Calcium Sensitivity of a Sodium-Activated Nonselective Cation Channel in Lobster Olfactory Receptor Neurons

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Bobkov, Yuriy V. and Barry W. Ache. Calcium sensitivity of a sodium-activated nonselective cation channel in lobster olfactory receptor neurons. J Neurophysiol 90: 2928–2940, 2003. We report that a Na+-activated nonselective cation channel described previously in lobster olfactory neurons, in which phosphoinositide signaling mediates olfactory transduction, can also be activated by Ca2+. Ca2+ activates the channel in the presence of Na+, increasing the open probability of the channel with a K1/2 of 490 nM and a Hill coefficient of 1.3. Ca2+ also increases the sensitivity of the channel to Na+. In some cells, the same channel is Ca2+-insensitive in a cell-specific manner. The nonspecific activator of protein phosphatases, protamine, applied to the intracellular face of patches containing the channel irreversibly eliminates the sensitivity to Ca2+. This effect can be blocked by okadaic acid, a nonspecific blocker of protein phosphatases, and restored by the catalytic subunit of protein kinase A in the presence of MgATP. The Ca2+-sensitive form of the channel is predominantly expressed in the transduction zone of the cells in situ. These findings imply that the Ca2+ sensitivity of the channel, and possibly its regulation by phosphorylation, play a role in olfactory transduction and help tie activation of the channel to the canonical phosphoinositide turnover pathway.

INTRODUCTION

In contrast to the well-established role of cyclic nucleotide signaling in vertebrate olfactory transduction, the involvement of phosphoinositide signaling in olfactory transduction is less clear even though elements of the canonical phosphoinositide turnover pathway have been localized to olfactory receptor neurons (ORNs) in various species, including vertebrates (Ache and Zhainazarov 1995; Schild and Restrepo 1998). New evidence implicating 3-phosphoinositide signaling in olfactory transduction (Ache and Zhainazarov 1995; Schild and Restrepo 1998) suggests the possible involvement of 3-phosphoinositide signaling in olfactory transduction.

METHODS

Cell preparations

SGC channels were studied in two different preparations of lobster (Panulirus argus) ORNs. Primary cultures of lobster ORNs were prepared as described previously (Fadool et al. 1991). Membrane patches were excised from the soma of these cells in the 30-mm culture cell dishes in which the cells were grown from 1 to 7 days. For other experiments, freshly isolated vesicles of outer dendritic membranes in vitro (Boekhoff et al. 1994) and blocking phospholipase C (PLC) blocks depolarizing receptor potentials in the cells in vivo (R. E. Doolin and B. W. Ache, unpublished results). An InsP3R has been cloned from lobster ORNs (Munger et al. 2000) and an InsP7-activated channel has been localized to the outer dendrites (Hatt and Ache 1994). Finally, lobster ORNs express a sodium-gated nonselective cation (SGC) channel (Zhainazarov and Ache 1997; Zhainazarov et al. 1998) that shares at least some properties with the growing transient receptor potential (TRP) family of ion channels that are commonly associated with PLC-mediated cell signaling (Minke and Cook 2002). More recently it was shown that 3-phosphoinositides activate the SGC channel and modulate gating of the channel by Na+ (Zhainazarov et al. 2001), suggesting the possible involvement of 3-phosphoinositide signaling in olfactory transduction.

We report that Ca2+ can activate the SGC channel in the presence of Na+ and increase the sensitivity of the channel to Na+ in some cultured lobster ORNs and that phosphorylation potentially regulates the Ca2+ sensitivity of the channel. Given that the Ca2+-sensitive form of the channel is predominantly expressed in the outer dendrites of lobster ORNs in situ, the Ca2+ sensitivity of the channel, and possibly its regulation by phosphorylation, presumably plays a role in olfactory transduction.

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brane were obtained by incubating the olfactory organ for 10–20 min in a solution containing (in mM) 210 NaCl, 696 glucose, 10 HEPES, 0.1 CaCl₂, and 1 EGTA buffered to a free calcium concentration of ~10 nM, and cutting the tips of the olfactory sensilla into the same solution, as described by Hatt and Ache (1994). Membrane patches were excised from these vesicles in the 30-mm culture cell dishes in which the vesicles were prepared.

**Electrophysiology**

Cells/vesicles were patch clamped in the inside-out or the outside-out configuration, as noted. Currents were measured with an Axopatch 200A patch-clamp amplifier (Axon Instruments) using AD-DA converter Digidata 1320A (Axon instruments), low-pass filtered at 5 kHz, sampled at 20 kHz, and digitally filtered off-line at 1–1.2 kHz. Data were collected and analyzed with pCLAMP 8.1(2) software (Axon Instruments) in combination with Microcal Origin 6.0 (Microcal Software) and SigmaPlot 5.0 (SPSS). The Clampex/Clampfit protocol parameters used are specified for each experiment. SGC channel activity was investigated in steady-state conditions at a holding potential of ~70 mV unless otherwise noted. The polarity of the currents is presented conventionally (i.e., relative to intracellular membrane surface) in spite of the membrane patch configuration. Patch pipettes were fabricated from borosilicate filament glass capillary (Sutter Instrument, BF150-86-10) using a Flaming-Brown micropipette puller, Model-P-87 (Sutter Instrument). The resistance of fire-polished pipettes was 2–10 MΩ when filled with 210 mM NaCl solution (see Solutions) in the pipette. The solution bathing the membrane patches was regulated and changed using a nine-channel rapid solution changer (RSC-100, Bio-Logic, France). Solution switching time was 50 ms unless otherwise noted. Application of the appropriate solution and data acquisition were synchronized. Liquid junction potentials were measured for different solution combinations, and the appropriate corrections were made when necessary. Activity of other types of channels sometimes observed in membrane patches comprised <1–1.5% of total SGC channel activity and was not correlated with SGC channels activity so it was ignored. Where noted, paired and unpaired Student’s t-tests were used to evaluate differences between two means. P < 0.05 was considered to indicate significance. The data are presented as the means ± SE of n observations. All recordings were performed at room temperature (~21°C).

**Solutions**

Panulirus saline (PS) contained (in mM) 458 NaCl, 13.4 KCl, 13.4 Na₂SO₄, 13.6 CaCl₂, 9.8 MgCl₂, 2 glucose, and 10 HEPES, pH 7.4 adjusted with 1 M NaOH or Tris-base. In some cases, the Na₂SO₄ in the SGC channel (data not shown).

**RESULTS**

**Intracellular Ca²⁺ activates the SGC channel and increases the sensitivity of the channel to Na⁺ in some, but not all, cultured lobster ORNs**

We explored the potential Ca²⁺ sensitivity of the lobster SGC channel in cell-free patches. We found that Ca²⁺ modulated the activity of the SGC channels in 137 of 211 patches tested (Fig. 1A). Earlier, it was shown that while intracellular Na⁺ reversibly activated the SGC, because, Li⁺ and other monovalent cations (K⁺, Rb⁺, Cs⁺) fail to activate the SGC channel (Zhainazarov and Ache 1995; Zhainazarov and Ache 1997), we used 210 mM LiCl as a control solution in these experiments. In the typical instance shown, increasing [Ca²⁺] augmented channel open probability (P_open) from 0.37 in the presence of 210 mM Na⁺ and 10 nM Ca²⁺ free to 0.95 in the presence of 210 mM Na⁺ and 100 μM Ca²⁺. Overall, the mean open probability of the channel, obtained from an approximation by the Hill equation, increased from 0.45 to 0.9 in the presence of 210 mM Na⁺ when Ca²⁺ free was elevated from 10 nM to 100 μM (Fig. 2). Ca²⁺ was never observed to activate the channel directly in the absence of Na⁺ (segments of current recordings marked by asterisks in Fig. 1, A and C). As shown by the all-points current amplitude histograms to the right of the data in Fig. 1A, the current noise appearing in the presence of Na⁺ is determined by channel activity. Each peak on the histogram reflects a discrete current level corresponding to a certain number of simultaneously open SGC channels, the patches in A and C appear to contain 10 and 3 SGC channels, respectively. Complete or partial replacement of monovalent ions or removing divalent cations from the extracellular side of the patch did not significantly alter the presence of the Ca²⁺ sensitivity of the SGC channel nor the kinetics of the Ca²⁺-induced effect, excluding the possibility that another ion transporting system that was directly sensitive to calcium mediated the SGC channel (data not shown).

In the remaining 74 patches, Ca²⁺ had no measurable effect on channel activity (Fig. 1C). In all cases in which Ca²⁺ modulated activity of the SGC channel, the patches were obtained from cells of a particular morphological type. These cells were larger (26 ± 8 vs. 15 ± 6 μm soma diameter, n = 72, P < 0.01), had more processes (2–5 vs. 0–2), and better defined cellular compartments than did cells yielding patches in which the SGC channels were Ca²⁺-insensitive (Fig. 1, B vs. D). We cannot resolve, however, whether these represent distinct types of neurons or the same type of neuron in a different developmental stage.

Increasing the cytoplasmic [Ca²⁺] also augments SGC channel activity by increasing the sensitivity of the channel to Na⁺ (Fig. 2, A vs. B). Measuring the dose-response relation of SGC channel activity to [Na⁺] at various cytoplasmic Ca²⁺ concentrations showed that Ca²⁺ increases the sensitivity of the channel to sodium by shifting the sodium concentration required for half-maximum effect ([Na⁺]₁/₂) from 113.4 ± 8.8 mM at 10 nM Ca²⁺ to 32.3 ± 14.6 mM at 100 μM Ca²⁺, with all inorganic salts were purchased from Fisher Scientific except for AlCl₃ and LaCl₃, which were purchased from Sigma Scientific. All organic compounds were obtained from Sigma except for calmodulin and 2-aminoethoxydiphenyl borate (2-APB), which were obtained from Calbiochem, and PIP2 and PIP3, which were obtained from Matreya.
FIG. 1. Representatives of 2 different morphological sub-
populations of lobster olfactory receptor neurons (ORNs; B and 
D) expressing sodium-gated cation (SGC) channels with dif-
ferent sensitivity to intracellular calcium ([Ca$^{2+}$]). Ca$^{2+}$-sen-
sitive (A) and Ca$^{2+}$-insensitive (C) SGC channel activity was 
recorded from inside-out membrane patches excised from both 
cell types. Patches in A and C contained 10 and 3 ion channels, 
respectively. Ca$^{2+}$ by itself did not activate the Ca$^{2+}$-sensitive 
SGC channel (portions of current traces marked by *). All-
points amplitude histograms presented in A were generated for 
12-s segments of current trace (in range: −120–0 pA, bin 
width: 0.1 pA) at 10 nM (○) and 100 μM (◇) Ca$^{2+}$. Time course 
of solution application is depicted by the bar under the current 
trace. Current traces were digitally filtered using Clampfit [low-
pass Bessel (8-pole) filtering at the −3 dB cutoff 1.2 kHz]. 
Current trace in C was not filtered. Membrane holding potential 
(HP) was −70 mV. Electrode solution: 210 mM NaCl + 10 nM 
Ca$^{2+}$. Scale bars in B and D are 30 μm. Unless noted otherwise, 
210 mM LiCl and 210 mM NaCl had 10 nM [Ca$^{2+}$]free.
corresponding Hill coefficients of 4.6 ± 1 at 10 nM Ca\(^{2+}\) and 0.92 ± 0.39 at 100 μM Ca\(^{2+}\) (Fig. 2C).

**Ca\(^{2+}\)-sensitive and -insensitive SGC channels are otherwise similar**

In spite of their profound difference in Ca\(^{2+}\) sensitivity, SGC channels in both types of cells had their other known properties in common, suggesting they were indeed the same type of channel (Fig. 3). Both Ca\(^{2+}\)-sensitive and -insensitive SGC channels had identical sensitivity to cytoplasmic Na\(^{+}\) (Fig. 3, A vs. B), the same single-channel current amplitude [means of single-channel amplitudes obtained at different voltages (n = 5–21)] and slope conductances (n = 5) are not significantly different at P < 0.05] and similar voltage dependence of the amplitude in the positive voltage range (Fig. 3, C and E vs. D and G), and similar voltage dependence of the open probability (Fig. 3, C and F vs. D and H). In addition to having the same kinetic properties, both Ca\(^{2+}\)-sensitive and -insensitive SGC channels had similar pharmacology, at least as it is known. Channels of both types were fully and reversibly blocked from both the intracellular and extracellular sides by W-7 (200 μM, n = 3, 7—numbers of experiments for Ca\(^{2+}\)-insensitive channels and Ca\(^{2+}\)-sensitive channels, respectively), TFP (200 μM, n = 8, 21), calmidozolium (100 μM, n = 3, 4), and mastoparan (5.6 μM, n = 4, 3) (data not shown). Both could also be fully and reversibly blocked from the extracellular and intracellular sides by high Mg\(^{2+}\) (3–5 mM only, n = 3,14), La\(^{3+}\) (10 μM, n = 3, 32), and Al\(^{3+}\) (10 μM, n = 1, 2) (data not shown). Blockade with trivalent cations was only reversible after incubation with chelating agents. Whereas it would seem that the channel should be permanently blocked in seawater, which contains 9.8 mM Mg\(^{2+}\), this doesn’t appear to be the case (Fig. 7B). This apparent discrepancy could reflect complex interaction between magnesium, calcium, and sodium ions in regulation of the SGC channel. Indeed, preliminary data demonstrate that the constant for magnesium inhibition in-

**FIG. 2.** Intracellular Ca\(^{2+}\) shifts the sodium-activation curve for SGC channels to lower sodium concentrations. A: Ca\(^{2+}\)-sensitive SGC channel activity in the presence of different [Na\(^{+}\)] and either 10 nM free calcium or saturating calcium (100 μM; B). ---, [Na\(^{+}\)]; - - -, [Ca\(^{2+}\)]. C: dependence of SGC channel open probability on [Na\(^{+}\)], in the presence of 10 nM (●) and 100 μM (○) Ca\(^{2+}\). ---, correspond to Hill-equation fit with the following parameters: [Na\(^{+}\)]\(_{1/2}\) = 113.4 ± 8.8 mM, h = 4.6 ± 1.6 (n = 3–5) for 10 nM Ca\(^{2+}\) and [Na\(^{+}\)]\(_{1/2}\) = 32.3 ± 14.6 mM, h = 0.92 ± 0.39 (n = 3–6) for 100 μM Ca\(^{2+}\). Open probability was estimated assuming P\(_o\) = I/Io. Experimental conditions: inside-out patch recordings; HP, –70 mV; electrode solution, 210 mM NaCl + 10 mM Ca\(^{2+}\) free.
creases with an increase in sodium and/or calcium concentration (data not shown). In our experiments, magnesium blockade occurred in the virtual absence of Ca\textsuperscript{2+}/H\textsubscript{11001} in the presence of 50\textmu M EGTA.

In addition to their common kinetic and pharmacological properties, both Ca\textsuperscript{2+}/H\textsubscript{11001}-sensitive and -insensitive SGC channels showed high sensitivity to pH. Decreasing internal pH from 7.4 to 5.7–6.0 reversibly inhibited SGC channels of both types without changing the current amplitude (n = 4, 15; Fig. 4A).

Finally, both Ca\textsuperscript{2+}-sensitive and -insensitive SGC channels were modulated by phosphoinositides. PIP3 (8.3\textmu M, n = 2, 3) activated channels with a P\textsubscript{open} of 0.5–0.6 (Fig. 4B) as did PIP2 (6\textmu M, n = 2, 3) (data not shown). Both ligands activated the SGC channels even in the absence of sodium (Zhainazarov and Ache 1999).

Ca\textsuperscript{2+} regulates activation of the SGC channel at physiological concentrations

The open probability of Ca\textsuperscript{2+}-sensitive SGC channels activated by Na\textsuperscript{+} has a bell-shaped dependence on [Ca\textsuperscript{2+}], (Fig. 5). As shown for a typical instance (holding potential,
–60 mV; symmetrical 210 mM NaCl with 10 nM [Ca\(^{2+}\)], the channel is activated between 100 nM and 100 μM Ca\(^{2+}\), whereas ≥3 mM Ca\(^{2+}\) inhibits the channel. Above 500 μM [Ca\(^{2+}\)], calcium simultaneously reduces the current amplitude (Fig. 5, A, B, and D). The Hill equation fit to the ascending phase of the concentration-response function gives an estimated half-maximal concentration, [Ca\(^{2+}\)]\(_{1/2}\), of 489 nM and a Hill coefficient of 1.25 (Fig. 5D). The dwell-time distributions for the channel in the open state could be described by a single-exponential probability distribution function with τ\(_{c1}\) growing from 15 ms at 10 nM to 330 ms at 350 μM [Ca\(^{2+}\)] (Fig. 5C). The dwell-time distribution for the closed state reflects two closed states with τ\(_{c1}\) ~ 17 ms and τ\(_{c2}\) < 1 ms. The slower component of the dwell-time distribution disappeared at saturating [Ca\(^{2+}\)] (Fig. 5C). The number of open and closed states are most likely underestimated due to the very brief and possibly extremely long life times in particular states. The reduction of the single channel current amplitude at higher [Ca\(^{2+}\)] (0.5–5 mM) was accompanied by significant increase in the channel current noise. The rms noise values (estimated for current recordings low-pass filtered at 5 kHz and sampled at 20 kHz) were 0.7 (10 nM Ca\(^{2+}\)), 1.075 (350 μM Ca\(^{2+}\)), and 1.53 (5 mM Ca\(^{2+}\)).

**Effect of Ca\(^{2+}\) on the SGC channel is voltage dependent and acts on the channel from the intracellular face.**

Exposing inside-out patches containing multiple SGC channels (Fig. 6A) to a voltage-ramp protocol showed that in all patches tested (n = 75) the calcium effect was voltage dependent (Fig. 6, B and C). In the presence of 100 μM Ca\(^{2+}\), the integral current exhibited so-called double-rectification characteristic or voltage-dependent biphasic inhibition. In a typical case, the conductance of membrane patches (calculated for average voltage-current characteristics) containing the SGC channel shows similar sensitivity to [H\(^+\)], pH 5.9 completely and reversibly blocked the channels (n = 7). Inset: dependence of Ca\(^{2+}\)-insensitive SGC channel open probability on [H\(^+\)]. — approximation by the Hill equation: P\(_o\) = 1 − [H\(^+\)]\(^h\)/[H\(^+\)]\(^h\) + [H\(^+\)]\(_0\)); h = 4.9 ± 1, [H\(^+\)]\(_0\) = 5.02e-8 ± 2.22e-9 (corresponding to pH 7.29; n = 4). The open probabilities were obtained using equation P\(_o\) = I/I\(_{Ni}\). B: phosphatidylinositol tris-3,4,5-phosphate (PIP3; 5.8 μM) activates Ca\(^{2+}\)-sensitive SGC channels without sodium. The application of testing solutions is noted on the line above the current trace. Experimental conditions: A and B, inside-out patches; HP, −70 mV; electrode (extracellular) solution, 210 mM NaCl + 10 nM [Ca\(^{2+}\)]. Inset: A different pH solutions contained 210 mM NaCl, 5 mM 2-N-morpholinino ethanesulfonic acid (MES), 5 mM HEPES and no added Ca\(^{2+}\)/EGTA. B: abbreviations used for superfusion solutions are LiCl, 210 mM LiCl + 10 mM Ca\(^{2+}\); NaCl, 210 mM NaCl + 10 mM Ca\(^{2+}\); NaCl + CaCl\(_2\), 210 mM NaCl + 100 μM CaCl\(_2\).
along with NaCl 210 mM in the electrode in outside-out patch recordings prevented activation of the channel by increasing extracellular calcium from 10 nM to 100 μM Ca^{2+}. Dwell-time histograms were generated from single-channel current traces of 6–20 s using logarithmic transformation of dwell-times (Sigworth and Sine 1987). To estimate τ_c and τ_o, the time distribution histograms were fit using exponential probability distribution functions and the maximum likelihood method. Closed and open time intervals were determined using half-amplitude threshold detection under visual inspection. All manipulations were performed with PSTAT software (Axon Instrument). D: dependence of open probability and single-channel current amplitude from [Ca^{2+}]. Data shown were obtained from 2 single-channel recordings. Current amplitudes are Gaussian fit to parameters (mean) of appropriate all-points amplitude histograms. —, result of Hill equation $P([Ca^{2+}]) = P_0 + (P_{max} - P_0) \frac{[Ca^{2+}]}{[Ca^{2+}]_{1/2} + [Ca^{2+}]^{b}}$ approximation of ascending phase of the dependence with following parameters: $P_0 = 0.4$, $[Ca^{2+}]_{1/2} = 489$ nM, $b = 1.25$. Where $P_0$ is basal open probability of the channel in the presence 210 mM NaCl only (nominal Ca^{2+}-free solution), $[Ca^{2+}]_{1/2}$ is half-effect calcium concentration; $b$ is Hill coefficient. Ca^{2+} concentrations exceeding 1 mM suppress channel activity. Time scales for each recording (B) correspond to time scale presented in A. Data were filtered at 2 kHz and reduced 10-fold. Electrode solution contained 210 mM NaCl, 1 mM EGTA, 1 mM EDTA. HP was –60mV.

Ca^{2+}-sensitive SGC channels predominate in cultured cells possessing these channels and in the outer dendrites of the cell in vivo

Multi-channel membrane patch recordings from the subset of ORNs containing Ca^{2+}-sensitive SGC channels almost always
demonstrated “stepwise” sensitivity to Ca\(^{2+}\) (Fig. 8), suggesting that the large majority of the channels either were sensitive to Ca\(^{2+}\) with the same activation parameters found in single-channel recording or were completely insensitive to Ca\(^{2+}\), i.e., could not be activated even by saturating concentrations (10–100 \(\mu M\)). Based on 140 ORNs of the morphological type (Fig. 1B) containing Ca\(^{2+}\)-sensitive SGC channels, we estimate no more than 20% of the SGC channels in a patch of membrane were of the Ca\(^{2+}\)-insensitive form and, in some instances, none. In only eight instances did we find multi-channel patches with intermediate Ca\(^{2+}\) sensitivity, i.e., patches in which 100 \(\mu M\) Ca\(^{2+}\) increased the open probability only to 0.5–0.6. We never found a single SGC channel with intermediate Ca\(^{2+}\) sensitivity. We conclude, therefore that the cells possess essentially a homogeneous population of Ca\(^{2+}\)-sensitive SGC channels and that this type of the channel predominates in the cells that possess it. Thus in all cases in which Ca\(^{2+}\) modulated activity of the SGC channels, the patches were obtained from cell type presented on Fig. 1D, while in all cases in which Ca\(^{2+}\) did not modulate activity of the SGC channels patches were obtained from cell type presented on Fig. 1D.

To determine the possible Ca\(^{2+}\) sensitivity of the SGC channel in lobster ORNs in vivo, we recorded from the SGC channel in inside-out patches obtained from outer dendrite vesicles (Hatt and Ache 1994) prepared from lobster ORNs in vivo. Ten to 100 \(\mu M\) Ca\(^{2+}\) increased the open probability of the SGC channel in 9 of 13 patches (i.e., 70%, quantitatively and kinetically similar to the cultured lobster ORNs; Fig. 8C), suggesting that the Ca\(^{2+}\)-sensitive form of the SGC channel plays a role in olfactory transduction. Transduction generally is assumed to occur in the outer dendritic compartment of these neurons.

Ca\(^{2+}\) sensitivity of the SGC channel appears to be regulated by phosphorylation

To begin to determine whether the difference in Ca\(^{2+}\)-sensitivity in the population of SGC channels is induced or inherent, we explored whether we could induce Ca\(^{2+}\) sensitiv-
Ca$^{2+}$-sensitivity of the SGC channels ($n = 3, 4$ data not shown). To determine if the lack of an effect of calmodulin could be explained by the persistent association of another, Ca$^{2+}$/calmodulin blockers are known to have nonspecific effects on ion channels, but the sensitivity of the SGC channel to them (Zhainazarov et al. 1998) suggests the potential involvement of Ca$^{2+}$/calmodulin or some other calcium-binding protein on the Ca$^{2+}$sensitivity of the channel. Exogenous calmodulin applied from intracellular side of membrane patch, however, did not modulate the Ca$^{2+}$-sensitivity of the SGC channel.}

FIG. 7. A: Ca$^{2+}$ activation of a Ca$^{2+}$-sensitive SGC channel in an outside-out patch. B: dependence of the open probability ($P_{\text{open}}$) and single-channel amplitude ($I_{\text{single}}$) on [Ca$^{2+}$]. $P_{\text{open}}$ values were calculated for 3 or 8 s intervals from 2 patches. Electrode solution: NaCl 210 mM +10 nM Ca$^{2+}$ free. Single $I_{\text{single}}$ corresponds to $P_{\text{open}}$ value obtained in the presence of 5 mM EGTA in the electrode solution with no calcium added to prevent activation of the channel by increasing extracellular calcium from 10 nM to 1 mM in the outside-out patch (0.52 ± 0.04, $n = 4$). C: SGC channel activity recorded under physiologically relevant ion conditions depends on [Ca$^{2+}$], and/or divalent cation chelator concentration. Inside-out multi-channel patch recording was obtained from an ORN possessing predominantly Ca$^{2+}$-sensitive SGC channels. All-points amplitude histograms were generated from current trace segments (as indicated by lines with symbols under trace), of 18 s, current range: −30 to 0 pA, step-size: 0.1 pA. Horizontal lines across the current trace correspond to different channel levels. Single-channel amplitude did not change in different conditions, mean of single SGC channel amplitude $-1.64 ± 0.04$ pA. HP in all cases was −70 mV.

FIG. 8. Typical multi-channel recording obtained from subset of cultured ORNs (Fig. 1B; A and B) and outer dendrite vesicles (C). SGC channel activity in the presence of different [Ca$^{2+}$] ($A$) and calcium dependency of SGC channels open probability determined for multi-channel patch recordings ($B$). Slight divergence between parameters of calcium dependence derived from single-channel recordings and patches containing 5–20 SGC channels can be explained by the simultaneous presence in the same patch of Ca$^{2+}$-sensitive and -insensitive channels. In A, the patch consisted of 10 SGC channels (9 Ca$^{2+}$-sensitive, 1 Ca$^{2+}$-insensitive channel). Hill equation (solid line in B) gives the following estimations: [Ca$^{2+}$]$^{1/2} = 704.7 ± 20.3$ nM, $h = 1.35 ± 0.07$ ($n = 4$). C: Ca$^{2+}$ activates SGC channels in inside-out patch obtained from outer dendrite vesicle. Current traces were filtered at 1 kHz and reduced 10-fold. Experimental conditions: inside-out patch recording; HP: −70mV (A and B); −80 mV (C); electrode solution: 210 mM NaCl + 10 nM Ca$^{2+}$ free. Note different current and time scale in $A$ and $C$. 

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endogenous Ca$^{2+}$-binding factor with the channel (e.g., Hac-Kos and Korenbrot 1997), we superfused the patch with a solution containing a high concentration of divalent cation chelators (5 mM EGTA + 5 mM EDTA, 2–4 min). This treatment, however, did not change the sensitivity of either the Ca$^{2+}$-sensitive or the Ca$^{2+}$-insensitive form of the SGC channel ($n = 6, 7$). We conclude, therefore, that the presence/absence of a suitable calcium binding protein did not underlie the differential Ca$^{2+}$ sensitivity of the SGC channel.

As phosphorylation/dephosphorylation is a known regulator of channel properties in different types of cells (Davis et al. 2001; Herzig and Neumann 2000), including other ORNs (Kroner et al. 1996; Muller et al. 1998; Wetzel et al. 2001), we explored if the observed difference in Ca$^{2+}$ sensitivity of the channel is induced by phosphorylation of the SGC channel or a tightly associated protein using particularly the nonspecific protein phosphatase activator, protamine (1–5 μg/ml) (Herzig and Neumann 2000). Applying protamine to the internal face of membrane patches containing Ca$^{2+}$-sensitive channels produced an irreversible loss in channel sensitivity to Ca$^{2+}$ in 10 of 17 patches tested (Fig. 9, A and E). After incubation with protamine, the SGC channels in 2 of the 17 patches could be activated in the presence of Na$^{+}$ and Ca$^{2+}$. In 3 of the 17 patches, incubation with protamine led to the loss of calcium sensitivity in some SGC channels while at the same time other SGC channels continued to stay active ($P_{\text{open}}$, 0.3–0.4), even after replacement 210 mM NaCl with 210 mM LiCl. In 2 of the 17 patches, incubation with protamine had no obvious effect. At concentrations in excess of 20–50 μg/ml, protamine blocked channel activity completely and was only partially reversible in all channels of both types (7 Ca$^{2+}$-sensitive, 9 Ca$^{2+}$-insensitive SGC channels), suggesting that the drug could have been acting nonspecifically (data not shown). We presume, however, that protamine was not acting nonspecifically at lower concentrations since 1–5 μg/ml protamine had no noticeable effect on Ca$^{2+}$-insensitive SGC channels in six of the patches in which it had effects on Ca$^{2+}$-sensitive SGC channel activity in equivalent conditions.

If the Ca$^{2+}$-sensitivity was induced by phosphorylation of the SGC channel or a tightly associated protein, the nonspecific protein phosphatase blocker, okadaic acid (OA) (e.g., Herzig and Neumann 2000) should block the effect of protamine. Pretreatment of membrane patches with OA (2 μM) could significantly slow the effect of protamine in all six patches.
tested (Fig. 9, B and E). If protamine and OA were targeting a phosphatase leading to dephosphorylation of a membrane protein/s, the conditions potentially providing phosphorylation should be able to restore the sensitivity of the channel to Ca$^{2+}$. The catalytic subunit of PKA (1,000 units/ml) in complex with 1 mM Mg-ATP restored the sensitivity of the channel to Ca$^{2+}$ in all four patches tested (Fig. 9, C and E). Mg-ATP (0.5–1 mM, n = 4, 16) or PKA (1,000 units/ml, n = 2, 22) itself did not occur any noticeable effect on SGC channel activity. As would be predicted from the preceding results, OA (2 μM) in combination with the catalytic subunit of PKA and Mg-ATP abolished the effect of protamine in all four patches tested (Fig. 9, D and E). Collectively, these findings are consistent with the interpretation that phosphorylation induces Ca$^{2+}$ sensitivity of the SGC channel, although attempts to impose Ca$^{2+}$ sensitivity on Ca$^{2+}$-insensitive SGC channels by incubating inside-out patches containing Ca$^{2+}$-insensitive SGC channels with the catalytic subunit of PKA and ATP failed to rescue the Ca$^{2+}$ sensitivity (n = 6 patches, data not shown).

**DISCUSSION**

We assume that both the calcium-sensitive and the calcium-insensitive SGC channels are variants of the same ion channel. 1. In both instances, intracellular Na$^{+}$ is required to activate the channels: for the Ca$^{2+}$-insensitive SGC channel [Na$^{+}$]$_{i/2}$ = 120.4 ± 12.1 mM and h = 5.1 ± 2.2 (n = 6), while for the Ca$^{2+}$-sensitive SGC channel [Na$^{+}$]$_{i/2}$ = 113.4 ± 8.8 mM and h = 4.6 ± 1.6 (n = 3–5) in equivalent conditions (free [Ca$^{2+}$], ~10 nM). 2. In both instances the channels have similar single-channel current amplitudes that deviate from linear voltage dependence at positive voltages, voltage-dependent single-channel gating parameters, exhibit subconductance states, and have similar ion selectivity at least for monovalent cations. 3. In both instances, the channels are extremely sensitive to pH: for the Ca$^{2+}$-insensitive SGC channel the inhibition coefficient (pH$_{1/2}$) = 7.29 and h = 4.9 ± 1 (n = 4), while for the Ca$^{2+}$-sensitive SGC channel pH$_{1/2}$ = 7.31 and h = 5.1 ± 1.4 (n = 6) under equivalent conditions in the absence of divalent cations. 4. In both instances, the channels are activated by phosphoinositides. There was no significant difference (P < 0.05) in comparable parameters. The similar gating parameters and pharmacological profile do not necessarily reflect molecular identity, and channels with different Ca$^{2+}$ sensitivity could reflect different gene products or splice forms of the same gene. Nonetheless, the fact that the Ca$^{2+}$-sensitive SGC channel could be reversibly converted to the Ca$^{2+}$-insensitive mode by manipulating the phosphorylation state of the channel argues strongly that the channels indeed are the same and that the Ca$^{2+}$ sensitivity of the channel is determined by the phosphorylation state of the channel and/or protein/s tightly associated with the channel.

Earlier, we reported that Ca$^{2+}$ had a different effect on the SGC channel than the one we report here in that 1 mM intracellular Cu$^{2+}$ almost completely blocked SGC channel activity (Zhainazarov and Ache 1995). It is becoming clear that in native conditions virtually all ion channels function as molecular clusters with functionally different integral membrane and peripheral proteins (Bauman and Scott 2002; Davis et al. 2002; Huber 2001; Ratcliffe et al. 2000). The multiple phosphorylation sites inherent in these molecular complexes allow the channel to be under the complex control of several types of protein kinases and/or protein phosphatases at the same time (Davis et al. 2001; Herzig and Neumann 2000). Multiple phosphorylation sites on the channel and/or on associated proteins in the present instance could explain not only the diverse effects of the nonspecific activation of phosphatases by protamine seen in some experiments (loss of both Na$^{+}$ and Ca$^{2+}$ sensitivity or only Na$^{+}$ sensitivity), and the inability to completely recover the native properties of the channel by cAMP/PKA mediated phosphorylation but also how the SGC channel could show opposite Ca$^{2+}$ sensitivity (i.e., inhibition vs. activation) in different experimental conditions. However, we are as yet unable to identify the specific experimental conditions under which we can show inhibition of the SGC channel by Ca$^{2+}$ or convert either the Ca$^{2+}$-sensitive or the Ca$^{2+}$-insensitive form of the SGC channel to the Ca$^{2+}$-inhibited form.

Phosphorylation is known to control the Ca$^{2+}$ sensitivity of other ion channels (Dzhura et al. 2000; Fuller et al. 1994; Ling et al. 2000; Reinhart et al. 1991; Wilson et al. 1998). In at least some instances, phosphorylation-dependent control of Ca$^{2+}$ sensitivity occurs in channels incorporated in lipid bilayers (Fuller et al. 1994), suggesting that the channel itself is phosphorylated. Although we were unable to induce Ca$^{2+}$ sensitivity by treating the Ca$^{2+}$-insensitive SGC channel with the catalytic subunit of PKA and MgATP (n = 4), this does not necessarily exclude that the SGC channel itself is phosphorylated because the specific lipid environment is known to influence phosphorylation of neuronal Ca$^{2+}$ channels (Lu et al. 2002). Interestingly, phosphorylation can, in turn, change the Ca$^{2+}$ sensitivity of ion channels to their lipid environment; PKA-mediated phosphorylation of ROMK1 channel increases the sensitivity of the channel to activation by PIP2 (Liou et al. 1999). Although we did not systematically analyze possible differential sensitivity of the SGC channel to phosphoinositides when phosphorylated, PIP3 increased the activity of the Ca$^{2+}$-blocked SGC channel (n$_{P_{O}}$ = 0.52, where n is the number of channels and P o is the open probability of the channel, corresponding to a P o ~ 0.26 with a minimum of 2 channels per patch). In comparison, PIP3 typically activated the Ca$^{2+}$-activated SGC channel to P o ~ 0.54 (Fig. 4B). While this apparent difference in activation of the SGC channel has several possible explanations, it is consistent with the idea that the differential sensitivity of the SCG channel to phosphoinositides is phosphorylation-dependent.

The apparent decrease of the unitary current we observed at higher [Ca$^{2+}$] is a common property of many nonselective cation channels that is conventionally interpreted in terms of the Woodhull model of fast channel block (Woodhull 1973) as done in earlier efforts in our lab to characterize interaction of this channel with cations (McCclintock and Ache 1990; Zhainazarov and Ache 1997). This interpretation is consistent with the increased open-channel noise we observed and the voltage dependence of the blocking effects of divalent cations. Given that we can reliably resolve channel substates (Fig. 3, A and B*), the decrease of single-channel amplitude potentially could reflect different substates of the channel and a tendency of the channel to stay in the low conductance state in the presence of high [Ca$^{2+}$]. Due to the very brief transitions between substates, such differences in channel conductance...
levels would only be seen occasionally at higher time resolution and obviously would not be reflected in the amplitude histogram. Confirmation of this alternate interpretation, however, would require further experimentation.

The lobster SGC channel has yet to be cloned and sequenced, but the functional properties of the channel are consistent with the hypothesis that the lobster SGC channel is a member of the growing family of TRP channels. 1) Both the SGC channel and TRP channels have similar ion selectivity and are differentially sensitive to Ca$^{2+}$ (Harteneck et al. 2000; Minke and Cook 2002). 2) Both the SGC channel and some members of the TRP family show distinctive double-rectification in their current-voltage relationship (Jung et al. 2002, 2003; Runnels et al. 2002). 3) Like most members of the TRP family (Benham et al. 2002; Hardie 2003; Minke and Cook 2002), the SGC channel is associated with the phosphoinositide signaling pathway.

Finding that the Ca$^{2+}$-sensitive form of the SGC channel is predominantly expressed in the outer dendrites (i.e., the transduction compartment) of lobster ORNs in vivo suggests that the Ca$^{2+}$ sensitivity of the channel, and possibly its regulation by phosphorylation, play a role in olfactory transduction. The Ca$^{2+}$ sensitivity of the channel provides a potential link to the canonical phosphoinositide turnover pathway and its target InsP$_3$R in these cells (Munger et al. 2001). Activation of the InsP$_3$R by odorants would be expected to increase [Ca$^{2+}$]i. Increased [Ca$^{2+}$]i presumably would activate the SGC channel and potentiate recurrent activation of the channel by permeant Na$^+$ as a result of the Ca$^{2+}$-dependent left-shift of the Na$^+$ concentration-response function. This scenario would be consistent with the proposed role of the lobster SGC channel in signal amplification (Zhaianazarov et al. 2001). Modulating Ca$^{2+}$-dependent facilitation of activation by controlling the extent of phosphorylation of the SGC would provide a potentially powerful mechanism to regulate the excitability of the cell, either in relation to longer-term adaptation or possibly short-term, dynamically fast adjustments in odorant sensitivity.

If, as suggested, the Ca$^{2+}$ sensitivity of the channel provides a potential link to the canonical phosphoinositide turnover pathway, the fact that the channel can also be modulated by exogenous 3-phosphoinositides (Zhaianazarov et al. 2001) suggests that the channel could be a common target for both arms of the phosphoinositide signaling pathway in lobster ORNs. The functional significance of having dual phosphoinositide signaling-dependent regulation of a common output channel in olfactory transduction remains to be explored, but could be of general relevance to other systems in light of the possibility that the lobster SGC is a TRP-related ion channel.

DISCLOSURES

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REFERENCES


