Differential Effects of Parathyroid Hormone and Its Analouges on Cytosolic Calcium Ion and cAMP Levels in Cultured Rat Osteoblast-like Cells

(Received for publication, September 8, 1987)

Henry J. Donahue, Michael J. Fryer, Erik F. Eriksen, and Hunter Heath III

From the Division of Endocrinology, Metabolism, and Internal Medicine, Mayo Clinic and Mayo Foundation, Rochester, Minnesota 55905

While the stimulatory effect of parathyroid hormone (PTH) on osteoblast-like cell adenylyl cyclase is well known, the effect of PTH on cytosolic calcium ion ([Ca\(^{2+}\)]) mobilization is controversial, one group finding no effect but others reporting various increases. We investigated the effects on [Ca\(^{2+}\)] of synthetic rat PTH fragment 1–34 (rPTH(1–34)) and two bovine PTH analogues that inhibit PTH's stimulation of adenylyl cyclase (bovine \(^{8\text{Nle}},34\text{Tyr}-\text{PTH}(3–34)\) and \(^{34\text{Tyr}-\text{PTH}(7–34)}\), [Ca\(^{2+}\)] was measured before, during, and after exposure to PTH analogues in perfused, attached osteoblast-like rat osteosarcoma cells (ROS 17/2.8) that had been scrape-loaded with the luminescent photoprotein aequorin. Resting [Ca\(^{2+}\)], was 0.094 ± 0.056 μM (mean ± S.D., n = 103) and rose in a time- and dose-specific way after exposure to rPTH(1–34). At 10–10 M rPTH(1–34), [Ca\(^{2+}\)] rose 100% within 30 s to a plateau; higher concentrations of PTH yielded increasing initial peaks of [Ca\(^{2+}\)], followed by lower plateaus. At 10–8 M, the initial peak was 5-fold basal, or 0.64 ± 0.07 μM. Both analogues of PTH were at least partial agonists for [Ca\(^{2+}\)], mobilization and did not reduce peak [Ca\(^{2+}\)] when co-perfused with rPTH(1–34). However, the analogues did reduce significantly rPTH(1–34)-induced cAMP accumulation and did not increase cAMP accumulation by themselves. Thus, rPTH(1–34) strongly mobilizes [Ca\(^{2+}\)], in ROS 17/2.8 cells, at near-physiologic concentrations. Failure of the PTH analogues to block the effect of PTH on [Ca\(^{2+}\)], while inhibiting the effect on cAMP accumulation suggests separate pathways for PTH activation of adenylyl cyclase and mobilization of calcium.

Parathyroid hormone (PTH)-mediated effects on target cells have been primarily attributed to stimulation of adenylyl cyclase activity (1, 2). Recent data indicate, however, that alterations in cytosolic ionized calcium levels ([Ca\(^{2+}\)]) may also play an important role in the actions of PTH on bone and kidney cells. While several studies have implicated calcium as a second messenger in the renal tubular cell response to PTH (3–5), the data on PTH-mediated alterations of [Ca\(^{2+}\)], in bone cells are still conflicting. Boland et al. (6) were unable to demonstrate any change of [Ca\(^{2+}\)], in rat osteosarcoma cells (ROS 17/2.8) after exposure to PTH. However, Lowik et al. (7) and Yamaguchi et al. (8) both reported increased [Ca\(^{2+}\)], levels in another rat osteosarcoma cell line (UMR-106) during exposure to PTH. Additional groups have recently reported that PTH stimulation results in a modest increase of [Ca\(^{2+}\)], in rat UMR-106 cells (9, 10) and mouse osteoblast-like MC3T3-E/cells (11).

The studies on bone cells cited above used fluorescent [Ca\(^{2+}\)], indicators (quin2, fura-2, or indo-1). Recent reports indicate, however, that these fluorescent indicators have some undesirable properties that may have confounded the data (12–14). Therefore, we chose a different indicator system to test the hypothesis that PTH mobilizes [Ca\(^{2+}\)], in bone cells. Employing scrape loading (15) of cells with the luminescent photoprotein aequorin (16–21), we investigated the effects of rat PTH fragment 1–34 (rPTH(1–34)) on the osteoblast-like rat osteosarcoma cell line, ROS 17/2.8 (22). We also studied the effects of two well known analogues of PTH that are inhibitory to PTH actions on adenylyl cyclase (bovine PTH fragments \(^{8\text{Nle}},34\text{Tyr}-\text{PTH}(3–34)\) and \(^{34\text{Tyr}-\text{PTH}(7–34)}\), or bPTH(3–34) and bPTH(7–34), respectively) (23, 24).

MATERIALS AND METHODS

RESULTS

[Ca\(^{2+}\)], in Unstimulated ROS 17/2.8 Cells—For ROS 17/2.8 cells perfused with culture medium only (Dulbecco’s Modified Eagle’s Medium, 10% fetal bovine serum, calcium 1 mM), we observed [Ca\(^{2+}\)] concentrations of 0.094 ± 0.056 μM (mean ± S.D.), calculated from all of the 103 experiments included in this paper; means for the individual groups of experiments ranged from 0.060 to 0.143 μM. In several experiments, the aequorin luminescence was stable over periods of 1–3 h in this system (data not shown). Perfusion with PTH vehicle in the medium had no effect on [Ca\(^{2+}\)] values.

Effect of rPTH(1–34) on [Ca\(^{2+}\)], in ROS 17/2.8 Cells—rPTH(1–34) increased [Ca\(^{2+}\)], in a dose-dependent fashion;

* Portions of this paper (including "Materials and Methods" and Figs. 6–8) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Fig. 1 shows examples of chart recorder tracings. During a 120-s perifusion with rPTH(1-34) at 10^{-6} M, [Ca^{2+}] rose almost immediately to values 4-5-fold resting levels (Fig. 2). The initial sharp peak was followed by a decrease to a stable plateau level, still above basal, until the rPTH(1-34) perifusion was stopped. After termination of exposure to rPTH(1-34), the [Ca^{2+}] returned to or near pretreatment levels with 1 min, with noticeable oscillations of [Ca^{2+}] for several minutes thereafter. Cells perifused with rPTH(1-34) at plateau level, still above basal, until the rPTH(1-34) perifusion was stopped. After termination of exposure to rPTH(1-34), the [Ca^{2+}] returned to or near pretreatment levels within 1 min, with noticeable oscillations of [Ca^{2+}], for several minutes thereafter. Cells perifused with rPTH(1-34) at 10^{-7} M for 20 min showed an initial transient [Ca^{2+}] peak identical to that seen during 2-min perifusions. However, the plateau phase was sustained during the entire 20 min until the hormone was removed (data not shown).

The initial peak height for [Ca^{2+}], decreased with decreasing concentrations of rPTH(1-34) in the medium (10^{-6}-10^{-10} M) (Fig. 2). Moreover, a change in the shape of the response curve occurred over this dose interval. At high doses, the initial peak tended to disappear and a uniphasic, plateau-shaped curve remained. While there was a significant dose-dependent increase of initial peak height (p < 0.05), the shoulder or plateau height remained constant (p = 0.48) over the dose interval studied. The dose-response relationship for height of the initial [Ca^{2+}] peaks is shown in Fig. 3. Preliminary results suggest that the threshold concentration of rPTH(1-34) for increasing [Ca^{2+}] lies between 10^{-12} and 10^{-12} M (data not shown).

**Effect of rPTH(1-34) on [Ca^{2+}] in Fibroblasts**—In a control study, rat skin-derived fibroblasts were exposed to rPTH(1-34), 10^{-7} M (n = 5). No significant changes in [Ca^{2+}] were recorded, but [Ca^{2+}], did increase dramatically (from about 0.06 to 0.50 μM) during perifusion of the aequorin-loaded fibroblasts with trifluoperazine (10^{-6} M), a putative calmodulin antagonist and inhibitor of membrane Ca,Mg-ATPase. [Ca^{2+}], returned toward basal rapidly upon removal of trifluoperazine. In previous studies, we found only small and inconsistent rises of cAMP content in similarly derived human fibroblast cells after exposure to rPTH(1-34) (25).

**Effects of PTH Analogues on [Ca^{2+}], in ROS 17/2.8 Cells**—To determine whether analogues of PTH could block the effects of rPTH(1-34) on [Ca^{2+}], ROS 17/2.8 cells were exposed to trivalent [Ca^{2+}]. There was no significant effect of rPTH(1-34) alone was similar over the dose range tested. However, the [Ca^{2+}], response to bPTH(3-34) was significantly greater (p < 0.05) at 10^{-5} M than at the two lower concentrations. Neither of the analogues caused the transient [Ca^{2+}] peak characteristic of rPTH(1-34). There was no significant effect of either analogue at any concentration tested on the [Ca^{2+}].

**Effect of PTH Analogues on [Ca^{2+}], in ROS 17/2.8 Cells**—To determine whether analogues of PTH could block the effects of rPTH(1-34) on [Ca^{2+}], ROS 17/2.8 cells were subjected to perifusion with rPTH(1-34), 10^{-8} M, in the presence of an excess of either one of two well characterized PTH analogues: bPTH(3-34) and bPTH(7-34) (10^{-6} M and 10^{-7} M, respectively) (26). The cells were perifused with an analogue for 120 s, followed by a 120-s perifusion of rPTH(1-34) plus analogue.

Perfusion with each analogue alone clearly elevated [Ca^{2+}]. (Fig. 4, a and b). There was no significant difference in initial [Ca^{2+}], response between the two analogues at these doses. During the following 120 s, wherein rPTH(1-34) was perifused along with analogues, the values for [Ca^{2+}], elicited were virtually identical to those recorded during exposure of the cells to rPTH(1-34) alone. We also examined the effects of PTH analogues at higher and lower concentrations (bPTH(3-34), 10^{-6}, 10^{-7}, and 10^{-8} M; and bPTH(7-34), 10^{-8}, 10^{-9}, and 10^{-10} M). Each PTH analogue alone, at every dose tested, increased [Ca^{2+}], (Table I). The response to bPTH (3-34) alone was similar over the dose range tested. However, the [Ca^{2+}], response to bPTH(7-34) was significantly greater (p < 0.05) at 10^{-5} M than at the two lower concentrations. Neither of the analogues caused the transient [Ca^{2+}] peak characteristic of rPTH(1-34). There was no significant effect of either analogue at any concentration tested on the [Ca^{2+}].
response to rPTH(1-34), 10^{-8} M (Table 1).

Effect of rPTH(1-34) and PTH Analogues on cAMP in ROS 17/2.8 Cells—Exposure of ROS 17/2.8 cells to 10^{-8} M rPTH(1-34) resulted in an almost 10-fold increase in cAMP (Fig. 5). This rPTH(1-34)-stimulated increase in cAMP was reduced 39% in the presence of 10^{-7} M bPTH(3-34) and 29% in the presence of 10^{-7} M bPTH(7-34). On the other hand, neither bPTH(3-34) nor bPTH(7-34) alone significantly increased cAMP accumulation when incubated alone for 2 min (data not shown) or 4 min (Fig. 5). rPTH(1-34)-stimulated cAMP accumulation was similar in cells lifted with trypsin-EDTA and cells scraped from the substrate with or without aequorin present (data not shown).

**DISCUSSION**

The present study verified that PTH has, among its actions on osteoblast-like bone cells, a powerful capacity to mobilize Ca^{2+} into the cytosolic compartment. The rapidity of this [Ca^{2+}]i response is similar to the adenylate cyclase response and is at least as sensitive. The Kd values for displacement of radiolabeled PTH from plasma membrane receptors (27, 28), activation of adenylate cyclase (27, 28), and release of cAMP from rat bone (29) are all about 10^{-9} M. While a Kd for the [Ca^{2+}]-mobilizing effect of rPTH(1-34) could not be derived from our studies, the plateau response at 10^{-10} M (a near physiologic concentration of rPTH (30)) was not less than that at higher doses. The [Ca^{2+}]-raising effect of PTH may therefore be one of the most sensitive indexes of PTH action on bone cells. The [Ca^{2+}]i response we observed was apparently tissue-specific, as it occurred in rat bone-derived ROS 17/2.8 cells, but not in cultured rat dermal fibroblasts.

Our findings differ considerably from published data on PTH modulation of [Ca^{2+}]i in osteoblast-like cells. For example, Boland et al. (6) used quin2-loaded ROS 17/2.8 cells to study the effects of various calcium-regulating hormones, and found no effect of PTH on [Ca^{2+}]i. In contrast, Lowik et al. (7), using the same fluorescent Ca^{2+} probe, found relatively small 25-50% increases of [Ca^{2+}]i during exposure of UMR-106 rat osteosarcoma cells to PTH. We attribute those authors' difficulties in seeing the large PTH-induced changes of [Ca^{2+}]i to the phenotypic drift of their cultured bone cells, with loss of PTH responsiveness, another possibility.

More recently, Yamada et al. (11) observed modest monophasic increases in [Ca^{2+}]i in mouse osteoblast-like MC3T3-E1 cells loaded with fura-2 and exposed to PTH as did Reid et al. (9) using UMR-106 cells loaded with indo-1. Yamaguchi

**TABLE I**

Effect of rPTH(1-34) and analogues on [Ca^{2+}]i.

Maximum initial effects on [Ca^{2+}]i in ROS 17/2.8 cells of 2-min perfusions with medium, bPTH(3-34), or bPTH(7-34) alone, followed by addition of rPTH(1-34) alone or in the presence of the PTH analogues (the protocol design is the same as that shown in Fig. 4). The basal period is designated period A. In all cases, the 2-min period immediately after the basal (period B) included perfusion with medium alone (first line) or PTH analogue alone (last six lines). The substances perfused in period B were continued in the third 2-min period (period C), but with addition in all experiments of rPTH (1-34), 10^{-8} M. Data are mean ± S.E. (n = 5 for each experiment) for calculated absolute concentrations of [Ca^{2+}]i in nanomoles per liter.

| Infused in periods B and C | Added in period C | A (basal) | B (medium or analogue alone) | C (rPTH(1-34) alone or + analogue) |
|---------------------------|-------------------|----------|-----------------------------|----------------------------------|
| Medium                    | rPTH(1-34), 10^{-8} M | 65.7 ± 19.4 | 78.8 ± 24.7 | 191.2 ± 23.2 |
| bPTH(3-34), 10^{-8} M     | rPTH(1-34), 10^{-8} M | 61.3 ± 13.6 | 117.6 ± 18.9a | 209.8 ± 40.4a |
| bPTH(3-34), 10^{-7} M     | rPTH(1-34), 10^{-8} M | 57.3 ± 9.2  | 95.6 ± 15.2 | 184.2 ± 27.7a |
| bPTH(3-34), 10^{-7} M     | rPTH(1-34), 10^{-9} M | 53.9 ± 8.7  | 104.9 ± 6.8 | 202.8 ± 9.2a |
| bPTH(3-34), 10^{-8} M     | rPTH(1-34), 10^{-7} M | 64.3 ± 8.0  | 182.8 ± 16.2b,b | 176.2 ± 13.8a |
| bPTH(3-34), 10^{-9} M     | rPTH(1-34), 10^{-6} M | 66.9 ± 10.3 | 101.1 ± 16.1 | 172.4 ± 9.6a |
| bPTH(3-34), 10^{-7} M     | rPTH(1-34), 10^{-9} M | 47.6 ± 10.0 | 98.4 ± 23.3 | 176.2 ± 7.0a |

a Significantly greater than period A (basal) within same dose and peptide (p < 0.05).

b Greater than all other values within period B.
Cytosolic Calcium in Bone Cells

et al. (8), using fura-2, found a transient initial PTH-mediated increase in [Ca++] in the UMR-106 cell line that may be mediated by protein kinase C (Ca++- and phospholipid-dependent protein kinase) (32), and, contrary to our findings, a later cAMP-dependent rise in [Ca++] . The [Ca++] response to rPTH(1-34) that we observed in ROS 17/2.8 cells was biphasic, with an initial peak resembling but larger than that seen by Yamaguchi et al. (8) and Reid et al. (9). In addition, we observed a sustained plateau elevation of [Ca++] after the initial peak and did not see the late increase of [Ca++] described by Yamaguchi et al. (8). Whether these discrepancies are due to differences in cell lines or indicator systems remains to be established.

We have not yet examined the sources for the Ca++ appearing in cytosol, but it is tempting to speculate that the curves seen at high PTH doses represent composites of two different responses. Perhaps the initial peak represents mobilization of Ca++ from intracellular membranes or organelles, and the plateau represents influx of extracellular calcium, as has been described for ascites tumor cells (33). The concept that PTH mobilizes both intracellular and extracellular Ca++ into the cytosol is supported by the findings of Yamaguchi et al. (8), showing that the removal of extracellular Ca++ or addition of calcium channel blocker reduced but did not abolish the [Ca++] response to bPTH(1-34) in UMR-106 cells. Reid’s data (f) are ambiguous: low-calcium medium (−1 μM) abolished the [Ca++] response to bPTH(1-34), whereas removal of extracellular Ca++ by EGTA had no effect on the response to PTH. Again, the reasons for these inconsistencies are unclear. However, numerous pharmacologic probes (34, 35) now available should permit us to address this issue in future studies.

The two amino-truncated analogues of parathyroid hormone, bPTH(3-34) and bPTH(7-34), both exert inhibitory effects on PTH-mediated adenylate cyclase stimulation (23, 24). We verified the ability of the two analogues to inhibit rPTH(1-34)-induced cAMP accumulation under our experimental conditions. In vivo weak agonism has been described for bPTH(3-34) in stimulation of adenylate cyclase, induction of hypercalcemia, and stimulation of urinary phosphate excretion (23, 24). Lowik et al. (7) also reported weak agonism of bPTH(3-34) in raising [Ca++] , but did not describe whether the analogue reduced [Ca++] mobilization by active PTH. On the other hand, Reid et al. (9) found that bPTH(3-34) alone had no effect on [Ca++] , and reduced the effect of bPTH(1-34) on [Ca++] in UMR-106 cells, while Yamaguchi et al. (8) obtained similar results with bPTH(7-34). In contrast to the above findings, our experiments showed bPTH(3-34) to be a partial agonist and bPTH(7-34) to be possibly a full agonist for [Ca++] mobilization. Neither analogue significantly antagonized rPTH(1-34) action on [Ca++] , at least in terms of absolute [Ca++] levels attained.

Our ability to detect increases in [Ca++] in response to bPTH(3-34) and bPTH(7-34) suggests that the aequorin method may have greater sensitivity to small or local changes in [Ca++] than the fluorometric techniques. We have no explanation for failure of the analogues to inhibit rPTH(1-34)-induced [Ca++] , elevations in our cells. It is unlikely that species differences in PTH structure played a role here; bPTH(1-34) and rPTH(1-34) are strongly homologous, differing at only 3 amino acid residues (24).

The [Ca++] indicator system used for these studies, aequorin luminescence, differs sharply from other methods used recently with bone cells. In cell systems studied so far, aequorin was distributed evenly throughout the cytosol and exerted only minimal buffering capacity on [Ca++] (16). The luminescence is unaffected by most other ions of biological significance, except for Mg++ (15), which may tend to decrease measured levels of [Ca++] . Unlike the fluorescent indicator quin2, which may undergo photobleaching on prolonged light exposure, the luminescence of aequorin stays essentially constant for many hours (15). The main disadvantage of aequorin is its relative scarcity. Other problems primarily encountered in studies on muscle cells, such as relatively slow response time (milliseconds) and slow diffusion rates of aequorin (16) were of no significance within the time frame of responses in this study.

Until recently, aequorin could only be used in systems wherein it could be loaded by techniques such as microinjection or hypotonic shock (16, 36, 37). This hindered its use in cultured adherent mammalian cells. Recently, however, this problem was solved with the development of scrape-loading of cells by McNeil and Taylor (15). Most techniques employing fluorescent indicators have involved the use of cells in suspension. Detachment of cells may lead to perturbations of membrane structure and receptor populations, which might alter responsivity to a given hormonal stimulus. Studies of cells in suspension necessitates the interruption of studies on muscle cells, such as relatively slow response time (milliseconds) and slow diffusion rates of aequorin (16) were of no significance within the time frame of responses in this study.

Studies of cells in suspension necessitates the interruption of studies on muscle cells, such as relatively slow response time (milliseconds) and slow diffusion rates of aequorin (16) were of no significance within the time frame of responses in this study.

In conclusion, our studies established that rPTH(1-34) elicits complex time- and dose-dependent effects on [Ca++] mobilization in cultured osteoblast-like ROS 17/2.8 cells. The
maximal responses were considerably greater than those observed with fluorescent indicators and included a pattern of [Ca^{2+}], response not previously described for those cells. Our data greatly strengthen the hypothesis that transduction of the PTH signal in bone cells involves not only formation of cAMP, but rapid activation of a [Ca^{2+}] signal. Furthermore, the methods we describe herein will permit a detailed exploration of PTH signal in bone cells. In contrast to their inhibitory effects on PTH stimulation of adenylate cyclase, the PTH analogues bPTH(3-34) and bPTH(7-34) were at least partial agonists for [Ca^{2+}], mobilization and ineffective antagonists of rPTH(1-34)-induced [Ca^{2+}] mobilization. We speculate that these data are clues to separate mediation of PTH’s effects on adenylate cyclase and [Ca^{2+}], whether it be by two separate PTH receptors (7) or differential regulation at a post-receptor level.

REFERENCES

1. Chase, L. R., and Aurbach, G. D. (1970) J. Biol. Chem. 245, 1520-1526
2. Hermann-Erlee, M. P. M., and Konijn, T. M. (1970) Nature 227, 177-178
3. Hruska, K. A., Goligorsky, M., Scoble, J., Tsutsui, M., Westbrook, S., and Moskowitz, D. (1986) Am. J. Physiol. F188-F194
4. Yanagawa, N., and Ono, O. D. (1986) Am. J. Physiol. 250, P942-P948
5. Hruska, K. A., Moskowitz, D., Esbrit, P., Civitelli, R., Westbrook, S., and Hruska, M. (1987) J. Clin. Invest. 79, 230-239
6. Boland, C. J., Fried, R. M., and Tashjian, A. H., Jr. (1986) Endocrinology 118, 960-969
7. Löwik, C. W. M., van Leeuwen, J. P. T. M., van der Meer, J. M., van Zeeland, J. R., Scheven, B. A. A., and Hermann-Erlee, M. P. M. (1985) Cell Calcium 6, 311-326
8. Yamaguchi, D. T., Hahn, T. J., Ida-Klein, A., Kleeman, C. R., and Muallem, S. (1987) J. Biol. Chem. 262, 7711-7718
9. Reid, I. R., Civitelli, R., Halstead, L. R., Avioli, L. V., and Hruska, K. A. (1987) Am. J. Physiol. 15, E40-E51
10. Fang, M. A., Ida-Klein, A., Greensfeld, M. W., Yamaguchi, D. T., Ituarte, H. G., and Hahn, T. J. (1987) J. Bone Mineral Res. 2(51), 236 (abstr.)
11. Yamada, H., Fukase, M., Tsunenari, T., Fujii, Y., Tsutsui, M., and Fujita, T. (1987) J. Bone Mineral Res. 2(S1), 115 (abstr.)
12. Scanlon, M., Williams, D. A., and Fay, F. S. (1986) J. Gen. Physiol. 88, 52A (abstr.)
13. Defeo, T. T., and Morgan, K. G. (1986) J. Gen. Physiol. 88, 18A (abstr.)
14. Johnson, P. C., Ware, J. A., Cliveden, P. B., Smith, M., Dvorak, A. M., and Salzman, E. W. (1985) J. Biol. Chem. 260, 2069-2076
15. McNeil, P. L., and Taylor, D. L. (1985) Cell Calcium 6, 83-93
16. Blinks, J. R., Wier, W. G., Hess, P., and Prendergast, F. G. (1982) Prog. Biophys. Mol. Biol. 4, 1-114
17. Allen, D. G., Blinks, J. R., and Prendergast, F. G. (1977) Science 195, 996-998
18. Allen, D. G., and Blinks, J. R. (1979) in Detection and Measurement of Free Ca^{2+} in Cells (Ashley, C. C., and Campbell, A. K., eds) pp. 159-174, Elsevier/North Holland, New York
19. Blinks, J. R. (1984) in Methods for Studying Heart Membranes (Dhalia, N. S., ed) Vol II, pp. 237-264, CRC Press, Inc., Boca Raton, FL
20. Blinks, J. R., Mattingly, P. H., Jewell, B. R., van Leeuwen, M., Harrer, G. C., and Allen, D. G. (1978) Methods Enzymol. 57, 292-326
21. Moore, E. D. W. (1986) Properties of Aequorin Relevant to Its Use in the Measurement of Intracellular [Ca^{2+}] in Skeletal Muscle Fibers. Ph. D. thesis, Graduate School of the University of Minnesota
22. Rodan, G. A., and Martin, T. J. (1981) Calcif. Tissue Int. 33, 349-351
23. Kubota, M., Ng, K. W., Murase, J., Noda, T., Moseley, J. M., and Martin, T. J. (1986) J. Endocrinol. 108, 261-265
24. Doppelt, S. H., Neer, R. M., Nussbaum, S. R., Frederico, P., Potts, J. T., Jr., and Rosenblatt, M. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7557-7560
25. Fryer, M. J., Fritz, S. F., and Heath, H. III (1986) Mayo Clin. Proc. 61, 263-267
26. Horiiuchi, N., Holick, M. F., Potts, J. T., Jr., and Rosenblatt, M. (1982) Science 220, 1052-1055
27. Nissenson, R. A., Nyiredy, K. O., and Arnaud, C. D. (1981) Endocrinology 108, 1949-1953
28. Rao, L. G., and Murray, T. M. (1985) Endocrinology 117, 1632-1638
29. Calvo, M. S., Fryer, M. J., Laakso, K. J., Nissenson, R. A., Price, P. A., Murray, T. M., and Heath, H. III (1985) J. Clin. Invest. 76, 2348-2354
30. Calvo, M. S., Laakso, K. J., and Heath, H. III (1986) J. Bone Mineral Res. 1(51), 338 (abstr.)
31. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 240, 3440-3448
32. Yamaguchi, D. T., Kleeman, C. R., and Muallem, S. (1987) J. Biol. Chem. 262, 14967-14973
33. Dubyak, G. R., and De Young, M. B. (1985) J. Biol. Chem. 260, 10653-10661
34. Rampe, D., Janis, R. A., and Trigg, D. J. (1984) J. Neurochem. 43, 1668-1692
35. Trigg, D. J., and Janis, R. A. (1984) in Modern Methods of Pharmacology (Back, N., and Specter, S., eds) Vol. I, pp. 1-28, Alan R. Liss, Inc., New York
36. Cobold, P. H., Cuthbertson, K. S. R., Goyns, M. H., and Rice, V. (1983) J. Cell Biol. 1073, 99-114
37. Morgan, J. P., and Morgan, K. G. (1982) Fed. Proc. 41, 854
Cytosolic Calcium in Bone Cells

SUGGESTIVE MATERIAL TO DIFFERENTIAL EFFECTS OF PARATHYROID HORMONE AND ITS ANALOGUES ON CYTOSOLIC CALCIUM LEVELS IN CULTURED RAT OSTEOBLAST-LIKE CELLS

Henny J. Donahue, Michael J. Fryer, Erik F. Erikren, and Hunter Heath Ill

MATERIALS AND METHODS

Cell culture. Cytosolic-like rat osteoclastoma cells (ROS 17/2.8) (generously supplied by Dr. J. Barret of NHP, and originally by Dr. Robin King of Merck, Sharpe, and Dohme) were cultured in 175 cm² plastic culture flasks (Falcon #3003). The cells were maintained in Ham’s F-12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 12 mM L-glutamine, 1120 mM sodium pyruvate, and 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco, Grand Island, NY). Cell cultures were maintained at 37°C with 5% CO₂. Stock solutions of forskolin were prepared in dimethyl sulfoxide (DMSO) and stored at -70°C.

Supersolution of ROS 17/2.8 cells and photoplates. Aequorin, a luminescent calcium indicator protein isolated from the jellyfish C. aequorea, was donated by Dr. J. King. The cells were loaded with aequorin using the technique of Neher (9) with some modifications. After removing the medium from the culture dish, the cell suspension was washed three times with Culture ( buffered to pH 7.4) prewarmed at 37°C. Aequorin loading was optimized to 30°C by perfusion with a solution containing 200 µg/ml aequorin in 15 mM KCl, 50 mM PIPES, pH 7.0. Aequorin was added slowly to the cell suspension and left in the dish overnight, then the sample was frozen at -10°C for later use.

After centrifugation, the cell suspension was resuspended in 1/250 ml solution (5) and an aliquot was used to test the sensitivity of the cells after the trypsinization was determined to yield 100 mg cells per ml, and 0.5% of the cell suspension was placed onto sterile 24-mm circular glass coverslips in 36-mm polyethylene culture dishes (Corning). Cells were transferred to a 95% humidified atmosphere at 37°C. Aequorin was added when the cultures were confluent or 24 hours, respectively. The light produced by the aequorin-labeled cells was proportional to the calcium concentration. The cells were washed three times with 1/200 ml solution (5) and an aliquot was used to test the sensitivity of the cells after the trypsinization was determined to yield 100 mg cells per ml, and 0.5% of the cell suspension was placed onto sterile 24-mm circular glass coverslips in 36-mm polyethylene culture dishes (Corning). Cells were transferred to a 95% humidified atmosphere at 37°C. Aequorin was added when the cultures were confluent or 24 hours, respectively. The light produced by the aequorin-labeled cells was proportional to the calcium concentration.

Schematic diagram of the calcium ionophore. The ionophore was used in the experiments to estimate the calcium concentration. The ionophore was used in the experiments to estimate the calcium concentration. The ionophore was used in the experiments to estimate the calcium concentration. The ionophore was used in the experiments to estimate the calcium concentration. The ionophore was used in the experiments to estimate the calcium concentration. The ionophore was used in the experiments to estimate the calcium concentration. The ionophore was used in the experiments to estimate the calcium concentration.

Calculation of (Ca²⁺). The calibration curve for the estimation of (Ca²⁺) was obtained by measuring the aequorin-labeled cells in 150 µM CaCl₂, 50 mM PIPES, 50 µM PMSF, with the aequorin signal recorded at 570 nm with a photomultiplier tube (PMT) and a temperature-controlled chamber. The PMT was connected to a multichannel recorder, which also recorded the temperature of the calcium ionophore, the temperature of the cell suspension, and the temperature of the reaction mixture. The temperature of the cell suspension was monitored with a thermistor connected to a thermometer. The temperature of the reaction mixture was monitored with a thermistor connected to a thermometer. The temperature of the cell suspension was monitored with a thermistor connected to a thermometer. The temperature of the reaction mixture was monitored with a thermistor connected to a thermometer.

Experiment construction of the aequorin-labeled cells. The cells were cultured in a 37°C, 5% CO₂ atmosphere. The aequorin-labeled cells were seeded onto sterile coverslips and cultured in 36-mm polyethylene culture dishes. The aequorin-labeled cells were cultured in 36-mm polyethylene culture dishes. The aequorin-labeled cells were cultured in 36-mm polyethylene culture dishes. The aequorin-labeled cells were cultured in 36-mm polyethylene culture dishes. The aequorin-labeled cells were cultured in 36-mm polyethylene culture dishes.

The mathematical model for Ca²⁺ binding to aequorin proposed by Albrecht et al. (11) was used to calculate (Ca²⁺). The aequorin was a polypeptide which contains three cysteine residues. The aequorin was a polypeptide which contains three cysteine residues. The aequorin was a polypeptide which contains three cysteine residues. The aequorin was a polypeptide which contains three cysteine residues. The aequorin was a polypeptide which contains three cysteine residues.