Induction of Endogenous Channels by High Levels of Heterologous Membrane Proteins in *Xenopus* Oocytes

T. Tzounopoulos,*‡ J. Maylie,§ and J. P. Adelman*‡
*Vollum Institute, ‡Department of Molecular and Medical Genetics, and §Department of Obstetrics and Gynecology, Oregon Health Sciences University, Portland, Oregon 97201 USA

ABSTRACT *Xenopus* oocytes are widely employed for heterologous expression of cloned proteins, particularly electrogenic molecules such as ion channels and transporters. The high levels of expression readily obtained permit detailed investigations without interference from endogenous conductances. Injection of min K mRNA into *Xenopus* oocytes results in expression of voltage-dependent potassium-selective channels. Recent data show that injections of high concentrations of min K mRNA also induce a chloride current with very different biophysical, pharmacological, and regulatory properties from the min K potassium current. This led to the suggestion that the min K protein acts as an inducer of endogenous, normally silent oocyte ion channels. We now report that high levels of heterologous expression of many membrane proteins in *Xenopus* oocytes specifically induce this chloride current and a hyperpolarization-activated cation-selective current. The current is blocked by 4,4′-diisothiocyanostilbene-2–2′-disulphonic acid and tetraethylammonium, enhanced by clofilium, and is pH-sensitive. Criteria are presented that distinguish this endogenous current from those due to heterologous expression of electrogenic proteins in *Xenopus* oocytes. Together with structure-function studies, these results support the hypothesis that the min K protein comprises a potassium-selective channel.

INTRODUCTION

Xenopus oocytes have become a widely employed avenue for expression of foreign proteins, most notably electrogenic molecules such as ion channels and transporters. Among the advantages of the oocyte expression system is the ability to obtain high levels of expression, permitting detailed quantitative analysis of currents essentially free of contaminating endogenous currents. Beginning with *Shaker*, voltage-gated potassium channels have been expressed and extensively studied in *Xenopus* oocytes, using depolarizing voltage protocols (Timpe et al., 1988; Christie et al., 1989; Pongs et al., 1989). Recently, a new family of potassium channel subunits has been described, the inward rectifiers, and investigation of the structure and function of this family of channels has become a central theme in many laboratories. Inward rectifiers readily pass current at potentials negative to E_K while current is decreased at more positive potentials, therefore hyperpolarizing voltage protocols are employed (Kubo et al., 1993a,b; Ho et al., 1993; Dascal et al., 1993; Bond et al., 1994). Because of the high levels of expression obtained in *Xenopus* oocytes, contributions by endogenous channels to current amplitudes are not considered significant. However, under some circumstances this assumption is incorrect and may lead to erroneous interpretations.

For example, Atali et al. (1993) reported that injection of high concentrations of min K mRNA into *Xenopus* oocytes induces a hyperpolarization-activated chloride current in addition to the depolarization-activated potassium current normally associated with expression of the min K protein. Moreover, the hyperpolarization-activated chloride current shows a pharmacological profile different from the potassium current, and two mutations in min K ablate the ability to specifically induce either the potassium or the hyperpolarization-activated chloride current. These results suggested that the min K protein does not itself form an ion channel, but acts as an inducer of endogenous oocyte channels (Attali et al., 1993).

This hypothesis contrasts with previous data. The characteristic feature of oocytes injected with min K mRNA is a depolarization-activated potassium current, I_{sK}, with activation kinetics on the order of seconds. Structurally, the 129-amino acid human min K protein is strikingly different from any other cloned potassium channel, containing only a single predicted transmembrane domain with the N-terminus residing outside and the C-terminus residing inside the cell (Takumi et al., 1988; Blumenthal and Kaczmarek, 1994; Williams et al., 1994). There is no obvious voltage sensor, and the hallmark pore sequence of other potassium channels is not present. However, mutations in the predicted transmembrane domain resulted in alterations of ion selectivity, open channel block and gating (Goldstein and Miller, 1991; Takumi et al., 1991), supporting the role of min K as a channel-forming protein.

We now report that high levels of expression of many membrane proteins in *Xenopus* oocytes induces a hyperpolarization-activated current with pharmacology similar to the current seen after injection of high concentrations of min K mRNA. The current can reach large amplitude and may be mistaken for currents flowing through experimental, heterologously expressed channels. Taken together, these

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Address reprint requests to Dr. John P. Adelman, Vollum Institute, mail code L-474, Oregon Health Sciences University, 3181 Sam Jackson Park Rd., Portland, OR 97201-3098. Tel.: 503-494-5450; Fax: 503-494-4976; E-mail: adelman@ohsu.edu.

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results support the role of the min K protein as a potassium channel pore-forming subunit, and provide a thorough set of criteria that distinguish the hyperpolarization-activated endogenous current from currents due to heterologously expressed electrogenic molecules.

MATERIALS AND METHODS

Oocyte handling and mRNA preparation were performed as previously described (Adelman et al., 1992). *Xenopus* care and handling were in accordance with the highest standards of institutional guidelines. Frogs underwent no more than two surgeries, separated by at least 3 weeks, and surgeries were performed using well established techniques by experienced members of the Animal Care staff. Frogs were anesthetized with an aerated solution of 3-aminobenzoic acid ethyl ester. In all cases, 50 ng of mRNA (in 50 nl water) was injected into each oocyte. Standard recording solution contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM CaCl, and 5 mM HEPES (pH 7.4), unless otherwise stated. Recordings were performed at 22°C with a CA-1 amplifier interfaced to a DEC LSI 1173 computer. The shift in reversal potential is given with standard error of estimate. Human min K was the generous gift of Dr. Eric Honore, and was cloned in the vector pBTG (Attali et al., 1993). *Shaker* H4-IRW434F was the generous gift of Dr. Rod MacKinnon and was subcloned in pKS. EAAT2 was provided by Drs. Mike Kavanaugh and Jeff Arriza, and was subcloned in pOTV (Bunzow et al., 1988); the rat dopamine D2 receptor was provided by Jim Bunzow and β-galactosidase was from Dr. Thom Segerson.

RESULTS

Expression of membrane and cytoplasmic proteins in *Xenopus* oocytes

To further investigate the relationship between expression of the min K protein and ion channel activity in *Xenopus* oocytes, six structurally and functionally distinct proteins were expressed: 1) the min K protein with a single transmembrane domain (TM; Takumi et al., 1988; Blumenthal and Kaczmarek, 1994; Williams et al., 1994); 2) the six-TM, voltage-dependent *Shaker* potassium channel, ShH4-IR, with a mutation, W434F (ShW434F), which eliminates potassium currents but retains gating currents (Timpe et al., 1988; Taglialatela et al., 1992); 3) BIR9, a nonconducting member of the inward rectifier potassium channel family clone predicted to possess two TMs (Bond et al., 1994); 4) the EAAT2 amino acid transporter, thought to contain 6–10 TMs (Arriza et al., 1994); 5) the dopamine D2 G-protein coupled receptor with seven TMs (Bunzow et al., 1988); and 6) the cytoplasmic protein, β-galactosidase. Currents were monitored in the two-electrode voltage clamp. Oocytes injected with min K mRNA showed slowly activating, potassium-selective currents upon prolonged depolarizing commands (Fig. 1 *a*). *Shaker* W434F did not demonstrate potassium currents, but short depolarizing commands evoked distinct gating currents (Fig. 1 *b*; Taglialatela et al., 1992). Oocytes injected with EAAT2 mRNA resulted in an inward transporter current only upon application of glutamate and at potentials negative to 0 mV (Fig. 1 *c*; Arriza et al., 1994). Oocytes injected with BIR9 mRNA did not produce K^+ currents different from noninjected oocytes, as previously reported (Fig. 1 *d*; Bond et al., 1994). Dopamine D2 receptors and β-galactosidase are non-conducting molecules, and oocytes injected with either of these mRNAs did not show currents different from noninjected oocytes following depolarizing commands (Fig. 1, *e* and *f*).

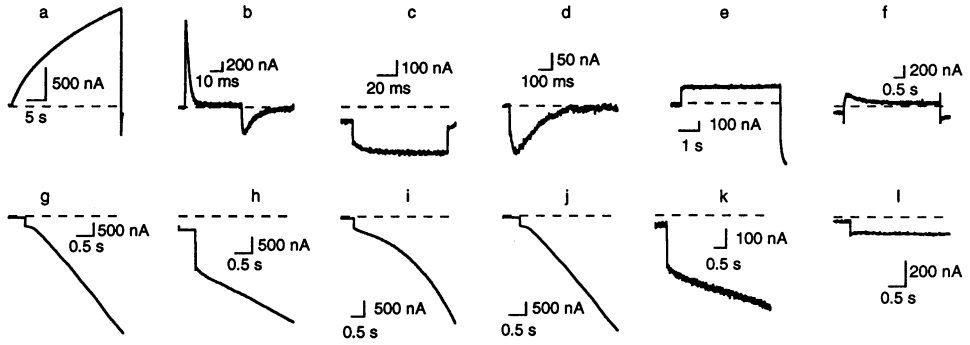

FIGURE 1 Current records from oocytes expressing (*a*) min K, (*b*) ShW434F, (*c*) EAAT-2, (*d*) BIR9, (*e*) D2 receptors, and (*f*) β-galactosidase. Min K currents were evoked by 30-s depolarizing commands to 20 mV from a holding potential of −50 mV. ShW434F gating currents were evoked by a 100-ms command to −10 mV from a holding potential of −100 mV; currents were leak-corrected. EAAT2 currents were elicited by a 100-ms command to −80 from a holding potential of −30 mV; traces represent the difference of the currents recorded in the presence and absence of 1 mM L-glutamate. Oocytes injected with BIR9 mRNA were recorded in 90 mM K^+ (substituted for sodium). 500-ms commands to −80 mV were delivered from a holding potential of −10 mV. Oocytes injected with D2 receptor or β-galactosidase mRNA were subjected to 5-s depolarizing commands to 20 mV from a holding potential of −50 mV. Hyperpolarization-activated inward currents were induced by high levels of expression of membrane proteins, but not β-galactosidase (*g–l*). Currents were elicited by a 3-s command to −130 mV from a holding potential of −50 mV. For the traces shown, calcium was replaced by cobalt (1 mM). No independent confirmation of the expression or membrane locale of the BIR9 protein was obtained. However, BIR9 is clearly a member of the inward rectifier potassium channel family, and in vitro translations of BIR9 mRNA yield a product of the predicted molecular mass (not shown). Efficient expression of dopamine receptors was verified in control experiments by application of dopamine (60 μM), in concert with hyperpolarizing commands, to oocytes coinjected with D2 receptor and GIRK mRNA (Kubo et al., 1993; Dascal et al., 1993); inward potassium currents were detected (not shown). β-galactosidase expression was verified by enzyme assay (Miller, 1972). These sequences, as well as BIR9, were subcloned into pSelect$^-$ (Adelman et al., 1992).

Hyperpolarization-activated currents

When oocytes were examined 2 days after injection, hyperpolarizing commands evoked little if any time-dependent current. However, when hyperpolarizing commands were delivered 4–8 days after injection, oocytes from each group except those expressing β-galactosidase showed slowly activating, inward currents that were usually not present in noninjected oocytes (Fig. 1, *g–l*). Decreasing the external chloride concentration shifted the tail current reversal potential consistent with the Nernst equation for a chloride-selective conductance; the slope of the reversal potential as a function of log $[Cl^-]_o$ was 53 ± 3 mV/10-fold change (n = 13, not shown).

The characteristics of this inward current were different among different batches of oocytes. Even within the same batch of oocytes, considerable variability was observed in the amplitude (1–10 μA) and time course of the appearance of the currents after injection. When calcium was removed from the bath solution, the hyperpolarization-activated current became distinctly more uniform in amplitude and kinetics. Fig. 2 shows current families and tail currents in the presence (Fig. 2, *A* and *B*) and absence (Fig. 2, *C* and *D*) of external calcium. With calcium present in the bath solution, large tail currents which reversed direction at approximately −20 mV were observed (Fig. 2 *B*). However, when calcium was removed from the extracellular solution, tail current amplitudes were greatly reduced and the reversal potentials were no longer shifted by altering the concentration of chloride in the bath solution (Fig. 2 *D*). Therefore, ion selectivity was investigated in the absence of extracellular calcium. Table 1 shows the reversal potentials of the hyperpolarization-activated current under biionic conditions for K^+, Na^+ and Cs^+. These results demonstrate that in the absence of external calcium, the hyperpolarization-activated current is mediated by a nonselective cation channel.

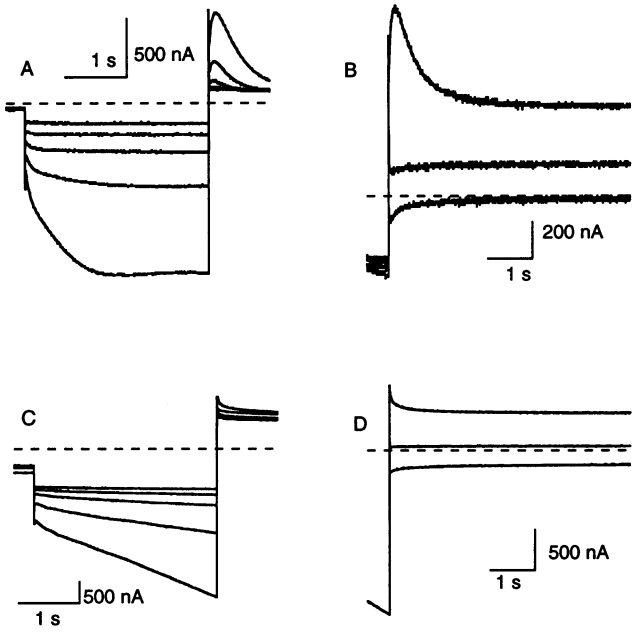

FIGURE 2 Current families and tail currents in the presence or absence of extracellular Ca^{2+}. (*A*) Currents elicited by 3-s commands from −80 to −160 mV in standard recording solution, tail currents measured at 20 mV. (*B*) Tail currents measured in standard recording solution at 20, −20, and −40 mV after a 3-s prepulse to −140 mV. (*C*) Same as in (*A*) except in the absence of extracellular Ca^{2+}. (*D*) Tail currents measured at 20, −40, and −60 mV after a 3-s prepulse to −140 mV. Bath solution contained 180 mM mannitol, 10 mM HEPES, 1 mM $MgCl_2$, and 10 mM KCl (predicted E_{Cl^-} = ~19 mV).

Pharmacology of the hyperpolarization-activated nonselective cation current

The pharmacology of the hyperpolarization-activated nonselective cation current was investigated in calcium-free bath solution. In all cases, the current was specifically blocked by 4,4′-diisothiocyanostilbene-2–2′-disulphonic acid (DIDS), with a 50% inhibitory concentration (IC_{50}) of 0.5 mM (n = 5, Fig. 3 *A*), whereas DIDS had no effect on *Shaker* W434F gating currents or EAAT2 transporter currents (not shown). In addition, 100 mM tetraethylammonium (TEA) blocked ~50% of the current (Fig. 3 *B*). As previously reported, 100 μM clofilium blocked ~50% of the min K potassium current (n = 5, not shown), while it had no effect on *Shaker* W434F gating currents or EAAT2 transporter currents (not shown), but in all cases increased the nonselective cation current (Fig. 3 *C*). Varying the pH of the external solution altered the nonselective cation current; increasing pH from 6.5 to 8.2 decreased the current by ~40% (n = 12; Fig. 3 *D*). Similar results were obtained in calcium-containing bath solution, demonstrating that this current is the same as that previously reported for oocytes expressing high levels of the min K protein (Attali et al., 1993).

The hyperpolarization-activated nonselective cation current was not usually detected until high levels of heterologous expression were achieved. Oocytes injected with the same amount of ShW434F mRNA were studied at progressively later times after injection. Fig. 4 shows the relationship between the amount of ShW434F off-gating charge, which presumably correlates with the number of channels in the membrane, and the appearance of the hyperpolarization-activated nonselective cation current. Although gating currents were clearly present, the nonselective cation current was not detected until gating charge movement surpassed 8 nanocoulombs (nC) in magnitude. Similar results were ob-

TABLE 1 Ion selectivity in the absence of extracellular Ca^{2+}

	K^+	Na^+	Cs^+
E_{rev} (mV)	−50 ± 3	−52 ± 2	−53 ± 2
P_X/P_K	1 ± 0.1	0.92 ± 0.1	0.88 ± 0.1

Reversal potentials were measured under biionic conditions, with 10 mM of the indicated cation in the external solution, which also contained 180 mM mannitol, 10 mM Hepes, and 1 mM $MgCl_2$. Values represent the mean ± SEM of four to six oocytes. Permeability ratios are defined as $P_X/P_K = \exp(F\Delta E_{rev(x-k)}/RT)$, where $\Delta E_{rev(x-k)} = E_{rev,X} - E_{rev,K}$.

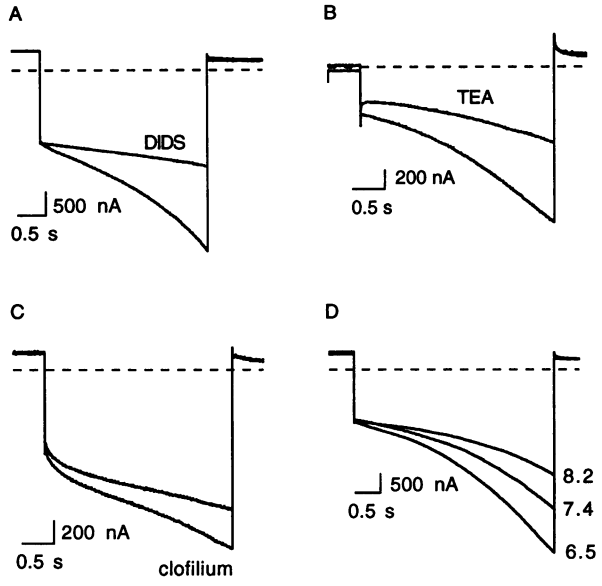

FIGURE 3 Pharmacology of the hyperpolarization-activated current. Effect of: (*A*) DIDS (1 mM), (*B*) TEA (100 μM), (*C*) clofilium (100 μM), (*D*) alteration of external pH. Oocytes injected with EAAT2 mRNA were recorded 4–7 days after injection in the absence of externally applied L-glutamate. Following a 3-s prepulse to 20 mV, currents were elicited by 3-s hyperpolarizing commands to −130 mV from a holding potential of −50 mV. To block residual calcium channels and screen surface charge, calcium was replaced by cobalt (1 mM) in the standard recording solution.

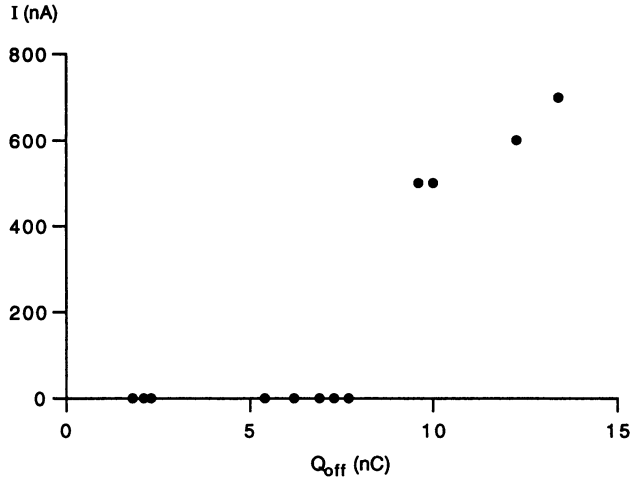

FIGURE 4 Relationship between the amount of ShW434F off-gating charge and the hyperpolarization-activated chloride current. Oocytes were injected with the same amount of mRNA and studied at progressively later times after injection. Currents were evoked as described in Fig. 1. The off-gating charge (Q_{off}) was obtained as the integral of the off-gating current. Total capacitance ranged from 200 to 225 nF. Each data point represents a single oocyte.

tained for each of the membrane proteins; only at relatively late times after injection was the hyperpolarization-activated nonselective cation current detected.

DISCUSSION

These results demonstrate that high levels of expression of many membrane proteins in *Xenopus* oocytes result in the induction of an endogenous hyperpolarization-activated current (Tzounopoulos et al., 1995; Shibo et al., 1995). In the presence of calcium, the current comprises two components, a calcium-activated chloride current and a nonselective cation current; when calcium is eliminated from the extracellular solution, only the nonselective cation current remains. It is possible that the calcium-activated chloride current is induced by calcium flowing into the cell through the nonselective cation channel. The hyperpolarization-activated current does not require extracellular calcium for activation, is blocked by DIDS and TEA, is accentuated by clofilium, and is pH-sensitive. The current was occasionally detected in some noninjected oocytes, but high levels of heterologously expressed membrane proteins consistently induced the current, while in oocytes expressing moderate levels, the current was either not detectable or present only in some oocytes.

Several chloride currents have previously been reported from oocytes (Miledi and Parker, 1984; Parker and Miledi, 1988). Recently, an endogenous chloride conductance of variable amplitude and kinetics in *Xenopus* oocytes, which resembles the conductance we observed in the presence of external calcium, has been described (Kowdley et al., 1994). Removal of external calcium dramatically reduced the variability in amplitude and kinetics, revealing the underlying nonselective cation current, suggesting that two distinct currents underlie the conductance described by Kowdley et al. (1994).

The hyperpolarization-activated current was consistently detected when heterologous expression of membrane proteins reached high levels. Although the membrane proteins were not specifically quantified, we assume that longer time after injection of a constant amount of mRNA is correlated with higher levels of expression. For electrogenic molecules this is clearly seen as a correlation between time after injection and current amplitudes. Alternatively, Attali et al. (1993) compared oocytes injected with different concentrations of min K mRNA at the same time post-injection and found that only when the concentration of injected mRNA exceeded 100 ng/μl was the hyperpolarization-activated current detected. As shown in Fig. 4, gating charge, presumably reflecting the number of channels on the oocyte surface, was quantitatively correlated with the appearance of the hyperpolarization-activated current. Therefore, it is important to examine currents elicited by hyperpolarizing protocols for contamination by this endogenous current. The dependence of the tail current reversal potential on external calcium and the distinct pharmacology of the current serve as defining characteristics that distinguish the endogenous hyperpolarization-activated current from those arising from heterologously expressed proteins.

Attali et al. (1993) reported that high levels of expression of the min K protein in *Xenopus* oocytes resulted in both a potassium and a chloride current. These results were inter-

preted as demonstrating that the min K protein is not a channel-forming protein, but rather an inducer of otherwise silent oocyte channels. However, the hyperpolarization-activated current observed by Attali et al. (1993) is pharmacologically indistinguishable from that reported here following high levels of expression of five structurally and functionally distinct membrane proteins. Therefore, the hyperpolarization-activated current described by Attali et al. (1993) is not specifically induced by the min K protein. These results, taken together with the results of Goldstein and Miller (1991) and Takumi et al. (1991), who demonstrated that point mutations in the hydrophobic domain of the min K protein result in alterations of conduction and pore properties as well as gating, strongly suggest but do not prove that the min K protein forms a potassium-selective ion channel.

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