Hormone-stimulated Ca\textsuperscript{2+} transport in rabbit kidney: multiple sites of inhibition by exogenous ATP

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Van Baal, Jürgen, Joost G. J. Hoenderop, Maarten Groenendijk, Carel H. Van Os, René J. M. Bindels, and Peter H. G. M. Willems. Hormone-stimulated Ca\textsuperscript{2+} transport in rabbit kidney: multiple sites of inhibition by exogenous ATP. Am. J. Physiol. 277 (Renal Physiol. 46): F899–F906, 1999.—Exogenous ATP markedly reduced 1-deamino-8-d-arginine vasopressin (dDAVP)-stimulated Ca\textsuperscript{2+} transport and cAMP accumulation in primary cultures of rabbit connecting tubule and cortical collecting duct cells. Similarly, ATP inhibited the stimulatory effect of 8-bromo-cAMP. At first sight, this is in agreement with the “classic” concept that dDAVP exerts its stimulatory effect via cAMP. However, dDAVP-stimulated Ca\textsuperscript{2+} transport was markedly reduced by the protein kinase C (PKC) inhibitor chelerythrine, reported previously to inhibit the cAMP-independent pathway responsible for parathyroid hormone-1 [Arg\textsuperscript{8}]vasopressin-, PGE\textsubscript{2}, and adenosine-stimulated Ca\textsuperscript{2+} transport. Chelerythrine also inhibited the increase in Ca\textsuperscript{2+} transport evoked by the cAMP-independent A\textsubscript{1} receptor agonist N\textsuperscript{6}-cyclopentyladenosine (CPA). Downregulation of phorbol ester-sensitive PKC isoforms by chronic phorbol ester treatment has been shown to be without effect on hormone-stimulated Ca\textsuperscript{2+} transport, indicating that the chelerythrine-inhibitable pathway consists of a phorbol ester-insensitive PKC isoform. Here, this maneuver did not affect ATP inhibition of dDAVP-stimulated Ca\textsuperscript{2+} transport and cAMP formation, while abolishing ATP inhibition of CPA-stimulated Ca\textsuperscript{2+} transport. These findings show that ATP acts via 1) a phorbol ester-sensitive PKC isoform to inhibit hormonal stimulation of Ca\textsuperscript{2+} transport at the level of the chelerythrine-inhibitable pathway involving a phorbol ester-insensitive PKC isoform and 2) a phorbol ester-insensitive mechanism to inhibit V\textsubscript{2o} receptor-mediated concomitant activation of this pathway and adenylyl cyclase.

The (patho)physiological relevance of exogenous ATP has been studied extensively. Under physiological conditions, ATP is released from nerve terminals, endothelial cells, and muscle cells (7, 9). Recently, ATP release associated with the cystic fibrosis transmembrane conductance regulator has attracted great deal of interest (14, 22, 28, 29, 31). Certain clinical syndromes are accompanied by the release of large quantities of ATP as a result of tissue injury (7, 9, 19). ATP binds to specific cell-surface receptors that are widely distributed throughout the body. Activation of these purinoceptors requires pericellular ATP concentrations in the high micromolar range (7, 9–11, 19, 21, 25, 30).

Purinoceptors are classified into two subtypes, designated P\textsubscript{1} and P\textsubscript{2} (9, 12, 19). P\textsubscript{1} purinoceptors exhibit a greater sensitivity to adenosine and AMP and are subdivided into A\textsubscript{1}, A\textsubscript{2}, and A\textsubscript{3} receptors (9, 12, 19, 24). A\textsubscript{1} and A\textsubscript{3} receptors have been demonstrated to inhibit adenylyl cyclase and to activate phospholipase C (23, 24), whereas A\textsubscript{2} receptors activate adenylyl cyclase (25, 33). P\textsubscript{2} purinoceptors are more sensitive to ATP and ADP and are subdivided into ionotropic P\textsubscript{2X} receptors and G protein-coupled P\textsubscript{2Y} receptors (12). P\textsubscript{2X} receptors are ligand-gated cation channels, whereas P\textsubscript{2Y} receptors increase the activity of phospholipase C.

Evidence for the involvement of P\textsubscript{2} receptors in the regulation of renal electrolyte and water transport was first provided by the observation that exogenous ATP stimulated Na\textsuperscript{+}-K\textsuperscript{+}-Cl\textsuperscript{−} cotransport activity in cultured A6 cells originally derived from the distal nephron of Xenopus laevis (25). More recently, ATP was shown to inhibit [Arg\textsuperscript{8}]vasopressin (AVP)-stimulated water transport in perfused tubules of the rat inner medullary collecting duct (IMCD; see Ref. 20) and rabbit cortical collecting duct (CCD; see Ref. 30). Similarly, ATP was shown to inhibit Na\textsuperscript{+} and Ca\textsuperscript{2+} reabsorption in primary cultures of rabbit connecting tubule (CNT) and CCD cells (21). The involvement of P\textsubscript{2X} receptors linked to the inositol 1,4,5-trisphosphate-mediated release of Ca\textsuperscript{2+} from the endoplasmic reticulum was confirmed from the observation that ATP readily increased the cytosolic Ca\textsuperscript{2+} concentration in the absence of extracellular Ca\textsuperscript{2+}. A similar observation was reached with rat IMCD (10) and mouse cortical thick ascending limb of Henle (27). In each case, the effect of ATP was shown to be mimicked by UTP, which is in agreement with the involvement of UTP-preferring P\textsubscript{2Y} receptors (12).

There is ample evidence that the distal nephron is the primary site of active Ca\textsuperscript{2+} reabsorption (1, 8, 13). To study the hormonal regulation of this process, we established a primary culture of immunodissected rabbit CNT and CCD cells (2–4, 16, 17, 21, 32, 33). We have shown that Ca\textsuperscript{2+} transport across these monolayers can be stimulated by parathyroid hormone (PTH; see Refs. 16 and 32), AVP (16, 32, 33), PGE\textsubscript{2} (16, 32), and adenosine (16, 17). Importantly, we recently demonstrated that the adenosine analog N\textsuperscript{6}-cyclopentyladenosine (CPA), which acts as an agonist at the A\textsubscript{1} receptor, can maximally stimulate Ca\textsuperscript{2+} transport without detectably increasing the cytosolic cAMP concentration (17). This led us to postulate the existence of a novel cAMP-independent pathway leading to stimulated Ca\textsuperscript{2+} transport. Moreover, in a follow-up study, we showed...
that also PTH, PGE₂, AVP, and adenosine act via this novel cAMP-independent pathway and that this pathway involves a chelerythrine-inhibitable protein kinase C (PKC) isotype that is not downregulated after chronic phorbol ester treatment (16).

Using this cell model, we previously reported that ATP can markedly inhibit Ca²⁺ transport, and evidence was provided for the involvement of PKC in the mechanism of action of ATP (2, 21). However, of eminent importance for a correct interpretation of the mechanism of action of ATP is our recent finding that primary cultures of CNT and CCD cells produce prostanoids that stimulate Ca²⁺ transport (32). This latter finding leaves the possibility that ATP might act by inhibiting this autostimulatory pathway.

The first aim of the present study is to assess whether the previously reported inhibitory action of ATP on Ca²⁺ transport is due to inhibition of the autostimulatory pathway. The second aim is to investigate whether ATP also inhibits the stimulatory effects of other hormones such as AVP and adenosine. Finally, the third aim of the present study is to investigate whether PKC is indeed involved in the inhibitory actions of ATP, as suggested by our previous work, and, if so, to elucidate the level(s) at which PKC exerts its inhibitory effects.

MATERIALS AND METHODS

Drugs. Collagenase A and hyaluronidase were obtained from Boehringer (Mannheim, Germany). 1-Desamino-8-arginine vasopressin (dDAVP) was from Bachem Feinchemikalien (Bubendorf, Switzerland). Pertussis toxin (PTX), chelerythrine, and Ro-20–1724 were purchased from Research Biochemicals International (Natick, MA). All other chemicals, including AVP, adenosine, CPA, PGE₂, 8-bromo-cAMP (8-BrcAMP), and 12-O-tetradecanoylphorbol 13-acetate (PMA), were obtained from Sigma (St. Louis, MO).

Primary cultures of rabbit CNT and CCD cells. New Zealand White rabbits weighing 0.5–0.6 kg were killed by decapitation. Kidney CNT and CCD cells were immunodissected with monoclonal antibody R2G9 and set in primary culture on permeable filter supports (0.33 cm²; Costar, Cambridge, MA), as described previously (32). The cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium-Ham's F-12 (GIBCO, Paisley, UK), supplemented with 5% (vol/vol) decomplemented FCS (Serva, Heidelberg, Germany), 50 µg/ml gentamycin, 0.5% (vol/vol) of a 100× mixture of nonessential amino acids (GIBCO), 5 µg/ml insulin, 5 µg/ml transferrin, 50 nM hydrocortisone, 70 nM PGE₂, 50 nM Na₃SeO₃, and 5 µM triiodothyronine, equilibrated with 5% CO₂/95% O₂ at 37°C. Cell monolayers reached confluency at day 3, and experiments were performed between 5 and 8 days after seeding the cells. Confluency of the monolayers was routinely checked by determining transepithelial potential difference and resistance with two "chopstick"-like electrodes connected to a Millicell-ERS meter (Millipore, Bedford, MA).

Measurement of transepithelial Ca²⁺ transport. Transepithelial Ca²⁺ transport was measured as described previously (32). Briefly, confluent monolayers were washed three times and preincubated in a physiological salt solution (PSS) containing (in mM): 140 NaCl, 2 KCl, 1 K₂HPO₄, 1 KH₂PO₄, 1 MgCl₂, 1 CaCl₂, 5 glucose, 5 l-alanine, and 10 HEPES (adjusted to pH 7.40 with Tris) for 15 min at 37°C. Long-term treatments with drugs were performed in culture medium. Subsequently, the monolayers were washed three times and incubated in PSS for another 90 min at 37°C to measure transepithelial Ca²⁺ transport. Drugs and hormones were added to either the apical and/or basolateral compartment, as indicated in the text. At the end of the incubation period, 25-µl samples were removed in duplicate from the apical compartment and assayed for Ca²⁺ with a colorimetric test kit (Boehringer). Ca²⁺ transport is expressed in nanomoles per hour per square centimeter.

Measurement of cAMP accumulation. To assess the effect of ATP on dDAVP-stimulated adenylyl cyclase activity, accumulation of cAMP was measured in the presence of an inhibitor of cyclic nucleotide phosphodiesterase activity, Ro-20–1724. Confluent monolayers were preincubated in PSS containing 0.1 mM Ro-20–1724 for 15 min at 37°C. Subsequently, hormones were added to either the apical and/or basolateral compartment as indicated in the text, and the monolayers were incubated for another 15 min. At 15 min, the filters were excised and immediately transferred to Erpender microtubes containing 500 µl of 5% (wt/vol) trichloroacetic acid. The samples were vigorously mixed and rapidly frozen in liquid nitrogen. Samples were frozen and thawed three times and centrifuged for 4 min at 10,000 g (Eppendorf minifuge). A 400-µl aliquot of the supernatant was removed and extracted three times with water-saturated diethyl ether. The aqueous phase was blown to dryness with nitrogen, after which the residue was dissolved in 500 µl of sodium acetate buffer (pH 6.2). Samples and standards were acetylated, and the cAMP content was determined by RIA (125I-cAMP Assay System; Amersham, Arlington Heights, IL). The amount of cAMP is expressed in nanomoles per 15 min per filter.

Statistics. Results are given as means ± SE. Overall statistical significance was determined by ANOVA and, in the case of significance, individual groups were compared by contrast analysis according to Scheffé. P values <0.05 were considered significant.

RESULTS

ATP inhibition of Ca²⁺ transport stimulated by endogenously produced prostanoids. We have shown that ATP interacts with apical and basolateral P₂Y purinoceptors to inhibit net apical-to-basolateral Ca²⁺ transport across monolayers of rabbit CNT and CCD cells (21). However, our recent finding that endogenously produced prostanoids potently stimulate this transport process (32) urged us to investigate the possibility that ATP might act by inhibiting this autostimulatory pathway. For maximal inhibition, ATP was added to both sides of the monolayer (21). Figure 1 shows the inhibitory action of ATP (100 µM; both compartments) on Ca²⁺ transport (P < 0.05; n = 8 filters). However, ATP did not have an effect in monolayers in which Ca²⁺ transport was reduced as a result of the inhibition of endogenous prostanoid production by the cycoxygenase inhibitor indomethacin (5 µM; both compartments). To investigate whether ATP exerts its inhibitory action on the autostimulatory pathway at or beyond the receptor level, its effect on PGE₂-stimulated Ca²⁺ transport was studied in indomethacin-treated monolayers. Figure 1 shows that ATP significantly (P < 0.005; n = 8 filters) reduced the stimulatory effect of exogenous PGE₂ (10 nM). The percentage inhibition was calculated to be 51%. These findings demonstrate that ATP
inhibits the autostimulatory pathway at least at or beyond the level of the prostanooid receptor. Subsequent experiments were routinely performed with indomethacin-treated monolayers.

ATP inhibition of AVP- and adenosine-stimulated Ca\(^{2+}\) transport. Both AVP and adenosine have been demonstrated to potently stimulate Ca\(^{2+}\) transport across monolayers of rabbit CNT and CCD cells (16, 17, 32, 33). Table 1 shows that ATP (100 µM; both compartments) significantly (P < 0.05) reduced the stimulatory effect of AVP (1 nM; basolateral compartment) and adenosine (10 µM; apical compartment) by 37 and 63%, respectively.

ATP inhibition of dDAVP- and CPA-stimulated Ca\(^{2+}\) transport. The stimulatory effect of AVP was shown to be mimicked by dDAVP (33), which suggests the involvement of the V\(_2\) receptor. Figure 2 shows that dDAVP (1 nM; basolateral compartment) increased Ca\(^{2+}\) transport by 71 ± 5 nmol·h\(^{-1}\)·cm\(^{-2}\) (P < 0.05; n = 12 filters) and that ATP (100 µM; both compartments) significantly decreased this value to 36 ± 4 nmol·h\(^{-1}\)·cm\(^{-2}\) (P < 0.05; n = 12 filters). The percentage inhibition was calculated to be 50%. ATP did not affect basal Ca\(^{2+}\) transport. As cAMP is generally implicated in the mechanism of action of dDAVP (5), we investigated the effect of ATP on cAMP-stimulated Ca\(^{2+}\) transport. Figure 2 shows that the membrane-permeable cAMP analog 8-Br-cAMP (0.1 mM; basolateral compartment) markedly increased Ca\(^{2+}\) transport by 71 ± 5 nmol·h\(^{-1}\)·cm\(^{-2}\) (P < 0.05; n = 9 filters) and that ATP significantly decreased this value to 39 ± 6 nmol·h\(^{-1}\)·cm\(^{-2}\) (P < 0.05; n = 9 filters). The percentage inhibition was calculated to be 45%. This demonstrates that, in case a hormone acts through cAMP, ATP acts, at least in part, at or beyond the level of protein kinase A. We recently showed that the adenosine analog CPA, which acts as an agonist at the A\(_1\) receptor (34), can maximally stimulate Ca\(^{2+}\) transport without detectably increasing the cytosolic cAMP concentration (17). This led us to investigate whether ATP can also inhibit this novel cAMP-independent stimulatory pathway. Figure 2 shows that CPA (10 µM; apical compartment) increased Ca\(^{2+}\) transport by 48 ± 3 nmol·h\(^{-1}\)·cm\(^{-2}\) (P < 0.05; n = 5 filters) and that ATP decreased this value to 14 ± 3 nmol·h\(^{-1}\)·cm\(^{-2}\) (P < 0.05).

Table 1. Inhibitory effect of exogenous ATP on AVP- and adenosine-stimulated Ca\(^{2+}\) transport across confluent monolayers of rabbit CNT and CCD cells

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Vehicle</th>
<th>ATP</th>
</tr>
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<tbody>
<tr>
<td>Vehicle</td>
<td>40 ± 2 (12)</td>
<td>41 ± 2 (12)</td>
</tr>
<tr>
<td>AVP</td>
<td>114 ± 5* (12)</td>
<td>80 ± 5* (12)</td>
</tr>
<tr>
<td>Adenosine</td>
<td>51 ± 5 (5)</td>
<td>43 ± 5 (6)</td>
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Values presented are means ± SE of the number of filters given in parentheses. CNT, connecting tubule; CCD, cortical collecting duct. Confluent monolayers were preincubated in the presence of indomethacin (5 µM; both compartments) and absence of ATP (100 µM; both compartments) for 15 min at 37°C. Subsequently, either vehicle or stimulant was added, and net apical-to-basolateral Ca\(^{2+}\) transport was measured for another 90 min. [Arg\(^6\)]vasopressin (AVP, 1 nM) was added to the basolateral compartment, whereas adenosine (10 µM) was applied apically. *Significantly different from corresponding unstimulated monolayers (P < 0.05). †Significantly different from corresponding monolayers stimulated in the absence of ATP (P < 0.05).
Inhibitory effect of chelerythrine on dDAVP- and CPA-stimulated Ca\textsuperscript{2+} transport across confluent monolayers of rabbit CNT and CCD cells

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Vehicle</th>
<th>Chelerythrine</th>
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<tbody>
<tr>
<td>Unstimulated</td>
<td>46 ± 5 (4)</td>
<td>ND</td>
</tr>
<tr>
<td>dDAVP</td>
<td>123 ± 4* (4)</td>
<td>83 ± 9† (4)</td>
</tr>
<tr>
<td>CPA</td>
<td>44 ± 4 (3)</td>
<td>ND</td>
</tr>
<tr>
<td>CPA</td>
<td>111 ± 18* (5)</td>
<td>54 ± 4† (4)</td>
</tr>
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Values presented are means ± 5E of the number of filters given in parentheses. Confluent monolayers were preincubated in the presence of indomethacin (5 µM; both compartments) and absence or presence of chelerythrine (5 µM; both compartments) for 15 min at 37°C. Subsequently, either vehicle or stimulant was added, and net apical-to-basolateral Ca\textsuperscript{2+} transport was measured for another 90 min. 1-Desamino-8-D-arginine vasopressin (dDAVP, 1 nM) was added to the basolateral compartment, whereas N\textsuperscript{6}-cyclopentyladenosine (CPA, 10 µM) was applied apically. *Significantly different from corresponding unstimulated monolayers (P < 0.05). †Significantly different from corresponding monolayers stimulated in the absence of chelerythrine (P < 0.05).

Table 2. Inhibitory effect of chelerythrine on dDAVP- and CPA-stimulated Ca\textsuperscript{2+} transport across confluent monolayers of rabbit CNT and CCD cells

ATP inhibition of the dDAVP-induced increase in adenylyl cyclase activity. To evaluate the effect of ATP on receptor-mediated adenylyl cyclase activation, the accumulation of cAMP was measured in the presence of the inhibitor of cyclic nucleotide phosphodiesterase activity, Ro-20-1724 (0.1 mM; both compartments). Under these conditions, cAMP accumulation in unstimulated monolayers amounted to 1.6 ± 0.3 nmol·15 min\textsuperscript{-1}·filter\textsuperscript{-1} (n = 4 filters). dDAVP (1 nM; basolateral compartment) increased the cAMP content by 6.3 ± 0.6 nmol·15 min\textsuperscript{-1}·filter\textsuperscript{-1} (P < 0.05; n = 4 filters), and ATP (100 µM; both compartments) significantly reduced this increase to 3.3 ± 0.6 nmol·15 min\textsuperscript{-1}·filter\textsuperscript{-1} (P < 0.05; n = 4 filters; Fig. 4). The percentage inhibition was calculated to be 48%. ATP did not affect basal cAMP accumulation (see also, Fig. 5).

Effect of PKC downregulation on ATP inhibition of dDAVP-induced adenylyl cyclase activation. To test the possibility that ATP acts via a phorbol ester-sensitive PKC isoform to inhibit dDAVP-induced adenylyl cyclase activation, monolayers were pretreated with PMA (0.1 µM; both compartments) for 120 h. Figure 4 shows that dDAVP readily increased cAMP by 4.9 ± 0.3 nmol·15 min\textsuperscript{-1}·filter\textsuperscript{-1} and that ATP still significantly lowered this increase to 2.6 ± 0.2 nmol·15 min\textsuperscript{-1}·filter\textsuperscript{-1} in PMA-treated monolayers (P < 0.05; n = 9). In the absence of ATP, the dDAVP-induced increase in cAMP was slightly but significantly (P < 0.04) reduced after chronic PMA treatment, whereas, in the presence of chronic PMA, the above findings can be taken as evidence that ATP acts via one or more PMA-sensitive PKC isoforms to inhibit the stimulatory effect of endogenously produced prostanoids.

Effect of PKC downregulation on ATP inhibition of dDAVP-, CPA-, and 8-BrCAMP-stimulated Ca\textsuperscript{2+} transport. Short-term activation of PKC by the phorbol ester PMA was shown to result in a rapid and marked inhibition of Ca\textsuperscript{2+} transport (2). In the continuous presence of PMA, however, Ca\textsuperscript{2+} transport gradually recovered. This recovery reflects the disappearance or downregulation of PMA-sensitive PKC isoforms. It was also demonstrated that ATP failed to inhibit Ca\textsuperscript{2+} transport in monolayers treated with PMA for a prolonged period of time (21). Because Ca\textsuperscript{2+} transport measurements were performed in the absence of indomethacin, the above findings can be taken as evidence that ATP acts via one or more PMA-sensitive PKC isoforms to inhibit the stimulatory effect of endogenously produced prostanoids.
ATP, no difference ($P > 0.2$) between the two groups was observed.

Effect of ATP on dDAVP stimulation of Ca$^{2+}$ transport and activation of adenylyl cyclase in PTX-treated monolayers. To investigate the possibility that ATP might act via the inhibitory GTP-binding protein (G$_i$) to inhibit dDAVP stimulation of Ca$^{2+}$ transport and activation of adenylyl cyclase, monolayers were pretreated with PTX (170 ng/ml) for 24 h. Figure 5 shows that this treatment did not interfere with the inhibitory effect of ATP on dDAVP-stimulated Ca$^{2+}$ transport (A; $P < 0.05$; $n = 4$ filters) and cAMP accumulation (B; $P < 0.05$; $n = 4$ filters). This demonstrates that ATP does not act via G$_i$ to exert its inhibitory effect. PTX did not affect basal cAMP accumulation but significantly potentiated the stimulatory effect of dDAVP both in the absence ($P < 0.05$; $n = 4$ filters) and presence ($P < 0.05$; $n = 4$ filters) of ATP. By contrast, PTX did not affect the dDAVP-induced increase in Ca$^{2+}$ transport either in the absence ($P > 0.8$; $n = 4$ filters) or presence ($P > 0.9$; $n = 4$ filters) of ATP. This is in agreement with the idea that cAMP does not play a role in dDAVP-stimulated Ca$^{2+}$ transport (16). As a control, we tested the effect of PTX on CPA inhibition of AVP-induced cAMP formation. CPA inhibited AVP-induced cAMP accumulation by 79% (see also, Ref. 17), and PTX abolished this inhibitory effect of CPA. PTX did not change CPA-stimulated Ca$^{2+}$ transport (transport rates of 110 ± 5 and 116 ± 8 nmol·h$^{-1}$·cm$^{-2}$ for untreated and PTX-treated monolayers, respectively, $n = 3$ filters, $P > 0.2$). This demonstrates that CPA does not interact with a G$_i$ protein to activate the novel cAMP-independent pathway leading to Ca$^{2+}$ transport.

**Fig. 4.** Effect of chronic phorbol ester treatment on ATP inhibition of dDAVP-induced cAMP accumulation in monolayers of rabbit CNT and CCD cells. Confluent monolayers were pretreated without (untreated) or with (PMA-treated) 12-O-tetradecanoylphorbol 13-acetate (PMA) for 120 h at 37°C. Indomethacin (5 μM; both compartments) and Ro-20–1724 (100 μM; both compartments) were added without (open bars) or with (filled bars) ATP (100 μM; both compartments) at 15 min before stimulation with dDAVP (1 nM; basolateral compartment). Accumulation of cAMP was measured for another 15 min. Basal cAMP accumulation in unstimulated monolayers amounted to 1.6 ± 0.1 nmol·15 min$^{-1}$·filter$^{-1}$ (n = 4 filters). Data presented show the change in cAMP accumulation above basal and are means ± SE of 4 (untreated) and 9 (PMA-treated) filters. *Significantly different from corresponding untreated monolayers ($P < 0.05$). #Significantly different from corresponding monolayers stimulated in the absence of ATP ($P < 0.05$).

**Fig. 5.** Effect of pertussis toxin (PTX) on ATP inhibition of dDAVP-stimulated Ca$^{2+}$ transport and cAMP accumulation in monolayers of rabbit CNT and CCD cells. Confluent monolayers were pretreated without (open bars) or with (filled bars) PTX (170 ng/ml) for 24 h at 37°C. A: monolayers were preincubated with indomethacin (5 μM; both compartments) and ATP (100 μM; both compartments) for 15 min before stimulation with dDAVP (1 nM; basolateral compartment). Ca$^{2+}$ transport was determined at 90 min after the onset of stimulation. B: Indomethacin (5 μM; both compartments), Ro-20–1724 (100 μM; both compartments), and ATP (100 μM; both compartments) were added 15 min before stimulation with dDAVP (1 nM; basolateral compartment), and accumulation of cAMP was measured for another 15 min. Data presented are means ± SE of 4 filters. *Significantly different from corresponding untreated monolayers ($P < 0.05$). **Significantly different from corresponding monolayers stimulated in the absence of ATP ($P < 0.05$). $Significantly different from corresponding monolayers not treated with PTX ($P < 0.05$).

**DISCUSSION**

The data obtained in the present study are incorporated in a model of the hormonal regulation of active Ca$^{2+}$ reabsorption in rabbit CNT and CCD. The model, schematized in Fig. 6, includes the interaction of ATP with apical and basolateral receptors to inhibit hormone-stimulated Ca$^{2+}$ transport via a phorbol ester-sensitive PKC isoform and vasopressin-stimulated Ca$^{2+}$ transport via a phorbol ester-insensitive mechanism. Of
Inhibition of AVP- and dDAVP-stimulated Ca\(^{2+}\) transport. This work shows that ATP can markedly inhibit AVP- and dDAVP-stimulated Ca\(^{2+}\) transport in a primary culture of rabbit CNT and CCT cells. Inhibitory effects of ATP have also been reported for AVP-stimulated water transport in perfused rabbit CNT (30) and rat IMCD (20). ATP inhibition of AVP- and dDAVP-stimulated Ca\(^{2+}\) transport was found to be paralleled by a reduction of dDAVP-stimulated cAMP accumulation. At first sight, the latter finding supports the "classic" concept that dDAVP interacts with V\(_2\) receptors to stimulate Ca\(^{2+}\) transport in a cAMP-dependent fashion. However, in a recent study, we provided evidence that cAMP is not involved in AVP-stimulated Ca\(^{2+}\) transport (16). Moreover, we showed that AVP-stimulated Ca\(^{2+}\) transport, although insensitive to PKC downregulation by chronic phorbol ester treatment, was inhibited by chelerythrine. Similarly, chelerythrine inhibited the effect of dDAVP in the present study. These findings suggest the involvement of a chelerythrine-inhibitable, phorbol ester-insensitive PKC isoform in the mechanism of action of AVP and dDAVP. In this context, the present observation that ATP reduces AVP- and dDAVP-stimulated Ca\(^{2+}\) transport indicates that the nucleotide inhibits the V\(_2\) receptor-mediated activation and/or action of this "novel" chelerythrine-inhibitable pathway.

ATP also inhibited the stimulatory effect of 8-BrcAMP on Ca\(^{2+}\) transport. This demonstrates the potential of ATP to inhibit also the classic cAMP-dependent pathway and that inhibition occurs at a level at or beyond protein kinase A. As far as the AVP-induced increase in cAMP is concerned, we previously speculated that it occurs in a compartment that does not affect Ca\(^{2+}\) transport, whereas a generalized increase in cAMP, as produced by 8-BrcAMP, does stimulate Ca\(^{2+}\) transport (16).
PKC to inhibit Ca\(^{2+}\) transport was demonstrated previously by means of the membrane-permeable diacylglycerol analog 1,2-dioctanoylglycerol (21) and the potent PKC activator PMA (2). Both PKC activators instantaneously inhibit dDAVP-stimulated Ca\(^{2+}\) transport (J. van Baal, S. E. van Emst-de Vries, R. J. M. Bindels, and P. H. G. M. Willems, unpublished observations) and Ca\(^{2+}\) transport stimulated via the autostimulatory pathway (2). However, this inhibition was only temporary and gradually diminished during continued PMA treatment. Finally, we showed that ATP inhibition of the autostimulatory pathway was abolished when phorbol ester-sensitive PKC isoforms were downregulated by chronic PMA treatment (21). Similarly, the present study shows that chronic PMA treatment abolished ATP inhibition of CPA-stimulated Ca\(^{2+}\) transport. These findings demonstrate that ATP acts via a phorbol ester-sensitive PKC isoform to inhibit stimulation of Ca\(^{2+}\) transport through the prostanoid receptor and the adenosine A\(_1\) receptor and, as can be deduced from the instantaneous inhibition of dDAVP-stimulated Ca\(^{2+}\) transport by PMA (see above), also through the vasopressin V\(_2\) receptor. Because all three receptors act via the novel chelerythrine-inhibitable pathway, these findings suggest that ATP acts via the phorbol ester-sensitive mechanism to inhibit the action of this novel pathway. However, chronic PMA treatment did not affect ATP inhibition of dDAVP-stimulated Ca\(^{2+}\) transport. This indicates that ATP acts in addition via a phorbol ester-insensitive mechanism to inhibit stimulation of Ca\(^{2+}\) transport through the vasopressin V\(_2\) receptor.

Chronic PMA treatment did not affect ATP inhibition of dDAVP-evoked cAMP formation. This suggests that ATP acts via a phorbol ester-insensitive mechanism to inhibit dDAVP-induced cAMP formation. It is tempting to speculate that the same phorbol ester-insensitive mechanism attenuates both V\(_2\) receptor-mediated adenylyl cyclase activation and activation of the chelerythrine-inhibitable pathway and therefore acts at the V\(_2\) receptor itself. Such a mechanism would also fit with the idea that the measured changes in cAMP are an epi-phenomenon unrelated to the stimulatory action of dDAVP on Ca\(^{2+}\) transport (16).

Chronic PMA treatment abolished ATP inhibition of 8-Br-cAMP-stimulated Ca\(^{2+}\) transport. This indicates that ATP acts via a phorbol ester-sensitive PKC isoform to inhibit stimulation of Ca\(^{2+}\) transport via the classic cAMP-dependent pathway at or beyond the level of protein kinase A.

ATP inhibition does not involve a PTX-sensitive G protein. The present work shows that PTX did not interfere with the inhibitory action of ATP on dDAVP-stimulated cAMP accumulation and Ca\(^{2+}\) transport. This lack of effect of the toxin on dDAVP-induced cAMP formation excludes the possibility that ATP interacts with a receptor that couples to G\(_i\) to inhibit adenylyl cyclase. By contrast, Rouse et al. (30) demonstrated that ATP inhibits AVP-stimulated water transport in rabbit CCD via the activation of both PKC and G\(_i\).

PTX markedly potentiated dDAVP-induced cAMP accumulation without affecting, however, dDAVP-stimulated Ca\(^{2+}\) transport. This is in agreement with the idea that dDAVP does not act via cAMP to increase Ca\(^{2+}\) transport. Presently, the mechanism underlying the potentiating effect of PTX on dDAVP-induced cAMP accumulation is unclear. One possibility is that dDAVP not only stimulates adenylyl cyclase via G\(_s\) but also inhibits the enzyme via G\(_i\).

In conclusion, the present study shows that ATP acts primarily via a phorbol ester-sensitive PKC isoform to inhibit hormonal stimulation of Ca\(^{2+}\) transport at the level of the novel chelerythrine-inhibitable pathway involving a phorbol ester-insensitive PKC isoform. Presently, it is unknown at which of the steps involved in transcellular Ca\(^{2+}\) transport, i.e., apical Ca\(^{2+}\) influx, cytosolic diffusion of Ca\(^{2+}\) bound to Ca\(^{2+}\)-binding protein, and basolateral Ca\(^{2+}\) extrusion, the chelerythrine-inhibitable pathway acts to exert its stimulatory effect. However, it is tempting to speculate that the Ca\(^{2+}\) influx step is rate limiting and that regulation takes place at this level. Recent elucidation of the primary structure of the apical Ca\(^{2+}\) influx channel (18) reveals the presence of seven potential PKC phosphorylation sites, and future studies may decide whether or not these sites are involved in the hormonal regulation of Ca\(^{2+}\) reabsorption. In addition, ATP acts via a phorbol ester-insensitive mechanism to inhibit V\(_2\) receptor-mediated concomitant activation of this novel pathway and adenylyl cyclase. Finally, ATP acts through a phorbol ester-sensitive PKC isoform to inhibit Ca\(^{2+}\) transport evoked by a generalized increase in cAMP. The latter finding demonstrates the potential of ATP to inhibit the classic cAMP-dependent pathway at a level at or beyond protein kinase A.

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