Special Communication

A rat kidney tubule suspension for the study of vasopressin-induced shuttling of AQP2 water channels

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Received 3 June 2002; accepted in final form 11 July 2002

Shaw, Stephen, and David Marples. A rat kidney tubule suspension for the study of vasopressin-induced shuttling of AQP2 water channels. Am J Physiol Renal Physiol 283: F1160–F1166, 2002. First published July 16, 2002; 10.1152/ajprenal.00207.2002.—AVP increases the osmotic water permeability of renal collecting ducts by inducing the translocation of specific aquaporin-2 (AQP2) water channels from cytoplasmic vesicles to the apical plasma membrane of the principal cells. Here, we report a novel inner medullary tubule suspension for the study of this phenomenon that overcomes some of the drawbacks faced by present techniques; both primary cultures of inner medullary collecting duct cells and cell lines expressing AQP2 show aberrant trafficking and/or signaling pathways. The tubule suspensions were prepared by proteolytic digestion of inner medullas dissected from freshly isolated rat kidneys. After drug treatment, cellular distribution of AQP2 was determined by membrane fractionation and Western blotting or by immunocytochemistry. Treatment of suspensions with 1 nM AVP caused redistribution of AQP2 to the apical plasma membrane of the principal cells, a process inhibited by microtubule disruption or PKA inhibition. We conclude that this method provides a valuable new approach to the study of the cellular mechanisms involved in the response of the collecting duct to AVP.

The peptide hormone 8-avp increases the osmotic water permeability of the collecting duct epithelium by causing the transfer of specific aquaporin-2 (AQP2) water channels from intracellular vesicles to the apical plasma membrane of the principal cells (18). This process has been shown to be dependent on an intact cytoskeleton and PKA (24, 27). However, many aspects of the intracellular mechanisms involved in AQP2 shuttling remain to be elucidated. It is difficult to study such processes in vivo, and present in vitro systems all have drawbacks, as discussed below. The purpose of the present study was to develop a suitable experimental model, using native tissue, in which the intracellular mechanisms of AVP-induced AQP2 trafficking can be studied.

There are several established techniques presently used to study the effects of AVP on water permeability in sensitive epithelia. Many early studies used anuran bladder or skin (2), which provide a functional analog of the mammalian collecting duct. The development of the use of isolated perfused tubules (7, 11) allowed the study of the response in mammalian systems, but this method is extremely technically demanding and takes a long time to produce results. Furthermore, although it is possible to fix such tubules after perfusion to study AQP2 distribution directly (18), this is yet more demanding and time-consuming.

Another obvious approach is to use cell culture models. Several groups have used cell lines stably expressing AQP2 or primary cultures of inner medullary collecting duct cells to try to elucidate the cellular processes involved in AQP2 shuttling. However, although excellent tools, they may not depict what is happening in a native epithelium. Primary cultures of inner medullary collecting duct cells rapidly downregulate expression of AQP2 unless grown in medium supplemented with dibutyryl cAMP, and they shuttle AQP2 to both basolateral and apical membranes (7, 15), whereas in the kidney, AQP2 shuttles almost exclusively to the apical membrane of the principal cells. LLC-PK1 porcine kidney cells were the first to be transfected with AQP2; they were stably transfected with cDNA encoding AQP2 tagged with a COOH-terminal c-Myc epitope (14). However, similar to the primary cultures, these cells show basolateral membrane localization of AQP2 in response to AVP treatment, possibly due to the c-Myc-tagged epitope. CD8 and Madin-Darby canine kidney cell lines have also been stably transfected with AQP2 and have been shown to shuttle...

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AQP2 to the apical plasma membrane in response to AVP stimulation (8, 28). However, the CD8 cells have been shown to shuttle AQP2 in the absence of AQP2 phosphorylation (29). Phosphorylation of AQP2 at Ser256 has been demonstrated to be of vital importance in its trafficking in both LLC-PK1 and intact rat renal tissue (10, 20), raising suspicions about the signaling and/or targeting pathways in these cells. Madison and Darby canine kidney cells stably expressing AQP2 show constitutive shuttling of AQP2 to the plasma membrane unless an inhibitor of prostaglandin synthesis is present (8), again suggesting abnormal signal transduction pathways.

To overcome the drawbacks presented by these methods, we have developed an isolated kidney tubule preparation that retains its epithelial polarity and AQP2 expression and behaves more like native tissue. We have demonstrated, using membrane fractionation and Western blotting, that AQP2 is shuttled to the plasma membrane after AVP stimulation and, using immunocytochemistry, that it is targeted to the apical plasma membrane. Using a microtubule-disrupting agent, [methyl-5-($\beta$-thienylcarbonyl)-1 H-benzimidazole-2-yl] carbamate; nocodazole], and an inhibitor of PKA, [N-$\beta$-bromo-cinnamylamino) ethyl]-5-isoquinolinesulfonamide, 2HCl H-89, we show that the shuttling of AQP2 in these isolated tubules displays the classic properties previously described. We conclude that this technique should prove to be a powerful tool with which to study the intracellular mechanisms involved in AQP2 shuttling.

**METHODS**

Reagents and antibodies. Standard laboratory reagents were purchased from either Sigma (Poole, Dorset, UK) or BDH (Poole) and were of analytic grade. For immunoblotting against AQP2, a polyclonal primary antibody (diluted 1:5,000; LL127) was used, which has been previously characterized (9). For immunocytochemistry, an affinity-purified polyclonal anti-AQP2 antibody (diluted 1:100; LL358AP) was used; this antibody has also been previously characterized (23). The immunoblotting against Na$^+$-K$^+$-ATPase was performed using a monoclonal antibody to the α$_i$-subunit (diluted 1:5,000; C62.4) previously characterized by Kashgarian et al. (13). For immunoblotting against AQP1, a polyclonal antibody was raised against the COOH-terminal 15 amino acids of rat AQP1 (diluted 1:5,000; AQP1a). Immunoblots revealed a 28-kDa band and a broad 35- to 50-kDa band corresponding to nonglycosylated and glycosylated AQP1, as previously described (25). Labeling was ablated by preadsorption with the immunizing peptide and was not seen with preimmune serum (data not shown).

Animals. Experiments were performed in male Wistar rats weighing 200–250 g (Central Biomedical Services, University of Leeds). Before experimentation, the rats were maintained on a standard rodent diet with free access to water.

Tubule isolation. Rats were terminally anesthetized with pentobarbital sodium (240 mg/kg ip) and killed by cervical dislocation. The kidneys were rapidly removed and split coronally, and the inner medulla was dissected out. The inner medulla was finely minced with a razor blade and suspended in 3–4 ml of isolation buffer ([mM] 140 K-gluconate, 10 NaCl, 1 MgCl$_2$, 2 CaCl$_2$, 10 KOH-HEPES, and 41 sucrose, as well as (in mg/ml) 0.01 DNase I, 1 collagenase, 1 hyaluronidase, and 0.1 pronase, pH 7.4, 340 mosm/kgH$_2$O, prewarmed to 37°C and aerated with 100% oxygen). A K-glucuronate solution was chosen to reduce the metabolic activity of the cells during isolation (1, 30). The suspension was then distributed between two 10-ml conical flasks (1.5–2 ml of tissue suspension/flask) and incubated for 60 min at 37°C in a shaking water bath while being top-gassed with 100% oxygen. Every 15 min, the suspension was gently trituated with a large-bore plastic Pasteur pipette to help tubule fragments to break off from the tissue pieces. After 60 min, the flasks were agitated gently, and the tubule suspension was drawn off into 1.5-ml microcentrifuge tubes. The suspension was pelleted at 800 g for 30 s and resuspended in 1 ml of Leibovitz L-15 culture medium containing 10 mM HEPES (buffered with NaOH to pH 7.4) and supplemented with 1% BSA. This centrifugation and resuspension step was repeated twice more to wash the tubules of remaining proteases.

Drug treatment. After resuspension, the tubules were divided into six aliquots, each was put in a 10-ml conical flask, and Leibovitz culture medium was added to a final volume of 1.5 ml in each flask. The following drugs were added to the six aliquots: control, 1 mM AVP, 33 μM nocodazole, 1 μM H-89, nocodazole+AVP, and H-89+AVP. Both nocodazole and H-89 were dissolved in DMSO; the amount of DMSO added was 0.1% of the total experimental solution. This amount of DMSO was also added to control and AVP-treated flasks. The experimental procedure involved a 30-min period in which tubules were pretreated with nocodazole or H-89 or with DMSO alone as a control, followed by AVP treatments, with the inhibitor still present, for 20 min. During this time, the tubules were incubated at 37°C, top-gassed with 100% oxygen.

Membrane fractionation. Membrane fractions were prepared using a modification of a previously described method (16). Briefly, after drug treatment, the tubules were pelleted at 800 g for 30 s and resuspended in 1 ml of ice-cold dissection buffer ([mM] 300 sucrose, 25 imidazole, and 1 EDTA, pH 7.2) prewarmed to 37°C and aerated with 100% oxygen. Every 15 min, the suspension was gently trituated, and the supernatant was transferred to a new microcentrifuge tube and centrifuged at 17,000 g for 30 min at 4°C. The pellets were then homogenized using a Polytron homogenizer (Kinematica), with a 10-s burst at setting 4, and centrifuged at 4,000 g for 15 min at 4°C in a refrigerated microcentrifuge (5417R, Eppendorf) to remove nuclei, mitochondria, and any remaining large cellular fragments. The pellet was discarded and the supernatant was transferred to new tubes and centrifuged at 17,000 g for 30 min at 4°C. The resulting pellet was dissolved in 100 μl of Laemmli sample buffer containing 2.5% sodium SDS. This fraction contains mostly plasma membranes (PM fraction). Six hundred microliters of supernatant were transferred to a new microcentrifuge tube and dissolved in 200 μl of 4× Laemmli sample buffer. The supernatant contained intracellular vesicles together with soluble proteins (ICV fraction). The samples were then heated at 85°C for 5 min and stored at 4°C.

Validation of the membrane fractionation protocol. The membrane fractionation protocol was validated in a similar manner to that of Marples et al. (16). Briefly, PM and ICV fractions were prepared from kidney inner medullary homogenates, using the method described above but with a range of secondary centrifugation speeds (15,000–21,000 g). The samples were immunoblotted for AQP1 or Na$^+$-K$^+$-ATPase. AQP1 is constitutively present in the cells of the descending thin limbs of the loop of Henle and in descending vasa recta (19), in which it is present almost exclusively in the plasma membrane, whereas Na$^+$-K$^+$-ATPase is present in the basolateral membranes of the principal cells of the collecting duct.
(13). The optimal centrifugation speed was considered to be the slowest at which there is maximal yield of AQP1 and Na\(^{+}\)-K\(^{+}\)-ATPase in the PM fraction.

**Electrophoresis and immunoblotting.** To determine AQP2 distribution, the PM and ICV fractions from each treatment group were run together in duplicate on either 9% (Na\(^{+}\)-K\(^{+}\)-ATPase samples) or 12% (AQP1 and AQP2 samples) SDS-polyacrylamide minigels on a Bio-Rad Mini Protean II system. On one gel, 10 \(\mu\)l each of PM and ICV fraction samples from each treatment group were loaded into consecutive lanes. This gel was silver-stained to allow quantification of protein loading. On the second gel, the PM fraction samples were diluted 1:5 with 1	imes Laemmli sample buffer before loading, whereas ICV samples were loaded at full strength. This arrangement results in approximately equal labeling of the two bands for AQP2, maximizing the accuracy of the estimation of the PM/ICV fraction ratio. This gel was used for immunoblotting; proteins were transferred to nitrocellulose paper by electroelution for 1 h at 100 V using a Bio-Rad Mini Protean II transblot apparatus. The blots were blocked for 1 h with 5\% skimmed milk in PBS-T (in mM) 80 Na\(_2\)HPO\(_4\), 20 NaH\(_2\)PO\(_4\), 100 NaCl, and 0.1\% Tween 20, pH 7.5) and then were washed three times in PBS-T. The blots were then incubated overnight at 4\(^\circ\)C with the appropriate antibody in PBS-T, 0.1\% BSA, and 2 mM azide. After three washes in PBS-T, the blots were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Pierce). AQP2 labeling within tubules was analyzed by immunocytochemistry. After the experimental period, the tubule suspensions were drawn off into 1.5-ml microcentrifuge tubes, pelleted at 800 g for 30 s, resuspended in 1 ml of PBS (in mM) 140 NaCl, 5.5 Na\(_2\)HPO\(_4\), and 1 NaH\(_2\)PO\(_4\), pH 7.4) containing 4\% paraformaldehyde, and fixed on ice for 30 min. The suspension was then pelleted as before and washed twice in PBS before finally being resuspended in 150 \(\mu\)l of PBS. Approximately 20 \(\mu\)l of the suspension were put on an electrostatically charged microscope slide (BDH) and allowed to settle for 5 min before immunocytochemical labeling was performed as follows. Tubules first underwent three 10-min washes in PBS containing 50 mM NH\(_4\)Cl and then were dehydrated and rehydrated in a series of graded alcohol solutions: 30\%>50\%>75\%>90\%>100\% (5 min in each). The tissue was then permeabilized by incubation in PBS containing 0.1\% Triton X-100 for 30 min. After three 10-min washes in PBS containing 1\% BSA, 0.05\% saponin, and 0.2\% gelatin (PBS-ASG), AQP2 was labeled by overnight incubation at 4\(^\circ\)C with the affinity-purified polyclonal anti-AQP2 antibody in PBS-T containing 0.1\% BSA and 2 mM sodium azide in a humid chamber. After three washes in PBS-ASG, the tubules were labeled with an FITC-conjugated mouse anti-rabbit antibody (P205, DAKO) diluted 1:40 in PBS containing 1\% BSA. The tubules underwent a further three washes in PBS-ASG before being mounted in Citifluor (Agar Scientific). AQP2 labeling within tubules was analyzed by laser scanning confocal microscopy (Leica TCS-SP).

**Data analysis.** Enhanced chemiluminescence films and silver-stained gels were scanned using an HP ScanJet 6200C and analyzed using Easygel software (16). The band densities on the immunoblots were corrected for protein loading by being normalized to the corresponding band density on the silver-stained gel. After densitometric analysis of the immunoblots, the ratio between the densities of the PM and ICV fraction bands were calculated. This gives an indication of the cellular distribution of AQP2 after drug treatments; a high PM/ICV fraction ratio indicates that most of the AQP2 was located in the plasma membrane. Experimental results are expressed as a percentage of the untreated control value, so values >100\% indicate a relative shift of AQP2 to the plasma membrane. Typically AVP stimulation increased the PM/IVC fraction ratio to 200–250\% of the control value. Experiments in which AVP stimulation alone failed to produce an increase of >175\% were disregarded; 30\% of experiments in this study were discarded in this way, although in subsequent projects the percentage of successful experiments has increased to \(\approx\)90\%. In the four experiments excluded under this criterion, the PM/ICV fraction ratio of the AVP-treated tubules was 101 ± 6\% of control (range 85–113\% of control), so there was a clear difference between experiments in which there was a response and those in which there was not. It is therefore appropriate to exclude experiments in which no response is seen. The relatively high cut-off threshold of 175\% was chosen to exclude experiments in which tubule viability was questionable, to ensure that any results included were reliable. Statistical comparisons were made using Student’s unpaired t-test. \(P\) values <0.05 were considered significant.

**RESULTS**

**Validation of the membrane fractionation technique: localization of AQP1 and Na\(^{+}\)-K\(^{+}\)-ATPase in PM and ICV fractions.** AQP1 is a water channel constitutively present in the apical and basolateral membranes of the cells of the descending thin limb and descending vasa recta in the inner medulla (19), whereas very little is found in intracellular membranes. It therefore provides a marker protein that can be used to test for separation of plasma membranes and intracellular vesicles. From previous studies (16), we know that all the plasma membrane should pellet at \(\approx\)17,000 \(g\), leaving the vesicles along with soluble proteins in the supernatant. To determine the centrifugal force at which there is optimum separation of plasma membranes from the intracellular vesicles, whole inner medulla homogenates were prepared and spun at 4,000 \(g\) to remove nuclei, mitochondria, and any remaining large pieces of cell debris. The resulting supernatants were then subjected to a further centrifugation step of 15,000, 17,000, 19,000, or 21,000 \(g\). The resultant PM and ICV fraction samples were used for immunoblotting for AQP1 or Na\(^{+}\)-K\(^{+}\)-ATPase. Figure 1A shows a representative immunoblot labeled for AQP1. Even at 15,000 \(g\), most of the AQP1 can be seen in the PM fraction, but there was a small increase in separation at 17,000 \(g\), with no further improvement at higher speeds, in agreement with previous findings (16). This conclusion is supported by similar experiments to determine the distribution of Na\(^{+}\)-K\(^{+}\)-ATPase (Fig. 1B), the majority of which is found in the basolateral plasma membrane of the collecting duct principal cells. Similar results were obtained when the experiment was repeated twice more.

**Effect of AVP on cellular AQP2 distribution.** It has previously been demonstrated that AVP causes the redistribution of AQP2 from intracellular vesicles to the apical plasma membrane in isolated perfused tubules (18). To determine whether AQP2 is shuttled to
the membrane in this system, tubules were incubated for 20 min in the presence of 1 nM AVP. Figure 2, A and B, shows that AVP produced a significant increase in the PM/ICV fraction ratio to 247 ± 22% compared with the untreated control value (n = 9, P < 0.01), indicating a relative shift of AQP2 to the plasma membrane. To visualize the distribution of AQP2 after AVP stimulation, fixed tubules were immunolabeled for AQP2. Figure 3B shows that AQP2 is redistributed from an intracellular location to the apical plasma membrane in response to AVP.

Effects of microtubule disruption on AVP-induced shuttling of AQP2. Previously, it has been demonstrated that microtubule disruption prevented the AVP-induced increase in osmotic water permeability in isolated perfused tubules (24) and AVP-induced re-localization of AQP2 to the apical plasma membrane in neonatal rat kidneys (26). To test for the involvement of microtubules in AQP2 shuttling in this tubule preparation, the suspensions were preincubated with the microtubule disrupting agent nocodazole (33 μM) for 30 min before stimulation with AVP. Figure 2, A and B, shows that nocodazole pretreatment completely inhibited the AVP-induced increase in the PM/ICV fraction ratio compared with control (nocodazole+AVP, 103 ± 15% of control, n = 5, P < 0.01), indicating the involvement of microtubules in AQP2 shuttling. When examined immunocytochemically, (Fig. 3C) it can be observed that microtubule disruption causes the diffuse distribution of AQP2 throughout the principal cells and prevents the expected apical redistribution produced by AVP.

Effects of PKA inhibition on AVP-induced shuttling of AQP2. Snyder et al. (27) first demonstrated the involvement of PKA in the AVP-induced increase in osmotic water permeability of the renal collecting duct, although its role had been inferred many years earlier from the identification of cAMP as the second messenger in this system (21). Since then, it has been demonstrated that the AQP2 protein itself is phosphorylated by PKA at Ser256 and that this process appears to be essential for AQP2 trafficking in LLC-PK1 cells (10). An inhibitor of PKA (H-89) has been used in cell culture models (3, 29) and isolated perfused tubules (27) to demonstrate the involvement of PKA in AVP-induced AQP2 trafficking and increased water permeability, respectively. Figures 2 and 3 demonstrate that pretreatment of tubules for 30 min with H-89 (1 μM) inhibited the ability of AVP to increase the PM/ICV fraction ratio (H-89+AVP, 125 ± 14% of control, n = 5, P < 0.01 with respect to AVP alone). Immunolabeling of tubules showed that H-89 pretreatment prevented the AVP-induced redistribution of AQP2 to the apical plasma membrane and produced a more diffuse distribution of AQP2 throughout the cell (Fig. 3D). Interestingly, treatment of tubules with 1 μM H-89 alone produced a small but statistically significant decrease in the PM/ICV ratio compared with control (H-89, 86 ± 5% of control, n = 5, P < 0.05).

DISCUSSION

The results presented here demonstrate a novel technique for studying the AVP-induced shuttling of AQP2 in native tissue. These studies show that treat-
ment of the tubule suspension with AVP causes an increase in the proportion of AQP2 associated with the plasma membrane. Furthermore, we have shown that the microtubule-disrupting agent nocodazole and H-89, a specific inhibitor of PKA, prevent the redistribution of AQP2 in response to AVP. Thus this method provides a useful way of studying the cellular mechanisms involved in AQP2 shuttling, in a preparation as close to the native epithelium as reasonably possible.

A number of attempts have been made to use suspensions of kidney tubules. One of the earliest came from Burg and Orloff (4), who used collagenase digestion to make a suspension of proximal tubules. They showed that the oxygen consumption of these cells was substantially greater than that seen in slices of cortical tissue, but the method was not subsequently widely used. A number of other attempts were made to use suspensions of proximal tubules or proximal tubule cells, including some by Whittembury and co-workers (1, 5), who found that the use of solutions containing low levels of sodium and chloride improved the viability of the cells, probably by unloading the sodium-potassium pump and preventing cell swelling (30). Recently, Chou et al. (6) have used a suspension of collecting ducts to measure cAMP production in response to antidiuretic agonists. We based our approach on that of Chou et al. but found that tubules prepared in a Ringer-based medium had most of their AQP2 in the plasma membrane even in the absence of AVP, probably because of cell swelling. The use of a solution on the basis of potassium gluconate for the proteolysis step improved this enormously.

Indirect evidence that microtubules are important in the hydrosmotic response to AVP has been available for many years (12, 17, 22, 24), and there has been some morphological evidence that disruption of microtubules impairs AQP2 shuttling in vivo (26). The results presented here using nocodazole highlight the importance of an intact microtubule network in the polarized delivery of AQP2 to the plasma membrane, in agreement with these previous findings.

It is well established that cAMP acts as the second messenger in the cellular response to AVP, immediately implicating PKA. It has been shown that PKA inhibition impairs the hydrosmotic response in isolated perfused tubules (27), and Valenti et al. (29) showed that H-89 prevented the AVP-induced redistribution of AQP2 in CD8 cells transfected with AQP2. It was therefore expected that inhibition of PKA would prevent AQP2 trafficking, but we believe that the results presented here represent the first direct demonstration of this in native epithelial cells. Interestingly, we observed a significant reduction in the PM/ICV fraction ratio in tubules treated with H-89 alone, suggesting that there is some constitutive PKA activity and membrane turnover of AQP2 in this tubule suspension.

It is very difficult to study the processes involved in the AVP-induced trafficking of AQP2 in vivo, because most drugs used to dissect the function of intracellular machinery will have widespread effects. As discussed

Fig. 3. Immunocytochemical localization of AQP2 within individual tubule fragments as revealed by confocal microscopy. Note that the images represent a single plane through a cylinder, so only part of the lumen is clearly visible, and the optical sections run through different regions of the cells in different parts of the tubule. A: in control tubules, AQP2 labeling is distributed throughout the cytoplasm. B: on treatment with 1 nM AVP, the labeling is now apical in localization. Note that the plane of the section goes through the apical pole of a cell projecting into the lumen (arrow). Pretreatment with either 33 μM nocodazole (C) or H-89 (D) prevents the apical redistribution of AQP2 in response to AVP. Similar results were obtained from at least 3 experiments.
in the introduction, a range of potential in vitro methods have been used to overcome this problem, but all bring their own problems. The use of isolated perfused tubules is probably the “gold standard,” providing direct measurements of water permeability that can be correlated with immunocytochemical studies if necessary, but this technique is extremely technically demanding and slow, making it suitable only for a limited range of projects. It has proven difficult to maintain endogenous AQP2 expression in cultured cells, and the dedifferentiation that these cells seem to undergo also results in the channels being targeted to the basolateral, as well as the apical, plasma membrane, making them unsuitable for studies on targeting or the directional aspects of the delivery process (for example, the role of cytoskeletal elements). Some stably transfected cell lines do appear to traffic AQP2 reproducibly to the apical plasma membrane (8, 28), but these cell lines appear to show differences in the regulatory pathways that control AQP2 shuttling, so any results obtained from them will need to be checked in native epithelial cells. Furthermore, because these cells are expressing AQP2 under the control of an exogenous promoter, they cannot be used to study the regulation of AQP2 expression in response to physiological stimuli; we hope that this will prove possible using modified versions of the method presented here.

The preparation described here was developed to overcome some of the drawbacks faced by the techniques described above. The tubule suspension is prepared from freshly isolated rat kidneys, and the tubules retain their epithelial structure and are naturally expressing AQP2. Thus they will presumably show, relatively faithfully, the events involved in AQP2 shuttling, from stimulation of the V2 receptor to exocytotic insertion into the apical plasma membrane of the principal cells. However, although improving on the in vitro methods presently available, it is probably inevitable that there will be differences from the response in vivo. The proteolysis required to free the tubules will cause some damage to the basement membrane surrounding the cells, which may lead to a partial dedifferentiation over time. We have deliberately chosen a defined culture medium to avoid the influence of the various hormones, etc., that are present in more sophisticated media, particularly those that contain serum or serum extracts. This means that various growth factors that may be essential to long-term stability of the cells will be missing. This protocol has been optimized for short-term studies, lasting not more than 2 h from the removal of the kidneys, and is now producing highly reproducible results under these conditions. If it is desired to maintain the suspension for longer periods, for example, to study the regulation of AQP2 gene expression, it is likely that different compromises will prove better; we are presently investigating this question.

In summary, we have developed a rat kidney tubule suspension that shows a shift of AQP2 from intracellular vesicles to the apical plasma membrane in response to AVP. This response can be prevented by the microtubule-disrupting agent nocodazole or by the PKA inhibitor H-89. This technique should prove to be a valuable tool in studying the intracellular mechanisms involved in the AVP-induced shuttling of AQP2.

The technical assistance of Julie Higgins is gratefully acknowledged. We thank Drs. Mark Kneppe (National Institutes of Health), Søren Nielsen (Aarhus University), and Michael Caplan (Yale University) for donating the AQP2 and Na+/K+/ATPase antibodies used in this study, and Dr. Malcolm Hunter for valuable discussions about the method and manuscript.

This work was supported by the Medical Research Council.

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AJP-Renal Physiol • VOL 283 • NOVEMBER 2002 • www.ajprenal.org


