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A receptor that is highly specific for extracellular ATP in developing chick skeletal muscle *in vitro*

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1 Extracellular adenosine 5'-triphosphate (ATP) activated an early excitatory conductance followed by a late potassium conductance in developing chick skeletal muscle. A series of ATP analogues were tested for their ability to activate these two conductances. All compounds tested were either agonists for both responses or for neither. Furthermore, the potency of agonists was similar for the two responses.

2 The order of potency for agonists was ATP ≈ adenosine 5'-O-(3-thiotriphosphate) (ATP-γ-S) ≈ 2-methylthio-ATP (2-CH_3S-ATP) > 2'-deoxy-ATP ≈ 3'-deoxy-ATP > adenosine 5'-tetraphosphate (ATP-OPO_3) ≈ adenosine 5'-diphosphate (ADP). Many other ATP analogues were not agonists.

3 Activation of the excitatory response did not require divalent cations. Furthermore, the concentration-response relation of the excitatory response was similar when ATP was applied as the free anion of ATP (ATP^{4-}) or complexed with a divalent cation ($M \cdot ATP^{2-}$).

4 Three antagonists of the ATP response were characterized. 8-Br-ATP was a weak antagonist, while 2',3'-dialdehyde-ATP and DIDS (4,4'-diisocyanatostilbene-2,2'-disulphonic acid) were potent irreversible inhibitors. The two conductances were equally affected by these antagonists.

5 These results suggest that both ATP responses are activated through the same receptor type, or two very similar receptors.

Keywords: ATP; receptor; chick skeletal muscle

Introduction

Extracellular adenosine 5'-triphosphate (ATP) is known to activate a variety of cellular responses in many different tissues (Gorden, 1986). For example, there is good evidence suggesting that ATP participates in synaptic transmission from autonomic neurones to smooth muscle cells (Burnstock, 1981). ATP is packaged in cholinergic vesicles (Zimmermann, 1982), and released upon stimulation of motor neurones (Silinsky & Hubbard, 1973), so ATP receptors could potentially play a role in synaptic transmission at the skeletal neuromuscular junction. In developing chick skeletal muscle in culture, micromolar concentrations of extracellular ATP activate an early excitatory conductance followed by a late potassium conductance (Hume & Thomas, 1988). To test whether these two responses are activated by different receptor types, a variety of ATP analogues were screened for their ability to activate or inhibit these two responses, and the potencies of those analogues that were agonist were compared. This study also allowed us to evaluate systematically the effect of various alterations to the structure of ATP on the ability to activate responses. Our results are consistent with the possibility that both ATP responses are activated by the same receptor type. In addition, two antagonists which may prove useful in determining the function of the ATP receptor and in identifying the macromolecule(s) that constitute this receptor have been characterized.

Methods

Cell culture

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. To prepare myotube cultures, cells were plated onto gelatin-coated tissue culture dishes (Corning) at 150,000 per 35 mm dish. Myoballs (spherical, multinucleate muscle cells) were made in an identical manner up to the cell plating stage. Cells were preplated onto uncoated tissue culture dishes at 750,000 per 35 mm dish and incubated at 37°C for 2 to 3 h. This procedure greatly reduced the number of fibroblasts, which adhere more rapidly to the dishes than the muscle precursor cells. The culture dishes were then swirled several times and the medium of each dish (containing an enriched population of myoblasts) was transferred to a fresh, uncoated tissue culture dish. The high cell density promoted 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. Recordings were made from myotubes after 6 to 10 days in culture, or from myoballs after 3 to 6 days in culture.

The culture medium, Eagle's minimum essential medium (MEM) with Earle's salts (Gibco), was supplemented with 10% heat-inactivated horse serum (Gibco), penicillin/streptomycin (50 units ml^{-1}, 50 $\mu g\,ml^{-1}$) and conalbumin (40 $\mu g\,ml^{-1}$, Sigma). Cultures were maintained in a humidified incubator at 37°C with an atmosphere of 95% air, 5% CO_2.

Solutions

Recording was performed over a period of up to several hours at room temperature (21–23°C) without perfusion of the bath. 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 exchanged about 3 ml. All solutions contained HEPES (as buffer) and 30 μM phenol red (as indicator) in order to maintain the pH near 7.3. NaOH and KOH were used to adjust the external and internal solutions respectively to pH 7.3.

The standard external solution contained cobalt, rather than calcium, to prevent the secondary activation of the calcium activated chloride current that is present in these cells (Hume & Thomas, 1989). External solutions containing ethylenediamine tetraacetate (EDTA) or ethyleneglycol-*bis*-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) were

Table 1 Composition of solutions (in mM)

Compound	Standard external	EDTA external	EGTA external	Internal
		Solution		
Na acetate	110	110	90	—
KCl	—	—	—	100
K acetate	4	4	4	—
TEA Cl	20	20	20	—
$CoCl_2$	4	—	—	—
$MgCl_2$	1	—	1, 3, or 9	2
Na_2H_2EDTA	—	5	—	—
Na_2H_2EGTA	—	—	11	—
K_4BAPTA	—	—	—	20
HEPES	12.5	12.5	12.5	10
Phenol red	0.03	0.03	0.03	0.03
Glucose	10	10	10	30

used to examine the role of extracellular magnesium. The EDTA solution was used to produce a solution essentially free of magnesium, while the EGTA solution was used to set the free magnesium concentration at different levels. The fraction of total ATP that was not complexed with Mg^{2+} was calculated from the equation:

$$ATP^{4-} = 1 - 1/(10^{x}*[Mg_{free}])$$

where x = 4.2, the association constant of $MgATP^{2-}$ under these conditions, and Mg_{free} was calculated assuming a K_d for Mg^{2+} of 300 μM (Walaas, 1958; Cockroft & Gomperts, 1979).

Intracellular and patch-clamp recording

Intracellular recordings were made with conventional glass microelectrodes filled with 3 M KCl as described previously (Hume & Honig, 1986). The high input resistance of myotubes bathed in the external solution 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 a single microelectrode with a balanced bridge circuit was used 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 myoballs and to gain access to the cell interior (Hamill et al., 1981). Myoballs were dialyzed with the internal solution and bathed in one of the external solutions (Table 1). Polished pipettes had resistances of 2 to 4 MΩ, and recordings were made from myoballs whose diameters ranged from 15 to 30 μm.

Drugs

All compounds came from Sigma except for 2-methylthio-ATP (2-CH_3-S-ATP) (Research Biochemicals), adenosine 5′-O-(3-thiotriphosphate) (ATP-γ-S) and β,γ-imido-ATP (AMP-PNP) (Boehringer Mannheim). Agonists were dissolved in the appropriate external solution and loaded into pipettes having tip diameters of 2–4 μm. Solution was ejected from the pipettes by pressure applied to the back of the pipette. 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. The compounds used in this study, and the abbreviations used were as follows: adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), inosine 5′-triphosphate (ITP), guanosine 5′-triphosphate (GTP), uridine 5′-triphosphate (UTP), cytidine 5′-triphosphate (CTP), 2-methylthio-ATP (2-CH_3-S-ATP), 8-bromo-ATP (8-Br-ATP), 2′- and 3′-O-(4-benzoylbenzoyl)-ATP (2′/3′-BB-ATP), adenosine 5′-tetraphosphate (ATP-OPO_3), adenosine 5′-O-(3-thiotriphosphate) (ATP-γ-S), α,β-methylene-ATP (AMP-CPP), β,γ-methylene-ATP (AMP-PCP), β,γ-imido-ATP (AMP-PNP), adenosine 5′-O-(3-thiotriphosphate) (ADP-β-S), adenosine 5′-diphosphoramidate (ADP-β-NH_2), adenosine 5′-diphosphomorpholidate (ADP-β-morpholidate), adenosine 5′-monophosphomorpholidate (AMP-α-morpholidate), adenosine 5′-phosphosulphate (AMP-OSO_3), adenosine 5′-monophosphoramidate (AMP-α-NH_2), P^1,P^2-di(adenosine 5′)pyrophosphate (A-P_2-A), P^1,P^3-di(adenosine 5′)triphosphate (A-P_3-A), P^1,P^4-di(adenosine 5′)tetraphosphate (A-P_4-A), P^1,P^5-di(adenosine 5′)pentaphosphate (A-P_5-A), P^1,P^6-di(adenosine 5′)hexaphosphate (A-P_6-A), 2′,3′-dialdehyde-ATP (oxidized ATP), 2′,3′-acyclic dialcohol-ATP (reduced ATP), 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), and 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid (SITS).

Measurement of ATP and analogue responsiveness

The responsiveness to ATP was measured in one of two ways. Intracellular recordings from myotubes were used when screening ATP analogues because many cells could be quickly studied with this technique. Cells were initially adjusted to −70 mV, and the input resistance (R_{in}) was measured with a 0.1 nA hyperpolarizing current pulse. The peak depolarization activated by a 1 s application of an agonist was measured, and then divided by R_{in} to obtain an estimate for the current activated. For all agonists that activated an excitatory response, a semi-quantitative concentration-response curve was prepared by applying several different concentrations of agonist to a series of myotubes. This method was quick, but produced only a rough estimate of the parameters of the concentration-response curve, because large responses are subject to considerable non-linear summation. For this reason, EC_{50}s obtained by this method are reported only by their approximate magnitude (Table 2), rather than as discrete values.

Quantitative estimates of ATP responsiveness were obtained from whole-cell recordings made from cultured myoballs, since the ATP-evoked currents could be measured directly. 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 (see Figure 1). Cell capacitance was measured to obtain an estimate for the surface area of the cell. The peak current activated by a 1 s application of an agonist was measured, and then normalized to 100 pF of cell capacitance.

To obtain concentration-response curves, it was necessary to record from a population of cells for each concentration of each agonist. Data from 5 to 20 cells were averaged for each concentration, and then at least four different concentrations were studied for each agonist. It was not possible to obtain concentration-response curves from individual cells because both the excitatory and potassium currents show long-term desensitization to repeated applications of ATP (Thomas &

Hume, 1990b). A sigmoid curve was fitted to the averaged data by a least-squares non-linear regression programme (SigmaPlot 4.0, Jandel Scientific). The bottom of the sigmoid curve was held constant at 0, while the maximum, the EC_{50}, and the Hill slope were allowed to vary. To test whether a single curve could describe the relationship between agonist concentration and the amplitude of both ATP responses, the method described by Motulsky & Ransnas (1987) was used. After the data for the two responses to each agonist had been individually fitted, the two data sets were pooled (after dividing each individual response by the estimated maximal response), and the F ratio calculated. The average curve was considered to be an adequate fit to both data sets if P was >0.05.

Results

Low micromolar concentrations of extracellular ATP had a dual effect on developing chick skeletal muscle (Figure 1). ATP caused excitation by activating a class of small conductance (0.3 pS) ion channels through which both cations and anions permeate (Thomas & Hume, 1990a). ATP also elicited a potassium conductance that had a longer latency to activation than the excitatory conductance (Hume & Thomas, 1988). Concentration-response curves for the two currents activated by ATP are shown in Figure 2. A single curve fitted the data for both the excitatory and potassium responses.

To characterize the pharmacological specificity of the ATP receptor that elicited the excitatory current, ATP analogues substituted at several different positions were tested. The structure of ATP, with key positions labelled, is shown in Figure 3. Each analogue was tested with intracellular recording, by applying the substance at 0.5 or 1 mM to myotubes set

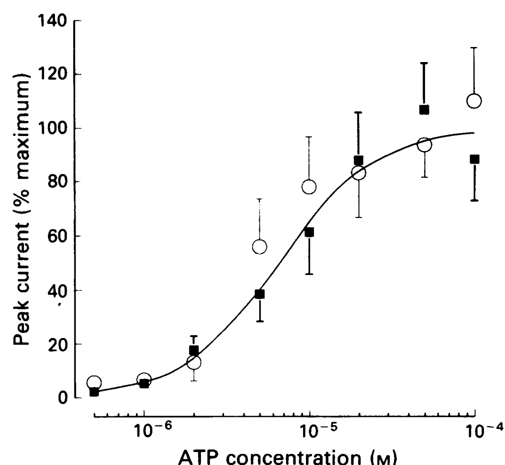

Figure 2 Concentration-response curves for the two currents activated by ATP. The peak current was measured in response to application of ATP from a nearby puffer pipette. Myoballs were voltage-clamped at −80 mV to isolate the excitatory current (■, thick error bars), or at +10 mV to isolate the potassium current (○, thin error bars). For each concentration of ATP, 5–20 cells were tested, and the ATP evoked currents were normalized (to pA/100 pF) and then averaged. To allow for presentation of concentration-response data for both responses on a single set of axes, least squares fits to the data for excitatory and potassium responses were performed separately. The average response and standard error for each concentration of ATP were divided by the maximal current indicated by the fitted curves. The solid line represents the least squares fit to the data for the excitatory response. The parameters of this curve were $EC_{50} = 6.6\,\mu M$, Hill coefficient = 1.5, maximal current = −1092 pA. Except for the expected difference in the maximal current reached, the curve fitted to the potassium current data was not significantly different ($EC_{50} = 5.1\,\mu M$, Hill coefficient = 1.6, maximal current = +773 pA).

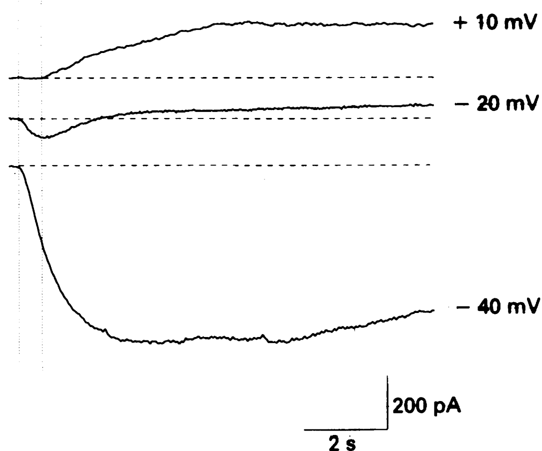

Figure 1 Activation of two distinct currents in chick skeletal muscle by ATP. Application of 10 µM ATP began at the time indicated by the first dotted line, and continued throughout the traces. For each trace, a horizontal dashed line indicates the holding current prior to the application of ATP. When a myoball was held at −40 mV (bottom trace), a large inward current was elicited with a delay of only a few milliseconds. After several seconds the current began to return towards the baseline. This return to baseline represented the activation of a second current. The presence of the second current could be seen more clearly when myoballs were held at more positive potentials. At −20 mV (middle trace) a biphasic current response was observed; the net current elicited by ATP was first inward and then outward. When a myoball was held at +10 mV (top trace), the early phase of the ATP response was absent, because the cell was at its reversal potential. ATP elicited only an outward current with a delay of several hundred milliseconds between the beginning of ATP application and the beginning of the response (indicated by second dotted line). At potentials positive to +10 mV (not shown), ATP evoked outward currents within a few milliseconds of application.

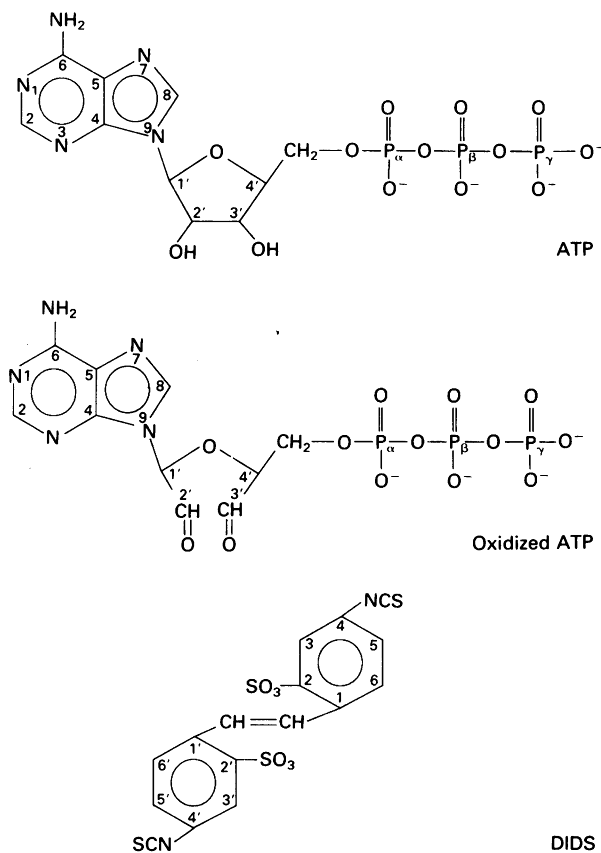

Figure 3 Primary structure of ATP, 2′,3′-dialdehyde-ATP (oxidized ATP) and 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS).

to a potential of −70 mV. At least 10 fibres from two different platings were tested for each analogue. The ability of ATP analogues modified at various positions to elicit a depolarization is summarized in Table 2.

Twenty six of the ATP analogues that were tested for their ability to activate the excitatory response were also tested for their ability to activate the potassium current. In these experiments, myoballs were voltage clamped at +10 mV, the reversal potential for the excitatory response. The potassium current had a pharmacological spectrum very similar to that for the excitatory current; each of the fifteen analogues that elicited the excitatory current also activated the potassium current and each of the eleven analogues that failed to activate the excitatory current also failed to activate the potassium current. The concentration-response relationships for both responses were examined semi-quantitatively for all 15 agonists (Table 2) and quantitatively for 8 agonists covering a wide spectrum of potencies (Table 3). For all agonists, there was great similarity in the EC_{50} for the excitatory and potassium currents activated by ATP. For 6 of the 8 agonists examined in detail, statistical analysis indicated that a single, averaged curve could describe the concentration-response relationship for either response as well as the individual curves, but statistical analysis indicated that, at the 5% confidence level, the best curves describing the two responses elicited by ATP-γ-S and by 3′-deoxy-ATP were not the same. One interpretation of these results is that the excitatory and potassium currents are activated by two distinct, but similar receptors. However, this conclusion may be incorrect. In both of these cases, one of the curves had a Hill coefficient that, based on the data for the other agonists, was aberrant. If the fits were constrained to have a Hill coefficient between 1 and 2, as suggested by the bulk of the data for agonists of high potency, then the best fitted curves for these two agonists were no longer significantly different. For this reason, there is at present no compelling reason to postulate that more than a single class of ATP receptors is present on the surface of chick skeletal muscle cells.

Table 2 Activity of ATP analogues

Site altered	*Agonist potency*			*Inactive compounds*
	EC_{50} $<50\,\mu M$	EC_{50} $>50\,\mu M$ and $<500\,\mu M$	EC_{50} $>500\,\mu M$	
Adenine	$2\text{-}CH_3S$-ATP!			8-Br-ATP*!
			N^1-oxide-ATP!	$1,N^6$-etheno-ATP
				ITP!; GTP!
				CTP!; UTP!
Ribose		2′-deoxy-ATP!		oxidized ATP!
		3′-deoxy-ATP!		reduced ATP!
		2′/3′-BB-ATP!		
Phosphate	ATP-γ-S!			ADP-β-S!
		$ATP\text{-}OPO_3$!		$ADP\text{-}\beta\text{-}NH_2$!
		ADP!		ADP-β-morpholidate*
			AMP-PNP!	ADP-ribose
			AMP-CPP!	ADP-glucose*
			AMP-PCP!	$A\text{-}P_2\text{-}A$
			$A\text{-}P_3\text{-}A$!	AMP!
			$A\text{-}P_4\text{-}A$!	$AMP\text{-}\alpha\text{-}NH_2$
			$A\text{-}P_5\text{-}A$!	$AMP\text{-}OSO_3$
			$A\text{-}P_6\text{-}A$!	AMP-α-morpholidate*
				Adenosine!
Multiple				2′-deoxy-ADP*

To test whether an analogue was an agonist for the excitatory response, the analogue was pressure-ejected (at 1 mM, except where indicated by *, when 0.5 mM was used) from a pipette for 1 s while recording the membrane potential from a nearby myotube. The initial membrane potential of the myotube was adjusted to −70 mV prior to application of an analogue. The analogue was scored as an agonist if it produced a depolarization in several myotubes. For analogues that did not produce a response, we confirmed that the cells depolarized in response to 10 μM ATP. The potency of all agonists was estimated by use of intracellular recording methods. Because this method was only semi-quantitative, EC_{50}s are given only by their approximate magnitude, rather than as discrete values. The potency of a subset of agonists was also assessed quantitatively by whole-cell recording (Table 3). Compounds indicated with an ! were also tested for their ability to elicit potassium currents in myoballs voltage clamped to +10 mV. All compounds tested for activation of the potassium current fell in the same potency class as indicated for the excitatory responses. For abbreviations, see Methods.

Table 3 Potency of the agonists

	EC_{50} (μM)		*Hill slope*	
Agonist	*Excitatory*	*Potassium*	*Excitatory*	*Potassium*
ATP*	7	5	1.5	1.6
ATP-γ-S	5	11	3.4	2.2
2′-deoxy-ATP*	79	78	1.7	2.4
3′-deoxy-ATP	110	47	1.8	2.9
$ATP\text{-}OPO_3$*	62	188	2.1	0.8
ADP*	231	282	1.9	4.9
AMP-PNP*	3280	6380	0.8	0.5
AMP-CPP*	6620	5600	0.8	0.4

For each agonist, data for concentration-response curves were collected and analyzed as described in Methods. This table presents the parameters that best fitted the excitatory and potassium responses generated by each agonist. However, for 6 of the 8 agonists (indicated by *), statistical analysis indicated that a single curve could describe both the excitatory and potassium responses as well as the individual curves. For abbreviations, see Methods.

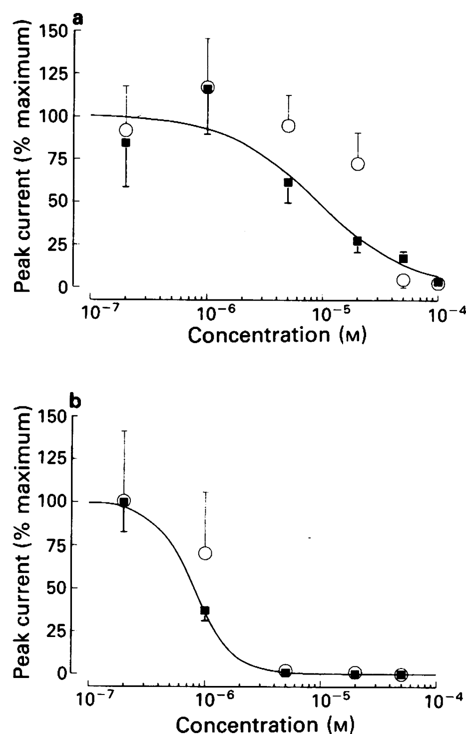

Figure 4 Inhibition of the two ATP responses by (a) 2′,3′-dialdehyde-ATP (oxidized ATP) and (b) 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS). Myoballs were incubated in the external solution with the indicated concentration of inhibitor for 1 h at room temperature prior to recording. The peak current activated by 50 μM ATP (applied from a pipette that did not contain the antagonist) was measured. The cells were voltage-clamped at −80 mV to study the excitatory current (■, thick error bars) or at +10 mV to study the potassium current (○, thin error bars). The smooth curves represent the curve fit to the data for the excitatory current. For oxidized ATP (a), the best curve for inhibition of the excitatory response had an IC_{50} of 9.4 μM and a Hill slope of −1.3, while the best curve for inhibition of the potassium conductance had an IC_{50} of 25 μM and a Hill slope of −4.2. For DIDS (b), the best curve for inhibition of the excitatory response had an IC_{50} of 0.8 μM and a Hill slope of −2.6, while the best curve for inhibition of the potassium conductance had an IC_{50} of 1.3 μM and a Hill slope of −2.9. In neither case did the two curves differ significantly.

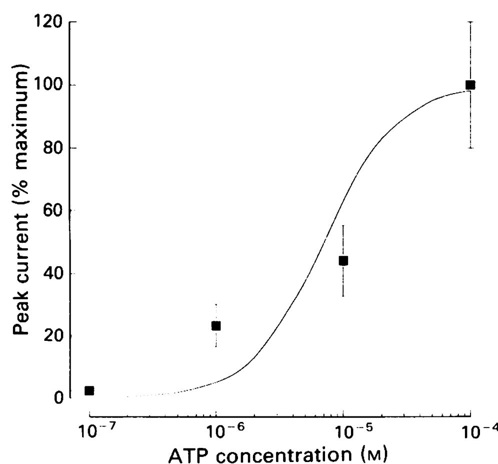

Figure 5 Activation of the excitatory response by ATP^{4-}. Myoballs were voltage clamped at −80 mV. The data points illustrated were taken from cells bathed in EDTA external, where virtually all the ATP was in the form of ATP^{4-}. The smooth curve is the one that was fitted to cells bathed in normal external solution, where virtually all the ATP was in the form of $M \cdot ATP^{2-}$. The curves were normalized to a single set of axes by assuming that the maximal current in these experiments was −295 pA/100 pF.

All analogues that were not agonists were tested as potential antagonists by adding the analogue (at 0.5 mM) to the external solution in the bath and to the solution in the pipette used to apply ATP. For these experiments, a concentration of 10 μM ATP was used, because it was in the steep region of the concentration-response curve, and thus would be most sensitive to antagonist activity.

Only two classes of ATP analogues were found to be antagonists. They were selective, since they did not inhibit the response of muscle cells to 10 μM acetylcholine. At a concentration of 0.5 mM, 8-Br-ATP reduced the response to 10 μM ATP and eliminated the response to 2 μM ATP. The triphosphate group was required for antagonism; neither 8-Br-ADP nor 8-Br-AMP were effective antagonists at 0.5 mM. Because it had to be present at high concentration to block the response to ATP, the antagonism by 8-Br-ATP was not analyzed in detail. A much more potent antagonist was the periodate-oxidized analogue of ATP: 2′,3′-dialdehyde-ATP (oxidized ATP). Inhibition by this molecule was both time- and temperature-dependent. With 100 μM oxidized ATP, almost complete antagonism developed in about 30 min at room temperature, and it developed more rapidly at 37°C. Inhibition could not be overcome with higher concentrations of ATP, and block was still effective after washing out the external solution containing oxidized ATP.

Approximately half of either the excitatory or the potassium conductance activated by ATP could be blocked by 10 μM oxidized ATP (Figure 4).

Interestingly, although ADP and AMP were much less potent agonists than ATP, oxidized ADP and oxidized AMP were as potent as oxidized ATP at blocking the response to ATP. However, oxidized adenosine had no effect on the ATP response. In addition, the oxidized forms of a number of nucleotide triphosphates that had no agonist activity (ITP, GTP, UTP, and CTP) blocked the response to ATP in a similar manner. However, these compounds required more time to be effective. For example, when a 1 h incubation at room temperature was used, these compounds were less effective than oxidized ATP, but they were almost as effective as oxidized ATP after a 1 h incubation at 37°C.

McMillian *et al.* (1988) reported that the stilbene derivative DIDS (4,4′-diisothiocyanatostilbene 2,2′-disulphonic acid) was a selective blocker of the ATP-induced increase in intracellular free calcium in rat parotid acinar cells. In chick skeletal muscle, DIDS was an even more potent antagonist of the ATP response than any of the ATP analogues tested. DIDS was similar to oxidized ATP in that its block was both time- and temperature-dependent, and could not be overcome with higher concentrations of ATP. Complete block of both conductances activated by ATP was observed with 20 μM DIDS, while approximately 1 μM DIDS was required to block half of each response (Figure 4). The related stilbene derivative SITS (4-acetamido-4′-isothiocyanatostilbene 2,2′-disulphonic acid) was approximately ten fold less potent in blocking the ATP response.

Activation: ATP^{4-} versus $MgATP^{2-}$

In a solution which contains divalent cations, the free anion ATP^{4-} is in equilibrium with $M \cdot ATP^{2-}$, where M represents a divalent cation. One class of ATP receptors is known to be much more sensitive to ATP^{4-} than to $M \cdot ATP^{2-}$ (Cockroft & Gomperts, 1979). Two sets of experiments were performed to evaluate the relative importance of ATP^{4-} and $M \cdot ATP^{2-}$. In one set of experiments, the total concentration of ATP was kept constant at 5 μM, but the ratio of ATP^{4-} to $Mg \cdot ATP^{2-}$ was varied by adding differing amounts of magnesium to solutions buffered with 11 mM EGTA. For the concentrations of total magnesium used, the free magnesium concentration varied from 36 μM to almost 1 mM, which produced concentrations of ATP^{4-} from 0.3 to 3.2 μM. Over this ten fold range of ATP^{4-}, the magnitude of the responses to ATP (estimated from intracellular recordings made from

myotubes) remained essentially constant (0.3 μM, -312 ± 38 pA; 1.7 μM, -349 ± 91 pA; 3.2 μM, -235 ± 25 pA). This result indicated either that the receptor for the excitatory response does not distinguish between ATP^{4-} and $Mg \cdot ATP^{2-}$, or that 0.3 μM ATP^{4-} is a saturating concentration. To test the latter possibility, cells were exposed to a solution containing 5 mM EDTA, without adding any divalent cations. In this solution ATP is almost entirely in its free anion form, ATP^{4-}. When myoballs were bathed in this solution and held at -80 mV, inward currents were activated by ATP over a concentration-range similar to that in the standard external solution, and the concentration-response relationship was not significantly different from that for ATP in normal external solution (Figure 5). Thus the receptor for ATP does not appear to discriminate between ATP^{4-} and $M \cdot ATP^{2-}$, nor does it require divalent cations for activation.

Discussion

ATP activates an excitatory conductance and a potassium conductance in developing chick skeletal muscle (Hume & Thomas, 1988). In the work described here, a series of ATP analogues was tested for their ability to activate these two responses. We found that the same set of analogues activated each conductance. Furthermore, the concentration-response relationship for each agonist was similar for the two responses. These results are consistent with the possibility that the two responses are activated by one receptor type for ATP, rather than by two distinct receptor subtypes.

There are at least three mechanisms by which a single receptor class could activate two distinct responses. One possibility is that activation of the second response is a consequence of activation of the excitatory response. For instance, calcium ions might flow into the cells during the early response and activate a calcium-dependent potassium current. However, this particular explanation cannot be correct, since the potassium current can be evoked in the absence of extracellular calcium. Recordings from cell-attached patches and excised outside-out patches indicate that the potassium current is activated by a diffusible second messenger and suggest that this messenger is not responsible for activation of the excitatory response (Hume & Thomas, 1990). Therefore, a second possibility is that binding of ATP to its receptor produces two different second messengers, one capable of only limited diffusion, which activates the excitatory response, and a second that can diffuse longer distances to activate the potassium current. A third possibility is that the ATP receptor is part of a molecular complex that comprises both an ion channel and an enzymatic activity that produces the second messenger that activates the potassium current. This third scenario is consistent with the observation that the excitatory current can be activated within a few milliseconds of application of ATP (Hume & Thomas, 1988). Finally, it is possible that there are two distinct, but very similar ATP receptors, one of which is coupled to the excitatory channel and the other coupled to the production of a second messenger.

The specificity of ATP

The receptor for ATP is relatively specific. ADP was about 20 times less potent as an agonist while AMP and adenosine were ineffective. Other nucleotide triphosphates were unable to activate the responses to ATP. Thus most if not all stimulation of this receptor *in vivo* probably results from exposure to ATP. Since most of the agonists had Hill slopes between 1 and 2, it seems likely that the receptor must bind 2 molecules of ATP to be activated.

By comparing the potency of the various analogues, one can learn what sites of the ATP molecule are important for activation of the receptor. The effect of modifying the adenine base of ATP depended on the site of modification. At least several sites of the adenine moiety appear to be responsible for nucleotide-specific recognition. These include N^1 and the amino group of C^6. Oxidation of the N^1 nitrogen created a low potency agonist. Reaction of ethene with N^1 and the amino group of C^6, which created a third ring, eliminated agonist activity. ITP, which is identical to ATP except that the amino group of C^6 is replaced by a carbonyl oxygen, was also ineffective. The area around C^2 seems to be relatively unimportant since the addition of a methylthio group to this carbon did not reduce potency. Additions to C^2 force ATP to stay in the 'anti' conformation, in which the base points away from the phosphates so that the molecule is fully extended (as shown schematically in Figure 3). In contrast, the addition of bromide to C^8 forces ATP to adopt the 'syn' conformation almost exclusively (the base points in the direction of the phosphates, so the molecule looks like a U). This addition eliminated agonist activity and instead produced an antagonist, suggesting that the 'anti' conformation is required for activation.

There also appeared to be important interaction sites between the receptor and the ribose of ATP. Both 2'- and 3'-deoxy-ATP were ten fold less potent than ATP as agonists. Interestingly, when these two sites were oxidized to aldehydes, the analogue became a fairly potent irreversible antagonist. These results suggest that normally the hydroxyls of the ribose hydrogen-bond to sites in the receptor. These sites might react to form a covalent bond when brought next to an aldehyde. When the aldehydes of oxidized ATP were reduced to alcohols, no antagonism was observed, demonstrating the importance of these aldehydes to antagonism. The lack of specificity for adenine as the base of the oxidized nucleotide, and the requirement that the nucleotide be only a monophosphate to block the response, suggests that the minimal molecular unit for an antagonist might be simply oxidized ribose 5'-monophosphate.

With respect to activation, the length of the phosphate chain was critical. Adenosine tetraphosphate and ADP were approximately ten fold less potent than ATP, and AMP and adenosine were without effect. Of the agonists tested, all modifications to the β phosphorous of ADP eliminated agonist activity. This may have been for steric reasons when large groups such as glucose or adenosine were added. However, the lack of any agonist activity with ADP-β-S and ADP-β-NH_2 in contrast to ADP suggests that the oxygen atoms in the second phosphate group may be important for activation. On the other hand, ATP-γ-S was as potent an agonist as ATP, indicating that the oxygens of the third phosphate are less crucial than those of the second. The two oxygens linking the three phosphates of ATP together must be important, since replacing them with either carbon or nitrogen lowered agonist potency dramatically. The di-adenosine phosphates (with the exception of the diphosphate) were low potency agonists. As might be expected for two separate sites of interaction between the receptor and ATP, combining two modifications that partially reduced the potency resulted in an analogue that was inactive (2'-deoxy-ADP).

Classification of ATP receptors on skeletal muscle

Since ATP but not adenosine is an agonist, then by definition the receptors must be in the second class of purinoceptors (P_2) (Burnstock, 1981). P_2 receptors have been broken down into several subtypes: T, X, Y, Z. The T and Z receptors have been described in circulating and immune cells, while X and Y have been found in smooth muscle, endothelium, liver, pancreas and parotid acinar cells (Gorden, 1986). The receptors on chick skeletal muscle do not meet the established criteria for belonging to any of these classes. The criteria for assigning a receptor to the P_{2X} class are that 2-CH_3S-ATP is approximately equipotent to ATP, (as was observed in chick skeletal muscle) and that AMP-PNP and AMP-PCP are more potent than ATP (contrary to what was observed in chick skeletal

muscle). The criteria for assigning a receptor to the P_{2Y} or P_{2T} classes are that AMP-PNP and AMP-PCP are less potent than ATP (as was observed in chick skeletal muscle) and that $2\text{-}CH_3S$-ATP is more potent than ATP (unlike chick skeletal muscle). In addition, the P_{2T} receptor responds to ADP as well as or better than ATP (unlike what was observed for chick skeletal muscle). Finally, the criteria for assigning a receptor to the P_{2Z} class are that the responses are largely or exclusively to ATP^{4-}, and that 2′/3′-BB-ATP is a more potent agonist than ATP. Both of these criteria are contrary to the results obtained for chick skeletal muscle.

The pharmacology of the chick skeletal muscle receptor appears to be most closely related to that described for cardiac muscle. ATP activates a small conductance cation channel in frog atrial myocytes (Friel & Bean, 1988), and increases intracellular free calcium in rat ventricular myocytes (De Young & Scarpa, 1987). The only pharmacological differences were that ADP was not effective at 100–200 μM, ATP-γ-S was slightly less potent than ATP, and AMP-CPP was an antagonist in frog cardiac muscle. ATP also activates a cation channel in arterial smooth muscle, but this receptor probably falls under the P_{2X} subtype since AMP-CPP was equipotent with ATP (Benham & Tsien, 1987).

ATP also elicits excitatory responses from several neuronal cell types. ATP activates cation channels in rat sensory neurones of spinal cord and brainstem ganglia (Krishtal *et al.*, 1983; 1988). These receptors have a similar pharmacology to that in chick skeletal muscle. However, for other neuronal ATP receptors the pharmacology is quite different. In the central nervous system, a subpopulation of dorsal horn neurones are excited by ATP. However, excitation is also elicited by AMP-PNP and AMP-PCP, suggesting that this receptor is of the P_{2X} subtype (Jahr & Jessel, 1983). A much less specific excitatory response is seen in bullfrog sympathetic neurones, where most nucleotide triphosphates are effective (Siggins *et al.*, 1977).

Finally, it has been shown that ATP stimulates the production of inositol phosphates in chick skeletal muscle (Haggblad & Heilbronn, 1987). However, the reported specificity of this response is different from what we have described for channel activation. Production of inositol phosphates is reported to be stimulated equally well by ATP and AMP-PNP, and less potently by AMP and adenosine. These results seem to suggest the possibility of two receptor subtypes for ATP in chick skeletal muscle. The alternative hypothesis is that activation of only a small number of receptors by AMP-PNP is sufficient to produce a significant increase in inositol phosphates, but not an electrical response. It would be interesting to test whether oxidized ATP or DIDS blocked the accumulation of inositol phosphates by ATP.

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