Parathyroid Hormone Analogues Inhibit Calcium Mobilization in Cultured Vascular Cells

Toru Hino, Michael D. Nyby, Marianne Fittingoff, Michael L. Tuck, Arnold S. Brickman

Abstract Parathyroid hormone and parathyroid hormone–related protein lower blood pressure and relax contracted arteries. Parathyroid hormone also antagonizes angiotensin II–induced vasoconstriction. To determine the cellular mechanism or mechanisms by which parathyroid hormone analogues antagonize pressor effects, we examined the effect of these peptides on angiotensin II–induced calcium mobilization in fura 2-AM–loaded cultured rat vascular smooth muscle cells. Either 100 nmol/L parathyroid hormone or parathyroid hormone–related protein significantly reduced the amount of calcium mobilized by 100 nmol/L angiotensin II. The attenuating effect of these peptides was mimicked by 10 nmol/L forskolin and 10 mmol/L isobutylmethylxanthine and was not dependent on the presence of extracellular calcium. This effect of the parathyroid hormone analogues was reduced when cells were pretreated with 100 mmol/L 2',5'-dideoxyadenosine, an adenylate cyclase inhibitor. Combined inhibition of cyclic nucleotide–dependent protein kinases eliminated the inhibitory effect of parathyroid hormone, whereas protein kinase C inhibition had no effect. Parathyroid hormone analogues decreased the amount of calcium released by inositol 1,4,5-trisphosphate in digitonin-permeabilized vascular smooth muscle cells. This effect was inhibited by treatment with 2',5'-dideoxyadenosine. These results suggest that these peptides attenuate inositol 1,4,5-trisphosphate–sensitive calcium mobilized by angiotensin II via an adenylate cyclase–dependent mechanism. This may be a mechanism by which acute administration of parathyroid hormone or parathyroid hormone–related peptide antagonizes vasoconstriction. (Hypertension. 1994;23:402–408.)

Key Words • parathyroid hormones • adenosine monophosphate • adenyl cyclase • vasodilator agents • vasoconstrictor agents • signal transduction • inositol 1,4,5-trisphosphate • dideoxyadenosine

Numerous studies have consistently demonstrated that parathyroid hormone (PTH), as either intact hormone or the N-terminal 1-34 fragment of PTH [PTH(1-34)], relaxes contracted vessels and lowers blood pressure in a variety of animal species.1–3 PTH can also attenuate vasopressor responses to angiotensin II (Ang II) and norepinephrine when coinjected with these pressors in rats.6 Although initially identified as a factor that decreases intracellular release of vascular smooth muscle Ca2+,4 many vasoconstrictors, such as Ang II, also mobilize Ca2+ from intracellular stores such as the sarcoplasmic reticulum. Any factor that decreases intracellular release of vascular smooth muscle Ca2+ may also induce vasodilation as a result of this decrease.

Based on this knowledge, we have examined the pathways for intracellular signaling produced by PTH(1-34) and PTHrp(1-34) to test the hypothesis that these peptides attenuate agonist-induced increases of calcium mobilized from intracellular stores in VSMCs through an adenylate cyclase–dependent mechanism.
Methods

Cell Cultures

Primary VSMCs were prepared by enzymatic dispersion of thoracic aorta segments obtained from male Sprague-Dawley rats (250 to 300 g, Bantin and Kingman) using methods previously employed in this laboratory. All animal procedures were approved by the Sepulveda Veterans Affairs Medical Center Animal Studies Subcommittee. The harvested cells were grown in Dulbecco's modified Eagle medium (DMEM, Sigma Chemical Co.) supplemented with 10% fetal calf serum (Hyclone Laboratories), 50 U/mL penicillin, and 50 mg/mL streptomycin (Sigma). For these studies, cells were grown in six-well plates and seeded at a density that reached confluence in 2 to 4 days. VSMCs were authenticated by randomly screening cultures for smooth muscle actin expression using immunofluorescent staining techniques.

Measurement of VSMC [Ca2+]i

Cells were seeded onto 25-mm-diameter glass coverslips in six-well plates. After the cells reached confluence, the medium was replaced with serum-free medium for 16 to 20 hours. Then the medium was replaced with a HEPES-buffered physiological salt solution (HPSS) containing (mmol/L) HEPES 10, NaCl 145, KCl 5, MgSO4 1, sodium phosphate 0.5, glucose 6, and CaCl2 2 (pH 7.4 at 37°C). Cells were incubated with 4 mmol/L fura 2-AM for 40 minutes at 37°C and then incubated with fresh HPSS for 20 minutes at 37°C. The coverslips were rinsed with fresh HPSS and inserted into a thermoregulated stage incubator (37°C) filled with 1 mL HPSS and mounted on an epifluorescence microscope (model IMT-2, Olympus Optical Co). The microscope was integrated into a PTL DeltaScan-1 (Photon Technology International) that rapidly alternated excitation wavelength between 340 and 380 nm and detected emission at 510 nm. A confluent field of cells was located using a ×40 oil immersion fluorescence lens, and the emissions ratio (340/380) resulting from the alternating excitation wavelengths was recorded every 0.5 seconds using an AT-compatible computer. Each coverslip was calibrated after experimentation by first treating the cells with 1 mmol/L ionomycin (Calbiochem) and 4 mmol/L EGTA in calcium-free HPSS to determine the minimal fluorescence ratio. The maximal fluorescence ratio was obtained by subsequently treating the cells with 10 mmol/L CaCl2 and 1 mmol/L ionomycin. Auto fluorescence of each field of cells was determined by quenching all fura 2 dye with 4 mmol/L MnCl2. After autofluorescence was subtracted from all recorded measurements, [Ca2+]i was calculated using the equation of Grynkiewicz et al., with a Kd of 224 for fura 2.

Determination of Effects of Parathyroid Hormone-(1-34) and Other Substances on Vascular Smooth Muscle Cell [Ca2+]i

VSMCs were preincubated with either 100 nmol/L rat PTH(1-34); 100 nmol/L PTHRp(1-34); 100 nmol/L Nea-,Tyr3/PTH(3-34)amide [PTH(3-34)], or 10 mmol/L forskolin (Calbiochem); 10 mmol/L isobutylmethylxanthine (IBMX, Sigma); or 100 mmol/L acetic acid (vehicle for PTH peptides) for 5 minutes. After basal [Ca2+]i was recorded for 60 seconds in each coverslip, 100 nmol/L Ang II (Sigma) was added to the medium covering the coverslip, and the resultant changes in [Ca2+]i were recorded. Because the magnitude of the Ang II–induced increase of [Ca2+]i varied between cell lines, experiments were always conducted using cells from the same line as controls. Cell lines that exhibited a low [Ca2+]i response (<100 nmol/L increase) to Ang II under control conditions were discarded. To test the dependence of the modulating effect of PTH(1-34) on extracellular calcium, we incubated some coverslips with calcium-free HPSS containing 5 mmol/L EGTA and repeated the above procedure.

To determine whether the attenuating effect of PTH(1-34) on Ang II–induced calcium mobilization was dependent on a Ca2+- or cAMP-dependent protein kinase or a protein kinase C (PKC) pathway, we preincubated VSMCs for 15 minutes with 50 mmol/L N-[2-(methylamino)-ethyl]-5-isouquinoline-sulfonamide, di-HCl (H-8, Calbiochem), which inhibits cAMP- and cGMP-dependent protein kinases, or with 1 mmol/L staurosporine (Calbiochem), which inhibits PKC. Then the effect of PTH(1-34) on the Ang II–induced calcium mobilization was examined as described above.

To determine the effect of adenylate cyclase inhibition on the ability of PTH(1-34) to attenuate the Ang II–induced calcium signal, we preincubated coverslips with VSMCs for 20 minutes with 100 mmol/L 2',5'-dideoxyadenosine (DDA, Pharmacia), an adenylate cyclase inhibitor, before treatment with PTH(1-34) and subsequent stimulation with Ang II.

Measurement of cAMP

VSMC cAMP concentrations were measured after 5 minutes of stimulation with PTH(1-34) or PTHRp(1-34) as described by Nickols. Briefly, confluent cultures of VSMCs in six-well plates were deprived of serum overnight. Culture medium was replaced with HPSS for 60 minutes, and then cells were washed and 1 mL HPSS was placed in each well. DDA or vehicle (100 nmol/L) was added to each well. After preincubation for 20 minutes, 100 mmol/L PTH(1-34), PTHRp(1-34), PTH(3-34), 10 mmol/L forskolin, or vehicle was added to the respective wells. After 5 minutes of incubation, HPSS was aspirated from all cultures, and cAMP was extracted from VSMCs with 0.1 N HCl for 10 minutes. These acidic extracts were evaporated and stored at −20°C until assayed for cAMP using a commercially available radioimmunoassay kit (Amer sham). Protein was dissolved with 0.2 N NaOH and measured using a bicinchoninic acid spectrophotometric method (BCA, Pierce Chemical Co).

Measurement of Inositol 1,4,5-Trisphosphate–Releasable Calcium

The inositol 1,4,5-trisphosphate (IP3)-sensitive calcium pool was estimated using a modification of previously described methods. Briefly, confluent VSMCs in 12-well culture dishes were loaded with 1 μCi/mL “Ca (Du Pont–NEN) for 30 minutes at 37°C. Loading and all subsequent steps were done in the presence of 5 μg/mL ruthenium red (Eastman Kodak Co) to prevent calcium uptake by the mitochondria and

![Fig. 1. Traces show effect of parathyroid hormone–(1-34) [PTH(1-34)] on angiotensin II–induced [Ca2+]i mobilization. Fura 2-loaded vascular smooth muscle cells were treated with either vehicle, 100 mmol/L PTH(1-34), or 100 mmol/L PTH(3-34) for 5 minutes. Then 100 mmol/L angiotensin II was added to the cells, which resulted in an immediate rise in [Ca2+]i in the vehicle-treated cells. PTH(1-34) preincubation attenuated the response, whereas PTH(3-34) had no effect. Each tracing is of a single experiment. All indicates angiotensin II.](http://hyper.ahajournals.org/)
With Hanks' buffered salt solution (Sigma) and incubated with VSMCs with 100 mmol/L DDA for 20 minutes and then 34) significantly attenuated this response.

Statistical Methods

Results

Basal [Ca²⁺] in VSMCs incubated in 2 mmol/L CaCl₂ containing HPSS was 88.2±14.5 nmol/L and was unaffected by additions of PTH(1-34), PTHrp(1-34), or PTH(3-34). In vehicle-treated VSMCs, 100 nmol/L Ang II caused an immediate rise of [Ca²⁺] that was attenuated by PTH(1-34) but not by the inactive PTH analogue PTH(3-34) (Fig 1). PTH(1-34) decreased the peak response from 285.8±45.8 to 84.3±17.3 nmol/L (P<.001), whereas PTH(3-34) did not alter the peak response (Fig 2). In a separate set of experiments, PTHrp(1-34) reduced the peak Ang II–induced [Ca²⁺] response from 463.9±40.9 nmol/L (n=6) to 102.6±13.0 nmol/L (n=6, P<.001).

The attenuating effect of PTH(1-34) and PTHrp(1-34) was mimicked by IBMX, which raises cellular cAMP by inhibiting phosphodiesterase activity, and forskolin, which raises cellular cAMP by direct stimulation of adenylate cyclase (Table 1). The effect of PTH(1-34) on Ang II–induced [Ca²⁺], transients was reversed by treatment with H-8 but not by staurosporine (Table 1). H-8, which preferentially inhibits cAMP- and cGMP-dependent protein kinases, had no effect itself on the Ang II–induced [Ca²⁺], response (288.3±33.9 nmol/L, n=11, with vehicle; 312.1±84.0 nmol/L, n=6, with H-8), whereas it did abolish the attenuating effects of PTH(1-34) when added in combination with the PTH(1-34).

Pretreatment of VSMCs with the adenylate cyclase inhibitor DDA decreased the attenuating effects of both PTH(1-34) and PTHrp(1-34) (Figs 3 and 4). DDA itself had no effect on the Ang II–induced [Ca²⁺], peak (467±67 nmol/L, n=5, for control; 516±40 nmol/L, n=4, for DDA-treated). This DDA concentration inhibited PTH(1-34)– and PTHrp(1-34)–induced cAMP accumulation in VSMCs in parallel studies. Basal VSMC cAMP concentration was 9.67±0.39 pmol/mg protein. PTH(1-34) and PTHrp(1-34) significantly increased cAMP concentrations (Table 2). Pretreatment of VSMCs with DDA attenuated these increases of cAMP concentration to near basal levels, as shown in Table 2. The PTH(1-34) analogue, PTH(3-34), did not increase cAMP concentration significantly (10.70±0.31 pmol/mg protein, n=4), whereas forskolin increased to determine significant differences between groups. A probability value less than .05 was considered significant. All results are expressed as mean±SEM.
Fig 3. Bar graph shows effect of 2',5'-dideoxyadenosine (DDA) on parathyroid hormone-(1-34) [PTH(1-34)] attenuation of angiotensin II-induced [Ca^{2+}] increases in vascular smooth muscle cells (VSMCs). Fura 2-loaded VSMCs were preincubated with either vehicle or 100 mmol/L DDA for 15 minutes. Then VSMCs were incubated with either vehicle or 100 nmol/L PTH(1-34) for 5 minutes. Angiotensin II (100 nmol/L) was added to each coverslip and peak [Ca^{2+}] determined. Each bar represents mean±SEM of peak [Ca^{2+}] increase in each group; number of experiments is shown in parentheses. PTH(1-34) significantly attenuated angiotensin II-induced [Ca^{2+}] response compared with vehicle-treated VSMCs. Pretreatment with DDA partially reversed this effect of PTH(1-34).

cAMP concentration to 16.51±0.54 pmol/mg protein (n=6, P<.01).

PTH(1-34) caused a reduction of Ang II-induced calcium responses in calcium-free conditions as well as when extracellular calcium was 2 mmol/L. The Ang II-induced peak [Ca^{2+}] responses were decreased from 830.5±87.2 (n=10) to 357.9±48.2 nmol/L (n=8, P<.01) by PTH(1-34) when external calcium concentration was 2 mmol/L and decreased from 497.1±81.3 (n=6) to 112.8±18.7 nmol/L (n=8, P<.01) in the presence of 5 mmol/L EGTA.

IP_{3}-releasable calcium, as measured by ^{45}Ca efflux, was decreased by both PTH(1-34) and PTHrp(1-34). PTH(1-34) reduced the percentage of IP_{3}-releasable ^{45}Ca from 13.9±2.1% in vehicle-treated cells to 3.0±1.9% (P<.002), whereas PTHrp(1-34) reduced the amount of IP_{3}-releasable ^{45}Ca to 1.4±0.9% (P<.001) (Fig 5). Forskolin, a direct activator of adenylate cyclase, yielded similar results in that IP_{3}-releasable ^{45}Ca was decreased from 12.5±1.5% to 1.3±0.2% (n=4). In a separate set of experiments, the ability of PTH(1-34) to attenuate IP_{3}-releasable calcium was inhibited by DDA, the adenylate cyclase inhibitor. PTH(1-34) significantly decreased the amount of IP_{3}-releasable calcium (Table 3). DDA had no effect on ^{45}Ca release itself but prevented the effect of PTH(1-34).

Discussion

This study was designed to investigate the intracellular mechanism or mechanisms by which PTH(1-34) and

![Table 2. Effect of 100 μmol/L 2',5'-Dideoxyadenosine on PTH(1-34)- and PTHrp(1-34)-induced Cyclic AMP Accumulation in Vascular Smooth Muscle Cells](image)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Vehicle-Treated</th>
<th>DDA-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>9.67±0.39 (15)</td>
<td>9.77±1.06 (4)</td>
</tr>
<tr>
<td>PTH(1-34) (100 nmol/L)</td>
<td>12.56±0.54* (15)</td>
<td>10.87±0.41† (16)</td>
</tr>
<tr>
<td>PTHrp(1-34) (100 nmol/L)</td>
<td>12.79±0.46* (6)</td>
<td>10.12±0.54† (7)</td>
</tr>
</tbody>
</table>

PTH(1-34) indicates rat parathyroid hormone-(1-34); PTHrp(1-34), parathyroid hormone-related protein-(1-34); and DDA, 2',5'-dideoxyadenosine. Values are in picomoles per milligram of protein; n is shown in parentheses.

*P<.05 compared with basal.
†P<.05 compared with vehicle-treated.

![Fig 5. Bar graph shows effect of parathyroid hormone (PTH) and PTH-related protein (PTHrp) on Inositol 1,4,5-trisphosphate (IP_{3})-releasable calcium in vascular smooth muscle cells (VSMCs). ^{45}Ca-loaded VSMCs were incubated with either vehicle, 100 nmol/L PTH(1-34), or 100 nmol/L PTHrp(1-34) for 5 minutes. Then this treatment buffer was removed, and VSMCs were incubated with 35 mmol/L digltonin and either vehicle or 10 nmol/L IP_{3} for 5 minutes. Resulting amounts of ^{45}Ca were counted, and the percentage of IP_{3}-releasable calcium was determined as described in "Methods." Each bar represents mean±SEM of the percentage of ^{45}Ca released by IP_{3} in each experimental group. Both PTH(1-34) and PTHrp(1-34) significantly decreased the amount of ^{45}Ca released by IP_{3} in digltonin-permeabilized VSMCs compared with vehicle-treated VSMCs.](image)
However, these results must be interpreted cautiously, as the possibility that PTH(1-34)-activation of PKC may be crucial by other investigators or in our studies. The results using DDA to inhibit adenylate cyclase provide additional support for the involvement of a cAMP-dependent mechanism in the PTH(1-34)- and PTHrp(1-34)-produced attenuation of the action of Ang II. The DDA concentration that inhibited cAMP production in VSMCs in response to PTH(1-34) and PTHrp(1-34) also partially reversed the effects of PTH(1-34) and PTHrp(1-34) on Ang II-induced calcium mobilization. The incomplete reversal of the attenuating effects of PTH and PTHrp on the Ang II-induced [Ca^{2+}] signal by DDA suggests that another mechanism not involving adenylate cyclase is partially responsible for this effect of these peptides in VSMCs. PTH(1-34) can inhibit L-type channels in cultured VSMCs, suggesting another mechanism by which this peptide decreases [Ca^{2+}]. However, it has been shown that cAMP can inhibit calcium influx in intact arteries, and it is thought that blockade of L-type channels by PTH(1-34) is also cAMP dependent. Therefore, it appears that an as yet unknown cAMP-independent mechanism accounts for some of the attenuating effects of PTH(1-34) and PTHrp(1-34) on the Ang II-induced [Ca^{2+}], increase in the present studies.

Because PTH(1-34) attenuated Ang II-induced changes of [Ca^{2+}] under extracellular calcium-free conditions, PTH(1-34) must have inhibited the mobilization or release of Ca^{2+} from intracellular stores, presumably the sarcoplasmic reticulum. It has been demonstrated that Ang II releases Ca^{2+} from intracellular stores via PLC activation, with IP_3 inducing release of Ca^{2+} from intracellular stores. As PTH(1-34) and PTHrp(1-34) significantly reduced the amount of 45Ca released by IP_3 in permeabilized VSMCs, one mechanism whereby PTH(1-34) and PTHrp(1-34) attenuate Ang II-induced increases of [Ca^{2+}] appears to be through regulation of IP_3-sensitive calcium stores. Furthermore, because this effect of PTH(1-34) and PTHrp(1-34) was completely abolished by treatment of VSMCs with DDA, this mechanism is apparently dependent on adenylate cyclase. cAMP may attenuate release of IP_3-sensitive calcium stores through the phosphorylation of IP_3 receptors by a cAMP-dependent protein kinase, as demonstrated in platelet membranes, or by other proposed mechanisms of cAMP action on intracellular calcium, such as increased sequestration of calcium into the sarcoplasmic reticulum by calcium ATPase. Alternatively, cAMP could also stimulate a cGMP-dependent kinase, as has been shown to occur in cultured rat VSMCs, which could also decrease the Ang II-induced [Ca^{2+}] transient. Whichever cyclic nucleotide-dependent protein kinase is involved, the present studies suggest a sequence of events whereby PTH(1-34) and PTHrp(1-34) stimulate adenylate cyclase in VSMCs, resulting in increased intracellular levels of cAMP that then inhibit the release of calcium from IP_3-sensitive stores. Our results do not exclude the possibility that PTH(1-34) or PTHrp(1-34)-generated cAMP may have other effects on the PLC-IP_3 axis, such as reduction of IP_3 production, as demonstrated by Wang et al.19
Our studies provide evidence for an additional intracellular mechanism by which PTH(1-34) and PTHrp(1-34) may attenuate Ang II-induced vasoconstriction. Increases of vascular smooth muscle [Ca$^{2+}$], are associated with contraction; in contrast, decreases are associated with relaxation. Increase of [Ca$^{2+}$] is thought to be the primary signal by which many vasoconstrictive agents, such as Ang II, contract vascular segments. Therefore, our results suggest that PTH(1-34) and PTHrp(1-34) relax contracted vessels in part by decreasing the amount of Ca$^{2+}$ mobilized by vasoconstrictive agonists such as Ang II. This action of PTH(1-34) and PTHrp(1-34) is independent of adenylate cyclase, and the resultant increase of cAMP inhibits the amount of calcium released by IP3, from intracellular stores. If these same mechanisms are operative in vivo, they may represent a cellular mechanism, in addition to blockade of L-type channels and reduction of IP3 production, by which acute administration of PTH(1-34) and PTHrp(1-34) dilates blood vessels and lowers blood pressure.

Finally, Ang II recently has been shown to induce expression of PTHrp mRNA in VSMCs, and it has been postulated that PTHrp may work as a local autocrine factor providing negative feedback to Ang II-induced VSMC growth and contraction. Our studies suggest that putative negative feedback by PTHrp on Ang II-induced contraction occurs through an adenylate cyclase-dependent mechanism. Although the antagonistic action of PTHrp on VSMC growth appears to reside in the more distal portions of the peptide beyond the 34th amino acid, we found that the ability of PTHrp (and PTH) to antagonize VSMC calcium mobilization was associated with the proximal 34-amino acid region of the peptide. PTH and PTHrp use the same receptor; a separate receptor or receptors for PTHrp have not been demonstrated. Because the presence of PTHrp has been demonstrated in vascular smooth muscle, it can be speculated that under physiological conditions, PTHrp but not PTH plays a role in the regulation of vascular smooth muscle tone and blood pressure in vivo. Although the physiological significance of the present in vitro observations is not yet established, it is suggested that under certain conditions PTHrp and/or PTH may be factors involved in the regulation of vascular reactivity and blood pressure.

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