J. Biochem. 120, 193-200 (1996)

Formation of the Functional Complexes of m2 Muscarinic Acetylcholine Receptors with GTP-Binding Regulatory Proteins in Solution[1]

Ago Rinken[2]

Department of Medical and Physiological Chemistry, University of Uppsala, BMC, Box 575, S-751 23 Uppsala, Sweden

Received for publication, March 11, 1996

Sixteen different detergents were studied for solubilization of functional complexes between m2 muscarinic acetylcholine receptors (mAChR) and guanine nucleotide-binding regulatory proteins (G proteins). More than 40% of solubilized mAChR retained their GTP-dependent high affinity for agonist binding after solubilization with sucrose monolaurate, whereas all other detergents studied gave considerably lower solubilization yields or caused the loss of the high affinity for agonist binding. The preformation of mAChR-G protein complexes in membranes revealed that a large excess of G proteins did not increase the portion of high-affinity binding sites, but caused GTP- and Mg^{2+}-dependent inhibition of the binding of radioactive antagonists to mAChR. The optimization of detergent concentration and other experimental conditions revealed that up to 47% of the solubilized receptors indicated the GTP-dependent high affinity for agonist binding after mixing solubilized mAChR with purified G proteins in sucrose monolaurate in the presence of Mg^{2+} and carbachol. These results give the first clear proof of the formation of functional complexes between mAChR and G proteins in solution and indicate that GTP-dependent high-affinity agonist binding is connected to the direct interactions between mAChR and G proteins and that other membrane components are not necessary.

Key words: detergents, G proteins, muscarinic receptors, receptor-G protein complex, solubilization.

The muscarinic acetylcholine receptors (mAChR) (m1-m5) are members of the family of receptors that are coupled to guanine nucleotide binding regulatory proteins (G proteins) (*1-3*). The interactions between receptors and G proteins can be detected by numerous methods, including GTP-sensitive high-affinity agonist binding, agonist-stimulated GTPase activity, agonist-dependent enhancement of GTPγS binding, GDP dissociation, and ADP ribosylation by toxins as well as by several G protein-activated events (activation/inhibition of adenylate cyclases and phospholipases C and D, ion channels, *etc.*) (*4-6*). Previous studies have indicated that m2 mAChR can interact with different subtypes of G proteins (*7-10*), whereas for effective coupling the presence of Mg^{2+} is necessary (*11*). Mutation analyses have revealed the critical amino acids and regions in the structure of receptor proteins for the coupling with G proteins, but the mechanism of signal transduction from receptor to effector is still not known (*12-14*). Solubilization of receptors from membranes minimizes the influence of lipid environment and facilitates the investigation of their general properties *per se*, including interactions with G proteins. mAChR are usually solubilized in digitonin or in a mixture of digitonin and sodium cholate (*15-17*), but several other detergents have also been successfully used [for review (*18*)]. At the same time, the data on solubilization of mAChR-G protein complexes are inconsistent: in many cases, agonist binding to solubilized mAChR is reported not to be affected by guanine nucleotides and to correspond to low-affinity binding sites (*19-25*), whereas in other cases the influence of GTP on the agonist binding and receptor mobility in sucrose density gradient centrifugation is described (*25-29*).

This report presents data on the formation of mAChR-G protein complexes in solution when these solubilized proteins are mixed. The optimal experimental conditions were found by screening 16 different detergents at different concentrations for solubilization of functional complexes between m2 mAChR and G proteins from different membranes.

[1] This work was supported by research grants from the Swedish Medical Research Council (Project 13X-50) and the Swedish Society for Medical Research and a Grant-in-Aid for Developmental Scientific Research from the Ministry of Education, Culture, Sports and Science of Japan to T. Haga (Project 01870003). Part of the work has been carried out at the Department of Biochemistry, Institute for Brain Research, Faculty of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113.

[2] Visiting Scientist from the Institute of Chemical Physics, Tartu University, Jakobi 2, EE-2400 Tartu, Estonia.

Abbreviations: mAChR, muscarinic acetylcholine receptor; G protein, guanine nucleotide-binding regulatory protein; [^{3}H]QNB, L-[^{3}H]-quinuclidinyl benzilate; [^{3}H]NMS, *N*-[^{3}H]methylscopolamine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; Deoxy-BIGCHAP, *N,N*-bis-(3-D-gluconamidopropyl)deoxycholamide; KPB, K-phosphate buffer.

MATERIALS AND METHODS

Materials—L-[^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB, 44 Ci/mmol) and *N*-[^{3}H]methylscopolamine ([^{3}H]NMS, 79.5 Ci/mmol) were obtained from New England Nuclear (Boston, MA, USA); 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), *N,N*-bis-(3-D-gluconamidopropyl)deoxycholamide (Deoxy-BIGCHAP), octanoyl-*N*-methylglucamide (O-MEG), decanoyl-*N*-methylglucamide (D-MEG), η-octyl-β-D-glucopyranoside (OGP), η-heptyl-β-D-thioglucopyranoside (HTGP), η-octyl-β-D-thioglucopyranoside (OTGP), 6-*O*-(*N*-heptylcarbamoyl)methyl-α-D-glucopyranoside (HECAMEG) from Kurita Water Industries (Tokyo); digitonin, cholic acid, carbachol, and atropine from Sigma Chemical (St. Louis, MO, USA). Catalase (EC 1.11.1.6, bovine liver) was from Worthington Biochemical (Freehold, NJ, USA), lactate hydrogenase (EC 1.1.1.28, porcine heart) from Boehringer Mannheim (Mannheim, Germany), malate dehydrogenase (EC 1.1.1.37, porcine heart) from Oriental Yeast (Osaka), and cytochrome *c* (horse heart) from Wako (Tokyo). Sucrose monocaprate (SM-1000), sucrose monocaproleate (SM-1080), and sucrose monolaurate (SM-1200) were kindly donated by Mr. Hajime Machida (Mitsubishi-Kasei Food, Japan).

Insertion of G Proteins into Membranes with mAChR—Porcine atrial membranes were prepared and purified as described by Haga *et al.* (*30*) and stored at −80°C. The Sf9 cells expressing m2 mAChR were grown and membranes prepared as described (*31, 32*) by Dr. K. Kameyama at the Department of Biochemistry, Institute for Brain Research, Faculty of Medicine, The University of Tokyo (Tokyo) and were kindly donated for the present studies. The m2 mAChR were purified from porcine atrial membranes by single-step affinity chromatography in digitonin as described (*30*) or in sucrose monolaurate as described (*18*). The specific [^{3}H]-QNB binding activity of the purified preparation was about 1.5 nmol/mg protein for digitonin-solubilized mAChR and 0.4 nmol/mg protein for sucrose monolaurate-solubilized mAChR. The mixture of G_i and G_o was solubilized from porcine brain with 1% sodium cholate and purified chromatographically using DEAE-Sephacel, Ultrogel AcA 34, and Heptylamine Sepharose columns, as described (*33*) with slight modifications (*34*). The concentration of G proteins was determined by the specific binding of [^{35}S]-GTPγS as described in Ref. *35*. The insertion of G proteins into atrial and Sf9 cell membranes was essentially as described (*36*): membranes (2-5 pmol [^{3}H]QNB binding sites in 1.8 ml) were incubated with purified G proteins (50-500 pmol) in a solution containing a final concentration of 0.4% sodium cholate, 5 mM $MgCl_2$, 20 mM potassium phosphate buffer (pH 7.0, KPB), and 5 mM imidazole in a total volume of 2 ml for 60 min at 4°C, then the mixture was slowly diluted with 7.5 ml of the KPB solution. Purified receptors were reconstituted with G proteins into lipid vesicles essentially as described for cerebral receptors (*34*).

Solubilization of mAChR and Their Complexes with G Proteins—For solubilization of mAChR, the thawed atrial membranes were diluted in 20 mM KPB (pH 7.5), 5 mM imidazole, and 1 mM EDTA to a protein concentration of 0.7-0.8 mg/ml and incubated with different concentrations of detergents for 30 min at 4°C. The supernatant fraction obtained by centrifugation at 100,000 × *g* for 90 min at 4°C was used as the solubilized fraction. The amount of solubilized mAChR was determined by specific binding of [^{3}H]-QNB (3.2 nM, 90 min incubation at 30°C), and solubilization yield was expressed as a percentage of membrane-bound mAChR, which was assessed under the same incubation conditions.

For solubilization of mAChR-G protein complexes, the suspension of atrial membranes, enriched with purified G proteins (1 pmol [^{3}H]QNB binding sites/ml and 0.7 mg protein/ml, 20 pmol [^{35}S]GTPγS binding sites/ml), was preincubated with 20 μM carbachol and 5 mM $MgCl_2$ for 60 min at 4°C before the treatment with detergents. The amount of functionally active mAChR-G protein complexes was determined by the difference of [^{3}H]QNB binding (1.2 nM, 90 min at 30°C) in the presence of 0.1 mM GTP and in the absence of GTP and was expressed as a percentage of mAChR solubilized under the same conditions.

Formation and Determination of Interactions of G Proteins with mAChR in Solution—The solubilized porcine atrial mAChR in 0.1% sucrose monolaurate (2 pmol of [^{3}H]QNB binding sites, 0.6 mg protein/ml) were incubated with purified G proteins (200 pmol) in a solution containing a final concentration of 0.06% of sucrose monolaurate, 0.01% sodium cholate, 20 mM KPB, 3 mM $MgCl_2$, and 10 μM carbachol for 60 min at 4°C. Aliquots of the mixture were incubated with 1.3 nM [^{3}H]QNB and different concentrations of carbachol in the presence of 0.1 mM GTP or in the absence of GTP for 90 min at 30°C or for 24 h at 4°C. After incubation the specifically bound [^{3}H]QNB was measured as described (*30*).

Sucrose Density Gradient Centrifugation of Solubilized mAChR and Its Complexes with G Proteins—Sucrose density gradient centrifugations were carried out using linear gradients of sucrose (5-20%) in 20 mM KPB, 0.1% sucrose monolaurate, and in the presence or absence of 10 μM carbachol, 3 mM $MgCl_2$, 0.1 mM GTP, as described (*18*). Samples of 0.3 ml were applied to 4.8-ml gradients and centrifuged at 180,000 × *g* for 18 h at 4°C. The [^{3}H]-QNB binding activity was measured for each collected fraction (20 × 0.25 ml). Catalase, lactate hydrogenase, malate dehydrogenase, and cytochrome *c* were used as internal standards and determined according to the protocol in Ref. *37*.

Ligand Binding to mAChR—The crude membrane homogenates, reconstituted vesicles or solubilized preparations were incubated with [^{3}H]NMS (2.1 nM) or [^{3}H]QNB (1.1-1.5 nM) and other ligands and components as indicated, for 90 min at 30°C or for 24 h at 4°C. Free ligands were removed by fast filtration through glass-fibre filter (GF/B, Whatman International, Madistone, UK) or by gel chromatography on Sephadex G-50 (fine, Pharmacia Fine Chemicals, Uppsala, Sweden) in the case of membrane-bound and solubilized mAChR, respectively, as described (*18, 30*). The specific binding was defined as the difference between total and nonspecific binding, measured in the absence of atropine and in the presence of 1 μM atropine, respectively. Protein concentrations were determined by the modified Lowry method (*38*) using bovine serum albumin as standard.

Data Analysis and Statistics—All binding data were

analyzed by nonlinear least-squares regression analysis using the commercial program GraphPad PRISM™ (GraphPad Software, San Diego, CA, USA). Results are presented as average mean±SE. The selection between a one- and two-site model was made according to the results of the F test. The K_I(K_H, K_L) values were calculated according to the equation of Cheng-Prusoff (*39*): $K_I = IC_{50}/(1+[L]/K_d)$, where [L] is the concentration of radioligand and K_d is the radioligand dissociation constant.

RESULTS

Interaction of G Proteins with m2 mAChR in Membranes—Purified atrial membranes and Sf9 cell membranes were used as sources of the m2 mAChR, and the interactions between mAChR and G proteins were studied in these membranes and in the artificial vesicles with the purified receptors. Figure 1 shows the displacement of [^{3}H]NMS binding to Sf9 cell membranes by carbachol. The curves of membranes without additional G proteins had $K_I = 12 \pm 3$ μM with Hill coefficient $n_H = 0.76 \pm 0.08$. Fitting these data to the model corresponding to two independent binding sites gave $K_H = 4.2 \pm 0.9$ μM and $K_L = 100 \pm 16$ μM, with the proportion of high-affinity sites $\alpha_H = 0.38 \pm 0.09$. Addition of 0.1 mM GTP had no significant influence on these parameters ($K_H = 3.3 \pm 0.7$ μM, $K_L = 78 \pm 14$ μM, $\alpha_H = 0.41 \pm 0.08$). Incubation of these membranes with G proteins (a mixture of G_i and G_o) in the presence of 0.4% sodium cholate initiated GTP-sensitive high-affinity binding of carbachol to mAChR. In the absence of GTP, $K_I = 1.4$ μM, which corresponded to 59% of high-affinity binding sites with $K_H = 0.11 \pm 0.03$ μM and 41% of low-affinity sites with $K_L = 8 \pm 3$ μM according to the two-site binding model. Addition of 0.1 mM GTP caused the shift of the displacement curve to the right ($K_I = 19 \pm 4$ μM), toward the curves without G proteins (Fig. 1), and the Hill coefficient remained significantly below unity ($n_H = 0.74 \pm 0.04$). Thus, the addition of G proteins increased the apparent carbachol affinity more than 10-fold, turning approximately 50% of mAChR to the high-affinity state, and this effect could be reversed by the addition of GTP. Essentially similar results were obtained with atrial membranes and with the lipid vesicles with purified receptors (data not shown). The atrial membranes had internally 10–15% of the mAChR in the GTP-dependent high-affinity state, but the insertion of G proteins increased this proportion to 65%. In the case of purified and reconstituted mAChR, the Hill coefficient of carbachol displacement curves in the absence of G proteins and also in the presence of GTP was not distinguishable from unity and fitted the equation corresponding to the one-binding-site model. Thus, in all studied preparations the insertion of G proteins initiated GTP-dependent high-affinity binding of carbachol to mAChR, which was thereafter interpreted as an indicator of the mAChR-G protein complexes.

To achieve a maximal level of mAChR-G protein complexes, the ratio of mAChR to G proteins was kept at 1 to 100 in the atrial and Sf9 cell membranes, but at 1 to 5 in the purified system. At lower concentrations of G proteins, the increase in G protein concentration increased the high-affinity portion of agonist binding to mAChR (data not shown), as has previously been described for atrial and Sf9 cell membranes and reconstituted vesicles (*7, 8, 34*). An increase of the G protein/receptor ratio over 100 in membrane preparations (data not shown) and 5 in vesicles with purified mAChR (Fig. 2) caused a decrease in apparent number of [^{3}H]QNB binding sites without effect on the [^{3}H]QNB binding affinity and without additional increase in affinity for carbachol. Addition of GTP restored the total [^{3}H]QNB binding and shifted the carbachol binding to the

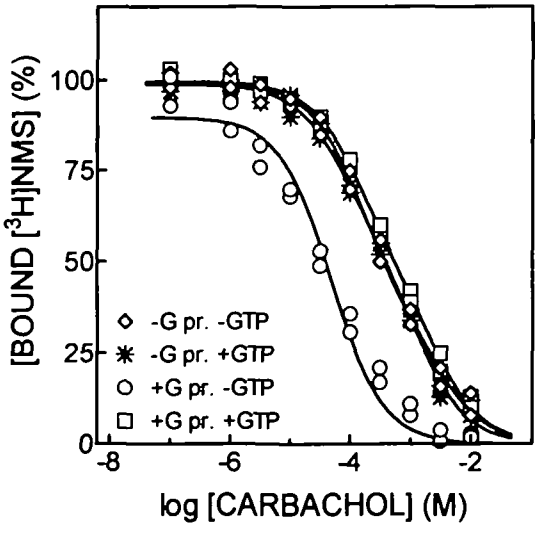

Fig. 1. **Effect of GTP and insertion of G proteins on the inhibition of [^{3}H]NMS binding by carbachol to m2 mAChR in Sf9 cell membranes.** Preparations of with (○, □) or without (◇, ∗) additional G proteins were incubated for 60 min at 30°C with different concentrations of carbachol and 2.1 nM [^{3}H]NMS in the presence (∗, □) or absence (◇, ○) of 0.1 mM GTP. Binding of [^{3}H]NMS is presented as the percentage of specific binding in the absence of carbachol.

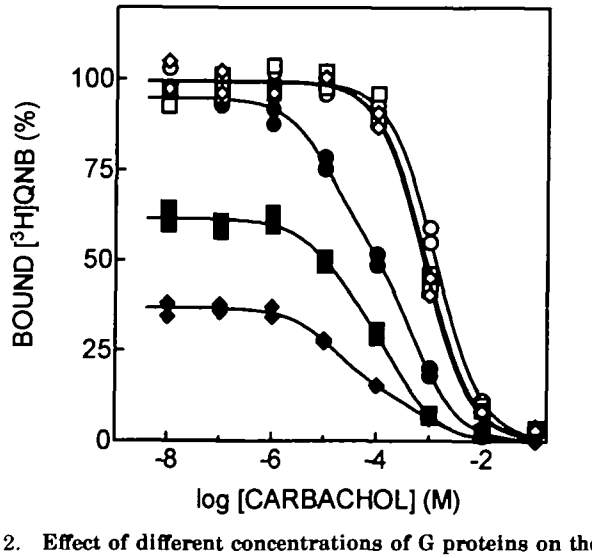

Fig. 2. **Effect of different concentrations of G proteins on the inhibition of [^{3}H]QNB binding by carbachol to purified atrial mAChR in artificial vesicles.** mAChR were reconstituted into the vesicles with 0.5 nM (●, ○), 1.7 nM (■, □), and 9.1 nM (◆, ◇) G proteins and incubated with different concentrations of carbachol and 1.1 nM [^{3}H]QNB in presence (○, □, ◇) or absence (●, ■, ◆) of 0.1 mM GTP for 60 min at 30°C. Binding of [^{3}H]QNB is presented as the percentage of specific binding in the presence of GTP and absence of carbachol and was 0.08 nM in these assays.

corresponding low-affinity binding site (Fig. 2). The decrease of [^{3}H]QNB binding sites by G proteins depended directly on the G protein concentration. The maximal inhibition was achieved at 10 nM G proteins for 80 pM [^{3}H]QNB binding sites of purified mAChR in vesicles, which corresponds to a G protein/mAChR ratio of 125 (Fig. 3). An additional increase in G protein concentration had only a slight influence on the [^{3}H]QNB binding. In the porcine atrial membranes, the G protein/mAChR ratio of 100 had no significant influence on the number of [^{3}H]QNB binding sites, whereas two- and threefold higher G protein concentrations (20 and 30 nM) caused a GTP-dependent reduction of [^{3}H]QNB binding sites by 15 ± 3 and 28 ± 4%, respectively. The generation of high-affinity binding of carbachol, but also the inhibition of [^{3}H]QNB binding, by G proteins required the presence of at least 1 mM free Mg^{2+}. In all cases studied the removal of Mg^{2+} by EDTA had a similar effect to addition of GTP, which restored the normal number of [^{3}H]QNB binding sites and shifted the carbachol displacement curves to the right, corresponding to the low-affinity state (data not shown).

In these and subsequent experiments, [^{3}H]QNB was used for determination of the amount of mAChR and carbachol affinity, as hydrophilic properties and fast dissociation of [^{3}H]NMS may lead to an underestimation of the number of mAChR. At the same time, it is important to note that binding of [^{3}H]QNB is partially irreversible (*40, 41*) and therefore the calculated constants indicate only the apparent potency of carbachol to inhibit [^{3}H]QNB binding.

Solubilization of the mAChR-G Protein Complexes—Sixteen different detergents, listed in Table I, were examined for solubilization of mAChR and mAChR-G protein complexes from atrial membranes. The best results were obtained with digitonin, sucrose monolaurate and the mixture of digitonin with sodium cholate (1%/0.08%), which solubilized 53-62% of mAChR with retention of the ligand-binding activity (Table I). CHAPS, CHAPSO, and Deoxy-BIGCHAP solubilized 8-13% of mAChR, but no ligand-binding activity was recovered in the supernatant with the other detergents studied (Table I). Similar results have been obtained for the m2 mAChR in Sf9 cell mem-

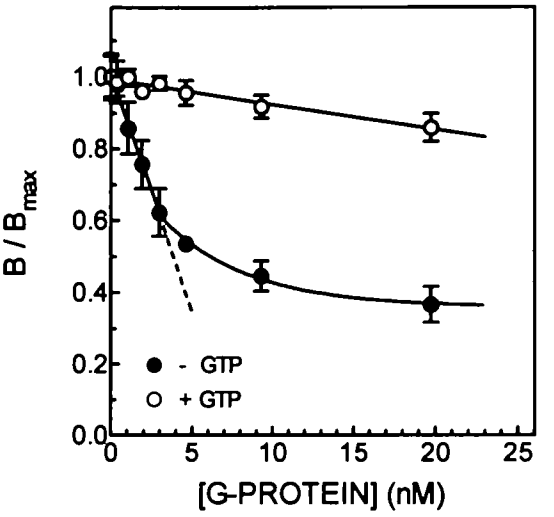

Fig. 3. **Effect of G protein concentration on the GTP-dependent [^{3}H]QNB binding to purified atrial mAChR in artificial vesicles.** mAChR were reconstituted into the vesicles in presence of different concentrations of G proteins and incubated in 20 mM K-Hepes (pH 8.0), 1 mM EDTA, 160 mM NaCl, 4 mM $MgCl_2$ with 2.5 nM [^{3}H]-QNB in the presence (○) or absence (●) of 0.1 mM GTP for 60 min at 30°C. Binding of [^{3}H]QNB is presented as the percentage of specific binding in the absence of G proteins (0.08-0.11 nM). Data are presented as mean ± SE of three independent experiments carried out in duplicate.

TABLE I. **Solubilization of mAChR and their complexes with G proteins from porcine atrial membranes.**

Detergent		Studied concentrations of detergent (%)	Maximal yield of mAChR[a] (%)	GTP dependence[b] (%)
Abbreviation	Chemical name			
Digitonin	Digitonin[c]	0.1-2.0	43	10
Na-cholate	3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid sodium salt	0.01-0.8	0	–
Na-deoxycholate	3α,12α-Dihydroxy-5β-cholan-24-oic acid sodium salt	0.01-0.8	0	–
Digitonin/cholate	Mixture of digitonin and Na-cholate	0.5-2.0/0.01-0.2	62	5
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate	0.1-2.0	8	ND
CHAPSO	3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate	0.1-1.5	12	40
Deoxy-BIGCHAP	*N,N*-Bis-(3-D-gluconamidopropyl)deoxycholamide	0.1-1.5	13	20
O-MEG	Octanoyl-*N*-methylglucamide	0.1-1.5	0	–
D-MEG	Decanoyl-*N*-methylglucamide	0.05-0.5	0	–
OGP	η-Octyl-β-D-glucopyranoside	0.1-1.5	0	–
HTGP	η-Heptyl-β-D-thioglucopyranoside	0.1-1.5	0	–
TritonX-100	Octyl-phenoxypolyethoxyethanol	0.02-2.0	0	–
TritonX-114	Octyl-phenoxypolyethoxyethanol	0.02-2.0	0	–
HECAMEG	6-*O*-(*N*-Heptylcarbamoyl)-methyl-α-D-glucopyranoside	0.05-0.5	0	–
SM-1000	Sucrose monocaprate	0.01-0.5	0	–
SM-1080	Sucrose monocaproleate	0.01-0.5	0	–
SM-1200	Sucrose monolaurate	0.01-2.0	55	30

Solubilization procedure and determination of solubilized mAChR and their complexes with G proteins are described under "MATERIALS AND METHODS." [a]The maximal solubilization yield with each detergent was determined by the specific binding of [^{3}H]QNB in the supernatant fraction and is expressed as a percentage of membrane-bound mAChR assessed under the same conditions. [b]The maximal proportion of the [^{3}H]QNB binding sites of the solubilized mAChR at carbachol concentration of 10 μM that was affected by the addition 0.1 mM GTP is expressed as a percentage of total solubilized [^{3}H]QNB binding sites. [c]Digitonin is a mixture of glycosides purified from plant the *Digitalis purpurea*, and the preparation from Sigma used contains at least 50% of digitonin.

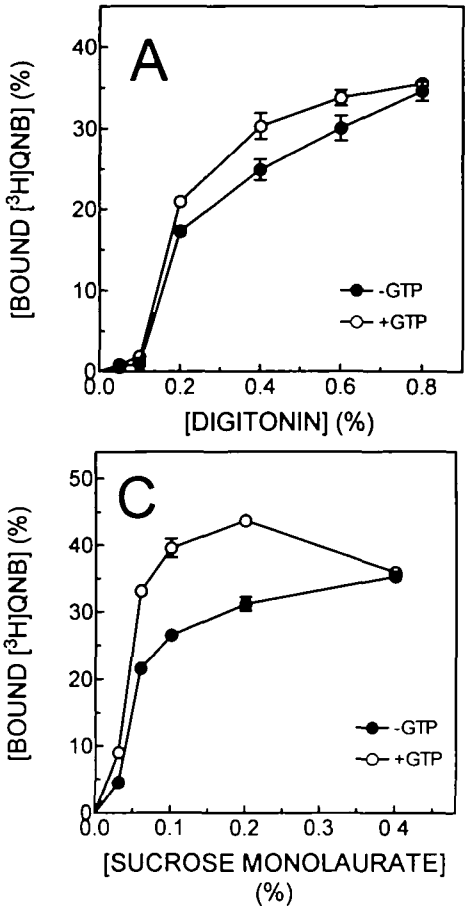

Fig. 4. **Solubilization of mAChR-G protein complexes by different detergents.** Porcine atrial membranes with additional G proteins were preincubated with 20 μM carbachol in KPB with 5 mM $MgCl_2$ and solubilized with different concentrations of digitonin (A), CHAPSO (B), or sucrose monolaurate (C). Aliquots of the supernatant fractions were incubated with 10 μM carbachol (final concentration) and 1.3 nM [^{3}H]QNB in the presence (○) or absence (●) of 0.1 mM GTP for 60 min at 30°C. Binding of [^{3}H]QNB is presented as the percentage of specific binding with membranes before solubilization in the absence of carbachol. Data are presented as mean ± SE at least of two independent experiments carried out in duplicate.

branes (*31*) and for mAChR from the rat cerebral membranes (*42*). Among the detergents studied, only Deoxy-BIGCHAP is a new detergent, which has been not reported to solubilize mAChR in active form (*31, 43*). The maximal yield of solubilization, 13%, was achieved in a narrow concentration range (0.15-0.25%) of the detergent, since higher concentrations caused decrease of the solubilization yield, as is also the case for CHAPS in the cortical membranes (*42*).

Solubilization of the mAChR-G protein complexes was determined using atrial membranes, which had been incubated with additional G proteins. The GTP dependence of the specific binding of [^{3}H]QNB in the supernatant fraction in the presence of 10 μM carbachol, which gave the maximal effect in membranes (Fig. 1), was used as an indicator of mAChR-G protein complexes. To detect a possible shift of the maximum of the GTP dependence during the solubilization, several control experiments in the presence of 3 and 100 μM carbachol were carried out, but in all cases the effect of GTP on the [^{3}H]QNB binding was less than in the presence of 10 μM carbachol. With digitonin as detergent, 35-43% of the [^{3}H]QNB binding sites could be solubilized, but the influence of GTP on the carbachol binding was small and did not exceed 10% of the total [^{3}H]QNB binding to the solubilized receptors (Fig. 4A). Use of sodium cholate in the mixture with digitonin increased the total solubilization yield of mAChR to 55-62%, but decreased the GTP-dependent binding to below 5%. On the other hand, the [^{3}H]QNB binding in the extract with 0.3% CHAPSO was higher by 40% in the presence of GTP than in its absence, but the total yield of solubilization of the [^{3}H]QNB binding sites was less than 12% (Fig. 4B). Similar results were obtained with Deoxy-BIGCHAP, but then the GTP dependence did not exceed 20% of the solubilized receptors. Sucrose monolaurate at the concentration of 0.1-0.2% solubilized more than 45% of the membrane-bound mAChR, and 30% of the [^{3}H]QNB binding to these solubilized receptors was inhibited by 10 μM carbachol in the absence of GTP in comparison with the [^{3}H]QNB binding in the presence of 0.1 mM GTP (Fig. 4C). This value of 30% is comparable to the 41% of the analogous value for the membrane preparation (Fig. 1), indicating that most of the mAChR solubilized with sucrose monolaurate retained their sensitivity towards GTP. An increase in the ionic strength of the buffer solution considerably decreased the proportion of the high-affinity binding, and in the presence of 200 mM NaCl no high-affinity carbachol binding in solution could be found. Thus, of the detergents studied, only sucrose monolaurate allowed a reasonable amount of mAChR to be solubilized in the functional complex with G proteins.

The sucrose gradient sedimentation profile of sucrose monolaurate-solubilized atrial mAChR-G protein complexes corresponded to the apparent sedimentation coefficient of 6.2±0.4 S (an average of 6 separate determinations). This value did not depend on the presence of GTP and carbachol after solubilization, but was slightly higher than the corresponding value of 5.7 S for atrial mAChR without additional G proteins (*18*).

Formation of mAChR-G Protein Complexes in Solution—The finding that a certain fraction of mAChR exists as a functional complex with G proteins after solubilization with sucrose monolaurate suggests that mAChR solubilized with this detergent may interact with G proteins in solution. To test this possibility, the mAChR were solubilized with 0.1% sucrose monolaurate from atrial membranes in which the internal G proteins had been inactivated by heat treatment as described (*36*), and the supernatant fraction obtained was mixed with purified G proteins. The displacement curve of [^{3}H]QNB binding by carbachol to the solubilized mAChR was affected by the addition of GTP

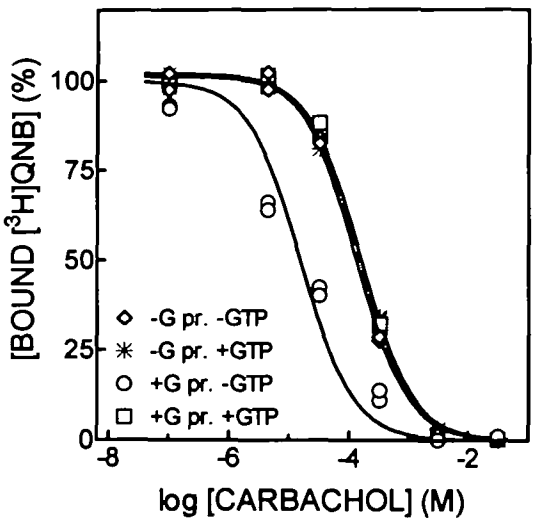

Fig. 5. **Effect of G proteins on the GTP-dependent inhibition of [^{3}H]QNB binding by carbachol to solubilized atrial mAChR.** Receptors were solubilized from atrial membranes with 0.1% sucrose monolaurate, then 15 nM (final concentration) purified G proteins was added (○, □) or omitted (◇, ✳) and the mixture was incubated with different concentrations of carbachol and 1.3 nM [^{3}H]QNB in presence (✳, □) or absence (◇, ○) of 0.1 mM GTP for 24 h at 4°C. Binding of [^{3}H]QNB is presented as the percentage of specific binding in the presence of GTP and absence of carbachol.

only in the preparations with G proteins. Under the usual incubation conditions (60 min at 30°C), the effect of GTP was small ($\Delta IC_{50} = 0.27$), but decrease of temperature and increase of incubation time revealed a clear GTP-dependent carbachol binding to solubilized mAChR (Fig. 5). Thus, in the presence of G proteins, the carbachol bound to solubilized mAChR with $K_I = 3.9 \pm 0.9$ μM and Hill coefficient $n_H = 0.58 \pm 0.04$. Of the different models compared, the two-binding-site model was preferred ($p < 0.0001$), and this revealed 47 ± 3% of high-affinity binding sites with $K_H = 0.70 \pm 0.24$ μM and 53% of sites with $K_L = 24.1 \pm 6.2$ μM. In the presence of 0.1 mM GTP, the binding curve shifted to the right, and the Hill coefficient was close to unity ($n_H = 1.09 \pm 0.05$), indicating that a single-binding-site model was preferred ($p > 0.05$) with a corresponding $K_I = 37.5 \pm 5.3$ μM. In these cases when G proteins were not added to the solubilized mAChR, the [^{3}H]QNB displacement curves by carbachol were not affected by GTP and were fitted to the single-binding-site model. The corresponding apparent binding constants were $K_I = 38.9 \pm 4.8$ μM and $K_I = 31.6 \pm 4.6$ μM in the presence and absence of GTP, respectively, corresponding to the low-affinity site of carbachol binding in the presence of G proteins (Fig. 5). With sucrose monolaurate-purified mAChR, the effect of the addition of G proteins was considerably smaller ($\Delta IC_{50} = 0.25$), but still 23 ± 5% of receptors indicated high affinity in carbachol binding ($K_H = 2.8 \pm 0.4$ μM; $K_L = 68 \pm 6$ μM), whereas in the presence of 0.1 mM GTP only low-affinity sites could be detected ($K_I = 59 \pm 8$ μM). Thus we can conclude that up to 50% of solubilized mAChR may form functionally active complexes with G proteins in a solution of sucrose monolaurate.

DISCUSSION

This study provided further confirmation that insertion of purified G proteins into m2 mAChR in porcine atrial or Sf9 cell membranes as well as into the purified receptors in lipid vesicles can form functional complexes and generate high-affinity binding for agonists. The overall increase in total apparent affinity for carbachol binding was more than 10-fold. At the same time, the Hill coefficient of carbachol displacement curves in atrial and Sf9 cell membranes was significantly below unity. A low Hill coefficient indicates heterogeneity of binding sites (*44*), and is usually connected with GTP-dependent high-affinity binding of agonist. In our study, the Hill coefficient remained significantly lower than unity after removal of Mg^{2+} with EDTA as well as after addition of GTP, even when all G proteins present were inactivated by heat treatment. Only receptor solubilization brought the Hill coefficients of carbachol displacement curves close to unity, and this remained so also after reconstruction of the receptors into lipid vesicles. Our previous data with different mAChR subtypes in Sf9 cell membranes indicated that this low, GTP-insensitive Hill coefficient occurs only for the m2 subtype (*24*). It has been shown that the lower Hill coefficient of carbachol binding to m2 mAChR is not directly caused by interactions with G proteins, but is still clearly dependent on the concentration of Mg^{2+} (*11, 45*). This unusual character of m2 mAChR in the carbachol binding considerably complicated the calculation of the receptors in the high-affinity state and interpretation of the data obtained, but the low solubilization yields and high lability in solutions of other mAChR subtypes (*24, 31*) together with their heterogeneous representation in mammalian tissues (*2*) prevented their use in this study. The reason for this anomalous carbachol binding to m2 mAChR is not clear and requires further investigation, but one possible explanation is that carbachol binds to both binding sites of mAChR which are proposed according to the two-site receptor model (*46*), while another possibility is that carbachol has different affinities for the receptors forming a receptor dimer as proposed by the models of dimerized receptors (*47, 48*).

Insertion of G proteins into membranes or vesicles with m2 mAChR initiated GTP-dependent agonist binding to the receptors. Up to 65% of the receptors had a high affinity for carbachol, and this is accords well with earlier data for purified mAChR (*11*), but is slightly lower than the maximal value of 82–83%, reported in the literature (*7*). Unlimited increase of the concentration of G proteins did not increase the proportion of the high-affinity binding sites over 65%, as higher G protein concentrations inhibited ligand binding to the receptor. This inhibition by G proteins seems to be dependent on the ratio of G protein concentration to the amount of total protein. Thus, 10 nM G proteins inhibited 56% of the [^{3}H]QNB binding in purified and reconstituted systems, but had no influence on the binding to mAChR in the atrial and Sf9 cell membranes. An additional increase in G protein concentration had also some influence on the [^{3}H]QNB binding in these crude membranes. At the same time, it is important to point out that the effect of high concentrations of G proteins was directly connected with their activity, as removal of Mg^{2+} with EDTA, as well as addition of GTP, abolished the

inhibitory effect. Thus we can propose that, at higher concentrations, G proteins form tight complexes with membranes and/or with mAChR, which prevent ligand binding to the receptor. Activation of G protein by GTP or removal of Mg^{2+} destroys such complexes and dissociates $\beta\gamma$-subunits from α subunits (*49*), thereafter allowing ligands to bind to the receptors. The physiological and biochemical essence as well as the mechanism of this effect are still not known and require further investigation.

Sixteen different detergents were studied for solubilization of mAChR, but none were more efficient than those earlier described. The best results for solubilization of functional mAChR-G protein complexes were achieved with sucrose monolaurate. In the supernatant fraction of this detergent, up to 16.5% of the membrane-bound specific [^{3}H]QNB binding sites could be inhibited in a GTP-dependent manner by 10 μM carbachol in comparison with up to 5% for all other detergents studied. This 16.5% corresponds to 30% of all solubilized receptors, and insofar as this one-point screening does not indicate the total proportion of high-affinity sites (Fig. 1), it is reasonable to conclude that more than 40% of solubilized mAChR were in functional complex with G proteins. The mAChR-G protein complex was quite labile in solution, as an increase in ionic strength of the solution or in detergent concentration led to its disappearance. Therefore, usually only low-affinity agonist binding sites could be detected after mAChR solubilization, and high-affinity binding was restored only after reconstitution into membranes with G proteins (*19-24*). At the same time, there are several reports in which the complex seems to be stable in solution and well regulated with GTP analogues (*25-29, 50*). In most of these studies, digitonin was used for solubilization of mAChR and its complexes with G proteins. This detergent has a different effect on membranes from other detergents, as it is proposed that it does not replace lipids in surrounding proteins, but only fragments membranes (*51, 52*). Therefore, the high values of apparent sedimentation coefficients for digitonin-solubilized mAChR (11.3-16.6 S) and the high stability of receptor-G protein complexes may be connected with their cosolubilized membrane fragments as well as with a high sedimentation coefficient of digitonin micelles (*53*). In addition, the composition of commercial digitonin varies from batch to batch, which influences the solubilization yield (*54*) and causes difficulties in obtaining reproducible results with this detergent. The sucrose monolaurate used in the present studies does not have these problems, and the sedimentation coefficients obtained (5.7-6.2 S) are in agreement with proposed values for mAChR-detergent and mAChR-G protein-detergent micelles.

The insertion of the additional G proteins into membranes increased the sedimentation coefficient for the sucrose monolaurate-solubilized mAChR from 5.7 to 6.2 S, indicating that these G proteins were solubilized with mAChR, as has also been shown for digitonin- and CHAPS-solubilized mAChR (*26, 29*). At the same time, the micelles formed seem to be quite stable and their size does not depend on the physiological coupling between mAChR and G proteins after solubilization, as neither addition of GTP nor removal of the agonist influenced the sedimentation coefficient. These results are in agreement with data indicating that mAChR are physically tightly coupled to G proteins (*25, 28*), and they indicate that the keeping together of physical complexes between these proteins is not enough for their physiological coupling.

Successful solubilization of mAChR-G protein complexes facilitated the next step of the study: the formation of this complex in solution. With sucrose monolaurate as detergent and with Mg^{2+} and carbachol present in the solution, 47% of the [^{3}H]QNB binding sites in solution were found to have GTP-dependent high affinity for carbachol binding. The requirement of a lower incubation temperature, low ionic strength and optimal detergent concentration for a larger effect suggested the high lability of the complexes formed. Thus, it can be concluded that the optimization of the experimental conditions facilitated, for the first time, the formation of a functional complex between m2 mAChR and G proteins in solution. These results indicate that GTP-dependent high-affinity agonist binding is connected to the direct interaction between mAChR and G proteins, and other membrane components are not necessary. Therefore, the data obtained about formation of functional complexes between mAChR and G proteins seem to be important and promising for further biochemical and biophysical studies of the mechanism of signal transduction.

The author is very grateful to Professor Lorentz Engström for his thorough support of this study and for very careful reading of the manuscript and useful comments. I also thank Professor Tatsuya Haga (The University of Tokyo, Tokyo) and members of his laboratory for help and very useful and critical discussions during the Tokyo period of the work. Special thanks are due to Dr. K. Kameyama for the m2 mAChR in Sf9 cell membranes and to Dr. K. Haga for the preparation of ABT-Sepharose column.

REFERENCES

1. Dohlman, H.G., Thorner, J., Caron, M.G., and Lefkowitz, R.J. (1991) Model systems for the study of seven-transmembrane-segment receptors. *Annu. Rev. Biochem.* **60**, 653-688
2. Caulfield, M.P. (1993) Muscarinic receptors—Characterization, coupling and function. *Pharmacol. Ther.* **58**, 319-379
3. Järv, J. and Rinken, A. (1993) Muscarinic acetylcholine receptors in *Neurotransmitter Receptors* (Hucho, F., ed.) pp. 199-220, Elsevier Science Publisher, Amsterdam
4. Poyner, D. (1990) Receptor—G-protein complexes in solution in *Receptor-Effector Coupling. A Practical Approach* (Hulme, E., ed.) pp. 31-58, IRL Press, Oxford
5. Milligan, G., Shah, B.H., Mullaney, I., and Grassie, M.A. (1995) Biochemical approaches to examine the specificity of interactions between receptors and guanine nucleotide binding proteins. *J. Rec. Sign. Transd. Res.* **15**, 253-265
6. Raymond, J.R. (1995) Multiple mechanisms of receptor-G protein signalling specificity. *Am. J. Physiol.* **38**, F141-F158
7. Ikegaya, T., Nishiyama, T., Haga, K., Haga, T., Ichiyama, A., Kobayashi, A., and Yamazaki, N. (1990) Interaction of atrial muscarinic receptors with three kinds of GTP-binding proteins. *J. Mol. Cell. Cardiol.* **22**, 343-351
8. Parker, E.M., Kameyama, K., Higashijima, T., and Ross, E.M. (1991) Reconstitutively active G protein-coupled receptors purified from baculovirus-infected insect cells. *J. Biol. Chem.* **266**, 519-527
9. Dell'Acqua, M.L., Carroll, R.C., and Peralta, E.G. (1993) Transfected m2 muscarinic acetylcholine receptors couple to $G_{\alpha i2}$ and $G_{\alpha i3}$ in Chinese hamster ovary cells. Activation and desensitization of the phospholipase-C signaling pathway. *J. Biol. Chem.* **268**, 5676-5685
10. Migeon, J.C., Thomas, S.L., and Nathanson, N.M. (1995) Differential coupling of m2 and m4 muscarinic receptors to inhibition of adenylyl cyclase by G_i and G_o subunits. *J. Biol. Chem.* **270**, 16070-16074
11. Shiozaki, K. and Haga, T. (1992) Effects of magnesium ion on the interaction of atrial muscarinic acetylcholine receptors and GTP-

binding regulatory proteins. *Biochemistry* **31**, 10634-10642
12. Wess, J. (1993) Mutational analysis of muscarinic acetylcholine receptors: Structural basis of ligand/receptor/G protein interactions. *Life Sci.* **53**, 1447-1463
13. Wong, S.K.-F. and Ross, E.M. (1994) Chimeric muscarinic cholinergic:β-adrenergic receptors that are functionally promiscuous among G proteins. *J. Biol. Chem.* **269**, 18968-18976
14. Birnbaumer, M. (1995) Mutations and diseases of G protein coupled receptors. *J. Rec. Sign. Transd. Res.* **15**, 131-160
15. Hurko, O. (1978) Specific [^{3}H]quinuclidinyl benzilate binding activity in digitonin-solubilized preparations from bovine brain. *Arch. Biochem. Biophys.* **190**, 434-445
16. Rinken, A.A., Langel, Ü.L., Tähepõld, L.J., and Järv, J.L. (1984) Kinetics of the inactivation of the muscarine cholinoreceptor, solubilized by digitonin. *Biokhimiia* (*Russ.*) **49**, 1533-1537
17. Peterson, G.L. and Schimerlik, M.I. (1984) Large scale preparation and characterization of membrane-bound and detergent-solubilized muscarinic acetylcholine receptor from pig atria. *Prep. Biochem.* **14**, 33-74
18. Rinken, A. and Haga, T. (1993) Solubilization and characterization of atrial muscarinic acetylcholine receptors in sucrose monolaurate. *Arch. Biochem. Biophys.* **301**, 158-164
19. Herron, G.S., Miller, S., Manley, W.-L., and Schimerlik, M. (1982) Ligand interactions with the solubilized porcine atrial muscarinic receptor. *Biochemistry* **21**, 515-520
20. Shreeve, S.M., Roeske, W.R., and Venter, J.C. (1984) Partial functional reconstitution of the cardiac muscarinic cholinergic receptor. *J. Biol. Chem.* **259**, 12398-12402
21. Vanderheyden, P., Andre, C., DeBacker, J.-P., and Vauquelin, G. (1984) Agonist mediated conformational changes of solubilized calf forebrain muscarinic acetylcholine receptors. *Biochem. Pharmacol.* **33**, 2981-2987
22. Florio, V.A. and Sternweis, P.C. (1985) Reconstitution of resolved muscarinic cholinergic receptors with purified GTP-binding proteins. *J. Biol. Chem.* **260**, 3477-3483
23. Peterson, G.L., Rosenbaum, L.C., and Schimerlik, M.I. (1988) Solubilization and hydrodynamic properties of pig atrial muscarinic acetylcholine receptor in dodecyl β-D-maltoside. *Biochem. J.* **255**, 553-560
24. Rinken, A. (1995) Subtype-specific changes in ligand binding properties after solubilization of muscarinic receptors from baculovirus-infected Sf9 insect cell membranes. *J. Pharmacol. Exp. Ther.* **272**, 8-14
25. Wreggett, K.A. and Wells, J.W. (1995) Cooperativity manifest in the binding properties of purified cardiac muscarinic receptors. *J. Biol. Chem.* **270**, 22488-22499
26. Berrie, C.P., Birdsall, N.J.M., Hulme, E.C., Keen, M., and Stockon, M. (1984) Solubilization and characterization of guanine nucleotide-sensitive muscarinic agonist binding sites from rat myocardium. *Br. J. Pharmacol.* **82**, 853-861
27. Flynn, D.D. and Potter, L.T. (1986) Effect of solubilization on the distinct binding properties of muscarinic receptors from rabbit hippocampus and brainstem. *Mol. Pharmacol.* **30**, 193-199
28. Matesic, D.F., Manning, D.R., Wolfe, B.B., and Luthin, G.R. (1989) Pharmacological and biochemical characterization of complexes of muscarinic acetylcholine receptor and guanine nucleotide-binding protein. *J. Biol. Chem.* **264**, 21638-21645
29. Singh, A.K. (1994) Regulation of the CHAPS-solubilized muscarinic receptors by an inhibitory GTP binding protein (G_i) in the brain of neonatal and adult rats. *Comp. Biochem. Physiol. A* **109**, 255-267
30. Haga, T., Haga, K., and Hulme, E.C. (1990) Solubilization, purification and molecular characterization of muscarinic acetylcholine receptors in *Receptor Biochemistry. A Practical Approach* (Hulme, E.C., ed.) pp. 51-78, IRL Press, Oxford
31. Rinken, A., Kameyama, K., Haga, T., and Engström, L. (1994) Solubilization of muscarinic receptor subtypes from baculovirus infected Sf9 insect cells. *Biochem. Pharmacol.* **48**, 1245-1251
32. Dong, G.Z., Kameyama, K., Rinken, A., and Haga, T. (1995) Ligand binding properties of muscarinic acetylcholine receptor subtypes (m1-m5) expressed in baculovirus-infected insect cells. *J. Pharmacol. Exp. Ther.* **274**, 378-384
33. Sternweis, P.C. and Robishaw, J.D. (1984) Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. *J. Biol. Chem.* **259**, 13806-13813
34. Haga, K., Haga, T., and Ichiyama, A. (1986) Reconstitution of the muscarinic acetylcholine receptor. Guanine nucleotide-sensitive high affinity binding of agonists to purified muscarinic receptors reconstituted with GTP-binding proteins (G_i and G_o). *J. Biol. Chem.* **261**, 10133-10140
35. Northup, J.K., Smigel, M.D., and Gilman, A.G. (1982) The guanine nucleotide activating site of the regulatory component of adenylate cyclase. Identification by ligand binding. *J. Biol. Chem.* **257**, 11416-11423
36. Haga, T., Ikegaya, T., and Haga, K. (1990) The interaction of acetylcholine receptors in porcine-atrial membranes with three kinds G proteins. *Jpn. Circ. J.* **54**, 1176-1184
37. Haga, T., Haga, K., and Gilman, A.G. (1977) Hydrodynamic properties of the beta-adrenergic receptor and adenylate cyclase from wild type and variant S49 lymphoma cells. *J. Biol. Chem.* **252**, 5776-5782
38. Peterson, G.L. (1983) Determination of total protein. *Methods Enzymol.* **91**, 95-119
39. Cheng, Y.C. and Prusoff, W.H. (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**, 3099-3108
40. Eller, M.H., Kõiv, A.H., Langel, Ü.L., Rinken, A.A., Tähepõld, L.J., and Järv, J.L. (1987) Quinuclidinyl benzilate can't be used as ligand in equilibrium binding studies with muscarinic receptor. *Neirokhim.* (*Russ.*) **6**, 605-610
41. Järv, J. and Eller, M. (1988) Kinetic aspects of L-quinuclidinyl benzilate interaction with muscarinic receptor. *Neurochem. Int.* **13**, 419-428
42. Rinken, A.A., Langel, Ü.L., and Järv, J.L. (1987) Solubilization by some detergents of muscarine cholinoreceptor and its complex with quinuclidinyl benzilate. *Biokhimiia* (*Russ.*) **52**, 263-269
43. Haga, T., Haga, K., and Hulme, E.C. (1990) Solubilization, purification, and molecular characterization of receptors: Principles and strategy in *Receptor Biochemistry. A Practical Approach* (Hulme, E.C., ed.) pp. 1-50, IRL Press, Oxford
44. Hulme, E.C. and Birdsall, N.J.M. (1992) Strategy and tactics in receptor binding studies in *Receptor-Ligand Interactions. A Practical Approach* (Hulme, E.C., ed.) pp. 63-176, IRL Press, Oxford
45. Peterson, G.L., Toumadje, A., Johnson, W.C., and Schimerlik, M.I. (1995) Purification of recombinant porcine m2 muscarinic acetylcholine receptor from Chinese hamster ovary cells. Circular dichroism spectra and ligand binding properties. *J. Biol. Chem.* **270**, 17808-17814
46. Järv, J. (1995) A model of non-exclusive binding of agonist and antagonist on G-protein coupled receptors. *J. Theor. Biol.* **175**, 577-582
47. Maggio, R., Vogel, Z., and Wess, J. (1993) Coexpression studies with mutant muscarinic/adrenergic receptors provide evidence for intermolecular "cross-talk" between G-protein-linked receptors. *Proc. Natl. Acad. Sci. USA* **90**, 3103-3107
48. Lemmon, M.A. and Schlessinger, J. (1994) Regulation of signal transduction and signal diversity by receptor oligomerization. *Trends Biochem. Sci.* **19**, 459-463
49. Neer, E.J. (1994) G proteins: Critical control points for transmembrane signals. *Protein Sci.* **3**, 3-14
50. Baron, B., Gavish, M., and Sokolovsky, M. (1985) Heterogeneity of solubilized muscarinic cholinergic receptors: Binding and hydrodynamic properties. *Arch. Biochem. Biophys.* **240**, 281-296
51. Lichtenberg, D., Robson, R.J., and Dennis, E.A. (1983) Solubilization of phospholipids by detergents. Structural and kinetic aspects. *Biochim. Biophys. Acta* **737**, 285-304
52. Hjelmeland, L.M. (1990) Solubilization of native membrane proteins. *Methods Enzymol.* **182**, 253-264
53. Haga, T. (1980) Molecular size of muscarinic acetylcholine receptors of rat brain. *FEBS Lett.* **113**, 68-72
54. Raidaru, G., Rinken, A., Järv, J., and Lõhmus, M. (1989) Chromatographic analysis of Digitalis glycosides and their relation to solubilization of muscarinic receptor. *Proc. Acad. Sci. Estonia Chem.* **38**, 103-114