J. Pharm. Pharmac., 1968, **20**, 368–372 Received October 23, 1967

Relaxation of the guinea-pig tracheal chain preparation by N^6, 2′-*O*-dibutyryl 3′,5′-cyclic adenosine monophosphate

P. F. MOORE, L. C. IORIO AND J. M. McMANUS

N^6,2′-*O*-Dibutyryl 3′,5′-cyclic adenosine monophosphate (dibutyryl 3′,5′-AMP), isoprenaline and theophylline relax the guinea-pig tracheal chain preparation; whereas 3′,5′-cyclic adenosine monophosphate (3′,5′-AMP) does not. The relaxant effect of isoprenaline, but not that of dibutyryl 3′,5′-AMP, was blocked by propranolol. 3′,5′-AMP is hydrolyzed rapidly by beef heart phosphodiesterase whereas dibutyryl 3′,5′-AMP is not. The presence of equimolar concentrations of dibutyryl 3′,5′-AMP does not alter the rate of phosphodiesterase mediated hydrolysis of 3′,5′-AMP. These data are consistent with the theories that relaxation of the guinea-pig trachea may be mediated by 3′,5′-AMP and that dibutyryl 3′,5′-AMP acts by mimicking 3′,5′-AMP at its site of action.

CATECHOLAMINES and other hormones may stimulate the enzyme adenyl cyclase to convert adenosine triphosphate (ATP) to 3′,5′-cyclic adenosine monophosphate (3′,5′-AMP), which is inactivated by hydrolysis mediated by a specific 3′,5′-AMP phosphodiesterase to 5′-adenosine monophosphate (AMP) (Sutherland & Rall, 1960). The production of 3′,5′-AMP has been associated with the responses of various tissues to hormones, but it has not always been possible to show a direct cause-effect relation between administration of exogenous 3′,5′-AMP and these same physiological effects. It was generally accepted that the reason for this lack of effect was that nucleotides, such as 3′,5′-AMP, penetrated the cell wall little, if at all (Roll, Weinfeld & others, 1956; Leibman & Heidelberger, 1955), and in direct support of this contention, Robison, Butcher & others (1965) demonstrated that 3′,5′-AMP, when added to the media perfusing the isolated rat heart, did not gain access to the intracellular fluid in concentrations comparable to those seen after the administration of adrenaline.

Posternak, Sutherland & Henion (1962) synthesized a number of derivatives of 3′,5′-AMP to ". . . obtain substances that might have a better action on the intact animal, on isolated organs, or on tissue slices, than the original nucleotide; and obtain substances that might act as antagonists to the original nucleotide". They found that the N^6,2′-*O*-dibutyryl derivative (dibutyryl 3′,5′-AMP) was, among others, more active than the parent 3′,5′-AMP in producing hyperglycaemia in intact dogs. Subsequently, Butcher, Ho & others (1965) showed dibutyryl 3′,5′-AMP to be a more potent stimulant of lipolysis in isolated fat cells than 3′,5′-AMP. Bdolah & Schramm (1965) reported that dibutyryl 3′,5′-AMP (1×10^{-3}M) stimulated amylase secretion in rat parotid cells whereas 3′,5′-AMP (9×10^{-3}M) was inactive. Pasten (1966) observed that dibutyryl 3′,5′-AMP (50–375 μg/ml), like TSH, stimulated both the oxidation of [1-^{14}C] glucose to $^{14}CO_2$ and the incorporation of ^{32}P into phospholipid whereas 3′,5′-AMP (250 μg/ml) was inactive, and Imura, Matsukura & others (1965) indicated

From the Medicinal Research Laboratories, Chas. Pfizer & Co., Inc., Groton, Conn., U.S.A.

that dibutyryl 3′,5′-AMP was more effective than 3′,5′-AMP in stimulating adrenal corticosterone secretion in rats *in vivo*. These authors concluded as did Posternak & others (1962) that the greater effectiveness of the derivative is related to its resistance to hydrolysis by phosphodiesterase and perhaps to increased entry into cells. However, no evidence has been presented to deny the possibilities that the dibutyryl 3′,5′-AMP might act by stimulating adenyl cyclase or inhibiting phosphodiesterase.

Bueding, Butcher & others (1966) have shown that a positive correlation exists between the relaxant effect produced by physiological concentrations of adrenaline upon muscle from the taenia coli of guinea-pigs and an increased concentration of 3′,5′-AMP in this tissue. A logical extension of this evidence is that the smooth muscle relaxant effects of catecholamines are mediated by 3′,5′-AMP. The following experiments were made to determine if this mechanism might exist in the guinea-pig trachea and to ascertain the mechanism of action of dibutyryl 3′,5′-AMP.

Experimental

METHODS

Male Reed Willett strain guinea-pigs, 300–400 g, were used for the paired tracheal chain preparation (Foster, 1960). The tissues were suspended under 1 g tension in 10 ml of Krebs-Henseleit solution, 37°, constantly perfused with oxygen 95% and carbon dioxide 5%. Contractions and relaxations were recorded on a smoked drum via a light wooden lever at fourteenfold magnification.

Dibutyryl 3′,5′-AMP was prepared essentially as outlined by Posternak & others (1962). To a suspension of 150 mg of 3′,5′-AMP in 4·5 ml of dry pyridine was added 2·25 ml of n-butyric anhydride, and the mixture heated to reflux until a solution was obtained (6 min). The reaction mixture was cooled to room temperature and allowed to stand for 10 days. The reaction mixture was hydrolysed with 6 ml of water followed by concentration *in vacuo* to a solid residue. The crude product was chromatographed on Whatman No. 1 paper in an ethanol–ammonium acetate (0·5M) (5 : 2) system. The product spot was eluted with methanol and the solvent removed *in vacuo*. An ether precipitation from methanol provided the pure product, 120 mg, λ_{max}^{MeOH} 270 (ϵ = 14,634).

Beef heart phosphodiesterase was prepared as outlined by Butcher & Sutherland (1962). The enzyme was partially purified as described using ammonium sulphate fractionations, dialysis and freezing but was not fractionated on DEAE-cellulose. Its protein content, determined by the method of Warburg & Christian (1941), was 6·4 mg/ml. Its K_M value was 10^{-4}M. Either or both substrates, 4×10^{-4}M were incubated at 30° with 0·02 ml enzyme in a total volume of 2 ml containing 0·2 μmole EDTA, 4·0 μmole $MgSO_4$ and 80 μmole Tris-HCl buffer, pH 7·5. The reaction was stopped by boiling. One mg of lyophilized *Crotolus atrox* venom dissolved in 1 ml of Tris-HCl buffer was then added to the incubation. This mixture was incubated for 30 min at 30° and the reaction stopped by addition of 1 ml 20% trichloroacetic acid. The tubes were centrifuged

and the supernatant was applied to a Dowex 1-X8 (Cl form, 100–200 mesh) column. After washing, the inorganic phosphate was eluted with 1 ml of saturated potassium chloride solution. Phosphate was determined colorimetrically by the method of Fiske & Subbarow (1925).

Results and discussion

As previously shown by Foster (1966), the guinea-pig tracheal chain preparation was relaxed by isoprenaline (0·001–0·030 μg/ml) or the phosphodiesterase inhibitor, theophylline (0·4–25·0 μg/ml), intimating the existence of a relaxant mechanism similar to that in the taenia coli muscle. Furthermore, dibutyryl 3′,5′-AMP (500 μg/ml) relaxed the preparation (Fig. 1); however, equimolar amounts of 3′,5′-AMP were inactive. The

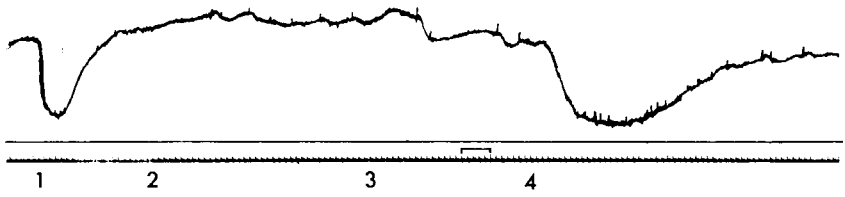

FIG. 1. Relaxation of the guinea-pig tracheal chain preparation by isoprenaline and dibutyryl 3′,5′-AMP. 1. Isoprenaline 0·03 μg/ml. 2, 3 and 4 Dibutyryl 3′,5′-AMP, 4, 160 and 500 μg/ml respectively. Time scale = 5 min.

onset and duration of response to dibutyryl 3′,5′-AMP were prolonged compared to those of isoprenaline. The response to 3′,5′-AMP in combination with submaximal concentrations of theophylline, was the same as that to theophylline alone, probably because 3′,5′-AMP did not enter the cell or did so at such an insignificant rate that partially inhibited phosphodiesterase was still capable of hydrolyzing it before it reached its active site.

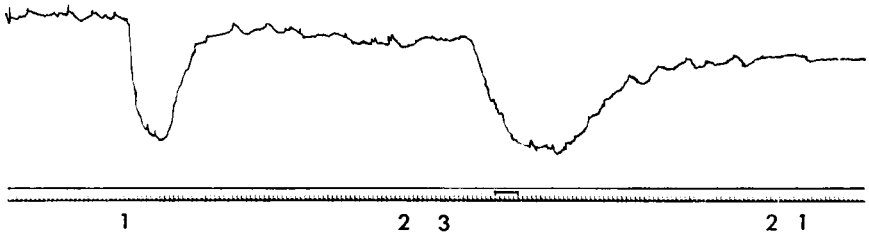

FIG. 2. The effects of the β-adrenergic blocking agent, propranolol, upon the responses of the guinea-pig tracheal chain preparation to isoprenaline and dibutyryl 3′,5′-AMP. 1. Isoprenaline, 0·03 μg/ml. 2. propranolol, 0·3 μg/ml. 3. Dibutyryl 3′,5′-AMP, 500 μg/ml. Time scale = 5 min.

Propranolol, in concentrations which blocked supramaximal concentrations of isoprenaline, did not alter the response to dibutyryl 3′,5′-AMP (Fig. 2).

The rate of hydrolysis of dibutyryl 3′,5′-AMP by phosphodiesterase was insignificant compared to that of 3′,5′-AMP (Fig. 3) and the presence of equimolar amounts of dibutyryl 3′,5′-AMP did not alter the rate of hydrolysis of 3′,5′-AMP by phosphodiesterase.

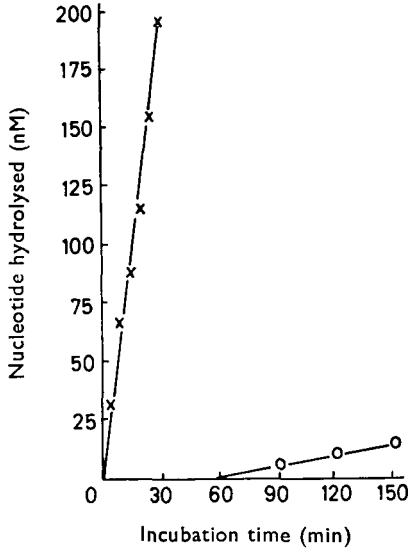

FIG. 3. Rates of hydrolysis of 3′,5′-AMP (×) and its dibutyryl derivative (○) in the presence of phosphodiesterase. Substrate concentrations, 4×10^{-4} M.

The data supports the contention that there is a relaxant mechanism in guinea-pig tracheal muscle similar to or identical with the mechanism in the taenia coli muscle wherein catecholamines induce the formation of 3′,5′-AMP accompanied by relaxation and the return to initial tone is governed by phosphodiesterase mediated hydrolysis of this 3′,5′-AMP.

Since the action of dibutyryl 3′,5′-AMP was not blocked by propranolol, it is improbable that it acts by releasing catecholamines which subsequently stimulate adenyl cyclase to form 3′,5′-AMP. It is also improbable that it unites with phosphodiesterase as a false substrate enhancing the activity of endogenously produced 3′,5′-AMP as do the methylxanthines, since it did not alter the rate of hydrolysis of 3′,5′-AMP by phosphodiesterase. The most logical explanation remaining for the activity of dibutyryl 3′,5′-AMP is that it mimics 3′,5′-AMP at its site of action. The longer onset of action observed with dibutyryl 3′,5′-AMP is probably the result of a slow entrance into the cell and the prolonged relaxation the result of its freedom from inactivation by phosphodiesterase and slow exit from the site of action.

Acknowledgements. We wish to thank Mrs. Edna Brucoli, Mrs. Lina Vlavianos and Mr. Charles Lamphere for their skilful assistance in the performance of these experiments.

References

Bdolah, A. & Schramm, M. (1965). *Biochem. biophys. Res. Commun.*, **18**, 452–454.
Bueding, E., Butcher, R. W., Hawkins, J., Timms, A. R., & Sutherland, E. W., Jr. (1966). *Biochim. biophys. Acta*, **115**, 173–178.
Butcher, R. W., Ho, R. J., Meng, H. C. & Sutherland, E. W. (1965). *J. biol. Chem.*, **240**, 4515–4523.
Butcher, R. W. & Sutherland, E. W. (1962). *Ibid.*, **237**, 1244–1250.
Fiske, C. H. & Subbarow, Y. (1925). *Ibid.*, **66**, 375.
Foster, R. W. (1960). *J. Pharm. Pharmac.*, **12**, 189–191.
Foster, R. W. (1966). *Ibid.*, **18**, 1–12.
Imura, H., Matsukura, S., Matsuyama, H., Setsuda, T. & Miyake, T. (1965). *Endocrinology*, **76**, 933–937.
Leibman, K. C. & Heidelberger, C. (1955). *J. biol. Chem.*, **216**, 823–830.
Pasten, I. (1966). *Biochem. biophys. Res. Commun.*, **25**, 14–17.
Posternak, T., Sutherland, E. W. & Henion, W. F. (1962). *Biochim. biophys. Acta*, **65**, 558–560.
Robison, G. A., Butcher, R. W., Oye, I., Morgan, H. E. & Sutherland, E. W. (1965). *Molec. Pharmac.*, **1**, 168–177.
Roll, P. M., Weinfeld, H., Carroll, E. & Brown, G. (1956). *J. biol. Chem.*, **220**, 439–454.
Sutherland, E. W. & Rall, T. W. (1960). *Pharmac. Rev.*, **12**, 265–299.
Warburg, O. & Christian, W. (1941). *Biochem. Z.*, **310**, 384.