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Binding Sites of Bromoacetylcholine in the Rat Diaphragm[1] (39520)

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Bromoacetylcholine, a halogenated analog of acetylcholine, has been shown to produce cholinergic effects similar to acetylcholine (1, 2). It has also been reported that bromoacetylcholine binds irreversibly to cholinergic receptors at nicotinic sites (3, 4). To explore the possibility of the use of bromoacetylcholine as a tagging agent for the isolation of cholinergic receptors, the binding sites of this compound in the rat diaphragm were studied. Since both bromoacetylcholine and its hydrolytic product, bromoacetate, may act as alkylating agent and bind at noncholinergic sites, the binding of bromoacetate in the rat diaphragm was also studied.

Materials and methods. Materials. Bromo-[1-^{14}C]acetylcholine perchlorate (^{14}C-BrACh) was synthesized according to the method described previously (5). Bromo-[1-^{14}C]-acetylbromide (specific activity: 1.1 mCi/mmole, New England Nuclear) was reacted with choline bromide to yield bromoacetylcholine bromide which was then converted into perchlorate salt with 70% perchloric acid in an absolute ethanol medium. The ^{14}C-BrACh synthesized had a specific activity of 1.0 mCi/mmole. Bromo-[1-^{14}C]-acetic acid (^{14}C-BrAcet) with a specific activity of 1.23 mCi/mmole was obtained from New England Nuclear. [^{3}H]Nicotine with a specific activity of 250 mCi/mmole and *N*-methyl-[^{3}H]acetylcholine with a specific activity of 250 mCi/mmole were obtained from Amersham/Searle.

Methods. Holtzman rats were anesthetized with ether. Their diaphragms were removed and weighed. The diaphragms were divided into three groups and each group of diaphragms was suspended in 5 vol of 100 m*M* phosphate buffer (pH 7.4) containing 0.95 μM diisopropylfluorophosphate (DFP). In group one, 0.2 m*M* (final concentration) of ^{14}C-BrACh was added. In group two, 0.2 m*M* ^{14}C-BrAcet was added. In group 3, 0.2 m*M* ^{14}C-BrACh and 0.2 m*M* bromoacetic acid (unlabeled) were added. The diaphragms were then incubated in these three media for 30 min at 25°C. Each group of diaphragms was then taken out of the incubation medium and resuspended in phosphate buffer to wash out the excess ^{14}C-BrACh or ^{14}C-BrAcet. The washing process was repeated two more times. The washed diaphragms from each group were then suspended in 5 vol of sucrose (250 m*M*)–phosphate (100 m*M*) buffer (pH 7.4) containing 0.95 μM DFP, minced, and homogenized with a Polytron (Brinkman Instruments) homogenizer. The homogenate from each group was centrifuged at 100,000*g* for 90 min at 4°. The pellet was resuspended in 5 vol of phosphate buffer and centrifuged again at 100,000*g* for 90 min. The process was repeated one more time to remove all the unbound ^{14}C-BrACh or ^{14}C-BrAcet. The pellet so obtained in each group was resuspended in 2 vol of phosphate buffer containing 1.5% Triton X-100 and kept overnight at 4°. It was centrifuged at 100,000*g* for 90 min at 4°C. The residue was discarded and the supernatant fractions containing ^{14}C-BrACh or ^{14}C-BrAcet and the solubilized membrane proteins were further purified for ^{14}C-BrACh-binding proteins by column chromatography.

The supernatant fractions containing ^{14}C-BrACh or ^{14}C-BrAcet and solubilized membrane proteins were passed through a Sephadex G-200 column (2.5 × 40 cm) preequilibrated with phosphate buffer containing 1.5% Triton X-100. The column was eluted with the same buffer and 1- to 3-ml fractions were collected using a fraction collector. The protein concentration in each

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fraction was determined by the method of Lowry *et al.* (6) with the modification that the white precipitates of Triton X-100 were removed by centrifugation before reading the blue color of the reaction. The validity of this method was confirmed by performing the determination on a known amount of albumin with and without Triton X-100 and also comparing this method with that of Wang and Smith (7). The radioactivity of each fraction was determined in a liquid scintillation counter.

In a fourth group of experiments, the ^{14}C-BrACh-binding proteins were isolated from rat diaphragms using the homogenization, centrifugation, and column chromatography on Sephadex G-200 procedures outlined above but without using ^{14}C-BrACh or ^{14}C-BrAcet as a tagging agent. The binding of [^{3}H]nicotine and [^{3}H]acetylcholine in this fraction was then studied by equilibrium dialysis. The inner compartment consisted of a bag of dialysis tubing containing 3.0 ml of binding protein solution (1 mg of protein/ml). The outer compartment contained 1000 ml of phosphate buffer with [^{3}H]nicotine (1.2×10^{-9} *M*) or [^{3}H]acetylcholine (5.4×10^{-10} *M*) in a stoppered Erlenmeyer flask. The dialysis was carried out in a cold room at 4° for 24 hr with constant stirring. Samples were taken from the dialysis bag and the outer compartment, and the radioactivity was counted.

Results. Figure 1 shows an elution profile of the rat diaphragm fraction containing solubilized membrane proteins and ^{14}C-BrACh when passed through a Sephadex G-200 column. It shows one peak of radioactivity (^{14}C-BrACh) and a parallel protein peak coming out immediatley after the void volume. The molar concentration of ^{14}C-BrACh in fraction No. 34 (peak of radioactivity) was 3.3 nmole/ml as calculated from the specific activity. The protein concentration in this fraction was 1.9 mg/ml. Assuming that ^{14}C-BrACh binds mole/mole to the receptor protein, the concentration of ^{14}C-BrACh-binding protein would be 1.7 nmole/mg of total proteins in this fraction.

Figure 2 shows an elution profile of the rat diaphragm fraction containing solubilized membrane protein and ^{14}C-BrAcet when passed through a Sephadex G-200 column. It shows one large and another small peak of radioactivity with parallel protein peaks. The first peak of radioactivity and protein was reached in fraction No. 43 because of the collection of smaller fractions as compared to the ^{14}C-BrACh column. The molar concentration of ^{14}C-BrAcet in fraction No. 43 was 2.1 nmole/ml as calculated from the specific activity. The protein concentration in this fraction was 2.4 mg/ml. Assuming that ^{14}C-BrAcet binds mole/mole to the receptor protein, the concentration of ^{14}C-BrAcet-binding protein would be 0.88 nmole/mg of total proteins in this fraction.

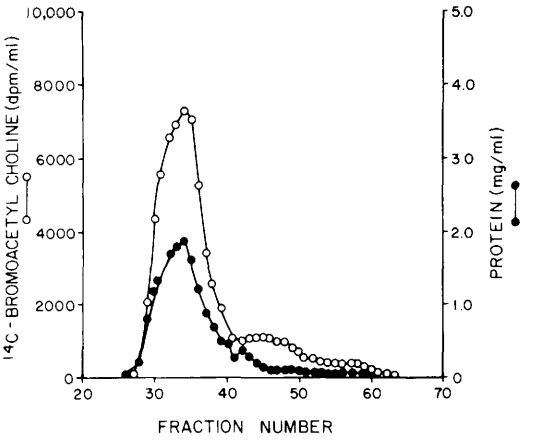

FIG. 1. Elution profile of ^{14}C-BrACh-treated rat diaphragm tissue on Sephadex G-200. the column was equilibrated with 100 m*M* phosphate buffer, pH 7.4, containing 1.5% Triton X-100. Elution was done with the same buffer. (●——●) Protein concentration; (○——○) ^{14}C-BrACh.

Figure 3 shows the elution profile of the rat diaphragm fraction containing solubilized membrane protein and ^{14}C-BrACh and bromoacetic acid (unlabeled) when passed through a Sephadex G-200 column. It shows one radioactivity peak of ^{14}C-BrACh and a parallel protein peak. The molar concentration of ^{14}C-BrACh in fraction No. 29 (peak of radioactivity) was 1.7 nmole/ml as calculated from the specific activity. The protein concentration in this fraction was 0.9 mg/ml. Assuming that ^{14}C-BrACh binds mole/mole to the receptor protein, the concentration of ^{14}C-BrACh-binding protein in the presence of bromoacetic acid would be 1.9 nmole/mg of total proteins. This value is not much different from the one obtained

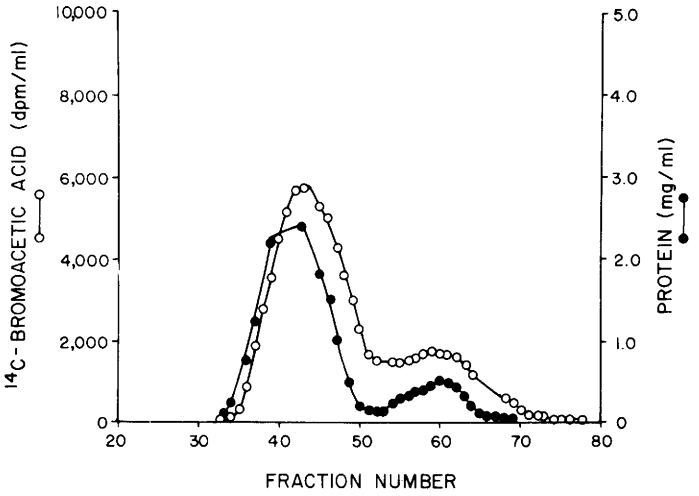

FIG. 2. Elution profile of ^{14}C-BrAcet-treated rat diaphragm tissue on Sephadex G-200. The column ws equilibrated with 100 m*M* phosphate buffer, pH 7.4, containing 1.5% triton X-100. Elution was done with the same buffer. (●——●) Protein concentration; (○——○) ^{14}C-BrAcet.

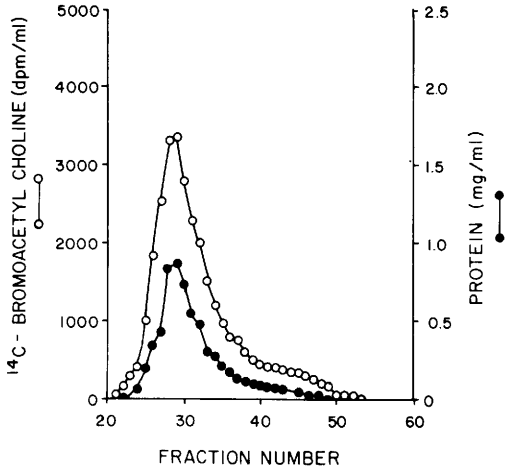

FIG. 3. Elution profile of ^{14}C-BrACh-treated (in presence of unlabeled BrAcet) rat diaphragm tissue on Sephadex G-200. The column was equilibrated with 100 m*M* phosphate buffer, pH 7.4, containing 1.5% Triton X-100. Elution was done with the same buffer. (●——●) Protein concentration; (○——○) ^{14}C-BrACh.

for binding of ^{14}C-BrACh in the absence of bromoacetic acid, indicating that bromoacetylcholine does not bind to bromoacetic acid-binding sites.

Table I shows the binding of [^{3}H]nicotine to the proteins of peak I (Fig. 1) when prepared without the tagging agent. The total protein concentration in the pooled fraction was 1.0 mg/ml. It is clear that there was a net increase of [^{3}H]nicotine inside the dialysis bag due to the binding of nicotine to the solubilized membrane proteins. Results obtained with ^{3}H-ACh were similar to those obtained with [^{3}H]nicotine (Table I).

Discussion. According to the results of the ^{14}C-BrACh-binding experiments, the solubilized fraction of rat diaphragms contained approximately 1.8 nmole of ^{14}C-BrACh-binding sites per milligram of total proteins. In our experiments 1% of rat diaphragm proteins was solubilized by Triton X-100 and collected in peak I of Sephadex G-200 column. Therefore, we estimate that the rat diaphragm has about 18 nmole of ^{14}C-BrACh-binding sites per gram of tissue. Our results indicate that ^{14}C-BrACh-binding sites are more than 1000-fold higher than that of α-bungarotoxin-binding sites which range from 1.3 to 4 pmole/g of rat diaphragm (8–12). The reason for more ^{14}C-BrACh-binding sites as compared to α-bungarotoxin is not known. One of the possible reasons would be that BrACh binds to nonspecific sites via alkylation just as BrAcet does. However, this possibility was ruled out because the binding of ^{14}C-BrACh was not affected by the combined treatment of the tissue with ^{14}C-BrACh plus cold BrAcet. Although BrACh produces biological effects of the cholinergic type which can be

TABLE I. BINDING OF [^{3}H]NICOTINE AND [^{3}H]ACETYLCHOLINE TO SOLUBILIZED CHOLINERGIC RECEPTOR PROTEIN.[a]

Compound	Free compound added into dialysis medium (A) (dpm/ml)	(n*M*)	Free compound plus compound bound to receptor proteins inside the dialysis bag (B) (dpm/ml)	Net amount of compound bound to receptor protein [(B)-(A)] (dpm/ml)	(n*M*)
[^{3}H]Nicotine	790 ± 50	1.2 ± 0.2	1180 ± 70	390 ± 22	0.69 ± 0.1
[^{3}H]Acetylcholine	300 ± 20	0.54 ± 0.1	664 ± 40	364 ± 22	0.66 ± 0.1

[a] The values given here are means ± SE of three different experiments.

blocked by cholinergic and cholinolytic agents (1–4), it is still possible that BrACh binds to proteins other than cholinergic receptors. On the other hand, it is also possible that the large molecule of α-bungarotoxin which binds to one of the cholinergic receptors also covers up the adjacent cholinergic receptors just like an umbrella (4) which tends to give a lower number of cholinergic binding sites.

Summary. To explore the possibility of using bromoacetylcholine as a tagging agent for the isolation of cholinergic receptors, the binding sites of this compound in the rat diaphragm were studied in the presence and absence of bromoacetate. The solubilized membrane fraction of rat diaphragm was found to contain 18 nmole of ^{14}C-BrACh-binding sites per gram of tissue. The presence of bromoacetate did not change the binding of ^{14}C-BrACh. The estimated binding sites of ^{14}C-BrACh were 1000-fold higher than those of α-bungarotoxin binding sites.

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