# Three different $Cl^-$ channels in the bovine ciliary epithelium activated by hypotonic stress

Jin Jun Zhang and T. J. C. Jacob*

*Physiology Unit, School of Molecular and Medical Biosciences, University of Wales, Cardiff CF1 3US, UK*

1. Hypotonic solution induced transient $Cl^-$ channel activity in both pigmented and non-pigmented ciliary epithelial cells in cell-attached patches.

2. The activation time constants for these currents were 63 and 97 s for non-pigmented and pigmented ciliary epithelial (NPCE and PCE) cells, respectively. The currents inactivated during the exposure to hypotonic solution with time constants of 59 and 304 s for NPCE and PCE cells, respectively.

3. Single channel analysis revealed the presence of a low-conductance $Cl^-$ channel in both the NPCE (7·3 ± 0·4 pS) and PCE (8·6 ± 0·9 pS) cells. In addition, an intermediate-conductance (18·8 pS) channel was found alone in the NPCE cells and a maxi $Cl^-$ channel (105 pS) was found in the PCE cells. The similarities between the 18·8 pS $Cl^-$ channel and the volume-activated organic osmolyte–anion channel (VSOAC) are discussed.

4. The hypotonic activation of all three $Cl^-$ channels was prevented by the inclusion of 100 $\mu$M 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) in the patch pipette.

5. Inclusion of tamoxifen in the patch pipette inhibited only the intermediate-conductance channel in the NPCE cells. This is consistent with the identification of the intermediate 18 pS $Cl^-$ channel, found only in NPCE cells, as VSOAC.

6. Negative pressure also evoked single $Cl^-$ channel activity but the activation kinetics were quite different, suggesting that, even if the channels are the same, the second messenger pathways involved are different.

7. The activation/inactivation kinetics of these $Cl^-$ channels correlate well with the time course of regulatory volume decrease (RVD) in these cells, suggesting that these channels may well participate in the process of RVD.

8. The asymmetrical distribution of $Cl^-$ channels in the ciliary epithelium may be significant for aqueous humour secretion. For instance, the presence of the VSOAC-like channel in the NPCE cells may allow one-way traffic of anions and organic osmolytes into the eye and, if these solutes were loaded by the PCE cells, then a vectorial flow from the blood into the eye would occur.

The ciliary epithelium is responsible for the secretion of aqueous humour. It consists of two cell layers of epithelial cells, a non-pigmented cell layer facing the inside of the eye and a pigmented layer facing the vasculature of the ciliary body. It has been suggested that the same mechanisms that are responsible for cell volume regulation are recruited for the secretion of aqueous humour (Farahbakhsh & Fain, 1987; Yantorno, Carre, Coca-Prados, Krupin & Civan, 1992). Thus, in the study of secretion, the activity of volume-activated channels is of particular relevance. Recently, using whole-cell recording techniques, Wu, Zhang, Koppel & Jacob (1996) described a volume-activated $Cl^-$ current that was regulated by P-glycoprotein in the non-pigmented cells, and Mitchell & Jacob (1995) have reported a volume-activated, phospholipase C-dependent $Cl^-$ current and a maxi $Cl^-$ channel in the pigmented cells (Mitchell & Jacob, 1994, 1995).

Relatively little is known about the single channel characteristics underlying the volume-activated currents involved in regulatory volume decrease (RVD). There are three main candidate $Cl^-$ channels for a role in RVD. The first is ClC-2, a member of the ClC family of $Cl^-$ channels, identified and cloned by Thiemann *et al.* (1992). Although

* To whom correspondence should be addressed.

ClC-2 is ubiquitously expressed, its macroscopic current–voltage relationship is unlike that observed for almost all swelling-activated currents. It has been reported as having a conductance of 3–5 pS (Thiemann, Grunder, Pusch & Jentsch, 1992; Pusch & Jentsch, 1994) and is not blocked by 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS); this distinguishes it from the swelling-activated currents in both pigmented and non-pigmented ciliary epithelial cells which are blocked by DIDS (Mitchell & Jacob, 1995; Wu *et al.* 1996). The second candidate is the outwardly rectifying $Cl^-$ channel which is found in almost all mammalian cells studied to date (Strange, Emma & Jackson, 1996). This channel has been variously reported as having a unitary conductance of 40–90 pS at large depolarizing potentials (Worrell, Butt, Cliff & Frizzell, 1989; Solc & Wine, 1991; Okada, Petersen, Kubo, Morishima & Tominaga, 1994). There is a body of evidence to suggest that this channel conducts both anions and organic osmolytes (Kimelberg, Goderie, Higman, Pang & Waniewski, 1990; Kirk, Ellory & Young, 1992; Sanchez-Olea, Pena, Moran & Pasantes-Morales, 1993; Strange *et al.* 1996) and it has thus been termed the 'volume-sensitive organic osmolyte–anion channel' or VSOAC by Strange & Jackson (1995). Boese, Wehner, Jackson, Strange & Kinne (1995) reported a unitary conductance for this channel of 40–50 pS at +120 mV and 15 pS at 0 mV. It is blocked by a range of pharmacological agents including NPPB, tamoxifen, dideoxyforskolin and various stilbene compounds (Strange *et al.* 1996), all of which block swelling-activated $Cl^-$ currents in non-pigmented ciliary epithelial cells, but of this list of inhibitors only NPPB, and to a lesser extent DIDS and SITS, block the swelling-activated $Cl^-$ currents in pigmented ciliary epithelial cells (Mitchell & Jacob, 1995; Wu *et al.* 1996).

The third candidate volume-activated $Cl^-$ channel is the maxi $Cl^-$ channel which has been shown to be activated by cell swelling in neuroblastoma cells (Falke & Misler, 1989), astrocytes (Jalonen, 1993) and renal cortical collecting duct cells (Schwiebert, Mills & Stanton, 1994). The maxi $Cl^-$ channel has a conductance of 200–400 pS (Falke & Misler, 1989; Jalonen, 1993; Schwiebert, Mills & Stanton, 1994) and is inhibited by NPPB, DIDS and diphenylamine-2-carboxylic acid (DPC) (Schwiebert *et al.* 1994). The presence of this channel in the pigmented cells of the ciliary epithelium has been demonstrated previously (Mitchell & Jacob, 1994).

In this report we describe the single channel characteristics of $Cl^-$ channels activated by cell swelling in the ciliary epithelium as determined by cell-attached patch clamp recording. We describe three $Cl^-$ channels that are found in the ciliary epithelial cells, and report that different $Cl^-$ channels are activated in the pigmented and non-pigmented ciliary epithelial cells by hypotonic-induced swelling. Some of this work has been presented in preliminary form (Zhang & Jacob, 1996).

# METHODS

### Preparation of cells

Pigmented and non-pigmented cells from the ciliary processes of bovine eyes, obtained from a local abattoir, were enzymatically dissociated using trypsin–EDTA and cultured in E199 medium (Sigma) supplemented with 10% newborn calf serum (Sigma) and plated onto sterile glass coverslips for 12–24 h, as described previously (Wu *et al.* 1996). After the cells had settled down, the coverslip with sub-confluent cells, was transferred to a recording chamber. The volume of the chamber was 0·3 ml and the flow rate of perfusion was regulated to approximately 3 ml $min^{-1}$. All measurements were carried out at room temperature (20–23 °C).

### Solutions

The cells were incubated in artificial aqueous humour (AAH) which had the following composition (mM): NaCl, 105; $CaCl_2$, 2; $MgCl_2$, 0·5; D-mannitol, 70 ; Hepes, 10. The pH was adjusted to 7·4 with 3 M NaOH and the osmolarity was adjusted to 295 mosmol $l^{-1}$ with sucrose. Hypotonic solutions were obtained by removing the mannitol from solution. The pipette solution consisted of (mM): *N*-methyl-D-glucamine chloride (NMDG-Cl), 105; $MgCl_2$, 0·5; D-mannitol, 70; Hepes, 10. The pH of the patch pipette solution was adjusted to 7·4 with Tris base and the osmolarity was adjusted to 295 mosmol $l^{-1}$ with sucrose.

5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) was a gift from SmithKline Beecham (Welwyn, Herts, UK). All other chemicals were obtained from Sigma unless otherwise stated.

### Patch clamp recording

The patch clamp recording methods have been described in detail previously (Zhang & Jacob, 1994). Cell-attached patch currents were recorded using a Dagan 8900 patch clamp amplifier (Dagan Corp., MN, USA). Data were filtered at 1 kHz and sampled at either 3 kHz (for amplitude data) or 300 Hz (for kinetic and blocking experiments). Recording electrodes were pulled from borosilicate glass capillaries (Clark Electromedical, Reading, UK) using a two stage vertical pipette puller (Narashige PB-7, Tokyo, Japan) and fire polished to a resistance of 4–8 MΩ when filled with the NMDG-Cl pipette solution. The bath electrode consisted of an Ag–AgCl pellet connected to the bathing solution via an agar bridge made up in normal pipette solution. Liquid junction potentials were measured with the electrode in the bath and if they were larger than 1 mV the appropriate adjustment was made. Voltage and current signals from the amplifier, together with synchronizing pulses, were digitized using a CED1401 interface (Cambridge Electronic Design, Cambridge, UK) and recorded on computer disks using a PC computer. The voltage pulse generation and voltage clamp analysis were achieved by using the EPC patch and voltage clamp software (version 6.0; Cambridge Electronic Design). Single channel amplitude was determined both by eye and by fitting Gaussians (using the least-squares method of the EPC software) to amplitude histograms.

Mean patch current was calculated, after leak subtraction, using SIGAVG, a signal averaging programme (Cambridge Electronic Design). Leak subtraction was performed by constructing a single channel current–voltage ($i$–$V$) curve from small voltage steps and measuring the holding current. The slope of this $i$–$V$ curve gives the leak conductance, and the appropriate value of current was subsequently subtracted from each single channel record.

The data are presented as means ± S.E.M. unless otherwise stated.

## RESULTS

### Activation and inactivation of hypotonic current

Recordings were made from cells using the cell-attached patch clamp technique. The patch pipettes contained $NMDG^+$ as the only cation and in the cell-attached configuration hyperpolarizing pulses resulted in inward currents – consistent with the outward movement of $Cl^-$ ions. When recordings were made from non-pigmented ciliary epithelial (NPCE) and pigmented (PCE) cells under resting conditions for up to 20 min, in the cell-attached mode, hyperpolarizing voltage steps failed to elicit single channel activity. However, when the cells were exposed to hypotonic solution, made hypotonic by removal of mannitol, channel activity began rapidly in 41/47 experiments in NPCE cells and in 29/33 experiments in PCE cells.

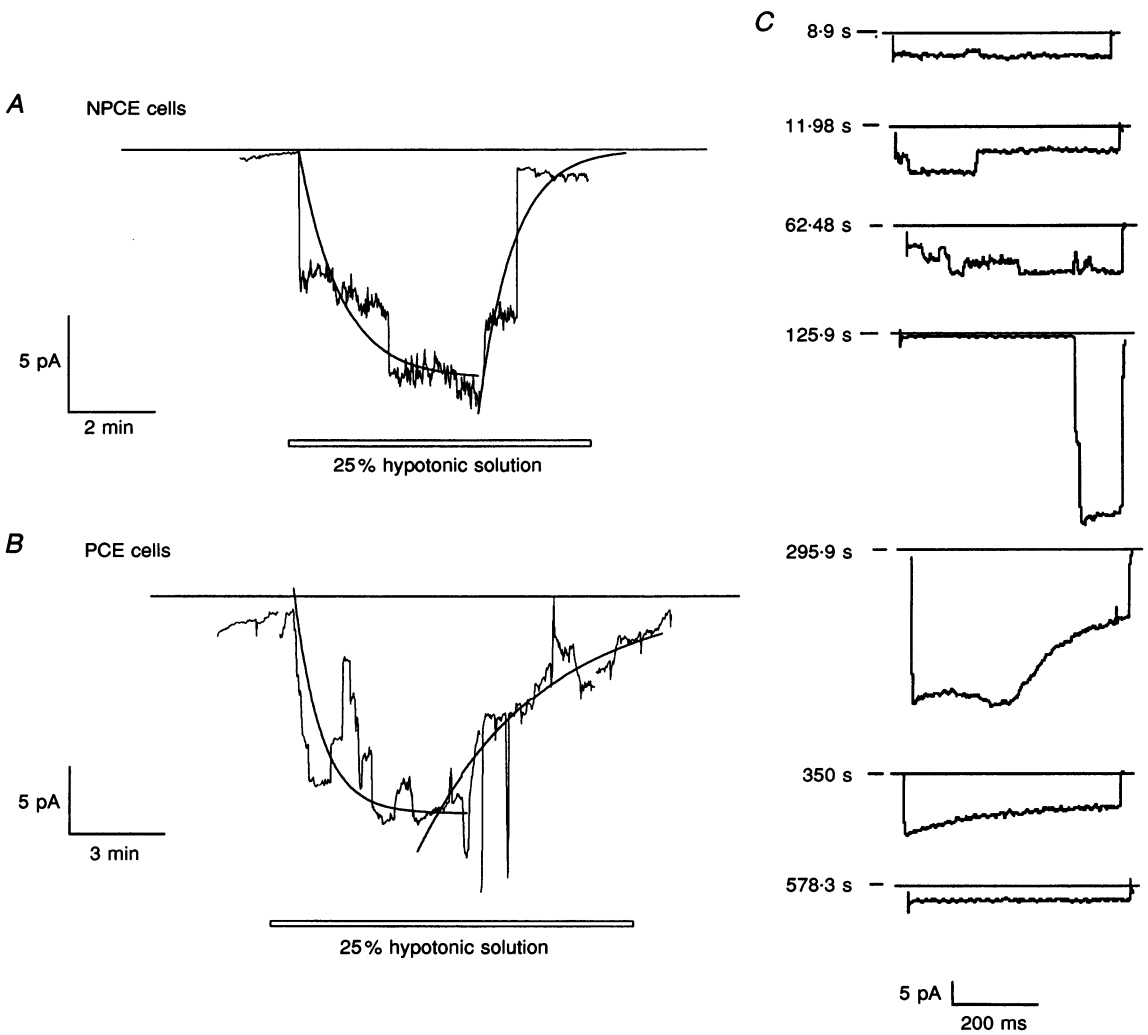


**Figure 1. Swelling-induced membrane patch current**

Mean cell-attached patch current responses of a non-pigmented ciliary epithelial (NPCE) cell (*A*) and a pigmented ciliary epithelial (PCE) cell (*B*) in response to 25% hypotonic solution. Downward deflection indicates inward current. The solution was made hypotonic by removal of mannitol (see Methods). The mean current for 500 ms sweeps at a pipette potential ($V_p$) of 40 mV (membrane hyperpolarization) was recorded every 5 s and leak subtracted and the data from 3 such experiments were averaged and plotted as a function of time. The pipette contained an NMDG-Cl-based solution (see Methods). Recordings were made at room temperature (20–23 °C). The curves represent exponential fits to the data. The activation ($\tau_a$) and inactivation ($\tau_i$) time constants for the hypotonic-activated currents were $\tau_a = 63$ s and $\tau_i = 59$ s in NPCE cells and $\tau_a = 97$ s and $\tau_i = 302$ s in PCE cells (see Results for further discussion). *C*, single channel data from a single NPCE cell-attached patch for $V_p = 40$ mV (membrane hyperpolarization) steps following exposure to hypotonic solution, recorded at the times shown to the left of the traces, illustrating the co-ordinated (non-independent) gating properties of the swelling-induced channels. The current jumped between open state levels and exhibited run-up and run-down (see text for further discussion).

The mean patch current for successive 500 ms data segments recorded at 5 s intervals in three experiments was leak subtracted and plotted as a function of time (Fig. 1*A* and *B*). The single channel activity rose to a peak in 265 s in NPCE cells with a time constant ($\tau_a$) of 63 s (determined from a single exponential fit to the mean data from 3 experiments) and in 160 s in PCE cells with $\tau_a = 97$ s. While still exposed to hypotonic solution the channel activity declined with a time constant ($\tau_i$) of 59 s in NPCE cells and 304 s in PCE cells.

An example of the single channel activity in a single cell, recorded from a NPCE cell in cell-attached mode, is given in Fig. 1*C*. The cell was exposed to hypotonic solution at time zero. Channel activity increased and then jumped in a fashion that suggests co-ordinated gating. Time-dependent inactivation was also evident as the current trace decayed, apparently in a non-stepwise fashion. However, this decay can be explained by the closure of many small channels – the single channel current, at a hyperpolarizing holding potential of 40 mV, would be around 0·4 pA for the low-conductance channel (see below) and therefore too small to resolve on this scale. A proposal that explains the unusual gating characteristics of some volume-activated $Cl^-$ channels has been put forward in which the channels are held just below the membrane by the cytoskeleton. Cell swelling releases the channels and they integrate into the membrane. Strange *et al.* (1996) term this the 'anchor-insertion' model. As the channels integrate they do so in the open state (Strange *et al.* 1996). This would explain co-ordinated opening but cannot explain co-ordinated closure.

Two types of single channel were observed in both NPCE and PCE cells. One channel type, the low-conductance channel, was seen in both cell types.

### Low-conductance chloride channel in both PCE and NPCE cells

A low-conductance $Cl^-$ channel was found in both PCE (Fig. 2) and NPCE cells in response to hypotonic solution and cell swelling. This channel was observed alone in 13/29 PCE cells and in conjunction with another channel type with a conductance of around 100 pS (see below) in 11/29 patches. The single channel *i–V* relationship for this low-conductance channel in PCE cells is presented in Fig. 2*B*. From a regression fit to the data from four experiments a conductance of 6·7 pS was determined. The mean slope of the *i–V* of eight low-conductance channels in NPCE cells gave a conductance of 7·3 ± 0·4 pS ($n = 8$), determined by linear regression fits to the data. The channel current reversed at a potential 23·3 ± 2·8 mV ($n = 4$) positive to the resting membrane potential. In a cell-attached patch the membrane potential is not known, but it can be assumed that the chloride reversal potential, $E_{Cl}$, will be between the resting membrane potential and zero. The membrane potential in these cells is between −60 and −80 mV (Gooch, Morgan & Jacob, 1992). Therefore the $E_{Cl}$ measured is within the range expected for a $Cl^-$ channel.

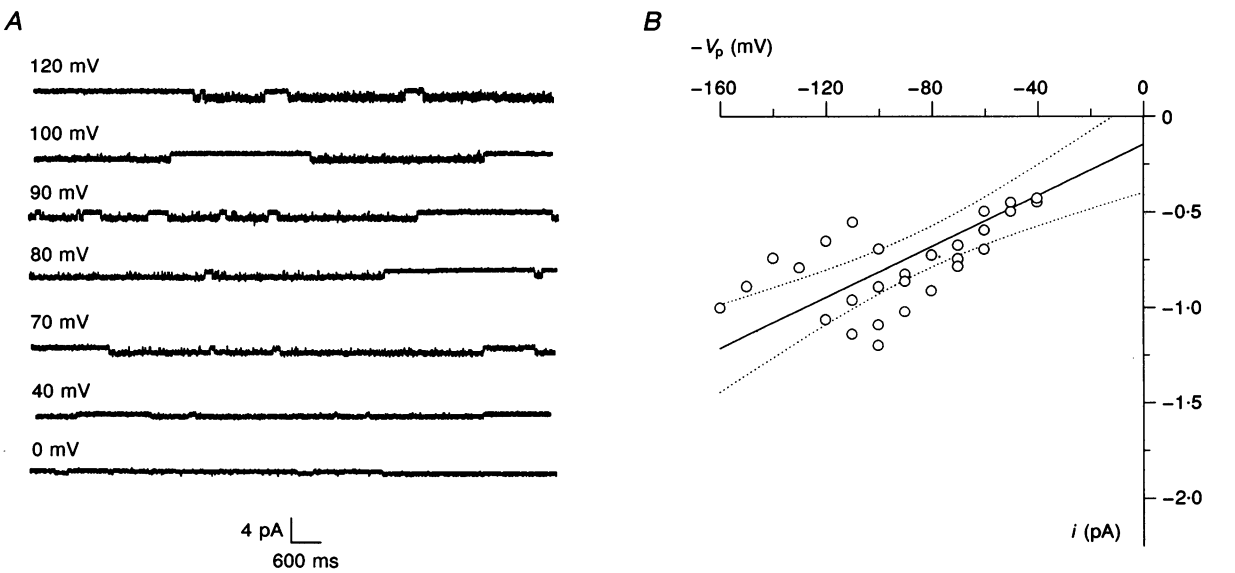


**Figure 2. Low-conductance channel in PCE cells**

*A*, the low-conductance $Cl^-$ channel recorded from a cell-attached patch on a PCE cell. The voltages above the traces are pipette potentials and result in patch membrane hyperpolarization. *B*, the pooled single channel amplitude data from 4 PCE cells. The continuous line represents a linear regression fit to the pooled data (with the 95% confidence limits shown by dotted lines) from which a conductance of 6·7 pS was determined. Regression fits to the individual experiments gave a conductance value of 8·6 ± 0·9 pS ($n = 4$).

In PCE cells the conductance of the low-conductance channel was found to be 8·7 ± 0·9 pS ($n = 4$).

### High-conductance chloride channel in PCE cells

A second, larger-conductance channel type was also observed in PCE cells in response to hypotonic swelling. In 5/29 patches this channel alone was observed and in 11/29 it was observed in conjunction with the low-conductance channel. Figure 3*A* illustrates the simultaneous appearance of both the low- and high-conductance channels in the same patch and the inset to Fig. 3*C* illustrates the high-conductance channel on its own. The slope of the *i–V* relation gave a conductance of 100·5 pS (Fig. 3*B*). From regression fits to the data from three experiments a conductance of 104·6 ± 2·6 pS was determined for this larger $Cl^-$ channel. The channel reversed at a potential 14·2 mV positive to the resting membrane potential, consistent with it being a $Cl^-$ channel (see Fig. 3*C*).

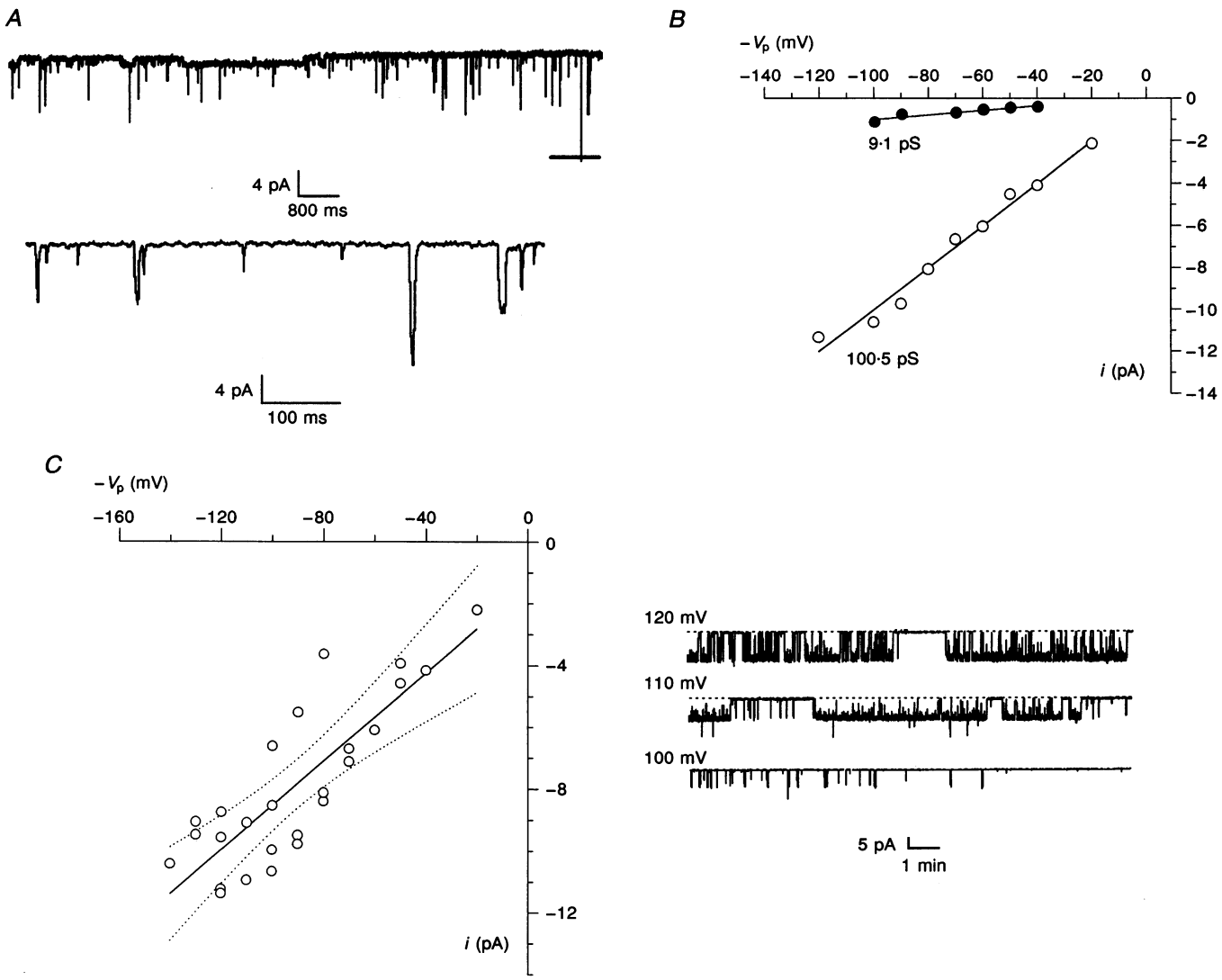


**Figure 3. PCE cell-attached patches**

*A*, single channel data illustrating the presence of at least two separate channels, a low-conductance channel and a second, larger-conductance channel. These channels were seen together in 38% of patches. The lower trace is an expansion of the upper trace at the region indicated by the bar. The pipette potential was 90 mV (membrane hyperpolarization). *B*, the *i–V* curves for the two channels in *A*. Linear regression fits gave conductance values of 9·1 and 100·5 pS, respectively. *C*, the pooled *i–V* data from four PCE patches. The continuous line is a linear regression fit to the data (with the 95% confidence limits shown by dotted lines) from which a conductance of 72 pS was determined. However, when the data from individual cells were fitted separately a mean conductance of 104·6 ± 2·6 pS was determined. Inset is a sample of the high-conductance channel activity recorded in cell-attached mode at hyperpolarizing potentials of 110 and 120 mV.

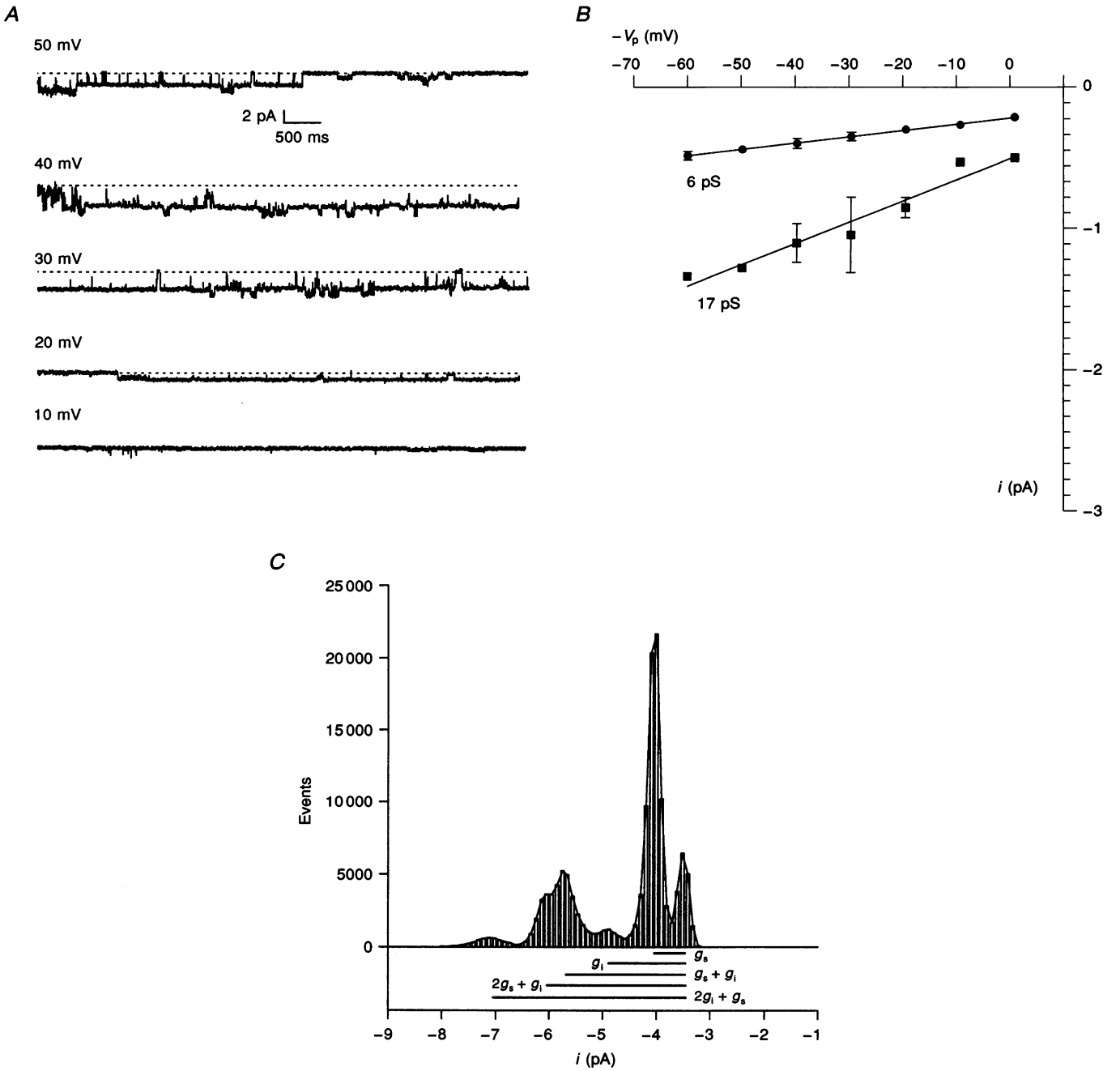


**Figure 4. NPCE cell patches**

*A*, cell-attached single channel data recorded from a NPCE cell. At least two distinct levels were visible in almost 59% of patches. The potentials above each trace are pipette potentials ($V_p$) and represent membrane hyperpolarization. *B*, pooled *i–V* data from three cells exhibiting both low- and intermediate-conductance channels. Linear regression fits gave conductances of 6 and 17 pS, respectively. The conductances determined from the slope of individual *i–V* curves were 7·3 ± 0·4 pS for the low conductance ($n = 8$) and 18·8 ± 2·7 pS for the intermediate conductance ($n = 4$). *C*, a detailed analysis of the single channel amplitudes recorded at a patch potential of 40 mV (membrane hyperpolarization). The simplest interpretation is that there are two independent channels whose amplitudes sum in the way illustrated below the amplitude histogram. The continuous line is the sum of six Gaussian distributions with peaks representing amplitudes of −3·45 (holding current = 0 pA), −0·54, −1·61, −2·19, −2·55 and −3·61 pA; with relative areas of 17, 59, 2·9, 10·7, 8·1 and 1·5%, respectively.

### Intermediate-conductance chloride channel in NPCE cells

The third type of channel, activated by hypotonic exposure, had a conductance intermediate between the other two. This intermediate-conductance channel was observed in conjunction with the low-conductance channel in 24/41 patches in NPCE cells and on its own on only three occasions. The single channel activity of this intermediate-conductance channel is shown in Fig. 4*A*. In four experiments the conductance was found to be 18·8 ± 2·8 pS and the current reversed at 24·3 ± 3·5 mV ($n = 4$) positive to the resting membrane potential. Figure 4*B* gives the mean $i$–$V$ curves for four experiments in which both the low- and intermediate-conductance chloride channels were active.

The presence of the two channels, of low ($g_s$) and intermediate ($g_i$) conductance, is further illustrated in the amplitude histogram (Fig. 4*C*) of data recorded at a hyperpolarizing holding potential of 40 mV. There are six clearly distinguishable peaks. The simplest interpretation of these data is that there are two channels present and that these peaks represent the single channel amplitudes of both these channels, singly, together and in various multiple combinations, as indicated below the histogram in Fig. 4*C*. The first peak, at −3·45 pA represents the holding current and can be taken as the baseline or 0 pA. The next peak, at −3·95 pA ($i = -3·95 - (-3·45)$ or −0·54 pA), represents openings of the low-conductance channel. The third peak, at −4·99 pA ($i = -1·6$ pA), represents openings of the intermediate-conductance channel. The fourth peak represents both the low- and intermediate-conductance channels open simultaneously; the sum of the two individual channels is −0·54 + (−1·6) or −2·14 pA and the actual measured current is −2·19 pA. The subsequent peaks can be similarly explained by sums of various multiples of the two channels. Applying Occam's razor we accepted this as the working hypothesis for the interpretation of the data.

### Inhibition of swelling-activated current

The presence of 100 μM NPPB in the pipette solution inhibited the swelling-induced current in both PCE (Fig. 5*A*) and NPCE (Fig. 5*B*) cells.

The mean patch currents for the 10 min exposure period to hypotonic solution without and with NPPB in the pipette were 11·00 ± 0·14 pA and 1·33 ± 0·02 pA ($n = 3$), respectively, for NPCE cells (a reduction of 89%) and 9·90 ± 0·20 pA and 0·41 ± 0·02 pA ($n = 3$) for PCE cells (a reduction of 96%).

The presence of 50 μM tamoxifen in the patch pipette caused a reduction in the peak swelling-induced current to 3·22 ± 1·22 pA (a 72% reduction) in NPCE cells but had no effect upon the swelling-induced current in the PCE cells (Fig. 6). For the current remaining after tamoxifen block in NPCE cells, the activation time constant was 29·4 s and the inactivation time constant was 47·9 min. The conductance of the remaining single channels in the NPCE cells (Fig. 6*A*) was 8·3 ± 0·8 pS ($n = 3$). This suggests that tamoxifen inhibits only the intermediate 18 pS conductance volume-activated $Cl^-$ channel (present only in the NPCE cells)

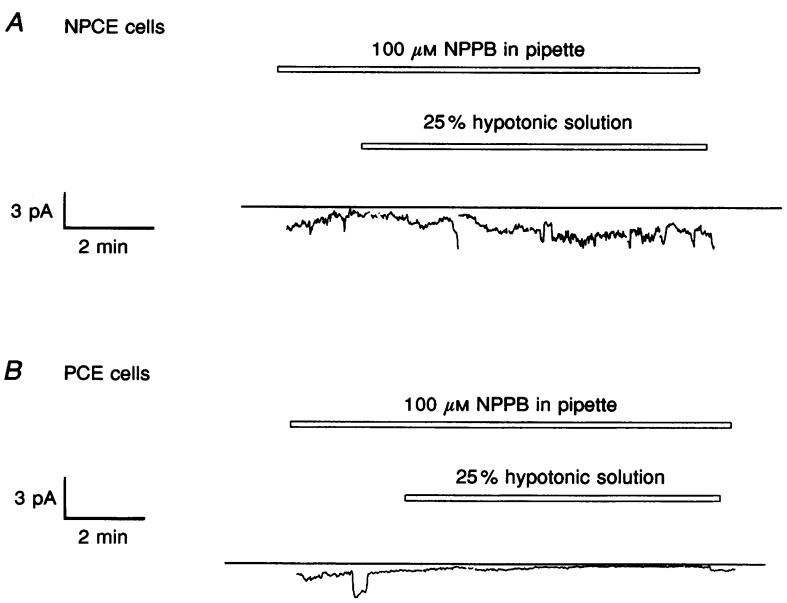


**Figure 5. Inhibition by NPPB of swelling-activated current**

Mean patch currents from cell-attached recordings were determined as described in the legend to Fig. 1 in three NPCE cells (*A*) and three PCE cells (*B*) in response to 25% hypotonic solution with 100 μM NPPB in the patch pipette at a (hyperpolarizing) holding potential of 40 mV.

leaving the low-conductance (present in both NPCE and PCE cells) and high-conductance (in PCE cells only) channels unaffected.

### Channel activation by suction

Channel activation occurred during an extended pulse of suction applied to the pipette. However, the kinetics of the channel activity were quite different to those of the hypotonically activated channels. The channel generally activated slowly and some time after the pressure had been applied. The mean response of four pigmented and four non-pigmented cells is given in Fig. 7. Following a delay in activation, the mean patch current increased, exhibiting 'noisy' characteristics which sometimes resolved into rectangular channel open–close events and often resulted in sudden rapid increases in patch current and patch loss. Because of the channel irregularity it was not possible to perform analysis of channel amplitudes or kinetics with any confidence. Suffice it to say that, if the same channels are activated by both hypotonic stress and membrane stretch, then the activation process, kinetics and/or second messenger pathways may be different.

The inference from these experiments is that membrane stretch is probably not the stimulus for activation of the hypotonic-evoked $Cl^-$ channels.

## DISCUSSION

We have identified three different $Cl^-$ channels activated by hypotonic stress in the ciliary epithelium. We have characterized them as $Cl^-$ channels on the basis of the patch pulse protocol combined with the composition of the patch pipette solution (NMDG-Cl), reversal potential and the observation that their activation was prevented by the inclusion of NPPB in the patch pipette.

The activation/inactivation kinetics of $Cl^-$ channels in cell-attached patches in response to hypotonic shock correlate well with the known parameters of regulatory volume decrease (RVD) in these cells. The duration of the activity of the hypotonic-activated channels was 298 s for non-pigmented (NPCE) cells and 509 s for pigmented (PCE). This compares with 375 and 400 s for the completion of RVD for NPCE and PCE cells, respectively, as determined by relative volume measurements (Walker, Miley, Camodeca, Stelling, Pollard & Jacob, 1995). It is therefore fair to assume that these $Cl^-$ channels are participating in the process of RVD.

The activation of these channels, following hypotonic stress, is rapid: 63 s in NPCE cells and 97 s in PCE cells. This rapid activation contrasts markedly with the delayed activation of channel activity following the application of suction to the

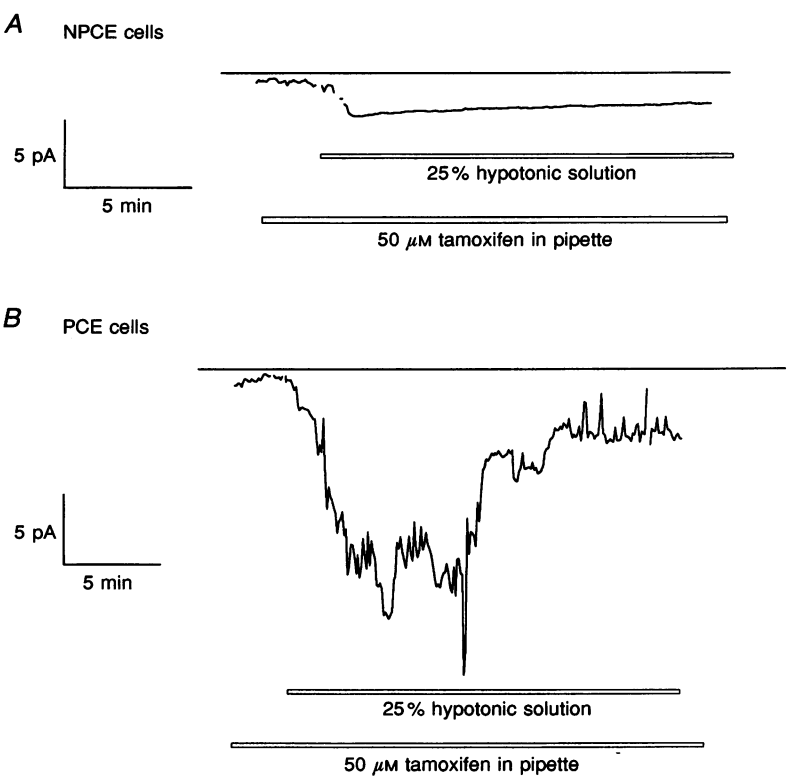


**Figure 6. Inhibition by tamoxifen of swelling-activated current**

Mean patch currents recorded as described in the legend to Fig. 1 from cell-attached recordings from three NPCE cells (*A*) and three PCE cells (*B*) in response to 25% hypotonic solution with 50 μM tamoxifen in the patch pipette at a (hyperpolarizing) holding potential of 40 mV.

patch pipette (see Fig. 7) and probably indicates that the stimulus for activation is not membrane stretch. It has been suggested that arachidonic acid metabolites may mediate the process of RVD and that epoxides activate the $Cl^-$ channel(s) involved (Civan, Coca-Prados & Peterson-Yantorno, 1994). Once activated, the channels inactivated while the cells were still exposed to the hypotonic stress. The inactivation time constant of the chloride currents was 59 s in non-pigmented cells and 304 s in pigmented cells. This difference may reflect the presence of different channels in these cells. The non-pigmented cells possess the low- and intermediate-conductance chloride channels, 7·3 and 18·8 pS, respectively, while the pigmented cells possess the low- and high-conductance channels, 8·6 and 105 pS, respectively. These cells have been found to respond differently to hypotonic shock in both volume studies and whole-cell patch clamp recordings (Miley, Alexander, Sau, Jacob & Pollard, 1995; Jacob & Mitchell, 1995; Mitchell & Jacob, 1995). The volume-activated chloride currents in the non-pigmented cells are blocked by NPPB, 4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulphonic acid (SITS), DIDS, verapamil, quinidine, dideoxyforskolin and tamoxifen – of which only NPPB, SITS and DIDS block the volume-activated chloride currents in the pigmented cells. This latter current is blocked by gadolinium (Miley *et al.* 1995).

In the Introduction, three candidate volume-activated $Cl^-$ channels are described. Do the three $Cl^-$ channels we have detected in the ciliary epithelial cells correspond with these candidates? Firstly, it is not possible to state categorically that the low-conductance $Cl^-$ channel, present in both PCE and NPCE cells, is ClC-2 since, while the conductances are similar (~5–6 pS), the low-conductance $Cl^-$ channel was inhibited by NPPB, which does not block ClC-2 and, in another study, no current with characteristics of ClC-2 currents, namely inward rectification and hyperpolarizing activation, was seen in these cells (Wu *et al.* 1996). These small channels may not therefore be ClC-2 channels but there are other possibilities. The first is the 'mini' $Cl^-$ channel in Ehrlich's cells described by Lambert & Hoffmann (1994), which has a conductance of 3–7 pS, is activated by cell swelling and inhibited by arachidonic acid and weakly by DIDS. A second possibility is the CFTR $Cl^-$ channel which has a similar conductance (Gray, Harris, Coleman, Greenwell & Argent, 1989; Gray, Pollard, Harris, Coleman, Greenwell & Argent, 1990) but is not thought to be volume activated. Thirdly, a 4 pS $Cl^-$ channel has been described in airway epithelia (Dusyk, French & Man, 1992) but whether this channel is volume activated is unknown.

As to the second, intermediate-conductance $Cl^-$ channel, found in the NPCE cells, this has a conductance (18 pS) and pharmacological sensitivity that could well identify it as VSOAC, the volume-activated organic osmolyte–anion channel described by Jackson, Morrison & Strange (1995). These authors reported that VSOAC was regulated by a

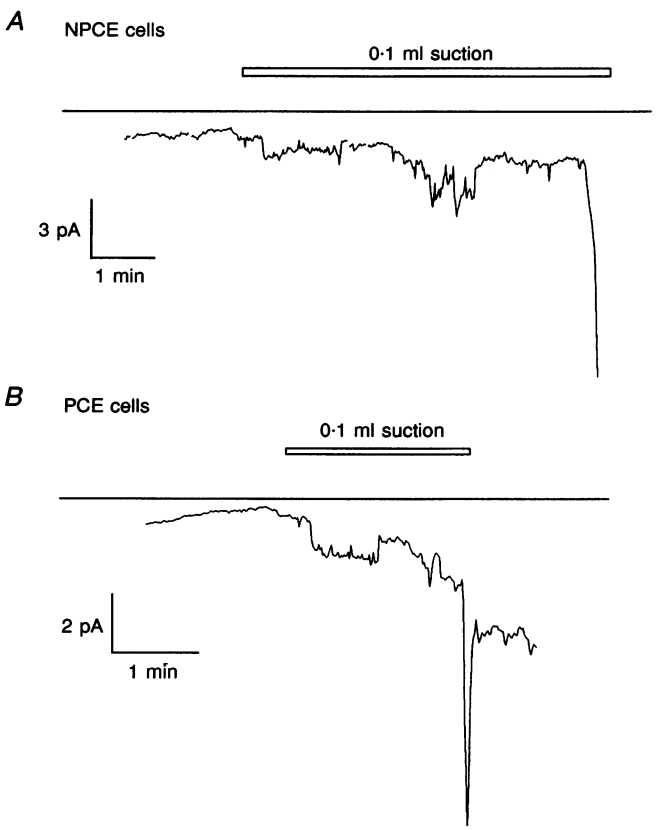


**Figure 7. Suction-induced channels**

Mean patch currents were determined as described in the legend to Fig. 1 in 4 NPCE cells (*A*) and 4 PCE cells (*B*) in response to 0·1 ml suction applied by syringe. Induction of the channel activity was delayed compared with hypotonic activation (see Fig. 1) and was often 'noisy' and resulted in loss of the patch. The pipette potential was 40 mV (representing membrane hyperpolarization).

non-hydrolytic binding of ATP, an observation which has also been made in NPCE cells (Wu *et al.* 1996). Further support for the identification of the intermediate conductance as VSOAC comes from the inhibition of the latter by tamoxifen – VSOAC is also blocked by tamoxifen. It has been proposed that PICln, first cloned by Paulmichl, Li, Wickman, Ackerman, Peralata & Clapham (1992), is VSOAC (Strange *et al.* 1996). In contrast, it has recently been suggested that ClC-3, first cloned from rat kidney by Kawasaki *et al.* (1994), is the volume-activated $Cl^-$ channel in a human non-pigmented ciliary epithelial cell line (ODM-2 cells) (Coca-Prados, Sanchez-Torres, Peterson-Yantorno & Civan, 1996) and that this channel is regulated by PICln (Coca-Prados *et al.* 1996). Further to this, but not necessarily in contradiction to it, Wu *et al.* (1996) demonstrated that this volume-activated current was regulated by P-glycoprotein. These regulators may affect different volume-activated $Cl^-$ channels. Future studies will be necessary to resolve and/or amplify this issue.

Finally, the third $Cl^-$ channel, that found in the pigmented cells, is almost certainly the same as the third candidate for the volume-activated $Cl^-$ channel – the maxi $Cl^-$ channel. It has a conductance of 105 pS and is inhibited by NPPB. It has also been found in excised inside-out patches in these cells (Mitchell & Jacob, 1995).

If we can equate the intermediate-conductance $Cl^-$ channel with VSOAC, it may of functional significance that the NPCE cells, which secrete aqueous humour directly into the eye, contain the anion–organic osmolyte channel (VSOAC) and the PCE cells, which must load solute from the blood side of the tissue, do not. The movement of anions ($Cl^-$, $HCO_3^-$) and organic osmolytes (amino acids, taurine, *myo*-inositol) into the eye through these channels may be intrinsic to the process of aqueous humour secretion. Future work will need to determine whether this channel in the NPCE cells conducts other anions and organic osmolytes. The PCE cells, on the other hand, must possess volume-activated channels since loading solute may well involve volume increases that will need to be compensated for if not all of the solute and fluid is transferred to the NPCE cells for transporting into the eye. Thus the asymmetrical distribution of $Cl^-$ channels in the ciliary epithelium may be necessary for the process of aqueous humour secretion. It will be interesting to see if immunocytochemical techniques locate the intermediate (VSOAC-like) $Cl^-$ channels on the membrane facing into the eye, that is, the basolateral membrane of the NPCE cells

In summary, we demonstrate that swelling activates a variety of chloride channels in cell-attached membrane patches of ciliary epithelial cells. Both the pigmented and non-pigmented cells contain a low-conductance channel (7–9 pS); the pigmented cells contain a larger 105 pS channel and the non-pigmented cells contain an intermediate-conductance 19 pS channel. All channels were volume activated, they inactivated while still exposed to the hypotonic stimulus and were inhibited by NPPB. Only the 19 pS channel of the NPCE cells was inhibited by tamoxifen. We speculate that this latter channel is the entry pathway into the eye for anions and organic osmolytes.

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**Author's email address**

T. J. C. Jacob: jacob@cardiff.ac.uk

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