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Potentiation of Postjunctional Cholinergic Sensitivity of Rat Diaphragm Muscle by High-Energy-Phosphate Adenine Nucleotides

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Summary. The cholinergic sensitivity of rat diaphragm muscle, measured as the magnitude of depolarization responses to repetitive, iontophoretic pulses of acetylcholine (ACh) onto neuromuscular endplates, is increased by addition of ATP to the perfusion medium. Depolarization responses begin to increase within the first min after addition of 10 mM ATP and plateau at 60% above control levels (mean value) after 4 to 6 min. Neither the magnitude nor the time course of the potentiations corresponds to changes in resting potential or membrane resistance. Other nucleotides are equally or less effective at the same concentration:

ATP = ADP > UTP > AMP = GTP (= no added nucleotide control)

The duration of the individual ACh responses does not increase during continuous exposure to the active nucleotides for up to 15 min except when the muscle is pretreated with eserine.

Mild enzymatic predigestion of the muscle with collagenase and then protease, increasing the availability of the postjunctional membrane to bath-applied drugs, decreases the variability and increases the magnitude of the potentiation to a given dose of ATP. The dose-response curve for ATP is then more than half-maximal at 1 mM and the ranking of the other nucleotides relative to ATP is the same as without predigestion.

There is an optimum Ca^{++} concentration for the potentiation between zero and 2 mM: potentiation is enhanced in Ca^{++}-free medium, partially blocked in twice-normal Ca^{++} medium, and totally blocked in Ca^{++}-free medium 10 min after a 5 min exposure to 2.5 mM EGTA. The similar Ca^{++} dependence of ACh receptor activation in the absence of added nucleotide suggests that ATP directly facilitates receptor activation by ACh. This facilitory action could be one of the physiological roles for the ATP released from stimulated phrenic nerve.

ATP[1] is released from rat phrenic nerve-diaphragm muscle preparations stimulated at physiological frequencies (Silinsky & Hubbard, 1973).

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1 *List of abbreviations:* ACh = acetylcholine; AChE = acetylcholinesterase; ADP = adenosine diphosphate; AMP = adenosine monophosphate; ATP = adenosine triphosphate; ATP·Ca = ATP titrated with $Ca(OH)_2$; $[Ca^{++}]$ = extracellular Ca^{++} concentration; cAMP = 3′:5′-cyclic adenosine monophosphate; EGTA = ethyleneglycol-bis-(amino ethyl ether)N,N′-tetra acetic acid; GTP = guanosine triphosphate; Na^+-K^+-ATPase = Na^+ and K^+ activated adenosine triphosphatase; RL = Ringer-Locke solution; UTP = uridine triphosphate.

Concomitant release of ATP is found in other stimulated cholinergic systems such as frog sartorius nerve-muscle preparation (Boyd & Forrester, 1968) and *Torpedo* electroplax tissue (Meunier, Israël, & Lesbats, 1975). In both cases the ATP is presumed to originate from postjunctional cells and with *Torpedo* electroplax the release is greatly reduced by curare, confirming this hypothesis. This evidence does not preclude the existence of prejunctional release which definitely occurs also: (i) ATP is found in the vesicle fraction of nerve endings (Dowdall, Boyne & Whittaker, 1974); (ii) stimulation which reduces vesicular ACh also reduces vesicular ATP; (iii) spinal nerves release ATP during stimulation (Abood, Koketsu, & Miyamoto, 1962; Zimmerman & Whittaker, 1974); and (iv) a residual release of ATP is seen after stimulation even in the presence of curare (Meunier *et al.*, 1975).

The physiological function of prejunctional release of ATP remains to be elucidated. It is not hydrolyzed in the release process itself (Douglas & Poisner, 1966), although it is rapidly degraded after release, unless the action of an extracellular, Ca^{++}-activated nucleotidase is inhibited (Silinsky & Hubbard, 1973). At synapses and junctions, where the target cell is in close proximity to the release site, the nucleotide may act in concert with the transmitter. For the neuromuscular junction such an action by ATP was demonstrated three decades ago on striated muscle (Buchthal, Deutsch, & Knappeis, 1944). Electrically stimulated contractions are potentiated during, but not after, a contraction induced by arterially-applied ATP. However, ACh-evoked contractions are potentiated for up to 15 min after exposure to ATP (Buchthal & Folkow, 1948). The former effect is due to direct activation of the contractile process. The following experiments demonstrate a potentiation of cholinergic sensitivity by ATP of sufficiently long time course to account for the latter effect.

Materials and Methods

Preparation

Whole diaphragm muscles with ribs still attached were excised from rats killed by a blow on the head. The muscle was drenched with oxygenated Ringer-Locke solution (RL) three or four times during this five-min operation. The dissection was completed in a bath of oxygenated RL by trimming the ribs close to the muscle origin and cutting the hemidiaphragms apart. Following this ten-min operation, the hemidiaphragms were digested in 20 ml of oxygenated RL solutions with collagenase (1.0 mg/ml) and then protease (0.1 mg/ml) for 40 and 30 min, respectively, or for 20 and 15 min, respectively. These

70- and 35-min digestions correspond to $^1/_2$ and $^1/_4$ of the digestion known to produce "disjunction" of the nerve endings from their postjunctional moorings (Betz & Sakmann, 1971). One hemidiaphragm was placed in the 4 ml experimental chamber where it was perfused with oxygenated RL at a rate of a few ml per min while the other half was kept in 50 ml of oxygenated RL until it replaced the first half in the rinsed chamber. Unless otherwise indicated, all experiments were on muscles which had not been exposed to drug solutions.

Electrodes, Stimulation, and Recording

A glass micropipette electrode was bent to fit the shank curve of a straight electrode, filled with 3 M ACh, and glued to a 3 M KCl-filled recording electrode with its tip about 15 to 25 μ back from the recording electrode tip (Oliver, 1971). Electrode resistances ranged from 10 to 20 MΩ for the recording electrodes and from 1 to 10 MΩ for iontophoretic electrodes when tested with 3 M KCl. Muscle fibers were impaled within a fiber-diameter of the fine, myelinated arborizations of the phrenic nerve which lie in an arc down the middle of each hemidiaphragm. If good miniature endplate potential amplitude was obtained (0.4 mV or larger), ACh stimulation was commenced. A *back-current* of 1 to 10 nA prevented leakage from the ACh electrode tip which was just extracellular and no more than 10 μ away from the point of recording electrode impalement. Iontophoretic ACh application was by repetitive pulses with a period of 15 sec, durations of 5 to 100 msec, and intensities of 2 to 5 V (Tektronix 160 series). Slight adjustments were sometimes made with the electrode assembly to obtain maximum amplitude and minimum duration and latency of the resulting ACh responses. Response durations, measured as the width at half-amplitude, were nearly all less than 1.5 sec. The repetitive responses were continuously monitored (WPI amplifier, Brush recorder) with no attenuation compared to oscilloscope traces.

Drug Addition

When resting potential and response magnitude were stabilized, the flow was turned off and 1.0 ml of nucleotide solution was added below the surface of the 4 ml bath over the center of the pinned-down hemidiaphragm near the stimulated endplate. Addition was at a rate of 1 ml per min by a micrometer-driven syringe through a polyethylene tube. After four or more min of exposure to the drug solution, the chamber was flushed at a rate of 5 ml per min for a few minutes before the normal flow rate was resumed.

Sensitivity and Desensitization

Changes in sensitivity at various time intervals after the beginning of drug addition were recorded as percent changes in response magnitude at the end of these intervals compared to the magnitude just preceding addition of the drug. Desensitization of the ACh responses was produced by reversing the back-current for 20 or 40 sec so that the ACh response immediately following the ACh-leakage flow was reduced to approximately half of the control magnitude. Desensitization was quantified as the ratio of the response magnitude immediately following the ACh flow to that just preceding it. Care was taken to keep the intervals between these ACh responses and the beginning and end of the ACh flow constant throughout a given experiment.

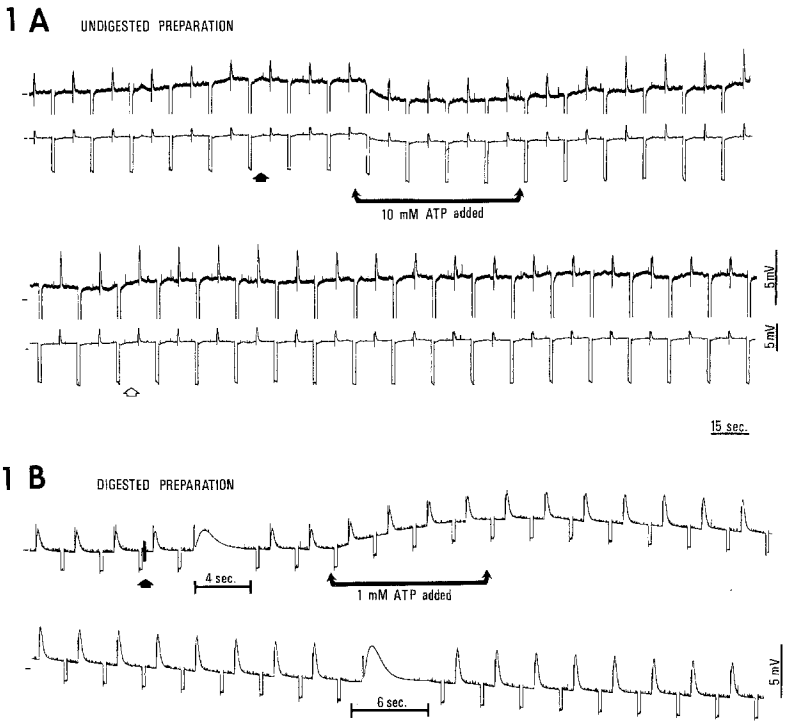

Fig. 1

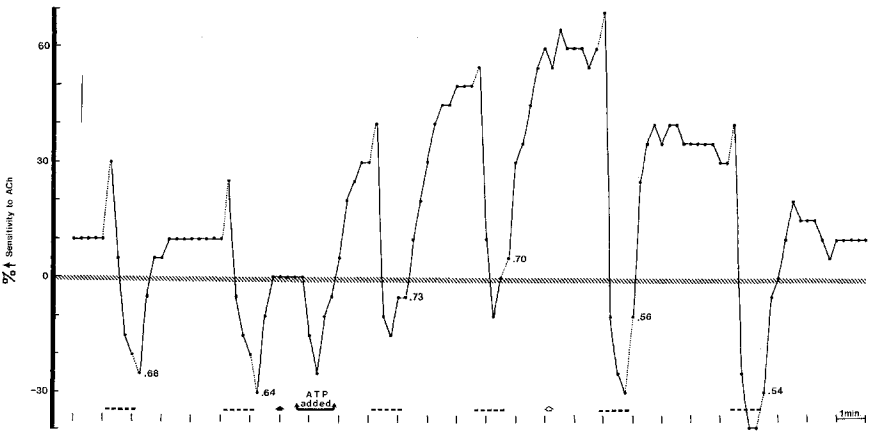

Fig. 2

Fig. 1. Potentiation of depolarization responses to iontophoretic pulses of ACh applied every 15 seconds near a neuromuscular endplate following addition of an ATP solution to the bath. Perfusion flow was turned off at the solid arrow and back on at the open arrow. Addition was at the bracket. (*A*). One ml of a 37.5 mM ATP solution was added to a 4 ml bath with constant-level overflow for a final bath concentration of 10 mM. (*B*). One ml of a 4 mM ATP solution was added to the 4 ml bath for a 1 mM concentration

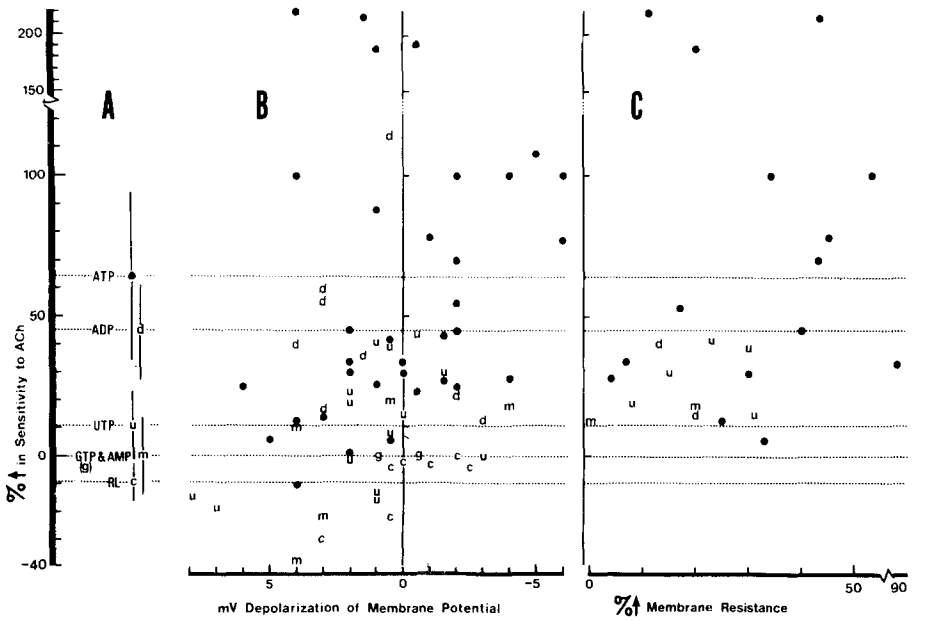

Fig. 3

on a preparation previously digested with collagenase (1.0 mg/ml for 20 min) and protease (0.1 mg/ml for 15 min). Resting potentials are 60 mV in (*A*) and 65 mV in (*B*). Calibration bars = 15 sec and 5 mV for both traces of (*A*) and for (*B*). Membrane resistance was also monitored every 15 sec in these records as the response to 1 sec, hyperpolarizing pulses from a current injection electrode in the same cell. The lower trace in (*A*) shows the increase in membrane resistance seen only on undigested preparations

Fig. 2. Sensitivity to ACh as a function of time following addition of 10 mM ATP to the bath (at bracket) measured as a percent increase in ACh response magnitude compared to the magnitude (2.0 mV) just preceding addition on an undigested preparation. Perfusion flow was turned off at solid arrow and back on at open arrow. Above the dashed lines the bucking current on the ACh electrode was reversed in polarity to desensitize the responses. The rate of desensitization is quantified by the ratio of the response magnitude just following the desensitizing current to that just preceding it. These ratios are given on the graph at the end of each desensitizing current. Of eleven experiments, this experiment had the median increase in this ratio due to addition of 10 mM ATP to the bath

Fig. 3. (*A*) Means $\pm {}^1/_2$ SD of the percent increase in ACh response magnitude four minutes after addition of 10 mM ATP ($n=34$), ADP ($n=8$), AMP ($n=5$), UTP ($n=14$), and an equivalent volume of Ringer-Locke solution ($n=7$) on undigested preparations. Percentage comparison was made to the magnitude just preceding addition of the nucleotide solution to the bath. (*B*) Individual sensitivity increases of all of the experiments in (*A*) as a function of their membrane potential changes (in mV) over the same time interval. Depolarizing change is taken as positive. These variables appear to be independent of each other. (*C*) Individual sensitivity increases as a function of membrane resistance increases (as percentage increases) over the same time interval for those experiments in (*A*) where resistance was concurrently measured. On undigested preparations these variables show an interdependence which is not seen on digested preparations (e.g., Fig. 1*B*)

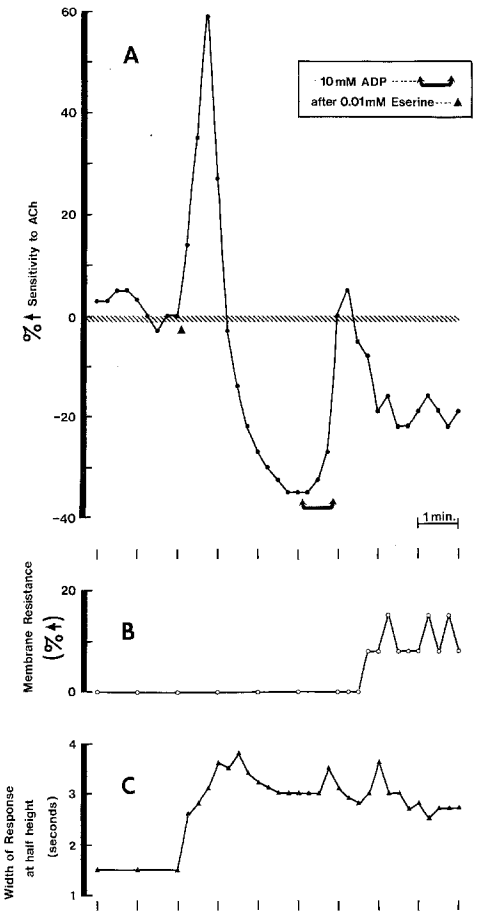

Fig. 4. (*A*) Sensitivity increases due to addition of 0.01 mM eserine to the bath (at triangle) followed after 3 min by addition of 10 mM ATP (at bracket) on an undigested preparation. Graphical format is as in Fig. 2 with a reference magnitude of 7.6 mV just before addition of eserine. Eserine pretreatment causes an eserine-like peak in the nucleotide response. (*B*) Relative increase in membrane resistance during the same time interval measured by passing hyperpolarizing pulses from a separate current injection electrode in the same cell. Resistance increases are not associated with addition of eserine. (*C*) Width of the responses to ACh in seconds measured at half magnitude over the same time interval. Some increase in response duration is associated with nucleotide addition following eserine pretreatment

Membrane Resistance

A separate 3 M KCl-filled electrode was inserted into the same muscle fiber for injecting current (via a Bioelectric Isolation Unit) with the same 15-sec period as the ACh stimulation but with a half-period lag. One sec hyperpolarizations of the same magnitude as the ACh responses were produced.

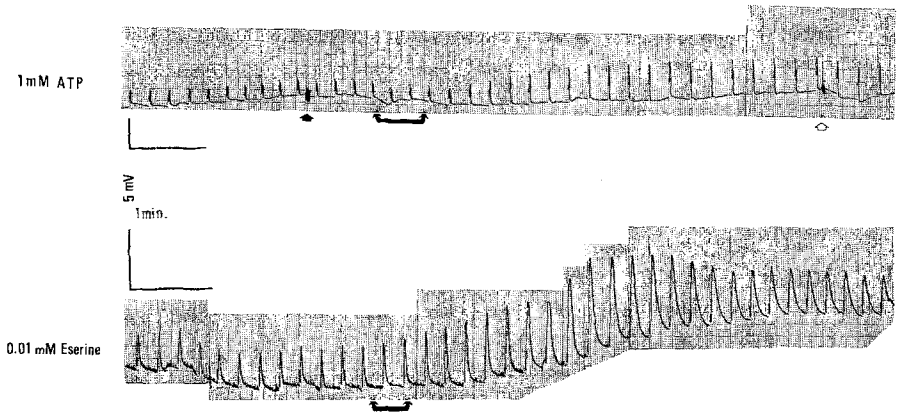

Fig. 5. Effect of 1 mM ATP (upper record) and 0.01 mM eserine (lower record) on depolarization responses to iontophoretic pulses of ACh on two diaphragm muscle preparations digested with collagenase (1 mg/ml for 40 min) and protease (0.1 mg/ml for 30 min). Brackets indicate addition of drug solutions. Perfusion flow of normal Ringer-Locke was turned off at solid arrow and on again at open arrow in upper record, but are off-record for the lower record. Eserine record was pieced together because of vertical-position adjustments and some responses were truncated when going off the vertical range of the recorder. Calibration bars for both records are 5 mV and 1 min

Drug Solutions

Nucleotide solutions were prepared in the following manner. 1.5×10^{-4} moles of nucleotide salt, dissolved in 1 ml of distilled water, was titrated to pH 7.0 with 0.5 M NaOH (except for the ATP·Ca solution which was titrated with equal moles of $Ca(OH)_2$). The Na^+ from the nucleotide salt and the NaOH corresponds to a volume (V) of 4 ml or less, V ml, which would have normal Na^+ (150 mM). Distilled water was added until the nucleotide solution was $V/2$ ml. The other half of V was then added as a Na^+-free solution with twice the normal concentration of all other ions in RL. Finally, $(4\text{-}V)$ ml of normal RL was added to yield a 37.5 mM concentration for all nucleotides. Osmotic adjustment was made with glucose for the missing anion for the polyvalent nucleotide salts (ATP, ADP, and UTP). These neutral pH, osmotically balanced solutions were diluted with RL for lower concentrations. Concentrations given in the text and figures refer to the final bath concentration.

Normal RL consisted of (in mM): 137, NaCl; 5, KCl; 1, $MgCl_2$; 2, $CaCl_2$; 11, glucose; 12, $NaHCO_3$; and 1, NaH_2PO_4 which was pH 6.8 to 7.0 with oxygenation. In the low Cl^- solution, 150 mM acetate replaced 150 mM Cl^- of the normal RL. The Ca^{++} chelating agents EGTA and oxalate replaced equimolar concentrations of NaCl and divalent cations, respectively.

Collagenase (type I), protease (type VII), oxalate, and EGTA were obtained from Sigma. Nucleotides and eserine were from Calbiochem.

All experiments were performed at room temperature of 18 to 20 °C and all solutions were equilibrated to that temperature for at least an hour before use.

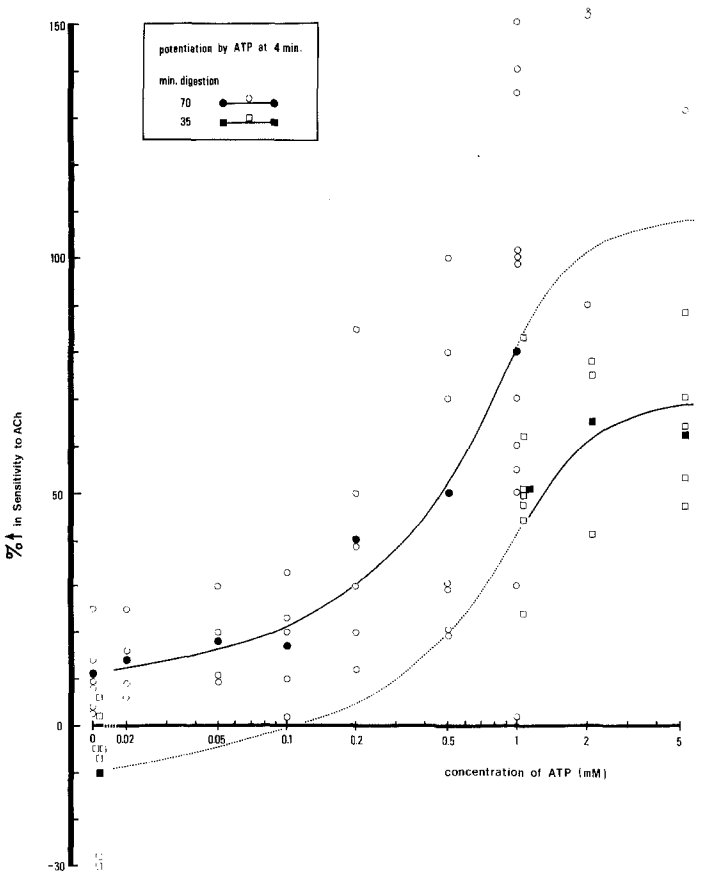

Fig. 6. Dose-response curve for concentrations of ATP of 1 mM or less drawn through the means (filled circles) of potentiations observed (open circles) four min after addition on preparations digested for 70 min. Dotted extrapolation above 1 mM (where addition of ATP causes twitching) follows the shape of the curve through the means (filled squares) of potentiations observed (open squares) on preparations digested for only 35 min (20 min collagenase, 15 min protease). The 35-min digestion curve is interpolated to its control data points following the shape of the 70-min curve. Small arrow above one data point signifies that it is out of the vertical range of the graph

Results

Undigested Preparations

Addition of concentrated ATP solution to a final bath concentration of 10 mM causes a gradual potentiation of iontophoretic ACh responses for three to four min reaching a plateau with a mean 60% above the control response (Fig. 1). The effect is slowly reversible when the flow

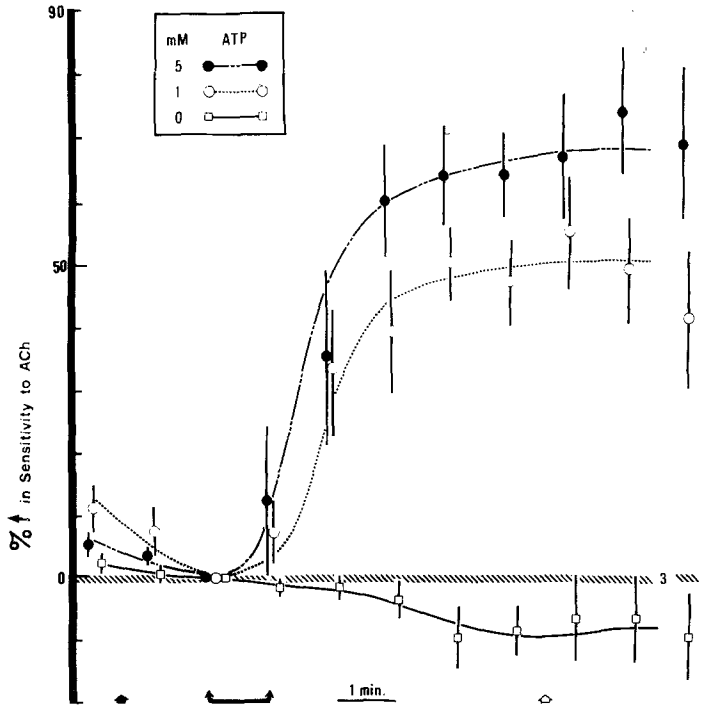

Fig. 7. Percent increase in the magnitude of depolarization responses to iontophoretic pulses of ACh applied every 15 sec to endplate regions of hemidiaphragms digested for 35 min after addition of 5 mM and 1 mM ATP or 1 ml of Ringer-Locke solution to the bath (at bracket). The response level just preceding addition is the reference for percentage comparisons. Mean percent increases are graphed at one min intervals (every fourth response) from −2 to 7 min and at 10 min with time equal to zero at the beginning of addition. Open circles are the means of 7 experiments with 1 mM ATP and filled circles are the means of 5 experiments with 5 mM ATP. One ml of Ringer-Locke solution was added as a control for addition artifacts in 7 experiments (open squares). Perfusion flow was turned off between −2 and −1 min (solid arrow) and back on between 5 and 6 min after the beginning of addition (open arrow). Smooth curves are drawn through the means ± SE for each case

is turned on again. No potentiations are observed after addition of the same volume of RL to the bath.

The duration of the responses is not increased as their magnitudes increase. An increase in response duration is seen only after 15 to 30 min of continuous exposure to the nucleotide solution. The membrane potential often increases a few millivolts during the minute following addition of the nucleotide solution and then tends to gradually decrease at a rate of less than a millivolt per min.

In some experiments the desensitization of the responses by 20 or 40 sec of continuous flow of ACh (back-current reversed) onto the end-

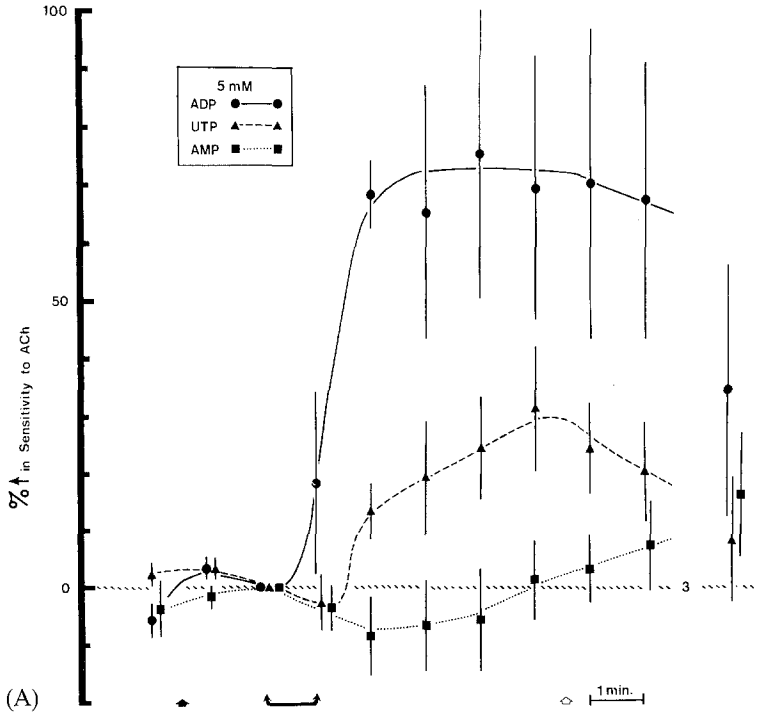

Fig. 8. Mean percent increases in sensitivity to ACh (±SE) after addition of 5 mM (*A*) or 1 mM (*B*) AMP (squares), ADP (circles), or UTP (triangles) to the bath of preparations digested for 35 min. Graphical format is as in Fig. 7 with 3 experiments for each curve except for 5 mM UTP which is the mean of 5 experiments

plate was measured before, during, and after exposure to ATP. In eleven experiments the rate of desensitization decreased to varying degrees during exposure to 10 mM ATP and then increased when the ATP was flushed out. The ratio of the response magnitude just following each desensitizing current to the one just preceding it was used to quantify these results. The experiment graphed in Fig. 2 has both the median increase in this ratio (0.09) during exposure to ATP and the median decrease (0.14) after the flow is resumed.

The mean potentiations by the other nucleotides, expressed as % increases from the response magnitudes just preceding addition at four min after addition, are summarized in Fig. 3*A*. Adenosine diphosphate (ADP) is nearly as potent as ATP; adenosine monophosphate (AMP) and uridine triphosphate (UTP) give mean potentiations of less than 10%; guanosine triphosphate (GTP) is without effect (2 experiments). So only the nucleotides with both an adenine moiety and a high-energy-phosphate bond are capable of the fullest potentiation of ACh action.

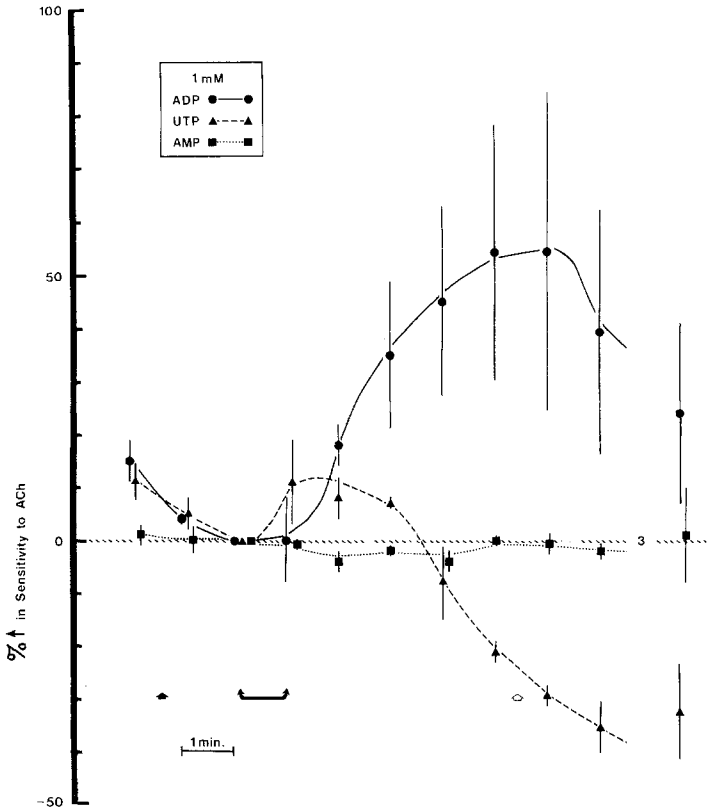

Fig. 8B

Potentiations in individual experiments show no correlation with membrane potential changes during the same time interval (Fig. 3*B*). Transmembrane resistance was monitored in fifteen ATP experiments. Four min after addition there is a mean increase of about 30%. Here also, the increases for the individual experiments do not correlate with the potentiations (Fig. 3*C*). Resistance increases due to exposure to other nucleotides (9 experiments) were all less than the mean increase due to ATP. Thus, part or all of the potentiation by ATP might be due to an enhanced effective resistance of the muscle fibers. However, results on enzymatically pretreated muscle show that this is not the case (*see* below).

The variability in the potentiation seen with each nucleotide may be caused by differences in pharmacological availability of the endplates chosen for each experiment, in spite of the attempt to choose only *surface* endplates. Eserine is a drug whose site (acetylcholinesterase) and mode (competitive inhibition) of action at the neuromuscular junction are well

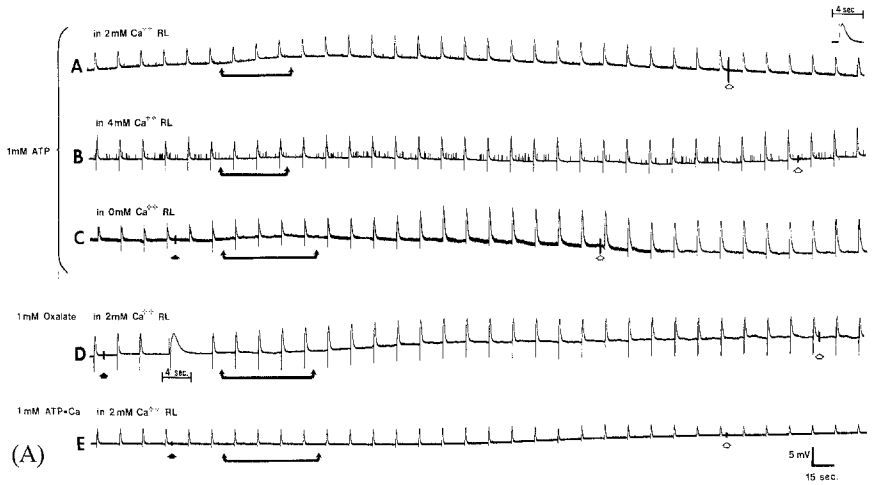

Fig. 9. (*A*) Continuous, eight-min records of the effects of 1 mM ATP on ACh depolarization responses in normal-Ca^{++} (A), double-Ca^{++} (B), and Ca^{++}-free (C) Ringer-Locke and the effects of 1 mM oxalate (D) and 1 mM ATP·Ca (E) in normal-Ca^{++} Ringer-Locke on preparations digested for 35 min. Note the expanded scale for one response in *A* and *D*. Addition is at the brackets. Flow is off at solid arrows and on at open arrows. Calibration bars are 5 mV and 15 sec. *See* Methods for description of preparation of ATP·Ca. (*B*) Mean percent increases in sensitivity to ACh (±SE) after addition of 1 mM ATP in Ca^{++}-Free (filled circles) and in double-Ca^{++} (half-filled circles) Ringer-Locke to bath of preparations digested for 35 min. Graphical format is as in Fig. 7 with 3 experiments in Ca^{++}-free and 6 experiments in double-Ca^{++} Ringer-Locke

known (Eccles, Katz & Kuffler, 1942). It also exhibits a variability in response with this experimental system which does not appear to be dose dependent. The results of 11 experiments fall into three categories: (i) a transient potentiation with an increase in response duration at 4 endplates; (ii) a gradual potentiation without plateau at 3; and (iii) a stable or slowly declining response magnitude with no increase in duration (similar to controls) at another 4. Final bath concentrations ranged from 0.01 to 0.2 mM with both high and low concentrations represented in the three groups. An experiment with ADP, and one with ATP, following eserine treatment produced eserine-like effects – transient potentiation and increased response duration (Fig. 4).

Addition of 1 ml of a RL solution with 150 mM acetate ion as a low-permeability substitute for Cl^- (to mimic the altered osmotic properties of the nucleotide solutions) produced a transient, hyperpolarization-linked increase in ACh responses of less than 10% in 2 experiments.

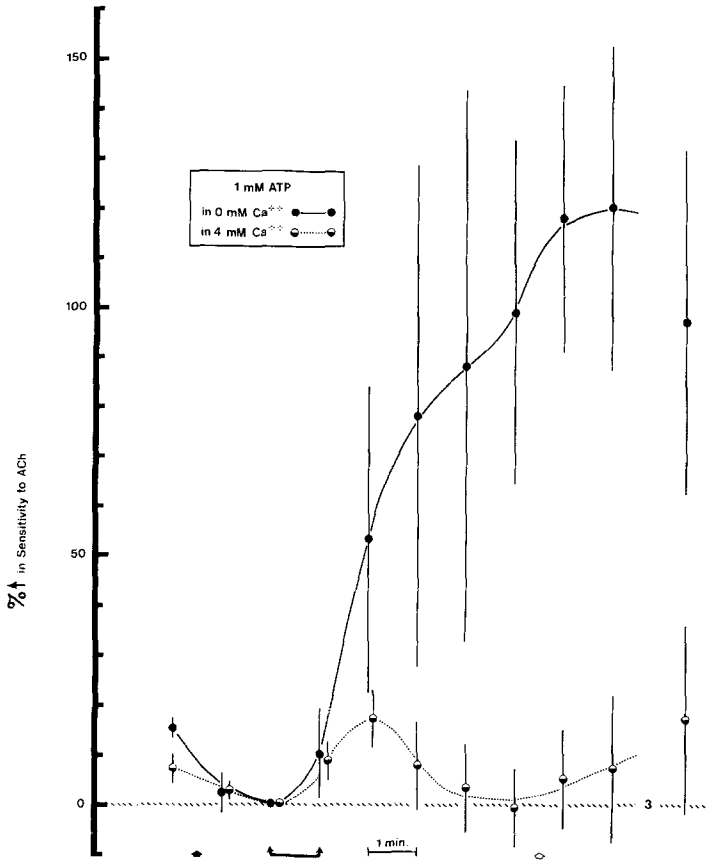

Fig. 9B

Digested Preparations

Digestion of the preparation for 70 min in oxygenated RL solutions of collagenase and then protease enhances the sensitivity to nucleotide-induced potentiations and reduces the variability at a given dose. Potentiation to 1 mM ATP after digestion is of the same magnitude seen with 10 mM ATP on the undigested preparation. Of five eserine experiments on digested preparations, none were of the gradual-potentiation category (ii), and most were of category (i). Most importantly, the increase in membrane resistance, which accompanies potentiation on undigested preparations, is no longer evident (6 experiments). This implies that enhanced effective resistance of the muscle cell membrane is not the electrophysiological mechanism for potentiation. Examples of these pharmacological characteristics of the digested preparation are shown in Figs. 1*B* and 5.

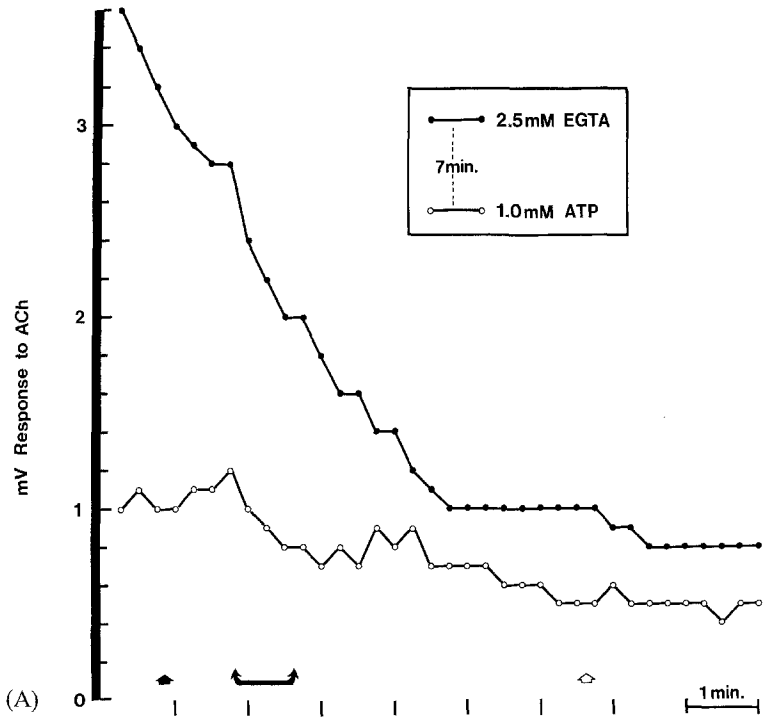

Fig. 10. (*A*) ACh response magnitude in mV as a function of time after adding 2.5 mM EGTA (filled circles) followed by 1.0 mM ATP (open circles) to the bath of a single hemidiaphragm preparation digested for 35 min. Perfusion flow is turned off at the solid arrow, addition of drug solutions is at the bracket, and flow is back on at the open arrow for both graphs. Seven min of normal perfusion flow elapses between graphs. (*B*) Percent increase in sensitivity to ACh after addition of 1 mM ATP in Ca^{++}-free (open symbols) or normal-Ca^{++} (solid symbols) Ringer-Locke on preparations which have been digested for 35 min and previously exposed to 1 mM ATP and perfused with Ca^{++}-free or normal Ringer-Locke respectively, for at least 10 min. Graphical format is as in Fig. 7 except data points for the five experiments in each case are shown (circles) instead of SE bars. Smooth curves are drawn through the means (stars)

In Fig. 6 a dose-response curve for the 70-min digestion treatment is drawn through the means of the potentiations observed after a 4-min exposure to ATP. At low doses, and even with addition of RL, there is a 10 to 20% potentiation which is often accompanied by an early hyperpolarization. This may be due to the improved rinsing of surface-accumulated K^+ by the flow of drug or RL solution directly onto the digested endplate. At doses of 1 mM or more, the muscle often twitched and abrupt changes in sensitivity were observed. No data was taken from such experiments. This high concentration data could be obtained from experiments on preparations digested for only 35 min which rarely

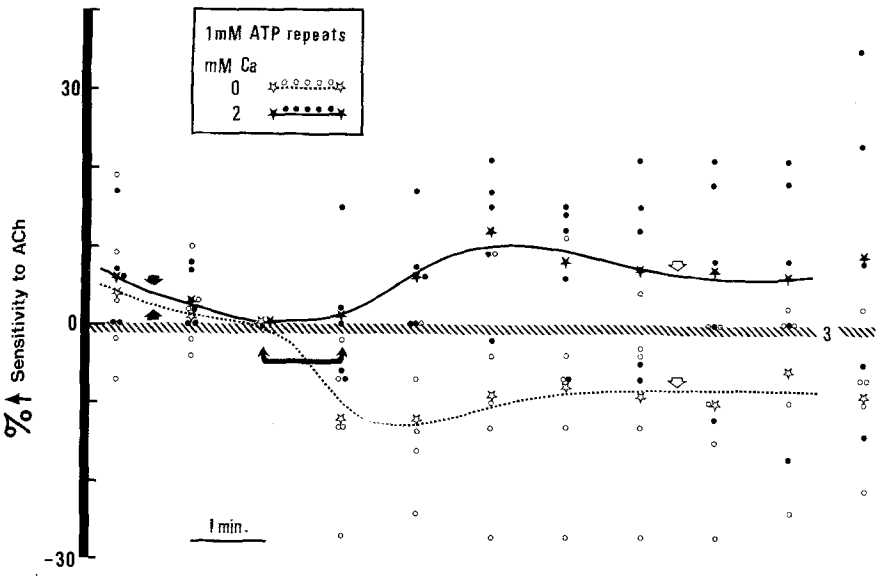

Fig. 10B

twitched when 1, 2, or 5 mM ATP was added. Potentiation on the 35-min preparation has the same maximum as on the undigested preparation, but a decreased variability at a given dose. The 70-min digestion treatment increases the nucleotide potentiation maximum without a further decrease in variability. Since a decrease in variability is what was sought with the digestion technique all of the following experiments were done on preparations digested for 35 min.

Fig. 7 is a minute-by-minute elaboration of the 1 and 5 mM ATP and control experiments (open squares in Fig. 6). The relative ranking of potentiation potency of the other nucleotides is the same as on the undigested preparation (Fig. 8).

Potentiation by 1 mM ATP is highly dependent on Ca^{++} concentration in the medium: accentuated in Ca^{++}-free medium and depressed by high-Ca^{++} medium (Figs. 9*A* and 9*B*). Potentiation is completely blocked under conditions which approach zero Ca^{++} in the medium: after exposure to 2.5 mM EGTA (Fig. 10*A*), and upon repeat exposure in Ca^{++}-free medium after 15 min of nucleotide-free, Ca^{++}-free perfusion (Fig. 10*B*). Thus the optimal Ca^{++} concentration for potentiation by ATP lies between zero and 2 mM, whereas the ACh receptor is maximally activated by ACh at 2 mM Ca^{++}. If ATP or its products act directly on the ACh receptors to potentiate sensitivity, then some auxiliary effect of low Ca^{++} must explain this lower optimum: inactivation of extracellular nucleotidases and/or reduced chelation of Ca^{++} to ATP.

To study the latter alternative, ATP was titrated with $Ca(OH)_2$ instead of NaOH. Five experiments using this ATP·Ca at 1 mM concentration caused transient increases in sensitivity (mean: 5%) for 1 to 2 min after the beginning of addition (example in Fig. 9*A*). Similarly, five experiments with 1 mM oxalate, a potent Ca^{++} chelator, produced transient potentiations with a maximum mean increase in sensitivity of 15% three min after the beginning of addition (example in Fig. 9*A*).

Discussion

Exposure of the diaphragm muscle to solutions of nucleotides (replacing Cl^- of the normal RL) potentiates the endplate depolarization responses to iontophoretic pulses of ACh:

ATP=ADP=>UTP>AMP=GTP (=no added nucleotide controls).

Addition of an equal volume of RL produces little or no change in the responses. The effects of a possible, nucleotide-induced contracture, which might thicken the muscle and shorten the vertical distance between the ACh electrode tip and the active spot, were minimized by stretching and securely pinning the muscle and by gluing the iontophoretic electrode onto the recording electrode.

There are many sites on the muscle membrane, particularly in the postjunctional area, which are potentially sensitive to exogenously applied nucleotides. However, the lack of relationship between membrane potential changes and ACh response potentiations eliminates a few of these as possible sites for ATP action to produce potentiation: the Na^+-K^+-ATPase and the resting permeability channels for Na^+, K^+, and Cl^-. The absence of measurable membrane resistance changes on digested preparations also suggests that resting permeability changes do not underlie the effects.

Noncompetitive inhibition of endplate acetylcholinesterase (AChE) by nucleotides (Kometiani & Kalandarishvili, 1969) would increase the mean lifetime of ACh in its unhydrolyzed form and could thus enhance the depolarization responses to ACh pulses. The addition of a competitive inhibitor of AChE, eserine, consistently produces effects on the digested preparation which are qualitatively different from nucleotide potentiation: increased response duration and a peak rather than a plateau in time course. The more subtle, noncompetitive inhibition by nucleotides might potentiate ACh responses without increasing their duration by

raising the maximum ACh concentration of each pulse to a level just short of desensitizing the receptors, whereas a competitive inhibitor with a high affinity for the active site of AChE would increase the concentration beyond this level. However, the eserine-like effects of nucleotides on eserinated muscle and the increased response duration with long exposure to nucleotides demonstrate that the expected effects of nucleotide inhibition of AChE appear only under conditions quite different from those under which nucleotide potentiation is produced. Furthermore, the specificity for adenine and high-energy-phosphate nucleotides is inconsistent with biochemical data which shows an equivalent inhibition of AChE activity by ATP, UTP, and GTP (Maheshwari, Shirachi & Trevor, 1971).

Finally, effects of the nucleotides on the ACh receptor could account for their ability to increase sensitivity to ACh. Direct action by bath-applied ATP at the same site where ACh activates the receptor would attenuate, rather than potentiate, ACh responses. Possible indirect actions are that ATP (i) affects the receptor allosterically, or is biochemically converted into a molecule with such an effect, or (ii) affects the concentration or availability of a cofactor which is involved in receptor activation.

Calcium ions are an essential cofactor in the process of ACh receptor activation. Maximal receptor activiation occurs at the normal extracellular Ca^{++} concentration (Lambert & Parsons, 1970). Potentiation of ACh responses by ATP is similarly dependent on extracellular Ca^{++} concentration, $[Ca^{++}]$, but has an optimum somewhat below normal. When $[Ca^{++}]$ is increased from 2 mM (normal) to 4 mM, or a Ca^{++}-titrated ATP solution is used, the potentiation is small and attenuated. In Ca^{++}-free medium (no added Ca^{++}), potentiation is greatly increased. However, a more complete depletion of extracellular Ca^{++} completely blocks potentiation. The shift of optimal $[Ca^{++}]$ to below normal is probably the result of the action of an extracellular Ca^{++}-activated nucleotidase.

Potentiation by ATP would have a $[Ca^{++}]$ dependence similar to receptor activation by ACh if ATP were facilitating the activation of the Ca^{++}-dependent receptor, or alternatively, if potentiation were the result of altered $[Ca^{++}]$ to which the receptors are sensitive. By the latter hypothesis the nucleotides could reduce $[Ca^{++}]$ due to the strong Ca^{++} chelation properties of their negatively charged phosphate groups. Attempts to mimic this effect using oxalate produced only a transient potentiation. With phospholipids, and presumably also with nucleotides, chelation strength is proportional to the net negative charge of the molecule at physiological pH (Dawson & Hauser, 1970). If a reduction of

[Ca^{++}] were the cause of nucleotide potentiation, then the nonadenine nucleotides, UTP and GTP, would be as effective as ATP. In fact, they are much less potent. The potentiation ranking of adenine nucleotides, ATP = ADP > AMP (1.6:1.6:1.0), is more closely related to their terminal phosphate bond energy ranking, ATP = ADP > AMP (2.0:2.0:1.0), than to their Ca^{++} chelation ranking, ATP > ADP > AMP (3.0:2.0:1.0).

The alternative hypothesis, allosteric facilitation of receptor activation, accomodates the observed potentiation ranking assuming an allosteric site requiring adenine nucleotides with high-energy phosphate bonds or their biochemical products. The site conceivably faces the cytoplasm of the postjunctional cell since exogenous ATP can activate intracellular metabolic processes of intact muscle fibers (Buchthal & Folkow, 1948) and endogenous ATP can *translocate* across plasma membranes (Abood, Koketsu, & Miyamoto, 1962; Trams, 1974). However, it is unlikely that extracellular ATP can significantly increase the intracellular ATP concentration which is already high. This problem is circumvented in the model for the biochemical mechanism of potentiation by ATP presented in the following paper which is based on the effects of an intracellular product of ATP – cyclic AMP.

Potentiation of sensitivity by ATP can also be interpreted as a reversal of receptor *desensitization* first described by Thesleff (1955). Certain drugs which increase the rate of desensitization inhibit ACh potentials at far lower concentrations than they inhibit endplate potentials (Magazanik & Vyskocil, 1973). The decrease in desensitization rate observed during exposure to exogenous ATP suggests that the prejunctional release of ATP (Silinsky & Hubbard, 1973) accounts for this difference.

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