行政院國家科學委員會專題計畫　成果報告

計畫名稱: PIP_2調控內流型整流鉀離子通道之分子機轉研究(2/2)

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中文

內流型整流鉀離子通道(Inward rectifier K^+ channel; Kir)的作用為穩定細胞膜內外電位，使細胞處於可激發狀態，其生理功能主要與維持細胞靜止膜電位、神經突觸之興奮性、胰島素的分泌、以及和腎臟內鉀離子的再吸收等有關。有許多證據指出，phosphatidylinositol 4,5-bisphosphate (PIP_2) 是調控 Kir 通道之重要分子，PIP_2可和 ROMK1 通道之 C 端作用，藉正負電荷之靜電關係，調控 Kir 通道之開啟或關閉。本計畫為二年期計畫，目標為確立 ROMK1 (Kir 1.1) 通道的 PIP_2 binding site 及其結構變化及對功能改變之影響研究。本研究以分子生物學和電生理方法，證明 ROMK1 通道的 PIP_2 binding site。以下是本研究之結果:

我們以下列方法: (1)<u>分子生物學方面:</u>在 ROMK1 通道 cDNA 的 C 端進行廣泛的單點氨基酸置換(尤其著重於 180～240 序列，此段具多數帶正電荷之氨基酸分子), 再用轉譯的方法,得到 mutants 的 mRNA。 (2)<u>電生理方面:</u> 將 mutant 的 mRNA 打入 *Xeuopus* oocytes 中,使其 express 之後,以 giant patch-clamp 之方式進行 inside-out patch 電生理實驗，利用單株抗體 PIP_2Ab 評估 mutant 和 wild type 之 ROMK1 通道對 PIP_2之 affinity，以確立 ROMK1 (Kir 1.1) 通道的 PIP_2 binding site。我們發現單點置換 ROMK1 通道 C 端 180 ~ 240 序列之氨基酸後在 *Xeuopus* oocytes 中 expression， 再以電生理實驗，觀察 ROMK1 通道結構變化後對功能改變的影響。結果證明其中 ROMK1 通道 C 端帶正電荷之氨基酸分子成電中性之 K181, R188Q, R216A, K217A mutants 後，對 PIP_2 的結合力明顯降低，因此我們證明 K181, R188, R216, K217 是 ROMK1 通道的 PIP_2 binding site。PIP_2可和 ROMK1 通道之 C 端作用，藉氨基酸分子正負電荷之靜電作用關係，調控 Kir 通道之開閉。

英文

Inwardly rectifying K^+ channels (Kir) exist in various excitable and nonexcitable cells. They are important for maintaining the resting membrane potential near the K^+ equilibrium potential (E_K) and for permitting long depolarizing response. Recent evidence implicates

phosphatidylinositol 4,5-bisphosphate (PIP_2) as a regulator of Kir channels. This direct interaction between PIP_2 and the channel is important for the constitutive opening of Kir channels. In this two years project, we focus our effort on the molecular mechanisms for regulation of Kir mediate by PIP_2. We have elucidated the structural motif of ROMK1 channel for interacting with PIP_2 .

The proximal C-terminal hydrophilic domain of Kir channels contain a region of many conserved basic residues. As the C-terminal domain may from part of the channel pore, binding of the basic (putative PIP_2-binding) region to membrane PIP_2 may be critical for stability of the open pore. We use frog eggs (*Xenopus* oocytes) for expression the wild and mutant ROMK1 channels. The strategies were performed as below: (1) Mutagenesis. To examine the role of these positive charged residues in the binding and activation of the channel by PIP_2. We extensive mutant the amino acids sequence from 180 to 240 with the neutral amino acid glutamine by *in vitro* mutagenesis. (2) Inside-out patch clamp study. The activity of the channels expressed in oocytes were measured using patch-clamp recording in giant excised inside-out membrane patches. The affinity of the ROMK1 mutants for PIP_2 were assessed using the ability of monoclonal PIP_2 antibody to bind PIP_2 and thus to inhibit the activity of the channel. We found that the binding affinity of PIP_2 were significant decreased in K181A, R188Q, R216A, and K217A mutant. Mutation of arginine 188 to glutamine (R188Q) in ROMK1 reduces its binding with PIP_2 to a negligible level. The differences in the affinities of inward rectifiers for PIP2 indicate that residues the conserved R188 are certainly important. The large decrease of PIP_2 binding induced by a single mutation, R188Q, suggests that binding of PIP_2 to multiple basic residues of the channel were cooperative, similar to the binding of PIP_2 to the pleckstrin homology (PH) domains. Proteins that are known to bind PIP_2 include proteins with the domain. The binding of PIP_2 to PH domain involves ionic interactions between the acidic headgroups of PIP_2 and the basic amino acids in the PH domain. Recently, X-ray and NMR studies of several PH domains have revealed that the structure of the PH domains are quite similar, featuring an antiparallel β-sheet consisting of seven strands and a C-terminal α-helix. The binding of PIP_2 to PH domains occurs between the 4-and 5-phosphates of PIP_2 and 3 basic residues at a positively charged cleft formed between β-strands 1-2 and 3-4. Binding of PIP_2 to these residues is cooperative. The putative PIP_2 binding region in ROMK1 is also rich in basic amino acids, which are conserved among inward rectifying K^+ channels. Our working model is that binding of PIP_2 (in the inner leaflet of the plasma membrane) to this

region of the inward rectifying K^+ channels regulate channel activity by stabilizing the open-pore structure of the channel. The electrostatic interaction occurs between the positively charged amino acid residues and the negatively charged head groups (phosphates) within the inositol 1,4,5-trisphosphate (IP_3) moiety of PIP_2.

We have previously shown that PIP_2 directly interacts with the proximal C-terminus of the Kir channels, including ROMK1, IRK1, and GIRK1. We also found the PKA activates ROMK1 channels by enhancing PIP_2-channel interaction. In the mutagenesis study, we have demonstrated that mutation of K181A, R188Q, R216A, and K217A in ROMK1 reduced the ability of ROMK1 to bind PIP_2. PIP_2 as the essential "gating molecule" for Kir channels. Our recent report that reduction of PIP_2-channel interaction decreases single-channel P_o of the channels as well as favors ROMK1 channels to enter subconductance states lends support to this hypothesis. Many hormones or growth factors alter PIP_2 metabolism through activation of PLC, phosphoinositide kinases, and/or phosphatases. The role of these hormones in regulating the activity of ROMK channels in the kidney via alteration of PIP_2-channel interaction awaits future investigation.

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180 AKISRPKKRAKTITFSKNAVISKRGGKLCLLIRVANLRKSLLIGSHIG 227

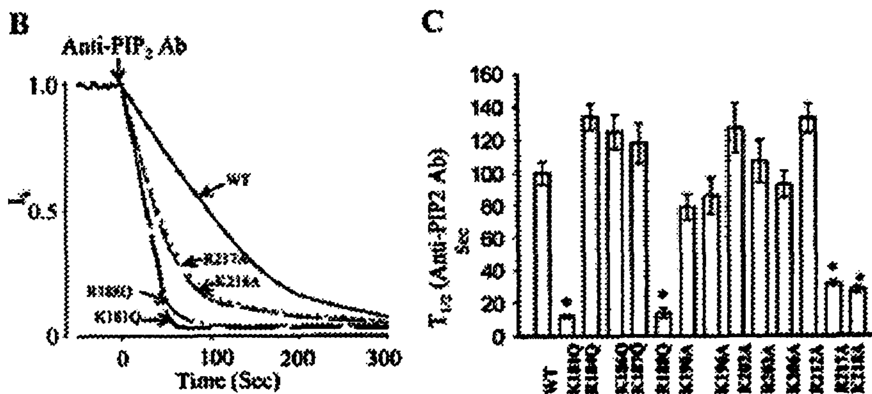

Fig. 1. Effects of charge neutralization on the sensitivity of ROMK1 channels to anti-phosphatidylinositol bisphosphate (PIP_2) antibodies. *A*: amino acid sequence (amino acids 180-227) of the proximal COOH terminus of ROMK1. Amino acids substituted by site-directed mutagenesis are shown in bold. The asterisks indicate amino acids that, when substituted by a neutral amino acid (glutamine or alanine), exhibit an increased sensitivity to inhibition by anti-PIP_2 antibody. *B*: representative tracings of anti-PIP_2 antibody-mediated inhibition of wild-type (WT) and leucine-181Q (K181Q), arginine-188Q (R188Q), arginine-217A (R217A), and leucine-218A (K218A) mutant channels. Inward K^+ currents (I_k; holding at −30 mV) from oocytes expressing the individual channel were recorded by giant patch-clamp recording first in cell-attached patches and subsequently in those in an excised inside-out configuration. Bath solutions contain mixtures of phosphatase inhibitors (FVPP solution) and no Mg^{2+} ions (see METHODS). Anti-PIP_2 antibodies (anti-PIP_2 Ab; 40 nM) were applied to the inside-out patches in FVPP solutions as indicated. Currents measured at all time points were normalized to the currents measured in cell-attached mode (currents in cell-attached patches are equal to 1). Currents before and after anti-PIP_2 antibodies for WT and mutant channels were superimposed for presentation. *C*: half-time ($T_{1/2}$; time to 50% inhibition by anti-PIP_2 antibody) for WT and all substitution mutants. Each bar is mean ± SE of at least 5 similar experiments for each channel. *$P < 0.05$ compared with WT ROMK1 by unpaired *t*-test.