EVIDENCE FOR A ROLE OF CYCLIC AMP IN NEUROMUSCULAR TRANSMISSION

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Abstract.—Experiments were undertaken to determine if the effects of epinephrine in promoting neuromuscular transmission were mediated by adenosine 3′:5′-cyclic phosphate (cyclic AMP). Dibutyryl cyclic AMP and the methyl xanthines, theophylline and caffeine (which inhibit cyclic AMP hydrolysis), were found to increase the amplitude of the end plate potential in the isolated rat diaphragm. Like epinephrine (which is known to promote cyclic AMP synthesis), these agents appear to facilitate the release of acetylcholine from the motor neuron. This interpretation is supported by the observation that theophylline and dibutyryl cyclic AMP markedly increased the frequency but not the amplitude of the spontaneous miniature end plate potentials. In addition, these drugs increased the number of transmitter packets released in response to nerve stimulation. These results are consistent with the view that cyclic AMP plays a role in the release of acetylcholine and in the "defatiguing effect" of epinephrine.

In a wide range of tissues, adenosine 3′:5′-cyclic phosphate (cyclic AMP) plays a central role in the regulation of metabolic processes and in the mediation of hormonal effects.[1, 2] A variety of observations suggest that this compound is also important in the function of nervous tissue, possibly in the release of neurotransmitters: (*a*) Sutherland *et al.*[3, 4] observed that the enzyme adenyl cyclase, which catalyzes the synthesis of cyclic AMP, and the phosphodiesterase that catalyzes its destruction are both present in higher concentrations in the brain than in any other tissue. Furthermore, both enzymes are found primarily in those regions of the brain richest in synapses; in cell homogenates they are localized in the fraction containing synaptic membranes.[5, 6] (*b*) The mechanism of release of neurotransmitters may be analogous to the secretion of subcellular vesicles from endocrine and exocrine glands, and cyclic AMP has been implicated in the release of insulin,[7, 8] salivary amylase,[9] and pancreatic amylase.[10] (*c*) Epinephrine is known to promote neuromuscular transmission by facilitating the release of acetylcholine from motor neurons.[11–14] (*d*) Epinephrine markedly increases the production of cyclic AMP by activating adenyl cyclase in various tissues, including brain.[1, 2, 15]

Thus it appears possible that facilitation of acetylcholine release by epinephrine involves cyclic AMP. A similar suggestion has been made recently by Breckenridge *et al.*,[16] who reported that theophylline, an inhibitor of cyclic AMP hydrolysis, augmented the increase in tension of the cat gastrocnemius muscle caused by epinephrine. Such changes in muscle tension, however, could also reflect the effects of these agents on contractile events and on muscle blood flow.[11]

To obtain more direct evidence for a role of cyclic AMP in neuromuscular transmission, we have examined the effects of dibutyryl cyclic AMP (the mono-

sodium salt), methyl xanthines, and epinephrine on the amplitude of both the end plate potential of single cells and the compound end plate potential (average population response). In addition, to determine whether the effects of these drugs on transmission involved a change in the release of acetylcholine from the motor neuron or in the sensitivity of the muscle cell membrane to this transmitter, we have studied the effects of these agents on (1) the spontaneous rate of release of acetylcholine (i.e., the frequency and amplitude of miniature end plate potentials) and (2) the amount released in response to a nerve action potential (i.e., the quantal content of the end plate potential).[11]

Method and Materials.—These experiments were carried out *in vitro* on the phrenic nerve diaphragm preparation of the rat. Male rats of the Charles River Strain were used in all experiments. The left hemidiaphragm with ribs and phrenic nerve attached was removed and mounted in 12 ml of Krebs-Ringer bicarbonate buffer[18] containing 11 mM glucose. For extracellular recordings, 95% O_2–5% CO_2 was continuously bubbled into the bath. For intracellular experiments, fresh oxygenated buffer flowed through the recording chamber at approximately 1 ml/min. Where specified, D-tubo-curarine was added to the bath in sufficient amount to abolish the muscle action potential but not the end plate potential. All other pharmacological agents were dissolved in 1 ml of the buffer (pH 7.2) and administered by syringe directly to the bath. Similar results were also obtained when the dibutyryl cyclic AMP was administered in isotonic Ringer's solution, prepared by replacing equimolar amounts of sodium chloride with the monosodium salt of the cyclic nucleotide (Schwarz BioResearch).‡ Final concentrations of the drugs in the bath are given. All recordings were carried out at 23°C.

To record the compound end plate potential, the tip of a recording electrode was placed above the end plate region, which was identified as the point of minimal latency following a supramaximal stimulus to the phrenic nerve. The recording electrode consisted of a 10 mil Ag-AgCl wire covered with polyethylene to its tip. The end plate potentials and miniature end plate potentials of single cells were recorded with glass pipettes filled with 3 *M* KCl (3–10 megohm resistance). Their amplitudes were measured from photographs of the oscilloscope traces. To count the total number of miniature end plate potentials, a standard electric pulse was triggered by each amplified miniature end plate potential, and the number of such pulses was counted on a Baird-Atomic scaler.

Results.—Various metabolic effects of epinephrine that involve cyclic AMP can be reproduced by the addition of theophylline or caffeine[1, 2] and have been shown to inhibit the hydrolysis of cyclic AMP by the cyclic phosphodiesterase of brain.[3] Hence, if the effect of epinephrine on transmission is mediated by cyclic AMP, it should be augmented by the addition of the methyl xanthines. In curarized preparations, the addition of 0.02 mM epinephrine was found to cause a marked increase in the height of the compound end plate potential (in accordance with earlier findings on the end plate potential of single cells).[12–14] The subsequent addition of 1 mM theophylline further increased the height of the compound end plate potential. The addition of more theophylline (1 mM) or more epinephrine (0.02 mM) further increased the amplitude and caused action potentials and muscle contractions to reappear in these curarized preparations. Moreover, even in the absence of added catecholamines, theophylline or caffeine augmented the amplitude of the compound end plate potential, in all eight preparations (Fig. 1*B*). This increase occurred within 30 seconds of administration and lasted for at least five minutes (at which time other agents were administered). Thus, the methyl xanthines and epinephrine have similar and additive effects on neuromuscular transmission.

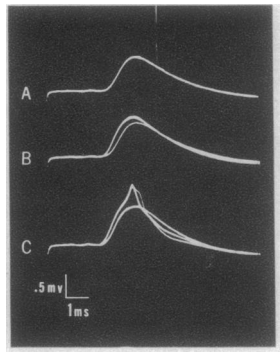

FIG. 1.—Recording of the compound end plate potential in a curarized rat diaphragm. Frequency of stimulation equaled 3 pulses/second, and each trace consisted of 4–5 sweeps of the oscilloscope. (*A*) Control amplitude of the end plate potential. (*B*) Superimposed records show increase in amplitude after the addition of 0.25 mM of theophylline (*upper trace*). Lower trace is control. (*C*) Addition of 4 mM of dibutyryl cyclic AMP 3 min after (*B*) caused an increase in the amplitude of the end plate potential to above threshold so that muscle action potentials became evident (*upper trace*). Lower trace is the same as upper trace in (*B*).

We examined the effects of N^6-2-*O*-dibutyryl cyclic AMP on the compound end plate potential to obtain more direct evidence for a role of cyclic AMP in transmission. This derivative has been found to be more potent than cyclic AMP itself in all tissues tested, presumably because of its greater ability to enter cells.[1] The amplitude of the compound end plate potential was increased in six of seven preparations tested by the addition of 4 mM dibutyryl cyclic AMP. The increase in amplitude became evident immediately after addition of the drug and lasted for several minutes. The effect subsequently decreased, presumably because of hydrolysis of the cyclic nucleotide. Similar results were obtained with recordings of single cells, in which the dibutyryl analog increased both the height and the rate of rise of the end plate potential (Fig. 2*B*).

Cyclic AMP itself, though occasionally promoting transmission, did not have as reproducible effects as the dibutyryl analog at equimolar concentrations. Large doses of AMP and ATP did not promote transmission and frequently even decreased the height of the compound end plate potential, probably as a result of their ability to chelate calcium.[21] The effects of the cyclic nucleotide and the methyl xanthines were additive (Fig. 1). Addition of 2 mM theophylline in the presence of 4 mM dibutyryl cyclic AMP increased the height of the compound end plate potential (in 4 of 5 preparations) so that action potentials were observed even in the presence of curare. Similarly, pretreatment of the phrenic nerve-diaphragm preparation with low doses of theophylline appeared to increase its sensitivity to the subsequent administration of the cyclic nucleotide in 3 of 3 preparations tested.

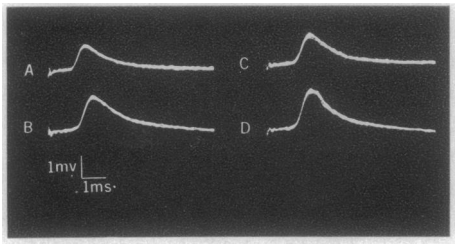

FIG. 2.—Intracellular recording of the end plate potential of single cells in a curarized rat diaphragm. (Frequency of stimulation was 1 pulse/2 sec.) Each trace consisted of 5 sweeps of the oscilloscope. (*A*) Control amplitude of the end plate potential. (*B*) Increase in the amplitude and rate of rise of the end plate potential following the addition of 4 mM dibutyryl cyclic AMP. (*C*) Same preparation 8 min after (*B*). Amplitude of end plate potential intermediate between (*B*) and control (*A*). (*D*) Increase in amplitude and rate of rise of end plate potential after the administration of 0.2 mM of caffeine.

Although the above studies suggest that cyclic AMP can promote neuromuscular transmission, they do not distinguish between possible presynaptic effects on the release of neurotransmitters and a postsynaptic effect on the sensitivity of the muscle membrane. We, therefore, studied the effects of dibutyryl cyclic AMP and theophylline on the frequency and amplitude of miniature end plate potentials. These spontaneous changes in membrane potential, recorded intracellularly in individual muscle cells, reflect the random release of individual packets of acetylcholine from the motor neuron.[17] An increase in the sensitivity of the muscle membrane should increase the amplitudes of the miniature end plate potential without markedly affecting their frequency. On the other hand, a change in the frequency of these potentials would indicate a presynaptic effect on the release of the transmitter.

Both dibutyryl cyclic AMP and theophylline, in doses which increased the amplitudes of the compound end plate potential (Figs. 1 and 2), were found to increase markedly the mean frequency of the spontaneous miniature end plate potentials (Tables 1 and 2). The frequency of these potentials (recorded continuously in single cells) increased in seven out of eight preparations treated with theophylline and in six out of seven preparations treated with the dibutyryl analog. In addition, highly significant increases were observed in the mean miniature end plate potential frequency of the preparation obtained by sampling many individual cells (Table 2, $p < 0.001$ by the Student's t-test). These changes were observed within one minute after the drugs were added and were still demonstrable 5 to 15 minutes later. The increase in frequency could be reversed by washing the drug from the bath with fresh buffer (Table 1). No effect was obtained with AMP. Finally, theophylline and dibutyryl cyclic AMP were found to have additive effects on the frequency of miniature end plate potentials (Tables 1 and 2), just as they had on the amplitude of the compound end plate potential (Fig. 1).

In contrast with these marked effects of miniature end plate potential frequency, no consistent change was observed in the amplitude of these signals (Table 1) or in the membrane potential of the muscle. In occasional experi-

TABLE 1. *Effects of theophylline and dibutyryl cyclic AMP on the frequency and amplitude of miniature end plate potentials.*

	Mean frequency (cps)	Mean amplitude (mv ± SEM)
Preparation A		
Control	116	0.34 ± 0.008
1.9 mM theophylline	147	0.37 ± 0.008
Additional 1.9 mM theophylline	174	0.33 ± 0.006
4.0 mM dibutyryl cyclic AMP	235	0.23 ± 0.006
Preparation B		
Control	120	0.56 ± 0.023
4.0 mM dibutyryl cyclic AMP	400	0.46 ± 0.008
20 min later	135	0.54 ± 0.018
4.0 mM dibutyryl cyclic AMP	207	0.55 ± 0.018

Miniature end plate potentials were recorded continuously in the same cell immediately prior to, or beginning 30 seconds after, addition of the drug. For Preparation A, the mean frequency and amplitude were averaged over 4 min and for Preparation B, 2 min. Both experiments were carried out in 22 mM Mg^{++}.

TABLE 2. *Effects of theophylline and dibutyryl cyclic AMP on the frequency of miniature end plate potentials.*

	Mean frequency ± SEM (counts/min)	Number of cells sampled
Preparation A		
Control	66 ± 8	15
1.4 mM theophylline	106 ± 12	9
20 min later	64 ± 3	5
4 mM dibutyryl cyclic AMP	112 ± 11	15
1.85 mM theophylline	216 ± 8	7
Preparation B		
Control	96 ± 14	11
4 mM dibutyryl cyclic AMP	172 ± 29	9
Preparation washed	114 ± 14	9
4 mM AMP	77 ± 10	10
4 mM dibutyryl cyclic AMP	179 ± 18	8
Preparation washed	84 ± 11	5

Miniature end plate potentials were counted for 30 seconds in Preparation A and 60 seconds in Preparation B in each cell sampled. All recordings were made from cells in the same area of the diaphragm. Prior to sampling and addition of drugs, the phrenic nerve was stimulated 1/sec for 30 min.

ments, dibutyryl cyclic AMP, while increasing miniature end plate potential frequency, actually appeared to decrease their amplitude (Table 1). These observations thus indicate that dibutyryl cyclic AMP and theophylline promote the spontaneous release of packets of acetylcholine from the motor neuron without significantly altering the sensitivity of the postsynaptic membrane.

Additional experiments were carried out to determine if the increase in amplitude of the end plate potential upon nerve stimulation (Figs. 1 and 2) also results from greater release of acetylcholine. Katz[17] has shown that each nerve impulse leads to the simultaneous discharge of many packets of transmitter. In high concentrations of Mg^{++}, fewer packets are released, and as a result, the end plate potential is reduced in height. The amplitude of the end plate potential varies markedly with individual nerve impulses, depending on the actual number of packets released. These variations follow a Poisson distribution, whose properties permit calculation of the mean number of transmitter packets released in response to nerve stimulation (i.e., the quantal content of the end plate potential).[19]

Table 3 demonstrates that in promoting transmission, dibutyryl cyclic AMP, theophylline, and caffeine increased the average quantal content of the end plate potential. These increases in quantal content (measured in 22 mM Mg^{++}) were highly significant ($p < 0.01$) and followed a similar time course to the changes in miniature end plate potential frequency described above. In addition, the cyclic AMP analog and the methyl xanthines were found to have additive effects on the number of packets released (Table 3). Similar results were obtained when the quantal content was estimated by different numerical methods.[19] Finally, additional evidence for increased transmitter release was obtained in certain preparations not blocked with high concentrations of Mg^{++}. In a typical curarized preparation, quantal content of the end plate potential[19] was calculated to be 204 ± 30 ($n = 120$). Following treatment with 4 mM dibutyryl cyclic AMP, this value appeared to be 423 ± 55 ($n = 194$, $p < 0.05$).

Discussion.—These experiments have provided several kinds of evidence sug-

TABLE 3. *Effect of theophylline, caffeine, and dibutyryl cyclic AMP on the quantal content of the end plate potential.*

	Mean quantal content ± SE (N)
Preparation 1	
Control	0.54 ± 0.05 (300)
0.46 mM theophylline	0.69 ± 0.06 (240)
4.0 mM dibutyryl cyclic AMP	1.05 ± 0.09 (240)
Preparation 2	
Control	0.50 ± 0.06 (180)
0.2 mM caffeine	0.90 ± 0.05 (540)
Preparation 3	
Control	3.18 ± 0.37 (240)
1.8 mM theophylline	18.00 ± 2.25 (143)
Preparation 4	
Control	1.00 ± 0.06 (300)
4.0 mM dibutyryl cyclic AMP	2.38 ± 0.21 (135)

These experiments were all carried out in Krebs-Ringer bicarbonate buffer[18] containing 11 mM glucose and 22 mM magnesium. The rate of stimulation was 1/sec. Quantal content was estimated, wherever possible, by different methods. The values given above were obtained by the method of counting misses for Preparations 1 and 2, by the covariance method for Preparation 3, and by dividing the mean end plate potential amplitude by the mean miniature end plate potential amplitude for Preparation 4.[19]

gesting that cyclic AMP can promote neuromuscular transmission by facilitating the release of acetylcholine from the motor neuron. Both dibutyryl cyclic AMP and the methyl xanthines were found to mimic the effects of epinephrine[13, 14] on the compound end plate potential, on the frequency of miniature end plate potentials, and on the quantal content of the end plate potential. Since epinephrine is known to cause the accumulation of cyclic AMP in nervous tissue,[15] these observations strongly suggest that the "defatiguing effects" of epinephrine are also mediated by cyclic AMP. This view is further supported by the finding that the effects of theophylline were synergistic with those of dibutyryl cyclic AMP or epinephrine.

Although these studies have presented strong evidence that acetylcholine release is promoted by agents that replace or raise the level of cyclic AMP, the possibility of an additional effect of these agents on the postsynaptic membrane has not been eliminated. Studies of the response to iontophoretically applied acetylcholine could better test this possibility.

Since theophylline appears only to inhibit the hydrolysis of the cyclic nucleotide, the marked effects obtained with this agent in the absence of added dibutyryl cyclic AMP or epinephrine further supports the view that endogenously synthesized cyclic AMP plays a role in transmitter release. In addition, it follows that the endogenous pool of the cyclic nucleotide is being turned over under the experimental conditions. Of additional interest is the observation that the effects of the dibutyryl analog and of theophylline appeared to be greatest when the preparations were subjected to long periods of stimulation or were in the bath for extended periods. The effects of epinephrine are also most easily demonstrated in fatigued preparations.[11, 19] These data suggest that the amount of cyclic AMP in the nerve ending may limit transmission during fatigue. It would thus be of interest to measure the levels of the cyclic nucleotide during fatigue and recovery.

The mechanism through which cyclic AMP might influence release of trans-

mitters remains to be elucidated. Cyclic AMP may promote glycolytic metabolism[1] in nerve endings which in turn may limit the rate of transmitter release.[22] Alternatively cyclic AMP might have a direct effect on Ca^{++} levels.[21] This ion plays a central role in release process,[17] and the methyl xanthines are known to alter calcium fluxes in muscle,[23] perhaps through cyclic AMP. These possibilities are under investigation.

The present results are consistent with earlier suggestions[16, 21] of a general cellular mechanism involving cyclic AMP in the release of intracellular packets from endocrine, exocrine, and neural tissues. Further experiments are necessary to determine whether the present observations on the neuromuscular junction are also applicable to transmission in the central nervous system. Since the enzyme systems for cyclic AMP production and destruction are localized in the synaptosome fraction of the brain, a role of cyclic AMP in transmitter release in the central nervous system appears likely. This result is of special interest since drugs known to influence cyclic AMP levels in the brain, such as the catecholamines, caffeine, and theophylline, have marked effects on cerebral functions.[24]

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Note added in proof: Since the completion of this manuscript, two articles have come to our attention which also demonstrate an effect of caffeine on the frequency of mEPPs: Elmquist, D., and D. S. Feldman, *J. Physiol. (London)*, **181**, 487 (1965), and Hofmann, W. W., *Am. J. Physiol.*, **216**, 621 (1969). Hofmann in addition observed an increase in the amplitude of the end plate potential in response to caffeine but in disagreement with the present study did not obtain this result in the presence of high concentrations of magnesium. The different findings may be due to the much faster rate of stimulation used in Hofmann's experiments.

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‡ In recent experiments, the effects of isotonic solutions of dibutyryl cyclic AMP on the frequency of miniature end plate potentials and on the quantal content of the end plate potential (measured in high concentrations of magnesium) have generally been smaller than these shown in Tables 1 and 3.

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