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ORIGINAL PAPER

Detection of dihydropyridine- and voltage-sensitive intracellular Ca^{2+} signals in normal human parathyroid cells

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Abstract We recently showed dihydropyridine- and voltage-sensitive Ca^{2+} entry in cultured parathyroid cells from patients with secondary hyperparathyroidism. To determine whether normal parathyroid cells have a similar extracellular Ca^{2+} entry system, cells were isolated from normal (non-hyperplastic) human parathyroid glands. Fluorescence signals related to the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_I$) were examined in these cells. Cells loaded with fluo-3/AM showed a transient increase in fluorescence (Ca^{2+} transient) following a 10-s exposure to a 150 mM K^+ solution in the presence of millimolar concentrations of external Ca^{2+}. The Ca^{2+} transient was reduced by dihydropyridine antagonists or 0.5 mM Cd^{2+}, but enhanced by FPL-64176, an L-type Ca^{2+}-channel agonist. Ca^{2+} transients induced by the 10-s exposure to 3.0 mM extracellular Ca^{2+} ($[Ca^{2+}]_o$) were also inhibited by dihydropyridine antagonists or 0.5 mM Cd^{2+}. These results provide the first evidence that normal human parathyroid cells express a dihydropyridine-sensitive Ca^{2+} entry system that may be involved in the $[Ca^{2+}]_o$-induced change in $[Ca^{2+}]_I$. This system might provide a compensatory pathway for negative feedback regulation of parathyroid hormone secretion under physiological conditions.

Keywords Parathyroid gland · Calcium · Dihydropyridine receptor · Calcium receptor · Calcium channel

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Introduction

The serum (extracellular) Ca^{2+} concentration ($[Ca^{2+}]_o$) is tightly controlled through negative feedback regulation by parathyroid hormone (PTH) secretion from the parathyroid gland. By targeting bones, kidneys and the small intestine, PTH leads to elevated $[Ca^{2+}]_o$. PTH secretion increases with decreasing $[Ca^{2+}]_o$ and vice versa [1, 2]. The cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_I$) in parathyroid cells is the key determinant of PTH secretion as indicated in studies on cultured bovine parathyroid cells showing that changes in $[Ca^{2+}]_I$ occur in parallel with changes in $[Ca^{2+}]_o$ [3, 4].

It is well established that signaling information from $[Ca^{2+}]_o$ is transduced to $[Ca^{2+}]_I$ via the Ca^{2+}-sensing receptor (CaR), a G protein-coupled transmembrane protein that is abundantly expressed in the surface membrane of parathyroid cells [5, 6] and other cell types [2]. The current view of this process is that CaR transduces $[Ca^{2+}]_o$ into $[Ca^{2+}]_I$ by regulating the amount of Ca^{2+} released from intracellular Ca^{2+} stores. This mechanism is supported by findings that inositol 1,4,5-trisphosphate (IP_3)

production is increased in bovine parathyroid cells in the presence of relatively high $[Ca^{2+}]_o$, and that HEK-293 cells exhibit IP_3-induced Ca^{2+} release when transfected with a plasmid carrying cDNA for CaR [7].

In addition to the IP_3-mediated mechanism, the results from several studies suggest that dihydropyridine-sensitive Ca^{2+} entry participates in the regulation (negative feedback) of PTH secretion [8–12] and $[Ca^{2+}]_I$ [11, 13–15]. We recently directly detected dihydropyridine- and voltage-sensitive Ca^{2+} entry in cultured parathyroid cells from patients with secondary hyperparathyroidism [16], thereby raising the question of whether such a Ca^{2+} entry system is a feature of normal parathyroid cells. The aim of the study reported here was to isolate parathyroid cells from normal (non-hyperplastic) parathyroid glands and to detect extracellular Ca^{2+} entry using fluo-3, a Ca^{2+}-sensitive fluorescent indicator dye.

Materials and methods

Ethical approval

The study follows procedures that are in accordance with the Helsinki Declaration of 1983 and which were approved by the Ethics Committee of Jikei University School of Medicine. Written informed consent was obtained from all three patients prior to surgery. In all cases, the removal of normal parathyroid glands was performed for therapeutic purposes and was not related in any way to the goals of this study.

Cell preparation

Cultured normal parathyroid cells were prepared essentially as described by Yokoyama et al. [16], although the isolation procedure for extremely small normal parathyroid glands was more difficult than that developed for large parathyroid glands in patients with secondary hyperparathyroidism. Normal parathyroid glands (diameter 5 mm) were surgically removed from patients with thyroid cancer and stored for 2–6 h in cold (<4 °C) culture medium [Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Gaithersburg, MD) supplemented with 10 % heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS/Thermo Trace, Noble Park, Victoria, Australia), 1 mM Na^+ pyruvate (Sigma, St. Louis, MO) and penicillin/streptomycin (100 U/ml; GIBCO BRL)]. Following removal of fat and connective tissues, the parathyroid glands were minced finely (fragments <0.3 mm) with scissors in ice-cold HEPES buffer solution containing 146 mM NaCl, 5.0 mM KCl, 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 10 mM HEPES, adjusted to pH 7.4 with NaOH. Digestion with 2 mg/ml collagenase (Sigma Type IA) was carried out for 50–60 min in a water bath shaker (130–140 cpm; TAITEC, Kubota, Japan) at 37 °C. The turbid solution was sedimented for 10 min in a centrifuge (KN-70; TAITEC) at 700–1000 rpm. Isolated cells plated on sterilized gelatin-coated glass cover slips (diameter 14 mm; thickness 0.08–0.12 mm; Matsunami Glass, Osaka, Japan) were cultured for 36–74 h in culture medium and incubated at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. In two patients from whom normal (non-hyperplastic) parathyroid glands were surgically removed, the serum levels of intact PTH were 39 and 58 pg ml^{-1}. These values fall within the normal range of serum PTH (10–65 pg ml^{-1}) in healthy adults. In patients with secondary hyperparathyroidism, the serum PTH was 180.0 ± 13.1 pg ml^{-1} [16].

Fluorescence measurements

Changes in $[Ca^{2+}]_I$ were estimated with a Ca^{2+}-sensitive fluorescent indicator, as described previously [16]. Cultured parathyroid cells were loaded for 40–60 min with fluo-3/AM at a final concentration of 10 μM (Invitrogen, Carlsbad, CA) at 37 °C in a standard bath solution containing 146 mM NaCl, 1.5 or 2.0 mM $CaCl_2$, 5.0 mM KCl, 1.0 mM $MgCl_2$ and 10 mM HEPES, adjusted to pH 7.4 with NaOH. One part of pluronic acid was added to five parts of dye dissolved in dimethyl sulfoxide (DMSO) before final dilution with saline. Preliminary experiments revealed that >90 % of the baseline fluorescence signal reflected $[Ca^{2+}]_I$ [16]. Fluorescence was detected with a modified Nipkow laser high-speed confocal scanning microscope system (CSU-21; Yokogawa Electric, Tokyo, Japan) attached to an inverted microscope (model IX70; Olympus, Tokyo, Japan). The excitation light was emitted from a laser unit (488 nm; argon laser, 4–13 mW). The light passing through the pinholes was focused by an objective lens (UPlanFl 40×, NA = 0.75; Olympus) to a point on the cells. Fluorescent light beams emitted from a cluster of cells were scanned by the pinholes and reflected by a dichroic mirror, passed through a barrier filter (>515 nm) and captured by a cooled digital EMCCD camera (C9100-12; Hamamatsu Photonics, Hamamatsu, Japan). Two-dimensional images composed of 512 × 512 pixels (204.8 μm × 204.8 μm) were recorded at 2 frames/s^{-1} with an exposure time of 495 ms. Data acquisition and image analysis were carried out on an IBM computer using the Aquacosmos image program (Hamamatsu Photonics); for details, see Yokoyama et al. [16]. All experiments were carried out at room temperature. A high K^+ solution or 3 mM Ca^{2+} was applied by local ejection from a small-tipped pipette using a pressure ejection device (PicoPump; World Precision Instruments, Sarasota, FL). The high K^+

solution contained 150 mM KCH_3O_3S (potassium methane sulfonate), 1.5 or 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10 mM HEPES, adjusted to pH 7.4 with KOH. Changes in fluorescence from the cytoplasm (excluding the nucleus) are shown relative to the baseline fluorescence ($\Delta F/F$), where F is the baseline fluorescence and ΔF is the change in fluorescence in response to stimulation. Background intensity was subtracted from the raw data before calculating $\Delta F/F$. Results are reported as the mean ± standard deviation (SD). In the experiments using 1 μM dihydropyridine antagonists, cells were pretreated with these agents for >25 min because the dihydropyridine-binding domains are not easily accessible at relatively hyperpolarized membrane potentials [17, 18].

Results

Voltage-dependent activation of the transient increase in fluorescence

We first examined whether depolarization evokes Ca^{2+} entry in cultured normal parathyroid cells. Cells were loaded with fluo-3/AM, and the surface membrane was depolarized to close to 0 mV (assuming a cytoplasmic K^+ concentration of about 140 mM) by external exposure to a 150 mM K^+ solution. A transient increase in fluo-3 fluorescence (Ca^{2+} transient) was detected in most cells following a 10-s exposure to the 150 mM K^+ solution containing 2 mM Ca^{2+} ($n > 74$ cells from 3 patients), indicating that a transient increase in $[Ca^{2+}]_I$ occurred in response to membrane depolarization (Fig. 1, left). We gently applied the solution from a small-tipped pipette to avoid opening mechanosensitive channels, resulting in slow onset of the Ca^{2+} transient following pressure application.

In a similar manner, nominally Ca^{2+}-free saline containing 150 mM K^+ was gently applied to targeted cells. However, depolarization failed to evoke a Ca^{2+} transient in the absence of $[Ca^{2+}]_o$ (Fig. 1, right; $n = 10$). Before application of nominally Ca^{2+}-free saline, the bath was switched to a 1 mM $[Ca^{2+}]_o$ solution to reduce $[Ca^{2+}]_o$ in the vicinity of the targeted cells; the average amplitude of fluo-3 fluorescence decreased significantly from 3.37 ± 1.16 to 0.17 ± 0.08 ($n = 10$). Application of the bath solution per se did not induce a Ca^{2+} transient ($n = 8$).

The high K^+-induced Ca^{2+} transient is sensitive to L-type Ca^{2+} channel modulators

We then examined whether Ca^{2+} entry is sensitive to L-type Ca^{2+} channel modulators. As shown in Fig. 2a, the depolarization-induced Ca^{2+} transient in 1.2–1.5 mM $[Ca^{2+}]_o$ markedly increased in the presence of 10 μM FPL-64176 ($n = 10$), a potent agonist of L-type Ca^{2+} channels that is known to bind to a domain distinct from that of dihydropyridines and exhibits exclusive Ca^{2+}-channel agonist action, even at very high concentrations [19, 20]. In contrast, exposure to the high K^+ solution failed to induce a noticeable Ca^{2+} transient following treatment with 1 μM nicardipine for >25 min (Fig. 2b). The high K^+-induced Ca^{2+} transient was also strongly inhibited by 0.5 mM Cd^{2+} ($n = 21$). The effects of L-type Ca^{2+} channel modulators on fluo-3 fluorescence in response to the high K^+ solution are summarized in Table 1. Taken together, these results suggest that normal parathyroid cells of human origin express an extracellular Ca^{2+} entry system that is physiologically similar to L-type Ca^{2+} channels.

Inhibition of the high $[Ca^{2+}]_o$-induced Ca^{2+} transient by Ca^{2+} channel inhibitors

Finally, we investigated the effects of L-type Ca^{2+} channel inhibitors on the high $[Ca^{2+}]_o$-induced increase in $[Ca^{2+}]_I$. This was a characteristic feature of the parathyroid cells in which $[Ca^{2+}]_o$ was ≥2.5 mM was able to induce this type of response under our experimental conditions. As shown

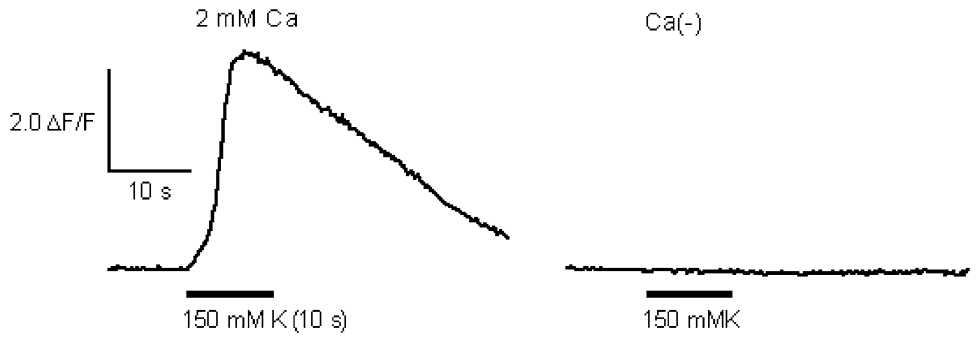

Fig. 1 Voltage-dependent activation of a transient increase in fluo-3 Ca^{2+} transients in a normal parathyroid cell. *Left* Application of a 150 mM K^+ solution (2 mM Ca^{2+}) evoked a transient increase in fluo-3 fluorescence (Ca^{2+} transient). *Right* Application of a nominally Ca^{2+}-free 150 mM K^+ solution failed to increase fluo-3 fluorescence. F Baseline fluorescence, ΔF change in fluorescence in response to stimulation

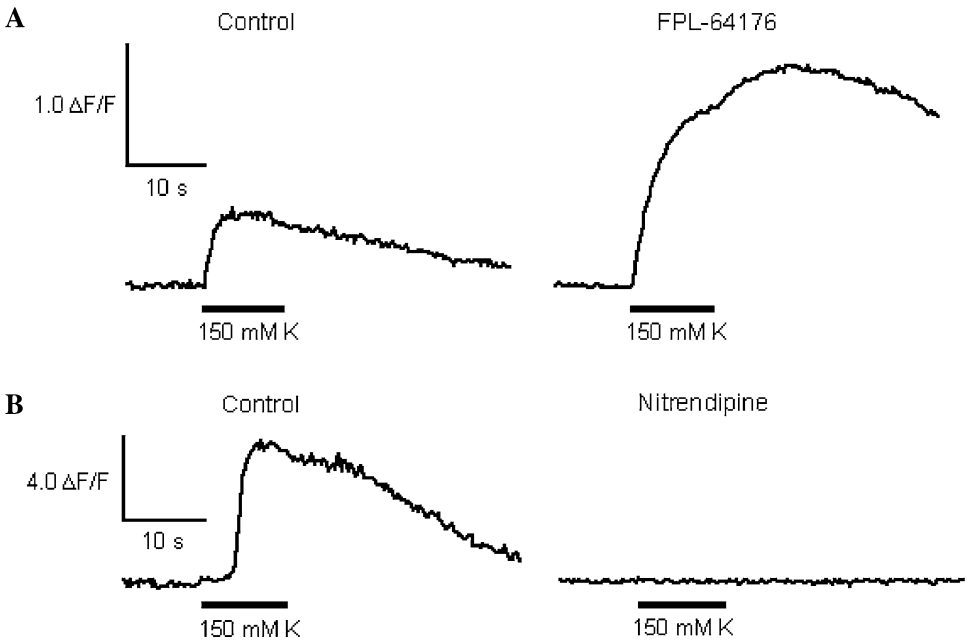

Fig. 2 The high K^+-induced Ca^{2+} transient is sensitive to L-type Ca^{2+} channel modulators. **a** The bath contained 1.2 mM Ca^{2+} to highlight the effect of the agent. *Left* Application of a 150 mM K^+ solution caused a small increase in fluo-3 fluorescence. *Right* In the presence of FPL-64176, fluo-3 fluorescence was markedly increased upon exposure to the high K^+ solution. **b** *Left* In the absence of nitrendipine, application of a 150 mM K^+ solution evoked a transient increase in fluo-3 fluorescence. The bath contained 2 mM Ca^{2+}. *Right* In the presence of 1 μM nitrendipine, exposure to the high K^+ solution failed to increase fluo-3 fluorescence

Table 1 Effect of Ca^{2+} channel modulators on the Ca^{2+} transient induced by extracellular application of 150 mM K^+ or 3 mM Ca^{2+}

Stimulator[a]	Modulator[b]	Control	With modulator	No. of cells
150 mM K^+	Nicardipine (1 μM)	2.68 ± 1.50	0.10 ± 0.04	15
	FPL-64176 (10 μM)	0.96 ± 0.90	6.91 ± 3.37	10
	Cd^{2+} (0.5 mM)	2.52 ± 0.93	0.03 ± 0.06	21
3 mM Ca^{2+}	Nitrendipine (1 μM)	4.30 ± 1.23	0.19 ± 0.30	20
	Nicardipine (1 μM)	4.88 ± 1.72	0.10 ± 0.08	20
	Cd^{2+} (0.5 mM)	2.13 ± 1.67	0.02 ± 0.02	12

Data are presented as the mean ± standard deviation. In each row, data {maximal change in change in fluorescence in response to stimulation/baseline fluorescence ($\Delta F/F$) from baseline fluo-3 fluorescence following 10 s exposure to 150 mM $[K^+]_o$ or 3 mM $[Ca^{2+}]_o$ solution} were obtained from the population of a single primary culture dish before and after exposure to the modulators

[a] $[Ca^{2+}]_o$ in the bath solution was 2 mM, except in the case of FPL-64176, for which $[Ca^{2+}]_o$ was 1.2 mM to highlight the effect of the agent

[b] For FPL-64176 and Cd^{2+}, data with a marked increase in baseline fluorescence were excluded from the table

in Fig. 3a (left), external application of the 3 mM $[Ca^{2+}]_o$ solution to cells bathed in 2 mM $[Ca^{2+}]_o$ solution evoked a transient increase in fluo-3 fluorescence. This did not occur following treatment with 1 μM nitrendipine for >25 min (Fig. 3a, right). In this population, all cells tested showed >95 % abrogation of the 3 mM $[Ca^{2+}]_o$-induced Ca^{2+} transient ($n = 20$). Similar inhibitory effects were obtained with 1 μM nicardipine ($n = 20$). The high $[Ca^{2+}]_o$-induced Ca^{2+} transient was also strongly inhibited by 0.5 mM Cd^{2+} ($n = 12$). The effects of L-type Ca^{2+} channel modulators on fluo-3 fluorescence in response to the 3 mM $[Ca^{2+}]_o$ solution are summarized in Table 1.

Discussion

In this study, we detected dihydropyridine-sensitive extracellular Ca^{2+} entry in normal parathyroid cells of human origin. The results in Fig. 1 suggest that the 150 mM K^+-induced Ca^{2+} transient observed in the presence of millimolar $[Ca^{2+}]_o$ reflects Ca^{2+} entry through voltage-dependent Ca^{2+} channels and that a high K^+ per se is not the cause of Ca^{2+} release from the endoplasmic reticulum via direct activation of the CaR. The fluorescence measurements in Fig. 2 and Table 1 further indicate that voltage-activated extracellular Ca^{2+} entry is sensitive to L-type Ca^{2+} channel modulators. Thus, voltage-sensitive Ca^{2+} entry appears to be an essential feature of normal parathyroid cells. These results are compatible with previous findings in patients with secondary hyperparathyroidism [16]. The Ca^{2+} transient induced by 150 mM K^+ was inhibited by 1 μM nitrendipine (from 3.0 ± 1.2 to 0.3 ± 0.3, $n = 19$), 1 μM nicardipine (from 2.8 ± 1.5 to 0.4 ± 0.6, $n = 18$), and 0.5 mM Cd^{2+} (from 2.6 ± 1.4 to 0.6 ± 0.7, $n = 10$)[16]. It should be noted that nearly complete abrogation was frequently observed in both

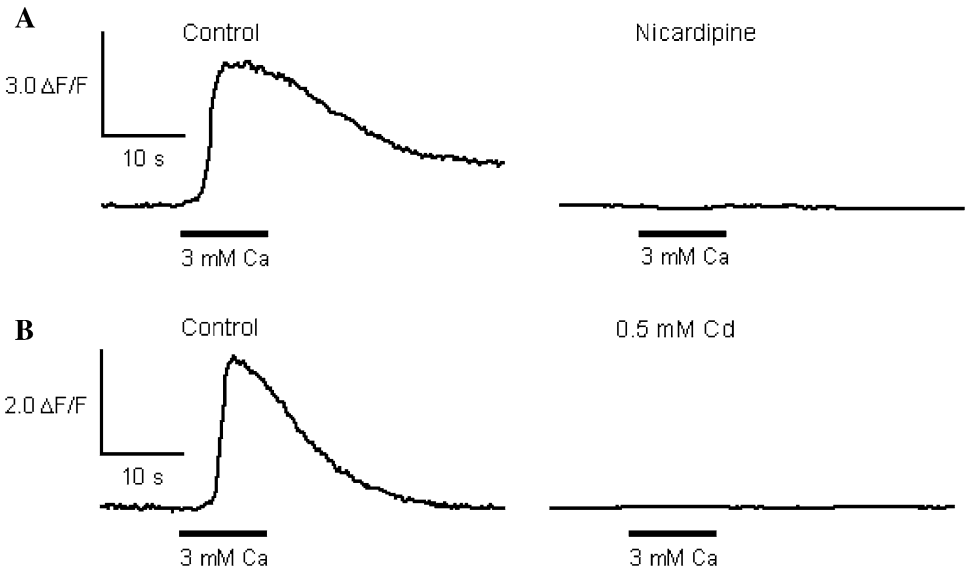

Fig. 3 Reduction of the high serum (extracellular) Ca^{2+} concentration ($[Ca^{2+}]_o$)-induced Ca^{2+} transient by L-type Ca^{2+} channel inhibitors. The bath contained 2 mM Ca^{2+}. **a** Application of the 3 mM $[Ca^{2+}]_o$ solution for 10 s evoked a Ca^{2+} transient (*left*). Following treatment with 1 μM nicardipine for >25 min, application of the 3 mM $[Ca^{2+}]_o$ solution no longer induced a Ca^{2+} transient (*right*). **b** In the presence of 0.5 mM Cd^{2+}, application of 3 mM $[Ca^{2+}]_o$ solution also failed to induce a Ca^{2+} transient (*right*)

populations. In contrast, FPL-64176 enhanced the depolarization-induced Ca^{2+} transient in parathyroid cells from patients with secondary hyperparathyroidism (from 2.7 ± 1.2 to 4.4 ± 1.6, $n = 10$) [16].

The cultured normal parathyroid cells were only exposed to excitation light during fluorescence measurements to reduce inactivation of the agents nitrendipine and nicardipine used in the study. Furthermore, both of these agents are resistant to light illumination, even at shorter wavelengths [18]. These features of the experiments are important, since earlier reports refuting dihydropyridine-sensitive Ca^{2+} entry in normal parathyroid cells did not address these points.

It would appear that the data presented in Fig. 3 and Table 1 support the previously proposed idea that dihydropyridine-sensitive Ca^{2+} entry is involved in regulation of $[Ca^{2+}]_I$ by $[Ca^{2+}]_o$ [16]. In cells from patients with secondary hyperparathyroidism, the Ca^{2+} transient induced by 3 mM $[Ca^{2+}]_o$ was also strongly inhibited by 1 μM nitrendipine (from 4.88 ± 2.6 to 0.15 ± 0.09, $n = 20$) and 0.2 mM Cd^{2+} (from 3.9 ± 1.1 to 0.44 ± 0.60, $n = 20$). Furthermore, extracellular application of 3 mM $[Ca^{2+}]_o$ or 150 mM K^+ solution failed to induce a noticeable Ca^{2+} transient just (e.g. 15 s) following the preceding 30 s exposure to the 150 mM K^+ solution. After a 10 min rest, however, the 3 mM $[Ca^{2+}]_o$ or 150 mM K^+ solution was again able to induce a Ca^{2+} transient with nearly the same amplitude (Figs. 2 and 6 in [16]). These results suggest that the cell had not deteriorated, but experienced a voltage-dependent inactivation process. This type of experiment was not conducted in the present study.

The mechanism through which voltage-dependent Ca^{2+} entry is activated under physiological conditions remains to be elucidated. The resting membrane potentials of normal parathyroid cells are more negative than −60 mV in the presence of physiological $[Ca^{2+}]_o$ [21, 22], and thus the membrane requires depolarization for voltage-dependent Ca^{2+} channels to be effectively opened. It is possible that activation of CaR by extracellular Ca^{2+} causes an increase in $[Ca^{2+}]_I$ via the IP_3-mediated pathway, which in turn inhibits Ca^{2+}-activated K^+ channels that may have a high affinity for Ca^{2+}, resulting in mild depolarization of the surface membrane [21, 23, 24]. Extracellular Ca^{2+} entry per se may further depolarize the membrane. Nevertheless, Ca^{2+}-activated K^+ channels with a low affinity for Ca^{2+} may compete with this effect [23–25], resulting in a relatively mild and slow membrane potential change during exposure to high $[Ca^{2+}]_o$ [21, 22]. It is also possible that the CaR directly or indirectly activates Ca^{2+} entry without causing a significant membrane potential change. Alternatively, window Ca^{2+} current flowing around the resting membrane potential of normal parathyroid cells may contribute to $[Ca^{2+}]_o$-activated Ca^{2+} entry. Regardless, there is no doubt that CaR is necessary in this process, since type II calcimimetic compounds, such as NPS R-568 and AMG 073 (cinacalcet HCl), potentiate the effects of $[Ca^{2+}]_o$ on $[Ca^{2+}]_I$ and PTH secretion in the presence of extracellular Ca^{2+} [26, 27]. Because CaR expression is downregulated in hyperparathyroidism [28, 29], Ca^{2+} entry that is not associated with CaR might play an important role in reducing PTH secretion in hyperparathyroidism.

Regardless of the mechanism through which dihydropyridine-sensitive Ca^{2+} entry is activated under physiological conditions, our study provides evidence that normal parathyroid cells express a pathway for extracellular Ca^{2+} entry. This may provide a compensatory mechanism for the negative feedback regulation of PTH secretion under physiological conditions, as well as pathophysiological conditions.

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