Pathophysiologic Changes in Extracellular pH Modulate Parathyroid Calcium-Sensing Receptor Activity and Secretion via a Histidine-Independent Mechanism

Katherine L. Campion,* Wanda D. McCormick,*† Jim Warwicker,* Mohd Ezuan Bin Khayat,*‡ Rebecca Atkinson-Dell,* Martin C. Steward,* Leigh W. Delbridge,§ Hee-Chang Mun,§ Arthur D. Conigrave,§ and Donald T. Ward*

*Faculty of Life Sciences, The University of Manchester, Manchester, United Kingdom; †Animal Welfare, Moulton College, Northamptonshire, United Kingdom; ‡Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Selangor, Malaysia; and §School of Molecular Bioscience, University of Sydney, New South Wales, Australia

ABSTRACT
The calcium-sensing receptor (CaR) modulates renal calcium reabsorption and parathyroid hormone (PTH) secretion and is involved in the etiology of secondary hyperparathyroidism in CKD. Supraphysiologic changes in extracellular pH (pHo) modulate CaR responsiveness in HEK-293 (CaR-HEK) cells. Therefore, because acidosis and alkalosis are associated with altered PTH secretion in vivo, we examined whether pathophysiologic changes in pHo can significantly alter CaR responsiveness in both heterologous and endogenous expression systems and whether this affects PTH secretion. In both CaR-HEK and isolated bovine parathyroid cells, decreasing pHo from 7.4 to 7.2 rapidly inhibited CaR-induced intracellular calcium (Ca2+) mobilization, whereas raising pHo to 7.6 potentiated responsiveness to extracellular calcium (Ca2+). Similar pHo effects were observed for Ca2+-induced extracellular signal-regulated kinase phosphorylation and actin polymerization and for L-Phe-induced Ca2+ mobilization. Intracellular pH was unaffected by acute 0.4-unit pHo changes, and the presence of physiologic albumin concentrations failed to attenuate the pHo-mediated effects. None of the individual point mutations created at histidine or cysteine residues in the extracellular domain of CaR attenuated pHo sensitivity. Finally, pathophysiologic pHo elevation reversibly suppressed PTH secretion from perifused human parathyroid cells, and acidosis transiently increased PTH secretion. Therefore, pathophysiologic pHo changes can modulate CaR responsiveness in HEK-293 and parathyroid cells independently of extracellular histidine residues. Specifically, pathophysiologic acidification inhibits CaR activity, thus permitting PTH secretion, whereas alkalization potentiates CaR activity to suppress PTH secretion. These findings suggest that acid-base disturbances may affect the CaR-mediated control of parathyroid function and calcium metabolism in vivo.

secondary hyperparathyroidism of CKD. In a heterologous human embryonic kidney 293 (HEK-293) cell-based expression system, Quinn et al.\(^3\) found that the Ca\(^{2+}\)\(_o\) potency of the human CaR is sensitive to supraphysiologic changes in extracellular pH (pH\(_o\)). By varying the pH of the experimental buffer in 0.5-unit steps from 5.5 to 9, the sensitivity of CaR to Ca\(^{2+}\)\(_o\) (and Mg\(^{2+}\)\(_o\)) was altered; external acidification decreased CaR sensitivity and alkalinization increased CaR sensitivity.\(^3\) However, blood pH levels (7.35–7.45 approximately 40 nM H\(^+\) concentration) rarely vary beyond ±0.4 pH units in vivo, even under extreme pathologic conditions. Even smaller changes (±0.2) are more generally seen, such as with the acidosis of CKD; however, even this still represents an approximately 58% increase in H\(^+\) concentration. Secondary hyperparathyroidism is another complication of CKD. In animal experiments, induction of metabolic acidosis results in increased serum PTH levels and hypercalcemia, whereas induction of alkalosis suppresses serum PTH levels.\(^4\)–\(^6\)

Therefore, because acidosis has been associated with increased serum PTH levels in vivo, we examined whether much smaller, pathophysiologically relevant changes in pH\(_o\) (±0.2) can alter the Ca\(^{2+}\)\(_o\) sensitivity of CaR-mediated signaling pathways in HEK-293 cells and whether such effects can be replicated in parathyroid cells. We also examined whether a specific histidine or free cysteine residue could account for CaR pH\(_o\) sensitivity and, finally, considered the effect of such pathophysiologic changes in pH\(_o\) on PTH secretion.

**RESULTS**

Fura 2–loaded CaR-HEK cells were stimulated with 2.5 mM Ca\(^{2+}\)\(_o\) (pH, 7.4) to elicit CaR-induced Ca\(^{2+}\)\(_i\) mobilization and were then switched to the same buffer at pH 7.2 or 7.6 before being returned to pH 7.4. At pH 7.6, CaR responsiveness (i.e., A 350/380 fura ratio area under the curve) was significantly increased (Figure 1A), an effect that was immediately reversible upon return to pH 7.4. In contrast, lowering pH\(_o\) to 7.2 significantly inhibited CaR responsiveness (Figure 1B), the effect being immediately reversible. The effect of ±0.2-unit pH changes on Ca\(^{2+}\)\(_i\) mobilization was then tested over a range of Ca\(^{2+}\)\(_o\) concentrations, from 0.5 to 10 mM. Again, CaR responsiveness was significantly increased with pH 7.2 (half maximal effective concentration for Ca\(^{2+}\)\(_o\) 3.6±0.2 mM in 7.2 versus 3.1±0.1 in 7.4; \(P<0.05\)) and significantly stimulated with pH 7.6 (2.8±0.0 mM in 7.6; \(P<0.05\)). In addition, similar effects of changing pH of (±0.2 unit) were also seen using bicarbonate/CO\(_2\) buffers (Figure 1D). See Supplemental Material for the complete methods.

To determine whether this apparent pH\(_o\) sensitivity of CaR is specific to intracellular Ca\(^{2+}\)\(_i\) mobilization or applies to other effector pathways, two other readouts of CaR activity were used: extracellular signal-regulated kinase (ERK) phosphorylation and actin polymerization.\(^9\)\(^,\)\(^10\) Indeed, mild acidosis (pH\(_o\) 7.2) significantly inhibited Ca\(^{2+}\)\(_o\)-induced ERK phosphorylation in CaR-HEK cells (3.5 mM Ca\(^{2+}\)\(_o\)) (Figure 2A), while mild alkalosis (pH\(_o\) 7.6) increased ERK activation. Similarly, a 0.2-unit reduction in pH\(_o\) inhibited 1.5 mM Ca\(^{2+}\)\(_o\)-induced actin polymerization while a 0.2-unit increase in pH\(_o\) potentiated 1.8 mM Ca\(^{2+}\)\(_o\)-induced actin polymerization (Figure 2B). Together these results suggest that the effect of altering pH\(_o\) affects the Ca\(^{2+}\)\(_o\) sensitivity of the CaR rather than the mechanism of Ca\(^{2+}\)\(_i\) mobilization. Indeed, in the presence of 0.5 mM Ca\(^{2+}\)\(_o\) (which

![Figure 1](https://example.com/figure1.png)

**Figure 1.** CaR-induced Ca\(^{2+}\)\(_i\) mobilization is sensitive to pathophysiologic pH\(_o\) changes in CaR-HEK cells. (A) Representative trace showing Ca\(^{2+}\)\(_i\) changes (Fura-2 ratio) in a single cell (upper trace, “cell”) and “global” (lower trace) cluster of (>10) cells in response to elevated [Ca\(^{2+}\)\(_o\)] (2.5 versus 0.5 mM control) when pH\(_o\) was changed from 7.4 to 7.6. Changes in [Ca\(^{2+}\)\(_i\)] shown as percentage control of the area under the curve. n=4 coverslips. (B) Identical except testing the effect of decreased pH\(_o\) (7.2). n=4 coverslips. *P<0.05 and ***P<0.001 versus first pH 7.4 treatment; ****P<0.001 versus pH 7.6 treatment by repeated-measures ANOVA (Tukey post-test) performed on the raw data. (C) Cells were exposed to buffers containing increasing concentrations of Ca\(^{2+}\)\(_o\) (0.5–10 mM) with pH\(_o\) 7.2, 7.4, or 7.6 with the resulting concentration-effect curves for Ca\(^{2+}\)\(_i\) mobilization shown (left) together with their half maximal effective concentration (EC\(_{50}\)) (right). While the EC\(_{50}\) values were significantly different, the maximal responses were not significantly different, despite the apparent trend. *P<0.05 versus pH 7.4 by repeated-measures ANOVA (Dunnett) performed on log EC\(_{50}\) values from four independent experiments (two to five coverslip replicates per data point, >15 cells per coverslip). (D) Similar results were obtained with use of bicarbonate/CO\(_2\) buffers, with pH 7.6 increasing Ca\(^{2+}\)\(_i\) mobilization and pH 7.2 decreasing mobilization in a single cell (upper trace) and “global” cluster of (>10) cells (lower trace) in response to 2.5 mM Ca\(^{2+}\)\(_o\) as before. Relative changes in [Ca\(^{2+}\)]\(_o\) are shown in the bar graph. The approximate pH values were obtained by varying the bicarbonate (and NaCl) concentrations in buffers gassed continuously with 5% CO\(_2\)/95% O\(_2\) (g). For the complete methods, see the Supplemental Material. *P<0.05 versus pH 7.4 treatment by one-tail paired t test; n=7 coverslips.
renders the CaR inactive), variations in pH₀ between 7.2 and 7.6 had no effect on actin polymerization. This finding indicates that changing pH₀ does not otherwise affect baseline signaling.

Next, we examined the effect of 0.2-unit pH₀ changes on the response of the CaR-positive allosteric modulator L-Phe (10 mM) to determine whether the pH₀ sensitivity is exclusive to orthosteric agonism with Ca²⁺. We observed significant potentiation of L-Phe-induced Ca²⁺ mobilization in pH₀ 7.6 and attenuation in pH₀ 7.2 (Figure 2C), similar to that observed for Ca²⁺₀.

We then assessed whether pathophysiologic changes in pH₀ also affect CaR-induced Ca²⁺ mobilization in parathyroid cells, in which the CaR is expressed endogenously. Indeed, raising pH₀ to 7.6 while stimulating CaR-induced Ca²⁺ mobilization in bovine parathyroid cells (2.5 mM Ca²⁺₀) significantly potentiated the response, an effect that was fully and immediately reversible upon return to pH₀ 7.4 (Figure 3A). In addition, lowering pH₀ to 7.2 promptly inhibited the Ca²⁺₀ mobilization elicited in parathyroid cells in response to 2.5 mM Ca²⁺ (Figure 3B).

In considering the potential (patho)physiologic relevance of CaR pH₀-sensitivity, it should be noted that serum albumin can bind calcium in a pH-dependent manner. Thus, albumin releases calcium under acidic conditions, which might tend to counteract the concomitant CaR inhibition, whereas alkaline conditions promote calcium binding to albumin and thus potentially counteract the CaR stimulation. Thus, we tested the effects of pH₀ on CaR-induced Ca²⁺ mobilization in the presence of a physiologic concentration of 5% (w/v) albumin. Exposure of CaR-HEK cells to 3 mM Ca²⁺₀ (in 5% [w/v] albumin) at pH 7.4 resulted in an increase in Ca²⁺ concentration, and as before, lowering pH₀ to 7.2 resulted in a reversible attenuation of the CaR response (Figure 4Ai). In addition, increasing pH₀ to 7.6 while stimulating CaR with 3 mM Ca²⁺₀ (in 5% albumin) potentiated the response (Figure 4Aii). This was further tested in bovine parathyroid cells; again, raising pH₀ to 7.6 potentiated CaR activity despite the presence of 5% (w/v) albumin (Figure 4B).

To test whether the changes in pH₀ might instead be acting by altering intracellular pH (pHᵢ), CaR-HEK and bovine parathyroid cells were loaded with the pH-sensitive dye BCECF and exposed to CaR stimulation in the presence or absence of even greater changes in pH₀, namely pH 7.0 for acidosis and pH 7.8 for alkalosis. However, in neither cell type did pH₀ 7 or 7.8 (or indeed CaR activation itself) affect pHᵢ over the timescales used (Figure 5). Therefore, these data suggest that the consequence of altering pH₀ on intracellular signaling is via an effect on the CaR per se. Ammonium chloride and sodium acetate were used as positive controls to demonstrate that pHᵢ changes could be detected in the BCECF-loaded cells. Indeed, ammonium chloride elicited intracellular alkalization in both cell types, whereas sodium acetate induced a marked intracellular acidification in CaR-HEK cells and had a much smaller effect in bovine parathyroid cells.

**Effect of Altered pH₀ on Parathyroid Hormone Secretion**

Parathyroid hormone secretion was measured in human parathyroid cells. In the presence of physiologic free Ca²⁺₀
concentration (1.2 mM), lowering pHo (7.2 then 7.0) caused an initial increase in PTH secretion whereas raising pH (7.6 then 7.8) suppressed PTH secretion (Figure 6). When pHo was lowered to normal (7.4), PTH secretion then rose again, suggesting that the suppression was fully reversible. Preliminary experiments investigating the effect of 0.4-unit pHo changes on PTH secretion from both bovine and human parathyroid cells (and conducted before those shown in Figure 6) showed a similar pHo sensitivity, with alkalosis suppressing PTH secretion significantly in both cases (not shown).

### Investigation of Extracellular Histidine/Cysteine Residues as Possible Sites of CaR pHo Sensitivity

Finally, modeling the homodimeric CaR extracellular domain using sequence alignment with the metabotropic glutamate receptor (mGlu) structure 2e4u \(^{11,12}\) revealed the proximity of certain extracellular histidine sites (in black) to clusters of aspartate and glutamate residues (in gray) previously proposed to be sites of CaR-HEK cell Ca\(^{2+}\) sensitivity, with alkalosis suppressing PTH secretion significantly in both cases (not shown).

Figure 3. CaR-induced Ca\(^{2+}\) mobilization is sensitive to pathophysiologic pHo changes in bovine parathyroid cells. (A) Trace showing Ca\(^{2+}\) changes (quantified as a bar graph, as before) in a single cell (upper trace; “cell”) or the “global” (lower trace) cluster of cells in the field of view, in response to elevated [Ca\(^{2+}\)]o (2.5 versus 0.8 mM control) at pHo 7.4 or 7.6 (resulting changes normalized to pH 7.4 control). (B) Identical experiment except examining the effect of lowering pHo (7.2) during CaR stimulation. n=6–8 coverslips. *P<0.05 and **P<0.01 versus initial pH 7.4 response; ††P<0.01 versus pH 7.6 by Kruskal–Wallis (Dunn post-test).

Concentration (3.5 mM), the exceptions being CaRH41V and CaRH595V (Table 1). Having failed to identify a sole histidine residue as being responsible for eliciting CaR pHo sensitivity, the two histidine mutants that produced the largest trend reductions in pHo sensitivity (at concentrations <10 mM), the exceptions being CaRH41V and CaRH595V (Table 1). See Supplemental Material for the complete methods.

Figure 4. Physiologic albumin concentration fails to prevent changes in pHo from modulating CaR activity. (A) Changes in CaR-HEK cell Ca\(^{2+}\) concentration (measured as before) in a single cell or the “global” cluster of cells in response to elevated [Ca\(^{2+}\)]o (3 versus 0.5 mM control) in buffer supplemented with 5% (w/v) BSA at pHo 7.6 (i) or 7.2 (ii). The resulting changes (area under the curve) are normalized to pH 7.4 control and displayed as a bar graph. (B) Identical experiment except using bovine parathyroid cells (2.5 versus 0.8 mM control at pHo 7.4 or 7.6). n=5 coverslips. *P<0.05 and **P<0.01 versus initial pH 7.4 response; †P<0.05 versus pH 7.6 (or 7.2) by Kruskal–Wallis (Dunn post-test).

and compared with their effects on wild-type CaR-expressing cells. For this, concentration-effect curves for Ca\(^{2+}\)-induced Ca\(^{2+}\) mobilization were generated to determine whether the mutations elicit substantial changes in Ca\(^{2+}\) potency/CaR responsiveness per se. All but two of the mutants exhibited significant, sigmoidal Ca\(^{2+}\) sensitivity (at concentrations <10 mM), the exceptions being CaRH41V and CaRH595V (Table 1). See Supplemental Material for the complete methods.

For the 15 CaR mutants that exhibited robust Ca\(^{2+}\)-induced Ca\(^{2+}\) mobilization were then tested after exposure to 3.5 mM Ca\(^{2+}\). Acidosis-elicited attenuation and alkalosis-mediated potentiation of CaR activity did not significantly decrease in any of the CaR mutants (relative to wild-type CaR), suggesting that none of them contribute to pHo sensitivity in CaR (Table 1). Having failed to identify a sole histidine residue as being responsible for eliciting CaR pHo sensitivity, the two histidine mutants that produced the largest trend reductions in pHo sensitivity, namely CaRH442V and CaRH449V, were then comuted to determine whether together they might inhibit CaR pHo sensitivity. However, the pHo sensitivity of CaRH442V/449V was not significantly different from that of wild-type CaR.
For the two remaining mutants that exhibited reduced Ca\textsuperscript{2+}\textsubscript{o} sensitivity (and lower protein abundance of the mature 160-kDa CaR protein), more robust stimulation was required, namely 5 mM Ca\textsuperscript{2+}\textsubscript{o} plus 1 mM R467 (positive allosteric modulator) for CaRH595V and 30 mM Ca\textsuperscript{2+}\textsubscript{o} plus 1 mM R467 for CaRH41V (Figure 7B, Table 1). Because these treatments represent supra-maximal stimuli for wild-type CaR and thus are unsuitable for testing the effects of altering pH\textsubscript{o} on the wild-type receptor, the resulting data for CaRH41V and CaRH595V cannot be compared directly to wild-type CaR but are appropriate for qualitative comparison by examining their signaling before and after the change in pH\textsubscript{o}. In the case of both mutations, lowering pH\textsubscript{o} significantly inhibited the CaR response while raising pH\textsubscript{o} significantly increased activity in the mutant CaRs as before. Therefore, taken together, there was no evidence that any of the 17 histidine residues or single free cysteine (Cys-482) contributed to the pH\textsubscript{o} sensitivity of CaR.

DISCUSSION

Ca\textsuperscript{2+}\textsubscript{o} potency for CaR is sensitive to large changes in ambient pH\textsubscript{o}.\textsuperscript{3,15} The pH of human plasma is maintained between 7.35 and 7.45, representing a 12.5% deviation above and below the normal H\textsuperscript{+} concentration of 4 \times 10^{-8} M (i.e., pH 7.40). Indeed, a 1-unit pH change would represent a lethal 10-fold change in H\textsuperscript{+} concentration,\textsuperscript{16} and even a smaller drop to 7.1 and below represents a medical emergency. This led us to investigate whether the CaR can also respond to smaller, pathophysiologically relevant (0.2-unit) changes in pH\textsubscript{o}. Because metabolic acidosis and secondary hyperparathyroidism are both common consequen-
tces of CKD, the current data may even provide a mechanistic link between the two.

Here we have demonstrated that pathophysiologically relevant (0.2-unit) changes in pH\textsubscript{o} significantly modulate Ca\textsuperscript{2+}\textsubscript{o}-induced Ca\textsuperscript{2+} mobilization in CaR-HEK cells and elicit similar effects on two other readouts of CaR-mediated signaling: ERK phosphorylation and actin polymerization. Thus, the effect of the pH\textsubscript{o} change is not specific for one particular signaling pathway and thus likely occurs at the level of the CaR. Indeed, no acute changes in pHi were detected in CaR-HEK or bovine parathyroid cells after exposure to larger (0.4-unit) changes in pH\textsubscript{o} over the timescale tested, supporting the idea that the change in pH\textsubscript{o} elicits an extracellular, as opposed to nonspecific intracellular, effect on CaR activity. Quinn et al.\textsuperscript{3} reported slow cytoplasmic acidification/alkalinization in CaR-HEKs in response to much larger 1- to 2-unit pH\textsubscript{o} decreases/increases, respectively; however, the slow rate of change failed to account for the much faster pH\textsubscript{o}-mediated change in CaR sensitivity.

Regarding the CaR agonist selectivity of the pH\textsubscript{o} effect, Quinn et al.\textsuperscript{3} found that Mg\textsuperscript{2+} was similarly affected by pH\textsubscript{o} as for Ca\textsuperscript{2+}, both of these cations being orthosteric CaR agonists. Here we found that L-Phe-induced Ca\textsuperscript{2+} mobilization

Figure 5. Acute pH\textsubscript{o} changes do not affect pH\textsubscript{i} levels in CaR-HEK or parathyroid cells. (A, i) Trace showing pH\textsubscript{i} changes (BCECF ratio) in a cluster of CaR-HEK cells in response to elevated [Ca\textsuperscript{2+}]\textsubscript{o} (2.5 versus 0.5 mM control) at pH\textsubscript{o} 7.4 and 7.8. Ammonium chloride (20 mM, NH\textsubscript{4}Cl) was used as a positive control. Quantification comparing baseline extrapolated and treated (actual) values for calculated pH\textsubscript{i}. (A, ii) Identical experiment except examining the effect of lowering pH\textsubscript{o} (7.0) and with sodium acetate (20 mM, NaAc) used as positive control. (B, i) Trace showing pH\textsubscript{i} changes (BCECF ratio) in a cluster of bovine parathyroid cells in response to elevated [Ca\textsuperscript{2+}]\textsubscript{o} (2.5 versus 0.8 mM control) at pH\textsubscript{o} 7.4 and 7.8 (NH\textsubscript{4}Cl positive control). (B, ii) Identical experiment except examining the effect of lowering pH\textsubscript{o} (7.0). n=3–7 coverslips. No significant change (by paired t test) in pH\textsubscript{i} occurred after acute changes in pH\textsubscript{o} in either cell type.

Figure 6. Extracellular pH modulates CaR-induced suppression of PTH secretion. (A) Human parathyroid cells were perifused in 1.2 mM calcium-containing buffers at pH\textsubscript{o} 7.0–7.8 (at 5-minute intervals) and PTH secretion quantified as fg per minute per cell. See Supplemental Material for the complete methods. (B) Quantification of these changes is shown as percentage control. n=3 independent experiments. *P<0.05 by unpaired t test (one-tail) on PTH values following preliminary experiments investigating the effect of 0.4-unit pH\textsubscript{o} changes on both human and bovine parathyroid cells (not shown).

 Calcium Receptor Modulation by pH\textsubscript{o}
was also sensitive to pathophysiologic changes in pHo, suggesting that allosteric CaR modulation is similarly sensitive to pHo and thus unlikely to counteract the effect in vivo.

Of note, the enhancement of CaR signaling with pHo 7.6 and inhibition with pHo 7.2 was also observed in bovine parathyroid cells, in which the CaR is expressed endogenously. Consistent with this observation was the clear suppression of PTH secretion from bovine and human parathyroid cells after perifusion in alkaline buffer (pHo 7.8; preliminary experiments not shown). Repeating these experiments with use of 0.2-unit pHo changes, we observed a transient rise in PTH secretion from human parathyroid cells that was not sustained when pHo was lowered to 7.2; we also noted sustained suppression of PTH secretion when pHo was increased to 7.6. These in vitro data are consistent with observations that metabolic acidosis is associated with increased serum PTH and calcium levels and that alkalosis suppresses serum PTH in vivo.4,6–8 The current findings suggest a CaR-based mechanism for these previous data from animal studies. It will be interesting therefore to determine therefore whether acidosis and/or alkalosis elicit chronic changes in PTH secretion in vivo. Interestingly, at age 80 years, human blood H+ concentration is 6%–7% higher than at age 20,17 and there is a concomitant rise in serum PTH levels.18

With regard to metabolic acidosis and secondary hyperparathyroidism in CKD, while secondary hyperparathyroidism is known to be due partly to hyperphosphatemia and partly to decreased calcitriol levels (resulting in part to lowered plasma free calcium concentration), it is interesting to speculate that acidosis may also contribute to elevated PTH secretion rates by suppressing CaR sensitivity, as observed here. In addition, the current data suggest that raising pHo promotes CaR-mediated suppression of PTH secretion and thus may provide the basis for a useful adjuvant therapy for secondary hyperparathyroidism in CKD. Clinically, low bicarbonate concentration in predialysis patients predicts subsequent coronary artery calcification.19

Because oral sodium bicarbonate supplementation slows the rate of decline of renal function in patients with CKD and low plasma bicarbonate concentrations,20 the effect of this co-therapy on the development of secondary hyperparathyroidism and vascular calcification might be considered for investigation. Of note, acidosis also increases fibroblast growth factor-23 expression in osteoblasts, raising the question of whether treating acidosis in CKD may lower fibroblast growth factor 23 and, indeed, PTH secretion.

Low pH displaces bound calcium from serum albumin and could compensate for the acid-mediated decline in CaR responsiveness by increasing the free calcium concentration.22 However, in the current study, inclusion of 5% (w/v) albumin in the physiologic saline solution (i.e., at a physiologically relevant concentration) had little or no effect on the pHo sensitivity of the CaR. Indeed, albumin was present in both sets of experiments measuring human and bovine PTH secretion, and this did not prevent detection of an alkalosis-induced suppression of PTH secretion. Thus, over the pathophysiologic pHo range, the effect of changing pHo on CaR activity appears greater than any potential effect on calcium buffering/displacement. Indeed, one study found that between pH 6.8 and 7.4, the calcium-binding affinity of albumin is not altered significantly, whereas at pH 8 the association constant increases 4-fold.23

Extracellular histidine residues account for proton sensitivity in several membrane proteins, including the anion exchange protein 2,24 ovarian cancer G protein–coupled receptor-125 and purinergic P2X4 receptor.26,27 Despite this, we found no evidence that any of the 16 extracellular histidine residues or the one free-cysteine residue (Cys-482) are individually responsible for CaR pHo sensitivity, at least over the pathophysiologic pHo range tested. Among members of the homologous family C GPCRs, mGluR4 is also inhibited by acidity and activated by alkalinity whereas mGlur Rs 1, 5 and 8 are pHo insensitive.28

Figure 7. CaR extracellular domain model showing histidine and free cysteine residue locations relative to aspartate and glutamate clusters. (A) Histidines and the free cysteine residues are shown in black with the three aspartate/glutamate clusters in light gray.21,22 Not shown are residues CaR1437 and CaR1766 (located in a transmembrane extracellular loop). (B) Immunoblots showing the mutant CaR proteins lacking each of the 17 extracellular histidine or free cysteine residues CaR immunoblots compared with wild-type (wt) CaR and β-actin loading controls.
### Table 1. Characterization of the Ca$^{2+}$o-sensitivity, relative maximal responses, and pHo sensitivity of the CaR histidine and free cysteine mutants versus wild-type

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<th>Mutant</th>
<th>EC$_{50}$ (mM)</th>
<th>E$_{\text{max}}$ (%)</th>
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<th>Inhibition by Acidosis (%)</th>
<th>Stimulation by Alkalosis (%)</th>
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<td>7</td>
<td>−35±6</td>
<td>26±8</td>
<td>7</td>
</tr>
<tr>
<td>H429V/H495V</td>
<td>5.0±0.4</td>
<td>53±7</td>
<td>7</td>
<td>−35±4</td>
<td>19±5</td>
<td>7</td>
</tr>
<tr>
<td>H411V</td>
<td>&gt; 10</td>
<td>&gt; 3</td>
<td>3</td>
<td>−43±6</td>
<td>22±11</td>
<td>8</td>
</tr>
<tr>
<td>H595V</td>
<td>&gt; 10</td>
<td>&gt; 22</td>
<td>3</td>
<td>−22±7</td>
<td>13±5</td>
<td>9</td>
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Two independent series of experiments tested (1) the Ca$^{2+}$o-sensitivity and maximal response for each CaR mutant (extracellular histidine or free cysteine residues replaced with valine or serine respectively) versus wild-type and (2) the effect of decreasing or increasing pHo by 0.2 unit on receptor responsiveness. All but four of the CaR mutants exhibited Ca$^{2+}$o-sensitivity not significantly different from wild-type CaR. For CaRH192V and CaRH359V the EC$_{50}$ values for Ca$^{2+}$o (mM) were increased significantly. Regarding maximal responsiveness to Ca$^{2+}$o, none of these CaR mutants responded differently to wild-type (Kruskal–Wallis, Dunn multiple comparison test) with the exception of CaRH41V and CaRH595V that required additional cotreatment with a positive allosteric modulator (1 mM R-467) to achieve robust Ca$^{2+}$o mobilization (not shown). Next, none of the mutants exhibited significantly altered CaR pHo sensitivity (one-way ANOVA with Dunnett) in response to pathophysiologic alkalosis (pHo 7.6) or acidosis (pHo 7.2) in the presence of 3.5 mM Ca$^{2+}$o. Because CaRH41V and CaRH595V lacked responsiveness to 3.5 mM Ca$^{2+}$o, the percentage change values quoted for them are not directly relative to wild-type CaR, but merely represent the percentage change of their CaR response from the immediately prior response in pH 7.4. EC$_{50}$, half maximal effective concentration; E$_{\text{max}}$, maximal responsiveness. n=coverslips, from a minimum of two independent transfections in the case of the pHo experiments.

$^a$P<0.05 by one-way ANOVA, Dunnett post hoc test.

$^b$P<0.001 by one-way ANOVA, Dunnett post hoc test.

However, no histamines are shared exclusively between CaR and mGlurR4. Therefore, we conclude, by exclusion, that the most likely molecular mediators of CaR pHo sensitivity are extracellular clusters of aspartate and glutamate residues.3 That is, despite the low pKa values of Glu and Asp side chains in their free amino acid forms (around 4), when clustered, their pKa values may lie closer to the physiologic pH range (approximately 7).29,30 Finally, while we cannot rule out the possible contribution of other pHo-sensitive membrane proteins to these data, the pHo changes had no effect in cells lacking the CaR (not shown) or incubated in low Ca$^{2+}$o concentration (Figure 2Bii).

In conclusion, the human CaR exhibits pHo sensitivity over the pathophysiologic range of pHo witnessed in vivo. This pHo sensitivity of the CaR exerts functionally significant effects on PTH secretion and thus might have wider relevance for whole-body calcium homeostasis and indeed for the secondary hyperparathyroidism of CKD.

### CONCISE METHODS

#### Cell Culture and Calcium-Sensing Receptor Functional Assays

HEK-293 cells, stably transfected with human parathyroid CaR, were cultured in DMEM (supplemented with 10% [v/v] FBS), loaded with Fura-2/AM and assayed for Ca$^{2+}$o, by dual-excitation wavelength microfluorometry in experimental buffer (20 mM HEPES, pH 7.4, 125 mM NaCl, 4 mM KCl, 1.2 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5.5 mM glucose) as described previously.10 Intracellular pH was quantified similarly using the pH-sensitive fluorescent dye BCECF. CaR-induced ERK phosphorylation was detected by semi-quantitative immunoblotting using a phospho-specific polyclonal antibody against the lysates of cells solubilized in RIPA buffer supplemented with protease and phosphatase inhibitors. To assess actin stress fiber assembly, paraformaldehyde-fixed cells were stained with Phalloidin-TRITC and imaged by fluorescence microscopy.10

**Site-Directed Mutagenesis**

CaR mutations were introduced into the human CaR by QuikChange (Stratagene) site-directed mutagenesis, then transiently transfected into HEK-293 cells using FuGENE-6.

**Parathyroid Gland Preparation and PTH Secretion Assay**

Bovine parathyroid cells (abattoir-sourced) were obtained by collagenase-digestion and cultured on collagen-coated coverslips. Normal human parathyroid cells were obtained by collagenase-digestion after neck surgery (procedures performed under institutional ethical guidelines and with patients’ written informed consent). PTH levels
were quantified by ELISA after perfusion with buffer containing 125 mM NaCl, 4 mM KCl, 1.25 mM CaCl₂, 1 mM MgCl₂, 0.8 mM Na₃HPO₄, 20 mM HEPES (pH 7.4, NaOH) supplemented with 0.1% D-glucose, 2.8 mM basal amino acid mixture (defined previously), and 1 mg/ml BSA.

Statistical Analyses
Data are presented as means±SEM, and statistical significance was determined using GraphPad Prism software. For the complete methods, see the Supplemental Material.

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DISCLOSURES
None.

REFERENCES


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