Environmental Medicine, Vol. 46 No.1, 2, 2002 (page 81 ~ 83)

Molecular Mechanisms for Regulation of the G Protein-activated Inwardly Rectifying K^+ (GIRK) Channels by Protein Kinase C

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Abstract: G protein-activated inwardly rectifying K^+ channels (GIRK) are inhibited by activation of protein kinase C (PKC), but the exact mechanisms are not known. GIRK channels in the heart are heterotetrama composed of GIRK1 and GIRK4 subunits. We investigated the phosphorylation sites by site-directed mutagenesis procedure in GIRK4 subunits heterologously expressed in *Xenopus* oocytes. Homomeric wild type (WT) GIRK4 current amplitude was inhibited by phorbor-1,2 myrisitate-acetate (PMA: a PKC activator), by 45.5±1.8% (n=5). Serine or theorine at five potential phosphorylation sites of GIRK4 channel were replaced by alanine to prepare five different single mutants and a penta mutant with replacement of all the sites. PMA (1 μM) application to these mutant GIRK4 channels resulted in a similar reduction of current amplitude (by 45–62%) as observed in WT channels (by 45.5±1.8%). From these observations, it is suggested that an inhibition of GIRK4 channels by PKC activation is not mediated by direct phosphorylation of the channel protein. Instead, some indirect PKC actions on other molecules may be involved.

Key words: G protein-activated inwardly rectifying K channels, protein kinase C, phosphorylation site

G protein-activated inwardly rectifying K^+ channels (GIRK) in the heart are composed of GIRK1 and GIRK4 subunits.[1,2] GIRK channels in the sinoatrial (SA) node play an important role in regulation of heart rate through activation by neurotransmitters, in particular by acetylcholine (ACh). Native ACh-sensitive K^+ currents are known to be suppressed by PKC activation, although it's molecular mechanism is unknown. As to the phosphorylation sites of K^+ channels by PKC, information available is still limited. In an inward rectifier K^+ channel (Kir2.3), one putative site has been proposed by Zhu G et al.[3] In the present study, we investigated the phosphorylation sites of GIRK4 channel by using site-directed mutagenesis procedure.

Methods

1. Mutagenesis and cRNA synthesis

Five possible cytosolic phosphorylation sites in GIRK4 channel were estimated from the consensus sequence reported by Woodgett et al.[4]: [S/T]-X-[R/K]. These are T37, T57, T70 in the N-terminal, and S209, S233 in the N-terminal (Fig. 1). Serine (S) or threonine (T) at the potential phosphorylation sites were replaced by alanine (A) to prepare five different single mutants and a penta mutant (all replaced by A) of GIRK channel.

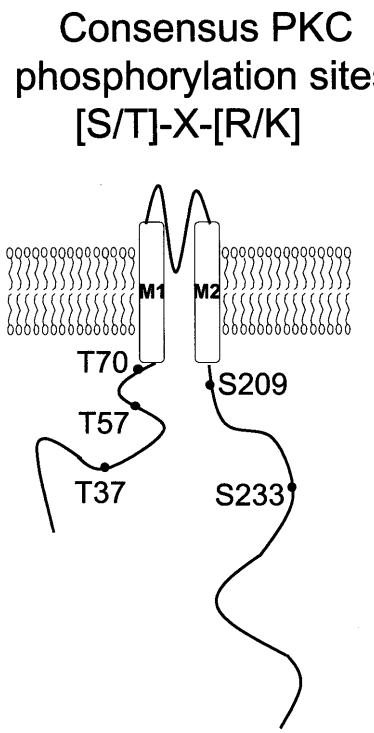

Fig. 1 Putative PKC phosphorylation sites in the GIRK4 channel. These sites are based on the consensus sequence of PKC phosphorylation ([S/T]-X-[R/K]), where X is generally an uncharged residue reported by Woodgett *et al.*[4]

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Full-length cDNAs encoding wild type (WT) and the six mutants of GIRK4 were subcloned into, together with $G_{\beta\gamma}$, pBluescript II SK+ and pAGA. Restriction enzymes *NotI* and *HindIII* were used to linearize the plasmid DNAs. Approximately 1μg purified linear DNA was used for *in vitro* T7 transcription reactions (mMessage mMachine, Ambion, Dallas, TX). The mutants were made using Quikchange site-directed mutagenesis kit (stratagene, USA).

2. Channel expression in oocytes and current recording

Oocytes in stage V-VI were obtained from mature female *Xenopus laevis* under anesthesia with 0.2% tricaine, and then isolated by enzymatic digestion using 1.22 mg/ml collagenase (Worthington, USA) in ND-96 solution. The oocytes were pressure-injected with 1–2 ng of cRNA of WT or mutant. The ratio of $G_{\beta\gamma}$ cRNAs co-injected with GIRK channels was 5:1. Electrophysiological studies were conducted 2–6 days after injection. Whole-cell currents were measured by a two-microelectrode voltage clamp at room temperature (22–25°C). The current was recorded in response to voltage steps to –140 ~ +40 mV from a holding potential at –20 mV.

3. Solutions, chemicals and statistic

Oocytes were isolated in ND96 (-) solution containing (mM): 96 NaCl, 2 KCl, 2 $MgCl_2$, 5 HEPES (pH 7.3–7.4 adjusted with NaOH). Current recordings were performed in KD 98 solution containing (mM): 98 KCl, 2 KOH, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 5.0 HEPES (pH 7.2 adjusted with MES). PMA (phorbol 12-myristate 14-acetate) was dissolved in dimethyl sulphoxide (DMSO) with final concentrations < 0.1%.

Data are presented as means ± S.E. Differences were tested with the Student's paired t test and were considered significant if $p<0.05$.

Results and Discussion

Fig. 2 shows representative effects of PMA (1 μM) on the WT GIRK4 channel current. The current amplitude (at –140 mV) was reduced remarkably in response to PMA application for 7–8 min and it reached to 45% of baseline at 15–20 min. This current change was not reversed by washing out of PMA.

Four single mutants (T37A, T57A, T70A, S209A) of GIRK4 channel expressed in oocytes had baseline current amplitudes comparable to those of WT GIRK channel. Another single mutant (S233A) and a penta mutant of GIRK4 channel, in contrast, had much less current amplitudes at baseline (Fig. 3). The effects of PMA (1 μM) on these six mutants of GIRK4 channel are more or less similar in terms of percentage reduction of the mutant amplitude from the respective baseline (Fig. 3).

From these results, we suggest that PKC inhibit GIRK4 channel not by a direct phosphorylation of a channel protein

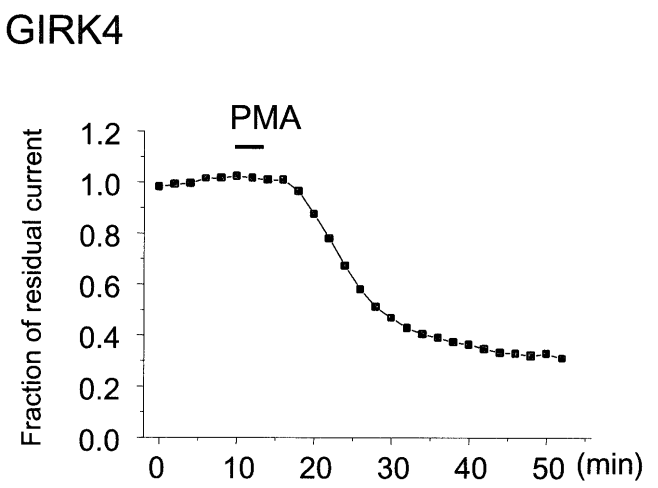

Fig. 2 The effect of PKC application on the current of the GIRK4 homomeric channel.
The current was inhibited by PKC activation with application of PMA (1 μM) for 3 min.

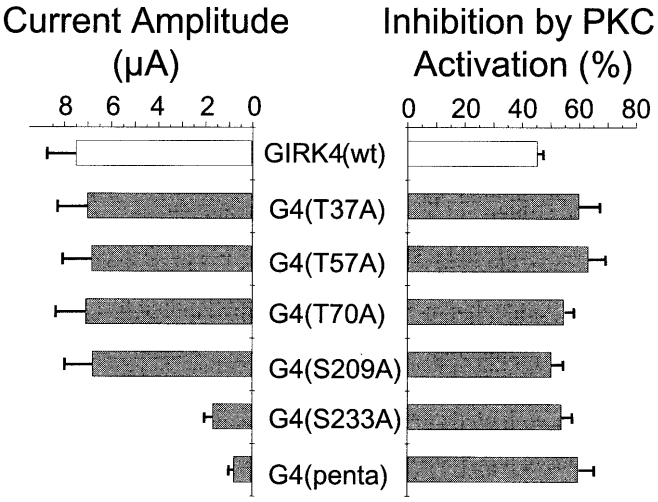

Fig. 3 PKC-induced inhibition of wild-type and mutant GIRK4 channels by PKC activation.
Left graphs show the current amplitude. Right graphs show % decrease of the current by PMA (1 μM).

at the consensus sites, but through activation of other molecules. However, there still remains a possibility that GIRK4 is inhibited directly by PKC at other unidentified sites in the channels, because several PKC-related phosphorylation sites has been found in other channels such as Kir2.3 or Kir6.2 channels which do not belong to consensus sequence of PKC ([S/T]-X-[R/K]). Experiments employing chimeric constructs of GIRK4 channels may provide information on this issue.

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Received June 3, 2002; accepted June 7, 2002