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Adverse effects of an active fragment of parathyroid hormone on rat hippocampal organotypic cultures

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1 Adverse effects of an active fragment of parathyroid hormone (PTH_{1-34}), a blood Ca^{2+} level-regulating hormone, were examined using rat hippocampal slices in organotypic culture.

2 Exposure of cultured slice preparations to 0.1 μM PTH_{1-34} for 60 min resulted in a gradual increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$); this effect was most obvious in the apical dendritic region of CA1 subfield.

3 When PTH_{1-34} at a lower concentration (1 nM) was added to the culture medium and its toxic effects examined using a propidium iodide intercalation method, significant toxicity was seen 3 days after exposure and increased with time. Cells in the CA1 region seemed more vulnerable to the hormone than cells in other regions. At 1 week of exposure, the toxic effects were dose-dependent over the range of 0.1 pM to 0.1 μM, the minimum effective dose being 10 pM.

4 The adverse effects were not induced either by the inactive fragment, PTH_{39-84}, or by an active fragment of PTH-related peptide ($PTHrP_{1-34}$), an intrinsic ligand of the brain PTH receptor.

5 The PTH_{1-34}-induced adverse effects were significantly inhibited by co-administration of 10 μM nifedipine, an L-type Ca^{2+} channel blocker, but not by co-administration of blockers of the other types of Ca^{2+} channel.

6 The present study demonstrates that sustained high levels of PTH in the brain might cause degeneration of specific brain regions due to Ca^{2+} overloading *via* activation of dihydropyridine-sensitive Ca^{2+} channels, and suggests that PTH may be a risk factor for senile dementia.
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Keywords: Parathyroid hormone; PTH_{1-34}; PTHrP; hippocampal organotypic culture; intracellular Ca^{2+} concentration; L-type Ca^{2+} channel; nifedipine; propidium iodide; senile dementia

Abbreviations: BSS, balanced salt solution; DG, dentate gyrus; PI, propidium iodide; PTH, parathyroid hormone ; PTHrP, parathyroid hormone related peptide

Introduction

Evidence demonstrating dysfunction of Ca^{2+} homeostasis during aging is accumulating, and a relationship with the incidence of dementia has been suggested (Fujita, 1990). It is now well established that an increase in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ leads to neurotoxicity (Choi, 1988; Ogura *et al*., 1988), and aging appears to be a major risk factor for Alzheimer's disease and several other neurodegenerative conditions, which suggests that an increase in $[Ca^{2+}]_i$ could contribute to the increased vulnerability of aged neurons (Kipen *et al*., 1995; Satrustegui *et al*., 1996; Thibault & Landfield 1996; Porter *et al*., 1997). The regulation of blood Ca^{2+} levels in normal subjects results from well-designed Ca^{2+} homeostatic mechanisms that depend on specific hormones, such as parathyroid hormone (PTH) and calcitonin, which regulate the absorption of dietary Ca^{2+}, reuptake of Ca^{2+} from the renal tube and Ca^{2+} retention in osteocytes. However, in elderly subjects, especially those with osteoporosis, and in Alzheimer patients, it has been shown that blood Ca^{2+} levels are lower and thus PTH levels higher, than in normal subjects (Desterhorft *et al*., 1994). In a previous study, we demonstrated that PTH causes a gradual increase in the $[Ca^{2+}]_i$ by activating dihydropyridine-sensitive Ca^{2+} channels *via* specific PTH receptors in the brain (Hirasawa *et al*., 1998). This result suggested to us that high blood PTH levels might cause an increase in the $[Ca^{2+}]_i$ in brain cells and thus induce degeneration of cells in the brain. Since the blood-brain-barrier in elderly subjects is reported to be less tight than in younger subjects (Ueno *et al*., 1997), the adverse effects of PTH may yield important clues in elucidating the causal relationship between the deterioration in Ca^{2+} homeostasis and the incidence of senile dementia.

In the present study, we examined the adverse effects of PTH_{1-34}, an active fragment of PTH, on organotypic hippocampal slice cultures using the propidium iodide (PI) intercalation method, and found that PTH_{1-34} had a marked toxic effect on the tissue due to dihydropyridine-sensitive Ca^{2+} channel activation.

Methods

Organotypic cultures of rat hippocampal slices

Organotypic slice cultures of rat hippocampus were prepared as described by Sakaguchi *et al*. (1994). The hippocampi were removed from Wistar rats of either sex 5–7 days after birth and 300 μm slices prepared using a McIlwain tissue chopper (Mickle Laboratory Engineering Co.). Each slice was placed on a filter (Millipore, Millicell-CM, 30 mm), which was then placed in a 6-well microplate (Iwaki Glass; code 3810-006) containing 1.0 ml of culture medium (Eagle's minimal essential

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medium, Gibco) supplemented with 25% heat-inactivated horse serum (Gibco). Three slices were placed in each well and cultured under the same conditions for 1 week before being used for the following experiments.

Image analysis of fura-2-loaded preparations

The cultured slice preparation was cut out of the filter and exposed for 60 min at room temperature to 7.5 μM fura-2/AM (a cell-permeable Ca^{2+} indicator, Dojin Chemical), dissolved in a balanced salt solution (BSS) (mM: NaCl 130, KCl 5.4, $CaCl_2$ 2.0, glucose 5.5, HEPES-NaOH 10, pH 7.3). The preparation was then transferred to fresh BSS and incubated at 32°C for at least 1 h before being mounted on the stage of a fluorescence microscope (Olympus, IX70) and continuously perfused with 32°C BSS at a rate of 2 ml min^{-1}.

Image analysis of the organotypic slice culture preparation was performed as previously described (Kudo *et al*., 1993). Fluorescence images of fura-2-loaded organotypic slice cultures were obtained using a low magnification objective lens (Olympus, UPlanApo 4×) and a silicon-intensified-target (SIT) video camera (Hamamatsu Photonics, C2400-8) and were fed into an image processor (Hamamatsu Photonics' Argus 50) for two-dimensional analysis in which a pair of fluorescence images (emission at >500 nm) produced by excitation at 340 nm (F340) and 380 nm (F380) were obtained alternatively once every 10–30 s for 60 min.

Examination of the toxic effects of PTH on rat hippocampus organotypic slice cultures

After 1 week of culture, various concentrations of PTH_{1-34} or related peptides and 2 μM propidium iodide (PI), a fluorescence-marker of degenerating cells, were added to the culture medium to examine the slow-onset toxic effects of the peptides (Vollenweider & Groscurth, 1992; Okada *et al*., 1995). The cultures were left in an incubator for different periods until examined for cell degeneration by measuring the PI fluorescence (emission at >580 nm) of the culture on excitation at 520–540 nm. Fluorescence was detected using a high sensitve video camera (SIT-camera, Hamamatsu Photonics, C2400-8) set at constant sensitivity, and quantified as the average intesity using an image analyser (Argus 50, Hamamatsu Photonics). The total intensity of the fluorescence for a given area (about 180×180 for whole slice images, about 30×30 for each subfiled of hippocampus), clipped from total of 256×241 pixels, was calculated by degitalizing the intensity of fluorescence by frame memory, and the average fluorescence intensity was obtained by dividing the intensity by the total pixels.

Cell deterioration was also confirmed by Nissl staining. The organitypic slice cutures were fixed in 4% paraform aldehyde for 6 h, dipped in 25% sucrose solution for 17–72 h, then mounted on a slide glass. After drying it in the air, the culture was washed twice with distilled water and incubated with Nissl solution at 56°C for 3 min. After dehydrated with ethanol, they were treated with xylene and mounted with Entellan (Merck KGa, Darmstadt, Germany).

Detection of mRNA for PTH/PTHrP and PTH_2 receptors in rat hippocampus organotypic slice cultures by PCR

Total RNA was prepared using the guanidine thiocyanate method from rat hippocampus organotypic slice cultures after 2 weeks in culture and from whole brain minus the cerebellum (used as a reference). First-strand cDNAs were generated from oligo (dT)-primed total RNA with M-MLV reverse transcriptase (TOYOBO). The reaction mixture was then subjected to PCR amplification with use of Taq polymerase (Programable thermal controller, PTC-100, MJ Research Inc.). Samples were heated to 94°C for 5 min; this was followed by 32 cycles, each of three phases of 94°C for 1 min, 59°C for 30 s and 72°C for 1 min. This protocol was followed by a final 10 min extension step at 72°C. The following primers, specific to the PTH receptor family, were designed on the basis of previously reported sequences (PTH/PTHrP receptor; Abou-Samura *et al*., 1992; PTH_2 receptor; Usdin *et al*., 1995) PTH/PTHrP receptor, TTGCCGTACACCGAGGTCTC (forward) and GGGCCCACATTGGTCACACT (reverse) for 251 bp from 1351 to 1601 among total mRNA size of 2065 bp; PTH_2 receptor, CTCCTTCTCGGGGCTCTGGT (forward) and GCAGTCTTGGCGGGTTTGCT (reverse) for 294 bp from 1360 to 1653 among total mRNA size of 1977 bp. PCR products were resolved on 5% acrylamide gels and visualized by ethidium bromide staining followed by UV transillumination.

Results

Effects of PTH_{1-34} on $[Ca^{2+}]_i$ in organotypic cultured slices

In a previous study using disperse-cultured hippocampal cells, we found that the increases in $[Ca^{2+}]_i$ produced by PTH_{1-34} and full-sized PTH were limited to certain populations of cells, suggesting that certain cells, located in a specific region, were sensitive to the hormone. To study possible regional specificity of the susceptibility to PTH_{1-34}, we examined the fura-2-loaded organotypic slice culture preparations under low magnification to view the whole preparation. As shown in Figure 1, during a 60 min exposure to PTH_{1-34} (0.1 μM), the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) gradually increased. This effect was most obvious in the apical dendritic region of CA1 subfield. Although the increase in $[Ca^{2+}]_i$ seen after administration of 0.1 μM PTH_{1-34} was quite high, it would take a lot of time to cause the neuronal cells death. To detect the toxicity of PTH_{1-34} we therefore incubated the preparation with various concentrations of the peptide for a much longer period in culture.

Long-term toxic effects of PTH_{1-34} on hippocampal organotypic slice cultures

After 7 days culture in normal medium, the organotypic slice culture preparations were exposed to a lower concentration (1 nM) of PTH_{1-34} in the presence of PI (2 μM) as an indicator of degenerating cells. One well, containing three slices, was removed each day after addition of PTH_{1-34} and PI, and the fluorescence of each preparation measured using an image analyser. Since the cells that died during the first 7 days of culture were removed by phagocytic microglia, only a faint fluorescence was seen in control preparations, even when exposed to PI for a further 5 days (control data in Figure 2). However, as shown in Figure 2, on exposure to PTH_{1-34}, significant intercalation of PI was seen 3 days after exposure, and the intensity of PI fluorescence increased with time, showing that the toxicity of PTH_{1-34} increased with exposure time. In agreement with the results for peptide-induced $[Ca^{2+}]_i$ increase, preferential vulnerability of cells in the CA1 region could be detected after 3 days exposure, and the toxicity

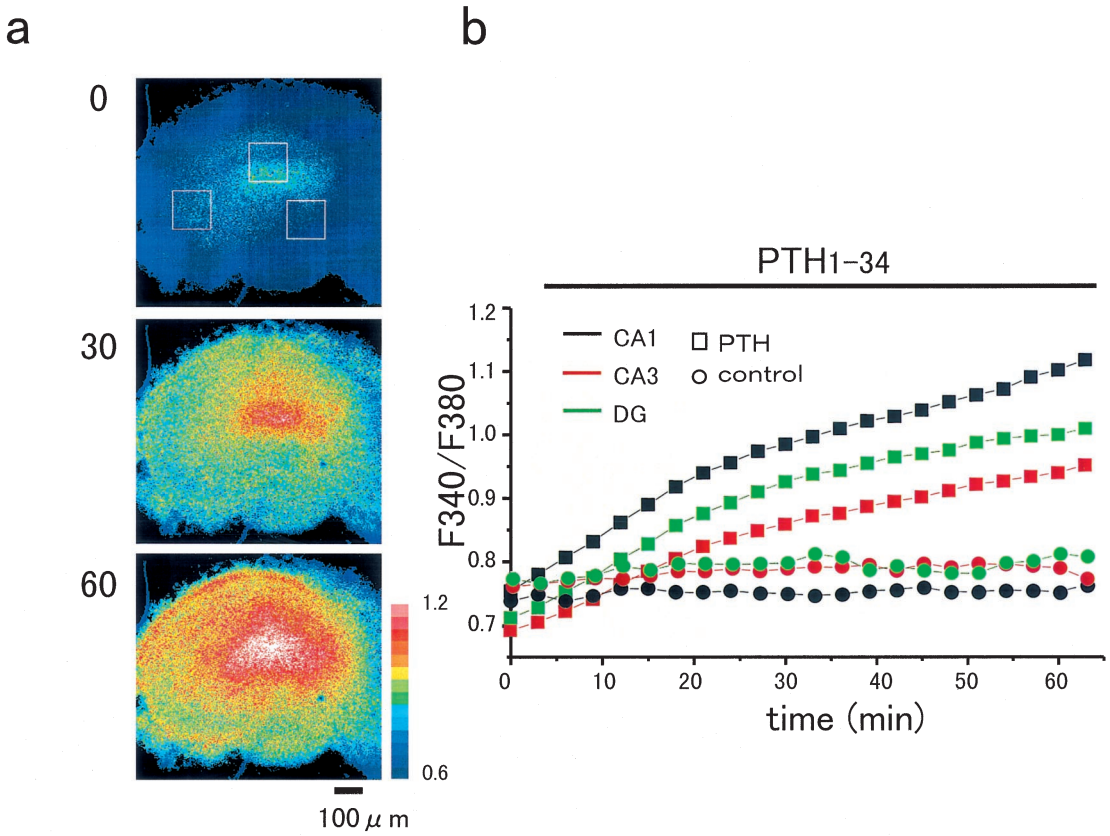

Figure 1 Effects of PTH_{1-34} on $[Ca^{2+}]_i$ in hippocampal organotypic slice cultures. (a) Pseudocolor ratio images (F340/F380) of hippocampal organotypic slices loaded with fura-2 as Ca^{2+} indicator during exposure to PTH_{1-34} (0.1 μM), using a low magnification objective lens (4 ×). The numbers indicate the time in min after exposure to PTH_{1-34}. The colored scale bar shows the F340/F380 ratio. (b) Time-course of the increase in $[Ca^{2+}]_i$ in the CA1, CA3 and dentate gyrus regions indicated as squares in the control image in (a) observed in PTH_{1-34} treated (filled squares) and untreated control preparations (filled circles).

became much more obvious after 5 days of exposure, at which time the cells in the CA3 and dentate gyrus (DG) region were also affected. Cell deterioration induced by PTH_{1-34} was confirmed by Nissl staining. As shown in Figure 3, massive cell loss was seen in CA1 region after incubation with 1 nM of PTH_{1-34} for 5 days, while the cells in CA3 region and DG seemed to be more resistant to PTH_{1-34} toxicity.

The toxic effects of the hormone after 1 week of exposure were dose-dependent over the range of 0.1 pM to 0.1 μM (Figure 4), with statistically significant toxicity being seen at, or above, a concentration of 10 pM. As shown in Figure 4b, the preferential PTH_{1-34} vulnerability of the cells in the CA1 subfield compared with the CA3 and DG subfield was demonstrated. The deterioration of the cells in the CA1 region was significant at 10 pM and reached a maximum at 1 nM, whereas concentration of more than 1 nM was required for significant deterioration of the cells in CA3 region and in DG subfield.

Confirmation of the specificity of PTH_{1-34} toxicity

In a previous study on disperse-cultured hippocampal cells, we confirmed that an increase in the $[Ca^{2+}]_i$ could be induced by either full-size PTH or its active fragment PTH_{1-34}, whereas an inactive fragment, PTH_{39-84}, or an active fragment of PTH-related peptide ($PTHrP_{1-34}$), an intrinsic ligand for the PTH receptor in the brain, had little effect on the $[Ca^{2+}]_i$ level, even after 60 min of continuous administration. We therefore examined the toxicity of these various peptides on organotypic slice culture preparations. As shown in Figure 5, neither PTH_{39-84} nor $PTHrP_{1-34}$ (1 nM) had any significant toxic effect on the preparations, even after 5 days of exposure, while 1 nM PTH_{1-34} caused a significant increase in PI fluorescence, indicating that the toxic effect of PTH_{1-34} is mediated *via* activation of specific PTH receptors.

The distribution of mRNAs coding for the PTH/PTHrP receptor and PTH_2 receptor in hippocampal organotypic slice cultures was examined by PCR. As shown in Figure 6 expected size of mRNAs coding for both of PTH receptors (251 bp for PTH/PTHrP receptor and 294 bp for PTH_2 receptor) could be found both in whole brain minus cerebellum and in hippocampal organotypic slice cultures. Although we have not yet succeeded in demontrating the distribution of these receptors by *in situ* hybridization, the present results indicate that the toxic effect of PTH_{1-34} was due to stimulation of specific PTH receptors expressed in the hippocampal region.

Protective effects of nifedipine on the adverse effects of PTH_{1-34}

In our previous study, we found that the increase in $[Ca^{2+}]_i$ induced by PTH_{1-34} or full-size PTH was almost completely blocked by pretreatment with nifedipine, a dihydropyridine Ca^{2+} channel blocker (Hirasawa *et al*., 1998). We therefore tested the effects of various types of Ca^{2+} channel antagonists on the toxicity of PTH_{1-34} (1 nM) to determine whether the effects were L-type Ca^{2+} channel-specific. The doses of the voltage operated Ca^{2+} channel blockers that were effective on the neurons were determined in our previous electrophysiological studies (Watanabe *et al*., 1998). As shown in Table 1,

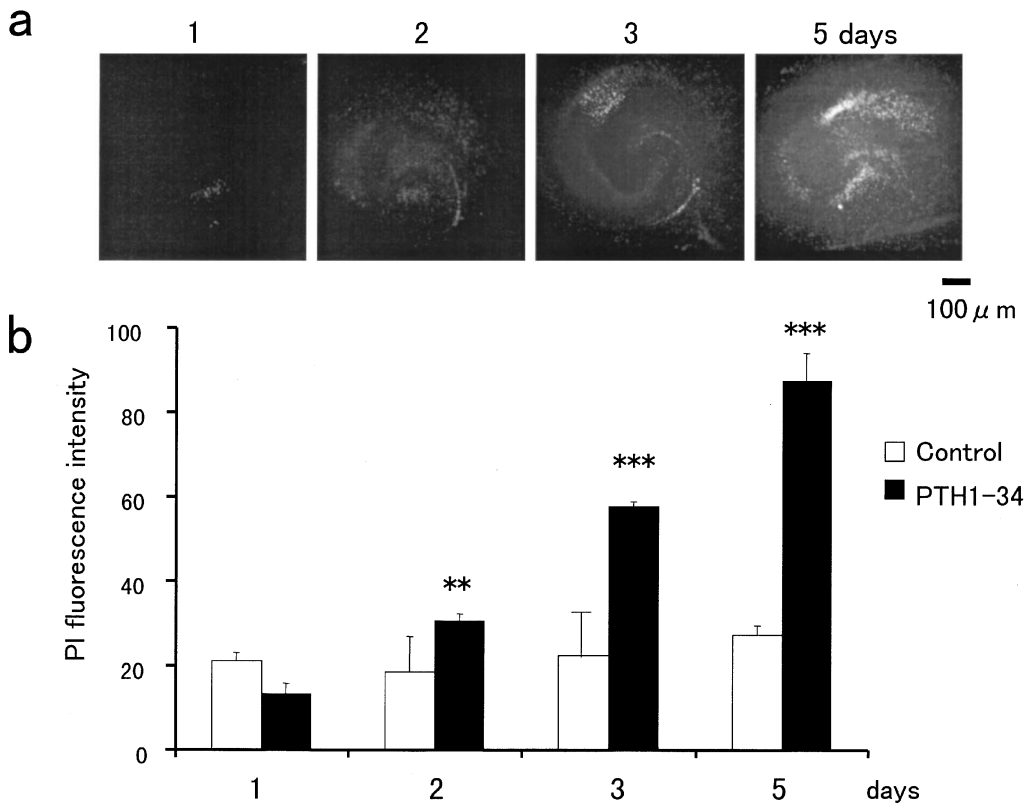

Figure 2 Time-course of the development of the toxic effects of PTH_{1-34} in hippocampal organotypic slice cultures, detected by PI intercalation. (a) Representative fluorescence images of PI-stained organotypic slice cultures exposed to PTH_{1-34} (1 nM) and PI (2 μM) for the number of days indicated on each panel. PI fluorescence (emission >580 nm) was measured using a SIT camera following excitation with 520–540 nm light. Organotypic slice culture preparations were prepared separately for each day tested. (b) The PI fluorescence intensity of the whole preparation was measured in arbitrary units, using an image analyser (Argus 50) from digitized images; the mean and standard error for the intensity are indicated ($n=6$). The open columns indicate the averaged fluorescence intensity of day-matched control preparations and the filled columns indicate the intensity after 1–5 days exposure to PTH_{1-34}. A statistically significant difference was seen from 3 days of exposure to PTH_{1-34} (**$P<0.01$ and ***$P<0.001$ as evaluated according to Tukey's multiple comparison method with control).

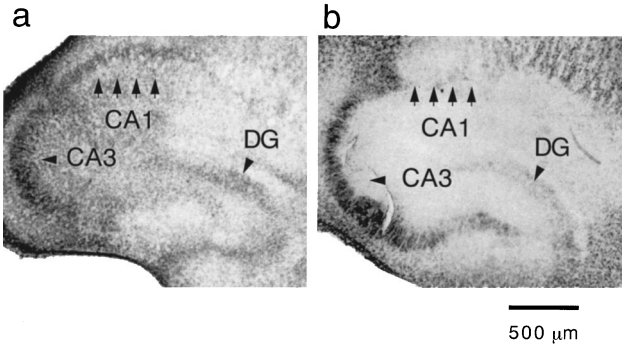

Figure 3 Histological observation on the organotypic slice culture exposed to PTH_{1-34}. Neuronal cells in the organotypic slice culture exposed to PTH_{1-34} (1 nM) was visualized by Nissl staining. (a) The control preparation cultured for 14 days. (b) The preparation cultured for 7 days and then exposed to PTH_{1-34} (1 nM) for another 7 days. Note the part shown by arrow heads. Neuronal cells located in the CA1 subfield were lost in the PTH treated prepration.

nifedipine (10 μM) significantly blocked the increase in PI fluorescence induced by PTH_{1-34}, while ω-conotoxin GVIA, a specific N-type Ca^{2+} channel blocker, and ω-conotoxin MVIIC, a specific P/Q-type Ca^{2+} channel blocker, both at effective dose of 5 mM, had no significant effects on the PTH_{1-34}-induced PI intercalation. Higher concentrations of those Ca^{2+} antagonists (more than 10 mM) were themselves toxic for the cultures. $NiCl_2$, a T/R-type Ca^{2+} channel blocker, in a minimum effective concentration, enhanced, rather than inhibited, the toxicity of PTH_{1-34}. We then used 0.1 μM S-(−)-BayK8644, a dihydropyridine Ca^{2+} channel agonist, to test for the facilitatory effect on agonist toxicity. Within 2 days of treatment, significant facilitation of the PI intercalation due to prolonged exposure to PTH_{1-34} (1 nM) was seen, but there were no significant differences between S-(−)-BayK8644/PTH_{1-34}-treated and PTH_{1-34} treated preparations after 5 days (data not shown). Those results indicated that dihydropyridine-sensitive channels in the preparation were fully activated by prolonged exposure to PTH_{1-34}.

Discussion

The present results indicate that PTH, an important hormone in Ca^{2+} homeostasis, might have adverse effects on hippocampal cells, when continually present at concentrations higher than 10 pM. Since the PTH_{1-34}-induced increase in $[Ca^{2+}]_i$ seen during a 60 min exposure period was most obvious in apical dendrite region of CA1 subfield, and since prerefential deterioration of the cells was also seen in the same subfield, the effects seemed to be caused by activation of the same type of PTH receptor. The PTH_{1-34}-induced increase in $[Ca^{2+}]_i$ developed gradually. The level reached after 60 min of exposure was rather high, but did not seem sufficient to cause

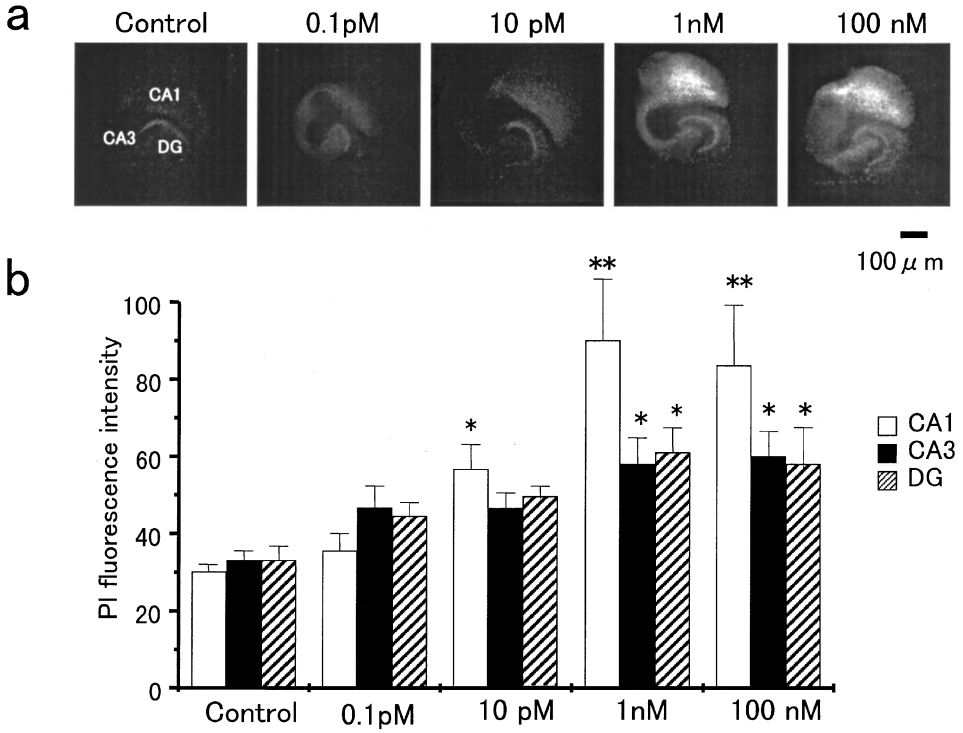

Figure 4 Dose-response relationship of the adverse effects of PTH_{1-34} on organotypic hippocampal slice culture preparations as detected by PI intercalation. (a) Representative PI fluorescence images in organotypic slice cultures exposed for 5 days to PTH_{1-34} (0.1 pM, 10 pM, 10 nM and 0.1 μM), together with PI (2 μM) were obtained as in Figure 2. (b) The intensities of the PI fluorescence in the CA1, CA3 and DG region was measured separately, as in Figure 2, to show the regional specificity of the adverse effects of PTH_{1-34}; the mean and standard error for the intensity are indicated ($n=6$). $^*P<0.05$ and $^{**}P<0.01$ as evaluated according to Tukey's multiple comparison method with control.

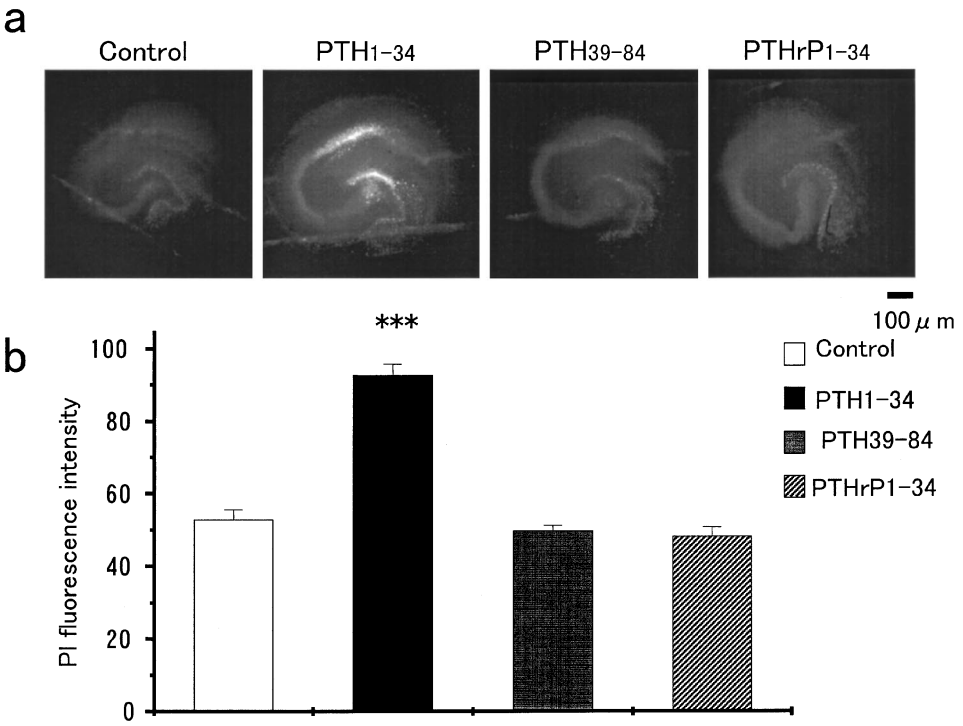

Figure 5 Effects of PTH_{1-34} and related peptides on organotypic slice cultures. (a) Representative fluorescence images of control samples and preparations exposed for 5 days to 1 nM PTH_{1-34}, PTH_{39-84} or $PTHrP_{1-34}$ plus PI (2 μM) as an indicator of degenerating cells. The images were obtained as described in Figure 2. (b) Averaged fluorescence intensity for six preparations treated with each peptide. A statistically significant difference was seen between the results for PTH_{1-34} and the control ($^{***}P<0.001$, Student's t-test), but no significant difference was seen using PTH_{39-84}, an inactive fragment of PTH, or $PTHrP_{1-34}$, an active fragment of PTHrP in the central nervous system.

acute neuronal cell death by itself. However, such an increase in $[Ca^{2+}]_i$ may trigger program for cell death. Since PTH_{1-34} toxicity was dependent upon dose and exposure time, a chronic increase in plasma PTH to a level only several times higher than normal would cause slow, but severe, toxicity to cells bearing the appropriate receptors.

Although our previous results on $[Ca^{2+}]_i$ levels showed that neuronal cells were more susceptible to the hormone than glial cells (which showed a small increase in $[Ca^{2+}]_i$ after prolonged exposure), some glial cells may also be affected by the hormone (Hirasawa *et al*., 1998). Since glial cells are involved in neuronal cell survivability through the release of neurotrophic factors, their deterioration could also affect the fate of neighbouring neuronal cells that express no, or little, PTH receptor. Deterioration of some neuronal cells during the exposure to PTH_{1-34} might be due to such an indirect mechanism.

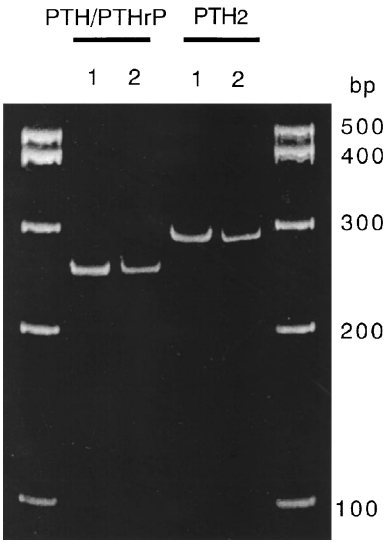

Figure 6 Detection of mRNA coding for PTH/PTHrP receptor and PTH_2 receptor in rat whole brain minus cerebellum and hippocampal organotypic slice culture using PCR method. As describe in Methods, specific mRNA primers for PTH/PTHrP receptor and PTH_2 receptor were used for whole brain minus cerebellum (1) and hippocampal organotypic slice culture (2). Expected size of mRNA for PTH/PTHrP receptor (251 bp) and for PTH_2 receptor (294 bp) detected by each primers in both preparations. Size markers were shown on both sides.

Molecular biological studies have shown that PTHrP receptors in the brain are identical to the classical type of PTH receptor found in peripheral tissues; this type of receptor has been classified as the PTH/PTHrP receptor (Abou-Samura *et al*., 1992). More recently, another PTH receptor, named PTH_2 receptor, was found in the brain (Usdin *et al*., 1995); this, however, is activated by PTH, but not by PTHrP (Behar *et al*., 1996; Turner *et al*., 1998). In the present study, PTHrP had no toxic effect on hippocampal organotypic cultures, while, in the previous study, we confirmed that PTHrP had little effect on the $[Ca^{2+}]_i$ of the dispersed hippocampal cells in culture (Hirasawa *et al*., 1998). Combining those two pieces of evidence, it would seem reasonable to ascribe the toxic effects of PTH on the hippocampal cells to the activation of PTH_2 receptor, found in this region. However, since the time-course of the increase in $[Ca^{2+}]_i$ seen in hippocampal cells using PTH_{1-34} was similar to that induced by PTH in distal renal tube epithelial cells containing the classical PTH/PTHrP receptor (Bacskai & Friedman, 1990; Gesek & Friedman, 1992), the classical PTH/PTHrP seems to be involved in the increase in $[Ca^{2+}]_i$ in, and the toxic effects on, hippocampal organotypic culture. Since the molecular homology of $PTHrP_{1-34}$ and PTH_{1-34} is not very high, the peptides may activate the same brain receptors by different mechanisms. Recent *in situ* hybridization studies on PTH receptors have shown that PTH/PTHrP receptor is found in CA1 subfield (Weaver *et al*., 1995) and that the PTH_2 receptor is widely distributed throughout the rat brain (Usdin *et al*., 1996). In the present study, using the PCR method and specific probes, we confirmed the existence of mRNAs coding for the PTH/PTHrP and PTH_2 receptors in hippocampal organotypic culture preparations. However, this merely demonstrates that these mRNAs are present and the question of whether active PTH receptor protein is expressed remained to be studied.

The PTH-induced $[Ca^{2+}]_i$ increase was found to be mediated by activation of L-type Ca^{2+} channels that are

Table 1 Effects of various types of voltage-dependent Ca^{2+} channel blockers on the PTH_{1-34} induced PI intercalation to organotypic slice culture of rat hippocampus

Treatment	*Dose*	n	*Average PI fluroescence with s.e.mean*	*Statistics (t-test)*
Control	–	12	30.0±4.6	
PTH_{1-34}	1 nM	12	51.2±3.3	$P<0.01$ vs control
PTH_{1-34} + Nifedipine	1 nM + 10 μM	12	39.5±1.2	$P<0.01$ vs PTH_{1-34} alone
PTH_{1-34} + ω-conotoxin GVIA	1 nM + 5 μM	12	56.7±5.0	N.S. vs PTH_{1-34} alone
PTH_{1-34} + ω-conotoxin MVIIC	1 nM + 5 μM	12	59.9±8.7	N.S. vs PTH_{1-34} alone $P<0.01$
PTH_{1-34} + $NiCl_2$	1 nM + 100 μM		82.1±11.2	vs PTH_{1-34} alone

Each drug was dissolved in culture medium and administered into the culture dish to make final concentration indicated. Average PI fluorescence was calculated according to the method described in Methods.

expressed on the hippocampal cell membrane during exposure to PTH_{1-34}. This was confirmed pharmacologically by studying the effects of common blockers for voltage-operated Ca^{2+} channels, such as nifedipine (an L-type antagonist), ω-conotoxin GVIA (an N-type antagonist), ω-conotoxin MVIIC (a P/Q-type antagonist) and $NiCl_2$ (a T/R-type antagonist). We examined the toxicity of these antagonists at doses shown, in our previous study (Watanabe *et al*., 1998), to be effective in blocking neuronal Ca^{2+} channels in fresh slice preparations and found that doses of those antagonists that are effective on channels showed little or no toxicity on cultures during 1 week of administration. However higher concentrations of those antagonists were themselves toxic. Although we have no evidence proving the activating effects of $NiCl_2$ on the PTH_{1-34} toxicity, T/R-type channel may be important in maintaining cell viability, since the T/R-type channel blocker facilitated the action of PTH_{1-34}.

The time required for an obvious increase in the $[Ca^{2+}]_i$ in organotypic slice cultures was similar to that seen using disperse-cultures of hippocampal cells (Hirasawa *et al*., 1998). Since the effect of PTH on the $[Ca^{2+}]_i$ in distal renal tube epithelial cells has been shown to be due to induction of L-type Ca^{2+} channels, the toxicity induced by PTH_{1-34} in organotypic culture preparations might be triggered by overloading of the $[Ca^{2+}]_i$, which might be induced by mechanisms analogous to the effect of PTH on renal tube epithelial cells (Bacsky & Freedman, 1990). Recently, an increase in L-type calcium channels in hippocampal neurons during aging has been reported, but the mechanism involved is unknown (Landfield, 1996; Thibault & Landfield, 1996; Porter *et al*., 1997). The present results may be a clue to identifying the mechanisms involved in this age-related change in hippocampal cell properties.

Since the blood PTH level in normal adult subject has been reported to be 43.6 ± 4.5 pg ml^{-1} (about 4.6 ± 0.5 pM, since molecular weight of PTH being 9424.7) (Fujita *et al*., 1997), the significant toxicity seen at a concentration of 10 pM may cause problems in regions of the brain containing high densities of PTH_2 receptors. Although we have no direct evidence to prove translocation of blood PTH to the central nervous system through the blood-brain barrier, the fact that the PTH level in the cerebrospinal fluid in hyperparathyroidism patients is higher than that in normal subjects seems to be indirect evidence for the penetration of PTH into the brain through the blood-brain barrier (Lindall *et al*., 1983; Joborn *et al*., 1991). Furthermore, recent studies have demonstrated degradation of the blood-brain barrier during senescence (Ueno *et al*., 1997). It is possible that certain populations of elderly subjects with defective blood-brain barrier, may be at some risk from the adverse effects of PTH.

The present results warn of the possible danger of a high PTH level causing hippocampal neuronal and glial cell deterioration through paradoxical Ca^{2+} overload. Although our data shown here were only concerned cultured preparations, and although it remains to examine the toxicity of high levels of PTH on the adult or senile rat brain, clinical evidence for toxicity of PTH has been shown in patients with uremia or hyperparathyroidism who show abnormality in psychological behaviour (Petersen, 1968) and on electro-encephalogram (Guisado *et al*., 1975); this evidence may support our present demonstration of the adverse effects of PTH on brain functions.

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