J. Pharm. Pharmacol. 1985, 37: 924–926
Communicated May 9, 1985

Inhibition of rabbit cardiac adenylate cyclase by theophylline

ALEXANDRA M. HOPWOOD*, SIAN E. HARDING, PETER HARRIS, *Cardiothoracic Institute, 2 Beaumont Street, London, W1N 2DX, UK*

Theophylline inhibits basal adenylate cyclase activity as well as cyclase stimulated by sodium chloride, sodium fluoride, GTP or 5′-guanylimidodiphosphate. This inhibition is dose-dependent and shows non-competitive inhibition, with respect to MgATP. The presence of adenosine deaminase does not alter the effect of theophylline. The inhibition produced by theophylline is not additive with that due to 2′-deoxyadenosine 3′-monophosphate (a P-site agonist). It is suggested that theophylline may act at the P-site to reduce adenylate cyclase activity.

Theophylline is a well known phosphodiesterase inhibitor that will generally increase cAMP in cells. However, it has been shown to affect adenylate cyclase through adenosine receptors (Londos et al 1981) and depending on whether it is blocking at inhibitory or stimulatory receptors, it can raise or lower the levels of cAMP. Under conditions where there is no adenosine and the cAMP phosphodiesterases are blocked, theophylline also exerts a direct inhibitory effect on adenylate cyclase. This has been noted for many tissues including rat erythrocyte ghosts (Shepperd 1970), lung (Weinryb & Michel 1971; Welton & Simko 1980), guinea-pig auricles (Kruse & Scholtz 1978), hepatic parenchymal cells (Pohl et al 1969), kidneys (Jakobs et al 1972), fat cells (Schönhöfer & Skidmore 1971), rabbit heart (Avdonin et al 1982) and sperm (Garbers 1977). The purpose of this study was to investigate further the nature of the inhibition in rabbit cardiac membranes.

Materials and methods

Dithiothreitol, creatine phosphate (Tris salt), adenosine deaminase type III, ATP (sodium salt prepared by phosphorylation of adenosine), GTP (sodium salt), 5′-guanylimidodiphosphate (GppNHp) and theophylline were all obtained from Sigma. ICI 63 197 (Davies 1973) (a non-methylxanthine phosphodiesterase inhibitor; 2-amino-6-methyl-5-oxo-4-n-propyl-4,5-dihydro-5-triazolo [1,5-*a*]pyrimide) was the kind gift of Dr M. G. Collis of Imperial Chemicals Industries plc, Macclesfield, UK.

Cardiac sarcolemmal membranes from male, New Zealand white rabbits were prepared by the method of Mas-Oliva et al (1979) and stored at −80 °C until required. A six-fold purification of adenylate cyclase activity was achieved by this procedure. The adenylate cyclase assay medium contained (mM): Tris acetate (pH 7·5) 22, dithiothreitol 0·72, EDTA 0·32, magnesium acetate 12, ICI 63 197 2, Sodium ATP 0·5, creatine phosphate 5 and creatine kinase 2 mg ml^{-1}. Sarcolemmal protein concentration was 0·1 to 0·2 mg ml^{-1}. All assay constituents except ATP and creatine kinase were preincubated with the membranes for 20 min before the reaction was started by the addition of ATP. The final assay volume was 250 µl. After 20 min incubation at 30 °C, 50 µl aliquots were transferred into 500 µl acetate buffer (0·1 M, pH 4·0) to stop the reaction. After neutralization with 3 M Tris, 2 × 50 µl aliquots were taken for cAMP measurement with the Amersham International assay kit. At least 3 separate membrane preparations were used in each series and assays were performed in triplicate.

One unit of adenosine deaminase will deaminate 1·0 µmol of adenosine to inosine min^{-1} at pH 7·5 and 25 °C (as defined by Sigma). Normalized adenylate cyclase activity is the enzyme activity expressed as a percentage of the control. The Michaelis constants were determined using the 'Wilkinson programme' (Wilkinson 1961). Statistical analysis was performed using the paired or unpaired Student's *t*-test.

Results

Theophylline (5 mM) inhibited rabbit cardiac adenylate cyclase in its basal state as well as when stimulated by 0·1 mM GTP, 0·1 mM GppNHp, 100 mM sodium chloride or 10 mM sodium fluoride (see Table 1).

As the most reproducible inhibition by theophylline was seen with adenylate cyclase stimulated by 0·1 mM GTP plus 100 mM sodium chloride, these conditions

Table 1. Inhibition of rabbit cardiac adenylate cyclase by 5 mM theophylline under various conditions.

Condition	Cyclase activity (pmol cAMP min^{-1} (mg protein)$^{-1}$) Control	+5 mM Theophylline
Basal (8)	74·9 ± 21·9	57·0 ± 17·0
100 mM NaCl (8)	231·2 ± 33·6	169·3 ± 30·2*
0·1 mM GTP + 100 mM NaCl (8)	336·6 ± 27·8	227·7 ± 30·7**
0·1 mM GTP (4)	102·4 ± 10·0	58·7 ± 20·6
0·1 mM GppNHp + 100 mM NaCl (4)	837·3 ± 110·0	717·1 ± 110·5
10 mM NaF (4)	328·7 ± 50·6	265·4 ± 41·3

Mean ± s.e.m. Number of experiments shown in parentheses.
*$P < 0·002$, **$P < 0·001$, paired *t*-test.
Theophylline inhibited activity in 34 out of 36 cases.

* Correspondence.

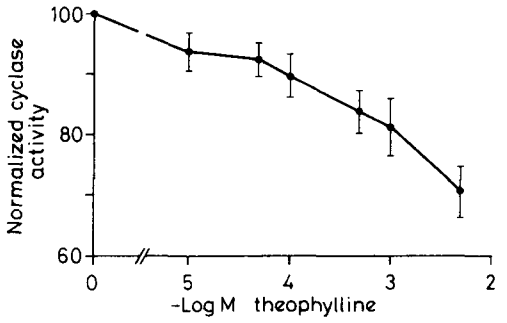

FIG. 1. Effect of increasing concentrations of theophylline on the activity of rabbit cardiac adenylate cyclase stimulated by 100 mM Na^+ and 0·1 mM GTP. n = 5, mean ± s.e.m.

Table 2. Effect of adenosine deaminase on the inhibition of rabbit cardiac adenylate cyclase (stimulated by 100 mM Na^+ and 0·1 mM GTP) by 5 mM theophylline.

Condition	Cyclase activity (cAMP min^{-1} (mg protein)$^{-1}$) Control	+ 5 mM Theophylline
GTP/Na stimulated	377·5 ± 22·3	265·3 ± 20·3†
+ Adenosine deaminase (1 u $ml)^{-1}$	367·2 ± 36·0	229·2 ± 21·8*

Mean ± s.e.m. of 5 experiments. *$P < 0.05$, †$P < 0.01$ paired t-test, against control.

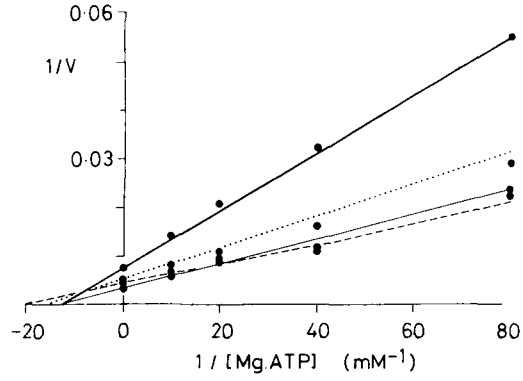

FIG. 2. Lineweaver–Burk plots showing the inhibition of rabbit cardiac adenylate cyclase by theophylline. Adenylate cyclase activity was stimulated by 100 mM Na^+ and 0·1 mM GTP. The lines shown are the average of 3 experiments. Key: —— control; – – – 0·1 mM; ····· 1 mM; ▬▬ 10 mM theophylline. 1/V = 1/cAMP min^{-1} (mg protein)$^{-1}$.

were used in the further characterization of the theophylline effect. Fig. 1 shows a dose-response curve for theophylline-induced inhibition of adenylate cyclase activity. Inhibition is apparent at 10 μM but is more marked at concentrations above 100 μM. Kinetic analysis revealed that this inhibition was not competitive with respect to MgATP (Fig. 2). In fact, the inhibition most closely resembled the non-competitive type.

Adenosine deaminase, at a concentration of 1 unit ml^{-1}, had no effect on the inhibition by theophylline (Table 2), thus ruling out the possibility that theophylline may be exerting its action by blocking stimulation of adenylate cyclase by endogenous adenosine. Adenosine deaminase at this concentration is effective in removing up to 1 mM adenosine (Table 3).

Table 3. Effect of adenosine deaminase on basal adenylate cyclase activity in the presence and absence of 1 mM adenosine.

Condition	Cyclase activity (cAMP min^{-1} mg $protein^{-1}$) Control	+ 1 mM Adenosine
Basal	47·2 ± 12·4	17·1 ± 4·0*
+ Adenosine deaminase (1 u ml^{-1})	46·1 ± 9·8	50·8 ± 12·7

Mean ± s.e.m. of 3 experiments. *$P < 0.02$. Group t-test (unmatched), against adenosine deaminase plus adenosine.

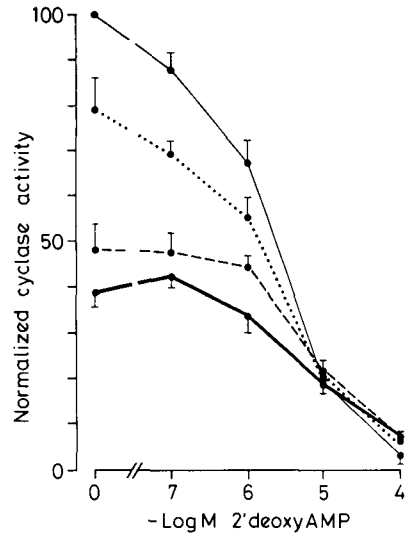

FIG. 3. Effect of increasing concentrations of 2′-deoxyadenosine 3′-monophosphate on the activity of rabbit cardiac adenylate cyclase stimulated by 100 mM Na^+ and 0·1 mM GTP in the absence and presence of 1, 5 and 10 mM theophylline. n = 5, mean ± s.e.m. Key: —— control; ····· 1 mM; – – – 5 mM; ▬▬ 10 mM theophylline.

Dose-response curves to 2′-deoxyadenosine 3′-monophosphate (a P-site agonist), in the absence and presence of 1, 5 and 10 mM theophylline are shown in Fig. 3. At 10 μM 2′-deoxyadenosine 3′-monophosphate, addition of theophylline produces no further reduction in adenylate cyclase activity and there is only partial additivity of the inhibition by theophylline with that of 2′-deoxyadenosine 3′-monophosphate at low concentrations (0·1–1 μM) of the latter. A similar, though not as pronounced, non-additivity was seen between 2′-deoxyadenosine and 2′-deoxyadenosine 3′-monophosphate, two compounds which are known to act at the P-site (results not shown).

Discussion

The results show that theophylline inhibits rabbit cardiac adenylate cyclase in a dose-dependent manner. The non-competitive nature of the inhibition shows that theophylline is not directly competing with MgATP for binding at the active site. In sea-urchin sperm (Garbers 1977), however, theophylline was shown to inhibit the adenylate cyclase in a competitive manner, with respect to MnATP. Whether this difference reflects different sites of action, for MnATP and MgATP, or is due to species and/or tissue differences is not known.

The inhibitory effect of theophylline is seen under basal conditions and when adenylate cyclase is stimulated with NaCl. In neither of these cases are guanine nucleotides included in the assay medium. This action of theophylline does not, therefore, depend on an activation of the stimulatory guanine nucleotide binding protein (Ns-protein).

The non-competitive nature of the inhibition resembles that seen with P-site agonsits (Welton & Simko 1980). The lack of additivity of inhibition by theophylline with that due to 2′-deoxyadenosine 3′-monophosphate provides evidence that the two may be acting at the same site. As the effect is also seen with caffeine (Jakobs et al 1972) and methyl isopropylxanthine (Garbers 1977) as well as isobutylmethylxanthine, the effect is likely to be common to all methylxanthines. If methylxanthines can indeed inhibit the adenylate cyclase through the P-site, this could give some insight into the structure of this site. It also follows that phosphodiesterase inhibitors other than methylxanthines ought to be used in studies where the P-site may be of interest. Furthermore, the high concentrations (1–10 mM) of methylxanthines, particularly theophylline, which are used routinely to block the phosphodiesterase in most adenylate cyclase studies could be inadvertently reducing the activity of the enzyme.

REFERENCES

Avdonin, P. V., Suitinia-Ultina, I. V., Tkachuk, J. A. (1982) Biochemistry USSR 47: 386–394

Davies, G. E. (1973) J. Pharm. Pharmacol. 25: 681–685

Garbers, D. L. (1977) Biol. Reprod. 16: 377–384

Jakobs, K. H., Schultz, K., Schultz, G. (1972) Pharmacology 273: 248–266

Kruse, E., Sholtz, H. (1978) Experientia 34: 504–505

Londos, C., Wolff, J., Cooper, D. M. F. (1981) in: Burnstock, G. (ed.) Purinergic receptors and recognition. Series B, vol. 12. Chapman and Hall, 287–324

Mas-Oliva, J., Williams, A. J., Nayler, W. G. (1979) Biochem. Biophys. Res. Commun. 87: 441–447

Pohl, S. L, Birnbaumer, L., Rodbell, M. (1969) Science 164: 566

Schönhöfer, P. S., Skidmore, I. F. (1971) Pharmacology 6: 109–125

Shepperd, H. (1970) Nature, London 228: 567–568

Weinryb, I., Michel, I. M. (1971) Experientia 27 (12): 1386

Welton, A. F., Simko, B. A. (1980) Biochim. Biophys. Acta 615: 252–261

Wilkinson, G. N. (1961) Biochem. J. 80: 324–332

J. Pharm. Pharmacol. 1985, 37: 926–927
Communicated September 16, 1985

Baclofen is a potent activator of brown fat metabolism

N. J. ROTHWELL, J. I. ADDAE, M. J. STOCK, T. W. STONE*, *Dept. of Physiology, St George's Hospital Medical School, University of London, London SW17, UK*

The injection of (±)-baclofen intravenously or directly into the ventromedial hypothalamus of urethane-anaesthetized rats, produced an activation of brown fat metabolism. This was seen as an increase of brown fat and rectal temperatures, and an increase of GDP binding in brown fat mitochondria. The activation was mediated by the sympathetic supply.

The thermogenic activity of brown adipose tissue (BAT) is a vital component in non-shivering and diet-induced thermogenesis (see Rothwell & Stock 1983 for reviews), but little is known of the central nervous control of this tissue. Electrical stimulation of the ventromedial hypothalamus will produce activation of BAT (Perkins et al 1981) but we have recently observed that injections of baclofen intravenously or directly into the hypothalamus will also cause a very marked stimulation of brown fat thermogenesis.

* Correspondence.

Methods

Injections of (±)-baclofen were made both intravenously (femoral vein) and directly into the hypothalamus of rats anaesthetized with urethane (1·5 g kg^{-1} i.p.) while temperature of the interscapular BAT and core (rectal) temperatures were continuously monitored. Central injections were made into the ventromedial hypothalamus at stereotaxic coordinates AP-0·8, L 0·5, V 8·8 using the atlas of Pellegrino et al (1981). Injections were made in a volume of not more than 1 µl over 1 min and injection sites subsequently confirmed histologically.