Toward Defining the Pharmacophore for Positive Allosteric Modulation of PTH1 Receptor Signaling by Extracellular Nucleotides

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Supporting Information

ABSTRACT: The parathyroid hormone 1 receptor (PTH1R) is a Class B G-protein-coupled receptor that is a target for osteoporosis therapeutics. Activated PTH1R couples through G_s to the stimulation of adenylyl cyclase. As well, β -arrestin is recruited to PTH1R leading to receptor internalization and MAPK/ERK signaling. Previously, we reported that the agonist potency of PTH1R is increased in the presence of extracellular ATP, which acts as a positive allosteric modulator of PTH signaling. Another nucleotide, cytidine 5'-monophosphate (CMP), also enhances PTH1R signaling, suggesting that ATP and CMP share a moiety responsible for positive allosterism, possibly ribose-5-phosphate. Therefore, we examined the effect of extracellular sugar phosphates on PTH1R signaling. cAMP levels and β -arrestin recruitment were monitored using luminescence-based assays. Alone, ribose-5-phosphate had no detectable effect on adenylyl cyclase activity in UMR-106 rat osteoblastic cells, which endogenously express PTH1R. However, ribose-5-phosphate markedly enhanced the activation of adenylyl cyclase induced by PTH. Other sugar phosphates, including glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-bisphosphate, also potentiated PTH-induced adenylyl cyclase activation. As well, some sugar phosphates enhanced PTH-induced β -arrestin recruitment to human PTH1R heterologously expressed in HEK293H cells. Interestingly, the effects of glucose-1-phosphate were greater than those of its isomer glucose-6-phosphate. Our results suggest that phosphorylated monosaccharides such as ribose-5-phosphate contain the pharmacophore for positive allosteric modulation of PTH1R. At least in some cases, the extent of modulation depends on the position of the phosphate group. Knowledge of the pharmacophore may permit future development of positive allosteric modulators to increase the therapeutic efficacy of PTH1R agonists.

KEYWORDS: allosteric site, arrestin, cyclic AMP, G-protein-coupled receptors, nucleotides, sugar phosphates

INTRODUCTION

The parathyroid hormone 1 receptor (PTH1R) is a Class B G-protein-coupled receptor (GPCR) that is implicated in bone development and remodeling, as well as the homeostasis of calcium and phosphate.^{1,2} Additionally, PTH1R agonists are used clinically as anabolic therapeutics for the treatment of osteoporosis.³ Activated PTH1R interacts with G_s and G_q protein heterotrimers to stimulate adenylyl cyclase and phospholipase C β , respectively, raising levels of cytosolic cAMP and calcium.^{4–6} Activated PTH1R is phosphorylated by GPCR kinase (GRK), which in turn promotes interaction between the receptor and β -arrestin.⁷ The recruited β -arrestin acts as a scaffolding protein to internalize the receptor and transduce MAPK/ERK signaling.⁸

Intracellularly, nucleotides serve as energy molecules and building blocks of nucleic acids. In the extracellular milieu, nucleotides can act as substrates for ectokinases⁹ and agonists at P2 purinergic receptors.¹⁰ Nucleotides consist of three moieties: nucleobase, ribose, or deoxyribose sugar, and phosphate(s); thus, every nucleotide includes a ribose/deoxyribose-5-phosphate. Many sugar phosphates exist as intermediate or final products in various metabolic pathways. For example, during glycolysis, glucose-6-phosphate (G6P),

fructose-6-phosphate (F6P), fructose-1,6-bisphosphate (F16bP), and other sugar phosphates are intermediate products. Similarly, during glycogenesis/glycogenolysis, glucose-1-phosphate (G1P) is an intermediate product.¹¹

Adenosine 5'-triphosphate (ATP) is coreleased with many other signaling molecules.^{12,13} Moreover, ATP is known to be released by cells in response to mechanical stimulation^{14,15} and is thought to play a role in mediating the anabolic effects of exercise on bone.^{16,17} In addition, ATP can be released from osteoblasts in response to PTH1R signaling.¹⁸ Recently, we reported that extracellular ATP increases agonist potency at PTH1R, through a mechanism independent of P2 nucleotide receptors and not involving ectokinases.¹⁹ Rather, ATP was found to enhance cAMP signaling and β -arrestin recruitment through a previously unrecognized allosteric mechanism at the level of the receptor or a closely associated protein. Thus, ATP could act as an autocrine factor to regulate PTH1R function in bone. It is also conceivable that the allosteric effects of ATP on PTH signaling account for the synergistic effects on bone of mechanical loading and PTH, which have been reported by

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Figure 1. Extracellular ribose-5-phosphate (R5P) enhances PTH-induced activation of adenylyl cyclase. (A) Two-dimensional structures of protonated forms of ATP, CMP, R5P and ribose. (B) UMR-106 osteoblastic cells were transfected with GloSensor cAMP biosensor plasmid. At time 0, cells were stimulated with PTH (0.1 nM) in the presence of ATP (1.5 mM, red), R5P (1.5 mM, green), or vehicle (Veh₂, blue). Luminescence intensity, which corresponds to the level of cytosolic cAMP, was measured from live cells every 1.5 min. Values are means of triplicate determinations from an individual experiment, representative of five independent experiments. (C) Cells were stimulated with PTH (0.1 nM) in the presence of vehicle (Veh₂, negative control) or the indicated test substance (1.5 mM): ATP, CMP, R5P, or ribose (Ri). The maximal rate of cAMP accumulation was determined from the greatest slope of the cAMP vs time curve (e.g., panel B). In the presence of the cyclic nucleotide phosphodiesterase inhibitor IBMX, the rate of cAMP accumulation reflects the relative activity of adenylyl cyclase. In the absence of PTH, none of the test substances altered adenylyl cyclase activity. Data were normalized to the average value within each individual experiment. Vertical bars illustrate means $\pm$ SEM, data points represent values from each independent experiment ($n = 5$ independent experiments, each performed in duplicate or triplicate). The asterisk (*) indicates significant difference from PTH + Veh₂ ($p < 0.05$, based on one-way ANOVA and Bonferroni test). (D) Cells were stimulated with indicated concentrations of PTH or its vehicle in the presence of R5P (1.5 mM). Note that R5P had no detectable effect on cAMP levels in the absence of PTH. Values are means of duplicate determinations from an individual experiment, representative of four independent experiments. (E) The maximal rate of cAMP accumulation was determined for the indicated concentrations of PTH (or its vehicle, Veh₁) in the presence of R5P (1.5 mM, green solid line), ATP (1.5 mM, red dashed line), or vehicle (Veh₂, blue solid line). Data were normalized to the maximal rate of cAMP accumulation induced by PTH alone (1 μ M). Values are means $\pm$ SEM ($n = 4$ independent experiments, each performed in duplicate). pEC₅₀ values for PTH in the presence of R5P and in the presence of ATP were both significantly greater than the pEC₅₀ for PTH alone (based on extra sum-of-squares F -test, Table 1). The maximum response to PTH was also enhanced significantly by R5P and ATP.

others *in vivo*.^{20,21} In our previous study, we found that both ATP and cytidine 5'-monophosphate (CMP) enhanced PTH1R signaling, suggesting that the pharmacophore that mediates positive allosteric modulation may be present in both ATP and CMP. These two nucleotides contain ribose-5-phosphate (R5P). Thus, we investigated whether R5P can function as a positive allosteric modulator (PAM) of PTH1R.

In the present study, we demonstrated that extracellular R5P potentiates PTH1R signaling. In addition, other sugar phosphates, including G1P, F6P, and F16bP, enhance signaling. The effects of these sugar phosphates are consistent with an increase in PTH potency, efficacy, or both. In contrast to the potentiating effects of G1P on PTH1R signaling, its constitutional isomer G6P showed comparatively weak effects,

Table 1. Summary of Parameters for PTH Concentration Dependence Data^a

figure	agent (mM)	cAMP accumulation			β -arrestin recruitment		
		$-\log EC_{50}$	max	(n)	$-\log EC_{50}$	max	(n)
Ribose Phosphate							
1E, S3	vehicle	7.63 $\pm$ 0.08	1.02 $\pm$ 0.04	(5)	7.41 $\pm$ 0.13	1.07 $\pm$ 0.07	(3)
	R5P (1.5)	8.82 $\pm$ 0.10***	1.28 $\pm$ 0.05***	(5)	7.50 $\pm$ 0.22	1.30 $\pm$ 0.14	(3)
	ATP (1.5)	9.14 $\pm$ 0.11***	1.38 $\pm$ 0.05***	(5)	7.72 $\pm$ 0.19	1.21 $\pm$ 0.10	(3)
Glucose Phosphates							
3B, 7B	vehicle	7.64 $\pm$ 0.07	1.02 $\pm$ 0.03	(3)	7.42 $\pm$ 0.13	1.06 $\pm$ 0.07	(3)
	G1P (0.3)	8.65 $\pm$ 0.15***	1.19 $\pm$ 0.06*	(3)	7.38 $\pm$ 0.13	1.21 $\pm$ 0.13	(3)
	G1P (1.0)	9.37 $\pm$ 0.12***	1.57 $\pm$ 0.06***	(3)	7.43 $\pm$ 0.15	1.29 $\pm$ 0.09*	(3)
	G1P (10.0)	9.99 $\pm$ 0.10***	1.52 $\pm$ 0.03***	(3)	7.64 $\pm$ 0.15	1.37 $\pm$ 0.09*	(3)
3C, 7C	vehicle	7.64 $\pm$ 0.09	1.02 $\pm$ 0.04	(3)	7.67 $\pm$ 0.20	1.03 $\pm$ 0.09	(3)
	G6P (0.3)	8.64 $\pm$ 0.33	1.04 $\pm$ 0.11	(3)	7.66 $\pm$ 0.18	1.21 $\pm$ 0.10	(3)
	G6P (1.0)	9.28 $\pm$ 0.25***	1.20 $\pm$ 0.10	(3)	7.76 $\pm$ 0.18	1.30 $\pm$ 0.10	(3)
	G6P (10.0)	9.73 $\pm$ 0.31***	1.14 $\pm$ 0.08	(3)	7.92 $\pm$ 0.11	1.39 $\pm$ 0.11*	(3)
3D, 7D	vehicle	7.69 $\pm$ 0.09	1.02 $\pm$ 0.04	(6)	7.22 $\pm$ 0.12	1.10 $\pm$ 0.07	(3)
	G1P (1.5)	8.37 $\pm$ 0.31	1.09 $\pm$ 0.10	(6)	7.38 $\pm$ 0.23	1.39 $\pm$ 0.16	(3)
	G6P (1.5)	8.39 $\pm$ 0.26*	1.10 $\pm$ 0.08	(6)	7.22 $\pm$ 0.16	1.39 $\pm$ 0.12*	(3)
	ATP (1.5)	9.25 $\pm$ 0.16***	1.26 $\pm$ 0.07*	(6)	7.55 $\pm$ 0.18	1.43 $\pm$ 0.12*	(3)
3E, 7E	vehicle	7.74 $\pm$ 0.14	1.01 $\pm$ 0.06	(3)	7.34 $\pm$ 0.09	1.10 $\pm$ 0.05	(4)
	G1P (6.0)	9.08 $\pm$ 0.15***	1.51 $\pm$ 0.08***	(3)	7.49 $\pm$ 0.13	1.49 $\pm$ 0.09***	(4)
	G6P (6.0)	8.65 $\pm$ 0.17***	1.39 $\pm$ 0.08***	(3)	7.35 $\pm$ 0.10	1.38 $\pm$ 0.07***	(4)
	ATP (1.5)	8.89 $\pm$ 0.13***	1.37 $\pm$ 0.07***	(3)	7.53 $\pm$ 0.09	1.52 $\pm$ 0.06***	(4)
Fructose Phosphates							
4B, 8B	vehicle	7.69 $\pm$ 0.07	1.02 $\pm$ 0.03	(3)	7.56 $\pm$ 0.10	1.06 $\pm$ 0.05	(3)
	F6P (0.3)	8.73 $\pm$ 0.21***	1.12 $\pm$ 0.08	(3)	7.59 $\pm$ 0.13	1.17 $\pm$ 0.07	(3)
	F6P (1.0)	8.84 $\pm$ 0.10***	1.29 $\pm$ 0.05***	(3)	7.74 $\pm$ 0.21	1.41 $\pm$ 0.13***	(3)
	F6P (10.0)	9.42 $\pm$ 0.14***	1.24 $\pm$ 0.05***	(3)	7.86 $\pm$ 0.30	1.91 $\pm$ 0.24***	(3)
4C, 8C	vehicle	8.11 $\pm$ 0.12	0.98 $\pm$ 0.04	(3)	7.36 $\pm$ 0.21	1.07 $\pm$ 0.11	(3)
	F16bP (0.3)	8.95 $\pm$ 0.17***	1.33 $\pm$ 0.09***	(3)	7.29 $\pm$ 0.20	1.29 $\pm$ 0.13	(3)
	F16bP (1.0)	9.49 $\pm$ 0.22***	1.71 $\pm$ 0.10***	(3)	7.36 $\pm$ 0.17	1.33 $\pm$ 0.11	(3)
	F16bP (10.0)	10.0 $\pm$ 0.2***	1.79 $\pm$ 0.07***	(3)	7.86 $\pm$ 0.30	1.46 $\pm$ 0.19	(3)
4D, 8D	vehicle	7.66 $\pm$ 0.09	1.02 $\pm$ 0.05	(3)	7.41 $\pm$ 0.11	1.08 $\pm$ 0.06	(3)
	F6P (1.5)	8.88 $\pm$ 0.09***	1.45 $\pm$ 0.05***	(3)	7.63 $\pm$ 0.14	1.49 $\pm$ 0.10***	(3)
	F16bP (1.5)	8.54 $\pm$ 0.12***	1.30 $\pm$ 0.05***	(3)	7.49 $\pm$ 0.11	1.36 $\pm$ 0.07***	(3)
	ATP (1.5)	9.25 $\pm$ 0.07***	1.42 $\pm$ 0.03***	(3)	7.82 $\pm$ 0.15*	1.50 $\pm$ 0.09***	(3)
4E, 8E	vehicle	7.52 $\pm$ 0.03	1.03 $\pm$ 0.02	(3)	7.28 $\pm$ 0.09	1.09 $\pm$ 0.05	(3)
	F6P (6.0)	8.77 $\pm$ 0.16***	1.49 $\pm$ 0.08***	(3)	7.59 $\pm$ 0.16	1.68 $\pm$ 0.12***	(3)
	F16bP (6.0)	9.15 $\pm$ 0.15***	1.59 $\pm$ 0.08***	(3)	7.45 $\pm$ 0.13	1.52 $\pm$ 0.10***	(3)
	ATP (1.5)	9.01 $\pm$ 0.14***	1.45 $\pm$ 0.08***	(3)	7.52 $\pm$ 0.14	1.63 $\pm$ 0.11***	(3)

^aFor each indicated figure panel, PTH concentration–response data from *n* independent experiments were fitted simultaneously to a three-parameter sigmoidal equation. Presented are best-fit values $\pm$ standard errors for EC_{50} and maximum response to PTH. The *F*-statistic (calculated using the extra sum-of-squares *F*-test) was used to assess the effect of the indicated sugar phosphate or ATP on these values. * *p* < 0.05. ** *p* < 0.01. *** *p* < 0.001 versus corresponding vehicle control (in italics).

suggesting selectivity at the allosteric site. Taken together, these effects and results show that PTH1R activation by its agonist PTH is enhanced by sugar phosphates, and that positive allosteric modulation by extracellular nucleotides is due at least in part to the presence of the R5P moiety.

RESULTS AND DISCUSSION

Extracellular Ribose-5-phosphate Enhances PTH-Induced Adenylyl Cyclase Activity. We have previously reported that extracellular ATP and CMP increase agonist potency at PTH1R.¹⁹ Structurally, ATP and CMP share the R5P moiety (Figure 1A). Therefore, we investigated the effect of R5P on PTH1R signaling.

UMR-106 cells, a rat osteoblast-like cell line that endogenously expresses PTH1R,²² were transfected with a plasmid encoding a luciferase-based cAMP biosensor.²³ To

suppress cAMP hydrolysis, cells were treated with the cyclic nucleotide phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX). Cells were then exposed to PTH (1–34) (0.1 nM) in the presence of ATP (1.5 mM), R5P (1.5 mM), or vehicle. At this concentration, PTH alone induced only a modest time-dependent increase in cAMP levels (Figure 1B). Notably, both ATP and R5P markedly enhanced the accumulation of cAMP induced by PTH, which peaked at about 15 min.

The maximal rate of cAMP accumulation was determined from the greatest slope of the time-course data (e.g., Figure 1B), as described previously.¹⁹ In the presence of IBMX, the rate of cAMP accumulation reflects the relative activity of adenylyl cyclase. On their own, ATP, CMP, ribose, and R5P (all at 1.5 mM) had no appreciable effect on adenylyl cyclase activity. On the other hand, as reported previously,¹⁹ ATP and

Figure 2. Effect of extracellular sugar phosphates on PTH-induced adenylyl cyclase activity. (A) Two-dimensional structures of the protonated forms of glucose, glucose-1-phosphate (G1P), glucose-6-phosphate (G6P), fructose, fructose-6-phosphate (F6P), and fructose-1,6-bisphosphate (F16bP). (B) UMR-106 cells were transfected with GloSensor cAMP biosensor plasmid. Parallel samples of cells were stimulated with PTH (0.1 nM) in the presence of vehicle (Veh₂, negative control) or the indicated test substance (1.5 mM): ATP (positive control), glucose (Glu), G1P, G6P, fructose (Fru), F6P, or F16bP. Data are the maximal rate of cAMP accumulation (maximal adenylyl cyclase activity) under each condition, normalized to the average value within each independent experiment. Vertical bars illustrate means $\pm$ SEM, data points represent values from each independent experiment ($n = 4$ independent experiments, each performed in triplicate). The asterisk (*) indicates significant difference from PTH + Veh₂ ($p < 0.05$, based on one-way ANOVA and Bonferroni test).

CMP significantly enhanced the ability of PTH to activate adenylyl cyclase (Figure 1C). Whereas the simple sugar ribose had no significant effect, RSP, like ATP and CMP, markedly enhanced PTH-induced activation of adenylyl cyclase.

We next evaluated how this effect of RSP depended on PTH concentration. In these experiments, cells were stimulated with various concentrations of PTH (1–34) in the presence or absence of RSP (1.5 mM), ATP (1.5 mM, as a positive control), or vehicle (as a negative control). Time course data (Figure 1D) revealed the dependence of cAMP elevation on PTH concentration, and the lack of effect of RSP alone. Again, the maximal rate of cAMP accumulation was quantified from the greatest slope of the time-course data. In the absence of PTH, neither ATP nor RSP stimulated adenylyl cyclase activity (Figure 1E, Veh₁). As expected, PTH alone yielded a sigmoidal concentration dependence curve (Figure 1E, blue curve). RSP, like ATP, significantly shifted the PTH concentration dependence curve to the left, indicating that RSP and ATP both enhanced the agonist potency at PTH1R (Figure 1E, Table 1). As well, RSP and ATP significantly enhanced the maximum

response to PTH. Thus, RSP recapitulates the ability of extracellular nucleotides to potentiate PTH1R signaling.

Effect of Extracellular Sugar Phosphates on PTH-Induced Adenylyl Cyclase Activity. To elucidate the pharmacophore responsible for potentiation of PTH1R signaling, we investigated the effects of other sugar phosphates. Glucose and fructose are hexose monosaccharides; glucose has a six-membered heterocyclic ring, whereas fructose has a five-membered heterocyclic ring (like ribose) (Figure 2A). G1P and G6P are intermediate products in energy metabolism. In G1P, a carbon within the ring is phosphorylated; whereas, in G6P, the carbon outside of the ring is phosphorylated. F6P and F16bP are intermediate products of glycolysis.¹¹ In both of these fructose phosphates, carbons outside of the ring are phosphorylated.

We first determined that all sugar phosphates had no appreciable effect on adenylyl cyclase activity on their own. (The lack of effect of sugar phosphates alone on adenylyl cyclase activity is evident from the data points labeled Veh₁ in Figure 3B–E and Figure 4B–E.) Then, we assessed their

Figure 3. Potentiation of PTH-induced adenylyl cyclase activity—dependence on the concentration of glucose phosphates and PTH. UMR-106 osteoblastic cells were transfected with GloSensor cAMP biosensor plasmid. (A) Parallel samples of cells were stimulated with PTH (0.1 nM) in the presence of vehicle (Veh₂) or the indicated concentrations of glucose-1-phosphate (G1P, orange symbols), or glucose-6-phosphate (G6P, brown symbols). Data are the maximal rate of cAMP accumulation under each condition, normalized to the maximal rate of cAMP accumulation induced by PTH in the presence of G1P at 3 mM. Values are means $\pm$ SEM ($n = 3$ independent experiments, each performed in duplicate). The asterisk (*) indicates significant difference between equivalent concentrations of G1P and G6P ($p < 0.05$, based on one-way ANOVA and Bonferroni test). (B–E) The maximal rate of cAMP accumulation was determined for the indicated concentrations of PTH (or its vehicle, Veh₁) in the presence of the indicated concentrations of G1P (orange lines), G6P (brown lines), ATP (red dashed lines), or their vehicle (Veh₂, blue lines). Data were normalized to the maximal rate of cAMP accumulation induced by PTH alone (1 μ M). (B,C) Values shown are means $\pm$ SEM ($n = 3$ independent experiments with duplicate determinations, performed separately for graphs B and C). (B) The pEC₅₀ values for PTH in the presence of G1P (316 μ M, 1 mM and 10 mM) were significantly greater than the pEC₅₀ for PTH alone (based on extra sum-of-squares F -test, Table 1). Maximal PTH activity was also significantly enhanced by G1P. (C) With G6P, maximal PTH activity was not significantly increased, while pEC₅₀ values for PTH in the presence of G6P (1 and 10 mM) were significantly greater than the corresponding pEC₅₀ for PTH alone. (D,E) Values shown are means $\pm$ SEM ($n = 3$ –6 independent experiments with duplicate determinations, performed separately for graphs D and E). In graph D, the pEC₅₀ values for PTH in the presence of 1.5 mM ATP and 1.5 mM G6P were significantly greater than the pEC₅₀ for PTH alone. In graph E, the pEC₅₀ values for PTH in the presence of 1.5 mM ATP, 6 mM G1P, and 6 mM G6P were significantly greater than the corresponding pEC₅₀ for PTH alone. As well, the maximum response to PTH was significantly enhanced by 1.5 mM ATP, 6 mM G1P, and 6 mM G6P.

effects on adenylyl cyclase activity induced by PTH (1–34) (0.1 nM) using ATP (1.5 mM) as a positive control and vehicle as a negative control. G1P, but not G6P or glucose, significantly enhanced PTH-induced adenylyl cyclase activation under these conditions (Figure 2B). Both fructose phosphates F6P and F16bP, but not fructose, significantly increased PTH-induced adenylyl cyclase activity. These results suggest differences in the relative ability of sugar phosphates to enhance PTH1R signaling.

As cAMP contains ribose with a cyclic phosphate, we examined whether extracellular cAMP also enhances PTH-induced adenylyl cyclase activation. Like ATP and G1P, cAMP (1.5 mM) significantly enhanced the response to PTH (0.1 nM). In contrast, neither glucose nor inorganic phosphate (1.5

mM) significantly altered the response to PTH (Supporting information, Figure S1).

Potentiation of PTH-Induced Adenylyl Cyclase Activity—Dependence on the Concentrations of Sugar Phosphates and PTH. Though G1P and G6P are constitutional isomers, their effects on PTH1R signaling were clearly not identical. To characterize their effects, we first evaluated the dependence of PTH-induced adenylyl cyclase activation on the concentration of glucose phosphates. In these experiments, cells were stimulated with PTH (0.1 nM) in the presence of varied concentrations of G1P, G6P, or vehicle. G1P significantly enhanced PTH-induced adenylyl cyclase activation at concentrations ≥ 1.5 mM (Figure 3A). Furthermore, the enhancing effect of G1P was 2.3-fold greater than that of

Figure 4. Potentiation of PTH-induced adenylyl cyclase activity—dependence on the concentration of fructose phosphates and PTH. UMR-106 osteoblastic cells were transfected with GloSensor cAMP biosensor plasmid. (A) Parallel samples of cells were stimulated with PTH (0.1 nM) in the presence of vehicle (Veh₂) or the indicated concentration of fructose-1,6-bisphosphate (F16bP, dark purple symbols), fructose-6-phosphate (F6P, light purple symbols), or fructose (black symbols). Data are the maximal rate of cAMP accumulation under each condition, normalized to the maximal rate of cAMP accumulation induced by PTH in the presence of F16bP at 3 mM. Values shown are means $\pm$ SEM (n = 6 independent experiments, each performed in duplicate). (*) Significantly greater effect than that induced by corresponding concentration of fructose; (†) significantly greater effect than that induced by corresponding concentration of F6P; at concentrations $\geq$ 3 mM, fructose significantly enhanced PTH-stimulated adenylyl cyclase activity; p < 0.05, based on one-way ANOVA and Bonferroni test. (B–E) The maximal rate of cAMP accumulation was determined for the indicated concentrations of PTH (or its vehicle, Veh₁) in the presence of the indicated concentrations of F6P (light purple lines), F16bP (dark purple lines), ATP (red dashed lines), or their vehicle (Veh₂, blue lines). Data were normalized to the maximal rate of cAMP accumulation induced by PTH alone (1 μ M). Values plotted are means $\pm$ SEM (n = 3 independent experiments with duplicate determinations, performed separately for each panel). (B,C) The pEC₅₀ values for PTH in the presence of F6P (316 μ M, 1 mM, and 10 mM) and F16bP (316 μ M, 1 mM, and 10 mM) were significantly greater than the corresponding pEC₅₀ for PTH alone, based on extra sum-of-squares F -test (Table 1). As well, the maximum response to PTH was significantly enhanced by F6P (1 and 10 mM) and F16bP (316 μ M, 1 mM, and 10 mM). In graph D, the pEC₅₀ values for PTH in the presence of 1.5 mM ATP, 1.5 mM F6P, and 1.5 mM F16bP were significantly greater than the pEC₅₀ for PTH alone. In graph E, the pEC₅₀ values for PTH in the presence of 1.5 mM ATP, 6 mM F6P, and 6 mM F16bP were significantly greater than the corresponding pEC₅₀ for PTH alone. As well, in graphs D and E, the maximum response to PTH was significantly enhanced at all concentrations of test substances.

G6P at a concentration of 6 mM, demonstrating selectivity of the allosteric site for particular pharmacophore structures. The dependence of potentiation on the concentration of glucose phosphates was similar to that reported previously for ATP- and CMP-stimulated enhancement of PTH-induced adenylyl cyclase activity.¹⁹

We next evaluated how the effects of glucose phosphates depended on PTH concentration. PTH alone yielded the expected sigmoidal concentration dependence curve. When tested at concentrations of 316 μ M, 1 mM, and 10 mM, G1P significantly shifted the PTH concentration dependence curve to the left and enhanced the maximum response to PTH (Figure 3B, Table 1). On the other hand, the effects of G6P became significant only at 1 mM (Figure 3C). In a separate

series of experiments, we directly compared the effects of G1P and G6P (Figure 3D,E). At 6 mM, both G1P and G6P significantly shifted the PTH concentration dependence curve to left and enhanced the maximum response to PTH.

F6P and F16bP differ structurally only by the presence of an additional phosphate group on the latter. To examine the contribution of phosphate, we evaluated the effects of varying concentrations of F6P, F16bP, or fructose on PTH-induced adenylyl cyclase activation, while holding the concentration of PTH constant at 0.1 nM. Both F6P and F16bP significantly enhanced PTH-induced adenylyl cyclase activation at concentrations of $\geq$ 1 mM (Figure 4A). In contrast, the effect of fructose was only significant at concentrations of $\geq$ 3 mM. The effect of F6P was significantly greater than that of fructose at 1

Figure 5. Fructose does not alter potentiation of PTH-stimulated adenylyl cyclase activity by fructose-1,6-bisphosphate (F16bP) or ATP. UMR-106 cells were transfected with GloSensor cAMP biosensor plasmid. Parallel samples of cells were treated with PTH (0.1 nM) or its vehicle in the presence or absence of the indicated concentrations of fructose, F16bP and ATP. Data are the maximal rate of cAMP accumulation (maximal adenylyl cyclase activity) under each condition, normalized to the average value within each independent experiment. Vertical bars illustrate means $\pm$ SEM; data points represent values from each independent experiment ($n = 7$ independent experiments, each performed in quadruplicate). The asterisk (*) indicates significant difference from PTH alone ($p < 0.05$, based on one-way ANOVA and Bonferroni test). Fructose had no significant effect on the ability of F16bP or ATP to potentiate signaling (as indicated by the horizontal bar labeled n.s.).

Figure 6. Effect of extracellular sugar phosphates on PTH-stimulated β -arrestin recruitment to PTH1R. HEK293H cells were cotransfected with plasmids encoding two chimeric proteins: N-terminal luciferase fragment- β -arrestin-1 and human PTH1R-C-terminal luciferase fragment. (A) At time 0, cells were stimulated with PTH (10 nM) in the presence of ATP (1.5 mM, red), F16bP (1.5 mM, dark purple), or vehicle (Veh₂, blue). Luminescence intensity, which reflects the extent of β -arrestin recruitment, was measured from live cells every 2 min. Values are means of triplicate determinations from an individual experiment, representative of three independent experiments. (B) The maximal rate of β -arrestin-1 recruitment was determined as the greatest slope of each luminescence vs time curve (e.g., panel A). Parallel samples of cells were stimulated with PTH (10 nM) in the presence of vehicle (Veh₂, negative control) or the indicated test substance (1.5 mM): ATP (positive control), glucose (Glu), glucose-1-phosphate (G1P), glucose-6-phosphate (G6P), fructose (Fru), fructose-6-phosphate (F6P), or fructose-1,6-bisphosphate (F16bP). Data are the maximal rate of β -arrestin-1 recruitment under each condition, normalized to the average value within each independent experiment. Vertical bars illustrate means $\pm$ SEM, data points represent values from each independent experiment ($n = 3$ independent experiments, each performed in triplicate). The asterisk (*) indicates significant difference from PTH + Veh₂ ($p < 0.05$, based on one-way ANOVA and Bonferroni test).

and 1.5 mM. Moreover, the effect of F16bP was significantly greater than that of F6P at concentrations ≥ 3 mM, and greater than that of fructose at concentrations ≥ 1 mM.

We next evaluated how the effects of fructose phosphates depended on PTH concentration. When tested at 316 μ M, 1 mM, and 10 mM, both F6P and F16bP significantly shifted the PTH concentration dependence curve to the left (Figure 4B and 4C, Table 1). As well, with the exception of 316 μ M F6P,

these sugar phosphates significantly enhanced the maximum response to PTH. In a separate series of experiments, we directly compared the effects of F6P and F16bP (Figure 4D,E). Again, F6P and F16bP (1.5 and 6 mM) significantly shifted the PTH concentration dependence curve to left and enhanced the maximum response to PTH. These data indicate that both F6P and F16bP potentiate PTH1R signaling, in a manner comparable to that of extracellular ATP.

Figure 7. Potentiation of PTH-stimulated β -arrestin-1 recruitment to PTH1R—dependence on the concentration of glucose phosphates and PTH. HEK293H cells were cotransfected with plasmids encoding components of the biosensor as described in the legend to Figure 6. (A) Parallel samples of cells were stimulated with PTH (10 nM) in the presence of vehicle (Veh₂) or the indicated concentrations of glucose-1-phosphate (G1P, orange symbols), or glucose-6-phosphate (G6P, brown symbols). Data are the maximal rate of β -arrestin-1 recruitment under each condition, normalized to the maximal rate of recruitment induced by PTH in the presence of G1P at 3 mM. Values shown are means $\pm$ SEM (n = three independent experiments, each performed in duplicate). The asterisk (*) indicates significant difference between equivalent concentrations of G1P and G6P (p < 0.05, based on one-way ANOVA and Bonferroni test). (B–E) Maximal rates of β -arrestin-1 recruitment were determined for the indicated concentrations of PTH (or its vehicle, Veh₁) in the presence of the indicated concentrations of G1P (orange lines), G6P (brown lines), ATP (red dashed lines) or their vehicle (Veh₂, blue lines). Data were normalized to the maximal rate of recruitment induced by PTH alone (1 μ M). (B,C) Values plotted are means $\pm$ SEM (n = 3 independent experiments with duplicate determinations, performed separately for graphs B and C). The pEC₅₀ for PTH in the presence of G1P or G6P did not differ significantly from the pEC₅₀ for PTH alone (based on extra sum-of-squares F -test, Table 1). In contrast, the maximal response to PTH was significantly enhanced by G1P (1 and 10 mM) and G6P (10 mM). (D,E) Values plotted are means $\pm$ SEM (n = 3–4 independent experiments with duplicate determinations, performed separately for graphs D and E). The pEC₅₀ for PTH in the presence of ATP, G1P, or G6P was not significantly different than the pEC₅₀ for PTH alone. On the other hand, the maximum response to PTH was significantly enhanced by 1.5 mM ATP, 6 mM G1P, and 1.5 and 6 mM G6P.

Overall, their potentiating effects on PTH-stimulated adenylyl cyclase activity suggest that the phosphorylated monosaccharides examined in the present study are PAMs at PTH1R. Indeed, when the PTH concentration–response data acquired at multiple concentrations of G1P (Figure 3B), G6P (Figure 3C), F6P (Figure 4B), and F16bP (Figure 4C) were reanalyzed in terms of an allosteric model, excellent fits were attained (Figure S2). The estimated values of the ternary complex constant α were greater than 1, indicating positive allosteric effects, and apparent affinities of allosteric modulators were in the range of 10 mM. However, it should be noted that the model used did not account for allosteric effects on efficacy, which are clearly evident for the sugar phosphates tested (Table 1).

At high concentrations, fructose enhanced PTH-induced adenylyl cyclase activation (Figure 4A). This raised the possibility that fructose could be a weakly efficacious PAM

or a neutral allosteric ligand. Therefore, we investigated the interaction between fructose and F16bP. However, the potentiating effects of F16bP and ATP were unaltered by increasing concentrations of fructose (Figure 5). On its own, 1 mM fructose had no significant effect on the response to PTH, whereas 6 mM fructose significantly enhanced activation of adenylyl cyclase. Taken together, these data suggest that fructose is a PAM with low affinity, rather than a weakly efficacious modulator.

Effects of Sugar Phosphates on PTH-Stimulated β -Arrestin Recruitment to PTH1R. The binding of PTH to PTH1R leads to G protein-dependent signaling events such as activation of adenylyl cyclase and also G protein-independent signaling triggered by the recruitment of β -arrestin.³ We have previously reported that extracellular ATP enhances PTH-induced recruitment of β -arrestin to PTH1R.¹⁹ Thus, we

Figure 8. Potentiation of PTH-stimulated β -arrestin-1 recruitment to PTH1R—dependence on the concentration of fructose phosphates and PTH. HEK293H cells were cotransfected with plasmids encoding components of the biosensor as described in the legend to Figure 6. (A) Parallel samples of cells were stimulated with PTH (10 nM) in the presence of vehicle (Veh₂), fructose-1,6-bisphosphate (F16bP, dark purple symbols), fructose-6-phosphate (F6P, light purple symbols), or fructose (black symbols). Data are the maximal rate of β -arrestin-1 recruitment under each condition, normalized to the maximal rate of recruitment induced by PTH in the presence of F16bP at 3 mM. Values are means $\pm$ SEM ($n = 3$ independent experiments, each performed in duplicate). (*) Significantly greater effect than that induced by corresponding concentration of fructose; (†) significantly greater effect than that induced by corresponding concentration of F6P; at concentrations ≥ 3 mM, fructose significantly enhanced PTH-stimulated recruitment of β -arrestin-1; $p < 0.05$, based on one-way ANOVA and Bonferroni test. (B–E) Maximal rates of β -arrestin-1 recruitment were determined for the indicated concentrations of PTH (or its vehicle, Veh₁) in the presence of the indicated concentrations of F6P (light purple lines), F16bP (dark purple lines), ATP (red dashed lines), or their vehicle (Veh₂, blue lines). Data were normalized to the maximal rate of signal increase induced by PTH alone (1 μ M). Values are means $\pm$ SEM ($n = 3$ independent experiments with duplicate determinations, performed separately for each panel). (B,C) The pEC₅₀ for PTH in the presence of F6P or F16bP was not significantly different than the pEC₅₀ for PTH alone (based on extra sum-of-squares F -test, Table 1). In contrast, the maximum response to PTH was significantly enhanced by F6P (1 and 10 mM). (D,E) The pEC₅₀ value for PTH in the presence of F6P or F16bP was not significantly different than the pEC₅₀ for PTH alone. In contrast, the maximal response to PTH was significantly enhanced at all concentrations of test substances.

evaluated the effect of sugar phosphates on the recruitment of β -arrestin-1.

Recruitment of β -arrestin-1 was monitored in real-time using a luciferase complementation assay.²⁴ HEK293H cells were transfected with plasmids encoding human PTH1R and β -arrestin-1, each connected to a luciferase fragment. Subsequently, cells were stimulated with PTH (1–34) at a threshold concentration (10 nM) in the presence of ATP (1.5 mM), F16bP (1.5 mM), or vehicle. PTH alone induced a time-dependent increase in β -arrestin-1 interaction with PTH1R (Figure 6A). Both ATP and F16bP markedly enhanced the recruitment of β -arrestin-1 induced by PTH under these conditions. Rates of β -arrestin recruitment were determined from the time-course data as the maximal slope, as described previously.¹⁹

The increases in PTH-stimulated signal shown in Figure 6 appear to require the presence of the orthosteric agonist since,

on their own, ATP or sugar phosphates did not promote recruitment of β -arrestin to PTH1R. (The lack of effect of sugar phosphates alone on β -arrestin recruitment is evident from the data points labeled Veh₁ in Figure 7B–E and Figure 8B–E.) As reported previously,¹⁹ ATP significantly enhanced the ability of PTH to induce β -arrestin recruitment (Figure 6B). Similarly, G1P, F6P, and F16bP all significantly enhanced PTH-induced β -arrestin-1 recruitment. In contrast, there was no significant effect of glucose, G6P, or fructose under these conditions, consistent with our findings using the cAMP accumulation assay (Figure 2). On the other hand, RSP did not significantly alter PTH-induced recruitment of β -arrestin-1 in these experiments (Figure S3).

Next, we evaluated the dependence of PTH-induced recruitment of β -arrestin-1 on the concentration of glucose phosphates. In these experiments, cells were stimulated with PTH (10 nM) in the presence of vehicle or concentrations of

G1P or G6P of up to 6 mM. G1P significantly enhanced recruitment at concentrations $\geq 316 \mu\text{M}$, whereas the effect of G6P was significant only at 6 mM (Figure 7A). Furthermore, the effect of G1P was significantly greater than that of G6P at concentrations $\geq 1 \text{ mM}$. We next evaluated how the effects of glucose phosphates depended on PTH concentration. The orthosteric agonist alone yielded the expected sigmoidal concentration dependence curve (Figure 7B,C). In contrast to their effects on PTH-induced adenylyl cyclase activation (Figure 3B,C), neither G1P nor G6P significantly shifted the curve (Figure 7B–E, Table 1). On the other hand, at higher concentrations, G1P and G6P did enhance maximum responses to PTH.

We also evaluated the dependence of PTH-induced recruitment of β -arrestin-1 on the concentration of fructose phosphates. Cells were stimulated with PTH (10 nM) in the presence of varied concentrations of F16bP, F6P, or fructose. F16bP and F6P significantly enhanced recruitment at concentrations $\geq 1 \text{ mM}$ (Figure 8A). In contrast, the effect of fructose was only significant at concentrations of $\geq 3 \text{ mM}$. The effect of F6P was significantly greater than that of fructose at 6 mM. Moreover, the effect of F16bP was significantly greater than that of F6P at concentrations $\geq 3 \text{ mM}$, and greater than that of fructose at concentrations $\geq 1 \text{ mM}$. When we examined how the effects of fructose phosphates depended on PTH concentration, neither F16bP nor F6P significantly shifted the concentration dependence curve to the left (Figure 8B–E, Table 1). On the other hand, in most cases, F6P and F16bP enhanced the maximum response to PTH.

The allosteric effects of sugar phosphates in the β -arrestin assays were not as striking as those observed in the cAMP assays. Reasons for this are unclear. It should be noted that, in the present study, assays for cAMP accumulation and β -arrestin recruitment were performed in different cell types (UMR-106 and HEK293H cells, respectively). In addition, UMR-106 cells endogenously express rat PTH1R, whereas HEK293H cells were transfected with a human PTH1R construct. However, we observed previously that transfected HEK293H cells show adenylyl cyclase responses to PTH very similar to those of UMR-106 cells, and that ATP has similar potentiating effects on cyclase signaling in both cell types.¹⁹ Consequently, it seems unlikely that species difference was a contributing factor. It is known that higher agonist concentrations are generally needed to promote β -arrestin recruitment in comparison to other end points of GPCR activation.²⁵ As well, compared to G protein-mediated signaling, the kinetics of β -arrestin recruitment may be more complex, as agonist occupancy may be required both for the targeting of GRKs to the intracellular face of the receptor and for increasing the affinity of the receptor for β -arrestin.^{26,27} In any event, sugar phosphates enhanced both PTH-induced cAMP signaling and β -arrestin recruitment to PTH1R.

Implications for Understanding Positive Allosteric Modulation of PTH1R. Recently, we reported that ATP can produce PAM-like effects at PTH1R.¹⁹ As well, other nucleotides, lacking an imidazole ring (CTP), a pyrophosphate moiety (i.e., in the β and γ phosphates) (AMP-PNP), or both of these elements (CMP), produced comparable effects, implying that the entire ATP molecule is not essential for potentiation of PTH signaling. The present study further defined the minimal pharmacophore that promotes stimulation of PTH1R signaling in response to PTH. The common core shared by all ribonucleotide structures is R5P, a molecule

which in the present study potentiated PTH-induced cAMP accumulation to a degree comparable to that of ATP. It follows that the PAM effects of extracellular nucleotides on PTH1R signaling¹⁹ can occur in the absence of a nucleobase moiety, and that R5P may be largely responsible for the potentiating effects of ATP and other nucleotides on PTH signaling. These findings further reinforce our previous conclusion that the effects of ATP on PTH1R signaling are not mediated through an action on P2 purinergic receptors.

In the present study, similar effects to those of R5P were observed with phosphorylated forms of glucose and fructose. Furthermore, the enhancement of PTH signaling was found to occur not only with cAMP signaling but also with agonist-induced β -arrestin recruitment to PTH1R. Taken together with our previous observations,¹⁹ the present findings point to the existence of an allosteric site on PTH1R that accommodates the binding of ATP and also binds productively to smaller molecules such as sugar phosphates.

The presence of at least one phosphate molecule appears to be an important property of PTH1R PAMs, as simple monosaccharide sugars minimally increased PTH signaling, if at all. This is in contrast to a previous report that glucose acts as a PAM at the calcium-sensing receptor, with maximal effects at 5–10 mM extracellular glucose.²⁸ However, if such an effect of glucose occurred in our system, it would likely not be evident as assays were carried out in medium that already contained $\sim 5 \text{ mM}$ glucose. We also found some evidence that the number and/or position of phosphate groups on the sugar phosphate influences its PAM effects. The present findings showed differences between the potentiating actions of G1P and G6P, with the former tending to produce greater effects when assessed at threshold concentrations of PTH (see for example Figure 3A and Figure 7A). Still, potentiating effects were readily observable with both isomers at higher agonist concentrations, and PTH concentration–response profiles showed clear increases in potency and/or maximal signal in the presence of G6P. Differences in PAM effects between G1P and G6P are unlikely to reflect a difference in charge, as pK_a values for the two are similar.²⁹ The carbon molecule that is phosphorylated in G1P lies within the ring structure, whereas the carbon at position 6 does not. Thus, it is possible that the location of negative charges relative to the rest of the molecule may be a determinant of PAM function.

In some cases, F16bP tended to produce a greater potentiating effect than F6P. This suggests that the number of negative charges may be a factor. Alternatively, it is possible that having phosphates at multiple positions increases the likelihood that one phosphate group will be in an appropriate position to interface with the site on PTH1R that conveys the observed PAM effects. Moreover, the total negative charge *per se* does not seem to be a crucial factor, as we have not found nucleotide triphosphates or diphosphates to be more efficacious than monophosphates.¹⁹

Potential Translational Significance. Sugar phosphates are not normally present extracellularly at high concentrations; in contrast, intracellular concentrations are typically in the millimolar or submillimolar range,³⁰ levels that are sufficient to potentiate PTH signaling. It has been proposed that, following activation and internalization, PTH1R continues to signal within endosomes.^{31,32} Therefore, it is conceivable that, physiologically, intracellular nucleotides/sugar phosphates could act on PTH1R following its internalization. Furthermore, several GPCRs,³³ including PTH1R,^{34,35} can be found in

the nucleus. However, in the present study, we report responses to the addition of extracellular PTH in the presence or absence of PAMs. The resulting cyclase activation and its potentiation are of such rapid onset as to rule out the possibility that these initial effects are mediated by internalized receptors.

In vivo, it is possible that other organic phosphates in the extracellular compartment may function like nucleotides and sugar phosphates as PAMs of PTH1R. Regardless of whether or not these PAMs are present at sufficient concentrations extracellularly to have physiological impact on PTH1R signaling, it may be possible to exploit our findings pharmacologically through the development of PAMs or bitopic ligands. In this regard, it has been difficult to target nonpeptide ligands to Class B GPCRs;³⁶ thus, the prospect of developing small molecule PAMs for PTH1R represents an advantageous therapeutic strategy. However, it should be noted that the precise site of action and specificity of such PAMs remain to be determined. As well, based on our findings, it is conceivable that purinergic receptor targeting compounds could have off-target effects at PTH1R.

If PTH1R PAMs or bitopic ligands were to be developed, then they could potentially modulate the catabolic and/or anabolic actions of PTH *in vivo*. The mode of administration determines whether the net effect of PTH on bone is catabolic (continuous PTH administration) or anabolic (intermittent PTH administration).³⁷ It is conceivable that the therapeutic administration of a short-acting PAM would transiently boost PTH1R signaling (perhaps even in the presence of basal levels of endogenous PTH) sufficiently to mimic intermittent PTH administration and induce an anabolic response in bone. Regardless of the future potential of such drug development, the present results provide insights into the functioning of a physiologically important receptor and also point to a possible mode of regulation by endogenous molecules.

METHODS

Materials and Solutions. α -Minimum essential medium, heat-inactivated fetal bovine serum, antibiotic–antimycotic solution (10 000 U/mL penicillin; 10 000 μ g/mL streptomycin; and 25 μ g/mL amphotericin B), trypsin solution, Dulbecco's phosphate-buffered saline, Dulbecco's modified Eagle medium (high glucose) (DMEM), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), and minimum essential medium (MEM, without bicarbonate and without phenol red) were obtained from Thermo Fisher Scientific (Waltham, MA). X-tremeGENE9 was from Roche Diagnostics (Laval, QC, Canada). Bovine albumin (BSA), Fraction V was obtained from MP Biomedicals (Solon, OH). D-(+)-glucose was obtained from VWR International (Radnor, PA). Adenosine 5'-triphosphate (ATP) disodium salt hydrate; adenosine 3',5'-cyclic monophosphate (cAMP) sodium salt monohydrate; cytidine 5'-monophosphate (CMP) disodium salt; D-glucose-1-phosphate (G1P) disodium salt hydrate; D-glucose-6-phosphate (G6P) sodium salt; D-fructose; D-fructose-6-phosphate (F6P) disodium salt hydrate; D-fructose-1,6-bisphosphate (F16bP) trisodium salt hydrate; and 3-isobutyl-1-methylxanthine (IBMX) were obtained from MilliporeSigma (St. Louis, MO). D-Luciferin sodium salt was obtained from Gold Biotechnology (St. Louis, MO). Rat PTH (1–34) was purchased from Bachem (Bubendorf, Switzerland). Nucleotides and sugars were dissolved in divalent cation-free buffer: 140 mM NaCl, 5 mM KCl, 20 mM HEPES,

and 10 mM glucose; and pH adjusted if necessary (Veh₂). PTH was dissolved in Dulbecco's phosphate-buffered saline, supplemented with 0.1% BSA (Veh₁).

Cells and Culture. UMR-106 rat osteoblast-like cells²² were obtained from the American Type Culture Collection (Rockville, MD). UMR-106 cells endogenously express PTH1R and downstream signaling components including adenylyl cyclase. UMR-106 cells were subcultured twice weekly and maintained at 37 °C and 5% CO₂ in α -MEM supplemented with 10% serum and 1% antibiotic–antimycotic solution. For the PTH1R- β -arrestin interaction assay, we used the HEK293H human embryonic kidney cell line (Gibco 293-H cells from Thermo Fisher Scientific), which does not endogenously express PTH1R. HEK293H cells were subcultured twice weekly and maintained at 37 °C and 5% CO₂ in DMEM supplemented with 10% serum and 1% antibiotic–antimycotic solution.

Transfections. Transfections were performed using X-tremeGENE 9 Reagent according to the manufacturer's protocol with a modification. Briefly, we prepared a DNA transfection complex consisting of DMEM, X-tremeGENE 9 Reagent, and plasmid vector. Next, a cell suspension was prepared by trypsinization followed by resuspension in fresh medium, and DNA transfection complex was added directly to the suspension. After mixing, the cell suspension was plated into multiwell plates as indicated for each experiment.

Live Cell cAMP Measurement. Cytosolic cAMP levels in live cells were monitored using GloSensor cAMP assay, as described previously.¹⁹ Briefly, UMR-106 cells were transfected with pGloSensor-22F cAMP plasmid (Promega)³⁸ and the cell suspension was seeded in a white 96-well plate (Corning, MilliporeSigma, Oakville, Canada; or Greiner Bio-One, Monroe, NC) at a density of 5.0×10^4 cells/well (1.5×10^5 cells/cm²). After a 24 h incubation at 37 °C/5% CO₂, cells were placed in fresh MEM (supplemented with 2 mM D-luciferin, 20 mM HEPES and 0.1% BSA; pH = 7.20 ± 0.02 ; 300 ± 5 mOsmol/L) and were incubated for 2 h at room temperature. Cells were treated with IBMX (200 μ M) and were further incubated for 20 min at room temperature. Next, cells were stimulated with agonists (time 0) and the emitted luminescence was measured using a Synergy HTX multimode reader (BioTek Instruments, Winooski, VT) at room temperature with 1 s integration time at 1.5 min intervals for a total of 45 min.

Live Cell β -Arrestin-1-PTH1R Interaction Assay. Agonist-promoted binding of β -arrestin-1 to PTH1R was assessed using a luminescent protein complementation assay, as described previously.^{19,24} Briefly, HEK293H cells were transfected with two plasmids. The first plasmid was PtGRN 415-ARRB1 in pcDNA3.1/myc-His B, which encodes N-terminal click beetle luciferase (1–415)- β -arrestin-1 chimeric protein; and the second plasmid was hPTH1R-linker20-PtGRC394 in pcDNA3.1(+), which encodes human PTH1R-C-terminal click beetle luciferase (394–542) chimeric protein. Cells were transfected with the two plasmids (1:1 mass ratio) in suspension and plated in a white 96-well plate at a density of 5.0×10^4 cells/well (1.5×10^5 cells/cm²). After 24 h incubation at 37 °C, 5% CO₂, cells were placed in fresh MEM (supplemented with 3.2 mM D-luciferin, 20 mM HEPES, and 0.1% BSA; pH = 7.20 ± 0.02 ; 300 ± 5 mOsmol/L) and were incubated for 1 h at 37 °C. Next, cells were stimulated with agonists (time 0) and luminescence was measured using a

Synergy HTX multimode reader, at 37 °C with 2 s integration time at 2 min intervals for a total of 80 min.

Data Analyses and Statistics. Data shown are means $\pm$ SEM. Differences among three or more groups were analyzed using one-way or two-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test. Data obtained in the live cell cAMP and β -arrestin-1-PTH1R interaction assays were in the form of response versus time curves. Data were analyzed as described previously.¹⁹ Briefly, for each time-course curve, we calculated an average slope beginning at every point using the next five consecutive data points (this average was the mean of the five individual slopes); we then selected the maximal slope, which was then normalized as indicated. PTH concentration–response data were fitted using GraphPad Prism 5 software (La Jolla, CA) to a 3-parameter sigmoidal equation (fixing Hill slope to 1 and varying minimum signal, EC₅₀, and maximum response) using simultaneous nonlinear regression analysis of multiple data sets. The *F*-statistic (calculated using the extra sum-of-squares *F*-test) was used to assess the effect of extracellular nucleotides/sugar phosphates on EC₅₀ and maximum response to PTH, by constraining this parameter to be the same between data sets acquired with and without extracellular nucleotides/sugar phosphates. Selected data sets were also fitted to an allosteric model³⁹ using GraphPad Prism 5 software (allosteric EC₅₀ shift equation), as detailed in the legend to Figure S2.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsptsci.8b00053.

Effects of extracellular nucleotides, glucose, glucose-1-phosphate and inorganic phosphate on PTH-stimulated adenylyl cyclase activity; analysis of PTH concentration–response data acquired at multiple sugar phosphate concentrations using an allosteric model; effect of extracellular ribose-5-phosphate on PTH-stimulated β -arrestin recruitment to PTH1R (PDF)

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Author Contributions

B.H.K. A.P., P.C., and S.J.D. conceived the study and designed experiments. P.C. and S.J.D. supervised the work. B.H.K. and F.I.W. performed the experiments and analyzed the data with help from A.P. B.H.K., P.C., and S.J.D. wrote the manuscript with edits from A.P. and F.I.W. All authors read and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ANOVA, analysis of variance; BSA, bovine albumin; cAMP, adenosine 3',5'-cyclic monophosphate; CMP, cytidine 5'-monophosphate; DMEM, Dulbecco's modified Eagle medium; F16bP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; GPCR, G-protein-coupled receptor; GRK, GPCR kinase; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; MEM, minimum essential medium; PAM, positive allosteric modulator; PTH, parathyroid hormone; PTH1R, parathyroid hormone 1 receptor; R5P, ribose-5-phosphate; SEM, standard error of the mean

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