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Swelling of the Vesicle Is Prerequisite for PTH Secretion

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Unlike most secretory cells, high extra cellular calcium inhibits rather than stimulates hormonal secretion in several cells such as parathyroid cells, Juxtaglomerular cells and osteoclast. To gain further insight into the common but unique stimulus-secretion coupling mechanism in these cells, bovine parathyroid slices were incubated in various conditions of Krebs-Ringer(KR) solution containing essential amino acids. Parathyroid cells showed the inverse dependency of secretion on extra cellular calcium concentration as we expected. Ammonium acetate overcame the inhibitory effect of 2.5 mM of calcium and the maximum effect was as much as the five times of the basal value, while there was a little additive effect under 0 mM $CaCl_2$. PTH secretion was biphasic according to the change of extra cellular osmolarity and the lowest response was observed at 300 mOsm/l. In Na-rich KR solution, high concentration of nigericin($>10^{-4}$ M) completely overcame the inhibitory effect of 2.5 mM $CaCl_2$ and the maximum stimulatory effect was 8 times greater whereas it was only 2 times greater without $CaCl_2$. In K-rich KR solution that abolished the K-gradient between the extra cellular solution and the cytoplasm, the rate of PTH secretion increased, and furthermore the addition of nigericin increased the rate of secretion significantly. The results above suggested that the osmotic swelling of the secretory vesicle in parathyroid cells might promote exocytosis as in Juxtaglomerular cells. We propose that the swelling of the vesicle is also prerequisite for secretion in several cells inhibited paradoxically by Ca^{++}, whatever the signal transduction pathway for swelling of the secretory granules induced by the lowering of Ca^{++} in cytoplasm are.

Key Words: Parathyroid hormone, secretion

The inverse sigmoidal control of hormone secretion in parathyroid cells by extra cellular calcium is unusual among many endocrine and exocrine cells(Patt and Luchhardt, 1942; Copp and Davidson, 1961; Sherwood *et al.* 1968, 1970). Parathyroid cells, therefore, appear to be highly anomalous in their secretory control, potentially involving novel mechanisms distinct from those present in most of other secretory cells, exhibiting more classic stimulus-secretion coupling.

By recognition of the change through the calcium receptor recently cloned, a decrease in the extracellular calcium in parathyroid cells stimulates the secretion of the hormone (Brown *et al.* 1994). However, the biochemical events to specific inhibitory step of exocytosis after Ca^{++} binding to its receptor are still unknown.

Recently, indirect evidence obtained in other

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secretory cells has also suggested that changes in the ionic permeability of secretory granules may play a key role in the secretory response. In the secretory granule membrane of neurohypophysis, a Ca^{++} activated K^+ channel and a Cl^- channel was also reported (Lemos *et al.* 1989). These ion channels activated by secretagogues may play an important role, especially in swelling of secretory granules implicit in the exocytosis. The swelling of secretory granule promotes close contact of swollen granules with plasma membrane.

As the calcium activated K^+ channels of the secretory granule membrane provides a driving force for osmotic swelling of secretory granules in other cells, the activation or inactivation of the same channel was speculated upon as a potential mechanism for the stimulation-secretion coupling in parathyroid cells. However, up to the present, there is no experimental evidence to support this (Kanazirska *et al.* 1995). Furthermore, the role of changes in the ionic permeability of plasma membrane and secretory granule membrane has not been extensively explored in parathyroid cells.

In order to test the above possibility as a mechanism of PTH secretion to the change of extracellular Ca^{++}, we have explored the potential role of changes in membrane ion permeability by assessing effects of osmotic swelling by mannitol or ammonium acetate, or the effect of K^+-H^+ ionophore, nigericin.

MATERIALS AND METHODS

Preparation of sliced parathyroid tissue

Even though cultured parathyroid cells have been used for analysis of the biological function, the main limitation are the changes of phenotypic characteristics of the cells during culture. To overcome this problem, we used a thin-sliced parathyroid tissue directly. Bovine parathyroid glands were obtained from the slaughter house and the thin connective capsule and fat covering the gland were removed with forceps and scissors. A piece of parathyroid tissue (5×5×0.3 mm) was sliced by using the tissue slicer blade, Stadie-Riggs microtome as described previously (Park and Marvin, 1978).

Preincubation and incubation

Sliced parathyroid tissue was incubated at 37℃ for 1hr in standard Krebs-Ringer (KR) solution (200 ml) containing essential amino acids to remove tissue debris and parathyroid hormone already secreted. It was continuously gassed with 100% O_2 during whole procedures of experiments. Under the same conditions, a second washing was performed for 60 minutes in 24 well culture dishes with an exchange of the solution (1 ml) every 20 minutes for three consecutive times. The composition of standard KR solution was (in mM): NaCl, 145.0; KCl, 5.0; $MgCl_2$, 1.0; $CaCl_2$, 2.5; glucose, 10.0; HEPES, 10.0. The K-rich KR solution used had identical composition except for 5.0 mM NaCl and 145mM KCl. The pH was adjusted to 7.0. The concentration of $CaCl_2$ was variably adjusted if necessary. Ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetractic acid (EGTA) was used to chelate calcium in media (Park and Malvin, 1978). Preincubation was performed for 15 minutes to 1 hour according to the purpose after washing. The last media during preincubation period were harvested and served as controls. After this, slices were incubated for 15 or 60 minutes each time in medium containing different ionic composition and test drugs for subsequent periods according to the different experiment. Media from each incubation period were collected and the amount of parathyroid hormone was determined by using the PTH-IRMA kit (Nichols Institute). The rate of PTH secretion is expressed as pico grams per ml per hour per 5×5×0.3 mm tissue, or as the fold ratio of PTH level during the experimental period per PTH level during the control period. All data are expressed as means±S.E.

PTH-calcium relationship

Parathyroid sliced-tissue was preincubated in standard KR solution (1 ml) for 60 minutes with four exchanges after washing at every 15 minutes in 24 well culture dish. The time

dependent rate of PTH secretion was studied by replacement of the standard KR solution (2.5 mM $CaCl_2$) with KR solution containing 0 mM $CaCl_2$. The last media of the preincubation and incubation period were harvested for PTH assay, and the slices were incubated for 15, 30, 45, 60 minutes respectively at KR solutions containing 0 mM $CaCl_2$. To test the effect of $CaCl_2$ on PTH secretion, fresh KR solutions (1 ml) containing different concentrations of $CaCl_2$ (0, 0.5, 1.0, 2.0, 3.0 mM, respectively) were applied and incubated for 15 minutes with four redundant exchanges at 37℃. The last media of preincubation and incubation periods were harvested for PTH assay.

The effect of ammonium acetate

Biological membranes show strong permeability to both the uncharged NH_3 and acetic acid. A greater net accumulation of NH_4^+ and AC^- in vesicle can induce a rapid swelling of secretory granules by osmotic gradient (Johnson and Scarpa, 1976; Delisle and Hopfer, 1986). After pre incubation for 60 minutes with three exchanges at every 20 minutes, the KR solutions containing different concentration of ammonium acetate were applied for 20 minutes to test the effect of ammonium acetate on PTH secretion. We also measured the rate of PTH secretion under the KR solutions containing 90 mM ammonium acetate with different concentrations of $CaCl_2$ (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mM).

The effect of mannitol

The incubation of cells into the hypo-osmotic media have been used as a standard method to induce the swelling of cells. After the first preincubation with standard KR solutions containing 2.5 mM $CaCl_2$ for 60 minutes, parathyroid slices were then preincubated for 60 minutes with KR solutions containing different concentrations of $CaCl_2$ respectively and then they were harvested to serve as a control. Finally the modified KR solutions(200, 300, 500, 600, 800 mOsm/l) with the same concentrations of $CaCl_2$ of the second preincubation were applied to a 24 well dish for 60 minutes. The modified KR solutions were prepared by 75 mM NaCl and different concentrations of mannitol respectively. The media were harvested for PTH assay. The standard KR solutions containing the same concentration of $CaCl_2$ were applied respectively at the end of experiment to test the recovery of PTH secretion from osmotic stress.

The effect of nigericin

To test the effect of nigericin on PTH secretion, the slices were pre incubated for 60 minutes in solution with or without $CaCl_2$ after washing, and the media were harvested as a control. Then the slices were incubated for 60 minutes with media containing different concentrations of nigericin (10^{-7}~5×10^{-3} M) at 2.5 mM or 0 mM of $CaCl_2$. We also tested the effect of the different concentrations of $CaCl_2$ at constant concentration of nigericin (10^{-4} M). To clarify the precise mechanism of stimulatory effects of nigericin, we abolished the K-gradient between extracelluar environment and cytoplasm by applying K-rich KR solution and analyzed the effect of nigericin.

Statistics

Results are expressed as the mean±SE. Statistical comparisons were made using Student t test between different groups. P less than 0.05 was accepted as the significance level.

RESULTS

PTH-$CaCl_2$ relationship

The time-dependent rate of PTH secretion was studied by replacement of the standard KR solution (2.5 mM $CaCl_2$) with KR solution containing 0 mM $CaCl_2$. PTH secretion was significantly stimulated and reached the maximal level at the 3rd incubation period and was maintained constantly thereafter (data not shown). When the concentrations of extracellular $CaCl_2$ were varied with the increments of 0.5 mM from 0 mM to 3 mM, the minimal rate of PTH secretion was observed at 2.5~3 mM $CaCl_2$. A gradual increase of

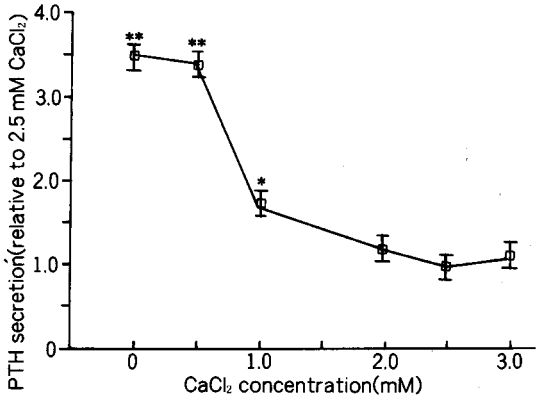

Fig. 1. *The pattern of concentration-dependent parathyroid hormone (PTH) secretion by $CaCl_2$. Values are mean SE from six observations. *P< 0.05, **P< 0.001 as compared to the value in 2.5 mM $CaCl_2$.*

PTH secretion was shown when the extracellular $CaCl_2$ increased from 2.5 mM to 1.0 mM, and the rate of PTH secretion was increased sharply by lowering $CaCl_2$ from 1 mM to 0.5 mM of $CaCl_2$. The maximal rate of PTH secretion was observed at about 0.5 mM $CaCl_2$. The ratio of PTH secretion in the each concentration of $CaCl_2$ to that in 2.5 mM $CaCl_2$ was 3.5 ± 0.25 ($P<0.001$), 3.42 ± 0.3 ($P<0.001$), 1.61 ± 0.2 ($P<0.05$), 1.25 ± 0.3 ($P>0.05$), 1.15 ± 0.25 ($P>0.05$) at 0, 0.5, 1, 1.5, 2 and 3 mM $CaCl_2$, respectively. In agreement with previous reports(Pocotte *et al.* 1991), the pattern of concentration-dependent PTH secretion by $CaCl_2$ showed a typical sigmoidal curve within the physiological range(Fig. 1).

Effect of ammonium acetate

For further exploration of the mechanism of PTH secretion, we examined the effects of ammonium acetate that has high permeability against cellular vesicle membrane. Compared to the basal secretion in KR solution without ammonium acetate, PTH secretion was increased rapidly by five times as much as basal values depend on the concentration of ammonium acetate (Fig. 2A). The ratio of PTH secretion before and after incubation in

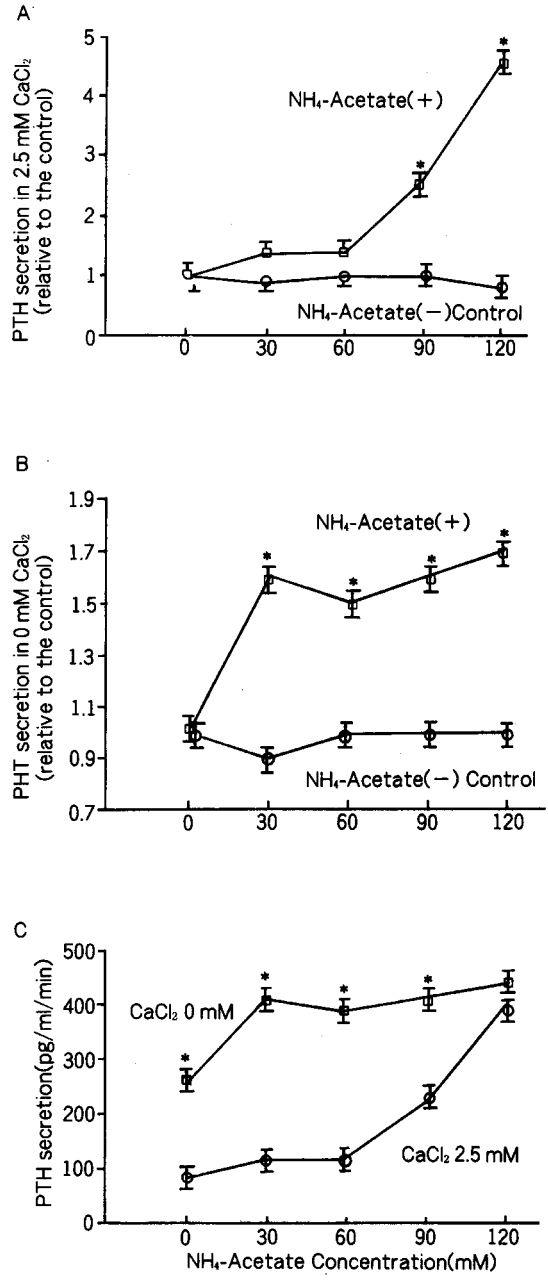

Fig. 2. *Concentration-stimulation of PTH by ammonium acetate in 2.5 mM $CaCl_2$ (A and C) or 0 mM $CaCl_2$ (Band C). Values are means±SE from six observation. *P<0.01 as compared to the value at 0 mM ammonium acetate in Fig. A and B. *P<0.001 as compared to the value at 0 mM ammonium acetate in Fig. C.*

the each concentration of ammonium acetate at 2.5 mM $CaCl_2$ was 1.05±0.08, 1.34±0.12, 1.32 ±0.14, 2.62±0.28($P<0.01$), 4.35±0.27($P<001$) at 0, 30, 60, 90, 120 mM ammonium acetate, respectively. Values are means±SE from six observation. Although ammonium acetate showed a little effect on PTH secretion within the range of 0 mM to 60 mM, it showed a significant effect on PTH secretion above 90 mM. When parathyroid slices were treated with various concentrations of ammonium acetate containing KR solution without $CaCl_2$, PTH secretion was significantly stimulated at 30 mM of ammonium acetate and was maintained up to 120 mM of ammonium acetate (Fig. 2B). The ratio of PTH secretion before and after incubation in the each concentration of ammonium acetate at 0 mM $CaCl_2$ was 0.98 ±0.09, 1.56±0.08 ($P<0.001$), 1.49±0.14 ($P<0.001$), 1.56±0.18 ($P<0.001$), 1.67±0.07 ($P<0.001$) at 0, 30, 60, 90, 120 mM ammonium acetate, respectively. Compared to the response in 2.5 mM of $CaCl_2$, PTH secretion was stimulated at a lower concentration of ammonium acetate without $CaCl_2$ (90 mM vs 30 mM) (Fig. 2C). This finding might suggest that increased concentration of intracellular calcium inhibits PTH secretion through the potential anti-swelling effect of secretory vesicle.

Effect of osmolarity

Although concentrations of extracellular $CaCl_2$ were primarily associated with PTH secretion, as shown in Fig. 3, when the osmolarities of the standard KR solution (2.5 mM $CaCl_2$) were changed by adding mannitol, the pattern of PTH secretion was biphasic according to the change of extra cellular osmolarity. The ratio of PTH secretion in the each mOsmol/L to that in 300 mOsm/L was 1.81 ±0.19 ($P<0.001$), 1.02±0.03, 1.55±0.2 ($P<0.001$), 1.98±0.14 ($P<0.001$) at 200, 300, 600, 800 mOsm/L, respectively (Fig. 3A). The lowest response was observed at 300 mOsm/l. In contrast, PTH secretion at 0 mM $CaCl_2$ was not changed in a steady state level except at 200 mOsm/l even though the osmolarity of media was changed. The ratio of PTH secretion in the each mOsmol/L to t at in 300 mOsm/L was 1.26±0.09 ($P<0.001$), 1.01±0.03, 0.92±0.08 and 1.02±0.04 at

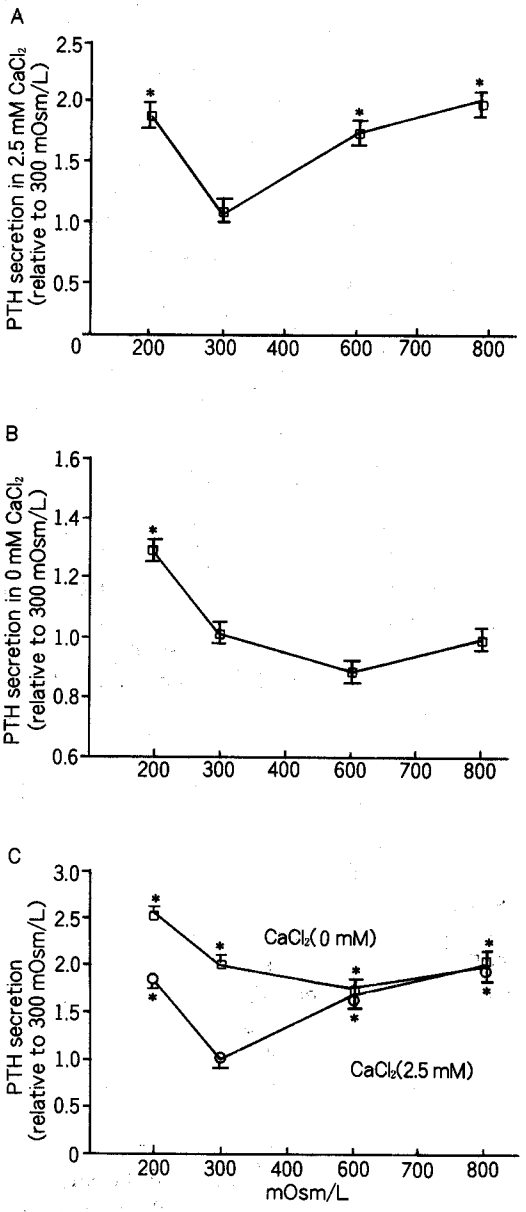

Fig. 3. *Concentration-stimulation of parathyroid hormone by mannitol in 2.5 mM $CaCl_2$ (A or C) or 0 mM $CaCl_2$ (B or C). In Fig. A and B, the value was expressed as the ratio of PTH secretion in each Osm/L to that in 300 mOsm/L, *P<0.05. In Fig. C, the value was expressed as the ratio of PTH secretion in each mOsm/L to that in 300 mOsm/L and 2.5 mM CaCl2, *P< 0.001.*

200, 300, 600, 800 mOsm/L, respectively (Fig. 3B). The ratio of PTH secretion in each mOsm/L at 0 mM $CaCl_2$ to that in 300 mOsm/L and 2.5 mM CaCl was 2.51±0.17 ($P<0.001$), 2.02±0.2 ($P<0.001$), 1.67±0.26($P<0.001$), 1.96±0.18 ($P<0.001$), respectively (Fig. 3C). In hyperosmotic media, water is removed from the space between the vesicle membrane and the plasma membrane of the cells and the removal of water from this space allows the two membranes to come closer and to fuse. These data suggest that osmotic swelling of the secretory vesicle could overcome the inhibition of PTH secretion at 2.5 mM $CaCl_2$. This conclusion may be an indirect evidence that osmotic swelling of the vesicle itself might requisite for PTH secretion at the challenge of decreased concentration of serum calcium (Breckenridge and Almers, 1987; Zimmerberg *et al.* 1987; Monck *et al.* 1991). Furthermore PTH secretion showed calcium-dependency at hypoosmolar solution (200 mOsmol/L) and calcium-independence at hyperosmolar solution(> 400 mOsmol/L).

Effect of nigericin

To clarify the relationship between vesicle swelling and PTH secretion further, we tested the effect of nigericin. When parathyroid slices were incubated with different concentrations of nigericin at 0 mM or 2.5 mM $CaCl_2$, PTH secretion was stimulated gradually according to the increase of nigericin. PTH secretion in each mM of nigericin at 2.5 mM $CaCl_2$ was 35±7.5, 27±7.7, 32±4.6, 37±3.7, 94±14.1 ($P<0.001$) and 202± 13.6 pg/ml ($P<0.01$), respectively(Fig. 4A). PTH secretion in each mM of nigericin at 0 mM $CaCl_2$ was 66±4.5, 62±2.7, 88±3.6 ($P<0.01$), 122±5.7 ($P<0.001$), 146±3.4 ($P<0.001$) and 126±5.6 pg/ml($P<0.001$), respectively. Values are mean±SE from six observations. The stimulatory effect was already observed at 10^{-6} M of nigericin in 0 mM $CaCl_2$, however the effect was observed at higher concentration of nigericin ($>10^{4-}$ M) in 2.5 mM $CaCl_2$ (Fig. 4B). The maximum stimulatory effect was only 2 times greater than basal rate in 0 mM $CaCl_2$, whereas 8 times greater in 2.5 mM $CaCl_2$. These data suggest that the swelling of vesicle could be induced by K^+-H^+ ionophore and intracellular calcium might inhibit the swelling of the vesicle. The rate of PTH secretion was increased, when the K-gradient between the extracelluar solution and the cytoplasm was abolished by changing from Na-rich KR solution to K-rich KR solution (Fig. 5A). The ratio of PTH secretion at each concentration of $CaCl_2$ in K-rich solution to that in Na-rich solution without nigericin was 0.93±0.06, 1.17±0.1, 1.48±0.17, 2.1±20.16, 2.1

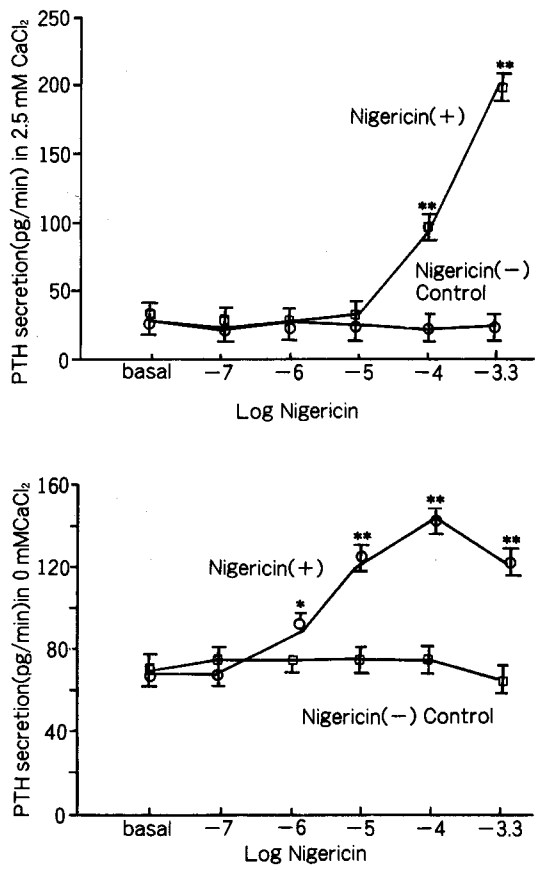

Fig. 4. *Stimulation of parathyroid hormone secretion by treatment of tissues with nigericin. The effect of nigericin on PTH secretion at 2.5 mM $CaCl_2$ (A) and 0 mM $CaCl_2$ (B). Values are mean±SE from six observations. *P<0.01, **P<0.001 as compared to mean PTH value of control wells not stimulated by nigericin at the same concentration of $CaCl_2$.*

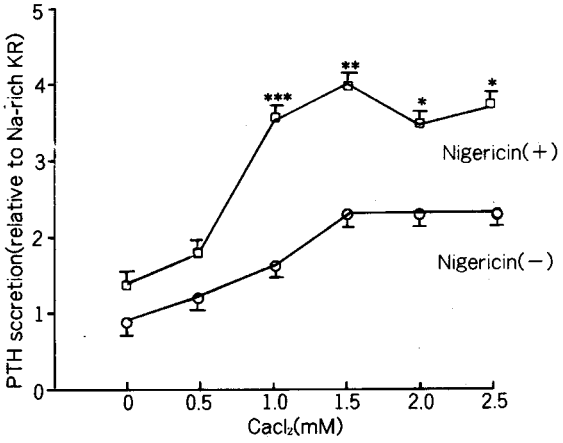

Fig. 5. *Stimulation of parathyroid hormone secretion by treatment of Nigericin in K-rich KR solution. Values are mean SE from six observations. *P<0.05, **P<0.01, ***P<0.001 as compared to the value not stimulated by nigericin.*

±0.17, 2.05±0.15 at 0, 0.5, 1, 1.5, 2, 2.5 and 3 mM $CaCl_2$, respectively. Furthermore the addition of nigericin stimulates the rate of PTH secretion significantly (Fig. 5B). The ratio of PTH secretion at each concentration of $CaCl_2$ in K-rich solution to that in Na-rich solution with nigericin was 1.35 ± 0.18, 1.77 ± 0.19, 3.38 ± 0.37 ($P < 0.001$), 3.8 ± 20.36 ($P < 0.01$), 3.3±0.42 ($P<0.05$), 3.62±0.32 ($P<0.05$) at 0, 0.5, 1, 1.5, 2, and 2.5 mM $CaCl_2$, respectively.

DISCUSSION

The present study demonstrated that osmotic swelling of PTH secretory granules could stimulate PTH secretion in bovine parathyroid cells. It also suggests that physiologic control of PTH secretion involves alterations in granular membrane ion permeability.

When osmotic gradients across the membrane were generated by the adding of ammonium acetate in the media or incubation of the slices into the hypo-osmotic media, the secretion of PTH was stimulated. These results suggest that an osmotic gradient across the membrane is also involved in membrane fusion and/or fission steps of exocytosis even in the cells showing the inverse sigmoidal control of secretion to extra cellular calcium such as parathyroid cells. Even though the mechanisms of which the swelling promotes exocytosis are yet undefined, the swelling might increase the tension of the secretory granule membrane, facilitate the release of the granule contents and increase the chance of the fusion between the secretory granules and plasma membrane.

Such exocytotic fusion events between plasma membrane and vesicular membrane were also shown in JG cells by the similar methods (Park *et al.* 1991).

How could secretory vesicles in parathyroid cells be swollen in physiologic condition? Nigericin, K^+/H^+ ionophore, overcame the inhibitory effect of 2.5 mM $CaCl_2$ completely and the level of PTH secretion reached close to the maximal value induced by lowering of extracellular Ca^{++} to 0 mM without nigericin. When the cellular K^+ concentration was elevated from 5 to 140 mM (K^+ rich KR solution) to reduce or abolish the K^+ gradient and Na^+ concentration gradients across the plasma membrane, the net movements of both Na^+ and K^+ induced by these ionophores across the plasma membrane should be reduced. However, the stimulatory effects of nigericin were still apparent. Therefore, the effects of this ionophore on PTH secretion is not likely a result of changes in cell volume secondary to their actions on the plasma membrane. Rather, the major effects of nigericin would involve alterations in ion permeability of intracellular organelles, most likely on the PTH secretory granule membrane.

Nigericin, an electroneutral exchanger of monovalent cations for H^+ (Pressman, 1976), would promote an inward movement of K^+ coupled with an outward movement of H^+ in secretory granules. In the presence of permeate anion such as Cl^- in cytoplasm, nigericin would cause net intragranular accumulation of KCl. The increase in the intragranular osmotic activity would provide a driving force for water movement into the granule, thereby

causing swelling of the granule (Park *et al.* 1991). In other studies, the calcium-activated potassium channel was postulated to be present in the secretory granules of parathyroid cells (Jia *et al.*1988; Pocotte and Ehrenstein, 1989). From our results, it is plausible that the decreased intracellular calcium level might trigger calcium dependent potassium channel in vesicle and lead to the swelling of vesicle in parathyroid cells. The changes in Cl- permeability of granular membrane and /or H^+-ATPase activity followed by the activation of K^+/H^+ exchange pump may be the sites of physiological regulation (Park *et al.* 1991). However this chemiosmotic regulation needs further proof in physiologic control of parathyroid cells.

The responses to ammonium acetate and nigericin were different at 0 mM $CaCl_2$ compared to that at 2.5 mM $CaCl_2$. The stimulatory effect was already observed at a low concentration of ammonium acetate (30 mM) and nigericin (10^{-6}M), whereas these were observed at high concentrations (90 mM and 10^{-4} M respectively) in 2.5 mM $CaCl_2$. However the maximal stimulatory effects in media containing ammonium acetate or nigericin were very close in both conditions. From these results, it is plausible that $CaCl_2$ may inhibit swelling of vesicle, and marked extracellular osmolar change or strong permeability of ammonium acetate could overcome the inhibitory effect of $CaCl_2$. However the downstream signals for inhibition of PTH secretion by Ca^{++} are not clarified yet in parathyroid cells, even though most effects of Ca^{++} in cells are mediated more indirectly via protein phosphorylation catalyzed by a family of Ca^{++}/calmodulin-dependent protein kinases (Park and Lee, 1994).

In summary, this study demonstrated that osmotic swelling could stimulate PTH secretion in vitro. The stimulatory effect of nigericin is an evidence that physiologic control of PTH secretion involves alterations in granular membrane ion permeability. We propose that the swelling of vesicle is also prerequisite for secretion in the cells inhibited paradoxically by Ca^{++}, even though the signal transduction pathway of the Ca^{++} induced inhibition of secretion needs to be addressed.

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