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# Modulation of Postjunctional Cholinergic Sensitivity of Rat Diaphragm Muscle by Cyclic Adenosine Monophosphate

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*Summary.* Addition of 2.5 mM cyclic adenosine monophosphate (cAMP) to the solution bathing a rat diaphragm muscle alters the magnitude of depolarization responses to iontophoretic pulses of acetylcholine (ACh) at neuromuscular endplates. Alterations are repeatable with small variability on a given preparation for initial and repeat experiments on both hemidiaphragms, but are different on each preparation. Five min after addition of the nucleotide solution, increases (potentiations) of up to 30% above control levels and decreases (attenuations) to 50% below control levels are observed. The effects on sensitivity to ACh of dibutyryl cAMP (1.25 mM), monobutyryl cAMP (0.25 mM), and cAMP (2.5 mM) in $Ca^{++}$-free solution are a function of whether the experiment is an initial one on that preparation or a repeat experiment after 10 or more minutes of perfusion flow. In all three cases, initial exposure attenuates sensitivity (means at 5 min: −30, −10, and −20%, respectively) and repeat exposure potentiates sensitivity (means: 20% at 5 min, 15% at 5 min, and 10% at 2 min respectively). A concentration of dibutyryl cAMP (0.25 mM) which is without effect on sensitivity alone, produces a large, transient potentiation (mean: 45% at 1 min) in conjunction with 0.5 mM theophylline. A decrease in the rate of desensitization is observed during exposure to 0.25 mM cAMP. These results are interpreted in terms of a physiological mechanism whereby receptor activity at the postjunctional membrane is modulated by cAMP formed from prejunctionally released ATP.

Electrophysiological investigations of the postsynaptic sensitivity to neurotransmitters at peripheral (McAfee & Greengard, 1972) and central (Siggins, Hoffer, & Bloom, 1969) synapses, as well as the postjunctional sensitivity of glands (Berridge & Patel, 1968) and muscles (Entman, Levey & Epstein, 1969; George, Polson, O'Toole & Goldberg, 1970), have shown a functional involvement of cyclic nucleotides in the membrane permeability changes underlying these sensitivities. Receptor activation apparently increases the synthesis of cyclic adenosine monophosphate

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(cAMP)[1] or cyclic guanosine monophosphate (cGMP) in the postsynaptic or postjunctional cells. The source of the precursor for the synthesis of cAMP, adenosine triphosphate (ATP), is assumed to be intracellular (Rasmussen, 1970). However, the source is conceivably extracellular since: (i) adenine nucleotides are released from electrically stimulated nerve endings (Pull & McIlwain, 1972); (ii) the enclosed extracellular space of a synapse or junction limits diffusion losses; (iii) ATP may *translocate* across plasma membranes (Trams, 1974); and (iv) stimulated release of adenine nucleotides is known to enhance the production of cAMP (Sattin & Rall, 1970).

At the neuromuscular junctions of rat diaphragm muscle, cholinergic *synapses* with nicotinic receptors, the ATP released by stimulated phrenic nerve endings (Silinsky & Hubbard, 1973) may serve to potentiate the response to ACh at the postjunctional muscle membrane (preceding paper). In this paper, two possible hypotheses for cAMP mediation of this potentiation are considered. Either receptor sensitivity is coupled to the production of cAMP from ATP by adenyl cyclase or it is modulated by cAMP or a cAMP-activated enzyme. The effects of cyclic nucleotides on cholinergic sensitivity favor the latter hypothesis.

## Materials and Methods

Rat diaphragm muscles were excised, separated into hemidiaphragms, and predigested in oxygenated Ringer-Locke solutions (RL) of collagenase (1 mg/ml) for 20 min and protease (0.1 mg/ml) for 15 min. All aspects of preparation treatment, intracellular recording, iontophoretic stimulation, drug addition, and desensitization are described in the preceding paper. Perfusion flow was turned off 1 to 2 min before addition of nucleotide solutions and turned on again 5 to 6 min after the beginning of addition. One ml of 10 mM or 1 mM cyclic nucleotide solution was added to the 4 ml bath to yield final bath concentrations of 2.5 and 0.25 mM respectively. As osmotic compensation, glucose (11 mM) was eliminated from the RL mixture in preparing the 10 mM solutions (1 mM solutions were not osmotically compensated). No titration was necessary for cyclic nucleotide solutions; with oxygenation, the buffer of normal RL maintained the pH of these solutions between 6.8 and 7.0. Cyclic nucleotides and enzymes were obtained from Calbiochem and Sigma. The $N^6$-, as opposed to the $O^{2'}$-, monobutyryl derivative of cAMP was used.

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1 List of abbreviations: ACh = acetylcholine; ATP = adenosine triphosphate; cAMP = 3′:5′-cyclic adenosine monophosphate; cGMP = 3′:5′-cyclic guanosine monophosphate; dibutyryl cAMP = $N^6$, $O^{2'}$-dibutyryl 3′:5′-cyclic adenosine monophosphate; monobutyryl cAMP = $N^6$-monobutyryl 3′:5′-cyclic adenosine monophosphate; monobutyryl cGMP = $N^6$-monobutyryl 3′:5′-cyclic guanosine monophosphate; RL = Ringer-Locke solution.

## Results

Addition of a concentrated solution of cyclic AMP (cAMP) to yield a final bath concentration of 2.5 mM changes the responsiveness to iontophoretic pulses of ACh with little change in membrane potential (Fig. 1*A*). On a given preparation (both hemidiaphragms), the effect is repeatable with low variance. However, the quantitative, and even qualitative, nature of this change is different for each preparation (Fig. 2). Experiments were performed on two preparations beyond those shown in Fig. 2 at this concentration; two experiments on a single hemidiaphragm potentiated the responses as in diaphragm #1, and 4 experiments on a final preparation showed decreases in sensitivity in two initial experiments and increases in repeat experiments. Experiments with 0.25 mM cAMP on a single preparation showed small, gradual decreases in sensitivity resembling controls in which only Ringer Locke solution (RL) was added (*see* preceding paper).

The effect of $Ca^{++}$ on the responsiveness to cAMP was studied by altering the $Ca^{++}$ concentration of the perfusion and drug solution

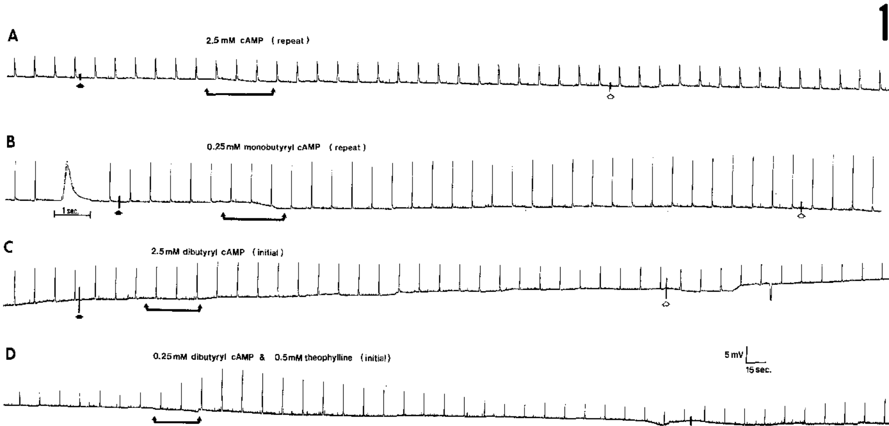


Fig. 1. Continuous, ten-min records of the effects of addition (at the brackets) of 2.5 mM cAMP (*A*), 0.25 mM monobutyryl cAMP (*B*), 2.5 mM dibutyryl cAMP (*C*), and 0.25 mM dibutyryl cAMP plus 0.5 mM theophylline (*D*) on the depolarization responses to iontophoretic pulses of ACh at neuromuscular endplates digested for 35 min. Perfusion flow of normal Ringer-Locke solution was turned off at the solid arrows and back on at the open arrows. *A* and *B* are repeat experiments following exposure to the same nucleotide (at the same concentration) and at least 10 min of normal perfusion. *C* and *D* are initial experiments on a hemidiaphragm. Stimulus artifacts are small compared to depolarization responses in *B*, *C*, and *D* but slightly larger in *A*. One response in *B* is expanded by a factor of five on the time axis and the gap in *C* is the result of a position adjustment on the recorder. Resting potentials are 70, 85, 90, and 80 mV respectively. Calibration bars, applying to all records, are 5 mV and 15 sec

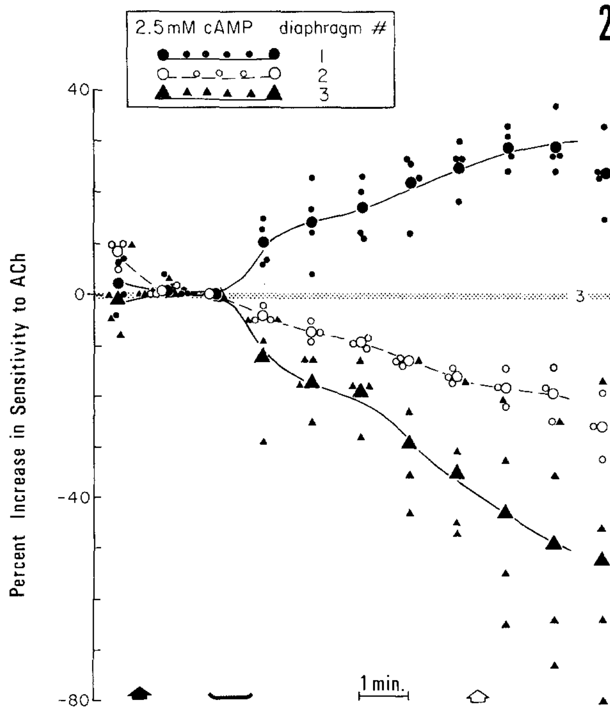


Fig. 2. Percent increase at one min intervals in the magnitude of depolarization responses to iontophoretic pulses of ACh applied every 15 sec to endplates of 3 diaphragm muscles digested for 35 min after addition of 2.5 mM cAMP to the bath (at bracket). The response level just preceding addition is the reference for percentage comparisons. Percent increases are graphed from -2 to 7 min and at 10 min with time equal to zero at the beginning of addition. A smooth curve is drawn through the means (large symbols) of the 3 or 4 experiments on each diaphragm which are represented by the corresponding small symbols. For each curve, two experiments were on a hemidiaphragm previously unexposed to cAMP. The remainder are experiments performed after at least 10 min of perfusion following these *initial* experiments. Diaphragms #1 and #2 were digested in fresh collagenase (1.0 mg/ml for 20 min) and protease (0.1 mg/ml for 15 min) solutions. For diaphragm #3 these solutions were re-used from a previous digestion

RL. In $Ca^{++}$-free RL (no added $Ca^{++}$), 2.5 mM cAMP caused a transient potentiation of sensitivity (mean: 10%) on repetition. In RL with twice the normal $Ca^{++}$ concentration, the ACh responses were attenuated well beyond the 10% attenuation seen in controls after 5 min. (Fig. 3)

The effects of butyryl derivatives of cAMP on sensitivity were a strict function of whether the experiment was an initial (no previous exposure to nucleotides) or a repeat (10 to 30 min of RL perfusion after an initial experiment). A low concentration of dibutyryl cAMP (0.25 mM) attenuated sensitivity beyond controls (22 and 16% at 5 min) in two initial experiments and was the same as controls in two repeat

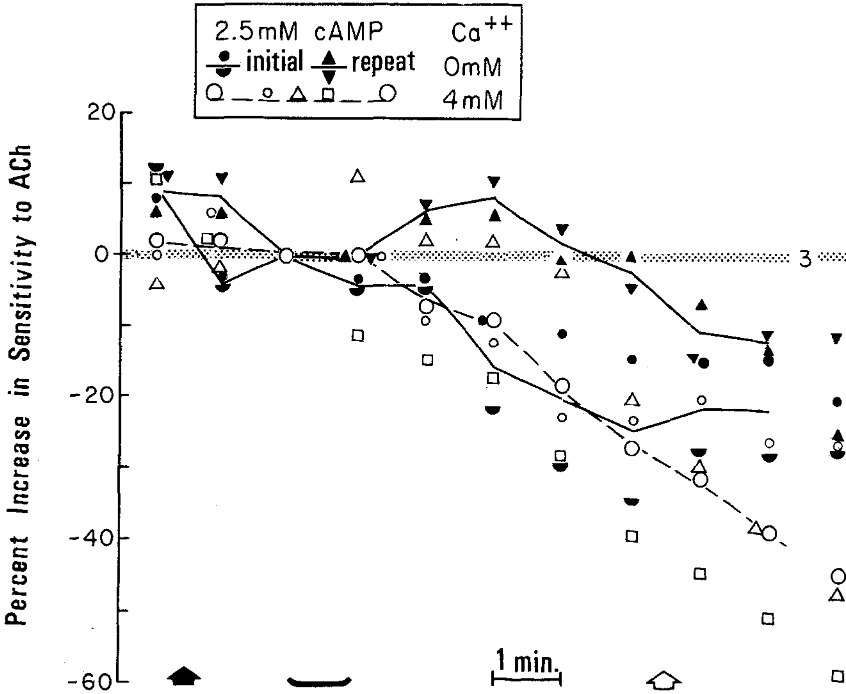


Fig. 3. Percent increase in sensitivity to ACh after addition of 2.5 mM cAMP in $Ca^{++}$-free (filled symbols) and in double-$Ca^{++}$ (open symbols, large circle means) Ringer-Locke on hemidiaphragms digested for 35 min. For $Ca^{++}$-free conditions lines are drawn through the averages of the two initial and the two repeat experiments on two hemidiaphragms from the same animal. Membrane potentials are 65 (circle), 70 (half-circle), 65 (triangle above line in insert), and 75 mV (triangle below line in insert). For the double-$Ca^{++}$ conditions the graphical format is as in Fig. 2 for 3 initial experiments on 3 hemidiaphragms from 2 animals. Membrane potentials, in the order of the insert, are 45, 50, and 70 mV

experiments. A higher concentration of dibutyryl cAMP (1.25 mM) attenuates 20 to 30% at 5 min in initial experiments and potentiates 20 to 30% at 5 min on repetition (Figs. 4 and 1*C*). Monobutyryl cAMP (0.25 mM) slightly attenuates sensitivity in initial experiments (mean: 10% at 5 min) and potentiates on repetition (mean: 20% at 5 min). This data is summarized in Fig. 5 and a record is shown in Fig. 1*B*.

The reversal of the effects of the butyryl derivatives of cAMP upon repeat exposure may indicate a dependence of their effects on the intracellular concentration of cAMP. To pursue this hypothesis theophylline was used to increase intracellular cAMP concentration (Butcher & Sutherland, 1962). Theophylline alone (0.5 mM) produced small, transient potentiations of sensitivity (mean: 5% at 1 min). In conjunction with dibutyryl cAMP, at a concentration (0.25 mM) which produces only a

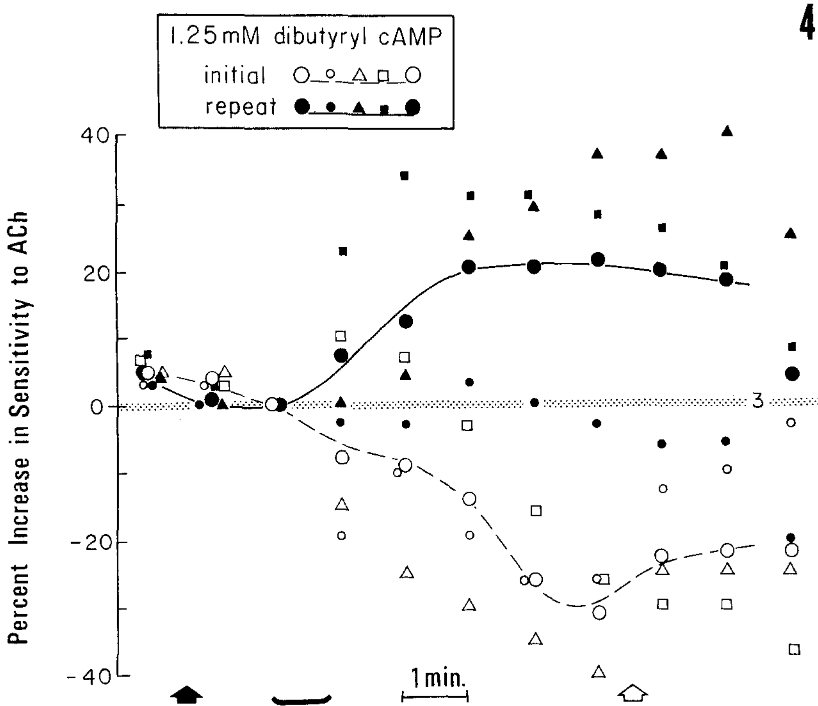


Fig. 4. Percent increase in sensitivity to ACh following addition of 1.25 mM dibutyryl cAMP in initial versus repeat experiments on hemidiaphragms digested for 35 min. Graphical format is as in Fig. 2 for 3 initial and 3 repeat experiments on 3 hemidiaphragms. Membrane potentials in mV are, in the order of the insert: *initial*—70, 60, 70, 80; and *repeat*—55, 50, 60, 55

small, gradual attenuation of sensitivity by itself, the transient potentiation is greatly increased (mean: 45% at 1 min). These results are summarized in Fig. 6 and a record of the largest transient potentiation is shown in Fig. 1*D*.

The ability of ATP to affect sensitivity (*see* preceding paper) is also altered by theophylline. Seven min after 1 mM theophylline was added, without resuming perfusion flow, 10 mM ATP increased sensitivity 175% after 3 min on the undigested preparation. The experiment was short due to muscle twitches which commenced 3 min after addition, but the potentiation was already well above the mean without theophylline. In other experiments the twitches appeared so early after ATP addition that no useful data were obtained. The following experimental protocol avoids the muscle twitches caused by simultaneous exposure to these two drugs, but also demonstrates that their synergistic action on sensitivity lasts for less that 10 to 20 min. After a 10-min exposure to 1 mM

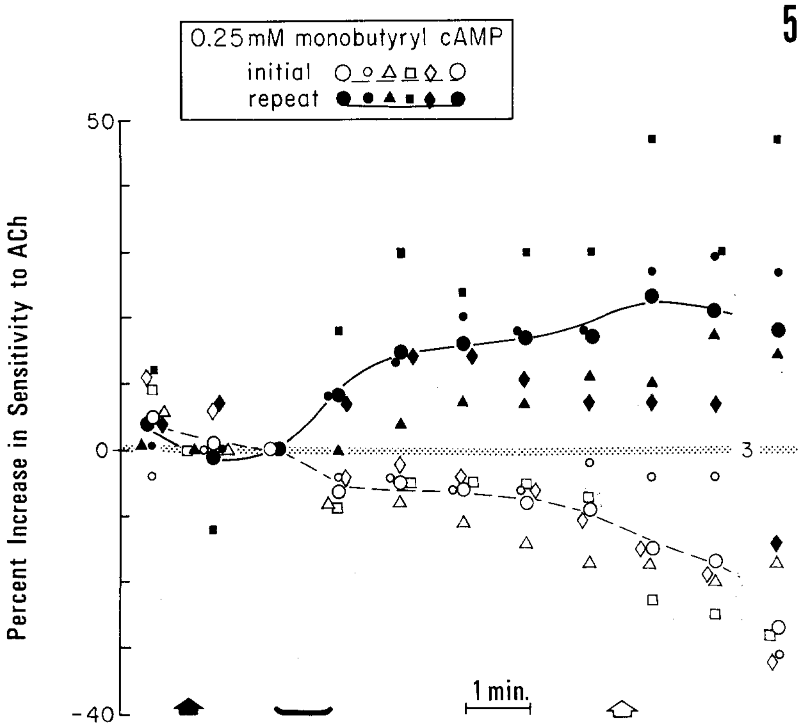


Fig. 5. Percent increase in sensitivity to ACh following addition of 0.25 mM monobutyryl cAMP in initial versus repeat experiments on hemidiaphragms digested for 35 min. Graphical format is as in Fig. 2 for 4 initial and 4 repeat experiments on 4 hemidiaphragms from 3 animals. In the order of the insert membrane potentials (in mV) are: *initial*—70, 60, 70, 80 and *repeat*—55, 50, 60, 55

theophylline and normal RL perfusion of 15, 25, and 35 min (three separate preparations), the potentiation by 10 mM ATP is blocked: −12, −8, and 0% increase in sensitivity at 4 min, respectively. After one hour of normal RL perfusion, the potentiation by ATP returns to its normal level (33% at 4 min).

The rate of desensitization was tested before, during, and after 0.25 mM and 2.5 mM cAMP and 0.25 mM monobutyryl cAMP (3 initial experiments each) in a manner similar to that described in the preceding paper. Only in the case of 0.25 mM cAMP was a significant decrease in the rate seen. This is reflected in the example of Fig. 7 where the ratio of the response magnitude just following the desensitization current to the magnitude just preceding it rose from 0.8 before addition, to 1.1 during exposure, and back down to 0.8 after normal perfusion was resumed. In the two other experiments at this concentration the ratio rose from 0.7 to 1.0 and from 0.6 to 0.8. No such increases in the

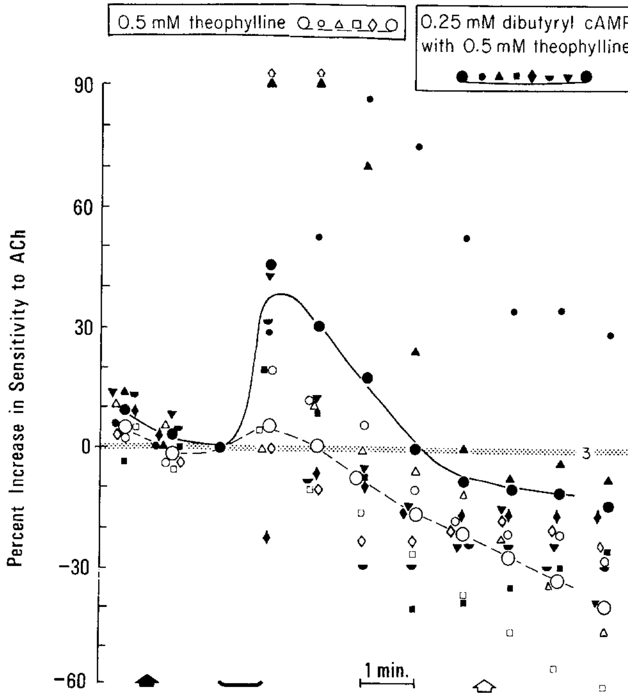


Fig. 6. Percent increase in sensitivity to ACh following addition of 0.5 mM theophylline (open symbols, large circle means) or 0.25 mM dibutyryl cAMP with 0.5 mM theophylline (filled symbols, large circle means) on hemidiaphragms digested for 35 min. Graphical format is as in Fig. 2 for 3 initial and 1 repeat experiment on 3 hemidiaphragms from 2 animals for theophylline alone and for 4 initial and 2 repeat experiments on 4 hemidiaphragms from 3 animals for dibutyryl cAMP plus theophylline. In the order of the inserts the membrane potentials (in mV) and initial (*i*) versus repeat (*r*) experiments are: theophylline alone—65*i*, 70*r*, 80*i*, 60*i* and theophylline plus dibutyryl cAMP—70*i*, 50*i*, 40*r*, 70*i*, 70*r*, 45*i*

ratio were seen during exposure to the same concentration of monobutyryl cAMP or to a higher concentration of cAMP.

Cyclic guanosine monophosphate (cGMP) has been implicated in the activation of muscarinic ACh receptors (George *et al.*, 1970). My results suggest that nicotinic ACh receptors are, instead, linked to intracellular cAMP metabolism. Two initial and two repeat experiments with cGMP (0.25 mM) produced no change in sensitivity. Two initial experiments with monobutyryl cGMP (0.25 mM) also showed no change and sensitivity changes of less than 10% at 5 min in repeat experiments. These results are consistent with the finding (*see* preceding paper) that GTP has no effect on sensitivity.

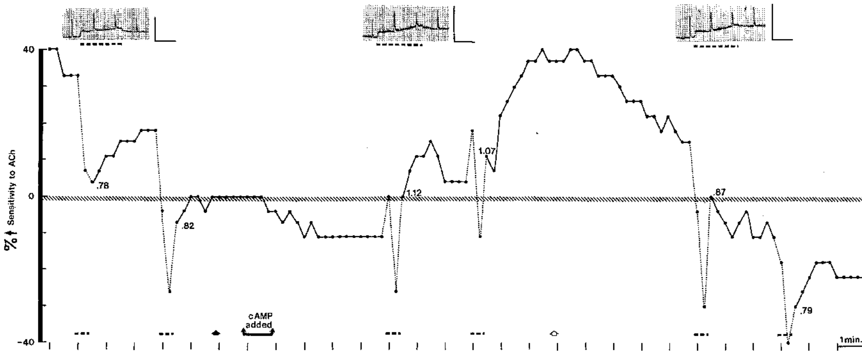


Fig. 7. Half-hour, graphical record of percent increase in ACh responses following addition of 0.25 mM cAMP (at bracket) in a single, initial experiment in which the ACh responses were desensitized six times on a hemidiaphragm digested for 35 min. The back-current on the iontophoretic ACh electrode was reversed during the dashed lines (*see* Methods) and the ACh responses during these desensitizing currents are connected by dotted lines in the graph. Perfusion flow was turned off at the filled arrow and on at the open arrow. Numbers at the end of each desensitization current are the ratios of he ACh response magnitude just following the current to the response magnitude just preceding it. Inserts show the actual records for the first of each pair of desensitization tests before, during, and after exposure to cAMP. Calibration bars are 5 mV and 15 sec. Two other experiments testing desensitization rate at this concentration of cAMP showed similar, but less pronounced, inhibition of desensitization

## Discussion

Cyclic AMP (2.5 mM) affects the sensitivity at endplates to iontophoretic pulses of ACh without changing membrane potential. The effect is different for each preparation (consisting of initial and repeat experiments on both hemidiaphragms), but repeatable on any given preparation. The time course, but not always the magnitude, is similar to the potentiation of sensitivity by comparable concentrations of ATP (*see* preceding paper). In initial experiments, cAMP causes a gradual attenuation of sensitivity in high-$Ca^{++}$ medium and a small, transient potentiation on repetition in $Ca^{++}$-free medium. These results are consistent with the hypothesis that cAMP and ATP affect sensitivity through the same biochemical mechanism. The lack of decrement of the response with repeated exposures to cAMP in normal RL, unlike the response to ATP, suggests that the cAMP effect is the more basic of the two.

An hypothesis to account for these effects is that extracellular receptor activation by ACh is potentiated by an increase in the intracellular cAMP

concentration up to an optimal concentration. The most likely molecular mechanism for this potentiation is phosphorylation or dephosphorylation of the cytoplasmic side of the receptor by a cAMP-activated protein kinase (Johnson, Ueda, Maeno & Greengard, 1972) or protein phosphatase (DeLorenzo, Walton, Curran & Greengard, 1973). At cAMP concentrations above this optimum a cAMP-activated $Ca^{++}$ release (Prince, Berridge & Rasmussen, 1972) would directly inhibit receptor activation (Lambert & Parsons, 1970). The $Ca^{++}$ release would also limit adenyl cyclase activity (Rasmussen & Nagata, 1971) and prevent exogenous or prejunctionally released ATP from increasing cAMP concentration beyond the optimum.

By this hypothesis high $Ca^{++}$ concentration would inhibit, and low $Ca^{++}$ concentration enhance, the potentiation of sensitivity by ATP or cAMP. Exogenous ATP would only potentiate sensitivity, whereas exogenous cAMP could increase intracellular levels beyond the optimum and produce either potentiation or attenuation depending on the endogenous concentration of cAMP in the preparation. Perfusion of muscle in high $K^{+}$ solutions will increase intracellular cAMP concentration (Lundholm, Rall & Vamos, 1967) and motor nerve activity increases the conversion of inactive phosphorylase into its active form (Posner, Stern & Krebs, 1965) which is a cAMP-mediated process (Rall & Sutherland, 1958). So my preparations might have different cAMP concentrations due to differences in (i) activity at the time of death, (ii) amount of nerve stimulation during the dissection, and (iii) digestion conditions. Attempts to standardize these procedures were not successful enough to yield preparations of uniform responsiveness to cAMP. One preparation was digested in used enzyme solutions. This preparation showed the largest decreases in sensitivity suggesting a high endogenous cAMP concentration due to excess $K^{+}$ in the digesting solutions. The effect of nerve stimulation on responsiveness to cAMP was not tested.

The butyryl derivatives of cAMP, having greater membrane permeability (Robison, Butcher & Sutherland, 1973), can apparently overcome the individual preparation differences and exceed the optimal intracellular cAMP concentration since sensitivity is consistently attenuated on initial exposure. This depresses adenyl cyclase activity by $Ca^{++}$ release and, after perfusion with normal RL, yields preparations with uniformly low cAMP concentrations. Thus, upon repeat exposure, the butyryl derivatives increase sensitivity.

From studies on other systems, theophylline is known to affect all the metabolic steps proposed to regulate receptor sensitivity in this sys-

tem: (i) it blocks the degradation of cAMP to AMP by phosphodiesterase (Butcher & Sutherland, 1962); (ii) it alters the balance between ionic and membrane-bound $Ca^{++}$ (Weber & Herz, 1968); and (iii) it inhibits adenyl cyclase activity at high cAMP concentrations (Sheppard, 1970). By itself, theophylline produces a small, transient potentiation of sensitivity followed by a gradual, long-lasting attenuation. In conjunction with a low concentration of dibutyryl cAMP, which by itself produces no significant change in sensitivity, the magnitude and duration of the transient potentiation is dramatically increased (reaching at one min the mean level of plateau potentiation by ATP) and the attenuation is delayed.

It was impossible to study the concurrent effect of theophylline and ATP at comparable concentrations with these methods since the muscle would immediately begin contracting and disrupt the measurement of sensitivity. This could not be avoided even by adding the ATP up to ten min after addition of theophylline. In one of these experiments, no contractions or abrupt changes in sensitivity were seen for three min after addition of ATP and at that point the sensitivity had more than doubled. A different experimental protocol gave the opposite result: potentiation by ATP was blocked by a 10-min exposure to theophylline followed by 15 to 45 min of normal RL perfusion and was partially recovered after an hour of perfusion. Thus theophylline affects the responses to both dibutyryl cAMP and ATP in a biphasic manner consistent with a short-term potentiation of receptor sensitivity by cAMP and a long-term blockage of this process due to the effects of intracellularly released $Ca^{++}$.

The rate of receptor desensitization (Thesleff, 1955) is decreased by 0.25 mM cAMP to a greater degree than by 1 mM ATP (*see* preceding paper). Calcium ion, which increases the rate of densitization (Manthey, 1966), depresses the potentiation of sensitivity by ATP and cAMP. Therefore, the regulatory pathways outlined above may be responsible for this phenomenon with the prejunctional release of ATP (Silinsky & Hubbard, 1973) serving to maintain the postjunctional receptors in their nondesensitized state.

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