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Extracellular 2-chloroadenosine and ATP stimulate volume-sensitive Cl^- current and calcium mobilization in human tracheal 9HTEo− cells

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The perforated-patch whole-cell technique was used to record membrane currents in epithelial cells (9HTEo−) obtained from the human tracheal epithelium. Extracellular application of 2-chloroadenosine and ATP (0.01–100 μM) caused activation of Cl^- currents similar to those regulated by cell volume in airway and intestinal cells. This response was inhibited by increasing extracellular osmolality, by omission of extracellular Ca^{2+}, or by the addition of the A_2 adenosine receptor antagonist 3,7-dimethyl-1-propargylxanthine (DMPX). Fluorimetric measurements with fura-2 reveal that 2-chloroadenosine and ATP elicited both a Ca^{2+} influx through the plasma membrane and a release from intracellular stores.

Airway epithelium; Cl^- current; Cell volume; 2-Chloroadenosine; ATP; Intracellular Ca^{2+}

1. INTRODUCTION

Two recent articles have shown that extracellular application of ATP, UTP and other related compounds causes stimulation of Cl^- secretion in the nasal epithelium of both normal individuals and cystic fibrosis patients [1,2]. Apical purinergic receptors have been proposed by these authors as possible targets of new drugs developed for the pharmacological therapy of cystic fibrosis.

In the present study, the ability of extracellular ATP and of an adenosine analogue to affect membrane currents, has been assessed, at the single cell level, by using the perforated-patch whole-cell technique [3,4] on a cell line (9HTEo−) derived from the human tracheal epithelium [5]. In addition, fluorimetric measurements with fura-2 were carried out to detect changes of intracellular free Ca^{2+} levels. The results indicate the presence of purinergic receptors whose activation causes mobilization of intracellular Ca^{2+} and appearance of 'volume-sensitive' Cl^- currents.

Abbreviations: 2-CADO, 2-chloroadenosine; DMPX, 3,7-dimethyl-1-propargylxanthine; EGTA, ethyleneglycol-*bis*-(β-aminoethyl)-*N,N,N',N'*-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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2. MATERIALS AND METHODS

9HTEo− cells have been obtained by transformation of human tracheal epithelium [5]. They were cultured as described elsewhere [6]. Measurements of membrane currents were carried out with the 'perforated-patch' modification of the patch-clamp technique [3,4]. With this method, which uses nystatin as perforating agent, the spontaneous activation of volume-sensitive Cl^- currents, detected during conventional whole-cell recordings on epithelial cells [7,8], was not observed.

The standard extracellular solution in electrophysiological experiments contained (in mM): 130 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 D-glucose, 10 Na-HEPES (pH 7.3). The pipette was filled with (in mM): 50 KCl, 10 NaCl, 45 K_2SO_4, 1 $MgCl_2$, 0.5 EGTA, 10 K-HEPES (pH 7.3) plus 200 μg/ml nystatin. The osmolality of extracellular and intracellular solutions was adjusted with mannitol to 295–305 mOsm/kg except where indicated.

Series resistance after patch perforation was 14.5 ± 0.8 MΩ (n=72). The voltage error resulting from currents flowing through this resistance was calculated to correct for the applied membrane potential. The holding potential was always −20 mV. To track the membrane conductance, a 500 ms long test potential to 100 mV was applied every 5 s. This stimulation was occasionally interrupted to construct current–voltage relationships by performing a protocol consisting in steps to membrane potentials in the range between −80 and +80 mV. The equipment for data acquisition and analysis has been described elsewhere [9].

For measurements of $[Ca^{2+}]_i$, 9HTEo− cells were trypsinized, centrifuged, resuspended (4×10^6 cells/ml) in DMEM containing 3% fetal calf serum (FCS) and loaded with 4 μM fura-2/AM at 37°C. At the end of the loading period (30 min) the cells were harvested, washed and resuspended (2×10^6/ml) in DMEM containing 1% FCS and 0.25 mM sulfinpirazone (to prevent Fura-2 leakage; [10]) and kept at room temperature until used. Immediately before use, an aliquot of the loaded cell suspension was centrifuged and resuspended in Ca^{2+}-free saline medium containing (in mM): 125 NaCl, 5 KCl, 1 $MgSO_4$, 1 KH_2PO_4, 5.5 D-glucose, 0.25 sulfinpirazone, 20 Na-HEPES (pH 7.4). Measurements of $[Ca^{2+}]_i$ were obtained with a Multiscan-2 spectrofluorimeter (AMKO-LTI) equipped with alternating dual wavelength excitation system. The 340/380 ratio was converted to an actual

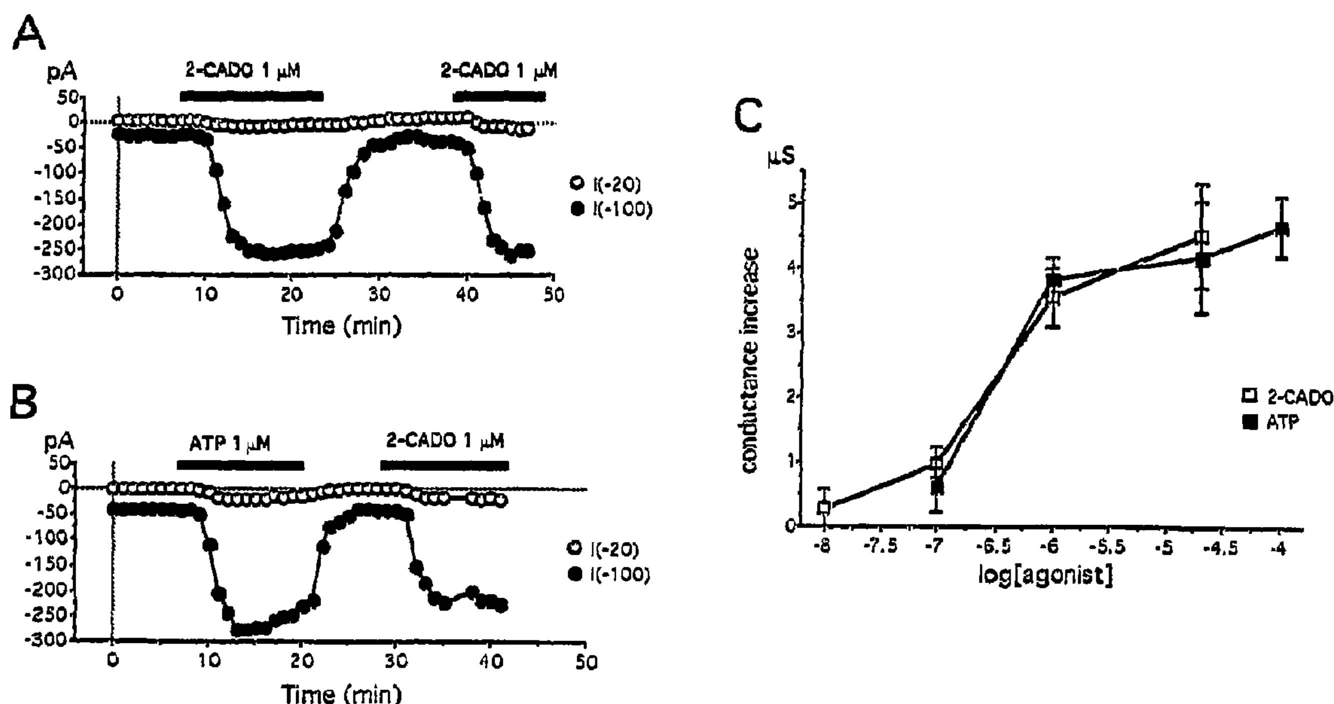

Fig. 1. Activation of membrane currents by 2-CADO and ATP. (A,B) Time course of membrane currents recorded at −100 and −20 mV from two representative cells upon application of ATP or 2-CADO 1 μM. (C) The graph shows the increase of chord conductance vs. the concentration of the agonist. Each point is the mean value of 4–22 separate experiments. Conductance was corrected for the series resistance error.

$[Ca^{2+}]_i$ by using the in situ calibration procedure described by Malgaroli et al. [11] after subtraction of cell background fluorescence. Data are shown as representative experiments or as mean values ± S.E.M.

3. RESULTS

Extracellular application of ATP and of the adenosine analogue 2-CADO caused a reversible increase of membrane conductance in 27/32 and 42/44 experiments, respectively. As shown in Fig. 1A and B, the current measured at −100 mV rose to a steady-state level which persisted as long as the agonist was applied. Occasionally, a small outward current was observed at −20 mV in the first minute of application thus indicating a transient activation of K^+ currents. In about 1/3 of experiments the current at −100 mV slowly decreased after having shown a peak. In these cases, lasting applications of the agonist revealed that a steady level (51 ± 4% of the peak) was reached within 10–15 min. Fig. 1A and B also demonstrate that a second application of the same or of the other agonist was equally effective as the first. Nevertheless, in 5/19 experiments a cell which responded to 2-CADO was insensitive to ATP. Fig. 1C illustrates the concentration–effect relationship for ATP and 2-CADO. Both agents have similar potency. Extracellular UTP (100 μM) failed to induce any activation of membrane currents (n=4; not shown).

The antagonist of the A_2 adenosine receptor, DMPX [12], partially or totally prevented the activation of membrane currents caused by 1 μM 2-CADO (n=12) as illustrated in Fig. 2A. Unexpectedly, DMPX was also a very effective antagonist of 1 μM ATP (n=9; Fig. 2B). If the current was already activated by the agonist, the subsequent application of DMPX caused a reversible inhibition (not shown). Fig. 2C demonstrates that DMPX is more potent on ATP than on 2-CADO-dependent currents. DMPX alone had no effect on Cl^- currents activated by hypotonic shock (not shown).

The increase of inward currents at −100 mV shown above is consistent with an activation of Cl^- or cation permeable channels. To characterize the ion selectivity and kinetics of these currents, experiments were performed with Na^+ instead of K^+ in the pipette solution. In this way K^+ currents, which have been described in a previous paper [6], could be excluded. Fig. 3 shows the main characteristics of currents activated by 2-CADO which were also displayed by those obtained with ATP. The peculiarities are the outward rectification of the current–voltage relationship and the time-dependent decrease of the current at highly positive potentials (Fig. 3B and D). Long-lasting depolarizations (i.e. >10 s) at +80 mV caused total inactivation of ATP- or 2-CADO-activated currents (not shown). A similar kinetic behavior has been described for volume-sensitive Cl^- currents in 9HTEo− and other epithelial cells [7,8,13]. The Cl^- selectivity was demonstrated by replacing 133 mM of extracellular Cl^- with 133 mM gluconate. Under this

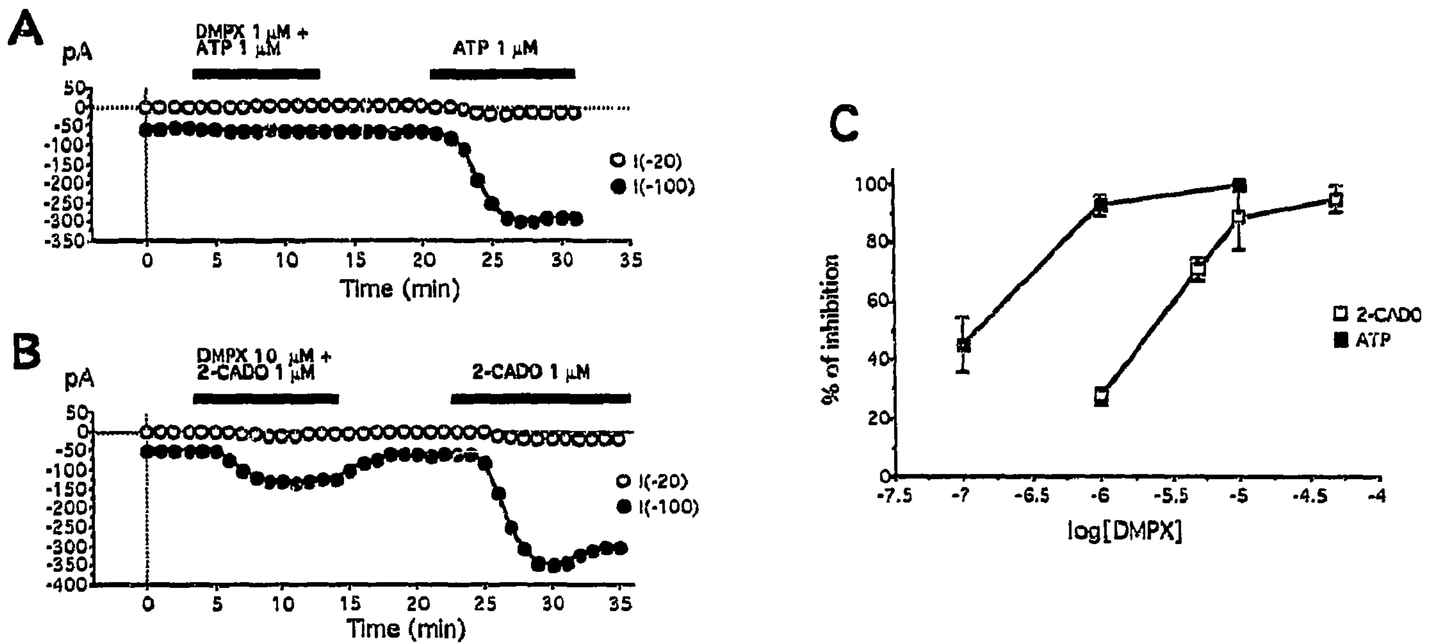

Fig. 2. Antagonizing effect of DMPX. (A,B) Time course of membrane currents following application of 2-CADO or ATP 1 µM in the presence and absence of DMPX in two representative cells. (C) Percentage inhibition of ATP- and 2-CADO-activated membrane currents vs. the concentration of DMPX. The concentration of agonists was 1 µM. Each point is the mean of 3 separate experiments.

condition (8 mM Cl^- outside/70 mM Cl^- inside) the Nernst potential for Cl^- should be +40 mV. In 5 experiments with 2-CADO and 4 with ATP the partial Cl^- replacement caused a strong reduction of outward currents and shifted the reversal potential from −16.3 ± 0.7 mV to +22.6 ± 2.0 mV and +23.9 ± 3 mV, respectively

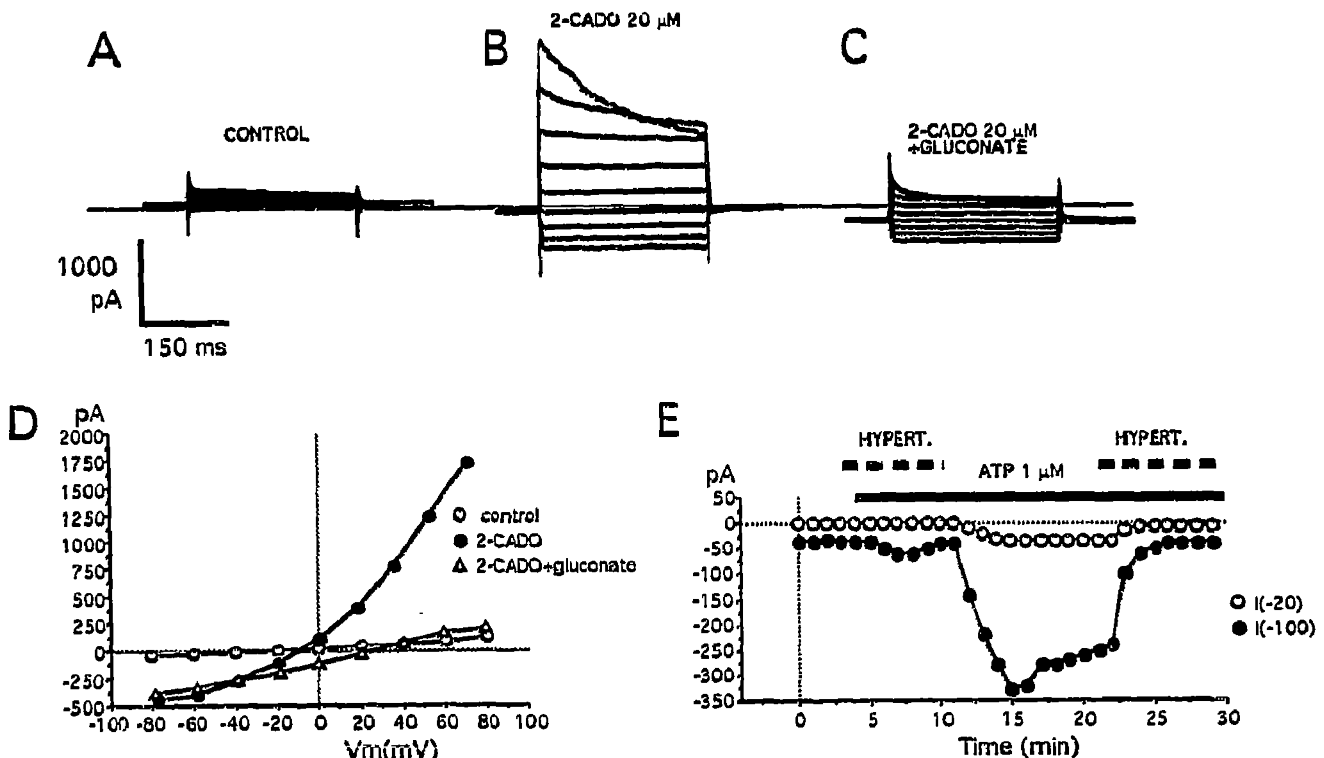

Fig. 3. Characteristics of membrane currents activated by 2-CADO. (A–C) Superimposed membrane currents measured at voltages between −80 and +80 mV with intervals of 20 mV. Experimental conditions are indicated in figure. (D) Current–voltage relationships taken from traces A–C. (E) Inhibition of Cl^- currents by extracellular hyperosmolality.

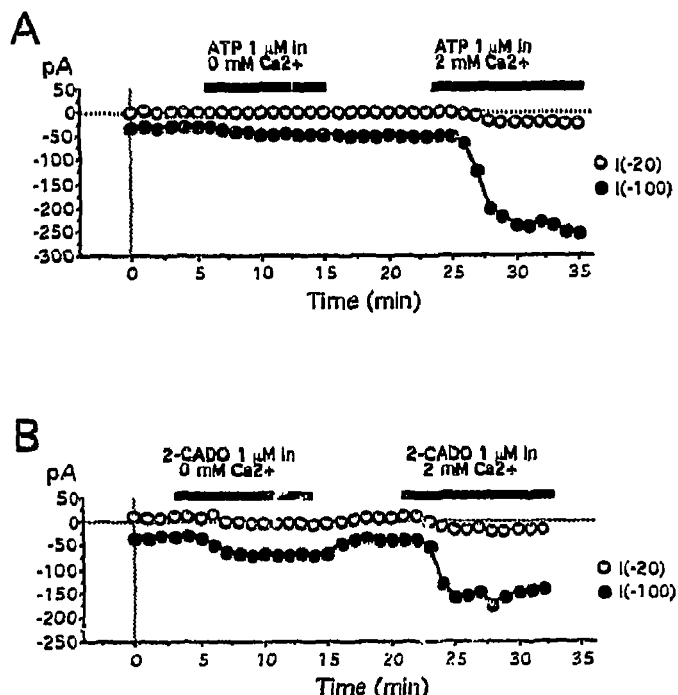

Fig. 4. Role of extracellular Ca^{2+} in the activation of Cl^- currents. (A,B) Time course of membrane currents upon application of 2-CADO or ATP 1 μM in the absence and in the presence of extracellular Ca^{2+}.

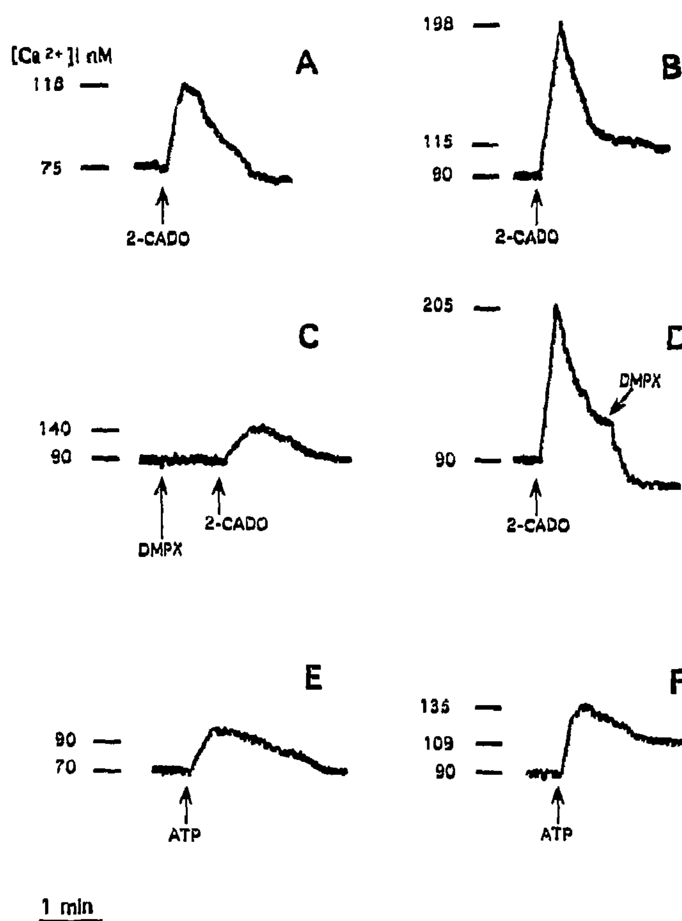

Fig. 5. Effect of 2-CADO, ATP, and DMPX on $[Ca^{2+}]_i$ in 9HTEo– cells. Cells were incubated in Ca^{2+}-free saline medium containing 0.2 mM EGTA (traces A and E); plus 1 mM $CaCl_2$ (traces B, C, D, F). The numbers represent the calibration of fura-2 signal in terms of $[Ca^{2+}]_i$ (nanomolar). Traces are representative of 4–6 similar experiments.

(Fig. 3C and D). These data suggest that 2-CADO and ATP activate Cl^- channels which are also slightly permeable to gluconate as also found for volume-sensitive Cl^- currents in 9HTEo– cells [14]. Replacement of extracellular Na^+ with *N*-methyl-D-glucamine had no effect on membrane currents (not shown).

The activation of volume-sensitive Cl^- currents suggests that purinergic agonists in 9HTEo– cells might cause cell swelling. This hypothesis seems confirmed by the finding, reported in Fig. 3E, that an hypertonic solution (340 mOsm/kg with mannitol) was able to prevent the activation and/or to inhibit Cl^- currents previously activated by ATP (n=8) and 2-CADO (n=4).

The role of extracellular Ca^{2+} in the purinergic activation of volume-sensitive Cl^- currents was investigated using a Ca^{2+}-free bath solution containing 1 mM EGTA. Under these conditions, ATP failed to activate Cl^- currents (n=5). In two of these experiments, a subsequent addition of ATP in 2 mM Ca^{2+}-containing medium was effective (Fig. 4). Conversely, 2-CADO was able to activate Cl^- currents in the absence of extracellular Ca^{2+}. The size of the currents was 41 ± 12% that obtained with 2 mM Ca^{2+} (n=4; Fig. 4).

Fig. 5A shows the effect on $[Ca^{2+}]_i$ of the addition of 5 μM 2-CADO in Ca^{2+}-free medium supplemented with 0.2 mM EGTA. The effect of 2-CADO was transient, since $[Ca^{2+}]_i$ returned to basal level within 1.5–2.0 min. At the peak, $[Ca^{2+}]_i$ was increased by 63 ± 7% (n=6) over the initial value. In the presence of excess EGTA, the increase in $[Ca^{2+}]_i$ is due to release from intracellular stores. In a medium containing 1 mM $CaCl_2$, the peak of $[Ca^{2+}]_i$ induced by 2-CADO was higher (+120 ± 8%, n=6) than that in Ca^{2+}-free/EGTA medium and was followed by a plateau which very slowly declined toward the resting level (trace B). This behaviour suggests the occurrence of Ca^{2+} influx through the plasma membrane, in addition to release from intracellular stores. Fig. 5C illustrates that Ca^{2+} containing media, preincubation with 10 μM DMPX reduced the increase in $[Ca^{2+}]_i$ caused by 2-CADO (+40 ± 3% over control, n=4). Addition of this compound after 2-CADO suddenly decreases $[Ca^{2+}]_i$ to a value even lower than the initial (trace D).

The addition of 10 μM ATP also induces an increase of $[Ca^{2+}]_i$ either in EGTA-containing medium (32 ± 5% over control, n=4, trace E) and in Ca^{2+}-containing medium (65 ± 9% over control, n=5, trace F). The effect of DMPX on the ATP-induced increase of $[Ca^{2+}]_i$ was

questionable since a weak inhibition was observed in 2/5 experiments (not shown).

4. DISCUSSION

Three types of Cl^- currents have so far been identified in human epithelial cells [15]. These currents are separately regulated by cAMP, Ca^{2+} and cell volume, and can be distinguished on the basis of the shape of their current–voltage relationship and kinetics. The present paper shows, for the first time, that volume-sensitive Cl^- currents can be activated by an extracellular agonist such as ATP and 2-CADO. The finding that the effects of ATP and 2-CADO are inhibited by increasing the extracellular osmolality might suggest that these compounds act indirectly by causing cell swelling.

Both electrophysiological and fluorimetric experiments suggest a role of Ca^{2+} in the purinergic activation of volume-sensitive Cl^- currents. The currents stimulated by ATP appear much more dependent on extracellular Ca^{2+} than those activated by 2-CADO. This may be due to the lower increase of $[Ca^{2+}]_i$ caused by ATP. If a critical $[Ca^{2+}]_i$ were required for current activation, this would be attained with ATP only through the influx from the plasma membrane.

At this stage, it is not possible to state which types of receptors are involved in the effects caused by 2-CADO and ATP. 2-CADO is an agonist which shows a slight selectivity for A_1 vs. A_2 adenosine receptors [16], while ATP is effective on P_{2x} or P_{2y} receptors [17] although a binding to adenosine receptors cannot be ruled out [18]. On the other hand, we have presented evidence that (i) some cells responded to 2-CADO but not to ATP; (ii) ATP was more dependent on extracellular Ca^{2+} than 2-CADO; (iii) DMPX showed a higher antagonizing effect on ATP than on 2-CADO. Therefore, it can be concluded that 2-CADO and ATP act on separate receptors in 9HTEo− cells.

Comparison of our results with those obtained by Mason et al. [1] reveals some differences. These authors have found little effect of adenosine application on Cl^- secretion and a response to UTP even higher than that obtained with ATP. In our experiments, UTP did not activate Cl^- currents whereas an adenosine analogue displayed a high effectiveness. It appears therefore that 9HTEo− cells have a pattern of purinergic receptors different from that found in nasal epithelium [1]. This may not be surprising since 9HTEo− derive from the tracheal epithelium. It has also to be taken into account that 9HTEo− may be representative of only a subpopulation of airway epithelial cells. Given the particular importance of purinergic compounds, in particular of adenosine, in many aspects of respiratory physiology [19], it will be interesting to define the role of the agonist-mediated activation of volume-sensitive currents in Cl^- secretion and other physiological and pathological processes.

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REFERENCES

[1] Mason, S.J., Paradiso, A.M. and Boucher, R.C. (1991) Br. J. Pharmacol. 103, 1649–1656.
[2] Knowles, M.R., Clarke, L.L. and Boucher, R.C. (1991) N. Engl. J. Med. 325, 533–538.
[3] Horn, R. and Marty, A. (1988) J. Gen. Physiol. 92, 145–159.
[4] Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflueger's Arch. 391, 85–100.
[5] Gruenert, D.C., Basbaum, C.B., Welsh, M.J., Li, M., Finkbeiner, W.E. and Nadel, J.A. (1988) Proc. Natl. Acad. Sci. USA 85, 5951–5955.
[6] Galietta, L.J.V., Rasola, A., Barone, V., Gruenert, D.C. and Romeo, G. (1991) Biochem. Biophys. Res. Commun. 179, 1155–1160.
[7] Worrell, R.T., Butt, A.G., Cliff, W.H. and Frizzell, R.A. (1989) Am. J. Physiol. 256, C1111–C1119.
[8] Galietta, L.J.V., Barone, V., Gruenert, D.C. and Romeo, G. (1991) Adv. Exp. Med. Biol. 290, 307–317.
[9] Galietta, L.J.V., Barone, V., De Luca, M. and Romeo, G. (1991) Pflueger's Arch. 418, 18–25.
[10] Di Virgilio, F., Steinberg, T.H., Swanson, J.A. and Silverstein, S.C. (1988) J. Immunol. 140, 9189–9195.
[11] Malgaroli, A., Milani, D., Meldolesi, J. and Pozzan, T. (1987) J. Cell Biol. 105, 2145–2155.
[12] Ukena, D., Shamim, M.T., Padgett, W. and Daly, J.W. (1986) Life Sci. 39, 743–750.
[13] Solc, C.K. and Wine, J.J. (1991) Am. J. Physiol. 261, C658–C674.
[14] Rasola, A., Galietta, L.J.V., Gruenert, D.C. and Romeo, G., submitted.
[15] Cliff, W.H. and Frizzell, R.A. (1990) Proc. Natl. Acad. Sci. USA 87, 4956–4960.
[16] Bruns, R.F., Lu, G.H. and Pugsley, T.A. (1986) Mol. Pharmacol. 29, 331–346.
[17] O'Connor, S.E., Dainty, I.A. and Leff, P. (1991) Trends Pharmacol. Sci. 12, 137–141.
[18] Williams, M., Braunwalder, A. and Erickson, T.J. (1986) Naunyn-Schmiederberg's Arch. Pharmacol. 332, 179–183.
[19] Griffiths, T.L. and Holgate, S.T. (1990) in: Adenosine and adenosine receptors (M. Williams, Ed.), The Humana Press, Clifton, NJ, pp. 381–422.