Parathyroid hormone stimulates calcium influx and the cAMP messenger system in rat enterocytes

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Picotto, Gabriela, Virginia Massheimer, and Ricardo Boland. Parathyroid hormone stimulates calcium influx and the cAMP messenger system in rat enterocytes. Am. J. Physiol. 273 (Cell Physiol. 42): C1349–C1353, 1997.—Direct effects of parathyroid hormone (PTH) on calcium uptake by isolated rat duodenal cell preparations enriched in enterocytes were investigated. PTH significantly stimulated enterocyte 45Ca2+ influx in a time-dependent (1–10 min) manner and at all doses tested (2 x 10−12 to 10−7 M). The Ca2+ channel antagonists verapamil (10 μM) and nitrendipine (1 μM) completely blocked the stimulation of Ca2+ influx by the hormone (10−8 M). PTH markedly increased CAMP levels in rat duodenal cells (88, 167, and 67% after 1, 2, and 3 min, respectively). In agreement with these observations, forskolin (adenylate cyclase activator), dibutyryl adenosine 3',5'-cyclic monophosphate (DBcAMP), and Sp-cAMPS (cAMP analogs) mimicked, whereas Rp-cAMPS (cAMP antagonist) suppressed PTH and DBcAMP activation of enterocyte calcium uptake. Furthermore, the effects of DBcAMP were abolished by nitrendipine. These results show direct rapid effects of PTH on duodenal cells' Ca2+ influx, which involve the activation of a dihydropyridine-sensitive Ca2+ influx pathway and the cAMP second messenger system.

injection; nongenomic effects; calcium channels; adenosine 3',5'-cyclic monophosphate signaling pathway

PARATHYROID HORMONE (PTH) is responsible for the regulation of calcium levels in blood and extracellular fluids in concert with other calcitropic hormones (12). PTH acts at its target tissues, kidney and skeleton, by enhancing calcium reabsorption at cortical sites within the distal portion of the nephron (1) or by stimulating bone resorption with the subsequent release of calcium and phosphate into the circulation.

Moreover, this peptide hormone exerts an indirect action on the intestine. Through its effects on 1α-hydroxylase, PTH stimulates the formation of 1,25-dihydroxyvitamin D3 (11), which in turn has a direct biological effect on the gut, increasing the absorption of dietary calcium.

Like other polypeptide hormones, PTH interacts with specific receptors on the cell plasma membrane in target tissues and modulates cellular responses mainly by activation of adenylate cyclase and the increase of adenosine 3',5'-cyclic monophosphate (cAMP) levels (17). Recent reports also suggest that PTH may act through other messenger systems that involve phosphoinositide breakdown, inositol trisphosphate accumulation, and protein kinase C (PKC) activation (16).

It is well known that 1,25-dihydroxyvitamin D3 is the principal regulator of duodenal calcium transport in mammalian intestine (23). However, early work also suggested certain direct actions of PTH on duodenum cells (21). Physiological concentrations of this peptide hormone increased lysosomal enzyme liberation and calcium uptake after 10–15 min of treatment. In the present work, using isolated rat enterocytes, we investigated the nongenomic action of PTH on calcium influx at very short treatment intervals (1–10 min) and tested the hypothesis that this event is related to the activation of intracellular second messenger pathways.

MATERIALS AND METHODS

Materials. Synthetic rat PTH (rPTH) (1–34), verapamil, N6,2'-O-dibutyladenosine 3',5'-cyclic monophosphate (DB-cAMP), and forskolin were purchased from Sigma Chemical (St. Louis, MO). 45CaCl2 was provided by New England Nuclear (Chicago, IL). Nitrendipine was provided by Bayer (Leverkusen, Germany). Rp and Sp isomers (Rp-cAMPS, Sp-cAMPS) were provided by Biolog Life Science Institute (Bremen, Germany). The cAMP 125I-radioimmunoassay kit was obtained from DuPont (Boston, MA), and (PH)cAMP assay kit was obtained from Diagnostic Products (Los Angeles, CA). PTH antagonist, PTH (7–34), was obtained from Bachem California (Torrance, CA).

Animals. Wistar rats (3–5 mo old) were fed with standard rat food (1.2% Ca:1.0% phosphorus), given water ad libitum, and maintained on a 12:12-h light-dark cycle. Animals were killed by cervical dislocation.

Duodenal cell isolation. Duodenal cells were isolated essentially as previously described (20). The method employed yields preparations that contain only highly absorptive epithelial cells and that are devoid of cells from the upper villus or crypt (26, 27). The duodenum was excised, washed with 0.9% NaCl, and trimmed of adhering tissue. The intestine was slit lengthwise and cut into small segments (2 cm length) and placed into solution A containing (in mM) 96 NaCl, 1.5 KCl, 8 Na2HPO4, 1.0 Na2HPO4, 27 sodium citrate, pH 7.3, for 10 min at 37°C. The solution was discarded and replaced with solution B (isolation medium) containing (in mM) 154 NaCl, 10 NaH2PO4, 1.5 EDTA, 0.5 dithiothreitol (DTT), 5.6 glucose, pH 7.3, for 15 min at 37°C with shaking (87 oscillations/min). The cells were sedimented by centrifugation at 750 g for 10 min; washed twice with 154 mM NaCl, 10 mM NaH2PO4 and 5.6 mM glucose at pH 7.4; and then resuspended in solution D (incubation medium) containing (in mM) 154 NaCl, 5 KCl, 1 Na2HPO4, 1 MgCl2, 10 3-(N-morpholino)propanesulfonic acid sodium salt, pH 7.4, 5.6 glucose, 0.5% bovine serum albumin, 1 CaCl2, and 2.5 glutamine. Duodenal cells were preequilibrated in the incubation medium before hormone treatment and measurement of calcium uptake for 20 min. All the above-mentioned steps were performed under an atmosphere of 95% O2:5% CO2 and using oxygenated solutions. Cell viability was assessed by Trypan blue exclusion in well-dispersed cell preparations. Exclusion of the dye in >90% of the cells was observed for at least 90 min after isolation. Morphological characterization was performed by phase-contrast microscopy. Although enterocytes isolated by this procedure have been shown to possess functional characteristics of intestinal cells (26, 27), the possibility that these
isolated cell preparations contain nonepithelial cells should not be ruled out.

Calcium uptake After the preequilibration period, duodenal cells were incubated in solution D with rPTH-(1—34), Sp-cAMPS [protein kinase A (PKA) activator], forskolin, or DBcAMP in the presence of $^{45}$CaCl$_2$ (0.2 µCi/ml; 1 mM). To the corresponding control cell suspensions, distilled water was added except in the case of forskolin, when ethanol ($<0.1\%$) was employed. When calcium channel blockers or cAMP antagonists (Rp-cAMPS) were used, they were added during the preequilibration period before hormone addition. Immediately after treatment, aliquots of cell suspension were diluted 25-fold in ice-cold unlabeled medium [in mM: 140 NaCl, 10 tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.4, 1 LaCl$_3$, and 1 CaCl$_2$] and quickly centrifuged for 45 s at 1,500 g, and then the pellet was solubilized in 1 N NaOH-0.1% sodium dodecyl sulfate. Under these conditions, extracellularly bound $^{45}$Ca$^{2+}$ is completely removed. Aliquots were taken for measurement of radioactivity and for protein determination by the method of Lowry et al. (19) using bovine serum albumin as standard.

Measurement of cAMP levels Immediately after hormone treatment, aliquots of cell suspension were quickly transferred to ice-cold 6% tricloroacetic acid and centrifuged at 1,200 g for 15 min (4°C), and the supernatant was washed six times with four volumes of water-saturated diethyl ether so that the final pH value was 5.5–6.0. The extract was used for cAMP measurements by a radioimmunoassay technique using a commercially available kit (14). The sensitivity of the method was 0.025 pmol/ml, and the variation between assays was indirect determination in vitro by a binding protein assay kit (9) measuring the amount of cAMP present in a sample after a timed incubation, using enterocyte microsomal membranes as an experimental model. Microsomes were obtained by centrifugation at 100,000 g for 60 min (4°C) of the postmitochondrial supernatant (12,000 g) of cells homogenized in 50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N, N', N'-tetraacetic acid, 0.5 µM phenylmethylsulfonyl fluoride, 1 mM DTT, 20 µg/ml aprotin, and 20 µg/ml leupeptin. Membranes were incubated for 3 min at 30°C with vehicle (distilled water), rPTH-(1—34), or rPTH-(7—34) in the assay buffer (50 µM ATP, 10 mM MgCl$_2$, 10 mM creatine phosphate, 50 U/ml creatine phosphokinase, 100 µM 3-isobutyl-1-methylxanthine, 1 mM DTT, 1 mM Tris-HCl, pH 7.4). The reaction was stopped with HClO$_4$, neutralized with KHCO$_3$ (so that the final pH was 5.5–6.0), and the solution was quickly centrifuged at 10,600 g for 15 min (4°C). Aliquots of the supernatant were taken for cAMP measurements with the [PH]cAMP assay kit. The results obtained represent the means ± SD of four experiments performed separately.

Determination of adenylate cyclase activity. The enzyme activity was indirectly determined in vitro by a binding protein assay kit (9) measuring the amount of cAMP present in a sample after a timed incubation, using enterocyte microsomal membranes as an experimental model. Microsomes were obtained by centrifugation at 100,000 g for 60 min (4°C) of the postmitochondrial supernatant (12,000 g) of cells homogenized in 50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N, N', N'-tetraacetic acid, 0.5 µM phenylmethylsulfonyl fluoride, 1 mM DTT, 20 µg/ml aprotin, and 20 µg/ml leupeptin. Membranes were incubated for 3 min at 30°C with vehicle (distilled water), rPTH-(1—34), or rPTH-(7—34) in the assay buffer (50 µM ATP, 10 mM MgCl$_2$, 10 mM creatine phosphate, 50 U/ml creatine phosphokinase, 100 µM 3-isobutyl-1-methylxanthine, 1 mM DTT, 1 mM Tris-HCl, pH 7.4). The reaction was stopped with HClO$_4$, neutralized with KHCO$_3$ (so that the final pH was 5.5–6.0), and the solution was quickly centrifuged at 10,600 g for 15 min (4°C). Aliquots of the supernatant were taken for cAMP measurements with the [PH]cAMP assay kit. The results obtained represent the means ± SD of four experiments performed separately.

Statistical evaluation. Data are presented as means ± SD and derived from at least two independently valid assays giving statistically homogeneous results. The significance of the results was evaluated by Student's t-test, and $P < 0.050$ was considered significant. In addition, for multiple comparisons, Bonferroni test and analysis of variance were employed (25).

RESULTS

Although PTH is a single-chain 84-amino acid peptide in several species, the structural requirements necessary for full biological activity are satisfied by the NH$_2$-terminal 34-amino acid fragment, which was used in our study (10, 12).

Figure 1 shows the dose-response profile of the rapid effects of rPTH-(1—34) on rat enterocyte calcium uptake. Results are expressed as percent of control values after a 5-min treatment interval. The hormone significantly increased $^{45}$Ca$^{2+}$ influx at all doses tested (10$^{-12}$ to 10$^{-7}$ M). At concentrations of 7 × 10$^{-13}$ and 2 × 10$^{-13}$ M, the stimulatory action of PTH on Ca$^{2+}$ uptake was still evident, whereas at 10$^{-13}$ M the hormone was without effects (control: $4.42 ± 0.58$, 7 × 10$^{-13}$ M: $4.42 ± 0.53$, P < 0.025; 2 × 10$^{-13}$ M: 3.98 ± 0.30, P < 0.025; 10$^{-13}$ M: 3.51 ± 0.21, not significant; values given in nmol Ca$^{2+}$/mg protein, n = 8, for each of 2 independent experiments). The fall observed at 10$^{-9}$ M PTH was not statistically different from the responses induced by the other PTH doses tested ($P > 0.05$; the possibility that this decline is due to a type II, β, error was ruled out). A hormone concentration of 10$^{-8}$ M was chosen for the following experiments because it has been widely used for studies of PTH actions in vitro (2, 17).

The stimulation of calcium uptake was time dependent (Fig. 2). Thus, 1 min after its addition, 10$^{-8}$ M rPTH-(1—34) elicited a significant increase (53% above control values) in calcium uptake, whereas no significant changes were observed at shorter treatment intervals (30 and 45 s; data not given). This elevation reached a maximum of 96% at 5 min after exposure of the cells to the hormone and decreased up to 10 min to 31%.

Voltage-sensitive calcium channels are modulated by intracellular second-messenger signaling systems (5). First, the possibility that the activation of a dihydropyridine- and phenylalkylamine-sensitive pathway was
involved in the early stimulation induced by the hormone of rat duodenal calcium influx and, second, the potential involvement of the cAMP-dependent pathway were investigated. As shown in Table 1, nitrendipine (1 µM) and verapamil (10 µM) completely abolished the increment in calcium uptake produced by the addition of the hormone after 3 min of treatment. These calcium channel antagonist concentrations have been previously shown to block calcium influx in similar rat enterocyte preparations (20).

Changes in enterocyte cAMP content induced by the peptide hormone and the effects of known activators and inhibitors of this messenger system on rat duodenal Ca\(^{2+}\) uptake were studied. Incubation of the cell suspension with 10^(-8) M rPTH-(1–34) rapidly increased cAMP levels. As shown in Fig. 3, already after 1 min of hormone addition a significant elevation (+88%) in enterocyte cAMP content was observed. Maximum response (+167%) was elicited by 2 min of treatment with the peptide. Although less markedly, cAMP levels remained higher (+67%) than basal values after 3–5 min of incubation of enterocytes with rPTH-(1–34). Treatment with 10^(-12) M rPTH-(1–34) for 2 min also markedly increased cAMP production (1.20 ± 0.12 vs. 4.41 ± 0.66 pmol/mg protein for control and PTH-treated enterocytes, respectively; P < 0.005; n = 3 for each of 2 independent experiments). Preincubation of cells with 10 µM verapamil did not abolish the fast increase in cAMP induced by PTH in enterocytes after 2 min of treatment [1.20 ± 0.12 vs. 3.00 ± 0.24 vs. 1.26 ± 0.20 vs. 3.26 ± 0.33 pmol/mg protein for control, 10^(-8) M rPTH-(1–34), 10 µM verapamil, and 10^(-8) M rPTH-(1–34) + 10 µM verapamil, respectively]. Moreover, the elevation of cAMP caused by 10 µM forskolin was not blocked by 10 µM verapamil either (data not shown).

Furthermore, calcium uptake was also measured in the presence of forskolin, an adenylyl cyclase activator, and the cAMP analogs 5′-AMP and 5′-cAMP. Three minutes of treatment of rat duodenal cells with these agents, similar to rPTH-(1–34), resulted in a stimulation of the Ca\(^{2+}\) uptake (Table 2). Rp-cAMP is a specific PKA-competitive inhibitor that blocks first messenger-stimulated phosphorylation by cAMP-dependent protein kinase (8). The presence of the analog at a concentration of 200 µM completely suppressed the increment in calcium uptake induced by 5 min of treatment with the hormone (Fig. 4). The inhibitory effect at lower concentrations of the cAMP antagonist was less pronounced (data not shown). Similar to the hormone, 200 µM Rp-cAMP completely blocked the stimulatory action of 100 µM 5′-AMP on the Ca\(^{2+}\) influx whereas 100 µM Rp-cAMP only inhibits by 61% the effect of the cAMP analog (3.1 ± 0.5, 4.3 ± 0.5, and 3.8 ± 0.4 nmol Ca\(^{2+}\)/mg protein for control, 5′-AMP, and 5′-AMP + 100 µM Rp-cAMP, respectively). Previous studies have shown that the optimal Rp-

### Table 1. Effect of calcium channel blockers on the rapid increase in enterocyte calcium uptake induced by rPTH-(1–34) and DBCAMP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Calcium Uptake, nmol Ca(^{2+})/mg protein</th>
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<tbody>
<tr>
<td>Control</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>Control + verapamil</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>Control + nitrendipine</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>PTH</td>
<td>5.8 ± 0.66</td>
</tr>
<tr>
<td>PTH + verapamil</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>PTH + nitrendipine</td>
<td>3.7 ± 0.6</td>
</tr>
<tr>
<td>DBCAMP</td>
<td>5.1 ± 0.9</td>
</tr>
<tr>
<td>DBCAMP + nitrendipine</td>
<td>3.8 ± 0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7. PTH, parathyroid hormone. Rat enterocytes were treated for 3 min with 10^(-8) M rat PTH (rPTH-(1–34)) or 100 µM dibutyryl cAMP (DBcAMP) in the absence or presence of the calcium channel blockers verapamil (10 µM) or nitrendipine (1 µM). The channel antagonist were added to the incubation medium 10 min before hormone treatment, during the pre-equilibration period. 45Ca\(^{2+}\) uptake was measured as described in MATERIALS AND METHODS. *P < 0.001, †P < 0.005 with respect to each control value.
cAMP concentration to block cAMP-dependent processes varies between 50 and 500 µM according to the cell type employed (8). In addition, the stimulation of 45Ca²⁺ influx in enterocytes by DBcAMP could be blocked by 1 µM nitrendipine (Table 1).

Finally, we measured adenylate cyclase activity using isolated enterocyte microsomes as an experimental model. As can be seen in Fig. 5, treatment of the membranes with 10⁻⁸ M rPTH-(1—34) for 2 min significantly increased the enzyme activity (188% above the control value). However, the NH₂-terminal-shortened fragment of the hormone, PTH-(7—34), was not able to activate the enzyme.

**DISCUSSION**

Despite the fact that the classical target tissues for PTH action on calcium fluxes are bone and kidney, the results reported in the present study provide evidence of a rapid (1–10 min), direct action of the peptide hormone on intestinal calcium uptake of mammals in a wide concentration range (2⁻³⁻¹⁻¹³ to 10⁻⁷ M). The mode of action elicited by the hormone involves the participation of the adenylate cyclase messenger system and calcium channel activation.

Fast alterations in Ca²⁺ fluxes induced by PTH in hepatocytes (13), connecting tubules (18), bone cells (10), and exocytosis of lysosomal enzymes (21) or cell Ca²⁺ by forskolin in the HT-29 intestinal cell line (7) have been reported. More important, it has also been shown that PTH rapidly stimulates Ca²⁺ transport in perfused duodena from normal chicks (22).

The mechanism of action of PTH in the classical bone and kidney target cells involves the interaction with hormone-specific receptors on the plasma membrane, which results in the activation of the adenylate cyclase-cAMP-PKA and/or the phospholipase C-diacylglycerol-PKC pathways (6, 10, 12).

However, observations of hormone receptor interaction and activation of adenylate cyclase have been expanded greatly to other cellular systems (13, 24). In the present study, we demonstrate that direct treatment with PTH rapidly induced a marked cAMP elevation in rat duodenal cells and an elevation of adenylate cyclase activity in isolated microsomal membranes derived therefrom. Future studies should also investigate whether the phospholipase C-PKC messenger system also mediates PTH effects in this cell system. In addition, coupling of these signaling pathways to a PTH receptor should be also experimentally addressed.

The increase in cAMP elicited by the hormone paralleled the stimulation of Ca²⁺ influx. Several lines of evidence in our study support the hypothesis that the production of cAMP by PTH activates calcium channels via cAMP-dependent phosphorylation and/or by a direct action on cyclic nucleotide-gated Ca²⁺ channels (28). First, the adenylate cyclase activator forskolin increased calcium uptake in rat duodenal cells. Second, cAMP analogs, DBcAMP and Sp-cAMPS, like PTH stimulation, induced similar effects on ⁴⁵Ca²⁺ influx. Third, the competitive inhibitor of cAMP binding to the

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<tr>
<th>Treatment</th>
<th>Calcium Uptake, nmol Ca²⁺/mg protein</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>Forskolin (100 µM)</td>
<td>4.8 ± 0.8*</td>
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<tr>
<td>cAMP analogs</td>
<td></td>
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<tr>
<td>DBcAMP (50 µM)</td>
<td>3.7 ± 0.4†</td>
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<tr>
<td>DBcAMP (100 µM)</td>
<td>4.3 ± 0.5†</td>
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<tr>
<td>Sp-cAMPS (50 µM)</td>
<td>4.9 ± 0.8*</td>
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<tr>
<td>Sp-cAMPS (100 µM)</td>
<td>4.6 ± 0.2*</td>
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Values are means ± SE, n = 5. Isolated rat enterocytes were exposed for 3 min to forskolin, DBcAMP, Sp-cAMPS, or vehicle at the concentrations indicated. Calcium uptake was measured as described in MATERIALS AND METHODS. *P < 0.005, †P < 0.050, ‡P < 0.010 with respect to controls.
R subunit of PKA, Rp-cAMPS, completely blocked PTH increment of calcium uptake at a concentration similar to that required for inhibition of the effects of DBCAMP on this process. Finally, the calcium channel blockers, nitrendipine and verapamil, fully suppressed PTH stimulatory action. Nitrendipine also inhibits DBCAMP-induced calcium uptake. However, electrophysiological data is required to conclusively establish the participation of Ca\(^{2+}\) channels in PTH stimulation of duodenal Ca\(^{2+}\) influx. The possibility that the increase in Ca\(^{2+}\) uptake is responsible for the elevation of cell cAMP levels is not supported by the fact that verapamil did not abolish the changes in cAMP induced by rPTH-(1—34).

It is widely known that the principal regulator of intestinal calcium transport is the biologically active form of vitamin D, 1,25-dihydroxyvitamin D\(_3\) (4). With regard to the physiological significance of the results obtained in this study, it should be considered that, because it was carried out with nonpolarized cells, it is not possible to distinguish apical versus basolateral Ca\(^{2+}\) transport. However, there is evidence indicating that voltage-dependent L-type Ca\(^{2+}\) channels are located at the basolateral membranes of mammalian duodenal cells (15). In addition, according to the currently accepted mechanism of transcalcitria, e.g., rapid modulation of intestinal Ca\(^{2+}\) transport by vitamin D (3), Ca\(^{2+}\) influx through basolateral membrane triggers calcium transfer to the circulation by exocytosis.

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