IRREVERSIBLE DESENSITIZATION OF ATP RESPONSES IN DEVELOPING CHICK SKELETAL MUSCLE

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SUMMARY

1. In developing chick skeletal muscle, extracellular adenosine 5′-triphosphate (ATP) elicits an early excitatory conductance increase followed by a late potassium conductance increase. Both of these responses desensitize profoundly. Intracellular recordings and whole-cell voltage-clamp recordings were made in order to examine the time course and mechanism of desensitization and the recovery from desensitization.

2. Most of the loss of responsiveness to ATP occurred during the first minute of exposure to ATP. For the excitatory conductance, the loss of responsiveness to ATP resulted in part from long-lasting activation of the ATP-sensitive channels and in part from entrance into an inactive (non-conducting) state. In contrast, desensitization of the potassium conductance was entirely the result of a relatively fast transition to an inactive state.

3. Recovery from desensitization took many hours for both responses and was quite sensitive to temperature.

4. Recovery from desensitization for both responses was prevented by pre-incubation with the glycosylation inhibitor, tunicamycin. Several lines of evidence suggest that tunicamycin treatment blocked the delivery of new ATP receptors to the cell surface.

5. The recovery of the early response to ATP following exposure to two non-competitive inhibitors of the ATP response was also examined. These two compounds are thought to covalently modify the receptor. After exposure to either of these inhibitors, responsiveness to ATP returned over a time course that was similar to the time course of recovery from desensitization.

6. These results indicate that, following activation, ATP receptors do not become available for reactivation, and that recovery from desensitization is due to the insertion of newly synthesized receptors into the plasma membrane.

INTRODUCTION

A property inherent to many physiological systems is desensitization of a response in the continued presence of the stimulus. Desensitization is thought to serve as a

negative feedback mechanism for preventing overstimulation. The rate of desensitization and recovery from desensitization can give important insights into the mechanism and physiological role of these processes. The time course of desensitization and recovery can range from milliseconds to days depending on the particular mechanism. For example, in the continuous presence of acetylcholine (ACh), desensitization of the nicotinic ACh receptor channel at the neuromuscular junction of the frog has two time constants (hundreds of milliseconds and tens of seconds), and recovery also has two time constants of 11–12 s and 4–5 min (Feltz & Trautmann, 1982). Desensitization and recovery on this time scale is thought to be due to conformational transitions between different states of the receptor. In contrast, a much slower time course of recovery from desensitization is observed for some receptors. For instance, in C_6 glioma cells, β-adrenergic receptors desensitized by exposure to isoprenaline recover with time constants of more than 12 h (Neve & Molinoff, 1986). It is thought that this form of desensitization represents removal of the receptors from the cell surface, and that recovery over such a slow time course is due to the insertion of new receptors.

Extracellular adenosine 5'-triphosphate (ATP) activates two membrane currents in developing chick skeletal muscle (Hume & Thomas, 1988). It has been shown previously that one of these currents desensitizes to repeated application of ATP, and does not recover for at least 10 min at room temperature (Hume & Honig, 1986). In this paper our goal was to determine whether the second ATP-activated current also shows such profound desensitization, and to characterize the time course and mechanism of desensitization and recovery from desensitization. We present three lines of evidence consistent with the idea that activation of ATP receptors is an irreversible process, and that recovery from desensitization requires the insertion of new receptors. We also discuss possible roles that such a profoundly desensitizing system might play in developing muscle.

METHODS

Cell culture

We made intracellular recordings from myotubes and whole-cell voltage-clamp recordings from myoballs. Standard chick embryo muscle cell cultures were prepared as described previously (Hume & Honig, 1986). Briefly, pectoral muscle was dissected from 11-day-old chick embryos, minced and incubated in a calcium- and magnesium-free saline (Puck's saline) for 20 min at room temperature. The tissue was then spun down for 5 min, resuspended in culture medium and triturated until the solution was cloudy. Cell density was determined with a haemocytometer after the suspension was filtered through lens paper to remove debris. Cells were plated onto gelatin-coated tissue culture dishes (Corning) at 150000 per 35 mm dish. The culture medium, Eagle's MEM with Earle's salts (GIBCO), was supplemented with 10% heat-inactivated horse serum (GIBCO), penicillin/streptomycin (50 U/ml, 50 μg/ml) and conalbumin (40 μg/ml, Sigma). Cultures were maintained in a humidified incubator at 37 °C with an atmosphere of 95% air, 5% CO_2. We made recordings from myotubes after 6–10 days in culture.

Myoballs (spherical, multinucleate muscle cells) were made in an identical manner up to the cell plating stage. Cells were pre-plated at 750000 per 35 mm dish onto uncoated tissue culture dishes and incubated at 37 °C for 2–3 h. The culture dishes were then swirled several times and the medium of each dish was transferred to a fresh, uncoated tissue culture dish. This procedure greatly reduced the number of fibroblasts, which adhere more rapidly to the dishes than the muscle precursor cells. The high cell density was used to promote the formation of clusters of muscle

precursor cells. The reduction in fibroblasts and the use of uncoated dishes made the culture dish surface poorly adhesive, which inhibited muscle cell elongation. Three- to six-day-old myoballs were used for recording.

Solutions

Just prior to recording, each culture dish was washed at least three times over a period of 5 min with the appropriate external solution (Table 1) to replace the incubating media. Each wash

TABLE 1. Composition of solutions (in mM)

Compound	Normal external	Standard recording	Low-Cl⁻ recording	Internal
		Solution		
NaCl	132	110	—	—
Sodium acetate	—	—	110	—
NaH_2PO_4	1·3	—	—	—
KCl	5	4	—	100
Potassium acetate	—	—	4	—
TEA Cl	—	20	20	—
$CaCl_2$	5·4	—	—	—
$CoCl_2$	—	4	4	—
$MgCl_2$	—	1	1	2
$MgSO_4$	1·6	—	—	—
K_4BAPTA	—	—	—	20
NaOH	4	5	5	—
KOH	—	—	—	2
HEPES	12·5	12·5	12·5	10
Phenol Red	0·03	0·03	0·03	0·03
Glucose	6·3	10	10	30

exchanged about 3 ml. Recording was then performed over a period of up to several hours at room temperature (21–23 °C) without perfusion of the bath. All solutions contained HEPES (as buffer) and 30 μM-Phenol Red (as indicator) in order to maintain the pH between 7·2 and 7·4. The pH was adjusted with the appropriate hydroxide as indicated in Table 1. To determine the time course of the onset of desensitization, we exposed cells to 20 μM-ATP dissolved in the normal external solution for a period of time, and then rapidly removed the solution from the dish with a glass pipette. The dish was flooded with external solution, and then washed two additional times before recording. For recovery experiments, cells were first treated with a test substance dissolved in the normal external solution. After such treatment, the cells were washed as described above with the culture medium and returned to the incubator for the period of recovery. Following recovery, the dishes were washed with the appropriate recording solution at room temperature and recordings were made from a series of cells in the dish over the next 60–90 min. The standard recording solution was used in experiments studying the excitatory current. The low-Cl⁻ recording solution was typically used in experiments measuring the ATP-activated potassium current because the solution enhanced the magnitude of the current. All recording from myoballs was done in the whole-cell configuration in which cells were dialysed with the internal solution (Table 1). All test substances were obtained from Sigma.

Intracellular and patch-clamp recording

Intracellular recordings were made using conventional glass microelectrodes filled with 3 M-KCl as described previously (Hume & Honig, 1986). The high input resistance of myotubes bathed in the recording solutions allowed the membrane potential to be varied between +20 and −100 mV by passing very small currents (< 1 nA). The resistance of electrodes was nearly constant when such small currents were passed; thus we used a single microelectrode with a balanced bridge circuit both to record voltage and pass current. The bridge circuit was balanced just prior to penetration of each cell.

Standard techniques were employed to form high resistance seals with pipettes onto the

membrane of myotubes or myoballs and to gain access to the cell interior (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Our polished pipettes had resistances of 2–4 MΩ, and we recorded from myoballs whose diameters ranged from 15–30 μm.

Agonists were dissolved in the appropriate recording solution and loaded into pipettes having tip diameters of 2–4 μm. The duration of the pressure pulse was accurately controlled by a solenoid valve in the pressure line. When the solenoid closed, it vented the pipette to the outside, so that no residual pressure could continue to force drug from the pipette. The tip of the pipette was placed approximately 30–60 μm from the test cell.

Measurement of ATP responsiveness

The responsiveness to ATP was measured in three ways. In the experiments comparing recovery at room temperature to recovery at 37 °C, the percentage of myotubes that contracted in response to a 1 s application of 50 μM-ATP was used. One myotube at a time was tested, and fifty myotubes were tested in each culture dish at each time point.

We made intracellular recordings from myotubes for the experiments measuring the time course of : (1) desensitization; (2) recovery of the excitatory ATP response from desensitization; and (3) recovery from treatment with DIDS (4,4'-diisothiocyanatostilbene-2, 2'-disulphonic acid). Cells were initially adjusted to −70 mV, and the input resistance (R_{in}) was measured with a 0·1 nA hyperpolarizing current pulse. We then applied 50 μM-ATP for 1 s and saved the resulting trace in a computer file (see Fig. 4). We used 50 μM-ATP to increase the sensitivity of our measurement, since this concentration is at the high end of the dose–response curve. We could not use the magnitude of the ATP-induced depolarization as an accurate measure of ATP responsiveness at this concentration since the cells often depolarized close to the reversal potential of the excitatory response. Instead, we measured the maximum rate of depolarization (dV/dt_{max}) and the potential (V_m) at which dV/dt_{max} occurred. The maximum rate of conductance increase due to ATP (dG/dt_{max}) was calculated from the equation:

$$dG/dt_{max} = dV/dt_{max}/(E_{ATP}-V_m)*R_{in},$$

where E_{ATP} is the reversal potential of the excitatory response. In these experiments V_m was typically in the range of −45 to −65 mV. Previous work has shown that the input resistance of myotubes is nearly constant over the range of −40 to −70 mV (Hume & Thomas, 1988). We used the rate of conductance increase as a measure of responsiveness because it compensated for differences in input resistance between fibres and for the decrease in driving force during large responses. This indirect method gave similar results to the direct method used to assess ATP-evoked currents in voltage-clamped myoballs.

After we developed the protocol for culturing myoballs, we used whole-cell recording from these cells to estimate the ATP responsiveness because it was more straightforward. Myoballs were voltage clamped at either −80 mV for measurement of the excitatory ATP-activated current or at +10 mV for measurement of the potassium current activated by ATP. Cell capacitance was measured to obtain an estimate for the surface area of the cell. We measured the peak current activated by a 1 s application of 50 μM-ATP, then normalized this value to 100 pF of cell capacitance. This method was used for measuring recovery of the potassium current from desensitization, recovery from desensitization of the excitatory response following tunicamycin treatment, and recovery of the excitatory response following treatment with oxidized ATP. In experiments in which ATP responsiveness was estimated with electrophysiological techniques (Figs 5 and 7) the time course of recovery of ATP responsiveness was approximated by a single exponential function fitted to the data by a non-linear, least-squares regression program (Sigmaplot 4·0, Jandel Scientific), with the constraint that the final value of the function returned to 100 % of the control level. For these experiments the time course of recovery is reported as the time constant of these exponential functions. In the experiments in which ATP responsiveness was estimated using the contraction assay, the time course of recovery is reported as the time until 50 % recovery, since the data were not well described by a single exponential function.

RESULTS

There are at least two distinct conductances activated by ATP in developing chick skeletal muscle. ATP rapidly activates a class of small conductance (0·3 pS) ion channels that conduct both cations and anions (Thomas & Hume, 1990). This

conductance is responsible for the excitation caused by ATP. In addition, ATP activates a class of potassium channels (25 pS) over a slower time course and with a longer latency (Hume & Thomas, 1988). It had been previously shown that ATP-induced depolarizations in myotubes exhibit pronounced long-term desensitization in response to a series of brief pulses of ATP (Hume & Honig, 1986). Typically, ATP responsiveness was almost completely eliminated by several brief applications (1 s or less) of 50 μM-ATP, and no recovery was observed at room temperature 10 min after an initial exposure to ATP. When we made whole-cell recordings from myoballs held at the reversal potential of either the excitatory current ($+10$ mV), or the potassium current (-80 mV), we found that both of these currents exhibited profound long-term desensitization in response to repeated 1 s applications of ATP.

Time course of the loss of responsiveness to ATP

To determine the time course of the loss of responsiveness to ATP, we made intracellular recordings from myotubes. We determined the responsiveness to ATP for individual cells by measuring the peak depolarizing response to a local application of ATP. We first sampled a series of control cells to estimate average responsiveness. We bathed other cells with 20 μM-ATP for a specified time, then rapidly washed the ATP out and measured the responsiveness of these myotubes. Using this protocol, the magnitude of the excitatory response to ATP was approximately half of the control value after a 30 s exposure to ATP, and the magnitude was only 23% of the control after 1 min (Fig. 1). It should be noted that we recorded from the test cells at room temperature for as long as 2 h following wash-out of the ATP. However, the responses did not tend to become either larger or smaller over this time. We take this to mean that there was neither significant additional desensitization following wash-out, nor was there significant recovery from desensitization (also see below).

Mechanism of desensitization

Desensitization is defined as the loss of the ability to respond to an agonist. This loss of responsiveness could come about by several different mechanisms. Two simple possibilities that would give similar physiological recordings are that the ion channels activated by ATP enter a long-lasting non-conducting state, or that the receptor enters an agonist-insensitive state. We refer to both of these mechanisms as inactivation. Inactivation is the major mechanism of desensitization of nicotinic ACh receptors. However, an alternative possibility is that cells fail to respond to subsequent applications of ATP because the ion channels opened by the initial stimulus are still open at much later times. In this mechanism, desensitization actually would be caused by prolonged activation, rather than by inactivation.

To determine the time course of inactivation, we examined the currents elicited by long applications of ATP. We applied 20 μM-ATP to myoballs for several minutes while recording membrane currents with a patch pipette in the whole-cell configuration. To study the excitatory current, we voltage clamped myoballs at the reversal potential for the potassium current (-80 mV). To study the potassium current, we voltage clamped myoballs at the reversal potential for the excitatory current ($+10$ mV). The excitatory current remained active for at least several minutes after the beginning of ATP applications, while the potassium current returned almost to baseline within 1 min (Fig. 2). On average, the excitatory current

was still at 60% (S.E.M. = 8, $n = 7$) of its peak after 1 min, while the potassium current had declined to only 3% (S.E.M. = 2, $n = 7$) of its peak after 1 min. It is very unlikely that the decline in the potassium current was due to depletion of potassium from within the cell. The cells were internally dialysed by the patch pipette, so there
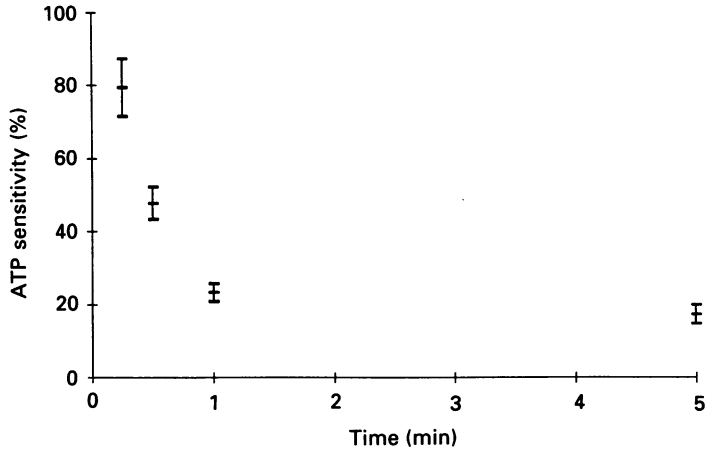

Fig. 1. The loss of responsiveness to ATP occurred mostly within the first minute of exposure to ATP. Myotubes were bathed in the normal external solution containing 20 μM-ATP for the indicated times. They were then rapidly washed several times with the standard recording solution at room temperature. Cells were then tested with brief pulses of 20 μM-ATP. ATP responsiveness was calculated from the maximum rate of depolarization during intracellular recording as outlined in Methods. Recording took place over approximately the next hour after ATP had been washed out. There was no difference between the responsiveness of cells recorded from in the first half-hour as compared to the second half-hour, indicating that desensitization did not continue in the absence of ATP, and that no significant recovery occurred during recording. All time points are the average responsiveness from twenty cells as compared to the control responsiveness from twenty other cells. Error bars represent the standard error of the mean (S.E.M.).

was a huge source to replenish internal potassium. Likewise, it is unlikely that the decline in the potassium current was due to accumulation of potassium outside of the cell. If accumulation of potassium was the cause of the decrease in current, then as the current declined, so would the amount of accumulated potassium. Potassium accumulation, therefore, could not lead to the decrease to nearly 0 current that was observed.

While the decline in the potassium current was approximately monophasic, the decline in the excitatory current was sometimes biphasic. There was always a slow component to inactivation of the excitatory current, but sometimes there was a faster component as well (Fig. 2B). It should be noted that the slow component of inactivation did not bring the excitatory current back to baseline even after many minutes. In some cells, as much as 75% of the peak inward current was still present after 10 min of continuous exposure to ATP. Interestingly, even brief applications of ATP elicit long-lasting excitatory currents (Thomas & Hume, 1990). It therefore seems likely that when ATP is applied as a series of brief applications, the long-term
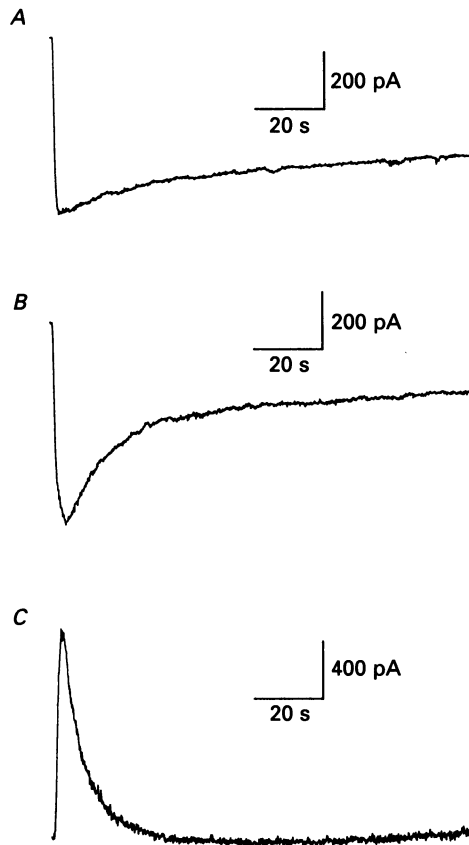
A

200 pA

20 s

B

200 pA

20 s

C

400 pA

20 s

Fig. 2. Inactivation of the excitatory and potassium conductances in the continuous presence of ATP. ATP (20 μM) was applied for 2 min to myoballs bathed in the low-Cl⁻ recording solution. *A*, inactivation of the excitatory conductance in this myoball was slow; 74% of the peak current was still active 1 min after the beginning of ATP application. *B*, inactivation of the excitatory conductance in this myoball was biphasic; some cells had a relatively fast component in addition to the slow component of inactivation. In this myoball 40% of the peak current was still active at 1 min. *C*, inactivation of the potassium conductance was uniformly monophasic and relatively rapid. The time constant of inactivation for this cell was 5·6 s. In all three parts, myoballs were dialysed with the internal solution during whole-cell patch-clamp recording. The cells were held at −80 mV to study the excitatory current and at +10 mV to study the potassium current.

desensitization of the excitatory conductance is due to both prolonged activation and slow inactivation. In contrast, the potassium conductance inactivates relatively quickly in the presence of ATP.

We did not examine in detail whether there was a voltage dependence to the desensitization of the ATP activated currents. However, in previously reported experiments we blocked the late potassium currents with internal caesium, so that we could examine the early current at a variety of membrane potentials (Thomas & Hume, 1990). It was clear from these experiments that the rate of inactivation of the

early current is quite slow at positive, as well as negative potentials. We have no information on the voltage dependence of inactivation of the potassium current, since it could only be studied in isolation at the reversal potential of the early current.
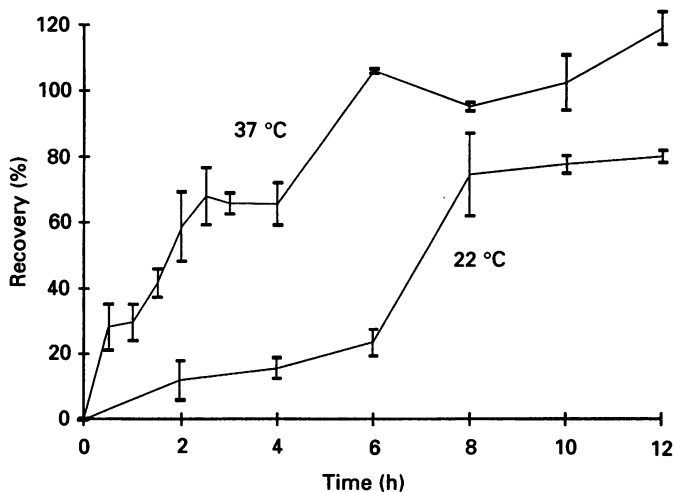

Fig. 3. Recovery of the ability to contract in response to ATP occurred over hours and was sensitive to temperature. At 37 °C, the time to recovery of half-maximal responsiveness was 2 h. Recovery at room temperature was considerably delayed. Myotubes were tested for the ability to contract in response to a 1 s application of 50 μM-ATP. In each culture dish, fifty myotubes were tested before all the cells in the dish were desensitized with a 5 min application of 50 μM-ATP in the normal external solution. ATP was washed out with the culture medium and the culture dish was left at either room temperature or 37 °C for the indicated times. The myotubes were then returned to the normal external solution at room temperature and again fifty fibres were tested for contraction. Fractional recovery was calculated as the number of fibres that contracted after the recovery period divided by the number that contracted prior to desensitization. Each time point is the average of three to six dishes from separate cultures. Error bars represent the S.E.M..

Recovery from desensitization to ATP

The experiments determining the onset of desensitization indicated that there was very little recovery of ATP responsiveness for many minutes at room temperature. We therefore decided to measure recovery over a much longer time course (12 h), both at room temperature and at 37 °C. We first used a simple contraction assay to monitor the return of ATP responsiveness. The basis of this assay is that a brief application of ATP caused most myotubes (75–95 %) to contract. After determining the proportion of fibres in each dish that contracted in response to ATP, the cells were bathed in 50 μM-ATP for 5 min. None of the myotubes contracted in response to ATP immediately after it was washed out. We then allowed the cells to recover in the culture medium for a designated time interval. Cells were again tested with ATP, and the percentage that contracted was divided by the percentage that initially contracted to give the fractional recovery. At 37 °C, the proportion of

myotubes contracting in response to ATP recovered to half its initial level in about 2 h (Fig. 3). However, at room temperature the return of contraction was substantially delayed. There was only minimal recovery during the first 6 h, but after 8 h there was significant recovery, although it was still less than that at 37 °C. The
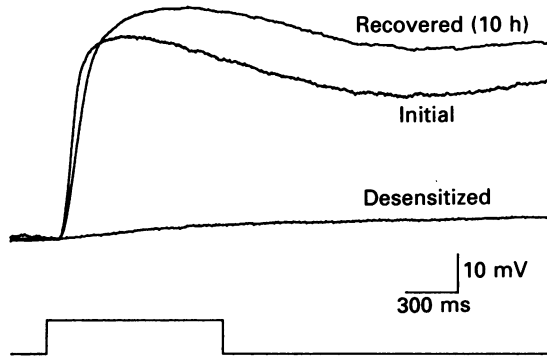

Fig. 4. Recovery of the ATP response in myotubes was almost complete 10 h after desensitization. ATP was applied at 50 μM, both to test and to desensitization myotubes. The initial responsiveness of this myotube was 45.4 nS/s. Shortly after desensitization the responsiveness was only 1·7 % (0·78 nS/s) of the initial response. After 10 h in the culture medium at 37 °C, the responsiveness had recovered to 98·4 % (44·7 nS/s) of the initial value. While it could not be quantified here, the ATP-activated potassium conductance also recovered over this time period. This was indicated by the partial repolarization in the initial and recovered records. Intracellular recordings from this myotube were made in the standard recording solution. To relocate the same muscle fibre, the position of the myotube in the culture dish was measured on the microscope stage, and the orientation of the myotube in the field was noted along with the orientation of the dish on the microscope.

observation that recovery from desensitization was quite sensitive to temperature suggested that there is at least one energy-dependent biochemical step mediating recovery.

Because it was so simple, the contraction assay allowed large numbers of cells to be studied quite quickly. However, this assay does not directly measure the recovery of ATP responsiveness, since the contraction of a myotube following application of ATP depends on many factors besides the number of functional receptors. Rather, this assay tests when enough receptors are on the cell surface for ATP to depolarize cells to the threshold for contraction. If there are normally significantly more receptors on the cell surface than are necessary to reach threshold, then receptor levels would be much less than 100 % of control at the time that this assay suggested that complete recovery had occurred. Thus this assay is likely to underestimate the true time constant of recovery. The apparently rapid recovery from desensitization observed at room temperature between 6 and 8 h was probably a manifestation of this tendency to underestimate the true time course of recovery.

A more precise assay would be to directly measure the ATP-activated currents. It seemed particularly appropriate to make measurements of the excitatory conductance, since evidence suggests that it is directly activated by ATP, without

mediation by a second messenger (Hume & Thomas, 1988). One method for assessing the recovery of the excitatory response was to make intracellular recordings from a series of myotubes before they were desensitized, and then to record from the same myotubes at specified time intervals following desensitization. An example of
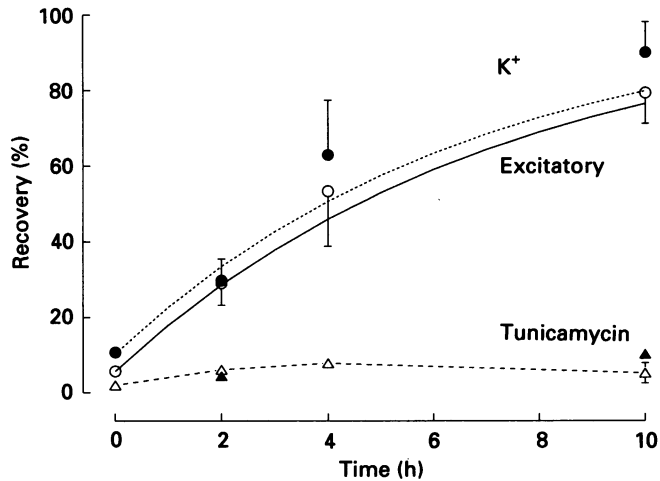

Fig. 5. Recovery of the ATP-activated currents was greatly reduced by treatment with tunicamycin. Treatment with 1 μg/ml of tunicamycin beginning 5 h prior to desensitization and continuing throughout the recovery period resulted in only minimal recovery of both the early and late responses. The ATP responses are expressed as the percentage of ATP responses measured prior to desensitization. For ATP responses in the absence of tunicamycin, the time course of recovery was fitted to a single exponential, by a non-linear, least-squares regression program. Exponential curves were fitted for the early excitatory response ($\bigcirc$ and continuous line) and the late potassium response ($\bullet$ and short dashed line). The time constants of recovery for the excitatory current and the potassium current were 7·2 and 6·7 h respectively. For the recovery of ATP responsiveness in the absence of tunicamycin, each time point is the average of three to five separate experiments. No curve was fitted to the data obtained in the presence of tunicamycin; rather, the data for the excitatory responses ($\triangle$) were simply connected by dashed lines. Recovery of the potassium current in tunicamycin ($\blacktriangle$) was only tested in a single experiment, so no error bars are indicated. For all the other points with no error bars, the s.e.m. was smaller than the symbols used.

recovery in an individual myotube is shown in Fig. 4. The initial responsiveness of this fibre was calculated by measuring the slope of the depolarizing phase of the response (see Methods). Shortly after bath application of ATP, the response was largely desensitized (2·4 % of the initial responsiveness). After 10 h in the culture medium at 37 °C, the excitatory response to ATP had almost completely recovered (98·4 %). In this fibre, the potassium conductance activated by ATP also recovered, as revealed by the dip in the membrane potential after peak depolarization in both the initial and the recovered traces. When we estimated the recovery of the excitatory response by making intracellular recordings from many different myotubes, we found that it recovered with a time constant of approximately 7·2 h (Fig. 5).

The second method we used to assess recovery from desensitization was to record

from myoballs using the whole-cell patch-clamp technique. With this method we recorded from a population of myoballs to obtain the initial responsiveness, but did not attempt to record from the same cells a second time. Instead we used a separate culture dish of myoballs for each recovery time point. To measure recovery of the potassium conductance we held cells at +10 mV, the reversal potential for the early response. We found that at 37 °C, the ATP-activated potassium conductance recovered with a time constant of 6·7 h, quite similar to the recovery time constant of the excitatory response.

Mechanism of recovery from desensitization

The slow time course of recovery from desensitization is compatible with the synthesis and insertion of new ATP receptors as a mechanism for recovery. A critical test of this idea would be to prevent the insertion of functional receptors into the plasma membrane and to see whether recovery from desensitization was blocked. We first attempted to accomplish this by adding inhibitors of protein synthesis, but found that the protein synthesis inhibitors cycloheximide and anisomycin were quite toxic to developing chick skeletal muscle at concentrations that typically block protein synthesis. Instead, we treated myoballs with tunicamycin, which blocks N-linked protein glycosylation by inhibiting the transfer of N-acetylglucosamine-1-P to the lipid carrier dolichol phosphate (for a review see Elbein, 1981). We chose tunicamycin for two reasons. First, most plasma membrane proteins are glycosylated, and the failure to glycosylate proteins has been shown to interfere with the function, or with the incorporation into the plasma membrane, of several integral membrane proteins. For example, block of glycosylation by tunicamycin prevents the secretion of IgA and IgE from plasma cells (Hickman, Kulczcki, Lynch & Kornfeld, 1977). Second, tunicamycin seemed likely to be less toxic than protein synthesis inhibitors over the period of many hours during which recovery would occur, because many intracellular proteins are not glycosylated, and therefore should be synthesized and processed normally. When we pre-treated muscle cells with 1–2 μg/ml of tunicamycin for several hours, desensitized the cells with ATP, and then allowed them time to recover in tunicamycin ($n = 4$), we found that recovery of the excitatory response was minimal (Fig. 5). The amplitude of the excitatory responses to ATP in the absence of tunicamycin was calculated from the rate of depolarization during intracellular recording from myotubes bathed in the standard recording solution. In the experiments with tunicamycin, the amplitude of the excitatory responses to ATP was measured as the peak current during whole-cell recording from myoballs bathed in the standard recording solution. Two experiments were performed to assess the full time course of the recovery of the excitatory response in the presence of 1 μg/ml tunicamycin and two very similar experiments (tunicamycin at 2 μg/ml in one, and pre-incubation for only 4 h in the other) confirmed the lack of recovery at 10 h. A similar lack of recovery of the potassium conductance following tunicamycin treatment was seen in the single experiment performed. In this experiment the amplitude of the potassium currents evoked by ATP was measured as the peak outward current during whole-cell recording from myoballs bathed in the low-Cl$^-$ recording solution.

There were three reasons for believing that tunicamycin interfered with recovery

by blocking glycosylation. First, tunicamycin had no direct effect on the ATP response. Myoballs bathed for 10 min or more in 1 μg/ml tunicamycin in the standard recording solution, and voltage clamped at -80 mV, had average inward currents of -530 pA (S.E.M. $= 161$, $n = 7$) activated by ATP as compared to
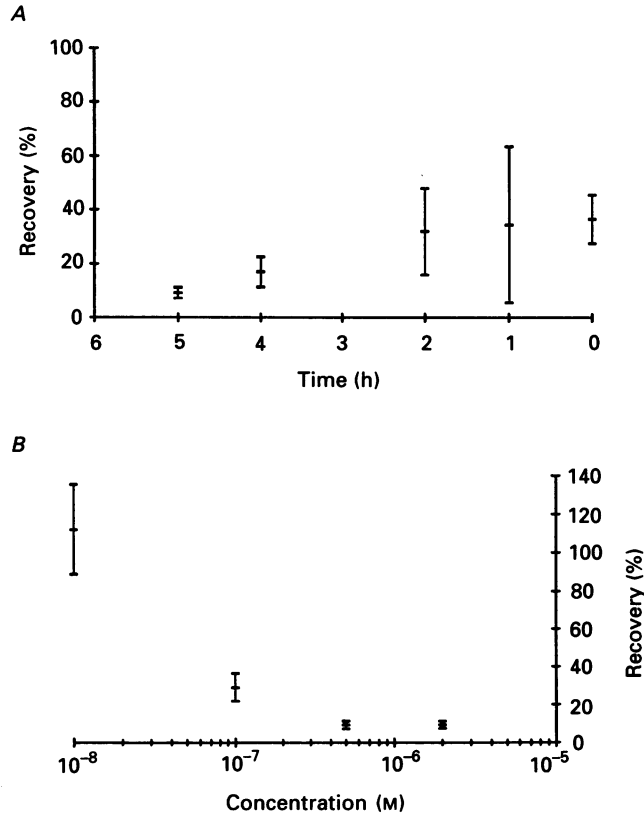

Fig. 6. Inhibition of recovery by tunicamycin depended on the pre-incubation period and was dose-dependent. *A*, tunicamycin was significantly more effective in blocking recovery if cells were pre-incubated for 5 h. All cells were allowed to recover for 10 h in the presence of 1 μg/ml tunicamycin. Prior to desensitization, the cells were also pre-incubated in tunicamycin for the times indicated. Whole-cell recording was used in these experiments. ATP responsiveness was measured as the peak inward current activated at -80 mV in myoballs bathed in the standard recording solution and dialysed with the standard internal solution. Each time point is the average response from at least five different cells. *B*, inhibition of recovery reached saturation at a concentration of tunicamycin around 1 μg/ml (1.25×10^{-6} M). ATP responsiveness was assayed as in part *A*. Error bars represent the S.E.M.

-401 pA (S.E.M. $= 75$, $n = 7$) for controls. Second, the dose–response relation for inhibition of recovery by tunicamycin was similar to what has been found in other cell types for block of glycosylation (Fig. 6*B*, see Elbein, 1981). Third, the duration of the pre-treatment period with tunicamycin affected the extent of inhibition of recovery (Fig. 6*A*). Significantly more recovery occurred without any pre-incubation

as compared to 5 h of pre-incubation with tunicamycin. If tunicamycin acts by blocking receptor glycosylation, then without pre-incubation in tunicamycin, there should be a population of glycosylated proteins that have not been inserted into the plasma membrane yet. These proteins would be inserted during the recovery period
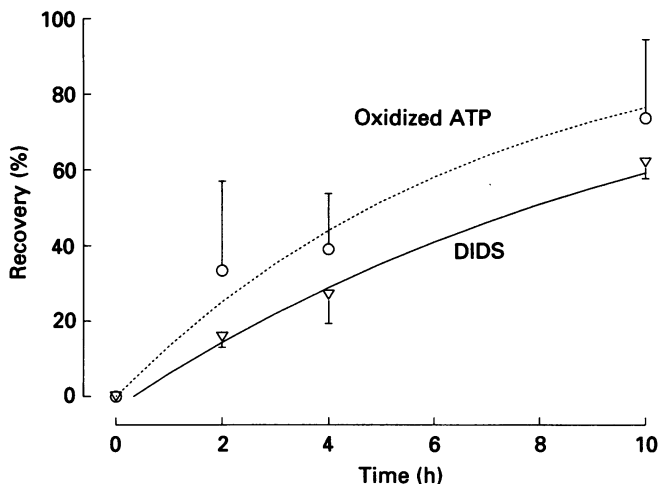

Fig. 7. Recovery of the excitatory ATP response after treatment with DIDS or oxidized ATP also required many hours. The time constants for recovery from blockade of the ATP response with DIDS and oxidized ATP were 10·8 and 6·9 h respectively. In these experiments we could not record from the same cells before and after exposure to antagonist because we did not want to test cells that had already been desensitized to ATP. For DIDS (∇, continuous line), ATP responsiveness was calculated from the rate of depolarization in myotubes. For oxidized ATP (○, dashed line), ATP responsiveness was estimated from the peak inward current evoked by ATP in myoballs voltage clamped at −80 mV. Cells were bathed in the standard recording solution and myoballs were dialysed with the internal solution. Each time point is the average of three to four separate experiments and error bars represent the s.e.m. The exponential curves were fitted by a least-squares, non-linear regression program.

until their supply was exhausted following block of glycosylation. If enough time is allowed after block of glycosylation before treatment with ATP, then most of the glycosylated proteins would be inserted into the membrane by the time the cell was desensitized, so less recovery would occur. These results support the idea that tunicamycin inhibits receptor incorporation.

Recovery from blockade by two antagonists of the ATP response

A third line of evidence also supports the idea that the mechanism of recovery from desensitization is by the insertion of new ATP receptors into the plasma membrane. Pharmacological studies have identified two molecules that block the response to ATP without activating it; they are oxidized ATP (2′,3′-dialdehyde-ATP) and the stilbene derivative DIDS (4,4′-diisothiocyanatostilbene-2, 2′-disulphonic acid) (S. A. Thomas and R. I. Hume, unpublished observations). Both of these compounds have reactive groups that can bind covalently to proteins; DIDS has a pair of

isothiocyanates and oxidized ATP has a pair of aldehydes. Both of these molecules are non-competitive blockers. Their block cannot be overcome by higher concentrations of ATP, and they both continue to block the response after they are washed out. It therefore seemed possible that these inhibitors might act irreversibly.

We treated cells with either 50 μM-DIDS or 100 μM-oxidized ATP for 30 min at room temperature to block all of the ATP responsiveness. We then measured recovery from antagonist blockade as we had measured recovery from desensitization. At 37 °C, recovery of the excitatory response after treatment with oxidized ATP had a time constant of 6·9 h, and recovery after treatment with DIDS had a time constant of 10·8 h (Fig. 7). Thus, recovery from exposure to non-competitive inhibitors that are presumed to covalently modify the receptor, and from desensitization by ATP, have very similar time courses. The simplest explanation of these results is that recovery from both treatments takes place by the same mechanism, insertion of newly synthesized receptors.

DISCUSSION

We have examined the time course of desensitization to ATP and the mechanism of recovery from desensitization. We found that inactivation of the excitatory current during continued application of ATP was slow and incomplete. However, the loss of responsiveness (desensitization) to ATP was faster, with the majority occurring within the first minute. Thus much of the loss of responsiveness to ATP as tested by subsequent applications of ATP is due to the continued activation of the excitatory channels. In contrast, inactivation of the ATP-activated potassium current was relatively rapid, being essentially complete within 1 min. Recovery from desensitization was a very slow process that occurred over many hours. Recovery was sensitive to temperature and could be inhibited by tunicamycin. Taken together, these results strongly suggest that insertion of newly synthesized receptors is required for recovery of both of the currents activated by ATP.

The different time courses of inactivation of the excitatory and inhibitory conductances suggests that they occur through different mechanisms. All of the available evidence suggests that the excitatory conductance is directly activated by ATP, without the mediation of second messengers (Hume & Thomas, 1988; and unpublished observations). A simple interpretation of the time course data would be that once the receptor binds ATP these channels stay open for a prolonged period of time before inactivating to a state from which they do not recover. Inactivation might be the result of a very slow process such as internalization of the receptor–channel complex. In contrast, there is very strong evidence that the potassium conductance is activated through an intramembranous second messenger system (S. A. Thomas and R. I. Hume, unpublished observations). In considering possible mechanisms, one needs to take the pharmacological evidence into account. Comparison of the activity of over thirty ATP analogues suggested that the excitatory conductance and the potassium conductance are activated through the same type of ATP receptor (S. A. Thomas and R. I. Hume, unpublished observations). The two different time courses for desensitization may indicate that there are actually two different ATP receptors with very similar pharmacology, but

different physiology. On the other hand, a single receptor could mediate both actions of ATP if the rapid inactivation of the potassium current occurred at some point down-stream of the receptor–channel complex. For example, the second messenger that activates the potassium channels may be short-lived and the supply of its precursor could be exhausted.

It is unlikely that the mechanism of receptor inactivation requires soluble intracellular factors. Desensitization has been observed for both the excitatory conductance and the potassium conductance in excised outside-out membrane patches (S. A. Thomas and R. I. Hume, unpublished observations). Since the cytoplasmic side of these patches was exposed to an internal solution which contained no nucleotides and a very low level of free calcium ($< 10^{-8}$ M), it seems unlikely that either soluble factors or phosphorylation of the receptor or ion channels on their intracellular face could play a role in inactivation. However, since ATP is the agonist, it is possible that extracellular phosphorylation is involved.

How might the rapid desensitization and slow recovery from desensitization relate to the physiological function of the ATP receptors on chick skeletal muscle? Since the role of these receptors is unknown, we can only speculate. Studies of the development of chick muscle *in vitro* indicate that these receptors are expressed on unfused myoblasts and on recently fused myotubes (Hume & Thomas, 1988), while studies of chick muscle *in vivo* suggest that these receptors are normally expressed in chick embryos, but not in hatched chickens (M. J. Zawisa and R. I. Hume, unpublished observations). We therefore presume that their normal function must occur during the period of muscle development. It is known that ATP is packaged in many neurotransmitter vesicles including cholinergic vesicles (Zimmermann, 1982), so ATP receptors could potentially play a role in synaptic transmission. To assess whether ATP plays a role during synaptogenesis or at the embryonic neuromuscular junction, one can record from co-cultures of cholinergic neurons and skeletal muscle in the presence of cholinergic blockers such as α-bungarotoxin. A set of such experiments has already been reported in which no indication of ATP-mediated synaptic events was observed (Hume & Honig, 1986). However, these experiments were done prior to learning the full extent of desensitization. Long-term desensitization of the ATP response must be taken into account when considering a role for the ATP response at the neuromuscular injunction. Since responses were not seen at mature synapses, the previous experiments seem to rule out the possibility that ATP only desensitizes a fraction of the receptors following release, or that these receptors are rapidly replaced by diffusion of extrajunctional receptors into the synapse. However, they leave open the possibility that ATP released from the presynaptic terminal might play a significant role during synaptogenesis, or after prolonged periods of inactivity. Alternatively, the source of endogenous ATP may not be nerve terminals and the ATP receptors may be involved in some other aspect of the development of muscle. In either case, the kinetics of ATP action (persistent activation followed by long-lasting desensitization) seem to indicate that the ATP response is likely to mediate a discrete event in development, rather than some on-going process that is part of normal synaptic transmission.

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