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# A Large-Conductance Chloride Channel in Pigmented Ciliary Epithelial Cells Activated by GTPγS

**C.H. Mitchell*, L. Wang, T.J.C. Jacob**

Eye Research Lab, Physiology Unit, School of Molecular and Medical Bioscience, University of Wales, Cardiff, CF1 3US UK

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**Abstract.** A large-conductance (or maxi-) chloride channel was identified in bovine pigmented ciliary epithelial (PCE) cells using inside-out excised patch clamp recording. The channel had a mean conductance of 293 pS when excised patches were bathed in symmetrical 130 mM NaCl although the conductance decreased to 209 pS when the solution bathing the cytoplasmic face of the patch contained only 33 mM NaCl. The channel was highly selective for chloride, with a $P_{Cl}/P_{Na} = 24$. A flickery, reversible block was produced by the diuretic stilbene 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), while 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) produced a permanent block. The channel was rarely active in cell-attached patches and usually required several minutes of polarization before activity could be detected in excised patches, a process known as metagenesis. Once activated, the channel was voltage-dependent and was mainly open within the voltage range −30 to +30 mV closing when the membrane was polarized to larger values. GTPγS (100 μM) activated the channel with a latency of 170 sec when applied to the cytoplasmic face of patches. This activation was not reversible upon return to control solution within the duration of the experiment. We assess the available evidence and suggest a role for this channel in volume regulation.

**Key words:** Ciliary epithelium — Maxi chloride channel — Patch clamp — Ion transport — Secretion — G-protein activated $Cl^-$ channel

## Introduction

In the process of aqueous humor formation, there is a net movement of ions and fluid from the blood vessels of the ciliary processes to the interior of the eye. This net secretion involves ionic transport through the ciliary epithelium, a bilayer of pigmented (PCE) and nonpigmented (NPCE) cells joined at their apical surfaces by gap junctions (Raviola, 1971). The cells are thought to act as a syncytium (Edelman, Sachs & Adorante, 1994), with PCE cells responsible for the loading of ions and solutes from the stroma while NPCE cells are responsible for the efflux as aqueous humor. In general, this hypothesis has been supported by the distribution of active and passive transport mechanisms. For example, the pigmented cells have been shown to possess a $Na^+/K^+/2Cl^-$ cotransporter (Edelman et al., 1994), a $Na^+/H^+$ exchanger (Helbig, 1988*a*), a $HCO_3^-/Cl^-$ exchanger (Helbig, 1988*b*) and an electrogenic $Na^+$/ascorbate exchanger (Helbig, 1989). Passive efflux through NPCE cells is supported by the presence of chloride channels (Coca-Prados et al., 1995; Wu et al., 1996) and $K^+$ channels (Edelman, Loo & Sachs, 1995; Yantorno et al., 1992).

In addition to active uptake mechanisms, the pigmented cells contain a number of ion channels. The interaction of calcium-activated potassium channels (Jacob, 1991*a*) and T-type calcium channels (Jacob, 1991*b*) were thought to underlie the slow sinusoidal oscillation identified in bovine PCE cells. In addition, these cells were shown to possess an inwardly rectifying potassium channel (Stelling & Jacob, 1992) and a nonselective channel (Mitchell & Jacob, 1996) which combined to produce larger, negative-going oscillations when the cells were injected with negative current (Stelling & Jacob, 1993). However, it was unclear whether the pigmented ciliary epithelial cells possessed a chloride conductance.

In the present study, the inside-out variant of the

* *Present address:* Department of Physiology, Richards Building, University of Pennsylvania, Philadelphia, PA 19104-6085, USA

*Correspondence to:* T.J.C. Jacob

patch clamp technique has been used to show that PCE cells do possess large-conductance chloride channels. These channels share many characteristics with maxi-chloride channels, including a conductance close to 300 pS, block by the diuretic stilbenes DIDS and SITS, and the voltage-dependent induction of channel activity.

Some of this work has previously appeared in abstract form (Mitchell & Jacob, 1994).

## Materials and Methods

### Cell Preparation

Ciliary epithelial tips were removed from the ciliary body of bovine eyes using a method described previously (Jacob, 1991*a*). The tissue was incubated at 37°C for 25 min in a solution containing 0.25% trypsin (Sigma, UK) and 0.02% EDTA. Cells were triturated in a solution of E199 + 10% foetal calf serum, spun at 800 rpm and washed twice before being plated on 13 mm glass coverslips. Cells were kept overnight in E199 + 10% serum.

### Electrophysiological Procedures

Electrodes were pulled on a two-stage puller (Sutter P30) and typically gave a resistance of 4–10 MΩ when filled with the pipette filling solution. Recordings were performed in a chamber connected to a Ag/AgCl-pellet via a 3 M KCl/2% agar bridge. Single-channel currents were recorded in the inside out mode using a patch-clamp amplifier (Dagan 8900, Dagan Corporation, Minneapolis, MN), filtered at 1 kHz and sampled at 1 kHz to 3 kHz using a laboratory interface (CED 1401, Cambridge, UK). Voltage stimuli were driven, and current analysis was performed with the EPC software package (CED, Cambridge, UK). Upward deflections represent outward current. Voltages are given as −Vp (mV). All experiments were performed at room temperature.

### Analysis Techniques

Single-channel currents were analyzed using the EPC software package (CED, Cambridge, UK). Channel amplitude measurements were obtained by constructing a histogram from the current trace at a particular voltage and taking the peaks as the corresponding closed and open levels. Occasionally, the channel amplitude was determined by fitting on-screen cursors to the open and closed levels of the current traces.

The channel open probability values used to show voltage-dependent activity (Fig. 5*A*) were determined by calculating the proportional area under each peak of the current histogram. The probability of finding at least one channel open, $P_o$, was defined as 1-$P_c$ ($P_c$ = probability of finding channel closed). For channel block (Fig. 6) open probability was calculated by the amount of time the current spent over a threshold set at 50% of the open current. Only records containing one channel were used for detailed analysis of block as these kinetic measurements also used measurements of the channel's mean open duration.

### Solutions

During seal formation, cells were bathed in standard extracellular solution containing (in mM): 125 NaCl, 4.4 KCl, 2 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES, 10 $NaHCO_3$, 5 glucose and 20 sucrose. The electrode solution contained (in mM) 130 NaCl, 20 sucrose, 10 HEPES, and 1.1 EGTA, 5 × $10^{-3}$ mM $CaCl_2$, for a free $Ca^{2+}$ concentration of $10^{-7}$ M. In most experiments using excised patches, the bath solution was identical to the electrode solution. In selectivity experiments, cells were bathed in (mM): 33 NaCl, 10 HEPES, 1.1 EGTA 5 × $10^{-3}$ $CaCl_2$ (free $Ca^{2+}$ of $10^{-7}$ M); 220 mM sucrose was added to maintain the osmolarity. The pH was adjusted to 7.4 with NaOH.

### Drugs

SITS, DIDS and GTPγS were perfused onto the cells or added directly to the bath. SITS was stored as a 100 mM stock solution at 5°C. DIDS and GTPγS were stored at −20°C at concentrations to 50 and 100 mM, respectively. All chemicals were obtained from Sigma, UK.

### Permeability Calculations

Channel selectivity was determined by exposing excised patches to an asymmetric mixture of NaCl. Ionic permeability was calculated by manipulating the constant field equation (Goldman, 1943). If only Na and Cl are present and $[Cl]_i = [Na]_i$, $[Cl]_o = [Na]_o$, then the ratio of anion to cation permeability is;

$$P_{Cl}/P_{Na} = (1 - \{[X]_i/[X]_o\} \cdot e(V_{rev} \cdot F/RT))/(e(V_{rev} \cdot F/RT) - \{[X]_i/[X]_o\}) \quad (1)$$

$V_{rev}$ = channel reversal potential
$[X]_i/[X]_o$ = the ratio of intracellular to extracellular ion activity and *R*, *T* and *F* have their usual meanings.

Potentials shown here have been corrected for offsets. All selectivity and offset calculations were based upon ion activities determined from activity coefficients (Robinson & Stokes, 1965). Values are given as mean ± SE.

### Abbreviations

DIDS = 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid: EGTA = ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; GTPγS = guanosine − 5′-O-(3-thiotriphosphate); HEPES = N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; SITS = 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid.

## Results

### Induction

Channel activity was rarely observed in the cell-attached mode. Square, large-conductance channel activity was seen in only 2 out of 90 cell-attached patches which were subsequently shown to contain a channel when excised. However, flickery, multiple conductance activity was observed in 20 out of these 90 records.

The vast majority of patches did not show any activity upon excision. Activity had to be induced by polarizing the patch >±30 mV and usually began 2–15 min after polarization began. Activity started as noisy events


**Fig. 1.** Induction of channel activity. This inside out patch was depolarized by 30 mV for >3 min before activity appeared. Upward deflections indicate outward current through an open channel. Activity began as noisy, multiple level events before developing into clean, square transitions.

of short duration and developed into square events with clean transitions (Fig. 1). This induction of channel activity, or ''metagenesis'' occurs within 1–5 sec. Once the square events had been induced, it was rare for a channel to re-enter the flickery transition mode while bathed in standard solution.

A large conductance channel characterized by sharp, clean transitions was observed in over 60% of excised patched examined (Fig. 2). Channels appeared to be clustered in the membrane; 62% of channels occurred with at least one other identical channel and at least 20% of patches with activity contained three or more channels.

## Conductance

When excised patches were bathed in symmetrical 130 mM NaCl, the channel conductance was 292.7 ± 2.4 pS ($n = 72$). Although this value is slightly lower than that reported for some chloride channels (Bosma, 1989; McCann et al., 1989; Schlichter et al., 1990), those measurements were obtained with a higher concentration of chloride bathing the patch, and conductance has been shown to be dependent upon the concentration of internal and external chloride (Nelson, Tang & Palmer, 1984; Schlichter et al., 1990; Woll & Neumcke, 1987). This is supported by the results of the present study, where the unitary conductance was only 209.2 ± 10.6 pS ($n = 39$) when the bath contained 33 mM NaCl. Under these conditions, inward current was rarely seen and openings were brief with flickery transitions (*see* Selectivity section).

The channels occasionally entered distinct subconductance levels which lasted at least 500 msec (Fig. 3). However, these sublevels were more commonly characterized by increased current noise, resembling the rapid fluctuations observed during the initial induction of channel activity. Transitions were observed to and from the main conduction level to each of the subconductance levels. The most frequently encountered level was at 52%, with others appearing at 38%, 62% and 140%. The full conductance level did not represent merely two 52% levels because the vast majority of transitions were between fully open and closed levels. This activity is highly unlikely if two independently gated channels were present (Sun et al., 1992).

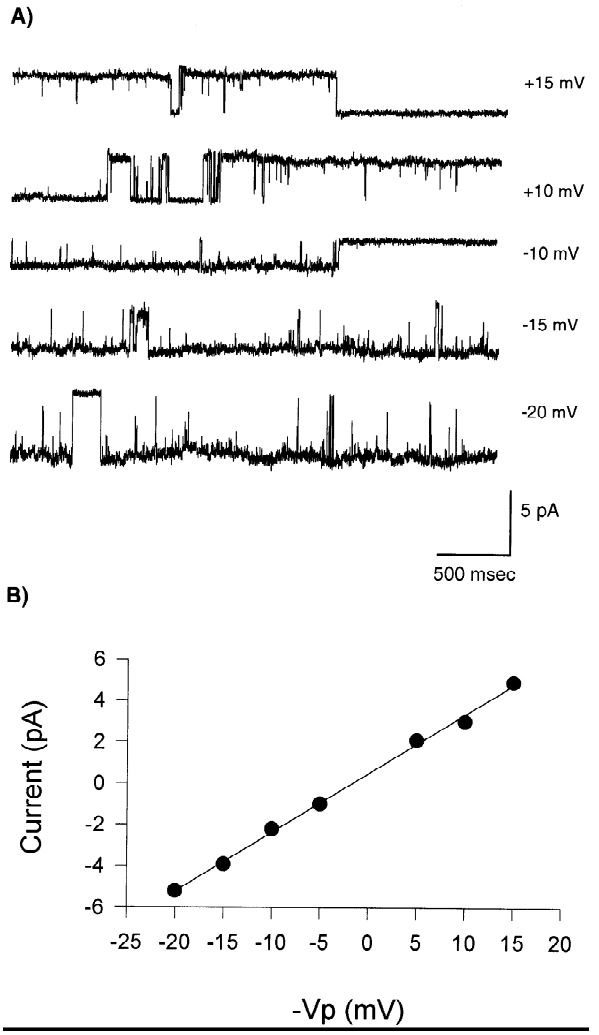


**Fig. 2.** Large-conductance chloride channel. (*A*) These current traces were obtained from an inside-out patch bathed in symmetrical 130 mM NaCl solutions. The patch was polarized to the potentials shown to the right of each trace. Upward deflections from this baseline indicate outward current through open channels, downward deflections are inward current. Traces were chosen to show transitions — the channel was actually open most of the time. (*B*) Current-voltage plot of this record. The conductance was 295 pS and the channel reversed at −2 mV.

## Selectivity

To determine the chloride to sodium selectivity ($P_{Cl}/P_{Na}$) patches were bathed in asymmetrical NaCl. With the

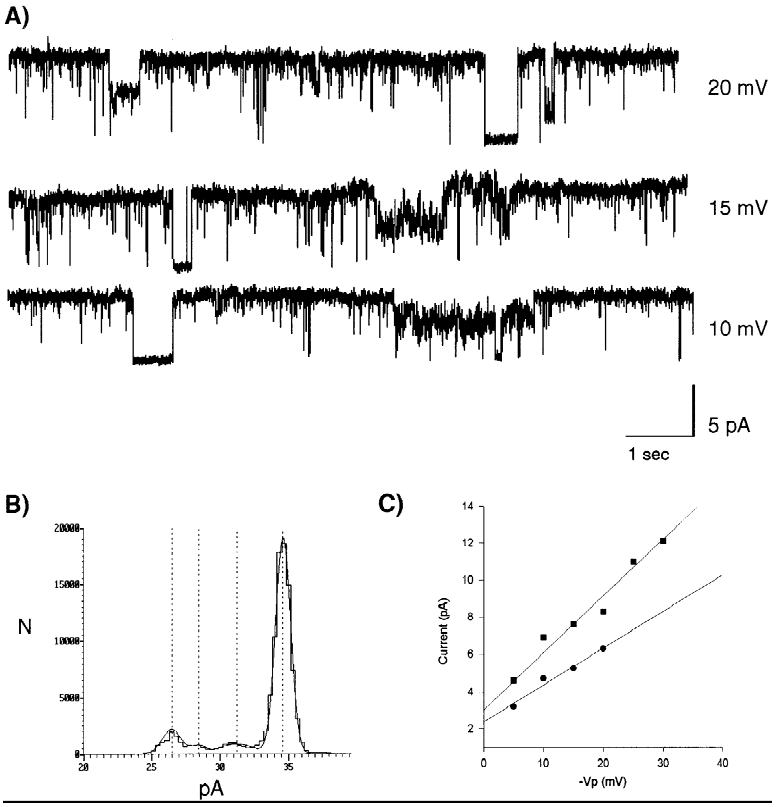


**Fig. 3.** Sub-conductance levels. (*A*) Current traces showing subconductance level in addition to full conductance level. The majority of transitions were to and from the fully open and fully closed levels. Upward deflections indicate outward current. (*B*) Histogram of current trace at 20 mV, from 60 sec worth of data. Peaks corresponding to the current levels when the channel is fully open and fully closed are clear, and a peak indicating the subconductance level is shown at 69% of the total. A smaller subconductance level at 26% of the maximum can also be detected. (*C*) Current-voltage plots of the main conductance level — ■ (306 pS) and the subconductance level — ● (197 pS).

cytoplasmic face of the patch in 33 mM NaCl, and standard 130 mM NaCl in the electrode, the chloride equilibrium potential ($E_{Cl}$) calculated from ionic activities was −32 mV and the sodium equilibrium potential ($E_{Na}$) was +32 mV. Initially, the reversal potential was determined from a first order regression fit to a current voltage plot based upon steady state records (Fig. 4*A* and *B*). However, because square transitions were rarely observed under these conditions, the regression had to be extrapolated and this could have led to an error in reversal potential measurements if the conductance was not completely linear. To avoid this problem, voltage ramps of 1–4 sec duration were applied, from −50 to +50 mV or −100 to +100 mV, to determine the precise potential at which the channel reversed (Fig. 4*C*). Apparently, the ramp speeds were sufficiently rapid for the amplitude to be measured at the hyperpolarized potentials before the channel entered the flickery mode. The reversal potential values obtained with both steady state and ramping voltages were similar, and gave a mean reversal potential of −31.3 ± 1.1 mV ($n = 36$) (Fig. 4). Using the permeability equation shown in the methods section, this corresponds to a $P_{Cl}/P_{Na} = 24$, similar to the ratio reported for the maxi-chloride channel in B lymphocytes (McCann et al., 1989).

## Kinetics

Although initial channel activity was induced by voltage pulses beyond ±30 mV, such voltage pulses had the opposite effect once the channel had been activated. Steady-state channel open probability ($P_o$) decreased as the patch was depolarized or hyperpolarized away from 0 mV (Fig. 5*A*). Channels usually closed when the patch was polarized by more than ±30–40 mV but this voltage dependence varied between channels, and the potential at which the channel closed often became further from 0 mV as the experiment progressed. The channel was either open most of the time, with $P_o > 0.95$, or closed, once the time-dependent activation and inactivation had taken place (*see below*). This sharp open/closed gating has also been observed in the maxi-chloride channel in T lymphocytes (Schlichter et al., 1990).

To obtain information rapidly from a variety of potentials, the patch potential was frequently cycled between 0 mV, −40 mV, −20 mV, 0 mV, + 20 mV and +40 mV, remaining at each voltage for 2 sec. With this protocol, channels were often seen to inactivate at ±40 mV and activate at ±20 mV (Fig. 5*B*). These changes would lead to the steady state voltage-dependent activity described above. The rates of activation and inactivation did vary—some channels inactivated more quickly than those illustrated in Fig. 5*B* while other channels remained fully open throughout this range of potentials. On some occasions, time constants changed considerably during a single recording. This variability precluded the use of more vigorous kinetic tests and meant that accurate time constant values could not be determined. However, kinetic changes were usually complete 2 sec

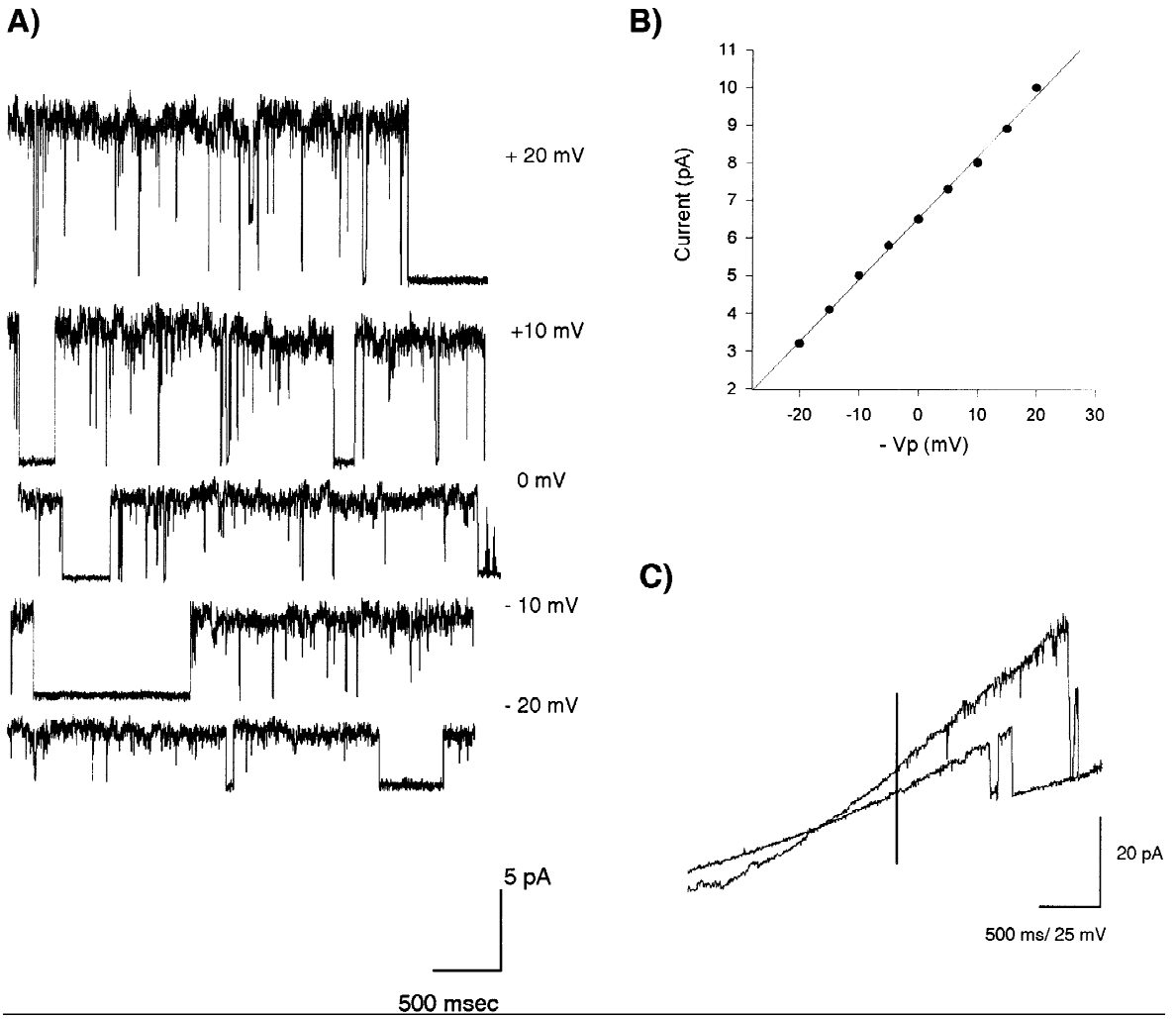


**Fig. 4.** Channel selectivity. (*A*) Current traces from an inside-out patch with 130 mM NaCl in the electrode and 33 mM NaCl in the bath. Under these conditions $E_{Cl} = -32$ mV. Patch potential is shown to the right of each trace. All traces show outward current. Traces were chosen to show transitions; the channel was actually open most of the time (*see* Results). (*B*) Current-voltage plot from the traces in *A* fit with a first order regression. The conductance of this channel was 169 pS and the extrapolated reversal potential was −39 mV. (*C*) Current traces from an inside-out patch evoked by 4-sec ramp from −100 to +100 mV. The electrode contained 130 mM NaCl and the bath contained 33 mM NaCl. The current traces from a single and double channel opening intersect at −30 mV, and give an accurate estimate of the reversal potential. The vertical bar shows indicates 0 mV.

after the presentation of a voltage pulse. Thus data from the first 2 sec of a recording from any potential was not used to determine the steady state open probability shown in Fig. 5*A*.

## Pharmacology

Many large-conductance anion channels are blocked by the diuretic stilbenes DIDS and SITS (Bettendorf, Holb, & Schoffeniels, 1993; Bosma, 1989; Light et al., 1990) and so a series of experiments was designed to test the sensitivity of the large-conductance chloride channels in PCE cells to these blockers. When applied to the cytoplasmic face of excised patches, SITS produced a flickery block in 12 of 15 trials. The drug was used in concentrations ranging from $10^{-4}$ M to $1.7 \times 10^{-3}$ M. No difference in the response could be attributed to the difference in concentration. The flicker, which occurred as soon as SITS was applied (Fig. 6*A*), is the characteristic response of maxi-chloride channels to these blockers (Bosma, 1989; Velasco et al., 1989) and has been reported for a range of concentrations from 10 μM (Coulombe & Coraboeuf, 1992) to 1 mM (Nelson et al., 1984). The presence of more than one channel in most of these experiments precluded a detailed kinetic analysis, but Fig. 6*B* shows that the flicker is due to a decrease in the mean open time of the channel. In 8/12 experiments, flicker was followed by complete channel closure between 30 sec to 5 min after drug application. Activity returned to normal once the SITS was washed off in all but one case.

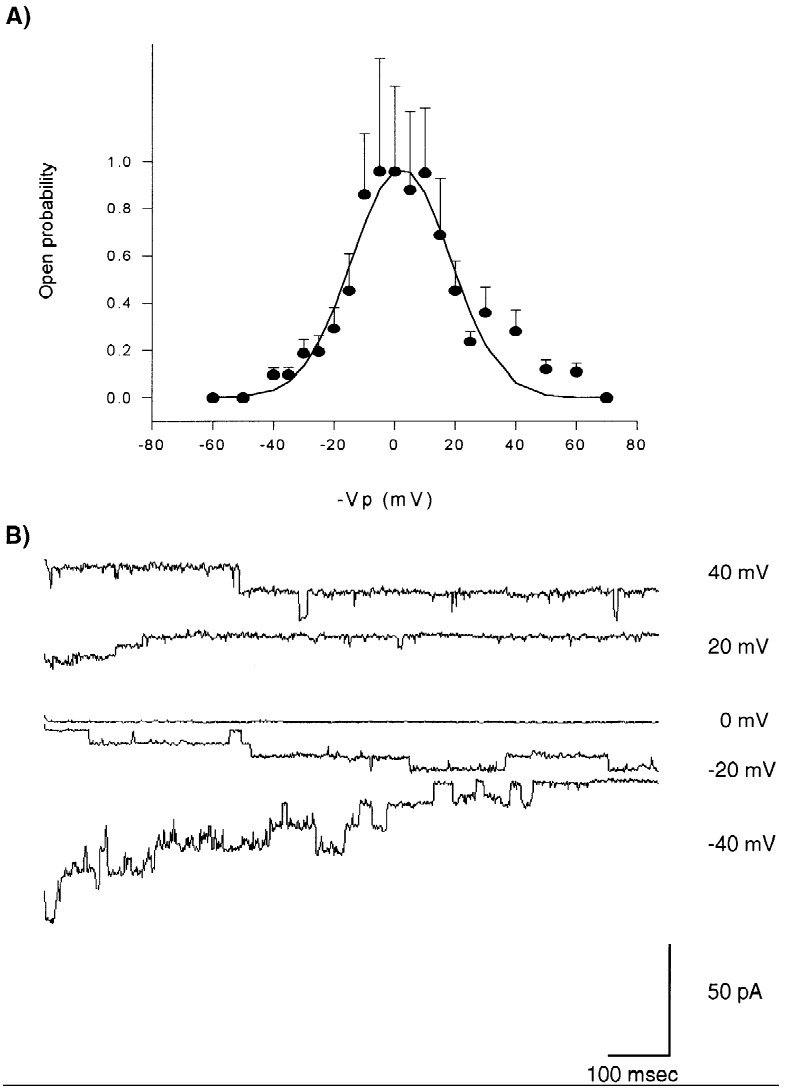


**Fig. 5.** Voltage dependence of channel open probability. (*A*) Steady-state open probability. The probability of at least one channel being open was determined. Only data obtained 2 sec after the changes in voltage were included to avoid complications from time dependent activation or inactivation. Each point is the mean ± SE of 8 to 13 experiments. Data are fit with a Gaussian distribution; mean = 2.1 mV, $\sigma$ = 16.7. The voltage-dependence of open probability did vary between experiments, and channels tended to open at more polarized potentials as experiments progressed. (*B*) The patch potential was frequently cycled between 0, −40, −20, 0, 20 and 40 mV. With this protocol, the channel frequently inactivated at −40 and +40 mV and activated at +20 and −20 mV. The patch potential is shown to the right of each trace.

DIDS produced a complete block of channel activity in 4 out of 4 trials when presented to the cytoplasmic face of the membrane at a concentration of 100 μM. The block was not as flickery as with SITS. In addition, channel activity failed to return after DIDS was washed off in all four cases.

### Channel Activation

Several reports have shown direct activation of a large-conductance anion channel with GTPγS, a nonhydrolyzable analogue of GTP (Mangel, Raymon & Fitz, 1993; Schwiebert et al., 1990; Sun et al., 1992). The theory that the maxi-chloride channel in PCE cells might also be regulated by G-proteins was tested by applying GTPγS to the cytoplasmic face of quiescent patches. GTPγS activated a large-conductance chloride channel in 17 out of 25 patches (68%) which were ultimately shown to possess such a channel. In Fig. 7 we illustrate the GTPγS activation of channel activity in excised inside-out patches. After excising the patch, recordings of current activity were made in response to 500 msec voltage steps of ±50 mV for 10 min to ensure that the activity of the patch was stationary. Then the patches were either exposed to GTPγS or sham solution. Following a latency of 168.8 ± 50.8 sec ($n$ = 11) the channel activated in response to 100 μM GTPγS (Fig. 7*A* and *B*). The mean, leak-subtracted patch current before and after activation is presented in Fig. 7*C*. The mean patch current increased from 5.44 ± 1.22 pA to 18.35 ± 1.65 pA ($n$ = 11) following activation by GTPγS, a 3.4-fold increase. There was no change in the mean current in the control (sham) patches given the same recording time and protocol. Sun et al. (1992) have also observed a delay in activation with GTPγS. They report that large-conductance chloride channels were activated in excised inside-out patches of rabbit colonic smooth muscle 5 sec following perfusion with 100 μM GTPγS. They ob-

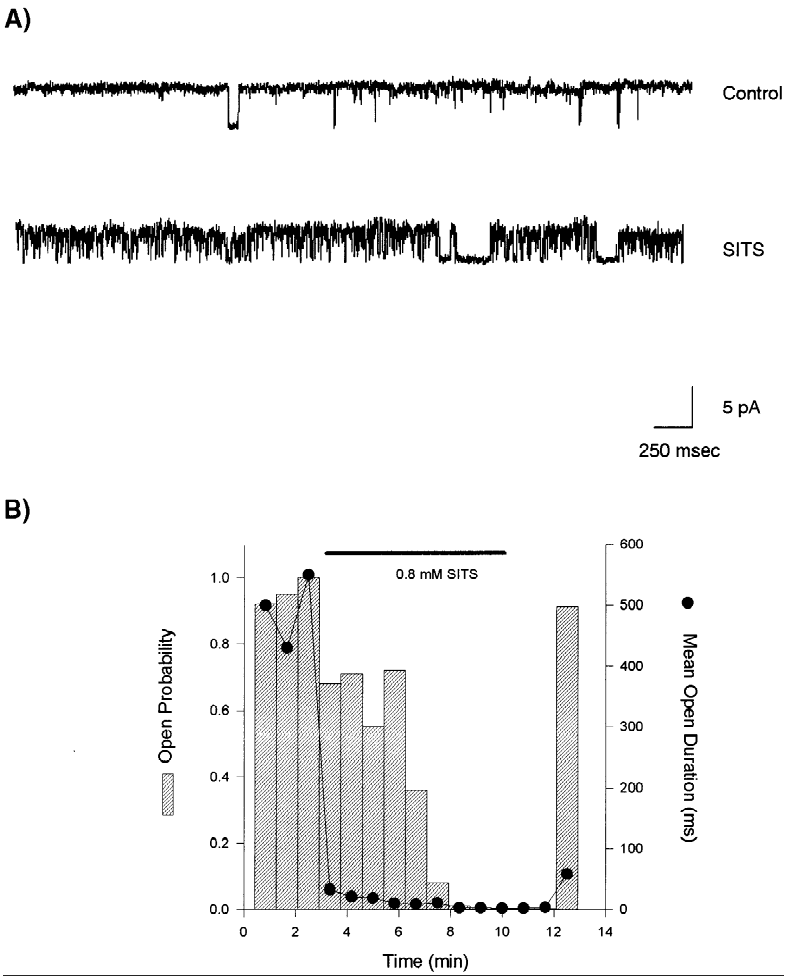


**Fig. 6.** Channel block by SITS. (*A*) The flickery block produced by SITS. Upper trace = control before SITS added; Lower trace = 1 min after the addition of 0.8 mM SITS. The patch was depolarized by 15 mV and upward deflections represent channel openings. Similar results were observed in 12 patches. (*B*) The flickery activity, due to a drop in the mean duration channel activity (●), occurs within a few seconds of adding 0.8 mM SITS. Full channel closure takes several minutes longer. This figure is representative of the three patches which contained only one channel and thus could be analysed in this way.

served a response in ten out of thirty patches which was not reversible following washout of the GTPγS.

## Discussion

In this study we demonstrate the existence of a large-conductance chloride channel on the membrane of fresh bovine pigmented ciliary epithelial cells. The channel shares many characteristics with other large-conductance anion channels including a large unitary conductance with multiple subconductance levels (Blatz & Magelby, 1983; Krouse, Schneider & Gage, 1986; Velasco et al., 1989; Woll & Neumcke, 1987) block by SITS and DIDS (Coulombe & Coraboeuf, 1992, Nelson et al., 1984) and voltage-dependent gating with maximum activity within ±30 mV of 0 mV (Bettendorf et al., 1993; McCann et al., 1989; McGill, Basavappa & Fitz, 1992).

The flickery transitions observed during the initial induction of channel activity have also been observed in other large-conductance, or ''maxi-'', chloride channels and possibly reflect a different kinetic state of this channel (Blatz & Magelby, 1983; Woll et al., 1987). The flickery and square modes of channel activity are unlikely to reflect distinct channels as the initial flickery behavior preceded square channel events (Fig. 1). In addition, the flickery mode was frequently mimicked by the noisy subconductance states (Fig. 2) and the inward current was flickery when the bath contained 33 mM NaCl. Together, these observations suggest that the large-conductance chloride channel in bovine PCE cells, like that in A6 cells (Nelson et al., 1984), can exist in different kinetic modes.

### Physiological Relevance

The large-conductance chloride channel was abundantly represented in the PCE membrane. The channel was present in 60% of excised patches, at an average of 2 channels per patch. A mean cell diameter of 10 μm, and an internal pipette diameter of 1 μm, predicts that there would be 480 maxi-chloride channels per cell. Even if one assumes that some of the cell membrane was sucked up into the electrode during gigaseal formation, thus increasing the relative area, this degree of cellular invest-

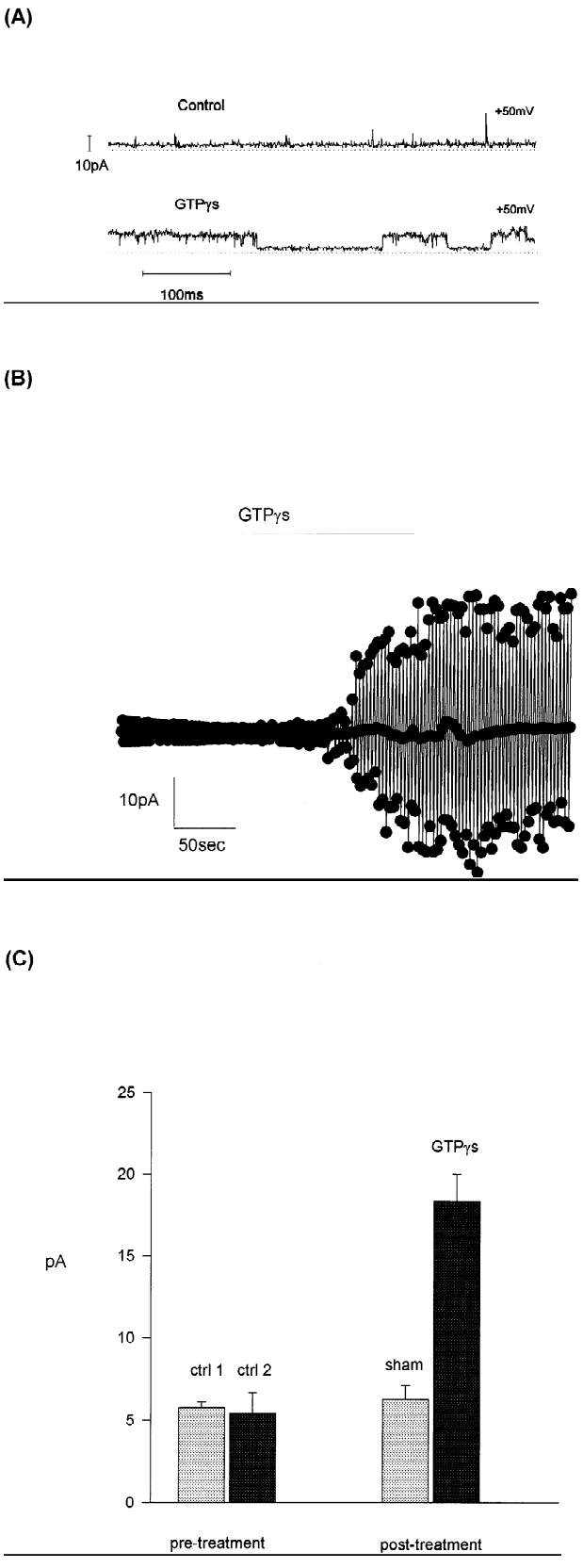


**Fig. 7.** GTPγS activation of the large-conductance chloride channel. (*A*) Single channel activity recorded from excised inside-out patches of PCE cell membrane before (top) and after (lower trace) activation by GTPγS (100 μM). (*B*) Mean patch current increased in response to 100 μM GTPγS. The data points represent the averaged current flowing through the patch. The patch was stepped between +50 and −50 mV for 500 msec with 1 sec at 0 mV between each step. The channel activated with a mean latency of 169 sec (*See* text). (*C*) Mean patch current before and after treatment with GTPγS (100 μM). The pretreatment currents, in response to a voltage step of +50 mV, were averaged (20 pulses averaged over 1 min) 10 min following excision of the patch in both cases. The post-treatment currents represent the average current, recorded over a 1-min period in response to the same voltage step, immediately following the latency period in response to either GTPγS or a sham solution ($n = 11$ in the case of the GTPγS currents and respective control, and $n = 3$ for the sham current and its respective control).

ment suggests that the channel must somehow make a considerable contribution to cellular function. If all these chloride channels open at once the total conductance would be 144nS. The volume-activated chloride conductance in a single PCE cell, determined from the slope of the outward *I/V* curve (from Fig. 1 of Mitchell et al., 1997), is about 18nS, thus the large-conductance chloride channel could readily account for all of this current (there are, however, additional chloride channels in these cells — *see* Zhang & Jacob, 1997). This channel was rarely seen in cell attached patches — a finding in common with other workers in the field (Light et al., 1990; McGill et al., 1992; Sun et al., 1992; Velasco et al., 1989) and the voltage-dependence found in excised patches predicts that the channel would be closed at the cell membrane potential (Hanrahan, Alles & Lewis, 1985; Light et al., 1990; Schlichter et al., 1990). These observations suggest that the maxi-chloride channel is not active under resting conditions. This has hampered attempts to assign a physiological role to the maxi-chloride channel. We are now able to suggest that this channel is under tight regulation by G-proteins and thus explain why it is so rarely seen in the cell-attached mode. Other studies have shown that maxi-chloride channels can be activated by particular stimuli including NK-1 agonists (Sun et al., 1992) and cell swelling (Schwiebert, Mills & Stanton, 1994).

G-proteins coupled to phospholipases C and $A_2$ have been shown to be involved in the coupling of cell swelling to increases in chloride conductance (Mitchell et al., 1997). This present study demonstrates that such coupling exists to a large-conductance chloride channel directly and, taken together with the identification of a large-conductance chloride channel activated by cell swelling in cell-attached patches of PCE cells (Zhang & Jacob, 1997), strongly suggests a role for this channel in volume regulation.

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