行政院國家科學委員會專題研究計畫　成果報告

台灣聽障患者中 KCNQ4 基因(鉀電流)變異表現在爪蟾卵母細胞之功能分析

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計畫主持人：林明忠
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中文摘要

本實驗主要是研究人類的KCNQ4基因的功能，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。 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約-18 mV ，此電流會被鉀離子阻斷劑 linopirdine (0.2 mM) 所抑制。給予 ionomycin (0.5 μM) 或 caffeine (1 mM) 會促進 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 –10 and –7 mV。ionomycin (0.5 μM) 或 caffeine (1 mM)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。ionomycin (0.5 μM) 或 caffeine (1 mM) 促進 KCNQ4鉀電流的作用會被穿膜性鈣離子螯合劑BAPTA-AM (0.3 mM)完美的逆轉。因此我們認爲 KCNQ4鉀電流可以被鈣離子所直接的調控這也可以解釋在聽神經的外毛細胞中鉀離子電流在比較負的電位下可被活化。

關鍵詞: 鉀離子管道; KCNQ4; 鈣離子; 爪蟾卵母細胞.

Abstract

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 expressed current showed the typical KCNQ4 voltage-dependence, with a voltage for half-maximal activation ($V_{1/2}$) of –18 mV, and was blocked almost completely by 0.2 mM linopirdine, a selective blocker of KCNQ4 current. Application of ionomycin (0.5 μM) or the caffeine (1 mM) shifted $V_{1/2}$ by approximately –10 and – 7 mV, respectively. Ionomycin or caffeine has no effect on the endogenous current of oocytes. These effects can be reverse by the addition of BAPTA-AM (0.3 mM), a membrane-permeable calcium-chelating agent. We suggest that KCNQ4 current is modulated by intracellular calcium directly can lead to the negative activation and the negative resting potential found in adult outer hair cells.

Keywords: Potassium channel, KCNQ4, Calcium, Xenopus oocytes

1. Introduction

KCNQ4 is expressed abundantly in the cochlea as well as in brain, heart and skeletal muscle. Mutations in the gene for KCNQ4 underlie a non-syndromes hereditary hearing loss, DFNA2. The channel is expressed in both inner hair cells (IHCs) and outer hair cells (OHCs). KCNQ4 has been identified tentatively as the molecular correlate of an 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. The potential eliciting half-maximal activation ($V_{1/2}$) seen in activation curves in OHCs is variable but, in general, very negative, –80 mV in guinea-pig and –66 mV in mouse at post-natal day (P)12. In contrast, $V_{1/2}$ for KCNQ4 in expression systems ranges from –10 mV in oocytes to –32 mV in HEK-293 cells. 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. We describe here the effect of a rise in $[Ca^{2+}]_i$ on KCNQ4 currents and show that KCNQ4 current modulated intracellular calcium.

2. Materials and Methods

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.

3. Results

3.1 Effect of ionomycin on the outward current of native Xenopus oocytes

The expressed current, although quite variable from cell to cell, was 20–70 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 (Fig.1B), linopirdine (200 μM) and ionomycin (10 μM) had no effect on this endogenous current.

3.2 *Effect of ionomycin on the KCNQ4 current expressed in Xenopus oocytes*

To investigate the mechanism by which intracellular calcium influenced KCNQ4 currents, ionomycin (0.5 μM) was added, at which the KCNQ4 currents at +30mV in Xenopus oocytes increased by 40% (FIG. 2B; Fig. 3A). Ionomycin also shifted the activation curve to more negative potentials. After exposure, $V_{1/2}$ was – 28mV, a negative shift of 10 mV ($V_{1/2}$ of control KCNQ4 current was – 18mV, Fig. 3B). BAPTA-AM (0.3 mM), a calcium-chelating agent reverse the effect of ionomycin (Fig. 2C; Fig. 3A; Fig. 3C), $V_{1/2}$ was – 18mV, suggest calcium modulate the KCNQ4 channel directly.

4. Discussion

To examine the mechanisms by which $[Ca^{2+}]_i$ could be having an effect, we studied the possible by adding the ionomycin, caffeine and BAPTA to the bath solution. Ionomycin increased KCNQ4 currents significantly (by 40% at +30 mV). The activation curve before and after application of ionomycin was fitted by Boltzmann function with voltage for half-maximal activation of –18 and –28 mV, respectively. Ionomycin do negative shift in activation about –10 mV from control. BATPA-AM a membrane-permeable chelating agent reverse the effect of ionomycin. The effects of BAPTA-AM showed intracellular calcium modulate the KCNQ4 ion channel directly.

Although the calcium binding proteins calmodulin and calcineurin when activated by Ca^{2+}, 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.

Acknowledgments

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6. Figures:

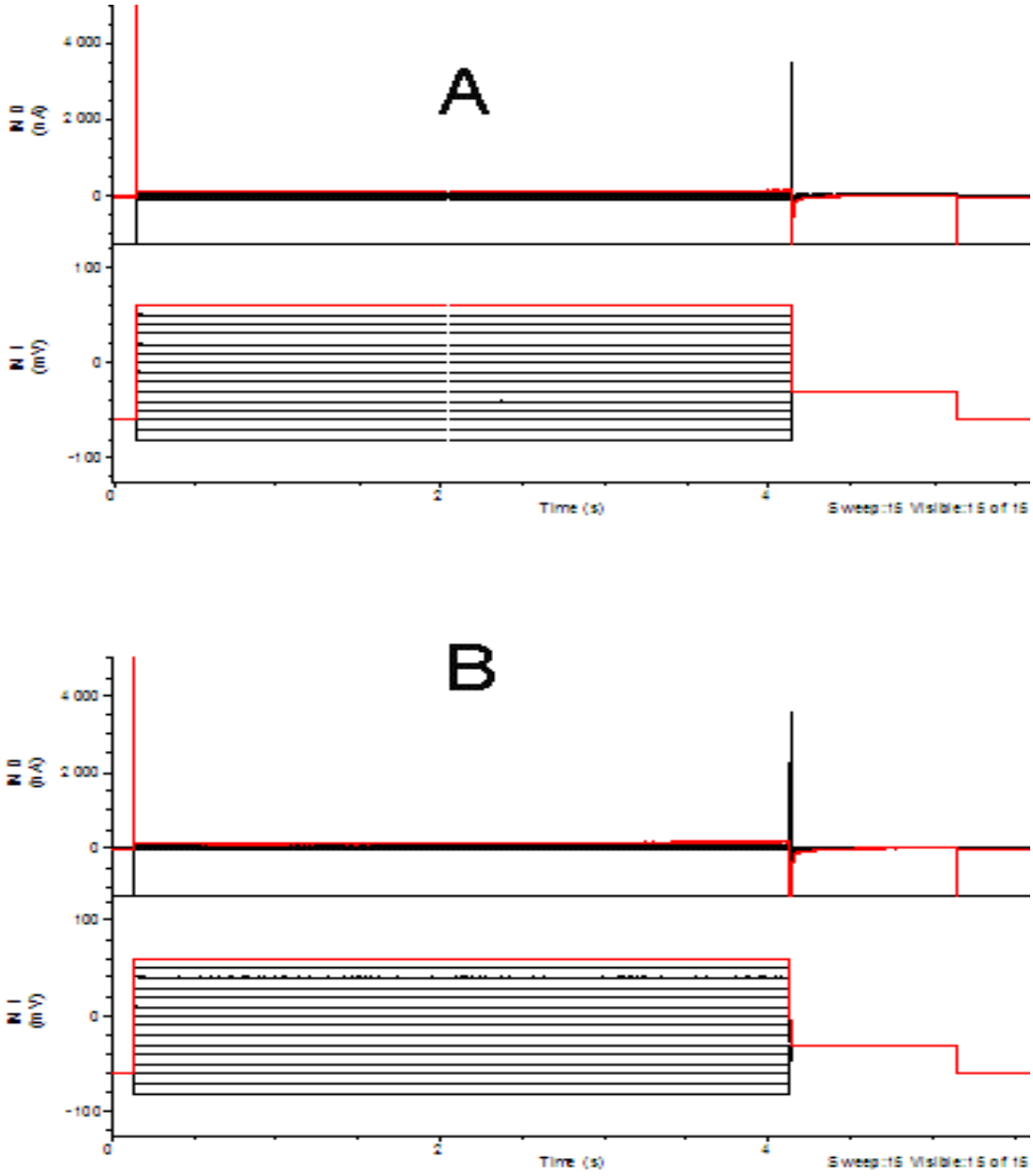

Fig.1 Ionomycin (10 μM) has not effect on the outward currents in native Xenopus oocytes. (A) Control native outward currents (< 0.2 μA). (B) The application of ionomycin for 10 mins. Currents were elicited by 4-s command steps from –80 to + 60 mV in 20 mV increments, followed by a 1-s step to – 30 mV.

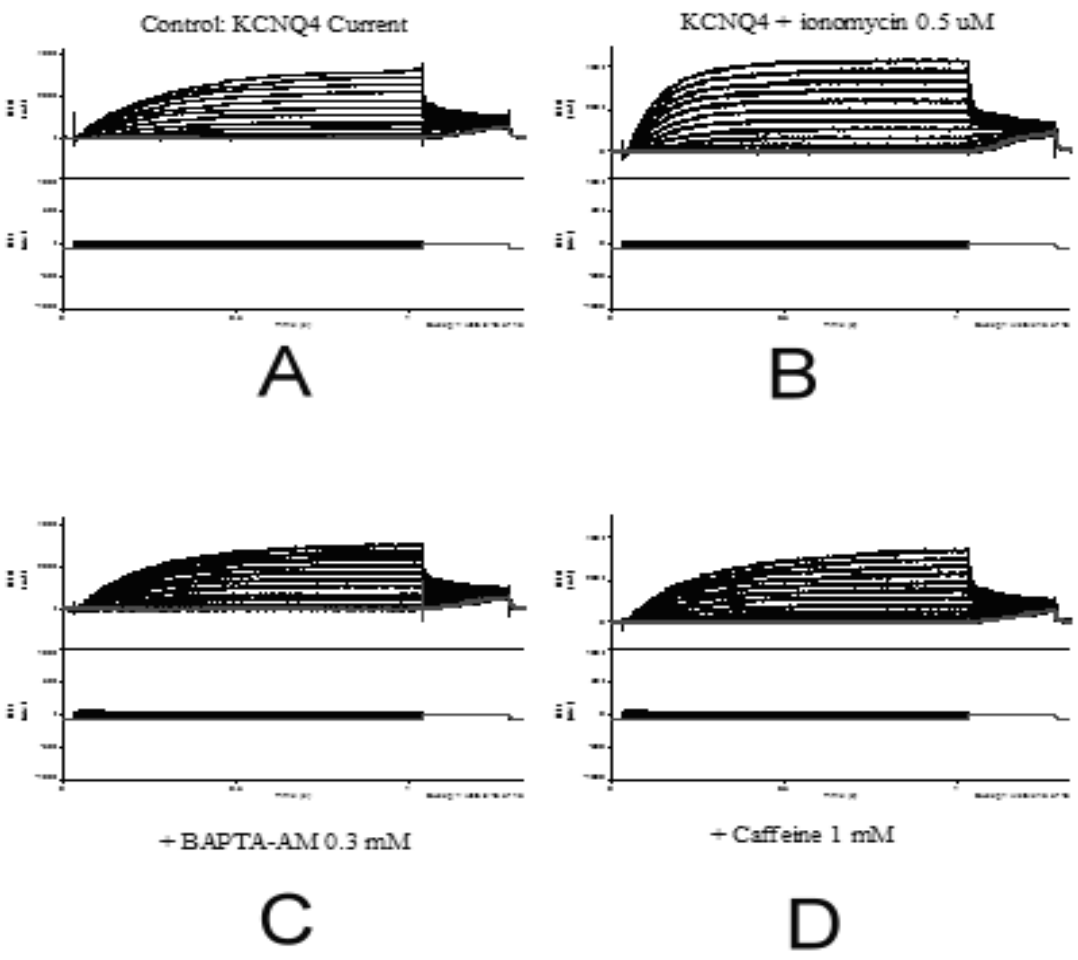

Fig.2 Transient expression of the human voltage-dependent K+ channel KCNQ4 in Xenopus oocytes. (A) Currents recorded from Xenopus oocyte cell injected with cRNA encoding KCNQ4. Holding potential – 60 mV. Currents were elicited by 1-s command steps from –80 to + 60 mV in 20 mV increments, followed by a 1-s step to – 10 mV. (B) 5 mins after the application of ionomycin (0.5 μM). (C) 10 mins after the application the BAPTA-AM (0.3 mM). (D) Further application the caffeine (1 mM) after the BAPTA-AM.

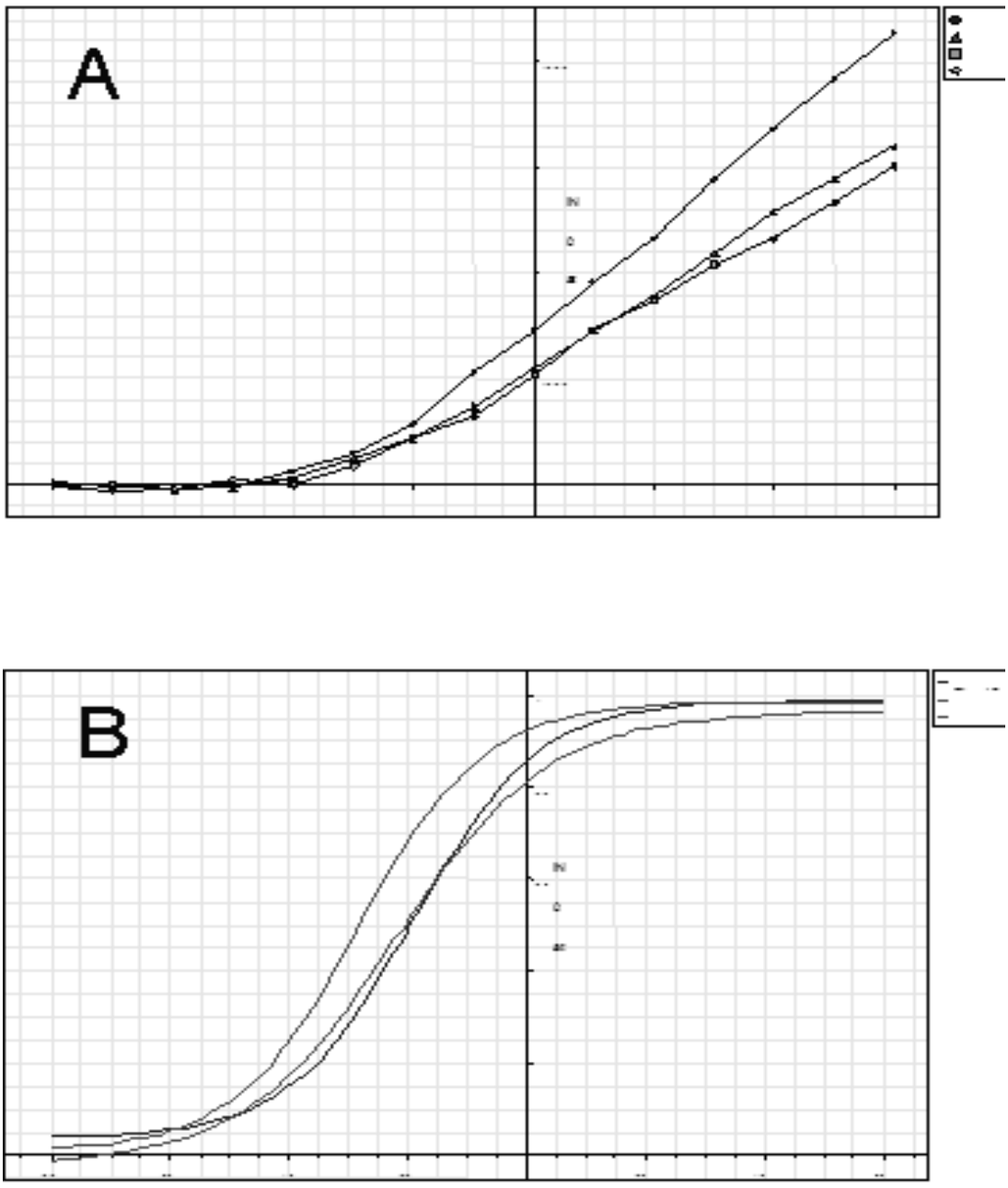

Fig.3 Effect of ionomycin on the I/V curves and activation curves of KCNQ4 channels. (A) Ionomycin enhances the KCNQ4 current and this effect is reversed by the addition of BAPTA-AM (0.3 mM). (B) The activation curve before and after application of ionomycin was fitted by Boltzmann function with voltage for half-maximal activation of –18 and –28 mV, respectively. BAPTA-AM reverse the effect of ionomycin on the KCNQ4 channel, half-maximal activation was –18 mV.

7. 計畫成果自評: 此報告部份已發表在: Hearing Research 203:172-179 (2005).