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Extracellular ATP Activates Both Ca^{2+}- and cAMP-dependent Cl^- Conductances in Rat Epididymal Cells

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Abstract. Activation of Ca^{2+} and cAMP-dependent Cl^- conductances by extracellular ATP was studied using the whole-cell patch clamp technique. Immediately after addition of extracellular ATP (10 μM), activation of whole-cell Cl^- current exhibiting delayed inactivation and activation kinetics at hyperpolarizing and depolarizing voltages, respectively, was observed. After prolonged activation, the kinetic characteristics of the ATP-induced Cl^- current became time- and voltage-independent. When applied to the later phase of the ATP-activated whole-cell current, the disulfonic acid stilbene DIDS (200 μM) could only inhibit 64% of the current while diphenylamine-dicarboxylic acid (DPC, 1 mM) completely inhibited it. Inclusion of a peptide inhibitor for protein kinase A (PKI, 10 nM) in the pipette solution blocked ATP-induced time- and voltage-independent current activation but did not affect the delayed activating and inactivating current activation but did not affect the delayed activating and inactivating current which could be totally blocked by DIDS. Anion selectivity sequence was determined in the presence of either PKI or DIDS and found to be significantly different. Increased pipette EGTA (10 mM) or treatment of the cells with trifluoperazine (40 μM), an inhibitor of calmodulin, suppressed both types of ATP-induced Cl^- currents. No current activation by ATP was observed when cells were dialyzed with the IP_3 receptor blocker, heparin (10 ng/ml). These results suggest that extracellular ATP activates IP_3-linked Ca^{2+}-dependent regulatory pathway, which in turn activates cAMP-dependent pathway, leading to activation of both Ca^{2+} and cAMP-dependent Cl^- conductances in epididymal cells.

Key words: ATP — Cl^- conductances — Epididymis — cAMP — Ca^{2+} — Calmodulin

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Introduction

It has been suggested that epididymal spermatozoa could control their own fluid environment by releasing certain substances such as ATP into the lumen of the epididymis to regulate electrolyte and fluid secretion across epididymal epithelium (Wong, 1990). Extracellular ATP has been shown to stimulate Cl^--mediated short-circuit current (I_{SC}) across rat epididymal epithelium (Wong, 1988*a*). Previous studies have also shown that ATP-stimulated I_{SC} exhibits a biphasic characteristic and that the two peaks of the ATP-induced I_{SC} show differential sensitivity to different Cl^- channel blockers, indicating possible activation of different Cl^- conductances (Chan et al., 1995). Increases in intracellular Ca^{2+} and cAMP in response to ATP stimulation have also been observed in the epididymal cells, further suggesting the involvement of different regulatory pathways in mediating the ATP response (Leung, Tai & Wong, 1993; Chan et al., 1995). However, only P_2 receptors, and not P_1 receptors, have been found to be involved in mediating the ATP stimulation in epididymal cells (Wong, 1988*a;* Chan et al., 1995);

It is generally believed that purinergic activation of cAMP and Ca^{2+} pathways involves P_1 and P_2 receptors, respectively. Therefore, it remains to be elucidated how the cAMP-dependent pathway is activated by extracellular ATP in epididymal cells since ATP activation in these cells has been found to involve P_2 receptors only. We have proposed that extracellular ATP stimulates a P_2 receptor-mediated increase in intracellular Ca^{2+} which activates the Ca^{2+}-dependent Cl^- conductance on one hand, and somehow stimulates cAMP production leading to activation of the cAMP-dependent Cl^- conductance on the other hand. To prove this hypothesis, it is important to demonstrate the activation of both Ca^{2+} and cAMP-dependent Cl^- conductances by ATP and to show that the activation of cAMP-dependent Cl^- conductance is Ca^{2+} dependent. The present study aimed to investigate the

details of this signal transduction mechanism in rat epididymal cells using the whole-cell patch clamp technique.

Previous whole-cell patch clamp studies have shown the presence of distinct types of Cl^- conductances in rat epididymal cells (Huang et al., 1993). These Cl^- conductances can be characterized and distinguished by their time- and voltage-dependent kinetic profiles (Cliff & Frizzell, 1990). While Ca^{2+}- activated Cl^- conductance exhibits delayed inactivation and activation at hyperpolarizing and depolarizing voltages, respectively, cAMP-activated Cl^- conductance is time- and voltage-independent. Therefore, the whole-cell patch clamp technique has the advantage over the short-circuit current measurement in that different conductances could be identified according to their whole-cell current characteristics.

Another advantage of the whole-cell patch clamp technique is that it allows us to introduce membrane impermeant agents, such as Ca^{2+} chelator and IP_3 receptor blocker, to the cytoplasm via patch pipette. The dependence of the ATP-activated conductances on Ca^{2+} and the involvement of IP_3 in the signal transduction were therefore investigated. The mechanism by which Ca^{2+} activates the cAMP-dependent pathway in rat epididymal cells was also studied.

Materials and Methods

Culture of Rat Epididymal Epithelial Cells

The procedures of tissue culture have been previously described (Cuthbert & Wong, 1986; Wong, 1988*b*). Male Sprague-Dawley rats weighing 210 to 230 g were used as the source of tissue. Rats were sacrificed and their lower abdomen was cut opened. The epididymis was separated from the rest of the organ. The tissue was finely chopped with scissors and then digested with 0.25% (W/V) trypsin followed by 0.1% collagenase I. The primary cultures were grown on coverslips in Essential Eagle Minimum Medium (EMEM) completed with fetal calf serum and other supplements. Cultures were incubated for 3–4 days at 32°C in 5% CO_2.

Whole-cell Current Measurement

After 3–4 days in culture, the epididymal cells formed colonies in dish. To isolate single epididymal cells for patch clamp study, the cells were immersed in a bath NaCl solution containing low Ca^{2+} concentration (120 nmol l^{-1}) for 10–20 min. The dissociated cells were then allowed to recover from low Ca^{2+} treatment in a solution with similar composition except for 1 mM Ca^{2+} for another 10–15 min before whole-cell current measurements.

Current recordings were obtained using the whole-cell patch clamp technique as described by Hamill and others (Hamill et al., 1981) with a patch clamp amplifier (Axopatch-200 or Axopatch-1D, Axon Instruments, Forster City, CA). Patch pipettes, made from borosilicate glass (Vitrex, Modulohm I/S, Herlev, Denmark), were prepared as previously described (Huang et al., 1992). After formation of whole-cell configuration, the series resistance and cell capacitance were compensated. The control of command voltages was carried out with an IBM-AT compatible computer equipped with interface (TL-1-125, Axon Instruments) and with the software *p*Clamp Version 6. The output current signals, after being filtered through an 8-pole Bessel filter (AI-2040, Axon Instruments) at a cutoff frequency of 1 kHz, were displayed on a chart recorder (Graphic, Yokohama, Japan).

Materials and Solutions

Culture media were from Gibco Laboratories (New York). HBSS, HEPES, *N*-methyl-*D*-glucamine (NMDG), glutamic acid, ethylene glycol-bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) were purchased from Sigma Chemical (St. Louis). Diphenylamine-2,2′-dicarboxylic acid (DPC) was purchased from Riedel-de Haen (Seelze, Germany). Peptide inhibitor for protein kinase A (PKI) and trifluoperazine were purchased from Calbiochem (San Diego, CA).

Unless stated otherwise, the following pipette and bath solutions were used, both containing (in mM): 70 NMDGCl, 1 $MgSO_4$, 1.2 NaH_2PO_4, 10 HEPES, 16 glucose except 0.1 EGTA in the pipette (pH 7.2) and 1 $CaCl_2$ in the bath (pH 7.4). Osmolarity of the solutions was raised to isotonic (300 mOsm) by addition of mannitol, using a vapor pressure osmometer (Wescor 5500, Logan, UT).

Results

ATP-induced Whole-cell Cl^- Currents

Whole-cell currents could be activated upon addition of 10 μM ATP. The current recordings were obtained using pipette and bath solutions containing symmetrical Cl^- concentration. Impermeant cation NMDG was used so that Cl^- was the major permeant ion species. Whole-cell currents were elicited by applying a series of command voltage pulses from a holding potential of −30 mV to potentials between −120 to 120 mV, starting from −120 with 20 mV increment. In 83% of the cells examined (n = 65), ATP-induced whole-cell current exhibited changes in kinetic profiles with time as shown in Fig. 1. Current activation was first observed in an average of 1 min after ATP addition exhibiting delayed activation and inactivation at depolarizing and hyperpolarizing voltages, respectively. Later, usually 3 min after, the whole-cell current profile changed to relatively time independent. The difference in whole-cell characteristics also reflected on their voltage dependence as shown in Fig. 1. The current showing delayed activation and inactivation had an outwardly rectifying current-voltage relationship (Fig. 1*A*), whereas, the time-independent currents exhibited relatively linear I-V relationship (Fig. 1*B*). The observed changes in ATP-activated whole-cell current profiles with time could be due to different Cl^- conductances activated at different times. In 11 out of 65 cells, ATP induced a transient current exhibiting delayed activation and inactivation characteristics as shown in Fig. 2, and no time and voltage-independent current was ob-

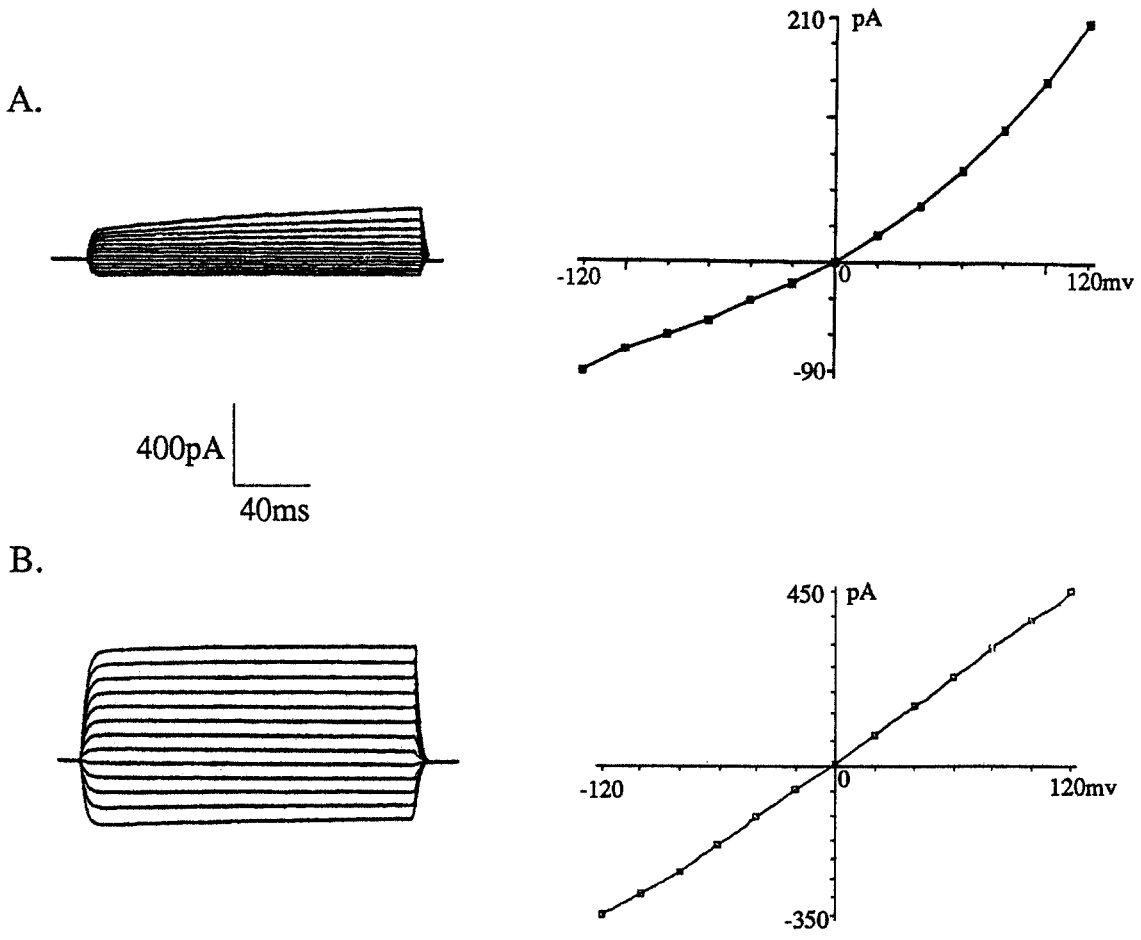

Fig. 1. ATP-activated whole-cell currents exhibiting different *I-V* relationships. Whole-cell currents obtained at different times after addition of 10 μM ATP, (*A*) t = 1 min and (*B*) t = 9 min, with corresponding *I-V* relationships obtained at peak current. The reversal potential is 0 mV (E_{Cl} = 0 mV). Currents were elicited by voltage pulses from a holding potential of −30 mV to potentials between −120 to 120 mV, 20 mV increment (representative of 54 experiments).

served, suggesting that only one type of Cl^- current was activated in these cells.

The reversal potential of the ATP-activated currents in symmetrical Cl^- solutions was close to the Cl^- equilibrium, 0 mV. To further identify that the ATP-activated whole-cell currents were mediated by Cl^- and not through any nonselective conductance, we performed experiments in which Cl^- concentration in the bath was changed from 70 to 140 mM while pipette solution containing 70 mM NMDGCl was used. As shown in Fig. 3 the reversal potential was shifted to a value close to the new equilibrium for Cl^-, 19.2 ± 0.4 mV (n = 4) as compared to the theoretic value of −17.6 mV. The results suggested that currents activated by extracellular ATP were mediated by Cl^-.

The ATP-activated whole-cell currents could be blocked by Cl^- channel blockers. As shown in Fig. 4*A*, when applied to the later phase, DIDS (200 μM) could partially inhibit the ATP-activated currents (64.4% ± 5.5, n = 21) and the residual currents could be totally blocked by DPC (1 mM, *data not shown*). Application of DPC without pretreatment of DIDS also completely suppressed the ATP-activated whole-cell currents (n = 5) as shown in Fig. 4*B*. The effects of DIDS and DPC on the ATP-activated currents are summarized in Fig. 4*C*.

Effect of Protein Kinase A Inhibitor

The above data suggest that two types of Cl^- conductances could be activated by ATP. Previous results from radioimmunoassay have shown an increase of cAMP in response to ATP stimulation (Chan et al., 1995); it remains to be determined whether cAMP-dependent Cl^- conductance could indeed be activated by ATP. The effect of a peptide inhibitor for protein kinase A (PKI, Cheng et al., 1989), contained in the pipette solution, on the ATP-activated whole-cell Cl^- currents was examined. Current activated by forskolin, an activator of adenylate cyclase (Seamon, Pudgett & Daly, 1981), exhib-

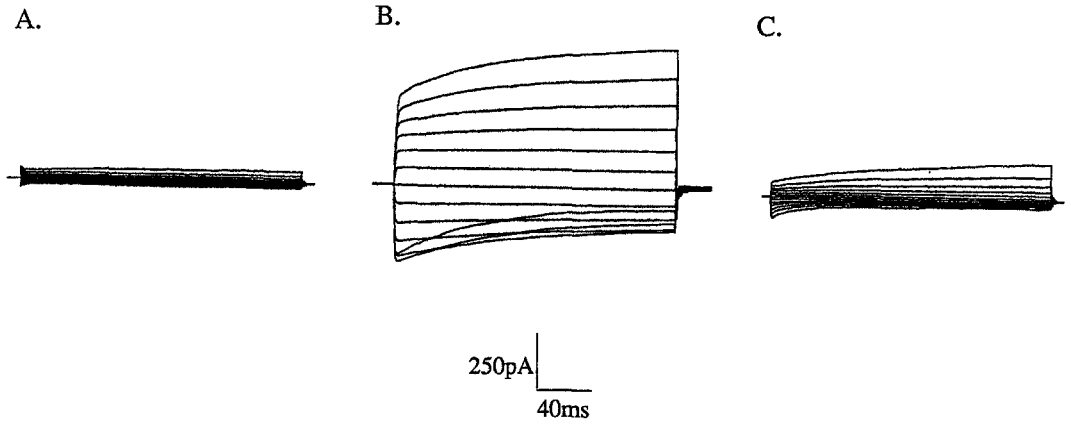

Fig. 2. ATP-activated transient whole-cell current. Transient whole-cell current recordings (11 out of 65 cells examined) obtained at $t = 0$ (*A*), $t = 3$ min (*B*) and $t = 10$ min (*C*). Experimental conditions were similar to that described in Fig. 1.

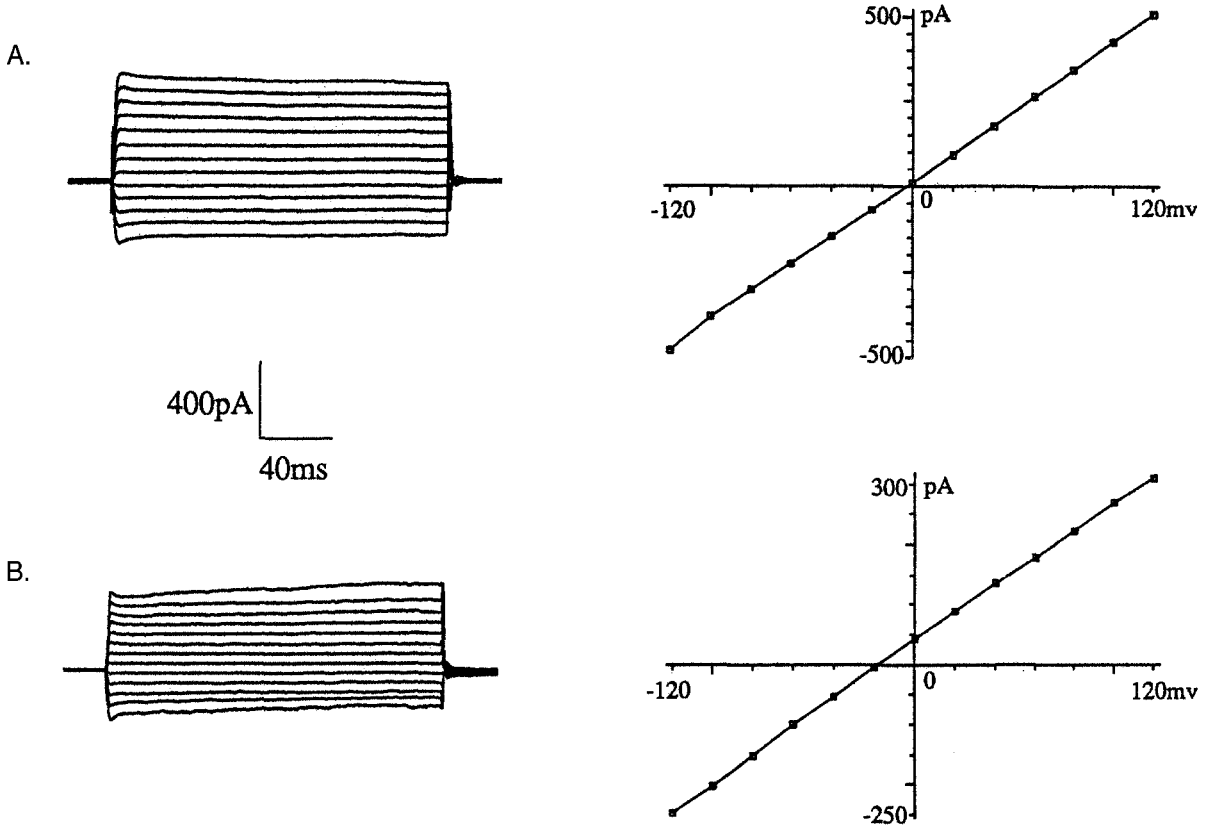

Fig. 3. Demonstration of the involvement of Cl^- in ATP-activated currents. Whole-cell recordings obtained 5 min after ATP stimulation from a cell patched with pipette containing 70 mM NMDGCl and bathed in 70 mM NMDGCl (*A*) and 140 mM NMDGCl (*B*) with corresponding *I-V* relationships. Note that reversal potential was shifted from 0 to −18.8 mV (−19.2 ± 0.4 mV, n = 4), close to the new $E_{Cl} = -17.6$ mV.

iting time and voltage independence as shown in Fig. 5*A*, could be completely suppressed by PKI (10 nM). However, as shown in Fig. 5*B*, it did not affect the ATP-activated current exhibiting delayed inactivation and activation at hyperpolarizing and depolarizing voltages, respectively. This PKI-insensitive current could be completely blocked by DIDS (n = 4, Fig. 5*C*). The changes in current characteristics with time, which were usually observed in the absence of PKI, were not observed in the presence of PKI (meaning that the current

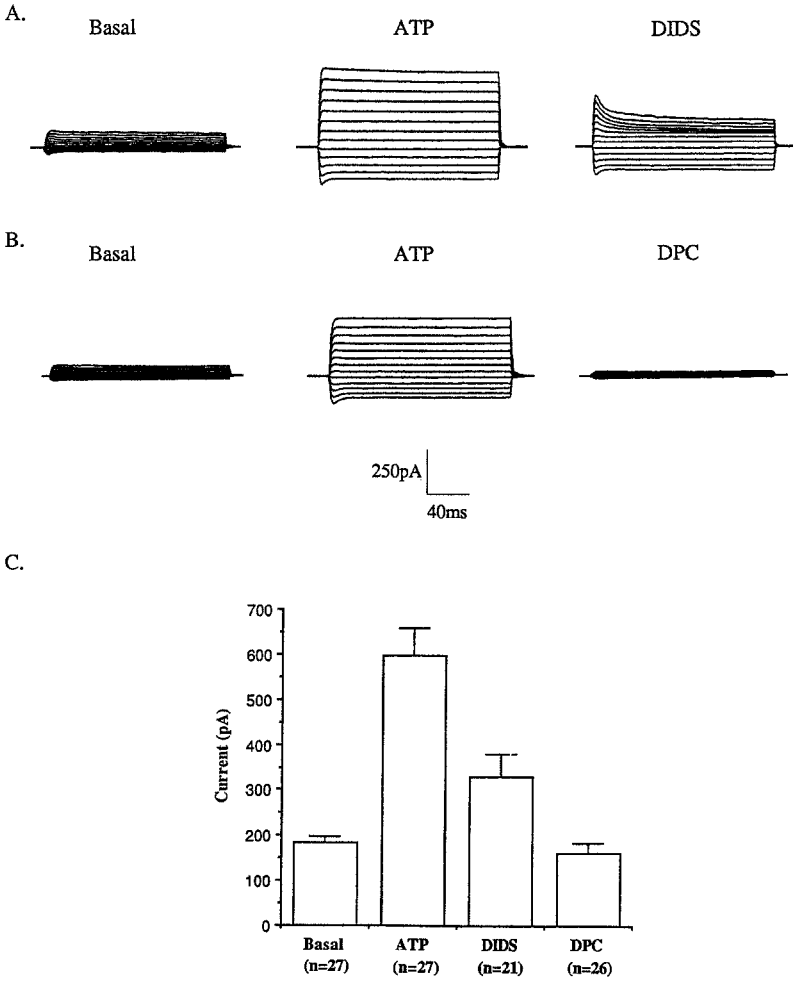

Fig. 4. Inhibition of the ATP-activated whole-cell currents by Cl^- channel blockers. Current recordings before and after exposure of single voltage-clamped cells to 200 μM DIDS (*A*) 7 min after ATP) and 1 mM DPC (*B*) 9 min after ATP). Experimental conditions are similar to those described in Fig. 1. (*C*). Summary of the effect of blockers on the mean current magnitude measured at ±120 mV. DIDS and DPC significantly reduced the ATP-activated currents ($P < 0.05$ and 0.001, respectively).

shown in Fig. 5*B* would not change with time). The lack of activation of time-independent current by ATP upon elimination of cAMP-dependent pathway by PKI suggested that this type of current was cAMP-dependent. The effect of PKI on forskolin and ATP-activated current magnitude, measured at +120 mV, and the effect of subsequent addition of DIDS on the current, are summarized in Fig. 5*C*.

Selectivity sequences of the Two Types of ATP-induced Cl^- Conductances

To further characterize the two types of ATP-activated Cl^- currents, their anion selectivity sequences were determined. Efforts were made to isolate one type of current from the other. Current with delayed activation and inactivation characteristics was obtained by using PKI in the pipette solution (*see above*). The selectivity sequence for this type of current was I^- (2.4) > Br^- (1.5 > Cl^- (1.0), (n = 5). DIDS was used in the experiments in which the selectivity sequence for the DIDS-insensitive ATP-activated current was determined: Cl^- (1.0) > I^- (0.8) > Br^- (0.6), (n = 6).

Inhibition of ATP-activated Cl^- Currents by Suppression of Intracellular Free Ca^{2+}

Since a rise of intracellular Ca^{2+} in response to ATP stimulation has been observed (Leung, Tai & Wong, 1993), it would be likely that Ca^{2+}-dependent Cl^- conductance could be activated. By increasing EGTA, a Ca^{2+} chelating agent, in the pipette solution to suppress intracellular free Ca^{2+}, the activation of Ca^{2+}-dependent Cl^- conductance should be blocked. In such experimental setting, the dependence of the ATP-induced cAMP-activated Cl^- conductance on intracellular Ca^{2+} could also be tested. If the cAMP-dependent pathway was independent of intracellular Ca^{2+}, increased pipette EGTA should only affect the Ca^{2+}-dependent ATP-activated current and not the cAMP-dependent current. When pipette EGTA was increased from 0.5 to 10 mM, neither type of currents was observed upon stimulation by ATP

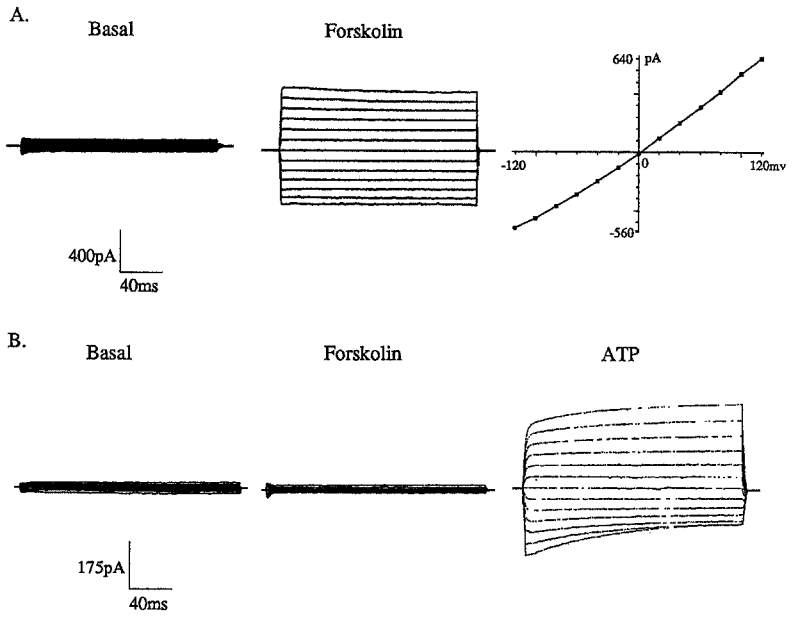

Fig. 5. Inhibition of the ATP-activated cAMP-dependent whole-cell current by PKI. (*A*) Current recordings before and after exposure of a single voltage-clamped cell to forskolin-containing solution (10 μM) with corresponding *I-V* relationship, exhibiting time and voltage independence. (*B*) Current recordings obtained in the presence of 10 nM PKI (in pipette) before and after addition of forskolin (10 μM) and ATP (10 μM, t = 7 min). (*C*) Summary of the effect of PKI and DIDS (200 μM) on the mean current magnitude measured at +120 mV. A value of $P < 0.05$ is obtained when the ATP-activated current is compared to each of other groups independently.

(*data not shown*, n = 7), indicating that both ATP-activated Cl^- conductances were Ca^{2+}-dependent. However, under high EGTA conditions, forskolin could activate Cl^- current (*data not shown*) suggesting that the cAMP pathway could be independently stimulated.

The Involvement of IP_3

Although an increase of intracellular Ca^{2+} in response to ATP, presumably via IP_3, has been shown (Leung, Tai & Wong, 1993), the involvement of IP_3 in the signal transduction pathway has not been demonstrated. Many reports have documented that heparin is able to block IP_3 receptors (Nilsson et al., 1988). By including heparin in the pipette solution, we wanted to test whether IP_3 was involved in mediating ATP response. When used at 10 ng/ml, heparin totally blocked ATP response (n = 6), as shown in Fig. 6; however, when adrenaline was added to 3 of the heparin-treated cells response was recorded, indicating that the effect of heparin on ATP response was specific. The ability of heparin to block both ATP-activated Ca^{2+} and cAMP-dependent Cl^- currents suggested that IP_3 was involved in an intermediate step before Ca^{2+} mobilization and cAMP activation by ATP.

Activation of cAMP-dependent Pathway by Ca^{2+} is Calmodulin-dependent

Previous study using radioimmunoassay (Chan et al., 1995) and the results from the present study (*see above*) indicated that activation of the cAMP-dependent pathway by extracellular ATP was Ca^{2+} dependent. We wanted to investigate how the cAMP cascade in epididymal cells could be activated by an ATP-stimulated increase in intracellular Ca^{2+}. Treating the cells with 40 μM of trifluoperazine, an inhibitor of calmodulin (Massom, Lee & Jarrett, 1990), resulted in total blockade of ATP-induced whole-cell currents; however, current ac-

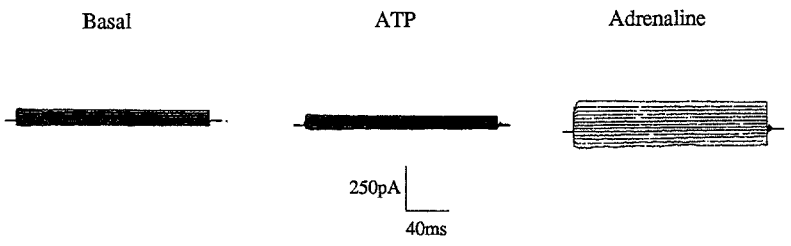

Fig. 6. Blocking effect of heparin on the ATP-activated whole-cell currents. Current recordings before and after exposure of a single voltage-clamped cell to ATP (10 μM, t = 7 min) and adrenaline (5 μM) in the presence of 10 ng/ml heparin (in pipette). Note that ATP-activated currents were completely abolished in the heparin-treated cell (n = 6) while the same cell still responded to adrenaline (n = 3), indicating that the effect of heparin was specific.

tivation by forskolin was still observed in the same cells (n = 3, *data not shown*), suggesting that ATP-activated cAMP cascade via Ca^{2+} in epididymal cells involved calmodulin.

Discussion

Activation of Cl^- channels or stimulation of Cl^- secretion by extracellular ATP has been reported in a number of secretory epithelia including the airway (Stutts et al., 1992; Fedan et al., 1993), colonic T84 cells (Dho, Stewatt & Foskett, 1992), breast epithelial cells MCF-7 (Flezar & Heisler, 1993) and the epididymis (Wong, 1988*a;* Leung, Tai & Wong, 1993). However, the action of ATP and the signal transduction pathways involved in mediating the ATP response are quite different in the tissues and cells examined. While direct stimulation of Cl^- channels by ATP binding has been reported in normal and cystic fibrosis airway epithelial cells, Ca^{2+} mobilization linked to P_2 receptors has been found in human breast tumor and rat epididymal cells. Additional involvement of P_1 receptors in mediating ATP-stimulated cAMP-dependent Cl^- secretion has also been reported in the colonic T84 cells.

Previous studies in rat cauda epididymal cells using the short-circuit current technique, fluorescence measurement and radioimmunoassay (Chan et al., 1995; Leung, Tai & Wong, 1993) have indicated the involvement of Ca^{2+} and cAMP in ATP-stimulated Cl^- secretion. The present study using the whole-cell patch clamp technique has further demonstrated that both Ca^{2+} and cAMP-dependent Cl^- conductances are indeed activated by extracellular ATP. (i) Cl^- currents with different kinetic characteristics can be observed upon ATP stimulation: one exhibiting delayed inactivation and activation at hyperpolarizing and depolarizing voltages, respectively, and the other exhibiting no dependence on either time or voltages. These kinetic characteristics have been reported for the Ca^{2+}-activated and cAMP-activated Cl^- conductances, respectively, in a number of epithelial cells including the epididymal cells (Cliff & Frizzell, 1990; Huang et al., 1993). (ii) The ATP-induced currents can be inhibited partially by DIDS and completely by DPC. While DPC has been reported to block different types of Cl^- channels, DIDS seems to have less effect on the cAMP-activated Cl^- channel in various epithelial tissues (*for review,* Fuller & Benos, 1992). Our previous study has also demonstrated that DIDS has less effect on the cAMP-activated I_{SC}in epididymal cells (Chan et al., 1995). The present study has also shown that the ATP-activated currents can be completely inhibited by DPC, or, by DIDS in the presence of PKI only (DIDS partially inhibits the ATP-activated currents in the absence of PKI). The differential sensitivity of the ATP-induced Cl^- currents to DPC and DIDS observed in the present study suggests the involvement of different Cl^- conductances, namely, the Ca^{2+} and cAMP-activated Cl^- conductances. (iii) Direct demonstration of the involvement of cAMP-activated Cl^- conductance comes from the experiments using PKI, in which only delayed activating and inactivating current, but not the time and voltage-independent current, was observed. (iv) The involvement of Ca^{2+}-activated conductance is evidenced by suppression of ATP-activated currents by either EGTA or calmodulin inhibitor. (v) The selectivity sequences for the two types of ATP-induced whole-cell Cl^- currents are different: $I^- > Br^- > Cl^-$ for current exhibiting delayed activation and inactivation and $Cl^- > I^- > Br^-$ for the time and voltage-dependent current. The selectivity sequence associated with the ATP-stimulated delayed activating and inactivating current, observed in the presence of PKI, is consistent with that observed for Ca^{2+}-activated Cl^- conductance in the epididymal cells (Huang et al., 1993) and other epithelial cells (Cliff & Frizzell, 1990; Anderson & Welsh, 1991; Chan, Goldstein & Nelson, 1992). The selectivity sequence associated with the time and voltage-independent current is somewhat different from that previously reported for the cAMP-activated Cl^- conductance in epididymal cells which is $Br^- > Cl^- = I^-$ (Huang et al., 1993) and other epithelia (Cliff & Frizzell, 1990; Anderson & Welsh, 1991; Chan, Goldstein & Nelson, 1992). It should be borne in mind that in an effort to eliminate the coexisted Ca^{2+}-activated Cl^- conductance, experiments were done in the presence of the Cl^- channel blocker, DIDS, which might somehow affect the results observed. The important observation is that this selectivity sequence is significantly different from that observed for Ca^{2+}-activated

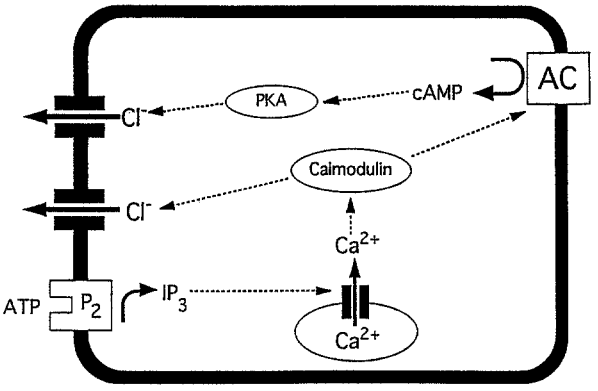

Fig. 7. Proposed model for ATP-coupled signal transduction pathways in rat epididymal cells. Both Ca^{2+} and cAMP Cl^- conductances are indicated by the arrows across the cell membrane. The following abbreviations are used: P_2, P_2-purinoceptor; AC, adenylate cyclase; PKA, protein kinase A.

Cl^- conductance. Taken together, our results have shown the activation of both Ca^{2+} and cAMP-dependent Cl^- conductances by extracellular ATP.

The present study has also demonstrated that the activation of cAMP-dependent Cl^- conductance by ATP is Ca^{2+}-dependent. The supporting evidence includes: (i) ATP-activated time and voltage-independent whole-cell Cl^- current occurs after the current exhibiting delayed activation and inactivation; (ii) increased EGTA concentration in the pipette totally abolishes both types of current activation by ATP while inclusion of PKI only eliminates the time and voltage-independent current. These results are consistent with the previous finding using radioimmunoassay that ATP-stimulated cAMP production is Ca^{2+}-dependent (Chan et al., 1995), suggesting that in the epididymal cells, an increase in $[Ca^{2+}]_i$ could activate the cAMP-dependent pathway. At this point, one may pose a question as to whether Ca^{2+} mobilizing agents could mimic the effect of ATP. Indeed, more than twofold increase in the level of intracellular cAMP has been observed in the epididymal cells in response to the Ca^{2+} ionophore, ionomycin. (Wong & Huang, 1990). Whole-cell current exhibiting time and voltage-independent characteristics, in addition to that exhibiting delayed activation and inactivation, has also been observed in response to ionomycin (*unpublished observation in authors' laboratory*), suggesting that the cAMP-dependent pathway in these cells could be activated by an increase in intracellular Ca^{2+}. It is interesting to note that the same observation of different whole-cell current profiles induced by the Ca^{2+} ionophore, A23187, has also been reported in airway epithelial cells (Chan, Goldstein & Nelson, 1992). It is also interesting to note that not all the cells examined in the present study responded to extracellular ATP with activation of both Ca^{2+} and cAMP-dependent Cl^- currents. Only transient Ca^{2+}-activated current characteristic, but not cAMP-activated current characteristic, was observed in some 15% of the cells examined. This could be explained by insufficient increase in intracellular Ca^{2+} by ATP to further activate the cAMP-dependent pathway in these cells.

Cytosolic Ca^{2+} has been recognized as one of the modulators of the cAMP cascade. When expressed in cultured cells, the type I adenylate cyclase displayed sensitivity to calmodulin as well as to forskolin and G protein s-α subunits (Tang, Krupinski & Gilman, 1991). The binding of Ca^{2+} to calmodulin has been shown to produce a conformational change that allows calmodulin to bind to adenylate cyclase (O'Neil & DeGrado, 1990), and affecting the cAMP cascade. The present results obtained using calmodulin inhibitor, trifluoperazine, also suggests the involvement of calmodulin in the activation of cAMP-dependent pathway via Ca^{2+} in epididymal cells. The total abolishment of ATP-activated currents by trifluoperazine can be explained by the involvement of calmodulin in activation of Ca^{2+}-dependent Cl^- conductance, as previously reported (Wagner et al., 1992; Chan et al., 1992), and in subsequent activation of cAMP cascade (*see above*). Since trifluoperazine has been reported to have other nonspecific effects, confirmation on the involvement of calmodulin in activation of cAMP production requires further study with different stereo isomers of calmodulin antagonists.

By including the inhibitor for IP_3 receptor, heparin, in the pipette, we have also demonstrated in our whole-cell patch clamp experiments that IP_3 is involved in the intermediate step of the ATP signaling pathway in epididymal cells. The fact that heparin completely inhibits the ATP-activated whole-cell currents suggests that IP_3 is involved in a step prior to activation of Ca^{2+} and cAMP cascades. This result is consistent with the well-documented action of P_2-purinoceptors, which are coupled to phosphoinositide hydrolysis leading to an increase in intracellular Ca^{2+} (*for review,* Dubyak & El-Moatassim, 1993).

Based on the current findings and previous results obtained in epididymal cells, we propose a signal transduction model which is schematically illustrated in Fig. 7. This model suggests that activation of cAMP cascade is through the interaction between calmodulin and adenylate cyclase. We have not yet tested the possibility that increased intracellular cAMP upon ATP stimulation could be due to inhibition of phosphodiesterase, which could also be mediated by calmodulin.

In closing, we have demonstrated in rat epididymal cells that both Ca^{2+} and cAMP-dependent Cl^- conductances are activated by extracellular ATP and that activation of cAMP cascade by extracellular ATP is Ca^{2+} and calmodulin dependent.

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