Disturbing GTP-Binding Protein Function through Microinjection into the Visual Cell of *Limulus*

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We have tested the action of three agents microinjected into the ventral nerve photoreceptor of *Limulus* on the electrical response to dim light. 1. A monoclonal antibody (mAb 4A) against the G_α subunit of frog transducin reduces the size of the receptor current to 60%, suggesting an interaction with G_α in the *Limulus* photoreceptor. 2. Injection of *Clostridium botulinum* ADP-ribosyltransferase C3 reduces the size to 46%; latency is not affected. The results imply that small GTP-binding proteins play a functional role in photoreception of invertebrates. 3. Injection of GDP-β-S reduces dose-dependently the size of the receptor current to 15% and prolongs the latency to 200%, presumably by reducing number and rate of G-protein activations.

Introduction

Heterotrimeric G-proteins have been shown to play a major role in transducing the light signal from rhodopsin to the downstream elements in the visual cascade of vertebrates. Analogous mechanisms of transduction on the G-protein level have also been proposed for the invertebrate visual system of *Limulus*. In photoreceptors of vertebrates many elements of the cascade have been identified and the terminal transmitter to open cation channels has been found to be cGMP [1, 2]. In photoreceptors of invertebrates it is known that through activation by light, rhodopsin triggers the activation of a G-protein which then activates a phospholipase C (PLC). PLC catalyses the breakdown of phosphatidylinositol bisphosphate (PIP_2) into inositoltrisphosphate (IP_3) and diacylglycerol (DAG). IP_3 causes the release of calcium from intracellular stores into the cytosol (for review see [3]). The terminal transmitter which causes the opening of ion channels has as yet to be identified; calcium and cGMP ions are likely to be involved ([4, 5], see however [6]).

More than one species of ion channels have been implicated in mediating the light-induced conductance of invertebrates [7–9]. The different species of channels may be regulated by different transmitters [8, 9]. Moreover, there is evidence for the existence of more than one light-activated G-protein in photoreceptors of the squid [10–12].

In this paper we describe the effects of microinjection of agents which affect the function of GTP-binding proteins. We used the ventral nerve photoreceptor cells of *Limulus*. These cells have a relatively large volume (400–500 pl) and are therefore suitable for microinjection studies.

We present evidence that more than one GTP-binding protein plays a functional role in the *Limulus* phototransduction pathway. Moreover, the experiments suggest that *Limulus* photoreceptors contain small GTP-binding proteins (20–30 kDa) which are known substrates for ADP-ribosylation by exoenzyme C3 [13] and also seem to take part in or influence the light-activated phototransduction cascade of vertebrate photoreceptors [14].

Materials and Methods

Ventral nerves of *Limulus polyphemus* were dissected under white light, treated with collagenase A (Boehringer, 10 min, 2 mg/ml) to ease electrode impalement and then superfused with physiological saline of 15 °C throughout the experiment [15] (HEPES buffer, pH 7.5).

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Microinjection pipettes were broken to a larger diameter by moving against a glass surface. Filled, the resistance was 6–16 MOhm. Pipettes prepared in this manner penetrated the photoreceptor membrane more easily than unbroken pipettes.

A ventral photoreceptor was then impaled with one microelectrode filled with one of the following agents dissolved in 500 mmol/l potassium chloride, 10 mmol/l HEPES, pH 7.5:

1. Fab fragments of monoclonal antibodies against frog G_α transducin, 20 µmol/l [16].
2. *Clostridium botulinum* ADP-ribosyltransferase C3, 10–40 µg/ml [17].
3. GDP-β-S, 10 mmol/l, as lithium salt.

With such a pipette a pressure pulse of 10–30 psi and 100–600 ms duration typically delivered about 7–10 pl of solution. Injection volumes were estimated by injection into a small pool of oil prior to each experiment [18]. The pressure injection of the solutions into the cells was monitored optically with an infrared video system.

The electrical light response of the photoreceptor was recorded either as receptor potential or as receptor current under single electrode voltage clamp conditions. In some of the experiments receptor current and receptor potential were recorded alternatingly. The method is described elsewhere in detail [15]. The photoreceptor cell was stimulated every minute by a light flash of 1 ms duration. Two different flash energies were applied alternatingly: One flash energy evoking an almost saturated electrical light response, the next one a response slightly below half-saturation. The drug effects are larger on the half-saturated response, therefore only these are discussed in this paper.

Controls

In a number of control experiments we pressure-injected different volumes of either heat-denatured antibodies (mAb 4A or anti-tetanus) or 30 mmol/l lithium chloride. Injection volumes of 5–15 pl of these control solutions caused no significant change in the response characteristics (see Fig. 1c). However, some variability in the response size was observed for a few minutes after injection of the controls. Therefore, we chose to compare the effect of the antibody, exoenzyme C3 or GDP-β-S 20–30 min after injection. For the data evaluation up to 5 values 20–30 min after injection were averaged and normalized to 3 to 5 averaged preinjection values. Table I summarizes the results.

Results and Discussion

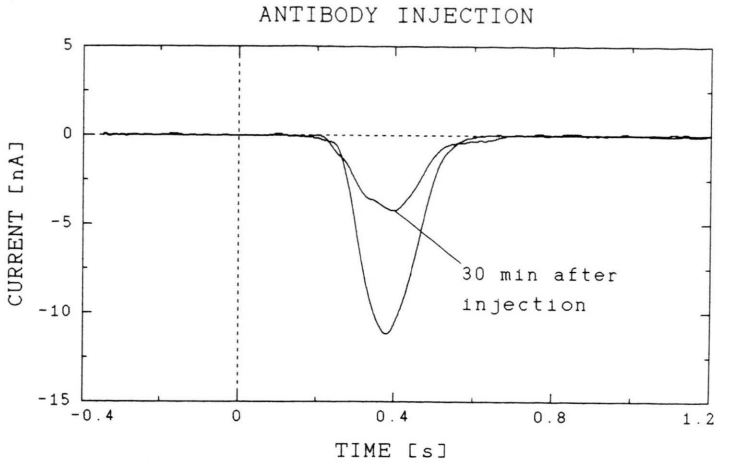
Injection of anti-transducin antibody

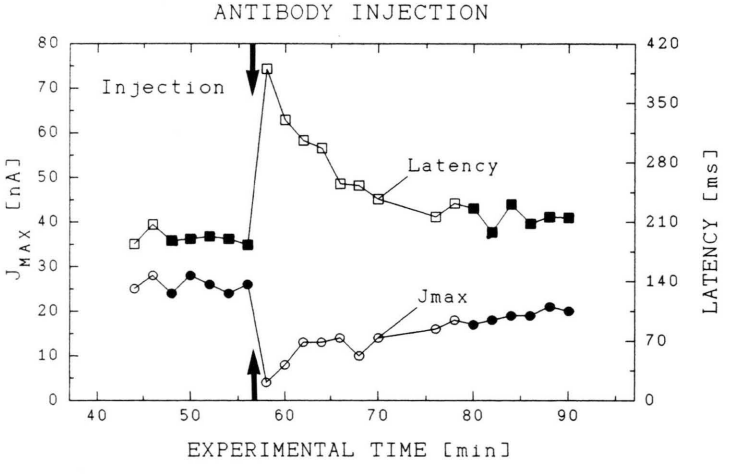
Transducin is the guanine nucleotide binding protein in photoreceptors of vertebrates. After interaction with photoexcited rhodopsin (metarhodopsin II) it exchanges GDP for GTP and the GTP-bound α-subunit dissociates from rhodopsin and the β γ-dimer. The GTP-containing α-subunit then activates a cGMP specific phosphodiesterase. In photoreceptors of vertebrates the antibody mAb 4A recognizes the G_α sequence Glu 311–Val 328 of transducin [19] and is able to block the interaction between rhodopsin and bovine transducin [16, 20].

To test the intracellular effect of the antibody mAb 4A in an invertebrate system, we injected the Fab fragments of the antibody into 8 cells. 6 of the cells showed desensitization, indicated by reduction in the size of the electrical light response which is seen in the receptor current or the receptor potential. In the experiments with the two not responding cells the antibody may have been ineffective because it had been thawed for more than two days.

Fig. 1a shows membrane currents evoked by dim light stimuli before and 30 min after injection of the antibody. Fig. 1b shows peak amplitude and latency of the receptor current in the course of an experiment. The amplitude Jmax of the receptor current is decreased by the injection and is still smaller than the original value 30 min after the injection. The latency is strongly prolonged by injection, but, 30 min after the injection, it is only slightly longer than the value before injection.

Table I summarizes the effects on the receptor current of several cells in the injection experiments. In a larger number of experiments the influence of the drug injection on the receptor potential was tested. They showed corresponding results. In Table I for the receptor potential only the values of the peak amplitude are listed. mAb 4A reduces the size of the receptor current in the average to ca. 60% of the reference response before injection under low intensity stimulus conditions ($E = 4 \times 10^{13}$ photons/cm^2). The average latency was prolonged to twice its preinjection value shortly after injec-





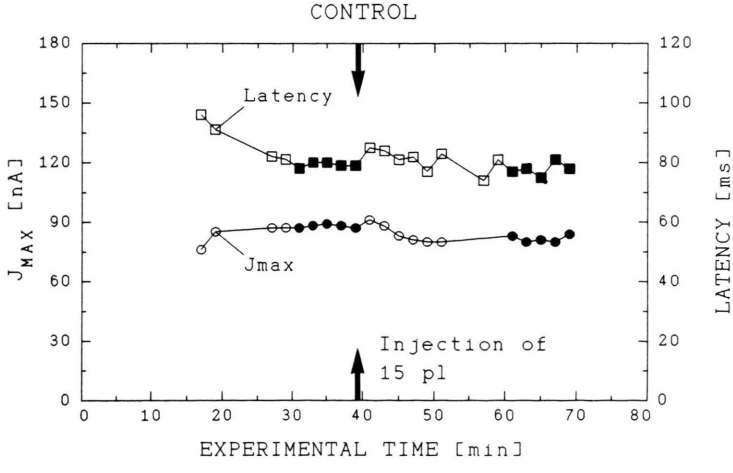


Fig. 1. Pressure injection of Fab-fragments of monoclonal antibodies (mAb 4A) against the G_α subunit of frog transducin in *Limulus* ventral photoreceptor.
a) Receptor current in response to a light flash before and 30 min after injection. Clamp potential −33 mV, flash energy E: 2×10^{13} photons/cm^2, applied at time zero, experiment G 7.
b) Peak amplitude Jmax and latency of the receptor current in the time course of an injection experiment. Clamp potential −50 mV; E: 2×10^{13} photons/cm^2, the arrows mark the injection; reference points that were included in Table I are marked solid; experiment G 10.
c) Peak amplitude Jmax and latency of the receptor current in the time course of an injection experiment. Control injection of 15 pl of heat-denatured anti-tetanus antibody. Clamp potential −33 mV, E: 1×10^{15} photons/cm^2, experiment G 9.

tion (2nd and 3rd min) and recovered to its reference value before injection about 30 min after injection (Fig. 1 b).

As we estimated the ratio of antibody to G-protein to be about 1:5 we did not expect a large effect onto the receptor response. Nevertheless, the results clearly indicate some disturbance of G-protein activation by the interaction between the antibody and a G-protein in the *Limulus* photoreceptor.

Injection of exoenzyme C3

In cells of vertebrates, *Clostridium botulinum* exoenzyme C3 inhibits the biological activity of small GTP-binding proteins of the rho-gene family by ADP-ribosylation but apparently does not block their GDP-GTP exchange [13, 21]. Substrates for ADP-ribosylation have been found in photoreceptors of vertebrates [14]. So far, the effectors of these GTP-binding proteins have not yet been identified. To investigate whether small GTP-binding proteins are of functional significance in photoreceptors of invertebrates, we injected the exoenzyme into ventral nerve photoreceptors of *Limulus*.

In this group of experiments the 8 injected cells showed a clear reduction of the light response after C3 injection. Fig. 2a demonstrates the effect for single responses, Fig. 2b shows the time course of the peak amplitude and latency during an experiment. Table I summarizes the results: Exoenzyme

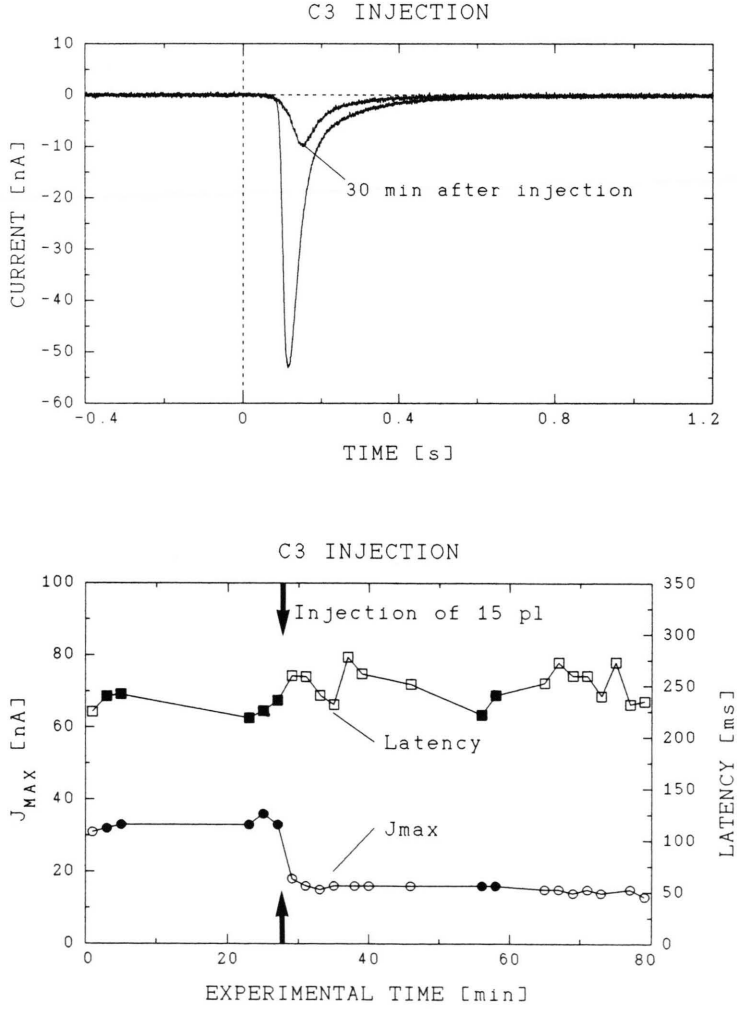


Fig. 2. Pressure injection of clostridial toxin C3 (40 μg/ml) in *Limulus* ventral photoreceptor.
a) Receptor currents in response to a light flash before and 30 min after injection. Clamp potential −31 mV, E: 1×10^{15} photons/cm^2, experiment T8.
b) Peak amplitude Jmax and latency of the receptor current in the time course of an injection experiment. Clamp potential −33 mV, E: 8×10^{13} photons/cm^2, experiment T7. Further details as in Fig. 1.

C3 reduces the size of the receptor current on the average to 46% of the reference response under low intensity stimulus conditions (stimulus energy 1×10^{15} photons/cm^2). The latency is not significantly affected.

Injection of GDP-β-S

Another way to interfere with GTP-binding protein activity in a general manner is to inject GDP-β-S [22–24]. To compare the effects of the antibody and exoenzyme C3 with a known G-protein blocker (for review see [25]), we injected GDP-β-S into 12 cells. All of the injected cells showed desensitization. Receptor currents of five cells were recorded under voltage-clamp conditions and included in Table I. Fig. 3a shows the receptor currents before and 20 min after injection, Fig. 3c shows peak amplitude of receptor current and latency of the electrical light response (receptor current and receptor potential) in the course of an experiment.

GDP-β-S reduces the size of the receptor current on the average to 15% of the reference response before injection (stimulus energy 6.4×10^{14} photons/cm^2). The latency was prolonged on the average to twice its reference value after 30 min.

The effect of GDP-β-S injection is dose-dependent. Fig. 3b shows the effect of two successive GDP-β-S injections on the light response. A second injection further diminishes the response size and further increases the latency.

Conclusions

In order to investigate the role of GTP-binding proteins in phototransduction chain of the invertebrate *Limulus*, we have used three different agents which are known to affect GTP-binding protein activity.

1. The monoclonal antibody against transducin apparently seems to recognize a G-protein of the *Limulus* photoreceptor cell and interferes with its interaction with photo-excited rhodopsin.

2. Assuming that *Clostridium botulinum* ADP-ribosyltransferase C3 inhibits only small GTP-binding proteins, our results show that these proteins interfere in some way with the transduction process. The exoenzyme could act as a modulator either directly or indirectly on the causal chain of phototransduction. C3 does not block the GDP-GTP exchange in cells of vertebrates [13, 21] and, as we have shown for the light response of *Limulus*, does not increase the response latency.

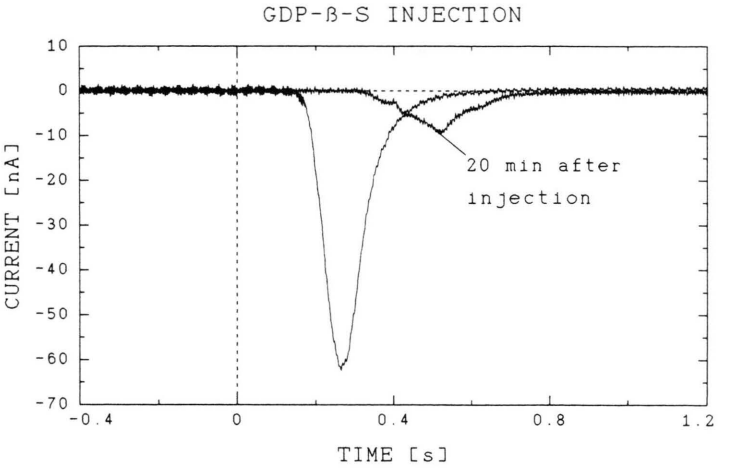
3. GDP-β-S reduces the size and prolongs the latency of the electrical light response in a dose-dependent manner. It acts by blocking those complexes of light-activated rhodopsin with GTP-binding protein which have "caught" a GDP-β-molecule. This reduces the number of GTP-binding protein molecules which are activated per flash and the overall rate of their formation.

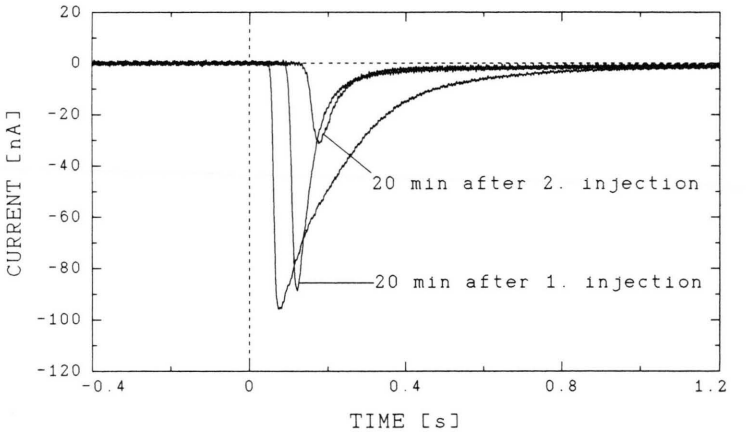
Whereas it can be expected that the monoclonal antibody mAb 4A and the exoenzyme C3 most likely act on specific GTP-binding proteins, GDP-β-S should inhibit all GTP-binding proteins.

As all three agents are expected to affect GTP-binding protein activity and as the results differ in their effect on size and latency of the electrical response, these results suggest that there are more than one species of GTP-binding proteins involved in the signal transduction of the *Limulus* photoreceptor cell.

Table I. Average of the results of receptor current parameters before (a) and 30 min after injection of the different agents (b). a: mean absolute values of all experiments; for each experiment at least three preinjection values were averaged. b: mean of 1 to 5 values in % of the reference value immediately before the injection, averaged over all experiments. Mean ± S.E.M. Flash energy between 4×10^{13} and 6×10^{14} photons/cm^2, evoking an almost half-saturated response, 15 °C. Jmax: peak amplitude, integral: current-time integral, latency: latency of receptor current; Hmax: peak amplitude of receptor potential.

	mAb	n = 3	C3	n = 3	GDP-β-S	n = 5	Control	n = 3
	a	b	a	b	a	b	a	b
x [nA]	16 ± 3.7	52 ± 8.7%	43 ± 4.6	41 ± 9.1%	42 ± 9.1	17 ± 10%	54 ± 15	96 ± 2.8%
gral [nC]	2.3 ± 0.6	59 ± 6.2%	3.3 ± 0.4	46 ± 7.3%	4 ± 1.2	15 ± 11%	4.3 ± 1	101 ± 2.9%
ency [ms]	187 ± 30	108 ± 3.9%	122 ± 43	107 ± 4.5%	150 ± 26	217 ± 24%	107 ± 13	102 ± 2.1%
		n = 6		n = 8		n = 7		n = 3
ax [mV]	31 ± 3.3	69 ± 9.4%	29 ± 3.3	56 ± 13%	28 ± 2	23 ± 12%	42 ± 6.6	101 ± 2.8%





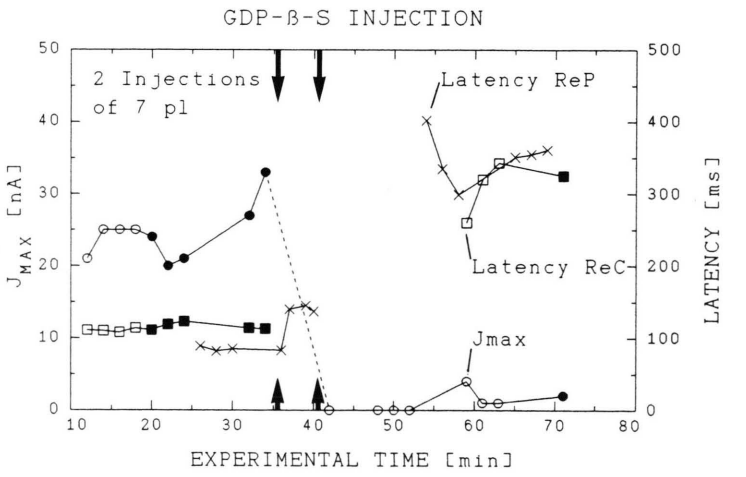


Fig. 3. Pressure injection of GDP-β-S (10–13 mM) into *Limulus* ventral photoreceptors.
a) Receptor current in response to a light flash before and 20 min after injection. Clamp potential −29 mV, E: 1×10^{13} photons/cm^2, experiment S6.
b) Receptor current before, 20 min after a first injection, and 20 min after second injection. Clamp potential −24 mV, E: 2×10^{16} photons/cm^2, experiment S5.
c) Peak amplitude Jmax and latency of the receptor current, ReC, and latency of the receptor potential, ReP, in the time course of an injection experiment. Clamp potential −25 mV, E: 1.25×10^{15} photons/cm^2, experiment S7. The arrows mark two injections. After the second injection the cell did not respond for 12 min. The ReP recovered earlier than the ReC. Further details as in Fig. 1.

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