Calcilytic Compounds: Potent and Selective Ca\textsuperscript{2+} Receptor Antagonists That Stimulate Secretion of Parathyroid Hormone

EDWARD F. NEMETH, ERIC G. DELMAR, WILLIAM L. HEATON, MICHAEL A. MILLER, LYSSA D. LAMBERT, REBECCA L. CONKLIN, MAXINE GOWEN, JOHN G. GLEASON, PRADIP K. BHATNAGAR, and JOHN FOX

NPS Pharmaceuticals, Inc., Salt Lake City, Utah (E.F.N., E.G.D., W.L.H., M.A.M., L.D.L., R.L.C., J.F.); and Departments of Bone and Cartilage Biology (M.G.) and Medicinal Chemistry (J.G.G., P.K.B.), SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania

ABSTRACT

Despite the discovery of many ions and molecules that activate the Ca\textsuperscript{2+} receptor, there are no known ligands that block this receptor. Reported here are the pharmacodynamic properties of a small molecule, NPS 2143, which acts as an antagonist at the Ca\textsuperscript{2+} receptor. This compound blocked (IC\textsubscript{50} of 43 nM) increases in cytoplasmic Ca\textsuperscript{2+} concentrations [Ca\textsuperscript{2+}], elicited by activating the Ca\textsuperscript{2+} receptor in HEK 293 cells expressing the human Ca\textsuperscript{2+} receptor. NPS 2143, even when tested at much higher concentrations (3 μM), did not affect the activity of a number of other G protein-coupled receptors, including those most structurally homologous to the Ca\textsuperscript{2+} receptor. NPS 2143 stimulated parathyroid hormone (PTH) secretion from bovine parathyroid cells (EC\textsubscript{50} of 41 nM) over a range of extracellular Ca\textsuperscript{2+} concentrations and reversed the effects of the calcimimetic compound NPS R-467 on Ca\textsuperscript{2+} and on secretion of PTH. When infused intravenously in normal rats, NPS 2143 caused a rapid and large increase in plasma levels of PTH. Ca\textsuperscript{2+} receptor antagonists are termed calcilytics and NPS 2143 is the first substance (either atomic or molecular) shown to possess such activity. The pharmacodynamic properties of NPS 2143 together with the recently demonstrated effects of this compound on bone formation support the view that orally active calcilytic compounds might provide a novel anabolic therapy for osteoporosis.

The cell surface Ca\textsuperscript{2+} receptor is the primary molecular entity regulating the secretion of parathyroid hormone (PTH). Activation of this receptor by increased levels of extracellular Ca\textsuperscript{2+} inhibits PTH secretion, whereas its presumed inactivation by lowered extracellular Ca\textsuperscript{2+} leads to an increase in PTH secretion. PTH regulates systemic Ca\textsuperscript{2+} homeostasis by acting on target cells in both the kidney and the skeleton to increase plasma levels of Ca\textsuperscript{2+} (Brown and MacLeod, 2001). In bone, PTH increases bone turnover but the resulting overall effect on bone mineral density is highly dependent on the temporal changes in the circulating levels of PTH. Thus, sustained elevations in plasma PTH levels, such as occur in hyperparathyroidism, result in increased bone resorption and a net decrease in bone mass at most skeletal sites (Antonsen and Sherrard, 1995; Silverberg et al., 1995). In contrast, temporary increases in plasma levels of PTH or its 1-34 fragment, achieved by the daily (or near daily) injection of either peptide, stimulate new bone formation in animal models of osteopenia (Dempster et al., 1993; Kimmel et al., 1993; Ejercet et al., 1995; Li and Wronska, 1995; Fox et al., 1997) and in clinical studies of osteoporotic patients (Reeve, 1996; Hodsmn et al., 1997; Lindsay et al., 1997; Lane et al., 1998; Fujita et al., 1999; Rittmaster et al., 2000). The results of these studies using peptides administered intermittently demonstrate that PTH is a potent anabolic agent that increases bone mineral density and bone strength to a greater extent than that achieved by antiresorptive therapies (Mosekilde et al., 1994; Hodsmans et al., 1997; Lindsay et al., 1997; Lane et al., 1998; Fujita et al., 1999; Rittmaster et al., 2000). The profound stimulatory effect of these peptides on bone formation has generated interest in the use of PTH or its fragments as a novel anabolic therapy for osteoporosis. However, the therapeutic use of these peptides is compromised by the need for systemic administration of a costly biological agent. An alternative approach that might overcome these drawbacks, and yet achieve similar anabolic effects on bone, is based on the use of small, orally active compounds that regulate plasma levels of endogenous PTH (Nemeth, 1996; Fox et al., 1997). This hypothesis holds that blocking Ca\textsuperscript{2+} receptor activity with small molecules will stimulate PTH secretion. With the appropriate pharmacokinetic profile, such compounds would be expected to cause a marked but transient increase in circulating PTH levels, sufficient to...
stimulate new bone formation. While this hypothesis is in line with conventional thinking, it remains untested because no ligand has been found that blocks activation of the Ca\(^{2+}\) receptor. In contrast, a wide variety of inorganic or organic polycations and certain phenylalkylamines have been shown to act as agonists or allosteric activators of this receptor (Nemeth and Fox, 1999). Like G protein-coupled receptor agonists in general, those that activate the Ca\(^{2+}\) receptor exhibit marked tissue selectivity (Lavigne et al., 1998; Fox et al., 1999) and are not ideal ligands to study Ca\(^{2+}\) receptor function, particularly in those tissues that are not involved in systemic Ca\(^{2+}\) homeostasis and that often express much lower levels of the Ca\(^{2+}\) receptor than classic "calcemic tissues" such as the parathyroid glands and kidney. In contrast, G protein-coupled receptor antagonists typically do not show profound tissue selectivity. Because of this, receptor antagonists are more valuable tools to study receptor function in a variety of different tissues. Such compounds, if capable of stimulating secretion of PTH, might also provide structures for novel drugs capable of transiently increasing levels of plasma PTH and stimulating new bone formation.

The need for such an anabolic therapy is underscored by the serious health problem posed by osteoporosis, the incidence of which is increasing as the general population ages. Already there are nearly 6 million women and about 2 million men with osteoporosis in the United States and a far greater number of individuals with osteopenia or low bone mineral density. While currently available antiresorptive therapies, such as estrogen or bisphosphonates prevent further bone loss, they cause relatively small increases in new bone formation. The ability to stimulate new bone formation and thereby increase bone mass to levels approaching those in young adults, would constitute a significant advance in the treatment of osteoporosis.

This report describes the salient pharmacodynamic properties of NPS 2143, a small molecule (Fig. 1) that blocks the parathyroid gland Ca\(^{2+}\) receptor and stimulates PTH secretion in vitro and in vivo. This compound, which is one member of a family of structurally similar compounds, is the first substance, either ionic or molecular, shown to possess inhibitory activity at the Ca\(^{2+}\) receptor.

### Materials and Methods

**Assays for Assessing Potency and Selectivity of Compounds on Ca\(^{2+}\) Receptor.** HEK 293 cells engineered to express the human parathyroid Ca\(^{2+}\) receptor have been described in detail previously (Nemeth et al., 1998). This clonal cell line, referred to as HEK 293 4.0-7 cells, has been used in a high-throughput screening format to detect agonists and allosteric activators (calcimimetics) of the Ca\(^{2+}\) receptor (Nemeth et al., 1998). Changes in the concentration of cytoplasmic Ca\(^{2+}\) ([Ca\(^{2+}\)]\text{cyt} ) provide a quantitative and functional assessment of Ca\(^{2+}\) receptor activity in these cells and the results using this assay parallel those obtained using a homologous expression system of bovine parathyroid cells. On-line continuous measurements of fluorescence in fluo-3- or fura-2-loaded HEK 293 4.0-7 cells were obtained using a custom-built spectrofluorimeter (Racke and Nemeth, 1993) or a fluorescence imaging plate reader instrument (Molecular Devices, Sunnyvale, CA). Test compounds were incubated with cells for 1 min before increasing the concentration of extracellular Ca\(^{2+}\) from 1.0 to 1.75 mM. Compounds were tested individually at a concentration of 100 μg/ml (20–80 μM) and those causing more than a 40% inhibition of the control response were considered to be biologically active.

To determine the potencies (IC\(_{50}\)) of compounds with biological activity, concentration-response curves were obtained and then, as an initial assessment of selectivity, the effects of compounds on [Ca\(^{2+}\)], evoked by other G protein-coupled receptors were examined at a concentration several times their IC\(_{50}\). Wild-type HEK 293 cells (and HEK 293 4.0-7 cells) express receptors for thrombin, bradykinin, and ATP, which couple to the mobilization of intracellular Ca\(^{2+}\). These responses can be studied to quickly assess any nonselective action of compounds on G protein-coupled receptors. Additional assays for selectivity included HEK 293 cells engineered to express receptors most homologous in sequence and topology to the Ca\(^{2+}\) receptor. These included native or chimeric receptors for various metabotropic glutamate (mGluRs) and γ-aminobutyric acid type B receptors (GABA\(_{B}\)Rs). Chimeric receptors were created using partial sequences of metabotropic glutamate receptors and Ca\(^{2+}\) receptors, engineered to couple to activation of phospholipase C and release of intracellular Ca\(^{2+}\) in HEK 293 cells as described in the legend to Fig. 4 and in Table 1. Compounds lacking pan-activity were then subjected to structural modifications and their potencies and selectivities monitored using these HEK 293 4.0-7 cell assays in an iterative process.

Screening of compound libraries using measurements of [Ca\(^{2+}\)]\text{cyt} in HEK 293 4.0-7 cells resulted in the identification of a number of structurally diverse compounds. Many of these compounds, however, failed to meet subsequent criteria and were eliminated. Several of

### TABLE 1

Cytoplasmic Ca\(^{2+}\) responses elicited by several G protein-coupled receptors that are homologous to the Ca\(^{2+}\) receptor are unaffected by NPS 2143. See Materials and Methods for description of cell lines used. Using the plate reader instrument, each cell line was treated with test compounds at the indicated concentrations prior to addition of the appropriate receptor agonist. The relative fluorescence units (RFU) shown are the means of 4 to 16 wells from two different plates and are not corrected for basal fluorescence.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RFU</th>
<th>% Control Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>mGluR1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 μM Glutamate (stable)</td>
<td>1125</td>
<td>100</td>
</tr>
<tr>
<td>+ 10 μM NPS 2190</td>
<td>341</td>
<td>30</td>
</tr>
<tr>
<td>+ 300 nM NPS 2143</td>
<td>1171</td>
<td>104</td>
</tr>
<tr>
<td>+ 3 μM NPS 2143</td>
<td>1166</td>
<td>104</td>
</tr>
<tr>
<td>20 μM Glutamate (transient)</td>
<td>1886</td>
<td>100</td>
</tr>
<tr>
<td>+ 300 nM NPS 2143</td>
<td>2262</td>
<td>120</td>
</tr>
<tr>
<td>+ 10 μM DHPG (transient)</td>
<td>1887</td>
<td>100</td>
</tr>
<tr>
<td>+ 10 μM NPS 2190</td>
<td>128</td>
<td>7</td>
</tr>
<tr>
<td>+ 300 nM NPS 2143</td>
<td>1924</td>
<td>102</td>
</tr>
<tr>
<td>+ 3 μM NPS 2143</td>
<td>1977</td>
<td>105</td>
</tr>
<tr>
<td>mGluR5d (transient)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 μM DHPG</td>
<td>882</td>
<td>100</td>
</tr>
<tr>
<td>+ 10 μM NPS 2190</td>
<td>139</td>
<td>16</td>
</tr>
<tr>
<td>+ 300 nM NPS 2143</td>
<td>848</td>
<td>96</td>
</tr>
<tr>
<td>+ 3 μM NPS 2143</td>
<td>923</td>
<td>105</td>
</tr>
<tr>
<td>GABA(<em>{B})R1 + GABA(</em>{B})R2 (stable)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 μM Baclofen</td>
<td>2142</td>
<td>100</td>
</tr>
<tr>
<td>+ 10 μM SCH 6279 \text{HCl}</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+ 300 nM NPS 2143</td>
<td>2144</td>
<td>100</td>
</tr>
<tr>
<td>+ 3 μM NPS 2143</td>
<td>1884</td>
<td>88</td>
</tr>
<tr>
<td>10 μM GABA</td>
<td>2334</td>
<td>100</td>
</tr>
<tr>
<td>+ 10 μM SCH 6279 \text{HCl}</td>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td>+ 300 nM NPS 2143</td>
<td>2225</td>
<td>95</td>
</tr>
<tr>
<td>+ 3 μM NPS 2143</td>
<td>2106</td>
<td>90</td>
</tr>
</tbody>
</table>
these original compounds did possess the requisite potency and selectivity; structural modifications to one of these (IC50 of 11 μM) led to a number of compounds that were profiled in detail. The pharmacodynamic properties of one of these, NPS 2143 (Fig. 1), is described in this report.

Assays of Ca2+ Receptor Activity Using Bovine Parathyroid Cells. The effect of NPS 2143 on Ca2+ receptor-dependent regulation of PTH secretion and cyclic AMP formation was assessed using primary cultures of dissociated bovine parathyroid cells. Following overnight culture, the cells were removed from the flasks by decanting and washing with buffer containing 126 mM NaCl, 4 mM KCl, 1 mM MgSO4, 0.5 mM CaCl2, 0.7 mM KH2PO4/K2HPO4, 20 mM Na-HEPES, pH 7.45, 1 mg/ml glucose, and 0.1% bovine serum albumin (ICN Biomedicals, Cleveland, OH). Portions (0.2 ml) of this cellular suspension were added to 12-× 75-mm polystyrene tubes with or without NPS 2143 and/or varying concentrations of CaCl2. Incubations (in triplicate) at 37°C for 20 or 30 min were terminated by placing the tubes on ice. Cells were pelleted by centrifugation (500g for 10 min at 4°C) and 0.1 ml of supernatant was immediately assayed for PTH content. A portion of the cellular suspension was left on ice during the incubation period and then processed in parallel with the other tubes. The amount of PTH in the supernatant from the tubes maintained on ice was defined as “basal release” and was subtracted from all other samples. PTH levels were quantified using a rat PTH(1-34) immunoradiometric assay kit, which also detects bovine PTH (Immutopics, San Clemente, CA). For each experiment, results were expressed as picograms of PTH released/10⁶ cells and then normalized to PTH released in 0.5 mM CaCl2. Cell numbers were determined by counting nuclei in a hemocytometer after lysing the cells and staining the nuclei with cresyl violet.

For measurements of cyclic AMP formation, parathyroid cells were incubated for 15 min at 37°C in 96-well plates (120,000 cells/well) in buffer containing 0.5 or 2 mM CaCl2 in the presence or absence of isoproterenol (1 μM) and/or NPS 2143 (300 nM) before cells were lysed. All wells additionally contained 0.5 mM isobutylmethylxanthine. Levels of total cyclic AMP (cells plus medium) were determined using the BIOTRAK cyclic AMP scintillation proximity assay kit (Amersham Pharmacia Biotech, Piscataway, NJ). Luminescence was measured on a Microbeta 1450 Tri-Lux instrument (Wallac, Gaithersburg, MD).

Plasma Levels of PTH in Rats. Normal male Sprague-Dawley rats (250–275 g) with unrestricted access to commercial rodent chow (Teklad 8640; Harlan Teklad, Madison, WI) and tap water were used. The animals were anesthetized by intraperitoneal injection of ketamine/xylazine (90:7 mg/kg) and chronic indwelling catheters were implanted in the inferior vena cava (for compound infusion) and in the abdominal aorta (for blood sampling) accessed by the femoral vein and artery, respectively. Following catheterization, the rats were housed individually and allowed to recover for at least 3 days before study. The protocol was approved by the Institutional Animal Care and Use Committee of NPS Pharmaceuticals, Inc. (Salt Lake City, UT).

On the day of study, the rats were infused intravenously (0.1 ml/kg · min) for 120 min with NPS 2143 (0.1 μmol/kg · min) or vehicle, a 20% aqueous solution of 2-hydroxypropyl-β-cyclodextrin (Sigma/RBI, Natick, MA). Blood samples (0.5 ml) were collected before and at various times after the start of the infusion for measurements of plasma levels of PTH and Ca2+. To prevent excessive blood volume loss during the course of the experiment, for each blood sample the erythrocyte pellet was suspended in an equal volume of normal rat plasma and reinfused. Plasma levels of Ca2+ were measured immediately after collection using a model 634 ionized calcium analyzer (Ciba Corning Diagnostics, Medford, MA). PTH levels were measured using the Immutopics rat PTH(1-34) immunoradiometric assay kit.

Statistical Analyses. The potency of NPS 2143 for stimulating PTH secretion in parathyroid cells (EC50) or inhibiting [Ca2+]i responses in HEK 293 4.0-7 cells (IC50) was determined by fitting a curve to the data from each series of experiments with the Levenberg-Marquardt algorithm using the KaleidaGraph program (Synergy Software, Reading, PA). The differences in PTH secretion and cyclic AMP accumulation by parathyroid cells treated with agonists and antagonists of the Ca2+ receptor were analyzed by analysis of variance followed by Fisher’s least-significant difference procedure (StatView; SAS Institute, Cary, NC). The plasma PTH and Ca2+ levels in the in vivo studies were analyzed by repeated measures analysis of variance, followed by a t-test to determine the significance of differences at each time point (StatView).

Results

Potency and Selectivity of NPS 2143. Increasing the concentration of extracellular Ca2+ from 1.0 to 1.75 mM caused a rapid and transient increase followed by a lower yet more prolonged increase in [Ca2+]i, in HEK 293 4.0-7 cells expressing the human Ca2+ receptor (Fig. 2a). Preincubation of these cells with NPS 2143 caused a concentration-dependent inhibition of the cytoplasmic Ca2+ response to extracellular Ca2+ (Fig. 2a). When NPS 2143 (300 nM) was added to cells during the prolonged phase of the response, there was an immediate fall in [Ca2+]i, to baseline values (Fig. 2a). Concentration-response curves, obtained using a change in agonist concentration equivalent to the EC50 (a 1.0–1.75 mM increase in the concentration of extracellular Ca2+) yielded an IC50 for NPS 2143 of 43 ± 5 nM.

The prompt fall in sustained levels of cytoplasmic Ca2+ caused by the addition of NPS 2143 (Fig. 2, a and c) is similar to that caused by inorganic trivalent cations, which at low micromolar concentrations, block influx of extracellular Ca2+ through both voltage-sensitive and -insensitive channels, including those permitting influx of extracellular Ca2+ in parathyroid cells (Nemeth and Scarpa, 1987). Thapsigargin was used to release Ca2+ from intracellular stores and to promote capacitive Ca2+ influx independently of receptor activation. Neither La3+ nor NPS 2143 affected the rapid increase in [Ca2+]i evoked by thapsigargin, which results from release of Ca2+ from intracellular stores (Fig. 3, b and c). In contrast, sustained increases in [Ca2+]i, elicited by thapsigargin were promptly lowered by the addition of La3+, but not by NPS 2143, whether added prior to or after thapsigargin (Fig. 3). NPS 2143 and La3+ thus lower sustained increases in [Ca2+]i, elicited by activation of the Ca2+ receptor by different mechanisms.

HEK 293 cells normally express purinergic, thrombin, and bradykinin receptors, all of which couple to phospholipase C and evoke the mobilization of intracellular Ca2+. NPS 2143 did not affect responses elicited by thrombin (Fig. 2b) or ATP (Fig. 2, c and d) even when tested at concentrations as high as 10 μM. Preincubation with ATP tended to reduce the cytoplasmic Ca2+ response to a subsequent challenge with extracellular Ca2+, a not unexpected result suggesting that both agonists draw on a common store of intracellular Ca2+. Again, the addition of NPS 2143 (300 nM) caused a prompt decrease in the sustained phase of [Ca2+]i, elicited by extracellular Ca2+ (Fig. 2c). Increases in [Ca2+]i, elicited by bradykinin were similarly unaffected by 10 μM NPS 2143 (data not shown).

More stringent tests for assessing the selectivity of NPS 2143 used those receptors that are more structurally homologous to the Ca2+ receptor, i.e., mGlurRs and GABARs. In this report we present the results with cell lines expressing...
Fig. 2. NPS 2143 blocks increases in [Ca\(^{2+}\)]\(_i\), elicited by increases in extracellular Ca\(^{2+}\) but not those induced by ATP or thrombin in HEK 293 cells expressing the human Ca\(^{2+}\) receptor. Fluorescence recordings were obtained from fura-2-loaded cells in a cuvette with continuous stirring. Receptor agonists and NPS 2143 were added to the cuvette as indicated by the arrows. Breaks in the fluorescence traces at the arrows are addition artifacts resulting from opening the cuvette compartment. In the traces shown here, and in those of Figs. 3 and 6, these artifacts have been intentionally eliminated. The recordings in a are superimposed traces in the absence of NPS 2143 (top trace) or after pretreatment with 30 (middle trace) or 300 nM NPS 2143 before increasing the concentration of extracellular Ca\(^{2+}\) from 1.0 to 1.75 mM. The recordings in b are superimposed traces showing responses to 1 U/ml thrombin in the absence or presence of 10 \(\mu\)M NPS 2143 and 1.0 mM extracellular Ca\(^{2+}\). Traces in c and d show responses to ATP (100 nM) and to extracellular Ca\(^{2+}\) (1.0–1.75 mM) in the presence or absence of 300 nM NPS 2143. The numbers accompanying each trace are estimates of [Ca\(^{2+}\)]\(_i\), in nanomolar concentration. Shown are representative recordings from two or three separate experiments.

Mechanism of Action of NPS 2143. Concentration-response curves to increasing concentrations of extracellular Ca\(^{2+}\) were generated using data derived from studies with HEK 293 4.0-7 cells in the presence or absence of NPS 2143. The addition of NPS 2143 shifted the extracellular Ca\(^{2+}\) concentration-response relationship to the right in a concentration-dependent manner (Fig. 5). The maximal response to extracellular Ca\(^{2+}\) was not affected by treatment with NPS 2143 nor did NPS 2143 decrease [Ca\(^{2+}\)]\(_i\), below that caused by lowered levels of extracellular Ca\(^{2+}\). The inhibitory potency of NPS 2143 is thus dependent on the concentration of extracellular Ca\(^{2+}\) and this compound has little effect at levels of extracellular Ca\(^{2+}\) outside the range of those occurring physiologically. In this respect, the effects of NPS 2143 parallel those of type II calcimimetic compounds.

Type II calcimimetic compounds act by a different mechanism of action than does extracellular Ca\(^{2+}\) and provide a means of activating the Ca\(^{2+}\) receptor without changing the concentration of extracellular Ca\(^{2+}\). The effects of NPS 2143 on responses elicited by one such calcimimetic, NPS R-467, were initially examined in HEK 293 4.0-7 cells. The addition of NPS R-467 caused a rapid increase in [Ca\(^{2+}\)]\(_i\), which was reduced in a concentration-dependent manner by pretreatment with NPS 2143 (Fig. 6b). Cumulative concentration-response curves to NPS R-467 were generated in the presence of increasing concentrations of NPS 2143 (Fig. 6, a–c). NPS 2143, when used at the lower concentrations, decreased the potency of NPS R-467 without affecting maximal responses.

NPS 2143 Affects Functional Responses of Parathyroid Cells. The above-mentioned results showed that NPS 2143 was a potent antagonist of the Ca\(^{2+}\) receptor with some degree of selectivity, enough, at least, to test the suitability of this compound as a tool to alter Ca\(^{2+}\) receptor activity in cells normally expressing this receptor. Bovine parathyroid cells were used as a homologous expression system, and two distinct functional readouts of Ca\(^{2+}\) receptor activity were monitored: secretion of PTH and formation of cyclic AMP.

The effect of NPS 2143 on the release of PTH in vitro was
studied using three different experimental designs. In the first series of experiments, cells were incubated under normocalcemic conditions (1.25 mM) in the absence or presence of increasing concentrations of NPS 2143. NPS 2143 augmented the amount of PTH appearing in the medium during a 20-min incubation (Fig. 7a). The threshold concentration for this effect was around 10 nM; the EC_{50} for NPS 2143 under these conditions was \( \frac{41}{11006} \) nM.

The second series of experiments examined the effects of NPS 2143 on the regulation of PTH secretion by extracellular \( \text{Ca}^{2+} \). The IC_{50} for extracellular \( \text{Ca}^{2+} \) on PTH secretion was increased from \( 0.99 \pm 0.02 \) mM in the absence to \( 1.29 \pm 0.07 \) mM in the presence of 300 nM NPS 2143 (Fig. 7b). Similar to cytoplasmic \( \text{Ca}^{2+} \) responses in HEK 293 4.0-7 cells, neither the minimal nor maximal PTH secretory responses to extracellular \( \text{Ca}^{2+} \) were affected by NPS 2143.

The final experiments assessed whether NPS 2143 would affect the inhibitory effects of NPS R-467 on PTH secretion. The results are presented in Table 2 and show that NPS 2143 reversed the depressive effect of NPS R-467 on the secretion of PTH.

In addition to secretion of PTH, the \( \text{Ca}^{2+} \) receptor in parathyroid cells couples through a G_\text{i}-like protein to the inhibition of adenylate cyclase and this effect is most pronounced when cyclic AMP levels have been increased by activating the \( \beta \)-adrenergic or D_1-dopaminergic receptor on parathyroid cells. In the present study, we used isoproterenol to activate the \( \beta \)-adrenergic receptor. Increases in cyclic AMP elicited by isoproterenol were inhibited by 80% when the concentration of extracellular \( \text{Ca}^{2+} \) was increased from 0.5 to 2 mM (Fig. 8). NPS 2143 did not affect the levels of cyclic AMP in the absence of isoproterenol when tested at either low (0.5 mM) or high (2 mM) concentrations of extracellular \( \text{Ca}^{2+} \) (data not shown). In the presence of isoproterenol, however, NPS 2143 tended to augment cyclic AMP levels at low concentrations of extracellular \( \text{Ca}^{2+} \) and completely blocked the inhibitory effects of 2 mM extracellular \( \text{Ca}^{2+} \) on evoked increases in cyclic AMP levels (Fig. 8).

**Fig. 3.** Influx of extracellular \( \text{Ca}^{2+} \) elicited by thapsigargin is blocked by \( \text{La}^{3+} \) but not by NPS 2143. Fluorescence was recorded from a cuvette containing HEK 293 4.0-7 cells loaded with fura-2. Thapsigargin, NPS 2143, and \( \text{La}^{3+} \) were each used at a final concentration of 1 \( \mu \)M. The numbers accompanying each trace are estimates of [\( \text{Ca}^{2+} \)]_{i} in nanomolar concentration. The traces shown are from a single preparation of cells and, with the exception of b, are representative of the results obtained using a separate cell preparation. In b, basal [\( \text{Ca}^{2+} \)]_{i} was slightly elevated compared with those in a or c and those in another cell preparation.

**Fig. 4.** NPS 2143 does not affect the activity of mGluR1. A chimeric rat mGluR1 receptor containing the human mGluR1 transmembrane domain was transiently expressed in HEK 293 cells. Recordings show the [\( \text{Ca}^{2+} \)]_{i} responses elicited by 3,5-dihydroxyphenylglycine (DHPG) (10 \( \mu \)M), a selective group 1 mGluR agonist, in the absence (a) or presence (b) of 10 \( \mu \)M NPS 2390, a small molecule antagonist of group I mGluRs, or 3 \( \mu \)M NPS 2143 (c). Experiments were performed in buffer containing 1.0 mM extracellular \( \text{Ca}^{2+} \) and fluorescence was recorded using the fluorescence imaging plate reader. Each trace is from a single well of a 96-well plate and is representative of that recorded in 3 to 11 other wells treated identically. The numbers accompanying each trace are arbitrary units of fluorescence and, when measured in wells from a single plate, are directly comparable. DMSO, dimethyl sulfoxide.
exocytotic PTH secretion or increased PTH synthesis or some combination of both. To help distinguish between these mechanisms, NPS 2143 was administered intravenously to normal rats. This route of administration was chosen to avoid pharmacokinetic features of this molecule that could limit its plasma concentration to subthreshold levels. This study, performed under physiological conditions, also provides information relevant to the therapeutic feasibility of using Ca\textsuperscript{2+} receptor antagonists to stimulate bone formation.

The intravenous infusion of NPS 2143 resulted in a rapid increase in plasma PTH levels that were maximal within 15 to 30 min of the start of infusion (Fig. 9). Plasma PTH levels were elevated throughout the 120-min infusion, but were approaching baseline levels 60 min after the infusion was terminated. There were no significant differences in plasma PTH levels between vehicle-treated animals and those infused with NPS 2143 at later time points (4–6 h).

**Fig. 5.** NPS 2143 shifts the concentration-response curve to extracellular Ca\textsuperscript{2+} in HEK 293 cells expressing the human parathyroid Ca\textsuperscript{2+} receptor. HEK 293 4.0–7 cells were incubated for 40 to 60 sec in the absence (○) or presence of 30 (■), 100 (●), 300 (▲), or 1000 (▼) nM of NPS 2143 before increasing the concentration of extracellular Ca\textsuperscript{2+} to the final indicated level. Each point is the mean of duplicate determinations from a single experiment and representative of the results obtained in two other separate experiments.

**Fig. 6.** NPS 2143 blocks cytoplasmic Ca\textsuperscript{2+} responses to NPS R-467 in HEK 293 4.0–7 cells. Cells were preincubated with 300 nM NPS 2143 or vehicle before cumulative additions of NPS R-467 (arrowheads) to final concentrations of 0.1, 1.1, or 4.1 μM. The numbers accompanying each trace are estimates of [Ca\textsuperscript{2+}]\textsubscript{i} in nanomolar concentration.

**Fig. 7.** NPS 2143 stimulates PTH secretion from bovine parathyroid cells. a, parathyroid cells were suspended in buffer containing 1.25 mM Ca\textsuperscript{2+} and incubated for 20 min in the absence (○) or presence (●) of increasing concentrations of NPS 2143. b, parathyroid cells were initially suspended in buffer containing 0.5 mM Ca\textsuperscript{2+} and then transferred to tubes containing a small volume of CaCl\textsubscript{2} to obtain the final indicated concentration of extracellular Ca\textsuperscript{2+}. Incubation in the absence (○) or presence (●) of 300 nM NPS 2143 was for 20 min. Each point is the mean ± S.E. of four separate experiments each performed in triplicate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PTH Secretion (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPS 2143 (300 nM)</td>
<td>124 ± 9\textsuperscript{c}</td>
</tr>
<tr>
<td>NPS R-467 (1 μM)</td>
<td>22 ± 4\textsuperscript{a,b}</td>
</tr>
<tr>
<td>NPS 2143 + NPS R-467</td>
<td>65 ± 9\textsuperscript{a,b,c}</td>
</tr>
</tbody>
</table>

\textsuperscript{a,b,c} p < 0.05, significance of difference from control, NPS 2143, and NPS R-467 treatments, respectively.
more slowly than those of PTH. Similarly, the return of plasma Ca\(^{2+}\) levels to baseline also appeared slower and levels were still significantly elevated 2 h after the end of infusion when plasma PTH levels were no longer elevated. The rapidity of the changes in PTH levels suggests that the appearance of PTH in culture medium in the in vitro experiments is associated with the secretion of PTH stored in the cells, and not to new hormone synthesis.

**Discussion**

The present study describes the salient pharmacodynamic properties of the first compound shown to possess antagonist activity at the Ca\(^{2+}\) receptor. For most G protein-coupled receptors, potent and selective antagonists have typically preceded the discovery of corresponding agonists. The converse has been the case with the Ca\(^{2+}\) receptor and a plethora of inorganic ions and organic compounds, acting either as true agonists or allosteric activators of this receptor, have been reported (Nemeth and Fox, 1999). Many of these calcimimetics lack potency and selectivity yet a ligand possessing even such meager properties, but acting as an antagonist, has not been identified. In fact, NPS 2143 is the first substance, either atomic or molecular, shown to block Ca\(^{2+}\) receptor activity. We call such blocking ligands “calcilytics” and, together with calcimimetics, they encompass the known pharmacological means of altering Ca\(^{2+}\) receptor activity.

While G protein-coupled receptors in general are hardly ideal candidates for receptor-based drug design, the Ca\(^{2+}\) receptor is considerably more challenging because Ca\(^{2+}\) ions fail to offer much structural information for molecular ligand-based drug design. High-throughput screening of small molecule libraries seemed to offer the best chance of discovering a Ca\(^{2+}\) receptor antagonist. Although the initial hit detected was not potent and clearly lacked specificity, it was not pan-active. Importantly, its structure suggested that it might have pharmaceutically acceptable properties. The compounds that emerged from the ensuing medicinal chemistry effort have potencies that span three orders of magnitude and differ markedly in selectivity. NPS 2143 was chosen as representative of the compounds in this family based on its potency and the initial results suggesting some degree of selectivity.

We initially tested for actions that might interfere with the readout of Ca\(^{2+}\) receptor activity and/or those that might generally affect the activity of G protein-coupled receptors. Because NPS 2143 did not inhibit responses to a number of different receptors, all of which couple to the mobilization of intracellular Ca\(^{2+}\) in HEK 293 cells, NPS 2143 affects neither receptor activity nor the signal transduction pathways leading from these receptors to the increase in [Ca\(^{2+}\)], Likewise, the failure of NPS 2143 to alter cytoplasmic Ca\(^{2+}\) responses to thapsigargin suggests that this compound does not affect ATPase activity or mechanisms comprising capacitive Ca\(^{2+}\) influx. In this respect, NPS 2143 differs from La\(^{3+}\), which decreases the sustained increases in [Ca\(^{2+}\)], induced by thapsigargin. Thus, NPS 2143 does not block extracellular Ca\(^{2+}\) influx under these conditions, as does La\(^{3+}\). This, in turn, suggests that the ability of NPS 2143 to rapidly lower [Ca\(^{2+}\)], (Fig. 2, a and c) does not result from inhibition of Ca\(^{2+}\) influx. If the sole action of NPS 2143 in these experiments is the inhibition of the Ca\(^{2+}\) receptor then

![Fig. 8. NPS 2143 blocks the inhibitory effects of extracellular Ca\(^{2+}\) on isoproterenol-stimulated increases in cyclic AMP formation in bovine parathyroid cells. Dissociated parathyroid cells were incubated with or without isoproterenol in buffer containing 0.5 or 2 mM CaCl\(_2\) in the presence or absence of 300 nM NPS 2143. Control levels of cyclic AMP (in buffer containing 0.5 mM CaCl\(_2\) and isoproterenol) were 5.6 ± 1.6 pg/well (n = 3). a, p < 0.05 versus control. b, p < 0.05 versus 2 mM calcium in the absence of NPS 2143.](image)

![Fig. 9. Plasma levels of PTH and Ca\(^{2+}\) in normal rats infused i.v. with vehicle (○) or NPS 2143 (○) (0.1 μmol/kg · min) for 2 h. Values are mean ± S.E., n = 3/group. *p < 0.05 versus vehicle-infused controls.](image)
the rapid fall in [Ca$^{2+}$]i, might indicate that continuous activation of the Ca$^{2+}$ receptor is required to maintain increased levels of [Ca$^{2+}$]i.

NPS 2143 did not affect the activity of several G protein-coupled receptors from class C that are structurally similar to Ca$^{2+}$ receptors. NPS 2143 neither activated nor inhibited group I mGlurS, which like the parathyroid Ca$^{2+}$ receptor, couple to phospholipase C and mobilize intracellular Ca$^{2+}$. 1 To test for actions on the GABA_B receptors, a GABA_B1 fusion construct was used. Like the native GABA_B receptor, expression of GABA_B2 and the R1 fusion construct was required to obtain a response to GABA or baclofen. The requirement for coexpression implies that affecting either receptor would alter the response, and the failure of NPS 2143 to do so suggests that it affects neither the R1 nor the R2 GABA_B receptors. In the aggregate, the results show that NPS 2143 is a compound with reasonable selectivity and, when used appropriately, might be a useful compound to probe the functions of Ca$^{2+}$ receptors in nonhuman animals. 2

Despite clear evidence for potent and selective inhibitory actions of NPS 2143 on the Ca$^{2+}$ receptor, it was not a certainty that a calcilytic compound would in fact stimulate PTH secretion. While intuitively appealing, this assumption rests solely on the finding that lowering the level of extracellular Ca$^{2+}$ stimulates PTH secretion. This is not, however, equivalent to blocking the activity of the receptor in a normocalcemic setting. Moreover, our screening assay used measurements of [Ca$^{2+}$]i as a functional readout of Ca$^{2+}$ receptor activity. In the parathyroid cell, increases in [Ca$^{2+}$]i are associated with an inhibition of PTH secretion. The mechanisms giving rise to this unusual relationship between [Ca$^{2+}$]i and hormone secretion are not understood and the possibility remains that the Ca$^{2+}$ receptor couples to an additional or alternative intracellular signal that regulates PTH secretion. Thus, it was not a certainty that compounds detected using measures of [Ca$^{2+}$]i would also affect PTH secretion. It was therefore rewarding to discover that NPS 2143 stimulated PTH secretion in vitro in the absence of changes in the level of extracellular Ca$^{2+}$ and also in normocalcemic rats.

Curiously, the effects of NPS 2143 on PTH secretion from bovine parathyroid cells mirror those of type II calcimimetic compounds. These calcimimetics are positive allosteric modulators that shift the concentration-response curve of extracellular Ca$^{2+}$ to the left without changing the maximum or minimum responses (Nemeth et al., 1998). NPS 2143 affects the agonist concentration-response curve in a converse manner: there is a shift to the right that is unaccompanied by changes in either the maximal or minimal response. These changes in the agonist concentration-response curves suggest that NPS 2143 decreases, whereas type II calcimimetic compounds increase the sensitivity of the Ca$^{2+}$ receptor to activation by extracellular Ca$^{2+}$. Although the rightward shift of the concentration-response curve could indicate competitive inhibition by NPS 2143, the shifts in the concentration-response curves shown in Fig. 5 would also be produced by a noncompetitive antagonist acting on a tissue with a large receptor reserve. Additional studies are underway to understand further the molecular actions of NPS 2143.

The stimulatory effects of NPS 2143 on PTH secretion in vitro and on circulating levels in vivo underscore the key role of the Ca$^{2+}$ receptor in controlling PTH secretion. These findings are additionally relevant for therapies, which by controlling circulating levels of endogenous PTH, seek to promote new bone growth. In this latter respect, it is instructive to compare the magnitude and rate of changes in plasma levels of PTH elicited by intravenous infusion of NPS 2143 with those elicited by subcutaneous administration of PTH in rats or humans. The 4-fold increase in plasma PTH levels following infusion of NPS 2143 falls within the range of levels produced by doses of exogenous PTH that stimulate new bone formation and increase bone mineral density in osteopenic rats (Fox et al., 1997) and osteoporotic humans (Lindsay et al., 1993, 1997). The increase in circulating levels of PTH is also rapid and indicates that NPS 2143 affects the regulated, rather than the constitutive pathway of PTH secretion. This finding mirrors that obtained with calcimetics, which inhibit only the secretory responses sensitive to extracellular Ca$^{2+}$ (Nemeth et al., 1998). The decrease in circulating levels of PTH following the end of infusion is likewise comparable with that seen following administration of exogenous hormone. Moreover, the elicited increase in plasma PTH levels induced the appropriate physiological response, i.e., a delayed increase in the plasma levels of Ca$^{2+}$. Thus, NPS 2143 causes a pattern of change in circulating levels of PTH that is quite similar to that seen following doses of PTH that stimulate bone growth. Calcilytic compounds might therefore prove useful as anabolic therapies for bone diseases such as osteoporosis. Indeed, we have shown that NPS 2143 when administered daily by oral gavage to estrogen-treated osteopenic rats for 5 weeks increases plasma PTH levels and bone formation sufficiently to increase bone mineral density and trabecular bone volume (Gowen et al., 2000).

References


Address correspondence to: Edward F. Nemeth, Ph.D., NPS Pharmaceuticals, Inc., 30 College St./Suite 301, Toronto, ON, Canada M5G 1K2. E-mail: enemeth@npsp.com