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Antiidiotypic antibodies against anti-cGMP polyclonal antibodies

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Affinity-purified polyclonal anti-cGMP antibodies were obtained from rabbit serum after immunization by succinyl derivative of cGMP coupled to bovine serum albumin. These antibodies were used to raise antiidiotypic antibodies in rats. Putative antiidiotypic serum inhibited the binding of [^{3}H]cGMP to affinity-purified anti-cGMP antibodies. The influence of immunoglobulins isolated from antiidiotypic serum on the ion conductance of rod outer segment plasma membrane fragments from frog retina was studied in patch-clamp experiments. These immunoglobulins increased the conductance of ion channels acting like a natural agonist (cGMP). Preimmune immunoglobulins did not act. The data obtained suggest that antiidiotypic antibodies interact with regulatory cGMP-binding sites of the plasma membrane channels.

Introduction

After the conductance of ionic channels of rod outer segment (ROS) plasma membrane had been discovered to be directly regulated by cGMP [1–3], attempts were made to identify and isolate this cGMP-dependent channel. The results obtained by several groups of researchers turned out rather contradictory. Cook et al. have purified a 63 kDa protein, which, being reconstituted in liposomes, results in cGMP-dependent conductance [4]. Subsequently cDNA encoded, this protein was isolated and then expressed in oocyte [5]. Matesic and Liebman have isolated a protein with a molecular mass of 39 kDa from the same object (bovine ROS). This protein, when being reconstituted in liposomes, revealed the cGMP-dependent conductance as well [6]. Clark and Stein have shown rhodopsin incorporated into lipid bilayer to exhibit cGMP-activated channel activity [7]. The attempt to identify cGMP-binding proteins using photoaffinity labelling by cGMP and its analogs showed a number of cGMP-binding proteins with apparent molecular masses from 250 to 41 kDa in ROS membranes [8–10]. Thus, the available data are rather conflicting.

To characterize and identify the cell receptors, the immunological approach based on using antiidiotypic antibodies, is widely developed in the last years [11–13]. Antiidiotypic antibodies were shown to be capable of specific binding with receptors, sometimes imitating the effects induced by naturally occurring agonists [14].

In the present work we have made an attempt to obtain antiidiotypic antibodies against affinity purified rabbit anti-cGMP polyclonal antibodies for their further use in identification of cGMP-binding proteins of the photoreceptor membrane.

Materials and Methods

Succinyl-cGMP (ScGMP). ScGMP was synthesized using the method described by Delage et al. [15]. The purity of the product was 90%, as determined by thin-layer chromatography on cellulose (Merck) in butanol/acetic acid/water (12 : 3 : 5, v/v).

ScGMP-BSA conjugate. ScGMP-BSA conjugate was synthesized according to Steiner et al. [16]. Unbound ScGMP was separated on Ultrogel GF-05 (LKB) column and the conjugate was dialyzed against 20 mM Na_2HPO_4/NaH_2PO_4, 120 mM NaCl (PBS) (pH 7.4) for 24 h. The molar ratio of cGMP/BSA, determined spectrophotometrically, was 4.4.

Abbreviations: ScGMP, 2′-*O*-succinyl-guanosine 3′,5′-cyclic phosphate; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ROS, rod outer segment(s).

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Immunization schedule. To develop anti-cGMP antibodies, the New Zealand white rabbits were intradermally immunized with 1 mg of ScGMP-BSA in 0.5 ml PBS emulsified with an equal volume of complete Freund adjuvant into multiple points. The boost injection was performed with 1 mg of conjugate in incomplete Freund adjuvant 4 weeks later in the same manner. Subsequent boosts were performed intradermally with 1 mg of conjugate in PBS each two weeks. To test antibodies, the rabbits were bled on day 9 after immunization. Maximal titer of antibodies was reached after the third boost.

To raise antiidiotypic antibodies, Wistar rats were primarily immunized intradermally with 100 μg of affinity isolated rabbit anti-cGMP immunoglobulins in complete Freund adjuvant into multiple points. The rats were boosted with 100 μg of antibodies in incomplete Freund adjuvant a month later. The rats were bled on day 7 after booster immunization.

Synthesis of cGMP-agarose. 3 ml of AH agarose (1 g) were washed with 700 ml of 0.5 M NaCl and with 150 ml of water. Then ScGMP (30 μmol in 6 ml of H_2O) was added to agarose. Afterwards 4 × 50 mg of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide were added stepwise (every 2 h). Suspension was stirred for 20 h at room temperature keeping pH between 4.5 and 6.0. cGMP-agarose was washed with 500 ml of 0.1 M Na-acetate (pH 4.0), 0.5 M NaCl and with 500 ml of H_2O. The amount of cGMP bound to agarose was determined by addition of [^{3}H]cGMP to the reaction medium and was 0.2 μmol/ml gel. cGMP was not released when the matrix was washed with the following solutions: PBS, 0.1 M glycine (pH 2.5), 0.1 M potassium succinate, 8 M urea, 3.5 M KSCN. cGMP-agarose was kept in Na-acetate (pH 4.0) and repeatedly used.

Affinity purification of anti-cGMP antibodies. 20–30 ml of serum were passed (5 ml/h) through cGMP-agarose column (1 ml). The column was washed with 100 ml of PBS containing 0.3% BSA to remove unbound protein. The antibody was eluted by 3 portions (20 min incubation) of 10 mM cGMP in washing medium. The combined eluate was dialyzed against 3 × 0.5 l of PBS for 24 h and then treated by stirring with charcoal (1/10 of solution volume containing 5% Norit A, 0.5% dextran-70 and 0.3% BSA) for 1 h at 0 °C. Charcoal was separated by centrifugation (12 000 × g for 15 min). To separate the immunoglobulins from BSA, they were precipitated by 40% ammonium sulfate followed by 24-h dialysis against PBS.

Determination of cGMP-binding activity of antibodies. 25 μl of serum or purified antibodies were incubated in total volume of 100 μl with 20 nM [^{3}H]cGMP (Amersham, 23.7 Ci/mmol) for 10 min at room temperature. Then an equal volume of saturated ammonium sulfate was added and the samples were centrifuged at 12 000 × g for 2 min. The precipitate was solubilized in 0.5 ml of water and counted in scintillation cocktail GS-8 (USSR). Unspecific binding was determined in the presence of a 100-fold excess of unlabelled cGMP.

Determination of antiidiotypic antibodies. The presence of antiidiotypic antibodies in serum was assayed by its ability to inhibit the binding of [^{3}H]cGMP to affinity purified anti-cGMP antobidies. Putative antiidiotypic serum at different dilutions was incubated in a total volume of 50 μl with 100 ng of affinity purified anti-cGMP antibodies overnight at 4 °C and then the binding of [^{3}H]cGMP was determined as described above.

cGMP-activated conductance. cGMP-activated conductance of isolated patches of ROS plasma membrane was determined by the patch-clamp method, as described previously [17]. All experiments were carried out with 8-Br-cGMP, which is a cGMP analog but more resistant to hydrolysis by phosphodiesterase as well as with immunoglobulins from antiidiotypic rat sera obtained by ammonium sulfate precipitation. The immunoglobulins isolated from preimmune serum were used as control ones.

Results and Discussion

After the third immunization of six rabbits the titre of anti-cGMP antibodies was the highest in two of them (half-maximal binding of [^{3}H]cGMP at dilution 1 : 200). Serum of these rabbits was used for further work. The apparent equilibrium constant (K_d) of cGMP binding to serum was 15 nM (Fig. 1). This constant was essentially lower than the K_d of cGMP-gated channels of ROS plasma membrane (25–50 μM, [18]). The dissociation rate of cGMP is an important parameter characterizing the functioning of cGMP-gated channels. The dissociation times of the cGMP-channel complex are in a millisecond range [17] and this enables the cGMP-regulated channels to 'monitor' quickly the light-induced decrease of the cGMP concentration in the photorecep-

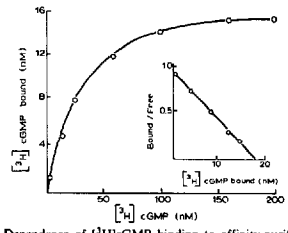

Fig. 1. Dependence of [^{3}H]cGMP binding to affinity-purified anti-cGMP antibodies on the [^{3}H]cGMP concentration. Concentration of immunoglobulins 10 μg/ml, sample volume 100 μl. The inset shows the Scatchard plot of the binding data.

tor cell [18]. Therefore, when specific antibodies are purified, it is desirable to isolate from serum the fraction of cGMP-binding immunoglobulins with a short period of keeping cGMP.

Rabbit anti-cGMP antibodies were isolated from serum by affinity chromatography on agarose with covalently coupled ScGMP. Anti-cGMP serum passed through the column did not contain cGMP-binding antibodies and, therefore, all cGMP-binding antibodies were sorbed on the matrix. For desorption we used a competitive removal of antibodies from the column by cGMP. This allowed us to obtain antibodies with kinetic parameters of dissociation tending to the parameters of a natural receptor. Such separation of antibodies was conditioned by the fact that upon affinity elution, antibodies with a higher dissociation rate of the hapten-antibody complex dissociated earlier. Indeed, as can be seen in Fig. 2, the dissociation rate of cGMP from affinity-isolated antibodies is higher than that from total antibodies in unfractionated serum.

5–10% of the cGMP-binding activity is eluted from the affinity column during the first hour of incubation with 10 mM cGMP. Comparison of the protein amount and the number of cGMP-binding sites in the affinity-isolated preparation shows that for different preparations 0.2 to 2 molecules of cGMP are bound to one molecule of immunoglobulin (supposing that all immunoglobulins belong to the G-class). The apparent K_d determined for the affinity-isolated antibodies does not greatly differ from the apparent K_d obtained for antiserum.

Application of a usual method of elution such as low pH values, high ion strength, concentrated solutions of urea and KSCN failed because antibodies eluted in such a way practically lost the ability to bind cGMP (the residual activity was 0.1–2% of the initial one).

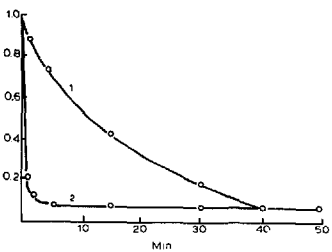

Fig. 2. Kinetics of [^{3}H]cGMP dissociation from the antibody-cGMP complex. Anti-cGMP serum (dilution 1:125, curve 1) or affinity-purified anti-cGMP antibodies (1.5 μg/ml, curve 2) were incubated with 20 nM of [^{3}H]cGMP for 10 min in 990 μl. Then 10 μl of 0.2 mM unlabelled cGMP were added to the sample and the amount of bound [^{3}H]cGMP was determined after different time intervals (see Methods). In the control sample, buffer was added instead of cGMP. The curves are normalized relative to values of maximal binding. The abscissa indicates the time (in min) after addition of unlabelled cGMP.

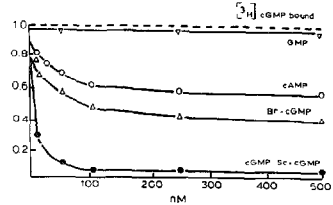

Fig. 3. Characteristics of specificity of affinity-isolated anti-cGMP antibodies. Specificity was determined by ability of the analogs to compete for binding sites with [^{3}H]cGMP. 1.5 μg/ml of antibodies were incubated in 100 μl with 20 nM [^{3}H]cGMP and the analogs at different concentrations. The binding is shown in arbitrary units. The abscissa indicates the concentration of unlabelled nucleotides.

To characterize the specificity of the affinity-purified antibodies, the competition of unlabelled analogs with [^{3}H]cGMP for binding sites was studied. Fig. 3 demonstrates the results of these experiments. It is seen that cGMP and ScGMP effectively compete with [^{3}H]cGMP for the binding sites. Among the affinity-isolated antibodies there are antibodies binding other analogs with a cyclophospnate group (cAMP). GMP without a cyclophosphate group does not compete with cGMP up to a concentration of 100 μM. The immunoglobulins obtained are, obviously, heterogeneous and contain antibodies against the ribose-cyclicphosphate part, which is common for all cyclic nucleotides. Thus, the obtained antibodies, like a cGMP-channel from the photoreceptor membrane, bind cGMP well and do not practically interact with GMP [17]. This preparation of antibodies was used to develop antiidiotypic antibodies in rats.

Serum of the rats immunized by affinity-isolated anti-cGMP antibodies inhibited the ability of anti-cGMP immunoglobulins to bind [^{3}H]cGMP (Fig. 4). This indicates the presence of antiidiotypic antibodies in rat serum which are capable of competing with [^{3}H]cGMP for a cGMP-binding site of anti-cGMP antibodies. Serum had antiidiotypic properties only in two rats from five immunized. Preimmune serum did not influence the [^{3}H]cGMP binding to anti-cGMP antibodies.

To determine the interaction of antiidiotypic antibodies with cGMP-binding sites of the ROS plasma membrane channels, we studied the influence of antiidiotypic immunoglobulins, isolated from serum (40% of ammonium sulfate followed by 24-h dialysis), on the conductance of plasma membrane patches. Three variants are possible here: (i) antiidiotypic antibodies do not interact with receptor and, therefore, do not

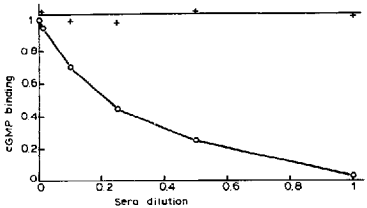

Fig. 4. Inhibition of [^{3}H]cGMP binding to affinity-purified anti-cGMP antibodies by rat preimmune (+) and antiidiotypic (○) serum. Anti-cGMP antibodies (2 μg/ml) were incubated overnight with antiidiotypic serum in 50 μl at 4°C and then the binding of 20 nM of [^{3}H]cGMP was determined as mentioned in Methods. The points are normalized relative to the binding in the absence of antiidiotypic serum. Maximal binding corresponds to $2.4 \cdot 10^3$ cpm (0.34 pmol) of bound [^{3}H]cGMP.

influence the cGMP-induced conductance; (ii) antiidiotypic antobidies hinder the binding of agonist with receptor and inhibit the agonist effect [19]; (iii) antiidiotypic antibodies interact with receptor imitating the agonist effect [15].

In our experiments application of antiidiotypic antibodies to the cytoplasmic surface of the inside-out patches of ROS plasma membrane leads to a slow rising of the patch conductance (Fig. 5). After the maximal conductance level has been reached, BrcGMP addition to the bathing solution does not result in any change of conductance (data not shown). The effect of antiidiotypic antibodies appeared to be irreversible. Immunoglobulins isolated from preimmune rat serum and those from rats immunized in the same manner by neutral rabbit immunoglobulins did not affect the membrane patch conductance.

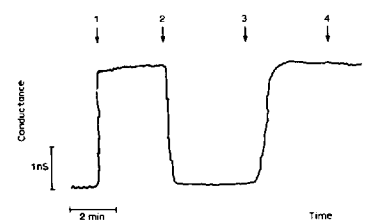

Fig. 5. Influence of antiidiotypic immunoglobulins on the conductance of ROS plasma membrane patch. Replacement of solution washing the cellular surface of plasma membrane is marked with arrows. 1, 5 μM BrcGMP; 2, buffer; 3, immunoglobulins isolated from antiidiotypic sera (1 mg/ml); 4 buffer.

These results allow us to assume that antiidiotypic antibodies bind to a cGMP-binding site of cGMP-gated ROS plasma membrane channels.

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