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A salt bridge between Arg-20 on parathyroid hormone (PTH) and Asp-137 on the PTH_1 receptor is essential for full affinity

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ABSTRACT

Parathyroid hormone (PTH) acts via the receptor PTH_1 and plays an important role in calcium homeostasis. PTH's interaction with the N-terminal domain of PTH_1 is mediated in part by Arg-20 on the peptide which forms a number of interactions with the receptor: a charge–charge interaction with Asp-137; hydrogen bonds with the backbone of Asp-29 and Met-32; and hydrophobic interactions with Met-32 and Gln-37. The aim of this work was to establish the importance of the charge–charge interaction through the combined use of modified peptide ligands, site-directed mutations of the receptor, and pharmacological assays. The substitution of Arg-20 with norleucine resulted in a 50-fold reduction in potency at PTH_1 and Asp-137-Glu while, in contrast, both Asp-137-Asn and Asp-137-Ala receptors were largely insensitive to this ligand modification. The effect of this removal of the positive charge as position 20 could be partially rescued at PTH_1 and Asp-137-Glu, but not Asp-137-Asn and Asp-137-Ala, through a substitution of peptide position 20 with ornithine. The latter two receptors, which have no negative charge at position 137, displayed potency for PTH that was reduced by 40- and 117-fold, respectively. These data demonstrate that a negative charge at residue-137 is important for interacting with ligands containing a positive charge at residue-20, and that the Arg-20 interaction with Asp-137, observed in the crystal structure of the isolated N-terminal domain of PTH_1, is likely to be present in the full length receptor where it provides an important affinity- and potency-generating interaction through a salt bridge.

Introduction

Parathyroid hormone (PTH) plays a central role in calcium homeostasis via its actions at osteoblasts in bone tissue and at renal tubule cells in the kidney [9]. The hormone acts via the Family B G protein-coupled receptor (GPCR) PTH_1 which also recognizes PTH-related protein (PTHrP), a hormone that plays a vital role in embryonic development [11]. Although PTH comprises 84 residues and PTHrP is 141 residues in length, their biological effects via PTH_1 can be replicated by synthetic peptides equivalent to the first 34 amino acids of each peptide [9]. Since daily administration of PTH or PTH(1–34) have been shown to increase bone formation and density in patients with osteoporosis, PTH_1 has become an important target for the development of potential new drugs to treat this widespread condition [10].

PTH_1 is activated by PTH(1–34) via a two site interaction model [2,3] in which the ligand's C-terminal α-helical region interacts with the receptor's N-terminal extracellular (N) domain to generate affinity, while the N-terminal region of the peptide activates the receptor via a second interaction with the receptor's transmembrane helices and connecting loops (juxta-membrane (J) or "core" domain). The nature of the first interaction has been detailed via the solution of the structure of the ligand-bound extracellular domain of PTH_1 via X-ray crystallography [7]. The crystal structure showed that the ligand forms an α-helix which docks into a long hydrophobic groove on the N domain via hydrophobic interactions formed by Val-21*, Trp-23*, Leu-24*, Leu-28*, Val-31* and Phe-34* of PTH (ligand residues will be distinguished from receptor residues by an asterisk following the residue number). In particular, the hydrophobic interaction between Trp-23* and Leu-41 has been shown to play an important role in ligand-receptor specificity [6]. In addition to these hydrophobic interactions, the

Abbreviations: PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; ^{125}I-rPTH(1–34), 125I-[Nle8,21,Tyr34]rPTH(1–34)NH2; MBS, membrane binding solution; GPCR, G protein-coupled receptor; N domain, N-terminal extracellular domain; PTH_1, PTH/PTHrP receptor 1; HEK, human embryonic kidney; PBS, phosphate-buffered saline; PTHrP(1–36), Tyr36-PTHrP(1–36).

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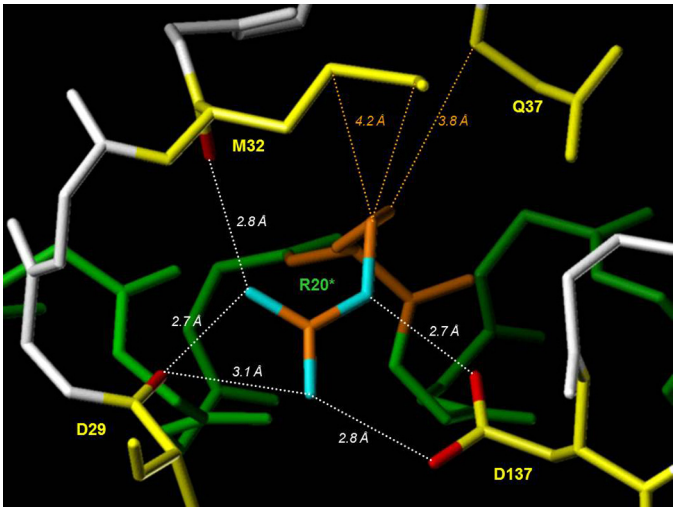

Fig. 1. View of part of the crystal structure of the PTH/PTH_1 complex showing the detail of the binding site around Arg-20*. Part of the PTH helix (green) is shown with Arg-20* projecting out of the page (brown, with side chain nitrogen atoms as blue). The main chain of PTH_1 is shown as white with those residues that interact with Arg-20* as yellow. Interactions are shown as dotted lines with inter-atom distances: the hydrophobic interactions are depicted as brown dotted lines while the five polar interactions are shown as white dotted lines that join the nitrogen atoms of PTH (blue) to the oxygen atoms of PTH_1 (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

crystal structure describes a number of polar interactions that may further strengthen the binding of PTH to PTH_1. The most notable of these interactions is mediated by Arg-20* which appears to form a charge–charge interaction with Asp-137, as well as additional hydrogen bonds with the backbone of Asp-29 and Met-32 (Fig. 1). Indeed, the role of Arg-20* in forming an important polar interaction has been supported by analyses of the structural requirements of residue 20* through its substitution by a number of natural and unnatural amino acids [1,4] which showed that the precise positioning of the cation of Arg-20* is critical for full activity. For example, while substitutions of Arg-20* by ornithine, 4-[(N-amidino)-piperidyl]-(S)-glycine or citrulline were tolerated, substitutions by lysine, 4-[(N-amidino)-piperidyl]-(L)-alanine or glutamine were not. However, since Arg-20* is completely buried in the PTH_1/PTH complex, its long aliphatic side chain also forms some hydrophobic interactions with the receptor via Met-32 and Gln-37 (Fig. 1). The use of cellulose-bound PTH peptide libraries suggests these hydrophobic interactions may be critical since Arg-20* can be replaced by Phe but not Lys, although this approach did not enable the determination of precise binding affinities [5]. Therefore, we set out to assess the importance of the interaction between Arg-20* of PTH and PTH_1 in order to explore whether the charge–charge interaction via Asp-137 was the critical component for ligand binding and ligand-induced receptor activation.

Materials and methods

Constructs

The full-length cDNA of human PTH_1 (gift from GlaxoSmithKline) in pcDNA3 (Invitrogen, Paisley, UK) was used to express wild type PTH_1 as described previously [6]. Mutant receptors were generated using QuikChange® site-directed mutagenesis (Stratagene, La Jolla, CA, USA) and confirmed by DNA sequencing. These various pcDNA3 constructs were used to express the wild type and mutant PTH receptors in Human Embryonic Kidney (HEK)-293 cells.

Cell culture

The HEK-293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, from Sigma, Poole, UK) supplemented with 10% fetal calf serum (Lonza Wokinham Ltd., Wokingham, UK) and containing 2 mM L-glutamine, 100 U/ml penicillin and 100 $\mu g\,ml^{-1}$ streptomycin (Invitrogen, Paisley, UK). Cells were transfected with pcDNA3 containing the cDNA encoding the receptors, using the SuperFect® Transfection Reagent (Qiagen Ltd., Crawley, UK) and stable clones were selected with G418 antibiotic (Invitrogen, Paisley, UK) as follows. Cells were seeded into a 25 cm^2 flask containing 10 ml of media and transfected when they reached 50–80% confluence. To do this, 20 μl of SuperFect® was mixed with a DNA solution consisting of 5 μg plasmid DNA in 150 μl DMEM. The DNA was incubated with the reagent for 10 min at room temperature after which 1 ml of media was then added and mixed gently. The cells were washed once with sterile PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) before the transfection mixture was added and incubated for 3 h at 37 °C. The cells were then washed 3 times with PBS before the addition of fresh media. Three days later, the supernatant was removed and the cells were washed with PBS before fresh media was added. Selection of transfected cells was achieved by addition of 800 $\mu g\,ml^{-1}$ G418. The media, containing G148, was replaced every 3 days until individual colonies were clearly visible. Approximately 10–20 individual colonies were detached from the flask using trypsin, seeded in a fresh plate and grown to confluence.

Peptides

PTH(1–34) and rat [$Nle^{8,21}$,Tyr^{34}] rPTH(1–34)NH_2 [called rPTH(1–34) throughout this paper] were from Bachem (Saffron Walden, UK). Orn^{20}-PTH(1–31) and Nle^{20}-PTH(1–31) were the kind gift of Gordon E. Willick and were synthesized as described previously [8]. The radioligand ^{125}I-[$Nle^{8,21}$,Tyr^{34}] rat PTH(1–34)NH_2 (called ^{125}I-rPTH(1–34) throughout the paper) was from Perkin Elmer Life and Analytical Sciences (Waltham, MA, USA).

Ligand binding to membranes

HEK-293 cells, cultured to confluence on five 160-cm^2 Petri dishes (pre-coated with poly-D-lysine), were washed with PBS, followed by the addition of 15 ml of ice-cold sterile double distilled water to induce cell lysis. Following 5 min incubation on ice, the ruptured cells were thoroughly washed with ice-cold PBS before being scraped from the plates and pelleted by centrifugation in a bench-top centrifuge (13,000 × *g* for 30 min). The crude membrane pellet was resuspended in 1 ml membrane binding solution (MBS; 20 mM HEPES pH 7.5, 100 mM $NaCl_2$, 1 mM EDTA, 3 mM $MgSO_4$, 50 mg L^{-1} bacitracin) and forced through a 23G needle after which 0.1 ml aliquots were snap-frozen in N_2(l) and stored at -70 °C. Membranes were slowly thawed on ice before diluting to a concentration that gave total radioligand binding of <10% of the total counts added. In a reaction volume of 200 μl, 75 pM (~60,000 cpm), ^{125}I-rPTH(1–34), various concentrations of an unlabelled competitor ligand and HEK-293 membranes expressing the receptor of interest were combined, all diluted in MBS supplemented with 0.3% non-fat milk powder (NFM). Assays were carried out in polypropylene v-bottomed 96-well plates (Nalge Nunc International, Rochester, NY, USA) for 1 h. MultiScreen 96-well filtration plates (polyvinylidene fluoride filters, 0.45 μm pore size, Millipore, Bedford, MA, USA) were pre-soaked in a 1% NFM/PBS solution for 30 min. After the incubation, membrane-associated radioligand was harvested by transferring the assay mixture to the filtration plate housed in a vacuum manifold. The wells of the assay plate were washed with 0.2 ml MBS, which was transferred to the filtration plate. The wells were then washed three times