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Single-Channel Recordings of Apical Membrane Chloride Conductance in A6 Epithelial Cells

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Summary. The apical membrane of epithelial cells from the A6 cell line grown on impermeable substrata was studied using the patch-clamp technique. We defined the apical membrane as that membrane in contact with the growth medium. In about 50% of the patches, channels with single-unit conductances of 360 ± 45 pS in symmetrical 105 mM NaCl solutions, and characteristic voltage-dependent inactivation were observed. Using excised membrane patches and varying the ionic composition of the bathing medium, we determined that the channels were anion selective, with a permeability ratio for Cl^- over Na^+ of about 9:1, calculated from the reversal potential using the constant-field equation. The channel was most active at membrane potentials between ±20 mV and inactivated, usually within a few seconds, at higher potentials of either polarity. Reactivation from this inactivation was slow, sometimes requiring minutes. In addition to its fully open state, the channel could also enter a flickering state, which appeared to involve rapid transitions to one or more submaximal conductance levels. The channel was inhibited by the disulfonic stilbene SITS in a manner characteristic of reversible open-channel blockers.

Key Words A6 cells · chloride channels · single-channel recording · inactivation · SITS

Introduction

Reabsorption of NaCl against a concentration gradient by tight epithelia involves the active transport of Na, while Cl is thought to move passively, driven by electrical gradients established ultimately by the Na pump (Ussing, 1960; Erlij, 1976). The mechanisms of transepithelial Cl transport, however, remain unclear. Macknight (1977) found that the apical membrane of the urinary bladder of *Bufo marinus* (Dominican origin) was impermeable to Cl, and suggested that the anion was reabsorbed through paracellular pathways. On the other hand, Narvarte and Finn (1980) claimed that the apical membrane of the toad bladder (Mexican origin) has a finite Cl conductance, raising the possibility that Cl transport may be in part transcellular. In amphibian skin, a voltage-dependent apical Cl conductance has been observed (Larsen & Kristensen, 1978; Kristensen, 1983). This conductance may reside in mitochondria-rich rather than granular cells (Voûte & Meier, 1978; Kristensen, 1981).

Cells from the A6 line derived from *Xenopus* kidney (Rafferty, 1969) form epithelia in culture which exhibit many of the properties shared by the toad bladder, frog skin and other tight, reabsorptive epithelia. These include high transepithelial electrical resistance, a short-circuit current which is accounted for by net Na reabsorption, and sensitivity of the Na transport system to the salt-retaining hormone aldosterone and the K-sparing diuretic amiloride (Handler et al., 1981; Perkins & Handler, 1981; Sariban-Sohraby, Burg & Turner, 1983). In this communication, we report observations of single Cl-selective channels in the apical membrane of A6 cells using the patch-clamp technique (Hamill et al., 1981). The channels have a large single-unit conductance (360 pS) and are voltage-dependent, being most active when the membrane potential difference is between ±20 mV. The characteristics are very similar to those of Cl channels from mitochondrial outer membranes (Schein, Colombini & Finkelstein, 1976; Columbini, 1979) and cultured rat muscle cells (Blatz & Magleby, 1983). The channels may be involved in transepithelial Cl transport.

Materials and Methods

Cell Culture

Starter A6 cultures were obtained from American Type Culture Collection as frozen cells in passage number 69. A stock flask culture was always maintained in a growing state. Cells were incubated at 26°C in an atmosphere of 4% CO_2 in air with constant humidity. The culture medium (Perkins & Handler, 1981) consisted of a mixture of seven parts Coon's modification of Ham's F-12 and three parts of Liebovitz's L-15 modified for

amphibian cells to contain 103 mM NaCl and 25 mM $NaHCO_3$ in the mixture (final osmolarity 276 mOsm). Additional media supplements included 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco). Cells were harvested approximately every week as the stock culture flask reached confluency and numerous domes could be observed. Cells were dissociated by incubation in Ca^{++}, Mg^{++}-free Hank's Basic Salt solution containing 0.25% trypsin (ICN). Once the removal of the cells from the flask was complete, the enzyme activity was inhibited by adding an equal volume of serum containing culture medium. The cells were then subsequently centrifuged, resuspended and seeded on plastic tissue culture dishes (Falcon) which had been previously coated with calf-skin collagen and allowed to air dry. Cells grown on tissue culture dishes were fed approximately twice a week and routinely used for patch clamp experiments at least three to four days after subculturing. In most experiments cells were between 69 and 78 passages. All experiments were carried out at room temperature.

Electrophysiology

Single-channel recordings were obtained from cells which had reached a state of confluency on plastic petri dishes according to standard protocols (Hamill et al., 1981). The dish on which the A6 cells were grown was placed in a chamber on the movable stage of a Leitz inverted microscope equipped with phase contrast optics. Experiments were carried out at a total magnification of 320×. The patch clamp electronics were designed by Dr. Richard Levis incorporating a U430 dual FET (Siliconix, Santa Clara, Calif.) input headstage.

Experiments were performed on excised patches in both the inside-out and outside-out configurations as well as on-cell patches. The recording solution in the pipette varied in ionic composition according to experimental protocol but in general contained 105 mM total chloride salt, was buffered to pH 7.4 using 25 mM HEPES and adjusted to a total osmolarity of 276 mOsm using sucrose. The bath solution contained (mM): 105 NaCl, 0.1 $CaCl_2$, 25 HEPES, 46 sucrose. The selectivity of the currents was determined by replacing some fraction of the total NaCl with an isosmolar amount of choline chloride (Sigma), TEA chloride (Sigma), Na_2SO_4 or sucrose. Solution osmolarities were monitored using a vapor pressure osmometer (Wescor, Inc., Model 5100). In a few specified experiments with inside-out patches, Ca^{++} was omitted from the bathing solution to prevent vesicle formation. Nominally Ca^{++}-free solutions led to rapid rounding of the cells and eventually detachment from the dish. In two experiments with outside-out patches 0.1 mM EGTA (Sigma) was added to regular nominally Ca^{++}-free solution in the pipette. In all but two experiments 1 to 10 mM dibutyryl cAMP (Sigma) was included in the recording bath solution. SITS (4-acetamino-4′-isothiocyano-2,2′-disulfonic acid stilbene) was obtained from Polysciences, Inc., Warrington, Pennsylvania.

Patch pipettes were formed from sodalime glass (Fisher Blue-Dot Hematocrit Glass) using a vertical puller. Pipettes were coated with Sylgard 184 (Dow-Corning, Midland, Mich.) and fire-polished to a final tip diameter of approximately 0.5 μm under a total magnification of 600× directly before use.

Data Analysis

Single-channel currents were recorded on analog tape with a bandwidth of DC-5 kHz. Signals were low-pass filtered at 1.0 kHz (4-pole Bessel response) and sampled at 500 μsec/point before transferring to nine-track digital tape for analysis. The data were analyzed and displayed using an automated pattern-recognition program (Sachs, Neil & Barkakati, 1982).

Bath Perfusion System

Solution changes at either the extracellular or intracellular surface of the patch membrane (which depended on the membrane patch configuration) was accomplished via a gravity-feed superfusion system. A fire-polished glass pipette with a 200 to 500 μm opening was placed into the bath directly opposite the tip of the patch pipette. Solution changes at the patch membrane were thus rapid and complete as fluid flowed out of the perfusion pipette into the bath. In those experiments in which the solution was changed, the ground and reference electrodes were connected to the bath solution via agar salt bridges in order to prevent electrode off-set potentials.

Results

We chose to study the conductive properties of the apical membrane of the A6 cell line, as these cells form epithelia in culture having many of the transport characteristics found in toad urinary bladder, frog skin and other tight epithelia (Perkins & Handler, 1981). We were able to form seals with resistances of 1 gigohm or greater with apical membranes of the A6 cells, although the success rate for forming good seals was considerably lower than that for other cell types using the same technique (20% with A6 cells compared with 80% with cultured muscle and nerve cells). The main problem seemed to be related to the low deformability of the epithelial cell membranes, making it difficult to pull omega-shaped patches of membrane into the lumen of the pipette. Grantham (1970) found that cAMP increased the deformability of the apical membranes of the mammalian cortical collecting tubule. Accordingly, we used 1 to 10 mM dibutyryl-cAMP in the bath in most of our experiments. It was our impression that this indeed improved the rate of seal formation but insufficient data comparisons precluded a rigorous statistical analysis. We checked that this agent did not alter the properties of the channels which we have studied. In two experiments done in the absence of cAMP we observed channels with single-channel conductance and kinetic properties identical to those found in its presence. Possible alterations in the density of the channels, however, are difficult to assess.

The most prevalent channel type in the apical membrane of the A6 cell turned out to be anion selective. This channel was observed quite frequently, in about 50% of the 49 membrane patches studied. We have characterized its conduc-

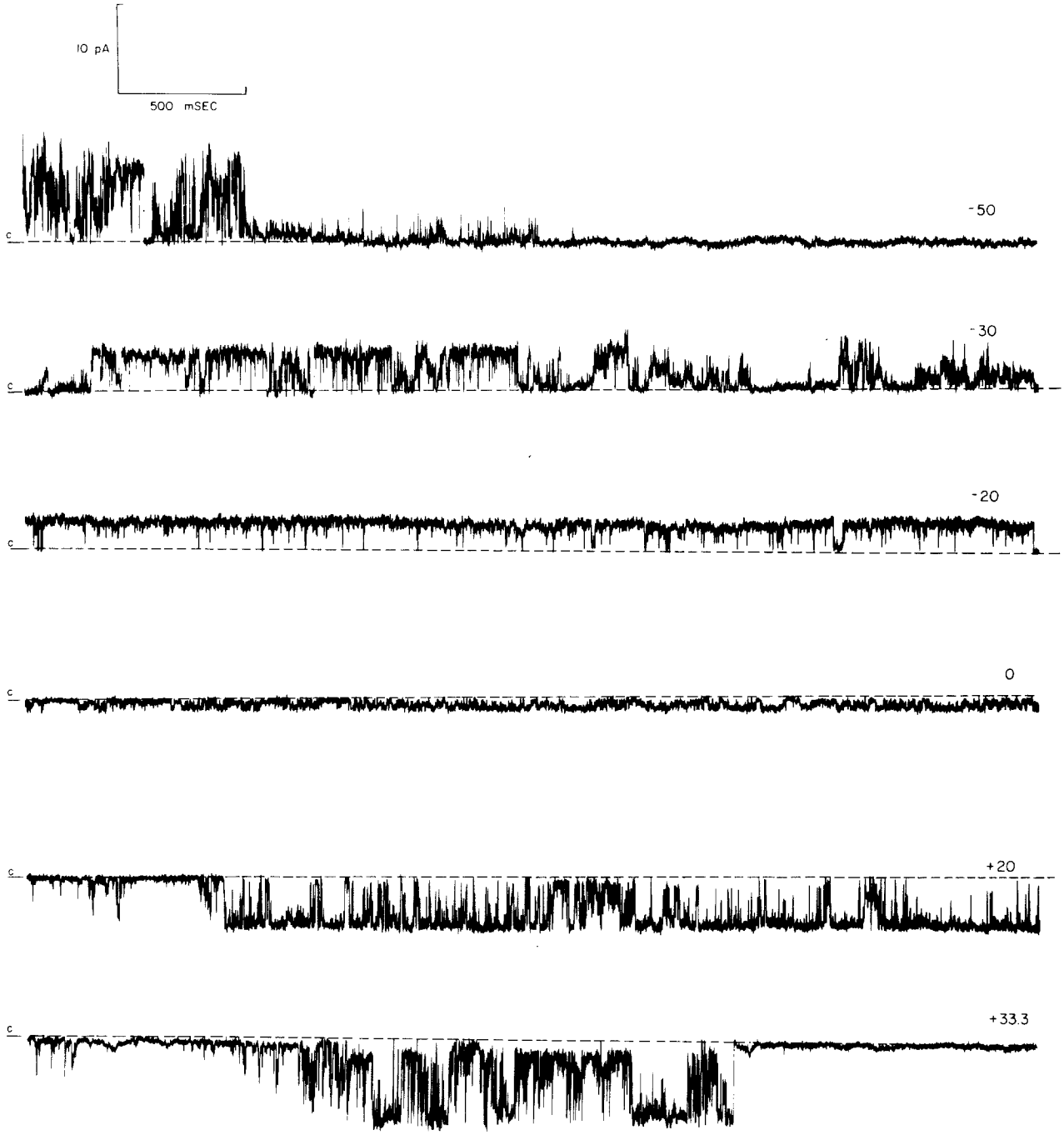

Fig. 1. Single-channel current recordings from the apical membrane of an A6 cell. This recording was made from an excised patch of membrane in the outside-out configuration (extracellular surface facing the bathing solution). Potentials are expressed as the potential at the intracellular surface of the membrane patch minus that in the bath. In each case, the membrane potential was returned to 0 mV before stepping to the desired command voltage. The patch electrode was filled with (mM): 35 NaCl, 35 Na_2SO_4, 25 HEPES and 91 sucrose. The bath solution contained (mM): 105 NaCl, 0.1 $CaCl_2$, 25 HEPES, 91 sucrose and 1 dibutyryl-cAMP. Upward deflections represent outward current. The closed-state current level is marked to the left of each recording with the letter "c"

tance, selectivity, and kinetics using a total of 13 membrane patches from cells whose age in culture ranged between 0 and 9 days. Its very large single-channel conductance and voltage-dependent gating properties gave it a characteristic signature. The anion channels were observed in membrane patches from confluent cells, from cells at the edge of a confluent area, and in freshly dissociated isolated

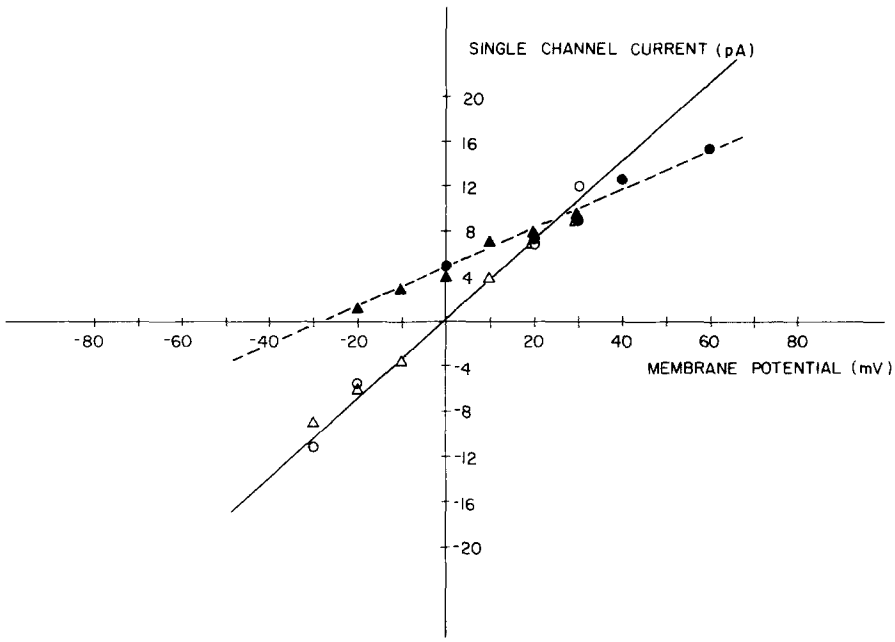

Fig. 2. Single-channel current-voltage curves from two excised inside-out (intracellular membrane surface facing the bathing solution) apical membrane patches. Initially both bath and pipette contained the normal bathing solution (105 mM NaCl). The bath additionally contained 8 mM dibutyryl-cAMP. The open symbols (○,△) represent current levels obtained under the initial symmetrical ionic conditions. The filled symbols (●,▲) plot currents obtained after the solution at the intracellular surface of the membrane patch was exchanged for one containing 21 mM NaCl. The solid and dashed lines represent linear regression analysis of the combined data points for the symmetrical and asymmetrical conditions, respectively

cells which had attached to the dish. Stable high resistance seals were difficult to form on cells in domes. In two instances where seals were satisfactory, no chloride channel activity was observed.

Our strategy in the experiments described below was to (1) determine the selectivity and magnitude of the anion conductive pathway, (2) to qualitatively describe the kinetic behavior of the channels in terms of voltage-dependent activation and inactivation which provides a kinetically identifiable fingerprint, and (3) to establish the pharmacology of channel inhibition.

Characteristic patch-clamp recordings of the channel at different membrane voltages are shown in Fig. 1. Here the patch was in an outside-out configuration and there was a gradient for Cl (3 : 1) but not for Na across the membrane. A number of characteristic features of the conductance pathway are evident in the tracings. First, single-unit conductance is quite large; the slope conductance measured in this experiment was 280 pS. The reversal potential was small (−5.7 mV) indicating a low selectivity for Cl over SO_4 and/or Na. Third, the channel switched rapidly between conducting and nonconducting states in the presence or absence of an applied potential. Finally, the mean channel open time, as well as the probability of being open, was greatly reduced at voltages of either polarity larger than 20 mV. Changes in potential to values greater than ±20 mV lead to channel inactivation in all 13 patches with a variable time course which was related to the magnitude of the command potential. In the experiment in Fig. 1 step voltage changes to −50 and +33.3 mV, respectively, produced only the one opening as illustrated. Reactivation of the channel from the inactivated state was brought about experimentally by returning to a holding potential of 0 mV. Note that at membrane potentials greater than or equal to ±30 mV channel opening and closure were not clear step functions. This could indicate passage through a number of subconductance levels or the existence of a fast flickering process producing slow rises or tail to the unitary events (*see* recordings at −50 and +33.3 in Fig. 1).

The ion selectivity of the channel was further defined in experiments illustrated in Fig. 2. *I-V* relationships were obtained in symmetrical NaCl solutions and in a 5 : 1 NaCl gradient. The reversal potential was close to zero in symmetrical solutions, as expected. In the presence of a gradient, there were currents through the channel at zero voltage in a direction indicating a net movement of Cl^- from pipette to bath. The reversal potential, obtained by linear regression, was −30 and −29 mV in the two experiments illustrated, implying that the permeability ratio for Cl/Na was about 9 : 1. For a perfectly selective Cl channel, the reversal potential would be −41 mV. The slope conductance with reduced NaCl in the bath was 165 pS.

The single-channel slope conductance and reversal potential under several different ionic conditions are given in the Table. Neither the conductance nor the reversal potential was greatly affected by substitution of TEA or choline for Na, by substituting 35 mM SO_4 for 70 mM Cl, or by changing the configuration of the patch. The single-channel conductance in three experiments with 105 mM Cl on both sides of the membrane was 359 ± 45 pS (mean ± SD).

While voltage-dependent inactivation was often

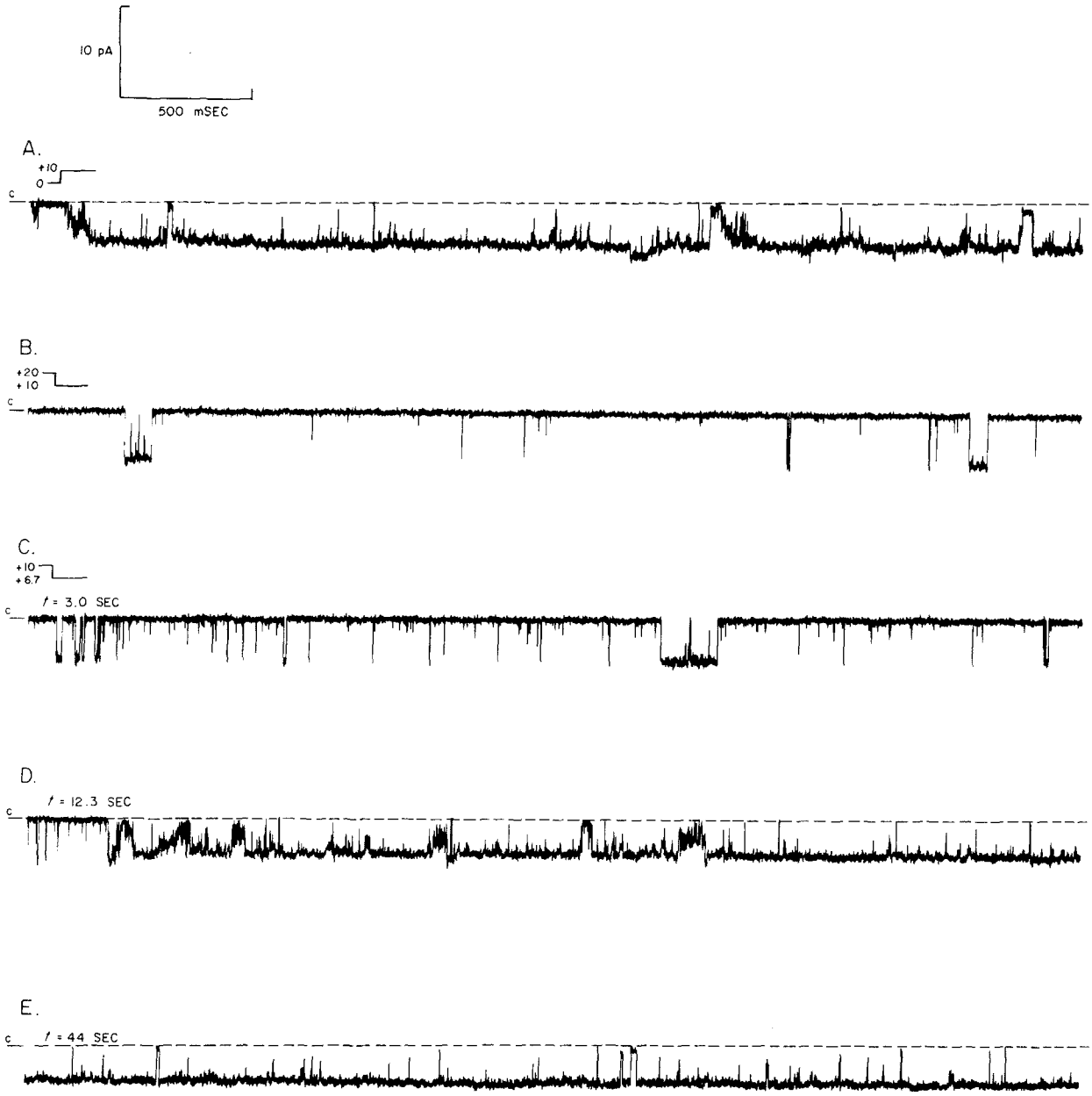

Fig. 3. Time course of return from inactivation. Recordings were taken from an outside-out excised apical membrane patch. The solutions in the pipette and bath were identical and contained (mM): 5 NaCl, 100 TEA chloride and 25 HEPES. The solution initially in the bath was the normal bath solution containing 1 mM dibutryl-cAMP and was changed to the TEA-chloride containing solution during the course of the experiment. The recording in *A* is a segment of record obtained after stepping to a command potential of +10 mV from 0 mV holding potential at which the channel was fully activated. *B*: The record was obtained approximately 10 sec after stepping to a command potential of +10 mV from a holding potential of +20 mV at which the channel was inactivated. The record remained stationary at this level of activation for approximately 1 min after which time the potential was decreased to +6.7 mV (*C* through *E*). Records *C* through *E* are discontinuous segments from the same potential record starting 3, 12.3 and 44 sec after the potential change was made to +6.7 mV. At 12.3 sec the channel was observed to undergo a state change, switching from a state where the channel remained closed, switching transiently to the open state, to one in which the channel remained continuously open and switched transiently to the closed state. In record *E* the transition to the fully activated state was complete

complete within a few seconds (Fig. 1), activation appeared to be a slower process varying in absolute time course from patch to patch. Figure 3 shows records from another outside-out patch at a potential of 10 mV coming from either the fully activated state (0 mV) or from the inactivated state (20 mV). The open state shows a strong memory of the previous state, being mostly open in the first

Table. Conductance and selectivity of the apical membrane chloride channel recorded from A6 epithelial cells in culture

Ionic conditions Inside/outside[a] (mM)		Conductance (pS)	Reversal potential (mV)	Patch type
105 NaCl	35 NaCl 35 Na_2SO_4	285	−8.6	Inside-out
35 NaCl	105 NaCl 35 Na_2SO_4	277	−5.7	Outside-out
105 Choline-Cl	105 NaCl	317	−1.5	Outside-out
—	105 Choline-Cl	290	+7.9	On-cell
—	105 NaCl	418	−1.3	On-cell
105 NaCl	105 NaCl	359 ± 45 (3)[b]	−1.7 ± 1.2 (3)	Inside-out
105 NaCl	21 NaCl	165(2)	−29.6 (2)	Inside-out
105 NaCl	55 Na_2SO_4	218	−9.5	Inside-out

[a] Inside and outside refer to the intracellular and extracellular surface of the membrane patch, respectively.

[b] Where a multiple number of experiments were performed for given experimental conditions values are reported as the mean ± standard deviation with the number of experiments in parentheses. Conductance and reversal potential were obtained from linear regression analysis of current-voltage plots.

case and mostly closed in the second (open probability = 0.02). The channel remained in this state for 60 sec, at which time the potential was further reduced to 6.7 mV. After a further delay of approximately 10 sec, the channel reactivated. As most epithelial transport systems operate in a steady state, the slow nature of the activation process does not preclude its playing a role in transport regulation.

A second aspect of the kinetics surrounding channel activation is illustrated in Fig. 4. Periods in which the channel is maximally open and maximally closed are interspersed with periods of rapid flickering between conductance states. This behavior was observed in nearly every patch (*see* Figs. 1, 3 and 4). These transitions may involve submaximal conductance levels, as suggested in the expanded time scale in Fig. 4*B*. In other cases, discrete subconductance states were not obvious, and transitions be-

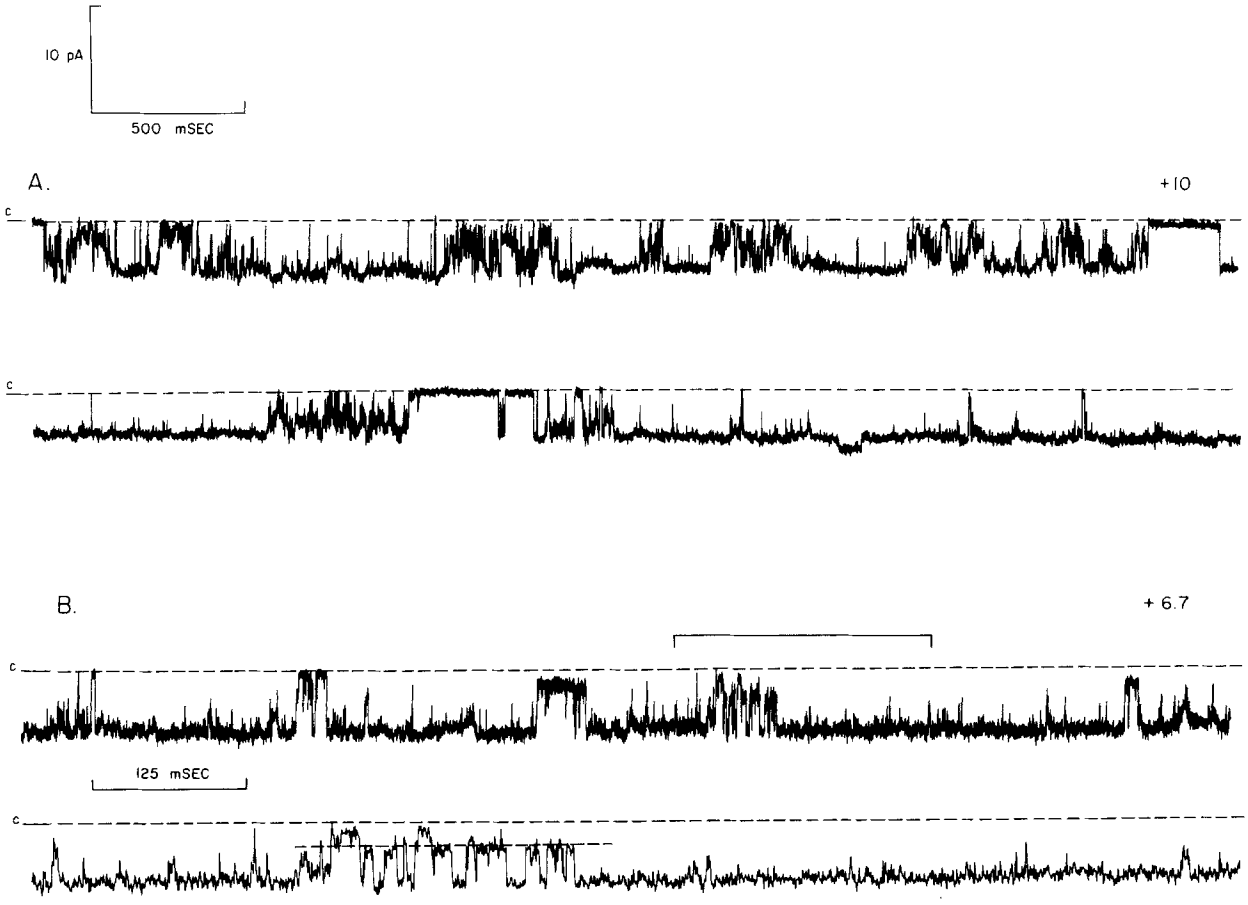

Fig. 4. Flickering of the channel to subconductance states. *A*: A continuous record from the same outside-out patch as depicted in Fig. 3. Note the presence of both fully open and flickering states which occur during a given channel opening. *B*: This record was obtained from an on-cell patch with (mM): 105 choline chloride, 10 HEPES, 0.1 EGTA and 60 sucrose in the pipette. The bottom trace in *B* is an expanded portion of the upper trace marked by the bar. Note the number of incomplete channel closures to a subconductance level, marked by the dashed line, which is significantly different from the zero conductance state. Although clearly seen in this record as discrete transitions to a conductance level approximately $\frac{1}{3}$ of the fully open-state, the kinetic process was frequently observed to be so rapid as to be unresolvable

tween fully open and fully closed conditions too rapid to be completely resolved with the limited bandwidth (1 kHz) may also account in part for the observed behavior.

As many anion transport systems are inhibited by disulfonic stilbene derivatives (Knauf & Rothstein, 1971; Russell & Boron, 1976; White & Miller, 1979) we investigated the effects of SITS on the epithelial anion channel. Figure 5 shows a record of a channel in an outside-out patch at a voltage of 6.7 mV. At this voltage the channel was observed to be continuously in the open state, closing only occasionally. After application of 1 mM SITS to the outside surface of the membrane by perfusion of the bath, the mean open time decreased markedly, as shown in the middle trace. As the perfusion was completed, the channel was open only in bursts of very rapid flickering, shown in the third and fourth traces, and finally became permanently inactivated. Similar irreversible channel inhibition was observed in both membrane patches in which SITS was applied. This rapid flickering process occurring throughout a given channel opening was never observed in the absence of SITS and may well be a consequence of open channel block, as with acetylcholine-induced single-channel currents measured in the presence of the local anesthetic QX222 (Neher & Steinbach, 1978; Neher, 1983). We were un-

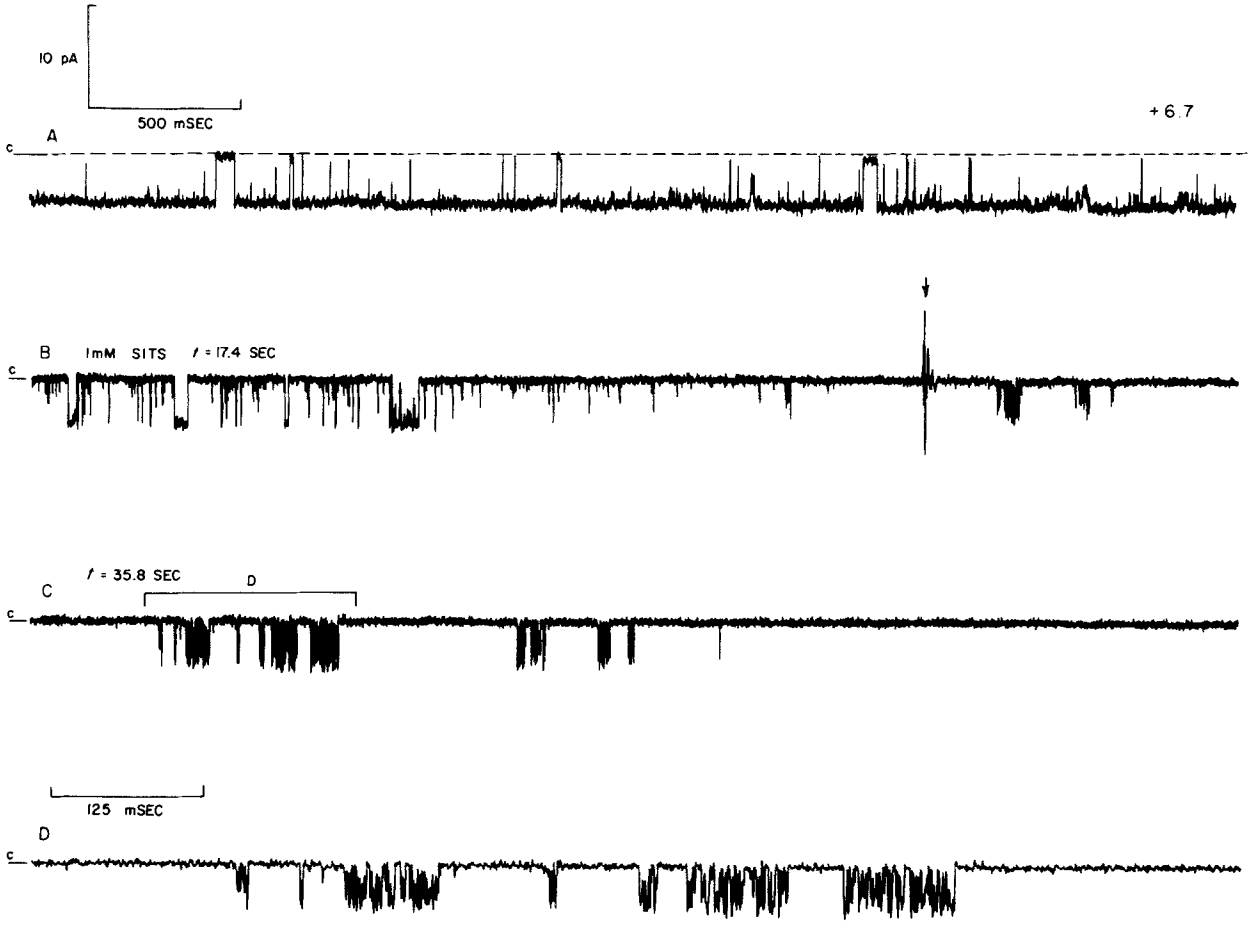

Fig. 5. SITS inhibition of single-channel currents. These current recordings were made from an excised outside-out patch where the solution in the pipette and bath contained (mM): 5 NaCl, 100 TEA chloride and 25 HEPES. *A*: Recording taken at a membrane potential of +6.7 mV where the channel was continuously open, interrupted by brief closures. *B*: This recording was made as the bath was initially being perfused with solution containing 1 mM SITS. The bath perfusion pipette was located within 100 μm of the patch pipette. The arrow marks the last of the interface bubbles between perfusion solutions. Channel inhibition can be seen to occur in stages. Note that in *B* the mean channel open time gradually decreases until the channel enters a very fast flickering state, as can be seen at the end of record *B*. Once these fast flickering events were observed, all subsequent channel openings exhibited this burst-like behavior. The recording in *C* was made directly prior to complete channel inhibition. Following the final event in this trace, no further channel openings were observed. *D*: This recording is of that portion of record *C* denoted by the bar displayed on expanded time scale in an attempt to resolve the fast flickering kinetics. This highly regular fast flickering occurring throughout a channel opening was never observed in the absence of SITS

able to reverse this inhibition upon removal of the SITS containing solution, perhaps due to inadequate removal of SITS from the bath or to the formation of a covalent adduct between the drug and the channel protein via the isothiocyanate group (Cabantchik & Rothstein, 1972; Rothstein, Cabantchik & Knauf, 1976).

Discussion

A6 cells as well as other epithelial cells in culture grow in oriented monolayers with the basolateral membranes facing the attachment substrate and the apical membranes facing the growth medium (*see* Handler, Perkins & Johnson, 1980). It was only the outer surface which was accessible to the patch pipette in the experiments described in this paper. Therefore, the channels described were, from the standpoint of geometry, located in the apical membrane. Although the channels we described were derived from what we define to be apical membrane patches, their distribution in the transporting epithelium may or may not be limited exclusively to the apical membrane. It should be noted that there is recent evidence to suggest that fully differentiated transport function, although not necessarily morphology, requires that cells be grown on permeable supports (Sariban-Sohraby et al., 1983). The distribution of membrane channels involved in membrane transport processes may be different under these growth conditions.

The anion selective channel found in A6 cells is certainly not unique to epithelia. Very similar channels have been observed in outer mitochondrial membranes (Schein et al., 1976; Columbini, 1978) and in cultured rat muscle (Blatz & Magleby, 1983). The similarities include the anion specificity, the open-channel conductance (360 pS in A6 cells, 430 pS in muscle and 450 pS in the mitochondrial channel under similar conditions) and inactivation as the membrane voltage is increased in either direction from zero. Both the voltage-dependent transitions shown in Fig. 1 and the slow return from inactivation shown in Fig. 4 were also described for the muscle channel. The noisy flickering state of the epithelial channel (Fig. 4) suggestive of submaximal conductance states was not reported by Blatz and Magleby (1983) although they did point out rapid jumps from the open towards the closed state and mentioned occasional current steps of less than unit magnitude. Another feature of this channel not previously reported for the large conductance anion channels is the inhibition by SITS. Cl channels from *Torpedo* electroplax also exhibit submaximal conductance levels and are sensitive to SITS; however, the open-channel conductances were observed to be 40-fold smaller than those reported here (White & Miller, 1979). The electroplax chloride channels are some 20-fold more permeable to Cl^- than K^+ and appear to be highly selective for Cl^- over all other anions tested (Miller & White, 1980). This is in sharp contrast to the permeability ratio for Cl^- over Na^+ of about 9 : 1 and the approximately twofold selectivity for Cl^- over SO_4^{-2} which was observed for the epithelial chloride channel.

It is conceivable that the channels which we have observed could be involved in the specialized function of transepithelial Cl transport. The Cl concentration in the epithelial cell water in toad bladder and frog skin is about 50 mM when the tissues are bathed with amphibian Ringer's solution on both sides (Macknight, 1977; Rick et al., 1978*a*,*b*; Nagel, Garcia-Diaz & Armstrong, 1981). The cell electrical potential, at least in the *Necturus* urinary bladder, is negative with respect to the mucosal medium at low transport rates but becomes close to zero or positive at high transport rates when the transepithelial potential exceeds 90 mV (Higgins, Gebler, & Frömter, 1977). Thus, the driving force for Cl across the apical membrane may be near zero or even outward at low transport rates, but when the transepithelial potential is high, both the electrical and the chemical driving forces for Cl should be inward. As pointed out by Macknight, DiBona and Leaf (1980), with high transepithelial potentials, the Cl permeability of the tissue can become rate-limiting for NaCl reabsorption. The Cl channels reported here activate at membrane voltages near zero, the condition that probably pertains when the driving force for Cl is inward, and when Cl transport is rate limiting.

While this reasoning is appealing as a teleological justification for a voltage-dependent apical membrane Cl channel, the quantitative contribution of such channels to overall salt transport in any epithelium under any conditions is unknown. However, Larsen and Kristensen (1978) and Larsen and Rasmussen (1982) have demonstrated a substantial Cl conductance in the apical membrane of toad skin that is voltage-dependent, being largest when the transepithelial potential is clamped to hyperpolarizing values (80 to 100 mV). The relationship between the large anion channel reported here and the Cl conductance of amphibian skin will have to be established. It will also be of interest to relate this channel to the cAMP-dependent apical Cl conductances which are thought to control fluid and electrolyte secretion in the corneal epithelium (Klyce & Wong, 1977) as well as other secretory epithelia (Frizzell, Field & Schultz, 1979) and are also found in *Necturus* gallbladder (Petersen & Reuss, 1983).

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