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The effect of a secreted form of β-amyloid-precursor protein on intracellular Ca^{2+} increase in rat cultured hippocampal neurones

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1 The effects of secreted forms of β-amyloid-precursor proteins (APP^Ss) on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were investigated in rat cultured hippocampal neurones. $APP695^S$, a secretory form of APP695, attenuated the increase in $[Ca^{2+}]_i$ evoked by glutamate. In addition, $APP695^S$ itself evoked an increase in $[Ca^{2+}]_i$ in 1 or 2 day-cultured hippocampal cells, but not in 7 to 13 day-cultured cells.

2 Eighty-one percent of neurones which were immunocytochemically positive for microtubule-associated protein 2 responded to $APP695^S$ with an increase in $[Ca^{2+}]_i$.

3 $APP695^S$ induced a transient rise in $[Ca^{2+}]_i$ even in the absence of extracellular Ca^{2+} and produced an elevation in inositol-1,4,5-trisphosphate (IP_3) in a concentration-dependent manner from 100 to 500 ng ml^{-1}. In the presence of extracellular Ca^{2+}, $APP695^S$ caused a transient rise in $[Ca^{2+}]_i$ followed by a sustained phase at high $[Ca^{2+}]_i$, suggesting Ca^{2+} entry from the extracellular space.

4 The $[Ca^{2+}]_i$ elevation was mimicked by amino terminal peptides of APP^S, but not by carboxy terminal peptides.

5 These results taken together suggest that $APP695^S$ induces an increase in $[Ca^{2+}]_i$ in hippocampal neurones through an IP_3-dependent mechanism that changes according to the stage of development.

Keywords: β-Amyloid-precursor protein (APP); intracellular Ca^{2+} concentration; inositol-1,4,5-trisphosphate

Introduction

Amyloid proteins are thought to be key molecules in the aetiology of Alzheimer's disease (see Selkoe, 1993). Recently, it has been shown that secreted forms of β-amyloid precursor protein (APP) (APP^Ss) as well as β-amyloid peptides (Aβ) exist in high levels in the brain and cerebrospinal fluid (Weidemann *et al*., 1989), suggesting a novel role for APP^Ss in the central nervous system. Several lines of evidence indicate that APP^Ss are involved in the regulation of neuronal activities including Ca^{2+} homeostasis. For instance, APP^Ss have been shown to inhibit increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by glutamate (Barger *et al*., 1995) and hypoglycaemia (Mattson, 1994), and to lower the resting $[Ca^{2+}]_i$ levels by guanosine 3′:5′-cyclic monophosphate (cyclic GMP)-mediated mechanisms (Furukawa *et al*., 1996) in rat cultured hippocampal neurones. These observations suggest that APP^Ss have a neuroprotective action against ischaemic or excitotoxic injury *in vivo* (Smith-Swintosky *et al*., 1993) and *in vitro* (Mattson *et al*., 1993a,b). It has been found that carboxy terminal peptides of APP^Ss are much more inhibitory than amino terminal peptides (Mattson *et al*., 1992; Furukawa *et al*., 1996). A specific APP^S receptor for these actions of APP^S (Mattson *et al*., 1993a) is assumed to exist in the plasma membrane because (1) both the APP^S-mediated inhibition of the $[Ca^{2+}]_i$ increase and the rise in intracellular cyclic GMP were rapid responses, (2) APP^S promoted these responses at low (nanomolar) concentrations and (3) the specific high-affinity binding of APP^S to fibroblasts has been demonstrated (Johnson-Wood *et al*., 1990). Neither the receptor nor the mechanism of signal-transduction of the protective function by APP^S has been clarified.

It has also been found that APP^Ss stimulate the $p21^{ras}$-dependent mitogen-activated protein kinase (MAPK) cascade in PC12 cells (Greenberg *et al*., 1994). It is very interesting that amino terminal peptides of APP^S are responsible for the stimulation of MAPK cascade (Greenberg *et al*., 1994). A polypeptide fragment that includes the Arg-Glu-Arg-Met-Ser (PERMS) sequence adjacent to the Kunitz-type serine protease inhibitor (KPI) domain was found to regulate fibroblast growth (Ninomiya *et al*., 1993) and promote neurite extension in a neuronal cell line (Jin *et al*., 1994). These findings suggest the existence of other signal-transduction systems coupled to specific receptor(s) for the amino terminal peptides of APP^S, which differ from the protective functions presumably stimulated by the carboxy terminal domains of APP^S.

During the course of experiments to clarify the function of APP^S by use of a recombinant APP^S ($APP695^S$), we have discovered not only inhibition of the glutamate-evoked rise in $[Ca^{2+}]_i$ but also stimulation of $[Ca^{2+}]_i$ by $APP695^S$ in cultured hippocampal cells. These reciprocal effects were seen in the same cells, suggesting the existence of at least two mechanisms in these cells. The aim of the present study was (1) to investigate the effects of $APP695^S$ on the increase in $[Ca^{2+}]_i$ in the cells, (2) to determine the type of cell (neurones or non-neurones responding to $APP695^S$), and (3) to clarify the mechanism of the increase in $[Ca^{2+}]_i$.

Methods

Cell culture

The culture of embryonic day 17 rat hippocampal cells was as described previously (Inoue *et al*., 1992). In brief, hippocampal

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cortices were dissected from cerebra of 17 day foetal Wistar rats and the tissues were incubated with a papain (9 u ml^{-1})-phosphate buffer saline solution containing 0.02% L-cysteine monohydrate, 0.5% glucose and 0.02% bovine serum albumin for 15 min at 37°C. The cells were dispersed, plated on a poly-L-lysine and collagen-coated glass coverslip and cultured for 1–9 days in Dulbecco's modified Eagle's medium supplemented with 5% precolostrum newborn calf serum, 5% heat-inactivated horse serum, 10 u ml^{-1} penicillin and 10 μg ml^{-1} streptomycin in a humidified atmosphere of 90% air and 10% CO_2 at 37°C. The majority of experiments were performed in cells cultured for 1 day.

Construction of the expression vectors for recombinant APP^S

Escherichia coli JM109 was used as a host for the construction of each plasmid. Plasmids pHIL-D2, pHIL-S1 and pPIC9 were purchased from Invitrogen. The expression vector for $APP695^S$ was described previously (Ohsawa *et al*., 1995). In brief, the DNA fragment encoding from Met^1 to Lys^{612} of human APP695 was inserted downstream of the alcohol oxidase promoter in pHIL-D2, yielding pAPE1. For expression of the amino-terminal region of APP^S (NAPP), the DNA fragment encoding from Arg^{16} to Val^{290} of human APP770 was obtained by PCR with primers 5′-CGGCTCGAGCGCTG-GAGG-3′ (5′ oligonucleotide) and 5′-GTTGAATTCA-CACCTCTCGAACC-3′ (3′ oligonucleotide) and subcloned into pHIL-S1 as a *Xho*I–*Eco*RI fragment, yielding plasmid pAPE3, an in-frame fusion of the *PHO1* secretion signal to Arg^{16}, and an insertion of the stop codon next to Val^{290}. For expression of the carboxyl-terminal of APP^S (CAPP), the DNA fragment encoding from Glu^{380} to Thr^{663} of human APP770 was obtained by PCR with primers 5′-CAAGTCTC-GAGAGACACCTGG-3′ (5′ oligonucleotide) and 5′-CAGA-GAATTCCTACGTCTTGAT-3′ (3′ oligonucleotide) and subcloned into pPIC9 as a *Xho*I–*Eco*RI fragment, yielding pAPE5, an in-frame fusion of the α-factor prepro region to Glu^{380}, and an insertion of the stop codon next to Thr^{663}. *Pichia pastoris* GS115 (*his4*) was transformed with each plasmid as described previously (Cregg *et al*., 1985). Briefly, after digestion of pAPE1 with restriction enzyme *Nco*I or of pAPE3 and pAPE5 with restriction enzyme *Bgl*II, the fragments obtained containing a gene cassette for the expression of a whole or part of APP^S and the *his4* gene were introduced into *P. pastoris*. Colonies of His^+ Mut^- phenotype, expected to be recombined at the *AOX1* sequence on the yeast chromosome, were then selected.

Expression and purification of recombinant proteins

The expression of $APP695^S$, NAPP and CAPP in the recombinant yeast was induced by the method described previously (Despreaux & Manning, 1993). $APP695^S$ containing from Leu^{18} to Lys^{612} of APP695, NAPP containing from Arg^{16} to Val^{290} of APP770, and CAPP containing from Glu^{380} to Thr^{663} following 6 amino acids derived from the α-factor prepro region, EAEAYI, were secreted into the culture medium. $APP695^S$ was purified according to the method described previously (Ohsawa *et al*., 1995). To purify NAPP and CAPP, the yeast cells were separated by centrifugation and 200 to 400 ml of the medium were concentrated to 15 ml by pressure filtration with a YM-10 Amicon ultrafilter. The concentrated sample was then loaded onto an anion exchange column, DE52 (1 × 5 cm; Whatman) and equilibrated with 20 mM phosphate buffer (pH 7.0). After washing with 25 ml of the buffer, the bound protein was eluted with 4 ml of 0.5 M NaCl in 20 mM phosphate buffer pH 7.0. The eluted protein was desalted with a HiTrap Desalting column (Pharmacia), mixed with one volume of 4.0 M $(NH_4)_2SO_4$, and then loaded onto an alkyl-Superose column (0.5 × 5 cm; Pharmacia) at a flow rate of 0.5 ml min^{-1}. After washing with 50 ml of 2.0 M $(NH_4)_2SO_4$ in 50 mM Na_2HPO_4 buffer (pH 7.0), the bound protein was eluted with a linear gradient of 2.0–0.0 M $(NH_4)_2SO_4$ at a flow rate of 0.5 ml min^{-1} over 40 min. To obtain NAPP, fractions corresponding to the first peak eluted with 1.4 M $(NH_4)_2SO_4$ were pooled. To obtain CAPP, fractions corresponding to the second peak eluted with 0.8 M $(NH_4)_2SO_4$ were pooled. After dialysis against phosphate-buffered saline

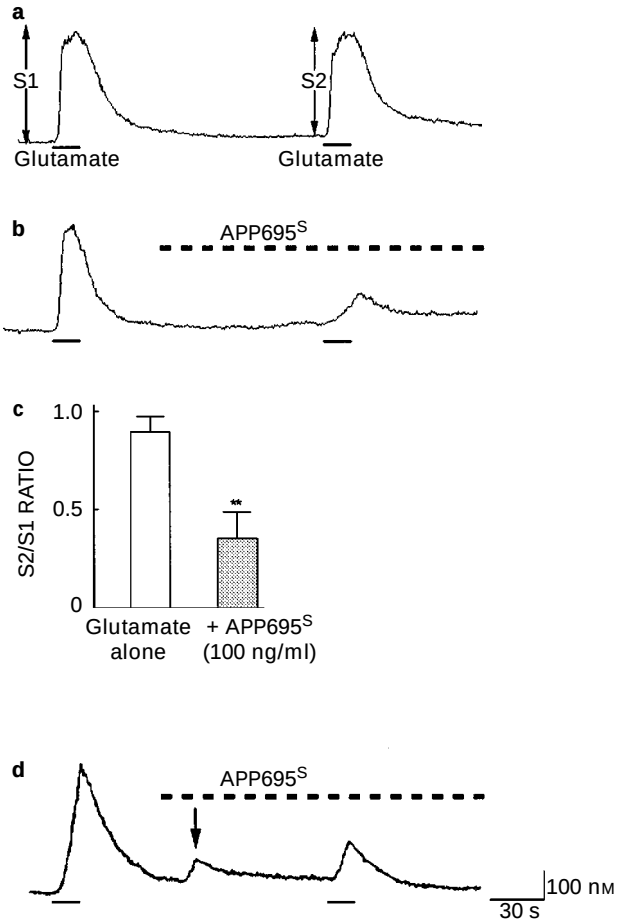

Figure 1 Effects of $APP695^S$ on the $[Ca^{2+}]_i$ in cultured rat hippocampal cells with or without glutamate stimulation. (a and b) Typical intracellular Ca^{2+} responses to glutamate (30 μM) in the absence and presence of $APP695^S$ (100 ng ml^{-1}). Glutamate was applied twice to the cells for 15 s, 2 min apart. The Ca^{2+} responses to the first and the second glutamate applications are designated S1 and S2, respectively. The first stimulation was always with glutamate alone and the second glutamate stimulation was performed in the absence (a) or presence of $APP695^S$ (b). In (b), $APP695^S$ was applied to the cells 90 s before, and during the second glutamate application. $APP695^S$ significantly reduced the glutamate-induced rise in $[Ca^{2+}]_i$. The $APP695^S$ inhibition of the glutamate-induced rise in $[Ca^{2+}]_i$ in these cells is summarized in (c). The ratio of S2 to S1 was calculated in individual cells and adopted as an index for comparing data in different cells. The data shown are means ± s.e.mean for 15 cells tested with glutamate alone and 17 cells tested with glutamate + $APP695^S$. $^{**}P < 0.01$ vs glutamate alone. The asterisks indicate a significant difference from the ratio obtained with glutamate alone. (d) $APP695^S$ was applied to the cells as in (b). Besides its inhibitory action on the glutamate-induced rise in $[Ca^{2+}]_i$ $APP695^S$ produced a rise in $[Ca^{2+}]_i$ (arrow). The vertical and horizontal bars represent 100 nM and 30 s, respectively.

(PBS), the purified proteins were monitored by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and analysed by staining with Coomassie Brilliant Blue (CBB) or Western blot with antibodies 22C11 (1 : 1000) and Alz90 (1 : 50). Binding of primary antibodies was detected with anti-mouse immunoglobulin, peroxidase-linked $F(ab')_2$ fragment (1 : 1000; Amersham), and visualized by the ECL technique (Amersham).

Measurement of $[Ca^{2+}]_i$ in the cells

Measurement of the increases in $[Ca^{2+}]_i$ in single cells was performed as previously described (Koizumi *et al*., 1994). All of the procedures, including incubation, washing and drug application, were carried out with balanced salt solution (BSS) having the following composition (in mM): NaCl 150, KCl 5.0, $CaCl_2$ 1.8, $MgCl_2$ 1.2, NaH_2PO_4 1.2, D-glucose 10 and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) 25, pH adjusted to 7.4 with NaOH. In the Ca^{2+}-free experiments, Ca^{2+} was omitted from the solution and 1 mM ethyleneglycol bis (2-aminoethylether) tetraacetic acid (EGTA) was added (Ca^{2+}-free BSS). The cells were washed with BSS and incubated with 10 μM fura-2 acetoxymethylester (fura-2 AM) in BSS at 37°C. After 30 min incubation, the cells were washed with 0.2 ml of BSS. The coverslips were mounted on an inverted epifluorescence microscope (TMD-300, Nikon) equipped with a 75 W xenon-lamp and 340-nm-wavelength band-pass filter (F340) to measure the Ca^{2+}-dependent signal and 360-nm-wavelength filter (F360) to measure the Ca^{2+}-independent signal. The measurements were made at room temperature. Image data were obtained by a high-sensitivity silicon intensifier target camera (C-2741-08, Hamamatsu Photonics, Co., Hamamatsu, Japan) and processed by a Ca^{2+}-analysing system (Furusawa Laboratory Applicance Co., Kawagoe, Japan). The absolute $[Ca^{2+}]_i$ was estimated from the ratio of emitted fluorescence (F340/F360) according to a calibration curve obtained by use of Ca^{2+}-buffers (Molecular Probes Inc., C-3712 with 1 mM $MgCl_2$).

Measurement of IP_3 concentration

For the IP_3 assay, hippocampus-containing cerebral hemispheres obtained from embryonic day 17 rats were mechanically dispersed and plated on 35 mm-diameter polyethylenimine-coated plates. The cultures were maintained in serum-free Dulbecco's modified Eagle's medium (DMEM)

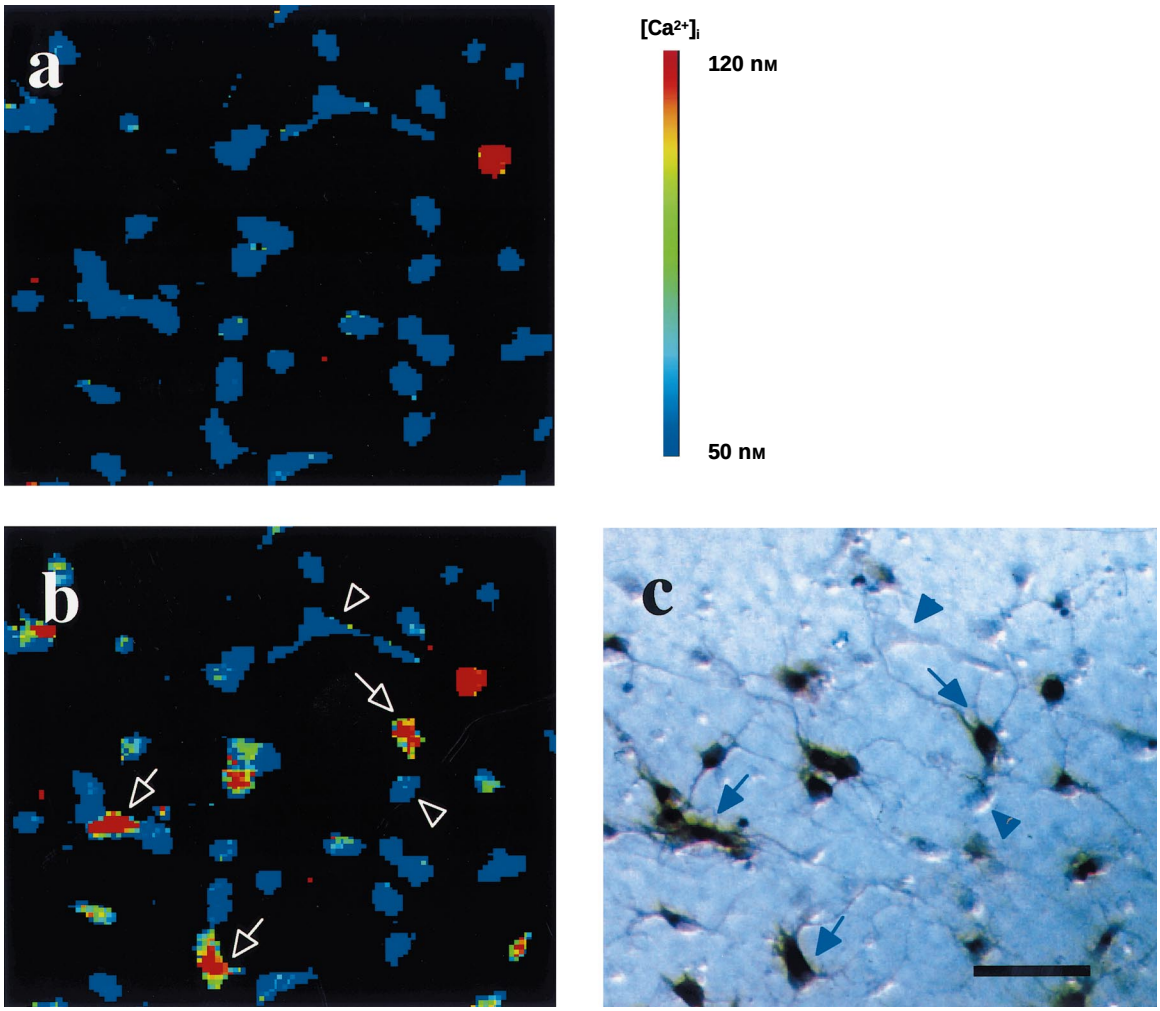

Figure 2 The rise in $[Ca^{2+}]_i$ induced by $APP695^S$ in rat dissociated hippocampal neurones cultured for 1 day. Pseudo-colour images of $[Ca^{2+}]_i$ in the neurones before (a) and 30 s (b) after application of $APP695^S$ (400 ng ml^{-1}). The colour bar shows the levels of $[Ca^{2+}]_i$ determined from a calibration curve (50–120 nM). (c) Immunocytochemical localization of the neurone-specific marker, microtubule-associated protein 2 (MAP2) in the cells. The cells were immunocytochemically stained by MAP2-antibody. All of the cells that responded to $APP695^S$ were MAP2-positive (neurones) and the representative cells are indicated by arrows. Arrowheads point to the MAP2-negative cells (non-neuronal cells), which did not respond to $APP695^S$. The bar represents 50 μm.

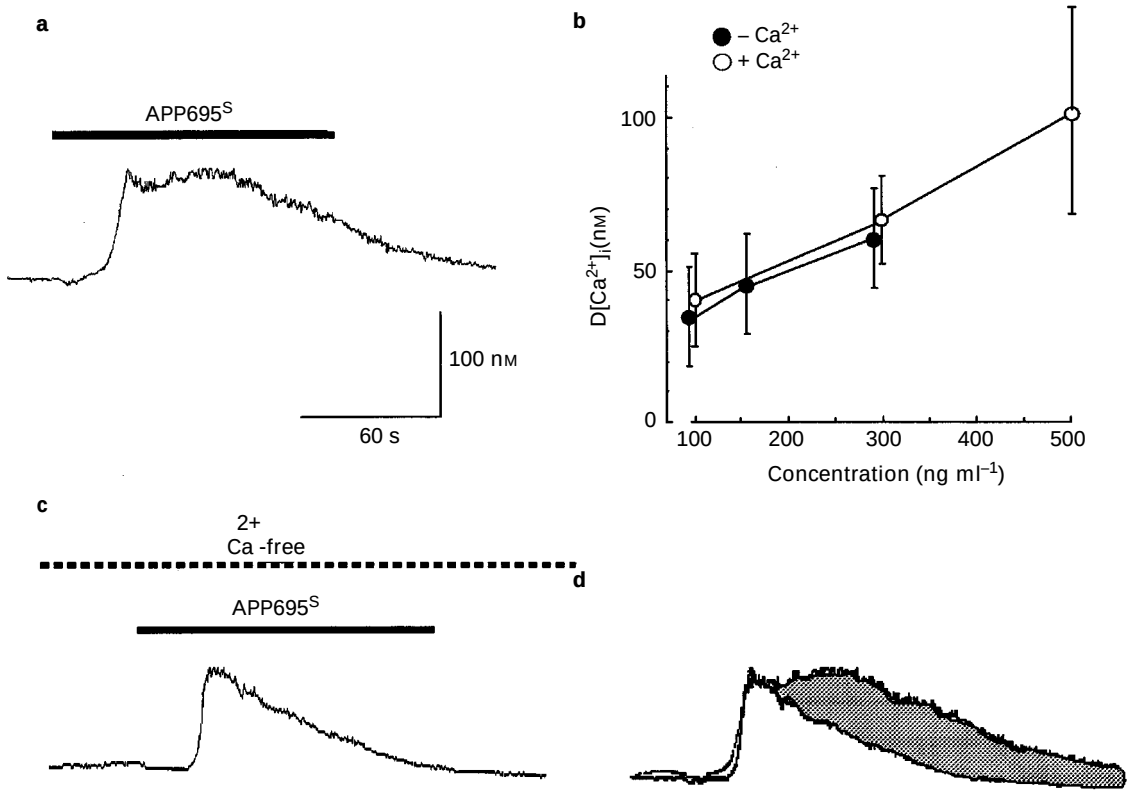

Figure 3 Characterization of the $APP695^S$-induced rise in $[Ca^{2+}]_i$ in hippocampal neurones. (a) A typical time-course of the $APP695^S$-evoked rise in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}. $APP695^S$ (300 ng ml^{-1}) was applied to the cells for 2 min. (b) Concentration-response relationships of the $APP695^S$-induced rise in $[Ca^{2+}]_i$. The data are means for 18–32 cells tested; vertical lines show s.e.mean. Values show the maximal responses above basal levels (51.6±2.4 nM, $n=111$). The Ca^{2+} responses to $APP695^S$ are shown in the presence and absence of extracellular Ca^{2+}. A typical time-course of the $APP695^S$-induced rise in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. The cells were pretreated with Ca^{2+}-free BSS (containing 1 mM EGTA) for at least 2 min, then $APP695^S$ (300 ng ml^{-1}) dissolved in Ca^{2+}-free BSS was applied to the cells for 2 min. (d) The response shown in (a) was superimposed on that from (c). The difference is indicated by the stippled area.

overnight at 37°C in a 5% CO_2, humidified incubator. The cells were preincubated with DMEM containing 10 mM LiCl for 5 min at 37°C and then exposed to various concentrations of $APP695^S$. After the various periods indicated, a 0.2 volume of ice-cold 20% perchloric acid was added, and the samples were kept on ice for 20 min to extract the IP_3. They were then transferred from the plates to microtubes and centrifuged at 2,000 × *g* for 15 min at 4°C. The supernatants were neutralized by the addition of ice-cold 1.53 M KOH for 20 min on ice, and the precipitated $KClO_4$ was sedimented by centrifugation (12000 × *g* for 15 min at 4°C) and removed. The supernatants were used for radioreceptor assay of IP_3 with an IP_3 assay kit (TRK-1000, Amersham). The amounts of IP_3 in the wells were calculated from a standard curve with authentic IP_3.

Determination of neurones

After the $[Ca^{2+}]_i$ changes had been measured, the cells were immunocytochemically stained with anti-MAP2 monoclonal antibody (Sigma) to determine whether the $APP695^S$-responders were of neuronal origin. Cells were fixed in 4% paraformaldehyde in PBS for 30 min, rinsed in PBS and incubated in blocking PBS solution containing 3% bovine serum, 3% goat serum and 0.3% TritonX100 for 60 min. They were then stained with anti-MAP2 monoclonal antibody and horseradish peroxidase-conjugated anti-mouse immunoglobulin G (Fab) according to the method described (Hamanoue *et al.*, 1996).

Drugs

Drugs and chemicals were obtained from the following sources: fura-2 AM, EGTA and HEPES were from Dojin, (Kumamoto, Japan). Other drugs were purchased from Wako Pure Chemical (Osaka, Japan).

Statistics

All the data were given as mean±s.e.mean. Statistical differences in values for changes in $[Ca^{2+}]_i$ were determined by analysis of variance and Dunnett's test for multiple comparisons.

Results

The rise in $[Ca^{2+}]_i$ induced by glutamate was significantly inhibited by $APP695^S$ in neurones obtained from embryonic day 17 rat hippocampus and cultured for one day (Figure 1). In the absence of $APP695^S$, the rise in $[Ca^{2+}]_i$ induced by the second glutamate application (S2) was almost the same as that induced by the first glutamate stimulation (S1) (Figure 1a), and the ratio of S2 to S1 was 0.92±0.067 ($n=15$, Figure 1c). The second Ca^{2+} response to glutamate was significantly inhibited by $APP695^S$ (Figure 1b) and the S2/S1 ratio was 0.38±0.11 ($n=17$, $P<0.01$ vs glutamate alone, Figure 1c). In addition to this inhibitory effect, $APP695^S$ itself increased

$[Ca^{2+}]_i$ in about 22% of all cells (neurones and glial cells) tested (95 out of 430 cells), as shown in Figure 1d (arrow). $APP695^S$ also induced an increase in $[Ca^{2+}]_i$ in 3 day-cultured cells, but not in 7–13 day-cultured cells (252 cells tested, data not shown).

Characterization of the cells in which $APP695^S$ evoked an increase in $[Ca^{2+}]_i$ was performed by an immunocytochemical method with an antibody to microtubule-associated protein 2 (MAP2). The cultured cells were composed of neurones and glial cells, and the MAP2-positive cells, which are thought to be neurones, represented 34% of the total number of cells (16 out of 46 cells), as shown in Figure 2c. From a pseudo-colour image of the $APP695^S$-evoked rise in $[Ca^{2+}]_i$ (Figure 2b), 81% of these neurones (13 out of 16 cells) responded to $APP695^S$ (arrows indicate representatives of responder). These data suggest that $APP695^S$ evokes an increase in $[Ca^{2+}]_i$ in most 1 or 2 day-cultured hippocampal neurones.

$APP695^S$ produced a rise in $[Ca^{2+}]_i$ in a concentration-dependent manner (Figure 3a and b). In the absence of extracellular Ca^{2+}, $APP695^S$ also induced a rise in $[Ca^{2+}]_i$ (Figure 3c). The peaks of both Ca^{2+} responses to $APP695^S$ in the presence and absence of extracellular Ca^{2+} were comparable (Figure 3d), suggesting that the $APP695^S$-evoked rise in $[Ca^{2+}]_i$ does depend on intracellular Ca^{2+} stores. However, the time-course of Ca^{2+} responses to $APP695^S$ in the absence of extracellular Ca^{2+} was quite different from that in the presence of Ca^{2+}. In the absence of extracellular Ca^{2+}, $[Ca^{2+}]_i$ increased transiently by the stimulation of $APP695^S$, then quickly decreased to the basal level, whereas, in the presence of extracellular Ca^{2+}, $[Ca^{2+}]_i$ initially increased in a manner similar to that seen in the absence of extracellular Ca^{2+}, but then remained at a high level (second phase). The difference between these two responses (Figure 3d, stippled area) suggests that $APP695^S$ could evoke not only Ca^{2+} release from internal Ca^{2+} stores but also Ca^{2+} influx from external sources.

Next we examined the effect of $APP695^S$ on IP_3 formation. As shown in Figure 4a, IP_3 levels increased rapidly after $APP695^S$ stimulation, peaking at 30 s, and then decreased to about 50% of the basal levels 60 s after stimulation. This time-course was almost the same as that of the $APP695^S$-induced rise in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} (Figure 3c). $APP695^S$ stimulated IP_3 formation in a concentration-dependent manner (100 to 500 ng ml^{-1}) (Figure 4b). Thus, $APP695^S$ probably produces the rise in $[Ca^{2+}]_i$ in hippocampal neurones by stimulating the formation of IP_3, which in turn leads to the release of Ca^{2+} from intracellular Ca^{2+} stores.

It has been shown that the carboxy terminal region of APP^S is required for the inhibitory actions of APP^S on the internal Ca^{2+} concentration (Mattson *et al*., 1993a,b; Furukawa *et al*., 1996). In contrast, the amino terminal region has been found to activate mitogen-activated protein kinase in rat PC12 cells (Greenberg *et al*., 1994). We examined which region of APP^S is required to stimulate the increase in $[Ca^{2+}]_i$ in the hippocampal neurones. The elevation in $[Ca^{2+}]_i$ was mimicked by an amino terminal peptide of APP^S (NAPP, 300 ng ml^{-1}, Figure 5a) but not by a carboxy terminal peptide (CAPP, up to 500 ng ml^{-1},

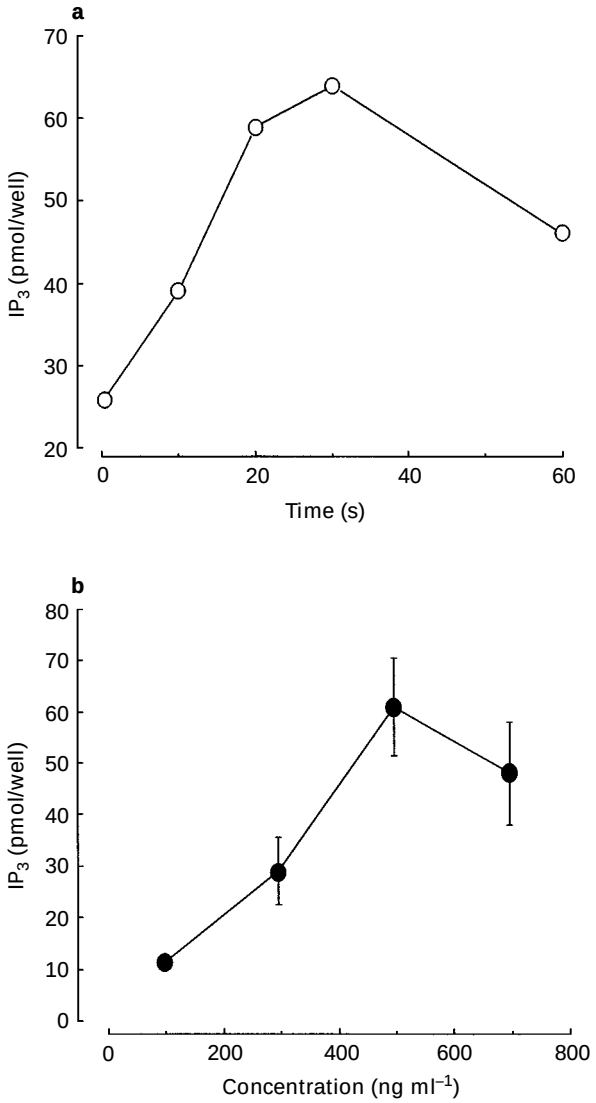

Figure 4 IP_3 formation evoked by $APP695^S$ in neurones dissociated from rat cerebral hemispheres, including the hippocampus. (a) Time-course of $APP695^S$ enhancement of IP_3 formation in the cells. IP_3 in the cells was determined at the times indicated after the addition of $APP695^S$ (500 ng ml^{-1}). These are the results of a typical experiment, with each data point representing the mean of duplicate measurements. Three independent experiments were performed and the results were reproducible. (b) Concentration-dependence of $APP695^S$ enhancement of IP_3 formation in the cells. IP_3 in the cells was determined 30 s after the addition of various concentrations of $APP695^S$. The data are means of three separate experiments; vertical lines show s.e.mean.

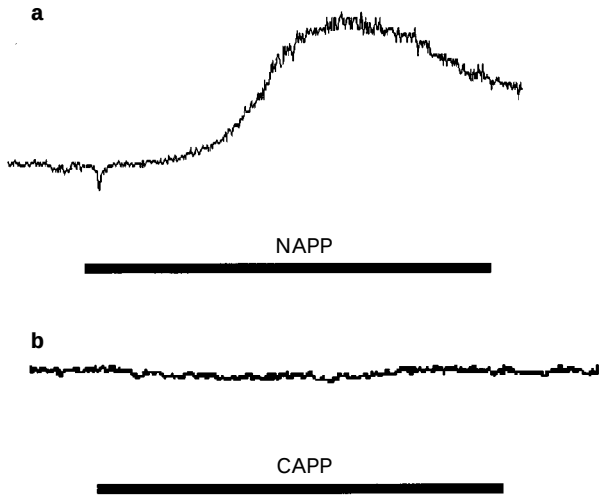

Figure 5 The effects of the amino termial peptide of APPs (NAPP) and the carboxy terminal peptide (CAPP) on the $[Ca^{2+}]_i$ in the hippocampal neurones. (a) A typical time-course of the NAPP-induced rise in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. NAPP (300 ng ml^{-1}) was applied to the cells for 2 min. (b) CAPP (up to 500 ng ml^{-1}, $n=125$) did not evoke any change in $[Ca^{2+}]_i$.

Figure 5b, $n=125$). The NAPP-evoked rise in $[Ca^{2+}]_i$ from the basal level in the presence of extracellular Ca^{2+} was 68 ± 50 nM (mean $\pm$ s.e.mean of 18 cells), which is comparable to the value seen when stimulated by $APP695^S$ with or without extracellular Ca^{2+} (Figure 3b).

Discussion

APP^Ss have been shown to lower resting $[Ca^{2+}]_i$ levels (Furukawa *et al*., 1996) or to inhibit rises in $[Ca^{2+}]_i$ induced by glutamate (Mattson, 1994), N-methyl-D-aspartate (Barger *et al*., 1995) and hypoglycaemia (Mattson *et al*., 1993b) in cultured hippocampal neurones. We demonstrated in this paper that, in addition to these inhibitory actions, $APP695^S$ can also stimulate an elevation in $[Ca^{2+}]_i$ in 1 or 2 day-cultured hippocampal neurones via an IP_3-sensitive mechanism. This effect of APP^S disappeared in 7–13 day-cultured neurones, suggesting that the sensitivity of the neurones to APP^S is dependent on the stage of development. A similar hypothesis has already been proposed for the increase in $[Ca^{2+}]_i$ induced by γ-aminobutyric acid (GABA) in cultured neurones from various brain regions, including the hippocampus of the rat (Obrietan & Van den Pol, 1995). Accumulation of phosphoinositides mediated by muscarinic acetylcholine receptors (Calvet & Ventura, 1995) and the rise in $[Ca^{2+}]_i$ mediated by P2U-purinoceptors (Sugioka *et al*., 1996) in embryonic chick retina drastically declined during maturation of the cells. Thus, differences in the developmental stage of the cells appear to have a crucial effect on the responses evoked by various endogenous substances. Changes in both the structure of the receptors and the functional coupling of receptors with effectors could occur when cells mature (Calvet & Ventura, 1995).

APP^S stimulates the $p21^{ras}$-dependent mitogen-activated protein kinase (MAPK) cascade in PC12 cells (Greenberg *et al*., 1994). A fragment that includes the RERMS sequence adjacent to the Kunitz-type serine protease inhibitor (KPI) domain regulates fibroblast growth (Ninomiya *et al*., 1993) and promotes neurite extension in a neuronal cell line (Jin *et al*., 1994). These findings suggest the involvement of novel receptor(s) for APP^S. For the $[Ca^{2+}]_i$-stabilizing action of APP^S, a specific APP^S receptor in the plasma membrane is also assumed (Mattson *et al*., 1993a) for the reasons mentioned previously (see Introduction). For similar reasons, it is speculated that APP^S may induce IP_3 formation and Ca^{2+} mobilization through a specific receptor coupled to phospholipase C. It has been shown that the carboxy terminal region is much more important than the amino terminal region with respect to the inhibitory action of APP^S (Mattson *et al*., 1993a,b; Furukawa *et al*., 1996). In contrast, the amino terminal region has been shown to stimulate mitogen activated protein kinase in rat PC12 cells (Greenberg *et al*., 1994) and promote neurite extension in rat neocortical explants (Ohsawa *et al*., 1997). From these data, it is speculated that the amino terminal region of APP^S transmits via another receptor different from that sensitive to the carboxy terminal region of APP^S. We showed here that an amino terminal peptide, NAPP, but not a carboxy terminal peptide, CAPP, produced a rise in $[Ca^{2+}]_i$ in the cells. In addition, $APP695^S$ showed both an elevation in $[Ca^{2+}]_i$ and inhibition of the glutamate-evoked $[Ca^{2+}]_i$ in the same hippocampal neurones (Figure 1d). These data suggest that $APP695^S$ stimulates at least 2 types of receptors: the one that is sensitive to the amino terminal fragment of $APP695^S$ evokes a $[Ca^{2+}]_i$ increase, and the other that is sensitive to the carboxy terminal fragment inhibits the $[Ca^{2+}]_i$ increase by glutamate.

Alternatively, APP^S themselves may function as a plasma membrane receptor since APP^Ss have a receptor-like architecture (Kang *et al*., 1987). The results of this study showed that both the $APP695^S$-induced rises in IP_3 and $[Ca^{2+}]_i$ in hippocampal neurones were rapid responses that peaked about 30 s after application. Based on these data, $APP695^S$ may act on its own specific receptors, presumably coupled to IP_3/Ca^{2+} systems.

The time-course of Ca^{2+} responses to $APP695^S$ in the absence of extracellular Ca^{2+} was quite different from that in the presence of Ca^{2+}. A sustained rise in $[Ca^{2+}]_i$ by the stimulation of $APP695^S$ was seen only in the presence of extracellular Ca^{2+}, suggesting that $APP695^S$ evoked a Ca^{2+} entry in the cells (Figure 3d). At least two explanations have been proposed to explain such a Ca^{2+} entry after stimulation of receptors coupled with IP_3/Ca^{2+} systems, i.e. capacitative Ca^{2+} entry (CCE), a Ca^{2+} entry in response to depletion of Ca^{2+} in the stores, and a second messenger operated Ca^{2+} entry (Berridge, 1995). Although CCE appears to be a universal feature of non-excitable cells (Putney, 1990), some studies suggest that excitable cells also display CCE. For example, activation of a P2U-purinoceptor which is coupled to a phospholipase $C\beta/IP_3$ system resulted in CCE in PC12 cells (Koizumi *et al*., 1995), and thapsigargin, an inhibitor of the Ca^{2+} ATPase pump in the endoplasmic reticulum, produced CCE in neuronal retina of chick embryo only when the neurones were in the development stage (Sakaki *et al*., 1997). Thus, it is possible that $APP695^S$ stimulated the sustained $[Ca^{2+}]_i$ rise by a mechanism dependent on the stored Ca^{2+} level. Alternatively, Ca^{2+} entry induced by the generation of some second messenger, such as IP_3 (Kuno & Gardner, 1987) or inositol 1, 3, 4, 5-tetrakisphosphate (Lückhoff & Clapham, 1992) could be involved in this process. In any case, hydrolysis of phosphoinositides seems to be a crucial event for both Ca^{2+} release and Ca^{2+} entry evoked by $APP695^S$. The contribution of a non-specific cationic entry activated by cytoplasmic Ca^{2+} (Loiland *et al*., 1991) to the sustained $[Ca^{2+}]_i$ rise also cannot be excluded.

We have demonstrated a reciprocal regulation of Ca^{2+} signalling by $APP695^S$ in rat cultured hippocampal neurones at an early developmental stage. $APP695^S$, in addition to its inhibitory action on glutamate-induced $[Ca^{2+}]_i$ increases, caused an increase in $[Ca^{2+}]_i$ through IP_3-sensitive mechanisms in these neurones.

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