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Adult Alveolar Type II Cells Lack cAMP and Ca^{2+}-Activated Cl^- Channels

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We investigated the presence of Ca^{2+}-activated Cl^-channels in adult rat alveolar type II (ATII) using patch-clamp techniques. Only one active channel each, with a single channel conductance of 50 pS and an opening probability (*Po*) of 0.76 was found among 130 successful cell-attached and 5 inside-out patches. Addition of CPT-cAMP into the bath (500 μM) induced one active patch from 33 silent cell-attached patches. Incubation of 9 ATII cells, with ionomycin (1 μM), failed to elicit chloride single currents in 9 cell-attached patches. Cl^- currents were also absent from 35 whole cell patches, even after the addition of 10 μM terbutaline in the bath or 1 mM ATP and 5 mM $MgCl_2$ in the pipette. These results indicate that only a very small fraction of adult rat ATII cells express CFTR and suggest that Cl^- ions are passively transported across the cell junctions.

Secretion of fetal lung fluid is critical to normal growth and differentiation of future gas exchange regions of the developing mammalian lungs. The major driving force for this process is the active transport of Cl^- ions across alveolar and airway epithelial cells (1,2).

Shortly before birth the Cl^-secretion ceases and sodium reabsorption commences (3,4). Previous reports indicate that the short-circuit current across fetal distal lung epithelial cells, cultured on polycarbonate filters and mounted in Ussing chambers is due to sodium reabsorption (5,6). Recent studies however, indicated the presence of chloride channels in fetal ATII cells (7) and in an ATII cell-like line (8,9). More important, Kim et al. (10) demonstrated that addition of terbutaline, a β-agonist, into the basolateral side of an Using chamber containing cultured adult rat ATII cells results in active chloride reabsorption. β-agonists are known to elevate intracellular cAMP and Ca^{2+} levels in adult and fetal ATII cells (11,12).

These observations led us to speculate that ATII cells contain Ca^{2+} or cAMP activated Cl^- channels. The potential existence of chloride channels in ATII cells is important because it may indicate the ability of these cells to secrete fluid; furthermore, a recent report indicates that cystic fibrosis transmembrane conductance regulator (CFTR), may modulate Na^+ reabsorption (13). We used patch clamp and indirect immunofluorescence techniques to investigate the possible existence and characterize the properties of Ca^{2+} or cAMP activated chloride channels in ATII cells in primary culture. Previous studies have shown that ATII cells express amiloride-inhibitable Na^+-selective ion channels (11), Na^+-K^+-ATPase (14), and are capable of vectorial ion transport. We have chosen to perform our measurements in ATII cells in primary culture because of well-known culture-induced alterations in ion channels (15). The results of our studies indicate that chloride channels are expressed very infrequently in adult ATII cells.

MATERIALS AND METHODS

ATII cell isolation. Adult male Sprague-Dawley rats were anesthetized and killed by pentobarbital injection (100 mg/kg). All procedures for the isolation of ATII cells have been discussed in detail (11,15). In brief, ATII cells were isolated by digesting lung tissue with elastase, mincing the tissue and centrifuging the homogenate on Percoll gradients. Isolated ATII cells were seeded on glass coverslips at density of 7.5-8.5X10^5/cm^2 and cultured at 37°C, 5% CO_2 and 95% air for 24 h.

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Immunofluorescence measurements. ATII cells grown on coverslips for 24 h were fixed and permeabilized by immersion in pure methanol at 4°C, rinsed with PBS 3 times, and then immersed in 1% bovine serum albumin (BSA) for 7 min to block nonantigenic binding sites (16). The cells were then incubated either with a polyclonal antibody to Ca^{2+}-activated Cl^- channel protein, purified from bovine tracheal epithelial cells (17), or a polyclonal antibody against a synthetic peptide of the R-domain of CFTR, at 37°C for 35 min [(18); both antibodies were a generous gift of Dr. D.J. Benos]. Cells were then washed with PBS and incubated with a fluorescent secondary antibody (goat anti-rabbit IgG conjugated to rhodamine) at 37°C for 35 min. In control experiments, the primary antibodies were substituted with an equal amount of non-immune rabbit IgG (Sigma). The cells were then washed with PBS once again, and the cell containing spot was covered by a coverslip, which was sealed to the glass slide with cytoseal mounting medium. Slides were viewed with an AusJena Sedival (Jenoptic Jena GmbH, Germany) inverted microscope equipped with a 50× planachromat objective and a standard rhodamine filter set. Cell fluorescent images were captured by Photometrics Series 250 cooled charge coupled device (CCD) camera system (Photometrics, Tucson, AZ), connected to Power Macintosh 8100/80 computer (Apple, Cupertino, CA).

Patch clamp studies. Patch clamp measurements were performed using standard techniques (19). Pipettes were fabricated from KG-12 glass capillaries (Wilmad Glass) on a two-stage vertical puller (Narishigi, Japan). The taper of the pipettes were coated with Q-dope and fire-polished, yielding tip resistance 5-10 Mω when filled with pipette solution (see below for composition). A piece of coverslip containing either freshly isolated or cultured ATII cells was placed into a one ml in vitro recording chamber mounted on a an inverted microscope, equipped with a standard FITC filter set (excitation: 485 ± 20 nm; emission: >510nm). Cells were patched in the cell-attached and inside-out modes. In all cases the bath solution contained in (mM): 145 N-methyl-D-glucamine-Cl ($NMDG^+$ an impermeant cation), 5 HEPES, 2 $CaCl_2$ and 1 EGTA (pH = 7.25). The pipette solution contained in (mM): 145 $NMDG^+$-Cl, 5 HEPES, 0.2 $CaCl_2$ and 1 EGTA (pH = 7.25; "cation-free solutions"). Voltage pulses (2 sec in duration) ranging from −80 mV to +80 mV were applied across the cells and the resulting inward and outward currents were measured with a EPC-7 amplifier using a 3 KHz filter, monitored with Nicolet 310 digital oscilloscope and stored on video tape for later analysis. The pCLAMP program (version 5.5.1, Axon) was used to analyze both whole cell and single channel data. Single channel currents were filtered at 300Hz before analysis.

To investigate whether increases in intracellular cAMP of Ca^{2+} stimulate Cl^- currents, a number of ATII cells were patched in cell-attached mode and treated with 500 μM 8−(4-chlorophenythio)-adenosine 3′:5′-cyclic monophosphate (CPT-cAMP), a cell-pemeant form of cAMP, or 1 μM ionomycin, a Ca^{2+} ionophore, known to increase intracellular Ca^{2+} concentration (20). Single channel recordings were performed 15 min after CPT-cAMP treatment or 3 min after ionomycin treatment. In addition, in a number of cases, ATII cells patched in whole-cell mode were treated for 15 min with 10 μM terbutaline, prior to measuring current-voltage relationships.

In the second series of experiments, we patched ATII cells in the whole cell mode using bionic, called "cation-rich" solutions, with the following composition (in mM): Bath: 150 NaCl, 5 HEPES, 2 $CaCl_2$, 1 EGTA; pipette: 150 KCl, 5 HEPES, 1 EGTA, 1 ATP. Using these solutions, the outward K^+ current were due to the movement of K^+ ions, while the inward currents were due to the movement of Na^+. Following measurement of current-voltage relationships, amiloride (1 μm) and $BaCl_2$ (5 mM) were added into the bath to block Na^+ and K^+ channels respectively. CPT-cAMP (500 μM) were added into the bath 10 min later, and current-voltage relationships were repeated in an attempt to detect changes in either the inward or outward currents which would be due to the movement of Cl^- ions.

RESULTS

I. Immunofluorescence studies. Permeabilized ATII cells demonstrated only background levels of fluorescence when immunostained with antibodies to either Ca^{2+}-activated Cl^- channel or CFTR. On the other hand, T84 cells, a human colonic crypt epithelial cell line, demonstrated a ten fold increase in fluorescence when immunostained with the CFTR Ab as compared with non-immune IgG (Figure 1; Table 1). These findings indicate the lack of proteins of ATII cells which are antigenically related to either Ca^{2+}-activated Cl^- channel, or CFTR, in either the regions of the plasma membrane or cytoplasm of ATII cells.

II. Patch clamp measurements. ATII cells were distinguished from macrophages and fibroblasts on the basis of the round granular appearance and low levels of fluorescein isothiocyanate fluorescence (FITC), when examined with fluorescence microscopy.

The results of patch clamp studies in ATII cells are summarized in Table 2. We obtained 130 successful cell-attached patches (seal resistance > 5 GΩ) and 5 inside-out patches in control conditions using "cation-free" solutions. Single channel currents were seen only in one cell-attached patch and one inside-out patch, in the same cell (Figures 2, 3). This channel had a conductance of 50 pS, an open probability of 0.76 and a mean open time of 33.5 ms. Addition of 100 μM DNDS (4,4′-dinitrostilbene-2,2′-disulfonic acid) in the bath, did not affect single channel

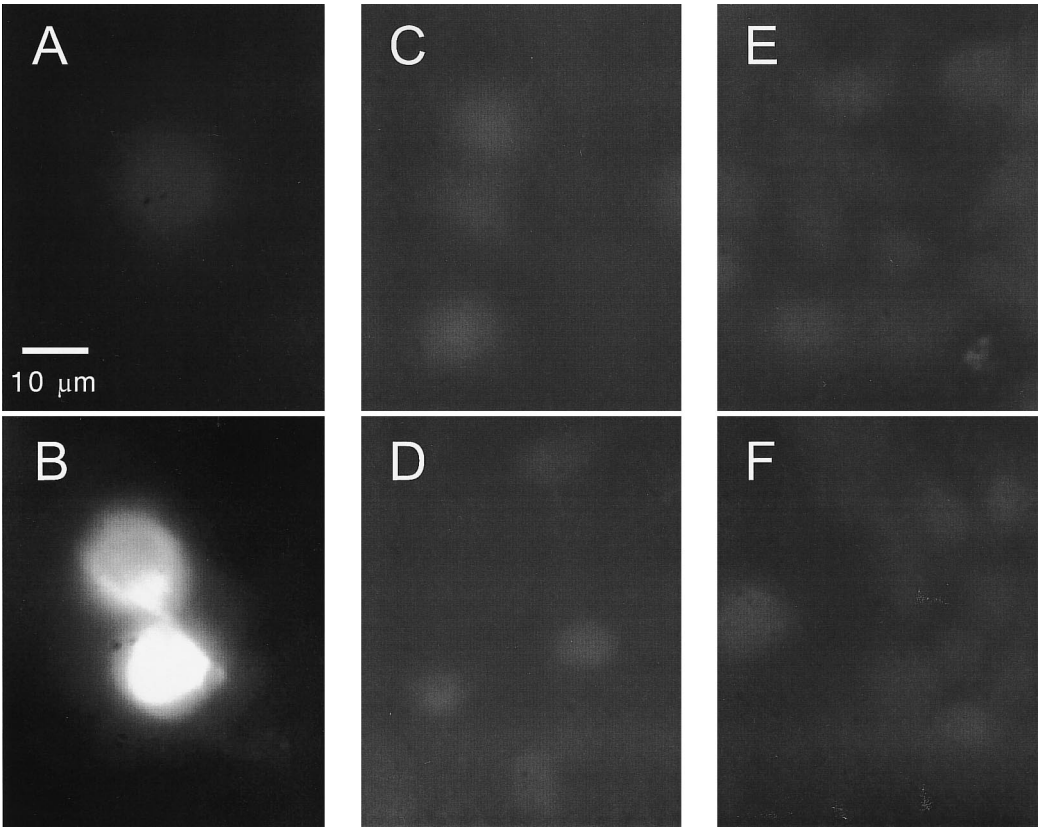

FIG. 1. Freshly isolated ATII cells or cultured T84 cells were immunostained with antibodies to Cl-channels (lower panels) or nonimmune IgG (upper panels), followed by goat antirabbit IgG conjugated to rhodamine. Typical fluorescence images were obtained at the same settings. White areas contain higher fluorescence levels than background (black). Left panels: T84 cells (A = IgG, B = CFTR Ab); Middle panels: ATII cells (C = IgG, D = Ca^{2+}-activated Cl^- channel Ab); Right panels: ATII cells (E = IgG, F = CFTR Ab).

conductance. However, the channel started to flicker, and its open probability and mean open time decrease to 0.64 and 4.6ms, respectively.

Treatment of ATII cells with 500 μm CPT-cAMP for 15 min, which results in a large increase in intracellular cAMP, failed to activate chloride channels: out of 33 successful patches, only one exhibited chloride channel activity. Even then, the channel was very unstable and was barely discernable above the baseline. Similarly, no single channels were seen in any of the cell-attached patches across 9 different ATII cells, following treatment with 1 μm ionomycin for up to 3 min,

TABLE 1
Pixel Fluorescence Intensity Following Immunostaining

	ATII cells	T84 cells
IgG	53 ± 7 (n = 18)	54 ± 7 (n = 25)
Ca^{2+}-activated Cl^- channel Ab	61 ± 14 (n = 7)	(not measured)
CFTR Ab	51 ± 7 (n = 14)	515 ± 35 (n = 20)**

Values are mean ± se; n = number of measurements.
** $P < 0.01$ compared with IgG.

TABLE 2
Detection of Chloride Channels in ATII cells

	C/A patches	I/O patches	W/C patches
Control	1/130	1/5	0/35
CPT-cAMP	1/33	NA	NA
Ionomycin	0/9	NA	NA
Terbutaline	NA	NA	0/16
Mg + ATP	NA	NA	0/2

Numbers of patches in which chloride currents were detected/ number of successful patches.

C/A = cell-attached; I/O = inside-out; W/C = whole-cell, NA = not available.

Concentrations of reactants CPT-cAMP = 500 μM; Ionomycin = 1 μm; Terbutaline = 10 μm $Mg^{+}ATP$ = 5 m $MgCl_2$ + 1 mM ATP (in pipette).

which causes significant elevation of intracellular Ca^{2+}. Finally, 16 ATII cells treated with 10 μM terbutaline and patched in the whole cell mode using "cation-free" solutions, showed only background levels of inward and outward currents for membrane potentials between −80 to +80 mV.

Both outward K^+ and inward Na^+ currents were observed in ATII cells patched in the whole-cell mode using "cation-rich" solutions (Fig. 3). The magnitude of these currents were decreased significantly following addition of amiloride (1 μm) and $BaCl_2$ (5 M) into the bath solution. However, the subsequent addition of 500 μM CPT-cAMP into the bath failed to induce whole-cell currents, indicating the absence of cAMP-activated Cl^- channels (Fig. 4).

DISCUSSION

Previous studies have shown that ATII cells contain amiloride sensitive Na^+ channels and Na^+-K^+-ATPase in their apical and basolateral membranes respectively and are capable of vectorial Na^+ transport (11,14-16,21-26). Na^+ currents were observed in approximately 10% of all cell-attached and inside-out ATII cell patches (11). Pretreatment of ATII cells with β-agonists increased

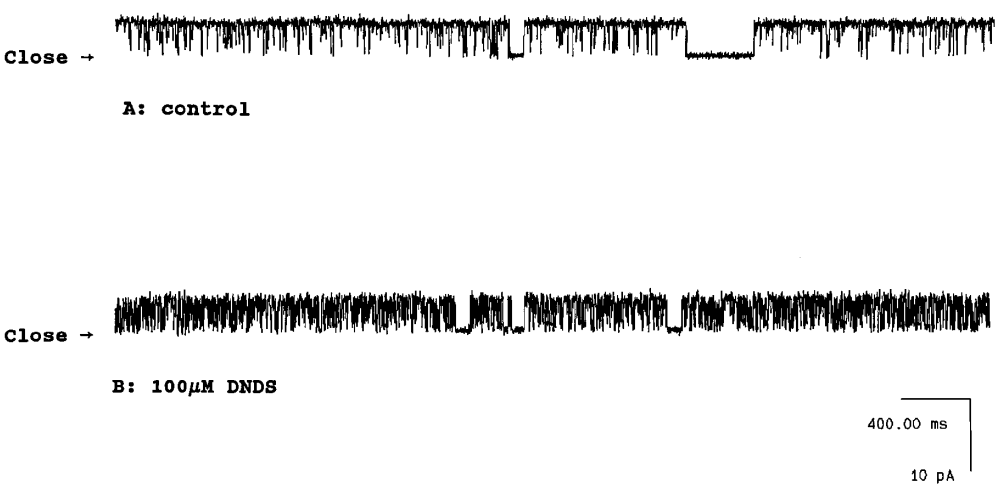

FIG. 2. Single channel recordings from inside-out patches of an ATII cell at +80 mV. The composition of the ionic solutions were as follows: Bath (in mM): 145 $NMDG^+$-Cl^-; 5 HEPES, 2 $CaCl_2$ and 1 EGTA (pH = 7.25). Pipette (in mM) 145 $NMDG^+$-Cl^-, 5 HEPES, 0.2 $CaCl_2$ and 1 EGTA (pH = 7.25). Panel A, Control; Panel B, Recording following addition of 100 μM DNDS in the bath solution.

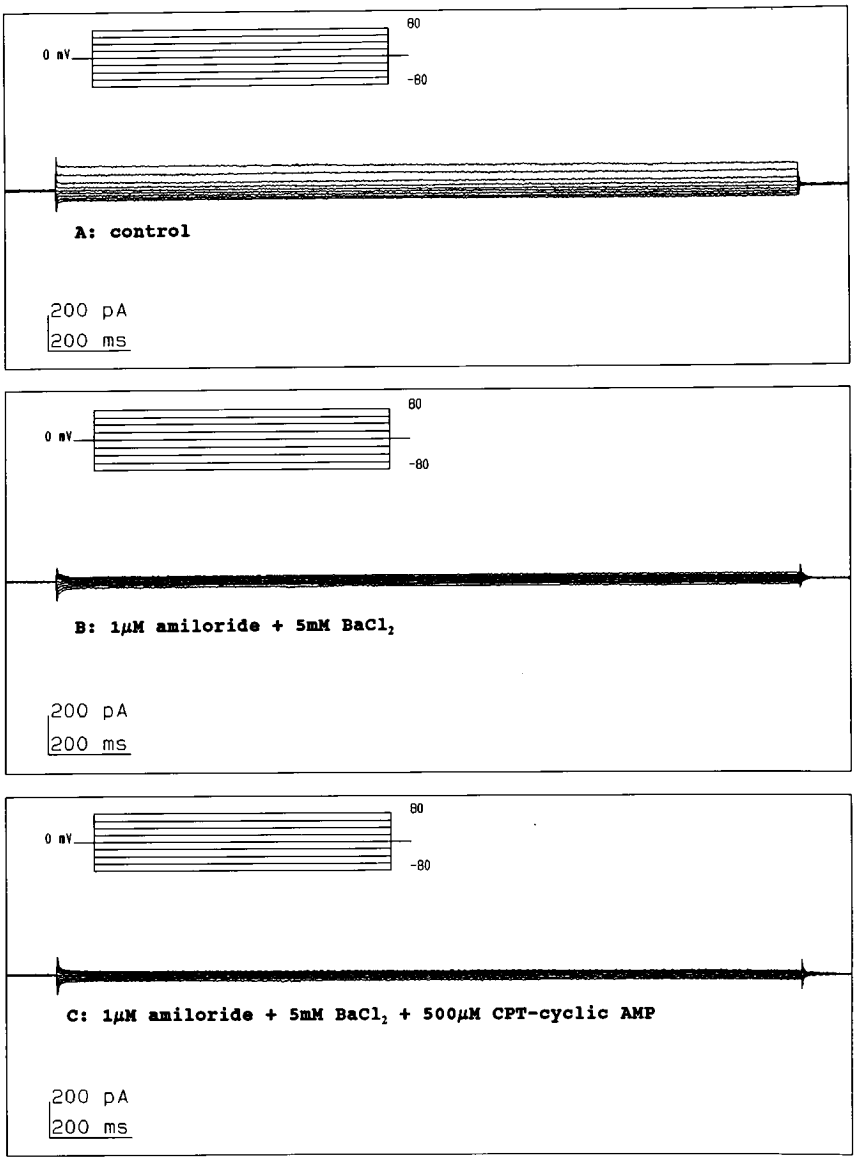

FIG. 3. Sample record of current–voltage relationships in a ATII cell patched in the whole-cell mode using the following solutions: bath (mM): 150 NaCl, 5 HEPES, 2 $CaCl_2$, 1 EGTA; pipette (mM): 150 KCl, 5 HEPES 1 EGTA, 1 ATP. Upper panel: Control. Middle panel: 1 μm amiloride +5 mM $BaCl_2$ in bath. Lower panel: 1 μ m amiloride+5 mm $BaCl_2$ + 500 μM CPT-cyclic AMP in bath.

the single channel mean open time without affecting their point conductance (11). Exposure of rats to sublethal hyperoxia, which leads to the development of tolerance to hyperoxia, resulted in increased ATII cell whole cell current (21), channel density (*NPo*) and αRENaC expression (22).

The expression of Cl^- channels in adult ATII cells will be highly significant because it will indicate their ability to act as both fluid absorbing and secreting cells. Chloride channels have been identified in fetal ATII cells (7), including apically located CFTR (27). However, other investigators reported that the short-circuit current across late gestational cultured rat ATII cells was due solely to the movement of Na^+ ions (6). Murray et al demonstrated both mRNA and protein

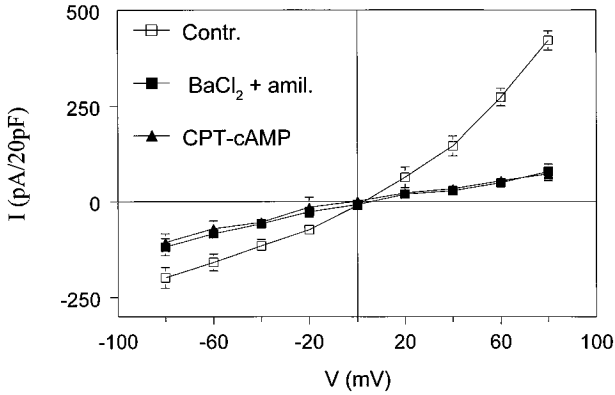

FIG. 4. Whole cell, current–voltage relationships for ATII cells, obtained after 10 min of exposure to the indicated agents (see legend). Cells were patched using bi-ionic solutions (bath (mM): 150 NaCl, 5 HEPES, 2 $CaCl_2$, 1 EGTA; pipette (mM): 150 KCl, 5 HEPES 1 EGTA, 1 ATP). Values are means ± 1 SEM; n ≥ 3.

expression of volume-activated Cl^- channel in fetal rat lungs (28). The expression of mRNA and protein significantly diminished after birth and disappeared in adult rat lung. The immunocytochemical study by McGrath et al. (29) indicated that CFTR was concentrated in the apical region of bronchiolar epithelial cells and absent from alveoli of fetal lungs of late gestation rabbits. In addition, Kemp et al identified an electroneutral anion exchange pathway for Cl^- transport but not Cl^- channels in adult ATII cells of guinea-pigs (30); however they identified Cl^- channels in fetal ATII cells from the same species of animals (7). Their findings represent another example of developmentally regulated Cl^- channels.

Chloride channels have been identified in mouse adult ATII cell lines, but not freshly isolated or primarily cultured ATII cells (8,9). Furthermore, the channels closed at membrane potential more negative than −10 mV (9). Thus their physiological significance is uncertain. Our results are in agreement with these findings and do not support the existence of functional Cl^- channels on primarily cultured ATII cells from adult rats. This is consistent with other investigators' findings. It should be stressed that a variety of Cl^- channels with different kinetic properties have been characterized in epithelial cells. It is possible that some silent Cl^- channels, different from CFTR and Ca^{2+}-activated Cl^- channels, do exist in ATII cells and become active in response to some stimuli. The two active Cl^- channels found in single channel recording may be examples of these channels.

In summary, our immunocytochemical and patch-clamp studies indicate the lack of cAMP and Ca^{2+} activated Cl^- channels in the apical membranes of ATII cells maintained in primary culture. These findings are important because they indicate that the well-documented Na^+ transport across these cells will not be modulated by CFTR, as proposed by Stutts et al (13).

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