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Phosphorylation-activated chloride channels in human skin fibroblasts

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A chloride-selective channel has been found using patch-clamp electrophysiology in human skin fibroblasts and it exhibits many of the biophysical properties of the Cl^- channel found in airway epithelia. As in the case of epithelial Cl^- channels, Cl^- channels in fibroblasts are activated at depolarized membrane potentials in excised patches, rectifying in an outward direction with a unit conductance of 33 pS at 0 mV. Furthermore, the agonists forskolin and prostaglandin E_2 evoke Cl^- channel activity in cell-attached patches. The effect of these agonists can be mimicked by direct application of catalytic subunit of protein kinase A with ATP and Mg^{2+} to the internal membrane surface of excised, inside-out patches. The Cl^- channel is also sensitive to inhibition by the stilbene derivative, DIDS. These results indicate that fibroblasts may provide a convenient and available model for the study of epithelial Cl^- channel regulation and accelerate efforts to determine the regulatory defect expressed in cystic fibrosis.

Chloride-selective channel; cyclic AMP-dependent protein kinase; DIDS inhibition; (Human fibroblast)

1. INTRODUCTION

In several types of epithelial tissue, including tracheal and sweat gland cells, agonists such as prostaglandin E_2 (PGE_2) and forskolin act through a cyclic AMP (cAMP) mediated process to stimulate chloride secretion [1,2]. This cAMP-induced activation can be reproduced using patch-clamp electrophysiology with the direct application of catalytic subunit of cAMP-dependent protein kinase A, Mg^{2+} and ATP to the internal membrane surface of excised, inside-out patches [3,4].

The biophysical characteristics of cAMP-activated Cl^- channels have been well documented in airway epithelial cells and in the model colonic cell line T84 using patch-clamp electrophysiology [1,3-5]. This channel is activated at depolarized membrane potentials, i.e. 80 mV, rectifies in an outward direction with symmetrical physiological salt solutions on either side of the membrane patch and has a unit conductance of approx. 35 pS close to 0 mV. In preliminary reports, the stilbene derivative DIDS (4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid) has been found to inhibit the Cl^- channel isolated from colonic mucosal cells [6].

In cystic fibrosis (CF), the primary defect leading to abnormal epithelial water and electrolyte secretion is thought to be the altered regulation of chloride channel activity. In contrast to normal epithelia, Cl^- channels in excised, inside-out membrane patches from CF airway epithelial cells fail to be activated by cAMP-dependent protein kinase [3,4]. Because they clearly express a specific transport defect, primary cultures of human airway cells have become (along with excised sweat glands) standard biological material for CF studies. Unfortunately, these human tissue samples are difficult to obtain.

Radiolabelled Cl^- flux studies have indicated that human fibroblasts contain a Cl^- conductance pathway which is regulated by cAMP [7]. In the present studies, patch-clamp electrophysiology has been used to describe a chloride-selective channel

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in cultures of human skin fibroblasts. This channel in fibroblasts is similar to that described in airway epithelia with respect to its physical properties and its modulation by protein kinase A phosphorylation. This is an important finding because it suggests that fibroblasts may provide a convenient, readily available cell culture model for the study of epithelial Cl^- channel regulation.

2. MATERIALS AND METHODS

2.1. *Cell cultures*

Primary cultures of human skin fibroblasts from 6 different subjects were obtained from T. Jensen and J.R. Riordan (Cystic Fibrosis Research Development Corporation, Hospital for Sick Children, Toronto). Cells were grown in Alpha medium (Princess Margaret Hospital, Toronto) plus 10% bovine calf serum on 35 mm plastic cell culture dishes. Single-channel currents were recorded from cells after confluency had been reached (3–7 days after plating). All experiments were conducted at room temperature (20–22°C).

2.2. *Current recordings*

Single-channel currents were recorded according to Hamill et al. [8] using a List EPC-7 amplifier (Medical Systems, Great Neck, NY). Pipettes were fabricated from borosillicate glass type no. 7052 (Garner Glass) using a two-stage Narishige pipette puller (PP-83). When filled with a high-Na^+ solution, pipette resistances were 2–3 M Ω. The bath electrode was an Ag-AgCl wire connected to the bathing solution via a KCl-agar bridge. Current output from the amplifier was monitored on a Tektronix oscilloscope and recorded on video tape after A/D conversion by a video adaptor (PCM2, Medical Systems).

2.3. *Solutions*

The standard pipette solution (Na^+ rich) contained (mM): 140 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 Hepes buffer (5). The pH was adjusted to 7.4 using NaOH. The standard bathing solution was (mM): 140 NaCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 1 EGTA and 10 Hepes. In some cases NaCl was replaced with 140 mM NMGCl (*N'*-methylglucamine chloride) in the bath. NaCl concentration was reduced in several experiments to 70 mM and the osmolarity adjusted with sucrose.

2.4. *Data analysis*

Single-channel currents, filtered at 1 kHz, were measured on the oscilloscope screen and corrected for junction potentials evoked by changes in bath composition. Junction potentials were calculated using the Henderson approximation of the Nernst-Planck equation. Channel open probability was determined as the area under the open-channel current divided by the area expected if all channels were open continuously [5]. Open probability was calculated from not less than 20 s of recorded data.

2.5. *Materials*

Purified catalytic subunit of protein kinase A was provided as a gift from Dr M.P. Walsh (Cell Regulation Group, University of Calgary). Specific activity of the catalytic subunit was 1.0 U/mg protein, where 1 unit of kinase catalyzes the transfer of 1 μmol ^{32}P from ATP to histone in 1 min. Forskolin and prostaglandin E_2 were obtained from Calbiochem (San Diego, CA) and DIDS from Pierce (Rockford, IL).

3. RESULTS

3.1. *Single-channel recordings in excised, inside-out patches*

Inside-out patches exposed to high-NaCl solutions in both the pipette and bath exhibit outward current steps, corresponding to channel openings, 2–3 min after membrane depolarization to 60 mV. Once the channel was voltage-activated, channel opening and closing events could be observed at various membrane potentials (fig.1A). In this figure it can be seen that channel open probability (OP) was greater at depolarized membrane potentials than at hyperpolarized potentials 1 min after activation. In 40% of patches, channel inactivation occurred within 4–10 min. This channel was evident in 36 of 88 successfully excised patches. The addition of 2 mM $CaCl_2$ to the bath originally containing 300 nM Ca^{2+} had no effect on channel activity at voltages ranging from -80 to 80 mV ($n=3$). The current-voltage (I-V) relationship of the channel displayed outward rectification (fig.1B) The conductance of the channel, measured close to 0 mV is 33.1 ± 0.2 ($n=36$). Substitution of the bath with a solution containing a mixture of NMGCl and NaCl in a ratio of 130:10 mM, respectively, resulted in no significant change in the reversal potential of the I-V curve ($n=4$). Changing the original high-NaCl bath solution to a solution containing 70 mM NaCl resulted in a shift of the reversal potential to -12 ± 2 mV, after correction for junction potentials ($n=5$). This shift indicates that the channel favours Cl^- over Na^+ with the permeability ratio $(P_{Na}/P_{Cl}) = 0.09 + 0.01$ as calculated from the Goldman-Hodgkin-Katz voltage equation.

3.2. *Channel recordings in the cell-attached, in-situ mode*

Channel activity was rarely observed when membrane patches were studied in the cell-attached configuration even at depolarized membrane potentials. The presence of Cl^- channels was confirmed after experiments in the cell-attached mode by excising the patch and examining the I-V relationship

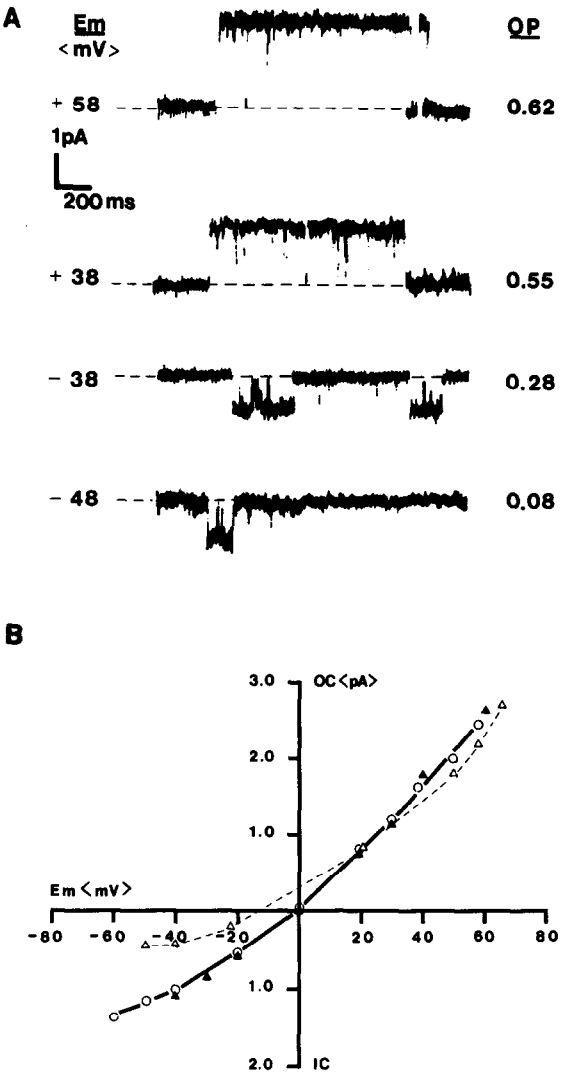

Fig.1. Single-channel recordings. (A) Single-channel currents recorded in an excised, inside-out patch with high-Na^+ solution in bath and pipette. The channel was activated by depolarizing voltage pulses to − 58 mV (a pulse injected once every 11s with a duration of 9 s) from a holding potential of 0 mV. (B) *I-V* relationships: (○) *I-V* relationship with symmetrical NaCl (140 mM) on both sides of excised, inside-out patch; (▲) NMGCl (130 mM) and NaCl (10 mM) in bath; (△) NaCl (70 mM) in bath solution. Each symbol represents the mean of 4–36 measurements. Lines drawn by hand.

of observed current steps activated either by agonist or voltage. Addition of forskolin (100 μM) evoked channel activity after 3–7 min in previously quiescent patches in 6 of 8 patches studied which

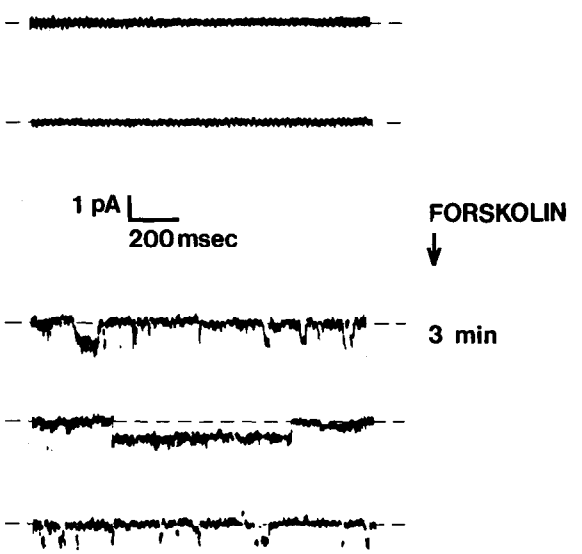

Fig.2. Activation of Cl^- channels by forskolin. No channels observed in patch in cell-attached mode for 4 min during voltage pulses to − 39 mV. 3 min after addition of forskolin (100 μM) to bath, inward current steps observed at − 39 mV.

contained the Cl^- channel (fig.2). PGE_2 (2–9 μM) stimulated Cl^- channel activity in 5 of the 5 patches containing channels. The activation was transient, lasting 1–2 min.

3.3. *Stimulation of Cl^- channels in excised, inside-out patches by the catalytic subunit of protein kinase A*

To examine phosphorylation-mediated activation directly, we exposed inside-out patches to a phosphorylation cocktail consisting of ATP (0.5–1.0 mM), Mg^{2+} (1.0 mM) and the catalytic subunit of protein kinase A in a concentration (0.03 μM) shown in previous experiments to evoke channel activity in airway epithelia [3,4]. Voltage pulses to − 40 mV from a holding potential of 0 mV were applied in order to assess channel activity in the patch prior to treatment with the phosphorylation cocktail for 4 min. This hyperpolarized potential was not expected to voltage-activate channels. If no channels were observed, ATP was added to determine membrane-bound kinase activity and in 3 experiments no channel stimulation was observed for 4 min. Addition of the catalytic subunit in the presence of ATP evoked channel activity in 6 of the 8 excised patches studied which

contained channels. Channel activation occurred in 1–5 min after treatment and at first appeared as irregular channel openings which became regular current steps within 10–20 s. Channel open probability determined at −40 mV was 0.32 immediately following phosphorylation-induced activation. 1–2 min after stimulation the channel became less active and eventually closed down completely. The *I-V* relationship of the phosphorylation-evoked channel was identical to that observed for the voltage-activated channel previously described suggesting that it is the same channel (fig.3).

3.4. *Channel inhibition by DIDS*

Following voltage activation, the chloride channel inhibitor DIDS was applied to the internal membrane surface of excised inside-out patches in a concentration (10 μM) previously shown to inhibit the Cl^- channel isolated from colonic mucosa [6]. DIDS caused a decrease in the duration of channel opening after 2 min. 4 min after DIDS addition only brief, incomplete channel openings were observed with a calculated open probability of 0.01 ± 0.02. The inhibitory effect of DIDS was irreversible in 4 experiments but could be washed out by control solution after 1 min in 1 of the 5 patches studied. In this experiment the channel open probability was only partially restored to control values (fig.4).

4. DISCUSSION

A cAMP-activated Cl^- transport pathway has been previously described by Lin and Gruenstein [7] in human fibroblasts using radiolabelled Cl^- efflux measurements. In these studies, conductive Cl^- transport was measured in fibroblasts exposed to medium in which Cl^- was substituted with the impermeant anion, gluconate and bumetanide was added in order to eliminate exchange and cotransport mechanisms for Cl^- respectively. Under these conditions dibutyryl cAMP was found to stimulate conductive Cl^- transport by 30%. Studies of this kind are limited because the biophysical characteristics of the conductive pathway cannot be adequately described. Furthermore, it is well known that there are several types of Cl^--selective channels in epithelial tissue and radioisotope flux experiments are not specific enough to distinguish between different channel types [9,10]. The present patch-clamp experiments provide the first direct evidence of a cAMP-activated Cl^- channel which shares many of the biophysical properties exhibited by the type of Cl^-

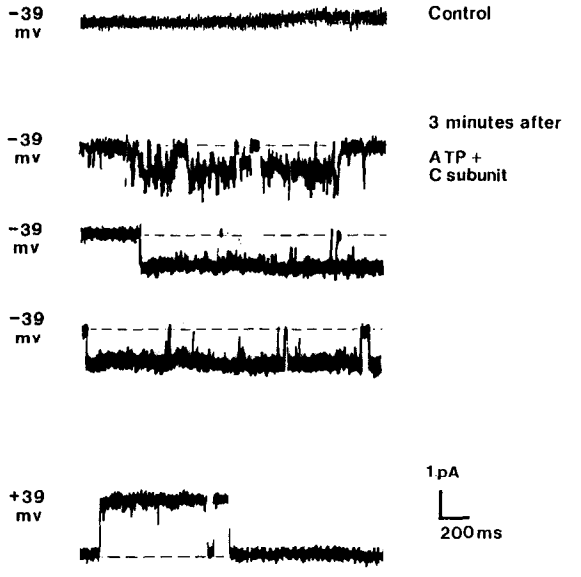

Fig.3. Channel activation by catalytic subunit of cAMP-dependent protein kinase. No channels observed for 4 min in excised, inside-out patches. 2 min after addition of 500 μM ATP, 1 mM Mg^{2+} and catalytic subunit (0.03 μM) inward current steps observed at −39 mV. Outward current steps observed with voltage pulses to −39 mV.

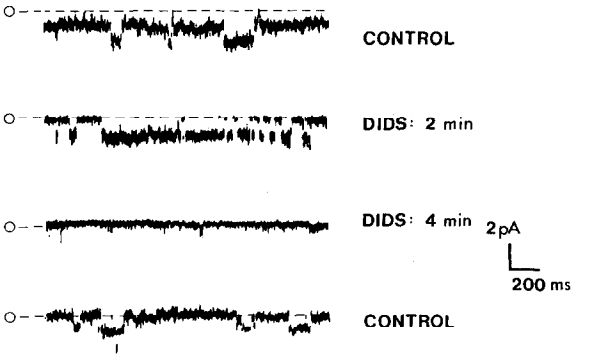

Fig.4. Inhibition of channels by DIDS. Following voltage activation, channels observed at −29 mV in excised, inside-out patches. Addition of DIDS (10 μM) to the bath resulted in decreased channel open probability after 2 and 4 min. Channel activity was partially restored in this patch, 1 min after washing patch with control solution.

channel involved in epithelial water and electrolyte secretion [1–6].

As stated previously, the primary abnormality in cystic fibrosis lies in the regulation of Cl^- channels by cAMP-mediated phosphorylation. Lin and Gruenstein [7] have shown in the previously described radioisotope studies that conductive Cl^- transport in fibroblasts taken from cystic fibrosis patients is not activated by dibutyryl cAMP and the authors suggested that the primary defect in cystic fibrosis (CF) is expressed in fibroblasts. However, the results showed significant overlap in conductive Cl^- transport measured between the normal and CF populations. Conclusive evidence that fibroblasts express the CF defect will come from patch-clamp experiments in which the regulation of the Cl^- channel described here will be investigated in fibroblasts from CF patients. If CF fibroblasts show altered Cl^- channel regulation then they will provide an excellent model for study of this disease. An extensive library of fibroblast cell lines is available at several hospitals including at the Hospital for Sick Children (Canada) and genetic studies can be readily correlated with functional, electrophysiological investigations.

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