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Cl^- Currents Activated by Extracellular Nucleotides in Human Bronchial Cells

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Abstract. The perforated-patch technique was used to study the response of human bronchial cells to extracellular nucleotides. ATP or UTP (100 μM) elicited a complex response consisting of a large transient membrane current increase followed by a relatively small sustained level. These two phases were characterized by different current kinetics. Throughout the transient phase (2–3 min) the membrane current (I_p) displayed slow activation and deactivation kinetics at depolarizing and hyperpolarizing potentials respectively. At steady-state (I_s) the relaxation at hyperpolarizing potential disappeared whereas at positive membrane potentials the current became slightly deactivating. The I_s amplitude was dependent on the extracellular Ca^{2+} concentration, being completely inhibited in Ca^{2+}-free medium. Cell preincubation with the membrane-permeable chelating agent BAPTA/AM prevented completely the response to nucleotides, thus suggesting that both I_p and I_s were dependent on intracellular Ca^{2+}. The presence of a hypertonic medium during nucleotide stimulation abolished I_s leaving I_p unchanged. On the contrary, niflumic acid, a blocker of Ca^{2+}-activated Cl^- channels, prevented completely I_p without reducing significantly I_s. 1,9-dideoxyforskolin fully inhibited I_s but also reduced I_p. Replacement of extracellular Cl^- with aspartate demonstrated that the currents activated by nucleotides were Cl^- selective. I_p resulted five times more Cl^- selective than I_s with respect to aspartate. Taken together, our results indicate that ATP and UTP activate two types of Cl^- currents through a Ca^{2+}-dependent mechanism.

Key words: Bronchial epithelial cells — Cl^- currents — Cl^- channels — Patch-clamp — Intracellular Ca^{2+} — ATP — UTP

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Introduction

Cystic Fibrosis (CF) patients have a defective Cl^- secretion and an increased Na^+ absorption in the airways due to mutations affecting a plasma membrane Cl^- channel termed CFTR [8]. The functional defect results in abnormal dehydration of the mucous blanket covering the epithelium surface. This causes bronchial obstruction and chronic bacterial infections. The impairment of respiratory function represents the main cause of death in this inherited disease.

The addition of extracellular nucleotides like ATP and UTP to the apical surface of airway epithelium is able to stimulate Cl^- secretion [15, 16, 19]. Given their ability to activate Cl^- channels different from CFTR, these compounds have been proposed as therapeutic drugs to increase the Cl^- secretion in the airway epithelium of cystic fibrosis (CF) patients. The interest in the effects of nucleotides on airway cells has increased after recent suggestions that ATP is able to permeate the CFTR pore [25, 29]. It has been proposed therefore, that the extrusion of ATP through the apical membrane of the epithelium might be a mechanism through which CFTR controls the activity of other Cl^- channels. However, the role of CFTR as an ATP channel has been recently questioned [6, 24]. The identity of the channels involved in nucleotide-induced Cl^- transport and the mechanism of activation are not clear. Indeed, besides CFTR, at least other four types of Cl^- currents have been described in epithelial cells. One type ($I_{Cl(Ca)}$) is activated after intracellular Ca^{2+} increase with a mechanism involving Ca^{2+}/calmodulin-dependent protein kinase [34]. This channel shows a marked voltage dependence and a peculiar kinetic behavior consisting of slow activation and deactivation kinetics at depolarizing and hyperpolarizing membrane potentials respectively [5, 7]. The second type, termed volume-sensitive Cl^- current ($I_{Cl(vol)}$), can

be elicited by means of a hypotonic shock and is characterized by deactivation at positive membrane potentials, outward rectification and inhibition in hypertonic medium [3, 14, 17, 22, 23, 26]. The third type of current is due to the activity of outwardly rectifying Cl^- channels (ORCCs) which do not show voltage-dependent relaxation [29]. Finally, hyperpolarization-activated currents [2, 9] have been recently described in epithelial cells. These last currents have biophysical characteristics resembling those of ClC-2 channels [13].

In previous studies, performed on immortalized airway epithelial cells, we found that ATP mobilizes intracellular Ca^{2+} and elicits the appearance of $I_{Cl(vol)}$-like currents [10, 24]. This result was unexpected since volume-sensitive currents have been considered Ca^{2+}-independent [1, 12, 17, 31]. Nevertheless, some recent indications point out that intracellular Ca^{2+} plays a role in the activation of such currents [32, 33].

Other investigators have found that extracellular nucleotides are able to activate ORCCs even in cell-free excised patches, apparently excluding the involvement of intracellular Ca^{2+} [29]. In the present paper, we have reexamined this problem by studying nontransformed human bronchial epithelial cells to determine the type(s) of Cl^- currents elicited by nucleotide application and the mechanism of activation involved. Our results show that ATP and UTP activate two types of Cl^- currents.

Materials and Methods

Cell Culture

Human bronchi, obtained from surgical specimens, were trimmed of connective tissue. Epithelial cells were detached by incubating bronchi in Hank's balanced salt solution containing 0.25% (w/v) type XIV protease for 24 hr at 4°C. Protease solution was then discarded and bronchial lumen was rinsed several times with a medium containing Dulbecco's MEM/Ham's F12 (1:1) plus 10% (v/v) fetal calf serum. Collected cells were pelleted by centrifugation, resuspended in the final culture medium and plated at a density of 5–10 × 10^4 cells/cm^2 on culture flasks coated with 1% (v/v) Vitrogen 100 for 1–2 hr at 37°C. The culture medium was a mixture of LHC9 [18] and RPMI 1640 (1:1) plus 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and, for the first days of culture, 2.5 μg/ml amphotericin B. Essential aspects of this medium, which sustained cell proliferation, were the absence of fetal serum, replaced by a large number of hormones and growth factors, and a Ca^{2+} concentration of 0.25 mM. Care was taken to avoid high density cultures as this resulted in squamous cell differentiation probably due to an autocrine production of TGF-β2 [28]. Accordingly, cells were detached by trypsinization when they were approximately 50% confluent and replated on flasks for further subculture or on 35 mm Petri dishes for patch-clamp experiments. At each subculture passage, various aliquots of cells were frozen in liquid nitrogen to keep a stock of the culture. The use of a monoclonal antibody against cytokeratin demonstrated that more than 95% of the cells were of epithelial origin. Experiments were carried out on cells at 3–6 passages.

Electrophysiology

Patch-clamp micropipettes were prepared from borosylicate glass capillaries with a L/M-3P-A puller and a L/M-CPZ101 microforge (List Medical, Darmstadt, Germany). Whole-cell membrane currents were measured using the perforated-patch technique with a List L/M-EPC7 patch-clamp amplifier. The reference electrode was connected to the bath solution through a 0.5 M KCl agar bridge. Currents were recorded directly on a L6512 chart recorder (Linseis, Selb, Germany) and saved on a conventional videotape recorder for further analysis. Voltage stimulation, acquisition and analysis of data were performed with a MEGA/STE computer (Atari, Sunnyvale, CA) interfaced with the patch-clamp amplifier through a 16-bit AD-DA converter and appropriated software (Instrutech, Elmont, NY). Currents were sampled each 0.5 msec. The series resistance and cell capacitance were read from the patch-clamp amplifier after analog compensation of the current artifact produced by the application of a small (10 mV, 4 msec) test pulse. Voltage-clamp experiments were performed with series resistance of 8–12 MΩ. The cell membrane capacitance was 23 ± 1.2 pF (n = 203).

The stimulation protocol consisted in three consecutive 500 msec voltage steps to +80, −20, and −100 mV repeated every 4 sec from a holding potential of −20 mV. Current-voltage relationships were obtained by applying voltage pulses from −100 to +120 mV each 20 mV from a holding potential of −20 mV. For selectivity studies, a 800 msec ramp from −80 to +80 mV was applied every 4 sec after a 800 msec prepulse at −80 mV. The holding potential was kept at −20 mV. Permeability ratio ($P_{aspartate}/P_{Cl^-}$) was calculated from the Goldman-Hodgkin-Katz equation:

$$E_r = (RT/zF)1n(P_{Cl^-}[Cl^-]_i/(P_{Cl^-}[Cl^-]_o + P_{aspartate}[aspartate]_o))$$

where E_r is the reversal potential obtained by partially replacing external Cl^- by asparate, R is the gas constant, T is the absolute temperature, z is the valency of the ion and F is the Faraday constant. All experiments were done at room temperature (18–22°C).

Solutions

Cells were bathed with a solution containing (in mM): 130 N-methyl-D-glucamine (NMDG), 0.25 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 36 mannitol, and 10 HEPES (pH = 7.3 with 122 mM HCl, 300 mosm/kg). In some experiments the extracellular $CaCl_2$ was increased to 2 mM. Ca^{2+}-free solution was prepared by omitting $CaCl_2$ and adding 1 mM EGTA. Low Cl^- solution was prepared by substituting NMDG-aspartate for NMDG-Cl. The liquid junction-potential change at the reference electrode caused by Cl^- substitution was calculated and used to correct reversal potentials [22]. The hypertonic medium was obtained by addition of further 60 mM mannitol whereas the hypotonic medium was prepared diluting the extracellular solution with 30% water (240 mosm/Kg). Extracellular solution was applied with a gravity perfusion system and experiments were done under continuous perfusion. The perfusion pipette was placed close to the cell so that solution change occurred in a few seconds.

The pipette solution contained (in mM): 60 CsCl, 45 Cs_2SO_4, 1 $MgCl_2$, 0.5 EGTA, 10 HEPES (pH = 7.3 with CsOH), and 250 μg/ml of amphotericin B. To facilitate giga-seal formation the tip of the pipette was filled with amphotericin B-free solution. Patch perforation was attained in 15–30 min by diffusion of the perforating agent towards the membrane.

Material

LHC medium was from Biofluids (Rockville, MD), whereas RPMI 1640, Dulbecco's MEM, Ham's F12, and Fetal Clone II serum were

from HyClone (Logan, UT). UTP and BAPTA/AM were purchased from Calbiochem (La Jolla, CA). All other chemicals were from Sigma (St. Louis, MO).

Analysis of Data and Statistics

Currents were normalized for the membrane capacitance. Results are presented as raw data or arithmetic mean ± SEM. Statistical analysis was carried out with the Student's *t* test for unpaired data.

Results

To study the response of bronchial cells to nucleotides we applied ATP or UTP extracellularly while measuring current changes in response to voltage steps as described in methods. In the presence of 0.25 mM extracellular Ca^{2+}, the response of nucleotides was characterized by a very fast increase of membrane currents elicited at either +80 and −100 mV. The currents reached a large peak (I_p) at the beginning of the stimulation and then decreased in approximately 3 min to a sustained phase (I_s). The maximal value of I_p, measured at −100 mV 10 to 15 secs after nucleotide application, was −75.7 ± 19.9 (n = 9) and −61.4 ± 15.1 (n = 11) pA/pF for 100 μM ATP or UTP, respectively. The value of I_s, measured after 3 min of nucleotide stimulation, was −5.7 ± 1.9 pA/pF with ATP and −5.9 ± 1.7 pA/pF with UTP. Close inspection of the traces elicited by voltage pulses showed that the kinetic behavior changed during the course of the stimulation (Fig. 1*A*). During the I_p phase, membrane currents showed slow activation at +80 mV and deactivation at −100 mV. As the response to the nucleotide decreased progressively, the aspect of the currents changed. Indeed, the relaxations at −100 and +80 mV disappeared. Actually, the current I_s displayed at +80 mV a slight deactivation that was more evident by further depolarization to +100/+120 mV (Fig. 1*A*).

The removal of extracellular Ca^{2+} significantly changed the cell response to nucleotides. The transient large increase of membrane currents was maintained but the following steady-state phase disappeared (Fig. 1*B*). In Ca^{2+}-free medium, ATP and UTP elicited only one type of kinetics behavior. Indeed, the currents showed slow relaxation at +80 and −100 mV for the entire course of nucleotide effect. This result suggests that two types of Cl^- currents are activated by nucleotide application and that the removal of extracellular Ca^{2+} can selectively inhibit one of these currents, namely I_s.

To look further at the role of extracellular Ca^{2+} we performed experiments with a higher concentration. The simple increase of Ca^{2+} to 2 mM caused *per se* a slight activation of membrane currents (Fig. 1*C*). These reached a steady-state and lasted as long as the high Ca^{2+} concentration was maintained. The currents activated under this condition had an amplitude of −6.6 ± 1.1 pA/pF (n = 46) at −100 mV. The kinetics of the currents activated by increasing the extracellular Ca^{2+} concentration was not homogeneous. In 15% of the experiments a slow activation at +80 mV and a deactivation at

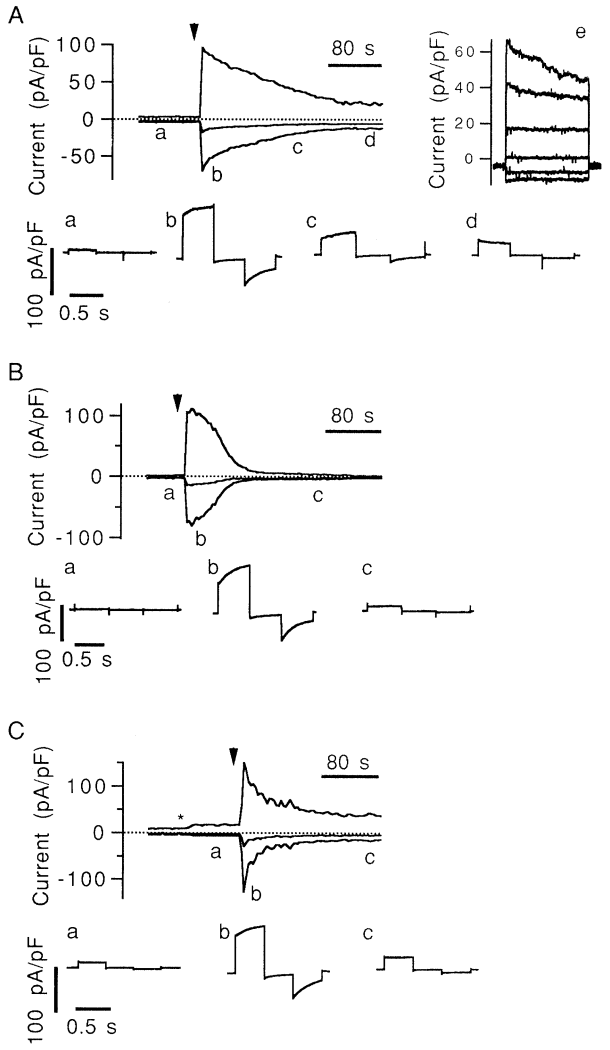

Fig. 1. Effect of extracellular Ca^{2+} on membrane currents elicited by extracellular nucleotides. Three representative experiments are shown in which UTP was applied in a medium containing 0.25 (*A*), 0 (*B*), or 2 mM Ca^{2+} (*C*). Alternate stimulation to +80 and −100 mV was continuously performed as described in Materials and Methods. Each of the main figures in *A*, *B*, and *C* represents the time course of membrane currents elicited at +80 (upper line), −20 (intermediate line), and −100 mV (lower line). These traces were generated by connecting with a continuous line the maximal current measured at each membrane potential. Expanded segments of the currents are depicted below each of the main figures to show current kinetics. The position of these sweeps in the experiment are indicated by lowercase letters. Superimposed currents elicited at the end of the experiment A by 40 mV voltage steps in the range −80 to 120 mV are also shown in *e* to display the voltage dependence of the steady-state current. UTP (100 μM) application is indicated by an arrowhead, whereas extracellular Ca^{2+} increase to 2 mM is indicated in *C* by an asterisk.

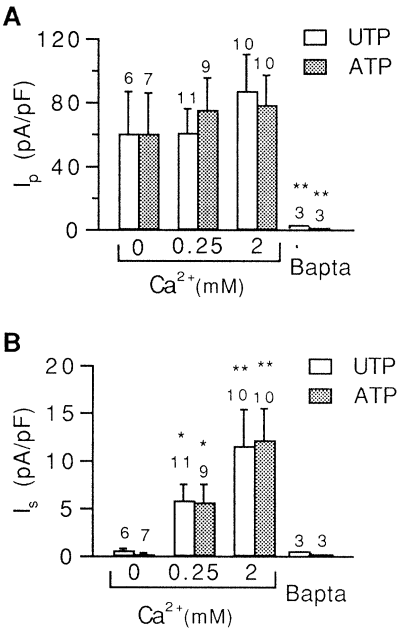

Fig. 2. Ca^{2+} dependence of nucleotide-evoked currents. Bars represent the mean current (±SEM) elicited at −100 mV at the peak, I_p (*A*) and 3 min after the nucleotide application, I_s (*B*). The extracellular Ca^{2+} concentration was 0, 0.25 or 2 mM as indicated in the figure. Experiments with Bapta/AM were carried out in 2 mM Ca^{2+}. Where the peak was absent (Bapta experiment) I_p was measured at 10 sec, a time at which the current was maximal in control experiments. The number of experiments is reported above each bar. Extracellular Ca^{2+} concentration affected I_s but not I_p (* significantly different from I_s in 0 Ca^{2+}, $P < 0.05$; ** significantly different from I_s in 0 Ca^{2+}, $P < 0.01$). Bapta preincubation strongly inhibited I_p (**, $P < 0.01$).

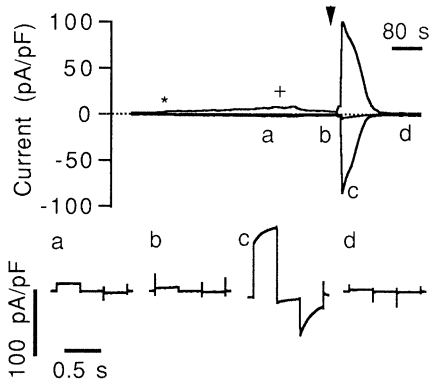

Fig. 3. Effect of hypertonic medium on nucleotide-induced currents. The main trace shows the effect of additive applications of 2 mM Ca^{2+} (asterisk), hypertonic medium (cross), and 100 μM UTP (arrowhead) from a representative experiment. Expanded segments of the currents, indicated by lower-case letters are shown below.

−100 mV were detected. In the remaining 85%, the current showed no relaxation at −100 mV. In these experiments, a slight deactivation could be observed at positive membrane potentials. This was not always detectable at +80 mV, but was evident by further depolarization to +100 and +120 mV (*not shown*).

The addition of nucleotides to the solution containing 2 mM Ca^{2+} increased further the membrane current. The response was qualitatively similar to that observed in 0.25 mM Ca^{2+}, with an initial large transient peak followed by a steady-state (Fig. 1*C*). This steady-state was significantly higher than the current level previously attained with 2 mM Ca^{2+} alone. As shown in the expanded traces, the current at the beginning of the nucleotide effect (I_p) had the characteristic slow activation and deactivation pattern at depolarizing and hyperpolarizing potentials, respectively. The I_s current instead showed no relaxation at −100 mM and a very slight deactivation at +80 mV. This deactivation became more evident in all the experiments by increasing the depolarizing step to +100 or +120 mV.

Figure 2 summarizes the results obtained with different extracellular Ca^{2+} concentrations. The currents I_p and I_s were measured at −100 mV at the peak (after 10–15 sec) and three min after nucleotide addition, the pre-nucleotide current being taken as zero. The net I_p did not significantly change with the variation of extracellular Ca^{2+} concentration (Fig. 2*A*). On the contrary, I_s was completely abolished by removal of extracellular Ca^{2+} (Fig. 2*B*).

To confirm the involvement of cytosolic Ca^{2+} increase in the activation of Cl^- currents, we used a membrane permeable Ca^{2+}-chelating agent. Preincubation of bronchial cells for 1–2 hr with 20 μM BAPTA/AM resulted in a complete inhibition of the effect caused by extracellular Ca^{2+} increase to 2 mM (*not shown*) and by subsequent nucleotide addition ($n = 3$ for ATP and 3 for UTP; Fig. 2*A* and *B*).

Our results suggested that two types of Cl^- currents are activated by nucleotide stimulation through an intracellular Ca^{2+} increase. With the attempt to inhibit selectively one of the two membrane current components at a time, we used different maneuvers. These experiments were carried out with 2 mM extracellular Ca^{2+} to increase the size of I_s.

In previous studies we found that extracellular hyperosmolality inhibited nucleotide-induced Cl^- currents [10]. Accordingly, we applied UTP or ATP in the presence of a hypertonic bath solution. As shown in Fig. 3, the hypertonic medium inhibited the small current that was previously activated by 2 mM Ca^{2+} but did not prevent the appearance of the transient current (I_p) elicited by nucleotide application. I_p at −100 mV was −66.6 ± 20.6 pA/pF with ATP ($n = 7$) and −80.4 ± 16.4 pA/pF with UTP ($n = 7$), not significantly different from I_p in isotonic conditions. Conversely, I_s was almost absent (80–90% inhibition 3 min after nucleotide addition). The behavior of the currents in the hypertonic medium was therefore similar to that seen in the absence of extracellular Ca^{2+}.

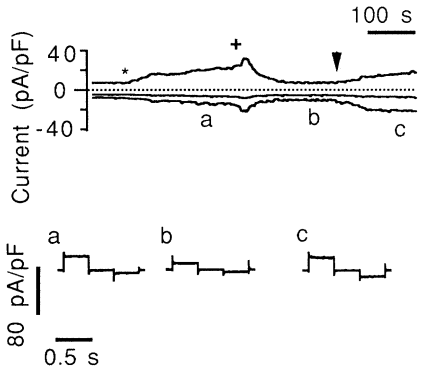

Fig. 4. Effect of niflumic acid on nucleotide-induced currents. As in the previous figures, the upper main trace depicts the time course of membrane currents at +80, −20, and −100 mV. Additive applications were: 2 mM Ca^{2+} (asterisk), 100 μM niflumic acid (cross), and 100 μM UTP (arrowhead). Expanded currents are shown in the lower part of the figure.

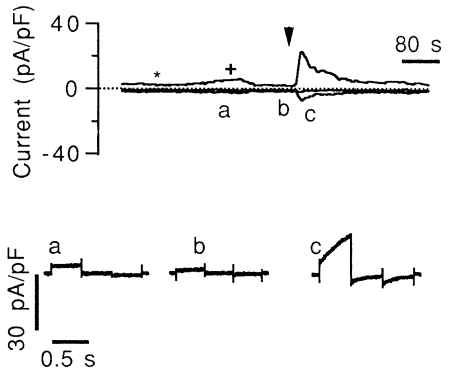

Fig. 5. Effect of ddFSK on nucleotide-induced currents. The upper main trace depicts the time course of membrane currents at +80, −20, and −100 mV. Additive applications were: 2 mM Ca^{2+} (asterisk), 100 μM ddFSK (cross), and 100 μM UTP (arrowhead). Expanded currents are shown below.

Niflumic acid has been described as a potent blocker of $I_{Cl(Ca)}$ [3]. Fig. 4 shows that niflumic acid caused a full inhibition of the I_p elicited by nucleotides but did not prevent the appearance of the I_s component. This current slowly developed upon nucleotide application and reached in 2–3 min a stable level which was not significantly different from that measured in the absence of the blocker. As also depicted in Fig. 4, niflumic acid caused 50% inhibition ($n = 8$, $P < 0.05$) of the current activated by extracellular Ca^{2+} increase alone.

To further characterize the currents activated by nucleotides we used 1,9 dideoxyforskolin (ddFSK), a strong blocker of $I_{Cl(vol)}$ [23]. This forskolin analogue blocked totally the current activated by 2 mM Ca^{2+} (Fig. 5). The subsequent addition of nucleotides resulted in a significant reduction of I_p (80%) and total inhibition of I_s (about 90% after 3 min and 100% after 5 min, $n = 10$). The current activated in the presence of ddFSK showed only the kinetics of I_p suggesting the presence of a single current type.

Fig. 6 summarizes the results obtained with the hypertonic medium, niflumic acid and ddFSK. The hypertonic medium and niflumic acid were selective in inhibiting I_s and I_p respectively (Fig. 6*A* and *B*). On the other hand ddFSK appeared much less specific since it blocked strongly I_s but significantly reduced also I_p. The current activated by the simple extracellular Ca^{2+} increase resembled the I_s activated by nucleotides since it was highly sensitive to the hypertonic medium and to ddFSK (Fig. 6*C*).

Since bath and pipette solution were designed to eliminate cation channels contribution to membrane conductance, our results suggest that extracellular nucleotides activate Cl^- currents in human bronchial cells. To further support this conclusion, we performed experiments in which extracellular Cl^- was partially replaced by aspartate. Two different protocols were used to study the selectivity of I_p and I_s activated by ATP and UTP. For the former current type, we decided to use voltage ramps to monitor the reversal potential throughout the rapid changes in current amplitude. These experiments were carried out in Ca^{2+}-free extracellular solution to eliminate the sustained component. Upon nucleotide application the reversal potential measured during maximal current activation was −7.3 ± 1.5 mV ($n = 8$; Fig. 7*A*) with 126 mM Cl^- in the extracellular solution (Nernst potential for $Cl^- = -17.9$ mV). The reversal potential was instead +59.3 ± 2.6 mV (Fig. 7*B*) when extracellular Cl^- was 4 mM by replacement with aspartate (Nernst potential = +69.2 mV). This gives a permeability ratio $P_{aspartate}/P_{Cl} = 0.015$.

The ion selectivity of I_s was investigated with a voltage-step protocol and in 2 mM extracellular Ca^{2+}. Lowering extracellular Cl^- to 8 mM by replacement with aspartate strongly reduced the outward currents and shifted the reversal potential from −3.4 ± 0.4 mV to +30.6 ± 1.7 mV (Nernst potential = +51.7 mV) concording with the activation of Cl^- selective channels by nucleotides (Fig. 7*C*). The permeability ratio $P_{aspartate}/P_{Cl}$ for I_s is 0.08.

Since the I_s component elicited by nucleotides resembled the volume-sensitive Cl^- current ($I_{Cl(vol)}$), we decided to investigate how bronchial cells respond to a hypotonic shock. Decreasing the extracellular osmolality to 240 mosm/kg elicited large currents characterized by outward rectification and slight deactivation at +80 mV. These currents were strongly inhibited by 100 μM ddFSK but only partially (about 50%) by 100 μM niflumic acid (Fig. 8).

Discussion

Our study shows that stimulation of bronchial epithelial cells with nucleotides causes the appearance of a com-

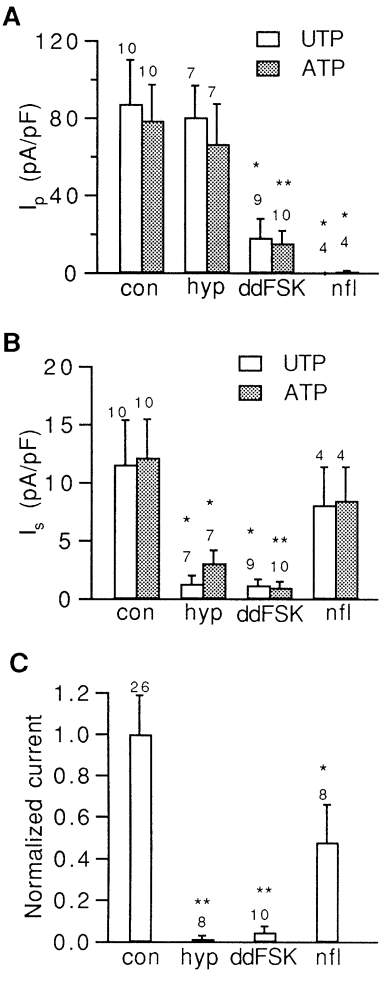

Fig. 6. Summary of the effects obtained with blockers and hypertonic medium. *A* and *B* show I_p and I_s respectively, in control conditions and in the presence of hypertonic medium (hyp), 100 μM ddFSK, and 100 μM niflumic acid (nfl). With niflumic acid the value of I_p was taken at 10 sec after nucleotide addition. Panel *C* shows the effect of blockers and hypertonic medium on the current activated by extracellular Ca^{2+} increase to 2 mM. Significant reduction of currents with respect to control conditions is indicated (*$P < 0.05$; **$P < 0.01$).

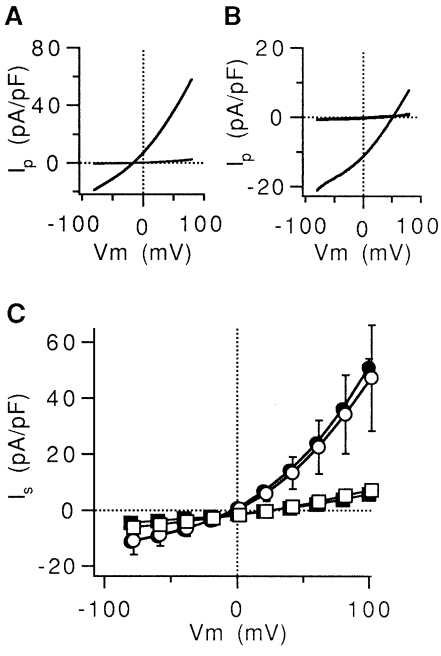

Fig. 7. Cl^- selectivity of nucleotide-evoked currents. The first two panels show I_p membrane currents elicited by 800 msec voltage ramps from −80 to +80 mV, before and after UTP addition in high Cl^- (*A*) or low Cl^- (*B*) extracellular solution. Data are from two representative experiments. *C* shows current-voltage relationships of I_s current activated by ATP (filled symbols) and UTP (open symbols) in high Cl^- (circles) or low Cl^- (squares) extracellular solution. Data are mean ± SEM of four experiments for each condition.

plex response. This response, identical to UTP and ATP, is characterized by a large and transient current increase followed by a sustained phase. The nucleotide effect requires an apparent increase in intracellular Ca^{2+}, since cell preincubation with the membrane permeable chelating agent BAPTA/AM completely abolishes both conductances. In contrast to the transient current, the sustained phase is strongly dependent on extracellular Ca^{2+}. Taken together these data argue that extracellular nucleotides elicit cytosolic Ca^{2+} increase with two mechanisms: release from intracellular stores and Ca^{2+} influx through the plasma membrane. The former mechanism should account for the initial large peak whereas the latter would be responsible for the steady state. The equipotency of UTP and ATP is consistent with nucleotides acting through a P_{2U} receptor as described in airway epithelium [19].

Interestingly, current kinetics change during the time course of nucleotide response. We interpret this behavior postulating that two types of Cl^- currents are activated by nucleotides. The first current type is mainly active at the beginning of the response and is responsible for the large transient current (I_p). As this current progressively decreases the second conductance becomes visible, but only when extracellular Ca^{2+} is present. Various experimental evidences support this interpretation: the different currents kinetics, their different permeability to Cl^-, their different sensitivity to extracellular Ca^{2+}, to hypertonicity and to niflumic acid.

I_p, the transient Cl^- current, has the characteristic kinetic behavior of $I_{Cl(Ca)}$, i.e., slow activation at positive membrane potential and slow deactivation at negative potentials. Furthermore, it is completely inhibited by 100 μM niflumic acid, a known blocker of $I_{Cl(Ca)}$ and is unaffected by hypertonic medium. Finally, replacement of Cl^- with aspartate shifts the reversal potential of I_p in a way that approximates the Nernst reversal potential for a perfect Cl^--selective channel. All these characteristics suggest that I_p is due to the activation of $I_{Cl(Ca)}$.

The identification of the sustained current, I_s, is less

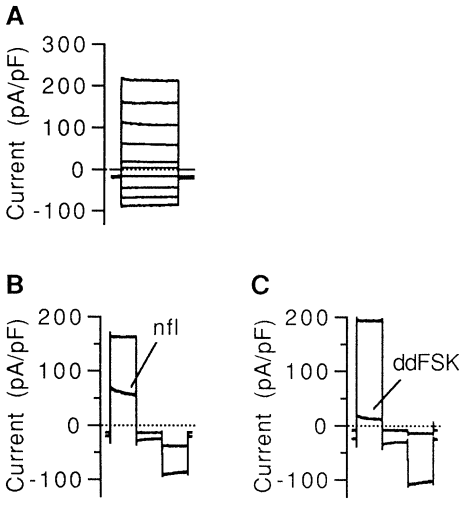

Fig. 8. Currents elicited by hypotonic shock. *A* shows superimposed membrane currents evoked by voltage steps in the range −80 to +80 mV with increments of 20 mV after stimulation with the hypotonic shock. *B* and *C* show the inhibitory effect of niflumic acid (nfl) and ddFSK, respectively, on currents elicited at +80, −20, and −100 mV.

clear. There are some attributes of this current that resemble the Cl^- current elicited by the hypotonic shock $I_{Cl(vol)}$, i.e., outward rectification of the current-voltage relationship and deactivation at strong depolarizing potentials [3, 14, 16, 22, 26]. Actually, the deactivation of I_s is very slow and is evident only at +80 mV or at more positive potentials. However, this is also the behavior of the current activated by the hypotonic shock (Fig. 8*A*). The hypertonic medium prevents the nucleotide activation of I_s. Furthermore, I_s is completely blocked by 100 μM ddFSK as $I_{Cl(vol)}$ in other cell preparations [23, 26]. Nevertheless, this inhibitor is not very selective since it also reduces the transient I_p current. From the anion substitution experiments it is clear that I_s is less Cl^- selective than I_p and has an aspartate permeability that is about 0.1 that of Cl^-, similar to that described for $I_{Cl(vol)}$ [22]. All these characteristics are in agreement with I_s being $I_{Cl(vol)}$.

Our data suggest that both I_p and I_s, here identified as $I_{Cl(Ca)}$ and $I_{Cl(vol)}$, are activated by ATP and UTP through an increase in intracellular Ca^{2+}. The transient behavior of I_p could indicate that its activation occurs only during the release of Ca^{2+} from intracellular stores, when the intracellular Ca^{2+} reaches a relatively high concentration. A similar transient activation of $I_{Cl(Ca)}$ by Ca^{2+}-mediated agonists has been described in other cell types [11, 21]. On the other hand, the Ca^{2+}-dependent activation of $I_{Cl(vol)}$ by nucleotides, which confirms data obtained from immortalized cells [10, 27], is less canonical. Indeed, previous studies have shown that the swelling-activation of $I_{Cl(vol)}$is not Ca^{2+}-dependent [1, 17, 31]. Nevertheless, recent data indicate that this current is indeed modulated by intracellular Ca^{2+}, at least in some cells [32, 33]. It appears therefore that the activation of $I_{Cl(vol)}$ by nucleotides is based on a signal transduction mechanism related in some way to cytosolic Ca^{2+}. One possible mechanism through which Ca^{2+} may activate $I_{Cl(vol)}$ is by inducing cell swelling. Cell volume increase could be based on a Na^+-coupled uptake of osmotically-active solutes from the extracellular medium, but in our conditions this is not possible given the lack of Na^+ in the extracellular solution. If cell swelling does not actually occur, an alternative mechanism to explain the effect of nucleotides on $I_{Cl(vol)}$ channels should be taken into account. Nevertheless, working hypotheses on this topic are difficult to formulate at the moment since the molecular process through which cell swelling activates ion channels is unknown. An obvious possibility is an involvement of the cytoskeleton in the signal transduction. Alternatively, the mechanism could be restricted to the plasma membrane, based on changes in the physical structure of the membrane. In such cases the nucleotides could mimick the effect of hypotonic shock by causing similar effects on the cytoskeleton or on membrane organization. With respect to the second possibility, it is interesting to note a recent observation that extracellular ATP causes a Ca^{2+}-dependent increase of the membrane fluidity in ciliary cells [4].

It has been recently suggested that ORCC is activated upon extracellular application of nucleotides [29]. The I_p current, activated at the beginning of the experiments, has kinetics quite different from ORCC. The steady-state phase is instead more similar since it is outward rectifying and, as ORCC, persists as long as the nucleotide is present in the extracellular solution. Nevertheless, I_s is dependent on intracellular Ca^{2+} unlike ORCC. In addition, I_s is inhibited by extracellular hypertonicity, a feature typical of $I_{Cl(vol)}$. Therefore, it appears that ORCCs are not activated by nucleotides in our cells.

A side finding of our study is that bronchial cells, at least in our culture conditions, respond to a simple extracellular Ca^{2+} increase with a slight activation of Cl^- currents. Since this effect is prevented by preincubation with BAPTA/AM, we conclude that a plasma membrane Ca^{2+} permeability is present in basal conditions. It is tempting to speculate that this permeability is related to the inhibitory effect that an extracellular Ca^{2+} increase shows on bronchial cell proliferation in a serum-free culture [18].

In conclusion, extracellular nucleotides activate at least two types of Cl^- currents in human bronchial cells. These data open important questions regarding the physiology of Cl^- secretion regulation in airway epithelia and the validity of nucleotides in the therapy of CF. Indeed, Ca^{2+}-dependent Cl^- conductance has been localized to the apical membrane in airway epithelia [5]. Its activation would therefore contribute to transepithelial Cl^-

transport. The localization of volume-sensitive Cl^- channels is instead uncertain. It has been suggested that such channels are also placed on the apical membrane of airway epithelium [20]. However, in other epithelia, such as kidney MDCK cells, swelling-induced Cl^- conductance is clearly basolateral [30]. In the latter case, activation of volume-sensitive channels would decrease Cl^- secretion by causing depolarization and by generating a shunt for Cl^- transport across the basolateral membrane. Study of these phenomena in polarized preparations and determination of the subcellular localization of $I_{Cl(vol)}$ would be of great help in the comprehension of functional aspects of the normal and the CF airway epithelium.

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