Epibatidine Binds with Unique Site and State Selectivity to Muscle Nicotinic Acetylcholine Receptors*  

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Ligand binding sites in fetal (αβγδ) and adult (αεβδε) muscle acetylcholine receptors are formed by αδ, γε, or αε subunit pairs. Each type of binding site shows unique ligand selectivity due to different contributions by the δ, γ, or ε subunits. The present study compares epibatidine and carbamylcholine binding in terms of their site and state selectivities for muscle receptors expressed in human embryonic kidney 293 cells. Measurements of binding to αγ, αδ, and αε intracellular complexes reveal opposite site selectivities between epibatidine and carbamylcholine; for epibatidine the rank order of affinities is αε > αδ > αγ, whereas for carbamylcholine the rank order is αδ = αε > αγ. Because the relative affinities of intracellular complexes resemble those of receptors in the closed activatable state, the results suggest that epibatidine binds with unique site selectivity in activating the muscle receptor. Measurements of binding at equilibrium show that both enantiomers of epibatidine bind to adult and fetal receptors with similar but monophasic binding curves. However, when receptors are fully desensitized, epibatidine binds in a biphasic manner, with dissociation constants of the two components differing by more than 170-fold. Studies of subunit-omitted receptors (αβδγ, αβγε, and αδβε) reveal that in the desensitized state, the αδ interface forms the low affinity epibatidine site, whereas the αγ and αε interfaces form high affinity sites. In contrast to epibatidine, carbamylcholine shows little site selectivity for desensitized fetal or adult receptors. Thus epibatidine is a potentially valuable probe of acetylcholine receptor binding site structure and of elements that confer state-dependent selectivities of the binding sites.

Epibatidine recently emerged as one of the most potent nicotinic receptor ligands thus far characterized. In addition to its potent analgesic activity for certain neuronal acetylcholine receptor (AChR)â subtypes, epibatidine also induces antihyperalgesia and is approximately 200 times more potent than morphine as an analgesic (1–3). Despite the intense interest in the neuronal actions of epibatidine, its interactions with muscle-type AChR are not well characterized. Muscle-type AChRs are heteropentamers of homologous but functionally distinct subunits with compositions αβγδ in fetal and αεβδε in adult muscle (4). Within the pentamer are two binding sites for acetylcholine (ACh); one is formed at the αδ subunit interface, whereas the other is formed at the αγ interface in the fetal receptor and at the αε interface in the adult. Each type of binding site displays distinct selectivities for agonists and competitive antagonists. For example, carbamylcholine binds 30-fold less tightly to the αγ binding site than to the αδ and αε binding sites owing to different contributions of the γ, δ, and ε subunits (5, 6). Previous studies used γ-δ and ε-δ subunit chimeras to identify residues in these subunits which confer site selectivity for curare, conotoxin M1, and carbamylcholine (6–9). The overall findings show that four loops, well separated in the primary sequence of the non-α subunits, contribute to the binding site interface (10).

To investigate further the structure of the ligand binding site, the present work examines binding of epibatidine to sites of fetal and adult muscle AChRs. We reasoned that a structurally constrained ligand such as epibatidine would be less able to accommodate differences in binding site structure and thus might show greater or different selectivity compared with flexible agonists such as carbamylcholine and ACh. We show that epibatidine binds with novel site selectivity to muscle AChRs, selecting strongly for the αε and αγ binding sites over the αδ site. Further, unlike carbamylcholine, epibatidine maintains strong site selectivity when the receptor is converted to the high affinity desensitized state. Owing to its unique site and state selectivity, epibatidine is a potentially valuable probe of binding site structure and of elements that confer state-dependent selectivity.

EXPERIMENTAL PROCEDURES  

Materials—[125I]-Labeled α-bungarotoxin (α-BTX) was obtained from NEN Life Science Products. Prolidifen and (−), (+), and (−)-epibatidine were purchased from Research Biochemicals Inc. The 293 human embryonic kidney-293 (HEK-293) cell line was obtained from the American Type Culture Collection. Sources of the mouse AChR subunits were as described previously (7, 11).  

Expression of Receptor Complexes—HEK-293 cells were transfected at about 50% confluence using calcium phosphate precipitation as described previously (7). Cells expressing intracellular αγ, αε, or αδ complex or cell surface pentamers (αβδγ or αβδε) were maintained at 37 °C for 48 h after transfection. Cells expressing triplet pentamers (αβδγ, αβγε, or αδβε) were maintained at 37 °C for 24 h after transfection and then at 31 °C for 48 h.  

Ligand Binding Measurements—Cells expressing surface pentamers were harvested by gentle agitation in phosphate-buffered saline containing 5 mM EDTA, centrifuged at 1000 x g for 1 min, and resuspended in potassium Ringer’s solution (140 mM KCl, 5.4 mM NaCl, 1.8 mM CaCl2, 1.7 mM MgCl2, 25 mM HEPES, 30 mg/ml bovine serum albumin, adjusted to pH 7.4 with 10–11 mM NaOH). Cells expressing intracellular complexes were permeabilized with saponin before harvesting (6). Agonist binding was determined by competition against the initial rate of [125I]-labeled α-BTX binding as described previously (6). In brief, the receptor complexes were equilibrated with agonist for 40 min before addition of 5 nM [125I]-labeled α-BTX. The cells were then incubated for a
further 20–40 min to allow occupancy of at most 50% of the binding sites by 125I-labeled α-BTX. The total number of sites was determined by incubating with 25 nM 125I-labeled α-BTX for 20–40 min and subtracting a blank determined in the presence of 10 mM carbamylcholine. The cells were harvested using a Brandell cell harvester and counted in a gamma counter.

Data Analysis—We fit the following two equations to our data using Prism 2.0 (GraphPad Software):

\[ 1 - \text{Fractional occupancy} = 1 - \frac{[L]^n}{[L]^n + K^e} \]  
\[ 1 - P \frac{[L]}{K_a + [L]} - (1 - P) \frac{[L]}{K_d + [L]} \]  

where [L] is the concentration of competing ligand, K is an apparent dissociation constant, n is the Hill coefficient, K_a and K_d are intrinsic dissociation constants, and P is the fraction of sites with dissociation constant K_a.

RESULTS

Binding to Intracellular Complexes—Transfection of HEK-293 cells with α and γ, δ, or ε subunit results in robust expression of agonist-displaceable intracellular α-BTX binding sites. To determine selectivity of epibatidine for these intracellular complexes, we measured binding by competition against 125I-labeled α-BTX binding. Both enantiomers of epibatidine bind with highest affinity to αε complexes, intermediate affinity to αγ, and lowest affinity to αδ (Fig. 1A and Table I). Although both isomers of epibatidine bind with the same affinity to αε complexes, the (+)-isomer binds with significantly higher affinity to αγ and αδ complexes compared with the (−)-isomer. By contrast, carbamylcholine shows opposite site selectivity to epibatidine, binding with high affinity to αδ and αε and low affinity to αγ complexes (Fig. 1B and Table I). In this and previous studies (6) we found that agonists bind to intracellular complexes with Hill coefficients significantly less than 1. Because complexes of an α and a non-α subunit should form only one type of binding site, the shallow binding curves we observe indicate some type of site heterogeneity. Despite this heterogeneity, selectivities of carbamylcholine and acetylcholine for intracellular complexes (5, 6) coincide with selectivities of receptors in the closed activable state determined by single channel kinetic analysis (12, 13). Further, intracellular complexes show the same rank order of affinity for the competitive antagonist d-tubocurarine to that observed in native receptors, αγ ≈ αε > αδ (14). Intracellular complexes, unlike fully assembled native receptors, do not enter a high affinity desensitized state and are presumably incapable of channel gating (6). Thus the binding site selectivity of intracellular complexes may most closely resemble that of closed activable receptors.

Binding to Cell Surface Pentamers—To assess site selectivity of epibatidine for receptors containing the full complement of subunits, we expressed adult or fetal receptors in HEK-293 cells and measured binding to intact cells under control and desensitization conditions (Fig. 2 and Table II). Under control conditions, both enantiomers of epibatidine displace 125I-labeled α-BTX with much higher affinity than carbamylcholine. Although fetal and adult receptors bind epibatidine with similar affinities, fetal receptors distinguish between (+)- and (−)-epibatidine, whereas adult receptors do not. Hill coefficients for epibatidine are only −0.8 compared with values for carbamylcholine of −1.3. Although a Hill coefficient less than 1 implies multiple binding sites, for agonists equilibrium binding cannot be interpreted using a simple two site model. At equilibrium, agonist binding is determined by affinities of the sites in the resting, open, and desensitized states as well as the allosteric constants governing channel opening and desensitization (see under “Discussion”).

To measure binding of agonist to a single functional state of the receptor, we used the local anesthetic proadifen to promote entry into the high affinity desensitized state (15, 16). When fully desensitized with proadifen, adult and fetal receptors bind carbamylcholine with high affinity and Hill coefficients close to 1, indicating binding to a single class of high affinity sites (Fig.
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FIG. 2. Binding of carbamylcholine and epibatidine to cell surface pentamers. Fetal (αβδγδ) (A) and adult (αβδε) (C) receptors bind (+)- and (−)-epibatidine in a monophasic manner under equilibrium conditions (no additions). In contrast, the desensitized states of these receptors have clearly biphasic binding curves for both isomers of epibatidine (+ proadifen). In all cases dissociation constants of the two desensitized epibatidine binding sites differ by more than 170-fold. B (fetal) and D (adult), under both equilibrium (no additions) and desensitizing (+ proadifen) conditions carbamylcholine binds in a monophasic manner. Receptors were transiently expressed in HEK-293 cells and agonist binding determined as described under “Experimental Procedures.” For fetal receptors, desensitization was induced by 200 μM proadifen and for adult receptors by 60 μM proadifen. The curves are fits of Equation 1 or 2 to the data (see under “Experimental Procedures”) with parameters given in Table II. In all cases the data are means of three to five experiments with standard errors of less than 5% of total.

TABLE II

| Agonist | Receptor type | K1 (nM) | K2 (nM) | P | n |
|---------|---------------|---------|---------|---|---|
| Carbamylcholine | Adult | 26320 ± 1431 | 29310 ± 1140 | 1.3 ± 0.08 | 60 μM for adult receptors and 200 μM for fetal receptors. |
| Carbamylcholine | Fetal | 1070 ± 85 | 191 ± 17 | 0.91 ± 0.06 | 60 μM for adult receptors and 200 μM for fetal receptors. |
| (+)-Epibatidine | Adult | 913 ± 55 | 1330 ± 135 | 0.7 ± 0.03 | 60 μM for adult receptors and 200 μM for fetal receptors. |
| (+)-Epibatidine | Fetal | 913 ± 55 | 1330 ± 135 | 0.7 ± 0.03 | 60 μM for adult receptors and 200 μM for fetal receptors. |
| (+)-Epibatidine | Adult | 3.8 ± 0.4 | 837 ± 4 | 0.41 ± 0.00 | 60 μM for adult receptors and 200 μM for fetal receptors. |
| (+)-Epibatidine | Fetal | 1.05 ± 0.09 | 182 ± 0.9 | 0.5 ± 0.00 | 60 μM for adult receptors and 200 μM for fetal receptors. |
| (+)-Epibatidine | Adult | 903 ± 67 | 182 ± 0.9 | 0.41 ± 0.00 | 60 μM for adult receptors and 200 μM for fetal receptors. |
| (+)-Epibatidine | Fetal | 513 ± 18 | 151 ± 20 | 0.45 ± 0.01 | 60 μM for adult receptors and 200 μM for fetal receptors. |

2, B and D, and Table II; however, see below). Epibatidine, on the other hand, binds in a distinctly biphasic manner in the presence of proadifen; fits to a two-site equation (Equation 2) yield intrinsic dissociation constants different by more than 170-fold (Fig. 2, A and C and Table II). Thus, whereas carbamylcholine does not distinguish between the αγ and αδ binding...
sites in the desensitized fetal receptor nor between the \( \alpha \varepsilon \) and \( \alpha \delta \) sites in the desensitized adult receptor, epibatidine selects strongly between the two binding sites of each receptor.

To determine which subunits form the high affinity sites in fetal and adult receptors, we expressed triplet \( \alpha_2\beta_2\gamma_2 \), \( \alpha_2\beta_2\varepsilon_2 \) or \( \alpha_2\beta_2\varepsilon_2 \) receptors and measured agonist binding under control and desensitizing conditions (Fig. 3 and Table III). Unlike native receptors, the two binding sites in triplet receptors are formed by \( \alpha \) and identical non-\( \alpha \) subunits (17). Under control conditions both enantiomers of epibatidine bind more tightly to \( \alpha_2\beta_2\gamma_2 \) and \( \alpha_2\beta_2\varepsilon_2 \) than to \( \alpha_2\beta_2\varepsilon_2 \) pentamers. Carbacholyloline, by contrast, binds more tightly to \( \alpha_2\beta_2\varepsilon_2 \) than to \( \alpha_2\beta_2\gamma_2 \) or \( \alpha_2\beta_2\varepsilon_2 \) pentamers. In the presence of proadifen, the affinities of epibatidine and carbacholyloline for \( \alpha_2\beta_2\gamma_2 \) pentamers increase markedly, as with native pentamers, whereas affinities for \( \alpha_2\beta_2\varepsilon_2 \) pentamers are only slightly affected; similar results were obtained for carbacholyloline by Sine and Claudio (17).

Comparison of epibatidine binding to desensitized fetal and the corresponding \( \alpha_2\beta_2\gamma_2 \) and \( \alpha_2\beta_2\varepsilon_2 \) triplet receptors shows that the high affinity component corresponds to the \( \gamma \) site, whereas the low affinity component corresponds to the \( \varepsilon \) site. Although expression of \( \alpha_2\beta_2\varepsilon_2 \) pentamers was too low to accurately measure under desensitizing conditions, at equilibrium epibatidine binds to these receptors 30-fold more tightly than to the low affinity binding site of desensitized adult receptors. Thus in the desensitized adult receptor, the high affinity component corresponds to the \( \varepsilon \) site, whereas the low affinity component corresponds to the \( \delta \) site.

**Effects of Proadifen: Concentration Dependence**—Our preliminary experiments with \( (\pm) \)-epibatidine tested a range of proadifen concentrations to determine an optimum concentration for promoting desensitization. As observed previously, increasing proadifen concentrations progressively increase carbacholyloline affinity to approach a limiting value corresponding to the agonist affinity for the desensitized state (15). For the fetal receptor, the limiting carbacholyloline affinity is reached at 200 \( \mu \)M proadifen; at all concentrations of proadifen carbacholyloline binding is well described by Hill coefficients of 1 or greater, implying binding to a single class of high affinity sites (Fig. 4 and Table IV). For the adult receptor the limiting affinity is achieved by only 30–60 \( \mu \)M proadifen; at proadifen concentrations up to 60 \( \mu \)M, carbacholyloline binding is described by Hill coefficients of 0.7–0.8. As carbacholyloline affinity reaches a limit at lower proadifen concentrations, the observed broadening of the binding curve at high proadifen concentrations likely results from an action distinct from enhancement of desensitization.

At both fetal and adult receptors, epibatidine binds in a distinctly biphasic manner in the presence of proadifen; biphasic binding is observed at all proadifen concentrations greater than 10 \( \mu \)M. Furthermore, for both sites of the adult receptor, epibatidine affinity increases to reach a limiting value with proadifen concentrations of 30 \( \mu \)M or greater, as observed for carbacholyloline (Table III). For the fetal receptor, the maximum increase in affinity is reached at 60–100 \( \mu \)M proadifen, similar to results with carbacholyloline (Table IV). Thus for both fetal and adult receptors, a given concentration of proadifen increases carbacholyloline and epibatidine affinities to similar extents.

We noticed for epibatidine, however, that the relative weights of the high and low affinity components depend on proadifen concentration (Fig. 4 and Table IV). For fetal receptors in the presence of 30 \( \mu \)M proadifen, the high affinity component dominates, whereas for adult receptors the two compo-

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**TABLE III**

| Agonist | Receptor | \( K_d \) | \( n \) |
|---------|----------|---------|--------|
| Carbacholyloline | \( \alpha_2\beta_2\gamma_2 \) | 7750 ± 477 | 1.0 ± 0.05 |
| | \( \alpha_2\beta_2\varepsilon_2 \) | 1711 ± 130 | 0.62 ± 0.03 |
| | \( \alpha_2\beta_2\varepsilon_2 \) | 6720 ± 82 | 0.75 ± 0.06 |
| | \( \alpha_2\beta_2\varepsilon_2 \) | 811 ± 97 | 0.81 ± 0.06 |
| | \( \alpha_2\beta_2\varepsilon_2 \) | 39 ± 2.6 | 0.9 ± 0.05 |
| | \( \alpha_2\beta_2\varepsilon_2 \) | 440 ± 35 | 0.54 ± 0.02 |
| | \( \alpha_2\beta_2\varepsilon_2 \) | 24 ± 2.3 | 0.7 ± 0.04 |
| | \( \alpha_2\beta_2\varepsilon_2 \) | 0.7 ± 0.06 | 1 ± 0.07 |
| | \( \alpha_2\beta_2\varepsilon_2 \) | 426 ± 34 | 0.7 ± 0.02 |
| | \( \alpha_2\beta_2\varepsilon_2 \) | 12 ± 0.8 | 1 ± 0.06 |
| | \( \alpha_2\beta_2\varepsilon_2 \) | 392 ± 33 | 0.59 ± 0.03 |
| | \( \alpha_2\beta_2\varepsilon_2 \) | 18 ± 2 | 0.73 ± 0.06 |
| | \( \alpha_2\beta_2\varepsilon_2 \) | 0.35 ± 0.00 | 1.3 ± 0.00 |
| | \( \alpha_2\beta_2\varepsilon_2 \) | 390 ± 38 | 0.68 ± 0.04 |
When proadifen concentration is increased to 200 μM, fetal receptors show equally weighted low and high affinity components, whereas adult receptors show a dominant low affinity component. These results indicate that in addition to promoting desensitization, proadifen noncompetitively inhibits the binding of 125I-labeled

![Figure 4](http://www.jbc.org/)

**Fig. 4. Concentration dependence of proadifen-induced desensitization.** A, binding of (±)-epibatidine to the fetal receptor. B, binding of carbamylcholine to the fetal receptor. C, binding of (±)-epibatidine to the adult receptor. D, binding of carbamylcholine to the adult receptor. The curves are fits of Equation 1 or 2 (see under “Experimental Procedures”) to the data (means of three to five experiments). Curve fit parameters are given in Table IV.

### Table IV

**Dependence of desensitized state agonist binding on proadifen concentration**

Adult or fetal receptors were incubated with the indicated concentration of proadifen, and agonist affinity was determined. The parameters (K₁, K₂; dissociation constants; n, Hill coefficient; P, fraction of sites with dissociation constant Kᵡ) are derived from fits of Equation 1 or 2 (see under “Experimental Procedures”) to the data in Fig. 4 and are expressed ± S.E. For the adult receptor, carbamylcholine binding curves at 100 and 200 μM proadifen were fit using both the Hill equation and the sum of two equally weighted sites.

| Receptor type | Proadifen (μM) | (±)-Epibatidine | Carbamylcholine |
|---------------|----------------|-----------------|-----------------|
|               | K₁ (nM) | K₂ (nM) | n | P   | K₁ (nM) | K₂ (nM) | n |
| Adult         |         |             |   |     |         |         |   |
| 0             | 1797 ± 68 | 0.76 ± 0.02 |   |     | 28410 ± 1350 | 1.1 ± 0.05 |
| 10            | 8.4 ± 1.2 | 580 ± 71 | 0.45 ± 0.03 |   | 6310 ± 400 | 1.46 ± 0.12 |
| 30            | 2.4 ± 0.17 | 807 ± 50 | 0.46 ± 0.01 |   | 1266 ± 62 | 1.1 ± 0.05 |
| 60            | 1.4 ± 0.14 | 778 ± 38 | 0.33 ± 0.01 |   | 1020 ± 61 | 1.03 ± 0.06 |
| 100           | 1.77 ± 0.25 | 789 ± 37 | 0.24 ± 0.01 |   | 858 ± 63 | 0.74 ± 0.04 |
| 200           | 1.14 ± 0.33 | 616 ± 39 | 0.18 ± 0.01 |   | 234 ± 35 | 3180 ± 493 |
| 100           | 216 ± 38 | 0.98 ± 0.03 |   |     | 873 ± 59 | 0.8 ± 0.04 |
| 200           | 216 ± 38 | 293 ± 47 | 2588 ± 417 |   | 293 ± 47 | 2588 ± 417 |
| Fetal         | 811 ± 24 | 0.98 ± 0.03 |   |     | 23720 ± 880 | 1.3 ± 0.06 |
| 10            | 13 ± 1.3 | 205 ± 52 | 0.72 ± 0.04 |   | 5030 ± 355 | 1.45 ± 0.13 |
| 30            | 1.9 ± 0.11 | 216 ± 38 | 0.75 ± 0.01 |   | 745 ± 76 | 1.14 ± 0.08 |
| 60            | 1.06 ± 0.01 | 252 ± 48 | 0.65 ± 0.02 |   | 388 ± 68 | 1.11 ± 0.07 |
| 100           | 1.57 ± 0.14 | 298 ± 36 | 0.57 ± 0.02 |   | 333 ± 56 | 1.1 ± 0.06 |
| 200           | 0.64 ± 0.07 | 215 ± 18 | 0.45 ± 0.01 |   | 218 ± 13 | 1.04 ± 0.07 |
Further 30 min. Nonspecific binding was determined in the presence of 300 B in the absence of proadifen was typically 200–600 fmol/10-cm plate of cells. 

125I-Labeled measurements do not directly reflect intrinsic agonist affinities of a particular binding site. 

The results show that increasing proadifen concentrations reduce the rate of toxin binding and the total number of sites approximately in parallel (Fig. 5). As these determinations reflect contributions of both sites in each receptor type, they do not reveal which of the αd, αε, or αγ sites are preferentially affected. However, our results on site selectivity of epibatidine show that proadifen decreases the rate and number of BTX sites in a site-selective manner (Fig. 4); site selectivity for proadifen follows the rank order, αε > αd > αγ.

**DISCUSSION**

The present study examines various combinations of AChR subunits to characterize the interaction of (+)- and (-)-epibatidine with the three types of binding sites of muscle AChRs. Our results reveal that epibatidine is unique among AChR agonists thus far characterized in that it binds with high affinity to αε and αγ sites and low affinity to αd sites. Further, unlike classical agonists, epibatidine selects between these binding sites in the desensitized state of the receptor. 

Binding of agonists to the AChR can be described by the following conventional scheme:

\[
2A + R \rightleftharpoons A + AR \xrightleftharpoons{K_2}{K_1} A_R \rightleftharpoons A_O \]

\[
2A + D \rightleftharpoons A + AD \xrightleftharpoons{K_{d2}}{K_{d1}} A_D \]

**SCHEME 1**

where R is the closed, activatable state of the receptor, O is the open state, D is the desensitized state, and A is agonist. K_1 and K_2 are the dissociation constants for the activatable state binding sites, and K_{d1} and K_{d2} are the dissociation constants for the desensitized state binding sites, M is an allosteric constant governing the transition between the activable and desensitized states, and θ is the channel opening equilibrium constant (18, 19). Because apparent affinity measured in equilibrium binding assays depends on the four intrinsic dissociation constants as well as the state equilibrium constants, such measurements do not directly reflect intrinsic agonist affinities of a particular binding site.

Nevertheless, estimates of parameters in the upper limb of Scheme 1 can be derived from the kinetics of single channel currents. Single channel kinetic analysis established that K_1 and K_2 for ACh differ by 30–100-fold in fetal mouse and Torpedo receptors (12, 20) and by 5-fold in adult human receptors (21) but are indistinguishable in adult mouse receptors (13). Here, we observe a similar selectivity pattern for carbachol binding to mouse intracellular complexes: αd and αε complexes bind agonist with high affinity but αγ complexes bind agonist 20–30-fold less tightly. As in previous studies (6) we find that intracellular complexes bind agonists with Hill coefficients less than 1. Because complexes of an α and a single type of non-α subunit should form only one class of binding site, a Hill coefficient less than 1 suggests heterogeneous binding sites. Heterogeneity of intracellular complexes could arise from 1) formation of tetrameric complexes of the form αααα, where x is γ, δ, or ε (22); 2) access of agonist to immature or partially degraded complexes due to permeabilization by saponin; 3) a fixed proportion of the receptors arrested in the low affinity activable and high affinity open channel and desensitized states. Despite this apparent heterogeneity, carbachol selectivity determined in our binding assay parallels selectivity of the activatable state of the native receptor determined from single channel kinetic analysis (12, 13). The overall results suggest that the ligand selectivities of αγ, αd, and αε complexes most closely resemble those of the corresponding closed activatable binding sites in native pentamers. Thus in activating the receptor, epibatidine likely binds with high affinity to the αγ and αε sites but with low affinity to the αd site.

Unlike the closed activatable state, the desensitized state can be studied in isolation by measuring agonist binding in the presence of a desensitizing agent such as proadifen (15, 16). Here we observe that carbachol does not distinguish between the desensitized αγ, αd, and αε binding interfaces. This is evidenced by the monophasic binding curves of desensitized fetal and adult receptors and the similar affinities of desensitized αβ_2δ_2 and αβ_2γ_2 triplet receptors. Similar results were obtained previously for carbachol and ACh (17, 23). Epibatidine, by contrast, distinguishes between the two sites in desensitized fetal and adult receptors.

Previous studies showed that αβ_2γ_2 and αβ_2δ_2 pentamers bind a range of ligands with affinities similar to those of the two sites in native fetal receptors (7, 17), suggesting that binding sites of subunit-omitted receptors are close in structure to binding sites in the corresponding native receptor. For epibatidine, we observe similar dissociation constants for desensitized αβ_2γ_2 receptors and the high affinity component of desensitized fetal receptors and similar dissociation constants for desensitized αβ_2δ_2 receptors and the low affinity components of Epibatidine Selectivity at Muscle Nicotinic Receptors

![Figure 5](http://www.jbc.org/)

**FIG. 5.** Proadifen inhibits binding of 125I-labeled α-bungarotoxin. A, proadifen reduces the total number of binding sites labeled by 125I-labeled α-BTX in a concentration-dependent manner. Cells expressing either fetal or adult AChR were preincubated for 40 min with the indicated concentration of proadifen. 125I-Labeled α-BTX was then added to a final concentration of 25 nM and the incubation continued for a further 30 min. Nonspecific binding was determined in the presence of 300 nM α-BTX. For both adult and fetal receptors, maximal binding in the absence of proadifen was typically 200–600 fmol/10-cm plate of cells. B, proadifen slows the initial rate of 125I-labeled α-BTX binding. Cells were preincubated with proadifen for 40 min, after which 125I-labeled α-BTX was added to a final concentration of 5 nM. The binding reaction was allowed to continue for 30 min. In both panels the data are the means of three experiments. The error bars represent the standard error.
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