Modulation of hybrid bass retinal gap junctional channel gating by nitric oxide

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1. To elucidate the role of the nitric oxide (NO) transmitter system in the regulation of gap junctional channel gating, we have examined the effects of the NO donor sodium nitroprusside (SNP) on the electrical synapses of hybrid bass H2-type horizontal cells.

2. SNP reversibly reduced the macroscopic junctional conductance without significantly changing voltage sensitivity.

3. Kinetic analyses showed that SNP made the voltage-dependent decay of junctional currents more rapid.

4. Single-channel data showed that SNP reduced channel open probability by reducing channel open frequency.

5. The action of SNP can be prevented or largely reduced by the NO scavenger, haemoglobin. NO release by SNP solutions was detected directly by a NO sensor.

6. NO appears to modulate the gap junctional conductance by activating the cGMP–cGMP-dependent protein kinase G (PKG) pathway. A membrane-permeable cGMP analogue, 8-Br-cGMP, mimics the action of SNP. A soluble guanylate cyclase inhibitor (LY-83583) and a highly specific cGMP-dependent protein kinase inhibitor (RKRARKE) blocked the action of NO. 3-Isobutyl-1-methylxanthine (IBMX), a non-specific phosphodiesterase inhibitor, potentiated the effect of SNP.

7. $[Ca^{2+}]_i$ image studies showed that NO donors did not change $[Ca^{2+}]_i$ in horizontal cells, suggesting that the regulation of junctional channels by NO is $[Ca^{2+}]_i$ independent.

Cell-to-cell transmission at electrical synapses is mediated by gap junctions. Gap junctions are widely distributed in all synaptic layers of the retina and are of fundamental importance in transmitting and shaping visual signals (Vaney, 1991). Horizontal cells are second order interneurons that form an electrically coupled network for the transmission of inhibitory signals in the outer plexiform layer of the retina (Dowling, 1987). They have been suggested to contribute to centre–surround antagonism in visual receptive fields (Werblin & Dowling, 1969). Horizontal cells are extensively electrically coupled to each other by gap junctions, or electrical synapses (Knapp & Dowling, 1987).

Electrical synaptic transmission can be modulated by many physiological transmitter and second messenger signals (Spray & Bennett, 1985). The modulation of retinal horizontal cell electrical synapses by the dopamine–cAMP pathway is well established (Lasater & Dowling, 1985; Lasater, 1987; DeVries & Schwartz, 1989; McMahon, Knapp & Dowling, 1989; McMahon, 1994; McMahon & Brown, 1994, McMahon & Mattson, 1996). The inorganic gas molecule nitric oxide (NO) has been revealed to act as a messenger in a variety of cell types, including the nervous system (Bredt & Snyder, 1994). The neuronal action of the NO system is mediated by activating a soluble guanylyl cyclase (Garthwaite, 1991) or by increasing ADP-ribosylation (Zhang, Dawson, Dawson & Snyder, 1994). In the retina, it has been reported that NO influences the light response of amphibian rods (Schmidt, Nöll & Yamamoto, 1992), modulates Ca^{2+} channels and transmission of the photoresponse to second-order cells (Kurenny, Moroz, Turner, Sharkey & Barnes, 1994), and gates cGMP-gated cation channels in retinal ganglion cells (Ahmad, Leinders-Zufall, Kocsis, Shepherd, Zufall & Barnstable, 1994). Immunocytochemical studies demonstrated that nitric oxide synthase (NOS) is widely distributed in both the inner and outer teleost retina, including horizontal cells (Weiler & Kewitz, 1993; Liepe, Stone, Koistinaho & Copenhagen, 1994). The NO system is involved in the modulation of gap junctions in the retina (DeVries & Schwartz, 1989, 1992; McMahon, 1994; Miyachi, Murakami & Nakaki, 1990; Mills & Massey, 1995). However, the current understanding of the cellular mechanisms of NO modulation on retinal electrical synapses is insufficient.

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This study has further examined the role and the mechanisms of the NO system in the modulation of gap junctional channel gating. The results show that NO reduces the overall open probability of gap junctional channels by a $[Ca^{2+}]_i$-independent activation of the cGMP–cGMP-dependent protein kinase G (PKG) pathway.

METHODS

Experimental animals

Experiments were performed using retinal neurons from hybrid striped bass resulting from breeding of white bass (*Roccus chrysops*) and striped bass (*Roccus morone*). Fish were obtained from the Minor Clark Fish Hatchery of the Kentucky Fish and Wildlife Department and were maintained on a 12 h light–12 h dark cycle.

Cell culture and identification

Dark-adapted adult hybrid bass were killed following National Institutes of Health guidelines for animal use. Fish were rendered insentient by immersion in an ice–water mix at 0 °C and then killed by rapid decapitation and pithing. Retinas were removed under dim red light and then incubated at room temperature (22 °C) for 20–30 min in Leibovitz L-15 media (Gibco) containing 20 units ml^{-1} papain (Worthington Biochemical Corporation, Freehold, NJ, USA), activated with 0·3 mg ml^{-1} cysteine. Retinas were then washed with six changes of fresh L-15 media, dissociated by repeated passage through a serological pipette, and plated onto plastic dishes containing fresh L-15 medium. Cultures were maintained at room temperature and were viable for 7–10 days. Culture medium was changed daily to eliminate metabolic products and minimize evaporation of solution. Cultured cells were used within 15–72 h in most experiments.

H2-type horizontal cells are cone-driven cells, which can be easily identified *in vitro* by morphological characteristics (Dowling, Pak & Lasater, 1982). We selectively used H2-horizontal cells, because they are larger in size and more easily recorded from for extended periods than other hybrid bass cone-driven horizontal cell (HC)-types (H1 and H3) in our culture conditions.

Junctional current recording

Recordings were performed using the double whole-cell patch-clamp technique (Neyton & Trautmann, 1985). Patch pipettes (3–5 MΩ) were made from Corning 7052 glass (AM Systems, Everett, WA, USA) and filled with a solution consisting of (mM): 120 potassium gluconate, 4 KCl, 1 $CaCl_2$, 11 EGTA, 10 Hepes, 1 MgATP and 0·1 NaGTP; adjusted to pH 7·5 with KOH. The extracellular solution contained (mM): 137 NaCl, 2·5 KCl, 2·25 $MgSO_4$, 2·25 $MgCl_2$, 2·5 $CaCl_2$, 1 sodium pyruvate, 0·5 $NaHCO_3$, 0·5 $NaHPO_4$, 10 Hepes, 16 glucose; and 0·1 mg ml^{-1} bovine serum albumin (fraction VII, Sigma). Whole-cell currents were recorded using an Axopatch-1D amplifier (Axon Instruments) in voltage-clamp mode.

[NO] measurement

Before measurement of NO released by NO donors, the instrument (ISO-NO: World Precision Instruments) was carefully calibrated by NO generated from the reaction:

$$2KNO_2 + 2KI + 2H_2SO_4 \rightarrow 2NO + 2H_2O + 2K_2SO_4.$$

Both calibration and actual measurement of NO released by NO donors were performed at room temperature. NO measurements were performed according to the manufacturer's instructions.

$[Ca^{2+}]_i$ measurement

For calcium imaging studies, cells were dissociated into glass-bottomed dishes coated with polyornithine (Sigma). Cells were cultured in L-15 medium. Intracellular Ca^{2+} measurements were made by the ratiometric measurement of fura-2 fluorescence. Cells were loaded at 22 °C for 45 min with 5 μM fura-2 AM (Molecular Probes). The loaded cells were then washed twice with the same solution as that used for the recording of coupling currents. Cells were incubated for an additional 20 min prior to mounting on the stage of a Nikon Diaphot inverted epifluorescence microsope. A dual-excitation (340 nm/380 nm) spectrofluorometric system (Photon Technologies Incorporated, Saverna Park, MD, USA) was used to quantify basal and agonist-induced changes in cytosolic Ca^{2+}. The fluorescence ratio was converted to $[Ca^{2+}]_i$ using an *in vitro* calibration determined with an external standard (Calcium Kit I, Molecular Probes) and 5 μM fura-2 potassium salt. K_d was 282 nM and the R_{min}/R_{max} fluorescence ratio was 0·077 for the system used (R_{min} and R_{max} are the fluorescence ratios at 0 Ca^{2+} and in saturating $[Ca^{2+}]$, respectively).

Data analysis

Macroscopic currents were analysed by pCLAMP 6 software (Axon Instruments). Single-channel data were low pass filtered (3 dB at 200–500 Hz) and then digitized at 2 kHz per channel using Axotape software and also analysed using pCLAMP 6. Typically, 30–60 s of pre- and post-treatment data were analysed for each cell pair. Analysis was performed during steady-state intervals in control and treated conditions. Steady-state behaviour during these sections was assessed by visual inspection of the records. Channel activity in the cell of each pair which had the best signal-to-noise ratio was analysed using the events list portion of the Fetchan program of pCLAMP. The events list function employs a half-amplitude threshold for event detection and is able to analyse up to six levels of conductance above baseline. To ensure the accurate determination of conductance levels necessary to calculate overall open times, only those records that exhibited an acceptably stable baseline and exhibited a maximum of six levels of conductance above baseline were used for analysis of single channel events. Event duration was defined by Fetchan as the time from the initial crossing of the half-amplitude threshold to the return of the signal below this threshold. Mean open times for multiple level recordings were determined using the correction for overlapping events of Fenwick, Marty & Neher (1982). Event amplitude was determined by Fetchan to be the mean amplitude of the signal between the two threshold crossings. By assigning a single mean amplitude to each channel opening and tracking baseline, this procedure reduces the variance in amplitude histograms when compared with the all-points histogram method. This is of particular importance for this study because whole-cell patch recordings, by nature, exhibit greater baseline drift than on-cell patch recordings. Initial recordings indicated that junctional conductance fluctuated from baseline predominantly in 60 pS steps, so the half-amplitude threshold for the first level of conductance was set at the current level corresponding to 32 pS. Events with durations shorter than 1·5 times the inverse of the filter frequency (200–500 Hz, i.e. 7·5–3·0 ms) were not used for analysis. In addition to 50–60 pS channels, we observed more rarely smaller junctional currents of 25–30 pS. We were unable to analyse these smaller junctional currents quantitatively because the half-amplitude threshold for these openings (15 pS) was too close to the mean baseline noise. However, all channel activity above our initial threshold of 32 pS above baseline was included for analysis, so 25–30 pS openings that occurred in conjunction with each other or

with 50–60 pS openings contribute to the data set. Amplitude and duration histograms of the events lists were produced using the pSTAT program of pCLAMP.

Reagent preparation

Sodium nitroprusside (SNP) was obtained from ICN Biochemicals (Cleveland, OH, USA). 8-Br-cGMP, IBMX and haemoglobin were purchased from Sigma. RKRARKE, a peptide inhibitor of protein kinase G with the sequence Arg-Lys-Arg-Ala-Arg-Lys-Glu (Glass, 1983), was purchased from Peninsula Laboratories, Inc. (Belmont, CA, USA) and was dissolved in internal pipette solution for direct back-filling of the pipette. SNP and 8-Br-cGMP were dissolved in extracellular recording solution. LY-83583 was obtained from Research Biochemicals Inc. and was dissolved as a stock in methanol. IBMX was dissolved as a stock in DMSO. The maximum final bath concentration of methanol or DMSO was less than 0·2%. Preparation of haemoglobin was as described by Martin *et al.* (1985). Unless otherwise noted, drugs were delivered by bath application. Concentrations given for these agents are the final bath concentrations. Washout was performed by exchange of the drug solution with fresh extracellular solution by gravity perfusion. A dose of less than 0·2% DMSO or methanol alone had no significant effects on junctional conductance.

RESULTS

Effects of SNP on electrical coupling–macroscopic junctional current analysis

Electrical coupling was recorded in homologous H2-type cell pairs. In the dual whole-cell recording configuration, electrical coupling was evident as transjunctional coupling currents induced by voltage steps in one cell of a pair. Application of the nitric oxide donor sodium nitroprusside (SNP) to the bath decreased the amplitude of macroscopic coupling currents in horizontal cells. Figure 1 (upper panel) are sample recording traces of coupling currents in an H2-type horizontal cell pair before and during treatment with SNP (0·3 mM). Junctional currents were measured by stepping one cell of the pair −20 mV from the common holding potential of 0 mV. The time course of the SNP effect on the macroscopic junctional current in the same cell pair is shown in the lower panel of Fig. 1. Maximum inhibition of junctional conductance was achieved within 1–2 min after SNP application. Full recovery of the amplitude of coupling current took 5–10 min after washout of SNP. In this experiment, the coupling current decreased from a control value of 17·5 nS to 1·3 nS at the end of SNP application. The average per cent decrease obtained from ten experiments with 0·3 mM SNP was 62·7 ± 6·5% (mean ± S.D.). Extracellular application of the NO scavenger haemoglobin, at 100–300 μM, prevented the action of SNP (0·5 mM, $n = 5$, Fig. 7*A*). This suggests that the action of SNP is via NO released in the solution.

The release of NO in the experimental solutions containing SNP was measured directly using an NO sensor. SNP, at the range of concentrations applied in this study (0·3–3 mM), produced NO concentrations that ranged from 110 to 1200 nM. The [NO] released by various doses of SNP is shown in Table 1.

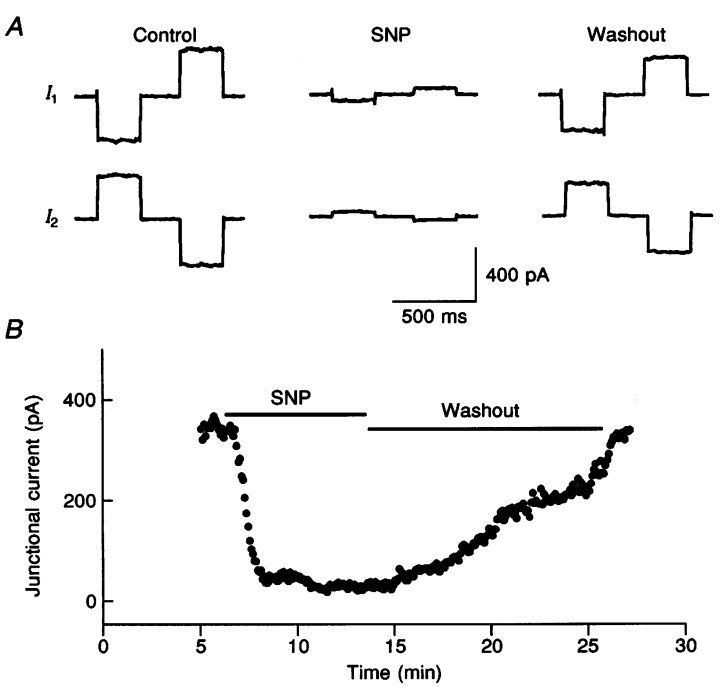

Figure 1. SNP reversibly decreased junctional conductance of bass retinal electrical synapses

A, coupling currents recorded from cell 1 (I_1) and cell 2 (I_2) of a cell pair before and after application of SNP (0·3 mM). Currents were acquired by stimulating one cell (driver cell) with a 20 mV, 250 ms pulse, and by holding another cell (follower cell) at 0 mV, then vice versa. The amplitudes of junctional currents were measured from the follower cell. *B*, time–effect curve before and after application of SNP.

Table 1. [NO] released by SNP

SNP (mM)	0·1	0·5	1	2	3
[NO] (nM)	69 ± 14	141 ± 21	410 ± 89	831 ± 193	1073 ± 211

The effects of SNP on voltage gating

The junctional current of bass horizontal cells exhibits voltage-dependent inactivation at elevated transjunctional voltages, as shown in Fig. 2*A*. In control conditions, the peak or instantaneous macroscopic junctional current (I_{inst}), was linear as transjunctional voltage was varied over ±90 mV, while the steady-state junctional current (I_{ss}) exhibited rectification due to voltage-dependent inactivation. After treatment with SNP ($n = 7$), both I_{inst} and I_{ss} were significantly decreased at all junctional voltages. Voltage sensitivity and equivalent gating charge of the junctional channels were derived by Boltzmann fits of the normalized steady-state current–voltage relationship. Figure 2*B* shows normalized junctional current (I_{ss}/I_{inst}) plotted *vs.* junctional voltage (V_j) and a Boltzmann fit of the data points with the equation:

$$G_{ss} = (G_{max} - G_{min})/(1 + \exp(A(V_j - V_0))) + G_{min}, \quad (1)$$

where G_{ss} is the normalized steady-state junctional conductance, G_{max} is the normalized maximum junctional conductance and G_{min} is the residual junctional conductance, which is resistant to voltage-dependent closure, A is the slope and V_0 is the half-maximal closure voltage. These parameters were obtained from cell pairs under control and SNP conditions for transjunctional voltages in which the driven cell of the pair was stepped to positive voltages while the follower cell was held at 0 mV. G_{ss} decreased with increasing transjunctional voltage (V_j) in both conditions. SNP decreased the apparent gating charge of junctional channels from approximately 2 to approximately 1, evident as a decrease in A from 0·104 ± 0·02 to 0·062 ± 0·01. However, SNP did not significantly change the sensitivity of horizontal cell gap junctions to voltage, as evident by the relatively small change in V_0 from 58·5 ± 6·4 to 56·4 ± 12·0 mV. An increase in the proportion of junctional current that was not closed by voltage (G_{min}) from 0·27 ± 0·10 to 0·33 ± 0·13 showed that SNP has a greater effect on I_{inst} than it does on I_{ss}.

Kinetic analysis of junctional current inactivation

The time course of voltage-dependent current inactivation was examined at 10 mV intervals of transjunctional voltage by fitting exponential functions to the decay phase of each

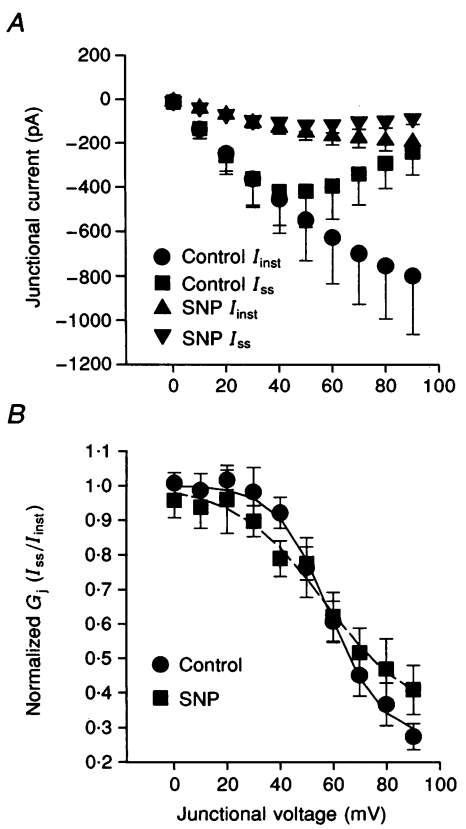

Figure 2. Modulation of SNP on voltage dependence of junctional currents

Junctional currents were recorded by stepping the voltage of cell 1 (V_1) from −90 to +90 mV in 10 mV increments and holding the voltage of cell 2 (V_2) at 0 mV. *A*, current–voltage relationships for I_{inst} and I_{ss} before and after treatment with SNP (0·3 mM, $n = 6$). Each data point represents the mean ± S.D. Only data at positive V_j values have been shown here. *B*, normalized steady-state junctional conductance (G_j) and transjunctional voltage (V_j) relation before and after treatment with SNP (0·3 mM). The steady-state junctional conductances (G_j) were derived from the ratio of I_{ss}/I_{inst}. The continuous and dashed lines are the theoretical fits of the data assuming a two-state Boltzmann distribution for control and SNP, respectively. Best fit of the Boltzmann relation was obtained for $V_0 = 58·5$ mV, $A = 0·104$ and $G_{min} = 0·27$ for control and $V_0 = 56·4$ mV, $A = 0·062$ and $G_{min} = 0·33$ for SNP. The slope (A) reflects the voltage sensitivity of the transition between two conductance states. The voltage axis intercept (V_0) is a function of the voltage sensitivity and the energy difference between these two states in the absence of a field.

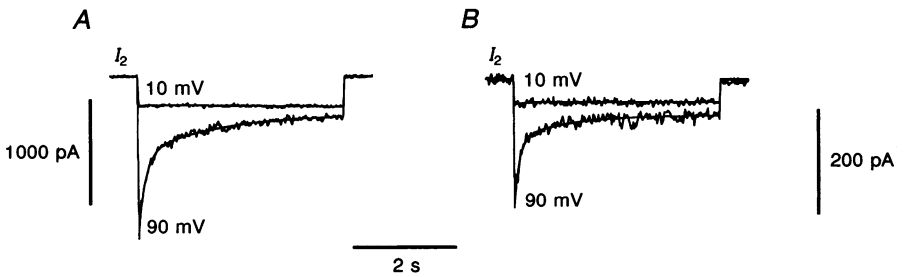

Figure 3. Junctional current decay accelerated by SNP

A and *B* show the current traces that are well fitted by two exponentials in control and SNP conditions. Current traces of junctional current (I_2) at V_j values of 10 and 90 mV are shown. SNP significantly decreased junctional current amplitude in both I_{inst} and I_{ss}. Note that the scales in *A* and *B* are different. SNP increased the rate of decay of junctional currents. The two fitted exponential time constants were 131·6 and 1349·8 ms for the control and 89·7 and 780·6 ms for SNP, respectively.

current trace. Current traces from voltage steps of less than ±50 mV were not included because of low correlation coefficients of fit, presumably due to the shallow slope of these functions. Traces could be fitted by one to two exponentials. In most cases, one exponential was sufficient to adequately fit control currents. After treatment with SNP, curve fitting for current decay became more difficult at lower values of V_j because SNP significantly reduced the junctional conductance, often to the point where single-channel events were evident, degrading the smooth nature of the closure function. In addition, one exponential was often not enough to describe the decay of current in the presence of SNP. Therefore, in the presence of SNP, only traces at ±90 mV V_j were used for curve fitting. After SNP treatment, the rate of current decay was increased in experiments performed with three cell pairs. An example was shown in Fig. 3, in which current decay is best fitted by two exponentials before and after SNP treatment. In this experiment, the two time constants were decreased from 131·6 and 1349·8 ms to 89·7 and 780·6 ms, respectively, indicating that channel closure has been accelerated by SNP.

Modulation of junctional currents at the single-channel level

To analyse further the interaction of gap junction channels and NO, single junctional channel currents were recorded in weakly coupled cell pairs. Each cell of the pair was voltage clamped to a different holding potential in order to maintain a constant transjunctional voltage difference. The gating of gap junction channels was evident as equal and opposite transitions in the holding current of the cells. Under control conditions, junctional conductance varied from baseline in conductance levels that were multiples of 50–70 pS. As with previous recordings from teleost retinal horizontal cells (McMahon *et al.* 1989; McMahon & Brown, 1994; McMahon & Mattson, 1996), these recordings can be interpreted as the result of independent gating of multiple gap junction channels, each with a unitary conductance of 50–70 pS. It is suggested that the higher conductance levels result from superposition of 50–70 pS conductances. Although the possibility that some of the higher conductance events represent a separate class of 130 or 200 pS channels cannot be rigorously excluded, most of the observed

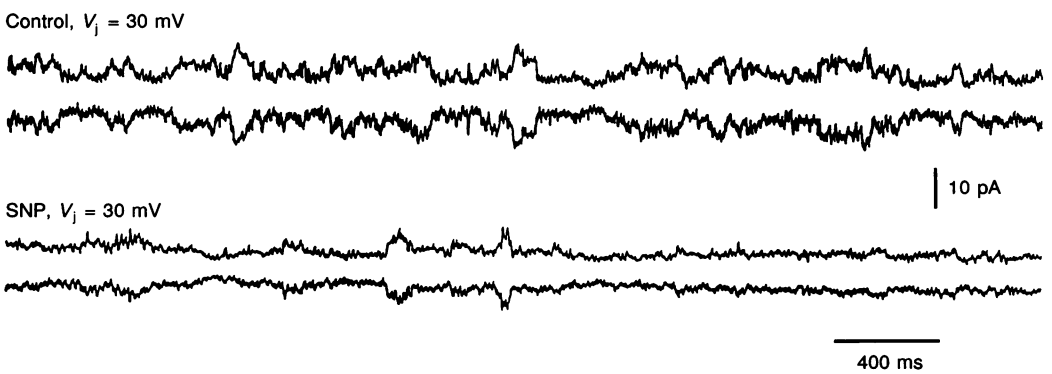

Figure 4. Single junctional channel recordings from a cell pair in control conditions (upper traces) and during application of 0·5 mM SNP (lower traces)

Gap junctional channel activity is evident as equal and opposite transitions of the two traces, with channel openings indicated by the traces moving apart. Currents were filtered at 200 Hz.

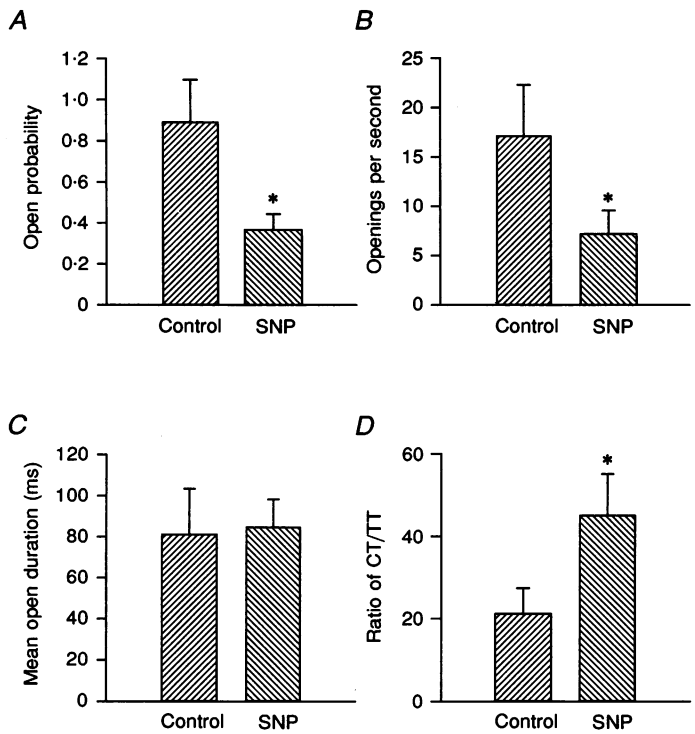

Figure 5. Effect of SNP on channel kinetics

Bar graph showing that SNP significantly decreased channel open probability (*A*), mainly by reducing channel open frequency (*B*), without significantly changing the mean channel open duration (*C*). *D*, SNP increased channel closure time. The ratio of the channel closing time/total recording time (CT/TT) was also increased. Each bar value represents the mean ± S.D. In comparison with control, * $P < 0{\cdot}05$ (Student's *t* test).

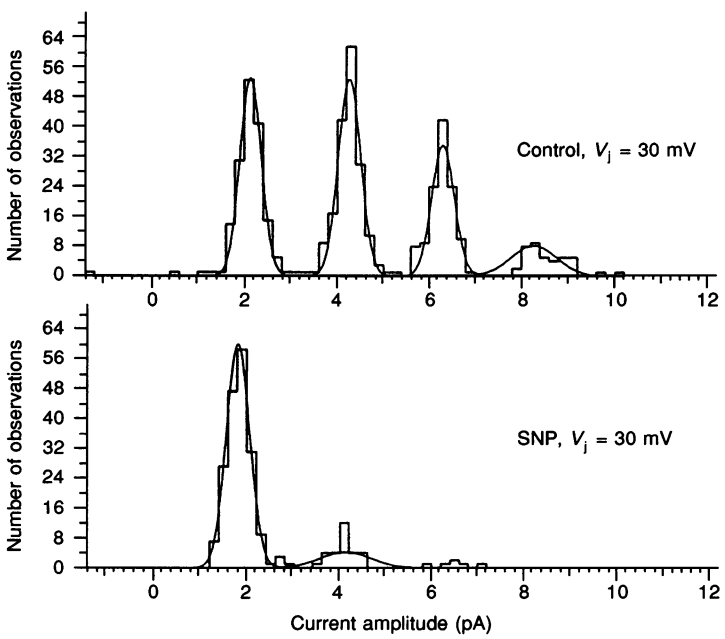

Figure 6. Amplitude histogram of channel openings before and after application of SNP (0·5 mM)

Bars indicate raw data, while the smooth curves indicate Gaussian fits with peaks of 70, 133, 210 and 273 pS for control and 61, 130 and 216 pS for SNP, respectively. SNP significantly reduced the frequency of events of high conductance.

transitions to 130 pS, 200 pS or higher levels were the result of a 50–70 pS step from the immediate lower conductance level rather than from baseline. At a transjunctional potential of 30 mV, the mean unitary conductance was 55·8 ± 6·9 pS ($n = 5$ cell pairs). This is essentially identical to the unitary conductances observed for teleost horizontal cell gap junction channels from three other species (McMahon *et al.* 1989; McMahon & Brown, 1994; McMahon & Mattson, 1996), and as expected less than half of the unitary conductance reported for catfish horizontal cell hemi-gap junction channels (DeVries & Schwartz, 1992), and similar to the unitary conductance observed for mammalian heart cell gap junctions (Burt & Spray, 1988).

After treatment with SNP (0·5–1 mM), gap junctional channel activity was significantly reduced, as shown in Fig. 4. Also shown in the example in Fig. 4 is a decrease in recording noise, which was often associated with NO modulation. This may be due to reductions in open channel noise from having, on average, fewer open gap junction channels or to effects of NO on ion channels in the extrajunctional membrane. As with uncoupling of the macroscopic junctional conductance, the decrease in channel activity induced by SNP was reversible when recordings were maintained for a sufficient duration.

Based on analysis of 170 s of recording from five cell pairs containing 3062 events in control conditions and 192 s of recording and 1264 channel openings after treatment with SNP, we found that, on average, SNP decreased overall gap junction channel open probability, calculated as the open probability of an individual channels (P_o) multiplied by the number of channels observed (N), from 0·88 ± 0·36 to 0·36 ± 0·07 ($n = 5$ cell pairs, $V_j = -30$ mV, $P < 0·05$, Student's unpaired t test, Fig. 5*A*). Further analysis showed that the decrease in overall junctional channel open probability by SNP is due to a reduction in the overall channel opening frequency rather than a decrease in the mean channel open duration. As shown in Fig. 5*B* and *C*, the opening frequency decreased from a control value of 17·2 ± 5·2 to 7·1 ± 2·4 openings per second and open duration did not change significantly from a control value of

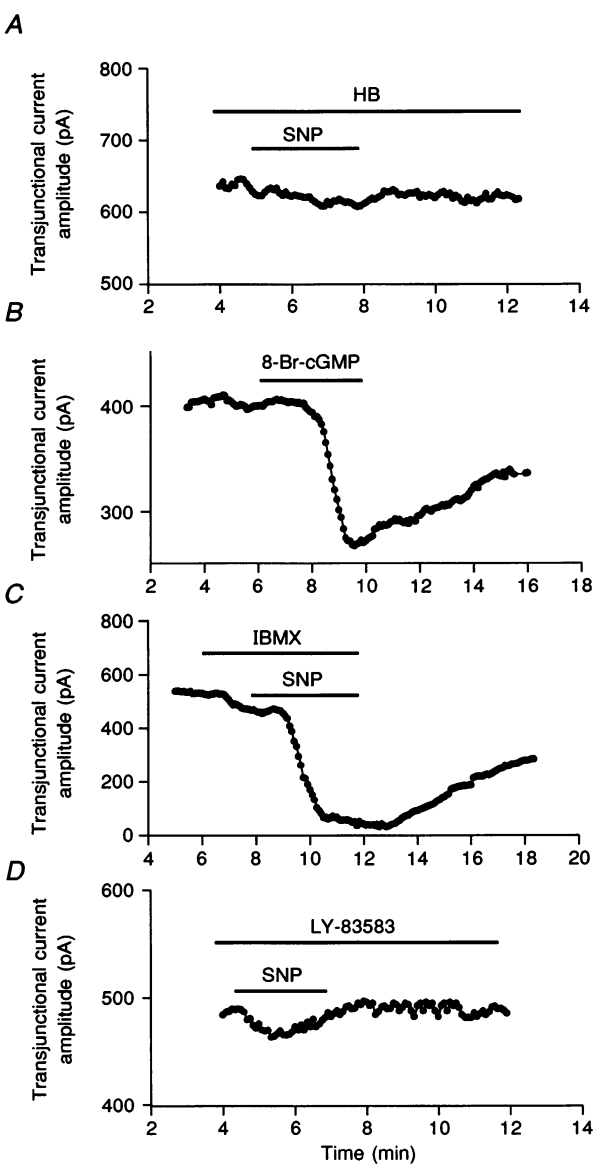

Figure 7. Time–effect curves of HB + SNP, 8-Br-cGMP, IBMX + SNP and LY-83583 + SNP on junctional currents of bass horizontal cells

A, pretreatment with haemoglobin (100–300 μM) prevented the effect of SNP. *B*, 8-Br-cGMP (0·3 mM) reduced junctional conductance. *C*, 0·1 mM IBMX alone produced a small decrease in junctional current but potentiated 0·1 mM SNP inhibition on junctional currents (see text for explanation). *D*, LY-83583 (0·3 mM) greatly reduced the effects of 0·5 mM SNP on junctional currents.

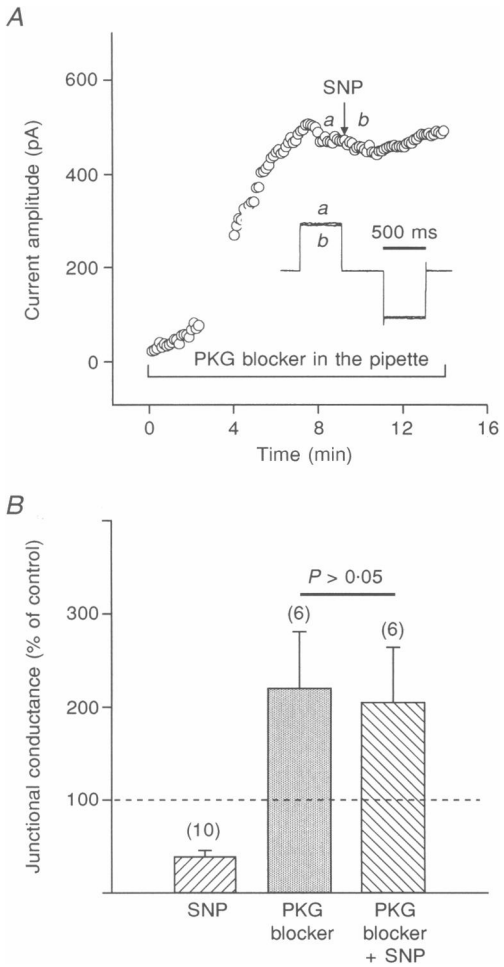

Figure 8. Effects of protein kinase G inhibitor on the action of SNP

A, time–effect curve before and after application of 3 mM SNP in the presence of PKG blocker (250 μM) in the pipette. Inset, original current traces acquired as in Fig. 1. *B*, bar graph shows that SNP (0·3 mM) alone significantly reduced junctional conductance. PKG blocker alone increased the basal coupling level of junctional conductance and inhibited the action of SNP. Dashed line is control level (set as 100 %), derived from an average of sixteen individual experimental control values. Each bar value represents the mean ± S.D. Numbers of experiments are indicated above each bar.

80·3 ± 23·4 to 84·5 ± 13·6 ms. SNP also increased channel closed time (dwell level zero) and the ratio of channel closed time/total recording time. The ratio of channel closed time/total recording time increased from an average of 0·2 ± 0·06 to 0·45 ± 0·1 ($n = 5$, $P < 0·05$, unpaired t test, Fig. 5*D*). SNP predominantly reduced the frequency of higher levels of conductance. As shown in Fig. 6, the number of multiple level openings to the third level (216 pS) and to the second level (130 pS) conductance were greatly reduced by SNP, and openings to the fourth level (273 pS) were eliminated. SNP also reduced the size of the unitary conductance from 70 to 61 pS in the individual experiment shown in Fig. 6. For the average of all five experiments, however, there was no statistical difference in unitary conductance following SNP application (55·8 ± 6·9 pS for control to 47·4 ± 4·4 pS for SNP, $n = 5$, $P > 0·05$, unpaired t test).

Modulation of junctional currents involves the NO–cGMP–PKG pathway

One mechanism of NO action is activation of the soluble form of guanylyl cyclase (Bredt & Snyder, 1989), thereby

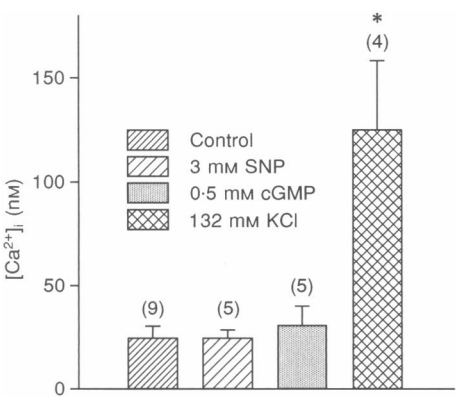

Figure 9. Effects of SNP, cGMP and KCl on basal $[Ca^{2+}]_i$

Bar graph shows that neither SNP (3 mM) nor cGMP (0·5 mM) has a significant effect on $[Ca^{2+}]_i$, but 132 mM KCl increases $[Ca^{2+}]_i$ of bass horizontal cells. Bars represent means ± S.D. The values above each bar give the number of channels observed.

mediating agonist-induced increases in intracellular cGMP levels in the central nervous system. To examine whether the guanylate cyclase is involved in the regulation of horizontal cell junctional currents by NO, cells were pretreated with a guanylate cyclase antagonist, LY-83583 (300 μM). In contrast to the strong effects of SNP when it was applied alone, when it was applied in the presence of LY-83583, 0·5 mM SNP produced an average of only 12 $\pm$ 7·8% ($n = 5$) inhibition on coupling currents (Fig. 7*D*). Application of the non-specific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) at 100 μM, produced a small decrease (less than 22% of control on average, $n = 4$) in coupling current, while SNP alone at 100 μM produced less than 26% ($n = 5$) inhibition on coupling currents. However, with concomitant use of IBMX plus SNP (100 μM each), the uncoupling effects of SNP were significantly potentiated (Fig. 7*C*). On average, concomitant use of IBMX and SNP resulted in 78 $\pm$ 14% inhibition ($n = 4$).

To determine whether NO acts on horizontal cell gap junction channels through cGMP-dependent protein kinase, 8-Br-cGMP (300 μM), a PKG stimulator, was applied and was found to mimic the effect of SNP (Fig. 7*B*). Furthermore, the highly specific cGMP-dependent protein kinase blocker, a seven amino acid peptide (RKRARKE, 250 μM in the pipette), blocked the effect of SNP (up to a dose of 3 mM; Fig. 8*A*). In addition, this PKG inhibitor increased the basal coupling level. Since no true control can be determined with internal dialysis of the PKG inhibitor, the mean value of controls taken from sixteen experiments was used as the control for the PKG inhibitor experiments. In comparison with this control, the coupling level following dialysis with RKRARRE is significantly higher (Fig. 8*B*, $n = 6$).

The SNP effect on junctional currents is $[Ca^{2+}]_i$ independent

We have examined the effects of SNP and cGMP on $[Ca^{2+}]_i$ using $[Ca^{2+}]_i$ imaging studies in cultured horizontal cell pairs. The basal calcium level in horizontal cells was 20–60 nM in nine experiments. Culture medium was then replaced with SNP (0·3–3 mM) or 8-Br-cGMP (200 μM–500 μM) or an external solution containing 132 mM KCl. Neither SNP nor 8-Br-cGMP had a significant effect on $[Ca^{2+}]_i$ in five experiments for each agent, but KCl (132 mM) in the external solution caused a fivefold increase in $[Ca^{2+}]_i$ ($n = 4$; see Fig. 9). These results suggest a $[Ca^{2+}]_i$-independent mechanism for NO–cGMP in the regulation of junctional channels.

DISCUSSION

There is accumulating evidence that the gas NO is involved in the modulation of cellular function and acts as a regulatory molecule in a variety of tissues (Garthwaite, 1991; Snyder & Bredt, 1991). In the nervous system, NO is thought to be a retrograde intercellular messenger, which is formed in response to activation of excitatory amino receptors (NMDA subtype) and plays an important role in the regulation of synaptic plasticity (Schuman & Madison, 1991). Recent evidence indicates that NO also plays a role in neurotoxicity in the retina (Geyer, Almog, Lupu-Meiri, Lazar & Oron, 1995). A primary target of NO appears to be the modulation of ion channels. While the actions of NO in the retina are just beginning to be explored, it has previously been shown to modulate ion channel function in horizontal cells (Miyachi *et al.* 1990), bipolar cells (Shiells & Falk, 1992), photoreceptors (Kurenny *et al.* 1994) and ganglion cells (Ahmad *et al.* 1994).

The present study has examined the biochemical and biophysical mechanisms by which NO modulates horizontal cell electrical synapses. Nitric oxide and cGMP have been shown previously to have modulatory effects on horizontal cell gap junctions. Stimulation of nitric oxide synthase (NOS) with L-arginine, exposure to nitric oxide donors, and application of membrane-permeable cGMP analogues have all been shown to reduce electrical coupling between retinal horizontal cells (DeVries & Schwartz, 1989; Miyachi *et al.* 1990).

It has been found that the NO-generating substance SNP uncoupled the junctional conductance of bass horizontal cells. The ability of haemoglobin to block SNP is consistent with the notion that nitric oxide itself induces the SNP effects on gap junctions. The spontaneous yields of NO in our SNP solutions with an NO microsensor has also been confirmed, again suggesting that it is NO that plays a role in the control of junctional channels. The fact that LY-83583, an inhibitor of soluble guanylate cyclase, greatly reduced the action of SNP suggests that nitric oxide acts on horizontal cell gap junctions by activating soluble guanylate cyclase to produce cGMP. The protein kinase G (PKG) activator, 8-Br-cGMP, mimicked the action of SNP, suggesting that cGMP-dependent protein kinase is present in horizontal cells. Application of the cGMP-dependent protein kinase inhibitor (RKRARKE) in the patch pipette blocked the action of SNP on the junctional conductance. In addition, RKRARKE produced a significant increase in the basal coupling level, suggesting a basal level of phosphorylation of the junctional channel by protein kinase G. These two aspects of PKG inhibitor action strongly suggest that NO modulates horizontal cell electrical synpases by inducing a cGMP-dependent phosphorylation of gap junctional channels.

NO affects the voltage-dependent closure and single-channel kinetics of horizontal cell gap junction channels. Voltage-dependent analysis of gap junctional currents suggests that NO reduces both the instantaneous and steady-state junctional currents, with greater inhibition of the instantaneous current at transjunctional voltages greater than 40 mV. However, voltage sensitivity of junctional channels, as expressed by the half-maximal closure voltage (V_0), was not significantly affected. The high half-maximal closure voltage of greater than $\pm$50 mV is unlikely to be obtained *in vivo* in the well-coupled horizontal cell network. Thus while voltage dependence is a useful parameter for

biophysical characterization of horizontal cell gap junction channels, its functional significance for retinal circuits is unclear. It has also been found that there are gap junction channels in bass horizontal cells that are insensitive to both voltage and SNP (i.e. there is a non-zero G_{min} after treatment with SNP). Whether this portion of current is derived from a subconductance state of a single population of gap junction channels (Moreno, Rook, Fishman & Spray, 1994) or a second type of gap junction channel expressed in horizontal cell junctions, cannot be determined from the Boltzmann analysis that assumes a single population of channels with only open and closed states. It is not yet known if the multiple connexins are expressed in bass horizontal cells.

Analysis of single-channel data showed that NO reduced channel open probability by reducing channel opening frequency without significantly affecting open duration. The changes in gap junction channel opening frequency induced by SNP (−60%) are appropriate in magnitude and direction to fully account for the reduction in macroscopic junctional currents induced by NO (−63%). Interestingly, it has been found previously that dopamine reduced the channel activity by reducing both channel open duration as well as opening frequency (McMahon *et al.* 1989; McMahon & Brown, 1994; McMahon & Mattson, 1996). Dopamine is known to act on horizontal cell gap junction channels through activation of the cAMP–PKA (protein kinase A) pathway (Lasater, 1987; DeVries & Schwartz, 1989). Therefore, phosphorylation by these two different kinases exhibits similar effects on the macroscopic junctional current, but different effects on the gating kinetics of junctional channels. One possible explanation of these observations is that PKA and PKG phosphorylate overlapping but not identical sites on horizontal cell gap junction channels. This notion is supported by preliminary experiments in this laboratory, which indicate a synergistic interaction of dopamine and NO on junctional conductance (C. Lu & D. G. McMahon, unpublished observations).

The functional effects of NO on retinal circuits and the adaptational conditions that induce NO release in the vertebrate retina are as yet unclear. Application of nitric oxide donors has been reported to induce morphological changes in fish horizontal cells, similar to those observed following light adaptation (Greenstreet & Djamgoz, 1994), and light adaptaion has been shown to induce changes in horizontal cell receptive fields that are not mediated by dopamine (Umino, Lee & Dowling, 1991). Alternatively, there is also evidence for a non-dopaminergic horizontal cell modulator released in the dark. Recent work has shown that there is an as yet uncharacterized non-dopaminergic signal that contributes to suppression of horizontal cell light responses during prolonged dark adaptation (Yang, Fau & Shen, 1994). Since the diffusion of NO is thought to be limited by its short half-life, NO may serve as a more local modulator than dopamine, which has been shown to diffuse widely in the retina. In addition to its effects on electrical synapses, NO–cGMP–PKG has also recently been shown to suppress responses from horizontal cell glutamate receptors (McMahon & Ponomareva, 1996). Thus the overall effect of NO on horizontal cell network function is likely to be complex and additional information on the release of NO in the retina and the precise mechanisms of the action of NO on horizontal cell synaptic conductances will be needed to understand its physiological role in the outer plexiform layer.

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