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GTP-BINDING PROTEIN COUPLES WITH METABOTROPIC GLUTAMATE RECEPTOR IN BOVINE RETINAL ON-BIPOLAR CELL

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SUMMARY: GTP-binding protein (G protein) linking to metabotropic glutamate receptor of bovine retinal on-bipolar cell was studied by use of pharmacologically selective ligands, 2-amino-4-phosphonobutyric acid (APB) on bacterial toxin-catalyzed ADP-ribosylation and GTPγS-binding. In contrast to the electrophysiological findings reported, G protein coupling to APB-sensitive glutamate receptor served as a substrate for pertussis toxin but did not for cholera toxin. Several glutamate analogues effective on on-bipolar cell, as well as APB, increased GTPγS binding to retinal membranes devoid of rod outer segments. The enhancement of GTPγS binding by APB was completely abolished when the membranes were pretreated with pertussis toxin and NAD. These results suggest that, in retinal on-bipolar cell, the G protein which couples metabotropic glutamate receptor to hyperpolarizing response of the cell is sensitive to pertussis toxin.

Photoreceptor cell releases glutamate as a neurotransmitter in the dark, which closes cation channels of on-bipolar cell. Light reduces the release of glutamate, resulting in opening of cation channels on on-bipolar cell and subsequent depolarization of the cell (1). In this respect, retinal on-bipolar cell is a novel neuron which hyperpolarizes on contact with neurotransmitter molecules. Since photoreceptor cell hyperpolarizes upon light illumination, similar molecular machinery may be involved in these two cellular systems.

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Abbreviations used: G protein, GTP-binding protein; ROS, rod outer segments; APB, 2-amino-4-phosphonobutyric acid; APV, 2-amino-5-phosphonovaleric acid; APMSF, 4-(amidinophenyl)methanesulfonyl fluoride; App(NH)p, adenosine 5'-(βγ-imido)triphosphate; Gpp(NH)p, guanosine 5'-(βγ-imido)triphosphate; GTPγS, guanosine 5'-(3-*O*-thio)triphosphate; cGMP, cyclic GMP; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Recently, it has been reported from several laboratories, based on electrophysiological studies, that cGMP acts as the second messenger mediating the action of glutamate through the G protein that has striking similarities to transducin in photoreceptor cell (2-4). Shiells and Falk demonstrated that inclusion of pertussis toxin in the patch-pipette solution resulted in a block of light-evoked current on retinal on-bipolar cell and that the opposite effect was observed with cholera toxin (5). These results led them to a conclusion that the G protein linking metabotropic glutamate receptor to the cGMP cascade in on-bipolar cell serves as a substrate both for pertussis toxin and for cholera toxin, as does transducin. However, there is no direct evidence reported for G protein participation in this signaling system. This is because isolation in a large scale for biochemical studies of on-bipolar cell from retina is quite difficult.

It has been shown that a glutamate analogue, 2-amino-4-phosphonobutyric acid (APB) is the highly selective reagent which acts solely on on-bipolar cell while leaves unaffected the other retinal neurons including off-bipolar cell (6,7). This pharmacological tool enabled us to characterize the signal coupling protein involved in the signaling system of on-bipolar cell by use of the retinal membranes devoid of rod outer segments (ROS).

In this paper, we characterized a G protein coupling to APB-sensitive glutamate receptor in ROS-depleted retinal membranes by bacterial toxin-catalyzed ADP-ribosylation and GTPγS-binding. From the results presented here, we suggest that the G protein serving as a substrate for pertussis toxin plays a key role in linking the metabotropic glutamate receptor to the response of retinal on-bipolar cell.

MATERIALS AND METHODS

Preparation of ROS-depleted membranes from bovine retina ——Fresh bovine eyes were obtained from a local slaughterhouse and kept on ice. The retinal membranes devoid of ROS were obtained by modification of the method for purification of ROS described by Papermaster (8). Briefly, removed retinas from 10 eyes were homogenized by shaking vigorously for 1 min in 20 ml of an ice-cold sucrose buffer containing 10 mM MOPS (pH 7.4), 30 mM NaCl, 60 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 50 μM APMSF, and 39% sucrose (w/w). Sheared ROS and the remainder of the retina were separated by centrifugation at 22,000 x g for 25 min. The pellet was resuspended by hand in 20 ml of the sucrose buffer and centrifuged. The pellet after repeated centrifugation was then homogenized with 2 passes of Teflon–glass homogenizer in 50 ml of an ice-cold buffer containing 10 mM MOPS (pH 7.4), 0.1 mM EDTA, 1 mM DTT, and 50 μM APMSF. The homogenate was first centrifuged at 1,400 x g for 5 min, the pellet was discarded, then the supernatant was further centrifuged at 40,000 x g for 30 min. The pellet was taken, rehomogenized with the same buffer, and centrifuged again at 40,000 x g for 30 min. The retinal membranes thus prepared were referred to henceforth as ROS-depleted membranes. ROS was prepared from dark adapted bovine retinas by following Papermaster's method. The membrane preparations were stored at –80°C until use.

ADP-ribosylation of G proteins by bacterial toxins ——The membrane preparations were incubated at 30°C with either pertussis toxin (5 μg/ml) or cholera toxin (200 μg/ml), which was activated prior to incubation (9), in a buffer each containing 2 μM [^{32}P]NAD and 1 mM NADP. The buffer for pertussis toxin-catalyzed reaction consisted of 100 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, and 10 mM thymidine; the one for cholera toxin consisted of 100 mM sodium phosphate (pH 7.5), 5 mM $MgCl_2$, 10 mM thymidine, and 0.1 mM GTP. At the indicated times, the reaction was terminated by addition of equal volume of sample buffer and the mixtures were subjected to SDS-PAGE on 10% gel

followed by autoradiography. In the experiments on Fig. 2, radio activities of the bands were quantified by means of BAS 2000 Bio Imaging Analyzer (Fuji Photo Film Co.,Ltd., Japan).

For ADP-ribosylation with unlabeled NAD, the membranes were incubated on ice for 1 hr with 5.5 μg/ml of pertussis toxin and 50 μM NAD. As a control the membranes were treated with the toxin alone.

GTPγS-binding assay ——ROS-depleted membranes (0.5 mg protein/ml) were incubated at 30°C with 1 μM [^{35}S]GTPγS (approx. 1 x 10^4 cpm/pmol) in the presence or absence of various glutamate ligands in 20 mM MOPS buffer (pH 7.4) containing 1.1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 10 μM GDP, and 100 μM App(NH)p. At the indicated times, 20 μl aliquots of the reaction mixture were diluted with 2 ml of ice-cold Tris-HCl buffer (pH 8.0) containing 25 mM $MgCl_2$ and 100 mM NaCl and were immediately filtered through glass filters (GF/B, Whatman). The filters, after being washed 3 times with each 2 ml of the same buffer, were counted for ^{35}S by liquid scintillation counter.

Miscellaneous ——Anti α-subunit antisera were generous gifts from Dr. Haga (Brain Institute, Tokyo University, Japan) and Dr. Asano (Institute for Developmental Research, Aichi Prefectural Colony, Japan). [^{35}S]GTPγS (44.4 TBq/mmol) and [α-^{32}P]NAD (29.6 TBq/ mmol) were purchased from DuPont-New England Nuclear. DL-APB, DL-APV, kainic acid, and ibotenic acid were purchased from Sigma. All other reagents used are the highest grades commercially available.

RESULTS AND DISCUSSION

Bacterial toxin-catalyzed ADP-ribosylation has been widely utilized as a versatile probe to characterize G proteins in signal transduction systems. To survey retinal G proteins in the sight of sensitivities to the toxins, we first conducted ADP-ribosylation of G proteins on the membranes prepared from bovine retina by pertussis toxin and cholera toxin. Since transducin, a well-characterized G protein which is sensitive to both pertussis toxin and cholera toxin, locates in rod outer segments (ROS), we separated retinal membranes into two fractions, ROS and the remainder (ROS-depleted membranes), through sucrose-flotation method. The membranes thus prepared were incubated with pertussis toxin or cholera toxin in the presence of [^{32}P]NAD under near-optimum conditions for each toxin as described in MATERIALS AND METHODS. As shown in Fig. 1, detection of radio-labeled proteins by autoradiography following SDS-PAGE separation revealed 39- and/or 40-kDa polypeptide(s) on ROS-depleted membranes and α-subunit of transducin in ROS membranes strongly labeled by pertussis toxin (lanes 1 and 3). Labeling of 39/40-kDa polypeptide(s) on ROS-depleted membranes was attenuated by APB as that of transducin by light (lanes 2 and 4). On the other hand, cholera toxin ADP-ribosylated 45- and 52-kDa polypeptides, probably α-subunits of G_s, but did not catalyze ADP-ribosylation of the 39/40-kDa pertussis-toxin substrate(s) on ROS-depleted membranes (lane 5). The presence of APB neither induced ADP-ribosylation of the pertussis-toxin substrate(s) nor enhanced that of 45- and 52-kDa polypeptides by cholera toxin (lane 6). Cholera toxin clearly catalyzed ADP-ribosylation of transducin α-subunit in a light dependent manner in ROS membranes (lanes 7 and 8). These results suggest that the G protein coupled with APB receptor on ROS-depleted membranes is sensitive to pertussis toxin but not to cholera toxin, in contrast to transducin on ROS membranes.

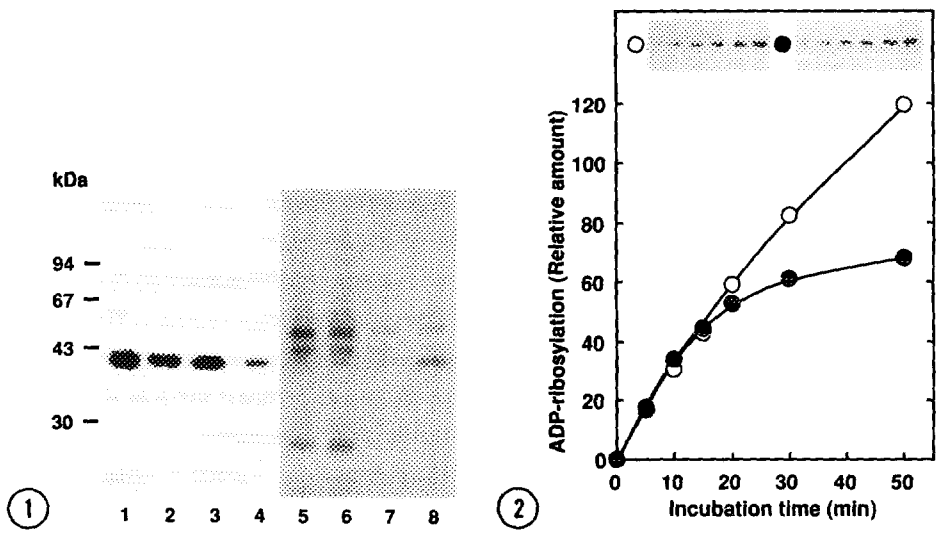

Fig. 1. ADP-ribosylation of ROS-depleted retinal membranes and ROS membranes by pertussis or cholera toxins. Membrane preparations (lanes 1, 2, 5, 6: ROS-depleted membranes, lanes 3, 4, 7, 8: ROS membranes) at 1 mg protein/ml were incubated at 30°C for 30 min with either 5 μg/ml pertussis toxin (lanes 1–4) or 200 μg/ml cholera toxin (lanes 5–8) in a buffer containing 2 μM [^{32}P]NAD and 1 mM NADP. APB at 1mM was included in the mixtures in lanes 2 and 6. The reactions for lanes 3 and 7 were conducted under dim red light, while the others under room light.

Fig. 2. Inhibition of pertussis toxin-catalyzed ADP-ribosylation by APB. ROS-depleted membranes (0.5 mg protein/ml) were incubated at 30°C with preactivated pertussis toxin (1.5 μg/ml) and 2 μM [^{32}P]NAD in the buffer supplemented with 0.5 mM $MgCl_2$, 1 μM Gpp(NH)p, and 100 μM App(NH)p in the presence (●) or absence (○) of 1 mM APB. At a given time, an aliquot was withdrawn into an equal volume of sample buffer, then analyzed as described in "MATERIALS AND METHODS". The autoradiogram is shown in *inset*.

Hereafter, our investigations were focused on the coupling between APB receptor and pertussis toxin-sensitive G protein.

We further investigated the effects of glutamate ligands on pertussis toxin-catalyzed ADP-ribosylation of G proteins on ROS-depleted membranes. The time-course of the reaction was demonstrated in Fig. 2. APB reduced ADP-ribosylation of ROS-depleted membranes by pertussis toxin in the presence of Mg^{2+} and guanosine 5'-(βγ-imido)triphosphate (Gpp(NH)p). As already mentiond above, APB did not alter ADP-ribosylation of α_s by cholera toxin (see Fig. 1, lanes 5 and 6). We also tested L-glutamic acid as an agonist, but the inhibition of ADP-ribosylation was not evident (data not shown). The results suggest that the glutamate receptor that is selective for APB couples to and activates a pertussis toxin substrate on ROS-depleted membranes. By western blotting analysis employing specific antisera for the α subunits, the polypeptides crossreacted with the antisera against either α_o or α_i comigrated with the pertussis toxin substrates on ROS-depleted membranes (data not shown). Though it is of our great interest, we have not yet been capable to determine which subtype of pertussis toxin substrates couples with APB receptor.

As a more direct approach to G-protein activation by the stimulated receptor, we examined whether GTPγS-binding to the membranes, the activation step of G proteins, is affected by glutamate agonists. ROS-depleted membranes were incubated with 1 μM [^{35}S]GTPγS in the presence or absence of various glutamate ligands. The radio activities bound to the membranes were measured after bound/free separation by rapid filtration. GTPγS bound to the membranes in a time-dependent manner and the binding reached apparent saturation at 20 min. As shown in Fig. 3, APB at 1 mM concentration accelerated GTPγS-binding to ROS-depleted membranes. APB enhanced GTPγS-binding to the membranes about 30% at 15 min (Fig. 3). Relatively high control GTPγS-binding, in the absence of APB, implies existence of large G-protein pool other than the one that couples with APB receptor. We also tested sensitivity to pertussis toxin of the G-protein activation by APB. GTPγS-binding experiments were conducted with the membrane preparations pretreated by pertussis toxin in the presence or absence of unlabeled NAD. Pretreatment of ROS-depleted membranes with pertussis toxin and NAD abolished the stimulated GTPγS-binding by APB without marked change in control GTPγS-binding level (Fig. 3, *inset*). It was thus confirmed that APB receptor couples to a pertussis-toxin substrate in bovine retina.

Next, we investigated pharmacological characteristics of the receptor with respect to the G-protein activation. The results are summarized in Fig. 4. Ibotenic acid and 2-amino-5-phosphonovaleric acid (APV), which selectively induce cellular responses to on-bipolar cell (10), increased GTPγS-binding to ROS-depleted membranes at 1 mM. Kainic acid also enhanced GTPγS-binding to the membranes to some extent. While L-glutamic acid did not show measurable effects on GTPγS-binding at 1 mM, it enhanced GTPγS-

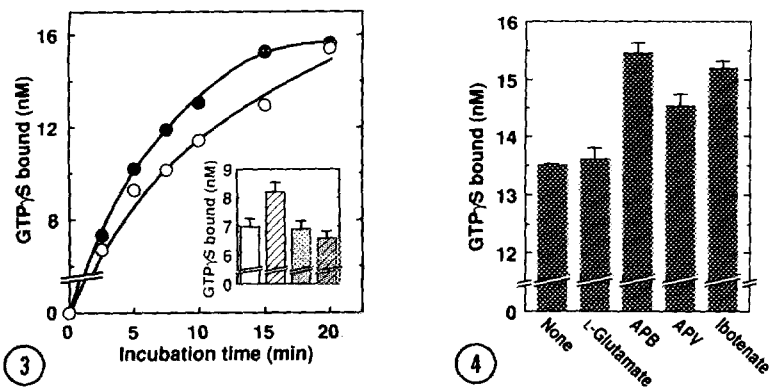

Fig. 3. Time course of GTPγS-binding to ROS-depleted membranes in the presence or absence of APB. Membrane preparation (0.5 mg protein/ml) was incubated at 30°C in the presence (●) or absence (○) of 1 mM APB in a buffer containing 1 μM [^{35}S]GTPγS, 10 μM GDP, and 100 μM App(NH)p. Essentially same experiments were carried out with pertussis toxin-treated or control membranes (*inset*). Toxin-treated (dotted bar) or control (open bar) membranes were incubated for 12 min in the presence (hatched) or absence (open) of 1 mM APB.

Fig. 4. Effects of various glutamate ligands on GTPγS-binding to ROS-depleted membranes. Membranes were incubated for 12 min with 1 μM [^{35}S]GTPγS in the presence of various glutamate ligands (1 mM) under the same conditions as in Fig. 3.

binding to the membranes at a higher concentration (10 mM; data not shown). These results show that APB is a more potent agonist than L-glutamic acid for the receptor that links to G protein in ROS-depleted membranes. It has been reported that APB is highly selective for retinal on-bipolar cell; APB does not induce physiological response to either horizontal cell or off-bipolar cell, both in lower vertebrates and in mammals (6,7,11). Besides, APB is a more potent agonist than L-glutamic acid on on-bipolar cell (6,10). Taken together these incidents, our results strongly suggest that the APB receptor which linked to pertussis toxin-sensitive G protein is present on bovine retinal on-bipolar cell. Very recently, Nakanishi *et. al.* have isolated a cDNA clone for a novel subtype of metabotropic glutamate receptor, namely, mGluR6 (12). This novel subtype shows high sequence similarity to APB-selective mGluR4 subtype and is specifically enriched in the postsynaptic region of the dendrites in retinal bipolar cell. Therefore, mGluR6 is thought to be the most probable candidate for the APB receptor of on-bipolar cell.

In the present paper, we demonstrated direct coupling of APB receptor to a pertussis toxin substrate, but not a cholera toxin substrate, on ROS-depleted membranes prepared from bovine retina. Since APB induces cellular response only to on-bipolar cell among vertebrate neural retina, it is very likely that the activation of G protein occurs on on-bipolar cell although we tested on mixed retinal membranes. Several reports based on electrophysiological studies on on-bipolar cell implies participation of G protein in phototransduction pathway (2-4). The G protein is thought to activate phosphodiesterase leading to decrease in cytosolic cGMP level. Subsequently, cGMP-gated channels close and cells hyperpolarize. This schema shows clear analogy to the phototransduction pathway in photoreceptors. Until now, this mechanism is thought to be unique in photoreceptors. So it is indispensable to clarify molecular machinery in signal transduction system of on-bipolar cell, although isolation of individual neuron from intact retina accompanies considerable difficulties. Furthermore, those findings may help understand signaling mechanisms of other hyperpolarizing neurons such as cerebellar Purkinje cell (13). At all events, this is the first biochemical report that suggests direct participation of pertussis toxin substrate in the response generation of retinal on-bipolar cell.

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