Published in final edited form as:

Eur J Haematol. 2013 July ; 91(1): 37–45. doi:10.1111/ejh.12110.

Parathyroid hormone ablation alters erythrocyte parameters that are rescued by calcium-sensing receptor gene deletion

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Abstract

The mechanisms by which parathyroid hormone (PTH) produces anemia, are unclear. Parathyroid hormone secretion is regulated by the extracellular Ca^{2+} -sensing receptor. We investigated the effects of ablating PTH on hematological indices and erythrocytes volume regulation in wild-type, PTH-null and Ca^{2+} -sensing receptor-null/PTH-null mice. The erythrocyte parameters were measured in whole mouse blood and volume regulatory systems were determined by plasma membrane K^+ fluxes and osmotic fragility was measured by hemoglobin determination at varying osmolarities. We observed that the absence of PTH significantly increases mean erythrocyte volume and reticulocyte counts, while decreasing erythrocyte counts, hemoglobin, hematocrit, and mean corpuscular hemoglobin concentration. These changes were accompanied by increases in erythrocyte cation content, a denser cell population and increased K^+ permeability, which were in part mediated by activation of the K^+/Cl^- cotransporter and Gardos channel. In addition we observed that erythrocyte osmotic fragility in PTH-null compared with wild-type mice was enhanced. When Ca^{2+} -sensing receptor gene was deleted on the background of PTH-null mice, we observed that several of the alterations in erythrocyte parameters of PTH-null mice were largely rescued, particularly those related to erythrocyte volume, K^+ fluxes and osmotic fragility, and became similar to those observed in wild-type mice. Our results demonstrate that Ca^{2+} -sensing receptor and parathyroid hormone are functionally coupled to maintain erythrocyte homeostasis.

Keywords

Parathyroid Hormone; Calcium-sensor receptor; erythrocytes; K^+ channels

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Declaration of interest: There are no conflicts of interest of the authors that could be perceived as prejudicing the impartiality of the research reported or any financial or other potential conflict of interest.

Introduction

PTH increases erythrocyte osmotic fragility in the absence of any detectable K^+ leakage or water movement across the erythrocyte plasma membrane (1). On the other hand, an *in vivo* analysis of a subset of patients with primary hyperparathyroidism indicated no difference in erythrocyte osmotic fragility when compared to normal control subjects (2) implying that PTH had no significant effects on hematological parameters. However, Sabatini found that PTH induces an increase in Ca^{2+} uptake after pre-incubation of erythrocytes with PTH *in vitro* that was accompanied by changes in water movement across the plasma membrane (3). It was also demonstrated that PTH increases intracellular Ca^{2+} levels mediated by changes in Ca^{2+} ATPase activity (4) suggesting its role as a potential mechanism for PTH effects on osmotic fragility. Consistent with these observations, a decrease in erythrocyte half-life was observed in patients with elevated PTH levels (5–7). Furthermore, it was reported that PTH enhanced DIDS-sensitive Cl^- fluxes in human erythrocytes (8). This is of importance for erythrocyte volume regulation as in erythrocytes, Cl^- transport is critical for cellular pH, volume and membrane potential regulation suggesting that PTH may indeed play a role in erythrocyte volume homeostasis.

In erythrocytes, influx of ions induces cellular volume changes that lead to swollen erythrocytes and eventually their destruction. Although, the PTH receptor does not seem to be present in erythrocytes (9), Saito et al found that the C-terminal of the PTH receptor interacts with cytoskeletal 4.1 protein that co-localizes to the plasma membrane (10). In erythrocytes, cytoskeletal 4.1 protein (4.1R) is important for maintaining plasma membrane stability, volume regulation and erythrocyte deformation capabilities. 4.1R is complexed to actin and spectrin to maintain the membrane-skeletal network (11). Indeed, modification of 4.1R protein in erythrocytes has been found to induce changes in erythrocyte ion transport and content. We previously reported that 4.1R protein deficiency causes a significant increase in Na/H exchanger activity, a major erythrocyte volume regulatory system (12). Thus, it is possible that erythrocytes respond to high levels of PTH by directly or indirectly modifying cytoskeletal proteins *via* a Ca^{2+} -dependent mechanism.

PTH secretion and gene expression as well as Ca^{2+} homeostasis are regulated in large part by the extracellular calcium-sensing receptor (CaSR) (13). CaSR regulates secretion of PTH in response to changes in extracellular calcium in parathyroid glands, the handling of calcium in the renal tubules and bone formation (13). Thus, the CaSR is critical in total calcium homeostasis. The presence of CaSR in erythrocytes has not been described as yet and its role in these cells is undefined. Therefore, the availability of mice with deletion of the gene for PTH alone, with or without deletion of the CaSR, provide a unique tool to study the *in vivo* roles of PTH and/or the CaSR in regulating hematological parameters and red blood cell physiology. We set out to explore the effects of PTH and CaSR on measures of erythrocyte volume regulation and hematological indices using PTH-null ($PTH^{-/-}$) (14) and mice with double knockout of both PTH and CaSR ($PTH^{-/-}CaSR^{-/-}$) mice (15).

Materials and Methods

Drugs and chemicals

Charybdotoxin (ChTX) was purchased from Calbachem (St. Louis, MO). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

Parathyroid hormone knockout (PTH^{-/-}) mice have been previously described (14, 16). The CaSR knockout mice were developed as described previously (15), and mice rescued from the lethal hyperparathyroidism and hypercalcemia that otherwise develops in the neonatal CaSR^{-/-} mice were generated by Kos, et al. (17) by crossing CaSR^{+/-} mice with PTH^{+/-} mice. The CaSR^{-/-} mice were created by deleting exon 5 of the CaSR. This results in some tissues, by alternative splicing, in an in-frame receptor lacking 77 amino acids within its extracellular domain(18). While it is possible that this alternatively spliced CaSR has some biological activity that could mitigate the phenotype of the CaSR^{-/-} mice, this has not been shown directly. Furthermore, the CaSR^{-/-} mice have clear abnormalities in parathyroid and renal function, consistent with near total or total functional knockout of the receptor (19).

These studies were part of an animal research protocol approved by the Brigham and Women's Hospital Animal Care and Use Committee. Animals were fed and given water *ad libitum* during the study. Animals were sacrificed according to the animal handling protocols and regulations of the Brigham and Women's Hospital and Harvard Medical School. In order to maintain normocalcemia at a level similar to that of the wild-type mice, the PTH^{-/-} and CaSR^{-/-}PTH^{-/-} mice were maintained on drinking water containing 1.5% and 0.75% calcium chloride, respectively. All three genotypes were fed *ad libitum* with PicoLab rodent diet 20, containing 0.8% calcium (Lab Diet, St. Louis, MO).

Hematological parameters

Blood cell counts were determined using the ADVIA 120 hematology analyzer (Bayer Diagnostics, Tarrytown, NY) as previously described (12). Briefly, freshly isolated whole blood from 4–5 weeks old mice was collected in EDTA-containing tubes, and an aliquot of 250 µL was used within one hour of blood drawing to perform erythrocyte and reticulocyte counts for each sample using a software program specific for mouse blood.

Preparation of erythrocytes

Blood was collected from 4–5 week old mice into heparin-containing tubes from the inferior vena cava at the time of sacrifice, passed through cotton to decrease the number of white cells, and centrifuged in a Sorvall RC 28S (Kendro Laboratory, Asheville, NC) at 2500 rpm for 4 min at 4°C as previously described (20). Briefly, the supernatant was removed by aspiration, and red blood cells were washed 4 times with ice-cold Choline Washing Solution (CWS) containing (mM): 150 Choline Cl; 10 Tris MOPS (3-[N-Morpholino]PropaneSulfonic acid), pH 7.4 (4°C), 20 sucrose and with or without 1 MgCl₂. A 50% cell (v/v) suspension was made in CWS, and hematocrits were measured manually. Aliquots of this suspension were diluted with 0.02% Acationox in double-distilled water to allow measurement of intracellular Na⁺K⁺ and Mg²⁺ by atomic absorption spectrometry (Perkin Elmer 800, Wellesley, MA) as previously described (20). Ion content was expressed as mmol/Kg Hb to correct for cellular volume and hemoglobin, the most abundant protein expressed in erythrocytes. These units allow for direct comparison of values across mouse strains as reported by us (21) and others(22, 23). In addition, we provided MCV and CHCM values that allow for estimation of cation levels expressed as either mmol/10¹³ cells or mmol/L RBC.

K/Cl co-transport activity

The K/Cl cotransport from freshly isolated cells was measured as volume-dependent K⁺ efflux following protocols previously described (24, 25). Briefly, blood collected in heparin was washed four times with an ice-cold choline wash solution (CWS) containing 172 mmol/L choline chloride, 1 mmol/L MgCl₂ and 10 mmol/L Tris-MOPS, pH 7.40, at 4°C.

Volume-induced K^+ flux was calculated as the difference between K^+ efflux in hypotonic NaCl (100 mmol/L)-containing and in isotonic NaCl (160 mmol/L)-containing media. Incubation times were 5 and 25 minutes at 37°C for flux measurements.

Gardos channel activity

Freshly isolated blood collected from mice was used to determine the Gardos channel activity (an intermediate conductance, Ca^{2+} -activated K^+ channel) in whole blood as previously described in detail (24). Briefly, the whole blood sample was mixed with Tris-MOPS, pH 8.0, at 25°C (final concentration 20 mM), 1 mM ouabain, and 10 μ M bumetanide in 3 separate Eppendorf microtubes with and without 50 nM charybdotoxin (ChTX, a potent and specific inhibitor of the Gardos Channel). The calcium ionophore, A23187, was added at a final concentration of 6 μ mol/L of cells, and the cell suspension was incubated for 1 hour at room temperature. ^{86}Rb (10 μ Ci/mL) was then added to each tube at time 0. A sample of 100 μ L was removed at 1, 3 and 5 min in duplicate and transferred to a 1.5 mL Eppendorf tube containing 0.3 mL phthalate oil and 0.8 mL normal saline with 5 mM EGTA. Samples were immediately spun down, and the cell pellet was counted for radioactivity. The suspension remaining after 3 min was spun down and the supernatant used to determine total specific activity.

Membrane Osmotic Fragility

Osmotic fragility was measured in cells washed three times in mouse isotonic saline (340 mOsm) and resuspended at a final hematocrit of $\approx$ 5%. Ten microliters of cells was added to two hundred and ninety microliters of lysis medium in microplates and was incubated at room temperature for 20 min. After gentle centrifugation, the supernatant was removed, and percent lysis was calculated from the absorbance at 540 nm. Lysis medium contained either 2 mM Tris-HEPES or 159 mM NaCl or combinations of these two media (range 120–325 mOsm, pH 7.5, at 20°C) (26).

Western Blot analysis of CaSR in normal erythrocytes

Erythrocyte plasma membranes or ghosts were prepared by hypotonic shock of freshly isolated erythrocytes in a 20-fold excess (10ml) of lysis media containing (mM): 10 Tris-HCl at pH 7.4, 0.1 EDTA, 5 DTT, 0.01 PMSF, and 1 mg/mL leupeptin. Lysates were centrifuged at 18,000 RPM (Sorvall SS34 rotor) for 20 minutes at 4°C and the pellets were washed four times in lysis media. Pellets that contained the plasma membrane fraction were suspended in 1ml lysis buffer. An aliquot was taken for protein determination using BCA method (Pierce). Membranes were suspended at a final concentration of 1 mg protein/mL. Each protein sample was subjected to SDS-PAGE on a linear gradient running gel of 4–12%. Membranes were blocked in 3% BSA for 1 h at room temperature, followed by overnight incubation with primary anti-CaSR-antibody at 4°C in T-TBS (0.05% Tween 20, 20 mM Tris, pH 7.5, 150 mM NaCl). CaSR detection was obtained by staining with either an anti-receptor antibody, 4641, or following pre-absorption of the antibody with a specific blocking peptide as a control for nonspecific staining. Membranes were then washed three times in T-TBS, incubated with a secondary antibody conjugated to horseradish peroxidase (goat anti-rabbit, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h, and washed extensively in T-TBS. The protein bands were then visualized by chemiluminescence (Amersham) and exposed to Kodak film.

Statistical analysis

Results were analyzed by 1-way ANOVA followed by the Tukey post hoc test for multiple comparisons. Differences in means with P values $\leq$ 0.05 were considered statistically significant. Values are expressed as mean $\pm$ SD.

Results

PTH deletion affects hematological parameters

We studied the *in vivo* effects of PTH ablation on hematological parameters in knockout mouse models. We found that in PTH^{-/-} mice, hemoglobin, erythrocyte counts and hematocrit were significantly decreased when compared to wild-type mice (Table I), suggesting a mild anemic state. These changes were accompanied by an increase in reticulocytes, red cell volume distribution width (RDW), and hemoglobin concentration distribution width (HDW) in mature erythrocytes. Measurements of the mean cellular volume (MCV) in mature PTH^{-/-} red cells showed no significant difference when compared to wild-type mice. In contrast, reticulocyte MCV (MCVr) was significantly elevated suggesting a macrocytic anemia. Furthermore, we observed that the reticulocyte RDW (RDWr) was reduced implying a greater homogeneity among reticulocytes.

Erythrocyte indices for the CaSR^{-/-}PTH^{-/-} mice are shown in Table 1. We found that hemoglobin, erythrocyte counts and hematocrit in the CaSR^{-/-}PTH^{-/-} mice were significantly different than wild-type but not different than the PTH^{-/-} (Table 1). However, we found that the reticulocyte count in CaSR^{-/-}PTH^{-/-} mice was significantly decreased (8.1×10^5 to 5.2×10^5 cell/mL, $n>12$, $p<0.01$) and was accompanied by an increase in reticulocyte hemoglobin levels when compared to PTH^{-/-} mice (16.1 to 17.0 pg, $n>12$, $p<0.02$).

CaSR is present in the plasma membrane of erythrocytes

Western immunoblotting of ghost erythrocytes was performed using a CaSR specific antibody (27). As shown in Figure 1A, two immunoreactive bands between 140 and 200 kDa were observed. The ~200 kDa bands may be due to differential degrees of glycosylation as reported by others (28). However, we did not detect smaller bands that have been reported in other cells and tissues and may represent monomeric forms of CaSR (29, 30). These bands were masked following pre-absorption of the specific antibody with blocking peptide (Fig. 1B). The pattern of immunoreactive bands observed in ghost membrane preparations was very similar to that in membranes prepared from bovine parathyroid glands (27).

Intracellular cation content in wild type, PTH^{-/-} and CaSR^{-/-}PTH^{-/-} mouse erythrocytes

Measurements of red cell indices and ion contents were carried out as detailed in the Methods section and are shown in Table 2. The content of the major intracellular cation, K⁺ was significantly elevated in the PTH^{-/-} mice compared to wild-type mice; however, significant decreases in these parameters were observed when CaSR was deleted in addition to the PTH gene. These results are in strong agreement with the decrease in RDW and osmotic fragility observed in the CaSR^{-/-}PTH^{-/-} mice relative to the PTH^{-/-} mice. Cellular Na⁺ and Mg²⁺ were significantly elevated in the PTH^{-/-} mice. No significant differences, however, were observed in the CaSR^{-/-}PTH^{-/-} mice when compared to the PTH^{-/-} (Table 2). Total cation content was significantly altered in PTH^{-/-} mice compared to wild-type mice ($p<0.002$, $n>7$), which is in agreement with the increase in MCVr observed under these conditions. Although we observed a smaller total cation content in the CaSR^{-/-}PTH^{-/-} when compared to the PTH^{-/-} it was not statistically significant. Together, these results strongly suggest an abnormality in the control of erythrocyte cellular volume when the PTH gene is inactivated, possibly via an alteration in K⁺ transport, which depends on the presence of CaSR for its expression.

K⁺ permeability is affected by PTH gene deletion in mouse erythrocytes

Since PTH deletion caused a change in erythrocyte densities, we tested whether these changes in cellular volume were associated with changes in volume regulatory systems. One

major red blood cell volume regulatory system is the K/Cl co-transporter, which is activated by cell swelling and, when active, increases K⁺ permeability to re-establish cellular volume. The activity of the K/Cl cotransporter was measured as detailed in *Methods* in freshly isolated erythrocytes from PTH^{-/-} and wild-type mice (Figure 2). We observed that deletion of the PTH gene induces an increase in volume-stimulated K⁺ transport in comparison to wild-type mice. In addition, CaSR deletion in combination with inactivation of the PTH gene reverses this effect (Figure 2). These results suggest that the absence of PTH is associated with changes in K⁺ transport via the K/Cl co-transporter thus providing a mechanism for volume/size changes in these red cells. As with the other parameters measured, the presence of CaSR is required, by some unknown mechanism(s), for expression of the alteration in K/Cl co-transport observed in the PTH^{-/-} mice.

To test if these effects of PTH loss on red cells only affected K⁺ transport mediated by the K/Cl co-transporter, we also measured the effects of PTH deletion on the red cell Ca²⁺-activated K⁺ channel, also known as the Gardos channel. Figure 3 shows that the activity of this channel was also elevated in the absence of PTH and was reduced when the CaSR was also deleted. Therefore, as with the parameters presented earlier, these results indicate that CaSR deletion, in the absence of PTH, blunted the observed abnormalities suggesting that CaSR and PTH receptor are functionally coupled to contribute to erythrocyte volume homeostasis.

Change in the osmotic fragility pattern in wild type, PTH and CaSR KO mouse erythrocytes

Changes in K⁺ permeability are associated with alterations in osmotic fragility. Analyses of osmotic fragility curves allow an estimation of the median osmotic fragility, which indicates the point at which 50% of the cells are hemolyzed. The effects of PTH deletion on osmotic fragility of mouse erythrocytes are given in Figure 4. We observed that deletion of PTH produces a significant increase in osmotic fragility. The median osmotic fragility was increased from 144 ± 4 mOsm to 162 ± 2 mOsm in the absence of PTH, thus rendering the cells more susceptible to lysis. Note that at the median osmotic fragility in wild-type erythrocytes, more than 80% of the red cells were lysed in the PTH^{-/-} mice. In contrast, deletion of the CaSR in combination with PTH (mean osmotic fragility = 148 ± 5) again reverses this effect of loss of PTH alone, thus suggesting interplay between PTH and CaSR in the regulation of water and surface area in mouse erythrocytes.

Discussion

The aim of the present study was to assess the potential role of PTH in erythrocyte volume regulation and the contribution of CaSR to this process. Modulation of erythrocyte volume by PTH ablation is evident from the abnormally increased erythrocyte K⁺ transport observed in these mice. These effects were accompanied with increased erythrocyte density. Red cell density is the result of hemoglobin, ion and water content. Changes in any of these parameters will induce a change in density and size/volume. Our results demonstrated parallel changes in MCV, a measure of the average size of the red blood cells, and total ion content in the PTH^{-/-} mouse when compared to wild-type mice strongly supporting the contention that PTH play a important role in volume regulation. Our results imply that the absence of the CaSR ameliorates some of the effects associated with loss of PTH in the circulation in the PTH^{-/-} mice. The effects of losing PTH on erythrocyte parameters and reticulocyte counts may require a permissive action of the CaSR. Furthermore, in the absence of both signals, e.g., in the double knockout mice, the maintenance of normality of these parameters of erythroid homeostasis uses an alternative/additional pathway(s). Unfortunately, it is not possible to study the hematological parameters being characterized here in CaSR^{-/-} mice, as mice of this genotype are severely hypercalcemic and hyperparathyroid at birth and die within the first 1–2 weeks of life.

Alterations in erythrocyte size could be due to nutritional deficiency (vitamin B12 or folate deficiency) or blood transfusion (31, 32). However, it is unlikely that the changes observed in the PTH^{-/-} mice are due to vitamin B12 and/or folic acid deficiency, since the diet of the mice was supplemented with these vitamins. In addition, no bone marrow fibrosis has been observed in these animals and the PTH^{-/-} mice have normal creatinine levels. Furthermore there is evidence that PTH may increase the hematopoietic stem cell population via parathyroid hormone receptor 1 (PTHr1) (33–37). Consequently, one could expect that in the absence of PTH there would be a reduction in erythrocyte production. However, our results show that lack of PTH results in reticulocytosis concomitant with increased osmotic fragility, reduced hemoglobin and decreased hematocrit, effects that are suggestive of a hemolytic anemia. Since parathyroid hormone-related peptide (PTHrP) can serve as a PTHr1 ligand, these results suggest that the effects we observe are specific effects of PTH on circulating erythrocytes.

Modulation of erythrocyte membrane ion channels is of fundamental importance for determining erythrocyte homeostasis, hydration status and volume regulation. Red cell membrane physiology studies have indicated that erythrocyte volume and hydration status is regulated by two major K⁺ transporters; namely the K/Cl co-transporter and the Ca²⁺-activated K⁺ channel (known in erythrocytes as the Gardos Channel) (38, 39). The K/Cl co-transporter is volume-stimulated and pH-sensitive as intracellular acidification and cell swelling activate this transporter. However, there is no pharmacological regulator or inhibitor of this system with sufficient specificity for potential therapeutic or clinical use. The Ca²⁺-activated K⁺ channels are activated by increases in ionized cellular Ca²⁺ that promotes K⁺ and water loss thus mediating red cell volume changes (38, 39). Upon activation, both cation transporters are known to induce significant changes in erythrocyte density profile as shown in the PTH^{-/-} and PTH^{-/-}CaSR^{-/-} erythrocytes. Human and mouse erythrocytes have a Gardos channel (hIK1 or hSK41–4) capable of mediating increases in K⁺ fluxes across the plasma membrane of 3 orders of magnitude thereby leading to significant volume changes in these cells (40, 41). Examination of the activities of these transporters demonstrated that PTH deletion induces an increase in the maximal activity of both K⁺ transporters through mechanisms that remain unexplained. Increases in cellular K⁺ have been shown to activate these transporters. Indeed, we demonstrate that PTH^{-/-} erythrocytes maintain a significant difference in intracellular cation content with increases in Na⁺K⁺ and Mg²⁺ levels that are in agreement with changes in MCV. Interestingly, ablation of the CaSR gene in the PTH^{-/-}CaSR^{-/-} mice decreases K⁺ content towards values observed in the wild-type mice, suggesting that the PTH/CaSR system is an important regulatory factor for cellular K⁺ homeostasis. Alternatively, it is possible that an increase in reticulocyte number might be responsible for the observed effects in K⁺ transport since reticulocytosis has been shown to be associated with increased K⁺ permeability (38). However, our data show that the PTH^{-/-}CaSR^{-/-} mice, which have a 3-fold increase in reticulocyte number when compared to wild-type mice, showed no difference in either K/Cl cotransport or Gardos channel activity. These results suggest that reticulocytosis in the PTH^{-/-} mice may not be responsible for the increased K⁺ transport observed. Therefore, in the absence of both genes, other currently undefined mechanisms are apparently capable of preserving the same balance normally promoted by PTH and the CaSR. We speculate that this effect could be due to a lack of PTH interacting directly with the red cells leading to an interruption of the normal regulation mediated by the coupling function of PTH and CaSR. Although the mechanism(s) underlying these effects are not defined, our observations suggest that the absence of PTH may induce effects at the level of progenitor cells that affect the expression, number and/or activities of K⁺ transporters or their regulatory proteins. Altogether, our results suggest that PTH and CaSR are functionally coupled to regulate K⁺ transport in mouse erythrocytes.

Deformability and mechanical stability are two properties of the erythrocyte membrane that are regulated by the cytoskeletal protein network and the interaction with various integral proteins. Osmotic fragility is a measure of the capacity of the erythrocyte to resist osmotic lysis and has been established as a sensitive assay to characterize the surface to volume ratio of erythrocyte. We observed that the membrane properties of fragility and stability were markedly different between $PTH^{-/-}$ and $PTH^{-/-}CaSR^{-/-}$ erythrocytes. While $PTH^{-/-}$ erythrocytes exhibited a marked increase in membrane fragility with a larger surface area (elevated MCV) during the osmotic challenge, erythrocytes from the double knockout mice, in contrast, exhibited only a marginal increase in cellular volume but a pronounced decrease in membrane fragility. There are at least two possible explanations for the differences between these two genotypes. First, CaSR deletion is permissive for the survival of populations of abnormal erythrocytes in $PTH^{-/-}$ mice that would ordinarily be removed by the spleen as a result of a reduction in the activity of the K^{+} transporter, in particular the Ca^{2+} -activated Gardos Channel. This possibility is supported by our results showing decreases in reticulocyte levels in the $PTH^{-/-}CaSR^{-/-}$ mice. Alternatively, expression of CaSR could alter the expression of membrane-skeletal proteins that modulate the size and deformability of erythrocytes. This is supported by reports from others showing that CaSR associates with the cytoskeletal protein, filamin (42, 43). CaSR mediates activation of the low molecular weight G protein, Rho, and its downstream effector, Rho kinase, with resulting changes in the actin cytoskeleton and cellular architecture (44). More recently, reports have shown that CaSR activation can induce actin stress fiber assembly and retraction of cellular processes via rho/rho kinase (45). Thus, PTH and CaSR deletion may be modulating assembly of the erythrocyte cytoskeleton leading to alterations in volume regulating cation transporters. Furthermore, changes in red cell membrane proteins that alter volume regulating cation transporters have been previously reported by us, and others (12, 46, 47).

Our observation that removal of the CaSR gene in the $PTH^{-/-}$ mice restores a number of hematological parameters towards values similar to those in wild-type mice suggests that the CaSR and PTH antagonize one another's actions on erythrocyte function and that the presence of the CaSR is permissive to the hematological abnormalities seen in the $PTH^{-/-}$ mice. A similar antagonistic relationship between CaSR and PTH is present in the thick ascending limb of Henle's loop, where PTH stimulates renal calcium reabsorption while the CaSR inhibits this action and promotes renal calcium excretion (48). Alternatively, the CaSR exerts actions on red cells that are normally masked by the presence of PTH, so that PTH can be seen as rescuing the CaSR effect in the wild-type mice. Since $CaSR^{-/-}$ mice die early in life, performing the converse experiment, i.e. removing CaSR first and then adding in the $PTH^{-/-}$ is not possible to distinguish between these possibilities. Thus our results have provided evidence that the CaSR and PTH are coupled to maintain erythrocyte homeostasis.

Acknowledgments

This work was supported by the National Institutes of Health grants HL090632 (to AR), HL096518, DK064841 and ES014462 (to JRR), DK67111 and DK67155 (to EMB) and by grants from the Canadian Institutes for Health Research (to DG and AK). We thank Ms. Jessica Alves for her excellent technical support.

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Figure 1. Western analysis of CaSR in erythrocyte membrane preparations

Red cell plasma membranes were prepared by hypotonic shock of freshly isolated erythrocytes as described in the *Materials and Methods* section. Each protein sample was subjected to SDS-PAGE on a linear gradient running gel of 4–12%. CaSR detection was obtained by staining with either a specific anti-CaSR antibody, 4641, (A) or with the same antibody following preabsorption with the specific blocking peptide against which it was raised as a control for nonspecific staining (B).

Figure 2. Functional characterization of K/Cl co-transport in wild type, PTH^{-/-} and PTH^{-/-}CaSR^{-/-} mouse erythrocytes

K/Cl co-transport was measured as the volume-sensitive K⁺ efflux in hypotonic conditions as described in the Methods section. A significant difference was found between wild-type and PTH^{-/-} mice but not between the wild-type and PTH^{-/-}CaSR^{-/-} mice. The results are expressed as mean ± SD of 3 experiments with duplicate determinations. N=3, *p<0.012.

Figure 3. Effects of PTH and CaSR deletion on the Ca^{2+} -activated K^+ channel activity (Gardos channel) in erythrocytes

The channel activity was expressed as the difference between the K^+ fluxes in the presence or absence of 50 nM ChTX as described in the *Methods* section. The results are expressed as mean $\pm$ SD of 3 experiments with duplicate determinations. The statistical analysis was performed comparing PTH^{-/-} vs wild-type (control,* $p < 0.05$) or PTH^{-/-}CaSR^{-/-} mice (** $p < 0.05$)

Figure 4. Erythrocyte osmotic fragility in wild type, $PTH^{-/-}$ and $PTH^{-/-}CaSR^{-/-}$ mice
 Freshly isolated cells were incubated at different osmolarities as described in the *Methods* section. Statistical analysis indicated a significant shift between $PTH^{-/-}$ vs. wild-type or $PTH^{-/-}CaSR^{-/-}$ mice, $n=3$, $*p<0.032$. The graph represents the mean of three different experiments.

Table 1

Hematological parameters of PTH^{-/-} and PTH^{-/-}CaSR^{-/-} mice compared to wild-type mice.

ID	Wild-type (n=16)	PTH ^{-/-} (n=12)	PTH ^{-/-} CaSR ^{-/-} (n=15)	p values
RBC, 10 ⁶ cells/ μ L	10.7 $\pm$ 1.0	8.5 $\pm$ 1.4 ^a	8.2 $\pm$ 0.8 ^C	a<0.0002 , c<0.0001
HGB, g/dL	16.2 $\pm$ 1.8	13.6 $\pm$ 2.7 ^a	14.3 $\pm$ 1.3 ^C	a<0.002 , c<0.003
Hct, %	52.5 $\pm$ 4.6	44.2 $\pm$ 8.5 ^a	46.0 $\pm$ 4.3 ^C	a<0.005 , c<0.01
<u>Reticulocytes</u>				
%	2.2 $\pm$ 0.6	9.4 $\pm$ 5.0 ^a	6.8 $\pm$ 2.6 ^b	a<0.001,b<0.03
Retic,10 ⁵ cells/ μ L	2.4 $\pm$ 0.7	8.1 $\pm$ 3.9 ^a	5.2 $\pm$ 2.3	a<0.002,b<0.01
MC Vr, %	57.0 $\pm$ 1.3	60.0 $\pm$ 2.9 ^a	61.3 $\pm$ 1.4	a<0.002
CHCMr, g/dL	27.6 $\pm$ 0.9	26.9 $\pm$ 1.4	27.8 $\pm$ 0.4 ^b	b<0.02
CHr, pg	15.6 $\pm$ 0.3	16.1 $\pm$ 1.4	17.0 $\pm$ 0.5 ^b	b<0.022
RDWr, %	18.1 $\pm$ 3.0	15.0 $\pm$ 2.0 ^a	12.5 $\pm$ 1.0 ^b	a<0.004, b<0.0 02
HDWr, g/dL	2.8 $\pm$ 0.6	3.2 $\pm$ 0.5 ^a	2.5 $\pm$ 0.2 ^b	a<0.04, b<0.0001
<u>Mature erythrocytes</u>				
MCV, %	49.6 $\pm$ 0.7	50.7 $\pm$ 3.6	55.3 $\pm$ 1.2 ^b	b<0.0001
CHCM, g/dL	29.4 $\pm$ 0.7	28.7 $\pm$ 1.3 ^a	29.2 $\pm$ 0.5	a<0.04
CH, pg	14.6 $\pm$ 0.3	14.6 $\pm$ 1.6	16.2 $\pm$ 0.3 ^b	b<0.0005
RDW, %	12.4 $\pm$ 0.3	17.1 $\pm$ 3. 0 ^a	13.5 $\pm$ 1.2 ^b	a<0.0001, b<0.0002
HDW, g/dL	1.9 $\pm$ 0.1	2.3 $\pm$ 0.5 ^a	1.9 $\pm$ 0.1 ^b	a<0.007, b<0.005

Determinations were done in whole blood collected in heparin-containing tubes from 4–5 week old isoflurane-anesthetized mice. Values represent the mean $\pm$ SD. RBC (Red Blood Cells), HGB (hemoglobin), Hct (hematocrit), MCVr (reticulocyte mean cellular volume), CHCMr (reticulocyte cellular hemoglobin concentration mean), CHr (reticulocyte cellular hemoglobin), RDWr (reticulocyte cell volume distribution width), HDWr (reticulocyte hemoglobin concentration distribution width)

^aWild-Type vs. PTH^{-/-};

^bPTH^{-/-} vs. PTH^{-/-} CaSR^{-/-} mice.

Measurements are performed in triplicate determinations for each mouse type.

Table 2

Cellular cation contents of erythrocytes from PTH^{-/-}, PTH^{-/-}CaSR^{-/-} and wild-type mice.

Cation	Wild-type	PTH ^{-/-}	PTH ^{-/-} CaSR ^{-/-}
mmol/ Kg Hb			
Na ⁺	14.3 ± 0.9	17.3 ± 2.3 ^a	15.4 ± 1.5
K ⁺	392.7 ± 20.4	460.4 ± 55.7 ^b	402.8 ± 38.0 ^c
Mg ²⁺	7.04 ± 0.49	10.70 ± 1.70 ^a	9.29 ± 1.4

Intracellular erythrocyte cation concentrations were determined by atomic absorption spectrometry utilizing freshly isolated erythrocytes as described in the *Materials and Methods* section. Total value represents the sum of all cation concentrations for each mouse genotype.

^a p<0.007,

^b p<0.05 wild-type vs. PTH^{-/-}; PTH^{-/-} vs. PTH^{-/-}CaSR^{-/-}

^c p<0.05.

The values are the mean ± SD of N >7 for each mouse type in triplicate determinations.