P-31

The gating mechanism of human voltage-gated proton channel Hv1 studied by fluorescence technology

［１］Organization
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［２］Research Progress（10.5 points）

In this project, we investigated the role of N- and C-terminal domain of human voltage-gated proton channel Hv1 in the gating mechanism using fluorescence technology. The plan in details is as follows:

(1) To determine the interaction between N- and C-terminal domains, the N-terminal domain was cloned, expressed and purified. (2) The interaction between N- and C-terminal domains was detected by GST-pull down and ITC. (3) The interaction between divalent metal ions, classical inhibitors of Hv1, with the C-terminal domain of Hv1 was studied by CD spectra and fluorescence lifetime spectroscopy.

［３］Results（10.5 points）

（３－１）Research results

(1) The N-terminal domain of Hv1 was successfully expressed and purified.

The N-terminus of Hv1 fused with GST was expressed in *E. coli*.. A specific band with a time-dependent manner at a molecular weight of about 38 kDa corresponding to the molecular weight of the protein fused with GST (38.2 kDa), was detected by the coomassie-stained SDS-PAGE and the anti-GST-HRP . The fusion protein induced with 0.5 mM IPTG was mainly expressed in including body, while reducing the concentration of IPTG could increase the soluble expression of the protein. Moreover, addition of 0.5% (v/v) Triton X-100 in lysis buffer obviously increased the content of the protein in supernatant. The GST-fused protein was successfully isolated from the GST moiety by preScission protease. The isolated N-terminus of Hv1 from GST was further purified with an ion exchange column and a gel filtration column.

(2) The interaction between N- and C-terminal domains was detected by GST-pull down and ITC

In GST pull-down experiments, we found that the N-terminal domain of Hv1 interacts with the C-terminal domain at pH 6.0. However, the N–terminal domain didn't interact with the C-terminal domain at pH 7.5. These results indicated that N-terminal domain interacts with C-terminal domain, and the interaction is pH-dependent.

To further confirm the interaction between the N- and C-terminal domain of Hv1, ITC experiments were carried out at 25 °C. The binding of N-terminal domain to C-terminal domain was an endothermic heat reaction. Fits of the heat data for the titration was carried out using the model which entails a single binding event. The association constant (Ka) was (2.51± 0.59)$\times 10^5 M^{-1}$ with a n value of 1.38 ± 0.03.

(3) Divalent metal ions induce a conformational change of the C-terminal domain.

The internal C-terminal domain of Hv1 forms a parallel, two-stranded coiled-coil architecture. The CD spectra in the range of 195–250 nm at 25 °C were used to monitor the effect of binding of divalent metal ions on secondary structure of the protein. At pH 5, the spectra of the protein without divalent metal ions showed a characteristic of a-helical secondary structure, and the molar ellipticities at 222 and 208 nm were about -33557.5 and -32614.5 degrees cm^2 $dmol^{-1}$, respectively. In the presence of divalent metal ions (75 μM), the molar ellipticities at 222 and 208 nm were both decreased. At pH 7, the molar ellipticities at 222 and 208 nm were more obviously changed by the titration of divalent metal ions. These results indicated that the interaction between divalent metal ions and the C-terminal domain induced an obvious secondary structural change of the protein at pH 7.

(4) Interaction of divalent metal ions with the C-terminal domain

To estimate the interaction between divalent metal ions and the C-terminal domain, we performed the fluorescence lifetime spectroscopy experiments. There are two His residues in the C-terminal domain of Hv1, His^{244} and His^{266}, both of which localize in the accessible region. Moreover, there is no Trp or Tyr residue within the C-terminal domain. Then either of Phe247 or Leu262, which is not in 'a' or 'd' position of the heptad repeat pattern but close to His^{244} or His^{266}, was mutated to Trp residue to generate the mutant F247W or L262W, respectively. The CD spectra of the two mutants were the same as the wild type, suggesting that the two mutants remained the characteristic of a-helical secondary structure.

The fluorescence lifetime of F247W upon addition of divalent metal ions were employed to investigate the binding of divalent metal ions to the site close to His244 (site 1). The addition of divalent metal ions quenched the fluorescence life time of F247W at pH 7, except for Zn^{2+} which enhanced the fluorescence life time of F247W. KSV were obtained from the Stern–Volmer plots. KSV is equal to kqs0 and the fluorescence lifetime of the biopolymer s0 is 10-8 s, so the quenching constant kq can be calculated. However, the maximum scatter collision quenching constant of various quenchers with the biopolymer is 2.0×10^{10} $lmol^{-1}s^{-1}$, and the rate constants of protein quenching procedure initiated by divalent metal ions were much greater than the kq of the scatter procedure, so the quenching should be initiated by the formation of complex rather than the dynamic collision. The dissociation constants (K_D) were estimated from the slopes. The order of the binding strength between divalent metal ions and the site close to His244 (site 1) from strong to weak is Co^{2+}, Ca^{2+}, Ni^{2+}, Mg^{2+} and Mn^{2+}.

(3－2) Ripple effects and further developments

(1) To investigate the role of the interaction between N- and C-terminal domains in the channel gating, the dynamic interaction between N- and C-terminal domains during the activation of Hv1 could be captured by Förster Resonance Energy Transfer (FRET).

(2) To determine the interaction between the N-terminal domain and PKC, the full-length Hv1 was used to be co-transfected with PKC. The yellow fluorescence protein was divided into two part reasonably and fused to Hv1 or PKC, respectively. The interaction could be detected by bimolecular fluorescence complementation technology.

(3) To investigate the role of the interaction between N-terminal domain and PKC in Hv1 enhance mold gating, the dynamic interaction between N-terminal domain and PKC during the activation of Hv1 could be captured by Förster Resonance Energy Transfer (FRET).

［4］Achievements (List of Publications) (10.5 points)

（1）Q. Zhao, S.J. Li, et al., The voltage-gated proton channel Hv1 is expressed in pancreatic islet β-cells and regulates insulin eecretion, Biochem. Biophys. Res. Commun., 468 (2015) 746-751.

（2）Q. Zhao, S.J. Li, et al., Proton pump inhibitors have pH-dependent effects on the thermostability of the carboxyl-terminal domain of voltage-gated proton channel Hv1, under review.