A Cyclic Adenosine Monophosphate Link in the Catecholamine Enhancement of Transmitter Release at the Neuromuscular Junction

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ABSTRACT The frequency of miniature endplate potentials (mepps) in rat diaphragms was markedly increased by epinephrine and norepinephrine in preparations exposed to 15 mM K^+. The effect was rapid in onset but gradually declined during continued exposure to the catecholamines. $N^6,O^{2'}$-dibutyryl adenosine $3',5'$-monophosphate (dibutyryl-cAMP) also caused transient frequency increases resembling in time-course those observed with catecholamines. Contrary to previous reports, catecholamines and dibutyryl-cAMP had little effect on mepp frequency in preparations not treated with K^+. Sustained increases with theophylline and decreases with adenosine were found in both K^+-treated and untreated preparations. Analysis of the data obtained with catecholamines showed the intensity of the response to be a function of nerve terminal polarization. The inability of catecholamines and dibutyryl-cAMP to affect mepp frequency of untreated preparations argues against an obligatory role for cAMP in the neurosecretory mechanism. The findings are consistent with an action of catecholamines and cAMP in the regulation of transmitter release at fatigued preparations.

INTRODUCTION

A role for cyclic adenosine $3',5'$-monophosphate (cAMP) in the release of neurotransmitters was initially postulated on the basis of the enhancement by theophylline of the epinephrine potentiation of neuromuscular transmission (Breckenridge et al., 1967). By analogy to other systems in which theophylline inhibited the hydrolysis of cAMP and catecholamines stimulated its synthesis (Sutherland and Robison, 1966), it was suggested that the facilitation of release might be due to cAMP. Subsequent microelelctrode investigations

have both supported (Goldberg and Singer, 1969; Singer and Goldberg, 1970) and questioned (Quastel and Hackett, 1971; Ginsborg and Hirst, 1972) this proposal. Discrepancies of interpretation appear to stem in part from considerations of whether cAMP is a *necessary* step in the sequence leading to neurosecretion rather than from the proposal that cAMP is a link in the catecholamine effect. Although catecholamines can cause small increases in transmitter release (Krnjević and Miledi, 1958; Jenkinson et al., 1968; Kuba, 1970), diverse effects of catecholamines on neuromuscular transmission have been observed at the nonfatigued junction (Bowman and Nott, 1969).

We have reexamined the evidence linking catecholamines, cAMP, and transmitter release at the neuromuscular junction. Analysis of transmitter release was made in terms of miniature endplate potential (mepp) frequency, a criterion which under properly controlled conditions is a sensitive and unequivocal index of nerve terminal activity. Compounds which have been reported to either mimic or increase cAMP were tested under resting (nonfatigued) and K^+-stimulated (fatigued) conditions. The results were consistent with the hypothesis that catecholamines and cAMP modify the release of transmitter in fatigued preparations but they failed to support an obligatory role for cAMP in the release process.

METHODS AND MATERIALS

Assessing Nerve Terminal Activity

The presynaptic spike is generally assumed to be a constant stimulus in evoking transmitter release from nerve terminals (see e.g., Hubbard et al., 1969, p. 113). However, the use of the endplate potential in assessing release may reflect drug actions on the presynaptic spike, a parameter well-known to affect the amount of transmitter released from nerve terminals. In the present study consideration of this factor was especially important in view of the ability of epinephrine to reverse nerve blockade produced by prolonged stimulation (Krnjević and Miledi, 1958).

Measurement of mepp frequency, on the other hand, is generally regarded as the most direct method of monitoring presynaptic activity. Since transmitter is released in discrete units, changes in mepp frequency must necessarily reflect nerve terminal activity (Katz, 1962). The need for conventional blocking agents is eliminated by tetrodotoxin (TTX) which may be used to prevent muscle twitching without alterations in transmitter output. However, several factors such as temperature (Fatt and Katz, 1952), osmotic pressure (Hubbard et al. 1968), and K^+ concentration (Liley, 1956) can drastically affect frequency, and employment of this technique must therefore include strict control over these parameters.

Potassium Stimulation and Fatigued Preparations

Neuromuscular fatigue, or a decline in junctional transmission, can normally be induced by prolonged nerve stimulation. Since the effect of catecholamines in reversing fatigue is much more pronounced in preparations exposed to K^+ (Bowman and

Raper, 1964), it appeared that K^+ might be mimicking the effects of fatigue. In lieu of prolonged nerve stimulation, preparations were therefore exposed to high concentrations of K^+. With 15 mM K^+, the concentration employed in this study, mepp frequency is raised from 1–2/s to about 50/s (Liley, 1956), and muscle resting potentials are lowered from -70 to about -50 mV. Higher concentrations of K^+ were undesirable due to the diminution in mepp amplitude as a result of the lowered resting potentials. To ensure a steady-state effect of K^+ on mepp frequency, preparations were equilibrated for at least 1 h.

Techniques

Left hemidiaphragms were removed from male Wistar rats (140–180 g) under ether anesthesia and mounted in a 5-ml chamber, continuously perfused with a 95 % O_2-5 % CO_2 gassed physiological saline solution. The control solution had a composition of (mM): NaCl 120; KCl 5.0; $CaCl_2$ 2.0; $MgCl_2$ 1.0; NaH_2PO_4 1.0; $NaHCO_3$ 24.0; glucose 17.0 and an osmolarity of 300 mosM as measured by cryoscopic techniques (Osmette S, Precision Systems, Inc., Natick, Mass.). Identical measurements of rat serum and heparinized blood gave values of 295 and 310 mosM, respectively. Saline solutions containing 15 mM KCl were made by substituting 10 mM KCl for equimolar NaCl with no resulting change in osmolarity.

Drugs were dissolved in portions of the control solution and the pH adjusted within ± 0.2 of control. The osmotic pressure of test solutions was adjusted to ± 2.0 mosM of control by the prior reduction of glucose when using nonelectrolytes or by the addition of NaCl when using electrolytes. In all cases total osmotic pressure did not exceed 320 mosM. Catecholamines were dissolved just before application. Drug concentration as a function of time was assessed by pumping a solution of 0.05 % Coomassie Blue into the chamber at 3 ml/min and measuring spectrophotometrically the concentration in 200 μl samples withdrawn from the center every 30 s. The increase in concentration with time approximated a first order exponential relationship with half-time of 1.1 min. In each figure arrows indicate the entry of drugs into the perfusion chamber.

The anhydrous, monosodium salts of mono- and dibutyryl-cAMP used in these experiments were obtained from Sigma Chemical Co., St. Louis, Mo. Solutions of these compounds were colorless and exhibited ratios of osmolarity/molarity of 1.9, indicating that the cyclic nucleotides had not undergone decomposition. Other compounds used were: adenosine; *l*-arterenol bitartrate; *l*-arterenol, free base; Na dibutyryl-cAMP; *l*-epinephrine bitartrate; Na glucose-6-phosphate; Na monobutyryl-cAMP; K_2 glucose-6-phosphate; and histamine dihydrochloride from Sigma; crystalline 3X TTX from Sankyo, Tokyo, Japan; and theophylline monohydrate from Mallinckrodt Chemical Works, St. Louis, Mo.

Recording apparatus consisted of an A-35L Medistor preamplifier (Medistor Instrument Co., Seattle, Wash.) coupled to a Tektronix 561B oscilloscope with 3A3 amplifier (Tektronix, Inc., Beaverton, Ore.). Photographs were made across the width of moving 35 mm film (Grass C-4 camera, Grass Instrument Co., Quincy, Mass.) and membrane potentials (E_m) monitored on one channel of a Brush 220 chart recorder (Brush Instruments Div. Clevite Corp., Cleveland, Ohio). Bath temperature was maintained between 32–34°C by heating the perfusing fluid through a

condensor coil before entry into the chamber. A bath thermistor was coupled to the second channel of the chart recorder, and for any given experiment, fluctuations were less than $\pm 0.5°C$. A constant flow of 3 ml/min was maintained using a roller pump. Microelectrodes were filled by diffusion under mild heating with 3 M KCl, and those employed ranged from 15–30 MΩ.

For maintained single cell recording, 2.5 to 5.0 $\times$ 10^{-7} M TTX was added to eliminate muscle twitching. Criteria for the selection of junctions were mepp rise times less than 1 ms, frequencies about 1/s for resting untreated preparations or at least 10/s for K$^+$-stimulated diaphragms, and high and stable muscle resting potentials. Photographs were taken approximately every minute, from 3 to 30 s in duration depending on basal mepp frequency. Except for Fig. 3, all experiments consisted of single junction recording, and results showing large frequency fluctuations or non-linear regression were rejected. All data presented indicate means $\pm$ 1 SEM. Comparisons of data with P values of 0.05 or less were considered to represent significant differences.

RESULTS

Drug Effects on Resting (Nonfatigued) Preparations

The actions of cAMP and its mono- and dibutyryl analogues, which are more lipid-soluble and more phosphodiesterase-resistant than cAMP, were tested on resting mepp frequency. Na butyrate was also tested since the reported increase in frequency by dibutyryl-cAMP might have been due to butyrate released from the cAMP analogues. The experimental protocol differed from Goldberg and Singer's (1969) in an attempt to attain more physiological conditions. Bath temperature was raised to 33° C and TTX used in place of 22 mM Mg^{++}. Because of the well-known actions of osmotic gradients in increasing mepp frequency (Hubbard et al., 1968), drugs were administered as isosmotic solutions using a system of gradual bath perfusion (3 ml/min).

The results from these experiments revealed no changes in mepp frequency on exposure to any of these compounds. Typical results of single experiments using dibutyryl-cAMP are shown in Fig. 1 A. No frequency increase was evident during the dibutyryl-cAMP perfusion, although as indicated by the sample inserts in Fig. 1 A, both mepp frequency and amplitude were greatly increased by theophylline. In a similar experiment with monobutyryl-cAMP, there was clearly no effect in a preparation showing a marked theophylline response (Fig. 1 B). Quantitative analysis of these results was in accord with the visual assessments in that no significant alteration in mepp frequency was found (Table I).

The above results with dibutyryl-cAMP were in disagreement with the frequency increase reported by Goldberg and Singer (1969). Their technique of directly injecting a concentrated dibutyryl-cAMP solution (52 mM) into the preparation bath so that the measured total osmotic pressure was 400 mosM suggested that the discrepancy might be due to osmotic effects. This
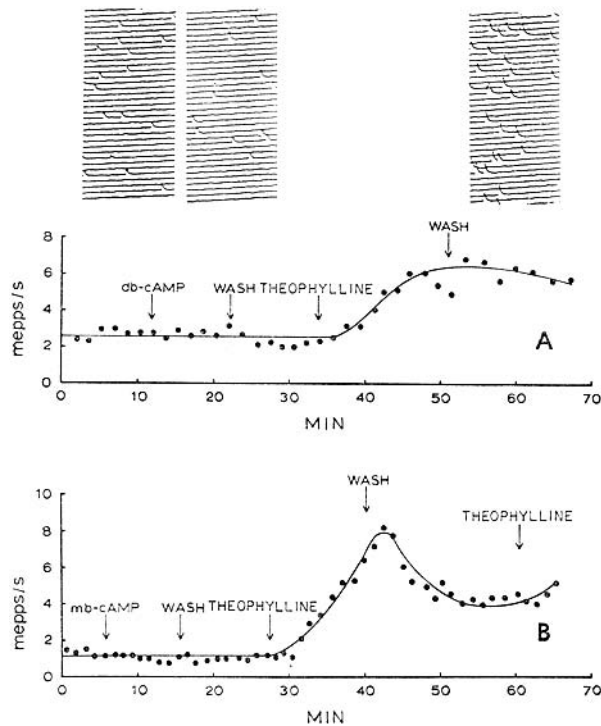
FIGURE 1.   Absence of effect of cAMP analogues on resting frequency of mepps. (A) 4 mM dibutyryl-cAMP was perfused onto a rat diaphragm for 10 min while recording from a single junction. After wash, 5.6 mM theophylline was introduced. Inserts from left to right represent sample recording during control, dibutyryl-cAMP, and theophylline periods, respectively. Interline distances are about 0.5 mV and horizontal sweeps are 110 ms long. (B) Experiment similar to the above, testing the effect of 4 mM monobutyryl-cAMP.

hypothesis was tested by duplicating the conditions of their study in both room temperature (25° C) and direct injection of drugs. As shown in Fig. 2 A, the application of control solutions containing added NaCl or sucrose (to make 400 total mosM) resulted in clear frequency increases to each application. This suggested that the technique of direct injection increased mepp frequency by creating large osmotic gradients within the chamber.

The effects of catecholamines on mepp frequency were also reexamined using the present system. Nonfatigued preparations were used and initial assessments were made from plots of frequency versus time. Inspection of these records showed that the application of supramaximal concentrations of catecholamines (1–50 $\times$ 10^{-5} M; cf. Jenkinson et al., 1968) did not reveal any changes distinguishable from base-line drift. Analysis of the data using the calculations described in Table I appeared to show an increase in mean frequency (Table II, columns 4 and 5) which was maintained during the wash

TABLE I

EFFECT OF CYCLIC NUCLEOTIDES ON MEPP FREQUENCY OF UNTREATED
(NONFATIGUED) PREPARATIONS

1	2	3	4	5	6
		No. of	Mean mepp frequency (%)		
Compound	Concentration	trials	Control	Test	Wash
	M				
Dibutyryl-cAMP	4×10^{-3}	4	100	98.7±7.9	91.7±4.5
			(5.3±1.5)	(7.3±1.2)	(12.0±1.5)
cAMP	4×10^{-3}	4	100	107.9±12.1	96.2±4.1
			(11.2±0.4)	(10.2±0.5)	(7.0±1.3)
Na butyrate	8×10^{-3}	3	100	85.6±9.6	91.7±20.7
			(9.6±0.4)	(9.8±0.2)	(8.7±0.8)

Each trial consisted of a continuous single cell recording. During each test or wash period indi-
vidual observations of mepp frequency were expressed as a percent of the mean frequency during
the control period of the same cell. The values so obtained were averaged to yield a mean value
for each trial under each condition. The set of such values derived from the number of trials
indicated (column 3) was then used to obtain a mean ± SE (columns 5 and 6). Numbers in
parentheses indicate duration in min (mean ± SE) for each period. In no instance was there a
significant change from the control frequency at the 0.05 level. Mean control frequency for all
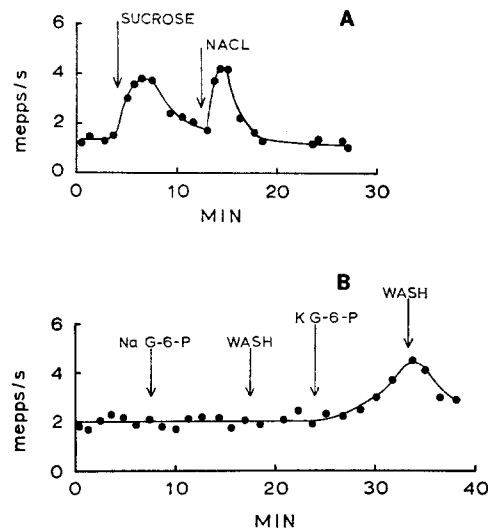data was 1.87 ± 0.29/s.

FIGURE 2. (A) Effect on mepp frequency of direct injection of concentrated drug
solutions into the preparation chamber. Increases in mepp frequency occurred upon
injection of solutions consisting of control saline plus added sucrose or NaCl. Injected
volume was 1/13 of total chamber volume, and osmolarities were 400 mosM, equal
to the dibutyryl-cAMP solution used by Goldberg and Singer (1969). Contractions
were blocked with 5 × 10⁻⁷ M TTX. Control solution was perfused throughout ex-
periment at 1 ml/min, and temperature was 25°C. (B) Actions of Na G-6-P and K₂
G-6-P on resting mepp frequency. Perfusion of 1.4 mM Na G-6-P onto a rat diaphragm
was followed by wash and 1.4 mM K₂ G-6-P.

TABLE II

EFFECT OF CATECHOLAMINES ON MEPP FREQUENCY OF UNTREATED
(NONFATIGUED) NEUROMUSCULAR JUNCTIONS

1	2	3	4	5	6
		No. of	Mean mepp frequency (%)		
Compound	Concentration	trials	Control	Test	Wash
	M				
Epinephrine	1×10^{-5}	5	100	117.7±10.2	131.2±13.6*
	to		(8.4±2.5)	(11.3±0.6)	(7.4±1.3)
	5×10^{-4}				
Norepinephrine	1×10^{-5}	6	100	122.6±15.0	127.7±21.1
	to		(8.4±1.6)	(12.4±1.1)	(7.1±1.1)
	5×10^{-4}				

Experimental methods and presentation of data are the same as in Table I. Mean frequencies
appeared to be slightly elevated during exposure to catecholamines and wash (columns 5 and
6). However, in only one instance (*) was this significant at the 0.05 level. Mean control mepp
frequency was 1.47 ± 0.19/s.

period (column 6), but the results from the t-test statistics showed this to be
significant in only one instance (asterisk).

Several additional compounds were tested which have been reported to
either increase cAMP levels in nervous tissue or to be implicated in cAMP fa-
cilitation of neuromuscular transmission. Histamine for example increases
cAMP in cerebral cortex (Kakiuchi and Rall, 1968) while adenosine stimu-
lates the formation of cAMP in cultured astrocytoma cells (Clark and Perkins,
1972) as well as in cerebral cortical slices (Sattin and Rall, 1970). Theophyl-
line raises cAMP levels by the inhibition of cyclic nucleotide phosphodiester-
ase. Glucose-6-phosphate (G-6-P) has been reported to facilitate muscle con-
tractions in both directly and indirectly stimulated preparations (Varagić and
Žugić, 1971). These authors interpreted the effect in terms of endogenous
G-6-P arising from cAMP-mediated glycogenolysis.

The results of testing these compounds on resting mepp frequency are sum-
marized in Table III. Adenosine, in accord with Ginsborg and Hirst (1972),
caused a significant decrease (25%) in mepp frequency. By contrast, the ap-
plication of both histamine and the sodium salt of G-6-P clearly had no effect
on frequency. However, since Varagić and Žugić (1971) had used the dipotas-
sium salt of G-6-P in their study, the possibility existed that the facilitation
might have been due to K^+ rather than to any specific action of G-6-P. The
effect of G-6-P on the nerve terminal was therefore again tested as shown in
Fig. 2 B. The application of 1.4 mM G-6-P resulted in no frequency changes
and was followed by wash. On exposure to 1.4 mM K_2 G-6-P however, there
was a marked increase in mepp frequency. These results taken together indi-
cate that the concentration of K^+ present was capable of facilitating trans-
mitter release but that G-6-P had no effect.

TABLE III

EFFECTS OF SELECTED COMPOUNDS ON MEPP FREQUENCY OF
UNTREATED (NONFATIGUED) PREPARATIONS

1	2	3	4	5	6
		No. of	Mean mepp frequency (%)		
Compound	Concentration	trials	Control	Test	Wash
	M				
Histamine	1×10^{-3}	3	100	96.0±2.8	93.1±11.6
			(9.8±1.1)	(9.8±0.6)	(9.6±3.1)
Adenosine	1×10^{-2}	3	100	74.6±8.4*	78.1±5.5*
			(8.4±1.7)	(10.1±0.2)	(7.2±3.0)
Na G-6-P	1.4×10^{-3}	3	100	98.3±2.6	104.3±0.4
			(9.0±0.3)	(9.3±0.1)	(6.0±1.4)
			Rate of increase	Time period to determine slope	
			mepp/min/min	*min*	
Theophylline	2.8×10^{-3}	2	3.5±0.1	10.2±0.2	
	5.6×10^{-3}	7	16.6±3.1	7.8±0.4	
	11.0×10^{-3}	2	12.0±1.2	8.4±0.1	

Experimental methods and presentation of data for the upper portion of the table are the same as in Table I. Mean control mepp frequency for all data was 1.47 ± 0.19/s. Differences recorded for adenosine (*) are significant at the 0.05 level.

Theophylline was consistently found to increase mepp frequency in un-
treated preparations (cf. Fig. 1 A and B). Within the time period observed,
there was no plateau in the frequency increase. The effect is described quanti-
tatively in terms of the rate of frequency increase, as determined by the slopes
of the linear regression through the test points (Table III, column 5). In the
range of concentrations tested, the rate of increase appeared to be maximal at
about 5 mM. This effect of theophylline was readily reversible as illustrated in
Figs. 1 A and B and 3. In the latter figure, 5.6 mM theophylline was applied
in the absence of TTX, but because of the resulting muscle fibrillation, re-
cording was carried out by random sampling of junctions with time.

Drug Effects at K^+-Stimulated (Fatigued) Preparations

Because a notable effect of catecholamines is to reverse neuromuscular fatigue,
experiments were also performed on preparations exposed to prolonged K^+
stimulation. Application of monobutyryl-cAMP or Na butyrate clearly had
no effect on these preparations (Table IV). With cAMP there appeared to be
a slow increase in mean mepp frequency, similar to that seen with catechol-
amines in untreated preparations (Table II). The statistical analyses, how-
ever, did not indicate this to be significant (Table IV). Dibutyryl-cAMP, on
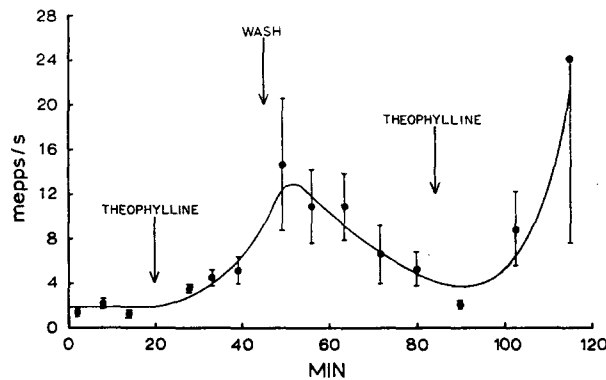the other hand, caused a conspicuous rise in mepp frequency (Fig. 4 B).

FIGURE 3. Effect of theophylline on mepp frequency in the absence of blocking drugs. Each point represents the mean frequency ± SE of from 2 to 7 junctions sampled at random and the average time at which each group of recordings was made. The probability of finding suitable junctions was increased by raising Ca^{++} to 4 mM.

TABLE IV

EFFECTS OF CYCLIC NUCLEOTIDES ON MEPP FREQUENCY OF
K^+-STIMULATED (FATIGUED) PREPARATIONS

1	2	3	4	5	6
		No. of		Mean mepp frequency (%)	
Compound	Concentration	trials	Control	Test	Wash
	M				
Monobutyryl-cAMP	4×10^{-3}	3	100 (11.4±0.9)	112.8±7.1 (10.2±0.1)	118.4±20.2 (11.6±0.4)
cAMP	4×10^{-3}	3	100 (11.4±1.0)	124.5±18.4 (13.3±0.9)	149.4±23.4 (10.2±3.1)
Na butyrate	8×10^{-3}	3	100 (12.4±1.7)	122.2±12.2 (13.8±0.6)	104.4±14.1 (9.1±0.5)

Experimental methods and presentation of data are the same as in Table I. Although mean frequencies during some test and wash intervals appear to be slightly elevated, none of the differences are significant at the 0.05 level. Mean control mepp frequency was 21.0 ± 3.6/s.

Clearly distinguishable were the slow rise in frequency, a peak after about 5 mins, and a decline to control levels during the continued application of the compound. A composite of effects for five such experiments is shown in Fig. 5 B, in which frequencies were converted to percentages to allow averaging (cf. Table I). It should be noted that the composite effect appears lower in intensity and the time-course more prolonged than in individual experiments because of variations in the time-course of effect. In three other experiments, no effect was found with dibutyryl-cAMP.

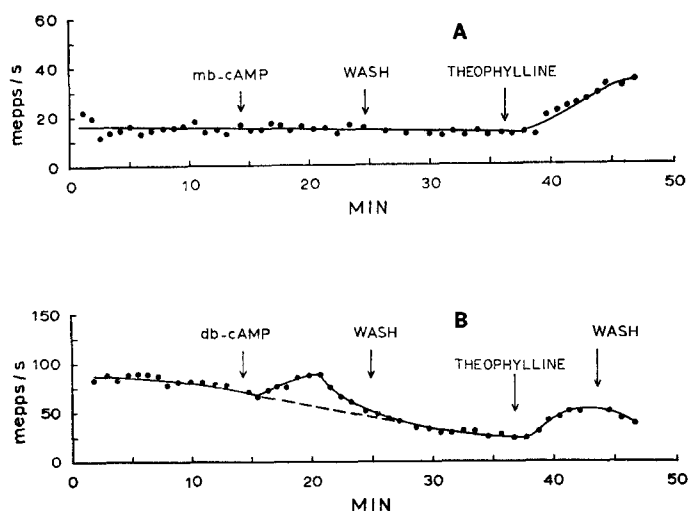No effect was found with histamine or Na G-6-P, but adenosine caused a

FIGURE 4. Actions of cAMP analogues and theophylline on mepp frequency in K+-stimulated preparations. Continuous recording from single junctions in which 4 mM monobutyryl-cAMP (A) and 4 mM dibutyryl-cAMP (B) were superfused for 10 min, followed by wash and 5.6 mM theophylline.

decrease in frequency in three experiments (Table V). Although no detailed examination of theophylline was made using K+-stimulated preparations, increases in mepp frequency (e.g., Fig. 4 A and B) similar to those seen with untreated diaphragms were always observed.

In contrast to untreated preparations, K+-treated junctions exhibited a striking enhancement of mepp frequency after application of norepinephrine. The onset of this effect was rapid, but like that observed with dibutyryl-cAMP, it declined during the continued application of the drug. Results of three experiments with epinephrine were identical to the above, and the data for the two catecholamines were therefore combined (Fig. 5 A). The similarities in time-course of the catecholamine and the dibutyryl-cAMP effects (Fig. 5 A and B) were clear, but the increase in mepp frequency with catecholamines was larger and the rate of increase more rapid than that with dibutyryl-cAMP.

The present findings with catecholamines were compared to those of a recent study in which similar effects were examined as a function of nerve terminal polarization (Kuba and Tomita, 1972). Those authors found mepp frequency was logarithmically related to electrophoretically applied current, and norepinephrine increased mepp frequency but not the slope of the relationship. Assuming that applied current alters nerve terminal E_m linearly (Cooke and Quastel, 1973) and that log mepp frequency is proportional to nerve terminal E_m (Liley, 1956; Katz, 1962), then log mepp frequency is proportional to applied current. These relationships were used in analyzing the
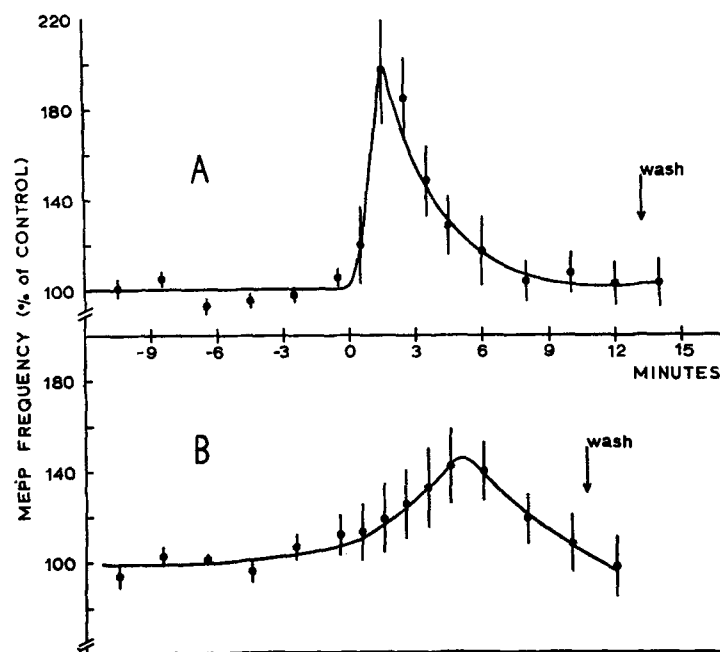
FIGURE 5. (A) Effects of catecholamines on mepp frequency under K^+-stimulated conditions. The figure represents a composite of results obtained in six single cell trials, three with 0.1 mM epinephrine and three with 0.1 mM norepinephrine. Drugs were applied at time = 0, and wash was begun at arrow. At each point the mean value ± SE is shown. (B) Similar composite of five experiments using 4 mM dibutyryl-cAMP on preparations under K^+-stimulated conditions.

TABLE V

EFFECTS OF HISTAMINE, ADENOSINE AND Na G-6-P ON MEPP FREQUENCY
OF K^+-STIMULATED (FATIGUED) PREPARATIONS

1	2	3	4	5	6
				Mean mepp frequency (%)	
Compound	Concentration	No. of trials	Control	Test	Wash
	M				
Histamine	1×10^{-3}	3	100	114.8±14.8	120.1±30.7
			(12.2±0.6)	(10.6±1.3)	(7.8±0.9)
Adenosine	2×10^{-3}	3	100	53.2±5.3*	54.0±6.0‡
			(11.4±0.5)	(13.0±0.3)	(11.8±2.1)
Na G-6-P	4×10^{-3}	3	100	92.4±3.8	90.6±6.0
			(11.0±1.2)	(8.8±2.5)	(11.6±0.7)

Experimental methods and presentation of data are the same as in Table I. Mean frequency for control data was 40.16 ± 8.69/s. Differences recorded for adenosine are significant at the 0.0005 level (*) and the 0.01 level (‡).

present results (Fig. 6). Log mepp frequency is plotted on the abscissa as the equivalent of applied current. Log of the peak mepp frequency attained on catecholamine application is plotted on the ordinate. As indicated by the solid line, the points for epinephrine and norepinephrine fall on the same line, suggesting a common response mechanism. The effect of added catecholamines is observed to be a parallel shift in frequency from the broken line toward the solid line with little change in the slope of the relationship. The agreement of the present data with those of Kuba and Tomita (1972) suggests that the variation in mepp frequency at individual junctions before adding catecholamines
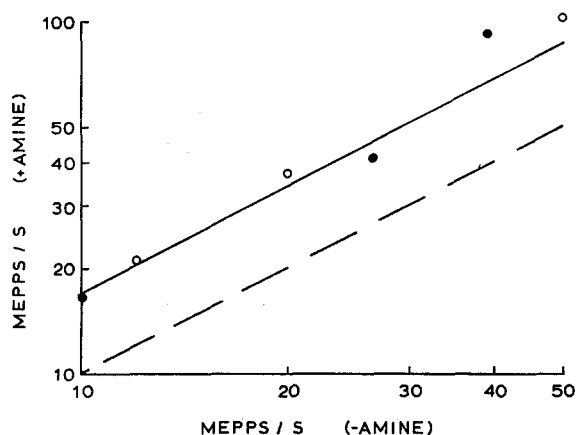

FIGURE 6. Dependence of the catecholamine-associated increase in mepp frequency upon basal mepp frequency. The data from Fig. 5 A were plotted at the peak frequency attained with catecholamines (ordinate) as a function of the initial mepp frequency before addition of catecholamines (abscissa). Note the log scales on both coordinates. Open circles represent results obtained with epinephrine and filled circles with norepinephrine. The broken line represents mepp frequency before catecholamines.

(Fig. 6, abscissa) was probably due to individual differences in nerve terminal E_m. The increased efficacy of catecholamines with decreased nerve terminal E_m supports a correlation between state of nerve terminal polarization and fatigue.

DISCUSSION

The present findings show a distinct similarity between the actions of dibutyryl-cAMP and catecholamines on transmitter release. Both are ineffective in altering resting mepp frequency in untreated preparations (Tables I and II) but cause significant increases under K^+-stimulated conditions (Fig. 5 A and B). Responses to both agents are characterized by the spontaneous reversal and by the similarity in their time-course. The rapid mepp frequency increase with catecholamines (Fig. 5 A) might be explained by the availability

of nerve terminal catecholamine receptors which would evoke a rapid intra-cellular increase in cAMP. The slower frequency increase with dibutyryl-cAMP (Fig. 5 B) might then be explained by a slow rise in intracellular cAMP because of the need for dibutyryl-cAMP to penetrate the nerve termi-nal membrane. The inability of cAMP to affect mepp frequency (Tables I and IV) may be due to the fact that it traverses membranes poorly and is rapidly hydrolyzed.

The generalization that cAMP is an obligatory intermediate in the release process (Rasmussen, 1970; Singer and Goldberg, 1970) requires additional critical analysis. On the basis of their findings, Singer and Goldberg (1970) correctly concluded that the effect of dibutyryl-cAMP could not be due to an action on nerve terminal E_m. The parallel increase in mepp frequency and evoked quantal content in high Mg^{++} argued instead for an action near the secretory step (Hubbard et al., 1969, pp. 122–123). However, the inability of dibutyryl-cAMP to alter resting mepp frequency was clearly demonstrated by the present results (Table I) as well as by the evidence (Fig. 2 A) indicating the osmotic nature of Goldberg and Singer's results. Added support for this explanation is provided by the fact that osmotic gradients increase both mepp frequency and the early quantal content, and do so by a mechanism inde-pendent of high Mg^{++} (Hubbard et al., 1968), two actions identical to those described by Goldberg and Singer (1969).

The available evidence suggests that cAMP is not a required factor in transmitter release at the motor nerve terminal. Such a role would require a sequential mechanism for cAMP in the events leading to the actual release of transmitter. However, studies on the kinetics of such a mechanism have shown this proposal to be highly unlikely (Quastel and Hackett, 1971). In addition, the rate-limiting step between excitation and secretion appears to be calcium influx (I_{Ca}) (Katz and Miledi, 1970) as determined by nerve terminal con-ductance to calcium (g_{Ca}). For a nerve terminal E_m at rest, g_{Ca} and thus I_{Ca} are low, and only a few spontaneous quanta are released. Therefore, if cAMP were a necessary component of the events occurring past the I_{Ca} step, intra-cellular increases in cAMP (or the application of dibutyryl-cAMP) should enhance release in resting preparations. However, this does not occur (Table I).

Because dibutyryl-cAMP and catecholamines facilitate transmitter release but are ineffective at resting nerve terminal E_m when I_{Ca} is minimal, the site of action must be before the I_{Ca} step. Furthermore, because catecholamines do not affect the action current at nerve endings, the effect is not due to mem-brane depolarization (Kuba, 1970). This implies that catecholamines, pre-sumably acting through cAMP, augment transmitter release by increasing g_{Ca}. Precedent for this type of mechanism can be found in atrial muscle fibers exposed to elevated K^+. In this system catecholamines restored action poten-

tials by an increase in g_{Ca} without affecting muscle E_m (Pappano, 1970). The increased catecholamine response with nerve terminal depolarization (Fig. 6; also Kuba and Tomita, 1972) may be explained by an increased g_{Ca} due to depolarization which would multiply with the increment in g_{Ca} due to catecholamines (cf. discussion in Kuba and Tomita, 1971). It is of interest that a similar dependence on K^+ of catecholamine action has been shown for duck erythrocytes. In this system, dibutyryl-cAMP or norepinephrine-promoted cation transport, but only in the presence of 15 mM K^+ (Riddick et al., 1971).

An unresolved question involves the spontaneous decline in mepp frequency increase at K^+-treated junctions exposed to dibutyryl-cAMP or catecholamines (Fig. 5 A and B). One possible explanation for the decline may be that the influx of calcium not only promotes transmitter release but also acts to lower cAMP by a feedback mechanism. Elevated levels of calcium inhibit brain adenyl cyclase (Bradham et al., 1970) and stimulate brain phosphodiesterase (Wolff and Brostrom, in press), so that calcium within the nerve terminal may regulate its own influx by altering cAMP levels.

Finally, the effects with adenosine and theophylline remain unclear. Although adenosine elevates cAMP levels in brain slices (Sattin and Rall, 1970), it causes a decrease in transmitter release (Ginsborg and Hirst, 1972; also Table IV). Possible explanations are competition by adenosine with cAMP at the level of protein kinase (Miyamoto et al., 1969) or inhibition of adenyl cyclase (McKenzie and Bär, 1973). This question is further complicated by the possibility that the increase in cAMP in brain slices exposed to adenosine may be due solely to glia (Clark and Perkins, 1972). Similarly, the actions of theophylline, while possibly due to the inhibition of phosphodiesterase and elevation of cAMP, may also be explained by a caffeine-like action in altering calcium influx (Blinks et al., 1972) at the nerve terminal.

In conclusion, the inability to measure cAMP levels at nerve endings imparts a degree of uncertainty to interpretation of these results. However, good evidence for a link between cAMP and catecholamines is provided by the overall similarity of effect on mepp frequency in K^+-stimulated preparations. Such findings are compatible with an action of catecholamines in increasing nerve terminal cAMP, and as a result, in accelerating the influx of calcium.

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