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Effects of Potassium-Free Media on ADH Action in Toad Urinary Bladder

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Summary. We studied the effects of potassium-free media on processes related to the hydro-osmotic response of toad bladder to ADH (20 mU/ml). Exposure of bladders to potassium-free media did not affect base-line osmotic water flow, but it promptly attenuated the level of osmotic water permeability induced by ADH. Both the frequency of hormonally induced intra(luminal)membrane particle aggregates (presumed sites for transmembrane water flow) and the number of luminal membrane fusion events (associated with aggregate delivery from the cytoplasm) were also reduced. Potassium-free media had no measurable effect either on cytoplasmic microtubule integrity or on mean aggregate size. Potassium repletion reversed the inhibitory effect of potassium-free media on ADH-related osmotic water permeability. For bladders fully stimulated with ADH in the presence of potassium, subsequent bathing media depletion of potassium led to an inhibition of ADH-related water flow and to reductions in membrane fusion sites and aggregates. We confirmed that the inhibitory effect of potassium-free media on ADH-induced osmotic water permeability results from serosal bathing medium potassium depletion alone and occurs at a post-cyclic AMP site. In addition, we found that ADH-stimulated water permeability was attenuated in bathing media containing a low potassium concentration (0.5 mM). The data are consistent with the view that potassium-free media or media containing low levels of potassium inhibit ADH-enhanced osmotic water permeability in toad bladder by interfering with the process of or leading to membrane fusion required for the delivery of water-conducting structures to the luminal membrane. In addition, some of our results imply that aggregates may turn over during sustained ADH stimulation.

Key Words antidiuretic hormone · toad urinary bladder · water permeability · intramembranous particle aggregates · aggregate turnover

Introduction

The isolated toad urinary bladder has been used extensively for studying the mechanism of action of antidiuretic hormone (ADH). When ADH is added to the medium bathing the serosal surface of this preparation, the water permeability of cell membranes comprising the bladder's luminal surface is enhanced. Water movement occurs passively in either direction across the bladder if a transbladder osmotic gradient is present. Under ordinary physiologic conditions the pathway for hormonally stimulated transbladder water flow is confined to granular cells alone [6, 7, 24].

ADH-enhanced toad bladder water permeability is a cyclic adenosine 5′-monophosphate (cyclic AMP)-mediated phenomenon [12, 23]. Post-cyclic AMP processes leading to this permeability alteration are of current interest and are beginning to be more fully understood. For example, freeze-fracture studies indicate that consequent to both ADH and cyclic AMP treatment structurally organized units of closely packed, linearly arranged intramembranous particles, termed "aggregates," appear in the luminal membrane of granular cells [2, 5, 19, 20]. Aggregates correlate specifically with and are markers of hormonally enhanced luminal membrane water permeability [2, 8, 13, 14, 16–21]; indeed they may be or contain actual sites for trans(luminal)membrane water movement. They are derived preformed from membranes of elongated tubules in the cytoplasm [22, 27]. The membrane fusion event leading to aggregate transfer to the luminal membrane depends on microtubules, while aggregate movement to areas in the luminal membrane remote to points of fusion seems to involve microfilaments [14, 22].

In the present investigation we combined physiologic and morphologic approaches to examine the effect of potassium-free media on the hydro-osmotic response of toad bladder to ADH. The results suggest that exposure of toad bladder to potassium-free media or to media containing a critically low potassium concentration inhibits ADH-

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enhanced osmotic water permeability in toad bladder by inducing interference with the membrane fusion process necessary for delivery of water-conducting structures to the luminal membrane. In addition, certain findings of this study imply that aggregates may turn over during the course of ADH stimulation.

Materials and Methods

Paired urinary hemibladders ($n=14$ pairs) from large, female toads (*Bufo marinus,* Dominican Republic) were prepared as sacs on the ends of glass tubes with serosal surface facing outward. One served as a control bladder, the other as an experimental bladder. Bladders were suspended in Ringer's solution (111 mM NaCl, 3.5 mM KCl, 2.5 mM $NaHCO_3$, and 1.0 mM $CaCl_2$; pH 7.6 to 8.2; 220 mosmol/kg H_2O) that was vigorously bubbled with room air, and they were filled to capacity (with the same solution) in order to standardize tissue distension [15]. After 30 min, transbladder electrical potential was assessed to ensure tissue viability. Only bladders with transepithelial electrical potentials of at least 20 mV were used for experimentation. Experimental bladders were then placed in potassium-free Ringer's solution whose osmolality was maintained at about 220 mosmol/kg H_2O by the addition of sodium. This same solution was used to replace the mucosal contents of these bladders.[1] Regular potassium-containing Ringer's solution was used as a sham replacement of the bathing media of the control bladders. After either 20 or 40 min the mucosal bathing medium of each bladder was replaced with a solution that corresponded to the serosal bathing medium (i.e., potassium-containing or not) except diluted 1:5 with distilled water, and base-line transbladder osmotic water movement was assessed gravimetrically for either 15 or 20 min, respectively. Thereafter, a maximally stimulating dose of ADH (Pitressin, Parke-Davis, Detroit, Mich.; 20 mU/ml) was added to the serosal bath and osmotic water flow was measured at 5-min intervals for 30 min. For these experiments bladder exposure time to potassium-free media prior to the addition of hormone was either 35 or 60 min. For every bladder studied, transbladder electrical potential was measured throughout the entire course of an experiment.

In 6 of these 14 experiments bladders were fixed after hormone treatment, in cacodylate-buffered glutaraldehyde [19]. These tissues were processed for both thin-section and freeze-fracture electron microscopy as described elsewhere [14, 19]. Tissue thin sections were used to assess cytoplasmic microtubule volume density [28], while freeze-fracture tissue replicas were used to quantitate both granular cell luminal membrane fusion events (frequency) [22] and aggregates (frequency and area) [19, 20]. In the other eight experiments, the further objective was to assess whether the inhibitory effect of potassium-free media on ADH-stimulated osmotic water flow was reversible. Following ADH exposure, bladders were washed of hormone by rinsing. Their bathing media (both mucosal and serosal) were then replaced with regular potassium-containing Ringer's solution. Mucosal baths were replaced 35 min thereafter with 1/5 strength Ringer's solution and osmotic water flow was re-evaluated, first for 15 min in the absence of ADH and then for 30 min in the presence of ADH (20 mU/ml). (These tissues were not saved for morphologic analysis.)

In another set of experiments, paired bladders ($n=6$ pairs) without prior exposure to potassium-free media were used to determine whether the inhibitory effect of potassium-free media on ADH-associated water flow (gross) was immediate. In these experiments full strength and diluted Ringer's solutions, containing potassium or not, as described above, were used. Bladders were stimulated with ADH for 40 min, beginning immediately after an osmotic gradient across them was established.

In two other series of experiments, paired bladders were used to confirm that cyclic AMP-stimulated osmotic flow was inhibitable by exposure to potassium-free media ($n=6$ pairs) [10] and to assess the extent to which the inhibitory effect of potassium-free media on ADH-stimulated water flow was evident with solutions containing smaller than ordinary concentrations of potassium (0.5 mM ($n=6$ pairs) and 1.0 mM ($n=4$ pairs)). In these experimental series, bladders were exposed to either regular Ringer's solution, potassium-free Ringer's solution, or Ringer's solution containing either 0.5 or 1.0 mM potassium for 60 min prior to stimulation for 30 min with either dibutyryl cyclic AMP (Sigma Chemical, St. Louis, Mo.; 2 mM final serosal concentration) or ADH (20 mU/ml). Before stimulation with either agent, the mucosal media were exchanged with diluted solutions in order to assess osmotic permeability.

In still other experiments, 12 pairs of bladders were studied to determine whether removal of potassium from the bathing media would inhibit hormonally stimulated osmotic water flow *after* a full maximal ADH effect had first been established. Following an initial 30-min equilibration period, a transbladder osmotic gradient across paired bladders was established with Ringer's solution as the serosal bath and 1/5 strength Ringer's solution as the mucosal bath. Osmotic water flow was measured for 30 min prior to and at 5-min intervals during 30 min of stimulation with ADH (20 mU/ml). Thereafter, the bathing media of the experimental bladders were replaced with potassium-free Ringer's solution while those of the controls were replaced with regular Ringer's solution containing potassium. Hormonal stimulation of these bladders was uninterrupted during the brief interval when bathing media were exchanged, and stimulation was extended for either 60 ($n=6$) or 30 min ($n=6$). Water flow was evaluated at 5-min intervals, beginning 5 min after media exchange. Bladders in the latter group were fixed in glutaraldehyde and processed by freeze-fracture electron microscopy to evaluate granular cell luminal membrane for fusion event frequency and the frequency and size of aggregates.

Measurements of water flow in this investigation were expressed per cm^2 of luminal membrane surface area. The latter was calculated from the volume of fluid required to fill bladders to maximal capacity [15], so that the assumption of a smooth sphere, circumscribed by the luminal membrane, could be most nearly approximated [11]. The physiologic and morphologic data from this investigation were analyzed for differences by Student's *t*-test for paired observations. Mean differences calculated to occur less than 5% of the time by chance were considered significant.

[1] Although it has been shown that the inhibitory effect of potassium-free media on ADH-stimulated water flow is the result of potassium removal from the serosal bathing medium alone [1], we deleted potassium from the mucosal bath to minimize the possibility of serosal bath potassium "contamination." In view of this, it seemed important for the experimental protocol we followed to verify that the effect of potassium removal on ADH-stimulated water flow was due to serosal bath medium potassium removal alone. In experiments ($n=3$) where potassium was removed only from the serosal bathing media (sodium being replaced to maintain osmolality), base-line flow was unaffected (0.5 ± 0.2 *vs.* 0.4 ± 0.1 mg $\times$ min^{-1} $\times$ cm^{-2}) and 30-min hormonally stimulated flow was less (139.8 ± 8.4 *vs.* 61.2 ± 4.8 mg $\times$ 30 min^{-1} $\times$ cm^{-2}, $P<0.005$) by 56.3 ± 1.8%. Removal of potassium from the mucosal bathing media in other bladders ($n=3$) had no effect on either base-line (0.5 ± 0.2 *vs.* 0.5 ± 0.2 mg $\times$ min^{-1} $\times$ cm^{-2}) or ADH-stimulated flow (123.6 ± 3.0 *vs.* 120.6 ± 7.2 mg $\times$ 30 min^{-1} $\times$ cm^{-2})

Results

Inhibitory Effect of Potassium-Free Media on ADH-Stimulated Water Flow and Related Morphologic Observations

The effect of bladder incubation in potassium-free media on water permeability is illustrated in Fig. 1. In accord with observations by others [1, 10], bladder exposure to potassium-free media did not affect basal water permeability, but it consistently prevented hormonally stimulated water flow from achieving a maximal level. The phenomenon of time-dependent, intrinsic inhibition of hormonally stimulated flow, which is ordinarily observed in toad bladder [3, 9], was also evident in bladders exposed to potassium-free media. For these preparations maximally achieved water flow (net) over the 30-min interval of ADH stimulation was less ($P<0.001$) with exposure to potassium-free media by $38.7\pm4.5\%$.

Figure 2 illustrates morphologic data for ADH-treated bladders exposed to potassium-free media. Hormonally stimulated (i.e., net) 30-min water flow in these bladders was inhibited by exposure to potassium-free media ($50.5\pm4.1\%$, $P<0.001$) to about the same general extent as that for bladders shown in Fig. 1. In experimental bladders the frequency of fusion events associated with aggregate delivery to the luminal membrane was less (10 ± 1 *vs.* 21 ± 3 per 235 μm^2, $P<0.005$) by $49.8\pm6.7\%$. Aggregate frequency in experimental bladders was also less (101 ± 19 *vs.* 246 ± 44 per 235 μm^2, $P<0.025$) by $54.8\pm7.7\%$, while the cumulative area of membrane occupied by aggregates was less (0.7 ± 0.1 *vs.* 2.1 ± 0.5 μm^2 per 235 μm^2, $P<0.025$) by $59.8\pm8.2\%$. Mean aggregate size in experimental bladders ($7.0\pm0.2\times10^{-3}$ μm^2) was not statistically different from that in controls ($8.0\pm0.5\times10^{-3}$ μm^2). Finally, stereologic analysis of 3 of these 6 pairs of bladders, suggested that cytoplasmic microtubule volume density was entirely unaffected (0.331 ± 0.001 *vs.* $0.328\pm0.001\%$) by exposure to potassium-free media. (On the basis of these data for microtubule volume density we considered it unnecessary to pursue further by additional measurements in the other bladder pairs whether exposure to potassium-free media affects microtubule integrity.)

Immediacy of the Inhibitory Effect of Potassium-Free Media on Water Flow During ADH Stimulation

In the experimental bladders of these studies in which bladders were simultaneously exposed to potassium-free media and treated with ADH, water flow (gross) was clearly attenuated during the first 5 min of stimulation. The level of inhibition approximated 20% during the first 20 min of stimulation, 35% during the next 10 min, and 45% during the last 10 min of the 40-min period of ADH treatment. The exact estimates of inhibition in relation to time are given in the Table.

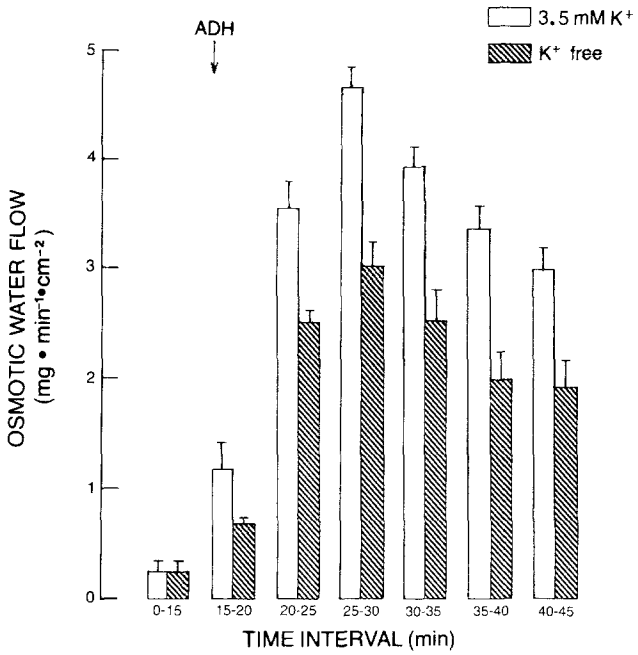

Fig. 1. Osmotic water flow (175 mosmol gradient) in bladders bathed with potassium-containing or potassium-free Ringer's solution before and during ADH stimulation (20 mU/ml). Mean and standard error of the mean shown. $n=8$

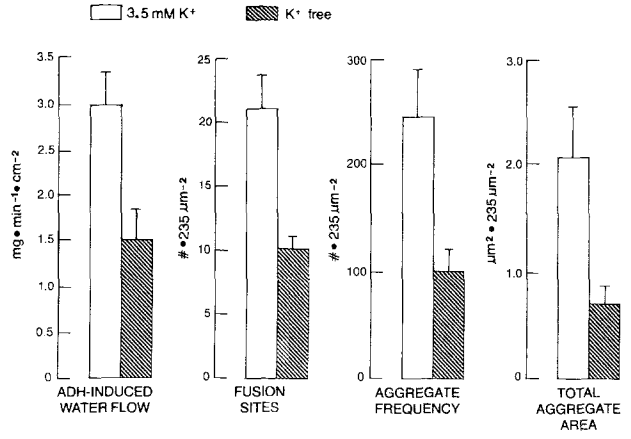

Fig. 2. ADH-stimulated (20 mU/ml) osmotic flow (175 mosmol gradient) and associated granular cell luminal membrane changes revealed by freeze-fracture electron microscopy in bladders bathed with potassium-containing or potassium-free Ringer's solution. Tissues were fixed in glutaraldehyde at the 30th min of ADH treatment. Mean and standard error of the mean shown. $n=6$

Table. Inhibition of water flow across bladders ($n=6$) simultaneously treated with ADH and exposed to media without potassium

Time interval of ADH stimulation (min)	Level of inhibition of hormonally related (gross) water flow (%)
0–5	22.8±8.8
5–10	18.0±2.2
10–15	19.0±7.0
15–20	22.5±6.3
20–25	34.4±6.1
25–30	37.4±5.5
30–35	45.7±4.9
35–40	46.8±4.0

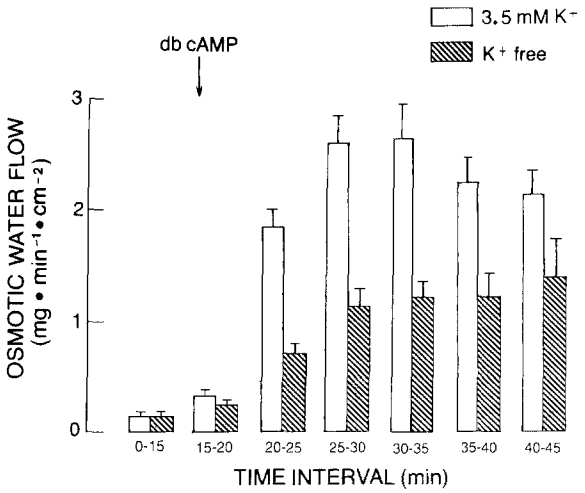

Fig. 3. Osmotic water flow (175 mosmol gradient) in bladders bathed with potassium-containing or potassium-free Ringer's solution before and during stimulation with 2 mM dibutyryl cyclic AMP (db cAMP). Mean and standard error of the mean shown. $n=6$

Effect of Potassium-Free Media on Dibutyryl Cyclic AMP-Stimulated Water Flow

The results of these experiments are illustrated in Fig. 3. Thirty-min cyclic AMP-stimulated water flow in bladders bathed with potassium-free media was inhibited by 53.7±3.6%. Inhibition became evident within 5 to 10 min following cyclic AMP addition. This is comparable to that observed for bladders stimulated with ADH (compare with Fig. 1).

Effect of Media Containing Lower than Ordinary Concentrations of Potassium on ADH-Stimulated Water Flow

In these studies, serosal Ringer's solution containing 0.5 mM potassium attenuated the water flow

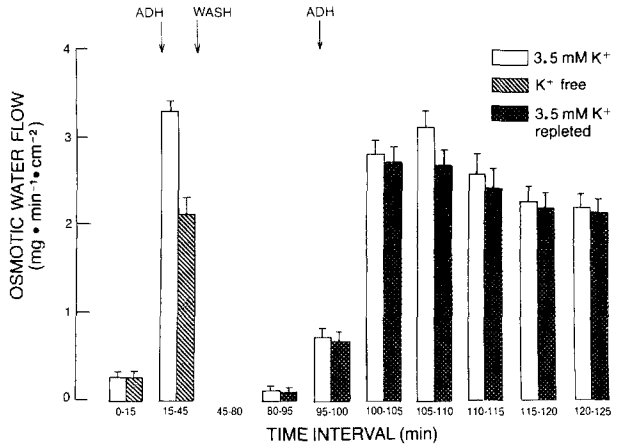

Fig. 4. Basal and ADH-stimulated (20 mU/ml) osmotic water flow (175 mosmol gradient) in bladders exposed either to potassium-free Ringer's solution followed by potassium-repleted Ringer's solution, or with potassium-containing Ringer's solution only. The same bladders used to generate the data in Fig. 1 were used to generate the extended data of this Figure. Data shown prior to "wash" summarize data in Fig. 1. Mean and standard error of the mean shown. $n=8$

response to ADH stimulation (from 109.2±9.6 to 89.4±6.6 mg × 30 min^{-1} × cm^{-2}, $P<0.005$); however, the reduction (18.4±2.6%) was considerably less than previously described for bladders exposed to potassium-free media. In the other similar experiments of this series, serosal bathing media containing 1.0 mM potassium did not prevent ADH-stimulated water flow over a 30-min interval from achieving a control level (93.6±7.2 in experimentals *vs.* 93.0±7.2 mg × 30 min^{-1} × cm^{-2} in controls).

ADH-Associated Water Flow in Bladders after Bathing Media Potassium Repletion

These experiments (extensions of those shown in Fig. 1) are illustrated in Fig. 4. As given above, exposure of the experimental bladders to potassium-free media attenuated the 30-min hydro-osmotic response of ADH by 38.7±4.5%. Following hormone washout with bathing media containing a normal concentration of potassium (3.5 mM), subsequent stimulation with ADH in the presence of the same media as used for washout, caused water flow to increase above a base-line value to the same extent in experimental bladders as in controls (59.4±2.4 (experimentals) *vs.* 64.8±4.2 (controls) mg × 30 min^{-1} × cm^{-2}). These data demonstrate that normal bladder responsiveness to ADH stimulation, after being diminished by tissue exposure to potassium-free media, can be (to the extent limited by normal refractoriness) re-established by potassium repletion.

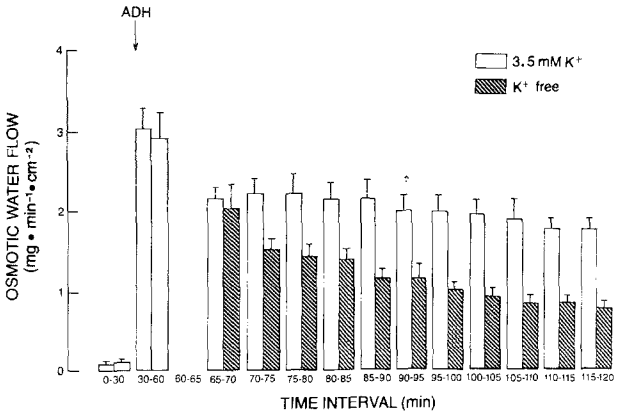

Fig. 5. Effect of bathing media potassium removal on sustained ADH-stimulated (20 mU/ml) osmotic water flow (175 mosmol gradient) across bladders whose ADH-stimulated water permeability was first fully established. Mean and standard error of the mean shown. $n=6$

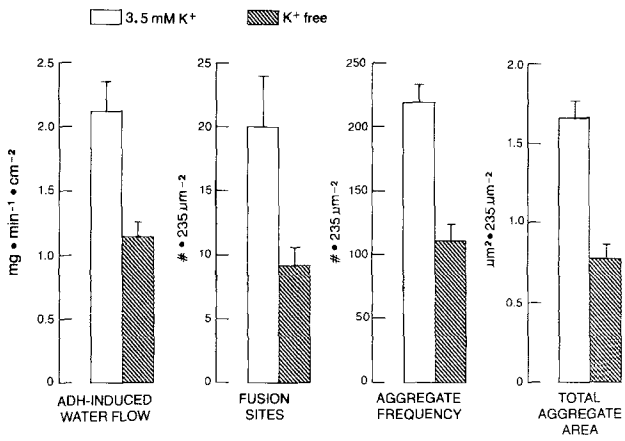

Fig. 6. ADH-stimulated (20 mU/ml) osmotic water flow (175 mosmol gradient) and granular cell luminal membrane changes revealed by freeze-fracture electron microscopy in bladders fully responding to ADH, then exposed to potassium-free bathing media. These experiments paralleled those illustrated in Fig. 5. Bladders were fixed with glutaraldehyde at the 60th min of ADH treatment, which was approximately 30 min after bathing media exchange. Mean and standard error of the mean shown. $n=6$

Effect of Potassium Removal from the Bathing Media of Bladders Fully Stimulated with ADH

Figure 5 illustrates that in bladders fully responding to ADH, bathing media potassium removal leads to prompt inhibition of the ongoing hydro-osmotic response. This inhibition is evident within 10 to 15 min following potassium removal and rather steadily becomes more evident until achieving a level of about 40 to 50%.

The data illustrated in Fig. 6 are from bladders that were treated identically to those used for generating the data in Fig. 5, except that at the 30th min following potassium removal from the experimental bladders, tissues were fixed in glutaraldehyde. The level of ADH-stimulated flow, measured from the 5th to 30th min following potassium removal, was reduced (from 59.0 ± 5.1 to 33.6 ± 1.9 mg $\times$ 25 min$^{-1}\times$cm^{-2}, $P<0.005$) with this maneuver by $41.5\pm4.5\%$. Corresponding to this, the frequency of fusion sites (which ordinarily persist unaffected as membrane invaginations throughout stimulation with hormone [22]) was reduced (from 20 ± 4 to 9 ± 1 per 235 µm^2, $P<0.01$) by $48.3\pm7.5\%$. The frequency of aggregates diminished (from 220 ± 13 to 111 ± 10 per 235 µm^2, $P<0.005$) by $47.9\pm6.5\%$ and the cumulative area of membrane occupied by aggregates decreased (from 1.7 ± 0.1 to 0.8 ± 0.1 µm^2 per 235 µm^2, $P<0.005$) by $50.8\pm7.1\%$. Mean aggregate size was not affected by potassium removal from the experimental bladders ($7.6\pm0.3\times10^{-3}$ in controls *vs.* $7.0\pm0.4\times10^{-3}$ µm^2 in experimentals).

Discussion

The established data of Bentley [1] and Finn et al. [10] indicate that potassium removal from the serosal bathing medium of toad bladder leads to a reduction in its hydro-osmotic response to ADH. Finn et al. [10] demonstrated that this effect occurs at a post-cyclic AMP site. The results of the present investigation confirm these observations.

In contrast to the earlier report of Finn et al. [10] which suggested a delay in the onset of inhibition, our data show that exposure of toad bladder to potassium-free media rapidly alters ADH-stimulated water flow. The reason for the apparent discrepancy is not altogether clear. In our protocol we attempted to maximize the sensitivity of assessing osmotic water flow by standardizing bladder distension [15] and by normalizing flow on the basis of luminal membrane surface area. The finding that the effect of potassium removal on ADH-stimulated water flow can be immediate and not necessarily delayed may be important for assessing the mechanism underlying the phenomenon. Had Finn et al. [10] observed the same immediate response they probably would not have concluded that the critical determinant of the inhibitory effect of potassium-free media on hormonally induced water flow directly involves, as an initiating factor, a reduction in intracellular potassium concentration. More specifically, though in those previous experiments intracellular potassium was reduced in bladders exposed to potassium-free media, the reduction was delayed by a considerable period of time (i.e., between 20 to 45 min) [10].

The possibility that a reduced level of intracellular potassium could have some other type of role

(e.g., potentiation) in the inhibitory effect of potassium-deficient media on the water flow response to ADH is not, however, invalidated by any of our findings. Consistent with this possibility and in agreement with the observations of Finn et al. [10], data from some of the separate groups of bladders we studied suggest that as the time of exposure to potassium-free media prior to ADH treatment increased, which would be expected to lead to greater reductions of intracellular potassium, so did the level of inhibition of ADH-stimulated osmotic water flow. Specifically, compared to the $24.7 \pm 4.2\%$ level of inhibition of 30-min stimulated water flow in bladders without prior exposure to potassium-free media (*see* Table), the level of inhibition in ADH-treated bladders preexposed to potassium-free media for 35 min (5 of the 8 in Fig. 1), 60 min (3 of the 8 in Fig. 1 and those in Fig. 2), and 155 min (5 additional bladders) appeared to become progressively more intense, increasing to $35.7 \pm 6.2\%$, $48.2 \pm 3.5\%$, and $69.3 \pm 1.9\%$, respectively.

Definition of the biochemical defect resulting from exposure of bladders to potassium-free media will require additional research. The possibility that this defect may involve an increase of intracellular calcium has had some indirect support [26]. We have not been able to provide compelling data that would resolve this problem; nevertheless our data argue against the further speculation of a connection between a postulated elevation of cytosolic calcium and microtubule dysfunction [25, 26]. Clearly, our observations indicate that microtubule integrity is unaffected by potassium-free media.

In relation to another possibility that may be pertinent, Carvounis and Carvounis demonstrated that the inhibitory effect of potassium-free media on ADH- and cyclic AMP-stimulated water flow could be instantly reversed by alkalinizing the serosal bath from a pH of 7.4 to 8.3 [4]. This may indicate that the inhibitory effect of potassium-free media on ADH action results from a change in cell pH towards an acid level and subsequent activity reduction at one or more post-cyclic AMP sites in the intracellular cascade leading to the hormonally related alteration of membrane water permeability. The precise relation of this to the issue at hand or to our findings, however, remains to be clarified. A change in intracellular pH is only one of a number of biochemical defects which may result from exposure of cells to a potassium-free environment.

Analysis of the freeze-fracture data provides not only structural evidence to indicate that the inhibitory effect of potassium-free media on ADH-stimulated water flow involves a decrease in luminal membrane water permeability per se, but it also supports the hypothesis that aggregates turn over during sustained ADH stimulation (at least during exposure to potassium-free media). Recall that when fully stimulated bladders were exposed to potassium-free media the number of fusion sites was reduced by about half (Fig. 6). Aggregate frequency in these same bladders was also reduced to the same extent. The reduction in aggregate frequency would suggest that the aggregates added to the luminal membrane during the initial phase of stimulation are not stable structures; if they were, they should have remained in place regardless of whether fusion sites persisted. The possibility that exposure to potassium-free media exerts some direct effect on aggregate stability, which would seem to deserve consideration, is not an issue here since our measurements indicate that the size of remaining aggregates did not change. To put it another way, if aggregates became structurally unstable (relative to controls) by exposure to potassium-free media, one would expect a larger number of smaller aggregates to remain, hence a reduction in (mean) aggregate size with experimental treatment. We tentatively conclude therefore, that during ADH stimulation luminal membrane aggregates turn over and that their normally constant frequency in relation to time depends on replacement of aggregates from the elongated cytoplasmic structures which remain fused with the luminal membrane during hormone exposure [13, 14, 22].[2]

The major findings of this study show that together with a reduction in ADH-stimulated water permeability, fusion events associated with aggregate delivery to the luminal membrane and aggregates in the luminal membrane are reduced by bathing media potassium removal, whether the maneuver precedes or occurs during stimulation. In neither of these cases is mean aggregate size altered. Since continuity between the luminal membrane and aggregate-carrying membranes is maintained during normal stimulation [22], the findings of this investigation point to the possibility that the inhibitory effect of potassium-free (or potassium-deficient) media on ADH-stimulated water flow results primarily from interference with the membrane fusion process necessary for continuous

[2] It is also interesting that in these experiments the percent reduction in aggregates and the percent reduction in fusion sites are always approximately the same. This suggests that the addition of aggregates to the luminal membrane probably occurs at a constant rate at all fusion sites and that this rate is not altered by exposure to potassium-free media.

delivery of water conducting structures to the luminal membrane.

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