Gating of Acid-sensitive Ion Channel-1: Release of Ca\textsuperscript{2+} Block vs. Allosteric Mechanism

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The acid-sensitive ion channels (ASICs) are a family of voltage-insensitive sodium channels activated by external protons. A previous study proposed that the mechanism underlying activation of ASIC consists of the removal of a Ca\textsuperscript{2+} ion from the channel pore (Immke and McCleskey, 2003). In this work we have revisited this issue by examining single channel recordings of ASIC1 from toadfish (EASIC1). We demonstrate that increases in the concentration of external protons or decreases in the concentration of external Ca\textsuperscript{2+} activate EASIC1 by progressively opening more channels and by increasing the rate of channel opening. Both maneuvers produced similar effects in channel kinetics, consistent with the former notion that protons displace a Ca\textsuperscript{2+} ion from a high-affinity binding site. However, we did not observe any of the predictions expected from the release of an open-channel blocker: decrease in the amplitude of the unitary currents, shortening of the mean open time, or a constant delay for the first opening when the concentration of external Ca\textsuperscript{2+} was decreased. Together, the results favor changes in allosteric conformations rather than unblocking of the pore as the mechanism gating EASIC1. At high concentrations, Ca\textsuperscript{2+} has an additional effect that consists of voltage-dependent decrease in the amplitude of unitary currents (EC\textsubscript{50} of 10 mM at −60 mV and pH 6.0). This phenomenon is consistent with voltage-dependent block of the pore but it occurs at concentrations much higher than those required for gating.

INTRODUCTION

The ASICs constitute a distinct group within the ENaC/DEG family of ion channels, which are expressed predominantly in neurons from the central and peripheral nervous systems. The function of the ASICs has not been definitively established but several lines of evidence suggest a role in nociception, in particular for ASIC3 (Sutherland et al., 2001; Chen et al., 2002; Shuka et al., 2003), in modulation of synaptic transmission (ASIC1) (Roza et al., 2004). In this work we have revisited this issue by examining single channel recordings of ASIC1 from toadfish (EASIC1). We demonstrate that increases in the concentration of external protons or decreases in the concentration of external Ca\textsuperscript{2+} activate EASIC1 by progressively opening more channels and by increasing the rate of channel opening. Both maneuvers produced similar effects in channel kinetics, consistent with the former notion that protons displace a Ca\textsuperscript{2+} ion from a high-affinity binding site. However, we did not observe any of the predictions expected from the release of an open-channel blocker: decrease in the amplitude of the unitary currents, shortening of the mean open time, or a constant delay for the first opening when the concentration of external Ca\textsuperscript{2+} was decreased. Together, the results favor changes in allosteric conformations rather than unblocking of the pore as the mechanism gating EASIC1. At high concentrations, Ca\textsuperscript{2+} has an additional effect that consists of voltage-dependent decrease in the amplitude of unitary currents (EC\textsubscript{50} of 10 mM at −60 mV and pH 6.0). This phenomenon is consistent with voltage-dependent block of the pore but it occurs at concentrations much higher than those required for gating.

The most salient feature of the ASICs is activation by a rapid increase in the concentration of external protons. The sensitivity to protons varies among the mammalian ASIC channels (ASIC1α, ASIC1β, ASIC2a, ASIC2b, and ASIC3), as well as the kinetics of activation and desensitization (Zhang and Canessa, 2002; Hesselager et al., 2004). There are also substantial differences between orthologues from different species, e.g., rat and fish ASIC1 (Waldmann et al., 1997; Coric et al., 2003), or rat and human ASIC3 (Lingueglia, et al., 1997; Babinski et al., 2000).

Immke et al. have shown previously that rat ASIC3 is also activated by a marked decrease of extracellular Ca\textsuperscript{2+}. They proposed that a Ca\textsuperscript{2+} ion occludes the channel pore outside the membrane electric field, thereby implying that the mechanism underlying gating consists of the release of Ca\textsuperscript{2+} block without involving conformational changes of the channel protein (Immke and McCleskey, 2003).

Fig. 1 shows two different schemes describing gating by release of block or by allosteric changes. The closed (C) and open (O) states have bound either Ca\textsuperscript{2+} and/or H\textsuperscript{+}. State D represents the desensitized, nonconducting state. The rate of channel opening in the block mechanism (Scheme 1) is determined only by the rate of Ca\textsuperscript{2+} unbinding, whereas in the allosteric mechanism (Scheme 2), the rate constant α determines the rate of channel opening. Increasing the concentration of Ca\textsuperscript{2+} decreases the time channels spend in the open state in Scheme 1, whereas increasing Ca\textsuperscript{2+} decreases the fraction of channels that open in Scheme 2.

According to the classical description of open-channel block, the residency time of Ca\textsuperscript{2+} at the entrance of the pore equals 1/κ\textsubscript{off}, independent of the Ca\textsuperscript{2+} concentration (Hille, 2001). If the affinity for Ca\textsuperscript{2+} is very high, the dwell time is long such that some of the channels will remain blocked during the period of observation (slow block). If the affinity for Ca\textsuperscript{2+} is intermediate (intermediate block), frequent shut events will induce “flickering” of the open channels and decrease in the mean open time as the concentration of Ca\textsuperscript{2+} increases. If the affinity for Ca\textsuperscript{2+} is low, the very fast block

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will be perceived as a decrease in the amplitude of the unitary currents. These predictions can be tested by single-channel recordings of ASIC currents.

In addition, Scheme 1 predicts that the latency time for the first channel opening remains constant at various Ca\(^{2+}\) concentrations because the delay for the first channel to open reflects the \(k_{Ca}\) of Ca\(^{2+}\), which is independent of the concentration. In contrast, in the allosteric mechanism, Scheme 2, the first latency arises from the time required for the passage from the closed state into the open state and reflects both the Ca\(^{2+}\) unbinding rate and the rate constant \(\alpha\) in the allosteric scheme. If the Ca\(^{2+}\) unbinding is rapid, the delay may decrease as [Ca\(^{2+}\)] decreases.

The goal of this work was to examine whether Scheme 1 or 2 best describes the kinetics of activation of ASIC1.

MATERIALS AND METHODS

Isolation and Injection of Xenopus Oocytes

Channels were expressed in stage V and VI Xenopus laevis oocytes injected with 4–8 ng of fASIC1 cRNA. Capped cRNA was synthesized with T7 RNA polymerase from linearized pCRII plasmid (In Vitrogen) containing the full-length cDNA using Message-Machine kit (Ambion). Oocytes were incubated at 19°C for 2–3 d before experiments. Before patching, oocytes were placed in a hypertonic solution for several minutes and the vitelline membrane was removed manually with fine forceps. Composition of hyperosmolar solution in mM: 220 N-methyl-D-glucamine, 220 aspartic acid, 2 MgCl\(_2\), 10 EGTA, 10 HEPES, adjusted to pH 7.4 with KOH.

Single-channel Recordings

Unitary currents were recorded using the outside-out configuration of the patch-clamp technique. Channels were activated by fast solution exchange from a pH of 7.4 to a lower pH or by reducing the Ca\(^{2+}\) concentration using a mechanical switching device SF-77B (Perfusion Fast-Step, Warner Instrument Corp.) modified according to Hinkle et al. (2003). In brief, gravity-fed solutions flowing at a rate of 1–1.5 ml/min through single-walled three-barrel square glass tubing provided continuous flow of control (central tubing) and two test solutions (adjacent to the central tubing). The three-barrel glass was heated and pulled to decrease the inner diameter to ~250 μm and the thickness of the septum width to ~35–45 μm. With this modification complete exchange of solutions, 10–90% rise time, were routinely achieved in the 0.4–0.8 ms range (see Fig. S1, available at http://www.jgp.org/cgi/content/full/jgp.200509396/DC1). Variations in solution exchange time were due to small differences in the position of the patch pipette in the flow of the central tubing. Steps of 100–200 μm moved the application tubing in front of a closely positioned patch tip. Patch pipettes were pulled from borosilicate glass (LG16, Dagan Corporation) using a micropipette puller (PP-83, Narishige, Scientific Instrument Lab) and fire polished to a final tip diameter of 1 μm. Pipettes were filled with solutions and had resistances of ~5–10 MΩ. Single-channel currents were recorded with an Axopatch-200B amplifier (Axon Instruments) using DigiData 1200 series interface and pClamp8.1 software both from Axon Instruments. The data were collected at 10 kHz, filtered at 1 kHz, and stored on a computer for analysis. For display, data were filtered with a digital Gaussian filter to 0.5 kHz.

The composition of the solutions is given in mM. Incubation solution for oocytes was as follows: 96 NaCl, 2 KCl, 1 MgCl\(_2\), 5 HEPES, adjusted to pH 7.4. Solutions in the recording chamber and in the pipette were identical: 150 NaCl, 1 EGTA, 10 MES-Tris, adjusted to pH 7.5. For activation of fASIC1 channels, outside-out patches were perfused with a standard preconditioning solution containing 150 NaCl, 10 MES-Tris, pH adjusted to 7.5, and 10 CaCl\(_2\) unless indicated. Activating solutions contained 150 NaCl, 1 CaCl\(_2\), 10 MES-Tris, pH adjusted from 7.4 to 5.0 as indicated. In experiments where activation was induced by lowering the external Ca\(^{2+}\) concentration, EGTA was added to the solutions to set the free Ca\(^{2+}\) at the desired concentration using the program CAMG, Ver 2 (W.H. Martin, Yale University, New Haven, CT), which takes into account pH, T = 23°C, and ionic strength = 0.15. Solutions with concentrations of free Ca\(^{2+}\) in the micromolar range were verified with a Ca\(^{2+}\)-sensitive electrode (Corning Pinnacle 555 pH/Ion meter, probe Orion 9700BN Thermo). When solutions are referred to as nominal 0 mM Ca\(^{2+}\) they actually contained ~10 nM Ca\(^{2+}\). All experiments were performed at room temperature.

When appropriate the data are expressed as the mean ± SD of n number of experiments indicated in the corresponding figure legend.

High Ca\(^{2+}\) Concentration in the Preconditioning Solutions

All the experiments in this work were conducted in outside-out patches of Xenopus oocytes expressing fASIC1. Previous observations indicated that high concentrations of Ca\(^{2+}\) in the preconditioning solution increase the extent and speed of recovery from desensitization of ASIC1 channels (Babini et al., 2002; Coric et al., 2003). Specifically, we previously showed that the EC\(_{50}\) of recovery from desensitization is 3 mM Ca\(^{2+}\) for fASIC1 (Coric et al., 2003); therefore, all patches were preconditioned with a solution

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**Figure 1.** Kinetic schemes for block (1) and allosteric (2) mechanisms of fASIC. C indicates the closed state with bound Ca\(^{2+}\) or H\(^+\). O denotes the open state and D the desensitized state. In Scheme 1 the rate of reaching the O state depends on the rate constants for binding and unbinding of Ca\(^{2+}\), whereas in Scheme 2, the rate depends on the rate constant \(\alpha\).
of pH7.4 containing 10 mM Ca²⁺, which is a concentration that achieves maximal effect. This protocol recovered all channels from the desensitized state, making it possible to submit the same patch to repetitive activating pulses with minimal channel rundown. Fig. 2 illustrates an example of continuous recording of a patch to repetitive activating pulses with minimal channel rundown from the desensitized state, making it possible to submit the same patch to repetitive activating pulses with minimal channel rundown.

Time constants of activation and desensitization were obtained by fitting the time course of currents to the function

\[ I(t) = I_o \left[ \exp \left( t / \tau_d \right) - \exp \left( t / \tau_a \right) \right], \]

where \( I_o \) is a scaling factor.

Fig. 4 A shows a representative current trace elicited by a single exposure to pH7.50. Here, the peak of the inward current is large because all channels opened after a very short delay, with a fitted time constant \( \tau_a \approx 1 \) ms, and the duration is short because channels desensitize rapidly, with \( \tau_d \) of 13.7 ms. Fig. 4 B represents the sum of 12 traces of the same patch shown in Fig. 4 A but here it was activated by pH7.0; four of the individual traces are shown.

Online Supplemental Material
The online supplemental material (available at http://www.jgp.org/cgi/content/full/jgp.200509396/DC1) consists of a description and a figure of the time resolution of the solution exchange devised.

RESULTS

Activation of fASIC1 Currents by Increasing Concentrations of External Protons
In these experiments the concentration of Ca²⁺ in the activation solution was maintained fixed at 1 mM, whereas the concentration of protons was increased stepwise from pH7.4 to 7.0. Patches were first exposed to pH7.50 to determine the total number of channels in the patch, followed by an exposure to a different low pH solution. The sequence was repeated three to five times and average currents were normalized to the value obtained with pH7.50 from the same patch. The normalization allowed expression of the data as fractional activation of channels in a particular patch, and to compare the results of many patches even as the level of expression of fASIC differed among patches.

Fig. 3 A shows representative examples of current traces for several pH7.4 tested. Increasing the concentration of external protons produced the following effects. First, we observed an increase in the number of open channels with higher concentration of protons. The top traces in Fig. 3 A show that pH7.2 elicited only a few single channel openings in contrast to ~100 channel openings with pH7.50 in the same patch. Second, the rise and the decay of the inward currents became progressively more rapid as the pH decreased. Inward currents were large (~100 pA) and of short duration (65–90 ms) at pH7.50 but small (1–12 pA) and of long duration (~1.4 s) at pH7.70 and 7.0. Third, there was a decrease in the delay of the first channel opening as proton concentration increased; down to pH7.68 it was possible to discern individual openings in the rising phase but at pH7.60 and 5.0 all channels open synchronously. Finally, the amplitude of the unitary currents, ~1.2 pA at ~40 mV, remained constant at different pH7.4 values.

Fig. 3 B shows the normalized peak current as a function of pH7.4 in Ca²⁺ concentrations of 1 mM (filled circles) and 0.1 mM (open circles). At the lower Ca²⁺ concentration the pH dependence is shifted to higher values and appears to be steeper. Time constants of activation \( \tau_a \) and desensitization \( \tau_d \) at various concentrations of H⁺ were obtained by fitting the time course of currents to the function

\[ I(t) = I_o \left[ \exp \left( t / \tau_d \right) - \exp \left( t / \tau_a \right) \right], \]

where \( I_o \) is a scaling factor.

Fig. 4 A shows a representative current trace elicited by a single exposure to pH7.50. Here, the peak of the inward current is large because all channels opened after a very short delay, with a fitted time constant \( \tau_a \approx 1 \) ms, and the duration is short because channels desensitize rapidly, with \( \tau_d \) of 13.7 ms. Fig. 4 B represents the sum of 12 traces of the same patch shown in Fig. 4 A but here it was activated by pH7.0; four of the individual traces are shown.

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The graph of Fig. 4 D shows that, as pHo decreases, the activation time constants decrease to the temporal resolution limit of ~1 ms. The desensitization time constant also decreases, but reaches a plateau below pHo 6.5.

From recordings showing up to four channels open simultaneously, we also estimated channel mean open times. From a multichannel trace, an idealization is first made by hand, yielding the number \( n(t) \) of channels open as a function of time. The number \( n_c \) of closing steps in the sweep is also counted. Then an estimate of the mean open time is obtained as

\[
t_o = \frac{1}{n_c} \int_0^T n(t) \, dt,
\]

where no channels are open at time zero, and the limit \( T \) of integration is a time near the end of a sweep where again no channels are open. The relative error in the estimate is approximately \( n_c^{-1/2} \). Using this method we were able to obtain estimates of \( t_o \) at pH values in the range 7.2 and 6.8. Over this range the peak open probability changes by fivefold but the \( t_o \) values show no significant variation, and are close to 20 ms (Tables I and II).

The single-channel events show no obvious flickers, so that we would tentatively assert that the open time follows a simple, single exponential distribution. The values of \( t_o \) are plotted as diamonds in Fig. 5 and are similar in value to the desensitization time constant at the same pH values.

To estimate the latency of the first opening we measured the time elapsed from a deflection of the baseline current induced by exposure of the patch to low Ca\(^{2+}\) concentrations to the first channel opening in the patch. This measurement is not influenced by the variability of the solution-exchange apparatus (e.g., position of the patch-pipette with respect to the perfusion pipette) because the deflection indicates the time point at which the new solution reached the patch. At pHo 7.2 we obtained values for the first latency of 240 ms with 1 mM Ca\(^{2+}\) and 14 ms with 0.1 mM Ca\(^{2+}\) (Table III).

A Very Simple Kinetic Model
The simple single-channel features displayed by fASIC1 (an invariant open time, no obvious flickers, and essentially complete desensitization) can be captured by the kinetic scheme

\[
C \xrightarrow{\alpha'} O \xrightarrow{\beta} D, \quad \text{(SCHEME 3)}
\]

where the channel moves irreversibly from a closed state, through the open state, to an absorbing desensitized state. Here \( \alpha' \) is analogous to the rate constant
α in Scheme 2, except that it is allowed to be concentration dependent. According to this scheme the probability of being in the open state at time \( t \), given state C at time zero, is

\[
p_{o}(t) = \frac{\alpha'}{\alpha' - \beta}[\exp(\beta t) - \exp(\alpha' t)]. \tag{3}
\]

An important feature of this expression is that if the rate constants \( \alpha' \) and \( \beta \) are interchanged, the time course of \( p_{o} \) is unchanged (Colquhoun and Hawkes, 1995). Except for an overall scaling factor, this equation is also the same as Eq. 1, which we used to fit the current time courses. This means that the apparent desensitization time constant \( \tau_{d} \) could be the reciprocal of either of the two rate constants, \( \alpha' \) or \( \beta \); the activation time constant \( \tau_{a} \) would be determined by the remaining rate constant.

In this scheme the channel open time is determined by the rate constant \( \beta \). Since the open time seems to be \( \sim 20 \) ms independent of pH, we associate \( \beta \) with the rate of either activation or desensitization, whichever is closer to a value of 50 s\(^{-1} \) (Fig. 5). The rate \( \alpha' \) is then associated with the steeply pH-dependent rates. The dependence of \( \alpha' \) and \( \beta \) on pH are obtained from power-law fits (straight lines in Fig. 5) to be

\[
\alpha'(1) = 440 \text{ s}^{-1}([\text{H}^{+}]/1 \mu\text{M})^{1.5}
\]

\[
\beta = 66 \text{ s}^{-1}([\text{H}^{+}]/1 \mu\text{M})^{0.07}, \tag{4}
\]

a fit to similar data, as pH was varied in 0.1 mM Ca\(^{2+}\), yielded the expression

\[
\alpha'(0.1) = 1600 \text{ s}^{-1}([\text{H}^{+}]/1 \mu\text{M})^{2.5} \tag{5}
\]

with the value of \( \beta \) remaining statistically indistinguishable.

Do these assignments of rate constants make sense? As a test, we used the fits of Eqs. 4 and 5 to predict the peak open probability in Eq. 3 as a function of pH. The resulting function is plotted as the solid curve in Fig. 3 B, which very successfully describes the peak current in 1 mM Ca\(^{2+}\). A curve calculated with Eq. 5 for the case of 0.1 mM Ca\(^{2+}\) also describes the peak current under this condition (dotted curve in the figure). We conclude that Scheme 3 describes the channel behavior with \( \alpha' \) being steeply dependent on H\(^{+}\) concentration, having an exponent of 1.5 or 2.5 at 1.0 and 0.1 mM Ca\(^{2+}\), respectively. The rate constant \( \beta \) is essentially insensitive to pH.

According to this scheme, the long time course of desensitization seen at high pH is actually due to a low activation rate \( \alpha' \). Thus the delayed openings seen for example in Fig. 4 C result from long first latencies to opening.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Proton dependence of apparent rates of activation and desensitization of fASIC. (A) An outside-out patch containing fASIC1 was activated first by a solution of pH\(_{o}\) 5.0 and 1 mM Ca\(^{2+}\). The continuous line represents the fit of the currents to Eqs. 4 and 5 with values for rise time, \( \tau_{a} \), of \( \sim 1.4 \) ms, and decay, \( \tau_{d} \), of 13.5 ms and peak current of 100 pA. (B) The same patch was subsequently activated by a solution of pH\(_{o}\) 7.0 containing 1 mM Ca\(^{2+}\). The trace represents the sum of 12 consecutive such pulses of pH\(_{o}\) 7.0. The current is of smaller amplitude, significantly noisier, and has slower time constants, \( \tau_{a} \) of 105 ms and \( \tau_{d} \) of 15 ms, than with pH\(_{o}\) 5.0. (C) Four of the individual responses to pH\(_{o}\) 7.0 are shown. (D) Plot of the log of the apparent time constants for activation (\( \tau_{a} \)) and desensitization (\( \tau_{d} \)) over pH\(_{o}\). Diamonds represent measurements of mean open time of individual channel openings at pHs in the range of 7.2 and 6.8. There is no theoretical meaning of the curves.
Activation of fASIC1 Currents by Decreasing Concentrations of External Ca2+

If protons and Ca2+ compete for the same binding sites in fASIC1, reducing the external Ca2+ concentration should recapitulate the results shown in the previous section. Additionally, the affinity for Ca2+ should decrease as proton concentration increases. We tested these predictions by examining the activation of fASIC1 at various concentrations of extracellular Ca2+ (from 1 mM to 10 nM) yet keeping the concentration of protons fixed. As in the previous experiments, the data were normalized to the total number of channels present in the patch, which were measured by first exposing the patch to the maximal activating stimulus of pHo 5.0. The same patch was subsequently activated by a test solution containing low Ca2+ concentrations. Initially, we tried to activate channels with solutions equilibrated with pHo 7.4, the same pHo as the preconditioning solution, but we could not detect single channel activity unless the Ca2+ concentration was decreased to the nanomolar range. Therefore, we conducted experiments with activation solutions of pHo 7.1 or 7.2 containing decreasing concentrations of Ca2+.

Fig. 6 A illustrates representative examples of patches first activated by pHo 5.0 with 1 mM Ca2+, by pHo 7.1 with the test Ca2+ concentration indicated on the bar above each trace. The top trace shows that pHo 5.0 and 1 mM Ca2+ induced a large (∼50 channel openings) and transient (70 ms) inward current. After recovery from desensitization the same patch responded to pHo 7.1 and 1 mM Ca2+ with only two channel openings, separated by a 1.6-s interval. The first opening was detected 200 ms after the application of the test pulse, i.e., much later than the completion of the activation and desensitization of all channels in the same patch by pHo 5.0.

The second trace in Fig. 6 A shows currents activated by pHo 7.1 and 0.3 mM Ca2++; this patch also contained ∼50 channels. The test pulse with 0.3 mM Ca2+ activated more channels than with 1 mM Ca2+. The openings were not simultaneous but occurred over 880 ms in contrast to 50 ms at pHo 5.0. With 10 nM Ca2+, inward currents were similar in magnitude to those activated by pHo 5.0 (bottom traces). Currents did not increase further with a combination of pHo 5.0 and 10 nM Ca2+, indicating that either of these treatments activates all the channels present in the patch. The magnitude of the unitary currents (1.5 pA at −40 mV) did not significantly change as Ca2+ concentration was decreased from 1 mM to nominally 0.

The Ca2+ dependence for activation at pHo 7.1 and 7.2 are presented in Fig. 6 B. The apparent EC50 for Ca2+ at pH 7.1 and 7.2 were 31 μM and 3.2 μM. These values are comparable to those found for rat ASIC3, 152 μM and 12 μM at pH 7.0 and 7.4, respectively (Immke and McCleskey, 2003).

Thus far, the results indicate that increasing proton or decreasing Ca2+ concentrations activates fASIC1 with similar kinetics and that maximal activation is achieved with either treatment. In addition, the decrease in Ca2+ affinity with increasing proton concentrations is consistent with competition of H+ and Ca2+ for the same sites (Immke and McCleskey, 2003).

Changes in time constants for activation and desensitization were calculated by fitting to Eq. 1 the current traces activated by different Ca2+ concentrations (Fig. 6 C). Again using the framework of Scheme 3 we associated...
the rate constant $\beta$ with a relatively Ca$^{2+}$-independent rate on the order of $50 \text{s}^{-1}$, and $\alpha'$ with the more Ca$^{2+}$-dependent rate. As in the case of H$^+$-activated channels, the estimates of mean open time (Table II) yielded values similar to those assigned to $\beta$ in Fig. 6 C, while estimates of first latency corresponded to our assignment of $\alpha'$. With these assignments the modeled dependence at pH 7.1 of the rates on [Ca$^{2+}$] (lines in Fig. 6 C) were

$$\alpha' = 140 \text{s}^{-1} \left(\frac{[\text{Ca}^{2+}]}{1 \mu\text{M}}\right)^{-0.37}$$
$$\beta = 17 \text{s}^{-1} \left(\frac{[\text{Ca}^{2+}]}{1 \mu\text{M}}\right)^{0.14}.$$  \hspace{1cm} (6)

The predicted peak open probabilities are shown as the solid curve in Fig. 6 B. Again the simple Scheme 3 is quite successful in predicting the peak open probability from the two rate constants.

The rate constant for activation $\alpha'$ decreases as the external Ca$^{2+}$ concentration increases. On the other hand, the rate constant $\beta$ for desensitization increases moderately with increasing Ca$^{2+}$ concentration. The latter observation is consistent with the previous finding that Ca$^{2+}$ may also participate in the desensitization process (Zhang and Canessa, 2002; Immke and McCleskey, 2003).

**Ca$^{2+}$ Dependence of Single-channel Currents**

At concentrations of external Ca$^{2+}$ >1 mM, we observed a reduction of peak inward currents elicited by low pH$_o$. This Ca$^{2+}$-mediated inhibition of fASIC1 was produced by a decrease in the amplitude of the unitary currents. Fig. 7 shows I-V curves of single channels activated with solutions of pH$_o$ 6.0 containing increasing concentrations of Ca$^{2+}$ (10 nM, 1 mM, and 10 mM). The magnitude of the unitary currents was measured at negative voltages from −20 to −100 mV. High Ca$^{2+}$ decreased the amplitude of the unitary currents in a voltage-dependent manner. The result is readily described by Ca$^{2+}$ block of the open pore in a concentration and voltage-dependent manner, but the effect is apparent decreasing concentrations of Ca$^{2+}$, from 1 mM to 10 nM, as indicated by the bars above the traces. Patches were preconditioned with pH$_o$ 7.4 and 10 mM Ca$^{2+}$ after exposure to activating solutions. Experiments were conducted with 150 mM symmetrical Na$^+$ and external solutions containing various concentration of Ca$^{2+}$ that were calculated from a mixture of Ca$^{2+}$ and EGTA according to the program CAMG, Ver 2 (W.H. Martin) and verified with a Ca$^{2+}$ electrode. The pipette solution contained 1 mM EGTA and no added Ca$^{2+}$.

**Figure 6.** Activation of fASIC1 by decreasing the concentration of external Ca$^{2+}$. (A) Paired current traces from outside-out patches expressing fASIC1 activated first by pH$_o$ 5.0 and 1 mM Ca$^{2+}$ followed by a second activation by solutions of pH$_o$ 7.1 containing 1 mM Ca$^{2+}$, 0.3 mM Ca$^{2+}$, 0.1 mM Ca$^{2+}$, 0.01 mM Ca$^{2+}$, 1 mM Ca$^{2+}$, 0.3 mM Ca$^{2+}$, 0.1 mM Ca$^{2+}$, and 0.01 mM Ca$^{2+}$.

(B) Peak currents as a function of [Ca$^{2+}$] measured at activating pH$_o$ of 7.1 and 7.2. Currents were normalized to the value obtained with pH$_o$ 5.0 in the same patch. Each point is the mean ± SD of three to five independent patches. The continuous (pH 7.1) and dotted (pH 7.2) lines represent predictions from Eqs. 3-6. To produce the curve at pH 7.2, $\beta$ was multiplied by 4. (C) Plot of the log of the time constants(s) for activation $\tau_\alpha$ (○) and desensitization $\tau_\beta$ (●) as a function of pH. Triangles are estimates of the first latency to channel openings. The straight lines show the fitted function Eq. 6.
only at Ca\(^{2+}\) concentration much higher (>1 mM) than the one required for activation. A fit of the Woodhull model for block yielded \(K_0 = 74\) mM and \(\delta = 0.35\).

**Discussion**

Our results are consistent with the notion that activation of fASIC1 is mediated by unbinding of Ca\(^{2+}\) from the extracellular domain either by reducing the concentration of extracellular Ca\(^{2+}\) or by competing with protons for the same binding sites. This is in agreement with previous findings reported for rASIC3 but our data differ from Immke in several respects (Immke and McCleskey, 2005). We observed that the number of open channels increased and the delay time for first opening decreased with increasing concentration of protons or decreasing concentration of Ca\(^{2+}\). We did not observe shortening of the mean open time or decreases in the magnitude of the unitary currents as would be expected for intermediate or fast block. The single channel conductance remained constant within the range of pH tested, 7.2 to 6.0, and decreased slightly only when Ca\(^{2+}\) concentrations were >1 mM; therefore, changes in conductance could not account for the total increase in current. Finally, contrary to Scheme 1, which predicts a constant latency for the first opening, we observed a marked decrease of the time for the first opening as \([\text{Ca}^{2+}]\) decreased at constant pH. Taken together, our observations favor the conclusion that release of Ca\(^{2+}\) from a site in the extracellular domain of the channel induces conformational changes that ultimately open fASIC1. Thus, we favor an allosteric mechanism (Scheme 2) over channel opening by release of Ca\(^{2+}\) blockade (Scheme 1).

**Gating Schemes**

The three-state scheme is very successful in describing the behavior of fASIC1 as indicated by the curves of peak currents at various H\(^+\) (Fig. 3 B) and Ca\(^{2+}\) concentrations (Fig. 6 B). The properties of this scheme are a variable \(\alpha\)'s, a fixed \(\beta\), and very small \(\gamma\) and \(\delta\) rates. A small value for \(\gamma\) was inferred from the absence of flickering in the openings of fASIC1, whereas the value of \(\delta\) was calculated from the number of reopening events in patches continuously exposed to pH\(_{5.0}\). We detected only 25 reopening events in 300 patches containing an average of 30 channels/patch. The number of reopening events divided by the product of the total number of channels observed over the 3 s duration of pH\(_{5.0}\) pulses yielded a value for \(\delta\) of 0.92 \(10^{-3}\) s\(^{-1}\). Activation by increasing concentrations of H\(^+\) results in a high \(\alpha\)', which is compatible with both Scheme 1 and 2. However, the difficulty arises when activation is induced by low Ca\(^{2+}\) concentrations, which become more rapid at fixed pH as [Ca\(^{2+}\)] decreases. The latter cannot be explained by Scheme 1. Instead, Scheme 2 assumes that Ca\(^{2+}\) binding and unbinding are fast, so that effective opening rate is the product of \(\alpha\) and the occupancy of \(C_{\text{state}}\). This can explain the steep Ca\(^{2+}\) dependence of \(\alpha\)' in our fits. At very low Ca\(^{2+}\) concentrations, the apparent activation rate \(\alpha\)' is expected to saturate as Ca\(^{2+}\) unbinding or \(\alpha\) becomes rate limiting. We did not observe such saturation, but our time resolution was limited to ~1 ms by the solution exchange.

It is reasonable to postulate an allosteric mechanism given the capacity of Ca\(^{2+}\) to be coordinated up to eight oxygen atoms, enabling it to induce large conformational changes upon unbinding from a protein. Indeed, Ca\(^{2+}\) activates many intracellular signaling proteins, including ion channels. Few examples are the large-conductance Ca\(^{2+}\)-activated K\(^+\) channels (BKCa) (Bao et al., 2004) and the bacterial K\(^+\) channel MthK (Jiang et al., 2002). What is unusual for the ASICs is that activation results from unbinding of Ca\(^{2+}\) and that the Ca\(^{2+}\)-binding site is located in the extracellular side of the protein where Ca\(^{2+}\) concentrations are usually much larger than in the cytosol. The primary sequence of the extracellular domain does not have any recognizable canonical Ca\(^{2+}\) binding motifs such as an EF hand or C2 domain. Without knowledge of the three-dimensional structure of the extracellular domain we cannot predict whether conserved acidic residues come to close proximity to form a Ca\(^{2+}\)-binding site in the native fold of the protein.

**Voltage-dependent Ca\(^{2+}\) Block**

Fish ASIC1 also exhibits an open-channel block effect at high external Ca\(^{2+}\) concentrations, EC\(_{50}\) of 10 mM at


pH, 5.0 and −60 mV (Fig. 5). A similar effect has been previously observed for the mammalian ASIC1 (Zhang and Canessa, 2002; de Weille and Bassilana, 2001). Most recently, Paukert et al. (2004) have shown that in the rat ASIC1 this effect depends on two negatively charged residues, E425 and D432, located immediately before the second transmembrane domain, which is considered to form the entrance of the pore. These two residues are conserved in the fASIC1 and are likely to be the entrance of the pore. This blocking mechanism appears to be unrelated to channel gating.

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