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BROMOACETYLCHOLINE AS AN AFFINITY LABEL OF THE ACETYLCHOLINE RECEPTOR FROM TORPEDO CALIFORNICA

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SUMMARY Bromoacetyl[methyl-^{3}H]choline is a highly specific label for the reduced acetylcholine binding site on the acetylcholine receptor from Torpedo californica. Only one of two binding sites per receptor monomer is susceptible to labeling. The labeled site is on the α chain of the receptor.

INTRODUCTION The affinity label 4-(N-maleimido)benzyltrimethylammonium iodide (MBTA) has been used to locate functionally significant acetylcholine binding sites on nicotinic acetylcholine receptors (1-4). The reaction of MBTA at the binding site depends on the prior reduction of a disulfide bond in the vicinity of the site; one of the sulfhydryls formed is specifically alkylated by MBTA. Reduction with dithiothreitol followed by alkylation with MBTA irreversibly inactivates receptor in intact cells from Electrophorus electric tissue (5,6). Other quaternary ammonium alkylating or acylating agents, however, activate reduced receptor (6,7). Bromoacetylcholine (BAC) is one such agent, which in addition to its effect in Electrophorus electroplax, has been shown to activate reduced receptor in frog muscle (8) and in rat sympathetic ganglia (9). These physiological results suggest that reduction followed by alkylation with radioactively tagged BAC may be an effective procedure for the specific labeling of the acetyl-

Abbreviations used: BAC, bromoacetylcholine; EDTA, ethylenediaminetetraacetic acid; MBTA, 4-(N-maleimido)benzyltrimethylammonium; TEA, triethanolamine; TLC, thin layer chromatography; Tris, tris-(hydroxymethyl)-aminomethane.

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choline receptors of electric tissue, skeletal muscle, and sympathetic ganglia.

METHODS Bromoacetylcholine bromide (BAC) was synthesized according to the method of Losee and Bernstein (unpublished), which is similar to the synthesis of the perchlorate salt (10). To 18.4 g of choline bromide is added dropwise, over 40 min, with stirring, 24.2 g of bromoacetyl bromide. The viscous mixture is stirred for an additional 75 minutes in an ice-bath. To the mixture is slowly added 75 ml of absolute ethanol. The white crystalline product is recrystallized twice from 400 ml of isopropanol. The yield is 67% and the product melts at 135°-136°C.

Bromoacetyl[methyl-^{3}H]choline ([^{3}H]BAC) bromide is synthesized on a microscale (2 µmoles) as follows: To a 10 ml screw-cap, conical, glass centrifuge tube are added 1 millicurie (14.4 nmoles) of [methyl-^{3}H]choline (New England Nuclear Corp.) and 100 µl of 20 mM (2 µmoles) choline bromide (recrystallized), both in ethanol. The ethanol is removed in vacuo. The tube is washed down with 100 µl of ethanol and the ethanol removed. This is repeated with 25 µl of ethanol. To the dry choline is added 25 µl of bromoacetylbromide; the tube is flicked gently and placed in ice for 60 to 80 min. Absolute ethanol (200 µl) is added to the tube, the contents of which are mixed and then partially dried in vacuo for about 15 minutes. Absolute ethanol (200 µl) is twice more added, mixed, and removed in vacuo, leaving a white product. The product is dissolved in 200 µl of water; 400 µl of ethyl ether is added; the tube is agitated and then centrifuged for 1 min in a clinical centrifuge. The ether phase is removed and discarded. The extraction with ether is repeated twice. The aqueous solution is lyophilized, and the product is finally dissolved in 2 ml of acetonitrile and stored in liquid nitrogen. The yield of [^{3}H]BAC is about 95% of theoretical.

To assay the concentration of [^{3}H]BAC in acetonitrile, an appropriate volume of the solution measured with a microcap is washed into additional acetonitrile and dried in vacuo. The [^{3}H]BAC is dissolved in water. The ester group was assayed by the method of Hestrin (11). To 100 µl of bromoacetylcholine (1 mM to 5 mM) is added 200 µl of a mixture of equal volumes of 2 M $NH_2OH \cdot HCl$ and 3.5 M NaOH. After at least 1 min, 100 µl of 4N HCl is added followed by 200 µl of 185 mM $FeCl_3$ in 50 mM HCl. The tubes are mixed after each addition. The absorbance at 540 nm is read promptly after the last addition. The concentration of [^{3}H]BAC is determined by comparison with a standard curve obtained with a fresh solution of recrystallized BAC.

The bromoacetyl group is assayed by reaction with dithiothreitol. To 50 µl of 1 mM dithiothreitol in water are added 25 µl of 200 mM NaCl, 100 mM triethanolamine (pH 8.0)(TEA buffer) and 25 µl of BAC (0.5 to 3 mM) in water. After 30 min at room temperature, 50 µl of 2 mM 5,5'-dithiobis(2-nitrobenzoate) (12) in TEA buffer is added. After 1 min, 2 ml of TEA buffer is added, and the absorbance is read at 412 nm. The absorbance is subtracted from that of the control prepared with no BAC, and the difference is compared with a standard curve obtained with a fresh solution of recrystallized BAC. A standard curve is necessary

because BAC reacts with less than the theoretically expected number of sulfhydryl groups due to the competing hydrolysis reaction. The product of hydrolysis, bromoacetate, reacts slowly with sulfhydryls compared with BAC. The agreement of the concentrations of [^{3}H]BAC determined by the two methods is a check on purity.

[^{3}H]BAC is also analyzed by ascending thin layer chromatography on Eastman cellulose chromagram sheets in a mixture containing n-butanol, acetic acid, water, 95% ethanol and ethyl acetate (80: 10: 30: 20: 14). The R_f values in this system are approximately 0.54 for BAC and 0.39 for choline. A small quantity of [^{3}H]BAC containing ~300,000 cpm is mixed with 10 nmoles of BAC and 20 nmoles of choline in acetonitrile and spotted on a 4 x 10 cm cellulose sheet. After the solvent front reaches the top of the sheet (~40 min), development is allowed to continue for another 20 min. The sheet is dried and placed in I_2 vapor to determine the positions of BAC and choline. The sheet is cut into thin strips which are counted in 100 µl of water and 5 ml of Scintisol Complete (Isolab). All radioactivity is recovered. Typically 10 to 15% of the radioactivity is associated with the choline spot and 85 to 90% with BAC.

The conditions for the use of [^{3}H]BAC to assay receptor were arrived at as with [^{3}H]MBTA (13,14) based on the rate constant for the reaction of BAC with reduced Torpedo receptor of 3 x 10^5 $M^{-1}min^{-1}$ and with 2-mercaptoethanol of 70 $M^{-1}min^{-1}$ determined as previously for MBTA (6,15). To quadruplicate 50 µl aliquots of receptor, containing 1 to 20 pmoles of α-neurotoxin binding sites, in 0.2% Triton X-100, 50 mM NaCl, 10 mM $NaPO_4$, 1 mM EDTA, 3 mM NaN_3 (pH 7.0) (TNP50) is added 50 µl 0.4 mM dithiothreitol in 0.2% Triton X-100, 150 mM NaCl, 20 mM Tris, 1 mM EDTA, 3 mM NaN_3 (pH 8.3) (TNT150). The final pH is 8.0. After incubation at 25° for 20 min, 25 µl of a mixture of 3 parts of TNP150 (like TNT150 except 10 mM $NaPO_4$ (pH 7.0) replaces Tris) and 2 parts of 0.56 M $NaPO_4$ (pH 6.75) are added to two of the samples. To the other two samples is added 25 µl of 8 µM α-neurotoxin (N. n. siamensis) in the same mixture of TNP150 and 0.56 M $NaPO_4$. Thus, the pH of the incubation is brought to 7.0 and half of the samples are protected with α-neurotoxin. After 20 min, 25 µl of 30 µM [^{3}H]BAC in TN150 (like TNP150 except no $NaPO_4$ or EDTA) is added, and after 3 min, 25 µl of 0.5 M 2-mercaptoethanol and 25 µl of 4 mg per ml succinylated, carboxymethylated lysozyme (13).

The samples are filtered by suction on glass fiber filters (Whatman GF/A) as follows: to the final incubation mixture is added 5 ml of a saturated solution of ammonium sulfate, the tube is shaken and the contents filtered. The tube is washed with 5 ml of 50% saturated ammonium sulfate. Five ml of 50% saturated ammonium sulfate is added directly to the filter. The filter is then washed twice with 5 ml of cold 1 M HCl and twice with 5 ml of acetone, and dried. The filters are placed in glass scintillation vials to which are added 100 µl water and 600 µl of NCS solubilizer. The vials are incubated for 1 hr at 50°, cooled, and, after addition of 10 ml of toluene based scintillant, counted.

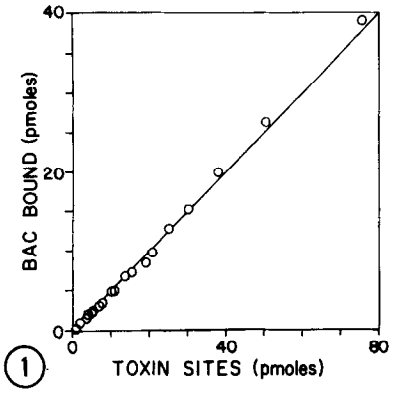
Aliquots of [^{3}H]BAC are added to washed filters in vials and prepared for counting as above in order to obtain the factor converting cpm to moles of [^{3}H]BAC. The total concentration of [^{3}H]choline in the standard, the sum of that in [^{3}H]BAC deter-

mined by assay and that in free [^{3}H]choline determined by TLC, is divided into the cpm to obtain the conversion factor.

When receptor is assayed in the presence of acetylcholinesterase, such as in membrane preparations, the esterase must be inhibited before incubation with [^{3}H]BAC. Pre-incubation of the samples for 30 minutes with 100 μM eserine or 100 μM diisopropylfluorophosphate are equally effective. BAC also spontaneously hydrolyzes at about 1% per min at pH 7.0 and 25°C. For this reason, stock aqueous solutions of BAC should be near pH 4 and kept cold. Preferably, an aliquot of [^{3}H]BAC in acetonitrile should be dried and redissolved in aqueous solution shortly before use.

RESULTS AND DISCUSSION The reaction of [^{3}H]BAC with reduced acetylcholine receptor under assay conditions is entirely specific in the sense that the reaction is completely blocked by prior saturation of receptor binding sites with α-neurotoxin. Even in impure preparations of receptor, the reaction of [^{3}H]BAC is highly specific; for example, in membrane containing 10% receptor, 80% of the total labeling by [^{3}H]BAC is blocked by α-neurotoxin. This high specificity is correlated with the enormously greater rate of reaction of [^{3}H]BAC with reduced receptor (5×10^3 $M^{-1}s^{-1}$) than with the sulfhydryl of, for example, 2-mercaptoethanol (1.2 $M^{-1}s^{-1}$). The rate of reaction of [^{3}H]BAC with average sulfhydryl groups on proteins is likely to be even slower than that with 2-mercaptoethanol. Even though MBTA reacts two orders of magnitude faster than BAC with reduced receptor, the ratio of the rate of reaction of MBTA with the reduced receptor binding site to its rate of reaction with "ordinary" sulfhydryls is less than this ratio for BAC; therefore under optimal conditions (13,14), there is less non-specific reaction of [^{3}H]BAC than of [^{3}H]MBTA.

The specific reaction of [^{3}H]BAC with reduced receptor is complete under assay conditions at 5 μM [^{3}H]BAC, final concentration, and is linear with receptor concentration (Fig. 1). The number of sites labeled by [^{3}H]BAC at saturation is one-half the number of α-neurotoxin binding sites, as with [^{3}H]MBTA (16).



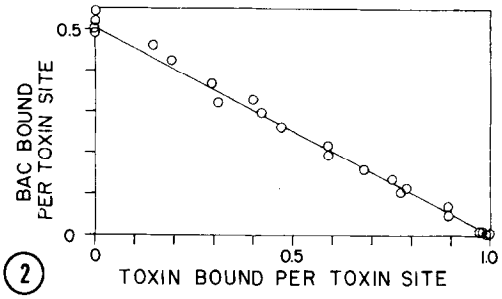


Figure 1. Specific labeling of purified receptor from Torpedo californica by [^{3}H]BAC as a function of receptor concentration expressed as α-neurotoxin binding sites (3). A line of slope 0.5 is drawn through the data points.

Figure 2. Degree of [^{3}H]BAC labeling of receptor as a function of the degree of occupancy of sites by toxin. Solutions containing 10 to 15 pmoles of toxin binding sites (1.5 to 2 μg of purified T. californica receptor) and 0 to 50 pmoles of N. n. siamensis α-neurotoxin were assayed with [^{3}H]BAC as described in Methods and for unoccupied toxin binding sites as described in ref. 3.

Prior reaction of the reduced receptor with BAC blocks all specific labeling with [^{3}H]MBTA and vice versa. Furthermore, as with [^{3}H]MBTA, the extent of specific reaction of reduced receptor with [^{3}H]BAC decreases linearly with the extent of occupation of the binding sites with α-neurotoxin (Fig. 2). This result implies that the two sites per receptor monomer which bind both acetylcholine and α-neurotoxins are different in that only one can react with [^{3}H]BAC and [^{3}H]MBTA (3). Finally, [^{3}H]BAC specifically and exclusively labels the α chain of Torpedo receptor (Fig. 3), just as does [^{3}H]MBTA (1,17).

[^{3}H]BAC and [^{3}H]MBTA appear to label the same site on the α chain; therefore, the activation of the receptor following reaction with BAC and the inhibition following reaction with MBTA are also both mediated by the same site. [^{3}H]BAC, which is

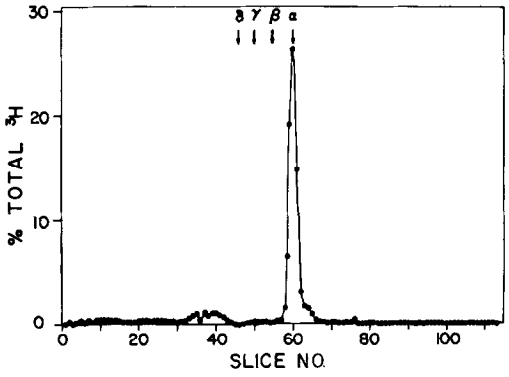


Figure 3. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of purified receptor from T. californica labeled with [^{3}H]BAC. Receptor (45 µg) was labeled under assay conditions, except with 10 µM [^{3}H]BAC, and the reaction was stopped with 5 mM N-ethylmaleimide. Succinylated, carboxymethylated lysozyme (40 µg) was added. The protein was precipitated and washed with acetone, dried, dissolved in sodium dodecyl sulfate solution, electrophoresed and the gels stained, sliced and counted as before (17). The positions of the receptor chains are shown by arrows.

easily synthesized and handled and is highly specific in its reaction with reduced receptor, should prove to be a valuable tool in further investigations of acetylcholine receptor binding sites and the mechanism of receptor activation.

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