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Regulation of Cl^- channels in normal and cystic fibrosis airway epithelial cells by extracellular ATP

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ABSTRACT **The rate of Cl^- secretion by human airway epithelium is determined, in part, by apical cell membrane Cl^- conductance. In cystic fibrosis airway epithelia, defective regulation of Cl^- conductance decreases the capability to secrete Cl^-. Here we report that extracytosolic ATP in the luminal bath of cultured human airway epithelia increased transepithelial Cl^- secretion and apical membrane Cl^- permeability. Single-channel studies in excised membrane patches revealed that ATP increased the open probability of outward rectifying Cl^- channels. The latter effect occurs through a receptor mechanism that requires no identified soluble second messengers and is insensitive to probes of G protein function. These results demonstrate a mode of regulation of anion channels by binding ATP at the extracellular surface. Regulation of Cl^- conductance by external ATP is preserved in cystic fibrosis airway epithelia.**

A normal function of airway epithelia is modulation of the volume and composition of liquid at the interface between air and the mucosal surface. The regulation of ion channels in the apical membrane of airway epithelial cells appears important for this function. In cystic fibrosis (CF), for example, defective cAMP-mediated regulation of Cl^- channels in airway epithelial cells (1–3) may limit the capacity to secrete Cl^- and contribute to the formation of viscous, underhydrated mucus (4, 5) that obstructs the airways of patients with this disease. Recently, we and others reported that regulation of Cl^- conductance by intracellular Ca^{2+} is functional in CF airway epithelia (6, 7). Thus, approaches to bypass the cAMP regulatory defect in CF could include stratagems to raise intracellular Ca^{2+} activity.

One means of modulating intracellular Ca^{2+} activity is through external receptors on airway epithelial cells. Earlier we demonstrated receptors on the surface of airway epithelial cells for bradykinin, histamine, and nucleotides that activate phospholipase C and raise intracellular Ca^{2+} activity (8). We also found that ATP receptors in the apical cell membrane regulated Cl^- secretion (9). Here, we have determined that the Cl^- secretion induced by ATP exposure of the luminal surface of airway epithelia is from activation of apical membrane Cl^- conductance. With excised patch-clamp techniques, we have found that the effect of luminal ATP may be mediated, in part, by direct stimulation of anion-selective channels.

MATERIALS AND METHODS

Solutions and Drugs. Cultured human airway epithelia for Ussing chamber and microelectrode studies were bathed in a Kreb's bicarbonate Ringer (KBR) solution that contained 140 mM Na^+, 120 mM Cl^-, 5.2 mM K^+, 25 mM HCO_3^-, 2.4 mM HPO_4^{2-}, 0.4 mM HPO_4^-, 1.1 mM Ca^{2+}, 1.2 mM Mg^{2+}, and 5.2 mM glucose. Na^+ salts were replaced by *N*-methyl-D-glucamine or tetraethylammonium chloride in Na^+-free, Cl^--replete KBR solution or by potassium gluconate in high-K^+, low-Cl^- KBR solution. The basic patch-clamp solution contained 140 mM Na^+, 142 mM Cl^-, 1 mM Mg^{2+}, and 5 mM *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), pH 7.4. Ca^{2+} activity and other modifications are described in the figure legends. 5-Nitro-2-(4-phenylpropylamino)benzoic acid (NPPB) was provided by Reiner Greger (Freiburg, F.R.G.). Nucleotides were purchased from Boehringer Mannheim. Amiloride and 4,4'-diisothiocyanatostilbene (DIDS) were purchased from Sigma.

Cell Culture. Human airway epithelial cells were disaggregated from surgical specimens, isolated, and cultured on permeable collagen supports as described (10). Some patch-clamp studies were done on cells grown on collagen-coated glass coverslips under the same conditions.

Ussing Chambers. Polycarbonate Ussing chambers, of otherwise conventional design, were milled to fit the plastic cups that supported the permeable collagen matrix upon which the cells were grown. The short circuit current (I_{sc}) or transepithelial potential difference was measured with a voltage-clamping device (UNC Electronics, Chapel Hill, NC) and plotted on a strip chart recorder. Pharmacologic probes were diluted from stock solutions into the luminal bath. Drug responses were measured as the difference between steady-state I_{sc} measured before drug exposure and the new steady state reached within 1–4 min after exposure.

Microelectrode Studies. Double-barreled Cl^--selective microelectrode techniques have been described in detail (11). Transepithelial current pulses (I, 1–10 μA for 0.5 s) were applied every 6 s, and the resulting deflections of V_t were measured to calculate transepithelial resistance (R_t) or conductance (G_t) as

$$R_t = 1/G_t = \Delta V_t/I. \quad [1]$$

Equivalent short-circuit current (I_{eq}) was calculated from the equation

$$I_{eq} = V_t \cdot G_t. \quad [2]$$

All impalements were perpendicular to the surface of the cell cultures. The fractional apical membrane resistance (fR_a) was calculated as

$$fR_a = R_a/(R_a + R_b) = \Delta V_a/\Delta V_t, \quad [3]$$

where R_a and R_b are resistances of the apical and basolateral membranes, respectively.

Abbreviations: KBR, Kreb's bicarbonate Ringer; MOT, mean open time; MCT, mean closed time; ORCC, outward rectifying Cl^- channel(s); P_o, open probability; DIDS, 4,4'-diisothiocyanatostilbene; NPPB, 5-nitro-2-(4-phenylpropylamino)benzoic acid; CF, cystic fibrosis; I_{sc}, short-circuit current; I_{eq}, equivalent I_{sc}; V_t, R_t, and G_t, transepithelial voltage, resistance, and conductance, respectively; fR_a, fractional apical membrane resistance; V_a, apical membrane potential.

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Patch Clamp. Cells grown on collagen membrane supports or coverslips were studied on the stage of an inverted microscope at 22°C by the techniques of Hamill *et al.* (12). Cl^--selective channels were provisionally identified in excised patches as channels with a reversal potential of −18 to −22 mV in the presence of a 2:1 gradient for Cl^- to leave the pipette. Pipette currents were amplified (List EPC-7) and stored on video cassette tape for later analysis. Most data were low-pass filtered [1 kilocycle (kc)] and digitized at 5 kc, but selected segments were filtered at 5 kc and digitized at 22 kc to detect any contribution of band-width limitation.

Open probability (P_o) was determined as the fraction of digitized points above a threshold set midway between the closed and open peaks of current–amplitude histograms. Each experiment analyzed for P_o contained 80–720 s of continuous data that began with a closed–open event and ended with an open–closed transition (>4800 s total for each group at +40 mV, and >2600 s for each group at −40 mV). Interval analyses were confined to experiments with one channel. Conventional 50% threshold analyses yielded distributions of dwell times that were fit by multiexponential (four or more) or power functions, consistent with multiple open and closed states. Given this complexity of basal kinetics, we report overall mean open time (MOT) and mean closed time (MCT). At −40 mV, MOT and MCT were calculated from 72,445 (control) and 109,670 events (ATP) (see Fig. 2*A Lower*). At +40 mV, outward rectifying Cl^- channels (ORCC) consistently displayed burst/gap behavior (for summary, see Fig. 2*B Lower*). Gaps were defined as closed intervals >500 ms. There were 331 gaps in 46 min of control recordings analyzed and 288 gaps in 45.7 min of ATP experiments. Bursts consisted of all channel activity between gaps. The density of low time resolution traces with ATP results from finite line width and the higher frequency of open–closed transitions within bursts.

We also assessed channel activity as the total current, integrated over time, that flowed through a membrane patch. For each digitized block of data, the closed state was determined from amplitude histograms. This basal current was subtracted from each digitized point and the difference accumulated over block length. The result, plotted as current in pA·mS against block time, is a clear visual indication of cumulative patch Cl^- current. This approach cirvumvents assumptions implicit in conventional P_o analysis.

RESULTS AND DISCUSSION

Cl^- secretion was measured as I_{sc} in cultured normal human airway epithelia pretreated with amiloride (1, 13, 14). Luminal ATP stimulated a sustained increase in current that was preceded in some experiments by a transient peak (Fig. 1*A*). The Cl^- current evoked by ATP was inhibited by a Cl^- channel blocker, NPPB (15). The relationship between maximum increase in current and ATP concentration (Fig. 1*B*) yields an ED_{50} of 44 μM, a value typical of receptor-mediated actions of ATP in solutions containing several millimolar Mg^{2+} (16–18). The change in I_{sc} with 100 μM ATP represents a 60% increase in Cl^- secretion.

The current induced by luminal ATP was associated with depolarization of the apical membrane potential (V_a) and decreases in fR_a and R_t (Fig. 1*C*). In amiloride-pretreated human nasal epithelium, these changes are consistent with a doubling of apical membrane Cl^- conductance and contrast to the pattern of electrical changes (hyperpolarized V_a and increased fR_a) induced by the well-studied Cl^- secretogogues histamine and bradykinin (8). These effects of ATP on V_a and fR_a were not sensitive to substitution of *N*-methyl-D-glucamine (19) (n = 3) or tetraethylammonium (20) (n = 4) for Na^+ in the luminal solution, indicating that ATP did not activate Na^+ or nonselective cation conductances (16).

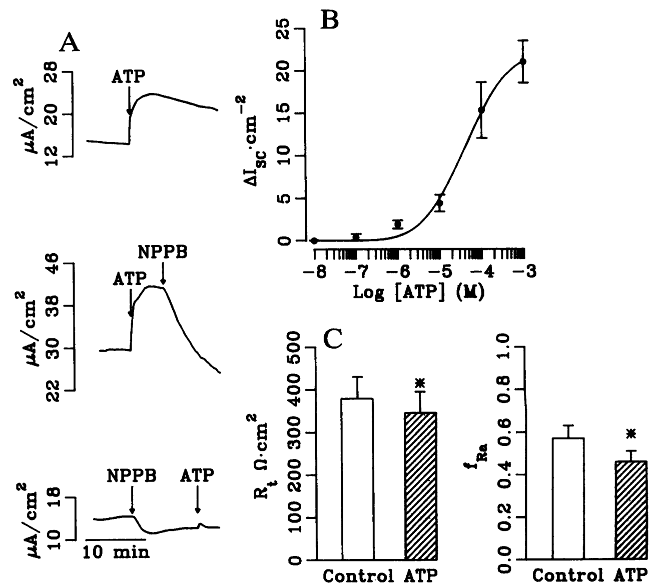

FIG. 1. Transepithelial Cl^- secretion induced by ATP added to the luminal surface. (*A*) Tissues were studied in Ussing chambers under short-circuit conditions and pretreated with amiloride (100 μM) alone or with amiloride with NPPB (100 μM). ATP (100 μM) was then added to the luminal bath. The ATP response after amiloride was 14.9 ± 1.3 $\mu A/cm^2$ (n = 9). The ATP response after amiloride and NPPB was 0.9 ± 0.9 $\mu A/cm^2$ (n = 4). (*B*) Concentration–effect relationships are for maximum change in current stimulated by luminal ATP with 100 μM amiloride. (*C*) Cultured epithelia were studied with microelectrodes. Luminal ATP decreased R_t (−34 ± 9 $\Omega \cdot cm^2$, n = 8) and fR_a (0.12 ± 0.3, n = 8) in amiloride-pretreated tissues. *, $P < 0.02$, paired t analysis.

The mechanism of ATP action on Cl^- conductance at the single-channel level was explored with patch-clamp techniques. In preliminary experiments with outside-out membrane patches, we had detected stimulation of ORCC by external ATP. We confirmed this result by studying the action of 100 μM ATP in the pipette solution of inside-out patches from normal cells (Fig. 2). Under control conditions ORCC were observed in 59 of 304 inside-out patches (19.4%) and in 56 of 243 inside-out patches (23%) with ATP (no difference). However, ATP at the extracellular face of the membrane patch dramatically altered the behavior of ORCC. The simplest analysis of the response shows that extracellular ATP increased overall P_o by 50% at 40 mV and by 40% at −40 mV. This result compares to the effect of luminal ATP on Cl^- secretion. In contrast, 2 mM ATP added to the cytosolic surface of patches containing ORCC had no effect on P_o (n = 3; data not shown).

The complex kinetics of ORCC, including multiple closed and open states (15), burst/gap behavior (21), and voltage-dependent P_o (22), preclude characterization of ATP effects in terms of simple kinetic models (23) (refer to legend for Fig. 2). Nonetheless, the changes in channel kinetics induced by ATP are striking. At 40 mV, the overall increase in P_o is from a 50% decrease in time the channels spent in the inactive state, and P_o within bursts was unaffected (Fig. 2*B*). These effects could represent a different mode of gating (23, 24) or mixed kinetics of activation and blockade (25). At a membrane voltage of −40 mV, near *in vivo* physiologic voltage, the increase in P_o evoked by ATP was from increased MOT; no change occurred in MCT (Fig. 2*A*). Thus, extracellular ATP acted at the external membrane surface to change ORCC gating with the net effect of increased P_o, and consequently, increased Cl^- flow across the patch membrane.

Distinct classes of "purinergic" receptors interact with adenosine (P_1 receptors) or ATP (P_2 receptors) (26, 27). We

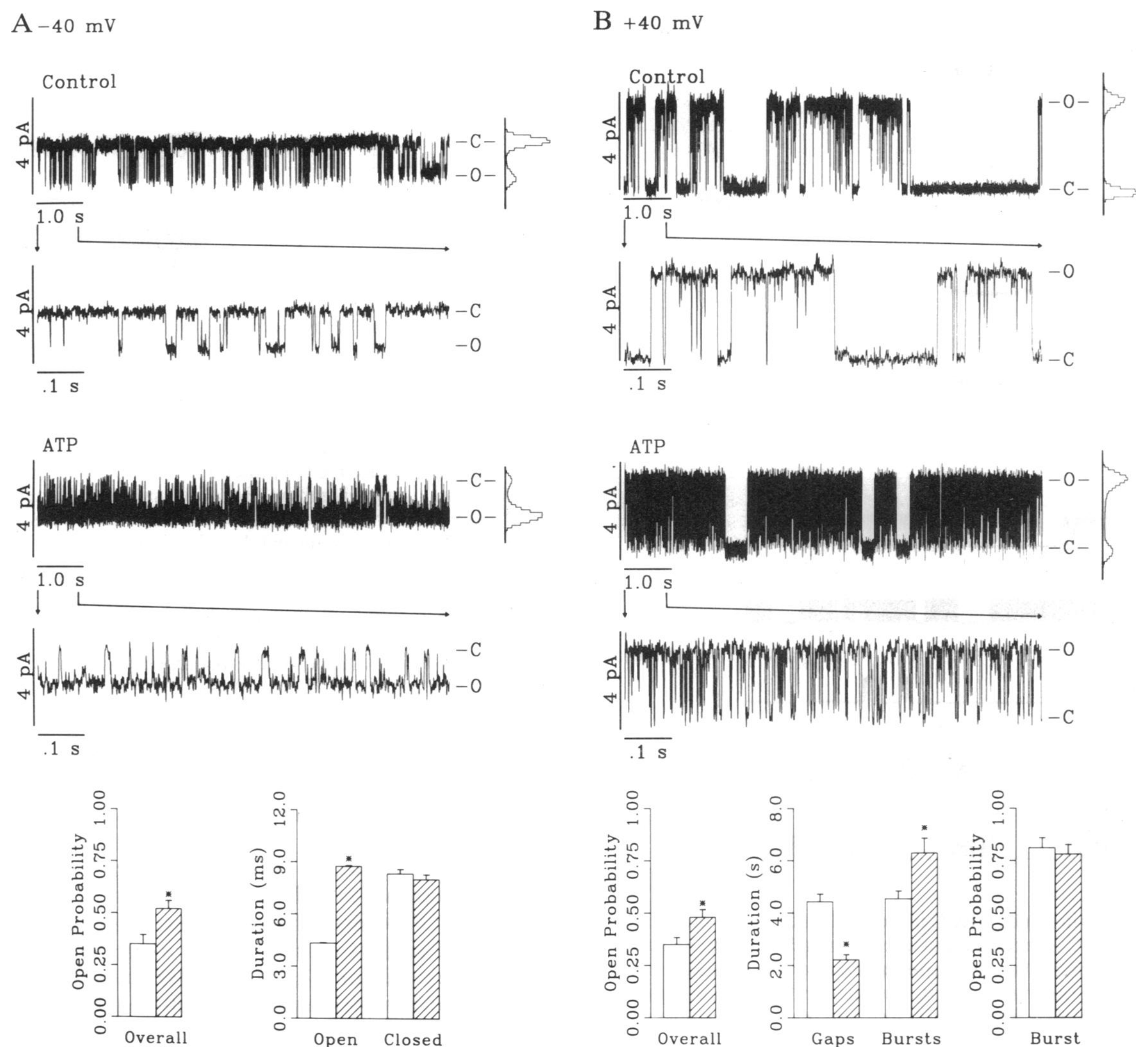

FIG. 2. Effect of external ATP on ORCC in excised, inside-out membrane patches held at −40 mV (*A*) or +40 mV (*B*). Upper portions show representative traces of ORCC activity without (Control) or with 100 μM ATP in pipette solution. The upper trace in each pair is 9 s of data aligned with the current–amplitude histogram for the displayed segment (O, open; C, closed); the lower trace in each pair is an expansion of the first 0.9 s of data from the preceding trace. Bar plots (lower parts of *A* and *B*) depict P_o and interval durations of ORCC from inside-out patches made with (hatched bars) or without 100 μM ATP in the pipette. At +40 mV, control MOT within bursts was 20.46 ± 0.14 ms, and MCT was 4.88 ± 0.10 ms, n = 56,443. With ATP, MOT was 8.41 ± 0.03 ms, and MCT was 2.40 ± 0.03 ms (n = 175,796) (data not shown). *, $P < 0.03$, by unpaired t analysis.

used outside-out membrane patches and sequential exposures to ATP and other nucleotides or nucleosides to study the role of such receptors in the ATP-induced increase in ORCC P_o. Although the characteristics of ORCC in this patch-clamp configuration had not been reported, we observed single-channel properties similar to published data from inside-out patches (15, 21) (Fig. 3*A*). The strong rectification of the channel verified the outside-out patch-clamp mode. In experiments like the one depicted in Fig. 3*B*, P_o was increased by 100 μM ATP (364 ± 86% of control, n = 10), and the effect was reversed by washing. In similar experiments, this reversible effect was observed with the poorly hydrolyzed analog adenosine 5′-[γ-thio]triphosphate at 100 μM/liter (272 ± 62%, n = 8). Thus, the action of ATP on ORCC is unlikely to require conversion of ATP to another form (28). Fig. 3*B* shows that 500 μM adenosine had no effect on ORCC P_o (104 ± 4%, n = 3), although subsequent exposure of the channel to 100 μM ATP again increased P_o. Kinetic stimulation of ORCC is not a general property of triphosphates because compounds such as cytosine triphosphate (500 μM, n = 2) and sodium metatriphosphate (1 mM, n = 2) were ineffective (data not shown). Thus, the action of ATP on single channel kinetics appears to be mediated by P_2 receptors but not by P_1 receptors (26). Moreover, the P_{2Y} agonist 2-methylthioadenosine 5-triphosphate (29) (100 μM) increased ORCC P_o (381 ± 143% of control, n = 3), but the P_{2X} agonist, adenosine 5′-[β,γ-methylene]triphosphate (30) (100 μM) was without effect (P_o was 99 ± 4% of control, n = 6), suggesting that ATP interaction with ORCC is not mediated by P_{2X} receptors.

In many cells (31, 32), including airway epithelial cells (9), ATP receptors may be coupled through guanine nucleotide-sensitive mechanisms to effectors that generate elevated cytosolic Ca^{2+} activity (Ca_i^{2+}). In our studies it was not necessary to supply exogenous GTP at the cytosolic surface of excised patches to observe the stimulation of ORCC by ATP or ATP analogs (see above). Moreover, stimulation of ORCC by external ATP in excised, inside-out patches was unaffected when GTP (n = 3) or guanosine 5′-[β-thio]diphosphate (n = 5), each at 1 mM/liter, were pulsed onto the cytosolic surface. Similarly, 1 mM guanosine 5′-[β-thio]-diphosphate in the pipette solution of outside-out membrane patches did not affect basal ORCC kinetics or the effect of external ATP (n = 3). We also found no effect of solution

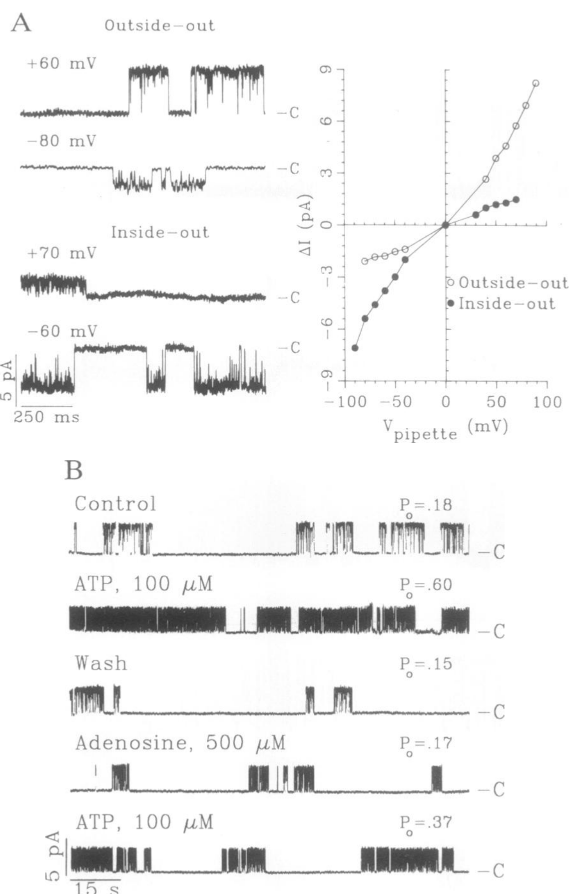

FIG. 3. ATP stimulates ORCC in excised, outside-out membrane patches. (*A*) ORCC in excised, outside-out membrane patches were identified by characteristic activation at depolarizing voltage, complex kinetics, and outward current rectification (21) Representative traces of data recorded from outside-out and inside-out patches are displayed and plotted as a function of pipette potential ($V_{pipette}$) to illustrate the easily recognized distinction between patch-clamp modes. In all other instances, data are displayed as if extracellular bath were ground and upward opening current jumps represent outward current (Cl^- entering cell). (*B*) Exposure of an ORCC in an outside-out membrane patch to ATP and adenosine. Solutions contained no added Ca^{2+} and EGTA at 1 mM/liter. All data were recorded from the same outside-out patch, which never displayed a second channel. Voltage of the membrane was 60 mV throughout. ATP and adenosine were added to the bath solution. ATP was washed out by a 5× change of bath volume (1 ml). Continuous blocks of data (2–2.5 min) after the indicated change in bath solution were analyzed for P_o as described for Fig. 2.

Ca^{2+} activity on ATP stimulation of ORCC, which was routinely observed with both pipette and bath solution Ca^{2+} activity buffered to 10 nM (Figs. 2 and 3). The effect of ATP was not enhanced by higher Ca^{2+} activity (1–1000 μM; data not shown) or lessened by the Ca^{2+} chelators EGTA (5–10 mM, $n = 4$) and bis(2-aminophenoxyethane-*N,N,N′,N′*-tetraacetate (BAPTA) (1 mM, $n = 3$). Although signal transduction involving G proteins and intracellular Ca^{2+} would contribute to the ATP-evoked Cl^- secretory response of the intact tissue, there is no readily apparent role for guanine nucleotides or Ca^{2+} in the action of external ATP on ORCC in excised patches.

Activation of Cl^- conductance by P_2 receptors on the apical membrane constitutes a previously unrecognized means of modulating airway epithelial Cl^- secretion. This regulation mode appears preserved in CF airway epithelial cells. Both Cl^- secretion (Fig. 4*A*) and P_o of ORCC in excised CF cell membrane patches (Fig. 4*B*) were increased by 100

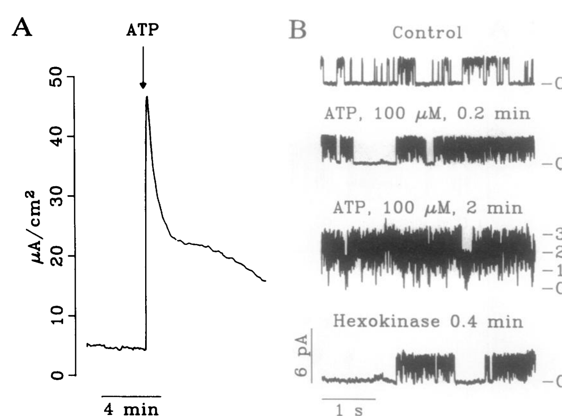

FIG. 4. Extracellular ATP induced Cl^- secretion and increased ORCC P_o in cultured CF epithelium. (*A*) I_{sc} with amiloride was measured in Ussing chambers. For three such experiments, 100 μM ATP increased current 26 ± 8 μA/cm². (*B*) ATP increased ORCC P_o in an outside-out membrane patch pulled from a CF cell. Initially, one channel was active. After 2-min exposure to ATP, at least three channels were active. The effect of ATP was reversed by washing. Hexokinase (0.1 unit/ml) was included to aid in removing ATP from the bath. In 20 excised, inside-out membrane patches from CF cells, P_o was 0.39 ± 0.04 with no ATP in the pipette. In 10 patches with 100 μM ATP in the pipette, P_o was 0.54 ± 0.04 ($P < 0.05$). C, closed.

μM ATP, suggesting that the domain of the protein that recognizes external ATP is functional in CF.

Our experiments identify ORCC as a Cl^--conductive element acted on by ATP in excised membrane patches. Previous reports implicated ORCC in defective cAMP regulation of Cl^- conductance in CF airway epithelia (33–36). However, the role of ORCC in epithelial Cl^- secretion has been recently questioned in light of cloning and expression of CF transmembrane regulator (37, 38). A cAMP-activated, 8-pS, linear Cl^- channel has been reported in cell-attached patches of T_{84} cells (39), which appears to match the characteristics of cAMP-dependent whole-cell Cl^- currents in heterologous cells expressing high levels of CF transmembrane regulator (37, 40). To complicate the issue further, ORCC is generally recognized not to be detected frequently in cell-attached membrane patches. However, even though the precise nature and relationship of all epithelial Cl^- channels has not been resolved, several explanations for our observation that external ATP activates ORCC exist. (*i*) ORCC could be an excised patch derivative of the smaller cAMP-dependent channels seen in cell-attached membrane patches. This explanation seems unlikely, given the markedly different conductance, kinetics, voltage dependence, and anion selectivity of the two channels. (*ii*) ORCC could be the channels associated with Ca^{2+}-activated Cl^- conductance. Although ORCC are not regulated by Ca^{2+} activity in excised patches, Ca^{2+}-dependent processes could regulate a Cl^- channel, perhaps with different conductive properties, in intact cells (41). (*iii*) ORCC could be a distinct component of apical membrane Cl^- conductance that is specifically regulated by extracellular nucleotides.

One approach used to relate single-channel behavior to macroscopic currents is inhibitor sensitivity. Ward *et al.* (38) showed inhibition of ORCC by DIDS acting at the external surface of excised membrane patches. We reasoned that if ORCC were related to the conductive element involved in ATP-stimulated Cl^- secretion, then DIDS should inhibit the Cl^- secretion induced in primary cultures of human nasal epithelia by ATP. The results in Fig. 5*A* confirm this expectation. DIDS, added to the mucosal bath during the plateau of ATP-stimulated Cl^- secretion, inhibited the stimulated current with an ED_{50} of 500 μM (Fig. 5*B*). This effect of DIDS

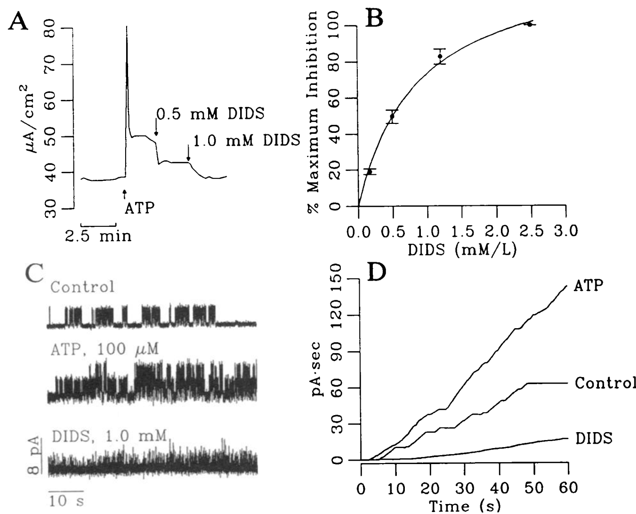

FIG. 5. Effect of DIDS on Cl^- secretion and patch current induced by external ATP. (*A*) Driving force for Cl^- secretion was increased by bathing tissues in a high-K^+, low-Cl^- KBR luminal bath containing amiloride. Luminal ATP stimulated I_{sc}. DIDS added during plateau phase reduced Cl^- secretion. (*B*) Data from six experiments similar to that depicted in *A* were analyzed as the partial decrement in current with increased DIDS concentrations and expressed as % maximum inhibition. L, liter. (*C*) ORCC in outside-out patches were stimulated by external ATP. Up to three channels were present during ATP exposure, which contributed to increased patch current; this stimulation was overcome by 1.0 mM DIDS. (*D*) Cumulative patch current calculated for 60-s segments of data displayed in *C*.

was specific for the ATP-induced current because 500 μM DIDS had no effect on basal or cAMP-stimulated Cl^- secretion and had no effects when present in the submucosal bath (data not shown). In outside-out membrane patches, external ATP increased patch current, and this current was blocked by DIDS (Fig. 5 *C* and *D*). Thus, macroscopic Cl^- secretion and ORCC are each stimulated by ATP and inhibited by DIDS. These findings point to some relationship between ORCC, as characterized in excised membrane patches, and the apical membrane conductance responsible for the ATP-induced Cl^- secretion.

A simple hypothesis fit by our data is that extracellular ATP increases ORCC P_o by binding to a P_2 receptor that is either a part of or intimately associated with the channel. Recently, extracellular ATP has been shown to activate a family of nonselective cation channels to a conducting state with complex, flickering kinetics (42–44). This activation follows the pharmacology of P_{2X} ATP receptors and, like the interaction of ATP with ORCC, does not involve soluble intracellular second messengers (27). Our results demonstrate a related mode of regulation for an airway epithelial Cl^- channel. The recent report that *in vivo* superfusion of ATP on nasal mucosa of normal and CF patients induces bioelectric changes consistent with Cl^- secretion points to a potential therapeutic application for agents that can modulate Cl^- conductance through purinergic receptors (45).

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