

Stabilization of Vasopressin-induced Membrane Events by Bifunctional Imidoesters

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ABSTRACT Vasopressin increases the water permeability of the luminal membrane of the toad bladder epithelial cell. This change in permeability correlates with the occurrence in luminal membranes of intramembrane particle aggregates, which may be the sites for transmembrane water flow. Withdrawal of vasopressin is ordinarily associated with a rapid reduction of water flow to baseline values and a simultaneous disappearance of the particle aggregates. The bifunctional imidoesters dithiobispropionimidate (DTBP) and dimethylsuberimidate (DMS), which cross-link amino groups in membrane proteins and lipids, slow the return of water flow to baseline after vasopressin withdrawal. Cross-linking is maximal at pH 10, and is reduced as pH is lowered. Freeze-fracture studies show persistence of luminal membrane particle aggregates in cross-linked bladders and a reduction in their frequency as water flow diminishes. Fusion of aggregate-containing cytoplasmic tubular membrane structures with the luminal membrane is also maintained by the imidoesters. Reductive cleavage of the central S—S bond of DTBP by β -mercaptoethanol reverses cross-linking, permitting resumption of the rapid disappearance of the vasopressin effect. Bladders that have undergone DTBP cross-linking and β -mercaptoethanol reduction respond to a second stimulation by vasopressin. Thus, the imidoesters provide a physiologic and reversible means of stabilizing normally rapid membrane events.

Rapid changes in membrane transport may involve the insertion of new material into the limiting cell membrane. This may be associated with exocytosis (1) or the appearance of specific transport proteins (2). An example of particular interest is the hormonally induced increase in water permeability of vasopressin-sensitive epithelial cells. Here, vasopressin initiates a series of cyclic AMP-mediated reactions ending with the appearance of aggregates of intramembrane particles in the luminal cell membrane (3, 4). The particles, presumably proteins, appear to be the sites of water transport (5–8). They originate from the membranes of cytoplasmic structures, which fuse with the luminal membrane under the stimulus of vasopressin (9–13). Upon withdrawal of vasopressin, aggregates rapidly disappear from the luminal membrane, and water flow falls to baseline values (14). This sequence has been shown in glutaraldehyde-fixed tissues, by both freeze-fracture and thin-section electron microscopy. Vasopressin-treated amphibian bladder cells have also been found to retain their high water permea-

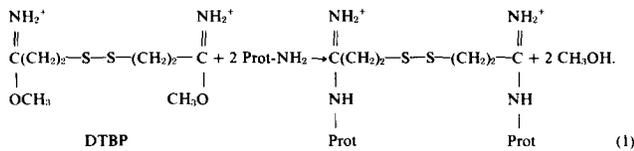
bility in an irreversible fashion after fixation with glutaraldehyde (15, 16). However, not only are there lethal effects of glutaraldehyde on cell function, but membrane proteins irreversibly cross-linked with glutaraldehyde cannot readily be introduced into gel electrophoresis systems for further analysis.

In the following study, we have employed the bifunctional cross-linking agents dithiobispropionimidate (DTBP) and dimethylsuberimidate (DMS) to immobilize aggregates in the luminal membrane of vasopressin-treated toad bladder epithelial cells. Unlike glutaraldehyde, DTBP does not kill the cell. In bladders treated with DTBP and DMS, water flow continued at a high rate even after the withdrawal of vasopressin, with a very slow decline toward baseline values. Intramembrane particle aggregates were retained by the cross-linking process, as shown by freeze-fracture. DTBP possesses a central disulfide bond that can be cleaved by the reducing agent β -mercaptoethanol, effectively reversing the cross-linking. This permitted water flow to return rapidly to baseline. Reversible cross-

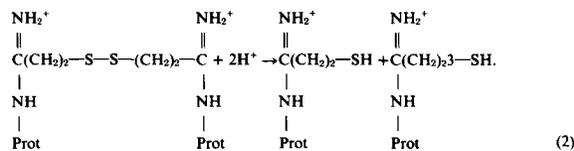
linking agents may provide a useful and physiologic technique for the study and control of rapid alterations in membrane structure and for the isolation of specific membrane proteins.

MATERIALS AND METHODS

Paired hemibladders from double-pithed female Dominican toads (*Bufo marinus*) (National Reagents, Bridgeport, Conn.) were mounted as sacs on the end of glass tubes; one from each pair served as an experimental bladder, the other as a control. The bladders were excised and washed inside and outside three times with amphibian phosphate-buffered Ringer's solution (120 mM Na⁺, 4 mM K⁺, 0.5 mM Ca²⁺, 116 mM Cl⁻, 5 mM phosphate, 220 mosmol/kg, pH 7.4). They were then filled with 5 ml of full-strength Ringer's solution, moved up on the glass tubes, and retied, and the excess tissue was removed, so that in each case 5 ml of solution filled the bladder. Thus, all the bladders were distended to a similar degree. They were then suspended in a bath containing 18 ml of full-strength Ringer's solution. Stirring and aeration of the serosal baths were accomplished by bubbling with room air. Bladders were stimulated by adding arginine vasopressin (Sigma Chemical Co., St. Louis, Mo.) to the serosal baths at a saturating concentration of 86 mU/ml, for a period of 15 min. The bladders were then emptied, and experimental hemibladders were filled with 5 ml of DTBP solution (Pierce Chemical Co., Rockford, Ill.; 2-4 mg/ml of DTBP in Ringer's solution, pH 10) and replaced for 10 min in the original serosal solution containing vasopressin. In a second series of experiments, the nonreversible cross-linking agent DMS (Pierce Chemical Co.) was used, at a concentration of 4 mg/ml, pH 9.0, in the mucosal solution for 30 min in the presence of vasopressin. Paired control hemibladders were filled with 5 ml of plain Ringer's solution at pH 10 (or pH 9, for the DMS experiments) and replaced in the original serosal baths for the same period as the experimental bladders. During this period, amino groups of membrane proteins (and possibly of lipids) were cross-linked by the imidoesters by the following reaction (17):



Reduction with β -mercaptoethanol:



After this period, all bladders were rinsed twice inside and out with fresh Ringer's solution. The inside solution was then replaced with 5 ml of Ringer's solution diluted 1:10, with 10 mM phosphate added (final osmolality, 40 mosmol/kg H₂O), and the bladders were placed in 18 ml of fresh full-strength Ringer's solution. Thus, there was now an osmotic gradient of 180 mosmol/kg H₂O across the bladder wall. Water flows were then determined gravimetrically by the method of Bentley (18) at intervals of 5-15 min for periods up to 90 min. It should be noted that water flows during the earlier periods of vasopressin stimulation and imidoester cross-linking were not measured, because there was no osmotic gradient. This procedure was adopted to simplify the experimental protocol, by avoiding weighing during the changes in solutions and washout steps, and to maximize the number of aggregates, because aggregate frequency has been reported to be increased in the absence of an osmotic gradient (7). Fig. 1 shows a single series of experiments in which an osmotic gradient was present throughout. The actual osmotic flow of water is seen for all phases of the procedure. In Figs. 2-4, where the simpler protocol was followed, no osmotic gradient was present during vasopressin stimulation and cross-linking; therefore, representative, rather than actual, water flows are shown in the initial phase of the experiment.

In another series of experiments, the protocol was similar, except that bladders were initially fixed with DTBP and then exposed to vasopressin, and water flows were determined.

Experiments were then conducted in which the DTBP effect was reversed by β -mercaptoethanol. The protocol was similar to that of the standard experiment using DTBP, except that after the 10-min DTBP treatment the experimental hemibladders were emptied and filled with 5 ml of 5% β -mercaptoethanol in Ringer's solution (pH 7.4) for a period of 15 min. The control hemibladders (which had received only DTBP treatment) were filled with plain Ringer's solution for 15 min. After this period, the bladders were rinsed inside and outside

once. They were filled with 5 ml of 1:10 Ringer's solution and placed in baths of 18 ml of fresh full-strength Ringer's solution, and water flows were measured gravimetrically for 90 min at 15-min intervals. At 60 min, we placed vasopressin (86 mU/ml) in the serosal bath to see whether the bladders were restimulatable. A similar protocol was carried out for the DMS-treated bladders, after 30 min of DMS treatment.

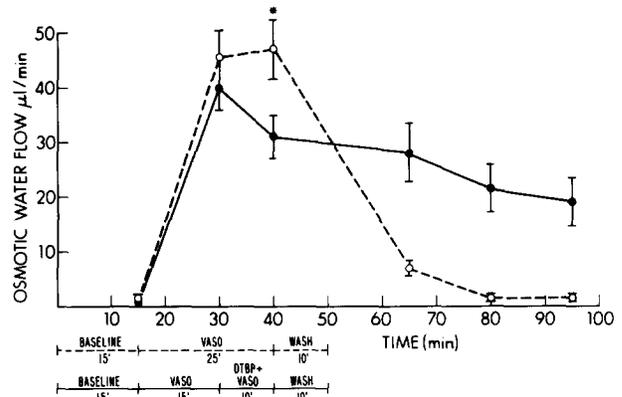


FIGURE 1 Experiment showing normal osmotic flow during vasopressin stimulation and after washout. An osmotic gradient was present throughout. All bladders were stimulated with vasopressin for 25 min. During the last 10 min of vasopressin stimulation, DTBP was added to experimental bladders. All bladders were then rinsed for 10 min, then osmotic water flows were measured during the washout period. Water flow rose sharply during the vasopressin period. After washout, water flow remained at high levels in the DTBP-treated bladders. $n = 8$. Vertical bars represent ± 1 SEM. For Figs. 1-4: (solid circles, solid lines) experimental bladders, (open circles, dotted lines) control bladders. (*) Experimental vs. control, $P < 0.05$.

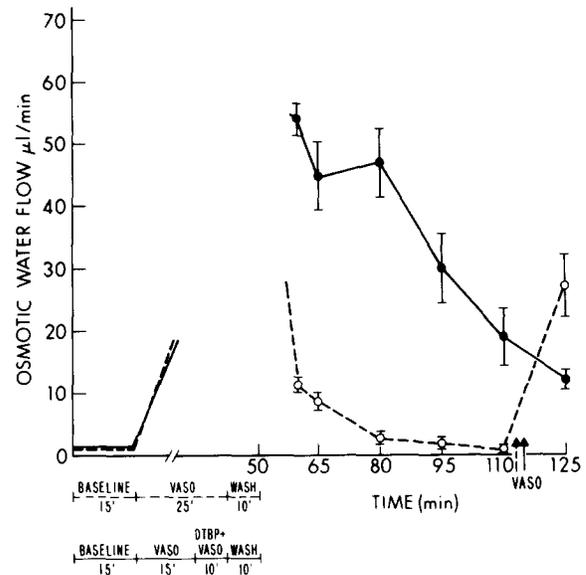


FIGURE 2 Osmotic water flows in paired bladders after stimulation and subsequent withdrawal of vasopressin. Protocol was the same as in Fig. 1, except that an osmotic gradient was present only after washout of vasopressin. Water flow in control bladders rapidly returned to baseline values and promptly increased after a second stimulation with vasopressin. In DTBP-treated bladders, water flow after the wash period remained significantly higher than control at all points beyond 55 min. There was no response to a second stimulation by vasopressin. $n = 6$. Vertical bars ± 1 SEM. (Note that representative baseline and early vasopressin-stimulation water flows are indicated at the beginning of this and the following figures.)

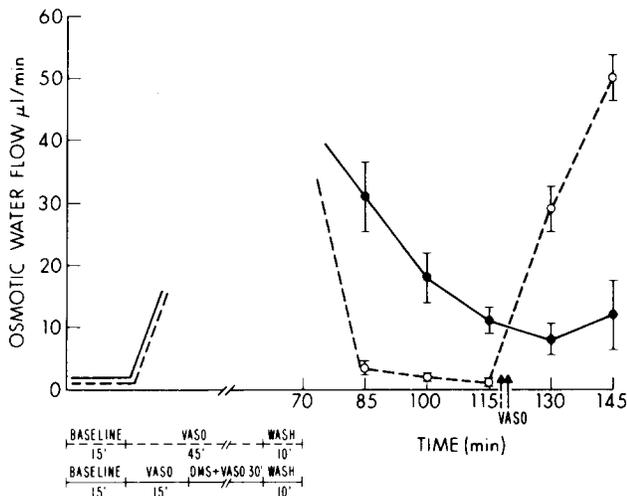


FIGURE 3 Osmotic water flows in paired bladders, in which the experimental bladder (solid circles) received DMS rather than DTBP. The experimental design is the same as Fig. 2. Exposure to DMS, however, was for 30 min. $n = 4$.

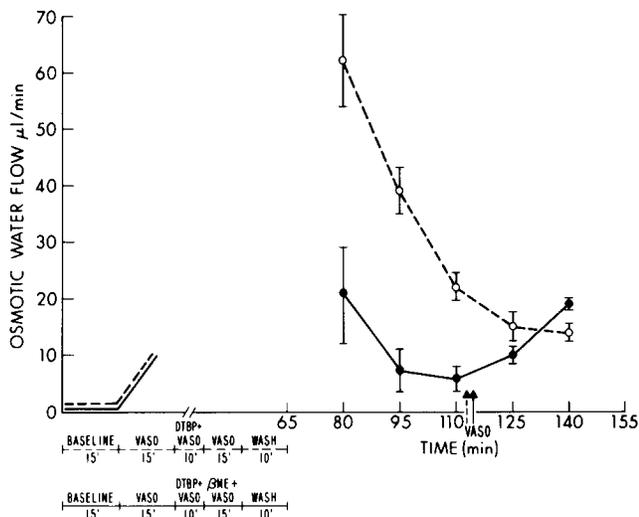


FIGURE 4 Osmotic water flows in paired bladders after stimulation and subsequent withdrawal of vasopressin. The control bladders (open circles) were exposed to DTBP for 10 min after vasopressin stimulation and showed the characteristic persistence of high water flows. The experimental bladders (solid circles) were exposed to β -mercaptoethanol after DTBP cross-linking and showed a significantly faster return toward baseline water flow. $n = 4$.

During some of the above experiments, we determined [^{14}C]sucrose permeability (K_{trans} sucrose) by placing the isotope in the luminal medium and sampling the serosal and luminal solutions at 15-min intervals. Samples were pipetted into Aquasol (New England Nuclear, Boston, Mass.) and counted in a liquid scintillation counter (Tricarb, Packard Instrument Co., La Grange, Ill.).

After the standard cross-linking protocol with DTBP, additional bladder pairs were prepared for freeze-fracture electron microscopy. After removal of vasopressin, washout water flows were measured for 15 and 30 min. Bladders were then rapidly stretched with Ringer's solution; placed in 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer, pH 7.4, for 1 min of serosal fixation; removed from the glass tubes; and placed in the above fixative for 15 min for both serosal and mucosal fixation. They were then rinsed in 0.1 M cacodylate buffer and stored in the same solution at 4°C. Freeze-fracture electron microscopy and counting of aggregates and fusion events were performed on these bladders as previously described (4), without knowledge of whether a given bladder was a control or experimental preparation.

RESULTS

Fig. 1 shows the results of a series in which an osmotic gradient was present throughout the entire experiment. All eight pairs

of bladders were filled with Ringer's diluted 1:10 throughout the experiment, except during the rinse period, and water flows were measured throughout. After a 15-min baseline period, bladders were stimulated with vasopressin for 25 min. Experimental bladders were treated with DTBP for the last 10 min of this period, during which control bladders received 1:10 Ringer's at pH 10. The bladders were rinsed for 10 min, and water flows were measured during the washout period. It can be seen that osmotic water flow rises very sharply after 15 min of vasopressin stimulation. After 10 min of DTBP treatment in the presence of vasopressin, water flow has decreased compared to control, indicating a small inhibitory effect of DTBP on vasopressin action. After washout of vasopressin, water flows in control bladders fell nearly to pre-vasopressin levels at 65 min (25 min after washout), whereas in DTBP-treated bladders flow persisted at ~70% of maximum and remained well above control values for 95 min. In the remaining experiments to be presented, the protocol was identical except that an osmotic gradient was present only during the washout period. Fig. 2 shows the results of this standard experiment. Osmotic water flows are shown at various periods after hormone washout. (Typical baseline and vasopressin-stimulated water flows are indicated for reference in Figs. 2-4.) As in Fig. 1, 20 min after washout of vasopressin, water flow across the control bladder had fallen to close to baseline value, whereas high water flows persisted in the fixed bladders, slowly declining over the next hour. It is of interest that post-vasopressin water flows in the DTBP bladders were substantially higher than in Fig. 1, where an osmotic gradient was present throughout vasopressin stimulation. This is in keeping with the findings of Eggena (16) and of Ellis et al. (7) that osmotic water flows and numbers of intramembrane particle aggregates in the luminal membrane are greater when vasopressin stimulation occurs in the absence of an osmotic gradient. On restimulation of the bladders with vasopressin, the control hemibladders responded, whereas DTBP cross-linked bladders did not.

If unstimulated bladders were first cross-linked with DTBP and then stimulated with vasopressin, there was no increase in water flow.

DMS

In a second series of experiments, the cross-linking agent DMS was used instead of DTBP. The results are shown in Fig. 3. The persistence of water flows in the DMS cross-linked bladders was similar to that in DTBP-treated bladders. Note that measurement of water flows began later after washout than in the DTBP experiments, hence water flows in the control bladders had already reached baseline. Again, the cross-linked hemibladders did not respond to restimulation with vasopressin.

Effect of Varying Luminal pH

Cross-linking by the imidoesters is optimal at high pH (16). We determined the effect of DTBP on the persistence of water flow over a pH range of 6-10 in the luminal bathing medium. Paired experiments were carried out, in which the luminal medium of one hemibladder was maintained at pH 10 and the luminal medium of the other hemibladder was set at pH 6.0, 7.0, or 8.0. All the bladders were cross-linked with DTBP, and the standard protocol for measuring water flow was followed. Table I shows water flows across paired bladders between 15 and 30 min after washout of vasopressin. There was a sharp reduction in water flow at pH 6.0 of 80% (compared with water

flow at pH 10) and reductions of 44% at pH 7.0 and 14% at pH 8.0. Thus, cross-linking was reduced as pH fell.

Reversal of DTBP Cross-linking

The effect of cleaving the DTBP cross-link with β -mercaptoethanol on residual water flow was determined in paired hemibladders, both of which were initially cross-linked with DTBP, and one of which was subsequently treated with 5% β -mercaptoethanol for 15 min (see Materials and Methods). The results are shown in Fig. 4. In the β -mercaptoethanol-treated hemibladders, there was a clear and significant fall in water flows compared with DTBP-treated hemibladders for any time interval after the treatments. These hemibladders responded significantly to restimulation with vasopressin (140-

min flows vs. 110-min flows; $P < 0.02$), whereas DTBP-treated hemibladders did not respond. β -Mercaptoethanol did not reverse the high water flows produced by cross-linking with DMS.

Effect of β -Mercaptoethanol Alone on Vasopressin-induced Water Flows

To determine whether the rapid fall in water flow in the β -mercaptoethanol-treated bladders described in the preceding experiment was the result of inhibition of the initial vasopressin effect, we investigated the effect of 5% β -mercaptoethanol solution alone for 15 min on the response of bladders to vasopressin. As shown in Table II, β -mercaptoethanol did not affect vasopressin-stimulated water flows in three 15-min periods.

Sucrose Permeability and Active Sodium Transport

To determine whether the integrity of the epithelium was maintained after cross-linking, we measured the permeability of the bladder to [14 C]sucrose. Permeability remained low under all experimental conditions. Sucrose permeability in DTBP-treated bladders was $5.2 \pm 1.2 \times 10^{-7}$ cm/s, whereas paired controls treated first with DTBP, then with β -mercaptoethanol, had a permeability of $2.7 \pm 0.7 \times 10^{-7}$ cm/s; the difference between the two paired sets was not significant. Sucrose permeability in DMS-treated bladders was slightly but significantly higher than controls (4.0 ± 0.8 compared with $2.3 \pm 0.5 \times 10^{-7}$ cm/s; $n = 10$, $P < 0.02$). The low values obtained under all conditions indicate preservation of epithelial integrity. Active sodium transport, as measured by short-circuit current, was almost completely blocked by the imidoesters.

Freeze-fracture Studies

Freeze-fracture electron microscopy was performed on paired bladders (cross-linked vs. control) at two time intervals after washout. Both control and test bladders were stimulated with vasopressin; one of each pair was then cross-linked with DTBP for 10 min. Both bladders were then washed rapidly in vasopressin-free Ringer's, and the rate of decline of water flow was determined after 15 and 30 min. Immediately after weighing, the bladders were fixed in glutaraldehyde (see Materials and Methods) and examined by freeze-fracture electron microscopy. The results are shown in Table III and Fig. 5. Persistence of water flow was seen in the cross-linked bladders, falling from $61 \pm 7 \mu\text{l}/\text{min}$ at 15 min, to $34 \pm 3 \mu\text{l}/\text{min}$ at 30 min. There was a striking difference in frequency of aggregates

TABLE I
Effect of Varying Luminal pH on Effectiveness of Cross-linking*

pH of luminal medium	Water flow at 30 min after washout $\mu\text{l}/\text{min}$	(Water flow at experimental pH/ water flow at pH 10) $\times 100\%$	$P\ddagger$
		%	
pH 6	7.6 ± 0.8	19.2	<0.01
pH 10	39.5 ± 3.6		
pH 7	18.4 ± 2.2	55.8	<0.05
pH 10	33.0 ± 3.7		
pH 8	42.6 ± 5.2	86.4	NS
pH 10	49.3 ± 6.7		

NS, not significant.

* $n = 4$.

\ddagger Experimental vs. pH 10.

TABLE II
Effect of β -Mercaptoethanol on Vasopressin-stimulated Water Flow

	Water flow*		
	I	II	III
	$\mu\text{l}/\text{min}$		
A. Control	36.0 ± 4.3	46.8 ± 3.7	32.4 ± 1.1
B. 5% β -Mercaptoethanol	35.2 ± 5.5	45.9 ± 1.7	30.6 ± 1.4

Water flow in four pairs of control and experimental bladders is shown for three sequential 15-min periods (I-III) after addition of 90 mU/ml vasopressin.

* P not significant (control vs. experimental, I-III).

TABLE III
Effect of DTBP Treatment on Frequency of Intramembrane Particle Aggregates and Fusion Events

	15 min after washout*			30 min after washout*		
	Control	DTBP-treated	$P\ddagger$	Control	DTBP-treated	$P\ddagger$
Water flow	$19 \pm 4\text{\S}$	$61 \pm 7\text{\S}$	<0.001	$3.6 \pm 0.6\text{\S}$	$34 \pm 3\text{\S}$	<0.01
Aggregate frequency (per $235 \mu\text{m}^2$)	17 ± 9	272 ± 44	<0.01	2 ± 2	$79 \pm 25\ \ $	<0.025
Fusion events (per $235 \mu\text{m}^2$)	9 ± 3	35 ± 9	<0.05	3 ± 1	$8 \pm 2\ \ $	<0.025

* $n = 6$.

\ddagger DTBP-treated vs. control.

\S $\mu\text{l}/\text{min}$.

$\|\|$ DTBP-treated bladders: 30 min vs. 15 min. For unpaired bladders, $P < 0.025$.

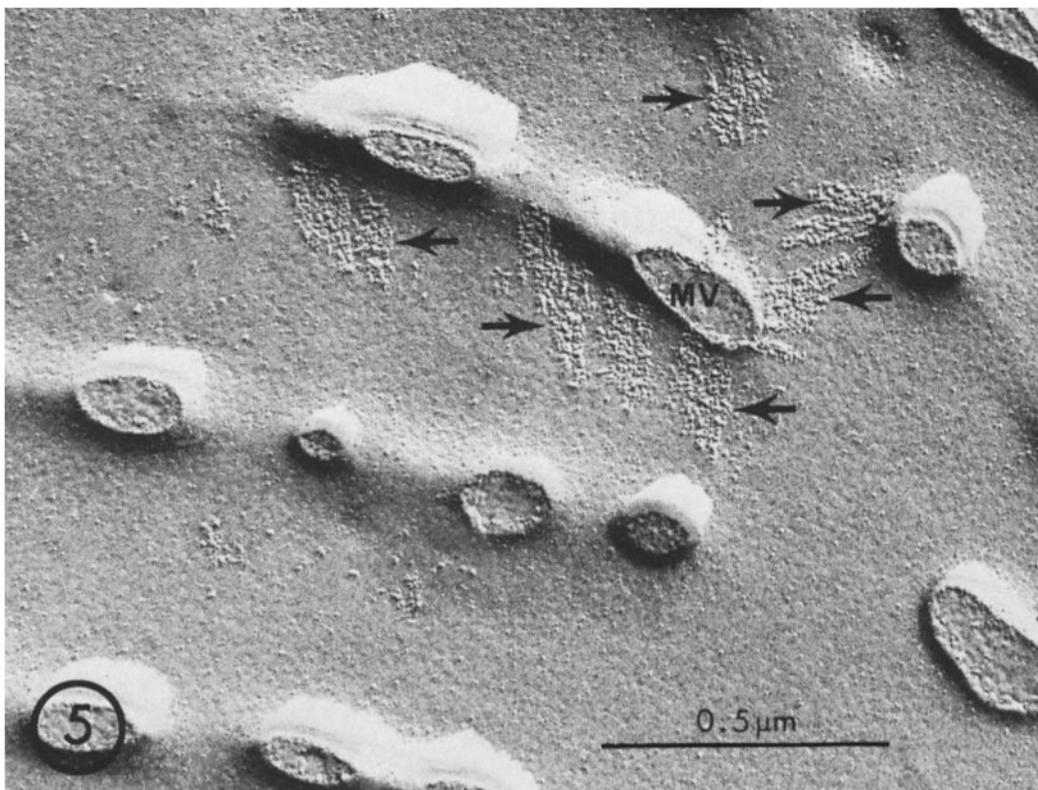


FIGURE 5 Protoplasmic fracture face of granular cell luminal membrane from a bladder fixed during ADH washout in the presence of DTBP. Many aggregates retain their normal organization of closely packed intramembrane particles in linear arrays (arrows). MV, microvillus. $\times 67,000$.

between control and DTBP-treated bladders at both time points, and a significant fall in the number of aggregates in DTBP-treated bladders at 30 min compared with 15 min, corresponding to the decrease in water flow. The frequency of fusion events¹ was also determined. The total number of fusion events was significantly greater in the cross-linked bladders at both time points. Thus, persistence of water flow in the cross-linked bladders was associated with persistence of both aggregates and fusion events in the luminal membrane. Both aggregate frequency and number of fusion events decreased with time, as did water flow.

DISCUSSION

Bifunctional imidoesters have been extensively used in the cross-linking of proteins (17). Their principal reaction is with ϵ - and α -amino groups to form amidines (17, 19). Imidoesters such as DTBP, which possess a central S—S bond, have the added advantage of undergoing cleavage in the presence of reducing agents. This type of reversal of cross-linking has been used to advantage in studies of erythrocyte surface proteins, which can be initially cross-linked, then returned to their original monomer state in the course of gel electrophoresis (20). Experience with the imidoesters has shown minimal interfer-

ence with the structure and function of the cross-linked proteins (17).

In the present studies, we have used imidoesters to stabilize the cell membranes in the course of the usually rapid reversal of a hormone-induced event. Ordinarily, vasopressin-stimulated water flow and particle aggregates disappear rapidly upon withdrawal of the hormone (14). If the membrane is cross-linked during vasopressin stimulation, however, the water-flow response and the presence of aggregates continue after hormone withdrawal, with an eventual slow decline of water flow towards baseline values. DTBP was most effective at pH 8–10, and had little effect at pH 6; this is typical of the pH dependence of cross-linking by the imidoesters (17) and indicates that cross-linking, rather than some other effect of the imidoesters, is involved in the present study. Reduction of the S—S bond of DTBP by β -mercaptoethanol permitted the return of water flow toward baseline to proceed at a nearly normal rate. Viability of the cells was demonstrated by the response of DTBP cross-linked, β -mercaptoethanol-treated bladders to a second serosal stimulation with vasopressin. Integrity of the epithelium was shown by its low permeability to [¹⁴C]sucrose while cross-linked by DTBP or DMS.

The cross-linking effect may be a general one, involving the entire luminal membrane. Evidence for this is the preservation of fusion events, which suggests that fusion of vesicles with the luminal membrane is preserved, as well as the aggregates within the membrane. We cannot say at this time whether cross-linking also takes place between aggregate particles. Cross-linking of the membrane by DTBP or DMS before administration of vasopressin prevented the water flow effect from taking place, again indicating that the imidoesters prob-

¹ Before vasopressin action, the particles that eventually form membrane aggregates are believed to exist on the membranes of cytoplasmic elongated tubular structures. On exposure to vasopressin, these tubular structures fuse with the luminal membrane and are seen as circular structures on freeze-fracture electron microscopy, known as "fusion events" (9, 10, 13).

ably have a widespread effect on components of the membrane surface. This is supported by the finding of Hassell and Hand (21) of a high proportion of liver cell membrane surface groups rendered insoluble by DMS.

We cannot be certain that the effects of the imidoesters are confined to the cell membrane alone. There is evidence that DTBP crosses the erythrocyte cell membrane and cross-links hemoglobin (20). By analogy, DTBP may cross the toad bladder cell membrane and exert a major part of its effect on the membrane indirectly, by cross-linking underlying cytoplasmic elements. It is entirely possible, for example, that submembrane structures such as microfilaments are modified by the imidoesters, with important consequences for aggregate appearance and disappearance.

Thus, the imidoesters greatly slow the rapid changes associated with hormone-induced changes in membrane structure. They may be employed at a physiologic pH. Variability of the system is preserved and, more importantly, reversibility of the cross-linking process affords an opportunity to return the membrane to its original state at desired points in the sequence, and to isolate membrane proteins under controlled conditions.

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REFERENCES

1. Palade, G. E., P. Siekevitz, and C. G. Caro. 1962. Structure, chemistry and function of the pancreatic exocrine cell. In CIBA Foundation Symposium on the Exocrine Pancreas. A.

2. V. S. de Reuck and M. P. Cameron, editors. J. & A. Churchill Ltd., London. 23-49.
3. Satir, B. H., and S. G. Oberg. 1975. Paramecium fusion rosettes: possible function as Ca²⁺ gates. *Science (Wash. D. C.)* 199:536-538.
4. Chevalier, J. J. Bourguet, and J. S. Hugon. 1974. Membrane associated particles: distribution in frog urinary bladder epithelium at rest and after oxytocin treatment. *Cell Tissue Res.* 152:129-140.
5. Kachadorian, W. A., J. B. Wade, and V. A. DiScala. 1975. Vasopressin: induced structural change in toad bladder luminal membrane. *Science (Wash. D. C.)* 190:67-69.
6. Kachadorian, W. A., S. D. Levine, J. B. Wade, V. A. DiScala, and R. M. Hays. 1977. Relationship of aggregated intramembranous particles to water permeability in vasopressin-treated toad urinary bladder. *J. Clin. Invest.* 59:576-581.
7. Kachadorian, W. A., J. Muller, S. W. Rudich, and V. A. DiScala. 1979. Temperature dependence of ADH-induced water flow and intramembranous particle aggregates in toad bladder. *Science (Wash. D. C.)* 205:910-913.
8. Ellis, S. J., W. A. Kachadorian, and V. A. DiScala. 1980. Effect of osmotic gradient on ADH-induced intramembranous particle aggregates in toad bladder. *J. Membr. Biol.* 52: 181-184.
9. Levine, S. D., W. A. Kachadorian, N. C. Verna, and D. Schlondorff. 1980. The effect of hydrazine on transport in toad urinary bladder. *Am. J. Physiol.* 239:F319-F327.
10. Humbert, F., R. Montesano, A. Grosso, R. C. DeSousa, and L. Orci. 1977. Particle aggregates in plasma and intracellular membranes of toad bladder (granular cell). *Experientia (Basel)*, 33:1364-1367.
11. Wade, J. B. 1978. Membrane structural specialization of the toad urinary bladder revealed by the freeze-fracture technique. III. Location, structure and vasopressin dependence of intramembranous particle arrays. *J. Membr. Biol.* 40:281-296.
12. Dratwa, M., C. C. Tisher, J. R. Sommer, and B. P. Croker. 1979. Intramembranous particle aggregation in toad urinary bladder after vasopressin stimulation. *Lab. Invest.* 40: 46-54.
13. Wade, J. B. 1980. Hormonal modulation of epithelial structures. *Curr. Top. Membr. Transp.* 13:123-147.
14. Muller, J., W. A. Kachadorian, and V. A. DiScala. 1980. Evidence that ADH-stimulated intramembranous particle aggregates are transferred from cytoplasmic to luminal membranes in toad bladder epithelial cells. *J. Cell Biol.* 85:83-95.
15. Kachadorian, W. A., C. Casey, and V. A. DiScala. 1978. Time course of ADH-induced intramembranous particle aggregation in toad urinary bladder. *Am. J. Physiol.* 234:F461-F465.
16. Eggena, P. 1972. Glutaraldehyde fixation method for determining the permeability to water of the toad urinary bladder. *Endocrinology*. 91:240-246.
17. Eggena, P. 1972. Osmotic regulation of toad bladder responsiveness to neurohypophyseal hormones. *J. Gen. Physiol.* 60:665-678.
18. Peters, K., and F. M. Richards. 1977. Chemical cross-linking: reagents and problems in studies of membrane structure. *Annu. Rev. Biochem.* 46:523-551.
19. Bentley, P. J. 1958. The effects of neurohypophyseal extracts on water transfer across the wall of the isolated urinary bladder of the toad *Bufo marinus*. *J. Endocrinol.* 17:201-209.
20. Hunter, M. J., and M. L. Ludwig. 1962. The reaction of imidoesters with proteins and related small molecules. *J. Am. Chem. Soc.* 84:3491-3508.
21. Wang, K., and F. M. Richards. 1974. An approach to nearest neighbor analysis of membrane proteins. Application to the human erythrocyte membrane of a method employing cleavable cross-linkages. *J. Biol. Chem.* 249:8005-8018.
22. Hassell, J., and A. R. Hand. 1974. Tissue fixation with diimidoesters as an alternative to aldehydes. I. Comparison of cross-linking and ultrastructure obtained with dimethylsulberimidate and glutaraldehyde. *J. Histochem. Cytochem.* 22:223-239.