Solubilization of the muscarinic acetylcholine receptor by sodium cholate: Stabilization of the receptor by muscarinic ligands

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ABSTRACT

Forty to fifty percent of the muscarinic receptor in membrane preparations of porcine caudate nucleus was solubilized by sodium cholate in its active form when the membranes were pretreated with carbamylcholine or other muscarinic ligands. The binding activity of solubilized receptors was assayed by using [3 H]quinuclidinylbenzylate ([3 H]QNB) after removal of cholate and carbamylcholine. Without the pretreatment, sodium cholate abolished most of the binding activity. The solubilized receptor had a high affinity for [3 H]QNB with a dissociation constant of 58 pM and an affinity for muscarinic ligands similar to that of the membrane receptor.

The muscarinic receptor has been studied in detail with respect to its binding with muscarinic ligands by using [3 H]-labeled ligands (3, 12) and several subclasses with different affinities for agonists (2) or selective antagonists (8) have been reported. It is not known, however, whether these subclasses represent several types of receptors which differ as chemical entities, or several states of a single receptor which differ in conformation and/or in interaction with other components in the membranes. The direct answer to this question would be obtained by purification and reconstitution of the receptor. The purification of the receptor should be preceded by solubilization of the receptor from membranes. Two kinds of detergents, digitonin (for review, see 10) and lysophosphatidylcholine (5) have been reported to partly solubilize the receptor but common detergents like Triton X 100 or sodium cholate have failed to solubilize it in its active form (9, 11). This paper is concerned with a finding that the muscarinic receptor can be solubilized in its active form when membranes are pretreated with muscarinic ligands and then treated with sodium cholate in their presence.

Microsomal fractions prepared from porcine caudate nucleus by the usual method were suspended in 0.32 M sucrose and stored at

-80°C until use. In the standard procedure of solubilization, the microsomal preparation (about 5 mg protein per ml) was incubated with 10 mM carbamylcholine in 50 mM Tris-HCl buffer (pH 7.5) at 30°C for 10 min, then a half volume of 3% sodium cholate in the Tris buffer was added and the incubation continued for 5 min at 30°C. The suspension was centrifuged for 1 h at 165,000 *g* and the supernatant was passed through Sephadex G-50 columns which were pre-equilibrated with the Tris buffer. An aliquot of the void volume fraction was incubated with 2 to 2.5 nM [3 H]QNB in the Tris buffer at 30°C for 30 min and the bound form of [3 H]QNB was separated by using small columns of Sephadex G-50 as described previously (5, 6). The binding of membrane receptor with [3 H]-QNB was assayed by using glass fiber filter paper (Whatman GF/C). Protein concentrations were determined by the Lowry method using bovine serum albumin as a standard.

Fig. 1 summarizes the effects of pretreatment of membranes with muscarinic ligands and other agents on solubilization of the muscarinic receptor by sodium cholate. About 40% of [3 H]QNB binding activity in the membranes was recovered in the supernatant when the membranes were pretreated with carbamylcholine and solubilized by sodium cholate in the

Fig. 1 Effects of the pretreatment of membranes with muscarinic ligands and other reagents on the solubilization of the muscarinic receptor by sodium cholate. Membrane preparations were first incubated at 30°C for 10 min in Tris-HCl buffer (pH 7.5) with the reagents shown in the figure. The concentrations of the reagents in the preincubation mixture were 1.5 times higher than those shown in the figure. To the pretreated membranes was added a half volume of 3% sodium cholate. The suspension was incubated for 30 min at 0°C and centrifuged at 165,000 *g* for 60 min. The supernatant was passed through a Sephadex G-50 column and the void volume fraction was used for the [³H]QNB binding assay as described in the text. The specific binding per 0.072 mg of original membrane protein was determined in the presence and absence of 1 μM atropine and plotted in the figure.

presence of carbamylcholine. On the other hand, less than 10% of the binding activity was recovered in the supernatant when the membranes were solubilized in the absence of carbamylcholine. In this and the following experiments, the supernatant was passed through a Sephadex G-50 column to remove compounds of small molecular weights including carbamylcholine and cholate, and then the void volume fraction was used for the binding assay. About 70% of the binding activity in the void volume fraction was recovered in the supernatant after centrifugation at 165,000 *g* for 1 h.

The effect of carbamylcholine was obtained also with atropine or pridinol indicating that the stabilization of the muscarinic receptor was attained by muscarinic ligands irrespective of whether they were agonists or antagonists. About 30% of [³H]QNB binding activity was recovered in the supernatant when the membranes were solubilized by cholate in the presence

of 1 M NaCl without addition of carbamylcholine. Coexistence of NaCl and carbamylcholine, however, did not increase the yield any more. It is possible, but remains to be proven, that Na stabilizes the receptor by binding to the same site as muscarinic ligands.

GTP did not affect the recovery of [³H]QNB binding sites with or without carbamylcholine.

The treatment of membranes with *N*-ethylmaleimide caused a decrease of the [³H]QNB binding activity in the cholate extracts to 30 to 50% of the control, although the same treatment did not affect the [³H]QNB binding activity of the membrane receptor but enhanced its affinity for agonists (1). On the other hand, treatment with *N*-ethylmaleimide resulted in an almost complete loss of [³H]QNB binding activity in the preparation solubilized by lysophosphatidylcholine (7).

Fig. 2 shows the effect of cholate concentrations on the solubilization of [³H]QNB binding

Fig. 2 Effects of sodium cholate concentrations on the solubilization of the muscarinic receptor. Membranes were treated with 10 mM carbamylcholine for 10 min at 30°C, and the subsequent solubilization and [³H]QNB binding assay were carried out as described in the text except that cholate concentration was varied as indicated. Absorbance was measured of the sample eluted in the void volume fraction of a Sephadex G-50 column. In the case of 100% recovery, the [³H]QNB binding activity and protein concentration in the void volume fraction should be 3.8 pmol/ml and 1.8 mg/ml, respectively.

sites. The recovery of [³H]QNB binding activity was maximal at the cholate concentration of 1%. Further increase of the cholate concentration was accompanied by a decrease in the recovery of the binding activity although the amount of solubilized protein was increased. The recovery of [³H]QNB binding sites at 1% cholate was $46 \pm 13\%$ (average $\pm$ SD; $n=9$) of that in membrane preparations. The recovery varied from 20 to 60% but usually ranged between 40 and 50%. When digitonin or lysophosphatidylcholine was used as solubilizing agents for the same membrane preparations, the yield of [³H]QNB binding sites was 40 to 50% and 20 to 30%, respectively (data not shown). The combination of two or three detergents or successive extraction did not increase the recovery.

The solubilized preparation showed a high affinity for [³H]QNB with a dissociation constant (K_d) of 58 pM. The affinity of the solubilized site for various muscarinic ligands was estimated from the displacement curves of

Fig. 3 Comparison of affinity for muscarinic ligands between membrane-bound and cholate-solubilized receptors. The value of pK_d ($= -\log K_d$) was calculated from the following equation: $K_d = IC_{50}/(1 + ([^3H]QNB)/K_d^*)$, where IC_{50} is the concentration of the ligand giving half maximal inhibition of [³H]QNB binding and K_d^* is the dissociation constant of the binding of the receptors with [³H]QNB (10 pM for the membrane receptor and 58 pM for the receptor solubilized by cholate). ([³H]QNB) is the concentration of [³H]QNB used (0.1 nM for the experiment with the membrane receptor and 2 to 2.5 nM for the experiment with the solubilized receptor). The correlation equation was $Y = 0.42 + 0.88X$ with a correlation coefficient of 0.99. The ligands used were as follows: 1, carbamylcholine; 2, acetylcholine; 3, arecoline; 4, *cis*-2-methyl-dioxolane; 5, oxotremoline; 6, diphenidol; 7, prometa-zine; 8, methixene; 9, triphenidyl; 10, atropine; 11, scopolamine; 12, isopropamide; 13, [³H]QNB

[³H]QNB binding and compared with those of the membrane receptor (Fig. 3). The K_d values obtained for the solubilized site correlated well ($r=0.99$) with those obtained for the membrane receptor and the former values were 4 times higher than the latter on the average. This good correlation constitutes evidence that the solubilized [³H]QNB binding site was derived from the membrane [³H]QNB binding site, that is the muscarinic receptor.

The value of 4 as the average ratio of K_d was much smaller than those obtained for the preparations solubilized by digitonin or lysophosphatidylcholine, which were 20 and 35, respectively (data not shown). Furthermore, preliminary experiments indicated that a displacement curve by carbamylcholine of [³H]QNB

bound to the receptor solubilized by cholate had a Hill coefficient of less than 1.0, though the displacement by atropine had a Hill coefficient of 1.0. On the other hand, the receptors solubilized by digitonin or lysophosphatidylcholine showed an apparent single affinity with a Hill coefficient of 1.0 for any given agonist or antagonist (5, 9). These findings indicated that the native state of the muscarinic receptor in membranes was more preserved in the receptor solubilized by cholate than in those solubilized by digitonin or lysophosphatidylcholine. It is also possible that the binding properties of the receptor solubilized by cholate reflected the interaction of the receptor with the other components in membranes. As sodium cholate was removed before the binding assay, it is more likely that the receptor exists as an aggregated form in the assay mixture rather than as a monodisperse form.

The fact that cholate is easily removed from the extracting medium is one of the advantages of using cholate as solubilizing agent. The cholate extract is, therefore, expected to be useful for the reconstitution of the receptor into lipid bilayers. The other advantages are that cholate has much fewer problems in the variation of products and in the solubility than digitonin and is much less expensive than digitonin or lysophosphatidylcholine. The cholate extract may provide good starting material for a large scale purification of the muscarinic receptor. After the completion of experiments described here, Carson reported the solubilization of the muscarinic receptor by a mixture of sodium cholate and 1 M NaCl (4). The present finding that muscarinic ligands stabilize the receptor in the cholate extract allows us to avoid the use of a high concentration of salts which may interfere with the application of ion exchange chromatography.

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