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

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## ABSTRACT

**The calcium-sensing receptor (CaR) modulates renal calcium reabsorption and parathyroid hormone (PTH) secretion and is involved in the aetiology of secondary hyperparathyroidism in CKD. Supraphysiological extracellular pH ($pH_o$) changes modulate CaR responsiveness in HEK-293 (CaR-HEK) cells. Therefore, since acidosis and alkalosis are associated with altered PTH secretion *in vivo*, we examined whether pathophysiological changes in $pH_o$ can alter CaR responsiveness significantly in both heterologous and endogenous expression systems and whether this affects PTH secretion.**

**In both CaR-HEK and isolated bovine parathyroid cells, decreasing $pH_o$ from 7.4 to 7.2 rapidly inhibited CaR-induced intracellular calcium ($Ca^{2+}_i$) mobilization while raising $pH_o$ to 7.6 potentiated extracellular calcium ($Ca^{2+}_o$) responsiveness. Similar $pH_o$ effects were observed for $Ca^{2+}_o$-induced extracellular signal-regulated kinase phosphorylation and actin polymerization and for L-Phe induced $Ca^{2+}_i$ mobilization. Intracellular pH was unaffected by acute 0.4 unit $pH_o$ changes while the presence of physiological albumin concentrations failed to attenuate the $pH_o$ effects. CaR**

**$pH_o$-sensitivity does not result from extracellular histidine or free cysteine ionization since point mutations of none of 17 such residues attenuated $pH_o$ sensitivity. Finally, pathophysiological $pH_o$ elevation reversibly suppressed PTH secretion from perifused human parathyroid cells, and acidosis transiently increased PTH secretion.**

**Therefore, pathophysiological $pH_o$ changes can modulate CaR responsiveness in HEK-293 and parathyroid cells, independently of extracellular histidine residues. Specifically, pathophysiological acidification inhibits CaR activity thus permitting PTH secretion, whereas alkalinization 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*.**

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Mammalian calcium homeostasis is maintained by the regulated secretion of parathyroid hormone (PTH) and renal calcium reabsorption under the control of the calcium-sensing receptor (CaR) (1,2). Hypercalcaemia stimulates the CaR to suppress PTH secretion while chronic underactivation of CaR can contribute to pathologically elevated PTH secretion such as in secondary hyperparathyroidism of chronic kidney disease (CKD). In a heterologous human embryonic kidney (HEK)-293 cell-based expression system, Quinn *et al* (3) found that the $Ca^{2+}_o$ potency of the human CaR is sensitive to supraphysiological 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 with external acidification decreasing CaR sensitivity and alkalinisation increasing CaR sensitivity (3). However, blood pH levels (7.35-7.45 ≈ 40nM $H^+$ concentration) rarely vary beyond ± 0.4 pH units *in vivo* even under extreme pathological conditions, with even smaller changes (± 0.2) more generally seen, such as with the acidosis of CKD however even this still represents a ~58% increase in $H^+$ concentration. Secondary hyperparathyroidism is another complication of CKD and interestingly in animal experiments, induction of metabolic acidosis results in increased serum PTH levels and hypercalcaemia, whereas induction of alkalosis suppresses serum PTH levels (4-8).

Therefore, since 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 pathophysiological changes in $pH_o$ on PTH secretion.

**RESULTS**

Fura 2-loaded CaR-HEK cells were stimulated with 2.5mM $Ca^{2+}_o$ (pH 7.4) to elicit CaR-induced $Ca^{2+}_i$ mobilisation and then switched to the same buffer at either pH 7.2 or 7.6 before being returned to pH 7.4. At pH 7.6, CaR responsiveness (i.e. Δ350/380 fura ratio area under the curve) was significantly increased (Fig.1*A*), an effect that was immediately reversible upon return to pH 7.4. In contrast, lowering $pH_o$ to 7.2 significantly inhibited CaR responsiveness (Fig.1*B*), the effect being immediately reversible. The effect of ± 0.2 pH unit changes on $Ca^{2+}_i$ mobilization was then tested over a range of $Ca^{2+}_o$ concentrations from 0.5-10 mM. Again, CaR responsiveness was significantly inhibited in pH 7.2 ($EC_{50}$ for $Ca^{2+}_o$, 3.6 ± 0.2 mM in 7.2 vs. 3.1 ± 0.1 in 7.4, $P<0.05$) and significantly stimulated in pH 7.6 (2.8 ± 0.0 mM in 7.6, $P<0.05$). In addition, similar effects of changing $pH_o$ (± 0.2 units) were also seen using bicarbonate/$CO_2$ buffers (Fig.1*D*).

To determine whether this apparent $pH_o$-sensitivity of CaR is specific to intracellular $Ca^{2+}_i$ mobilisation or applies to other effector pathways, two other readouts of CaR activity were used; namely 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 (Fig.2*A*; 3.5 mM $Ca^{2+}_o$), 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 polymerisation while a 0.2 unit increase in $pH_o$ potentiated 1.8 mM $Ca^{2+}_o$-induced actin polymerisation (Fig.2*B*). 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 renders the CaR inactive) variations in $pH_o$ between 7.2 and 7.6 had no effect on actin polymerisation indicating that changing $pH_o$ does not otherwise affect baseline signalling. Next, we examined the effect of 0.2 unit $pH_o$ changes on the response to the CaR positive allosteric modulator L-Phe (10 mM) to determine whether the $pH_o$ sensitivity is exclusive to orthosteric agonism with $Ca^{2+}_o$. We observed significant potentiation of L-Phe-induced $Ca^{2+}_i$ mobilization in $pH_o$ 7.6 and attenuation in $pH_o$ 7.2 (Fig.2*C*) similar to that observed for $Ca^{2+}_o$.

Next, we examined whether pathophysiological changes in $pH_o$ also affect CaR-induced $Ca^{2+}_i$ mobilisation in parathyroid cells, in which the CaR is expressed endogenously. Indeed, raising $pH_o$ to 7.6 while stimulating CaR-induced $Ca^{2+}_i$ mobilization in bovine parathyroid cells (2.5mM $Ca^{2+}_o$) significantly potentiated the response, an effect that was fully and immediately reversible upon return to $pH_o$ 7.4 (Fig.3*A*). In addition, lowering $pH_o$ to 7.2 promptly inhibited the $Ca^{2+}_i$ mobilization elicited in parathyroid cells in response to 2.5mM $Ca^{2+}_o$ (Fig.3*B*).

In considering the potential (patho)physiological relevance of CaR $pH_o$-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. In consequence, we tested the effects of $pH_o$ on CaR-induced $Ca^{2+}_i$ mobilisation in the presence of a physiological concentration of albumin (5% w/v). Exposure of CaR-HEK cells to 3 mM $Ca^{2+}_o$ (in 5% (w/v) albumin) at pH 7.4 resulted in an increase in $Ca^{2+}_i$ concentration, and as before, lowering $pH_o$ to 7.2 resulted in a reversible attenuation of the CaR response (Fig.4*Ai*). In addition, increasing $pH_o$ to 7.6 while stimulating CaR with 3 mM $Ca^{2+}_o$ (in 5% albumin) resulted in potentiation of the response (Fig.4*Aii*). This was further tested in bovine parathyroid cells and again raising $pH_o$ to 7.6 potentiated CaR activity despite the presence of 5% (w/v) albumin (Fig.4*B*).

To test whether the changes in $pH_o$ might instead be acting by altering intracellular pH ($pH_i$), 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 $pH_o$, namely pH 7.0 for acidosis and pH 7.8 for alkalosis. However, in neither cell type did either $pH_o$ 7 or 7.8 (or indeed CaR activation itself) affect $pH_i$ over the timescales employed (Fig.5). Therefore, these data suggest that the consequence of altering $pH_o$ on intracellular signalling is via an effect on the CaR *per se*. Ammonium chloride and sodium acetate were used as positive controls to demonstrate that $pH_i$ changes could be detected in the BCECF-loaded cells. Indeed, ammonium chloride elicited intracellular alkalinisation 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 $pH_o$ on parathyroid hormone secretion*

Next, parathyroid hormone secretion was measured in human parathyroid cells where it was shown that in the presence of physiological free $Ca^{2+}_o$ concentration (1.2 mM), lowering $pH_o$ (7.2 then 7.0) caused an initial increase in PTH secretion whereas raising pH (7.6 then 7.8) suppressed PTH secretion (Fig.6). When $pH_o$ 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 $pH_o$ changes on PTH secretion from both bovine and human parathyroid cells (and conducted prior to those shown in Fig.6) showed a similar $pH_o$ sensitivity, with alkalosis suppressing PTH secretion significantly in both cases (not shown).

*Investigation of extracellular histidine / cysteine residues as possible sites of CaR $pH_o$ sensitivity*

Finally, modelling 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 grey) previously proposed to be sites of $Ca^{2+}_o$ binding (13,14) (Fig.7*A*). The amino acids whose sidechain p*K* values are closest to the physiological $pH_o$ and thus that might account for $pH_o$-sensitivity in CaR, are histidine (~6.5) and free cysteine (~8.5). Indeed the human CaR contains 16 extracellular histidines plus 1 free cysteine residue (Cys-482). Therefore, each of these residues was mutated individually, histidines to valines and the cysteine to serine, with the resulting 17 CaR mutants expressed transiently in HEK-293

cells. The effects of ± 0.2 $pH_o$ unit changes on CaR-induced $Ca^{2+}_i$ mobilization were then tested in the mutant CaR-expressing cells and compared to their effects on wild-type CaR-expressing cells. For this, concentration-effect curves for $Ca^{2+}_o$-induced $Ca^{2+}_i$ mobilization were generated to determine whether the mutations elicit substantial changes in $Ca^{2+}_o$ potency / CaR responsiveness *per se*. All but two of the mutants exhibited significant, sigmodial $Ca^{2+}_o$ sensitivity (at concentrations <10mM), the exceptions being $CaR^{H41V}$ and $CaR^{H595V}$ (Table 1).

For the 15 CaR mutants that exhibited robust $Ca^{2+}_o$ sensitivity, the effect of ± 0.2 $pH_o$ unit changes on CaR-induced $Ca^{2+}_i$ mobilization was then tested following exposure to 3.5 mM $Ca^{2+}_o$. There were no significant reductions in either acidosis-elicited attenuation or alkalosis-mediated potentiation of CaR activity in any of the CaR mutants (relative to wild-type CaR) suggesting that none of them contribute to $pH_o$ sensitivity in CaR (Table 1). Having failed to identify a sole histidine residue as being responsible for eliciting CaR $pH_o$ sensitivity, the two histidine mutants that produced the largest trend reductions in $pH_o$ sensitivity, namely $CaR^{H429V}$ and $CaR^{H495V}$, were then co-mutated to determine whether together they might inhibit CaR $pH_o$ sensitivity. However, the $pH_o$ sensitivity of $CaR^{H429V/H495V}$ was not significantly different from wild-type CaR.

For the two remaining mutants that exhibited reduced $Ca^{2+}_o$ sensitivity (and lower protein abundance of the mature 160kDa CaR protein), more robust stimulation was required, namely 5 mM $Ca^{2+}_o$ plus 1 μM R467 (positive allosteric modulator) for $CaR^{H595V}$ and 30 mM $Ca^{2+}_o$ plus 1 μM R467 for $CaR^{H41V}$ (Fig.7*B*, Table 1). Since these treatments represent supramaximal stimuli for wild-type CaR, and thus are unsuitable for testing the effects of altering $pH_o$ on the wild-type receptor, the resulting data for $CaR^{H41V}$ and $CaR^{H595V}$ cannot be compared directly to wild-type CaR but are appropriate for qualitative comparison by examining their signalling before and after the change in $pH_o$. In the case of both mutations, lowering $pH_o$ significantly inhibited the CaR response while raising $pH_o$ 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 $pH_o$ sensitivity of CaR.

**DISCUSSION**

$Ca^{2+}_o$ potency for CaR is known to be sensitive to large changes in ambient $pH_o$ (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 $H^+$ concentration of $4x10^{-8}$ M (i.e., pH 7.40). Indeed, a 1-unit pH change would represent a lethal ten-fold change in $H^+$ concentration (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 changes in $pH_o$. Since metabolic acidosis and secondary hyperparathyroidism are both common consequences 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 $pH_o$ modulate significantly $Ca^{2+}_o$-induced $Ca^{2+}_i$ mobilization in CaR-HEK cells and elicit similar effects on two other readouts of CaR-mediated signalling, namely ERK phosphorylation and actin polymerisation. Thus, the effect of the $pH_o$ change is not signalling pathway-specific and thus likely occurs at the level of the CaR. Indeed, no acute changes in $pH_i$ were detected in either CaR-HEK or bovine parathyroid cells following exposure to larger (0.4 unit) changes in $pH_o$ over the timescale tested, supporting the idea that the change in $pH_o$ elicits an extracellular, as opposed to non-specific intracellular, effect on CaR activity. Quinn *et al* (3) reported slow cytoplasmic acidification / alkalinisation in CaR-HEKs in response to much larger 1-2 $pH_o$ unit decreases / increases respectively, however the slow rate of change failed to account for the much more rapid $pH_o$-mediated change in CaR sensitivity.

Regarding the CaR agonist selectivity of the $pH_o$ effect, Quinn *et al* (3) found that $Mg^{2+}_o$ was similarly affected by $pH_o$ as for $Ca^{2+}_o$, both of these cations being orthosteric CaR agonists. Here we found that L-Phe-induced $Ca^{2+}_i$ mobilization was also sensitive to pathophysiological changes in $pH_o$ suggesting that allosteric CaR modulation is similarly sensitive to $pH_o$ and thus unlikely to counteract the effect *in vivo*.

Importantly, the enhancement of CaR signalling in $pH_o$ 7.6 and inhibition in $pH_o$ 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 following perifusion in alkaline buffer ($pH_o$ 7.8; preliminary experiments not shown). Repeating these experiments using 0.2 unit $pH_o$ changes, we observed a transient rise in PTH secretion from human parathyroid cells which was not sustained when $pH_o$ was lowered to 7.2, and, more sustained suppression of PTH secretion when $pH_o$ 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, 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 regards 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 $pH_o$ promotes CaR-mediated suppression of PTH secretion and thus may provide the basis of a useful adjuvant therapy for secondary hyperparathyroidism in chronic kidney disease. Indeed clinically, low bicarbonate concentration in pre-dialysis patients predicts subsequent coronary artery calcification (19). Since oral sodium bicarbonate supplementation slows the rate of decline of renal function in CKD patients with 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. Interestingly, acidosis also increases fibroblast growth factor (FGF)23 expression in osteoblasts raising the question of whether treating acidosis in CKD may lower FGF23 (21) 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 physiological saline solution, i.e., at a physiologically relevant concentration, had little or no effect on the $pH_o$-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 pathophysiological $pH_o$ range, the effect of changing $pH_o$ 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, there is a four-fold increase in the association constant (23).

Extracellular histidine residues have been shown to account for proton sensitivity in a number of membrane proteins including the anion exchange protein 2 (24), ovarian cancer G protein-coupled receptor-1 (25) 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 $pH_o$ sensitivity, at least over the pathophysiological $pH_o$ range tested. Amongst members of the homologous family C GPCRs, mGluR4 is also inhibited by acidity and activated by alkalinity whereas mGluRs 1, 5 and 8 are $pH_o$-insensitive (28). However, no histamines are shared exclusively between CaR and mGluR4. Therefore we conclude, by exclusion, that the most likely molecular mediators of CaR $pH_o$ 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 physiological pH range (~7) (29,30). Finally, while we cannot rule out the possible contribution of other $pH_o$-sensitive membrane proteins to these data, it should be noted that the $pH_o$ changes were without effect in cells either lacking the CaR (not shown), or, incubated in low $Ca^{2+}_o$ concentration (Fig.2Bii).

In conclusion, the human CaR exhibits $pH_o$ sensitivity over the pathophysiological range of $pH_o$ witnessed *in vivo*. This $pH_o$ 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 Dulbecco's Modified Eagle's medium (supplemented with 10% v/v fetal bovine serum), loaded with Fura-2/AM and assayed for $Ca^{2+}_i$ by dual-excitation wavelength microfluorometry in Experimental Buffer [20mM HEPES, pH 7.4, 125mM NaCl, 4mM KCl, 1.2mM $CaCl_2$, 0.5mM $MgCl_2$, 5.5mM 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 (9) against the lysates of cells solubilised 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 (9) and cultured on collagen-coated coverslips. Normal human parathyroid cells were obtained by collagenase-digestion following neck surgery (9; procedures performed under institutional ethical guidelines and with patients' written informed consent). PTH levels were quantified by ELISA following perifusion with buffer containing (mM) 125 NaCl, 4 KCl, 1.25 $CaCl_2$, 1 $MgCl_2$, 0.8 $Na_2HPO_4$, 20 HEPES (pH 7.4, NaOH) supplemented with 0.1% D-glucose, 2.8mM basal amino acid mixture (defined previously (9)) and 1 mg/ml bovine serum albumin.

*Statistical analysis* - Data are presented as means ± S.E. and statistical significance determined using GraphPad Prism software.

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**STATEMENT OF COMPETING FINANCIAL INTERESTS**

The authors have no conflicts of interest to declare regarding the current study. We can confirm that neither the manuscript nor any significant part of it is under consideration for publication elsewhere, or has appeared elsewhere in a manner that could be construed as a prior or duplicate publication of the same, or very similar, work. Portions of this work were presented in Abstract form at the World Congress of Nephrology in Milan and at the International Congress of Endocrinology in Florence.

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## FIGURE LEGENDS

FIGURE 1. **Effect of changes in $pH_o$ on CaR-induced $Ca^{2+}_i$ mobilisation 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 vs 0.5mM control) when $pH_o$ was changed from 7.4 to 7.6. Changes in $[Ca^{2+}]_i$ shown as % 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, ***P<0.001 vs first pH 7.4 treatment; +++P<0.001 vs 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) in either $pH_o$ 7.2, 7.4 or 7.6 with the resulting concentration-effect curves for $Ca^{2+}_i$ mobilization shown (left) together with their $EC_{50}$s (right). While the $EC_{50}$ values were significantly different, the maximal responses were not significantly different, despite the apparent trend. *P<0.05 vs pH 7.4 by repeated measures ANOVA (Dunnett's) performed on log $EC_{50}$ values from 4 independent experiments (2-5 coverslip replicates per data point, >15 cells per coverslip). *D*) Similar results were obtained using bicarbonate / $CO_2$ buffers, with pH 7.6 increasing $Ca^{2+}_i$ mobilisation and pH 7.2 decreasing mobilisation 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 $[Ca^{2+}]_i$ are shown in the bar graph. The appropriate pHs were obtained by varying the bicarbonate (and NaCl) concentrations in buffers gassed continuously with $5\%CO_2/95\%O_2$ (g). *P<0.05 vs pH 7.4 treatment by one-tailed paired t-test; n=7 coverslips.

FIGURE 2. **Effect of $pH_o$ on CaR-induced ERK activation, actin polymerization and L-Phe responsiveness in CaR-HEK cells.** *A*) Representative immunoblot showing ERK phosphorylation (pERK) following treatment with 3.5mM $Ca^{2+}_o$ for 10-min under different $pH_o$ conditions (7.4 ± 0.2). Mean changes in ERK phosphorylation were determined by densitometry and expressed on a bar graph in arbitrary units. n=9; *P<0.05, **P<0.01 vs $pH_o$ 7.4 by one-way ANOVA (Dunnet's). *B*) Representative immunofluoresence images (*i*) showing phalloidin-TRITC staining following CaR-HEK treatment with 1.5 mM $Ca^{2+}_o$ for 3 hours in buffers of $pH_o$ ranging from 7.2 to 7.6. Quantification presented on a bar graph as pixels (Phalloidin-TRITC) per unit area in five randomly selected regions of the cells is shown below (*iii*). Also shown are bar graphs from separate experiments where the cells were exposed to either 0.5 (*ii*) or 1.8 (*iv*) mM $Ca^{2+}_o$. n≥5; *P<0.05, **P<0.01 vs $pH_o$ 7.4 by one-way ANOVA (Dunnet's). *C*) Representative $Ca^{2+}_i$ trace (*i*) showing CaR-HEK cells stimulated first with 10mM L-Phe (in 1.8 mM $Ca^{2+}_o$) and then with 3 mM $Ca^{2+}_o$ under various $pH_o$ conditions as shown (0.5 $Ca^{2+}_o$, baseline, pH 7.4 unless otherwise stated). The traces include a typical response in a single cell (grey) as well as the total response in the cell cluster (>10 cells; "global", black). Quantification (panel *ii*) is shown as a bar graph

(% change in L-Phe responsiveness relative to $pH_o$ 7.4 control). n=7; **P<0.01 by Repeated Measures ANOVA, (Bonferroni's).

FIGURE 3. **Effect of $pH_o$ on CaR-induced $Ca^{2+}_i$ mobilization in parathyroid cells.** *A*) Trace showing $Ca^{2+}_i$ 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 vs 0.8mM control) at $pH_o$ 7.4 or 7.6 (resulting changes normalised to pH 7.4 control). *B*) Identical experiment except examining the effect of lowering $pH_o$ (7.2) during CaR stimulation. n=6-8 coverslips; *P<0.05, **P<0.01 vs initial pH 7.4 response; ++P<0.01 vs pH 7.6 by Kruskal-Wallis (Dunn's post-test).

FIGURE 4. **Physiological albumin concentration fails to prevent changes in $pH_o$ from modulating CaR activity.** *A*) Changes in CaR-HEK cell $Ca^{2+}_i$ concentration (measured as before) in a single cell or the "global" cluster of cells in response to elevated $[Ca^{2+}]_o$ (3 vs 0.5 mM control) in buffer supplemented with 5% (w/v) BSA at either $pH_o$ 7.6 (*i*) or 7.2 (*ii*). The resulting changes (area under the curve) are normalised to pH 7.4 control and displayed as a bar graph. *B*) Identical experiment except using bovine parathyroid cells (2.5 vs 0.8mM control at $pH_o$ 7.4 or 7.6). n≥5 coverslips; *P<0.05, **P<0.01 vs initial pH 7.4 response; +P<0.05 vs pH 7.6 (or 7.2) by Kruskal-Wallis (Dunn's).

FIGURE 5. **Effect of acute $pH_o$ changes on $pH_i$ in CaR-HEK and parathyroid cells.** *Ai*) Trace showing $pH_i$ changes (BCECF ratio) in a cluster of CaR-HEK cells in response to elevated $[Ca^{2+}]_o$ (2.5 vs 0.5mM control) at $pH_o$ 7.4 and 7.8. Ammonium chloride (20mM, $NH_4Cl_2$) was used as a positive control. Quantification comparing control (baseline extrapolated) and treated (actual) values for calculated $pH_i$. *Aii*) Identical experiment except examining the effect of lowering $pH_o$ (7.0) and with sodium acetate (20mM, NaAc) used as positive control. *Bi*) Trace showing $pH_i$ changes (BCECF ratio) in a cluster of bovine parathyroid cells in response to elevated $[Ca^{2+}]_o$ (2.5 vs 0.8mM control) at $pH_o$ 7.4 and 7.8 ($NH_4Cl$ positive control). *Bii*) Identical experiment except examining the effect of lowering $pH_o$ (7.0). n=3-7 coverslips. No significant change (by paired t-test) in $pH_i$ occurred following acute changes in $pH_o$ in either cell type.

FIGURE 6. **Extracellular pH modulates CaR-induced suppression of PTH secretion.** Human parathyroid cells were perifused in 1.2 mM calcium-containing buffers at $pH_o$ 7.0-7.8 (at 5-min intervals) and PTH secretion quantified as fg per min per cell (*A*). Quantification of these changes is shown as % control in panel *B*. N=3 independent experiments; *P<0.05 by unpaired t-test (1-tail) on PTH values following preliminary experiments investigating the effect of 0.4 unit $pH_o$ changes on both human and bovine parathyroid cells (not shown).

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 are shown in black with the three aspartate / glutamate clusters in light grey (21,22). Not shown are residues $CaR^{H377}$ and $CaR^{H766}$ (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 to wild-type (wt) CaR and β-actin loading controls.

| Mutant | $EC_{50}$ (mM) | $E_{max}$ (%) | n | % Inhibition by acidosis | % Stimulation by alkalosis | n |
|---|---|---|---|---|---|---|
| **WT-CaR** | **4.3 ± 0.3** | **100 ± 19** | **16** | **-20 ± 2** | **14 ± 3** | **20** |
| **H134V** | **4.3 ± 0.3** | **93 ± 19** | **6** | **-22 ± 6** | **9 ± 4** | **9** |
| **H192V** | *****7.6 ± 1.0** | **118 ± 16** | **5** | **-22 ± 9** | **34 ± 13** | **7** |
| **H254V** | **3.9 ± 0.6** | **90 ± 22** | **6** | **-10 ± 5** | **35 ± 9** | **6** |
| **H312V** | **3.3 ± 0.5** | **80 ± 8** | **5** | **-26 ± 5** | **32 ± 10** | **6** |
| **H338V** | **5.7 ± 0.1** | **162 ± 17** | **4** | **-22 ± 7** | **16 ± 4** | **6** |
| **H344V** | *** 6.1 ± 0.5** | **82 ± 6** | **8** | **-38 ± 10** | **55 ± 26** | **6** |
| **H359V** | **5.2 ± 0.5** | **152 ± 22** | **8** | **-24 ± 13** | **11 ± 5** | **6** |
| **H377V** | **5.8 ± 0.7** | **143 ± 11** | **8** | **-43 ± 6** | **19 ± 12** | **7** |
| **H413V** | **3.9 ± 0.4** | **104 ± 20** | **5** | **-16 ± 9** | **13 ± 3** | **9** |
| **H429V** | **5.7 ± 0.7** | **91 ± 11** | **3** | **-13 ± 13** | **9 ± 3** | **7** |
| **H463V** | **5.9 ± 1.1** | **90 ± 13** | **6** | **-40 ± 5** | **23 ± 6** | **6** |
| **H463V/H466V** | **4.7 ± 0.3** | **70 ± 9** | **9** | **-17 ± 8** | **21 ± 5** | **7** |
| **H495V** | **4.8 ± 0.6** | **180 ± 52** | **3** | **-11 ± 7** | **11 ± 6** | **8** |
| **H766V** | **3.3 ± 0.2** | **131 ± 9** | **9** | **-22 ± 4** | **20 ± 8** | **9** |
| **C482S** | **3.7 ± 0.7** | **81 ± 39** | **3** | **-36 ± 5** | **26 ± 8** | **7** |
| **H429V/H495V** | **5.0 ± 0.4** | **53 ± 7** | **7** | **-35 ± 4** | **19 ± 5** | **7** |
| **H41V** | **>10** | **-** | **9** | **-43 ± 6** | **22 ± 11** | **8** |
| **H595V** | **>10** | **-** | **9** | **22 ± 7** | **13 ± 5** | **9** |

TABLE 1. **Characterization of the $Ca^{2+}_o$-sensitivity, relative maximal responses and $pH_o$ sensitivity of the CaR histidine and free cysteine mutants versus wild-type.** Two independent series of experiments tested a) the $Ca^{2+}_o$-senstivity and maximal response (shown to the left of the thick vertical line) for each CaR mutant (extracellular histidine or free cysteine residues replaced with valine or serine respectively) versus wild-type (wt) and also b) the effect of decreasing or increasing $pH_o$ by 0.2 units on receptor responsiveness (as shown to the right of the line). All but 4 of the CaR mutants exhibited $Ca^{2+}_o$ sensitivity not significantly different from wild-type (WT) CaR. For $CaR^{H192V}$ and $CaR^{H344V}$ the $EC_{50}$ values for $Ca^{2+}_o$ (mM) were increased significantly. *P<0.05, ***P<0.001 by one-way ANOVA, Dunnett's post-hoc test. Regarding maximal responsiveness to $Ca^{2+}_o$ ($E_{max}$), none of these CaR mutants responded differently to wild-type (Kruskal-Wallis, Dunn's multiple comparison test) with the exception of $CaR^{H41V}$ and $CaR^{H595V}$ that required additional cotreatment with a positive allosteric modulator (1mM R-467) to achieve robust $Ca^{2+}_i$ mobilisation (not shown). Next, none of the mutants exhibited significantly altered CaR $pH_o$ sensitivity (1-way ANOVA with Dunnett's; ns, not significant) in response to either pathophysiological alkalosis ($pH_o$ 7.6) or acidosis ($pH_o$ 7.2) in the presence of 3.5mM $Ca^{2+}_o$. Since $CaR^{H41V}$ and $CaR^{H595V}$ lacked responsiveness to 3.5mM $Ca^{2+}_o$, the % change values quoted for them are not directly relative to wild-type CaR, but merely represent the % change of their CaR response from the immediately prior response in pH 7.4.