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Pharmacological and Immunological Identification of Native $\alpha 7$ Nicotinic Receptors: Evidence for Homomeric and Heteromeric $\alpha 7$ Receptors

Raed A. El-Hajj, Susan B. McKay, and Dennis B. McKay

Division of Pharmacology The Ohio State University College of Pharmacy 500 West 12th Avenue Columbus, Ohio 43210

Abstract

Controversy surrounds the expression of $\alpha 7$ nicotinic acetylcholine receptors (nAChRs) in adrenal chromaffin cells. In these studies, $\alpha 7$ nAChRs expressed in bovine adrenal chromaffin cells are investigated. Using radiolabeled ligand binding techniques, [125 I] α -bungarotoxin (α BGT) binding reaches equilibrium within 4 hours and is saturable with a K_d value of 4.2 nM. Using homologous competition experiments, the K_i for binding of α BGT was 1.9 nM. These data are consistent with the expression of homomeric $\alpha 7$ nAChRs. Methyllycaconitine (MLA), which binds $\alpha 7$ nAChRs with high affinity, inhibits [125 I] α BGT binding in a concentration-dependent manner with a K_i of 30.6 nM; this value is ~ 10 fold higher than the reported affinity of MLA for $\alpha 7$ nAChRs. We also document the ability of bromoacetylcholine (brACh) to alkylate $\alpha 7$ nAChRs, as has been previously demonstrated for bovine adrenal $\alpha 3\beta 4$ nAChRs. When adrenal nAChRs are immunoprecipitated with mAb 319, an antibody which recognizes $\alpha 7$ nAChR protein, and then probed with mAb 319 using Western blot analysis, a single band of ~ 53 kD is identified. When adrenal nAChRs are immunoprecipitated with mAb35, an antibody which recognizes $\alpha 3$ and $\alpha 5$ nAChR proteins, and then probed with mAb319 using Western blot analysis, a single band of ~ 53 kD is identified. Together, these results support the expression of $\alpha 7$ nAChRs in bovine adrenal chromaffin cells. However, these data suggest that the subunit composition of some of these receptors may include heteromeric $\alpha 7$ nAChRs.

Keywords

Nicotinic acetylcholine receptor; binding; adrenal medulla; methyllycaconitine; α -bungarotoxin; $\alpha 7$

INTRODUCTION

Neuronal nAChRs are composed of multiple subunits; currently, nine neuronal nAChR α subunits and three β subunits have been described in vertebrates. These subunits combine into pentameric ligand-gated ion channels with distinct pharmacological and functional properties. Because of this nAChR subunit diversity, the determination of precise subunit compositions of native neuronal nAChRs has been difficult. The chromaffin cells isolated from the adrenal medulla provide a unique model to investigate native neuronal nAChRs. In bovine adrenal chromaffin cells, immunological and pharmacological characterization supports the expression

Correspondence should be addressed to: Dennis B. McKay, Ph.D. Division of Pharmacology The Ohio State University College of Pharmacy 500 West 12th Avenue Columbus, Ohio 43210 Telephone #: (614) 292-3771 Telefax #: (614) 292-9083 Email: mckay.2@osu.edu.

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of heteromeric, $\alpha 3\beta 4^*$ nAChRs (Free et al., 2002; Free et al., 2003; Gu et al., 1996), the asterisk indicating the possible presence of additional subunits (Lukas et al., 1999). These $\alpha 3\beta 4^*$ nAChRs are the principal receptors mediating adrenal neurosecretion.

$\alpha 7$ nAChRs are homomeric nAChRs found both in the central nervous system (CNS) and peripheral nervous system (PNS). These receptors are distinguished among nAChRs by their high permeability to Ca^{++} , their rapidly activating and desensitizing currents, and their high affinity for α -bungarotoxin (α BGT) and methyllycaconitine (MLA) (Seguela et al., 1993). Several studies have shown that $\alpha 7$ nAChRs contribute to a number of neurological functions, such as improving memory (Meyer et al., 1998), sensory gating (Martin et al., 2004) and control of the cholinergic anti-inflammatory pathway (for review, Ulloa, 2005). Despite the identification and characterization of homomeric $\alpha 7$ nAChRs in the CNS, $\alpha 7$ nAChRs in the PNS appear more diverse, where evidence exists for the expression of heteromeric $\alpha 7$ -containing nAChRs (Virginio et al., 2002; Yu and Role, 1998).

The adrenal medulla is thought of as part of the sympathetic nervous system, sharing several characteristics with sympathetic neurons (Marley and Prout, 1965). Acetylcholine released from the splanchnic nerve activates nAChRs of the adrenal medulla, releasing epinephrine into the bloodstream, triggering the 'flight and fight' response. The principal receptors mediating adrenal neurosecretion are $\alpha 3\beta 4^*$ nAChRs. Evidence also exists for the expression of $\alpha 7$ nAChRs in adrenal medullary cells. In bovine adrenal medulla, molecular studies have shown the presence of RNA for $\alpha 7$ nAChR subunits (Campos-Caro et al., 1997), although expression of $\alpha 7$ proteins in these cells has yet to be documented. Electrophysiological studies have shown that bovine medullary cells express nAChRs with channel characteristics similar to $\alpha 7$ nAChRs with some limited involvement with neurosecretion (Lopez et al., 1998). However, [^{125}I] α BGT binding studies have also suggested that adrenal chromaffin cells from rat do not express $\alpha 7$ subunits (Di Angelantonio et al., 2000). In the studies reported here, pharmacological and immunological approaches are used to characterize native $\alpha 7$ nAChRs expressed in bovine adrenal medullary cells. These studies document the utility of bovine adrenal chromaffin cells and [^{125}I] α BGT for the study of native $\alpha 7$ nAChRs and support the expression of homomeric $\alpha 7$ nAChRs and possibly a smaller population of heteromeric, $\alpha 7$ -containing, nAChRs.

MATERIALS AND METHODS

Materials

(-)-nicotine hydrogen tartrate, methyllycaconitine, α -bungarotoxin, mAb319 (anti-nAChR monoclonal antibody), iodoacetamide, polyethyleneimine (PEI), Nonidet-P40 (NP-40), Bradford reagent, were obtained from Sigma Chemical Co. (St. Louis, MO). Ethylenebis (oxyethylenenitrilo)tetraacetic acid (EGTA) was purchased from Amresco. All other reagents were purchased from Fisher Scientific (Pittsburgh, PA). [^{125}I]- α -bungarotoxin ([^{125}I] α BGT) was purchased from Perkin-Elmer (Boston, MA). Glass fiber filters were obtained from Brandel (Gaithersburg, MD). Mammalian protein extraction reagent (MPER) reagent was obtained from Pierce (Rockford, IL). mAb35 (anti-nAChR monoclonal antibody) was isolated and purified from a hybridoma cell line obtained from the American Type Culture Collection (Rockville, MD) using techniques described previously (Gu et al., 1996).

Isolation and Primary Culture of Bovine Adrenal Chromaffin Cells

Adrenal chromaffin cells were dissociated from intact glands and plated in supplemented DMEM, as previously described (Free et al., 2005). Cells were plated on 24 well plates at a density of 5×10^5 cells per well for intact cell binding studies. One day prior to experimentation, culture media were removed and replaced with media free of amphotericin B and 5-fluoro-2'-deoxyuridine. Cells were used 3-7 days after isolation.

[¹²⁵I]α-Bungarotoxin Binding

Membranes from bovine adrenal medullae were prepared as previously described by our laboratory (Free et al., 2002). [¹²⁵I]αBGT binding was performed by modification of techniques previously described by our laboratory (Bryant et al., 2002). Briefly, adrenal membranes (500-750 μg per assay tube) were incubated for 4 hrs at 37°C in buffer containing 1 nM [¹²⁵I]αBGT. After the 4 hr incubation, the assay mixtures were filtered and rapidly washed. Nonspecific binding was determined in the presence of 1 mM nicotine. Specific binding was determined by subtracting nonspecific binding from total binding. Binding to adrenal nAChRs in intact cells was performed by modification of techniques previously described by our laboratory (Free et al., 2005). Briefly, cells were incubated as described for the membrane preparation. After the 4 hr incubation, the binding buffer was aspirated and the cells were rapidly washed. Cells were extracted in 1M NaOH and the cells scraped from the plates. The cellular extracts were neutralized and counted using liquid scintillation spectroscopy. For both membrane binding and intact cell binding, each observation (n) was performed in duplicate or triplicate. Experiments were performed on at least three different membrane preparations or cell preparations. K_d and B_{max} values were determined from saturation binding assays by non-linear regression fit of the data to a model incorporating a single class of non-interacting binding sites (Prism 4.02, GraphPad, San Diego, CA). IC_{50} values were obtained by averaging values generated from nonlinear regression analyses (Prism 4.02, GraphPad, San Diego, CA) of individual concentration-response curves. Dissociation constants (K_i) for each drug were calculated using the Cheng-Prusoff relationship, $K_i = IC_{50} / \{1 + [^{125}I\text{-}\alpha\text{BGT}]/K_d \text{ of } ^{125}I\text{-}\alpha\text{BGT}\}$ (Cheng and Prusoff, 1973).

Alkylation of nAChRs from Cultured Adrenal Chromaffin Cells

nAChRs expressed in cultured bovine adrenal chromaffin cells were irreversibly alkylated with 100 μM brACh using techniques previously published by our laboratory (Free and McKay, 2001). Briefly, intact cells were first treated with 1 mM DTT in buffer (pH 8) for 15 min at 37°C in order to reduce nAChR disulfide bonds. Cells were then washed, followed by treatment with brACh for 6 min at room temperature. After washing, the disulfide bonds were reoxidized by incubating the cells with 1 mM DTNB in buffer for 15 min at 37°C.

Immunoprecipitation of Adrenal nAChRs

Cultured adrenal chromaffin cells were lysed in MPER supplemented with protease inhibitors ("Complete Mini" from Roche) and centrifuged (18,000 × g) for 15 min at 4°C. The supernatant was precleared with 50 μl protein G-agarose by incubating for 3 hours at 4°C on a rocking platform. The suspension was centrifuged briefly and the supernatant transferred to a new microcentrifuge tube. Antibodies (mAb35 or mAb319) were added to the samples and gently rotated overnight at 4°C. Protein G-agarose (50 μl) was then added and incubated for at least 3 hours at 4°C with rocking. The complexes were collected by brief, low spin, centrifugation and the supernatant carefully removed and stored at -80°C. The pellet was washed a total of five times: twice in 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P40 (a nonionic surfactant used in the isolation of membrane complexes), twice in a high salt buffer containing 50 mM Tris (pH 7.5), 500 mM sodium chloride and 0.1% Nonidet P40, and once in a low salt buffer of 50 mM Tris (pH 7.5) and 0.1% Nonidet P40. Bound proteins were eluted with SDS sample buffer.

Western Blot Analyses

The immunoprecipitated proteins were separated on 10% SDS-polyacrylamide gels and then electroblotted onto Hybond membranes (Amersham Pharmacia Biotech, UK). The blots were incubated at room temperature for 1 hour in blocking buffer, consisting of phosphate buffered saline, 0.1% Tween-20, and 10% dry powdered milk (PBS-T), and then incubated overnight

with the appropriate antibody at 4°C. After three 15 min washes with PBS-T, horseradish peroxidase-linked secondary antibody was incubated with the blot for 1 hour. After another 3 washes in PBS-T, bound antibodies were visualized using enhanced chemiluminescence (ECL, AmershamPharmaciaBiotech, Little Chalfont, UK). Images of the blots were visualized by autoradiography. Molecular weights were determined using LabWorks™ imaging software (UVP, Inc. Upland, CA).

Calculations and Statistics

Results were calculated from the number of observations (n) performed in duplicate or triplicate. IC₅₀ values were obtained by averaging values generated from nonlinear regression analyses (Prism 4.02, GraphPad, San Diego, CA) of individual concentration-response curves. Results are expressed as arithmetic means ± SEM, except for IC₅₀ values which are expressed as geometric means (95% confidence limits).

RESULTS

In these studies [¹²⁵I]αBGT is used as the radioligand to characterize bovine adrenal nAChRs. As illustrated in Figure 1, specific binding of [¹²⁵I]αBGT is saturable with a K_d value of 4.2 ± 0.82 nM. [¹²⁵I]αBGT binding fits a model for a single site. The B_{max} value for [¹²⁵I]αBGT binding is 49.9 ± 7.6 fmol/mg protein. The concentration-response effects of two selective α7 nAChR antagonists, MLA and αBGT, were investigated (Figure 2). Hill coefficients are always near unity, indicating no cooperative interactions among sites. The K_i value for αBGT is 1.9 nM and is similar to our reported K_d value for [¹²⁵I]αBGT (4.2 nM). MLA also inhibits [¹²⁵I]αBGT binding in a concentration-dependent manner with a K_i value of 30.6 nM.

In the next series of studies, [¹²⁵I]αBGT binding experiments were performed on intact bovine adrenal cells in culture. The effects of several cholinergic drugs on binding of [¹²⁵I]αBGT are illustrated in Figure 3. At concentrations predicted to inhibit all specific [¹²⁵I]αBGT binding, nicotine, carbachol, MLA, αBGT and cytisine inhibit total binding of the radioligand by similar amount (~20%). Next, we investigated the ability of bromoacetylcholine (brACh) to alkylate bovine adrenal α7 nAChRs in intact cells. We have previously documented that α3β4* nAChRs expressed in cultured adrenal chromaffin cells are able to be alkylated (Free and McKay, 2001). Alkylation produces a 24.0% ± 1.9% reduction of total [¹²⁵I]αBGT binding (Figure 4). This reduction in [¹²⁵I]αBGT binding is not significantly different from the reduction produced by high concentrations of nicotine or carbachol on non-alkylated cells or from the inhibition observed with other nicotinic receptor drugs (Figure 3). To determine whether the site of action of nicotine and alkylation are on the same nAChR population, nicotine competition experiments were performed on alkylated cells. As illustrated in Figure 4, nicotine is unable to displace any additional [¹²⁵I]αBGT binding to cells that had been previously alkylated. Likewise, the number of nicotine-insensitive receptors did not change following receptor alkylation (Figure 4). These studies support the identification and expression of α7 nAChRs on the cell surface of intact chromaffin cells.

Finally, the anti nAChR monoclonal antibody, mAb319, which recognizes α7 nAChR subunit protein, was used in immunoprecipitation experiments on bovine adrenal cell extracts to confirm the expression of α7 nAChR subunit protein. Western blot analyses show that mAb319 pulls down a single protein with a molecular weight of ~53 kDa, identifiable using mAb319 as primary antibody. (Figure 5). This size is consistent with the calculated molecular weight of 56kDa for α7 nAChR subunit protein. mAb35, an anti-nAChR monoclonal antibody which recognizes α3, and possibly α5, nAChR subunit proteins in adrenal cells, was also used in pull down experiments on bovine adrenal cell extracts. Western blot analysis identified a single protein with a molecular weight of ~53 kDa when probed with mAb319 (Figure 5).

DISCUSSION

$\alpha 7$ nAChRs are located both in the CNS and PNS. While the molecular, biochemical, physiological and pathophysiological elucidation of $\alpha 7$ nAChRs in the CNS is unfolding, $\alpha 7$ nAChRs expressed in the PNS are much less characterized. Experimental models to study 'native' (vs. recombinant) $\alpha 7$ nAChRs will greatly facilitate the characterization of peripheral $\alpha 7$ nAChRs. The studies reported here characterize bovine adrenal $\alpha 7$ nAChRs; they also document the utility of an easily obtainable membrane preparation from bovine adrenal medulla and the use of adrenal chromaffin cells in culture for the study of native $\alpha 7$ nAChRs.

Our intact cell binding studies support the surface expression of $\alpha 7$ nAChRs; both nicotine, an agonist capable of crossing plasma membranes, and carbachol, an impermeant agonist, produce equivalent amounts of displacement of [125 I] α BGT binding. We found that all surface $\alpha 7$ nAChRs were able to be alkylated, as are adrenal $\alpha 3\beta 4^*$ nAChRs (Free and McKay, 2001). From our membrane binding studies, we found that the K_d value of α BGT (4.2 nM) is similar to those reported for rat brain (1.5 nM) (Macallan et al., 1988), rat hippocampal membranes (0.54 nM) (Garcia-Guzman et al., 1995), native $\alpha 7$ receptors from SH-SY5Y cells (1.1 nM) (Xiao et al., 1998), and human $\alpha 7$ homomers expressed in oocytes (0.8 nM) (Ward et al., 1990). In addition, our K_i value for α BGT was found to be 1.9 nM and is similar to our K_d value of [125 I] α BGT derived from saturation experiments. The B_{max} for [125 I] α BGT binding to bovine adrenal membranes is 49.9 ± 7.6 fmol/mg protein, corresponding to that observed for rat hippocampal membranes of 51.1 fmol/mg protein (Garcia-Guzman et al., 1995). In competition binding experiments using MLA, we have found that the K_i of MLA is ten-fold higher than values reported in other tissues [e.g., rat brain, K_i value of ~ 1.4 nM (Amar et al., 1993) or chick ciliary ganglion, K_i value of 2.8 nM (Vijayaraghavan et al., 1992)], but is similar to values obtained for invertebrate locust ganglion (K_i value, ~ 18 nM) (Macallan et al., 1988). Functional studies in bovine adrenal cells have also documented a relatively lower affinity of MLA for $\alpha 7$ nAChRs, the IC_{50} for MLA inhibition in these electrophysiological pulse recordings was reported to be ~ 100 nM (Lopez et al., 1998). These studies suggest that MLA interactions with $\alpha 7$ nAChR subtypes in adrenal cells are atypical, having a lower binding affinity.

Immunoprecipitation experiments using mAb319 and mAb35 support the expression of $\alpha 7$ nAChR subunit proteins in bovine adrenal chromaffin cells. Our data suggest the presence of both a homomeric and heteromeric population of $\alpha 7$ containing receptors. The identification of a 53 kDa protein, similar to that reported by others for $\alpha 7$ proteins (Del Toro et al., 1994; Orr-Urtreger et al., 1997), in mAb35 immunoprecipitated nAChRs suggests that at least some mAb35 nAChRs contain $\alpha 7$ nAChR proteins. In these studies multiple antibodies against $\alpha 7$ nAChR protein were eliminated for use because of their cross-reactivity with other nAChR subunits, resulting in the use of mAb319 both to precipitate the sites and to detect the protein on Western blots. Interpretation of these studies may be problematic and rests on the subunit specificity of the antibodies used. Most antibodies currently available that are directed against 'specific' nAChR subunits show cross-reactivity with other subunits, species preferences, and/or technique limitations. Therefore, data obtained using mAb35 and mAb319 should be interpreted with caution. mAb35 has been shown to interact with $\alpha 1$, $\alpha 3$, and $\alpha 5$ nAChR subunits. We have previously shown that mAb35 interacts with $\alpha 3\beta 4^*$ nAChRs expressed in bovine adrenal cells (Gu et al., 1996). Commonly used for detection of $\alpha 7$ protein, mAb319 has recently been reported to cross-react with nonspecific proteins (Herber et al., 2004), but affinity purification prior to Western blotting eliminates most of the specificity problems.

Evidence exists for the expression of heteromeric $\alpha 7$ -containing nAChRs. Listerud and colleagues were the first to document that $\alpha 7$ nAChR subunits can substitute for $\alpha 3$ nAChR subunits and be incorporated into native $\alpha 3$ -containing nAChRs expressed in chick sympathetic

ganglia neurons after antisense-induced deletion of the $\alpha 3$ nAChR subunit (Listerud et al., 1991). Using electrophysiological, pharmacological and antisense approaches, Yu and Role have proposed three native heteromeric $\alpha 7^*$ nAChRs in embryonic chick sympathetic neurons (Yu and Role, 1998). In the rat pheochromocytoma cell line (PC12), a tumor cell line of the adrenal medulla, Virginio et al. (2002) also proposed the functional expression of heteromeric nAChRs containing $\alpha 7$ nAChR subunits.

CONCLUSIONS

Our studies support the expression of homomeric $\alpha 7$ nAChRs and possibly a smaller population of heteromeric, $\alpha 7$ -containing, nAChRs in bovine adrenal medulla. Saturation studies and homologous competition experiments using α BGT, as well as the immunoprecipitation and Western blot studies using mAb319 support the expression of homomeric $\alpha 7$ nAChRs. We demonstrate that $\alpha 7$ nAChRs are expressed on the surface of cultured bovine adrenal cells and are able to be completely alkylated. This will allow for receptor protection approaches (Furchgott, 1954; Grider and Makhoulouf, 1991; Oriowo et al., 1992; Free and McKay, 2001) to distinguish functional contributions of $\alpha 3\beta 4$ and $\alpha 7$ nAChRs in adrenal neurosecretion and provide insights into their physiological roles.

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REFERENCES

- Amar M, Thomas C, Johnson C, Lunt GG, Wonnacott S. Agonist pharmacology of the neuronal $\alpha 7$ nicotinic receptor expressed in *Xenopus* oocytes. *FEBS Letters* 1993;327:284–288. [PubMed: 8348955]
- Bryant DL, Free RB, Thomasy SM, Lapinsky DJ, Ismail KA, McKay SB, Bergmeier SC, McKay DB. Structure-activity studies with ring E analogues of methyllycaconitine on bovine adrenal $\alpha 3\beta 4^*$ nicotinic receptors. *Neuroscience Research* 2002;42:57–63. [PubMed: 11814609]
- Campos-Caro A, Smillie FI, Dominguez del Toro E, Rovira JC, Vicente-Agullo F, Chapuli J, Juiz JM, Sala S, Sala F, Ballesta JJ, Criado M. Neuronal nicotinic acetylcholine receptors on bovine chromaffin cells: cloning, expression, and genomic organization of receptor subunits. *Journal of Neurochemistry* 1997;68:488–497. [PubMed: 9003033]
- Cheng Y-C, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. *Biochemical Pharmacology* 1973;22:3099–3108. [PubMed: 4202581]
- Del Toro E, Juiz J, Peng X, Lindstrom J, Criado M. Immunocytochemical localization of the $\alpha 7$ subunit of the nicotinic acetylcholine receptor in the rat central nervous system. *Journal of Comparative Neurology* 1994;349:325–342. [PubMed: 7852628]
- Di Angelantonio S, Nistri A, Moretti M, Clementi F, Gotti C. Antagonism of nicotinic receptors of rat chromaffin cells by N,N,N-trimethyl-1-(4-*trans*-stilbenoxy)-2-propylammonium iodide: a patch clamp and ligand binding study. *British Journal of Pharmacology* 2000;129:1771–1779. [PubMed: 10780985]
- Free RB, Bryant DL, McKay SB, Kaser DJ, McKay DB. [3 H]Epibatidine binding to bovine adrenal medulla: evidence for $\alpha 3\beta 4^*$ nicotinic receptors. *Neuroscience Letters* 2002;318:98–102. [PubMed: 11796195]
- Free RB, McKay DB. Receptor protection studies to characterize neuronal nicotinic receptors: tubocurarine prevents alkylation of adrenal nicotinic receptors. *Brain Research* 2001;891:176–184. [PubMed: 11164821]
- Free RB, von Fischer ND, Boyd RT, McKay DB. Pharmacological characterization of recombinant bovine $\alpha 3\beta 4$ neuronal nicotinic receptors stably expressed in HEK 293 cells. *Neuroscience Letters* 2003;343:180–184. [PubMed: 12770692]

- Free RB, McKay SB, Gottlieb PD, Boyd RT, McKay DB. Expression of native $\alpha 3\beta 4^*$ neuronal nicotinic receptors: binding and functional studies investigating turnover of surface and intracellular receptor populations. *Molecular Pharmacology* 2005;67:2040–2048. [PubMed: 15772292]
- Furchgott RF. Dibenzamine blockade in strips of rabbit aorta and its use in differentiating receptors. *Journal of Pharmacology and Experimental Therapeutics* 1954;111:265–284. [PubMed: 13175108]
- Garcia-Guzman M, Sala F, Sala S, Campos-Caro A, Stuhmer W, Gutierrez LM, Criado M. α -Bungarotoxin-sensitive nicotinic receptors on bovine chromaffin cells: molecular cloning, functional expression and alternative splicing of the $\alpha 7$ subunit. *European Journal of Neuroscience* 1995;7:647–655. [PubMed: 7620615]
- Grider JR, Makhlof GM. Identification of opioid receptors on gastric muscle cells by selective receptor protection. *American Journal of Physiology: Gastrointestinal and Liver Physiology* 1991;260:G103–G107.
- Gu H, Wenger BW, Lopez I, McKay SB, Boyd RT, McKay DB. Characterization and localization of adrenal nicotinic receptors: evidence that mAb35-nicotinic receptors are the principal receptors mediating adrenal catecholamine secretion. *Journal of Neurochemistry* 1996;66:1454–1461. [PubMed: 8627298]
- Herber DL, Severance EG, Cuevas J, Morgan D, Gordon MN. Biochemical and histochemical evidence of nonspecific binding of alpha $\alpha 7$ nAChR antibodies to mouse brain tissue. *Journal of Histochemistry and Cytochemistry* 2004;52:1367–1376. [PubMed: 15385583]
- Listerud M, Brussaard AB, Devay P, Colman DR, Role LW. Functional contribution of neuronal AChR subunits revealed by antisense oligonucleotides. *Science* 1991;254:1518–1521. [PubMed: 1720573]
- Lopez MG, Montiel C, Herrero CJ, Garcia-Palmero E, Mayorgas I, Hernandez-Guijo JM, Villarroya M, Olivares R, Gandia L, McIntosh JM, Olivera BM, Garcia AG. Unmasking the functions of the chromaffin cell $\alpha 7$ nicotinic receptor by using short pulses of acetylcholine and selective blockers. *Proceedings of the National Academy of Sciences USA* 1998;14184–14189.
- Lukas RJ, Changeux J-P, le Novère N, Albuquerque EX, Balfour JK, Berg DK, Bertrand D, Chiappinelli VA, Clarke PBS, Collins AC, Dani JA, Grady SR, Kellar KJ, Lindstrom JM, Marks MJ, Quik M, Taylor PW, Wonnacott S. International union of pharmacology. XX. Current status of the nomenclature for nicotinic acetylcholine receptors and their subunits. *Pharmacological Reviews* 1999;51:397–401. [PubMed: 10353988]
- Macallan DRE, Lunt GG, Wonnacott S, Swanson KL, Rapoport H, Albuquerque EX. Methyllaconitine and (+)-anatoxin-a differentiate between nicotinic receptors in vertebrate and invertebrate nervous systems. *FEBS Letters* 1988;226:357–363. [PubMed: 3338564]
- Marley E, Prout GI. Physiology and pharmacology of the splanchnic-adrenal medullary junction. *Journal of Physiology* 1965;180:483–513. [PubMed: 5846790]
- Martin LF, Kem WR, Freedman R. Alpha-7 nicotinic receptor agonists: potential new candidates for the treatment of schizophrenia. *Psychopharmacology* 2004;174:54–64. [PubMed: 15205879]
- Meyer EM, Tay ET, Zoltewicz JA, Meyers C, King MA, Papke RL, De Fiebre CM. Neuroprotective and memory-related actions of novel alpha-7 nicotinic agents with different mixed agonist/antagonist properties. *Journal of Pharmacology and Experimental Therapeutics* 1998;284:1026–1032. [PubMed: 9495863]
- Oriowo MA, Nichols AJ, Ruffolo RR. Receptor protection studies with phenoxybenzamine indicate that a single $\alpha 1$ -adrenoceptor may be coupled to two signal transduction processes in vascular smooth muscle. *Pharmacology* 1992;45:17–26. [PubMed: 1354876]
- Orr-Urtreger A, Göldner FM, Saeki M, Lorenzo I, Goldberg L, De Biasi M, Dani JA, Patrick JW, Beaudet AL. Mice deficient in the $\alpha 7$ neuronal nicotinic acetylcholine receptor lack α -bungarotoxin binding sites and hippocampal fast nicotinic currents. *Journal of Neuroscience* 1997;17:9165–9171. [PubMed: 9364063]
- Seguela P, Wardiche J, Dineley-Miller K, Dani JA, Patrick JW. Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium. *Journal of Neuroscience* 1993;13:596–604. [PubMed: 7678857]
- Ulloa L. The vagus nerve and the nicotinic anti-inflammatory pathway. *Nature Reviews. Drug Discovery* 2005;4:673–684.

- Vijayaraghavan S, Pugh PC, Zhang Z, Rathouz MM, Berg DK. Nicotinic receptors that bind α -bungarotoxin on neurons raise intracellular free Ca^{2+} Neuron 1992;8:353–362. [PubMed: 1310863]
- Virginio C, Giacometti A, Aldegheri L, Rimland JM, Terstappen GC. Pharmacological properties of rat alpha 7 nicotinic receptors expressed in native and recombinant cell systems. European Journal of Pharmacology 2002;445:153–161. [PubMed: 12079679]
- Ward JM, Cockcroft VB, Lunt GG, Smillie FS, Wonnacott S. Methylycaconitine: a selective probe for neuronal α -bungarotoxin binding sites. FEBS Letters 1990;270:45–48. [PubMed: 2226787]
- Xiao Y, Meyer EL, Thompson JM, Surin A, Wroblewski J, Kellar KJ. Rat $\alpha 3/\beta 4$ subtype of neuronal nicotinic acetylcholine receptor stably expressed in a transfected cell line: pharmacology of ligand binding and function. Molecular Pharmacology 1998;54:322–333. [PubMed: 9687574]
- Yu CR, Role LW. Functional contribution of the alpha7 subunit to multiple subtypes of nicotinic receptors in embryonic chick sympathetic neurones. Journal of Physiology 1998;509(Pt 3):651–665. [PubMed: 9596789]

FIGURE 1.

Saturation binding of [¹²⁵I]αBGT to adrenal medullary membranes. Membranes were incubated at 37 °C for four hours with various concentrations of [¹²⁵I]αBGT. Total and non-specific binding were determined in the absence or presence of 1 mM of nicotine, respectively. Insert. Total, nonspecific, and specific binding of [¹²⁵I]αBGT to adrenal medullary membranes. Specific binding was determined by subtracting non-specific from total binding for each value. Values represent means ± SEM (n = 5). Non-linear regression analysis of specific binding indicates a K_d value of 4.2 ± 0.8 nM and a B_{max} value of 49.9 ± 7.6 fmol/mg protein.

FIGURE 2.

Concentration-response effect of αBGT and MLA on [¹²⁵I]αBGT binding to adrenal membranes. Competition binding experiments were performed using the indicated concentrations of αBGT (A) or MLA (B). Data are expressed as a percentage of control specific binding. Values represent means $\pm$ SEM (n = 8). Non-linear regression analyses of specific binding indicate K_i values of 1.86 $\pm$ 0.5 nM and 30.6 $\pm$ 4.9 nM for αBGT and MLA, respectively.

FIGURE 3.

Binding of [¹²⁵I]αBGT to intact bovine adrenal chromaffin cells. Binding experiments using [¹²⁵I]αBGT were performed on cultured bovine adrenal chromaffin cells. Total binding of the radioligand was determined either in the absence (Control) or presence of nicotine (1 mM), carbachol (12 mM), MLA (100 nM), αBGT (1 μM), or cytisine (140 μM). Data are represented as means ± SEM (n = 7) of a percentage of total binding of the control group.

FIGURE 4.

Effects of alkylation on binding of $[^{125}\text{I}]\alpha\text{BGT}$ to intact bovine adrenal chromaffin cells. Cultured adrenal cells were either not alkylated (control) or alkylated using 100 μM brACh prior to the binding reaction. Specific binding to control nAChRs and to alkylated nAChRs were determined using nicotine (NIC, 300 μM). Data are represented in fmol/mg protein $[^{125}\text{I}]\alpha\text{BGT}$ binding (means $\pm$ SEM, $n = 3-5$).

FIGURE 5.

Western blot of immunoprecipitated proteins from bovine adrenal chromaffin cells. Immunoprecipitated proteins were separated by 10% PAGE and analyzed by Western blotting, as described in methods section. The blot was probed with the 1° antibody, mAb319 (1:250 dilution), followed by incubation with HRP- linked anti-rat 2° antibody (1:1000). The blot was visualized by autoradiography.