Volume 219, number 2, 293–295 FEB 04919 July 1987

Detection of Ca^{2+}-dependent cyclic GMP binding protein in frog rod outer segments

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Received 26 April 1987

For the identification of the cGMP-sensitive ion channel protein of frog rod outer segments (ROS), we analyzed cGMP binding proteins in the ROS by photoaffinity labeling with [^{3}H]cGMP. We found three cGMP binding polypeptides (66 kDa, 92 kDa and 100 kDa) in the membrane protein fraction of ROS. cGMP binding to the 66 kDa polypeptide required the addition of 2 mM $CaCl_2$. We propose that this polypeptide corresponds to the cGMP-activated channel protein reported by Cook et al. [(1987) Proc. Natl. Acad. Sci. USA 84, 585–589]. The 100 kDa and 92 kDa polypeptides are subunits of the cGMP phosphodiesterase.

Photoreceptor; Rod outer segment; cyclic GMP binding protein; Visual transduction

1. INTRODUCTION

On absorption of light, vertebrate photoreceptor cells generate a hyperpolarized receptor potential owing to the closure of Na^+ channels in the plasma membrane of the outer segment [2]. Recently, the regulation mechanism of this ion channel has been clarified; cGMP regulates the Na^+ channel from the inside and Ca^{2+} regulates it from the outside of the plasma membrane of ROS [3,4]. Cook et al. [1] reported the purification of a 63 kDa polypeptide which constitutes the cGMP-dependent channel. However, they did not show the cGMP binding to this 63 kDa polypeptide.

The present paper describes the Ca^{2+}-dependent cGMP binding to a 66 kDa polypeptide in the frog ROS.

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Abbreviations: ROS, rod outer segments; UV_{254}, ultraviolet light at 254 nm; DTT, dithiothreitol; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Solutions*

Ringer's solution: NaCl (90 mM), KCl (10 mM), $MgCl_2$ (2 mM), $CaCl_2$ (0.1 mM), DTT (1 mM) and Na-phosphate (10 mM), pH 7.5. Ca-deleted Ringer's solution: Ringer's solution without $CaCl_2$. EDTA solution: EDTA (3 mM), DTT (1 mM) and Tris-HCl (10 mM), pH 7.5. SDS sample solution: SDS (7%), DTT (70 mM), EDTA (20 mM) and bromophenol blue (0.05%).

2.2. *Preparation of EDTA-washed membrane suspension*

ROS were isolated from the retinas of dark-adapted bull frogs (*Rana catesbeiana*) by a 43% (w/w) sucrose flotation method [5], under an infrared light, with the use of an image converter. The pasty layer of ROS formed at the air-sucrose interface was collected and was then suspended in Ringer's solution. The ROS in the suspension was disrupted by passing it 5 times through a 25-gauge needle. We called this preparation membrane suspension. The ratio of absorption at 280 nm to

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00145793/87/$3.50

that at 500 nm (A_{280}/A_{500}) for the membrane suspension dissolved in 50 mM cetyltrimethylammonium bromide was 2.0–2.4. For the preparation of EDTA-washed membrane suspension, membrane suspension was washed by centrifugation, at 15000 × *g* for 30 min, with EDTA solution (twice) and Ca-deleted Ringer's (twice). These washings removed most of the soluble proteins and peripheral-membrane proteins. The pellet was suspended in the Ca-deleted Ringer's solution.

2.3. *[^{3}H]cGMP labeling of ROS proteins*

[^{3}H]cGMP labeling was carried out by the direct labeling method reported by Nath et al. [6]. The EDTA-washed membrane in 100 μl of Ca-deleted Ringer's, or Ringer's solution supplemented with 2 mM $CaCl_2$, was irradiated on a whole glass slide on ice by UV_{254} from a fluorescence inspection lamp (TOPCON F11-31S; Tokyo Kogaku Kikai KK) at a distance of 2.5 cm (27 $\mu W \cdot cm^{-2}$ (3.4×10^{13} photons $\cdot s^{-1} \cdot cm^{-2}$)) in the presence of [^{3}H]cGMP (New England Nuclear, USA) for 7.5 min. Then 1 ml of ice-cold Ringer's solution was added to the membrane suspension, followed by centrifugation at 15000 × *g* for 30 min. The pellet containing membrane protein was dissolved in 150 μl SDS sample solution by incubation for 8 min in a boiling water bath. The supernatant of the washing was supplemented with 0.1 volume of 100% (w/v) trichloroacetic acid solution. The proteins in the supernatant were precipitated by centrifugation. After washing the precipitates with ice-cold acetone, they were dissolved in SDS sample solutions (we could not detect ^{3}H-labeled proteins in this fraction). The separation of the proteins was carried out by SDS-PAGE containing 10% acrylamide in the resolving gel as described [7]. The proteins in the gel were stained with Coomassie blue R-250. After shaking the gel in Amplify solution (Amersham, England), the gel was dried. The [^{3}H]cGMP-labeled proteins were identified by autoradiogram exposing a Fuji RX film for 14 days at −80°C (fig.1). The molecular masses of the ^{3}H-labeled proteins were determined by using BioRad's and Pharmacia's molecular mass standards.

2.4. *Protein determination*

Protein concentrations were determined according to Lowry et al. [8].

3. RESULTS

3.1. *Ca-dependent cGMP-binding protein in the membrane protein fraction of ROS*

For labeling of ROS protein with [^{3}H]cGMP, the EDTA-washed membrane suspension was irradiated with UV_{254}, in the presence of [8,5′-^{3}H]cGMP in the Ringer's solution, in the absence or presence of $CaCl_2$. As shown in fig.1 (lane 2), autoradiograms of SDS-PAGE obtained from the irradiated sample without $CaCl_2$ displayed two ^{3}H-labeled polypeptides with molecular masses of 100 and 92 kDa. A difference

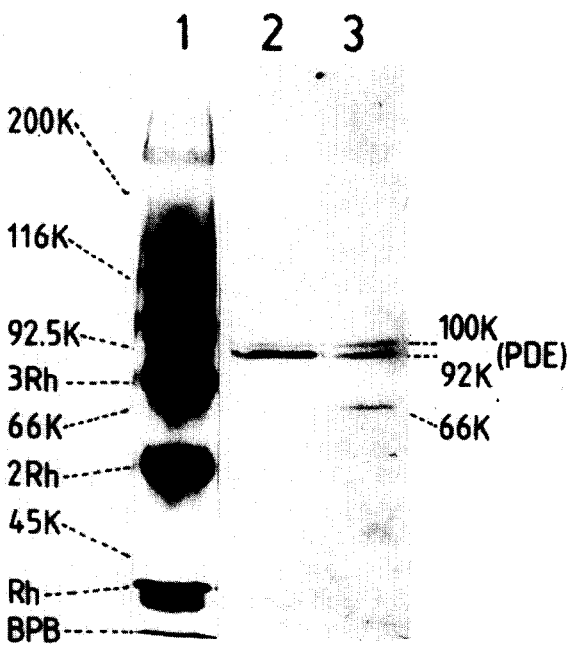

Fig.1. SDS-PAGE of frog ROS proteins labeled with [^{3}H]cGMP by UV_{254}-irradiation. EDTA-washed membrane (containing 350 μg protein) was irradiated by UV_{254} in the presence of 90 μM [^{3}H]cGMP (500 μCi) in the absence or presence of 2 mM $CaCl_2$ for 7.5 min and then the cGMP binding was analyzed by SDS-PAGE and autoradiography. Lanes: 1, membrane proteins stained with Coomassie blue; 2, autoradiogram of the membrane proteins (100 and 92 kDa proteins) irradiated in the absence of $CaCl_2$; 3, autoradiogram of the membrane proteins (100, 92 and 66 kDa proteins) irradiated in the presence of $CaCl_2$. The positions of molecular mass standard proteins, rhodopsin monomer (Rh), rhodopsin dimer (2Rh), rhodopsin trimer (3Rh) and bromophenol blue (BPB) are shown on the left.

of ^{3}H-labeling between 100 and 92 kDa polypeptides was sometimes observed. However, this phenomenon did not depend on the addition of Ca^{2+}. The irradiation in the presence of $CaCl_2$ labeled three polypeptides with [^{3}H]cGMP (100, 92 and 66 kDa; lane 3). These three ^{3}H-labeled proteins were not detected without UV_{254} irradiation; the binding of cGMP to these polypeptides was not inhibited by the addition of 20-fold cAMP but was clearly inhibited by that of cGMP (not shown). The 100 and 92 kDa polypeptides between the trimer and tetramer of rhodopsin appear to be the subunits of cGMP phosphodiesterase (PDE) through the following result: the activity of partially purified PDE by sucrose density gradient centrifugation correlated with the 100 and 92 kDa proteins. Labeling of the two subunits of cGMP PDE with [^{32}P]cGMP was also reported by Yuen et al. [9]. The hydrolysis of [^{3}H]cGMP, measured by the method of Miki et al. [5], by PDE contained in the EDTA-washed membrane during the irradiation, was 10%.

4. DISCUSSION

The patch-clamp studies of frog ROS showed that Na^+ channel proteins are located in the plasma membrane containing cGMP binding sites on the inside of the plasma membrane and the Ca-binding site on the outside [3,4]. From the purification and reconstitution of cGMP-dependent ion channel activity, Cook et al. [1] suggested that this channel protein has subunits of 63 kDa and is also stabilized by the addition of Ca^{2+}. We have detected a cGMP-specific binding protein in the membrane protein fraction with a molecular mass of 66 kDa (which is close to 63 kDa) and the binding of cGMP to that protein required the addition of Ca^{2+}. These facts suggest that this 66 kDa protein corresponds to the cGMP-sensitive ion channel protein with a molecular mass of 63 kDa which was reported by Cook et al. [1]. In addition to these three polypeptides (66, 92 and 100 kDa), we also detected the cGMP binding to a 250 kDa polypeptide. However, the cGMP binding to this polypeptide did not require Ca^{2+} (not shown). The relationships between this 250 kDa polypeptide and those polypeptides are now under investigation.

ACKNOWLEDGEMENTS

We thank Otsuka Pharmaceutical Factory Inc. for their help in our experiments with the use of [^{3}H]cGMP. This work was supported by Grants-in-Aid for Special Research on Molecular Mechanisms of Bioelectrical Response (60115001), Co-operative Research (60304098) and for Scientific Research (61580223) to T.S. from the Japanese Ministry of Education, Science and Culture.

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