A novel compound, 3-(2'-aminobenzhydryloxy)-tropane (ABT), and an ABT-agarose gel were synthesized and used for the purification of solubilized muscarinic receptors. ABT had a high affinity with an apparent dissociation constant ($K_d$) of 7 nM for the muscarinic receptors solubilized from the porcine brain by digitonin. An ABT-agarose gel was prepared by coupling ABT with epoxy-activated Sepharose 6B, and the degree of substitution to the gel was determined to be 4–5 µmol/ml of the gel by UV absorption spectrum. During affinity chromatography using 10 ml of the ABT-agarose gel and 100 ml of the digitonin-solubilized preparation, 70% of muscarinic receptors were adsorbed to the gel, marked contrast with the adsorption of only 2% of proteins. Approximately 25% of muscarinic receptors applied to the gel were eluted specifically with 1 mM muscarinic ligands. The purified fraction showed a high affinity for $[^3H]$quinuclidinyl benzylate with a $K_d$ of 0.4 nM and similar specificity for muscarinic ligands to that of unpurified soluble receptors. The protein concentration of the purified fraction was too low to be determined accurately, but very approximately a purification of 104-fold was indicated.

Muscarinic receptors have been studied in detail with regard to their binding with muscarinic ligands by using $[^3H]$ QNB (1) and other labeled ligands (2). Recently, muscarinic receptors have been shown to be solubilized in their active forms by several detergent systems (Refs. 3–7 and see references of Ref. 5), and their molecular weight has also been estimated by different methods (8–12). However, little progress has been made in the purification of the receptor mainly because of the minute quantities of muscarinic receptors. The high affinity and great specificity of ligand-receptor interactions make affinity chromatography an attractive method for purification. Most muscarinic ligands do not contain a functional group that is necessary for the coupling to gels and can be used in their active form. The purification of muscarinic receptors solubilized by digitonin from porcine cerebral membranes.

**EXPERIMENTAL PROCEDURES**

**Materials**

Tropane, 3-quinuclidinyl, N-piperidinemethanol, benzophenone, and 2-amino benzophenone were purchased from Aldrich; digitonin was from Wako Co. Ltd., Osaka, $[^3H]$QNB (33 Ci/mmol) was from New England Nuclear; and epoxy-activated Sepharose 6B was from Pharmacia Fine Chemicals. Pirenzepine and aprotinin were donated from Nippon Boehringer Ingelheim Co., Ltd., Tokyo, and Mochida Pharmaceutical Corp., Tokyo, respectively.

**Synthesis of Muscarinic Ligands**

2-Aminobenzhydryloid—To a solution of 2-aminobenzophenone (30 g, 152 mmol), methanol (300 ml), and benzene (100 ml) in a 500-ml round bottom flask which was cooled in an ice bath, NaBH$_4$ (4.6 g, 125 mmol) was added with stirring in 0.5-g portions. After addition was completed, the reaction mixture was stirred for 2 h at room temperature with the flask loosely stopped. The solvent was removed in vacuo and the oily pellet was poured into ice-cold water. The white solid which was formed was collected by filtration and washed repeatedly with water. The white solid (2-aminobenzhydryl) was dried in vacuo over P$_2$O$_5$.

**ABT—**A 100-ml two-necked round bottom flask containing 10 ml of concentrated sulfuric acid was equipped with a sealed stirrer and chilled in an ice-salt bath. To the flask was added 14.1 g (100 mmol) of powdered tropine. After the addition was completed, the mixture was vigorously homogenized at room temperature. To the resulting mixture in an ice bath, 4 g (20 mmol) of 2-aminobenzhydryl were gradually added, and the mixture was stirred to homogeneity. The mixture was then warmed in an oil bath at 60–70 °C with stirring for 30 min. After cooling, it was poured onto a slurry of ice and water. The pH of the mixture was adjusted to 7.0 with 5 M NaOH, and the resulting insoluble yellowish solid was extracted with benzene. The fraction contained 2-aminobenzhydryl and by-products with a little ABT and was discarded. The aqueous layer was separated, washed with benzene, and made alkaline with an excess of 5 M NaOH in an ice bath. The resulting insoluble yellowish oil was extracted with benzene. The benzene extract was washed with water until the water phase became neutral, dried over anhydrous sodium sulfate, and evaporated under reduced pressure. The oily residue (3.7 g) soon became crystalline (yield 58%). It was recrystallized from methanol.

**RESULTS AND DISCUSSION**

The final product showed a single spot in TLC (chloroform:methanol, 9:1) which absorbed UV and reacted with iodine vapor. The starting materials (2-aminobenzhydryl) absorbed UV but did not react with iodine, and the other (tropine) reacted with iodine but did not absorb UV.

GC-MS analysis was carried out with a Nihon Denahi-JMS-D300 and JMA-200E under the following conditions: 1% OV-1 column on Chromosorb W (60–80 mesh), helium as the carrier gas at a flow rate of 30–40 ml/min, 250 °C at the injector, 200 °C in the column, and an ionizing potential of 20 eV. GC-MS analysis of the final product showed a single peak with GC and a molecular peak with MS at m/z 232 (mass to charge ratio) = 322 (calculated value, 322). The most abundant ions appeared at m/z 140, 83, and 125, which were identical with the values expected for the ions of tropine minus H, tropine minus HOCH(CH$_3$)$_2$, and tropine minus OH, respectively. $[^1]$H NMR
dioxane (24 ml) and aqueous 0.1 M K$_2$CO$_3$ (36 ml). The pH of the volumes of the Tris buffer just before use. The content of bound ABT was washed with 2 liters of 50% aqueous dioxane and then 2 liters of water. After suspension in 50 ml of 1 M ethanolamine overnight, the suspension was adjusted to above 10.5 by adding 9 ml of 1 M KOH and 9 ml of 0.6 M Tris-HCl buffer (pH 8). The suspension was homogenized in 2 liters of 0.32 M sucrose, 5 mM Hepes buffer (pH 7.5) which had been equilibrated with digitonin:phosphate buffer and bovine serum albumin dissolved in the same buffer (0.2 ml each) was reacted with the digoxigenin-phosphate buffer and bovine serum albumin which had been equilibrated with digitonin:phosphate buffer (0.2 ml) and then centrifuged at 11,000 g for 90 min. The pellet was washed with a Silverson homogenizer in 4 liters of 50 mM Tris-HCl buffer (pH 7.5) and centrifuged at 11,000 × g for 90 min, and this process was repeated once more. In these procedures, the loosely packed pellet was collected and the darker brown portions of the pellet which stuck tightly to the walls of the bottles were discarded. The final pellet was suspended in 0.5 liter of 0.5 M NaCl buffer (pH 7.5) supplemented with 0.5 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml of leupeptin, chymostatin, antipain, and aprotinin in a total volume of 300 ml. These protease inhibitors were added to eliminate a possible action of endogenous proteases on muscarinic receptors. The suspension was stirred for 60 min at 4 °C and then centrifuged at 98,000 × g for 60 min. The supernatant was stored at -80 °C. Concentrations of proteins and [H]QNB-binding sites were 1.5-2.3 mg/ml and 1.1-1.5 pmol/ml, respectively. The recoveries of protein and the [H]QNB binding activity from the membrane preparation were 37-57 and 21-28%, respectively.

Affinity Chromatography

Typically 100 ml of the solubilized preparations were thawed and extracted with the addition of 2% digitonin, 20 mM Tris-Cl buffer (pH 7.5) at 4 °C. In some experiments, protease inhibitors used in the solubilization were included in the washing medium. Muscarinic receptors were eluted with 50-100 ml of 1 mM BP or ABQ at 4 °C and collected in polypropylene tubes. All procedures after the elution with muscarinic ligands were carried out in plastic tubes or plastic columns instead of glass vessels. Muscarinic ligands used for the elution were removed from receptors by using small columns (0.5 × 5 cm, 2 ml) of Sephadex G-50 fine which had been equilibrated with digitonin:phosphate buffer (0.1% digitonin, 20 mM K phosphate (pH 7.0)) or digitonin:Tris buffer (0.1% digitonin, 20 mM Tris-Cl (pH 7.5)). Typically 0.2 ml of the eluate was applied to the column and the column was washed with 0.6 ml and then 0.4 ml of the buffer. The second effluent of the eluate was collected and used for assays of the [H]QNB binding activity and protein. The recovery of the [H]QNB binding activity after the Sephadex column procedure was 86 ± 7% (average ± S.D., n = 6) when crude solubilized preparations were used.

Binding Assays

Solubilized preparations (usually 0.2 ml) or the effluents from the Sephadex columns following the elution from the ABT-agarose gel (usually 0.4 ml) were incubated with 2.5-3 nm [H]QNB in the digoxigenin:phosphate or digitonin:Tris buffer in a total volume of 0.5 ml at 30 °C for 60 min. No appreciable difference was found between the two buffers with regard to [H]QNB binding activity. After the incubation, 0.2 ml of the reaction mixture was applied in duplicate on the same Sephadex column used for the separation of muscarinic ligands and the bound form of [H]QNB was eluted with 1.1 ml of the buffer. The whole eluate was collected in a vial, and the radioactivity was assayed in a liquid scintillation spectrometer with an efficiency of 38%.

Estimation of Protein Concentrations

Protein concentrations were determined as described by Lowry et al. (13) using bovine serum albumin as standard. Special care was taken for the fraction eluted from the ABT-gel by muscarinic ligands because protein content was very low. The effluent from the Sephadex column equilibrated with the digoxigenin:phosphate buffer and bovine serum albumin dissolved in the same buffer (0.2 ml each) was reacted with Polin regents in polypropylene tubes in a total volume of 1.3 ml, and the absorbance at 700 nm was read with Union-Gigen High Sensitivity Spectrophotometer SM 401 at a full scale setting of 0.1.

RESULTS AND DISCUSSION

Synthesis of Ligands and Their Affinities for the Receptor—In a series of initial attempts, muscarinic ligands with an amino group and an ether bond were synthesized by the
The binding of $[^3H]$QNB to digitonin-solubilized preparations was inhibited by newly synthesized ligands, and the displacement curves showed an apparent Hill coefficient of unity (data not shown). Concentrations giving a half-maximal inhibition were 630 nM for BP, 250 nM for ABQ, and 50 nM for ABT in the presence of 2.5 nM $[^3H]$QNB; the $K_I$ values were calculated to be 84, 33, and 7 nM, respectively, by assuming a simple mass action.

Adsortion of the Receptor to the ABT-Agarose Gel—The adsorption of solubilized receptors to the ABT-agrose gel was examined in the presence of different concentrations of NaCl (Fig. 2). More than 90% of $[^3H]$QNB binding activity was adsorbed to the gel in the absence of NaCl whether the solubilized preparations were preincubated with $[^3H]$QNB or not. In the presence of 0.1 mM NaCl, however, less than 5% of the $[^3H]$QNB-receptor complex was adsorbed to the gel when the solubilized preparations were pretreated with $[^3H]$QNB, in marked contrast with the adsorption of 75% of the $[^3H]$QNB binding activity to the gel without pretreatment. About 55% of proteins in solubilized preparations were adsorbed to the gel in the absence of NaCl, but less than 10% were adsorbed in the presence of 0.1 mM NaCl.

Adsortion of the $[^3H]$QNB-receptor complex as well as proteins to the ABT gel in the absence of NaCl may be caused by nonspecific electrostatic interaction with a positive charge of ABT. On the other hand, the adsorption of the ligand-free receptor in the presence of 0.1 M or higher concentrations of NaCl appears to be due to the biospecific ligand-receptor interaction because the $[^3H]$QNB-receptor complex is hardly adsorbed under the conditions.

The adsorbed receptor was found to be eluted with 30 nM $[^3H]$QNB, 0.1 mM atropine, 10 mM oxotremorine, and other muscarinic ligands in small scale experiments. The recovery of $[^3H]$QNB binding activity was dependent on species and concentrations of ligands and was the highest among conditions examined when 1 mM BP or ABQ was used (data not shown).

Affinity Chromatography—Fig. 3 indicates elution profiles of protein and the $[^3H]$QNB binding activity when 100 ml of digitonin-solubilized preparations were applied to 10 ml of the ABT-agarse gel in the presence of 0.1 mM NaCl. About 98% of proteins and 29% of $[^3H]$QNB binding activity in the solubilized preparations were not adsorbed to the gel and recovered in a flow-through fraction. Very small amounts of proteins and $[^3H]$QNB binding activity were eluted by further washing of the gel with buffers containing 0.1 or 0.25 mM NaCl. The gel was then eluted with the buffer containing 1 mM BP, and the eluates were passed through Sephadex columns to remove BP. The sharp elution of the $[^3H]$QNB binding activity was detected without elution of appreciable amounts of proteins. The $[^3H]$QNB binding activity in these fractions amounted to 22% of that applied to the gel unless the correction was made for the recovery through the Sephadex column and to 26% when the correction was made by using the recovery factor obtained with crude solubilized preparations.

The reason for the nonquantitative recovery of the $[^3H]$QNB binding activity is not known at present. In connection with this problem, it should be mentioned that the $[^3H]$QNB binding activity in the fraction eluted from the ABT-agarse column with muscarinic ligands was greatly decreased when glass vessels were used or when digitonin was omitted from the medium of the Sephadex column for the binding assay, although these factors hardly affected the $[^3H]$QNB binding activity of crude solubilized preparations. During the course of this study, Andre et al. (14) reported an application of affinity chromatography with dextemide as a ligand for purification of the muscarinic receptor. They suggested an extreme instability of the purified receptors because the radioligand binding activity was not detected in the purified preparations eluted by atrpine. In our experiments, the $[^3H]$QNB binding activity of the purified preparations was hardly decreased during the storage at 4°C for 2 days, although the activity was very likely to be lost by contact with glass vessels or by omission of digitonin from the medium as described above.

![Graph](image-url)
Affinity Chromatography of the Muscarinic Receptor

FIG. 3. Affinity chromatography of solubilized preparations on the ABT-agarose gel. Digitoninsolubilized preparations (100 ml) were incubated with 10 ml of the ABT gel in the presence of 0.1 M NaCl for 2 h at 0 °C. The incubated gel was packed in a column, and then muscarinic receptors were eluted from the column and the [3H]QNB binding activity (○) and protein (●) were determined as described under "Experimental Procedures." The eluting medium was initially 0.1 M NaCl, 0.1% digitonin, 20 mM Tris buffer (pH 7.5) and was changed to 0.15 M NaCl, 0.1% digitonin, 20 mM Tris buffer at the point indicated by arrow 1 and finally to 1 mM BP, 0.1 M NaCl, 0.1% digitonin, 20 mM Tris buffer at the point indicated by arrow 2. Each fraction contained 5 ml except that the flow-through fraction was collected in a single flask. Broken and dotted lines indicate protein concentrations and the [3H]QNB binding activity of original solubilized preparation, respectively.

FIG. 4. [3H]QNB binding of the purified receptor as a function of increasing concentrations of [3H]QNB. An aliquot of the purified fraction (40 μl and 0.11 μg of protein/assay) was incubated with various concentrations of [3H]QNB in the digitonin:phosphate buffer with (●) or without (○) 1 μM atropine for 60 min at 30 °C, and 0.2 ml of the incubated solution was used for the assay of the bound form of [3H]QNB. Inset, Scatchard plot of the specific [3H]QNB binding that is the difference of the binding in the absence and presence of 1 μM atropine. The dissociation constant was calculated to be 408 pm, and the maximum binding capacity to be 65 fmol, which corresponded to 0.6 nmol/mg of protein.

Protein concentrations of the fraction eluted with BP were too low to be estimated in a routine assay using the Lowry (13) procedure. We tried to estimate an approximate amount by statistical analysis of the scattered data that were obtained when minute amounts of proteins were subjected to the Lowry procedure. When 0, 0.2, 0.4, and 0.6 μg of bovine serum albumin were used, average absorbances at 700 nm and standard deviations for 10 determinations each were 0.037 ± 0.004, 0.042 ± 0.004, 0.046 ± 0.005, and 0.049 ± 0.006, respectively. The average values correlated well with amounts of albumin with a correlation coefficient of 0.98. In addition, the values for 0 and 0.2 μg of albumin were significantly different with p < 0.05 and the values for 0 and 0.4 μg of albumin with p < 0.001. When 0.2 ml of the fraction with the highest [3H]QNB binding activity was assayed, an average absorbance and standard deviation was 0.048 ± 0.005 (n = 7) and the amount of protein was estimated to be 0.54 μg from the above correlation equation.

In preliminary experiments, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified fraction followed by silver staining revealed several bands, suggesting the necessity for further purification steps.

Binding of the Purified Receptor with Muscarinic Ligands—Fig. 4 shows the binding of [3H]QNB with the purified receptor at different concentrations of [3H]QNB. A straight line
obtained by the Scatchard analysis was consistent with a single class of binding sites with a $K_d$ for $[^3H]QNB$ of 408 pM. The $K_d$ value was comparable to those reported by others (15) and obtained for unpurified soluble receptors. The specific $[^3H]QNB$ binding activity was estimated to be 0.6 nmol/mg of protein by using the protein concentration determined as described above. This value means a 920-fold purification of the muscarinic receptor as the specific $[^3H]QNB$ binding activity of unpurified soluble preparations is 0.65 pmol/mg of protein.

Fig. 5 shows the displacement curves by atropine, pirenzepine, and acetylcholine of the $[^3H]QNB$ binding with the purified receptor. Apparent $K_d$ values were calculated by assuming a simple mass action and found to be 3 nM for atropine, 144 nM for pirenzepine, and 67 pM for acetylcholine. These values were comparable with those determined for unpurified soluble preparations.

In conclusion, affinity chromatography using the ABT-agarose gel newly developed and described here should be useful for large scale purification of the muscarinic receptor.

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