Changes in free cytosolic calcium were measured in UMR-106 cells in response to parathyroid hormone (PTH) stimulation. Bovine PTH-(1-34) induced an increase in [Ca\(^{2+}\)], with the contour of the rise in [Ca\(^{2+}\)], occurring in three successive phases: 1) a rapid increase in [Ca\(^{2+}\)], occurring within seconds, 2) rapid decrement in [Ca\(^{2+}\)], to near-resting levels within 1 min, and 3) slow increment in [Ca\(^{2+}\)]. Phase one and phase three increases in [Ca\(^{2+}\)] were dependent on medium calcium. The phase one rise in [Ca\(^{2+}\)], was inhabitable by the calcium channel blockers lanthanum and verapamil. Only the phase one rise in [Ca\(^{2+}\)], was blocked by preincubation of the cells with the phorbol ester, phorbol 12-myristate 13-acetate. This channel was also blocked when cellular Ca\(^{2+}\) levels were increased prior to PTH stimulation. The phase two decrement of [Ca\(^{2+}\)], was due to the rapid inactivation of the phase one calcium channel. The phase three rise in [Ca\(^{2+}\)], was mediated by cellular Ca\(^{2+}\) levels. This Ca\(^{2+}\)-dependent Ca\(^{2+}\) channel was insensitive to pretreatment of the cells with phorbol diesters and showed low sensitivity to Ca\(^{2+}\) channel blockers. It is concluded that UMR-106 cells respond to PTH stimulation by the activation of a Ca\(^{2+}\)-independent Ca\(^{2+}\) channel. This channel rapidly inactivates. The subsequent PTH-dependent increase in cellular Ca\(^{2+}\) is followed by activation of a Ca\(^{2+}\)-dependent Ca\(^{2+}\) channel resulting in a slow rise in [Ca\(^{2+}\)].

Parathyroid hormone's (PTH) main, if not only, function is to maintain the calcium ion concentration of the body.

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The abbreviations used are: PTH, parathyroid hormone; bPTH-(1-34), bovine parathyroid hormone-(1-34); [Tyr\(^{9}\)]bPTH-(7-34) amide, [tyr\(^{9}\)]bovine parathyroid hormone-(7-34) amide; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; PMA, phorbol 12-myristate 13-acetate; PDA, phorbol 12,13-diacetate; Bt\(^{4}\)cAMP, dibutyryl cyclic AMP; [Ca\(^{2+}\)], free cytosolic calcium concentration; IBMX, 3-isobutyl-1-methylxanthine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

fluids. All its varied effects seem directed toward this end. With respect to bone, PTH stimulates bone resorption (1, 2), suppresses collagen synthesis (2, 3), inhibits citrate decarboxylase activity (4), and in higher doses inhibits proliferation of osteoblast-like cells but in lower doses may stimulate proliferation of these cells (5).

All these actions of PTH in bone are associated with a change in cell cAMP (3). Previous studies provided indirect evidence which suggests that PTH effects may be mediated by a change in cytosolic Ca\(^{2+}\) (6, 7). The ability to accurately measure changes in free cytosolic calcium concentration ([Ca\(^{2+}\)]) using fluorescent probes in osteoblast-like cells (8-10) can provide direct evidence for the involvement of changes in [Ca\(^{2+}\)], in the PTH effects. In the osteoblast-like cell line, UMR-106, Lowik et al. (9) showed that PTH caused a dose-related increase in [Ca\(^{2+}\)]. However, in the osteoblast-like rat osteosarcoma cell lines ROS 25/1 and ROS 17/2.8, the human osteoblast-like osteosarcoma cell line G-292, and in primary cultures of neonatal mouse calvaria, PTH did not change [Ca\(^{2+}\)]. (10). Only in SaOS-2 cells, a human osteoblast-like osteosarcoma cell line, did PTH cause a 10-15% increase of [Ca\(^{2+}\)], over basal levels (10).

The osteoblast has receptors for PTH (11, 12) and appears to be the only bone cell type directly responsive to PTH (1). We examined the effects of PTH on [Ca\(^{2+}\)], in UMR-106 cells since this cell line possesses many osteoblastic characteristics including cAMP response to PTH but not to calcitonin at early passages (13, 14), high alkaline phosphatase activity (14), exclusive synthesis of type 1 (bone-specific) collagen, insulin sensitivity (15), and the ability to form bone in vivo (16). The mechanism by which PTH changes [Ca\(^{2+}\)], in UMR-106 cells has not been fully elucidated. In this paper, we describe PTH activation of different types of calcium channels in UMR-106 cells and characterize each channel.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine PTH-(1-34) (6800 units/mg) was obtained from Beckman Instruments (Palo Alto, CA) and the PTH antagonist [Tyr\(^{9}\)]bPTH-(7-34) amide; [tyr\(^{9}\)]bovine parathyroid hormone-(7-34) amide; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; PMA, phorbol 12-myristate 13-acetate; PDA, phorbol 12,13-diacetate; Bt\(^{4}\)cAMP, dibutyryl cyclic AMP; [Ca\(^{2+}\)], free cytosolic calcium concentration; IBMX, 3-isobutyl-1-methylxanthine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

**Culture Conditions**—UMR-106 cells were cultured between passages 7-9. Cells were seeded at a density of 0.5-1.0 × 10\(^5\) cells/cm\(^2\) area flasks and grown at 37 °C in a humidified 95% air, 5% CO\(_2\) atmosphere in Ham's F12/Dulbecco's modified Eagle's medium (1:1) supple-
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mestet with 14.3 mM NaHCO3, 1.2 mM L-glutamine, 10% fetal bovine serum, 0.1 mg/ml streptomycin, and 100 units/ml penicillin. The cells reached confluence within 7–9 days and were used on day 8 or 9 of growth.

**Determination of [Ca2+]i**—Measurements of free cytosolic calcium concentration were made by incorporating a calcium-sensitive, fluorescent probe, Fura2, into UMR-106 cells which had been released from tissue culture flasks by rapid treatment with trypsin (0.05%) and EDTA (0.02%) in Dulbecco’s phosphate-buffered saline (PBS). Trypsin-EDTA treatment took place for approximately 3 min at 37 °C followed by a rapid 25-fold dilution with medium A (composition in mM: 140 NaCl, 5 KCl, 1 MgCl2, 1.5 CaCl2, 10 HEPES/Tris, pH = 7.4; 5 glucose; bovine serum albumin, Fraction V (0.1%)) and washing twice by centrifugation at 600 × g for 3 min at 25 °C. Longer trypsinization times resulted in greater propensity for the leakage of Fura2 out of cells and loss of PTH responses. Cells were resuspended in medium A and incubated with 2 μM of Fura2/AM in a shaking water bath at 37 °C for 30 min. After completion of Fura2 loading, the cells were washed in medium A and resuspended in this medium. Approximately 1 million cells/ml were used in each set of experiments. Fluorescence was measured in a Perkin-Elmer 650-40 spectrophotometer at excitation and emission wavelengths of 340 and 360 nm, respectively, with slits of 3 and 12 nm, respectively.

Calibration of the Fura2 signal was performed by methods similar to those described for the calibration of another fluorescent intracellular Ca2+ probe, Quin2 (17). Briefly, the cells were lysed with digitonin (25 μg/ml) to obtain the maximum fluorescence. Next, 10 mM EGTA and sufficient NaOH to elevate the pH to 8.5 were added to obtain the minimum fluorescence. The dissociation constant for Ca2+-Fura2 was assumed to be 220 nM (18). Calculation of [Ca2+]i was determined as described by Tsien et al. (17).

To ensure that PTH or other additives to the fluorometer cuvettes did not give nonspecific fluorescence or interact with the dye itself to alter fluorescence independent of changes in [Ca2+]i, the tetrapotassium salt of Fura2 in medium A, in the absence of cells or in the presence of unloaded cells, was mixed with concentrations of the various reagents used in these experiments and the fluorescence determined. None of the additives in the concentrations used in these experiments had any significant effect on dye fluorescence or cell autofluorescence.

During the course of this study, it was found that the calcium signal obtained with PTH was dependent on the presence of 0.1% bovine serum albumin in the medium. As shown in Fig. 1A, the change in fluorescence and therefore [Ca2+]i, was more pronounced than that observed in the absence of bovine serum albumin (Fig. 1B). This observation was due to the ability of bovine serum albumin to prevent the adherence of PTH to the fluorometer cuvettes.

A previous study suggested that in an in vitro system extracellular phosphate may be important in facilitating the amount of calcium taken up across the plasma membrane (19). In some of our experiments we used lanthanum (La3+) as a calcium channel blocker. Phosphate in the media binds to La3+ and thus decreases the effect of La3+ to act as a calcium channel antagonist. For these two reasons, we tested to see if the PTH-induced rise in [Ca2+]i was similar in the presence or absence of phosphate in the media. As shown in Fig. 1, A and C, at least in these acute measurements of [Ca2+]i, the presence or absence of 1 mM phosphate in the media did not significantly change the PTH-stimulated increase in [Ca2+]i. Unless otherwise stated, all the experiments reported here are one of at least 10 or more similar observations.

**Determination of Cellular cAMP**—Determination of cellular cAMP was done in culture plates containing 12 multiwells/plate. Incubation times with hormone or vehicle were for approximately 3 min at 37 °C. At the end of the experiment the cells were lysed by the addition of 50 μg of digitonin to determine maximum fluorescence (Fmax) followed by EGTA addition and enough NaOH to raise the pH to greater than 8.5 to obtain minimum fluorescence (Fmin). Results are expressed as picomoles of cAMP/well.

**RESULTS**

**Effect of PTH on [Ca2+]i**—UMR-106 cells between passages 7–9 were used in the experiments. The results were independent of passage so that effects of PTH could be compared in experiments done with cells from these different passages. Fig. 2 shows the effect of PTH on [Ca2+]i in UMR-106 cells in the presence or absence of ambient Ca2+. In the presence of 1.5 mM CaCl2, the PTH-induced change in [Ca2+]i has three
components. Initially, [Ca\(^{2+}\)], increased rapidly from a resting level of 138 nM (147 ± 19 nM (n = 38) to 258 nM (267 ± 17 nM (n = 30)) within 15–30 s. In the second phase of 30–45 s, [Ca\(^{2+}\)], decreased towards near-resting levels. However, in many experiments (e.g. Fig. 2) [Ca\(^{2+}\)], was reduced to below resting levels. In the third phase [Ca\(^{2+}\)], increased at a much slower rate compared to the increase in [Ca\(^{2+}\)], observed in the first phase. Fig. 2 shows that within 10 min of incubation with PTH, [Ca\(^{2+}\)], rises to 384 nM. In three separate experiments, even after 40 min of incubation with PTH, [Ca\(^{2+}\)], continued to increase, although at a lesser rate than that observed between 1–10 min. The precise effect of an incubation period longer than 10 min of the cells with PTH on [Ca\(^{2+}\)], was precluded due to a significant dye leak at 37 °C. During the first 10 min of incubation at 37 °C, approximately 3% of the dye leaked from the cells. This results in a 20% overestimation of the actual increase in the [Ca\(^{2+}\)]. The data shown in this and subsequent figures were not corrected for dye leak.

That the apparent increase in [Ca\(^{2+}\)], observed in phase three was not due to dye leak, measurements of [Ca\(^{2+}\)], were made in cells washed immediately prior to [Ca\(^{2+}\)], measurement. At designated intervals up to 40 min, samples of control or PTH-treated cells were washed and then [Ca\(^{2+}\)], was measured immediately. In all experiments [Ca\(^{2+}\)], in the PTH-treated cells was reduced to below control by 256 ± 37% (n = 3). Fig. 3 shows the dose-response curve for PTH and the effect of the PTH antagonist, [Tyr\(^{39}\)]bPTH-(7-34) amide, on the initial increase in [Ca\(^{2+}\)]. The dose of PTH that gave half of the maximal increase in [Ca\(^{2+}\)], was approximately 0.3 units/ml which corresponds to approximately 10 nM. The PTH antagonist inhibited the PTH-stimulated increase in [Ca\(^{2+}\)], in a dose-dependent manner. 10 \(^{-9}\) M [Tyr\(^{39}\)]bPTH-(7-34) amide which, by itself, had no effect on [Ca\(^{2+}\)], inhibited 74 ± 5% (n = 3) of the initial increase in [Ca\(^{2+}\)], induced by 1 units/ml of bPTH-(1-34). Although not studied in as much detail, the late rise in [Ca\(^{2+}\)], mediated by PTH was also inhibited by the PTH antagonist.

We then proceeded to study the Ca\(^{2+}\) signal in each period separately. To identify the source of Ca\(^{2+}\) contributing to the observed increase in [Ca\(^{2+}\)], we first tested the effect of Ca\(^{2+}\) removal from the medium. When cells were added to Ca\(^{2+}\)-free medium containing 0.2 mM EGTA, (Fig. 2B), PTH stimulation caused only a rapid, very small increase in [Ca\(^{2+}\)]. This experiment indicates that most of the PTH-dependent increase in [Ca\(^{2+}\)], is due to Ca\(^{2+}\) entry from the medium into the cytosol.

Since Ca\(^{2+}\) entry down its electrochemical gradient is likely through a Ca\(^{2+}\) channel, we tested the effect of La\(^{3+}\), an inorganic Ca\(^{2+}\) channel blocker and verapamil, an organic Ca\(^{2+}\) blocker, on the initial PTH-dependent increase in [Ca\(^{2+}\)], (Fig. 4). 0.8 \(\mu\)M La\(^{3+}\) and 1.5 \(\mu\)M verapamil inhibited the increase in [Ca\(^{2+}\)], by 50%. Nicardipine was more potent than verapamil (see below), but diltiazem was about five times less potent than verapamil in blocking the PTH-mediated increase in [Ca\(^{2+}\)]. As can be seen in Fig. 4, the [Ca\(^{2+}\)], channel blockers did not completely inhibit the PTH-stimulated increase in [Ca\(^{2+}\)]. This Ca\(^{2+}\) channel blocker-insensitive rise in [Ca\(^{2+}\)], is probably due to some PTH-mediated Ca\(^{2+}\) release from the intracellular stores (see also Fig. 2B).

The reduction in [Ca\(^{2+}\)], to or below resting levels during phase two can be a consequence of rapid inactivation of the Ca\(^{2+}\) channel, an increase in Ca\(^{2+}\) extrusion rate from the cell, sequestration of Ca\(^{2+}\) by intracellular organelles, or a combination of the above. To test whether the Ca\(^{2+}\) channel was rapidly inactivated, we measured the effect of Ca\(^{2+}\) addition to the medium before or after PTH stimulation (Fig. 5). When cells suspended in medium containing 1.5 mM CaCl\(_2\) were stimulated with PTH, [Ca\(^{2+}\)], increased to 278 nM (Fig. 5A). PTH stimulation of cells suspended in medium containing only EGTA but no added Ca\(^{2+}\) caused only a small increase in [Ca\(^{2+}\)]. Addition of 1.7 mM CaCl\(_2\) (1.5 mM free Ca\(^{2+}\)) to the medium 1.5 min after PTH addition increased [Ca\(^{2+}\)], from 131 to only 164 nM (Fig. 5B) which was similar to the increase measured after addition of 1.7 mM CaCl\(_2\) to unstimulated cells (Fig. 5C). Moreover, an increase in medium Ca\(^{2+}\) concentration from 1.5 to 5 mM before or after PTH stimulation had the same effect on [Ca\(^{2+}\)]. (Fig. 5, D and E). If the PTH-activated Ca\(^{2+}\) channel remained open 1 min after PTH addition, then a significant increase in [Ca\(^{2+}\)], should have

![Graph A](image1.png)  ![Graph B](image2.png)

**Fig. 3. PTH dose response and effect of [Tyr\(^{39}\)]bPTH-(7-34) amide.** A, UMR-106 cells in medium A were stimulated with different concentrations of PTH and [Ca\(^{2+}\)], was measured as in Fig. 1. In the three separate experiments shown, the increase in [Ca\(^{2+}\)], over the resting level caused by 2.5 units/ml PTH was taken as control response (100%) and the measured increase in [Ca\(^{2+}\)], by different concentrations of PTH was calculated as percent of that control. B, the cells were also exposed to the indicated concentrations of [Tyr\(^{39}\)]bPTH-(7-34) amide for 1 min before the stimulation with 1 unit/ml of PTH. In this experiment the response to 1 unit/ml was taken as the control response (100%). The data are expressed as mean ± S.E. from three separate experiments.
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**FIG. 4.** Effect of verapamil and La^{3+} on PTH-dependent increase in [Ca^{2+}]. UMR-106 cells loaded with Fura2 and suspended in medium A were incubated with the indicated concentrations of verapamil or La^{3+} for 1 min before stimulation with 2.5 units/ml PTH. The effect of PTH on [Ca^{2+}] in the absence or presence of the blockers was measured during the first 2 min of incubation. The PTH-mediated increase in [Ca^{2+}] above basal level was measured at each blocker concentration and calculated as percent of that induced by PTH in the absence of blockers. The figure shows the inhibition of PTH-dependent increase in [Ca^{2+}], during the first phase of three separate experiments with the calculated S.E.

**FIG. 5.** PTH-dependent increase in [Ca^{2+}], effect of added Ca^{2+}. Fura2-loaded UMR-106 cells were suspended in medium A containing 1.5 mM CaCl_2 (A, D, and E) or 0.2 mM EGTA (B and C). After 30-60 s of incubation in the appropriate medium, the cells were stimulated with 2 units/ml PTH and [Ca^{2+}] was measured. In B and C after 1.5 min of incubation with or without PTH, respectively, 1.7 mM CaCl_2 (1.5 mM free Ca^{2+}) was added to the medium. In D and E medium CaCl_2 concentration was elevated to 5 mM before or after stimulation with PTH.

been observed upon addition of 1.7 or 5 mM Ca^{2+}. This was not seen (Fig. 5, B and E).

Further evidence for rapid inactivation of the PTH-dependent Ca^{2+} channel is given in Fig. 6. Here we compared the effect of Ca^{2+} channel blockers and the PTH antagonist when added before and after PTH. In case the channel remains active 1.5 min after PTH stimulation and the reduction in [Ca^{2+}], is only due to activation of Ca^{2+} efflux, addition of the blockers after PTH should reduce [Ca^{2+}], more dramati-

**FIG. 6.** Effect of antagonists on PTH-mediated increase in [Ca^{2+}], when added before or after PTH. Fura2-loaded cells in medium A were exposed to 10^-8 M [Tyr^{38}]bPTH-(7-34) amide, 0.1 mM verapamil, or 50 μM LaCl_3 before or after stimulation with 1 unit/ml PTH. The calculated resting [Ca^{2+}] in the experiments shown was 127 ± 6 nM (n = 4). PTH increased [Ca^{2+}], to 241 ± 14 nM (n = 4).

Fig. 7 suggests a possible mechanism for channel inactivation. Fig. 7 shows the effect of phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-diacetate (PDA), and 4α-phorbol on...
the PTH-dependent increase in \([\text{Ca}^{2+}]\). Incubation of the cells for 6 min with 0.1 nM PMA almost completely blocked the PTH-dependent increase in \([\text{Ca}^{2+}]\). Five min of preincubation with 0.1 \(\mu\)M PDA had the same effect, whereas 10 \(\mu\)M of 4-phorbol had no effect on the PTH-dependent increase in \([\text{Ca}^{2+}]\). The potency of PMA and PDA varied somewhat between experiments, but a 5-min preincubation with 0.5 nM PMA or 0.5 \(\mu\)M PDA always completely inhibited the PTH-stimulated increase in \([\text{Ca}^{2+}]\). The effect of PMA and PDA was time- and concentration-dependent. With 10 nM PMA, maximum inhibition was achieved within 45 s (not shown), whereas with 0.1 nM PMA, 6 min of incubation was required for maximum inhibition (Fig. 7, B and C).

The PTH-stimulated increase in \([\text{Ca}^{2+}]\), in phase three was slower than that observed in phase one. However, similar to phase one, the increase in \([\text{Ca}^{2+}]\) was dependent on extracellular \(\text{Ca}^{2+}\). In order to study the nature of the phase three \(\text{Ca}^{2+}\) pathway, we investigated the ability of PTH to stimulate cAMP production in trypsinized cells and the possibility that cAMP may stimulate \(\text{Ca}^{2+}\) influx. In four separate experiments, 1 unit/ml PTH increased cAMP levels in the trypsinized cells by about 25-fold (not shown), which is similar to that found with PTH stimulation of cells attached to culture plates (Table I). Cyclic AMP accumulates to a maximum in osteoblasts and osteoblast-like cells within 3-5 min after PTH stimulation (22-23); the time frame of cAMP production and that of the rise in \([\text{Ca}^{2+}]\) was similar, suggesting a relationship between intracellular cAMP accumulation and the rise in \([\text{Ca}^{2+}]\).

Fig. 8 shows that addition of 1 mM Bt\(\text{cAMP}\) to cells suspended in medium 1.5 mM \(\text{CaCl}_2\) was followed by a slow increase in \([\text{Ca}^{2+}]\). The effect of Bt\(\text{cAMP}\) has a lag period of 15-45 s, and like the phase three effect of PTH, it was not completed after 10 min of incubation at 37 \(^\circ\) C. Removal of \(\text{Ca}^{2+}\) from the medium completely prevented the Bt\(\text{cAMP}\)-dependent increase in \([\text{Ca}^{2+}]\), similar to that noted for the PTH-stimulated phase three increase in \([\text{Ca}^{2+}]\). Fig. 9 shows the effect of different concentrations of PTH on \([\text{Ca}^{2+}]\) and cellular cAMP levels in UMR-106 cells. The initial and late increase in \([\text{Ca}^{2+}]\) and cAMP levels had a similar dose-response pattern. The measured ED\(50\) for PTH-mediated initial \([\text{Ca}^{2+}]\), increase, late \([\text{Ca}^{2+}]\), increase, and cAMP production were 0.30 ± 0.04 \((n = 3)\), 0.25 ± 0.03 \((n = 3)\), and 0.21 ± 0.17 unit/ml \((n = 4)\), respectively. This suggests that the late PTH-dependent increase in \([\text{Ca}^{2+}]\), is related to the cellular level of cAMP.

### Table I

<table>
<thead>
<tr>
<th>Condition</th>
<th>cAMP pmoles/well</th>
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<tr>
<td>1.5 mM (\text{CaCl}_2)</td>
<td>0.10 ± 0.08 ((n = 4))</td>
</tr>
<tr>
<td>0.2 mM EGTA</td>
<td>0.17 ± 0.14 ((n = 5))</td>
</tr>
<tr>
<td>2.5 units/ml PTH</td>
<td>1.95 ± 0.54 ((n = 6))</td>
</tr>
<tr>
<td>2 mM PMA</td>
<td>2.48 ± 1.07 ((n = 6))</td>
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<tr>
<td>PMA + PTH</td>
<td>NT</td>
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</table>

Although cAMP appears to mediate an increase in \([\text{Ca}^{2+}]\), during phase three, pretreatment of cells with cAMP appears to inhibit phase one increase in \([\text{Ca}^{2+}]\). Preincubation of cells with 1 mM Bt\(\text{cAMP}\) for 7.5 min blunted the phase one increase in \([\text{Ca}^{2+}]\), induced by 2 units/ml PTH (not shown).
To further study this effect of cAMP under conditions where cellular levels of cAMP can be rapidly increased, we examined the effect of forskolin on [Ca\(^{2+}\)]
. Fig. 10 (top) shows that 15 μM forskolin increased [Ca\(^{2+}\)], in a pattern similar to that observed with Bt2cAMP. The effect of forskolin was also dependent on the presence of extracellular Ca\(^{2+}\) (not shown). Incubation of the cells at 37 °C with forskolin inhibited the phase one PTH-dependent increase in [Ca\(^{2+}\)]. The time dependency of the effect of forskolin to inhibit phase one in-[phase one PTH-dependent increase in [Ca\(^{2+}\)]. The time dependency of the effect of forskolin to inhibit phase one increase in [Ca\(^{2+}\)]. It is important to indicate here that these specific pretreatment protocols with PMA, forskolin, or Bt2cAMP had no effect on the ionomycin mobilizable pool of intracellular Ca\(^{2+}\) or Ca\(^{2+}\) influx and efflux from the cells after ionomycin treatment in Ca\(^{2+}\)-containing medium (not shown). This indicates that the pretreatment was specific in blocking the PTH-mediated increase in [Ca\(^{2+}\)].

In an attempt to demonstrate that two separate Ca\(^{2+}\) channels are involved in the PTH-mediated phases one and three increase in [Ca\(^{2+}\)], we compared the effect of channel blockers on both activities. Fig. 11 shows that 1 μM verapamil and 0.1 μM nicardipine inhibited the phase one increase in [Ca\(^{2+}\)], by about 84%, whereas the phase three increase in [Ca\(^{2+}\)], was inhibited by only approximately 50%. Verapamil and La\(^{3+}\) at concentrations of 25 μM each completely inhibited the initial increase in [Ca\(^{2+}\)], but the late increase was inhibited by only 65%. Therefore, the two phases of PTH-dependent increase in [Ca\(^{2+}\)], appear to have different sensitivities to the Ca\(^{2+}\) channel blockers tested.

Since preincubation of the cells with phorbol esters prevented the activation of the phase one Ca\(^{2+}\) channel, we tested the effect of phorbol ester treatment on the phase three increase in [Ca\(^{2+}\)]. In trace A, cells suspended in medium A containing 1.5 mM CaCl\(_2\) were stimulated with 2 units/ml PTH. In trace B, 1 μM verapamil was added 1 min prior to addition of 2 units/ml PTH. In trace C, 25 μM verapamil was added 1 min prior to addition of 2 units/ml PTH. In trace D, 25 μM La\(^{3+}\) was added prior to addition of 2 units/ml PTH. In trace E, 0.1 μM nicardipine was added prior to PTH. Extracellular CaCl\(_2\) content was 1.5 mM in traces B–D.

The experiment in Fig. 12 suggests that removal of medium Ca\(^{2+}\) or PMA treatment have no effect on the ability of PTH to increase cAMP levels in cells. Table 1 shows that PTH-dependent increases in cAMP levels were independent of medium Ca\(^{2+}\) concentration. Furthermore, pretreatment of the cells with 2 nM PMA for 5 min did not affect the PTH-dependent increase in cAMP.

**DISCUSSION**

In these studies PTH clearly caused a triphasic change in [Ca\(^{2+}\)], in UMR-106 cells. In the resting, nonstimulated state, the cells have a mean [Ca\(^{2+}\)], of 147 ± 19 nM (n = 30), a value very similar to the basal value of 177 ± 11 nM found by Lowik et al. (9) in UMR-106 cells. Rizzoli et al. (8) found that resting [Ca\(^{2+}\)], was 143 nM in ROS 17/2.8 cells.

The PTH-stimulated triphasic response of [Ca\(^{2+}\)], is fundamentally different from the PTH-stimulated rise in [Ca\(^{2+}\)], observed in SaOS-2 cells. In these cells Boland et al. (10) reported that PTH induced only a 10–15% increase in [Ca\(^{2+}\)]. There was no acute transient increase in [Ca\(^{2+}\)], noted as we observed in UMR-106 cells, although similar doses in
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SaOS-2 and UMR-106 cells resulted in similar CAMP generation.

In the rat osteosarcoma cell line ROS 17/2.8, no PTH-stimulated rise in [Ca\(^{2+}\)], was observed when Quin2 was used as the Ca\(^{2+}\)-sensitive dye. Differences in the biological responses of the different primary or cell line osteosarcomas have been reported before and may be due to the presence or absence of PTH receptors. However, another contributing factor to the differences observed in different laboratories is likely to be from the selection of the Ca\(^{2+}\)-sensitive dye. We were able to show that PTH has similar effects in UMR-106 and ROS 17/2.8 cells when [Ca\(^{2+}\)] was measured with Fure2 rather than Quin2. Since PTH has profound effects on bone resorption in vivo, the UMR-106 cells appear to be a good model system to study the effect of PTH on osteoblasts. It would be important to compare normal rat osteoblasts or even normal human osteoblasts with UMR-106 cells in terms of PTH stimulation of [Ca\(^{2+}\)], increase to see if the Ca\(^{2+}\) signal we are reporting here is unique to the UMR-106 cell line or to malignant tissue arising from bone cells.

Sensitivity to the calcium channel blockers nicardipine and verapamil was different for the phase one and phase three channels with the phase one channel being more sensitive to calcium channel blockers. Furthermore, the phase three channel appeared to be mediated by CAMP, whereas CAMP addition in the form of BtzcAMP or forskolin stimulation of adenylate cyclase did not reproduce a phase one Ca\(^{2+}\) transient as with PTH. The phase one channel could be blocked by preincubation of cells with BtzcAMP or forskolin but also with the phosphor ester PMA. Previous treatments with PMA, however, did not block the phase three increase in [Ca\(^{2+}\)], by PTH. This difference strongly suggests that PTH activates two independent Ca\(^{2+}\) channels which are regulated by different mechanisms.

These two PTH-activated Ca\(^{2+}\) channels in UMR-106 cells probably serve different purposes in these cells. The phase three response is not dependent on the phase one or two responses. The transient phase one channel may be involved only in rapid rises in [Ca\(^{2+}\)], that may serve as a trigger function of PTH action in osteoblasts. Those biochemical or physiologic functions of the osteoblast (e.g. collagen production, citrate decarboxylase activity) have been previously correlated with varying external calcium concentration (4) or blocking calcium entry into cells with calcium antagonists (26). The exact mechanisms of how intracellular Ca\(^{2+}\) changes affect these functions, however, is still unclear.

Cyclic AMP-dependent Ca\(^{2+}\) channels have been described in AtT-20 cells. Both forskolin and 8-bromo-CAMP were shown to cause an increase in [Ca\(^{2+}\)], that was dependent on extracellular Ca\(^{2+}\) and blocked by nifedipine, nisoldipine, or verapamil (27). Somatostatin, a blocker of adenylate cyclase, also blocked the forskolin-induced increase in [Ca\(^{2+}\)]. The CAMP-activated Ca\(^{2+}\) channel (phase three) may be related to transcellular Ca\(^{2+}\) transport. It has been hypothesized that PTH may act on bone to enhance calcium release (28). The increase in Ca\(^{2+}\) in bone interstitial fluid could then increase the Ca\(^{2+}\) gradient across the osteoblast layer lining bone from fluid to blood. The downhill movement of Ca\(^{2+}\) from bone interstitial fluid to cell may occur via a CAMP-activated Ca\(^{2+}\) channel. Studies using radiolabeled 45Ca have demonstrated that PTH stimulates a 30–40% increase in unidirectional Ca\(^{2+}\) uptake relative to control values in bone cells isolated by collagenase digestion from fetal rat calvariae (6). The increased uptake was observed 1–10 min after PTH stimulation, a time frame that is similar to the phase three increase in [Ca\(^{2+}\)], stimulated by PTH. The Ca\(^{2+}\) entering the cells thus can be extruded into the blood which will result in Ca\(^{2+}\) exit from bone.

Such transcellular Ca\(^{2+}\) transport in the osteoblast can be hypothesized to occur in the following manner. PTH-activated Ca\(^{2+}\) channels may be localized to the side of the cell facing mineralizing matrix. Ca\(^{2+}\) entering the cell can be extruded from the side of the cell facing the blood by means of either a Ca\(^{2+}\)–ATPase or a Na\(^{+}\)/Ca\(^{2+}\) exchanger. A Ca\(^{2+}\)-ATPase has been shown to be located on the plasma membrane facing the blood in osteoblasts (29).

It is important to note here that the rate of [Ca\(^{2+}\)], reduction in phase two was very rapid and usually to concentrations below resting levels. In experiments to be published elsewhere, we also noted that unlike other cells, UMR-106 cells were able to regain normal resting [Ca\(^{2+}\)], even in the presence of as high as 1 μM ionomycin. These observations reflect the
potency of the Ca\textsuperscript{2+} extrusion mechanisms in the plasma membrane of these cells.

Krieger and Tashjian (30, 31) have indirectly shown the presence of a Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in bone cells, although such an exchanger has not been exclusively localized to the osteoblast. We have observed that in the absence of extracellular Na\textsuperscript{+} or in ouabain-treated cells, the phase two decline in [Ca\textsuperscript{2+}] is still rapid suggesting that Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange, if present in UMR-106 cells, is not responsible for phase two observations. Hence, one would conclude that UMR-106 cells possess a very potent Ca\textsuperscript{2+} pump.

As demonstrated in these studies, PTH can increase the cellular levels of two second messengers, Ca\textsuperscript{2+} and cAMP. The two effects of PTH could be separated from each other by pretreatment of the cells with phorbol esters or cAMP. It was also reported previously that the PTH fragment bPTH-(3-34) can increase [Ca\textsuperscript{2+}], without any effect on cellular cAMP levels (9). On the basis of these observations it is possible that UMR-106 cells have two subtypes of PTH receptors, one of which induces an increase in [Ca\textsuperscript{2+}]; (first phase), and the other increases cAMP and consequently [Ca\textsuperscript{2+}] (phase three). It may be possible that pretreatment of the cells with PMA down-regulated the PTH receptors responsible for the initial increase in [Ca\textsuperscript{2+}]. Hormone or antigen-activated receptors on cells have been shown to be down-regulated by PMA (32-34).

Activation of the two signal transduction systems, Ca\textsuperscript{2+} and cAMP, by one hormone have recently been shown in glucagon-stimulated hepatocytes (41). Each signal transduction system had different ED\textsubscript{so} for glucagon, suggesting the presence of two subclasses of glucagon receptors. The similar ED\textsubscript{so} values for PTH effect on [Ca\textsuperscript{2+}] and cAMP production, however, suggest that these effects are related and may involve binding of PTH to a single class of receptors. Therefore, an alternative explanation for the selective effect of PMA on the phase one increase in [Ca\textsuperscript{2+}]; could be the direct inhibition of the Ca\textsuperscript{2+} channel by PMA or PDA. Both PMA and PDA have been shown to activate protein kinase C as described in many cell systems (35, 36). Similar inhibition of plasma membrane Ca\textsuperscript{2+} channel activity by protein kinase C activation has been reported for agonist (37) or depolarization-activated (38) Ca\textsuperscript{2+} channels. The possible mechanism of PMA inhibition of the channel is not clear yet. However, it is likely to involve protein kinase C-mediated phosphorylation of the Ca\textsuperscript{2+} channel. Since stimulation of two different protein kinase systems (cAMP and protein kinase C) resulted in inhibition of the phase one Ca\textsuperscript{2+} channel, it is likely that kinase-dependent phosphorylation of the channel results in channel inactivation. It is therefore possible that PTH-dependent activation of the channel might involve a dephosphorylation step. If one compares the time course of phospholipase C activation and therefore protein kinase C stimulation and cAMP accumulation in the cells, (39, 40) it is likely that the rapid inactivation of the channel after PTH-stimulation (within 15-30 s) is mediated by protein kinase C rather than cAMP-dependent kinase phosphorylation.

In summary we have described two types of Ca\textsuperscript{2+} channels stimulated by PTH in UMR-106 cells and have characterized them according to their activation and inactivation by cAMP and stimulators of protein kinase C activity. The functional utility of these channels in the UMR-106 cell needs further elucidation. Using the UMR-106 system and the sensitivity of only the rapidly inactivated channel to PMA, it should now be possible to study the contribution of changes in [Ca\textsuperscript{2+}]; to physiological functions of osteoblasts.