Guanine nucleotides are potent secretagogues in permeabilized parathyroid cells

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We studied the effects of GTP and its' analogues on PTH release in permeabilized parathyroid cells to assess their role in mediating the unusual inverse relationship between Ca^{2+} and PTH release in intact parathyroid cells. Both 10–5 M GppNHp and GTPγS, nonhydrolysable analogues of GTP, produce up to an 8-fold enhancement of PTH release, which is dose-dependent. This effect is specific for GTP analogues as we could not mimic it with other nucleotides. 10^{-3} M GDPβS, a nonhydrolysable GDP analogue, completely abolishes GppNHp-stimulated hormone release, providing further support for mediation of this effect by a guanine-nucleotide regulatory protein. In GppNHp-stimulated cells, PTH release is maximal at free [Ca^{2+}] less than 200 nM and progressively decreases as the free [Ca^{2+}] increases from 300 nM to 100 μM. These results suggest the presence of a guanine-nucleotide binding protein in the parathyroid cell that may play an important role in the regulation of PTH secretion by Ca^{2+} and perhaps other secretagogues.

PTH release *Permeabilized cell* *Guanine nucleotide* *Ca^{2+}*

1. INTRODUCTION

The parathyroid cell is unusual for its inverse relationship between both the extracellular [1] and cytosolic [2,3] Ca^{2+} concentrations and PTH release. This relationship is the opposite of classical stimulus-secretion coupling [4] in that high Ca^{2+} concentrations inhibit rather than stimulate PTH secretion. The mechanisms underlying the inhibitory effects of Ca^{2+} on PTH release remain obscure. Recent work has suggested that extracellular Ca^{2+} and other polyvalent cations may interact with a 'receptor' or 'sensor' on the plasma membrane of the parathyroid cell, which regulates the permeability of the membrane to K^+ [5] as well as Ca^{2+} [3,6,7]. In addition, PTH secretion appears to be closely related to a variety of intracellular second messengers, including cAMP [8], cytosolic Ca^{2+} [2], and diacylglycerol [9]. In many systems, the interactions of extracellular ligands with cell surface receptors is transduced into intracellular signals via guanine-nucleotide binding proteins (N proteins) [10]. It is possible, therefore, that N proteins might play a role in the recognition of changes in the extracellular Ca^{2+} concentration by the parathyroid cell. In fact, Fitzpatrick and Aurbach [11] have reported recently that preincubation with pertussis toxin prevents the inhibitory effects of Ca^{2+} on PTH release. In the present studies, we have investigated further the potential role of N proteins in PTH release using parathyroid cells permeabilized by electroshock [12]. In this model system, extracellular guanine nucleotides have direct access to the secretory apparatus, and we demonstrate that nonhydrolysable analogues of GTP have po-

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Guanine nucleotide-stimulated PTH release is maximal at low free Ca^{2+} (10^{-9} to 2.4×10^{-7}) (see fig.5, below). Low free Ca^{2+} concentrations were used, therefore, in these and subsequent experiments unless otherwise indicated

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tent stimulatory effects on PTH release which are inhibited by physiologically relevant Ca^{2+} concentrations.

2. MATERIALS AND METHODS

Dispersed bovine parathyroid cells are prepared by enzymatic digestion of minced parathyroid tissue from calves as described [8]. The cells are then washed three times with Eagle's minimal essential medium (bicarbonate, Ca^{2+} and Mg^{2+} deleted) with 20 mM Hepes, 1.5 mM $CaCl_2$, 0.5 mM $MgSO_4$ and 0.1% (w/v) BSA, pH 7.4 (274 mOsm). After the final wash, the cells needed for a given experiment are resuspended in 1 ml of a solution containing 119.3 mM potassium glutamate, 5 mM ATP, 7 mM Mg acetate, 4.3 mM glucose, 17.2 mM K Pipes, pH 6.9, 1 mM EGTA and 0.1% BSA ('K-Glu'; 272 mOsm). The cell suspension is then placed in a plexiglass well, 1.5 cm deep and 1.5 cm wide, containing two stainless steel electrodes positioned 0.5 cm apart. The cells are shocked 5 times at 10-s intervals using a reconditioned Hewlett-Packard cardiac defibrillator which delivers 2 kV over 200 μs. Cells permeabilized in this fashion are unchanged morphologically by light microscopy. In addition, at least 90% of the permeabilized cells take up trypan blue (M_r 961) for a minimum of 60 min, versus 5% or less of control (nonpermeabilized) cells incubated in the same medium. Finally, shocked cells lose 90% of cellular ^{86}Rb taken up during an hour preincubation within 1.5 min after electroshock (vs 5–10% for control cells), thereby showing bidirectional movement of small solutes across the plasma membrane following cell permeabilization.

After permeabilization the cells are removed and collected at a final concentration of 2×10^6 cells/ml into 5 ml incubation vials containing K-Glu with varied Ca^{2+} concentrations with or without other agents as detailed in section 3. After incubation for varying durations at 37°C in a metabolic incubator, the cells and media are sedimented in a desktop centrifuge at $100 \times g$ for 2 min. PTH in the supernatant is determined by RIA as published before, using either an antiserum recognizing intact bPTH(1–84) and C-terminal fragments of the hormone (GW-1) or another antiserum (CK-13; generously provided by Dr G.V. Segre) recognizing the intact hormone and N-terminal fragments of PTH [13]. Unless otherwise indicated, the results given below were measured by C-terminal assay. cAMP in the cellular pellet was extracted with TCA and measured by RIA [14]. The Ca^{2+} concentrations in incubation media were determined by a Ca^{2+} sensitive microelectrode as used by Levy and Fein [15]. Lactate dehydrogenase (LDH) activity was measured using a kit (Sigma, St. Louis, MO, cat.no.500). Results were evaluated using the unpaired Student's t-test and statistical significance was assigned to P values < 0.05.

3. RESULTS

As shown in fig.1, PTH release from permeabilized parathyroid cells under basal conditions gradually increases from 0.38 ± 0.08 at 5 min to 1.26 ± 0.14 ng/10^5 cells at 60 min. Conversely, in the presence of 10^{-5} M GppNHp, there is an 8.2-fold enhancement of PTH release above basal values to 3.16 ± 0.55 ng/10^5 cells at 5 min, and hormone release rises slightly to 3.75 ± 0.39 ng/10^5 cells at 15 min. The stimulation of PTH release with GppNHp is dose-dependent between 3×10^{-7} and 10^{-4} M (fig.2). In contrast to the effects of GppNHp in permeabilized cells, incubation of intact, nonpermeabilized parathyroid cells with 10^{-5} M GppNHp had no effect on PTH secretion. As shown in fig.2, GTPγS, another

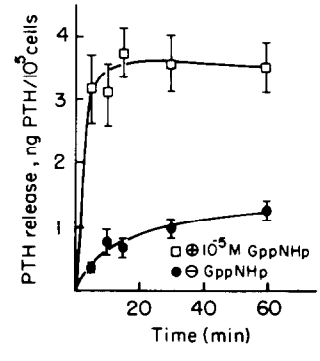

Fig.1. Time course for basal and GppNHp-stimulated PTH release. Parathyroid cells were permeabilized as described in section 2 and incubated in K-Glu with or without 10^{-5} M GppNHp for 5 to 60 min. At the end of the experiment, the cells were sedimented and PTH in the supernatant was assayed by RIA. Each point represents the mean ± SE for 3 experiments each performed in triplicate and assayed in duplicate.

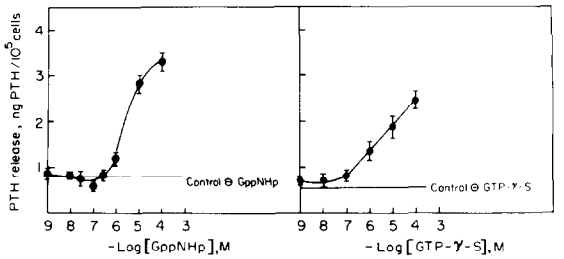

Fig.2. Dose-response curves for the effects of GppNHp and GTPγS on PTH release. Permeabilized parathyroid cells were incubated in varied concentrations of the nonhydrolysable GTP analogues for 15 min and PTH was measured by RIA as described above (N = 4–15).

nonhydrolysable analogue of GTP, also produces a dose-dependent rise in PTH release between 10^{-9} and 10^{-4} M. At 10^{-4} M GTPγS, the increment in PTH release above control is similar to that observed with equimolar concentrations of GppNHp.

In 3 experiments, the release of LDH from permeabilized parathyroid cells was slightly, but not significantly, higher than from unshocked cells. GppNHp (10^{-5} M) had no significant effect on LDH release (not shown), suggesting that GppNHp did not increase hormone release by causing nonspecific release of cellular contents. In addition, GppNHp-enhanced PTH release as measured by an N-terminal assay was comparable to or greater than that observed using a C-terminal assay (not shown), consistent with stimulation of the release of intact hormone.

Guanine nucleotide-enhanced hormone release is specific for guanine nucleotide triphosphates as the effect is not mimicked by the GDP analogues GDPβS, GDP, CTP, or UTP (fig.3). Of note, GTP does not significantly increase PTH release in permeabilized cells, most likely because it is rapidly cleaved by intracellular GTPases. cGMP (not included here) also had no significant effect on PTH release compared to control cells ($P > 0.05$). To assess whether GDPβS might compete with GTP for an intracellular guanine-nucleotide binding site, we incubated permeabilized parathyroid cells with 10^{-5} M GppNHp and varied concentrations of GDPβS. As shown in fig.4, 10^{-5} M GDPβS attenuates the GppNHp-enhanced hormone release by 50% (from 2.73 ± 0.38 to 1.36 ± 0.36 ng/105 cells) and 10^{-3} M GDPβS totally abolishes GppNHp-stimulated PTH release (fig.4).

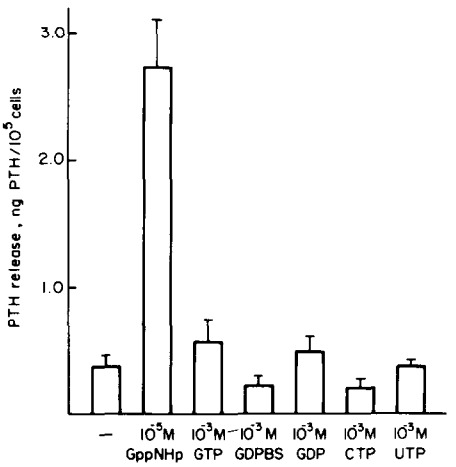

Fig.3. The effects of different nucleotides on PTH release. Permeabilized parathyroid cells were incubated with each of the nucleotides shown for 15 min and PTH was measured by RIA. None of these nucleotides produced any significant stimulation of PTH release compared to the control (N = 9–18).

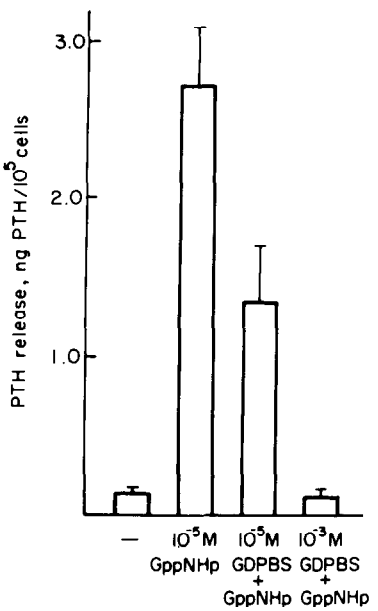

Fig.4. The effects of the GDP analogue GDPβS on GppNHp-stimulated PTH release. Incubations of permeabilized parathyroid cells were carried out under control conditions as well as in the presence of 10^{-5} M GppNHp with or without varied concentrations of GDPβS for 15 min using the methods described above (N = 9–12).

To evaluate the possibility that this guanine nucleotide-stimulated hormone release in permeabilized cells might account for the enhanced secretion of PTH at low free Ca^{2+} and inhibition at higher Ca^{2+} concentrations in intact parathyroid cells, we examined the effects of Ca^{2+} on basal and GppNHp-stimulated hormone release (fig.5). As demonstrated previously, cells incubated with Ca^{2+} alone exhibit little change in PTH release at Ca^{2+} concentrations less than 10^{-6} M, and PTH release increases 2-fold at higher Ca^{2+} concentrations, with half-maximal and maximal values at 10^{-5} and 10^{-4} M, respectively [12] (fig.5). Conversely, in GppNHp-stimulated cells, PTH release is maximal at low free Ca^{2+} concentrations, ranging from 10^{-9} to $\sim 2.4 \times 10^{-7}$ M, and is inhibited progressively as the free $[Ca^{2+}]$ increases from 3×10^{-7} M to 100 μM.

To investigate possible cellular mechanisms underlying the stimulatory effects of GppNHp on hormone release, we evaluated the effect of this GTP analogue on cAMP content because of the known role of guanine-nucleotide regulatory proteins in coupling the interaction between receptors and adenylate cyclase [10]. GppNHp (10^{-5} M) produced a 1.98-fold rise in cellular cAMP content in permeabilized parathyroid cells at 5 min (N = 8–9), in the presence of a 6-fold rise in PTH release. Isoproterenol (10^{-5} M), however, which elevates cAMP in intact parathyroid cells [8] increased cAMP levels 5-fold in permeabilized cells at 5 min but had little or no effect on PTH release. Thus, changes in cAMP cannot account quantitatively for the effects of GppNHp on PTH release.

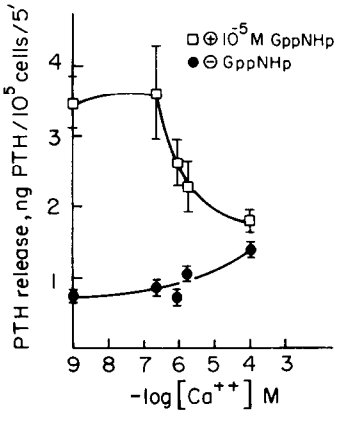

Fig.5. The effects of varied free Ca^{2+} concentrations on basal and guanine nucleotide-stimulated PTH release. Permeabilized parathyroid cells were incubated for 5 min in K-Glu with Ca-EGTA buffers so as to attain free Ca^{2+} concentrations ranging from 10^{-9} to 10^{-4} M and PTH was determined in the supernatant (N = 9).

4. DISCUSSION

Unlike classical stimulus-secretion coupling, high Ca^{2+} concentrations inhibit rather than stimulate PTH release in the parathyroid cell due to poorly defined mechanisms. Previous studies of parathyroid cell secretory function have been performed using intact cell preparations and have been hindered by the complex ionic gradients across the plasma membrane and the lack of direct access to the internal cellular environment and the secretory apparatus. To elucidate the mechanisms underlying the control of exocytosis in the parathyroid cell, we have recently developed a system of permeabilized parathyroid cells using a method of electroshock [16]. In examining the effects of Ca^{2+} on PTH release, we were surprised to observe that in the parathyroid cell, like other cells showing classical stimulus-secretion coupling, high free Ca^{2+} concentrations increase PTH release 2–3-fold, with half-maximal stimulation occurring at 10^{-5} M Ca^{2+} [12]. However, over the physiologic range of cytosolic free Ca^{2+} concentrations in the intact parathyroid cell (10^{-7} to 10^{-6} M as measured with quin2 [2]), PTH release is low and Ca^{2+} has no effect on the secretion of hormone. Moreover, these low secretory rates for PTH cannot account for the enhanced PTH secretion observed at reduced extracellular Ca^{2+} concentrations in the intact cell, which are associated with intracellular free Ca^{2+} concentrations of 100–200 nM. These studies reveal that the basic blueprint for secretion in the parathyroid cell is similar to other exocytotic cells, and therefore, mediators other than or in addition to cytosolic Ca^{2+} must invert the secretory response to Ca^{2+} in the intact parathyroid cell.

In the present investigations, we demonstrate that the nonhydrolysable analogues of GTP, GppNHp and GTPγS, produce a dose-dependent stimulation of PTH release which is specific for GTP analogues, providing support for mediation of this effect by a guanine-nucleotide regulatory

protein. In addition, 10^{-3} M GDPβS completely abolishes the GppNHp-enhanced hormone release. These data concur with similar observations in other permeabilized cell systems and further suggest that GDPβS may be interacting with a GTP binding site on a guanine-nucleotide regulatory protein in the parathyroid cell [17].

We also examined the effects of Ca^{2+} on GppNHp-stimulated hormone release. At low Ca^{2+} concentrations ($\leq 2.4 \times 10^{-7}$ M), equivalent to those in the intact cell at reduced extracellular Ca^{2+}, PTH release is maximal at ~3–4 ng/10^5 cells (figs 1,5). This rate of PTH release in the presence of guanine nucleotide can readily account for that observed at low extracellular Ca^{2+} concentrations (~3–5 ng/10^5 cells/h) in the intact parathyroid cell [18]. Moreover, there is a progressive decrease in guanine nucleotide-stimulated hormone release as the free Ca^{2+} concentrations increase from 300 nM to as high as 100 μM in permeabilized parathyroid cells. This is phenomenologically similar to the Ca^{2+}-induced suppression of PTH secretion in nonpermeabilized cells. The inhibitory effects of Ca^{2+} on PTH release in intact parathyroid cells, therefore, may result from a Ca^{2+}-induced reduction in guanine nucleotide-stimulated PTH secretion either via inhibition of a stimulatory N protein and/or, as suggested by Fitzpatrick and Aurbach [11], activation of an antagonist guanine-nucleotide regulatory protein.

Guanine-nucleotide binding proteins transduce extracellular signals into a variety of intracellular events. The GppNHp-induced rise in intracellular cAMP in permeabilized cells, presumably mediated via activation of Ns [10] (the guanine-nucleotide regulatory protein activating adenylate cyclase) is one possible mechanism for the effects of guanine nucleotides on PTH release. Isoproterenol, however, which produces an even larger rise in cAMP, has essentially no effect on PTH, indicating that GppNHp must act via other mechanisms. The lack of effect of isoproterenol on PTH release in permeabilized cells contrasts with its potent stimulatory effects on PTH secretion from intact parathyroid cells, suggesting that the intracellular mechanisms underlying the actions of cAMP on PTH release are not active in permeabilized cells. Other possible mechanisms through which GppNHp might modify PTH release include effects on an ion channel [19], activation of phospholipase C [20] resulting in stimulation of protein kinase C and/or direct effects on secretion [17].

In summary, nonhydrolysable GTP analogues are potent secretagogues in permeabilized parathyroid cells. The nucleotide specificity of this effect and the inhibition of guanine nucleotide-stimulated hormone release by GDPβS and by high Ca^{2+} suggest that a guanine-nucleotide regulatory protein in intact parathyroid cells may play an important role in the regulation of PTH secretion.

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