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A C-terminally truncated human parathyroid hormone receptor is functional and activates multiple G proteins

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Abstract We have investigated the role of the C-terminal cytoplasmic domain of the human PTH receptor in effector coupling. Following transient expression in COS-1 cells, coupling to both AC and PI-PLC was observed with the full-length receptor. Progressive C-terminal truncations did not dissociate activation of the two signalling systems. In stably transfected 293 cells, however, the full-length receptor as well as the majority of truncated constructs stimulated AC exclusively but failed to activate PI-PLC. Activation of both signalling systems was again observed following stable expression of a severely truncated receptor (R483) in 293 cells. In this case, pertussis toxin was also found to potentiate the cAMP response to hPTH-(1–38) significantly, indicating functional coupling of R483 to G_i proteins. Our results suggest that a core region of the human PTH receptor (first, second, third intracellular loop) can interact promiscuously with different G proteins and that the C-terminus of the full-length receptor directs the receptor towards an interaction with G_s.

Key words: Parathyroid hormone; Receptor; G protein; Adenylyl cyclase; Phosphoinositide

1. Introduction

PTH is a major regulator of bone and mineral metabolism. In its target cells, which are primarily osteoblasts and kidney proximal tubule cells, the hormone elicits a multitude of responses which are not readily explained solely on the basis of its capacity to stimulate AC (see [1,2] for review). Receptors for PTH have been cloned from opossum, rat, and human cDNA libraries [3–6]. These receptors bind PTH, PTH-related peptide, and N-terminal fragments of these hormones with nanomolar affinities. Only one PTH receptor gene exists in the mouse, rat, and human genome [7] and so far, there is no evidence for receptor subtypes mediating different physiological effects [5].

Importantly, the rat and human receptor activate both cAMP and IP production following transient expression in COS cells [4,6]. Stimulation of both signal transduction systems has also been observed with the structurally related calcitonin receptor [8]. Studies presented in abstract form [9] concerning the full-length and a truncated opossum PTH receptor (OK-H, see Fig. 1) had suggested that regions within the C-terminus of the PTH receptor play an important role in the activation of PI turnover, whereas they are dispensable for the coupling to AC.

Given these results, we set out to study the coupling specificity of the full-length human PTH receptor, which we had recently cloned from a kidney cDNA library [6], as well as a number of receptor constructs truncated in the C-terminal intracellular tail region. Coupling to AC and PI-PLC was investigated both in transiently transfected COS cells as well as in stably transfected 293 cells.

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Abbreviations: AC, adenylyl cyclase; G_s and G_i, stimulatory and inhibitory G protein of adenylyl cyclase, respectively; G_q, stimulatory G protein of PI-PLC; IP, inositol phosphate; PI, phosphoinositide; PI-PLC, phosphoinositide-specific phospholipase C; PTH, parathyroid hormone; hPTH, human parathyroid hormone; cPTHrP, chicken parathyroid hormone-related protein; PTX, pertussis toxin; HEPES, 4-(2-hydroxyethyl)-piperazineethanesulfonic acid; IBMX, isobutylmethylxanthine; TCA, trichloroacetic acid; 293 cells, human embryonic kidney 293 cell line.

2. Materials and methods

2.1. Construction of truncated PTH receptors

Construct pR2 is identical to pRezex-2 as described in [6]. It contains the full-length cDNA of the human PTH receptor in the pcDNAIneo expression vector (Clontec). For the structure of the various truncated receptors described in the following, please refer to Fig. 1.

To obtain pR3, pR2 was cut with *Xho*I which releases a small sequence between nucleotide 1,832 of the receptor cDNA and a *Xho*I site in the pcDNAIneo polylinker. The vector was then religated, which results in a new C-terminus as indicated in Fig. 1.

All other constructs were generated starting from the receptor cDNA cloned into pUC19 (pUCR2). To obtain pR4, the unique *Sfi*I site at nucleotide 1,904 was used to insert a synthetic sequence containing a stop-codon which terminates translation after E579. Plasmids pR5-pR8 were constructed using polymerase chain reaction (PCR). The region between the unique *Sac*I and *Xho*I sites (nucleotides 1,246 and 1,832, respectively) of pUCR2 was replaced by different PCR products. As 5′ primer, oligonucleotide PTRec-3 [6] was used. Different 3′ primers were designed to place a stop-codon at the desired position followed by a *Xho*I site. Using *Bam*HI and *Xho*I, the entire receptor coding regions were then subcloned into pcDNAIneo. The fragments generated by PCR were verified by DNA sequencing.

2.2. Transfections

All cells were grown in Dulbecco's modification of Eagles medium (DMEM)/Ham's F12 (1:1) medium containing 10% fetal calf serum. For transient transfections, COS-1 cells were seeded in 10 cm plates at a density of 40,000 cells/cm^2. The following day, cultures were transfected with 20 μg/plate of the receptor constructs or empty vector (control) using the diethylaminoethyl (DEAE)-dextran method [10]. Three plates per construct were used. After 24 h, cells were reseeded into three 24 well plates at a high cell density of ≈100,000 cells per well. The following day, these plates were assayed in parallel for binding and for agonist-induced cAMP and IP formation.

Stable transfections were carried out using the calcium phosphate coprecipitation method [11]. 293 cells were seeded in six well plates at a density of 2×10^5 cells per well and transfected the following day with 10 μg/well of the receptor constructs linearized at the *Sac*II site of the pcDNAIneo vector. After 24 h, normal medium was added and the cells were left to recover for one day before starting selection for G418 resistance. Cell populations resistant to the antibiotic (1,000 μg/ml of

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the material provided by Sigma, Buchs) were obtained within two weeks, expanded and frozen. Per receptor construct, five independent transfections were carried out in parallel wells. Populations showing strongest binding and cAMP response to hPTH-(1–38) were selected for further experiments. The identity of the expressed receptors in the selected cell populations was independently verified using PCR.

2.3. Binding experiments

As radioligand, iodinated chick [Tyr^{36}]PTH related peptide-(1–36) ([Tyr^{36}]cPTHrP-(1–36)) was used (2200 Ci/mmol, Anawa Ag, Wangen) and experiments were carried out as described in [6]. Affinities and B_{max} values were determined by scatchard analysis. Nonspecific binding was below 10% of total binding in all experiments.

2.4. Measurements of AC and PI-PLC activities

To measure cAMP production, cells in 24 well plates were prelabelled with [^{3}H]adenine (Amersham) at 2–4 μCi/ml for 2 h. Thereafter, cells were washed and incubated in HBS containing 1 mM IBMX and the indicated agonists at 37°C for 15 min. The cells were then extracted with 5% ice-cold TCA. [^{3}H]ATP and [^{3}H]cAMP were separated by sequential chromatography on Dowex and alumina columns as described in [12].

Inositol phosphate formation was measured following prelabelling of cells with [^{3}H]inositol (Amersham) for 24 h at 2–4 μCi/ml. 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 as described in [13]. Total inositol phosphates were collected.

Pretreatments with PTX (Sigma, 100 ng/ml) were carried out for 4 h prior to agonist application. Both for cAMP and IP formation, data are presented as means ± S.E.M. for duplicate or triplicate determinations.

3. Results

3.1. Truncations at the C-terminus of the human PTH receptor do not change binding affinities and do not abrogate coupling to both AC and PI-PLC in COS-1 cells

A series of receptor cDNAs truncated at the C-terminus were constructed (Fig. 1) and transiently expressed in COS-1 cells as described in section 2. The transfected cells were assayed for PTH binding and/or PTH-stimulated cAMP and IP formation. With the exception of the shortest construct (pR8), all were functionally expressed. The receptor encoded by pR8 lacks a number of charged amino acids (E469, K471, K472), which we anticipated to be important for the correct positioning of transmembrane domain seven (TM7) in the membrane. All other truncated receptors showed binding affinities which were not significantly different from the full-length receptor (Fig. 2). Likewise, all receptors were still able to stimulate both cAMP and IP formation, as is summarized in Table 1. Thus, based on transient expression in COS cells, no significant changes in binding or coupling properties between the full-length and functionally expressed truncated receptors became apparent.

It should be pointed out that the stimulation of PI-PLC induced by the PTH receptor is relatively weak. In general we observed a less than two-fold increase over basal IP levels (Table 1), but when we transfected the hamster 5-HT$_{2A}$ receptor [14] into COS-1 cells under identical experimental conditions, we observed an 8-fold increase with serotonin (not shown).

3.2. The full-length human PTH receptor stably expressed in 293 cells activates G_s only, whereas a severely truncated receptor activates G_s, G_q and G_i

Working with UMR 106–06 and ROS 17–2.8 osteosarcoma cells as well as with secondary cultures of rat and human osteoblasts, we had been unable in the past to detect a significant stimulation of PI turnover by PTH peptides, whereas formation of cAMP was clearly observed (unpublished results). We were therefore interested to determine whether the full-length PTH receptor as well as the truncated receptor constructs were able to show dual coupling to both AC and PI-PLC also in stably transfected cells. 293 cells were transfected with the different receptor constructs and G418 resistant cell populations were isolated. No subcloning was undertaken in order to avoid artefacts due to clonal selection. At least two independently transfected populations per construct were tested for PTH-induced cAMP and IP formation. As in COS cells, pR8 was not expressed as a ligand-binding, functional protein. All other cell populations tested showed a strong cAMP response to hPTH-(1–38), as shown for instance in Fig. 3A for the full-length receptor. However, neither cells expressing the latter receptor (Fig. 3B) nor cells expressing constructs pR3, pR5 or pR6 (not shown) showed a significant PTH response in PI assays, whereas a strong stimulation of the system was detectable with carbachol, which activates endogenous m3-muscarinic receptors in 293 cells [8].

We were surprised, therefore, to find that cells expressing pR7 stimulated IP formation strongly in response to hPTH-(1–38) (Fig. 4B) in all experiments carried out. We set out to compare second messenger formation stimulated by the wild type receptor and by R483 in more detail, using the cell populations designated 293 p5 and 293 pR7 p1, respectively. These experiments are summarized in Table 2. The two cell populations were found to express a comparable mean number of receptors per cell showing similar binding affinities for our radioligand. Also, activation of AC by hPTH-(1–38) occured with similar EC_{50}s (0.39 and 0.12 nM, respectively). Whereas no stimulation of IP formation was detected in the cells expressing the full-length receptor, it occured in the cells expressing R483 with an EC_{50} of 10 nM. The activation of PI turnover was only minimally sensitive to PTX (Fig. 4B), suggesting activation of G_q proteins by the truncated receptor. It is important to underscore that the activation of PI turnover observed in 293 pR7 p1 cells can not be attributed to higher levels of receptor expression compared to 293 p5 (Table 2).

Table 1
AC and PI-PLC responses to hPTH-(1–38) in COS-1 cells transfected transiently with different receptor constructs

Receptor construct	cAMP Formation			IP Formation		
	Max. stimulation (% of control)	pEC_{50} (−log(M))	n	Max. stimulation (% of control)	pEC_{50} (−log(M))	n
pR2 (full length)	558 ± 96	9.33 ± 0.10	6	198 ± 23	8.26 ± 0.13	5
pR5	265 ± 4	9.03 ± 0.29	2	163 ± 15	8.03 ± 0.33	2
pR6	209 ± 12	8.7 ± 0.14	2	140 ± 21	7.85 ± 0.35	2
pR7	410 ± 106	9.1 ± 0.11	3	176 ± 3	8.1 ± 0.32	3

Data are given as means ± S.E.M.; n = number of independent experiments.

In an attempt to determine whether the receptor encoded by pR7 activated other G proteins as well, we checked whether PTX was able to potentiate the cAMP response to hPTH-(1–38), which would be indicative of an activation of G_i [15]. Indeed, following pretreatment of cells with PTX, a significantly stronger cAMP response was observed in 293 pR7 pl at high concentrations of agonist (Fig. 4A), whereas no such effect was observed in the cells expressing the full-length receptor (Fig. 3A). The potentiating action of PTX in 293 pR7 pl was specific for the PTH- response, as the toxin did not affect the response elicited by the β-adrenergic agonist isoproterenol or by forskolin. As expected, PTX reversed the inhibitory effect of the G_i-activator aluminum fluoride in both cell systems, which demonstrates functionality of the toxin. Stimulation of the PTH-response by PTX in the cells expressing R483 was readily reproducible (increase to 139 ± 5% of control at

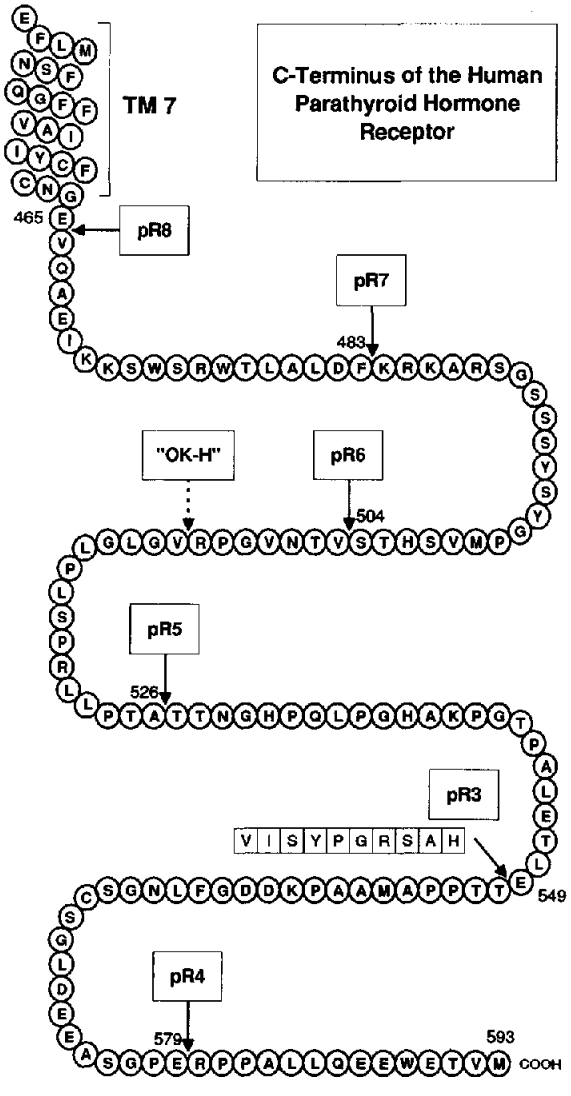

Fig. 1. Amino acid sequence of the C-terminal cytoplasmic domain of the human PTH receptor. Sites of truncation are marked. In the case of construct pR3, the cloning procedure results in a new C terminus which is not present in the full-length receptor (boxed residues, see methods section). 'OK-H' refers to the truncated receptor described in [3] which was isolated from opossum kidney cells. This receptor is truncated at a site corresponding to R511 of the human receptor. Construct pR4 was not used in the present study.

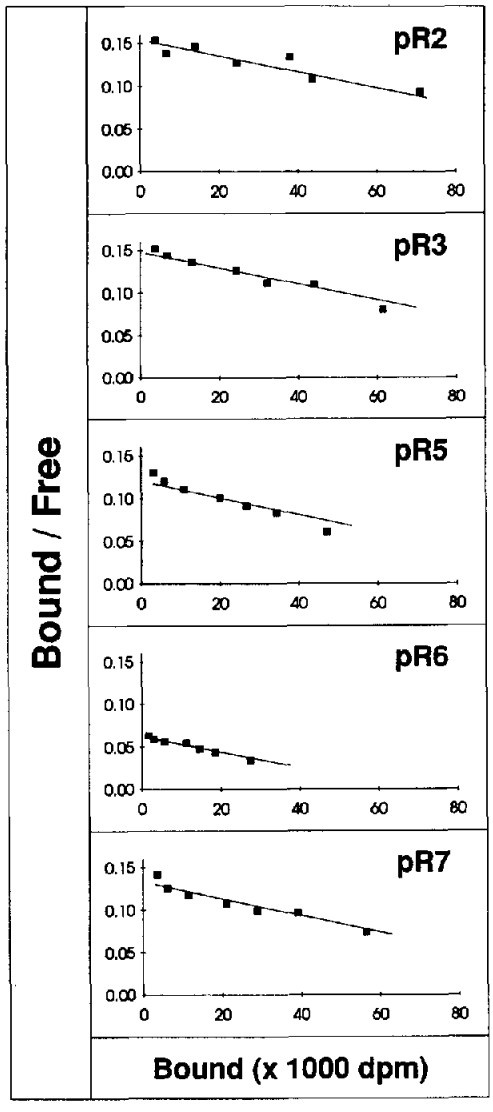

Fig. 2. Ligand binding in COS-1 cells transiently transfected with different receptor constructs. Construct pR2 codes for the full-length receptor. The slope of the lines used to fit the scatchard data corresponds to a dissociation constant of 0.9 nM in all cases.

10^{-8}M hPTH-(1–38); mean ± S.E.M., n = 7). This is a relevant effect, as inhibition of stimulated cAMP formation by mainly G_i- coupled receptors rarely exceeds 50% in comparable experimental settings [16,17]. The small inhibition of PTH-stimulated IP- formation by PTX (Fig. 4B) in 293 pR7 pl is also in agreement with an activation of G_i proteins which may stimulate the activity of isoforms of PI-PLC through the release of G protein $\beta\gamma$ subunits [18].

There was no evidence for a basal activity of the unliganded receptors. Cells expressing pR7, like untransfected cells or cells expressing the full-length receptor, showed only minimal increases of cAMP or IPs following addition of IBMX or lithium alone, respectively.

4. Discussion

How PTH elicits its diverse physiological effects in target

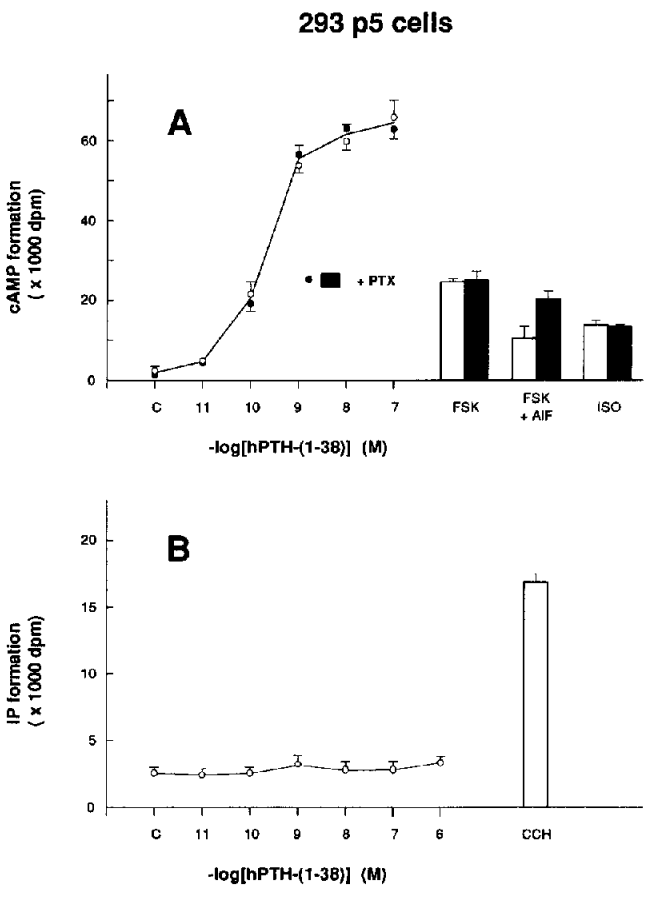

Fig. 3. Stimulation of cAMP (A) and IP formation (B) by hPTH-(1–38) and other agonists in 293 cells expressing the full-length receptor (293 p5 cell population). Cells were pretreated (black symbols and bars) or not (open symbols and bars) with PTX prior to the experiment. Forskolin (FSK), isoproterenol (ISO), and carbachol (CCH) were used at 10^{-5}, 10^{-5}, and 10^{-4} M, respectively. To stimulate with aluminum fluoride (AlF), 10^{-5} M Al^{3+} and 5 mM F^- were added.

cells is a matter of debate. There is a general consensus that the hormone stimulates cAMP formation, but in addition, several laboratories reported activation of PI turnover, PKC or calcium mobilization [1, 19–23]. The latter signal has been proposed to arise independently of the formation of IP_3 [24], and studies carried out in our hands are in agreement with this notion (K. Seuwen and H. Boddeke, manuscript in preparation).

Obviously, our experiments carried out with stably transfected 293 cells do not support the notion that the full-length human PTH receptor activates PI turnover significantly under physiological conditions. These data confirm results obtained before in our laboratory with ROS 17/2.8 and UMR106 06 osteosarcoma cells as well as with secondary cultures of human and rat osteoblasts. In none of these cultures were we able to detect a significant inositol phosphate formation in response to PTH peptides (unpublished results). To our knowledge, there are no reports which have so far demonstrated activation of PI turnover by PTH in cells stably transfected with one of the PTH receptors cloned. A recent study on LLC-PK1 cells transfected with the rat receptor dealt with calcium signals [25].

Yet, expression of the full-length PTH receptor in COS cells can lead to PTH- induced IP formation, as demonstrated in this study and before [4,6]. However, the amplitude of this response is small compared to the one observed with other mainly PI-coupled receptors ($5HT_{2A}$ receptors, for instance) and requires higher agonist concentrations than cAMP formation. The high levels of receptor expression achieved in COS cells may lead to unspecific coupling and we attribute the weak signal seen in these cells to such an effect. In fact, estimating a transfection efficiency of 30%, we calculate from our COS cell binding data that the transfected cells express > 10^6 receptors, which is ten times more than the 293 cells. Also, in transient expression experiments, COS-7 cells have been shown to be a much more permissive system than 293 cells to demonstrate coupling to PI turnover of α_2-adrenergic receptors [26].

Our data obtained with the severely truncated receptor R483 stably expressed in 293 cells are particularly interesting. Not only was this receptor functional as an activator of AC, but it also stimulated IP formation and a PTX- induced potentiation of the cAMP response was observed, thus suggesting coupling to G_s, G_q and G_i proteins. Coupling of one receptor to two different signal transduction pathways has been described in several cases, examples include coupling to G_i and G_q of the α_2-adrenergic receptor [16] and to G_s and G_q by the calcitonin receptor [8]. Activation of G_s and G_i could be demonstrated with chimeras of β_2- and α_2-adrenergic receptors [15]. However, to our knowledge there is only one example of a receptor where evidence for promiscous coupling to all three G proteins has been obtained. This is the case for the prostaglandin EP_3 receptor subtype, where splice variants of the C-terminal cytoplasmic tail exist which engender coupling to G_s or G_i or $G_s/G_i/G_q$, respectively [27]. In this case, however, the receptor variant with the shortest cytoplasmic tail shows the highest degree of coupling specificity, interacting exclusively with G_s.

As far as the PTH receptor is concerned, our data indicate that sequences within the receptor C terminus direct the receptor towards an interaction with G_s. Splice variants of the PTH

Table 2
Ligand binding and activation of second messenger systems in 293 cells stably transfected with the full-length human PTH receptor cDNA (pR2 construct, 293 p5 cell population) and with a truncated receptor (pR7 construct, 293 pR7p1)

	Cell type	
	293 p5 (full length R.)	293 pR7 p1 (R483)
Binding sites per cell (× 1,000)	155 ± 44 (n = 4)	45.8 ± 5.3 (n = 3)
Affinity for radioligand (× 10^{-10} M)	6.4 ± 0.9 (n = 4)	6.9 ± 0.6 (n = 3)
pEC_{50} cAMP Formation (−log(M))	9.41 ± 0.09 (n = 8)	9.92 ± 0.10 (n = 7)
pEC_{50} IP Formation (−log(M))	no response detected	8.11 ± 0.05 (n = 6)

Binding affinities and number of sites per cell were determined by scatchard analysis using iodinated [Tyr^{36}]cPTHrP-(1–36) as ligand. Data are given as means ± S.E.M.; n = number of independent experiments.

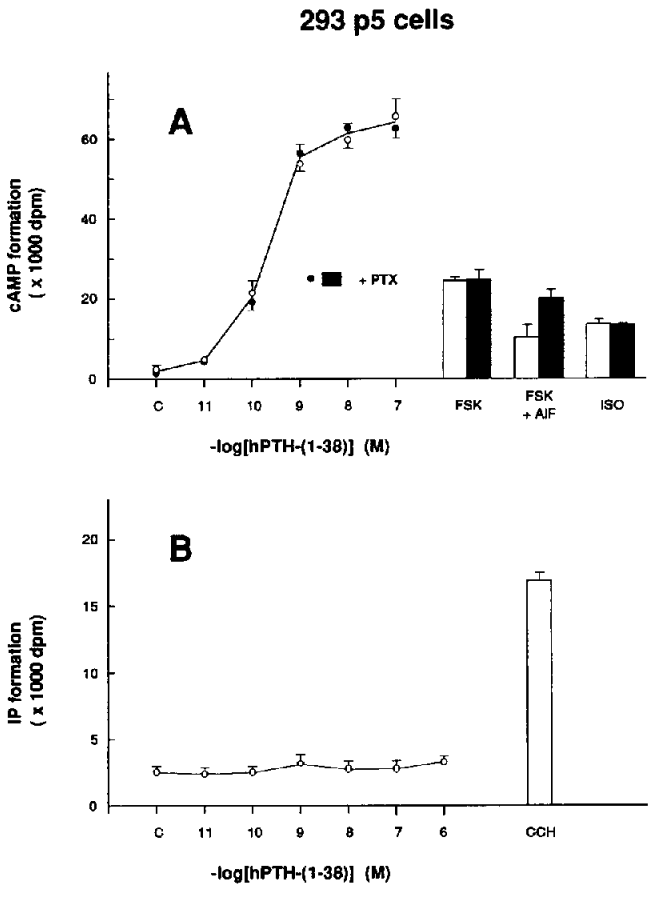

Fig. 4. Stimulation of cAMP (A) and IP formation (B) by hPTH-(1–38) and other agonists in 293 cells expressing R483 (293 pR7 pl cell population). Cells were pretreated (black symbols and bars) or not (open symbols and bars) with PTX prior to the experiment. Agonists were applied as described in the legend to Fig. 3.

receptor seem to exist in tissues other than bone [28] and it may turn out that some of them show coupling to several signalling systems. Also, it can not be excluded that unknown mechanisms acting on the receptor C-terminus modulate coupling in a specific cellular setting.

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