

P2 Receptor-mediated Inhibition of Vasopressin-stimulated Fluid Transport and cAMP Responses in AQP2-transfected MDCK Cells

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We cultured canine kidney (MDCK) cells stably expressing aquaporin-2 (AQP2) on collagen-coated permeable membrane filters and examined the effect of extracellular ATP on arginine vasopressin (AVP)-stimulated fluid transport and cAMP production. Exposure of cell monolayers to basolateral AVP resulted in stimulation of apical to basolateral net fluid transport driven by osmotic gradient which was formed by addition of 500 mM mannitol to basolateral bathing solution. Pre-exposure of the basolateral surface of cell monolayers to ATP (100 μ M) for 30 min significantly inhibited the AVP-stimulated net fluid transport. In these cells, AVP-stimulated cAMP production was suppressed as well. Profile of the effects of different nucleotides suggested that the P2Y₂ receptor is involved in the action of ATP. ATP inhibited the effect of isoproterenol as well, but not that of forskolin to stimulate cAMP production. The inhibitory effect of ATP on AVP-stimulated fluid movement was attenuated by a protein kinase C inhibitor, calphostin C or pertussis toxin. These results suggest that prolonged activation of the P2 receptors inhibits AVP-stimulated fluid transport and cAMP responses in AQP2 transfected MDCK cells. Depressed responsiveness of the adenylyl cyclase by PKC-mediated modification of the pertussis-toxin sensitive G_i protein seems to be the underlying mechanism.

Key Words: P2 receptor, ATP, Vasopressin, Adenylyl cyclase, Cyclic AMP

INTRODUCTION

Extracellular ATP acts as an autocrine and paracrine regulator of a broad range of physiological processes by interacting with the P2 receptors (Dubyak & El-Moatassim, 1993; North & Barnard, 1997; Williams & Burnstock, 1997). The IUPHAR Purine Nomenclature Subcommittee grouped the P2 receptors into 2 families (Fredholm et al, 1997), P2X and P2Y. The P2X is a ligand-gated ion channel receptor family, whereas the P2Y is a G protein-coupled receptor family. Among the P2Y receptors, the P2Y₁ and P2Y₂ have most widely been studied in relation with their expression and functions in various tissues.

The P2Y receptors are expressed in the inner medullary collecting duct cells (McCoy et al, 1999; Kishore et al, 2000; Vallon, 2008), and are involved in regulation of ion transport across the epithelium. Studies with gene knockout mice provided insights including an antihypertensive activity of P2Y₂ receptors that is linked to an inhibitory effect on renal Na⁺ and water reabsorption (Rieg et al, 2007).

Upon activation of P2Y receptors, G protein-dependent activation of phospholipase C and subsequent increase of intracellular Ca²⁺ have been believed to be a major signaling mechanism to evoke cellular responses (Dubyak & El-Moatassim, 1993; Harden et al, 1995). However, there is accumulating evidence indicating that adenylyl cyclase

is also involved in the signaling mechanism of the P2Y receptor-mediated responses in a certain type of cells.

Inhibition of cAMP accumulation by activation of the P2 receptor was reported in rat hepatocytes (Okajima et al, 1987), mouse ventricular myocytes (Yamada et al, 1992), FRTL-5 thyroid cells (Sato et al, 1992), and C6 glioma cells (Pianet et al, 1989; Lin & Chuang, 1993). In contrast, however, it was reported that the P2 receptor agonists enhance cAMP production in cells of distal nephron origin (Post et al, 1996; Post et al, 1998; Woo et al, 1998). These contradictory results make it difficult to predict the precise effect of P2 receptor activation on different cell or tissue functions in relation with cAMP-dependent signaling.

In the course of our preliminary study using several types of cells derived from the distal nephron, it was observed that a prolonged exposure to ATP modifies the responsiveness of adenylyl cyclase to certain agonists. This finding appears to have a wide range of physiological significance because of the fact that cAMP is an important mediator of hormonal regulation of urinary concentrating mechanism in the distal nephron. In the present study, therefore, we cultured MDCK cells stably expressing aquaporin-2 (AQP2) on permeable membrane filters and examined the effect of extracellular ATP on AVP-dependent fluid transport and cAMP responses.

ABBREVIATIONS: AQP2, aquaporin-2; ATP, adenosine 5'-triphosphate; ATP- γ S, adenosine 5'-O-(3-thiotriphosphate); AVP, [Arg⁸]-vasopressin; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; MeATP, β , γ -methylene-ATP; MeSATP, 2-methylthio-ATP; PPADS, pyridoxal-phosphate-2'-4'-disulphonic acid; UTP, uridine 5'-triphosphate.

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METHODS

Cell culture

MDCK cells obtained from American Type Culture Collection were routinely maintained on plastic culture flasks in MEM supplemented with 10% fetal bovine serum (FBS), 50 IU/ml penicillin G and 50 μ g/ml streptomycin. Cells were trypsinized when they became confluent (approximately every 4~5 days) using 0.05% trypsin-0.53 mM EDTA solution and re-seeded at one-sixth the original density. For fluid movement studies cells were plated at a density of 5×10^5 cells on 12 mm polycarbonate membrane filters (Snapwell, Costar, Cambridge, MA). Before plating, the filters were coated with 1% Vitrogen 100 (Collagen, Palo Alto, CA) and 1 mg/ml human fibronectin (Collaborative Research, Bedford, MA) dissolved in MEM supplemented with 1% FBS. Cells which were plated on Snapwells were evaluated for formation of tight junctions by measuring transepithelial resistance using transepithelial voltometer (EVOM, World Precision Instruments, Sarasota, FL) and chopstick electrodes. Usually, transepithelial resistance reached its peak ($4,310 \pm 730$ ohm \cdot cm²) 5 to 6 days after plating. Experiments were carried out on the 6th to 8th day after plating. Cells used for this study were between passages of 86 to 110.

Preparation of AQP2-transfected cells

Full length cDNA (1.2 Kb) of AQP2 was subcloned first into a mammalian expression vector pcDNA3.1/Neo using HindIII and XbaI site, and then transfected into MDCK cells using lipofectamine plus (Gibco). AQP2 transfected cells were maintained in culture media containing G418 (Gibco). Expression of AQP2 was assayed by Western blot analysis.

Western blot analysis

Cells were washed twice with ice-cold phosphate-buffered saline at 4°C and resuspended in 1 ml of extraction buffer (50 mM PIPES, pH 7.0, 50 mM KCl, 5 mM MgCl₂, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin and 10 μ g/ml pepstatin A). Cells were lysed by five cycles of freezing in liquid nitrogen and thawing at 37°C. After verifying by microscopic examination that more than 90% of cells were lysed, the lysates were centrifuged at 100,000 g for 1 hr at 4°C. The resulting supernatant, which consisted of the cytosol, was separated from the pellet containing the cellular membrane and organelles, and was analyzed for aquaporin 2 by Western blots using polyclonal anti-AQP2 antibody.

Measurement of transepithelial fluid movement

Transepithelial fluid movement was measured in cells grown as confluent monolayers on Snapwell filters. Monolayers were used only when transepithelial resistance reached over 4,000 ohm \cdot cm². Culture medium bathing the cell monolayer was replaced with MEM supplemented with 2% FBS. Apical to basolateral transepithelial fluid movement was measured under osmotic gradient. The osmotic gradient was formed by addition of 500 mM mannitol into the basolateral bathing medium. Net apical to basolateral fluid movement was measured by monitoring the increase

in the radioactivity of [¹⁴C]-inulin which was added into the apical bathing medium at an initial concentration of around 1,000 cpm/ml. Every monolayer used in this study was checked out, and it was confirmed that [¹⁴C]-inulin did not appear in the basolateral bathing medium during the fluid movement study. This procedure confirmed that the functional tight junction was maintained throughout the experimental procedure.

Measurement of cAMP content

After exposure to the agonists in the presence of a phosphodiesterase inhibitor IBMX (10^{-4} M), the uptake media were rapidly removed from the incubation wells and immersed in ice-cold ethanol-HCl (ethanol solution containing 20 mM HCl and 0.2% Triton X-100) for 2 hr to disrupt cell membranes and to precipitate proteins. Disrupted cell extracts were then transferred to microcentrifuge tubes and sonicated to completely disrupt the cell membrane and extract intracellular cAMP. The cell suspension was then centrifuged (12,000 g) for 10 min at 4°C to precipitate the protein, and the supernatant was collected. The supernatant was freeze-dried and dissolved in an adequate volume of 50 mM Tris/1 mM EDTA (pH 7.5). Cyclic AMP content was determined by radioimmunoassay using [³H]cAMP assay kit.

Chemicals

[¹⁴C]inulin and [³H]cAMP assay kit were obtained from Amersham international (Amersham, UK). Adenosine 5'-triphosphate (ATP, disodium salt), uridine 5'-triphosphate (UTP, sodium salt), adenosine 5'-O-3-thiotriphosphate (ATP- γ S, tetralithium salt), [Arg⁸]-Vasopressin (AVP) and forskolin were purchased from Sigma Chemical (St. Louis, MO). 2-methylthio-ATP (MeSATP), β , γ -methylene-ATP (MeATP), pyridoxal-phosphate-2'4'-disulphonic acid (PPADS), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) pertussis toxin and calphostin C were obtained from Calbiochem (La Jolla, CA).

Statistical analysis

Data are expressed as mean \pm S.E. Comparison between two groups was made using the unpaired t test. Multiple group comparison was done using one-way analysis of variance followed by the Tukey post hoc test. p values lower than 0.05 were considered statistically significant.

RESULTS

Effect of AVP on fluid movement in AQP2 transfected cells

Enhanced and stable expression of AQP2 in cells transfected with full length cDNA (1.2 Kb) of AQP2 was confirmed by Western blot analysis. In control MDCK cells, there was negligible expression of AQP2 and AVP-stimulated response (Fig. 1A left lane, and B). In AQP2-transfected MDCK cells, however, there was a remarkable increase in the expression of AQP2 (Fig. 1A right lane). In these cells, the radioactivity of [¹⁴C]-inulin in the apical bathing media increased significantly when stimulated with AVP (basolateral side, 10^{-6} M) in the presence of an osmotic gra-

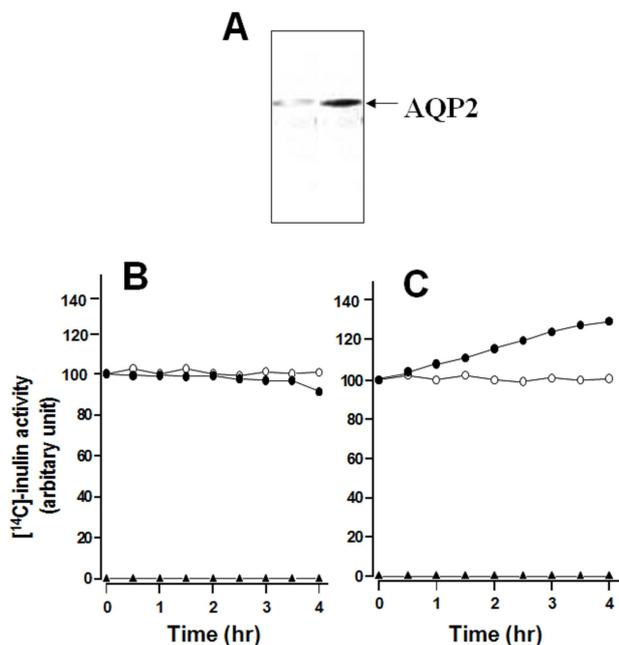


Fig. 1. (A) Western blot analysis of AQP2 expression in control (left lane) and AQP2 transfected (right lane) MDCK cells. Cells were transfected with full length cDNA of AQP2. (B, C) Changes in [^{14}C]inulin activity in the apical bathing medium were monitored as an index of net apical to basolateral fluid movement in the presence (closed circle) and absence (open circle) of 10^{-6} M AVP in control (B) and AQP2 transfected (C) cells. Each point represents mean of 5 experiments.

dient (basolateral > apical, 500 mOsm) which was formed by addition of mannitol into the basolateral bathing media (Fig. 1B). Apical to basolateral leak of [^{14}C]inulin radioactivity was negligible throughout the experimental period, indicating that there was no significant paracellular leak of the solutes or fluid, thus confirming that the increase of the [^{14}C]inulin radioactivity in the apical bathing media reflects net apical to basolateral fluid movement.

Net fluid movement which was estimated from the changes in the apical [^{14}C]inulin radioactivity, was presented in Fig. 2. There was a time-dependent increase in the apical to basolateral fluid movement in AQP2 transfected MDCK cells when stimulated with AVP in the presence of an osmotic gradient. We used the 10^{-6} M AVP concentration which showed maximum effect in the preliminary studies (data not shown). It stimulated net apical to basolateral fluid movement in a time-dependent manner up to 4 hr of incubation. We did not determine the fluid movement for longer than 4 hr, because some monolayers tended to lose the functional integrity of their tight junction when exposed to experimental procedures for longer than 4 hr. We used 10^{-6} M AVP and 3 hr of incubation time for the fluid movement studies in the following study.

Effect of ATP on AVP-stimulated fluid movement

To determine whether activation of P2 purinergic receptors affects AVP-stimulated fluid movement, the ability of AVP (basolateral, 10^{-6} M) to stimulate the net apical to basolateral fluid movement was measured in the pres-

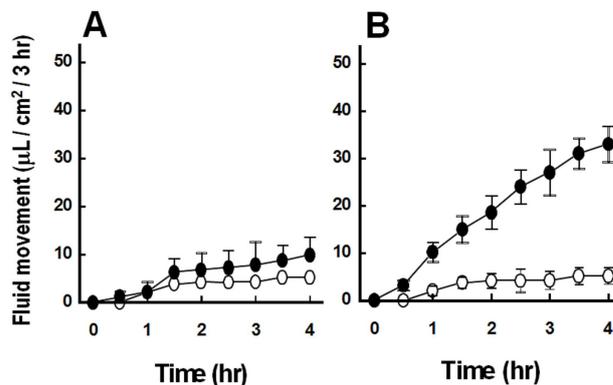


Fig. 2. Effect of AVP on net apical to basolateral fluid movement in control and AQP2 transfected MDCK cells. Net apical to basolateral fluid movement was calculated from the changes in [^{14}C]inulin activity in the apical bathing medium. (A) In control cells, in the presence (closed circle) and absence (open circle) of 10^{-6} M AVP. (B) In AQP2 transfected cells, in the presence (closed circle) and absence (open circle) of 10^{-6} M AVP. Each point represents mean \pm S.E. of 5 experiments.

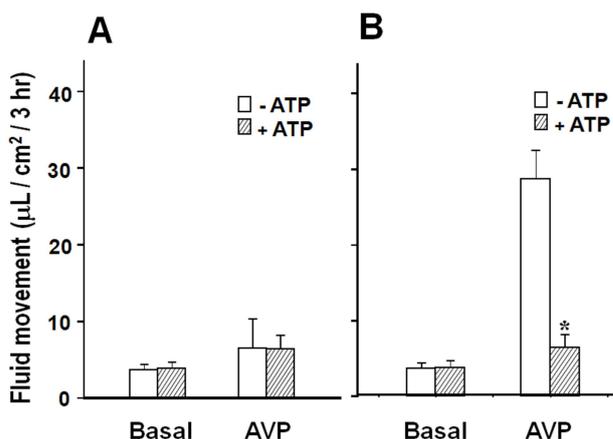


Fig. 3. Effect of ATP on AVP-stimulated net apical to basolateral fluid movement. Net apical to basolateral fluid movement was calculated from the changes in [^{14}C]inulin activity in the apical bathing medium in control (A) and AQP2 transfected (B) cells. ATP (10^{-4} M) was added to basolateral bathing medium 30 min prior to the stimulation with AVP. Data are mean \pm S.E. of 6 experiments. * $p < 0.01$ vs. without ATP.

ence of ATP (basolateral, 10^{-4} M). Fig. 3B shows that ATP significantly attenuated the AVP-induced stimulation of the net apical to basolateral fluid movement (27.2 ± 4.8 to 4.3 ± 1.9 $\mu\text{L}/\text{cm}^2/3$ hr) in AQP2-transfected cells. In control cells, there was no significant changes in the fluid movement by either AVP or ATP (Fig. 3A).

Effect of ATP on cellular cAMP

As AVP-induced stimulation of the fluid movement is mediated by an enhancement of cellular cAMP production, the effect of ATP on AVP-stimulated cAMP production was determined. Treatment of cells with AVP alone resulted in

4.3 fold increase in cellular cAMP level (Fig. 4). In the presence of ATP which was added 30 min prior to the stimulation with AVP, AVP-induced increase in cAMP production was remarkably attenuated. This result strongly suggested that the effect of ATP to attenuate the AVP response is closely related with its modulation of adenylyl cyclase activity. We next examined whether ATP affects the activation of adenylyl cyclase by another G protein dependent modulator isoproterenol and a direct activator of catalytic subunit, forskolin. Fig. 4 shows that, in contrast to

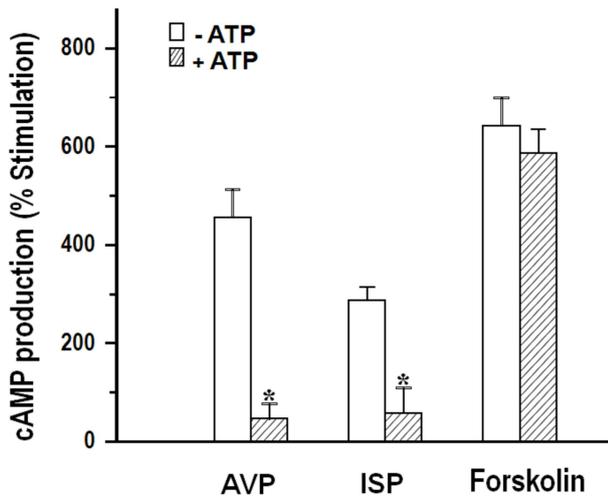


Fig. 4. Effect of ATP on cAMP production in AQP2 transfected cells stimulated with AVP, isoproterenol and forskolin. The magnitude of stimulation in cellular cAMP production by exposure for 5 min to 10^{-6} M each of AVP, isoproterenol (ISP) and forskolin was measured in the presence and absence of ATP (10^{-4} M). Data are mean \pm S.E. of 4 experiments. * $p < 0.01$ vs. without ATP.

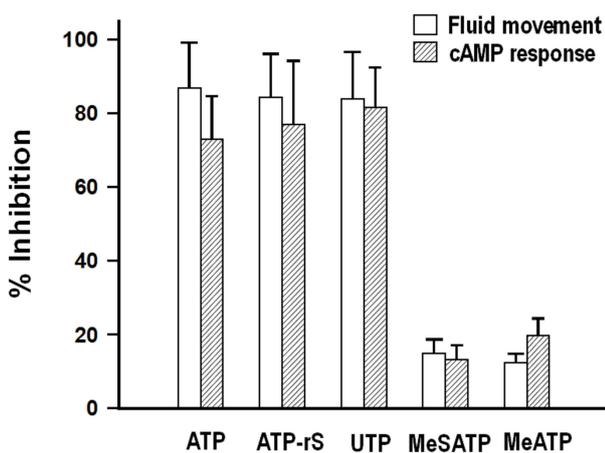


Fig. 5. Effects of different P2 nucleotide receptor agonists on AVP-stimulated responses. The magnitude of inhibition of AVP-stimulated responses by each nucleotide (10^{-4} M) was measured via 3-hr fluid movement and 5-min cAMP production. Data are mean \pm S.E. of 5 experiments. ATP- γ S, 5'-O-3-thiotriphosphate; UTP, uridine 5'-triphosphate; MeSATP, 2-methylthio-ATP; MeATP, β, γ -methylene-ATP.

the effect on AVP and isoproterenol-induced stimulation, ATP did not affect the forskolin-induced stimulation of cAMP production (Fig. 4).

Effects of different nucleotides on AVP-stimulated cAMP production

We tested the effects of ATP, ATP γ S, UTP, 2-methylthio-ATP (MeSATP), and β, γ -methylene-ATP (MeATP) to elucidate the nature of receptors involved in the inhibition of AVP-stimulated fluid movement and cAMP production. When cell monolayers were pre-exposed to 10^{-4} M each of nucleotides for 30 min, both the AVP-stimulated fluid movement and cAMP production were inhibited. The magnitudes of inhibition by these nucleotides were in decreasing order: ATP \geq ATP γ S \geq UTP \gg MeSATP = MeATP suggesting the involvement of the P2Y₂ receptor (Fig. 5).

Effect of P1 and P2 receptor blockers

The P1 receptor is not likely to be responsible for the inhibitory effect of ATP, because ATP- γ S showed an equipotent effect. Nevertheless, we tested this possibility by determining the effect of the P2 receptor blocker (PPADS) and P1 receptor blocker (DPCPX) on the action of ATP to inhibit AVP-stimulated fluid movement and cAMP production. PPADS or DPCPX by itself did not affect the basal or AVP-stimulated cAMP production. PPADS, when present during pre-exposure of cells to ATP and subsequent stimulation by AVP, significantly attenuated the inhibitory effect of ATP on AVP-stimulated fluid movement and cAMP production. In contrast, DPCPX did not affect the action of ATP to inhibit the AVP-stimulated responses (Fig. 6).

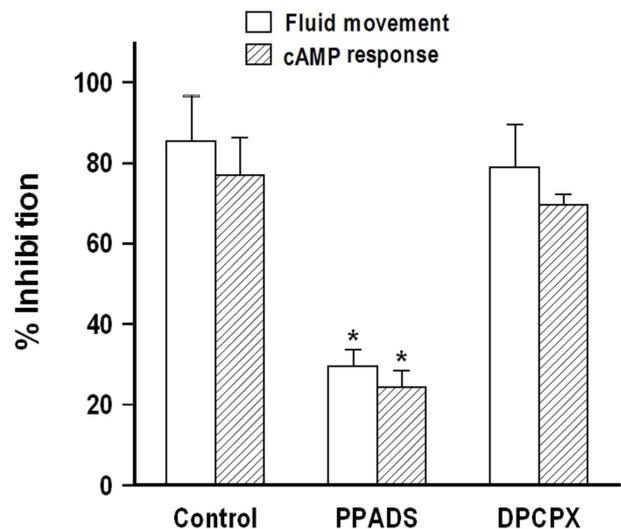


Fig. 6. Effects of purinergic antagonists on the ATP-induced inhibition of AVP-stimulated responses. The magnitude of ATP-induced inhibition of AVP-stimulated responses of 3-hr fluid movement and 5-min cAMP production was measured in the presence and absence of the P2 receptor antagonist pyridoxal-phosphate-24-disulphonic acid (PPADS, 10^{-5} M) and the P1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 10^{-5} M). The data represent mean \pm S.E. of 4 experiments. * $p < 0.01$ compared to the respective control.

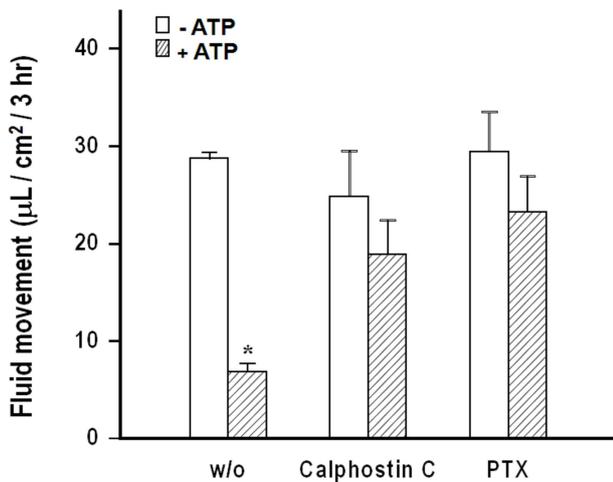


Fig. 7. Effects of calphostin C and pertussis toxin on ATP-induced inhibition of AVP-stimulated fluid movement. AVP-stimulated fluid movement was measured in the presence and absence of ATP (10^{-4} M) in cells pretreated with calphostin C (20 μ M) or with pertussis toxin (PTX, 1 μ g/ml) for 2 hr. Data are mean \pm S.E. of 5 experiments. * $p < 0.01$ vs. the respective values without ATP.

Role of PKC and G_i protein

The fact that ATP did not affect forskolin-stimulated cAMP suggested that underlying mechanism of the ATP-induced inhibition of AVP-stimulated cAMP responses did not involve the catalytic subunit of adenylyl cyclase. Several investigators have suggested that activation of protein kinase C can modify G_i protein and result in inhibition of the responsiveness of adenylyl cyclase to agonists (Beckner & Farrar, 1987; Budnik & Mukhopadhyay, 1987; Teitelbaum, 1993). Furthermore, evidence indicates that activation of protein kinase C occurs upon activation of P2Y receptors and subsequent stimulation of phospholipase C (Dubyak & El-Moatassim, 1993; Harden et al, 1995). Therefore, we evaluated the possible role of protein kinase C and G_i protein in the ATP-induced attenuation of the AVP-stimulated responses, by determining the effect of a well-known PKC inhibitor, calphostin-C (Kobayashi et al, 1989), and the effect of ATP on cells treated with pertussis toxin as well. As shown in Fig. 7, pretreatment of cells with calphostin C or pertussis toxin significantly blocked the inhibitory effect of ATP on the AVP-stimulated fluid movement.

DISCUSSION

Arginine vasopressin (AVP) plays a major role in maintaining body fluid volume and blood pressure by regulation of renal water and solute transport. The biologic action of AVP to increase water permeability is mediated through the AVP type 2 (V2) receptors in the collecting duct cells (Knepper, 1997). The short-term regulation involves rapid AVP-dependent increase of water permeability as a consequence of shuttling AQP2 into the apical membrane. AVP also mediates long-term regulation of water and solute transport in collecting duct cells through a transcriptional/translational mechanism.

In this study, we tried to reconstitute the short-term reg-

ulation of water permeability by AVP using MDCK cells stably expressing AQP2. MDCK cells are a polarized cell line derived from canine kidney collecting duct, and they have been shown to express V2 receptor in a polarized manner (Andersen-Beckh et al, 1999). Western blot analysis demonstrated negligible expression of AQP2 in control cells, whereas enhanced and stable expression in AQP2-transfected cells. It has earlier been demonstrated that these AQP2-transfected MDCK cells have normal AQP2 routing to apical membrane when treated with AVP and could provide a valuable model to study the regulation of AVP on AQP2 (Deen et al, 1997). In these cells, net apical to basolateral fluid movement is significantly increased when stimulated with AVP in the presence of an osmotic gradient, whereas there is no significant AVP-induced responses in the absence of an osmotic gradient. These results indicate that the AVP-stimulated fluid movement can be ascribed to an osmosis-driven transcellular water movement through AQP2 water channel, but not to a fluid movement secondary to a solute transport or paracellular leak.

In the present study, the ability of extracellular ATP to modify the AVP-stimulated fluid movement and cAMP production was evaluated. In cells pretreated with ATP, AVP-stimulated fluid movement was remarkably inhibited. Likewise, AVP-stimulated cAMP production was significantly suppressed in these cells. In this study, the exact nature of the receptors involved in the stimulatory and antagonizing effect of ATP were not examined, nevertheless, the results with different nucleotides and antagonists suggest that the P2 receptors might be responsible for the action of ATP. Furthermore, the ability of UTP, a P2Y₂ receptor agonist (Fredholm et al, 1997; Heilbronn et al, 2000), to attenuate the AVP-stimulated responses suggests that the P2Y₂ or a P2Y₂-like receptor is the major transducer to mediate the effect of ATP.

In this study, it is clear that the attenuation of AVP-stimulated responses by ATP is mediated via a mechanism involving adenylyl cyclase. Catalytic subunit of adenylyl cyclase is not a site responsible for the inhibition by P2 receptor activation with ATP, because it did not affect the stimulation of cAMP generation by direct activation of the enzyme by forskolin. Our results strongly suggested the role of G_i protein and PKC in the attenuation of AVP-stimulated responses by ATP. In pertussis toxin-treated cells, ATP-induced inhibition of AVP response was significantly attenuated. The effect of pertussis toxin is not likely due to stimulation of basal cAMP production, because pertussis toxin by itself did not change the basal cAMP content (data not shown). It has been reported that activation of G_i protein might be an important target for several hormones and autacoids that antagonize the Gs protein-coupled effects of AVP. G_{ai1} , G_{ai2} and G_{ai3} are known to be present in the nephron cells (Buhl et al, 1992). It seems, therefore, very likely that these pertussis toxin-sensitive species constitute underlying desensitization mechanism of ATP-stimulated cAMP responses. The present study also suggested that PKC might be the mediator responsible for the modification of adenylyl cyclase upon activation of P2 receptors, because the effect of ATP to attenuate the AVP-stimulated response was significantly blocked by calphostin C. This finding is in good agreement on the role of PKC to inhibit AVP-stimulated cAMP production with the report by Teitelbaum (1993).

It has become clear that the P2 receptors play important roles in the regulation of the renal solute and fluid transport (Vallon, 2008). Studies with gene knockout mice pro-

vided evidence that an antihypertensive activity of these receptors is linked to an inhibitory effect on renal Na⁺ and water reabsorption (Rieg et al, 2007). Among the innate activators of the P2 receptors, ATP has the most important physiological and pathophysiological significance. It is released as a cotransmitter at adrenergic nerve endings, through a conductive pathways such as cystic fibrosis transmembrane conductance regulator (CFTR), and via a non-specific pathways from inflammatory and damaged cells etc. (Dubyak & El-Moatassim, 1993; North & Barnard, 1997; Williams & Burnstock, 1997). The circulating concentration of ATP (to 1 micromolar) is much lower than those which we used in this study. However, local concentration of ATP can rise to near milimolar levels when ATP release via the above routes is stimulated. Furthermore, in certain pathological conditions following massive cell destruction such as ischemic strokes, there should be abrupt increase in extracellular ATP concentration.

The present finding that the activation of the P2 receptor could modify the AVP-stimulated adenylyl cyclase activity and fluid transport provided us an insight into the mechanism of the P2 receptor-mediated regulation of the renal solute and water transport. It might also provide a clue to understanding of the mechanism of defective renal concentrating ability in some pathological conditions including acute renal failure. Moreover, as adenylyl cyclase is an important mediator of hormonal regulation of renal tubular function not only for AVP but also for other diverse hormones such as parathyroid hormone and catecholamines, our present finding has wide range of physiological and pathophysiological relevance.

In conclusion, our results suggest that prolonged activation of the P2 receptors inhibits AVP-stimulated fluid transport and cAMP responses in AQP2 transfected MDCK cells. Depressed responsiveness of adenylyl cyclase by PKC-mediated modification of the pertussis-toxin sensitive G_i protein seems to be the underlying mechanism.

ACKNOWLEDGEMENT

This work was supported for two years by Pusan National University Research Grant.

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