Calcium sensitivity of a sodium-activated non-selective cation channel in lobster olfactory receptor neurons

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Abstract

We report that a Na\(^+\)-activated non-selective cation channel described previously in lobster olfactory neurons (Zhainazarov et al. 1998), in which phosphoinositide signaling mediates olfactory transduction, can also be activated by Ca\(^{2+}\). Ca\(^{2+}\) activates the channel in the presence of Na\(^+\), increasing the open probability of the channel with a $K_{1/2}$ of 490 nM and a Hill coefficient of 1.3. Ca\(^{2+}\) also increases the sensitivity of the channel to Na\(^+\). In some cells the same channel is Ca\(^{2+}\)-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 Ca\(^{2+}\). This effect can be blocked by okadaic acid, a nonspecific blocker of protein phosphatases, and restored by the catalytic subunit of PKA in the presence of MgATP. The Ca\(^{2+}\)-sensitive form of the channel is predominantly expressed in the transduction zone of the cells in situ. These findings imply that the Ca\(^{2+}\) 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 in both arthropods and mammals (Zhainazarov et al. 2001; Spehr et al. 2002) suggests that the role of phosphoinositide signaling may be more complex than initially appreciated, and that to understand the potential involvement of phosphoinositide signaling in olfactory transduction it will be necessary to better understand the relationship of the 3-phosphoinositide pathway to the canonical phosphoinositide turnover pathway, as well as to identify the downstream target(s) of these pathways in ORNs.

The participation of phosphoinositide signaling in olfactory transduction is perhaps best established in lobster ORNs, where the canonical phosphoinositide turnover pathway mediates excitation (Fadool and Ache 1992). Odors elevate IP3 in lobster olfactory outer dendritic membranes in vitro (Boekhoff et al. 1994) and blocking PLC blocks depolarizing receptor potentials in the cells in vivo (RE Doolin and BW Ache, unpublished results). An IP3R has been cloned from lobster ORNs (Munger et al. 2000) and an IP3-activated channel has been localized to the outer dendrites (Hatt and Ache 1994). Finally, lobster ORNs express a sodium-gated non-selective cation (SGC) channel (Zhainazarov and Ache 1997; Zhainazarov et al. 1998) that shares at least some properties with the growing 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 lobster olfactory transduction. In order to determine if and how the SGC channel could serve as a functional link between canonical phosphoinositide signaling and 3-phosphoinositide signaling in lobster ORNs, and to better understand the downstream target(s) of phosphoinositide signaling in general, we explored the potential Ca$^{2+}$-sensitivity of the lobster SGC channel.

We report that Ca$^{2+}$ 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 Ca$^{2+}$ sensitivity of the channel. Given that the Ca$^{2+}$-sensitive form of the channel is predominantly expressed in the outer dendrites of lobster ORNs \textit{in situ}, the Ca$^{2+}$ sensitivity of the channel, and possibly its regulation by phosphorylation, presumably plays a role in olfactory transduction.

\textbf{Materials and Methods}

\textit{Cell preparations}

SGC channels were studied in two different preparations of lobster (\textit{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 1-7 days. For other experiments, freshly isolated vesicles of outer dendritic membrane were obtained by incubating the olfactory organ for 10-20 min in a solution containing (mM): 210 NaCl,
696 glucose, 10 Hepes, 0.1 CaCl₂, 1 EGTA buffered to a free calcium concentration of approximately 10nM, 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), lowpass 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, Inc.) and SigmaPlot 5.0 (SPSS, Inc). 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 Co., BF150-86-10) using a Flaming-Brown micropipette puller, Model-P-87 (Sutter Instrument Co.). 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 50ms 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 less than 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-test was used to evaluate differences between two means. P<0.05 was considered to indicate significance. The data are presented as the mean ± SE of n observations. All recordings were performed at room temperature (~21°C).

**Solutions**

Panulirus saline (PS) contained (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 PS was replaced with equimolar NaCl. Low-calcium sodium solution contained (mM): 210 NaCl, 1 EGTA, 0.1 CaCl₂, 696 glucose, and 10 HEPES, pH 7.4 adjusted with Tris-Base. Low-calcium lithium solution consisted of (mM): 210 LiCl, 1 EGTA, 0.1 CaCl₂, 696 glucose, and 10 HEPES, pH 7.4 adjusted with Tris-Base. Solutions of different sodium concentrations were prepared by appropriate substitution of [Li⁺] for [Na⁺] as noted. The estimated free calcium concentration ([Ca²⁺]_{free}) in low-calcium sodium/lithium solutions was ~10 nM. Solutions containing more than 10 µM Ca²⁺/Mg²⁺ were prepared without chelating agents. Divalent-cation free solutions consisted of 1-5mM EGTA/1-5mM EDTA and no added Ca²⁺/Mg²⁺. Solutions below pH 7 (adjusted with 1N HCl or Tris-HCl) contained 5mM MES (2-(N-Morpholino ethanesulfonic acid) and 5mM HEPES. Stocks of phosphatidylinositol bis-4,5-phosphate (PIP2, 585 µM) and phosphatidylinositol tris-3,4,5-phosphate (PIP3, 415 µM) were prepared by dispersing the phosphoinositides in distilled water with 30 min sonication on ice, aliquoted, and stored at -20°C for use within 3 d. Stock solutions were diluted to the
working concentration indicated and sonicated for an additional 30 min on ice immediately before use.

All inorganic salts were purchased from Fisher Scientific, except for AlCl$_3$ and LaCl$_3$, 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, Inc.

Results

_Intracellular Ca$^{2+}$ 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 Ca$^{2+}$ sensitivity of the lobster SGC channel in cell-free patches. We found that Ca$^{2+}$ 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, since, 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 [Ca$^{2+}$], augmented channel open probability ($P_{\text{open}}$) from 0.37 in the presence of 210 mM Na$^+$ and 10 nM Ca$^{2+}_{\text{free}}$ to 0.95 in the presence of 210 mM Na$^+$ and 100 µM Ca$^{2+}$. 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 Ca$^{2+}_{\text{free}}$ was elevated from 10 nM to 100 µM (Fig. 2). Ca$^{2+}$ was never observed to activate
the channel directly in the absence of Na\(^+\) (segments of current recordings marked by asterisks in Fig. 1A,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 Ca\(^{2+}\) sensitivity of the SGC channel nor the kinetics of the Ca\(^{2+}\)-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, Ca\(^{2+}\) had no measurable effect on channel activity (Fig. 1C). In all cases in which Ca\(^{2+}\) 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\(\mu\)m 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 Ca\(^{2+}\)-insensitive (Fig. 1B 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 [Ca\(^{2+}\)] also augments SGC channel activity by increasing the sensitivity of the channel to Na\(^+\) (Fig. 2A vs B). Measuring the dose-response relation of SGC channel activity to [Na\(^+\)] at various cytoplasmic Ca\(^{2+}\) concentrations showed that Ca\(^{2+}\) 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 10nM Ca\(^{2+}\) to 32.3±14.6 mM at 100µM Ca\(^{2+}\), with corresponding Hill coefficients of 4.6±1 at 10nM Ca\(^{2+}\) and 0.92±0.39 at 100µM Ca\(^{2+}\) (Fig. 2C).

**Ca\(^{2+}\)**-**sensitive and Ca\(^{2+}\)**-**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 Ca\(^{2+}\)-insensitive SGC channels had identical sensitivity to cytoplasmic Na\(^{+}\) (Fig. 3A 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. 3C,E vs D,G), and similar voltage dependence of the open probability (Fig. 3C,F vs D,H). In addition to having the same kinetic properties, both Ca\(^{2+}\)-sensitive and Ca\(^{2+}\)-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 (200µM, n=3, 7 - indicate numbers of experiments for Ca\(^{2+}\)-insensitive channels and Ca\(^{2+}\)-sensitive channels, respectively), TFP (200µM, n= 8, 21), calmidozolium (100µM, n= 3, 4), and Mastoparan (5.6µM, n= 4, 3) (data not shown). Both could also be fully and reversibly blocked from the extracellular and intracellular sides by high Mg\(^{2+}\) (3-5mM only, n= 3,14), La\(^{3+}\) (10µM, n=3, 32), and Al\(^{3+}\) (10µM, n= 1, 2) (data not shown). Blockade with trivalent cations was only reversible following incubation with chelating agents. While 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 increases with an increase in sodium and/or calcium concentration (data not shown). In our experiments, magnesium blockade occurred in the virtual absence of Ca\(^{2+}\) in the presence of 50\(\mu\)M EGTA.

In addition to their common kinetic and pharmacological properties, both Ca\(^{2+}\)-sensitive and Ca\(^{2+}\)-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\(^{2+}\)-sensitive and Ca\(^{2+}\)-insensitive SGC channels were modulated by phosphoinositides. PIP3 (8.3\(\mu\)M, n= 2,3) activated channels with a \(P_{open}\) of 0.5-0.6 (Fig. 4B), as did PIP2 (6 \(\mu\)M, n= 2,3) (data not shown). Both ligands activated the SGC channels even in the absence of sodium (Zhainazarov and Ache 1999).

**Ca\(^{2+}\) regulates activation of the SGC channel at physiological concentrations**

The open probability of Ca\(^{2+}\)-sensitive SGC channels activated by Na\(^+\) has a bell-shaped dependence on [Ca\(^{2+}\)]\(_i\) (Fig. 5). As shown for a typical instance (holding potential, -60mV; symmetrical NaCl 210mM with 10nM [Ca\(^{2+}\)]\(_o\)), the channel is activated between 100nM and 100 \(\mu\)M Ca\(^{2+}\), whereas 3mM Ca\(^{2+}\) and higher inhibits the channel. Above 500 \(\mu\)M [Ca\(^{2+}\)]\(_i\), calcium simultaneously reduces the current amplitude (Fig. 5A,B,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 489nM 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 \(\tau_o\)
growing from 15ms at 10nM to 330ms at 350µM [Ca²⁺]i (Fig. 5C). The dwell-time
distribution for the closed state reflects two closed states with τ_c1~17ms and τ_c2 less
than 1ms. The slower component of the dwell-time distribution disappeared at saturating
[Ca²⁺] (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 [Ca²⁺] (0.5-5mM) was
accompanied by significant increase in the channel current noise. The rms noise values
(estimated for current recordings lowpass filtered at 5 KHz and sampled at 20 KHz) were
0.7 (10nM Ca²⁺), 1.075 (350µM Ca²⁺), 1.53 (5mM Ca²⁺).

*The effect of Ca²⁺ 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. 6B,C). In the presence of 100µM Ca²⁺, 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 channels decreased at
depolarizing voltages more positive than -40 mV (Fig. 6B,C). A detailed analysis of the
possible mechanism underlying the observed rectification was not pursued.

For SGC channels activated by 210mM NaCl + 10 nM Ca²⁺ free on the intracellular
face, increasing [Ca²⁺]o could also enhance activity of the channels in both single-
channel recordings (Fig. 7A,B) and 42 multi-channel patches (data not shown) recorded
in the outside-out configuration, suggesting that Ca²⁺ permeating the SGC channel
potentially could interact with Ca$^{2+}$-binding sites located close to the pore region of the channel. In this context, SGC channel activity was dependent on the Ca$^{2+}$ concentration and divalent buffering capacity of the intracellular solution. Having EGTA 5mM along with NaCl 210mM in the electrode in outside-out patch recordings prevented activation of the channel by increasing extracellular calcium from 10nM to 1µM ($n=4$, Fig. 7B). Also, in multi-channel patches in the inside-out configuration ($n=5$) with PS in the electrode, the mean steady-state current was (mean±SD): 12.6±3.2pA (70%) at NaCl210mM + Ca$^{2+}$$_{\text{free}}$10nM, 9.7±2.7pA (54%) at NaCl210mM + EGTA 2mM + EDTA 2mM, and 18.0±3.6pA (100%) at NaCl210mM + Ca$^{2+}$100µM (Fig. 7C). This example also provides evidence that Ca$^{2+}$ can participate in SGC channel regulation in near physiological conditions. As indicated by the all-points current amplitude histograms, the current noise observed in each ionic condition is determined by channel activity; the single channel amplitude was not changed by application of solutions with different chelator concentrations.

\textit{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 (10-100µM). 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 $\text{Ca}^{2+}$-insensitive form and, in some instances, none. In only eight instances did we find multi-channel patches with intermediate $\text{Ca}^{2+}$-sensitivity, i.e., patches in which $100\mu\text{M} \text{Ca}^{2+}$ increased the open probability only to 0.5-0.6. We never found a single SGC channel with intermediate $\text{Ca}^{2+}$-sensitivity. We conclude, therefore, that the cells possess essentially a homogeneous population of $\text{Ca}^{2+}$-sensitive SGC channels, and that this type of the channel predominates in the cells that possess it. Thus, in all cases in which $\text{Ca}^{2+}$ modulated activity of the SGC channels, the patches were obtained from cell type presented on Fig. 1B, while in all cases in which $\text{Ca}^{2+}$ did not modulate activity of the SGC channels patches were obtained from cell type presented on Fig. 1D.

To determine the possible $\text{Ca}^{2+}$ 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. 10-100$\mu\text{M} \text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$-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.

$\text{Ca}^{2+}$-sensitivity of the SGC channel appears to be regulated by phosphorylation

To begin to determine whether the difference in $\text{Ca}^{2+}$-sensitivity in the population of SGC channels is induced or inherent, we explored whether we could induce $\text{Ca}^{2+}$-sensitivity through the action of known regulators or post-translation modifiers of channel properties. $\text{Ca}^{2+}$-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 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, endogenous Ca$^{2+}$-binding factor with the channel (e.g., Hackos and Korenbrot 1997), we superfused the patch with a solution containing a high concentration divalent cation chelators (EGTA 5mM + EDTA 5mM, 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 (Herzig and Neumann 2000; Davis et al. 2001), 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. 9A, E). After incubation with protamine, the SGC channels in two of the 17 patches could be activated in the presence both Na$^{+}$ and Ca$^{2+}$. In three 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 NaCl 210mM with LiCl 210mM. In two of the 17 patches, incubation
with protamine had no obvious effect. At concentrations in excess of 20-50µg/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 1-5µg/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 (2µM) could significantly slow the effect of protamine in all six patches tested (Fig. 9B,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 (1000units/ml) in complex with 1mM Mg-ATP restored the sensitivity of the channel to Ca\(^{2+}\) in all four patches tested (Fig. 9C,E). Mg-ATP (0.5-1mM, n=4,16) or PKA (1000 units/ml, n=2,2) itself did not occur any noticeable effect on SGC channel activity. As would be predicted from the above results, OA (2µM) in combination with the catalytic subunit of PKA and Mg-ATP abolished the effect of protamine in all 4 patches tested (Fig. 9D,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 $[\text{Na}^+]_{1/2} = 120.4\pm12.1$ mM and $h=5.1\pm2.2$ (n=6), while for the Ca$^{2+}$-sensitive SGC channel $[\text{Na}^+]_{1/2} = 113.4\pm8.8$ mM and $h=4.6\pm1.6$ (n=3-5) in equivalent conditions (free [Ca$^{2+}$]$\sim 10$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 $h=4.9\pm1$ (n=4), while for the Ca$^{2+}$-sensitive SGC channel pH$_{1/2}=7.31$ and $h=5.1\pm1.4$ (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\textsuperscript{2+} had a different effect on the SGC channel than the one we report here in that 1mM intracellular Ca\textsuperscript{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 (Ratcliffe et al. 2000; Huber 2001; Bauman and Scott 2002; Davis et al. 2002). 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 (Herzig and Neumann 2000; Davis et al. 2001). 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\textsuperscript{+} and Ca\textsuperscript{2+} sensitivity or only Na\textsuperscript{+} 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\textsuperscript{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\textsuperscript{2+} or convert either the Ca\textsuperscript{2+}-sensitive or the Ca\textsuperscript{2+}-insensitive form of the SGC channel to the Ca\textsuperscript{2+}-inhibited form.

Phosphorylation is known to control the Ca\textsuperscript{2+}-sensitivity of other ion channels (Reinhart et al. 1991; Fuller et al. 1994; Wilson et al. 1998; Dzhura et al. 2000; Ling et al. 2000). In at least some instances, phosphorylation-dependent control of Ca\textsuperscript{2+}-sensitivity occurs in channels incorporated in lipid bilayers (Fuller et al. 1994), suggesting that the channel itself is phosphorylated. While we were unable to induce Ca\textsuperscript{2+}-sensitivity by treating the Ca\textsuperscript{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.
since 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 (NP\(_o\) = 0.52, where N = the number of channels and 
P\(_o\) = the open probability of the channel, corresponding to a P\(_o\)~0.26 with a minimum of 
two 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 
(McClintock 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 (asterisks, Fig. 3A,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 (Runnels et al. 2002; Jung et al. 2002, 2003). (3) Like most members of the TRP family (Benham et al. 2002; Minke and Cook 2002; Hardie 2003), the SGC channel is associated with the phosphoinositide signaling pathway. (4) Also, like some members of the TRP family (Liman et al. 1999; Perez et al. 2002), the SGC channel has been implicated in chemosensory transduction (Zhainazarov et al. 2001). (5) Finally, although there are no specific agonists or antagonists for TRP channels, pharmacological blockers generally used to characterize TRP channels (Minke and Cook 2002) also blocked the SGC channel, including full and reversible blockade by (10$\mu$M): La$^{3+}$, Gd$^{3+}$, SKF-96365 [1-2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl-1H-imidazole], 2-APB. We suggest, therefore, that the lobster SGC channel is a TRP-related ion channel.

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 IP3R in these cells (Munger et al. 2001). Activation of the IP3R by odorants would
be expected to increase $[\text{Ca}^{2+}]$. Increased $[\text{Ca}^{2+}]$ presumably would activate the SGC channel and potentiate recurrent activation of the channel by permeant $\text{Na}^+$ as a result of the $\text{Ca}^{2+}$-dependent left-shift of the $\text{Na}^+$ concentration-response function. This scenario would be consistent with the proposed role of the lobster SGC channel in signal amplification (Zhainazarov et al. 2001). Modulating $\text{Ca}^{2+}$-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 $\text{Ca}^{2+}$ sensitivity of the channel provides a potential link to the canonical phosphoinositide turnover pathway, the fact that the channel can also be modulated by exogeneous 3-phosphoinositides (Zhainazarov 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 SCG is a TRP-related ion channel.
Acknowledgements

This work was supported by grant DC01655 from the NIDCD. We thank Ms. Jocelyn Tulsian for preparation of the cultured cells, and Ms. Lynn Milstead for assistance with the illustrations.


**Figure legends**

Fig.1. Representatives of two different morphological subpopulations of lobster ORNs (B, D) expressing sodium-gated cation (SGC) channels with different sensitivity to intracellular calcium ([Ca$^{2+}$]). Ca$^{2+}$-sensitive (A) and Ca$^{2+}$-insensitive (C) SGC channel activity was recorded from inside-out membrane patches excised from both cell types. Patches in A, 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 asterisks). All-points amplitude histograms presented in (A) were generated for 12s segments of current trace (in range -120 to 0 pA, bin width – 0.1 pA) at 10 nM (gray squares) and 100 µM (white circles) Ca$^{2+}$. Time course of solution application is depicted by the bar under the current trace. Current traces were digitally filtered using Clampfit (Lowpass Bessel (8-pole) filtering at the –3 Db cutoff 1.2 kHz). Current trace in C was not filtered. Membrane holding potential (HP) was –70mV. Electrode solution – NaCl 210mM + Ca$^{2+}$ 10nM. Scale bars in B and D are 30 µm. Unless noted otherwise, LiCl210mM and NaCl210mM had 10nM [Ca$^{2+}$]free.

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 10nM free calcium or saturating calcium (100µM) (B). Solid lines, [Na$^+$]; dashed lines, [Ca$^{2+}$]. C- Dependence of SGC channel open probability on [Na$^+$], in the presence of 10 nM (filled circles) and 100 µM (empty circles) Ca$^{2+}$. Solid lines 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±14.6mM, h=0.92±0.39 (n=3-6) for 100µM Ca$^{2+}$. Open probability was estimated assuming $P_o=I/N_i$ Experimental
conditions: inside-out patch recordings; HP - –70mV; electrode solution - NaCl 210mM + Ca$^{2+}_{\text{free}}$ 10nM.

Fig.3. General features of single Ca$^{2+}$-insensitive (A, C, E, F) and and Ca$^{2+}$-sensitive (B, D, G, H) SGC channels. A, B - Ca$^{2+}$ effect on the single channel activity, HP - –60mV. C, D - Portions of single-channel recordings obtained at different potentials (indicated over current traces) under the identical control ion conditions: symmetrical NaCl 210mM + Ca$^{2+}$ 10nM. All-points amplitude histograms presented on the right of every recording were obtained using equivalent current range for all cases (bin width 0.05 pA). Amplitude distributions were fit by the sum of two Gaussian equations to determine single-channel current amplitude. E, G - Voltage dependence of single-channel current amplitudes. Amplitude values were obtained from the Gaussian distribution fitting the parameters. The linear approximations for mean values of single-channel current amplitudes in the voltage range from –70 to –20 mV correspond to slope conductances of 197±16 pS (n=5-12) for Ca$^{2+}$-insensitive channels (E) and 208±7 pS (n=5-21) for Ca$^{2+}$-sensitive channels (G). Lines were extrapolated up to 60mV to demonstrate a similar voltage dependence of single-channel amplitude in the positive voltage range for both channel types. F, H - Similar voltage dependences of the open probability $P_o$. Solid lines approximate a Boltzmann distribution with the following parameters: half-maximal amplitude $P_o(V)$ is at $V_{1/2}$=52.25±9.23, slope factor $t$=19.33±6.97 (n=3) for F and $V_{1/2}$=-66.4±26.7, $t$=18.6±10.75 (n=3) for H. Data presented were filtered at 1.2 kHz and reduced tenfold. A, C and B, D are recordings from the same patches, respectively. Current and time scales in A, B differ from C, D. Asterisks (C, D) mark subconductance levels.
Fig. 4. Other properties of the SGC channel. **A** – Ca$^{2+}$-insensitive SGC channel activity is reversibly inhibited by increasing [H$^+$]. Application of solutions of different pH is depicted by the line over the current trace. The Ca$^{2+}$-sensitive 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$^+$]. Solid line reflects approximation by the Hill equation: $P_o = 1 - ([H^+]^h/([H^+]^h + [H^+]_{1/2}^h))$. $h = 4.9 \pm 1$, $[H^+]_{1/2} = 5.02 \times 10^{-8} \pm 2.22 \times 10^{-9}$ (corresponding to pH 7.29) (n=4). The open probabilities were obtained using equation $P_o = I/Ni$. **B** – 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**– inside-out patches; HP – – 70mV; electrode (extracellular) solution – NaCl 210mM + 10nM [Ca$^{2+}$]$_{free}$. **A**– Different pH-solutions contained 210mM NaCl, 5mM MES, 5mM HEPES and no added Ca$^{2+}$/EGTA. **B** – Abbreviations used for superfusion solutions: LiCl = LiCl 210mM + Ca$^{2+}$ 10nM; NaCl = NaCl 210mM+Ca$^{2+}$ 10nM; NaCl + CaCl$_2$ = NaCl 210mM + CaCl$_2$ 100µM.

Fig. 5. Effect of [Ca$^{2+}$]$_i$ on single Ca$^{2+}$-sensitive SGC channel activity. General current trace (**A**) and portions of single-channel current recording (**B**) used for subsequent analysis. All-points amplitude histograms presented on the right of the recordings were fit by two Gaussian distributions. The analysis was corrected for changes in single-channel amplitude and current baseline shift at increasing [Ca$^{2+}$]. **C** – Distributions of open ($\tau_o$) and closed ($\tau_c$) times (for SGC channel shown in **A, B**) at 10nM and 350µM Ca$^{2+}$. Dwell-time histograms were generated from single-channel current traces of 6-20 sec using logarithmic transformation of dwell-times (Sigworth and Sine 1987). To estimate $\tau_o$ and $\tau_c$ the time distribution histograms were fit using exponential probability distribution function/s 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 Inc.). **D** - Dependence of open probability and single-channel current amplitude from [Ca$^{2+}$]. Data shown were obtained from two single-channel recordings. Current amplitudes are Gaussian fit to parameters (mean) of appropriate all-points amplitude histograms. Solid smooth line – result of Hill equation 

$$ P([Ca^{2+}]) = P_b + \frac{P_{\text{max}} h}{([Ca^{2+}]_{1/2}^h + [Ca^{2+}]^h)} $$

approximation of ascending phase of the dependence with following parameters: $P_b = 0.4$, $[Ca^{2+}]_{1/2} = 489\text{nM}$, $h = 1.25$. Where $P_b$ – basal open probability of the channel in the presence 210 mM NaCl only (nominal Ca$^{2+}$-free solution), $[Ca^{2+}]_{1/2}$ – half-effect calcium concentration, $h$ – Hill coefficient. Ca$^{2+}$ concentrations exceeding 1mM suppress channel activity. Time scales for each recording (B) correspond to time scale presented in A. Data were filtered at 2 kHz and reduced tenfold. Electrode solution contained NaCl 210 mM, EGTA 1mM, EDTA 1mM. HP was –60mV.

**Fig.6. Voltage-dependence of Ca$^{2+}$ effects on SGC channel.** A- Representative macro-patch recording demonstrating effect of Na$^+$ and Ca$^{2+}$ on SGC-channel activity in steady state conditions (HP –70mV). Bar diagram above current trace shows time course of solution application. B – Current-voltage characteristics obtained by voltage ramp series application from inside-out patch in the presence of different Ca$^{2+}$ concentration: in gray – superposition of 40 ramps generated in the presence of 10nM Ca$^{2+}$, in black – superposition of 40 ramps at the 100µM Ca$^{2+}$. Solid lines are average currents of a total number of ramps, black line for 10nM and gray line for 100µM Ca$^{2+}$. Every graph dot corresponds to single sampling value. Visible discrete current levels correspond to current through a various number of simultaneously open SGC channels. Voltage ramp protocol used is presented by diagram. A 30ms hyperpolarizing step to –100mV
preceded linear voltage changing (ramp). Ramp duration is 200ms. A, B show data obtained from the same patch and have the same current scale. Corresponding corrections were introduced by successive subtraction of leakage ramp current obtained in conditions without sodium. Electrode solution: NaCl 210mM+Ca$^{2+}_{\text{free}}$ 10nM. C – Normalized conductances calculated for average ramp currents from B given reversal potential as 0 mV at both calcium concentrations: 10nM Ca$^{2+}$ (black line) and 100µM (gray line).

Fig.7. A - Ca$^{2+}$ activation of a Ca$^{2+}$ sensitive SGC channel in an outside-out patch. B - Dependence of the open probability (open circles) and single channel amplitude (filled circles) on [Ca$^{2+}$]. $P_{\text{open}}$ values were calculated for 3 or 8 sec intervals from 2 patches. Electrode solution – NaCl 210mM + 10nM Ca$^{2+}_{\text{free}}$. Single open circle corresponds to $P_{\text{open}}$ value obtained in the presence of 5mM EGTA in the electrode solution with no calcium added to prevent activation of the channel by increasing extracellular calcium from 10nM to 1µM 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 sec, current range 0 --(–30) pA, step-size 0.1pA. 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. 1C) (A, B) and outer dendrite vesicles (C). SGC channel activity in the presence of different \([\text{Ca}^{2+}]\) (A) and calcium dependence 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 \(\text{Ca}^{2+}\)-sensitive and \(\text{Ca}^{2+}\)-insensitive channels. In (A), the patch consisted of 10 SGC channels (9 \(\text{Ca}^{2+}\)-sensitive, 1 \(\text{Ca}^{2+}\)-insensitive channel). Hill equation (solid line in B) gives the following estimations: \([\text{Ca}^{2+}]_{1/2} = 704.7\pm20.3\text{nM}, h=1.35\pm0.07\ (n=7)\). C - \(\text{Ca}^{2+}\) activates SGC channels in inside-out patch obtained from outer dendrite vesicle. Inside-out patch recordings. Current traces were filtered at 1kHz and reduced tenfold. Experimental conditions: inside-out patch recording; HP - -70mV (A, B); –80mV (C); Electrode solution: NaCl 210mM + 10nM Ca\(^{2+}\) free. Note different current and time scale in A and C.

Fig. 9. Current traces showing the behavior of a \(\text{Ca}^{2+}\)-sensitive SGC channel in the presence of potential regulators of phosphorylation. A - Protamine (5\(\mu\)g/ml) irreversibly changes the SGC channels sensitivity to \(\text{Ca}^{2+}\). B – Incubation with the catalytic subunit of protein kinase A (PKA) (1000 units/ml) and Mg-ATP (1mM) recovered the “native” channel’s properties (C). D – Okadaic acid (OA) 2\(\mu\)M in combination with the catalytic subunit of PKA (1000 units/ml) and Mg-ATP (1mM) abolished the protamine effect. A, B, C were obtained from the same patch. (E) – Comparison of the open probability of the \(\text{Ca}^{2+}\)-sensitive SGC channel in control conditions, and within 2 min after application of different combinations of the phosphorylation-regulating agents noted. Open probability values were determined as \(P_o = I/N_i\). Data on the histogram are presented as the mean\(\pm\)SEM of 3-12 patches.
Figure 3

**Ca\(^{2+}\)-insensitive SGC channel**

**Ca\(^{2+}\)-sensitive SGC channel**

E. Single channel amplitude, pA vs. Membrane potential, mV

F. Open probability vs. Membrane potential, mV
Figure 4

A

B
Figure 6

A

B

C

LiCl 210mM

NaCl 210mM

NaCl 210mM

+ Ca^{2+} 10nM

+ Ca^{2+} 100μM

LiCl 210mM

Current, pA

-100

-200

0

100

Membrane potential, mV

Normalized conductance

-100

-80

-60

-40

-20

0

20

40

60

80

HP -70mV

NaCl 210mM + Ca^{2+} 10nM

NaCl 210mM + Ca^{2+} 100μM

Membrane potential, mV
Figure 7