Calcium Channel Activation Facilitated by Nitric Oxide in Retinal Ganglion Cells

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Calcium channel activation facilitated by nitric oxide (NO) in isolated salamander retinal ganglion cells with the goals of determining the type of Ca channel affected and the signaling pathway by which modulation might occur. The NO donors, S-nitroso-N-acetyl-penicillamine (SNAP, 1 mM) and S-nitroso-cysteine (1 mM) induced modest increases in the amplitude of Ca channel currents recorded with ruptured- and permeabilized-patch techniques by causing a subpopulation of the Ca channels to activate at more negative potentials. The Ca channel antagonists ω-conotoxin GVIA and nisoldipine each reduced the Ca channel current partially, but only ω-conotoxin GVIA blocked the enhancement by SNAP. The SNAP-induced increase was blocked by oxadiazolo-quinoxaline (50 μM), suggesting that the NO generated by SNAP acts via a soluble guanylyl cyclase to raise levels of cGMP. The membrane-permeant cGMP analog 8-(4-chlorophenylthio) guanosine cyclic monophosphate also enhanced Ca channel currents and 8-bromo guanosine cyclic monophosphate (1 mM) occluded enhancement by SNAP. Consistent with these results, isobutyl-methyl-xanthine (IBMX, 10 μM), which can raise cGMP levels by inhibiting phosphodiesterase activity, increased Ca channel current by the same amount as SNAP and occluded subsequent enhancement by SNAP. Neither IBMX, the cGMP analogs, nor SNAP itself, led to inhibiting phosphodiesterase activity, KT5823 (1 μM), a specific protein kinase G (PKG) inhibitor, and a peptide inhibitor of PKG (200 nM) blocked SNAP enhancement, as did 5′-adenylylimidophosphate (1.5 mM), a nonhydrolyzable ATP analog that prevents protein phosphorylation. A peptide inhibitor of protein kinase A (10 nM) did not block the facilitory effects of SNAP. Okadaic acid (1 μM), a phosphatase inhibitor, had no effect by itself but increased the enhancement of Ca channel current by SNAP. These results suggest that NO modulates retinal ganglion cell N-type Ca channels by facilitating their voltage-dependent activation via a mechanism involving guanylyl cyclase/ PKG-dependent phosphorylation. This effect could fine-tune neural integration in ganglion cells or play a role in ganglion cell disease by modulating intracellular calcium signaling.

INTRODUCTION

There is strong evidence suggesting that nitric oxide (NO) and cyclic GMP (cGMP) together play a role in the regulation of the intracellular concentration of Ca$^{2+}$ ($[Ca^{2+}]_i$), $[Ca^{2+}]_i$ plays a vital role in the regulation of many physiological events in cells and organs including fertilization, cell growth, muscle contraction, neuronal signal transduction, and bile production (Bygrave et al. 1994; Putney and Bird 1993). Because increases of $[Ca^{2+}]_i$, and depletion of calcium stores may lead to cell death (Sippel et al. 1993), it is essential for cells to maintain $[Ca^{2+}]_i$, within strict limits. Cycling of Ca$^{2+}$ occurs through the movement of Ca$^{2+}$ into and out of cells and their intracellular compartments and often is initiated when agonists such as hormones, growth factors and neurotransmitters interact with specific receptors. The retina provides a valuable model for studying the physiological and pathological roles of NO in the nervous system. NO synthase, the enzyme responsible for synthesizing NO from L-arginine (Bredt and Snyder 1989, 1992), has been shown to be localized in specific retinal cells by the histochemical marker NADPH-diaphorase (Dawson et al. 1991; Hope et al. 1991; Kurenny et al. 1994; Liepe et al. 1994). Enzyme histochemistry for NADPH-diaphorase in the rabbit retina has detected a small subpopulation of amacrine cells at the border of the inner plexiform layer (Sagar 1986; Sandell 1985) and a similar subpopulation of cells also has been detected by immunocytochemical labeling with antibodies raised against NO synthase (Yamamoto et al. 1993).

There is great interest in the role of NO as an intercellular messenger in the nervous system and in its ability to activate cGMP as an intracellular second-messenger (Bredt and Snyder 1992; Garthwaite et al. 1988; Vincent and Kimura 1992). The present study investigates the regulation of Ca channels in isolated ganglion cells, showing that retinal ganglion cells express L- and N-type voltage-gated Ca channels and that the latter type undergo facilitation of voltage-dependent activation when exposed to NO. Our results further indicate that NO is transduced by a soluble guanylyl cyclase to produce an increase in cGMP that in turn acts via a cGMP-dependent protein kinase to enhance Ca channel activity.

METHODS

Preparation and identification of isolated ganglion cells

Whole cell patch recordings were made from isolated, identified ganglion cells of larval tiger salamanders following the methods of Lukasiewicz and Werblin (1988). Animals were treated in accordance with guidelines set forth by the Canadian Council of Animal Care. Salamanders were killed by decapitation and the eyes were removed. Crystals of Lucifer yellow were applied to the cut end of the optic nerve, and the eye was incubated for 18–24 h at 5°C. Isolated ganglion cells were obtained from retinas by trituration after treatment with papain (0.5 mg/ml, 15 min, 20°C; Sigma, St. Louis, MO) and were visualized in a Nikon Diaphot microscope equipped for epifluo-
resonant illumination. For cell isolation, the bathing solution contained (in mM) 90 NaCl, 2.5 KCl, 3 CaCl₂, 15 HEPES, and 10 glucose at pH 7.6. Round cells of ~10–15 µm diameter that were fluorescent in the isolated mixed tissue of retinal neurons were taken to be ganglion cells (Fig. 1).

**Patch-clamp recording**

Ganglion cell currents were recorded with patch electrodes having 2.5–6 MΩ resistance. Patch electrodes were filled with a solution containing either (in mM) 95 CsCl, 3 MgCl₂, 10 HEPES, and 1 EGTA (pH 7.6) for recording Ca channel currents or 95 KCl, 3 MgCl₂, 10 HEPES, and 1 EGTA (pH 7.6) for recording K channel currents. For permeabilized patch experiments, the pipette solution was sonicated with 150 µg/ml nystatin, then back-filled into the recording pipette. In this case, access occurred usually within 5 min, and series resistance was compensated partially (Axopatch 1-D). Signals were filtered and then digitized with an Indec Systems interface for storage in a 486 computer running BASIC-FASTLAB acquisition software. Because slow rundown of Ca channel currents occurred in ruptured patch recordings, data acquisition was completed in all experiments within 5 or 6 min.

The bath solution used for recording Ca channel currents contained (in mM) 70 NaCl, 2.5 KCl, 5 CaCl₂, 15 TEAC, 15 HEPES, and 10 glucose (pH 7.6). Ca channel antagonists were diluted in external bathing solution from concentrated stocks and applied via gravity-driven bath flow with suction pump removal. Nisoldipine and Bay K 8644 were diluted from 100 or 10 mM stocks, respectively, in 100% ethanol and diluted in Ba²⁺ external solution. Ganglion cells expressed, to varying extents, transient sodium currents as defined by sensitivity to block with TTX (Fig. 2). Because TTX was not routinely included in the superfusate, some recordings of ganglion cell currents include the transient sodium current, whereas others do not. Because all measurements for current-voltage (I-V) relations were made near the end of each test voltage step, well after the time when Na channels are inactivated (Fig. 2), the presence or absence of this current component has no bearing on our analysis. Potassium currents were recorded in a solution containing (in mM) 90 NaCl, 2.5 KCl, 3 CaCl₂, 15 HEPES, and 10 glucose (pH 7.6).

**Drugs and chemicals**

The following drugs were obtained from Sigma Chemical: 1H-[1, 2, 4]oxadiazolo[4, 3-a]quinonealine-1-one (ODQ), 3-isobutyl-1-methyl-xanthine (IBMX), 8-(4-chlorophenyl)thio) guanosine cyclic monophosphate (CPT-cGMP), 8-bromo guanosine cyclic monophosphate (8-Br-cGMP), S-nitroso-N-acetyl-penicillamine (SNAP), 5'-adenylylimidophosphosphate (AMP-PNP), tetrodotoxin (TTX). Okadaic acid, KT5823, protein kinase G inhibitor (No. N370654), and protein kinase A inhibitor, 6–22 amide (No. N539684) were obtained from Calbiochem-Novabiochem (San Diego, CA), Na,[2-(methylamino) ethyl]-5-isouquinoline-sulfonamide (H-8) was from RBI (Oakville, Ontario), iberotoxin was from Peninsula Labs (San Carlos, CA), and nisoldipine was a gift of Miles (Kankakee, IL). S-nitroso-cysteine (SNC) was prepared from equimolar L-cysteine and sodium nitrite mix in HCl and immediately diluted to the final desired concentration. All chemicals were dissolved in water, dimethyl sulfoxide (DMSO), or ethanol to make stock solutions, which then were diluted to the final concentrations designated in the text and figures. The final DMSO concentration never exceeded 0.1%. The final ethanol concentration never exceeded 0.2%. Experiments with 0.1% DMSO (n = 4) or with 0.2% ethanol (n = 4) alone in the bathing solution produced no change in Ca channel currents parameters in comparison with control.

**Quantitative evaluation**

Activation curves were constructed from leak subtracted, steady-state I-V relations by dividing by the driving force or from isochronal tail current analysis (as illustrated in Fig. 3). Activation curves were fit with the Boltzmann function \( f = \frac{1 + \exp[(V - V_{1/2})/m]}{1 + \exp[-(V - V_{1/2})/m]} \) to permit characterization of the voltage for half activation (activation midpoint, \( V_{1/2} \)) and slope factor (m). Percentage block and enhancement were defined as \( [1 - (I_{test}/I_{control})] \cdot 100 \), and were assessed at the peak I-V relation values, which were measured near the end of each depolarizing pulse. All statistical data are presented as means ± SE unless otherwise noted and analyzed using an independent Student’s t-test where appropriate. Statistical significance was considered with \( P < 0.05 \).

**RESULTS**

**Modulation of Ca channels by the NO donor, SNAP**

An action of NO on voltage-gated Ca channels has been suggested previously in several different cell types (Collins et
functions, accounting for 2 separate populations of Ca channels as described in factors. Dashed lines, fit to the control data made with the sum of 2 Boltzmann steps were made in 10-mV increments to potentials between 200 K. HIROOKA, D. E. KOURENNYI, AND S. BARNES

2 steps were made in 10-mV increments to potentials between 0 and 10 mV from a holding potential of 70 mV. Solid lines, fits to the I-V relations made by multiplying fitted activation curves by a linear driving force. Inset: currents recorded during the voltage step to −10 mV. B: tail currents recorded on return of membrane potential to −70 mV from depolarized test potentials revealed a negative shift in the activation curve in the presence of SNAP. Same cell as in A. Figure shows the last 1.8 ms of each recording at test potentials of −30, −20, −10, 0, 10, and 20 mV, followed by a blanked point, and then each tail current for 5.7 ms after the transition to −70 mV. Difference currents were obtained through subtraction of recordings obtained in 100 μM cadmium from those in control (top) or SNAP (bottom) to minimize capacitance artifacts. Current trace highlighted in each panel was recorded at −10 mV before and during SNAP application and indicates greater current activation at −10 mV in the presence of SNAP than in control. Exponential fits to these current trajectories had time constants averaging 0.97 ms. C: activation curves constructed from the tail currents shown in B. *, averaged from tail current measurements in control; †, averaged measurements obtained in the presence of SNAP; solid lines, single Boltzmann fits to these data. Activation curves derived from the I-V relations in A gave nearly identical midpoints and slope factors. Dashed lines, fit to the control data made with the sum of 2 Boltzmann functions, accounting for 2 separate populations of Ca channels as described in the text.

al. 1986; Gurney and Clapp 1994, Kurenny et al. 1994). Here we focused on the actions of NO on retinal ganglion cells by superfusing the NO-generating substance SNAP over single identified ganglion cells and measuring changes in ion channel currents using both ruptured-patch whole cell techniques and nystatin perforated-patch techniques. Application of fresh NO-containing solution reversibly increased current in Ba2+-conducting Ca channels of ganglion cells (Fig. 3A). Similar results were obtained from 13 ganglion cells treated with SNAP. In six of those recorded with perforated-patch techniques, Ca channel currents increased by 21.5 ± 0.6%, whereas in seven recorded with ruptured patch techniques, Ca channel currents increased by 22.8 ± 5.1%. Another NO donor, SNC increased Ca channel currents in a comparable manner (41 ± 9%, n = 3) when the donor was applied at a concentration of 1 mM to cells recorded with the permeabilized patch technique. On the basis of our previously published measurements, it can be estimated that cells treated with SNC in this manner may be exposed to continuous NO concentrations of ~5 μM (Kurenny et al. 1994).

The SNAP-induced increase in current was associated with a negative shift of the Ca channel activation curve. After fitting with the Boltzmann function, the voltage for half activation shifted on averaged by −3.7 ± 0.3 mV (n = 13). To illustrate this phenomenon, Fig. 3, B and C, provides analysis of Ca channel tail currents elicited on repolarization of a ganglion cell to −70 mV after a series of steps, each lasting 60 ms, to more positive potentials. The highlighted current traces in Fig. 3B, which are associated with the step to −10 mV, show greater inward current development in the presence of SNAP, which is indicative of a greater degree of activation. The analysis of tail current magnitudes shown in Fig. 3C confirms this assessment. These tail current-derived activation curves were assessed at three different time points during the decay of the tail currents and show a negative shift of 4.9 mV at the activation midpoint.

SNAP, being freely soluble in water, was applied normally without the aid of ethanol or DMSO. In some of the experiments to follow, certain drugs requiring ethanol or DMSO for dissolution were applied in conjunction with SNAP. Because some drug effects on Ca channels seem sensitive to the type of solvent used (Wu et al. 1992a, b), we compared the magnitude of SNAP enhancement when ethanol or DMSO were present in the bathing solution. From the same holding potential (−70 mV), application of 1 mM SNAP with ethanol or DMSO controls produced 22.0 ± 6.0% (n = 4) and 17.9 ± 3.3% (n = 4) enhancement respectively, which was indistinguishable (0.8 < P < 0.9 for ethanol, 0.3 < P < 0.4 for DMSO) from the experiments in which no ethanol or DMSO had been used.

SNAP enhances ω-conotoxin GVIA-sensitive Ca channels

Nisoldipine (5 μM), a dihydropyridine L-type Ca channel antagonist, blocked the Ca current by about one-third (−33.5 ± 10.3%, n = 6). The current remaining was enhanced with 1 mM SNAP by 25.1 ± 13.0% (n = 4, Fig. 3, A and D). We then tested the Ca channel antagonist, ω-conotoxin GVIA, a peptide toxin the blocking actions of which tend to define the presence of N-type Ca channels (Olivera et al. 1994). Partial block of Ca channel current occurred when 1 μM ω-conotoxin GVIA was applied to the ganglion cells (−30.9 ± 6.8%, n = 5), and the remaining inward current was not enhanced in the presence of 1 mM SNAP (−3.6 ± 3.4%, n = 5, Fig. 4, B and D).

In some neurons, especially those of lower vertebrates, ω-conotoxin GVIA has been shown to block non-N-type Ca channels (Olivera et al. 1994). For example, in salamander retinal photoreceptors, ω-conotoxin GVIA has been shown to block Bay-K-8644-augmented L-type Ca channel current (Wilkinson and Barnes 1996). To establish the specificity of ω-conotoxin GVIA action on salamander ganglion cell N-type Ca channels, we used the dihydropyridine agonist Bay K 8644. Bay K 8644 produces prolonged openings of single L-type Ca
channels, resulting in an increase in the whole cell Ca current (Plummer et al. 1989). In these salamander neurons, 1 μM Bay K 8644 increased the peak Ca channel current (282.5 ± 56.5%, n = 4), an amount similar to that seen in other cell types (Regan et al. 1991; Wilkinson and Barnes 1996). In the presence of 1 μM Bay K 8644, addition of 1 μM ω-conotoxin GVIA slightly decreased the whole cell current (−8.5 ± 3.3%, n = 4; Fig. 4, C and D). Supposing that N-channel current had made up 31% of the total current in control (the amount blocked by ω-conotoxin GVIA), and that Bay K 8644 increased selectively L-channel current to produce a net Ca channel current of 282% of control, the N component would amount to 11% of the new total. Because ω-conotoxin GVIA block was reduced to approximately this level in the presence of Bay K 8644, these data suggest that ω-conotoxin GVIA specifically blocked a non-L-type Ca channel current, one presumably arising from the activity of N-type Ca channels. Further, because SNAP enhancement was eliminated in the presence of ω-conotoxin GVIA, the results indicate that the channels enhanced in control by SNAP were of the N-type.

In view of the fact that SNAP only modulated Ca channel current in the absence of ω-conotoxin GVIA, we reconsidered the effects of SNAP on the activation curves by assuming that there were two populations of channels and that only one, the N-type channels, underwent modulation by SNAP. For the example shown in Fig. 3C, it is apparent that a greater negative shift occurred in the presence of SNAP in the upper portion of the curves (corresponding to the voltage range between approximately −10 to +10 mV) than in the lower portion (voltage range between −10 and −30 mV). To fit these data, Boltzmann functions with different midpoints and slope factors had to be used (Fig. 3C, solid line). In the cell shown, the slope factor shifted from 6.0 mV in control to 3.9 mV in SNAP. In five of six of the cells recorded with perforated patch and in three of seven recorded with ruptured patch techniques, a steepening of the activation curve also was observed. The slope factors were reduced by an average of 0.8 mV ±0.3 mV (n = 8). For this same group of cells, the negative shift in the activation curve midpoint was −5.4 ±2.6 mV (n = 8).

To account for the overall reduction in slope factor, Fig. 3C, dashed line, shows the result of a model in which two populations of Ca channels were assumed. Thirty percent of the channels were designated N-type and allowed to undergo a negative shift in activation curve in the presence of SNAP, but the slope factor was held constant at 4 mV. The activation midpoint and slope factor of the remaining 70% of the channels were held constant at −15 and 4 mV, respectively. In control conditions, the dashed line fits the data with the subpopulation of N channels having an activation midpoint at +1 mV. This midpoint shifts negative by 16 mV in the presence of SNAP, such that the two populations of channels now have identical activation midpoints and slope factors. This simple model provides a reasonable explanation for the broader range over which activation occurs in control than in SNAP. It accounts for selective modulation of N-type Ca channels by SNAP, which in this case amounts to a facilitation of voltage-dependent activation wherein the channels activate at significantly more negative potentials. We note that depolarization-induced facilitation of Ca channels, as described in other cells (Boland and Bean 1993), which also features a shift of voltage-dependent gating to negative potentials, was not observed under control conditions in the ganglion cells (data not shown).

**SNAP acts via guanylate cyclase to increase cGMP**

According to many reports (reviewed in Garthwaite 1991), NO activates soluble guanylate cyclase and effects changes in cell function via an increase in cGMP concentration. Soluble guanylate cyclase has been shown to be present in ganglion cells (Ahmad and Barnstable 1993), suggesting that the modulation of Ca channel currents described in the preceding text might be mediated by guanylate cyclase activation.

In the presence of 50 μM ODQ, an inhibitor of NO-sensitive guanylyl cyclase (Garthwaite et al. 1995), enhancement by
SNAP was blocked (−2.6 ± 3.4%, n = 4; Fig. 5A). To further examine the role of cGMP in Ca channel enhancement, we examined the effects of two membrane permeant cGMP analogs (CPT-cGMP and 8-Br-cGMP). Tested during recordings with permeabilized patch recording techniques, CPT-cGMP (1 mM) mimicked the facilitatory actions of SNAP, enhancing Ca channel current by 24.2 ± 1.6% (n = 3; Fig. 5C). Tested alone, 1 mM 8-Br-cGMP produced no modulation of Ca channel properties; however, when SNAP was applied, 8-Br-cGMP greatly reduced the enhancement normally seen with SNAP (4.6 ± 2.8%, n = 4).

If cGMP was involved in Ca channel modulation, then inhibition of the enzyme that breaks cGMP down ought to augment the effects of activating this pathway. Indeed, potentiation of Ca channel currents by inhibition of phosphodiesterase (PDE) was seen with IBMX. IBMX (10 μM) enhanced Ca current itself (27.3 ± 8.5%, n = 4), and occluded subsequent enhancement by SNAP (3.6 ± 1.7%, n = 4; Fig. 5B). From this result, we conclude that retinal ganglion cells contain an active PDE that can regulate the intracellular concentration of cGMP.

In rat retinal ganglion cells, activation of cyclic nucleotide gated (CNG) channels by NO has been demonstrated (Ahmad et al. 1994). We found no evidence for activation of CNG channels in salamander ganglion cells by NO in the present study. Before leak subtraction, there was no increase in voltage-independent inward current as would be expected if CNG channels were activated. Furthermore, treatment with CPT-cGMP, 8-Br-cGMP, or the other manipulations that would be expected to increase levels of cGMP in the cells did not produce evidence of a conductance increase, suggesting indeed that CNG channels are not even present in salamander ganglion cells. This result may stand as an important difference between the ganglion cells of the mammalian and lower vertebrate retinas studied in these two reports.

**Phosphorylation by cGMP-dependent protein kinase is required for Ca channel enhancement by SNAP**

We next investigated the role of protein kinases in the modulation caused by SNAP. Because we had established a role for cGMP in Ca channel enhancement, we tested two inhibitors of cGMP-dependent protein kinase (PKG) to see if they. Applied via the recording pipette in ruptured patch recording mode, 1 μM KT5823 abolished the enhancement normally seen with SNAP, which now failed to produce a change in Ca channel current (−0.2 ± 1.5%, n = 3, Fig. 5, D and F). KT5823 has been reported to inhibit PKG with a K_i of 234 nM (Grider 1993), a value approximately fourfold smaller than the concentration tested here. The second PKG inhibitor tested was a peptide (Arg-Lys-Arg-Ala-Arg-Lys-Glu), reported to inhibit PKG with a K_i of 86 μM and PKA with a K_i of 550 μM (Glass 1983). Used in the present work at 200 μM, a value well above the K_i for inhibition of PKG yet well below the K_i for PKA inhibition, this inhibitor blocked the enhancement normally seen with SNAP, allowing only a 2.1 ± 2.1% (n = 3) increase of Ca channel current (Fig. 5F). However, a peptide inhibitor of PKA, applied intracellularly at 10 nM, had no effect on the enhancement produced by SNAP (25.3 ± 2.4%, n = 3, Fig. 5F). This concentration is at least five times the reported K_i for inhibition of PKA (Glass 1989). Previously, activation of PKA by forskolin was shown to lead to Ca channel suppression in retinal ganglion cells (Zhang et al. 1997), which represents a modulation of opposite sign to that shown in our present work, so we would not have expected activation of PKA to enhance Ca channels. Finally, 2 μM H-8, a broader spectrum protein kinase inhibitor (Hidaka et al. 1994).
1984), also blocked SNAP enhancement (2.6 ± 4.9%, n = 4, Fig. 5D), although our use of this agent at 2 μM does not discriminate between cAMP- or cGMP-dependent kinases. Taken together, the action of these kinase inhibitors suggests that Ca channel facilitation is dependent on a protein kinase, in particular, PKG.

Another result suggested that phosphorylation and dephosphorylation could be active processes in the ganglion cells during our period of recording and that by inhibiting phosphatase activity, SNAP-induced phosphorylation was increased. Okadaic acid, an inhibitor of serine/threonine phosphatases, (1 μM) had no effect by itself but tended to increase the enhancement of Ca channel current by SNAP (30.9 ± 4.6%, n = 4). Exposure to okadaic acid was in each case limited to under 1 min owing to the need to proceed briskly through the experimental paradigm, e.g., application of SNAP and then wash, to minimize potential problems with rundown. It is possible that a longer exposure to okadaic acid, by itself, could result in an increase in Ca channel current due to basal levels of kinase activity. AMP-PNP, a nonhydrolyzable ATP analog that prevents protein phosphorylation, (1.5 mM) also greatly reduced SNAP enhancement (6.7 ± 1.5%, n = 4; Fig. 5C). While not pin-pointing the specific actions of AMP-PNP that might interfere with the regulation of Ca channels, this result is consistent with AMP-PNP out-competing endogenous ATP, producing an environment in which no phosphorylation could occur, and thus blocking SNAP-induced phosphorylation of the Ca channel or an associated protein as required for the development of facilitation of activation.

Effects of SNAP on potassium channels in ganglion cells

A direct consequence of enhanced Ca channel activity might be reflected in a number of other calcium-dependent functions in ganglion cells, including enhancement of calcium-activated potassium current. Thus we investigated the effects of SNAP on potassium currents found in ganglion cells: calcium-dependent potassium current (I_K(Ca)) and slowly inactivating voltage-dependent potassium currents (I_R and I_C) (Lukasiewicz and Werblin 1988).

SNAP (1 mM) applied to ganglion cells during recording conditions designed to highlight K channel activity produced strong, reversible enhancement of the potassium currents in the cell shown in Fig. 6A. In this cell, increased outward current was evident over the entire voltage range that has been attributed to K channel activity in ganglion cells (positive to about −40 mV) (Lukasiewicz and Werblin 1988). In four responding cells, SNAP increased potassium current by 32.7 ± 7.7% (measured at +40 mV). Two other cells that showed no response to SNAP were not included in the analysis. To test whether the outward current SNAP enhanced was a calcium-activated potassium current, we applied 100 μM Cd2+ to block Ca channels and calcium-activated currents and then applied SNAP. Figure 6B shows that in the presence of Cd2+, SNAP no longer increased outward current. On its own, Cd2+ reduced outward current by 65.8 ± 4.6% from control (measured at +20 mV; n = 4), an amount indistinguishable from the current blocked when SNAP and Cd2+ were applied together (70.3 ± 5.3%, n = 4; P = 0.55). Because Cd2+ blocked both inward calcium and outward potassium currents, these percentage reductions may represent an underestimate of the potassium current affected. This result is consistent with the notion that potassium current enhancement by SNAP is secondary to the facilitation of Ca channel activation shown to be caused by SNAP in the previous sections of this work.

To test the specificity of the SNAP enhancement of calcium-activated potassium currents, we checked the actions of SNAP in the presence of 10 nM IBTX, a selective inhibitor of large-conductance, calcium-activated K channels. IBTX by itself had a pronounced effect on the outward current of ganglion cells, blocking 76.8 ± 4.2% of the current measured in control at ±40 mV (n = 4; data not shown), an amount similar to that blocked by Cd2+ as described in the preceding text. In the presence of IBTX, SNAP had no effect on the outward current, with 78.7 ± 4.2% of the control current blocked in the presence of the two agents together (n = 4; data not shown). We

![Fig. 6](http://jn.physiology.org/figure)
might have expected an increase in inward current when SNAP was applied due to the facilitation of Ca channels and the block of some K channels. However, because these experiments were designed to study potassium currents and used 3 mM Ca\(^{2+}\) in the bath, not 10 mM Ba\(^{2+}\) as was used in the experiments for isolating and enhancing Ca channel currents, the 20% increase of Ca channel current might have been too small to be detected under these conditions. Like the previous experiment with Cd\(^{2+}\), this result with IBTX occluding SNAP-induced enhancement of outward current is consistent with the notion that SNAP-induced facilitation of Ca channel activation can lead to the secondary effect of enhancement of calcium activated potassium current.

**Time course of channel modulation in ganglion cells**

Data describing the temporal actions of drug effects often provide important information and insights about the dynamics of signaling processes. In some cells, data were collected to show the time course with which the actions of SNAP and IBMX occurred (Fig. 7). In the examples shown, the onset of SNAP or IBMX actions were indistinguishable, being complete in <8 s. Because the changes in current magnitude were sampled every 4 s, resolution of the time constant of the current increase was not possible. The offset, or reversal, of SNAP and IBMX actions differed in that the current increase elicited by IBMX returned to control levels at a rate approximately twice that for SNAP, which was the same for Ca and K currents under the conditions of our recordings.

**DISCUSSION**

**NO modulation of Ca channels**

Our results suggest that modulation by NO of Ca channel current in salamander ganglion cells results in facilitation of voltage-dependent activation of N-type Ca channels. This important kinetic alteration is mediated through a rise in intracellular cGMP. Recent reports indicate that NO modulates Ca channels in cardiac cells, in which concentration-dependent increases or decreases of Ca channel current are observed (Mery et al. 1993). In sympathetic neurons, NO produces only an increase in Ca channel current (Chen and Schofield 1993), and in retinal rods, NO enhanced L-type Ca channel current (Kurennny et al. 1994). One might expect that other factors in the retina, capable of stimulating either NO production (e.g., neurotransmitters and possibly circulating agents) or intracellular cGMP formation (possibly atrial natriuretic peptide) would lead to facilitation of N-type Ca channel voltage-dependent activation in ganglion cells.

Modulation of the high-threshold calcium channels in lower vertebrate ganglion cells by neurotransmitters such as glutamate and GABA has been demonstrated previously. Suppression of L-type Ca channels was found to be mediated via metabotropic glutamate (Akopian and Witkovsky 1996; Shen and Slaughter 1998) and GABA (Zhang et al. 1997) receptors, whereas suppression of N-type Ca channels was shown to be mediated by different second-messenger pathways stimulated by a separate metabotropic GABA receptor (Zhang et al. 1997) or by ionotropic glutamate receptor agonists (Shen and Slaughter 1998). Enhancement of the GABA-mediated suppression of barium current in the presence of NO donor sodium nitroprusside or by 8-Br-cGMP was reported (Zhang et al. 1997). In this report, sodium nitroprusside was reported to have a modest inhibitory effect on barium current by itself, an action in contrast to our present findings. The difference might be due to differences in the cell environment (Zhang et al. used cells in tissue slices that have extensive neuritic processes; we used enzymatically isolated cells devoid of any neurites) or properties of the NO donor (Zhang et al. used sodium nitroprusside; we used SNAP).

**Ca channel subtypes in ganglion cells**

An important observation of this study is that tiger salamander retinal ganglion cells express high-threshold Ca channel currents that can be blocked by \(\omega\)-conotoxin GVIA and nisoldipine and augmented by Bay K 8644. This observation is consistent with an existing characterization of Ca channel subtypes in ganglion cells of tiger salamander retina (Shen and Slaughter 1998). Because in cone photoreceptors of this same species a single type of Ca channel appears sensitive to both \(\omega\)-conotoxin GVIA and dihydropyridines (Wilkinson and Barnes 1996), we showed here that \(\omega\)-conotoxin GVIA and dihydropyridines act on separate types of Ca channels, consistent with expression of N- and L-type, respectively. Results obtained in other studies of retinal ganglion cells are mostly consistent with the notion that L-type Ca channels and other types of Ca channels are present in these cells. For example, dihydropyridine-sensitive Ca channel current coexists with \(\omega\)-conotoxin GVIA-insensitive current in turtle retinal ganglion cells (Liu and Lasater 1994), but coexists with \(\omega\)-conotoxin
GVIA-sensitive current in rat retinal ganglion cells (Guenther et al. 1994; Karschin and Lipton 1989).

Regulation of Ca channel activity in ganglion cells

The present work investigates one pathway by which Ca channels in ganglion cells might be regulated by NO. CNG channels previously had been shown to be activated by NO in rat retinal ganglion cells, so we sought to determine whether voltage-gated Ca channels also were affected by a cGMP-based second-messenger cascade in ganglion cells. Also, other work has detected transcripts for both a transmembrane (the GC-A or atrial natriuretic factor receptor form) and soluble form of guanylate cyclase in the ganglion cell layer of the rat retina (Ahmad and Barnstable 1993). NO donor application to rat ganglion cells induced a conductance increase that appeared to be the same as that induced by direct application of cGMP (Ahmad et al. 1994), but our data show that CNG channels are not activated by these substances in salamander ganglion cells.

Application of IBMX (10 μM), a nonselective PDE inhibitor, enhanced Ca channel activation, mimicking the action of SNAP and, indeed, occluding it. In contrast, Shen and Slaughter (1998) found that IBMX applied by itself at 1 mM decreased the magnitude of whole cell barium currents in ganglion cells. To further the comparison, we also tried higher concentrations, finding that both 500 μM and 2 mM IBMX decreased Ca channel current. However, with the IC₅₀ for IBMX inhibition of type-V cGMP phosphodiesterase often reported to lie in the single-digit micromolar range (Beavo and Reifsnyder 1990; Jeffery and Wanstall 1998), we are satisfied to have a substantial response to the agent at 10 μM concentration. Elsewhere in the literature, PDE inhibitors have been reported to increase Ca channel currents by elevating the intracellular cGMP concentration through inhibition of cGMP hydrolysis by PDE (Fischmeister and Hartzell 1987; Simmons and Hartzell 1988). 8-Br-cGMP occluded the actions of SNAP, but by itself, and in contrast to our result with CPT-cGMP, another membrane permeant cGMP analog, Ca channel activation was not enhanced as we expected it would. It remains possible that 8-Br-cGMP interfered with the normal stimulatory actions of cGMP, thus occluding the actions of SNAP, but failed to activate the effector enzyme sufficiently to induce changes to the Ca channels on its own. Taken together with the block of SNAP enhancement by KT5823, an inhibitor of cGMP-dependent protein kinase, as well as the PKG inhibitor peptide, we conclude that increased levels of cGMP in the ganglion cells, normally brought about by NO stimulation of soluble guanylate cyclase, activates PKG, and this enzyme in turn phosphorylates the Ca channels or some regulatory protein with the net result being a facilitation of the voltage-dependent activation of the channels.

Our observation that ganglion cells treated with okadaic acid displayed an increased response to SNAP suggests an involvement of phosphatase activity. Okadaic acid is a potent inhibitor of serine/threonine phosphatase 1 and 2A with a Kᵢ in the nanomolar range (Hardie et al. 1991). However, okadaic acid at 1 μM also may inhibit phosphatase 2B (calcineurin), a Ca²⁺/calmodulin-dependent phosphatase (Hardie et al. 1991). Further experiments with additional phosphatase inhibitors are warranted to determine with more specificity the type of phosphatases involved in this pathway. It is interesting to note that okadaic acid also reduces the kainate-induced suppression of N-type channels in ganglion cells, suggesting that this ionotropic receptor mediated mechanism could rely on dephosphorylation to suppress Ca channel activity (Shen and Slaughter 1998). Calcium signaling in ganglion cells: potential roles in neuronal integration and pathophysiology

There is currently a great deal of interest in the possible roles of Ca²⁺-stimulated production of NO as an anterograde, retrograde, or dendrodendritic messenger that modulates the efficacy of synaptic interactions in other regions of the mammalian CNS (Bliss and Collingridge 1993; Breer and Shepherd 1993). It is important to determine whether NO, which appears to have a key role in ion channel regulation in other brain regions (Bohme et al. 1991; Izumi et al. 1992; Schuman and Madison 1991; Shibuki and Okada 1991), is a modulator of retinal ganglion cell activity with subsequent relevance to retinal signal processing and pathology.

It seems likely that NO produced in the vicinity of ganglion cells would have the potential to modulate calcium signaling via changes to the voltage-dependent gating of Ca channels as shown in the present work. Increases in NO would facilitate the activation of Ca channels during depolarizations and in turn affect all calcium-mediated pathways, including the activation of Ca-dependent ion channels, altering the integration of synaptic signals impinging on ganglion cells. It is possible that, in some manner, plasticity of the synapses made onto ganglion cells could be dependent on postsynaptic Ca signaling, which appears here to be itself NO dependent in these cells.

Finally, pathophysiological alterations of NO levels in the inner retina could directly affect ganglion cell calcium signaling. If Ca channel activity was enhanced sufficiently to allow destructive levels of intracellular calcium to accumulate, perhaps in concert with other actions leading to ganglion cell depolarization, the consequences of this overburden might be the loss of ganglion cell function and long-term visual impairment.

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