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ACCELERATED COMMUNICATION

Activation of a Heart Chloride Current During Stimulation of Protein Kinase C

KENNETH B. WALSH

Department of Pharmacology, University of South Carolina, School of Medicine, Columbia, South Carolina 29208

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SUMMARY

The whole-cell arrangement of the patch-clamp technique was used to examine the effect of protein kinase C (PKC) stimulation on ion channels in isolated guinea pig ventricular cells. In the presence of appropriate external solutions and drugs to reduce contamination from sodium, calcium, and potassium ion currents, application of phorbol 12-myristate 13-acetate or phorbol 12,13-dibutyrate, to stimulate PKC, activated a time-independent background current. The current-voltage relation for the PKC-activated current was linear over the voltage range of −90 to +60 mV. Alteration of the chloride equilibrium potential, brought about through changes in external and internal Cl^- concentrations, shifted the reversal potential for the background current in a manner expected for a Cl^--selective ion channel. The PKC-activated current was reversibly inhibited by the *S*-(−)-enantiomer of the monocarboxylic acid derivative 8-chlorophenoxyproprionic acid, at a concentration that did not affect Ca^{2+} or delayed rectifier K^+ currents. Dialysis of ventricular cells with partially purified PKC obtained from rat brain resulted in the activation of a large (>1-nA) time-independent background current after addition of external phorbol 12,13-dibutyrate. In the presence of the β-adrenergic receptor antagonist propranolol, norepinephrine activated a background current with properties similar to those of the PKC-sensitive current. It is concluded that cardiac ventricular cells contain PKC-activated Cl^- channels, which may be regulated during α-adrenergic stimulation.

Cl^- channels are found in a large variety of tissues, where they play important roles in controlling electrical excitability and in maintaining normal intracellular pH and cell volume (1). In the disease CF, Cl^- secretion by airway epithelium cells is severely impaired (2, 3). Recent electrophysiological experiments have indicated that the activity of Cl^- channels in normal but not CF epithelial cells is augmented by both PKA (4, 5) and PKC (6, 7). Thus, the major defect in CF may be related to an inability of membrane Cl^- channels to be regulated by cAMP-dependent and -independent protein kinases (8).

In analogy to the Cl^- channel found in airway epithelial cells, a β-adrenergic-sensitive Cl^- current has recently been identified in guinea pig ventricular cells (9–12). The assignment of this current as a Cl^- current was based on several observations. The reversal potential for this current was found to vary with changes in the external and internal Cl^- concentrations, as predicted by the Nernst equilibrium potential. The current was not blocked by barium or cesium, suggesting that it was not a K^+ current. Finally, the current could be reduced by addition of anthracene-9-carboxylic acid (12) and 4,4′-dinitrostilbene-2,2′-disulfonic acid (10, 11), which have been shown to block Cl^- channels in other tissues (13, 14). Of additional importance, this current could be activated during dialysis of the ventricular cell with the catalytic subunit of PKA (10), suggesting that phosphorylation might play a role in regulating this channel.

The purpose of this study was to determine whether heart Cl^- channels, like those found in epithelium cells, could be activated by PKC. Stimulation of PKC with low concentrations of the phorbol esters PMA and PDB activated a background current with ionic and pharmacological properties consistent with those of a Cl^- current. This current was strongly activated during dialysis of cells with partially purified PKC, indicating a functional role for PKC in regulating this channel.

Materials and Methods

Isolation of ventricular cells and recording procedures. An enzymatic dissociation procedure modified from the method of Mitra and Morad (15) was used to isolate the myocytes. Briefly, hearts were removed from adult guinea pigs, mounted on a Langendorf-type column, and perfused for 10 min with a Ca^{2+}-free Tyrode's solution containing collagenase (0.25–0.32 units/ml) (type B; Boehringer Mann-

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ABBREVIATIONS: CF, cystic fibrosis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CPPA, 8-chlorophenoxyproprionic acid; PKA, cAMP-dependent protein kinase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PDB, phorbol 12,13-dibutyrate.

heim Biochemicals) and protease (0.2 mg/ml) (type 14 or 25; Sigma Chemical Co.). After 20 min of perfusion with 0.2 mM Ca^{2+}-containing Tyrode's, the heart was dissected into small pieces and single cells were obtained by gentle agitation. Cells were stored at room temperature (22–25°) in normal Tyrode's solution (see below) and used between 1 and 10 hr after isolation.

The patch-clamp method of Hamill *et al.* (16) was used to record whole-cell ventricular currents using a Warner PC-501 amplifier (Warner Instrument Corp.). Pipettes were made from Gold Seal Accu-fill 90 Micropets (Clay Adams Inc.) and had resistances of 1–3 M-Ω when filled with KCl/glutamate internal solution. Series resistance compensation (1–2 M-Ω) was used throughout the experiments. Data were sampled at 10 kHz, filtered at 2–5 kHz with a low-pass Bessel filter, and stored using a Softek 386 computer.

Measurement of PKC-sensitive current. Isolated cells were initially placed in a normal Tyrode's solution consisting of (in mM) 132 NaCl, 5 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 5 dextrose, and 5 HEPES, pH 7.4. After establishment of the whole-cell voltage clamp, the solution was changed to a K^+-free external solution containing 140 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM dextrose, 200–500 nM nisoldipine, and 10–50 μM tetrodotoxin, pH 7.4. In some experiments, 1 mM $BaCl_2$ was added to the external solution. Because activation of the β-adrenergic-sensitive Cl^- current appears to be dependent on external Na^+ (11, 12), this cation was present in all experiments. A 10 mM external Cl^- solution was made by substituting sodium aspartate for NaCl. Patch electrodes were filled with an internal solution containing (in mM) 50 KCl, 60 potassium glutamate, 2 $MgCl_2$, 0.1 $CaCl_2$, 11 EGTA, 5 ATP (K^+ salt), 10 HEPES, and 25 KOH, pH 7.3. This ratio of EGTA/$CaCl_2$ sets the free intracellular Ca^{2+} concentration at approximately 1 nM (17). Use of this solution allowed measurement of the background current and the delayed rectifier K^+ current (I_K) (using a 2–3-sec voltage step to +50 mV) in the same cell. Because I_K is augmented by PKC (18, 19), measurement of this current was useful in monitoring cellular PKC responsiveness to phorbol esters. Internal [Cl^-] was varied by adjusting the amounts of KCl and potassium glutamate. In some experiments, CsCl/glutamate was substituted for KCl/glutamate.

Ion currents were recorded during 40-msec voltage steps applied to −90 through +60 mV from a holding potential of −40 mV. Use of this voltage protocol, along with the external and internal solutions described above, greatly reduces or completely eliminates ion currents that arise from Na^+ channels (tetrodotoxin and a holding potential of −40 mV), Ca^{2+} channels (nisoldipine and a holding potential of −40 mV), inward rectifier (no external K^+, 1 mM external $BaCl_2$), delayed rectifier (little activation during 40-msec voltage pulse at room temperature), and ATP-sensitive (5 mM internal ATP) K^+ channels, Na^+/K^+ pump (no external K^+), and Na^+/Ca^{2+} exchanger (no internal Na^+, 1 nM internal Ca^{2+}). The reversal potential (E_{rev}) was defined as the potential at which the PKC-sensitive current was zero. In those cases in which the exact zero current was not recorded, the E_{rev} was determined by fitting a straight line through the points on the *I-V* curve directly above and below this potential.

Preparation of drugs. The phorbol esters PMA, PDB, and 4β-phorbol (Sigma) were prepared as 1 mM stock solutions in dimethyl sulfoxide (100%) and were used at a concentration of 10–50 nM. These concentrations fall into the range of concentrations shown to be required for phorbol esters to stimulate PKC in isolated myocytes (20). Dimethyl sulfoxide (0.01%), when used alone, produced no effect on ion currents measured in the −90 to +60 mV range. The *S*-(−)-enantiomer of CPPA was a generous gift of Dr. Shirley H. Bryant (Department of Pharmacology and Cell Biophysics, University of Cincinnati) and was prepared as a 50 mM stock in external solution.

Dialysis of PKC. A mixed isozyme preparation of PKC, partially purified from rat brain, was generously supplied by Ms. Muriel C. Maurer and Dr. Julianne J. Sando (Department of Pharmacology, University of Virginia). The PKC preparation was purified as described previously (21, 22), using DE-52 and threonine-Sepharose chromatography. For internal cellular dialysis, a solution of the enzyme was included in the internal pipette solution. After disruption of the cell membrane, PKC moves from the pipette into the cell by diffusion. Based on theoretical studies (23, 24), a large molecular weight substance such as PKC (molecular mass, approximately 80,000 Da) should reach equilibrium at a slow rate (τ = 10–15 min), given the size of the cells and electrodes used in this study. This time course closely matches experimental rates determined for augmentation of I_K in these cells by PKC.[1] Thus, use of this procedure allowed adequate time for the measurement of control and PKC-sensitive currents.

Results

Fig. 1, *left*, shows whole-cell currents obtained during 40-msec voltage steps from a holding potential of −40 mV to −60 mV and +60 mV, in the presence and absence of the phorbol ester PMA. The experiment was conducted in a K^+-free external solution containing tetrodotoxin (50 μM) and nisoldipine (400 nM), with a $[Cl^-]_o$ of 146 mM. The dialyzing pipette contained a K^+-glutamate/Cl^- internal solution with a $[Cl^-]_i$ of 54 mM. After application of 20 nM PMA, the outward current recorded at +60 mV was found to double in size. At −60 mV, PMA induced a small inward current that was not present under control conditions. This PKC-sensitive background current could also be activated when internal K^+ was substituted with Cs^+ and the external solution contained 1 mM $BaCl_2$.

Fig. 1, *right*, shows the current-voltage (I-V) relation for the PKC-sensitive current recorded over the voltage range of −90 to +60 mV. The amplitude of the current was obtained by subtracting the control records from the records obtained in the presence of PMA at each potential. The resulting *I-V* was linear and E_{rev} was −22 mV. Overall, in 10 cells, the PKC-activated current reversed at −24 ± 4 mV (mean ± standard error) under these conditions ($[Cl^-]_o$ = 146 mM, $[Cl^-]_i$ = 54 mM). This value is consistent with the theoretical Nernst equilibrium potential of −25 mV for a Cl^--selective current (E_{Cl}), suggesting that the PKC-activated current may arise from the activity of membrane Cl^- channels.

This same background current could also be activated with the phorbol ester PDB. However, it was not present after exposure of cells to 4β-phorbol, a PMA analogue that does not stimulate PKC (20). The background current was present in the majority of tested cells (60–70%) but varied greatly in size, ranging in amplitude from 56 to 471 pA at +60 mV (mean ± standard error = 275 ± 35 pA). Activation of the current occurred within 2 to 5 min after application of PMA or PDB to the bath and was reversible after prolonged periods (10–15 min) of washout.

One important prediction for a current proposed to arise from Cl^--selective channels is that changes in internal and external concentrations of Cl^- should shift the E_{rev} for the current according to E_{Cl}. With 126 mM $[Cl^-]_i$, the E_{rev} of the background current was found to increase to −6 ± 3 mV (n = 4) (E_{Cl} = −4 mV). Reduction of $[Cl^-]_i$ to 10 mM decreased E_{rev} to −58 ± 2 mV (n = 2) (E_{Cl} = −68 mV). Fig. 2 shows the *I-V* relation for the PKC-sensitive current first recorded in external solution containing 10 mM $[Cl^-]_o$ (E_{rev} = +30 mV, E_{Cl} = +43 mV). Increasing the $[Cl^-]_o$ to 146 mM, without any change in the external cation concentration, caused the E_{rev} for the current to shift by −65 mV (E_{rev} = −35 mV, E_{Cl} = −25 mV). This change in $[Cl^-]_o$ did not alter the linearity of the *I-V* plot (Fig. 2).

[1] K. B. Walsh, unpublished results.

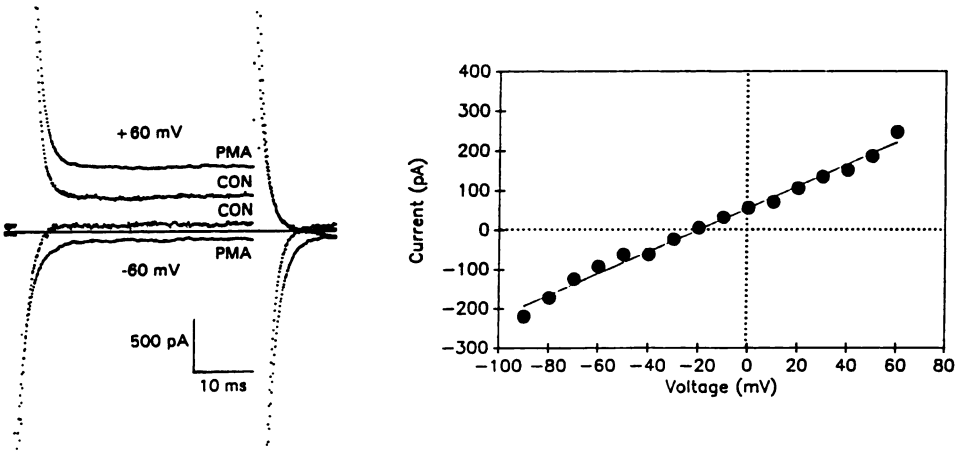

Fig. 1. Activation of background current by phorbol esters. *Left*, currents recorded during 40-msec voltage steps to −60 mV and +60 mV, in the presence and absence of 20 nM PMA. *CON*, control. *Right*, *I-V* relation for PKC-sensitive current (146 mM external Cl^-/54 mM internal Cl^-, E_{Cl} = −25 mV). A straight line was fit to the data points to emphasize the linearity of the *I-V* relation. Temperature = 22°. Cell A35.

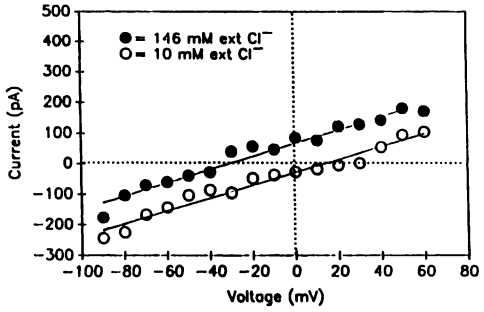

Fig. 2. Changes in external [Cl^-] shift the reversal potential for the PKC-sensitive background current. *I-V* relation for PKC-sensitive current recorded in 146 mM and 10 mM external Cl^- (146 mM external Cl^-/54 mM internal Cl^-, E_{Cl} = −25 mV; 10 mM external Cl^-/54 mM internal Cl^-, E_{Cl} = +43 mV). Straight lines were fit to the *I-V* relations. Temperature = 24°. Cell A20. Changes in $[Cl^-]_o$ produced only small changes (<2 mV) in the liquid junction potential.

Various aromatic monocarboxylic acids have been shown to be effective in reducing Cl^- channel activity. One such compound, CPPA, substantially inhibits skeletal muscle Cl^- channels (25). Fig. 3 shows the effect of the *S*-(−)-enantiomer of CPPA (1 mM) on the PKC-sensitive background current recorded at −60 and +60 mV. In three experiments, CPPA reduced the PKC-sensitive current by 61 ± 4% at −60 mV and by 53 ± 8% at +60 mV. Inhibition by CPPA was partially reversible in each of these experiments. Although CPPA reduced the PKC-sensitive background current, it did not alter E_{rev} for the current (Fig. 3, *right*). In separate experiments, 1 mM CPPA produced no reduction in the amplitudes of I_{Ca} and I_K.

The results suggest that PMA and PDB activate a heart Cl^- current. In order to determine whether this activation results specifically from a stimulation of PKC, partially purified PKC was dialyzed into the ventricular cells and the background current was recorded. Fig. 4 shows the *I-V* relation for the PKC-sensitive current obtained 15 min after establishment of the whole-cell configuration. With exogenous PKC in the cell, PDB activated large inward and outward background currents. The increase in outward current obtained at +60 mV (1.4 nA) was >5-fold larger than the average current obtained at this potential in the absence of exogenous PKC (see above). The E_{rev} of −28 mV for this current was close to E_{Cl} (−23 mV) and, despite the large amount of current activated, the *I-V* relationship remained fairly linear. Similar results were obtained in two other experiments.

Because α-adrenergic stimulation facilitates PKC translocation in cardiac myocytes (26), the ability of norepinephrine to activate the background current, via the α_1-adrenergic receptor, was determined. Fig. 5 shows the effect of 10 μM norepinephrine on membrane currents recorded in the presence of the β-adrenergic receptor antagonist propranolol (1 μM). As was the case with phorbol esters and PKC, norepinephrine activated a time-independent background current that displayed a linear *I-V* relation and a E_{rev} (−21 mV) close to E_{Cl} (−25 mV) (Fig. 5). In three experiments, norepinephrine increased the outward current measured at +60 mV by 324 ± 78 pA. This augmenta-

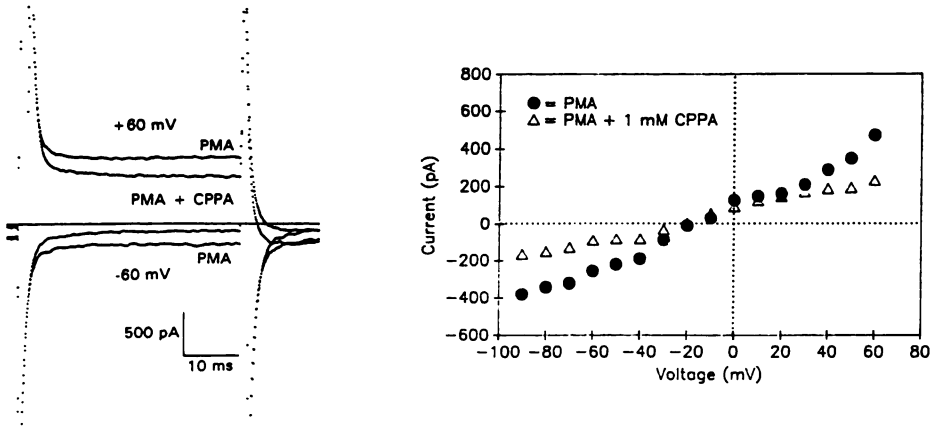

Fig. 3. Reduction of PKC-sensitive background current by monocarboxylic acid derivative. *Left*, currents recorded during 40-msec voltage steps to −60 and +60 mV, with 50 nM PMA and PMA plus 1 mM *S*-(−)-enantiomer of CPPA. *Right*, *I-V* relation for PKC-sensitive current in the presence and absence of CPPA (146 mM external Cl^-/54 mM internal Cl^-, E_{Cl} = −25 mV). Temperature = 24°. Cell A90.

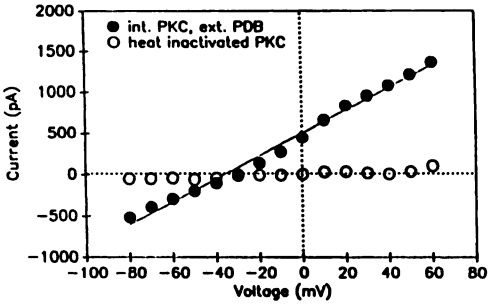

Fig. 4. Activation of background current by partially purified PKC. ●, *I-V* relation for background current in the presence of PDB during internal dialysis with PKC (activity = 0.4 nmol of P_i/min/ml). PKC-sensitive current was determined by subtracting currents recorded during the first minute after breakthrough of the pipette into the cell from currents recorded 15 min later. PDB was added to the bath 10 min after breakthrough (148 mM external Cl^-/60 mM internal Cl^-, E_{Cl} = −23 mV). A straight line was fit to the *I-V* relation. Temperature = 27°. Cell A130. ○, Membrane currents recorded in another cell of comparable size after 30 min of dialysis with heat-inactivated PKC. Temperature = 24°. Cell A151.

tion was not statistically different ($p > 0.5$, using Student's *t* test) from the increase in current obtained with phorbol esters at the same voltage.

Discussion

The results of this study indicate that stimulation of PKC with phorbol ester compounds activates a time-independent background current, in guinea pig ventricular cells, with properties expected for a current arising from the opening of membrane Cl^- channels. Previous studies have shown that stimulation of PKC augments the delayed rectifier K^+ current (18, 19) but is without effect on Ca^{2+} (19, 27) and inward rectifier K^+ (27) currents in these cells. However, phorbol esters do increase Ca^{2+} channel activity in neonatal rat myocytes (28, 29).

A β-adrenergic-activated Cl^- current has recently been identified in heart ventricular cells (9–12). The measurement of a 13-pS conductance single channel, which may be responsible for this current, has also been reported (30). Activation of this channel is mediated through PKA. As is the case for the PKC-sensitive current reported in this study, the PKA-sensitive current does not appear to be present in the absence of kinase stimulation and is not dependent on internal Ca^{2+} for activation. This current is sensitive to changes in external and internal [Cl^-] and can be inhibited by Cl^- channel-blocking agents such as anthracene-9-carboxylic acid (12) and 4,4′-dinitrostilbene-2,2′-disulfonic acid (10, 11).

However, there was one important difference found to exist between the β-adrenergic-activated current and the PKC-sensitive current. In the presence of nonsymetrical concentrations of internal and external Cl^-, as used in the present experiments, the β-adrenergic-sensitive current displays strong outward rectification (9, 10, 30). In the majority of experiments, the PKC-sensitive current *I-V* relation was found to be linear over the voltage range of −90 to +60 mV. It is unlikely, based on the sensitivity of the PKC-sensitive current to changes in the Cl^- gradient, that the linearity results from the permeability of this channel to an internal anion, such as glutamate, during activation by PKC. A more likely possibility is that PKA and PKC activate different types of Cl^- channels. Numerous types of Cl^- channels are present in epithelial cells (8). One such channel, found in colonic tissue, is activated by cAMP and displays a linear *I-V* relation (31). The prediction of multiple channel types in heart should be readily tested by accessing whole-cell PKA/PKC additivity effects and measuring single-channel currents activated by PKA and PKC.

In the presence of the β-adrenergic antagonist propranolol, norepinephrine was found to activate a background current with properties (i.e., linear *I-V* relation, time-independent kinetics) similar to those of the current activated by phorbol esters and PKC. Because α-adrenergic stimulation in isolated cardiac myocytes leads to an increased translocation of PKC from cytosolic to membrane compartments (26), the present results may indicate that norepinephrine, acting via PKC, serves as a physiological stimulus for Cl^- channel activation. However, these results must be interpreted carefully, because α-adrenergic stimulation increases production of inositol triphosphates in ventricular cells (26, 32), which are known to regulate ion channels (33). In addition, further experiments, using α-adrenergic-selective agonists, will be required in order to define the exact pharmacological basis for norepinephrine action.

Cl^- channels in airway epithelial cells are activated by PKA (4, 5) and PKC (6, 7). Riordan *et al.* (34) have reported the cloning of the CF gene. The predicted protein product of the CF cDNA shares structural homology with several ion channel proteins that have been sequenced. Expression of the CF cDNA has been shown to generate Cl^- channel activity in cells lacking the CF gene (35), suggesting that this gene codes for a functional Cl^- channel. Thus, knowledge gained in the study of

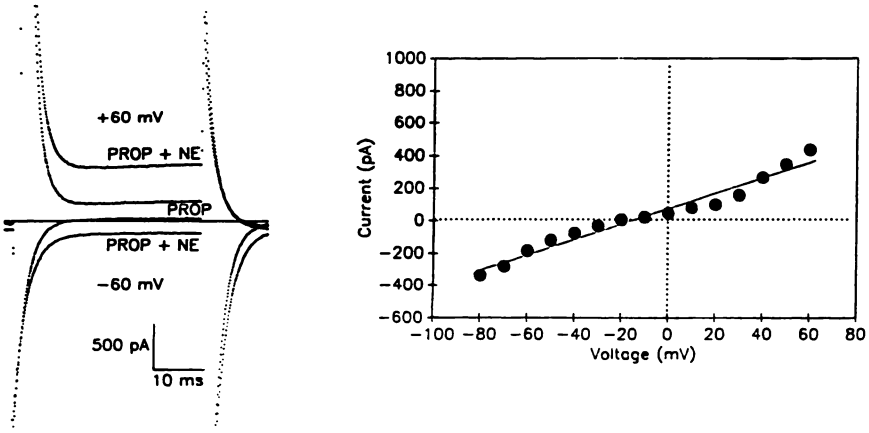

Fig. 5. Activation of background current by norepinephrine. *Left*, currents recorded during 40-msec voltage steps to −60 and +60 mV, in the presence of 1 μM levels of the β-adrenergic antagonist propranolol (*PROP*) and propranolol plus 10 μM norepinephrine (*PROP + NE*). *Right*, *I-V* relation for current activated by norepinephrine in the presence of propranolol (146 mM external Cl^-/54 mM internal Cl^-, E_{Cl} = −25 mV). Temperature = 25°. Cell A140.

epithelial Cl^- channels may provide a important starting point for the molecular analysis of heart Cl^- channels.

Acknowledgments

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Send reprint requests to: Kenneth B. Walsh, Ph.D., Department of Pharmacology, University of South Carolina, School of Medicine, Columbia, SC 29208.