Anion, Cation, and Zwitterion Selectivity of Phospholemman Channel Molecules

Gopal C. Kowdley,* Stephen J. Ackerman,* Zhenhui Chen,§ Gabor Szabo,# Larry R. Jones,§ and J. Randall Moorman*#

*Departments of Internal Medicine (Cardiovascular Division), #Molecular Physiology and Biological Physics, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908, and §Department of Internal Medicine, Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, Indiana 46202 USA

ABSTRACT Phospholemman (PLM), a 72-amino acid membrane protein with a single transmembrane domain, forms taurine-selective ion channels in lipid bilayers. Because taurine forms zwitterions, a taurine-selective channel might have binding sites for both anions and cations. Here we show that PLM channels indeed allow fluxes of both cations and anions, making instantaneous and voltage-dependent transitions among conformations with drastically different ion selectivity characteristics. This surprising and novel ion channel behavior offers a molecular explanation for selective taurine flux across cell membranes and may explain why molecules in the phospholemman family can induce cation- or anion-selective conductances when expressed in *Xenopus* oocytes.

INTRODUCTION

The sulfonic amino acid taurine is present in high concentration in many tissues (Huxtable, 1992). It does not diffuse passively through lipid membranes and acts as an osmolyte in the regulation of cell volume. Taurine flux through swelling-activated anion-selective channels has been observed (Jackson and Strange, 1993; Szucs et al., 1996; Voets et al., 1995), suggesting that taurine efflux through membrane ion channels may be a mechanism for the regulatory volume decrease seen in many types of cells responding to swelling under hypotonic conditions. The most notable feature of the taurine molecule is its zwitterionic character, which makes it extremely polar. The nearby and oppositely charged amino and sulfonate groups, however, complicate the problem of selective transport of taurine through ion channels, which employ charged binding sites to select among and between cations and anions. A possible solution is that a taurine-selective pore might contain binding sites for both the negatively and positively charged moieties. Phospholemman (PLM), a major cell membrane substrate for protein kinases (Palmer et al., 1991), forms ion channels selective for taurine when reconstituted in synthetic lipid bilayers (Moorman et al., 1995). To test the hypothesis that PLM channels have binding sites for both anions and cations, we studied currents carried through PLM channels in the absence of taurine.

Received for publication 19 July 1996 and in final form 25 October 1996.

Address reprint requests to Dr. J. Randall Moorman, Box 6012, MR4 Building, University of Virginia Health Science Center, Charlottesville, VA 22908. Tel.: 804-982-3367 Fax: 804-982-3162; E-mail: rmoorman@virginia.edu.

0006-3495/97/01/141/05 $2.00

METHODS

Recombinant and canine cardiac PLM, and PLM-specific antibodies were prepared as previously described (Moorman et al., 1995). Mass spectroscopic analysis demonstrated greater than 98% purity of the protein preparation (kindly provided by D. Cafiso, University of Virginia). Bilayer recordings were made using conventional techniques (Moorman et al., 1995). Briefly, the recording chamber was fabricated from Teflon. The aperture measured about 0.45 mm, and a lipid bilayer had a capacitance of about 1 nF, depending on lipid composition and the size of the black lipid membrane. In a typical experiment, a drop of lipid (1,2-diphytanoyl-*sn*-glycero-3-phosphocholine, Avanti Polar Lipids, Birmingham, AL; 20 mg/ml in decane, Sigma) was applied to both sides of the aperture with a clean Pasteur pipette. Then 1.1 ml of salt solution was added simultaneously to both sides of the chamber. After 10 to 30 min of equilibration, bilayers were formed by brushing air bubbles over the aperture. The back chamber was connected to the headstage. In this configuration, positive currents flow from front to back.

One to four micrograms of immunoaffinity-purified recombinant PLM in 20 mM glycine and 0.1% Triton X-100 (pH 7.2, 3-(*N*-morpholino)propanesulfonic acid) were added to one of the chambers. Bilayer membrane currents were amplified and filtered (10 mV/pA and 100 Hz; Warner Corporation and Axon Instruments) and digitized (200 Hz, equivalent to 1 point/0.05 to 0.4 mV during voltage ramps; Axon Instruments) and analyzed (Origin, Microcal) using conventional means. To eliminate passive elements of leak and capacitance currents, the average of null traces was subtracted. Because the probability of opening is so high after channel insertion, we have routinely used voltage ramps rather than prolonged records at single potentials. From currents during a voltage ramp, we are able to obtain single-channel conductance (from the slope) and ion selectivity (from E_{rev}).

For the experiments depicted in Figs. 1, 2, and 3, one chamber held a solution containing (mM) 200 KCl and 10 HEPES (pH 7.4, titrated with KOH); the other chamber held a solution of 50 KCl and 10 HEPES (pH 7.4, KOH). For the experiments depicted in Fig. 4, one chamber held a solution containing (mM) 50 taurine, 10 HEPES (pH 7.4, 2 mM NaOH); the other chamber held a solution of (mM) 50 NaCl, 10 HEPES (pH 7.4, NaOH). Lines were fit through conductance states of more than 200 ms duration to yield conductance and extrapolated E_{rev}. Permeability ratios were calculated using the Goldman-Hodgkin-Katz equation (Hille, 1992).

The pH of taurine solutions was adjusted using NaOH, and [Na] was measured directly using an Na-selective electrode (Corning). For 50 mM taurine, 2 mM NaOH was required. In the permeability ratio calculations,

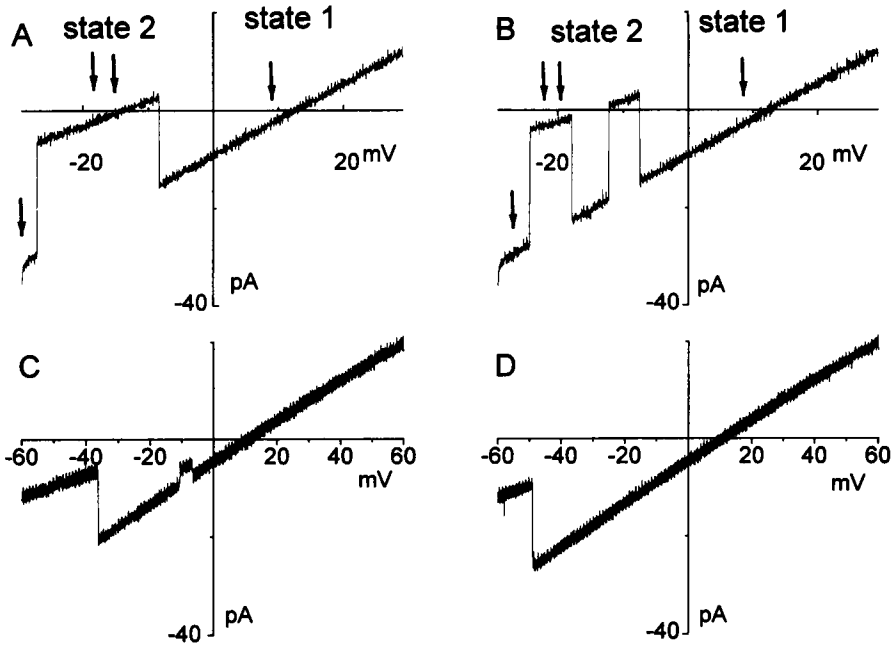

FIGURE 1 Anion-selective and cation-selective conformations of PLM molecules reconstituted in planar lipid bilayers. In a fourfold KCl gradient there were transitions between K-selective (*double arrows*) and Cl-selective (*single arrows*) conformations. (*A, B*) Recombinant PLM. Voltage ramps are 6 mV s^{-1}. (*C, D*) Canine cardiac PLM. Voltage ramps are 10 mV s^{-1}

P_{Na}:P_{Cl} was arbitrarily set to 0.1. In fact, P_{Na}:P_{Cl} varies according to voltage but cannot be measured directly under these conditions.

RESULTS AND DISCUSSION

Two kinds of conductance states in phospholemman channels

Fig. 1 shows currents through immunoaffinity-purified recombinant PLM channel molecules reconstituted in a phosphatidylcholine bilayer membrane in the presence of a fourfold asymmetry of KCl concentration (200:50 mM KCl). Under these experimental conditions, anion currents reverse at positive voltages (current through a perfectly selective anion channel would reverse at 35 mV), and cation currents reverse at negative voltages. At the beginning of the 10-s voltage ramp from −30 to 30 mV in Fig. 1 *A*, the 730-pS conductance (*single arrow*) has an extrapolated E_{rev} of 13 mV. This is an anion-selective conductance with P_K:$P_{Cl} \cong$ 0.43 and is denoted state 1, because it was the predominant conductance state at 0 mV. There was an instantaneous transition to a 500-pS conductance (*double arrows*) with an E_{rev} of −14 mV; this is a cation-selective conductance with P_K:$P_{Cl} \cong$ 2.6 and is denoted state 2. There was then a second transition back to state 1, the anion-selective conformation. In Fig. 1 *B*, two such transitions occurred. Transitions among conformations have been common in our studies of reconstituted PLM channels and have been observed in more 400 bilayer experiments under varying ionic conditions. The findings suggest that both anion- and cation-binding sites are present in the PLM channel.

Another possible explanation of these instantaneous changes in ion selectivity of reconstituted PLM channels is that two channel proteins with different ion selectivity are present. This is unlikely, as the currents appear as single initial jumps in conductance (Moorman et al., 1995), and mass spectroscopic analysis of the protein shows that it is more than 98% pure. A third explanation is that it is an artifact of protein processing by the *Baculovirus* expression system. Fig. 1, *C* and *D*, shows similar behavior of ion currents across a bilayer to which PLM purified from canine cardiac sarcolemmal vesicles was added, supporting the idea that this behavior is a unique physiological feature of the PLM channel molecule.

Voltage dependence of the conductance states and the effect of antibodies

Both anion-selective and cation-selective conformations were present in 63 of 96 bilayer experiments in a KCl gradient; the others showed a stable anion- or cation-selective conductance with no transitions to another state. The transitions were voltage dependent. As shown in Fig. 1, transitions to state 2 were more likely at negative voltages. To examine the voltage dependency, we idealized current traces to form plots of $P_{state\ 2}$, the probability of the cation-selective conformation, as a function of voltage. For the idealization and normalization of the current records, voltages over which cation-selective currents were present were assigned the value 1, and voltages over which anion-selective currents were present were assigned the value 0. The normalized ensemble average of the idealized traces is thus a plot of $P_{state\ 2}$ as a function of voltage. Fig. 2 *C* is such a plot and demonstrates that transitions occurred predominantly over negative voltages in this experiment. $P_{state\ 2}$ fell from its maximum (0.7) to less than 0.05 over a 35–40 mV range. Transitions were seen predominantly at negative voltages in 52 of 59 experiments; four experiments showed the opposite voltage dependence. These findings suggest

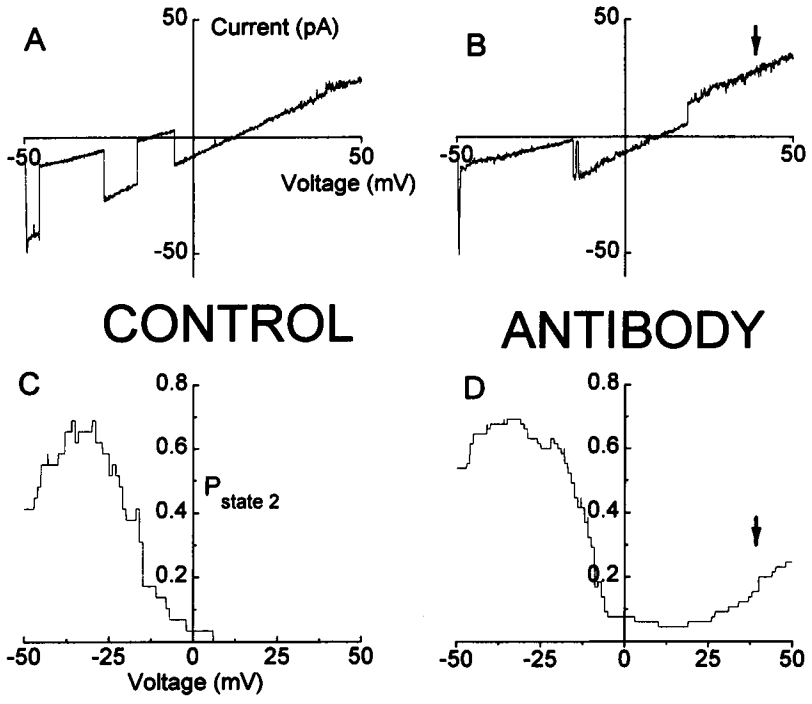

FIGURE 2 Voltage dependence of transitions between anion-selective and cation-selective PLM conformers. (*A, B*) Currents before and after addition of polyclonal antibodies directed against the N-terminal 15 residues of PLM. (*C, D*) Plots of $P_{\text{state 2}}$ as a function of voltage from the 60 traces in this experiment. After antibody addition, transitions to cation-selective conformations were observed (*arrow* in *B* and *D*). Voltage ramps are 10 mV s^{-1}

that the accessibility of the cation- and anion-binding sites of the PLM channel vary with membrane potential-induced changes in channel molecule conformation.

The voltage-dependent nature of the transitions could be modulated by PLM-specific antibodies. The addition of antibodies after channel formation often resulted in flickering or complete channel block (Moorman et al., 1995). This block was usually transient. Afterward there were frequent transitions to state 2 at the opposite end of the voltage range. In Fig. 2 *A* transitions occurred only in the negative voltage range until antibodies were added, after which transitions were observed at positive voltages (Fig. 2 *B*). The normalized ensemble averages of the idealized currents are shown in Fig. 2, *C* and *D*. The addition of PLM-specific antibodies resulted in a change in voltage-dependent behavior in 18 of 32 experiments.

Variability of conductance states

Whereas most experiments showed two conductance states, several experiments showed a larger number. Fig. 3 *A* shows current during a 20-s ramp from −75 to 75 mV in a fourfold KCl gradient, the same experimental conditions as shown in Fig. 1. There are many more than two conductance states marked by different conductance and reversal potentials. A frequency histogram (Fig. 3 *B*) of E_{rev} from many experiments demonstrated that a large number of discernibly different conductance states exist. In any experiment, though, there was remarkable uniformity of the conductance level we defined to be state 1, that which is present at 0 mV in the majority of traces. Fig. 3 *C* is a plot of E_{rev} as a function of conductance in 33 current traces from a single bilayer experiment. Whereas most of the points for the state 1 currents overlap, each state 2 conductance varies.

Effects of taurine

Taurine modulated the voltage dependence of the channels' conformational changes. The currents shown in Fig. 4 *A* were recorded with 150 mM taurine and 4 mM Na in one chamber, and 150 mM NaCl in the other. The equilibrium potential under these conditions is 94 mV, and currents reversing at potentials of less than 94 mV denote P_{taurine}:$P_{\text{Cl}} > 1$. In these experimental conditions, the channels were always more selective for taurine, and the transitions were among taurine-selective states exclusively. For example, states 1 and 2 in the left-hand half of Fig. 4 *A* were 5- and 50-fold selective for taurine over Cl, respectively. In the right-hand half of the tracing, where the voltage was held at 0 mV, there was a transition between the two states that resulted in a reversal in the direction of current flow. Fig. 4 *B* is a frequency histogram of P_{taurine}:P_{Cl} values for 160 current epochs in three bilayer experiments. State 1 conductances were 2.7- to 11.3-fold more selective for taurine (median 4.5; *unshaded portions of histogram*). State 2 conductances were 13- to 170-fold more selective for taurine (median 47; *shaded portions*).

The presence of taurine affected the voltage dependence of transitions among conformations. Fig. 3 *C* is a plot of the probability of state 2 as a function of voltage in the same experiments. Taurine led to increased $P_{\text{state 2}}$ in the positive voltage range, in the same fashion as did PLM-specific antibodies. In 8 of 12 experiments in taurine, transitions in the positive voltage range were observed in more than 30%

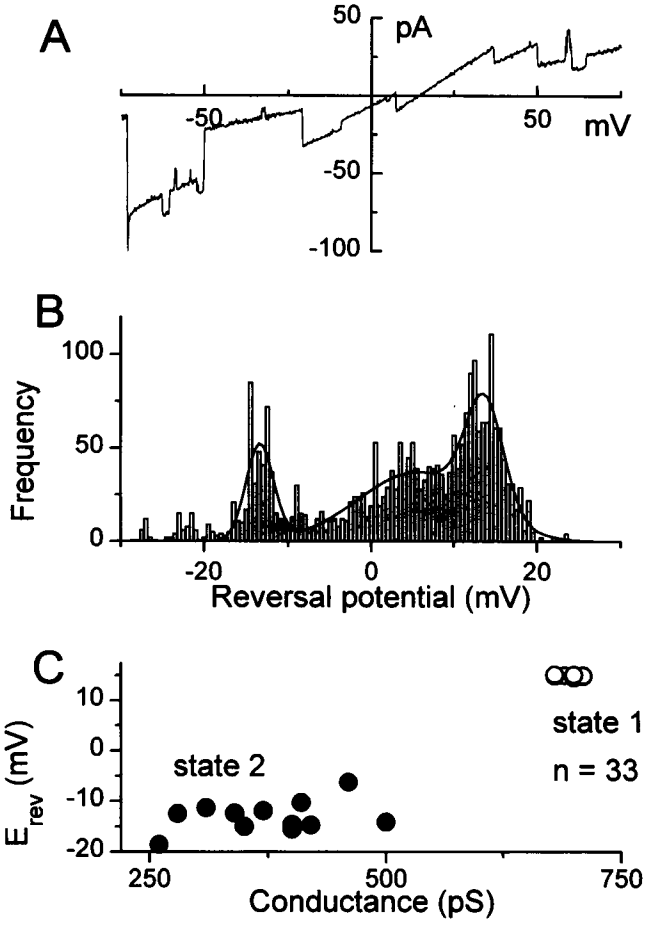

FIGURE 3 Variability of conductance states of phospholemman channels. (*A*) In this bilayer experiment with many conductance states, conductance varied from 0.47 and 1.02 nS, and E_{rev} varied from −18 to 16 mV. (*B*) Frequency histogram of E_{rev} measured in 2548 sweeps from 96 bilayer experiments using four preparations of purified PLM. The smooth line is a sum of three Gaussian functions and has peaks at −13, 5, and 13 mV, corresponding to P_K:P_{Cl} ≅ 2.4, 0.7, and 0.4, respectively. The areas were 17%, 54%, and 29%.

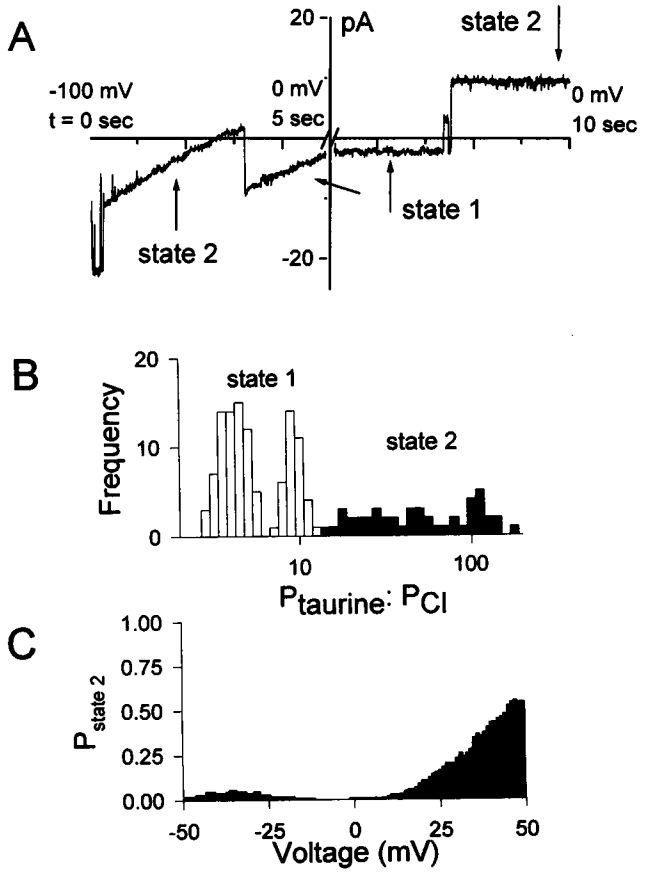

FIGURE 4 Zwitterion-selective conformations of PLM molecules reconstituted in planar lipid bilayers. (*A*) Transitions between conformations in which $P_{taurine}$:P_{Cl} was 50 (state 2) or 5 (state 1). In the left-hand half of the current record, a voltage ramp of 20 mV s^{-1} from −100 to 0 mV was applied. The right-hand half of the record is continuous and shows the current at 0 mV. The entire record is 10 s in duration. (*B*) Frequency histogram of $P_{taurine}$:P_{Cl} in 160 current epochs of more than 200 ms duration in three bilayer experiments in which voltage ramps from −50 to 50 mV were applied. (*C*) Taurine alters the voltage dependence of channel states. The plots show $P_{state\ 2}$ as a function of voltage for the same three bilayer experiments.

of sweeps compared with 4 of 59 in the KCl experiments ($p < 0.0001$, Fisher Exact test). This finding suggests that taurine ions not only permeate the channel, but might also interact with the channel molecule in such a way as to alter properties of the voltage sensor.

We have no direct evidence that the "state 1" demonstrated in Fig. 1 is the same as the "state 1" shown in Fig. 4. That is to say, we have no direct evidence that Cl binds to the same site that binds the sulfonic group of taurine, or that K binds to the same site that binds the amino group. The speculation centers on two findings. First, negative voltages bring enhanced K selectivity over Cl, and enhanced taurine selectivity over Cl. Second, selectivity and permeation of taurine through PLM channels is critically determined by the positively charged amino group, as substitutions here severely limited permeation of the ethanesulfonates isethionate, propanesulfonic acid, and 2-bromoethanesulfonic acid (Moorman et al., 1995). We thus conjecture that there may be a single negatively charged site that is more prominently exposed at negative bilayer voltages and selects the amino group of taurine in preference to K. An equally viable explanation is that voltage-induced conformational changes may expose multiple new binding sites, some specific for taurine and others specific for Cl or K. This hypothesis is supported by the multiplicity of permeability ratios, suggesting multiple conformations or arrangements of subunits.

Selectivity of currents when PLM family members are expressed in *Xenopus* oocytes

The PLM family has three members to date: itself (Palmer et al., 1991; Moorman et al., 1992; Kowdley et al., 1994; Attali et al., 1993), Mat-8 (Morrison and Leder, 1994; Morrison et al., 1995), and channel-inducing factor (CHIF) (Attali et al., 1995). The first two induce Cl-selective cur-

rents when expressed in *Xenopus* oocytes; CHIF, on the other hand, induces K currents. Oocytes have endogenous channels that are candidates for regulation by these molecules (Kowdley et al., 1994; Ackerman et al., 1994; Tzounopoulis et al., 1995; Shimbo et al., 1995), limiting the use of this expression system as a means of resolving the role of PLM as ion channel or regulator. If all three PLM family members form zwitterion-selective pores, then all might be expected to display both anion- and cation-selective conformations. The paradox of anion selectivity of hyperpolarization-activated currents induced by PLM and Mat-8 and of cation selectivity of depolarization-activated currents induced by CHIF is resolved if PLM and Mat-8 assume stable anion-selective forms in oocytes, and CHIF assumes a stable cation-selective form.

REFERENCES

Ackerman, M., K. Wickman, and D. Clapham. 1994. Hypotonicity activates a native chloride current in *Xenopus* oocytes. *J. Gen. Physiol.* 103:153–179.

Attali, B., E. Guillemare, F. Lesage, E. Honore, G. Romey, M. Lazdunski, and J. Barhanin. 1993. The protein IsK is a dual activator of K^+ and Cl^- channels. *Nature.* 365:850–852.

Attali, B., H. Latter, N. Rachamim, and H. Garty. 1995. A corticosteroid-induced gene expressing an "IsK-like" K^+ channel activity in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA.* 92:6092–6096.

Hausdorff, S. F., S. A. N. Goldstein, E. E. Rushin, and C. Miller. 1991. Functional characterization of a minimal K^+ channel expressed from a synthetic gene. *Biochemistry.* 30:3341–3346.

Hille, B. 1992. Ionic Channels of Excitable Membranes. Sinauer Associates, Sunderland, MA.

Huxtable, R. J. 1992. Physiological actions of taurine. *Physiol. Rev.* 72: 101–163.

Jackson, P. S., and K. Strange. 1993. Volume-sensitive anion channels mediate swelling-activated inositol and taurine efflux. *Am. J. Physiol.* 265:C1489–C1500.

Kowdley, G. C., S. J. Ackerman, J. E. John, L. R. Jones, and J. R. Moorman. 1994. Hyperpolarization-activated chloride currents in *Xenopus* oocytes. *J. Gen. Physiol.* 103:217–230.

Moorman, J. R., S. J. Ackerman, G. C. Kowdley, M. P. Griffin, J. P. Mounsey, Z. Chen, S. E. Cala, J. J. O'Brian, G. Szabo, and L. R. Jones. 1995. Unitary anion currents through phospholemman channel molecules. *Nature.* 377:737–740.

Moorman, J. R., C. J. Palmer, J. E. John, M. E. Durieux, and L. R. Jones. 1992. Phospholemman expression induces a hyperpolarization-activated chloride current in *Xenopus* oocytes. *J. Biol. Chem.* 267:14551–14554.

Morrison, B. W., and P. Leder. 1994. *neu* and *ras* initiate murine mammary tumors that share genetic markers generally absent in c-*myc* and *int*-2-initiated tumors. *Oncogene.* 9:3417–3426.

Morrison, B. W., J. R. Moorman, G. C. Kowdley, L. R. Jones, and P. Leder. 1995. Mat-8, a novel phospholemman-like channel protein expressed in human breast tumors. *J. Biol. Chem.* 270:2176–2182.

Palmer, C. J., B. T. Scott, and L. R. Jones. 1991. Purification and complete sequence determination of the major plasma membrane substrate for cAMP-dependent protein kinase and protein kinase C in myocardium. *J. Biol. Chem.* 266:11126–11130.

Shimbo, K., D. L. Brassard, R. A. Lamb, and L. H. Pinto. 1995. Viral and cellular small integral membrane proteins can modify ion channels endogenous to *Xenopus* oocytes. *Biophys. J.* 69:1819–1829.

Szucs, G., S. Heinke, C. De Greef, L. Raeymaekers, J. Eggermont, G. Droogmans, and B. Nilius. 1996. The volume-activated chloride current in endothelial cells from bovine pulmonary artery is not modulated by phosphorylation. *Pflugers Archiv./Eur. J. Physiol.* 431:540–548.

Tzounopoulis, T., J. Maylie, and J. P. Adelman. 1995. Induction of endogenous channels by high levels of heterologous membrane proteins in *Xenopus* oocytes. *Biophys. J.* 69:904–908.

Voets, T., G. Szucs, G. Droogmans, and B. Nilius. 1995. Blockers of volume-activated Cl-currents inhibit endothelial cell proliferation. *Pflugers Archiv./Eur. J. Physiol.* 431:132–134.