HERG Potassium Channel Activation Is Shifted by Phorbol Esters via Protein Kinase A-dependent Pathways*

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We investigated the effects of the phorbol ester phorbol 12-myristate 13-acetate (PMA) on the rapid component of the delayed rectifier potassium current, \( I_{Kr} \), in guinea pig cardiomyocytes and found that the \( I_{Kr} \) current amplitude was reduced by 20% with 10 nM PMA and 44% with 100 nM PMA. The ether-a-go-go-related gene (HERG) encodes \( I_{Kr} \) in human heart. We expressed HERG heterologously in Xenopus oocytes and investigated the effects of PMA on the delayed rectifier potassium current. Upon application of PMA in a concentration of 100 nM, we found a similar reduction of HERG outward current amplitude by 59%. This reduction was due to a shift in the HERG activation curve by 37 mV. The \( ED_{50} \) for the PMA-induced shift was 9.0 nM. The inactive 4a-phorbol 12-myristate 13-acetate (4a-PMA) had no effect. PMA is known to act by stimulating distinct protein kinase cascades. Additional application of the specific protein kinase C inhibitors chelerythrine (10 \( \mu \)M) or bisindolylmaleimide (1 \( \mu \)M) could not attenuate the PMA-induced shift. In contrast, the shift by PMA was reduced significantly when the specific protein kinase A (PKA) inhibitors H89 (50 \( \mu \)M) or KT5720 (2.5 \( \mu \)M) were applied. Forskolin (400 \( \mu \)M), an activator of the adenylate cyclase that results in PKA activation, shifted the HERG activation curve by 14 mV. Moreover, the specific protein kinase C activator 1-stearoyl-2-arachidonoylglycerol (10 \( \mu \)M) showed no effect. Our data suggest that mainly PKA is mediating the shift of the HERG activation kinetics.

Repolarization of the cardiac action potential involves many potassium currents (1). One crucial repolarizing potassium current is the rapid component of the delayed rectifier potassium current (\( I_{Kr} \))\(^3\) (2–4). Recently, it has been shown that the underlying gene of the \( I_{Kr} \) current is the human ether-a-go-go-related gene (HERG) (5, 6). HERG expressed in Xenopus oocytes produces a potassium current largely indistinguishable from native cardiac \( I_{Kr} \) (6, 7). The HERG channel is the molecular target of antiarrhythmic therapy with Class III antiarrhythmic drugs, with the result that \( I_{Kr} \) is blocked, and the cardiac action potential is prolonged (7, 8). Congenital mutations in the HERG gene produce one form of the congenital long QT syndrome (LQT2) (9). Electrophysiological studies have shown that these HERG mutations result in a reduced HERG current with the consequence that the cardiac action potential and QT interval on the surface electrocardiogram of this patient is prolonged (10). These patients have a high risk for sudden cardiac death because of torsade de pointes ventricular arrhythmias (11).

Protein kinases have been found in almost all cell membranes, and it has been demonstrated that they regulate ion channels (12–16). The biophysical properties of ion channels can be affected by protein kinases in native tissue (12, 14) and when ion channels were expressed heterologously in Xenopus oocytes (13, 16). Among the various protein kinases that have been found in almost all cells are protein kinase C (PKC) and protein kinase A (PKA). The PKC is a family of serine/threonine kinases that is implicated in intracellular signaling events triggered in response to a variety of agonists. At least 11 different PKC isofoms have been identified (17). The effects of PKC on an effector protein can be tested via phorbol ester activation of the PKC cascade, which involves many steps with lipids and proteins of the plasma membrane. PMA is a very potent activator of PKC but not specific for PKC. Besides activation of the PKC cascade, it also has effects on other protein kinases (18, 19).

PKA is a serine/threonine kinase that is cAMP-dependent. PKA can be stimulated by extracellular signals that elevate the intracellular cAMP concentration. cAMP binds to the regulatory subunit of the enzyme, and the catalytic subunit phosphorylates the substrate. The effector protein can be phosphorylated directly, or other proteins may be phosphorylated that act on the effector protein. An increased intracellular cAMP concentration and finally PKA activation can be achieved experimentally with the adenylate cyclase activator forskolin (20). Effects of PKA on substrates can also be investigated by the use of specific PKA inhibitors.

Protein kinases play an essential role in the regulation of many cellular processes under physiological and pathophysiological conditions. The aim of this study was to investigate whether protein kinases, specifically PKA and PKC, are involved in the regulation of the HERG potassium channel, which underlies \( I_{Kr} \). Because protein kinases are present endogenously in Xenopus oocytes, we could use protein kinase activators and inhibitors. Using combinations of various activators and inhibitors, we provide evidence that PKA is a key enzyme involved in the PMA-induced shift of the HERG activation kinetics.

EXPERIMENTAL PROCEDURES

Solutions and Drug Administrations—Patch-clamp measurements of guinea pig cardiomyocytes were performed in a bath solution containing

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The abbreviations used are: \( I_{Kr} \), delayed rectifier potassium current; PKC, PKA, and PKG, protein kinases C, A, and G, respectively; PMA, phorbol 12-myristate 13-acetate; DAG, 1-stearoyl-2-arachidonoylglycerol.
**RESULTS**

**PMA Reduces I_{Kr} Currents in Guinea Pig Cardiomyocytes**—We investigated the effects of PMA on I_{Kr} in isolated guinea pig cardiomyocytes using a two-step protocol. From a holding potential of −40 mV (where the sodium current is inactivated), we stepped to a variable test pulse from −40 to 40 mV in 20-mV increments and then back to a constant second step to −40 mV to measure tail currents (Fig. 1A). After a 10-min equilibrium time, the control measurements were done. Then we perfused 100 nM PMA into the bath and made measurements every 3 min. After 15 min, peaks of the I_{Kr} tail currents were reduced as shown in Fig. 1, B and C. Fig. 1D shows the concentration dependence of the effect of PMA on the peak tail current amplitudes after the test pulse to 40 mV. 1 nM PMA showed no significant current reduction (2.7 ± 8.7% (n = 3), 10 nM significantly reduced the peak tail current amplitude by 20 ± 7.3% (n = 3), and 100 nM PMA reduced the peak tail current significantly by 44 ± 7.4% (n = 6).

PMA Reduces Outward Currents of HERG—When HERG is expressed in Xenopus oocytes, potassium outward currents were measured in a two-step protocol, a variable first step (test pulse potential) to measure activating outward currents and a second step to constant −60 mV to measure tail currents. The control HERG current had an activation threshold of −40 mV, and the current amplitude reached a maximum at 0 mV and decreased at test pulse potentials from 20 to 70 mV because of inward rectification that is characteristic to this channel (2, 6, 7) (Fig. 2, A and C). In the second step of the protocol the tail current amplitude depends on the preceding test pulse and is a
The peak tail current was virtually not changed (I_decreased the maximal outward current to 25%), whereas by PMA reduces HERG outward currents. The numbers at the end of the tail currents indicate the corresponding test pulse potential preceding the tail current. C shows the current amplitude at the end of the test pulse as a function of the test pulse potential in control and after stimulation with PMA. The maximum current amplitude at 0 mV is reduced in the measurements with PMA to 41%. D shows the peak tail current amplitude as a function of the test pulse potential during the first step of the protocol. The resulting activation curve of the channel is shifted by 27.8 mV. Holding potential, −80 mV; test pulse, −80 to 80 mV (400 ms) in 10-mV increments; return pulse constant, −60 mV (400 ms). Bath, 5 mM K.

The Biophysical Mechanism of Current Reduction by PMA—We investigated in more detail the mechanism of HERG current reduction by PMA. Inward tail currents at −120 mV were larger and could therefore be investigated more precisely than outward tail currents, which are close to the potassium equilibrium potential of −75 mV. Fig. 3A shows an I-V measured with a protocol similar to that in Fig. 2A, except that the tail currents were measured at −120 mV. 30 min PMA (100 nM) decreased the maximal outward current to 25 ± 4.7% (n = 8). The peak tail current was virtually not changed (I_tail, PMA/I_tail, control = 90.8 ± 10.5% (n = 8)), but the activation curve was shifted ΔV_1/2 = 37 ± 6.5 mV (n = 8) (Fig. 3E). The slope k of the activation curve was not significantly changed (k_control = −9.5 ± 1.2 mV versus k_30 min PMA = −11.7 ± 1.1 mV (n = 8)).

To address the question whether the current shifted by PMA is a pure HERG current, we chose two different approaches. First, we determined the reversal potential for the control measurement and for the measurement with 100 nM PMA in a tail current protocol, where we activated the current with a first step to 40 mV and stepped back to various potentials between −140 and −20 mV to measure tail currents. The peaks of the tail currents were plotted as an I-V (Fig. 3F). The reversal potential was nearly identical in control (−76 mV) versus the measurements after 30 min 100 nM PMA (−74.6 mV). The tail current amplitudes with PMA were generally smaller because the activation at 40 mV with PMA was smaller compared with control. This result was obtained in four experiments and indicates that the currents affected by PMA were pure potassium currents with a predicted potassium equilibrium potential of −75 mV. The second approach to demonstrate that PMA acts on the HERG channel was to block the currents after the PMA measurements almost completely with 10 μM dofetilde, which is a specific blocker of HERG. Under the given experimental conditions, the block of HERG by dofetilde is expected to be about 95% (7). This was found in four experiments (Fig. 3C).

PMA might change the inactivation properties of the HERG channel as well. To measure inactivation, we used a special protocol that inactivates the channel at a holding potential of 20 mV, recovers the current from inactivation at various potentials from −120 to 30 mV (20 ms) in 10 mV steps, and measures the resulting peak outward current at constant 20 mV as a measure of steady-state inactivation (6, 22). Having
obtained the control measurement shown in Fig. 4A, we washed in 100 nM PMA for 30 min and held the cell during this period of time at −80 mV. In this set of experiments, a holding potential of −80 mV was necessary to prevent destruction of the oocyte that usually occurs when holding the oocyte 30 min at 20 mV. Finally we made the measurements with PMA, shown in Fig. 4B. In Fig. 4C, the inactivating outward current amplitude (measured at constant 20 mV) was normalized and plotted against the test pulse potential, giving the steady-state inactivation curve. This curve could be fitted with a Boltzmann distribution and gave the voltage for half-maximal inactivation of 68.4 ± 1.8 mV (n = 4) for control and −69.8 ± 2.0 mV (n = 4) for the PMA measurements. There was only a small shift of 4.6 mV in the inactivation curves. The pronounced shift in the activation curve by 37 mV together with the small shift in the inactivation curve explains why the current amplitude during the test pulse was reduced, whereas the maximum tail current amplitude was not.

To test whether the effect of PMA was specific, we measured a dose-response curve for the effect of PMA on the activation curve shift (ΔV1/2) (Fig. 4E). The half-maximal effective dose (ED50) was 9 nM and demonstrated that the effect of PMA on ΔV1/2 was very potent. The stability of the experimental setup and the onset of the PMA effect is demonstrated in Fig. 4F. We further tested whether the effect of PMA could be washed out. In three experiments the effect of PMA was partially reversible and shifted back the activation curve by ΔV1/2 = −12.3 ± 1.9 mV after a 120-min washout.

Protein Kinase A Is Involved in the Activation Shift of HERG upon Stimulation with PMA—To elucidate the role of protein kinases for the PMA-related activation shift of HERG, we perfused 100 nM PMA together with protein kinase inhibitors into the bath for 30 min. After 30 min, the measurements for the activation curve were performed. First we measured the effects of 100 nM PMA plus the wide range protein kinase inhibitor staurosporine (1 μM), which inhibits protein kinases unspecifically with an IC50 of 8–20 nM (Table 1 line B) (23). PMA (100 nM) plus staurosporine (1 μM) gave a significantly smaller shift in the activation curve (ΔV1/2 = 10.7 ± 7.7 mV (n = 4)) than PMA (100 nM) alone (ΔV1/2 = 37.3 ± 6.5 mV (n = 8)) (Table 1, line B versus line A). This demonstrates that protein kinases in general are involved, without specifying the types of protein kinases further. The staurosporine derivative bisindolylmaleimide is known to inhibit specifically PKC by acting as a competitive inhibitor for ATP on PKC without strong effects on other protein kinases (Table 1, line C) (24). Even very high concentrations up to 1 μM showed no significant reduction in the PMA-induced shift (Table 1, line C). The structurally different very specific PKC inhibitor chelerythrine (25) also
showed no significant reduction in the PMA-induced shift in a high concentration of 10 μM (Table I, line D).

Different from the PKC inhibitors, the specific PKA inhibitor H89 (26) reduced the PMA-induced shift to $D_{1/2}V = 26.7 ± 2.6$ mV ($n = 5$) (Table I, line E) in a concentration of 5 μM and to $D_{1/2}V = 5.8 ± 2.4$ mV ($n = 5$) in a concentration of 50 μM (Table I, line F). KT5720 is an even more specific inhibitor of PKA (27).

KT5720 (2.5 μM) significantly reduced the PMA-induced shift to $D_{1/2}V = 7.0 ± 5.4$ mV ($n = 5$) (Table I, line G). The protein kinase G (PKG) inhibitor Rp-8-pCPT-cGMPS (28) (20 μM) and the broad range tyrosine kinase inhibitor genistein (29) could not attenuate the PMA-induced shift (Table I, lines H and I). This indicates that mainly PKA and not PKC, PKG, and tyrosine kinases are involved in the PMA-induced shift of HERG activation.

Another approach to test whether PKA is involved in the PMA-induced shift is to determine whether PKA activation could induce a shift. In this set of experiments we used forskolin, which activates the adenylate cyclase with an ED<sub>50</sub> of 5–10 μM (20) and subsequently PKA. We found that forskolin alone (400 μM) induced a shift of the HERG activation kinetics of $ΔV_{1/2} = 14.1 ± 4.2$ mV ($n = 4$) after an application time of 60 min. These results indicate that PKA is involved in the shift of the HERG activation kinetics.

In contrast the physiologic and very specific PKC activator DAG (30) could not produce a shift in HERG activation by itself. DAG is a neutral lipid that is able to activate the PKC cascade much more specific than PMA (30). We used DAG in a very high concentration of 10 μM (without PMA). In five experiments DAG caused no positive shift of the half-maximal activation voltage ($ΔV_{1/2} = –4.9 ± 2.8$ mV ($n = 5$)). This indicates that activation of PKC is not causing the shift in the HERG activation curve.

**DISCUSSION**

The phorbol ester PMA reduces $I_{Kr}$ currents in guinea pig cardiomyocytes in a concentration-dependent manner. A more detailed analysis of the effects by PMA was performed on the cloned HERG channel, which was expressed in *Xenopus* oocytes. Here we found a similar current reduction as in the

**FIG. 4.** A–D, PMA induces only a small shift in the inactivation curve of HERG. Control measurement (A) and the effect of 100 nM PMA after 30 min (B) in the same oocyte. C shows the inactivation curve (i.e., normalized current amplitude at the beginning of the inactivating current at 20 mV). There is only a small shift of 4 mV between control and the PMA-stimulated current. D shows an overlay of the activation and inactivation curve. The marked area below the curves indicates approximately where the channel is open. The area in the control measurements is larger than in the PMA measurements. This explains the smaller outward current in the measurements with PMA during the test pulses. A and B: holding potential, 20 mV; test pulse, −120 to 30 mV in 10-mV increments; inactivating pulse constant, 20 mV. For better illustration, only the traces at −110, −80, −50, −20, and 10 mV are displayed. E: shift of the half-maximal activation potential ($V_{1/2}$) of HERG as a function of the PMA concentration. The $ED_{50}$ was 9.0 nM, and the hill coefficient was 1.05. F: time course of the change in half-maximal activation potential ($V_{1/2}$) by 100 nM PMA as a function of time. Measurements were done every 3 min. From $t_1 = 7$ min to $t_3 = 58$ min after the beginning of the experiment we measured control currents. At $t_3 = 60$ min, PMA was perfused into the bath up to $t_3 = 120$ min, 7 min after the beginning of the perfusion of PMA into the bath, the shift of $V_{1/2}$ began and reached a maximum approximately 24 min later. Up to 60 min of perfusion of PMA into the bath did not further increase the change in $V_{1/2}$. Bath, 5 mM K.
Regulation of HERG by Protein Kinase A

### Table I

| Shift (ΔV_{1/2}) | Significance from A | PKA (IC50) | PKC (IC50) | PKG (IC50) | Tyrosine kinase (IC50) |
|------------------|---------------------|------------|------------|------------|------------------------|
| A 100 nM PMA     | 37.3 ± 6.5 (n = 8)  | Significant | 0.007      | 0.007      | 0.085                  |
| B 100 nM PMA + 1 mM staurosporine | 10.7 ± 7.7 (n = 4)  | Not significant | 2.0        | 0.01       |
| C 100 nM PMA + 1 mM bisindolylmaleimide | 31.9 ± 2.7 (n = 4)  | Not significant | 170        | 0.66       |
| D 100 nM PMA + 10 μM chelerythrine | 33.1 ± 2.1 (n = 3)  | Not significant | 0.048      | 31         | 0.48                   |
| E 100 nM PMA + 5 μM H89 | 26.7 ± 4.4 (n = 5)  | Not significant | 0.048      | 31         | 0.48                   |
| F 100 nM PMA + 50 μM H89 | 8.3 ± 5.4 (n = 5)   | Significant  | 0.056      | >2         | >2                     |
| G 100 nM PMA + 2.5 μM KT5720 | 1.0 ± 5.4 (n = 5)   | Significant  | 0.048      | 31         | 0.48                   |
| H 100 nM PMA + 20 μM Rp-8-pCPT-cGMPS | 31.8 ± 2.7 (n = 4)  | Not significant | 0.048      | 31         | 0.48                   |
| I 100 nM PMA + 100 μM genistein | 32.1 ± 5.6 (n = 5)  | Not significant | >100       | >100       | 2.6                    |

cardiomyocytes. This reduction is the result of a pronounced shift in the activation curve of 37 mV and a small shift (4.6 mV) in the inactivation curve. The area below these two curves, which reflects open, conducting channels, is reduced by PMA (Fig. 4D). The number of activable channels in the membrane is likely to be constant upon stimulation with PMA because the maximal tail current amplitude at very positive potentials is not reduced (Fig. 2, D and E).

Our results suggest that mainly PKA mediates the PMA effect on HERG. PMA stimulates the PKC cascade, but it is also known that other protein kinases are stimulated by PMA as well (18, 19). The involvement of PKA upon PMA stimulation could be demonstrated by additionally adding PKA inhibitors into the bath. The specific PKA inhibitors H89 and KT5720 reduced the PMA-induced shift. In support of this view forskolin alone, an adenylate cyclase activator that leads to activate PKA could induce a shift of 14 mV in the HERG activation curve. This is independent evidence that PKA is an important mediator, leading to a shift in HERG activation. PKC appears not to be involved directly in the PMA-induced shift in the HERG activation kinetics. This could be demonstrated by the use of the specific PKC inhibitors bisindolylmaleimide and chelerythrine, which failed to inhibit the PKA response. Furthermore DAG, the physiologic and highly specific activator of PKC, even in a very high concentration of 10 μM failed to induce a shift in HERG activation. These results suggest that PKC is not involved in a major way in the PMA-induced shift. Other effects of PMA, such as the activation of other kinase cascades that result in the activation of PKA, produce the effect. Also, cross-talk between different protein kinase systems might be involved (31). At this point it is not possible to identify the exact biochemical pathway of this process. PMA is known to affect many proteins and lipids in the membrane, and many of its effects are not fully understood (17). Nevertheless our results clearly demonstrate that PKA is a key enzyme in this process of HERG activation shift, which can be inhibited or stimulated specifically.

The concentrations of the protein kinase inhibitors that we used in the experiments were relatively high. In general, higher concentrations for various drugs are necessary in *Xenopus* oocyte experiments when applied to the external surface of whole oocytes. For example, the block of HERG by the antiarrhythmic drug dofetilide gave an IC50 that was 20-fold higher when the drug was applied to the bath compared with the application of the drug to the internal surface of the membrane in inside-out membrane patches (7). This difference in sensitivity is due to the vitelline membrane and the yolk that will reduce the concentration of many drugs in the cell membrane of the oocytes.

The effects of protein kinase activation by PMA on HERG are similar in *in vitro* and *in vivo*, demonstrated by our measurements in *Xenopus* oocytes and guinea pig cardiomyocytes. Because we found the effects *in vitro*, these effects may be of physiological significance. PKA is affected in many cellular processes under physiological and pathophysiological conditions (32, 33). The HERG potassium channel is expressed in many cells, most importantly in excitable tissue such as heart and brain. Our results demonstrate for the first time a link between the protein kinase system and the electrophysiological function of the HERG potassium channel. This link could be important in better understanding its physiological and pathophysiological role.

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