Voltage-dependent blockade of HERG channels expressed in *Xenopus* oocytes by external Ca^{2+} and Mg^{2+}

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1. We expressed the human *eag*-related gene (*HERG*), which is known to encode the delayed rectifier K^+ current (I_{Kr}) in cardiac muscle, in *Xenopus* oocytes. Using a two-microelectrode voltage clamp technique, the effect of external Ca^{2+} and Mg^{2+} on the HERG current (I_{HERG}) was investigated.

2. When $[Ca^{2+}]_o$ was increased, the amplitude of outward I_{HERG} elicited by depolarization decreased, and the rate of current onset slowed. The rate of current decay observed on repolarization was greatly accelerated. The threshold and fully activated potential of I_{HERG} shifted to a more positive potential. On the other hand, the inactivation property represented by the negative slope of the *I–V* curve and the instantaneous conductance of I_{HERG} were little affected by $[Ca^{2+}]_o$.

3. The effect of $[Ca^{2+}]_o$ on I_{HERG} can be interpreted using the channel blockade model. The blockade is voltage dependent; smaller dissociation constants (K_M) at more negative potentials indicate that block is facilitated by hyperpolarization. K_M changes e-fold for 14·5 mV and the fractional electrical distance of the binding site calculated from this value is 0·86.

4. Blockade by a low concentration of Ca^{2+} (0·5 mM) was inhibited by increasing $[K^+]_o$ (from 2 to 20 mM), whereas blockade by a high concentration of Ca^{2+} (5 mM) was not affected by varying $[K^+]_o$, indicating that there is competition between permeating ions and blocking ions.

5. The effect of $[Mg^{2+}]_o$ on I_{HERG} was qualitatively similar to that of $[Ca^{2+}]_o$, but the potency was lower.

6. These results suggest that external Ca^{2+} and Mg^{2+} block the HERG channel in a voltage- and time-dependent manner, resulting in a voltage dependence which has been regarded as a property of the activation gate.

It is well known that intracellular Mg^{2+} and polyamines block the outward flow of K^+ through inward rectifier K^+ channels in a voltage-dependent manner (Matsuda, Saigusa & Irisawa, 1987; Lopatin, Makhina & Nichols, 1994): block is facilitated by depolarization, but is removed by hyperpolarization, resulting in an inward rectification. By this voltage-dependent block mechanism, inward rectifier K^+ channels behave like voltage-dependent channels, in spite of the absence of a voltage-sensing S4 region in the channel proteins. A similar gating mechanism but operating in an opposite way was found in NMDA receptor channels (Nowak, Bregestovsk, Ascher, Herbet & Prochiantz, 1984), where external Mg^{2+} blocks the channels upon hyperpolarization, and in cardiac delayed rectifier K^+ channels which carry the current I_{Kr}. Ho, Earm, Lee, Brown & Noble (1996) have shown that, in rabbit sino-atrial node cells, voltage dependence and the kinetics of I_{Kr}, which have been regarded as a result of voltage-dependent gating, are very sensitive to external Ca^{2+} and Mg^{2+} concentrations, and this effect can be explained by a voltage- and time-dependent block of I_{Kr} by external cations. This re-interpretation is functionally important, considering the significant role of this channel in repolarization and also in the pacemaker depolarization (Irisawa, Brown & Giles, 1993).

It has recently been shown that I_{Kr} is encoded by the *HERG* gene which belongs to the *eag* (*ether-a-go-go*)-like K^+ channel family (Sanguinetti, Jiang, Curran & Keating, 1995; Trudeau, Warmke, Ganetzky & Robertson, 1995; Kiehn, Lacerda, Wible & Brown, 1996). The gating mechanism of HERG channels has generally been considered not to be

different from that of other voltage-gated K^+ channels, except for the fact that inactivation is very fast. The removal of inactivation on hyperpolarization is known to be responsible for inward rectification of the HERG channel (Smith, Baukrowitz & Yellen, 1996; Spector, Curran, Zou, Keating & Sanguinetti, 1996), as it is for the delayed rectifier K^+ channel (Shibasaki, 1987). The activation mechanism has not yet been investigated in detail, however, and whether or not Ca^{2+} acts as a gating molecule as it does in the delayed rectifier K^+ channel has not been determined. In the view of the fact that HERG channels, like other voltage-gated channels, possess a voltage-sensing S4 region, this question is of particular interest. In the present study, we have demonstrated that the HERG channel is blocked by external Ca^{2+} and Mg^{2+} in a voltage- and time-dependent manner, resulting in a voltage dependence which has been regarded as a property of the activation gate.

METHODS

Expression of *HERG* in oocytes

Complementary RNA of *HERG* was synthesized by *in vitro* transcription from 1 μg of linearized cDNA using T7 message machine kits (Ambion, Austin, TX, USA) and stored in 10 mM Tris-HCl (pH 7·4) at −80 °C. Stage V–VI oocytes were surgically removed from female *Xenopus laevis* (Nasco, Modesto, CA, USA) anaesthetized with 0·17% tricaine methanesulphonate (Sigma). Following suture, frogs were allowed to recover in isolation in a tank. Theca and follicle layers were manually removed from the oocytes by using fine forceps. Oocytes were then injected with 40 nl of cRNA (0·1–0·5 μg μl^{-1}). After injection, oocytes were maintained in modified Barth's solution containing (mM): 88 NaCl, 1 KCl, 0·4 $CaCl_2$, 0·33 $Ca(NO_3)_2$, 1 $MgSO_4$, 2·4 $NaHCO_3$, 10 Hepes (pH 7·4), supplemented with 50 μg ml^{-1} gentamicin sulphate. Currents were studied 2–7 days after injection.

Two-microelectrode voltage clamp of oocytes

Normal Ringer solution contained (mM): 96 NaCl, 2 KCl, 1·8 $CaCl_2$, 1 $MgCl_2$, 5 Hepes (pH adjusted to 7·4 with NaOH). In experimental solutions for varying Ca^{2+}, $MgCl_2$ was omitted and the concentration of $CaCl_2$ was varied as indicated. In experimental solutions for varying Mg^{2+}, $MgCl_2$ was varied as indicated and the concentration of $CaCl_2$ was kept at 0·5 mM. To make a 20 mM K^+ solution, the concentration of NaCl was reduced to 78 mM NaCl. Currents were measured at room temperature (21–23 °C) with a two-electrode voltage clamp amplifier (Warner Instruments, Hamden, CT, USA). Electrodes were filled with 3 M KCl and had a resistance of 2–4 MΩ for voltage-recording electrodes and 0·6–1 MΩ for current-passing electrodes. Stimulation and data acquisition were controlled with Digidata and pCLAMP software (Axon Instruments). Unless otherwise stated, data were expressed as mean values ± S.D. (n = number of experiments).

RESULTS

HERG currents (I_{HERG}) expressed in *Xenopus* oocytes were recorded and the effect of external Ca^{2+} concentration ($[Ca^{2+}]_o$) was investigated. As shown in Fig. 1*A*, depolarizing pulses from a holding potential of −60 mV induced activation of outward I_{HERG}. In 0·5 mM $[Ca^{2+}]_o$ with 2 mM $[K^+]_o$, the amplitude of outward currents measured at the end of a 5 s pulse (I_{ss}) grew larger as the membrane was depolarized, reached its maximum at −40 mV, and then decreased progressively with further depolarization, resulting in a bell-shaped I–V relationship (Fig. 1*B*). The negative slope of the I–V curve is considered as a unique property of HERG channels distinct from other delayed rectifier K^+ channels, and has been explained by assuming very fast (almost instantaneous) and voltage-dependent inactivation on depolarization (Smith *et al.* 1996; Spector *et al.* 1996).

On returning to the holding potential of −60 mV, outward tail currents (I_{tail}) developed. The amplitude of I_{tail} was larger than that of I_{ss} elicited by the preceding depolarizing pulse, reflecting the inwardly rectifying property of HERG channels. The amplitude of tail currents (I_{tail}) increased progressively and reached its maximum at 0 mV in 0·5 mM $[Ca^{2+}]_o$. The I_{tail}–V relationship was well fitted by a Boltzmann equation with $V_{½} = -40$ mV and $dx = 7·6$ mV ($V_{½}$, membrane potential at half-maximal inactivation; dx, steepness of the curve; Fig. 1*C*). This curve has been considered to represent the voltage-dependent property of the activation gate of HERG channels. In other words, the development of I_{HERG} on depolarization has been known as activation, and the decay of current on repolarization as deactivation.

When external Ca^{2+} was increased to 5 mM, the I_{HERG} which developed on depolarization decreased significantly and the rate of current onset slowed (Fig. 1*A*, lower panel). By contrast, the amplitude of I_{tail} decreased slightly and the decay rate of I_{tail} was accelerated significantly. (Initial transient outward currents in the early part of depolarization were observed only in $[Ca^{2+}]_o \geqslant 5$ mM. Since they were not accompanied by outward tail currents when short pulses were applied, they were not considered as I_{HERG}.) On the other hand, reducing $[Ca^{2+}]_o$ to 0·1 mM induced changes in I_{HERG} opposite to those induced by increasing $[Ca^{2+}]_o$. The decay of I_{tail} became very slow and the holding current level at −60 mV shifted outward, indicating that at this potential, channels were already open. The holding potential was therefore changed to −80 mV in order to maintain the holding current near zero (data not shown).

The effect of various concentrations of $[Ca^{2+}]_o$ on I_{ss} and I_{tail} is demonstrated in Fig. 1*B* and *C*. As $[Ca^{2+}]_o$ was progressively increased, activation of outward current started at a more positive potential (shown in the I_{ss}–V relationship as a rightward shift, Fig. 1*B*), and the amplitude of I_{ss} decreased. The I_{tail}–V curve, which represents the voltage dependence of conductance increase induced by depolarization, shifted progressively to the right (Fig. 1*C*). The steepness of the curve (dx) was also changed ($dx = 5·3$ mV in 0·1 mM, 8·7 mV in 5 mM), suggesting that

the effect of Ca^{2+} is not a simple shift of voltage dependence. The amplitude of I_{tail} also decreased progressively for $[Ca^{2+}]_o > 0{\cdot}5$ mM. The negative slope of the $I_{ss}-V$ curve, which represents the inactivation property of I_{HERG} (conductance decrease induced by depolarization), was, however, little affected by $[Ca^{2+}]_o$. In Fig. 2, the maximum conductance of HERG channels was obtained by applying two step pulses: a varying level of test pulses following the prepulse to +20 mV which is given to induce a full activation of I_{HERG}. Test pulses induced a transient current increase before deactivation occurred (outward current for $V > -80$ mV, inward current for $V < -80$ mV). The amplitude of the currents was measured at its peak (I_{peak}) and plotted against the membrane potential, showing a strong inward rectification which is typical of HERG channels (Fig. 2*B*). The $I_{peak}-V$ relationship and the reversal potential were hardly affected by $[Ca^{2+}]_o$.

The above results show that external Ca^{2+} does not affect the inactivation property of I_{HERG}, but affects only activation/deactivation. The effect is dose dependent: at higher $[Ca^{2+}]_o$, further depolarization is needed to activate the current and deactivation is facilitated. These effects are similar to those found in the rapidly activating delayed rectifier K^+ current (I_{Kr}) of sinoatrial node cells of the rabbit heart as described by Ho *et al.* (1996). They interpreted this action of external Ca^{2+} as a voltage-dependent block on the basis of Woodhull's analysis (1973), and proposed a new interpretation for the I_{Kr} channel: activation/deactivation of the I_{Kr} channel is in fact controlled by voltage-dependent block/unblock by external Ca^{2+}. It is still possible, however, that the action of Ca^{2+} is a result of a simple shift of voltage dependence of gating due to the surface charge effect. In order to test this possibility, we investigated the effect of $[Ca^{2+}]_o$ on I_{HERG} in different K^+ concentrations where ionic

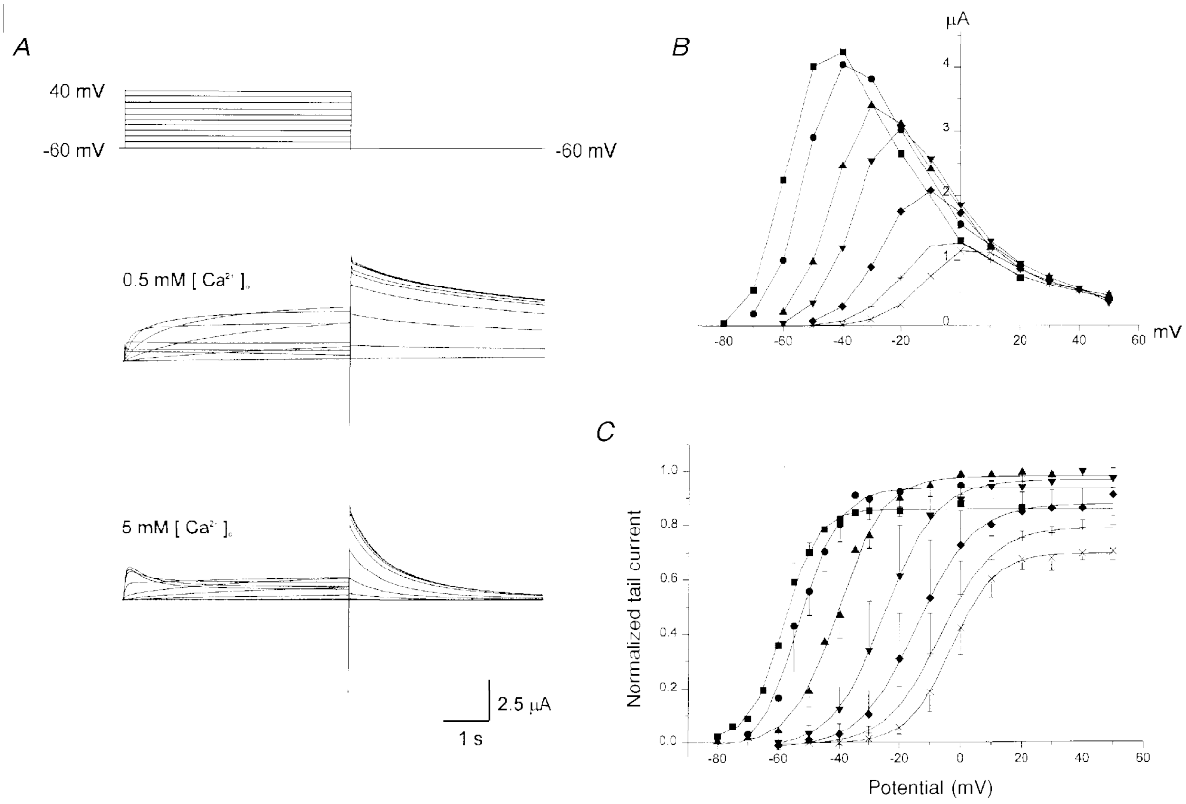


Figure 1. Effect of external Ca^{2+} concentration on HERG currents elicited by depolarizing voltage pulses

A, superimposed current traces elicited by depolarizing voltage pulses (5 s) in 10 mV steps (upper panel) from a holding potential of −60 mV in 0·5 mM (middle panel) and in 5 mM (lower panel) $[Ca^{2+}]_o$. $[K^+]_o$ is 2 mM. *B*, plot of the steady-state current measured at the end of depolarizing pulses against the pulse potential in different external $[Ca^{2+}]_o$ (obtained from the same cell shown in *A*). *C*, plot of the normalized tail current measured at its peak just after repolarization. The amplitude of the tail current was usually maximal at 0·5 mM $[Ca^{2+}]_o$ and was taken as 1. Symbols with error bars represent mean ± S.D.: data obtained from 4 cells. Symbols in *B* and *C*: ■, 0·1 mM; ●, 0·2 mM; ▲, 0·5 mM; ▼, 1 mM; ◆, 2·5 mM; +, 5 mM; ×, 10 mM $[Ca^{2+}]$. Lines in *C* are the fits to the Boltzmann equation, $y = 1/\{1 + \exp((-V + V_{½})/dx)\}$ ($V_{½}$ from left to right, −58, −53, −40, −25, −14, −7, and −3 mV; dx from left to right, 5·3, 6·7, 7·6, 8·7, 9·2, 8·7, and 7·1 mV).

strength was maintained constant (2 mM $[K^+]_o$–96 mM $[Na^+]_o$, 20 mM $[K^+]_o$–78 mM $[Na^+]_o$). If the effect of Ca^{2+} is mainly caused by the surface charge effect, Ca^{2+} should produce the same effect in 2 mM $[K^+]_o$ and in 20 mM $[K^+]_o$, since the surface charge effect produced by changing $[Ca^{2+}]_o$ is expected to be same in the two solutions. The result is demonstrated in Fig. 3.

When $[K^+]_o$ was increased to 20 mM, the holding potential was changed to −40 mV in order to minimize the continuous current flow. But the tail current was recorded at −60 mV, as it was in 2 mM $[K^+]_o$, in order to compare the effect of Ca^{2+} in different $[K^+]_o$ under the same conditions. I_{tail} in 2 mM and in 20 mM $[K^+]_o$ was measured and normalized values were plotted in Fig. 3*B* (using the same method as in Fig. 1*C*). In 5 mM Ca^{2+}, I_{tail}–V curves in 2 mM (○) and in 20 mM (□) $[K^+]_o$ almost overlapped. When $[Ca^{2+}]_o$ was reduced to 0·5 mM, the shift in the I_{tail}–V curve was far more pronounced in 20 mM $[K^+]_o$ (■) than in 2 mM $[K^+]_o$ (●): the voltage at which the HERG channel is half-open ($V_{½}$) is −36 mV in 2 mM $[K^+]_o$, and −68 mV in 20 mM $[K^+]_o$. This discrepancy strongly contradicts the prediction based on the surface charge effect. A possible explanation can be found, if we suppose that the I_{tail}–V relationship, which was formerly thought to represent steady-state activation, represents a steady-state block of I_{HERG} by external Ca^{2+}. If this is the case, the discrepancy implies that when the concentration of blocking ion (Ca^{2+}) is low, a high concentration of permeating ion (K^+) interferes with the blockade and the same degree of block is obtained at more negative voltages. It agrees well with the assumption that permeating ions (K^+) compete with blocking ions (Ca^{2+}) at the same site.

It was reported that external Mg^{2+} blocks I_{Kr} channels of SA node cells in the same way as external Ca^{2+} (Ho *et al.* 1996). We tested the effect of varying $[Mg^{2+}]_o$ on I_{HERG} and the result is demonstrated in Fig. 4. $[Ca^{2+}]_o$ was kept constant at 0·5 mM to prevent the development of a leak conductance in oocytes in the absence of external Ca^{2+}. The effect of increasing $[Mg^{2+}]_o$ was similar to that of Ca^{2+}. When $[Mg^{2+}]_o$ was increased progressively, the I_{HERG} which developed on depolarization started at a more positive potential and the amplitude of I_{ss} decreased. The decay rate of I_{tail} was accelerated significantly (Fig. 4*A*, lower panel), and the I_{tail}–V curve shifted progressively to the right (Fig. 4*C*). The result, however, revealed that the effect of Mg^{2+} is less potent than Ca^{2+}: addition of 10 mM Mg^{2+} shifted $V_{½}$ by 20 mV, whereas addition of 4·5 mM Ca^{2+} shifted it by 33 mV.

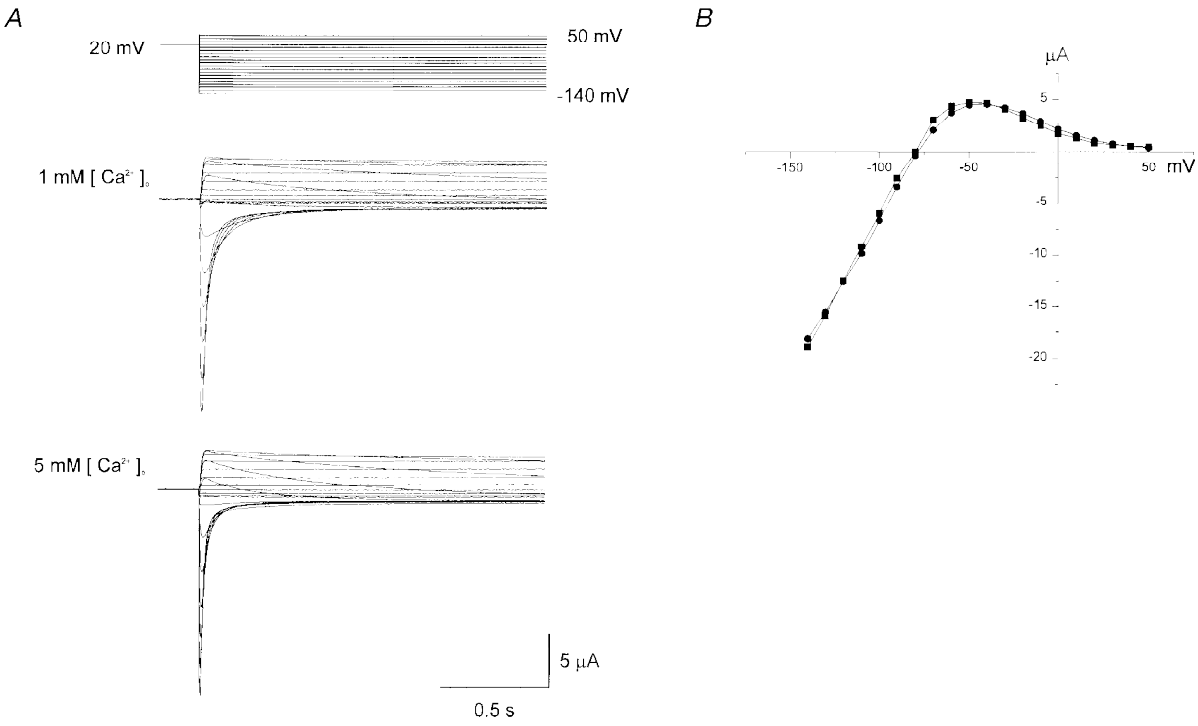


Figure 2. Effect of $[Ca^{2+}]_o$ on the inward rectification of the fully activated I–V relationship of HERG channels

A, superimposed current traces elicited by various levels of test pulses ranging from −140 to +50 mV following the prepulse to +20 mV for 5 s (upper panel, front part of prepulse is not shown) in 1 mM (middle panel) and 5 mM (lower panel) $[Ca^{2+}]_o$. *B*, plot of the amplitude of peak currents measured at the beginning of the test pulse in 1 mM (●) and in 5 mM (■) $[Ca^{2+}]_o$.

DISCUSSION

The action of external Ca^{2+} and Mg^{2+} on the HERG channel presented in this paper is similar to that on the rapidly activating delayed rectifier K^+ current (I_{Kr}) of sinoatrial node cells of the rabbit heart (Ho *et al.* 1996). In the present study, we not only confirmed that their action is common to both I_{Kr} and I_{HERG}, but also discovered several important features which were not easily tested in native I_{Kr}: competition with $K^+$and a flow-independent effect.

The gating characteristics of the HERG channel are depicted as follows:

$$\text{Close} \rightleftharpoons \text{Open} \rightleftharpoons \text{Inactivated}$$

Scheme 1

Two different approaches are possible for modelling the effect of Ca^{2+} on the HERG channel presented in the present paper. The first approach is to regard the action of Ca^{2+} as a modifier of intrinsic voltage-dependent gating (see also Discussion in Ho *et al.* 1996). This has been widely investigated and it is well known that various divalent cations can modify gating of the voltage-dependent channels either by screening the surface charge of the membrane (Green & Andersen, 1991; Hille, 1992), or by direct modulation (Armstrong & Lopez-Barnes, 1987; Spires & Begenisich, 1992, 1994). This approach requires the presence of intrinsic voltage-dependent gating which persists even in a Ca^{2+}-free solution. However, it has not been possible to determine the intrinsic gating properties experimentally, since in Ca^{2+}-free or low-Ca^{2+} solutions (less than 0·1 mM), a non-specific leak conductance developed progressively, which prevented further study. The disappearance of channel properties, including gating and selectivity, in Ca^{2+}-free solution has also been observed in studies on the delayed rectifier K^+ channel in squid giant axon (Armstrong & Lopez-Barnes, 1987), and it was suggested that Ca^{2+} ions act as a cofactor for channel gating. It is, however, not certain whether this phenomenon implies that the HERG channel also requires Ca^{2+} ions to preserve its properties. On the other hand, the increase of monovalent cationic conductance in divalent cation-free solutions has been observed in control oocytes (Arellano,

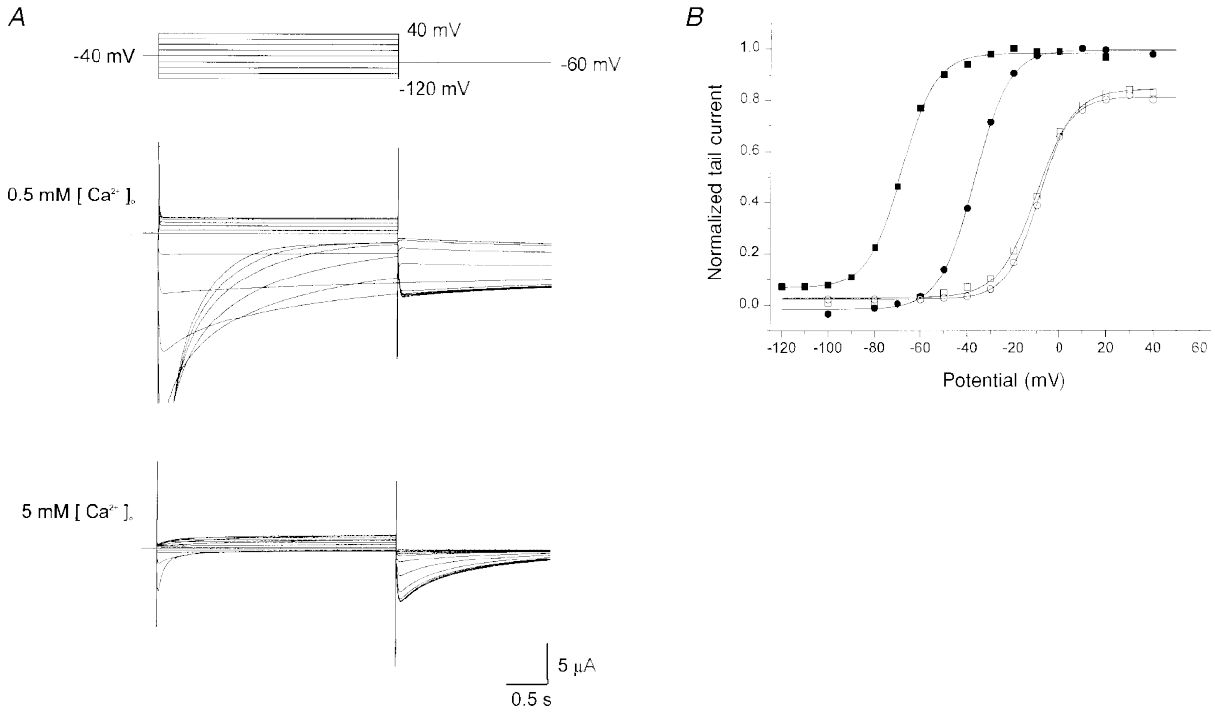
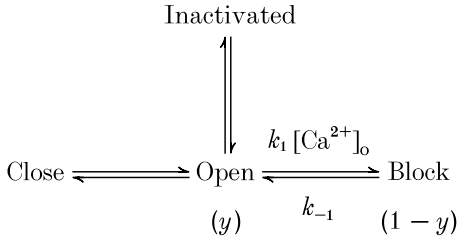


Figure 3. The effect of $[K^+]_o$ on the action of Ca^{2+} on HERG currents

A, superimposed current traces elicited by depolarizing voltage pulses (3 s) in 10 mV steps (upper panel) from the holding potential of −40 mV in high-K^+ solution (20 mM) with 0·5 mM (middle panel) and 5 mM (lower panel) $[Ca^{2+}]_o$. Some of the initial parts of the current in 0·5 mM $[Ca^{2+}]_o$ are out of scale. Tail currents were recorded on repolarization to −60 mV. *B*, plot of the normalized tail current measured at its peak just after repolarization. The same experiment shown in *A* was performed in 2 mM $[K^+]_o$ in the same cell and the data are shown in the plot. ■, 0·5 mM $[Ca^{2+}]_o$–20 mM $[K^+]_o$; □, 5 mM $[Ca^{2+}]_o$–20 mM $[K^+]_o$; ●, 0·5 mM $[Ca^{2+}]_o$–2 mM $[K^+]_o$; ○, 5 mM $[Ca^{2+}]_o$–20 mM $[K^+]_o$. Lines are the fits to the Boltzmann equation, $y = 1/\{1 + \exp(-(V - V_{½})/dx)\}$ ($V_{½}$ from left to right, −68, −36, − 10, and −9 mV; dx from left to right, 7·3, 7·3, 8·1, and 6·8 mV). Same observation from three cells.

Woodward & Miledi, 1995), suggesting that it may be related to a native property of oocytes.

Another approach is to interpret the action of Ca^{2+} independently of intrinsic gating. In cardiac SA node cells, I_{Kr} was recorded in Ca^{2+}-free solutions and the intrinsic gating properties were described (Ho *et al.* 1996). However, the effect of Ca^{2+} and Mg^{2+} was not satisfactorily explained by a simple shift of the voltage dependence of gating. A significant difference in the steepness of the activation curve ($dx = 4{\cdot}2$ mV in Ca^{2+}-free and 9·1 mV in 5 mM Ca^{2+}) was observed, suggesting that the Ca^{2+}-dependent process is fundamentally different from intrinsic voltage-dependent gating. This tendency was also observed in the present paper. Ho *et al.* (1996) interpreted the action of external Ca^{2+} and Mg^{2+} on I_{Kr} as a voltage-dependent block, on the basis of Woodhull's analysis (1973), and successfully modelled the effect of Ca^{2+} and Mg^{2+} on I_{Kr}. Since the effects on I_{HERG} presented in the present paper are similar to those on I_{Kr}, we applied the same model to the HERG channel for quantitative analysis. The Ca^{2+}-dependent process can be added to Scheme 1:



Scheme 2

Since the intrinsic gating (open–close, open–inactivated) in the absence of Ca^{2+} was not determined in the present experiment, we only dealt with the condition in which the

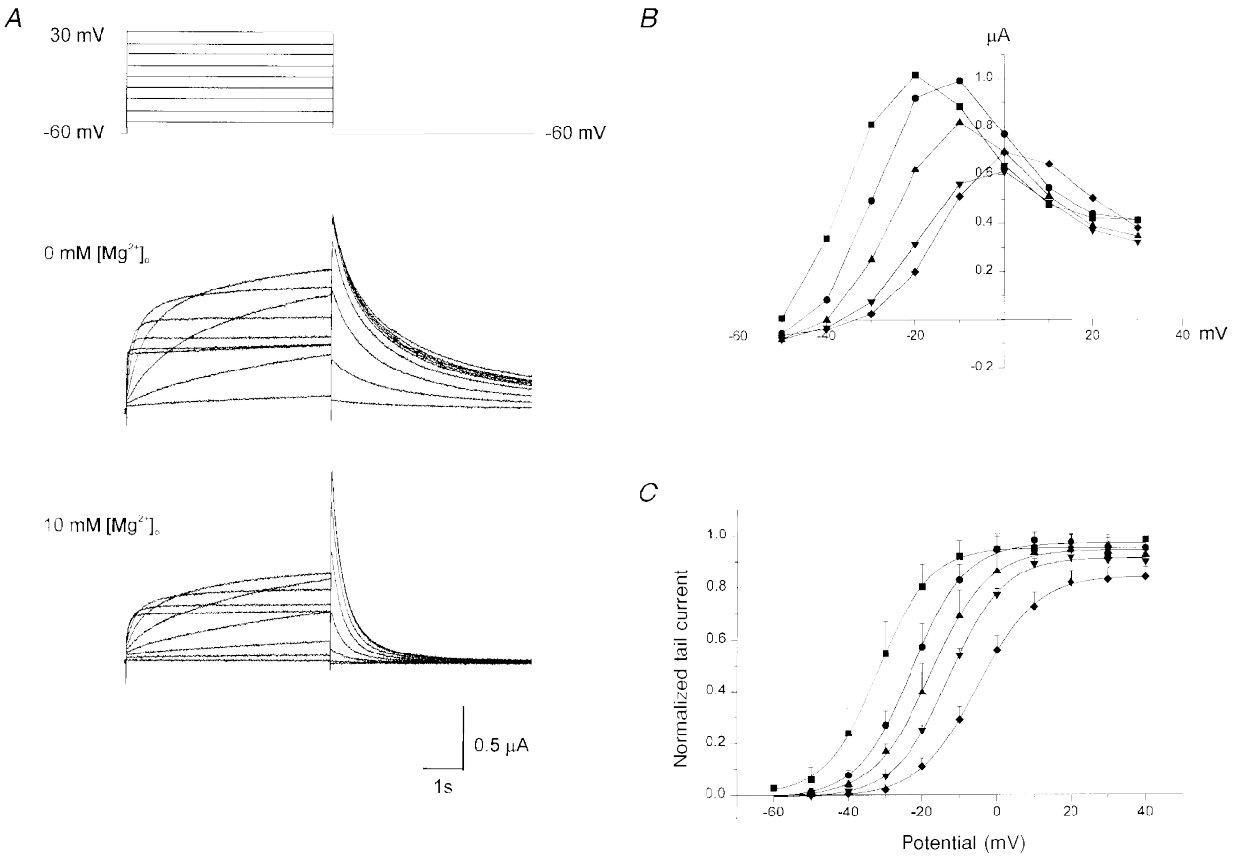


Figure 4. Effect of $[Mg^{2+}]_o$ on HERG currents elicited by depolarizing voltage pulses

A, superimposed current traces elicited by depolarizing voltage pulses (5 s) in 10 mV steps (upper panel) from the holding potential of −60 mV in 0 mM (middle panel) and in 10 mM (lower panel) $[Mg^{2+}]_o$. $[Ca^{2+}]_o$ was 0·5 mM and $[K^+]_o$ was 2 mM. *B*, plot of the steady-state current measured at the end of depolarizing pulses against the pulse potential in varying external $[Mg^{2+}]_o$ (obtained from the same cell shown in *A*). *C*, plot of the normalized tail current measured at its peak just after repolarization. Symbols with error bars represent mean ± S.D. Data obtained from 4 cells. Symbols in *B* and *C*: ■, 0 mM; ●, 2·5 mM; ▲, 5 mM; ▼, 10 mM; ◆, 20 mM $[Mg^{2+}]_o$. Lines in *C* are the fits to the Boltzmann equation, $y = 1/\{1 + \exp((-V + V_{½})/dx)\}$ ($V_{½}$ from left to right, −32, −23, −17, −12, and −5 mV; dx from left to right, 7·0, 7·2, 7·7, 7·3, and 7·8 mV).

conversion between open and inactivated and open and closed states can be neglected. We analysed I_{tail} at a fixed potential (−60 mV), so that the inactivation parameter was constant. From the results obtained at the lowest $[Ca^{2+}]_o$ used (0·1 mM, Fig. 1*C*; I_{tail} reaches it maximum at −60 mV), we can assume that the fraction of closed states is negligible for $V > -60$ mV. Thus, over the voltage range $V > -60$ mV, the Ca^{2+}-dependent block process is a major factor only in determining the fraction of conducting channels. The amplitude of I_{tail} thus represents the fraction of conducting channels (y) at the end of the test pulse. The effect of $[Ca^{2+}]_o$ on y at various membrane potentials was obtained by dividing I_{tail} by the maximum I_{tail} (which indicates when $y = 1$). The dose–response relationship between y and $[Ca^{2+}]_o$ was fitted by the following equation:

$$y = 1/(1 + [Ca^{2+}]_o{}^n/K_M).$$

Dissociation constants (K_M) at −50, −40, −30, −20, −10, 0, 10 and 20 mV were 0·2, 0·4, 0·8, 1·4, 3·0, 6·7, 13·8 and 26·6 mM, respectively. Hill coefficients, n, were between 0·8 and 1·4. This value is compatible with previous results (Ho *et al.* 1996) showing that the number of binding sites for Ca^{2+} in I_{Kr} channel is one. The decrease in K_M following hyperpolarization (e-fold change of 14·5 mV) shows that blockade is facilitated by hyperpolarization. From the voltage dependence of the dissociation constants, the fractional electrical distance (δ) was calculated to be 0·86. The effect of $[Mg^{2+}]_o$ was subject to same analysis. The result shows that Mg^{2+} is less potent than Ca^{2+}, and the K_M value for Mg^{2+} at 0 mV is 24·8 mM (6·7 mM for Ca^{2+}). K_M is also voltage dependent (e-fold change of 16·3 mV) and the fractional electrical distance (δ) is calculated to be 0·67.

This analysis supports the hypothesis that external Ca^{2+} and Mg^{2+} block the HERG channel in a voltage- and time-dependent manner, and these properties have been regarded as properties of the activation gate. The idea is related to the gating particle theory, originally suggested by Frankenhaeuser & Hodgkin (1957) to explain the decrease in membrane conductance induced by increased $[Ca^{2+}]_o$. The gating particle theory supposes that Ca^{2+} ions are an essential component in voltage-dependent gating, acting as a voltage sensor or charged gating particles for the channel.

Although we have tested the channel blockade model as the most likely mechanism for the action of external Ca^{2+} and Mg^{2+}, there are several points both in the present study and in the previous study on I_{Kr} (Ho *et al.* 1996) which do not agree with this model. The decrease in maximum conductance at positive potentials (Figs 1*C* and 4*C*) and the slowing of current onset with increasing $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ are not easily explained by a simple block model. In order to reproduce the decrease in maximum conductance, it may be necessary to introduce another blocked state which is voltage independent. The slowing of current onset with increasing $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ does not conform to a simple block model, in which the unbinding rate constant (k_{-1}) does not depend on the concentration of blocking ions. This result may possibly be explained by introducing multiple blocked states (B1, B2 and B3), since the rate of current onset depends not only on k_{-1} but also on the fraction of channels in each state. If a higher concentration of blocking ions increases the fraction of B3 over B1, current onset will be slowed. The presence of multiple non-conducting states is also supported by a recent study by Wang, Liu, Morales, Strauss & Rasmusson (1997). They observed that the time course of the onset of I_{HERG} on depolarization is sigmoidal, and suggested that at least three closed states (C1, C2 and C3) were required to reproduce the sigmoidal time course. Another interpretation is, however, still possible, namely that Ca^{2+} and Mg^{2+} modify the activation gate in such a way that they accelerate channel closure and slow channel opening.

In spite of such disagreement, the results in Fig. 3 showing the dependence of the Ca^{2+} effect on $[K^+]_o$ can only be explained by the channel blockade model. Various K^+ channels are known to be affected by $[K^+]_o$ and the effect of $[K^+]_o$ on HERG channels has already been examined (Baukrowitz & Yellen, 1995; Wang, Morales, Liu, Strauss & Rasmusson, 1996; Yang, Snyders & Roden, 1997). However, the effect of $[K^+]_o$ in relation to varying $[Ca^{2+}]_o$ has never been tested. The results shown in Fig. 3 clearly demonstrate the competition between K^+ and Ca^{2+}. This feature can be found when permeating ions and blocking ions bind to the same site. The other theories, such as the surface charge and gating modifier theories, do not predict such interaction. Details of the interaction between the two ions need to be investigated further.

A question which is functionally very important but which could not be investigated in detail in cardiac I_{Kr} is whether the effect of external cations depends on the direction of current flow. It is well known that voltage-dependent blockade of inward rectifier K^+ channels by internal Mg^{2+} depends on E_K (reversal potential for K^+) since the blockade is flow dependent; Mg^{2+} blocks only outward currents (Matsuda *et al.* 1987). If this is also true for Ca^{2+} block of I_{Kr}, the results obtained from inward I_{Kr} in high-K^+ solution cannot be extrapolated to normal conditions under which I_{Kr} flows outwards. It is not easy, however, to investigate I_{Kr} in normal K^+ solution, since outward I_{Kr} is very small due to its inward rectifying property. Furthermore, it is difficult to isolate I_{Kr} from other outward currents in cardiac myocytes. Ho *et al.* (1996) therefore recorded I_{Kr} in symmetrical K^+ solutions as an inward current. This study involving the expression of *HERG* allows these limits to be overcome, and we have found that outward I_{HERG} is controlled by external Ca^{2+} and Mg^{2+} in the same way as inward I_{Kr}.

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