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Evidence that cyclic nucleotides are not mediators of fever in rabbits

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1 The N^6-2′-0-dibutyryl derivative of adenosine 3′,5′-monophosphate (db cyclic AMP) and related compounds have been micro-injected into the preoptic/anterior hypothalamic nuclei (PO/AH) of the unanaesthetized, restrained rabbit and the effects on deep body temperature observed.

2 Db cyclic AMP (100–400 μg) produced hypothermia of rapid onset in rabbits at an ambient temperature of 20–23°C. Hypothermia was also produced by N^2-2′-0-dibutyryl guanosine 3′,5′-monophosphate (db cyclic GMP), but not by saline, sodium *n*-butyrate, adenosine 3′,5′-monophosphate (cyclic AMP), guanosine 3′,5′-monophosphate, adenosine 5′-mono-, di- or triphosphate.

3 The initial hypothermic response to db cyclic AMP and db cyclic GMP was followed by a sustained rise in temperature. However, all compounds injected into the PO/AH produced a similar hyperthermia which was attenuated by paracetamol. Development of this tissue-damage fever abolished the hypothermic response to db cyclic AMP in some rabbits.

4 The effects of db cyclic AMP on body temperature and behaviour were not reproduced by the adenylate cyclase activators, cholera toxin (0.125–5 μg) and guanyl imidodiphosphate (5–400 μg).

5 It is concluded that hypothermia is the principal effect of db cyclic AMP on body temperature when injected into the PO/AH in rabbits. These data do not support the proposal that endogenous cyclic AMP in the rabbit brain mediates pyrexia.

Introduction

Studies on the possible involvement of the intracellular modulator, adenosine 3′,5′-monophosphate (cyclic AMP) in central thermoregulatory neurones have included documentation of the effects of N^6-2′-0-dibutyryl cyclic AMP (db cyclic AMP) on deep body temperature. Db cyclic AMP is more active than exogenous cyclic AMP on intact cells, presumably because of its greater lipid solubility and resistance to enzymatic hydrolysis (Posternak *et al.*, 1962; Henion *et al.*, 1967). Db cyclic AMP injected intracerebroventricularly (i.c.v.) in rabbits produces mixed hypo- and hyperthermic effects (Duff *et al.*, 1972; Philipp-Dormston & Siegert, 1975) but these complex responses may be the results of db cyclic AMP affecting numerous sites in the CNS after its introduction into the cerebral ventricles. After local application to the preoptic/anterior hypothalamic nuclei (PO/AH), which contain neurones sensitive to both temperature and pyrogens (Eisenman, 1982), db cyclic AMP produces only sustained hyperthermia in rabbits (Woolf *et al.*, 1975; Willies *et al.*, 1976). This observation gives substantial support to the proposal by Rosendorff (1976) that endogenous cyclic AMP is involved in the neurochemical events mediating fever. However, this theory cannot be applied to other species used in the study of pyretics and antipyretics, namely the cat and the rat, which develop hypothermia in response to db cyclic AMP in the PO/AH (Dascombe & Milton, 1975; Dascombe & Parkes, 1981).

The difference between these reported responses of the rabbit and the cat to db cyclic AMP may not be an interspecies difference because cats respond to db cyclic AMP i.c.v. with mixed hypo- and hyperthermic responses similar to those in rabbits (Varagić & Beleslin, 1973; Clark *et al.*, 1974). The purpose of this study was to re-examine the effects of db cyclic AMP and related drugs on body temperature in rabbits after micro-injection into the PO/AH. A preliminary account of part of this work has been presented to the British Pharmacological Society (Dascombe, 1981).

Methods

Animals and body temperature measurement

Albino half lop rabbits of either sex, weighing from 2 to 3.5 kg at the start of the study, were supplied by the Manchester Medical School Animal Unit. Experiments were conducted between 09 h 30 min–17 h 00 min, each animal being used at intervals of not less than 3 days. Body temperature was measured with a Yellow Springs Instrument model 401 thermistor inserted about 10 cm past the anus while each rabbit was restrained in stocks at an ambient temperature of 20–23°C.

Micro-injection studies

Injections were made unilaterally into the PO/AH through a modified Collison cannula with a 21 gauge guide tube. The guide cannula was implanted chronically and aseptically at least 2 weeks earlier under general anaesthesia produced by pentobarbitone sodium (30 mg kg^{-1} i.v.) injected 5–10 min after chlorpromazine hydrochloride (5 mg kg^{-1} i.v.). Stereotaxic co-ordinates were taken from the atlas by Urban & Richard (1972). Upon completion of experiments, the injection site in each rabbit was verified *post mortem*. Drugs were dissolved in Sodium Chloride Injection B.P. 0.9% w/v (Travenol Laboratories Ltd) except cholera toxin, which was dissolved in Water for Injections B.P. (Phoenix Pharmaceuticals Ltd), unless otherwise stated. Drug solutions were injected into the PO/AH through a 26 gauge injection cannula passed through the implanted guide cannula to a site 1 mm beyond the tip of the outer tube (Myers, 1966). The volume of fluid injected over about 5 s was 1 μl unless otherwise stated. Procedures minimizing contamination by extraneous pyrogens were used as described previously for the preparation and injection of drug solutions (Dascombe & Milton, 1975), with the exception that solutions were not passed through a 0.22 μm filter.

Statistical analysis

Temperature responses were assessed as changes from pre-injection values (Δ°C) and as a temperature response index (TRI) integrating Δ°C against time (h), a single TRI unit (TRI 1°C. h) being equivalent to a 1°C rise in temperature lasting 1 h. The value of x in TRI_x is the period of time (h) for which the response has been assessed. Results are expressed as the mean ± s.e.mean for n experiments. The probability (P) of the significance of the difference between different groups was determined by a 2-tailed Student's t test (Armitage, 1971).

Drugs

The following drugs were used: adenosine 3',5'-monophosphate, adenosine 5'-monophosphate, adenosine 5'-diphosphate, adenosine 5'-triphosphate, N^6-2'-0-dibutyryl adenosine 3',5'-monophosphate, N^2-2'-0-dibutyryl guanosine 3',5'-monophosphate, guanosine 3',5'-monophosphate and 5'-guanyl imidodiphosphate as sodium salts (Sigma Chemical Co.), paracetamol (4-acetamidophenol) and sodium n-butyrate (BDH Chemicals Ltd), cholera toxin (Wellcome Research Laboratories, Sigma Chemical Co.).

Results

Studies with adenosine nucleotides

Db cyclic AMP (dose range 10–400 μg) produced a dose-dependent fall in deep body temperature after injection into the PO/AH of rabbits (Figure 1). Hypothermia was maximal about 60 min after injection and was associated with heat loss by ear skin vasodilatation and, in some animals, tachypnoea. Some rabbits responded to 400 μg db cyclic AMP

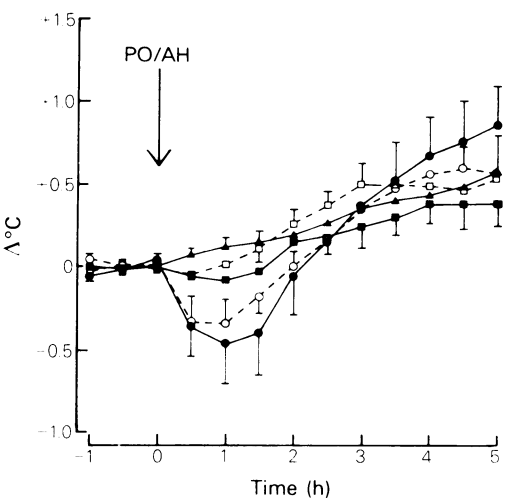

Figure 1 Effects of N^6-2'-0-dibutyryl adenosine 3',5'-monophosphate (db cyclic AMP) on rectal temperature in rabbits at an ambient temperature of 20–23°C. At the time indicated by the arrow, drug solutions (1 μl) were injected unilaterally into the preoptic/anterior hypothalamic nuclei (PO/AH). Points represent the mean change in temperature (Δ°C) for n experiments, vertical lines show the s.e.means. (▲) Saline, $n = 15$; (□) 50 μg db cyclic AMP, $n = 10$; (■) 100 μg db cyclic AMP, $n = 13$; (○) 200 μg db cyclic AMP, $n = 12$; (●) 400 μg db cyclic AMP, $n = 7$.

during the first hour with increased motor activity manifest as struggling. Release from stocks resulted in reduced excitation. Body temperature returned to pre-injection values about 2 h after drug administration; the hypothermia over these 2 h was statistically significant ($P<0.05$) for 100, 200 and 400 μg db cyclic AMP but not for 10 μg or 50 μg. The fall in temperature was followed by a sustained hyperthermia of late onset (Figure 1) associated with ear skin vasoconstriction and quiescent behaviour.

The delayed rise in body temperature produced by db cyclic AMP (400 μg) was attenuated by paracetamol (50 mg kg^{-1} i.p.). Db cyclic AMP appeared to have no effect on temperature in a few rabbits but when injected 15 min after paracetamol db cyclic AMP did induce hypothermia.

Saline (1 μl 0.9% w/v NaCl) or sodium *n*-butyrate (85 μg, equivalent sodium and butyrate content to 200 μg db cyclic AMP) injected into the PO/AH produced a rise but no fall in temperature (Figure 2). The late rise in body temperature produced by db cyclic AMP after the initial hypothermia (Figure 1) was not significantly different from responses to saline or sodium *n*-butyrate which were also associated with peripheral vasoconstriction and sedation, and were attenuated by paracetamol

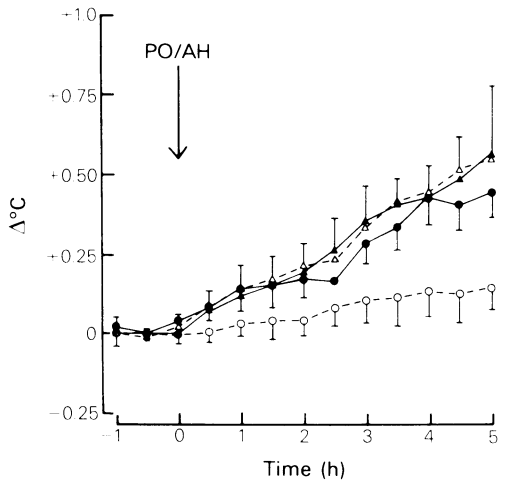

Figure 2 Effects of 'control' injections into the preoptic/anterior hypothalamic nuclei (PO/AH) on rectal temperature in restrained rabbits at an ambient temperature of 20–23°C. At the time indicated by the arrow, drug solutions (1 μl) were injected into the preoptic/anterior hypothalamic nuclei (PO/AH) as described in Methods. Sham injected rabbits had injection cannulae placed into the PO/AH but without the introduction of fluid into the tissue. Points represent the mean change in temperature (Δ°C) for *n* experiments, vertical lines show the s.e.means. (○) No injections, $n=9$; (●) sodium butyrate 85 μg, $n=12$; (△) sham injections, $n=8$; (▲) 0.9% w/v saline 1 μl, $n=15$.

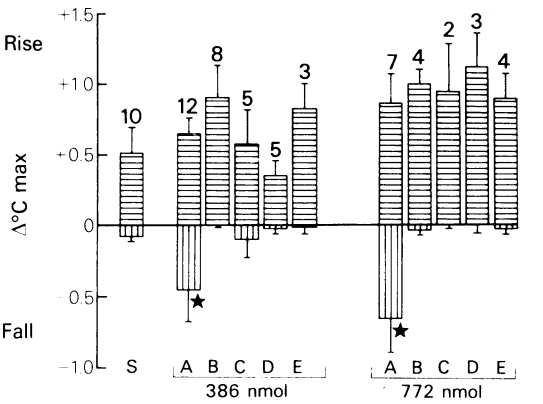

Figure 3 Effects of adenine nucleotides on rectal temperature in rabbits at an ambient temperature of 20–23°C. Drugs were injected into the preoptic/anterior hypothalamic nuclei dissolved in 1 μl saline (S). Bars represent the greatest rise and fall in body temperature from pre-injection values (Δ°C max) over a 5 h period following injection. Vertical lines show the s.e.means for the number of experiments shown over the bar. (A) N^6-2′-0-dibutyryl adenosine 3′,5′-monophosphate; (B) adenosine 3′,5′-monophosphate; (C) adenosine 5′-monophosphate; (D) adenosine 5′-diphosphate; (E) adenosine 5′-triphosphate.
* $P<0.05$ compared with response to saline (S).

(50 mg kg^{-1} i.p.). A similar pyrexia was observed after placement of an injection cannula into the PO/AH without the introduction of fluid ('sham injection') but not in rabbits restrained for the experimental period without receiving either a sham or a drug injection (Figure 2).

Cyclic AMP in doses equimolar with 100, 200 and 400 μg db cyclic AMP, as well as adenosine 5′-monophosphate (AMP), adenosine 5′-disphosphate (ADP) and adenosine 5′-triphosphate (ATP) in doses equimolar with 200 μg and 400 μg db cyclic AMP, all induced a prolonged hyperthermia (duration > 5 h) usually of late onset (Figure 3). Unlike db cyclic AMP, the nonbutyrated adenine compounds did not induce rapid onset hypothermia, ear skin vasodilatation or motor excitation (Figure 3).

Studies with cyclic guanine nucleotides

N^2-2′-0-dibutyryl guanosine 3′,5′-monophosphate (db cyclic GMP, dose range 10–200 μg) produced initial hypothermia followed by a later hyperthermia (Figure 4). Hypothermia in response to db cyclic GMP was associated with ear skin vasodilatation, but was shorter in duration than the response to db cyclic AMP in the same rabbits (Figure 4).

The late rise in body temperature after injection of

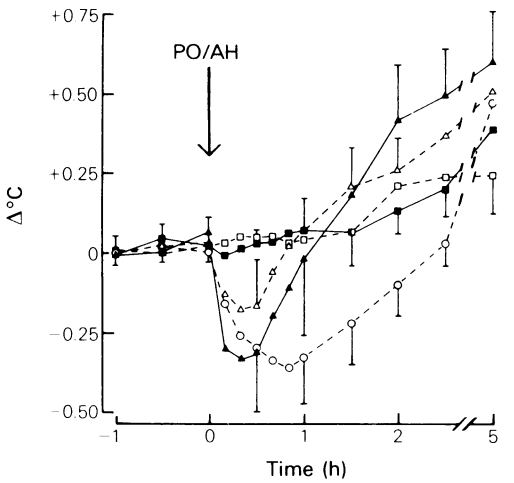

Figure 4 Effects of N^2-2′-0-dibutyryl guanosine 3′,5′-monophosphate (db cyclic GMP) on rectal temperature in rabbits at an ambient temperature of 20–23°C. At the time indicated by the arrow, drug solutions (1 μl) were injected unilaterally into the preoptic/anterior hypothalamic nuclei (PO/AH). Points represent the mean change in temperature (Δ°C) for n experiments, vertical lines show the s.e.means. (□) 10 μg db cyclic GMP, $n=4$; (■) 50 μg db cyclic GMP, $n=6$; (△) 100 μg db cyclic GMP, $n=9$; (▲) 200 μg (362 nmol) db cyclic GMP, $n=3$; (○) 200 μg (386 nmol) db cyclic AMP, $n=8$.

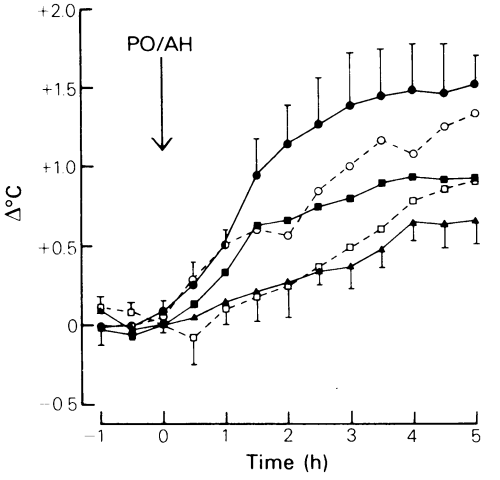

Figure 5 Effects of cholera toxin on rectal temperature in rabbits at an ambient temperature of 20–23°C. At the time indicated by the arrow, drug solutions (2 μl) were injected unilaterally into the preoptic/anterior hypothalamic nuclei (PO/AH). Points represent the mean change in temperature (Δ°C) for n experiments, vertical lines show the s.e.means. (▲) Water for Injection 2 μl, $n=7$; (□) 0.125 μg cholera toxin, $n=4$; (■) 0.5 μg cholera toxin, $n=5$; (○) 1 μg cholera toxin, $n=5$; (●) 5 μg cholera toxin, $n=4$.

db cyclic GMP was similar to that produced by saline (1 μl), sodium n-butyrate (85 μg) or sham injection. Guanosine 3′,5′-monophosphate (cyclic GMP, doses equimolar with 50 μg and 100 μg db cyclic GMP) had no effect on body temperature when compared with responses to saline, sodium n-butyrate or sham injection over 5 h after injection.

Studies with activators of adenylate cyclase

Cholera toxin (dose range 0.125–5 μg) injected into the PO/AH produced an increase in body temperature associated with ear skin vasodilatation and sedation (Figure 5).

The febrile response to cholera toxin was unaffected by heating the toxin for 1 h at 98–100°C before injection; the TRI_5s for 0.25 μg and 1 μg cholera toxin without heating were 3.99 ± 1.84 °C.h ($n=3$) and 4.38 ± 1.40 °C.h ($n=4$) respectively, and after heating 3.67 ± 1.70 °C.h ($n=3$) and 4.71 ± 1.52 °C.h ($n=4$) respectively. Db cyclic AMP (400 μg) dissolved in cholera toxin vehicle (2 μl Water for Injection B.P.) and injected into the PO/AH of these rabbits produced hypothermia similar to that shown for this dose in Figure 1. Cholera toxin (25 $\mu g\,kg^{-1}$) injected i.v. in rabbits produced a monophasic rise in temperature (TRI_5 3.21 ± 0.36 °C.h, $n=5$) significantly different ($P<0.001$) from controls (TRI_5 0.29 ± 0.24 °C.h, $n=5$), beginning within 30 min and lasting about 4 h. Heat-treated cholera toxin (25 $\mu g\,kg^{-1}$) injected i.v. was pyrogenic (TRI_5 2.72 ± 0.88 °C.h, $n=5$).

Guanyl imidodiphosphate 400 μg injected into the PO/AH induced a small but sustained increase in temperature (TRI_5 3.23 ± 0.75 °C.h, $n=5$) compared with responses to saline 1 μl (TRI_5 1.55 ± 0.55 °C.h, $n=5$, $P<0.05$). Smaller doses of guanyl imidodiphosphate (5, 100 and 200 μg) had no significant effect on body temperature.

Discussion

Db cyclic AMP injected into the PO/AH in rabbits produced an initial dose-dependent hypothermia associated with increased heat loss by peripheral vasodilatation and tachypnoea, followed by a later sustained hyperthermia. Db cyclic GMP produced a similar biphasic response although the initial fall in temperature was smaller than that caused by db cyclic AMP. This similarity is presumably due to high concentrations of cyclic GMP activating cyclic AMP-dependent protein kinase activity (Goldberg *et al.*, 1973). No fall in body temperature was produced by

central injection of either cyclic AMP or cyclic GMP, which is consistent with cyclic nucleotides in the extracellular space having little or no biological activity. However, cyclic AMP and cyclic GMP did produce a sustained hyperthermia similar to that induced by db cyclic AMP and db cyclic GMP but these rises in temperature above preinjection values were not significantly different from responses to vehicle. Similar rises in temperature were produced by all other compounds injected in this study including AMP and sodium *n*-butyrate. Precautions were taken to prevent pyrogenic contamination of drug solutions, and sham injections also induced pyrexia, which indicates the febrile response to drugs injected into the PO/AH was a consequence of tissue damage caused by introduction of the injection cannula. Similar tissue damage fevers have been reported in the cat (Dey *et al.*, 1974) and the rat (Ackerman & Rudy, 1980) and have been attributed to prostaglandin release from damaged tissue. The sustained hyperthermia following the initial hypothermic response to db cyclic AMP was attenuated in this study by the prostaglandin synthetase inhibitor, paracetamol. Rapid development of tissue damage fever masked the hypothermic response to db cyclic AMP in a few rabbits but hypothermia was evident in these animals after pretreatment with paracetamol.

These results do not agree with the findings of Woolf *et al.* (1975) who observed hyperthermia without prior hypothermia in response to db cyclic AMP, the hyperthermia being unaffected by sodium salicylate (Willies *et al.*, 1976). The apparent discrepancy between these reports as to the sensitivity of the sustained hyperthermia to antipyretic drugs may be attributable to sodium salicylate (120 mg then 0.75 mg min^{-1} i.v.) being less antipyretic than paracetamol (50 mg kg^{-1} i.p.) in the rabbit. The absence of hypothermia in the study of Woolf *et al.* (1975) may be due to the doses of db cyclic AMP employed (10 μg or less bilaterally into the hypothalamus) being too small. Similar doses did not cause hypothermia in this study. The need to use large doses of db cyclic AMP to induce hypothermia introduces doubt as to whether the fall in temperature mimics a function of endogenous cyclic AMP in the PO/AH.

Levels of endogenous cyclic AMP can be increased by cholera toxin, an activator of adenylate cyclase in various tissues including those in the brain (Miller & Kelly, 1975; Quenzer *et al.*, 1977; Nistico *et al.*, 1978). Clark *et al.* (1974) observed hyperthermia in cats receiving cholera toxin into the cerebral ventricles and attributed this effect to adenylate cyclase activation. In this study, cholera toxin caused hyperthermia in rabbits after injection into the PO/AH, in contrast with the hypothermic response to db cyclic AMP. It seems unlikely, therefore, that the temperature effects of both cholera toxin and db cyclic AMP in the rabbit are mediated by raised concentrations of intracellular cyclic AMP. Indications are that the response to cholera toxin was not due to activation of adenylate cyclase. The latency to onset of hyperthermia was shorter than the characteristic lag period of several hours before responses are evident in other intact systems (Gill, 1977). In addition, the ability of cholera toxin to induce hyperthermia in rabbits was unaffected by heat although heat-denatured toxin does not activate adenylate cyclase (Schafer *et al.*, 1970; Sharp & Hynie, 1971). Cholera toxin was a heat-stable pyrogen after intravenous administration to rabbits, and consequently the hyperthermic response to toxin injected directly into the pyrogen-sensitive PO/AH is attributable to an endotoxin-like property of the bacterial product. In keeping with this interpretation, guanyl imidodiphosphate, a metabolically stable analogue of guanosine triphosphate, which produces a persistent activation of adenylate cyclase similar to that by cholera toxin (Londos *et al.*, 1974; Spiegel & Aurbach, 1974; Pfeuffer & Helmreich, 1975; Schramm & Rodbell, 1975), had no effect on body temperature in doses up to 200 μg. Guanyl imidodiphosphate 400 μg induced a small rise in temperature but this can be attributed to physiochemical properties of the concentrated solution (about 0.6 M) exacerbating the inflammatory response to central injections.

It is concluded that db cyclic AMP and db cyclic GMP injected into the PO/AH cause hypothermia in rabbits at an ambient temperature of 20–23°C. The initial fall in body temperature is followed by a later hyperthermia which can be attributed to tissue damage at the injection site. Similar responses to db cyclic AMP have been reported for the cat (Dascombe & Milton, 1975) and the rat (Dascombe & Parkes, 1981). The fall in temperature produced by db cyclic AMP was not mimicked by two activators of adenylate cyclase, cholera toxin and guanyl imidodiphosphate, indicating the hypothermia is of pharmacological rather than pathophysiological interest. Accordingly, these data do not substantiate the theory (Rosendorff, 1976) that endogenous cyclic AMP mediates the neuronal events leading to fever in rabbits.

Samples of cholera toxin were kindly donated by Dr R.O. Thomson, The Wellcome Research Laboratories, Beckenham, England.

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