with distilled water; in addition, that of the experimental bladders was adjusted with raffinose<sup>1</sup> to a final osmolality between 397 and 488 mosmol ( $453 \pm 27$  [SD]<sup>2</sup>). All bladders were then resuspended in fresh serosal Ringer baths. After a 30-min equilibration period, they were treated for 5 min with a maximally stimulating dose (final serosal concentration = 20 mU/ml) of ADH (Pitressin, Parke-Davis, Detroit, Mich.), and then quickly fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Thereafter, tissues were processed for freeze-fracture or thin-section electron microscopy by routine procedures (12, 13). Random micrographs of granular cell luminal membrane (protoplasmic fracture face) were evaluated for fusion events and for aggregates as described elsewhere (12, 13).

5 min was selected as the time of exposure to ADH for two reasons: first, under the conditions existing for control bladders, this time suffices for induction of a maximal number of fusion events (unpublished observations of bladders reported in reference 9) and luminal membrane aggregates (8, 9). Second, it was critical for our purpose to assess the early effects of hyperosmolality on the experimental bladders, before the water permeability of the luminal membrane could increase to a level associated with possible cell shrinkage (and its confounding complications). In parallel experiments (see Discussion) additional bladders ( $n = 6$  pairs) were exposed to ADH for the longer interval of 20 min.

## RESULTS

The number of ADH-induced fusion events between luminal membrane and aggregate-containing membrane structures was approximately two-fold less when the mucosal bathing medium was hyperosmotic than when it was hypo-osmotic (Fig. 1A). (Some of our earlier observations, included as the isosmotic group in Fig. 1, show that the number of aggregates and the number of fusion events resulting from 5 min of ADH treatment do not depend on mucosal osmolality in the range of 42 [control condition] to 220 [isosmotic condition] mosmol [9, and unpublished observations of bladders in reference 9].) The decreased number of fusion events was accompanied by a reduction in the number of aggregates appearing in the luminal membrane (Fig. 1B).

Conceivably, the 30-min exposure of experimental bladders to hyperosmotic raffinose solutions, before the 5-min ADH treatment, could have made the luminal membrane refractory to ADH-induced fusion. To assess this possibility, we performed three experiments in which both control and experimental bladders were exposed for 30 min on their mucosal sides to  $476 \pm 3$  (SD) mosmol hyperosmotic raffinose solution before ADH treatment. During the subsequent 5-min ADH treatment, the mucosal medium of the control bladders contained one-fifth strength Ringer solution without raffinose. Thus, control bladders were exposed to raffinose for 30 min, and their paired experimental bladders were exposed to raffinose for 35 min. In these ancillary experiments, the response of control bladders was the same as that of the previous controls (that lacked 30-min pre-ADH exposure to raffinose) both in the number of fusion events ( $28 \pm 4$  vs.  $34 \pm 4$ ) and in the number of aggregates ( $318 \pm 69$  vs.  $285 \pm 33$ ) per reference area of luminal membrane ( $235 \mu\text{m}^2$ ). Therefore, 30-min exposure to raffinose before the 5-min ADH treatment does not

<sup>1</sup> We chose raffinose as the solute to make the mucosal bathing medium hyperosmotic because it does not produce structural alterations in the junctional region under our experimental conditions (6, 21, and confirmed here); such alterations do occur with smaller solutes such as urea and sucrose (6, 21).

<sup>2</sup> Mean  $\pm$  standard deviation (SD) are given for descriptive purposes. Mean  $\pm$  standard error of the mean (the latter undesignated in the text) are given for the purpose of fiducial inference.

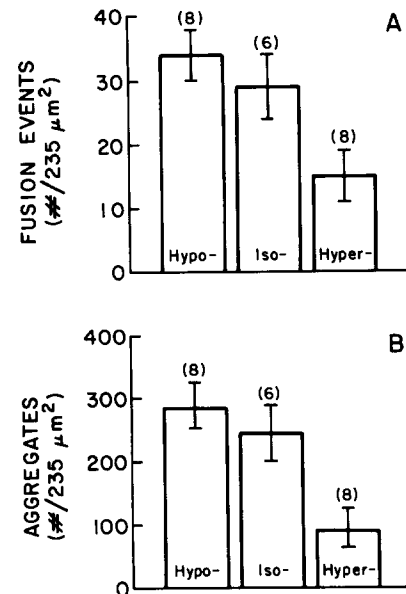


FIGURE 1 Effect of mucosal bathing medium osmolality on ADH-induced morphologic changes in the granular cell luminal membrane of toad bladder. All bladders were bathed on their serosal surfaces with isosmotic (220 mosmol) Ringer solution and were stimulated for 5 min (see text) with ADH. Analysis of variance (24) reveals that neither the number of fusion events (A) nor the number of aggregates (B) is statistically different in bladders whose mucosal bathing media are hypo-osmotic (one-fifth strength Ringer) and isosmotic (full strength Ringer). (Data for the isosmotic case come from [9].) In bladders whose mucosal bathing media are hyperosmotic (397–488 mosmol), both the number of fusion events and the number of aggregates are appreciably reduced compared with either of the other bladder groups ( $P < 0.05$ ).

make the luminal membrane refractory to ADH-induced fusion. In confirmation of the data illustrated in Fig 1A and B, the experimental bladders in this series had fewer fusion events ( $8 \pm 1/235 \mu\text{m}^2$ ) and fewer luminal membrane aggregates ( $40 \pm 17/235 \mu\text{m}^2$ ) than their paired controls.

In additional experiments ( $n = 3$  pairs of bladders), we investigated whether the inhibitory effects of mucosal hyperosmolality could possibly represent a pharmacologic action of raffinose. The same procedure as in the original experiments was used, except that the mucosal bathing media of the control and experimental bladders were adjusted to  $251 \pm 1$  (SD) and  $429 \pm 1$  (SD) mosmol, respectively, by adding an identical amount of raffinose to either one-fifth strength or to full strength Ringer solution. In response to ADH stimulation for 5 min, control bladders, whose mucosal bathing medium was approximately isosmotic to cytoplasm, demonstrated the same number of fusion events as our original control samples ( $30 \pm 9$  vs.  $34 \pm 4/235 \mu\text{m}^2$ ). In the experimental bladders, the frequency of ADH-induced fusion events was reduced to a level comparable to our original experiments ( $9 \pm 3/235 \mu\text{m}^2$ ). This series of experiments indicates that the inhibitory effect of hyperosmotic mucosal bathing media on the frequency of ADH-induced fusion events is an effect of osmolality rather than of raffinose itself.

## DISCUSSION

### *Vesicle-membrane Fusion Is Affected by Osmotic Gradients across the Plasma Membrane*

The model of vesicle-membrane fusion and exocytosis proposed by Pollard and co-workers (1, 14, 16) and by Cohen et

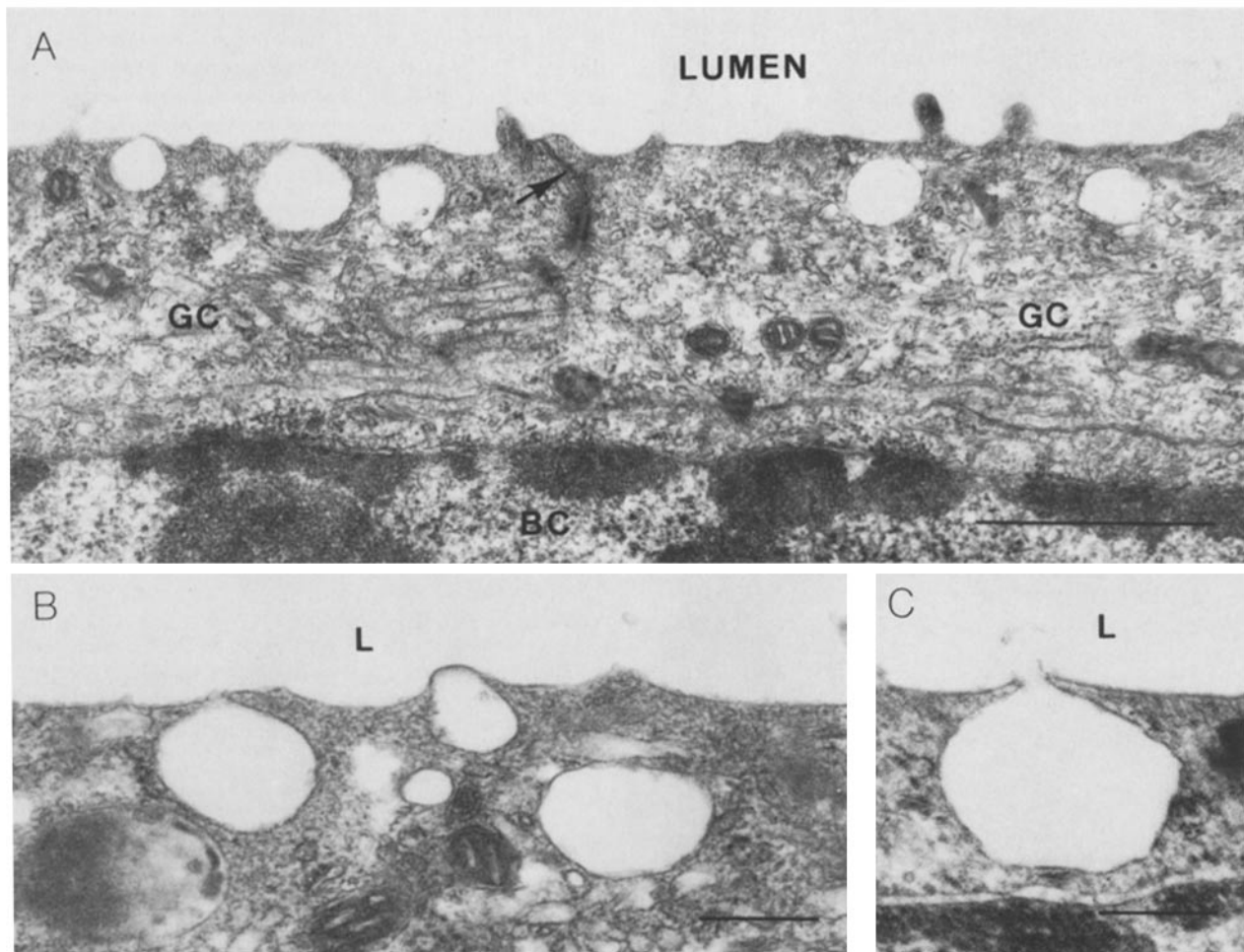


FIGURE 2 Bladder with hyperosmotic mucosal solution and treated with ADH for 5 min. (A) Large, clear vesicles are found in the vicinity of the luminal plasma membrane in two granular epithelial cells (GC). Tight junction morphology is not altered (arrow). BC, basal cell. Bar, 1  $\mu\text{m}$ .  $\times 32,000$ . (B and C) Swollen vesicles fused with the luminal membrane. From freeze-fracture observations (see Fig. 3) they are presumed to be the aggregate-carrying structures whose shape has been altered by the experimental procedures. L, lumen. Bar, 0.25  $\mu\text{m}$ .  $\times 63,000$ .

al. (5, 23) includes a crucial role for osmotic bursting of vesicles in the region of contact with the plasma membrane. One prediction of this model is the inhibition of fusion when the extracellular medium is made sufficiently hyperosmotic to the cytoplasm. This condition is generally not easy to establish in biological systems (see Introduction), but it is attainable across the luminal membrane of toad bladder because of this membrane's low water permeability in comparison with that of other series barriers in the tissue. Our results demonstrate that during a 5-min exposure to a maximally stimulating dose of ADH, bladders whose mucosal medium is hyperosmotic show fewer fusion events than do controls whose mucosal medium is either hypo-osmotic or isosmotic. These experiments provide a direct demonstration of the predicted effect of osmotic gradients across the plasma membrane on fusion—uncomplicated by osmotically induced changes in cell volume.

In the toad bladder, mucosal hyperosmolality ( $\sim 450$  mosmol) apparently delays fusion without reducing the number of fusion events that can ultimately occur. Thus, after 20-min (instead of 5-min) exposure to ADH in the presence of mucosal hyperosmolar bathing solutions ( $446 \pm 38$  (SD) mosmol), we observed no decrease in the number of fusion events. To the contrary, there were more fusion events ( $48 \pm 5$  vs.  $37 \pm 3/235 \mu\text{m}^2$ ;  $P < 0.05$ ) and proportionally even more aggregates ( $400$

$\pm 83$  vs.  $177 \pm 36/235 \mu\text{m}^2$ ;  $P < 0.05$ ) than in controls. We believe these data are explained either by sequelae to decreased cell volume (caused by prolonged exposure to hyperosmotic mucosal media) or by changes in the elongated vesicles, which become evident only when their fusion is suppressed by mucosal hyperosmolality (see below).

#### Swelling of Elongated Vesicles upon Suppression of Fusion

Fusion of phospholipid vesicles with planar phospholipid bilayer membranes requires an osmotic gradient across the planar membrane, with the side containing the vesicles (the *cis* side) hyperosmotic to the opposite (*trans*) side (5, 22, 23). Swelling is then presumed to occur, in vesicles contacting the planar membrane, by osmotic water entry from the hypo-osmotic *trans* solution (23). In biological fusion and exocytosis, however, the cytoplasm is generally isosmotic to the surrounding medium. If osmotic swelling of vesicles is also crucial to biological fusion, alternative mechanisms must exist for generating osmotic gradients across cytoplasmic vesicular membranes. For example, the fusion stimulus might mobilize osmotically inactive particles within the vesicle, alter vesicle membrane solute permeability, thus leading to colloid osmotic swelling, or induce active pumping of ions into the vesicle.

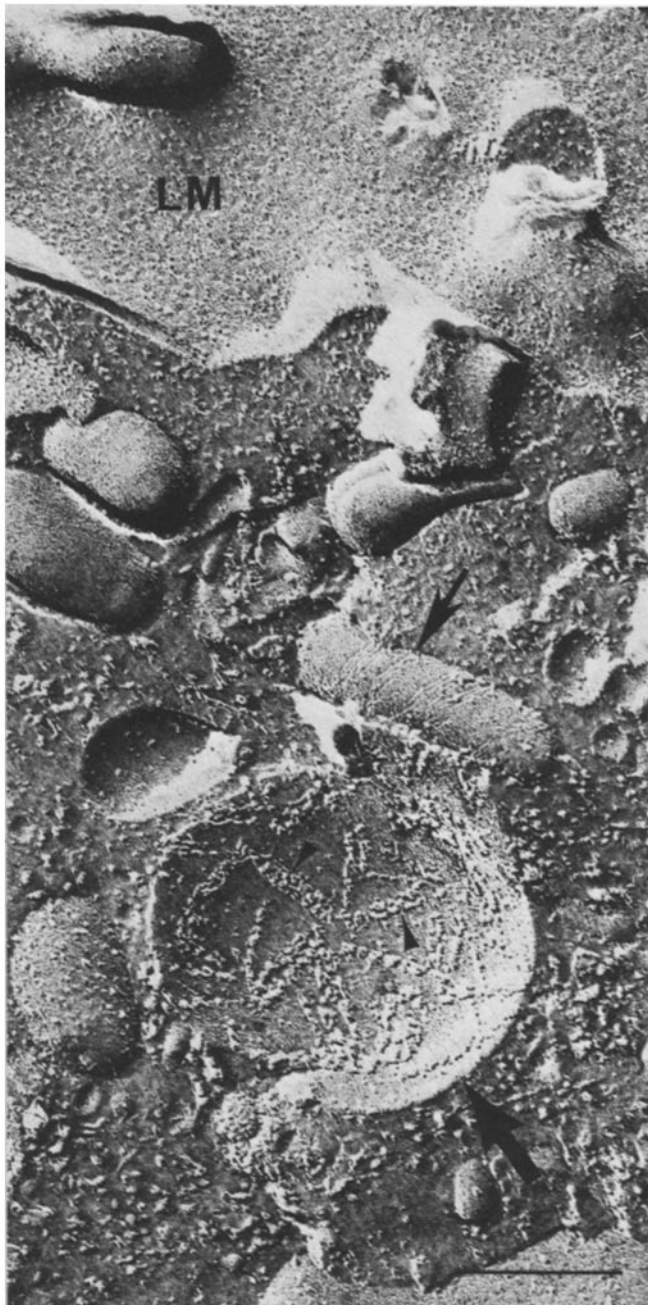


FIGURE 3 Bladder treatment, same as in Fig. 2. Protoplasmic fracture face of a spherical vesicle (thick arrow) with intramembrane particle aggregates (arrowheads). Adjacent to it (thin arrow) is a normal-appearing tubular structure whose exoplasmic fracture face has the parallel impressions of the intramembrane particle aggregates. From this cross-fracture it is not possible to ascertain the distance of either structure from the luminal membrane (LM). Bar,  $0.25 \mu\text{m}$ .  $\times 87,500$ .

Vesicular swelling has been associated with biological exocytosis (e.g., 17; W. W. Douglas, unpublished lecture quoted in reference 16), which supports the osmotic theory of fusion. Swelling has not been reported so far for the elongated vesicles which fuse with the luminal membrane of the bladder in response to ADH stimulation under usual experimental conditions. Large, rounded vesicles do appear, however, within the cytoplasm of the granular cells of those bladders in which ADH-induced fusion has been inhibited with hyperosmotic

mucosal bathing media<sup>3</sup> (Fig. 2). This has also been reported by Civan and DiBona (4) for comparable experimental conditions. The occurrence of characteristic aggregates in the membrane of these rounded vesicles, as we now show in Fig. 3, indicates that these vesicles are the elongated, aggregate-carrying structures after becoming enormously swollen.<sup>4</sup> Possibly, this represents an extreme exaggeration of the slight osmotic swelling that might normally accompany fusion. Further studies of this fusion-inhibited system may reveal the cAMP-induced biochemical and biophysical changes in vesicular contents and/or membrane function that induce the osmotic entry of water into the vesicles.

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<sup>3</sup> In separate studies we found no swelling of aggregate-containing membrane structures in unstimulated bladders ( $n = 3$ ) exposed to mucosal hyperosmolality.

<sup>4</sup> Assuming that the change in shape from cylindrical to spherical of the aggregate-carrying structures does not involve a change in membrane surface area, we have estimated the normal length of these structures as follows: from measurements done on thin-section electron micrographs, the diameter of swollen structures in proximity to the luminal membrane (see Fig. 2) was found to be  $0.51 \pm 0.06 \mu\text{m}$  ( $n = 47$ ). Measurements made of swollen structures containing aggregates in freeze-fracture preparations (Fig. 3) gave a diameter of  $0.47 \pm 0.04 \mu\text{m}$  ( $n = 15$ ). Thus, the mean length of an unswollen cylinder with a diameter of  $0.13 \mu\text{m}$  (thin section) or  $0.11 \mu\text{m}$  (freeze fracture) (see reference 13) would be  $\sim 2 \mu\text{m}$ .

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