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PROPERTIES OF 1-METHYLADENINE RECEPTORS IN STARFISH OOCYTE MEMBRANES: Involvement of Pertussis Toxin-sensitive GTP-binding Protein in the Receptor-mediated Signal Transduction

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SUMMARY: In response to a meiosis-inducing hormone, 1-methyladenine (1-MA), starfish oocytes undergo reinitiation of meiosis with germinal vesicle breakdown. The 1-MA-initiated signal is, however, inhibited by prior microinjection of pertussis toxin into the oocytes, suggesting that a guanine nucleotide-binding protein (G protein) serving as the substrate of pertussis toxin is involved in the 1-MA receptor-mediated signal. We thus investigated properties of 1-MA receptors by means of binding of the radiolabeled ligand to the oocyte membranes. There were apparently two forms of 1-MA receptors with high and low affinities in the membranes. The high-affinity form was converted into the low-affinity one in the presence of a non-hydrolyzable analogue of GTP. A 39-kDa protein, which had been identified as the α-subunit of the major substrate G protein for pertussis toxin, was also ADP-ribosylated by cholera toxin only when 1-MA was added to the membranes. The ADP-ribosylated 39-kDa α-subunit could be immunoprecipitated with antibodies raised against the carboxy-terminal site of mammalian inhibitory G-α. These results indicate that 1-MA receptors are functionally coupled with the 39-kDa pertussis toxin-substrate G protein in starfish oocyte membranes.

Starfish oocytes are arrested at the first prophasic stage of meiosis. In response to a polypeptide hormone from the radial nerve, the follicle cells surrounding immature oocytes release a hormone, 1-methyladenine (1-MA), which induces meiosis reinitiation and maturation of the cells (1,2). When 1-MA binds to a cell surface of the oocytes (3), the signal is transferred to cytoplasm where the maturation (or M phase) promoting factor (MPF) is activated. MPF, which has been identified with cdc2 kinase in the M phase of fission yeast (4,

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Abbreviations used: 1-MA, 1-methyladenine; G proteins, GTP-binding proteins consisting of αβγ-subunits; G_s and G_i, G proteins of adenylate cyclase that mediate stimulation and inhibition, respectively; G_o, a G protein of unclear function purified from mammalian brain tissues; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfate; DTT, dithiothreitol; guanosine 5'-[γ-thio]triphosphate; MPF, maturation (or M phase) promoting factor; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis.

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5), can reinitiate meiotic maturation upon its microinjection into unstimulated oocytes (2, 6). The meiosis reinitiation is first observed as germinal vesicle breakdown occurring at about 20-30 min after the treatment of 1-MA. The 1-MA-induced maturation of the oocytes was interestingly inhibited by microinjection of an ADP-ribosylating enzyme of G proteins, pertussis toxin, and that the pertussis toxin-induced inhibition could be bypassed by transfer of MPF into the oocytes (7). These findings suggest that a pertussis toxin-sensitive G protein may be involved in the signal transduction arising from the 1-MA binding to the cell surface.

In the previous paper (8), we have purified an $\alpha\beta\gamma$-trimeric G protein serving as the substrate of pertussis toxin-catalyzed ADP-ribosylation from the membranes of immature starfish oocytes. Biochemical properties of the starfish G protein, such as guanine nucleotide-binding and GTPase reactions or substrate activity for pertussis toxin-induced modification, were quite similar to those observed with mammalian G proteins, G_i, and G_o. The 39-kDa α-subunit of the starfish G protein was recognized by antibodies raised against the carboxy-terminal ADP-ribosylation site of mammalian G_i-α. Moreover, a cDNA encoding the starfish G protein α has recently been cloned from a cDNA library of the immature ovary (9). The deduced amino acid sequence of the α-subunit was identical 89% to the α-subunit of rat G_{i-1}. Thus it is very likely that the pertussis toxin-substrate G protein purified in the previous paper is indeed the signal transducing protein from the putative 1-MA receptors to effector molecules in starfish oocytes.

There are some reports showing the existence of a receptor-like, specific 1-MA binding activity in starfish oocyte membranes (3, 10). However, the 1-MA receptor has not yet been characterized well in terms of its protein purification or its possible interaction with G proteins. In this paper, we first report that the 1-MA receptors are coupled with the pertussis toxin-sensitive G protein. The binding property of radiolabeled 1-MA has been characterized in a preparation obtained from the oocyte membranes.

MATERIALS AND METHODS

Preparation of membrane fractions from immature starfish oocytes

Starfishes, *Asterina pectinifera*, were collected near Hashirimizu, and Otsuchi, Japan. Ovaries were removed from 20-30 starfishes and allowed to release oocytes into a calcium-free artificial seawater as described previously (8). The oocytes (total volume of 100 ml), after being washed several times with the seawater at 4°C, were diluted with 3 volumes of hypotonic buffer consisting of 20 mM Tris/HCl (pH 7.5), 1 mM EDTA, 5 mM $MgCl_2$, 1 mM benzamidine, 25 kallikrein inhibitory units/ml of aprotinin, and 1 μM GDP and then gently suspended with a Dounce-type homogenizer. The homogenate was centrifuged at 500 x *g* for 2 min. The pellet was washed three times with the same buffer and used as a preparation of the plasma membranes.

1-MA binding assay

1-[methyl-^{3}H] methyladenine ([^{3}H]1-MA; Code # TRQ 5656, the specific activity of 433 GBq or 11.7 Ci/mmol and the radioactive concentration of 37 MBq or 1 mCi/ml) was purchased by a custom order from Amersham. The membranes (approximately 100 μg of protein) were

incubated with various concentration of [^{3}H]1-MA (1-1000 nM) at 20°C for 30 min in 100 µl of buffer A consisting of 20 mM Tris/HCl (pH 7.5), 1 mM EDTA, 2.5 mM $MgCl_2$, 50 µM adenosine 5'-[β,γ-imido]triphosphate, and 50 kallikrein inhibitory units/ml of aprotinin. The reaction mixture further contained 100 µM GTPγS, 1 mM DTT and 0.02% Chaps where indicated. The reaction was terminated by dilution with 3 ml of an ice-cold buffer consisting of 5 mM Tris/HCl (pH 7.5) and 5 mM $MgCl_2$ and then applied to a Whatman GF/C glass filter (2.4 cm diameter). The filter, after being washed four times with the same buffer, was dried in an oven at 100°C, and radioactivity retained in the filter was measured in a liquid scintillation spectrometer as the amount of the ligand bound to the membranes. The specific binding was obtained as a difference between the total binding and the non-specific binding which was measured in the presence of an excess concentration (100 µM) of non-radioactive 1-MA. In typical experiments, the non-specific binding was less than 20% of the total binding.

ADP-ribosylation of G protein by pertussis toxin or cholera toxin

Prior to use for ADP-ribosylation of G proteins in the membranes, cholera toxin (2 mg/ml) and pertussis toxin (1 mg/ml) were activated by incubation at 30°C for 15-30 min with an equal volume of 50 mM NaP_i (pH 7.4) containing 50 mM DTT and with 9 volumes of 50 mM Tris/HCl (pH 7.5) containing 100 mM DTT and 0.1 mM ATP, respectively (11). The membranes (20 µg of protein) were incubated at 20°C for 60 min with either the activated cholera toxin (5 µg) or pertussis toxin (0.5 µg) in 25 µl of a reaction mixture consisting of 100 mM NaP_i (pH 7.4), 5 µM [α-^{32}P]NAD (5,000-10,000 cpm/pmol), 1 µM guanosine 5'-[β,γ-imido]triphosphate, 2.5 mM $MgCl_2$, 1 mM EDTA, 10 mM thymidine, 0.2 mM benzamidine, and 5 kallikrein inhibitory units/ml of aprotinin in the presence or absence of 1 µM 1-MA. The ADP-ribosylation was terminated by the addition of 6.25 µl of 10% SDS containing 10 mM DTT followed by boiling at 90°C for 3 min. Each sample, after being alkylated with 3.3 µl of 100 mM *N*-ethylmaleimide for 15-30 min at 20°C, was mixed with 35 µl of 2-fold concentrated Laemmli buffer. An aliquot (10-15 µl; 3-4 µg of protein) of the sample was then subjected to SDS/PAGE. The gel was stained with Coomassie Brilliant Blue R-250, destained, dried and exposed to Kodak X-Omat AR film with an intensifying screen at -85°C.

Immunoprecipitation of G protein with anti-α antibodies

[^{32}P]ADP-ribosylated G protein in the membranes (25 µl) was mixed with 6.25 µl of 10% SDS containing 10 mM DTT. The aliquot (10 µl) was incubated with 0.5 mg/ml of a polyclonal antibody raised against the carboxy-terminal site (KNNLKDCGLF) of mammalian G_{i-1} (8) or normal rabbit immunoglobulin G at 30°C for 2 h in 100 µl of phosphate-buffered saline (PBS) containing 0.5% NP-40. After the incubation, 20-µl suspension of protein A cellulofine (Seikagaku Kogyo) was added, and they were further incubated for 30 min at room temperature. The antibody-protein A complex, after being washed several times with 500 µl of PBS, was denatured by the addition of the SDS/DTT sample buffer and then subjected to SDS/PAGE and autoradiography.

RESULTS AND DISCUSSION

In many types of mammalian cells, it has been widely observed that membrane receptors containing a seven membrane-spanning domain are capable of coupling with G proteins and that such receptors have two different affinities with a high and a low for the receptor agonists (11-13). The interaction of receptor molecules with G proteins result in the high-affinity state for the agonists, while receptors resolved from the signal coupling proteins are characterized as the low-affinity form. The high-affinity receptors can be converted to the low-affinity ones in the presence of GTP or its non-hydrolyzable analogues, GTPγS, due to the dissociation of the activated G protein from the receptors.

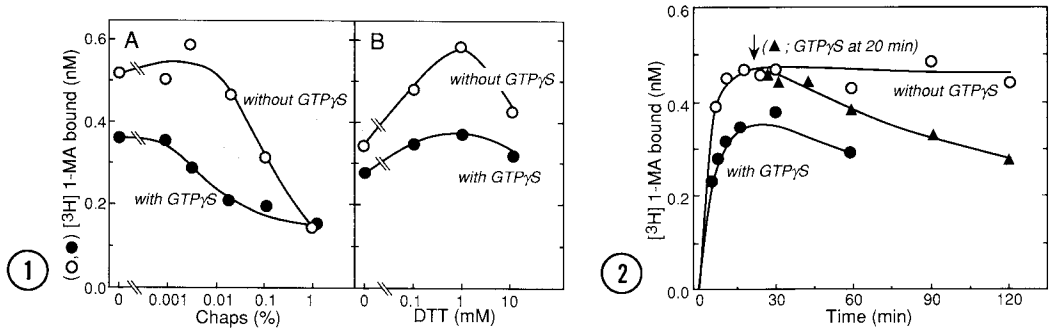

Fig. 1. Effect of Chaps or DTT on 1-MA binding to starfish oocyte membranes. Membrane fractions isolated from starfish oocytes were incubated with (●) or without (○) of 100 μM GTPγS in a reaction mixture containing 20 nM [^{3}H]1-MA and various concentrations of Chaps (*A*) or DTT (*B*). The specific binding of [^{3}H]1-MA was then determined as described in MATERIALS AND METHODS.

Fig. 2. Time courses of 1-MA binding to starfish oocytes membranes. The membranes were incubated with (●) or without (○) of 100 μM GTPγS for the indicated times in a reaction mixture containing 20 nM [^{3}H]1-MA, and the specific binding of [^{3}H]1-MA was then determined as described in MATERIALS AND METHODS. In an experiment shown by *closed triangles* (▲), GTPγS was added after 20-min incubation.

Thus, we first investigated how the specific binding of ^{3}H-radiolabeled 1-MA to starfish oocyte membranes was modified by GTPγS under various assay conditions. A high-affinity 1-MA binding could be readily observed at the agonist concentration of 20 nM under the present assay conditions (see Fig. 3 later). When GTPγS were added to the reaction mixture in the absence of a detergent, Chaps, there was a decrease in the specific binding to the membranes. Specific bindings observed in the presence and absence of GTPγS were both inhibited as the concentration of Chaps added to the reaction mixture was increased (Fig. 1A); this might be due to the solubilization of the receptor and/or G protein from the membranes. An extensive difference in the two bindings obtained with the presence of GTPγS and its absence was, however, observed at the detergent concentration around 0.01%.

Since the previous paper has reported that DTT exerts its influence on the 1-MA binding (10), the action of DTT was also investigated under the present conditions, and the results are illustrated in Fig. 1B. The addition of 0.1-1 mM DTT to the reaction mixture apparently stimulated the [^{3}H]1-MA binding to the membranes in the absence of GTPγS. However, such a marked stimulation by DTT was not observed in the presence of GTPγS. Further characterization of [^{3}H]1-MA binding was thus carried out in the presence of 0.01-0.02% Chaps and 1 mM DTT.

Fig. 2 show time courses of specific [^{3}H]1-MA binding to the oocyte membranes. The binding of [^{3}H]1-MA in the absence of GTPγS reached a plateau within 10 min, and the maximum activity was retained even after 2-h incubation. If GTPγS was simultaneously added to the initial reaction mixture, the rate of [^{3}H]1-MA binding as well as its maximum binding

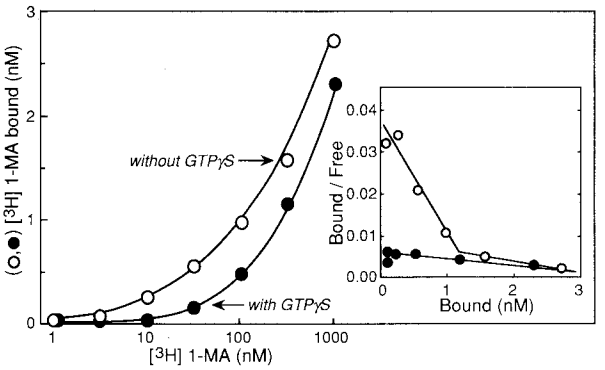

Fig. 3. Concentration-dependent binding of 1-MA to starfish oocytes membranes. The membranes were incubated with (●) or without (○) of 100 μM GTPγS in a reaction mixture containing various concentrations of [^{3}H]1-MA, and the specific binding of [^{3}H]1-MA was then determined as described in MATERIALS AND METHODS. The *inset* shows Scatchard plots of the data obtained with and without GTPγS.

was inhibited. When GTPγS was added at 20 min after the initiation of [^{3}H]1-MA binding, there was a progressive decease in the binding activity, as the incubation time was further increased. It would be important to note here that the dissociation rate of [^{3}H]1-MA from the membrane receptors in the presence of GTPγS (see *closed triangles* in Fig. 2) was much slower than that of typical agonists from mammalian membrane receptors. This might be due to the property of a slow rate of GTPγS binding to the starfish oocyte G protein (8).

Properties of [^{3}H]1-MA binding were further investigated with various concentrations of the radiolabeled ligand in the presence and absence of GTPγS. As shown in Fig. 3, there were progressive increases in the two binding activities, as the concentration of the agonist was increased. As expected, agonist bindings were markedly inhibited by GTPγS at the agonist concentration of 10-100 nM. Scatchard analysis (14) was conducted to the two binding curves, and the results are illustrated in the *inset* of Fig. 3. There were two affinities for [^{3}H]1-MA with apparent K_d's of approximately 30 nM and more than 1 μM in the absence of GTPγS. However, the high-affinity fraction observed in the absence of GTPγS was completely abolished when the GTP analogue was added. Inhibition of the [^{3}H]1-MA binding was rather selective to guanine nucleotides such as GTP and GTPγS; ATP or its analogue did not exert its inhibitory action on the [^{3}H]1-MA binding (data not shown). Thus it was evident that 1-MA receptors in starfish oocyte membranes displayed a GTPγS-sensitive, high-affinity state for the agonist as had been observed with typical membrane receptors coupling to G proteins.

We next investigated the G protein responsible for the formation of the high-affinity 1-MA bindings. For this evaluation, cholera toxin-induced ADP-ribosylation of G proteins was utilized. The toxin ADP-ribosylates the α-subunit of G_s in mammalian cells, and a 44-kDa protein is identified as the cholera toxin substrate in starfish oocyte membranes (ref. 8, and also see Fig. 4). In addition, it has been reported that the α-subunits of pertussis toxin-substrate G

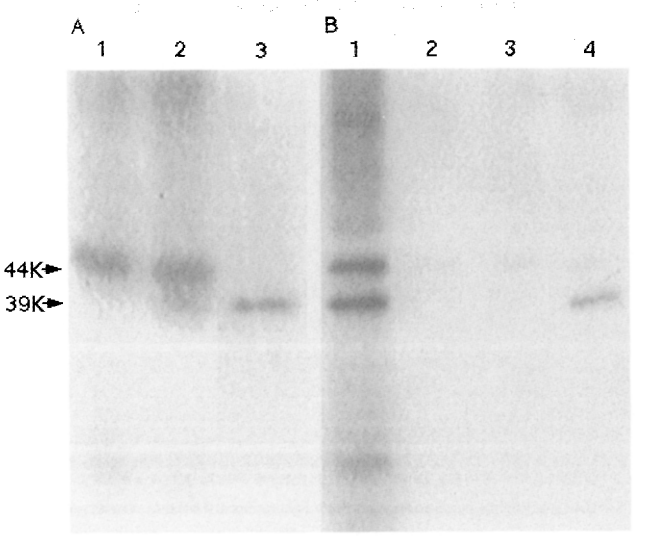

Fig. 4. Bacterial toxin-catalyzed ADP-ribosylation of G proteins in starfish oocytes membranes. *Panel A*: The membranes were [^{32}P]ADP-ribosylated by cholera toxin in the presence (*lane 2*) or absence (*lane 1*) of 10 μM 1-MA, and the radiolabeled proteins were then analyzed by SDS/PAGE and autoradiography as described in MATERIALS AND METHODS. The membranes were also [^{32}P]ADP-ribosylated by pertussis toxin. *Panel B*: The membranes were [^{32}P]ADP-ribosylated by cholera toxin in the presence (*lane 1*) of 10 μM 1-MA, and the solubilized proteins were then incubated without (*lane 2*) or with normal rabbit immunoglobulin G (*lane 3*) or anti-G_i α antibody (*lane 4*) as described in MATERIALS AND METHODS.

proteins can be also modified by cholera toxin only when receptors coupled to the G protein are stimulated by the agonist. Such examples are chemotactic peptide receptors in HL-60 cells (11, 13, 15) and opiate receptors in NG108-15 cells (16).

The oocyte membranes were incubated with activated cholera toxin and [^{32}P]NAD in the absence of 1-MA, and [^{32}P]ADP-ribosylated proteins were then analyzed by means of SDS-PAGE and autoradiography (Fig. 4A). A G_s α-like, 44-kDa protein in the membranes was indeed [^{32}P]ADP-ribosylated by cholera toxin (*lane 1*). When 1-MA was added to the incubation mixture, a protein with the molecular weight of 39,000 appeared to be radiolabeled by cholera toxin; the 44-kDa ADP-ribosylation was not modified by the addition of 1-MA (*lane 2*). The 39-kDa band radiolabeled by cholera toxin co-migrated with [^{32}P]ADP-ribosylated α-subunit of pertussis toxin-substrate G protein in the membranes (*lane 3*), suggesting that the 39-kDa protein ADP-ribosylated by cholera toxin is the α-subunit of G_i-like G protein purified from starfish oocytes by us. The more direct evidence was obtained by means of immuno-precipitation with anti-G_i-α antibodies (Fig. 4B). The 1-MA-supported radiolabeled 39-kDa protein could be immunoprecipitated by a polyclonal antibody raised against the carboxy-terminal site of mammalian G_i-α (*lane 4*), but not by normal rabbit immunoglobulin G (*lane 3*).

The present study demonstrated, for the first time, that 1-MA receptors in starfish oocytes membranes were functionally coupled with pertussis toxin-substrate G protein of which

molecular weight of the α-subunit was 39,000. Scatchard analysis of the 1-MA binding property suggested that there were two forms of binding sites with a high and a low affinities for 1-MA in the absence of GTPγS and that the high-affinity receptors could be converted into the low-affinity ones in the presence of GTPγS (see Fig. 3). The K_d's for 1-MA were approximately 30 nM and 1 μM. The lower value of K_d appeared to be reasonably high as a characteristic of general G protein-coupled membrane receptors for the agonists. The high K_d value of micromolar range was consistent with a concentration previously reported by others (3). The B_{max} value of 3-4 nM under the present assay conditions (100 μg of membrane protein in a 100-μl solution) corresponded to 3-4 pmol of binding site/mg of protein. This number is comparable to the values of typical membrane-bound receptors in mammalian cells, though it was lower than a value (approximately 100 pmol of mg protein) of the pertussis toxin-sensitive G protein in the same membranes (8).

In summary, the properties of the 1-MA binding characterized in the present study are in accordance with the biological features of the hormone-induced maturation of starfish oocytes. Identification of an effector(s) that functionally interacts with the G protein activated by the 1-MA receptors and that should be responsible for MPF induction is a crucial next step in confirmation of the physiological importance in the hormone-mediated signal transduction.

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