Limulus ventral photoreceptors contain a homologue of the α-subunit of mammalian N_s

Craig C. Malbon*, U.B. Kaupp and J.E. Brown

*Department of Pharmacological Sciences and Department of Physiology and Biophysics, The State University of New York, Stony Brook, NY 11794-8651, USA, Schwerpunkt Biophysik, Universität Osnabrück, D-4500 Osnabrück, FRG and The Marine Biological Laboratory, Woods Hole, MA 02543, USA

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Membranes from ventral photoreceptors of *Limulus* were incubated with cholera toxin and [^{32}P]NAD$^+$. Cholera toxin catalyzes a specific ADP-ribosylation of a 43-kDa peptide from *Limulus* ventral photoreceptors. Possible homologies between the 43-kDa peptide of *Limulus* and the α-subunits of mammalian stimulatory, guanine nucleotide-binding regulatory component of adenylate cyclase (N_s) were investigated by comparing the electrophoretic patterns of proteolytic fragments derived from each of these peptides that are radiolabeled by [^{32}P]NAD$^+$ and cholera toxin. Evidence is provided for structural homology between this invertebrate peptide and mammalian N_s.

Limulus *Photoreceptor* *Cholera toxin* *ADP-ribosylation*
Stimulatory guanine nucleotide-binding regulatory component

1. INTRODUCTION

Light-induced activation of cyclic GMP phosphodiesterase in vertebrate retinal rod outer segments is mediated by a guanine nucleotide-binding protein ('ROS G-protein') [1–3]. Activation of adenylate cyclase by hormones is also mediated by a guanine nucleotide-binding protein, termed N_s [4]. These two proteins are structurally homologous [5] and both can be radioactively labeled by the covalent binding of the ADP-ribose moiety of [^{32}P]NAD$^+$ ('ADP-ribosylated'); this reaction is catalyzed by activated cholera toxin [4,6–8]. Light induces a positive-going change in membrane voltage (the 'receptor potential') of the ventral photoreceptors of *Limulus polyphemus* [9]. The receptor potential is generated by the summation of small changes of membrane conductance ('discrete events') each arising from the absorption of a single photon [10,11]. Recently we and others have observed that intracelluar injection of the non-hydrolyzable GTP analog, guanosine-5′-*O*-(3-thiotriphosphate) (GTP S) greatly increases the frequency of discrete events recorded from ventral photoreceptors in darkness [12,13]. That is, injection of GTP S mimics dim illumination. In addition, the adenylate cyclase of ventral photoreceptors has been shown to be regulated by guanine nucleotides and cholera toxin [14,15]. These observations suggest the possible involvement of guanine nucleotide-binding proteins similar to vertebrate N_s and ROS G-protein in both the activation of adenylate cyclase and visual excitation of *Limulus* ventral photoreceptors.

Incubating membranes of ventral photoreceptors with cholera toxin and NAD$^+$ influences the action of GTP on the adenylate cyclase [15]. GPT inhibits adenylate cyclase activity in control membranes. After cholera toxin treatment, GTP activates adenylate cyclase in the photoreceptor membranes. That is, the response of invertebrate adenylate cyclase to GTP is regulated by the action of cholera toxin. Similarly, cholera toxin has been shown to regulate the response to GTP of other adenylate cyclases [4]. Toxin treatment reduces both hormonal stimulation of GTPase activity of

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mammalian N_s [16] and light-induced activation of GTPase activity of ROS G-protein [8]. The α(42–45 kDa)-subunit of N_s [4–7] and the α(39 kDa)-subunit of ROS G-protein are radiolabeled by [^{32}P]NAD$^+$ in the presence of cholera toxin. The ability of cholera toxin to regulate the response of adenylate cyclase of ventral photoreceptors of *Limulus* to GTP suggested that invertebrate counterparts of mammalian N_s and ROS G-protein might also be identified via autoradiography following their radiolabeling by [^{32}P]NAD$^+$ in the presence of cholera toxin. To investigate these possibilities we employed cholera toxin-catalyzed ADP-ribosylation of membrane proteins of ventral photoreceptors. We show that cholera toxin catalyzes the ADP-ribosylation of a 43-kDa peptide from *Limulus* ventral photoreceptors and provide evidence for structural homology between this invertebrate peptide and mammalian N_s.

2. METHODS

Membranes were prepared from rat hepatocytes [16], rat fat cells [17], and ventral eyes of *Limulus* [14] and incubated for 30 min at 30°C in a buffer containing 250 mM KH_2PO_4 (pH 7.5), 10 mM thymidine, 10 mM arginine, 0.1 mM guanylyl imidodiphosphate, 0.5 mM ATP, 0.35 μM [^{32}P]-NAD$^+$ (70 μCi/assay), and 0–50 $\mu g \cdot ml^{-1}$ preactivated cholera toxin. The toxin was preactivated with 20 mM dithiothreitol [17]. After the incubation, the membranes were diluted 20-fold with ice-cold 250 mM KH_2PO_4 (pH 7.5) buffer, collected by centrifugation, and solubilized in 50 μl Laemmli sample buffer. The proteins were subjected to electrophoresis in SDS on 10% homogeneous polyacrylamide gels [18]. Peptide maps of substrates for cholera toxin-catalyzed ADP-ribosylation were generated as in [19]. The gels were stained, destained, dried and used to expose Kodak XAR-5 X-ray film.

3. RESULTS AND DISCUSSION

We investigated the proteins that are ADP-ribosylated by cholera toxin as follows: we incubated membranes from ventral photoreceptors of *Limulus* with cholera toxin and [^{32}P]NAD$^+$, separated the membrane proteins on polyacrylamide gels by electrophoresis in SDS, and identified the radiolabeled proteins by autoradiography. A 43-kDa membrane peptide of *Limulus* ventral photoreceptor was the major target for ADP-ribosylation catalyzed by cholera toxin (fig.1). Membranes of isolated rat hepatocytes or fat cells were also incubated in the presence of cholera toxin and [^{32}P]NAD$^+$ and the radiolabeled proteins were identified in gels by autoradiography. As noted in [16,17], a 42-kDa membrane peptide of both hepatocyte and fat cells was the predominant peptide that becomes ADP-ribosylated in the presence of cholera toxin and [^{32}P]-NAD$^+$ (fig.1). Membrane proteins of 46 and 48 kDa were also radiolabeled [17,20]. All of these peptides that were targets for ADP-ribosylation catalyzed by cholera toxin are α-subunits of mammalian N_s [4,21].

Illumination of membranes from rod outer segments enhances the ADP-ribosylation of the

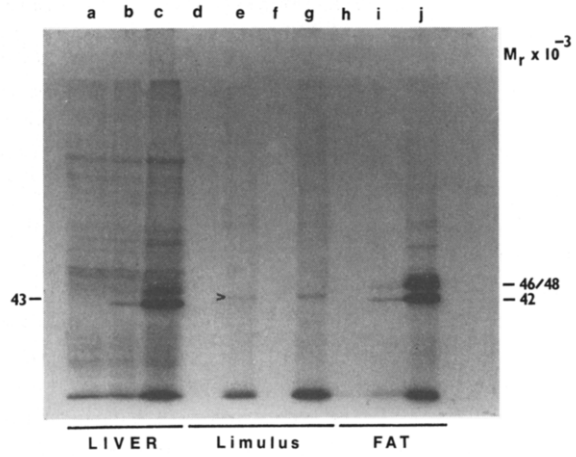

Fig.1. Autoradiogram of a polyacrylamide gel prepared from membranes both from mammalian cells and from *Limulus* ventral eyes that had been incubated with cholera toxin and [^{32}P]NAD$^+$. The amount of cholera toxin used in the labeling reaction was 0 (lanes A,D,F,H), 5 (lanes B,I), or 50 (lanes C,E,G,J) $\mu g \cdot ml^{-1}$. Membranes were prepared from ventral eyes that were dark-adapted or light-adapted for 4 h prior to preparation and labeling. The amount of protein loaded onto the polyacrylamide gels was 0.2 mg for membranes from rat hepatocytes and fat cells and light-adapted photoreceptors, and 0.13 mg for membranes from *Limulus* photoreceptors. Note that the major labeled band from the *Limulus* membranes is a peptide of 43 kDa.

α(39 kDa)-subunit of ROS G-protein that is catalyzed by cholera toxin [8]. We determined if the labeling of ventral photoreceptor membranes of *Limulus* by the action of cholera toxin was also influenced by light. Membranes were prepared from ventral photoreceptors that had been previously dark- or light-adapted. These membranes were then incubated immediately with cholera toxin and [^{32}P]NAD$^+$ (fig.1). The amount of ADP-ribose incorporated into the 43-kDa peptide of *Limulus* was measured and found to be approximately the same in membranes from dark-adapted (8.8 fmol/mg membrane protein) and light-adapted (9.2 fmol/mg membrane protein) photoreceptor preparations.

To investigate possible homologies between the 43-kDa peptide of *Limulus* ventral photoreceptors and the α-subunit of mammalian N_s we compared electrophoretic patterns of proteolytic fragments derived from each of these peptides. Membranes of both *Limulus* ventral photoreceptors and rat fat cells were radiolabeled by [^{32}P]NAD$^+$ in the presence of the cholera toxin. The 43-kDa peptide of *Limulus* and the 42-kDa peptide of fat cell membranes were isolated electrophoretically, treated with either elastase or *S. aureus* V8 protease and electrophoresed concurrently (fig.2). The gel was autoradiographed for different exposure times to allow comparison of the M_r of fragments despite the differences in the total amount of label incorporated into the two different tissues. There is a striking similarity between the electrophoretic patterns of the fragments derived from the radiolabeled peptides of *Limulus* ventral photoreceptor membranes and those of fragments derived from rat fat cell membranes. The many similarities between peptide maps of 43-kDa peptide of *Limulus* and those of the 42-kDa subunit of rat fat cell N_s generated by either elastase or *S. aureus* V8 protease suggest that the toxin target in *Limulus* ventral photoreceptors and the α-subunit of mammalian N_s are homologous proteins.

Lubrol extracts from ventral photoreceptor membranes reconstitute the adenylate cyclase system of membranes of S49 mouse lymphoma *cyc*$^-$ mutants [15] which lack functional N_s [22]. Lubrol extracts from *Limulus* membranes pretreated with cholera toxin cause enhanced stimulation of adenylate cyclase activity by GTP in this reconstituted system [15]. These data together

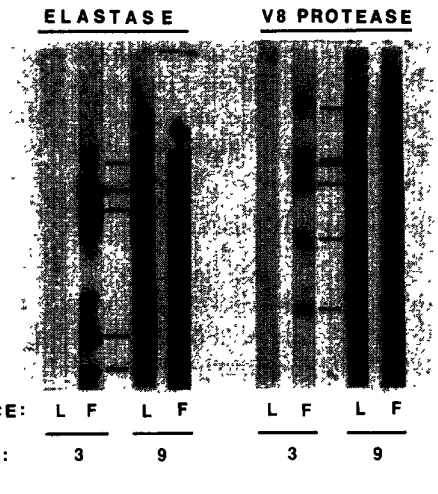

Fig.2. Autoradiograms of polyacrylamide gels prepared from partial proteolytic digests of both the 43-kDa peptide of *Limulus* ventral photoreceptors and the 42-kDa subunit of N_s from rat fat cells that had been radiolabeled in the presence of cholera toxin and [^{32}P]NAD$^+$. Membranes were prepared and labeled as described in the legend to fig.1. The labeled membranes were solubilized in SDS and subjected to electrophoresis on 10% polyacrylamide tube gels in the first dimension. The first dimension tube gel was bonded to a 5% stacking gel using agarose containing either 8 μg elastase or 12.5 μg *S. aureus* V-8 protease. The samples were moved into the stacking gel by electrophoresis; then the current was turned off sufficiently long to allow partial proteolysis of the peptides. The proteolytic fragments were separated by electrophoresis in the second dimension on a 14% polyacrylamide-SDS gel. Autoradiography was performed for 3 and 9 days on gels prepared as described in legend to fig.1. Peptides common to both digests are indicated by horizontal lines.

with the similarities found in the electrophoretic mobilities in SDS (fig.1) and partial peptide maps (fig.2) suggest that the 43-kDa peptide of *Limulus* ventral photoreceptors is an invertebrate counterpart to mammalian N_s. That is, the target for cholera toxin-catalyzed ADP-ribosylation appears to be a highly conserved protein. A labeled peptide with molecular mass similar to the α(39 kDa)-subunit of ROS G-protein was not detected in ventral photoreceptor membranes radiolabeled by [^{32}P]NAD$^+$ in the presence of cholera toxin (fig.1). VertebrateN$_s$ and ROS G-protein are homologous proteins [5] that are ADP-ribosylated by the action

of cholera toxin [4–8] and share common subunits [5], but have very different functions. Similarly, the function of the 43 kDa peptide of *Limulus* ventral photoreceptors may not be restricted only to the activation of adenylate cyclase.

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