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ROLE OF CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE IN THE ISOPRENALINE-INDUCED RELAXATION OF THE OESTROGEN DOMINATED RABBIT UTERUS

BRITT-INGJERD NESHEIM, J.-B. OSNES & I. ØYE
Institute of Pharmacology, University of Oslo, Blindern, Oslo 3, Norway

1 The role of cyclic adenosine 3',5'-monophosphate (cyclic AMP) in the relaxation produced by isoprenaline in muscle strips from the oestrogen dominated rabbit uterus has been investigated.

2 Isoprenaline 2×10^{-8} M produced an inhibition of the mechanical activity but no increase in cyclic AMP. Isoprenaline 2×10^{-6} M produced both inhibition of mechanical activity and increase in cyclic AMP.

3 The increase in cyclic AMP, but not the inhibition of mechanical activity, was blocked by propranolol 3.4×10^{-6} M.

4 Dibutyryl-cyclic AMP produced a relaxation which mimicked that produced by isoprenaline, in that the longitudinal strips were more sensitive than the circular ones.

5 It is concluded that cyclic AMP may be a mediator of the β-adrenergic effect in the oestrogen dominated rabbit myometrium. However, it seems not to be an obligatory link between stimulation of β-adrenoceptors and relaxation. Other mechanisms may also exist.

Introduction

Strips from rabbit myometrium show spontaneous contractions *in vitro*. Isoprenaline inhibits this spontaneous activity (Nesheim, 1972). This inhibition is not blocked by either propranolol or practolol in concentrations which usually block β-adrenoceptors (Nesheim, 1975).

In all organ systems examined, stimulation of β-adrenoceptors is followed by an increase in the level of cyclic adenosine 3',5'-monophosphate (cyclic AMP) in the tissue. A substantial amount of evidence now supports the hypothesis that β-adrenergic effects are mediated by cyclic AMP (Robison, Butcher & Sutherland, 1971).

It was therefore thought of interest to study the level of cyclic AMP in the rabbit myometrium where the mechanical effect produced by isoprenaline is not blocked by conventional β-adrenoceptor antagonists.

Methods

Seven rabbits were ovariectomized, and a tablet of 25 mg diethylstilboestrol was implanted subcutaneously. One week later the animals were killed and the uteri removed and stored in modified Ringer solution as described previously (Nesheim, 1972). Muscle strips 10-15 mm long and 2 mm wide, were cut in the circular and longitudinal direction, and transferred to organ baths containing the same solution at 39° C. The strips were allowed to equilibrate in the bath for one hour. Adrenoceptor antagonists were added immediately after the strips were mounted. Phentolamine 5.3×10^{-7} M was used for blocking α-adrenoceptors in all experiments.

Muscle tension was recorded isometrically, and the degree of inhibition calculated as described by Nesheim (1972).

For measurement of cyclic AMP the muscle strips were rapidly removed from the organ baths and frozen in liquid nitrogen.

Determination of cyclic adenosine 3',5'-monophosphate

The frozen strips of uterus (average weight about 100 mg) were homogenized in 5% ice-cold TCA (19 μg per mg tissue weight) with a glass homogenizer. Hydrochloric acid was added to 1000 μl of the supernatant to give 0.1 M HCl. The sample was extracted 7 times with 2 ml ether and evaporated to dryness under a stream of air at room temperature. The dried extracts were dissolved in 300 μl 50 mM sodium acetate, pH 4.0, and cyclic AMP in 50 μl aliquots was determined by the method of Gilman (1970). The incubation volume was 200 μl. The millipore filters were

dissolved directly in 10 ml of the scintillation fluid: BBOT-toluene-ethyleneglycolmonomethyl-ether-naphthalene (4 g : 600 ml : 400 ml : 80 g). The cyclic AMP content was calculated as pmol/mg protein.

Determination of homogenate protein

Samples (50 μl) of the tissue TCA homogenate were dissolved in 550 μl 0.18 N NaOH, and the protein content was determined by the method of Lowry using bovine albumine as standard (Lowry, Rosebrough, Farr & Randall, 1951). The protein contents of the uterine strips were about 0.09-0.13 mg protein/mg tissue.

Drugs and chemicals

Isoprenaline sulphate, phentolamine (Regitin), propranolol (Inderal) (obtained through Norsk Medisinaldepot); [^{3}H]-cyclic 3′,5′-AMP (24.1 or 22.1 Ci/mmol, New England Nuclear); cyclic 3′,5′-AMP and bovine albumin (fraction V) (Sigma Chemical Co.); Folin-Ciocalten's phenol reagent (E. Merck, Darmstadt); 2,5-bis-(5′-*t*-butyl-benzoxaxolyl (2′))-thiophene (BBOT), naphthalene, ethyleneglycolmonomethylether and toluene (Koch-Light Laboratories Ltd); N^6,$O^{2'}$-dibutryryl-adenosine 3′,5′-cyclic monophosphate (D 0627, Sigma Chemical Company).

Results

Basal concentration of cyclic adenosine 3′,5′-monophosphate

The basal concentration of cyclic AMP after equilibration for 1 h in the organ baths varied between the different rabbits, as shown in Table 1. Propranolol 3.4 x 10^{-6} M had no influence on the basal concentration of cyclic AMP.

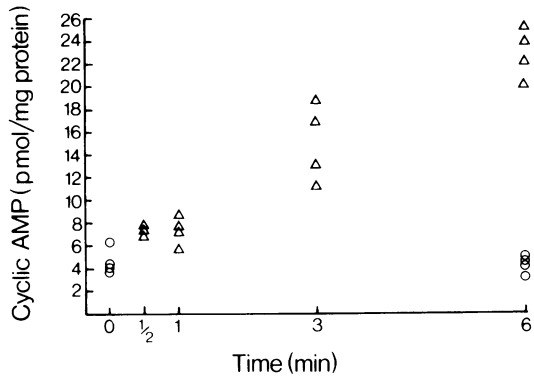


Figure 1 Concentration of cyclic 3′,5′-adenosine monophosphate (cyclic AMP) in 24 myometrium strips from one rabbit (no. 2, Table 1). At time 0, isoprenaline 2 x 10^{-6} M was added. Controls (○) taken before addition of isoprenaline and 6 min after addition of an equal amount of water.

Effect of isoprenaline 2 x 10^{-8} M

Twenty-three circular muscle strips from one rabbit (rabbit no. 1 in Table 1), were examined. Twelve of the strips had been equilibrated with propranolol 3.4 x 10^{-6} M. Cyclic AMP was measured in controls, and 0.5, 1 and 6 min after addition of isoprenaline 2 x 10^{-8} M. In no instance was the concentration of cyclic AMP above the control level.

In the four strips examined after 6 min, the degree of inhibition of mechanical activity could be calculated. In one of the strips there was no inhibition, in the others the inhibition was 21, 27 and 73%, respectively.

Effect of isoprenaline 2 x 10^{-6} M

Figure 1 shows the concentration of cyclic AMP 0.5, 1, 3 and 6 min after the addition of

Table 1 Concentration of cyclic 3′,5′-adenosine monophosphate (cyclic AMP) (pmol/mg protein) in rabbit uterine strips incubated for 1 h in aerated Ringers solution at 39°C with phentolamine 5.3 x 10^{-7} M and with or without propranolol 3.4 x 10^{-6} M.

Rabbit No.	*Propranolol*	*Number of muscle strips*	*Cyclic AMP (median)*	*Range*
1	–	3	2.2	1.2-2.3
	+	4	1.9	1.8-2.0
2	–	8	4.2	3.1-6.3
3	–	4	7.7	6.9-8.4
	+	4	7.2	6.6-7.8
4	+	8	4.6	4.1-5.4

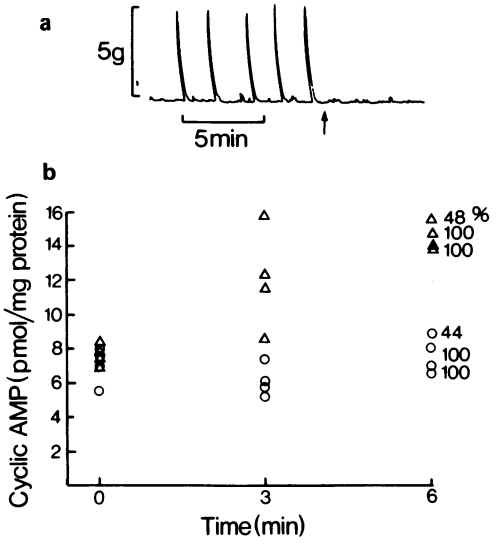


Figure 2 (a) Tracing from an experiment where propranolol 3.4×10^{-6} M was present in the bath. At the arrow, isoprenaline 2×10^{-6} M was added. The concentration of cyclic adenosine 3',5'-monophosphate (cyclic AMP) in this particular strip is plotted as no. 2 from the bottom to the right in Figure 2b.
(b) Concentration of cyclic AMP in 24 myometrium strips in one rabbit (no. 3, Table 1). At time 0, isoprenaline 2×10^{-6} M was added. Controls were taken before addition of isoprenaline. Propranolol 3.4×10^{-6} M present in the bath (○), no β-blocker (△). Numbers to the right on the figure indicate % inhibition of mechanical activity in that particular muscle strip.

isoprenaline 2×10^{-6} M (rabbit no. 2 in Table 1). Controls taken before addition of isoprenaline and 6 min after the addition of an equal volume of water are included. All strips were cut circularly from the uterus. After 0.5 min the median concentration of cyclic AMP was 172% of the median control value ($\alpha = 0.002$, Wilcoxon's two sample test, one-sided), after 1 min 177% ($\alpha = 0.004$), after 3 min 359% ($\alpha = 0.002$) and after 6 min 552% ($\alpha = 0.002$).

The inhibition of the mechanical activity measured in the four strips where isoprenaline was allowed to act for 6 min, ranged from 54% to 79%. No correlation was found between the degree of inhibition of mechanical activity and the concentration of cyclic AMP.

Figure 2b shows the concentration of cyclic AMP in controls, and 3 and 6 min after the addition of isoprenaline 2×10^{-6} M with and without propranol 3.4×10^{-6} M present in the bath (rabbit no. 3 in Table 1). All were circular strips from the uterus. After 3 min, the median concentration of cyclic AMP without propranolol present, was 156% of the median control value ($\alpha = 0.01$, Wilcoxon's two sample test, one-sided), with propranolol 83% (not significantly different from control, $\alpha = 0.4$, two-sided test). After 6 min the concentration of cyclic AMP without propranolol was 188% ($\alpha = 0.01$, one-sided test), and with propranolol 104% (not significant, $\alpha = 0.6$, two-sided test).

The inhibition of the mechanical activity could be measured in 6 of the 8 strips where isoprenaline was allowed to act for 6 min (one strip with and one without propranolol did not have spontaneous activity). The inhibition of the mechanical activity for each particular muscle strip is indicated to the right in Figure 2b. Inhibition was the same in strips with and without propranolol, and there was no correlation between cyclic AMP concentration and inhibition of contractions. Figure 2a shows the tracing from an experiment with propranolol present in the bath, where the concentration of cyclic AMP was 6.9 pmol/mg protein (second point from bottom at 6 min in Figure 2b), and inhibition of mechanical activity was 100%.

To determine whether there might be a small rise in cyclic AMP 6 min after addition of isoprenaline when propranolol was present in the bath (104%, but not significantly different from control in Figure 2), additional experiments were done with another rabbit (rabbit no. 4 in Table 1). Strips were taken for cyclic AMP determination from controls, and 3 and 6 min after the addition of isoprenaline, with propranolol present in the bath. Each group consisted of 8 muscle strips. After 3 min there was no rise in the cyclic AMP level. After 6 min there was a small rise, 109% of controls, which was statistically significant ($\alpha = 0.04$, two-sided test).

Effect of dibutyryl-cyclic AMP

The action of dibutyryl-cyclic AMP 5×10^{-5}, 10^{-4} 5×10^{-4} and 10^{-3} M was tested on 38 circular and 31 longitudinal muscle strips from 3 rabbits. Sodium-butyrate in the same concentrations was used as control in the same number of muscle strips. The effect of dibutyryl-cyclic AMP developed in 2-5 minutes. The mechanical activity was measured 10-15 min after addition of the drug, and compared to the mechanical activity during the last 5 min before addition of the drug.

Sodium butyrate inhibited the mechanical activity to a certain extent (median approximately 20%). The effect of each concentration of dibutyryl-cyclic AMP was calculated by subtracting the median inhibition caused by the same concentration of sodium butyrate from the median inhibition caused by dibutyryl-cyclic AMP.

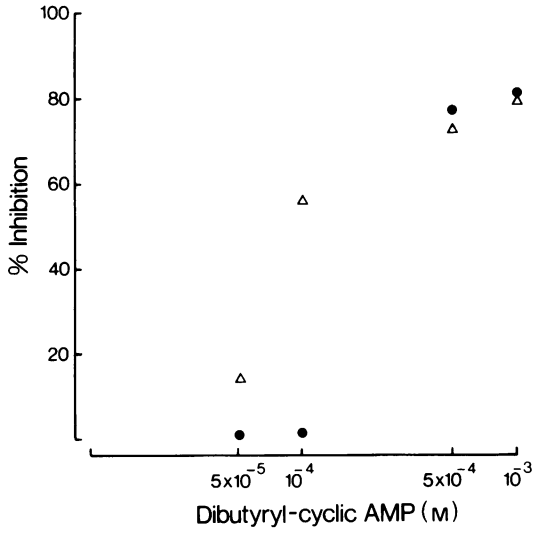


Figure 3 Inhibition of spontaneous mechanical activity in circular (•) and longitudinal (△) muscle strips from rabbit uterus by dibutyryl-cyclic AMP. Abscissae: concentrations of dibutyryl-cyclic AMP (M). Ordinates: % inhibition of spontaneous activity. Each point represents the median of 6-14 individual muscle strips from one rabbit.

The values obtained in this way are plotted in Figure 3. For each concentration of dibutyryl-cyclic AMP, the values from the circular and longitudinal strips are from the same rabbit.

The significance of the difference from controls was tested with a one-sided Wilcoxon two sample test. For dibutyryl-cyclic AMP 5×10^{-5} M there was no inhibition of the circular strips, and the inhibition of the longitudinal strips was not significant at the 5% level ($\alpha = 0.08$). For 10^{-4} M there was no inhibition of the circular strips. The inhibition of the longitudinal strips was statistically significant ($\alpha = 0.0003$). For 5×10^{-4} M and 10^{-3} M all inhibitions were significant ($\alpha \leqq 0.006$).

Discussion

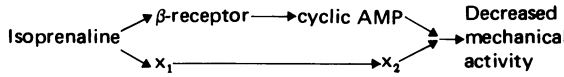
Isoprenaline inhibited the mechanical activity and increased the cyclic AMP level in the oestrogen dominated rabbit uterus. This is in agreement with previous findings in other smooth muscle preparations (Robison *et al.*, 1971), including rat uterus muscle (Dobbs & Robison, 1968; Polacek & Daniel, 1971; Marshall & Kroeger, 1973). The levels of cyclic AMP 6 min after isoprenaline were quite different in the two rabbits tested (188% and 552%). The full effect of isoprenaline on the mechanical activity developed in the course of 1 min, and at this time cyclic AMP had increased to 177% of basal levels in the uterus where the ultimate level was 552%. The large increase in cyclic AMP content observed between 1 and 6 min was thus not associated with further inhibition of the contractile activity.

Under the conditions used, the inhibition of mechanical activity varied between different muscle strips and between different rabbits (Nesheim, 1972; 1975). The rise in cyclic AMP content showed much less variation between the different muscle strips of one uterus. No quantitative correlation was observed between the degree of inhibition of the mechanical activity and the rise of cyclic AMP. Because the longitudinal layer is more sensitive to isoprenaline than the circular layer (Nesheim, 1972; 1975), a strict quantitative correlation between the degree of mechanical inhibition and degree of rise in cyclic AMP content might be difficult to detect. In addition, only part of the cyclic AMP in each cell might be involved in regulation of contractile activity, which would also explain the different time-response relationships discussed above. Finally, the ability of the different muscle strips to respond to cyclic AMP might vary.

In the oestrogen dominated rabbit uterus we have found decreased mechanical activity without a measureable increase of cyclic AMP in two experimental situations: (1) with isoprenaline 2×10^{-8} M; (2) with isoprenaline 2×10^{-6} M in the presence of the β-adrenoceptor antagonist, propranolol. The ability of propranolol to block the isoprenaline-induced increase in cyclic AMP is in agreement with previous investigations in other smooth muscle preparations (Robison *et al.*, 1971). However, in the rabbit uterus the decrease of mechanical activity caused by isoprenaline was not affected by propranolol, as reported before (Nesheim, 1975). This is to our knowledge the first report on dissociation between decreased mechanical activity and cyclic AMP accumulation in uterine muscle after adrenoceptor stimulation. In the rat uterus, which has been most extensively studied, propranolol antagonizes both the mechanical and the metabolic effects of isoprenaline (Dobbs & Robison, 1968) or adrenaline (Polacek & Daniel, 1971).

Our findings may be interpreted in different ways: (1): The lack of correlation may reflect an apparent and not a functional dissociation. A local increase in cyclic AMP concentration sufficient to elicit the mechanical response could have escaped detection by the methods used. The small rise in cyclic AMP measured 6 min after the addition of isoprenaline with propranolol present in the bath, might support this interpretation. This rise might represent the 'overshoot' of cyclic AMP synthesis

in a small intracellular compartment. The effect of dibutyryl-cyclic AMP strongly indicates an involvement of cyclic AMP in the decreased mechanical activity after β-adrenoceptor stimulation. (2): The results may represent a functional dissociation between cyclic AMP and decreased mechanical activity. Then two possibilities exist: (a) cyclic AMP is not a mediator of adrenergic relaxation, (b) cyclic AMP is not an obligatory mediator; other mechanisms may contribute or even substitute for cyclic AMP under certain conditions. Confirmation of the former alternative would require the finding of a primary increase in cyclic AMP not accompanied by decreased mechanical activity. Such findings have never been reported. Polacek & Daniel (1971) observed a reversal of the inhibiting mechanical effects of isoprenaline in the rat uterus by addition of propranolol, without change in the elevation of cyclic AMP. This, however, might have represented a stimulation of α-receptors by isoprenaline in the presence of propranolol, as the experiments were performed without an α-blocker present. When intracellular cyclic AMP was elevated by addition of dibutyryl-cyclic AMP (Polacek & Daniel, 1971) decreased mechanical activity was observed. Our results with dibutyryl-cyclic AMP confirm these findings. Thus, alternative 2a above seems rather improbable, while alternative 2b accounts for the available information. This model includes a dual mechanism of the action of isoprenaline on the oestrogen-dominated rabbit uterine muscle:



This model has the following characteristics: cyclic AMP is involved as a mediator when it is increased; but it is not the only mediator and it is not an obligatory mediator. Cyclic AMP accumulation is blocked by propranolol, but the mechanical response is not affected. There is an alternative chain of processes leading to decreased mechanical activity. This mechanism is propranol-resistant. The common pathway beyond cyclic AMP may be responsible for the similar pattern of susceptibility of the longitudinal and circular muscle layers to both dibutyryl-cyclic AMP and isoprenaline. The nature of the non-cyclic AMP chain is unknown.

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