Water Transport in Cultured Cells from the Rat Inner Medullary Collecting Duct

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ABSTRACT
In this study, cells derived from rat inner medullary collecting duct formed a polarized monolayer when grown in wells on a membrane. When the membrane was sealed in an Ussing apparatus, the cells passed water from their apical to basolateral surfaces in the presence of an osmotic gradient. This was detected by the movement of the meniscus (1 mm = 1 μL) in a capillary tube connected to the basolateral chamber. The movements were measured by a travelling microscope, and the effects of vasopressin, oxytocin, and kappa opioids were explored at 37 ± 1°C. In the presence of vasopressin and oxytocin, water movement increased to between the threshold of 0.2 pM and the maxima of 100 pM (vasopressin) and 20 pM (oxytocin). Higher concentrations had a lesser effect (vasopressin) or no greater effect (oxytocin). It was possible to get a similar effect with each of two doses of 10 pM vasopressin at intervals as short as 3 min, but tachyphyllaxis lasting 70 min followed a dose of 20 pM oxytocin. The effects of vasopressin and oxytocin could be blocked completely by the cAMP antagonist adenosine-3',5'-cyclic monophosphorothioate Rp isomer. The benzenacetamide kappa agonist U-50488 had no effect on the response to 1 pM vasopressin but the kappa antagonist norbinaltorphimine significantly increased the effects of 1 pM vasopressin; this action was exerted earlier on in the initiation of water transport, as norbinaltorphimine did not affect the response to Sp-cAMPS, an activator of cAMP-dependent kinases.

Key Words: Vasopressin, oxytocin, collecting ducts, kappa opioids

Primary cell cultures of the rat inner medullary collecting duct (IMCD) have been established recently (1). The cells display certain features of the segment from which they were derived. For instance, IMCD cells respond to vasopressin (AVP) by a four- to fivefold increase in permeability to urea; this effect can also be brought about by 8-bromo-adenosine 3,5 cyclic monophosphate, which suggests that the V2 receptor for AVP is involved. A major physiological function of the IMCD in vivo is the absorption of water from the lumen of the collecting duct (2). Recently, this ability has been demonstrated in these cultured cells maintained at room temperature, and water transport has been shown to increase in the presence of AVP and its cyclic analogues (3,4).

Oxytocin (OT) also has an antidiuretic effect on rats in vivo (5,6) although this effect may be minor. Although the plasma level of OT in rats is about 10 times higher than that of AVP (7), and the levels of both rise when plasma osmolality rises (8) or during water deprivation (9), AVP exerts its antidiuretic effect at concentrations about 500 to 1000 times lower than that of OT. In humans, however, AVP is only about twice as potent as OT (10); in obstetric practice, in which OT is infused to induce uterine contractions, levels of OT that are high enough to cause water intoxication at the end of a long infusion are sometimes obtained (11).

Experiments in vivo suggest that OT acts as a partial agonist having a weak antidiuretic action in the presence of low levels or a lack of AVP and by being an antagonist of an AVP-induced antidiuresis (9,12,13). However, the threshold concentration for OT, the biochemical pathway for its action, and the responses to a range of doses have not been defined. The finding that ethylketocyclazocine blocked AVP-stimulated water flow in the toad urinary bladder (14) suggests that opioids may suppress AVP action in the mammalian IMCD. Specific binding of kappa opioid agonists has been demonstrated in the rat kidney by some researchers (15,16) but not others (17). Furthermore, spirodoline mesylate (U-62066) and the benzenacetamide amide U-50488, both potent kappa agonists (18,19), produce a diuresis in humans and rats with no effect on the plasma AVP concentration. The diuresis depends on a permissive action of adrenal medullary hormones (20). Close analysis of events in the kidney indicates that the diuresis is the result of an increase in GFR (21,22); however, an effect on the peripheral action of AVP was not ruled out. In contrast, Yamada et al. (23) reported that U-62,066E had
no effect on AVP-stimulated osmotically induced water flux in rat IMCD perfused in vitro.

In this article, I first report on the threshold for AVP-evoked water transport in cultured IMCD cells, the response to repeated doses of AVP, and the dose-response curve. Evidence is also provided that the AVP-induced water transport requires active cAMP. Second, I report on the determination of the threshold concentration for OT, the response to repeated doses of OT, the dose-response curve, and the investigation of whether the action of OT can be inhibited by blocking the biochemical pathway by which AVP produces an antidiuresis. Third, I report on the effects of U-50488, which is a more selective kappa agonist than U-62066 (24), and of norbinaltorphimine (BNI), a selective kappa opioid antagonist (25), on AVP-induced, osmotically driven water transport in the cultured IMCD cells.

METHODS

Cell Culture

Stocks of IMCD cells (3) from Dartmouth College Medical School, Hanover, NH, were imported into New Zealand under Permit Number 89/BIO/332 and kept in liquid nitrogen. As required, they were grown to confluence in 75-cm² vented culture flasks (Costar Corporation, Cambridge, MA) at 37°C in an atmosphere of 95% O₂ and 5% CO₂. The cells were bathed with a sterile solution consisting of Dulbecco's modified Eagle medium with L-glutamine (Gibco Life Technologies Inc. Grand Island, NY) to which 10% fetal calf serum, 1% glutamine, and a 1% antibiotic solution of penicillin and streptomycin was added. The medium was changed every 2 to 3 days.

For studies of water transport, cells were grown to confluence on 25-mm diameter "Cyclopore" membranes (0.45-μm pore size, Falcon 3090; Becton Dickinson Inc, Lincoln Park, NJ) placed in six-well, flat-bottomed culture plates (Falcon 3046, Becton Dickinson). The membranes were seeded with approximately 10⁵ cells and bathed on both sides with culture medium. Under these conditions, the cells formed a polarized monolayer, with the basolateral surface of the cells facing the medium and the apical surface attached to the membrane. Age and passage number were noted for the monolayers used in each experiment. Cells, 6 to 20 days old, from Passes 13 to 18 were used in all experiments.

Measurement of Water Flux

For experiments, the Cyclopore membranes, with their attached sheets of cells, were cut from their wells with a sharp razor blade and transferred to an Ussing chamber that had been modified by the addition of inlet and exit ports (Figure 1). A membrane was put into the chamber and held by a rubber O-ring when the two halves of the chamber were clamped together. Both apical and basolateral membranes were bathed with Ringer's solution. Its composition (in mM) was: Na⁺, 140; K⁺, 5; Ca²⁺, 1; Cl⁻, 145; creatinine, 4; N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer was added to a final concentration of 10 mM. Except when noted otherwise, the basolateral solution contained an additional 150 mM NaCl to provide an osmotic gradient of about 300 mosmol·kg⁻¹ H₂O across the monolayer. The solutions were titrated to pH 7.4 with NaOH. The osmolality of both solutions was determined by vapor-pressure osmometry (Wescor, Logan, UT).

Basolateral and apical perfusing solutions were held in syringe barrels (Figure 1). Stopcocks controlled fluid flow down a small hydrostatic gradient from the barrels to the preparation. During perfusion, with the stopcocks open, the levels of the perfusing fluids were maintained at approximately equal heights in the barrels; during recording with the stopcocks closed, the fluid level of the apical perfusion system was brought down to that of the apical entry into the recording chamber.

Water flow across a monolayer was measured by determining the displacement of fluid in a glass capillary tube connected to the fluid bathing the basolateral surface. Fluid flow...
movement was observed through the eyepiece of a travelling microscope fitted with a graticule and a vernier with 20-μm graduations. In the capillary tube, 1 mm of movement corresponded to the addition of 1 μL of fluid. Bathing solutions were preheated in a waterbath and the chamber was temperature-controlled by heaters applied to each side of the chamber. The chamber temperature was monitored by a thermistor, the sensor of which was embedded in the wall of the apical chamber, and experiments were conducted at 37°C ± 1.

Conduction of Experiments

Changes of fluid (each 5 mL) took 3 to 4 min. The position of the meniscus in the capillary tube was usually recorded after 1, 2, 4, 6, 8, and 10 min. After the last recording, the solutions were replaced with the basic solution for the apical bath and the hyperosmotic or test solution for the basolateral bath. In separate experiments (Hubbard, unpublished), I determined that 99% of the bath fluid was exchanged in 3 min.

Drugs

AVP and OT were obtained from Sigma, St. Louis, MO. These substances were developed in the hyperosmotic solution as required, by dilution from a stock of 10−4 M kept under refrigeration. Test solutions were applied to the basolateral surface of each preparation. Tests for responses to doses of AVP or OT were done at least 1 h apart unless closer intervals were required. The cAMP antagonist adenosine-3',5'-cyclic monophosphorothioate, Rp isomer (Rp-cAMPS), and the cAMP agonist adenosine-3',5'-cyclic monophosphorothioate, Sp isomer (Sp-cAMPS), described by Rothermel and Botelho (26), were obtained from Biobog Life Science Institute, Bremen, Germany. U-50488 and BNI were obtained from Research Biochemcabs, Natick, MA.

Calculation of Results

Results are expressed as osmotic water permeability (P1, in μm·s−1). P1 was calculated using the formula of Knepper et al. (27), which relates water flux to surface area and osmotic gradient:

\[
P_1 = \frac{JV(A \times Vw \times Osm)}{m s^{-1}}
\]

where \( Jv \) = volume of water (mm³·min⁻¹), \( A \) = area of monolayer (cm²), \( Vw \) = molar volume of water (55.5 L mol⁻¹), \( Osm \) = osmotic gradient × osmotic coefficient (0.15 M × 1.82).

Data were analyzed using an analysis of variance (ANOVA) after appropriate transformation of the data (arc sine for ratios; 28) or by nonparametric tests if the data could not be normalized. \( P \) was set at 0.05; tests were two-tailed.

RESULTS

Response to an Osmotic Gradient

At the beginning of every experiment, water transport under the influence of an osmotic gradient was measured over 10 min. When the data were converted to P1 (which corrects for slightly different osmotic gradients in different experiments), the responses from monolayers of Passes 13 to 18 were not significantly different (\( F = 1.87, P = 0.11 \)). The mean permeability was 2.72 ± 0.25 μm·s⁻¹, \( N = 80 \). In all experiments, monolayers from at least two different passes were used.

Response to AVP

The criterion selected for a response to AVP was water transport over 10 min that was greater than 120% of the average of the water transport evoked by the preceding and following osmotic gradients, each over 10 min. Doses of 0.1 pM met this criterion only once in 10 trials. Doubling the dose of AVP to 0.2 pM increased the response rate to 6 of 12 trials. The threshold concentration of AVP, therefore, was 0.2 pM.

In all experiments, the response to AVP was largest when initially measured, and then declined, at first rapidly and then more slowly. Responses to OT and to an osmotic gradient had the same form. Figure 2 shows successive responses to an osmotic gradient before and after the lowest effective concentration of AVP (0.2 pM) was applied, and the successive responses after 10 pM was applied 1 h later.

When testing the effect of repeated doses, experiments were carried out at a concentration of 10 pM. It was possible to repeat this dose 3 min after measuring the response to a first dose and obtain a response of similar magnitude. This was true at all intervals up to and including 80 min, except that the response to 10-pM doses given 15 min after the first dose tended to be larger. A regression of response ratios on time (Figure 3A), after conversion of response ratios to their arcsines, showed that the slope of the regression was not significantly different from zero (\( F = 0.1196, P = 0.7533, N = 21 \)).

A dose-response curve for AVP (Figure 3B) was constructed by examining at least ten responses to seven concentrations (0.1, 0.2, 1, 10, 100, 1000, and 10,000 pM). There were two interesting aspects of the curve. First, over the physiological range (0.1 to 10 pM), there was a progressive increase in the mean response. Second, at higher concentrations, the response fell off markedly, becoming undetectable at the highest dose. Because there were significant differences between the variances of the different means, a Kruskal-Wallis nonparametric test was used. This test showed significant differences between the medians of the responses to the different doses (Kruskal-Wallis statistic = 58.24; \( P < 0.0001 \)). Dunn’s multiple comparison test showed that there were significant differences (\( P < 0.05 \)) between the mean response to the subthreshold (0.1 pM) doses and the mean responses to doses of 1 pM or more, except that there was no significant difference between the response to the subthreshold dose and the response to 10,000 pM. The mean responses to the 1000- and 10,000-pM concentrations were less than the mean responses to concentrations of 1, 10, and 100 pM, and the difference was significant (\( P < 0.01 \)) for the response to 10,000 pM.
Dependence of AVP on cAMP

The effects of AVP on water transport are dependent on cAMP. Figure 4A shows the mean response of nine preparations to 1 pM AVP contrasted with the mean response to an osmotic gradient (OG) before and afterwards. An ANOVA showed a significant difference between the means ($F = 15.88$, $P = 0.0002$) and the response to AVP was significantly greater than the initial and final responses to an osmotic gradient (Dunnett test, $P < 0.01$). Figure 4B shows the lack of effect of AVP in the same nine preparations when AVP was applied in the presence of 10 nM Rp-cAMPS, a potent antagonist of cAMP-dependent protein kinases I and II ($F = 0.178$, $P = 0.839$). The effect of Rp-cAMPS can be reversed by washing and, as Figure 5C shows, AVP was again effective in increasing water flow when compared with the initial effect of the osmotic gradient ($F = 4.82$, $P = 0.03$, $N = 6$; Dunnett test, $P < 0.05$).

Response to OT

The threshold concentration of OT was assessed using the same criterion as for AVP, i.e., 10-min water transport after OT administration greater than 120% of the average of the 10-min water transport evoked by the preceding and following osmotic gradients. Responses to doses of 0.1 pM met this criterion once in 10 trials, responses to doses of 0.2 pM met the criterion in 5 of 10 trials, and responses to doses of 0.5 pM met the criteria in 9 of 10 trials. The threshold concentration was therefore 0.2 pM.

The effect of repeated doses of OT was assessed using a dose of 20 pM. Tachyphylaxis lasting about 70 min was produced by the initial dose and, as Figure 5A shows, when a regression of the ratio of the test response to the control response on time was carried out, after arcsine transformation, it was significant ($N = 20$, $r^2 = 0.56$, $P = 0.0002$).

A dose-response curve for OT (Figure 5B) was constructed, with eight concentrations—0.1, 0.2, 0.5, 2, 20, 200, 2000, and 20,000 pM. There was a progressive increase in mean Pf as the concentration increased from 0.1 to 20 pM, but higher doses were not more effective. There were significant differences between the variances of the different means, so a Kruskal-Wallis nonparametric test was applied to the data. This showed significant differences between the medians of the responses to the different doses (Kruskal-Wallis statistic = 32.446; $P < 0.0001$). Dunn's multiple comparison test showed there were significant differences ($P < 0.05$) between the mean response to the subthreshold concentration (0.1 pM) and the mean response to concentrations of 20 pM or more. The mean responses to these higher concentrations were not significantly different from each other, indicating that the maximum response was attained at 20 pM.

Figure 6 indicates that, like AVP, the effects of OT on water transport are dependent on cAMP. Figure 6A shows the mean response of seven preparations to 20 pM OT contrasted with the mean response to an OG
before and afterwards. The response to OT was significantly greater than the initial response to an osmotic gradient ($F = 4.0861$, $P = 0.043$; Dunnett test, critical value, $q = 2.81$, $P < 0.05$). Figure 6B shows the lack of effect of OT in the same seven preparations when OT was applied in the presence of 20 nM of Rp-cAMPS ($F = 0.2842$, $P = 0.7575$). The effect of Rp-cAMPS can be reversed by washing, and as Figure 6C shows, OT was then again effective in increasing water flow ($F = 7.5452$, $P = 0.01$; Dunnett test, $q = 3.8373$, $P < 0.01$).
Effects of Kappa Opioids

Figure 7A shows the mean effects of 1 pM AVP in eight preparations before, in the presence of 0.01 mM of the kappa agonist U-50488, and again 1 h later. There was no significant difference between the responses, indicating no effect of U-50488 ($F = 2.09, P = 0.17, N = 8$). When the procedure was repeated with the kappa antagonist BNI (1 nM) (Figure 7B), there was a significant difference between the responses to 1 pM AVP before, during, and after the introduction of BNI ($F = 3.583, P = 0.0434, N = 13$). Tests performed after the procedure showed that the response in the presence of BNI was significantly larger than the response before the introduction of drug (Dunnett test, $q = 2.503, P < 0.05$). The mean response to AVP was still larger 1 h later, but this effect was more variable and was not significant.

Neither U-50488 (0.1 mM) nor BNI (1 nM) modified water transport in the absence of AVP in six experiments. In these experiments, $P$, over 10 min in the presence of the test drug was compared with the mean
The effect of BNI must be exerted on earlier steps in the AVP-induced initiation of water transport.

**DISCUSSION**

Three problems are discussed in this section: the form of the response to AVP, OT, and an OG, the effects of the hormones themselves, and the effects of kappa opioids.

In regard to the time course of the response, an example of which is shown in Figure 2, the effects of AVP, OT, or an OG are maximal at the time measurements are initiated, and decline thereafter. This time course is probably a result of the stopflow method of measurement; Hall and Grantham (29), using a similar technique on isolated rabbit collecting ducts at 37°C, found that when AVP was applied, there was a rapid rise of Pf to a peak and then a monotonic decline. In my experiments, the initial rise is presumably lost, as AVP is present for 3 to 4 min before measurements begin. In contrast, if collecting ducts are perfused continuously and exposed to AVP, the Pf increases steeply over 10 min after a lag period, and more slowly over the next 40 min (30).

If Pf in the presence of AVP is measured in toad bladder by using stopflow methods, the response shows—albeit with a much slower time course—the same rise to a peak and then a decline (31), as is found in mammalian experiments using similar techniques. The response to AVP could be regenerated in these inhibited preparations if the serosal surface was bathed with fresh solution containing fresh AVP. It has been suggested (31) that soluble inhibitors of AVP accumulate, causing the observed decline in the response to AVP. I obtained evidence indicating a similar explanation for my results. As Figure 3A shows, a response to AVP could be obtained at short intervals,
provided that fresh solution with fresh AVP was added to the basolateral (serosal) surface. It has been suggested that the inhibitory products may be adenosine and prostaglandins (30).

The responses to both AVP and OT (Figures 2, 3B, and 5B) are also small compared with that obtained in isolated tubules (30,32,33). A similar small response is found when Madin-Darby canine kidney cells are exposed to AVP (34). Such small responses may have several causes, which may be the result of inhibition, as mentioned earlier. Figure 2 indicates that the response is greatest when first recorded and, if inhibition is present, this initial response may be the best marker of the true size of the response. The first minute of recording in ten experiments in which the preparation was exposed to 100 pM of AVP gave a mean P of 40.2 ± 5.3 μm·s⁻¹. This time corresponds to 4 to 5 min after exposure of the preparation to AVP. The time course of the P response to the same concentration of AVP in perfused tubules (30; Figure 3) indicates that at 5 min, 40% of the response to AVP has been achieved, corresponding to 40% of 618.3 ± 184 μm·s⁻¹ or 247.32 μm·s⁻¹, which is much larger than my result. Inhibition cannot, therefore, fully explain the smaller size of the response in monolayers.

Although the P changes in response to AVP are small, the baseline P is also very small, so that a response to AVP is easily detected. In the tubules, baseline P was 78.8 ± 28.8 μm·s⁻¹ (30), so that the AVP effect at 5 min was 247/78, or 3.14 times the baseline, whereas in my preparation the ratio was 40.2/2.72, or 14.78. Monolayers are, therefore, more sensitive preparations than tubules.

Studies on a sensitive measuring system are interesting in that they permit physiological studies that are close to resting levels of AVP and OT in rat plasma and close to the limits of current RIA and bioassays (0.2 pM) to be made (35). Indeed, investigators interested in the plasma levels of AVP and OT often work with rats that have been made hyperosmotic to increase the plasma levels of the peptides (e.g., 36). The threshold that I found in this investigation is approximately 0.2 pM, which agrees well with the previously reported threshold for an effect of AVP on water permeability in rat collecting ducts (between 0.1 to 1.0 pM) (30,37). Baerwolf and Bie (38), in studies of the effects of subciclonal changes in AVP on urinary concentration in dogs, calculated that their lowest effective concentration produced an increase in plasma AVP of between 0.05 and 0.13 pM.

The form of the dose-response curve for AVP (Figure 3B) was quite conventional for responses to doses of 100 pM or less. At greater concentrations, the response became progressively smaller, so that at 10,000 pM, there was no significant response. Reports that pharmacological doses of AVP have less effect than physiological doses have been made by other authors (97,38). One possible explanation could be that collecting ducts have V₁ₐ receptors for AVP that are activated at high AVP concentrations (39). Activation of these receptors inhibits the hydro-osmotic action of the V₂ receptors; thus, blocking them would restore response to higher AVP concentrations. If OT had no effect on such postulated V₁α receptors, this would account for the lack of falloff in the dose-response curve at high OT concentrations (Figure 5B). However, a recent study by Firsov et al. (40) found no evidence for expression of a V₁α receptor in the IMCD of rat nephrons.

Comparison of AVP and OT

The two hormones had a similar threshold for evoking a response, and over the physiological range (compare Figures 3B and 5B), there was no significant difference between the effects of AVP and OT present at twice the concentration. This would indicate that, in this preparation, AVP is twice as effective as OT. The results with OT are fully compatible, with its action occurring solely by activation of the V₂ receptors for AVP. The finding that OT action can be completely blocked by Rp-cAMPS (Figure 6), which also blocks the response to AVP (Figure 4), and that OT and AVP both cause cAMP accumulation in IMCD cells (41) indicate that the normal pathway for V₂ action is activated by OT. It is noteworthy that the pathway can be activated repeatedly by AVP (Figure 3A) but is refractory for 60 to 80 min after OT activation (Figure 5A). These results support a physiological role for OT in antidiuresis when the AVP concentration is low.

Opioid Activity in IMCD Cells

The drug that excited interest in possible kappa opioid activity in the kidney, ethylketocyclazocine, has been found to have almost equally high affinities for mu, delta, and kappa receptors (24). It was, however, the kappa receptors that were responsible for the diuresis observed in experimental animals. The results shown in Figure 7A, together with the results of Yamada et al. (23), might suggest that there is no kappa opioid interference with the action of AVP in the collecting duct. However, there was a long-lasting stimulant effect of the kappa antagonist BNI (Figure 7B), which was exerted on the events that preceded the action of c-AMP (Figure 8). This is an interesting result because BNI is a much more potent antagonist than U-50488 is an agonist: U-50488 was used at a concentration of 10,000 nM, and BNI was effective at 1 nM.

These experiments establish that IMCD cells in culture respond to physiological doses of AVP and OT by increased osmotic permeability to water, and that the response can be studied quantitatively by using a direct volume measurement. The ease with which this system can be prepared and operated suggests that it is a useful technique for investigating the fluid transport properties of the IMCD.
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Editor's Note: John Hubbard died unexpectedly while this paper was under primary review. The revision was prepared by Dr. Carolyn Burns and Dr. John Leader.

JOHN INGRAM HUBBARD (1930-1995)

John Hubbard. Professor of Neurophysiology in the Department of Physiology, University of Otago Medical School, died peacefully in his sleep on the 1st of October, 1993, a few weeks before his retirement. Born and educated in New Zealand, he first became a student of physiology during his medical studies at Otago. After completing his medical qualifications at Oxford University, he moved to the John Curtin School of Medical Research at the Australian National University in Canberra, where he studied for a PhD to 1970. He returned to Dunedin in 1972 as the first Professor of Biological Sciences and Engineering Sciences at North Dunedin, New Zealand.

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