The calcium-sensing receptor (CaR) is activated by small changes in the ionic extracellular calcium concentration ($C_{\text{a}}$) within the physiological range, allowing the parathyroid gland to regulate serum $C_{\text{a}}$; however, the CaR is also distributed in a number of other tissues where it may sense other endogenous agonists and modulators. CaR agonists are polycationic molecules, and our previous studies suggest that charged residues in the extracellular domain of the CaR are critical for receptor activation through electrostatic interactions. Therefore, pH could also potentially modulate CaR activation by its polycationic agonists. Changes in the concentration of extracellular $H^+$ substantially altered the activation of the CaR by $C_{\text{a}}$ and other CaR agonists. The effects of external pH on the CaR’s sensitivity to its agonists were observed for both acidic and basic deviations from physiological pH of 7.4, with increases in pH rendering the receptor more sensitive to activation by $C_{\text{a}}$ and decreases in pH producing the converse effect. At pH values more acidic than 5.5, CaR sensitivity to its agonists showed some recovery. Changes in the intracellular pH could not account for the effects of external pH on CaR sensitivity to its agonists. Other G-protein-coupled receptors, which are endogenously expressed in human embryonic kidney 293 cells, showed little change in activity with alterations in external pH or effects opposite those found for the CaR. Extracellular pH directly alters the CaR in the case of $C_{\text{a}}$, and Mg$^2+$ activation; however, the charges on many organic and inorganic agonists are pH-dependent. Activating CaR mutations show reduced pH modulation, suggesting a molecular mechanism for increased CaR activity at physiological pH. Several CaR-expressing tissues, including regions of the stomach, the kidney, bone, and the brain, could potentially use the CaR as a sensor for pH and acid-base status.

A calcium-sensing receptor (CaR)$^1$ has been cloned that allows cells expressing this receptor to sense the level of external $C_{\text{a}}^{2+}$ ($C_{\text{a}}$) within its physiological range of 1–1.5 mM (1, 2). Initially cloned from bovine parathyroid cells, the CaR is highly expressed in the tissues involved in regulating $C_{\text{a}}$, including the parathyroid, calcitonin-secreting cells of the thyroid (C cells), and several regions of the kidney (1, 3, 4). It is noteworthy that CaR is also distributed in a number of other tissues that do not have well-established roles in the control of $C_{\text{a}}$. These include several regions of the brain (e.g., the subfornical organ and hypothalamus), the pituitary, collecting duct of the kidney, lung, and the intestines (1, 5–8). In many of these tissues, the physiological role of the CaR is not understood. One possibility is that the CaR senses endogenous ligands other than $C_{\text{a}}$, thus allowing the CaR to function in a number of specialized capacities in different CaR-expressing tissues.

The CaR is activated by both polyvalent cations and polycationic molecules that interact with its extracellular domain (1, 9). This might take place through the screening of charged side chains of acidic or basic amino acids, rather than more classical binding motifs, including hydrogen bonding and salt bridges. If its endogenous agonists act by screening charges on the CaR, then activation of the receptor by these ligands should be modulated by conditions such as changes in pH (10). With changes in pH, the charge on the acidic and basic amino acids can be altered, thus affecting the ability of the polycationic ligand to activate the CaR. It is noteworthy that the N-methyl-D-aspartate receptor shares some regions of homology with the CaR, and divalent cations and polycationic molecules, such as neomycin and spermine, can modulate both receptors (11–14). Furthermore, activation of the NMDA receptor is susceptible to modulation by pH and ionic strength, suggesting that they may also act through charge screening (10).

pH can have substantial effects on a number of different cell types, including those involved in the regulation of the gastrointestinal tract, bone, and the kidney. Many pH effects are believed to act intracellularly, thereby modifying various cellular processes; however, there is a growing appreciation of the extracellular actions of pH on cellular functions, including the behavior of calcium channels and enzymatic activity. In a limited number of cases, small pH changes in the physiological range have been observed to alter specific cellular functions. However, the effects of external pH on membrane receptors have been less well characterized.

Extracellular pH is well regulated in the body; most tissues and organs encounter systemic pH values between 7.0 and 7.8. In contrast, some tissues normally experience large changes in external pH, such as the collecting duct of the kidney, where pH is highly dependent on the level of dietary protein intake. In addition, the area near the plasma membrane of some nephron segments may experience large changes in local pH when the rates of transport of protons and bicarbonate are high, as in the renal distal tubule.

The results of the studies carried out here indicate that pH is an important modulator of CaR activation. Changes in pH can alter the charge on acidic and basic amino acids of the CaR that are important for sensing of polycationic CaR agonists, thus providing a potential mechanism for a change in the sensitivity
of the CaR to its agonists. In addition, pH will also modify the charge state of organic CaR agonists and some polyvalent cations, therefore altering their potencies as CaR agonists. Many activating mutations of the CaR show a reduced modulation by pHo, which may explain the increased activity of these mutations at physiological pHo. The ramifications of these actions of pH are far-reaching and must be considered wherever the CaR is expressed.

**EXPERIMENTAL PROCEDURES**

**Culturing and Maintenance of CaR-transfected and Untransfected HEK 293 Cells.—**These cell lines were the generous gift of Dr. Kimberly Rogers (NPS Pharmaceuticals Inc., Salt Lake City, UT). The CaR-expressing HEK 293 cells were stably transfected with the human parathyroid CaR (15) and selected by hygromycin resistance. The transfected HEK 293 cells express the CaR on the cell surface and are responsive to addition of CaR agonists to the external medium (15). Cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 200 μg/ml hygromycin.

**Transient Expression of Mutant CaRs in HEK 293 Cells.—**Site-directed mutagenesis was used to modify previously (15) to produce mutated receptors in which a residue substitution was made that yielded an activating CaR mutant receptor. CaR cDNA was prepared with the Midi plasmid kit (QIAGEN). LipofectAMINE (Invitrogen) was employed as a DNA carrier for transfection. The DNA-liposome complex was prepared by mixing DNA and LipofectAMINE in Opti-MEM I reduced serum medium (Invitrogen) and incubating the mixture at room temperature for 30 min. The DNA-LipofectAMINE mixture was then diluted with Opti-MEM I reduced serum medium and added to 90% confluent HEK 293 cells plated on glass coverslips using 2.5 μg of DNA. After the cells were incubated for 5 h at 37 °C, an equivalent amount of Opti-MEM I reduced serum medium with 20% fetal bovine serum was added to the medium overlying the transfected cells, and the latter was replaced with fresh Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 24 h after transfection. Experiments were performed on transiently transfected HEK 293 cells 48 h after the start of transfection.

**Measurement of Ca2+ Using Cell Population System.—**Coverslips with nearly confluent HEK cells were loaded with Fura-2/acetoxymethyl ester and placed diagonally into cuvettes equipped with thermostats and magnetic stirrers, using a modification of techniques we used previously (15). The bath solution was stirred at 37 °C, and CaR agonists were added to the desired final concentration. Excitation monochrometers were centered at 340 and 380 nm, with emission light collected at 490° using a long-pass emission filter. The 340/380 excitation ratio of emitted light and in vitro calibrations were used to calculate cytosolic calcium (Ca2+) as described previously (16).

**Measurement of pH Using Cell Population System.—**Coverslips with nearly confluent HEK cells were loaded with 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein/acetoxymethyl ester and placed diagonally into cuvettes equipped with thermostats and magnetic stirrers, using a modification of techniques we used previously (16). The bath solution was stirred at 37 °C, and CaR agonists were added to the desired final concentration. Excitation monochrometers were centered at 490 and 440 nm, with emission light collected at 90° using a long-pass emission filter. The 490/440 excitation ratio of emitted light and in vitro calibrations were used to calculate pH, as described previously (16).

**RESULTS**

**Effect of pHo on Activation of the Calcium-sensing Receptor Evoked by Ca2+ and Mg2+.—**The effect of pHo on activation of the calcium-sensing receptor by Ca2+ was studied by adding NaOH or HCl to the HEPES-buffered, extracellular media followed by the elevation of Ca2+. Changes in Ca2+ were used as an indicator of CaR activation in CaR-expressing HEK cells. Fig. 1 shows the effect of changing the extracellular pH on activation of the CaR by 3.0 mM Ca2+ in HEK 293 cells that have been stably transfected with the human CaR. When extracellular pH is made more acidic, the Ca2+ response to 3.0 mM Ca2+ is attenuated, whereas an extracellular pH more basic than 7.4 produces an enhanced response.

The concentration-response relationships for activation of the CaR by Ca2+ were compared in the presence of varying extracellular pH values (Fig. 2A). With a physiological pH of 7.5, the threshold for cell activation is ~1.5 mM Ca2+ with an EC50 of ~3.5 mM Ca2+. With addition of HCl to produce an acidic pH, the threshold and EC50 both shift to the right, whereas addition of NaOH to yield a basic pH produced a left shift in threshold and EC50, perhaps because of a change in charge distribution on the CaR. The change in EC50 seemed relatively linear with changes in extracellular pH between 9.0 and 6.5. A similar pH dependence was observed with stimulation of the CaR by Mg2+ (Fig. 2B).

At pH values more acidic than 5.5, the CaR seems to regain sensitivity toward Ca2+ and Mg2+ (Fig. 2B). This partial recovery of receptor sensitivity for its polycationic agonists is difficult to assess because placement in the acidic medium will activate the CaR-expressing HEK 293 cells in the presence of 0.5 mM Ca2+. At pH values below 5.5, there was a CaR-dependent, agonist-independent increase in Ca2+ (Fig. 3). In non-transfected HEK 293 cells, there were only modest changes in Ca2+ when tested with the same set of pHo values (Fig. 3). At a pH of 4.5, CaR-transfected HEK 293 cells are no longer stimulated by...
CaR agonists, probably because of agonist-independent activation of the CaR (data not shown).

**Effect of Extracellular pH on Other Receptors**—To determine whether the effect of pH on receptor activation was specific to the CaR, activation of endogenous G protein-coupled receptors on the HEK 293 cells were examined in solutions of varying pH values (Fig. 4, A and B). Purinergic and thrombin receptors were tested using ATP and a peptide agonist (SRLLRNP) of the thrombin receptor, respectively, as ligands. Determination of the EC50 for thrombin receptor activation indicates that optimal activation of the thrombin receptor was found at physiological pH of 7.4, and extracellular pH had little effect until extremely acidic or basic values were reached (Fig. 4D). With extracellular pH values of 5.5 or less, the thrombin receptor became much less sensitive to agonist stimulation. Activation of the purinergic receptor by ATP showed a trend toward reduced sensitivity with basic pH and enhanced sensitivity with acidic pH between 7 and 5 (Fig. 4C). Thus, extracellular pH had an effect on purinergic receptors opposite that seen for the CaR (Fig. 2). Bradykinin produced only a small Ca2+ response in HEK 293 cells, which made it difficult to test the effects of extracellular pH on the activation of its endogenous receptor. However, variations in extracellular pH had little effect on the sensitivity toward bradykinin (data not shown).

**Effect of Extracellular pH on Calcium Influx during Receptor Activation**—The dose-dependence of the effects of external pH on CaR activation by Ca2+ is shown in Fig. 5A. It indicates that peak Ca2+ transients caused by Ca2+ mobilization, as well as sustained Ca2+ increases caused by modification of Ca2+ movement across the plasma membrane, are modulated in a similar direction and magnitude by changes in extracellular pH. The sustained increase in Ca2+ associated with receptor activation is caused primarily by the stimulation of Ca2+ influx across the plasma membrane. This sustained Ca2+ increase was sensitive to pH regardless of which receptor was activated during the experiment. In the cases of the CaR (Fig. 5A), as well as the thrombin receptor (Fig. 5B) and purinergic receptor (data not shown), alkaline pH increased the sustained Ca2+ phase whereas acidic pH decreased it. The experimental conditions that showed this pH sensitivity of Ca2+ influx most clearly was stimulation by a saturating concentration of receptor agonist, which would minimize the effects of pH on Ca2+ transients produced by CaR activation.

**Extracellular pH and the Relative Degree of Activation of the CaR and ATP and Thrombin Receptors**—At a constant concentration of its agonist, changes in extracellular pH will modulate the degree of CaR activation. This is represented in Fig. 6 as the change in the magnitude of receptor activation at an agonist concentration equal to the EC50 concentration in our standard medium. The CaR shows a simple linear relationship between the change in extracellular pH and the extent of receptor activation by Ca2+. These data suggest that pH can both positively and negatively modulate CaR activation in the presence of a constant calcium concentration. In this way, the CaR could act as a pH sensor. Thrombin receptor activation by its peptide agonist shows a more complex relationship, indicating optimal or nearly optimal receptor activation at physiological pH and reduced activation with large changes in pH. With ATP stimulation, an opposite effect is observed compared with the
CaR, thus, the purinergic receptors expressed on HEK 293 cells can also sense external pH, with acidic pH facilitating receptor activation by ATP.

**Role of Extracellular versus Intracellular pH in Modulation of the Calcium-sensing Receptor**—To determine the effects of extracellular pH versus intracellular pH on activation of the CaR, experiments were performed at extracellular pH values after preincubation under conditions that would alter the intracellular pH of CaR-transfected HEK 293 cells. Intracellular pH was monitored using the fluorescent pH probe 2'-7'-bis(carboxyethyl)-5(6')-carboxyfluorescein as described under Experimental Procedures. Changing extracellular pH led to predictable alterations in intracellular pH, with acidic extracellular solutions resulting in cytosolic acidification and the converse occurring in alkaline extracellular media (Fig. 7A). These intracellular pH changes were slow to develop and reach equilibrium; a half-maximal change in cytosolic pH required more than 5 min. Experiments examining activation of the CaR could be performed immediately after a change in extracellular pH, at a time when little change in intracellular pH had taken place, to evaluate the role of extracellular pH *per se* on CaR activation. Likewise, cells could be preincubated with varying levels of extracellular pH to modulate intracellular pH, with CaR activation subsequently being tested at one extracellular pH value to determine the effects of the level of intracellular pH on CaR activation. Experiments examining the effects of extracellular pH values of 6.5 and 8.5 showed that modulation of CaR activation was dependent on extracellular pH but independent of intracellular pH achieved after preincubation in these acidic or alkaline media (Fig. 7B). It is noteworthy that the sustained Ca<sup>2+</sup> responses observed with CaR activation or stimulation of endogenous receptors were sensitive to both extracellular and intracellular pH, with alkaline pH values potentiating and acidic pH values attenuating these sustained Ca<sup>2+</sup> responses to receptor activation (data not shown).

**pH Modulation of the Calcium-sensing Receptor with Activating Mutations**—Alkaline pH levels make the CaR more sensitive toward its physiological agonists, Ca<sup>2+</sup> and Mg<sup>2+</sup>, whereas acidic pH has the opposite effect. One possible mechanism by which activating mutations could alter the sensitivity

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**Fig. 4. Effect of external pH on agonist stimulation of purinergic and thrombin receptors endogenously expressed in HEK 293 cells.** HEK 293 cells were incubated with ATP or thrombin receptor agonist peptide (SRLLRNP) to stimulate purinergic or thrombin receptors, respectively, and Ca<sup>2+</sup> changes were monitored in CaR-transfected HEK 293 cell populations. A and B, concentration-response curves were determined for stimulation with ATP or SRLLRNP in the presence of different pH<sub>e</sub> values. For stimulation with ATP, the pH<sub>e</sub> values were 9.0 (■), 8.0 (●), 7.0 ( ■ ), 6.0 ( ■ ), and 5.0 ( ■ ). For stimulation with SRLLRNP, the pH<sub>e</sub> values were 8.5 (■), 7.5 (■), 6.5 (■), and 5.5 (■). C and D, the EC<sub>50</sub> was determined for each agonist-receptor combination at varying pH<sub>e</sub> levels. The experiments were performed in media containing 135 mM NaCl with an osmolality of 280–285 mOsm. The data represent the mean ± S.E. of four to eight separate experiments.
The CaR could involve changes in this external pH modulation of CaR sensitivity. Physiological concentrations of protons may have a more limited effect on agonist sensitivity, leading to a more active receptor. To test this hypothesis, we examined the activation of wild-type CaR and a set of activating mutations at pH values of 9.5, 8.5, 7.5, and 6.5 (Fig. 8). The CaR mutations were all point mutations, except for del543, which has an in-frame deletion of 181 amino acids between serine 895 and valine 1075. Both wild-type CaR and activating CaR mutations were transiently transfected into HEK 293 cells, as described previously (15). We found that the weakest activating mutation (E127A) had modest changes in pH modulation of sensitivity. Two of the most active mutations (del543 and E127K) retained some pH modulation between 9.5 and 7.5 but little pH modulation between 7.5 and 6.5. However, all the potent activating mutations showed much less pH modulation of CaR activation over the entire range of pH (Fig. 8). These data suggest that activating mutations alter the pH modulation of agonist sensitivity, resulting in greater activation of the CaR at physiological pH. Several inactivating CaR mutations were examined for changes in pH modulation. These inactivating mutations showed robust pH modulation of receptor activity, which was not clearly greater than that found in the wild-type CaR (data not shown).

Fig. 5. Effect of external pH on the sustained Ca_o responses resulting from receptor activation. A, the pH_o-Ca_o response relationship was determined over a range of pH values during stimulation by 15 mM Ca_o. Both maximal peak and sustained Ca_o responses showed a decline as the test solution was set to more acidic values. 15 mM Ca_o was used to maximally activate the CaR at any given pH_o minimizing the influence of shifts in CaR sensitivity to these Ca_o responses. B, the pH_o-Ca_o response relationship was determined over a range of pH values during stimulation by 10 μM thrombin peptide in the presence of 0.5 mM Ca_o. Sustained Ca_o responses showed a decline as the test solution was set to more acidic values, while the peak response remained largely unchanged until pH_o reached highly acidic levels. 10 μM thrombin was used to maximally activate the CaR at any given level of pH_o, minimizing the influence of shifts in CaR sensitivity to these Ca_o responses. Similar results were found for CaR-transfected and untransfected HEK 293 cells. The data represent the mean ± S.E. from 4 to 6 separate experiments.

Fig. 6. Effect of external pH on the activation of the CaR, ATP, and thrombin receptors. The change in the relative extent of receptor activation at an agonist concentration equal to the EC_{50} concentration at pH_o value of 7.5 was plotted for the calcium sensing (○), purinergic (□), and thrombin (△) receptors using the concentration-response relationship in varying pH_o values (see Figs. 2A and 4, A and B). The CaR shows a simple linear relationship between CaR activation by Ca_o with pH_o. The thrombin receptor shows little change in activity with different pH_o values, whereas purinergic receptors show modulation by pH_o in the opposite direction and over a narrower pH_o range than for the CaR.

Additional diagrams and text related to the effect of external pH on the sustained Ca o responses and the change in receptor activation are included in the figure captions. The figure illustrates the relationship between external pH and the activation of the CaR, ATP, and thrombin receptors, highlighting the differing sensitivities and responses across different pH ranges.
pH. Aluminum, a group IIIB trivalent cation with a $pK_a$ of 4.8, showed little change in EC$_{50}$ between pH 9.5 and 7.5 (Fig. 9). At these alkaline pH values, aluminum acts like a poor divalent cation agonist for CaR activation. However, aluminum was very sensitive to external pH below 7.5 and was an effective CaR agonist at micromolar concentrations in acidic pH. Similar effects were seen for scandium. Trivalent cations with acidic $pK_a$ values below 4, such as gallium and lead, were less potent than other trivalent cations in activating the CaR and showed little effect of pH in the range of 9.5 to 5.5 (data not shown).

Cadmium is a divalent cation with a $pK_a$ near 7.5, yet cadmium activation of the CaR showed little effect of changes in external pH (Fig. 9). This may be the result of competing actions of alkaline pH, making the CaR more sensitive to charged agonists, and acidic pH, causing greater protonation of cadmium in aqueous solution. Together, these data suggest that extracellular pH can have a profound effect on many polyvalent cations, probably because of changes in ionization of these cations, but have little impact on the charge of the physiological divalents, calcium and magnesium.

**Effect of External pH on CaR Activation by Polycationic Agonists with Titratable Groups**—Another broad class of CaR agonists is polycationic molecules, including such endogenous molecules as spermine (a polyamine) and histidine 5 (a basic defensive peptide), as well as antibiotics such as neomycin. Organic polycations have titratable groups with positive charges that are a function of the external pH. For these organic polycations, the number of positive charges will increase as the external pH becomes more acidic, although there will be a loss of positive charge at more basic pH. To test the generality of the effects of pH on CaR activation, the CaR-transfected HEK cells were stimulated by spermine, histatin 5, or neomycin in the presence of varying extracellular pH.

Spermine is a polyamine produced by most cells and found at particularly high concentrations in the lumen of the small and large intestines, where it is generated by bacteria. In the case of spermine, four different positive charge groups are distributed along the molecule, each with its own $pK_a$ value. As external pH becomes more acidic, the EC$_{50}$ for spermine activation decreases, indicating greater sensitivity of CaR toward the more positively charged spermine (Fig. 10). This shift in sensitivity takes place despite the opposite effect that external pH apparently has on the CaR when assessed for inorganic divalent cation activation. The impact of positive charge on spermine activation is not surprising given the observation that spermidine and putrescine are much less effective agonists for the CaR. As seen by us (data not shown) and described by others (19, 20), neomycin activation of the CaR shows sim-
ilar modulation by external pH, with acidic pH greatly reducing the concentration of neomycin needed for half-maximal stimulation of the CaR, probably because of the extent of protonation of its amine groups (data not shown).

Many defensive molecules in the gastrointestinal system as well as other organs are highly charged molecules because of a large number of basic amino acid residues in their primary structure. Histidine 5 is one of a group of antimicrobial molecules produced by the salivary glands of many mammalian species. Histatin 5 has several histidine residues considered critical for its antimicrobial effects (17). These histidine residues are more positively charged at extracellular pH values more acidic than 7.4. Activation of the CaR by histatin 5 is observed at pH values more acidic than 6.5 and was found to be more sensitive to histatin 5 at more acidic pH values (data not shown).

**DISCUSSION**

The CaR is activated by agonists possessing multiple positive charges, including polyvalent cations (i.e. Ca$^{2+}$ and Gd$^{3+}$) and polycationic molecules (i.e. spermine and neomycin) (1, 2). Given this shared characteristic of CaR ligands, it is quite likely that these agonists act on the extracellular domain through an electrostatic mechanism. One possibility is that these cationic agonists may screen negatively charged residues of the CaR’s extracellular domain, thus altering the conformation of the receptor. Another possibility is that several negatively charged side groups form one or more binding pockets for calcium and other polycationic agonists. Indeed, multiple negative residues are grouped together within this domain, particularly in the amino acid sequence encompassing residues 126 to 251 along with many single glutamate and aspartate residues (1, 4, 5, 9). Multiple binding sites for Ca$^{2+}$ interactions with the CaR are suggested by the steep activation curves with Hill coefficients between 3 and 4. Protons may modulate several of these putative Ca$^{2+}$ binding sites.
If electrostatic interactions on the extracellular domain of the CaR are an important mechanism of agonist action, then activation of the receptor should be modulated by extracellular pH. Changing pH in the alkaline direction will increase the net negative charge or, conversely, decrease the net positive charge associated with a protein through loss of protons bound to side chains of amino acids. The amino acids most susceptible to changes in the ionization of side chains in the pH range of 6.5 to 8.5 will be histidine and cysteine, although a small number of other amino acids are likely to have pK<sub>a</sub> values in this range. Increased negative charge of the extracellular head of the CaR at alkaline pH would suggest that extracellular polycationic agonists would need to screen more charge on the receptor; intuition suggests that more agonist would be needed to effect a half-maximal activation of the receptor. This is the opposite of our finding that alkaline pH<sub>i</sub> increases the sensitivity of the CaR toward its divalent agonists. Therefore, more general, unspecific electrostatic interactions may not be critical for agonist activation of the CaR.

With regard to specific interactions between the charged agonists and the CaR, two possibilities are coordinated binding sites or critical, specific sites of electrostatic interactions. Examples of critical sites of electrostatic interactions between polycations and proteins have not been clearly identified in other proteins. However, coordinate binding sites for polycations have been demonstrated in a number of proteins, which are often high-affinity protonation sites that are titratable between pH 9.0 and 6.0. In these Ca<sup>2+</sup>-dependent proteins, clusters of acidic amino acid residues form the polycationic binding site, which also serves as a protonation site sensitive to physiological changes in pH.

This is found in t-type calcium channels, where a set of four glutamate side chains forms a proton binding site with a pK<sub>a</sub> near 8.5 (21–23). This carboxylate cluster acts to shift the typical pK<sub>a</sub> of the glutamate side chains in the alkaline direction. This protonation site allows for the blocking of the calcium channel with an IC<sub>50</sub> similar to its pK<sub>a</sub>. Thus, the same set of glutamate residues that is important for calcium selectivity of the channel is also the site of pH modulation. The CaR may represent an analogous situation in which the sites determining the selectivity and affinity of polycationic agonists also form the sites of protonation, leading to its pH dependence. In the case of the t-type calcium channel, mutational analysis of these glutamate residues suggest that several carboxylates contribute to the binding of the titratable H<sup>+</sup> and that substitutions of alanine, glutamine, and aspartate all interfere with the protonation of this site, lowering its pK<sub>a</sub> and relieving H<sup>+</sup> blockade of the channel. Other molecules with protonation sites with pK<sub>a</sub> values between 7.5 and 8.5 that are formed by clusters of carboxylate side chains include the cyclic nucleotide-gated channel and the photosynthetic reaction center of *Rhodopseudomonas viridis* (24–26). In these cases, mutation of single glutamate residues leads to a lower affinity H<sup>+</sup> binding site and changes in molecular behavior.

Although a similar mutational analysis of glutamate residues of the CaR has not been reported, mutations of the human CaR identified from patient populations with inherited diseases of Ca<sub>s</sub>-sensing show five separate mutations of glutamate residues in the extracellular domain. Of these five mutations, four substitutions of glutamate residues with other residues lead to activating mutations of the CaR in which the sensitivity toward extracellular calcium has increased at neutral pH. This activating characteristic may be a result of the lowering of the affinity of an agonist binding site for protons, thus lessening the degree of the H<sup>+</sup> inhibition at pH 7.4. Furthermore, these four mutations occur in glutamate residues found in groups of acidic amino acids in the primary sequence. We examined three of these glutamate mutations that lead to activation mutations and found that each showed reduction in pH modulation, and the most active mutations had the greatest attenuation of this pH modulation. Other activating mutations also demonstrated reduced pH modulation, suggesting that this may be a common characteristic of this type of mutation. Most activating mutations in the extracellular domain are clustered in a region of the receptor in which dimeric interactions take place. Because many of the activating mutations involved charged amino acid residues, it is highly likely that electrostatic interactions influence dimeric interactions in this region and allow for shifts in the sensitivity of the CaR.

The effect of pH on the activation of the CaR depends, in part, on the agonist under investigation. The shifts in the EC<sub>50</sub> for activation by inorganic polyvalent cations, such as Ca<sup>2+</sup> and Mg<sup>2+</sup>, whose positive charges will be largely unaffected by pH, seems linear between pH<sub>i</sub> of 8.5 and 6.5. In these cases, the external pH effects seem to be largely caused by changes in charged residues of the CaR. For organic polycationic molecules containing several titratable primary amino groups, such as polyamines, aminoglycosides, and some antimicrobial peptides, external pH can have the opposite effect. Their protonation is increased at acidic pH<sub>i</sub> values, leading to greater positive charge on the agonist and more effective activation of the CaR. The converse would hold for more alkaline pH. It is clear that the CaR is very sensitive to the number of positive charges on an organic polyvalent cation. For example, the polyamine spermine with four amine groups is a much better CaR agonist than spermidine, with three amine groups, and putrescine, with two amine groups (9). Because protonation of titratable groups on these molecules extends across the effective range of pH<sub>i</sub> over which the CaR is modulated, these two actions of pH may in part offset each other. For spermine, acid pH clearly promotes greater sensitivity for CaR activation, opposite from the effects of acid pH on Ca<sup>2+</sup> or Mg<sup>2+</sup> activation of the CaR. In the case of organic polycationic molecules with numerous histidine residues, such as histatin 5, pH<sub>i</sub> values of 6 and below are required to protonate the molecule sufficiently for it to act as a CaR agonist (20). This is the same acidic pH range that can produce additional activation of the CaR in the absence of ligand, suggesting that protonation of histidine residues on the receptor could potentially be responsible for this CaR activation. This external pH effect on receptor activation seems specific to the CaR, because the sensitivity of the endogenous purinergic and thrombin receptors in HEK 293 cells to activation by external ATP and a peptide agonist of the thrombin receptor, respectively, showed either a modest effect with changes in pH<sub>i</sub> for the thrombin receptor or modulation opposite that of the CaR for stimulation of purinergic receptors. Part of the pH sensitivity toward ATP stimulation of HEK 293 cells may be caused by changes in magnitude of the negative charge of the ATP molecule.

The importance of modulation of the CaR by external pH on tissue and whole body physiology remains to be established. The CaR is expressed in several tissues and organs, including the kidney, gastrointestinal tract, and bone cells, where sensitivity toward external pH could have important physiological implications. Several tissues offer interesting possibilities for interactions between pH<sub>i</sub> and Ca<sub>s</sub>. Alterations in external pH will have additional effects on most CaR-expressing cell types through changes in the activity of calcium channels, especially during cell stimulation, which will lead to substantial differences in calcium influx and intracellular calcium.

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* M. Bai, unpublished observations.
The CaR is often found localized to the basolateral membrane of epithelial tissues. For acid-secreting organs such as the stomach and the kidney, the CaR will probably experience the alkaline tide associated with acid secretion from the apical membrane. In the stomach, the CaR is expressed in G (gastrin-secreting) cells as well as the acid-secreting gastric parietal cells, in which activation of the CaR seems to be associated with acid secretion (27–29). The alkalization of the interstitial fluid adjacent to the basolateral membrane could lead to facilitation of acid secretion under conditions in which local Ca\(^{2+}\) may remain largely unchanged. In the kidney, the CaR is strongly expressed on the basolateral membrane of the cortical thick ascending limb, where acid secretion takes place in association with bicarbonate reabsorption (4, 30). Here, the alkalization near the basolateral membrane would enhance CaR activation, thereby stimulating a negative feedback that would reduce Na\(^+\) reabsorption and, therefore, Ca\(^{2+}\) reabsorption (30). This may be a factor that leads to hypercalciuria with an increased acid load and a resultant acid urine (31, 32). In the collecting duct, the CaR is located on the apical membrane, and the pH level in the urine could have a direct effect on CaR modulation of water handling (8). Persons with high protein intake will have acidic urine, whereas vegetarians typically have less acidic or even alkaline urine; thus, metabolism and diet could influence the role of CaR in water handling.

Bone metabolism may also be affected by pH modulation of CaR activation. Osteoclasts have been shown to express the CaR in some studies and to be activated by high concentrations of Ca\(^{2+}\), typically above 10 mM. Osteoclasts are known to secrete acid at the apical surface to help resorb bone matrix, and this acidification may also reduce the sensitivity of the CaR toward Ca\(^{2+}\). Finally, parathyroid hormone secretion is known to be modulated by serum pH (33, 34). With acidic pH, parathyroid hormone secretion is elevated in relation to the serum calcium level. Likewise, alkaline pH may lead to reduced circulating parathyroid hormone (33). This pH modulation of parathyroid hormone secretion is consistent with our findings that acidic pH would diminish the sensitivity of the CaR to Ca\(^{2+}\), leading to a decrease in the receptor activation at a given level of Ca\(^{2+}\), which would lead to greater parathyroid hormone secretion.

In summary, the CaR can sense changes in pH, through direct effects on the receptor itself, possibly occurring through high-affinity proton binding sites associated with negatively charged glutamate and aspartate side chains. External pH can also have independent effects on inorganic and organic polycationic agonists, with acidic external media resulting in more positively charged CaR agonists. The potency of calcium and magnesium for activation of the receptor is inversely related to the proton concentration. This pH modulation is diminished in many activating mutations, resulting in greater receptor activity at physiological pH. This may be a common mechanism for altering receptor behavior in activating CaR mutants. The ability of pH to modify CaR function seems to be related to the site of agonist activation or its dimeric interactions.

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