行政院國家科學委員會專題研究計畫 成果報告

以異源性表達方式探討補骨脂成份衍生物與KCNQ阻斷藥物對於 KCNQ4 基因與多型性功能的作用之研究
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

我們已知KCNQ4基因蛋白存在內耳毛細胞中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，AS-84，AS-77，PAP-10，PAP17a對於人類的KCNQ4基因(與polymorphism)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。此電流會被鉀離子阻斷劑 linopirdine (0.25 mM) 所抑制。實驗發現某些補骨脂衍生物 (psoralen derivatives) 具有抑制 KCNQ4電流的作用，其中PAP-1與PAP17a 具有顯著的抑制作用。PAP-1 (30 μM) 與PAP17a (30 μM) 可分別抑制 KCNQ4電流約50%與40%。AS-84，AS-77與PAP-10對於KCNQ4電流並無顯著的抑制作用。PAP-1與PAP17a 對於KCNQ4 鉀離子電流電位依賴性的二分之一活化電位無明顯偏移作用。PAP-1與PAP17a 對於KCNQ4的變異型(F182L;V404I)與wild type比較並無顯著的差異。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PAP-1與PAP17a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; PAP-1; PAP17a; KCNQ4; 鉀離子管道; 爪蟾卵母細胞.

Abstract

It is has been known that KCNQ4 is expressed in inner and outer hair cells of the inner ear and plays an important role for auditory transmission. In this study the effects of psoralen derivatives, PAP-1, AS-84, AS-77, PAP-10 and PAP17a on the function of KCNQ4 (and mutants) 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. This current was blocked almost completely by 0.25 mM linopirdine, a selective blocker of KCNQ4 channel. These experiments have shown that psoralen derivatives PAP-1 and PAP17a have inhibiting effect on the KCNQ4 current. The inhibiting effect of KCNQ4 current about 50 % and 40% by treated with PAP-1 (30 μM) and PAP17a (30 μM) respectively. The other psoralen derivatives AS-84, AS-77 and PAP-10 were no inhibiting effect of KCNQ4 current. Furthermore, PAP-1 and PAP17a did not produce a shifted effect of half-maximal activation ($V_{1/2}$) voltage. There are no difference in the inhibiting effect of KCNQ4 current between the mutants (F182L and V404I) and wild type. The result reveals that psoralen derivatives PAP-1 and PAP17a possess the effect on the inhibiting KCNQ4 channels. The clinical implication of these observations is a need to further evaluation.

Keywords: Psoralen derivatives; PAP-1; PAP17a; KCNQ4; potassium channel, Xenopus oocytes

1. Introduction

Human potassium (K^+) channels are encoded about 76 genes [1]. Each K+ channel has a unique expression pattern that allows cells to fine-tuning their membrane potential. Several pharmaceutical companies started screening their libraries for more potent and more selective small molecule Kv1.3 blockers and identified a number of compound classes [2]. Psoralen derivative, PAP-1 blocks Kv1.3 in a use-dependent manner with an EC50 of 2 nM. These effects of psoralen derivatives on KCNQ4 channel are still unknown. KCNQ4 is expressed abundantly in the inner ear as well as in brain and heart muscle [3]. Mutations in the gene for KCNQ4 underlie a non-syndromes hereditary hearing loss, DFNA2 [4, 5, 6, 7, 8, 9]. 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 [10] and ultimately excitotoxicity of IHCs and afferent fibres [4]. The sensitivity of KCNQ channels to intracellular Ca^{2+} ($[Ca^{2+}]_i$) and the physiological role of such sensitivity have been highly debated questions in this K^+ channels family that underlie neuronal M current (KCNQ2, 3, 5), cardiac (KCNQ1), epithelial (KCNQ1) and other important K^+ currents. Although M-type channels were sensitive to $[Ca^{2+}]_i$, muscarinic activation of superior cervical ganglion (SCG) sympathetic neurons does not raise $[Ca^{2+}]_i$. However, in the same cells, other $G_{q/11}$-coupled receptors that can modulate the M current via $[Ca^{2+}]_i$ signals. Previous studied we have shown that ionomycin can activate the KCNQ4 currents, and may imply the role of calcium on the channel activities [11]. $I_{K,n}$ is sensitive to elevated intracellular calcium that have been also described the effects of Ca^{2+}-dependent modulation of KCNQ4 currents via calmodulin (CaM) and calcineurin (CaN). As so far the effects of psoralen derivatives 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. It has been described that a small molecule Kv1.3 blocker Psora-4 [2] as a template, and have now identified a number of new phenoxyalkoxypsoralens that potently

inhibit the lymphocyte K+ channel Kv1.3 and display 2 to 50-fold selectivity over the cardiac K+ channel Kv1.5. In this studied the psoralen derivatives were test on the KCNQ4 channels expressed in Xenopus oocytes. The results showed that PAP-1 and PAP17a have an inhibiting effect on KCNQ4 channel. The molecular mechanism of psoralen derivatives on the the channel protein and its clinical implication need to further evaluation.

2. Materials and Methods

Molecular Cloning and Expression of KCNQ4. KCNQ4-containing PTLN plasmid vector was linearized with HpaI, and in vitro transcription was performed using SP6 RNA polymerase (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 2–3 days after injection using an Axoclamp-2B amplifier (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 pClamp 9.0 software (Axon Instruments) and Sigmaplot 8.0.

Xenopus laevis oocytes and cRNA injection. *Xenopus* oocyte was used as a model system for the functional expression of plasma membrane protein in this study**.** The frog was 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 (~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-I, 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). The injected oocytes were kept in sterile MBS (with gentamycin 50 mg/L) at 16-18 °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.

Electrophysiology. The ionic 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 the resistance of 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 experiment, 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. The condition of each single oocyte was controlled before measurements by recording membrane potentials. Only oocyte with membrane potential below -30 mV was 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 (22-28°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, k is the slope factor and $I_{tail\ (max)}$ is the maximal tail current. Current and membrane potential levels are expressed as means ± S.E.M. The differences in the mean were tested with the Student's *t*-test or ANOVA. *P* value < 0.05 were accepted as significant.

3. Results

3.1 Effect of KCNQ4 blocker linopirdine on KCNQ4 channel 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. The 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) as shown in Fig.1. The injection of distilled water or without injection of oocytes are no expressed the K+ currents.

3.2 *Effect of psoralen derivatives on the human voltage-dependent KCNQ4 channel.*

To investigate whether the PAP-1 is act on KCNQ4 channels, PAP-1 30 μM was tested on KCNQ4 channels expressed in Xenopus oocytes. The experiment indicated that PAP-1 was able to produce the inhibiting effect of KCNQ4 (Fig. 2). The maximal inhibiting effect was significantly inhibited to about 50% of KCNQ current. Another psoralen derivative, 5-[4-(4-Oxopyrimidin-3-yl)butoxy]psoralen (PAP-17a, 30 μM) also produce a significant inhibiting effect on the KCNQ current by 40% (Fig. 3). The other three psoralen derivatives PAP-10, AS-77 and AS-84 had no the inhibiting effects on KCNQ4 current. PAP-1 and PAP-17a did not produce the shifted effect of V1/2 (half-maximal activation potential) of KCNQ4 channels (Fig. 4; Table 1).

3.3 *Effect of psoralen derivatives on the mutant human voltage-dependent KCNQ4 channels.*

The application of either PAP-1 or PAP-17a on the mutant KCNQ4 channels, F182L and V404I indicate an inhibiting effect on mutant channels. PAP-1 can inhibit the F182L and V404I channels by 47.2 ± 4.7% and 46 ± 5.1% respectively. PAP-17a can inhibit the F182L and V404I channels by 39.2 ± 4.1% and 41 ± 3.6% respectively. There are no statically difference in the percentage of inhibition of KCNQ4 channels between wild-type and mutants by treatment of PAP-1 or PAP17a.

4. Discussion

In this study we demonstrated that psoralen derivatives, PAP-1 and PAP-17a but not other psoralen derivatives can produce the inhibited effect of the KCNQ4 channel. Moreover the PAP-1 and PAP-17a also can induce the inhibiting effect on mutant KCNQ4 channels to the same extent as wild-type channels. Inhibition of KCNQ4 channels in auditory hair cells may influence the auditory transmission pathway. It has been shown PAP-1 can inhibiting the Kv1.3 channel [12]. 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 [12]. In the present study, we found that PAP-1 and PAP17a possess the effect on the inhibiting KCNQ4 channels without the effect of shift *G–V* relationship of KCNQ4 channels to the right; in other words, PAP-1 and PAP17a made KCNQ channels to close on channel pore binding site. The second messenger, protein kinase C (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, phosphatidylinositol bisphosphate (PIP2) significantly affected current amplitude with little change in the gating properties [13]. Because PIP2 is required for the activity of KCNQ channels, there would be decrease the channel activity if PIP2 were completely depleted. The availability of PKC may be different depending on the intracellular or molecular environment, which includes not only PKC itself, but also auxiliary proteins. Recently, AKAP150 was shown to bind to KCNQ2 channels [14], 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 [15]. 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 inactivation of the channel. Calmodulin is a 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 [11]. Those effects of PKC and Ca^{2+}/calmodulin can produce the shifted effect of half-maximal activation curve of KCNQ4. The acting mechanism of PAP-1 and PAP17a on KCNQ4 are quite different from PKC and Ca^{2+}/calmodulin. In summary, we here describe the psoralen derivative PAP-1 and PAP17a possess the inhibiting effect of KCNQ4 channels. It has been reported that 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 psoralen derivatives on the KCNQ4 channels need to further elucidation.

Acknowledgments

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5. References:

[1] Gutman GA, Chandy KG, Grissmer S, Lazdunski M, McKinnon D, Pardo LA *et al*. (2005). International Union of Pharmacology. LIII. Nomenclature and molecular relationships of voltage-gated potassium channels. Pharmacol Rev 57: 473–508.

[2] Vennekamp J, Wulff H, Beeton C, Calabresi PA, Grissmer S, Hansel W, and Chandy KG (2004) Kv1.3-blocking 5-phenylalkoxypsoralens: a new class of immunomodulators. Mol Pharmacol 65: 1364-1374.

[3] Kharkovets T, Hardelin JP, Safieddine S, Schweizer M, El-Amraoui A, Petit C, Jentsch TJ (2000) KCNQ4, a K^+ channel mutated in a form of dominant deafness, is expressed in the inner ear and the central auditory pathway. Proc Natl Acad Sci USA 97:4333–4338.

[4] Kubisch C, Schroeder BC, Friedrich T, Lutjohann B, El Amraoui A, Marlin S, Petit C, Jentsch TJ (1999) KCNQ4, a novel potassium channel expressed in sensory outer hair cells, is mutated in dominant deafness. Cell 96:437-446.

[5] Coucke PJ, Van Hauwe P, Kelley PM, Kunst H, Schatteman I, Van Velzen D, Meyers J, Ensink RJ, Verstreken M, Declau F, Marres H, Kastury K, Bhasin S, McGuirt WT, Smith RJ, Cremers CW, Van de HP, Willems PJ, Smith SD, Van Camp G (1999) Mutations in the KCNQ4 gene are responsible for autosomal dominant deafness in four DFNA2 families. Hum Mol Genet 8:1321-1328.

[6] Talebizadeh Z, Kelley PM, Askew JW, Beisel KW, Smith SD (1999) Novel mutation in the KCNQ4 gene in a large kindred with dominant progressive hearing loss. Hum Mutat 14: 493-501.

[7] Oliver D, Knipper M, Derst C, Fakler B (2003) Resting potential and submembrane calcium concentration of inner hair cells in the isolated mouse cochlea are set by KCNQ-type potassium channels. J Neurosci 23:2141-2149.

[8] Sogaard R, Ljungstrom T, Pedersen KA, Olesen SP, Jensen BS (2001) KCNQ4 channels expressed in mammalian cells: functional characteristics and pharmacology. Am J Physiol Cell Physiol 280:C859-C866.

[9] Hougaard C, Klaerke DA, Hoffmann EK, Olesen SP, Jorgensen NK (2004) Modulation of KCNQ4 channel activity by changes in cell volume. Biochim Biophys Acta 1660:1-6.

[10] Platzer J, Engel J, Schrott-Fischer A, Stephan K, Bova S, Chen H, Zheng H, Striessnig J (2000) Congenital deafness and sinoatrial node dysfunction in mice lacking class D L-type Ca2+ channels. Cell 102:89-97.

[11] Su Ching-Chyuan, Li Shuan-Yow, Yang Jiann-Jou, Su Mao-Chang and Lin Min-Jon (2006) Studies of the effect of ionomycin on the KCNQ4 channel expressed in Xenopus oocytes. Biochem Biophys Res Commun 348: 295-300.

[12] Alexander Schmitz, Ananthakrishnan Sankaranarayanan, Philippe Azam, Kristina Schmidt-Lassen, Daniel Homerick, Wolfram Hänsel, and Heike Wulff (2006) Design of PAP-1, a Selective Small Molecule Kv1.3 Blocker, for the Suppression of Effector Memory T Cells in Autoimmune Diseases, Mol. Pharmacol. 68 :1254-1270.

[13] Koichi Nakajo and Yoshihiro Kubo (2005) Protein kinase C shifts the voltage dependence of KCNQ/M channels expressed in *Xenopus* oocytes, J Physiol 569: 59–74.

[14] Hoshi N, Zhang JS, Omaki M, Takeuchi T, Yokoyama S, Wanaverbecq N, Langeberg LK, Yoneda Y, Scott JD, Brown DA&Higashida H (2003). AKAP150 signaling complex promotes

suppression of the M-current by muscarinic agonists. Nat Neurosci 6: 564–571.
[15] Andersen OS (2005). The 58th annual meeting and symposium of the Society of General Physiologists: lipid signaling in physiology. J General Physiol 125: 103–110.

Figures:

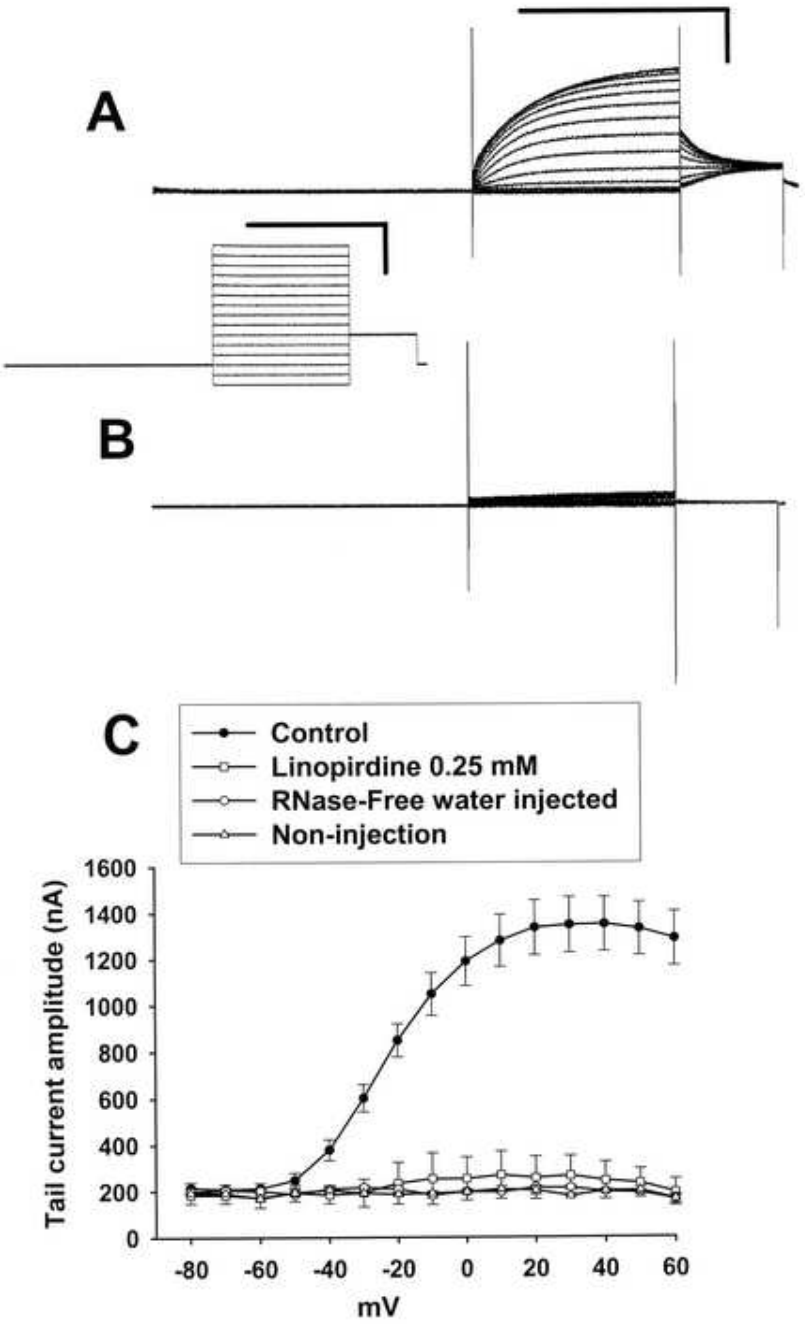

Fig. 1 The expressed currents of KCNQ4 were blocked by the addition of linopirdine in *Xenopus* oocytes. (*A*) Typical current traces recorded from an oocyte cell expressed with KCNQ4 channels with a voltage step protocol as following: oocyte was clamped at -60 mV for 3-s and the channel activated by a 2-s command steps from -100 mV to +60 mV in 10 mV increments, followed by a 1-s step to -30 mV. Calibration scale of all current traces was in upper right corner of (*A*): 2 s and 1 A. Calibration scale of voltage step protocol was shown in the between (*A*) and (*B*): 2 s and 50 mV. (*B*) The expressed KCNQ4 current was almost completely blocked by the application of linopirdine (0.25 mM) after 10 min. (*C*) The summarized curves of tail current-voltage relationship were shown in the absence (●) and presence of linopirdine (□). Two negative control curves, with oocytes of RNase-free water injected (○) and non-injection (△) also were shown for the comparison. The tail-current amplitude was measured at the peak current of the tail potential (-30 mV).

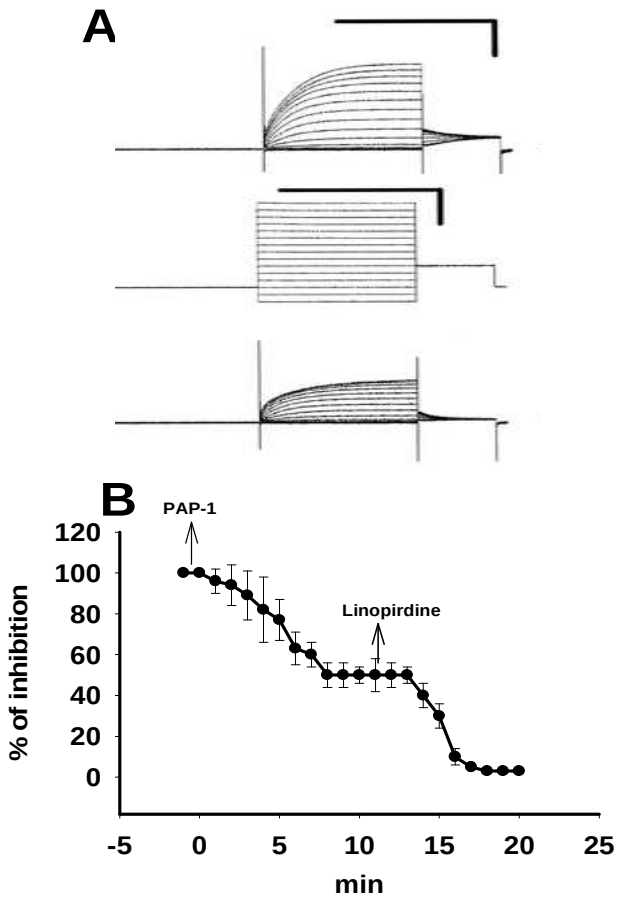

Fig.2 The inhibition effect of 5-(4-phenoxybutoxy)psoralen (PAP-1) on the human voltage-dependent KCNQ4 channel expressed in *Xenopus* oocytes. (A) The control current was showed in upper trace, after the application of PAP-1 (30 μM) in lower trace. (B) The time course of inhibiting curve treated with PAP-1. 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. The percent of inhibition was obtained from the tail current at -30 mV.

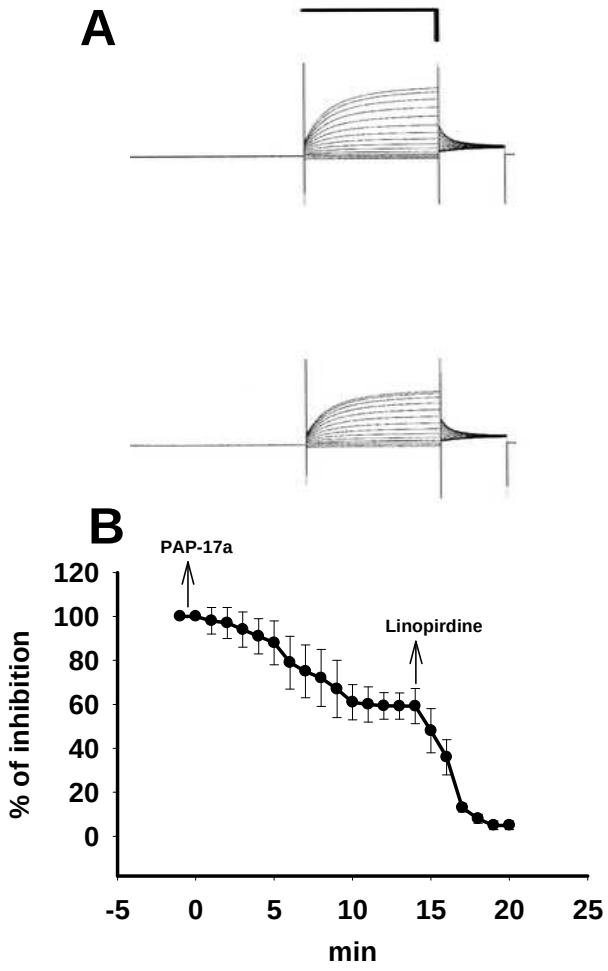

Fig.3 The inhibition effect of 5-[4-(4-Oxopyrimidin-3-yl)butoxy]psoralen (PAP-17a) on the human voltage-dependent KCNQ4 channel expressed in *Xenopus* oocytes. (A) The control current was showed in upper trace, after the application of PAP-17a (30 μM) in lower trace. (B) The time course of inhibiting curve treated with PAP-1. 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. The percent of inhibition was obtained from the tail current at -30 mV.

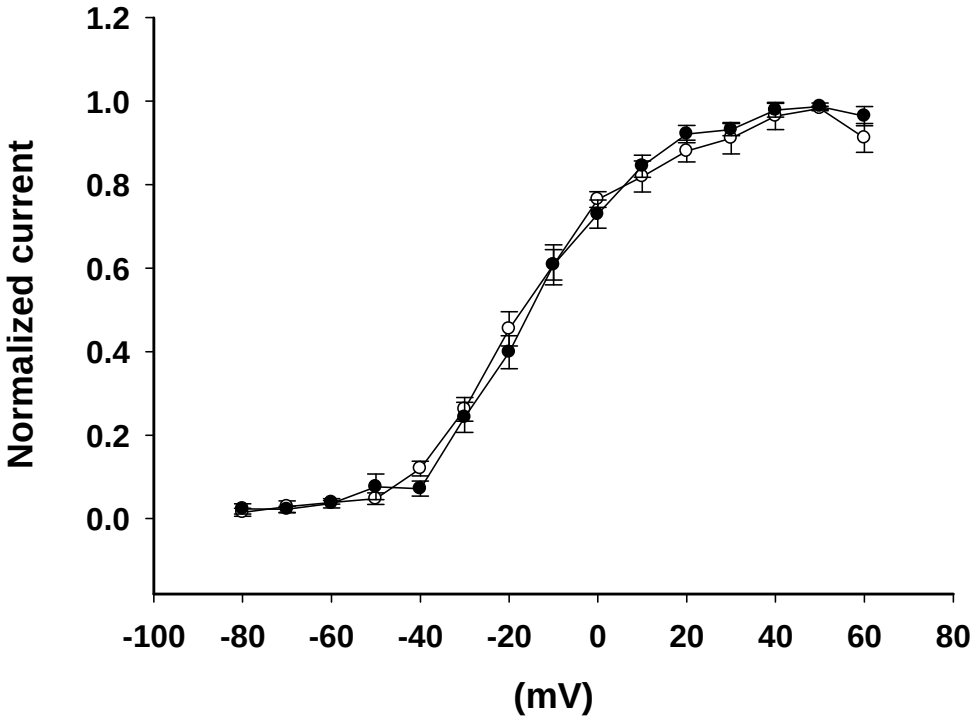

Fig.4 Effect of5-(4-phenoxybutoxy)psoralen (PAP-1) on steady-state activation curves of KCNQ4 channels. PAP-1 did not produce the shifted effect of V1/2 (half activation potential) of KCNQ4 channels. Steady-state activation curves were fitted to a two-state Boltzmann functions as described in materials and methods.

Table 1. Effects of psoralen derivatives on the electrophysiological properties of KCNQ4 channels.

psoralen derivatives	KCNQ4 channel properties	
	% of inhibition	V1/2 (mV)
PAP-1 (30 μM)	51.2 ± 5.6	-15.3 ± 5.6
PAP-17a (30 μM)	40.2 ± 4.4	-18.3 ± 6.1
PAP-10 (50 μM)	3.2 ± 0.6	-16.6 ± 5.1
AS-84 (50 μM)	2.3 ± 0.5	ND
AS-77 (50 μM)	2.1 ± 0.5	ND

Datas are presented as mean ± S.E.M. ND: not determine.

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會議名稱：10^{th} the International Neuroscience Winter Conference (INWC), 2008
日期： 2008 年 4 月 5- 10 日
會議地點：奧地利 Solden
發 表 方 式：Poster
會議內容與心得:

The subject of conference is focus on the synaptic tramsmission and cannabioid research. For example, the $Ga_{i/o}$ protein-coupled CB_1 cannabinoid receptor is the primary neuronal target of the phytocannabinoid Δ^9-tetrahydrocannabinol. It can also be activated by the endogenous cannabinoids (endocannabinoids) anandamide and 2-arachidonylglycerol. The CB_1 receptor is widely distributed in the central nervous system. The analysis of the effects of exogenous and endogenous cannabinoids on synaptic transmission between identified neurons in the central nervous system. Furthermore, another interesting theme was the effects of channel-modulating agents in the conference. In this conference we reported 15 point mutation of C-terminal of CLCN1, and found that serine 892 is an important site in response to the treatment of PKC activator PMA . The poster of conference is shown in the next page.

Functional study of the possible phosphorylation sites of human ClCN1 by protein kinase C in the heterologous expression system.

Min-Jon Lin, Ren-Yu Huang, Pei-Hua Tang, Kuang-Ming Hsiao

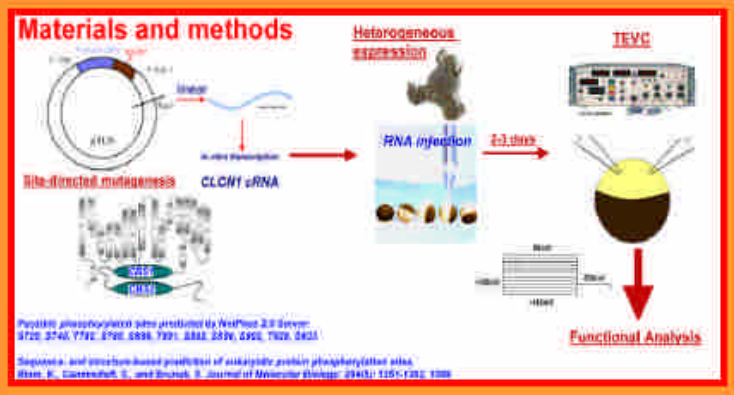
Department of Biomedical Sciences, Chung Shan Medical University, TaiChung, Taiwan

Introduction

ClCN1 voltage-dependent chloride channel play an important role in membrane potential repolarization of mammalian skeletal muscle. Mutations of ClCN1 lead to the myotonia congenita which is an inherited human disease. It has been known protein kinase C can modulate the ClCN1 channel, however, the phosphorylated site is still unclear. PMA (phorbol 12-myristate 13-acetate) a potent protein kinase C (PKC) activator was to treat hClCN1 channel and various mutants expressed in *Xenopus* oocytes. Human wild-type ClCN1 and various C-terminal mutants were heterologous expressed in *Xenopus* oocytes. The functional properties were obtained by using the two-electrode voltage clamp (TEVC) method.

In this study we have surveyed 15 point mutation of C-terminal of ClCN1, and found that serine 892 is an important site in response to the treatment of PKC activator PMA.

Materials and methods



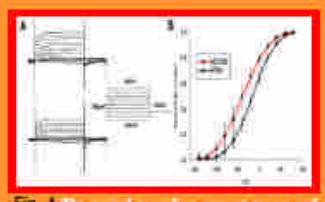


Fig. 1 The typical recording current traces and electrophysiological analysis of the voltage dependence of ClCN1 in *Xenopus* oocytes. (A) The typical recording current trace of ClCN1 (upper trace), and representative current trace after a 10 min application of 2 μM PMA (lower trace). The voltage step protocol (right trace), oocytes were clamped at a holding potential of -100 mV and stepped from -140 to +80 mV in 20mV increment for 0.5s followed by repolarization to a tail potential of -80mV. (B) Current/voltage (I/V) relationship for the ClCN1 channels in the absence (red) and present (black) of PMA.

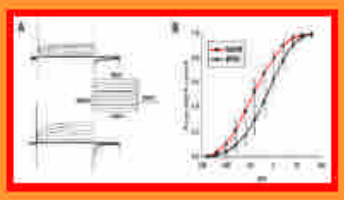


Fig.2 The recording current traces and electrophysiological analysis of the voltage dependence of ClCN1 mutant S896K in *Xenopus* oocyte. (A) the recording current trace of ClCN1 mutant S896K (upper trace), and representative current trace after a 10min application of 2 μM PMA (lower trace). (B) Current/voltage (I/V) relationship for the ClCN1 mutant S896K in the absence (red) and present (black) of PMA. The voltage-dependent activation curve of S896K is shifted to right similar to that of wild-type ClCN1 when present of PMA.

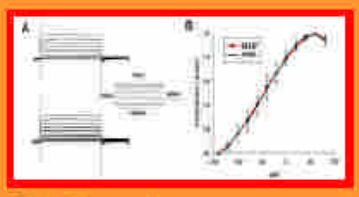


Fig.3 The recording current traces and electrophysiological analysis of the voltage dependence of ClCN1 mutant S892P in *Xenopus* oocyte. (A) the recording current trace of ClCN1 mutant S892P (upper trace), and representative current traces after a 10min application of 2 μM PMA (lower trace). (B) Current/voltage (I/V) relationship for the ClCN1 mutant S892P in the absence (red circle) and present (black circle) of PMA (2 μM). Please note that the voltage-dependent activation curve of S892P is no shifted after the application of PMA.

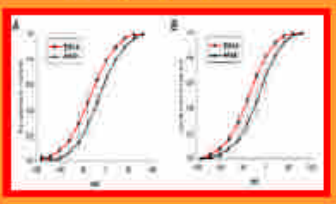


Fig.4 Current/voltage (I/V) relationship for the ClCN1 mutant T891A and T893A in *Xenopus* oocytes. (A) Current/voltage (I/V) relationship for T891A in the absence (red circle) and present (black circle) of PMA (2 μM). (B) Current/Voltage (I/V) relationship for T891A in the absence (red circle) and present (black circle) of PMA (2 μM). The voltage-dependent activation curves of T891A and T893A were shifted to right, but they are less extent shifted effect than that of wild-type.

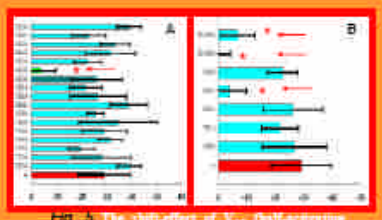


Fig. 5 The shift-effect of $V_{1/2}$ (half-activation voltage of open probability) to wild-type and various mutants by the treatment with PMA (2 μM). (A) The value of potential shift of wild-type is red column as indicated, the other mutants are cyan color columns. The value of S892P is significant lower than that of wild-type. (B) The values of potential shift of double mutants, S892A plus T893A and T891A plus T893A, are significant lower than that of wild-type.

Summary and Conclusion

The expressed current of ClCN1 showed the half-maximal activation potential ($V_{1/2}$) was -42.9 mV and shifted to -13.7 mV in the present of 2 μM PMA.

The expressed current of S892P showed the half-maximal activation potential ($V_{1/2}$) was -52.3 mV. The $V_{1/2}$ of S892P was no significantly changed after application of 2 μM PMA.

The voltage-dependent activation curves of other mutants except S892P, S892A plus T893A and T891A plus T893A produced a shifted of $V_{1/2}$ in the present of 2 μM PMA.

This result indicates that S891~T893 of ClCN1 may serve a site for phosphorylation by protein kinase C. The presence of P892 instead of S892 causes loss of sensitivity of ClCN1 to the treatment of PMA, which could be probably explained as a structural change in C-terminal of ClCN1.

Acknowledgments

We thank Dr. Thomas Jentsch for providing the ClCN1 cDNA. This study was supported by grant from National Science Council, Taiwan (NSC 95-2320-B-040-007 and NSC 95-2320-B-040-002).

參加國際會議論文發表心得報告

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This result indicates that S891~T893 of CLCN1 may serve a site for phosphorylation by protein kinase C. The presence of P892 instead of S892 causes loss of sensitivity of CLCN1 to the treatment of PMA, which could be probably explained as a structural change in C-terminal of CLCN1.

The poster of this conference is shown in the following page (附件).

附件 (Poster)

Functional study of the possible phosphorylation sites of human CICN1 by protein kinase C in the heterologous expression system.

Min-Jen Lin, Ren-Yu Huang, Pei-Hua Tang, Kuang-Ming Hsiao

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Introduction

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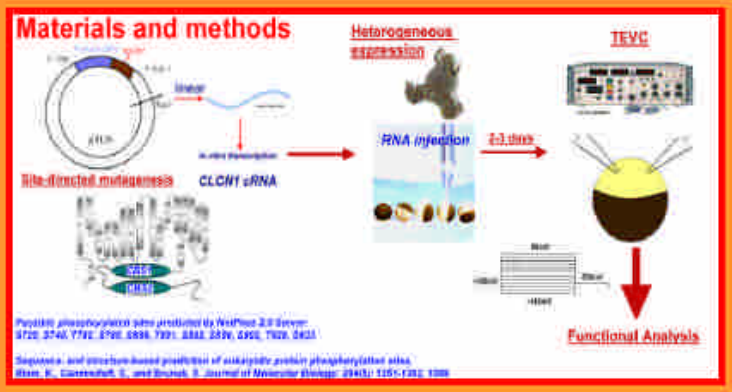
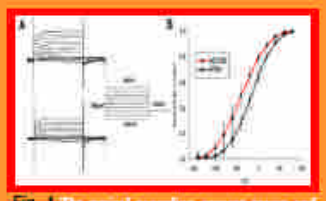
In this study we have measured 15 point mutation of C-terminal of CLCN1, and found that serine 892 is an important site in response to the treatment of PKC activator PMA.

Materials and methods





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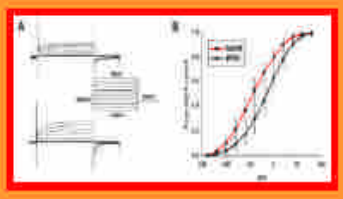

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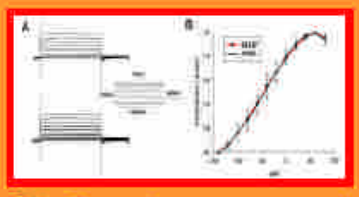

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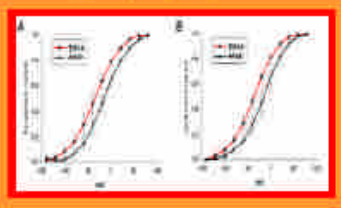

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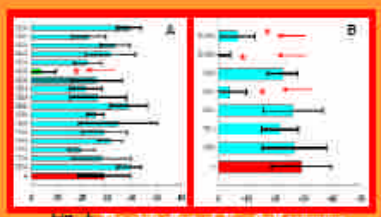

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