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PTH2 receptor-mediated inhibitory effect of parathyroid hormone and TIP39 on cell proliferation

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Abstract

The parathyroid hormone (PTH) has dual mitogenic and inhibitory effects on cell proliferation, depending on the cell type and experimental conditions. PTH can signal via two different receptors, both positively coupled to the adenylyl cyclase/cyclic AMP pathway which can mimic some of the proliferative effects of PTH. We evaluated the role of the type-2 PTH (PTH2) receptor on cell proliferation in clonal human embryonic kidney HEK293 cells stably expressing the human PTH2 receptor. Using a cyclic AMP-responsive gene-reporter, we confirmed that the tuberoinfundibular peptide (TIP39) and various human (h) PTH fragments including hPTH-(1–34) were potent agonists (EC_{50} in the range of 0.01–0.3 nM) whereas the bovine (b) PTH peptides b(Tyr^{34})PTH-(7–34) and its tryptophan derivative b[D-Trp^{12},Tyr^{34}]PTH-(7–34) behaved as antagonists (IC_{50} = 117 and 249 nM, respectively). hPTH-(1–34) produced a dose-dependent inhibition of cell proliferation (EC_{50} = 8.5 ± 0.4 nM) after 3 days and this effect was fully reversed by the tryptophan derivative antagonist. The same effect was observed with TIP39 which caused a 30% maximal inhibition. These findings reveal that PTH2 receptor activation can inhibit cell proliferation and might explain the dual functionality of PTH on cell proliferation.

Keywords: PTH (Parathyroid hormone); Tuberoinfundibular peptide (TIP39); PTH2 receptor; Proliferation; Luciferase gene-reporter assay; Bioluminescence assay

1. Introduction

The parathyroid hormone (PTH) is an 84-amino-acid polypeptide hormone which has been demonstrated to be a major regulator of bone formation (Strewler et al., 1987). Besides its regulatory effects on osteoblastic cell proliferation (MacDonald et al., 1986) and differentiation (Ishizuya et al., 1997), PTH seems to have dual mitogenic and inhibitory effects on proliferation depending on the cell type, species, and experimental conditions (Partridge et al., 1985; MacDonald et al., 1986; Kano et al., 1993; Turner et al., 2000; Ishizuya et al., 1997).

A number of studies have tried to address the molecular mechanisms underlying the versatility of PTH activity on cell proliferation. Both the adenylyl cyclase/cAMP/protein kinase A and the phospholipase C/protein kinase C/Ca^{2+} signal transduction pathways, which have been demonstrated to transduce the PTH signal (Swarthout et al., 2002), are able to mimic the PTH proliferative effects in vitro. Although there is still uncertainty about the relative role of these pathways, recent studies by Fujita et al. (2002) showing that the cAMP-stimulated activation of ERK through B-Raf has determinant implications on the modulation of cell proliferation by PTH activity, further support the importance of cAMP signaling in the proliferative effects of PTH.

Both the type-1 (PTH1) receptor which binds PTH and PTH-related peptide (PTHrP) (Abou-Samra et al., 1992) and the type-2 (PTH2) receptor which binds PTH and the tuberoinfundibular peptide (TIP39) (Usdin et al., 1995, 1999; Behar et al., 1996; Hoare et al., 2000b) signal through the cAMP signaling pathway. Whereas PTH1 receptor is believed to be responsible for PTH-induced regulatory effects in mammalian osteoblasts, little is known about the functional role of PTH2 receptor and its putative

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endogenous ligand TIP39 (Usdin, 2000). While PTH1 receptor appears to be the only one expressed in osteoblasts, PTH2 receptor is expressed in the central nervous system, preferentially in the hypothalamic regions (Usdin et al., 1995) and in the dorsal horn of the spinal cord (Usdin et al., 1999). Recent evidence demonstrating that PTH but not PTHrP regulates hippocampal cell degeneration/apoptosis further suggests that PTH could have larger regulatory activities on cell survival through a PTH1 receptor-independent mechanism (Hirasawa et al., 2000; Turner et al., 2000).

In the present study, we evaluated the role of PTH2 receptor on cell proliferation using a newly established recombinant cell system which stably and selectively expresses the human PTH2 receptor. We report the pharmacological profile of PTH2 receptor and the activity of a series of PTH-derived peptides using a cAMP-responsive luciferase gene-reporter assay and provide the first evidence that PTH and TIP39 can inhibit cell proliferation by a PTH2 receptor-mediated mechanism.

2. Materials and methods

2.1. Biochemicals

Biochemicals reagents were obtained from Sigma-Aldrich (Milan, Italy) and Geneticin (G418 sulfate) was from Calbiochem Inalco (Milan, Italy). Cell culture supplies, reagents for cDNA synthesis and transfection studies were obtained from GIBCO-BRL-Life Technologies (San Giuliano Milanese, Italy). Cell culture™ microplates and reagents for luciferase assay were from Packard Bioscience (Milan, Italy). The human (h) and bovine (b) peptides hPTH-(1–34), h[Ile^{5},Trp^{23},Tyr^{36}]PTHrP-(1–36), hPTH-(1–31), h(Leu^{27})cyclo[Glu^{22}-Lys^{26}]PTH-(1–31), hPTHrP-(1–34), b[D-Trp^{12},Tyr^{34}]PTH-(7–34), b(Tyr^{34})PTH-(7–34) and b[Nle^{8,18},Tyr^{34}]PTH-(3–34) were purchased from Bachem (Bubendorf, Switzerland). All peptides were resuspended in sterile water as 1 mM stock solutions and stored at −20 °C. TIP39 was synthesized in-house (Discovery Research Chemistry and Screening Sciences, GlaxoSmith-Kline Pharmaceuticals, Harlow, UK).

2.2. Generation of a stable cell line expressing the human PTH2 receptor

The full-length 1746 bp cDNA encoding for human PTH2 receptor (accession no. U25128) was obtained from human placenta mRNA (Clontech, Heidelberg, Germany) by reverse transcription followed by polymerase chain reaction (RT-PCR) using the specific primers 5′-TTCT-TCCTACAGCCGTTCC-3′ and 5′-AGTATCAGCC-AAGCCCTCTC-3′. Reverse transcription reactions were performed using standard procedures and commercial kits (GIBCO-BRL-Life Technologies). The RT-PCR fragment was cloned into the mammalian expression vector pcDNA3.1-Hygro(+) (Invitrogen, Milan, Italy). Clonal cell lines expressing the hPTH2 receptor were established from the human embryonic kidney (HEK) 293 cells (American Type Culture Collection no. 85120602) stably expressing a luciferase gene-reporter construct under the control of a multiple responsive element (MRE) and cyclic AMP responsive element (CRE) thereafter refered as MRE/CRE-luciferase gene-reporter (Fitzgerald et al., 1999). Briefly, cells were transfected with the hPTH2 receptor-containing plasmid using the FuGENE6™ transfection reagent (Roche Molecular Biochemicals, Monza, Italy) according to the manufacturer's recommendations. Stable transfectants were selected by growth selection using Hygromycin B (300 μg/ml) and screened for PTH2 receptor expression by RT-PCR using the specific primers 5′-TATGCCTGCCTCACTCCTTCA-3′ and 5′-TCT-CCCTGCCTCCCACTATCTTCC-3′. Clonal cell lines expressing the hPTH2 receptor were named HEK293-hPTH2R cells. The lack of expression of hPTH1 receptor was verified by RT-PCR using the specific primers 5′-GACCGCCTGGGCATGATTTACACC-3′ and 5′-ACCCGGACGATATTGATGAAGAGG-3′. For both primer combinations, a 35-cycle PCR was run in a GeneAmp PCR System 9700 thermal cycler (Perkin Elmer, Monza, Italy) using a standard thermal profile consisting of 30 s denaturation at 95 °C, 30 s annealing at 55 °C, 50 s extension at 72 °C and a 5-min final extension step at 72 °C. DNA products were separated on ethidium bromide-stained agarose gel and analyzed with a Fluor-S Multimager (BioRad, Segrate, Italy). Parental cells did not show any detectable expression of either PTH1 or PTH2 receptors, as evaluated by RT-PCR using the above specific primers.

2.3. Cell culture

HEK293-hPTH2R cells were grown in plastic cultureware precoated with poly-D-lysine hydromide (Sigma-Aldrich). Cells were routinely grown in Eagle's minimal essential (EMEM) medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 400 μg/ml geneticin, 300 μg/ml hygromycin B, 1% non-essential amino acids, at 37 °C in a humidified atmosphere with 5% CO_2. For all experiments, cells were used between passage 10 and 20 to minimize cell culture-induced modifications. Cells were split at subconfluency by trypsinization (0.5 mg/ml trypsin and 0.2 mg/ml EDTA) and dissociation in supplemented EMEM medium.

2.4. CRE-luciferase gene reporter assay

PTH2 receptor signaling assays were performed in 96-well microtiter plates using the luciferase gene-reporter assay as previously described (Fitzgerald et al., 1999) and an improved procedure which allows a rapid, sensitive and high-throughput assay (Garnier et al., 2000). Briefly,

cells (1×10^4/well) were plated in 60–70 μl of culture medium without phenol red in poly-D-lysine-hydrobromide-coated white polystyrene CulturPlate™. Twenty-four hours after seeding, cells were pre-incubated for 30 min in the presence of 0.5 mM phosphodiesterase inhibitor 3-isobutyl-1-methylxantine. The stimulatory activity of agonist peptides on luciferase expression was then assessed. Peptides at various concentrations were added as 10-fold concentrated solutions to a final volume of 100 μl/well. When mentioned, cells were preincubated with antagonist peptides 30 min prior to the agonist. Luciferase expression was measured after 4 h of incubation at 37 °C (5% CO_2), by addition of 100 μl of reconstituted LucLite™ reagent and measurement of chemiluminescence using a TopCount microplate scintillation and luminescence counter (Packard Bioscience) for 1 s/well.

2.5. Proliferation studies

For proliferation studies, cells (8×10^2/well) were seeded in 100 μl of medium in 96-well tissue culture plates and grown for the indicated periods of time in the absence or presence of hPTH-(1–34), TIP39 and/or b[D-Trp^{12},Tyr^{34}]PTH-(7–34). Drugs were added every day with medium change every 2 days. Cell proliferation was measured by assessment of cell viability using the bioluminescent ATP Detection Assay Kit (ATPLite-M, Packard Bioscience). Briefly, cells were lysed by addition of 50 μl of cell lysis solution with shaking for 2 min followed by subsequent adding of the substrate solution (50 μl/well) containing luciferase and luciferin. In this assay, ATP which is present in all metabolically active cells and proportional to cell number, catalyses the transformation of D-luciferin by luciferase resulting in the emission of luminescence. Plates were dark-adapted for 10 min with shaking and the intensity of the luminescent signal (CPS) was measured using a TopCount microplate scintillation and luminescence counter (Packard Bioscience) for 1 s/well.

2.6. Data analysis

The luminescence data from the luciferase-assays were analysed by nonlinear fitting analysis using the data analysis software GraFit version 4.09 (Eriacus Software, Horley, UK) and results expressed as EC_{50} values. Data from multiple experiments were averaged and expressed as the mean ± S.E.M. Antagonist potency was assessed by determination of the pK_B value which represents the negative logarithm value of the concentration of the antagonist that would produce a twofold increase of agonist EC_{50} using the following equation:

$$pK_B = \log[(EC'_{50}/EC_{50}) - 1] - \log[\text{antagonist}]$$

where EC_{50} and EC'_{50} represent the agonist concentration which produces half of maximal effect in the absence and in the presence of a fixed concentration of the antagonist. Statistical analysis were made by analysis of the variance (one-way ANOVA) and if overall significance was obtained, followed by Dunnett's test. Differences between groups with a P value of < 0.05 were considered statistically significant.

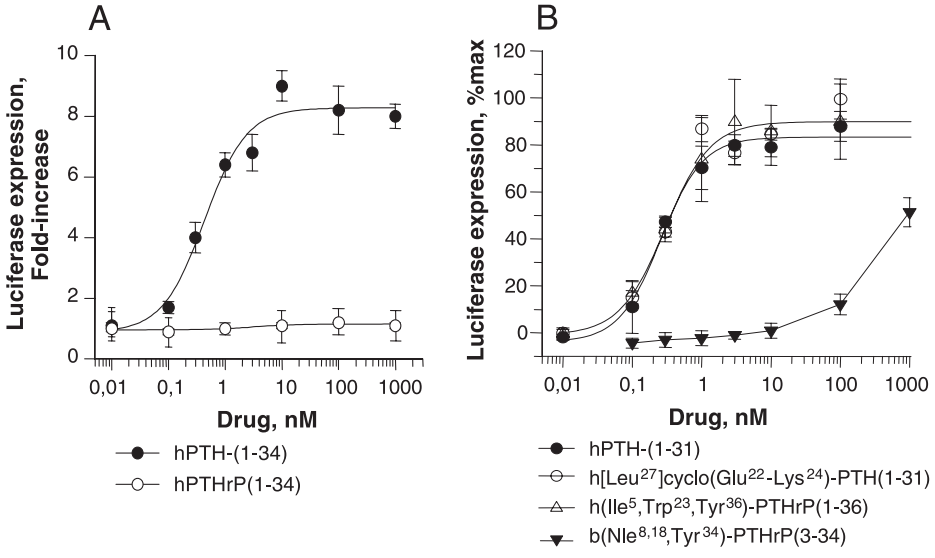

Fig. 1. Functional characterization of HEK293-hPTH2R cells: agonist activity of PTH peptide fragments. The dose–response effect of PTH peptide fragments on intracellular cAMP levels was studied using the CRE-Luciferase-gene reporter assay by determination of the chemiluminescence signal after 4 h incubation of cells with increasing concentrations of peptides. Results are expressed as fold-increase over basal line and are mean ± S.E.M. from three to six independent experiments, each performed in triplicates. (A) Effect of hPTH-(1–34) and of the related peptide hPTHrP-(1–34). (B) Agonist activity of PTH peptide fragments.

3. Results

3.1. Pharmacological activity of PTH-derivative peptides on HEK293-hPTH2R cells using a CRE-luciferase gene reporter assay

A novel clonal HEK293-hPTH2R cell line was established to generate a suitable cell system to specifically evaluate PTH2 receptor activity and assess the receptor signaling response using a gene-reporter assay. Three clones showed highest level of expression of hPTH2 receptor as assessed by RT-PCR analysis, and were further characterized in functional assay. In all three clones, hPTH-(1–34) produced a dose-dependent increase in luciferase expression with similar EC_{50} values in the range of 0.3 nM and a maximal stimulation of five- to eightfold relative to basal level. The clone which produced the highest maximal increase in luciferase expression was chosen for further studies. The EC_{50} value for hPTH(1–34) was 0.350 ± 0.037 nM ($n = 6$). Consistent with the absence of expression of the PTH1 receptor, we observed no response to hPTHrP-(1–34) up to 1 μM (Fig. 1A), a peptide which is a potent and selective agonist at PTH1 receptor with EC_{50} value of 0.32 nM (Gardella et al., 1996).

The pharmacological profile of PTH2 receptor was further characterized by studying the activity of a series of PTH peptide fragments. As shown in Fig. 1B, the non selective agonist h[Ile^5,Trp^{23},Tyr^{36}]PTHrP-(1–36) was equipotent to hPTH-(1–34) with an EC_{50} value of 0.300 ± 0.034 nM ($n = 3$) similar to that previously reported for the activity of this peptides at PTH1 receptor (Gardella et al., 1996). The hPTH-(1–31) peptide and its cyclic derivative h[Leu^{27}]*cyclo*[Glu^{22}-Lys^{26}]PTH-(1–31) which are known agonists of the PTH1 receptor (Rixon et al., 1994; Whitfield et al., 1997) have previously been shown to exert a strong anabolic response, presumably through regulation of cell proliferation. We investigated their activity at the PTH2 receptor and found that both peptides exhibited a strong and equipotent PTH2 receptor agonist activity similar to that of hPTH-(1–34). In comparison to their published activity at PTH1 receptor with EC_{50} values of 19.9 and 3.30 nM (Whitfield et al., 1997), these peptides displayed a significantly greater agonist activity for PTH2 receptor. The EC_{50} values were 0.260 ± 0.045 nM ($n = 3$) for hPTH-(1–31) and 0.302 ± 0.009 nM ($n = 4$) for the cyclic derivative. The b[Nle^{8,18},Tyr^{34}]PTH-(3–34) fragment was initially discovered as a potent PTH1 receptor antagonist with an IC_{50} value of about 12 nM (Rosenblatt et al., 1977). Incubation of HEK293-hPTH2R cells with the peptide alone produced a reproducible 51% stimulation of luciferase expression at the highest dose tested of 1 μM, revealing a weak agonist activity of the peptide at PTH2 receptor (Table 1).

The dose–response effect of the PTH analogue b[D-Trp^{12},Tyr^{34}]PTH-(7–34) and of its tryptophan derivative b(Tyr^{34})PTH-(7–34) which are moderate to weak antagonists at the PTH1 receptor with IC_{50} values of about 70 and 900 nM, respectively (Goldman et al., 1988) behaved as antagonists at the PTH2 receptor. These peptides were capable of reversing the increase in luciferase expression induced by 1 nM hPTH-(1–34) in HEK293-hPTH2R cells. In comparison with published data for the activity of the peptides at PTH1 receptor, b[D-Trp^{12},Tyr^{34}]PTH-(7–34) appeared twofold less potent at PTH2 receptor ($IC_{50} = 117 \pm 15$ nM, $n = 5$) and the tryptophan derivative potency was in the same range to that of the parent peptide ($IC_{50} = 249 \pm 19$ nM, $n = 4$).

Table 1
Pharmacological characterization of PTH-derived peptide activity at PTH2 receptor

Peptide	Activity	EC_{50}, nM (mean ± S.E.M.)	IC_{50}, nM (mean ± S.E.M.)
hPTH-(1–34)	agonist	0.350 ± 0.037	–
hPTHrP-(1–34)	none	–	–
h[Ile^5,Trp^{23},Tyr^{36}] PTHrP(1–36)	agonist	0.300 ± 0.034	–
hPTH-(1–31)	agonist	0.260 ± 0.045	–
h[Leu^{27}]*cyclo* [Glu^{22}-Lys^{26}] PTH-(1–31)	agonist	0.302 ± 0.090	–
TIP39	agonist	0.014 ± 0.001	–
b[Nle^{8,18},Tyr^{34}] PTH-(3–34)	agonist	>1 μM	–
b[D-Trp^{12},Tyr^{34}] PTH-(7–34)	antagonist	–	117 ± 15
b(Tyr^{34})PTH-(7–34)	antagonist	–	249 ± 19

The dose–response effect of various PTH-derived peptides on luciferase expression has been studied in HEK293-hPTH2R cells using the CRE-luciferase gene reporter assay. The agonist (peptide alone) or antagonist (peptide active in the presence of 1 nM hPTH-(1–34)) activity of the peptides is reported with the measured EC_{50} and IC_{50} values (mean ± S.E.M. from three to six independent experiments).

3.2. TIP39 activity at PTH2 receptor

TIP39 which exhibits only limited amino acid sequence homology with PTH has been shown to stimulate cAMP accumulation in cells expressing PTH2 receptor (Usdin et al., 1999). As shown in Fig. 2, we confirmed that TIP39 was a potent PTH2 receptor agonist in our system, producing a dose-dependent increase in luciferase expression with a potency ($EC_{50} = 0.014 \pm 0.001$ nM, $n = 4$) about 20-fold greater than that of the reference peptide hPTH (1–34). The addition of 1 μM of the antagonist b[D-Trp^{12},Tyr^{34}]PTH-(7–34) produced a rightward shift of the dose–response curves of both peptides with similar pK_B values of 8.74 (TIP39) and 8.62 (hPTH-(1–34).

3.3. Inhibitory activity of PTH-(1–34) and TIP39 on HEK293-hPTH2R cell proliferation

To investigate the role of PTH2 receptor in the regulation of proliferation, we tested the activity of hPTH-(1–34) and TIP39 using an ATP bioluminescence assay previously used as a mean to assess cell proliferation and cytotoxicity (Crouch et al., 1993). A linear relationship was obtained between the

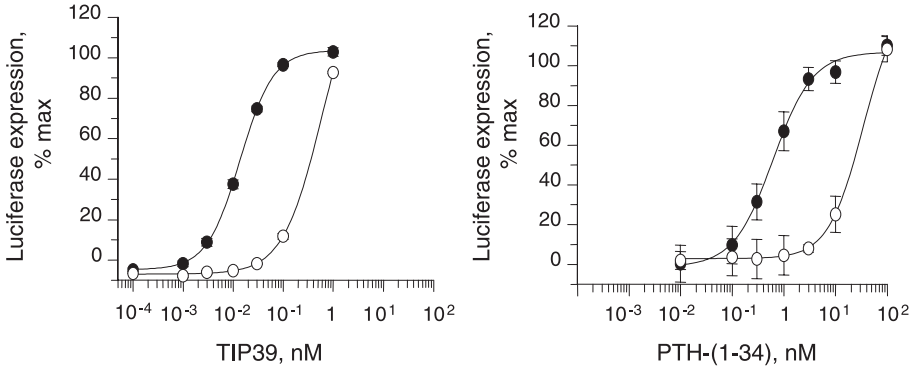

Fig. 2. Pharmacological characterization of TIP39 activity at PTH2 receptor. Cells were incubated with increasing concentrations of either PTH(1–34) or TIP39 alone (filled circle) or following a 30 min-preincubation with 1 μM of b[D-Trp^{12},Tyr^{34}]PTH(7–34) (open circle). Luciferase expression was assessed by chemiluminescence and was expressed in percentage of the maximal effect induced by hPTH-(1–34). Results are mean ± S.E.M. from three independent experiments, each performed in triplicate.

luminescence measured (CPS) and cell number in the range of 95–6250 cells per well (correlation coefficient r^2 = 0.9963) using the luciferin–luciferase reaction. All further experimental conditions were set up to fit within this range. Preliminary proliferation studies using control cells showed that the luminescence signal increased with the incubation time over a 4-day period of time, corresponding to an increase in cell number from about 500 to 2800 cells per well from day 1 to day 4. When cells were incubated in the presence of a saturating (100 nM) concentration of hPTH-(1–34) or TIP39, luminescence values significantly decreased after 2 and 3 days of incubation relative to control cells (Fig. 3). At day 3, PTH-(1–34) produced a maximal 35% inhibition as compared to control cells, significantly greater than that produced after 1 day of incubation ($P<0.05$). This effect was less pronounced at longer times of incubation showing an inhibition of cell growth of about 15% at day 4, a value which did not reach statistical significance. TIP39 produced a time-dependent decrease in luminescence signal to the same extent with a maximal 30 ± 7%, relative to control cells. However, the time-course inhibitory effect of TIP39 differed slightly from that produced by hPTH-(1–34). Maximal inhibition was observed after 4 days of incubation and was maintained over a 6-day incubation period of time. The inhibitory effect of TIP39 after 4 and 6 days of incubation was significantly greater than that produced after 1 day of incubation ($P<0.05$). Direct measurement of cell cytotoxicity using standard colorimetric techniques did not reveal significant cell cytotoxicity following incubation of cells for up to 4 days in the presence of 100 nM PTH-(1–34) (data not shown). All together, these data indicate that PTH2 receptor agonists can inhibit cell proliferation through activation of PTH2 receptor.

Moreover, the inhibitory effect of hPTH-(1–34) on cell growth was dose-dependent with an EC_{50} value of 8.5 ± 0.4 nM ($n=3$). As shown in Fig. 4, the inhibitory effect produced by 100 nM hPTH-(1–34) on HEK293-hPTH2R cell proliferation was blocked by the PTH2 receptor antagonist peptide b[D-Trp^{12},Tyr^{34}]PTH-(7–34). In the presence of 10 μM of the antagonist, the inhibitory effect of PTH-(1–34) on cell proliferation was almost completely abolished

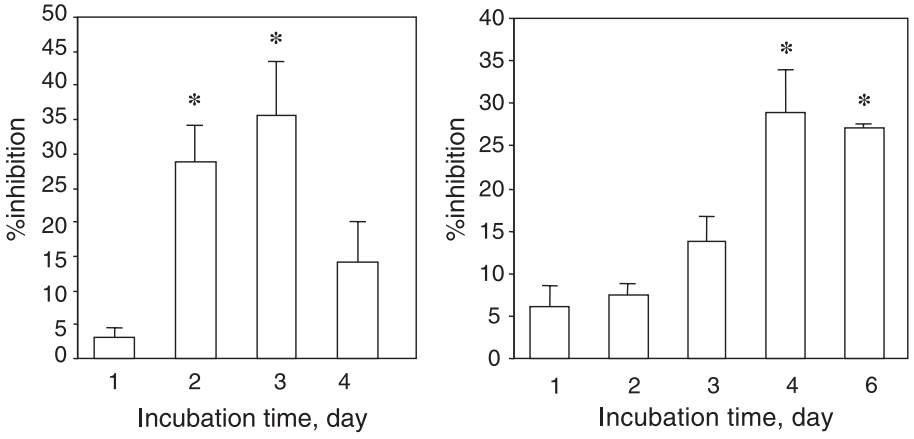

Fig. 3. Effect of hPTH-(1–34) and TIP39 on HEK293-hPTH2R cell proliferation. Cells (800 cells/well) were incubated in the absence (control cells) or in the presence of 100 nM PTH-(1–34) (left panel) or 100 nM TIP39 (right panel). The chemiluminescent signal was measured every day using the ATP-LiteM assay. Results were expressed as % inhibition relative to control cells at each time point and data are mean ± S.E.M. from at least three independent experiments, each performed in triplicate. *$P<0.05$ vs. Day 1 using Dunnett's test.

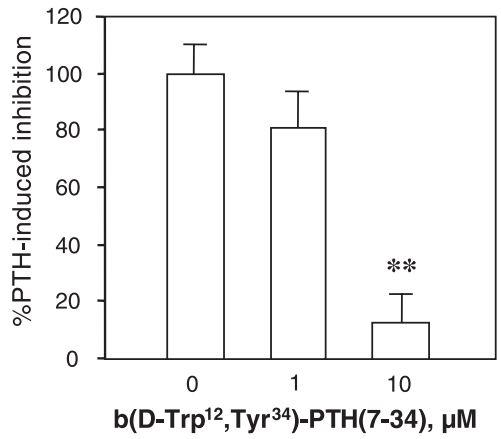

Fig. 4. Antagonism of hPTH-(1–34)-induced inhibition of cell proliferation. Cells were treated with vehicle (control), 100 nM hPTH-(1–34), the PTH antagonist b[D-Trp^{12},Tyr^{34}]PTH-(7–34) at 1 or 10 μM, alone or in combination. Chemiluminescent signal was measured at day 3. Results were expressed as % PTH-(1–34)-induced inhibition. Each bar represents the mean ± S.E.M. from four independent experiments, each performed in triplicate. $^{**}P<0.01$ vs. hPTH-(1–34)-treated group using Dunnett's test.

($P<0.01$). The peptide alone had no significant effect (data not shown). These data further confirm that hPTH-(1–34) is able to inhibit cell growth through a PTH2 receptor-mediated mechanism.

4. Discussion

In the present study, we report the pharmacological activity of PTH2 receptor in a novel cell-based system and address its role in the regulation of cell proliferation. In addition, the pharmacological profile of PTH2 receptor was extended with the novel characterization of the activity of a series of PTH peptide fragments. The cyclization and Lys^{27} to Leu substitution in PTH-(1–31) peptide leading to the h[Leu^{27}]*cyclo*[Glu^{22}-Lys^{26}]PTH-(1–31), did not affect the peptide agonist potency at PTH2 receptor, a result which differs from previously reported activity of the peptides at PTH1 receptor where the peptide modifications increased agonist potency (Barbier et al., 1997). The b[Nle^{8,18},Tyr^{34}]PTH-(3–34) derivative, a PTH1 receptor antagonist (Rosenblatt et al., 1977) or partial agonist depending on the cell system utilised (McKee et al., 1990; Pines et al., 1996) exhibited a weak agonist activity at micromolar concentration at PTH2 receptor, possibly reflecting a partial agonist activity in this cell system. Finally, the two PTH1 receptor antagonists, b(Tyr^{34})PTH-(7–34) and its tryptophan derivative b[D-Trp^{12},Tyr^{34}]PTH-(7–34) (Goldman et al., 1988) behaved as moderate PTH2 receptor antagonists with IC_{50} values in the 100–200 nM range. These peptides represent new valuable pharmacological tools to assess PTH2 receptor biological function in heterologous expression systems.

The PTH2 receptor endogenous ligand TIP39 appeared 25-fold more potent than PTH (1–34) in our assay, a result which differs from the initially reported characterization of the peptide activity (Usdin et al., 1999; Hoare and Usdin, 2000a). This greater potency of TIP39 could be explained by the recruitment of additional signaling pathway(s) leading to the activation of the cyclic AMP (cAMP) responsive element binding (CREB) protein and its subsequent transcriptional activity on the luciferase gene-reporter, through cAMP-independent intracellular pathway(s) (Wu et al., 2001). Although PTH2 receptor was shown to be positively coupled to Ca^{2+}-dependent pathways (Behar et al., 1996) which could activate CREB, it is unlikely that such pathways could be responsible for the greater potency of TIP39 in this system, as the gene-reporter construct was not modulated by direct activators of calcium release (not shown).

Turner et al. (2000) found that PTH treatment of HEK-293 expressing PTH1 receptor produced a marked reduction in cell number. We report, for the first time, that PTH2 receptor activation by either PTH-(1–34) or TIP39 can also result in a decrease in cell growth in the context of our model system. The EC_{50} value of hPTH-(1–34) activity on cell proliferation was about 10 nM and 20-fold higher than that determined in receptor signalling assay. This result is in agreement with previous studies showing an effect of PTH on cell growth in cultured bone explants cell (Sabatini et al., 1996). The magnitude of the response observed, with a maximal 25–35% inhibition, is also in the range of that previously reported by others (Van der Plas et al., 1985; Verheijen and Defize, 1995), and likely represents the maximal biological response that PTH can exhibit in in vitro cell systems. Our observation that hPTH-(1–34) and TIP39 exhibit different inhibitory time-course profiles further suggests that PTH2 receptor activation could results in the recruitment of different signaling pathways and downstream cascades depending on the agonist, and that TIP39 could trigger a more sustained inhibitory effect than PTH.

The ATP bioluminescence assay used in this study allows the assessment of cell metabolic activity and represents a highly sensitive methodology to assess changes in cell proliferation. Clearly, a reduction in luminescence signal could also result from cell cytotoxicity. It is unlikely that this represents a major component of the response observed as we have not detected significant cell cytotoxicity using standard colorimetric techniques. Clearly, the response observed could be a balance of activatory and inhibitory influences on cell growth, the resulting activity being a net decrease in cell growth. The b[D-Trp^{12},Tyr^{34}]PTH-(7–34) peptide derivative which behaved as a full antagonist on both hPTH-(1–34) and TIP39 activity in signaling assays, also reversed PTH-(1–34) inhibitory activity on cell growth, confirming that the effect observed is mediated through a PTH2 receptor specific mechanism.

Clearly, these results might be associated to the cell context and cell-based system used in this study and further studies would be needed to further investigate the activity

of the PTH/PTH2 receptor system in other cell models. Nevertheless, these results provide new insights to support a role for the PTH2 receptor in the control of cell proliferation/survival processes and raise the question on the functional role of PTH2 receptor in vivo. Whether or not PTH and TIP39 regulate such processes in PTH2 receptor-expressing cells under normal physiological conditions is still unkown and would need to be further elucidated. However, the fact that the time-course profile of hPTH-(1–34), showing a maximal effect during the first 2–3 days with a following decline, is similar to previously published results for PTH activity on osteoblastic cell growth (Sabatini et al., 1996) and suggests that the current cell system can reproduce some of the PTH effects observed in a more physiological system with endogenously expressed PTH receptors.

Such inhibitory activity of PTH2 receptor may well explain some of the reported dual activity of PTH on proliferation. It is clearly possible that the expression of one or both types of the PTH receptors might trigger different intracellular pathways and determine distinct biological responses. Further studies in this direction will continue to increase our understanding of the physiological role of PTH2 receptor and may open up new therapeutic applications.

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