Gating of I_{sK} Expressed in *Xenopus* Oocytes Depends on the Amount of mRNA Injected

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ABSTRACT I_{sK} is a K^+ channel of the delayed rectifier type widely distributed throughout both excitable and nonexcitable cells. Its structure is different from other cloned K^+ channels and molecular details of its gating remain obscure. Here we show that the activation kinetics of I_{sK} expressed in *Xenopus* oocytes depend upon the amount of its mRNA injected, with larger amounts resulting in slower activation kinetics with a longer initial delay during activation. Similar changes in activation kinetics occur with time after a single injection of I_{sK} mRNA. We present two kinetic schemes which illustrate how our experimental results could arise. Both imply an interaction among individual channel proteins during I_{sK} activation. The dependence of channel gating on mRNA concentration provides a novel mechanism for long term regulation of ion current kinetics.

INTRODUCTION

Potassium channels are found ubiquitously throughout all cell types. Among their many physiological roles they are important determinants of the cell's excitability (Rudy, 1988; Gintant, Cohen, Datyner, and Kline, 1991). Potassium channels are functionally a diverse family. Not only are there various types (delayed rectifier, transient A-type, calcium dependent, and inward rectifier, et cetera) but also each class of channels can exhibit a variety of different biophysical properties. Potassium channels coexist in different proportions; cells in different tissues and at different developmental stages have their own blend of potassium channels to suit their physiological function. This functional diversity of potassium channels derives from their structural diversity: different channel proteins can be the products of different genes or the products of alternative splicing of the same gene, these different gene products can also coassemble into heteromultimeric potassium channels (Jan and Jan, 1990). Recently, several investigators reported that *Xenopus* oocytes injected with different amounts of a single cRNA expressed potassium channels with differing

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gating and pharmacology (Honoré, Attali, Romey, Lesage, Barhanin, and Lazdunski, 1992; Guillemare, Honoré, Pradier, Lesage, Schweitz, Attali, Barhanin, and Lazdunski, 1992; Moran, Schreibmayer, Weigl, Dascal, and Lotan, 1992).

I_{sK} protein is a potassium channel that is different from all other cloned potassium channels. It has merely 130 amino acids and a single putative transmembrane domain. The cDNA for I_{sK} was first cloned from rat kidney (Takumi, Ohkubo, and Nakanishi, 1988) and subsequently cloned from neonatal rat heart (Folander, Smith, Antanavage, Bennett, Stein, and Swanson, 1990) and rat uterus (Pragnell, Snay, Trimmer, MacLusky, Naftolin, Kaczmarek, and Boyle, 1990) with an identical putative protein sequence. The cDNA of a similar channel protein was also cloned from mouse heart (mI_{sK}, Honoré, Attali, Romey, Heurteaux, Richard, Lesage, Lazdunski, and Barhanin, 1991) and human heart (Krafte, Dugrenier, Dillon, and Volberg, 1992). Unlike *Shaker* type potassium channels whose structure-function relationship has been extensively studied and some of their molecular mechanisms such as the inactivation, the ion pore, and the oligomeric configuration are reasonably well understood (Miller, 1991), the structure-function relationship of I_{sK} is largely unknown. This lag in knowledge is partly due to experimental difficulties in recording single channel currents (Blumenthal and Kaczmarek, 1992) and the lack of a working hypothesis of how the 130 amino acid protein forms a potassium channel.

In this paper we report that the I_{sK} current expressed in *Xenopus* oocytes injected with different amounts of I_{sK} mRNA had differing current amplitudes and gating properties. Similar changes also occurred with time after a single injection of mRNA. This observation implies that channel protein levels can alter gating properties of the channel. In the previous studies of the *Shaker* type channels (Honoré et al., 1992; Moran et al., 1992) there were two pharmacologically and kinetically distinct modes of gating. Only the relative proportions were altered with variations in mRNA levels. However, we report that the changes in I_{sK} were graded with increasing mRNA in a fashion suggesting that the kinetics for all I_{sK} channel proteins were equally affected by the change in mRNA. One explanation for our results is that I_{sK} channel proteins may interact during the activation process.

An abstract of this work was presented at the 1993 American Heart Association meeting (Cui, Mandel, Kline, Pennefather, and Cohen, 1993).

METHODS

RNA preparation. mRNA was prepared from the pKI27 cDNA (Takumi et al., 1988) by in vitro transcription with T7 polymerase using the Riboprobe kit (Promega Corp., Madison, WI) in the presence of the capping analog G(5′)ppp(5′)G (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). pKI27 is a full-length cDNA clone derived from rat kidney and was kindly provided to us by Dr. S. Nakanishi (Kyoto University, Japan).

The concentration of the pKI mRNA stock solution was roughly 1 mg/ml. It was diluted in series 5, 100, 500, 2,500, and 25,000 times. In this paper, the concentration corresponding to the five dilutions above are expressed as 200, 10, 2, 0.4, and 0.04 pg/nl, respectively, assuming the initial concentration of stock mRNA was exactly 1 mg/ml.

Oocyte preparation and injection. Ovarian lobes were dissected from mature female *Xenopus* laevis that had been anesthetized for 20–30 min. in a shallow bath containing 0.2% tricaine solution (3-aminobenzoic acid ethyl ester, Sigma Chemical Co., St. Louis, MO). They were then treated with collagenase (2 mg/ml, Type Ia, Sigma Chemical Co.) to remove the follicles

(Dascal, Ifune, Hopkins, Snutch, Lübbert, Davidson, Simon, and Lester, 1986). Stage V and VI oocytes were selected for microinjection. 50 nl of the various dilutions of the mRNA solution was injected into each oocyte with a Drummond digital microdispenser. Oocytes were then incubated at 18°C in ND96 solution (in millimolar: 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES-NaOH, pH = 7.5) with addition of 2.5 mM pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Electrophysiology. Currents were recorded with a conventional two-electrode voltage clamp (Dagan, Minneapolis, MN) (Kusano, Miledi, and Stinnakre, 1982). Intracellular glass microelectrodes were filled with 3 M KCl and had tip resistances between 0.5 and 3 MΩ. All external solutions contained (in millimolar): 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 $MnCl_2$, 5 Hepes-NaOH, pH 7.5. All recordings were obtained at room temperature (24–26°C). The voltage clamp steps

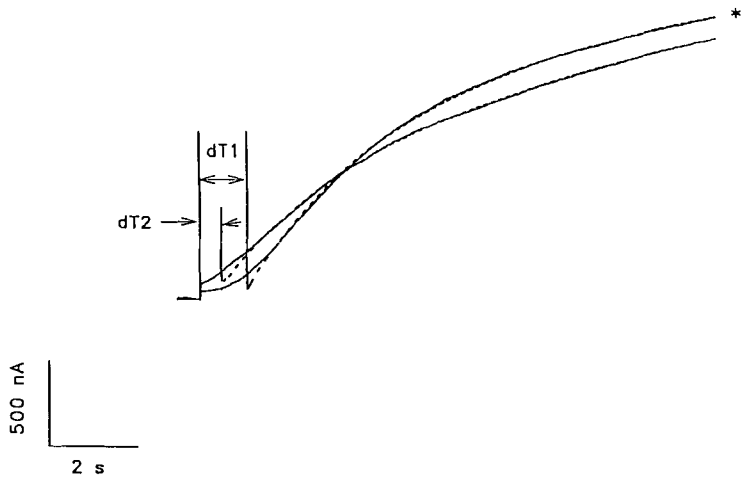

FIGURE 1. *Xenopus* oocytes injected with pKI mRNA expressed I_{sK} that activated with an apparent delay. The oocyte was injected with 2 pg/nl of pKI mRNA. It was recorded 4 d after injection. The current was activated by a depolarizing voltage pulse to 30 mV from holding potentials of −70 or −110 mV (indicated by *), respectively. The dashed curves are the fits of two exponentials to the current traces. The vertical bars at the beginning of the current traces demonstrate the measurement of the delay. The delays of the current traces recorded from the holding potentials of −110 and −70 mV are indicated as dT1 (1,008 ms) and dT2 (404 ms), respectively.

were delivered from a programmable pulse generator. The voltage and current were recorded using a PCM adaptor (VR-10, Instrutech, Elmont, NY) and VCR. Data were filtered at 50 Hz and analyzed with the pCLAMP program (Axon Instruments, Foster City, CA).

The time dependent current relaxations were fit to obtain a fast and a slow time constant of activation as well as an initial delay. The fitting procedure was as follows: after the current had risen significantly above the instantaneous step (~10–20% of the fast component time constant), a two exponential fitting process was initiated. As indicated in Fig. 1, an excellent fit to this portion of the relaxation was obtained, but an obvious deviation existed at earlier times when the fit is extrapolated. We defined the delay in activation as the time between the start of the relaxation and the time at which the extrapolated fit obtains the same level of current as the instantaneous current step at the start. All experimental data presented are averages from

three to seven oocytes from the same donor. Each experiment was repeated an additional two times with different donors to verify the reproducibility of the basic findings.

We also investigated four oocytes from each batch injected with water instead of mRNA. In one batch we also investigated uninjected oocytes. These controls were studied on the same day (3 or 4 d after injection) as the mRNA injected oocytes which were removed from the same *Xenopus* donor at the same time. In none of these batches were large endogenous delayed rectifier currents observed. In general, the time dependent current in response to a 35-s depolarizing pulse to all test potentials from a holding potential of −80 mV is less than 100 nA in the water injected oocytes, whereas the I_{sK} amplitudes we are reporting frequently exceed 1 μA at 3 or 4 d post injection. Also, the time course of the endogenous current is much more rapid than I_{sK}.

Computer simulation. The kinetic models were developed and solved using the Axon Engineer program (Axon Software, Eugene, OR). The simulation modeling process was straightforward with one exception worthy of note. In both models the effect of mRNA concentration on the delay in activation is dissociated from its effects on peak current. The latter is simulated by assuming that in addition to increasing the number of channels in the membrane, increasing the amount of mRNA injected also leads to a decrease in the efficacy of those channels. To achieve this aim, we postulate that all fully open states must be entered or left through a "preopen state" that is in rapid equilibrium with the open state. Thus, the relative proportion of these two states can be defined by an isomerization ratio ([open]/[preopen]) which we propose is inversely proportional to the mRNA concentration (x): [open]/[preopen] = r/x. We assume $x = 1$ at the lowest mRNA concentration so r is the isomerization ratio for this lowest mRNA concentration. Given these initial assumptions, the maximal possible absolute conductance when all channels are either in the preopen or open state will be given by the relation:

$$x[\text{open}]/([\text{open}] + [\text{preopen}]) = x/(1 + x/r).$$

If r is set at 1 then the maximal fraction in the open state for the lowest mRNA concentration = ½. As x is increased this value assymptotes to 1. This means that the maximal increase in current magnitude when $r = 1$ is 100% even if the mRNA concentration is increased several hundred-fold.

RESULTS

mRNA Concentration Determines Gating Properties of I_{sK} Currents

The I_{sK} current expressed in *Xenopus* oocytes injected with pKI mRNA activated with an apparent delay in response to a depolarizing voltage pulse (Fig. 1). Fig. 1 shows two current traces recorded from an mRNA injected oocyte at 30 mV but from different holding potentials of −70 mV and −110 mV (indicated by *), respectively. The delay (see Methods) in current activation was longer with the more negative holding potential. Therefore, to accentuate this kinetic component we held the membrane potential at −110 mV in experiments designed to measure the delay.

Superimposed I_{sK} current traces recorded from two *Xenopus* oocytes injected with 0.04 and 2 pg/nl pKI mRNA, respectively, are provided in Fig. 2 *A* (water injected oocytes never exhibited large outward currents). The current expressed from the higher concentration of pKI mRNA activated with a pronounced delay. However, the delay in activation of I_{sK} was much less prominent in oocytes injected with the lower concentration of pKI mRNA. The difference in kinetics and in current amplitude resulted in a cross-over phenomena: at the beginning of the voltage pulse the current

expressed from the lower concentration of pKI mRNA was larger than the current expressed from the higher concentration, whereas at the end of the pulse the latter current was larger. In Table I the amplitudes of the time dependent current measured at 336 ms (I_1) and at 35 s (I_2) after the beginning of the depolarizing pulse, respectively, are presented for various mRNA concentrations (0.04, 0.4, 2, and 200 pg/nl). I_1 progressively decreases with increases in mRNA concentration while I_2 progressively increases. These mRNA dependent kinetic changes which gave rise to the crossover of the observed currents (Fig. 2 *A*) are more clearly demonstrated by the ratio I_1/I_2 (Table 1).

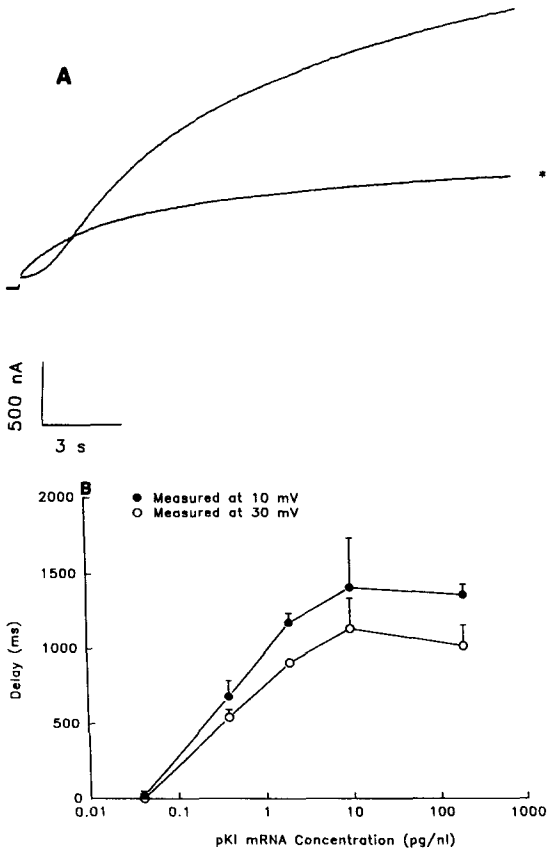

FIGURE 2. *Xenopus* oocytes injected with different concentrations of pKI mRNA expressed I_{sK} that activated with different delays. The oocytes were incubated for 3 d after injection. The membrane potential was held at −110 mV. (*A*) Superimposed current traces recorded from two oocytes injected with 0.04 pg/nl (indicated by *) and 2 pg/nl of pKI mRNA, respectively. In both oocytes the current was elicited by a depolarization voltage pulse to 30 mV. (*B*) The dependence of the delay on the concentration of pKI mRNA in a semi-log plot. The concentrations were 0.04, 0.4, 2, 10, and 200 pg/nl. The delays were measured with the depolarization voltage pulses to 10 and 30 mV, respectively. The error bars show the standard deviation and have the same meaning in all figures. The standard deviation is not shown when it is smaller than the symbol size.

Fig. 2 *B* illustrates the dependence of the activation delay on the concentration of pKI mRNA. The delay increased with increasing concentration of mRNA and reached a maximum at 10 pg/nl. The delay in the activation of the current was somewhat voltage dependent. At more positive test membrane potentials the delay was shorter. Fig. 2 *B* shows that the current elicited by a voltage pulse to 10 mV had a longer delay than the current elicited at a voltage of 30 mV for each concentration of pKI mRNA. However, the dependence of the delay on the mRNA concentration was similar at both potentials.

After an initial delay the I_{sK} current traces could be well fit with two exponentials (see Methods for details) (Fig. 1):

$$I(t) = I(\infty) + A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2),$$

where $I(\infty)$ is the steady state current amplitude, τ_1 and τ_2 are the time constants of the fast and slow components, respectively, and A_1 and A_2 are the amplitudes of the fast and slow components, respectively. The I_{sK} current expressed from different concentrations of pKI mRNA activated with different kinetics. In Fig. 3 *A* the dependence of the fast time constant τ_1 on the concentration of pKI mRNA at test

TABLE I

The Dependence of Expressed I_{sK} Current on the mRNA Concentration

	[RNA]	Tau1	Tau2	A_1	A_2	A_2/A_1	Delay	I_1	I_2	I_1/I_2
	pg/nl	*ms*	*ms*	*nA*	*nA*		*ms*	*nA*	*nA*	
	0.04	2506	21635	−352	−616	1.78	0	65	856	0.08
		± 146	± 1514	± 120	± 177	± 0.10		± 13	± 244	± 0.01
	0.4	3158	31771	−774	−1681	2.28	488	32	1750	0.018
		± 198	± 2170	± 281	± 327	± 0.32	± 45	± 12	± 480	± .004
30 mV	2	3786	36464	−967	−2104	2.24	907	10	1991	0.005
		± 94	± 3007	± 322	± 540	± 0.20	± 118	± 2	± 565	± .002
	200	3347	26199	−1364	−2058	1.55	1020	13	2422	0.005
		± 312	± 3818	± 224	± 90	± 0.28	± 62	± 5	± 218	± .002
	0.04	2872	24503	−255	−430	1.71	5	43	589	0.07
		± 320	± 1963	± 80	± 115	± 0.09	± 9	± 10	± 154	± 0.01
	0.4	4186	32912	−474	−908	2.01	648	16	981	0.017
		± 481	± 3504	± 200	± 258	± 0.24	± 57	± 8	± 371	± .006
10 mV	2	5167	48299	−650	−1391	2.24	1198	5	1179	0.004
		± 192	± 3980	± 235	± 335	± 0.27	± 115	± 2	± 331	± .002
	200	4919	37290	−1037	−1238	1.25	1381	5	1474	0.004
		± 271	± 6337	± 192	± 125	± 0.34	± 37	± 1	± 140	± .000

The current was measured at 30 mV and 10 mV with depolarizing voltage pulses from the holding potential of −110 mV. The duration of the pulse was 35 s. Tau1 (τ_1), Tau2 (τ_2), A_1, and A_2 were obtained by fitting the current trace with two exponentials as described in the text. The tabulated values of the intercepts A_1 and A_2 were determined roughly at the time marking the end of the delay as defined in the text. I_1 and I_2 are the amplitudes of the time dependent current measured at 336 ms and at 35 s, respectively, after the beginning of the depolarizing pulse. The results are presented as mean ± SD from four oocytes with each RNA concentration. The ratio A_2/A_1 and I_1/I_2 were calculated for each oocyte and then averaged.

Please note that the absolute value of $A_1 + A_2$ is not equal to I_2 because the current did not reach steady state at 35 s.

potentials of 10 and 30 mV is provided. At both potentials τ_1 was larger with higher mRNA concentrations from 0.04 to 2 pg/nl and reached a maximum at 2 pg/nl. The results are more fully demonstrated in Fig. 3 *B* where τ_1 is plotted against membrane potential for the currents expressed from three different concentrations of pKI mRNA: 0.04, 0.4, and 2 pg/nl. With each mRNA concentration, τ_1 was larger when the membrane potential was less depolarized and the voltage dependence of τ_1 was steeper with higher mRNA concentration. Therefore, the difference in τ_1 with different mRNA concentrations was much greater at less depolarized potentials: At 50 mV, the τ_1 from 2 pg/nl was ~50% larger than the τ_1 from 0.04 pg/nl, whereas at

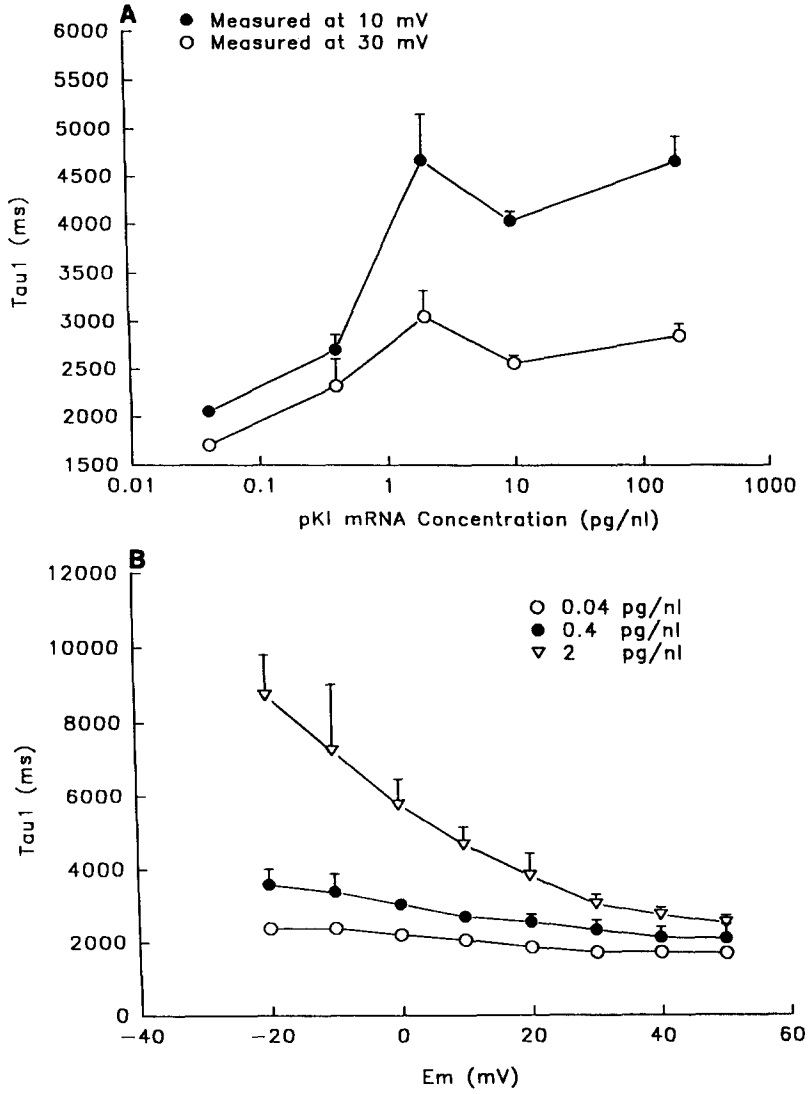

FIGURE 3. I_{sK} expressed from different concentrations of pKI mRNA activated with different kinetics. The oocytes were incubated for 3 d after injection. The membrane potential was held at −70 mV. Depolarizations of 35-s duration were applied to the potential indicated in the figures. The activation time constants were obtained by fitting current traces with two-exponentials as described in Results. (*A*) The dependence of the fast time constant (Tau1) on the concentration of pKI mRNA in a semi-log plot. The concentrations were 0.04, 0.4, 2, 10, and 200 pg/nl. The time constants were measured with voltage pulses to 10 and 30 mV, respectively. (*B*) Fast (Tau1) component of time constants versus membrane potential (E_m). Results were obtained from oocytes injected with 0.04, 0.4, or 2 pg/nl of pKI mRNA.

−20 mV, the former was more than fourfold larger. The slow time constant τ_2 had a similar dependence on the mRNA concentration as τ_1: it was larger with higher mRNA concentration from 0.04 to 2 pg/nl and reached its maximum at 2 pg/nl as shown in Table I. In Table I the amplitudes of fast and slow component A_1 and A_2 are also presented. Consistant with the measured current amplitude (I_2 in Table 1 and also Fig. 4 *A*) both A_1 and A_2 are larger with the increase of mRNA concentration from 0.04 to 2 pg/nl. Although both A_1 and A_2 varied from oocyte to oocyte, the ratio A_2/A_1 was appreciably less variable among oocytes injected with the same concentration of mRNA (Table 1).

Fig. 4 *A* plots the *I-V* relationship for the I_{sK} current expressed from different concentrations of pKI mRNA. Because the current did not saturate for test pulses that were minutes in duration (Busch, Kavanaugh, Varnum, Adelman, and North, 1992) the *I-V* relation was determined isochronally for 35-s depolarizing voltage pulses. With this batch of oocytes, the current at all potentials saturated at a concentration of 2 pg/nl. Qualitatively similar observations were made in the other two batches of oocytes from different donors. However, the saturating mRNA concentration varied slightly between 2 pg/nl and 10 pg/nl from batch to batch. The amount of current expressed also varied between oocytes from different donors, for example, the saturating current measured at 40 mV varied from 1 to 5 μA. Fig. 4 *A* demonstrates that the observed current at different mRNA concentrations was not proportional at various voltages. The difference was larger at more depolarized voltages. For example, the current expressed from 0.04 pg/nl was more than twofold smaller than the current expressed from 2 and 10 pg/nl at 40 mV but these currents had about the same amplitude at −20 mV. This indicates that the current expressed from 0.04 pg/nl might activate at a more negative voltage range than the current expressed from 2 and 10 pg/nl.

Fig. 4 *B* shows the isochronal activation curves for these currents. The curves were constructed from normalized conductances that were calculated from the onset current amplitude. The activation curves were also constructed from tail currents and the curves from both methods superimposed (data not shown). In Fig. 4 *B* the activation curve from 0.04 pg/nl occurred over a more negative voltage range exhibiting a ~−14 to −16 mV shift compared to those from other mRNA concentrations. It was also slightly steeper than the others (see figure legend). The results obtained in Fig. 4 *B* are different from the conventional steady state activation curve because our voltage pulses did not enable the current onset to reach a constant level. Because the current expressed from 0.04 pg/nl had faster activation kinetics than the currents expressed from other mRNA concentrations, especially when the voltage was less depolarized (see Fig. 3), the apparent voltage shift of the isochronal activation curve may be due partly to the differences in activation time course.

The Gating Properties of the I_{sK} Current Vary with Time after Injection of a Single mRNA Concentration

The above experiments demonstrate that in *Xenopus* oocytes, the expressed I_{sK} current properties depend on the amount of mRNA injected. Because the mRNA was derived from the pKI cDNA these results suggest that the properties of the I_{sK} current depend on the amount of the channel protein expressed in oocytes. Another

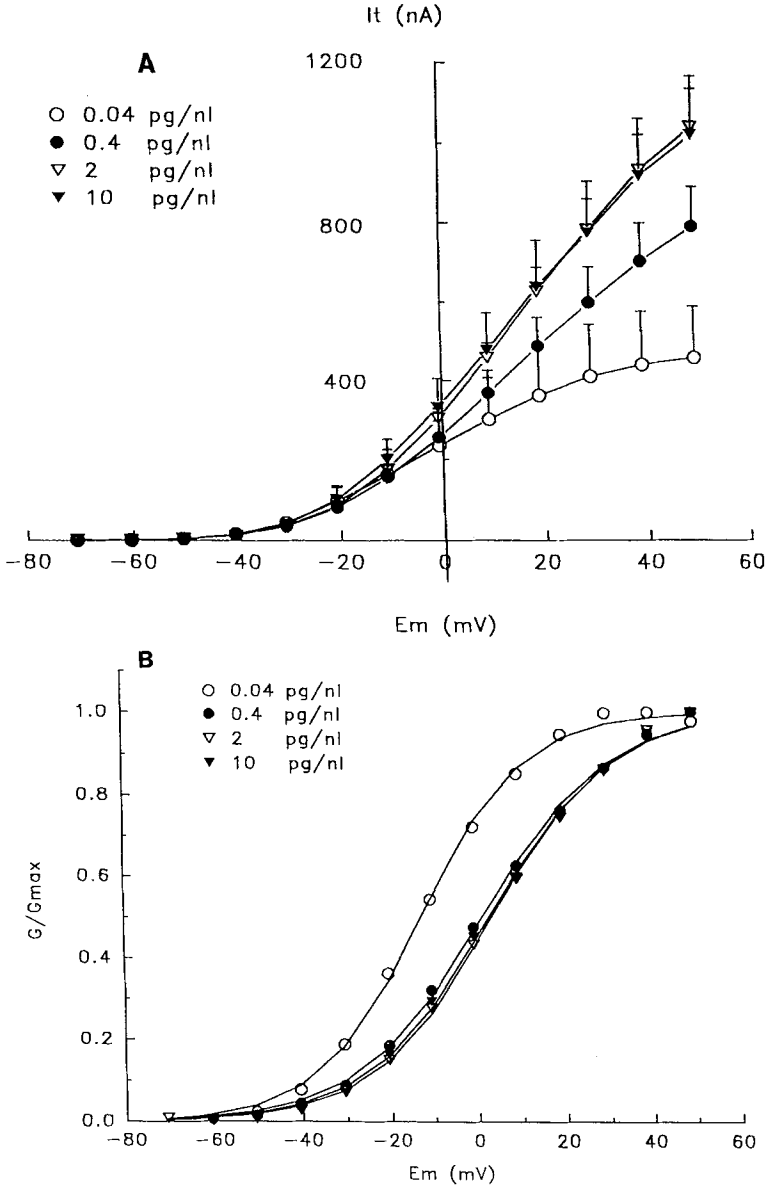

FIGURE 4. The *I–V* curves and the activation curves of the I_{sK} current expressed from different concentrations of pKI mRNA. The oocytes were incubated for 4 d after injection. The voltage protocol was as follows: the holding potential was −70 mV. Voltage pulses of 35-s duration were applied from −70 to +50 mV with a ~10-mV increment. Membrane potential was then stepped back to the holding potential after each pulse. (*A*) The *I–V* curves of the currents expressed from 0.04, 0.4, 2, and 10 pg/nl of pKI mRNA. The time dependent I_{sK} current amplitude was measured for each voltage pulse. (*B*) The activation curves. The conductance was calculated from the current amplitude in *A* (assuming E_K = −107 mV, see Cui, Mandel, DiFrancesco, Kline, Pennefather, Datyner, Haspel, and Cohen, 1992) and normalized for each oocyte, then averaged and plotted versus the pulse potential. The continuous lines are the best nonlinear curve fit to the Boltzmann relation with the half activation at −12.8, 1.3, 3.4, and 2.8 mV and a slope factor of 11.8, 14.3, 13.5, and 13.9 mV for 0.04, 0.4, 2, and 10 pg/nl of pKI mRNA.

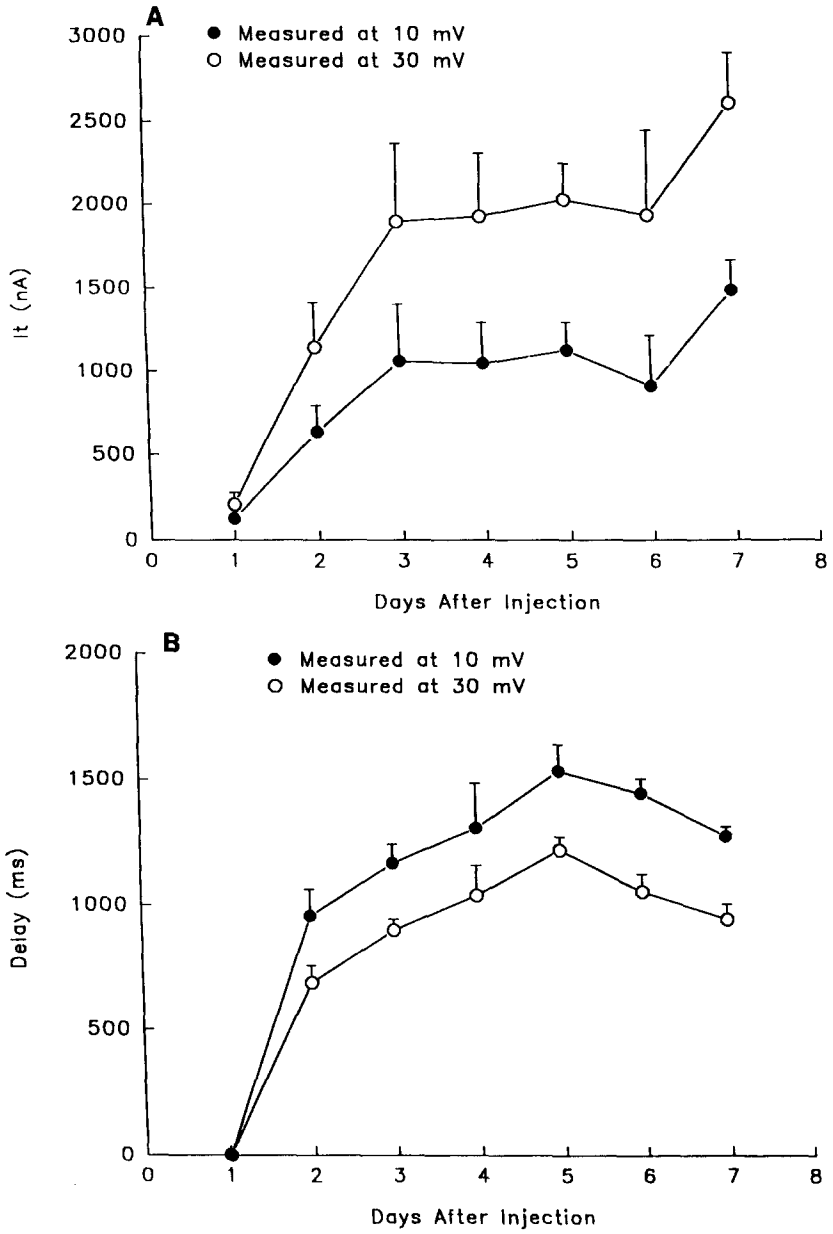

FIGURE 5. The activation kinetics of the I_{sK} current depended on the incubation time of the oocytes after mRNA injection. The oocytes were injected with 2 pg/nl of pKI mRNA on day 0 and I_{sK} currents were recorded from day 1 through day 7. The results in *A–C* were obtained from the same oocytes. (*A*) The relationship of the expressed current amplitude to the incubation time. The membrane potential was held at −110 mV. The current was activated by depolarizations of 35-s duration to 10 and 30 mV. The time dependent current was measured and plotted against the day of recording. (*B*) The dependence of delay on the incubation time. The delays were measured from the same protocol as in *A* and plotted against the day of recording.

way to examine the dependence of the biophysical properties on the amount of channel protein expression is to study the current properties over a period of time after the mRNA injection. This assumes that the channel protein accumulates in the membrane over a certain period after the mRNA injection. Fig. 5 presents the results of just such an experiment. The oocytes were injected with 2 pg/nl pKI mRNA. Fig. 5 *A* graphs the amplitude of the expressed current as a function of days after injection (from day 1 to day 7). Current increased during the first 3 d after injection and reached a plateau that lasted to the seventh day. The delay in activation of the current also changed during the same time period (Fig. 5 *B*). Most of the increase occurred between day 1 and 2. However, a slower monotonic increase was observed between day 2 and 5. The delay then decreased slightly over the next two days (day 6

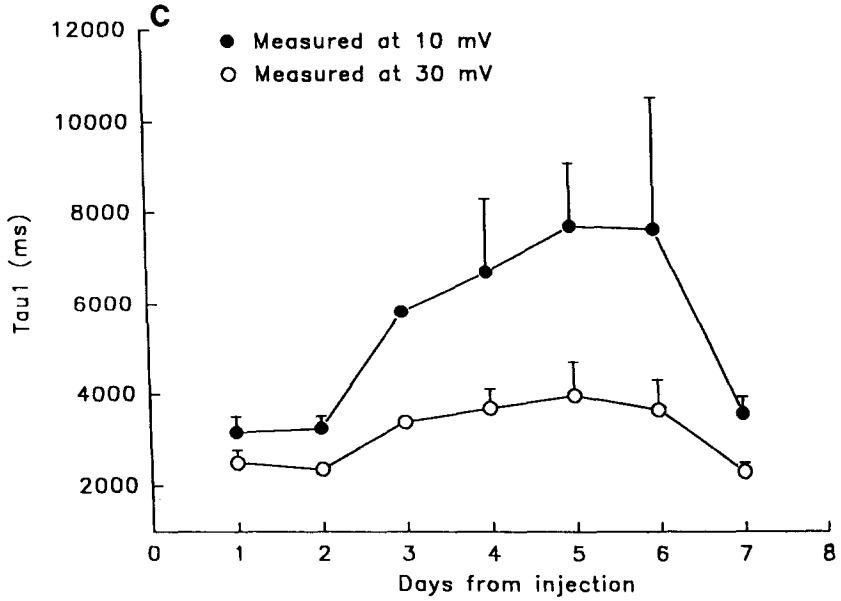

FIGURE 5. (*C*) The dependence of the fast time constant (Tau1) on the incubation time. The membrane potential was held at −70 mV and depolarized to 10 or 30 mV for 35 s. Current traces were fit with two exponentials and the fast time constant is plotted.

and day 7). Fig. 5 *C* shows that the fast time constant (τ_1) increased between day 2 and 3. It reached a maximum at day 5, and between day 6 and 7 decreased back to the day 1 value. The fast time constant measured at 30 mV on day 5 was about twice as long as it was on day 1. Generally, like the current amplitude, both the delay and the fast time constant increased after injection and then reached a plateau or a maximum. However, the time course of the changes was different for the three channel properties.

Models of I_{sK} Kinetics and the Concentration of mRNA

Our results have demonstrated an alteration in gating properties of I_{sK} as mRNA concentration is increased. There are a variety of alternative explanations for this finding. These alternatives include: (*a*) some channel protein when produced at high

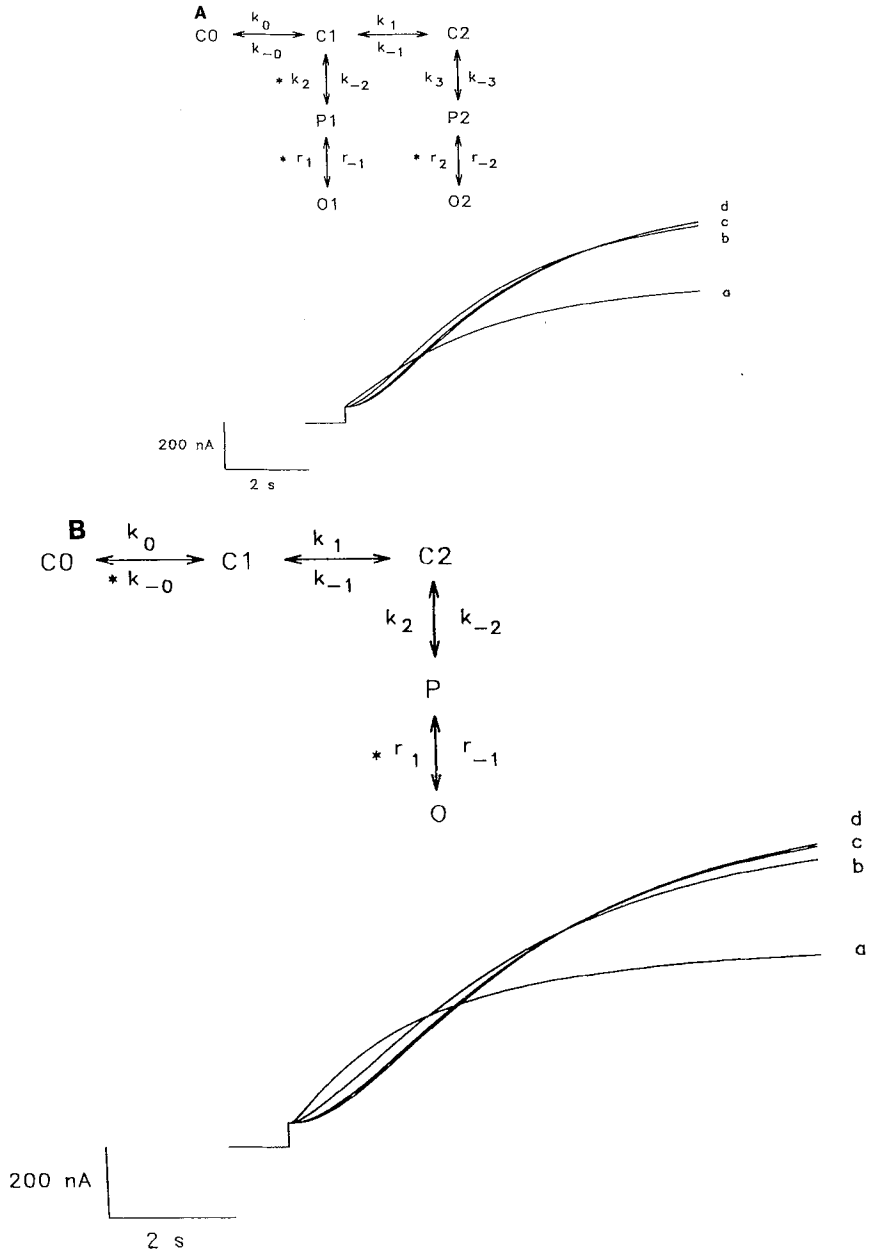

FIGURE 6. Computer simulations of the kinetic properties of I_{sK} as a function of mRNA concentration. The simulated currents in both *A* and *B* are for the lowest mRNA concentration and for concentrations 5-, 50-, and 250-fold higher (indicated by *a*, *b*, *c*, and *d*, respectively). Note the increased delay with increasing mRNA concentration, the current cross-over, and the modest increase in current amplitude. In the kinetic schemes, *C*, *O*, and *P* stand for closed, open, and preopen states, respectively. And the * indicates mRNA concentration dependent

rates may be abnormal; (*b*) an endogenous oocyte cofactor may be used up; (*c*) post-translational modification of some channel proteins may be abnormal; and (*d*) certain interactions between normal channel proteins may become increasingly prevalent as membrane channel density increases. The first three alternatives provide a structural basis for our experimental findings, while the last depends on a change in gating kinetics of the normal channel. At present, none of these alternatives can be entirely rejected. However, if normal production of the channel protein proceeds at a rate equivalent to that at the lowest mRNA concentration then the structural alternatives (1–3) would not predict the observed reduction of time dependent current in the first second following depolarizations (the cross-over in Fig. 1). Also, since the increase in current delay and time constants is graded with increasing mRNA concentration and is not precisely correlated with changes in peak current, we favor at present a kinetic explanation of our experimental results. It is also worth pointing out that kinetic models can be more easily created and tested with our available data than the structural alternatives.

In this section we present two kinetic schemes which we examined using computer simulations to provide insight into the types of physical mechanisms that might

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rate constants. (*A*) The first model has three closed and two open states. The rate constants for the transitions are:

$$k_0 = 0.30\ s^{-1};\ k_{-0} = 0.0162\ s^{-1} \exp[-1.50\ FV/RT];$$

$$k_1 = 1.16\ s^{-1} \exp[1.50\ FV/RT];$$

$$k_{-1} = 1.11\ s^{-1} \exp[0.072\ FV/RT];$$

$$k_2 = (2.00/x)\ s^{-1} \exp[1.65\ FV/RT];$$

$$k_{-2} = 0.30\ s^{-1} \exp[-0.60\ FV/RT];$$

$$r_1 = 1{,}000/x\ s^{-1};\ r_{-1} = 1{,}000\ s^{-1};$$

$$k_3 = 0.63\ s^{-1} \exp[0.22\ FV/RT];\ k_{-3} = k_{-2};$$

$$r_2 = 1{,}000/x\ s^{-1};\ r_{-2} = 1{,}000\ s^{-1};$$

V is membrane potential; F/RT have their standard meanings; and x is relative concentration of mRNA (ranging from 1 to 250). (*B*) The second model has three closed and one open states. The rate constants are:

$$k_0 = 0.30\ s^{-1};\ k_{-0} = (0.000272\ x)\ s^{-1} \exp[-1.50\ FV/RT];$$

$$k_1 = 1.16\ s^{-1} \exp[1.50\ FV/RT];$$

$$k_{-1} = 1.11\ s^{-1} \exp[0.072\ FV/RT];$$

$$k_2 = 0.63\ s^{-1} \exp[0.22\ FV/RT];$$

$$k_{-2} = 0.30\ s^{-1} \exp[-0.60\ FV/RT];$$

$$r_1 = 1{,}000/x\ s^{-1};\ r_{-1} = 1{,}000\ s^{-1};$$

x has the same meaning and values as in *A*.

underlie the mRNA concentration related changes in biophysical properties of I_{sK}. To proceed we made two simplifying assumptions: first, for the range of mRNA amount used here (2–500 pg/oocyte) the amount of channel protein inserted into the membrane is linearly proportional to the amount of mRNA injected (This assumption has been investigated and confirmed in the range 1–50 ng by Blumenthal and Kaczmarek (1993); and second, all the protein synthesized as a result of the mRNA injection is functionally identical (i.e., no protein of altered function due to uneven processing and assembly is produced).

The major findings which we have attempted to reproduce are (*a*) the increased delay and slower time course of current activation, and (*b*) the relatively small increases in current amplitude (twofold) for up to a 250-fold increase in the amount of mRNA injected. These two effects result in a cross-over of the current activations as the mRNA concentration is increased. Two kinetic schema which produce the desired outcomes are provided in Fig. 6, along with computer generated outputs. In both cases the delay and cross-over exist and the current increases with increasing mRNA concentration, but much less than linearly. Both models have some characteristics in common, but also differ in important ways.

Let us first consider the similarities. Both have three closed states, which are necessary to produce the delay and multiexponential time course of activation. Both have transitions dependent on the relative amount of mRNA injected. These transitions are indicated by an * in the kinetic schemes. These concentration dependent rate constants help to produce the increasingly smaller fraction of channels in the open state, the longer delay, and the slower activation with increasing mRNA concentration. An additional preopen state is present in both models in order to help generate the nonlinear increases in current amplitude with mRNA concentration (see Methods for details on preopen state). Both models use identical rate constants as far as possible to link analogous states. The core of both schemes used here (i.e., the rates linking C1, C2, and O2) are based on parameters derived by Balser, Bennett, and Roden (1990) to describe the slowly activating delayed rectifier channels found in guinea pig ventricular myocytes which is likely similar in structure to the I_{sK} channels studied here (see Discussion). With the original parameters deduced by those authors the voltage for half activation was 40 mV. We therefore shifted those parameters by −40 mV. This also gave rise to a slowing of the activation kinetics. With the three-state model of Balser et al., (1990) the amount of delay in activation was insensitive to the holding potential. Therefore an additional closed state (C0) was added to our schemes and the magnitude and voltage dependence of k_0 and k_{-0} were adjusted to give appropriate delays that shifted as observed with changes in holding and command potentials. The rate of conversion of C0 to C1 (k_0) is the prime determinant of the magnitude of the delay in both models. As the delay was only slightly voltage dependent between +10 and +70 mV, we made k_0 voltage independent. The rate of conversion of C1 to C0 (k_{-0}) was given an effective gating charge of 1.5. This allowed for changes in the delay with changes in holding potential similar to those observed in Fig. 1.

However, there are also significant differences. The parallel kinetic scheme in A has two open states and achieves the delay by favoring activation to the open state that must be preceeded by a greater number of closed states. This type of model

makes one think of oligomerization of the channel proteins, with larger size oligomers having more closed states and favored as the I_{sK} channel (protein) increases in the membrane. In the model illustrated we have assumed the two open states were of equal conductance, but this assumption is not required.

The sequential kinetic scheme in B has only a single open state which follows three sequential closed states. As the mRNA concentration is increased, the voltage range favoring C_0 is expanded to more positive values. In contrast to the parallel model in *A*, the sequential model predicts a delay will be observed even at the lowest [mRNA] provided the initial holding potential is sufficiently negative.

Besides their basic properties, both models also produce a positive shift of the activation curve on the voltage axis with increases in [mRNA] (Fig. 4 *B*). The time constants are concentration and voltage dependent, but their detailed characteristics do not closely resemble the results of the experiments (Fig. 3 *B*). In neither model have we attempted a detailed fit to experimental data. Rather it was our aim to investigate the minimal models that might qualitatively reproduce distinguishing characteristics. To provide a more detailed fit, the models would have to produce the slow increases in I_{sK} current that proceed for many seconds and even minutes upon depolarization. Undoubtedly many other kinetic schemes and sets of parameters could reproduce the relatively limited sets of observations on I_{sK} that we have made.

DISCUSSION

In the present study, our basic finding is that the amplitudes and kinetics of the I_{sK} current expressed in *Xenopus* oocytes depend on the amount of pKI mRNA injected and on the time after injection. These results suggest that the kinetics of the I_{sK} current depend on the density of the channel protein in the membrane. The most apparent characteristic of this dependence is that I_{sK} current associated with higher levels of expression activated with a longer delay and a slower time course. This dependence combined with a nonlinear relation between channel expression and current magnitude resulted in a cross-over such that the peak current from the higher expression was larger, but the lower expression generated more current at the beginning of a depolarizing voltage step (Fig. 2 *A*).

Based on our experimental results, we presented three structural and one kinetic alternatives to explain our results. For the kinetic alternative we have further presented two alternative kinetic schemes that reproduce our results. We provided two of these models just to demonstrate that the schemes are not exclusive. Both models predict the essential property of the I_{sK} current provided in our experimental results: the mRNA concentration dependent delay and activation time course and consequently the cross-over. This cross-over occurs because when the density of channel proteins becomes higher in the membrane more channels will reside in either a closed or a preopen state that opens with only a very low probability. It is worth pointing out that the model parameters derived from cardiac data (Balser et al., 1990) were employed to simulate our results with only minor modifications. These models assume that the interaction among channel proteins modulates their function and a higher channel density increases this interaction. This later proposal deviates from a basic assumption common in previous analyses of ion channel properties: that each channel is independent of all others (Hille, 1992). However, this

mechanism is consistent with a recent report (Varnum, Busch, Maylie, and Adelman, 1993) that chemical cross-linking of the I_{sK} protein could alter the current activation.

It is worth pointing out that at present it is unknown how many individual I_{sK} channel subunits combine to form a functional channel, or indeed whether all functional channels are composed of the same number of subunits. Single-channel data is lacking. The models proposed in the manuscript are primarily for illustrative purposes to demonstrate how by making a simple assumption (namely that the individual channels can interact with other channels or channel subunits in a manner that increases with channel protein density) it becomes quite easy to simulate our results. These models are not proof of interaction but rather are provided to encourage thought about the consequences of allowing individual channels to interact with one another.

The lack of an apparent voltage sensor and the small size of I_{sK} with a single membrane spanning domain created skepticism of its potassium channel function when its clone first appeared (Hausdorff, Goldstein, Rushin, and Miller, 1991; Goldstein and Miller, 1991). The study of mutations of this channel even revealed that only part of this protein was sufficient to generate its potassium channel activity (Takumi, Moriyoshi, Aramori, Ishii, Oiki, Okada, Ohkubo, and Nakanishi, 1991). The unique structure of the channel suggests that previous results on structure-function relations of other potassium channels (e.g., *Shaker*) may not apply to I_{sK}. Recently, Ahali et al. (1993) have questioned the function of the I_{sK} protein. They suggested it serves as an activator of endogenous channels within the oocyte membrane. Their evidence is largely based on the ability of large mRNA injections >50 ng/oocyte to produce increases in Cl^- conductance as well as K^+ conductance. However, whether I_{sK} is a channel activator or the channel protein itself, our results and conclusions in this study remain unaffected.

The cDNA of I_{sK} has been cloned from the hearts of rat (Folander et al., 1990), mouse (Honoré et al., 1991), and human (Krafte et al., 1992). An I_{sK} like potassium current has also been expressed in *Xenopus* oocytes by injecting canine ventricular mRNA (our unpublished data). Honoré et al. (1991) showed that I_{sK} mRNA was present throughout the whole heart. They also demonstrated that the delayed rectifier potassium channel in cardiac myocytes had a similar *I-V* relationship and similar pharmacological properties. Considering that cardiac delayed rectifiers activate one to three orders of magnitude more slowly than those in nerve (Gintant et al., 1991) and than those *Shaker* type potassium channels cloned in heart (Paulmichl, Nasmith, Hellmiss, Reed, Boyle, Nerbonne, Peralta, and Clapham, 1991), it seems likely that I_{sK} is a major contributor to the cardiac delayed rectifier. In a recent study, Freeman and Kass (1992) showed that the guinea pig ventricular delayed rectifier was insensitive to intracellular trypsin and therefore appeared to be structurally different from the trypsin sensitive *Shaker* type potassium channels. This result led the authors to suggest that the structure of the guinea pig ventricular delayed rectifier was more compatible with I_{sK}. They later solidified this suggestion with immunolocalization of I_{sK} protein in guinea pig heart (Freeman and Kass, 1993).

Our finding that the properties of the I_{sK} current depend on the mRNA level may have some physiological importance. The levels of I_{sK} mRNA in neonatal heart are much higher than in adult heart in both rat and mouse (Folander et al., 1990;

Honoré et al., 1991). It is interesting that the action potential duration in adult rat heart is much shorter than the action potential in neonatal rat heart (Bernard, 1975). Because the major physiological role of the delayed rectifier potassium current in heart is to terminate the action potential (Gintant et al., 1991) it is possible that the different levels of mRNA may contribute to the changing action potential durations observed during development, possibly through channel interactions.

Finally, it is worth considering more general implications of our results. It is well known that ion channel properties can be regulated on millisecond to second time scales by phosphorylation or ligand binding, and that intracellular ion concentration changes resulting from cell activity can alter ion currents for periods of seconds to minutes. Our results suggest that long term regulation of ion channel properties might occur through changes in mRNA levels which can change both ion channel density and biophysical properties. Such long term changes in I_{sK} mRNA are induced in rat uterus by estrogen (Pragnell et al., 1990). It is interesting that changes in the concentration of one intracellular ion, Ca^{2+}, has already been shown to alter enzyme activity and phosphorylation (Kaczmarek, 1986), directly control channel permeability (Tillotson, 1979), and alter the level of mRNA in cells (Bading, Ginty, and Greenberg, 1993). Thus, it is possible that a single intracellular messenger (for example Ca^{2+}) might be effective in initiating both short and long term regulation of ion channels. This intriguing possibility awaits further investigation.

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