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Short communication

# Effect of substance P and protein kinase inhibitors on β-amyloid peptide-induced proliferation of cultured brain cells

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**Abstract**

The present study investigated the effect of substance P (SP) and protein kinase inhibitors (H7 and HA1004) on β-amyloid peptide-induced proliferation of neonatal rat brain cells in primary cultures The β-amyloid peptide$_{1-28}$ (designated as βAP28), at nanomolar concentrations ($10^{-9}$ M), significantly ($P \leq 0.05$) increased the proliferation of brain cells (presumably non-neuronal) as measured by [$^3$H]thymidine uptake into DNA (mitogenesis). The effect was dependent on time of culture, concentration of βAP28, and presence of fetal calf serum. The supplementation of SP into cell cultures at time zero reversed the proliferative response of βAP28. Moreover, the βAP28-induced proliferation was inhibited by protein kinase inhibitor H7, but not by HA1004. Since H7 is a selective protein kinase C (PKC) inhibitor and SP action involves PKC, we conclude that βAP28 induces normal brain cell proliferation through PKC pathway of cell signaling.

*Keywords* β-Amyloid protein; Brain cell proliferation; Growth factor; Signal transduction, Alzheimer's disease

Cerebral amyloidosis or accumulation of β-amyloid protein$_{1-40}$ (designated as βAP40) in brain occurs during normal aging. However, the amount of βAP40 increases several fold in Alzheimer's disease (AD), suggesting a pathological link of the protein with disease [22]. The βAP40, a small molecular weight (4 kDa) protein containing approximately 40 amino acids [4], is produced by normal proteolytic processing [14] of a larger precursor molecule (90 to 130 kDa) known as β-amyloid precursor protein (βAPP). Both βAP40 and βAP28 fragment thereof have been shown to induce neurotrophic and neurotoxic effects on primary cultures of hippocampal cells [20,21]. Moreover, βAP40 induces growth factors in glial cell cultures [1] and is localized extraneuronally in the skin fibroblasts [7], eukaryotic cultured cells [2,5] and biological fluids [13]. This finding suggested that βAP40 is produced during normal cell metabolism presumably having a physiological function throughout life. The present study describes the effect of βAP28 on the proliferation of primary brain cell cultures and its sensitivity to SP and protein kinase inhibitors.

The primary brain cell cultures were prepared according to our previous work [15]. Sprague–Dawley neonatal rats (18 to 24 h old) were sacrificed under ether anesthesia. By using pre-sterilized dissecting tools, the brain was removed quickly and collected in RPMI-1640 growth medium. Brain tissue was dissociated and passed through a nylon sieve under aseptic conditions of a Laminar flow-hood and the cell suspension was passed through a 21G needle. The cell suspension was centrifuged at $800 \times g$ for 10 min. The cell pellet, after one washing, was resuspended in RPMI-1640 containing penicillin (100 U/ml)-streptomycin (100 μg/ml), 10% heat-inactivated fetal calf serum (FCS) and 1% glucose. The cells were seeded into tissue culture flasks (25 cm$^3$ size) at a count of $2.5 \times 10^5$ cells in 5 ml per flask and allowed to grow for 4 to 5 days inside the humified chamber of a $CO_2$ (5%)-incubator at 37°C. The confluent cultures were subcultured by trypsin (0.2%) treatment for about 5 min and

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the cells were washed three times with RPMI-1640 medium. They were either subcultured or used to set up the experiments.

The cell proliferation was measured by [$^3$H]thymidine uptake as described before [16]. The cell suspension from freshly trypsinized cultures was pipetted into microwells of a flat-bottomed 96-well microtiter plate (Corning). Triplicate microwells were set up in the absence or presence of βAP28 in a final volume of 200 μl which included 100 μl of cell suspension containing approximately 10,000 cells per well. After 5 days of incubation in $CO_2$-incubator, the cells were labelled for 4 h with 0.4 μCi/well of [methyl-$^3$H]thymidine (spec. act. 2 Ci/mmol, purchased from NEN, Boston, MA) and harvested using a semi-automatic cell harvester (Titertek model, Flow Labs.) onto Whatman glass-fiber filter paper The filter paper discs (10 mm in diameter) were transferred into counting vials, mixed with 2 ml of Ready Safe scintillant (Beckman) and counted for radioactivity using a liquid scintillation spectrometer (Packard Model Tri-Carb 1500) with approximately 58% counting efficiency. The data are presented as the counts per minute (CPM) of radioactivity or the per cent of growth medium control. The statistical significance ($P \leq 0.05$) was assessed by Student's *t*-test using Statview software for the Macintosh computer

Neuropeptide βAP28 (cat. #PNPE269) was a synthetic product of > 99% purity (HPLC analysis) purchased from Bachem California, Torrance, CA. Synthetic substance P (code 7451) was purchased from Penninsula Labs., Inc., Belmont, CA. The protein kinase inhibitors H7 (code 120805) and HA1004 (code 120804) were purchased from Seikagaku America, St. Petersburg, FL. The fetal calf serum (FCS) and penicillin-streptomycin mixture were purchased from Hyclone Labs. (Logan, UT) and Gibco (Grand Island, NY), respectively. The βAP28 and all other reagents were dissolved aseptically in RPMI containing penicillin-streptomycin but no FCS and stored frozen at −70°C. Whenever needed, they were thawed and used fresh.

As shown in various figures, βAP28 displayed a stimulatory effect on the proliferation of brain cells in primary cultures. The proliferative response was significantly higher in the presence of βAP28 as compared to growth medium control (Fig. 1); the *P* values were 0.003, 0.05 and 0.006 for 31 nM, 153 nM and 765 nM concentrations, respectively. However, it required fetal calf serum (FCS) since in the absence of FCS there was virtually no cell proliferation. The βAP28-induced cell proliferation increased up to 5 days of incubation that was used in subsequent experiments. The stimulatory effect of βAP28 was observed both pre- and post-cell plating (Fig. 2) although the overall response was slightly lower if the cells had been pre-seeded overnight

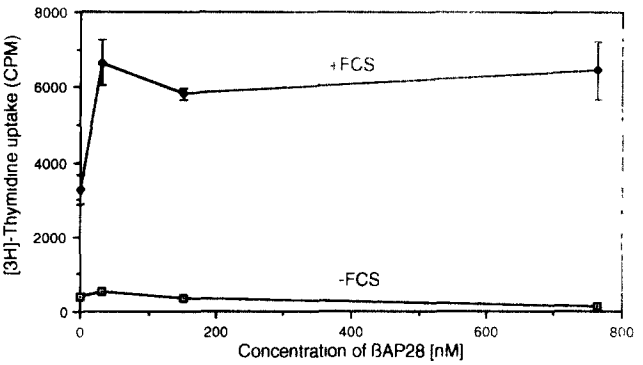


Fig 1 Effect of βAP28 on brain cell proliferation in primary cultures In this experiment, 100 μl of cell suspension (approximately 8,000 cells per microwell in triplicate) was plated in the presence or absence of 10% (v/v) FCS As desired, 100 μl of growth medium (blank) or a sterile solution of βAP28 in RPMI-1640 was pipetted After 5 days, the cell proliferation was measured as described in the text The data represent mean values ± standard error (S.E.) In the presence of FCS, the *P* values were 0 003, 0 05 and 0.006 at 31 nM, 153 nM and 765 nM concentrations, respectively

(16 to 18 h). The supplementation of substance P (SP) into cultures completely blocked the proliferative response of βAP28 (Fig. 3). In addition, the βAP28-induced stimulation of cell proliferation was inhibited by protein kinase inhibitor H7, but not by HA1004; the two inhibitors were tested at a concentration of 20 μM against the three concentrations of βAP28 (Fig. 4).

Based on their patholgocial link with Alzheimer's disease [22], the amyloid proteins of neuritic plaques have been intensely studied during the last decade. However, the pathological role of βAP40 in AD continues to be a matter of debate and controversy especially in light of genetic associations of apolipoprotein E [12] and non-neuronal expression of βAP40 during

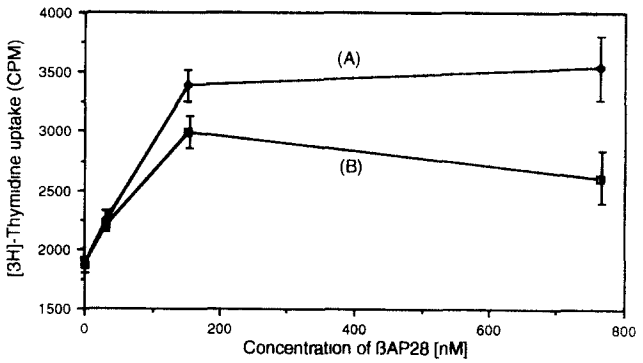


Fig 2 Effect of time of cell plating on the proliferative effect of βAP28 Triplicate cultures of approximately 10,000 cells per microwell were plated In one set up, the βAP28 was added at time zero (A) and incubated for five days Alternately, the cells were first allowed to differentiate (B) overnight (about 18 h), followed by the addition of βAP28 and incubation for a total period of 5 days At low to high concentrations of βAP28, the *P* values were 0 016, 0 015 and 0.0001 for conditions under A and 0 09, < 0 0001 and 0 003 for conditions under B

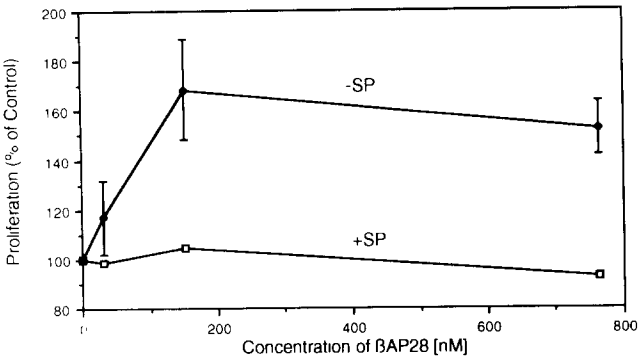


Fig 3 Effect of substance P on the action of βAP28 The cell concentration was approximately 10,000 cells per microwell All other conditions of incubation were the same as those given under Fig 1 Whenever present, the substance P ( + SP) was at a concentration of 20 μM The data are given as the per cent of control (100%) that did not contain βAP28

normal cell metabolism [2,5,7,13]. While a causative role of βAP40 in AD continues to be established, this protein may have some biological function during normal cell metabolism. This line of thinking stems from several studies: (i) βAP40 and its fragment βAP28 exert trophic and toxic effects on hippocampal neurons [20,21]; (ii) βAP40 expression occurs predominantly in astrocytic glial cells [2]; (iii) βAP40 occurs naturally in cells that are not brain-derived [5,7,13]; (iv) βAP40 induces glia-derived growth factors [1]; (v) βAP40 eliminates a functional potassium channel but not calcium channel in human fibroblasts [3]; (vi) βAP40 and βAP28 induce DNA synthesis in immunocytes of normal blood donors but not AD patients [18]; and (vii) βAP28 increases proliferation of neonatal rat brain cells as reported in our present study. Since neuronal proliferation ceases early in embryogenesis whereas glial cells continue to divide into postnatal periods of development, the βAP28-induced proliferative activity would be related to non-neuronal cells presumably

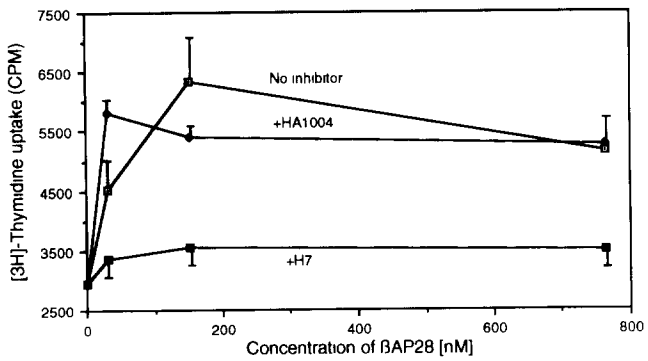


Fig 4 Effect of protein kinase inhibitors on βAP28-stimulated cell proliferation Three different concentrations of βAP28 were set up with approximately 10,000 cells per microwell Depending on design, the H7 or HA1004 was added at a concentration of 20 μM and the response was measured as described under Fig. 1

astrocytes. Based on this consideration and the fact that astrocytes are a major source of βAP in brain [2], we suggest that this peptide acts as an autocrine hormone on glial cells and a paracrine hormone on neurons under physiological conditions in vivo.

Our finding of a proliferation-enhancing effect by βAP28 is similar to a neurotrophic effect [20,21] but whether the two involve same or completely different mechanisms is not presently known One notable difference, however, is that the proliferative response can be induced by 100- to 3000-fold lower concentrations of βAP28 than the concentrations required for neurotrophic effect; we used a concentration range of 30 to 765 nM as compared to 3 to 30 μM in one study [20] and 100 μM in another [21]. The latter study [21] showed that the trophic effect of βAP40 was maximal by approximately 0 1 nM, but βAP28 was much less potent than βAP40 and required a 100 μM concentration to yield only 29% of the trophic response Furthermore, the βAP28-induced proliferative response required serum since there was no proliferation in the absence of fetal bovine serum (FCS). The reason for this requirment is presently not known but we suggest that βAP28 may interact with some serum protein(s) ensuing a proliferative response. Also, the morphological differentiation was not a prerequisite since the stimulation of cell proliferation took place irrespective of the time of addition (at time zero or after overnight seeding) of βAP28 into cultures

SP has been shown to prevent neurotrophic and neurotoxic effects of βAP40 on hippocampal neurons [21] We also found that SP completely reverses βAP28 action on cell proliferation. In contrast, SP did not modify $^{125}$I-βAP40 binding to brain homogenates [9] or truncated βAP22–35-induced neurotoxicity [19]. This discrepancy may be related to assay differences in different studies: (i) $^{125}$I-βAP40 binding was assayed with pathological tissue (AD brain) [9] but not with normal tissue and the homgenate contained bovine serum albumin and protease inhibitors (bacitracin, chymostatin, leupeptin and dimethyl sulfoxide) that may alter binding characteristics; and (ii) a truncated βAP22–25 form [19], instead of βAP40 or βAP28, was added to cultures after 5 days of cell differentiation but not at time zero. Since the βAP28-induced response was counteracted by SP, we suggest that this peptide interacts with a molecule structurally similar to SP receptor or functionally coupled to it but the nature of this putative molecule remains unknown.

Indirectly through the use of protein kinase inhibitors, an approach recently used by us for second messenger studies of another neuropeptide [17], we found that the proliferative response of βAP28 was preferentially inhibited by H7, a selective inhibitor of PKC [6], but not by HA1004 which is a PKA inhibitor and intracellular $Ca^{2+}$ antagonist without any in-

hibitory effect on PKC [6]. This finding implies that the mechanism of proliferation-enhancing action of βAP28 involves a PKC-activated pathway of signal transduction; a mechanism strengthened by a recent study [8] which showed that in the presence of glutamate βAP40 activates tachykinin receptors and phosphatidylinositol (PI) turnover. PKC is a key enzyme in mediating the action of neurotrophic factors to maintain neuronal survival and βAP28 certainly behaves like a growth factor [1,20,21, present study]. In addition, SP, which prevents βAP28-induced proliferation, also involves PKC-activated phosphoinositide turnover [10], indicating that the two peptides might compete with each other for interaction to PKC system. Based on PKC involvement in βAP28 action [present study] and PKC (βII isoform) reduction in AD brains [11], we postulate that βAP28 controls cellular (PKC activity) and molecular (DNA synthesis) functions of normal brain cells but aberration of its function at a purported site of action may form neuritic plaques in the CNS. Thus, our study probes into the mechanism of cellular action of this peptide which may help in understanding the pathological link between β-amyloid protein and Alzheimer's disease.

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