Permeation of Both Cations and Anions through a Single Class of ATP-activated Ion Channels in Developing Chick Skeletal Muscle

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ABSTRACT Micromolar concentrations of extracellular adenosine 5'-triphosphate (ATP) elicit a rapid excitatory response in developing chick skeletal muscle. Excitation is the result of a simultaneous increase in membrane permeability to sodium, potassium, and chloride ions. In the present study we quantify the selectivity of the ATP response, and provide evidence that a single class of ATP-activated ion channels conducts both cations and anions.

Experiments were performed on myoballs using the whole-cell patch-clamp technique. We estimated permeability ratios by measuring the shift in reversal potential when one ion was substituted for another. We found that monovalent cations, divalent cations, and monovalent anions all permeate the membrane during the ATP response, and that there was only moderate selectivity between many of these ions. Calcium was the most permeant ion tested. To determine if ATP activates a single class of channels that conducts both cations and anions, or if ATP activates separate classes of cation and anion channels, we analyzed the fluctuations about the mean current induced by ATP. Ionic conditions were arranged so that the reversal potential for cations was +50 mV and the reversal potential for anions was −50 mV. Under these conditions, if ATP activates a single class of channels, ATP should not evoke an increase in noise at the reversal potential of the ATP current. However, if ATP activates separate classes of cation and anion channels, ATP should evoke a significant increase in noise at the reversal potential of the ATP current. At both +40 and −50 mV ATP elicited a clear increase in noise, but at the reversal potential of the ATP current (−5 mV), no increase in noise above background was seen. These results indicate that there is only a single class of excitatory ATP-activated channels, which do not select by charge. Based on analysis of the noise spectrum, the conductance of individual channels is estimated to be 0.2–0.4 pS.

INTRODUCTION

As ion channels continue to be discovered and characterized, their diversity grows with the variety of physiological roles that they serve. Much of the earliest work examined the properties of channels that are selective for a single ion, such as volt-age-dependent sodium channels and delayed-rectifier potassium channels. Their voltage dependence, ionic selectivity, and kinetics are all crucial to their functioning as the propagators of action potentials along axons. Other channels, also highly selective for potassium ions, are thought to set the resting membrane potential in many cells. Perhaps the most striking example of ionic selectivity is shown by volt-age-dependent calcium channels. Channels of this class may be >1,000-fold more selective for calcium ions over potassium or cesium ions (Reuter and Scholz, 1977; Lee and Tsien, 1984). Many other channels select by charge but are not specific for any particular ion. For example, the acetylcholine channel at the neuromuscular junction conducts many different cations while excluding anions (Adams et al., 1980; Dwyer et al., 1980), and the GABA-gated channel of the central nervous system conducts anions while excluding cations (Bormann et al., 1987).

Adenosine 5'-triphosphate (ATP) has an excitatory effect on developing chick skeletal muscle (Hume and Honig, 1986). Interestingly, excitation is the result of a simultaneous increase in membrane permeability to sodium, potassium, and chloride ions (Hume and Thomas, 1988). However, it is not known whether ATP activates a single class of channels that conducts both cations and anions, or whether it activates two separate classes of channels, one that is selective for cations and another that is selective for anions. One approach to answering this question would be to study the properties of single channels activated by ATP. However, since the effect of ATP on the whole-cell current noise of myoblasts is quite small (Hume and Honig, 1986), it seemed likely that the single-channel currents would be difficult to resolve from the background noise. Instead we decided to study the selectivity of the ATP response by measuring the reversal potential of whole-cell currents in dialyzed myoballs. We found that monovalent cations, divalent cations, and monovalent anions permeate the membrane during the ATP response. To determine whether one class of channels or separate classes of cation and anion channels are activated during the excitatory ATP response, we analyzed the current noise elicited by ATP under special ionic conditions. ATP appears to activate a single class of channels with a unitary conductance we estimate to be 0.2–0.4 pS. A preliminary report on some of this work has appeared (Thomas and Hume, 1988).

METHODS

Cell Culture

Standard chick embryo muscle cell cultures were prepared as described previously (Hume and Honig, 1986). Briefly, pectoral muscle was dissected from 11-d 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 hemocytometer after the suspension was filtered through lens paper to remove debris. Cells were plated onto gelatin-coated tissue culture dishes (Corning) at 150,000 per 35-mm dish. The culture

medium, Eagle's MEM with Earle's salts (Gibco, Grand Island, NY), was supplemented with 10% heat-inactivated horse serum (Gibco), penicillin/streptomycin (50 U/ml, 50 μg/ml), and conalbumin (40 μg/ml; Sigma Chemical Co., St. Louis, MO). Cultures were maintained in a humidified incubator at 37°C with an atmosphere of 95% air, 5% CO_2. We made recordings from mononucleate myoblasts after 1–2 d in cell culture, and from myotubes after 6–10 d in culture.

Myoballs (spherical, multinucleate muscle cells) were made in an identical manner up to the cell plating stage. Cells were preplated at 750,000 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. 3–6-d-old myoballs were used for recording.

Solutions

Just before recording, each culture dish was washed at least three times over a period of 5 min with the appropriate external solution (Table I) to replace the incubating media. Each wash exchanged ~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) to maintain the pH between 7.2 and 7.4. The pH was adjusted with the appropriate hydroxide as indicated in Table I. To determine permeability ratios, a standard recipe for the external solution was used. Monovalent ions were tested at 100 mM, cations were tested as their chloride salts, and anions were tested as their cesium salts. The divalent anion sulfate was tested using 50 mM Cs_2SO_4 in the external solution. Divalent cations were tested by adding their chloride salt at 5 mM to a 100-mM CsCl external solution. When fluoride was tested, a CsF internal solution was used, since it was extremely difficult to obtain high resistance seals when fluoride was present in the external solution. The pH was set at 7.2 with 10 mM HEPES and ~3 mM CsOH, and the osmolarity was adjusted to 300 mosmol with ~100 mM sucrose. We used the nominal ion concentrations in the calculations of permeability ratios since activity coefficients were not available for some of the ion pairings we tested. When calculations were made with available coefficients, the permeability ratios changed by at most 4%, and none of the orderings in the permeability series were altered.

Whole-Cell Patch-Clamp and Intracellular Recording

All experiments except those documenting block by some divalent and trivalent cations (e.g., Fig. 4) were performed using the whole-cell patch-clamp technique. Standard techniques were used to form high resistance seals with pipettes onto the membrane of myoblasts or myoballs and to gain access to the cell interior (Hamill et al., 1981). Our polished pipettes had resistances of 2–4 MΩ, and we recorded from myoballs whose diameters ranged from 15 to 30 μm. Liquid junction potentials were measured by filling a patch pipette with the internal solution, adjusting the zero current potential to 0 mV in internal solution, then measuring the zero current potential in each external solution. A 3 M KCl broken-tip microelectrode was used for reference.

For the experiments measuring block of the ATP response by some divalent and trivalent cations, intracellular recordings were made using conventional glass microelectrodes filled with 3 M KCl as described previously (Hume and Honig, 1986). The high input resistance of

TABLE I

Composition of Solutions (in Millimolar)

Solution	Internal solutions					
	KCl	CsCl	CsF	High CsNO$_3$	Low CsNO$_3$	NaCl
KCl	100	—	—	—	—	—
CsCl	—	115	—	—	—	—
CsF	—	—	115	—	—	—
CsNO$_3$	—	—	—	150	21	—
NaCl	10	—	—	—	—	140
HCl	—	4	4	—	—	—
KOH	5	—	—	—	—	—
TEA·OH	—	—	—	43	43	43
K$_4$BAPTA	10	—	—	—	—	—
Cs$_4$BAPTA	—	10	10	—	—	—
H$_4$BAPTA	—	—	—	10	10	10
CaCl$_2$	1	2	2	1	1	1
MgCl$_2$	2	—	—	—	—	—
HEPES	10	10	10	10	10	10
Phenol red	0.03	0.03	0.03	0.03	0.03	0.03
Glucose	60	40	40	10	—	10
Sucrose	—	—	—	—	200	—

Solution	External solutions					
	Standard blocking	Low Cl⁻ blocking	Low CsCl	Low CsCl/BaCl$_2$	CsNO$_3$	NaCl
NaCl	110	—	—	—	—	140
Na·acetate	—	110	—	—	—	—
NaOH	5	5	—	—	—	—
KCl	4	—	—	—	—	—
K·acetate	—	4	—	—	—	—
CsCl	—	—	20	20	—	—
CsNO$_3$	—	—	—	—	150	—
CsOH	—	—	3	3	—	—
TEA·OH	—	—	—	—	3	3
TEA·Cl	20	20	—	—	—	—
MgCl$_2$	1	1	—	—	—	—
CoCl$_2$	4	4	—	—	—	—
BaCl$_2$	—	—	2	—	1	1
Tetrodotoxin	10^{-4}	10^{-4}	—	—	—	—
HEPES	12.5	12.5	10	10	10	10
Phenol red	0.03	0.03	0.03	0.03	0.03	0.03
Glucose	10	10	—	—	40	45
Sucrose	—	—	235	240	—	—

myotubes bathed in the blocking 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 we used a single microelectrode with a balanced bridge circuit both to record voltage and to pass current. The bridge circuit was balanced just before the penetration of each cell.

Agonists were dissolved in the appropriate external solution and loaded into puffer pi-

pettes having tip diameters of 2–4 μm. The duration of the pressure pulse was accurately controlled by a solenoid value 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 ~30–60 μm from the test cell. The latency of the ATP response varied from ~100–500 ms depending on the distance of the ATP-containing pipette from the cell, and the concentration of ATP.

Noise Analysis

Fluctuations in current were recorded from myoballs using the whole-cell voltage-clamp configuration. The current signal from the patch-clamp amplifier (List EPC 5) was fed into two channels of a Neuro Data neurocorder, each channel sampling at 44 kHz, and stored on video tape. One channel sampled the unfiltered signal at low gain, while the second channel sampled the signal at high gain, usually after it had been AC-coupled with a high-pass filter (4-pole Butterworth, f_c = 3 Hz). In some cases records collected at high gain were not high-pass filtered, and we found that AC-coupling did not alter the results. We analyzed the stationary current noise in a manner similar to that originally described by Anderson and Stevens (1973). Records were low-pass filtered at 600 Hz with an 8-pole Butterworth filter and sampled at 2 kHz from tape with a 12-bit analog-to-digital converter. The power spectrum of the data was obtained with the fast Fourier transform algorithm of BASIC-23 (INDEC). The data were cosine-tapered, and 50% overlapped processing was used to limit data loss (Bendat and Piersol, 1986). We applied 1 μM ATP or ACh by pressure-ejection from a nearby pipette for 16 s. In all cases the background spectrum, obtained during the application of external solution without agonist, was subtracted from the spectrum obtained with agonist to give the spectrum of the noise induced by the agonist. This procedure was followed because we found that the background noise sometimes increased during application of the external solution. Such an effect could be due to stretch-activated channels present in the muscle cell membrane (Guharay and Sachs, 1984).

Using the equations outlined by Anderson and Stevens (1973), spectra were fit with the following Lorentzian function:

$$S(f) = S(0)/(1 + f^2/f_c^2) \tag{1}$$

where f is the frequency and $S(f)$ is the spectral density as a function of frequency. The values for the zero-frequency asymptote, $S(0)$, and the corner frequency, f_c, were adjusted by eye to fit the curve to the data. These values were used to calculate the single-channel conductance, γ, and the mean open time of the channel, τ, with the following equations:

$$\gamma = \pi^* f_c^* S(0)/2I^* E_{Ag} \tag{2}$$

$$\tau = 1/2^* \pi^* f_c \tag{3}$$

where I is the steady-state current, and E_{Ag} is the driving force for the agonist-induced current. This method for estimating the single-channel conductance depends on the assumption that p, the probability that any given channel is in the open state, is much less than 1. Data are presented in Results which indicate that p was low.

Unitary conductance was also calculated by a second method. At steady-state, the mean current, I, and the variance of the current, σ_i^2, are given by the equations:

$$I = N^* p^* \gamma^* E_{Ag} \tag{4}$$

$$\sigma_i^2 = N^* p^* (1 - p)^* \gamma^2 * E_{Ag}^2 \tag{5}$$

where N is the total number of channels. If $p \ll 1$ so that $1 - p$ can be approximated as 1, then the following equation can be used to calculate the single-channel conductance:

$$\gamma = \sigma_i^2/I^*E_{Ag}. \tag{6}$$

This method typically gave estimates for the single-channel conductance that were 80–90% of the spectral estimates, probably due to the low- and high-pass filtering of the signal. All single-channel values reported are those obtained from the spectral estimates.

RESULTS

To study the selectivity of the ATP response, we needed to voltage clamp the membrane potential and dialyze the intracellular compartment of muscle cells. We developed a simple procedure for culturing large numbers of myoballs, which are multinucleate muscle cells that have not elongated (see Methods). These relatively small,
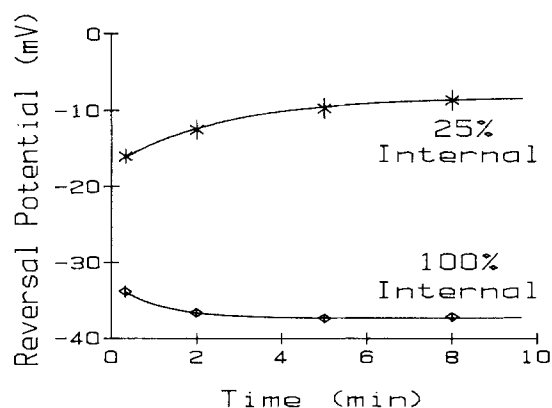

FIGURE 1. Time course of intracellular dialysis as measured by the reversal potential of ACh-activated currents. Dialysis was rapid within the first 20 s of rupturing the cell membrane, and was essentially complete by 5 min. Myoballs were bathed in the low CsCl/BaCl$_2$ external solution and dialyzed with the CsCl internal solution either at full strength (100%, diamonds), or diluted to one-quarter strength with isotonic sucrose (25%, *). ACh was applied to each cell at 20 s, 2, 5, and 8 min after the beginning of dialysis, and to most cells at 15 min. The reversal potential was measured as described in Fig. 3. The data for the 100% internal solution is the average of 7 cells while that for the 25% internal solution is the average of 10 cells. In this experiment we were interested only in the time course of dialysis, so we did not correct for liquid junction potentials. This may explain why the final reversal potential indicated for each internal solution was positive to that calculated from the Nernst equation for cesium. Error bars represent the SEM.

spherical muscle cells could be voltage-clamped and dialyzed with patch pipettes. The cells had diameters which ranged from 15 to 30 μm, which is larger than the diameter for many other cell types studied using patch-clamp techniques, so it was necessary to determine the dialysis time for these cells. This was done by measuring the change in the reversal potential of acetylcholine (ACh)-induced currents while dialyzing cells with one of two internal solutions. Cells were bathed in the low CsCl/BaCl$_2$ external solution and dialyzed with the CsCl internal solution either at full strength, or diluted to 25% ionic strength with an isotonic sucrose/10 mM HEPES solution. The reversal potential became more positive as cells were dialyzed with the low ionic strength solution, and more negative as cells were dialyzed with the full ionic strength solution (Fig. 1). There was very little change in the reversal potential

after 5 min, and for the cells we examined at 15 min, the reversal potential was almost identical to that at 8 min. Based on these results, we allowed at least 5 min to pass between gaining access to the cell interior and data collection.

Currents Activated by ATP

We first dialyzed myoballs with a simple salt solution in which the internal free calcium was buffered at 10^{-8} M (KCl internal solution), to test whether the response to ATP was affected by long periods of dialysis. There was no significant difference in the magnitude of the ATP response when cells were tested after short or long dialysis times. ATP elicited an average inward current of -133 pA (SEM $=$ 39, $n = 7$) after 15 s of dialysis, and -163 pA (SEM $=$ 59, $n = 7$) after 5 min of dialysis when cells were held at -80 mV. Furthermore, cells that had been dialyzed for 15 min or longer could also exhibit large responses to ATP. Thus ATP sensitivity does not appear to depend on intracellular factors such as nucleotides or soluble proteins, which can be dialyzed away by the pipette solution. However, as had been previously shown with ATP-induced depolarization (Hume and Honig, 1986), the ATP-induced currents exhibited pronounced long-term desensitization. Typically, ATP responsiveness was completely eliminated by several applications of 50 μM ATP, and greatly reduced by several applications of 1 μM ATP. No recovery was observed 10 min after an initial exposure to ATP. For this reason, we usually studied the responses of a series of cells, rather than studying multiple responses of individual cells.

In addition to an early inward (excitatory) current, ATP also activates a potassium conductance after a delay of $\sim$1 s (Hume and Thomas, 1988). We found that by substituting cesium for potassium in the internal solution, the late increase in potassium conductance was eliminated (Fig. 2 *A*). The reversal potential for the excitatory current in these solutions was $\sim +10$ mV. When cells were held at 0 mV and dialyzed with the KCl internal solution, ATP activated a small inward current that was followed by a larger outward current. However, when cells were held at 0 mV and dialyzed with the CsCl internal solution, only an inward current was activated by ATP. Furthermore, the current noise in these cells was much smaller, presumably due to the blockage by cesium of resting and ATP-activated potassium channels.

The duration of ATP action was quite long. For instance, in Fig. 2 *A*, a short (1 s) application of ATP activated an inward current that was still at 56% of its peak at 16 s. The long-lasting action of ATP was not due to the slow diffusion of the pressure-ejected agonist away from the cell. The approximate time course of diffusion of agonist away from a cell in our system was determined in experiments in which ACh was applied to cells. ACh-activated currents decayed with a time constant of 1–3 s, and completely returned to baseline within 10 s after a pressure pulse ended (Fig. 2 *B*). Since the ATP-evoked currents persisted long after free ATP had most likely diffused away, we conclude that a transient application of ATP activates a long-lasting current.

Selectivity of the Excitatory ATP Conductance

Because we needed to have cesium present in the internal solution to block the late increase in potassium conductance, we chose to use cesium as the reference ion for

all our permeability measurements. Since the excitatory response to ATP causes an increase in membrane permeability to sodium, potassium, and chloride (Hume and Thomas, 1988), and the internal solution usually contained CsCl, we first determined the relative permeability of chloride to cesium. Reversal potentials for agonist-induced currents were measured by depolarizing cells with linear ramp stimuli before and during the application of agonist (Fig. 3).
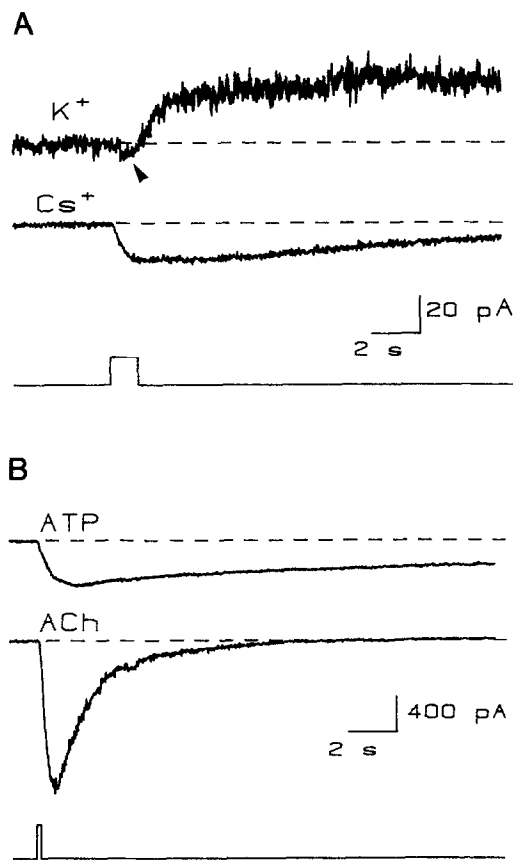

FIGURE 2. Isolation of the early excitatory current. (A) Myoblasts were bathed in the low Cl⁻ blocking external solution and dialyzed with either the KCl or the CsCl internal solution. The cells were held at 0 mV, just negative to the reversal potential for the early excitatory current, and 10 μM ATP was applied for 1 s as indicated by the bottom trace. With the KCl internal solution, the ATP response consisted of an early inward current (*arrow*) followed by a larger outward current (which overwhelmed the initial inward current). With the CsCl internal solution, only inward currents were observed, and the current noise was greatly reduced, presumably because of the blockage of resting and ATP-activated potassium channels. (B) ATP-activated currents are long-lasting. Pressure ejection of ATP (50μM) or ACh (10μM) from a nearby pipette for 200 ms (indicated by the bottom trace) led to long-lasting activation of ATP- but not ACh-activated currents, which suggests that long-lasting activation is intrinsic to the ATP response. These myoballs were bathed in the standard blocking solution and dialyzed with the CsCl internal solution. The cells were held at −80 mV. In A and B the dotted lines indicate the current in the absence of agonist.

In our initial experiments, cells were dialyzed with the CsCl (115 mM) internal solution and bathed in the low CsCl (20 mM) external solution. Under these conditions the expected reversal potential for cesium is quite negative (−48.0 mV), while the expected reversal potential for chloride is quite positive (+45.8 mV). On average, the reversal potential for the ATP-activated currents was −21.7 mV

(SEM = 1.3, n = 17). To gain an independent estimate of the cesium reversal potential, we determined the reversal potential to ACh, since the ACh receptor (AChR) channel is highly selective for cations over anions (Adams et al., 1980). The reversal potential of the ACh-activated currents was −41.5 mV (SEM = 0.2, n = 26). We also measured reversal potentials when cells were bathed in an external solution containing 100 mM CsCl (plus 3 mM CsOH, see Methods). In this solution, the average reversal potential of the ACh-gated currents was −5.3 mV (SEM = 0.5, n = 12), while the reversal potential for the ATP-gated currents was −1.5 mV (SEM = 0.4, n = 15).

In both the 20 mM CsCl and 100 mM CsCl external solutions, the measured reversal potentials of the ACh-gated currents were 5–6 mV positive to those calculated from the Nernst equation. To compensate for this offset, we calculated perme-
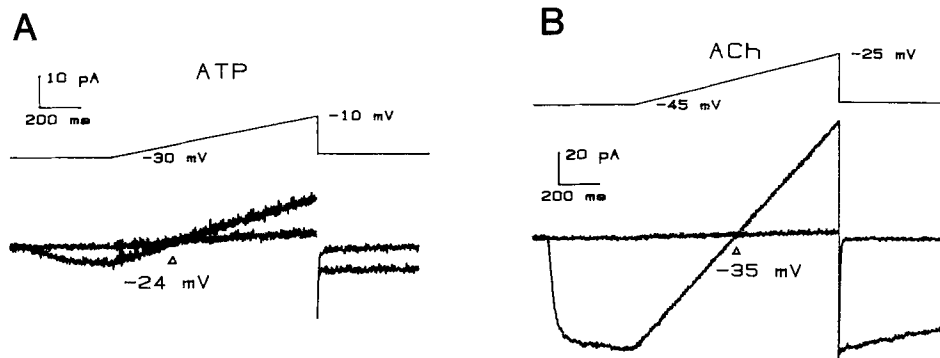

FIGURE 3. Method for determining the reversal potential of ATP-and ACh-activated currents. To determine the reversal potential for agonist-activated currents, cells were first depolarized with a linear ramp stimulus of 10 or 20 mV for 1 s. The agonist was then applied, and 0.5 s later the cells received a second identical ramp stimulus. Reversal occurred at the potential where the two traces crossed, i.e., where the agonist-induced current went from inward to outward. This myoball was bathed in the low CsCl external solution and dialyzed with the CsCl internal solution. First 10 μM ATP (*top*) and then 10 μM ACh (*bottom*) was tested. In this cell, the reversal potential for the ATP-activated current was 11 mM positive to that for the ACh-activated current. The membrane potentials listed here have not been corrected for the −5.0 mV liquid junction potential between the internal and external solutions.

ability ratios for the ATP-activated current from the difference in reversal potentials of the ACh- and ATP-induced currents. Assuming there is no chloride permeability through the ACh channel (Adams et al., 1980), the 19.8-mV difference between the reversal potentials in the low CsCl external solution gives a permeability ratio, P_{Cl}/P_{Cs}, of 0.24 for the ATP-activated conductance, while the 3.8-mV difference in the 100 mM CsCl solution gives a P_{Cl}/P_{Cs} of 0.39.

We made measurements of the reversal potentials of the ATP responses in a series of solutions to obtain permeability ratios (P_X/P_{Cs}) for cations and anions (Table II). The difference between the ATP reversal potential in the test and the reference solutions was used to solve the Goldman equation for P_X/P_{Cs}. To make this calculation one needs a value for P_{Cl}/P_{Cs}. We used P_{Cl}/P_{Cs} = 0.39 rather than

TABLE II

Both Cations and Anions Permeate the Membrane in Response to ATP

Ion	$E_{rev} \pm$ SEM	N	P_{ion}/P_{Cs}
	mV		
Potassium	+2.9 ± 1.2	8	1.28
Lithium	+2.6 ± 0.5	8	1.26
Rubidium*	+1.9 ± 0.4	9	1.15
Cesium	−1.5 ± 0.4	15	1
Sodium	−2.0 ± 0.6	10	0.97
Methylammonium*	+11.1 ± 0.4	10	1.93
Dimethylammonium*	+5.6 ± 0.6	10	1.43
Trimethylammonium*	−6.4 ± 0.6	12	0.19
Tetramethylammonium*	−20.0 ± 0.9	14	0.10
Tetraethylammonium	−35.0 ± 2.6	17	0
Nitrate	−11.8 ± 0.3	9	1.34
Iodide	−9.9 ± 0.5	10	1.13
Bromide	−3.0 ± 0.5	10	0.50
Chloride	−1.5 ± 0.4	15	0.39
Fluoride (internal)	−3.6 ± 0.5	12	0.22
Sulfate	+5.1 ± 1.3	11	0
Glucuronate	+5.6 ± 1.5	12	0
Calcium	+1.5 ± 0.2	10	2.09
Nickel	+0.9 ± 0.4	9	1.69
Strontium	+0.4 ± 0.2	10	1.38
Barium	−0.2 ± 0.2	10	0.99
Cobalt	−0.5 ± 0.3	10	0.84
Magnesium	−1.5 ± 0.2	10	0.30

Reversal potentials were estimated by depolarizing cells with linear ramp stimuli during the application of 10 μM ATP (see Fig. 3), and were corrected for liquid junction potentials between the internal and external solutions. The external solutions all contained 100 mM of a monovalent test ion as either the cesium or chloride salt. Sulfate was tested with 50 mM Cs_2SO_4 in the external solution. When solutions contained divalent cations, they were added at 5 mM as the chloride salt. All external solutions contained 10 mM HEPES and ~3 mM CsOH so that the pH was buffered at 7.2, and ~100 mM sucrose so that the osmolarity was adjusted to 300 m/smol. Except when fluoride was being tested, myoballs were dialyzed with the CsCl internal solution and bathed in the appropriate external solution. Because extracellular fluoride made it very difficult to obtain the whole-cell recording configuration, we estimated fluoride permeability by bathing the cells in the 100 mM CsCl/5mM $BaCl_2$ solution, and dialyzing them with an internal solution in which CsCl was replaced with equimolar CsF. Permeability ratios were calculated with the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949; Spangler, 1972) using the shift in reversal potential between the solution containing the test ion and the CsCl solution.

*For these ions, 5 mM $BaCl_2$ was also included in the external solution to reduce large leakage currents and aid in the formation of high-resistance membrane seals. In these cases the reference solution used to determine shifts in reversal potential contained 100 mM CsCl and 5 mM $BaCl_2$.

0.24, since the latter value was obtained with a solution of much lower ionic strength than that used in our test and reference solutions.

We found that there was very little selectivity among the alkali metals. The sequence of permeabilities we obtained was K > Li > Rb > Cs > Na, but the differ-

ences between potassium and lithium, and between cesium and sodium, were quite small. We also tested a series of organic cations, and found that their permeability decreased as their size increased. Methylammonium and dimethylammonium were more permeant than any of the alkali metals, while trimethylammonium and tetra-methylammonium were considerably less permeant, and tetraethylammonium was impermeant. A similar result was found for the permeability ratios of anions. Their permeability decreased as their hydration radius increased. In order of decreasing
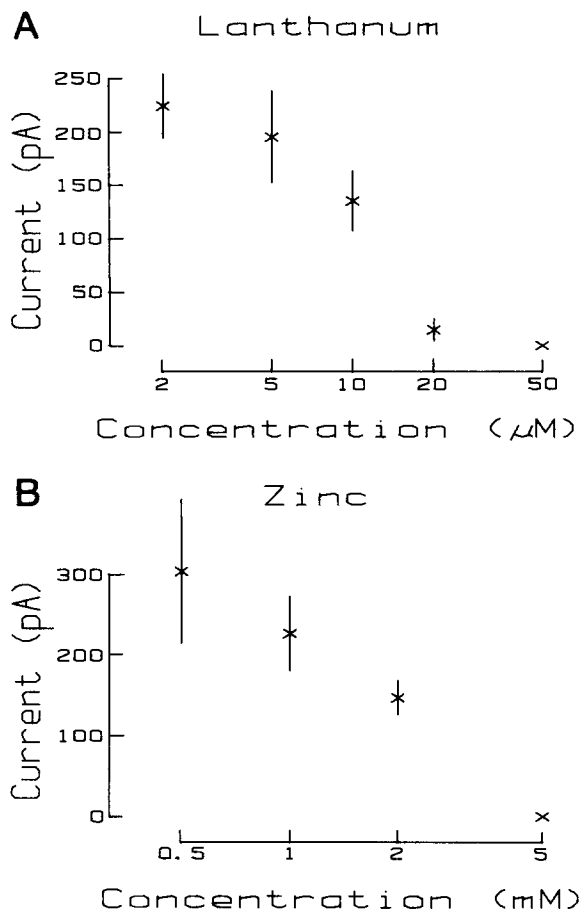

FIGURE 4. The dose-response relationship for block of the ATP response by lanthanum and zinc. (A) Lanthanum blocked the response to ATP in a dose-dependent manner, with complete block occurring at 50 μM. Lanthanum was added at the concentrations indicated to the standard blocking solution. (B) Zinc also exhibited a dose-dependent block of the ATP response. Zinc replaced the magnesium and cobalt in the standard blocking solution at the concentrations indicated. Cadmium and manganese had similar dose-response curves; each completely blocked the ATP response at 5 mM. The data were collected by recording intracellularly from myotubes. Each cell was initially adjusted to −70 mV, and then 10 μM ATP was applied for 1 s. The peak depolarization was divided by the initial input resistance to obtain an estimate for the current activated by ATP. Each point is the average current from at least five cells; error bars represent the SEM.

permeability, these ions were NO_3 > I > Br > Cl > F; glucuronate and sulfate were impermeant.

In addition, we measured the permeabilities to divalent cations, each being added at a concentration of 5 mM to the 100 mM CsCl external solution. We chose this concentration because it is near that for calcium and magnesium in vivo. Some divalent cations completely blocked the ATP response at 5 mM; these included cadmium, manganese, and zinc (Fig. 4). The ATP response was also completely blocked

by 50 μM lanthanum. Interestingly, of all the ions tested, calcium was the most permeant. P_{Ca}/P_{Cs} was 2.09, seven times that for magnesium (Table II). Overall however, the conductance activated by ATP did not select very strongly between divalent cations; other alkaline earth and transition metal divalent cations had permeabilities similar to those for the monovalent cations.

Ions with high permeability tended to produce large currents. For instance, the currents observed in the LiCl, CsI, and CsNO$_3$ external solutions were much larger than the currents observed in the CsCl external solution. Similarly, the addition of 5 mM calcium, strontium, or barium (but not the other divalent cations tested) to several different external solutions increased the size of the ATP-activated currents. An exception to this tendency is that only moderate currents were observed in the KCl external solution, even though potassium had a relatively high permeability. It is also interesting to note that the currents activated by ATP were not noticeably smaller when impermeant ions such as TEA or glucuronate were used in the external solution. However, this is not too surprising, since both cesium and chloride ions from the internal solution were still available to carry current.

ATP Activates a Single Class of Excitatory Channels

The fact that tetraethylammonium, glucuronate, and sulfate were all impermeant during the ATP response suggested that ATP activates channels with rather narrow pores. An interesting question follows from this idea. Does ATP activate a single class of channels capable of selecting by size but not by charge, or does ATP simultaneously activate two separate classes of ion channels, one that is selective for cations and another that is selective for anions? To test these two possibilities, we measured the variance of the current noise during the application of ATP. Dionne and Ruff (1977) used a similar approach to demonstrate that both sodium and potassium permeate the AChR channel. This analysis is based on the principle that the variance of the current noise from a single class of ion channels will be zero at their reversal potential. At potentials positive and negative to reversal, the variance will be positive since variance is proportional to the square of the driving force. If more than one class of channels is present, the current noise from each would sum to give the total current noise. Thus if an agonist-gated current is due to the activation of two channel classes, and the reversal potential for the current from each channel class is far from the reversal potential of the agonist-gated current, then there will be an increase in noise at the reversal potential of the agonist-gated current.

To test whether ATP activated one or two channel classes, we used solutions containing CsNO$_3$ because cesium eliminates the late increase in potassium conductance, and the permeability of nitrate is comparable to that of cesium. The internal solution was made low in ionic strength (21 mM) relative to the external solution (150 mM) so that the reversal potential for cations would be quite positive (calculated E_{Cs} = +50 mV), and the reversal potential for anions would be quite negative (calculated E_{NO_3} = −50 mV). Under these conditions, if ATP activates a single class of channels, then there should not be an increase in current noise when ATP is applied at the reversal potential of its current (−5 mV). However, if ATP activates separate cation and anion channel classes, then there should be an increase in current noise when ATP is applied at the reversal potential of its current.

We measured the change in the variance of the current noise elicited by application of 1 μM ATP, which activated ~12% of the conductance activated by 100 μM ATP (near saturation). We chose this concentration because the spectral noise analysis described below assumes that only a small fraction of the total conductance is activated. ATP was applied for the duration of each 16-s sample record. We calculated the variance of the current noise once the current had reached a plateau, and

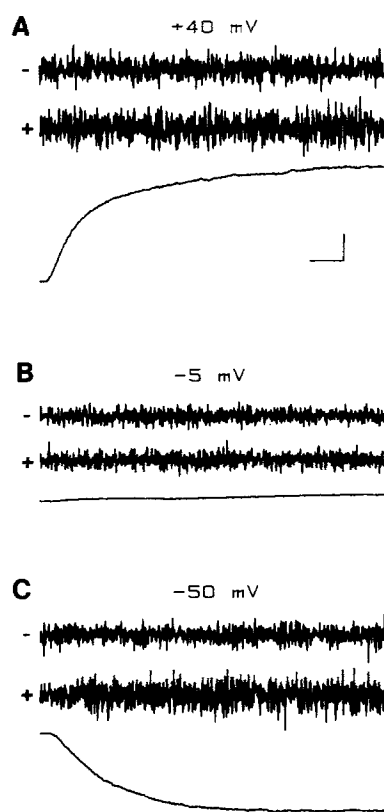**A**   +40 mV

B   −5 mV

C   −50 mV

FIGURE 5. ATP induces an increase in current noise at potentials positive and negative to the reversal potential, but not at the reversal potential. For each part of the figure, the top trace (−) is the high-gain, AC-coupled record before application of ATP, the middle trace (+) is the high-gain, AC-coupled record during the application of ATP, and the bottom trace is the low-gain record of the DC-coupled current induced by ATP. (A) ATP was applied first when the cell was held at +40 mV, causing an increase of 0.65 pA^2 in the variance of the current noise. (B) ATP was applied a second time when the cell was held at −5 mV, very close to the reversal potential. In this case there was a 0.01 pA^2 increase in the variance of the current noise. (C) ATP was applied a third time when the cell was held at −50 mV, causing an increase of 1.15 pA^2 in the variance of the current noise. The cell was bathed in the CsNO$_3$ external solution and dialyzed with the low CsNO$_3$ internal solution. Under these ionic conditions, the reversal potential for cations was +50 mV and the reversal potential for anions was −50 mV. All traces were low-pass filtered at 2 kHz, and the current noise traces were also AC-coupled with a 3-Hz high pass filter. ATP was applied for the duration of the trace at a concentration of 1 μM. The horizontal scale bar represents a time of 1.6 s, and the vertical scale bar represents a current of 2 pA for the high-gain traces and 100 pA for the low-gain traces.

then subtracted the variance of the previously recorded background current noise to obtain the change in variance due to ATP. At the reversal potential for the ATP-activated current (−5 mV), there was on average no change in the variance during the application of ATP (Fig. 5, Table III). However at +40 and −50 mV, ATP elicited small but measurable increases in the variance. These results indicate that current through the ATP-activated channels reversed at the reversal potential of the

macroscopic current, implying that these channels conduct both cations and anoins. We conclude from these results that ATP activates a single class of channels that do not select by charge.

ATP Activates Channels of Small Conductance

The ratio of the variance to the mean current activated by ATP suggested that the single-channel conductance was quite small. To obtain an estimate for the single-channel conductance, we analyzed the spectral density of the stationary current noise elicited by ATP. Records were accumulated as described above using 1 μM ATP. A computer-based fast Fourier transform was performed on the data after it

TABLE III

ATP-induced Current Noise Is Negligible at the Reversal Potential for the Macroscopic Current

Potential	−50 mV	−5 mV	+40 mV
Current (pA)	−223 ± 31	−1 ± 6	+200 ± 86
Variance (pA^2)	1.83 ± 0.61	0.00 ± 0.04	0.66 ± 0.12
No. of cells	10	11	4

Myoballs were bathed in the high $CsNO_3$ external solution and dialyzed with the low $CsNO_3$ internal solution. Under these conditions the reversal potential for cations was +50 mV and the reversal potential for anions was −50 mV. The variance of the background current was subtracted from the variance of the mean steady-state current during the application of 1 μM ATP to give the variance of the current elicited by ATP. This was done for three membrane potentials: at −5 mV, very close to the reversal potential; and at −50 mV and +40 mV, where the driving force was 45 mV. There was no increase in the variance at the reversal potential, while there was a clear increase in the variance for inward currents at −50 mV and outward currents at +40 mV. Based on the values obtained at +40 and −50 mV, one can calculate the predicted increase in noise at −5 mV if there were separate classes of cation and anion channels using the Goldman-Hodgkin-Katz current equation and the equations for variance of the current noise outline by Dionne and Ruff (1977). These calculations predict an increase in current noise of 0.44 pA at −5 mV, a value significantly greater than what we observed. These results demonstrate that ATP activates a single class of channels that does not select by charge. For each of the 11 cells tested, ATP-induced currents were studied at −5 mV and at either −50 or +40 mV, or both. Because the ATP response exhibits long-term desensitization with repeated applications, we verified that ATP could still elicit a current after its application at the reversal potential (−5 mV). All values are ±SEM.

had been cosine tapered. Estimates for the single-channel conductance were derived from the zero-frequency asymptote of Lorentzian functions visually fit to the data (Fig. 6).

For the data collected with the low $CsNO_3$ internal solution, the average single-channel conductance was 0.22 pS when the external solution also contained 1 mM $BaCl_2$ (Table IV). Barium was included in the external solution to promote the formation of high resistance seals between the membrane and pipette. We also recorded from a few cells when there was no barium in the external solution, and found that the single-channel conductance was similar. The small single-channel conductance was not a result of the low ionic strength internal solution. When the

internal solution contained 150 mM $CsNO_3$, the average conductance was 0.24 pS. Because neither cesium nor nitrate bathe muscle cells in vivo, we also measured single-channel properties in symmetrical NaCl solutions. Once again the conductance we obtained was ~0.2 pS. In the simple model outlined by Anderson and
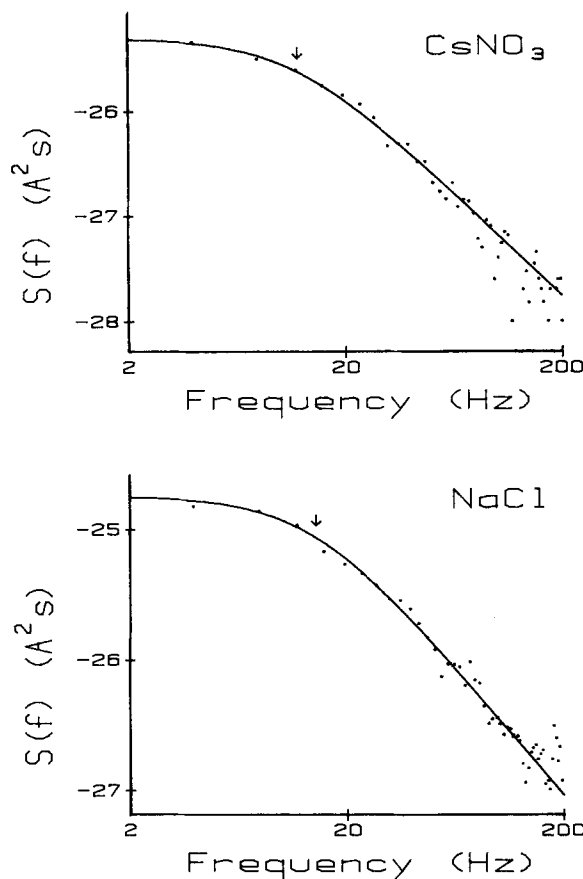

FIGURE 6. Power spectra of the ATP-induced current noise can be approximated by a Lorentzian function. (*A*) This is the same cell as that shown in Fig. 5, with the same $CsNO_3$ solutions. The cell was held at −50 mV, and the current activated by ATP was similar to that shown in Fig. 5 *C*. The arrow indicates the corner frequency (half-power level), which was 12.0 Hz. The spectral estimate for the single-channel conductance was 0.30 pS. As was the case here, occasionally we did not AC-couple the high-gain current noise, a procedure which did not alter the results. (*B*) This cell was bathed in the NaCl external solution and dialyzed with the NaCl internal solution. The membrane potential was held at −50 mV. The corner frequency was 14.4 Hz and the spectral estimate of the single-channel conductance was 0.35 pS. As demonstrated in the previous figure, we collected records of current noise both before and during ATP application, and a low-gain record of whole-cell current that was not AC-coupled. Power spectra of the background current noise were subtracted from the spectra of current noise during ATP application to give the spectra of the noise induced by ATP. Lorentzian curves were fit by eye to the data. This procedure did not introduce significant additional variability into the results. For example, refitting the data in *B* with values of $S(0)$ that appeared to be maximal or minimal for producing a good fit, led to changes in $S(0)$, f_c, and γ of ±10, 7, and 5% respectively. This variance was well within the variation between cells.

Stevens (1973), the corner frequency of the power spectrum is inversely proportional to the mean open time of the channel. Using this model, the mean open time of the ATP-activated channel was calculated to be between 10 and 14 ms depending on the solution used.

To check the accuracy of our spectral analysis programs, we estimated the single-channel conductance of AChR channels from the ACh-activated current noise in these cells, and then measured the conductance of single AChR channels in outside-out membrane patches under identical conditions. At −50 mV, the single-channel conductance, calculated by averaging several separate spectral estimates for each of six cells, was 23.9 pS (SEM = 2.6) while the average conductance for 37 separate single-channel openings in a patch was 37.0 pS (SEM = 3.2). This value is similar to the value of 42 pS obtained by Dwyer and Farley (1984) for the conductance of single AChR channels in chick skeletal muscle. Their measurements were made in symmetrical CsCl solutions, while ours were made with the KCl internal solution and the standard blocking solution (except that tetraethylammonium was replaced by sodium). Thus, our spectral estimate for the AChR channel conductance underesti-

TABLE IV

Estimates of the Single-Channel Conductance and Mean Open Time for the ATP-activated Channel

Solutions	Conductance	Open time	No. of cells
CsNO₃	pS	ms	
Internal = 150 mM			
Ext: 150 mM + 1 mM BaCl₂	0.24 ± 0.04	11.2 ± 0.3	4
Internal = 21 mM			
Ext: 150 mM + 1 mM BaCl₂	0.22 ± 0.07	13.8 ± 0.9	6
Ext: 150 mM (no BaCl₂)	0.19 ± 0.04	10.3 ± 0.9	6
NaCl			
Internal = 140 mM			
Ext: 140 mM + 1 mM BaCl₂	0.16 ± 0.04	11.9 ± 0.8	12
Ext: 140 mM (no BaCl₂)	0.24 ± 0.05	9.8 ± 0.3	3

Single-channel conductance and mean open time were calculated from the zero-frequency asymptote, $S(0)$, and the corner frequency, f_c (see Methods). Theoretical Lorentzian curves were fit by eye to the power spectra by adjusting these values of $S(0)$ and f_c. The power spectrum of the background current noise was calculated, and then subtracted from the spectrum of the noise during ATP application to give the power spectrum of the current noise induced by ATP. Cells were bathed in the external (*Ext*) solutions and dialyzed with the internal solutions indicated above. Currents were elicited with 1 μM ATP while the cells were held at −50 mV. All values are ± SEM.

mated the actual conductance by 36%. Spectral analysis underestimates the unitary conductance of AChR channels because it is based on a kinetic model that is simpler than the actual kinetics of the channel. In the absence of detailed knowledge about the kinetics of the ATP-activated channels, it is not possible to determine whether we have also underestimated the unitary conductance to ATP. Such knowledge could only be obtained from recording single-channel currents, which is very difficult for currents as small as these. However, in a number of studies of agonist-activated currents, spectral analysis has produced values for unitary conductance that are from one half to two thirds that determined from single-channel recordings (Gardner et al., 1984). By analogy, the spectral estimate of 0.2 pS that we obtained for the ATP-activated channels would suggest that the actual conductance is in the range of 0.3–0.4 pS.

In summary, ATP activates a single class of channels that is responsible for the rapid excitatory effect of ATP on developing chick skeletal muscle. This class of channels conducts both cations and anions, yet excludes larger ions such as tetra-ethylammonium. We estimate the single-channel conductance to be 0.3–0.4 pS, which would produce unitary currents of about 21–28 fA at the normal resting potential of ~ -70 mV.

DISCUSSION

This work has expanded on initial results which demonstrated that the early excitatory response to ATP increases membrane permeability to sodium, potassium, and chloride (Hume and Thomas, 1988). The development of culture conditions that promote myoball formation has provided us with muscle cells that can be readily voltage clamped and internally dialyzed using the whole-cell patch-clamp techinque. These methods were used to quantify the selectivity of the excitatory response for a large number of ions. We found that there was very little selectivity between inorganic monovalent cations, that the permeability of organic cations depends inversely on the size of the molecule, that the permeability of anions depends inversely on their hydration radius, and that divalent cations also have significant permeabilities.

We tested whether ATP activates a single class of channels or separate classes of cation and anion channels by measuring the increase in the variance of the current during the application of ATP. We made the ionic conditions such that if two separate channel classes were activated, one selective for cations and the other selective for anions, then there should be a measurable increase in noise at the reversal potential for the ATP-activated current. However, we found that there was no increase in the noise during ATP application at the reversal potential of the current, even though there was a clear increase in noise at potentials both positive and negative to reversal.

Since the evidence indicated that there is only a single class of channels activated during the excitatory response to ATP, we analyzed the spectral density of the noise in order to estimate the single-channel conductance. We found that for either $CsNO_3$ or NaCl solutions, the conductance was $\sim$0.2 pS. Our spectral estimate for single ACh channel conductance was approximately two thirds the actual conductance as measured in membrane patches. This result confirms previous observations that spectral estimates tend to slightly underestimate the actual value for single-channel conductance (Gardner et al., 1984), and so if the same is true for the single channels activated by ATP, we estimate the actual single-channel conductance to be $\sim$0.3–0.4 pS.

A possible source of concern was whether cesium completely eliminated the late increase in "potassium" conductance activated by ATP. Potassium channels discriminate strongly between monovalent cations. For instance, the permeability of sodium or cesium through several potassium channel types is approximately one hundredth of that for potassium (Hille, 1984). If the conductance of cesium through the late, ATP-activated potassium channels was significant, we should have detected it when determining whether there were one or two channels activated by ATP. Cesium current through the late-activating potassium channels would have reversed at $\sim +50$

mV under those conditions. Since there was no increase in noise at the reversal potential of the ATP-activated current (−5 mV), then cesium conductance through the potassium channels must have been insignificant. We did not directly test whether sodium conductance through the potassium channels was also insignificant. Thus it remains possible that the conductance of the excitatory ATP channel was even smaller than 0.3 pS when cells were bathed in NaCl, and that some portion of the noise we measured represented the conductance of sodium through ATP-activated potassium channels.

The properties of the ion channels responsible for the whole-cell current activated by ATP are quite different from the ion channels in chick skeletal muscle that Kolb and Wakelam (1983) described as being activated by ATP. Our estimates for the single-channel conductance are approximately 100 times smaller than the conductance of the single-channel currents they observed in cell-attached patches. Also, our permeability measurements demonstrated that ATP increases conductance to anions as well as to cations; the channels Kolb and Wakelam observed were cation selective. Thus either the large conductance cation channels described by Kolb and Wakelam are not activated by extracellular ATP or were not present in the cells we studied.

Our estimate for the conductance of the ATP-activated channel is smaller than the conductances of most other ion channels. Interestingly however, Friel and Bean (1988) have estimated that the single-channel conductance of a current activated by extracellular ATP in bullfrog cardiac muscle is also <1 pS. By making several assumptions, one can estimate the density of the excitatory ATP channels. Application of near-saturating concentrations of ATP (100 μM) elicit peak inward currents that range from several hundred picoamps to several nanoamps at 100 mV of driving force. If one assumes that all of the channels are open at the peak current, then based on an estimate of the single-channel conductance of 0.3 pS, there are a minimum of 10,000–100,000 channels per myoball. Since a typical myoball has a capacitance of ~100 pF, and the capacitance of biological membranes is close to 0.01 pF/μm^2, then the average density is 1–10 channels/μm^2. Such a density is three to four orders of magnitude lower than the most tightly packed membrane proteins. For example, the AChR channel has a density of ~10,000 channels/μm^2 at the mature motor endplate (Mathews-Bellinger and Salpeter, 1978). We have not yet investigated what fraction of channels are actually open at saturating ATP concentrations, or whether the channels are randomly distributed or clustered.

Another channel that has a very small conductance is the cyclic GMP-activated cation channel of vertebrate photoreceptors. Noise analysis of the light-sensitive whole-cell current indicated that the single-channel conductance is ~0.1 pS (Bodoia and Detwiler, 1985). The small conductance of these channels is due to the "blocking" activity of divalent cations, which are also permeant. When divalent cations are buffered to micromolar concentrations on both sides of the membrane, cyclic GMP-activated single-channel currents with conductances of up to 25 pS are observed (Haynes et al., 1986; Zimmerman and Baylor, 1986). We found that divalent cations permeate the excitatory ATP channels, but do not appear to be responsible for the small conductance of these channels. No difference in the single-channel conductance was observed when measurements were made in solutions with 1 mM barium and solutions containing no added divalents.

The fact that cations and anions permeate the same ATP-activated channel is interesting. Since larger ions like tetraethylammonium, glucuronate, and sulfate are not permeant, the channel must have a fairly narrow pore and yet not discriminate between positive and negative ions. Dwyer et al. (1980) estimated the size for the narrowest region of the ACh channel based on the molecular dimensions of permeant and impermeant organic cations. They predicted a pore ~6.5 Å square, which would just barely exclude tetraethylammonium. Thus the narrowest region of the ATP channel should be no larger than that for the AChR channel, but not much smaller either since tetramethylammonium is permeant. Charge selectively is thought to be the result of polar and ionized groups which line the inside of the channel with charge of opposite sign to that of the permeant ions. A channel with very few of these charges, or with an even balance of charges would be much less likely to discriminate between ions of opposite charge. A lack of charges may also explain the small conductance of the single channel. Site-directed mutagenesis of the AChR channel has revealed that a reduction in the number of negative charges believed to be lining the vestibule of the channel leads to a reduction in the single-channel conductance (Imoto et al., 1988). Another explanation for the small conductance of the ATP channel could be that the pore is narrow for much of the channel length, reducing the mobility of permeant ions considerably when inside the channel. A third possibility is that there are very strong ion binding sites within the channel, which would slow the rate of ion exchange. This possibility seems less likely since the channel is not very selective by charge.

Only a few channels have been found to be charge nonselective. The channel with a selectivity most similar to the ATP channel is the mechanosensitive ion channel in yeast. Gustin et al. (1988) showed that these channels conduct anions and monovalent and divalent cations. The permeabilities of the inorganic ions are quite similar, while larger organic ions (glutamate and arginine) are much less permeant. The conductance of this ion channel (36 pS) is much greater than that for the ATP channel, and Gustin et al. were able to demonstrate stretch-activated single-channel currents that were carried by either cesium or chloride. It is also known that several anion channels have measurable permeabilities to cations. Blatz and Magleby (1983, 1985) have demonstrated that two different chloride channels in rat skeletal muscle have cation permeabilities that are approximately one fifth of that for anions. Franciolini and Nonner (1987) found that an anion channel from rat hippocampal neurons also conducts cations, which had one quarter to one third the permeability of chloride. They noticed that cations did not permeate when only impermeant anions were present, and proposed that cation flux was coupled to anion flux by the presence of a cation binding site within the channel. While passing through the channel, anions would interact with the cation bound at this site. Cation permeability was suggested to be the result of the anion-cation complex dissociating from the cation binding site.

Of all the ions tested, calcium had the highest permeability through the ATP-activated channel. One can calculate calcium's share of the total current using the Goldman current equation. For cells exposed to normal physiological conditions (extracellular solution containing 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, and 2 mM $MgCl_2$, and an intracellular solution containing 100 mM K^+, 10 mM Na^+, 20 mM Cl^-, 2 mM Mg^{++}, and 94 mM of an impermeant anion), the values for rela-

tive permeabilities presented in Table II predict that calcium would carry ~10% of the ATP-activated inward current. Thus, ATP directly elicits calcium influx, which may lead to a significant rise in intracellular free calcium. During the initial depolarizing phase of the ATP response, additional calcium may also enter the cell through voltage-activated calcium channels. The combination of depolarization and a rise in internal free calcium is known to have at least one consequence in developing chick skeletal muscle. These cells have chloride channels that require both depolarization and a rise in internal free calcium to activate (Hume and Thomas, 1989). Under normal physiological conditions, activation of these channels leads to a large increase in chloride conductance which clamps the membrane potential between -40 and -50 mV for many seconds. This conductance, combined with the late potassium conductance that ATP activates, will serve to maintain the inward driving force and thus the calcium influx through the excitatory ATP conductance. The influx of calcium is likely to trigger other intracellular calcium-dependent processes as well, since the excitatory ATP conductance remains active for over a minute after a brief (1 s) application of ATP. Calcium may also be a second messenger for ATP in other cells. In smooth muscle cells of the rabbit ear artery, ATP activates a channel that has a calcium permeability approximately three times that of sodium (Benham and Tsien, 1987) and calcium may also permeate an ATP-activated channel in cardiac muscle of the bullfrog (Friel and Bean, 1988). Intracellular loading of fluorescent calcium indicators would provide a direct means for measuring the magnitude of the changes in intracellular free calcium elicited by ATP.

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