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Stimulation of the hypothalamo-pituitary-adrenal axis in the rat by the type 4 phosphodiesterase (PDE-4) inhibitor, denbufylline

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1 Preliminary studies in our laboratories showed that the synthetic xanthine analogue denbufylline, a selective type 4 phosphodiesterase (PDE-4) inhibitor, is a potent activator of the hypothalamo-pituitary-adrenal (HPA) axis when given orally to adult male rats. This paper describes the results of experiments in which well established *in vivo* and *in vitro* models were used to (a) examine further the effects of denbufylline on HPA function and (b) identify the site and mode of action of the drug within the axis.

2 *In vivo*, administration of denbufylline (0.1-2.5 mg kg^{-1}, i.p.) produced a significant increase in the serum corticosterone concentration; maximal responses were attained at a dose of 1.0 mg kg^{-1} ($P<0.01$ vs. vehicle control, Scheffe's test). However, when denbufylline was administered by intracerebroventricular injection (0.05-1 $\mu g\ kg^{-1}$) it failed to influence significantly the serum corticosterone concentration ($P>0.05$ vs. vehicle control, Scheffe's test). The adrenocortical responses to peripheral injections of denbufylline (1 mg kg^{-1}, i.p.) were reduced in rats in which the secretion of endogenous corticotrophin releasing factors (CRFs) from the hypothalamus was blocked pharmacologically ($P<0.01$ vs. controls, Scheffe's test). However, denbufylline (0.1 mg kg^{-1}, i.p.) potentiated the significant ($P<0.01$) increases in serum corticosterone concentration provoked in 'CRF blocked rats' by hypothalamic extract (5 hypothalamic extracts kg^{-1}, i.v.) although it failed to influence ($P>0.05$) the relatively moderate increases in corticosterone secretion evoked by CRH-41 (2 mg kg^{-1}, i.v.).

3 *In vitro*, denbufylline (0.01-1 mM) evoked small but significant ($P<0.05$) increases in the release of ACTH from rat anterior pituitary segments; furthermore, at these and lower concentrations (0.01 μM-1 mM), it potentiated the adrenocorticotrophic responses to sub-maximal concentrations of hypothalamic extract ($P<0.01$) and forskolin (0.1 mM, $P<0.01$) but not those to CRH-41 (10 nM) or 8-bromo-cyclic AMP (1-100 μM). In addition, denbufylline (0.1 mM) increased the anterior pituitary cyclic AMP content ($P<0.05$) and potentiated the rises in tissue content of the cyclic nucleotide induced by hypothalamic extract (0.1 hypothalamic equivalents ml^{-1}, $P<0.01$) and forskolin (0.1 mM, $P<0.01$) but not by CRH-41 (10 nM, $P<0.05$). By contrast, denbufylline (1 μM-1 mM) failed to influence the release of AVP from rat isolated hypothalami and stimulated the secretion of CRH-41 ($P<0.01$) release only at the highest concentration tested (1 mM).

4 The results suggest that the stimulatory actions of denbufylline on the hypothalamo-pituitary-adrenocortical axis are exerted predominantly at the level of the anterior pituitary gland and that they may be attributed, at least in part, to inhibition of type 4 phosphodiesterase enzymes.

Keywords: Denbufylline; type 4 phosphodiesterase; ACTH; signal transduction; HPA axis

Introduction

Cyclic nucleotide phosphodiesterase enzymes (PDEs) are responsible for the degradation of the cyclic nucleotide second messengers, adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP), in cells throughout the body. They thus serve to terminate cyclic nucleotide-dependent protein kinase activation and hence, play a critical role in the regulation of the cellular responses which are driven by these pathways (Beavo, 1988). Biochemical, pharmacological and molecular biology studies have shown that the phosphodiesterases comprise a heterogeneous family of enzymes. To date, seven distinct classes of PDE have been identified (Beavo & Reifsnyder, 1990; Bentley & Beavo, 1992; Michaeli *et al.*, 1993; Loughney *et al.*, 1994) and gene splicing and post translational events provide opportunity for further variants which may be tissue/cell specific (Beavo & Reifsnyder, 1990; Repaske *et al.*, 1992; Bolger, 1993). Particular interest has focused on the type 4, cyclic AMP specific phosphodiesterase (PDE-4) for which a number of inhibitors has been developed. These compounds are of potential therapeutic value in the treatment of certain allergic conditions, notably asthma (Nicholson & Shahid, 1994; Sullivan *et al.*, 1994). However, since PDE-4 has been identified in a variety of cells and tissues in the body (Beavo & Reifsnyder, 1990), these drugs inevitably also have the potential to exert more widespread actions. Of particular interest in this regard is that the endocrine system for PDE-4 has been identified in several peripheral endocrine organs (e.g. thyroid) where it plays a critical role in terminating cyclic AMP-dependent activation of the secretory cells (Conti *et al.*, 1991; Sette *et al.*, 1994b; Koch & Lutz-Bucher, 1995). Indeed, it may provide a mechanism by which endocrine cells alter their responsiveness to specific stimuli since such cells possess mechanisms of short and long term activation of PDE, the latter involving *de novo* synthesis of PDE-4 (Sette *et al.*, 1994a).

The role of PDE-4 in the regulation of hypothalamo-pituitary function has scarcely been addressed despite the fact that cyclic AMP is known to play a critical part in the signal transduction mechanisms which effect the release of most, if not all, of the hypothalamic and anterior pituitary hormones. The positive influence of the nucleotide on the secretion of corticotrophin (ACTH) and the two major hypothalamic corticotrophin releasing hormones, CRH-41 and arginine va-

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sopressin (AVP), has been particularly well characterized. It is thus apparent that drugs which stimulate adenyl cyclase activity directly or indirectly initiate the release of CRH-41 and AVP and that cyclic AMP response elements are included in the promoter region of the genes which encode these peptides (Seasholtz *et al.*, 1988; Emanuel *et al.*, 1990). Similarly, the ability of CRH-41 to stimulate the synthesis and release of ACTH by the corticotrophs and to induce proliferation of these cells is dependent on the generation of cyclic AMP (Labrie *et al.*, 1982a, b; Aguilera *et al.*, 1983). Furthermore, although the increase in ACTH release induced by AVP is effected by receptors which are positively coupled to phospholipase C (Aguilera *et al.*, 1983; Todd & Lightman, 1987), the powerful synergistic actions of CRH-41 and AVP on the corticotrophs (reviewed in Buckingham *et al.*, 1992), which are critical to adrenal function, involve a pronounced augmentation of the cyclic AMP response to CRH-41 (Giguère & Labrie, 1982). Not surprisingly, systemic administration of non-selective PDE inhibitors (e.g. caffeine, isobutyl-methylxanthine (IBMX)) produces a significant increase in hypothalamo-pituitary-adrenocortical (HPA) activity in rodents (Sobel, 1985; Nicholson, 1987; 1989). Furthermore, these agents promote the release *in vitro* of ACTH and CRH-41 from the adenohypophysis and hypothalamus respectively. We are unaware of any published studies in which the effects of selective PDE-4 inhibitors on HPA function have been examined *in vivo*. However, two recent *in vitro* studies point to a role for the type 4 isoform in the regulation of corticotrophic function in the rat (Hadley *et al.*, 1993; Koch & Lutz-Bucher, 1995). Furthermore, in preliminary studies, we found that oral dosing of the selective PDE-4 inhibitor, denbufylline (1,3-di-n-butyl-7-(2'-oxopropyl)-xanthine), induced pronounced sequential increases in the plasma ACTH and serum corticosterone concentrations which were blocked readily by pretreatment of the animals with dexamethasone. The present study describes the results of experiments in which we exploited a number of well established *in vivo* and *in vitro* models to examine more fully the influence of denbufylline on HPA function in the rat and to identify its site and mode of action.

Methods

Animals

Adult, male Sprague Dawley rats derived from closed specific pathogen-free colonies and weighing approximately 200 g (*in vitro* studies) or 225–275 g (*in vivo* studies) were used. They were either bred in house (i.c.v. injection of denbufylline and *in vitro* studies) or purchased from Harlan Olac, Banbury, England (i.p. injection of denbufylline) and housed post weaning in groups of 2 (*in vivo*) or 5 (*in vitro*) per cage. All animals were kept in a quiet room maintained at 19–23°C with controlled lighting (lights on 08 h 00 min–20 h 00 min) and humidity and had free access to food and water. Rats for all *in vivo* studies were handled and weighed daily for at least 7 days prior to the experiments by the individual who undertook the subsequent studies. The experiments were started between 08 h 00 min and 09 h 00 min in order to avoid changes associated with the circadian rhythm.

In vivo *studies*

The effects of denbufylline given peripherally or centrally on the serum corticosterone concentrations were examined in conscious rats. Further experiments examined the ability of peripheral injections of denbufylline to initiate the release of corticosterone in anaesthetized rats in which the release of endogenous corticotrophin releasing factors was blocked pharmacologically.

Administration of denbufylline Single doses of denbufylline were administered either intraperitoneally (i.p.) in doses of 0.1–2.5 mg kg^{-1} and a volume of 1.0 ml kg^{-1} or intracerebroventricularly (i.c.v.) via an indwelling cannula in doses of 0.05–1.0 μg kg^{-1} and volumes of 3 μl per rat. In all instances controls received a corresponding volume of the vehicle (0.9% NaCl solution). The rats were killed by decapitation 20 min after the injections and the trunk blood collected for hormone determination.

Cannulation of the third ventricle In a preliminary operation, guide cannulae with stoppers were implanted stereotaxically (Loxley *et al.*, 1993a) into the third ventricle of the brain of young adult male rats anaesthetized with sodium pentobarbitone (60 mg kg^{-1} body weight i.p., 2.0 ml kg^{-1}). The animals were allowed a period of 7–10 days to recover from the trauma of the operation during which they were handled and weighed daily. Any animal which did not gain weight normally or appeared unhealthy postoperatively was killed humanely. On the day of the experiment, the rats were lightly restrained (hand held) and the stopper removed from the guide cannula. An injection cannula, which protruded 1 mm beyond the guide cannula and was fixed via polypropylene tubing to a 10 μl Hamilton microsyringe, was then inserted and drug solutions injected slowly over a 30–60 s period. Blood was collected 20 min later and the position of the cannula was verified post-mortem.

Pharmacological blockade of endogenous CRF release Pharmacological blockade of endogenous CRF release from the hypothalamus was effected according to the method of Arimura *et al.* (1967). Rats were thus subjected to sequential injections of chlorpromazine hydrochloride (10 mg kg^{-1} s.c., 2.0 ml kg^{-1}, time 0 min), morphine sulphate (20 mg kg^{-1}, s.c., 2.0 ml kg^{-1}, time 3 h), and sodium pentobarbitone (25 mg kg^{-1}, i.p., 1.0 ml kg^{-1}, time 3 h 15 min), each of which was diluted in sterile NaCl solution (0.9% w/v, BDH Chemicals Ltd., Dagenham, U.K.). Denbufylline (0.1 mg kg^{-1}, i.p.) or an equal volume (1.0 ml kg^{-1}) of its vehicle was injected 45 min after administration of sodium pentobarbitone. After a further 5 min, the anaesthetized rats were challenged with a submaximal dose (Buckingham, 1984) of either the 41-amino acid corticotrophin releasing hormone, CRH-41 (2 μg kg^{-1}, i.v.) or hypothalamic extract (HE, 5 hypothalamic equivalents per kg, i.v.); controls received a corresponding volume (1.0 ml kg^{-1}) of the saline vehicle. Blood was collected after a further 15 min.

Collection of blood The animals were killed by stunning with subsequent decapitation. Blood was collected from the trunk, placed in cooled plastic tubes and allowed to stand on ice for 15–20 min before centrifugation (2,500 r.p.m., 10 min, 4°C). The resulting serum samples were separated and stored at −20°C.

In vitro *studies*

Static incubation of hypothalamic tissue The method employed was a modification of that described by Buckingham & Hodges (1977) using tissue from rats which had been adrenalectomized 7–10 days previously (under sodium pentobarbitone anaesthesia, 60 mg kg^{-1}, i.p., 2 ml kg^{-1}), a process which increases the amount of CRH-41 and AVP available for release (Loxley *et al.*, 1993a,b). Briefly the rats were decapitated; the hypothalamus was dissected out and the peritoneum examined to verify the completeness of adrenalectomy. The hypothalamic blocks were transferred immediately to tubes containing 1 ml artificial cerebrospinal fluid (CSF, Buckingham & Hodges, 1977) enriched with aprotinin (100 kiu ml^{-1}, Bayer UK Ltd.) and incubated for 60 min in a gently shaking water bath at 37°C. Throughout this period the tubes were gassed constantly with 95% O_2/5% CO_2; the medium was replaced after 30, 45 and 60 min. The hypothalami were then subjected to two successive 15 min incubations. During the first, the hypothalami were exposed to test substances; the final 15 min

incubation was used to verify the viability of the tissue by exposure to 56 mM K^+. The medium from each of the final two incubations was collected; 300 μl was aspirated from each and stored at −80°C for determination of AVP. The remainder was either assayed immediately for CRH-41 or freeze dried for subsequent determination of the peptide.

Static incubation of anterior pituitary segments

Anterior pituitary glands were removed from intact rats immediately after decapitation, divided into 4–6 pieces of approximately equal size and incubated individually according to a method described previously (Hadley *et al.*, 1993) using modified 24-well culture plates (Tissue Cluster 24, Costar, Cambridge, U.S.A.). Each tissue piece was thus placed in 1 ml Earle's balanced salts solution [EBSS (Sigma Chemical Co., Poole, Dorset, U.K.) enriched with aprotinin 100 kiu ml^{-1}, Bayer U.K. Ltd) prewarmed to 37°C and pre-gassed with 95% O_2/5% CO_2] and incubated for 2.5 h at 37°C in an atmosphere saturated with 95% O_2/5% CO_2 gas. The medium was changed after 2 h and again 15 min later. The segments were then transferred to fresh medium (1.0 ml) containing a secretagogue (HE, CRH-41 or forskolin) and/or graded concentrations of denbufylline or, in the case of controls, an equal volume of medium alone, and the incubation continued for a further 30 min (for hormone determination) or 2 min (for cyclic AMP measurement). The medium was collected and either assayed immediately for ir-ACTH or stored at −20°C for later hormone measurement. Pituitary segments were weighed on a torsion balance and then discarded. For tissue cyclic AMP determination, segments were rapidly frozen on dry ice, thawed and boiled for 10 min in 250 μl EBSS containing theophylline (2 mM, Sigma Chemical Co., Poole, Dorset, U.K.) and aprotinin (100 kiu ml^{-1}). The resultant medium was cooled and assayed immediately for cyclic AMP content. In order to minimize potential problems of variability associated with inter-animal differences, the segments were randomized throughout the treatment groups such that no one treatment group contained more than one segment from any one animal.

Hormone determination

ACTH, CRH-41, AVP, corticosterone and cyclic AMP were each determined in duplicate by radioimmunoassay using modifications of methods described elsewhere (Rees *et al.*, 1971; Negro-Vilar *et al.*, 1979; Al Dujaili *et al.*, 1981; Hillhouse & Milton, 1989; Loxley *et al.*, 1993a) and, in the case of cyclic AMP, a commercial kit (*Rianen* cyclic AMP ^{125}I kit; Du Pont Medical Products Department, U.K.). Samples from any one experiment were always run in single assays so as to avoid inter-assay variance; in all cases the samples diluted in parallel with the respective standard curves.

The ACTH assay employed human $ACTH_{1-39}$ (National Institute for Biological Standards and Control, South Mimms, Herts., U.K.) as a standard, [^{125}I]-$ACTH_{1-39}$ as a tracer and an antibody raised in rabbit against $ACTH_{1-24}$. The antibody (Ab; coded Tom 8/3) had negligible cross-reactivity with β-endorphin, β-lipotrophin, corticotrophin-like intermediate lobe peptide, α or γ melanocyte stimulating hormones but bound readily with $ACTH_{1-24}$ and $ACTH_{1-39}$. The senstivity of the assay was 20 pg ml^{-1} and the inter- and intra-assay coefficients of variation were 9.5% and 11.3% respectively.

The reference preparation for the CRH-41 assay was rat/human CRH-41 (Peninsula Laboratories, St. Helens, U.K.) and the tracer [^{125}I]-tyrosine rat/human CRH-41 (Tyr-CRH-41 from Bachem, Saffron Walden, U.K. and ^{125}I from Amersham International plc., Amersham, U.K.). The antibody (raised in rabbit against rat CRH-41 conjugated with β-gamma-globulin, BGG) cross-reacted with rat/human CRH-41 (100%), oxidised rat/human CRH-41 (75%), rat/human CRH_{21-49} (35%), rat/human CRH_{1-20} (0.1%), rat/human CRH_{6-33} (0.02%), ovine CRH-41 (9%) and sauvagine (0.035%). It had no cross reactivity with somatostatin, luteinising hormone releasing hormone or thyrotrophin releasing hormone. The sensitivity of the assay was 10 pg ml^{-1} and the inter and intra-assay coefficients of variation were 4.4% and 9.2% respectively. Samples freeze dried for CRH-41 determination were reconstituted in CRH-41 assay buffer immediately prior to assay.

The AVP assay employed an antibody with defined specificity (Negro-Vilar *et al.*, 1979) raised in rabbit against AVP_{1-9}. The reference preparation was synthetic AVP (National Institute for Biological Standards and Control, South Mimms, Herts., U.K.) and the tracer was ^{125}I-labelled AVP (Amersham International plc). The sensitivity of the assay was 2 pg ml^{-1} and the inter- and intra-assay coefficients of variation were 3.6% and 11.9% respectively.

The direct assay for corticosterone used a well characterized antibody (Al-Dujaili *et al.*, 1981) raised in rabbit, corticosterone (Sigma London Chemical Company, U.K.) as a reference preparation and ^{125}I-labelled corticosterone conjugate (I.D.S., U.K.) as a tracer. The sensitivity of the assay was 0.4 pg ml^{-1} and the intra- and inter-assay coefficients of variation were 4.8 + 0.2% and 4.8 + 0.3% respectively.

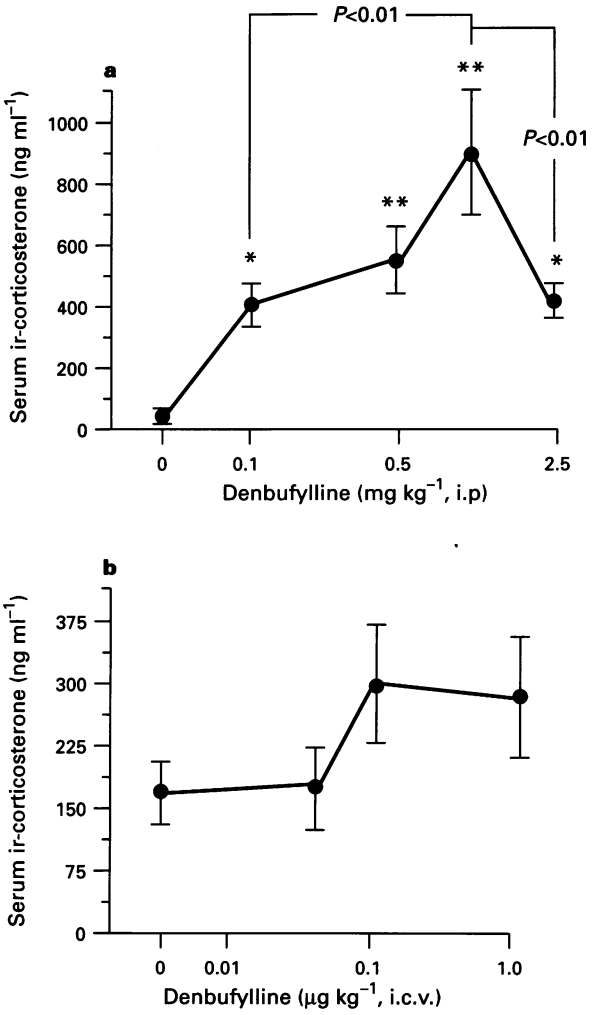

Figure 1 Serum ir-corticosterone concentrations in conscious rats 20 min after a single injection of denbufylline given (a) peripherally (0.1–2.5 mg kg^{-1}, i.p. in a volume of 1.0 ml kg) or (b) centrally (0.05–1.0 μg kg^{-1}, i.c.v. in a volume of 3 μl). Controls received an equivalent volume of the sterile saline vehicle. Values represent the mean ± s.e.mean: in (a) vehicle-treated controls $n = 18$; drug-treated groups $n = 6$; in (b) $n = 5–6$ in all groups $^*P < 0.05$, $^{**}P < 0.01$ vs. vehicle control (ANOVA plus Scheffe's test). Resting serum corticosterone concentrations in untreated control rats varied from 10–30 ng ml^{-1}.

Drugs

Denbufylline was supplied by SmithKline Beecham Pharmaceuticals, Harlow, Essex, U.K. It was dissolved at 45°C in sterile 0.9% NaCl solution and stirred magnetically for 30 min to aid dissolution. Working concentrations were obtained by diluting the stock solution in 0.9% NaCl for *in vivo* use or in EBSS for *in vitro* use. CRH-41 for *in vitro* use was a gift from Whittier Institute for Diabetes & Endocrinology, U.S.A. and for *in vivo* use was obtained from Shire Pharmaceuticals, Andover, U.K. Forskolin and 8-bromoadenosine 3′:5′-cyclic monophosphate were obtained from Sigma London Chemical Company.

Statistical analysis

The results, which were normally distributed, were analysed using ANOVA and Duncan's multiple range test (*in vitro* studies) or Scheffe's test (*in vivo* studies). Differences were considered to be significant if $P<0.05$. Since the rate of basal peptide release *in vitro* varied between experiments, statistical comparisons were made only within experiments.

Results

A single intraperitoneal injection of denbufylline produced within 20 min a marked, significant increase in the serum ir-corticosterone concentration (0.1 mg kg^{-1} and 2.5 mg kg^{-1}: $P<0.05$; 0.5 and 1.0 mg kg^{-1}: $P<0.01$ vs. vehicle control). Over the range 0.1–1 mg kg^{-1}, the effects of denbufylline appeared to be dose-related but the responses to a higher dose (2.5 mg kg^{-1}) were less pronounced (Figure 1a). By contrast, when denbufylline (0.05–1 μg kg^{-1}) was injected directly into the third ventricle of the brain no significant ($P>0.05$ vs. vehicle) changes in serum corticosterone concentrations were apparent (Figure 1b).

When the release of endogenous CRFs from the hypothalamus was blocked by administration of chlorpromazine hydrochloride, morphine sulphate and sodium pentobarbitone, the stimulatory effects of denbufylline (0.1 mg kg^{-1}, i.p.) on corticosterone release were reduced ($P<0.01$ vs. intact rats) but not abolished ($P<0.05$ vs. 'CRF-blocked' rats treated with saline the vehicle); these results are shown in Figures 2a and b. A single intravenous injection of either hypothalamic extract (5 HE kg^{-1}; Figure 2a) or CRH-41 (2 μg kg^{-1}; Figure 2b) increased the serum corticosterone concentration ($P<0.05$, Scheffe's test) in the 'CRF-blocked' rats. The adrenocortical responses to hypothalamic extract were potentiated markedly by pretreatment of the 'CRF blocked' rats with denbufylline (0.1 mg kg^{-1}, i.p. $P<0.01$, Scheffe's test, Figure 2a). By contrast, the responses to CRH-41 and denbufylline were only additive in 'CRF-blocked' rats (Figure 2b).

Figure 3 demonstrates the effects of denbufylline (0.01–1 mM) and K^+ (56 mM) on the release of CRH-41 and AVP from rat isolated hypothalami *in vitro*. At the highest concentration tested, denbufylline (1 mM) stimulated the release of CRH-41 ($P<0.01$ vs. basal) but lower concentrations (0.01 and 0.1 mM) were without effect; subsequent exposure of the tissue to K^+ (56 mM) precipitated a 3–5 fold increase in CRH-41 release irrespective of the previous drug treatment (Figure 3a). By contrast, the release of AVP release was unaffected by the inclusion of denbufylline (0.01–1 mM) in the medium (Figure 3b); however, while control hypothalami and hypothalami pre-exposed to denbufylline (0.01–0.1 mM) re-

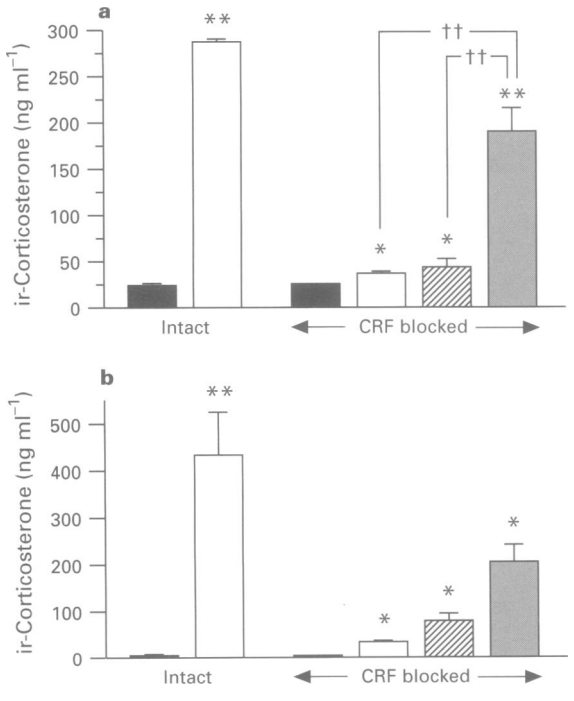

Figure 2 Effects of denbufylline (0.1 mg kg^{-1}, i.p.) on the resting serum ir-corticosterone concentrations in intact rats and in rats in which endogenous CRF release was blocked pharmacologically (a and b) and on the ability of (a) hypothalamic extract (5 HE kg^{-1}, i.v.) and (b) corticotrophin releasing hormone-41 (CRH-41, 2 μg kg^{-1}, i.v.) to increase the serum ir-corticosterone concentration in the 'CRF-blocked' rats. Solid columns = vehicle controls; open columns = denbufylline; hatched columns = HE (a) or CRH-41 (b); stippled columns = denbufylline + HE (a) or denbufylline + CRH-41 (b). Each column represents the mean ± s.e.mean ($n=6-8$). *$P<0.05$; **$P<0.01$ vs. vehicle control; ††$P<0.01$ bars indicated on the figure (ANOVA plus Scheffe's test).

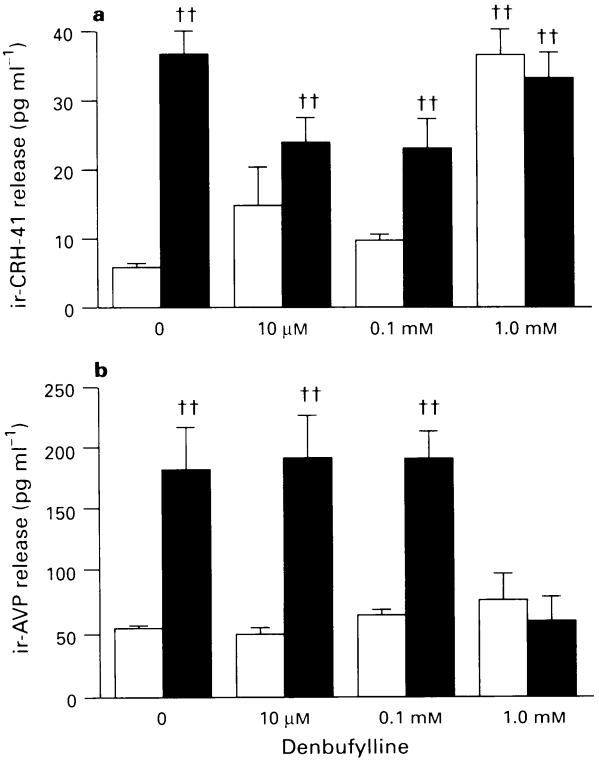

Figure 3 The effects of graded concentrations of denbufylline and subsequent stimulation with K^+ (56 mM) on the release of (a) ir-CRH-41 and (b) ir-AVP from isolated hypothalami *in vitro*. Open columns = denbufylline or its vehicle as indicated on the abscissa scale; solid columns = K^+. Each column represents the mean ± s.e.mean ($n=6$) ††$P<0.01$ vs. vehicle control (ANOVA plus Duncan's test).

sponded to K^+ (56 mM) with the anticipated hypersecretion of AVP, ($P<0.01$ vs. unstimulated control), those pre-exposed to denbufylline (1 mM) did not (Figure 3b).

Preliminary studies (data not shown) confirmed the ability of hypothalamic extract (HE; 0.05–0.4 HE ml^{-1}, CRH-41 (0.1–100 nM) and forskolin (0.01–1 mM) to elicit concentration-dependent increases in the release of ACTH from static incubates of anterior pituitary segments *in vitro*. The secretory responses to each of these agents were accompanied by overt increases ($P<0.05$) in the tissue cyclic AMP content which were maximal within 2 min of the application of the stimulus and returned to near control values within 4 min. On the basis of these studies, submaximal concentrations of the three secretagogues were selected for further studies and determinations of the tissue cyclic AMP content were made 2 min post stimulation.

Denbufylline (10 μM–1 mM) stimulated the ir-ACTH release from static incubates of anterior pituitary segments *in vitro* ($P<0.05$) but was ineffective at lower concentrations (0.1–1 μM, $P>0.05$, Figure 4a–c). However, at all concentrations tested (0.1 μM–1 mM) denbufylline potentiated the corticotrophic responses to a submaximal concentration of HE (0.1 HE ml^{-1}, $P<0.01$; Figure 4a). Denbufylline (1.0 μM and 1.0 mM) also potentiated ($P<0.001$) markedly the increases in ACTH release induced by forskolin (0.1 mM; Figure 4b). However, the significant ($P<0.01$) increases in ACTH release evoked by CRH-41 (10 nM) were unaffected by denbufylline (0.1 μM–1.0 mM, $P>0.05$; Figure 4c).

The profile of the cyclic AMP responses to denbufylline, HE, CRH-41 and forskolin was similar to that observed for ir-ACTH release. Thus, hypothalamic extract (HE; 0.1 HE ml^{-1}; Figure 5a), CRH-41 (10 nM; Figure 5b) and forskolin (0.1 mM; Figure 5c) each caused significant ($P<0.05$) increases in the ir-cyclic AMP content of the tissue. Furthermore, denbufylline (0.1 mM), which alone also produced an increase in ir-cyclic AMP accumulation ($P<0.05$), potentiated the increase in pituitary ir-cyclic AMP content induced by hypothalamic extract ($P<0.01$; Figure 5a) and forskolin ($P<0.01$; Figure 5c) but not by CRH-41 (Figure 5b).

The stable cyclic AMP analogue, 8-bromo-cyclic AMP (1 μM–1 mM), also caused significant ($P<0.05$ vs. basal) increases in ir-ACTH release from rat anterior pituitary tissue *in vitro*. The responses to this secretagogue were unaffected by denbufylline (1 μM or 0.1 mM) which itself produced significant ($P<0.01$) concentration-dependent increases in ir-ACTH release (Figure 6).

Discussion

The results show clearly that denbufylline, like other xanthines such as caffeine and IBMX (Sobel, 1985; Nicholson, 1989), stimulates HPA activity in the rat. They also suggest that the actions of denbufylline are exerted predominantly at the level of the anterior pituitary gland through mechanisms which are dependent, at least in part, on inhibition of PDE-4.

It is evident from our *in vivo* studies that a single intraperitoneal injection of denbufylline produces a profound,

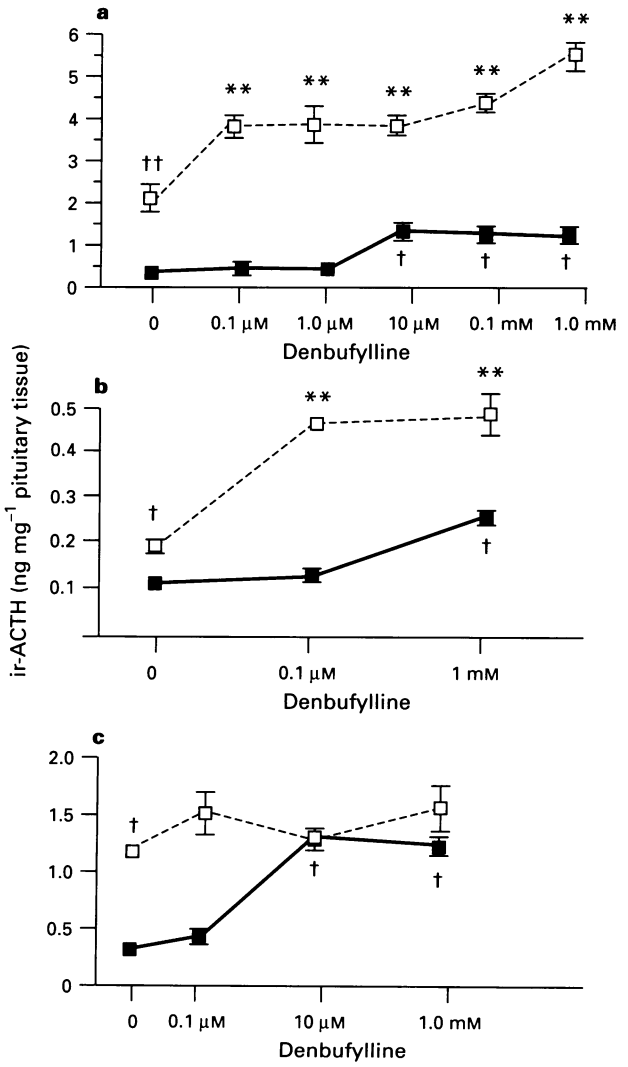

Figure 4 The effects of graded concentrations of denbufylline on the release of ir-ACTH from anterior pituitary segments *in vitro* in the presence and absence of submaximal concentrations of (a) hypothalamic extract (HE 0.1 ml^{-1}), (b) forskolin (0.1 mM) and (c) CRH-41 (10 nM): (■) basal peptide release; (□) secretagogue-stimulated. Each point represents the mean±s.e.mean ($n=6$). †$P<0.05$, ††$P<0.01$ vs. vehicle control; **$P<0.01$ vs. secretagogue alone (ANOVA plus Duncan's test).

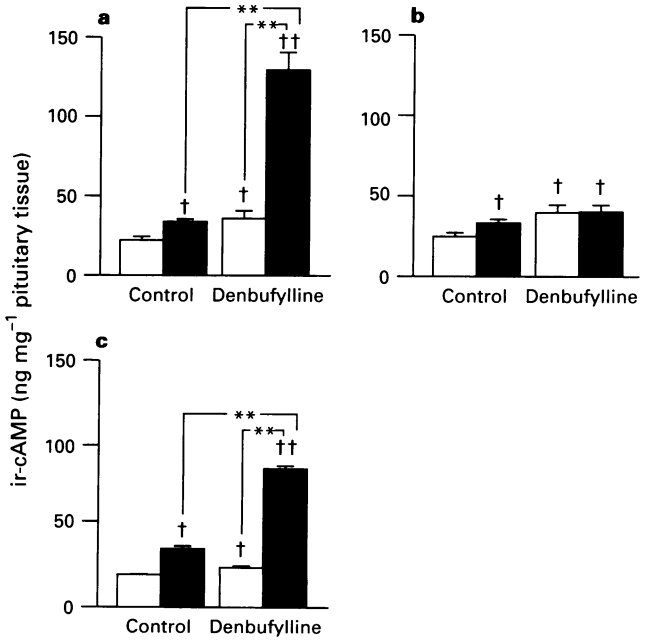

Figure 5 The effects of denbufylline on ir-cyclic AMP accumulation in anterior pituitary segments *in vitro* in the presence (solid columns) and absence (open columns) of (a) hypothalamic extract (HE 0.1 ml^{-1}) (b) CRH-41 (10 mM) and (c) forskolin (0.1 mM). Each column represents the mean±s.e.mean ($n=6$). $P<0.05$, ††$P<0.01$ vs. control; **$P<0.01$ as indicated on the figure (ANOVA plus Duncan's test).

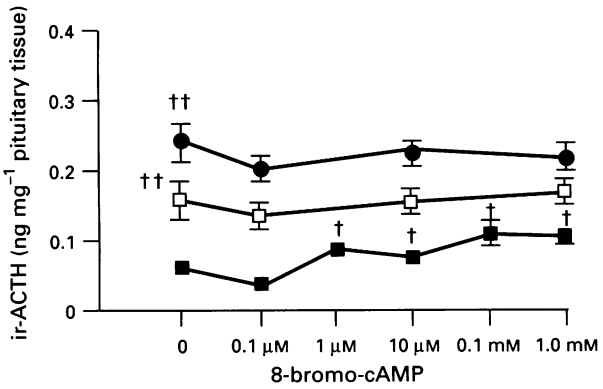

Figure 6 The effects of graded concentrations of 8-bromo-cyclic AMP on the release of ir-ACTH from anterior pituitary segments *in vitro* in the presence and absence of denbufylline: (■) controls; (□)+denbufylline (1 μM); (●) denbufylline (0.1 mM). Each point represents the mean±s.e.mean ($n=6$). †$P<0.05$, ††$P<0.01$ vs. drug free control (ANOVA plus Duncan's test).

dose-dependent increase in the serum corticosterone concentration. It is unlikely that this is due to a direct action of the drug on the adrenal cortex for in a preliminary study we noted that the hypersecretion of corticosterone induced by a single oral injection of denbufylline (24 mg kg^{-1}; serum corticosterone = 195±8 ng ml^{-1} (vehicle controls) vs. 472±9 ng ml^{-1} (denbufylline-treated); $P<0.01$) was preceded by a significant ($P<0.01$) ~5 fold increase in the plasma ACTH concentration (plasma ACTH = 16±3 iu ml^{-1} (vehicle controls) vs. 65±15 iu ml^{-1} (denbufylline treated); furthermore, the pituitary adrenocortical response to denbufylline (24 mg kg^{-1}, orally) was blocked ($P<0.01$) by pretreatment of the rats with dexamethasone (1 mg kg^{-1} day^{-1}, s.c. for 2 days; serum corticosterone = 472±9 ng ml^{-1} (denbufylline alone) vs. <25 ng ml^{-1} (denbufylline + dexamethasone or dexamethasone alone)) which suppresses the secretion of corticosterone *in vivo* through actions at the anterior pituitary gland, hypothalamus and elsewhere in the central nervous system (for review see Buckingham *et al.*, 1992). In principle it could be argued that the overt stimulatory effects of denbufylline and related xanthines on HPA activity reported here and elsewhere (DePasquale *et al.*, 1979; Spindel *et al.*, 1983; Nicholson, 1987; 1989) are merely a consequence of non-specific 'stressful' effects of the drugs. The present data militate against this and point to a primary action of denbufylline at the pituitary level. Thus, although denbufylline alone produced only a small rise in serum corticosterone concentration when injected i.p. into rats in which the endogenous CRF release was blocked pharmacologically (i.e. in rats in which the normal ACTH responses to stress were blocked), its effects were marked when co-administered with physiological ACTH secretagogues (i.e. by restoration of the normal drive to the corticotrophs). Thus, denbufylline readily potentiated the adrenocortical responses of the 'CRF blocked' rats to exogenous hypothalamic extract, a potent cocktail of ACTH releasing factors, while producing effects which were additive with those of CRH-41. Furthermore, *in vitro* denbufylline stimulated the release of ACTH from anterior pituitary tissue and potentiated the increases in ACTH and cyclic AMP accumulation provoked by hypothalamic extracts and forskolin although, paradoxically, it failed to modify those induced by CRH-41. A complementary series of experiments on enzymatically dispersed, perifused cells suspended in Sephadex (procedures which minimize cell-cell interactions) yielded a similar profile of data (Hadley *et al.*, 1990), suggesting that the actions of denbufylline are effected on the corticotrophs. Interestingly, earlier workers have argued that non-selective xanthine PDE inhibitors (e.g. caffeine, IBMX) act principally on the hypothalamus, or possibly elsewhere in the CNS, to provoke the release of CRH-41/AVP (Spindel *et al.*, 1980a, b; 1983; Nicholson, 1989). By contrast, we could find little evidence of an action of denbufylline at the hypothalamic level. Thus, denbufylline had no significant effect on the serum corticosterone concentration when injected intracerebroventricularly into conscious rats. Furthermore, it failed to elicit the release of AVP from isolated hypothalami *in vitro* and increased CRH-41 release only at a very high concentration (1.0 mM). The latter response is unlikely to reflect specific blockade of PDE-4 and its significance is unclear. It may be explained by non-specific inhibition of other PDEs. On the other hand, although hypothalami treated with the high concentration of denbufylline responded to a subsequent depolarizing stimulus (56 mM K^+) with a pronounced increase in CRH-41 release, our finding that the concomitant release of AVP was markedly impaired suggests the functional integrity of the tissue was at least partially disrupted by this drug treatment.

Denbufylline is a potent ($K_i \sim 1$ μM) selective inhibitor of PDE-4 (Nicholson *et al.*, 1989) but, like other xanthines (e.g. caffeine, IBMX) it possesses other pharmacological properties which include significant adenosine receptor blocking activity (K_d at A_1 receptors = 20±5 μM; K_d at A_2 receptors = 46±2 μM; Nicholson *et al.*, 1989). Several lines of evidence suggest the ACTH releasing activity of denbufylline reported here is due primarily, although not necessarily exclusively, to blockade of PDE. Firstly, data from *in vitro* studies using rolipram as a probe advocate a role for PDE-4 in intracellular mechanisms which control ACTH secretion (Hadley *et al.*, 1993; Koch & Lutz-Bucher, 1995). Secondly, our data show clearly that the rises in the resting and pharmacologically-evoked release of ACTH caused by denbufylline are accompanied by parallel increases in the tissue content of cyclic AMP. By contrast, denbufylline failed to augment the increases in ACTH release produced by 8-bromo cyclic AMP, a membrane-permeable cyclic AMP analogue which is resistant to phosphodiesterase action (Chneimeiss *et al.*, 1991). Finally, by analogy with data on other cells/tissues such as neutrophils (Nielson *et al.*, 1986; Keuhl *et al.*, 1987), our demonstration of a pronounced difference in potency between the actions of denbufylline on basal and secretagogue (HE or forskolin) stimulated ACTH *in vitro* is indicative of an inhibitory action on phosphodiesterase. Thus, in resting cells only high concentrations of denbufylline effect the marked inhibition of PDE-4 necessary to raise the low intracellular cyclic AMP concentration to a level sufficient to evoke peptide release. By contrast, in cells in which the cyclic AMP levels are raised by secretagogues, the degree of PDE inhibition produced by a lower drug concentration is sufficient to augment the intracellular concentration of the cyclic nucleotide and thereby potentiate ACTH release. Notwithstanding these arguments, we cannot fully eliminate the possibility that stimulatory actions of denbufylline on ACTH secretion are due in part to blockade of the adenosine receptors identified in the anterior pituitary gland (Anand-Srivastava *et al.*, 1989; Hadley *et al.*, 1990). However, such a mechanism seems unlikely for, while treatment of pituitary segments *in vitro* with adenosine deaminase and/or a variety of selective adenosine receptor ligands modifies ACTH secretion (Kumari *et al.*, 1994a), the responses are very small compared with those of denbufylline.

Our finding that denbufylline failed to modify the corticotrophic responses to CRH-41 *in vivo* and *in vitro* is difficult to explain. The fact that CRH-41 *per se* is a relatively weak secretatogue may be important in this regard for the moderate rise in cyclic AMP accumulation which it causes (Giguère *et al.*, 1982) may, for the reasons rehearsed above, be insufficient for the actions of denbufylline to be expressed. However, in contrast to our data, Koch & Lutz-Bucher (1995) have recently reported that the rises in cyclic AMP accumulation and ACTH release induced by CRH-41 in a mouse corticotroph tumour cell line are potentiated both by rolipram and by the non-selective PDE inhibitor, IBMX. This apparent discrepancy may reflect fundamental differences between the biochemical

mechanisms effecting peptide secretion by the immortalised tumour cells and by the 'normal' corticotrophs present in our preparation. Heterogeneity within the inherent pituitary corticotroph population may also be a further significant factor for, although CRH-41 receptors are found on all corticotrophs, AVP receptors are not (Schwartz *et al.*, 1989; 1990). Thus, the powerful synergistic actions of CRH-41 and AVP on intracellular cyclic AMP accumulation and ACTH release, which are readily mimicked by HE (whose principal active components are AVP and CRH-41) and forskolin (for review see Buckingham *et al.*, 1992), are exerted on a specific subpopulation of corticotrophs. Little is known of the distribution of PDE isozymes within these cells but, since at least four distinct cyclic AMP-PDE genes have been identified in the rat (Repaske *et al.*, 1992) which differ in their sensitivity to hormonal and cyclic AMP stimulation (Conti *et al.*, 1991) and which may thus also differ in their responsiveness to 'selective' inhibitors, it is possible that cell specific expression of the isoforms within the corticotroph population may be a significant factor underlying the differential effects of denbufylline on the corticotrophic responses to HE/forskolin and CRH-41. In this event, the synergistic effects of denbufylline with HE or forskolin may reflect actions of the secretagogues on convergent pathways within target cells (i.e. via PDE and increased adenyl cyclase activity respectively). By contrast, the additive effects of denbufylline and CRH-41 may be indicative of actions of the two drugs on distinct cellular targets and thus on divergent mechanisms leading to ACTH release.

In conclusion, our results show clearly that systemic administration of the xanthine derivative denbufylline causes a marked activation of the HPA axis in the rat. They also provide novel evidence that the actions of denbufylline are exerted predominantly at the level of the anterior pituitary gland through mechanisms which are at least partially dependent on inhibition of PDE-4 and thus provide new insight to the molecular mechanisms controlling ACTH secretion. Further studies involving comparisons of the actions and relative potencies of other selective PDE-4 inhibitors are now necessary to verify this view. Whether denbufylline exerts similar effects in other species is unknown although interestingly when given orally neither denbufylline (Garside & Harvey, 1992) nor caffeine (8 mg kg^{-1}, Spindel & Wurtman, 1984) produce noticeable effects on serum cortisol in man. Nonetheless, our preliminary findings (Kumari *et al.*, 1994b) that two further selective PDE-4 inhibitors, rolipram and 1,3-dicyclopropylmethyl-8-xanthine (BRL 61063), produce overt increases in ACTH and corticosterone secretion in the rat support our premise (Buckingham *et al.*, 1996) that this area warrants careful and thorough investigation.

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