Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 10182–10186, October 1995
Neurobiology

Signaling by N- and C-terminal sequences of parathyroid hormone-related protein in hippocampal neurons

SHOICHI FUKAYAMA*†, ARMEN H. TASHJIAN, JR.*, JOHN N. DAVIS‡, AND JANE C. CHISHOLM‡

*Department of Molecular and Cellular Toxicology, Harvard School of Public Health, and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115; and ‡Institute for Dementia Research, Bayer Corp., West Haven, CT 06516

Communicated by Gerald D. Fischbach, Harvard Medical School, Boston, MA, July 19, 1995

ABSTRACT Parathyroid hormone-related protein (PTHrP) is synthesized in the brain, and a single type of cloned receptor for the N-terminal portion of PTHrP and PTH is present in the central nervous system. Nothing is known about the physiological actions or signaling pathways used by PTHrP in the brain. Using cultured rat hippocampal neurons, we demonstrate that N-terminal PTHrP[1–34] and PTH[1–34] signal via cAMP and cytosolic calcium transients. The cAMP response showed strong acute (≤6 h) homologous and heterologous desensitization after preincubation with PTHrP or PTH. In contrast, the acute calcium response did not desensitize after preincubation with PTHrP; in fact, preincubation dramatically recruited additional responsive neurons. Unexpectedly, C-terminal PTHrP[107–139], which does not bind or activate the cloned PTH/PTHrP receptor, signaled in neurons via cytosolic calcium but not cAMP. Although some neurons responded to both PTHrP[1–34] and PTHrP[107–139], others responded only to PTHrP[1–34]. We conclude that certain hippocampal neurons exhibit dual signaling in response to PTHrP[1–34] and that some neurons have a receptor for C-terminal PTHrP that signals only via cytosolic calcium.

The gene encoding the human parathyroid hormone-related protein (hPTHrP), a polypeptide of 141 amino acid residues, was first identified and cloned from malignant tumor cells and tissues from patients with the syndrome of humoral hypercalcemia of malignancy (1–4). Subsequently, it was shown that the PTHrP gene is expressed at the protein and/or mRNA level in several normal tissues, including bone, kidney, brain, placenta, skin, and certain fetal organs (5–9). N-terminal PTHrP[1–34] and PTH[1–34], which have a high degree of sequence identity in their most N-terminal portions, act via a single species of cloned PTH/PTHrP receptor (PTH/PTHrPR) (10–15), although certain PTHrP-specific actions have been described (16–21). The PTH/PTHrPR is also widely distributed (22, 23), implying that PTHrP acts physiologically in normal tissues (24). It has recently been shown that the PTH/PTHrP receptor and/or its mRNA are expressed in the rat brain (25, 26). Targeted disruption of the PTHrP gene is lethal (27).

Biological responses to PTHrP can be elicited by the N-terminal 1–34 or 1–36 fragments, although multiple forms including mid-portion and C-terminal fragments have been identified in the circulation (7, 9). These PTHrP fragments are derived from the cleavage of the intact 1–141 molecule by specific enzymes (7, 9). It is not known whether the fragments have physiological actions, although some unique effects of the C-terminal 107–139 peptide, which does not bind to the cloned PTH/PTHrPR (10, 15), have been described (28, 29). No mechanistic studies of PTHrP signaling in the central nervous system have been reported. In this paper, we demonstrate that both N- and C-terminal PTHrP peptides act directly on hippocampal neurons to cause cAMP production and/or Ca^{2+} influx. Unlike classical PTH/PTHrP target tissues, there was no acute homologous desensitization of neuronal Ca^{2+} responses to these peptides; in fact, preincubation with PTHrP dramatically recruited additional responsive neurons.

MATERIALS AND METHODS

Primary Cell Culture. Neurons, dissociated from newborn rat brains (<48 h old) were maintained in primary cell culture as described (30, 31) with minor modifications. Briefly, pups were decapitated and their brains were placed in oxygenated HBSS buffer (118 mM NaCl/4.6 mM KCl/0.4 mM $CaCl_2$/10 mM D-glucose/20 mM Hepes, pH adjusted to 7.2). The hippocampal formation (including the dentate gyrus) was dissected, stripped of meninges and blood vessels, and incubated for 30 min (37°C, 5% CO_2) with gentle shaking in HBSS buffer containing 1.5 mM $CaCl_2$, 0.5 mM EDTA, 0.2 mg of L-cysteine per ml, and 7.5 units of papain per ml. The tissue was then rinsed and gently triturated with a fire-polished glass pipette in an oxygenated solution containing 137 mM NaCl, 5.3 mM KCl, 1.0 mM $MgCl_2$, 25.0 mM D-glucose, 10.0 mM Hepes, 3.0 mM $CaCl_2$, 1.5 mg of trypsin inhibitor per ml, and 1.5 mg of bovine albumin per ml. Cells were plated and maintained in 45% Dulbecco's modified Eagle medium, 45% Ham's F12 medium, 10% fetal bovine serum, 100 μg of penicillin and streptomycin per ml, and 0.5 μg of fungizone per ml in a humidified atmosphere at 37°C and 5% CO_2. As reported previously (30), these primary cultures from the hippocampus contain >98% neuronal and glial cells.

Calcium Imaging. Cells were plated in 35-mm culture dishes on 22 × 22 mm^2 Corning glass coverslips coated with poly(L-ornithine) and laminin. For measurement of cytosolic free calcium concentration ($[Ca^{2+}]_i$) using epifluorescence microscopy, $[Ca^{2+}]_i$ was monitored as the Ca^{2+}-dependent fluorescence emission from neuronal soma in cells loaded with fura-2, using an image system and protocol as described (32). Briefly, cells on coverslips were incubated with 5 μM fura-2 AM, at a final concentration of 0.5% dimethyl sulfoxide for 30 min at 37°C. The monolayers were rinsed in buffer and placed in a slide chamber for microscopic viewing and measurement at room temperature (23°C). Fluorescence was detected with a Nikon Diaphot inverted microscope fitted with a 40× Fluor objective (numerical aperture = 1.3; Nikon). Excitatory illumination was provided by a 150-W xenon arc lamp. The beam was split, filtered at 340 nm or 380 nm (Omega Optical, Brattleboro, VT), and continuously monitored for constant

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; PTH/PTHrPR, PTH/PTHrP receptor; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; Bt_2cAMP, $N^6,O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphate; PMA, phorbol 12-myristate 13-acetate; Fsk, forskolin; h, human.

†To whom reprint requests should be addressed at: Department of Molecular and Cellular Toxicology, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115.

intensity using a photodiode before passing to the sample. Fura-2 emission, during alternate excitation at 340 nm and 380 nm, was filtered by long-pass and barrier filters for 490–510 nm and quantified using a photomultiplier tube (Hamamatsu, Bridgewater, CT).

For $[Ca^{2+}]_i$ measurements using scanning laser confocal microscopy, cells were cultured for 5–6 days and then incubated with 5 μM fluo-3AM for 60 min at 37°C, rinsed free of unaccumulated dye, and placed in a microscope chamber in Hepes buffer (118 mM NaCl/1 mM $CaCl_2$/1 mM KCl/2 mM glucose, pH adjusted to 7.0). The chamber was positioned on a microscope (Zeiss Axiovert), fitted with a Bio-Rad MRC 600 confocal laser scanning system, and a representative field, containing 12–20 neurotypic cells, was monitored at room temperature, as described (31). Excitation was provided by a krypton/argon laser filtered for the 488-nm line. Images presented are averages of five frames collected at 1.8-sec resolution. Additions were applied directly to the open slide chamber. In each experiment, we established that a significant increase in $[Ca^{2+}]_i$ was induced in virtually all neurons in response to high K^+ depolarization.

Measurement of cAMP. Cell monolayers in 6- or 24-well plates were preincubated with vehicle alone (control) or with test agents or forskolin for the times indicated. After washing with HBSS buffer, cells were incubated without (control) or with peptides for 15 min at 37°C in the presence of 1 mM isobutylmethylxanthine. The concentration of cAMP in acid (50 mM HCl) extracts of the cells was measured by radioimmunoassay as described (33).

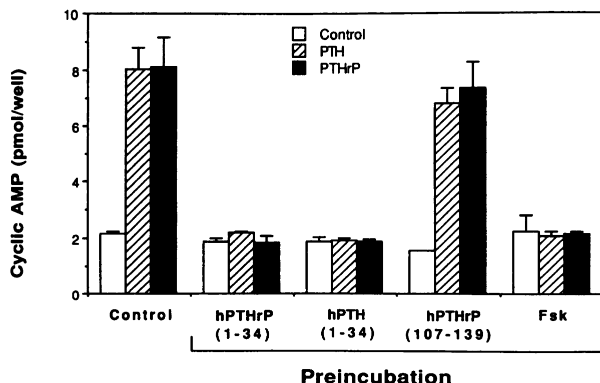

FIG. 1. Homologous and heterologous cAMP desensitization in primary cultures of neuronal and glial cells from the newborn (2-day-old) rat hippocampus. Cells in 6- or 24-well plates were preincubated with vehicle alone (Control) or with 0.1 μM hPTHrP[1–34], 0.1 μM hPTH[1–34], 0.1 μM hPTHrP[107–139], or 0.01 mM Fsk for 6 h. After washing with HBSS buffer, cells were incubated without (Control) or with hPTHrP[1–34] (0.01 μM), hPTH[1–34] (0.01 μM), or Fsk (0.01 mM) for 15 min at 37°C in the presence of 1 mM isobutylmethylxanthine, and the concentration of cAMP in the cells was measured by radioimmunoassay. hPTHrP[1–34] and hPTH[1–34] each induced complete homologous (to the same peptide) and heterologous (to the other peptide) cAMP desensitization, whereas hPTHrP[107–139] was without effect. cAMP concentrations in response to Fsk (0.01 mM) after preincubation without (Control) or with hPTHrP[1–34], hPTH[1–34], hPTHrP[107–139], and Fsk were 14 ± 1.1, 43 ± 2.2, 23 ± 0.7, 14 ± 1.1, and 4.0 ± 0.5 pmol per well, respectively. Thus, as in osteoblastic cells (34), there is significant (≈73%) Fsk-induced homologous cAMP desensitization to Fsk in these primary neuronal cultures. Each bar gives the mean value ± SE for four cultures. Similar results were obtained in two additional experiments of similar design. For a majority of experiments, neurons were dissociated from newborn (<48-h-old) rat brains and maintained in primary cell culture. In several cAMP experiments, we have also used cells freshly dissociated from 4-day-old rats, and the results were not significantly different from those obtained from 2-day-old rats. The cAMP desensitization after preincubation with PTHrP[1–34], PTH[1–34], and Fsk was statistically significant in each case ($P < 0.01$).

Materials. Long Evans rats were purchased from Charles River Breeding Laboratories. Papain was from Worthington. Culture medium, serum, and fungizone were obtained from GIBCO. Culture and plasticware were from Corning. Synthetic PTH and PTHrP were purchased from Bachem California (Torrance, CA) as hPTH[1–34], lot ZL 216; hPTHrP, lots E 599 and QE 599; and hPTHrP[107–139], lots ZJ 949 and QJ 949. Phorbol 12-myristate 13-acetate (PMA) was from LC Services (Woburn, MA). The fluo-3 AM and fura-2 AM were from Molecular Probes. ^{125}I-labeled cAMP and cAMP antiserum were obtained from Biomedical Technologies (Stoughton, MA). $N^6,O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphate (Bt_2cAMP) and forskolin (Fsk) were from Sigma, as were other reagent grade chemicals.

RESULTS

Actions of PTHrP on cAMP Production. We first examined the cAMP responses in hippocampal cultures to several PTH/PTHrP peptides. hPTHrP[1–34] and hPTH[1–34] stimulated cAMP production to similar magnitudes (Fig. 1), while hPTHrP[107–139], at concentrations up to 0.1 μM, was without effect (data not shown). Preincubation with hPTHrP[1–34] or hPTH[1–34] for 6 h induced almost complete homologous and heterologous cAMP desensitization (Fig. 1). Similar desensitization was induced by preincubation with Fsk, a direct stimulator of adenylate cyclase (35), while preincubation with hPTHrP[107–139] did not induce desensitization (Fig. 1). Similar results were obtained when the cells were pretreated with these peptides for 24 h (data not shown). These findings demonstrate that the PTH/PTHrPR is expressed on neuronal and/or glial cells from the newborn rat hippocampus.

Actions of PTHrP on $[Ca^{2+}]_i$. We next examined the action of hPTHrP on $[Ca^{2+}]_i$ in hippocampal neurons. Incubation with hPTHrP[1–34] induced acute elevations in $[Ca^{2+}]_i$ in some (10–20%), but not all, neurons (Figs. 2*A* and 3*A* and *B*). There was a significant time lag (2–5 min) between the addition of peptides and the $[Ca^{2+}]_i$ responses that were characterized by reproducible transient and variable sustained phases (Fig. 2). The same pattern of neuronal $[Ca^{2+}]_i$ responses was observed after

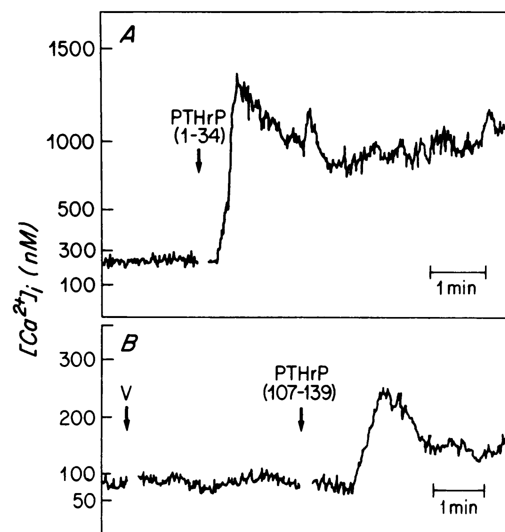

FIG. 2. Action of PTHrP on $[Ca^{2+}]_i$ in a single neuron. Cultures were treated with hPTHrP[1–34] (0.1 μM) (*A*) or hPTHrP[107–139] (0.1 μM) (*B*). Typical patterns of change in $[Ca^{2+}]_i$ in single neurons, measured by epifluorescence microscopy, are shown. Neuronal $[Ca^{2+}]_i$ elevations were characterized by both transient and sustained phases. Vehicle (V) alone was without effect. A significant lag (≈2 min) between the addition of peptides and neuronal $[Ca^{2+}]_i$ responses was observed. Similar results were obtained in multiple additional independent experiments of similar design.

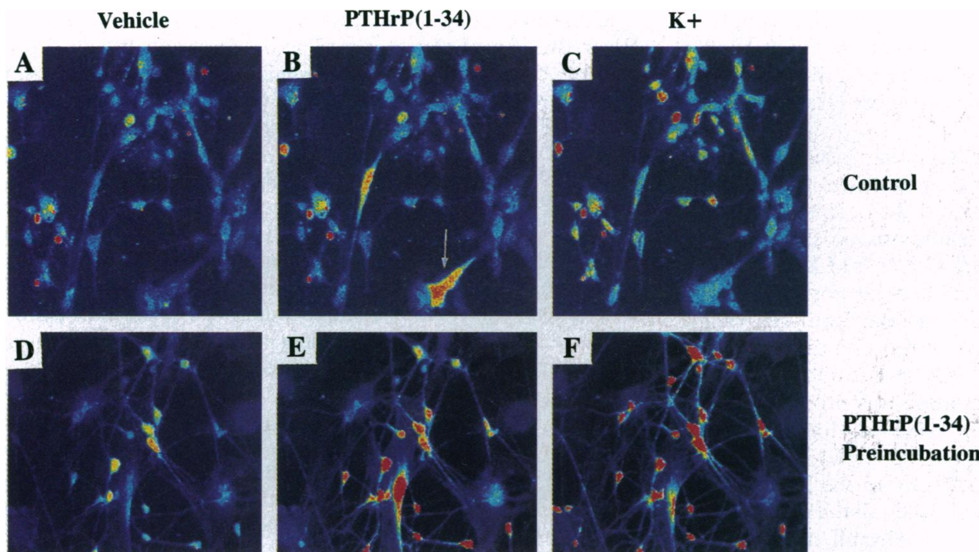

FIG. 3. Calcium responses of individual cells to PTHrP[1–34] and high K^+ in control cultures and in cultures preincubated with PTHrP[1–34]. Cells were preincubated without (Control, *A–C*) or with 0.1 μM hPTHrP[1–34] (*D–F*) for ≈7 h. After washing with HBSS buffer, cells were treated acutely with 0.1 μM hPTHrP[1–34] (*B* and *E*) or 50 mM K^+ (*C* and *F*). Confocal images of individual cells are shown 5 min after exposure (*A* and *B*), 2 min after exposure (*D* and *E*), and 30 sec after exposure (*C* and *F*). The arrow in *B* indicates a responding glial cell. Vehicle alone was without effect. Similar results were obtained in two additional experiments of similar design.

addition of hPTH[1–34] (data not shown), and a similar pattern was induced in a subpopulation of neurons after addition of Bt_2cAMP (Fig. 4) or the protein kinase C activator PMA (data not shown). As in neuronal $[Ca^{2+}]_i$ responses to the peptides, there was a significant time lag (2–5 min) between the addition of Bt_2cAMP (or PMA) and the $[Ca^{2+}]_i$ responses. In all cases, vehicle alone was without effect.

Unexpectedly, incubation with hPTHrP[107–139] also activated neuronal $[Ca^{2+}]_i$ responses (Figs. 2*B*, 5 *A* and *B*). Like the response to hPTHrP[1–34], hPTHrP[107–139] elicited $[Ca^{2+}]_i$ spikes in only a subpopulation (10–20%) of neurons (Fig. 5 *A* and *B*). Although we have not yet performed complete dose–response experiments, we have observed neuronal $[Ca^{2+}]_i$ responses to each of these peptides at concentrations as low as 0.1 pM and regularly obtain responses at 1 nM for hPTHrP[1–34] and hPTHrP[107–139]. PTHrP[107–139] cannot be significantly contaminated with PTHrP[1–34] because it elicits no cAMP response even at 0.1 μM. Cells that did not respond to peptides with elevations in $[Ca^{2+}]_i$ were capable of $[Ca^{2+}]_i$ responses to depolarization, indicating that they were responsive and capable of $[Ca^{2+}]_i$ signaling and supporting our conclusion that PTHrP peptides induced an elevation of $[Ca^{2+}]_i$ in a subpopulation of neurons. Chelation of extracellular calcium with EGTA completely eliminated the neuronal $[Ca^{2+}]_i$ responses to these peptides (data not shown), indicating that they required entry of extracellular calcium.

Heterogeneity of Neuronal $[Ca^{2+}]_i$ Responses to PTHrP. To determine whether the same cells were responding to hPTHrP[1–34] and hPTHrP[107–139], we compared the pattern of $[Ca^{2+}]_i$ responses in individual neurons as a function of time. Addition of hPTHrP[1–34], after initial exposure to hPTHrP[107–139], caused elevation of $[Ca^{2+}]_i$ in the same neuron as well as in other neurons (Fig. 6). Addition of hPTHrP[107–139], after initial exposure to hPTHrP[1–34], also induced $[Ca^{2+}]_i$ spikes in the same neuron as well as in additional neurons (data not shown). These findings demonstrate that hPTHrP[1–34] and hPTHrP[107–139] elicited $[Ca^{2+}]_i$ transients in the same and different neurons, presumably via different receptors, because PTHrP[107–139] does not bind to the known PTH/PTHrPR. Periodic spikes in neuronal $[Ca^{2+}]_i$ persisted (≥3 min) in the continued presence of PTHrP and often extended to additional neurons that did not respond initially (Fig. 6). Many of the $[Ca^{2+}]_i$ responses occurred in synchrony (Fig. 6), suggesting intercellular coupling. Delayed $[Ca^{2+}]_i$ responses were occasionally observed in some, but not all, glial cells (Fig. 3*B*).

Homologous Ligand-Induced Regulation of Neuronal $[Ca^{2+}]_i$ Responses in Hippocampal Cultures. As shown in Fig. 1, preincubation with hPTHrP[1–34] or hPTH[1–34] induced complete cAMP desensitization to a second challenge with these peptides. However, preincubation with hPTHrP[1–34] or hPTHrP[107–139] for 6–8 h did not desensitize the neuronal $[Ca^{2+}]_i$ changes to a second challenge with the same peptides; rather, preincubation dramatically increased the number of neurons responding (compare Fig. 3 *D* and *E* with *A* and *B* and Fig. 5 *D* and *E* with *A* and *B*). Prolonged preincubation (20 h) with hPTHrP[1–34]/hPTH[1–34] dramatically decreased neuronal $[Ca^{2+}]_i$ responses to an acute rechallenge with the same peptide (homologous $[Ca^{2+}]_i$ desensitization) but not to acute rechallenge with hPTHrP[107–139], and vice versa (Fig. 7), results consistent with the concept that hPTHrP[1–34] and hPTHrP[107–139] activate, at least in part, different populations of neurons. An increase in $[Ca^{2+}]_i$ was induced by high K^+ in virtually all neurons, including those that did or did not respond to PTHrP peptides, indicating that they were responsive and capable of $[Ca^{2+}]_i$ signaling.

DISCUSSION

From the results of this study, we conclude that hPTHrP[1–34] and hPTHrP[107–139] induce acute elevations in $[Ca^{2+}]_i$ in hippocampal neurons apparently via distinct receptors, at least some of which are expressed on different subpopulations of cells. We do not yet know the second messenger signaling pathways used by these peptides to elicit neuronal $[Ca^{2+}]_i$ transients. A single species of the cloned N-terminal PTH/

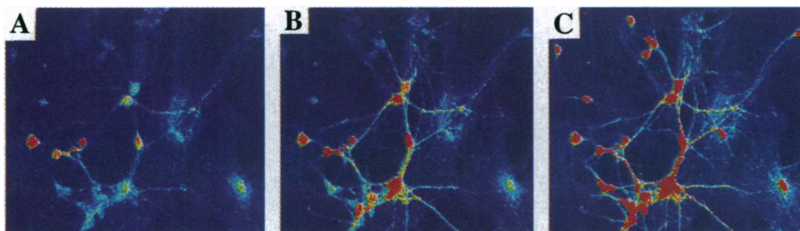

FIG. 4. Action of Bt_2cAMP and K^+ on $[Ca^{2+}]_i$ in primary cultures of hippocampal neurons. Confocal images of individual neurons in response to vehicle (*A*, 3 min after addition), 1 mM Bt_2cAMP (*B*, 3 min after addition), and 50 mM K^+ (*C*, 30 sec after addition) are shown. Similar results were obtained in two additional experiments of similar design.

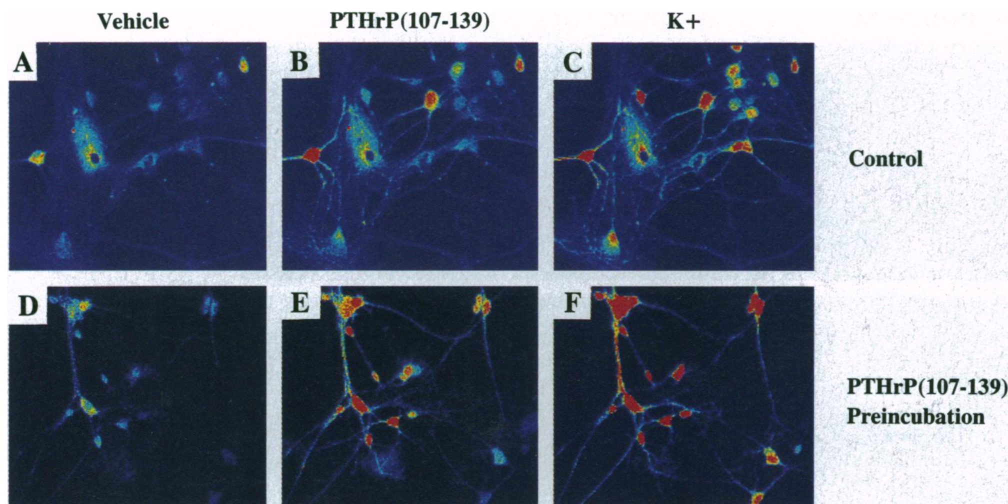

FIG. 5. Calcium responses of individual cells to PTHrP[107–139] and high K^+ in control cultures and in cultures preincubated with PTHrP[107–139]. Cells were preincubated without (Control, *A–C*) or with 0.1 μM hPTHrP [107–139] (*D–F*) for ≈7 h. After washing with HBSS buffer, cells were treated acutely with 0.1 μM hPTHrP[107–139] (*B* and *E*) or 50 mM K^+ (*C* and *F*). Confocal images of individual cells are shown 3 min after addition (*A*), 2 min after addition (*B*), 3 min after addition (*D*), 1 min after addition (*E*), and 30 sec after addition (*C* and *F*). Vehicle alone was without effect. Similar results were obtained in two additional experiments of similar design.

PTHrPR can couple to the cAMP/protein kinase A (PKA) and inositol lipid/Ca^{2+} pathways in classical PTH target cells (11, 13, 36). For receptors that couple to phospholipase C, the inositol 1,4,5-trisphosphate (IP_3)-mediated $[Ca^{2+}]_i$ transients occur rapidly (≤10 sec) (37). In neuronal cultures, we note a significant time lag (1–5 min) between addition of the peptides and the $[Ca^{2+}]_i$ responses. Moreover, there was no neuronal Ca^{2+} response in the absence of extracellular Ca^{2+}. These findings argue against the involvement of the classical action of IP_3 in the effect of PTHrP peptides on $[Ca^{2+}]_i$ in the hippocampal neurons. Delayed $[Ca^{2+}]_i$ responses to PTH have been demonstrated in some osteoblastic cells (33). Because incubation with Bt_2cAMP induced a similar pattern of neuronal $[Ca^{2+}]_i$ transients, alterations in $[Ca^{2+}]_i$ in hippocampal neurons by hPTHrP[1–34] may be mediated by the cAMP/PKA pathway. The mechanism by which hPTHrP[107–139] elicits neuronal $[Ca^{2+}]_i$ responses does not appear to involve the cAMP pathway, because PTHrP[107–139] did not affect cellular cAMP concentrations.

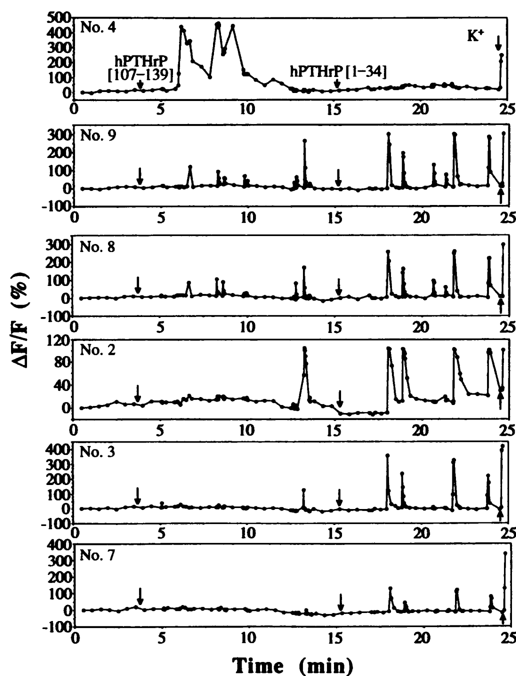

FIG. 6. Traces of fluo-3 AM fluorescence intensity in individual hippocampal neurons. Eleven neurons (six are shown), which revealed no spontaneous changes in $[Ca^{2+}]_i$, were randomly selected from the same monolayer. Additions of 0.1 μM hPTHrP[107–139], 0.1 μM hPTHrP[1–34], and 50 mM K^+ are indicated (arrows). Neuron 4 gave a $[Ca^{2+}]_i$ response to hPTHrP[107–139] but not hPTHrP[1–34], whereas neuron 7 responded to hPTHrP[1–34] but not hPTHrP[107–139]. Other neurons (nos. 9, 8, 2, and 3) responded to both peptides. Five neurons (not shown) in the same monolayer did not respond to either hPTHrP[107–139] or hPTHrP[1–34], although increases in $[Ca^{2+}]_i$ in all of these cells were induced by K^+. $[Ca^{2+}]_i$ responses to these peptides were often synchronized (compare the pattern of $[Ca^{2+}]_i$ spikes in neurons 4, 9, and 8 for hPTHrP[107–139] and neurons 9, 8, 2, 3, and 7 for hPTHrP[1–34]). Similar results were observed in two additional experiments of similar design. F, fluorescence.

The neuronal Ca^{2+} responses to these peptides require extracellular Ca^{2+}, indicating that some plasma membrane Ca^{2+} channel is importantly involved in the responses. It is intriguing to note that, as was the case in neuronal $[Ca^{2+}]_i$ responses to PTH/PTHrP, treatment with Bt_2cAMP or PMA activated $[Ca^{2+}]_i$ spikes only in subpopulations of the neurons. Taken together, these findings suggest that any putative intermediate neurotransmitter signaling pathway, potentially involved in the action of PTHrP, is also locally activated in a subpopulation of hippocampal neurons.

A particularly unexpected finding was that preincubation with hPTHrP[1–34] or hPTHrP[107–139] for 6–8 h did not induce homologous neuronal $[Ca^{2+}]_i$ desensitization but dramatically enhanced the $[Ca^{2+}]_i$ responses to a second challenge with the homologous peptide. Prolonged pretreatment (≥20 h), however, did induce homologous $[Ca^{2+}]_i$ desensitization. Homologous desensitization has been well recognized in the PTH/PTHrP-induced cAMP and calcium signaling pathways in classical PTH/PTHrP target tissues such as bone (38, 39). In fact, we have found that acute (≤6 h) preincubation of neuronal cultures with hPTHrP[1–34] or hPTH[1–34] did induce dramatic homologous cAMP desensitization. We do not yet have an explanation for the discordance between the cAMP and $[Ca^{2+}]_i$ desensitization responses in neurons. It is possible that the difference between cAMP and calcium desensitization might be a reflection of cell-specific dual signaling by a single receptor type or signaling via different specific receptors.

Our findings that N-terminal and C-terminal PTHrP elicit cAMP and/or $[Ca^{2+}]_i$ spikes in different subpopulations of neurons suggest a differential role for these peptides in selected hippocampal cells and imply a functional role for PTHrP in the regulation of central nervous system neurons.

Note. After submission of this manuscript, Usdin *et al.* (40) described a type of PTH receptor (PTH2 receptor) that is activated by PTH but not by PTHrP.

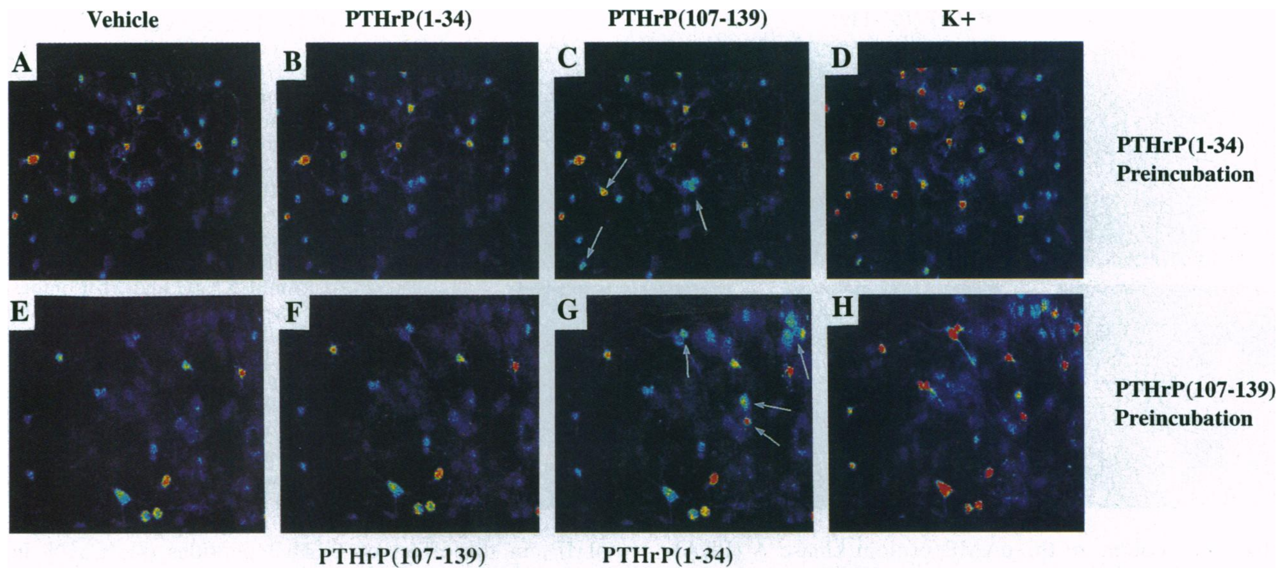

FIG. 7. Ligand-specific homologous $[Ca^{2+}]_i$ desensitization in hippocampal neurons to hPTHrP[1–34] and hPTHrP[107–139]. Cells were preincubated with 0.1 μM hPTHrP[1–34] (*A–D*) or 0.1 μM hPTHrP[107–139] (*E–H*) for 20 h. After washing with HBSS buffer, cells were treated acutely with 0.1 μM hPTHrP[1–34] or 0.1 μM hPTHrP[107–139]. Confocal images of individual neurons in response to vehicle (*A*, 10 min after addition), hPTHrP[1–34] (*B*, 5 min after addition), hPTHrP[107–139] (*C*, 1.5 min after addition), or 50 mM K^+ (*D*, 30 sec after addition) are shown. Preincubation with hPTHrP[1–34] for 20 h essentially abolished the neuronal $[Ca^{2+}]_i$ response to the second challenge with hPTHrP[1–34] (*B*) but not with hPTHrP[107–139] (*C*). Arrows indicate the responding neurons. Similarly, confocal images of individual neurons in response to vehicle (*E*, 2 min after addition), hPTHrP[107–139] (*F*, 2 min after addition), hPTHrP[1–34] (*G*, 2 min after addition), and 50 mM K^+ (*F*, 30 sec after addition) are shown. Pretreatment with hPTHrP[107–139] essentially abolished the neuronal $[Ca^{2+}]_i$ response to the second challenge with hPTHrP[107–139] (*F*) but not with hPTHrP[1–34] (*G*). Before each experiment, we confirmed that both peptides stimulated Ca^{2+} transients in a subpopulation (10–20%) of neurons in control cultures without preincubation. These responses were indistinguishable from those shown in Fig. 3 (for N-terminal PTHrP) and Fig. 5 (for C-terminal PTHrP). Arrows indicate the responding neurons. Similar results were obtained in two and three additional experiments of similar design for preincubation with N-terminal PTHrP and C-terminal PTHrP, respectively.

We thank Jean Foley for assistance in the preparation of this manuscript. This investigation was supported in part by a research grant from the National Institute of Diabetes, Digestive and Kidney Diseases (DK 46655).

1. Suva, L. J., Winslow, G. A., Wettenhall, R. E. H., Hammonds, R. G., Moseley, J. M., Diefenbach-Jagger, H., Rodda, C. P., Kemp, B. E., Rodriguez, H., Chen, E. Y., Hudson, P. J., Martin, T. J. & Wood, W. I. (1987) *Science* **237,** 893–896.
2. Moseley, J. M., Kubota, M., Diefenbach-Jagger, H., Wettenhall, R. E. H., Kemp, B. E., Suva, L. J., Rodda, C. P., Ebeling, P. R., Hudson, P. J., Zajac, J. D. & Martin, T. J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 5048–5052.
3. Strewler, G. J., Stern, P. H., Jacobs, J. W., Eveloff, J., Klein, R. F., Leung, S. C., Rosenblatt, M. & Nissenson, R. A. (1987) *J. Clin. Invest.* **80,** 1803–1807.
4. Mangin, M., Webb, A. C., Dreyer, B. E., Posillico, J. T., Ikeda, K., Weir, E. C., Stewart, A. F., Bander, N. H., Milstone, L., Barton, D. E., Francke, U. & Broadus, A. E. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 597–601.
5. Moniz, C., Burton, P. B. J., Malik, A. N., Dixit, M., Banga, J. P., Nicolaides, K., Quirke, P., Knight, D. E. & McGregor, A. M. (1990) *J. Mol. Endocrinol.* **5,** 259–266.
6. Campos, R. V., Asa, S. L. & Drucker, D. J. (1991) *Cancer Res.* **51,** Pt. 1, 6351–6357.
7. Martin, T. J., Moseley, J. M. & Gillespie, M. T. (1991) *Crit. Rev. Biochem. Mol. Biol.* **26,** 377–395.
8. de Papp, A. E. & Stewart, A. F. (1993) *Trends Endocrinol. Metab.* **4,** 181–187.
9. Orloff, J. J., Reddy, D., de Papp, A. E., Yang, K. H., Soifer, N. E. & Stewart, A. F. (1994) *Endocr. Rev.* **15,** 40–60.
10. Jüppner, H., Abou-Samra, A.-B., Freeman, M., Kong, X. F., Schipani, E., Richards, J., Kolakowski, L. F., Jr., Hock, J., Potts, J. T., Jr., Kronenberg, H. M. & Segre, G. V. (1991) *Science* **254,** 1024–1026.
11. Abou-Samra, A.-B., Jüppner, H., Force, T., Freeman, M. W., Kong, X. F., Schipani, E., Urena, P., Richards, J., Bonventre, J. V., Potts, J. T., Jr., Kronenberg, H. M. & Segre, G. V. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 2732–2736.
12. Schipani, E., Karga, H., Karaplis, A. C., Potts, J. T., Jr., Kronenberg, H. M., Segre, G. V., Abou-Samra, A.-B. & Jüppner, H. (1993) *Endocrinology* **132,** 2157–2165.
13. Bringhurst, F. R., Jüppner, H., Guo, J., Urena, P., Potts, J. T., Jr., Kronenberg, H. M., Abou-Samra, A.-B. & Segre, G. V. (1993) *Endocrinology* **132,** 2090–2098.
14. Kaufmann, M., Muff, R., Born, W. & Fischer, J. A. (1994) *Mol. Cell. Endocrinol.* **104,** 21–27.
15. Pines, M., Adams, A. E., Stueckle, S., Bessalle, R., Rashti-Behar, V., Chorev, M., Rosenblatt, M. & Suva, L. J. (1994) *Endocrinology* **135,** 1713–1716.
16. Rodda, C. P., Kubota, M., Heath, J. A., Ebeling, P. R., Moseley, J. M., Care, A. D., Caple, I. W. & Martin, T. J. (1988) *J. Endocrinol.* **117,** 261–271.
17. Thiede, M. A. & Rodan, G. A. (1988) *Science* **242,** 278–280.
18. Thiede, M. A. (1989) *Mol. Endocrinol.* **3,** 1443–1447.
19. Insogna, K. L., Stewart, A. F., Morris, C. A., Hough, L. M., Milstone, L. M. & Centrella, M. (1989) *J. Clin. Invest.* **83,** 1057–1060.
20. Stewart, A. F., Elliot, J., Burtis, W. J., Wu, T. & Insogna, K. L. (1989) *Endocrinology* **124,** 642–648.
21. Thurston, A. W., Cole, J. A., Hillman, L. S., Im, J. H., Thorne, P. K., Krause, W. J., Jones, J. R., Eber, S. L. & Forte, L. R. (1990) *Endocrinology* **126,** 1183–1190.
22. Urena, P., Kong, X.-F., Abou-Samra, A.-B., Jüppner, H., Kronenberg, H. M., Potts, J. T., Jr., & Segre, G. V. (1993) *Endocrinology* **133,** 617–623.
23. Tian, J., Smogorze, M., Kedes, L. & Massry, S. G. (1993) *Am. J. Nephrol.* **13,** 210–213.
24. Amizuka, N., Warshawsky, H., Henderson, J. E., Goltzman, D. & Karaplis, A. C. (1994) *J. Cell Biol.* **126,** 1611–1623.
25. Weaver, D. R., Deeds, J. D., Lee, K. & Segre, G. V. (1995) *Mol. Brain Res.* **28,** 296–310.
26. Struckhoff, G. & Turzynski, A. (1995) *Brain Res.* **676,** 1–9.
27. Karaplis, A. C., Luz, A., Glowacki, J., Bronson, R. T., Tybulewicz, V. L. J., Kronenberg, H. M. & Mulligan, R. C. (1994) *Genes Dev.* **8,** 277–289.
28. Fenton, A. J., Martin, T. J. & Nicholson, G. C. (1994) *J. Bone Miner. Res.* **9,** 515–519.
29. Whitfield, J. F., Isaacs, R. J., Chakravarthy, B. R., Durkin, J. P., Morley, P., Neugebauer, W., Williams, R. E., Willick, G. & Rixon, R. H. (1994) *J. Cell. Physiol.* **158,** 518–522.
30. Banker, G. A. (1980) *Science* **209,** 809–810.
31. Cornell-Bell, A. H., Finkbeiner, S. M., Cooper, M. S. & Smith, S. J. (1990) *Science* **247,** 470–473.
32. Tsien, R.-Y. & Harootunian, A. T. (1990) *Cell Calcium* **11,** 93–109.
33. Fukayama, S. & Tashjian, A. H., Jr. (1990) *Endocrinology* **126,** 1941–1949.
34. Fukayama, S., Tashjian, A. H., Jr., & Bringhurst, F. R. (1992) *Endocrinology* **131,** 1770–1776.
35. Seamon, K. B. & Daly, J. W. (1986) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **20,** 1–150.
36. Schneider, H., Feyen, J. H. M., Seuwen, K. & Movva, N. R. (1993) *Eur. J. Pharmacol.* **246,** 149–155.
37. Berridge, M. J. & Irvine, R. F. (1989) *Nature (London)* **341,** 197–205.
38. Fukayama, S., Tashjian, A. H., Jr., & Bringhurst, F. R. (1992) *Endocrinology* **131,** 1757–1769.
39. Lee, S. K. & Stern, P. H. (1994) *J. Bone Miner. Res.* **9,** 781–789.
40. Usdin, T. B., Gruber, C. & Bonner, T. I. (1995) *J. Biol. Chem.* **270,** 15455–15458.