Cyclic GMP–gated Channels in a Sympathetic Neuron Cell Line

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ABSTRACT The stimulation of IP_3 production by muscarinic agonists causes both intracellular Ca^{2+} release and activation of a voltage-independent cation current in differentiated N1E-115 cells, a neuroblastoma cell line derived from mouse sympathetic ganglia. Earlier work showed that the membrane current requires an increase in 3′,5′-cyclic guanosine monophosphate (cGMP) produced through the NO-synthase/guanylyl cyclase cascade and suggested that the cells may express cyclic nucleotide–gated ion channels. This was tested using patch clamp methods. The membrane permeable cGMP analogue, 8-br-cGMP, activates Na^+ permeable channels in cell attached patches. Single channel currents were recorded in excised patches bathed in symmetrical Na^+ solutions. cGMP-dependent single channel activity consists of prolonged bursts of rapid openings and closings that continue without desensitization. The rate of occurrence of bursts as well as the burst length increase with cGMP concentration. The unitary conductance in symmetrical 160 mM Na^+ is 47 pS and is independent of voltage in the range −50 to +50 mV. There is no apparent effect of voltage on opening probability. The dose response curve relating cGMP concentration to channel opening probability is fit by the Hill equation assuming an apparent K_D of 10 μm and a Hill coefficient of 2. In contrast, cAMP failed to activate the channel at concentrations as high as 100 μm. Cyclic nucleotide gated (CNG) channels in N1E-115 cells share a number of properties with CNG channels in sensory receptors. Their presence in neuronal cells provides a mechanism by which activation of the NO/cGMP pathway by G-protein–coupled neurotransmitter receptors can directly modify Ca^{2+} influx and electrical excitability. In N1E-115 cells, Ca^{2+} entry by this pathway is necessary to refill the IP_3-sensitive intracellular Ca^{2+} pool during repeated stimulation and CNG channels may play a similar role in other neurons.

KEY WORDS: Ca^{2+} homeostasis • cGMP • CNG channel • N1E-115 neuroblastoma cells • patch clamp

INTRODUCTION

The stimulation of inositol 1,4,5-trisphosphate (IP_3) production by neurotransmitters results in the release of calcium from intracellular storage compartments associated with the endoplasmic reticulum (Berridge, 1993). During repetitive stimulation, much of the calcium that is released into the cytosol is removed from the cell by the action of Ca^{2+}-ATPases and Na^+/Ca^{2+} exchangers (Carafoli, 1987; Dipolo and Beauge, 1988; Reeves, 1992). This leaves the intracellular compartments partially depleted of Ca^{2+} and the deficit must be restored by the activation of a membrane Ca^{2+} current. Identifying the Ca^{2+} currents and the regulatory processes involved in refilling intracellular Ca^{2+} stores is important for understanding Ca^{2+} homeostasis in neurons and other excitable cells.

In N1E-115 neuroblastoma cells, a cell line derived from mouse sympathetic ganglion neurons, the activation of M1 muscarinic receptors stimulates phospholipase-Cβ to produce IP_3 and causes both intracellular Ca^{2+} release and activation of a Ca^{2+} current (Mathes and Thompson, 1995). Membrane permeable analogs of 3′,5′-cyclic guanosine monophosphate (cGMP) activate a current with similar properties and activation of the current by agonist is prevented by inhibitors of cGMP production. In addition, this cGMP regulated pathway is both necessary and sufficient to replenish intracellular Ca^{2+} stores during repeated stimulation of muscarinic receptors (Harrington and Thompson, 1996). These observations suggested that cGMP may provide the link between the activation of M1 receptors and Ca^{2+} influx (Mathes and Thompson, 1996).

This proposal was tested using single channel recording methods and it was found that N1E-115 cells express cation channels that are directly activated by cGMP. The single channel currents share features in common with the cyclic nucleotide–gated (CNG)[1] currents responsible for phototransduction in retinal rods and cones and odor transduction in olfactory receptor cells (Zagotta and Siegelbaum, 1996; Zufall, 1996). The increase in cGMP that occurs during muscarinic activation in N1E-115 cells requires nitric oxide (NO) which is generated by NO-synthase specifically in response to the increase in $(Ca^{2+})_i$ (McKinney et al., 1990; Arroyo et al., 1991; Hu and El-Fakahany, 1993; Thompson et al., 1995). This suggests that Ca^{2+} entering via cGMP-

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[1]*Abbreviations used in this paper:* CNG, cyclic nucleotide–gated.

gated channels may participate in positive feedback regulation of NO synthase, leading to an amplification of the NO and cGMP signals (Thompson et al., 1995). Positive feedback may explain the large amplitudes of cGMP signals measured in this cell line. A similar process may occur in other neurons that are known to experience increases in cyclic nucleotide production during stimulation, for example in the cerebellum, forebrain, hippocampus, and sympathetic ganglia.

MATERIALS AND METHODS

N1E-115 neuroblastoma cells (Amano et al., 1972) were obtained from the UCSF Cell Culture facility and used in passages 3–8. Cultures were maintained in DMEM with 10% FBS (HyClone, Logan, UT) at 37°C with 10% CO_2, plated on glass cover-slips, and grown to ~60% confluence before differentiation with DMSO for 5–21 days (Kimhi et al., 1976). The cultures were fed every 2–3 d and used 1–2 d after feeding.

Single channel currents were recorded in cell-attached and inside out patch configurations using Axopatch 200A or List EPC/7 amplifiers. Records were filtered before digitizing with a 8-pole bessel filter (Frequency Devices, Inc., Haverhill, MA) and data acquisition and analysis was done using PCLAMP (Axon Instruments, Foster City, CA) and DEMPSTER software. Pipettes were pulled from fiber filled borosilicate capillary tubing (Sutter Instruments Co., Novato, CA) just before experiments, coated to near the tip with Silgard elastomer (Dow Corning Corp., Indianapolis, IN), and fire polished. The experimental chamber was maintained at a temperature of 30°C by a peltier device. The normal external saline contained (mM); 146 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgS0_4$, 0.4 KH_2PO_4, 0.3 Na_2HPO_4, 5 glucose, 20 HEPES, and 1 μM TTX (pH 7.4). In experiments on excised inside out patches, both sides of the membrane were exposed to a low divalent solution containing (mM); 160 NaCl, 10 EGTA, 1 EDTA, 10 HEPES (pH 7.2). Stimulus solutions containing cyclic nucleotides were applied to the inner membrane face by positioning the tip of the patch pipette in the continuous stream from a delivery tube. The solution flowing in the delivery tube was switched with a solenoid valve and required about 10 s to fully equilibrate. Na^+-cGMP, Na^+-cAMP and 8-br-cGMP (Sigma Chemical Co., St. Louis, MO) were stored as 10 mM stock solutions in distilled water. Final solutions were made by serial dilution in the appropriate saline.

RESULTS

8-br-cGMP Activates Na^+ Permeable Channels in Cell-attached Patches

Discrete openings of cyclic nucleotide–gated (CNG) channels in retinal rods and cones are difficult to observe in normal physiological saline because the channels are subject to block by divalent cations. In the absence of divalent ions, however, unitary currents carried by sodium can be resolved and analyzed (Haynes et al., 1986; Zimmerman and Baylor, 1986; Matthews and Watanabe, 1987, 1988; Haynes and Yau, 1990; Taylor and Baylor, 1995). The same approach was used to measure single channel currents activated by cyclic nucleotides in differentiated N1E-115 mouse neuroblastoma cells.

Single channel currents were recorded in cell attached patch configuration during the application of 8-br-cGMP, a membrane permeable analogue of cGMP. The pipette solution contained 153 mM NaCl, 10 mM EGTA, 1 mM EDTA, 1 μM TTX, and 10 HEPES (pH 7.4; 30°C), and the potential across the patch was equal to the resting potential, estimated from microelectrode measurements to be between −20 and −40 mV. Fig. 1 *A* shows records taken before and at successive times after adding 1 mM 8-br-cGMP in the external medium. The cGMP analogue activates a channel permeable to Na^+. Channel activity occurs in bursts of brief openings, and the rate of occurrence of bursts as well as the

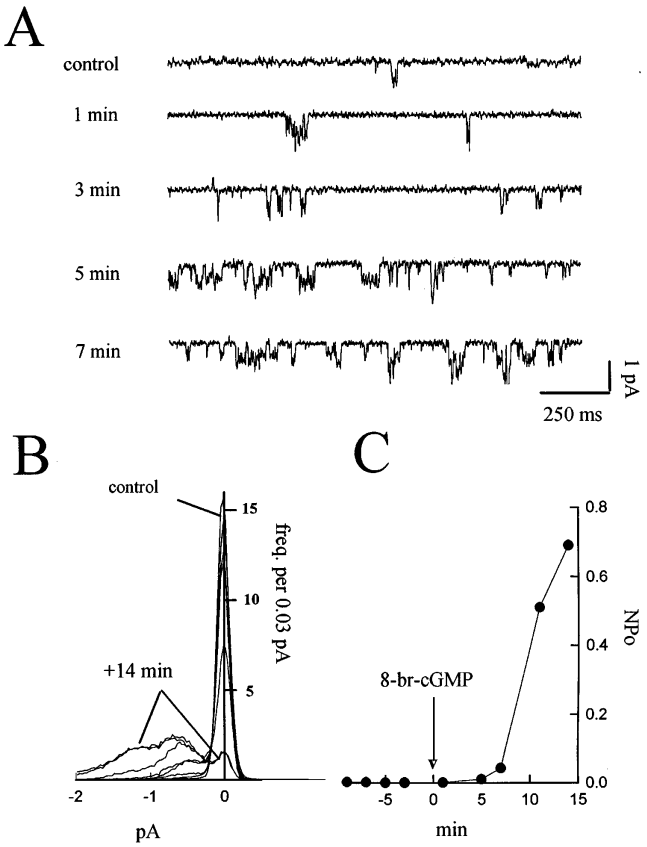

FIGURE 1. The membrane permeable cGMP analogue, 8-br-cGMP, activates cation channels in cell attached patches in differentiated N1E-115 cells. (*A*) Current records taken from a continuous recording before and 1, 3, 5, and 7 min after adding 1 mM 8-br-cGMP in the external saline. The pipette contained 153 mM NaCl, 10 mM EGTA, 1 mM EDTA, 1 μM TTX, and 10 HEPES (pH 7.4; 30°C). The pipette voltage was 0 mV and inward Na^{2+} currents are shown as downward deflections. Filter corner frequency = 1.5 kHz. (*B*) Amplitude histograms generated during 10-s recordings taken before and 1, 3, 5, 7, 11, and 14 min after adding 1 mM 8-br-cGMP (bin width 0.031pA). (*C*) Increase in the probability of one or more channels being open (NP_o) as a function of time before and after adding 1 mM 8-br-cGMP. NP_o was estimated by dividing the mean open time by the mean closed time during 10-s records taken at the times indicated. A 50% criterion was used to identify openings.

burst length increase with time during exposure to 8-br-cGMP. Amplitude histograms constructed from 10-s segments recorded at various times after adding 8-br-cGMP are shown in Panel *B*. The peak at zero current represents the background noise. Additional peaks at approximately −0.6 and −1.2 pA begin to appear and increase in amplitude with time. The progressive change in the amplitude histogram indicates that this patch contained at least two inward current channels that are activated by 8-br-cGMP and that each channel contributes a current of ~0.6 pA. Opening probability for the multiple channels in this patch (NP_o) was estimated from the ratio of mean open time to mean closed time using a 50% criterion for detection of unitary events. NP_o increased gradually, reaching approximately 0.7 after 14 min (Panel *C*). The accuracy of this plot is limited because the number of channels in the patch is not known precisely and because brief openings may have been missed due to low pass filtering. Nevertheless, the increase in opening probability follows approximately the same time course as the increase in whole cell inward current measured with perforated patch voltage clamp under similar conditions (Mathes and Thompson, 1996). In both cases, gradual activation probably results from slow entry and gradual intracellular accumulation of the cGMP analogue.

cGMP-gated Channels in Inside Out Patches

Single channel currents were recorded from inside out patches exposed to symmetrical Na^+ solutions. In the example in Fig. 2, the voltage across the patch was 40 mV, pipette positive, and the inside face of the membrane was perfused with saline containing 10 μM cGMP for the period indicated (Panel *A*). This rapidly and reversibly activated a Na^+ permeable channel. Channel openings occurred in bursts separated by silent periods and only rarely as isolated single openings. There was no evidence of desensitization during exposures to cGMP lasting several minutes. A portion of the record is shown on an expanded time scale in Panel *B* to illustrate the burst structure. In the presence of cGMP, the channel flickers rapidly between open and closed states and some of these events are too brief to fully resolve. Panel *C* shows the distribution of patch current amplitudes during exposure to 10 μM cGMP. In this and each of the following examples the amplitude histogram was free of points that might indicate the presence of more than one channel. The solid line represents the sum of two gaussian distributions fitted to the data. The channel undergoes transitions from a closed state to an open state with a mean current amplitude of −1.44 pA at this voltage. In the presence of 10 μM cGMP the opening probability (P_o) estimated from the areas under the fitted curves was 0.43. The I(V) curve for single channel currents was measured by varying the pipette potential and is linear in the range −50 to +60 mV in symmetrical Na^+ solutions with an intercept near zero mV and a slope conductance of 47pS (Fig. 3 *A*). There was no obvious effect of pipette potential on opening probability.

The kinetic behavior of single channel currents was estimated from 20-s long recordings of channel activity in the presence of 10 μM cGMP using a filter cutoff frequency of 1.5 kHz and a 50% criterion for the identification of channel openings. Open and closed time histograms are shown in Fig. 3 *B*. The open time histogram was fitted by the sum of two exponentials with time constants of 0.57 (~40% of openings) and 4.9 ms (r = 0.43). This is consistent with the flickery appearance of single channel currents. The closed time histo-

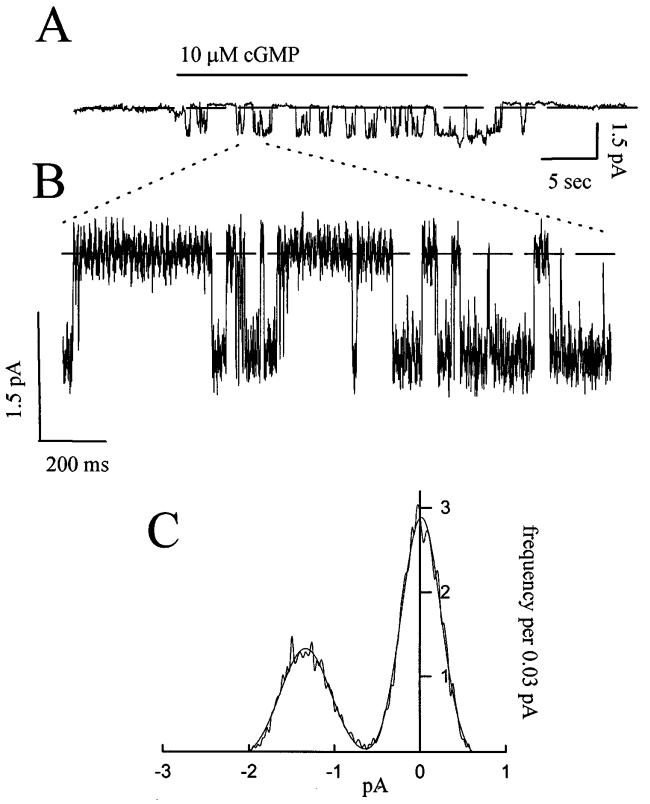

FIGURE 2. Cyclic GMP activates single channel currents in excised, inside out membrane patches. (*A*) Patch current during exposure to 10 μM cGMP for the period indicated by the horizontal bar. Both sides of the membrane were exposed to the same Na^+ saline (composition in mM; 160 NaCl, 10 EGTA, 1 EDTA, and 10 HEPES, pH 7.4) and the potential across the patch was 40 mV, pipette positive. A dashed line shows the zero current level (filter cut-off frequency = 200 Hz). (*B*) A segment of the record is shown on an expanded time base to illustrate the burst structure (filter cutoff frequency = 1.5 kHz; sampled at 100 μs/pt). (*C*) Current amplitude histogram during exposure to 10 μM cGMP (filter cutoff frequency 1.5 kHz; bin width 0.03 pA). The distribution was fitted by the sum of two gaussian functions with mean amplitudes of zero and −1.44 pA using the method of least squares. The opening probability in 10 μM cGMP was 0.43.

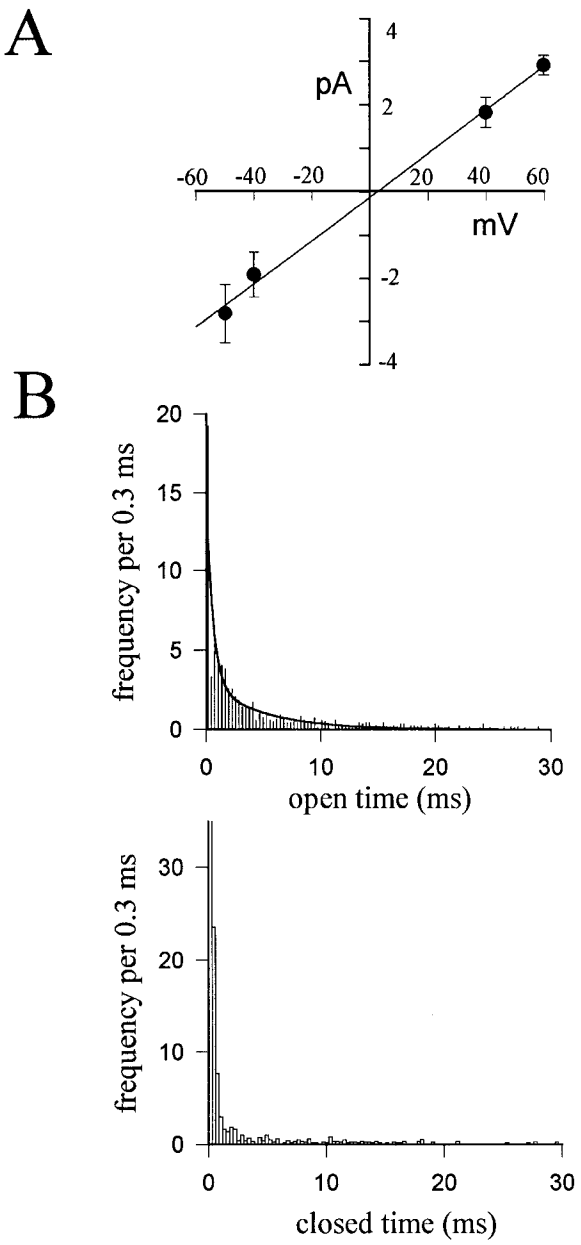

FIGURE 3. (*A*) Current-voltage relationship for single channel currents in symmetrical Na^+ solutions. Measurements were taken from the same patch during exposure to 10 μM cGMP applied to the inner membrane face. Points represent the mean current amplitude (±SD) for 10–12 well defined single openings at each voltage (filter cut-off frequency = 1.5 kHz). The solid line was fitted to the data by least-squares linear regression. (*B*) Open and closed time histograms of channel activity in response to 10 μM cGMP (0.3 ms bin size). The 20-s record used in this example contained 2431 events (filter cut-off frequency 1.5 kHz; 100 μs/pt.). The open time histogram was fitted by the sum of two exponentials (*solid line*) with time constants of 0.57 and 4.9 ms, accounting for 40 and 51% of all openings, respectively. The opening probability (P_o) calculated from the all points histogram was 0.46.

gram shows that the majority (86%) of channel closures are less than 10 ms in duration but there are also much longer closures, a feature consistent with the observation that openings tend to occur in bursts. The longer closures are described in more detail below. At an agonist concentration of 10 μM, the average burst duration, assuming a critical closed period of 10 ms and excluding single openings, was 83 ms with an average of 10.8 openings per burst.

Dose-Response Curve for Channel Activation by cGMP

Solutions containing different concentrations of cGMP were applied to the inner face of membrane patches. Fig. 4 *A* shows examples of channel activity at four

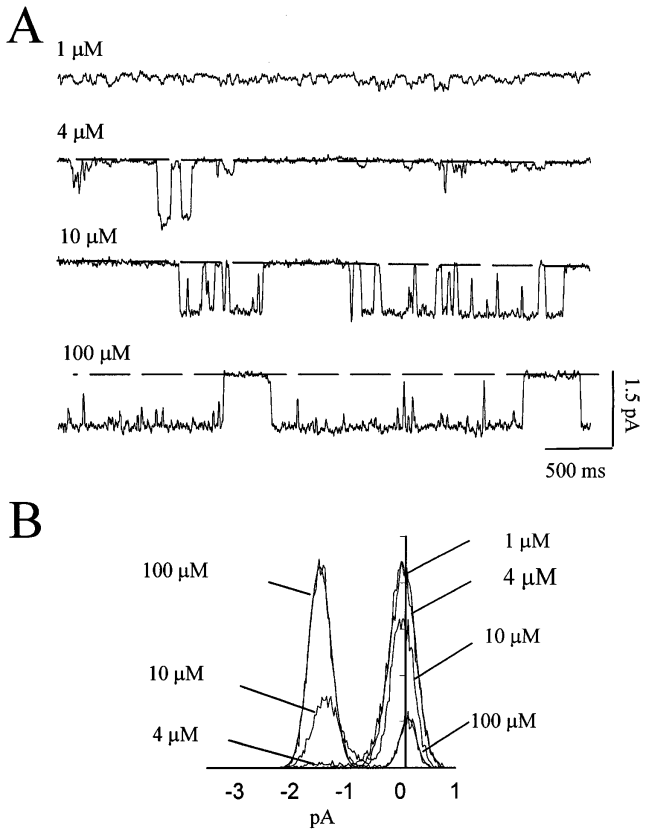

FIGURE 4. Effect of increasing cGMP concentration on channel activity. (*A*) Sample records from an inside out patch in symmetrical Na^+ solutions. Solutions containing different concentrations of cGMP were applied to the inner face of the membrane by a multichannel perfusion device and channel activity is shown at four cGMP concentrations. The zero current level is indicated by dashed lines (pipette voltage = 40 mV; analog filter cutoff frequency = 500 Hz). (*B*) Amplitude histograms taken at four cGMP concentrations (0.026 pA/bin). The individual histograms were made from 10-s record segments (filter frequency = 1.5 kHz). The amplitude distribution with 100 μM cGMP was fitted by the sum of two gaussian functions and the fitted curve is shown by a smooth line superimposed on the data. The unitary current amplitude was −1.5 ± 0.2 pA (mean ± S.D.; 40 mV, pipette positive). P_o at 100 μM cGMP was 0.84.

cGMP concentrations in a patch containing a single cGMP dependent channel. It demonstrates that channel opening increases with cGMP concentration. From an analysis of 10-s segments taken during long records at each cGMP concentration it was found that the mean open time increases from 0.46 ms in 1 μM cGMP to between 4.86 and 7.0 ms in 100 μM cGMP while the mean closed time decreases from ~20 ms to between 0.8 and 3.7 ms over the same concentration range. Burst length increases dramatically from a mean of 8–20 ms in 1 μM cGMP to 316–941 ms in 100 μM cGMP, as does the average number of openings per burst (3–7 vs. 101–119). The principle factor responsible for the increase in opening probability at higher cGMP concentrations, therefore, is increased burst length. Fig. 4 *B* shows current amplitude histograms taken at four different cGMP concentrations. It is apparent from the histograms that the channel undergoes transitions from a closed state to one predominant open state with a unitary current amplitude of −1.5 ± 0.2 pA at the 40 mV pipette potential and that the time spent in the open state increases with cyclic nucleotide concentration.

The amplitude distributions for single channel currents at different cGMP concentrations were fitted with the sums of two gaussian functions corresponding to the closed and open states of the channel. Values of opening probability (P_o) were calculated from the areas under the fitted curves and used to generate a dose-response curve for channel activation by cGMP. Fig. 5 shows the results from five patches, each containing a single CNG channel, normalized to the opening probability measured in 100 μM cGMP. The solid line in the figure is the solution of the Hill equation for K_D = 9.4 μM and n = 2 where K_D is the apparent dissociation constant for channel activation by cGMP and n is the Hill coefficient. The opening probability at 100 μM cGMP, which is assumed to be a saturating concentration, was 0.84. This suggests that cGMP dependent channels continue to open and close even when agonist binding sites are fully occupied.

Channel activation is much more sensitive to cGMP than to cAMP. Fig. 6 shows results obtained from an in-

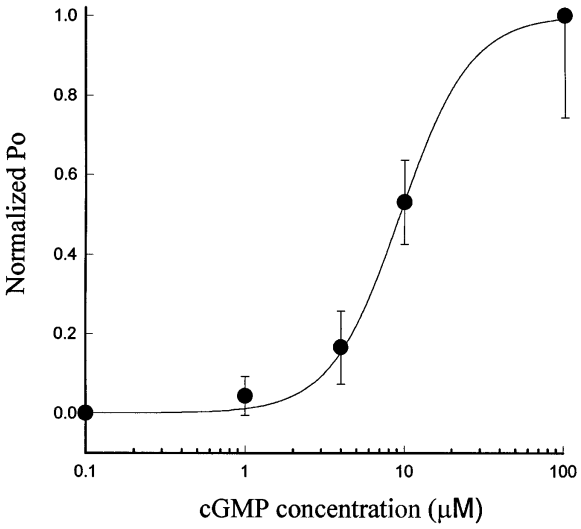

FIGURE 5. Dose-response curve for single channel currents. Opening probability (P_o) is plotted as a function of cGMP concentration. Data were obtained by sampling 2-s to 2-min long record segments taken at four different cGMP concentrations (filter cut-off frequency; 1 kHz). Records with long closed periods were excluded from the analysis. Amplitude histograms were made from each record and fitted by the sum of two gaussian distributions. P_o was obtained either from the areas under the fitted curves or from measures of mean closed and mean open time using a 50% amplitude criterion. The points represent the mean (±S.D.) value of P_o from five single channel patches. The solid line is the nonlinear least-squares fit to the Hill equation ($P = P_{max} \cdot [cGMP/K_D]^n/(1 + [cGMP/K_D]^n)$) using a dissociation constant, K_D, of 9.4 μM and Hill coefficient, n, of 2, assuming $P_{max} = 1$.

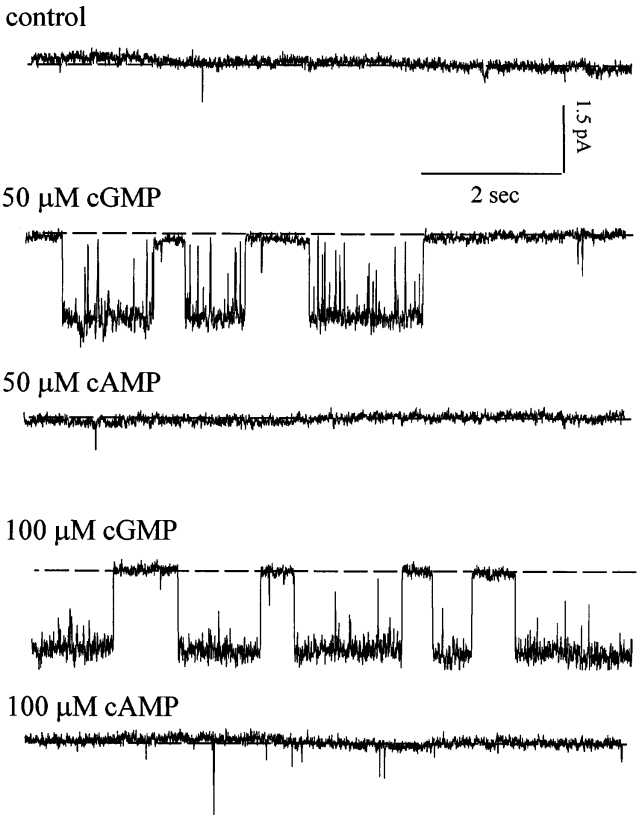

FIGURE 6. Sensitivity to cGMP and cAMP measured in the same single channel patch. A membrane patch was excised in symmetrical Na^+ saline. The solution perfusing the inside face was changed every 30 s, switching between control saline and salines containing cGMP or cAMP at 50 and 100 μM concentrations. Records were taken 20 s after changing the solution flowing in the delivery pipette. Channel opening probability was 0.47 in 50 μM cGMP and 0.7 in 100 μM cGMP. cAMP failed to activate channel activity (filter corner frequency 100 Hz; 50 mV pipette potential; zero current level shown by dashed lines).

dividual membrane patch containing a single CNG channel that was exposed alternately to cGMP, cAMP, and control solutions. Records were taken 20 s after changing solutions. Channel opening probability was 0.47 in 50 μM cGMP and 0.7 in 100 μM cGMP. In contrast, the same concentrations of cAMP failed to activate channel activity in this experiment or in any of 23 other experiments on inside out patches. The insensitivity to cAMP is consistent with the results of Ca^{2+} imaging experiments by Mathes and Thompson (1996).

Evidence for Multiple Open and Closed States

There is evidence that cGMP-dependent channels in N1E-115 cells undergo transitions to a long lived closed state. In the example shown in Fig. 7 *A*, 100 μM cGMP was applied to the inside of a patch containing a single CNG channel. The most common behavior at high cGMP concentrations is continuous rapid flicker between closed and open states without noticeable desensitization. Periodically, however, the record is interrupted by long channel closures. This suggests additional closed states that are entered with low probability even in the presence of a saturating concentration of agonist. Recovery from these states occurs slowly. The open time distribution in 100 μM cGMP is shown in Fig. 7 *B*. It is well fitted by the sum of two exponential relaxations with time constants of 5 and 32 ms. When compared with the data in Fig. 3 *B* this shows that one effect of increased cGMP concentration is an increase in channel open time. Fig. 7 *C* shows the closed time distribution plotted as the square root against the log of time to emphasize longer closures. Greater than 85% of closures in this example are less than 10 ms in duration but there are a number of longer closures lasting for up to two seconds.

There is also evidence for multiple open states of the channel. The current amplitude distribution from the same experiment is shown in Fig. 7 *D*. The most frequently observed single channel current has an amplitude of −1.55 ± 0.2 pA (mean ± SD; 40 mV) but the distribution is best fit by the sum of three gaussians due to the presence of a less commonly observed component with an amplitude of −0.29 ± 0.14 pA. The sublevel is illustrated in Fig. 8 which shows activity in a patch containing a single CNG channel during exposure to 100 μM cGMP. The majority of openings were to a conductance level of 42 pS and for most of the record the channel flickered between this state and the fully closed state. The segment of the record shown in Fig. 8 *A* illustrates a less frequent event, a transition to an intermediate level lasting long enough to be unambiguously identified as a subconductance of ~14 pS, one-third of the conductance of the fully open channel. The figure shows transitions from the fully open state to either the closed state or the subconductance state as well as transitions from the closed state to either the substate or the fully open state. The current amplitude histogram from this experiment (Fig. 8 *B*) was fitted with the sum of three gaussian distributions and the areas under the fitted curves were compared to determine the relative probability of occupying the fully open state ($P = 0.93$), the subconductance state ($P = 0.05$), or the closed state ($P = 0.008$) at an agonist concentration of 100 μM. Clear examples of subconductance events were rare probably because openings and closings to this level tend to be short lived at high agonist concentrations. There was an indication that openings to the subconductance level occur more frequently at low cGMP concentrations (see Fig. 4 *A*, 1 μM trace).

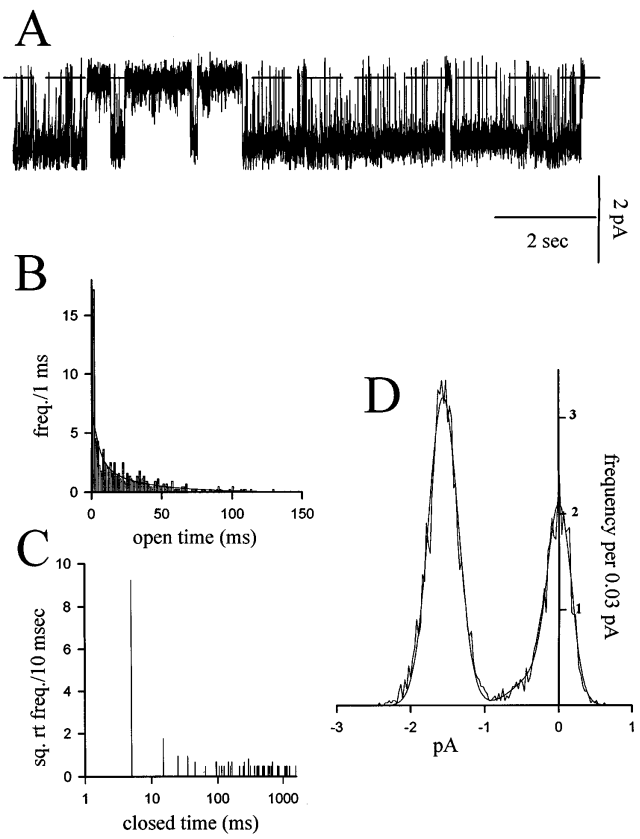

FIGURE 7. Evidence for multiple open and closed states. (*A*) Inside-out patch recording in symmetrical Na^+ saline during continuous application of 100 μM cGMP to the inner face (filter corner frequency 1.5 kHz; 40 mV pipette potential). The patch contained a single CNG channel. The zero current level is shown by a dashed line. (*B*) Open time distribution (1 ms bin size). The sum of two exponential relaxations was fitted to the data by the method of least squares (time constants, 5 and 32 ms). (*C*) Closed time distribution. The square root of frequency is plotted against the log of time to emphasize longer closures. Greater than 85% of closures are less than 10 ms in duration in this example. (*D*) Amplitude histogram from the same experiment. The distribution was fitted by the sum of three gaussian functions with peaks at 0.0, −0.29, and −1.55 pA (*solid line*).

DISCUSSION

Cyclic nucleotide–gated (CNG) channels are heteromultimeric membrane proteins that share features in common with voltage-dependent Na^+ and K^+ channels (Chen et al., 1993; Bradley et al., 1994; Liman and Buck, 1994; Menini, 1995; Pugh, 1996). They play a well known role in sensory transduction in photoreceptors and olfactory receptors (Yau and Baylor, 1989; Zufall et al., 1994; Zufall, 1996; Zagotta and Siegelbaum, 1996; Finn et al., 1996). In addition, members of the family of CNG channel proteins are found in a variety of other cells including retinal bipolar and ganglion cells (Ahmad et al., 1994), pineleocytes (Shaad et al., 1995; Dryer and Henderson, 1991), kidney cells (Distler, et al., 1994), aortic muscle (Biel et al., 1994), epithelial cells and sperm (Weyand et al., 1994). CNG channels are also expressed in neurons within the CNS (Distler et al., 1994; Lienders-Zufall et al., 1995; Zufall, 1996; Bradley et al., 1997) which raises the possibility that CNG channels may function in neuronal signal transduction in brain regions where cAMP and cGMP signals are known to occur, notably the cerebellum, hippocampus, and forebrain, and in sympathetic ganglia (Southam and Garthwaite, 1993).

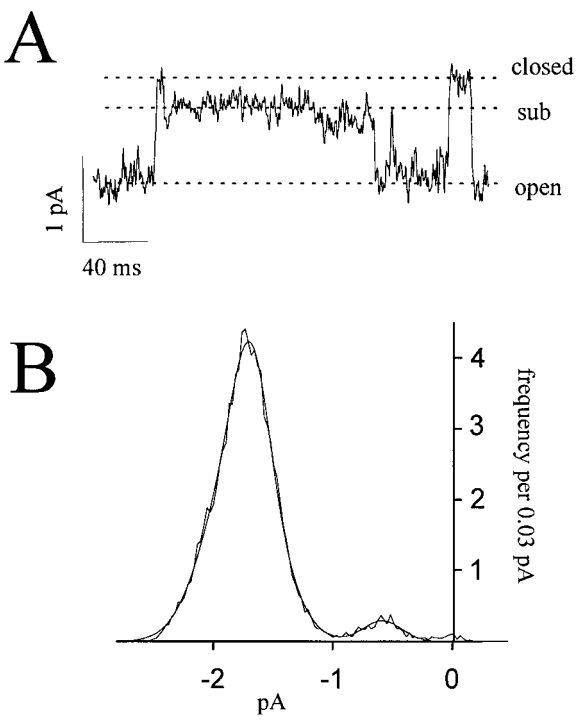

FIGURE 8. Evidence for a subconductance level. (*A*) Single channel currents in an inside out patch in symmetrical Na^+ during stimulation with 100 μM cGMP. The zero current level is shown by the dashed line labeled *closed* and the maximum current is shown by the line labeled *open*. This figure illustrates a particularly long lasting transition to a level about 1/3 the maximum current amplitude (labeled *sub*). (*B*) Amplitude histogram generated from a 3-s record from the same experiment (bin width = 0.026 pA; filter corner frequency = 500 Hz). The sum of three gaussian functions was fitted to the data by the method of least squares (*continuous line*). From the areas under the fitted curves the probabilities of being in the closed state, the subconductance state, and the open state were 0.008, 0.05, and 0.93, respectively.

Whole cell voltage clamp and fura-2 imaging experiments on N1E-115 neuroblastoma cells showed that the stimulation of M1 muscarinic receptors activates a cation current that is carried in part by Ca^{2+} (Mathes and Thompson, 1995). The membrane permeable cGMP analogue 8-br-cGMP activates a current with similar properties and the currents activated by agonist and 8-br-cGMP are not additive which suggests that the same pathway is involved (Mathes and Thompson, 1996). Activation of cation current by muscarinic agonist is prevented by inhibitors of NO-synthase and guanylyl cyclase and the inhibition is overcome by adding 8-br-cGMP (Mathes and Thompson, 1996). These observations led to the hypothesis that N1E-115 cells express cyclic nucleotide gated channels. The present experiments confirm this by demonstrating the presence of cGMP gated channels in excised patches. Since channel activity can be turned on and off repeatedly by applying cGMP in the absence of ATP and Mg^{2+}, it is concluded that activation does not involve cGMP-dependent kinases or phosphatases but instead results from a direct effect of the cyclic nucleotide on channel gating.

Cyclic nucleotide gated channels in N1E-115 cells have a number of features in common with the channels in receptor cells. Channel activity is characterized by bursts of brief openings and does not desensitize during prolonged exposure to cGMP (Kurahashia and Kaneko, 1993; Zufall et al., 1991; Zimmerman and Baylor, 1986; Haynes and Yau, 1986; Matthews and Watanabe, 1988; Haynes and Yau, 1990). In symmetrical solutions containing 160 mM Na^+ the unitary conductance in N1E-115 cells is 47 pS. Yau and Baylor (1989) report a unitary conductance of 25 pS for the rod CNG channel in 110 mM Na^+ (see Haynes et al., 1986; Zimmerman and Baylor, 1986) which is equivalent to the conductance in N1E-115 cells when correction is made for the difference in ion concentration. The cone isoform, on the other hand, has a unitary conductance of 45 pS in 110 mM Na^+, roughly twice the conductance of the rod channel under the same ionic conditions (Haynes and Yau, 1990). The unitary conductance of CNG channels in olfactory receptors is similar to the value measured in N1E-115 cells, with reported values ranging from 29 to 45 pS (Kurahashia and Kaneko, 1993; Zufall et al., 1991; Bradley et al., 1994). Channel activation in N1E-115 cells is very much more sensitive to cGMP than to cAMP which suggests a possible affinity with photoreceptor channel isoforms. This interpretation is complicated, however, because the beta subunit of the heteromeric protein plays a significant role in determining the sensitivity to cyclic nucleotides (Bradley et al., 1994;

Liman and Buck, 1994). Because of this complication, definitive identification of the channel expressed in N1E-115 cells will require sequence information.

The dose response curve for channel activation by cGMP in N1E-115 cells was fitted using an apparent dissociation constant (K_D) of 9.4 μM and a Hill coefficient of 2. The K_D is similar to the value of 8.6 μM reported for rod channels by Taylor and Baylor (1995) and comparable to estimates ranging from 1 to 20 μM for olfactory receptor channels (Kurahashia and Kaneko, 1993; Frings et al., 1992; Zufall et al., 1991). A Hill coefficient of 2 suggests multiple cGMP binding sites but because of variability in the measurements this value is only tentative. Estimates of the Hill coefficient for olfactory receptor channels range from 1.4 to 2 (Kurahashia and Kaneko, 1993; Frings et al., 1992; Zufall et al., 1991) while Haynes and Yau (1990) report a Hill coefficient of 2.4 for the activation of cone channels. Taylor and Baylor (1995) give a value of 2.8 for rod channels but other investigators report values as large as 4, which may indicate that binding sites on all four subunits must be occupied by ligand to fully activate the channel (Karpen and Brown, 1996).

The maximum opening probability measured at saturating cGMP concentration in N1E-115 cells is ~0.8. At high agonist concentrations, openings occur in nearly continuous bursts with infrequent longer closures. This is similar to what is seen in cone photoreceptors where the maximum opening probability is 0.8–0.9 (Haynes and Yau, 1990). Single channel openings in both rods and cones also occur in bursts and bursting continues at saturating cGMP concentrations (Zimmerman and Baylor, 1986; Haynes and Yau, 1986; Hanke et al., 1988). Bursts of brief openings characterize the gating of toad olfactory receptors as well where the maximum opening probability is again ~0.8 (Kurahashia and Kaneko, 1993). In each of these examples, it appears that the fully bound channel continues to undergo transitions between open and closed states. In N1E-115 cells, the mean open time during bursts is about the same as the mean open time for individual openings suggesting that the major effect of increasing agonist concentration is an increase in burst length.

The prolonged bursts of channel openings observed at high cGMP concentrations are occasionally interrupted by long closures which suggests the possibility of additional closed states of the channel. Taylor and Baylor (1995) made a similar observation in rods. Evidence was also obtained for a subconductance state of the CNG channel in N1E-115 cells similar to what is seen in rods (Haynes et al., 1986; Zimmerman and Baylor, 1986; Tanaka et al., 1987). In rod channels the smaller conductance is roughly 1/4 of the conductance of the dominant open state (Taylor and Baylor, 1995) while in N1E-115 cells it is about 1/3 of the maximum conductance. In both cases the fraction of time spent in the substate appears to decrease with increasing cGMP concentration suggesting that it may result from the opening of a partially ligated channel (Haynes and Yau, 1990; Taylor and Baylor, 1995; Karpen and Brown, 1996).

In N1E-115 neuroblastoma cells, as in olfactory receptor cells and photoreceptors, signal transduction begins with the activation of a G-protein–coupled receptor and ends with a change in membrane current due to modulation of a CNG cation channel. The signaling pathway in N1E-115 cells involves receptor dependent activation of intracellular Ca^{2+} release followed by activation of NO-synthase by Ca^{2+}-calmodulin, activation of guanylyl cyclase by NO, cGMP production, and activation of cation channels by cGMP. Whole cell voltage clamp experiments showed that Ca^{2+} makes a substantial contribution to the cyclic nucleotide–gated current (Mathes and Thompson, 1996). Leinders-Zufall et al. (1995) and Bradley et al. (1997) reached the same conclusion for the current activated by cGMP in hippocampal neurons. Attempts to record single channel currents carried by Ca^{2+} have not been successful using N1E-115 cells, however, possibly because rapid blocking and unblocking of the channel by Ca^{2+} makes the opening events too brief to resolve. This problem is compounded by the possibility that the ions used as substitutes for Na^+ (TEA, TMA, and Tris) may also block the channel. This is similar to the situation in rod channels where it has not been possible to measure unitary Ca^{2+} currents even though it is estimated that Ca^{2+} carries about 15% of the current under normal conditions and that the permeability to Ca^{2+} is several times higher than the permeability to Na^+. Calcium influx through CNG channels in N1E-115 cells would provide an opportunity for positive feedback since increases in intracellular Ca^{2+} stimulate further cGMP production by activating NO-synthase (Fostermann et al., 1990). It is especially interesting that Ca^{2+} influx, rather than internal Ca^{2+} release, makes the major contribution to the activation of cGMP production (Thompson et al., 1996). Positive feedback involving Ca^{2+} entry through cGMP gated channels may help to explain the very large and rapid increases in cGMP that occur in regions such as the hippocampus and cerebellum.

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REFERENCES

Ahmad, I., T. Leinders-Zufall, J.D. Kocsis, G.S. Shepherd, F. Zufall, and C.J. Barnstable. 1994. Retinal ganglion cells express a cGMP-gated cation conductance activatable by nitric oxide donors. *Neuron.* 12:155–165.

Amano, T., E. Richelson, and M. Nirenberg. 1972. Neurotransmitter synthesis by neuroblastoma clones. *Proc. Natl. Acad. Sci. USA.* 69:258–263.

Arroyo, C.M., and C.M. Forray. 1991. Activation of cyclic GMP formation in mouse neuroblastoma cells by a labile nitroxyl radical: an electron paramagnetic resonance spin trapping study. *Euro. J. Pharm. Mol. Pharm.* 6:157–161.

Berridge, M.J. 1993. Inositol trisphosphate and calcium signalling. *Nature (Lond.).* 361:315–325.

Biel, M., X. Zong, M. Distler, E. Bosse, N. Klugbaur, M. Muraami, V. Flockerzi, and F. Hofmann. 1994. Another member of the cyclic nucleotide-gated family, expressed in testis, kidney, and heart. *Proc. Natl. Acad. Sci. USA.* 91:3505–3509.

Bradley, J., J. Li, N. Davidson, H.A. Lester, and K. Zinn. 1994. Heteromeric olfacory cyclic nucleotide-gated channels: a subunit that confers increased sensitivity to cAMP. *Proc. Natl. Acad. Sci. USA.* 91:8890–8894.

Bradley, J., Y. Zhang, R. Bakin, H.A. Lester, G.V. Ronnett, and K. Zinn. 1997. Functional expression of the heteromeric "olfactory" cyclic nucleotide-gated channel in the hippocampus: a potential effector of synaptic plasticity in brain neurons. *J. Neurosci.* 17: 1993–2005.

Carafoli, E. 1987. Intracellular calcium homeostasis. *Annu. Rev. Biochem.* 56:395–433.

Chen, T.-Y., Y.-W. Peng, R.S. Dhallan, B. Ahmed, R.R. Reed, and K.-W. Yau. 1993. A new subunit of the cyclic nucleotide-gated cation channel in retinal rods. *Nature (Lond.).* 362:764–767.

Dipolo, R., and L. Beauge. 1988. Ca^{2+} transport in nerve fibers. *Biochim. Biophys. Acta.* 947:549–569.

Distler, M., M. Biel, V. Flockrzi, and F. Hofmann. 1994. Expression of cyclic nucleotide-gated cation channels in non-sensory tissues and cells. *Neuropharmacology.* 33:1275–1282.

Dryer, S.E., and D. Henderson. 1991. A cyclic GMP-activated channel in dissociated cells of the chick pineal gland. *Nature (Lond.).* 353:756–758.

Finn, J.T., M.E. Brunwald, and K.-W. Yau. 1996. Cyclic nucleotide-gated ion channels: an extended family with diverse functions. *Annu. Rev. Physiol.* 58:395–426.

Fostermann, U., L.D. Gorsky, J.S. Pollock, K. Ishii, H.H.H.W. Schmidt, M. Heller, and F. Morad. 1990. Hormone-induced biosynthesis of endothelium-derived relaxing factor/nitric oxide-like material in N1E-115 neuroblastoma cells requires calcium and calmodulin. *Mol. Pharmacol.* 38:7–13.

Frings, S., J.W. Lynch, and B. Lindemann. 1992. Properties of cyclic nucleotide-gated channels mediating olfactory transduction: activation, selectivity, and blockage. *J. Gen. Physiol.* 100:45–67.

Hanke, W. , N.J. Cook, and U.B. Kaupp. 1988. cGMP-dependent channel protein from photoreceptor membranes: single-channel activity in the purified and reconstituted protein. *Proc. Natl. Acad. Sci. USA.* 85:94–98.

Harrington, M.A., and S.H. Thompson. 1996. Activation of the nitric oxide-cGMP pathway is required for refilling intracellular Ca^{2+} stores in a sympathetic neuron cell line. *Cell Calcium.* 19: 399–407.

Haynes, L.W., A.R. Kay, and K.-W. Yau. 1986. Single cyclic GMP-activated channel activity in excised patches of rod outer segment membrane. *Nature (Lond.).* 321:66–70.

Haynes, L.W., and K.-W. Yau. 1990. Single-channel measurement from the cyclic-GMP-activated conductance of catfish retinal cones. *J. Physiol. (Lond.).* 429:451–481.

Hu, J., and E. El-Fakahany. 1993. Role of intercellular and intracellular communication by nitric oxide in coupling of muscarinic receptors to activation of guanylate cyclase in neuronal cells. *J. Neurochem.* 61:578–585.

Karpen, J.W., and R.L. Brown. 1996. Covalent activation of retinal rod cGMP-gated channels reveals a functional heterogeneity in the ligand binding sites. *J. Gen. Physiol.* 107:168–181.

Kimhi, Y., C. Palfrey, I. Spector, Y. Barak, and U.Z. Littauer. 1976. Maturation of neuroblastoma cells in the presence of dimethlysulfoxide. *Proc. Natl. Acad. Sci USA.* 73:462–466.

Kurahashi, T., and A. Kaneko. 1993. Gating properties of the cAMP-gated channel in toad olfactory receptor cells. *J. Physiol. (Lond.).* 466:287–302.

Leinders-Zufall, T., H. Rosenboom, C.J. Barnstable, G.M. Shepherd, and F. Zufall. 1995. A calcium-permeable cGMP-activated cation conductance in hippocampal neurons. *NeuroReport.* 6: 1761–1765.

Liman, E.R., and L.B. Buck. 1994. A second subunit of the olfactory cyclic nucleotide-gated channel confers high sensitivity to cAMP. *Neuron.* 13:511–621.

Mathes, C., and S.H. Thompson. 1995. The relationship between depletion of intracellular Ca^{2+} stores and activation of Ca^{2+} current by muscarinic receptors in neuroblastoma cells. *J. Gen. Physiol.* 106:975–994.

Mathes, C., and S.H. Thompson. 1996. The nitric oxide-cyclic GMP pathway couples muscarinic receptors to activation of Ca^{2+} influx. *J. Neurosci.* 16:1702–1709.

Mathes, C., S.S.-H. Wang, H.M. Vargas, and S.H. Thompson. 1992. Intracellular calcium release in N1E-115 neuroblastoma cells is mediated by the M1 muscarinic receptor subtype and is antagonized by McN-A-343. *Brain Res.* 585:307–310.

Matthews, G., and S. Watanabe. 1987. Properties of ion channels closed by light and opened by 3′5′-cyclic guanosine monophosphate in toad retinal rods. *J. Physiol. (Lond.)* 389:691–715.

Matthews, G., and S. Watanabe. 1988. Activation of single ion channels from toad retinal rod inner segments by cyclic GMP: concentration dependence. *J. Physiol. (Lond.).* 403:389–405.

McKinney, M., C. Bolden, C. Smith, A. Johnson, and E. Richelson. 1990. Selective blockade of receptor-mediated cyclic GMP formation in N1E-115 neuroblastoma cells by an inhibitor of nitric oxide synthesis. *Eur. J. Pharmacol.* 178:139–140.

Menini, A. 1995. Cyclic nucleotide-gated channels in visual and olfactory transduction. *Biophys. Chem.* 55:185–196.

Pugh, E.N., Jr. 1996. Cooperativity in cyclic nucleotide-gated ion channels. *J. Gen. Physiol.* 107:165–167.

Reeves, J.P. 1992. Molecular aspects of sodium-calcium exchange. *Arch. Biochem. Biophys.* 292:329–334.

Southam, E., and J. Garthwaite. 1993. The nitric-oxide-cyclic GMP signalling pathway in rat brain. *Neuropharmacol.* 32:1267–1277.

Tanaka, J.C., R.E. Furman, W.H. Cobbs, and P. Mueller. 1987. Incorporation of retinal rod cGMP-dependent conductance into planar bilayers. *Proc. Natl. Acad. Sci. USA.* 84:724–728.

Taylor, W.R., and D.A. Baylor. 1995. Conductance and kinetics of single cGMP-activated channels in salamander rod outer segments. *J. Physiol. (Lond.).* 483:567–582.

Thompson, S.H., C. Mathes, and A.A. Alousi. 1995. Ca^{2+} requirement for cGMP production during muscarinic activation of N1E-115 neuroblastoma cells: weighing the contributions from Ca^{2+} release and Ca^{2+} influx. *Am. J. Physiol.* 269:C979–C985.

Weyand, I., M. Godde, S. Frings, J. Weiner, F. Müller, W. Aten-

hofen, H. Hatt, and U.B. Kaupp. 1994. Cloning and functional expression of a cyclic-nucleotide-gated channel from mammalian sperm. *Nature (Lond.)*. 368:859–863.

Yau, K.-W., and D.A. Baylor. 1989. Cyclic GMP-activated conductance of retinal photoreceptor cells. *Annu. Rev. Neurosci.* 12:289–327.

Zagotta, W.N., and S.A. Siegelbaum. 1996. Structure and function of cyclic nucleotide-gated channels. *Annu. Rev. Neurosci.* 19:235–263.

Zimmerman, A.L., and D.A. Baylor. 1986. Cyclic GMP-sensitive conductance of retinal rods consists of aqueous pores. *Nature (Lond.)*. 321:70–72.

Zufall, F., S. Firestein, and G. Shepherd. 1991. Analysis of single cyclic-nucleotide-gated channels in olfactory receptor cells. *J. Neurosci.* 11:3573–3580.

Zufall, F., S. Firestein, and G.M. Shepherd. 1994. Cyclic nucleotide-gated ion channels and sensory transduction in olfactory receptor neurons. *Annu. Rev. Biophys. Biomol. Struct.* 23:577–607.

Zufall, F. 1996. Cyclic nucleotide-gated channels, nitric oxide, and neural function. *The Neuroscientist.* 2:24–32.