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Cyclic nucleotide-activated channels in carp olfactory receptor cells

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When applied from the cytoplasmic side, cyclic 3′,5′-adenosine and guanosine monophosphates reversibly increased the ion permeability of inside-out patches of carp olfactory neuron plasma membrane. The cAMP (cGMP)-induced permeability via cAMP (cGMP) concentration was fitted by Hill's equation with the exponents of 1.07 ± 0.15 (1.12 ± 0.05) and $EC_{50} = 1.3 \pm 0.6$ μM (0.9 ± 0.3 μM). Substitution of NaCl in the bathing solution by chlorides of other alkali metals resulted in a slight shift of reversal potential of the cyclic nucleotide-dependent (CN) current, which indicates a weak selectivity of the channels. Permeability coefficients calculated by Goldman-Hodgkin-Katz's equation corresponded to the following relation: $P_{Na}/P_K/P_{Li}/P_{Rb}/P_{Cs} = 1:0.98:0.94:0.70:0.61$. Ca^{2+} and Mg^{2+} in physiological concentrations blocked the channels activated by cyclic nucleotides (CN-channels). In the absence of divalent cations the conductance of single CN-channels was equal to 51 ± 9 pS in 100 mM NaCl solution. Channel density did not exceed 1 μm^{-2}. The maximal open state probability of the channel (P_o) tended towards 1.0 at a high concentration of cAMP or cGMP. Dichlorobenzamil decreased P_o without changing the single CN-channel conductance. CN-channels exhibited burst activity. Mean open and closed times as well as the burst duration depended on agonist concentration. A kinetic model with four states (an inactivated, a closed and two open ones) is suggested to explain the regularities of CN-channel gating and dose–response relations.

Introduction

A new type of cation channels activated by cyclic nucleotides has been revealed in plasma membranes of photoreceptor, olfactory and mechanoreceptor cells [1–6]. Indetectability of single cyclic nucleotide-activated channels under physiological conditions is their peculiarity, since the channels were partially blocked by divalent cations. However, in the absence of divalent cations an obvious channel activity induced by cyclic nucleotides was detected from inside-out patches of rods, cones [3,7–9] as well as from those of frog [5], salamander [11] and catfish [12] olfactory neurons. This kind of ionic channels mediates phototransduction in retinal rods and cones [13–16] and, probably, transduction of stimuli in other receptor cells. More exactly, some findings indicate that in olfactory cells these channels are stimulus-dependent. So it was shown that olfactory epithelium exhibits a high adenylate cyclase activity [17,18]. The enzyme was activated by various odorants [19–21] and the activation proceeded as rapidly as needed to underlie the receptor potential generation [22,23]. Injection of the cyclic nucleotides into isolated olfactory receptors of frog [24], newt [25] and salamander [26] depolarized cells and, moreover, the properties of cyclic AMP-sensitivity conductance were close to those of the odorant-dependent one [27,28]. And finally, as it was shown recently, the channels regulated by odor is gated by cyclic nucleotides as well [29].

In the present work we have studied CN-channels of carp olfactory receptor cells. As expected, the channels were partially blocked under physiological conditions. However, when Ca^{2+} and Mg^{2+} were removed, the single CN-channel activity could be detected quite well. The channel described are in many respects similar to CN-channels of other receptor cells although there are some peculiarities. At moderate agonist concentrations the single CN-channel, as a rule, manifests a burst of rapid openings and closure (flickering). At low agonist concentrations the channels predomenantly exhibit single openings, whereas at saturating concentrations they practically remain open all the time. This type of channel gating as well as the dependence of open probability on the agonist concentration are predicted by a simple kinetic model with four states.

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Methods

Preparation

The experiments were carried out using carp *Cyrpinus carpio*. The animals were decapitated and pithed. The nasal cavities were opened and the olfactory epithelium was scraped. The scrapings were collected into plastic tubes containing trypsin solution (0.5 mg/ml). Trypsin was solved in 0.5 ml of Ringer solution (RS) (mM): NaCl, 100; KCl, 2.5; $CaCl_2$, 0.1; $MgCl_2$, 1.0; glucose, 5.0; Na-pyruvate, 5.0; Hepes, 10.0 (pH 7.5). After a 5–10-min incubation the trypsin solution was removed, the pieces of mucosa were rinsed in 0.5 ml of RS and then pipetted several times by a plastic pipette 0.5 ml in volume. The sample obtained contained the great amount of single cells which were then transferred by a plastic pipette into the electrophysiological experimental chamber. All procedures were performed at room temperature (20–23°C). The cell suspension was maintained up to three hours at 4°C when needed.

Native carp olfactory neurons similar to those of other vertebrates can be easily identified according to their peculiar morphology characterized by an axon and a ciliated bulbous dendrite [30]. Yet, when the cells are isolated, some dendrites as well as (and more frequently) cilia and axon can be broken. Such 'reduced' receptor cells can be hardly distinguished among some other isolated cells in a sample. That is why the experiments were only performed on the receptors with pronounced axons and dendrites crowned by cilia.

Solutions

We used the solutions of the following content. Solution A (mM): NaCl, 100; $MgCl_2$, 1.0; $CaCl_2$, 0.1; Hepes, 10 (pH 7.5). Solution B (mM): NaCl, 100; EGTA, 0.1; EDTA, 0.1; Hepes, 10 (pH 7.5). When studying the CN-channel selectivity, (i) NaCl in solution B was replaced by an equimolar amount of chlorides of alkali metals; (ii) 50 mM of NaCl was substituted by 100 mM of sucrose in solution B. In the present study we used cGMP, cAMP, from Boehringer (Austria); ATP, GTP, EDTA, EGTA from Serva (Germany); trypsin from Sigma (USA). Other chemicals were of chemical grade from Reachim (Russia).

Chamber

Two parallel thin glass plates fixed by the holder served as an experimental chamber with a volume of about 100 μl. Surface tension prevented the solution to leave the space between the plates providing thereby the possibility to perfuse the chamber with a velocity up to 100 μl/s. The perfusion solutions flowed by gravity into the chamber through a thin pipe with a volume of about 50 μl. The surplus solution was sucked out of the chamber.

Pipettes

Patch pipettes were made from thin-walled Pyrex tubes (o.d. = 1.1 mm; i.d. = 1 mm). Prior to fire polishing the outer tip diameter was about 0.5–1.5 μm. The electrodes were polished and filled with solution B just before the experiment. The chamber was filled with solution A which was replaced by solution B immediately after gigaohm seal formation (5–10 GΩ, as a rule).

Reference electrode

The reference electrode was constructed as follows. The opaque plastic tube was filled with 3% agar/2 M KCl gel. The Ag-AgCl electrode was placed onto the end of the tube. Nylon wick was mounted on the opposite end providing a galvanic contact with bath solution. Such a construction originated a negligible diffusion potential making thereby the potential of the reference electrode insensitive to the saline composition of a chamber solution. The reference potential could be corrected for the junctional potentials between a pipette (sealed or unsealed) and the reference electrode. For symmetrical bath and pipette solutions, the zero pipette current was considered to indicate a full correction.

The current and potential across the patch membrane were described with the usual sign convention. That is, the external side of the membrane was taken as a reference; currents flowing into a pipette were given a positive sign.

Recording procedure and data analysis

We used a hand-made patch-clamp amplifier with a feed-back resistance of 22 GΩ and a frequency compensation circuit. The output of the amplifier was stored on a FM tape recorder (MR-10C, Teac) at bandwidth equal to 0–5000 Hz or digitized by the A/D converter DT2821 (Data in personal computer (IMB PC-AT).

Integral current data. As a measure of CN-channel selectivity we used the shift of CN-current reversal potential (ΔV) originated by substitution of NaCl by chlorides of other alkali metals. The permeability coefficients were estimated using the adapted Goldman-Hodgkin-Katz's equation:

$$\Delta V = \frac{RT}{F} \ln \frac{P_{Na} a_{Na}}{P_x a_x} \quad (1)$$

where R, T, F have their common meaning; P and a are permeability coefficient and activity of an ion type, respectively. We believe that such approach is quite correct: (i) the potential of the reference electrode is independent of the bath solution composition (see above), (ii) the liquid junction potential between pipette and bath solution is absent in the case of gigaseal patch

formation, (iii) the potential shift is independent of what potential is taken as the reference one.

Single channel data. The data from the tape recorder were filtered at 3 kHz (corner frequency), digitized at 20 kHz and analyzed with the use of Single Channel Current Analysis Program (J. Dempster, University of Strathclyde, Glasgow). The program provided setting the base and unitary channel current level by eye. Thereafter, an idealized list of open and closed states were generated by scanning the data file. The times of transition were defined as crossings of the mid-line between adjacent levels. A list of duration of the idealized channel current states was used for design of the histograms of channel open and closed times and burst durations as well as for calculation of mean open and closed times. The open probability, P_o, was taken as the sum of all open intervals divided by the duration of data segment. A burst was defined as starting with any opening separated from the foregoing one by a closed time of no less than 1 ms. The burst was terminated by any closure lasting longer than 1 ms. A single-, double- or triple-exponential probability density function can be fitted to the dwell time histograms using Levenberg-Marquadt's method of non-linear least squares.

As a rule, several CN-channels operated in a patch. The multi-channels recording was subdivided into current levels to calculate the time interval, t_j, for which the patch current was equal to j. Here, the open probability and mean open time, τ_o, were estimated according to:

$$P_o = \sum j t_j / TN \tag{2}$$

$$\tau_o = \sum j t_j / K \tag{3}$$

where T is a complete recording time, N is the number of channels operating in a patch, K is a number of all channel openings (transitions between a given level, j, and subsequent open level, $j + 1$). The sum extends over all interval countered.

To estimate the conductances of single channels we selected well resolved open-closed transitions and plotted single channel current histograms. The histograms were fitted by a sum of two Gaussians:

$$F(i) = A \cdot \exp(-(i - i_1)^2/\sigma_1^2) + B \cdot \exp(-(i - i_2)^2/\sigma_2^2) \tag{4}$$

The amplitude of the single channel current was estimated as $\delta i = i_1 - i_2$.

Results

As established earlier, CN-channels operate in ciliary membranes as well as in plasma membranes of dendrites and cell somata of toad and frog olfactory receptors [4,5]. The diameter of olfactory receptor cilia is about 0.25 μm, so the probability of gegaseal patch formation is extremely low. For these reasons CN-channels of carp olfactory cells were exclusively recorded on dendrites and cell somata. We succeeded in obtaining 97 stable inside-out patches from plasma membranes of olfactory receptors, 34 of them responded to application of cAMP or cGMP.

When the patch pipette was filled with solution A, the cyclic nucleotides only slightly increased the patch conductance. In the absence of divalent cations on both sides of the patch cyclic nucleotide-dependent (CN) conductance was rather expressed, whereas magnesium applied at physiological concentrations (≈ 1 mM) from the cytoplasmic side of the membrane almost entirely blocked CN-permeability (the data not shown). So, in most cases we used bathing solutions without divalent cations. Although cAMP was mainly employed for the experiments described below, no essential difference between the effects induced by cGMP or cAMP were observed. Using an agonist at nanomolar concentrations or pipettes with submicrometer tip diameter, we succeeded in recording of single CN-channel activity. As a rule, several channels operated in a patch, but sometimes a single channel was recorded.

Dose–response relations

As illustrated in Fig. 1A, the CN-current of excised patches varied with agonist concentration in a dose-dependent manner. Each dose–response relation was approximated in Hill's coordinates according to the equation

$$I/I_{max} = [cA]^n / ([cA]^n + K_{1/2}^n) \tag{5}$$

to estimate the Hill coefficient (n) and a half-effect concentration ($K_{1/2}$). Fig. 1B summarizes the results of all experiments where continuous and dotted curves correspond to Hill's equation with the exponents equal to 1 and 2, respectively. It is seen that experimental points are grouped near the curve with Hill coefficient equal to 1. For cAMP (cGMP) the mean ($\pm$S.E.) of all experiments gives $n = 1.07 \pm 0.15$ (1.12 ± 0.05), $K_{1/2} = 1.3 \pm 0.6$ (0.9 ± 0.3) μM. Thus, both cyclic AMP and GMP increase inside-out patches conductance approximately with an equal efficiency just as for toad [4], frog [5], salamander [11] and catfish [12] olfactory receptors.

The Hill coefficient of concentrational curves is commonly used as a measure of cooperativity of ligand–receptor interaction. This characteristic of carp CN-channels is obviously different from that of cGMP-gated channels of rods and cones as well as CN-channels of toad, salamander and newt olfactory receptors whose Hill coefficients are equal to 2–3 [1,4,7,11,25]. It should be noted that for the CN-chan-

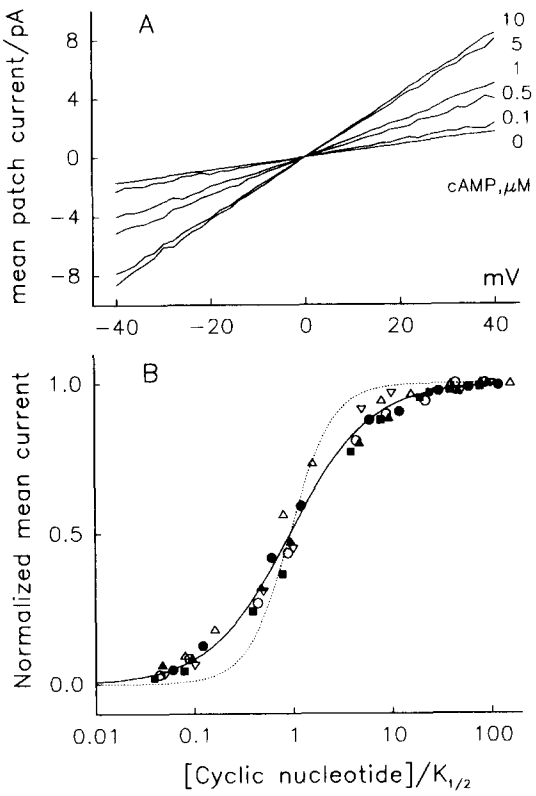

Fig. 1. Effects of cyclic nucleotides on permeability of inside-out patches. (A) Current–voltage relations of a membrane patch at various cAMP concentrations. (B) Normalized CN-current (at −40 mV) versus normalized cAMP (■ ▲ ● ○) and cGMP (△ ▽) concentration.

nels of catfish olfactory cells the hill coefficient was estimated to be 1.3–1.4 [12].

Selectivity of CN-channels

When the patch pipettes were filled with 100 mM NaCl, the substitution of 100 mM NaCl by 50 mM NaCl in bathing solution (two experiments, data not shown) shifted the reversal potential of CN-current by 17 ± 0.5 mV (mean ± S.E.); this value is close to the potential of an ideal Na^+-selective electrode (17.6 mV at 20°C) and points to the cation selectivity of CN-channels. When NaCl was substituted by LiCl, KCl, RbCl or CsCl (four experiments), the mean values (±S.E.) of the reversal potential shifts were equal to 0.9 ± 1.5; 1.3 ± 1.2; 9.9 ± 1.8; 13.5 ± 3.2 mV, respectively. Thus, taking into account the coefficients of ion activity, the sequence of selectivity can be written as follows:

$$P_{Na}/P_K/P_{Li}/P_{Rb}/P_{Cs} = 1:0.98:0.94:0.70:0.61$$

This result is not very surprising since all known cyclic nucleotides-activated channels of receptor cells, namely cGMP-gated channels of photoreceptor cells [1,31–34], CN-channels of frog and newt olfactory cells [5,25] and cAMP-gated channels of guinea-pig cochlea hair cells [6] are weakly selective for alkali cations.

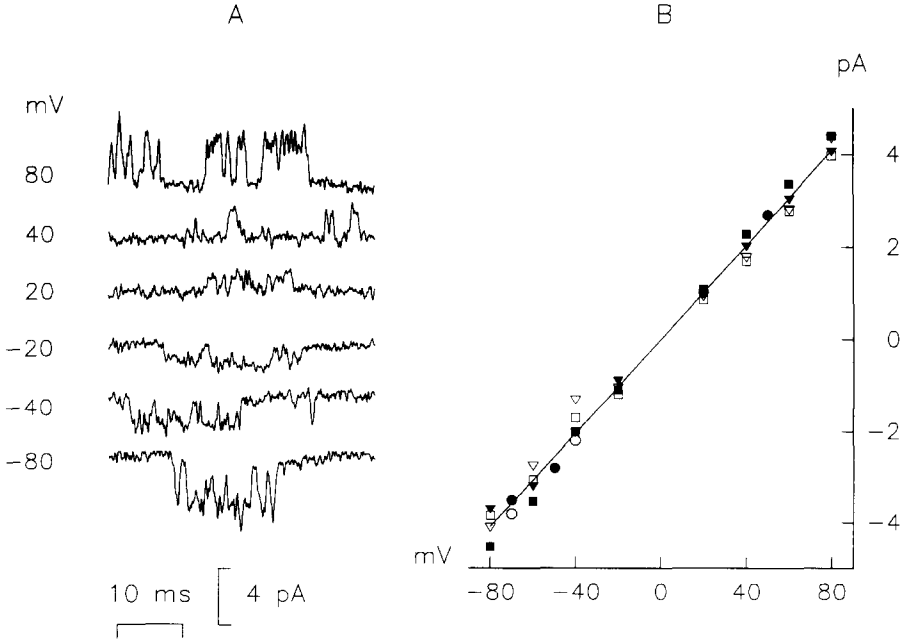

Fig. 2. Single channel voltage–current relation. (A) Single channel current fluctuations at various holding potentials. (B) Amplitudes of single channel currents versus membrane voltage. The straight line corresponds to the equation $i = \gamma \cdot V$ with a slope of 51 pS. The following agonist concentrations were used: 10 nM cAMP (▼ ■ ● ▲), 100 nM cAMP (▽ ○) and 100 nM cGMP (□).

Single channel conductance

In most experiments several CN-channels operate simultaneously in a patch. However, at nanomolar agonist concentrations single channel openings can be recorded at various membrane voltages. On the base of these experiments and using the data obtained from the patches with the only functioning channel, we estimated the channel current amplitude at various potentials (see Methods). As illustrated in Fig. 2B, the single channel conductance is practically independent of membrane voltage in the range of -80 to 80 mV and linear regression yields its value equal to 51 ± 9 pS (mean $\pm$ S.E.). By comparison, a single CN-channel conductance in frog, salamander and catfish olfactory cells were estimated at 19 pS [5], 45 pS [11] and 55 pS [12], respectively, in similar ionic conditions.

The open state probability (P_o)

A number of experiments was carried out to reveal the dependence of P_o on the agonist concentration. CN-current fluctuations similar to those usually observed at various cAMP concentrations are shown in Fig. 3. As illustrated, at a saturating concentration of cAMP the channels are open during all the time of recording. This behavior of CN-channels is just that observed in both cases: for the patches containing several operating channels or a single one (see Fig. 5), irrespective of whether cAMP or cGMP were used.

P_o via cAMP concentration is shown in Fig. 4. To estimate $K_{1/2}$ and n, the data were fitted by Hill's equation:

$$P_o = [cA]^n / ([cA]^n + K_{1/2}^n) \quad (6)$$

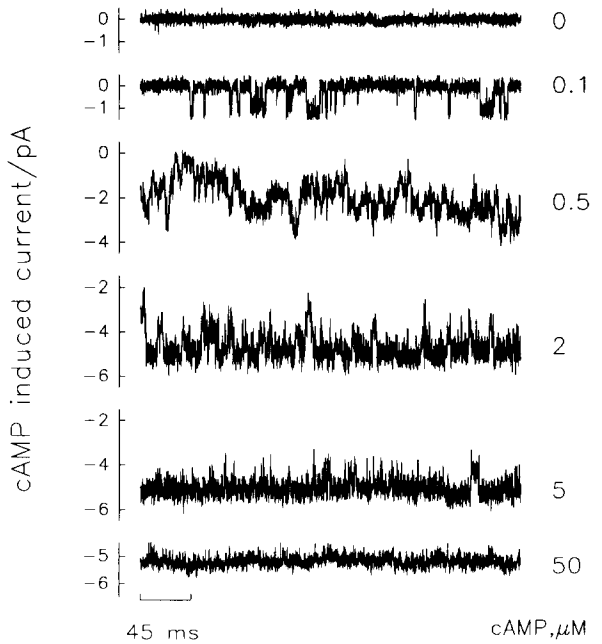

Fig. 3. Fluctuations of CN-current at various cAMP concentrations. The membrane voltage was of -30 mV. Four CN-channels operated in the patch.

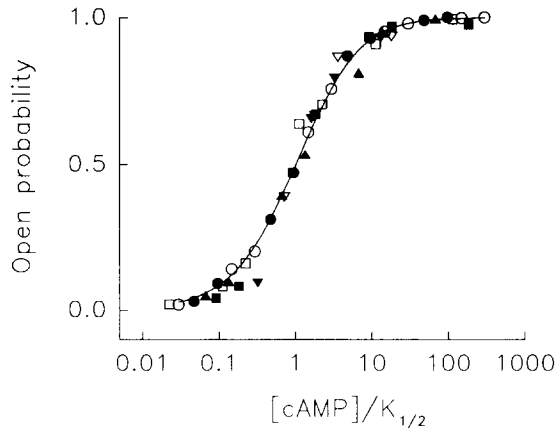

Fig. 4. Open probability of CN-channels versus normalized cAMP concentration. The solid line corresponds to the Eqn. 6 with $n = 1$.

The following mean values ($\pm$S.E.) of the parameters were obtained: $K_{1/2} = 0.73 \pm 31$ μM and $n = 1.06 \pm 0.4$. Thus, in agreement with integral current data, the P_o as the cAMP concentration function was fitted by Hill's equation with an exponent of about 1. Besides, the findings indicate the tendency o fP_o to equal 1 at saturating concentrations of cAMP. This may show a fully liganded channel not to perform any open-closed flickering. It should be noted that although P_o was estimated at the patch potential equal to -40 mV or -30 mV, the results obtained hold for other potential values as well. The latter is supported by the fact that the current–voltage curves were practically linear (see Fig. 1A).

Density of CN-channels

With the tip diameter of about 1 μm, the pipette resistance was 3–6 MΩ in 0.1 M NaCl. This agrees with the data of Sakmann and Neher [35] who mentioned the thin-wall pipettes with the tip diameter of 1 μm to have a resistance of 2–5 MΩ in 150 mM KCl. They estimated the patch area equal to 5–20 μm^2 for such a case.

In our experiments at saturating concentrations the agonist induced an increase of the integral patch conductance to 130–850 pS. An averaged CN-conductance accounted for 187 pS (11 experiments) or there were four channels operating in a patch (P_o was taken equal to 1). Basing on the data described we may conclude that the channel density in dendrite and cell body is about 0.2–1 μm^{-2}, whereas for newt the channel density was reported to be equal to 2–3 μm^{-2} [25].

Concentrational dependence of channel kinetics

A single channel operating in a patch is needed to provide a complete analysis of CN-channel gating. The density of the channels estimated above is just that hoped for in advance. For a high probability of the only operating channel in a patch the area of it must not

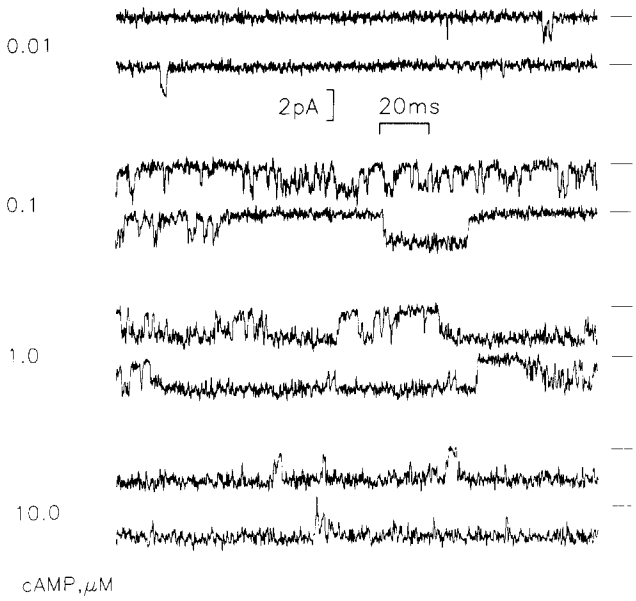

Fig. 5. Dependence of single channel activity on cAMP concentration. Membrane voltage was of −40 mV. The bars to the right of sample recordings show the closed state of a channel. The traces shown at each cAMP concentration were continuous in time.

exceed several μm^2. Equivalently, the patch pipette tip diameter needs to be equal to about 0.5 μm. While using the pipettes of this sort, the probability of gegaseal formation was very low and vesicles quite frequently appeared. This was the reason why in three cases only (from more than sixty attempts) inside-out patches with single operating CN-channel had been obtained.

As expected, the activity of the single CN-channel

TABLE I

Temporal parameters of CN-channels gating at different cAMP concentrations (mean ± S.E.)

cAMP	τ_o (ms)	τ_c (ms)	τ_b (ms)
10 nM	1.3±0.2	63.7 ±10.6	4.5± 0.7
100 nM	1.9±0.9	6.4 ± 2.8	4.7± 0.4
1 μM	8.5±4.1	2.7 ± 1.2	24.6± 5.4
10 μM	20.2±2.6	0.62± 0.31	252 ±93

depends on the agonist concentration and manifests itself as bursts of channel openings (Fig. 5). The distribution of channel open time (τ_o), closed time (τ_c), and burst duration (τ_b) at various cAMP concentrations were examined. While measuring a burst duration, all closings less than 1 ms in duration were ignored, so the burst consisted of series of one or more openings separated by closures less than 1 ms. Fig. 6 illustrates distributions of open, closed states and burst durations at 100 nM and 1 mM cAMP. The number of extended openings of the channels is seen to increase with cAMP concentration, whereas the number of prolonged closures decreases (Fig. 6B). The results summarized in Table I suggest that the open lifetime and the burst duration increase with an increasing agonist concentration while the closed lifetime decreases.

It should be noted that the open lifetime of photoreceptor cGMP-gated channels does not depend on cGMP concentration [9]. Taking into account this fact as well as an insufficient number of recordings from single channel patches, the mean open time at various cAMP concentrations was estimated by using the

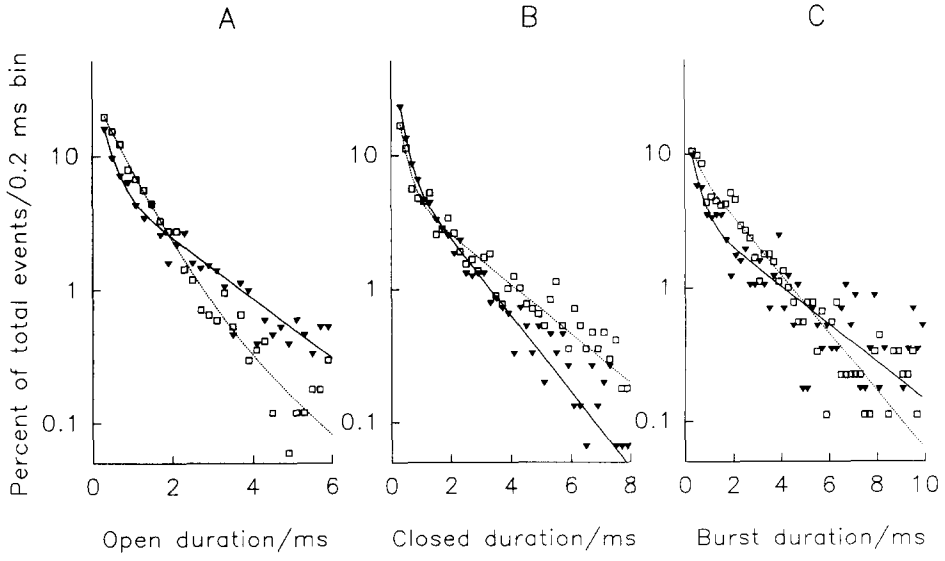

Fig. 6. Distributions of open (a), closed (B) and burst (C) durations of a single CN-channel at 100 nM (□) and 1 μM (▽) cAMP. All data were fitted with a two-exponential equation, dotted and solid lines correspond to 100 nM and 1 μM cAMP, (A) 100 nM: $\tau_1 = 0.71$ ms, $\tau_2 = 1.94$ ms; 1 μM: $\tau_1 = 0.28$ ms, $\tau_2 = 1.8$ ms. (B) 100 nM: $\tau_1 = 0.26$ ms, $\tau_2 = 2.35$ ms; 1 μM: $\tau_1 = 0.25$ ms, $\tau_2 = 1.52$ ms. (C) 100 nM: $\tau_1 = 0.32$ ms, $\tau_2 = 2.01$ ms; 1 μM: $\tau_1 = 0.36$ ms, $\tau_2 = 3.08$ ms.

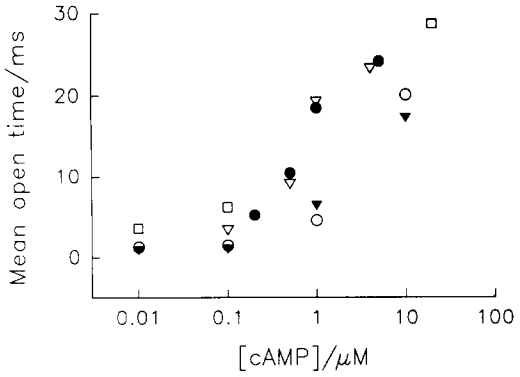

Fig. 7. Mean open time versus cAMP concentration. Membrane potentials were −40 or −30 mV.

patches with several operating CN-channels (see Methods). Fig. 7 summarizes all open lifetime data. As it can be seen, the mean open time of CN-channels actually depends on cAMP concentration. This fact is highly importent to design a correct model of CN-channel gating.

Block of CN-channels by dichlorobenzamil

As it has been established earlier, 3′,4′-dichlorobenzamil (DCBA), derivative of amiloride, blocks the CN-channels of frog olfactory cells [5]. So, in a number of experiments we studied the effects of DCBA on carp CN-channel activity and found that the blocker effectively inhibits the CN-current ($ES_{50} \approx 4$ μM). As shown in Fig. 8, the decrease of the channel open probability

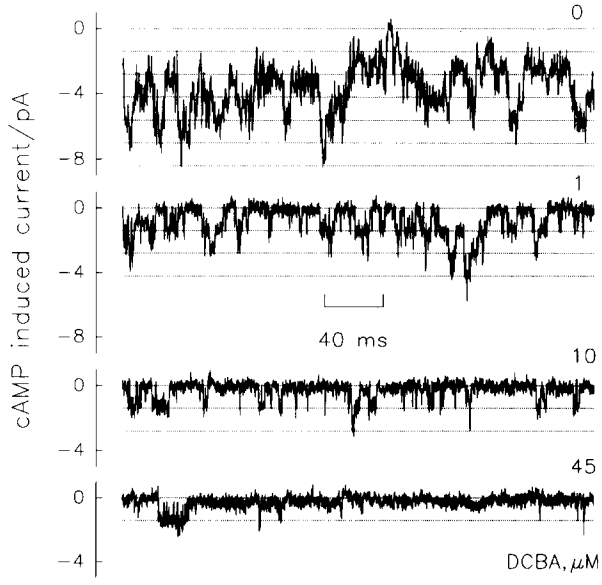

Fig. 8. Fluctuations of CN-current at various DCBA concentration and 0.5 μM cAMP. Membrane voltage was of −30 mV. Six CN-channels operated in the patch.

seems to underlie the CN-current blocking by DCBA without altering the single channel conductance.

Discussion

In the present work the CN-channels of carp olfactory receptors have been investigated. The results obtained show that the properties of one more variety of cyclic nucleotide-activated channels are similar to those of other receptor cells. The resemblance is characterized by their (i) efficiency to transport cations of alkali metals, (ii) activation at micromolar concentrations of cyclic nucleotides, (iii) inhibition by Ca^{2+} and Mg^{2+}. At the same time, some peculiarities of the carp CN-channels should be marked.

The dose–response relations of the channels described turned out to be surprising. The mean CN-current as well as the open state probability expressed in Hill's coordinates are proportional to the first degree of an agonist concentration (see Fig. 1 and Fig. 4). Traditional interpretation of this fact makes us assume one molecule of cAMP or cGMP to be required to activate the carp CN-channel. At the same time CN-channels of olfactory cells of toad [4], frog [5], salamander [11], newt [25] and catfish [12] as well as cGMP-gated channels of photoreceptors [1,3,6,9,36] must bind no less than two agonist molecules to be completely activated.

The cGMP-activated channels of vertebrate photoreceptors as well as the CN-channels of olfactory cells of some species manifest the open-closed flickering within the whole range of agonist concentrations [9,10,12,36]. As a result, P_o is obviously less than 1 even at saturating agonist concentrations. The maximum value of P_o for cGMP-gated channels of toad accounts for 0.42 [9], whereas for those of salamander rods and goldfish cones it is equal to 0.9 [36]. In the case of catfish olfactory CN-channels, maximal P_o ranges from 0.2 to 0.8 [12]. P_o as a function of an agonist concentration for the frog and salamander CN-channel has not been studied yet. It is, however, noteworthy that the flickering of frog and salamander CN-channels was observed at saturating agonist concentrations [5,11]. In contrast to this, for the carp CN-channels P_o was about 0.98 and no evident flickering was observed at 200 μM cAMP. Consequently, the kinetics of fully liganded CN-channels of carp olfactory cells and that of other cyclic nucleotide-gated channels seem to be different.

And finally, our experiments have not revealed any clear sublevels of the CN-channel conductance. Contrary to this, one subopen level of cGMP-gated channels of salamander rod [3], catfish cone [35], and CN-channels of catfish olfactory receptors [12] were detected. Besides, reconstituted cGMP-gated channels of

bovine rods manifested four discrete conductance levels [37].

Thus, there are essential differences in gating between the channels described here and the variety of other CN-channels.

The model of carp CN-channel gating

Usually the gating of agonist-dependent channels is explained by kinetic models. The first kinetic model appears to be proposed by Del Castillo and Katz [38]. It served for description of the effects of acetylcholine (ACh) on the end plate membrane potential:

$$R \rightleftharpoons AR \rightleftharpoons AR^* \qquad (7)$$

where R denotes a shut conformation of the ACh-receptor, R* denotes the open state; and A represents the ACh. For such a scheme of agonist–channel interaction the open state probability versus agonist concentration is described by the Hill equation with an exponent equal to 1, whereas both the open lifetime and burst duration do not depend on the agonist concentration [39]. However, in our case these temporary parameters depend on cAMP concentration (see Table I and Fig. 7), so the scheme mentioned cannot be used to describe CN-channel gating. At the same time a cooperative mechanism of interaction between the agonist and the channel exhibits the dependence of the open lifetime and burst duration on agonist concentration [39], hence, no less than two molecules of cAMP or cGMP are required to activate CN-channels. It should be, however, noted that the model of the agonist-channel binding must provide a fitting of dose–response relations by the Hill equation with an exponent close to 1.

The following scheme seems to be quite suitable to satisfy the above statement:

$$R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} AR^* \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} AR \underset{k_4}{\overset{k_3}{\rightleftharpoons}} A_2R^* \qquad (8)$$

Here R, AR and AR* correspond to the same symbols used in scheme (7); A_2R^* denotes an open state of the channel upon binding of two agonist molecules. It should be noted that the channel conductances in states AR* and A_2R^* are assumed to be equal. The present study was not aimed at carrying out a complete investigation of model (8) properties, nevertheless, some conclusions could be made. First, according to this scheme, the channel has a single conductance level and its activity is of a burst character. At present we cannot say with assurance in what manner open and closed lifetimes and burst duration depend on the agonist concentration, however, the dependence of these values on the agonist concentration is an intrinsic property of model (8) [39]. Second, the model is capable of correct predicting the channel behavior in limiting cases. For instance, if $k_2 \gg \beta$, then at small agonist concentrations the channel activity will be mainly observed as single openings, whereas the burst activity will arise at moderate agonist concentrations. Actually, at 10 nM cyclic AMP about 80% of channel openings were single and only the remaining ones occurred as bursts. As the cyclic AMP concentration increased, the channel activity aquired a burst character (Fig. 5).

In the frames of model (8) the open state probability can be expressed as:

$$P_o = ak_1(\beta k_3 a + \alpha k_4)/(a^2\beta k_1 k_3 + ak_1 k_4(\alpha + \beta) + \alpha k_2 k_4) \qquad (9)$$

where a denotes the concentration of agonist A. Note, that $P_o \Rightarrow 1$ at $a \Rightarrow \infty$. Simple transformation results in:

$$P_o(1 - P_o) = a(k_3/k_4)(1 + (\alpha/k_3) \times (k_1 k_4 - k_2 k_3)/(\beta k_1 a + \alpha k_2)) \qquad (10)$$

If in expression (10) the factor:

$$B = 1 + (\alpha/k_3)(k_1 k_4 - k_2 k_3)/(\beta k_1 a + \alpha k_2) \qquad (11)$$

is practically independent of a over the experimental range of agonist concentration, then

$$P_o/(1 - P_o) = a(k_3/k_4)B = \text{const} \cdot a \qquad (12)$$

Eqn. 12 implies that the open state probability as a function of agonist concentration will be linearized in Hill's coordinates with an exponent equal to 1.

Two obvious cases exist when factor B is nearly constant. First, if the condition

$$|k_1 k_4 - k_2 k_3| \ll k_2 k_3, \quad \text{i.e. } |k_4/k_3 - k_2/k_1| \ll k_2/k_1 \qquad (13)$$

takes place, then $B = 1$. Relation (13) indicates that the both agonist molecules bind to the channel with a similar affinity. Second, if the relation

$$\beta k_1 a \ll \alpha k_2, \text{ i.e. } a \ll (\alpha/\beta)(k_2/k_1) \qquad (14)$$

is true, the factor is equal to $B = k_1 k_4/k_2 k_3$.

As a presumable test of model (8) experimental P_o values were fitted by equation:

$$P_o = a(a + m)/(a^2 + pa + q) \qquad (15)$$

which is the consequence of Eqn. 9, where $m = \alpha_4/\beta k_3$, $p = k_4(\alpha + \beta)/k_3\beta$, $q = \alpha k_2 k_4/\beta k_1 k_3$. The data shown in Fig. 9 are an example for such an approximation. As seen, the fitting accuracy of experimental P_o by relation (15) is not worse than that by Hill's equation (Eqn. 6). Similar results were obtained in two other cases.

If m, p and q are known, it is possible to estimate

TABLE II

Parameters of Eqns. 6 and 15

Patch	$K_{1/2}$ (μM)	n (μM)	m (μM)	p (μM)	q (μM^2)	K_1 (μM)	α/β	K_2 (μM)
1	0.34	1.06	3.66	3.81	1.33	0.36	25.4	0.14
2	0.71	1.05	2.86	3.70	2.17	0.80	5.61	0.51
3	0.43	1.09	1.02	1.34	0.49	0.48	3.10	0.33

the dissociation constants of agonist molecules binding (in our case it is cAMP):

$$K_1 = k_2/k_1 = q/m \tag{16}$$

$$K_2 = k_4/k_3 = p - m \tag{17}$$

as well as

$$\alpha/\beta = m/(p - m) \tag{18}$$

The values of the parameters in Eqns. 6 and 15 resulting from P_o approximation and estimated values of the constants are listed in Table II (the data from single channel patches were used). On the base of these data we obtained that $(\alpha/\beta)(k_2/k_1) = 8.9$; 4.3 and 1.5 μM, respectively. This means that within the agonist concentration range of the highest CN-channels activity variation the condition (14) is almost satisfied.

Thus, model (8) describes a possible interaction of cAMP (or cGMP) and CN-channel and it is suitable in many respects. First of all it is correct for experimental dose–response relations and explains why the Hill coefficient for concentrational curves may be close to 1 whereas the channel binds two agonist molecules. The model predicts a burst character of CN-channel gating, the dependence of open lifetime on agonist concentration and the absence of flickering in the fully liganded channel. In conclusion it should be noted that our experimental findings are insufficient to say whether model (8) describes all peculiarities of CN-channel gating or not. A more thorough analysis of single CN-channel fluctuations is required.

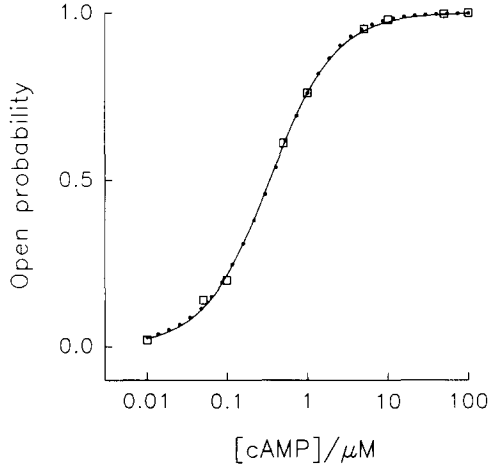

Fig. 9. Open probability of single CN-channel versus cAMP concentration. Membrane voltage was −40 mV. Experimental data (□) were fitted by Eqns. 6 and 15 (solid line and cycles, respectively). The following values for the parameters were obtained; Eqn. 6: $K_{1/2}$ = 0.34, n = 1.06; Eqn. 15: m = 3.66 μM, p = 3.81 μM, q = 1.33 μM^2.

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