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Role of anions in parathyroid hormone release from dispersed bovine parathyroid cells

(exocytosis/secretion/osmotic lysis/chloride/hydroxyl ion)

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ABSTRACT **It is known that permeant anions are required for the release of epinephrine from isolated chromaffin granules and of serotonin from intact platelets. We have now investigated the role of anions in the release of a polypeptide hormone, parathyroid hormone, from dispersed bovine parathyroid cells. The release is inhibited 60%–80% by decreasing either [Cl^-] or [OH^-] and 60%–70% by replacement of NaCl with the impermeant anion isethionate. By contrast, substitution of various monovalent cations in the medium had no effect on the release. Disodium 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS) and probenecid, which are known to block anion transport in the erythrocyte, also cause a dose-dependent 90%–100% inhibition of release. Moreover, kinetic analysis of inhibition by probenecid suggests that it is competitive with respect to either OH^- or Cl^-. These results suggest that anions and the anion transport system may play a role in exocytosis of a polypeptide hormone. The proton ionophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was also found to block hormone release, and the possibility is discussed of a "chemosmotic" mechanism for exocytosis in this system similar to that previously postulated for chromaffin granules and platelets.**

Parathyroid hormone (PTH) is released from dispersed bovine parathyroid cells upon exposure to β-adrenergic agonists and to decreased ambient calcium concentration (1); similar responses occur *in vivo*. The presence of secretory granules within the parathyroid cells (2, 3) as well as the particulate nature of PTH in cell homogenates (4) have led to the conclusion that PTH is stored in the granules and that the mechanims of hormone release might be via exocytosis.

The chemical basis of exocytosis in parathyroid and other cells is poorly understood although recent studies have implicated anion transport in this general process. Permeant anions are required for the release of epinephrine from isolated chromaffin granules (5–7) and for serotonin secretion from human platelets (8). In addition, anion transport blocking drugs, which do not readily penetrate plasma membranes, such as disodium 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS) (9, 10), probenecid (11), and others, block hormone release from both systems (7, 8). Kinetic analysis with the blockers suggested that the anions of physiologic importance were Cl^- for granules and OH^- for platelets. The actual motive force for release from both granules and platelets appeared to be osmotic lysis because stimulated release was also suppressed by increased osmotic strength of the medium. It was thus possible that PTH release might prove sensitive to similar agents. In the present report we show that PTH release from dispersed parathyroid cells is inhibited by drugs known to block anion transport and that both Cl^- and OH^- appear to support hormone release in this system.

MATERIALS AND METHODS

Dispersed parathyroid cells were prepared by digestion of tissue with collagenase and DNase as described (1). Incubations were carried out in 20-ml polypropylene scintillation vials (Beckman) in a 37° metabolic shaker (Dubnoff-Precision Scientific Instruments), with Eagle's medium number 2 (bicarbonate deleted) containing 0.02 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethansulfonic acid (Hepes) (pH 7.5), 0.5 mM $MgSO_4$, variable $CaCl_2$ as indicated, and 0.2% heat-inactivated bovine serum albumin (medium A). In experiments in which sodium chloride, sodium isethionate, or sucrose were varied, the following medium (medium B) was used: dextrose, 1 g/liter; K_2HPO_4, 0.25 g/liter; KCl, 0.3 g/liter; Hepes, 20 mM, pH 7.5 or as indicated; and 0.2% bovine serum albumin. Less than 10% of the calcium is protein-bound in either medium.

Supernatant samples were collected in 400-μl Microfuge tubes (Beckman) and assayed for PTH by using a guinea pig anti-bovine PTH antiserum. The drugs used had no effect on the radioimmunoassay at the concentrations in these experiments. Cell counts were carried out with a hemocytometer just prior to incubation. Cell viability by trypan blue exclusion was routinely 95%–100%. Reagents were of the best grade commercially available and were obtained from sources previously cited (1). SITS was obtained from British Drug House; sodium isethionate and probenecid were obtained from Sigma Chemical Co.; and FCCP was a gift from G. Weissbach.

RESULTS

Effects of Alterations in Anion Concentration on PTH Release. PTH release, in response to low[Ca], was suppressed 70%–80% when [Cl^-] was decreased from 150 mM to 15 mM by isotonic replacement of NaCl by sucrose (Fig. 1). The complete titration of release by chloride revealed a sigmoid curve with apparent threshold at 15–20 mM Cl^- and a $K_{1/2}$ of ~50 mM. Replacement of NaCl by sodium isethionate also led to a 70% inhibition of PTH release [isethionate is a large, polar anion that does not readily cross plasma membranes (12, 13)].

Because OH^- has been shown to be the important permeant anion in regulating serotonin release from platelets (8), we tested the effect of pH variation on PTH release. Decreasing the pH from 7.5 to 6.3–6.4 in Eagle's medium 2 caused 60%–70% in-

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Abbreviations: PTH, parathyroid hormone; SITS, disodium 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

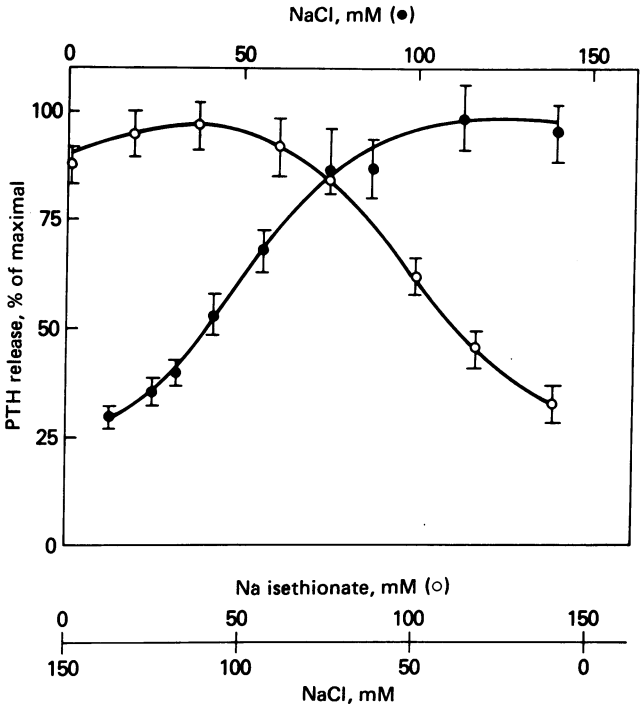

FIG. 1. Effect of chloride and isethionate on PTH release from dispersed bovine parathyroid cells. Bovine parathyroid cells (3–4 × 10^5/ml) were incubated in medium B with 0.5 mM $CaCl_2$, 0.5 mM $MgSO_4$, and 0.2% heat-inactivated bovine serum albumin for 1 hr at 37°. Points indicate percentage of maximal release observed with isosmotic replacement of 150 mM NaCl by sucrose (●) or isosmotic replacement of 150 mM NaCl by sodium isethionate (○). Data shown are means ± SEM for duplicate determinations on triplicate incubation vials.

hibition of low [Ca]-stimulated PTH release, and 50% of maximal inhibition was obtained at pH 6.7–6.8 (Fig. 2). The exposure of cells to pH 6.4 was not in itself toxic because incubation for 90 min at this pH did not affect the activity of cells upon their return to pH 7.5 (Fig. 3). The pH effect was also observed when bicarbonate was used in place of Hepes.

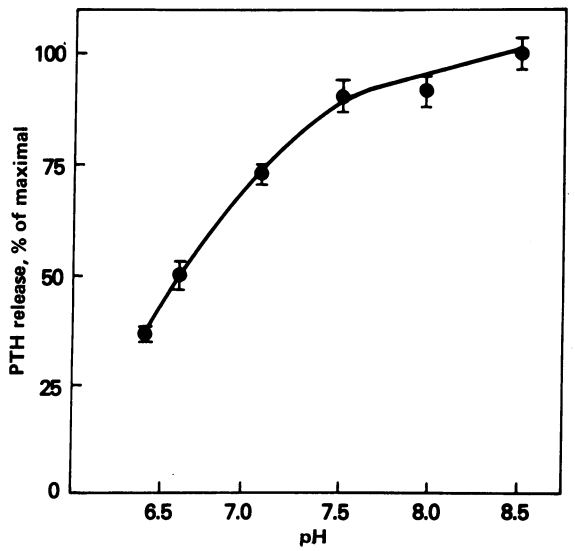

FIG. 2. Effect of altering [OH^-] on PTH release from dispersed bovine parathyroid cells. Bovine parathyroid cells (3 × 10^5/ml) were incubated in medium A at 37° with 0.5 mM $CaCl_2$ at the indicated pH for 1 hr. Points indicate the means ± SEM for duplicate determinations on six incubation vials in two separate experiments.

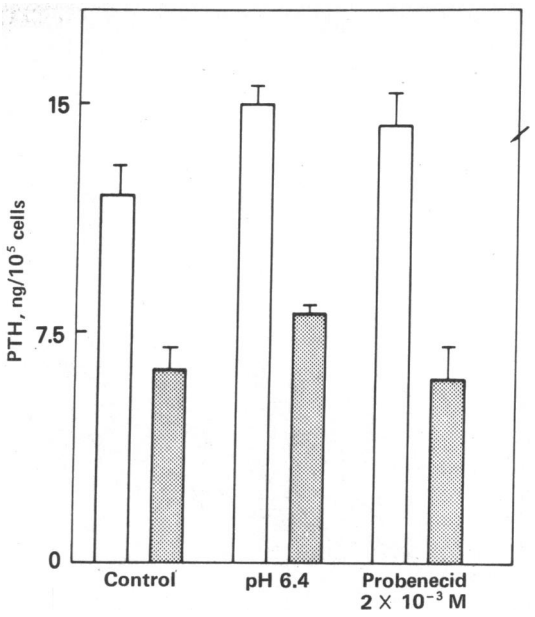

FIG. 3. Reversibility of the inhibitory effect of low pH and probenecid in PTH release from dispersed bovine parathyroid cells. Dispersed cells (4 × 10^5/ml) were incubated for 90 min at 37° with 0.5 mM $CaCl_2$ in medium B at pH 7.5 (Control), in medium B at pH 6.4 (pH 6.4), or in medium B at pH 7.5 with 2 mM probenecid (Probenecid). The cells were then sedimented and resuspended three times in medium B and incubated for 30 min at 37° in medium B at pH 7.5 with either 0.5 mM $CaCl_2$ (open bars) or 1.5 mM $CaCl_2$ (stippled bars). Results are shown as means ± SEM for six determinations.

Effect of Anion Transport Blocking Drugs on PTH Release. Studies with isolated chromaffin granules (7) and platelets (8) have shown that drugs known to inhibit anion transport in erythrocytes block epinephrine and serotonin release, respectively. We therefore examined the effect of these drugs on PTH release. As shown in Fig. 4, both SITS and probenecid inhibited PTH release in a dose-dependent fashion. The curves appeared to be parallel, suggesting that the agents were acting on similar sites.

The nature of the inhibition by probenecid was then analyzed by the method of Dixon (14) with Cl^- and OH^- treated as possible substrates. As indicated in Fig. 5, the inhibition curves

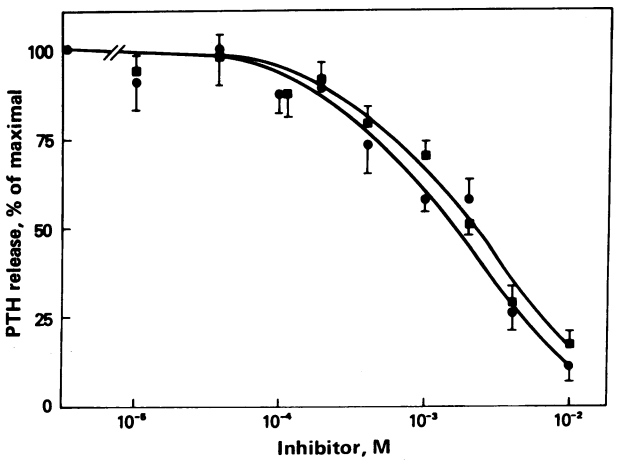

FIG. 4. Effect of anion transport blockers on PTH release from dispersed cells. Parathyroid cells (3 × 10^5/ml) were incubated at 37° for 1 hr in medium A with 0.5 mM $CaCl_2$, 0.2% heat-inactivated albumin, and indicated concentrations of SITS (●) or probenecid (■). Points indicate the mean ± SEM for two determinations on triplicate incubation vials.

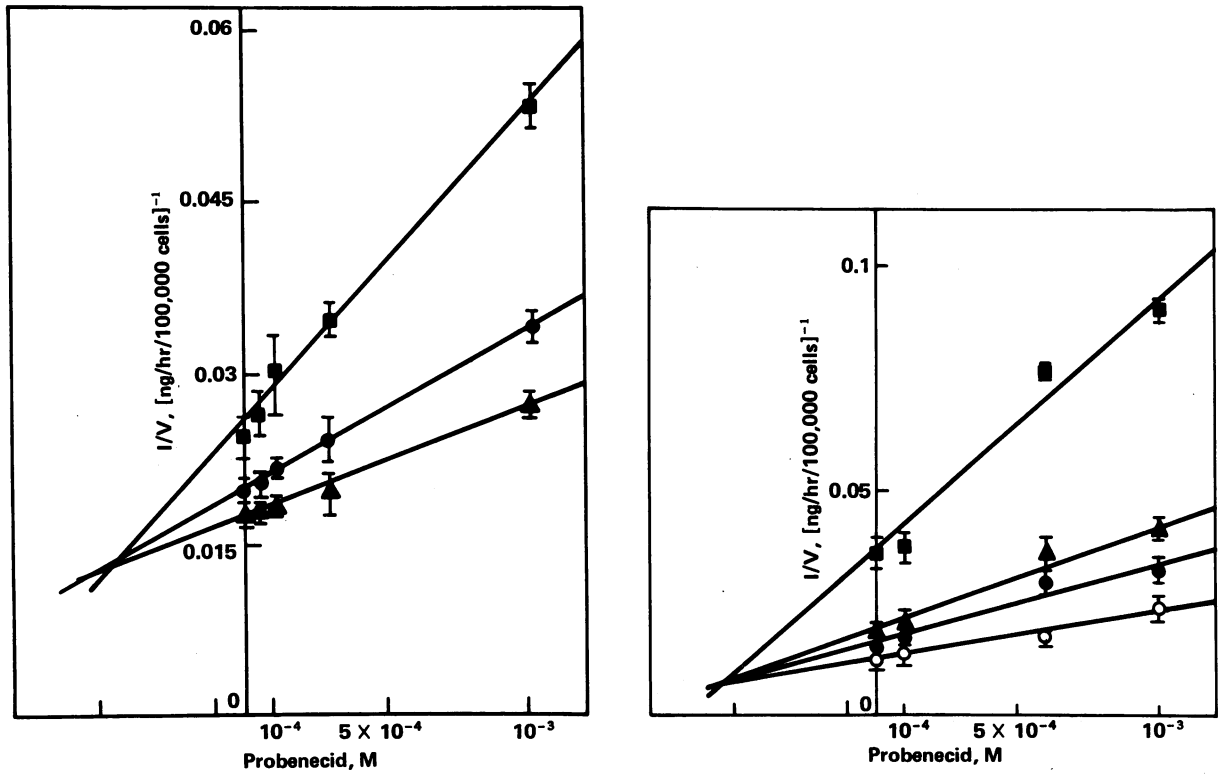

FIG. 5. Kinetic analysis (14) of the inhibition of PTH release from dispersed bovine parathyroid cells by probenecid. Results are shown as means ± SEM for six determinations on triplicate samples. (*Left*) As a function of OH^- concentration. Dispersed cells (4 × 10^5/ml) were incubated at 37° for 60 min in medium A with 0.5 mM $CaCl_2$ and varying concentrations of probenecid at pH 7.1 (■), 7.5 (●), or 7.9 (▲). (*Right*) As a function of Cl^- concentration. Dispersed cells (4 × 10^5/ml) were incubated at 37° for 60 min in medium B with 0.5 mM $CaCl_2$ and varying concentrations of probenecid and 25 mM (■), 50 mM (▲), 75 mM (●), or 150 mM (○) Cl^-. Chloride concentration was varied by replacing 150 mM $NaCl_2$ isotonically with sucrose.

intersected in the fourth quadrant. This result suggested that probenecid was a competitive inhibitor with respect to both $[Cl^-]$ and $[OH^-]$. The K_i for probenecid with respect to both anions was 4–6 × 10^{-4} M in several experiments.

Cells exposed to 2 mM probenecid for 90 min maintained the capacity for secretory response to low or high calcium when washed free of the drug. In addition, exposure to probenecid or pH 6.4 caused no effect on cellular ultrastructure (not shown).

Table 1. Effect of cation vs. anion substitution on PTH release

Medium*	PTH release (mean ± SEM), %
NaCl, 150 mM	100 ± 8
KCl, 150 mM	91 ± 8
Choline Cl, 150 mM	104 ± 11
TEA Cl, 150 mM	98 ± 12
NaCl, 150 mM + FCCP, 2 μM	4 ± 10
Sucrose, 300 mM	36 ± 7
Eagle's medium no. 2, pH 6.0	15 ± 2

Dispersed parathyroid cells (3 × 10^5/ml) were incubated in saline-containing media [medium (150 mM NaCl) or Eagle's medium number 2 (salt composition includes NaCl 6.8 g/liter (116 mM), KCl 0.4 g/liter, $NaH_2PO_4 \cdot H_2O$ 0.15 g/liter, and Na Hepes 20 mM, pH 7.5.)] or in media in which NaCl was replaced with the constituents indicated. PTH release was determined from control (1.0 mM Ca^{2+}, 0.5 mM Mg^{2+}) or in isoproterenol-stimulated [1.0 mM Ca^{2+}, 0.5 mM Mg^{2+} plus 1 μM (−)isoproterenol] cells after a 15-min incubation at 37°. Results are shown as percentage of isoproterenol-stimulated release occurring in 150 mM NaCl.
*TEA, tetraethylammonium.

The effects of probenecid were not limited to calcium-regulated secretion because PTH release stimulated by either (−)isoproterenol (15) or dopamine (16) was 90%–95% inhibited by 10^{-2} M probenecid (not shown).

Effect of Cation Substitution on PTH Release. As shown in Table 1, replacement of NaCl by KCl, tetraethylammonium chloride, or choline chloride had no effect on PTH release, indicating that the species of monovalent cation in the medium was of little importance. By contrast, 2 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), a proton ionophore, decreased secretion to less than 10% of control.

Influence of Osmotic Strength on PTH Release. Increased osmolality suppresses release from chromaffin granules (17, 18) and platelets (8) and we therefore studied the influence of extracellular osmotic strength on PTH release. As shown in Table 2, increasing the osmotic strength up to 600–800 mOsmol of sucrose per kg caused a striking increase in baseline release (high

Table 2. Effect of osmolality on basal and stimulated PTH release

Osmolality, mOsmol/kg	PTH, ng/10^5 cells		
	ISO + 0.5 Ca	1.5 Ca	Difference
300	24.0	8.2	15.8
450	29.2	18.0	11.2
600	37.0	32.6	4.4
1000	17.0	18.6	−1.6

Dispersed cells (4 × 10^5/ml) were incubated for 30 min in medium B with either 1 μM isoproterenol and 0.5 mM Ca (ISO + 0.5 Ca) or 1.5 mM Ca (1.5 Ca). Results are the mean of six determinations on three incubation vials. Difference equals (ISO + 0.5 Ca) − 1.5 Ca.

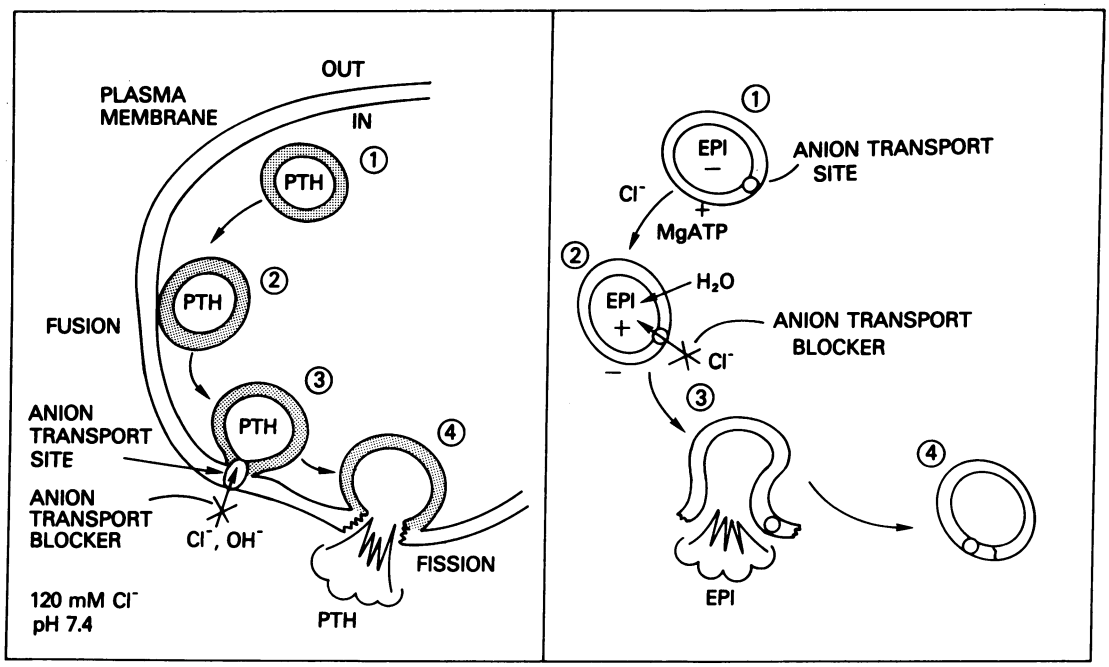

FIG. 6. Proposed analogy between PTH secretion and epinephrine release from isolated chromaffin granules. Both panels depict the time sequence of hormone release. (*Left*) Parathyroid cells. (*1*) An isolated PTH granule free in the cytoplasm becomes (*2*) intimately associated with the plasma membrane in a "fusion" complex. The fused granule becomes more intimately associated with the plasma membrane, and an anion transport site then spans the membrane separating intragranular space and the medium (*3*). The anion transport site may be derived from the granule membrane by analogy with the chromaffin granule system. As with the chromaffin granule, anion transport into the PTH granule might provoke fission (*4*), and anion transport blockers could inhibit this step. By analogy with the chromaffin granule system, this last step may be due to osmotic lysis. (*Right*) Isolated chromaffin granules (5–8). Isolated granules (*1*) are stable until exposed to ATP and Cl^-. ATP makes the granule's interior positive, possibly by means of an inwardly directed electrogenic proton pump. This induces Cl^- entry through an anion transport site (*2*). The osmotic content of the granule increases, possibly due either to increase in ion concentration or to the solubilization of the granule core, and thus the granule bursts (*3*).

calcium); at 1000 mOsmol/kg, PTH release returned to rates near those found at 300 mOsmol/kg. The difference between baseline and maximally stimulated release (low-calcium plus isoproterenol), however, was significantly depressed at both 600 and 1000 mOsmol/kg. Nearly identical results were obtained when osmotic strengths were changed with NaCl rather than sucrose.

DISCUSSION

These results show that PTH release from dispersed bovine parathyroid cells is inhibited by low Cl^- or OH^- concentration or by the addition of drugs known to block anion transport (9–11). The erythrocyte anion transport system is known to transport not only Cl^- but also OH^- (19). Probenecid blocked PTH release in a manner consistent with competitive inhibition of both Cl^- and OH^-, suggesting the possibility that transport of both Cl^- and OH^- might be involved in the PTH release process.‡ The potential importance of anion permeation in this system was further suggested by the observation that media with 150 mM sodium isethionate, an impermeant anion (12, 13), could also suppress release and that monovalent cation substitution had no effect on PTH release. The inhibition constant for probenecid was similar for both Cl^- and OH^- ($4–6 \times 10^{-4}$ M) and was similar to the K_i for probenecid in the OH^--dependent platelet secretion system (6). However, it was higher than inhibition constants reported for inhibition of erythrocyte Cl^- transport (30 μM) or for release from the chromaffin granule (125 μM) (5). This difference in part may reflect the high concentration of nonvariant substrate in the kinetic experiments (pH 7.5 or 150 mM Cl^-), nonspecific interaction of drugs with albumin in the medium,§ tissue variations in transport affinity, or other factors.

In this regard, it was of particular interest that the shape of the curve describing the dependence of PTH release on Cl^- concentration was nearly identical to the Cl^- titration of epinephrine release from chromaffin granules (7). This evidence suggests direct mechanistic relationships between release from granules and secretion of PTH. A difference, however, lies in the additional sensitivity of parathyroid cells to OH^-. These different specificities for anions may reflect an additional constraint upon the granule release system when it is enclosed in the plasma membrane of the intact cell. Alternatively, serotonin granules, PTH granules, and chromaffin granules may be sufficiently distinct chemically to explain the difference. A detailed comparison of these granule systems is clearly indicated.

Increasing the osmotic strength of the medium up to 600 mOsmol/kg led to an increase in baseline PTH release from dispersed cells, although this was followed by a secondary decrease at 1000 mOsmol/kg. The difference between maximally stimulated and baseline release, however, was nearly completely suppressed by osmotic strengths of 600–1000 mOsmol/kg. Although the variation in baseline release made it difficult to interpret these results unambiguously, the inhibition of stimulated release indicated that an osmotic mechanism for PTH release could not be excluded.

‡We cannot exclude the possibility that the effect of lowering pH results from titration of a site both inhibiting PTH release and facilitating probenecid binding.

§ Parathyroid cells require a protein-containing medium for proper cellular function.

In isolated chromaffin granules, epinephrine release is inhibited by increased osmotic strength (17, 18) and by low doses of the proton ionophore FCCP (17). These findings have led to the consideration of a "chemosmotic hypothesis" for release in this system (7, 17). In this formulation (Fig. 6 *right*), ATP-dependent proton transport into granules is accompanied by anion and consequent water uptake, dissolution of granule contents, and osmotic lysis. The striking similarity of results obtained with serotonin release from platelets and PTH release from dispersed parathyroid cells suggests the possibility that a similar chemosmotic mechanism might be involved in exocytosis *in vivo*. A proposed version of such a mechanism is shown in Fig. 6 *left*, using the morphologic model of Palade (20) for exocytosis. As indicated by this model, the artificial anion gradients required to observe release in the isolated chromaffin granule preparations occur naturally across the fused PTH granule–plasma membrane complex. By analogy with the chromaffin granule system, anion uptake from the medium by the fused granule might be accompanied by solubilization of the granule contents, increase in osmotic content, and lysis. Inhibition of PTH release by FCCP is consistent with a possible role of protons as the accompanying counterion required for both electroneutrality and elevation of the osmotic content of the granule. In order to demonstrate anion and proton transport directly, however, and their direct involvement in granule dynamics, further studies with isolated PTH granules will be necessary. Our experiments do not allow us to assess the possible action of this compound on mitochondrial function. The small fraction of the total parathyroid cell volume occupied by fused PTH granules and the transient nature of this complex obviates direct studies of granule anion and cation flux in the intact cell.

Acid–base status has been postulated to affect parathyroid function (21), but we know of no studies on PTH release *in vitro* as a function of pH. On the basis of *in vivo* studies, however, it has been suggested that low pH might stimulate PTH release (21). The present results indicate that, with bovine glands at least, lowered pH results in a highly significant inhibition of PTH release. PTH is known to cause bicarbonaturia as well as phosphaturia *in vivo* (22–24). A decrease in PTH release during acidosis, therefore, might results in a retention of the buffers bicarbonate and phosphate. Further studies on parathyroid function in the intact animal would be necessary to establish if such a mechanism plays a significant role *in vivo*.

The present results are consistent with the prediction of Pollard *et al.* (6, 8) and Pazoles and Pollard (7) that anion transport might play a role in exocytosis from intact cells. The parathyroid cell thus represents a second exocytotic system in which drugs known to block anion transport inhibit secretion in a manner consistent with direct competition with a specific anion.

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