Degradation of Acetylcholine Receptors in Muscle Cells: Effect of Leupeptin on Turnover Rate, Intracellular Pool Sizes, and Receptor Properties

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ABSTRACT The cellular mechanisms of degradation of a transmembrane protein, the acetylcholine receptor (AChR), have been examined in a mouse muscle cell line, BC3H-1. The half-time of degradation of cell surface receptors labeled with [^{125}I]α-Bungarotoxin ([^{125}I]α-BuTx) is 11–16 h. Leupeptin, a lysosomal protease inhibitor, slows the degradation rate two- to sixfold, depending on the concentration of inhibitor used. The inhibition is reversible since the normal degradation rate is regained within 20 h after removal of the inhibitor. Cells incubated with leupeptin accumulate AChR. Little change in the number of surface AChR occurs but the amount of intracellular AChR increases two- to threefold. Accumulated AChR are unable to bind [^{125}I]α-BuTx if excess, unlabeled α-BuTx is present in the culture medium during leupeptin treatment. Thus, leupeptin causes the accumulation of a surface-derived receptor population not previously described in these cells. Subcellular fractionation studies utilizing Percoll and metrizamide gradient centrifugation in addition to molecular exclusion chromatography suggest that the accumulated AChR reside in a compartment with lysosomal characteristics. In contrast, the subcellular component containing another intracellular pool of AChR not derived from the surface is clearly separated from lysosomes on Percoll gradients. The sedimentation properties of AChR solubilized from the plasma membrane and the lysosomal fraction have been compared. The plasma membrane AChR exhibits a sedimentation coefficient of 9S in sucrose gradients containing Triton, whereas the AChR derived from the lysosomal fraction exists in part in a high molecular weight form. The large aggregate and the organelle in which it resides may represent important intermediates in the degradative pathway of this membrane protein.

Large changes occur in the amount, metabolic stability, and distribution of the AChR during the maturation process of the neuromuscular junction as well as after muscle denervation (for reviews, see references 26, 31, 56, 15). Some of these changes involve alterations in the rates of synthesis, degradation, and incorporation of the receptor into the surface membrane. The biochemical nature of both synthesis (2, 19, 20, 30, 35, 53, 54) and degradation (12, 22, 23, 25, 27, 46, 47, 57, 59) has been the focus of numerous studies.

In addition to their possible involvement in synaptogenesis, changes in the degradation rate of AChR may play an important role in the cause of the neuromuscular disease, myasthenia gravis. Sera from patients with this disease contain antibodies to the AChR (48), and the interaction of antibodies with the receptor is thought to be at least partly responsible for the pathogenesis of the disease (for reviews, see references 29, 49, 50). Antibodies to AChR induce an abnormally rapid rate of turnover of the AChR in muscle (5, 14, 35, 37, 43, 55). The subsequent decrease in the synaptic content of AChR contributes to the failure of synaptic transmission at affected muscles. Much of the information concerning the cellular degradation pathway of AChR is based on morphological studies. A more detailed understanding of the biochemical mechanisms of degradation could be beneficial to efforts in developing treatments for myasthenia gravis.

A study of the cellular mechanism of AChR turnover is of interest in a more general sense as a model system for the degradation of transmembrane proteins. The metabolic fate of cell surface membrane receptors and their ligands has been studied in a variety of experimental systems including the

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asialoglycoprotein receptor (7, 16, 62), the insulin receptor (8, 32, 36, 41, 42, 58), the low density lipoprotein receptor (3, 4, 21), and polypeptide hormone receptors (9, 38). Antibody-induced internalization has been used as a tool to study the degradation of cell surface membrane constituents as well (11, 39). For several reasons, the AChR may provide an ideal model system for the study of the turnover of membrane proteins. Its normal degradative process is not induced by ligand binding, nor is antibody induction of its turnover necessary to facilitate biochemical analysis. The availability of [^{125}I]α-BuTx, a high affinity specific ligand for the receptor, provides an extremely convenient means for its detection throughout the degradative pathway and thus facilitates analysis of the turnover process. Finally, the AChR is rapidly becoming one of the most thoroughly characterized of all multisubunit transmembrane proteins. This information will eventually be very useful in examining the biochemical properties responsible for the regulation of degradative rates.

The focus of this study is a biochemical analysis of the steps involved in the degradation pathway of the AChR in the mouse cell line, BC3H-1. Four approaches were utilized for this purpose: (*a*) measurement of the degradation rate of the AChR and the effects of blocking the degradation with the protease inhibitor leupeptin; (*b*) quantitation of the distinct receptor pools present in these cells and determination of the effect of leupeptin treatment on pool sizes; (*c*) subcellular fractionation to identify the cellular organelles involved in degradation; and (*d*) biochemical characterization of internalized toxin-receptor complexes. The experiments detailed below demonstrate that leupeptin treatment inhibits the degradation of AChR and causes accumulation of a population of receptors derived from the cell surface which has not been previously described in these cells. This leupeptin-augmented receptor appears to reside in a lysosomal compartment and exists in at least two forms differing in sedimentation properties. A preliminary report of this work has appeared (40).

MATERIALS AND METHODS

Materials: Sephacryl S-1000, Percoll, and the density bead marker kit were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Metrizamide and maxidens oil were obtained from Nyegaard, distributed through Accurate Chemical & Scientific Corp., Westbury, NY. Leupeptin was kindly provided by Dr. Alfred Stracher, Department of Biochemistry, Down State Medical Center, Brooklyn, NY, or was purchased from the Peptide Institute, Minoh-Shi, Osaka, Japan. Fetal calf serum (FCS) was obtained from M. A. Bioproducts, Walkersville, MD. Na^{125}I was obtained from Amersham Corp., Arlington Heights, IL. A 17-ml zero clearance homogenizer was purchased from Kontes Co., Vineland, NJ. All other reagents were obtained from Sigma Chemical Co., St. Louis, MO. [^{125}I]α-BuTx was prepared as previously described (33).

Tissue Culture: BC3H-1 cells were cultured as described by Patrick et al. (57). Cells were passaged every 4 d in order to maintain logarithmic phase of growth. Cells were plated at 2×10^3/cm^2 on 60-mm plastic tissue culture dishes. For turnover studies, cells were allowed to enter stationary phase growth and were used within 10 d of passage.

Enzyme Assays: *N*-Acetyl-β-glucosaminidase (NAβGase) was assayed using the methylumbelliferyl-derivatized substrate according to the method of Barrett (10) with slight modifications. The final incubation mixture contained 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminidine (0.5 mM), sucrose (0.25 M), Triton X-100 (0.33%), 0.1 M sodium citrate, pH 5.0, and bovine serum albumin (BSA) (0.17 mg/ml). The reaction was initiated by the addition of enzyme and terminated by the addition of bicarbonate-carbonate stop solution after incubation for 9 min at 37°C. Reaction product was detected fluorometrically with an excitation beam of 375 nm and emission measured at 460 nm. To construct a standard curve, 0.25 ml of methylumbelliferone solutions in the concentration range of 0.0025–0.025 mM were included in reaction mixtures instead of the enzyme. Protein was determined according to the method of Bradford (17) with minor modifications. Samples (100 μl) containing 0.15–2.5 ng protein were mixed with 0.5 ml of the protein reagent and the A_{595} was determined. BSA standards were used to generate a standard curve. To measure the protein content of samples that contained Percoll, aliquots were made 0.5% in SDS and incubated at 25°C for 1 h. The samples were then centrifuged in a Beckman LP42 rotor (Beckman Instruments, Inc., Fullerton, CA) at 28,500 rpm for 1 h to pellet the Percoll. Samples (20 μl) of the supernatants were analyzed as described by Bramhall et al. (18).

AChR Turnover Experiments: Procedures similar to those described by Patrick et al. (57) were used. To label surface AChR of BC3H-1 cells, cultures were incubated in Dulbecco's Modified Eagle's Medium (DME), 0.2% FCS (0.2% DME) containing 1×10^{-8} M [^{125}I]α-BuTx for one h at 37°C, washed to remove unbound toxin, and placed in growth medium. At various time intervals, duplicate aliquots of culture medium were removed and replaced with fresh medium. Radioactivity in the aliquots was determined in a gamma 4000 counter (Beckman Instruments Inc.). At the end of the incubation period, the cultures were washed two times with 2 ml of 138 mM NaCl, 3 mM KCl, 8.15 mM Na_2HPO_4, 1.47 mM KH_2PO_4 (NaCl/P$_i$) and the cells were collected by centrifugation in a microfuge (Beckman Instruments Inc.). Cell pellets were then counted. The radioactivity associated with cells at the beginning of the experiment was calculated by summing that found in the cell pellet with that in the medium. Chromatography of an aliquot of the medium on a Bio-Gel P-2 column verified that the radioactivity excreted by the cells had the properties of [^{125}I]tyrosine (13) (data not shown). Degradation half-times of toxin-receptor complexes were determined by plotting the percentage of radioactivity which remained cell-associated on a semilog scale against time of incubation. The rate of [^{125}I]α-BuTx dissociation from the receptors is slow compared with the rate of degradation (59).

Quantitation of AChR Pools: BC3H-1 cells have, in addition to a surface population of AChR, two discrete internal pools of AChR (57). Methods similar to those described by Patrick et al. (57) were used to quantitate these AChR receptor populations. Surface AChR was determined by binding [^{125}I]α-BuTx (10^{-8} M for 60 min) to cells and washing out unbound toxin as described above. Nonspecific [^{125}I]α-BuTx binding, defined as the binding that occurred in the presence of 2×10^{-8} M α-BuTx or 0.1 mM *d*-tubocurarine (dTc), was < 10%. The labeled cells were harvested from the dish and centrifuged in a table top clinical centrifuge for 10 min at 1,000 rpm. The cell pellet was extracted for 3 h on ice in 0.2 ml NaCl/P$_i$ containing 1% Triton X-100, 1 mM EDTA (1% NaCl/P$_i$). After centrifugation of the extract at 1,000 rpm for 10 min, aliquots of the labeled extract were then counted directly or after filtration through DEAE filters. To quantitate total internal AChR, cell surface receptors were first blocked with unlabeled α-BuTx (10^{-8} M for 60 min) and excess toxin was removed by washing. Triton extracts were then prepared, 20 μl was incubated with 10^{-8} M [^{125}I]α-BuTx for 30 min at 37°C, and the amount of complex formed was determined by DEAE filter assay similar to that described by Schmidt and Raftery (61). The internal AChR is composed of two pools, a precursor to surface component and a hidden pool of unknown characteristics (57). The hidden pool was quantitated after cycloheximide depletion of the precursor to surface population from the total internal pool as in Patrick et al. (57). Following saturation of surface AChR with α-BuTx, cells were washed, harvested, and extracted as above. Quantitation of the AChR in the extracts was carried out by DEAE filter assay. The size of the precursor to surface component was calculated as the difference between the total internal pool and the hidden pool. Total cellular AChR was determined by DEAE filter assay of an untreated cell extract.

In some cases, a spinning column toxin binding assay was employed. Samples of Triton extracts (400 μl) containing 3×10^{-9} M [^{125}I]α-BuTx were incubated at 37°C for 90 min. Bio-Gel P-30 columns were prepared in 3-ml syringes, equilibrated with 50 mM Tris Cl, 0.1% Triton X-100, 1 mg/ml BSA, pH 7.4, and then centrifuged for 5 min at 1,000 rpm. The reaction mixture was applied to the column and the spin was repeated. The fluid that passed through the columns was counted. Lower molecular weight components such as unbound α-BuTx were retained by the column, whereas higher molecular weight material such as toxin-receptor complexes eluted in the void volume. Nonspecific binding (the binding that occurred after preincubation in the presence of saturating concentrations of α-BuTx or 0.1 mM dTc) was subtracted.

Subcellular Fractionation of BC3H-1 Cells: Cells were washed, harvested from the plates, and centrifuged in a clinical centrifuge as described above. The cell pellet was then suspended in 6 ml 1 mM triethanolamine, 0.25 M sucrose, pH 7.2 (tetraethylammonium [TEA] sucrose), and homogenized in a 17-ml zero clearance homogenizer for three strokes at 4°C using a motor-driven pestle. The homogenate was then spun in a centrifuge (model JA 20; Beckman Instruments Inc.) for 10 min at 1700 *g*. The supernatant was recovered and centrifuged at 33,000 *g* for 60 min. The high speed pellet was resuspended in 1.0 ml TEA sucrose and applied to a 25% vol/vol Percoll gradient which was prepared as follows: 2.5 ml 2.5 M sucrose was placed in the bottom of type 60 Ti centrifuge tube and was overlaid with 20 ml of a 25% vol/vol isoosmotic Percoll (d = 1.126 g/cm^3)/TEA sucrose solution. The gradient was then centrifuged in a type 60 Ti rotor for 20 min at 45,000 *g* with low acceleration. 1-ml fractions were collected from the top. Densities were determined by the use of a density

marker bead kit. Fractions were assayed for radioactivity and NAβGase activity. In some experiments, the desired fractions were pooled and chromatographed on a 0.7 × 75 cm Sephacryl S-1000 column. Fractions (1.5 ml) were collected and assayed for radioactivity and NAβGase. The appropriate fractions were pooled and concentrated by one of two methods. Pooled material was centrifuged over a 1.0-ml cushion of 2.5 M sucrose for 60 min at 33,000 *g*. The material at the interface was collected in a minimal volume and diluted with 1.0 mM triethanolamine, pH 7.2, to the desired final sucrose concentration. Samples prepared in this manner were subsequently used for further fractionation purposes. Alternatively, the sample was centrifuged over a 1.0-ml cushion of maxidens oil for 60 min at 33,000 *g*. The material at the interface was collected and made 1.0% in Triton X-100, and extracted on ice for 1 h. This material was used in subsequent velocity gradient sedimentation experiments. Samples intended for further subcellular fractionation studies were layered over 11.0 ml, 20–42% (wt/vol) metrizamide gradients and centrifuged at 50,000 *g* for 18 h as described by Aas (1). 1-ml fractions were collected from the top and analyzed for radioactivity, NAβGase activity, and protein. The extracted samples to be analyzed by velocity gradient sedimentation were layered on 4.4 ml, 5–20% (wt/vol) sucrose gradients prepared in 1% NaCl/P_i. The gradients were centrifuged at 102,000 *g* for 20 h in a rotor (model SW 56; Beckman Instruments, Inc.) and 180-μl fractions were collected by puncturing the bottom of the tube. The fractions were then analyzed for radioactivity.

RESULTS

Work from several laboratories has shown that the degradation rate of cell surface AChR can be measured by reacting them with [^{125}I]α-BuTx and then monitoring the appearance in the culture medium of ^{125}I-monoiodotyrosine (12, 13, 57). The turnover rate of the toxin-receptor complex is very similar to that of the unbound receptor (34, 52). In reasonable agreement with results from other laboratories, we found that AChR in BC3H-1 cells are degraded with a half-time of ~11–16 h (Figs. 1 and 2). Radioactivity in the medium was identified as monoiodotyrosine by comparing its elution position on Bio-Gel P-2 with that of a standard (data not shown). Treatment of the cells with the lysosomal protease inhibitor, leupeptin, inhibits AChR degradation in a dose-dependent manner (Fig. 1). The $t_{1/2}$ for degradation was increased two- to sixfold, depending on the concentration of leupeptin present. The half-time of degradation as well as the sensitivity to leupeptin varied somewhat with the stock of cells used and the time after plating.

It was important to determine whether this inhibition was reversible. For this purpose a turnover-recovery experiment (Fig. 2) was carried out. Surface AChR were labeled with [^{125}I]α-BuTx and incubated in medium containing 60 μM leupeptin. Half of the cultures exposed to leupeptin were washed free of inhibitor after 19-h exposure and were incubated in control media. The results show that cultures treated in this manner regain a turnover rate similar to that of the control cultures within 20 h of the media change.

In addition to AChR on the cell surface, there exists an internal pool of receptor which can be measured after blocking cell surface AChR with unlabeled α-BuTx (57). Previous work has shown that the internal pool is composed of at least two components. One component is a precursor to surface pool which is depletable from its internal location by cycloheximide treatment and presumably represents newly synthesized AChR in transit to the plasma membrane. The other component, termed the hidden pool, appears to be at no time accessible to the external milieu and has no assigned function. Since cultures exposed to leupeptin contained more AChR than control cells, it was important to determine which population of receptor was augmented. After a 19-h incubation in the presence of 40 μM leupeptin, the amount of internal AChR measured after depleting the surface precursor component was increased approximately two-fold (Table I). Little effect on either surface or precursor to surface pools was found. This series of experi-

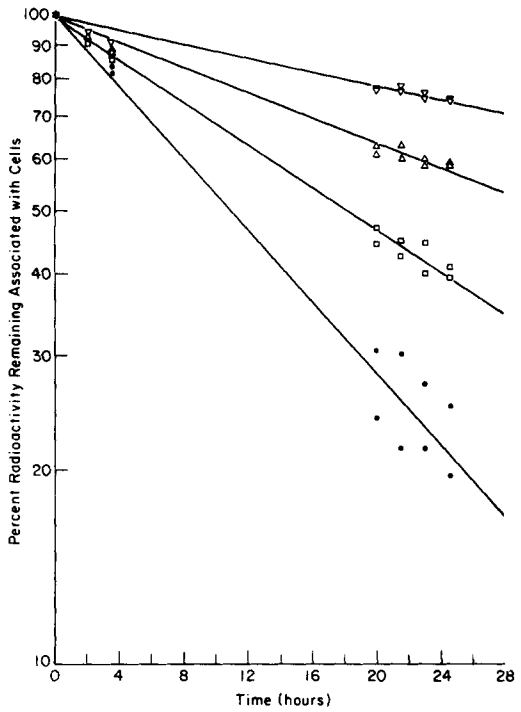

FIGURE 1 Leupeptin inhibition of BC3H-1 AChR turnover. BC3H-1 cells were labeled with [^{125}I]α-BuTx, washed and incubated in control culture medium ($t_{1/2}$ = 11.1 h) (●), or medium containing 10 μM leupeptin ($t_{1/2}$ = 18.2 h) (□), 20 μM leupeptin ($t_{1/2}$ = 31.2 h) (△), or 40 μM leupeptin ($t_{1/2}$ = 59.1 h) (▽). The radioactivity released into the media was measured at the times indicated, and the data were used to calculate the turnover rates.

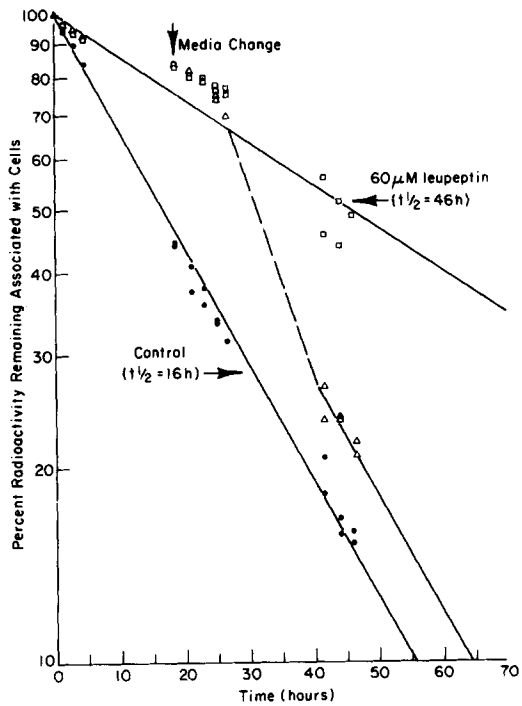

FIGURE 2 Recovery of AChR turnover from leupeptin inhibition. Cells were labeled with [^{125}I]α-BuTx, washed and incubated in control medium (●) or medium containing 60 μM leupeptin (□) for the entire time course of the experiment. Other cells (△) were labeled and washed as above, incubated in the presence of 60 μM leupeptin for 18 ½ h, and then washed free of inhibitor and incubated in control medium for the remainder of the time. AChR degradation was measured as described in Materials and Methods.

ments demonstrates that an internally located pool with some of the characteristics of the hidden pool is augmented by leupeptin treatment.

Additional results indicate, however, that the leupeptin-augmented pool is distinct from the pre-existing hidden pool. Cultures were exposed to control or leupeptin-containing media (60 μM), in both the presence and absence of excess, unlabeled α-BuTx. After depletion of the surface precursor pool by cycloheximide treatment, the amounts of internal receptors were measured. Receptors that are derived from the cell surface during the exposure to unlabeled α-BuTx would be blocked and, therefore, silent in a soluble toxin binding assay. Table II shows that a 24-h incubation in the presence of 60 μM leupeptin causes a three-fold buildup in the internal AChR. In control cultures, the size of the hidden pool is unaffected by the presence of excess α-BuTx in the culture medium. This is in agreement with the results of Patrick et al. (57) and indicates that the hidden pool in control cells is not derived from the surface. In contrast, α-BuTx in the medium during leupeptin exposure reduces the amount of internal receptors capable of binding [^{125}I]α-BuTx after Triton solubilization to the level observed in control cells. This result is consistent with the idea that leupeptin promotes the accumulation of AChR internalized from the muscle cell surface and that these represent a new population of AChR not previously described in BC3H-1 cells.

Subcellular fractionation studies were carried out to identify the organelle in which the leupeptin-augmented AChR reside. Cultures treated in four ways were analyzed. Cells were labeled with [^{125}I]α-BuTx and then maintained for 15 h in control or leupeptin-containing medium (prelabeled cultures). Alternatively, unlabeled cells were incubated for 15 h in control or leupeptin-containing medium and then labeled for a short time with [^{125}I]α-BuTx (postlabeled cultures). In the latter case, only surface receptors should be labeled with [^{125}I]α-BuTx since the incubation is too short to allow detectable internalization. Finally, the cells were harvested, homogenized, and analyzed by Percoll rate zonal gradient centrifugation. Cultures that were prelabeled and treated with control media show a single peak of radioactivity near the top of the gradient (Fig. 3 *A*). This material represents plasma membrane since it is found also in postlabeled cells (Fig. 3 *C*). The amounts of plasma membrane AChR in the latter two cultures are very nearly identical, substantiating the results from the pool quantitation experiments (Table I).

TABLE I

Effect of Leupeptin on AChR Populations

Population	AChR* Control	AChR* Leupeptin‡	Ratio (leupeptin/control)
	fmol		
Surface	160	179	1.1
Total internal	118	201	1.7
Hidden	96	174	1.8
Precursor to surface	22	27	1.2
Total	283	377	1.3

* Average of duplicate determinations (2–11% variation in range).
‡ Cells were exposed to 40 μM leupeptin for 19 h.

TABLE II

AChR in Leupeptin-augmented Pool Are Surface Derived

Treatment	AChR*¶ Control	AChR*¶ Leupeptin‡	Ratio, (leupeptin/control)
	fmol		
−α-BuTx	279	845	3.0
+α-BuTx§	268	261	1.0

* Internal AChR pools were measured after depletion of surface precursor component by cycloheximide treatment.
‡ Cells were exposed to 60 μM leupeptin for 24 h.
§ Unlabeled toxin (2×10^{-8} M) was present in the medium.
¶ Average of duplicate determinations (4–7% variation in range).

Gradient profiles from prelabeled cultures treated with leupeptin contain, in addition to the surface membrane AChR, a denser peak of radioactivity (Fig. 3 *B*). This material sediments reproducibly as a peak in fractions 16–20 (1.07–1.13 g/cm^3), which is coincident with the NaβGase activity, a marker enzyme for lysosomes. The enzyme activity of the peak fractions was 70% latent as measured by its activity in the absence of Triton. The dense material does not arise from artifactual vesiculation of specialized regions of the plasma membrane during homogenization since it is not found in postlabeled, leupeptin-treated cultures. Thus, leupeptin inhibition of AChR turnover causes a build up of a population of AChR which co-migrates with the lysosomal component of these cells on Percoll gradients.

Further purification of the lysosomal component from prelabeled leupeptin-treated cultures has been carried out in an attempt to determine whether the leupeptin-augmented AChR reside in lysosomes or in some other cellular compartment which co-migrates with lysosomes on Percoll gradients. Before additional purification techniques could be employed, however, it was necessary to remove the Percoll from the NAβGase-containing cellular material. Gel filtration chromatography on Sephacryl S-1000 was used to separate the subcellular material from the Percoll. Percoll particles have a mean diameter of 35 nm and therefore elute within the included volume while the cellular material elutes in the void volume measured by the elution position of 795-nm-diam beads (arrow, Fig. 4 *A*). Both the radioactivity and the NAβGase activity from the Percoll gradient dense material of prelabeled cultures treated for 16 h with 15-μM leupeptin elute in the void volume (300–400-nm-diam exclusion limit). The peak fractions were pooled, concentrated, and applied to continuous 20–42% metrizamide gradients for further fractionation by equilibrium density gradient centrifugation. Profiles of the radioactivity, NAβGase activity, and protein distribution of this purification step are shown in Fig. 4 *B*. Radioactivity peaks and NAβGase activity, although rather broad, remain coincident. The density of the peak fraction is 1.15 g/cm^3, which is in agreement with the values found by others for the density of lysosomes in this medium (1). Total purification of the lysosomal fraction was 10–17-fold, based on the relative specific activity of NAβGase.

Separation of the plasma membrane AChR from those in the presumptive lysosomal compartment on Percoll gradients allowed a comparison of the biochemical characteristics of the two receptor populations in leupeptin-treated cells. The sedimentation behavior of the two populations on sucrose velocity gradients containing Triton was examined. Following chromatography on Sephacryl S-1000 columns, samples were concentrated, solubilized with Triton, and applied to 5–20% sucrose gradients. Purified *Torpedo californica* AChR, BC3H-1 plasma membrane receptor samples, and leupeptin-augmented material were run in parallel (Fig. 5). As expected, virtually all of the plasma membrane receptor sediments coincidentally

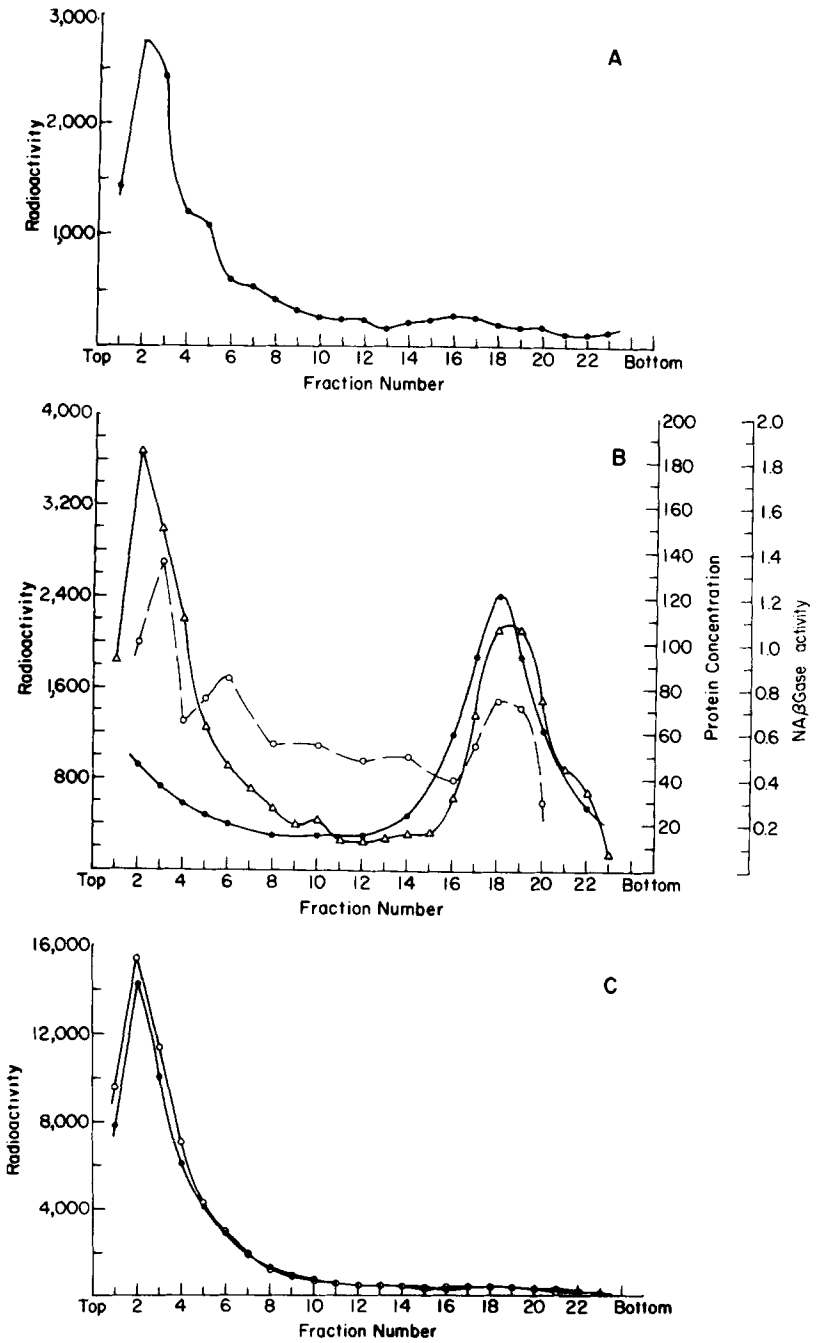

FIGURE 3 Subcellular fractionation of control and leupeptin-treated BC3H-1 cells on Percoll gradients. Subcellular fractionation was carried out as described in Materials and Methods. (*A*) Cells were incubated with [^{125}I]α-BuTx to label surface AChR, washed to remove unbound toxin, and maintained for 18 h in control medium. Fractions from the Percoll gradient were analyzed for radioactivity (cpm, ●). (*B*) Cells were labeled with [^{125}I]α-BuTx, washed, and maintained for 18 h in medium containing 120 μM leupeptin. Fractions from the Percoll gradient were analyzed for radioactivity (cpm, △), Naβ Gase activity (nanograms product per minute per microliter, ●) and protein concentration (nanograms per microliter, ○). (*C*) Cells were incubated in control medium (●) or medium containing 120 μM leupeptin (○) for 18 h, and then surface AChR were labeled with [^{125}I]α-BuTx. Fractions from the Percoll gradients were analyzed for radioactivity (cpm).

with the 9S form of *Torpedo* AChR. The results are identical with AChR solubilized from plasma membrane obtained by Percoll gradient centrifugation of homogenates of prelabeled control, prelabeled leupeptin-treated, or postlabeled control cultures. The leupeptin-augmented AChR from the lysosomal fraction, however, has two forms. Approximately two-thirds of this material sediments in the 9S position, while the remainder sediments to the bottom of the centrifuge tube. This indicates that a very high molecular weight form of the BC3H-1 receptor exists in the leupeptin-augmented population. The plasma membrane AChR samples tested contained either very little (~5–10%) or no high molecular weight form. In three separate experiments, 27–34% of the leupeptin-augmented AChR was large molecular weight.

Turnover of mammalian AChR has been shown to be energy dependent in other systems (12). In BC3H-1 cells, 5 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP) causes a 3.5-fold increase in the $t_{1/2}$ for degradation of the receptor (data

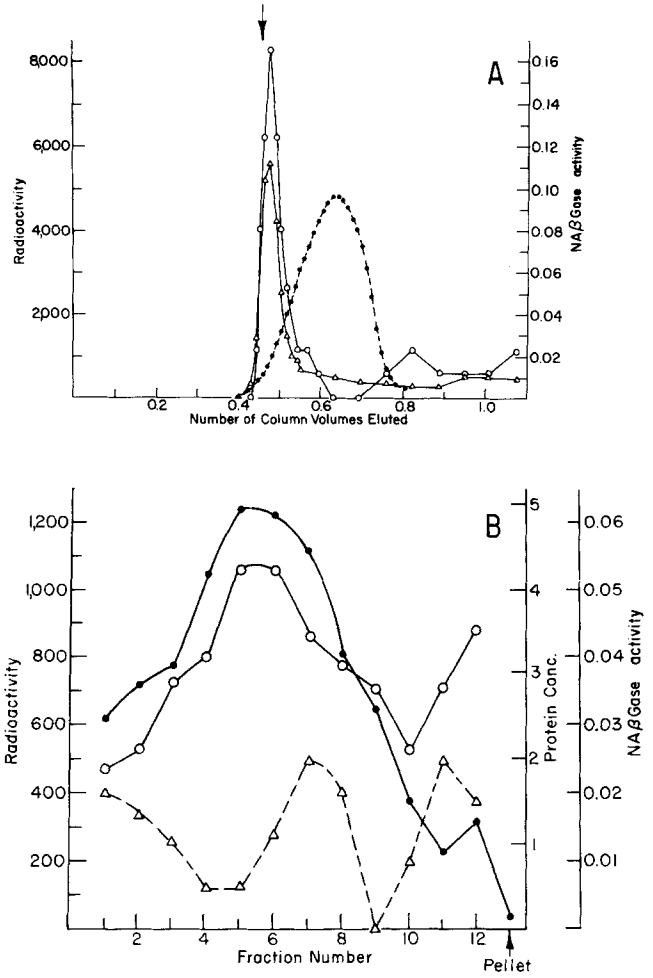

FIGURE 4 (*A*) Chromatography of subcellular material on Sephacryl S-1000. A sample derived from prelabeled cultures that had been exposed to 15 μM leupeptin for 16 h was fractionated by Percoll gradient centrifugation. Fractions 15–19 containing the leupeptin-augmented material were pooled and applied to a Sephacryl S-1000 column (0.7 × 75 cm) equilibrated with TEA sucrose buffer. The column was eluted with the same buffer at a flow rate of 10 ml/cm²/h. Fractions (1.5 ml) were analyzed for radioactivity (cpm, △) and NaβGase activity (nanograms product per minute per microliter, ○). In a separate experiment, [^{125}I]Percoll (●) was analyzed identically. The void volume of the column (arrow) was determined by the elution position of beads with a diameter of 795 nm. (*B*) Purification of lysosomal fraction on a metrizamide gradient. The dense peak of radioactivity from a Percoll gradient (fractions 15–19) of prelabeled, 15 μM leupeptin-treated cells was chromatographed on a Sephacryl S-1000 column, concentrated, and fractionated on a continuous 20–42% wt/wt metrizamide gradient as described in Materials and Methods. Fractions were analyzed for radioactivity (cpm, ●), NaβGase activity (nanograms per minute per microliter, ○), and protein concentration (nanograms per microliter, △).

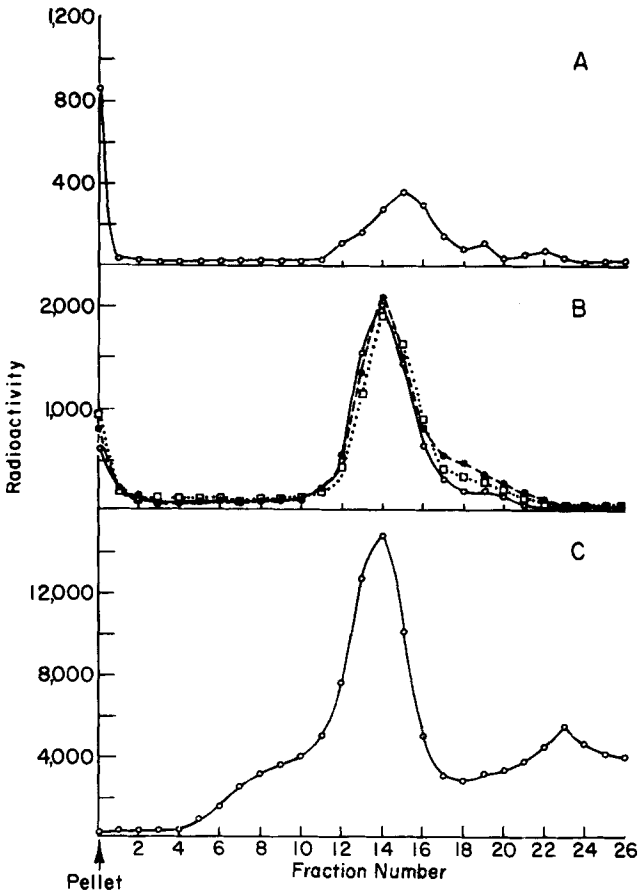

FIGURE 5 Comparison of AChR from plasma membrane and lysosomal fraction by sucrose velocity gradient sedimentation. Fractions were recovered from Percoll gradients, chromatographed on Sephacryl S-1000 columns, concentrated, and solubilized with Triton X-100. Samples (200-μl) were applied to 5–20% (wt/vol) sucrose gradients and fractionated as described in Materials and Methods. Fractions were analyzed for radioactivity (cpm). (*A*) Lysosomal fraction prepared from prelabeled cells treated with 15 μM leupeptin (○). (*B*) Plasma membrane fraction from control, prelabeled cells (○), prelabeled cells treated with 15 μM leupeptin (□), or control, postlabeled cells (●). (*C*) AChR standard prepared by incubation of affinity-purified *Torpedo* receptor (0.26 μg) with 1×10^{-8} M [^{125}I]α-BuTx for 60 min followed by four-fold dilution with 1% NaCl/P_i (○).

not shown). The mechanism by which metabolic poisons cause the inhibition of AChR turnover may differ from the mechanism of inhibition by leupeptin. Thus, the cellular site of accumulated toxin-AChR complexes may also differ. Therefore, prelabeled cultures were treated with 2-deoxyglucose and CCCP for 15 h and then analyzed by subcellular fractionation (Fig. 6). No accumulation of label occurs in the dense region of the Percoll gradient where the lysosomal marker is known to sediment. However, the plasma membrane peak of radioactivity of the treated cells is approximately twofold larger than that of control cells. Thus, in contrast to leupeptin treatment, the inhibition of AChR turnover by metabolic poisoning leads to an accumulation of receptor at the plasma membrane.

We considered the possibility that the hidden pool of AChR may reside in lysosomes, even though it is apparently not derived from the surface and its size is not affected by leupeptin. Cell cultures were treated such that only the hidden pool of AChR would be detectable in a solubilized [^{125}I]α-BuTx binding assay and then subjected to the subcellular fractionation procedure. Fractions from a Percoll gradient were solubilized with Triton and tested for [^{125}I]α-BuTx binding activity. Since the concentration of AChR in the Percoll gradient fractions was low, an alternative spinning column assay for binding was developed. As shown in Fig. 7, the majority of the receptor occurs in a peak that is well-resolved from the NAβGase activity. The hidden pool component has a density of 1.046–1.050 g/cm^3, whereas the NAβGase activity shows a characteristic peak at a density of 1.079 g/cm^3. The results clearly demonstrate that the hidden pool is well-resolved from the

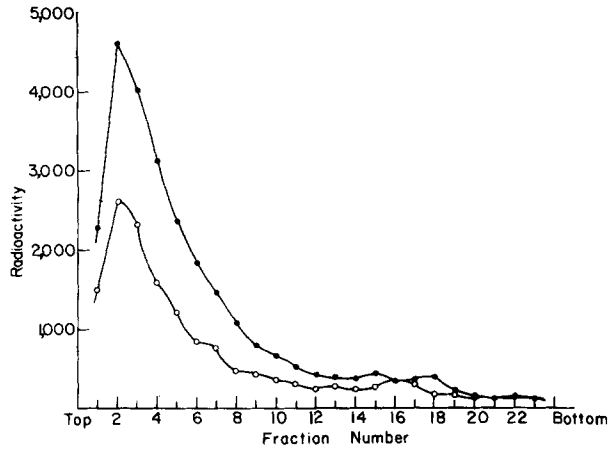

FIGURE 6 Subcellular fractionation of control and energy-inhibited cultures. Cells were labeled with [^{125}I]α-BuTx, washed, and maintained for 18 h in control medium (○) or in medium containing 10 μM CCCP and 94 mM 2-deoxyglucose (●). Subcellular fractionation was carried out as described in Materials and Methods. Fractions from the Percoll gradient were analyzed for radioactivity (cpm).

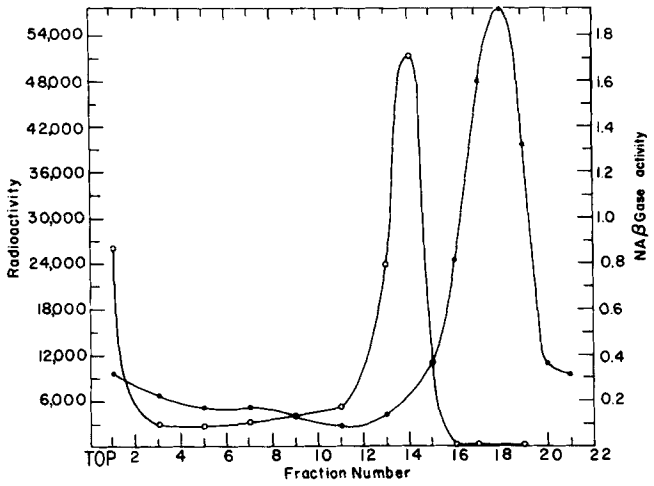

FIGURE 7 Subcellular fractionation of hidden AChR pool on Percoll gradient. Cultures were treated with 50 μg/ml cycloheximide for 3 h to deplete the cells of the surface precursor pool and incubated with 2×10^{-8} M α-BuTx for 1 h to block surface AChR. After washing away unbound α-BuTx, cells were homogenized and subcellular fractionation was carried out by centrifugation on a Percoll gradient. Aliquots of fractions obtained were assayed for NaβGase activity (nanograms per minute per microliter, ●) and [^{125}I]α-BuTx binding activity (cpm, ○) as described in Materials and Methods.

lysosomal component but its sedimentation position does not indicate in which cellular organelle it resides.

DISCUSSION

Our measurement of the turnover rate for the AChR in the BC3H-1 system ($t_{1/2}$ = 9–16 h) is in reasonable agreement with the value of 8 h reported previously by Patrick et al. (57). In this system, we detected no degradative intermediates during the course of normal AChR turnover, possibly because the rate-limiting step is endocytosis. For this reason, leupeptin was utilized in an attempt to accumulate such an intermediate. Leupeptin treatment caused the inhibition of the turnover of surface AChR in a dose-dependent and reversible manner. An internal pool of receptor accumulated as a consequence of this treatment, and this population was shown to be surface derived.

Our results suggest that AChR in leupeptin-treated BC3H-1 cells accumulates in lysosomes. The Percoll gradients used in this study afforded a clear resolution of plasma membrane from lysosomes. The accumulated labeled material observed in leupeptin-treated, prelabeled cultures co-migrated with a lysosomal enzyme marker, NAβGase, and these activities remained coincident throughout two additional purification steps. The possibility that the radio-labeled material is contained in a contaminating cellular compartment which co-migrates with lysosomes is not excluded by these findings since lysosomes were not purified to homogeneity. In view of the coincident behavior of accumulated AChR and NAβGase activity however, it is reasonable to assume that the AChR accumulation in leupeptin-treated BC3H-1 cells occurs in the lysosome. Other laboratories have reported that leupeptin plus pepstatin inhibits AChR degradation and suggested on this basis that receptor turnover proceeds via a lysosomal pathway (55). Our results showing accumulation of internalized AChR in a compartment with lysosomal characteristics provide further support for this proposal.

Libby et al. (46) reported a study of the effects of leupeptin and other inhibitors on AChR turnover in cultured chick muscle cells. In their system, leupeptin inhibited AChR degradation and caused the accumulation of an internal pool of receptors. The cellular compartment containing these AChR was sedimentable at 35,000 *g*. Ultrastructural studies indicated that leupeptin-treated cells contained three times as many coated vesicles as control cells and, on this basis they suggested that the accumulated AChR reside in coated vesicles. Using horseradish peroxidase conjugated-α-BuTx, Bursztajn and Fischbach (24) identified coated vesicles containing AChR in chick muscle cells treated with an extract of chick brain. However, only ~20% of these coated vesicles became labeled in a 1–6-h incubation of the myotubes with the toxin conjugate. Thus, most of them are not derived from the surface. These results neither demonstrate nor discount a possible involvement of coated vesicles in the AChR degradation pathway. Similarly, the evidence we present here does not rule out a coated vesicle step in the turnover process. Untreated chick myotubes are known to contain an internal pool of AChR derived from the cell surface (27). This permitted an autoradiographic study which demonstrated that, in cells labeled with [^{125}I]α-BuTx, structures resembling secondary lysosomes contain radioactivity (28). A final resolution of the cellular location of internalized AChR in leupeptin-treated cells as well as elucidation of the steps in the degradative pathway will require an autoradiographic study at the ultrastructural level.

The possibility that the radioactivity observed to accumulate due to leupeptin treatment does not represent internalized α-BuTx-AChR complexes but instead merely traces the path of dissociated or degraded toxin is excluded by the result obtained in the sucrose velocity gradient centrifugation studies. Two-thirds of the label from the NAβGase coincident material recovered from the Percoll gradients sedimented in a position near 9S, the characteristic sedimentation behavior of monomeric *Torpedo* and mammalian AChR. It should be noted that in some cases the sedimentation patterns of intact and proteolyzed AChR are very similar (51). Thus, this portion of the AChR from the lysosomal fraction is not necessarily unmodified. The remainder sedimented as a very high molecular weight form which may be an important degradative intermediate. In a previously published report (44), the study of the degradation of proteins containing an amino acid analogue in

the rabbit reticulocyte system revealed the formation of intracellular aggregates, one-third of which was sedimented by a 10,000 *g* centrifugation. This high molecular weight form may be an analogous degradative intermediate to that portion of the leupeptin-augmented, NAβGase coincident AChR that behaves as an aggregate. Whether AChR aggregates actually exist in the cell or whether they represent modified receptors that are more prone to aggregation after Triton solubilization remains to be determined. Further studies will be required in order to examine in more detail the characteristics of this material.

A hidden pool of AChR in BC3H-1 cells has been described (57) as a portion of an internal pool which does not appear to function as a surface precursor and is not accessible to the external milieu at any time. This receptor population has no assigned function, nor is its cellular location known. After solubilization, receptors from the hidden pool have a sedimentation coefficient 0.5–0.6S lower than surface receptors (57). Leupeptin inhibition of AChR turnover does not change the hidden pool size but rather causes an accumulation of a receptor subpopulation that is surface derived. Subcellular fractionation studies demonstrated a further distinction between the hidden and leupeptin-augmented pools. A clear resolution of the hidden pool from the NAβGase coincident, leupeptin-augmented AChR was obtained on Percoll gradients. The hidden pool of receptor migrated to a position that is less dense than the lysosomal peak and of greater density than the position of plasma membrane. It is not known however, in what cellular compartment the hidden pool resides. More sensitive assays for other enzyme markers are needed in this system before this question can be answered.

Morphological studies of internalized α-BuTx-AChR complexes have provided most of the information concerning the pathway of AChR degradation. Electron microscopic autoradiographic studies of AChR labeled with [^{125}I]α-BuTx have shown that most receptors reside on the cell surface (45, 60), although some cytoplasmic staining has been observed as well. Intracellular AChR have been observed associated with Golgi apparatus (6, 30), lysosomes (28), coated vesicles (24), and multivesicular bodies (6). The details of the involvement of these intracellular compartments in AChR degradation remain to be elucidated. The biochemical approach that we have described here should be a useful complement to the morphological studies. In particular, it should now be possible to examine the effects of other inhibitors that may block the pathway at different steps.

We thank our colleagues in the Department of Biochemistry for helpful discussions during the course of this work.

This research was supported by U. S. Public Health Service grant NS 14871 and a grant from the Muscular Dystrophy Association.

Received for publication 20 September 1982, and in revised form 10 January 1983.

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