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Involvement of a putative G-protein-coupled receptor and a branching pathway in argipressin (4-8) signal transduction in rat hippocampus[1]

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KEY WORDS argipressin; vasopressins; G-proteins; vasopressin receptors; hippocampus; protein kinases; signal transduction

AIM: To study the signal transduction pathway induced by argipressin (4-8) (AVP_{4-8}) in rat hippocampus. **METHODS**: Rat hippocampi were sectioned transversely at 300 μm with a tissue chopper and transferred to fresh incubation solution circulated with a humidified gas mixture of 95 % O_2 + 5 % CO_2 at 36 ± 0.5 ℃. After incubation with various drugs, MAP kinase (MAPK) activity and Ca^{2+}/calmodulin-dependent protein kinase Ⅱ (CaMKⅡ) autophosphorylation were measured. **RESULTS**: The main findings are: (1) The AVP_{4-8}-stimulated MAPK activity and the CaMKⅡ autophosphorylation were blocked by ZDC(C)PR, an antagonist of AVP_{4-8}, and also completely inhibited by pertussis toxin, a selective inhibitor of the G-protein-coupled receptor (GPCR). But, AVP-induced MAPK activation was not sensitive to ZDC(C)PR or PTX. (2) Polymyxin B (PMB), an inhibitor of protein kinase C (PKC), markedly suppressed the peptide-activation of MAPK, but did not affect CaMKⅡ autophosphorylation. Phorbol myristate acetate (TPA), an activator of PKC, elicited an increase of MAPK activity, but did not further influence the level of AVP_{4-8}-enhanced MAPK activity; Nevertheless, the extent of CaMKⅡ activation was attenuated by TPA. (3) The enhancement of MAPK activity was not reduced by KN-62, a specific inhibitor of CaMKⅡ. (4) AVP_{4-8} did not show any influence on cAMP production. **CONCLUSION**: AVP_{4-8} stimulated signal transduction via a GPCR and a branching pathway in rat hippocampus.

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Argipressin(4-8), as a metabolite of argipressin (AVP) and namely AVP_{4-8}, has been found in animal brain to have a high affinity receptor in rat hippocampus[1]. The distribution pattern of AVP_{4-8} binding sites found in rat brain by autoradiography[1] and binding assay[2] are distinct from that of AVP and AVP_{4-8} is devoid of the peripheral effects of AVP, but much more potent than AVP in facilitating the acquisition and maintenance of learning and memory in rats[3], in potentiating synaptic transmission[4] and in enhancing the accumulation of the second messenger IP_3 in rat hippocampal slices[5]. Therefore, AVP_{4-8} is thought to be a new neuropeptide with certain effects different from those of AVP.

It has been reported that AVP-induced activation of 42 kDa mitogen-activated protein kinase (p42 MAPK) was mediated through protein kinase C (PKC) in vascular smooth muscle cells[6]. In the nervous system, AVP enhanced cell growth or other cell response via V_1 receptor which coupled to G_q protein[7]. In order to answer the question of the receptor involved in the AVP_{4-8} signaling pathway, in the present work, the changes of MAPK activity and Ca^{2+}/calmodulin-dependent protein kinase Ⅱ (CaMKⅡ) phosphorylation[8] were estimated and the relationship between AVP and AVP_{4-8} signaling pathway was investigated.

MATERIALS AND METHODS

Materials Wistar rats ♂ weighing 150 g were provided by Shanghai Institute of Radiomedicine, China. 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^{-1}) was purchased from Amersham (UK). AVP, tetradecanoylphorbol acetate (TPA), polymyxin B (PMB), myelin basic protein (MBP), and protein kinase inhibitor peptide were from Sigma (St Louis MO). Leupeptin and aprotinin were from Fisher (Fair Lawn NJ). Pertussis toxin (PTX) was from Gibco (Grand Island NY). Cyclic AMP RIA assay kit was

from Shanghai Second Medical University. KN-62 was a generous gift from Prof HIDAKA Hiroyoshi, Nagoya University School of Medicine, Japan. All the other chemicals were of AR.

Hippocampus slicing and stimulation Rat brain was rinsed with cold (on ice) incubation solution A (containing final concentration of NaCl 124, KCl 3, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 2, $NaHCO_3$ 26, and glucose 10 mmol·L^{-1}, pH 7.4) and dissected. Isolated hippocampi were sectioned vertically at 300 μm with a tissue chopper and transferred to fresh solution A gassed with 95 % O_2/5 % CO_2 at 36 ± 0.5 ℃. After pre-incubating the slices for 20 – 30 min, fresh solution A pre-warmed at 36 ± 0.5 ℃ was added. Incubation was carried out for a further 25 min before the addition of effectors.

Preparation of tissue extracts The stimulation was stopped by aspirating the buffer, addition of 0.5 mL·ice-cold lysis buffer (containing sucrose 250, Tris-HCl 10, $Na_4P_2O_7$ 10, NaF 100, edetic acid 5, egzatic acid 5, Na_3VO_4 4, DTT 1, PMSF 0.5 mmol·L^{-1} and leupeptin 10, aprotinin 100 mg·L^{-1}, pH 7.4), followed by immediate freezing with liquid nitrogen. After thawing on ice, the slices were sonicated for 30 s. The cytosolic fraction was obtained by centrifugation at 100 000 × g for 60 min and stored at – 70 ℃ for assays of MAPK activity and CaMK Ⅱ autophosphorylation. Proteins were determined using the Bradford microassay procedure.

MAPK activity assay The MAPK activity was determined by the phosphorylation of the specific substrate $MBP^{[6,9]}$. The reaction was performed at 30 ℃ with 3 μg protein in a final volume of 30 μL containing (final concentration) pH 7.0, HEPES 10, DTT 1, $MgCl_2$ 10, egtazic acid 2 mmol·L^{-1}, MBP 1 g·L^{-1}, and cAMP-dependent protein kinase inhibitor (PKI) 0.25 μmol·L^{-1}. Pre-incubated for 1 min, reactions were initiated by the addition of [γ-^{32}P]ATP 18.5 kBq. After 10-min incubation, reactions were halted by adding SDS-PAGE sample buffer and subjected to 15 % polyacrylamide gel electrophoresis in the presence of 0.1 % SDS[9].

Autophosphorylation of CaMK Ⅱ The autophosphorylation of CaMK Ⅱ was carried out as a standard 2-min assay (30 μL) without an exogenous substrate, and at 30 ℃ in reaction buffer of (final concentration) HEPES 10, $MgCl_2$ 10, $CaCl_2$ 2.5, DTT 0.5 mmol·L^{-1}, and [γ-^{32}P]ATP 18.5 kBq. The amount of α and β subunits was densitometrically determined after exposing the 10 % SDS-PAGE samples to Kodak X-ray film for 24 h.

Estimation of cAMP production After stimulated for 10 min, hippocampal slices were chilled on ice by the addition of 1/1 (vol/vol) perchloric acid (1 mmol·L^{-1}) and spun at 12 000 × g for 5 min. Supernatants were neutralized to pH 7.4, then spun at 12 000 × g for 5 min before cAMP generation was quantified. Aliquots of extract were used for the determination of cAMP by the [^{3}H]cAMP RIA assay kit.

Statistical evaluation Data were compared by t test.

RESULTS

Activation of MAPK and CaMK Ⅱ by AVP_{4-8} in rat hippocampal slices Exposure of rat hippocampal slices to AVP_{4-8} increased MAPK activity and CaMK Ⅱ autophosphorylation, as measured by the phosphorylation of the specific substrate MBP and in the absence of an exogenous substrate, respectively. The concentration of AVP_{4-8} required for maximal responses was 10 nmol·L^{-1}, and the maximal stimulation was 1.93 ± 0.18 times (P < 0.01 *vs* control) at 5 – 10 min for MAPK activity, 2.15 ± 0.14 times (P < 0.01 *vs* control) at 2 – 5 min for CaMK Ⅱ autophosphorylation, with both returned to control values by 30 min (Fig 1).

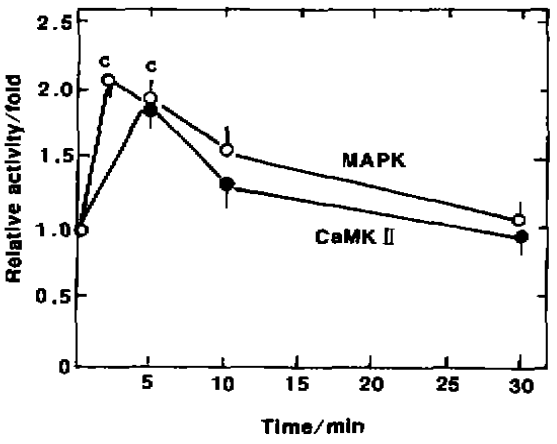

Fig 1. MAPK and CaMK Ⅱ autophosphorylation in rat hippocampal slices after incubation with AVP_{4-8} 10 nmol·L^{-1}. Relative activities were represented by the optical density of autoradiogram after SDS-PAGE electrophoresis of phosphorylated MBP and CaMK Ⅱ α subunit, and expressed as induction fold above the levels found in control (0 min). n = 3 assays. $\bar{x} \pm s$. ^cP < 0.01.

Blocking effects of ZDC(C)PR and pertussis toxin (PTX) treatments on kinase activation MAPK activity and CaMK Ⅱ autophosphorylation level were evaluated after treatment with ZDC(C)PR, AVP_{4-8}, PTX, or their combination. ZDC(C)PR (500 nmol·L^{-1}), an antagonist with Asp2 replacement of AVP_{4-8}, while it *per se* has no effect on MAPK activity and CaMK Ⅱ autophosphorylation levels, nevertheless did markedly blocked the AVP_{4-8}-enhanced protein kinases activa-

tion ($P < 0.05$ *vs* AVP_{4-8} group) when present in the hippocampal slices culture medium. In consistent with the stimulation of MAPK in vascular smooth muscle cells[5], the concentration of AVP required for maximal responses in rat hippocampal slices was 100 nmol·L^{-1}. However, 50-fold of ZDC(C)PR (5 μmol·L^{-1}) had no effect on the AVP-induced MAPK activation (Fig 2).

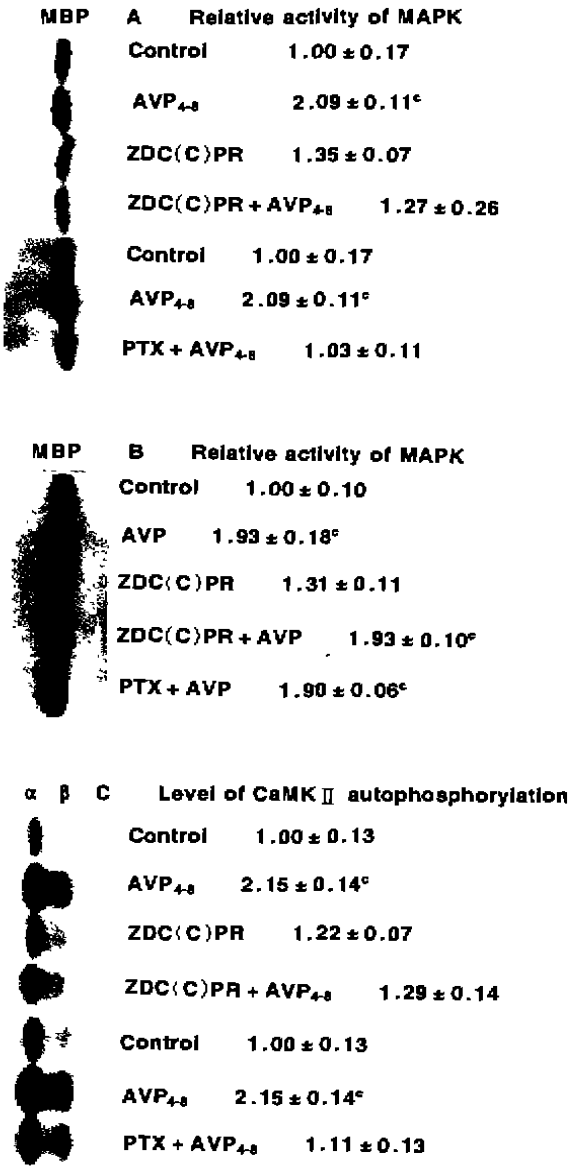

Fig 2. Effects of ZDC(C)PR and PTX on MAPK activities (A, B) and CaMK II autophosphorylation (C) in hippocampal slices incubated with AVP (B) or AVP_{4-8} (A, C). Autoradiogram of MBP and CaMK II α subunit is illustrated in the below panel. Relative activity was expressed as fold *vs* control. $n = 4$ assays. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$.

To see if a subfamily of AVP_{4-8} receptor was involved, the hippocampal slices were pre-incubated with PTX 100 μg·L^{-1} (pre-activated at 30 ℃ for 30 min in DTT 50 mmol·L^{-1}-NaCl 0.05 mol·L^{-1}-sodium phosphate 0.01 mol·L^{-1}, pH 7.0) for 30 min before the exposure to AVP_{4-8} (10 nmol·L^{-1}). The phosphorylation of MBP (Fig 2A and 2B) revealed that AVP_{4-8}-(but not AVP-)induced MAPK activation was sensitive to PTX treatment, because AVP_{4-8} could no longer enhance the protein phosphorylation after pre-treatment of slices with PTX, but AVP did. Furthermore, PTX also markedly inhibited the level of CaMK II autophosphorylation enhanced by AVP_{4-8} (Fig 2C).

Upstream regulation of MAPK and CaMK II To elucidate the role of PKC in the PI signaling pathways stimulated by AVP_{4-8} (PTX-sensitive) and by AVP (PTX-insensitive), the effects of the PKC activator TPA and its inhibitor PMB on MAPK and CaMK II were examined. As shown in Fig 3, there was a threshold in the enhancement of MAPK activity stimulated by AVP_{4-8}, AVP, and TPA. Treatment of hippocampal slices with TPA (1 μmol·L^{-1}) for 5 min elicited an increase of MAPK activity. Nevertheless, stimulation by AVP_{4-8} or AVP, together with TPA was not statistically different from that by each agent alone. The treatment with PMB 100 μmol·L^{-1} for 5 min before exposure to the peptides completely abolished MAPK activation both by AVP_{4-8} (Fig 3A) and AVP (Fig 3B).

The extent of CaMK II autophosphorylation induced by AVP_{4-8} was attenuated by the simultaneous activation of PKC with TPA, but did not affected by PMB treatment (Fig 3C).

Effect of KN-62 on peptide-activation of protein kinases KN-62 is a relatively selective inhibitor of CaMKII[10]. The experiments were performed through pre-incubating hippocampal slices with KN-62 0.5 μmol·L^{-1} for 5 min, followed by the addition of AVP_{4-8} 10 nmol·L^{-1} for further incubation. Densitometry scanning of the autophosphorylation showed that the total density of α and β subunits of CaMKII enhanced by AVP_{4-8} stimulation was completely blocked by KN-62 (Fig 4A). The simultaneous measurement of MAPK activity indicated that the inhibition of CaMKII activity by KN-62 did not influence the activation of MAPK by AVP_{4-8} (Fig 4B).

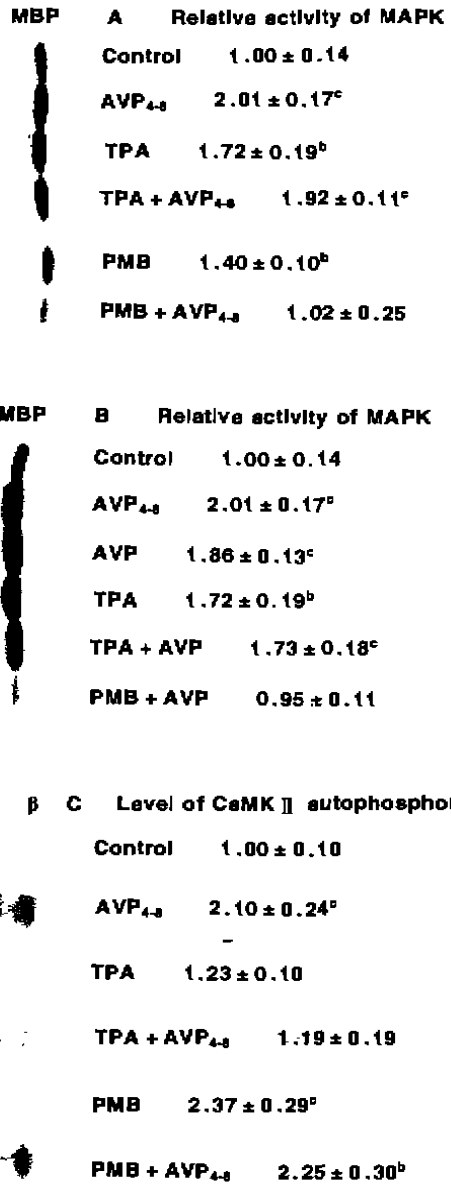

Fig 3. Effects of TPA and PMB on MAPK activity (A, B) and CaMKⅡ autophosphorylation (C) in hippocampal slices in the presence of AVP (B) or AVP_{4-8} (A, C). Autoradiogram of MBP and CaMKⅡ α subunit is illustrated in the below panel. Relative activity was expressed as fold *vs* control. $n = 4$ assays, $x \pm s$, $^bP < 0.05$, $^cP < 0.01$.

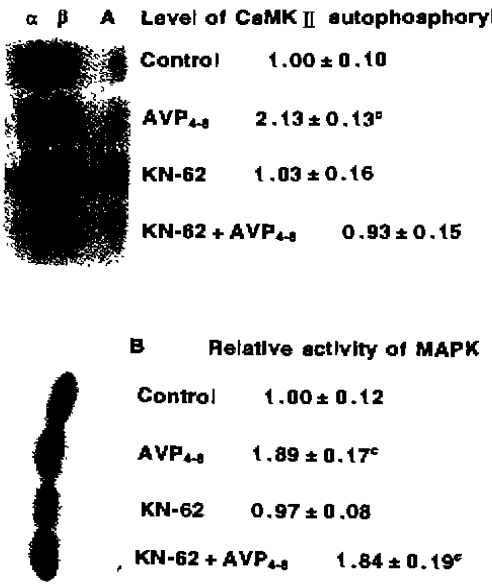

Fig 4. Effect of KN-62 on CaMKⅡ autophosphorylation (A) and MAPK activity (B) in hippocampal slices in the presence of AVP_{4-8}. Autoradiogram of MBP and CaMKⅡ α subunit is illustrated in the below panel. Relative activity was expressed as fold *vs* control. $n = 4$ assays, $x \pm s$. $^bP < 0.05$, $^cP < 0.01$.

DISCUSSION

In this study we have examined the peptide-activation of MAPK and CaMKⅡ in rat hippocampal slices. Our results indicated that AVP_{4-8} activated mitogenic signaling pathway (via p44 MAPK) and CaMKⅡ autophosphorylation through its specific receptor which was specifically competed by ZDC(C)PR, an antagonist of AVP_{4-8}. Moreover, AVP_{4-8} signaling pathway could be completely blocked by PTX, a selective inhibitor of G-protein-coupled receptor (GPCR). It was suggested that AVP_{4-8} receptor coupled to G-protein sensitive to PTX but might not be a known AVP receptor, since AVP-stimulated MAPK activation was not sensitive to PTX (Fig 2B).

It is well known that several second messenger pathways are activated in response to PTX-sensitive receptor, which include G_0-activated IP_3 and G_i-activated cyclic AMP pathways. Since AVP_{4-8} signaling in rat brain was mediated by IP_3[5] and no detectable change of the cAMP concentration could be found in hippocampal slices in the presence of AVP_{4-8} in this work, while forskolin ($100\ \mu mol \cdot L^{-1}$) significantly increased cyclic AMP generation (2.2 ± 0.3, $P < 0.05$, $n = 3$). It is believed that in rat hippocampus, the putative G-protein coupled to AVP_{4-8} receptor may be G_0, not PTX-sensitive G_i nor PTX-insensitive G_q.

Other evidence that G_0 was involved in AVP_{4-8} signaling pathway comes from the comparison of

AVP_{4-8} with AVP signal transduction. The activation of MAPK by AVP_{4-8} occurred via a PKC-dependent pathway. This was similar to AVP[6]. In nerve cells, AVP enhanced cell growth or other cell response via V_1 receptor which coupled to G_q protein[7]. The GTP-bound-subunit of the G_q protein stimulated phosphoinositide hydrolysis[11] and consequent PKC activity was irresponsive to PTX[12]. Actually, in our experiment, AVP-induced MAPK activation was not blocked by ZDC(C)PR and PTX. This is in contrast to AVP_{4-8}. Furthermore, studies on the synaptic transmission have demonstrated that AVP_{4-8} induced long-term potentiation (LTP) via a non-NMDA receptor mechanism with a much lower dose than AVP[3]. In rat hippocampus, tetanus-induced LTP was sensitive to PTX by a presynaptic mechanism, this G-protein was postulated to be G_0 protein[13]. Therefore, the receptor of AVP_{4-8} must be different from that of AVP. It is specific to AVP_{4-8} and may be coupled to G_0.

Interestingly, a negative cross-talk was found between PKC and CaMK Ⅱ during the stimulation of the phosphatidylinositol signaling system with AVP_{4-8}. As a PKC activator, TPA suppressed peptide-stimulation of CaMK Ⅱ while PMB, the well known PKC inhibitor facilitated CaMK Ⅱ autophosphorylation. A reasonable explanation for this phenomena is that PKC (MAPK) and CaMK Ⅱ are located in two separate branches of AVP_{4-8} signaling pathway. In the PTX-sensitive signaling pathway induced by AVP_{4-8}, activation of MAPK is mediated by PKC and there is a positive regulation between them, while AVP_{4-8} stimulates CaMK Ⅱ autophosphorylation by another independent pathway which is negatively regulated by PKC. Therefore, AVP_{4-8} signaling pathways from its receptor and IP_3 to CaMK Ⅱ and to MAPK were thought to be of two branches reaching two effectors, LTP and gene expression. The expression of some genes, such as NGF[14] and BDNF (Zhou *et al*, in preparation) may be regulated by this mitogenic signaling pathway.

In general, present study demonstrates that an unknown GPCR and G_0 protein mediate the branching signaling pathway induced by AVP_{4-8} in rat hippocampus.

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REFERENCES

1 Du YC, Guo NN, Chen ZF. Autoradiographic approach to the developmental study on the binding sites of AVP_{4-8} in rat hippocampus. Acta Physiol Sin 1994; 46: 435 - 40.

2 Du YC, Wu JH, Jiang XM, Gu YJ. Characterization of binding sites of a memory-enhancing peptide AVP(4 - 8) in rat cortical synaptosomal membranes. Peptides 1994; 15: 1273 - 9.

3 Lin C, Liu RY, Du YC. Cysteinyl methyl ester of AVP(4 - 8), a potent agonist on the maintenance of passive avoidance in rats. Peptides 1990; 11: 633 - 9.

4 Rong XW, Chen XF, Du YC. Potentiation of synaptic transmission by neuropeptide AVP_{4-8} (ZNC (C) PR) in rat hippocampal slices. NeuroReport 1993; 4: 1135 - 8.

5 Gu BX, Du YC. Arginine-vasopressin C-terminal peptide stimulates inositol phospholipid metabolism in rat hippocampus. Chin J Biochem Biophys 1992; 24: 287 - 94.

6 Kribben A, Wieder ED, Li X, Putten VV, Granot Y, Schrier RW, *et al*. AVP-induced activation of MAP kinase in vascular smooth muscle cells is mediated through protein kinase C. Am J Physiol 1993; 265 (4 Pt 1): C989 - 45.

7 Brinton RD, Monreal AW, Fernandez JG. Vasopressin-induced neurotrophism in cultured hippocampal neurons via V_1 receptor activation. J Neurobiol 1994; 25: 380 - 94.

8 Hanson PI, Schulman H. Neuronal Ca^{2+}/Calmodulin-dependent protein kinases. Annu Rev Biochem 1992; 61: 559 - 602.

9 Qiao LY, Du YC. Increase of mitogen-activated protein kinase activity in rat brain after injection of argipressin(4 - 8). Acta Pharmacol Sin 1997; 18: 380 - 4.

10 Tokumitsu H, Chijiwa T, Hagiwara M, Mizutani A, Terasawa M, Hidaka H. KN-62, 1-[*N*, *O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-*L*-tyrosyl]-4-phenylpiperazine, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase Ⅱ. J Biol Chem 1990; 265: 4315 - 20.

11 Wu D, Lee CH, Rhee SG, Simon MI. Activation of phospholipase C by the α-subnits of the G_q and G_{11} proteins in transfected cos-7 cells. J Biol Chem 1992; 267: 1811 - 7.

12 Smrcka AV, Hepler JR, Brown KO, Sternweis PC. Regulation of polyphosphoinositide-specific phospholipase C activity by purified G_q. Science 1991; 251: 804 - 7.

13 Goh JW, Pennefather PS. A pertussis toxin-sensitive G protein in hippocampal long-term potentiation. Science 1989; 244: 980 - 3.

14 Zhou AW, Guo J, Du YC, Chen XF. Effects of the AVP_{4-8} analogs on NGF mRNA transcription in rat brain. Chin J Physiol Sci 1996; 12: 81 - 6.

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