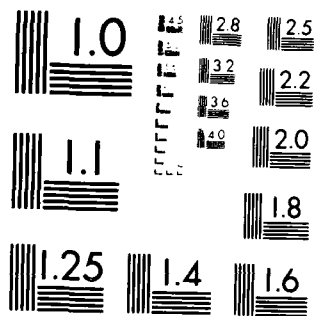END

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AD-A142 442

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THE EFFECTS OF HYPERBARIC PRESSURE

ON <u>IN</u> <u>VITRO</u> NEURAL RECEPTORS

Final Report
August 1980 to September 1983
Contract No. N00014-80-C-0707

to

Office of Naval Research
Department of the Navy
800 N. Quincy Street
Arlington, VA 22217

by

Richard F. Taylor
Arthur D. Little, Inc.
Acorn Park
Cambridge, MA 02140

June, 1984

Reference 85398

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Cholinergic-Anticholinergic agents
Organophosphorus agents

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

In vitro preparations of the nicotinic acetylcholine receptor from rat muscle and eel electroplax were chemically and pharmacologically characterized and then subjected to compression/decompression to/from 800 psi. Pressure causes a reversible decrease in cholinergic agent binding to the receptor. This decrease is prevented if agents such as hexamethonium and diisopropylfluorophosphate are present. These studies imply that pressure effects on molecular interactions at neural receptors could account for hyperbaric neurological disorders. In addition, hyperbaric therapy could be successful in cases of toxin

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and organophosphorus agent intoxication.

PREFACE

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ACKNOWLEDGEMENT

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SUMMARY

In vitro preparations of the nicotinic acetylcholine receptor (AChR) from rat gastrocnemius tissue were chemically and pharmacologically characterized and then subjected to compression/decompression to/from 800 psi. The binding of the natural cholinergic agonist, acetylcholine (ACh), and a number of other cholinergic agonists and antagonists to the AChR preparations was determined at varying pressure levels. In addition, the AChR protein from the electric organ of Electrophorus electricus was prepared and its binding of ACh at varying pressure levels was also studied. Our studies established that:

1. The nicotinic AChR from rat gastrocnemius muscle, isolated either as a protein or proteoglycolipid, is altered in its binding toward cholinergic agents by pressure. Such pressure alteration of binding follows a dose-response relationship.

2. The pressure effect on cholinergic agent binding to the AChR is reversible upon decompression.

3. The decreased binding of ACh to the AChR induced by increasing pressure is prevented by certain agents, including hexamethonium and diisopropylfluorophosphate, which appear to stabilize the receptor toward pressure.

4. The effect of pressure on the AChR appears due to conformational changes at the ACh binding sites.

5. The AChR protein from E. electricus appears more resistant to pressure induced loss of ACh binding than does the mammalian AChR protein from rat gastrocnemius muscle.

Our studies imply that hyperbaric pressure effects on molecular interactions at neural receptors could account for neurological disorders incurred at high pressure. Further, our studies suggest that hyperbaric therapy could be applied in cases of acute intoxication by toxic agents including marine toxins and organophosphorus nerve agents.

1. INTRODUCTION

Man is presently limited in his ability to explore and live in the oceans due primarily to neurophysiological effects. These effects are exemplified by the conditions of narcosis and high pressure nervous syndrome (HPNS). As the demand grows for increasing depth achievement for human divers such neurophysiological conditions must be at least minimized if not eliminated. Toward this end, many current hyperbaric studies are directed at the development of diving gas mixtures and drug protocols which could extend the present limits of manned dives (1-5). While such studies are necessary and valuable for the immediate practical extension of diving limits, eventual control of neurophysiological conditions in human divers must require definition of the biological effects of hyperbaric pressure on the macromolecular components of neural cell membranes responsible for neural transmission. The present proposal is concerned with such studies.

Research directed at the effects of hyperbaric pressure on animal neural tissues dates back nearly a century to the work of Regnard (6), who reported paralysis of animals at pressures in excess of 100 ATA. Continuing studies to date have exposed a variety of animal tissues or isolated nerve preparations to hyperbaric pressure. For example, tissues such as rat phrenic nerve diaphragm (7,8), cat gastrocnemius soleus muscle (9), and isolated frog sciatic nerve and gastrocnemius muscle (10) have been used to monitor neural transmission at various pressure levels. Other studies have attempted increased definition of pressure effects at neural synapses and receptors by utilizing squid axon (11) and isolated rabbit deuodenum (12). The overall conclusions derived from these experiments indicate (a) that neurotransmission is proportionately decreased by increasing pressure, (b) that pressure can affect the sensitivity of normal neural transmitters to blockade by antagonists, and (c) that such effects may be due to pressure influences on the conformation of neural junctional receptors. Such conclusions support the postulate that neurophysiological disorders occurring in human divers are caused by pressure effects at the molecular level.

Other studies have been directed at the biomolecular basis of pressure effects on neural processes. For example, it has been shown that hyperbaric helium-oxygen atmospheres affect the interaction of α- and β-receptors with norepinephrine and antagonists such as pentolamine and propranolol (13) and that increasing pressure can affect the binding of acetylcholine and α-bungarotoxin to the acetylcholine receptor proteoglycolipid (14). More basic studies concerned with the effects of pressure and anesthetics on artificial lipid or lipid-protein membranes have shown that inert and narcotic gases appear to affect membrane volume and permeability (15,16) and to cause expansion of lipoprotein-water interfacial films (17), while high pressure causes perturbations in phospholipid bilayers (18) and the binding of drugs to such bilayers (19). These studies as well as others concerned with pressure effects on protein activity and structure (20), lend further support to a theory that pressure-induced neurological effects may be due to the compression and alteration of critical cell membrane

proteins or proteolipid complexes (16,18,21). Such changes at neural membrane receptors would be expected to result in changes in neurotransmission with increasing pressure. These results also stress the need for further investigations concerning the effects of pressure on neural cell membrane macromolecules.

The fundamental macromolecule involved in neurotransmission is the so-called "receptor" located at post-junctional neural synapses and which, when stimulated by the appropriate neurotransmitter, initiates ionic membrane processes resulting in neurotransmission and tissue response to internal and external stimuli. While the exact chemical structure of neural receptors is not known, it is known that, in most cases, such receptors are comprised of a protein component, in which specificity for the appropriate neurotransmitter resides, and associated components, including lipids and carbohydrate, which aid in membrane integration and functional orientation of the protein. Current research on neural receptors has led to the actual isolation of portions of these membrane complexes which retain in vitro the binding kinetics and specificity of the intact, in vivo, receptor. Studies utilizing such in vitro receptor preparations can thus yield information which is directly applicable to the understanding of both normal and abnormal neurotransmission in vivo. For example, the successful isolation of the acetylcholine receptor protein and the use of antibodies raised against this protein have led to the postulate that the neuromuscular disorder, myasthenia gravis, is an auto-immune disease involving the post-synaptic cholinergic receptor (22). This understanding now makes possible the development of new drug treatments for the disease (23).

Neural receptor macromolecules have been isolated from a variety of tissues as either detergent-solubilized proteins or protein-lipid complexes (24,25). For example, receptor macromolecules have been isolated from tissues and retain the binding characteristics of in vivo cholinergic (muscarinic and nicotinic), adrenergic (α- and β-), opiate, serotoninergic and amino acid receptors. While most of these receptors have been successfully isolated as either a protein or as a proteolipid complex, questions have been raised concerning the validity of isolating neural receptors as proteolipids utilizing organic solvents (26). These questions appear answered by recent studies (25,27) which support the postulate that an isolated proteolipid receptor preparation more closely represents the true in vivo neural complex since the integration and function of the protein portion of the receptor in the neural membrane must require lipid (25,28). This postulate is further supported by the requirement for detergent in the purification of any intact neural receptor protein in order to dissociate that protein from its associated membrane (lipid) components. A final and definitive proof that neural receptor proteins and proteolipids represent but steps in active site purification must, however, await further studies on direct comparisons of the two preparations from the same tissue. Such a comparison was carried out in the present study.

We previously reported the first, direct evidence that pressure can affect the acetylcholine receptor (AChR). Utilizing the isolated nicotinic AChR proteoglycolipid (PGL) from rat gastrocnemius muscle, we showed that increasing pressure caused a decreased binding of cholinergic ligands, including acetylcholine (ACh), to the receptor, apparently due to pressure-induced binding site changes (14). Our observations were subsequently confirmed by other workers using an AChR membrane preparation from the electric tissue of Torpedo californica (29). We also showed that the pressure-induced decrease in receptor affinity for ACh was prevented by hexamethonium, thus providing a starting point for studies on drug control of pressure-induced neurological disruption. Our approach toward defining pressure effects on neuronal membranes/receptors also illustrated that studies on the effects of pressure on receptors can provide new insights into our understanding (and control) of the function of biological molecules (receptors, enzymes, hormones, etc.) in general.

The present study was directed at continuation of our investigations on hyperbaric pressure induced changes in neural receptor function. We again utilized isolated components (protein and PGL) or the nicotinic cholinergic receptor. Our primary source of the receptors was rat gastrocnemius muscle, although studies were also carried out with the AChR protein from the electric eel, Electrophorus electricus. In the course of these studies, a number of new methods were developed for purification of the AChR, including a rapid high pressure affinity chromatography method. In addition, for the first time, both a protein and PGL AChR were isolated from the same tissue and studied in parallel. Significantly, both preparations were similar in their physical and chemical characteristics and in their response to pressure. A preliminary report of our work has been presented (30) and a more detailed report is currently in press (31). All information in the latter reports is contained in this report.

2. MATERIALS AND METHODS

2.1 Materials

Gastrocnemius muscle was dissected immediately from sacrificed male and female, 140-170g Sprague-Dawley rats. After washing with 0.9% aqueous NaCl, the tissue was blotted dry, weighed and stored at -40°C until extracted.

All solvents and reagents were analytical grade or better. Acetylcholine chloride, d-tubocurarine chloride (dTC), nicotine, muscarine, decamethonium, hexamethonium, atropine, diisopropylflurophosphate (DFP), physostigmine, tetrodotoxin, veratridine and α-bungarotoxin (αBTX) were all obtained from Sigma Chemical Co., St.Louis, MO. Bromoacetylcholine (BAC) was synthesized from bromoacetyl bromide and ch ... e chloride (both from Aldrich Chemical, Milwaukee, WS) according to s dard methods (32). Its identity was established by chromatographic anc ...emical analysis (32-34). [^{3}H]BAC (873 µCi/µmol) was synthesized in t same way utilizing [methyl-^{3}H] choline chloride (80.0 Ci/mmol; New Eng. Nuclear, Boston, MA). [^{14}C] Acetylcholine iodide (4.8 mCi/mmol), [^{5} u-bungarotoxin (15.9 µCi/µg) and [13^1-^{3}H(N)]d-tubocurarine chloride (15.8 Ci/mmol) were purchased from New England Nuclear.

2.2 Preparation of the AChR

Our general approach for isolation of the AChR-PGL or AChR protein is illustrated in Fig. 1. The AChR-PGL was isolated from rat gastrocnemius using CHCl$_3$-MeOH extraction and purification to homogenecity according to our standard method (27). For example, in a typical run the AChR-PGL in the total lipid extract was purified using sequential columns of Sephardex LH-20, Sepharose CL-4B and an affinity support containing trimethyl(p-aminophenyl)ammonium chloride (p-TAPA) as the binding ligand. The synthesis of this support utilizing Sephadex LH-20 as the starting support is illustrated in Fig. 2. A typical purification run using this affinity support is shown in Fig. 3, where the pulse used can be either 0.02 M ACh or 0.10 M HCl in 5 ml of CHCl$_3$-MeOH (1:1, v/v). A more rapid method for purifying the AChR-PGL is discussed below in Section 2.2. The purity of the PGL was established by rechromatography on Sephadex or Sepharose columns; or by high pressure gel permeation chromatography (HP-GPC) and polyacrylamide gel electrophoresis (PAGE) according to our standard methods (35). For example, the purified AChR-PGL eluted as a single peak from a bank of five µStyragel HPLC columns (Waters Associates, Milford, MA) eluted with CHCl$_3$ at a flow rate of 1 ml/min. The columns were linked in the order of pore size (inlet to outlet) of 100,000 to 10,000 to 1000 to 1000 to 500 Å, allowing separation of molecules with molecular weights ranging from 10^3 to 8 x 10^6.

Aqueous preparations of the AChR were prepared in 50 mM NaHPO$_4$ buffer (pH 7.4) containing 50 mM NaCl, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride according to standard methodologies (36,37). After homogenization of the tissue and an initial centrifugation at 1,000 x g

HOMOGENIZED TISSUE

ORGANIC MEDIUM

AQUEOUS MEDIUM

$CHCl_3$ - MeOH
SOLUBLE MATERIAL

DETERGENT-SOLUBLE
MATERAL

CHROMATOGRAPHY
(SEPHADEX LH-20; AFFINITY)

CHROMATOGRAPHY
(SEPHADEX; ION-EXCG; AFFINITY)

RECEPTOR PROTEOLIPID

RECEPTOR PROTEIN
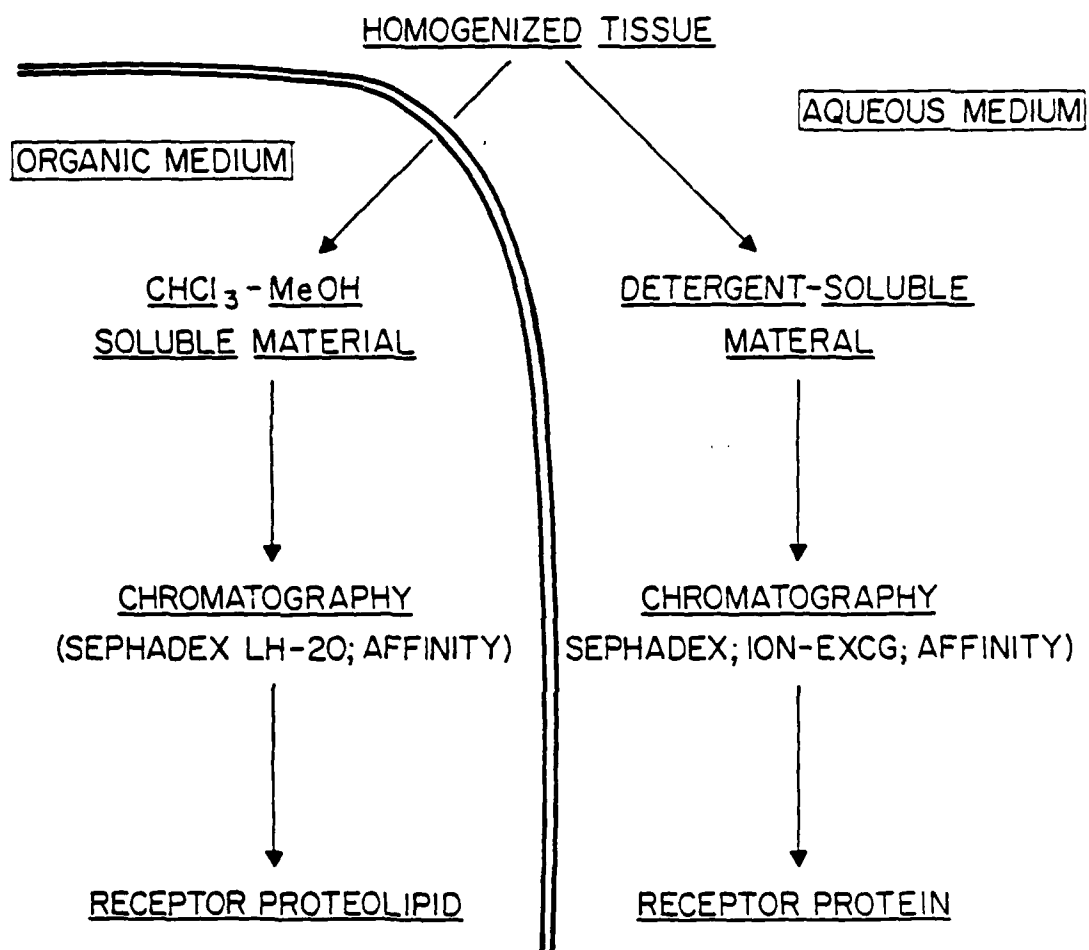
Fig. 1. Comparison of the methods used for isolation of the AChR as either a proteolipid or a protein.

ACTIVATED LH-20

$$\text{C}=\text{NH} \xleftarrow[\text{OH}^-]{\text{CNBr}} \text{LH-20}$$

$$\text{OH}^- \qquad \text{H}_2\text{N}-(\text{CH}_2)_3-\overset{\text{H}}{\text{N}}-(\text{CH}_2)_3\text{NH}_2$$

3,3'-DIAMINODIPROPYLAMINE

AMINOALKYL LH-20

$$\text{OH}$$
$$\text{O}\overset{\text{H}}{\text{C}}-\text{N}(\text{CH}_2)_3-\overset{\text{H}}{\text{N}}-(\text{CH}_2)_3\text{NH}_2$$
$$\overset{\|}{\text{NH}}$$

$$\text{H}^+ \qquad \text{O}=\text{O} \quad =\text{O}$$

SUCCINIC ANHYDRIDE

SUCCINYLAMINOALKYL LH-20

$$\text{OH}$$
$$\text{O}\overset{\text{H}}{\text{C}}-\text{N}(\text{CH}_2)_3\overset{\text{H}}{\text{N}}(\text{CH}_2)_3\overset{\text{H}}{\text{N}}-\overset{}{\text{C}}\text{CH}_2\text{CH}_2\text{COOH}$$
$$\overset{\|}{\text{NH}} \qquad\qquad\qquad \overset{\|}{\text{O}}$$

$$\text{H}^+ \atop \text{CARBODIIMIDE} \qquad \text{H}_2\text{N}-\bigcirc-\overset{+}{\text{N}}\overset{\text{CH}_3}{\underset{\text{CH}_3}{-\text{CH}_3\text{Cl}^-}}\cdot\text{HCl}$$

p-TAPA

$$\text{OH}$$
$$\text{O}\overset{\text{H}}{\text{C}}-\text{N}(\text{CH}_2)_3\overset{\text{H}}{\text{N}}(\text{CH}_2)_3\overset{\text{H}}{\text{N}}-\text{C}\text{CH}_2\text{CH}_2\text{C}-\overset{\text{H}}{\text{N}}-\bigcirc-\overset{+}{\text{N}}\overset{\text{CH}_3}{\underset{\text{CH}_3}{-\text{CH}_3\text{Cl}}}$$
$$\overset{\|}{\text{NH}} \qquad\qquad\qquad \overset{\|}{\text{O}}\quad\overset{\|}{\text{O}}$$
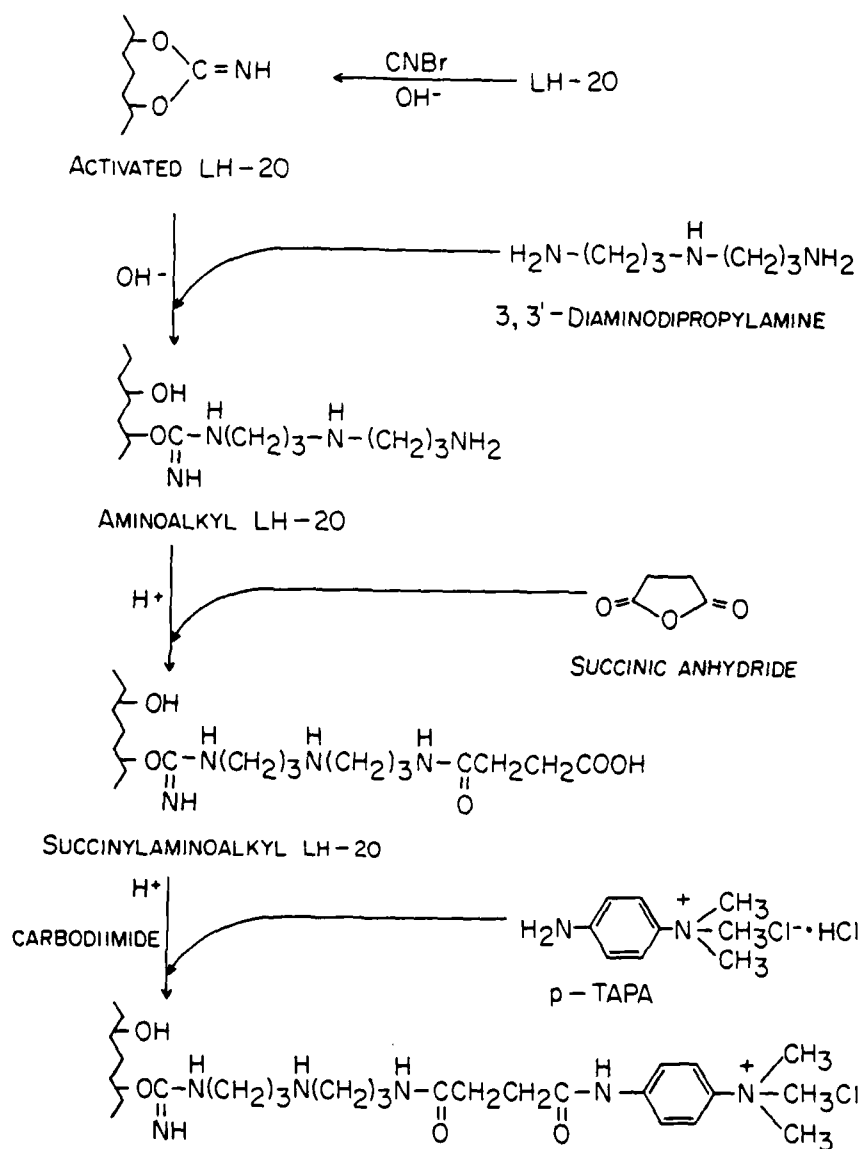
Fig. 2. Synthesis of the low pressure affinity chromatography support
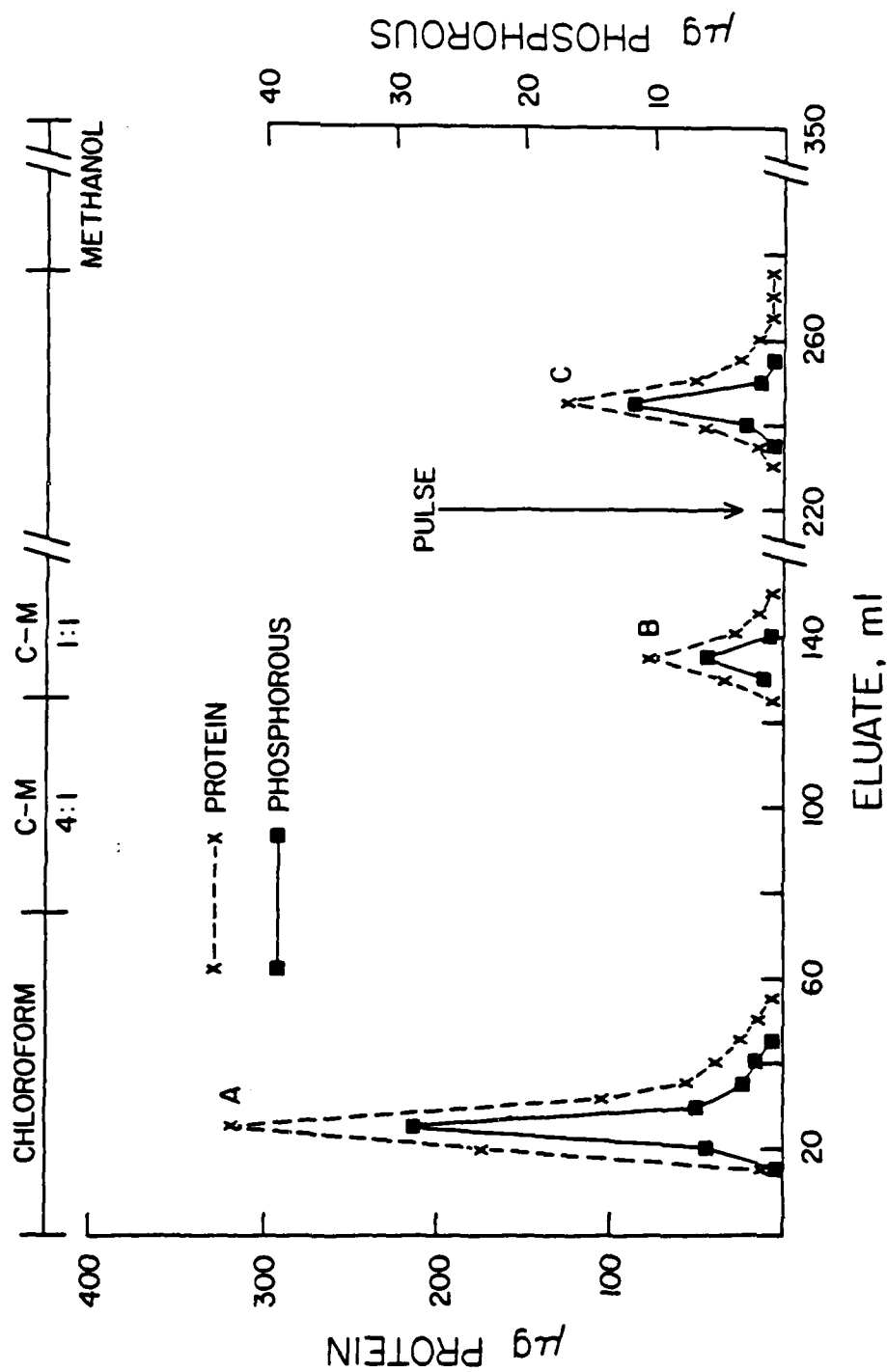utilized for isolation of the AChR-PGL or the AChR protein.

Fig. 3. Typical elution profile resulting from low pressure affinity chromatography of the AChR-PGL. The PGL from 10 g wet weight gastrocnemius tissue was first partially purified by Sephadex LH-20 chromatography and then applied to the affinity column. The pulse applied was 0.02 M ACh in C-M, 1:1 (from Reference 27).

for 30 min, the crude membrane fractions were isolated by centrifugation of the 1,000 x g supernatant at 40,000 x g for 60 min. The membrane-associated AChR protein was extracted from the resulting pellet with the original homogenizing buffer containing 2% Triton X-100 for 60 min at 4°C. After centrifugation of this mixture at 30,000 x g for 30 min, >90% of specific cholinergic binding activity was found in the supernatant. This crude membrane receptor preparation ("AChR membrane") was used in initial pressure studies and as the starting material for purification of the AChR protein utilizing affinity and DEAE-Sephadex chromatography (27,37). Affinity chromatography utilized a support comprised of p-TAPA linked to 6-aminohexanoic acid-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) using carbodiimide coupling (27). The purified AChR protein was shown to be homogenous by polyacrylamide gel electrophoresis (with and without SDS) and by HP-GPC. For example, the AChR protein eluted as a single peak from a Biol-Sil TSK 250 HPLC column (BioRad Laboratories, Richmond, VA) eluted with 0.1 $\underline{M}$ Na$_2$SO$_4$ in 0.02 $\underline{M}$ NaH$_2$PO$_4$ pH 6.8 at a flow rate of 1 ml/min.
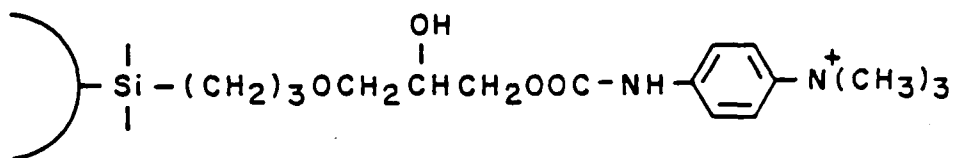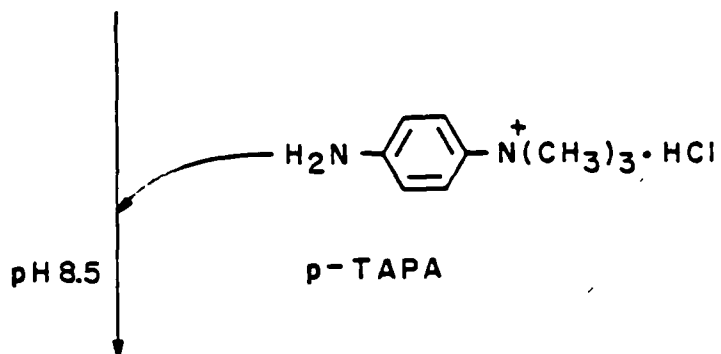
2.3 High Pressure Affinity Chromatography (HPAC)

HPAC was evaluated as a means for rapid purification of the AChR components. The support for HPAC was synthesized (Fig. 4) by direct reaction of p-TAPA ($\cong$800 mg) with 15g of a glycerol-coated controlled-pore glass bead (74-125μ) activated with carbonyldiimidazole (CDI-activated glycophase, Pierce Chemical Co., Rockford, IL). After reaction overnight in 0.1 $\underline{M}$ Na borate-boric acid pH 8.5, the support was washed and the amount of p-TAPA bound to the support was quantitated spectrophotometrically from unbound p-TAPA remaining in the reaction supernatant and washes (27). Typically, from 10 to 20 μmol of p-TAPA was bound per g of support.

The washed support was dehydrated by sequential washings in MeOH and acetone, and then dried at 50°C overnight in a vacuum oven. The dry support was packed into 8 x 250 mm stainless steel HPLC columns and equilibrated at 1 ml/min in either CHCl$_3$ or 0.01 $\underline{M}$ Na$_2$HPO$_4$-NaH$_2$PO$_4$ pH 7.2 depending on which receptor component was to be purified. After sample application (typically 50-250 μg of protein for analytical runs and up to 25 mg of protein for preparative runs) to the column, the column was eluted with the equilibration solvent until all non-adsorbed protein/proteolipid eluted ($\cong$10-12 min at 1 ml/min flow). After this time, a 5 ml pulse of 0.02 $\underline{M}$ ACh in CHCl$_3$ (for the AChR-PGL) or the addition of 1 $\underline{M}$ NaCl to the equilibration buffer (for the AChR protein) was used to desorb the receptor from the column. For example, as shown in Fig. 5, the partially purified AChR-PGL from the initial purification of a gastric total lipid extract on a Sephadex LH-20 column was purified on the TAPA-glycophase HPAC column in $\cong$30 min. The purified AChR-PGL was comparable in homogeniety and retention of cholinergic binding activity to AChR-PGL prepared using Sephadex LH-20, Sepharose CL-4B and TAPA-LH-20 affinity columns, a sequence requiring three additional days past the Sephadex LH-20 step. Similar results were obtained for the purification of the AChR protein from solubilized membranes using the aqueous HPAC system described above.
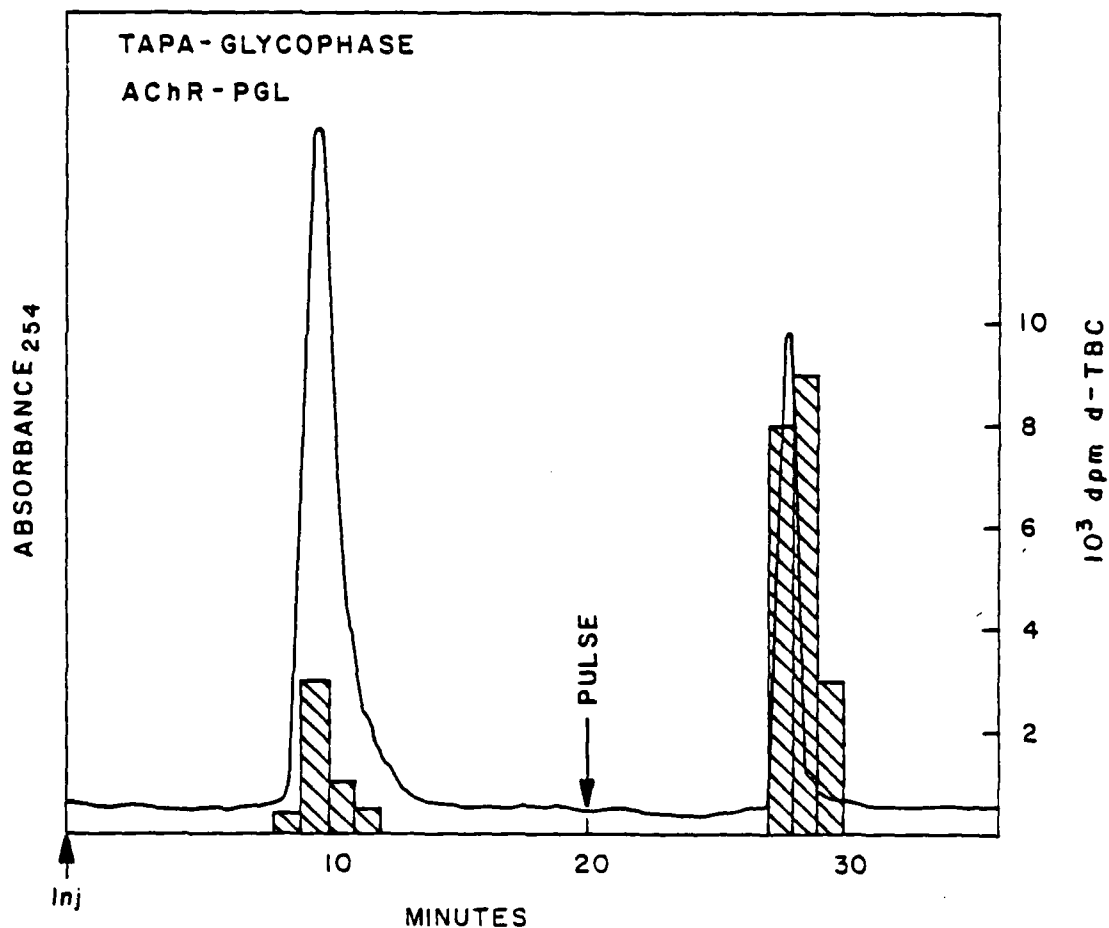
CDI-GLYCOPHASE

pH 8.5

p-TAPA
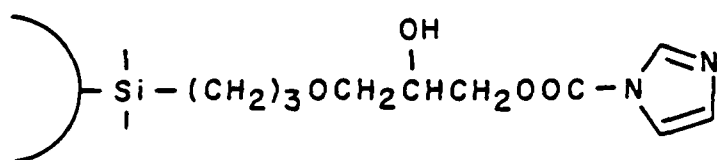
TAPA-GLYCOPHASE

Fig. 4. Synthesis of the high pressure affinity chromatography support used for purification of the AChR.

10

Fig. 5. Purification of the AChR-PGL from rat gastrocnemius muscle by high pressure affinity chromatography utilizing the TAPA-Glycophase support (Fig. 4). Approximately 50-100 µg of protein was applied to the column after pre-reaction with 1 µM [3H]d-tubocurarine and eluted at 1 ml/min with $CHCl_3$ until all UV-absorbing material had eluted from the column (≈20 min). At that time, a 5 ml pulse of 0.01 $\underline{M}$ in $CHCl_3$-MeOH (4:1) was applied to the column and elution was continued until the receptor eluted from the column. Analysis of the column fractions for radioactivity identified dTC-receptor binding.

2.4 Binding Assays

Two different approaches are necessary to *assay cholinergic ligand*
binding to the AChR preparations. For the organic *solvent soluble*, AChR-
PGL, a biphasic (chloroform-aqueous) partition method is used (14,27,35)
in which the (hydrophobic) PGL is dissolved in the lower, *organic phase*;
and the (hydrophilic) ligand is dissolved in the (buffered) upper,
aqueous phase. Migration of radioactive ligand from the upper to the
lower phase directly correlates to ligand-PGL binding after correction
for non-specific migration with appropriate controls. Typically, PGL
samples containing from 10 to 100 µg of protein were made up in 5 ml of
$CHCl_3$ saturated with 0.05 $\underline{M}$ Tris-buffer pH 7.2 and placed in a reaction
vessel containing a teflon-coated stirring bar. An equal colume of 0.05
$\underline{M}$ Tris-HCl pH 7.2 saturated with $CHCl_3$ and containing the ligand was then
added to the vessel. The lower $CHCl_3$ phase of the mixture was then
stirred using the magnetic stirrer, while the upper aqueous phase was
stirred at the same rate using a glass stirring rod attached to an
overhead stirring unit. After 30 min at 25°C, the aqueous phase was
removed and both phases were assayed for radioactivity. The amount of
ligand bound by the PGL was determined from the amount of radioactivity
found in the $CHCl_3$ phase after correcting for the normal partitioning
behavior determined for each ligand in non-proteolipid containing control
runs (27). In studies using [^{125}I]α-BTX as the aqueous phase ligand,
100µg of bovine serum albumin (BSA) was added per ml in order to prevent
non-specific binding of the BTX to the glass walls of the reaction vessel
(27).

Assays of ligand binding to the aqueous AChR membrane and protein
preparations utilized an ultrafiltration assay with Amicon PM-30 filters
(Amicon Corp., Lexington, MA). Receptor-ligand complex is retained on
these filters and the amount of ligand bound to the AChR preparation is
quantitated from free ligand in the filtrate, corrected for non-specific
binding of ligand to the *filter* as determined from control runs with no
AChR preparation present. Typically, *from* 10 to 100µg of protein was
made up into 5 ml of 5 m$\underline{M}$ Na_2HPO_4-NaH_2PO_4 buffer, pH 7.4, containing 0.1%
Triton X-100 (TX-100) and 100 µg/ml of BSA. Ligand was added to this
solution and, after 30 min, bound ligand was determined by radioactivity
present in the reaction mixture filtrate.

2.5 Pressure Studies

Studies of pressure effects on the receptor preparations were
carried out in a temperature-controlled, 1500 psi, 3 ft x 1.5 ft stain-
less steel pressure vessel (Pressure Products Industries, South Hacken-
sack, NJ) equipped with a remote oxygen analyzer, gas mixing capabilities
and an on-line diaphragm compressor (Figs. 6 and 7). The diving gas used
was helium-oxygen (heliox). Assays for the binding of radioactive
ligands to the AChR preparations utilized the same methodologies de-
scribed in Section 2.4. To carry out such assays in the chamber, the
apparatus illustrated in Fig. 8 was used (14). This apparatus allows
duplicate ligand binding assays to be carried out at any pressure level.
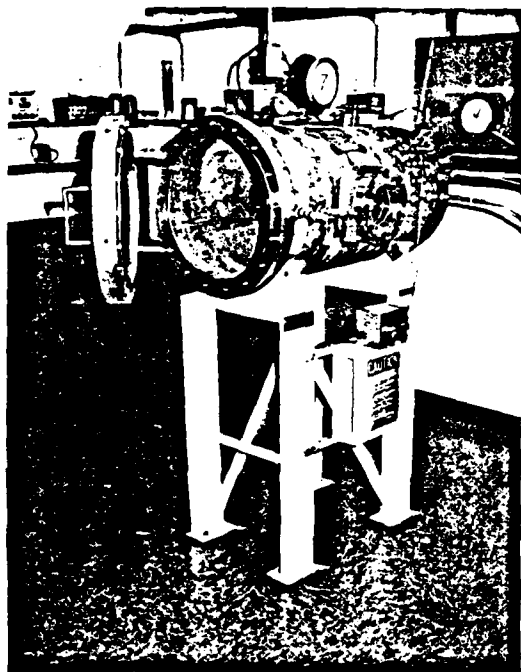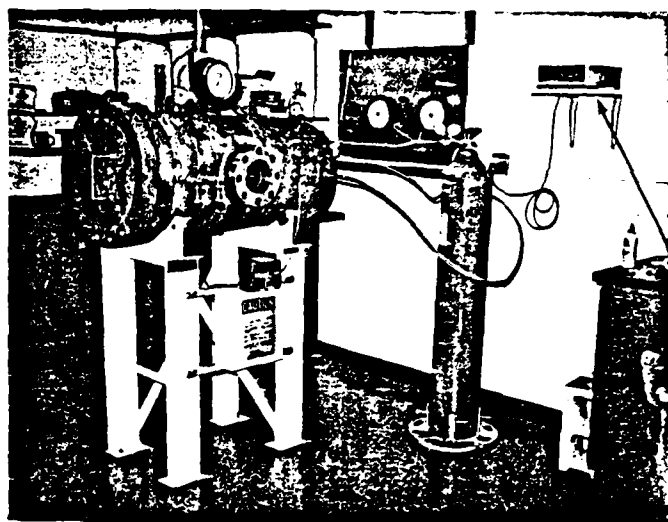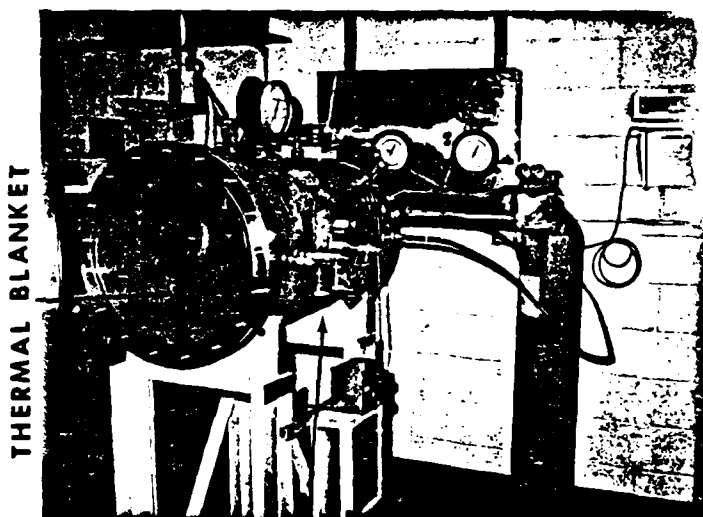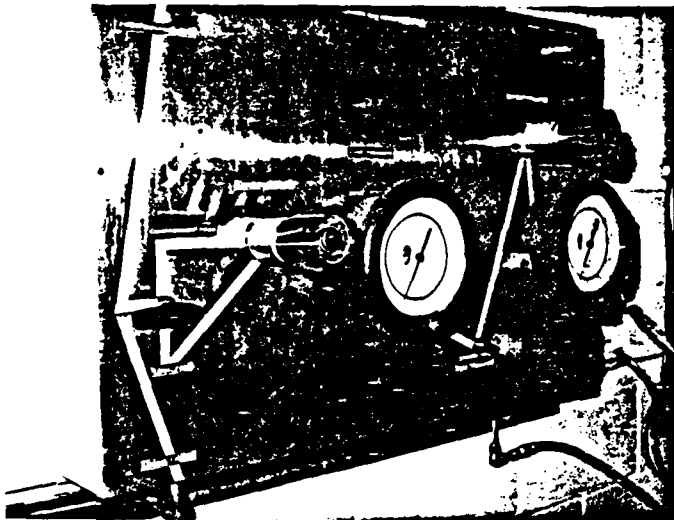
OXYGEN ANALYZER

CHAMBER

CHAMBER

THERMAL BLANKET

SAMPLING VALVES

<u>Fig. 6.</u> Arthur D. Little, Inc., 1500 psi research hyperbaric chamber.

CHAMBER GAS CONTROL
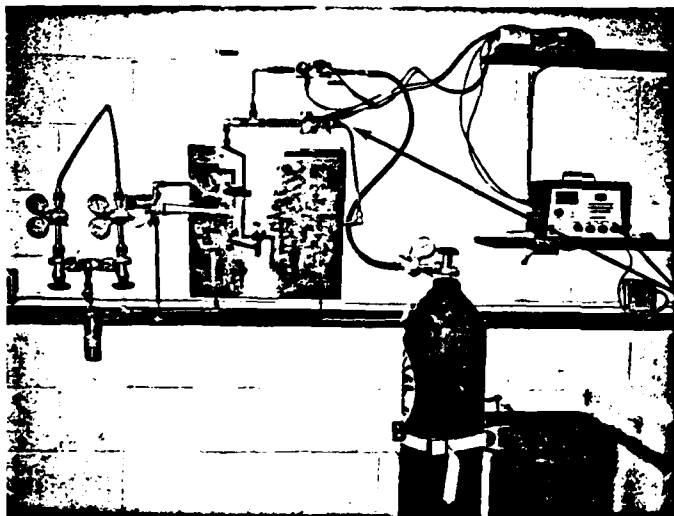
GAS INLET CONTROL

GAS MIXER
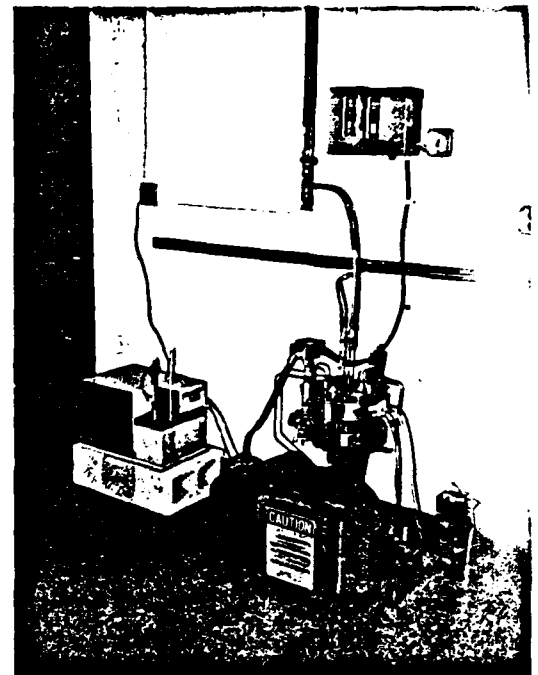
DIAPHRAGM COMPRESSOR

Fig. 7. Support equipment for the Arthur D. Little, Inc., 1500 psi research hyperbaric chamber.

AQUEOUS PHASE

CHCl$_3$ PHASE

SPARKLESS MOTOR

GLASS CYLINDER

GLASS STIRRING ROD

TO SAMPLING VALVE

STIRBAR

SPARKLESS MOTOR

TO EXTERNAL CONTROL
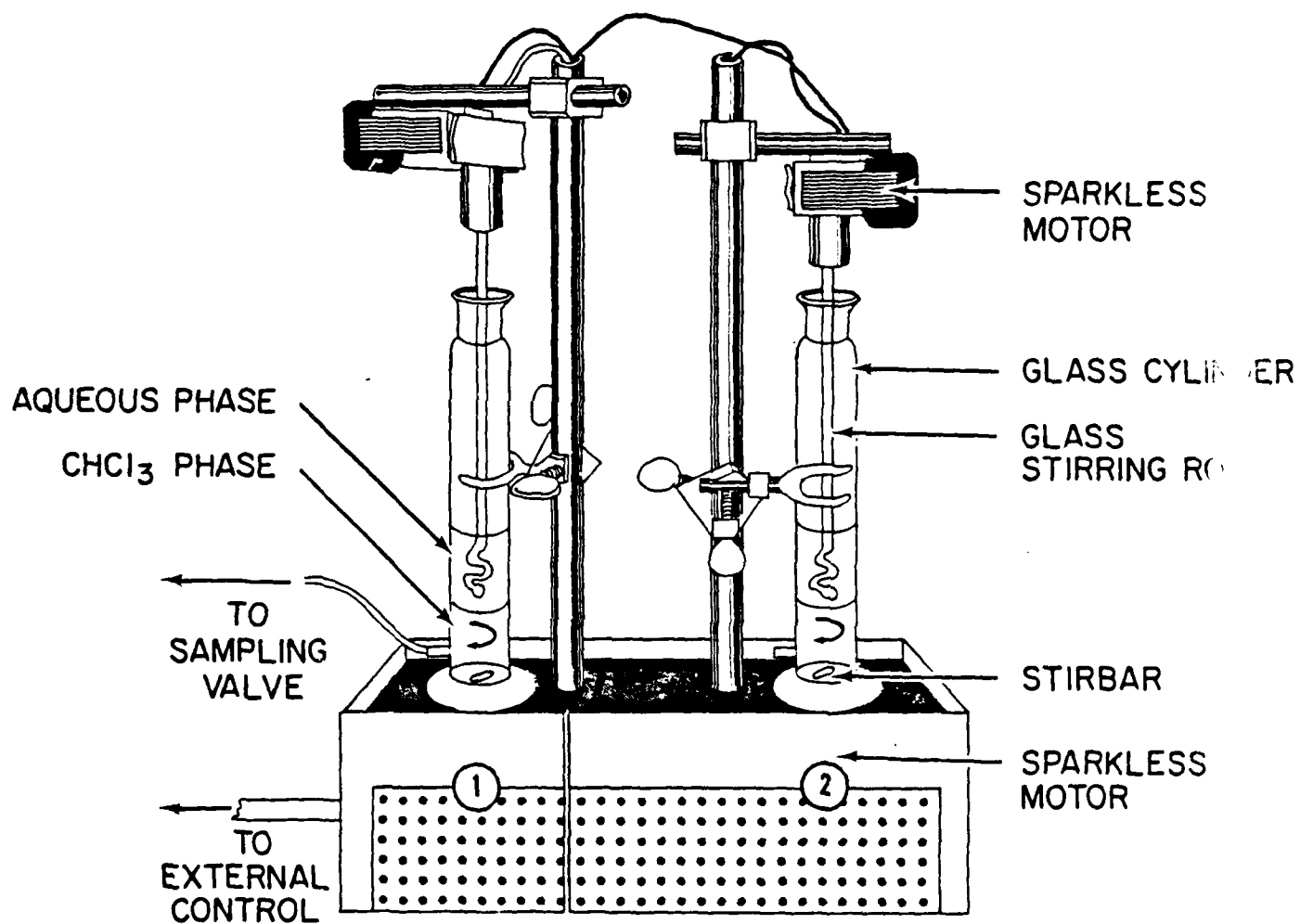
Fig. 8. In-chamber apparatus used for studies on the effect of hyperbaric pressure on the binding of cholinergic ligands to the AChR-PGL and AChR protein (from Reference 14).

The glass cylinders used with the apparatus have been modified to contain 3 mm (i.d.) glass outlet tubes near their bases to allow sampling of the reaction mixtures under pressure through stainless steel/teflon micro-metering sampling valves installed on the chamber exterior (Fig. 6).

In studies with the AChR-PGL, both phases of the biphasic partition mixture are stirred by means of overhead and bottom stirring units, and samples for receptor-ligand quantitation are withdrawn directly from the lower phase through the sampling lines. For studies with the aqueous AChR preparations, the (monophasic) reaction mixture is also sampled through the in-chamber lines but the lines are modified to contain an in-chamber, stainless steel Swinney filter containing a 13 mm Amicon PM-30 filter to allow for separation of free ligand (which passes through the filter) and ligand-receptor complex (which is retained on the filter). The small pressure drop across the filter (estimated at <5% of the internal chamber pressure) was not found to affect the PM-30 filters used.

Each pressure test run employed four identical samples, two inside and two outside the chamber. The two samples inside the chamber were those actually sampled at the elevated pressure levels studied, while the two samples outside the chamber were sampled at the beginning and end of the pressure run, thus serving as atmospheric pressure controls. During all runs, the hyperbaric chamber was maintained at an internal temper-ature of 25°C. After installation of the assay apparatus into the chamber, the reaction mixtures were equilibrated by bubbling helium into the solutions for 1 min through the two exterior sampling valves. The chamber was then pressurized with helium as the diluent gas following a diving protocol in which a rate of 5 psi/min was used to achieve the 50-psi pressure level and then a rate of 10 psi/min to achieve all subsequent pressure levels. Such rates are similar to those established for diving experiments with live animals (38). A surface equivalent content of 0.20 atm of oxygen was maintained in the chamber at all times as determined by monitoring with a Beckman Model OM-14 Oxygen Analyzer. Decompression was carried out using the same protocols. A summary of the diving and sampling times used is presented in Table 1. After a given pressure level was achieved, the partition phases were allowed to equil-ibrate at that level for 30 min and then samples were taken. Sampling was carried out by withdrawing 0.1 ml aliquots of the $CHCl_3$ phase (for the AChR-PGL) or the reaction mixture (for the aqueous AChR preparations) and analyzing for radioactivity as described in Section 2.4. Both prior to and after sampling, the sample lines were backflushed using an ex-ternal helium tank to ensure proper mixing of samples and resuspension of receptor-ligand complex from the (aqueous assay) filters.

2.6 Chemical and Physical Assay Methods

Protein carbohydrate and phosphorus contents in the receptor prepa-rations were determined using standard methods (27,39-41). PAGE analysis was carried out both in the presence and absence of SDS on 7.5% cross-linked gels according to standard methods (42,43) using standard protein

TABLE 1

Diving and Sampling Protocol[a]

Sample	Diving Time (min)	Final Pressure (psi)	Sampling Time (min)
1	0	14.7	+30
2	0-10	50	+40
3	+40-45	100	+75
4	+75-85	200	+115
5	+115-135	400	+165
6	+165-185	600	+215
7	+215-235	800	+265
8	+265-285	600	+315
9	+315-335	400	+365
10	+365-385	200	+415
11	+415-425	100	+455
12	+455-464	14.7	+495

[a]In any experimental run, duplicate samples were withdrawn from the reaction mixtures after the mixture had equilibrated for 30 min at the appropriate pressure level. Thus, for example, in proceeding from 50 to 100 psi, the vessel was pressurized at 10 psi/min for 5 min (diving time, 40-45 min). The reaction mixtures were allowed to equilibrate for 30 min and duplicate samples were then withdrawn (sampling time 75 min).

markers to assign tentative molecular weights to the receptor preparations (35). HP-GPC analysis of the receptor preparations was carried out as described in Section 2.2 using a Micromeritics Model 701 liquid chromatograph equipped with a variable UV-visible detector and a Schoffel FS 970 fluorometer. UV-visible spectroscopic analysis of the receptor preparations was carried out on a Varian Cary 118C UV-visible spectrophotometer and fluorescence spectra were determined on a Perkin-Elmer Hitachi MPF-44 fluorescence spectrophotometer.

3. RESULTS AND DISCUSSION

3.1 Characterization of the AChR Preparations

3.1.1. Chemical and Structural Characterization

Three primary AChR preparations from rat gastrocnemius muscle were isolated and used in these studies, the AChR-PGL, an enriched AChR membrane fraction and the A hR protein. Of these, the proteolipid and protein preparation proved homogeneous upon PAGE and HP-GPC analysis. As shown in Table 2, both similarities and differences were found upon analysis of the two preparations. For example, the protein contains more carbohydrate than the PGL but contains no phosphorus. The occurrence of carbohydrate in the receptor preparations was expected and agrees with the findings of other workers that the AChR is a glycoprotein (44,45). The phosphorus in the PGL preparation is presumably part of its lipid component(s) and this component is lost if the AChR protein is isolated using detergent solubilization.

By weight, there is more PGL than protein in the tissues. This is expected due to the associated lipid/glycolipid/lipoprotein components in the PGL. Such an increased molecular size of the PGL is not apparent, however, from its estimated molecular weight (Table 2). The molecular weight reported may be misleading since it is estimated in comparison to standard polystyrenes. We have shown that the molecular weights of lipid-protein complexes cannot be accurately determined by comparison to such standards (35) and may be underestimated by 2-10X. Pending full characterization of protein-lipid complexes such as the AChR-PGL, an accurate molecular weight for such complexes cannot be determined.

We also compared isolation of the PGL from wet and dry (lyophilized) tissue since a question remains as to which method is the most appropriate for isolating the receptor, i.e., while extraction from dry tissue is much easier, there are indications that the drying process leads to lipid oxidation/degradation and irreversible changes in the AChR-PGL (27). As shown in Fig. 9, we found that such changes do occur. The two preparations analyzed were carried through identical pre-purification steps prior to HP-GPC. It was found that the major, receptor site-containing peak isolated from wet tissue apparently degraded to a number of lower molecular weight components during the drying process. For this reason, we routinely extracted the AChR-PGL from wet tissue during our studies.

Analysis of the AChR protein and PGL by SDS-PAGE allows characterization of the subunits comprising the receptor. As reported in Table 2, the receptor protein is comprised of five distinct polypeptide chains with molecular weights of $\cong 100,000$, 83,200, 61,900, 53,700 and 43,200. As illustrated in Fig. 10, the 43,200 subunit contained the active receptor binding site. These results are in agreement with those reported by other workers for the AChR protein from rat muscle (36,37, 59-61).

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TABLE 2

Characterization of the Receptor Preparations[a]

| Parameter	AChR Protein	AChR-PGL
% Cell weight	0.00044	0.001
Molecular weight (HP-GPC)	232,000	39,800[b]
Subunit molecular weights (SDS-PAGE)	100,000	115,000
	83,200	
	61,900	69,200
	53,700	54,300
	43,200[c]	47,800[c]
		18,200
Component composition (wt %)		
Protein	94	72
Carbohydrate	6.0	2.1
Phosphorus	<0.01	0.25

[a]Each value is the mean of at least three determinations with no single value deviating from the mean by more than ±8.3%.

[b]Compared to polystyrene standards, see the text for details.

[c]Active site component
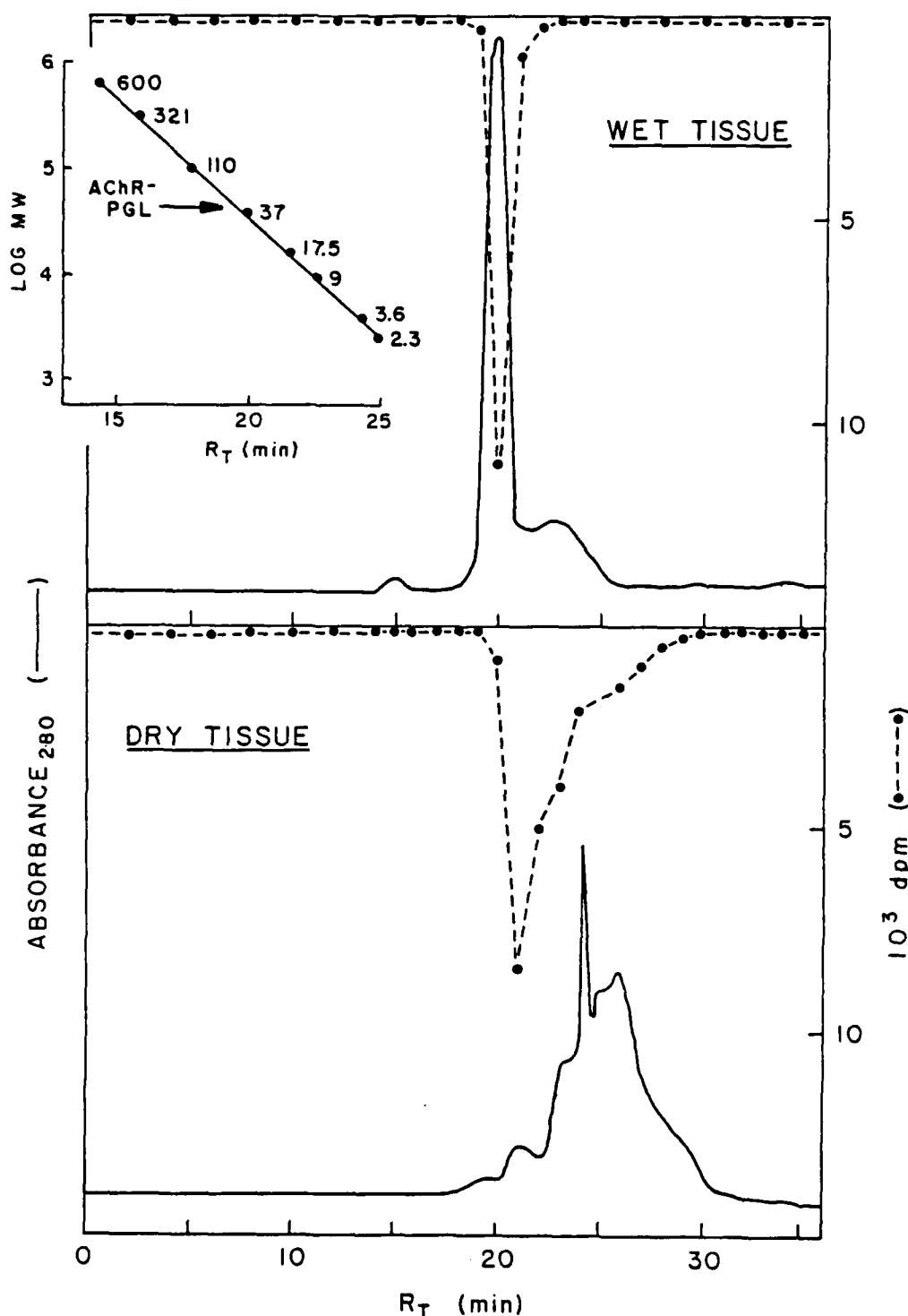
Fig. 9. HP-GPC analysis of the AChR-PGL extracted from wet or lyophilized (dry) gastrocnemius tissue. The preparations were purified through Sepharose CL-4B chromatography and analyzed on a bank of five µStyragel HPLC columns described in Section 2.2. Prior to chromatography, 100-200 µg (protein) of each preparation was reacted for 60 min with 5 µM [^{3}H]d-tubocurarine to label the receptor active site. The column was eluted with $CHCl_3$ methanol (4:1) at 2 ml/min. Collected fractions were analyzed for radioactivity to locate the receptor active site. The insert locates the AChR-PGL MW (X1000) in comparison to known polystyrene standards analyzed on the same HP-GPC system.

21
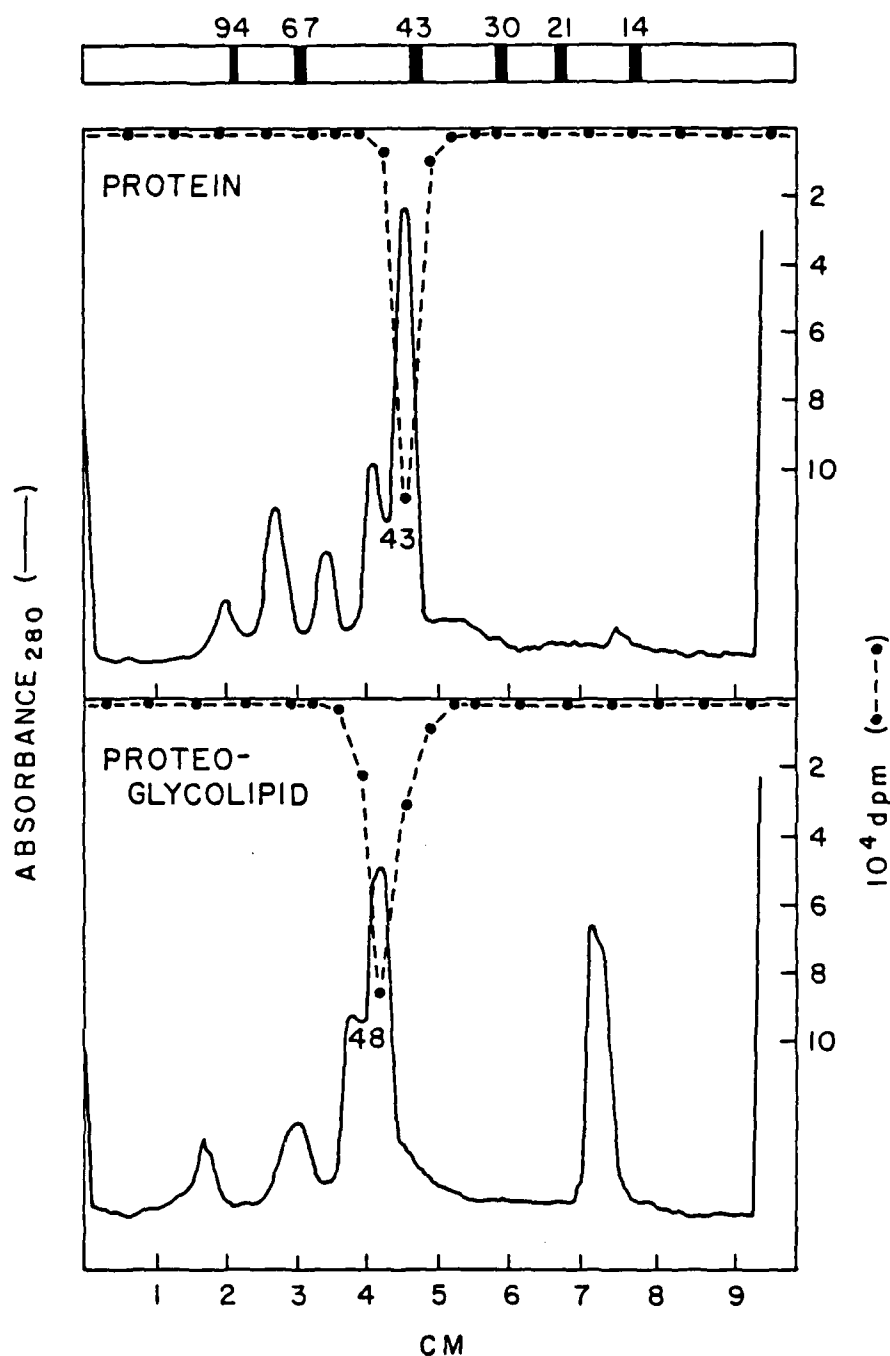
Fig. 10. SDS-PAGE analysis of the AChR protein and proteoglyco-
lipid from rat gastrocnemius muscle. Approximately
100 μg of receptor protein was pre-reacted with 5 μM
[125-I]αBTX before application to the gel. After elec-
trophoresis was completed, the gels were scanned at
280 nm to locate protein/lipid; and then were sliced
into 50 portions. Each gel slice was extracted with
1 ml 20 mm Tris-HCl pH 8 containing 1% SDS for 24 h
and the resulting extract was assayed for radioactivity.
The migration of standard protein molecular weight
markers is illustrated for reference purposes.

Analysis of the AChR PGL by SDS-PAGE also resulted in five components but with approximate molecular weights differing from those of the protein, i.e., ≅115,000, 69,200, 54,300, 47,800 and 18,200. Except for the last component, which may represent a hitherto unknown receptor component, the increased molecular weights observed suggest that these components may be comprised of at least four of the polypeptides found in the receptor protein with the additional weight due to associated lipid and/or carbohydrate (see Table 2). As shown in Fig. 10, the 47,800 subunit of the AChR-PGL contained the active binding site. This is the first known report of the direct comparison of a protein and PGL receptor by SDS-PAGE. Our results confirm that these two preparations represent different portions of the same biological receptor.

A further SDS-PAGE analysis was carried out on the AChR-PGL. It is common knowledge that during the extraction and isolation of proteo-lipids, removal of all the $CHCl_3$ from the extract can lead to dissoci-ation of the protein and lipid components and precipitate formation. During the course of one PGL extraction, this occurred. The precipitate, however, was successfully resolubilized in $CHCl_3$ and we proceeded to carry out our routine first PGL purification step using preparative Sephadex LH-20 chromatography. As reported previously, dissociated/modified PGL receptor is retained on this column until elution with methanol is carried out (27). In this particular extraction run, a significant amount of such material was eluted with methanol. Upon concentrating this material in vacuo to a small volume, a preciptate formed which was comprised of both $CHCl_3$ soluble and insoluble material. We washed this precipitate sequentially with $CHCl_3$, methanol and water (which dissolved the remaining precipitate) and analyzed each fraction separately for [^{125}I]α-BTX binding. As reported in Table 3, each frac-tion bound the ligand indicating the presence of a fragment containing the AChR active site. Upon SDS-PAGE analysis of the [^{125}I]α-BTX labeled fractions, we found that the toxin bound to very specific components in the fractions. From the results presented in Fig. 11, we conclude that disruption of the AChR-PGL resulted in a water-soluble receptor with subunit characteristics (Table 3) midway between the AChR pro··in and AChR-PGL (Fig. 10). The major toxin binding component found in the $CHCl_3$ fraction has a much lower molecular weight than the binding component of the AChR-PGL and may represent a fragment of the native component. The methanol fraction appears to contain a very low molecular weight toxin-binding component which appears, in addition, a very minor component as determined by UV_{280} detection. It is notable that the sum of the molecular weights of the toxin-binding components in the $CHCl_3$ and methanol fractions is 43,000 (Table 3), suggesting that these are two portions of the water-soluble, 45,200 toxin binding component. This experiment thus further supports the direct relationship of the AChR protein and proteolipid. Further studies of this nature could definitively characterize the differences between these two preparations.

3.1.2. Binding Characteristics

The most critical test of the AChR preparations is their ability to bind cholinergic ligands. As shown in Table 4, all three receptor preparations bound cholinergic ligands. As reported elsewhere (27), such binding is comprised of both specific and non-specific components. The
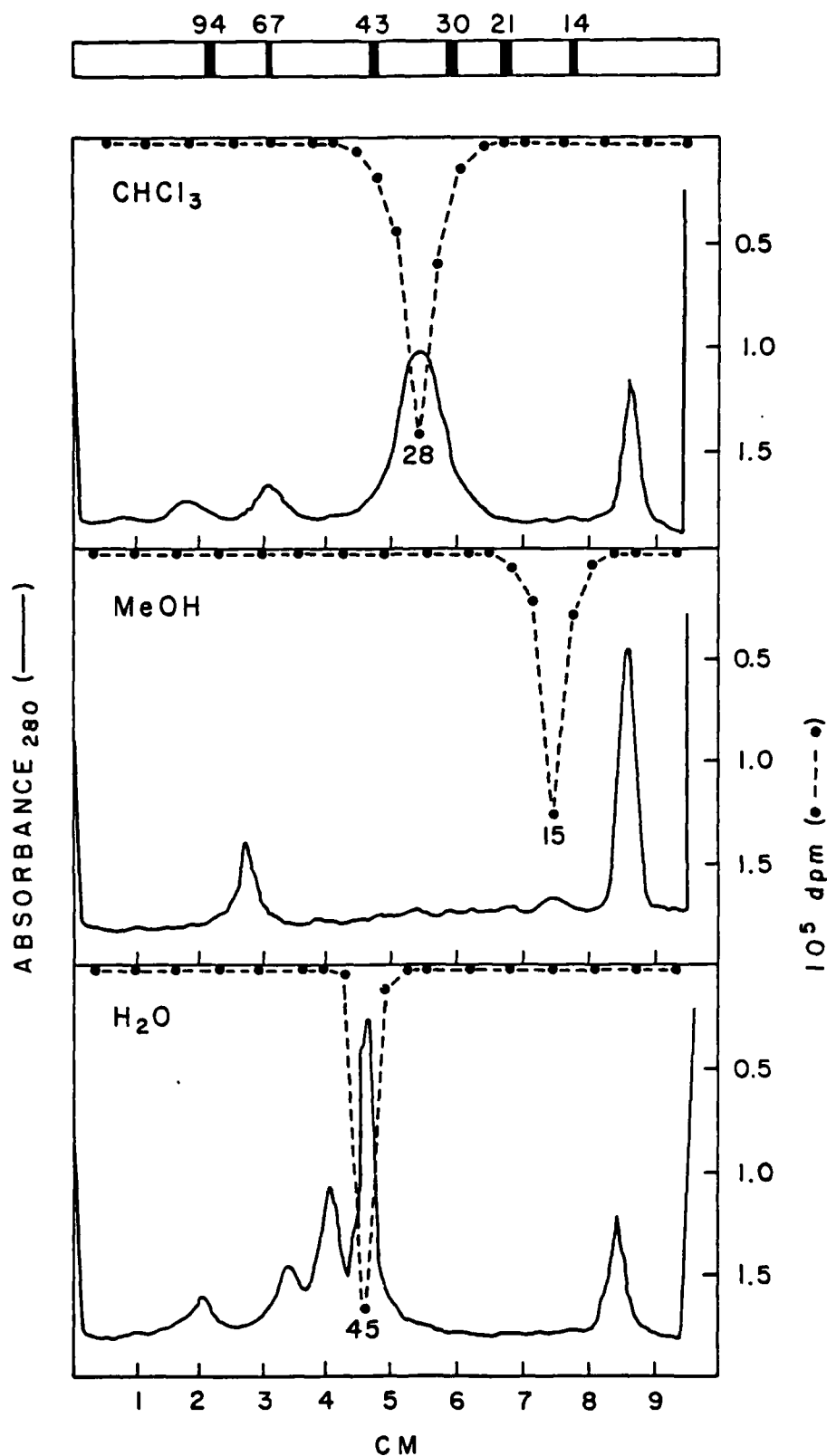
Fig. 11. SDS-PAGE analysis of three fractions resulting from dissociation of the AChR-PGL. The analysis methods are detailed in Fig. 10.

TABLE 3

Characterization of Three Fractions Resulting From
Dissociation of the AChR-PGL[a]

Fraction	pmol $[^{125}]\alpha$-BTX Bound/μg Protein[b]	Approximate Subunit Molecular Weights[c]
CHCl$_3$	23.3	107,000 67,600 28,000[d] 15,500
Methanol	12.2	77,000 15,000[d] 11,500
Water	8.8	100,000 65,000 54,300 45,200[d] 15,500

[a]See the text for preparation of the fractions.

[b]As determined using the binding assays described in Section 2.4, the final concentration of α-BTX in the reaction mixtures was 5 μM.

[c]Using SDS-PAGE, see Fig. 11.

[d]Toxin-binding component.

latter component may be quantitated utilizing α-BTX pre-treatment which blocks all specific binding sites. As expected, the AChR-PGL and AChR membrane preparations exhibited the highest amounts of non-specific binding, presumably due to their lipid components. In our studies on hyperbaric effects on ligand binding to the receptor preparations, no attempt is made to distinguish between the effects on specific vs non-specific binding.

Table 4 presents, for the first time, the direct comparison of both an AChR-PGL and an AChR protein isolated from the same tissue. It is notable that in spite of their very different chemical and physical properties, both preparations have very similar binding properties and both exhibit the ligand binding specificity of the nicotinic cholinergic receptor.

The saturation binding of ACh to the AChR-PGL and protein was also used to characterize the two preparations. As shown in Fig. 12, the double reciprocal plots resulting from such studies showed two saturable sites on each receptor preparation. This finding of two ligand sites on the AChR is in agreement with other workers (27,46-48). Also in agreement with other workers, the dissociation constants (K_d) for the PGL receptor are larger than those for the protein receptor ($\cong 0.16$ and 9.2 μM vs 0.052 and 1.7 μM, respectively) indicating that, as expected, the isolated AChR protein has a higher affinity for cholinergic ligands. Studies with the AChR membrane preparation showed K_d values closer to the AChR-PGL ($\cong 0.19$ and 7.2 μM).

3.2 Hyperbaric Effects on Ligand-Receptor Binding

We have previously shown that studies on the binding of cholinergic ligands to the AChR-PGL at varying hyperbaric pressures are not significantly affected due to changes of ligand partition/solubility characteristics as pressure changes (14). Additionally, the small changes in reaction mixture volume due to the multiple sampling technique used during a dive does not affect ligand-receptor binding. Control studies on these potential interferences with the AChR protein also proved negligible. Thus, any change observed in the binding of cholinergic ligands to the receptor preparations is due to pressure effects on the ligand-receptor complex.

3.2.1 Ligand Binding at Varying Pressures

The binding of a cholinergic ligand to the AChR is disrupted by pressure. This process is reversible upon decompression. For example, Fig. 13 shows the binding of [^{14}C]ACh to the AChR membrane and protein preparations. As compression proceeds from 14.7 to 800 psi, binding to the membranes and protein decreases by $\cong 55$ and 80%, respectively. Decompression results in re-association of the ACh with the receptor preparations. The apparent greater stability of the ACh-membrane AChR association may be due to stabilization of the AChR toward pressure effects by other membrane components in the (crude) membrane preparation. This, in turn, may reflect the natural stability of the in vivo receptor

TABLE 4

Cholinergic Ligand Binding by the AChR Preparations[a]

| | pmol Bound/g Tissue[b] | | | | | |
| | AChR-PGL | | AChR-Membrane | | AChR Protein | |
Ligand	Total	Specific[c]	Total	Specific	Total	Specific
[^{14}C]ACh	110	74	130	73	72	61
[^{125}I]αBTX	71	71	92	80	51	51
[^{3}H]BAC	102	65	128	69	65	57

[a]As determined using the binding assays described in Section 2.4. Final ligand concentrations in the reaction mixtures were 0.33 μM for ACh and BAC, and 0.10 μM for αBTX.

[b]The values reported are the means of at least two determinations with no single value deviating from the mean by more than ±8.4%. Tissue weight is wet weight.

[c]Determined by pre-reacting the receptor preparation with 1 μM αBTX for 30 min and then adding the radioactive ligand. The amount of ligand bound after such αBTX pre-treatment represents non-specific binding to the receptor preparation (see Ref. 27).
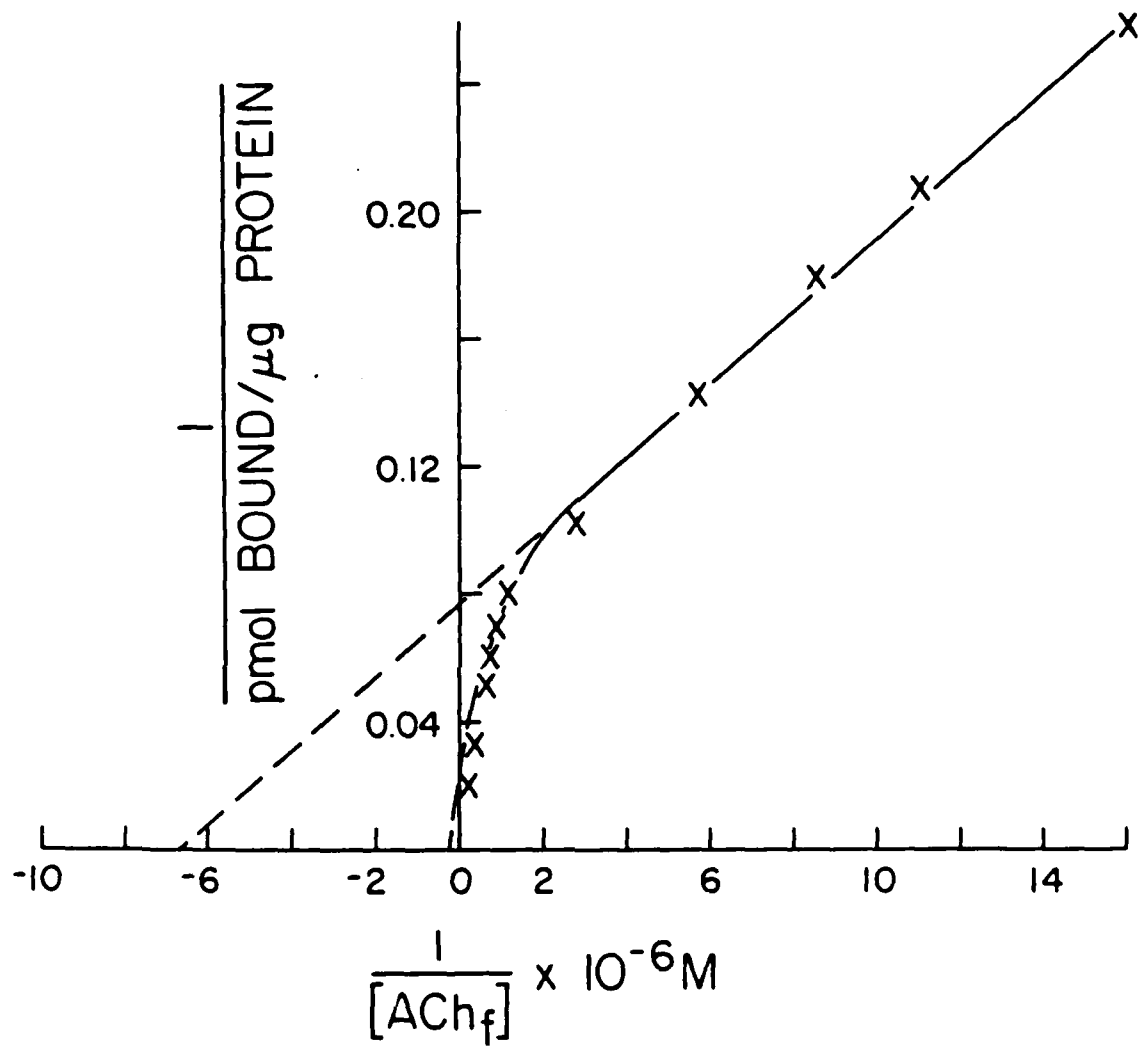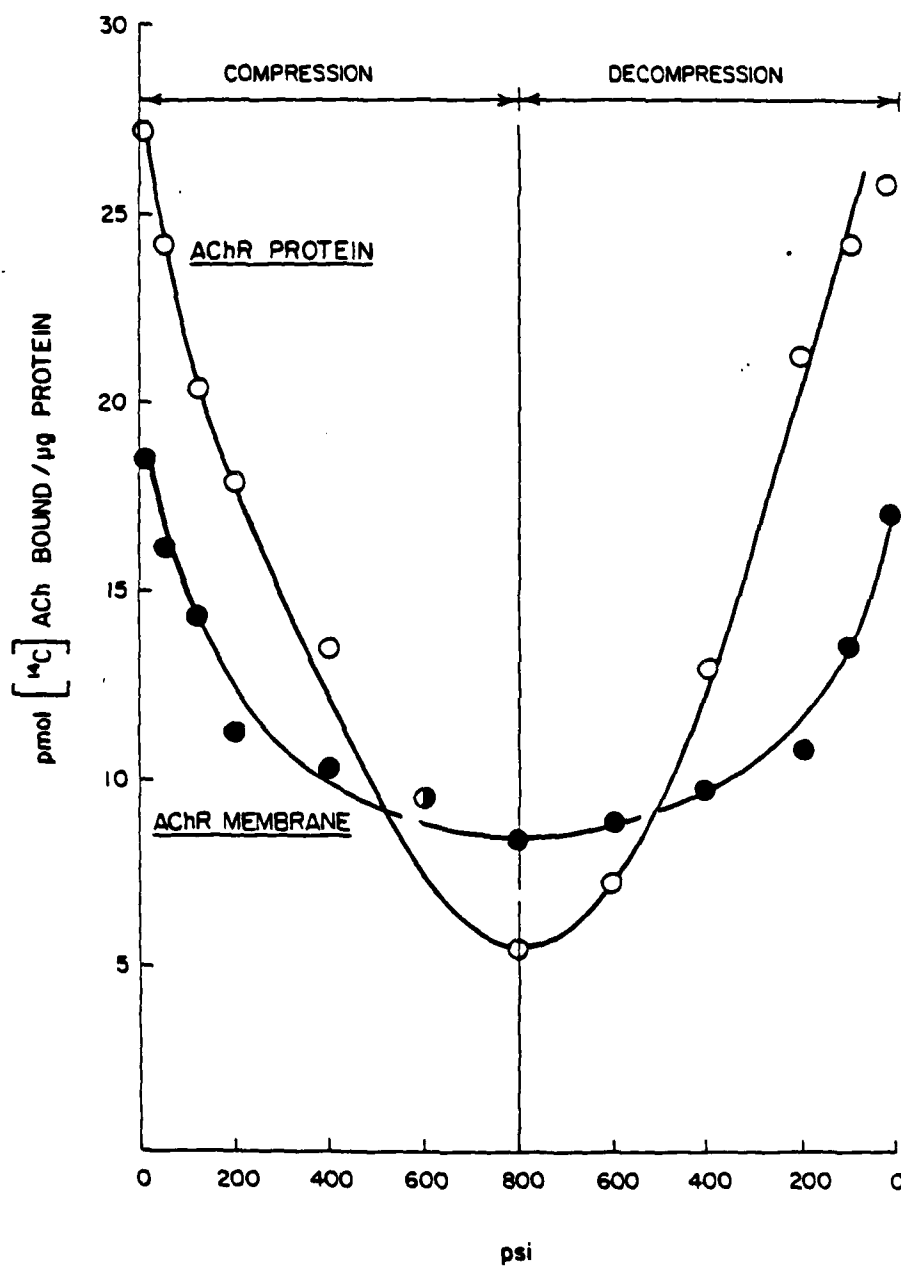
Fig. 12. Double-reciprocal plot resulting from saturation of the AChR-PGL with [^{14}C]ACh (from Reference 27).

$\left[{}^{14}C\right]$ ACh + AChR PROTEIN

Fig. 13. Effect of pressure on the binding of ACh to the AChR membrane and AChR protein preparations. The reaction mixtures contained ≈50 μg of protein and 1 μM ACh.

since if a dramatic loss of binding to the receptor (as occurs with the purified receptor) occurred in divers, total neurological dysfunction would rapidly occur under hyperbaric conditions. Thus, the effects of pressure on neural transmission appear tempered by the natural organization of neural cell membranes.

Table 5 reports the effects of compression/decompression on the binding of four cholinergic ligands, ACh, α-BTX, bromoacetylcholine (BAC) and d-tubocurarine (dTC), to the AChR protein and PGL. In all cases, pressure causes a decreased binding of ligand to the receptors. This binding is recovered upon decompression, although complete recovery of binding activity was only observed with the AChR protein. With both receptor preparations, ACh binding was most affected by pressure, resulting in $\cong 90\%$ or greater inhibition at 800 psi.

We have carried out further studies to define the binding kinetics of ACh to the AChR at varying pressure levels. As discussed above, it is known that the AChR has two saturable binding sites for ACh termed "high" and "low" based on their binding affinity for ACh. Previously we had shown, using the AChR-PGL, that the AChR-ACh dissociation constant (K_d) for the high affinity binding site only was affected by pressure, resulting in an apparent increased affinity for ACh even though total ACh binding to the receptor decreased (14). We have repeated these experients with the AChR protein and observed similar results (Table 6) although the total change in the K_{d1} for the protein from 14.7 to 400 psi was less than for the PGL, -54% vs -81%, respectively. Thus, it appears that compression directly alters the suprastructure of the AChR, resuting in changes not only in its binding capacity for ACh, but also in its (high) binding site affinity for ACh.

We also have carried out preliminary studies on the binding of BAC to the AChR at normo- and hyperbaric pressure. BAC presents unique characteristics for AChR studies since it can bind to the AChR per se, or be covalently linked to the reduced AChR via an alkylation reaction (Fig. 14). Our studies have shown the BAC can bind to both the AChR-PGL and protein preparations (Tables 4 and 5) and that such binding is affected by pressure (Fig. 15). Further studies on BAC binding to reduced receptor at normo- and hyperbaric pressure could aid in defining the nature of pressure at the receptor active site.

3.2.2 Agonist/Antagonist Effects on ACh-AChR Binding at Varying Pressures

We have previously reported (14,30,31) that pressure effects on the AChR can be further potentiated or inhibited by cholinergic drugs/agents. As shown in Table 7, we have tested a variety of drugs/agents for their effect on pressure-induced inhibition of ACh binding to its receptor. For example, Figs. 16 and 17 illustrate the binding of two concentrations of ACh to either the AChR-PGL or protein, and the effect of BAC (a competitive agonist) on binding of ACh at the higher concentration to the receptors. These results typify those found with other cholinergic

TABLE 5

Binding of Cholinergic Ligands to the AChR at Normo- and Hyperbaric Pressures[a].

Receptor Preparation	Ligand (μM)	pmol Bound/ μg Protein[b] (14.7 psi; 0 time)	ΔZ Bound[c]										
			50	100	200	400	600	800	600	400	200	100	14.7 psi
Protein	[14C]ACh (0.5)	16.2	-7	-32	-42	-58	-75	-89	-81	-60	-35	-14	2
	[125I]α-BTX (0.5)	24.7	20	1	-9	-11	-16	-71	-59	-45	-31	-18	-4
	[3H]BAC (0.5)	17.9	-6	-27	-36	-57	-73	-84	-78	-58	-39	-23	1
	[3H]dTC (2.0)	96.2	-13	-14	-20	-32	-44	-75	-57	-44	-20	-15	3
Proteoglyco- lipid	[14C]ACh (1.0)	15.4	-30	-51	-65	-81	-90	-94	-93	-80	-69	-48	-14
	[125I]α-BTX (0.5)	18.3	-20	-36	-52	-67	-75	-83	-79	-74	-59	-44	-10
	[3H]BAC (1.0)	14.7	-20	-43	-61	-75	-82	-88	-86	-77	-68	-37	-12
	[3H]dTC (2.0)	295	-20	-36	-42	-48	-51	-55	-53	-49	-46	-41	-6

[a]Protein binding assays were carried out in 5 ml of 5mM NaHPO$_4$ buffer (pH 7.4) containing 0.1% TX-100, 100 μg/ml of BSA and from 25 to 50 μg of protein. PGL binding assays utilized the biphasic partition method in which a 3 ml chloroform solution of proteoglycolipid containing 30 to 50 μg of protein was partitioned against the appropriate ligand in 3 ml of 50 mM tris-HCl buffer (pH 7.2) containing 100 μg/ml BSA. See the text for details.

[b]Each value is the mean of at least two assays at that pressure level with no single value deviating from the mean by more than ±8.3%.

[c]The ΔZ bound represents the loss (gain) of ligand bound as compared to the amount bound at 14.7 psi (0 time).

TABLE 6

ACh Binding Kinetics to the AChR at Normo- and Hyperbaric Pressures

psi	AChR Protein[a]			AChR Proteoglycolipid[b]		
	ACh Bound[c]	$K_{d1}(\mu M)$	$K_{d2}(\mu M)$	ACh Bound	$K_{d1}(\mu M)$	$K_{d2}(\mu M)$
14.7	26.3	0.052	1.7	21.4	0.16	9.2
50	18.2	0.049	1.7	16.1	0.76	9.2
100	15.6	0.046	1.7	15.7	0.039	9.2
200	10.4	0.034	1.7	15.0	0.033	9.2
400	9.1	0.024	1.7	9.3	0.030	9.2
600	8.1	0.023	1.7	d	-	-

aEach saturation experiment utilized six assays, each containing ≅50 μg of protein and increasing amounts of [^{14}C]ACh from 0.01 to 1.0 μM. The amount of ACh bound at each concentration and pressure was determined and used to construct double reciprocal plots. The amounts of ACh bound at saturation and the apparent K_d values were taken from the plots.

bValues from Reference 14.

cpmol [^{14}C]ACh bound/μg protein at saturation.

dNot determined.

BAC ACTION ON THE ACETYLCHOLINE RECEPTOR

AChR (-S-S-) $\xrightarrow{\text{DTT}}$ AChR (-SH)

$+$

$$BrCH_2COCH_2CH_2\overset{+}{N}(CH_3)_3$$

BROMOACETYLCHOLINE (BAC)

REVERSIBLE AGONIST

IRREVERSIBLE ALKYLATION (40K SUBUNIT)
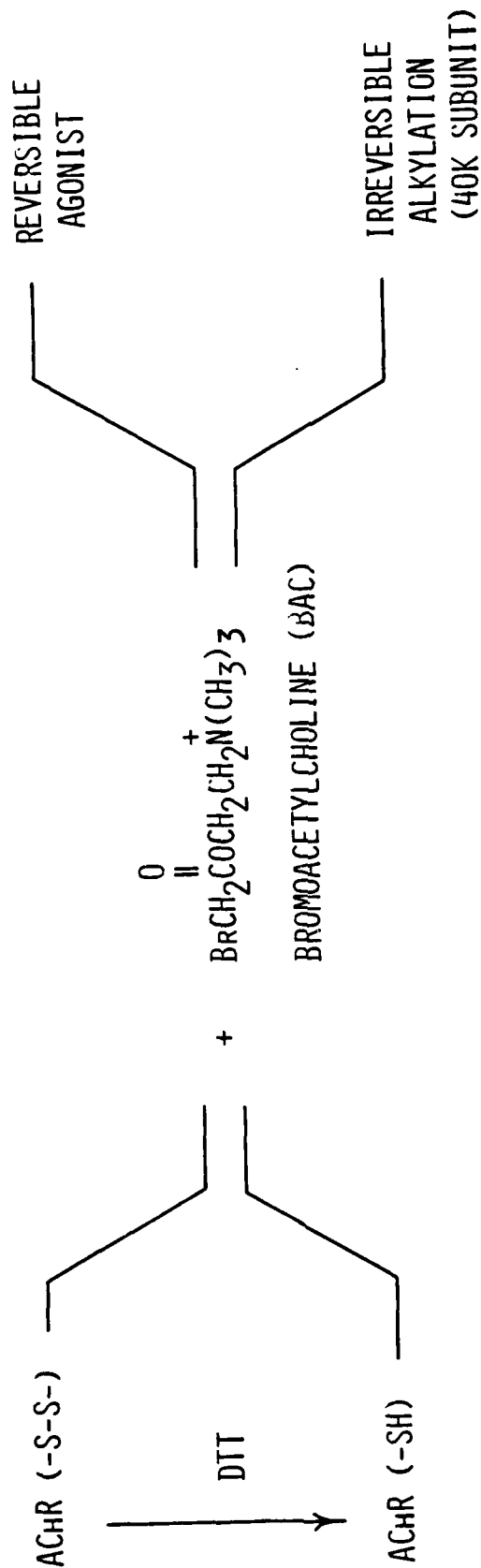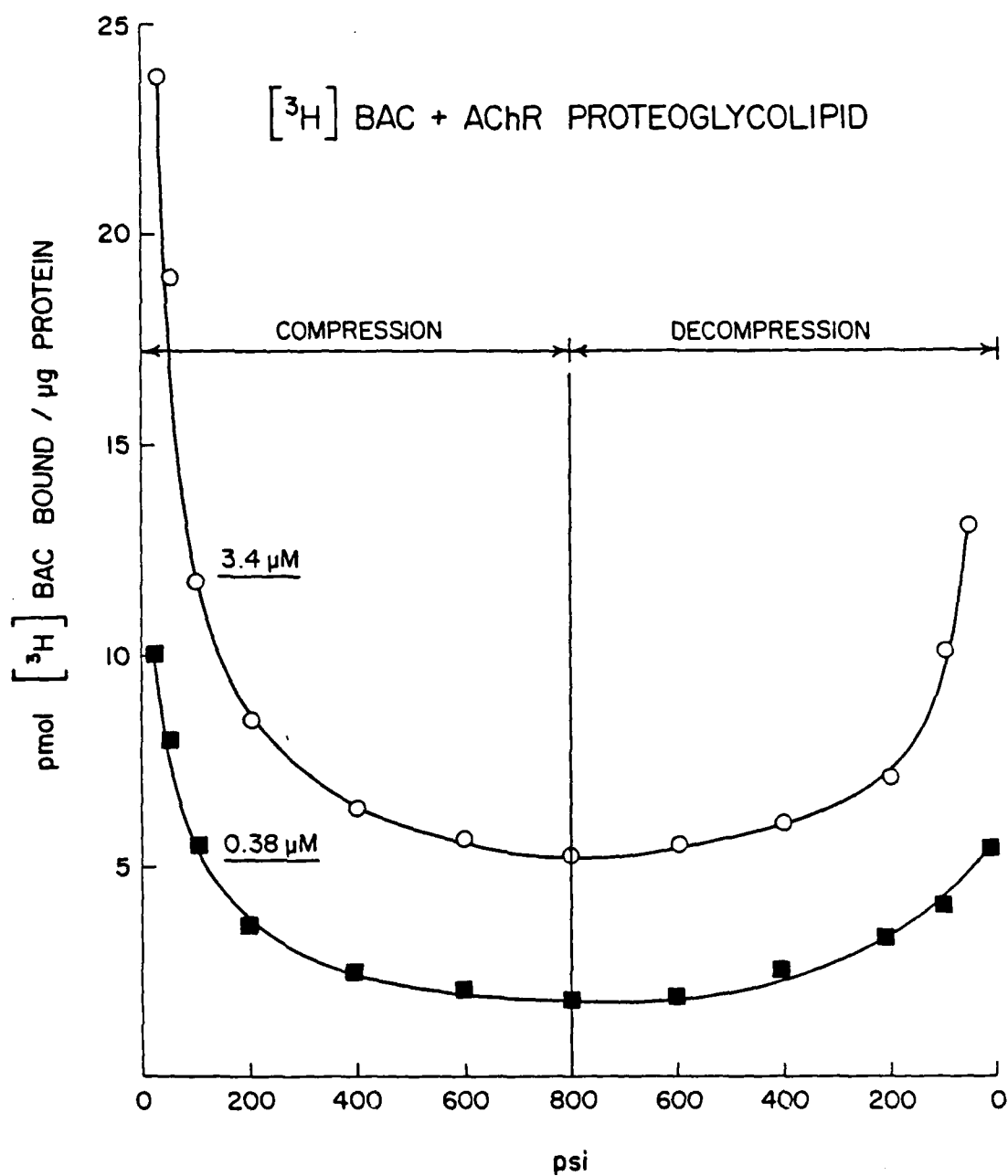
Fig. 14

Fig. 15. BAC binding to the AChR-PGL at varying pressure levels. The final BAC concentration in the reaction mixtures was 3.4 μM or 0.38 μM. Bound BAC was determined by the standard biphasic partition assay.

TABLE 7

Effects of Various Drugs and Agents on ACh Binding to the
AChR Preparations at Normo- and Hyperbaric Pressures.[a]

Receptor Preparation	Drug/ Agent[b]	Δ% pmol [^{14}C]ACh Bound/µg Protein[c]						
		14.7	100	400	800	400	100	14.7 psi
Protein	None	–	-29	-46	-82	-54	-15	-5
	Nicotine	-58	-77	-81	-92	-86	-89	-72
	Muscarine	7	-30	-70	-68	-73	-59	-13
	d-Tubocura- rine	-5	-27	-36	-60	-33	-32	-30
	Decamethonium	-38	-64	-83	-96	-84	-82	-42
	Hexamethonium	45	35	36	-52	-41	-36	29
	Atropine	5.4	-27	-36	-60	-33	-32	-30
	DFP	2.1	-7	-8	-11	-10	-5	-3
	Physostigmine	-53	-56	-65	-70	-71	-71	-62
	Tetrodotoxin	32	-14	-42	-53	-44	-44	-2
	Veratridine	12	-5	-7	-21	-16	-19	-7
Proteo- glycolipid	None	–	-39	-71	-78	-74	-49	4
	Nicotine	-15	-12	-26	-54	-34	-19	-16
	Muscarine	-19	-14	-29	-39	-34	-23	-16
	d-Tubocura- rine	-27	-68	-79	-90	-84	-70	-12
	Decamethonium	-4	-63	-82	-87	-81	-62	-14
	Hexamethonium	-3	10	-6	4	3	10	3
	Atropine	-7	-19	-42	-40	-34	-29	-24
	DFP	-10	-47	-49	-63	-59	-53	-23
	Physostigmine	4	-24	-34	-52	-29	-7	-6
	Tetrodotoxin	1	-20	-33	-66	-44	-3	-2
	Veratridine	-4	-5	-18	-29	-21	-11	-9

[a]Assays were carried out using the ultrafiltration or biphasic partition method described in Section 2.4 except that the aqueous buffer in both assays was 5 m$\underline{M}$ NaHPO$_4$ (pH 7.4). In each reaction mixture, 45 µg (AChR protein) or 12 µg (AChR-PGL) of protein was reacted with drug/agent for 15 minutes prior to addition of [^{14}C]ACh. Final ACh concentrations were 1.0 µM (AChR protein) or 2.5 µM (AChR-PGL). Under these conditions, AChR protein bound 18.5 pmol ACh/µg protein and the AChR-PGL bound 33.6 pmol ACh/µg protein.

[b]For the AChR protein, all agents and drugs, except DFP, were at a final concentration of 5 µM in the reaction mixtures. DFP was at a final concentration of 10 µM. For the AChR-PGL, all agents and drugs were at a final concentration of 15 µM.

[c]The Δ% binding represents the loss (gain) of [^{14}C]ACh bound as compared to the amount bound at 14.7 psi in the absence of any drug/agent.
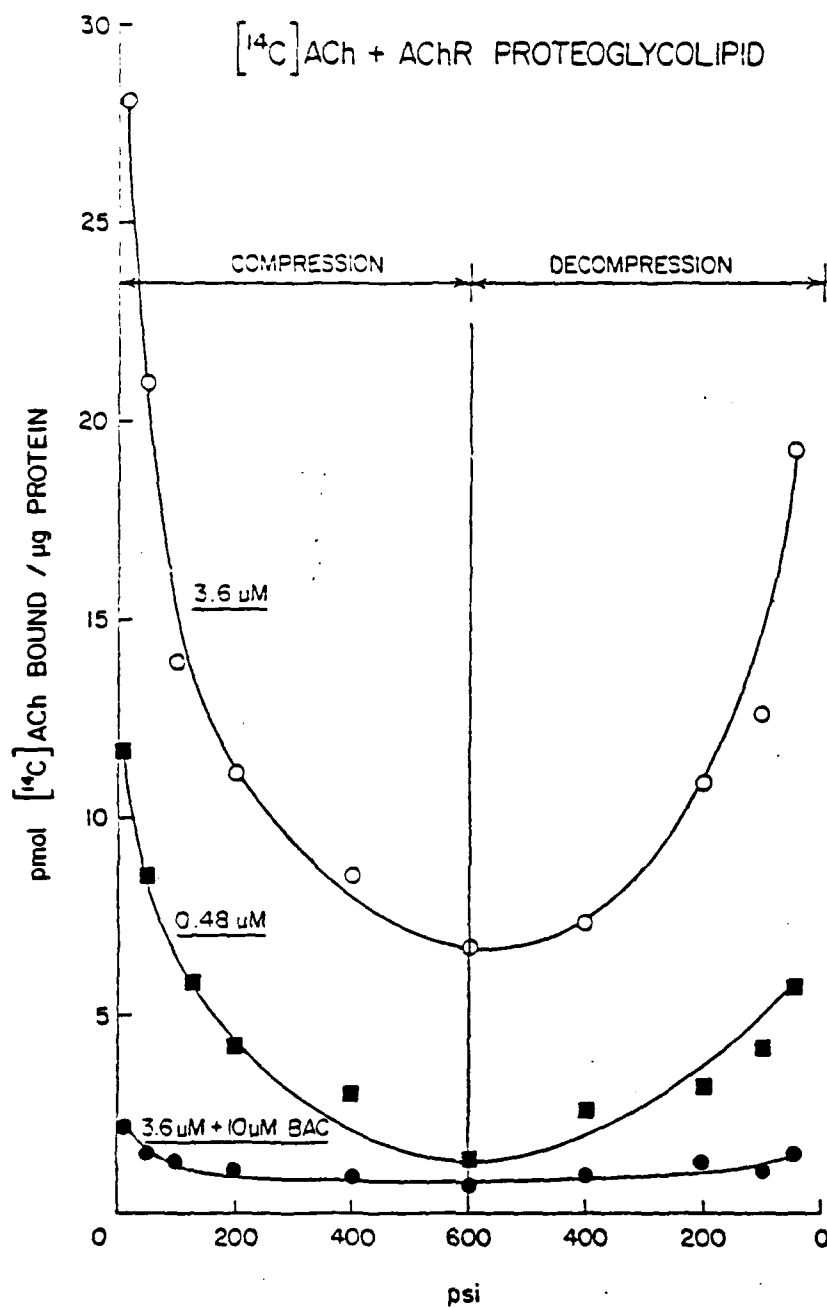
Fig. 16. Binding of ACh to the AChR-PGL at varying pressure levels, with or without BAC present.
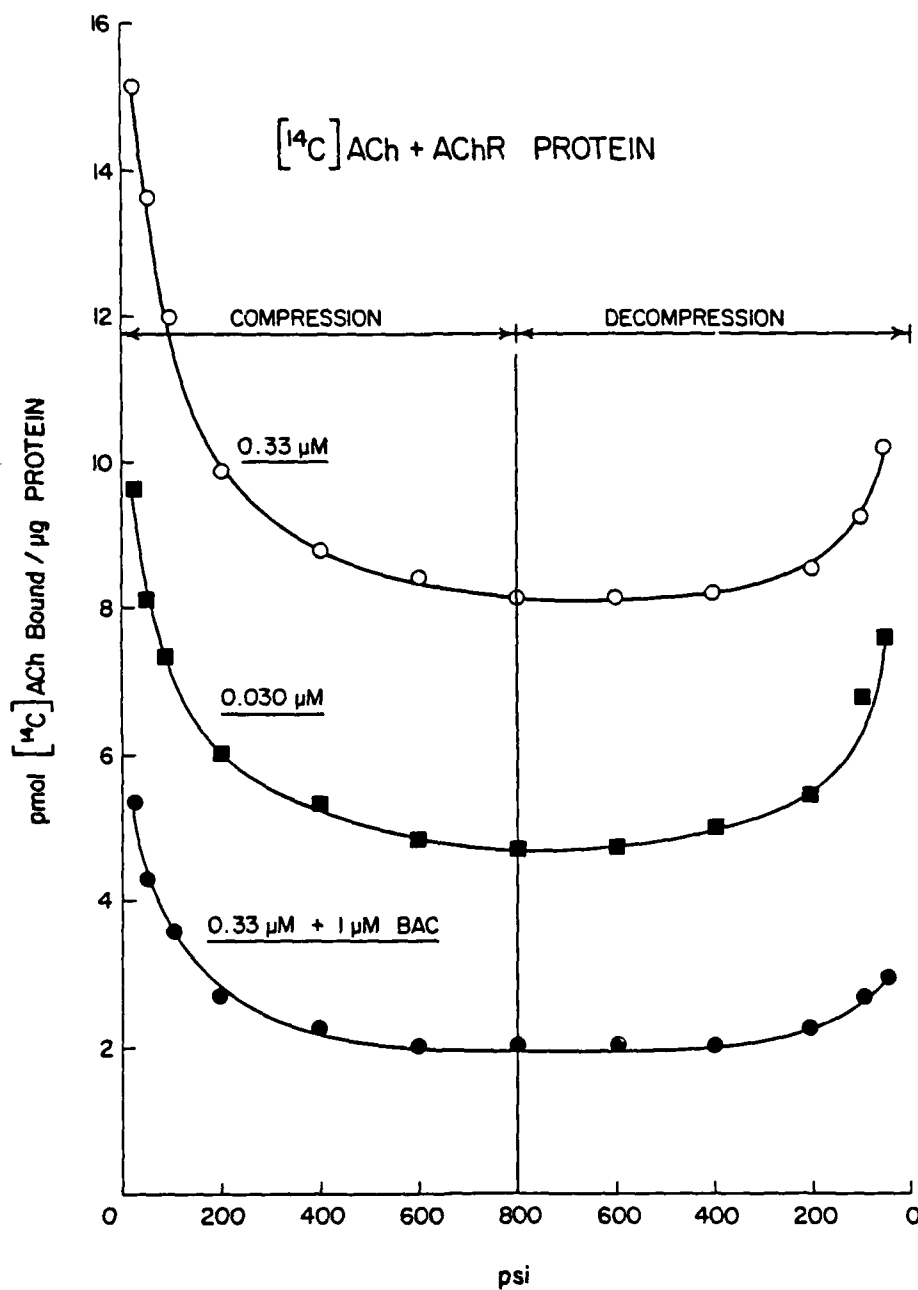
Fig. 17. Binding of ACh to the membrane AChR at varying pressure levels, with or without BAC present. The standard aqueous binding assay method was used to determine bound ACh.

agonists/antagonists (e.g., d-tubocurarine, decamethonium and nicotine), i.e., such agents maintain their competition/inhibition action throughout the pressure levels studied.

Other agents showed different effects on the pressure-induced ACh-AChR disruption. Of particular interest was the effect of hexamethonium. As shown in Fig. 18, decamethonium (DMet; a neuromuscular, nicotinic, cholinergic antagonist) further inhibits, as expected, ACh binding to the AChR-PGL thus potentiating the pressure effect; while hexamethonium (HMet; a ganglionic, nicotinic, cholinergic antagonist) exhibits the surprising ability to inhibit pressure-induced loss of ACh binding. In the case of the AChR protein (Fig. 19), DMet again potentiates binding while HMet inhibits loss of binding up to ≅400 psi. This action of HMet is intriguing, suggesting that the drug may be binding to another (non-ACh) binding site on the AChR resulting in stabilization of the receptor toward pressure and/or activation of the receptor to a more avidly (ACh) binding form. This hypothesis is supported by other studies which have shown that agents, including HMet, may interact directly with the AChR resulting in the conversion of the receptor to a higher affinity form in vitro able to bind more AChR. This high affinity, in vitro state parallels the in vivo state of receptor desensitization (49,50). Thus, our observations may reflect a balance between pressure-induced loss of ACh binding with drug-induced potentiation of ACh binding until, as in he case of the AChR protein (Fig. 19), the drug effects are overcome by pressure effects. It would be interesting to see if the in vitro observations on HMet action at various pressure levels correlate with in vivo studies using the drug in animals under hyperbaric conditions.

It is also interesting that veratridine, an alkaloid toxin which specifically interacts with the regulatory component of the AChR sodium action potential ionophore (51,52) antagonizes pressure-induced loss of ACh binding; while tetrodotoxin, which specifically interacts with the ion-transport components of the ionophore provides significantly less protection (Table 7). Also, atropine, a muscarinic cholinergic agonist, appears to afford some protection against pressure effects on the nicotinic AChR. These results suggest that other classes of neural-active drugs besides cholinergic desensitizing agents may provide protection against hyperbaric disruption of the AChR.

3.2.3 Organophosphorus Agent Effects on ACh-AChR Binding at Varying Pressures

Diisopropylfluorophosphate (DFP), a potent anti-cholinesterase, is also known to bind to a non-ACh binding site on the AChR, and can cause desensitization of the in vivo AChR (53,54). Thus, we studied the effect of DFP on the pressure-induced loss of ACh binding to the AChR. In the case of the AChR-PGL, DFP offered only moderate protection against the pressure effect (Table 7). In the case of the AChR protein, however, DFP significantly protected the receptor from pressure effects at all levels tested (Fig. 20). Since DFP is a reversible binding agent at the AChR (54), we also monitored [^{3}H]DFP at each pressure level. As shown in Fig.
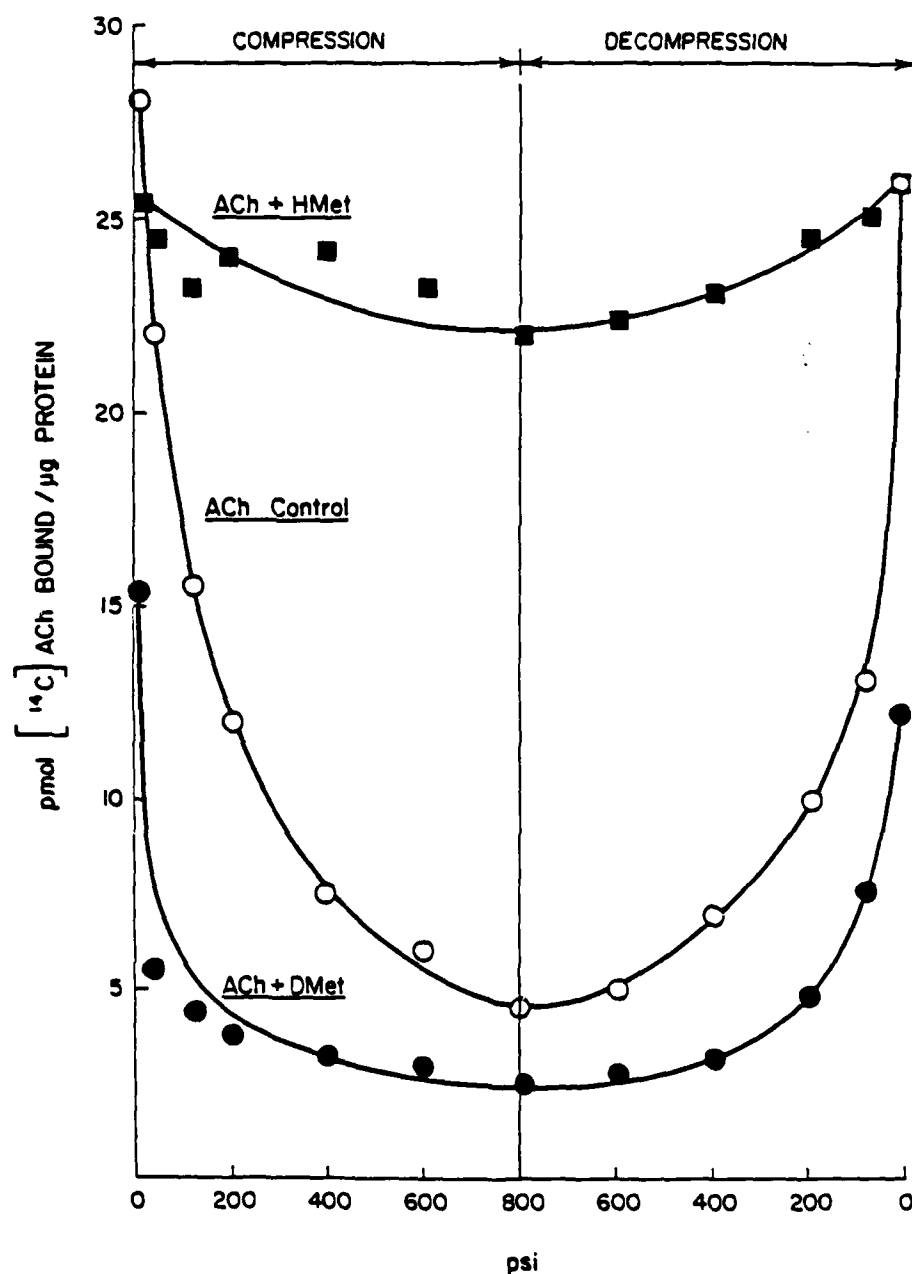
$[^{14}C]$ ACh + AChR PROTEOGLYCOLIPID

Fig. 18. Effect of pressure on the binding of ACh to the
AChR-PGL _per se_ (ACh control) and in the presence
of decamethonium bromide (DMet) and hexamethonium
bromide (HMet). Each reaction mixture contained
≈25 µg of PGL protein and 2.5 µM ACh.
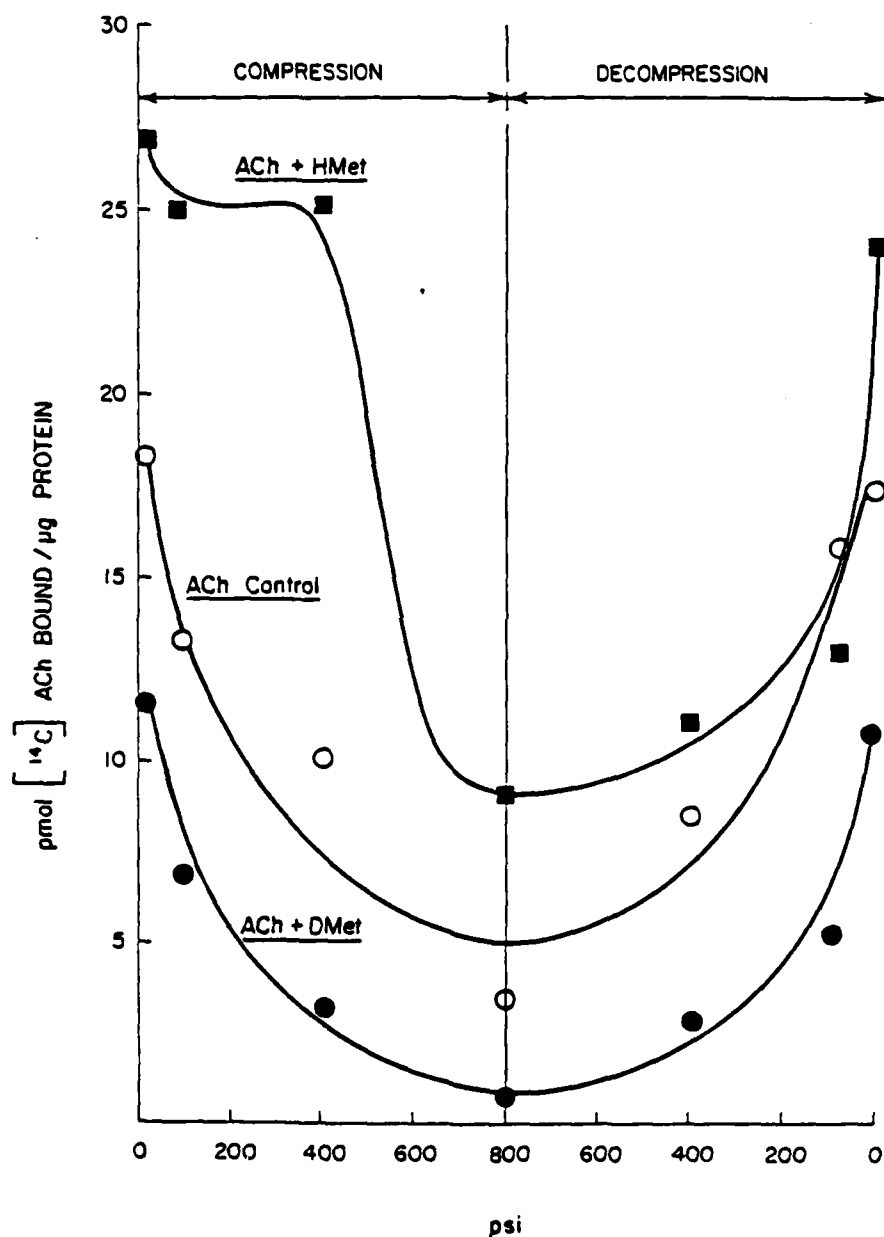
39

$\left[^{14}C\right]$ ACh + AChR PROTEIN

Fig. 19. Effect of pressure on the binding of ACh to the AChR protein _per se_ (ACh Control) and in the presence of DMet and HMet. Each reaction mixture contained ≈50 μg of protein and 1 μM ACh.

ACh PROTEIN
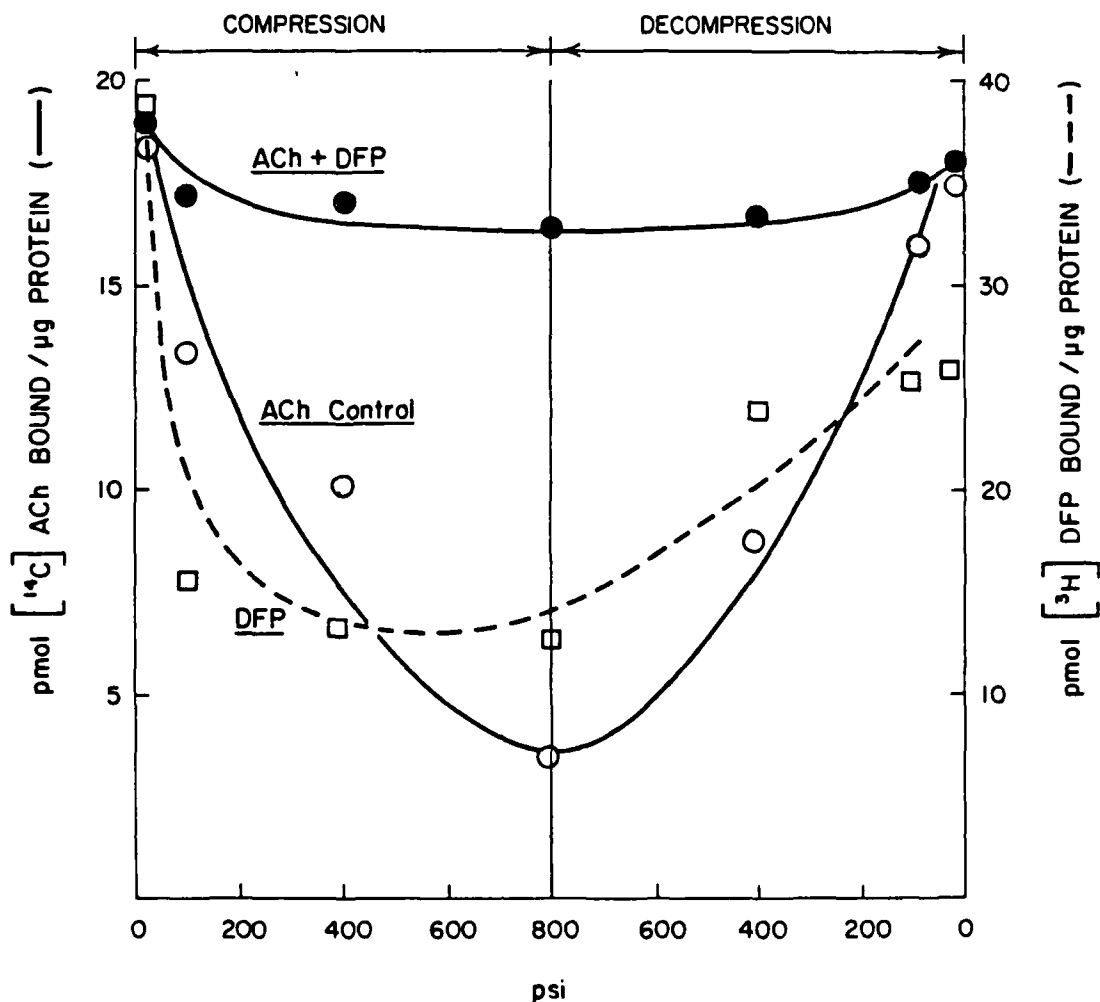
Fig. 20. Effect of pressure on the binding of ACh and DFP to the AChR protein individually (ACh control and DFP, respectively) and together (ACh + DFP). Each reaction mixture contained ≈50 µg protein. Ligand concentrations were 1 and 10 µm for ACh and DFP, respectively.

20, significant amounts of DFP were displaced from the receptor with increasing pressure. This suggests that while DFP can protect the AChR from pressure effects, at least a portion of the agent binds to pressure-sensitive regions of the receptor. These studies with DFP further support the hypothesis that agents which can interact with the AChR at non-ACh binding sites and cause receptor desensitization may provide the means to protect the AChR against pressure effects.

In addition, our results present a potential means of treating casualties of organophosphorus poisoning. If such a casualty is compressed (using standard human diving tables) in a hyperbaric chamber (such as the portable, one-man chambers currently in use) to ≅400-800 psi, the net effect would be a decrease in ACh affinity for the AChR and dissociation of the organophosphorus agent from the AChR. Both these actions are required for successful organophosphorus agent treatment. Decompression would occur after a (defined) compression period during which time the organophosphorus agent is detoxified/cleared within the victim to non-toxic levels. While we have not carried out studies on pressure effects on agent-acetylcholinesterase binding, preliminary studies on other serine esterases (R.F. Taylor, unpublished results) indicate that, again, pressure would disrupt such binding. Thus, hyperbaric pressure therapy may be applicable to organophosphorus and other toxic agent therapy. Animal studies are now required to test this theory.

3.2.4 Pressure Effects on ACh Binding to the AChR from Electric Eel

In the only other studies to date on hyperbaric pressure effects on the isolated AChR, it was reported that ACh binding to an AChR membrane preparation from Torpedo californica decreased by ≅30% at 300 atm (≅4409 psi) (29). These authors concluded that pressure does act on the AChR active site and that such action is independent of the two ACh binding sites of the AChR.

While these studies support our conclusions on pressure-induced inhibition of ACh to the AChR, it is obvious that the magnitude of the effect we observed with the rat gastrocnemius AChR is far greater than that observed with the Torpedo AChR, i.e., as shown in Fig. 13 and Table 5, at 800 psi (54.4 atm) we observed ≅55% loss in ACh binding to the receptor. While different experimental conditions were used (e.g., different receptor and ACh amounts and a different diving protocol) further investigations were deemed warranted in an attempt to account for the differences in results between the gastroc and Torpedo preprations. We thus chose to isolate the AChR protein from another source, the electroplax of Electrophorus electricus and to study its binding of ACh at hyperbaric pressures.

Specimens of E. electricus (World Wide Scientific Animals,Ardsley, NY; 1.2-1.6 Kg, 90-100 cm) were decapitated and their electric organs (300-400g) were immediately removed, weighed, quick-frozen in liquid

nitrogen and stored at -80°C. For extraction of the AChR protein
(55-58), ≅50-75g of electric organ was homogenized with 3 vol cold 50 m$\underline{M}$
NaH$_2$PO$_4$-K$_2$HPO$_4$ buffer pH 7.4 containing 0.02% NaN$_3$ and 0.1 m$\underline{M}$ phenyl-
methylsulfonyl fluoride (Buffer 1). The latter inhibitor is not used if
active acetylcholinesterase is to be extracted from the tissue as well.
After filtering through cheesecloth, the homogenate was centrifuged at
20,000 x g for 60 min at 4°C. The pellet ("crude membrane" preparation)
was suspended in 5 vol of Buffer 1 containing 10 m$\underline{M}$ NaCl (Buffer 2).
This suspension was briefly homogenized and then centrifuged at 100,000 x
g for 60 min at 4°C. The resulting pellet was extracted with 2 vol of
Buffer 2 containing 1% Triton X-100 for 2 h at 25°C. The mixture was
then centrifuged at 30,000 x g for 60 min and the supernatant was used
for purification of the AChR by affinity chromatography on a cobrotoxin-
Sepharose support. The support was prepared by covalently linking 10 mg
of α-cobrotoxin (Sigma Chemical Co.) to 5 g of cyanogen bromide activated
Sepharose 4B (Pharmacia Fine Chemicals) in 0.1 m$\underline{M}$ Na borate-boric acid
buffer pH 8.5 for 16-18 h at 4°C (57,58). The final toxin content on the
gel was ≅1.7 mg/g gel. The column was developed with Buffer 2 containing
0.2% Triton X-100 (Buffer 3) until no further protein eluted. The column
was then eluted with Buffer 3 containing 0.2 $\underline{M}$ carbamylcholine chloride
to recover the AChR protein. The resulting AChR was applied to a 0.9 x
30 cm DEAE-Sepharose 6B column (Pharmacia Fine Chemicals). After eluting
with Buffer 3 to remove non-receptor proteins and carbamylcholine, the
purified AChR protein was recovered from the column using a 0.1 to 1.5 $\underline{M}$
gradient of KCl in Buffer 3. The resulting AChR protein was shown to be
homogeneous by PAGE and HP-GPC (see Section 2.2) and was used in our
binding and pressure studies. At a final concentration of 0.5 or 1μ$\underline{M}$
ACh, and using 50 μg of this AChR protein, 2.6 and 13.5 pmol ACh were
bound per μg of protein.

We subjected the eel AChR protein to our standard compression/decom-
pression method (Section 2.5). As reported in Table 8, this resulted in
loss of ligand binding with increase pressure but ina manner and degree
different from that observed with our mammalian AChR protein. Most
notable was the apparent stability of the ACh-receptor complex to pres-
sure up to 400 psi. Significant loss of binding only occurred at 600 and
800 psi (Fig. 21). This is in marked contrast to the binding profile
seen for ACh during compression of the gastroc AChR protein (Fig. 13).
It appears that the eel AChR is much more stable toward pressure induced
ACh binding loss. This observation is significant for two reasons.
First, from an evolutionary and life habitat viewpoint, the natural ocean
environment and activities of the eel may have led to changes in its AChR
to accomodate its routine exposure to varying pressure levels. Second,
from a molecular viewpoint, if such changes in AChR affinity are present,
further studies comparing the AChR from mammals and non-mammals could
further define the molecular architecture of the AChR. These latter
studies are especially important if differences also exist between other
components of the eel and mammalian AChR system, e.g., acetylcholines-
terase. Such differences would require definition to assess whether
studies on non-mammalian AChR components are pertinent to mammalian AChR
function, inactivation and therapy. Further pressure studies could aid
in such an assessment.

TABLE 8

Binding of Cholinergic Ligands to the AChR Protein from
E. electricus at Normo- and Hyperbaric Pressures[a]

| Pressure | Δ% Bound[b] | | | | |
| | [^{14}C]ACh | | [^{125}I]αBTX | | |
(psi)	0.5	1.0	0.2	0.5	μM
14.7[c]	–	–	–	–	
50	27	20	-34	-30	
100	12	6	-35	-61	
200	6	2	-42	-66	
400	2	-8	-46	-68	
600	0	-16	-48	-76	
800	-51	-73	-70	-100	
600	-23	-42	-61	-93	
400	-1	-8	-57	-81	
200	-3	-7	-51	-76	
100	2	-6	-35	-73	
14.7	2	-0.3	-25	-65	

[a]See table 4 for the assay method. Each reaction mixture contained 40-60 μg protein.

[b]The Δ% bound represents the loss (gain) of ligand bound as compared to the amount bound at 14.7 psi (0 time).

[c]At 0 time, the amounts of ligand bound were 2.6 and 13.5 pmol ACh/μg protein at 0.5 and 1.0 μM ACh; and 3.2 and 6.8 pmol αBTX/μg protein at 0.5 and 0.2 μM αBTX.

$[^{14}C]$ ACh + AChR PROTEIN (EEL)
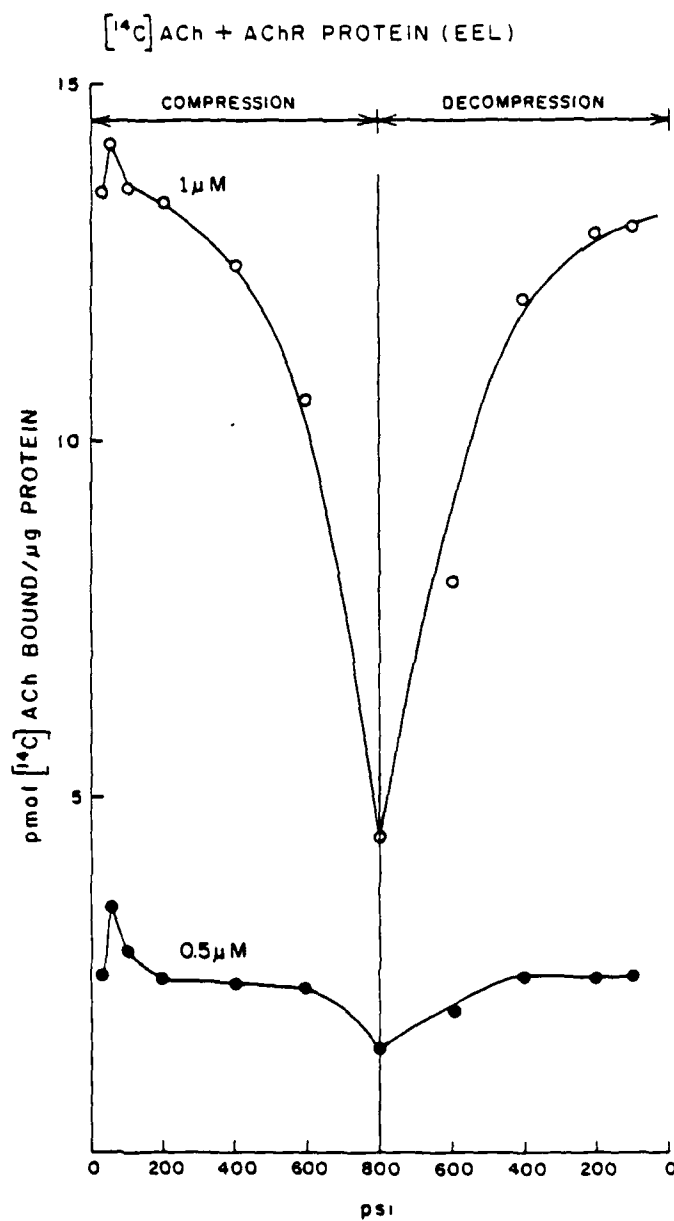
Fig. 21. Effect of pressure on the binding of ACh to
the AChR protein from E. electricus. Each
reaction mixture contained ≈50 µg protein and
final ACh concentrations as noted.

The binding of αBTX to the eel AChR protein (Table 8) also differs from that observed with the gastroc AChR protein (Table 5) at varying pressures. Toxin binding to the eel protein appears less resistant to pressure, i.e., more toxin is displaced with increasing pressure. Also, upon decompression, toxin binding does not return to its pre-compression level, suggesting that compression has caused a confirmational change in the eel AChR. Further studies on the nature of this change could lead to data defining the AChR active site interaction with high affinity toxins such as αBTX.

In summary, our studies with pressure effects on the eel AChR protein have shown that significant differences do occur between mammalian and non-mammalian AChR responses to pressure. This may aid in explaining the differences reported by other workers using the (non-mammalian) Torpedo AChR (29).

4. CONCLUSION

Our studies have confirmed that the nicotinic AChR system is affected by increasing pressure, resulting in a reversible loss of ACh binding. This ACh binding loss can be prevented or potentiated if other cholinergic agents are present. For example, competitive binding inhibitors of ACh at the AChR such as bromoacetylcholine or d-tubocurarine, potentiate pressure-mediated ACh loss. Alternatively, agents which do not specifically bind to the nicotinic AChR active site, such as hexamethonium, diisopropylflurophosphate and veratridine, appear to stabilize the AChR against pressure mediated loss of ACh binding. This observation suggests that specific drugs can be used to offset the neurophysiological effects of pressure. Further studies utilizing such drugs could result in effective therapies for diving disorders such as HPNS and allow much more rapid compression of humans than is now possible. This would be of importance from practical, economic and strategic viewpoints.

Our studies also allow us to further describe molecular events occurring at the AChR. For example, there is confusion in the literature concerning the two saturable sites on the AChR known to bind ACh. Questions have been raised concerning not only the interaction (if any) between these sites, but also their very existence. Our studies and those of others have established that two sites do, indeed, exist (27,46-48). Our saturation studies under pressure (Section 3.2.1) now allow further insights into the nature of these two sites. It is proposed that pressure affects the AChR as depicted in Fig. 22. At normobaric pressure, ACh binds at both high (partially buried) and low (surface) affinity receptor sites (H and L, respectively). At elevated pressure, deformation of the receptor causes decreased total ACh binding, presumably affecting binding to the L site since this site binds the majority of ACh under normal conditions. At the same time, compression causes an increase in ACh affinity at the H site, presumably due to increased accessibility of the ligand to the site. These pressure effects are reversible up to at least 800 psi, and may be inhibited by agents such as HMet and DFP. Our studies thus show that each of the two AChR specific binding sites are acted on independently by pressure although the overall state of the AChR (i.e., ACh binding) is a sum of the two. There appears to be no allosteric interaction between the two sites during compression.

The protection of the AChR in its natural, membrane environment precludes the dramatic pressure effects we have observed for ACh-AChR binding occurring in vivo. Such dramatic effects are, however, not required for in vivo dysfunction. Rather, more subtle changes in AChR function caused by compression would be sufficient for the causation of pressure-induced neurological disorders. We conclude that such neurological disorders have, as their primary lesion, changes in the suprastructure of membrane-bound neural receptors. Such changes at the molecular level are then translated, via disruption of normal neurotransmission, into macro responses leading to tissue dysfunction and the observed disorder.
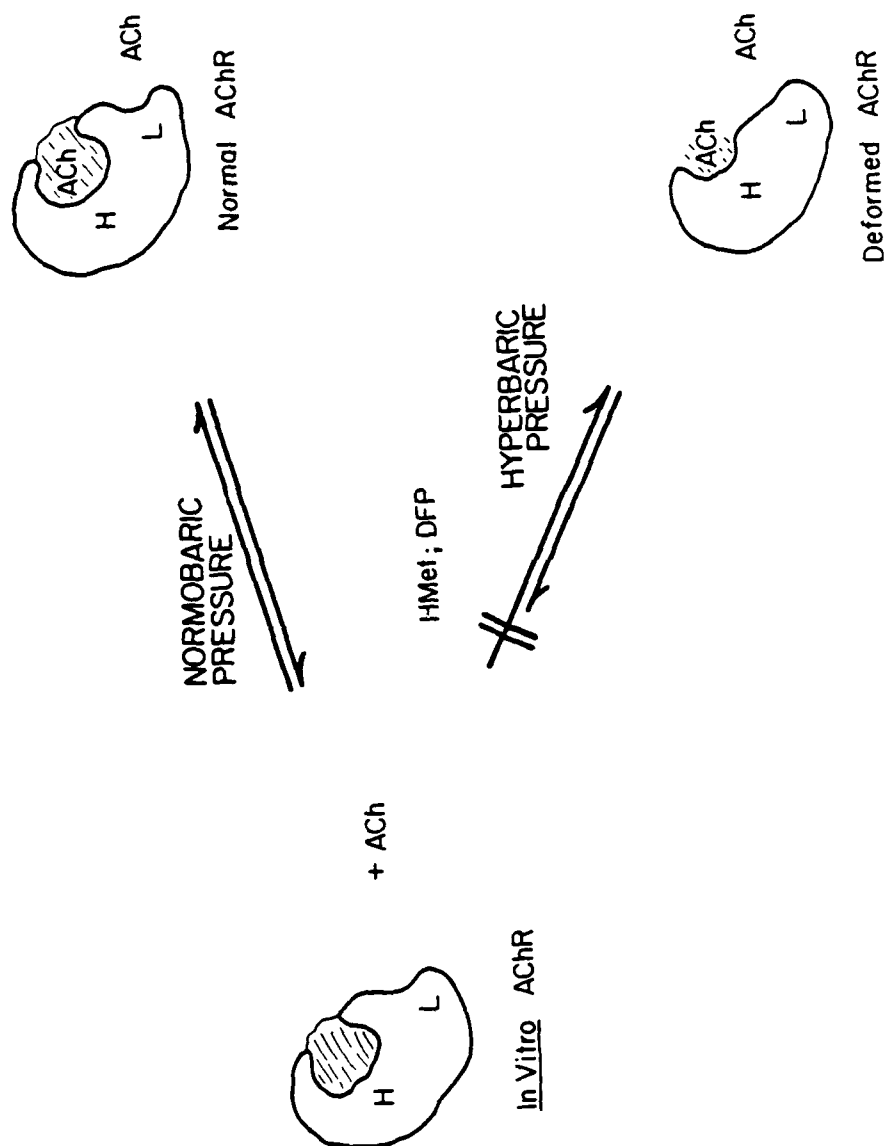
Fig. 22. Hypothetical representation of ACh interaction with the isolated AChR at normo- and hyperbaric pressure. See the text for details.

Our studies with various toxins acting on the AChR system could also have far-reaching consequences for treating persons affected by such toxins. For example, we found that DFP, a model compound for organophosphorus nerve agents, can inhibit pressure-induced loss of ACh binding to the AChR. DFP itself, in turn, can be displaced from the AChR with increasing pressure. Thus, hyperbaric therapy could be useful in treating acute organophosphorus poisoning, displacing agent from its target sites and allowing clearance/metabolism to occur for detoxification. Similarly, acute intoxication with toxins such as d-tubocurarine, muscarine and tetrodotoxin could be treated using hyperbaric therapy. Such treatment could be based in portable hyperbaric chambers and compression tables already routinely in use by the diving community. Further studies both in vivo and in vitro are now required to test this therapeutic use of hyperbaric pressure. Such studies could lead to significant advancements in our knowledge and control of physiological disorders based in neural receptor dysfunction.

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