Time, voltage and ionic concentration dependence of rectification of h-*erg* expressed in *Xenopus* oocytes

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Abstract The rapid delayed rectifier, I_{Kr}, is believed to have h-*erg* (human ether-à-go-go related gene) as its molecular basis. A recent study has shown that rectification of h-*erg* involves a rapid inactivation process that involves rapid closure of the external mouth of the pore or C-type inactivation. We measured the instantaneous current to voltage relationship for h-*erg* channels using the saponin permeabilized variation of the cut-open oocyte clamp technique. In contrast to C-type inactivation in other voltage-gated K^+ channels, the rate of inactivation was strongly voltage dependent at depolarized potentials. This voltage dependence could be modulated independently of activation by increasing $[K^+]_o$ from 2 to 98 mM. These results suggest that inactivation of h-*erg* has its own intrinsic voltage sensor.

Key words: Potassium; Delayed rectifier; Repolarization; Cardiac myocyte

1. Introduction

In cardiac myocytes, at least two types of K^+ channels have been demonstrated to contribute to delayed rectification and the development of repolarization in cardiac muscle [1]. One of these activates slowly (I_{Ks}) and the other activates much more rapidly and shows strong inward rectification (I_{Kr}) [2,3]. In particular, I_{Kr} is believed to have h-*erg* (human ether-à-go-go related gene) as its molecular basis [4,5] because it expresses a current with characteristics similar to native I_{Kr} observed in myocytes. Furthermore, mutations in the h-*erg* gene have been linked to a familial form of long QT Syndrome [6] which emphasizes the physiological importance of this gene product.

Rectification in ion channels can result from asymmetries in the permeation pathway, block due to asymmetric distributions of permeant ions and impermeant blocking ions or particles, or intrinsic gating properties [7]. The strong inward rectification of h-*erg* and I_{Kr} has recently been proposed to result from a time-dependent inactivation-like process [2,4,5,8]. This inactivation process has recently been shown to result from closure of the external mouth of the pore, or C-type inactivation [9]. However, the time and voltage dependence of the putative inactivation process has yet to be quantitatively described for the positive potentials where rectification is observed. In this study, we demonstrate that inactivation of h-*erg* has a strong voltage dependence at positive potentials. This potential dependence is shifted strongly (~40 mV) by elevation of extracellular $[K^+]_o$ independently of shifts in the threshold for activation. C-type inactivation in other voltage-gated K^+ channels is voltage insensitive at positive potentials, with voltage dependence at intermediate potentials reflecting a strong coupling between activation and inactivation. Our results suggest that inactivation of h-*erg* has a unique intrinsic voltage sensor which differentiates inactivation of this channel from previously described mechanisms of C-type inactivation.

2. Materials and methods

Defolliculated *Xenopus laevis* oocytes (stage V–VI) were injected with 50 nl cRNA solution prepared as described [10], containing up to 50 ng h-*erg* cRNA made from cRNA kindly provided by G. Robertson at the University of Wisconsin [5]. This clone contained two point mutations in the 5′ end: T595A, yielding V198E, and C607T, yielding P202L (G. Robertson, personal communication, see also [9]). Voltage-clamp experiments were performed at room temperature, within 3–6 days of injection, using a cut-open oocyte clamp amplifier (CA-1a, Dagan Corp.) [11] as described previously [10]. Extracellular solution (ND-96) contained: (in mM) 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES-NaOH, pH 7.4, high K^+ extracellular solution contained: (in mM) 98 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES-NaOH, pH 7.4, and intracellular solution contained: (in mM) 98 KCl, 1.8 $MgCl_2$, 1 EGTA, 5 HEPES-NaOH, pH 7.4. Current traces were leakage and capacitive subtracted using a P/4 pulse protocol, unless otherwise noted. Data was filtered at 5 kHz. Data are shown as mean ± S.E.M. Confidence levels were calculated using a paired *t*-test.

3. Results

Fig. 1A shows typical h-*erg* currents recorded in response to a series of depolarizing P1 pulses for 1000 ms to potentials ranging between −80 and +50 mV followed by a 200 ms P2 pulse to −40 mV ($V_{holding}$=−80 mV). The final current measured at the end of the P1 pulse shows the typical [4] inward rectification observed for h-*erg* (Fig. 1B). This inward rectification can be seen much more clearly in the fully activated tail currents. As shown in Fig. 1C h-*erg* channels were depolarized with a P1 pulse from a holding potential of −80 to +50 mV for 600 ms to fully activate the channel, which was followed by a P2 pulse to different potentials ranging between −110 and +40 ms to elicit tail currents. Note that there is a time-dependent lag between the onset of the P2 pulse and the peak of the P2 tail current. This time-dependent delay has been previously described [4,5] and interpreted to be recovery from an inactivation-like process which mediates rectification. This rectification can be seen more clearly in the *I-V* relationship of the peak tail currents shown in Fig. 1D.

The fully activated peak current to voltage relationship measured above reflects both time-dependent and instanta-

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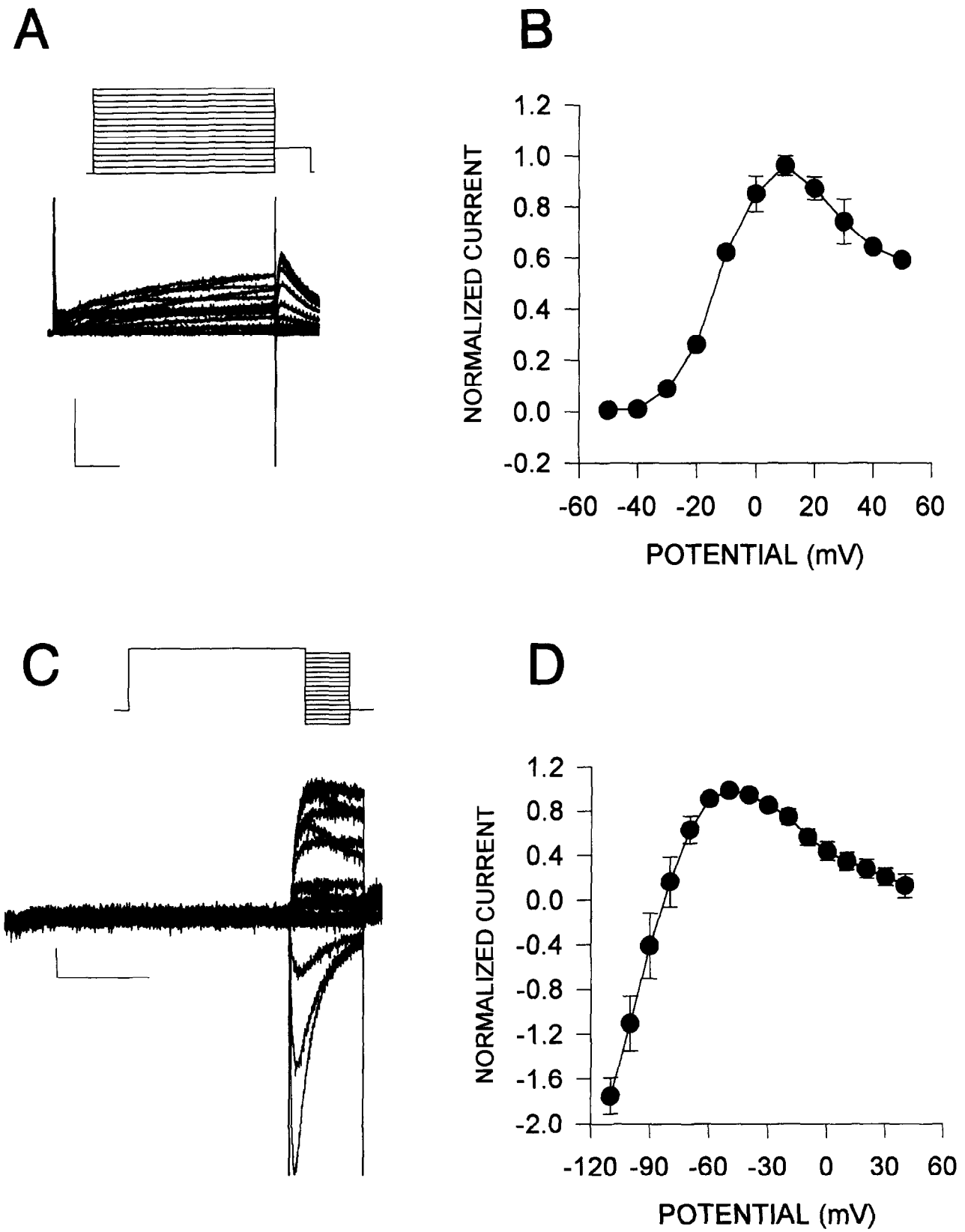


Fig. 1. Properties of the h-*erg* current measured using the cut-open oocyte clamp technique. (A) Typical h-*erg* currents recorded in response to a series of depolarizing P1 pulses for 1000 ms to potentials ranging between −80 and +60 mV followed by a 200 ms P2 pulse to −40 mV ($V_{holding}$=−80 mV; normal extra- and intracellular solutions, no P/4 subtraction was applied in this record). (B) Steady-state current measured at the end of the P1 pulse. Data were normalized by the maximal outward current and averaged (n=4). (C) Fully activated peak tail current. Typical currents recorded from a P1 pulse to +50 mV for 600 ms to fully activate the channel, followed by a P2 pulse to different potentials ranging between −110 and +40 ms to elicit tail currents (HP=−80 mV). (D) Average fully activated peak tail currents. Data obtained using the protocol shown in (C) were normalized by the maximal outward current (−50 mV) and averaged (n=4). Calibration bars: 40 nA, 200 ms for (A) and 50 nA, 200 ms for (C).

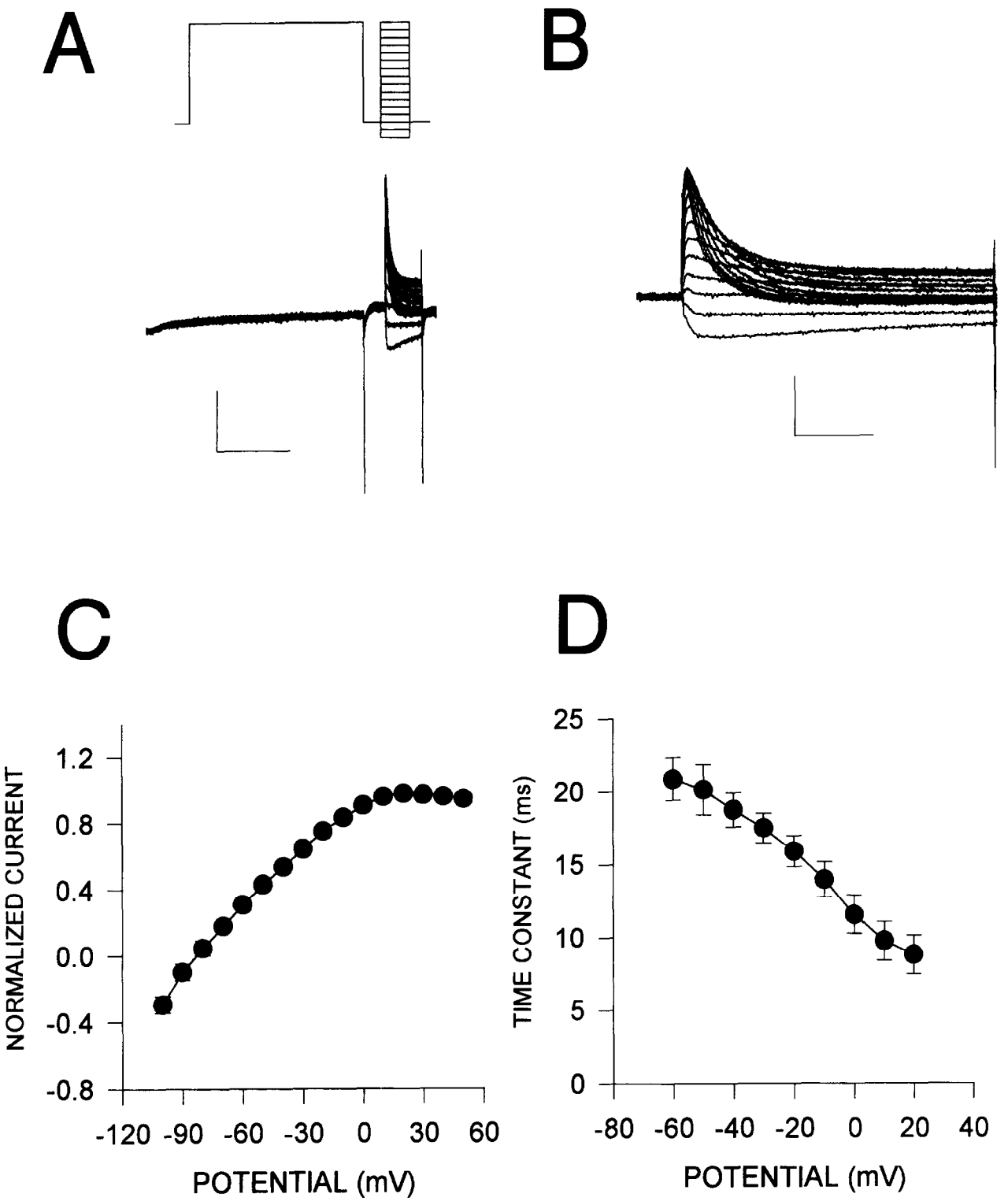


Fig 2. Instantaneous and time-dependent components of rectification. (A) Currents elicited during a three-pulse protocol. A 600 ms P1 pulse to −50 mV was applied, followed by a P2 pulse to −80 mV for 60 ms to remove inactivation without allowing deactivation to occur, followed by a P3 pulse to potentials from −100 to +50 mV. (B) Currents during the P3 pulse of (A) expanded to show the instantaneous peak currents and the time-dependent on rate of inactivation. (C) Average instantaneous current to voltage relationship for h-*erg*. The instantaneous currents obtained during P3 were normalized to maximum outward current and averaged (n=13). (D) Voltage dependence of time constants of inactivation of h-*erg*. Time constants of inactivation were estimated from the P3 current traces by fitting either single or double exponentials to the time course of decay. In ranges where a double exponential fit was required because of the overlap of other processes (e.g. residual capacitative current or deactivation) only the dominant component was plotted. Calibration bars: 400 nA, 200 ms for (A) and 400 nA, 25 ms for (B).

neous processes. Previous analysis of this current has assumed that the instantaneous current to voltage relationship for this channel is linear [4]. We sought to address this assumption by measuring the instantaneous current to voltage relationship directly using a three-pulse protocol. A P1 pulse to +50 mV was initially applied for 600 ms to fully activate h-*erg*, which

was followed by a P2 pulse to −80 mV for 60 ms to remove inactivation without allowing sufficient time for deactivation to occur. A final P3 pulse was then applied to different potentials from −100 to +50 mV. An example of currents recorded using this protocol for a P3 pulse to +50 mV is shown in Fig. 2A. Representative currents (for potentials of −100 to +50 mV) during P3 are shown on an expanded scale in Fig. 2B. Instantaneous currents at the beginning of the P3 pulse were measured for 13 oocytes and resulted in the average instantaneous current to voltage relationship shown in Fig. 2C. Inward rectification of the instantaneous *I-V* relationship was clearly evident for potentials positive to −20 mV. The time constants of development of inactivation during P3 from the same voltage-clamp protocol were measured directly by fitting single exponential curves to current decay in the range −60 to +20 mV. The average time constants of development of inactivation are shown in Fig. 2D and show that inactivation of h-*erg* is strongly voltage dependent, becoming increasingly faster with increasing depolarization. This is in marked contrast to the voltage insensitvity of both N- and C-type inactivation in other voltage-gated K^+ channels [12,13].

Increased external K^+ concentration has been previously shown to strongly increase the magnitude of h-*erg*, particularly with respect to the size of inward tail currents [4,5]. Therefore, we examined the effects of $[K^+]_o$ concentration on the instantaneous current voltage relationship and the time-dependent development of inactivation of h-*erg*. Fig. 3A shows the changes in instantaneous current to voltage relationship for h-*erg* when the oocyte is exposed to 2, 25, 50 and 98 mM $[K^+]_o$. Currents were normalized relative to the amount of current measured at +20 mV in 2 mM $[K^+]_o$. Clearly, increasing $[K^+]_o$ increases the magnitude of the instantaneous current. We also examined if the degree of instantaneous rectification was sensitive to $[K^+]_o$ in doubly normalized data from Fig. 3A (normalized for both peak and final current) where the relative shapes of the rectification curves could be observed (Fig. 3B). These curves show a weak dependence of instantaneous inward rectification on $[K^+]_o$. This relative insensitivity to $[K^+]_o$ is in marked contrast to the $[K^+]_o$ sensitivity of I_{K1} or IRK1 where rectification due to Mg^{2+} is strongly sensitive to $[K^+]_o$ and the direction of current flow[7,14].

An important, but untested, consequence of the finding of Smith et al. [9] that inactivation is mediated through a C-type inactivation mechanism is that it will be strongly sensitive to extracellular K^+ concentration. Therefore, we examined the effects of $[K^+]_o$ on the time-dependent inactivation rate at positive potentials. As shown in Fig. 3C the rate of inactivation was strongly dependent on $[K^+]_o$, becoming much slower when $[K^+]_o$ was increased from 2 to 98 mM and resulting in an approximate 40 mV shift of voltage dependence to more depolarized potentials (from -64 ± 9 to -23 ± 5 mV, n=3). This increase in $[K^+]_o$ also resulted in a parallel approximate 40 mV shift of steady-state inactivation. Thus, when one examines the changes in steady-state currents that accompany an increase in $[K^+]_o$, the change in magnitude is due to increased instantaneous conductance and time-dependent changes in gating, while the apparent depolarizing shift of the negative slope of steady-state rectification (Fig. 3D) is due to changes in time-dependent inactivation.

Intracellular Mg^{2+} has been shown to produce inward rectification in I_{K1} [14] and to produce a much weaker inward rectification in $I_{K,ATP}$ channels [15]. Our standard intracellular solution contained 1.8 mM $MgCl_2$. However, because our intracellular solution did not contain anything which might bind Mg^{2+} (e.g. ATP or creatine phosphate) this may have resulted in an abnormally high free intracellular Mg^{2+} concentration which might give rise to a non-physiological rectification of the instantaneous current. We examined this possibility by exchanging the contents of the intracellular compartment with solution in which either Mg^{2+} was replaced with 10 mM EDTA or Mg^{2+} was elevated to 10 mM for 15 min. As shown in Fig. 4, there was a small but significant effect of Mg^{2+} on the instantaneous current to voltage relationship, with increased rectification in 10 mM Mg^{2+} and decreased but did not abolish rectification in 10 mM EDTA. The time constants of inactivation were completely insensitive to manipulations of intracellular Mg^{2+} (Fig. 4C). These results suggest that while intracellular Mg^{2+} causes some voltage-dependent block of the h-*erg* channel, Mg^{2+} block may not necessarily account for all of the observed rectification of the instantaneous current. More importantly, the instantaneous inward rectification observed is not an artifact of the intracellular solutions employed in this study.

4. Discussion

The time-dependent component of inactivation displayed some interesting properties in this study which will aid in our understanding of the biophysical basis of inactivation in h-*erg* channels. Time-dependent inactivation of h-*erg* has been previously demonstrated to be strongly voltage dependent in the hyperpolarized range of potentials with recovery from inactivation becoming stronger with increasing hyperpolarization [4]. We also found that inactivation of h-*erg* was strongly voltage dependent in the positive range of potentials with the on-rate becoming faster at depolarized potentials. Thus the time constant of inactivation of h-*erg* has a classical 'bell' shaped voltage dependence.

This bell-shaped voltage dependence is distinct from C-type inactivation mechanisms described for voltage-gated K^+ channels. In both *Shaker* and Kv1.4 channels C-type inactivation is a voltage-insensitive process at positive potentials. Any voltage dependence arises from direct coupling to activation. Such a coupling-type mechanism is unlikely to explain the voltage dependence noted here; inactivation continues to be voltage dependent at potentials where activation is essentially complete. Similarly, the slopes of steady-state activation and inactivation relationships differ by more than a factor of 2 [4,9]. In contrast, the slope factor of steady-state inactivation of other K^+ channels roughly mirrors steady-state activation [13]. Similarly, the threshold for activation of h-*erg* is largely insensitive to increased extracellular K^+ (Fig. 3D) while the inactivation rate (Fig. 3C) and $V_{1/2}$ of inactivation are shifted positive by approx. 40 mV. These non-parallel shifts of activation and inactivation also indicate that the voltage dependence of inactivation is independent of the voltage dependence of activation. Thus, it seems likely that inactivation of h-*erg* has its own intrinsic voltage sensitivity.

Our analysis of the rectification process of h-*erg* expressed in *Xenopus* oocytes indicates that the net rectification of this channel results from two processes, one time-independent and the other time-dependent. Of these two components, it is clear

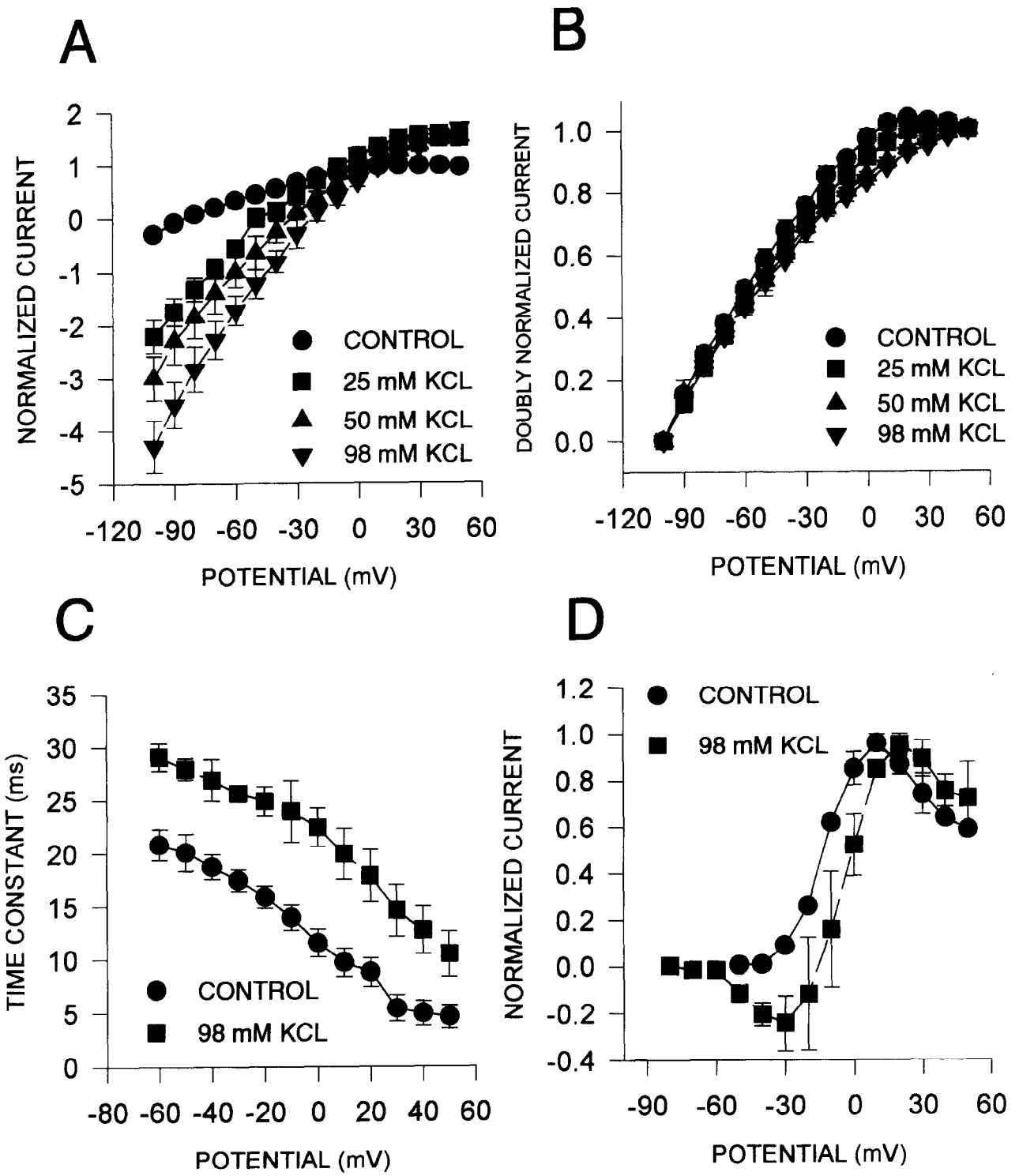
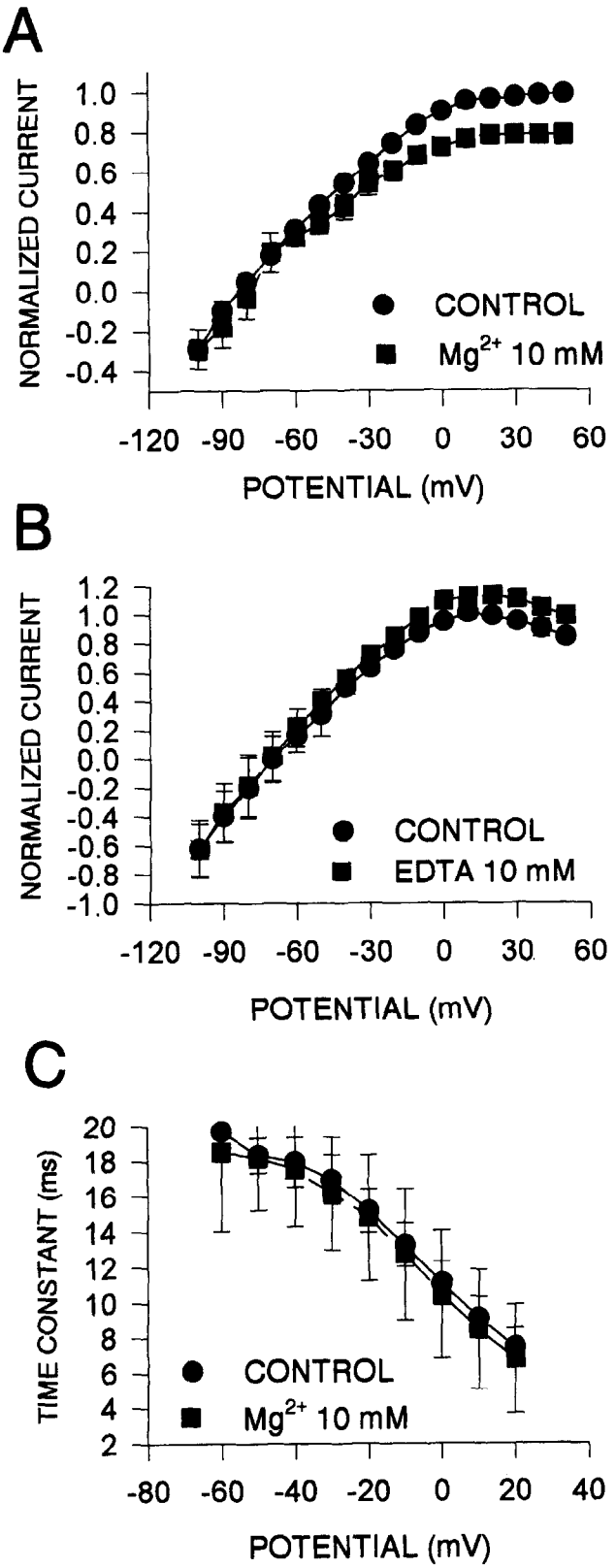


Fig. 3. Effects of extracellular K^+ on rectification. (A) Instantaneous current to voltage relationship for h-*erg* channels exposed to varying $[K^+]_o$. The instantaneous current to voltage relationship was estimated using the protocol described in Fig. 2A and currents were normalized to the peak outward current obtained under control (2 mM) $[K^+]_o$. Increasing K^+ concentration from 2 to 25, 50 and 98 mM increased the average conductance of the current but inward rectification remained (n=3, 4 and 3). (B) Comparison of the shape of the current voltage relationship of instantaneous h-*erg* current. The data from (A) were renormalized so that current at −100 mV equals 0 and current at +50 mV equals 1 and then averaged. This double normalization showed that increasing $[K^+]_o$ altered rectification slightly, with 2 mM K^+ showing more rectification and a more negative slope region than 98 mM K^+. (C). Increasing extracellular K^+ from 2 to 98 mM strongly slowed the rate of inactivation (n=3). (D) Steady-state current in 2 and 98 mM extracellular K^+. Data obtained using a similar protocol to that shown in Fig. 1A were normalized to peak outward current and averaged (n=4). Note that the negative slope region of rectification is shifted towards positive potentials.

that the time-dependent component causes much more of the inward rectification at positive potentials than does the instantaneous component. This time-independent component of rectification has not been reported previously. Studies on intact



oocytes using two-electrode voltage clamp would lack the rapid voltage-clamp response time necessary to effectively separate the inactivation at positive potentials from the capacitance current. A rapid method of voltage clamp such as cut-open oocyte clamp or torn-off patch clamp technique is required to resolve the instantaneous *I-V* relationship. The study of Smith et al. [9] examined the current to voltage relationship of h-*erg* expressed in HEK293 cells using torn-off macropatches, with an intracellular solution containing 0.5 mM Mg^{2+} and 150 mM K^+. These conditions may tend to linearize the current due to competition between intracellular Mg^{2+} and K^+ at an internal site. The use of the torn-off patch technique will also eliminate the contribution of any endogenous lipophilic cellular components (e.g. polyamines, [16,17])

Fig. 4. Effects of intracellular chamber Mg^{2+} and EDTA on instantaneous rectification. (A) Effects of 10 mM Mg^{2+} in the intracellular chamber. Instantaneous currents were obtained using the protocol described in Fig. 2 in control intracellular solution. The intracellular chamber was then washed with approx. 30 ml of intracellular solution containing 10 mM Mg^{2+} and was allowed to equilibrate for 15 min and the instantaneous current to voltage relationship was obtained again. (B) Effects of 10 mM EDTA in the intracellular chamber. Instantaneous currents were obtained using the protocol described in Fig. 2 in control intracellular solution. The intracellular chamber was then washed with approx. 30 ml of intracellular solution containing 10 mM EDTA and was allowed to equilibrate for 15 min and the instantaneous current to voltage relationship was obtained again using the same protocol. (C) Elevation of intracellular Mg^{2+} has no effect on the time constants of inactivation. The time constants of inactivation were measured from the same data as in (A). No change in inactivation rate or in the voltage dependence of inactivation was observed.

which may also be acting to block the channel from an intracellular site. Thus, the choice of expression system or the choice of measurement conditions may be of consequence to the linearity of the instantaneous *I-V* relationship. This instantaneous component is unlikely to be of important physiological consequence given the dominance of the time-dependent component. However, care should be taken in interpreting data concerning steady-state inactivation from steady-state *I-V* relationships at positive potentials from intact oocytes.

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