Desensitization and Sequestration of Human m2 Muscarinic Acetylcholine Receptors by Autoantibodies from Patients with Chagas’ Disease*

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Chronic Chagas’ disease is associated with pathologic changes of the cardiovascular, digestive, and autonomic nervous system, culminating in autonomic denervation and congestive heart failure. Previously, circulating autoantibodies that activate signaling by cardiac muscarinic acetylcholine receptors (mAChRs) have been described. However, it remains unclear whether the chagasic IgGs directly interact with the m2 mAChRs (predominant cardiac subtype), and, if so, whether chronic exposure of the mAChRs to such activating IgGs would result in receptor desensitization. Here we performed studies with purified and reconstituted ham2 mAChRs and demonstrate that IgGs from chagasic serum immunoprecipitated the mAChRs in a manner similar to an anti-m2 mAChR monoclonal antibody tested in parallel. The chagasic antibodies did not directly interact with the ligand binding site, because the binding of radiolabeled antagonist was unchanged by the addition of the chagasic IgG. In intact cells stably expressing the hm2 mAChR, the chagasic IgGs, but not normal IgGs, mimicked the ability of the agonist acetylcholine to induce two effects associated with agonist-induced receptor desensitization: a decrease in affinity for agonist binding to m2 mAChR and sequestration of the hm2 mAChRs from the cell surface. The results demonstrate that the chagasic IgGs can directly interact with and desensitize m2 mAChRs and provide support for the hypothesis of autoimmune mechanisms having a role in the pathogenesis of Chagas’ cardiomeuropathy.

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The abbreviations used are: mAChR, muscarinic cholinergic receptor; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; hm2, human m2; DMMF-F-12, Dulbecco’s modified Eagle’s medium; Ham’s F-12; Gpp(NH)p, 5’-guanylylimidodiphosphate; NMS, N-methyloxopamine; QNB, quinuclidinyl-benzilate.

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Purification of m2 Muscarinic Acetylcholine Receptors—Human m2 mAChRs (hm2 mAChR) from Spodoptera frugiperda (Sf9) insect cells infected with recombinant baculovirus encoding the human m2 mAChR were purified and reconstituted as described (22–24). The hm2 mAChR purified from Sf9 cells is expressed as a 55-kDa protein, which is smaller than that observed in mammalian cells because it lacks the extensive glycosylation associated with the mammalian expressed receptor (24). Recombinant virus was kindly provided by Drs. Eric M. Parker and Elliot Ross (University of Texas, Dallas, TX). Culture of m2 Chinese Ovary Cells—Chinese hamster ovary cells stably transfected with hm2 mAChRs (m2 CHO cells; Ref. 25) were kindly provided by Dr. Ernest Peralta (Harvard University, Cambridge, MA). Cells were grown in Dulbecco’s modified Eagle’s medium:Ham’s F-12 (DMEM:F-12) supplemented with 10% dialyzed fetal bovine serum, 100 units/ml of penicillin and streptomycin, and 2 μg/ml glutamine in the presence of 250 mM methotrexate. Cells were plated in 60- or 100-mm dishes and used at about 80% confluency. The density of mAChRs per cell was determined by saturation binding of [3H]QNB to intact cells (26).

Immunoprecipitation Assays—Purified reconstituted hm2 mAChRs were immunoprecipitated with chagasic antibodies or a monoclonal anti-m2 mAChR antibody (27) generously provided by Dr. M. Schmerlik from Oregon State University (Corvallis, OR). Chagasic or normal sera or anti-m2 hybridoma supernatant were precoupled to protein A-agarose beads (50 μl, Pierce) overnight at 4 °C. The beads were then incubated with ~0.7 pmol of reconstituted mAChRs at 4 °C for 5 h and washed thoroughly. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis using 8.5% gels and then transferred to nitrocellulose membranes. Proteins were visualized by means of the anti-m2 monoclonal antibody and a goat anti-mouse IgG conjugated to peroxidase followed by the ECL reaction (Amersham Corp.) as described (26).

Desensitization and Sequestration of the mAChR—The m2 CHO cells were grown in 100-mm dishes, washed with PBS, and then allowed to stabilize for 10 min at 37 °C with serum-free fresh DMEM:F-12 containing 10 μM eserin (a cholinesterase inhibitor) before the addition of treatments to induce desensitization or sequestration. To assess the effects of chagasic sera to cause decrease in high affinity agonist binding, cells were treated with serum-free medium containing either PBS (as a no drug/no IgG control), the agonist acetylcholine, or chagasic or normal IgGs. Cells were incubated for 1 h at 37°C in a 5% CO2 atmosphere with one of the above reagents, and after extensive washing, they were harvested in 10 mM phosphate buffer (pH 7.4), 1 mM EDTA, 0.25 M sucrose. All steps were performed at 4 °C. Cells were centrifuged at 400 × g for 15 min, and crude membranes were prepared as described previously (27). To test for effects of pretreatments with agonist or IgGs on the affinity of the receptors for agonist, the agonist carbachol was used to compete for [3H]QNB binding to mAChR in the membrane preparations. Membranes were incubated at 37°C for 75 min in 20 mM phosphate buffer (pH 7.4), 1 mM EDTA, 2 mM MgCl2, [3H]QNB (0.5–0.6 nM), and varying concentrations of carbachol in the presence or the absence of the guanine nucleotide analogue 5′-guanylimidodiphosphate (Gpp[NH]p, at 100 μM). The data were analyzed with the curve-fitting program LIGAND (28).

To assess for sequestration of hm2 mAChRs, experiments were performed on intact m2 CHO cells using the hydrophilic ligand [3H]NMS-methylscopolamine (NMS) to determine changes in cell surface m2 mAChR number, whereas the hydrophobic ligand [3H]QNB was used to assess total receptors. After pretreatment with drugs or IgGs as described above, the culture dishes were placed on ice, and the cells were thoroughly washed with cold PBS. They were then spun down at 400 × g, resuspended in ice-cold DMEM:F-12 and assessed for viability by trypan blue exclusion, and suspensions with more than 95% viable cells were used for binding assays. Saturating concentrations of [3H]NMS or [3H]QNB were incubated with cells in Hepes-buffered DMEM:F-12 in the presence or the absence of 1 μM atropine to calculate specific binding for 2 h at 4 °C for [3H]NMS and for 75 min at 37 °C for [3H]QNB. Cells were filtered onto GPC glass fiber filters, and radioactivity was counted in a Beckman spectrometer. Control levels of surface and total receptors were defined as the levels in cells treated the same as above but with the corresponding volume of PBS instead of drug or IgG. These values were considered as 0% internalization of each assay. The percentage of internalization due to drug or IgG treatments was calculated as the percentage of loss of surface receptors measured with [3H]NMS in treated samples compared with 0% internalization controls. Down-regulation was defined as a net decrease in the total receptor pool as assessed by [3H]QNB binding assays. Variations in cell density per plate were controlled by normalizing all data on the basis of mg protein/plate.

RESULTS AND DISCUSSION

Molecular Interaction of Chagasic Antibodies with Purified Human m2 mAChRs—To test the ability of chagasic antibodies to directly interact with purified hm2 mAChRs, immunoprecipitation experiments were performed with several chagasic or normal sera and purified hm2 mAChRs reconstituted in vesicles. As a control, an equivalent amount of the hm2 mAChR were also immunoprecipitated with a known monoclonal anti-m2 mAChR antibody (26). The immunoprecipitates were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose, and the immunoprecipitated receptors were identified by immunoblotting with the anti-m2 monoclonal antibody. The results are representative of six other experiments with different chagasic and normal sera. M, indicates the position of the molecular weight standards.
Desensitization of Muscarinic Receptors by Chagasic IgG

Membranes were prepared from CHO cells expressing the hm2 mAChR as described in the text and incubated in the presence or the absence of normal or chagasic IgG (0.4 mg/ml) for 120 min at 37 °C. The membranes were then washed four times with binding buffer and used in saturation binding assays with \(^{3}H\)QNB (25–500 pm). The results are the means ± S.E. of four experiments. The data were analyzed with the LIGAND program (28).

| Addition   | \(K_d\) (pm) | \(B_{max}\) (pmol/mg protein) |
|------------|-------------|-------------------------------|
| None       | 96 ± 32     | 14.7 ± 1.6                    |
| Chagasic IgG| 89 ± 16     | 13.1 ± 1.1                    |
| Normal IgG | 103 ± 26    | 12.9 ± 0.9                    |

**TABLE I**

Effect of normal and chagasic IgG on the binding of the antagonist \(^{3}H\)QNB

The possibility that the persistence of the activation of the G-protein and its subsequent dissociation of G-protein-coupled receptors is associated with several events, in particular, receptor/G-protein uncoupling and sequestration of the receptors away from their normal membrane environment. A hallmark of receptor uncoupling from G-proteins is the conversion of receptors from a high to low affinity state. The high affinity state for agonists is considered to be a receptor/G-protein complex, whereas the lower affinity state is thought to represent the free receptor. In agonist binding studies in vitro a loss of high affinity agonist binding can be induced upon the addition of GTP or its analogs, presumably due to the activation of the G-protein and its subsequent dissociation of the G-protein from the receptor. Agonist-induced desensitization of the \(\beta\)-adrenergic receptor has been associated with the loss of high affinity agonist binding and conversion of receptors to a low affinity state (29).

To test for desensitizing effects of chagasic antibodies on agonist binding properties, we compared the effects of pretreatment of intact cells with either the agonist acetylcholine or normal or chagasic IgG on agonist affinity. After each pretreatment, membranes were prepared and used in carbachol/\(^{3}H\)QNB competition binding experiments in the presence or the absence of Gpp(NH)p, reflecting both high and low affinity agonist binding (Fig. 2a). The addition of Gpp(NH)p right shifted and steepened the curves, as expected for the guanine nucleotide-dependent conversion of high affinity sites into low affinity sites (Fig. 2a, untreated). The corresponding IC\(_{50}\) values and Hill coefficients reflected these changes (Table II). In addition, analysis of the data with the curve fitting program LIGAND (28) demonstrated the expected results, in that a high and low affinity state were observed and Gpp(NH)p caused a conversion of receptors from the high to low affinity state (Table III). Pretreatment of cells either with the agonist acetylcholine under conditions that lead to desensitization (27) or with chagasic IgG produced rightward shifts of the concentration-displacement curves of carbachol in the absence of Gpp(NH)p compared with untreated cells (compare Fig. 2b and d, to Fig. 2a; Table II). The effects caused by either pretreatment were not as extensive as those induced by the addition of Gpp(NH)p to the in vitro assays. The protease digestion fragment of chagasic IgG, F(ab')\(_2\), had similar effects to the nondigested chagasic IgG (data not shown). The shift induced by chagasic IgG was no longer observed when incubations were performed with normal IgG (Fig. 2c, Table II). Analysis of the data with the LIGAND program indicated that the decrease in overall affinity caused by the chagasic IgG was due to an increase in the value of \(K_d\), whereas there was no detectable change in the percentage of receptors in the higher affinity state (Table III). Treatment of the cells with agonist produced a similar trend (Table III). The LIGAND analysis also suggested that the pretreatment of the

**FIG. 2.** Reduction in agonist affinity of human m2 muscarinic receptors by chagasic IgG. CHO cells expressing m2 mAChRs were incubated with serum-free DMEM:F-12 containing eusine and no additions (panel a, untreated, continuous lines) or with acetylcholine (1 mM, panel b), normal IgG (panel c), or chagasic IgG (0.2 mg/ml, panel d). In panels b–d, the dashed lines are drawn through the data points for the treated cells, and the solid lines from the control (panel a) are included as a reference to facilitate comparisons. After 1 h of incubation, cells were washed and harvested, and the membranes were prepared as described under “Materials and Methods.” Carbachol displacement curves were obtained in the absence (open symbols) or the presence (filled symbols) of 100 \(\mu\)M Gpp(NH)p. The results are representative of 6–8 separate experiments performed with different chagasic and normal IgGs and with similar results.

**TABLE II**

IC\(_{50}\) values and Hill coefficients for the agonist competition studies

| Cell treatment | Without Gpp(NH)p | With Gpp(NH)p |
|----------------|------------------|---------------|
|                | \(IC_{50}\) \(H\) | \(IC_{50}\) \(H\) |
| Untreated      | 71 ± 18 0.41 ± 0.05 | 230 ± 24 0.92 ± 0.15 |
| Acetylcholine  | 202 ± 40 0.62 ± 0.07 | 313 ± 62 0.68 ± 0.06 |
| Chagasic IgG   | 160 ± 23 0.66 ± 0.12 | 193 ± 17 0.82 ± 0.06 |
| Normal IgG     | 87 ± 16 0.47 ± 0.06 | 241 ± 20 0.87 ± 0.07 |

\(a\) Significantly different from untreated, \(p < 0.025.\)

\(b\) Significantly different from untreated, \(p < 0.01.\)
The present results also were similar to those we previously obtained for the expected decrease in high affinity states of the receptor, respectively, whereas \( R_H \) and \( R_L \) refer to the percentage of receptors in each affinity state. Shown are the means ± S.E. from 6–8 experiments similar to the one shown in Fig. 2. ND, not detectable.

| Pretreatment of m2 CHO cells | Addition of Gpp(NH)p | \( K_H (\mu M) (R_H, \%) \) | \( K_L (\mu M) (R_L, \%) \) |
|-----------------------------|---------------------|-----------------|-----------------|
| None                        | –                   | 0.33 ± 0.12     | 34 ± 9          |
|                             | –                   | (46 ± 5)        | (54 ± 13)       |
|                             | +                   | ND              | 20 ± 2          |
|                             | –                   | ND              | (100)           |
| Acetylcholine               | –                   | 3.8 ± 1.8       | 49 ± 18         |
|                             | +                   | 0.41 ± 0.19     | 38 ± 10         |
|                             | –                   | (50 ± 9)        | (50 ± 11)       |
| Chagasic IgG                | –                   | 2.6 ± 0.6       | 62 ± 9          |
|                             | +                   | (48 ± 9)        | (52 ± 11)       |
|                             | –                   | ND              | 21 ± 6          |
|                             | –                   | (11 ± 5)        | (69 ± 15)       |
| Normal IgG                  | –                   | 0.49 ± 0.06     | 37 ± 6          |
|                             | +                   | (52 ± 5)        | (48 ± 6)        |
|                             | –                   | ND              | 17 ± 3          |
|                             | –                   | ND              | (100)           |

* Significantly different from no treatment, \( p < 0.01 \).

TABLE III
Binding parameters obtained from curve-fitting of competition data

The data shown in Fig. 2 were analyzed with the curve-fitting program LIGAND (28). \( K_H \) and \( K_L \) refer to \( K \) values for carbobcholine binding to high and low affinity states of the receptor, respectively, whereas \( R_H \) and \( R_L \) refer to the percentage of receptors in each affinity state. Shown are the means ± S.E. from 6–8 experiments similar to the one shown in Fig. 2. ND, not detectable.

![Fig. 3. Internalization of m2 mAChRs by chagasic IgG.](image)

a. CHO cells expressing m2 muscarinic receptors were incubated with serum-free medium containing eserine and 1 mM acetylcholine (filled triangles) or 0.2 mg/ml chagasic (filled circles) or normal (empty circles) IgGs. Cells were incubated for the indicated times and then washed and harvested, and radioligand binding assays were performed as described under "Materials and Methods." The data are the means ± S.E. of five experiments performed in triplicate with different IgGs. b, cells were preincubated with varying concentrations of acetylcholine (ACH), chagasic IgG (ch IgG), chagasic F(ab′)2 (ch F(ab′)2), or normal IgG (n IgG) as indicated. After 2 h of incubation cells were washed and harvested, and radioligand binding was performed. The data are the means ± S.E. of five experiments with different IgGs.
involved remain unexplained. Thus, the major point of our report is that early events of agonist-promoted desensitization of m2 mAChR can be initiated by autoantibodies that bind to and persistently activate the receptors. Further studies will be required to address the issue of whether early molecular alterations in the function of the cardiac autonomic nervous system during Chagas’ disease are related to the presence of autoantibodies promoting receptor desensitization. Interestingly, the existence of autoantibodies against autonomic receptors has been reported in other cardiomyopathies such as human dilated cardiomyopathy (32–34) and congenital heart block (35), but evidence is still lacking about the mechanisms underlying their potential pathological role. Whether or not autoantibodies play a general role in other cardiomyopathies remains to be determined.

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