Biochimica et Biophysica Acta, 774 (1984) 26–34
Elsevier

BBA 72125

MAXIMAL FLUX RESPONSES AFTER MULTIPLE CHALLENGES WITH VASOPRESSIN

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(Received August 31st, 1983)
(Revised manuscript received January 3rd, 1984)

Key words: Water transport; Hydro-osmotic response; Vasopressin; Membrane recycling (shuttling); (Toad bladder)

Antidiuretic hormone (ADH) increases transepithelial flux of water and particular solutes across the amphibian urinary bladder and mammalian collecting duct by increasing the permeability of the apical surface. We find that if each challenge with ADH is ended by replacing the medium bathing both the mucosal and serosal surfaces of the toad bladder, then rechallenge with the same supramaximal dose of ADH 36–100 min later produces flux equivalent to or greater than the original response, but rechallenge after 15 min produces only 68% of the original response. If the medium bathing the mucosal surface is neither replaced nor returned to its original volume, complete recovery of the osmotic flux response to ADH does not occur. Maximal restimulation by ADH occurs with transepithelial osmotic gradients between 119 and 180 mosmol/kg during both challenges (the serosal bath is always isotonic amphibian Ringers). In addition, ADH-containing serosal baths that have maximally activated transport across bladders for 30–60 min can be reused and again produce maximal activation of ADH responses in fresh bladders or in the original bladders after washing. These results are in contradistinction to reports of desensitization of transepithelial flux upon rechallenge with ADH after an initial stimulation under many conditions. Our findings suggest that desensitization in vitro may result from experimental design rather than intrinsic biological characteristics of the system.

Introduction

Antidiuretic hormone (ADH, vasopressin) binds to receptors on the serosal (S) surface of amphibian urinary bladders to bring about rapid loss of water (and solutes) from the stored urine (or mucosal bath, M) [1–3]. Similar responses to ADH occur in the collecting duct of mammalian nephron [2,3].

We now report that bladders can produce equivalent responses to successive ADH challenges, and describe the experimental conditions we used.

Desensitization to repeated challenges with ADH of osmotic flux across toad bladder epithelium has been reported many times [4–14], but our studies are more consonant with recent evidence [15–20] on the significant role that membrane-recycling shuttle mechanisms probably play in ADH-stimulated transport as well as other hormonal processes.

Our findings suggest that bladders have adequate reserves of receptor and effector units to

Abbreviations: ADH, antidiuretic hormone or vasopressin; PD, transepithelial potential difference, a measure of Na^+ flux.

support second (and third) responses in vitro, and/or that bladders quickly recycle previously used units.

Methods

Female Dominican toads (*Bufo marinus*) were obtained from National Reagents (Bridgeport, CT) and maintained either in dripping tap water or on damp peat moss. Toads were killed by double-pithing, dissected and bladder lobes (hemi-bladders) tied to glass tubes as sacs in situ and then excised [1].

The experimental protocol is shown in Scheme I.

Amphibian Ringers was buffered with Hepes or phosphate and was 230–247 mosmol/kg. Hepes-buffered Ringers contained 10 mM Hepes at pH 7.4–7.6 and 110 mM NaCl, 3 mM $KHCO_3$, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM glucose. Phosphate-buffered Ringers contained 5 mM phosphate at pH 7.3–7.4 and 110 mM NaCl, 4 mM KCl, 0.5 mM $CaCl_2$ and no $MgCl_2$ or glucose. 'Ca^{2+}-free phosphate-buffered Ringers' contained 1.5 mM EGTA, a Ca^{2+} chelator, and no added $CaCl_2$.

To define the period after which equivalent responses to ADH could be obtained, experiments were performed with different intervals between ending one ADH challenge (after 30, 45 or 60 min) and starting the next. Ending one ADH challenge meant changing the serosal medium at least once and usually three times. The serosal medium was replaced again at the start of equilibration and the next ADH challenge was begun by adding fresh ADH to the serosal medium. The intervals ranged from 15 to 100 min. For intervals shorter than 45 min, washout and equilibration times were shortened, and basal osmotic water loss measured for 5–8 min before the second stimulation. In all studies in which the length of the interval between challenges was the variable, both

Scheme I. Experimental protocol for osmotic flow experiments. The composition of phosphate-buffered Ringers (PR) and Hepes-buffered Ringers (HR), the dilutions of amphibian Ringers (R) used for mucosal medium (M) (the transepithelial gradients), the length of washout periods, the number of replacements of serosal medium (S) and/or mucosal medium during washout, and other experimental details are described in the text.

elapsed time (min)	
	Dissect bladders from fresh-killed toads
	Wash both surfaces (M and S) of hemibladders with PR or HR three times (30–60 min total)
0	Replace M with 5 ml diluted R (e.g. R/5) *; suspend in 35 ml well-aerated R to establish transepithelial gradient (equilibration)
15	Weigh bladder (t_0)
	Basal water loss (μl/min) = ($t_0 - t_1$) μg/15 min
30	Weigh bladder (t_1)
	Measure PD and discard tissue if PD < 5 mV (basal PD)
	Add 50 mU ADH/ml to S
45	Weigh bladder (t_2).
	Measure PD
	ADH-stimulated water loss (μl/min) = ($t_1 - t_2$) μg/15 min − basal water loss (μl/min)
	ADH-stimulated PD = ($t_2 - t_1$) PD (mV)
60–75	Weigh bladder at 15-min intervals during ADH stimulation of 30–60 min.
	Measure PD at 30-min intervals
60–75	Washout (15 min) to end ADH challenge.
	Replace S with fresh hormone-free R one to three times.
	Replace M with fresh R or diluted R (e.g. R/5) * one to three times.
	Return to $t = 0$ and repeat sequence from equilibration to washout at least once.

* In other experiments, the diluted R used for the mucosal bath at both t_0 and t_{60} was R/2 or R/10.

mucosal and serosal media were changed at the end of each challenge, and bladders were filled with 5 ml of fifth-strength amphibian Ringers (R/5) and suspended in 35 ml of well-aerated amphibian Ringers.

The extent to which the transepithelial gradient during the first challenge affected osmotic water loss during another ADH challenge in that gradient was assessed. The starting measured transepithelial gradient ranged from 119 to 180 mosmol/kg with mucosal baths prepared as half-, fifth-, or tenth-strength amphibian Ringers (R/2, R/5 or R/10). Serosal baths were undiluted isotonic amphibian Ringers, between 220 and 240 mosmol/kg. Osmolarity of the mucosal bath was also measured after ADH-stimulated transport. Osmolarities were measured with a Wescor 5100-C vapor phase osmometer.

The different compositions of Hepes-buffered Ringers and phosphate-buffered Ringers also allowed us to note whether buffer composition, pH, Ca^{2+} and Mg^{2+} content or glucose content affected reactivity to ADH (re)challenge.

Solute transport. ADH specifically and rapidly increases transepithelial flux of sodium and urea [1–3]. The transepithelial potential difference (PD) depends primarily on Na^+ flux and was quickly measured potentiometrically (open circuit) at intervals during successive ADH stimulations. Hemibladders with non-hormone-stimulated (basal) PD < 5 mV were discarded as metabolically inactive. Hemibladders without transepithelial potential difference responses to ADH were also discarded.

[^{14}C]Urea flux from the mucosal to the serosal bath during successive stimulations was measured by liquid scintillation counting of aliquots removed at 15 min intervals [29].

Data analysis. Experimental results are expressed as means ± S.E.; in the figures, bars show the S.E. The data were analyzed for pair differences or differences between groups by Student's *t*-test or Wilcoxon's rank test, tests of equal statistical power [30]. Significance of $P < 0.05$ is noted. The rank test was used when the range of pair differences or individual responses was very large.

The response to successive ADH challenges was compared in single hemibladders or after treating each of a hemibladder pair differently in the second stimulation. Successive challenges were of the same length and with the same supramaximal dose, 50 mU ADH/ml serosal medium, unless otherwise stated. ADH was Pitressin (Parke-Davis) or 8-arginine vasopressin (Bachem).

Results

To observe the effects on equivalent hydro-osmotic responses to ADH, we varied
(1) the interval between successive ADH challenges,
(2) the transepithelial osmotic gradient during ADH challenges,
(3) the buffer composition of amphibian Ringers, and
(4) the bath replacements used to end an ADH stimulation: only the serosal (hormone-binding), or only mucosal (transporting), or both surfaces of the epithelium.

TABLE I

INTERVAL BETWEEN ADH CHALLENGES AFFECTS ADH-STIMULATED OSMOTIC WATER LOSS

Hemibladders containing HR/5 and bathed in well-aerated Hepes-buffered Ringers (HR) were treated identically through the first ADH stimulation. Washout was initiated by replacing the serosal medium three times with hormone-free Hepes-buffered Ringers and replacing the mucosal medium one to three times with HR/5. Mucosal and serosal media were replaced again at appropriate times to start equilibration, and basal osmotic water loss measured. Then 50 mU ADH/ml serosal medium was added to begin the second challenge. All values are given as mean ± S.E. for *n* determinations and the Δ is calculated for paired observations by Student's *t*-test. [a] $P < 0.05$; [b] $P < 0.02$; [c] $P < 0.002$.

Interval (min) (*n*)	μl water loss/min during 30 min ADH stimulation		
	First ADH challenge	Second ADH challenge	Δ
15 (8)	38.6 ± 3.0	26.1 ± 6.7	−12.5 ± 1.1 [c]
21 (3)	17.2 ± 1.4	15.0 ± 1.4	−2.3 ± 0.1 [b]
31 (6)	52.2 ± 8.0	48.7 ± 8.2	−3.6 ± 3.4
36 (6)	17.8 ± 1.6	17.6 ± 1.7	−0.2 ± 0.1
45 (14)	32.7 ± 3.4	34.7 ± 3.6	1.4 ± 1.7
50 (7)	44.8 ± 2.9	39.1 ± 6.8	0.0 ± 2.4
75 (3)	48.1 ± 2.7	49.5 ± 6.5	1.4 ± 4.9
85–90 (6)	32.0 ± 3.2	30.9 ± 3.3	−1.1 ± 5.3
100 (3)	43.4 ± 2.5	46.7 ± 2.5	3.2 ± 0.6 [a]

Interval length

Each challenge was ended by replacing the mucosal and serosal baths (washout), again replacing both baths so the tissue equilibrated in the freshly imposed transepithelial gradient, and then measuring osmotic water flow (or solute flux) in the absence of ADH (basal measurements). These three steps comprised the interval between challenges. Immediately after measurement of basal hydroosmotic flow, fresh ADH was added to serosal medium to initiate the next challenge.

After a 36 min interval, osmotic water flux after ADH rechallenge is equal to that in the first challenge (Table I). Longer intervals, to 100 min, permit second ADH responses equivalent to or larger than the original.

However, after a 21 min interval, restimulation with ADH produces a statistically significant water flow decrease of 13% from original levels. After a 15 min interval, water flow is 32% less than during the first challenge.

Thus with the experimental conditions we use, very little osmotic flux desensitization to ADH rechallenge is apparent, and that is quickly recoverable *.

Transepithelial osmotic gradients

We studied the extent to which ADH-stimulated osmotic water flow affects the response to ADH rechallenge in the same transepithelial gradient. That is, osmotic flux was measured from hemibladder sacs filled with R/2 or R/5 or R/10 with the serosal medium always being isotonic amphibian Ringers. We find that with imposed osmotic gradients between 119 and 180 mosmol/kg, rechallenge with fresh solutions and the original gradient evokes second responses equal to the first (Fig. 1).

Solute transport

Mucosal osmolarity does not change appreciably during 15–60 min of ADH-stimulated osmotic water loss, presumably because ADH stimulates mucosal-to-serosal transport of both water and sodium [1–3], the principal cation of the baths. Measured mucosal osmolarities are 128 mosmol/kg for PR/2, 88 for HR/5 and 63 for HR/10, and are higher than those calculated from the dilution since the measured osmolarity of amphibian Ringers is 247 for phosphate-buffered Ringers (PR) and is 230 or 243 for Hepes-buffered Ringers (HR).

In many experiments with PR/2 vs. phosphate-buffered Ringers, Na^+, urea and osmotic fluxes were all measured at once. The same conditions permit repeated maximal osmotic

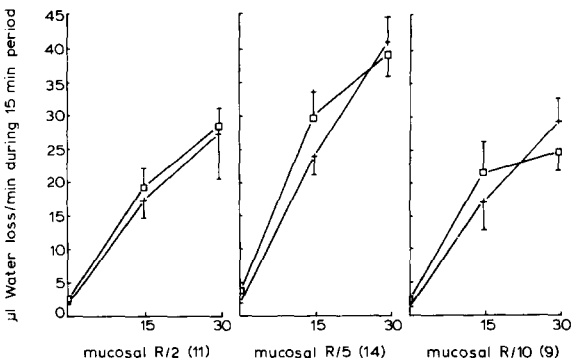

Fig. 1. Osmotic water loss during two successive ADH stimulations. Hydro-osmotic flux increases similarly in first (+) and second (□) ADH stimulations when the imposed gradient during each stimulation is PR/2 vs. phosphate-buffered Ringers (PR) (first panel, 119 mosmol/kg), HR/5 vs. Hepes-buffered Ringers (HR) (second panel, 142 mosmol/kg) or HR/10 vs. HR (third panel, 180 mosmol/kg). Similar results were also obtained with PR/5 vs. phosphate-buffered Ringers. Osmotic flux decreases during the next 30 min (not shown) are also the same in both challenges. The apparent increase in water flow during the first 15 min of the second ADH stimulation is not statistically significant. The first ADH challenge was ended after 30, 45 or 60 min by replacing the mucosal and serosal media, replacing serosal medium twice more during the next 15 min and then reimposing the same gradient by replacing the serosal and mucosal media with fresh amphibian Ringers and dilute amphibian Ringers baths again for 15 min. Basal water loss was measured, and then 50 mU ADH/ml serosal medium was added to start the second challenge (total interval 45 min). Replacement of mucosal medium with PR/2 or phosphate-buffered Ringers for washout of bladders filled with PR/2 and challenged with ADH did not affect subsequent ADH responses. Usually mucosal baths were replaced with fresh Ringers of the dilution already present. The number of experiments is listed in parenthesis after the mucosal medium dilution.

* The effects of interval length, transepithelial gradient and bath replacement on flux responses to ADH rechallenge are independent of a second source of variations seen in the osmotic responses to the first challenge. Osmotic water losses in response to ADH follow a striking, repetiive and statistically significant seasonal pattern. Minimal responses occur in February, and maximal in early October (Rubin, M.S. and Masur, S.K., unpublished data).

water and solute fluxes in responses to ADH rechallenge. Thus hemibladders with equal osmotic water loss to ADH (re)challenge also have equivalent transepithelial potential difference responses (sodium fluxes) (Table II) and mucosal osmolarities remain unchanged during each challenge.

Early in our studies we noted that hemibladders in which mucosal → serosal sodium flux is not increased by ADH have much lower osmotic water flows than their pairs, whether in a first or later ADH stimulation. In those instances, mucosal osmolarity increases considerably during ADH stimulation and basal PD before the second challenge usually falls to < 5 mV (data not shown).

With only 21 min between challenges, sodium flux (PD) recovers only partially upon ADH rechallenge (Table II), as had been noted for water flux.

Second challenge urea flux was assessed only after 45 min between challenges, and again responds maximally to ADH (Table II).

Washout conditions affect subsequent ADH responses

Experiments in this section were designed to compare subsequent ADH responses when (1) neither mucosal medium nor serosal medium, or (2) only mucosal medium, or (3) only serosal medium was replaced. This is in contrast to the experiments described until now in which both mucosal and serosal baths were replaced repeatedly to end the first challenge and further changes with fresh solutions reproduced the original transepithelial gradient.

When neither mucosal medium nor serosal medium is replaced, the rate of ADH-stimulated osmotic water loss peaks after 15–30 min and then falls. Thus, in the continued presence of ADH osmotic water loss between 75 and 105 min is only 49% that between 0 and 30 min ($P < 0.05$) (Fig. 2). Both the decrease in rate of osmotic water loss and the plateau above basal levels after 90 min corroborate early reports [4–10].

When only mucosal medium is replaced after 30 min of ADH stimulation, the rate at which osmotic water loss falls temporarily slows (Fig. 2) as compared to bladders with neither serosal or mucosal medium replaced. In fact, water loss plateaus for about 30 min at the rate prevailing when the mucosal medium was replaced, and then resumes the rate occuring in hemibladders still in their original baths. After another 45 min replacing the mucosal medium again produces a plateau at the lower flux rate occurring at the time of replace-

TABLE II

TRANSEPITHELIAL SOLUTE FLUX

With HR/5 and HR/10, transepithelial potential differences were measured after 45 min challenges. With PR/2, the ADH challenges were only 30 min long. All values are given as mean ± S.E. for n determinations, and the Δ is calculated for paired samples (Student's t-test). [a] $P < 0.05$.

Interval between challenges (n)	ADH-stimulated flux above basal level			
	Basal	First ADH response	Second ADH response	Δ
	Transepithelial potential difference (Na^+ flux) (mV)			
45 min				
PR/2 (11)	47± 7	16± 5	19± 7	4±12
HR/5 (6)	22± 5	16± 5	30± 6	17±17
HR/10 (9)	23±10	6±10	9± 5	13± 8
36 min				
HR/5	22± 9	7± 7	6± 7	−1± 6
21 min				
HR/5 (9)	4± 2	14± 4	8± 3	−4± 2 [a]
	K_{trans} urea (cm/s) ($\times 10^7$)			
45 min				
PR/2 (11)	38±10	596±91	568±59	−27±71

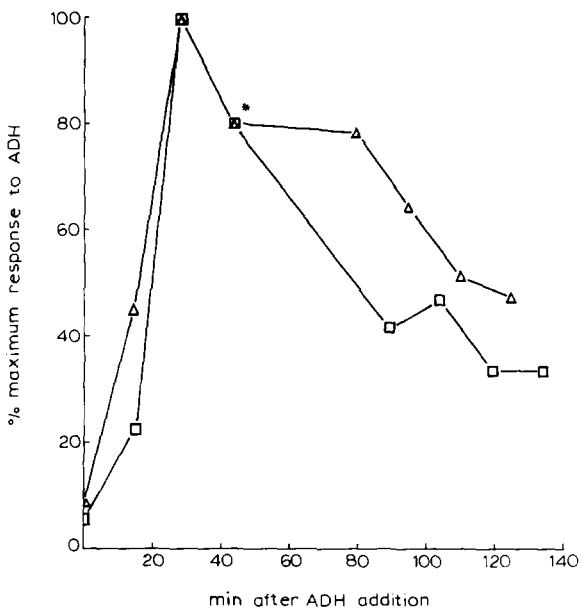

Fig. 2. Osmotic water loss during continuous incubation in ADH. Bladders were suspended in ADH-containing amphibian Ringers for two to three hours. At * mucosal medium was replaced with fresh mucosal medium (△, $n = 6$). The rate of water loss was maintained for approx. 30 min. In the other group (□, $n = 4$) mucosal medium was not replaced at all, and osmotic flux fell continuously.

ment. When both the mucosal medium and the serosal medium containing ADH were replaced every 20 min [10], maximal flow rates were maintained for several hours, providing additional evidence for the importance to restimulability of mucosal medium replacement.

If full responsiveness to ADH restimulation depended only on the receptor (serosal) side of the cell, repeated replacement of serosal medium only would duplicate the timing of washout and also would permit complete restimulability 36 min after the end of the first challenge. We find that if only the serosal bath is replaced (three times) to end the first ADH challenge, (1) the washout is enough for osmotic flux to fall to basal levels during the last third of the 45 min interval (Table III), but (2) recovery is incomplete still, only 82% of the original (0–30 min) flux when the second ADH response is initiated at 75 min by replacing the serosal medium with serosal medium containing ADH and replacing the mucosal medium for the first time ($P < 0.01$, $n = 8$). On a third rechallenge after the same washout and rechallenge pattern, ADH-stimulated osmotic water loss (150–180 min) is again 82% ($P < 0.01$, $n = 4$). Thus when both the mucosal and the serosal media are replaced 36 min is already enough for recovery, but when only the serosal medium is replaced to end the first challenge, 45 min is insufficient.

Mucosal osmolarity remains constant even after 60 min ADH-stimulated transport. However, response to subsequent ADH stimulations could be affected by altered Ringers constituents other than Na^+, or by release or secretion of material from the apical surface of the transporting cells (Ref. 31 and Rubin, M.S., et al., unpublished data *). Thus, in eight pairs of hemibladders, serosal medium replacement as usual ended the first ADH challenge but mucosal medium was replaced only in control hemibladders. In experimental hemibladders fresh Ringers of the original dilution was added to return the mucosal medium to its original volume (thus also eliminating volume effects as a variable). Osmotic and sodium fluxes in the second stimulation equalled those in the first (in the first stimulation 35.5 ± 1.6 vs. 34.2 ± 3.3 μl/min water loss in 45 min and in the second, with fresh mucosal medium 34.2 ± 3.3 and with old mucosal medium 38.2 ± 3.2).

Bath composition and osmotic flux

After a 45 min interval, equivalent osmotic water losses occur during ADH (re)stimulation in phosphate-buffered Ringers, Hepes-buffered Ringers or Tris-acetate Ringers (pH 7.2–7.8, data not shown), and appear not to require $MgCl_2$ or

* We have used exogenous proteases in the mucosal medium to discretely modify membrane constituents before and during ADH-stimulated water and urea transport. The lysosomal aspartic proteinase Cathepsin D specifically increased ADH-stimulated osmotic water flow by 25–50% ($P < 0.05$) without affecting urea transport or PD (M.S.R., C.F.K. and J.D.W., manuscript in preparation).

Also, we have added thiol reagents to the mucosal medium to modify membrane-associated or secreted sulfhydryl groups, so as to assess their importance in transport. Many of the lysosomal proteinases are thiol-dependent. However, dithiothreitol, *p*-chloromercuribenzoate, *p*-chloromercuribenzoylsulfonate and the cleavable cross-linker dithiobis(succinimidyl propionate) were all ineeffective as modifiers of osmotic flux (M.S.R., J.D.W. and C.F.K., unpublished data), although some of these agents significantly alter ADH-dependent sodium flux [38].

TABLE III

BASAL OSMOTIC FLUX RECOVERY AFTER ENDING ADH CHALLENGE

All bladders were filled with R/5 and suspended in amphibian Ringers. At the end of each basal flux measurement, 50 mU ADH was added per ml serosal bath. All values are means ± S.E. for n determinations; P values are calculated from Student's t-test for paired observations. [a] $P < 0.05$, [b] $P < 0.005$. M, mucosal medium; S, serosal medium.

Period of second measurement (min after ADH removal)	Bath replaced	Water lost (μl/min)	
		First basal period	Second basal period
35–45	S	3.5 ± 0.8 (8)	2.3 ± 0.6
10–15	M+S	0.6 ± 0.4 (8)	1.0 ± 3.6 [a]
14–21	M+S	1.0 ± 0.7 (17)	7.8 ± 1.7 [b]
21–36	M+S	1.4 ± 0.3 (6)	1.7 ± 0.3
30–45	M+S	1.7 ± 0.4 (12)	2.1 ± 0.4

glucose in the baths (compare panels of Fig. 1). The serosal medium always contains 0.5 or 1 mM calcium. Equivalent osmotic flux occurs in both first and second stimulations when mucosal Ca^+ is between 0.2 and 1 mM during transport, or when mucosal washout is performed with 'Ca^{2+}-free Ringers' (containing EGTA but no added Ca^{2+}, data not shown). Undiminished ADH restimulation of osmotic flow occurs whether the mucosal medium is replaced with amphibian Ringers or dilute amphibian Ringers to end the previous challenge (Fig. 1 and other data).

Ability of 'preincubated ADH + serosal medium' to stimulate osmotic flow

To determine whether bladders secrete an inhibitor into the serosal bath [4–7] during 45 min of ADH-stimulated transport, or whether ADH is damaged significantly during transport [13], four fresh paired hemibladders were stimulated with fresh ADH and serosal medium, or with 'preincubated ADH + serosal medium' taken from other bladders at the end of a 45 min ADH stimulation. Since both treatments evoked the same response (46.1 ± 4.2 vs. 51.1 ± 4.7 μl water loss/min for 30 min) neither damage nor inhibitor secretion into serosal medium are important during short (1–2 h) stimulations, although both have been reported during long incubations.

Recovery of basal flow after ADH challenge

As already mentioned, only replacement of serosal medium is required at the end of an ADH challenge to decrease osmotic flux to basal levels again (Table III). In these studies the serosal medium was always replaced three times during washout, and then a fourth time at the start of reequilibration. Hydroosmotic flux does not return to basal levels during measurement 10–15 min after the start of washout even though the serosal bath is replaced four times, but is at the original basal level by 21 to 36 min.

Discussion

We have described conditions in which ADH induces multiple undiminished osmotic flow responses in vitro. The phenomena called flux inhibition, refractoriness and resistance to ADH restimulation [4–14] need not occur in vitro under appropriate experimental conditions. In fact, replacement of mucosal and serosal media had already been shown to permit complete restimulability of ADH-dependent osmotic water flow [39] after 1 h washout and we now report that 35 min washout is sufficient for maximal restimulation. In vivo, normal fluid movement over both surfaces of the bladder may suffice to maintain the high level of hormone responsiveness; in vitro, we changed mucosal and serosal baths repeatedly to approximate this fluid movement.

Initiation of ADH responses at the serosal surface is regulated principally by circulating ADH levels and the number and affinity of ADH receptors [2,3,32]. In vivo, hormone secretion is pulsatile and circulating ADH has a short half-life, less than 10 min [3], whereas in vitro experimental exposure to ADH has often been for many hours.

Many hypotheses have been invoked to explain diminished sensitivity to ADH restimulation as evidenced by diminished responses. ADH might be chemically altered or bound irreversibly to receptors [13] that are then internalized, degraded and replaced by new receptors at the basolateral surface. But it seems likely that physiologically relevant ADH receptors are still available, since we find that ADH fully activates osmotic water flow again after 45 min preincubation with hemibladders, and that basal flow rates are recovered by 21–36 min after washout (Table III).

Others have described in vitro refractoriness and resistance, experimental desensitization of hydroosmotic flux to ADH restimulation, for hours to days after ADH removal [4–14]. From their experiments they suggested that desensitization followed alteration in components of the serosal surface, in the transmission of information of receptor-hormone interaction along the reaction sequence leading to altered transport, or from inhibitors secreted into the serosal medium. Even for long ADH stimulations, decreased responsiveness as a direct result of damage or degradation of ADH receptors at the basolateral surface is mitigated against by the probable existence of intracellular pools of available receptors (as is common for other (poly)peptide hormones [25–28]). In addition, toad bladder has reserves of ADH receptors in great excess over those required for maximal physiological effect [2].

Desensitization or refractoriness to repeated ADH challenge [4–14] usually involved much longer ADH stimulations and/or washout intervals without concomitant evaluation of tissue viability during these incubations (see below). Long incubations may not affect ADH-stimulated water and solute losses from mucosal medium primarily or specifically, but rather maintenance of other aspects of cell structure and function [33–35], i.e., poor utilization of energy sources, substrates and precursors for maintaining cell viability or for specific processes like membrane recycling [21–28, 33–35] and/or nonremoval of waste products that would normally be further metabolized or lost to the circulation.

We always measure transport in hemibladders in fresh mucosal and serosal baths; our conditions maintain transport responses over short times in baths of Ringers. We have confirmed that longer incubations in Ringers do not permit (re)stimulation by ADH (data not shown). But ADH-stimulated water and solute flows are maintained at the original levels for 8 h by repeatedly providing fresh mucosal and serosal baths at short intervals (King, C.F., Samuels, A.W. and Rubin, M.S., unpublished data) (see also Ref. 10) or for at least 24 h by incubation in medium more adequate for long term cell survival, Wolf and Quimby's amphibian tissue culture medium [36] with 2–5% fetal calf serum. (Eagle's minimal and Ham's F-12 culture media with 2–5% fetal calf serum were less effective.) Eggena [11] also mentions increased responsiveness after overnight incubation in a tissue culture medium. Clearly, bladders can be kept viable and responsive for long periods, but this is a different question from the minimal requirements (including time) for recovery of full responsiveness to ADH rechallenge.

Because tissues vary greatly in their responses to lower ADH doses even in an initial ADH stimulation [9,37], we cannot interprete studies in which submaximal ADH concentrations were used only for restimulation (see Ref. 13, or our unpublished data).

Flux inhibition

Flux inhibition is the hypothesis [6,8–12] that the amount of ADH-induced osmotic flux across the apical surface (or the bladder) produces parallel loss of the osmotic gradient and of essential constituent(s) of the apical surface or subapical cytoplasm. Thus a longer challenge or a steeper imposed transepithelial gradient should increase flux inhibition and decrease restimulability by ADH. We found neither dissipation of the transepithelial gradient nor flux inhibition, as tested by varying the transepithelial gradient imposed across individual bladders but stimulating each tissue repeatedly in its original gradient. These data, and those [10] showing transport rate maintenance for hours with repeated changes of the mucosal medium and the ADH-containing serosal medium at 20-min intervals suggest there may be other explanations for 'flux inhibition', and that sufficient receptors or effectors are present to provide maximal response on repeated stimulation.

Our major goal was to find in vitro conditions adequate for multiple maximal ADH responses. The second goal was to find the shortest period

which permitted full restimulation, especially important because the previously reported inability to fully restimulate in vitro had led to hypotheses of ADH action incorporating limiting concentration(s) of receptor or effector units. We find that when both receptor and effector surface baths are refreshed, the bladder has either enough stored receptor and effector units and/or conserves previously used units via retrieval and recycling to respond maximally again within 36 min.

Acknowledgements

This work was supported by the National Institute of Health of Grants AM-18128 (M.S.R.), AM-25110 (S.K.M.) and AM-10080 (IL Schwartz), and Research Career Development Awards AM-00010 (M.S.R.) and AM-00547 (S.K.M.). We thank Maria Rodriguez for secretarial assistance.

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