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MODIFICATION OF G REGULATORY PROTEIN MEDIATED ACTIONS BY THE INTERACTION OF HISTOCOMPATIBILITY ANTIGENS WITH CARDIAC MUSCARINIC CHOLINERGIC RECEPTORS

GRACIELA CREMASCHI, GABRIELA GORELIK, ANA GENARO, ENRI BORDA and LEONOR STERIN-BORDA*

Centro de Estudios Farmacológicos y Botánicos (CEFYBO), Consejo Nacional de Investigaciones Científicas y Téctinicas de la República Argentina (CONICET), 1414 Buenos Aires, Argentina

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Abstract—In this work we characterized the interaction of class I histocompatibility (HC) antigens (Ag) with cardiac cholinergic receptors by means of specific radioligand binding and by production of cholinergic-mediated cellular transmembrane signals. Alloimmune as well as anti-class I but not anti-class II antibodies were able to inhibit in an allosteric manner the binding of [^{3}H]quinuclidinyl benzilate to cardiac membrane. Moreover, alloantibody could modify all of the muscarinic cholinergic effects mediated by a G regulatory protein, i.e. decrement of atria contractility, inhibition of cAMP stimulation, and activation of the turnover of phosphoinositides via phospholipase C. The cGMP production was not altered by the alloantibody. The data indirectly indicated that HC-Ag–muscarinic cholinergic interactions trigger all the cholinergic functions related to G proteins. The induction of intracellular second messengers by class I antigens and hormone–receptor interactions is discussed.

The stimulation of muscarinic cholinergic receptors in atria results in the activation of a series of transmembrane signalling systems that lead to regulation of atrial rate and conduction [1, 2]. The biochemical events that accompany this phenomenon are: (1) an increase in potassium permeability together with a decrease in the slow inward calcium/sodium current, events that are mediated by the regulatory GTP binding protein G_k [3]; (2) a decrease in cyclic AMP (cAMP) formation coupled to a G_i protein that inhibits the adenylate cyclase [2, 4]; (3) an increase in cyclic GMP (cGMP) production by a direct stimulation of the guanylate cyclase [1]; and (4) the activation through a pertussis toxin nonsensitive G protein, namely G_p, of the phospholipase C (PLC†) that leads to an increase in inositol triphosphate (IP_3) and 1,2-diacylglycerol (DAG) cellular concentrations [5, 6]. It was demonstrated recently that hormone receptor and class I histocompatibility (HC) antigen (Ag) interactions induce the production of intracellular second messengers in atria, namely, cAMP induction by specific alloantibodies through β-adrenoceptor activation in cardiac [7] and smooth muscle [8] preparations and an increase in phosphoinositide turnover via cardiac cholinergic muscarinic receptors [9].

The interaction between β-adrenoceptors and HC Ag was fully characterized: class I Ag participation in the phenomenon was demonstrated as well as the involvement of β-adrenoceptors by alloimmune IgG competitive inhibition of specific β-radioligand binding in purified cardiac membranes [10, 11] and the increase in cAMP formation [7, 8].

The aim of the present work was to characterize the interaction of HC Ag with cardiac muscarinic cholinergic receptors by means of specific radioligand binding and by production of cholinergic-mediated cellular transmembrane signals. Here we show that alloantibody could competitively inhibit the binding of [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) and could modify all the muscarinic cholinergic effects mediated by G regulatory proteins.

MATERIALS AND METHODS

Materials. [^{3}H]Quinuclidinyl benzilate (sp. act. 43.3 Ci/mmol) was obtained from the Amersham Co. *myo*-2[^{3}H]Inositol ([^{3}H]MI) precursor (sp. act. 15 Ci/mmol), adenosine-3′,5′-cyclic [^{3}H]monophosphate (sp. act. 30.5 Ci/mmol) and a cyclic GMP [^{125}I]RIA kit were from Dupont/New England Nuclear. Prostaglandin E_1 (PGE_1), carbachol, atropine and 2-nitro-4-carboxy-phenyl-*N*,*N*-diphenyl-carbamate (NCDC) were from the Sigma Chemical Co. All chemicals used were of analytical grade. Alloimmune mouse anti-serum against H-2^K HC Ag was obtained by immunizing BALB/c (H-2^d) mice with C3H (H-2^k) lymphoid cells as described [10]. Purification of alloimmune IgG was achieved by DEAE-cellulose ion-exchange chromatography, and the degree of purification was analyzed by immunoelectrophoresis. Monoclonal anti-class II alloantigen of k haplotype I-E^k (Ia7) was from

* Corresponding author: Leonor Sterin-Borda, M.D., CEFYBO, Serrano 665, 1414 Buenos Aires, Argentina. Tel. (54) 1-855-7194; FAX (54) 1-856-2751.

† Abbreviations: PLC, phospholipase C; IP_3, inositol triphosphate; DAG, 1,2-diacylglycerol; HC, histocompatibility; Ag, antigen(s); [^{3}H]QNB, [^{3}H]quinuclidinyl benzilayte; [^{3}H]MI, *myo*-2-[^{3}H]inositol; PGE_1, prostaglandin E_1; NCDC, *N*,*N*-diphenylcarbamate; KRB, Krebs–Ringer bicarbonate; MIX, methyl-isobutyl-xanthine; IP, inositol phosphate; and TCA, trichloroacetic acid.

Cederlane; it is specific for Ia Ag coded by the E subregion of the k haplotype. Monoclonal anti H-$2K^k$ purified antibody was from Becton Dickinson, and its cytotoxic effect upon C3H thymocytes is shown in Results (see Table 1).

Muscarinic cholinergic binding assay. Purification of cardiac membranes was performed as described before [11]. Briefly, BALB/c mice were killed by decapitation and their hearts were mixed with 4 vol. of cold buffer containing 0.25 M sucrose, 50 mM Tris–HCl (pH 7.8), 10 mM $MgCl_2$. Tissues were homogenized twice with a Polytron PT 20 at maximum speed for 15 sec. Homogenates were spun at 700 *g*, 4° for 15 min and the resulting supernatants were centrifuged at 10,000 *g* for 15 min at 4° and then at 40,000 *g* for 30 min. The pellet was then resuspended in buffer [50 mM Tris–HCl (pH 7.8), 10 mM $MgCl_2$] at a protein concentration of approximately 6 mg/mL.

For [^{3}H]QNB binding, 200 μg of membrane protein, pretreated or not with normal (IgG_N) or alloimmune (IgG_I) IgG (concentrations indicated in Results) for different times at 30°, was incubated with stirring in a final volume of 150 μL 50 mM Tris–HCl (pH 7.8), 10 mM $MgCl_2$, with different concentrations of [^{3}H]QNB. When preincubation times with alloantibody exceeded 30 min, some protease inhibitors (0.1 mM phenylmethlysulfonyl fluoride, 0.01 mg/mL leupeptin, 1 mM bacitracin and 0.1 mg/mL soybean trypsin inhibitor) were added; none of them interfered in radioligand binding. Samples were left for 45 min at 37° and they were filtered through GF/c glass fiber filters. Filters were washed twice with 4 mL of buffer, dried and counted in Triton-liquid scintillation fluid. Nonspecific binding was determined in the presence of 10^{-6} M atropine, and it never exceeded 10% of specific binding. In competition studies, a fixed radioligand concentration of 0.2 nM and different concentrations of IgG or muscarinic agents were used. In saturation studies, IgG (10^{-7} M) was added, and the [^{3}H]QNB concentration range was 0.05 to 2 nM.

Atrial preparations. Mice were decapitated and their auricles were quickly removed and placed in a glass chamber containing a modified Krebs–Ringer bicarbonate (KRB) solution, gassed with 5% CO_2 in O_2 (pH 7.4) and maintained at 30°, as described [12]. A constant resting tension of 500 mg was applied and the activity of spontaneously beating atria was assessed, recording the maximum rate of isometric force development (dF/dt in g/sec) above externally applied resting tension. Tissues were allowed to stabilize for 60 min. Experimental data obtained with normal or alloimmune IgG were determined 20 min after each IgG addition and are expressed as a percentage of control values (3.6 ± 0.1 g/sec). For each experiment (either repeat or different treatment), a separate, not previously manipulated tissue was used.

Inhibition of PGE_1-induced cAMP formation by alloantibody. C3H mice auricles were incubated in the presence of 1 mM methyl-isobutyl-xanthine (MIX), 5×10^{-6} M propranolol in a final volume of 1 mL of KRB for 20 min. For kinetic analysis of alloantibody action, MIX was not used. Samples were left for 5 min alone or with 5×10^{-7} M alloimmune BALB/c anti C3H IgG and then for an additional 5 min with different concentrations of PGE_1. After incubation, tissues were homogenized in 2 mL of absolute ethanol (EtOH), and centrifuged at 6000 *g* for 10 min. Supernatants were collected and the pellets were rehomogenized with EtOH : H_2O (2 : 1) and centrifuged again as before. Supernatants were adjoined to the others and evaporated to dryness. Residues were resuspended in 50 mM Tris–HCl buffer (pH 7.4) containing 8 mM thoephylline, 0.45 mM EDTA and 6 mM 2-mercaptoethanol, and cAMP levels were determined by the competitive protein-binding assay described by Brown *et al.* [13] using [^{3}H]cAMP as tracer.

cGMP formation. Tissues were incubated alone or at different times with alloantibody or drugs in the presence or absence of 10^{-6} M propranolol as was indicated. After incubation, hearts were homogenized in 6% ice-cold trichloroacetic acid (TCA) and centrifuged at 2500 *g* for 15 min at 4°. The TCA supernatants were extracted with 4 mL of water-saturated ethyl ether three times. The aqueous phase was heated at 56° to remove the ether and evaporated to dryness under a stream of nitrogen gas. cGMP in the residue was dissolved in 400 μL of 0.05 M sodium acetate buffer, (pH 6.2). Aliquots of 100 μL were taken for the nucleotide determination using a radioimmunoassay (RIA) procedure with a cyclic GMP [^{125}I]RIA kit from Dupont/New England Nuclear.

Inositol phosphate (IP) measurement. C3H mice hearts were incubated for 120 min in 0.5 mL of KRB gassed with oxygen containing 5% CO_2 with 1 μCi [^{3}H]MI, and 10 mM LiCl was added for inositol monophosphate accumulation, according to Berridge *et al.* [14]. Alloantibody or agonist drugs were added 30 min before the end of the incubation periods except for kinetics studies where the times used are indicated in the Results. When blockers were used, they were added 30 min before the addition of alloantibody and drugs. In each experimental case a not previously manipulated atria was used. Water-soluble IPs were extracted and separated by ion exchange chromatography on Bio-Rad AG-X8 columns as described before [9].

RESULTS

Alloimmune IgG inhibition of [^{3}H]QNB binding to purified cardiac membranes. [^{3}H]QNB is a cholinergic muscarinic radioligand that binds to purified mice cardiac membranes in a saturable form with a K_d of 0.12 ± 0.01 nM and a B_{max} of 76.5 ± 9.4 fmol/mg protein. When purified C3H cardiac membranes were incubated for different times with 10^{-7} M alloimmune IgG, an inhibition of [^{3}H]QNB binding was obtained which reached its maximal effect at 30 min preincubation time (Fig. 1). When cardiac membranes were pretreated with different concentrations of alloimmune IgG and used to measure [^{3}H]QNB binding, a concentration-dependent inhibition was obtained (Fig. 2A) with a 50% maximal inhibition of radioligand binding. No inhibition was observed with normal IgG (Fig. 2A) or with C3H anti BALB/c IgG (data not shown).

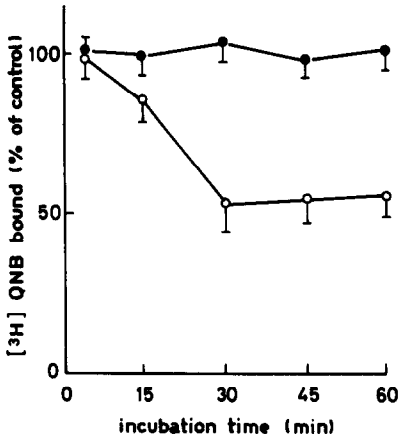

Fig. 1. [^{3}H]QNB binding to cardiac membranes pre-incubated for different times with alloimmune IgG. C3H cardiac membranes were incubated at 30° in the presence of 10^{-7} M alloimmune (○—○) or normal (●—●) IgG for different times, washed, and the used to determine the binding with 0.2 nM [^{3}H]QNB. Data are means ± SEM of four independent experiments, and are given as a percentage of control values: 43.5 ± 4.3 fmol/mg protein (membranes alone were kept at 30° for the same time as the IgG-treated membranes).

Similar results were obtained, as shown in Table 1, with monoclonal antibody (anti-H-2K^k) directed against class I K alloantigens, but not with monoclonal anti-I-E^k antibodies directed to class II products. This suggests that class I alloantigens specifically participate in hormone–receptor interactions. It is worth noting that the class I monoclonal antibodies do not cross-react with class II products and vice versa, and that both monoclonal antibodies had cytotoxic titers compatible with k haplotype from the C3H strain (Table 1).

Varying the radioligand concentration to perform the binding assay with membranes pretreated with 10^{-7} M IgG_N or IgG_I antibodies, saturation curves were obtained whose Scatchard plots are shown in Fig. 2B. Alloantibody significantly modified the K_d value (from 0.19 ± 0.01 nM with IgG_N to 0.64 ± 0.07 nM with IgG_I) without a significant change in the B_{max}. These data suggest that alloantibodies interact at an allosteric site on the muscarinic receptor. We also analyzed whether alloimmune IgG inhibition of [^{3}H]QNB binding is regulated by guanine nucleotides. As shown in Fig. 3B, the muscarinic cholinergic agonist carbachol inhibition of [^{3}H]QNB binding was affected by 1 mM GTP which shifted to the right the competition curve obtained in the presence of carbachol but not in the presence of the antagonist atropine. In Fig. 3A it can be seen that 1 mM GTP did not modify the interactions of alloimmune IgG with cholinergic receptors, showing that IgG inhibition of [^{3}H]QNB binding does not to be regulated by guanine nucleotides. As a control, the binding of an alloantibody directed against alloantigens not related to those expressed in the cardiac membrane is shown, stressing the fact that a specific immunologic recognition of cardiac HC Ag took place. As alloimmune IgG was able to interact with cardiac muscarinic cholinergic receptors, we investigated

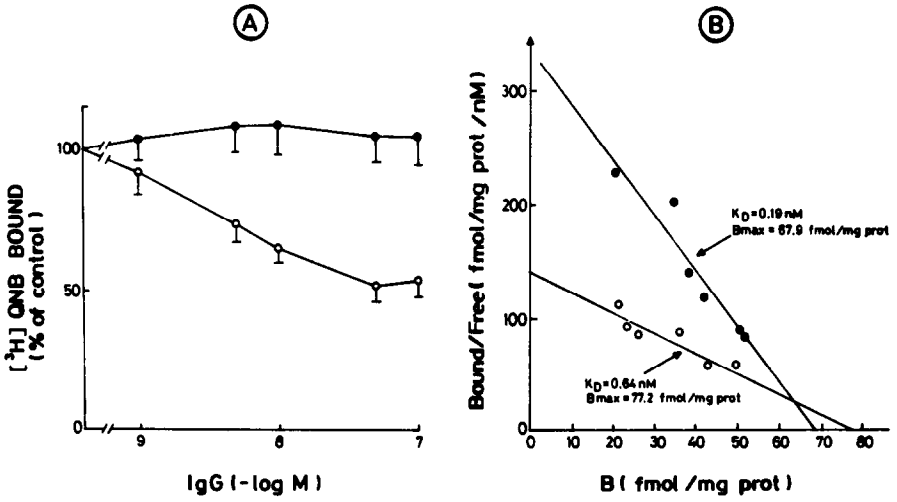

Fig. 2. Alloimmune IgG inhibition of [^{3}H]QNB binding to purified cardiac C3H membranes. (A) Competitive binding assay: C3H cardiac membranes were incubated for 30 min at 30° alone, with increasing concentrations of normal (IgG_N) (●—●) or with alloimmune BALB/c anti C3H (IgG_I) (○—○) IgG; they were then used to determine the binding of a single concentration of [^{3}H]QNB (0.2 nM). Means ± SEM of five experiments are plotted and are expressed as a percentage of control values (membranes alone: 49.3 ± 2.5 (fmol/mg protein). (B) Scatchard plots from saturation assays. Membranes were incubated with 10^{-7} M IgG_N (●—●) or IgG_I (○—○) and were then used to perform the binding assay with increasing amounts of [^{3}H]QNB as described in Materials and Methods.

Table 1. Effects of monoclonal anti-class I and anti-class II MHC products on [^{3}H]QNB binding to C3H cardiac membranes

Antibody treatment*	[^{3}H]QNB specific binding† (% of variation)	Cytotoxicity titers‡
IgG_N	+ 4.8 ± 0.3	10
IgG_I	− 49.0 ± 5.1§	320
Mo anti H-2K^k	− 29.0 ± 2.3§	40–80
Mo I - E7	− 0.1 ± 0.02	80
Anti-murine IgG	− 0.2 ± 0.01	160

* C3H cardiac membranes were incubated for 30 min at 30° either alone or with one of the following: 1×10^{-7} M normal IgG (IgG_N) or alloimmune BALB/c anti C3H IgG (IgG_I) or monoclonal anti-class I antibody (Mo anti H-2K^k) or monoclonal anti-class II antibody (Mo I - E7).

† After antibody treatment, membranes were assayed for 0.2 nM [^{3}H]QNB binding as described for competition studies in Materials and Methods. Values are expressed as a percentage of the control value obtained with membranes alone. Control value: 49.3 ± 2.5 (fmol/mg protein). Results are means ± SEM of four independent experiments for each treatment.

‡ Complement-mediated cytotoxicity of each reagent was measured by the two-stage trypan blue assay performed over C3H spleen cells as described before [10]. Titers are reported as the reciprocal of the highest 2-fold antibody dilution producing more than 50% of maximum specific lysis.

§ Differs significantly from normal IgG ($P < 0.01$).

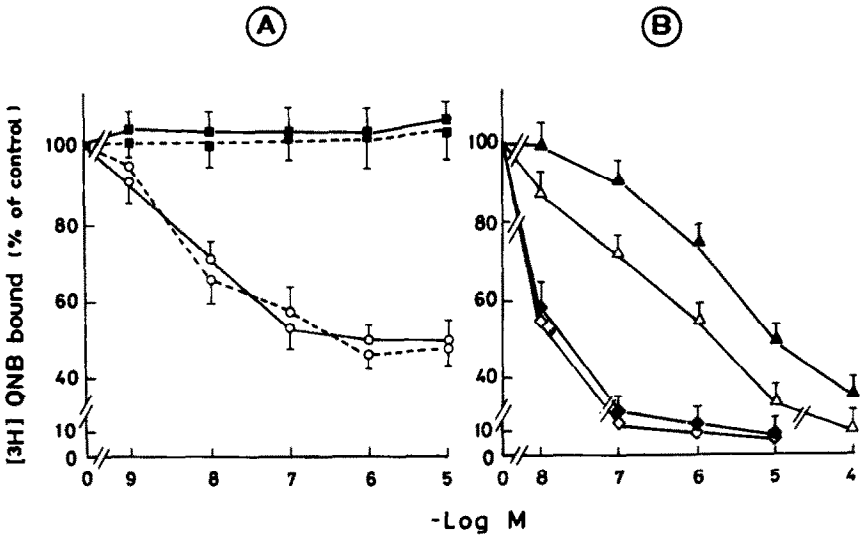

Fig. 3. Inhibition of [^{3}H]QNB binding to C3H cardiac membranes by increasing concentrations of (A) BALB/c anti C3H IgG ((○—○) and this alloimmune IgG plus GTP (○- -○), C3H anti-BALB/c (non-related) IgG (■—■), non-related IgG plus GTP (■- -■) and (B) carbachol (△—△), carbachol plus GTP (▲—▲), or atropine in the absence (◇—◇) or presence of GTP (◆—◆). GTP was used at 1 mM. Membranes were incubated with different concentrations of IgG, carbachol or atropine during 30 min at 30° prior to the binding assay. All assays were carried out simultaneously with membranes without any additions and data were compared to control binding (100%). Absolute values (fmol/mg protein) for the control were: membrane alone: 50.4 ± 2.7; and membranes in the presence of 1 mM GTP: 48.2 ± 2.5. Means ± SEM of five independent experiments in each group performed in triplicate are plotted.

which cholinergic-mediated effects were triggered by this interaction.

Changes in atrial contractility by alloimmune IgG. We have already demonstrated that alloimmune antibody is able to stimulate atrial contractility via β-adrenoceptors and that this effect is blunted by 10^{-7} M propranolol [7, 8, 11]. When higher doses of propranolol were used, not only the inhibition but also a decrease in atrial contractility was observed (Fig. 4). This effect obtained with 5×10^{-7} M

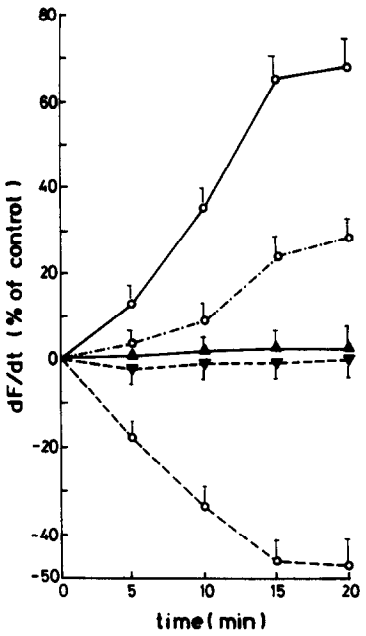

Fig. 4. Alloimmune IgG actions upon C3H mice atria contractility in the presence of the blocker propranolol. C3H auricles were suspended in a KRB bath as described and after equilibration the following concentrations of drugs were added: none (O—O), 5×10^{-8} M propranolol (O-·-O), 1×10^{-7} M propranolol (▲—▲), 5×10^{-7} M propranolol (O--O), and 5×10^{-7} M propranolol plus 1×10^{-6} atropine (▼--▼). After 30 min of incubation, 5×10^{-7} M alloimmune BALB/c anti C3H IgG was added and dF/dt values were recorded at the indicated times as described in Materials and Methods. The dF/dt control value was 3.6 ± 0.1 g/sec. Means ± SEM of five independent experiments are plotted.

propranolol was inhibited by 10^{-6} M atropine, showing that the inhibition of cardiac contractility could be due to HC Ag and muscarinic cholinergic receptor interactions.

Modification of cyclic nucleotides by alloantibody. We first analyzed whether alloantibody was able to inhibit the adenylate cyclase through its interaction with cardiac muscarinic cholinergic receptors. As we have demonstrated before [8] and as shown in Fig. 5A, alloantibody was able to induce an increase in cardiac intracellular cAMP concentration. This was due to the activation of β-adrenoceptors as it was inhibited by propranolol and mimicked by the β-agonist isoproterenol (Fig. 5A). Higher doses of propranolol (5×10^{-7} M), which lead to a decrease in cardiac contractility due to HC Ag–muscarinic cholinergic receptor interactions, did not diminish cAMP cardiac levels in the presence of alloantibody (Fig. 5A).

To assess the possible inhibition of adenylate cyclase by alloimmune IgG, we studied its effect on the PGE_1 concentration-increment of cAMP cardiac levels, in the presence of 5×10^{-7} M propranolol. It is well known that PGE_1 increases cAMP levels, activating the adenylate cyclase system [15]. Propranolol did not affect PGE_1 action upon cAMP levels (data not shown). As can be seen in Fig. 5B, alloantibody inhibited the PGE_1-dependent increase in cardiac cAMP levels, showing in this way its capacity to inhibit the adenylate cyclase system. It is worth noting that the alloimmune IgG inhibition of the PGE_1 increase in cAMP levels was blocked by atropine (10^{-6} M): PGE_1 (10^{-7} M) in the presence of propranolol (5×10^{-7} M) plus alloimmune IgG (5×10^{-7} M): 3.5 ± 0.3 pmol/mg wet weight; PGE_1 + propranolol + atropine + alloimmune IgG: 6.1 ± 0.5 pmol/mg wet weight. Neither propranolol nor atropine modified the stimulatory effect of PGE_1 (6.5 ± 0.5 pmol/mg wet weight).

Next we studied alloantibody action over cardiac cGMP intracellular levels. In the absence of propranolol, a decrease in cGMP levels was observed that correlated in time with the peak increment of cAMP (Fig. 6A). Furthermore, a similar decrease was obtained with the β-agonist isoproterenol at the concentration that mimicks the alloantibody effect upon cAMP levels. When using 5×10^{-7} M propranolol, alloantibody gave a slight non-significant increase of cGMP levels in contrast with the increment obtained with the cholinergic agonist carbachol. This slight effect of alloimmune IgG on cGMP was not modified by atropine. As soluble guanylate cyclase could be activated via the release of arachidonic acid (AA) that takes place during a G protein-mediated activation of phospholipase C (PLC) with subsequent hydrolysis of PI [16], we extended the kinetic study until 1 hr of incubation in the presence and absence of 5×10^{-7} M propranolol. In both cases, cGMP values were similar to basal values (data not shown). We also analyzed the effect of a PLC blocker (NCDC) upon the slight increase in cGMP triggered by alloimmune IgG, but no differences were observed (Fig. 6B). These data pointed to the fact that alloantibody interactions with cardiac muscarinic cholinergic receptor did not induce the direct activation of guanylate cyclase of myocardium, or indirectly through a PLC dependent-PI turnover activation with subsequent release of AA metabolites.

Increase in PI turnover mediated by cholinergic muscarinic activation of PLC. For studying alloantibody actions on the production of PLC-induced intracellular second messengers in cardiac preparations. C3H auricles were first incubated with ([^{3}H]MI) precursors in the presence of 10 mM LiCl and the water-soluble radiolabeled IP formed in the presence or absence of BALB/c anti C3H IgG. In Fig. 7 the amounts of IPs induced in C3H auricles by alloimmune IgG are shown. Maximum stimulation (98%) (Fig. 7A) was obtained between 30 and 45 min of incubation and normal IgG had no effect upon PI hydrolysis. At longer incubation times (> 60 min), stimulations varied between 70 and 85%. All the experiments were performed using a 30-min incubation time. The effect of 5×10^{-7} M alloantibody was also compared to that of 5×10^{-6} M carbachol on PI turnover in cardiac tissue (Fig. 7B). It can be observed that alloantibody induced an increase in IP formation similar to that observed with the muscarinic cholinergic agonist. This increase was abolished by pretreating auricles with 10^{-6} M atropine or with 5×10^{-6} M PLC-inhibitor NCDC,

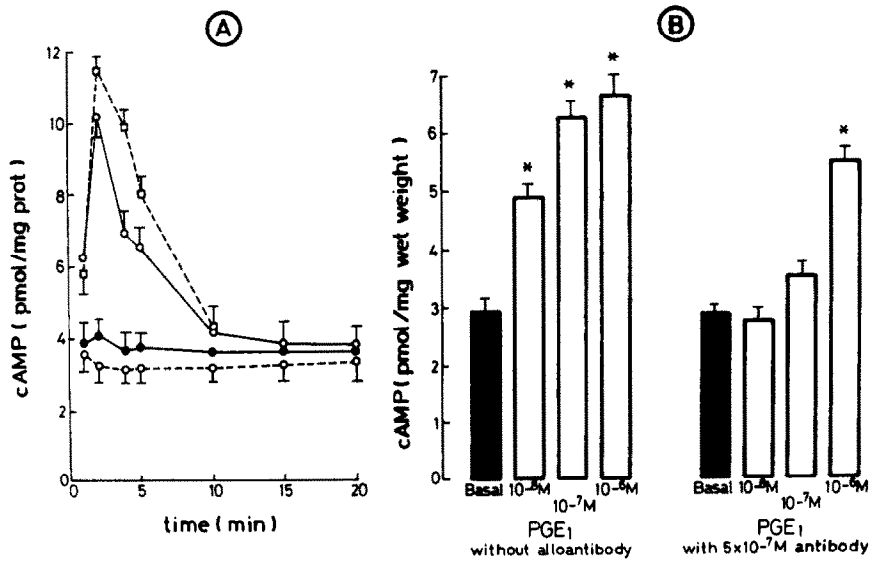

Fig. 5. Modifications of cardiac cAMP levels by alloimmune IgG. (A) C3H auricles were incubated in KRB alone (control), in the presence of 5×10^{-7} M normal IgG (●—●), 5×10^{-7} M alloantibody (○—○) or 5×10^{-8} M isoproterenol (□ - - □) for the times indicated in the figure. C3H auricles were also pretreated with 5×10^{-7} M propranolol and then exposed to alloantibody (○ - - ○). Tissues were then homogenized and cAMP contents were assayed as indicated in Materials and Methods. The basal cAMP value for control was: 4.1 ± 0.3 pmol/mg protein. Results are means ± SEM of four independent experiments. (B) C3H auricles were incubated in KRB plus propranolol (5×10^{-7} M) alone or in the presence of 5×10^{-7} M IgG and then exposed to increasing concentrations of PGE_1. Data are means ± SEM of four experiments. An asterisk (*) indicates a significant difference from basal value ($P < 0.05$).

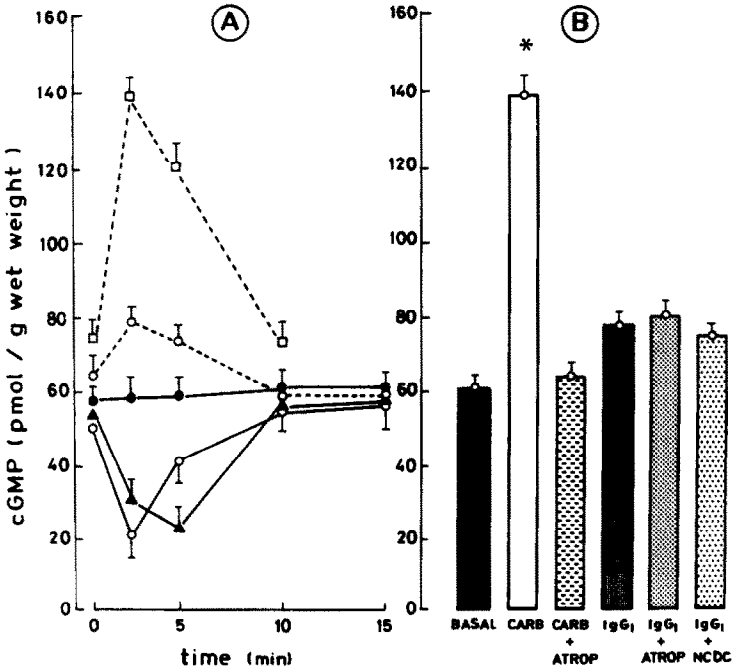

Fig. 6. Effect of alloimmune IgG on cGMP levels: Comparison with cholinergic muscarinic and β-adrenergic agonists. (A) C3H auricles were incubated in KRB alone, with 5×10^{-7} M normal IgG (●—●) and 5×10^{-7} M alloimmune BALB/c anti C3H IgG (○—○), 10^{-6} M carbachol (□ - - □) or 5×10^{-8} M isoproterenol (▲—▲) for the times indicated in the figure. C3H auricles were also pretreated with 5×10^{-7} M propranolol and then exposed to alloantibody (○ - - ○). (B) cGMP levels were determined on auricles preincubated for 3 min at 37° with 10^{-6} M carbachol (CARB), 10^{-6} M carbachol + 10^{-6} M atropine (CARB + ATROP), 5×10^{-7} M alloimmune IgG (IgG_I), 5×10^{-7} M alloimmune IgG plus 10^{-6} M atropine (IgG_I + ATROP) or 5×10^{-7} M IgG_I plus 5×10^{-6} M NCDC. (IgG_I + NCDC). Tissues were then homogenized as indicated and cGMP values were determined by RIA. The control cGMP value was: 56.0 ± 4.0 pmol/g wet weight. Results are means ± SEM of five independent experiments. An asterisk (*) indicates a significant difference from basal value ($P < 0.05$).

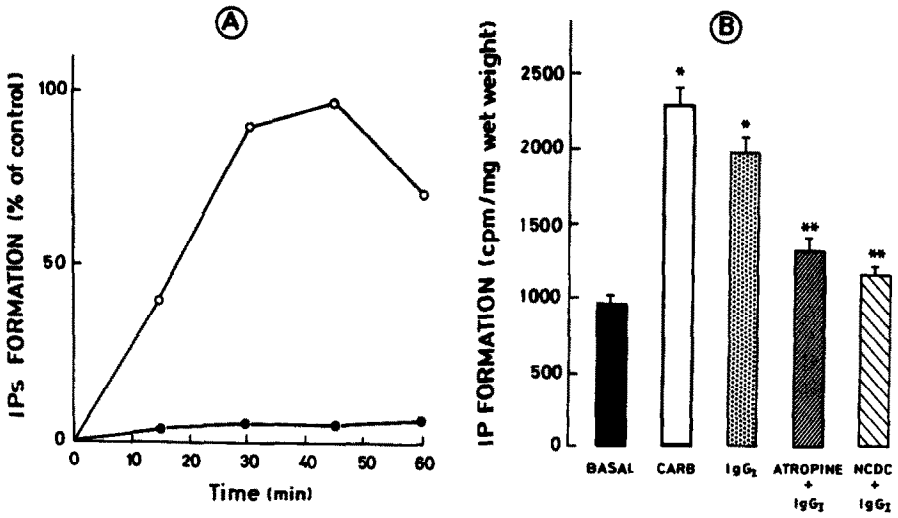

Fig. 7. Alloantibody increased IP formation in C3H atria (A) Auricles were labeled with the [^{3}H]MI precursor for 60 min, washed and left alone (control) or treated with 5×10^{-7} M normal IgG (●—●) or BALB/c anti C3H IgG (○—○) for different times. Formation of IPs was analyzed as described in Materials and Methods. Results are expressed as a percentage of radioactivity with respect to control values. (B) Auricles were incubated for 60 min with [^{3}H]MI precursor and for an additional 30 min in the absence or in the presence of the following blockers: 10^{-6} M atropine or 5×10^{-6} M NCDC. Tissues were then left for 30 min more alone (basal) or in the presence of 5×10^{-7} M alloimmune IgG or 5×10^{-6} M carbachol. Values represent the amount of radioactivity per mg of wet weight of atria. Each value is the mean ± SEM of three independent experiments. Key: (*) $P < 0.001$ versus control and (**) $P < 0.001$ versus alloimmune IgG alone by one-way analysis of variance and the Dunnett test.

but were not modified in the presence of 5×10^{-7} M propranolol (data not shown). These data pointed to an increase in PI turnover due to HC Ag-muscarinic cholinergic receptor interactions in atria.

DISCUSSION

Hormone receptor and class I HG Ag interactions have been described [17, 18], but their physiological significance is still unknown. We have demonstrated those sorts of interactions for cardiac β_1-adrenoceptors [7, 10, 11], and smooth muscle [7] and splenocyte [19] β_2-adrenoceptors with the induction of cAMP as an intracellular signal of receptor activation. We have also observed that alloantibody triggers other second messengers as a consequence of receptor-mediated increased PI turnover [9]. Therefore our purpose was to characterize this last interaction in which muscarinic cholinergic receptors participate. To accomplish this objective, we first analyzed the actions of alloantibody on specific muscarinic cholinergic receptor radioligand [^{3}H]-QNB binding to cardiac membrane. We observed a concentration-dependent inhibition of [^{3}H]QNB binding to purified cardiac membrane of mice carrying the appropriate H-2 haplotype. Moreover, the involvement of class I but not class II antigens in the phenomenon was demonstrated specifically by using monoclonal alloantibodies. Furthermore, when analyzing the effect of guanine nucleotides in alloantibody-mediated inhibition of [^{3}H]QNB binding to cardiac membrane, we observed that GTP (1 mM) did not modify the antibody effect, although it shifted to the right the competition curve of the full agonist carbachol. It was reported that little regulation by guanine nucleotide occurred for partial agonist binding and that the difference between full and partial agonists would be that a high affinity GTP-sensitive state is formed in the presence of full agonists but not with partial agonists [20]; this effect also occurred with the antagonists. As alloantibody was able to trigger cholinergic muscarinic receptor activation, we cannot consider its behavior to be similar to that of cholinergic antagonists, but rather it behaves as an allosteric inhibitor of radioligand binding. It can be suggested that while interacting with HC Ag the alloantibody induces a modification in the receptor affinity as was also demonstrated for cardiac [11] and splenocyte [19] β-adrenoceptors.

Our interest was then focused on the atrial muscarinic cholinergic receptor-mediated responses that could be triggered during this sort of interaction. For this, we have studied its effects on the described muscarinic cholinergic related biochemical events, namely: (a) decrease in atrial contractility, which reflects changes in membrane ion permeability via a G_k regulatory protein; (b) cAMP formation, which is diminished by a G_i coupled inhibition of the adenylate cyclase; (c) cGMP production, through a direct activation of guanylate cyclase; and (d) activation of PLC production of IP_3 and DAG second messengers via a G_p regulatory protein.

Thus, we analyzed atrial contractility in the presence of increasing concentrations of the β-adrenergic antagonist propranolol, as we have previously demonstrated that alloimmune IgG is able to stimulate cardiac contractility through its interactions with cardiac β_1-adrenoceptors [8]. When 5×10^{-7} M propranolol was used, a cholinergic mediated decrease of contractility was obtained which was blunted by 10^{-6} M atropine. These data support previous affirmations [17, 18] pointing to the pluripotenciality of HC Ag to interact with different hormone receptors. It is worth noting that the "target" receptor(s) in each model is (are) the most important in modulating the physiology of the corresponding tissue, so this points to the biological relevance of this type of interaction.

With respect to cAMP formation, we were unable to demonstrate the inhibition of adenylate cyclase by means of a decrease in basal cardiac cAMP levels, even in the presence of 5×10^{-7} M propranolol. These data are in agreement with those found in mice atria and in cardiomyocytes where the cholinergic agonists do not alter cAMP levels but inhibit the increase in cAMP formation induced by the β-agonist isoproterenol [21, 22]. As we have to work in the presence of 5×10^{-7} M propranolol to avoid the β-adrenoceptor-mediated stimulation of cAMP formation, we used PGE_1 stimulation of adenylate cyclase to analyze alloantibody muscarinic cholinergic receptor interaction-mediated inhibition of the cyclase. As shown in the Results, alloantibody shifted to the right of PGE_1 concentration-dependent increase of cAMP levels, and this inhibition was blocked by the muscarinic cholinergic antagonist atropine, pointing to an inhibition of the adenylate cyclase.

Analyzing the production of cGMP, we observed that even though we studied cAMP formation in the presence of propranolol, we only found a slight non-significant increase in cGMP levels when compared with those induced by the cholinergic agonist carbachol. This slight increase was not abrogated by pretreating the cardiac tissue with atropine. Therefore, alloantibody does not seem to induce the direct activation of guanylate cyclase.

With respect to the activation of PLC, we have demonstrated before [9] and confirm here that alloantibody is able to increase the turnover of phosphoinositides in murine atria by activating PLC (as its actions are blocked by the PLC inhibitor NCDC), through its interaction with muscarinic cholinergic receptors, as indicated by the inhibition of the alloantibody effect by atropine. The possible indirect activation of cardiac guanylate cyclase through a G protein-mediated activation of PLC with subsequent hydrolysis of phosphoinositides and release of AA metabolites, which then activate guanylate cyclase, seems improbable for several reasons: (1) in the kinetic studies we have seen the effect of alloantibody upon PI turnover at least after 10 min; (2) we have also analyzed the effect of alloimmune IgG upon cardiac cGMP levels (both in the presence and absence of propranolol) for 1 hr; no differences from basal values were obtained; and (3) the slight increase in cGMP after a 3-min exposure of cardiac preparations to alloimmune IgG was not abrogated by NCDC.

With the exception of the direct activation of the guanylate cyclase, all the other biochemical events that take place during muscarinic cholinergic receptor activation are mediated by GTP-binding regulatory proteins. The data presented here point out that HC Ag–hormone receptor interactions trigger all the cholinergic functions related with G proteins. We previously proved indirectly the participation of G proteins in the phenomenon [18]. We have proposed that hormone-receptor and HC Ag interactions are through or affect these types of regulatory proteins. This would explain the extensive capacity of alloantibody to interact with such different hormone-receptors (i.e. β-adrenergic, muscarinic cholinergic, LH/hCG, and insulin receptors). In addition, all the hormone receptors that have been described to interact with HC Ag are coupled to G protein-mediated biochemical events to exert their biological actions. It is probably in this HC Ag–hormone receptor–G protein interaction that we can find the answer for its physiological significance.

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