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Indirect neurotrophic effect of neuropeptide ZNC(C)PR on PC12 cells via peptide-stimulation of C6 cells[1]

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KEY WORDS argipressin; C6 cells; binding sites; PC12 cells; cell differentiation; conditioned culture media; radioligand assay; neurites

ABSTRACT

AIM: To understand the mechanism of neurotrophic action of neuropeptide ZNC(C)PR and its effect on which could affect both growth and apoptosis of C6 cells. **METHODS**: Effects of ZNC(C)PR-treated C6 conditioned medium was observed on on culture of PC12 cells. The development of PC12 cells was determined by ratio of neurite-bearing cells in the total cells. The specific binding of ZNC(C)PR on C6 cells was determined by radioligand binding assay (RBA). **RESULTS**: ZNC(C)PR-treated C6 conditioned medium increased the ratio of neurite-bearing PC12 cells by 36 % compared to the untreated C6 conditioned medium or to a mixture of ZNC(C)PR with the untreated C6 conditioned medium. RBA showed a specific binding site of ZNC(C)PR on C6 cells with K_d value of 2.74 nmol·L^{-1} and B_{max} value of 19 pmol·g^{-1} protein. **CONCLUSION**: ZNC(C)PR enhanced C6 cells induced secretion of some neurotrophic factors which acted as enhancers for PC12 cells differentiation, through its specific receptor sites on the neuroglioma cell.

INTRODUCTION

The pentapeptide pGlu-Asn-Cyt-Pro-Arg-OH [ZNC(C)PR], a metabolite of argipressin (AVP), is a new neuropeptide[1] which does not exert any peripheral activity as AVP but shows much more potency than AVP in facilitating the acquisition and maintenance of learning and memory in rats[2]. It is well documented that neuropeptides as well as neurotransmitters can act as be neurotrophic factors, during development and possibly also in the adult nervous system[3]. In our previous study, we have found that ZNC(C)PR could play a trophic role not only in the rat brain by enhancing the expression of neurotrophic factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF)[4,5] but also on cultured neuroglioma C6 cells. ZNC(C)PR markedly stimulated C6 cells growth in the early stages of cell culture and retarded its apoptosis process[6]. All these results implied that ZNC(C)PR might play a multi-functional role in rat brain, as a neuromodulator and in some cases as a neurotrophic factor. Although the signaling pathway of ZNC(C)PR for facilitating memory process in rat brain has been elucidated[7], the process regarding its trophic functions still remains unclear. In this paper, the mechanism of trophic actions of neuropeptide ZNC(C)PR on C6 cells were studied by observing the effects of C6 conditioned medium on growth of PC12 cells and by investigating the specific binding of ZNC(C)PR to C6 cell membranes.

MATERIAL AND METHODS

Reagent DMEM, F12, newborn calf serum from Gibco BRL; DMSO, Gelatin, BSA, argipressin, Coomassie Brilliant Blue G 250 from Sigma; ZNC(C)PR, ZDC(C)PR ZNCPR were synthesized and purified by HPLC in the lab; inhibitors of protease leupeptin, pepstatin A, benzamidine from Sigma; [^{35}S] cysteine from Amersham. All other reagents were of analytic or biochemical grade.

Cell lines Rat neuroglioma C6 cell line was purchased from the Cell Bank in Shanghai Institute of Cell Biology. PC12 cells were a kind gift from Dr ZHOU Yuan-Cong from the same Institute.

C6 culture and cell harvesting Rat C6 glioma

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cells were maintained in DMEM supplemented with 10 % newborn calf serum，benzylpenicillin 100 kU·L^{-1}，streptomycin 50 mg·L^{-1}，gentamicin 25 mg·L^{-1} in a humidified incubator containing 5 % CO_2. Cells were harvested in the log-phase growth by centrifugation（250 × g，4 ℃，30 min），resuspended in 5 pellet volumes of PBS at 4 ℃，and counted by hemocytometer in the presence of trypan blue. The cells were discarded if fewer than 95 % excluded the dye.

Serum-free culture，exposure to drugs and collection of CM Lyophilized peptides were dissolved in water and diluted 1∶50 directly by fresh serum-free medium of DMEM∶F12（1∶1）.

C6 cells were seeded onto the 100 mL flask at the density of 10^9 cells/m^2，after culture for 3 h，the serum-containing medium was removed by aspiration，and cells were washed with PBS，and peptide-containing serum-free medium was added（control groups included only the serum free medium）. Following culture for 5 d，the conditioned media were centrifuged and the resulting supernatants were collected，passed through 0.22-μm filters to remove cell debris and stored at −20 ℃.

Culture of PC12 cells and assay for the cell growth and differentiation Rat pheochromocytoma PC12 cells were cultured in DMEM containing 10 % newborn calf serum and 6 % horse serum. The cells were grown on gelatin-coated culture dishes and incubated in a humidified atmosphere containing 5 % CO_2 at 37 ℃. The medium was changed every 2 d.

Cells were seeded onto 35 mm dishes at the density of 10^9 cells/m^2. After adhesion to the flask wall，cells were washed with PBS，and the medium was replaced with serum free conditioned medium. Three or four random photographs were taken per dish. Neurites were identified as having processes longer than two cell diameters in length，and the percentage of neurite-bearing cells was calculated. Cell clumps containing more than six cells were excluded. With 20 – 30 cells per photograph，and testing every culture condition in three dishes；a total of 200 – 300 cells were counted. At least three separate experiments were performed.

Preparation of ^{35}S-labeled ZNC（C）PR The synthesis and labeling of ZNC（C）PR was performed according to the procedures described previously[7]. In brief，The lyophilized [^{35}S] cysteine，which was purified by RP-HPLC，was codissolved with fresh synthetic ZNCPR（SH-form）in 0.25 % acetic acid and titrated with iodine/ethanol 0.1 mol·L^{-1}. After condensing to a small volume，the reaction solution was fractioned on the same column type. The second radioactive fraction with a typical ZNC（C）PR retention time was collected. Further purification was required to remove side-products such as $(ZNCPR)_2$. Purified [^{35}S] ZNC（C）PR was finally obtained by rerunning the HPLC on the same column and collecting the main peak. The specific activity of [^{35}S] ZNC（C）PR thus generated was 8.6 PBq·mol^{-1}.

Homogenization Cells obtained as described above were centrifuged（250 × g，4 ℃，10 min）and the pellet was resuspended in 8 volumes of hypotonic buffer（Tris-Cl 10 mmol·L^{-1}，leupeptin 1 mg·L^{-1}，pepstatin A 1 mg·L^{-1}，benzamidine 5 mmol·L^{-1}，pH 7.4）at 0 ℃. Cells were allowed to swell for 10 min，centrifuged（12 000 × g，4 ℃，15 min），when 90 % of the cells were broken they were determined by phase-contrast microscopy. The pellet was resuspended in the binding buffer（Tris-HCl 50 mmol·L^{-1}，BSA 1 g·L^{-1}，$NiCl_2$ 5 mmol·L^{-1}，pH 7.4），homogenized by a #4 needle syringe. The concentration of protein was determined according to the Bradford procedure，with BSA as the standard.

Binding assay Unless indicated otherwise，the binding assays were performed in Tris-HCl 50 mmol·L^{-1} buffer containing 0.1 % bovine albumin（BSA）and $MgCl_2$ 5 mmol·L^{-1} at pH 7.4. The competing unlabeled ZNC（C）PR was added 5 min before adding 5 μL of [^{35}S] ZNC（C）PR（16.7 kBq；final concentration 28.3 MBq·L^{-1}）. Incubation with [^{35}S] ZNC（C）PR was carried out at 37 ℃ for 40 min；then 200 μL of the mixture was filtered through a 0.22-μm membrane filter which had been presoaked in 0.2 % BSA in PBS at 0 ℃. The filter was washed three times with 0.4-mL aliquots of same BSA solution at 0 ℃ prior to counting in a gamma counter.

RESULTS

Effects of ZNC（C）PR on the differentiation of PC12 cells PC12 cell is a clonal cell line derived from rat pheochromocytoma. When cultured in the presence of neurotrophic factors，these cells extend long branching neurites and undergo several biochemical changes characteristic of sympathetic neurons[8]. So，it has been widely used to investigate the mechanisms underlying neuronal differentiation. In contrast to C6 cells[6]，ZNC（C）PR did not show any significant effect on morphological changes of PC12 cells in the culture（Fig 1A）. Surprisingly，in the presence of C6 condi-

tioned medium treated with ZNC(C)PR [ZNC(C)PR-C6-CM], PC12 cells showed evident morphological changes from pheochromocytoma to sympathetic neurons, such as enlargement of cell body and elongation of the neurites (Fig 1B).

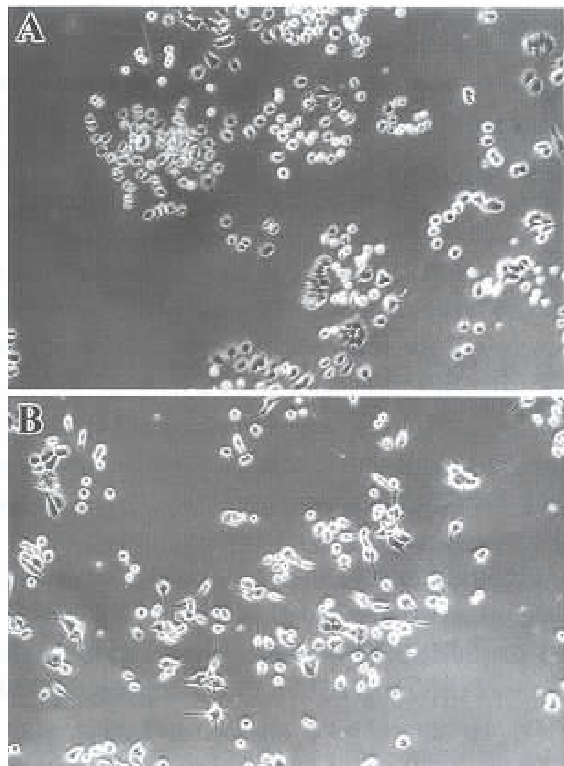

Fig 1. PC12 cells after treatment with ZNC(C)PR-C6-CM for 24 h. A) PC12 cells in normal serum-free medium. B) PC12 cells in ZNC(C)PR-C6-CM. The ratio of CM and fresh serum-free medium was 1∶10.

In comparison to the control group, the ratio of neurite-bearing cells increased in a time-dependent manner. No evident changes were observed in the PC12 cells from the culture supplemented with C6-conditioned medium without neuropeptide treatment. To exclude the effects of ZNC(C)PR existing in the culture of PC12 cells, in the control group we supplemented the conditioned medium of untreated C6 (cultured for as long as the experimental group) with fresh peptide just before culture. The results showed that the mixture of ZNC(C)PR and C6 medium could not induce the development of PC12 cells (Fig 2). The enhancement of treated C6-CM to the development of PC12 cells was positively correlated with the ratio of CM in the culture of PC12 cells. After incubation for 24 h, the percentages of neurite-bearing PC12 cells were 11.8 ± 1.9, 12.7 ± 0.9, 17.9 ± 2.8, and 24.3 ± 1.8 in the presence of different concentrations of ZNC(C)PR-C6-CM 0.08 %, 0.40 %, 2 %, and 10 % respectively.

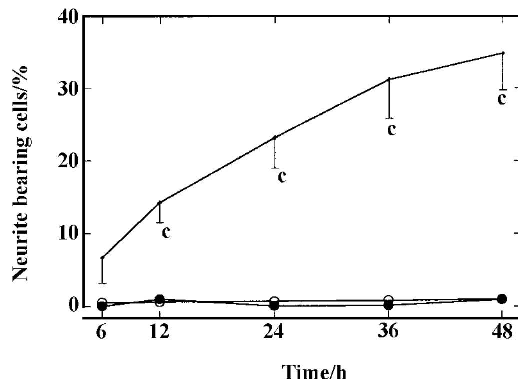

Fig 2. Effects of ZNC(C)PR-treated conditioned medium on differentiation of PC12 cells. The ratio of CM and fresh serum-free medium was 1∶10. (○), untreated CM; (●), untreated CM plus ZNC(C)PR; (+), ZNC(C)PR-treated CM. Concentration of ZNC(C)PR supplemented to mediums was identical as 0.1 μmol·L^{-1}. $n=6$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* untreated CM plus ZNC(C)PR.

Indirect neurotrophic activity of ZNC(C)PR analogs The indirect neurotrophic effect of ZNC(C)PR analogs was compared in the identical peptide concentration (10 nmol·L^{-1}) and the same medium condition (the ratio of ZNC(C)PR-C6-CM and fresh medium being 1∶10). The results as shown in Tab 1 indicate that among ZNC(C)PR, ZDC(C)PR and AVP, only ZNC(C)PR was active as it increased neurite bearing cells to 18 fold as that of control.

Tab 1. Effects of ZNC(C)PR analogs on PC12 cells differentiation. Peptides, 10 nmol·L^{-1}. The ratio of CM and fresh serum-free medium was 1∶10. Incubation for 24 h. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* ZNC(C)PR-treated.

Neurites-bearing cells/%	ZNC(C)PR	AVP	ZDC(C)PR
Basal	1.2 ± 2.1	1.8 ± 1.6	1.7 ± 1.5
Peptide-treated	25.1 ± 2.5^c	1.9 ± 1.7^f	2.0 ± 1.9^f

Characterization of ZNC(C)PR binding to C6 cell membranes In the standard reaction condition as described in METHODS, a specific binding of [^{35}S]ZNC(C)PR to the membranes of C6 cells was found. From the saturable experiments and Scatchard plot (Fig 3), the

dissociation constant K_d and B_{max} values were 2.74 nmol·L^{-1} and 19 pmol·g^{-1} protein. In comparison with the parameters (K_d, 3.12 nmol·L^{-1} and B_{max}, 30.8 pmol·g^{-1} protein) of the specific binding of [^{35}S] ZNC(C)PR to the rat cortical membranes[7], the K_d values are very close to each other and the binding sites on cell membranes are slightly lower than that from the rat cortex but close to the value from rat hippocampus (21 pmol·g^{-1} protein). Furthermore, in consistence with the characterization of its binding to rat brain, the binding of [^{35}S] ZNC(C)PR to C6 cell membranes was reversible and could be completely competed by the unlabeled ZNC(C) PR (Tab 2) in the same range as found previously in the rat cortical membrane[7]. In comparison with the displacement values on [^{35}S] ZNC(C) PR binding, 10 μmol ZDC (C) PR showed high binding activity [approximately 91 % *vs* ZNC(C) PR] to the cell membranes, while AVP, at the same concentration, did not show any displacement (<0.3 %).

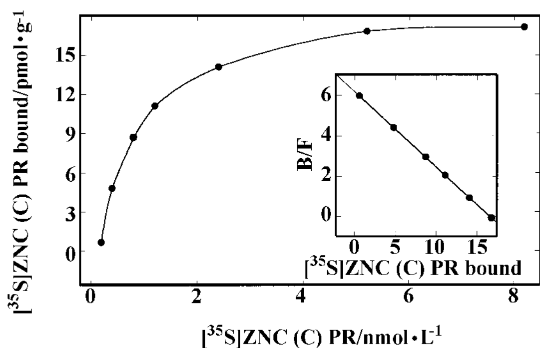

Fig 3. Saturation binding of [^{35}S] ZNC(C)PR to membranes from C6 cells in Tris buffer (50 mmol·L^{-1}, pH 7.4) at 37 ℃ for 40 min. Inset, Scatchard transformation of the data. The membranes used in these experiments were purified by two centrifugations with the first one in 500 × *g*, at 4 ℃ for 10 min. This is a representative experiment, which was replicated three times.

Tab 2. Unlabeled ZNC(C) PR competing for [^{35}S] ZNC (C)PR binding sites on crude C6 cell membranes. *n* = 3. $\bar{x} \pm s$.

Competing ligand/mol·L^{-1}	Displacement/%
1.93×10^{-5}	94.5 ± 3.0
3.73×10^{-6}	89.1 ± 0.6
7.19×10^{-7}	66.5 ± 3.6
1.38×10^{-7}	23.8 ± 3.0
2.60×10^{-8}	11.7 ± 0.6
5.00×10^{-9}	1.91 ± 0.0

DISCUSSION

In our previous study, we have reported that ZNC (C)PR not only plays an important role in rat brain as a neuromodulator for regulating learning behavior[2], but also exerts a neurotrophic function on the growth of C6 cells in the serum-free culture[6]. In both cases, ZNC (C) PR showed a high activity with a lowest effective dosage range round 0.1 nmol·L^{-1} which was hundreds of times lower than that of AVP, while ZDC(C)PR showed no activity or a significant inhibition[6]. It has been suggested that this trophic function may be either a direct or an indirect one. In the latter the peptide acts as a secretagogue releasing a trophic effector from nonneural cell line. It was necessary to investigate mechanism of the trophic action of this peptide. We found that the differentiation of PC12 cells was markedly facilitated by the ZNC(C)PR-C6-CM medium but not by ZNC(C)PR or C6 medium alone or in combination. It implies that certain neurotrophic factor had been synthesized and secreted into the medium by peptide-treated C6 cells. Further investigation with receptor binding studies demonstrated that the main characters of specific binding of ZNC(C)PR to C6 cells were very similar to that in rat brain. AVP did not compete with ZNC(C)PR but ZDC(C)PR did. Our findings indicate that a specific receptor on the C6 membrane mediated the secretagogue action of ZNC(C) PR and the receptor site could be antagonised by ZDC(C) PR.

Regarding the characteristic similarities in binding sites of ZNC(C)PR and its action as a stimulator of neurotropic factors in both rat brain and cultured glioma cell line, it might be possible that glia cells may be one of its target sites besides other known target neurons such as pyramidal cells and granular cells[9]. So, further investigations need to be done on the primary culture of glial cells from rat hippocampus and the trophic factor released by ZNC(C)PR-treated C6 cells should be identified.

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REFERENCES

1 Burbach JPH，Kovacs GL，de Wied D. A major metabolite of arginine vasopressin in the brain is a highly potent neuropeptide. Science 1983；221：1310－2.
2 Liu RY，Lin C，Du YC. Facilitation of arginine-vasopressin analogs on learning and memory in rats. Acta Pharmacol Sin 1990；11：97－100.
3 Schwartz JP. Neurotransmitters as neurotrophic factors：a new set of functions. Int Rev Neurobiol 1992；34：1－23.
4 Zhou AW，Guo J，Wang HY，Gu BX，Du YC. Enhancement of NGF gene expression in rat brain by the memory-enhancing peptide AVP（4－8）. Peptides 1995；16：581－6.
5 Zhou AW，Li WX，Guo J，Du YC. Facilitation of AVP（4－8）on Gene Expression of BDNF and NGF in Rat Brain. Peptides 1997；18：1179－87.
6 HE M，Miao HW，Chen XF，Du YC. Effect of Arginine - vasopressin short analogs on the growth of C6 cells. Chin J Cell Biol 1995；17：176－80.
7 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.
8 Greene LA，Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci USA 1976；73：2424－8.
9 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.

神经肽 ZNC（C）PR 通过刺激 C6 细胞而对 PC12 起间接营养作用[1]

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关键词 精氨酸加压素；C6 细胞；结合位点；PC12 细胞；细胞分化；条件化培养基；放射配位体测定；神经突

目的：研究神经肽 ZNC（C）PR 通过 C6 细胞发挥的神经营养作用和机制. 方法：以ZNC（C）PR处理 C6 细胞的条件培养液对 PC12 细胞生长的影响来观察该肽的间接营养作用，并用受体结合分析来确证该肽在此系统中作用的靶细胞类型. 结果：ZNC（C）PR处理的 C6 细胞的条件培养液能够促进 PC12 细胞由嗜铬瘤细胞向交感神经元转化；放射性配体分析进一步指出在 C6 细胞上存在着该肽的特异性结合位点. 结论：ZNC（C）PR能够通过位于 C6 细胞上的受体促进该细胞分泌某种营养性因子，通过这种因子促进 PC12 细胞的发育.

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