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Activation of calcium-sensing receptor accelerates apoptosis in hyperplastic parathyroid cells

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Abstract

Calcimimetic compounds inhibit not only parathyroid hormone (PTH) synthesis and secretion, but also parathyroid cell proliferation. The aim of this investigation is to examine the effect of the calcimimetic compound NPS R-568 (R-568) on parathyroid cell death in uremic rats. Hyperplastic parathyroid glands were obtained from uremic rats (subtotal nephrectomy and high-phosphorus diet), and incubated in the media only or the media which contained high concentration of R-568 (10^{-4} M), or 10% cyclodextrin, for 6 h. R-568 treatment significantly suppressed medium PTH concentration compared with that of the other two groups. R-568 treatment not only increased the number of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay-positive cells, but also induced the morphologic changes of cell death determined by light or electron microscopy. These results suggest that CaR activation by R-568 accelerates parathyroid cell death, probably through an apoptotic mechanism in uremic rats in vitro.

Keywords: Calcimimetic compound; Parathyroid hyperplasia; Secondary hyperparathyroidism; Apoptosis; Uremia

Secondary hyperparathyroidism (SHPT) is a well-known feature of chronic kidney disease. SHPT is characterized by parathyroid hormone (PTH) oversecretion and hyperplastic parathyroid glands mainly due to disturbances of calcium (Ca), phosphate (P), and vitamin D metabolism. The reduced calcium-sensing receptor (CaR) expression in parathyroid cells are implicated in the pathogenesis of SHPT [1–4]. The reduced CaR expression impairs the sensitivity of parathyroid cells to extracellular Ca^{2+}, and consequently parathyroid cell proliferation is accelerated.

The CaR plays a critical role in the regulation of PTH synthesis and secretion, and parathyroid cell proliferation [4–6]. Current studies indicate that a calcimimetic compound NPS R-568 (R-568), which acts as an allosteric activator against parathyroid CaR, has a potential to ameliorate SHPT in uremic subjects [7–11]. R-568 inhibits not only PTH synthesis and secretion, but also parathyroid cell proliferation in animal experiments [7–11]. However, the precise mechanism of the inhibitory effect on parathyroid cell proliferation and survival mediated by the CaR has not been fully elucidated.

The aim of the present study is to elucidate whether the calcimimetic compound R-568 induces apoptosis in hyperplastic parathyroid cells in uremic milieu. This study was performed in vitro because the direct effect of the calcimimetic compound on the parathyroid cells could be observed.

Materials and methods

All experimental protocols were approved by the Animal Studies Committee of Wakayama Medical University.

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Experimental animals and diets

Seven-week-old male Sprague–Dawley rats weighing 200–250 g were used in this study. Renal insufficiency was induced in the rats by 5/6 subtotal nephrectomy as previously described [12]. These rats were fed a high-phosphorus (HP) diet (1.2% P, 0.8% Ca) for 8 wk to accelerate SHPT. Normal control rats, which had undergone sham-operations, were fed a standard diet (0.8% P, 1.1% Ca) for 8 wk. Both diets were obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan). After 8 wk the nephrectomized rats were randomly allocated into three groups as follows:

(1) Uremic rats whose parathyroid glands were incubated only in the medium described as below (UC group; $n = 10$).
(2) Uremic rats whose parathyroid glands were incubated in the medium containing Vehicle (10% cyclodextrin) of R-568 (Vehicle group; $n = 9$).
(3) Uremic rats whose parathyroid glands were incubated in the medium containing R-568 (R-568 group; $n = 10$).

Parathyroid glands of normal rats were treated with the same medium as the UC group (NC group; $n = 6$). Blood samples were obtained by aortic puncture for determinants of serum chemistries. Serum chemistries were measured using standard laboratory methods with an automated multiparametric analyzer (DRI-CHEM 3030; Fuji Film, Tokyo, Japan). The serum PTH level was determined using the Rat intact PTH ELISA kit (Immutopics, San Clemente, CA). The parathyroid glands were removed using the microscopic surgical technique, weighed by microbalance (LA 230S; Sartorius, Tokyo, Japan) and then incubated for 6 h.

Incubation medium

After the rat parathyroid glands were washed with 1% phosphate buffered saline (PBS), they were placed in individual wells containing 1 ml of each incubation medium; glands were incubated at 37 °C with 5% CO_2. The incubation medium which has been previously reported by several investigators [14] was applied. The incubation medium was buffered (pH 7.4) and prepared as follows: DME (HG)/Ham's F-12 medium (1:1) containing 15 mM Hepes, 100 IU/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 2 mM glutamine, 1% nonessential amino acids, 1 mg/ml bovine serum albumin, and 5 μg/ml transferrin. The Ca concentration (1.25 mM) in the medium was modified by adding $CaCl_2$. P was added in the form of NaH_2PO_4 and Na_2HPO_4 in a 1:2 proportion to achieve a final concentration of 1 mM. To investigate the effects of a high concentration of the calcimimetic compound, R-568 was added to the medium to achieve the concentration of 10^{-4} M. For the Vehicle group, 2 μl of 10% cyclodextrin (2-hydroxypropyl cyclodextrin; Research Biochemical International, Natick, MA) was added to the medium. This volume is equal to 10^{-4} M of R-568 per 1 ml of medium. After 6 h, the parathyroid glands were washed three times with 1% PBC and were fixed in 4% paraformaldehyde for hematoxylin and eosin stain and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. The PTH concentration of the each medium was determined by the Rat Intact PTH ELISA kit (Immutopics, San Clemente, CA). Because of the positive correlation between DNA content and tissue weight in parathyroid glands [13], we measured the medium PTH concentration which was standardized by parathyroid gland weight. Three glands of each group were used for the electron microscopy to detect apoptotic cells.

Evaluation of apoptotic cells

Apoptotic parathyroid cells were determined by TUNEL assay, and light and electron microscopy.

TUNEL assay. TUNEL assay was performed on 4% paraformaldehyde-fixed, paraffin-embedded parathyroid tissues. Briefly, the tissues were deparaffinized in xylene and dehydrated through an ethanol series. Then the tissue sections were treated with proteinase K and washed with 1% PBS. The *in situ* apoptosis detection kit ApopTag (Serologicals Co., Spaulding Drive Norcross, GA) was used for labeling the free 3′-OH terminus. These procedures were automatically performed using the Ventana Discovery XT system (Ventana Medical Systems, Inc., Tucson, AZ). The number of TUNEL-positive nuclei was determined in the entire tissue specimen; thereafter, this number was divided by the total cell number in the same tissue specimen, yielding the number of the TUNEL-positive nuclei per 1000 parathyroid cells. Six to 8 sequential sections of tissue from each parathyroid gland were analyzed, and the average staining value was regarded as the TUNEL index.

Light and electron microscopy. Paraffin-embedded tissue blocks were cut at 5 μm and stained with hematoxylin and eosin. Apoptotic cells were also investigated by electron microscopy, for which the tissues were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 48 h at 4 °C. The samples were then dehydrated and embedded. Thin sections were examined with a Philips CM 10 electron microscope (Philips, Eindoven, The Netherlands). The morphologic changes including cell shrinkage and nuclear pyknosis were examined by the light microscopy. The characteristic changes of apoptotic cells (intact cell membranes with nuclear changes including chromatin condensation and fragmentation) were examined by electron microscopy.

Statistical analysis

All results were expressed as means ± SEM. Statistical differences between the groups were assessed by one-way ANOVA, and a post hoc test (Fisher protected least significant difference) was used for the comparison of groups. A value of $P < 0.05$ was considered statistically significant.

Results

Biochemical data of rats

Body weight was significantly lower in the uremic rats (the UC, Vehicle, and R-568 groups) than in the normal controls. No significant difference in body weight was noted among the UC, Vehicle, and R-568 groups (Table 1). The serum urea, creatinine, P and PTH levels were significantly higher in the uremic rats than in the normal controls (Table 1). These parameters were comparable among the uremic groups. No significant difference in the serum Ca level was noted among all groups (Table 1).

PTH concentration of medium after 6 h incubation

The medium PTH concentration after 6 h incubation in the UC (1432.8 ± 239.8 pg/ml/μg; $P < 0.01$) and Vehicle (1436.6 ± 375.7 pg/ml/μg; $P < 0.01$) was higher than that in either the NC (413.6 ± 170.8 pg/ml/μg) or R-568 group (420.4 ± 128.5 pg/ml/μg). There was no significant difference in the medium PTH concentration between the R-568 and NC groups. During 6 h incubation, R-568 suppressed PTH release from parathyroid cells (Fig. 1).

Effect of R-568 on parathyroid cell death in uremic rats

Morphologic changes in cell death were observed by light microscopy. Many parathyroid cell nuclei in the R-568 group showed morphologic changes including cell shrinkage and nuclear pyknosis (Fig. 2A). Apoptosis was also demonstrated by the TUNEL-positive cells in parathy-

Table 1
Serum biochemistries of rats

Group	Body weight (g)	SUN (mg/dl)	Cr (mg/dl)	Ca (mg/dl)	P (mg/dl)	PTH (pg/ml)
NC ($N = 6$)	570.1 ± 14.2	22.9 ± 1.0	0.4 ± 0.0	9.6 ± 0.1	6.3 ± 0.1	84.7 ± 6.5
UC ($N = 10$)	441.1 ± 27.6a	63.7 ± 11.1a	1.2 ± 0.2a	8.1 ± 0.7	10.5 ± 0.5b	672.0 ± 136.4b
Vehicle ($N = 9$)	457.2 ± 40.1a	63.2 ± 7.7a	1.0 ± 0.2a	8.4 ± 0.7	10.2 ± 0.9b	802.1 ± 225.7a
R-568 ($N = 10$)	475.6 ± 18.7a	62.8 ± 13.7a	1.0 ± 0.2a	8.8 ± 0.8	10.6 ± 2.3b	755.6 ± 245.2b
ANOVA	$P < 0.01$	$P < 0.01$	$P < 0.05$	$P = 0.19$	$P < 0.05$	$P < 0.05$

SUN, serum urea nitrogen; Cr, creatinine; Ca, calcium; P, phosphorus; PTH, parathyroid hormone. Values are means ± SEM ($N = 6$–10 rats). a, $P < 0.01$ and b, $P < 0.05$ compared with the control group (post hoc test by Fisher's PLSD).

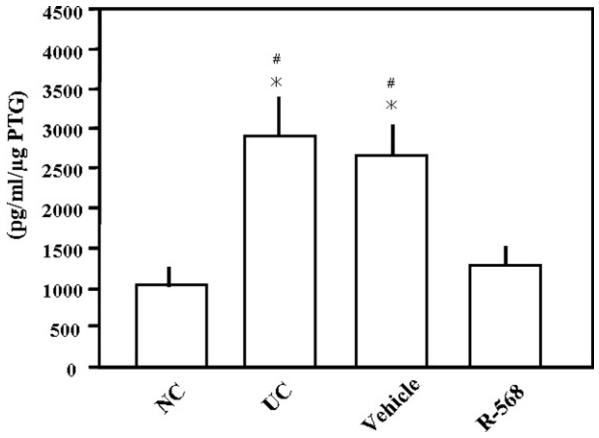

Fig. 1. PTH concentration of medium at 6 h incubation of whole parathyroid glands. Values are means ± SEM ($N = 6$–12 parathyroid glands). $P < 0.05$ by ANOVA. $^{*}P < 0.05$ versus the control group. $^{\#}P < 0.05$ versus the R-568 group by post hoc test (Fisher's PLSD).

roid tissue (Fig. 2B). The number of TUNEL-positive cells per 1000 parathyroid cells in the R-568 group (108.6 ± 14.0 per 1000 cells) was much higher than that in the other three groups: the UC (26.3 ± 0.3 per 1000 cells; $P < 0.01$), Vehicle (20.8 ± 5.1 per 1000 cells; $P < 0.01$), and NC group (12.2 ± 4.0 per 1000 cells; $P < 0.01$) (Fig. 2C). As shown in Fig. 3, R-568 did not have a dose-dependent effect on apoptosis. Few TUNEL-positive cells were detected at the concentration of 10^{-7} and 10^{-6} M while a dramatic increase in the number of TUNEL-positive cells was noted in that of 10^{-5} and 10^{-4} M. No significant increase in the number of TUNEL-positive cells was noted in the incubation with R-568 at low Ca concentration (0.05 mM) compared with the number in the other three groups (data not shown). Electron microscopy showed intact cell membranes, cellular and nuclear shrinkage, and nuclear changes including chromatin condensation and fragmentation in the R-568 group (Fig. 4).

Discussion

It has been a longstanding issue whether therapeutic options, including the active vitamin D analogues, P restriction, calcimimetics, and parathyroid intervention, could induce apoptosis in hyperplastic parathyroid glands of uremic patients with SHPT. Evidence that indicates the induction of apoptosis in hyperplastic parathyroid glands by the calcimimetic compounds has not been reported yet. This study demonstrated that the high concentration of a calcimimetic compound, R-568, could increase cell death of parathyroid cells in uremic rats in vitro. Several in vivo studies have revealed that the calcimimetic compounds suppress PTH secretion and parathyroid cell proliferation, although no data indicating the induction of parathyroid cell death has been reported [7–10]. Wada et al. showed that oral R-568 treatment suppresses the serum PTH level and parathyroid cell proliferation determined by 5-bromo-2′-deoxyuridine staining in 5/6 nephrectomized rats [10]. However, apoptosis was not detected in the study. Chin et al. demonstrated in vivo that intermittent or continuous R-568 treatment for 8 wk to uremic rats which are associated with established SHPT suppresses both the serum PTH level and parathyroid cell proliferation [7]. The suppressive effect of R-568 on parathyroid gland enlargement is correlated to the induction of morphologic difference in parathyroid cells. In the study, R-568 suppresses not only parathyroid cell number, but also parathyroid cell volume.

There are several reasons for the difficulty of detecting parathyroid cell death in vivo. The first possibility is related to biological characteristics of parathyroid cells. The turnover of normal parathyroid cells is very low. The mean life span of adult rats has been reported to be approximately 2 yr [15]. Even though parathyroid gland growth in uremic rats is accelerated by hyperphosphatemia and hypocalcemia, the cell turnover may be essentially so low that the induction of cell death will be very rare, even in cases treated with calcitriol or the calcimimetic compounds. Moreover, Uda et al. reported that apoptotic process even in SHPT, in which parathyroid cells proliferate vigorously, might be suppressed [16]. The second possibility is related to the conditioning of the tissue to be examined. For example, it has been reported that protein digestive process is very harmful to cryopreserved parathyroid tissue [17]. The third possibility is related to the tissue distribution and the concentration of the drugs. The higher concentration of R-568 might be necessary to induce cell death in hyperplastic parathyroid cells. No data has revealed what amount of each drug will be enough to induce apoptosis in parathyroid cells. These problems make it difficult to detect cell death of parathyroid cells in vivo.

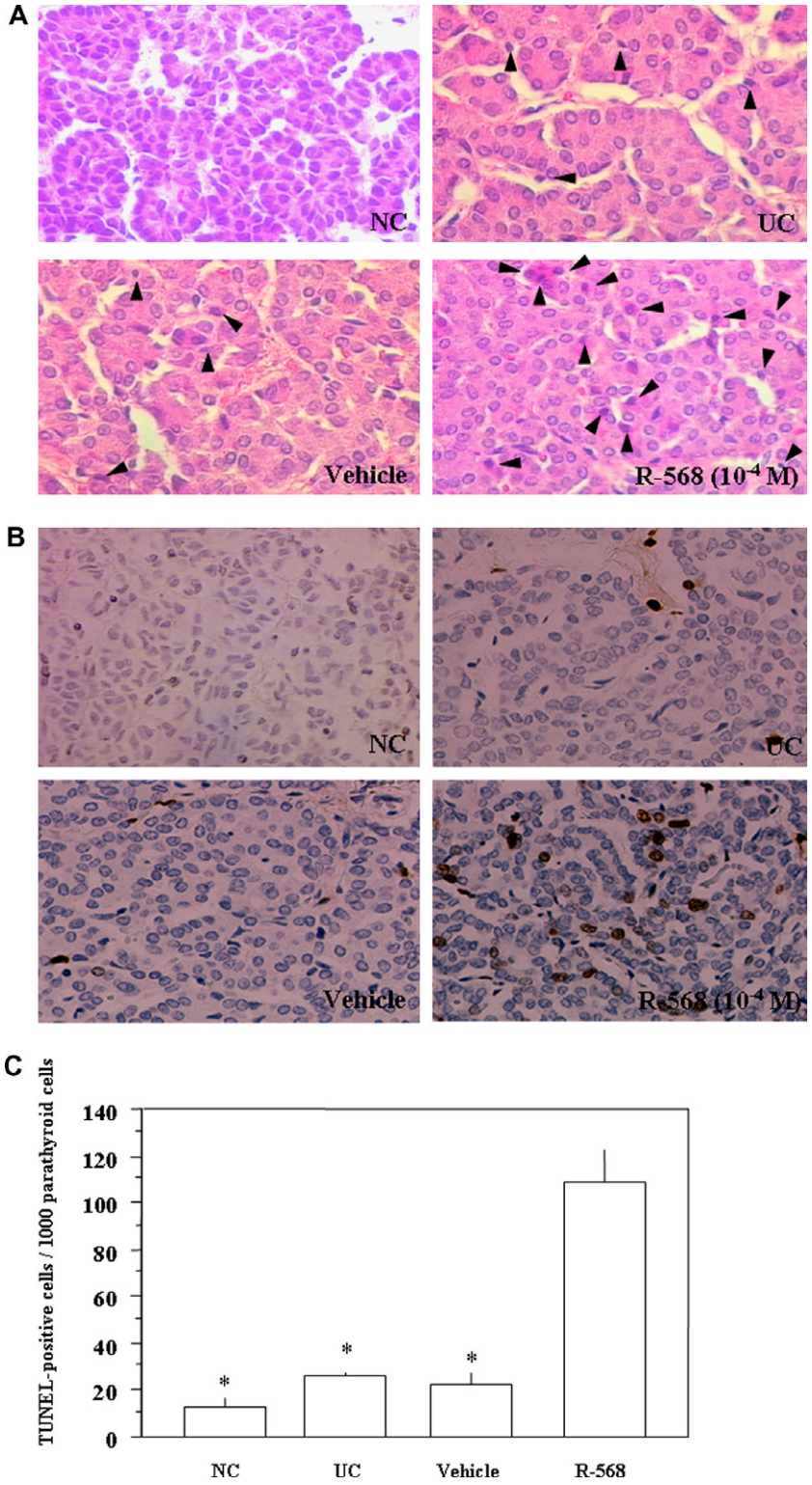

Fig. 2. Representative images of hematoxylin and eosin (HE) staining (A) and TUNEL-positive parathyroid cells of the NC, UC, Vehicle, and R-568 groups (B) and quantification of TUNEL-positive parathyroid cells reported per 1000 parathyroid cells. Values are means ± SEM ($N = 6$–12 parathyroid glands). $P < 0.01$ by ANOVA. $^{*}P < 0.01$ versus the R-568 group by post hoc test (Fisher's PLSD) (C). The arrows represent parathyroid cell death with nuclear pyknosis. Magnification at 400×.

In the current in vitro study we could test the direct action of R-568 on the parathyroid cells. Canalejo et al. reported that the tissue culture of the parathyroid gland easily induces apoptosis, which is detected by flow cytometric analysis in the parathyroid cells [18]. According to their study, not only hyperplastic parathyroid cells (from SHPT

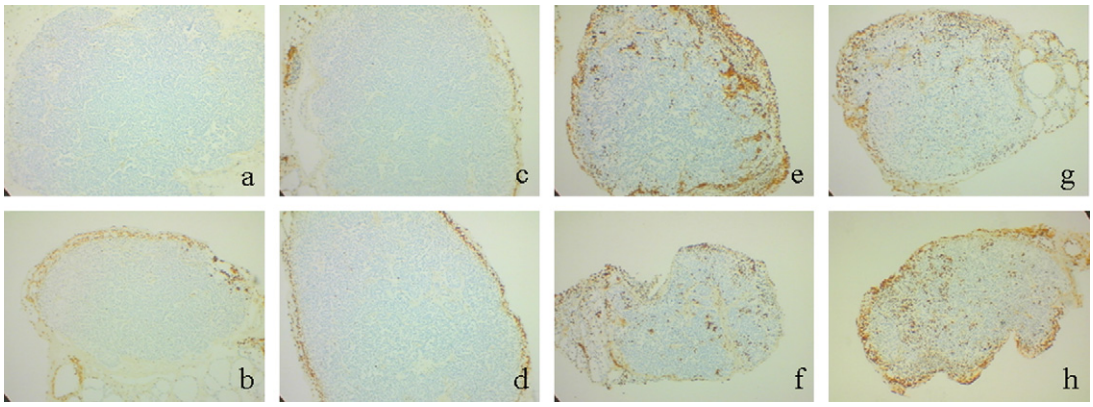

Fig. 3. Representative images of TUNEL-positive parathyroid cells in different concentrations of R-568 (10^{-7} M: a and b, 10^{-6} M: c and d, 10^{-5} M: e and f, and 10^{-4} M: g and h). Magnification at 40×.

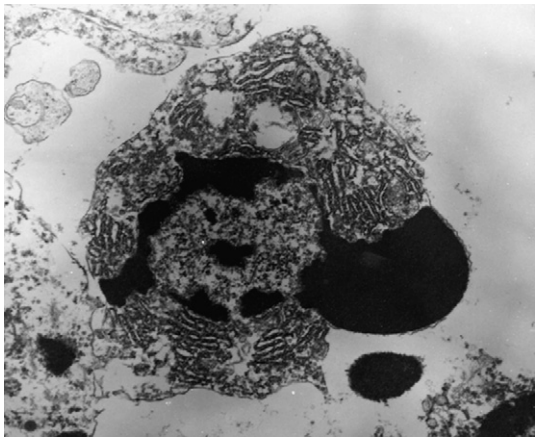

Fig. 4. Representative images of parathyroid cell apoptosis in the R-568 group confirmed by electron microscopy. Magnification at 7000×.

patients), but also normal parathyroid cells (from normal dogs) have more than 10% of apoptotic parathyroid cells after tissue incubation for 24 h. On the other hand, in our study the ratio of the TUNEL-positive cells in the NC and the UC groups was about 1% and 2%, respectively. The reason that these ratios of cell death in our study were different from those in their study may be mainly due to the different methodologies to detect cell death including apoptosis. However, the trend of facile induction of cell death in the present study was consistent with that in their study.

The precise mechanism by which parathyroid CaR activation by high concentrations of calcimimetic compound induces parathyroid cell death could not be fully elucidated in the present study. We set up several concentrations of R-568 from 10^{-7} to 10^{-4} M. While the TUNEL-positive cells in the uremic parathyroid tissues were not increased in either 10^{-7} or 10^{-6} M of R-568, these cells were dramatically increased in both 10^{-5} and 10^{-4} M, compared with those in the UC group. Moreover the ratio of the TUNEL-positive cells in 10^{-5} M was similar to that in 10^{-4} M (Fig. 4). There was a bifurcation point of an increase in the TUNEL-positive parathyroid cells between 10^{-6} and 10^{-5} M of R-568. Nemeth et al. demonstrated by using bovine parathyroid cells that a calcimimetic compound (NPS R-467) increases intracellular Ca^{2+} in a concentration-dependent manner but not in a linear pattern [9]. Intracellular Ca^{2+} concentration remains at the minimum level with the calcimimetic concentration of less than 10^{-7} M. From 10^{-7} to 10^{-5} M, however, the intracellular Ca^{2+} concentration increases dramatically to the maximum level and finally, at more than 10^{-5} M, the intracellular Ca^{2+} concentration remains at the maximum level. Their study demonstrates that R-568 also increased the intracellular Ca^{2+} concentration in HEK 293 cells expressing human parathyroid CaR. The property of calcimimetic compounds is very important in considering the mechanism by which apoptosis was induced by the calcimimetic compound in the present study. Previous studies have demonstrated that changes in intracellular Ca^{2+} concentration play a critical role in the process of cell proliferation and apoptosis [12,19–21]. Ca^{2+} release from endoplasmic reticulum leads to Ca^{2+} accumulation in mitochondria. This accumulation in mitochondria relates to programmed cell death. Recently, Choi et al. demonstrated by using the cell line of mouse beta cells (MIN6N8a cells) that cell death which is mediated by the increase in intracellular Ca^{2+} occurs within 6 h in vitro [22]. Lin et al. reported that extracellular Ca^{2+} concentrations mediate apoptosis via CaR [23]. Accordingly, our study showed that parathyroid cell apoptosis can be induced within 6 h. Taking these facts into consideration, apoptosis induced by high concentration of R-568 may be partly associated with elevation in intracellular Ca^{2+} concentration. However, the precise mechanism by which parathyroid cell apoptosis was accelerated by R-568 was not clarified in the present study.

In conclusion, we demonstrated that high concentration of the calcimimetic compound accelerated parathyroid cell apoptosis from uremic rats in vitro. It is evident that apoptotic process is involved in regulating CaR-mediated cell survival. Further studies are necessary to clarify the mechanisms by which the calcimimetic compounds mediate apoptotic process in parathyroid cells via CaR.

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