

# High extracellular $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ stimulate accumulation of inositol phosphates in bovine parathyroid cells

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Received 15 April 1987

We examined the effects of the divalent cations  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  on inositol phosphate accumulation in bovine parathyroid cells prelabelled with [ $^3\text{H}$ ]inositol to determine whether the high extracellular  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -evoked transients in cytosolic  $\text{Ca}^{2+}$  in these cells might result from increases in cellular  $\text{IP}_3$  levels. In the presence of  $\text{Li}^+$ , both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  produced rapid, 2–6-fold increases in  $\text{IP}_3$  and  $\text{IP}_2$  and a linear increase in IP of 6–8-fold at 30 min. Smaller (1.5–2-fold) increases in  $\text{IP}_2$  and  $\text{IP}_3$  were evident within 7.5–15 s upon exposure to high (3 mM)  $\text{Ca}^{2+}$  in the absence of  $\text{Li}^+$ . The relative potencies of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ( $\text{Ca}^{2+}$  3-fold more potent than  $\text{Mg}^{2+}$ ) in elevating inositol phosphates were similar to those for their effects in inhibiting PTH release. Fluoride (5 and 10 mM) also produced similar increases in inositol phosphate accumulation, presumably through activation of phospholipase C by a guanine nucleotide (G) protein-dependent process. Thus, high extracellular  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -induced spikes in cytosolic  $\text{Ca}^{2+}$  in bovine parathyroid cells may be mediated by increases in  $\text{IP}_3$ , perhaps through a receptor-mediated process linked to phospholipase C by a G-protein.

Inositol phosphate; PTH release;  $\text{Ca}^{2+}$ ;  $\text{Mg}^{2+}$

## 1. INTRODUCTION

In many exocytotic systems, receptor-mediated activation of hormonal secretion is accompanied by increases in the cytosolic  $\text{Ca}^{2+}$  concentration, which frequently arise both from extracellular as well as intracellular  $\text{Ca}^{2+}$  [1]. The mobilization of intracellular  $\text{Ca}^{2+}$  often results from receptor-mediated activation of phospholipase C via a guanine nucleotide (G)-protein, thereby hydrolyzing phosphatidylinositol 4,5-bisphosphate ( $\text{PIP}_2$ ) to diacylglycerol and inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ). The latter liberates  $\text{Ca}^{2+}$  from nonmitochondrial stores, predominantly the endoplasmic re-

ticulum [2]. The parathyroid gland is unusual among secretory systems in that increases in the extracellular and cytosolic  $\text{Ca}^{2+}$  concentrations are associated with inhibition, rather than stimulation, of hormonal secretion [3]. In fura-2-loaded parathyroid cells,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  evoke an initial spike in cytosolic  $\text{Ca}^{2+}$ , which arises from intracellular  $\text{Ca}^{2+}$  stores, followed by a sustained increase in the cytosolic  $\text{Ca}^{2+}$  concentration due to uptake of extracellular  $\text{Ca}^{2+}$  [4]. These changes in cytosolic  $\text{Ca}^{2+}$  are analogous to those in other secretory systems and suggest that divalent cation-induced transients in cytosolic  $\text{Ca}^{2+}$  might arise from generation of cellular  $\text{IP}_3$ , perhaps resulting from interaction of extracellular divalent cations with a putative cell-surface 'receptor' [4–6]. In the present studies, we have directly examined the effects of extracellular  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  on inositol phosphate accumulation in bovine parathyroid cells.

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## 2. MATERIALS AND METHODS

Bovine parathyroid cells were prepared by digestion with collagenase and DNase using sterile conditions as described [3]. Cells were then cultured in fibronectin-coated cluster wells in medium 199 with 15% neonatal calf serum, penicillin and streptomycin, and 10 mM Hepes, pH 7.45, as outlined [3] for 20–28 h with 30  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]inositol (New England Nuclear, Boston, MA). Cells cultured in this fashion routinely showed  $\text{Ca}^{2+}$ -regulated PTH release which was nearly indistinguishable from that of acutely dispersed cells [3] and fewer than 10% fibroblasts on the light microscope. The cells then were washed free of the labelled precursor with Eagle's MEM ( $\text{NaHCO}_3$ ,  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$  deleted) containing 0.5 mM  $\text{Ca}^{2+}$ , 0.5 mM  $\text{Mg}^{2+}$  and 0.2% (w/v) bovine serum albumin and equi-

librated with 1 ml of the same medium for 5–10 min at 37°C. In experiments in which  $\text{Li}^+$  was included, 10 mM LiCl was added to the cultured cells 30–60 min prior to washing and was also included in the wash and incubation solutions. At the beginning of the experimental period, the appropriate  $\text{Ca}^{2+}$  concentration was added to the cells as  $\text{CaCl}_2$ , and the incubation was continued for the desired time interval (7.6 s to 30 min). The reaction was terminated by the addition of a final concentration of 1 M perchloric acid. The cells were then scraped off the culture dish with a rubber policeman and sedimented for 10 min at 1000 $\times g$ . The supernatant was neutralized with a mixture of 2 N KOH and 1 M Tris base and frozen at  $-20^\circ\text{C}$ . For determination of [ $^3\text{H}$ ]inositol phosphates, the neutralized supernatant was applied to disposable polypropylene columns containing 0.8 ml of

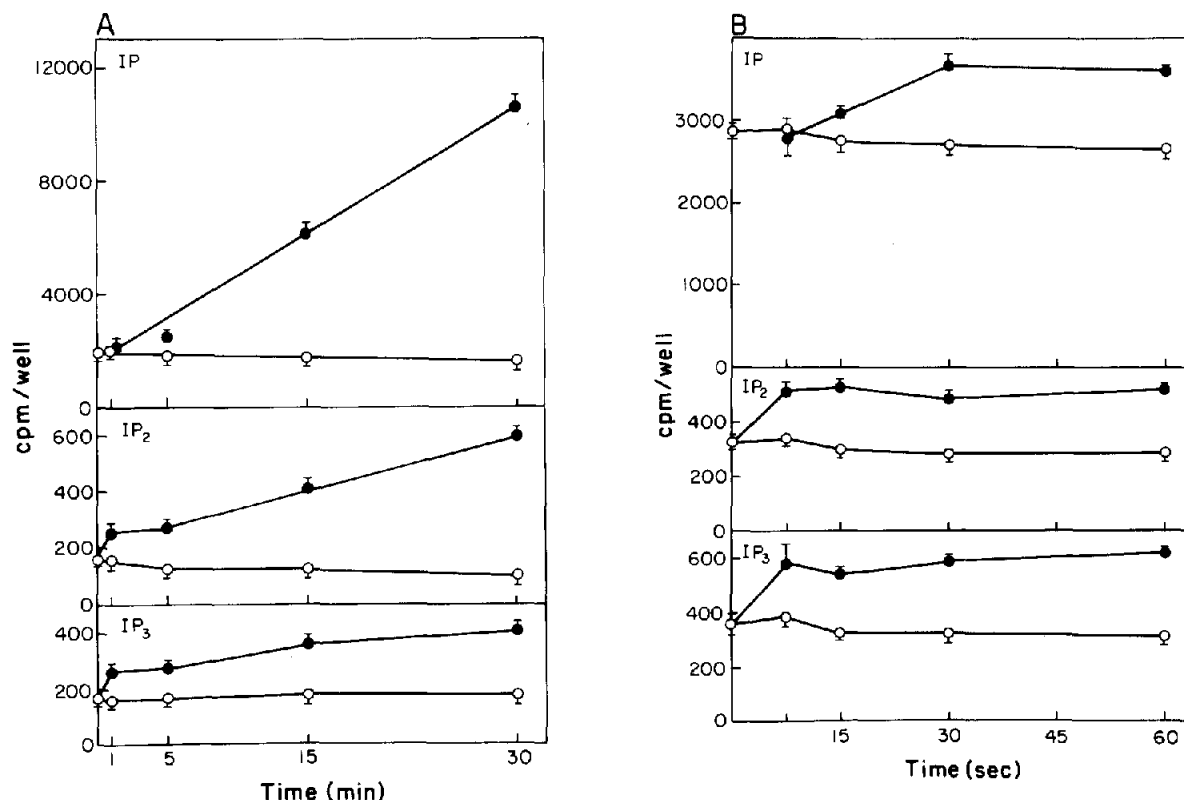


Fig.1. Time course for the stimulation of inositol phosphate accumulation by high  $\text{Ca}^{2+}$ . After parathyroid cells were cultured with [ $^3\text{H}$ ]inositol and were washed free of the labelled precursor, the cells were incubated with either 0.5 mM  $\text{Ca}^{2+}$  and 0.5 mM  $\text{Mg}^{2+}$  (open symbols) or 3.0 mM  $\text{Ca}^{2+}$  and 0.5 mM  $\text{Mg}^{2+}$  (closed symbols) in the presence (A) or absence (B) of 10 mM  $\text{Li}^+$  for the times shown. Results in A are the mean  $\pm$  SE for 4–6 wells in 2–3 experiments, while those in B are the mean  $\pm$  SE for three wells from one of three similar experiments.

Dowex AG-1X8 in the formate form and eluted sequentially with distilled water followed by 0.2, 0.4, and 1.0 M ammonium formate in 0.1 M formic acid [7]. PTH in supernatant samples was determined by radioimmunoassay using an antiserum recognizing the intact hormone as well as carboxy-terminal fragments [3]. Statistical comparisons were performed using the unpaired *t*-test.

### 3. RESULTS

#### 3.1. Time course for the high $\text{Ca}^{2+}$ -induced accumulation of inositol phosphates

We initially employed medium containing 10 mM  $\text{Li}^+$  to assess the effects of high extracellular  $\text{Ca}^{2+}$  on inositol phosphate accumulation in bovine parathyroid cells, since the recycling of inositol phosphates into phosphoinositides is impaired by the lithium-induced inhibition of the inositol-1-phosphatase. In addition, cultured cells were utilized because preliminary studies revealed that insufficient [ $^3\text{H}$ ]inositol was incorporated in short-term experiments (2–4 h) to detect reproducibly changes in  $\text{IP}_2$  and  $\text{IP}_3$ . A high  $\text{Ca}^{2+}$  concentration maximally suppressing PTH release (3 mM) produced a 1.5–2-fold rise in  $\text{IP}_3$  within 1 min, which increased further to 2–3-fold in 30 min, while levels of  $\text{IP}_3$  remained constant or declined slightly in cells incubated with a low, maximally stimulatory  $\text{Ca}^{2+}$  concentration (0.5 mM) (fig. 1A). Levels of  $\text{IP}_2$  also rose rapidly within 1 min, while the level of IP did not increase significantly at 1 min but rose linearly thereafter to a 6–8-fold increase by 30 min. The temporal sequences of the changes in  $\text{IP}_3$ ,  $\text{IP}_2$ , and IP are consistent with extracellular  $\text{Ca}^{2+}$ -induced hydrolysis of polyphosphoinositides with subsequent conversion of  $\text{IP}_3$  and  $\text{IP}_2$  to IP. In the absence of  $\text{Li}^+$ , rapid increases in  $\text{IP}_2$  and  $\text{IP}_3$  were also noted which preceded those in IP (fig. 1B).

#### 3.2. Dose response for the effects of $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ on inositol phosphate accumulation

In the presence of  $\text{Li}^+$ , IP rose significantly at 1 mM  $\text{Ca}^{2+}$ , and IP accumulation increased progressively up to 3 mM  $\text{Ca}^{2+}$  (fig. 2).  $\text{IP}_2$  and  $\text{IP}_3$  showed similar dose response relationships to  $\text{Ca}^{2+}$ . High  $\text{Mg}^{2+}$ , which inhibits PTH release with a potency 3-fold less than  $\text{Ca}^{2+}$  [8,9], also increased inositol phosphate accumulation (fig. 2).

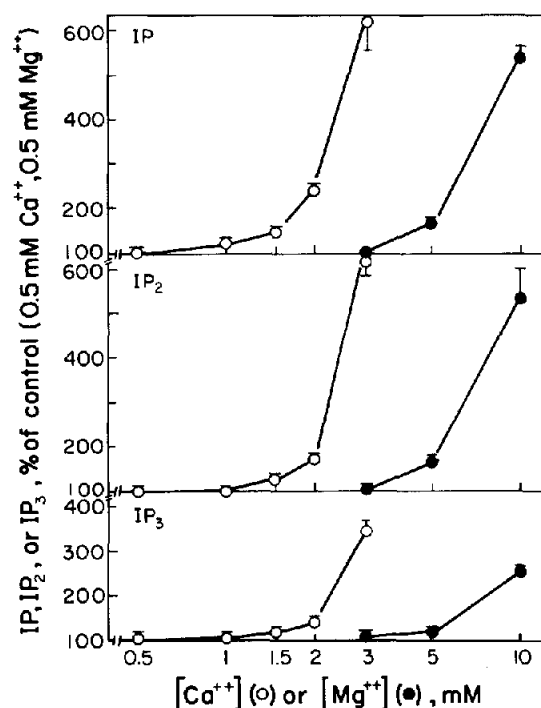


Fig. 2. Dose-response for the effects of high extracellular  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  on inositol phosphate accumulation. Cells were preincubated with 10 mM  $\text{Li}^+$  for 30–60 min, washed, and subsequently incubated with 10 mM  $\text{Li}^+$  as well as varying concentrations of  $\text{Ca}^{2+}$  (○) or  $\text{Mg}^{2+}$  (●) in the presence of 0.5 mM  $\text{Mg}^{2+}$  or 0.5 mM  $\text{Ca}^{2+}$ , respectively. Results show the percent of the value obtained with 0.5 mM  $\text{Ca}^{2+}$  and 0.5 mM  $\text{Mg}^{2+}$  in 4–10 wells from 2–5 experiments. The lowest doses of  $\text{Ca}^{2+}$  increasing IP,  $\text{IP}_2$ , and  $\text{IP}_3$  accumulation significantly were 1.0, 2, and 2 mM, respectively, while for  $\text{Mg}^{2+}$  the lowest concentration significantly increasing inositol phosphate accumulation was 5 mM in all three cases.

Comparable changes in inositol phosphate levels required about 3-fold higher levels of  $\text{Mg}^{2+}$  than of  $\text{Ca}^{2+}$ .

#### 3.3. Effect of fluoride on inositol phosphate accumulation

Fluoride ion has been found to activate G-proteins in intact cells and has recently been shown to activate the G-protein mediating phosphoinositide hydrolysis via phospholipase C [10]. Fluoride (5 and 10 mM) increased inositol phosphates in bovine parathyroid cells (fig. 3) and also inhibited PTH release to an extent similar to

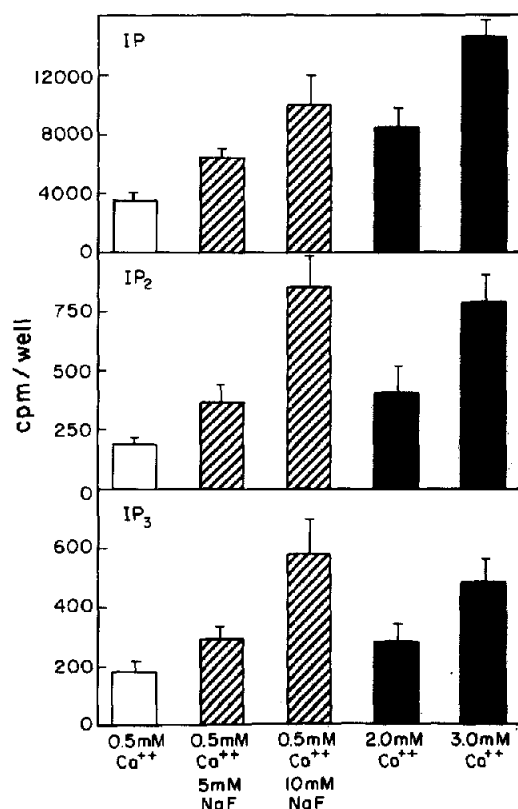


Fig.3. Effect of fluoride on inositol phosphate accumulation. Cells were preincubated for 30–60 min with 10 mM Li<sup>+</sup>, washed, and then incubated with 10 mM Li<sup>+</sup>, 0.5 mM Mg<sup>2+</sup> with or without 5 or 10 mM NaF. For comparison, results with 2 or 3 mM Ca<sup>2+</sup> and 0.5 mM Mg<sup>2+</sup> are also shown. Results are the mean  $\pm$  SE for 7 wells in three experiments. Fluoride significantly ( $p < 0.01$ ) increased the accumulation of IP and IP<sub>2</sub> at both 5 and 10 mM and IP<sub>3</sub> at 10 mM.

that observed with high Ca<sup>2+</sup>: PTH release at 0.5 and 2 mM Ca<sup>2+</sup> were  $1.58 \pm 0.08$  and  $0.37 \pm 0.05$  ng/10<sup>5</sup> cells per 30 min, respectively, while PTH secretion with 0.5 mM Ca<sup>2+</sup> and 5 mM fluoride was  $0.59 \pm 0.06$  ng/10<sup>5</sup> cells per 30 min.

#### 4. DISCUSSION

The parathyroid gland is unusual among exocytotic systems in that high extracellular Ca<sup>2+</sup> inhibits, rather than stimulates, PTH release. The effects of Ca<sup>2+</sup> on parathyroid function are pleiotropic, including not only inhibition of secretion

but also reduction in the levels of mRNA for PTH and inhibition of parathyroid cellular proliferation, as well as modulation of several putative mediators, including cAMP, membrane potential, PI turnover, and the cytosolic Ca<sup>2+</sup> (review [6]). Other divalent cations produce similar effects on parathyroid function, including Mg<sup>2+</sup>, which is about 3-fold less potent than Ca<sup>2+</sup> on a molar basis [9]. The mechanisms underlying these diverse actions of polyvalent cations on parathyroid function, however, have remained obscure.

Recent data have suggested that Ca<sup>2+</sup> and other polyvalent cations exert their effects on the parathyroid gland via a cell surface receptor for these ions. The divalent cation-induced depolarization of rat parathyroid cells could not be explained simply by a change in electromotive force across the plasma membrane, making it necessary to postulate the existence of a cell-surface receptor regulating membrane potential [5]. Furthermore, the increase in cytosolic Ca<sup>2+</sup> associated with elevations in extracellular Ca<sup>2+</sup> are reminiscent of those produced by a variety of secretagogues interacting with cell-surface receptors in other types of cells [1]. That is, Ca<sup>2+</sup> produces an initial 'spike' in cytosolic Ca<sup>2+</sup>, which arises from intracellular Ca<sup>2+</sup> stores, followed by a sustained increase in cytosolic Ca<sup>2+</sup> which derives from uptake of extracellular Ca<sup>2+</sup> [4,6]. Finally a variety of factors binding to the parathyroid cell surface, such as lectins [11], naturally occurring autoantibodies [12], and monoclonal antibodies [13] modulate PTH release [11–13] and the cytosolic calcium concentration [13], consistent with a role for cell-surface moieties in regulating parathyroid function.

The present results further suggest that extracellular divalent cations modulate parathyroid function, at least in part, through a cell-surface receptor or 'sensor'. Ca<sup>2+</sup> and Mg<sup>2+</sup> produce rapid increases in IP<sub>3</sub> which may account for the Ca<sup>2+</sup> or Mg<sup>2+</sup>-induced spikes in cytosolic Ca<sup>2+</sup> via release of cellular Ca<sup>2+</sup> [14] and which occur with a potency which parallels the relative effectiveness of Ca<sup>2+</sup> and Mg<sup>2+</sup> in inhibiting PTH release. It is of interest that these ions continue to raise inositol phosphate content at concentrations which already maximally suppress PTH release, although such discrepancies (e.g. 'spare receptors') are common in the regulation of cellular function by second

messengers. Moreover, lithium, which was included in these dose response studies, has been shown to reduce the sensitivity of PTH release to suppression by high extracellular  $\text{Ca}^{2+}$  [15]. The temporal relationship of the divalent cation-induced changes in  $\text{IP}_1$ ,  $\text{IP}_2$ , and  $\text{IP}_3$  are consistent with phospholipase C-mediated hydrolysis of polyphosphoinositides, particularly  $\text{PIP}_2$ . Furthermore, fluoride ion, which has been found to activate the G-protein mediating activation of phospholipase C in other cells [10], produces changes in inositol phosphates in parathyroid cells which are similar to those brought about by high extracellular  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . By analogy with such other cells, therefore, extracellular divalent cations may bind to a cell-surface receptor which is coupled to phospholipase C via a G-protein.

In addition to changes in  $\text{IP}_3$ , calcium-induced changes in other second messengers might also be mediated by a receptor coupled to one or more G-proteins. Although the lowering of intracellular cAMP content by high extracellular  $\text{Ca}^{2+}$  [16] could be related to concomitant changes in cytosolic  $\text{Ca}^{2+}$ , secondarily inhibiting adenylate cyclase or activating phosphodiesterase, activation of  $\text{G}_i$  by a putative  $\text{Ca}^{2+}$  receptor could also reduce cellular cAMP. Similarly, G-proteins have also been found recently to modulate potassium [17] and  $\text{Ca}^{2+}$  channels [18], thereby providing potential mechanisms for  $\text{Ca}^{2+}$ -induced changes in membrane potential [5] and in cytosolic  $\text{Ca}^{2+}$  due to uptake of extracellular  $\text{Ca}^{2+}$  [4,6], respectively. It is also possible that the sustained increases in cytosolic  $\text{Ca}^{2+}$  at high extracellular  $\text{Ca}^{2+}$  might result from alterations in PI metabolism, since  $\text{IP}_4$  (inositol 1,3,4,5-tetrakisphosphate) has recently been implicated in mediating uptake of extracellular  $\text{Ca}^{2+}$  [19].

We employed cultured cells in these studies to achieve sufficient incorporation of [ $^3\text{H}$ ]inositol to label the pool of polyphosphoinositides yielding  $\text{IP}_3$  and  $\text{IP}_2$ . Although cells were preincubated for 20–28 h with [ $^3\text{H}$ ]inositol, it is possible that complete isotopic equilibrium was not achieved. Thus, changes in [ $^3\text{H}$ ]inositol phosphate accumulation may not have provided a totally accurate reflection of changes in the mass of these compounds. Moreover, it is possible that the  $\text{IP}_3$  measured in this study may represent a mixture of inositol 1,4,5- and 1,3,4-trisphosphate. Nevertheless, the rapidity

and temporal sequence of the appearance of  $\text{IP}_3$ ,  $\text{IP}_2$  and  $\text{IP}_1$  suggest hydrolysis of phosphoinositides, and inositol 1,3,4-trisphosphate is thought to arise from this process via  $\text{IP}_4$  [20].

In contrast to other secretory cells, activation of PI hydrolysis in the parathyroid cell by divalent cations or fluoride is associated with inhibition, rather than of stimulation, of PTH release. This relationship is particularly intriguing, since in parathyroid cells, as in other cells, agents activating protein kinase C, such as the phorbol ester TPA, stimulate hormone secretion [21]. Moreover, Morrissey has reported that high extracellular  $\text{Ca}^{2+}$  concentrations lower cellular diacylglycerol (DG) levels [22], rather than raising them as might be expected from activation of phosphoinositide cleavage. Presumably, other mechanisms are responsible for any divalent cation-induced lowering of cellular DG, and these may ultimately determine the secretory response to high extracellular divalent cation concentrations. It is also conceivable that a divalent cation receptor could be directly linked to the exocytotic process via an inhibitory mechanism, without any obligatory linkage to intracellular second messengers.

## ACKNOWLEDGEMENTS

The authors gratefully acknowledge the expert secretarial assistance of Connie Chisari and Joan Munroe. These studies were supported by USPHS grants AM36796 and AM36801 and grant no. PDT230 from the American Cancer Society.

## NOTE ADDED IN PROOF

Shoback [23] has recently reported in abstract form that high extracellular  $\text{Ca}^{2+}$  enhances accumulation of  $\text{IP}_1$  in parathyroid cells.

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