*Journal of Bioenergetics and Biomembranes, Vol. 23, No. 4, 1991*

# MINI REVIEW

# Four Cases of Direct Ion Channel Gating by Cyclic Nucleotides

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*Received June 20, 1990; revised December 3, 1990*

## Abstract

Four different nucleotide-gated ion channels are discussed in terms of their biophysical properties and their importance in cell physiology. Channels activated directly by cGMP are present in vertebrate and invertebrate photoreceptors. In both cases cGMP increases the fraction of time the channel remains in the open state. At least three cGMP molecules are involved in channel opening in vertebrate photoreceptors and the concentration of the cyclic nucleotide to obtain the half maximal effect is about 15 $\mu$M. The light-dependent channel of both vertebrates and invertebrates is poorly cation selective. The vertebrate channel allows divalent cations to pass through 10–15-fold more easily than monovalent ions. In agreement with their preference for divalent cations, this channel is blocked by *l-cis* Dialtazem, a molecule that blocks certain types of calcium channels. In olfactory neurons a channel activated by both cAMP and cGMP is found and, as in the light-dependent channel, several molecules of the nucleotide are needed to open the channel with a half maximal effect obtained in the range of 1–40 $\mu$M. The channel is poorly cationic selective. A $K^+$ channel directly and specifically activated by cAMP is found in *Drosophila* larval muscle. At least three cAMP molecules are involved in the opening reaction. Half-maximal effect is obtained at about 50 $\mu$M. This channel is blocked by micromolar amount of tetraethylammonium applied internally. Interestingly, this channel has a probability of opening 10–20-fold larger in the mutant *dunce*, a mutant that possesses abnormally elevated intracellular cAMP level, than in the wild type.

**Key Words:** Ion channels; gating; cyclic nucleotides, olfactory neurons, photoreceptors; *Drosophila* muscle.

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0145-479X/91/0800-0577$06.50/0 

## Introduction

Ion channel behavior can be modified in a long-term manner (seconds to hours) via intracellular second messengers, a prime example of which is cyclic AMP (cAMP). In some cases cAMP has been found to act through the activation of protein kinases which in turn induce phosphorylation of the channel protein (for reviews see Levitan, 1985; Reuter, 1987). However, patch clamp and reconstitution studies have provided evidence that second messengers also can act independently of protein phosphorylation (for a review see Dani, 1989). Examples of direct regulation of ion channel gating by second messengers include modulation of $K^+$ channels by $Ca^{2+}$, cGMP activation of cation-selective channels in vertebrate rods and cones (Fesenko *et al.*, 1985; Matthews and Watanabe, 1987; Nakatani and Yau, 1988) and in invertebrate photoreceptors (Bacigalupo *et al.*, 1990), and the cyclic nucleotide activation of cationic channels in olfactory neurons (Nakamura and Gold, 1987). Furthermore, recent work by Delgado *et al.* (1990a, b) shows that cAMP directly and specifically activates a $K^+$ channel present in *Drosophila* larval muscle. In addition, ATP has also been found to gate directly potassium channels as discussed by Stanfield in this same issue.

In most cases, the existence of second messenger binding sites in the channel protein has not been proven and a closely associated membrane component could serve as the site for interaction. However, the examples mentioned above make it clear that direct activation of ion channels by cyclic nucleotides independent of phosphorylation represents an important mechanism in the regulation of membrane conductance. Since, in some cases, the intracellular concentration of cyclic nucleotide can remain elevated for periods that outlast the turn off of the external stimulus (e.g., light), receptor activation will persist longer than in the case of classical chemical transmitters like, for example, acetylcholine. On the other hand, due to the presence of cytoplasmic buffers and enzyme systems, cyclic nucleotide concentration will be lowered in a matter of seconds to minutes and channel activation will not be a long-lasting one as that induced by channel phosphorilation. This may prove to be advantageous when controlling cell responses of intermediate duration.

In this review we will discuss and compare, in terms of their importance in signal transduction, the properties of four channels activated by cyclic nucleotides (cAMP, cGMP). We start by describing the characteristics of cationic channels involved in visual transduction, both in vertebrates and invertebrates. Cationic channels involved in chemical transduction in olfactory neurons are analyzed next. We end this review with a description of a novel cAMP-activated channel we recently detected in *Drosophila* larval muscle.

## cGMP-Gated Channels of Photoreceptors

Photoreceptors of both vertebrates and invertebrates exhibit "light-dependent conductances." The effect of light on this conductance is mediated by an enzyme cascade which controls the cytoplasmic concentration of a second messenger that directly interacts with the ion channels underlying the light-dependent conductance.

In vertebrate photoreceptors, the light-dependent conductance stays open in the dark, and light causes its inactivation. In invertebrates, on the contrary, the light-dependent conductance is negligible in the dark and is activated by light. The difference resides in the mechanisms of photo-transduction of both types of organisms (see Bacigalupo *et al.*, 1990). In vertebrates, cyclic GMP (cGMP) activates light-dependent channels. Its concentration in the cytoplasm of rods and cones is about 120 $\mu$M, and light induces rapid hydrolysis of cGMP (Cote *et al.*, 1984; Liebman *et al.*, 1987; Pugh and Cobbs, 1986), leading to channel closure. In invertebrates, recent evidence suggests that cGMP also has the role of the intracellular messenger that causes the opening of the light-dependent channels (Johnson *et al.*, 1986; Bacigalupo *et al.*, 1990). However, in this case light triggers an increase in cGMP concentration. (Johnson *et al.*, 1986). In what follows we will describe and compare the properties of light-dependent channels of vertebrate and invertebrate photoreceptors.

## Evidence for cGMP as the Molecule That Gates the Light-Dependent Channels

### *Invertebrates*

Evidence showing that cGMP is the intracellular chemical messenger that opens the channels in invertebrates has been derived mainly from macroscopic current measurements in *Limulus* photoreceptors. Pressure injection of cGMP in the dark into the light-sensitive part of the cell leads to an increase in a membrane conductance with the same properties as the light-dependent conductance (Johnson *et al.*, 1986). The effect of cGMP appears to be specific. cAMP did not cause an effect when injected at the same site of a successful cGMP injection. Calcium and inositol tris-phosphate are two other second messengers that also participate in the transduction process (Payne *et al.*, 1986; Payne, 1990). However, there is at present strong evidence against an involvement of these messengers in direct light-dependent channel activation during illumination (Bolsover and Brown, 1985; Bacigalupo *et al.*, 1990). Actually, based on macroscopic and

single-channel measurements, calcium appears to be a blocker of light-dependent channels (Lisman and Brown, 1971; Bacigalupo *et al.*, 1987).

### *Vertebrates*

The evidence that definitively established the role of cGMP in vertebrate phototransduction came from patch clamp experiments in amphibian rods. Fesenko *et al.* (1985) first showed that perfusion of the intracellular side of the rod membrane with micromolar concentrations of cGMP caused an increase in the patch conductance. The relation between the relative magnitude of the cGMP effect on the conductance and the cGMP concentration was found to be best described by a sigmoidal function, with an apparent dissociation constant of about 15 $\mu$M. On the other hand, the Hill coefficient needed to fit the dose–response curve suggests that at least three molecules of cGMP are required for channel activation. Further evidence for cGMP being the messenger for excitation was provided by experiments done in truncated rod outer segments. This was done by drawing a large fraction of the outer segment of a photoreceptor into a suction electrode and then breaking away the part of the cell left out of the pipette. What remains is the truncated end of the rod outer segment, in which the cytoplasmic side of the membrane is now in contact with the bath solution. When cGMP was added to the bath solution, the membrane conductance of the truncated rod outer segment increased. Light had no effect on the cGMP-sensitive conductance unless GTP was also added to the solution. A GTP-binding protein, known as transducin, has been shown to have a key role in the phototransduction cascade (Fung and Stryer, 1980). In the presence of GTP, a light flash reversibly suppressed the cGMP-sensitive conductance, indicating that the cGMP-sensitive conductance and the light-sensitive conductance are the same conductance (Nakatani and Yau, 1988).

## Properties of the Light-Dependent Channels

### *Invertebrates*

Light-dependent single-channel currents have been recorded from the light-sensitive membrane of *Limulus* ventral photoreceptors by means of the patch clamp technique (Bacigalupo and Lisman, 1983, 1984; Bacigalupo *et al.*, 1986). Unitary light-dependent currents can be resolved by using a cell-attached patch electrode containing normal extracellular solution (artificial seawater). These channels are closed in the dark and activate upon stimulation by light (Fig. 1A). Channel activity is preceded by a latency lasting tens to hundreds of milliseconds, after which channel activity develops

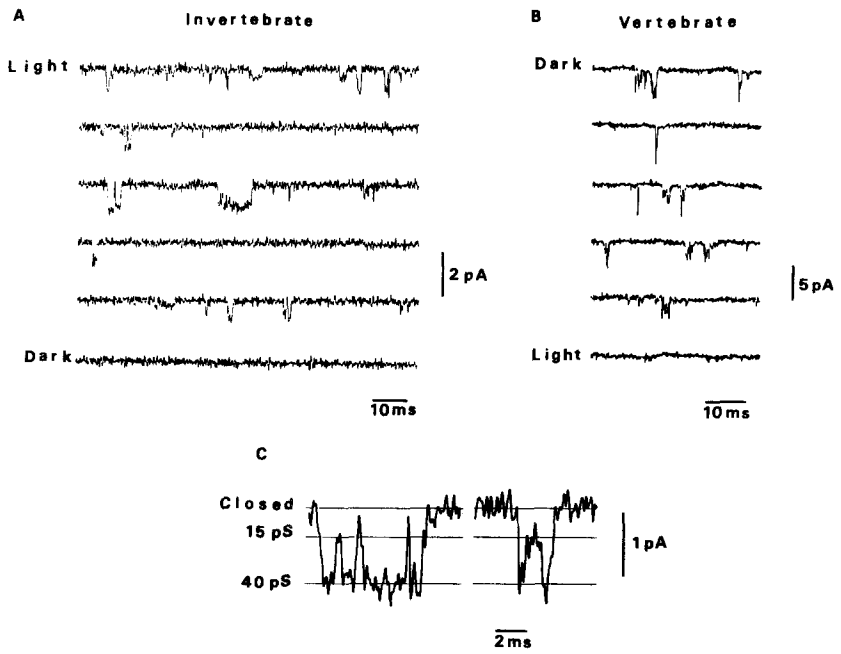


**Fig. 1.** Light-dependent channels of invertebrate and vertebrate photoreceptors. (A) Recording obtained from the R-lobe of a *Limulus* ventral photoreceptors after the response reached steady-state. Channels opened with light and they close in the dark. Downward current deflections correspond to channel openings. Individual events of two sizes can be distinguished corresponding to the two open states of the same channel. Pipette solution was artifical seawater. Membrane potential −30 mV. 2 kHz filtering. (B) Recording obtained in the dark and during saturating illumination from the outer segment of an intact toad rod. The patch was hyperpolarized by 148 mV. Pipette solution was divalent-free Ringer solution. 4 kHz filtering. (C) Detailed view of two different current records showing direct transitions between the two conductance states of the *Limulus* light-dependent channel. This type of transitions occur at a much higher frequency than predicted if they were due to the opening of two independent channels, indicating that they are substates of the same channel. 2 kHz filtering. (A and C modified from Johnson *et al.*, 1990. C modified from Matthews and Watanabe, 1987).

and rapidly reaches a maximum, droping subsequently to a lower level which is maintained until the end of the light stimulus (Bacigalupo and Lisman, 1983; see Bacigalupo, 1986). This time course of channel activation roughly resembles the waveform of the macroscopic light-dependent inward current, which shows a peak that decays to a maintained level due to light adaptation (Millecchia and Mauro, 1969; see Fain and Lisman, 1981). This process is mediated by calcium that induces the light-dependent conductance decrease during a prolonged light stimulus (Lisman and Brown, 1972).

The light-dependent channel of *Limulus* photoreceptors has two open conductance states, of 14 and 40 pS, which have mean open times of 2.3 and 1.3 ms, respectively (Fig. 1C). Even under bright light at physiological

voltages, the probability of the channel being open ($P_0$) during the maintained part of the response is quite low ($\approx 0.001$). Transitions between the two different conductance states are rather infrequent. Statistical analysis of these recordings indicate that they correspond to two open conductance states of a single class of light-dependent channel (Johnson *et al.*, 1991). The occurrence of 14 and 40 pS events is not even during the response to light. The channel behavior observed during the response to a prolonged light stimulus varies with time. During the first few hundreds of milliseconds after the onset of light the channel preferentially enters a mode in which it only fluctuates between the closed and the 40 pS state. This behavior is followed by a shift to a mode in which the 14 pS state has a much higher probability. The channel modes are probably related to light adaptation (Johnson *et al.*, 1991).

The light-dependent channel of *Limulus* is strongly voltage-dependent. Voltage cannot open the channel in the absence of light, but the probability of the channel being open in the presence of light increases as the membrane is depolarized. This is mainly accounted for by a remarkable increase in the channel mean open time at depolarizing voltages (Bacigalupo *et al.*, 1986). Both conductance states of the channel show similar voltage dependence (Johnson *et al.*, 1991).

The ionic selectivity of the light-dependent channel from *Limulus* ventral photoreceptors has been obtained from reversal potentials of the macroscopic currents. The selectivity sequence is: $Na^+ : Li^+ : K^+ = 1.0 : 1.0 : 0.5$ (Brown and Mote, 1974). The channel is, therefore, a poorly selective cationic channel. This is reflected on the reversal potential of the light-dependent current at both the macroscopic and single-channel levels (+ 10 mV; Millecchia and Mauro, 1969; Bacigalupo and Lisman, 1983).

The role of cGMP in light transduction in invertebrates was studied in inside-out membrane patches found to contain only light-dependent channels (Bacigalupo *et al.*, 1990). In the absence of cGMP the patches were silent. However, upon perfusion with a pseudointracellular solution supplemented with 20 to 100 $\mu$M cGMP an inward current was induced. The current returned to baseline upon washing out the cGMP. Single-channel currents have also been recorded under these experimental conditions and supporting, but not conclusive, evidence that cGMP is involved in channel gating was obtained. In the presence of cGMP events of 15 pS conductance, mean open times in the millisecond range and reversal potentials near zero have been observed. However, only a small fraction of the patches studied were affected by this cyclic nucleotide.

Nasi and Gomez (1990) have reported the incorporation of a cGMP-activated channel from squid outer segment membrane fractions into planar lipid bilayers. Further characterization of this conductance is necessary to

establish whether it corresponds to the light-dependent conductance of squid photoreceptors.

The evidence presented above is summarized in a model in which the light-dependent channel has an excitatory site for cGMP which binds the nucleotide leading to channel opening and an inhibitory site that binds calcium. This last conclusion is based on the observations that intracellular $Ca^{2+}$ levels can reach a concentration of 100 $\mu$M under bright lights (Levy and Fein, 1985; Payne *et al.*, 1988), suppressing the light-dependent conductance and causing light adaptation (Lisman and Brown, 1972), and on single-channel measurements that show a blocking effect of $Ca^{2+}$ (Bacigalupo *et al.*, 1987). The rise in cGMP concentration would precede the rise in calcium concentration, which is also triggered by light; the light-induced rise in internal calcium has been detected with aequorin, and shown not to occur before the receptor potential (Payne *et al.*, 1988). Therefore, all channels would be available for cGMP at the onset of the light stimulus and, subsequently, calcium would reduce the number of activatable channels.

### *Vertebrates*

Noise analysis indicated a peculiar characteristic of the light-dependent vertebrate channel (Detwiller *et al.*, 1982). The vertebrate channel was found to have a unitary conductance in the femtosiemen range, smaller by over two orders of magnitude than that of all other known ion channels (Detwiller *et al.*, 1982; Bodola and Detwiller, 1985; Gray and Atwell, 1985; Zimmermann and Baylor, 1985), leaving open the possibility that the mechanism for ionic permeation could consist of a carrier rather than an ion channel (Detwiller *et al.*, 1982; Fesenko *et al.*, 1985, 1986). An answer to this question remained elusive until conditions were found that allowed the resolution of unitary currents through the light-dependent channel. External divalent cations were found to cause a significant blockage of the light-dependent current. Removal of divalent cations from the external solution increased by 10-fold the light-dependent current, also called "dark current" (Yoshikami and Hagins, 1973; Yau *et al.*, 1981; Bodola and Detwiller, 1985; Lamb and Matthews, 1988; Nakatani and Yau, 1988). Removal of divalent cations from the extracellular solutions also increased the magnitude of unitary events to a few picoamperes. These observations demonstrated that channels rather than carriers were responsible for the light-dependent current (Haynes *et al.*, 1986; Zimmermann and Baylor, 1986; Matthews and Watanabe, 1987, 1988).

The vertebrate light-dependent channel is, as the invertebrate channel, poorly selective among cations. The reversal potential of the light-dependent current is near 0 mV (Baylor *et al.*, 1979). Early results of experiments in

which external sodium was replaced by other cations had shown that the dark current dropped after sodium removal, indicating that even though other cations can permeate the channel, the presence of sodium was necessary for the maintenance of the dark current (Cervetto, 1973; Brown and Pinto, 1974).

In order to study the ionic selectivity of the light-dependent conductance, the inner segment of the photoreceptor was sucked into a suction pipette, leaving the outer segment facing the bath which was perfused with a different extracellular solution (Yau and Nakatani, 1984; Hodgkin *et al.*, 1985). A method that allowed a fast solution exchange made it possible to measure approximate permeability ratios of the channel for various cations. The relative initial magnitudes of the current obtained in the presence of the different ions that replaced sodium gave an approximate measure of the selectivity ratios of the channel for the different cations. The selectivity sequence obtained with this method is: $Li^+ : Na^+ : K^+ : Rb^+ : Cs^+ : Ca^{2+} : Mg^{2+} = 1.4 : 1.0 : 0.8 : 0.6 : 0.15 : 12 : 2.5$ (Yau and Nakatani, 1984; Hodgkin *et al.*, 1985). These results showed that the channel allowed divalent cations to permeate the pore much better than monovalent cations. However, under physiological conditions, the dark current is carried mainly by sodium. Calcium carries a smaller fraction of this current (15%), and magnesium is responsible for 5% of the total current. Similar to other $Ca^{2+}$ channels (Tsien *et al.*, 1987), the light-dependent channel allows the flow of divalent and monovalent ions. However, unlike "orthodox" $Ca^{2+}$ channels, the light-dependent channel let sodium go through in the presence of millimolar amounts of $Ca^{2+}$.

The pharmacological properties of the light-dependent channels have some resemblance to calcium channels. *l-cis*-Diltiazem, a compound that blocks certain types of $Ca^{2+}$ channels (Lee and Tsien, 1983), is also effective in blocking the light-dependent channel at micromolar concentrations (Koch and Kaupp, 1985; Stern *et al.*, 1986). Verapamil and dehydropyridines, which also block calcium channels, have no effect on the light-dependent channel (Koch and Kaupp, 1985). Another compound, 3′,4′-dichlorobenzamil, a derivative of amiloride, a well-known epithelial sodium channel blocker, blocks the light-dependent channel in the micromolar range (Nicol *et al.*, 1987).

The vertebrate channel has been chiefly characterized in inside-out membrane patches excised from rod and cone outer segments in the absence of divalent cations. Under these conditions, the channel events present a flickering behavior. Two types of events can be distinguished, with conductances of about 8 and 25 pS in rods and 17 and 50 pS in cones. As in the invertebrate channel, these distinct levels correspond to two different conductance states of the same channel rather than two independent channels

(Haynes and Yau, 1990). Single-channel currents have recently been resolved in cell-attached patches of vertebrate rods using pipettes filled with divalent-free solution. The individual events are indistinguishable from the cGMP-dependent events that were subsequently observed after the same patches were excised. As expected, on-cell channel activity was present in the dark and was suppressed by light (Matthews, 1987; Matthews and Watanabe, 1988).

Divalent cations can block the light-dependent channels from either side of the membrane in a voltage-dependent way. Therefore, blockade of the channel by divalent ions is likely to occur within the aqueous pore. A two-barrier, one-site ion conduction model has been proposed to explain $Ca^{2+}$ blockade of monovalent ion movement across the channel. In this model, blockade is a consequence of a much longer residence time (larger well depth) of $Ca^{2+}$ than monovalent ions in the channel (Zimmermann and Baylor, 1988; Yau and Baylor, 1989). The effect of the divalents present in the extracellular solution is to keep the light-dependent conductance 98% closed in complete darkness. The physiological significance of this surprising finding has been discussed elsewhere (Yau and Nakatani, 1985; Nakatani and Yau, 1988; Yau and Baylor, 1989).

As in invertebrates, the current–voltage relation of the light-dependent channel is non-linear. However, the nature of the voltage-dependent conductance is different in both cases. In vertebrates, $P_o$ is rather independent of voltage (Matthews and Watanabe, 1987; see Table I), as opposed to *Limulus* (see above). Rectification is due mainly to the voltage-dependent blockade by divalent cations. At negative potentials, the driving force is favorable for external cations to enter the channel, thus reducing the current. At positive potentials, however, divalent cations are kept largely away from the channel and the current is therefore larger (Yau and Baylor, 1989). Removal of divalent cations from both sides of the membrane almost completely removes the rectification in the current–voltage relation (Haynes and Yau, 1990).

The molecular characterization of the vertebrate light-dependent channel has received an important impulse from the purification and subsequent reconstitution of the channel protein in artificial lipid bilayers (Tanaka *et al.*, 1987; Cook *et al.*, 1987; Hanke *et al.*, 1988, 1990), and from the elucidation of the primary structure and expression from complementary DNA of the channel (Kaupp *et al.*, 1989). Purification of the channel protein has been attained by three different laboratories, which have obtained different values for the molecular weight of the protein: 63 kD (Cook *et al.*, 1987), 39 kD (Matesic and Liebman, 1987), or 250 kD (Shinozawa *et al.*, 1987). The reasons for the discrepancy remain unclear. Since the native channel requires the binding of three or more cGMP molecules (Haynes *et al.*, 1986; Zimmermann and Baylor, 1986), the channel would be formed by either a single

**Table I.** Characteristics of Ion Channels Activated by Cyclic Nucleotides

| Preparation | Activator[a] | Selectivity | Channel[b] conductance (pS) | Voltage dependence | Pharmacology | References[d] |
|---|---|---|---|---|---|---|
| *Drosophila* larval muscle | cAMP ($51\,\mu M$, $N = 3$) | $K^+ \gg Na^+$ | 47 | no | TEA | 1 |
| Photoreceptors | | | | | | |
| (a) Vertebrate | cGMP ($15\,\mu M$, $N = 3$) | $Ca^{2+} : Na^+ : K^+ = 12 : 1 : 0.8$ | 8 and 25 (rods) 17 and 50 (cones) | yes[c] | Diltiazem; amiloride analogs | 2–5 |
| (b) Invertebrates | cGMP $< 100\,\mu M$ | $Na^+ : K^+ = 1 : 0.5$ | 14 and 40 | yes | | 6,7 |
| Olfactory neuron | cGMP, cAMP ($1–40\,\mu M$, $N = 1.1–1.8$) | $Na^+ \approx K^+$ | 30 | ? | amiloride? | 8 |

[a] Numbers in parentheses are $K_{50}$ and Hill's numbers, respectively.
[b] Conductances were measured in 120 Na/150 K (*Drosophila*), 118 Na/118 Na (vertebrate photoreceptors), and in cell-attached patched (invertebrate photoreceptors).
[c] Voltage dependence is due to blockade by divalent cations.
[d] 1. Delgado *et al.* (1990); 2. Stern *et al.* (1986); 3. Haynes and Yau (1990); 4. Yau and Baylor 1989; 5. Nicol *et al.* (1987); 6. Brown and Mote (1974); 7. Bacigalupo *et al.* (1990). 8. Nakamura and Gold (1987).

polypeptide having three (or more) cGMP binding sites or three (or more) subunits, each having a single cGMP binding site. The 63-kD polypeptide has been incorporated into lipid bilayers, showing characteristics similar to the native channel (Hanke *et al.*, 1988, 1990). However, neither the sensitivity to divalent cations nor to *l-cis*-Diltiazem are present in the reconstituted 63-kD channel, indicating that the native and the purified channels are not identical, perhaps as a consequence of the purification procedure or the lipid bilayer composition. The 39-kD polypeptide purified by Matesic and Liebman is blocked by *l-cis*-Diltiazem, as shown in flux studies performed on vesicles containing the purified protein (Matesic and Liebman, 1987).

Kaupp *et al.* (1989) determined the primary sequence of the cGMP-gated channel. The protein has 690 amino acids and a molecular weight of 79 kD, reasonably close to the 63 kD of the purified polypeptide. The hydrophobicity profile suggests that the protein would have 4 or 6 transmembrane segments. The 80 amino acid sequence at the C-terminal side of the protein shows remarkable similarity with two tandem cGMP-binding domains of the cGMP-dependent protein kinase. From this observation, it was proposed that in this region of the channel protein resides a single cGMP binding site.

The channel was expressed into *Xenopus* oocytes by injecting mRNA obtained from cDNA. This procedure induced in the oocyte a cGMP-dependent conductance similar to the rod conductance. Unitary currents were recorded from the mRNA injected oocytes. The conductance and current–voltage relation of the channels in divalent-free solutions were the same as the native rod channel under the same conditions. It was concluded that the rod channel is formed by three or more identical subunits, each of which binds a single cGMP molecule (Kaupp *et al.*, 1989).

In conclusion, both vertebrate and invertebrate photoreceptors contain light-dependent channels that are directly gated by cGMP. However, substantial differences in the properties of these channels indicate that they are different proteins.

## Cyclic-Nucleotide-Gated Conductances in Olfactory Transduction

In what follows we will discuss evidence supporting the notion that cyclic nucleotide-gated conductances represent one of the mechanisms that operate in chemotransduction in vertebrate olfactory receptors. It is widely accepted that a crucial step in olfactory transduction, following the interaction of odorants with chemosensory membranes in the cilia of olfactory neurons, is the activation of conductances that give rise to the receptor potential (Getchell, 1986). Until recently little was known on the mechanisms underlying the modulation of conductances by odorants in olfactory receptors.

However, the development in recent years of methods to isolate functional olfactory receptors from olfactory epithelium has rendered these small chemosensory devices amenable to patch-clamp studies (Labarca and Bacigalupo, 1988). Furthermore, biochemists are now able to obtain highly enriched fractions of chemosensory membranes derived from olfactory cilia which are expected to have their transduction properties intact. Since olfactory cilia are the places where the first events in olfactory transduction are thought to take place, these membrane fractions provide the starting material in which dissection of the biochemical machinery involved in transduction can be attempted (Anholt, 1987; Lancet, 1986). Vesicular fractions of purified ciliary membranes can also be fused to planar lipid bilayers (Labarca *et al.*, 1988; Bruch and Teeter, 1989), contributing an alternative approach to investigate the conductance mechanisms present in these structures whose small dimensions (0.2 $\mu$m; Menco, 1980) make them difficult to patch-clamp.

## Olfaction and Second Messenger Mechanisms

Electrophysiological studies using the electroolfactogram (EOG) developed by Ottoson in the fifties (Ottoson, 1956, 1958) yielded the first evidence that cyclic nucleotides might play a role in olfactory transduction when it was demonstrated that cAMP can trigger olfactory responses and that inhibitors of phosphodiesterase activity affect the response to odorants as measured by the EOG (Minor and Sakina, 1973; Meneuse *et al.*, 1977). More recently, biochemical work in chemosensory membranes derived from the olfactory cilia of vertebrates revealed that they display a high activity of adenylate cyclase (Meneuse *et al.*, 1977; Pace *et al.*, 1985; Sklar *et al.*, 1986; Breer *et al.*, 1990), the enzyme catalyzing the formation of cAMP from ATP. In addition, olfactory cilia also possess GTP-binding proteins (G-proteins) including $G_o$, $G_i$, and, in particular $G_s$, a form of G-protein coupling cell surface receptors to the activation of adenylate cyclase (Pace *et al.*, 1985; Pace and Lancet, 1986; Anholt *et al.*, 1986; Bruch and Kalinoski, 1987). Recently, the notion that olfactory receptors display a unique form of $G_s$, named $G_{olfactory}$, has been developed (Jones and Reed, 1989).

Olfactory cilia from fish provide a particularly valuable material to carry on studies of olfactory transduction since, in addition to a high activity of adenylate cyclase and G-proteins, they are known to contain surface receptors that are specific to certain stimuli (amino acids) (Bruch and Teeter, 1989). Odorant receptors have not yet been resolved in amphibia or terrestrial vertebrates.

The studies by Pace *et al.* (1985) provided the first evidence that odorants are able to stimulate the adenylate cyclase in olfactory cilia obtained from

amphibia. Cyclase stimulation was found to depend on GTP. These observations led Pace and coworkers to propose that the early events in olfactory transduction involve an enzyme cascade resembling that operating in visual transduction (see above). Later on, studies by Sklar *et al.* (1986) on the stimulation of the olfactory adenylate cyclase by different odorants demonstrated that many, but not all, fruity, floral, minty, and herbaceous odorants were effective in stimulating cAMP formation. Stimulation could be observed at odorant concentrations of 100 $\mu$M. Cyclase stimulation was enhanced by odorants with the longest hydrocarbon chain, in agreement with the observation of Ottoson (1958) that hydrophobicity is a factor in odorant potency. Putrid odorants, organic solvents, and some odorants belonging to the above classes were ineffective in stimulating adenylate cyclase activity. Recently, Breer *et al.* (1990) were able to measure odorant-induced cAMP formation in the millisecond range, using fast kinetic techniques, in membrane fractions derived from rat olfactory cilia. This approach allowed these authors for the first time to demonstrate rapid and significant changes in cAMP formation induced by submicromolar concentrations of odorants. cAMP accumulation is maximal 20–50 ms after the odorant pulse and rapidly decays to values near the pre-stimulation levels. The rates of cAMP formation measured some 300 ms after stimuli were found to depend on odorant concentration. This last observation might explain why previous measurements made at longer times after stimuli could detect significant stimulation of cAMP only at relatively high odorant concentration (Pace *et al.*, 1985; Sklar *et al.*, 1986).

## Cyclic Nucleotide-Gated Conductances in Olfactory Cilia

The idea that cyclic nucleotides are relevant to olfactory transduction through the modulation of conductances received important support from the impressive work of Nakamura and Gold (1987) who patch-clamped olfactory cilia in isolated olfactory neurons. Their work demonstrated that olfactory cilia, dendrite, and soma of olfactory receptors display a cyclic nucleotide-gated conductance. Activation by cyclic nucleotides is direct and reversible and the conductance pathway was found to be equally permeable to $Na^+$ and $K^+$, making it compatible with an excitatory role. Divalent cations, at millimolar concentrations, block the cyclic nucleotide-gated conductance, similar to the effect they exert on cGMP-gated channels in photoreceptors. Interestingly, as first demonstrated by Nakamura and Gold (1987), the ciliary conductance can be gated by cGMP as well as cAMP, cGMP exhibiting a higher apparent affinity. Moreover, cCMP was also reported to be able to activate, albeit with a lower affinity. $K_{1/2}$ for cGMp ranged from 1 to 20 $\mu$M and 3–37 $\mu$M for cAMP, with Hill coefficients

ranging from 1.1 to 1.8, suggesting that activation is cooperative. Since at high concentrations of either ligand the maximal conductance was the same as in the presence of high concentrations of both ligands, it was concluded that they must act on the same rather on different conductance pathways.

Results similar to those reported by Nakamura and Gold (1987) were obtained by Bruch and Teeter (1989) who fused olfactory cilia membranes from channel catfish to planar lipid bilayers. Fusion inserts cyclic nucleotide-gated channels exhibiting conductances of 45 pS (in 100 mM salt) that do not select between $Na^+$ and $K^+$. Channel activation was achieved at micromolar concentrations of either cGMP or cAMP. In agreement with the results of Nakamura and Gold (1987), channel activation by cyclic nucleotides seems to be direct since the bilayer assay is done in the absence of exogenous nucleotide triphosphates. Cyclic nucleotide-gated conductances have also been reported in chemosensory membranes derived from rat olfactory epithelium inserted into planar bilayers (Vodyanoy and Vodyanoy, 1987). Thus, the experimental evidence indicates that the presence of cyclic nucleotide-gated conductances is a general feature of vertebrate olfactory receptors. However, we still lack direct proof that exposure of olfactory neurons to physiological concentrations of odorous stimuli actually results in the activation of nucleotide-gated conductance pathways. Such an evidence, when available, will be extremely important in defining the precise role they play in olfactory transduction. Moreover, it would help to determine which of the many different chemicals that are able to stimulate olfactory neurons do so through the control of cAMP levels in the ciliary lumen, particularly so since other transduction mechanisms, including second messenger mechanisms other than cAMP, have been postulated to operate in olfactory transduction (Labarca *et al.*, 1988; Bruch and Teeter, 1989). These experiments would be easier to perform if agents that block cyclic nucleotide-gated channels in olfactory cells are identified. In this respect, work in planar bilayers doped with chemosensory membranes might help to find such blockers. Some authors have suggested that amiloride, the classical blocker of epithelial sodium channels, could be a blocker of odorant-modulated conductances (Frings and Lindemann, 1988). Recent studies by Ugarte *et al.* (1990) indicate that amiloride blocks voltage-gated inward currents in frog olfactory receptors in the absence of odorants.

The studies of odorant-activated currents reported by Firestein and Werblin (1989) suggest strongly that intermediate steps separate the moment an odorant interacts with chemosensory membranes from the development of the accompanying conductance change. In olfactory neurons under whole-cell clamp conditions Firestein and Werblin (1989) demonstrated that there is a considerable time interval ($> 100$ ms) separating the moment an

odorant hits an olfactory cell from the time an inward current is detected. Such a delay can be explained if one assumes that odorants trigger an enzyme cascade which must operate to raise cAMP levels in the cilia previous to the development of a conductance change. In short, olfactory neurons are competent in linking the interaction between odorants and their chemosensory membranes with an enzyme cascade that modulates cAMP levels in the olfactory cilia. This cyclic nucleotide, in turn, activates directly cation-selective channels in the cilia, dendrite, and soma of the receptor cell. However, as evident from the above considerations, much work will be needed in all fronts before we can proclaim a full understanding of the mechanisms operating in olfactory transduction. The evidence seems to suggest that several mechanisms, of which the cyclic nucleotide mechanism is the most strongly supported by experimental data, might actually operate in the first events of olfaction. Taking into account the important progress achieved in recent years, one can be reasonably confident that a combination of biochemical and electrophysiological approaches, seasoned with the tools of molecular biology, should allow us to make an adequate assessment of the role played by cyclic nucleotide-gated conductances in the receptor cell and how this and other mechanisms interplay to give rise to the receptor potential in olfactory receptors.

## Potassium Channels in *Drosophila* Muscle

In *Drosophila* larval muscle, membrane excitability is in part controlled by several $K^+$ currents that are voltage- and/or $Ca^{2+}$-activated. Voltage clamp, the use of specific blockers, and genetics have shown the presence of four different $K^+$ currents. Two of them show inactivation and the other two are sustained. One of the inactivating currents is pharmacologically similar to the *A* current present in nerve cells, one of the sustained currents show properties of the delayed rectifier, and the remaining currents share the property of being modulated by intracellular $Ca^{2+}$ (Salkoff and Wyman, 1981; Gho and Mallart, 1986; Singh and Wu, 1989). On the other hand, patch clamp studies in cultured embryonic myotubes derived from *Drosophila* indicate the presence of at least six different types of $K^+$ channels (Solc and Aldrich, 1988; Zagotta *et al.*, 1988; Solc *et al.*, 1987; Komatzu *et al.*, 1990). Three of these display voltage-dependent kinetics, and ensemble current average indicates kinetic correspondence with the macroscopic currents of the *A* and delayed rectifier type. A fourth type is voltage independent and stretch independent. The fifth type is $Ca^{2+}$ activated and correlates well with the macroscopic inactivating $Ca^{2+}$ activated current. Finally, we describe below some of the properties of a cAMP-activated $K^+$ channel (Delgado *et al.*, 1990; Delgado *et al.*, 1991).

## Potassium Channels and *Drosophila* Mutants

An attractive feature of electrophysiological studies in *Drosophila* is that is allows one to combine studies on genetics and membrane ion currents. This is due to the availability of mutants with defects in one conductance pathway. The single-gene mutants *shaker* and *slowpoke* have been of particular importance for these studies. *Shaker* lacks the voltage-dependent *A* current, and *slowpoke* is defective in the transient $Ca^{2+}$-activated $K^+$ current (Salkoff and Wyman, 1981; Elkins *et al.*, 1986). *Drosophila* mutations impairing a specific ion channel have provided the key for obtaining the channel primary structure by gene cloning and DNA sequencing (e.g., Papazian *et al.*, 1988). We were attracted by a *Drosophila* mutant showing poor levels of associative learning and rapid short-term memory losses (e.g., Dudai, 1988; Tully, 1987). The mutant *dunce* is a single-gene mutant that lacks a form of phosphodiesterase leading to abnormally high levels of intracellular cAMP (Byers *et al.*, 1981). Therefore, it is expected that in this mutant any cAMP-dependent channel phosphorylation and/or direct channel gating by cAMP should be greatly affected.

## A cAMP-Activated Channel in Wild Type *Drosophila* and *Dunce* Larval Muscle

In cell-attached membrane patches in wild-type larval muscle we have found a channel that opens very infrequently with an average probability of opening of about 0.01 (Fig. 2A; Delgado *et al.*, 1991). This channel shows a high selectivity for $K^+$ ($P_{Na}/P_K \leqslant 10$) and is blocked by internal TEA at micromolar concentration. Thus, the TEA site resembles those found in the delayed rectifier of the node of Ranvier and in certain $Ca^{2+}$-activated $K^+$ channels (Latorre and Miller, 1983). Under physiological conditions the channel shows a maximum conductance of 50 pS. In inside-out patches the probability of opening remains low, but is enhanced 10–15-fold when cAMP is added to the internal side without affecting the number of channels present in the patch or the channel current (Fig. 2B). A dose–response curve reveals an apparent dissociation constant of 51 $\mu$M and a Hill number of 3, indicating that several cAMP molecules are involved in channel opening. These numbers are similar to those reported for the cyclic nucleotide-activated conductances in vertebrate photoreceptors and olfactory neurons (Table I). Other nucleotides, such as AMP, ATP, or cGMP, inositol trisphophate, and $Ca^{2+}$, are not able to mimic the effects of cAMP.

Measurements of channel activity in a series of *dunce* mutants indicated that a channel with similar conductance was persistently activated in these mutants (Fig. 2C). The intracellular concentration of cAMP in these mutants

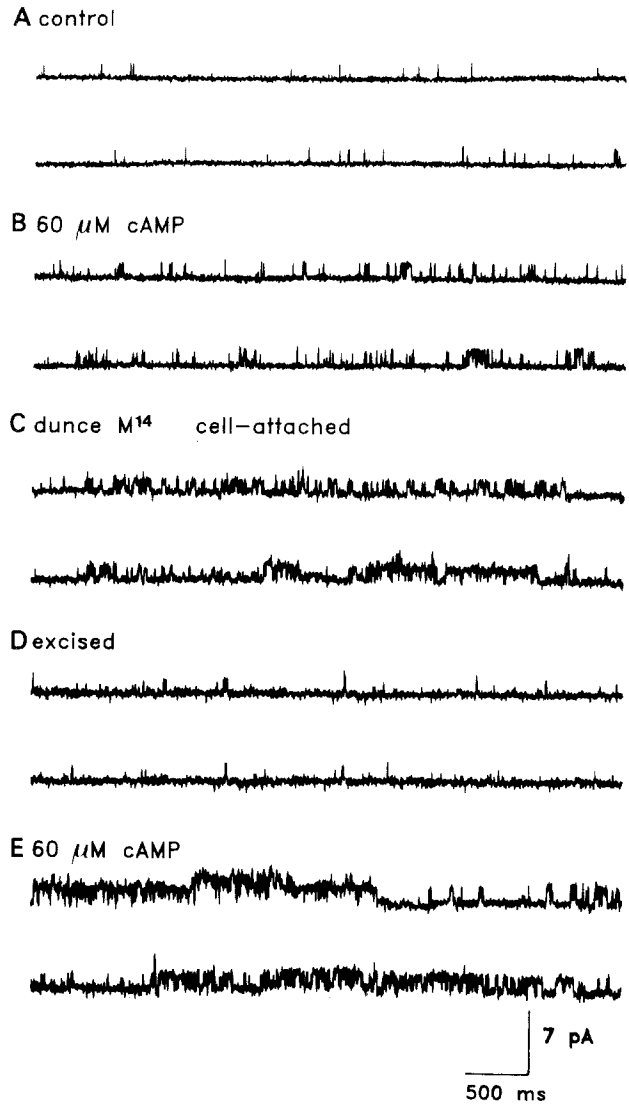


**Fig. 2.** Potassium channel activation by cAMP in excised membrane patches of *Drosophila* and *dunce* larval muscle. (A) Channel activity in a larval muscle membrane patch of *Oregon-R* (wild type). (B) Channel activity in the same patch shown in A, but in the presence of 60 μM cAMP. In this experiment channel open probability increased from 0.01 in the absence to 0.10 in the presence of cAMP. Upward current deflection correspond to channel openings. The patch pipette contained a 128 mM NaCl saline buffer, pH 7, and the bath (pseudointracellular buffer) contained 150 mM potassium acetate. 1 kHz filtering. The membrane voltage was 10 mV. (C) Channel activity in a male larva muscle from *dunce*$^{M14}$ culture. Current record from a cell-attached patch containing 2 cAMP-activated channels. (D) The patch showed in C was excised and channel activity was measured. Notice the remarkable decrease in activity. (E) The same patch showed in D was exposed to 60 mM cAMP. Membrane voltage: 10 mV.

is 2–6-fold that found in the wild type fly (Byers *et al.*, 1981; Davis and Kiger, 1981). The increase in probability of opening appears to be related to the cytoplasmic concentration of cAMP present in these mutants. Thus, *dunce*$^{M14}$, with an intracellular concentration of cAMP 3-fold larger than the [cAMP] in *dunce*$^{1}$, shows an open probability 3–4-fold larger than in the latter mutant. As in the wild type, the channel opens very infrequently when the membrane patch is excised from the cell and is activated by cAMP added to the internal side (Fig. 2D, E). The physiological importance of this channel is yet unclear, but resting potential measurements indicate that *dunce* shows a 7 mV larger membrane potential than the wild-type muscle. This observation suggests that this channel contributes in *dunce* muscle to the total membrane resting conductance.

In summary, *Drosophila* larval muscle is the first excitable cell found to possess a $K^+$-selective channel that is activated directly and reversibly by cAMP. In *dunce* muscle this channel shows a much increased probability of opening compared to the wild type. The possibility that cAMP exerts its effect indirectly by activating a kinase is unlikely since channel activation occurs in cell-free patches in the absence of ATP. Moreover, the effect is obtained rapidly after exposing the patch to cAMP and disappears readily when the chamber is perfused with a cAMP-free solution. This channel adds to those examples of direct activation of ionic conductances by intracellular messengers which are supported by single-channel recording, and is the first, to our knowledge, to be specifically activated by cAMP. It will be of interest to determine whether or not this $K^+$ channel also operates in neurons of wild type and *dunce* mutants and, if so, how its increased activity affects cell excitability in the mutant. Such studies might be of importance in helping to establish a link between alterations in the regulation of the electrical activity in the nervous system of *Drosophila* neurological mutants and the deficiencies they exhibit in associative learning and retention.

## Acknowledgments

This work was financed in part by the Fondo Nacional de Investigacion, grants 1167-88, 451-88, and 1116-90, NIH GM-35981, and the Tinker foundation. R.L. is recipient of a John S. Guggenheim Fellowship. R.L. wishes to thank the Dreyfus Bank (Switzerland) for generous support from a private foundation that they made available to him.

## Note Added in Proof

Recently, Buck and Ascel (1991) (Cell **65**: 175–187) have provided evidence for 18 different members of a large multigene family likely encoding for a diverse family of odorant receptors.

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