行政院國家科學委員會專題研究計畫 成果報告

壓力荷爾蒙對鉀離子通道之調控
研究成果報告(精簡版)

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計 畫 主 持 人 ：賴桂珍

計畫參與人員：此計畫無其他參與人員

處 理 方 式 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 99 年 01 月 22 日

附件一

行政院國家科學委員會補助專題研究計畫

壓力荷爾蒙對鉀離子通道之調控

成果報告

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成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

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執行單位：政治大學

中　華　民　國　99 年　01 月　18 日

Principal Investigator: Guey-Jen Lai（賴桂珍）

本研究報告將於其他市數據補齊後投稿國際期刊，本研究結果與預期相似

研究報告內容：**The regulation of BK channel alternative splicing: effects of stress hormones**

1. OBJECTIVES

To understand how the previous stress experiences shape the stress response system for the future response in both adult and neonate during postnatal development is the main theme of my research. In this proposal, the aim is to improve our understanding of how stress hormones might have a lasting effect on future chromaffin cell excitability and adrenaline release by changing the pattern of BK channel alternative splicing. Alternative splicing allows a single gene to give rise to multiple mRNA variants each encoding proteins with different properties. It is estimated that transcripts of more than 75% of human genes are alternative spliced. However, it remains largely unclear how alternative splicing is controlled.

Stress hormones (corticosterone, dehyroepiandrosterone, mineralocorticoid, other androgens), secreted from the adrenal cortex differentially regulate the alternative splicing of BK channels (big potassium, calcium-voltage activated potassium channels) in the adrenal medulla (Lai and McCobb 2002). I have previously developed two experimental systems to study hormone-induced alternative splicing. One experimental system relies on primary bovine adrenal chromaffin cell culture and will form the basis of this project, while the second experimental system utilizes neonatal rats in the stress hypo-responsive period (SHRP) of development. This proposal extends and expands my previous work on characterizing how stress hormones regulate alternative splicing of RNA transcripts.

Overall Hypothesis

The major objective of the proposed project is to determine the validity of the hypothesis that DHEA (stress hormones) and KCl-mediated depolarization alter the alternative splicing of *slo* by acting through the second messenger, calcium.

Results and Discussion

Establishing a primary cell culture system to study hormone-induced alternative splicing in Taiwan

Chromaffin cells are neurosecretory cells located in the adrenal medulla. These cells are embedded by the adrenal cortex, the source of stress hormones. Thus, chromaffin cells are subjected to very high levels of stress hormones, a situation which is difficult to replicate *in vivo* using systemically introduced steroids. Thus to test for direct regulation of *slo* splicing in adrenal chromaffin cells by adrenal

hormones and other factors, I established an experimental system using primary culture of dissociated bovine chromaffin cells when I was a doctoral student at Cornell University. I reset up this system in Taiwan to further investigate the regulation of RNA alternative splicing by stress hormones.

Depolarization leads to changes in the alternative splicing pattern of BK channel in adrenal chromaffin cells. Calcium is a potential secondary messenger in mediating the changes in alternative splicing.

To understand whether depolarization can affect the STREX inclusion of Slo transcripts, a high concentrations of KCl was used to depolarize chromaffin cells. This was followed by analysis of changes in the splicing pattern of transcripts of the slo gene from chromaffin cells. Cells were cultured in either control serum-free medium, serum-free medium that was osmolarity-adjusted after addition of high KCl saline, or the same after addtion of high NaCl saline. After 24 hours cells were harvested, RNA extracted, and RT-PCR performed. The relative STREX percentage (STREX/(STREX+ZERO))% was calculated as described in materials and methods. KCl-induced depolarization was found to increase STREX inclusion (Figure 1).

Increased intracellular calcium levels is sufficient for increasing the inclusion of the STREX exon.

KCl depolarization opens voltage dependent Ca channels and allows calcium to flow into the cells. I thus hypothesized that the KCl-induced STREX percentage increase was caused by calcium influx into the cells. Ca ionophores A23187 and Ionomycin were added to separate dishes to raise intracellular calcium levels under normal KCl condition. Consistent with the KCl experiments, incubation in either calcium ionophore significantly increased STREX inclusion (Figure 2). Thus, intracellular calcium levels should alter the splicing pattern of the slo gene in the absence of depolarization and stress hormone.

Characterization of calcium binding proteins that might be involved in the regulation of STREX inclusion.

Calcium binding to calmodulin, and subsequent activation of calcium-calmodulin dependent protein kinase (CaMK) is a likely pathway for stress hormone-induced alternative splicing. Immunocytochemistry were performed using an assortment of CaMK antibodies to determine the type(s) of CaMK that exist in adrenal chromaffin cells.

Immunocytochemical experiments revealed no CaMK IV in bovine chromaffin cells, while CaMK II was very abundant (Figure 3). This suggests that

CaMK II rather than CaMK IV contributed to the STREX inclusion in chromaffin cells.

Future Experiments

1. Characterization of KCl (depolarization) and DHEA (stress hormones) induced kinetic changes in intracellular calcium levels
2. Determine if increased intracellular calcium levels is necessary for KCl and DHEA's effect on alternative splicing

II. BACKGROUND AND SIGNIFICANCE

Stress hormones (corticosterone, dehyroepiandrosterone, mineralcorticoid, other androgens), secreted from the adrenal cortex differentially regulate the alternative splicing of BK channels (big potassium, calcium-voltage activated potassium channels) in the adrenal medulla (Lai and McCobb 2002). Alternative splicing which allows pre-mRNA from one gene to be spliced in many ways is an efficient way for a single gene to give rise to multiple proteins with different properties. Transcripts from the majority of human genes undergo alternative splicing. Alternative splicing at splice site 5 of the slo transcript results in two types of BK channel splice variants (STREX and ZERO). Ion channels of the STREX type are activated at more negative voltage than the ZERO type. Therefore ion channels of the STREX type allow faster sodium channel recovery after activation and thus increase an excitable cell's capability for repetitive firing. For example, adrenal chromaffin cells (source of adrenaline) with more STREX type BK channels are more likely to fire repetitively and thus release more adrenaline. This is an example of how previous stress experience which result in an increase in stress hormone levels can reset the intrinsic property of neuroendocrine cells at the molecular level, and affect future response to stress.

STREX and ZERO configurations can alter cell firing properties

The 2 types of BK channel splice variants at splice site 5 are named STREX and ZERO. STREX includes an extra 58 amino acid residues that ZERO does not have. Expression of Slo cDNAs (STREX and ZERO) in oocytes showed STREX has faster activation and slower deactivation than ZERO. Ion channels of the STREX type can be activated at more negative (~20mV) voltages than the ZERO type at a given Ca concentration (Xie and McCobb 1998). At a constant voltage, STREX type ion channels are proposed to be more sensitive to Ca concentrations (Xie and McCobb 1998). Interestingly, hair cells in the inner ear of turtles and chickens are electrically tuned to different frequencies by BK channels, and mRNAs of STREX

and ZERO splice variants are differentially distributed along the tonotopic map of the cochlea. It is believed that BK channels help determine the corresponding intrinsic frequencies of oscillatory resonance (Navaratnam, Bell et al. 1997; Rosenblatt, Sun et al. 1997; Jones, Laus et al. 1998; Ramanathan, Michael et al. 1999). STREX variants have been shown to modulate responses of BK channels, including sensitivity to protein kinase A phosphorylation and genomic actions of glucocorticoids on pituitary cells (Shipston, Kelly et al. 1996; Tian, Duncan et al. 2001; Tian, Hammond et al. 2001; Erxleben, Everhart et al. 2002).

Hormonal and social stress regulation of Slo Alternative splicing

In rats, removal of pituitary glands results in a decrease in STREX inclusion in chromaffin cells, and this decrease can be reversed by adrenocorticotropin hormone (ACTH)-injection (Xie and McCobb 1998). ACTH regulates secretion of multiple adrenal steroids. Addition of corticosterone to cultured primary bovine chromaffin cells down-regulates STREX inclusion, while adrenal androgens, including DHEA and testosterone, increases STREX inclusion (Lai and McCobb 2002). Thus, the regulation of Slo splicing is controlled by multiple steroids that can act in opposite directions.

I have also previously shown that subordination stress affect the ratios of STREX and ZERO channels in the adrenal glands of tree shrews (McCobb et al. 2003). Male tree shrews subjected to chronic stress by exposure to a dominant male develops robust symptoms with parallels to human depression. This type of neuron plasticity modulation occurs in both the adrenal medulla and the anterior pituitary in adults and during neonatal development (Lai and McCobb 2006).

Evidence of calcium involvement in the decision of splicing

Elevated calcium levels caused by depolarization with elevated KCl saline results in a drop in the relative abundance of STREX-containing transcripts, and a corresponding increase in the abundance of ZERO transcripts in cultured pituitary cells (Xie and Black 2001). In a series of elegant experiments, the authors demonstrated that calcium/calmodulin-dependent kinase IV (CamKIV) was a key intermediary in the signal transduction pathway, and identified regions within the STREX exon and an adjoining intron that were critical to the CamKIV-mediated regulation.

Adrenal chromaffin cells are part of the neural crest-derived sympathetic nervous system. The amount of catecholamines they secrete depends on the frequency of inputs to these cells via the splanchnic nerve, and on the intrinsic firing properties of chromaffin cells themselves. One important implication of the

evidence presented by my previous work is that experiences of stress, by activating the hypothalamic-pituitary-adrenal axis (HPA, stress axis) and stimulating changes in the synthesis and release of steroid hormones, might have a lasting effect on future chromaffin cell excitability and adrenaline release. If calcium is a critical regulator of slo splicing in chromaffin cells, as suggested by studies using pituitary cells, this may mean that electrical activity, triggered by splanchnic nerve inputs to chromaffin cells, provides another important pathway for experience-dependent plasticity in the excitable properties of chromaffin cells.

DHEA(dehydroepiandrosterone), like CORT (corticosterone), is a steroid secreted from the adrenal cortex. Synthesis of both hormones are controlled by the HPA stress axis, especially adrenocorticotropin hormone (ACTH) from pituitary. DHEA and CORT both influence Slo splicing when applied directly to bovine chromaffin cells in culture (Lai and McCobb 2002). Several links between CORT and calcium levels in chromaffin cells have been suggested (Fuller, Lu et al. 1997; Fuller, Lu et al. 1997; Wagner, Jorgensen et al. 1999). DHEA was shown to have the ability to increase the intracellular calcium level in the cerebral cortex (Compagnone and Mellon 1998).

III. EXPERIMENTAL DESIGN AND METHODS

Chromaffin cell preparation

Fresh bovine adrenal glands from slaughterhouses will be perfused sequentially with buffer I (118 mM NaCl/3.3 mM KCl/1 mM NaH2PO4/1 mM MgSO4·7H2O/10 mM glucose/25 mM Hepes, pH 7.4), buffer II (buffer I plus 1X MEM vitamins, 1X MEM amino acids, 0.11g/liter sodium pyruvate, and 2 mM L-glutamine), and collagenase type B (100 mg/100 ml of buffer II plus 0.0975% BSA). After 1 h of collagenase perfusion, the glands will be sliced open and the medullary tissue will be removed for a second collagenase treatment. During the whole process 95% oxygen/5% carbon dioxide will be bubbled into the solution. After the second digestion, cells will be filtered through a 70-µm cell strainer (Falcon), centrifuged at 500 x g for 10 min, and counted. Cells will then be resuspended in RPMI 1640 supplemented with 10% horse serum, 5% FBS, penicillin (100 units/ml), streptomycin (100 µg/ml), and nystatin (100 units/ml). Approximately 10^6 cells will then be plated on to sterile 35-mm Falcon dishes coated with Vitrogen (0.53 mg/ml, 400 µl per dish). After 24 hours, the culture medium will be replaced with RPMI 1640, to which steroids, KCl, antagonists, or vehicle will be added.

KCl, DHEA or Antagonist treatment

Treatment with high K^+ (38mM KCl final concentration in a serum-free defined medium, RPMI 1640) will be carried out in cells 24 hours after plating. NaCl, and mocked-treated control cells will be handled in parallel as for the experimental cells, except that control cells will be incubated in a medium in which high KCl has been replaced by NaCl, and RPMI 1640, respectively
Cells will be pretreated with blockers for 30 minutes before the cotreatment of KCl and blockers.

RT-PCR and Data analysis

The STREX/ZERO ratio of Slo transcripts in chromaffin cells will be determined by quantitating RT-PCR amplified products. Bovine chromaffin cells (~10^6 cells per 35mm dish) will be cultured in serum containing medium for 24 hours, after which serum-containing medium will be replaced with serum-free medium containing appropriate chemicals or vehicle. Total RNA will be extracted and reverse transcribed at the appropriate time (what is it). STREX and ZERO products will be coamplified by PCR using primers flanking the splice site and analyzed on 3.5% agarose gels. The middle and lower bands of the three expected bands contain STREX (597bp) and ZERO (423bp) homoduplexes, respectively. The top band consists of heteroduplexes with one strand of each type (Lai and McCobb 2002; Mahmoud, Bezzerides et al. 2002). Relative copy numbers of the two splice variants will be determined based on the intensity of the appropriate homoduplex band, plus half of the intensity value of the heteroduplex band. The intensity of STREX will be further divided by 1.41 to correct for the length difference of the two isoforms.

Immunocytochemistry

Cells will be fixed with 4% paraformaldehyde at 5 hours post-plating, washed with 1% BSA/PBS, and permeabilized with 0.2 % Triton-X for 10 minutes at room temperature. Cells will then be incubated in a blocking solution, primary antibodies (CaMK II or IV) and appropriate secondary antibody conjugated with alexa 594 or 488 fluores for visualization by fluorescence microscopy.

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Figure 1

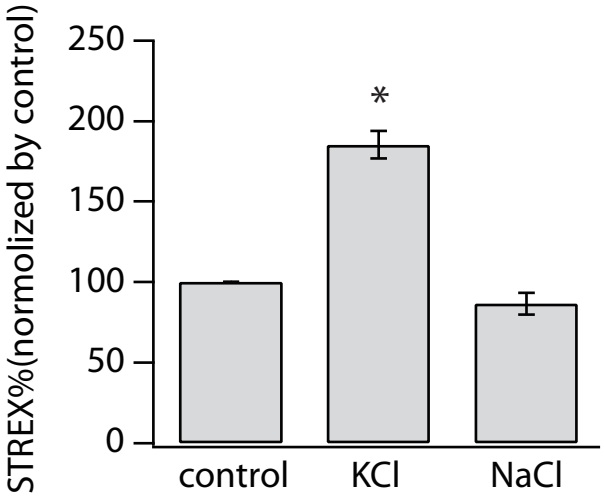


Figure 2

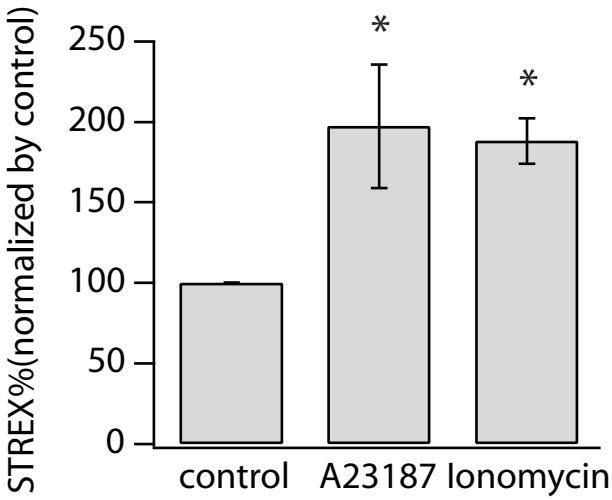
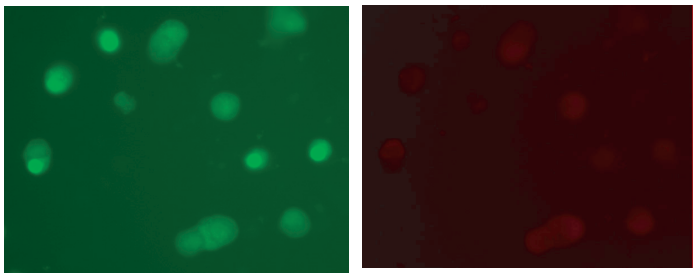


Figure 3



CaMK II

CaMK IV