Volume 270, number 1,2, 157-164 FEBS 08833 September 1990

Low-conductance chloride channel activated by cAMP in the epithelial cell line T_{84}

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Received 2 July 1990

We have studied the modulation and pharmacological properties of two anion channels in T_{84} cells by recording single channel and transepithelial currents. One channel had an outwardly rectifying current-voltage I/V curve, was rarely active in cell-attached patches, and was unaffected by cAMP. The other channel had lower conductance (8.7 pS at 37°C) and a more ohmic I/V relationship. Exposure to cAMP increased the probability of observing low-conductance channel activity in cell-attached patches > 6-fold. Extracellular DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid) or IAA-94 (an indanyloxyacetic acid) inhibited the outward rectifier but did not affect the low-conductance channel or cAMP-stimulated transepithelial current. These results suggest the low-conductance Cl channel may contribute to apical membrane conductance during cAMP-stimulated secretion.

Epithelial secretion; Patch clamp; Chloride channel; Cystic fibrosis; T_{84} cell line; 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid inhibition

1. INTRODUCTION

Many epithelia share a common mechanism for chloride secretion which involves electroneutral coentry of NaCl or NaK2Cl through the basolateral membrane and passive exit of Cl through a cAMP-activated conductance in the apical membrane [1]. The differentiated colonic tumor cell line T_{84} provides a convenient model preparation for studying the regulation of epithelial Cl secretion because it develops into monolayers with apical-basal polarity and relatively high transepithelial resistance (> 1000 ohms · cm^2) and responds to a variety of secretagogues including vasoactive intestinal peptide, prostaglandin E_1 and carbachol [2]. Agents that elevate cAMP also increase the short-circuit current (I_{sc}) from near zero to about 40 $\mu A/cm^2$. Previous radiotracer flux studies [3] have shown that this current is carried exclusively by Cl transport from the serosal to mucosal side (i.e. in the basolateral-to-apical direction). Carbachol and calcium ionophores also induce Cl secretion, probably by activating a basolateral potassium conductance, which would hyperpolarize the cells and increase the electrical potential favoring Cl efflux at the apical membrane [4]. The T_{84} cell line is also useful for genetic analyses; it contains relatively large amounts of mRNA encoding the protein that is defective in cystic fibrosis (CF) and was used as the control cell line during cloning of the CF gene [5,6].

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The pathways responsible for apical Cl conductance have not yet been identified with certainty. Patch clamp studies of airway, T_{84} and other cultured cells have revealed outwardly rectifying anion channels having conductances between 30 and 40 pS (150 mM NaCl, 20°C) and the selectivity sequence $SCN > I > NO_3 > Br > Cl > HCO_3 >$ acetate $> SO_4 >$ gluconate (see [7] for review). They are sensitive to several inhibitors including DIDS and other disulfonic stilbenes [8], diphenylamine-2-carboxylate [9], 5-nitro-2-(3-phenylpropylamino)-benzoic acid [10,11] and Hepes-related buffers [12]. In some preparations, outward rectifiers can be activated in cell-attached patches by cAMP and may therefore contribute to apical Cl conductance [13-15]. This proposed role, and its relevance to CF, is supported by reports that outward rectifiers excised from normal cells can be activated by exposure to A- or C-kinase whereas those from CF cells are not responsive [16,17].

We have used the patch clamp technique to study anion channel regulation in T_{84} cells. Parallel studies of transepithelial transport were carried out in Ussing-type chambers under comparable conditions. We found that cAMP greatly increased the activity of a low-conductance Cl channel in T_{84} cells but had little effect on the outwardly rectifying anion channel. The low-conductance Cl channel and cAMP-stimulated I_{sc} were both insensitive to DIDS and IAA-94 although these compounds were potent inhibitors of the outward rectifier. We conclude that the low-conductance Cl channel in T_{84} cells may contribute to apical membrane conductance during secretagogue stimulation.

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00145793/90/$3.50

2. MATERIALS AND METHODS

2.1. *Cell culture and materials*

The T_{84} line was obtained from American Type Culture Collection (Rockville, MD) and studied between passages 77 and 115. Cells were plated on glass coverslips or type 1 collagen membranes at a density of 400 000/cm^2. The growth medium was 50:50 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 supplemented with 15 mM Hepes (hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), fetal bovine serum (5%), penicillin (100 IU/ml) and streptomycin (100 μg/ml). Culture media and most supplements were purchased from Gibco (Burlington, Ont.) except collagen, which was from Collagen Corp. (Palo Alto, CA). IAA-94 (2-[(2-cyclopentyl-6,7-dichloro-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl) oxy]acetic acid) was a gift from Merck Sharp and Dohme Research Laboratories (Rahway, NJ). DIDS and other biochemicals were from Sigma (St. Louis, MO). Solutions containing DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic) acid were prepared fresh and kept in the dark at 4°C until used. All experiments were performed at 37 ± 1°C.

2.2. *Patch clamp experiments*

Cells were transferred to a solution containing (mM): NaCl 150, KCl 4, $CaCl_2$ or $MgCl_2$ 2, glucose 10, mannitol 100, TES 10, pH 7.4. The pipette solution contained 150 mM NaCl, 2 mM $CaCl_2$ and 10 mM TES, pH 7.4, unless indicated otherwise. To stimulate Cl secretion via the cAMP path, cells were exposed to a mixture of 10 μM IBMX (3-isobutyl-1-methylxanthine), 10 μM forskolin, and 0.5 mM dibutyryl-cAMP. The temperature was maintained at 37°C by a locally built Peltier-type chamber and proportional controller. Currents were recorded using cell-attached and inside-out patches [18]. Pipettes were pulled in two stages (PP-83, Narishige Instr. Lab., Tokyo) and had resistances of 4–6 MΩ when filled with 150 mM NaCl solution. The pipette contained a chlorided Ag wire; the bath was grounded through an agar bridge having the same ionic composition as the pipette solution. Liquid junction potentials at the agar bridge were measured against a flowing 3 M KCl electrode. Single-channel currents were amplified (Axopatch, Axon Instr. Inc., Burlingame, CA), recorded on video cassette tape by a pulse-coded modulation-type recording adapter (DR384, Neurodata Instr. Co., NY) and low-pass filtered during playback using an 8-pole Bessel filter (902LPF, Frequency Devices, Haverhill, MA). The overall bandwidth at the A/D converter was 150 or 514 Hz and records were sampled at 0.5–2.0 kHz. The data were analyzed using a laboratory microcomputer system (Indec Systems, Sunnyvale, CA) as described previously [12,19]. V_p refers to the command voltage applied to the pipette interior with reference to the bath; for example, $V_p = -20$ mV would indicate the potential difference across the membrane patch is 20 mV more positive than the cell membrane potential. Current-voltage (I/V) relationships were calculated by a semi-automated procedure in which amplitude histograms were computed for short segments of record and displayed on a split screen next to the data so that peaks could be verified using cursors. At least 10 open events were measured at each steady-state potential, averaged, and entered into an I/V curve which was displayed at the end of the run. Reversal potentials were obtained by interpolation after fitting a polynomial function to the I/V curve. Slope conductance was determined by linear regression over the voltage ranges specified in the Results. The mean number of channels open during successive segments of the record ($<I>/i$) was computed from the fraction of time spent at each multiple of the single-channel current.

2.3. *Transepithelial experiments*

Studies were carried out in Ussing-type chambers (Vangard International, Neptune, NJ) that had been fitted with teflon adaptors for collagen disks. The control solution contained (mM) NaCl 115, K_2HPO_4 2.5, $CaCl_2$ 1.5, $MgSO_4$ 1, glucose, 10, $NaHCO_3$ 25, and was gassed with 95% O_2/5% CO_2. Monolayers were short-circuited using a conventional voltage clamp (DVC-1000, WPI Instr., Hamden, CT). Brief voltage pulses (10 mV, 2 s duration) were applied every 150 s to monitor transepithelial resistance. I_{sc} was plotted on a strip chart recorder (SE 400, Goerz Electro GmbH, Vienna, Austria) and transferred to a computer spread sheet program for calculations and graphics.

Significance was determined at the 95% confidence level using paired Student's *t*-tests.

3. RESULTS

In the first series of experiments we compared the effects of cAMP and membrane excision on two anion channels, an outwardly rectifying channel similar to those described in earlier studies of T_{84} cells [15,19] and a low-conductance Cl channel, which has not been reported previously in this cell line.

3.1. *Outwardly rectifying anion channel*

Under control conditions, outward rectifiers were active in 1 of 39 cell-attached patches (2.5%; Fig. 1a). Incubating the cells with the cAMP solution for more than 5 min did not increase the fraction of cell-attached patches containing outward rectifier activity, in fact this decreased slightly (Fig. 1b). Nevertheless, outwardly rectifying anion channels were present at relatively high density, and became active in 30 of 99 patches (30%) after they had been excised (Fig. 1c).

3.2. *Activation of a low-conductance Cl channel by cAMP*

A low-conductance channel was also observed in unstimulated cells at approximately the same low frequency as the outward rectifier (3/39 cell-attached patches; Fig. 1a). However, in marked contrast to the outward rectifier, the probability of observing the low-conductance channel in seals increased more than 6-fold when cells were exposed to the cAMP mixture (Fig. 1b). Activity of the low-conductance channel disappeared when patches were excised (Fig. 1c). Ac-

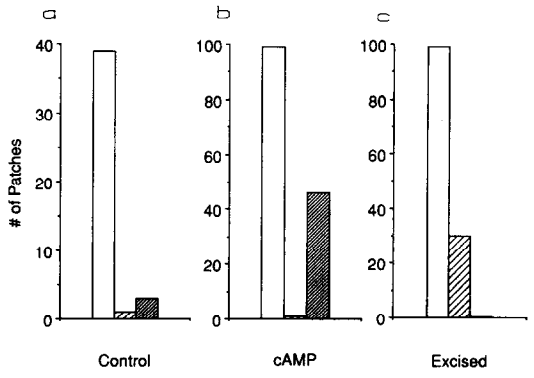

Fig. 1. Histograms showing the number of patches (open bars), the number containing outward rectifier activity (light hatched bars), and the number containing low-conductance Cl channel activity (dark hatched bars). (a) Cell-attached patches using unstimulated cells. (b) Cell-attached patches after more than 5-min exposure to 0.5 mM db-cAMP, 10 μM IBMX and 10 μM forskolin. (c) Same patches as in (b) after they had been excised. All recordings were at 37°C.

tivation was not observed during continuous recordings in the cell-attached configuration. Similar results were obtained using confluent or subconfluent cells, whether they had been cultured on glass or porous collagen supports.

Fig. 2a shows typical cell-attached recordings of the low-conductance channel at different potentials. Open probability was not obviously voltage-dependent although we noticed the currents flickered more at hyperpolarizing potentials despite low-pass filtering at 150 Hz. Fig. 2b shows mean current-voltage relationships determined on-cell using different NaCl solutions in the pipette. Outward currents were slightly larger than inward currents as expected when the Cl activity in the cell is lower than in the pipette solution (150 mM NaCl). Single channel currents reversed direction at $-V_p = +7.0$ mM ± 1.5 mV suggesting intracellular Cl is slightly above electrochemical equilibrium. The reversal potential (E_{rev}) shifted to −3.4 ± 1.4 mV when recordings were made with 270 mM NaCl in the pipette, which confirms that the channel is selective for anions over cations. P_{Cl}:$P_{cations}$ was calculated to be 8.1 ± 2.4 using the Goldman-Hodgkin-Katz (GHK) equation and assuming $[Cl]_i \sim 40$ mEq/liter. The reversal potential increased to +30.6 ± 2.7 mV ($^{\Delta}E_{rev} = 23.6$ mV) when a solution containing 40 mM NaCl and 110 Na gluconate was used in the pipette, similar to the shift expected predicted from the GHK equation assuming $P_{Cl} >> P_{gluconate}$ (28.2 mV). Slope conductances at zero current flow were 8.7 ± 0.4 pS, 9.1 ± 0.4 pS and 7.4 ± 0.3 pS when the pipette solution contained 150, 270 and 40 mM Cl, respectively.

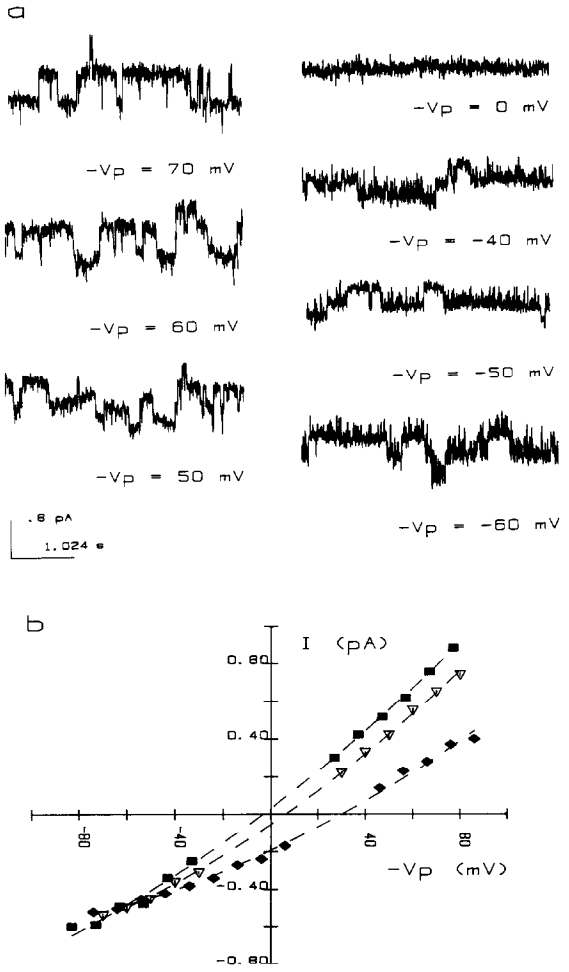

Fig. 2.(a) Low-conductance Cl channels in a cell-attached patch. (b) Mean current-voltage relationships determined with ▽ 150 mM NaCl, (■) 270 mM NaCl or (◆) 40 mM NaCl + 110 mM sodium gluconate in the pipette. Dashed lines indicate best fits with second degree polynomials. Standard errors are smaller than the symbols. Applied potentials are shown in (a); the *I/V* data in (b) are shown after corrections for liquid junction potentials.

3.3. *Spontaneous rundown of low-conductance Cl channel in cell-attached patches*

Patches on cAMP-stimulated cells usually had at least two low-conductance Cl channels and sometimes had more than eight. Calculating open probability (P_o) was problematic because channel activity often declined spontaneously during long cell-attached recordings. For example in Fig. 3a, up to nine multiples of the unitary current are apparent at the beginning of the trace; however, the mean number of channels open ($<I>/i$) during successive 30 s segments declines from 6.4 ($P_o \sim 0.71$) to approximately 0.7 ($P_o \sim 0.08$) within 10 min (Fig. 3b). Fig. 3c shows excerpts from Fig. 3a at higher time resolution along with the average number of channels open. The transitions are difficult to resolve in segment 'i' but become more obvious in 'ii' and 'iii' when fewer channels are open. Fig. 3d shows a current amplitude histogram for 1 min of data beginning at 320 s (see Fig. 3a). The histogram can be described very well as a sum of five Gaussian functions having means at −3.44, −3.10, −2.73, −2.40 and −2.09 pA, which corresponds to 0–4 open channels, respectively. According to the χ^2 test, the distribution of peak areas (0.02, 0.20, 0.53, 0.22 and 0.04, respectively) agrees with predictions for a binomial distribution assuming nine independent channels and $P_o = 0.23$ (0.10, 0.26, 0.29, 0.21 and 0.10, respectively) even without correction for overlap. It is still possible that individual channels are not gated independently, despite the χ^2 test. This is suggested by the observation that low-conductance Cl channels often opened and closed in a coordinated manner, producing irregular, wave-like patterns of activity (for example see traces at −50 and −60 mV in Fig. 4b). These waves typically lasted a few seconds each and were superimposed on the slow rundown, which had a time scale of minutes.

3.4. *Comparison of the effects of DIDS and IAA-94 on the outward rectifier and the low-conductance Cl channel*

We examined the sensitivity of the outward rectifier, the low-conductance Cl channel, and the cAMP-

stimulated I_{sc} to anion transport inhibitors to further identify the channel responsible for Cl secretion. DIDS (10 μM) reduced the open probability of the outward rectifier by ~35% when added to the pipette solution and inhibition increased to 70% when the pipette solution contained 30 μM DIDS (Fig. 4a). Outward rectifiers were never active when the pipette solution contained 100 μM DIDS although >30% of the patches contained the channel according to control experiments. By contrast, the gating and conductance of the low-conductance Cl channel were not noticeably affected by 100 μM DIDS (Fig. 4b,c). IAA-94, which inhibits anion conductance in membranes from bovine renal cortex and trachea [21], was more potent than DIDS in blocking the outward rectifier. IAA-94 (10 μM) reduced the open-state probability by ~90% (Fig. 5a) and 100 μM abolished activity completely. By contrast, 100 μM IAA-94 in the pipette solution did not affect the low-conductance Cl channel noticeably (Fig. 5b).

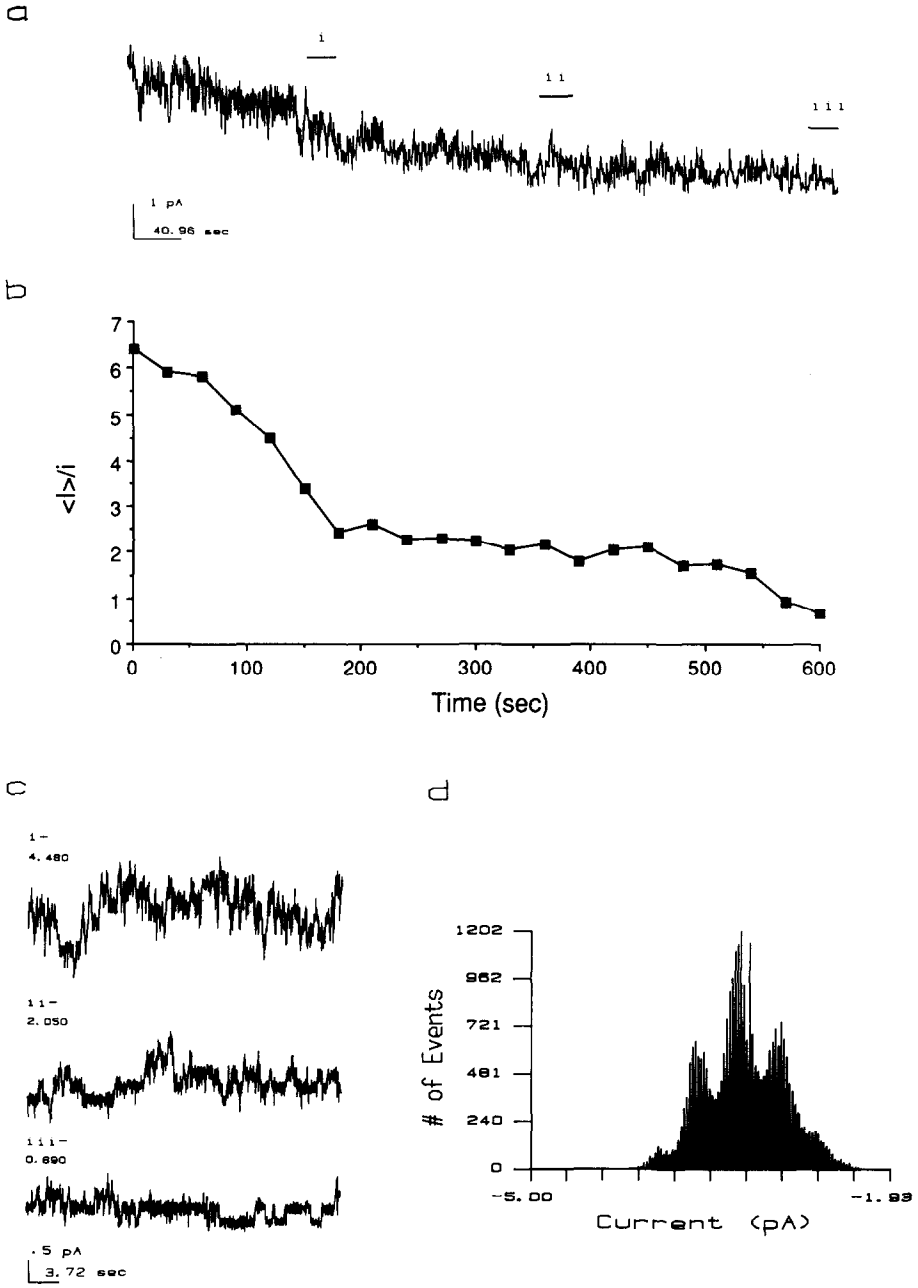

Fig. 3.(a) Continuous, cell-attached recording of Cl channels during cAMP stimulation (V_p = −40 mV). (b) Mean number of channels open as a function of time for the record shown in (a). (c) Segments of the data indicated by i–iii in (a). (d) An amplitude histogram for the current recorded between 320 and 380 s in (a).

3.5. *Temperature dependence of low-conductance Cl channel*

The low-conductance channel in T_{84} cells is similar to those described previously in pancreatic duct [21,22] and thyroid gland [23]; except for its somewhat higher conductance (8.7 vs 5–7 pS). To determine if temperature could account for this difference we measured single-channel currents as the bath was allowed to cool. V_p was clamped at −60 mV to minimize the impact of possible alterations in intracellular [Cl]. Lowering bath temperature from 36 to 21.5°C reduced the current amplitude measured at −60 mV and decreased the slope of the *I/V* relationship (Fig. 6a). The conductance at room temperature was 6.0 pS, similar to the values obtained for channels in cultured pancreatic duct and thyroid cells. The Q_{10} estimated by linear regression was 1.36 (Fig. 6b).

3.6. *Transepithelial studies*

In parallel Ussing chamber experiments, exposure of

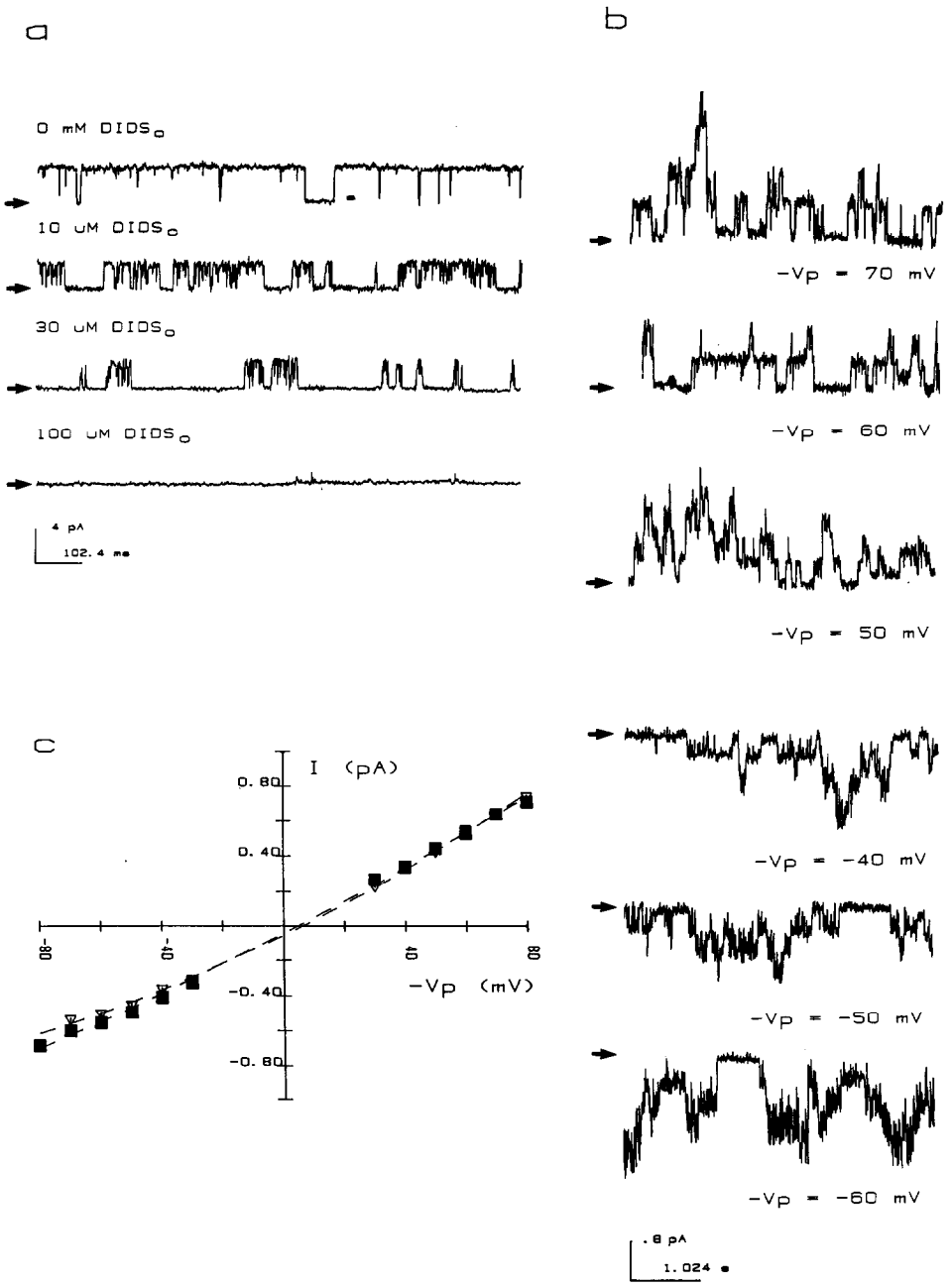

Fig. 4.(a) Inhibition of outward rectifier by external DIDS. Records were obtained using excised patches with 0, 10, 30 or 100 μM DIDS in the pipette solution. (b) Low-conductance Cl channels recorded in a cell-attached patch with 100 μM DIDS in the pipette solution. Arrows indicate the current level at which all channels weer closed. Note wave-like pattern of activity, especially at negative potentials. (c) Comparison of the *I/V* relationship for the low-conductance channels in the (▽) absence and (■) presence of 100 μM external DIDS.

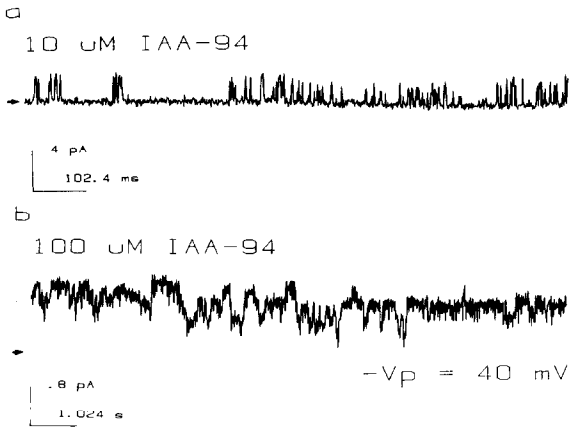

Fig. 5.(a) Effect of 10 μM external IAA-94 on activity of the outwardly rectifying anion channel (V_p = −40 mV). Compare with control trace in Fig. 4a (0 mM $DIDS_o$). (b) Low-conductance Cl channels recorded with 100 μM IAA-94 in the pipette solution. Arrows indicate the current level at which all channels are closed.

T_{84} monolayers to cAMP solution on the serosal side increased the short-circuit current (I_{sc}) from near zero to about 45 μM/cm^2 (Fig. 7a). Carbachol (100 μM) added to the serosal side in the presence of cAMP caused a further increase in I_{sc} by more than 100%, although I_{sc} declined back to the cAMP-stimulated level within 20 min. Large carbachol responses were invariably preceded by large cAMP responses so that the ratio ($^{\Delta}I_{carbachol}/^{\Delta}I_{cAMP}$) for any particular monolayer remained between 1 and 2. This suggests that variations in carbachol responsiveness among monolayers may simply reflect variations in their transport capacity.

DIDS (100 μM) had no effect on the cAMP-stimulated I_{sc} when added to the mucosal solution ($P > 0.2$) and further addition of 100 μM carbachol to the serosal side (with mucosal DIDS present) caused a large, transient stimulation of I_{sc} from 34.2 to 72.0 μA/cm^2. The ratio of carbachol:cAMP responses was similar with and without 100 μM DIDS present on the mucosal side (1.12 and 1.28, respectively). IAA-94 (100 μM) also had no effect on the cAMP-stimulated I_{sc} in similar experiments ($P > 0.2$), although the subsequent carbachol response was depressed by approximately 54%. However, in view of the high dose (100 μM) and long exposure to IAA-94 in our protocol (20 min), this reduction in carbachol response could indicate a

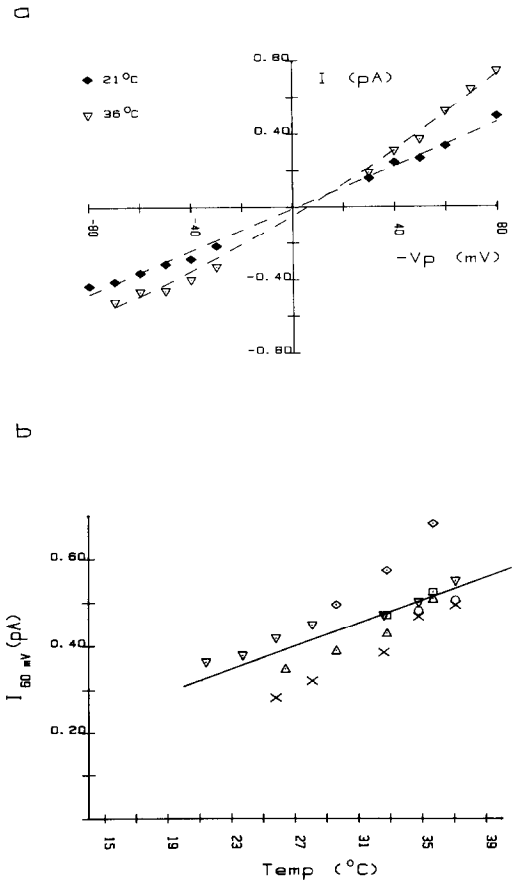

Fig. 6. Temperature dependence of current flowing through the low-conductance Cl channel (a) Comparison of I/V relationships determined at 21 and 36°C. (b) The effect of temperature on single-channel currents recorded with V_p = −60 mV. Linear regression of data from 6 patches indicates the Q_{10} ~ 1.4.

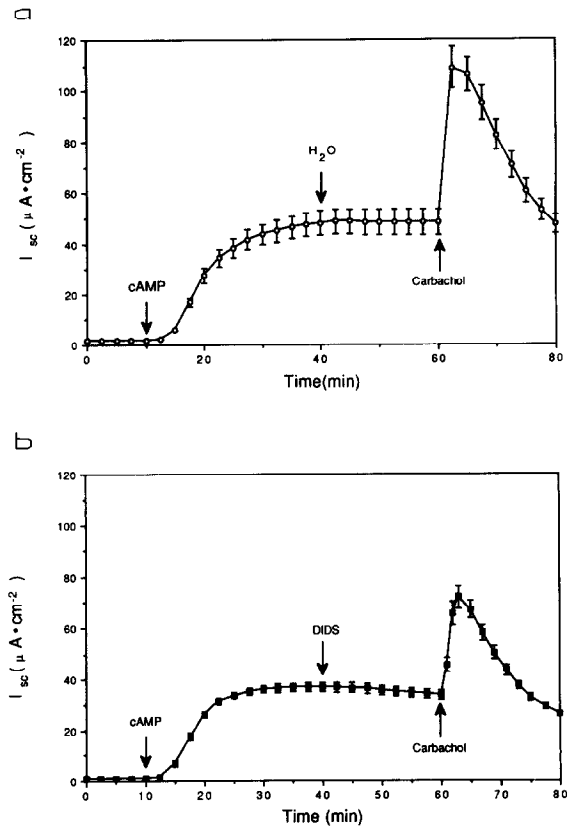

Fig. 7.(a) Effects of sequential addition of cAMP mixture (see Materials and Methods), distilled water (vehicle), and 100 μM carbachol on the I_{sc} (mean ± SEM, n = 5). (b) Same protocol except mucosal DIDS solution was added instead of vehicle (100 μM final concentration). DIDS had no effect on the cAMP-stimulated I_{sc} or the ratio $^{\Delta}I_{carbachol} \cdot :I_{cAMP}$ (mean ± SEM; n = 6).

generalized toxic effect of IAA-94 rather than specific inhibition of apical Cl channels.

In summary, two inhibitors which abolish activity of the outward rectifier when applied to the external surface of membrane patches had no effect on the low-conductance Cl channel or cAMP-stimulated, transepithelial Cl secretion.

4. DISCUSSION

We have described a low-conductance (8.7 pS at 37°C) Cl channel in the apical membrane of T_{84} cells and compared its properties with those of the outwardly rectifying anion channel and transepithelial Cl secretion. The probability of observing this channel in cell-attached patches increases >6-fold during exposure to cAMP and the channel was not affected by 100 μM DIDS or 100 μM IAA-94 in the extracellular (pipette) solution. On the other hand, outwardly rectifying anion channels which were also present in approximately one-third of the patches were rarely active in cell-attached recordings and were not responsive to cAMP. The outward rectifier was strongly inhibited by addition of DIDS or IAA-94 to the pipette solution. I_{sc} was increased by maneuvers that elevate cAMP, and this cAMP-stimulated current was insensitive to high concentrations of DIDS or IAA-94 on the mucosal side. Thus, the properties of transepithelial Cl secretion are more similar to those of the low-conductance Cl channel than to the outward rectifier, and it seems likely that the low-conductance Cl channel mediates a component of the cAMP-stimulated Cl efflux from T_{84} cells.

It is interesting that the transient response evoked by carbachol, which probably involves mobilization of cell calcium, was also insensitive to DIDS. This may indicate that carbachol stimulates Cl secretion indirectly by activating a basolateral K conductance [4], rather than by activating another apical Cl conductance. Although distinct cAMP- and carbachol-induced Cl channels cannot be excluded, if separate pathways do exist in T_{84} cells they are not readily distinguishable using DIDS. IAA-94 had no effect on cAMP-stimulated I_{sc} but depressed the subsequent response to carbachol but it is not known if the reduced carbachol response is a non-specific (i.e. metabolic) inhibition caused by prolonged exposure to this compound. A study of acute IAA-94 effects during the course of the carbachol response might give some insight into its mechanism of action. The most important result for the present study is the observation that neither DIDS nor IAA-94 affected the cAMP induced I_{sc}.

The low-conductance Cl channel described in this paper is clearly distinct from the 20 pS channels described in cultured airway cells by Frizzell et al. ([13]; 20°C) and Duzcyk et al. ([24] 37°C). A low-conductance Cl channel has been reported in the shark rectal gland which might have similar conductance under comparable experimental conditions ([25]; 11 pS, 20°C, 280 Cl); however, its gating behaviour is different from the low-conductance channel in T_{84} cells and it is frequently observed in excised patches. Another Cl channel which strongly resembles the one described here occurs in the apical membrane of rat (4 pS) and human (7 pS) pancreatic duct, where it is believed to mediate Cl recycling at the apical membrane during secretin-stimulated HCO_3 secretion [21,22]. A similar channel has also been demonstrated recently in cultured thyroid gland epithelium (5.5 pS), where it may be involved in thyrotrophin-modulated fluid transport [23]. The Cl channel in T_{84} cells has somewhat higher conductance compared to these other preparations; however, this could be explained by the higher temperature (37°C) used in the present study. The low-conductance Cl channel in T_{84} cells may underlie a linear, cAMP-activated Cl conductance observed recently using the whole-cell patch clamp technique [26].

The physiological roles of outwardly rectifying anion channels appear to be complex. An outwardly rectifying conductance, which inactivates at positive membrane potentials, has been described in T_{84} cells [27]. This osmotically sensitive conductance may be important in cell volume regulation, although activation of single outward rectifiers by swelling has not yet been demonstrated. Another outwardly rectifying Cl conductance which does not inactivate at positive membrane potentials has been described in lymphocytes during the G_1-S phase of the cell cycle, suggesting a role in cell division [28]. Different modulators may influence the voltage dependence of the outward rectifier or there may be a family of closely related channels subserving different functions.

Acknowledgements: Supported by the US and Canadian Cystic Fibrosis Foundations and by the Medical Research Council (Canada). W.L. is the recipient of a postgraduate scholarship from the Canadian CF Foundation. J.W.H. is an MRC Research Scholar.

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