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THE EFFECTS OF CYCLIC *N*-2-*O*-DIBUTYRYL-ADENOSINE 3′,5′-MONOPHOSPHATE, ADRENALINE AND AMINOPHYLLINE ON THE ISOMETRIC CONTRACTILITY OF THE ISOLATED HEMIDIAPHRAGM OF THE RAT

D. KENTERA & V.M. VARAGIĆ

Department of Pharmacology, Faculty of Medicine, and Institute for Medical Research, PO Box 662, Belgrade, Yugoslavia

1 *N*-2-*O*-dibutyryl adenosine 3′,5′-monophosphate (db cyclic AMP), adrenaline and aminophylline produce a potentiation of the tension developed (T_d) and the maximum rate of rise of tension (dT/dt *max*) in the rat isolated diaphragm during indirect electrical stimulation. Aminophylline and db cyclic AMP also produce the same effect during direct stimulation.

2 Propranolol produced a depression of the action of adrenaline on T_d and dT/dt *max* during indirect stimulation of the diaphragm. On the other hand, the potentiating actions of db cyclic AMP and of aminophylline on T_d and dT/dt *max* during indirect stimulation were unaffected by propranolol.

3 The results support the idea that cyclic AMP may be involved not only in regulating the processes associated with synthesis, mobilization and storage of transmitter in the motor nerve terminal, but also in modifying some metabolic processes which regulate the function of the contractile elements.

Introduction

It has been shown previously that cyclic *N*-2-*O*-dibutyryl-adenosine 3′,5′-monophosphate (db cyclic AMP) potentiates the response of the rat isolated diaphragm preparation to indirect stimulation (Varagić & Žugić, 1972). Parallel to this, db cyclic AMP has also been found to produce a significant decrease in the concentration of glycogen in the diaphragm (Varagić, Žugić & Mršulja, 1972). Cyclic adenosine-monophosphate (cyclic AMP) itself did not affect either the response of the diaphragm to phrenic nerve stimulation, or the concentration of glycogen in the diaphragm.

The dibutyryl derivative of cyclic AMP has also been found to increase the amplitude of the endplate potential in the isolated diaphragm. It has therefore been suggested that cyclic AMP might play a role in the process of acetylcholine release and also in the well known 'defatiguing effect' of adrenaline (Goldberg & Singer, 1969).

The interaction of catecholamines and methylxanthines in the cyclic AMP system has been described by Sutherland and his collaborators (Robison, Butcher & Sutherland, 1971).

In the present experiments attention was paid to recording of more precise parameters of the isometric muscle contractility: tension development (T_d) and maximum rate of rise of tension (dT/dt *max*). The effects of three substances, adrenaline, aminophylline and cyclic AMP, known to affect the adenyl cyclase-cyclic AMP system, were recorded and correlated.

Methods

The isolated phrenic nerve-diaphragm preparation was set up as described by Bülbring (1946). The diaphragm preparation was suspended in a 10 ml bath at 36° C, in Tyrode solution with a double amount of glucose and bubbled with pure oxygen. The composition of the Tyrode solution was as follows (mM): NaCl 136.7, KCl 2.81, $CaCl_2$ 1.8, $MgCl_2$ 0.105, NaH_2PO_4 0.417, $NaHCO_3$ 11.9, glucose 11.101.

The diaphragm was stimulated indirectly with pulses of 0.8 ms duration and a frequency of 0.1 to 0.15 Hz. The isometric contractions were recorded with a Statham Universal Transducing Cell and displayed on a Physiograph IV polygraph paper. Both tension development (T_d) and maximum rate of rise of tension (dT/dt *max*) were recorded simultaneously. The differential of tension with respect to time was recorded by

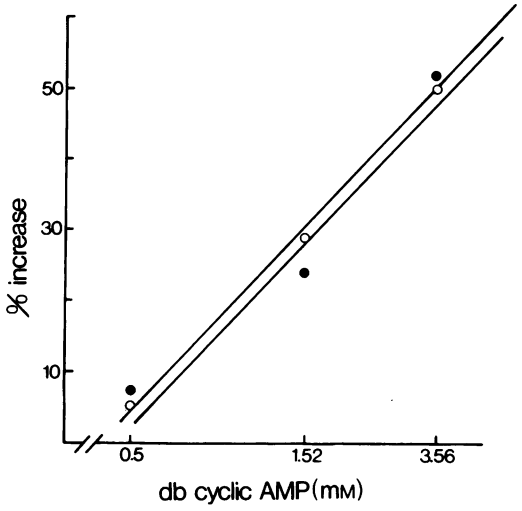

Figure 1 The effects of dibutyryl (db) cyclic AMP on tension development (T_d) (•) and the maximum rate of rise of tension (dT/dt *max*) (○) in the isolated hemidiaphragm of the rat during indirect stimulation. Abscissae: concentrations of db cyclic AMP in the bath in mM (log scale). Ordinates: percentage increase in the parameters measured.

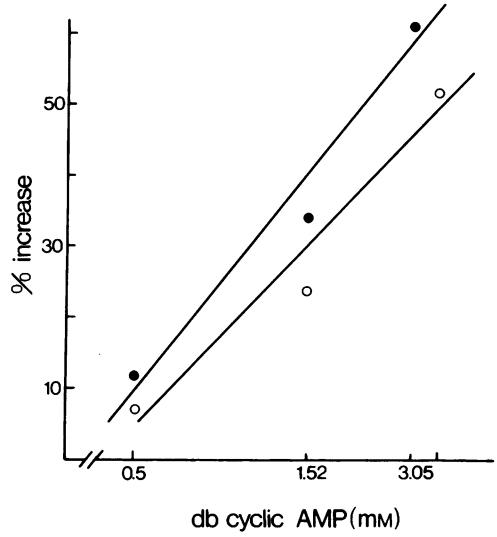

Figure 2 The effects of dibutyryl (db) cyclic AMP and propranolol on T_d of the isolated hemidiaphragm of the rat during indirect stimulation. Abscissae: concentrations of db cyclic AMP in the bath in mM (log scale). Ordinates: percentage increase in T_d. (○) effect of db cyclic AMP; (•) effect of db cyclic AMP in the presence of propranolol (1 μg/ml).

means of a Differentiator Coupler Type 7301 (Narco-Bio-Systems, Inc.), which provides the true mathematical derivative of analog signal voltages.

Direct stimulation with pulses of 1-1.5 ms duration, 0.1 to 0.15 Hz, was applied by means of two palador wires; the diaphragm was secured to one wire and the other was in the bathing solution. In these experiments (+)-tubocurarine was added to the bath in a concentration sufficient to block neuromuscular transmission completely (3 μg/ml, 4×10^{-6} M).

The following substances were used: cyclic *N*-2-*O*-dibutyryl adenosine 3′,5′-monophosphate-monosodium salt (Boehringer), cyclic adenosine 3′,5′-monophosphate, aminophylline, adrenaline hydrochloride, (+)-tubocurarine chloride, adenosine 5′-triphosphate sodium salt (Koch-Light Laboratories), nicotinic acid and propranolol hydrochloride.

Results

The effests of db cyclic AMP on indirect stimulation

Dibutyryl cyclic AMP was added to the bath in concentrations ranging from 0.5 to 3.56 mM in nine experiments. These concentrations of db cyclic AMP produced simultaneously an equal and parallel dose-dependent increase both in tension development (T_d) and in the maximum rate of rise of tension (dT/dt *max*) during indirect stimulation. When the percentage increase of these two parameters was plotted against the molar concentration of db cyclic AMP on a log scale, two close parallel lines were obtained (Figure 1).

The maximum increase in T_d and dT/dt *max* was reached 10 to 20 min after addition of db cyclic AMP to the bath and lasted for about 40 minutes. The percentage increase of these two parameters of contractility varied from preparation to preparation even when the same standard concentration of db cyclic AMP was present in the bath.

Cyclic AMP itself (not the dibutyryl derivative) in concentrations from 0.5 to 3 mM did not affect either T_d or dT/dt *max*.

Adenosine 5′-triphosphate (ATP) (0.5-3 mM) produced in three experiments a short lasting increase both in T_d and dT/dt *max*, followed by a depression. The increase in these two parameters lasted only a few minutes. It was evident that the effects of ATP and of db cyclic AMP differed both qualitatively and quantitatively.

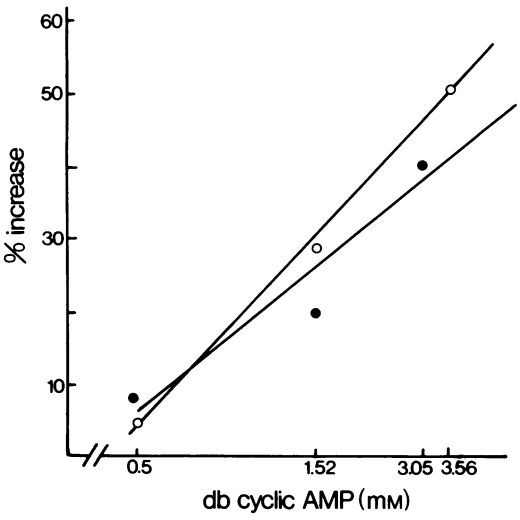

Figure 3 The effect of dibutyryl (db) cyclic AMP and propranolol on dT/dt *max* of the isolated hemidiaphragm of the rat during indirect stimulation. Abscissae: concentrations of db cyclic AMP in the bath in mM (log scale). Ordinates: percentage increase in dT/dt *max*. (○) effect of db cyclic AMP; (●) effect of db cyclic AMP in the presence of propranolol (1 μg/ml).

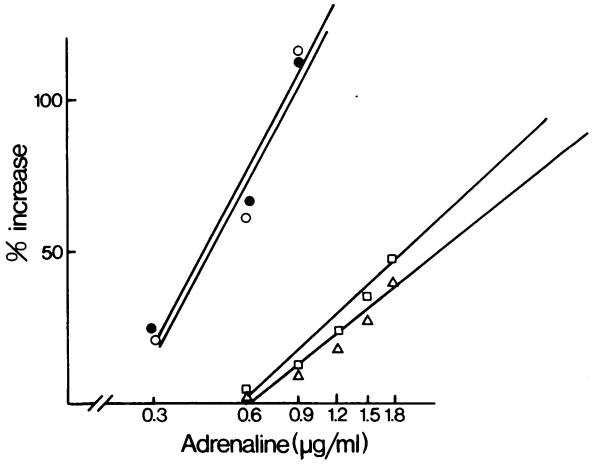

Figure 4 The effects of adrenaline and propranolol on T_d and dT/dt *max* of the isolated hemidiaphragm of the rat during indirect stimulation. Abscissae: concentration of adrenaline, in μg/ml (log scale). Ordinates: percentage increase in the parameters measured. (●) effects of adrenaline on T_d and (○) dT/dt *max*; (□) effect of adrenaline on T_d in the presence of propranolol (1 μg/ml); (△) effect of adrenaline on dT/dt *max* in the presence of propranolol (1 μg/ml).

Sodium butyrate was added to the bath in amounts corresponding to the amount of butyrate in the db cyclic AMP molecule (three experiments). These concentrations of butyrate (0.1-0.4 mg/ml) did not affect either T_d or dT/dt *max*.

Dibutyryl cyclic AMP and propranolol

To investigate the site of action of db cyclic AMP, propranolol was used in order to block the β-adrenoceptors. In a concentration of 1 μg/ml propranolol itself did not affect the response of the diaphragm to indirect stimulation.

This concentration of propranolol did not significantly alter either the effect of db cyclic AMP on T_d or its action on dT/dt *max*. The results of four experiments are presented in Figures 2 and 3. In all these experiments propranolol was added to the bath 5-7 min before the addition of db cyclic AMP.

The effect of adrenaline

Adrenaline has long been known to produce facilitation during indirect stimulation of the diaphragm (Bülbring, 1946). In the present experiments adrenaline (0.3 to 0.9 μg/ml) produced an equal and parallel increase both in T_d and dT/dt *max*. If the percentage increase of these two parameters was plotted against the concentration of adrenaline on a log scale, two parallel straight lines were obtained (Figure 4).

In the presence of propranolol (1 μg/ml) the responses to adrenaline, both in T_d and dT/dt *max*, were significantly smaller, the log dose lines being shifted to the right (Figure 4). This type of response indicates the competitive type of antagonism between adrenaline and propranolol. The results shown in Figure 4 were obtained from five experiments.

The effect of aminophylline

In 15 experiments aminophylline, in concentrations ranging from 250 to 1000 μg/ml, was found to increase both tension development and the maximum rate of rise of tension during indirect stimulation of the diaphragm. The increase in both parameters was equal and parallel. When the percentage increase in T_d and dT/dt *max* was plotted against the concentration of aminophylline, two parallel straight lines were obtained (Figure 5).

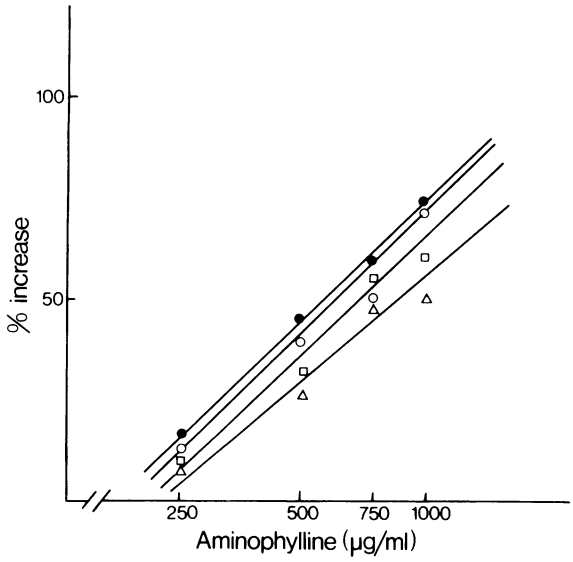

Figure 5 The effects of aminophylline and propranolol on T_d and dT/dt *max* of the isolated hemidiaphragm of the rat during indirect stimulation. Abscissae: concentrations of aminophylline in μg/ml (log scale). Ordinates: percentage increase in the parameters measured. (●) effects of aminophylline on T_d and (○) dT/dt *max*; (□) effect of aminophylline on T_d in the presence of propranolol (1 μg/ml); (△) effect of aminophylline on dT/dt *max* in the presence of propranolol (1 μg/ml).

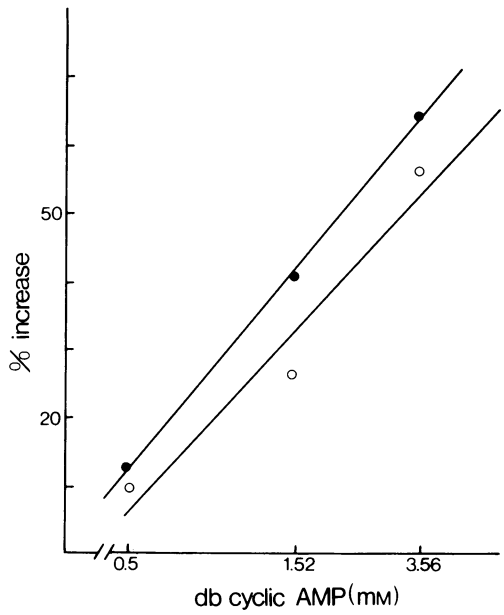

Figure 6 The effect of increasing concentrations of dibutyryl (db) cyclic AMP (log scale) on the percentage increase in T_d (●) and dT/dt *max* (○) of the isolated hemidiaphragm of the rat during direct stimulation.

Similar effects were obtained in the presence of propranolol (1 μg/ml). This concentration of propranolol produced no change in the response to aminophylline, the values for T_d and dT/dt *max* were similar to those obtained with aminophylline alone with no significant shift of the dose-response curves (Figure 5).

Dibutyryl cyclic AMP and direct stimulation

In 9 of a series of 12 hemidiaphragms, taken from six animals, db cyclic AMP (0.5 to 3.56 mM) produced an increase both of T_d and dT/dt *max* during direct stimulation. The results from these nine experiments are shown in Figure 6. It can be seen that parallel lines are obtained if the percentage increase is plotted against concentrations of db cyclic AMP on a log scale. In the other 3 of the 12 hemidiaphragms the same concentrations of db cyclic AMP produced no change in either T_d or dT/dt *max*. It should be noted that in these three preparations even aminophylline (0.5 mg/ml) produced only an insignificant increase in T_d and dT/dt *max*.

Nicotinic acid (0.5 to 2 mg/ml) was found to depress or completely block the effect of db cyclic AMP on the isometric contractility of the iolated hemidiaphragm (four experiments).

Aminophylline and direct stimulation

In seven experiments aminophylline (250 to 1000 μg/ml) was tested during direct stimulation. These concentrations of aminophylline regularly produced a parallel increase both in T_d and dT/dt *max*.

Discussion

The three substances studied in the present experiments, db cyclic AMP, adrenaline and aminophylline, all potentiated the tension developed (T_d) and the maximum rate of rise of tension (dT/dt *max*) of the rat isolated diaphragm when stimulated indirectly. Dibutyryl cyclic AMP and aminophylline also produced this effect during direct stimulation. In the original concept of the

cyclic AMP system, as proposed by Robison *et al.* (1971), all three substances are intimately interconnected. Catecholamines have been shown to stimulate adenyl cyclase, whereas xanthine derivatives including aminophylline inhibit the enzyme phosphodiesterase and thus produce an accumulation of cyclic AMP (Butcher & Sutherland, 1962). Adrenaline and aminophylline supposedly act through the intracellular cyclic AMP produced. It might therefore be expected that the general intracellular mediator, i.e. cyclic AMP, would produce the same type of response as the substances which produce either its increased synthesis (adrenaline) or decreased breakdown (aminophylline).

Although cyclic AMP alone did not produce any change in response of the isolated diaphragm to indirect stimulation, this is to be expected because nucleotides and other anionic phosphorylated compounds penetrate cell membranes poorly, if at all (Robison *et al.*, 1971). This generalization may not apply equally to all nucleotides and to all cells (Robison, Butcher, Oye, Morgan & Sutherland, 1965). Dibutyryl cyclic AMP contains lipid-soluble fatty acid residues which facilitate its passage across cellular membranes and at the same time increase its resistance to enzymatic hydrolysis by phosphodiesterase (Posternak, Sutherland & Henion, 1962; Falbriard, Posternak & Sutherland, 1967). This may explain why db cyclic AMP potentiated T_d and dT/dt *max* in the present experiments, whereas cyclic AMP itself did not.

It was also shown that propranolol, a β-adrenoceptor blocking agent, affected the action of adrenaline on T_d and dT/dt *max*, causing a significant shift of the log dose-response lines to the right. This possibly indicates a competitive type of antagonism between adrenaline and propranolol on β-adrenoceptors, present in the diaphragm somewhere on the excitation-contraction pathway. It has already been suggested that adenyl cyclase itself might serve a β-adrenoceptor function (Robison, Butcher & Sutherland, 1967). On the other hand, propranolol did not affect either the effect of db cyclic AMP or the response to aminophylline of the diaphragm when stimulated indirectly. Both db cyclic AMP and aminophylline produced practically the same effects on T_d and dT/dt *max*, no matter whether propranolol was present or not. This is hardly surprising since the processes affected by aminophylline (Haugaard & Hees, 1966) and by cyclic AMP (Robison *et al.*, 1971) are distal to the adenyl cyclase system.

Breckenridge, Burn & Matshinsky (1967) were the first to suggest that cyclic AMP within the motor nerve ending might augment the release of acetylcholine at the terminal membrane. Evidence supporting this hypothesis is the finding that addition of cyclic AMP or db cyclic AMP to the bathing medium of an isolated nerve muscle preparation increases transmitter release (Goldberg & Singer, 1969). The frequency of miniature end plate potentials is increased in response to db cyclic AMP and theophylline (Goldberg & Singer, 1969). It has also been shown that adrenaline increases the frequency and amplitude of the miniature endplate potentials (Krnjević & Miledi, 1958). Increased quantities of acetylcholine are released in response to adrenaline at the neuromuscular and ganglionic junctions (Krnjević & Miledi, 1958; Birks & MacIntosh, 1961). Therefore our results in which db cyclic AMP, adrenaline and aminophylline were found to increase T_d and dT/dt *max* during indirect stimulation could be explained in terms of an increased release of transmitter at the neuromuscular junction in the isolated diaphragm. This conclusion is strongly supported by the finding of Wilson (1974) that db cyclic AMP causes a significant increase in the quantum content of the first endplate potential in the mobilization rate and in the releasable store of transmitter.

On the other hand, it has been reported that adenosine reduces transmitter release (Ginsborg & Hirst, 1972). Adenosine also increases the amount of cyclic AMP in brain tissue (Sattin & Rall, 1970) and probably a similar effect might be expected at the nerve terminal. All these data are taken to indicate that actions of db cyclic AMP, adrenaline and aminophylline on the transmitting process are not the sole factors contributing to the potentiation of T_d and dT/dt *max* in the diaphragm during indirect stimulation. We have shown that db cyclic AMP and aminophylline also produce an increase in these two parameters even after direct stimulation. Cyclic AMP is known to have a role in the processes of glycogenolysis and lipolysis (Sutherland & Rall, 1960; Butcher, Ho, Meng & Sutherland, 1965; Reynolds & Haugaard, 1967). In the presence of ATP, cyclic AMP converts phosphorylase b to active phosphorylase a, the levels of which are increased by adrenaline (Hornbrook & Brody, 1963). It is therefore possible that the potentiating effects of db cyclic AMP and aminophylline on T_d and dT/dt *max*, particularly during direct stimulation of the diaphragm, might be due, at least partly, to activation of metabolic processes which provide the energy for contraction.

Besides inhibiting phosphodiesterase, the methylxanthines can induce calcium release from intracellular stores (Bianchi, 1961; Isaacson & Sandow, 1967). The transmitter release is known to be calcium-dependent (Hubbard, Jones &

Landau, 1968), but calcium also activates the contractile mechanism. In cardiac tissue db cyclic AMP, as well as adrenaline, has been shown to produce a significant increase in ^{45}Ca uptake (Meinertz, Nawrath & Scholz, 1973). These actions mean that more calcium is available both for transmitter release and to the contractile elements. Thus the potentiation of T_d and $dT/dt\ max$ by aminophylline and by db cyclic AMP, particularly during direct stimulation, might be due to calcium-dependent changes in the muscle action potential, with increase in calcium release during the spike and depression of the mechanical threshold for activation of the contractile elements. Such an effect has already been shown for caffeine (Sandow, 1970).

Our experiments therefore indicate that cyclic AMP may be involved not only in regulating the processes associated with synthesis, mobilization and storage of transmitter in the nerve terminal, but also in modifying some metabolic process (or processes), which regulates the function of the contractile elements.

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