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Heparin-insensitive calcium release from intracellular stores triggered by the recombinant human parathyroid hormone receptor

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1 In the present study we have characterized the parathyroid hormone (PTH)-induced calcium signalling in 293 cells stably transfected with the human PTH receptor cDNA. In these cells, human PTH-1(1-38) strongly stimulates adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation ($EC_{50} = 0.39$ nM) but fails to activate phosphoinositide (PI) turnover. The latter pathway is strongly activated, however, by carbachol (CCh) acting through endogenous M_3-muscarinic receptors.

2 Despite the lack of detectable inositol phosphate (IP) formation, hPTH-(1-38) elicited calcium transients ($EC_{50} = 11.2$ nM) which were comparable to the signals evoked by CCh. These signals are independent of cyclic AMP generation as cyclic AMP elevating agents did not mimic or modify the PTH response.

3 The PTH-stimulated calcium signal still occurred in calcium-free medium but was absent in cells pretreated with thapsigargin, an inhibitor of the calcium pump of the endoplasmic reticulum (ER). hPTH-(1-38) did not accelerate Mn^{2+}-influx through the plasma membrane. These data indicate that PTH releases calcium from intracellular stores.

4 Using heparin, an inhibitor of the IP_3-activated calcium release channel of the ER, we tested whether the formation of a low amount of IP_3, escaping detection by our biochemical assay, might be the origin of the PTH-induced calcium response. However, intracellular infusion of heparin through patch pipettes in voltage clamp experiments failed to block hPTH-(1-38)-induced calcium signals, whereas it abolished the CCh response.

5 The PTH response, like the CCh response, was insensitive to micromolar concentrations of ryanodine and ruthenium red, eliminating the possibility that hPTH-(1-38) stimulates calcium-induced calcium release through ryanodine receptors.

6 We conclude that the recombinant human PTH receptor stimulates calcium release from intracellular stores through a novel pathway not involving IP_3- or ryanodine receptors.

Keywords: Parathyroid hormone; cyclic AMP; inositol trisphosphate; G protein; heparin; ruthenium red; ryanodine

Introduction

Parathyroid hormone (PTH) stimulates adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation in target cells. In addition, the hormone has also been reported to increase intracellular free calcium ($[Ca^{2+}]_i$) in various cell types, as first demonstrated by Löwik *et al.* in 1985. Today, some controversy persists concerning the heterogeneity of responses and the origin of the calcium signal (Yamaguchi *et al.*, 1987; Reid *et al.*, 1987; Babich *et al.*, 1991; Civitelli *et al.*, 1992; Wiltink *et al.*, 1993). Data arguing both for PTH-stimulated calcium influx (Yamaguchi *et al.*, 1987) as well as release from intracellular stores (Babich *et al.*, 1991) have been reported. The fact that some groups found activation of protein kinase C and formation of IP_3 in response to PTH peptides provided support for the latter concept (Tamura *et al.*, 1989; Abou-Samra *et al.*, 1989; Fujimori *et al.*, 1992).

The molecular cloning of a PTH receptor from several species opened new opportunities to study PTH signal transduction. As there is no evidence for receptor subtypes, a single receptor seems to account for the different intracellular responses described for PTH (Schipani *et al.*, 1993; Kong *et al.*, 1994). Under conditions of transient expression in COS cells, the rat and human PTH receptor stimulate both cyclic AMP and inositol phosphate (IP) formation (Abou-Samra *et al.*, 1992; Schneider *et al.*, 1993). We were unable, however, to demonstrate coupling of the human receptor to the latter signalling system under the more physiological condition of stable receptor expression in 293 cells (Schneider *et al.*, 1994) or hamster fibroblasts (unpublished data). Likewise, we have observed previously that PTH failed to stimulate phosphoinositide (PI) turnover significantly in osteoblasts and various osteosarcoma cell lines, whereas other hormones like endothelin-1 were active in these cells (unpublished data). These results are in agreement with those of Babich and coworkers (1991), which suggested that thrombin and PTH elicit calcium transients through different pathways in rat osteosarcoma cells.

In the present study we have analysed the calcium signal triggered by the recombinant human PTH receptor stably expressed in 293 cells. We found that this receptor elicits calcium release from intracellular stores in the absence of detectable IP formation through a pathway which is insensitive to heparin, ryanodine, and ruthenium red.

Methods

Cells and culture conditions

293 p5 cells are human embryonic kidney 293 (HEK 293, American type culture collection) cells transfected with the human PTH receptor cDNA (Schneider *et al.*, 1994). They express approximately 1.5×10^5 receptors per cell. Cultures were grown in a 1:1 mixture of Dulbecco's modification of Eagles medium (DMEM) and Ham's F12 medium containing 10% foetal calf serum.

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Measurements of $[Ca^{2+}]_i$ in cell suspensions

293 p5 cells were detached from culture plates by incubation for 10 min in calcium/magnesium-free phosphate buffered saline containing 50 μM EDTA. No trypsin was used in order to avoid a partial destruction of membrane receptors. Following centrifugation, cells were resuspended in serum-free DMEM medium and loaded with fura-2 acetoxymethylester (2.5 μM) for 45 min at 37°C. After loading, cells were washed three times with HEPES-buffered salt solution (HBS composition, mM: NaCl 130, KCl 5.4, NaH_2PO_4 0.8, $CaCl_2$ 1.8, $MgSO_4$ 0.9, glucose 10 and HEPES 20, pH 7.4) and cell density was adjusted to $\approx 5 \times 10^5$ cells ml^{-1}. Experiments were carried out in HBS using a Perkin Elmer LS 50B spectrofluorimeter equipped with a thermostated cuvette holder (37°C).

In the standard ratio mode, excitation wavelength was changed once per second between 340 and 380 nm, emission was recorded at 500 nm. Fluorescence ratios R = F340/F380 were recorded and the concentration of intracellular free calcium calculated from the equation

$$[Ca^{2+}]_i = K_d \times S_{f2}/S_{b2} \times ((R - R_{min})/(R_{max} - R))$$

as given by Grynkiewicz *et al.* (1985), using K_d = 135 nM and S_{f2}/S_{b2} = 5.6 (determined experimentally for our system). Values for R_{min} and R_{max} were determined after each experiment by successive addition of ionomycin (3 μM) and EGTA (10 mM), respectively.

Quenching of fura-2 fluorescence by Mn^{2+} was measured by exciting the dye at 360 nm, where emission measured at 500 nm is independent of ambient calcium concentration (isosbestic point). This technique detects agonist-stimulated opening of cation channels in different cell types (Merritt *et al.*, 1989; Fasolato *et al.*, 1993).

Combined patch-clamp and $[Ca^{2+}]_i$ measurements in single cells

Cells seeded on glass coverslips were loaded for 45 min with 5 μM fura-2 acetoxymethylester prior to experiments. Coverslips were mounted on a Zeiss Axiovert-M microscope and perfused with oxygenated buffer (composition, mM: NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 5.6, HEPES 5, pH 7.4).

Whole cell recordings were performed with a List LM-PC amplifier. Patch electrodes were prepared from thinwall capillary borosilicate glass. The intracellular pipette solution contained (mM): K-gluconate 150, $MgCl_2$ 2, $CaCl_2$ 0.1, EGTA 0.1, Mg-ATP 5, Li-GTP 0.1 and HEPES 5, adjusted to pH 7.2 with KOH. Where indicated, this solution was supplemented with 45 USP units ml^{-1} of heparin which corresponds to an approximate concentration of 10^{-4} M. Pipette resistances were 3–10 MΩ.

Calcium responses were recorded from cells, voltage-clamped at −50 mV (Boddeke *et al.*, 1993). hPTH-(1-38) or CCh were applied by low pressure (3–4 psi, 3 s) from a micropipette placed 5–10 μm from the cell. Changes in fura-2 fluorescence were determined with a photodiode array connected to a set of amplifiers. Excitation was alternated at 340 and 380 nm, emission was recorded at 509 nm. Fluorescence ratios $R = F_{340}/F_{380}$ were calculated on line. Calibration was performed with 5 μM ionomycin and EGTA at the end of each experiment as described above. The calcium responses were recorded and analysed using pClamp software (Axon Instruments).

Measurements of cyclic AMP and inositol phosphate formation

To measure cyclic AMP production, cells in 24 well plates were prelabelled with [2-^{3}H]-adenine at 2–4 μCi ml^{-1} for 2 h. Thereafter, cells were washed and incubated in HBS containing 1 mM isobutylmethylxanthine (IBMX) and the indicated agonists at 37°C for 15 min. The cells were then extracted with 5% ice-cold trichloroacetic acid (TCA). [^{3}H]-ATP and [^{3}H]-cyclic AMP were separated by sequential chromatography on Dowex and alumina columns as described by Salomon (1979).

IP formation was measured following prelabelling of cells with *myo*-[2-^{3}H]-inositol for 24 h at 2–4 μCi ml^{-1}. Cells were then washed and incubated in HBS containing 20 mM Li^+ and the indicated agonists at 37°C for 20 min. The cells were then extracted with ice-cold 10 mM formic acid and samples were processed by anion exchange chromatography as described by Seuwen *et al.* (1988). IP_1, IP_2, and IP_3 were eluted successively from the columns with 300, 800, and 2000 mM formate, respectively. Total inositol phosphates were collected by adding 2 M formate buffer immediately.

Chemicals

[2-^{3}H]-adenine and *myo*-[2-^{3}H]-inositol were purchased from Amersham, Dübendorf, Switzerland. Human PTH-(1-38) and rolipram were synthesized at Sandoz, Basel, Switzerland. Carbachol (CCh), isoprenaline, forskolin, fura-2 acetoxymethylester, heparin (low molecular weight, sodium salt), IBMX, ruthenium red (oxychloride), ryanodine, and thapsigargin were obtained from Sigma, Buchs, Switzerland. Ionomycin (calcium salt) was from Serva, Heidelberg, Germany. Cell culture media were obtained from Bioconcept, Basel. Foetal calf serum was from Gibco, Basel.

Results

Effects of hPTH-(1-38) and carbachol on cyclic AMP and inositol phosphate (IP) formation in 293 p5 cells

hPTH-(1-38) strongly stimulated adenylyl cyclase in 293 p5 cells but failed to activate PI turnover (Figure 1; Schneider *et al.*, 1994). CCh, on the other hand, was a potent inducer of IP formation and stimulated cyclic AMP production only very weakly. Combining data from 8 independent experiments we could not detect a statistically significant increase of IP production relative to control in hPTH-(1-38)-treated cells whereas the CCh response was clearly significant ($P < 0.01$ at 10^{-4} and 10^{-5} M; two-tailed t test). In order to detect possible transient effects of hPTH-(1-38) on IP production, we carried out time course experiments with high concentrations of hPTH-(1-38) (100 nM) and CCh (100 μM), and we collected individual IPs separately. The results of one of three experiments, all of which produced similar results, are shown in Figure 2. There was no significant increase of IP_1, IP_2, or IP_3 detectable with hPTH-(1-38), whereas CCh strongly stimulated the accumulation of all three IP species.

hPTH-(1-38) stimulates calcium transients which are not due to increases in cyclic AMP

Despite undetectable changes in PI turnover, hPTH-(1-38) stimulated calcium transients in 293 p5 cells. We quantified the responses to hPTH-(1-38) and CCh using fura-2-loaded cells in suspension. Figure 3 shows typical traces obtained with different concentrations of both agonists. High concentrations of CCh produced signals which were higher in amplitude and of longer duration than those elicited by maximal concentrations of hPTH-(1-38). The maximal amplitude of the PTH-response corresponded roughly to that obtained with 1 μM CCh, which stimulates a weak but detectable formation of IPs (Figure 1). Figure 4 compares dose-response data for the two agonists as obtained by averaging peak changes in $[Ca^{2+}]_i$ from several recordings. The EC_{50} for hPTH-(1-38)-induced calcium signalling was 11.2 nM, which is considerably higher than the EC_{50} for cyclic AMP formation in the same cells (0.39 nM; Schneider *et al.*, 1994; Figure 1).

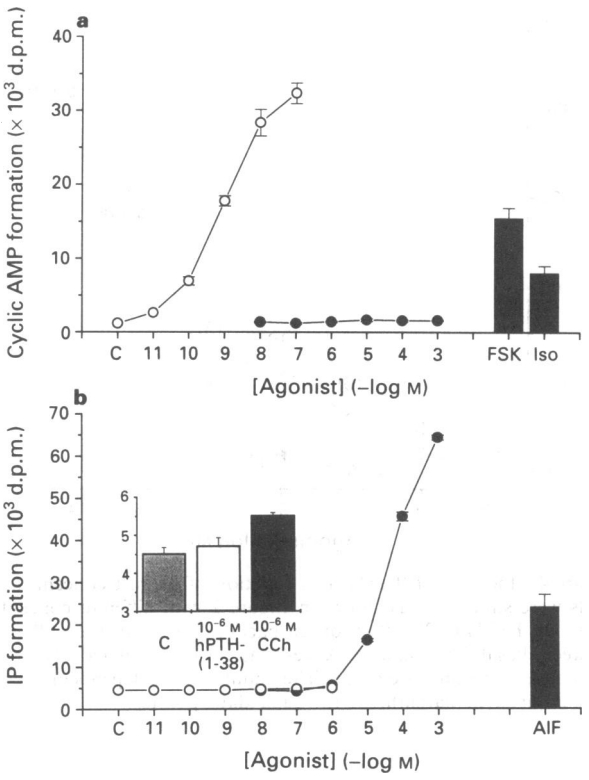

Figure 1 Stimulation of cyclic AMP (a) and inositol phosphate (IP) formation (b) by hPTH-(1-38) (○) and carbachol (CCh, ●) and other agents in 293 p5 cells. Forskolin (FSK) and isoprenaline (Iso) were used at 3 and 10 μM, respectively. To stimulate cells with the AlF_4^- complex (AIF), 10 μM $AlCl_3$ and 5 mM NaF were added. Control values (C) refer to the signal measured in the presence of isobutyl methylxanthine (a) or lithium (b) alone, respectively. The inset in (b) shows the responses to 1 μM of the agonists in greater detail. S.e.mean of triplicate determinations are shown except where exceeded by the symbol size.

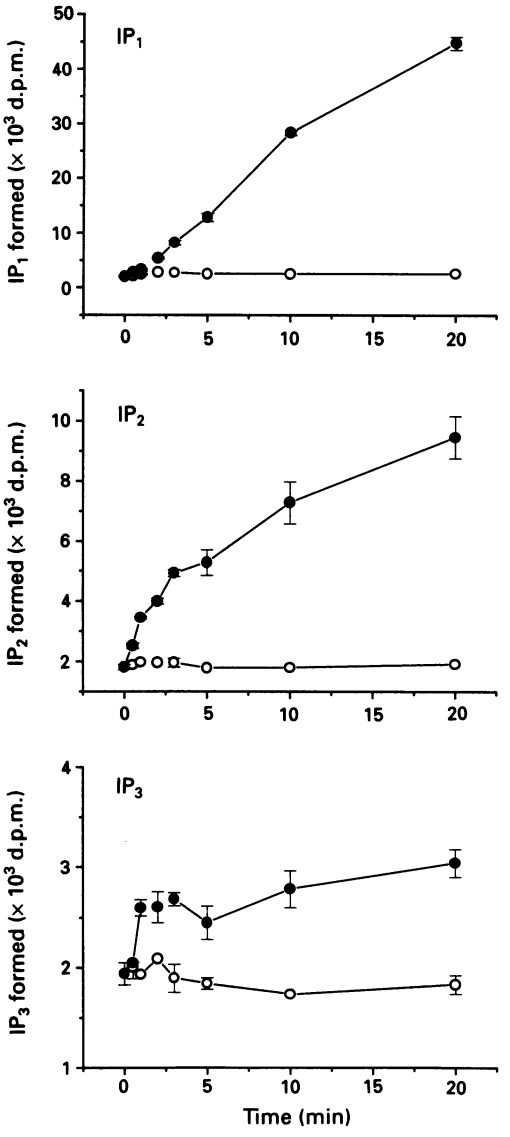

Figure 2 Time course of formation of IP_1, IP_2, and IP_3 following stimulation of cells with 100 nM hPTH-(1-38) (○) or 100 μM carbachol (●). S.e.mean of triplicate determinations are shown except where exceeded by the symbol size.

We next tested whether the PTH-induced calcium signal could be a consequence of cyclic AMP formation, as described for instance for thymocytes (Stojceva-Taneva *et al.*, 1993) and pancreatic islet cells (Fadda *et al.*, 1993). 293 p5 cells were therefore stimulated with the cyclic AMP-elevating agent, forskolin, in the presence or absence of the phosphodiesterase inhibitor, rolipram. Rolipram is equally active as IBMX as an inhibitor of phosphodiesterase activities in 293 cells (unpublished results). As is shown in Figure 5, forskolin with or without rolipram did not evoke a rapid calcium transient in 293 p5 cells and did not abrogate the subsequent responses to hPTH-(1-38) and to CCh. Thus, the calcium transients stimulated by hPTH-(1-38) seem not to be triggered by an elevation of intracellular cyclic AMP. In some experiments, however, a slow increase of intracellular free calcium was observed following forskolin (FSK) administration (Figure 5a), which may reflect sensitization of IP_3 receptors by PKA (Bird *et al.*, 1993).

Calcium transients induced by hPTH-(1-38) or CCh were not sensitive to a 4 h pretreatment of cells with 100 ng ml^{-1} of pertussis toxin (data not shown).

hPTH-(1-38) stimulates release of calcium from intracellular stores

To determine whether the PTH-induced calcium transient was caused by release from intracellular stores and/or due to stimulated influx across the plasma membrane, three different types of experiments were carried out. First, we tested whether the calcium signal persisted in calcium-free medium. To this end, an excess of the calcium chelator EGTA (3 mM vs. 1.8 mM Ca^{2+} in HBS) was added to the cell suspension shortly before the addition of hPTH-(1-38). As is shown in Figure 6a, hPTH-(1-38) still produced a calcium transient. It should be noted that longer preincubations with EGTA (>10 min) tend to deplete exchangeable intracellular calcium stores. Neither hPTH-(1-38) nor CCh were able to increase $[Ca^{2+}]_i$ following prolonged (>10 min) incubation of cells in calcium-free medium.

In a second set of experiments, the calcium pump inhibitor, thapsigargin (Lytton *et al.*, 1991) was used to empty the rapidly exchangeable calcium pool of the endoplasmic reticulum. As is shown in Figure 6b, thapsigargin led to a transient increase of $[Ca^{2+}]_i$ by itself, as expected. Subsequent addition of hPTH-(1-38) or CCh remained without effect.

A third series of experiments was carried out to test whether hPTH-(1-38) accelerated the influx of Mn^{2+} across the plasma membrane, which would indicate an opening of calcium-permeable cation channels (Merritt *et al.*, 1989; Fasolato *et al.*, 1993). The influx of Mn^{2+} into fura-2-loaded cells can be monitored conveniently due to the Mn^{2+}-induced quenching of fura-2 fluorescence. In these experiments,

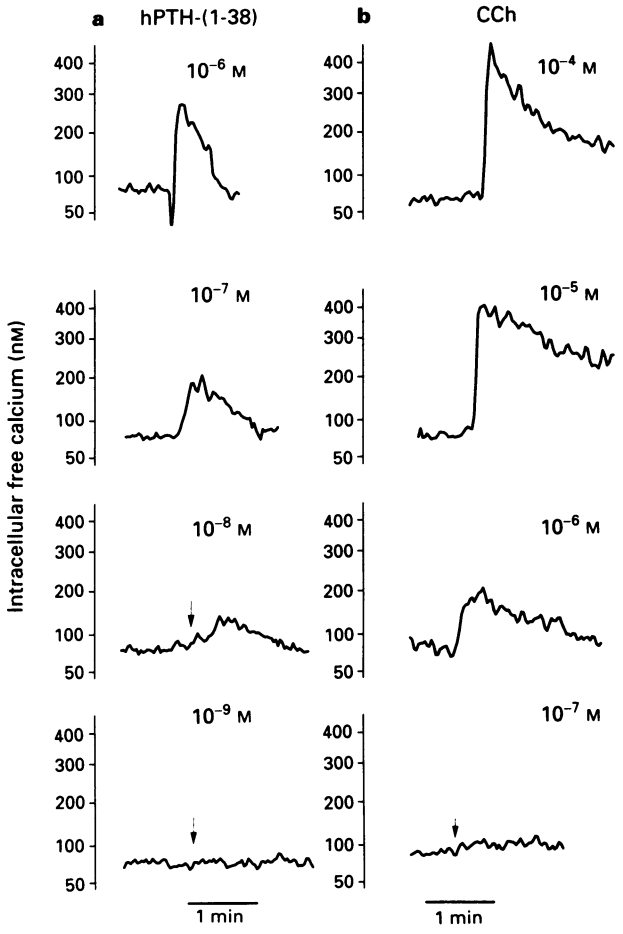

Figure 3 Changes of $[Ca^{2+}]_i$ observed following addition of the indicated concentrations of hPTH-(1-38) (column a) or carbachol (CCh, column b) to 293 p5 cells. The traces shown are representative of 4–20 independent recordings.

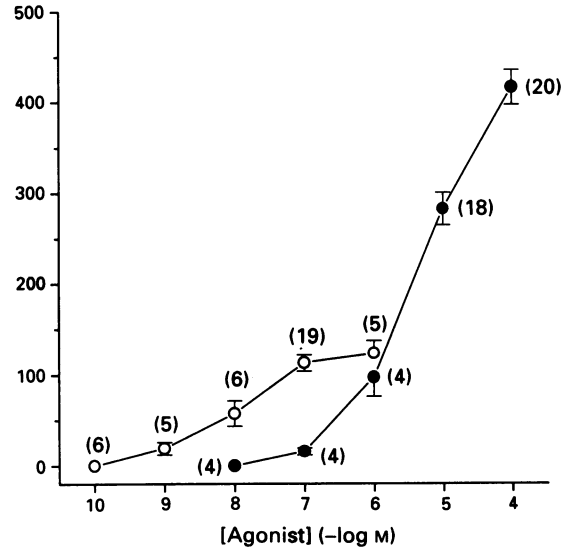

Figure 4 Increase of $[Ca^{2+}]_i$ as a function of agonist concentration. Cells were stimulated as shown in Figure 2 with different concentrations of hPTH-(1-38) (○) or carbachol (●) and the difference between basal level and peak response was calculated. Data are presented as mean ± s.e.mean. The number of independent recordings is given in parentheses for each data point.

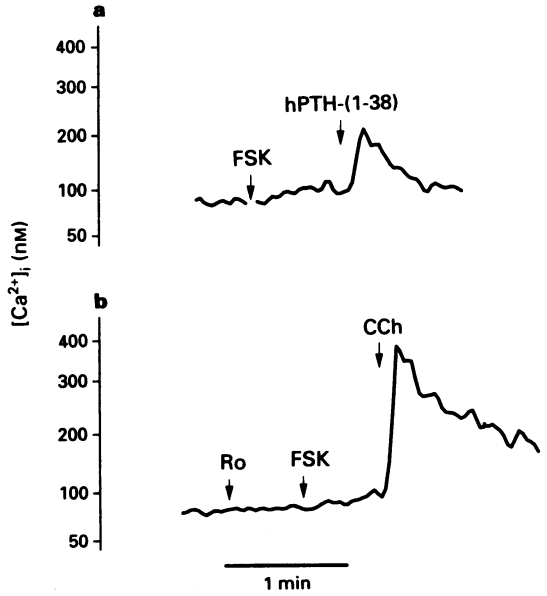

Figure 5 Effects of forskolin (FSK, 3 μM) added alone or in the presence of rolipram (Ro, 3 μM) on $[Ca^{2+}]_i$. After the addition of the cyclic AMP-elevating agents, cells were stimulated with hPTH-(1-38) (100 nM) or carbachol (CCh, 100 μM). The traces shown are representative of 4 independent experiments.

fluorescence is measured at the isosbestic point of the excitation spectrum as opposed to the ratio technique used for the determination of free intracellular calcium. The results of a typical experiment are shown in Figure 6c. hPTH-(1-38) did not accelerate Mn^{2+}-induced quenching of fura-2 fluorescence.

Using conventional voltage clamp experiments (Boddeke *et al.*, 1994) we found no evidence of an activation of voltage-gated calcium channels in 293 p5 cells in response to CCh or hPTH-(1-38) (data not shown).

Taken together, the data presented in this section show that hPTH-(1-38) can mobilize calcium from a thapsigargin-sensitive intracellular store. There is no evidence for PTH-stimulated calcium influx in 293 p5 cells.

Calcium signalling by hPTH-(1-38) is not affected by intracellular application of heparin

Heparin is an inhibitor of the IP_3-triggered calcium release channel of the endoplasmic reticulum (Gosh *et al.*, 1988; Bird *et al.*, 1993). In order to check the possibility that the hPTH-(1-38)-induced calcium signal was due to the formation of a small quantity of IP_3 which was not detected by our biochemical assay, we set out to test whether the polyanion interfered with calcium signalling. As the cell membrane is impermeable to heparin, these experiments were carried out with single cells in whole cell patch clamp mode. Heparin was included in the patch pipette solution. Figure 7a,b shows the result of these experiments. Infusion of heparin blocked calcium transients stimulated by CCh, as expected, but left the response to hPTH-(1-38) intact. Interestingly, in single cell recordings 100 nM hPTH-(1-38) produced signals which were of equal amplitude as those evoked by 100 μM CCh. The reasons for the discrepancies between experiments with cell suspensions and single cells are not yet clear.

At first glance, the data obtained with heparin seemed to rule out a participation of IP_3 receptors in PTH-induced calcium signalling. However, although unlikely, one possible explanation for this observation could be the sensitization of IP_3 receptors to low concentrations of the second messenger due to activation of protein kinase A, as has been demonstrated in hepatocytes (Bird *et al.*, 1993). hPTH-(1-38), but not CCh, strongly stimulates cyclic AMP formation in 293

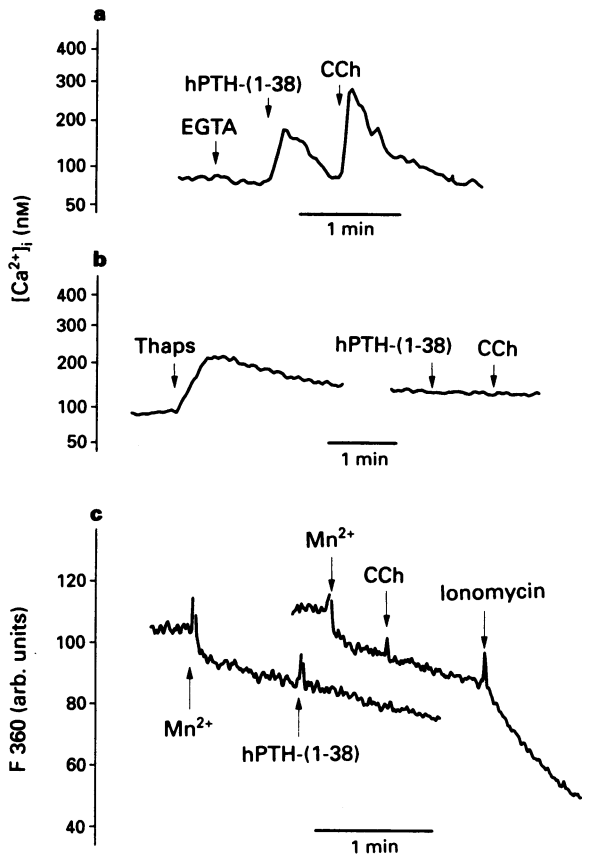

Figure 6 Effects of pretreatment of cells with EGTA (3 mM) (a) or thapsigargin (Thaps, 1 μM) (b) on the $[Ca^{2+}]_i$-response to hPTH-(1-38) and carbachol (CCh). (c) Effects of hPTH-(1-38) and CCh on Mn^{2+} (100 μM)-induced quenching of Fura-2 fluorescence. hPTH-(1-38) and CCh were used at 100 nM and 100 μM, respectively. Mn^{2+} was added as $MnCl_2$ from a 100 mM stock solution. The traces shown are representative of 4–6 independent experiments.

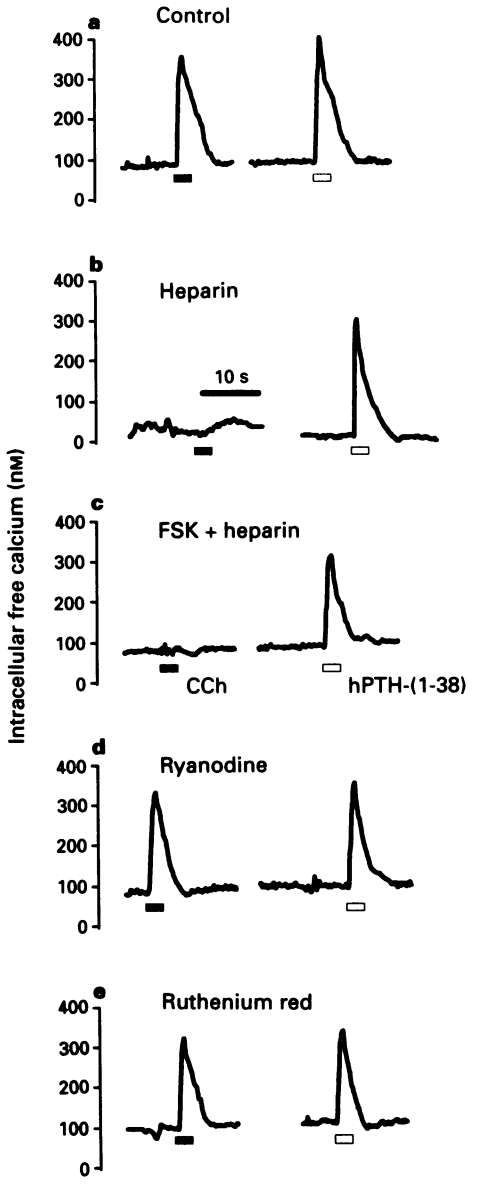

Figure 7 Single cell recordings of $[Ca^{2+}]_i$. Cells were patch clamped (a,b,c) and dialyzed with electrode buffer either with or without heparin (≈100 μM) as indicated. In (c), forskolin (FSK, 3 μM) was added to the bath solution 15 min before recording of a hPTH-(1-38) (100 nM; open bars) and carbachol (CCh, 100 μM; black bars) response. (d,e) Single cell recording without patch clamp. Ryanodine (10 μM) and ruthenium red (10 μM) were added to the bath solution 15 min before recording of a hPTH-(1-38) and CCh response. The traces shown are representative of 5–22 independent recordings.

p5 cells, and we hypothesized that such a sensitization mechanism might render the response heparin-insensitive, if the local heparin concentration near the IP_3 receptors was in a critical range. If this was the case we would expect that cyclic AMP elevating agents should render the CCh response heparin-insensitive. However, as is shown in Figure 7c, this is clearly not observed. Forskolin was unable to induce a heparin-resistant CCh signal. As CCh at 100 μM leads to the production of readily detectable amounts of IP_3 (Figure 2), it seems extremely unlikely that the lack of heparin sensitivity of the hPTH-(1-38) response can be attributed to a PKA-mediated sensitization of the IP_3-receptor.

Calcium signalling by hPTH-(1-38) is not affected by treatment of cells with ruthenium red and ryanodine

In several cell systems, cyclic adenosine diphosphate ribose (cADP-R) is a second messenger stimulating calcium release from intracellular stores in an IP_3-independent manner (Galione, 1994). This response seems to be mediated by a calcium-induced calcium release mechanism involving non-skeletal type ryanodine receptors (McPherson & Campbell, 1993). Calcium release through these receptors can be blocked by ruthenium red and by high concentrations of ryanodine. In order to verify whether this mechanism was involved in PTH-induced calcium signalling, cells were incubated in the presence of 10 μM ruthenium red or 10 μM ryanodine and the responses to hPTH-(1-38) and CCh were measured. As shown in Figure 7c and d, treatment with these two inhibitors interfered neither with the hPTH-(1-38)- nor with the CCh-response.

Heparin-insensitive calcium transients observed in UMR 106-06 rat osteosarcoma cells

In this study we have used a cell line expressing the recombinant human PTH receptor. In order to rule out that the heparin-insensitive calcium signals stimulated by hPTH-(1-38) observed were due to a peculiarity of this cell system, we tested whether the same results could be obtained in UMR 106-06 rat osteosarcoma cells, which express high numbers of PTH receptors and are widely used to study PTH signal transduction (Babich *et al.*, 1991; Wiltink *et al.*, 1993). As in 293 p5 cells, 100 nM hPTH-(1-38) also stimulated heparin-insensitive calcium transients in UMR 106-06 cells (Figure 8). These signals were of longer duration than in 293 p5 cells.

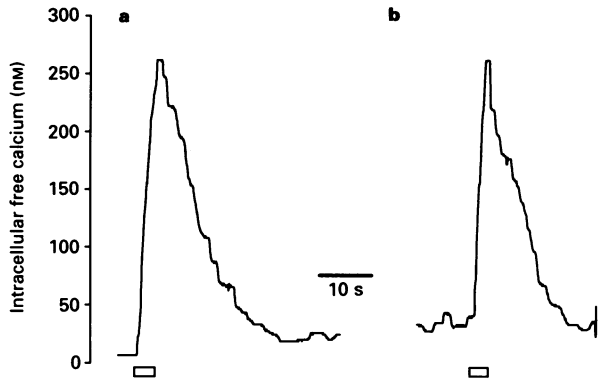

Figure 8 Single cell recordings of $[Ca^{2+}]_i$ in UMR 106-06 rat osteosarcoma cells. Cells were patch clamped and dialyzed with electrode buffer with (b) or without (a) heparin (≈100 μM). hPTH-(1-38) (100 nM, open bars) was applied and calcium responses were recorded. The traces shown are representative of 11 independent recordings, respectively.

Discussion

PTH, PTHrP and peptide analogues have been reported to increase intracellular free calcium rapidly in various cell types by different mechanisms. In osteoblasts and osteosarcoma cell lines, release from intracellular stores (Babich *et al.*, 1991; Civitelli *et al.*, 1992) as well as stimulated influx of calcium across the plasma membrane (Yamaguchi *et al.*, 1987; Bizzarri *et al.*, 1994) have been described. In kidney proximal tubule cells, PTH induces slow changes in $[Ca^{2+}]_i$ which reflect recruitment and activation of specific plasma membrane channels (Bacskai & Friedman, 1990; Friedman & Gesek, 1994).

The physiological relevance of the rapid PTH-induced calcium signals remains uncertain. Normal plasma levels of PTH are in the low picomolar range. At these concentrations, small effects on cyclic AMP formation become apparent, but effects on intracellular calcium are clearly not measurable *in vitro*. In most studies, the EC_{50} for PTH-induced calcium signalling was reported to be considerably higher than the EC_{50} for cyclic AMP formation (Babich *et al.*, 1991; Bringhurst *et al.*, 1993). With our recombinant 293 cells expressing the human receptor we obtained half maximal activation of the two signals at 11.2 and 0.39 nM, respectively. Interestingly, whereas constant infusion of low doses of PTH or analogues strongly activates bone resorption in rats *in vivo*, short intermittent application of high doses, where plasma levels in the nanomolar range are reached, produce anabolic effects (Tam *et al.*, 1982; Mosekilde *et al.*, 1994; Dempster *et al.*, 1994). It is possible that the PTH-induced calcium signals play an important role in this bone forming process.

According to current knowledge the various responses to PTH and analogues are triggered by only one type of PTH/PTHrP receptor (Schipani *et al.*, 1993; Kong *et al.*, 1994), although the existence of functionally different splice variants cannot be excluded at present (Urena *et al.*, 1993; Schneider *et al.*, 1994). A clear picture seemed to emerge when both the recombinant rat and human receptor were found to stimulate cyclic AMP as well as IP formation following transient expression in COS cells (Abou-Samra *et al.*, 1992; Schneider *et al.*, 1993). This dual coupling to second messenger systems was in keeping with results obtained with other receptors of this family, notably the calcitonin receptor (Chabre *et al.*, 1992). It therefore seemed legitimate to speculate that IP_3 generated in response to a PTH challenge was responsible for calcium release from intracellular stores and that the parallel activation of protein kinases A and C could lead, in certain cell types, to an opening of plasma membrane channels.

However, we have been unable to detect a significant formation of inositol phosphates in response to PTH in osteoblasts and osteosarcoma cell lines (unpublished results) as well as in cells stably transfected with the recombinant human PTH receptor cDNA (Schneider *et al.*, 1994; this paper). On the other hand, we could clearly demonstrate activation of this signalling pathway by other stimuli like CCh or AlF_4^- in the transfected 293 cells used in this study. It is possible, therefore, that the IP formation detected in transiently transfected COS cells is an artefact due to severe receptor overexpression. To our knowledge, activation of phosphoinositide turnover has so far not been demonstrated in cells stably expressing one of the cloned PTH receptors.

Despite undetectable IP formation, however, we observed a robust calcium signal in response to hPTH-(1-38) in our transfected 293 cells. This signal occurs independently of the cyclic AMP response and is largely due to release from intracellular stores, as it resists quenching of calcium in the extracellular buffer and is abolished by pretreatment of cells with thapsigargin. Also, hPTH-(1-38) did not stimulate the influx of Mn^{2+}, which can enter cells through certain types of agonist-regulated cation channels.

As the amplitudes of the PTH-induced calcium responses were relatively small, it seemed possible that they were due to the formation of a low amount of IP_3, escaping biochemical detection. We could rule this out using intracellular application of heparin, a strong inhibitor of the IP_3-triggered calcium release channel of the ER. The polyanion completely blocked the calcium responses to high doses of CCh, which elicit strong IP formation, but left the PTH signal intact. Heparin-independent calcium transients stimulated by hPTH-(1-38) were also observed in UMR 106-06 osteosarcoma cells, demonstrating that they are not a peculiarity of the recombinant 293 cells.

Our data provide strong evidence for a PTH-stimulated calcium release mechanism independent of IP_3 and thus confirm a conclusion drawn before by Babich *et al.* (1991). They had compared the calcium signalling mechanism stimulated by bovine PTH-(1-34) and thrombin in rat osteosarcoma cells. Thrombin, but not the PTH peptide, strongly stimulated IP formation. Nevertheless, both agents elicited similar and additive calcium signals.

What could be the nature of the PTH signal? One obvious candidate mechanism was calcium-induced calcium release involving non skeletal type ryanodine receptors. This mechanism was first demonstrated in sea urchin eggs and is now known to exist also in several mammalian cell types (Lee, 1993; Meszaros *et al.*, 1993; Galione, 1994; McPherson & Campbell, 1994). It is strongly inhibited by micromolar concentrations of ruthenium red and ryanodine. However, like heparin, these compounds had no effect on the PTH-induced calcium transients in our cells.

Other potential pathways will now have to be investigated. Whereas a few years ago IP_3 seemed to account for most, if not all cases of calcium release from intracellular stores in non-muscle cells, it is becoming increasingly clear today that several independent pathways seem to exist in different cell types (Mattie *et al.*, 1994; Rosales *et al.*, 1994).

To our knowledge, the present study is the first to demonstrate heparin-insensitive calcium signals triggered by a G protein coupled receptor. However, there is evidence that other receptors of this superfamily also elicit calcium responses in the absence of detectable IP formation (Michel *et al.*, 1992; Vassaux *et al.*, 1992; Frelin *et al.*, 1993). It is important to determine whether our observation can be generalized to a subclass of G protein coupled receptors.

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