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Influence of AVP_{4-8} on MAPK activity in astrocytic glial and fetal neuronal cells in primary culture[1]

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KEY WORDS argipressin; hippocampus; cerebral cortex; mitogen-activated protein kinase; signal transduction

ABSTRACT

AIM: To study the effect of argipressin (4 - 8) (AVP_{4-8}) on the mitogen-activated protein kinase (MAPK) activity in astroglial culture and fetal neuronal culture from rat cerebral cortex and hippocampus. Some protein kinases involved in this signal pathway were also addressed. **METHODS**: Rat brain primary cells were cultured in serum free medium or starved for 24 h before use. Cells were transferred to Ca^{2+} and Mg^{2+} free Dulbeco's phosphate buffer (D-PBS) with various drugs. MAPK activity was measured. **RESULTS**: The main findings were: (1) AVP_{4-8} induced the MAPK activity in rat brain astroglial culture but not in fetal neuronal cultures. And this was blocked by ZDC (C) PR, an antagonist of AVP_{4-8}. (2) PD98059, a potent selective inhibitor of MAPK/ERK kinase (MEK) and GF109203X, a specific inhibitor of protein kinase C (PKC) abolished AVP_{4-8}-evoked MAPK activity on astrocytes. **CONCLUSION**: AVP_{4-8} can activate the MAPK activity in astrocytes but not in fetal neuronal culture. MEK and PKC may be involved in the AVP_{4-8}-evoked cascade.

INTRODUCTION

AVP_{4-8} is a metabolite of argipressin (AVP) and has high affinity receptor sites in rat brain[1]. Evidence has demonstrated that AVP_{4-8} induces long term potentiation (LTP) via a non-*N*-methyl-*D*-aspartic acid (NMDA) receptor transmission mechanism[2]. Previous work in our laboratory indicated that AVP_{4-8} could induce the enhancement of MAPK activity *in vivo* or *in vitro* through a pathway mediated by an unknown G-protein coupled receptor (GPCR) and protein PKC[3]. Therefore, it is indisputable that AVP_{4-8} has an effect on the brain function of adult animals.

Recent studies[4] suggested that MAPK cascade in neurons and astrocytes may play different roles in cellular physiological function. It is interesting to investigate the effect of AVP_{4-8} on these two cell types and its possible mechanism. In the present work, the action of AVP_{4-8} on the MAPK activity of primary cultures was detected and the possible signal pathway was investigated.

MATERIALS AND METHODS

Materials Sprague-Dawley rats (0 - 3 d old; Grade II, Certification № 005) of either sex were from Shanghai Experimental Animal Center, Chinese Academy of Sciences. Peptides pyroglutamylasparaginylcystinylprolylarginine (AVP_{4-8}) and pyroglutamylaspartylcystinylprolylarginine [ZDC (C) PR] were synthesized with solid-phase method and purified to > 98 % purity by HPLC. [γ-^{32}P] ATP (specific activity, 110 PBq/mol) was purchased from Amersham (UK). Myelin basic protein (MBP) was from Sigma (St Louis Mo). PD98059 and GF109203X were from Calbiochem (San Diego). All the other chemicals were of AR.

Cell culture and treatment Primary astrocytes were obtained from neonatal rat cerebral cortex and hippocampus according to McCarthy and de Vellis[5] (1980). The cells were kept in DMEM supplemented with 10 % FCS, penicillin 100 kU/L and gentamicin 100 kU/L, streptomycin 125 kU/L and grown in 5 % CO_2/95 % air at 37 ℃. The cells grew to confluenoy in about two weeks. Purified astrocytes culture were obtained by the

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following procedure: oligodendrocytes and microglial cells growing on top of astrocyte monolayer were removed by shaking the culture flask on a rotary shaker overnight at 250 rpm at 37 ℃. Detached cells were removed. The culture was then subjected to 0.25 % trypsin. The resulting cell suspension was centrifuged and resuspended in DMEM with 10 % FCS until it was confluent. For investigating the effect of AVP_{4-8}, the cultures were kept in medium containing 0.5 % FCS for 24 h before the addition of stimulant.

Neurons were prepared from the hippocampus and cerebral cortex of 17-d old rat embryos and cultured in 2 % B27, 0.5 $\mu mol \cdot L^{-1}$ glutamine in neurobasal medium. The neurons were cultured for 7 d before used.

Cells were rinsed with D-PBS, then transferred to Ca^{2+} and Mg^{2+} free D-PBS containing stimulant.

Preparation of cell extracts The stimulation was stopped by aspirating the medium, addition of 0.20 mL ice-cold lysis buffer (containing sucrose 250, Na_3VO_4 4, DTT 1, PMSF 0.5 $mmol \cdot L^{-1}$, pH 7.4), followed by immediate freezing with liquid nitrogen. After thawing on ice, the cells were sonicated for 30 s. The cytosolic fraction was obtained by centrifugation at 10 000 × g for 60 min and stored at −70 ℃ for assays of MAPK activity. Proteins were determined using the Lowry procedure.

MAPK activity assay[6,7] The MAPK activity was determined by the phosphorylation of the MBP. The reaction was performed at 30 ℃ with 3 μg protein in a final volume of 30 μL containing (final concentration), HEPES 10, DTT 1, $MgCl_2$ 10, egtazic acid 2 $mmol \cdot L^{-1}$, MBP 1 $g \cdot L^{-1}$ pH 7.0. Preincubated for 1 min, reactions were initiated by the addition of [γ-^{32}P] ATP 18.5 kBq. After a 5 − 10-min incubation, reactions were halted by adding SDS-PAGE sample buffer and subjected to 10 % polyacrylamide gel electrophresis in the presence of 0.1 % SDS.

Statistical evaluation *Data were expressed as* $\bar{x} \pm s$ *and compared by* t *test.*

RESULTS

Activation of MAPK by AVP_{4-8} in rat brain astrocytes but not in fetal neuronal culture After treatment with AVP_{4-8}, the MAPK activity of cells was measured by the phosphorylation of the specific substrate MBP. Time course studies showed that AVP_{4-8} enhanced MAPK activity of astrocytes and exerted maximal effect at 5 min, and returned to the basal level by 30 min (*Fig 1*). AVP_{4-8} *10 nmol* $\cdot L^{-1}$ had the similar effect to 500 nmol $\cdot L^{-1}$ concentration, indicating that 10 nmol $\cdot L^{-1}$ was adequate to induce the maximal response. The maximal stimulation on cortical astrocytes was 2.46 ± 0.24 ($n = 4$, $P < 0.01$ *vs* control) while that of hippocampal was 2.10 ± 0.20 ($n = 4$, $P < 0.01$ *vs* control). The MAPK activity of fetal neuronal culture seemed not to be influenced by the addition of AVP_{4-8} regardless of the cell source.

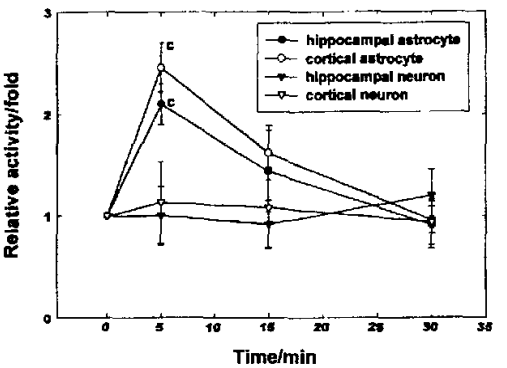

Fig 1. MAPK activity in the neuronal cells and astrocytes from cortex and hippocampus after incubation with AVP_{4-8} 100 nmol $\cdot L^{-1}$. Relative activities were presented by the optical density of autoradiogram after SDS-PAGE electrophoresis of phosphorylated MBP, and expressed as induction fold above the levels found in control (0 min). $n = 4$ assays. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Blocking effect of ZDC (C) PR MAPK activity was evaluated after treatment with ZDC (C) PR, AVP_{4-8}, or their combination. ZDC (C) PR, an antagonist with Asp2 replacement of AVP_{4-8}, had no effect on the cellular MAPK activity but blocked the AVP_{4-8}-enhanced MAPK activity in hippocampal and cortical astrocytes [Fig 2, AVP_{4-8} + ZDC (C) PR (5 min) *vs* AVP_{4-8}(5 min), $P < 0.01$]. The maximal effect induced by AVP_{4-8} at a 5-min interval could be reduced to 1.22 ± 0.11 and 1.33 ± 0.19, respectively (Fig 2, $P > 0.05$ *vs* control) in the presence of 50-fold ZDC (C) PR.

Upstream regulation of MAPK in astrocytes According to our previous results[3], the putative receptor of AVP_{4-8} is a member of GPCR and the signaling from GPCR to the MAPK (ERK) is preceded by several distinct pathways including PKC-dependent or -independ-

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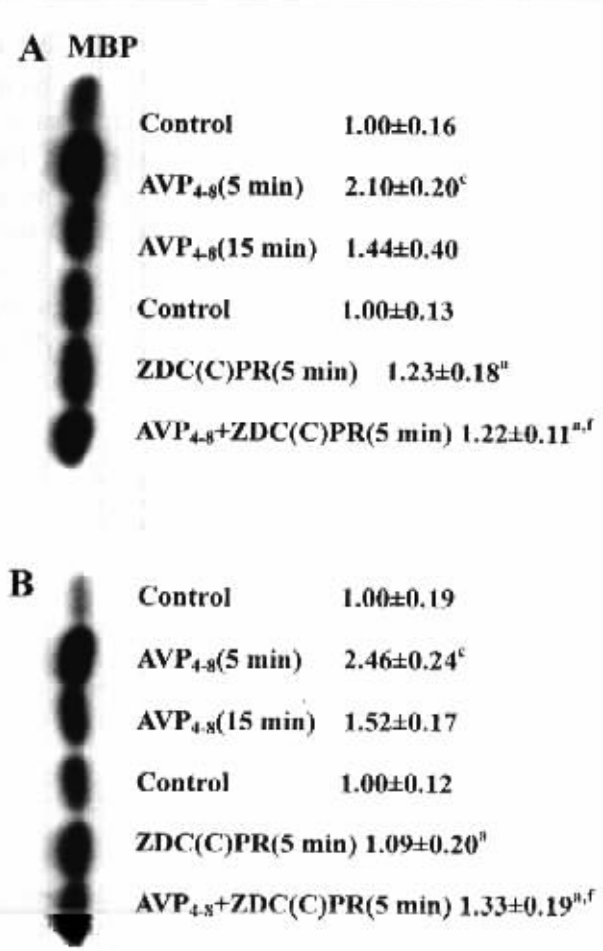

Fig 2. Effect of ZDC (C) PR on MAPK activity in hippocampal astrocytes (A) and cortical astrocytes (B) incubated with AVP_{4-8}, ZDC (C) PR, or their combination. Autoradiogram of MBP is illustrated in the lower panel. Relative activity was expressed as fold *vs* control. $n = 3$ assays. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control; $^fP < 0.01$ *vs* AVP_{4-8} (5 min).

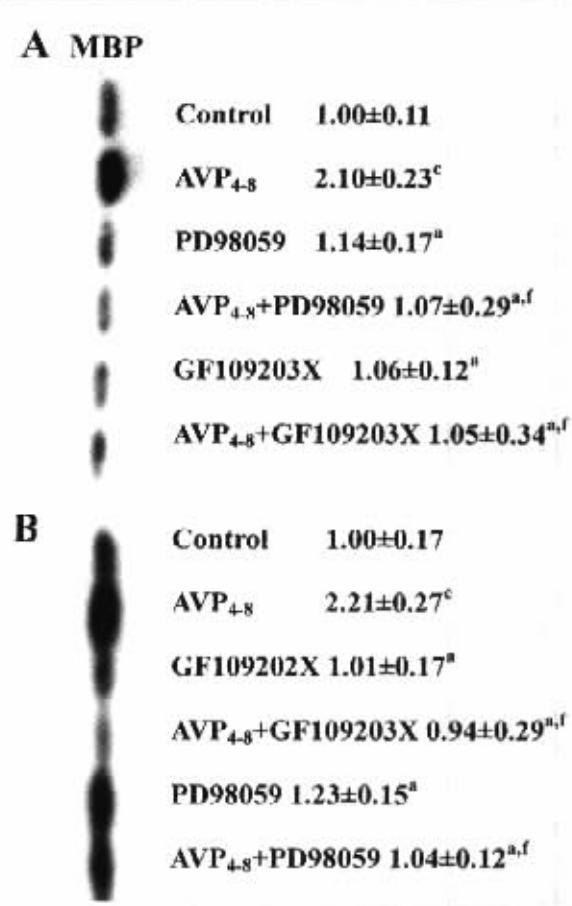

Fig 3. Effects of PD98059 and GF109203X on AVP_{4-8}-evoked maximal MAPK activity in hippocampal (A) and cortical astrocytes (B) in the presence of AVP_{4-8}: Autoradiogram of MBP is illustrated in the lower panel. Relative activity was expressed as fold *vs* control. $n = 4$ assays, $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control; $^fP < 0.01$ *vs* AVP_{4-8} (5 min).

ent pathways. In order to determine whether PKC was involved in the MAPK signaling induced by AVP_{4-8}, GF109203X was used to treat astrocytes before the application of AVP_{4-8} for 0.5 h to down-regulate PKC activity. The result indicates that the pretreatment of GF109203X could completely block the AVP_{4-8}-evoked MAPK activity (Hippocampal astrocyte: 1.05 ± 0.34 *vs* 2.10 ± 0.23; Cortical astrocyte: 0.94 ± 0.29 *vs* 2.21 ± 0.27, all $P < 0.01$).

MAPK has several subfamilies, each of which have distinct pathways. To determine which kind of MAPK cascade has been followed by AVP_{4-8}, we examined the inhibitory ability of PD98059 on AVP_{4-8}-evoked MAPK pathway. As shown in Fig 3, PD98059 50 $\mu mol \cdot L^{-1}$ abolished the increase of MAPK activity stimulated by AVP_{4-8} (Hippocampal astrocyte: 1.07 ± 0.29 *vs* 2.10 ± 0.23; Cortical astrocyte: 1.04 ± 0.12 *vs* 2.21 ± 0.27, all $P < 0.01$). It implies that the MAPK kinase in this cascade is MEK. Generally, the AVP_{4-8}-evoked MAPK cascade in astrocytes is mediated by PKC and MEK.

DISCUSSION

The main findings of our studies presented here are that (1) AVP_{4-8} can activate mitogenic signaling pathway (via ERK) in hippocampal and cortical astrocytes through its specific receptor, which can be specifically competed by ZDC (C) PR, an antagonist of AVP_{4-8} in hippocampal and cortical astrocytes but not in fetal neural culture. (2) Signaling from putative AVP_{4-8} receptor to MAPK in astrocytes is dependent on PKC and MEK.

It has been reported that AVP_{4-8} can induce the enhancement of MAPK activity in the brain of adult rat

through its specific receptor[7]. However, it is unknown how the neuron and astrocytes, responded to the stimulation of AVP_{4-8}. Evidence presented here indicated that the MAPK activity in astrocyte was enhanced by AVP_{4-8} in a receptor-specific way, suggesting that the putative AVP_{4-8} receptor also existed in astrocytes. To our knowledge, MAPK cascade in astrocytes mainly has relationship with mitogenic signaling whereas neural culture predominantly plays a role in the formation of LTP, neural plasticity, differentiation, and so on. According to this, the influence of AVP_{4-8} on MAPK activity in these two cell types may has a different function. Unfortunately, the stimulation by AVP_{4-8} on the fetal neuronal culture failed to induce the change in MAPK activity. The main explanation is that the fetal neuronal culture is not as well-differentiated as the adult culture so it is devoid of some responsibility to AVP_{4-8}, since the AVP_{4-8} binding sites in hippocampal neuron do not appear until 5 d after birth according to autoradiograph experiments[1].

So far, the role of PKC in GPCR/ MAPK (ERK) signaling pathway is not well-established. In some cases, GPCR can be coupled to ERK by both PKC-dependent and -independent pathways suggesting that there existed a multiple signaling receptor to ERK. Our previous work[3] implicated the involvement of PKC in signaling for the putative GPCR of AVP_{4-8} to ERK in hippocampus. Consistent with it, GF109203X, an inhibitor of both Ca^{2+}-dependent and -independent PKC isoforms, displays very obvious inhibitory effects on the AVP_{4-8}-evoked MAPK (ERK) activity in astrocytes. In addition, the pretreatment with PD98059, a specific inhibitor of MEK, can cancel the AVP_{4-8}-evoked activation of MAPK (ERK). The results provide evidence that AVP_{4-8} stimulates a signaling cascade involving MAPK kinase or a MAPK kinase-related protein that subsequently activates ERK. It suggests AVP_{4-8} may play more roles in cellular function than we have known. Furthermore, the regulation of some genes such as nerve growth factor (NGF) may be regulated by this mitogenic signaling pathway.

Generally, our study indicated that an AVP_{4-8}-evoked, receptor-specific, PKC-dependent MAPK pathway existed in astrocytes.

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精氨酸升压素(4－8)对原代培养星状胶质细胞及胚胎神经元细胞中 MAPK 活性的影响[1]

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关键词 精氨酸升压素；海马；大脑皮质；促细胞分裂剂活化的蛋白激酶；信号传递

目的：研究精氨酸升压素(4－8) (AVP_{4-8})对来自大鼠大脑皮层和海马的原代培养星状胶质细胞及胚胎神经元细胞中促细胞分裂素活化的蛋白激酶(MAPK)活性变化的影响．同时探讨了这种影响经由的信号传递途径中可能牵涉的蛋白激酶．**方法**：来自大鼠脑的原代细胞培养于无血清培养基(胚胎神经元细胞)或于给药前无血清饥饿 24 h，转入加药物的无钙镁磷酸缓冲液中温育，测定 MAPK 激酶活性．**结果**：(1) AVP_{4-8}能刺激原代培养的星状胶质细胞中 MAPK 活性但对胚胎神经元中 MAPK 活性没有影响．AVP_{4-8}在星状胶质细胞中诱导的 MAPK 活性增强作用可被 AVP_{4-8}的拮抗剂 ZDC(C)PR 阻断．(2) MEK (MAPK/ERK 激酶)的选择性抑制剂 PD98059 以及 PKC 专一性抑制剂 GF109203X 能消除星状胶质细胞中 AVP_{4-8}诱导的活性增强作用．**结论**：AVP_{4-8}能以受体特异的方式激活星状胶质细胞中的 MAPK 活性，MEK 和 PKC 这两个激酶参与了这个 MAPK 信号传递途径．AVP_{4-8}不影响原代培养胚胎神经元中的 MAPK 活性．

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