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Evidence for a cGMP gated cation channel in photoreceptor cell membranes of *Sepia officinalis*

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Abstract **It is assumed that cyclic nucleotides are involved in signal transduction of invertebrate photoreceptors. In this study, membranes of photoreceptor cells from freshly caught cuttlefish were isolated, and the membrane proteins were reconstituted into proteoliposomes. With the dye Neutral red it was possible to measure cyclic nucleotide induced Na^+ fluxes into the liposomes. cGMP and cAMP concentrations for half maximal activation of Na^+ fluxes are 77 μM and 224 μM, respectively, with Hill coefficients of 2.0 for cGMP and 2.4 for cAMP. These fluxes may demonstrate the presence of at least one cyclic nucleotide gated cation channel in the membranes of the photoreceptor cells of the invertebrate *Sepia officinalis*.**

Key words: Invertebrate photoreceptor; cGMP; Cation channel; Reconstitution; *Sepia officinalis*

1. Introduction

In invertebrate photoreceptors the absorption of a photon by rhodopsin starts an enzyme cascade which results in a conductance increase of the photosensory membrane [1]. It is generally assumed that this is mediated by an increase of intracellular transmitter concentrations. The nature of the transmitters is still unknown, and different possible transmitters such as Ca^{2+}, inositol trisphosphate, cGMP, or cAMP have been postulated [2].

There is evidence for the presence of cyclic nucleotide gated cation channels in the photosensory membrane of invertebrate photoreceptors. Experiments on squid photoreceptors [3] and on the ventral nerve photoreceptor of *Limulus* [2,4] suggest that cGMP is involved in the transduction cascade of the photoreceptor cell. Furthermore, recent patch clamp experiments [5] show results demonstrating cGMP gated channels in photoreceptor cells of *Octopus vulgaris*.

In the present study, the membrane proteins of the photoreceptor cells from freshly caught cuttlefish were reconstituted into proteoliposomes. As described by Schnetkamp [6] the dye Neutral red was used to measure cyclic nucleotide (cGMP and cAMP) induced Na^+ fluxes into the proteoliposomes. The cooperative activation of cation fluxes by the two cyclic nucleotides suggests that there may be at least one type of cyclic nucleotide gated cation channel in the membrane of the photoreceptor cells of *Sepia officinalis*.

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Abbreviations: HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphon ic acid; OD_{540}, optical density at 540 nm.

2. Materials and methods

2.1. Preparation of photosensory membranes

Most important for the present study is the freshness of the material. Cuttlefish dead for more than about 10 h are not useful for these studies. Freshly killed cuttlefish (*Sepia officinalis*) were purchased directly from a fisherman's boat and the dissected eyeballs were kept on ice for no longer than 0.5 h. The retinae were collected in preparation buffer (10 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphonic acid (HEPES)/KOH, pH 7.4, 400 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 15% (w/w) sucrose) and shock-frozen in liquid nitrogen. After thawing the suspension was shaken for 90 s and centrifuged for 10 min at 4,000 × *g*. The supernatant was layered on a two-step discontinuous sucrose gradient of 20% and 40% (w/w) sucrose in preparation buffer and centrifuged for 75 min at 90,000 × *g*. The band containing the photosensory mem-branes was collected from the interface between the two sucrose solutions and diluted 1:2 with preparation buffer without sucrose. Aliquots containing 3 mg protein were centrifuged for 15 min at 70,000 × *g*. The supernatants were discarded and the pellets with the photosensory membranes were frozen in liquid nitrogen and stored at −80°C.

2.2. Electron microscopy

To test the composition of the isolated cells aliquots containing the photosensory membranes were analyzed under the electron microscope. The aliquots were fixed for 2 h at 4°C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and 0.4% tannic acid, pH 7.4. After washing (5 times with cacodylate buffer) the aliquots were postfixed in 2% OsO_4 in 0.1 M cacodylate buffer for 2 h at room temperature. After fixation the aliquots were dehydrated in an acetone series and embedded in Spurrs resin. Ultrathin sections were made with an Ultrome Nova ultramicrotome (LKB), mounted on Formvar-coated copper grids and observed under a Philips EM 300.

2.3. Preparation of proteoliposomes

A pellet with photosensory membranes was hypotonically shocked and washed twice in 1 mM HEPES/arginine, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol (centrifugations for 25 min at 37,000 × *g*). The pellet was washed again in dialysis buffer (40 mM HEPES/arginine, pH 7.4) for 15 min at 26,000 × *g*. The resulting membrane containing pellet was solubilized for 10 min in 4 ml dialysis buffer containing 50 mM *N*-octyl-β-D-glucopyranoside, 8 mg soybean phosphatidylcholine (type IV-S, Sigma) and for 5 min in 1 ml dialysis buffer containing 65 mM *N*-octyl-β-D-glucopyranoside, 10 mg soybean phosphatidylcholine at room temperature. The solubilized proteins were diluted 1:2 with dialysis buffer to a final volume of 10 ml. Dialysis was carried out using a Liposomat (Diachema, Munich) with a membrane of high permeability (molecular mass cut-off: 10 kDa). The liposome solution was kept on ice and pumped with a flow rate of 0.5 ml/min, dialysis buffer was pumped with a flow rate of 2.5 ml/min. The removal of the non-ionic detergent octylglucopyranoside below its critical micellization concentration in order to form proteoliposomes was achieved in a total dialysis time of 4 h.

2.4. Measurement of cation fluxes across the liposome membranes

Na^+ fluxes were measured with the optical probe Neutral red as described by Schnetkamp [6]. Cation fluxes into the liposomes were monitored with 30 μM Neutral red using a spectrophotometer (Cary 1E, Varian). Kinetic studies with a sampling rate of 33 ms were carried out at 540 nm with control measurements at 650 nm to correct light scattering artifacts. NaCl was applied after 1 min by adding 25 μl of

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a 2 M stock solution in dialysis buffer. Cyclic nucleotides were added from stock solutions with varying concentrations in dialysis buffer (always 4 μl to keep the dilution artifact constant). The solution in the cuvette (1 ml) was permanently stirred with a magnetic bar, temperature was kept constant at 15°C.

3. Results

The band from the sucrose-gradient was taken for electron microscopical investigations. The fragments of the isolated cells resemble the shape of invertebrate photoreceptor cells: besides the formation of vesicles there are cell fragments left with clearly identifiable microvillar structures (Fig. 1).

Kinetic measurements of cation fluxes into proteoliposomes containing the membrane proteins of these cells were performed at 540 nm. Stray light artifacts were compensated by monitoring the absorbance changes also at 650 nm where Na^+ fluxes cannot be detected by Neutral red and only light scattering can be seen [6]. Addition of Na^+ ions into the bulk medium of the liposome suspension causes the release of Neutral red from the extravesicular surfaces of the liposomes and results in a decrease of the optical density at 540 nm ($OD_{540\ nm}$) (Fig. 2). With this Na^+ gradient cyclic nucleotide induced Na^+ fluxes into the liposomes were investigated, releasing Neutral red from the intravesicular surfaces and thereby leading to a decrease of $OD_{540\ nm}$ (Fig. 2).

If an ionophore (gramicidin with Na^+, valinomycin with K^+) was applied at the end of the measurements to allow the cations to equilibrate into all liposomes, the effect of the ionophore was greatest without preceding additions of cyclic nucleotides (not shown).

From Na^+ flux measurements like that shown in Fig. 2 the change of $OD_{540\ nm}$ after addition of cyclic nucleotides was calculated. Fig. 3 shows the dependence of the change of $OD_{540\ nm}$ on the concentration of two different cyclic

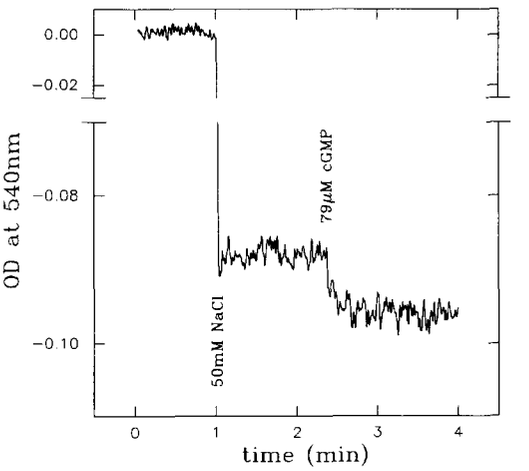

Fig. 2. An original recording from a Na^+ flux experiment monitored at 540 nm with 30 μM Neutral red in a permanently stirred cuvette kept at 15°C (0.2 mg protein/ml). After 1 min 25 μl of a 2 M Na^+ stock solution were added (final concentration 50 mM). After stabilization of the decrease of $OD_{540\ nm}$ 4 μl of a 20 mM cGMP stock solution were applied eliciting a further decrease of the optical density.

nucleotides. At concentrations higher than about 250 μM the change of $OD_{540\ nm}$ reaches a plateau both for cAMP and cGMP. However, the two cyclic nucleotides initiate Na^+ fluxes with different activation behaviours.

A Hill plot (Fig. 4) using the data shown in Fig. 3 quantifies the concentration dependence of the absorbance changes caused by cyclic nucleotide induced Na^+ fluxes. A Hill coefficient of 2.0 and a value for half maximal activation of 77 μM were calculated for cGMP. However, it takes a higher concentration of cAMP to activate a half maximal Na^+ influx, namely 224 μM with a Hill coefficient of 2.4.

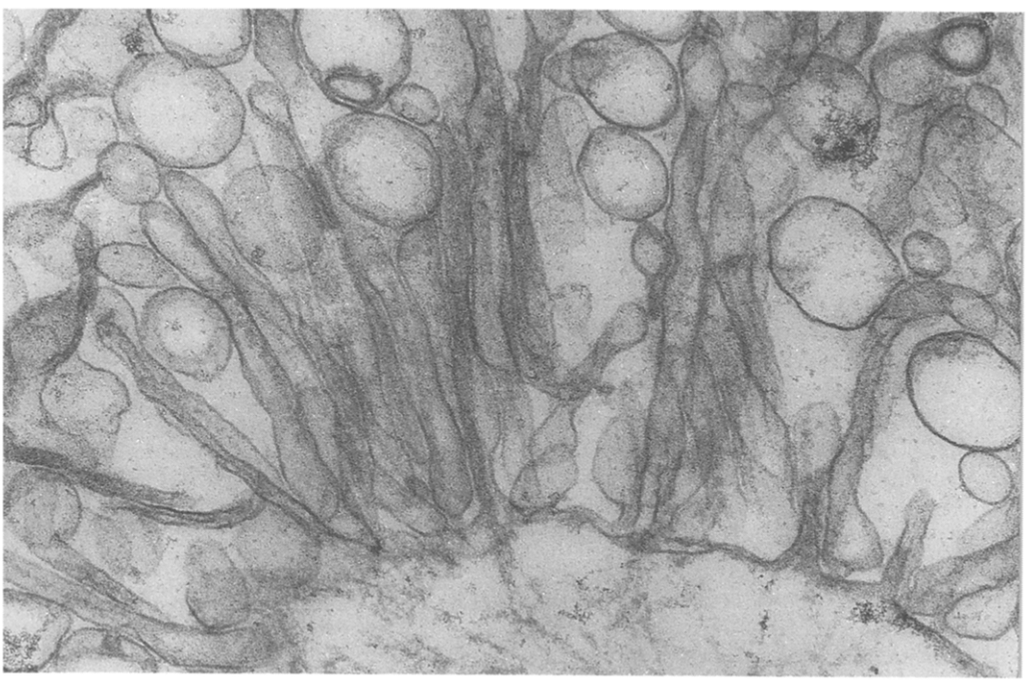

Fig. 1. Electron micrograph of cell fragments isolated on a sucrose gradient. Cuttlefish retinas were freeze-thawed and layered on a two-step sucrose gradient. The band on the interface contained the photoreceptor cells as indicated by the microvillar structures that in the retina are only present in photoreceptor cells (×87,500).

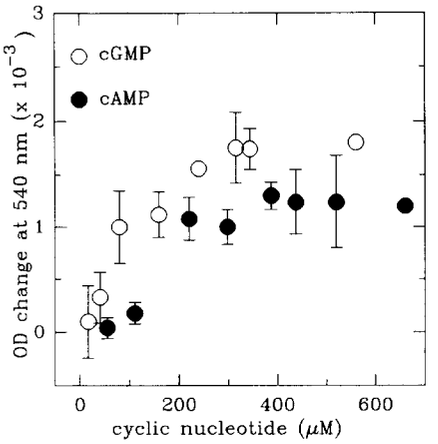

Fig. 3. Changes of $OD_{540\,nm}$ elicited by increasing concentrations of cGMP (open circles) and cAMP (closed circles). After adding 50 mM NaCl into the liposome suspension (30 μM Neutral red, 0.2 mg protein/ml) Na^+ fluxes were initiated by the application of 4 μl dialysis buffer containing different concentrations of cyclic nucleotides. The open and filled circles are the mean values, the error bars the standard deviations of three to twenty measurements per point.

4. Discussion

The most critical point of the experiments reported in this study is the freshness of the material. Even with cuttlefish lying on ice after death for about 10 h it was impossible to measure any reproducible Na^+ flux. Therefore, it was necessary to collect the eyes from animals that were dead for no longer than 2–4 h. The experiments of this study were performed using such freshly prepared retinae.

Under an electron microscope several approaches were undertaken to control the composition of the membranes used for this study. In one control the shape of the membranes was investigated showing the clearly identifiable structure of microvilli (Fig. 1). In a second control (not shown) the proteoliposomes were labeled with an anti-Sepia rhodopsin antibody. With a gold-labeled secondary antibody a fraction of the liposomes could be identified as containing rhodopsin. It cannot be excluded, however, that membrane fragments from other retinal cells are also included within the preparation.

The dye Neutral red can be utilized as a probe for surface potentials at phospholipid membranes. With this dye cation fluxes across liposome membranes containing the cGMP gated cation channel from bovine retinal rod outer segments were measured by Hsu and Molday [7] and Schnetkamp [6] who extensively described the physicochemical basis of this method. Here, this system was adapted to measure Na^+ fluxes into proteoliposomes. The fluxes were quantified by evaluating the change of $\dot{O}D_{540\,nm}$. Calculation comprises the correction for stray light measured at 650 nm. Therefore, the values for half maximal activation and the Hill coefficients given in this paper rely on the saturation plateau and not on the initial velocity of the change of $OD_{540\,nm}$, similar to the calculations of Bauer [8].

As described by Schnetkamp [6], different control experiments were carried out to ensure that cyclic nucleotide induced changes of $OD_{540\,nm}$ were due to changes in the binding of Neutral red to the membranes. After repeating these control experiments, further controls were carried out to proof the cGMP/cAMP induced changes of $OD_{540\,nm}$ to be Na^+ influx effects into the liposomes: (i) The change of $OD_{540\,nm}$ induced by the application of an ionophore alone had the same size as the sum of the changes of $OD_{540\,nm}$ of ionophore and preceding addition of cyclic nucleotides. (ii) Liposomes were prepared solely with phosphatidylcholine, without the addition of proteins. Using these lipid vesicles no change of $OD_{540\,nm}$ except the dilution artifact could be induced by adding cGMP or cAMP to the medium. With an ionophore again changes of $OD_{540\,nm}$ were induced.

The gating properties of the cGMP gated cation channels from vertebrate photoreceptor cells were calculated by several authors: The cGMP gated channels from retinal rod and cone cells have values for half maximal activation (EC_{50} values) between 11 and 70 μM cGMP, and Hill coefficients between 1.6 and 3.3 [7,9–12]. For cAMP the EC_{50} value for the rod channel was found to be between 740 and 1210 μM with a Hill coefficient between 1.1 and 1.8 [10,11].

The dependence of the Na^+ fluxes on cGMP concentrations measured with Neutral red shows properties similar to those of the cGMP gated channels described above. Both, the value for half maximal activation (77 μM cGMP) and the Hill coefficient (2.0) are in the ranges of the values for these vertebrate ion channels. This strongly suggests that there is a cGMP gated channel in the photosensory membrane of the cuttlefish.

The values obtained with cAMP allow two explanations: If the estimated EC_{50} value is originated from only one type of channel in our preparation then one can explain the lower affinity to cAMP compared to cGMP (factor 2.9) with the presence of a cGMP gated channel that has a lower affinity also to cAMP. On the other hand, it cannot be excluded that there are two different channels in the photosensory membrane gated by cyclic nucleotides. It is possible that the EC_{50} value for cAMP calculated from the experiments is due to different affinities implying the existence of another channel gated by cAMP. In some experiments it was possible to elicit cAMP induced

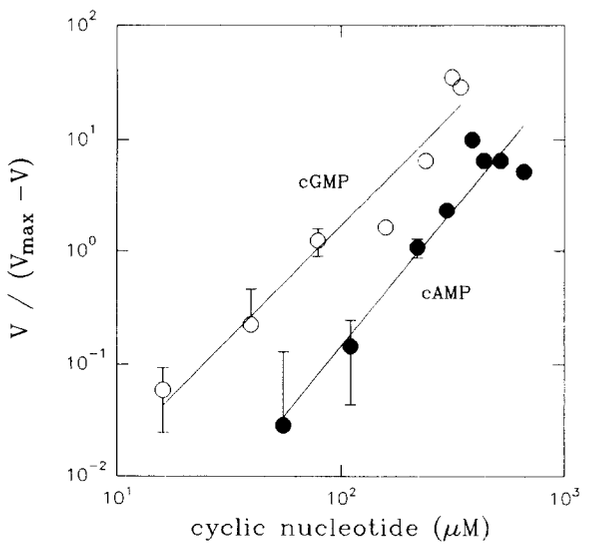

Fig. 4. Hill plot of the data shown in Fig. 3. Data are fitted by linear regression with a slope of 2.0 for cGMP (open circles) and 2.4 for cAMP (closed circles). Values for half maximal activation calculated from these regressions are 77 μM cGMP and 224 μM cAMP. The term V is the cGMP or cAMP induced change of $OD_{540\,nm}$, V_{max} corresponds to the maximal change of $OD_{540\,nm}$ evoked by cGMP or cAMP.

Na^+ fluxes even after applying saturating concentrations of cGMP. Furthermore, this hypothesis is supported by electrophysiological experiments with photoreceptors of *Limulus* [2].

The results indicate that there is at least one new member in the family of cyclic nucleotide gated cation channels. It has to be elucidated whether there are two different channels in the photosensory membrane of the cuttlefish *Sepia officinalis* that are gated by cyclic nucleotides and of which nature these channel proteins are.

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