Stabilization of Acetylcholine Receptors by Exogenous ATP and Its Reversal by cAMP and Calcium

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Abstract. Innervation of the neuromuscular junction (nmj) affects the stability of acetylcholine receptors (AChRs). A neural factor that could affect AChR stabilization was studied using cultured muscle cells since they express two distinct populations of AChRs similar to those seen at the nmjs of denervated muscle. These two AChR populations are (in a ratio of 9 to 1) a rapidly degrading population (Rr) with a degradation half-life of $\sim$1 d and a slowly degrading population (Rs) that can alternate between an accelerated form (half-life $\sim$3–5 d) and a stabilized form (half-life $\sim$10 d), depending upon the state of innervation of the muscle.

Previous studies have shown that elevation of intracellular cAMP can stabilize the Rs, but not the Rr. We report here that in cultured rat muscle cells, exogenous ATP stabilized the degradation half-life of Rr and possibly also the Rs. Furthermore, pretreatment with ATP caused more stable AChRs to be inserted into the muscle membrane. Thus, in the presence of ATP, the degradation rates of the Rr and Rs overlap. This suggests that ATP released from the nerve may play an important role in the regulation of AChR degradation. Treatment with either the cAMP analogue dibutyryl-cAMP (dB-cAMP) or the calcium mobilizer ryanodine caused the ATP-stabilized Rr to accelerate back to a half-life of 1 d. Thus, at least three signaling systems (intracellular cAMP, Ca^{2+}, and extracellular ATP) have the potential to interact with each other in the building of an adult neuromuscular junction.

T HE metabolic stability of the acetylcholine receptor (AChR)[1] of skeletal muscle is regulated developmentally and is dependent on innervation (for reviews see Schuetze and Role 1989; Salpeter et al., 1993). However, the nature of the neural regulation in building an adult neuromuscular junction (nmj) having stable AChRs is not yet fully understood. One major source of confusion is the fact that during critical periods of development or during the denervation/reinnervation cycle, two distinct AChR populations with very different inherent degradation rates and different responses to stabilizing influences can coexist, interspersed at the neuromuscular junction (Shyng and Salpeter, 1989, 1990). These two distinct receptor entities are (*a*) a rapidly degrading AChR (Rr) (degradation half-life $\sim$1 d) that is synthesized and expressed predominantly in embryonic and denervated muscle and (*b*) a more slowly degrading AChR (Rs) that can exist in either a stabilized (degradation half-life $\sim$10 d) or accelerated form (degradation half-life $\sim$3–4 d) and is synthesized and expressed predominantly in adult innervated muscle. The exact half-lives vary between muscles and between animal species (Bevan and Steinbach, 1983; Andreose et al., 1993), but the relative effect of different stabilizing and destabilizing conditions is remarkably consistent. To date, much more information is available on the regulation of Rs than of Rr degradation. A well-established instance of Rs regulation is its stabilization by either innervation or by intracellular cAMP to the adult degradation rate of $\sim$10 d (Salpeter et al., 1986; Shyng et al., 1991; O'Malley et al., 1993; Andreose et al., 1993). Under these conditions, neither stabilization of Rr to adult degradation values nor interconversion between the Rr and Rs AChRs has been observed.

The present paper deals with new observations on the stabilization of AChRs. We find that under the influence of ATP, Rr can be stabilized to overlap the Rs degradation rate. Thus, under certain circumstances, perhaps during development or reinnervation of denervated muscle, ATP released from the nerve could stabilize the embryonic Rr to bridge the time when the Rr are replaced by the adult Rs. Evidence is also given that the action of ATP may be mediated by a P2 purinergic receptor. However, the

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1. *Abbreviations used in this paper*: α-BTX, α-bungarotoxin; AChR, acetylcholine receptor; dB-cAMP, dibutyryl cAMP; nmj, neuromuscular junction; PLA$_2$, phospholipase A$_2$; Rr, rapidly degrading acetylcholine receptors; Rs, slowly degrading acetylcholine receptors.

unique and independent nature of Rr and Rs metabolic stability is still maintained since, unlike the cAMP stabilized Rs, purine-stabilized Rr are accelerated back to a faster decay rate by dibutyryl-cAMP (dB-cAMP) as well as by the Ca^{2+} activator ryanodine.

These data, plus earlier experimental evidence (for review see Salpeter et al., 1993), have led us to the working hypothesis that the formation of an adult neuromuscular junction containing stable AChRs may involve two processes. One is a replacement of Rr by Rs, which requires down regulation of Rr (Andreose et al., 1993) and/or up regulation of Rs. The second involves posttranslational modifications of AChRs either during or after insertion into the sarcolemma. The data presented here indicate that ATP may mediate one such modification, and, by its ability to stabilize Rr, ATP may play an important role at a time when the Rr predominates and the Rr to Rs content of the muscle is changing. In addition, our data emphasize the potential interactions between different signaling pathways involved in the production of a stable AChR population at the innervated nmj.

Materials and Methods

Muscle Cell Culture

Collagenase-dissociated rat muscle cells were plated in 1.5 ml medium consisting of 20% FBS and 80% culture medium (10% horse serum, 90% Dulbecco's minimum essential medium) and at 3×10^5 cells/ml onto 35-mm tissue culture dishes (Corning Glass Works, Corning, NY) coated with a thin layer of 0.05% Matrigel (Collaborative Biomedical Products, Waldham, MA) using a previously published method (O'Malley et al., 1993, 1996). The day of seeding was designated day 1 of culture. On day 2 of culture, the medium was changed to culture medium alone. Drug treatment was initiated on day 4 of culture, after which the culture medium was changed at daily intervals.

Labeling of AChRs with [125I]α-bungarotoxin and Estimation of AChR Degradation

For experiments in which the effects of drugs were measured on AChRs already in the membrane, the AChRs were labeled with [125I]α-bungarotoxin ([125I]α-BTX) on culture day 4 as described earlier (O'Malley et al., 1993). In experiments where treatment with drugs preceded [125I]α-BTX labeling, the AChRs were labeled on culture day 7.

The medium was changed at daily intervals for 14 d after labeling, and its radioactivity was measured. After the medium was removed for the last time, the remaining [125I]α-BTX bound to the tissue was recovered by wiping the culture dish twice with cotton swabs. The loss of AChR during the sampling period was calculated by summing all the radioactivity released into the media plus that remaining on the dish at the end of the sampling period. Calculated residual radioactivity per sample day was plotted on a semi-logarithmic scale as described previously (Burden, 1977; Bevan and Steinbach, 1983; Shyng et al., 1991; O'Malley et al., 1993). In a variant of the procedure used previously (Shyng and Salpeter 1989; O'Malley et al., 1993), the degradation rate(s) and relative proportions of the fast and slow components were determined by fitting the logarithmic decay of labeled receptors to a double exponential using a computer program based on the Levenberg-Marquart method (Press et al., 1992). The use of a double exponential fit to describe the degradative behavior of acetylcholine receptors in muscle relies on the assumption that the two components, Rr and Rs, are distinct and that their half-lives remain invariant throughout the observation period. However, in the present study these assumptions do not always hold, since some Rr were stabilized to values that overlap those of the Rs (see Results). The double exponential fitting was nevertheless used for the purpose of providing a description of the different degradation rates of the AChR populations. However, these curves cannot necessarily make any definitive claims regarding the relative content of Rr and Rs in the slow component.

Drug Treatment of Cell Cultures

Myotubes were treated on alternating on and off cycles (2 h each) with 50 mM ATP (Sigma Chemical Co., St. Louis, MO), supplied by a syringe pump (Sage model 362; VWR Scientific Products, Bridgeport, NJ) at a rate of 5 μl/h (250 nmol/h) into the center of a 35-mm dish containing 1 ml of culture medium to give a potential maximal dose of 500 μM per cycle. It should be noted that when 1 mM ATP is added to cultures, the media become slightly acidic, sufficiently so to cause a change in the phenol red indicator that could be seen by eye. This effect is temporary (presumably due to enzymatic degradation of the ATP) and is gone within a few hours of application. No such pH shift was ever seen with syringe application, indicating that ATP did not accumulate and presumably was degraded by the end of each cycle. Treatment with ATP was initiated on culture day 4 either immediately after, or 3 d before, labeling of AChR with [125I]α-BTX and was maintained throughout the sampling period. In some experiments, dB-cAMP (Sigma Chemical Co.) or ryanodine (Calbiochem, La Jolla, CA) at final concentrations of 2 mM and 1 μM, respectively, was added to the culture medium 3 d after the labeling of AChRs and the initiation of syringe application of ATP. Both drugs were then maintained throughout the experiment. Labeling the AChRs on culture day 4 before the addition of any drugs allowed the observation of the effects of the drug treatment on preexisting AChRs.

In other experiments, myotubes were treated on culture day 4 immediately after [125I]α-BTX labeling with 50 U/ml of phospholipase A_2 (Sigma Chemical Co.), 5 μg/ml melittin (Sigma Chemical Co.), 2 mM dB-cAMP, or 1 μM ryanodine. In all experiments, control myotubes were labeled at the same time as those treated with drugs.

35S Incorporation and Measurement of Overall Protein Loss

Myotubes were treated on culture day 3 with 10 μl of a 11 mCi/ml 35S-protein labeling mix (EXPRE35S35S, New England Nuclear, Boston, MA) in 1 ml of culture medium and cultured for a further 24 h. On culture day 4, they were washed three times with 1 ml Earle's balanced salt solution, after which they were cultured in 1 ml of culture medium in the presence of the test drug at the concentration indicated in the results. The medium was changed at daily intervals for 14 d and, after the addition of an equal volume of scintillant (Liquiscint; National Diagnostics, Inc., Manville, NJ), counted on a beta spectrometer (model LS230; Beckman Instruments, Fullerton, CA). On the last day of sampling, the medium was removed for counting, after which the cells were lifted in 1 ml of culture medium using a rubber policeman before counting. Protein degradation was then determined in the same manner as that for AChR degradation described above.

Results

We were concerned that the expression of adenosine deaminase by cultured myotubes could minimize the effect of bath application of purines requiring high doses of these drugs. To circumvent this problem, ATP was applied via a syringe pump in a cyclical manner (2 h continuous release followed by 2 h with no release) to allow for enzymatic removal of the drug between cycles.

Fig. 1 compares the AChR degradation curves of control and ATP-treated muscle cultures. The data is analyzed by fitting a two-component exponential curve to the experimental data as given in Materials and Methods. AChRs from untreated control myotubes, which were labeled with [125I]α-BTX on culture day 4, behaved as previously reported (O'Malley et al., 1993), but with a slightly slower slow component. They degraded in a biphasic manner with the majority (90%) of the AChRs decaying with half-lives of 1 d (representing the Rr AChR) and the remaining (10%) with a half-life of 4.2 d (representing the Rs AChRs). When muscle cells were treated with ATP after the AChRs were labeled with [125I]α-BTX, a biphasic degradation of AChRs was again obtained. However, in

the ATP-treated cultures, the relative content of the slow component was almost doubled, and the half-life of the slow component stabilized to 11 d (Fig. 1). Preliminary experiments showed a stabilization of AChRs after treating the cells with adenosine but not with the adenosine degradation product inosine (data not shown). Since the AChRs were labeled with $[^{125}I]\alpha$-BTX before treatment with ATP, the increased slow component could not reflect newly inserted AChRs and must indicate a stabilization of the preexisting Rr. As indicated in Materials and Methods, the use of the two-component exponential decay curve allows the determination of the relative content and the degradation rates of two independent populations of AChRs.
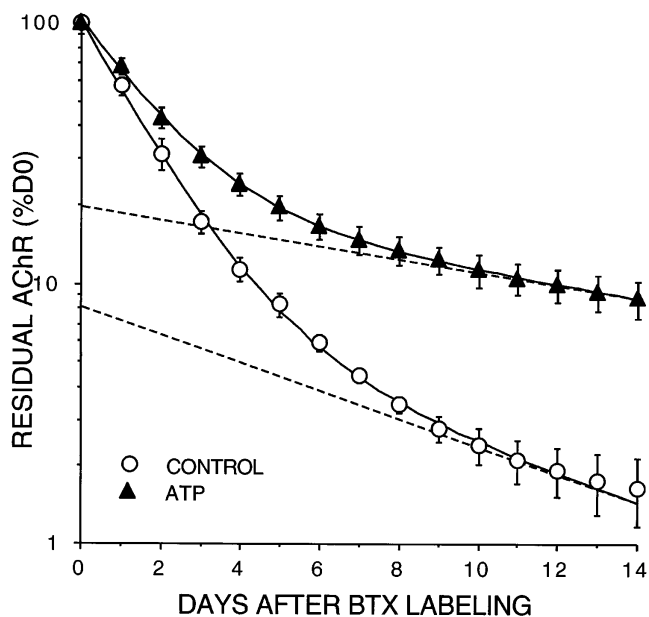

Figure 1. ATP stabilizes preexisting AChRs. Acetylcholine receptors were labeled with $[^{125}I]\alpha$-BTX before muscle cells were treated cyclically with a potential maximal dose of 500 μM ATP per cycle. The degradation curves of the AChRs is the best fit of two decay rates. When the experimental data extends far enough so as not to include significant input from the fast component, the degradation curves consist almost exclusively of the slow component. The slopes of the two components on a semi-logarithmic plot give their half-lives. Provided the assumptions underlying the use of two-component exponential as described in Materials and Methods is fulfilled, the intercept of the slow component with the y-axis can then be used to estimate the fraction of the total receptor population that constitutes the slow component at day 0. The 9% of total AChRs constituting the slow component of controls (○) decayed with a half-life of 4.2 d, while the slow component of the ATP-treated cells (▲) (20% of the total) decayed with a half-life of 11.1 d. The double exponential curve fitted to the ATP data (*solid line*) assumes that the Rs was also stabilized by ATP. To test whether it is possible to determine if the Rs were not stabilized, a single exponential decay of 4.2 d constituting 9% of the total AChRs (the half-life of the Rs in the controls) was subtracted from the ATP data, and a double exponential was fitted to the remaining values. Half-lives of 1.2 (80% of total) and 14.0 d (11% of total) were obtained. Addition of the three components gave a triple exponential fit that was identical in shape to the double exponential fit shown. Thus, it is not possible with this data to state whether the Rs was also stabilized. Values are mean ± SEM of seven experiments.

However, in the case where there is stabilization of the degradation of the fast component, the two-component curve contains some uncertainties in interpretation. There could be a very early stabilization of a small fraction of the fast component, which would be most analogous to the two population case. Alternatively, there could be a delayed stabilization of all the remaining fast component or a gradual stabilization throughout the assay period.

In the last two cases, the assumption that the Rr half-life is the same throughout the degradation period no longer holds, and the disclaimer regarding the ability of obtaining an accurate assessment of the relative content of stabilized Rr would apply. Since we do not know which case is true in the present study, we can only claim unequivocally that because of the increase in the slow component when AChRs were prelabeled with $[^{125}I]\alpha$-BTX, a stabilization of some Rr must have occurred.

The question of whether the Rs are also stabilized by ATP arises. With the analysis used in this study, it is not possible to answer this question since the total amount of Rs is normally small, and when interspersed with the stabilized Rr, a difference between a half-life of 3 and 10 d is difficult to resolve. Explicitly, we cannot distinguish between a three-component curve in the ratio of half-lives of 1:3:10 d and a two-component curve.

To test if the action of the purines was mediated by a P2 class purinergic receptor, as has been previously reported for other effects of ATP on muscle (Haggblad & Heilbronn, 1988; Fu and Poo, 1991), we mimicked P2 receptor activity by treating myotubes with either phospholipase A_2 (PLA$_2$) or a PLA$_2$ activator melittin. AChRs from myotubes that were treated with PLA$_2$ after $[^{125}I]\alpha$-BTX labeling decayed with a double exponential of 1 and 8 d with the slow component constituting 33% of the AChR population (Fig. 2). A similar result was obtained from cells treated with 5 μg/ml melittin, in which the slow component with a half-life of 10 d constituted 58% of the receptor population (Fig. 2). Indeed, of all the putative second messengers associated with purinergic signal transduction that were tested (PLA2 [Fig. 2], calcium and cAMP [Fig. 6], and cGMP [data not shown]), only activators of the PLA$_2$ pathway mimicked the effect of ATP on AChR stabilization.

To determine whether agents that stabilized AChRs had a general effect on total protein degradation, loss of ^{35}S-labeled protein was measured in the presence of dB-cAMP, PLA$_2$, melittin, and ATP at the concentrations used in this study. No significant difference was observed with any of these treatments relative to controls (Fig. 3). Thus, the stabilizing effect of ATP, PLA$_2$, melittin, and dB-cAMP appear to be specific to the AChR.

If muscle cells were pretreated with ATP from day 4 to 7, and the AChRs were labeled with $[^{125}I]\alpha$-BTX on culture day 7, the proportion of slowly degrading receptors was 34% of the total AChR population with a half-life of 12 d. To eliminate any influence of culture age on AChR degradation, a control group was labeled on culture day 7 without ATP treatment. The proportion of slowly degrading AChRs remained similar to that in control cells labeled on culture day 4 (Fig. 4). These data indicate that AChRs inserted into the muscle membrane in the presence of ATP are more likely to be stable.

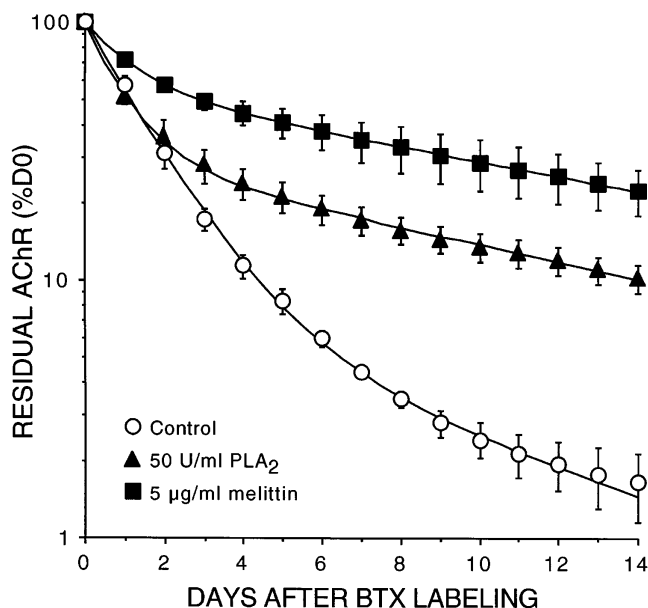We found, however, that the purine-induced stabilization

Figure 2. Melittin or PLA$_2$ stabilize preexisting AChRs. Acetylcholine receptors were labeled with [^{125}I]α-BTX before muscle cells were treated with 5 mg/ml melittin or 50 U of PLA$_2$. The degradation curves of the AChRs is the best fit of two decay rates. The slow component of controls ($\bigcirc$) (13% of total) decayed with a half-life of 4.0 d, while the slow component of melittin- ($\blacksquare$) (58% of total) or PLA$_2$- ($\blacktriangle$) (32.8% of total) treated cells decayed with a half-life of 9.73 and 7.6 d, respectively. Values are mean $\pm$ SEM from four experiments.

Figure 3. Treatments that slow AChR degradation do not affect total protein turnover. Myotubes were treated for 24 h with ^{35}S-protein labeling mix, after which they were washed and cultured in the presence of 2 mM dB-cAMP ($\blacksquare$), 50 U PLA$_2$ ($\bullet$), 5 mg/ml melittin ($\triangle$), or 500 nmol/cycle ATP ($\blacktriangle$). Parallel control ($\bigcirc$) cultures were labeled with ^{35}S but not treated with drugs. Medium was changed at daily intervals, and after counting, the radioactivity degradation profiles were constructed as described in Materials and Methods. Values are mean $\pm$ SEM from three experiments.

appears different from that reported for the stabilization of Rs (to a half-life of ~10 d) by cAMP analogues (Shyng et al., 1991; O'Malley et al., 1993). If the ATP-stabilized AChRs were treated with 2 mM dB-cAMP in the continued presence of ATP, the stabilization was reversed, and the profile of the curve inclined towards that of control cultures (Fig. 5 *a*). A similar result was seen with 1 μM ryanodine (Fig. 5 *b*). Lower concentrations of the cAMP analogue or the calcium mobilizer (1 mM or 100 nM, respectively) were not able to reverse the ATP-induced stabilization of these AChRs. However, in preliminary experiments, such lower concentrations cAMP or ryanodine did reverse AChR stabilization by bath-applied purines (data not shown). Thus dB-cAMP and ryanodine reversal of purine stabilization appears to be critically dependent on either the concentration or the periodicity of the purine signal.

The acceleration of ATP-stabilized AChRs by both dB-cAMP and ryanodine is particularly interesting since both compounds behave very differently when applied to control cells. We previously reported that treatment of muscle cells in culture with 1 mM dB-cAMP results in the stabilization of preexisting Rs to a half-life of ~10 d (O'Malley et al., 1993) without altering the degradation rate of the Rr. We obtained a similar result in the present study (Fig. 6). In addition, when control AChRs are treated with ryanodine, little effect on AChR degradation is seen, although a slight acceleration of the AChR degradation may occur. (Preliminary results show that ryanodine causes a significant increase in cytoplasmic calcium levels within 24 h of application [O'Malley, J.P., W.R. Zipfell, and M.M. Salpeter, manuscript in preparation].)
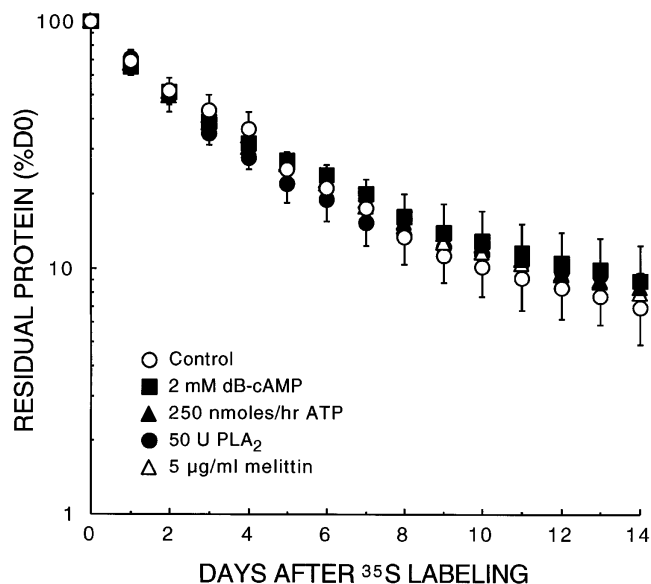
Discussion

Extensive literature has established that the Rr and Rs AChRs are distinct molecules that do not interconvert (for review see Salpeter et al., 1993). The data from the present study confirm this conclusion and add a complexity that helps clarify existing information on the construction of an adult nmj with stable AChRs. The information from earlier studies showed that both Rs and Rr can coexist at the nmj (as, for instance, after denervation; Shyng and Salpeter, 1989, 1990) and in tissue culture cells (O'Malley et al., 1993) while degrading simultaneously, each at its own rate. It was also shown that in both denervated junctions and tissue culture cells, stabilization of Rs can be accomplished by elevating cAMP (O'Malley et al., 1993; Xu and Salpeter, 1995) without affecting the degradation rate of naive Rr. Thus, the mechanisms of stabilization are clearly distinct for Rs and Rr.

In the present paper, we report the novel observation that in tissue cultured muscle cells, Rr AChRs can be stabilized by exogenously applied purines and that this stabilization is reversed by the cAMP analogue dB-cAMP and by the calcium activator ryanodine. Previous reports that Rr can be stabilized by electrical stimulation (Rotzler and Brenner, 1990) have been disputed (Andreose et al., 1993), and the report that calcium can stabilize AChRs (Rotzler et al., 1991) is not confirmed by our own observations (O'Malley, J.P., W.R. Zipfell, and M.M. Salpeter, manuscript in preparation). However there is additional new evidence that Rr stabilization may be present in vivo during reinnervation (Salpeter, M.M., and M. Szabo, manuscript
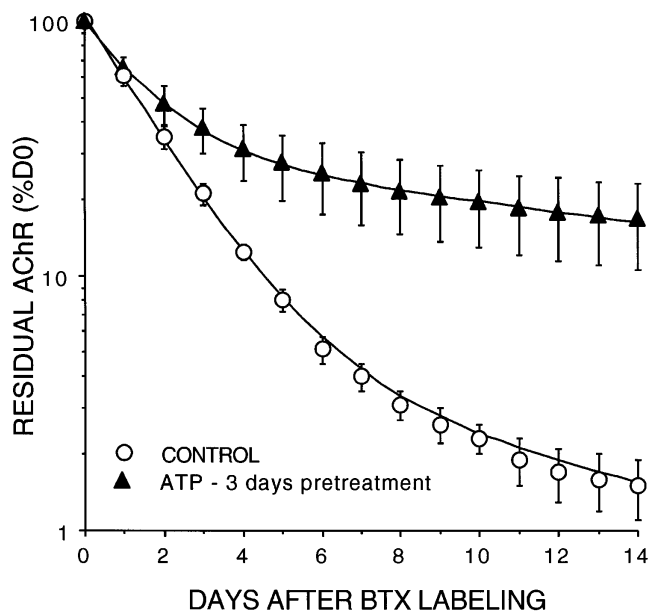
Figure 4. Chronic pretreatment with ATP increases the proportion of slowly degrading AChRs. Treatment with ATP was initiated on culture day 4, and AChRs were labeled with [^{125}I]α-BTX 3 d later. The degradation curves of both control and ATP-treated cultures were fitted with a double exponential decay. For both the controls (○) and ATP- (▲) treated cells, the solid line is the sum of the two exponentials. In addition to slowing the rate of degradation of the slow component, ATP treatment caused an increase in the proportion of slowly degrading AChRs from 9% in controls to 33% in ATP-treated cells. Values are mean ± SEM from five experiments.

in preparation). Purines may also stabilize the Rs, but such an effect is difficult to determine in our present study, given the small relative content of Rs in cultured muscle cells (Figs. 1 and 4) and the difficulty, when the t1/2 values are similar, in distinguishing a three-component (if the Rs was not stabilized) from a two-component (if the Rs was stabilized to the same half-life as the stable Rr) curve. However, a residual slow component is seen in cultures where ATP stabilization of Rr is reversed by cAMP or calcium. Such a residual slow component suggests that an ATP stabilization of Rs may have been present and was not reversed by the dB-cAMP or ryanodine at the concentrations used (Fig. 5). Thus, under special circumstances the degradation rates and stabilization mechanisms of Rr and Rs could overlap.

Because of when they are expressed in vivo, the Rs is the adult and thus an ε-containing AChR, while the Rr is the embryonic and thus a γ-containing AChR (Mishina et al., 1986; Gu and Hall, 1988; Brenner et al., 1990; Duclert and Changeux, 1995; Missias et al., 1996). However, contradictory data in nonmuscle systems expressing AChRs with different subunits (Gu et al., 1990; Jaywickreme and Claudio, 1994; Kopta and Steinbach, 1994; Liu et al., 1994) do not resolve the question of whether the different stabilization mechanisms of the Rs and Rr depend on the presence of the gamma versus epsilon subunits. However, a recent study (Sala, C., J.P. O'Malley, R. Xu, G. Fumagalli, and M.M. Salpeter, manuscript in preparation) shows that the ε AChR is of the slowly degrading Rs population.

The concentration of ATP applied in the syringe application was derived empirically as being the lowest concentration of ATP capable of slowing the degradation of a pre-existing AChR population. Thus, it represents a threshold dose. Higher concentrations of ATP did indeed induce greater stabilization effects (data not shown). However, because protein turnover is dependent upon the metabolic state of the cells and purines have been reported to affect the metabolic rate of muscle in vivo, we chose to use a dose that we determined to have no nonspecific effect on total protein turnover (Fig. 3).

To determine which purinergic receptor system may mediate the ATP effect on AChR stability, we tested second messengers reported to be elevated by purinergic receptor activation. Only factors that upregulated the activity of the PLA$_2$ pathway mimicked the effects of ATP on AChR stabilization (Fig. 2). Thus, it is not unreasonable to suggest that the effect of ATP on AChR stability is probably mediated through a P2 purinergic receptor via PLA$_2$. This conclusion is consistent with reports of the presence of P2 purinergic receptors in muscle cells (Haggblad and Heilbronn, 1988). In addition, ATP, in the range of 100 μm to 1 mM, potentiates spontaneous synaptic currents in *Xenopus* nerve–muscle cell cultures (Fu and Poo, 1991) via a G protein–linked production of arachidonic acid by PLA$_2$ (Harish and Poo, 1992). In the present study, PLA$_2$ was able to stabilize AChRs when applied exogenously. Thus, unless the exogenous PLA$_2$ added to myotubes was incorporated into their membrane, the site for PLA$_2$ cleavage must be extracellular. This may prove critical in determining the molecular basis of the purinergic stabilizing activity.

The ability of adenosine to stabilize AChRs with apparently equal efficiency to ATP is puzzling. Normally, purinergic receptors can be pharmacologically differentiated on the basis of preferred affinity for either AMP and adenosine (P1 class receptor) or ATP and ADP (P2 class receptor). However, at sufficiently high doses, adenosine may still activate a P2 class receptor (Gordon and Martin, 1983; Gordon, 1986).

The cultured muscle cells exhibited spontaneous contractile activity after culture day 5. Muscle contraction would be expected to be accompanied by transient elevation of intracellular calcium, which might then be expected to reverse the effect of ATP-induced AChR stabilization. However, ATP stabilization of AChRs is maintained throughout this period. This implies either that calcium released from intracellular sources does not affect the transduction of the ATP signal or that calcium is not elevated in spontaneous cultures to the same level as in ryanodine-treated cultures and is thus not sufficient to reverse the effect of ATP. It has been suggested that calcium entering the cell from extracellular sources plays an important role in suppressing AChR expression (Walke et al., 1994). Such a relationship between the site of calcium entry may also exist for AChR stability. However, ryanodine can also suppress AChR expression (Rubin, 1985). Also, our unpublished data seems to be able to mimic the effects of extracellular Ca^{2+} influx by simply elevating intracellular calcium to a level that is effective, regardless of its site of release or entry.

The results in this report demonstrate that the action of calcium and cAMP on AChR stability are dependent upon the condition of the myotube before the initiation of these
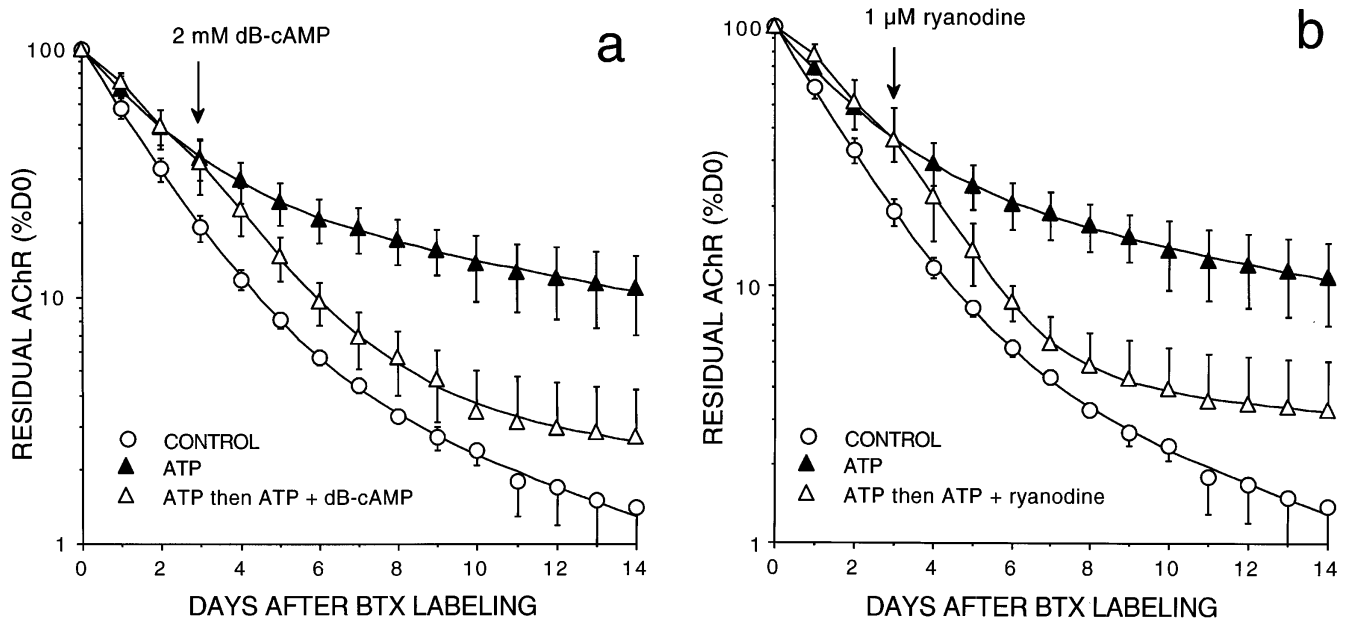
Figure 5. ATP-induced stabilization of Rr is reversed by both dB-cAMP (*a*) and ryanodine (*b*). AChRs were labeled with $[^{125}I]\alpha$-BTX, and ATP treatment (▲) was initiated on culture day 4. 3 d later, some ATP-treated plates were treated with 2 mM dB-cAMP or 1 μM ryanodine in combination with the ATP (△). Some untreated control cultures (○) were also labeled on culture day 4. The degradation curves of control and ATP-treated cells gave double exponential fits as also seen in Fig. 1. When ATP is followed by treatment with dB-cAMP (*a*) or ryanodine (*b*), a distinct acceleration of the Rr AChR towards the control curve is seen. From sample day 8, these curves begin again to diverge away from control curves, suggesting that the Rs may not have been accelerated by these treatments. Values are the mean ± SEM of four experiments.

signals. In naive muscle cells calcium mobilization with ryanodine has little effect on AChR degradation, while cAMP analogues induce a stabilizing influence only on the Rs AChRs. However, elevation of calcium or cAMP results in a destabilization of an ATP-stabilized AChR population. These observations illustrate that, unless the second messenger state of the muscle is known, it is difficult to predict the action of any agent on the degradation behavior of AChRs. It may be that the complicated interaction between the three signaling pathways (Ca^{2+}, cAMP, and ATP) accounts for the overall responses of AChRs to signals at different stages of development and innervation.

The relationship between Rr AChR stabilization by ATP and the action of Ca^{2+} provides an interesting complexity. The preliminary observation that more calcium mobilizer is required to reverse the effect of syringe pump than bath-applied ATP suggests that the calcium and ATP signaling pathways may compete with each other. A time when such a competition between ATP and Ca^{2+} on AChR stability may be important is during the period of synapse elimination, when embryonic AChRs are still present. It has been reported that the loss of synaptic contact is preceded by a loss of AChRs in reinnervating (Rich and Lichtman, 1989) and developing (Balice-Gordon and Lichtman, 1993) and adult muscle (Balice-Gordon and Lichtman, 1994). Thus, it appears that the maintenance of an AChR population in the postsynaptic membrane is essential for a neuron to maintain contact. If, during the period of synapse elimination, the calcium signal associated with AChR activation is sufficiently large and diffuse, an active neuron may be able to contribute to the destabilization of the AChRs of an ad-

jacent neuron while restabilizing its own AChR population by the release of ATP. This model requires that the ATP stabilization effect is confined to the region immediately below the point of release and that the calcium destabilizing activity spread to adjacent sites, and that the pharmacological relationship between calcium and ATP be competitive. If true, activity-dependent (Hebbian) competition between neurons could result. Although highly hypothetical, such conjectures warrant further experiments.

The fact that Rr can be stabilized under the influence of ATP provides a possible resolution to a lingering controversy. It has been argued that AChR stability is independent of subunit composition since AChRs become stabilized soon after innervation, before the adult epsilon subunit becomes predominant (for review see Hall and Sanes, 1993). Our data provide a possible missing link in the events occurring during the development of a stable adult nmj. The ingrowing nerve, by releasing ATP, could stabilize the embryonic or Rr AChRs, thus providing a bridging degradation rate to the adult receptors. Recent data (Salpeter, M.M., and M. Szabo, manuscript in preparation) has strengthened this conjecture by showing that AChRs inserted during reinnervation of denervated muscle have a partially stabilized half-life of 3–4 d while most likely being γ-containing. Such a bridging degradation value could prevent a dip in AChR density that could occur if the rapidly degrading embryonic receptors (Rr) were directly replaced by slowly degrading adult receptors (Rs). One possible scenario for the events leading to the establishment of a stable neuromuscular junction without a major loss of AChR number during critical periods of
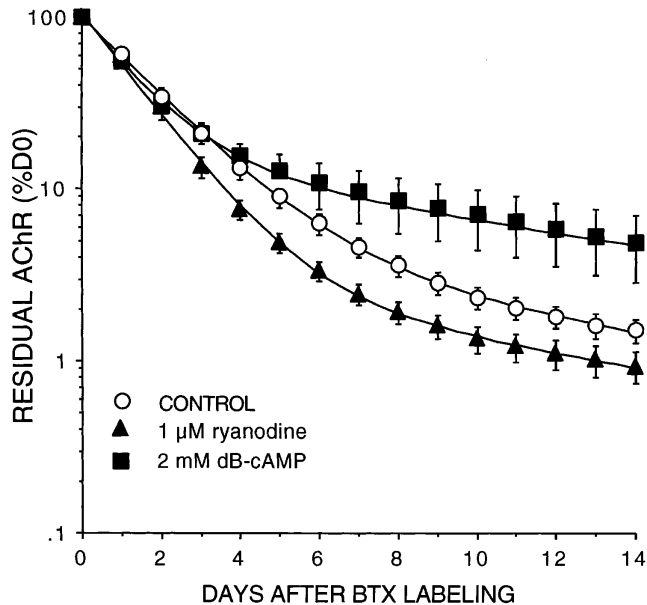
Figure 6. dB-cAMP stabilizes preexisting Rs AChRs while ryanodine does not. Acetylcholine receptors were labeled with [^{125}I]α-BTX before muscle cells were treated with 1 mM ryanodine or 2 mM dB-cAMP. The degradation curves of the AChRs are the best fit of two decay rates. Values are mean ± SEM from three experiments.

development or reinnervation could involve an early purinergic stabilization of Rr when AChRs are still γ-containing, followed by a down regulation of Rr synthesis induced by muscle activity (for review see Hall and Sanes, 1993). The Rr would then be replaced by stable Rs, presumably stabilized by cAMP or some other as yet unidentified neural factor.

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