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中藥補骨脂改善聽覺功能分子機制之研究
研究成果報告(精簡版)

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行政院國家科學委員會補助專題研究計畫 ■ 成果報告 □期中進度報告

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中文摘要

已知KCNQ4基因蛋白對於聽覺傳訊過程扮演著重要的角色。本實驗主要是研究補骨脂衍生物(PAP-1)與磷酸化酶抑制劑對於人類的KCNQ4基因的功能的影響。人類KCNQ4基因純化之 cRNA 打入爪蟾卵母細胞並於打入後 2-4天的期間以雙電極電壓鉗定技術記錄其所表現的電流。補骨脂衍生物 (psoralen derivatives) 具有抑制 KCNQ4電流的作用，所測得的KCNQ4 鉀離子電流顯示是電位依賴性的其二分之一活化電位向右偏移，此電流會被鉀離子阻斷劑 linopirdine (0.25 mM) 所抑制。給予 phosphatase 抑制劑 okadaic acid and calyculin A (3 μM)亦具有對KCNQ4相似的作用，會抑制 KCNQ4鉀電流並使其二分之一活化電位曲線圖向左偏移分別是。Psoralen衍生物 或 phosphatase 抑制劑對於未表現有KCNQ4爪蟾卵母細胞之內源性電流不具有影響性。Psoralen衍生物對於KCNQ4電流之調控是否經由phosphatase仍須進一步的證實。因此我們認為 KCNQ4鉀電流可以被Psoralen衍生物所抑制，而在臨床上的作用須待進一步的評估。

關鍵詞: Psoralen衍生物; phosphatase; KCNQ4; 鉀離子管道; 爪蟾卵母細胞.

Abstract

It is has been known that KCNQ4 plays an important role for auditory transmission. In this study the effects of psoralen derivatives and phosphatase inhibitor on the function of KCNQ4 were investigated. The human potassium channel KCNQ4, expressed in the Xenopus oocytes injected with KCNQ4 cRNA and currents were recorded using the two-electrode voltage clamp technique. The psoralen derivatives have inhibiting effect on the KCNQ4 current, the expressed current showed the typical KCNQ4 voltage-dependence, with a voltage for half-maximal activation ($V_{1/2}$) shifted to right, and was blocked almost completely by 0.25 mM linopirdine, a selective blocker of KCNQ4 current. Application of phosphatase inhibitors, okadaic acid (0.5 μM) or calyculin A (3 μM) have the similar effect with psoralen derivatives on the KCNQ4 current, shifted $V_{1/2}$ to the right. Either psoralen derivatives or phosphatase inhibitors have no effect on the endogenous current of oocytes. If the effect of psoralen derivatives on KCNQ4 by the phosphatase activities needs to further investigate. The result reveals that psoralen derivatives possess the effect on the inhibiting KCNQ4 channels.

Keywords: Psoralen derivatives; phosphatase; KCNQ4; potassium channel, Xenopus oocytes

1. Introduction

KCNQ4 is expressed abundantly in the cochlea as well as in brain, heart and skeletal muscle [1]. Mutations in the gene for KCNQ4 underlie a non-syndromes hereditary hearing loss, DFNA2 [2, 3, 4]. The channel is expressed in both inner hair cells (IHCs) and outer hair cells (OHCs). KCNQ4 has been identified as the molecular relative with OHC potassium current, termed $I_{K,n}$. $I_{K,n}$ is distinguished by an activation curve which contributes to the large negative resting potential of OHCs. This activation does not match that of KCNQ4 found in expression systems [5, 6]. The reasons for this discrepancy remained elusive but may include unknown interacting proteins like scaffolding proteins, KCNEs, or regulation by ions, kinases, PIP2 and/or further signaling mechanisms. Furthermore, cell volume, the protein kinases PKA and PKC, G proteins and intracellular Ca^{2+} have been reported to regulate KCNQ4 [7]. In OHCs, the large KCNQ4 currents are the dominating K^+ currents both at rest and at depolarized voltages and thus form the major exit route for K^+ ions entering the OHCs through the transduction channels. Lack of functional KCNQ4 channels in DFNB 2 most likely leads to IHC degeneration and deafness because use-dependent depolarization causes Ca2+ influx through voltage-gated Ca^{2+} channels [8] and ultimately excitotoxicity of IHCs and afferent fibres [2]. Immunoprecipitation and immunoblot analysis showed Src-dependent phosphotyrosine signals associated with KCNQ3, KCNQ4, and KCNQ5 but not with KCNQ1 or KCNQ2 that may be tyrosine phosphorylation of the channel subunits. Expression of a dominant negative Src that cannot phosphorylate substrates had no effect on the current and did not induce phosphotyrosine signals associated with KCNQ3-5 subunits, further indicating that Src actions on KCNQ currents are mediated by tyrosine phosphorylation. Immunostaining and confocal analysis showed no effect of Src overexpression on the abundance of KCNQ3 protein in CHO cells. Previous studied we have shown that ionomycin can activate the KCNQ4 currents, and may imply the role of calcium on the channel activities [9]. To parallel findings on $I_{K,n}$ in OHCs and, in particular, that $I_{K,n}$ is sensitive to elevated intracellular calcium, we also describe the effects of Ca^{2+}-dependent modulation of

KCNQ4 currents via calmodulin (CaM) and calcineurin (CaN). The universal sensor CaM is a small protein with four EF-hand-type Ca^{2+}-binding sites, and has been detected in hair cells. However, the role of protein kinase and phosphatase on KCNQ4 are still unclear. Psoralen is the effective ingredient extracted from a Chinese herb, bu gu zhi. It has been known that psoralen crude extract have the ability to improve the impairment of hearing function as well as inhibiting the Kv channels. In this studied the phosphatase inhibitors and psoralen derivatives were test on the KCNQ4 channels expressed in Xenopus oocytes. The results showed that both phosphatase inhibitors and psoralen derivatives possess the inhibiting effect on KCNQ4 channel. The molecular mechanism of phosphatase inhibitors and psoralen derivatives on the the channel protein need to further clarify.

2. Materials and Methods

The preparation of Xenopus laevis oocytes and cRNA injection. Xenopus oocytes were used as a model system for the functional expression of plasma membrane proteins in this study. The frogs were anaesthetized by immersion in 0.15% tricaine (Ethyl 3-aminobenzoate, methanesulfonic acid salt, Sigma-Aldrich) which dissolved in 0.1% sodium bicarbonate solution. The ovarian lobe was then surgically removed from the abdominal cavity through a small (0.5-1 cm) incision and placed in modified Barth’ s solution (MBS), which contained (in mM) 90 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 15 HEPES-Tris; pH 7.6 at room temperature. Then, the abdominal incision was closed and the frogs were washed by fresh water for 1-2 min and allowed to recover from the anesthesia in a plastic container for distilled water. Small pieces of ovarian lobe were defolliculated enzymatically by gently agitating the oocytes in a sterile modified Barth’ s solution, with collagenase (2 mg / ml, type 1, Gibco) for about 2 h followed by five to six washes in MBS containing 0.1% BSA (Sigma). Defolliculated

oocytes (Stage V or VI) were then stored and kept overnight at 17 °C before injection with cRNA. Approximately 50 nl cRNA (10-15 ng) was injected into each defolliculated oocyte using a Drummond Nanojector (Drummond Scientific, Broomall, PA). Injected oocytes were kept in sterile MBS (with gentamycin 50 mg/L) at 17-18 °C for 2-3 days before electrophysiological measurements were performed. The MBS medium was changed once a day. All experiments and animal maintenance were approved by Institutional Animal Care and Use Committee at the Chung Shan Medical University.

Molecular Cloning and Expression of KCNQ4. After linearization of the KCNQ4-containing PTLN vector with HpaI, capped cRNA was transcribed in vitro using the mMessage mMachine kit (Ambion). Usually 5 – 15 ng of cRNA was injected into *Xenopus* oocytes previously isolated by manual defolliculation and short collagenase treatment. Oocytes were kept at 17°C in modified Barth’ s solution (90 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 10 mM HEPES, 40 mg gentamycin /l [pH 7.6]). Two-electrode voltage-clamp measurements were performed at room temperature 2 – 4 days after injection using an Axoclamp-2B amplifier (Axon instruments) and pClamp 9.0 software (Axon Instruments). Currents were usually recorded in ND96 solution. Reversal potentials were determined from tail currents after a 2 s depolarizing pulse to +60 mV and corrected for liquid junction potentials. Data analysis used pClamp9 and Sigmaplot 8.0.

Electrophysiology. Current through expressed KCNQ4 channels was recorded using a two-electrode voltage-clamp amplifier (AxoClamp-2B, Axon Instrument Inc., Foster City, CA, USA). Electrodes were pulled from borosilicate glass capillaries on a vertical electrode puller

(Model PP-830, Narishige Scientific Instrument Lab, Japan) and had tip resistances between 0.5 and 2.0 MΩ when filled with 3 M KCl. One of the electrodes was used as a voltage recording which was connected to an HS-2 x 1L headstage, and the other electrode was used for current recording connected to an HS-2 x10 MG headstage. During the experiments oocytes were placed in a small chamber (volume, 3 ml). KCNQ4 channels were activated by membrane depolarization and channel activity was measured in ND 96 solution consisting of (mM): 96 NaCl, 1 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 Hepes; pH was 7.4. For the intracytoplasmic injection of intracellular calcium or ionomycin medium, the third glass electrode was used. Intracellular calcium or ionomycin medium for intracytoplasmic injection was consist of the following respectively (mM): 96 KCl 1 $MgCl_2$, 1 $CaCl_2$, 5 Hepes or 96 KCl 1 $MgCl_2$, 1 ionomycin, 5 Hepes, pH was 7.4. The condition of each single oocyte was controlled before measurements by recording membrane potentials. Only oocytes with membrane potentials below -30 mV were used for current recordings. A steady current level was always obtained before electrophysiological analysis or drug application. All experiments were carried out at room temperature (22-26°C). Data were digitized at 5 KHz and stored using Digidata 1322A (Axon Instruments) and analysis were accomplished with the pClamp 9.0 software (Axon Instruments). To determine the current/voltage (I/V) relations, a step protocol was employed, whereby the oocytes were clamped at -80 mV for 3 s and depolarized at +60 mV with 20 mV increments to -100 mV. Tail current analysis for conductance-voltage (*G-V*) relations was measured at -30 mV for 2 s.

Calculations. Steady-state activation curves were fitted to a two-state Boltzmann functions as follows

$$I_{tail}(V_m) = I_{tail\ (max)} / \{1 - \exp[(V_{1/2} - V_m) / k]\}$$

where V_m is the membrane potential, $V_{1/2}$ is the half-activation potential, and k is the slope factor. $I_{tail\ (max)}$ is the maximal tail current. Current and membrane potential levels are stated as means ± S.E.M, and differences in the mean were tested with the Student's t test or ANOVA and P value < 0.05 were accepted as significant.

3. Results

3.1 Effect of PKC activator on the KCNQ4 expressed in Xenopus oocytes

The expressed current, although quite variable from cell to cell, was 30–70 times larger than that in non-injected cells. Indeed, native *Xenopus* oocytes expressed endogenous K^+ current with an amplitude of no more than 0.2 μA at 0 mV. The expressed KCNQ4 current was blocked by the treatment of the KCNQ4 blocker, linopirdine (250 μM) (Fig.1). Protein kinase C activator, phorbol 12-myristate 13-acetate (PMA, 1 μM), was shown a significant effect on the inhibition of KCNQ4 channel and shifted the voltage-activation curve to the right (Fig. 2). Preatment of PKC inhibitor, bisindolylmaleimide I (BIMI, 1 μM) can prevent the inhibiting effect of KCNQ current induced by PMA (Fig. 3). The result demonstrated that PKC mediate the KCNQ4 current.

3.2 *Effect of phosphatase inhibitor and psoralen derivatives on the KCNQ4 current expressed in Xenopus oocytes.*

To investigate whether the phosphatase is involving the modulation of KCNQ4 channels, the phosphatase inhibitors, okadaic acid and calyculin were used in this studied. Calyculin (3 μM) was able to produce the inhibiting effect of KCNQ4 (Fig. 4). Okadaic acid has the similar effect with calyculin. A psoralen derivative, 5-(4-phenoxybutoxy)psoralen (PAP-1, 10 μM) produce a significant effect on the KCNQ current (Fig. 5), the other three psoralen derivative have the similar effect with PAP-1. Both the PAP-1 and phosphatase inhibitors have the inhibiting effect on KCNQ4 current and shifted the voltage-dependent activation curves to the right.

4. Discussion

In this study we demonstrated that PKC and phosphatase can modulate the KCNQ4 channel. Moreover the psoralen derivatives (e.g., PAP-1) have the inhibiting effect on KCNQ4 channels. Inhibition of KCNQ4 channels in auditory hair cells may influence the auditory transmission pathway. However, the clinical application of psoralen derivatives is

still need to further clarify. It has been shown PAP-1 can inhibiting the Kv1.3 channel [10]. The good selectivity of PAP-1 over other ion channels, receptors, and transporters, together with its lack of cytotoxicity, mutagenicity, and acute toxicity in animals, suggests that PAP-1 might indeed be safe for in vivo use. Its low affinity to P450-dependent enzymes also makes it unlikely that PAP-1 should cause any drug-drug interactions that are mediated through P450 inhibition. PAP-1 could be therefore potentially developed into a therapeutically useful immunomodulator [10]. In the present study, we found that PKC shifts the *G–V* relationship of KCNQ4 channels to the right; in other words, PKC made KCNQ channels reluctant to open. If the major role of PKC is changing the gating properties of KCNQ4 but not the number of available channels, it is reasonable that PKC only partially suppresses the KCNQ4-current. By contrast, PIP2 significantly affected current amplitude with little change in the gating properties [11]. Because PIP2 is required for the activity of KCNQ channels, there would be no channel activity if PIP2 were completely depleted. The availability of PKC may be different depending on the cellular or molecular environment, which includes not only PKC itself, but also auxiliary proteins. Recently, AKAP150 was shown to bind toKCNQ2 channels [12], where it can recruit cytoplasmic PKC to the cytoplasmic domain of KCNQ channels, which should activate PKC pathway. It is thus obvious that AKAP150 make the alteration of the molecular environment of KCNQ2 channels. On the other hand, the density or concentration of PIP2 is controlled by the activities of numerous kinases and phosphatases [13]. Both the activities of PKC and some phosphatases are calcium dependent. Although the calcium binding proteins calmodulin and calcineurin when activated by Ca^{2+}, was to interact with KCNQ4 in the membrane and lead to channel inactivation. Calmodulin is an ubiquitous Ca^{2+} binding protein that controls many cellular events including the activation of several proteins, enzymes and ion channels. It is certainly known to be present in OHCs. Calmodulin interacts with members of the KCNQ family binding to an IQ domain motif on the protein, either controlling the tetrameric assembly into the membrane or by direct binding and conferring Ca^{2+} sensitivity. It is unresolved whether the Ca^{2+}/ calmodulin complex or the Ca^{2+}-free apocalmodulin form binds to this sequence. The simplest model here compatible with the data is that

Ca^{2+}/calmodulin both binds to a site on the channel and to a site on calcineurin to activate the phosphatase. The results show that calcium is involved in the basal modulation of KCNQ4 as we previous reported [9]. In summary, we here describe the psoralen derivative and phosphatase possess the inhibiting effect of KCNQ4 channels. This selectivity and PAP-1's effectiveness in suppressing KCNQ4 channel is similar with that of effect on Kv1.3 which as a target for the prevention and treatment of autoimmune diseases in animal models of multiple sclerosis, type-1 diabetes, rheumatoid arthritis, transplant rejection, and graft-versus host disease. However, the clinical application of PAP-1 on the hearing function is need to further elucidation.

Acknowledgments

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6. Figures:

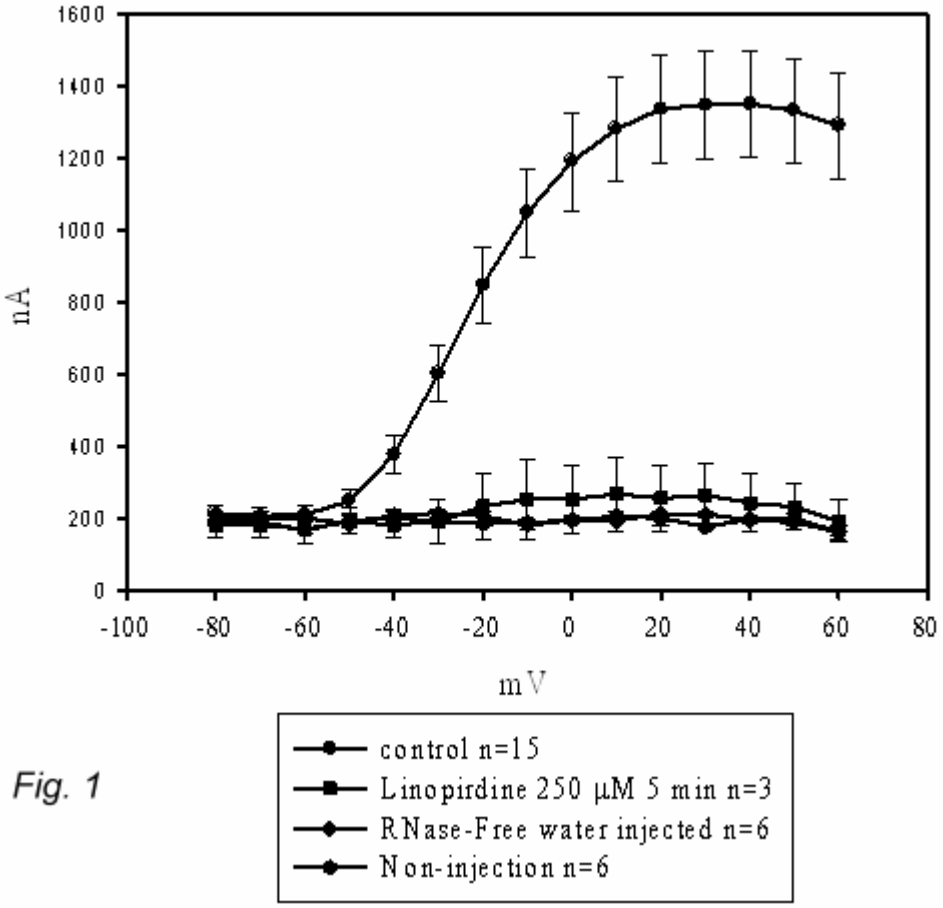

Fig. 1

Fig.1 Linopirdine (250 μM) block the KCNQ4 current expressed in *Xenopus* oocytes.The before (filled circle) and after the treatment of linopirdine (filled square) as shown in above. There are no current (< 0.2 μA) produced both in RNase-free water injected and non-injection oocytes. Currents were elicited by 4-s command steps from –80 to + 60 mV in 20 mV increments, followed by a 1-s step to – 30 mV.

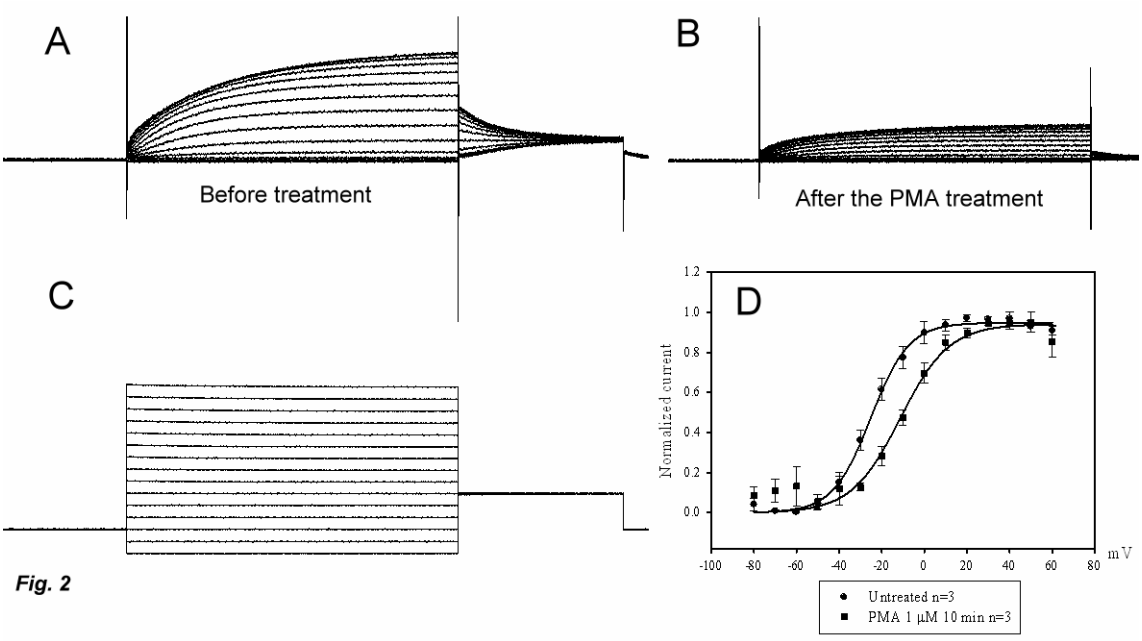

Fig.2 Transient expression of the human voltage-dependent KCNQ4 channel in *Xenopus* oocytes. (A) The control currents recorded from Xenopus oocyte cell injected with cRNA encoding KCNQ4. (B) The treatment of phorbol 12-myristate 13-acetate (PMA, 1 μM) for 10 min. (C) Voltage-step protocol. Holding potential is -60 mV. Currents were elicited by 1-s command steps from –80 to + 60 mV in 20 mV increments, followed by a 1-s step to – 30 mV. (D) The activation curve of KCNQ4 is shown. PMA shifts the activation curve to the right. . Steady-state activation curves were fitted to a two-state Boltzmann functions as described in materials and methods.

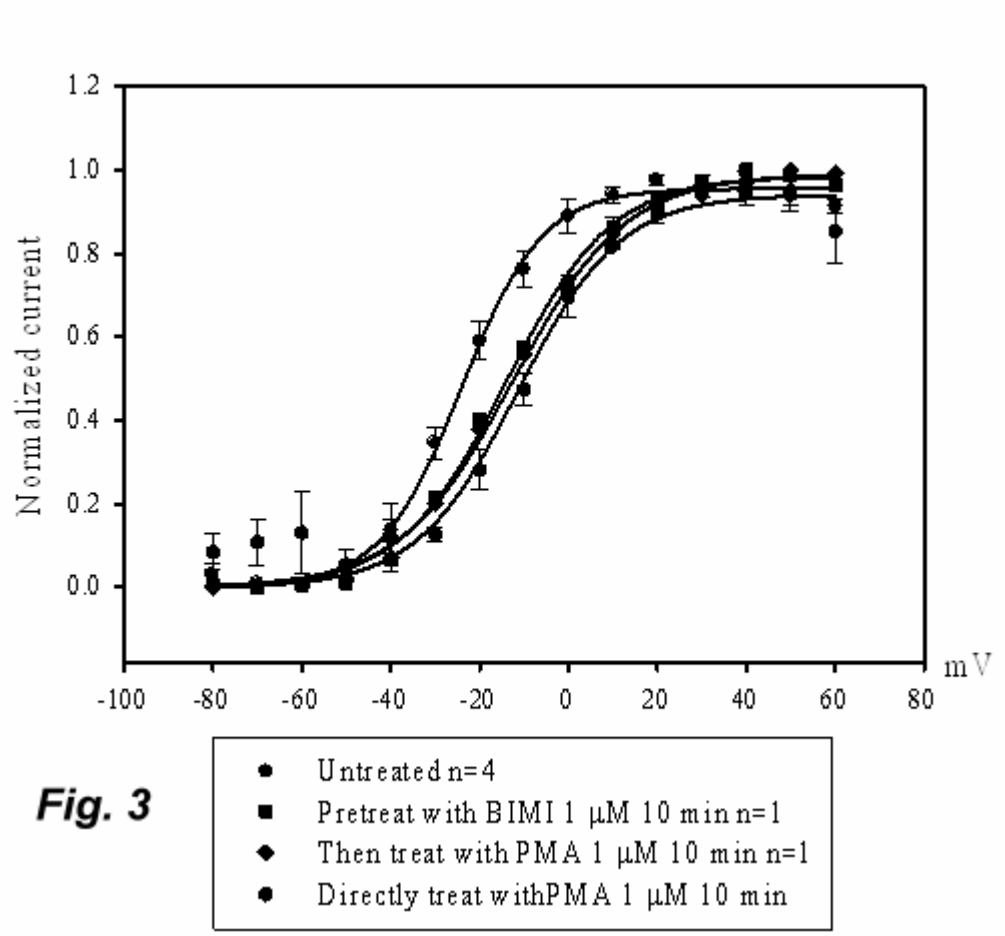

Fig. 3

Fig.3 Effect of protein kinase C inhibitors on the effect of PMA the activation curves of KCNQ4 channels. The pretreatment of Bisindolylmaleimide I (1 μM), a protein kinase C inhibitor, prevent the inhibiting effect of KCNQ4 induced by PMA. The activation curve do not shift by PMA when the pretreatment of BIMI. Steady-state activation curves were fitted to a two-state Boltzmann functions as described in materials and methods.

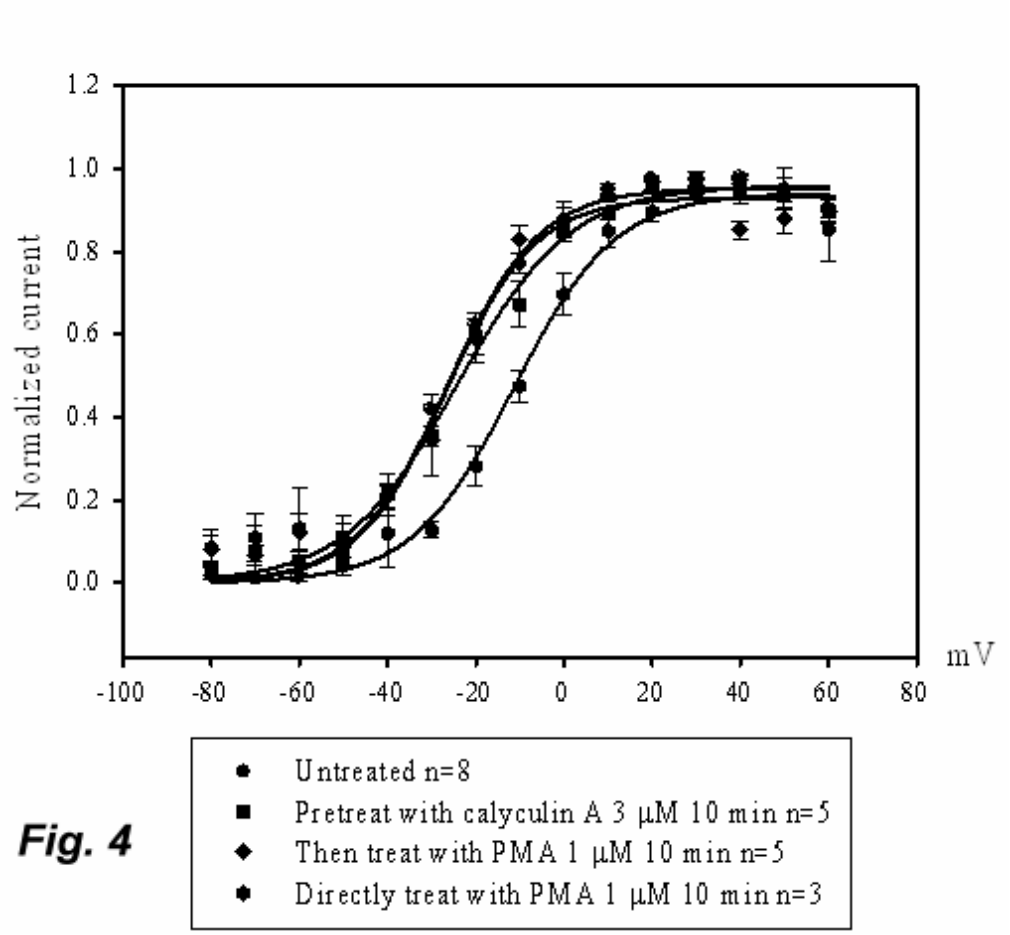

Fig. 4

Fig.4 Effect of phosphatase inhibitors on the activation curves of KCNQ4 channels. The pretreatment of calyculin A (3 μM), a phosphatase C inhibitor, prevent the further inhibiting effect of KCNQ4 induced by PMA.. Steady-state activation curves were fitted to a two-state Boltzmann functions as described in materials and methods.

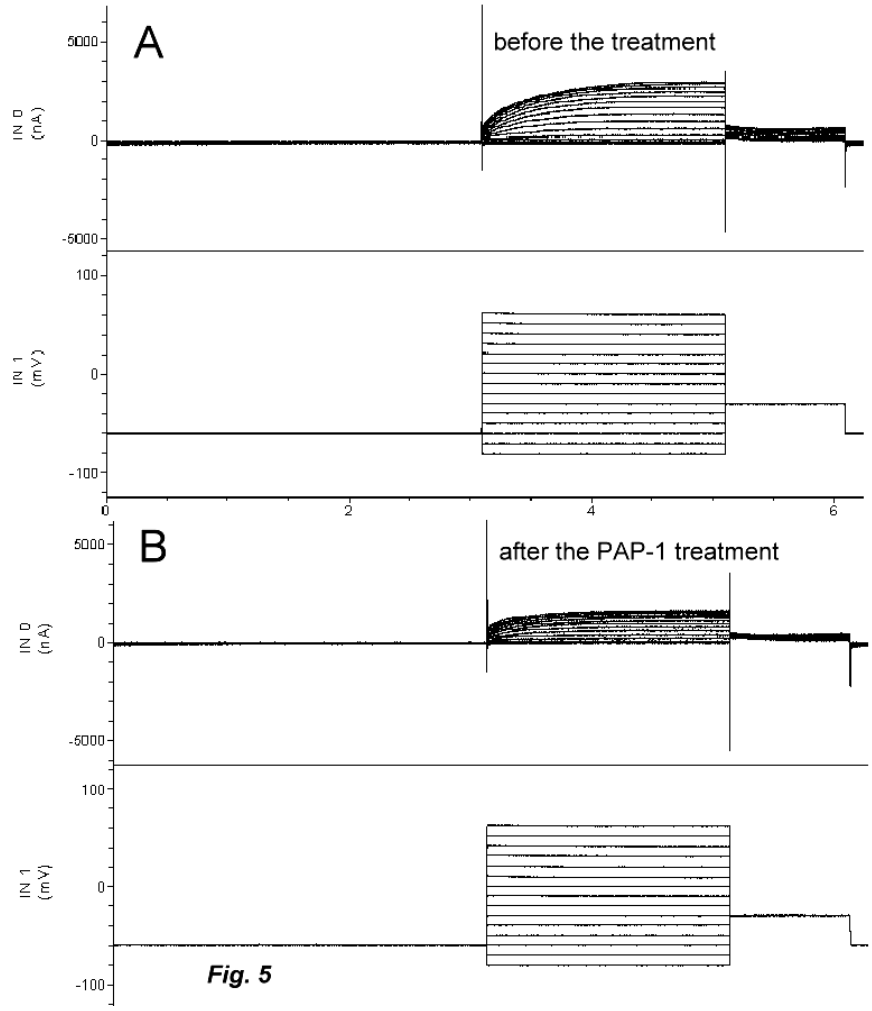

Fig.5 Effect of psoralen derivative, PAP-1 on the KCNQ4 currents. The treatment of PAP-1 (10 μM), produce the inhibition effect of KCNQ4. Voltage-step protocol. Holding potential is -60 mV. Currents were elicited by 1-s command steps from –80 to + 60 mV in 20 mV increments, followed by a 1-s step to – 30 mV.

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