A New Action of Parathyroid Hormone

RECEPTOR-MEDIATED STIMULATION OF EXTRACELLULAR ACIDIFICATION IN HUMAN OSTEOBLAST-LIKE SaOS-2 CELLS

Mark G. Barrett, Glenn S. Belinsky, and Armen H. Tashjian, Jr.

From the Department of Molecular and Cellular Toxicology, Harvard School of Public Health, and the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

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The major physiological function of parathyroid hormone (PTH) is the maintenance of Ca\(^{2+}/\)P homeostasis via the parathyroid hormone/parathyroid hormone-related protein receptor (PTHR) in kidney and bone. An important consequence of PTH activation in bone is enhanced local acidification of the extracellular space. Agonist activation of some seven transmembrane-domain receptors increases the extracellular acidification rate (ECAR). We utilized microphysiometry to investigate PTH-stimulated, receptor-mediated increases in ECAR in human osteoblast-like SaOS-2 cells. PTH-(1–34) elicited a large, acute, dose-dependent increase in ECAR with an EC\(_{50}\) of about 2 nM. The PTH-induced increase in ECAR was specific to cells expressing the PTHR and was inhibited by PTHR antagonists. Rapid, partial, homologous desensitization of the PTH-induced increase in ECAR was observed. Incubation of SaOS-2 cells with 8-bromo-cyclic AMP neither mimicked nor abrogated the PTH effect, and PTH stimulated an acute increase in ECAR in cAMP-resistant SaOS-2 Ca\(^{2+}/\)A cells. Stimulation of ECAR by PTH was independent of transient increases in cytosolic free calcium. Both inhibition and down-regulation of PKC reduced the PTH-induced increase in ECAR. Inhibition of Na\(^+/\)H\(^+\) exchange did not affect the PTH-induced ECAR response. We conclude that PTH caused a receptor-mediated, concentration-dependent, increase in ECAR, which was not dependent on the cAMP/PKA or inositol lipid/Ca\(^{2+}/\)PKC signaling pathways by the PTHR in osteoblasts (4). Receptor regulation is also important in the action of PTH on osteoblasts (4–9). Homologous desensitization of PTH-stimulated increases in intracellular cAMP accumulation ([cAMP]i) and in cytosolic free calcium concentration ([Ca\(^{2+}\)i]) and down-regulation of the PTHR have been described in several target cell systems (4–9).

Previous investigations of the physiological actions of PTH have largely used animal and whole bone experimental systems (10–14), while studies of PTHR signaling have concentrated on monitoring the activation of either adenylyl cyclase and the cAMP/PKA or phospholipase C and inositol lipid/Ca\(^{2+}/\)PKC signaling pathways in osteoblastic cells in culture (4–9, 15). Microphysiometry is a novel method for monitoring cellular metabolism (16) and has been effectively adapted to monitor acid secretion and metabolic rates in small populations of cultured cells in real time (17, 18). Because local pH homeostasis is physiologically important in mineral ion metabolism, and PTH-stimulated bone resorption has been linked to acid production by osteoclasts (13), we utilized microphysiometry to monitor the effect of PTH on extracellular acidification in osteoblasts, the primary target cell for PTH in bone.

The Cytosensor microphysiometer utilizes silicon-based, light-addressable potentiometric sensors to make rapid, precise determinations of extracellular pH. The Cytosensor monitors the rate at which cells secrete the acidic byproducts of metabolism, the extracellular acidification rate (ECAR). Cells acidify the environment via ion exchangers and pumps such as the Na\(^+/\)H\(^+\) antiporter and the H\(^+\)-ATPase and by transporting metabolites such as lactic acid across the plasma membrane (19). Several seven-transmembrane G protein-coupled receptors such as adrenergic, dopaminergic, and muscarinic receptors elicit concentration-dependent increases in ECAR in response to agonist binding (17, 18, 20); however, the physiological significance of receptor-mediated stimulation of extracellular acidification in these cell systems is not clear. In contrast, acidification of the bone microenvironment is known to be...
functionally important in skeletal metabolism and Ca\(^{2+}\)/P homestasis (13). Osteoclast-mediated bone resorption depends on the accumulation of acid at the site of resorption (13, 21, 22), and small fluctuations in extracellular pH in bone affect osteoclastic bone resorption (23–25). Stimulation of bone resorption by PTH is also linked to increased glycolytic acid production (13), but the cellular and molecular mechanisms of this action of the hormone are unknown. For these reasons, we utilized microphysiometry to demonstrate that agonist activation of the PTHR stimulates a large, acute, dose-dependent increase in ECAR, which we have utilized for a new analysis of PTHR signaling and regulation in osteoblastic cells.

EXPERIMENTAL PROCEDURES

Materials—Culture media and sera were obtained from Life Technologies, Inc. (Grand Island, NY). hPTH-(1–34) and human vasopressin (VIP) were obtained from Peninsula Laboratories (Belmont, CA). [Nle\(^8\),Trp\(^{12}\), Tyr\(^{24}\)]bPTH-(7–34)NH\(_2\) was a generous gift from Dr. Michael Chorev (Beth Israel Hospital, Boston, MA). Porbo 12-myristate 13-acetate (PMA), thapsigargin, and nigericin were purchased from Calbiochem (La Jolla, CA), and Ro-20–1724 was obtained from Biomial (Plymouth Meeting, PA). Fura-2, acetoxymethyl ester (Fura-2/AM) and 2,7’-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein, acetoxymethyl ester (BCECF/AM) were prepared by Molecular Probes, Inc (Eugene, OR). 1-O-Hexadecyl-2-O-methyl-rac-glycerol (AMC-C\(_2\)), 8-bromo-cyclic AMP (8-Br-cAMP), (-)-isoproterenol, and additional chemicals were purchased from Sigma. The Cytosensor microphysiometer and supplies were obtained from Molecular Devices (Sunnyvale, CA). Tissue culture plasticware was purchased from Beckton Dickinson (Lincoln Park, NJ).

Cell Culture—Culture of the cells used in this study has been described previously. In brief, human osteoblast-like SaOS-2 (26–28) and rat osteoblast-like UMR-106 (29) cells were grown as monolayers in minimal essential medium supplemented with 5% horse serum and 5% fetal bovine serum (MEM\(^-\)). Rat pituitary GH\(_4\)C\(_1\) (30) and F\(_4\)C\(_1\) (31) cells were cultured as monolayers in Ham’s F-10 nutrient mixture supplemented with 15% horse serum and 2.5% fetal bovine serum (Ham’s F-10). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO\(_2\) and 95% air. The cells were subcultured 1:5 every fifth day or as necessary for experiments.

Microphysiometric Analysis—Experiments were performed as detailed previously (17, 18, 20). Briefly, 12–24 h before an experiment, cells were subcultured onto the polycarbonate membranes of transwell capsule cups (Corning Costar, Cambridge, MA) in the following manner. Cells were detached from the plastic culture dish with 0.5 mg/ml trypsin and then suspended in MEM\(^-\), and the concentration of cells in suspension was determined. The cell suspension was diluted to a concentration of 5 × 10\(^4\) cells/ml (1 × 10\(^5\) cells/ml for GH\(_4\)C\(_1\) cells), and 1 ml of the suspension was seeded into each transwell capsule cup placed in a 12-well culture plate. The cells were then incubated at 37 °C until the beginning of the experiment. Immediately preceding an experiment, the low buffered MEM running buffer (MEM lacking NaHCO\(_3\) to minimize buffering capacity), supplemented with 30 \(\mu\)M H\(_2\)CO\(_3\) (the PTH vehicle) and 0.1% bovine serum albumin or 5% horse serum (carrier proteins)) was prepared. The Cytosensor system was warmed to 37 °C and flushed with running buffer. The transwell capsule cups containing the cells were fitted with a spacer gasket and capsule cup insert to create a microvolume flow chamber containing the cells. The assembled capsule cups were then placed on the sterile silicon sensors and mounted on the Cytosensor. The Cytosensor was programmed to perfuse the cells with running buffer at a rate of approximately 100 \(\mu\)l/min for periods of 60 s interrupted by 22-s periods of no flow. The pH of the extracellular media was measured at 1-s intervals, and the rate of acidification was determined by calculating the least squares fit to the slope of the pH profile while the flow of buffer was stopped. After being loaded into the Cytosensor, cells were allowed to equilibrate until a stable base-line ECAR was reached, and then they were exposed to pharmacological and agonist agents as described under “Results.” The low buffered, balanced salt solution (BSS) used in experiments containing Na\(^{+}\)/H\(^+\) exchange consisted of 5 mM NaCl, 0.6 mM MgCl\(_2\), 0.5 mM KH\(_2\)PO\(_4\), 3 mM KCl, 5 mM glucose, and 130 mM either C\(_6\)H\(_5\)CINO or NaCl.

For the purpose of comparing ECARs from many different experiments, microphysiometer data were normalized utilizing CytoSoft software (Molecular Devices, Sunnyvale, CA). A minimum of five consecutive data points from the base-line ECAR of each cell population were selected and averaged, and then all other data points were expressed as a percentage of this basal average.

Spectrofluorometric Measurements of Cytosolic Free Ca\(^{2+}\) and Intracellular pH—SaOS-2 cells were prepared for analysis of pH, and [Ca\(^{2+}\)]\(_i\), as described previously (32, 33). In brief, the cells in the monolayer were detached from the culture dish using Ca\(^{2+}\)-free Hanks’ balanced salt solution (HBSS) containing 120 mM NaCl, 4 mM KCl, 10 mM HEPES, pH 7.2, supplemented with 5 mM EDTA. The cells were then washed three times and resuspended in HBSS containing 1 mM CaCl\(_2\) at a concentration of approximately 2 × 10\(^5\) cells/ml. Fura-2/AM or BCECF/AM was added to the suspension to a final concentration of 1 or 5 \(\mu\)M, respectively. The suspension was incubated at 37 °C in the dark with gentle shaking for 60 min. After incubation, the cells were washed three times in HBSS containing 1 mM CaCl\(_2\) and then were resuspended in HBSS containing 0.5 mM EDTA and resuspended in the low buffered BSS at a concentration of 5 × 10\(^5\) cells/ml. The suspension was loaded in a UV grade acrylic cuvette (Spectrocell, Oregon, PA) in a Hitachi F-2000 spectrofluorometer (Rye, NH) warmed to 37 °C and stirred constantly. [Ca\(^{2+}\)]\(_i\) was determined using the ratio of Fura-2 emission fluorescence at 510 nm at excitation wavelengths of 340 and 380 nm as described previously (32). pH was determined by monitoring the fluorescence emission of BCECF at 535 nm at an excitation wavelength of 500 nm.

Each spectrofluorometric experiment shown was repeated at least three times with qualitatively and quantitatively similar findings.

RESULTS

ECAR in SaOS-2 Cells Is Stimulated by PTH—Baseline ECAR in serum-free, low buffered MEM was 47 ± 15 µV/s (mean ± S.D., n = 55) when 5 × 10\(^4\) cells were seeded in each transwell capsule cup. Human PTH-(1–34) elicited an acute increase in ECAR (Fig. 1). The maximum PTH-stimulated increase in ECAR was 68 ± 24 µV/s (mean ± S.D., n = 55) above basal, a 2.4-fold increase above the resting acidification rate, and the EC\(_{50}\) was about 2 nM (Fig. 2). The peak in ECAR occurred within 45 s of agonist addition and usually returned to basal levels within 5 min even in the persistent presence of PTH (Fig. 1). In some experiments, the ECAR dropped below the original basal level or remained slightly above it following a PTH exposure. Such modest variations in the pattern of ECAR responses to agonists have been reported in analogous studies in intact cell types (18). Similar results were obtained when cells were also elicited by hPTPrP-(1–34)NH\(_2\) (data not shown). These results demonstrate that PTH elicited large, acute, dose-dependent increases in ECAR in SaOS-2 cells. The specificity of the ECAR response was investigated in the following experiments.

Cell Type Specificity of the PTH-induced Increase in ECAR—Human osteoblast-like SaOS-2 cells, which endogenously ex-
Fig. 2. Dose response to PTH in SaOS-2 cells as measured by ECAR. Cells were equilibrated in low buffered MEM supplemented with 5% horse serum (to prevent nonspecific adsorption of PTH) and sequentially exposed to increasing concentrations of hPTH-(1–34) (0.01–500 nM) as denoted at the top for 2-min intervals marked by the arrows at the bottom of the figure. Between treatments, cells were perfused with hormone-free buffer for a minimum of 25 min, allowing the ECAR to return to basal levels and the cells to recover responsiveness (see Fig. 6). The basal ECAR was elevated to the level seen in this experiment by the presence of 5% horse serum. The ECAR response of the SaOS-2 cells to PTH was dose-dependent with an EC50 of about 2 nM. The trace depicted is the composite of two independent experiments.

Press the PTHR, and rat pituitary F4C1 and GH4C1 cells, which do not express the PTHR, were examined. Each cell type was incubated with 100 nM hPTH-(1–34) for 2 min, and ECAR was monitored (Fig. 3). PTH induced an increase in ECAR in SaOS-2 cells, but no response was observed in the two PTHR-deficient cell strains. F4C1 and GH4C1 cells were responsive to an appropriate peptide hormone in the Cytosensor system, because thyrotropin-releasing hormone, for which these cells express the receptor, induced large increases in ECAR in these two cell types (data not shown). In addition, hPTH-(1–34) stimulated rapid increases in ECAR in rat osteoblast-like UMR-106 cells, which also express the PTHR (data not shown). These results suggest that the PTH-induced increase in ECAR is mediated by the PTHR. Additional evidence to support this conclusion is given below.

Peptide Specificity of the PTHR-mediated Increase in ECAR—SaOS-2 cells were incubated with the PTHR antagonist [Nle6,18,D-Trp12,Tyr34]bPTH-(7–34)NH2 (35). A 25-min pretreatment of cells with 200 nM [Nle6,18,D-Trp12,Tyr34]bPTH-(7–34)NH2 elicited no increase in ECAR, and it reduced the ECAR response elicited by 20 nM hPTH-(1–34) to about 25% of the control value, while the response of the cells to 20 nM VIP was unaffected (Fig. 4). The inhibition of the PTH-induced increase in ECAR by [Nle6,18,D-Trp12,Tyr34]bPTH-(7–34) was reversed by washing the cells with buffer for 90 min (data not shown). A 25-min preincubation of SaOS-2 cells with 100 nM [Nle6,18,D-Trp12,Tyr34]bPTH-(7–34)NH2 caused a shift in the ECAR dose-response curve for hPTH-(1–34) about 10-fold to the right, increasing the EC50 to about 10 nM (Fig. 5). These results demonstrate that the PTH-induced ECAR response is mediated by the PTHR in SaOS-2 cells. The following experiments were designed to investigate the response of osteoblast-like cells to repeated exposures to PTH.

Desensitization of the PTH-induced ECAR Response—Microphysiometry was used to analyze desensitization of the receptor-mediated, PTH-induced ECAR response. Because the increase in ECAR induced by PTH was acute in nature and the acidification rates of the cells returned rapidly to the basal level, it was possible to monitor repeated sequential responses to PTH in real time. An initial response to a 2-min challenge with 100 nM hPTH-(1–34) was followed by a refractory period of approximately 15 min during which the response of the same cells to a second challenge with 100 nM hPTH-(1–34) was reduced relative to the first response. When two 2-min agonist treatments were separated by a 2.5-min wash, the second PTH-induced response was reduced to about 13% of the first response (Fig. 6). As the wash time between treatments was increased, the magnitude of the second response to PTH increased, with nearly full recovery after 14.5 min of hormone-free perfusion (Fig. 6).

Interestingly, decreased responsiveness after the initial exposure to PTH was not progressive. In cells treated with 100 nM hPTH-(1–34) for 2-min intervals separated by 7-min washes, the first response to hPTH-(1–34) was the largest, and the second response was reduced to about 50% of the first response; however, no further reduction in responsiveness was observed in up to nine subsequent, sequential agonist exposures (data not shown).

The effect of continuous long term agonist exposure on the acute PTH-induced ECAR response was also investigated. In preparation for these experiments, SaOS-2 cells were subcul-

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The acute maximal response of PTH-pretreated cells to fresh agonist exposure was reduced to about 50% of the control response, while the cells still exhibited an increase in ECAR relative to the first exposure. The magnitude of the ECAR response to 100 nM hPTH-(1–34) remained consistent for up to 50 min after washout, indicating that this cAMP analog was active in cells pretreated with hPTH-(1–34) for 2 min. The PTH-induced ECAR response was then examined.

The cAMP/PKA Signaling Pathway and ECAR in SaOS-2 Cells—When SaOS-2 cells were incubated for 5 min with 1 mM 8-Br-cAMP, there was no large, acute increase in ECAR such as that elicited by PTH in the same cells; only a transient 5–20% increase in ECAR was observed (Fig. 7). When UMR-106 cells were exposed to 1 mM 8-Br-cAMP, a persistent increase in ECAR resulted (data not shown). The increase in ECAR in UMR-106 cells stimulated by 8-Br-cAMP peaked at 1.7-fold above basal levels and returned to basal levels only after a 50-min wash, indicating that this cAMP analog was active in the Cytosensor system.

The effect of a high concentration of exogenous cAMP on the PTH-induced increase in ECAR was then examined in SaOS-2 cells. Cells were pretreated for 30 min with either 1 mM 8-Br-cAMP, clamping the intracellular cAMP concentration at supramaximal levels above those generated by PTH exposure, or control running buffer, and then the cells were exposed to 10 nM hPTH-(1–34) for 2 min. The PTH-induced ECAR response in SaOS-2 cells was not affected by the simultaneous presence of 1 mM 8-Br-cAMP (Fig. 7). SaOS-2 cells were also incubated with 100 nM forskolin to stimulate endogenous cAMP production, but no acute increase in ECAR was observed (data not shown).

Finally, SaOS-2 Ca#4A cells were used to analyze further the role of the cAMP/PKA signaling pathway in the PTH-stimulated increase in ECAR. SaOS-2 Ca#4A cells are a subclone of SaOS-2 cells in which enhancement of cAMP formation does not activate PKA due to the overexpression of a mutant PKA regulatory subunit (36). hPTH-(1–34) (100 nM) stimulated an increase in [cAMP]_i, or activation of PKA.
calcium stores with 1 mM thapsigargin elicited a transient increase in [Ca^{2+}]_{i} in SaOS-2 cells in suspension (5 × 10^6 cells/ml). B, SaOS-2 cells in suspension were incubated with 1 μM thapsigargin. Once cytosolic free calcium concentrations equilibrated at an elevated level, the concentration of thapsigargin in the cell suspension was increased to 2 μM (at the arrow labeled Th) to ensure that all thapsigargin-sensitive Ca^{2+} stores were depleted. The cells were then exposed to 100 nM PTH (arrow labeled PTH). Under these conditions, PTH elicited no further increase in [Ca^{2+}]_{i}. C, SaOS-2 cells were first exposed to 100 nM hPTH-(1–34) as indicated by the arrow at the left. The cells were then incubated with 1 μM thapsigargin for the period indicated by the bar at the bottom. After the ECAR of the cells returned to basal levels, the cells were rechallenged with 100 nM hPTH-(1–34), marked by the arrow at the right. The PTH-stimulated ECAR response was not diminished by the prior depletion of intracellular Ca^{2+} pools. The ECAR trace depicted is a composite of data derived from four independent experiments. The brackets represent 1 S.E.

ECAR elicited by 20 nM PMA to 35% of the control PMA response (data not shown) and reduced the response to 100 nM hPTH-(1–34) to 37% of the control PTH response, while the increase in ECAR stimulated by 1 mM (-)-isoproterenol was unaffected (Fig. 9). In addition, SaOS-2 cells were pretreated with PMA (100 nM) for 24 h, which down-regulates PKC expression in these cells (38), and then exposed to 100 nM PTH. Down-regulation of PKC resulted in a 73% decrease in the PTH-stimulated increase in ECAR (Fig. 10) and a reduction of about 50% in a subsequent PMA-stimulated increase in ECAR (data not shown). Taken together, these results indicate that activation of PKC is involved in the PTH-stimulated increase in ECAR in SaOS-2 cells.

Role of Na^{+}/H^{+} Exchange in the PTH-induced Increase in ECAR—It has been demonstrated that the increase in ECAR stimulated by some G protein-coupled receptors is mediated by activation of Na^{+}/H^{+} exchange (20, 39). The PTHR has been shown to modulate the activity of Na^{+}/H^{+} exchange in certain osteoblast-like cell lines (40–42). Therefore, we equilibrated SaOS-2 cells in either Na^{+}-containing, low buffered BSS or Na^{+}-free, low buffered BSS, which inhibits Na^{+}/H^{+} exchange. The PTH-stimulated ECAR response was equal in Na^{+}-free and Na^{+}-containing buffer (Fig. 11). To ensure that the Na^{+}-free BSS effectively inhibited Na^{+}/H^{+} exchange, SaOS-2 cells were loaded with the pH-sensitive fluorescent probe BCECF/AM and then acidified with 1 μM nigericin (a K^{+}/H^{+} ionophore). In nigerin-acidified SaOS-2 cells, PMA induces alkalization via a Na^{+}/H^{+} exchanger-dependent mechanism (42). This PMA-induced alkalization was completely blocked in Na^{+}-free buffer (data not shown). Furthermore, the large, acute ECAR response of GH_{4}C_{1} cells to the neuropeptide agonist thyrotropin-releasing hormone was abolished in the Na^{+}-free buffer (data not shown). These results demonstrate that the PTH-stimulated increase in ECAR is independent of Na^{+}/H^{+} exchange in SaOS-2 cells.

**FIG. 8. Role of thapsigargin-sensitive Ca^{2+} pools in the PTH-induced increase in ECAR.** SaOS-2 cells were prepared for measurement of [Ca^{2+}]_{i} as described under “Experimental Procedures.” A, 100 nM hPTH-(1–34) elicited a transient increase in [Ca^{2+}]_{i} in SaOS-2 cells in suspension (5 × 10^6 cells/ml). B, SaOS-2 cells in suspension were incubated with 1 μM thapsigargin. Once cytosolic free calcium concentrations equilibrated at an elevated level, the concentration of thapsigargin in the cell suspension was increased to 2 μM (at the arrow labeled Th) to ensure that all thapsigargin-sensitive Ca^{2+} stores were depleted. The cells were then exposed to 100 nM PTH (arrow labeled PTH). Under these conditions, PTH elicited no further increase in [Ca^{2+}]_{i}. C, SaOS-2 cells were first exposed to 100 nM hPTH-(1–34) as indicated by the arrow at the left. The cells were then incubated with 1 μM thapsigargin for the period indicated by the bar at the bottom. After the ECAR of the cells returned to basal levels, the cells were rechallenged with 100 nM hPTH-(1–34), marked by the arrow at the right. The PTH-stimulated ECAR response was not diminished by the prior depletion of intracellular Ca^{2+} pools. The ECAR trace depicted is a composite of data derived from four independent experiments. The brackets represent 1 S.E.

**FIG. 9. The effect of AMG-C_{16} on the PTH-stimulated increase in ECAR.** Identical populations of SaOS-2 cells were first exposed to 100 nM hPTH-(1–34) to ensure that they were equally responsive to hormone. Subsequently, one population was treated with control running buffer ( ■ ) and another with running buffer supplemented with 200 μM AMG-C_{16} ( ⊕ ) during the period indicated by the bar at the bottom. All of the cells were then exposed to 100 nM hPTH-(1–34) (center) and, subsequently, 100 μM (-)-isoproterenol (Iso, right). The two traces are composites of the data derived from four independent measurements of control and AMG-C_{16} treated groups. Each point gives the mean value, and the brackets indicate 1 S.E. (n = 4). The asterisk indicates a significant decrease (p < 0.05) of PTH-stimulated ECAR in the presence of AMG-C_{16}.

**FIG. 10.** The effect of AMG-C_{16} on the PTH-stimulated increase in ECAR. Identical populations of SaOS-2 cells were first exposed to 100 nM hPTH-(1–34) to 37% of the control PTH response, while the increase in ECAR stimulated by 1 mM (-)-isoproterenol was unaffected (Fig. 9). In addition, SaOS-2 cells were pretreated with PMA (100 nM) for 24 h, which down-regulates PKC expression in these cells (38), and then exposed to 100 nM PTH. Down-regulation of PKC resulted in a 73% decrease in the PTH-stimulated increase in ECAR (Fig. 10) and a reduction of about 50% in a subsequent PMA-stimulated increase in ECAR (data not shown). Taken together, these results indicate that activation of PKC is involved in the PTH-stimulated increase in ECAR in SaOS-2 cells.
PTH in osteoblasts has been linked to osteoclast-mediated bone resorption (43, 44); however, the precise biochemical link between osteoblasts and osteoclasts in bone resorption has not been elucidated. PTH also has an anabolic effect on bone when administered intermittently, but the molecular mechanisms of this action are not understood (3). Additionally, acid production and pH homeostasis in the bone microenvironment are important in regulation of mineral metabolism and bone resorption (23–25), and PTH action has been linked to acid production in resorbing bone (13); therefore, the data presented here, demonstrating that PTH stimulates ECAR in human osteoblast-like SaOS-2 cells, has important consequences in bone physiology and mineral metabolism.

Early investigations of mineral metabolism linked acid production by glycolysis to stimulation of bone resorption mediated by PTH (13). In vitro exposure of whole bones to PTH resulted in the production of lactate and citrate (13, 45–47), and local pH is important in controlling bone mineral content (23–25). Activated osteoclasts concentrate acid in the resorptive compartment, resulting in the solubilization of Ca$^{2+}$ and P from hydroxyapatite crystals, allowing for subsequent degradation of matrix proteins by proteases (13, 21, 22). In addition, in vivo and in vitro studies show that decreased pH results in acid production by osteoblasts (23, 25). Although osteoblasts are the primary cellular target for PTH in bone and local pH homeostasis is known to be important, evidence is lacking concerning the role of osteoblasts in the production of acid in the bone environment. Based on the findings described in this report, we conclude that PTH directly enhances acid production by osteoblasts. This effect occurs in addition to osteoclast secretion of acid, which is an indirect action of PTH.

We investigated the signaling mechanisms involved in stimulation of increases in ECAR by PTH in SaOS-2 cells. PTH causes acute increases in cAMP accumulation in these cells (7, 8); therefore, the role of the cAMP/PKA signaling pathway in the PTH-induced increase in ECAR was examined. Activation of PKA with 8-Br-cAMP produced a small increase in ECAR, which did not mimic stimulation of ECAR by PTH either temporally or quantitatively. It is possible that the inability of 8-Br-cAMP to mimic the large, acute PTH-induced increase in ECAR was due to slow diffusion of the analog into cells relative to the acute increase in [cAMP] stimulated by PTH. However, the PTH-induced increase in ECAR was not altered by clamp-cAMP, at supramaximal concentrations by preincubation with 8-Br-cAMP. In addition, increases in ECAR induced by submaximal concentrations of PTH were neither augmented nor abrogated by the presence of 8-Br-cAMP (data not shown). If the PTH-induced increase in ECAR was the result of a receptor-mediated increase in intracellular cAMP, the presence of high concentrations of 8-Br-cAMP would be expected to abrogate subsequent receptor-mediated increases in ECAR as seen in other receptor-mediated increases in ECAR that are dependent on increases in [cAMP] (20, 48). Furthermore, PTH was as efficacious in stimulating an acute increase in ECAR in cAMP-resistant SaOS-2 Ca$^{2+}$/A cells as it was in wild-type SaOS-2 cells, and forskolin treatment, which increased [cAMP], did not induce an increase in ECAR. Finally, the phosphodiesterase inhibitor Ro-20–1724 was used to elevate basal [cAMP], but no potentiation of the PTH-induced increase in ECAR resulted (data not shown). Other cAMP-dependent actions of PTH are known to be potentiated by inhibition of phosphodiesterase activity. Based on these data collectively, we conclude that activation of the cAMP/PKA signaling pathway is not involved in the acute, PTH-stimulated increase in ECAR in SaOS-2 cells.

Previous studies on SaOS-2 cells reported that PTH did not stimulate acute increases in [Ca$^{2+}$] (33). However, PTH has been shown to elicit transient increases in [Ca$^{2+}$], and generate inositol polyphosphates in other osteoblast-like cell lines (4, 5, 15); therefore, we examined the role of the inositol lipid/Ca$^{2+}$/PKC signaling pathway in the stimulation of ECAR by PTH. Using dual wavelength spectrofluorometry, transient increases in [Ca$^{2+}$], were measured in the subclone of SaOS-2 cells used in the investigations presented in this report. Heterogeneity of intracellular calcium responses to PTH in osteoblasts has been reported (49, 50). Therefore, we believe that we isolated a subpopulation of SaOS-2 cells that does respond to PTH with an increase in [Ca$^{2+}$], allowing us to consider the contribution of this signaling event to stimulation of increased ECAR elicited by PTH. The increases in [Ca$^{2+}$], were dependent on the release of Ca$^{2+}$ from intracellular stores, because pharmacological depletion of these pools with thapsigargin blocked subsequent PTH-stimulated increases in [Ca$^{2+}$]. The PTH-induced increase in ECAR, however, was not blocked by thapsigargin pretreatment. PTH also stimulated a similar, acute increase in ECAR, but not [Ca$^{2+}$], in the wild-type SaOS-2 cells from which the subclone used in this study was...
derived (data not shown), indicating that the increase in ECAR was not dependent on the transient increase in [Ca\(^{2+}\)]. We then investigated the role of PKC in the PTH-induced increase in ECAR. Treatment of cells with the PKC inhibitor AMG-C\(_{16}\) significantly reduced the PTH-stimulated increase in ECAR. In control experiments, AMG-C\(_{16}\) inhibited the PMA-induced increase in ECAR, while it did not decrease basal ECAR or affect stimulation of ECAR by isoproterenol, indicating that AMG-C\(_{16}\) was inhibiting PKC and was not nonspecifically toxic to the cells. Additionally, pretreatment of SaOS-2 cells with PMA resulted in a substantial reduction in the PTH-induced increase in ECAR. Pretreatment of SaOS-2 cells with PMA down-regulates PKC (38) and it reduced PMA-stimulated increases in ECAR to about 50% of control (data not shown), demonstrating the specificity of the effect of the pretreatment on PKC. Taken together, these results indicate that stimulation of ECAR by PTH is dependent on the activity of PKC but independent of transient increases in [Ca\(^{2+}\)], demonstrating that PTHR activation of the inositol lipid/PKC signaling pathway may be functionally relevant to osteoblast physiology.

Receptor-mediated increases in ECAR have been linked to Na/H exchange in several systems (20, 39), and proton extrusion by these exchangers could account for acute acidification of the extracellular environment. In addition, in vitro experiments have suggested that PTH can modulate Na/H exchangers via multiple signaling pathways (40–42, 51). To determine the contribution of Na/H exchange in the PTH-induced increase in ECAR, SaOS-2 cells were incubated in Na\(^{-}\)-free buffer, which blocks Na/H exchange, and then exposed to PTH. The PTH-induced increase in ECAR was not reduced relative to control in Na\(^{-}\)-free buffer. In addition, the Na/H exchanger inhibitor, 5-(N-methyl-N-isobutyl)amiloride, did not inhibit the PTH-induced increase in ECAR (data not shown), indicating that stimulation of ECAR by PTH was independent of Na/H exchange in SaOS-2 cells.

Desensitization of the PTHR-mediated increase in ECAR was also investigated. The PTH-stimulated increase in ECAR peaked within 45 s of PTH addition and returned rapidly to basal levels. The acute nature of the increase allowed us to monitor the ECAR response of cells to repetitive exposures to hormone on a time scale of minutes. Prolonged pharmacological activation of PKC in SaOS-2 cells resulted (data not shown), indicating that the increase in ECAR by PTH was independent of Na/H exchange in SaOS-2 cells.
