Viral and Cellular Small Integral Membrane Proteins Can Modify Ion Channels Endogenous to *Xenopus* Oocytes

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ABSTRACT A slowly activated, inward current could be evoked from *Xenopus* oocytes in response to application of a strong (~−190 mV) hyperpolarizing pulse. However, a much lesser hyperpolarization (~−130 mV) was able to evoke a similar current from oocytes that expressed the cellular proteins IsK and phospholemman, the synthetic protein SYN-C, and the NB protein of influenza B virus. All of these currents were carried principally by Cl^-, and they had similar blocker profiles. The time course (the function of time that described the current increase during a hyperpolarizing voltage-clamp pulse, i.e., activation kinetics) varied from one batch of oocytes to another, but did not vary within each batch with the type of protein expressed. This slowly activated, inward current evoked by hyperpolarization to ~−130 mV required the expression of a characteristic, minimum level of each of the proteins IsK, SYN-C, and NB. However, not every integral membrane protein expressed in oocytes allowed substantial inward currents to be generated at −130 mV. Oocytes that expressed large amounts of the M_2 protein of influenza A virus, which is known to possess an intrinsic cation channel activity, did not display a Cl^- current when hyperpolarized to −130 mV. These results suggest that expression of any of the four proteins—IsK, phospholemman, SYN-C, or NB— acts as an activator of an endogenous Cl^- conductance.

INTRODUCTION

When mRNA encoding a transmembrane protein is expressed in a *Xenopus* laevis oocyte and a current is present in that oocyte, the current can in principle result from 1) activity intrinsic to the protein, 2) background channels that are endogenous to the oocyte and normally expressed in control oocytes, or 3) normally silent ion channels that are not activated in control oocytes but for which the expressed protein acts as an activator (Attali et al., 1993). Evidence for the latter possibility comes from experiments in which high levels of the integral membrane proteins IsK and phospholemman were observed to allow the expression of a Cl^- current that is not normally expressed in the oocyte (Attali et al., 1993).

We have been studying the ion channel activity of a small, type III, 97-residue, homotetrameric, integral membrane protein, M_2, encoded by influenza A virus. The activity of this cationic channel is modulated by pH, and the channel can be blocked by the antiviral drug amantadine (Pinto et al., 1992). The activity of the M_2 channel can be reconstituted from purified protein in lipid bilayers (Tosteson et al., 1994) and is thus intrinsic to the protein. To understand the properties of the M_2 channel, a synthetic derivative of the M_2 channel, SYN-C, formerly called M_2-$A_{30}T$, +V (Pinto et al., 1992), was constructed and expressed. SYN-C differs from the M_2 protein by containing the change $A_{30}T$ and the introduction of a valine residue to the transmembrane domain, to change the pitch of the presumptive α-helical transmembrane domain pore region with the expectation that this change would ablate the activity of the M_2 channel. We have also been attempting to determine whether the NB glycoprotein of influenza B virus has an intrinsic ion channel activity. The NB protein resembles M_2 protein in size and membrane orientation (Shaw et al., 1983; Williams and Lamb, 1986; reviewed in Lamb, 1989), and it has been speculated that NB is the influenza B virus counterpart of the influenza A virus M_2 ion channel protein (Duff and Ashley, 1992; Hay, 1992).

In the course of studying the currents in oocytes that expressed either the SYN-C or the NB proteins, we observed a Cl^- current with the unusual properties of enhancement by lowering either $[Ca^{2+}]$ or pH. The Cl^- current that had been observed with overexpression of IsK (Attali et al., 1993) shared these unusual properties. More importantly, we also observed that these currents all showed several key properties of a current that can be elicited from water-injected oocytes that were strongly hyperpolarized. Thus, we were interested in testing the emerging hypothesis that many small integral membrane proteins may be capable of up-regulating an endogenous oocyte Cl^- current.

Here we describe experiments in which we compared the properties of the Cl^- conductance of oocytes that express small integral membrane proteins with the properties of the Cl^- conductance evoked in control oocytes and quantified the relative amount of protein required to activate the Cl^- conductance. We conclude that four of the proteins tested, including the influenza B virus NB protein, SYN-C, IsK, and phospholemman, are capable of modifying the activation properties of an endogenous Cl^- conductance of the oocyte.

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MATERIALS AND METHODS

Microinjection and culture of oocytes

Xenopus were individually identified with an implanted marker (Basic Medic Data Systems, Maywood, NJ). Oocytes were defolliculated and injected with synthetic mRNA as described previously (Pinto et al., 1992). The synthetic cDNA to human IsK was cloned in a derivative of pGEM9Zf(−) such that T7 RNA polymerase transcripts would contain a poly(A) tail. This plasmid was a generous gift of Dr. R. Swanson (Merck Research Laboratories, West Point, PA). For in vitro transcription, plasmid DNA was linearized with Not I before synthesis by T7 RNA polymerase, as described previously (Pinto et al., 1992). The cDNA encoding the SYN-C protein was cloned in pGEM3Zf(+), and T7 mRNA transcripts were made after linearization with *Xba*I. The cDNA of phospholemman was obtained from the pSelect Dog PLM plasmid (Moorman et al., 1992) (kindly provided by Dr. L. Jones, University of Indiana, Indianapolis, IN) and subcloned into pGEM9Zf(−), and T7 mRNA transcripts were made after linearization with Not I. cDNA encoding the influenza B/Lee/40 virus protein NB (Williams and Lamb, 1986) was subcloned into pGEM9Zf(−) after PCR generation of *Nco*I and *Hind* III sites at the 5′ and 3′ ends, respectively, and T7 mRNA transcripts were made after linearization with Not I. Oocytes were maintained in ND96 for 1–3 days at 19°C before use.

Electrophysiological measurements

Whole-cell current was measured with a two-electrode voltage-clamp apparatus 1–3 days after mRNA injection (Pinto et al., 1992). The electrodes were filled with 3 M KCl, and the oocytes were bathed in either Barth's solution (88.0 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $NaNO_3$, 0.71 mM $CaCl_2$, 0.82 mM $MgSO_4$, and 15 mM HEPES, pH 7.5), or a modified solution, during the recording.

Western blot analysis

Immediately after electrophysiological recording, the oocytes were individually lysed in 25 μl of RIPA buffer containing a cocktail of protease inhibitors (aprotinin, antipain, pepstatin A, leupeptin, and chymostatin) (Paterson and Lamb, 1987), frozen, and extracted once with 1,1,2-trichloro-trifluoroethane to remove yolk and pigment proteins. For oocytes expressing the SYN-C and NB proteins, 0.2 U of *N*-glycosidase F (Boehringer-Mannheim Biochemicals, Indianapolis, IN) was added to the lysate and incubated overnight at 37°C to remove *N*-linked carbohydrate chains and thus create a more discrete band on SDS-PAGE. To adjust for differences in the amount of protein expressed, 1–40% of the individual lysate was placed in reducing protein lysis buffer and run on SDS-PAGE on 17.5% + 4 M urea polyacrylamide gels. The proteins were electrotransferred to PVDF membranes (Millipore, Bedford, MA) by using a Trans-Blot Semidry transfer cell (Bio Rad, Richmond, CA). Membranes were incubated with 5% milk/0.3% Tween-20/PBS^- for 60 min to block nonspecific sites and then were incubated for 60 min with a primary antibody in the same solution. For the IsK protein, a rabbit antiserum raised against a peptide corresponding to the 12 C-terminal amino acids was used at a 1:500 dilution (a kind gift of Dr. R. Swanson). For the SYN-C protein, a 1:2000 dilution of M_2-specific 14C2 ascites fluid was used (Zebedee and Lamb, 1988). For the NB protein, a rabbit antiserum raised against a peptide corresponding to residues 58–74 was used at a 1:500 dilution. Membranes were washed in PBS^-/0.3% Tween-20 and incubated for an additional 60 min with 1:1000 dilution of horseradish peroxidase conjugated goat anti-mouse or anti-rabbit immunoglobulin G secondary antibody (Cappel, Organon Teknika, Malvern, PA) in a solution of PBS^- with 5% milk and 0.3% Tween-20. Blots were extensively washed in PBS with 0.3% Tween-20, and the immune complexes were detected using the ECL system (Amersham International, Arlington Heights, IL), and the luminescent signal was then detected using preflashed x-ray film (Laskey and Mills, 1975). The strength of the signal was quantified by laser scanning densitometry on an LKB Ultroscan XL Densitometer (Pharmacia-LKB, Piscataway, NJ). For quantification of SYN-C, 25–200 pg of immunoaffinity purified M_2 protein was applied to each Western blot, and a standard curve was generated from densitometric measurements of these standards (Holsinger and Lamb, 1991). For the quantification of IsK and NB proteins, a standard curve was generated for each Western blot using a series of dilutions of pooled oocyte lysates expressing the proteins of interest. The amount of SYN-C or relative amount of IsK and NB expressed in each oocyte was then calculated by interpolation using the standard curve.

RESULTS

Messenger RNAs encoding four small integral membrane proteins, the influenza B virus NB protein, the cellular proteins IsK and phospholemman, and a synthetic integral membrane protein (SYN-C), were expressed in oocytes, and the currents of these oocytes evoked by depolarization or hyperpolarization were studied 1–3 days postinjection. For oocytes injected with relatively large amounts of the mRNA encoding the IsK, phospholemman, SYN-C, and NB proteins, hyperpolarization to ~−130 mV evoked a slowly activated, inward current that attained an amplitude of 0.5–20 μA with a 3-s activating pulse (Fig. 1, *A–D*). This current was observed in ~70–80% of the oocytes. However, in control experiments (control experiments always employed water-injected oocytes) with every batch of oocytes, hyperpolarization to more negative voltages (~−190 mV) always evoked an inward current with a similar amplitude and time course (Fig. 1 *E*). This current never inactivated, even when activating pulses as long as 1 min were applied. This current was not associated with expression of all small integral membrane proteins in oocytes because oocytes that expressed the bona fide influenza A virus M_2 ion channel protein never exhibited a similar current with hyperpolarization to ~−130 mV (Fig. 1 *F*), even when large amounts of M_2 protein were expressed (see below and Fig. 7 *D*). The current-voltage relationship of the slowly activated, inward current was similar for each of the oocytes from a given batch, regardless of the protein that it expressed (IsK, phospholemman, SYN-C, or NB), but was shifted by about +60 mV from the current-voltage relationship of the control oocytes (Fig. 2).

Ion selectivity of inward current

To determine whether the slowly activated, inward currents found in the control oocytes and those found in the oocytes expressing the four small integral membrane proteins were the result of the same conductance mechanism, we studied the ion selectivity, effect of blockers, and activation of the current from each. We identified the ion responsible for the current by measuring the reversal voltage of tail currents that flowed after cessation of a strong activating pulse while the oocyte was bathed in solutions of various ionic compositions. This was done for control oocytes and oocytes that expressed the SYN-C and NB proteins. Each experiment was performed with at least four oocytes, and the experimental details are described in the legends of Tables 1 and 2. We found that the reversal voltage was altered only

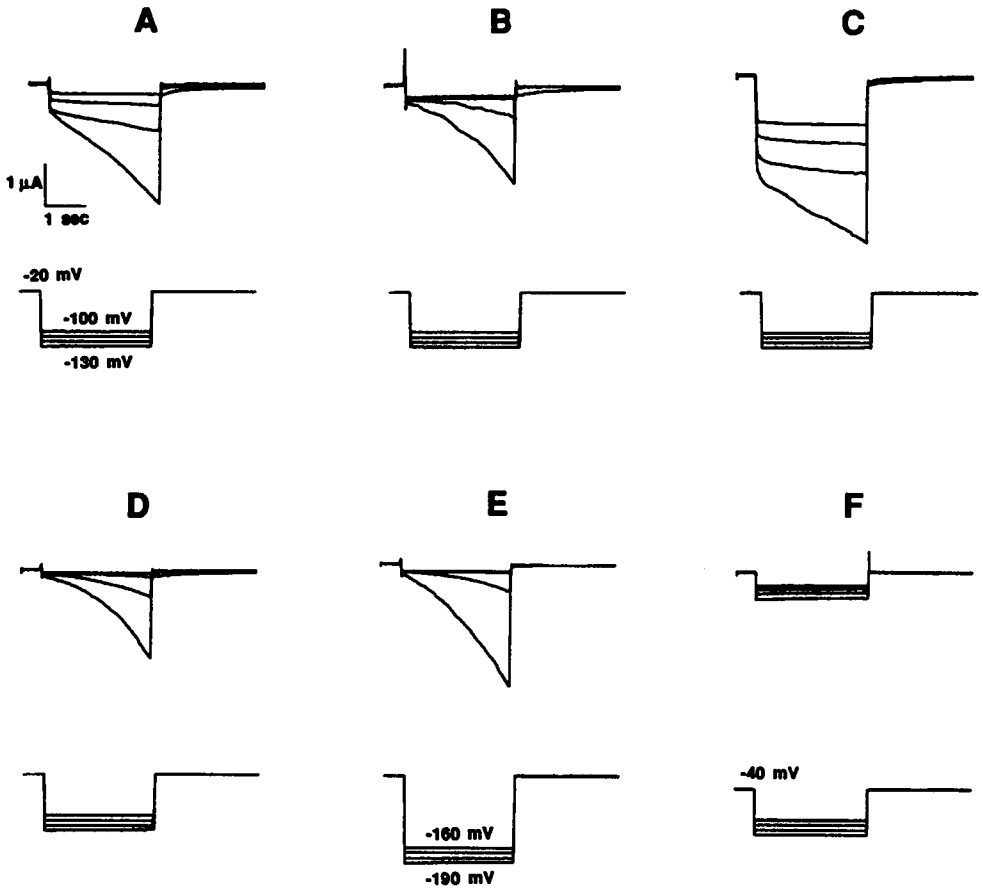


FIGURE 1 Time course of ionic currents of control oocytes and oocytes expressing small integral membrane proteins of viral and cellular origin (*upper*) evoked by hyperpolarizing voltage clamp steps to the voltages indicated (*lower*). (*A*) IsK, (*B*) phospholemman, (*C*) SYN-C, (*D*) NB, (*E*) control oocyte injected with water, and (*F*) M_2 protein of influenza A virus. All oocytes were bathed in Barth's solution, pH 7.5. Note that extreme hyperpolarization evokes a slowly activated current in control oocytes but that more modest hyperpolarization serves to activate a similar current for oocytes that expressed all of these proteins except the M_2 protein. For oocytes expressing the SYN-C protein (*C*), a time-independent current was also observed.

slightly (<5 mV) by reduction of $[Na^+]$ achieved by replacement of Na^+ with either K^+ or *N*-methyl-D-glucamine$^+$ but that the reversal voltage was altered to a greater extent by a 10-fold reduction of $[Cl^-]$, achieved by replacing Cl^- with methansulfonate$^-$ (Table 1). The ranges of the increase in reversal voltages for control oocytes and for oocytes that expressed either the SYN-C or the NB proteins overlapped (Table 1) and were slightly smaller than the previously reported values for IsK (Attali et al., 1993) and phospholemman (Moorman et al., 1992). This greater dependence of reversal voltage upon $[Cl^-]$ than upon $[Na^+]$ suggested that an anion conductance was activated by hyperpolarization. We tested the anion selectivity by replacing Cl^- with other halide anions for control oocytes and oocytes that expressed the IsK, SYN-C, and NB proteins. We found that the anion permeability for the current generated in each of these cases was identical ($I^->Br^->Cl^->F^-$; Table 2) and it was the same as that previously reported for phospholemman (Kowdley et al., 1994). The similarity of the alteration of reversal voltage when Cl^- was replaced with methansulfonate, I^-, Br^-, or F^-, but not when Na^+ was replaced, suggests that all of these currents are anionic, with Cl^- being the principal charge carrier.

Blocker profile of inward current

The similarity of ionic mechanism of the slowly activated, inward current that we observed for oocytes expressing all four small integral membrane proteins was not sufficient evidence to conclude that the current was the result of the same conductance mechanism in each case. Thus, we compared the effects of the channel modulating agents anthracine-9-carboxylic acid, DIDS, SITS, Ba^{2+}, Ca^{2+}, Mg^{2+}, and H^+ in control oocytes and oocytes that expressed the IsK, phospholemman, SYN-C, and NB proteins. These experiments were performed for at least four oocytes for each blocker, and the details of the experimental conditions are given in the legend of Table 3. The current of control oocytes and oocytes expressing each of the four small integral membrane proteins was blocked ~40–70% by anthracine-9-carboxylic acid (3 mM), DIDS (1 mM), and

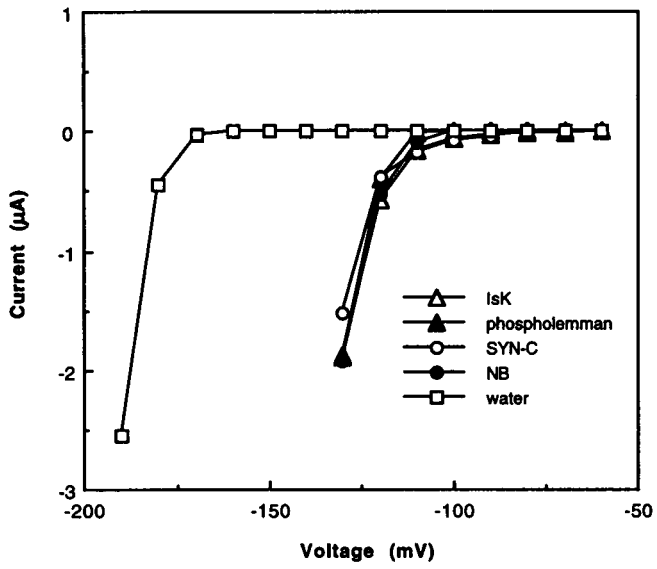


FIGURE 2 Current-voltage relationship of control oocytes (□) and oocytes expressing the small integral membrane proteins IsK (△); phospholemman (▲); SYN-C (○); and NB (●). Note that an inward current is evoked by a much smaller hyperpolarization for oocytes expressing each protein than for the control oocytes.

SITS (2 mM) in a manner that did not depend on membrane voltage (data not shown). The effects of anthracine-9-carboxylic acid and SITS were reversible over a period of 10–20 min for control oocytes and oocytes that expressed each of the four small integral membrane proteins. However, the effects of DIDS were not reversible for as long as 30 min for oocytes that expressed each of the four small integral membrane proteins. The reversibility of the effects of DIDS could not be tested for control oocytes because these oocytes could not withstand repeated application of the −190 mV test pulse during the 30-min period required for possible reversibility of the effects of DIDS. The current of control oocytes and oocytes that expressed each of the four proteins was also reversibly blocked by Ba^{2+} and Ca^{2+}. The IC_{50} for blockade by Ba^{2+} and Ca^{2+} was nearly equal for control oocytes and oocytes that expressed each of the four small integral membrane proteins (Table 3). Elevating Mg^{2+} (by 4 mM) did not alter the current of either

TABLE 1 Chloride ion selectivity of control oocytes and oocytes expressing small integral membrane proteins

	mV change in reversal voltage/10-fold decrease in $[Cl^-]_o$ (mean ± SEM) (N)			
IsK	Phospholemman	SYN-C	NB	Control oocytes
53*	38‡	30§	10.5 ± 3.9 (8)	19.6 ± 2.3 (6)

Reversal voltage was measured by the method of tail currents with the number of oocytes shown in parentheses. Cl^- (82 mEq/l) was replaced with methansulfonate$^-$ to achieve a 10-fold reduction of $[Cl^-]$. Measurements were made at pH 7.5.

*Attali et al., 1993.

‡Moorman et al., 1992.

§Pinto et al., 1992. Note: SYN-C was formerly called M_2-A_{30}T, + V.

TABLE 2 Anion selectivity of control oocytes and oocytes expressing small integral membrane proteins

Ion varied	Reversal voltage (mV) (mean ± SEM)			
	IsK (4)	SYN-C (4)	NB (7)	Control oocytes (4)
NaF	−2.7 ± 2.7	1.0 ± 4.0	−9.7 ± 3.1	−6.5 ± 1.6
NaCl	−14.3 ± 1.3	−18.0 ± 1.8	−19.8 ± 3.1	−14.0 ± 0.6
NaBr	−26.8 ± 2.1	−30.2 ± 3.8	−26.4 ± 2.7	−23.8 ± 0.4
NaI	−32.9 ± 3.0	−43.2 ± 2.9	−29.4 ± 0.1	−29.2 ± 0.3

Reversal voltage was measured by the method of tail currents with the number of oocytes shown in parentheses. NaCl was replaced with NaF, NaBr, or NaI to alter ionic composition. Measurements were made at pH 7.5. Number of cells shown in parentheses.

control oocytes or oocytes expressing each of the four small integral membrane proteins (data not shown). Thus, the effects of blocking agents and divalent ions were indistinguishable for control oocytes and oocytes that expressed each of the four small integral membrane proteins.

It has long been known that lowering the external pH of many ion channels, including the voltage-activated Na^+ and K^+ channels (Hille, 1992), results in a decrease in channel activity. On the other hand, lowering the pH of the medium bathing either control oocytes or oocytes expressing each of the four small integral membrane proteins resulted in a small reversible increase in the current amplitude that can be seen in a plot of relative current amplitude against pH in Fig. 3. The lowest pH that could be tested without encountering irreversible changes in the membrane currents was pH 5.8; this limitation made it impossible for us to determine the pK_a of the titratable group that effected the changes brought about by lowering pH. The lowering of pH thus affected the currents of control oocytes and oocytes expressing each of the four small integral membrane proteins in a qualitatively similar way.

Gating of inward current

The above results show that the ionic mechanism and blocker profile of the currents of control oocytes and oocytes expressing each of the four small integral membrane proteins are remarkably similar. This suggests that all of these currents may be the result of the action of a single conductance mechanism. To test this possibility, we measured an activation property of the currents, the equivalent gating charge, for control oocytes and oocytes that expressed the IsK, SYN-C, and NB proteins. Each experiment was performed with at least four oocytes, and the experimental details are described in the legend of Table 4. We found that the equivalent gating charge did not differ significantly among these currents (Table 4). Moreover, the measured equivalent gating charge for control oocytes and oocytes expressing these three proteins did not differ markedly from that reported for phospholemman (Kowdley et al., 1994). This similarity of an activation property supports the notion that a single conductance mechanism provided the current that had been measured in each case. However,

TABLE 3 Inhibitory effects of divalent cations on inward current of control oocytes and oocytes expressing small integral membrane proteins

Divalent cation	IC_{50} (mM) (mean ± SEM) (N)				
	IsK	Phospholemman	SYN-C	NB	Control oocytes
Ca^{2+} (total calcium concentration)	0.98 ± 0.09 (6)	1.07 ± 0.12 (6)	0.97 ± 0.11 (5)	0.95 ± 0.12 (5)	1.62 ± 0.07 (7)
Ba^{2+}	0.27 ± 0.11 (6)	0.31 ± 0.09 (6)	0.33 ± 0.16 (4)	0.35 ± 0.14 (4)	0.36 ± 0.10 (4)

Percent inhibition of the current was calculated for each of seven or eight concentrations of blocking ion from the ratio of the current evoked by a −130 mV pulse during application of the blocking ion to the average of the currents evoked by the same pulse before and after application of the blocking ion. Only those cells for which recovery was 90% complete were used for the calculation. IC_{50} was calculated by interpolation of the plot of percent inhibition versus concentration of blocking ion. Measurements made at pH 7.5.

these experiments cannot distinguish between the possibilities that this single conductance mechanism is a property of an endogenous oocyte protein or is intrinsic and common to each of the expressed proteins.

Time course of inward current

We made an observation which suggested that the slowly activated, inward current results from a conductance endogenous to the oocyte. We noted that the time course (activation kinetics) of the inward current during the activating pulse (−130 mV for oocytes expressing the IsK, phospholemman, SYN-C, or NB proteins and −190 mV for water-injected oocytes) did not vary with the protein expressed but did vary with the maximum amplitude of the current (which always occurred at the end of the voltage-clamp pulse) and with the batch of oocytes.

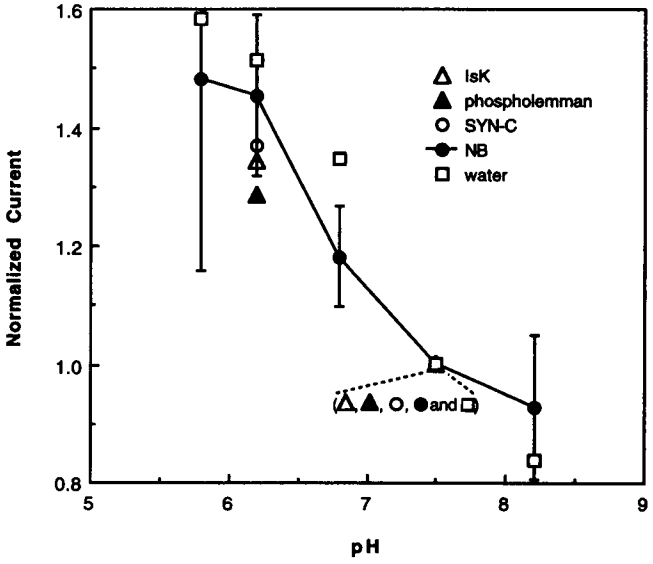


FIGURE 3 Dependence of inward current amplitude upon external pH. Current (normalized to value at pH 7.5) is plotted as a function of pH for control oocytes (□) and oocytes expressing the proteins IsK (△), phospholemman (▲), SYN-C (○), and NB (●). Solid line is for oocytes expressing NB (mean + SEM), and individual points are for control oocytes and oocytes expressing other proteins. Note that in each case the current is increased by lowered pH.

The variation of time course with the maximal amplitude of the current (at the end of a −130 mV activating pulse) is shown in Fig. 4, *A–D*, for oocytes from one batch that expressed the IsK, phospholemman, SYN-C, and NB proteins. In each of the upper traces of Fig. 4, *A–D*, the maximal current attained at the end of the pulse was ~−1 μA. The amplitude of the current of each of these oocytes during the first few hundred milliseconds of the voltage clamp pulse was relatively constant and then rose at an accelerated rate at the end of the pulse, regardless of the expressed protein. On the other hand, for oocytes that attained a larger maximal current of ~−16 μA at the end of the same voltage clamp pulse (lower traces of Fig. 4, *A–D*, indicated by *), the current initially began to rise at a rapid rate but later in the pulse the rate of rise lessened, regardless of the expressed protein. Results similar to this were obtained with a total of >1800 oocytes from 15 batches. We quantified the shape of the time course of the currents of at least four oocytes expressing small and large amplitude currents after injection with each of the four mRNAs by fitting to the time course of the current during the voltage-clamp pulse an equation of the form

$$I(t) = C - A \exp(1 - t/\tau), \quad (1)$$

where $I(t)$ is the time course of the current evoked by a hyperpolarizing pulse applied at $t = 0$; C and A are constants, and τ is the time constant.

TABLE 4 Equivalent gating charge of control oocytes and oocytes expressing small integral membrane proteins

	Equivalent gating charge (e^-) (mean ± SEM)			
IsK	Phospholemman	SYN-C (4)	NB (7)	Control oocytes (7)
3.3 ± 0.5	1.3*	2.0 ± 0.8	3.3 ± 0.8	3.2 ± 0.7

*Kowdley et al., 1994.

Equivalent gating charge was measured by 1) applying hyperpolarizing pulses in 2-mV increments of negativity from −60 mV to −130 mV (−110 mV to −180 mV for control oocytes) and measuring the voltage-gated current for each pulse; 2) measuring the reversal voltage for the current of each cell; 3) calculating the conductance associated with each voltage-activated current; and 4) plotting the natural logarithm of conductance against voltage. These plots were all well-fitted by a straight line for Vm > −130 mV (Vm > −180 mV for control oocytes). The slope of the line was taken as the equivalent gating charge. Number of cells shown in parentheses.

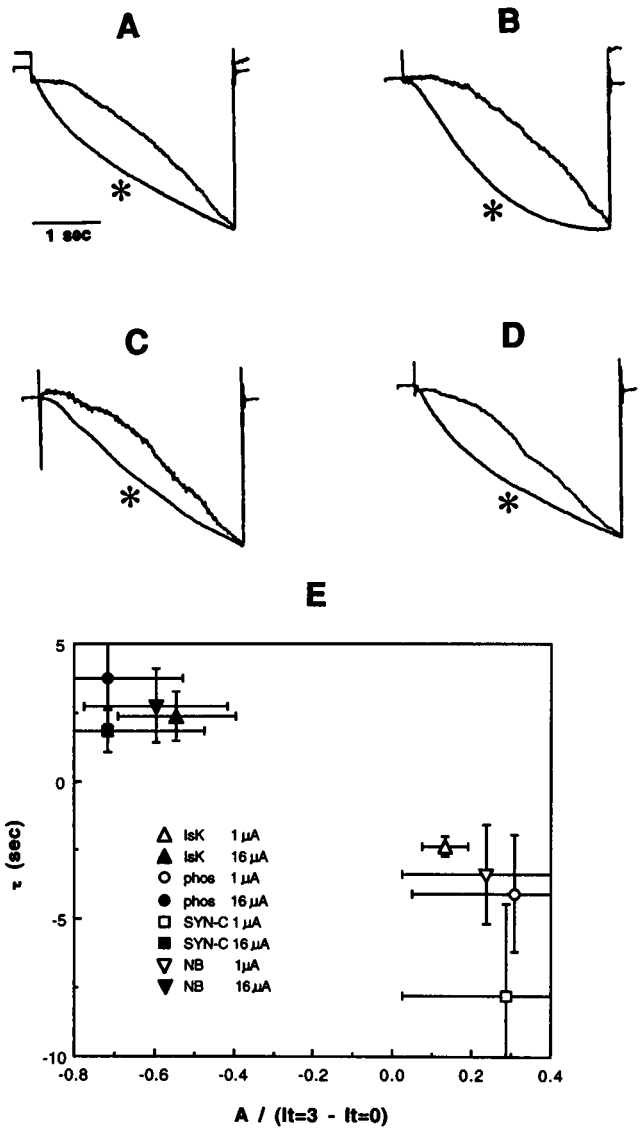


FIGURE 4 Dependence of time course of current upon current amplitude within a given batch of oocytes. The scaled time courses of the currents (evoked by a pulse to −130 mV) from four pairs of oocytes from the same batch are plotted together. For each pair the same type of mRNA, IsK (*A*), phospholemman (*B*), SYN-C (*C*), or NB (*D*) was injected. One oocyte from each pair had a small (~1 μA) and the other had a large (~16 μA) total current (*). (*E*) Quantification of shape of the current time courses from oocytes expressing small (*open symbols*) and large amplitude (*filled symbols*) currents from the same batch. The time constant τ is plotted against the quantity $[A/(I_{(t = 3\ sec)} - I_{(t = 0\ sec)})]$ from the equation $I(t) = C - A\ \exp(1-t/\tau)$, where $I(t)$ is the time course of the current and C is a constant. Note that the maximal rate of rise of current occurred later for the oocytes expressing the smaller currents. These differences were observed for each mRNA.

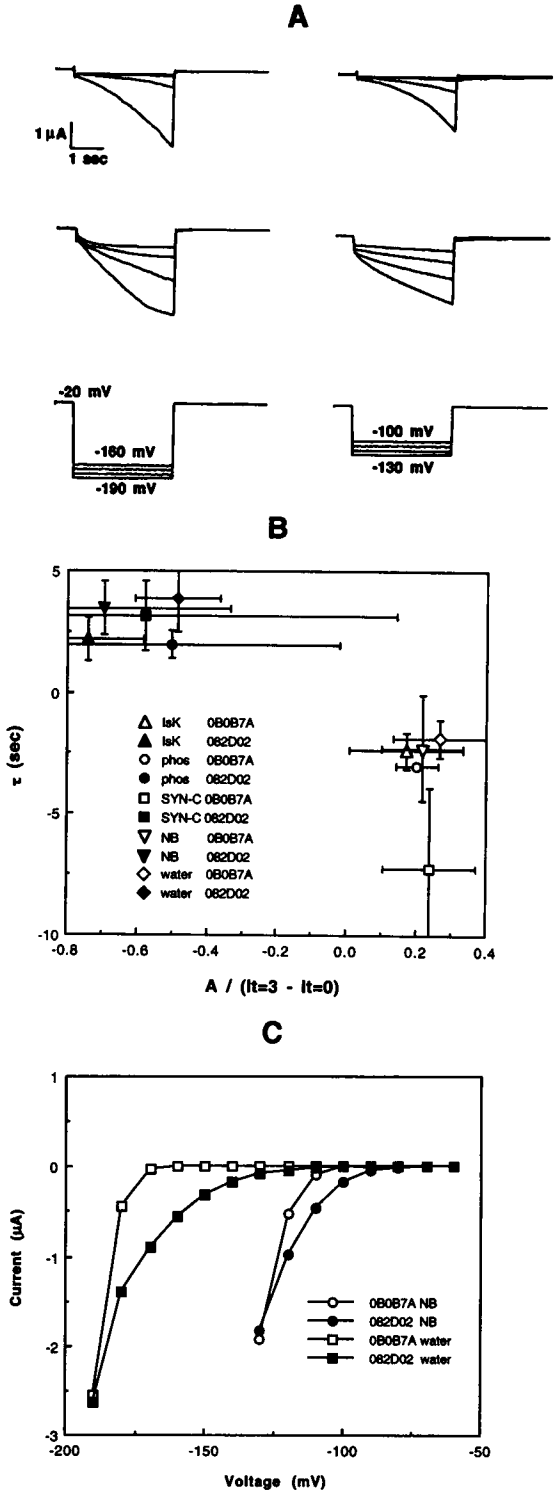
The time course of the currents could be well-fitted with this equation (R^2 >0.999). To distinguish the current time courses we plotted the quantity $[A/(I_{(t = 3\ sec)} - I_{(t = 0\ sec)})]$ against the time constant τ (Fig. 4 *E*). The data from the oocytes with small current amplitude (e. g., upper traces of Fig. 4, *A–D*) are seen to cluster in the lower right of the plot, regardless of which protein each oocyte expressed. A similar clustering in the upper left of the graph is seen for the oocytes expressing a high level of current. This quantitative analysis shows that the current amplitude, and not the nature of expressed protein, influences the time course of the voltage-activated current.

The dependence of the time course of the voltage-induced current on the batch of oocytes is illustrated for a pair of control oocytes and a pair of oocytes injected with mRNA encoding the NB protein in Fig. 5 *A*. The amplitude of the current evoked by a −190 mV activating pulse in the control oocytes and the amplitude of the current evoked by a −130 mV activating pulse in the NB-expressing oocytes were similar for these two batches (~−2 μA), but the time courses differed noticeably. For the first batch (*upper trace*), the maximal rate of rise of current could be seen for both the control oocyte and the NB-expressing oocyte to occur at the end of the pulse. For the second batch (*lower trace*), the maximal rate of rise occurred at the beginning of the pulse. We also quantified the shape of the time course of the currents by fitting Eq. 1 to the time courses for at least four oocytes injected with each mRNA or water from each batch. The time course of the currents could be well-fitted with this equation (R^2 >0.999). The data from two batches are compared in Fig. 5 *B*, where the time constant τ is plotted against the quantity $[A/(I_{(t = 3\ sec)} - I_{(t = 0\ sec)})]$. It can be seen that the data for the currents of all of the oocytes of batch 082D02 (*closed symbols*), regardless of whether they expressed one of the four proteins or were from control oocytes, are clustered in the upper left of the plot, and that the data from batch 0B0B7A (*open symbols*) are clustered in the lower right of the plot. Moreover, the shape of the current-voltage relationship (Fig. 5 *C*) depended on the batch of oocytes and not on the nature of the expressed protein. Results similar to these were obtained from a total of >200 oocytes from three pairs of batches. Thus, the time course of the slowly activated, inward current was a function of both the amplitude of the expressed current and the batch of oocytes that expressed the current but not the particular exogenous protein expressed in the oocyte.

Ion channel activity associated with IsK and SYN-C

For oocytes expressing the IsK protein and the SYN-C protein, we observed a current in addition to the slowly activated, inward current shown above (Fig. 1). For the former oocytes, this current was the slowly activated K^+ current evoked by depolarization that has been reported previously (Blumenthal and Kaczmarek, 1994; Follander et al., 1990; Pragnell et al., 1990; Takumi et al., 1988; Varnum et al., 1995), and for the latter oocytes, it was a time-independent current that summed with the voltage-activated, slow inward current described above (Fig. 1). We compared the ion selectivity of the time-independent current of oocytes that expressed the SYN-C protein (using ramp

analysis) with that of the voltage-activated, slow inward current (using tail-current analysis) and found it to differ in one respect: whereas the latter current was little affected by replacement of Na^+ in the bathing medium, the current-voltage relationship of the time-independent current was altered by replacement of external Na^+ (data not shown). We compared the effects of channel modulators and found that both components were inhibited by DIDS (1 mM, data not shown), increased by reduction of pH, and unaffected by amantadine (100 μM, data not shown). Finally, we noted that a change in the ion selectivity of the time-independent current occurred when the pH of the bathing medium was lowered. This change in ion selectivity was detected with ramp analysis and was evidenced by a shift in the reversal voltage to a more positive value (Fig. 6, *solid lines*) when the pH of the bathing medium was lowered from pH 7.5 to pH 6.2. On the other hand, no alteration in the ion selectivity was observed for the voltage-activated current, as determined by tail-current analysis (Fig. 6, ○ versus ●). This pH-induced alteration of the ion selectivity of the time-independent current, but not of the voltage-activated slow inward current, demonstrates that these two components of current of ooyctes expressing the SYN-C protein are generated by different conductance mechanisms.



Dependence of inward current on amount of expressed protein

The above observations suggested that the slowly activated, inward current may be influenced by the expression of certain exogenous proteins. If this were so, then it would be expected that the extent of this influence would depend on the quantity of exogenous protein expressed. Therefore, we used quantitative immunoblotting to measure the amount of the IsK, SYN-C, and NB proteins expressed in individual oocytes. This was done after measurement of the amplitude of the time-dependent inward current for oocytes that expressed each of these proteins. In addition, the amplitude of the outward K^+ current of oocytes that expressed the IsK protein and the amplitude of the time-independent current of oocytes expressing the SYN-C protein (Fig. 6) were also measured. To compare these results with those from a protein that has been shown to possess intrinsic ion channel activity, the amantadine-sensitive current of the M_2 protein at pH 6.2 was plotted against the mass of M_2 protein expressed in individual oocytes (Fig. 7 *D*). For oocytes that expressed the IsK protein (Fig. 7 *A*), the amplitude of the

FIGURE 5 Time course and current-voltage relationship of inward current depended upon oocyte batch and not expressed protein. (*A*) Time course of inward current for two batches that had currents of approximately the same amplitude. Upper traces are from one batch (0B0B7A) and middle traces are from another batch (082D02) of oocytes. Lower traces show the applied voltages. Left records are from oocytes that were injected with water, and right records are from oocytes that expressed NB protein. (*B*) Quantification of shape of the current time courses from oocytes from two batches. The time constant τ is plotted against the quantity $[A/(I_{(t = 3\ sec)} - I_{(t = 0\ sec)})]$ from the equation $I(t) = C - A \exp(1 - t/\tau)$, where $I(t)$ is the time course of the current and C is a constant. (*C*) Current-voltage relationship for oocytes shown in (*A*). Control oocytes: batch 0B0B7A (□), batch 082D02 (■). Oocytes expressing NB protein: batch 0B0B7A (○), batch 082D02 (●). Note that the maximal rate of rise of current occurs later for the oocytes from batch 0B0B7A, regardless of the expressed protein, that the parameters describing the shape of the time course of the current were clustered for a given batch of oocytes, and that the shape of the current-voltage relationship depended upon the batch of oocytes.

outward current was proportional to the amount of protein expressed for small amounts of protein, and it decreased for larger amounts of expressed protein, as observed previously (Attali et al., 1993). For oocytes that expressed the SYN-C protein (Fig. 7 *B*), the amplitude of the time-independent inward current was also proportional to the amount of protein expressed. However, for oocytes that expressed the IsK and SYN-C proteins (Fig. 7, *A* and *B*), the slowly activated, inward current was not detected in oocytes that expressed a small amount of protein, and only for oocytes that expressed more than a characteristic minimum amount of protein was it possible to detect this current. For oocytes that expressed more than this characteristic minimum amount of protein, further increases in expression resulted in proportional increases in the amplitude. For oocytes that expressed the NB protein, the amplitude of the inward current tended to increase with increased protein expression (Fig. 7 *C*); however, when the amount of NB protein expressed was just above the lower limit of detection, the inward current was not consistently observed, suggesting that for the NB protein as well there is a characteristic minimum amount of protein above which the inward current is consistently expressed. In contrast, oocytes that expressed the M_2 protein of influenza A virus did not demonstrate a voltage-activated current, regardless of the mass of M_2 protein they expressed, and the amplitude of current that these oocytes expressed was proportional to the mass of expressed protein (Fig. 7 *D*). Thus, the amplitude of the slowly activated, inward current of an oocyte depended on the amount of protein expressed: for oocytes expressing more than a certain characteristic critical amount of the proteins IsK, SYN-C, and NB, the current amplitude was proportional to the amount of protein, but for oocytes that expressed less than the characteristic minimum amount of these proteins, no inward current could be detected.

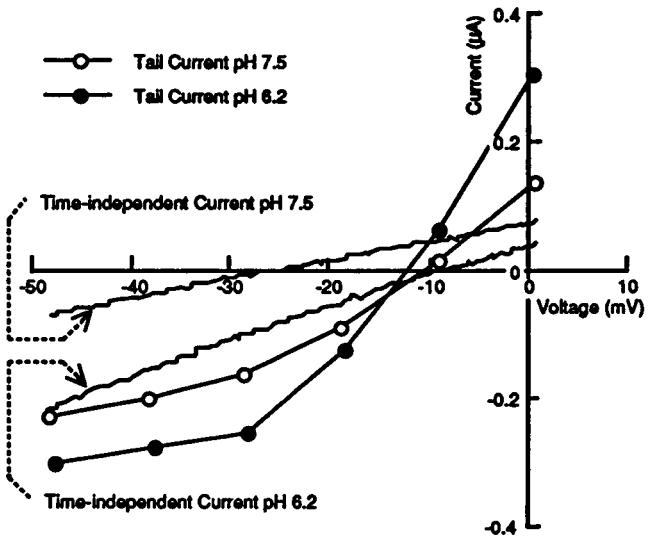


FIGURE 6 Separation of currents of an oocyte expressing the SYN-C protein. The ion selectivity of the slowly activated, inward current was measured by tail current analysis. The current-voltage relationship for the tail currents was measured while the oocyte was bathed at pH 7.5 (○) or pH 6.2 (●). The ion selectivity of the time-independent component of current was measured by ramp analysis and the current-voltage relationship plotted in the lines for the same two values of pH. Note that lowering pH did not alter the reversal voltage of the tail currents but did shift the reversal voltage of the time-independent current to more positive values.

DISCUSSION

Mechanism for the slowly activated, inward current

We found that an inward current was slowly activated by strong hyperpolarization (to ~−190 mV) of control *Xenopus* oocytes. A current with similar time course could be activated by lesser hyperpolarization (to ~−130 mV) of oocytes that expressed any one of four small integral membrane proteins (two cellular in origin, IsK and phospholemman; one synthetic, SYN-C; and one viral in origin, NB) in amounts greater than a characteristic minimum level. The currents recorded from control oocytes and oocytes expressing these proteins had similar ion selectivity (for the halides); were modulated in similar ways by several organic ion channel blockers, divalent cations, and pH; and had similar equivalent gating charge. However, voltage-activated currents were not recorded from oocytes that expressed the M_2 protein of the influenza A virus. These results can be explained by two reasonable possibilities: 1) each of the small integral membrane proteins is a channel per se and is intrinsically capable of generating a conductance that is activated by modest hyperpolarization but is otherwise indistinguishable from the conductance formed by an endogenous protein that can be activated only by very large hyperpolarizations; and 2) each of these small integral membrane proteins modifies the activation properties of an endogenous conductance in a similar way, while leaving unaltered its ion selectivity and blocker profile.

One key observation suggests that the latter possibility is correct. We noted that the time course of the slowly activated, inward current during a hyperpolarizing voltage clamp pulse (the activation kinetics) varied from batch to batch (Fig. 5). This variation could not be explained by the dependence of time course on the maximum amplitude of the current (Fig. 4). The time course varied from batch to batch of oocytes. The oocytes of some batches had a maximal rate of rise of current that occurred early during the voltage clamp pulse, whereas the maximal rate of rise of the current of other batches with comparable amplitude occurred later in the pulse (Fig. 5, *A* and *B*), independent of the identity of the expressed protein. The variation of the time course from batch to batch was confirmed by quantitative analysis of the time courses (Fig. 5 *B*). These observations show that the time course of the current was not an exclusive property of the expressed protein (and in fact did not depend on the expression of exogenous protein at all), but rather depended on both the amplitude of the current and the batch of oocytes. This suggests that the time course depends

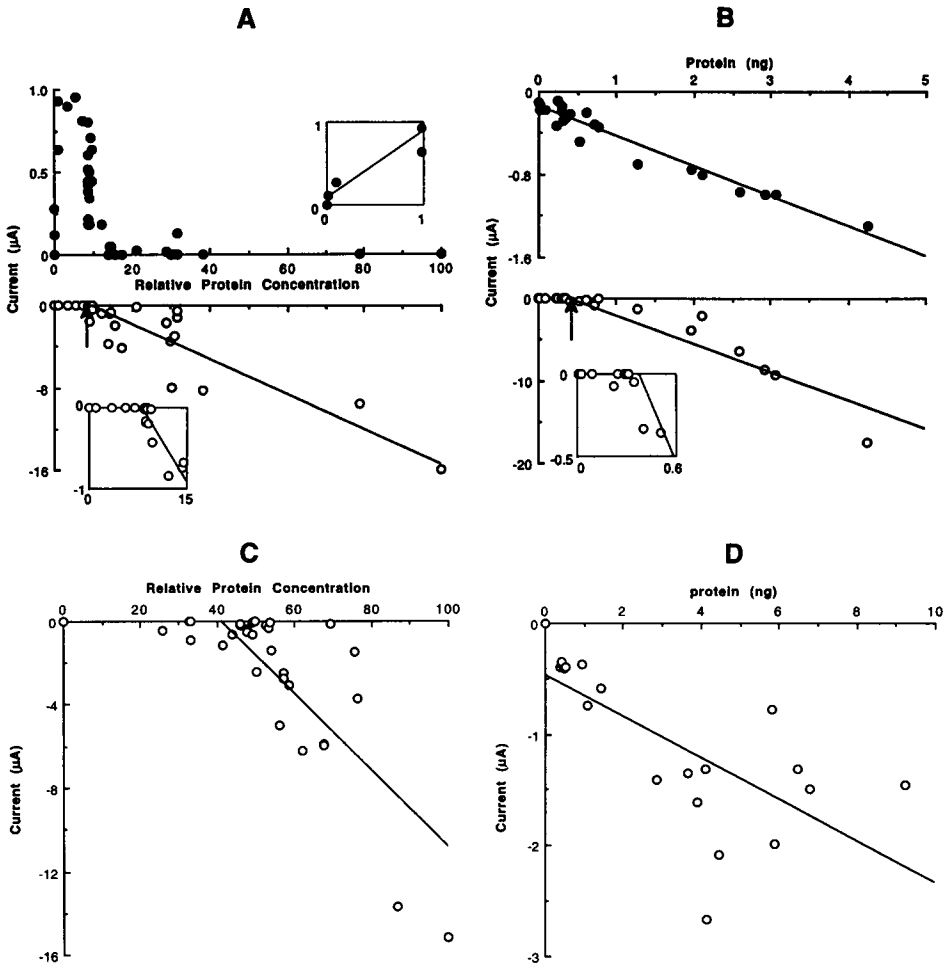


FIGURE 7 Relationship between current amplitude and amount of protein expressed for individual oocytes expressing the IsK, SYN-C, NB, and M_2 proteins. (*A*) (IsK) The amplitudes of the outward current evoked by depolarization to +40 mV (●, *upper panel*) and the slowly activated, inward current evoked by hyperpolarization to −130 mV (○ *lower panel*) are plotted against the relative amount of IsK protein expressed. Insets show the relationships near the origin. (*B*) (SYN-C) The amplitude of the time-independent, pH sensitive (●, *upper panel*) and slowly activated, inward (○, *lower panel*) components of current are plotted against the amount of SYN-C protein expressed. Inset shows the relationships near the origin. (*C*) (NB) The amplitude of the slowly activated, inward current is plotted against the relative amount of NB protein expressed. (*D*) M_2 protein of influenza A virus. The amplitude of the amantadine-sensitive current at pH 6.2 is plotted against the mass of M_2 protein expressed. Note that for IsK, SYN-C, and NB proteins the inward, voltage-activated currents were elicited only from oocytes that expressed an amount of protein greater than a certain characteristic minimum level (*arrows* in *A* and *B*). The amplitudes of the outward current for IsK (*inset in A, upper panel*), the time-independent current for SYN-C (*B, upper panel*), and the amantadine-sensitive current of the M_2 protein (*D*) were proportional to the amount of protein expressed in the oocyte.

on a factor intrinsic to the oocyte. We suggest that this factor is the protein or proteins responsible for the endogenous current.

Our results are consistent with but do not prove that SYN-C has an intrinsic ion channel activity. This conclusion is contrary to our initial expectation that this mutant form of influenza virus M_2 protein would have its ion channel activity ablated because an extra residue was added to the transmembrane domain. Oocytes expressing the SYN-C protein expressed a time-independent current that differed in ion selectivity from the slowly activated, inward current (Fig. 6). For SYN-C-expressing oocytes, the amplitude of the time-independent current was proportional to the amount of expressed protein, but the slowly activated current was observed only for oocytes that expressed amounts of SYN-C protein greater than a characteristic minimum level (Fig. 7 *B*). The properties of the slowly activated current were similar to those of the endogenous current, but the properties of the time-independent current differed from those of the endogenous current because reduction of $[Na^+]$ shifted the reversal voltage. These results are consistent with the interpretation that the time-independent current was intrinsic to the SYN-C protein. The simplest explanation for the various currents we measured is that 1) the IsK and SYN-C proteins each have intrinsic ion channel activity that can be seen with low levels of protein expression and 2) higher levels of expression of each of these proteins permit modest hyperpolarization to induce a slowly activated, inward current that is capable of being activated only by strong hyperpolarization in control oocytes.

Our principal findings can be explained by an endogenous anion channel that is activated only by strong hyperpolarization in the absence of exogenous proteins but is modified by large amounts of certain exogenous proteins to allow activation with modest hyperpolarization. The exogenous proteins may either possess intrinsic ion channel activity (IsK, SYN-C) or not. In this model, the exogenous proteins are thought to act in a manner similar to auxiliary subunits of the Na^+ channel, which shift the activation curve without modifying the other properties of the channel significantly (reviewed in Isom et al., 1994). We find it intriguing that the four small integral membrane proteins we found able to modify the activation properties of the endogenous Cl^- channel are all type III integral membrane proteins. However, there is no significant sequence homology among these proteins, nor did we undertake experiments, e.g., those involving the use of deletion mutants of the IsK protein (Attali et al., 1993), to determine which portion of the phospholemman, SYN-C, and NB proteins interact with the endogenous Cl^- channel to modify its activation properties.

Implications for IsK and phospholemman

Our results cannot definitively distinguish among the possibilities that the K^+ current of IsK-expressing oocytes results from 1) ion channel activity intrinsic to the IsK protein (Goldstein and Miller, 1991; Varnum et al., 1995), 2) association of the IsK protein with a factor endogenous to the oocyte (Blumenthal and Kaczmarek, 1994), or 3) activation of an otherwise silent endogenous K^+ channel by the IsK protein (Attali et al., 1993).

Our results are not consistent with the IsK and phospholemman proteins having intrinsic anion channel activity that can be evoked by modest hyperpolarization (to ~ -130 mV) of *Xenopus* oocytes. This conclusion differs from that proposed for the phospholemman protein on the basis of an altered time course of current resulting from mutation of the presumed transmembrane domain (Moorman et al., 1992), a conclusion that is supported by the recording of ion channel activity in phospholemman-containing lipid bilayers (Ackerman et al., 1994a). However, time course is not a defining property of the ion channel, and ion channel activity in bilayers can result from the expression of nonchannel peptides (Lear et al., 1988; Tosteson et al., 1989; Tosteson et al., 1988). Thus, we feel that the more likely explanation is that the IsK and phospholemman proteins modify the properties of an endogenous oocyte ion channel. It may well be that a modification of other channels also occurs in the cells in which the IsK and phosopholemman proteins are endogenous.

We do not know the identity of the protein that causes the slowly activated current we measured in every one of hundreds of control oocytes from every batch studied. However, the properties of the current we measured do not match those of any of a number of hyperpolarization-activated Cl^- currents that have been described for the *Xenopus* oocyte (Ackerman et al., 1994b; Kowdley et al., 1994; Parker and Miledi, 1988; Peres and Bernardini, 1983).

Implications for influenza A and B viruses

It is emphasized that although the experiments described here grew from studying the possible ion channel activity of viral small integral membrane proteins, the data reported here for the IsK, phospholemman, SYN-C, or NB proteins have no relationship to the influenza A virus M_2 protein ion channel activity. The M_2 protein of influenza A virus has intrinsic ion channel activity in oocytes (Pinto et al., 1992), in mammalian cells (Wang et al., 1994), and when reconstituted in lipid bilayers (Tosteson et al., 1994). However, expression of this protein did not permit modest hyperpolarization (to ~ -130 mV) to evoke a slowly activated, inward current (Fig. 1 *F*), even for oocytes that expressed a large mass of M_2 protein (Fig. 7 *D*). In fact, hyperpolarization as strong as that needed to evoke the inward current from control oocytes (to ~ -190 mV) had to be applied to oocytes that expressed the M_2 protein in order to evoke the slowly activated, inward current. Although an intrinsic ion channel activity of the influenza B virus NB protein has been anticipated (Duff and Ashley, 1992; Hay, 1992), it has not been demonstrated here. However, the experiments described here, which suggest that a viral protein can mimic cellular proteins in modifying the activation properties of an ion channel, have important implications for the consequences of viral infection. First, it is possible that other viral proteins modify the properties of ion channels of the host cell. Secondly, the possibility of modification of host cell ion channels by viral proteins should be considered when using viral vectors for gene therapy.

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