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# pH dependence of extracellular calcium sensing receptor activity determined by a novel technique

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**pH dependence of extracellular calcium sensing receptor activity determined by a novel technique.**

*Background.* Increasing evidence points to the role of the extracellular *Ca*lcium *S*ensing *R*eceptor (CaSR) as a multimodal receptor responding to diverse physiologic stimuli, such as extracellular divalent and polyvalent cations, amino acids, and ionic strength. Within the kidney, these stimuli converge on the CaSR to coordinate systemic calcium and water homeostasis. In this process, the impact of urinary pH changes on the activity of the CaSR has not yet been defined. We therefore performed the present study to analyze the pH sensitivity of the CaSR.

*Methods.* To assess the activation state of the CaSR, we developed a new method based on the functional coupling between CaSR activity and gating of calcium sensitive potassium currents mediated by SK4 potassium channels. Two-electrode voltage clamping was used to determine whole cell currents in *Xenopus* oocytes heterologously expressing rat CaSR and rat SK4 potassium channels.

*Results.* Coexpression of CaSR and SK4 gave rise to potassium currents that were dependent on CaSR-mediated intracellular calcium release, and thereby corresponded to the activation state of the CaSR. In presence of extracellular calcium, ambient alkalinization above pH 7.5 increased CaSR activity. Evaluation of the CaSR calcium sensitivity at various ambient proton concentrations revealed that this effect was due to a sensitization of the CaSR towards extracellular calcium.

*Conclusion.* Coexpression with SK4 potassium channels provides a fast and sensitive approach to evaluate CaSR activity in *Xenopus* oocytes. As disclosed by this novel technique, CaSR activity is regulated by extracellular pH.

The extracellular calcium–sensing receptor (CaSR) functions as a sensor for parathyroid and kidney to determine the extracellular calcium concentration and, thus, helps to maintain a stable plasma calcium concentration [1]. The physiologic importance of the CaSR for the regulation of extracellular calcium homeostasis is impressively illustrated by hyper- and hypocalcemic disorders resulting from mutations in the *CaSR* gene. Mutations resulting in CaSR inactivation cause familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism (NSHPT) [2, 3] when present in the heterozygous and homozygous state, respectively, whereas mutations that activate the CaSR are the cause of autosomal-dominant hypocalcemia (ADH) [4].

In the kidney, activation of CaSR by hypercalcemia, hypermagnesemia, or gain-of-function mutations inhibits divalent cation reabsorption along the nephron, which results in urinary loss of calcium and magnesium [5]. Moreover, the CaSR, which is expressed along essentially the entire renal tubule, integrates calcium- and magnesium-homeostasis with renal salt and water handling [5, 6]. As observed with pronounced gain-of-function mutations in ADH patients, who may suffer from Bartter-like renal salt wasting [7, 8], CaSR activation by an increased tubular calcium load reduces urinary concentrating ability via a decrease in NaCl reabsorption along the thick ascending limb (TAL), and in water reabsorption along the inner medullary collecting duct (IMCD) [5, 6, 9]. During physiologic variations of glomerular calcium delivery to the nephron, this mechanism promotes the excretion of the increased calcium load in more dilute urine, thereby reducing the risk of supersaturation-dependent calcium precipitation in the form of nephrocalcinosis and/or nephrolithiasis.

With respect to calcium phosphate, however, formation of precipitates is not only dependent on the absolute ion concentrations within the tubular lumen, but also on the urinary pH, which—when elevated—favors calcium phosphate precipitation. Thus, in the event of an alkaline urine, a stronger activation of the CaSR would be required to keep calcium at a concentration below saturation. Although allosteric modulation of CaSR activity has been shown for various factors like amino acids [10] and ambient ionic strength [11], the effect of extracellular pH on CaSR activity has not yet been investigated in detail. To clarify this point, we developed a novel technique

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to determine the influence of ambient proton concentration on CaSR activity by electrophysiologic means after heterologous expression in *Xenopus* oocytes.

As a member of the G-protein coupled receptor family, the CaSR signals via pertussis toxin–sensitive and –insensitive G proteins to regulate second messengers that include cAMP, diacylglycerol, inositol trisphosphate, and intracellular calcium [12]. The latter was monitored by coexpression of the calcium-activated potassium channel SK4 (KCNN4), which is tightly regulated by intracellular calcium concentration [13, 14]. As disclosed by this novel method, CaSR activity strongly increased upon extracellular alkalinization, and decreased upon extracellular acidification. Urinary alkalinization thus acts in synergism with elevated urinary calcium on CaSR activity, which, to a certain degree, might prevent intrarenal calcium phosphate precipitation.

## METHODS

### Cloning of the CaSR gene from rat kidney

Freshly prepared rat kidney tissue was homogenized in cold Trizol reagent (Invitrogen; www.invitrogen.com, Carlsbad, CA, USA), and total RNA was prepared according to the manufacturer's recommendations. After mRNA purification with oligo-dT coupled beads (Oligotex mRNA purification system; Qiagen, www.qiagen.com, Valencia, CA, USA), reverse transcription to cDNA was performed with SuperScript reverse transcriptase (Invitrogen). We used a proofreading polymerase (pfuPolymerase; Stratagene, www.stratagene.com, La Jolla, CA, USA) to amplify the complete open reading frame of the kidney-type CaSR cDNA (NCBI GenBank Acc. No. NM_016996) with the primers (in 5′-3′ direction) GCT **ATG** GCA TCG TAC AGC TGC TGT TTG (5′-end) and A ACT GGA TAG CCT TTC CCT CCA **TTA** GG (3′-end) (the start ATG and the reverse complementary stop TTA are indicated in bold). The PCR product was cloned into the pCR-TOPO vector (Invitrogen) and sequenced in both directions.

### Expression in *Xenopus laevis* oocytes and voltage clamp analysis

Ten ng of in vitro transcribed cRNA (mMessage mMachine kit; Ambion, www.ambion.com, Austin, TX, USA) of the CaSR construct and 1 ng of rSK4 cRNA (the cDNA construct was kindly provided by Professor Richard Warth, University of Regensburg, Germany) was injected in defolliculated *Xenopus* oocytes, which were kept at 16°C in ND96 storage solution containing 96 mmol/L NaCl, 2 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 5 mmol/L HEPES (pH 7.4), 2.5 mmol/L sodium pyruvate, 0.5 mmol/L theophylline, and 20 μg/mL gentamicin. Two to five days after injection, 2-electrode voltage clamp measurements were performed at room temperature with a GeneClamp 500 amplifier (Axon Instruments; www.axon.com, Foster City, CA, USA). Currents were recorded in ND96 solution without sodium pyruvate, theophylline, and gentamicin. For pH experiments, the indicated proton concentrations were adjusted by titration with HCl or NaOH. Data from at least 2 different batches of oocytes derived from different frogs are shown. Statistical analysis was performed on $N$ oocytes derived from 1 preparation ($N$ = number of experiments). The error bars in the diagrams were calculated from the standard error of the mean (SEM). Statistical significance was analyzed with Student $t$ test (unpaired test with unequal variance), and was assumed at a $P$ value $\leq 0.05$.

## RESULTS

The CaSR gene was cloned from a cDNA preparation reverse transcribed from oligo-dT purified rat kidney mRNA. PCR amplification with oligonucleotides (27 mers) covering the start ATG and the stop TAA, respectively, gave rise to a full-length cDNA with sequence identity to the NCBI GenBank entry NM_016996 (not shown). As determined by 2-electrode voltage clamp analysis, expression of CaSR cRNA in *Xenopus* oocytes did not induce currents different from noninjected oocytes when extracellular calcium concentration was gradually changed from 0 to 5 mmol/L, which is in the concentration range usually used for CaSR activation (Fig. 1). A small outward current at a holding voltage of −30 mV appeared after expression of rat SK4 calcium-activated potassium channels, which slightly decreased upon removal of extracellular calcium. Increasing extracellular calcium to 5 mmol/L, on the other hand, was without effect on current amplitude. However, when SK4 was expressed together with the CaSR, the outward current dramatically increased when extracellular calcium was shifted from 0 to 5 mmol/L (Fig. 1). SK4 current amplitude thus served as a highly sensitive and fast accessible surrogate parameter for CaSR activity.

The signaling cascades initiated by the CaSR include the liberation of calcium from intracellular stores by inositol trisphosphate ($IP_3$). The stimulation of other G-protein coupled receptors signaling via $IP_3$ release thus similarly should activate SK4 currents when expressed in *Xenopus* oocytes. We therefore tested exogenously expressed m3 muscarinic acteylcholine receptors and endogenously expressed lysophosphatidic acid receptors, which indeed activated SK4 currents upon application of the respective agonists (Fig. 2). To prove that the CaSR-dependent activation of SK4 induced currents is mediated by an intracellular calcium release and not by a calcium influx from the outside, we stimulated CaSR and SK4 expressing oocytes with 10 mmol/L magnesium in

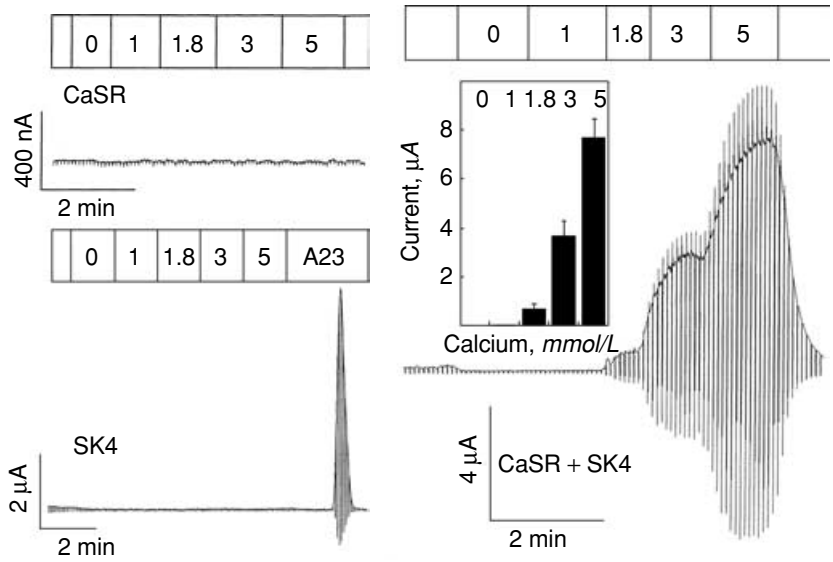


**Fig. 1. Coexpression of CaSR and SK4 confers extracellular calcium sensitivity to SK4 mediated whole cell currents.** When expressed alone, neither CaSR nor SK4 expression in *Xenopus* oocytes alters current amplitude upon stepwise increasing extracellular calcium concentration from 0 to 5 mmol/L (top and bottom left). In case of SK4, functional expression is verified by adding the calcium-ionophore A23187 (A23, 1 µmol/L), which increases intracellular calcium and thereby activates SK4 currents (bottom left). When expressed together with the CaSR (CaSR+SK4; right panel), SK4 currents strongly increase upon elevation of extracellular calcium. The inset shows a statistical analysis derived from 6 oocytes. Currents were recorded at a holding voltage of −30 mV with 500 ms hyperpolarizing pulses to −100 mV at a rate of 0.2 Hz.

presence and in absence of extracellular calcium. Magnesium, which acts as an agonist on CaSR but does not activate SK4, indeed provoked an increase in SK4 current amplitude, even in the absence of extracellular calcium. Moreover, chelating intracellular calcium by preincubation with a membrane-permeable calcium chelator (BAPTA-AM) potently reduced the SK4 response upon activation of the CaSR with 5 mmol/L calcium (Fig. 3). The link between CaSR stimulation and SK4 activation thus was mediated via a release of intracellular calcium.

To evaluate whether the functional coupling between CaSR and SK4 was useful for the determination of pH-sensitivity of the CaSR, we first analyzed the effect of extracellular pH changes from pH 6.5 to pH 8.5 on whole cell currents of oocytes expressing either CaSR or SK4. As shown in Figure 4, currents of CaSR- or SK4 cRNA injected oocytes were not altered by extracellular pH changes. After coexpression of CaSR and SK4, however, a huge increase in current amplitude was observed during elevation of extracellular pH from pH 6.5 to pH 8.5, with the first increase starting at pH 7.5 (Fig. 4). This indicated that the CaSR reacted on extracullar pH, and was activated upon alkalinization above pH 7.5. A similar effect of ambient pH changes on SK4 activation was not observed in oocytes stimulated with lysophosphatidic acid (Fig. 4, inset), arguing against unspecific effects of pH on G-protein coupled receptor signaling or SK4 activation by intracellular calcium.

Allosteric modulation of CaSR calcium sensitivity has been described for factors as different as aromatic amino acids and ambient ionic strength. To test whether CaSR stimulation by extracellular alkalinization is mediated by an effect of pH on CaSR calcium sensitivity, we analyzed the calcium effect on CaSR at acidic and alkaline ambient pH (pH 6.5 and 8.0, respectively). As is evident from Figure 5, at an extracellular pH of 6.5, a distinct increase in SK4 current amplitude was observed after raising the extracellular calcium concentration to 3 mmol/L. At pH 8.0, however, SK4 currents already activated at an extracellular calcium concentration of 1.8 mmol/L. Extracellular alkalinization thus shifted the sensitivity of the CaSR towards lower ambient calcium concentrations. Moreover, as is also shown in Figure 5, removal of extracellular calcium completely abolished the stimulatory effect of alkalinization on CaSR activity. Ambient proton concentration thus modulated the calcium sensitivity of the CaSR.

## DISCUSSION

In this study we exploited the functional coupling between the CaSR and SK4 potassium channel to efficiently access the CaSR activation state in *Xenopus* oocytes. The calcium sensitivity, as determined by this technique, is well within the range of published values established by various other methods like measurements of intracellular cAMP- or intracellular calcium levels after heterologous expression of CaSR in mammalian cells [15, 16].

The advantages of an immediate access to the activation state of the CaSR, as enabled by the whole cell current measurements described here, over time-consuming biochemical methods are clearly evident. Compared to fluorescence-optical methods, which are applied to analyze the intracellular calcium release upon CaSR activation and, likewise, permit an instantaneous assessment of CaSR activity, the electrophysiologic method offers the advantage that it can easily be used also in *Xenopus* oocytes. Their nontransparent pigmentation normally hinders fluorescence-based measuring methods, so that the advantages of oocyte expression like the exact control of the expression level or the simplicity of coexpression

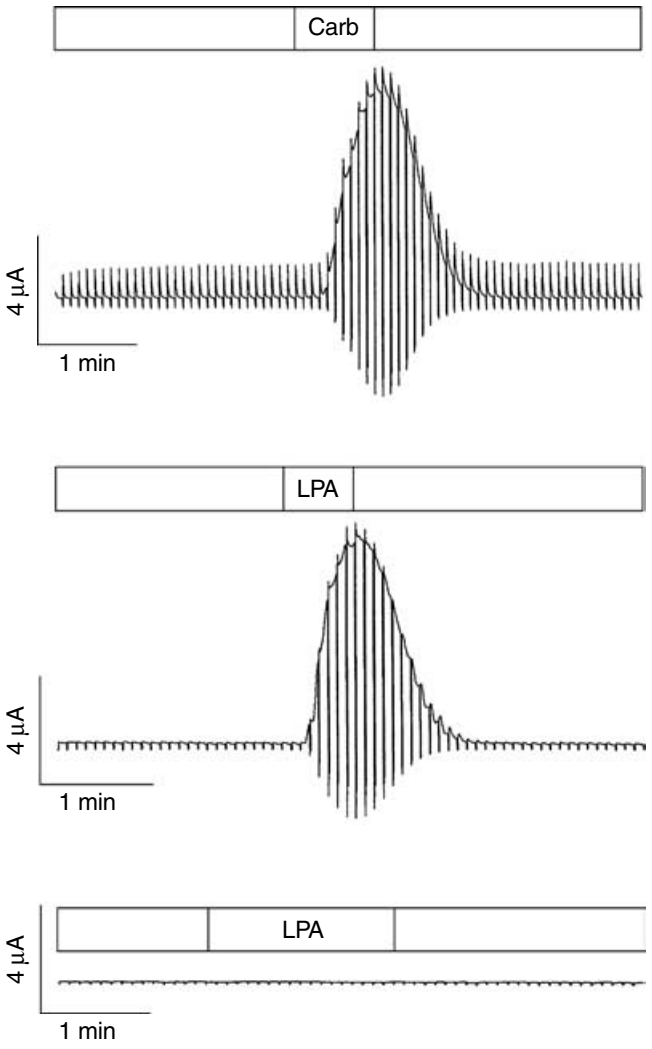


**Fig. 2. Activation of SK4 currents by other G-protein coupled receptors.** Coexpressed m3 muscarinic acetylcholine receptors (upper panel) and endogenous lysophosphatidic acid receptors (middle panel) couple to $IP_3$ formation and activate SK4 currents upon application of their agonists carbachol (Carb; 5 nmol/L) and lysophosphatidic acid (LPA; 50 nmol/L), respectively. In the absence of SK4 expression, application of LPA (100 nmol/L) does not induce a change in whole cell current amplitude (lower panel).

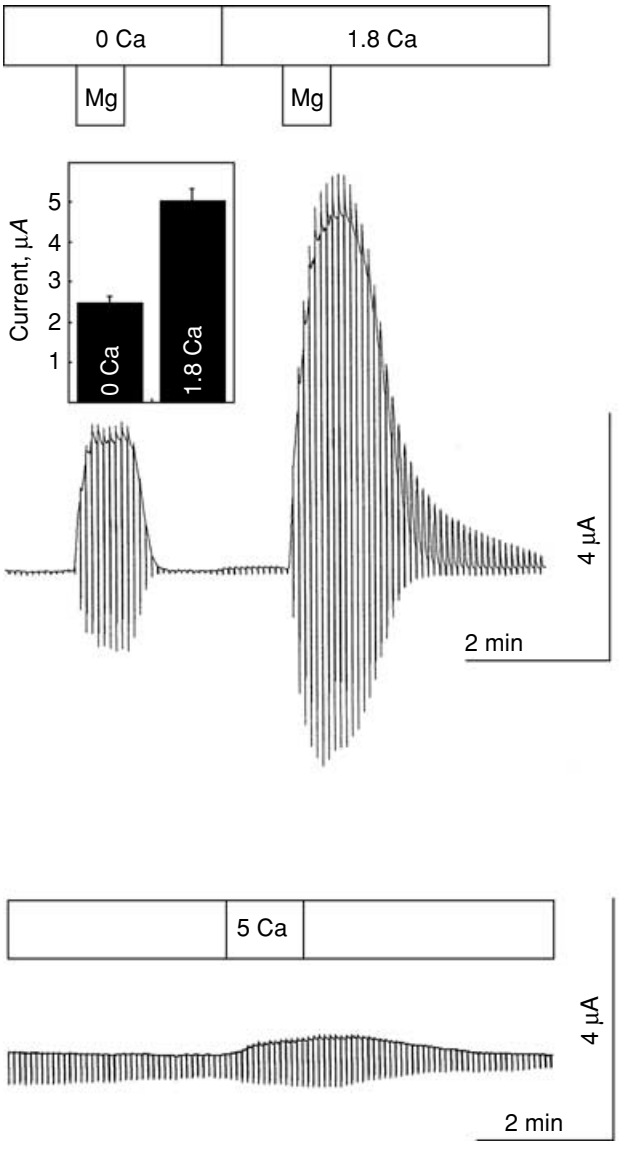


**Fig. 3. CaSR-dependent SK4 activation is mediated by a release of intracellular calcium.** Original whole cell current record of a CaSR and SK4 expressing oocyte stimulated with 10 mmol/L magensium (Mg) in absence (0 Ca) and presence of extracellular calcium (1.8 mmol/L) (upper panel). A significant reduction of current amplitude as shown in the inset ($N = 7$) may be explained by a weaker overall effect on the CaSR, which responds to both divalent cations. Preincubation of CaSR and SK4 expressing oocytes with the membrane permeable calcium chelator BAPTA-AM almost completely abolishes the SK4 response on CaSR activation (lower panel).

of other proteins cannot be exploited for the analysis of CaSR function. Noteworthy, the first molecular identification of a CaSR gene succeeded with a method analogous to the described electrophysiologic technique. For expression cloning of the CaSR from bovine parathyroid mRNA, Brown et al used as an indirect signal a chloride current, which is endogenous to *Xenopus* oocytes, and which is regulated by intracellular calcium [17]. In our experiments, however, even though an intracellular calcium release was convincingly suggested by the SK4 activation, we could not observe chloride currents elicited by CaSR stimulation. This might be explained by a different calcium sensitivity of the endogenous chloride current compared to the SK4-induced current or by qualitative differences of the oocytes, which might not constantly express calcium-activated chloride channels. A defined coexpression of SK4 as a calcium-sensitive indicator channel in *Xenopus* oocytes thus enables more reliable measurements of CaSR activity independent of endogenous chloride channel expression.

As with any indirect signal, the SK4 current amplitude does not necessarily correspond to the activation state

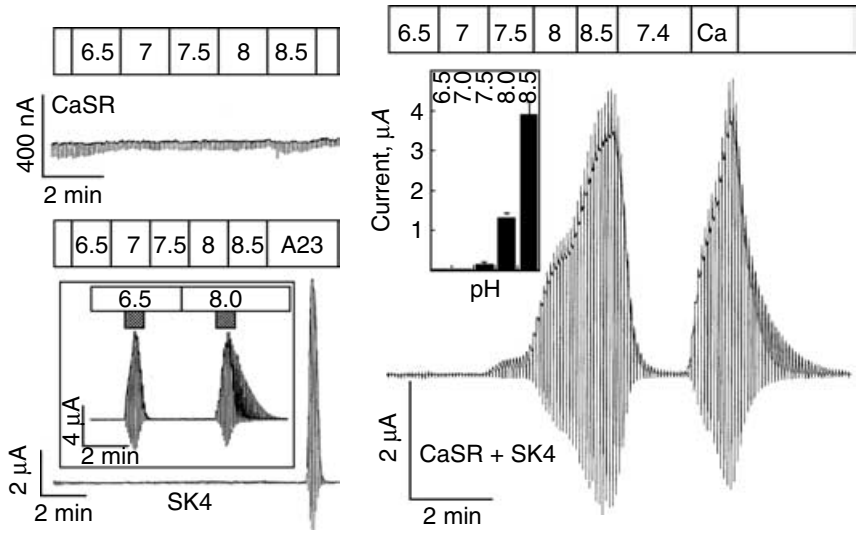


**Fig. 4. Extracellular alkalinization activates the CaSR.** Isolated expression of CaSR or SK4 does not alter whole cell current amplitude when extracellular pH is shifted from 6.5 to 8 (top and bottom left). As illustrated in Figure 1, functional expression of SK4 is verified by adding the calcium-ionophore A23187 (A23, 1 µmol/L; bottom left). As shown in the inset (bottom left), SK4 current activation by lysophosphatidic acid (10 nmol/L; dark boxes) is insensitive towards ambient pH changes. When expressed together with the CaSR (CaSR+SK4; right panel), SK4 mediated currents strongly increase upon elevation of extracellular pH (at 1.8 mmol/L extracellular calcium concentration). For comparison, the depicted original tracing includes the effect of 5 mmol/L calcium on current amplitude. The inset shows a statistical analysis of the outward current amplitude at −30 mV derived from 6 oocytes.

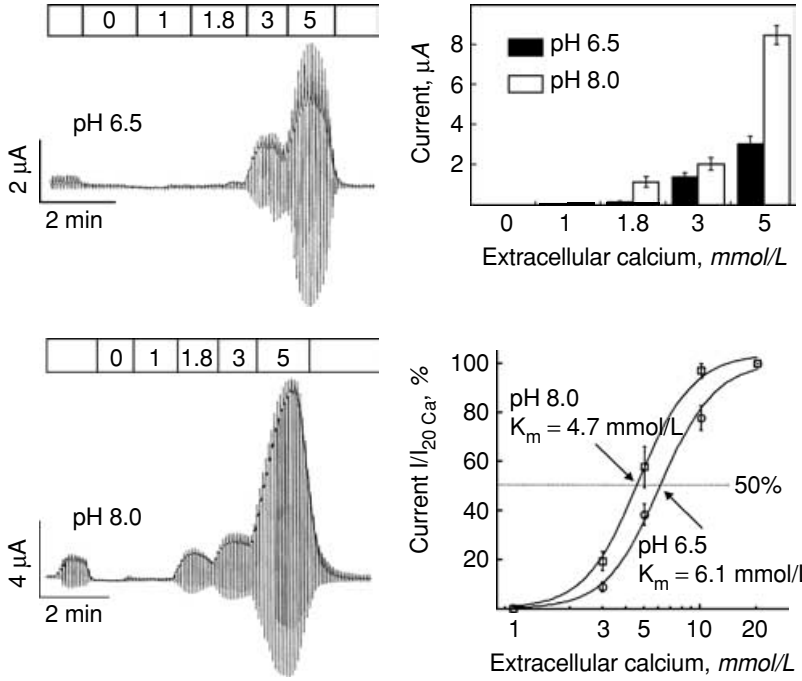


**Fig. 5. Extracellular alkalinization increases the calcium sensitivity of the CaSR.** Original current tracings from oocytes expressing CaSR and SK4 at acidic (pH 6.5) and akaline (pH 8.0) extracellular pH (upper left and lower left panel). The extracellular calcium concentration was increased from 0 to 5 mmol/L. Significantly higher currents ($P < 0.01$; $N \geq 7$) upon 1.8 mmol/L calcium were observed at pH 8.0 as compared to pH 6.5 (upper right panel). Relative SK4 current amplitudes in dependence of extracellular calcium concentration fitted with the Hill equation at pH 8.0 and pH 6.5 (lower right panel). A steep concentration-response relationship with a Hill-coefficient close to 3 is observed at acidic and alkaline ambient pH. Alkalinization by 1.5 pH units reduces the $K_m$ value for half-maximal activation of the CaSR by more than 1 mmol/L extracellular calcium concentration.

of the CaSR in a straight proportional manner. There are several enzymatic steps between CaSR- and SK4 activation that have their own kinetics, and may be saturated even before saturation occurs for agonist binding to the CaSR. For this reason, we cannot provide a true concentration-response relationship reflecting the binding of the agonist to the receptor. Instead, in order to determine the calcium sensitivity of the CaSR at different ambient proton concentrations, we evaluated the minimal calcium concentration needed to provoke a distinct increase in SK4 current amplitude at a defined pH. The sensitization of the CaSR towards extracellular calcium, as observed upon ambient alkalinization, parallels the effect of various other factors, like certain amino acids [10] and extracellular ionic strength [11], which were shown to allosterically modify CaSR calcium sensitivity. There is indeed increasing evidence that the CaSR is capable of responding to several different agonists or modulators that are present in vivo, and whose concentrations are within a range capable of modulating the receptor's activity [18]. In this regard, at a constant extracellular calcium concentration the CaSR would act as a pH sensor, being activated upon alkalinization. pH values sufficiently high to activate the CaSR in vivo indeed are found in tissues where the CaSR is expressed, such as in the pancreatic acini [19], which produce an alkaline secretion, or renal medullary collecting ducts [20], which face urinary pH values normally ranging between 4.6 and 8.0.

In the pancreas, activation of acinar CaSR expressed at the luminal membrane potently stimulates bicarbonate secretion [19]. There, the CaSR thus was suggested to sense the level of calcium in pancreatic juice in order to mitigate the risk of the formation of calcium carbonate stones by adapting the acinar secretion rate on luminal calcium concentration. In the kidney, on the other hand, CaSR activation at the luminal membrane of collecting duct cells reversibly inhibits vasopressin-elicited, transepithelial water flow presumably by redistributing AQP-2 water channels from the membrane to the cytosol [6, 9]. Together with the CaSR-mediated inhibition of transepithelial NaCl reabsorption in the thick ascending limb, this may be part of a diuretic strategy to prevent calcium salts from precipitation in case of elevated urinary calcium concentrations. Sensitization of the CaSR towards calcium upon urinary alkalinization, as suggested by our findings, would further increase the diuretic response and thereby could act against the risk for calcium phosphate precipitation that increases with urinary pH.

## CONCLUSION

We have shown that coexpression of SK4 potassium channels allows for the fast and sensitive assessment of CaSR activity in *Xenopus* oocytes. With this method, we could show that ambient pH modulates the activity of the CaSR with alkalinization acting as a sensitizer towards extracellular calcium.

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