行政院國家科學委員會補助專題研究計畫成果報告

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※ACTH抗癲癇作用其機制之探討，第2, 3年※
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計畫主持人： 楊千立

共同主持人：

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□國際合作研究計畫國外研究報告書一份

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ACTH抗癲癇作用其機制之探討，第2, 3年

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中文摘要：

雖然醫學界用ACTH來治療點頭癲癇已經有數十年之久，但到目前為止，尚無人能解釋其作用機制。同時，亦無人能解釋，為何這些病人在接受ACTH 治療時，會有厲害的情緒障礙。我們使用細胞電生理的研究方法，直接測試ACTH對於神經細胞電訊傳導底影響。在第一年的報告裡我們已經指出，ACTH對於幼年鼠海馬回之神經傳導有抑制作用。在第二年的實驗裡，我們試著探討ACTH對於amygdala鈣離子電流的影響，結果發現，ACTH會增大鈣離子電流。而其主要作用對象為L-type calcium channel。其作用機制為經由PKA的活化，使得鈣離子通道被磷酸化，進而使鈣離子流量增大。在第三年的實驗裡，我們測試ACTH對於海馬回長程抑制效應的影響，結果發現雖然ACTH會抑制突觸傳導，但是並不影響長程抑制作用的形成。

關鍵詞：腎上腺皮質促素，突觸傳導，突觸可塑性，離子通道，海馬回，杏仁核

Abstract: ACTH was demonstrated to affect animal behavior. We also found that patients with infantile spasm often become irritable when they were receiving ACTH. Several evidences suggested that amygdala may be a target for ACTH to affect behavior and emotion. We studied the effect of ACTH on ion channels of amygdala neurons in the 2nd year of this project, and studied the effect of ACTH on synaptic plasticity, mainly LTD in hippocampus in the 3rd year of the project. We found that by acting through PKA, ACTH augmented L type voltage gated calcium currents in amygdala. But in hippocampus, ACTH inhibits baseline synaptic response, but do not affect low frequency stimuli-induced long term depression.

Key Words: adrenocorticotropic hormone, synaptic transmission, synaptic plasticity, ion channels, hippocampus, amygdala.

Introduction:

During stress or psychiatric disturbance, adrenocorticotropic hormone (ACTH), a 39-amino acid polypeptide, is released from the anterior pituitary in response to corticotropin-releasing factor (CRF) stimulation. ACTH then acts on zona fasciculata cells of the adrenal cortex to activate steroidogenesis and stimulate cortisol secretion (Bondy, 1985). In addition to its hypothalamus-pituitary-adrenal (HPA) axis activating property, ACTH has been implicated in many neural and behavioral functions (Hol et al., 1995; van Rijzingen et al., 1996). ACTH originates from the precursor molecule pro-opiomelanocortin (POMC). POMC is synthesized mainly in the pituitary and to a lesser degree in the neurons of the hypothalamus, the nucleus tractus solitarius and the amygdala (de Wied and Jolles, 1982). After intraventricular administration of ACTH analog, ACTH-radiolabelled cells were observed in the amygdala (Rees et al., 1980). Behavioral studies demonstrated that intra-amygdala administration of ACTH analog counteracted changes in social behavior induced by isolation, an effect comparable to subcutaneous injection (Hol and Spruijt, 1992) suggesting that amygdala is a possible site of action.

The amygdala plays a key role in the modulation of endocrine function, visceral effecter mechanisms and complex patterns of integrated behavior such as defense, aggregation, epilepsy, learning and memory (Gallagher et al., 1981; Racine et al., 1981). A primary model for studying the neural substrates of emotional memory related to the function of the amygdala is Pavlovian fear conditioning (Davis et al., 1994; LeDoux, 1994). During fear conditioning, sensory inputs from the auditory thalamus and cortex reaches the lateral and basolateral amygdala, which in turn project to central nucleus. The central nucleus of the amygdala projects to the several brain areas involved in autonomic and endocrine regulation, such as the paraventricular nucleus of the hypothalamus which links the amygdala directly to the HPA axis (Pitkanen et al., 1997; Maren, 1999). It has been

shown that CRF causes a pronounced facilitation of acoustic startle and an enhancement of Ca++ currents in the amygdala neurons (Liang et al., 1992; Yu and Shinnick-Gallagher, 1998). It is, therefore, of interest to see whether ACTH exerts similar action on brain cells.

Transmitter and hormone release by many secretory cells is tightly coupled to depolarization-dependent Ca++ entry. In pituitary corticotrophs, CRF increases cytosolic Ca++ through voltage-dependent Ca++ channels (VDCCs), and this Ca++ entry is responsible for the CRF-mediated ACTH release from those cells (Guerineau et al., 1991). In adrenocortical cells, ACTH inhibited a noninactivating K+ current leading to depolarization-dependent Ca++ entry and cortisol secretion (Capponi et al., 1984; Enyeart et al., 1996). Cortisol secretion induced by ACTH could be inhibited by antagonists of T-type Ca++ channels, the primary Ca++ channel subtype expressed by these cells (Enyeart et al., 1993). These results suggest that VDCCs are the targets modulated by ACTH, leading to hormone release. In the present study, we analyze the action of ACTH1-24 and ACTH4-10 on the VDCCs in isolated amygdala neurons using patch-clamp recording in a whole-cell configuration.

Materials and Methods

The techniques used to prepare brain slices and cell dissociation were similar to those described previously (Wang et al., 1996). In brief, male Sprague-Dawley rats of 10-18 days old were decapitated and transverse slices (500 μm) were cut from tissue block of the brain using a Vibroslice (Campden Instruments, Silbey, UK). Lateral and basolateral amygdala regions were visualized under a stereomicroscope and dissected from the brain slices with a scalpel. The pieces of brain tissue containing the amygdala were transferred to 10 ml of 1,4-piperazine(ethanesulfonic acid) (PIPES) saline solution in a spinner flask. The temperature was maintained at 32±1 0C and 2-3 mg of protease XIV (Sigma) was added. The amygdalar minislices were stirred gently at a rate sufficient to prevent them from settling, and the solution was bubbled continuously with 95% O2-5% CO2. The PIPES saline solution contained (in mM): NaCl 120, KCl 5, CaCl2 1, MgCl2 1, glucose 25 and PIPES 20, pH 7.0.

One hour later, minislices were washed with 10 ml of PIPES saline solution (3 times), transferred for storage to a holding chamber filled with continuously bubbled PIPES saline solution at room temperature. When needed, a slice was transferred to 1 ml of external solution and the neurons were dissociated by trituration using a fire-polished Pasteur pipette with an 1 mm tip diameter. The suspension of dissociated amygdalar neurons then was transferred to a 0.5-1 ml recording chamber mounted on an Olympus IMT-2 inverted microscope. Neurons were allowed to settle on poly-L-lysine-coated coverslips.

Whole-cell Ca++ currents were recorded using a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA) and filtered at 10 KHz. Patch pipettes were pulled from borosilicate glass and fire polished with a resistance of 2-5 MΩ. The external solution contained (in mM): CaCl2 3, glucose 10, tetraethylammonium hydrochloride (TEA) 120, 4-aminopyridine (4-AP) 5 and PIPES 10; tetrodotoxin (2 μM) was always added. Pipette solution contained (in mM): MgCl2 2, PIPES 10, glucose 10, TEA 20, Cs methanesulphonate 100, EGTA 10, Mg-ATP 4, Na-GTP 0.3, phosphocreatine 20 and leupetine 0.2. All the experiment were performed at room temperature (22-25 0C). After establishing a gigaseal, the membrane underlying the pipette was ruptured by a gentle suction to obtain whole-cell recording. Experimental tests were initiated when the size of ICa became stable which usually took 5 min. When studying the effect of ACTH on the voltage-dependent steady-state activation, we used the voltage ramps and Ba++ as the charge carrier (3 mM CaCl2 was replaced by 5 mM BaCl2 in the external solution).

Online and offline data acquisition and analysis were accomplished using a DigiData 1200 interface (Axon Instruments) between a Axopatch 200B preamplifier and a PC 486 computer using pClamp 6.0 softward program. Current traces (not including those of the current-voltage curves) shown in the figures were the averaged responses of two identical voltage steps elicited consecutively at 7-sec intervals. Statistical significance was determined at the level of $p \leq 0.05$ using paired or unpaired Student's t-tests. All data were expressed as

mean±SEM.

ACTH and its analogues were obtained from Sigma (St. Louis, MO). Nimodipine and ω-CgTX were obtained from Research Biochemicals (Natick, MA). ω-AgTX and TTX were obtained from Calbiochem (La Jolla, CA). ACTH and neurotoxins were dissolved in water and were aliquoted and frozen. Each of the stocks were diluted to the appropriate concentrations in the external solution immediately before the experiment. Nimodipine was dissolved in dimethylsulfoxide (DMSO) and protected from light. Final dilution of nimodipine resulted in DMSO concentration of 0.1%, which when delivered alone had no effect on calcium currents.

Results:

The 2nd Year

The modulation of voltage-dependent calcium currents (I_{Ca}) by ACTH was then studied in rat acutely dissociated amygdala neurons using whole cell patch-clamp recording technique. $ACTH_{4-10}$ increased I_{Ca} in a concentration-dependent manner with a ED_{50} of about 100 nM and a maximal facilitation of about 50%. The enhancement was mimicked by other ACTH analogs suggesting a receptor-mediated mechanism. Among all the fragments tested, $ACTH_{1-24}$ is the most potent one to potentiate Ca^{2+} current.

From the literatures we know that $ACTH_{1-24}$ will keep animals awake, but $ACTH_{18-39}$ will cause them to sleep. In our study we found that $ACTH_{18-39}$ actually can also potentiate Ca^{2+} current but to a lesser degree. $ACTH_{4-10}$ can block its effect, and it can also block the facilitatory effect of $ACTH_{4-10}$. These results suggested that $ACTH_{18-39}$ is actually a agonist. ACTH must act on other receptors or channels to affect sleep pattern.

There are several types of Ca^{2+} channels in amygdala neurons. To see which type was the target of ACTH, we performed the following experiments. Nimodipine (1μM), a selective L-type Ca^{2+} channel blocker, by its own reduced the I_{Ca} by about 30%. Subsequent application of ACTH in the presence of nimodipine failed to produce an enhancement of I_{Ca}. Conversely, application of ACTH increased the I_{Ca} to 153±3% of control and subsequent addition of nimodipine reduced the I_{Ca} back to 68±2% of the initial control. These results suggest that ACTH acts selectively on the L-type channels. Furthermore, ACTH-mediated enhancement of I_{Ca} after exposure to ω-conotoxin-GVIA (ω-CgTX) and ω-agatoxin-IV (ω-AgTX) was not significantly different from that observed in the control neurons, ruling out the involvement of N- and P-/Q- type channels. The effect of ACTH was markedly reduced in neurons intracellularly dialyzed with Rp-cyclic adenosine 3'5'-monophosphothioate (Rp-cAMPS), a regulatory site antagonist of cAMP-dependent protein kinase (PKA). Similarly, a catalytic site antagonist of PKA KT 5720 blocked ACTH-induced I_{Ca} enhancement. Taken together, these results show for the first time that ACTH enhances voltage-dependent Ca^{2+} currents in brain neurons and that this increase is mediated through L-type channels and involves a cAMP-dependent mechanism.

The 3rd year:

Since we found in the 2nd year that ACTH can enhance the voltage gated calcium currents in neurons. And intracellular calcium level is crucial for the expression or establishment of synaptic plasticity. We thus test the effect of ACTH on long-term depression in hippocampus, which is related to the NMDA response and intracellular calcium level.

Extracellular recording was performed in the CA1 region of hippocampus, with the stimulating electrode placed in the stratum radiatum, and the fEPSP was recorded in the stratum radiatum. For steady LTD, 2 trains of low frequency stimulation were given to the slice after a baseline recording of 10 min with a recording frequency of 0.05 Hz. Each train of LFS cosisted of stimuli of the same intensity at 1Hz for 15 min. There was a 15 min recording between these 2 LFS. In control Wistar rats of 4 weeks old, 40 min after the 2nd LFS, the fEPSP was 44.7±4.3% of the basline response.

To see the effect of ACTH on LTD, we first look at its effect on baseline synaptic transmission. After 10 minutes of perfusion, ACTH(1μM) decreased the synaptic response to 64.8±6.4% of baseline. The effect of ACTH on baseline synaptic

transmission diminished 20 min later, despite continuous perfusion of fresh prepared ACTH. By 40 mins after perfusion, the fEPSP almost returned to the baseline level.

In the presence of ACTH (1μM), the 2 train LFS protocal still induced stable LTD. The fEPSP 40 min after 2nd LFS was 44.9±3.5%, not significantly different from the control group.

Discussion

Although there were several studies about the effect of ACTH on ion channels in the literature, but most of them studied the endocrine system. The effect of ACTH on ion channels in the neuron was actually never studied, despite the fact that ACTH has been used to treat patients with infantile spasms, a severe seizure disorder in infants, for several decades. There are theories about how ACTH can suppress the seizure activity, but a conclusion is not yet reached. On the other hand, despite the severe impact of ACTH on the emotion, mood and appetite of these patients, no study has been performed so far to know why.

In the first year of this project, we have demonstrated that ACTH attenuated the synaptic response in hippocampal neurons (report already sumitted). That is the first direct evidence of how ACTH can possibly affect the seizure activity in the brain. In the 2nd year of this project, we try to explore the reason why ACTH can affect the mood so dramatically. Amygdala, the center for the mood, was thus the best target to be studied. Here we showed that ACTH, including various fragments, augmented the voltage gated calcium currents. As calcium is an important messenger for intracellular signaling, it is very likely that ACTH may profoundly affect the signal transduction pathways and the excitability of the neurons. It can be one of the mechanisms why ACTH affects mood and emotion.

We explored deeply to see which type of calcium channel was mainly affected by ACTH. By using pharmacological dissection, we are able to tell that L-type calcium channel is the target of ACTH which after binding to its receptor, activats PKA and phosphorylates the ion channel protein and increases probability of opening.

Since the magnitude of calcium influx and level of intracellular calcium concentration is a very important regulator for the expression of synaptic plasticity. We thus try to determine if ACTH also affects synaptic plasticity in the 3rd year of this project. But in hippocampus CA1 region, the expression of LTD was not affected by ACTH. We also studied the effect of ACTH on LTP in amygdala (data not shown, ongoing study), but again the affect is not significant. The factors to be considered are 1), we don't know whether ACTH also affects the calcium channel in hippocampus. As in different region of the brain, the percentage and subtype of calcium channels may vary; 2), ACTH may also have effects on other channels or neurotransmitter receptors which may act on the opposite way of calcium current so that the net effect of ACTH on synaptic plasticity is not significant. But there are many forms of synaptic plasticity each developes under cirtain condition, we may need to test more conditions to reach a conclusion whether ACTH affects synaptic plasticity in young brain or not.

成果自評：

1. We successfully provided the first evidence that ACTH attenuates synaptic response in hippocampus.
2. This is the first study to show that ACTH augmented L-type calcium current in amygdala, which is the center of emotion and mood.
3. We provided the first clue about why ACTH may affect the behavior and mood of patients with infantile spasms under ACTH treatment.
4. We showed that ACTH does not affect the LTD formation in hippocampus.
5. We still need to test other conditions and regions of the brain to determine how ACTH may act on synaptic plasticity.
6. It is very likely that ACTH may also affect other ion channels or neurotransmitter receptors, and we need to continue in the future project to explore its effects. But due to limitation of facility and time, our initial plan to study the effect of ACTH on K channels was not performed.
7. Part of the results were already published in Molecular Pharmacology, 2000.

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