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# Protein Kinase A-regulated $Cl^-$ Channel in ML-1 Human Hematopoietic Myeloblasts

**B. Xu, L. Lu**

Department of Physiology and Biophysics, Wright State University School of Medicine, Dayton, Ohio 45435

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**Abstract.** Using the inside-out patch clamp technique, we identified a $Cl^-$ channel in patches from the membrane of cultured human hematopoietic myeloblastic leukemia ML-1 cells. The $Cl^-$ channel was not seen at negative membrane potentials in excised patches until the membrane potential was depolarized to greater than +40 mV. The channel was also activated by addition of cAMP-dependent protein kinase (PKA) catalytic subunit at physiological membrane potential (–40 mV). Biophysical studies of the $Cl^-$ channel revealed that the current-voltage (*I-V*) relationship of the $Cl^-$ channel was outwardly rectifying in symmetrical 142 mM $Cl^-$ solutions. Single channel conductances were 48 pS for the outward current measured at +60 mV and 27 pS for the inward current at –60 mV. The open time constant of the channel was dependent on the membrane potential and was significantly prolonged at positive membrane potentials. Channels activated by cAMP-dependent protein kinase spent a significantly longer time in the open state compared to those channels activated by depolarization pulses. Pharmacological properties of the $Cl^-$ channel were also studied. Two anion transport inhibitors, anthracene-9-carboxylic acid (9-AC) and 4,4-diisothiocyanatostilbene-2,2-disulfonic acid (DIDS) caused a flickering block of the channel. Half-inhibitory concentrations ($IC_{50}$) for 9-AC and DIDS were 174 ± 20 and 70 ± 16 μM, respectively. Blockade of the $Cl^-$ channel by 9-AC or DIDS was completely reversible. Our findings suggest that outwardly rectifying $Cl^-$ channels (ORCC) are present in human hematopoietic myeloblasts. The function of ORCC may be involved in hormone-regulated cell growth, cell volume regulation and immune responses.

**Key Words:** Patch clamp—Chloride channel—cAMP-dependent protein kinase—Human hematopoietic myeloblast

*Correspondence to:* L. Lu

## Introduction

Apical membrane $Cl^-$ channels are important in the regulation of salt and water transport in a variety of salt-secreting epithelia. In most epithelial cells, $Cl^-$ ions are taken up from the basolateral membrane of epithelial cells by a $Na^+/2Cl^-/K^+$ cotransport mechanism and secreted into the lumen through apical membrane $Cl^-$ channels (for review, *see* Welsh, 1987; Gogelein, 1988). Using patch clamp techniques, outwardly rectifying $Cl^-$ channels (ORCC) have been found in the apical membrane of epithelial cells. The activities of ORCC in excised patches were not found until after depolarizing the membrane potential to large positive voltages (e.g., over +40 mV) (Schoumacher et al., 1987). The channels can also be regulated by multiple intracellular pathways acting through the cAMP-dependent protein kinase A (PKA) phosphorylation and increased $Ca^{2+}$ concentration (Frizzell, Rechkemmer & Shoemaker, 1986; Li et al., 1988; Willumsen & Boucher, 1989). Furthermore, protein kinase C (PKC) can cause activation of ORCC when $Ca^{2+}$ concentration is low and inactivation when it is high (Hwang et al., 1989; Li et al., 1989).

Outwardly rectifying $Cl^-$ channels with conductances ranging from 20 to 400 pS have been characterized in various cell types. It has been proposed that ORCC play a crucial role in $Cl^-$ secretion and cell volume regulation (Frizzell et al., 1986; Bear, 1988; Gray et al., 1989; Garber, 1992; Vaca & Kunze, 1993). In the past several years, ORCC have received increasing attention since a defect in regulation of this channel is associated with human genetic disease cystic fibrosis (CF). Studies of airway epithelial cells and lymphocytes prepared from CF patients reveal a defect in the activation of an outwardly rectified $Cl^-$ channel by PKC and PKA (Li et al., 1988; Chen, Schulman & Gardner, 1989; Hwang et al., 1989). The cystic fibrosis transmembrane conductance regulator (CFTR), a product of the gene mutated in CF patients, is an epithelial anion channel that

may be involved in both secretion and reabsorption of $Cl^-$. The conductance of CFTR, however, is much smaller than that of ORCC (Berger et al., 1991). Recently, it has been reported that CFTR and ORCC are distinct proteins with a regulatory relationship because ORCC were present in CFTR (-/-) mouse nasal epithelial cells. In the patched membrane, the regulation of ORCC was intact from normal mouse nasal epithelial cells, but not CFTR (-/-) cells (Gabriel et al., 1993). The other evidence is that the defective regulation of ORCC in CF epithelial cells can be repaired by transfecting CFTR into the cell (Egan et al., 1992).

Human myeloblastic ML-1 cells can differentiate into granulocytes or macrophages upon specific stimulation (Craig et al., 1984). These cells play important roles in the immune defense system, and require membrane-mediated transduction of cell-cell and cell-environment signals. Many studies have implied that various ion channels are related to cell growth and function. In the present study, we have identified an ORCC in ML-1 cells by using the inside-out patch clamp technique. Our results provide new information about the characterization and distribution of ORCC. This will be a useful model to extend our understanding of the function of ORCC in cells of the immune system and to further study the relationship between ORCC and CFTR.

## Materials and Methods

### Cell Culture

ML-1 cells were received as a generous gift from Dr. R. W. Craig's laboratory (Dartmouth Medical School, NH). The cells were maintained in suspension culture in RPMI 1640 medium containing 25mM HEPES buffer (GIBCO, Grand Island, NY), essentially as described previously (Lu et al., 1993*b*). Briefly, the culture medium was supplemented with 7.5% heat-inactivated fetal bovine serum (FBS). ML-1 cells were grown in a humidified incubator with 5% $CO_2$ at 37°C and passed at a seeding density of $3 \times 10^5$/ml. Cells were washed twice with phosphate buffer solution (PBS) before they were transferred for patch clamp experiments.

### Reagents

The catalytic subunit of cAMP-dependent protein kinase (PKA C-subunit), $Mg^{2+}$ 5′-adenosine triphosphate ($Mg^{2+}$-ATP), dithiothreitol (DTT), anthracene-9-carboxylic acid (9-AC) and 4,4-diisothiocynatostilbene (DIDS) were purchased from Sigma Chemical (St. Louis, MO). The C-subunit of PKA was diluted in NaCl bath solution, and 1 mg/ml DTT was added to the mixture. The mixture was then allowed to stand for 10 min at room temperature (22°C) before use or being stored in a −80°C freezer. DIDS and 9-AC were prepared as stock solutions with a concentration of 100 mM in dimethylsulfoxide (DMSO). The concentrations of 9-AC and DIDS used in blocking experiments were 20–400 and 20–200 μM, respectively.

### Patch Clamp Studies

The inside-out patch clamp technique was utilized to study the single channel current in excised membranes from cultured ML-1 cells. Patch pipettes were made from microhematocrit capillary tubes (VWR Scientific, West Chester, PA) using a two-step puller (PP-83, Narishige Scientific Instrument Lab., Tokyo, Japan). After fire-polishing, the resistances of pipettes ranged from 4 to 8 MΩ when filled with 136 mM CsCl solution. The bath electrode was an Ag-AgCl wire connected to the bath solution via a NaCl-agar bridge. Single channel currents were recorded using an Axopatch 200A amplifier (Axon Instruments, Burlingame, CA). Junction potentials were compensated to 0 mV. Single channel data were filtered through a 4-pole low-pass filter at 1 or 2 kHz and digitized at 22 kHz by a pulse-code modulator (A.R. Vetter, Rebersburg, PA). Data for kinetic analysis of the channel blockade was recorded from a 2 kHz low-pass filter. Data were stored on video tapes using a video cassette recorder (JVC, Japan). The pCLAMP program (Axon Instruments, Foster City, CA) was used to analyze the single channel data. Data on video tapes were transferred to an IBM compatible 486 DX2-66 computer using the Fetchex* program. Analysis of the single channel kinetics were performed by using Fetchan* and pSTAT* programs. All experiments were done at room temperature (21–23°C). Data were presented as original values, or as means ± standard error/deviation (SE/SD) when it was indicated. The significant differences were determined by using paired *t*-tests.

### Solutions

The solutions used in these experiments were (1) NaCl bath solution containing (in mM): 137 NaCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 1 EGTA and 10 *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.2; (2) KCl bath solution containing (in mM): 137 KCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 1 EGTA and 10 HEPES, pH 7.2; (3) high $Cl^-$ bath solution containing (in mM): 295 NaCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 1 EGTA and 10 HEPES, pH 7.2; (4) pipette solution containing 136 CsCl, 2 $MgCl_2$, 1 $CaCl_2$ and 10 HEPES, pH = 7.4.

## Results

### Single Channel Recording from Excised Inside-Out Patches

Membrane patches excised from cultured ML-1 cells displayed no channel activity when they were held at negative membrane potentials. After the membrane potential was depolarized to +40 mV or greater, the channel activities were observed in 30% (37 out of 122) stable patches. Usually, the channel activities were elicited by continuously applying a depolarization pulse for several seconds to minutes. Once the channel was activated by the depolarization pulse, it remained in the activated state even at negative membrane potentials. Figure 1*A* shows single channel current traces recorded at different membrane potentials in the symmetrical concentration of $Cl^-$ ions. Channel kinetics were characterized as burst-like openings interrupted by very brief flickers and short-lived closed periods. The current-voltage (*I-V*) relationships of the $Cl^-$ current (Fig. 1*C*) showed an outwardly

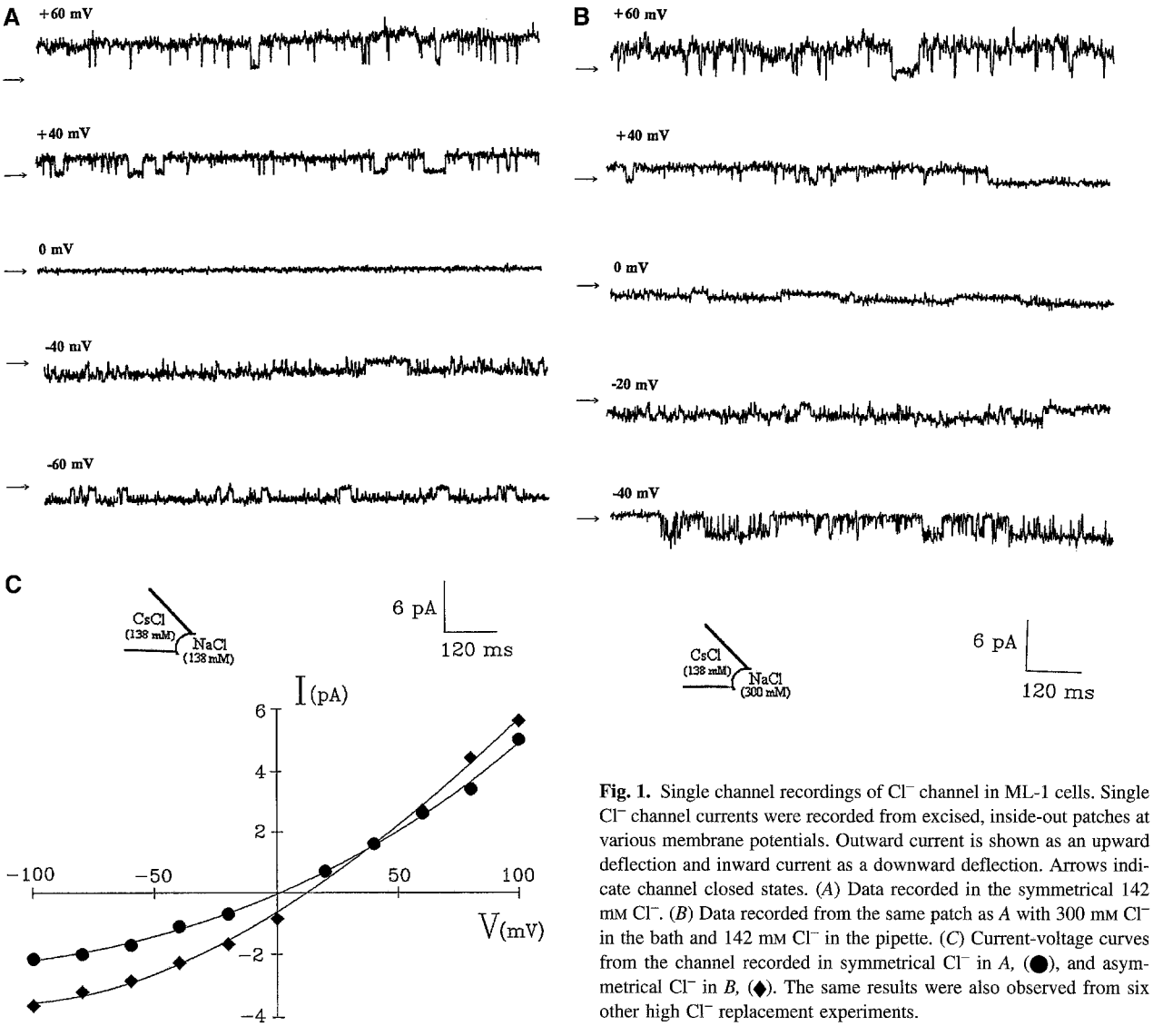



**Fig. 1.** Single channel recordings of $Cl^-$ channel in ML-1 cells. Single $Cl^-$ channel currents were recorded from excised, inside-out patches at various membrane potentials. Outward current is shown as an upward deflection and inward current as a downward deflection. Arrows indicate channel closed states. (*A*) Data recorded in the symmetrical 142 mM $Cl^-$. (*B*) Data recorded from the same patch as *A* with 300 mM $Cl^-$ in the bath and 142 mM $Cl^-$ in the pipette. (*C*) Current-voltage curves from the channel recorded in symmetrical $Cl^-$ in *A*, (●), and asymmetrical $Cl^-$ in *B*, (◆). The same results were also observed from six other high $Cl^-$ replacement experiments.

rectifying fashion in symmetrical $Cl^-$ solutions (142 mM $Cl^-$ on both sides of the membrane). Single channel conductances calculated from the slope pf the *I-V* curve were 48 pS (at +60 mV) in the outward direction and 27 pS (at −60 mV) in the inward direction. The outwardly rectifying current-voltage relationship made this channel easy to recognize in excised patches.

To confirm the ion selectivity of the channel, the bath solution was replaced by 300 mM $Cl^-$ solution (six independent experiments were performed). The current traces shown in Fig 1*B* were obtained in 300 mM $Cl^-$ bath solution. After high concentration, $Cl^-$ solution was perfused in the bath and the reversal potential was shifted from 0 to +13 mV (Fig. 1*C*). The value of the shifted reversal potential was close to the value that would be expected for the $Cl^-$ equilibrium potential estimated by the Nernst equation. The current-voltage relationship was not changed when NaCl bath solution was isotonically replaced by the same concentration of KCl (*data not shown*).

### Activation of ORCC in Excised Inside-Out Patches by cAMP-dependent Protein Kinase

Phosphorylation-mediated channel activation was examined by directly exposing inside-out patches to the phosphorylation mixture solution containing $Mg^{2+}$-ATP and PKA catalytic subunit. Since application of a depolarization pulse to patched membranes could cause activation of ORCC, experiments to study the effect of the phosphorylation on channel activation were performed in the physiological voltage range. Excised patches were held at −40 mV for 10 min to ensure the absence of channel activity; 1 mM $Mg^{2+}$-ATP was then added to the bath solution. No channel opening was detected during

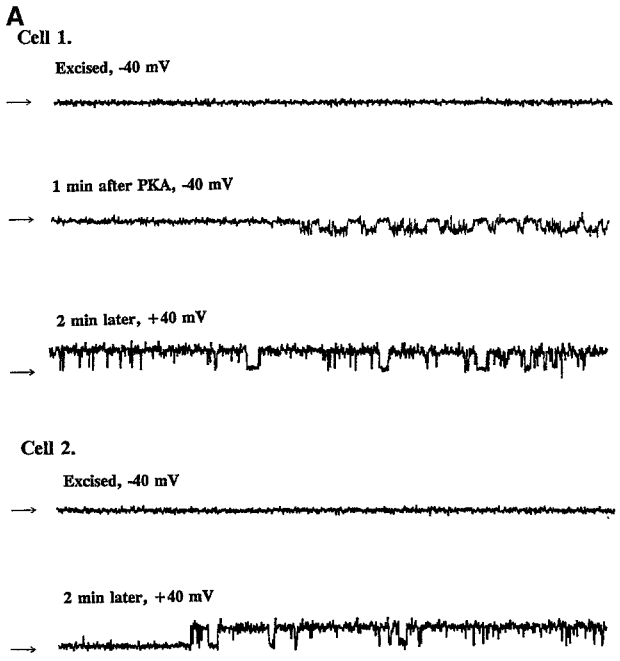
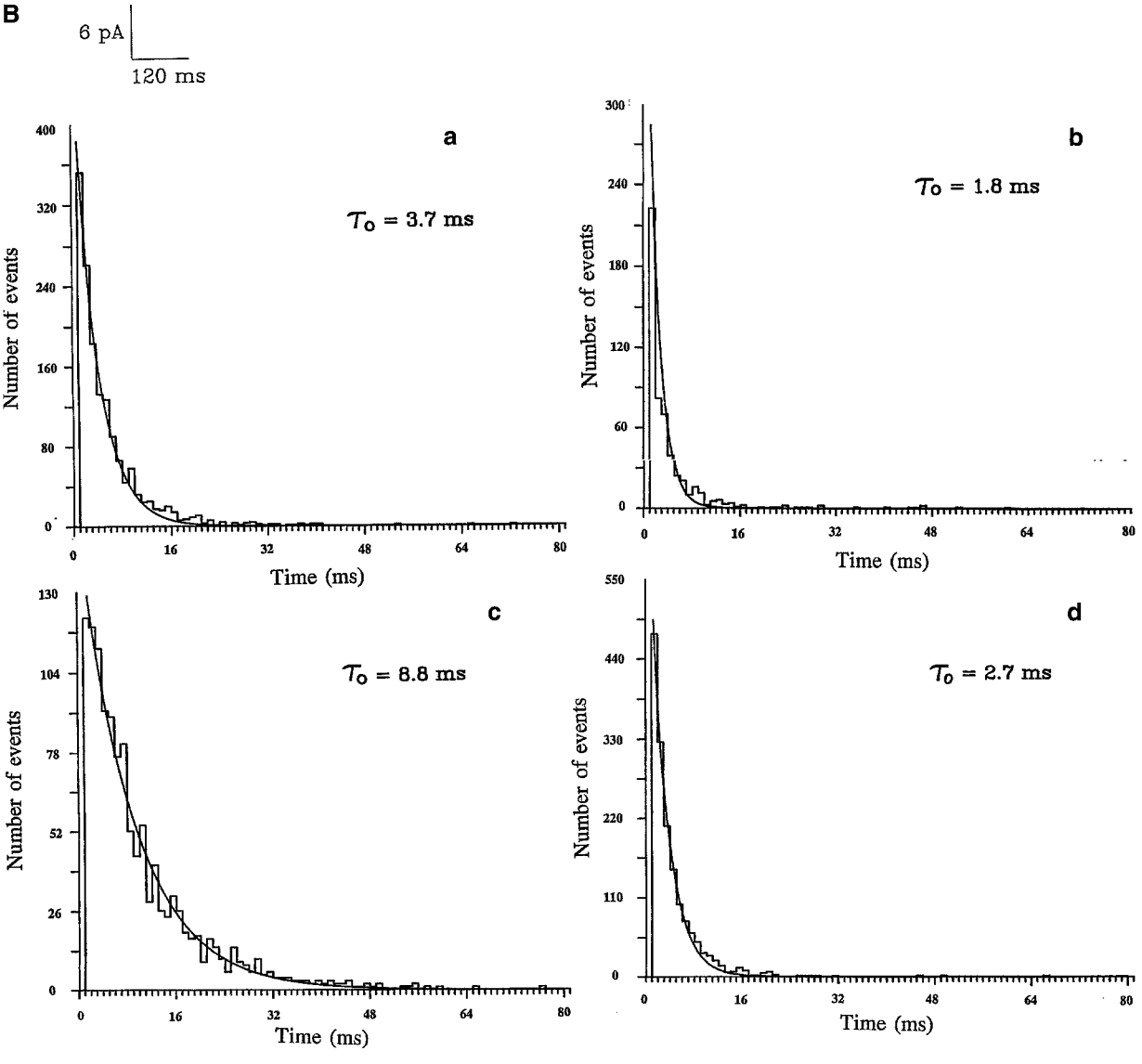



**Fig. 2.** Comparison of the activation of ORCC by cAMP-dependent protein kinase and by depolarization in ML-1 cells. (*A*) Activation of the $Cl^-$ channel in the excised inside-out patches before and after addition of catalytic subunit of PKA and $Mg^{2+}$-ATP in the bath at –40 mV; and activation of the $Cl^-$ channel by depolarizing the membrane potential to +40 mV. Arrows indicate the closed state of the channel. (*B*) Comparison of open time constants obtained from open time histograms between depolarization- (*a* and *b*, $n = 8$) and enzyme-activated (*c* and *d*, $n = 3$) channels at +40 mV (*a* and *c*) and –40 mV (*b* and *d*). (*C*) Voltage dependence of channel open time constants. Data were plotted as means ± SE. (●) Enzyme activated ($n = 3$) and (○) voltage activated ($n = 8$).



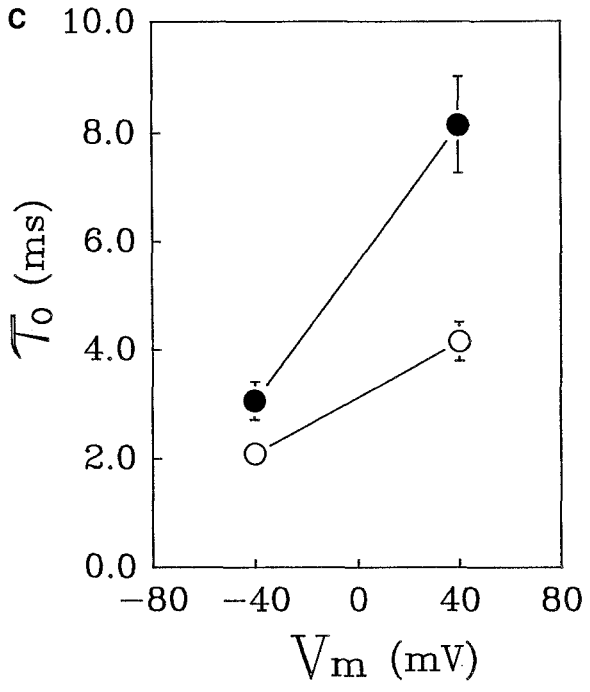



**Fig. 2.** Continued.

the first 5 min. The channel was activated by the subsequent addition of 50 nM PKA catalytic subunit to the bath (Fig. 2). After channel activity was observed, the membrane potential was switched to +40 mV to verify the outward rectification property of the channel. The only instance where phosphorylation had no effect was in two patches lacking a functional channel (no openings even at +80 mV). Usually, channel activations occurred within 3 min after treatment with PKA. The *I-V* relationship of enzyme-activated ORCC was identical to that obtained from the depolarization-activated channel (Fig. 1*C*).

The average lifetimes (open time constant, $\tau_o$) of channels activated by depolarization pulses and by PKA phosphorylation at membrane potentials of +40 mV were compared by using event-duration histograms (Fig. 2*B*). Open time constants obtained from enzyme-activated channels were significantly increased when comparing to those from channels activated by depolarization pulse at membrane potentials of −40 mV ($P < 0.01$) and +40 mV ($P < 0.005$). The values were 8.1 ± 0.8 msec ($n = 3$) *vs.* 4.1 ± 0.4 msec ($n = 8$) at +40 mV and 3.1 ± 0.3 msec ($n = 3$) *vs.* 2.0 ± 0.1 msec ($n = 8$) at −40 mV, respectively. Furthermore, open time constants of the channel showed a voltage-dependent behavior and the channel lifetime was much longer at positive membrane potentials than at negative membrane potentials ($P < 0.005$). The voltage dependence was exaggerated in enzyme-activated channels (Fig. 2*C*).

Subconductance states of ORCC in ML-1 cells were observed at both positive and negative membrane potentials (Fig. 3). Amplitude histograms constructed from 30 sec recordings show two peaks in the distribution corresponding to single channel conductances. The proportion of events in each peak in the histogram is the probability of finding the channel in each conductance state. Examples of subconductance states are demonstrated in the inserted traces in Fig. 3. The amount of subconductance states is not statistically different when comparing voltage- and PKA-activated channels (*data not shown*).

### Effects of 9-AC and DIDS on ORCC in ML-1 Cells

Inhibitory effects of $Cl^-$ transport inhibitors on ORCC have been reported in a variety of cell types. Application of 40 μM 9-AC to the cytoplasmic face of excised patches from ML-1 cells resulted in increased flickering of the channel open events which interrupted normal long-duration open states. The frequency of closures increased in parallel with increasing concentrations of 9-AC (Fig. 4*A*). The increased flickering of the channel opening caused by 9-AC led to a decrease in the open time constant ($\tau_o$, Table 1). The blocking effects of 9-AC on the channel were completely reversible after 3-5 min washing with normal bath solutions (*data not shown*). A concentration-response relationship of 9-AC blockade was obtained by plotting the mean values of fractional open time constants $\tau_{ob}/\tau_o$ *vs.* 9-AC concentration (Fig. 4*B*), where $\tau_{ob}$ represents open time constants obtained from duration-event histograms in the presence of various concentrations of 9-AC, and $\tau_o$ represents the open time constant obtained from control experiments at the same membrane potential. Data were fit with the Michaelis-Menten saturation function, and calculated half-maximal inhibitory concentration ($IC_{50}$) was 174 ± 20 μM ($n = 6$). The effect of the membrane potential on 9-AC blockade was examined over a voltage range of 20 to 100 mV (Fig. 4*C*). Our data suggest that 9-AC had a more potent blocking effect at more positive potentials ($P < 0.01$ when comparing the fractional block between +20 and +80 mV).

Since we did not observe the presence of channel inactivation states during long recording periods in membrane potentials ranging from −100 to +100 mV, results from experiments of 9-AC blockade suggested that the increase in the number of transitions within a burst period indicated blocking and unblocking states of the channel by 9-AC. We applied a simple linear model (shown below) for the kinetic analysis of channel blockade studies.

$$C \underset{\alpha}{\overset{\beta}{\rightleftarrows}} O \underset{K^{-1}}{\overset{K^{1}}{\rightleftarrows}} B$$

In this scheme, β is the reciprocal of the open time con-

stant ($1/\tau_o$), referred to the open rate constant; $\alpha$ is the reciprocal of the closed time constant ($1/\tau_c$), referred to the closed rate constant; $K^1$ is the on-rate constant of the blocker ($K_{on}$) and $K^{-1}$ is the off-rate constant of the blocker ($K_{off}$). To satisfy the closed-open-blocked linear model, the kinetic analysis has to meet several requirements: (i) only a single $\tau_o$ is found in the presence of the blocker; (ii) $\tau_o$ decreases with increasing blocker concentration, so that $1/\tau_o$ varies as a function of the blocker concentration with a slope corresponding to the on-rate constant ($K_{on}$) of the blocker; (iii) an additional exponential component is present in the closed time histograms corresponding to the blocked time constant ($\tau_b$); and (iv) $1/\tau_b$ is independent of blocker concentration (Venglarik et al., 1993).

Analysis of the open and closed time histograms after addition of 9-AC revealed a single open time constant and an extra exponential distribution in the closed time histogram. Data presented in Fig. 5*A* show that both closed and blocked rate constants were independent of 9-AC concentrations. However, open rate constants were increased following increases in 9-AC concentration (Fig. 5*B*). The slope calculated from the concentration-rate curve is $1.6 \times 10^6$ $M^{-1}sec^{-1}$ corresponding to on-rate constant of 9-AC blockade. Results of the kinetic analysis from 9-AC blockade are summarized in Table 1.

The other anion transport inhibitor, DIDS, also blocks ORCC in ML-1 cells. A time scale expansion method was used to demonstrate the inhibitory effect of DIDS on the channel opening (Fig. 6*A*). DIDS caused a dramatic increase in the frequency of current transitions between open and closed states. The $IC_{50}$ of DIDS blockade calculated by using the Michaelis-Menten equation is $70 \pm 16$ μM. The effects of DIDS blockade on ORCC in ML-1 cells can be fully reversed after 5 min washing out from the bath solution (Fig. 6*A*). Open and closed time histograms before and after addition of DIDS are shown in Fig. 6*B*. The channel open probabilities ($P_o$). are significantly reduced when 100 μM of DIDS is added to the intracellular side of patches. The open time constants are shortened by more than half after addition of 100 μM DIDS. Table 2 summarizes the results obtained from both control and 9-AC / DIDS blockade experiments. As would be expected, the closed time constants ($\tau_c$) of the channel remained unchanged after applications of 9-AC and DIDS to the patches.

## Discussion

Outwardly rectifying $Cl^-$ channels are widely distributed in epithelial and nonepithelial cells. They are thought to play a role in the mediation of transepithelial transport, cell volume regulation and signal transduction (Lukacs & Moczydlowski, 1990; Solc & Wine, 1991; Lu, Markakis & Guggino, 1993*a*). The present study demonstrates that ORCC are also present in human hematopoietic myeloid leukemia ML-1 cells. We used the excised, inside-out patch clamp technique to identify and characterize an outwardly rectifying $Cl^-$ channel in human ML-1 cells. The ORCC presented here share many similarities with those described in epithelial cells, endothelial cells, lymphocytes and fibroblasts (Bear, 1988; Halm et al., 1988; Garber, 1992; Vaca & Kunze, 1993). These channels, for example, are activated in inside-out patches by either depolarizing voltages or by cAMP-dependent protein kinases, display an outwardly rectifying current-voltage relationship, and their activity can be blocked by various derivatives of carboxylic acid and disulfonic stilbenes.

The regulation of ORCC is a complex process. It has been shown that ORCC can be activated by many physiological or nonphysiological maneuvers, such as depolarization, increasing temperature, swelling, elevating ionic strength or pH, trypsinization, raising cytoplasmic $Ca^{2+}$ concentration, as well as altering intracellular cAMP or cAMP-dependent protein kinase in both cell-attached and inside-out configurations (Frizzell et al., 1986; Schoumacher et al., 1987; Welsh, Li & McCann, 1989; Tabcharani & Hanrahan, 1991). We report here that ORCC in the excised membrane from ML-1 cells were activated by phosphorylation induced by addition of PKA C-subunit to the cytoplasmic side of the patches at the physiological membrane potential. When compared to voltage-activation, kinetic studies reveal that the enzyme-activated channel demonstrates prolonged channel lifetime (increase in average open time, $\tau_o$). Kinetic differences in enzyme-activated channels clearly increase the capacity for transmembrane $Cl^-$ movement at the resting membrane potential. These results seem reasonable since channels activated by depolarization have no physiological relevance in nonexcitable cells, while enzyme activation is physiologically significant. The mechanism of PKA activation is thought to be via an electrostatic interaction between the voltage sensor of the channel protein and the phosphate group transferred to the regulatory domain of the channel protein (Perozo & Bezanilla, 1990).

ORCC can be inhibited by numerous carbolic, sulfonic organic acids and arachidonic acid (Singh, Afink & Venglarik, 1991; Tilmann et al., 1991; Hwang, Guggino & Guggino, 1990). In addition, it has been reported that the channel can also be inhibited from both sides of the membrane by an ATP derivative, trinitrophenyl-ATP (TNP-ATP), and by high concentrations of HEPES (Hanrahan & Tabcharani, 1990; Venglarik et al., 1993). Our data indicate that both 9-AC and DIDS induced a concentration-dependent flickering-type block with $IC_{50}$ of $174 \pm 20$ and $70 \pm 16$ μM, respectively. The effects of 9-AC and DIDS blockade on ORCC in ML-1 cells are

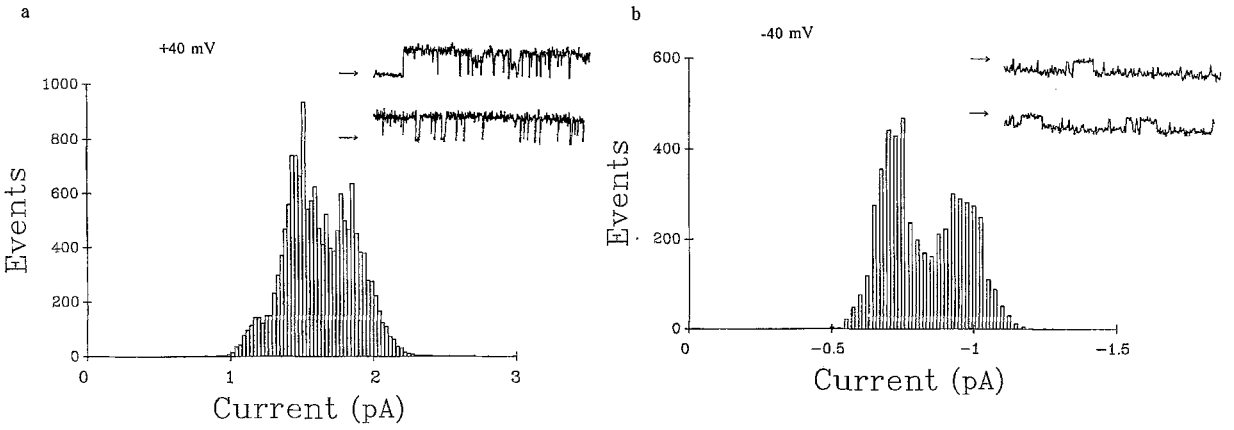



**Fig. 3.** Substates of the single channel conductance of ORCC in ML-1 cells. Amplitude histograms of depolarization-activated ORCC reveal substates of conductance at (*a*) +40 mV and (*b*) −40 mV. Single channel currents (inset) of the $Cl^-$ channel showed many subconductance states at different membrane potentials.

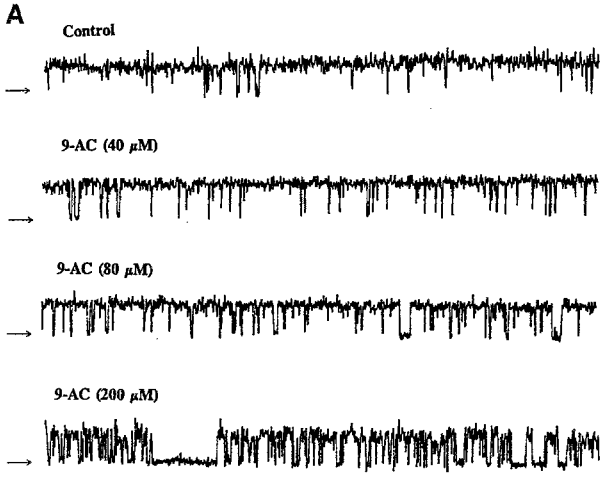
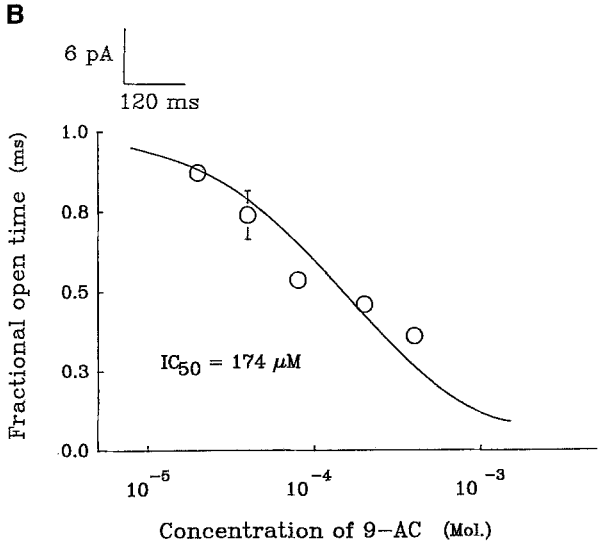



**Fig. 4.** Blocking effect of 9-AC on ORCC from excised inside-out patches at membrane potential of +60 mV. (*A*) Single channel current traces before and after addition of various concentrations of 9-AC. Arrows indicate the closed state of the channel (current traces shown were filtered at 1 kHz). (*B*) Fractional channel open time constant ($\tau_{ob}/\tau_o$) by 9-AC plotted as a function of the concentration of 9-AC in the bath. Data points were collected from 4–5 independent experiments and plotted as means ± SD. The half-inhibitory concentration ($IC_{50}$) of 9-AC is 174 ± 20 μM ($n = 5$). (*C*) Fractional block of channel open time constant ($1 - \tau_{ob}/\tau_o$) by 200 μM 9-AC plotted as a function of the membrane potential to demonstrate voltage dependence of block.



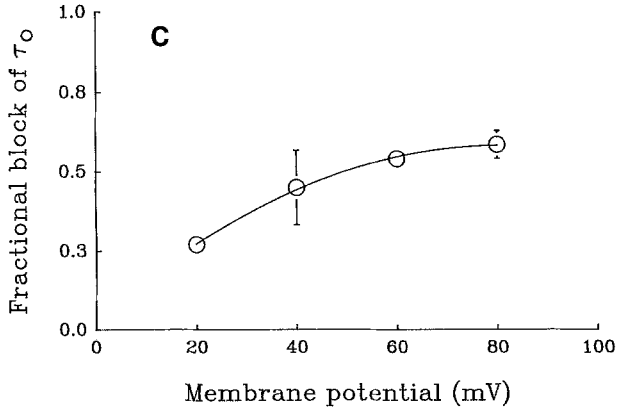



**Table 1.** Kinetic constants of 9-AC block of ORCC in ML-1 cells

| $K_i$ (μM) | $K_{on}$ ($M^{-1}$ $sec^{-1}$) | $K_{off}$ ($sec^{-1}$) | $K_D$ (μM) |
|---|---|---|---|
| 174 ± 20 | 1.6 × $10^6$ | 243 ± 54 | 165 ± 23 |

Data are presented as means ± SE from 4–6 independent experiments. $K_i$ was calculated by using the Michaelis-Menten function as described in Fig. 4*B*. $K_{on}$ is the slope of the dose-response curve in Fig. 5*B*. $K_{off}$ is the reciprocal block-time constant ($\tau_b^{-1}$). $K_D$ was calculated from the ratio of the off-rate and on-rate constants. All values were obtained at a holding potential of +60 mV.

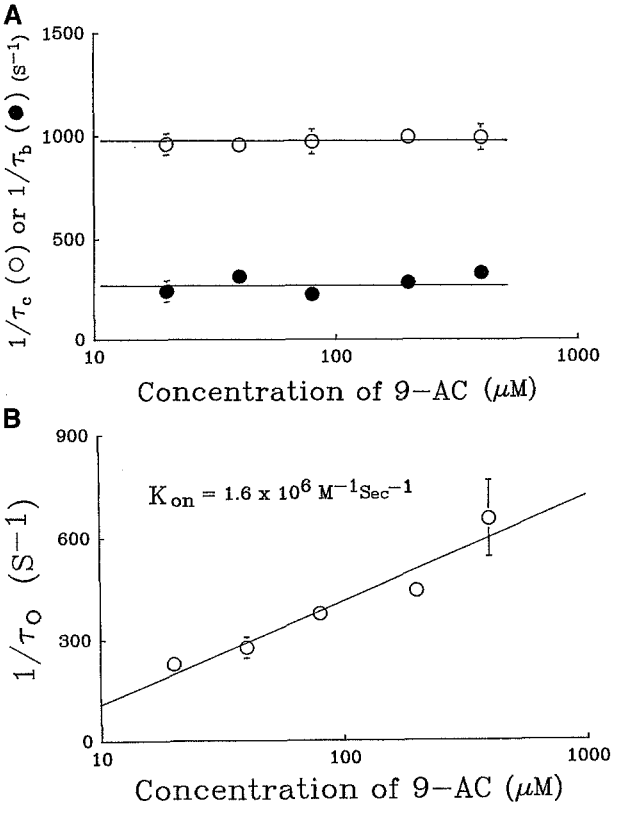



**Fig. 5.** Kinetic analysis of 9-AC blockade. (*A*) Reciprocal time constant of the closed ($\tau_c^{-1}$) or reciprocal time constant of the block ($\tau_b^{-1}$) plotted as a function of 9-AC concentration to show the dose independence of the closed-rate and the off-rate of the blocking. (*B*) Reciprocal time constant of the channel open ($\tau_o^{-1}$) plotted as a function of 9-AC concentration to demonstrate the dose dependence of the channel open-rate. The slope of the curve corresponding to the on-rate constant ($K_{on}$) was 1.6 × $10^6$ $M^{-1}$ $sec^{-1}$. All time constants used in the kinetic analysis were obtained from event-duration histograms similar to those shown in Fig. 2*B*.

completely reversible. This is consistent with previous studies which demonstrate that 9-AC caused a reversible blockade of $Cl^-$ channels (Lu, Markakis & Guggino, 1991). Blockade of DIDS is not a reversible process in most $Cl^-$ channels (Bridges et al., 1989; Paulmichl et al., 1992). The variation of DIDS blockade might result from structural differences on the mouth region or the region within the channel pore, which affect the depth of charged blockers entering the channel pore. The blockade of 9-AC showed a voltage-dependent characteristic and the possible mechanism for this effect has been described previously (Lu et al., 1991).

Kinetics analysis of 9-AC block revealed that the decrease of channel open time constant ($\tau_o$) is concentration dependent. The blocker did not increase the closed time constant ($\tau_c$), but produced an additional component corresponding to the blocked time constant ($\tau_b$). Both closed and blocked rates are independent of the 9-AC concentration. This suggests that there is no direct connection between the closed and blocked states. Therefore, the channel can only close from the open state, but not from the blocked state. Results from 9-AC blocking experiments are consistent with the proposed linear kinetic model in which 9-AC is only able to bind to an open channel. The equilibrium dissociation constant ($K_D$) calculated from the ratio of on-rate and off-rate constants was 165 ± 23 μM. This value is in general agreement with the $IC_{50}$ calculated from the Michaelis-Menten equation. Our data fully support the proposed model of the channel, and the model fitted with kinetic parameters is shown below. Open rate for channels activated by PKA-induced phosphorylation has been altered from 270 to 114 $sec^{-1}$, and the closed rate remains relatively constant compared to that of voltage activation. The relationship between the two activated states remains to be investigated.

$$C \underset{270\ sec^{-1}}{\overset{1{,}018\ sec^{-1}}{\rightleftarrows}} O \underset{243\ sec^{-1}}{\overset{1.6 \times 10^6\ M^{-1}\ sec^{-1}}{\rightleftarrows}} B$$

The physiological function of the outwardly rectifying $Cl^-$ channel appears to be complicated. In cystic fibrosis (CF) patients, evidence that ORCC in the apical membrane lack phosphorylation-dependent activation while containing normal conductive properties, suggests that one of the likely physiological roles of ORCC in epithelial cells is regulating fliud secretion. ORCC might also regulate the pH of the secretion since this channel is permeable to bicarbonate and cAMP-mediated agonists are able to stimulate bicarbonate secretion across normal, but not CF, airway epithelia (Tabcharani et al., 1989; Smith & Welsh, 1992). The other possible roles, such as cell volume regulation, maintenance of the resting membrane potential and cell proliferation have been proposed in epithelial and/or nonepithelial cells (Worrell et al., 1989; Bubien et al., 1990; Solc & Wine, 1991; Garber, 1992; Vaca & Kunze, 1993).

ML-1 cells proliferate in tissue culture media as immature myeloblasts. They can be induced to differentiate along the monocyte/macrophage pathway by treatment with phorbol ester, 12-O-tetradecanoylphorbol-13-

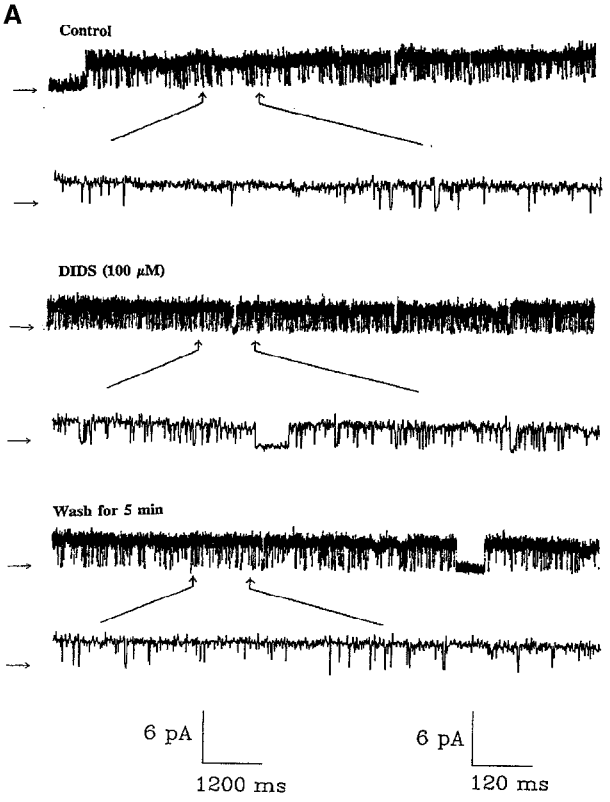



**Fig. 6.** Blocking effect of DIDS on ORCC from excised inside-out patches. (*A*) Current traces of the $Cl^-$ channel activity from the control, addition of 100 μM DIDS to the cytoplasmic side of the patch and after removal of DIDS. Top traces for each condition are at low time resolution (12 sec/trace) and indicated portions of the trace are shown below at higher time resolution (1,200 msec/trace). Arrows indicate closed states of the channel. (*B*) Open time and closed time histograms of the $Cl^-$ channel in the absence (*a* and *c*) and presence of 100 μM DIDS (*b* and *d*). Bin width was 1 msec and fit range is 1–80 msec. DIDS blocking produced an additional distribution in closed time histogram corresponding to the blocking time constant ($\tau_b$).

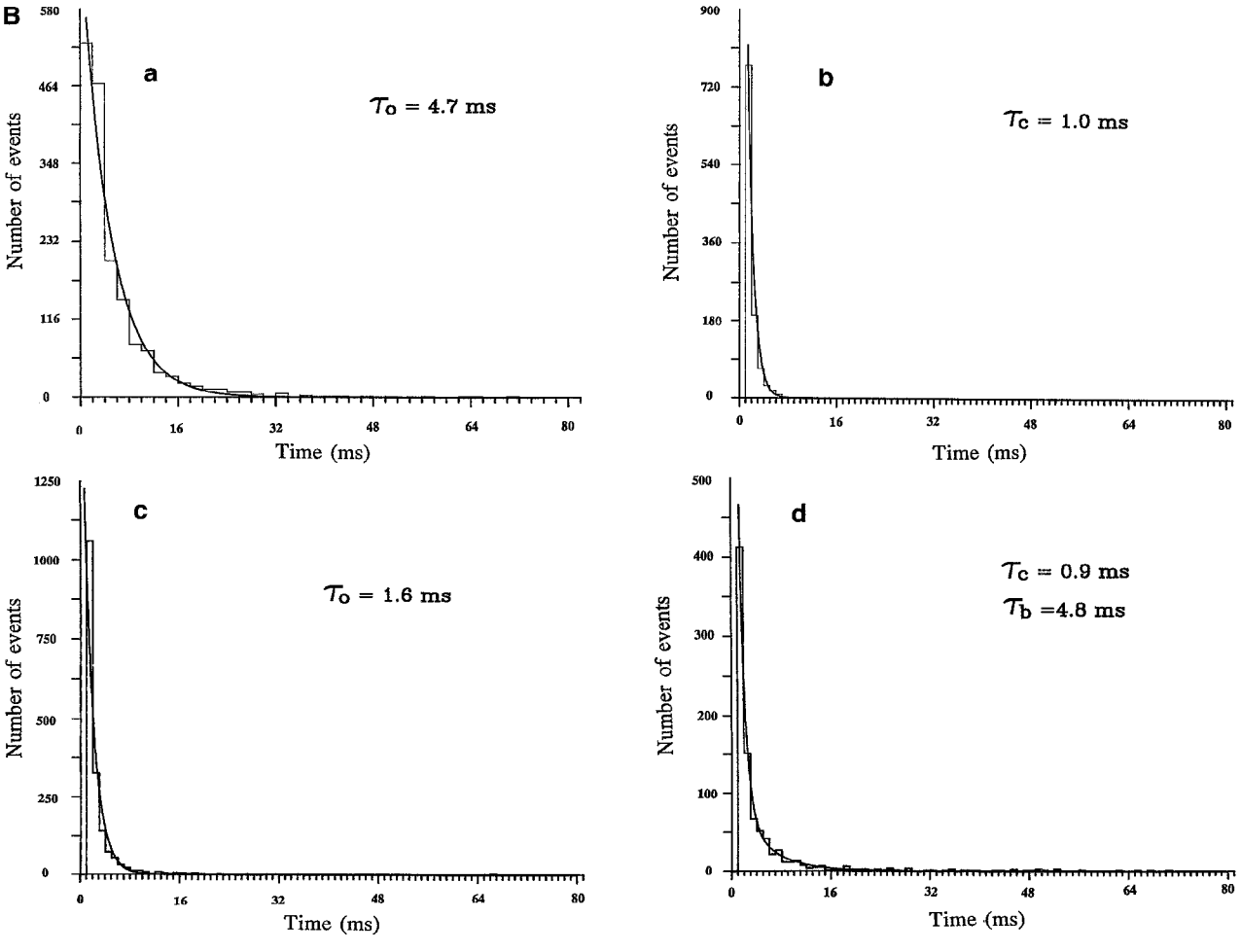


**Table 2.** ORCC open probability, open and closed time constants before and after addition of 9-AC or DIDS

| | $P_o$ (%) | $\tau_o$ (msec) | $\tau_c$ (msec) | $\tau_b$ (msec) |
|---|---|---|---|---|
| Control | 61.9 ± 7.1 | 5.6 ± 0.7 | 1.0 ± 0.1 | |
| 200 μM 9-AC | 46.6 ± 11.2 | 2.6 ± 0.1* | 1.0 ± 0.1 | 4.3 ± 0.9 |
| 100 μM DIDS | 41.3 ± 5.3* | 2.5 ± 0.4* | 1.3 ± 0.2 | 5.3 ± 0.7 |

Values are presented as means ± SE with experimental numbers. Holding potential was +60 mV. *Significant when compared to the control group at $P < 0.025$ level ($n = 4$–6).

acetate (Craig et al., 1984). Earlier studies have demonstrated that differentiation is associated with alterations in a voltage-gated $K^+$ channel. The activation of the $K^+$ current was suppressed and inactivation was accelerated during the early stages of differentiation, while the $K^+$ current was completely suppressed in the later stages (Lu et al., 1993*b*). Alterations in ion channel activity linked to cell differentiation are found in several other cells (Wieland, Chou & Chen, 1987; Mauro et al., 1993). Moreover, the expression of CFTR was dramatically increased in the differentiated human colon adenocarcinoma cells (HT29) accompanied by a large increase in second messenger-regulated and $Cl^-$ channel blocker-sensitive $Cl^-$ fluxes (Rafizadeh-Montrose, Guggino & Montrose, 1991), which supports a possible regulatory relationship between CFTR and ORCC (Egan et al., 1992; Gabriel et al., 1993). Since proliferation/differentiation of ML-1 cells can be regulated by some cytokines, such as interferon-gamma (INF-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF), the PKA-regulated ORCC in ML-1 cells may somehow play a role in the regulation of cell growth. Further studies will focus on examining CFTR expression in ML-1 cells before and after cell differentiation, the relationship between CFTR and ORCC as well as the possible roles of CFTR/ORCC in cell differentiation.

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