Suppression of Kir2.3 Activity by Protein Kinase C Phosphorylation of the Channel Protein at Threonine 53*

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Kir2.3 plays an important part in the maintenance of membrane potential in neurons and myocardium. Identification of intracellular signaling molecules controlling this channel thus may lead to an understanding of the regulation of membrane excitability. To determine whether Kir2.3 is modulated by direct phosphorylation of its channel protein and identify the phosphorylation site of protein kinase C (PKC), we performed experiments using several recombinant and mutant Kir2.3 channels. Whole-cell Kir2.3 currents were inhibited by phorbol 12-myristate 13-acetate (PMA) in Xenopus oocytes. When the N-terminal region of Kir2.3 was replaced with that of Kir2.1, another member in the Kir2 family that is insensitive to PMA, the chimerical channel lost its PMA sensitivity. However, substitution of the C terminus was ineffective. Four potential PKC phosphorylation sites in the N terminus were studied by comparing mutations of serine or threonine with their counterpart residues in Kir2.1. Whereas substitutions of serine residues at positions 5, 36, and 39 had no effect on the channel sensitivity to PMA, mutation of threonine 53 completely eliminated the channel response to PMA. Interestingly, creation of this threonine residue at the corresponding position (I79T) in Kir2.1 lent the mutant channel a PMA sensitivity almost identical to the wild-type Kir2.3. These results therefore indicate that Kir2.3 is directly modulated by PKC phosphorylation of its channel protein and threonine 53 is the PKC phosphorylation site in Kir2.3.

Inward rectifier K⁺ channels (Kir) play an important role in controlling membrane excitability (1, 2). Kir2.3 is a member of the Kir superfamily and is expressed in several tissues including the central nervous system, heart, and kidney (3–5). Unlike other members in the Kir2 family, Kir2.3 is directly coupled to G proteins, a coupling that enables this channel to contribute to neurotransmission and cell-cell communications (6). Also, Kir2.3 is known to be modulated by several intra- and extracellular signal molecules including Mg2⁺, polyamines, protons, and protein kinase C (PKC) (7–9). The modulation of channel activity by PKC is remarkable not only because PKC activators strongly inhibit channel activity by almost 50% but also because the signal transduction pathway of PKC is so common that a large number of extracellular messengers can act on this K⁺ channel through the activation of PKC. This is particularly important when Kir2.3 channel modulation is considered in the central nervous system, because the control of neuronal membrane excitability by numerous neurotransmitters and hormones can occur via this K⁺ channel.

Although Kir2.3 activity is affected by PKC activators and inhibitors, the critical PKC phosphorylation site is unidentified (8). Without this information, it remains debatable whether the modulation is mediated by a direct phosphorylation of the channel protein or indirectly by other molecules that are activated by PKC. To determine the molecular substrate for the PKC phosphorylation, we perform experiments in which channel sensitivity to a PKC activator PMA was studied in several chimeras constructed between Kir2.3 and Kir2.1, another member in the Kir2 family that is insensitive to PMA (8). The PKC phosphorylation site was identified using site-directed mutagenesis.

MATERIALS AND METHODS

Oocytes from female frogs (Xenopus laevis) were used in these studies. Frogs were anesthetized by bathing them in 0.3% 3-aminobenzoic acid ethyl ester. A few lobes of ovaries were removed after a small abdominal incision (~5 mm) was made. The surgical incision was closed, and the frogs were allowed to recover from the anesthesia. Xenopus oocytes were treated with 2 mg/ml collagenase (type I, Sigma) in OR2 solution (82 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 5 mM HEPES, pH 7.4) for 90 min at room temperature. After 3 washes (10 min each) of the oocytes with OR2 solution, cDNA (40–50 ng in 50 nl of double distilled water) was injected into the oocytes. The oocytes were then incubated at 18 °C in ND-96 solution containing 96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, and 2.5 mM sodium pyruvate with 100 mg/liter gentamicin added, pH 7.4.

Kir2.1 (KirK1) and Kir2.3 (KirK3) cDNAs were generously provided by Drs. Lily L. Jan and Carol A. Vandenberg. These cDNAs were subcloned in a vector for eukaryotic expression (pcDNA3.1, Invitrogen Inc., Carlsbad, CA) and used for expressions in the Xenopus oocytes. Chimerical constructs between Kir2.1 and Kir2.3 were prepared by overlap extension at the junction of the interested domains using the polymerase chain reaction using the Pfu DNA polymerase (Stratagene, La Jolla, CA). The resulting polymerase chain reaction products were subcloned into pcDNA3.1. Site-specific mutations were made using a site-directed mutagenesis kit (Quickchange, Stratagene, CA). Correct mutations in the cDNAs were confirmed with DNA sequencing. All plasmids for oocyte injection were prepared with a QIAfilter Plasmid Midi Kit (QIAGEN, Chatsworth, CA).

Whole-cell currents were studied on the oocytes 2–5 days after injection. The two-electrode voltage clamp procedure was performed using an amplifier (Geneclamp 500, Axon Instruments Inc., Foster City, CA) at room temperature (22–24 °C). The extracellular solution (KD-90) contained: 90 mM KCl, 3 mM MgCl₂, and 5 mM HEPES (pH 7.4). Cells were impaled using electrodes filled with 3 M KCl. One of the electrodes (1.0–2.0 MΩ) was used as a voltage recording, which was connected to an HS-2 ×10M headstage (input resistance, 10¹¹ Ω), and the other electrode (0.3–0.6 MΩ) was used for current recording connected to an HS-2 ×10M headstage (maximum current, 130 μA). Oocytes were accepted for further experiments only if their leak currents measured as the difference before and after a leak subtraction were less than 10% of the
Peak currents. The leak subtraction was not applied. Current records were low-pass filtered (Bessel, 4-pole filter, 3 db at 5 kHz), digitized at 5 kHz (12-bit resolution), and stored on computer disk for later analysis (pClamp 6.0.3, Axon Instruments) (10, 11).

PMA was purchased from Sigma and dissolved in dimethyl sulfoxide (Me2SO) for a stock concentration of 10 mM. Immediately before experiments, PMA was diluted to a concentration of 100 μM in the KD-90 solution. Thirty microliters were added to the recording chamber to reach a concentration of 1 μM. The final concentration of Me2SO was 0.01% or less in the present study (8). PKC inhibitor chelerythrine (RBI, Natich, MA) was dissolved in double distilled water with a final concentration of 10 μM. All data for PMA and chelerythrine chlorides were obtained with an exposure period of 8–20 min.

Data are presented as means ± S.E. (n, number of oocytes), and differences in the mean were tested with the Student’s t test or ANOVA (analysis of variance) and were accepted as significant if p ≤ 0.05.

RESULTS

Kir2.3 but Not Kir2.1 Is Sensitive to PMA—Whole-cell currents were studied in Xenopus oocytes that had received an injection of either Kir2.3 or Kir2.1 cDNA 2–5 days earlier. A high concentration of K+ (90 mM) was applied to the extracellular solution. The membrane potential was held at 0 mV and stepped to a series of depolarizing and hyperpolarizing potentials. Under such an experimental condition, strong inward rectifying currents were observed in both Kir2.3- and Kir2.1-injected oocytes. Exposure of these oocytes to PMA (1 μM) produced an inhibition of Kir2.3 currents by 49.9 ± 4.8% (mean ± S.E., n = 6) (Fig. 1). The inhibition of PMA had a long lasting effect. As has been observed previously by Henry et al. (8), we did not see evident recovery of channel activity in 20–30 min of washout. The inhibition of Kir2.3 was voltage-independent. The profile of the I/V relationship was identical in the presence and absence of PMA after they were scaled to the same size (Fig. 2). Unlike the Kir2.3, however, a similar exposure caused only modest inhibition of Kir2.1 currents by 10.1 ± 4.4% (n = 4). This small change in current amplitude is insignificant in comparison with that of the vehicle control using 0.01% Me2SO in the KD-90 solution (p > 0.05, n = 4). These results are therefore consistent with previous observations on these two K⁺ channels.

The PKC-sensitive Motif Is Located in the N-terminal Region of Kir2.3.—To identify the PKC phosphorylation site in Kir2.3, we took advantage of the following established experimental results and constructed several chimeras between Kir2.3 and Kir2.1. 1) The effect of PKC on Kir2.3 and Kir2.1 has been well documented, and there is a clear difference in PKA sensitivity between Kir2.3 and Kir2.1. Thus, extensive pharmacological manipulations using PKC activators and inhibitors can be avoided. 2) Kir2.3 has a high homology with Kir2.1 in their peptide sequences, suggesting that recombinant proteins are likely to produce functional channels. 3) It is known that PKC phosphorylation sites are only located in the cytosolic side of the plasma membranes. 4) According to the widely accepted transmembrane topology, both Kir2.3 and Kir2.1 have their N and C termini inside the membrane. Hence, it is possible that the N- and/or C-terminal region contains the critical motif(s) for PKC phosphorylation.

When the entire N-terminal region in Kir2.3 was replaced with a Kir2.1 counterpart (NI-HIR), the mutant channel lost its sensitivity to PMA (5.0 ± 3.9%, n = 6). In a chimera with its N-terminal sequence from Kir2.3 and the rest of the sequence from Kir2.1 (NH-IRK), we found that this mutant channel gained sensitivity to PMA (38.4 ± 2.3%, n = 4, p < 0.01 compared with wild-type Kir2.1), suggesting that the PMA phosphorylation site(s) is located in the N-terminal region of Kir2.3 (Fig. 3).

Threonine 53 Is the PKC Phosphorylation Site—Four potential PKC phosphorylation sites have been found in the N-terminal region of Kir2.3. To identify which one of them is the true PKC phosphorylation site, we focused on these potential PKC phosphorylation sites and generated several chimeras and site-specific mutations in which one or two of these sites were eliminated. We found that the serine residue at position 5 was not involved in PKC phosphorylation, because a removal of this residue by replacing the first 19 residues with the first 45 residues in IRK (N1–19HIR) did not significantly change the PKA sensitivity (34.7 ± 4.4%, n = 4, p > 0.05 compared with wild-type Kir2.3) (Fig. 3). Serine 36 and serine 39 had been previously proposed to be the PKC phosphorylation sites (8). Hence, these two residues were examined. Substitution of the middle part of the N-terminal region, including these two serine residues and the other 18 amino acids (N25–44HIR), did not reduce the PMA sensitivity of the chimerical channel (46.2 ± 6.2%, n = 3, p > 0.05 compared with wild-type Kir2.3) (Fig. 3). Site-specific mutation of both of these serine residues together (S36G,S39G) also had no effect on the inhibition of
PKC Phosphorylation in Kir2.3

FIG. 2. Voltage independence of Kir2.3 inhibition by PMA. A, currents were recorded from an oocyte in the same condition as Fig. 1. B, these currents were inhibited by exposure to 1 μM PMA (8 min). C, I/V plots show that only the inward rectifying currents were affected by PMA. D, when currents in A and B are scaled to the same magnitude at −160 mV, the I/V relationship of the currents recorded in these two conditions becomes identical, indicating that the effect of PMA on Kir2.3 currents is voltage-independent. Open circles, control; solid triangles, PMA exposure.

FIG. 3. The PKC phosphorylation site is identified at threonine 53 in Kir2.3. Left panel, when the threonine residue was mutated to isoleucine (T53I-HIR), the PMA (1 μM) sensitivity was completely abolished in the mutant Kir2.3. Right panel, when isoleucine 79 at the corresponding position to the threonine 53 in Kir2.3 was replaced with threonine, the mutant Kir2.1 (I79T-IRK) obtained PMA sensitivity. Note that the PMA exposure periods were 18 and 20 min for T53I-HIR and I79T-IRK, respectively.

FIG. 4. The PKC phosphorylation site is located in the N-terminal region of Kir2.3. Percentage inhibition of the inward rectifier currents of HIR, IRK, and their mutants created by the addition of 1 μM PMA (10–20 min exposure) is plotted. These chimerical and mutant channels are clearly distributed in two groups with one showing a similar PMA sensitivity to Kir2.3 and the other to Kir2.1. Dotted lines indicate levels of PMA sensitivity of Kir2.3 and Kir2.1. Open circles, Kir2.3-based mutations; open triangles, Kir2.1-based mutations; closed diamond, experimental control with 0.01% Me2SO alone. HIR, Kir2.3 (n = 6); IRK, Kir2.1 (n = 4); NI-HIR, the N-terminal region of HIR was replaced with that in IRK (n = 6); NH-IRK, the N-terminal region of IRK was substituted with that in HIR (n = 4); N1–19HIR, the first 19 residues in the N-terminal end of Kir2.3 were substituted with the first 19 residues in the N-terminal end of Kir2.1 (n = 4); N25–44HIR, residues 25–44 in Kir2.3 were replaced with residues 51–70 of Kir2.1 (n = 3); S36G/S39G, serine 36 and serine 39 in Kir2.3 were mutated to glycine (n = 4); N53–60HIR, residues 53–60 in Kir2.3 were replaced with residues 79–86 in Kir2.1 (n = 4); T53I-HIR, threonine 53 in HIR was mutated to isoleucine (n = 4); N79–86IRK, residues 79–86 in Kir2.1 were replaced with residues 53–60 in Kir2.3 (n = 4); 179T-IRK, isoleucine 79 in IRK was changed to threonine (n = 4); DMSO, control using Me2SO alone in the same concentration as used in all other experiments (n = 4). Data are presented as mean ± S.E.

this channel by PMA (38.1 ± 1.5%, n = 4, p > 0.05 compared with wild-type Kir2.3) (Fig. 3). This suggests that neither of these two serine residues is the PKC phosphorylation site. Threonine 53 is another residue that appears to be a PKC phosphorylation site. It has a motif (VDTRWR) and is located near the first transmembrane (M1) domain. Site-specific mutation of this threonine into isoleucine (T53I-HIR) with all of the rest of the sequence intact completely abolished the channel sensitivity to PMA (1.6 ± 1.0%, n = 4, p < 0.001 compared with wild-type Kir2.3) (Figs. 3 and 4). Interestingly, the amino acid sequence around this residue is quite similar between Kir2.3 and Kir2.1. To see if the creation of this threonine can
PKC Phosphorylation in Kir2.3

Affect Kir2.1 sensitivity to PMA, a sequence including eight amino acid residues surrounding this threonine was introduced to Kir2.1 by substituting a corresponding sequence in the same region (N79–86IRK). We found that the chimerical channel became PMA-sensitive (37.8 ± 5.7%, n = 4, p < 0.01 compared with wild-type Kir2.1), although only three residues in this segment are different between Kir2.3 and Kir2.1. We further mutated the isoleucine residue to threonine in Kir2.1 at the same position as the threonine 53 in Kir2.3. This mutation (I79T-IRK) led to an emergence of the PMA sensitivity in the Kir2.1-based channel (42.6 ± 3.7%, n = 4, p < 0.05 compared with wild-type Kir2.1) (Fig. 4). This inhibitory effect of PMA was totally blocked with 10 μM chelerythrine, a PKC inhibitor (0.1 ± 2.1%, n = 3, 20 min of chelerythrine preincubation followed by 20 min of PMA exposure). Thus, these results indicate that threonine 53 is the only PKC phosphorylation site involved in the modulation of Kir2.3 channel activity.

Discussion

A number of neurotransmitters and hormones has been shown to modulate inward rectifier K+ channels via phosphorylation of the channel protein by PKC (12–16). For example, substance P and neurotransin cause a slow excitation of neurons of the nucleus basales and substantia nigra. This is mediated by inhibition of inward rectifying K+ channels through the activation of PKC (17, 18). In cardiac myocytes and renal epithelial cells, activation of PKC also produces an inhibition of Kir currents, although detailed functions of this inhibition are not fully understood (12, 19–23). In addition to the covalent activation of channel activity, PKC has also been shown to affect channel expression of these Kir channels (15, 24).

Characterization of specific Kir channels that are modulated by PKC has been carried out recently. Macica et al. (23) have shown that exogenous protein kinase C inhibits the wild-type but not the S4A mutant Kir1.1 channel. Fakler et al. (25) have found that N-heptyl-5-chloro-1-naphthalenesulfonamide, a specific PKC stimulator, causes Kir2.1 rundown. Henry et al. (8) have indicated that Kir2.3 is strongly inhibited by PKC. The latter investigators have also shown no effect of PKC activators on Kir2.1. In the present study, we have observed a strong inhibition of Kir2.3 when the oocytes are exposed to PMA. However, the same exposure causes only a small and insignificant change in Kir2.1 currents, a result consistent with previous observations (8). Therefore, Kir2.3 but not Kir2.1 appears to be modulated by PKC.

Identification of the PKC phosphorylation site in Kir2.3 was attempted previously by Henry et al. (8) who studied Ser-36 and Ser-39 as potential PKC phosphorylation sites. By replacing these two serine residues with cysteine and alanine (S36C, S39A), they could not see channel expression and thus were unsuccessful in demonstrating the PKC phosphorylation site in Kir2.3 (8). In the present study, we have mutated Ser-36 and Ser-39 to glycine residues located in the corresponding positions in Kir2.1. These mutations have no effect on channel expression and its sensitivity to PMA, indicating that they are not PKC phosphorylation sites. The other serine residue at position 5 is not involved in PKC phosphorylation, because mutation of this residue does not change the PMA sensitivity as shown in our current study. Our results indicate that threonine 53 is the only PKC phosphorylation site that controls channel activity, because a substitution of this residue with isoleucine completely eliminates channel sensitivity to PMA. This is further strengthened by our results showing that a creation of this threonine residue in Kir2.1 at the same location leads to a strong inhibition of the mutant channel by PMA.

In conclusion, the modulation of Kir2.3 by PKC phosphorylation of the channel protein has been demonstrated in our current studies. The PKC phosphorylation site is located in the N-terminal region. Threonine 53 with a VDTRWR motif is the substrate of PKC.

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