MOLECULAR PHARMACOLOGY, 13, 283-290

Drug Binding Properties of an α-Bungarotoxin-Binding Component from Rat Brain

JAKOB SCHMIDT

Department of Biochemistry, State University of New York at Stony Brook, Stony Brook, New York 11794

(Received June 28, 1976)
(Accepted October 15, 1976)

SUMMARY

SCHMIDT, JAKOB (1977) Drug binding properties of an α-bungarotoxin-binding component from rat brain. *Mol. Pharmacol.*, 13, 283-290.

The drug binding properties of an α-bungarotoxin-binding component in a crude membrane preparation from whole rat brain were investigated by analyzing the effects of various neuroactive drugs on the rate of toxin binding. High affinities were found for nicotinic ligands such as *d*-tubocurarine, nicotine, gallamine, and dihydro-β-erythroidine, whereas interaction with choline and muscarinic compounds was observed to be weak and presumably due to nonspecific electrostatic forces. Little interaction was seen with other putative neurotransmitters. No evidence was obtained for more than one type of toxin binding site. The findings are interpreted as supporting the notion that the α-bungarotoxin-binding macromolecule in the central nervous system is a nicotinic acetylcholine receptor.

INTRODUCTION

Numerous studies employing microelectrophoretic application of the putative transmitter acetylcholine and its agonists and antagonists have suggested the presence of nicotinic acetylcholine receptors on specific cells in various regions of the vertebrate central nervous system (1, 2); thus the nicotinic nature of a population of acetylcholine receptors on spinal Renshaw cells is well established (3, 4).

The biochemical analysis of cholinergic receptors in brain is still at an early stage. An important development has been the demonstration that central binding sites exist for α-bungarotoxin, a snake neurotoxin which is considered to be a highly specific probe for nicotinic acetylcholine receptors in peripheral organs (for reviews of nicotinic receptors in electric organs and skeletal muscle, see refs. 5 and 6). Several laboratories have now reported the existence of α-bungarotoxin binding sites in rat brain, at concentrations comparable to those in rat skeletal muscle (7-10). Whether the toxin-binding macromolecule is an acetylcholine receptor and, more specifically, a nicotinic one, remains an intriguing question. Perhaps the most convincing demonstration of the nicotinic nature of this binding component would be through an analysis of its drug affinities. The present communication describes studies on the pharmacological properties the α-bungarotoxin receptor from rat brain exhibits *in vitro*.

This research was supported by National Science Foundation Grant BMS 7418607 and a grant from the Muscular Dystrophy Associations of America, Inc.

METHODS

α-Bungarotoxin was purified from *Bungarus multicinctus* venom (Miami Serpentarium) and iodinated with ^{125}I as described previously (10). A chromatographi-

ISSN 0026-895x

cally homogeneous fraction, tentatively identified as monoiodo-α-bungarotoxin, and 100% biologically active as tested by its ability to bind to the *Torpedo californica* acetylcholine receptor, was used. The specific radioactivity of [^{125}I]BuTX[1] initially was 10^6 Ci/mole and declined with the half-life of ^{125}I, 60 days; no loss of binding activity of [^{125}I]BuTX was observed over a period of up to three half-lives.

Most drugs were obtained from Sigma, except for acetylcholine (Aldrich), acetylthiocholine (Boehringer/Mannheim), and gallamine triethiodide (K & K); dihydro-β-erythroidine, mecamylamine, and dimethylphenylpiperazonium were gifts from Merck Sharp & Dohme.

Male outbred albino rats, 100–200 g, were used. Following light ether anesthesia, animals were decapitated and their brains were excised and homogenized with a glass tissue grinder in 15 volumes of 10 mM sodium phosphate, 1 mM EDTA, 0.02% sodium azide, and 0.4 mM phenylmethylsulfonyl fluoride, pH 7.2 (assay buffer). Aliquots of 0.2 ml of the homogenate were added to equal volumes of assay buffer containing drugs at the desired concentration; range-finding experiments were conducted to determine optimal concentrations of each ligand. Incubations of homogenates with drugs were carried out for approximately 1 hr in 1.5-ml Eppendorf centrifuge tubes. Then 0.1 ml of 1 nM [^{125}I]BuTX was added, and the toxin binding reaction was allowed to proceed for 20 min. The incubation was terminated by addition of 0.8 ml of 0.2 M NaCl, immediately followed by centrifugation for 2 min in an Eppendorf model 3200 centrifuge. The supernatant fluid was removed by aspiration, and the pellet was washed by resuspension in 1.4 ml of 0.2 M NaCl and centrifugation. After two washes the particulate fraction was digested in 0.2 ml of Protosol (New England Nuclear) at room temperature overnight. Then 1.2 ml of 0.4% Permablend in toluene were added, and samples were analyzed in a Packard model 3330 liquid scintillation spectrometer, using the disposable centrifuge tubes as minivials. Ligands sensitive to acetylcholinesterase, such as acetylcholine, acetylthiocholine, butyrylcholine, acetyl-β-methylcholine, and succinylcholine, were investigated in the presence of 50 μM eserine.

[1] The abbreviations used are: BuTX, α-bungarotoxin; pI, isoelectric point.

RESULTS

Binding of labeled toxin to particulate brain preparations was determined by centrifugation for 2 min in the Eppendorf model 3200 centrifuge. More than 95% of the specifically bound toxin sedimented under these conditions (Fig. 1). A small fraction was lost in the supernatant and could be recovered by ultracentrifugation; the postribosomal supernatant was found to contain only free toxin, as judged by gel permeation chromatography on Sephadex G-75. The amount of specifically bound radiotoxin in the washed pellet thus reflects the number of toxin receptors reasonably well, provided that the binding sites are indeed all occupied under the conditions of the assay. To ascertain this, a binding equilibrium analysis was carried out, employing a modified version of the centrifuge assay, in which the washing steps were omitted (Fig. 2). Ostensibly brain particulate preparations contain a component saturable with [^{125}I]BuTX; the dissociation constant is estimated to be 0.05 nM, and the tissue concentration, 2.3 nM. In 24 independent measurements using the standard assay (which involves removal of unbound toxin by repeated washes), the concentration of BuTX receptors in rat brain was found to be 2.19 $\pm$ 0.35 nM.

The association of BuTX and acetylcholine receptor sites is a bimolecular process whose rate is proportional to the concentration of either reactant. When cholinergic drugs bind to the receptor they reduce the number of sites available for toxin binding, which is reflected in a decreased rate of toxin-receptor association. The drug concentration at which the toxin binding rate is lowered to 50% of its original value is a quantitative measure of the affinity of the drug for the receptor; toxin binding inhibition analysis has been used to investigate pharmacological properties of peripheral acetylcholine receptors (11,

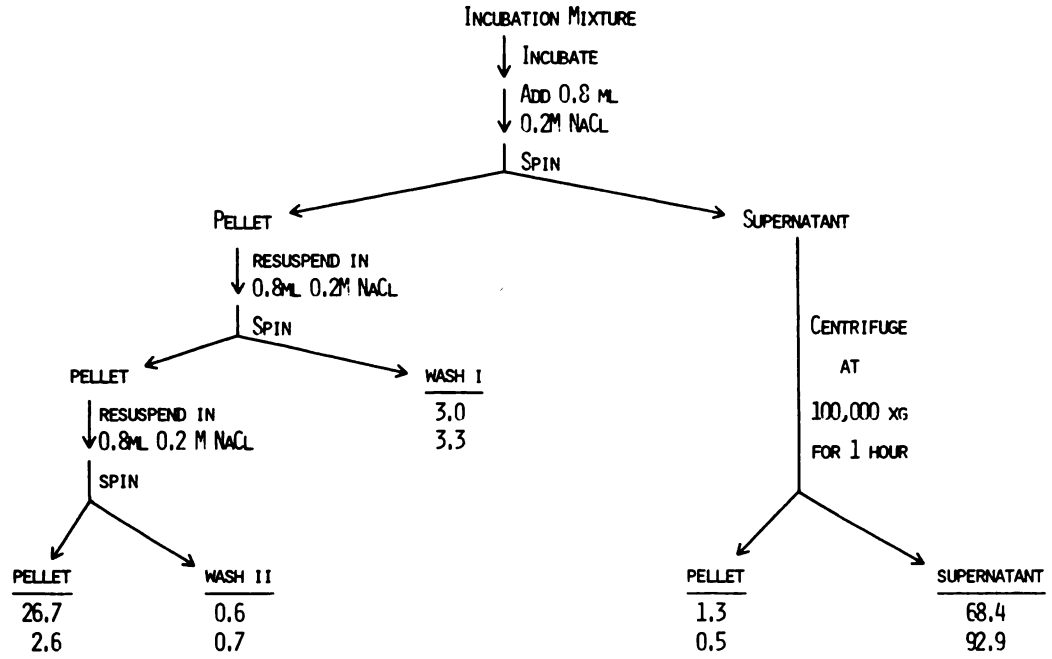

FIG. 1. *Validity of centrifugal assay*

Brain homogenate corresponding to 13.3 mg of original tissue was incubated in 1.5-ml Eppendorf centrifuge tubes with 100 fmoles of [^{125}I]BuTX in a final volume of 0.5 ml of assay buffer for 2 hr at room temperature and then subjected to the fractionation procedure outlined here. "Spin" designates centrifugation in an Eppendorf model 3200 centrifuge for 2 min. In the control experiment the incubation mixture contained 100 pmoles of native toxin. Results are presented for individual fractions as a percentage of all [^{125}I]BuTX used in the experiment. Control values are shown beneath experimental data.

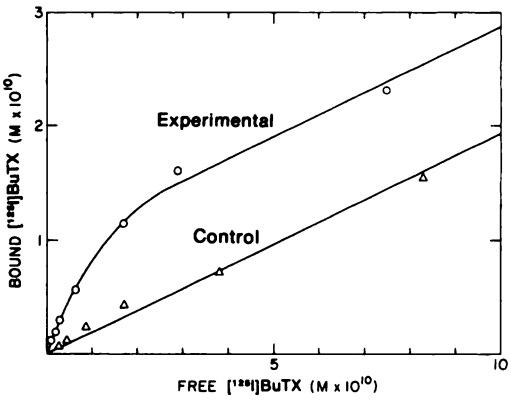

FIG. 2. *Toxin binding equilibrium analysis*

Samples of brain homogenate, representing 13.3 mg of wet tissue, were incubated at various concentrations of [^{125}I]BuTX in a total volume of 0.4 ml of assay buffer. After 2 hr at room temperature the suspensions were centrifuged. Ten-elevenths of the sample was removed by aspiration and its radioactivity was determined; the remaining $^1/_{11}$, containing the particulate fraction, was solubilized in 0.2 ml of Protosol and counted. Radioactivity in the supernatant was taken to represent free toxin; bound toxin was determined from radioactivity in the sediment by correcting for free toxin present in it. In the control experiment specific binding of [^{125}I]α-bungarotoxin was prevented by first exposing the homogenate to native toxin.

12). In order to apply this technique to a study of drug binding properties of central toxin receptors, the rate constants for toxin binding and toxin release were determined. Under standard conditions binding of [^{125}I]BuTX to brain particulate preparations is half completed in less than 30 min and levels off after about 2 hr (Fig. 3). Upon addition of an excess of native BuTX radiotoxin is released with a half-life of about 3$^1/_2$ hr. Kinetic constants were obtained by replotting the data of Fig. 3, and were found to be 1.35×10^6 M^{-1} sec^{-1} ("on" rate) and 5.8×10^{-5} sec^{-1} ("off" rate). The dissociation constant derived from the rate constants is 0.043 nM, in good agree-

ment with the results of the equilibrium analysis.

This experiment aided in deciding how long to carry out incubations. To minimize deviations from initial binding rate and to maximize the signal-to-noise ratio, an incubation time of 20 min was chosen. In addition, the experiment revealed that during the wash procedure, which took less than 10 min, only a very small fraction of specifically bound toxin was lost because of the reversibility of the binding reaction. This conclusion is in agreement with the experiment described in Fig. 1. Finally, Fig. 3 also shows that in rat brain particulate fractions, as in solubilized preparations from *Torpedo californica* (12), inorganic cations compete effectively for the BuTX binding site of the acetylcholine receptor. Therefore, in the standard assay, addition of 0.2 M NaCl was used to terminate the toxin binding reaction.

Toxin binding rate (i.e., toxin bound in 20 min) as a function of ligand concentration is shown for acetylcholine and for the agonist nicotine in Fig. 4; the drug concentration resulting in 50% inhibition is termed protection constant (K_P). Protection constants were determined for a number of neuroactive drugs (Table 1). It is obvious that a large percentage of the noncholinergic ligands have no inhibitory potency except at very high concentrations. Among the cholinergic drugs two categories can be discerned: a series that act at concentrations of approximately 10 μM, comprising the nicotinic ligands *d*-tubocurarine, nicotine, gallamine, and dihydro-β-erythroidine, and another group with protection constants in the millimolar range, including the muscarinic compounds pilocarpine, atropine, oxotremorine, scopolamine, acetyl-β-methylcholine, and muscarine.

Several small basic proteins were also tested for their inhibitory effect on the binding of BuTX, which itself is a small basic protein. Both horse heart cytochrome *c* (mol wt 13,400; pI[1] 10.0) and hen egg white lysozyme (mol wt 13,930; pI 11.1) displayed affinities about six orders of magnitude lower than [^{125}I]BuTX (mol wt 8000; pI 9.0; K_D 0.05 nM; see ref. 13). β-Nerve growth factor (mol wt 26,518; pI 9.3), on the other hand, was found to bind with a protection constant of 0.2 μM. Bo-

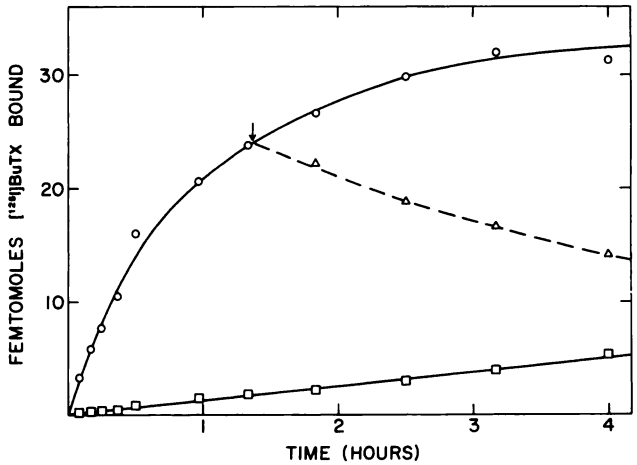

FIG. 3. *Binding of [^{125}I]BuTX to particulate brain preparations as a function of time and ionic strength*

Brain homogenate, corresponding to 270 mg of original tissue, was incubated at room temperature with 2.4 pmoles of [^{125}I]α-bungarotoxin in a total volume of 10 ml of assay buffer, without (○) or with (□) 0.2 M NaCl. The binding reaction was started by the addition of [^{125}I]α-bungarotoxin; 0.5-ml aliquots were removed at the indicated times thereafter and processed by centrifugation and washing as described in METHODS. The low ionic strength sample was divided into two equal portions after 82 min (arrow), with one half receiving 1.2 nmoles of native toxin in 0.01 ml of buffer (△), and the other half receiving buffer only. Values have been corrected for nonspecific binding.

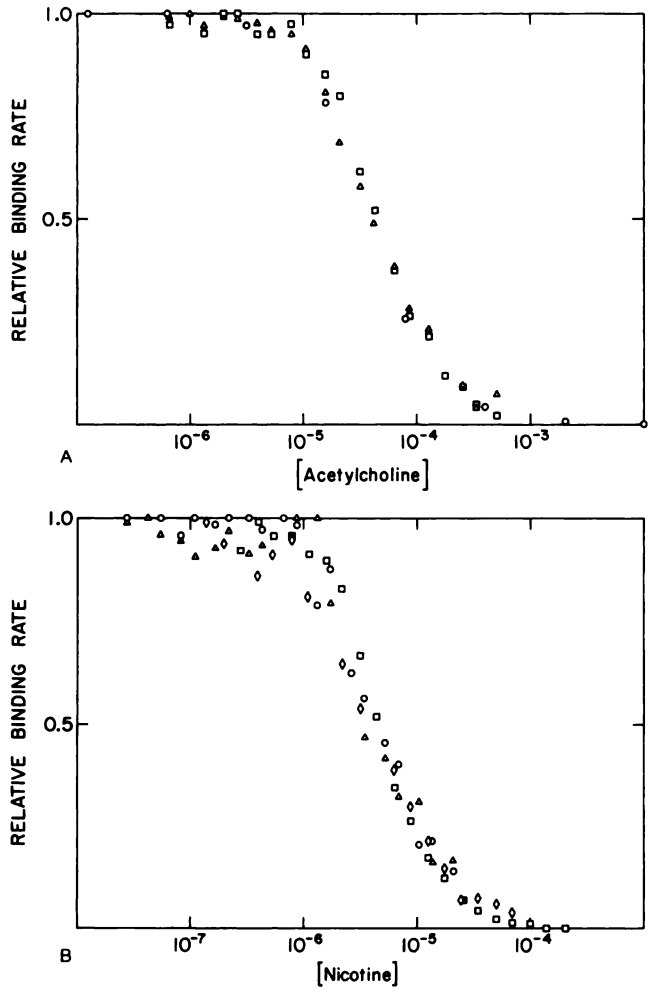

FIG. 4. *Effect of acetylcholine and nicotine on the rate of binding of [^{125}I]BuTX.*
The rate of [^{125}I]BuTX binding was determined as described in Methods, in the presence of the indicated drug concentrations. Data were corrected for background and normalized to binding rates seen in the absence of drugs. Different symbols refer to independently conducted experiments.

vine serum albumin (mol wt 65,400; pI 5.4) had no effect at millimolar concentrations.

DISCUSSION

There can be little doubt concerning the existence of a BuTX receptor in the central nervous system of the rat, at concentrations comparable to those in rat skeletal muscle (7–10). The level of receptor sites reported in the present study (2.19 nM) is higher than an earlier value obtained with Triton X-100-solubilized preparations [1.4 nM (10)]; the apparent increase may be due to the use of younger animals and a different assay procedure which avoids the risk of incomplete extraction. The dissociation constant for the membrane-embedded receptor–BuTX system (0.043 nM) is similar to that obtained with detergent extracts [0.056 nM (10)]. Thus data obtained with soluble and insoluble preparations are comparable; the centrifugal assay appears preferable for drug binding analysis, in that it obviates the consideration of detergent effects.

In view of the peripheral specificity of the snake α-neurotoxins, it is tempting to

TABLE 1

Affinities of rat brain BuTX binding sites for various drugs

Drug	Protection constant
	mM
Cholinergic	
d-Tubocurarine	0.0019
Nicotine	0.0031
Gallamine	0.0035
Butyrylcholine	0.0035
Dihydro-β-erythroidine	0.010
Dimethylphenylpiperazonium	0.010
Tetramethylammonium	0.020
Acetylthiocholine	0.025
Acetylcholine	0.030
Lobeline	0.050
Tetraethylammonium	0.080
Carbamylcholine	0.090
Decamethonium	0.50
Hexamethonium	0.90
Pilocarpine	1.0
Choline	1.2
Succinylcholine	1.5
Atropine	1.6
Oxotremorine	2.0
Eserine	2.2
Mecamylamine	3.0
Acetyl-β-methylcholine	3.5
Muscarine	10
Noncholinergic	
Procaine	1.3
Histamine	10
Cyclic AMP	10
Dopamine	>10
Serotonin	>10
Ouabain	20
Veratridine	20
Glutamate	30
Norepinephrine	40
γ-Aminobutyric acid	>100
Diethyl ether	>400
Glycine	>600
Ethanol	>1400

speculate that the toxin-sensitive macromolecule in the brain is a nicotinic acetylcholine receptor. Such a conclusion, based on analogy alone, appears premature, and a further analysis of the properties of the toxin receptor is therefore needed. Let us briefly consider the available information. The BuTX-binding component from rat brain is known to be located in membranes (10); associated with, and enriched in, synaptosomal preparations (9, 14); present within the central nervous system in a clearly nonuniform distribution (9);[2] and it resembles the peripheral acetylcholine receptor with respect to radius of gyration and isoelectric point (10). Analysis of the ligand properties of the central toxin receptor also provides important information, some of which is already available. Salvaterra and Moore observed an inhibitory effect of *d*-tubocurarine on toxin binding to rat brain receptors while atropine was over two orders of magnitude less effective (7); Polz-Tejera *et al.* (15), in a radioautographic study of rat and chick brain, were able to suppress toxin binding with nicotine. A more systematic analysis of the pharmacological properties of the toxin receptor in rat brain, however, has not been undertaken. While direct binding studies may have to await complete or partial purification of the receptor, the effect of drugs on the binding of BuTX to the receptor can be investigated in crude preparations. In the case of electric tissue, direct binding studies have been conducted with purified acetylcholine receptors; Changeux and collaborators have demonstrated that the dissociation constants obtained by procedures such as equilibrium dialysis are in excellent agreement with the "protection constants" derived from toxin binding inhibition assays. This agreement reveals that drug and toxin compete for the same site and justifies the use of the competition assay for the evaluation of the binding properties of the toxin receptor. Such an analysis has recently been used by Vogel and Nirenberg (16) in a study of chick retina toxin receptors; they tested seven cholinergic drugs and observed that the nicotinic compounds nicotine and *d*-tubocurarine were the most powerful inhibitors.

The survey of cholinergic compounds and other neuroactive substances presented here indicates that only classical nicotinic drugs display high affinity for the toxin receptor in rat brain, with nicotine itself ranking second, behind only *d*-tubocurarine. It is noteworthy that the ganglionic stimulating drugs nicotine, tetramethylammonium, dimethylphenylpiper-

[2] N. Schechter, manuscript in preparation.

azonium, and lobeline bind considerably more strongly than the ganglionic blockers hexamethonium, tetraethylammonium, and mecamylamine. Whether this is due to a desensitization effect *in vitro*, as recently described for the interaction of the *Torpedo* acetylcholine receptor and agonists (17), remains to be seen. The protection constants of the typical muscarinic drugs fall in the millimolar range; their inhibitory potency thus resembles that of the physiologically inactive choline, whose receptor affinity probably is a consequence of its positive charge. There is reason to assume that the α-toxin binding site comprises an anionic element which will interact with simple inorganic cations; the acetylcholine receptor from *Torpedo californica* electric organ has been shown to bind monovalent cations with apparent K_D values of approximately 5 mM (12). Although no systematic investigation of such effects on the rat brain receptor was carried out, the effect of high concentrations of sodium chloride on binding rate seems to indicate that the same holds for the central toxin receptor. The binding of the small basic proteins cytochrome *c* and lysozyme appears remarkably strong compared with that of ligands of low molecular weight, but negligible when contrasted with that of BuTX itself. Among all compounds tested, β-nerve growth factor displayed the highest affinity apart from BuTX itself; the physiological significance (if any) of this puzzling observation remains to be established.

What physiological role can be assigned to the central BuTX receptor with its unquestionably nicotinic binding characteristics? There is, at present, no physiological evidence for the tempting conclusion that BuTX binds to a postsynaptic neurotransmitter receptor in the central nervous system. On the contrary, two recent attempts to block the well-established nicotinic acetylcholine receptor in the spinal cord of the frog (13) and the cat (18) by administration of BuTX failed.

The biochemical and physiological results are best reconciled by assuming the existence of at least two kinds of central nicotinic receptor, only one of which is sensitive to BuTX. Peripheral nicotinic receptors have long been classified into two pharmacologically distinct groups, those occurring in skeletal muscle and those found in autonomic ganglia. Likewise the central nervous system may contain different classes of nicotinic receptors and even receptors of an intermediate nicotinic-muscarinic type, as has been argued most strongly for neurons of the medial geniculate nucleus of the cat (19). BuTX seems to bind to one kind of receptor site only. The presence of multiple types of toxin receptors with different ligand affinities should have manifested itself in multiphasic inhibition curves; however, no deviation from the simple S-shape was ever seen, thus corroborating the results of the biochemical analysis, according to which the central toxin receptor is likely to be a unique macromolecule (10).

ACKNOWLEDGMENTS

I gratefully acknowledge the expert technical assistance of Mrs. Indira Handy and the help of Mr. Joseph Lowy and Mr. Marshall Dawer in early phases of this work. β-Nerve growth factor from mouse submaxillary glands was a gift of Dr. Alexander Wlodawer.

REFERENCES

1. Curtis, E. F. & Crawford, J. M. (1969) *Annu. Rev. Pharmacol.*, 9, 209-240.
2. Krnjevic, K. (1975) in *Handbook of Psychopharmacology*, Vol. 6 (Iversen, L. L., Iversen, S. D. & Snyder, S. H., eds.), pp. 97-126, Plenum Press, New York.
3. Curtis, D. R. & Eccles, J. C. (1958) *J. Physiol. (Lond.)*, 141, 435-445.
4. Curtis, D. R. & Ryall, W. (1966) *Exp. Brain Res.*, 2, 49-65.
5. Hall, Z. (1972) *Annu. Rev. Biochem.*, 41, 925-952.
6. Changeux, J. P. (1975) in *Handbook of Psychopharmacology*, Vol. 6, (Iversen, L. L., Iversen, S. D. & Snyder, S. H., eds.), pp. 235-301, Plenum Press, New York.
7. Salvaterra, P. M. & Moore, W. J. (1973) *Biochem. Biophys. Res. Commun.*, 55, 1311-1318.
8. Eterovic, V. A. & Bennett, E. L. (1974) *Biochim. Biophys. Acta*, 362, 346-355.
9. Salvaterra, P. M., Mahler, H. R. & Moore, W. J. (1975) *J. Biol. Chem.*, 250, 6459-6475.
10. Lowy, J., McGregor, J., Rosenstone, J. & Schmidt, J. (1976) *Biochemistry*, 15, 1522-1527.
11. Weber, M. & Changeux, J.-P. (1974) *Mol. Pharmacol.*, 10, 15-34.

12. Schmidt, J. & Raftery, M. A. (1974) *J. Neurochem.*, **23**, 617–623.
13. Miledi, R. & Szczepaniak, A. C. (1975) *Proc. R. Soc. Lond., Ser. B, Biol. Sci.*, **190**, 267–274.
14. Bartfai, T., Berg, P., Schultzberg, M. & Heilbronn, E. (1976). *Biochim. Biophys. Acta*, **426**, 186–197.
15. Polz-Tejera, G., Schmidt, J. & Karten, H. J. (1975) *Nature*, **258**, 349–351.
16. Vogel, Z. & Nirenberg, M. (1976) *Proc. Natl. Acad. Sci. U. S. A.*, **73**, 1806–1810.
17. Weber, M., David-Pfeuty, T. & Changeux, J.-P. (1975) *Proc. Natl. Acad. Sci. U. S. A.*, **72**, 3443–3447.
18. Duggan, A. W., Hall, J. G. & Lee, C. Y. (1976). *Brain Res.*, **107**, 166–170.
19. Tebecis, A. K. (1972) *J. Physiol. (Lond.)*, **226**, 153–172.