

# Acetylcholine Receptor: Effects of Proteolysis on Receptor Metabolism

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**ABSTRACT** Previous studies (Miskin, R., T. G. Easton, and E. Reich, 1970, *Cell*. 15:1301-1312) have shown that sarcoma virus transformation and tumor promoters reduced the cell surface concentration of acetylcholine receptors (AChR) in differentiating chick embryo myogenic cultures. Both of these agents also induced high rates of plasminogen activator (PA) synthesis in myogenic cultures (Miskin, R., T. G. Easton, A. Maelicke, and E. Reich, 1978, *Cell*. 15:1287-1300), and the present work was performed to establish whether proteolysis might significantly affect receptor metabolism.

Proteolysis in myogenic cultures was modulated by one or more of the following: stimulation of PA synthesis, direct addition of plasmin, removal of plasminogen, or addition of plasmin inhibitors. The results were: (a) When the rates of proteolysis were raised either by addition of plasmin or by stimulating PA synthesis in the presence of plasminogen, both the steady-state concentration and the half-life of surface AChR decreased, but the rate of receptor synthesis was unaffected. (b) The magnitude of these effects, and their dependence on added plasminogen, indicated that proteolysis initiated by plasminogen activation could account almost entirely for the reduction in receptor half-life produced by sarcoma virus transformation and phorbol ester. (c) The rate of receptor synthesis, which is also reduced by viral transformation and tumor promoters, was not modified by proteolysis; hence plasmin action may be responsible for a large part, but not all of the change in surface receptor under these conditions. (d) The plasmin catalyzed changes in receptor parameters appear to occur in response to modified membrane metabolism resulting from proteolysis of surface components other than AChR itself.

The acetylcholine receptor (AChR) in differentiating myogenic cultures provides an accessible and attractive experimental system for analyzing many parameters of membrane structure and function. The receptor is well-characterized chemically, pharmacologically, and metabolically, and it can be measured accurately by use of specific, high-affinity, radioactive neurotoxins. As a protein that mediates transmembrane signaling and local permeability, the AChR may serve as a model for other integral membrane structures such as hormone receptors, and it may also reflect diverse aspects of surface membrane metabolism.

Previous studies of differentiating myogenic cultures have established a correlation between plasminogen activator (PA) production and the rate of acetylcholine receptor (AChR) degradation: conditions associated with elevated PA produc-

tion, such as sarcoma virus transformation, exposure to tumor-promoting phorbol esters, or early stages of culture, uniformly accelerated AChR degradation and reduced receptor half-life (14, 15). These results, derived from cultures maintained and propagated in plasminogen-containing media, suggested that proteolysis by plasmin could modulate the metabolism of cell surface structures. This possibility could be tested experimentally and it prompted the work reported here.

To characterize the influence of plasmin on AChR metabolism we have determined receptor half-life, receptor concentration, and the rate of receptor synthesis in myogenic cultures exposed to conditions in which proteolysis was controlled either by exclusion of plasminogen, addition of plasmin, addition of inhibitors, or some combination of these. The results show that plasmin-catalyzed proteolysis can indeed affect the rate of

receptor degradation; further, the reduction of receptor half-life after sarcoma virus transformation or exposure to tumor promoters is largely, if not completely, due to formation of plasmin secondary to enhanced production of PA. While this manuscript was in preparation, Libby et al. (11) published a report concerning the role of lysosomal proteolysis in AChR metabolism. Their work, which deals with intracellular proteolysis, is both relevant and complementary to the experiments described below.

## MATERIALS AND METHODS

### Materials

Sephacryl S-200, Sepharose 4B, and Bio-Gel P-2 were obtained from Pharmacia Fine Chemical Co. (Piscataway, N.J.); 6-aminocaproic acid and diisopropyl fluorophosphate (DFP) from Aldrich Chemical Inc. (Milwaukee, Wisc.); urokinase from Leo Pharmaceutical Products (Ballerup, Denmark); Trasylol from Bayer A.G. (Wuppertal-Elberfeld, W. Germany); glycerol from Fisher Scientific Co. (Pittsburgh, Pa.); aquacide from Calbiochem-Behring Corp. (San Diego, Calif.); and chicken plasma from Pel-Freezer. Leupeptin was a gift from Dr. W. Troll (New York University Medical Center).  $^{125}\text{I}$ -labeled  $\alpha$ -bungarotoxin ( $^{125}\text{I}$ - $\alpha$ -BuTx) (specific radioactivity, 30,000 and 136,000 cpm/pmol, respectively) was prepared as described by Miskin et al. (14) or was a gift from Dr. B. Fulpus. Rabbit anti-AChR serum was a gift from Dr. B. Fulpus (University of Geneva, Switzerland). Other materials were as described by Miskin et al. (14, 15).

### Methods

**CELL CULTURE:** Chick embryo myogenic cultures were prepared and maintained as previously described (15) but the procedures were modified, when required, to exclude plasminogen, especially in experiments designed to examine the effects of this zymogen on AChR metabolism. Because plasminogen adheres to plastic surfaces and a wide variety of macromolecules, it was necessary to use plasminogen-depleted media from the initial steps in the preparation of cultures. Thus, plasminogen-depleted horse serum and fetal bovine serum (FBS) were used throughout. Passage over lysine-Sepharose columns (Deutsch and Mertz [3]) largely reduced the ability of chicken embryo extract to promote myoblast fusion, an effect that could not be reversed by the addition of purified chicken plasminogen. Hence, ordinary chicken embryo extract (final concentration, 3% [vol/vol]) was used during the first 4 d of culture and then replaced by plasminogen-depleted embryo extract (1%).

**DEPLETION OF PLASMINOGEN FROM CULTURE SUPPLEMENTS:** Plasminogen was removed from culture supplements according to Deutsch and Mertz (3). (a) Sera: 500 ml of horse serum or FBS was centrifuged at 19,000 g for 20 min and loaded into a lysine-Sepharose column (30  $\times$  1.5 cm, flow rate of 20 ml/h, 4°C) previously equilibrated with 0.1 M phosphate buffer pH 7.4 (buffer A). The depleted horse serum was dialyzed against native horse serum (5 vol) to restore low molecular weight substances removed by the column. (b) Embryo extract: embryo extract was purified in the same way as horse serum after incubation at 37°C for 1 h with deoxyribonuclease (20  $\mu\text{g}/\text{ml}$ ), ribonuclease (10  $\mu\text{g}/\text{ml}$ ), and hyaluronidase (5  $\mu\text{g}/\text{ml}$ ). After passage over lysine-Sepharose the extract was also dialyzed against an excess of nonpurified embryo extract. (c) Antisera: 1 ml each of rabbit anti-AChR serum and control rabbit antiserum (anti-bovine serum albumin) was passed through lysine-Sepharose columns (2 ml) at a flow rate of 1 ml/10 min, at room temperature. Both antisera were heat inactivated (56°C, 30 min) and stored at -70°C.

**PURIFICATION OF CHICKEN PLASMINOGEN:** Chicken plasma (500 ml) was filtered on gauze mesh to remove fibrin clots, and centrifuged for 30 min at 25,000 g. The plasma was then brought to 20% of saturation by addition of solid ammonium sulfate, centrifuged for 20 min at 10,000 g, left on ice for a few hours, and recentrifuged to remove additional aggregates. The supernatant was loaded onto a lysine-Sepharose column (30  $\times$  1.5 cm, flow rate of 50 ml/h, 4°C) previously equilibrated with buffer A, and the column was washed with buffer A and with 0.3 M phosphate buffer, pH 7.4, successively. Plasminogen was eluted with 0.2 M 6-aminocaproic acid in buffer A, precipitated at 50% of saturation with ammonium sulfate, dialyzed against 50 mM ammonium bicarbonate, and then lyophilized. 20 mg of plasminogen were dissolved in 2 ml of 0.1 M Tris/HCl buffer, pH 8.0, containing 0.5 M NaCl, loaded onto a Sephacryl S-200 column (100  $\times$  1.5 cm, flow rate of 20 ml/h, 4°C), and eluted with the same buffer. The fractions containing plasminogen were pooled, concentrated to 1-2 mg/ml in a dialysis bag by packing in aquacide, dialyzed against PBS, treated with DFP (13), and stored at -70°C.

**ACTIVATION OF PLASMINOGEN:** Human plasminogen (purified according to Deutsch and Mertz [3]) was diluted to a final concentration of 7-10 mg/ml

in 0.05 M Tris/HCl buffer, pH 8.0, containing 50% glycerol, and incubated with 100 Ploug units/ml urokinase for 24 h at 30°C.

**LABELING OF CELL SURFACE ACHR WITH  $^{125}\text{I}$ - $\alpha$ -BuTx AND ESTIMATION OF ACHR DEGRADATION RATE:** The measurements were made exactly as described by Miskin et al. (15). For plasminogen-depleted cultures, the following modification was introduced: ~16 h before labeling of surface AChR the culture medium was changed to Dulbecco's modified Eagle's medium containing 1 mM additional glutamine and 10% of plasminogen-depleted and acid-treated FBS (18). The same mixture was used for the  $^{125}\text{I}$ - $\alpha$ -BuTx binding reaction and throughout the determination of AChR degradation. AChR labeling was achieved by incubation with  $2 \times 10^{-8}$  M  $^{125}\text{I}$ - $\alpha$ -BuTx for 60 min. Unless otherwise specified, all incubations were at 37°C. All experimental points are the means of duplicate or triplicate cultures.

**SYNTHESIS OF NEW SURFACE ACHR:** Measurement of the rate of synthesis of new surface AChR was as described by Miskin et al. (14), with the following modification: the culture medium was changed as described above and was also supplemented with nonradioactive  $\alpha$ -BuTx ( $2 \times 10^{-8}$  M) for ~16 h, to block surface AChR. The same medium was used after removal of nonradioactive  $\alpha$ -BuTx. All experimental points are the mean of duplicate cultures.

## RESULTS

Three experimental approaches were used to delineate the potential role of plasmin in AChR metabolism: (a) cultures were deprived of plasminogen, thereby blocking the formation of plasmin; these were compared with control cultures in which the desired level of plasminogen had been restored by addition of pure proenzyme; (b) protease inhibitors, capable of blocking PA and/or plasmin were added to culture media; (c) plasmin was added to cultures for various lengths of time. The steady-state level of receptor, the rate of receptor degradation, the rate of receptor synthesis, or some combination of these, was determined for each set of conditions.

### Effect of Plasminogen on AChR Half-life

Previous work having shown that the tumor-promoter phorbol myristate acetate (PMA) both reduced receptor half-life and increased PA production (14, 15), we explored the effect of plasminogen on the rates of AChR degradation in both untreated and PMA-treated cultures. The results of a typical set of observations are presented in Fig. 1, and the findings obtained from eight separate experiments are summarized in Table I.

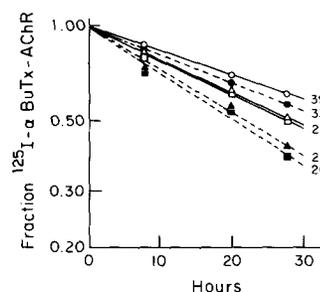


FIGURE 1 Effect of plasminogen, ts-68, and PMA on half-life of AChR in muscle cultures. Uninfected and ts-68 infected companion cultures were grown at 42°C for 6 d in media containing plasminogen-depleted horse serum and embryo extract. Medium was then changed, PMA (50 ng/ml) and/or plasminogen (42  $\mu\text{g}/\text{ml}$ ) were added to some of the cultures, and all cultures were shifted to 37°C. Determination of AChR half-life was performed in the usual way beginning 16 h after temperature shift, and when indicated, in the presence of PMA and/or plasminogen. (○, ●) Control without and with plasminogen, respectively; (△, ▲) ts-68 infected without and with plasminogen, respectively; (□, ■) PMA treated without and with plasminogen. Surface AChR was, in the same order: 371, 347, 69, 73, 295, and 286 fmol/culture. The numbers indicate the value of the receptor half-life for each curve.

The profiles in Fig. 1 demonstrate that typical decay curves, with single first-order slopes, were observed under all of the experimental conditions tested; hence, the presence or absence of plasminogen or PMA merely altered the rate, but did not grossly distort the characteristic pattern of AChR degradation. Fig. 1 also shows that both sarcoma virus transformation and PMA accelerated receptor degradation in comparison with the control, and that the addition of plasminogen shortened AChR half-life in all three types of culture. These results are reinforced by the data in Tables I and II. Here it can be seen:

(a) There is a basic rate of AChR turnover which persists in the absence of added plasminogen (Table I, column I). Although we could not rigorously exclude every last trace of plasminogen (see Materials and Methods) it appears unlikely that the small amounts remaining from the initial supplement of embryo extract could account entirely for the observed rate of receptor degradation, especially since the effect of much larger supplements of plasminogen (column II) was consistently modest.

(b) The addition of plasminogen to control cultures accelerated receptor degradation perceptibly, but only to a limited extent, in six of eight experiments (compare columns I and II). The magnitude of this effect was compatible with the low levels of PA production, and hence of plasmin formation, observed in such cultures (15).

(c) PMA treatment alone (column III) caused only a minor reduction in receptor half-life. This decrease was less frequent, occurring in only four of eight experiments, than that produced by the addition of plasminogen alone. Since this PMA treatment induces high levels of PA production (15), the inconsistent

TABLE I  
Effect of Plasminogen and PMA on Half-life of AChR in Muscle Cultures

Experiment number	Half-life of control cultures (h)		Half-life of PMA-treated cultures (h)	
	Plasminogen			
	Absent (I)	Present (II)	Absent (III)	Present (IV)
1	32 (1.00)	32 (1.00)	32 (1.00)	22 (0.69)
2	34 (1.00)	29 (0.85)	29 (0.85)	18 (0.53)
3	43 (1.00)	36 (0.84)	29 (0.81)	22 (0.51)
4	43 (1.00)	44 (1.02)	42 (0.98)	37 (0.86)
5	34 (1.00)	29 (0.85)	27 (0.79)	20 (0.59)
6	40 (1.00)	33 (0.83)	28 (0.70)	21 (0.53)
7	43 (1.00)	38 (0.88)	41 (0.95)	26 (0.60)
8	41 (1.00)	36 (0.88)	42 (1.02)	27 (0.66)
Average ratio:	(1.00)	II/I (0.89)	III/I (0.89)	IV/I (0.62)

Plasminogen-depleted cultures were prepared and maintained as described in Materials and Methods. AChR half-life determination was performed in the usual way in the presence and absence of PMA and/or plasminogen. When present, PMA (50 ng/ml) and chicken plasminogen (28 µg/ml in experiment 1, and 42 µg/ml in experiments 2-8) were first added to the cultures ~16 h before labeling AChR with radioactive α-BuTx, and then maintained throughout the experiment. Culture ages on days of labeling were: 4 and 5 d in experiments 1 and 2, respectively, 7 d in experiments 3-7, and 8 d in experiment 8. In experiments 6 and 7, cultures were incubated for 5 d at 42°C and then for 1 d at 37°C before labeling with <sup>125</sup>I-α-BuTx. The numbers in parentheses represent the ratio of AChR half-life under the particular culture conditions (Column II, III, or IV) to that in control (column I). The value for the control (column I) was arbitrarily set at unity for each experiment. When these data were analyzed by Student's *t* tests (both separate and pooled data) groups I, II, and III were not significantly different (*P* > 0.1), whereas the difference between IV and III (and therefore between IV and II or I) was highly significant (*P* < 0.001).

TABLE II  
Effect of ts-68 on Half-life of AChR in Muscle Cultures

	Temperature	Half-life (h)	
		Plasminogen	
		Absent	Present
Control	42°C	25 (1.00)	20 (0.80)
ts-68	42°C	23 (0.92)	21 (0.84)
Control	37°C	43 (1.00)	38 (0.88)
ts-68	37°C	43 (1.00)	32 (0.74)

Uninfected and companion ts-68 cultures were incubated at 42°C for 7 d in media containing plasminogen-depleted horse serum and embryo extract as described in Materials and Methods. 16 h before AChR was labeled with radioactive α-BuTx, the medium was changed, chicken plasminogen (42 µg/ml) was added to some of the cultures, and the indicated cultures were shifted to 37°C. Determination of AChR half-life was performed at 37° or 42°C, with or without plasminogen, as indicated. The values in parentheses represent the ratio of the AChR half-life under the particular conditions to that of the control, uninfected cultures, in the absence of plasminogen, at 42° or 37°C, respectively.

effect on receptor degradation might have been due to small variations in residual plasminogen levels, and thus of plasmin activity, in the different experiments. It also follows from this result that proteolysis by PA itself, in the absence of plasminogen, was not a significant factor in AChR turnover.

(d) Substantial reductions in AChR half-life occurred in all cultures exposed to both PMA and plasminogen (column IV). Thus the PMA-stimulated acceleration of receptor turnover was plasminogen dependent and therefore largely, and perhaps entirely, due to the proteolytic action of plasmin.

The data in Table II present an analogous pattern for myogenic cultures infected with ts-68. The results of this experiment are complicated by the fact that receptor degradation increases as a function of increasing temperature (4), thereby limiting comparisons of the different cultures to those incubated at a single temperature. Here PA production was increased (15) and receptor half-life decreased (14) at 37°C, the permissive temperature for sarcoma virus transformation. It can be seen, as in Table I, that small reductions in AChR half-life occurred when plasminogen was added to control cultures at both 37° and 42°C, and to the ts-68 infected culture at 42°C, the nonpermissive temperature for transformation. A large reduction in AChR half-life was observed only at 37°C in the presence of plasminogen, conditions that ensured the generation of substantial amounts of plasmin since PA production is strongly enhanced by and coupled with transformation.

#### Effect of Plasmin on AChR Turnover

The plasminogen dependence of the changes in AChR half-life after ts-68 transformation and exposure to PMA implied that comparable effects on receptor metabolism should be produced simply by addition of plasmin. The results of the four experiments summarized in Table III show that this is the case. Both concentrations of plasmin tested were well within the range that can be regarded as physiological, considering the level of plasminogen in plasma (>200 µg/ml), and both markedly shortened half-life when added after receptor was labeled with <sup>125</sup>I-α-BuTx. The effect of plasmin pretreatment (16 h, 50 µg/ml) before labeling with <sup>125</sup>I-α-BuTx was tested in companion cultures to those of experiments 2 and 3: this treatment gave values for AChR half-life intermediate between those of the control and plasmin-treated samples (34 and 40 h,

TABLE III  
Effect of Plasmin and Trasylol on Half-life of AChR in Muscle Cultures

Treatment	Medium supplement	Half-life (h)			
		Exp. 1	Exp. 2	Exp. 3	Exp. 4
1	Control	29 (227)	41 (197)	48 (388)	42 (198)
2	Trasylol, $5 \times 10^{-6}$ M	—	—	48 (386)	—
3	Trasylol, $2 \times 10^{-4}$ M	—	—	—	42 (195)
4	Plasmin, 25 $\mu$ g/ml	—	—	30 (396)	—
5	Plasmin, 50 $\mu$ g/ml	19 (238)	27 (187)	24 (367)	—
6	Plasmin, 50 $\mu$ g/ml + Trasylol, $5 \times 10^{-6}$ M	28 (228)	40 (191)	43 (367)	—

Cultures were grown with plasminogen-depleted horse serum and embryo extract (experiments 1 and 2) or with plasminogen-containing supplements (experiments 3 and 4) and analyzed for AChR half-life in the presence of the indicated supplements. Supplements were first added to culture medium immediately after labeling AChR with radioactive  $\alpha$ -BuTx. Supplements were maintained throughout the period in which AChR degradation was measured. Numbers in parentheses represent surface AChR in fmoles per culture.

respectively, for experiments 2 and 3); the combination of plasmin pretreatment, followed by toxin labeling and subsequent plasmin treatment yielded half-life values close to those of the plasmin posttreatment alone. The plasmin effects on AChR half-life were abolished by the addition of the trypsin and plasmin inhibitor Trasylol, showing that these were due to the proteolytic action of the enzyme (Table III). Trasylol itself was inert with respect to receptor metabolism and, specifically, did not affect receptor half-life in the absence of plasminogen; this is evidence that a significant fraction of AChR metabolism proceeds by a plasmin-independent route.

It was previously observed (14) that when AChR half-life was modulated by environmental variables such as serum or embryo extract the rates of receptor degradation were matched by corresponding changes in synthesis to preserve a relatively constant level of surface AChR. It was therefore of interest also to examine the effect of plasmin both on surface AChR concentration and on receptor synthesis. The results of such an experiment are presented in Fig. 2: after a 5-h lag period, surface AChR decreased progressively for 24 h in the plasmin-containing culture, reaching a level approximately one third below that of the control (upper curves). Plasmin is known to be autolytic and thus to lose its catalytic activity progressively under conditions of cell culture resembling those used here (20). The 5-h lag period preceding any measurable decrease in surface AChR is therefore noteworthy, since it coincided with the period of highest plasmin concentration and most intense proteolytic activity. This suggests that the subsequent reduction of receptor concentration was due to a process which was initiated gradually during the first few hours and persisted thereafter for a longer period, rather than to direct proteolysis of membrane-bound receptor itself. The concurrent measurement of AChR synthesis (lower curves) showed no significant difference between the plasmin-treated and control cultures. The decrease in surface AChR that accompanied plasmin treatment therefore reflected the increased rate of degradation (Table III) which, in this case, did not induce any reciprocal change in receptor synthesis.

A number of control experiments were performed to test for other potential sources of artifacts. Since  $\alpha$ -BuTx contains a number of basic amino acid residues that are potential targets for plasmin action, the decreased half-life might have been due to direct proteolysis of  $\alpha$ -BuTx in receptor complexes, followed by toxin dissociation. Had this occurred, the putative toxin fragments released by such a process should have been detectable in the supernatant as peptides of molecular weight intermediate between those of native toxin and iodotyrosine, the

radioactive degradation product normally produced by metabolic turnover of receptor-toxin complexes (4). The data in Fig. 3 show that this was not the case: as in control cultures, iodotyrosine was essentially the only toxin degradation product observed in plasmin-treated cultures, a finding which indicates that plasmin-stimulated degradation proceeds by the lysosomal pathway. A second possibility, namely, that a rate-limiting endoproteolytic nick by plasmin, followed by exopeptidase action due to serum protease, could generate free iodotyrosine as an  $\alpha$ -BuTx degradation product, was excluded by the finding that plasmin does not attack the toxin.<sup>1</sup> A third alternative was that proteolysis by plasmin could have modified the receptor to increase the rate of dissociation of intact  $^{125}$ I- $\alpha$ -BuTx; the released toxin would in turn have been more or less degraded either by plasmin outside the cells, or, after pinocytosis, in some cellular compartment. This possibility is excluded by three facts: free  $^{125}$ I- $\alpha$ -BuTx, at the concentrations that could be generated by dissociation of receptor toxin complexes, is neither internalized nor degraded in myogenic cultures (4, 14), nor is it attacked by plasmin (Hatzfeld and Miskin, unpublished observations). Moreover, had significant dissociation and subsequent partial degradation occurred in the supernatant these should have been detected (in the experiment presented in Fig. 3), as an increase either of free  $^{125}$ I- $\alpha$ -BuTx or of partial degradation products, but neither was observed. We therefore conclude that the proteolytic mechanism by which plasmin accelerates receptor degradation is not based on direct proteolysis of bound or dissociated toxin.

As a further test of protease involvement in AChR metabolism we investigated the effects of the protease inhibitor leupeptin. The action of this inhibitor is somewhat complex because it blocks serine proteases, including plasmin and PA

<sup>1</sup> The resistance of  $\alpha$ -BuTx to plasmin was demonstrated in the following experiment: 500 fmol of  $^{125}$ I- $\alpha$ -BuTx were incubated for 15 h at 37° in 2 ml of Dulbecco's modified Eagle's medium containing the following supplements: (a) human plasmin (50  $\mu$ g/ml); (b) human plasmin (50  $\mu$ g/ml) plus 10% of fetal bovine serum (acid-treated to destroy protease inhibitors); (c) chicken plasmin (50  $\mu$ g/ml, generated as described in Materials and Methods for human plasmin, but using 10-fold more urokinase) plus 10% of acid-treated fetal bovine serum. At the end of the incubation, the samples were reduced by the addition of dithiothreitol ( $5 \times 10^{-2}$  M) and analysed on a Bio-Gel P-2 column as described in the legend to Fig. 3. All three samples gave identical profiles in which the bulk of radioactivity emerged, as did authentic  $\alpha$ -BuTx, at the void volume of the column; there was a small amount of residual contaminating  $^{125}$ I<sup>-</sup>, and no evidence of proteolytic fragments in any sample.

(22), as well as lysosomal proteases (8, 12). When added to cultures in the presence of PMA and/or plasminogen, appropriate concentrations of leupeptin invariably prolonged apparent AChR half-life; two examples, representing a control series in the absence of either PMA or plasminogen, and a set of cultures exposed to both of these agents, are illustrated in Fig. 4. Cultures containing either PMA or plasminogen alone yielded analogous profiles intermediate between and parallel with the two curves in Fig. 4. Beginning at  $\sim 5 \times 10^{-7}$  M, increasing concentrations of leupeptin progressively raised apparent AChR half-life both in control cultures and in cultures containing PMA and plasminogen. Nevertheless, the difference in the AChR half-life between the two cultures, which exists in the absence of leupeptin (Table I, columns I and IV), was maintained at all concentrations of leupeptin tested. We note also that the ordinate in Fig. 4 represents a half-life determination for each point on both curves. Thus the overall process of receptor degradation follows first-order kinetics throughout the range of experimental conditions explored here. To obtain additional information about leupeptin effects, we also measured both surface AChR and the rate of receptor synthesis under conditions identical to those for Fig. 4. There was no change either in the steady-state surface AChR level or in the rate of receptor synthesis and incorporation into plasma membrane during a 24-h period (Fig. 5). The results confirm the findings described by Libby et al. (11), which were obtained under slightly different conditions. These findings also represent a reasonable foundation for the reported intracellular

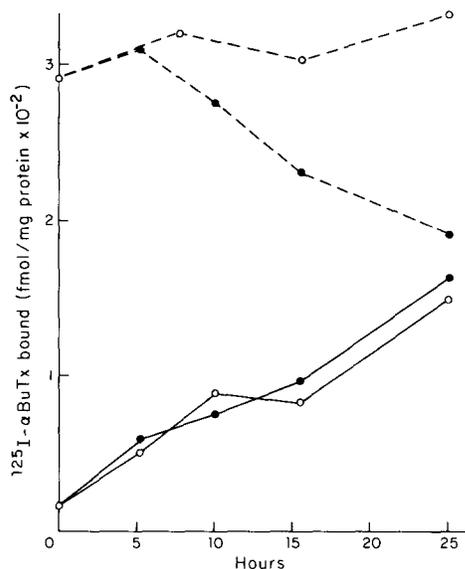


FIGURE 2 Effect of plasmin on surface levels of AChR and on the synthesis of new surface receptors. Plasminogen-depleted cultures were used for the experiment. To examine the effect of plasmin on the levels of surface AChR, human plasmin was first added to some of the cultures at zero time, and the medium was renewed after 16 h. At different times after the first addition, levels of surface AChR were measured at the indicated times in some cultures, and the rate of synthesis of new surface receptors was determined in companion cultures, likewise in presence and absence of plasmin. In the latter case, plasmin was first added just after removal of nonlabeled  $\alpha$ -BuTx from culture medium (time 0), and the medium was changed once after 16 h. Protein was determined by the method of Lowry et al. (13). (○—○, ●—●) Surface AChR in control and in plasmin-treated cultures, respectively. (○—○, ●—●) Synthesis of AChR in control and plasmin-treated cultures, respectively.

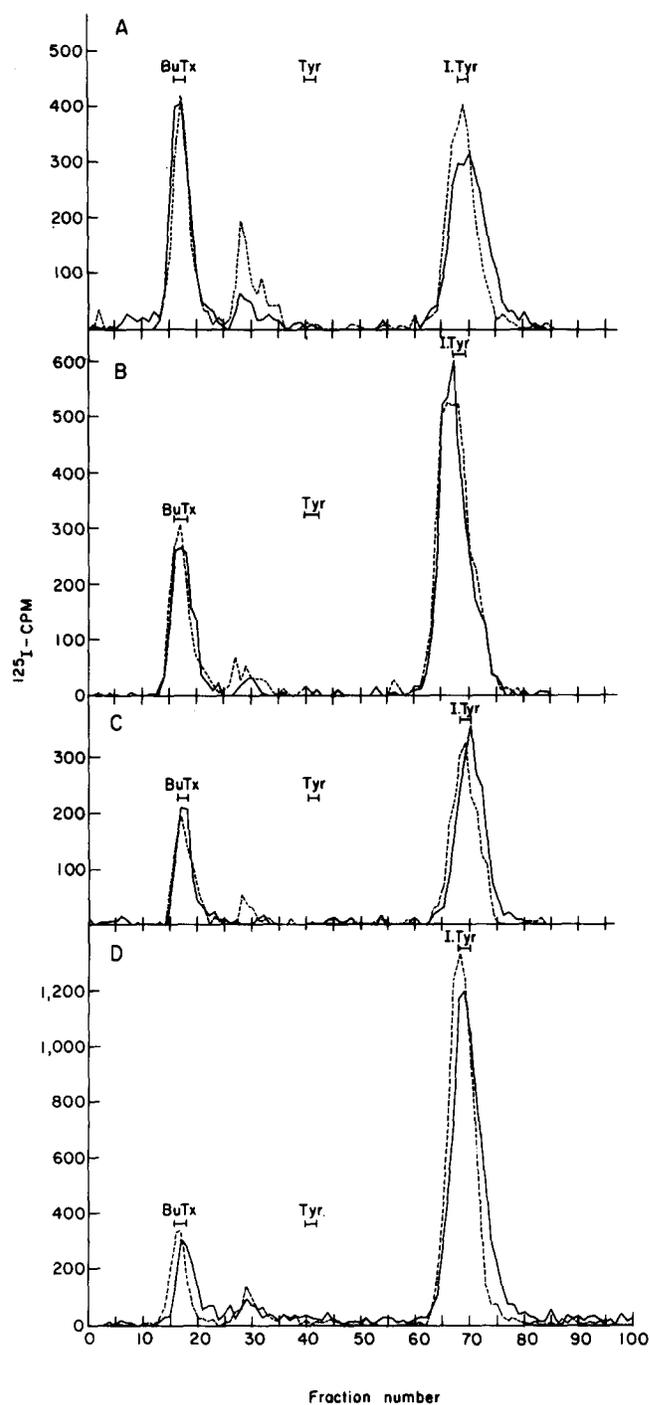


FIGURE 3 Analysis of degradation products of AChR- $^{125}$ I- $\alpha$ -BuTx complexes from control and plasmin-treated muscle cultures. Radioactivity released into culture medium in control and plasmin-treated cultures was analyzed for degradation products of AChR- $^{125}$ I- $\alpha$ -BuTx complexes by gel filtration on a Bio-Gel P-2 column. Samples taken from experiment 3 in Table IV (treatments 1 and 4) were loaded onto a column (25  $\times$  1 cm) previously equilibrated with 0.5 M ammonium acetate, pH 5.0, at 4°C, and eluted with the same buffer. (A and B) Samples containing  $\sim 5,000$  cpm, collected 2–10 h (A) and 10–18 h (B) after labeling of AChR with  $^{125}$ I- $\alpha$ -BuTx. (C) Samples containing  $\sim 1,000$  cpm collected 18–26 h after labeling. (D) Samples containing  $\sim 9,000$  cpm, collected 25 h after labeling, without any change of medium. Solid lines, control; dashed lines, plasmin-treated. The recovery of radioactivity applied to the column was always  $\sim 100\%$ .

(presumably intralysosomal) accumulation, in leupeptin-treated cells, of AChR in a form capable of binding  $\alpha$ -BuTx (11). However, several attempts to obtain evidence also confirming this observation failed; leupeptin at concentration of  $5 \times 10^{-6}$  M and  $2 \times 10^{-4}$  M gave no detectable increase of intracellular toxin-binding components during incubations lasting up to 38 h.

AChR degradation in various myogenic cultures can be accelerated by antisera obtained from myasthenic patients and from animal models of myasthenia produced by injection of receptor preparations (1, 6, 7). It was thus of interest to establish whether this method of modulating receptor metabolism might be related to the induction of PA. We used for this purpose a high-titer rabbit antiserum raised against highly purified *Torpedo marmorata* receptor. As seen in Table IV, this antiserum greatly reduced receptor half-life, but the effect did not depend on the presence of plasminogen. This is in accord with the finding that a number of myasthenic sera and other rabbit antisera directed against *Torpedo* receptor either failed to increase PA production, or stimulated only minimally (Miskin and Fulpius, unpublished observations).

## DISCUSSION

That plasmin catalyzed proteolysis stimulated AChR degradation in myogenic cultures is established by several lines of evidence: first, when added at modest concentrations, highly purified plasmin produced a substantial and persisting acceleration of receptor degradation; second, this effect of plasmin

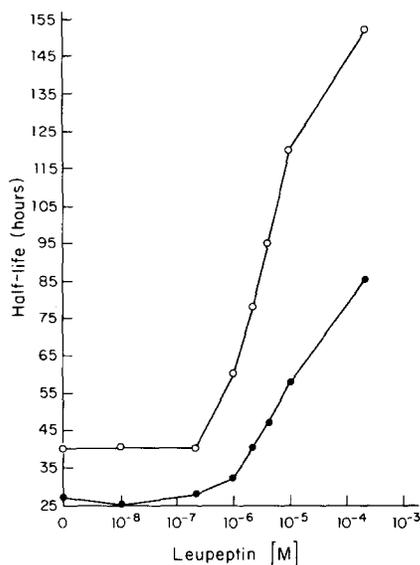


FIGURE 4 Effect of leupeptin on AChR degradation in control and PMA-treated cultures. Plasminogen-depleted cultures were prepared and maintained as described in Materials and Methods. 16 h before labeling of AChR with radioactive  $\alpha$ -BuTx, some of the cultures were treated with PMA (50 mg/ml), with plasminogen (42  $\mu$ g/ml), and/or with different concentrations of leupeptin. The respective treatments were maintained throughout the period during which AChR degradation was being measured. (O) Control without plasminogen; (●) PMA-treated cultures with plasminogen. Surface AChR was in the range 220–259 and 153–192 fmol/culture for control and PMA-treated cultures, respectively. Not shown in the figure: half-lives of control cultures with plasminogen were 34 and 60 h, respectively, in the absence and in the presence of  $2 \times 10^{-6}$  M leupeptin. For PMA-treated cultures without plasminogen, the half-lives were 42 and 58 h, respectively, in absence and presence of leupeptin.

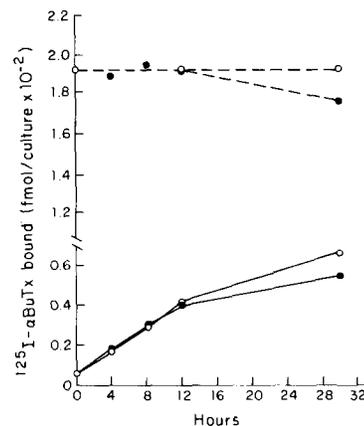


FIGURE 5 Effect of leupeptin on surface levels of AChR and on the rate of synthesis of surface receptors. Cultures were grown with plasminogen containing horse serum and embryo extract under standard conditions. Surface AChR levels and the rate of synthesis of surface AChR were determined in cultures maintained in the absence and presence of leupeptin ( $2 \times 10^{-4}$  M). In experiments concerned with rates of AChR synthesis, leupeptin was added immediately after the removal of nonradioactive  $\alpha$ -BuTx (time 0). (●—●, O—O) Surface levels of AChR in control and in leupeptin-treated cultures, respectively. (●—●, O—O) Synthesis of surface AChR in control and in leupeptin-treated cultures, respectively.

TABLE IV  
Effect of Plasminogen and Rabbit Anti-*Torpedo*-AChR Serum On Half-life of AChR in Muscle Cultures

Medium supplement	Half-life (h)	
	Plasminogen	
	Absent	Present
None	42 (1.00)	36 (0.86)
Control rabbit serum	43 (1.02)	37 (0.88)
Rabbit anti- <i>Torpedo</i> -AChR serum	21 (0.50)	19 (0.45)

Plasminogen-depleted cultures were prepared and maintained as described in Materials and Methods. AChR half-life was measured by the standard procedure using cultures maintained with or without chicken plasminogen (42  $\mu$ g/ml), rabbit anti-AChR serum, and/or control rabbit serum (70  $\mu$ l/ml each). The indicated supplements were first added immediately after labeling surface AChR with  $^{125}$ I- $\alpha$ -BuTx, and then maintained throughout the experiment. Surface AChR was in the range of 201–257 fmol/culture. The values in parentheses represent the ratio of AChR half-life under the particular conditions to that of the control culture, in the absence of plasminogen or rabbit serum.

required proteolysis since it was blocked by the potent trypsin inhibitor Trasylol; third, the acceleration of AChR degradation by sarcoma virus transformation or by PMA was almost completely plasminogen dependent. Since both of these agents also markedly enhance PA production (15), it seems safe to conclude that the plasmin thus generated was responsible for the increased rate of receptor turnover.

How might proteolysis by plasmin influence receptor degradation? Although the available evidence is limited, several facts imply that this perturbation of AChR turnover is probably a secondary consequence of altered membrane metabolism resulting from proteolysis of surface components other than the receptor itself. Thus, plasmin did not reduce the number of externally accessible, toxin-binding sites for at least 5 h after addition to myogenic cultures (Fig. 2), the period during which plasmin action should be most pronounced. Further, the reduced level of surface AChR that was ultimately reached developed slowly; it coincided with and reflected the accelerated first-order receptor degradation, and therefore did not

follow the kinetics expected of direct enzymatic inactivation. These considerations suggest that plasmin did not attack free AChR. Likewise, judging both from the rate of toxin dissociation and the spectrum of  $^{125}\text{I}$ -toxin degradation products, there is no evidence that plasmin could attack either the receptor or toxin components of surface complexes. We conclude tentatively that plasmin probably affects the receptor indirectly, by modulating some aspect of membrane metabolism which influences the rate of AChR degradation. It is also clear that AChR degradation can be modulated by a variety of means, including antisera containing antibodies directed against receptor and probably other membrane components, and by cross-linking agents (6, 7); at least some of these effects are not mediated by membrane proteolysis. Thus, although plasminogen activation is not an obligatory factor in all pathways of AChR degradation, receptor half-life is appreciably shortened when this enzymatic system is mobilized by myogenic cells.

Our data, taken together with those of Devreotes and Fambrough (4) and Libby et al. (11), provide a framework for visualizing the metabolic cycle of AChR and identifying likely sites of regulatory modulation. Because receptor degradation follows overall first-order kinetics, each step in the pathway must also obey the first-order rate law. With this in mind, a reasonable minimal sequence would be internalization of surface receptor by formation of endocytic vesicles, followed by vesicle fusion with lysosomes wherein receptor proteolysis is thought to occur; each of these stages would conform to a first-order process. The first could be modulated by events occurring at the cell surface, and we propose that plasmin accelerates internalization by proteolysis of some as yet unidentified plasma membrane constituent(s). This plasmin-catalyzed reaction provides an experimentally accessible starting point for attempting to dissect some of the initial stages in receptor turnover. The second step in AChR degradation, vesicle fusion and intralysosomal digestion, is apparently inhibited by several protease inhibitors including pepstatin, leupeptin, and trypan blue, and by other inhibitors of lysosomal functions such as chloroquine (4, 11). Two of these, leupeptin and trypan blue, also inhibit plasmin and PA (2, 22), and thus would slow the reaction rate at both ends of the degradative pathway.

It is probably significant that neither the acceleration of receptor degradation by proteolysis (plasmin alone, or PMA in presence of plasminogen) nor its inhibition by leupeptin or chloroquine (11) produced any change in the rate of AChR synthesis. Hence, perturbations of the degradative limb of receptor metabolism, at least by way of the particular agents tested so far, are apparently not reflected in changes of receptor synthesis; and the latter process is, therefore, not coupled to degradation and must be subject to independent regulation. Moreover, proteolysis by plasmin and by lysosomal enzymes are both nonspecific processes, and specific regulation is likely to occur predominantly at some stage in the biosynthetic portion of receptor metabolism. Thus the compensating increases observed to accompany accelerated AChR degradation at early culture age, or stimulated by embryo extract and FBS (14) appear to depend on additional factors that modulate the rate of receptor synthesis more directly. Likewise, protease-catalyzed increase in AChR degradation cannot by itself account for all of the PMA-stimulated reduction in surface receptor: the full magnitude of this change is the consequence of two additive but independent processes, both of which are due to PMA. One of these is the inhibition of AChR biosynthesis (14) and the second is accelerated degradation of surface AChR secondary to proteolysis initiated by PA induction (15).

We have previously shown that sarcoma virus transformation and tumor promoters shorten AChR half-life in myogenic cultures and the present results show that this is achieved by production of PA, leading to the formation of plasmin. This effect may be of general significance, since oncogenic viruses and tumor promoters have been reported to reduce the levels of receptors for epidermal growth factor (EGF) (10),  $\beta$ -adrenergic compounds (17), and insulin (19), and to increase the turn over of fibronectin (16). Some or all of these phenomena, and the recent finding that down-regulation of EGF receptors is serum dependent (21), could be either primary or secondary consequences of elevations in activity of the PA system. Indeed, since induction of PA synthesis by EGF (9), and down-regulation (21) take place under similar conditions in the same cells, the decreased cellular sensitivity to EGF may be an example of a negative feedback response by mediated proteolytic reduction of receptors. All of these observations suggest that proteolysis generated by high levels of PA production may influence the metabolism and concentration of surface receptors and, thereby, the hormone responsiveness of neoplastic cells, a possibility that deserves further study.

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#### REFERENCES

- Appel, S. H., R. Anwyl, M. W. McAdams, and S. Elias. 1977. Accelerated degradation of acetylcholine receptor from cultured rat myotubes with myasthenia gravis sera and globulins. *Proc. Natl. Acad. Sci. U. S. A.* 74:2130-2134.
- Dang, K., and E. Reich. 1979. Plasminogen activator from cells transformed by an oncogenic virus. Inhibition of the activation reaction. *Biochim. Biophys. Acta.* 566:138-151.
- Deutsch, D. G., and E. T. Mertz. 1970. Plasminogen purification from human plasma by affinity chromatography. *Science (Wash. D. C.)* 170:1095-1096.
- Devreotes, P. N., and D. M. Fambrough. 1975. Acetylcholine receptor turnover in membrane of developing muscle fiber. *J. Cell. Biol.* 65:335-358.
- Drachman, D. B., C. W. Angus, R. N. Adams, J. P. Michelson, and G. J. Hoffman. 1978. Myasthenic antibodies cross-link acetylcholine receptors to accelerate degradation. *New Engl. J. Med.* 298:1116-1122.
- Heinemann, S., S. Bevan, R. Kullberg, J. Lindstrom, and J. Rice. 1977. Modulation of acetylcholine receptor by antibody against the receptor. *Proc. Natl. Acad. Sci. U. S. A.* 74:3090-3094.
- Kao, I., and D. B. Drachman. 1977. Myasthenic immunoglobulin accelerates acetylcholine receptor degradation. *Science (Wash. D. C.)* 196:527-529.
- Kirschke, H., F. Langner, B. Wiederanders, S. Ansoorge, and P. Bohley. 1977. Cathepsin L: a new proteinase from rat-liver lysosomes. *Eur. J. Biochem.* 74:293-301.
- Lee, L.-S., and I. B. Weinstein. 1978. Epidermal growth factor, like phorbol ester, induces plasminogen activator in HeLa cells. *Nature (Lond.)* 274:696-697.
- Lee, L.-S., and I. B. Weinstein. 1978. Tumor promoting phorbol ester inhibits binding of epidermal growth factor to cellular receptors. *Science (Wash. D. C.)* 202:313-315.
- Libby, P., S. Bursztain, and A. L. Goldberg. 1980. Degradation of the acetylcholine receptor in cultured muscle cells: selective inhibitors and the fate of undegraded receptors. *Cell.* 19:481-491.
- Libby, P., and A. L. Goldberg. 1978. Leupeptin, a protease inhibitor, decreases protein degradation in normal and diseased muscles. *Science (Wash. D. C.)* 199:534-536.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Miskin, R., T. G. Easton, A. Maelicke, and E. Reich. 1978. Metabolism of acetylcholine receptor in chick embryo muscle cells: effects of RSV and PMA. *Cell.* 15:1287-1300.
- Miskin, R., T. G. Easton, and E. Reich. 1978. Plasminogen activator in chick embryo muscle cells: induction of enzyme by RSV, PMA and retinoic acid. *Cell.* 15:1301-1312.
- Olden, K., and K. M. Yamada. 1977. Mechanism of the decrease in the major cell surface protein of chick embryo fibroblasts after transformation. *Cell.* 11:957-969.
- Sheppard, J. R. 1977. Catecholamine receptor differences identified on 3T3 and similar virus-transformed 3T3 cells. *Proc. Natl. Acad. Sci. U. S. A.* 74:1091-1094.
- Strickland, S., E. Reich, and M. I. Sherman. 1976. Plasminogen activator in early embryogenesis: enzyme production by trophoblast and parietal endoderm. *Cell.* 9:231-240.
- Thomopoulos, P., J. Roth, E. Lovelace, and I. Pastan. 1976. Insulin receptors in normal and transformed fibroblasts: relationship to growth and transformation. *Cell.* 8:417-423.
- Walther, P. J., H. M. Steinman, R. L. Hill, and P. A. McKee. 1974. Activation of human plasminogen by urokinase. *J. Biol. Chem.* 249:1173-1181.
- Wolfe, A., R. Wu, and G. H. Sato. 1980. Epidermal growth factor-induced down-regulation of receptor does not occur in HeLa cells grown in defined medium. *Proc. Natl. Acad. Sci. U. S. A.* 77:2735-2739.
- Zimmerman, M., J. P. Quigley, B. Ashe, R. Goldfarb, and W. Troll. 1978. Direct fluorescent assay of urokinase and plasminogen activators of normal and malignant cells: kinetics and inhibitor profiles. *Proc. Natl. Acad. Sci. U. S. A.* 75:750-753.