British Journal of Pharmacology (1997) 121, 459–468 © 1997 Stockton Press All rights reserved 0007–1188/97 $12.00

Stimulation of the hypothalamo-pituitary-adrenal axis in the rat by three selective type-4 phosphodiesterase inhibitors: *in vitro* and *in vivo* studies

Meena Kumari, [1]Patricia O. Cover, *Robert H. Poyser & Julia C. Buckingham

Department of Pharmacology, Charing Cross and Westminster Medical School, Fulham Palace Road, London W6 8RF and *SmithKline Beecham Pharmaceuticals, Brockham Park, Surrey RH3 7AJ

1 Previous studies in our laboratory have shown 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 or intraperitoneally (i.p.) to adult male rats. This paper describes the results of experiments in which well established *in vivo* and *in vitro* methods were used to compare the effects of denbufylline on HPA function with those of two other selective PDE-4 inhibitors, rolipram and BRL 61063 (1,3-dicyclopropylmethyl-8-amino-xanthine). For comparison, parallel measurements of the immunoreactive- (ir-) luteinising hormone (LH) were made where appropriate.

2 When injected intraperitoneally, rolipram (40 and 200 $\mu g\ kg^{-1}$, $P<0.005$), denbufylline (0.07–0.6 $\mu g\ kg^{-1}$, $P<0.05$) and BRL 61063 (30 $\mu g\ kg^{-1}$, $P<0.005$) each produced marked rises in the serum ir-corticosterone concentrations. However, lower doses of rolipram (1.6 and 8 $\mu g\ kg^{-1}$) and BRL 61063 (0.25–6 $\mu g\ kg^{-1}$) were without effect ($P>0.05$). By contrast, intracerebroventricular (i.c.v.) injection of rolipram (8 ng–1 $\mu g\ kg^{-1}$) or denbufylline (50 ng–1 $\mu g\ kg^{-1}$) failed to influence the serum ir-corticosterone concentration. BRL 61063 (8–120 $ng\ kg^{-1}$, i.c.v.) was also ineffective in this regard although at a higher dose (1 $\mu g\ kg^{-1}$, i.c.v.) it produced a small but significant ($P<0.05$) increase in ir-corticosterone release. Denbufylline also increased the serum ir-LH concentration when given peripherally (0.2–0.6 $\mu g\ kg^{-1}$, i.p., $P<0.05$) or centrally (100 $ng\ kg^{-1}$, i.c.v., $P<0.05$) but rolipram (1.6–200 $\mu g\ kg^{-1}$, i.p. or 8 ng–1 $\mu g\ kg^{-1}$, i.c.v.) and BRL 61063 (0.25–30 $\mu g\ kg^{-1}$, i.p. or 1 ng–1 $\mu g\ kg^{-1}$, i.c.v.) did not ($P>0.05$).

3 *In vitro* rolipram (10 μM, $P<0.01$), denbufylline (1 mM, $P<0.001$) and BRL 61063 (1 and 10 μM, $P<0.05$) stimulated the release of corticotrophin releasing hormone (ir-CRH-41) but lower concentrations of the drugs were without effect as also was BRL 61063 at 100 μM ($P>0.05$); the rank order of potency was thus BRL 61063 > rolipram > denbufylline. The adenylyl cyclase activator forskolin (100 μM, $P<0.01$) also stimulated the release of ir-CRH-41, producing effects which were additive with those of rolipram and denbufylline but not with those of BRL 61063. The secretory responses to forskolin (100 μM) were accompanied by a highly significant increase in the cyclic AMP content of the hypothalamic tissue ($P<0.005$). Rolipram (10 μM) also significantly ($P<0.05$) elevated the hypothalamic cyclic AMP but denbufylline (10 mM) and BRL 61063 (10 μM) did not. However, all three PDE-inhibitors potentiated the rise in cyclic AMP induced by forskolin ($P<0.05$). None of the drugs tested, alone or in combination, modified the release of arginine vasopressin (ir-AVP) from the hypothalamus.

4 Rolipram (100 μM), denbufylline (100 μM) and BRL 61063 (100 μM) stimulated the release of corticotrophin (ir-ACTH) from pituitary tissue *in vitro* ($P<0.05$) but in lower concentrations they were without significant effect. In addition, rolipram (10 μM, $P<0.05$), denbufylline (0.1 μM, $P<0.05$) and BRL 61063 (10 μM, $P<0.05$) potentiated the significant ($P<0.05$) rises in ir-ACTH secretion induced by forskolin (100 μM). Forskolin (100 μM) also produced a highly significant increase ($P<0.01$) in the tissue cyclic AMP content which was further potentiated by rolipram (10 μM), denbufylline (10 μM) and BRL 61063 (10 μM) which, alone did not affect the cyclic AMP content of the tissue.

5 Since both denbufylline and BRL 61063 possess significant adenosine A_1 receptor blocking activity, further studies examined the potential influence of these receptors on the secretion *in vitro* of CRH-41, AVP and ACTH. The release of ir-CRH-41 was increased significantly by adenosine deaminase (ADA, 5 $u\ ml^{-1}$, $P<0.05$) and the A_1-receptor antagonist, 1,3-dicyclopropyl-8-cyclopentylxanthine (DPCPX, 0.1–10 nM, $P<0.05$). The responses to ADA were abolished by the A_1 receptor agonist N^6-cyclohexyladenosine (CHA, 100 nM, $P<0.05$) which alone had no significant effect on ir-CRH-41 release. ADA (0.1–10 $u\ ml^{-1}$) and DPCPX (1 nM) had weak stimulant and inhibitory effects, respectively, on the release of ir-ACTH from the pituitary gland while CHA (0.1–10 nM) was without effect. Ligand binding studies with [^{3}H]-DPCPX as a probe demonstrated the presence of specific high affinity A_1 binding sites in the hypothalamus ($K_d=0.7$ nM; $B_{max}=367\pm32$ fmol mg^{-1} protein) and in the hippocampus ($K_d=1$ nM; $B_{max}=1165\pm145$ fmol mg^{-1} protein). In both tissues binding of the ligand was displaced by CHA ($IC_{50}=1$ nM (hypothalamus) and 2 nM (hippocampus)), BRL 61063 ($IC_{50}=80$ nM (hypothalamus) and 100 nM (hippocampus)) and denbufylline ($IC_{50}=5$ μM (hypothalamus) and 9 μM (hippocampus)) but not by rolipram.

6 The results suggest that rolipram, denblufylline and BRL 61063 stimulate the HPA axis in the rat, acting at the levels of both the hypothalamus and the pituitary gland. Their actions may be explained, at least in part, by inhibition of PDE-4 but additional actions including blockade of hypothalamic adenosine A_1 receptors by denbufylline and BRL 61063 cannot be excluded.

Keywords: Type 4 phosphodiesterase; HPA axis; ACTH; CRH; hypothalamus; pituitary; rolipram; denbufylline; BRL 61063

[1] Author for correspondence.

Introduction

The cyclic nucleotide adenosine 3′:5′-cyclic monophosphate (cyclic AMP) is known to play a critical role in the intracellular signal transduction mechanisms which control the secretory activity of the hypothalamus, the pituitary gland and the peripheral endocrine organs (Sette *et al*., 1994). The positive influence of the nucleotide on the activity of the various components of the hypothalamo-pituitary-adrenal (HPA) axis is particularly well characterized. Thus, the release of corticosterone from the adrenal gland initiated by corticotrophin (ACTH) is mediated by cyclic AMP (Grahame-Smith *et al*., 1967). Similarly, the ability of the 41 amino acid corticotrophin releasing hormone (CRH-41) to stimulate the synthesis and release of ACTH from the pituitary gland and to induce proliferation of the corticotrophs is dependent on the generation of cyclic AMP (Labrie *et al*., 1982a,b; Aguilera *et al*., 1983). Moreover, although the release of ACTH driven by the second corticotrophin releasing factor, arginine vasopressin (AVP), is effected by receptors coupled to phospholipase C, the powerful synergistic actions of CRH-41 and AVP on the corticotrophs, which are critical to normal HPA function, are dependent on potentiation of the cyclic AMP response to CRH-41 (Giguere & Labrie, 1982). The secretion of CRF-41 and AVP by the hypothalamus is also positively influenced by cyclic AMP (Suda *et al*., 1985; Widmaier *et al*., 1989; Hu *et al*., 1992) and indeed cyclic AMP response elements are contained in the promotor region of the genes which encode these peptides (Seasholtz *et al*., 1988; Emanuel *et al*., 1990). Furthermore, the nucleotide is likely to contribute to transduction in the various ascending and descending pathways which impinge on the hypothalamus to regulate the secretion of the hypophysiotrophic hormones.

The actions of cyclic AMP are terminated by cyclic nucleotide phosphodiesterase (PDE) enzymes which catalyse the intracellular hydrolysis of the nucleotide to 5′-adenosine monophosphate (Butcher & Sutherland, 1962). PDE enzymes thus play a critical role in regulating the cellular responses which are driven by cyclic AMP. It is now well established that the PDEs represent a heterogeneous family of enzymes. To date seven nucleotide phosphodiesterase gene families (PDE-1 – PDE-7) have been identified and gene splicing and post translational modifications provide opportunity for further variants which may be tissue-cell specific (Bolger *et al*., 1993).

Many selective PDE inhibitors have been developed (Beavo, 1988; Beavo & Reifsnyder, 1990; Conti *et al*., 1995); some of these have provided invaluable tools with which to examine the physiological and pathophysiological roles of the various isoforms of the enzyme and thus to identify new therapeutic targets. Particular attention has focused on the type 4, cyclic AMP specific phosphodiesterase (PDE-4). PDE-4 is widely distributed being particularly abundant in the central nervous system, immune/inflammatory cells and the reproductive system. Inhibition of this isozyme produces diverse functional effects including airway smooth muscle relaxation, inhibition of the release of inflammatory mediators, modulation of mood and behaviour and gastric acid secretion (Nicholson *et al*., 1991). Early studies focused on the development of selective PDE-4 inhibitors for the treatment of depression, dementia and cerebral vascular disease, with rolipram, denbufylline and 1,3-dicyclopropylmethyl-8-amino-xanthine (BRL 61063) reaching clinical trial but not proceeding to development. More recently, the therapeutic potential of selective PDE-4 inhibitors in the treatment of certain allergic conditions, notably asthma, has been recognised (Nicholson & Shahid, 1994; Sullivan *et al*., 1994). Much research is now focused in this area, with the aim of maintaining therapeutic usefulness but reducing or eliminating unwanted effects.

In view of the importance of cyclic AMP in the regulation of endocrine function, it may be expected that inhibition of PDE activity may lead to significant hormonal imbalance. In accord with this view, the non-selective PDE inhibitors, caffeine and theobromine, have been shown to have marked effects on neuroendocrine function in the rat and other non-primate species, producing increases in serum corticosterone, while thyrotrophin and growth hormone concentrations are lowered (Spindel *et al*., 1983; Spindel & Wurtman, 1984; Nicholson, 1987; 1989). Similarly, isobutyl-methylxanthine (IBMX) produces significant increases in HPA activity in the rat and, indeed, is used routinely in neuroendocrine studies to augment pituitary hormone release *in vitro*. Surprisingly few published studies have addressed the possible influence of selective PDE inhibitors on neuroendocrine function. However, two recent *in vitro* studies point to a role for PDE-4 in the regulation of ACTH release from the rat anterior pituitary gland (Hadley *et al*., 1993; Koch & Lutz-Bucher, 1995). Furthermore, we have recently shown that oral or intraperitoneal administration of denbufylline (1,3-di-n-butyl-7-(2′-oxopropyl)-xanthine) causes a pronounced increase in HPA activity through actions at the levels of the pituitary gland and, to a lesser extent, the hypothalamus (Hadley *et al*., 1996). In the present study we have exploited a combination of well established *in vivo* and *in vitro* techniques to extend this study by comparing the effects of denbufylline on HPA function in the rat with those of two other selective PDE-4 inhibitors, rolipram and BRL 61063. In addition, since BRL 61063 and denbufylline both possess significant adenosine A_1 receptor blocking activity, we have performed further *in vitro* studies to examine the potential influence of A_1 receptors in the regulation of the release of CRH-41, AVP and ACTH.

Methods

Animals

Adult male Sprague Dawley rats weighing 200 ± 10 g and bred from closed, specific pathogen free colonies at Charing Cross and Westminster Medical School, London (CFY substrain, *in vitro* studies) or Harlan Olac (Oxford, *in vivo* studies) were used. They were housed either in pairs (*in vivo* experiments) or groups of five per cage (*in vitro* experiments) in a room with controlled lighting (lights on 08 h 00 min – 20 h 00 min) and humidity in which the temperature was maintained at 21 – 23°C. Food and water were available *ad libitum*. Rats used for *in vivo* experiments were weighed daily for at least 7 days before the experiment by the individual who undertook the subsequent studies. Experiments were started between 08 h 00 min and 10 h 00 min to avoid changes associated with the circadian rhythm.

In vivo *experiments*

Intraperitoneal administration of drugs Rolipram (8 – 200 μg kg^{-1}), denbufylline (70 – 600 μg kg^{-1}) and BRL 61063 (0.25 – 30 μg kg^{-1}) or a corresponding volume of saline (0.9% sodium chloride solution, 1 ml kg^{-1}) were administered by intraperitoneal injection. The animals were killed by decapitation 20 min (rolipram and denbufylline) or 1 h (BRL 61063) later, the trunk blood was collected into chilled plastic tubes and allowed to stand on ice for 10 – 20 min. The tubes were then centrifuged (2,000 rpm, 4°C 10 min) and the serum separated and stored at −20°C for estimation of corticosterone and luteinising hormone (LH).

Intracerebroventricular (i.c.v.) administration of drugs In a preliminary operation, guide cannulae with stoppers were implanted stereotaxically into the third ventricle of the brain of intact adult rats anaesthetized with a 1:1 mixture (v/v, 2 ml kg^{-1}) of Hypnorm (fentanyl citrate 0.315 mg ml^{-1} and fluanisone 10 mg kg^{-1}, Janssen Pharmaceutical Ltd., Oxford) and diazepam (5 mg kg^{-1}, Phoenix Pharmaceuticals Ltd., Gloucester). The animals were allowed at least 7 days to recover from the trauma of the operation and weighed daily. Any animal which did not gain weight normally or which appeared unhealthy in any way was killed humanely (<5%).

On the day of the experiment the rats were removed from their cages, 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 a drug solution (rolipram: 8 – 1000 ng kg^{-1}, denbufylline: 50 – 1000 ng kg^{-1} and BRL 61063: 1 – 600 ng kg^{-1}) or an equivalent volume of saline (3 μl), was injected. The animals were decapitated 20 min later and trunk blood collected in chilled plastic tubes. The serum was separated and stored as described above. The position of the cannulae was verified *post mortem*.

In vitro *experiments*

Static incubation of anterior pituitary segments The method used was a modification (Taylor *et al*., 1993) of that described by Buckingham and Hodges (1977a). Briefly, rats were decapitated in the room in which they were housed and the anterior pituitary glands were removed immediately. Each gland was cut into four segments of approximately equal size which were then transferred to the wells of modified 24 well culture plates (Tissue Cluster 24, Costar, Cambridge, U.S.A., 1 segment per well) containing 1 ml per well freshly oxygenated Earle's Balanced Salt Solution (EBBS, phenol red free, Sigma Chemical Co. Poole, Dorset, U.K.) enriched with aprotinin (100 kiu ml^{-1}, Bayer, U.K. Ltd., Newbury, Berkshire) and prewarmed to 37°C. The plates were incubated at 37°C in an atomosphere saturated with 95% O_2/5%CO_2 for 2 h 30 min with medium changes at 2 h and 2 h 15 min. The segments were then transferred to fresh medium containing either a secretagogue (forskolin) or its vehicle and incubated for 1 h (for hormone determination) or 2 min (for cyclic AMP measurement). Rolipram, denbufylline and BRL 61063 were added as appropriate after the 2 h preincubation, while adenosine receptor ligands were present if required during the final 1 h incubation only. The medium was collected and either assayed immediately for ir-ACTH and ir-LH or stored in aliquots (300 μl) at −80°C until assay. The pituitary segments were weighed and discarded. For tissue cyclic AMP determination, segments were rapidly frozen on dry ice, thawed and boiled for 10 min in 250 μl EBBS 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 an attempt to reduce inter-animal variation, pituitary segments were randomized so that no one treatment group contained more than one segment from one animal.

Incubation of the hypothalamic tissue The method used was a modification (Loxley *et al*., 1993a,b) of that described by Buckingham & Hodges (1977b) with tissue from rats which had been adrenalectomized 7 – 10 days previously (under sodium pentobarbitone anaesthesia, 60 mg kg^{-1} body weight, i.p., in a volume of 2 ml kg^{-1}, Rhone Poulenc, Dagenham, U.K.), a process which increases the amount of CRH-41 and AVP available for release from the parvocellular neurones of the paraventricular neucleus (Loxley *et al*., 1993a,b). Briefly, the adrenalectomized animals were decapitated; the hypothalamus was dissected out and the peritoneum examined to verify the completeness of adrenalectomy. The hypothalamic tissue taken was bordered rostrally by the anterior border of the optic chiasma, laterally by the hypothalamic fissures and caudally by the mammillary bodies. The ventral border of the preparation was delineated by the median eminence and the dorsal extent of the cut was a depth of 2 mm. The tissue blocks were transferred immediately to tubes containing 1 ml prewarmed (37°C) freshly oxygenated artificial cerebrospinal fluid (Bradbury *et al*., 1974) plus aprotinin (100 kiu ml^{-1}, Bayer U.K. Ltd) and ascorbic acid (0.0175% w/v, Sigma Chemical Co, U.K.) and incubated for 75 min at 37°C in a gently shaking water bath. Throughout this period the tubes were gassed 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, they were exposed to forskolin or, in the case of controls, medium alone, the final 15 min incubation was used to verify the viability of the tissues by exposure to K^+ (56 mM). Rolipram, denbufylline and BRL 61063 were added as appropriate after the initial 30 min incubation and replenished with each medium change; controls received a corresponding volume of medium alone. Adenosine receptor ligands were present if required during the two final 15 min incubations only. The medium from each of the 15 min incubations was collected; 300 ml was aspirated from each and stored (−80°C) for ir-AVP determination. The remainder was either assayed immediately for ir-CRH-41 or freeze dried for subsequent determination of the peptide. In experiments where alterations in tissue cyclic AMP were determined, the final incubation period was reduced to 4 min; the tissue was rapidly frozen, thawed and boiled for 10 min in 500 μl sodium azide buffer (Dupont Chemical Co. Germany) containing 100 kiu ml^{-1} aprotinin and 2 mM theophylline (Sigma Chemical Co. Poole, Dorset). The cyclic AMP content was then determined.

Hormone assays

Corticosterone The concentration of corticosterone in serum samples was measured by radioimmunoassay (Al-Dujaili *et al*., 1981) by use of a well characterized antibody raised in rabbit and ^{125}I-labelled histamine-corticosterone conjugate as a tracer. Separation of bound and free corticosterone was achieved by addition of dextran coated charcoal. The sensitivity of the assay was 5 ng ml^{-1} and the intra- and inter-assay coefficients of variation were both 5%.

Corticotrophin (ACTH) ACTH was measured by radioimmunoassay by use of a method based on that described by Rees *et al*. (1971) and an antibody raised in sheep against $ACTH_{1-24}$. The reference preparation was human $ACTH_{1-39}$ (National Institute for Biological Standards and Control, South Mimms, Herts, U.K.) and the tracer was ^{125}I-labelled ACTH. Separation of bound from free ACTH was achieved by addition of dextran coated charcoal. The sensitivity of the assay was 78 pg ml^{-1} with intra- and inter-assay coefficients of variation of 9.5% and 11.3%, respectively.

Luteinising hormone (LH) LH was determined by radioimmunoassay (Kilpatrick *et al*., 1976) by use of reagents supplied by the National Hormone and Pituitary Programme (Bethesda, MD U.S.A.) and a second antibody (IDS, Washington, U.K.) for the separation of bound and free peptide. The standard preparation was coded NIDDK-LH-rat RP-3 and the tracer was ^{125}I-labelled rat LH. The sensitivity of the assay was 0.13 ng mg^{-1} with intra- and inter-assay coefficients of variation of 9% and 11%, respectively.

Corticotrophin releasing hormone (CRH-41) CRH-41 was measured by radioimmunoassay (Hillhouse & Milton, 1989) with an antibody raised in rabbit against rat/human (r/h) CRH-41 conjugated with β-γ-globulin. The antibody cross-reactivity was 100% with r/h CRH_{1-20}, 35% with r/h CRF_{21-49}, 0.1% with r/h CRF_{1-20}, 0.02% with r/h CRF_{6-33}, 75% with oxidized r/h CRH-41, 9% with ovine CRH-41 and 0.03% with sauvagine. It was found to have no cross reactivity with AVP, somatostatin, luteinising hormone-releasing hormone or thyrotrophin releasing hormone. The reference preparation was r/h CRH-41 (Peninsula Labs. U.K.) and the tracer was ^{125}I-labelled tyrosine r/h CRH-41. Separation of bound and free CRH-41 was achieved by addition of a second antibody (Donkey, anti-rabbit, IDS Ltd.). The sensitivity of the assay was 10 pg ml^{-1} with intra- and inter-assay coefficients of variation were 5% and 9%, respectively.

AVP The AVP assay employed an antibody of defined specificity (Negro-Vilar *et al*., 1979) raised in rabbit against AVP.

The reference preparation was synthetic AVP (National Institute for Biological Standards and Control, South Mimms, Herts, U.K.) and the tracer was [^{125}I]-AVP (Amersham International plc, Amersham, U.K.). The sensitivity of the assay was 2 pg ml^{-1} and the intra- and inter-assay coefficients of variation were 3.6% and 11.9%, respectively.

Tissue cyclic AMP measurement Tissue cyclic AMP was measured by radioimmunoassay with a kit purchased from Dupont International (Stevenage, Herts, U.K.). The sensitivity of the assay was 1 pmol ml^{-1} and the antibody supplied showed negligible cross-reactivity with cyclic GMP, adenosine, guanosine or theophylline. Intra- and inter-assay coefficients of variation were 6% and 9%, respectively.

For all assays the dilution curves of the samples were parallel with those of the standard curve. Samples from a single experiment were always run in the same assay in order to avoid the inter-assay variation inherent to these methods. Specificity studies showed that none of the drugs employed interfered with any of the above assay methods.

A_1 receptor binding studies

Tissue preparation Hypothalami and hippocampi were rapidly removed *post mortem* from rats adrenalectomized 7–10 days previously and placed immediately in Tris-HCl (BDH Chemicals Ltd., Dagenham, U.K., 50 mM, 4°C). After sonification, the homogenates were centrifuged (3,000 *g*, 20 min, 4°C), the pellet was retained and the supernatant fluid was further centrifuged (50,000 *g*, 20 min, 4°C). The resultant pellets were pooled and resuspended (2 hypothalami ml^{-1} and 4 hippocampi ml^{-1}) in 50 mM Tris-HCl containing 5 iu ml^{-1} adenosine deaminase (Sigma Chemicals Co., Poole, U.K.) and incubated at room temperature for 30 min. The protein content of the homogenate was determined (Lowry *et al*., 1951) and the receptor binding experiments were carried out immediately.

Receptor binding experiment Solutions of [^{3}H]-1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]-DPCPX, 0.05–10 nM) were prepared by dilution of stock solution (88.2 Ci mmol^{-1} stored in ethanol) in Tris-HCl and aliquoted (25 μl) into two triplicate sets of assay tubes together with 200 μl protein extract. Aliquots of buffer (25 μl) were added to the first set of tubes (total binding) and DPCPX (25 μl, 100 nM) to the second set (non-specific binding). The tubes were vortexed and incubated for 1 h at 25°C with continuous shaking. The reaction was terminated by filtration with a Brandel cell harvester (Semat Technical UK Ltd., St. Albans, U.K.) with ice-cold Tris-HCl and 3 further washes with ice-cold Tris HCl. The filter pads were placed in scintillation tubes containing 0.5 ml Triton X100 (BDH Chemicals Ltd., Dagenham, U.K.) in toluene (BDH Chemicals Ltd., Dagenham, U.K.) and 45 ml scintillation fluid (Solvene E). After incubation overnight at room temperature in the dark, the tubes were counted (4 min) on a β-automatic counter (LKB, Turku, Finland).

Displacement studies Solutions of CHA (20 pM–100 nM), rolipram (20 nM–100 μM), denbufylline (20 nM–100 μM) and BRL 61063 (1 nM–5 μM) were prepared by dilution of stock solutions (1 mM in ethanol) in Tris-HCl and aliquoted (25 μl) into triplicate sets of tubes together with 25 μl [^{3}H]-DPCPX (final concentration 1 nM) and 200 μl protein extract. Further triplicate tubes containing either 25 μl [^{3}H]-DPCPX/25 μl CHA (final concentration 100 nM)/200 μl protein extract or 25 μl [^{3}H]-DPCPX/25 μl buffer/200 μl protein extract were set up to determine specific and non-specific binding, respectively. The tubes were then vortexed, incubated and counted exactly as described above.

Drugs

BRL 61063 (1,3-dicyclopropylmethyl-8-amino-xanthine) and denbufylline were obtained from SmithKline Beecham Pharmaceuticals (Harlow, Essex, U.K.), rolipram was a generous gift from Schering AG (Berlin, Germany) and forskolin was purchased from Sigma Chemical Co. (Poole, U.K.). They were dissolved in either sterile saline (0.9% sodium chloride solution, Charing Cross Hospital Pharmacy) for *in vivo* experiments or initially in ethanol with subsequent dilutions in incubation medium for *in vitro* experiments. In all cases appropriate vehicle controls were prepared and used. Previous experiments have shown that, when given peripherally, the onset of action of BRL 61063 is delayed in comparison with rolipram or denbufylline. Measurements were therefore made 1 h after i.p. injection of BRL 61063 but 20 min after rolipram and denbufylline. Adenosine deaminase, DPCPX and CHA were purchased from Sigma Chemical Co. (Poole, U.K.) and dissolved and diluted in medium before use. [^{3}H]-DPCPX (88.2 Ci mmol^{-1} stored in ethanol) was purchased from Dupont Ltd. (Stevenage, Herts, U.K.).

Statistical analysis

The results, which were normally distributed, were analysed by one way analysis of variance followed by Fischer's test of least difference. In those instances where only two groups were compared Student's *t* test was used. Data were considered to be significantly different if $P<0.05$. In view of the variation between experiments in basal peptide release which is inherent to our *in vitro* preparations, statistical comparisons were made only within experiments. In all instances, repeat experiments produced data which were similar qualitatively.

Results

Figure 1 demonstrates the effects of single intraperitoneal injections of rolipram (1.6–200 μg kg^{-1}), denbufylline (0.07–0.6 μg kg^{-1}) and BRL 61063 (0.25–30 μg kg^{-1}) on the serum ir-corticosterone and ir-LH concentrations in adult male rats. Both rolipram (8–200 μg kg^{-1}, i.p., Figure 1a) and denbufylline (0.07–0.6 μg kg^{-1}, i.p., Figure 1c) produced significant ($P<0.005$) dose-dependent increases in serum ir-corticosterone. At the highest dose tested BRL 61063 (30 μg kg^{-1}, i.p., Figure 1e) also stimulated the release of ir-corticosterone ($P<0.005$) but the lower doses tested were without effect. Denbufylline (0.2 and 0.6 μg kg^{-1}, i.p., Figure 1d) also produced a small but significant rise in serum ir-LH ($P<0.05$) but no such response was apparent in rats treated with rolipram (1.6–200 μg kg^{-1}, i.p., Figure 1b) or BRL 61063 (0.25–30 μg kg^{-1}, i.p., Figure 1f). When given centrally, the saline vehicle produced a significant ($P<0.01$) rise in serum ir-corticosterone versus values in untreated controls but neither rolipram (8–1000 ng kg^{-1}, i.c.v.) nor denbufylline (50–1000 ng kg^{-1}, i.c.v.) produced any further change in steroid release; BRL 61063 (1–120 ng kg^{-1}, i.c.v.) was also ineffective in this regard although at a higher dose (1000 ng kg^{-1}, i.c.v.) a significant ($P<0.005$) rise in serum ir-corticosterone was apparent (data not shown). The ir-LH serum concentrations were unaffected by central injections of rolipram (8–1000 ng kg^{-1}, i.c.v.) or BRL 61063 (1–1000 ng kg^{-1}, i.c.v.) but were increased ($P<0.05$ vs. vehicle control) in rats treated with denbufylline (100–1000 ng kg^{-1}, i.c.v., data not shown).

Preliminary *in vitro* studies examined the effects of graded concentrations of rolipram (0.1–100 μM), denbufylline (10–1000 μM) and BRL 61063 (1–100 μM) on the release of ir-CRH-41 and ir-AVP from rat isolated hypothalami. None of these drugs influenced the release of ir-AVP (Table 1) although subsequent exposure of the tissue to K^+ (56 mM) precipitated a highly significant ($P<0.01$), approximately 3 fold increase in the release of this peptide (data not shown) analogous to that obtained previously (Loxley *et al*., 1993a; Hadley *et al*., 1996). By contrast, all three drugs stimulated the release of CRH-41; BRL 61063 was the most potent in this regard producing significant ($P<0.01$) increases in peptide release at concentrations

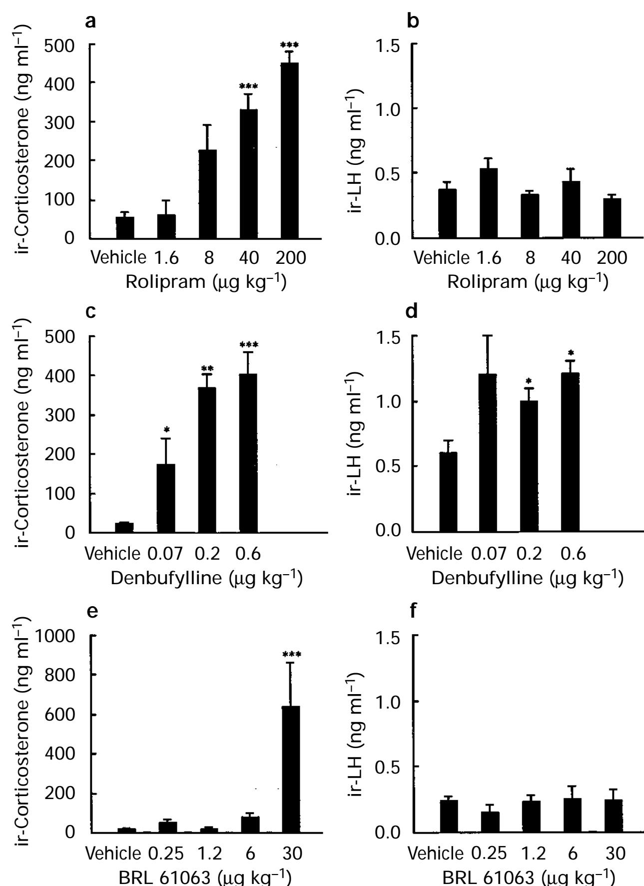

Figure 1 Effects of a single intraperitoneal injection of rolipram (1.6–200 μg kg^{-1}, a and b), denbufylline (0.07–0.6 μg kg^{-1}, c and d) and BRL 61063 (0.25–30 μg kg^{-1}, e and f) on the serum concentrations of ir-corticosterone (a, c and e) and ir-LH (b, d and f) in adult male rats. Controls received an equal volume of the vehicle (sterile saline, 2 ml kg^{-1}, i.p.). Values represent the mean±s.e.mean (n=5–6). *$P<0.05$, **$P<0.01$, ***$P<0.005$ vs corresponding vehicle control. (ANOVA+Fischer's test). N.B. the experiments for each drug were performed on different days.

Table 1 Effects of graded concentrations of rolipram (0.1–100 μM), denbufylline (10–1000 μM) and BRL 61063 (1–100 μM) on the release *in vitro* of ir-CRH-41 and ir-AVP from isolated hypothalami

Drug (μM)	*ir-CRH* (pg ml^{-1})	*ir-AVP* (pg ml^{-1})
Rolipram		
Vehicle	31±5	50.0±10.7
0.1	36±5	48.6±10.0
1.0	27±6	37.1±14.3
10.0	52±7*	61.4±8.6
100.0	47±6	27.9±5.7
Denbufylline		
Vehicle	6±1.0	62.7±11.4
0.1	–	74.7±10.7
10.0	15±5.5	-
100.0	9±1.0	68.7±10.7
1000.0	34±4.0*	67.3±12.9
BRL 61063		
Vehicle	14±0.1	53.5±17.5
1.0	40±5.0**	41.3±3.8
10.0	62±37.0**	62.5±27.5
100.0	17±2.0	85.0±7.5

Values represent the mean±s.e.mean (n=5–6). *$P<0.05$, **$P<0.01$ (ANOVA and Fischer's test).

of 1 and 10 μM, while rolipram and denbufylline were effective only when their concentrations were raised to 10 μM and 1 mM, respectively ($P<0.05$, Table 1). Subsequent stimulation of the tissue with K^+ (56 mM) produced the anticipated (Loxley *et al*., 1993a; Hadley *et al*., 1996) 2–3 fold increase in CRH-41 irrespective of the previous drug treatments (data not shown). On the basis of these studies the following drug con-

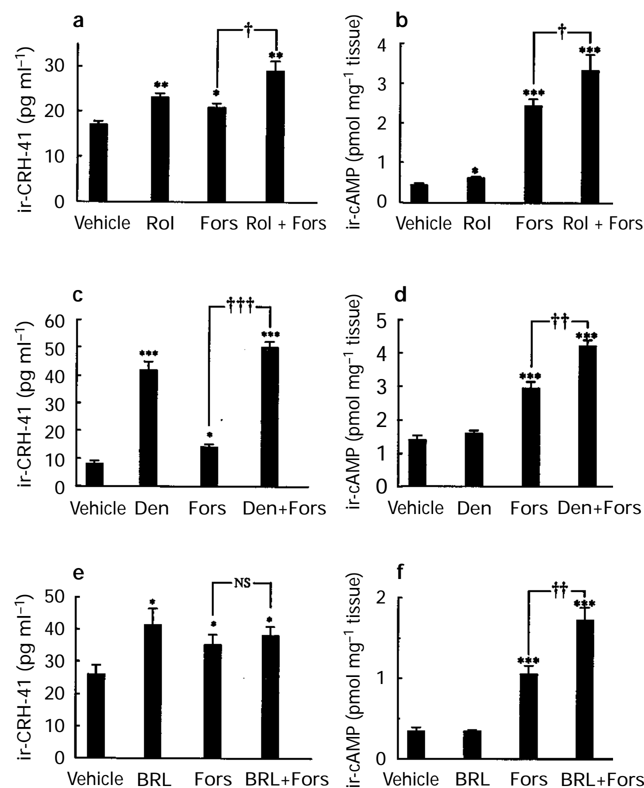

Figure 2 Effects of rolipram (Rol, 10 μM, a and b), denbufylline (Den, 1 mM, c and d) and BRL 61063 (BRL, 10 μM, e and f) in the presence and absence of forskolin (Fors, 100 μM) on the release of ir-CRH-41 (a, c and e) from isolated hypothalami *in vitro* and on the cyclic AMP content (b,d and f) of the tissue. Values represent the mean±s.e.mean (n=5–6). *$P<0.05$, **$P<0.01$, ***$P<0.005$ vs vehicle control. †$P<0.05$, ††$P<0.01$, †††$P<0.005$, forskolin vs forskolin+drug (ANOVA+Fischer's test). NS=not significant. N.B. the experiments for each drug were performed on different days.

Table 2 Effects of graded concentrations of rolipram, denbufylline and BRL 61063 on the release *in vitro* of ir-ACTH from anterior pituitary tissue

Drug (μM)	*ir-ACTH* (ng ml^{-1} pituitary tissue)
Rolipram	
Vehicle	0.32±0.06
1.0	0.35±0.10
10.0	0.41±0.09
100.0	0.52±0.08*
Denbufylline	
Vehicle	0.31±0.07
1.0	0.36±0.01
10.0	0.29±0.005
100.0	0.47±0.02*
BRL 61063	
Vehicle	0.32±0.06
1.0	0.49±0.12
10.0	0.37±0.09
100.0	0.49±0.06*

Values represent the mean±s.e.mean (n=4–6). *$P<0.05$ vs control (ANOVA and Fischer's test).

centrations were selected for further study; rolipram (10 μM), denbufylline (1 mM) and BRL 61063 (10 μM). Figure 2 illustrates the effects of these drugs alone and in combination with forskolin on the release of ir-CRH from the hypothalamus *in vitro* and on the cyclic AMP content of the tissue. The submaximal concentration of forskolin employed (100 μM) produced a small but significant increase in CRH-41 release ($P<0.05$, Figure 2a, c and e) and in the tissue cyclic AMP content ($P<0.005$, Figure 2b, d and f). Rolipram (10 μM, $P<0.01$, Figure 2a), denbufylline (1 mM, $P<0.001$, Figure 2c) and BRL 61063 (10 μM, $P<0.05$, Figure 2e) also increased ir-CRH-41 release; the responses to rolipram and denbufylline were additive with those of forskolin but those to BRL 61063 were not. Neither denbufylline (1 mM, $P>0.05$, Figure 2d) nor BRL 61063 (10 μM, $P>0.05$, Figure 2f) affected the tissue cyclic AMP content although rolipram (10 μM) produced a small but significant increase ($P<0.05$, Figure 2b). However, all three drugs potentiated the rise in cyclic AMP induced by forskolin (rolipram, $P<0.05$, Figure 2b; denbufylline, $P<0.01$, Figure 2d; BRL 61063, $P<0.01$, Figure 2f).

Concentration-response studies showed that rolipram (100 μM), denbufylline (100 μM) and BRL 61063 (100 μM) also produced small ($\sim 50\%$ above basal) but significant ($P<0.05$) increases in the release of ir-ACTH from pituitary tissue *in vitro* but that lower concentrations of the drugs were without effect (Table 2). By contrast, a submaximal concentration of forskolin (100 μM, Taylor *et al.*, 1993) caused a marked ($P<0.05$) increase in ir-ACTH secretion (Figure 3a, c and e). The responses to forskolin were potentiated by rolipram (10 μM, $P<0.05$, Figure 3a), denbufylline (0.1 μM, $P<0.01$, Figure 3c) and BRL 61063 (10 μM, $P<0.05$, Figure 3e). Forskolin (100 μM) also produced a marked rise in the tissue cyclic AMP content ($P<0.01$, Figure 3b, d and f). The cyclic AMP responses were further potentiated by rolipram (10 μM, $P<0.001$, Figure 3b), denbufylline (10 μM, $P<0.01$, Figure 3d) and BRL 61063 (10 μM, $P<0.001$, Figure 3f) each of which, alone, had no discernable effect on the cyclic nucleotide.

Figure 4 shows effects of adenosine deaminase (ADA), DPCPX (an A_1 receptor antagonist) and N^6-cyclo-hexyladenosine (CHA, an A_1 receptor agonist) on the secretion of

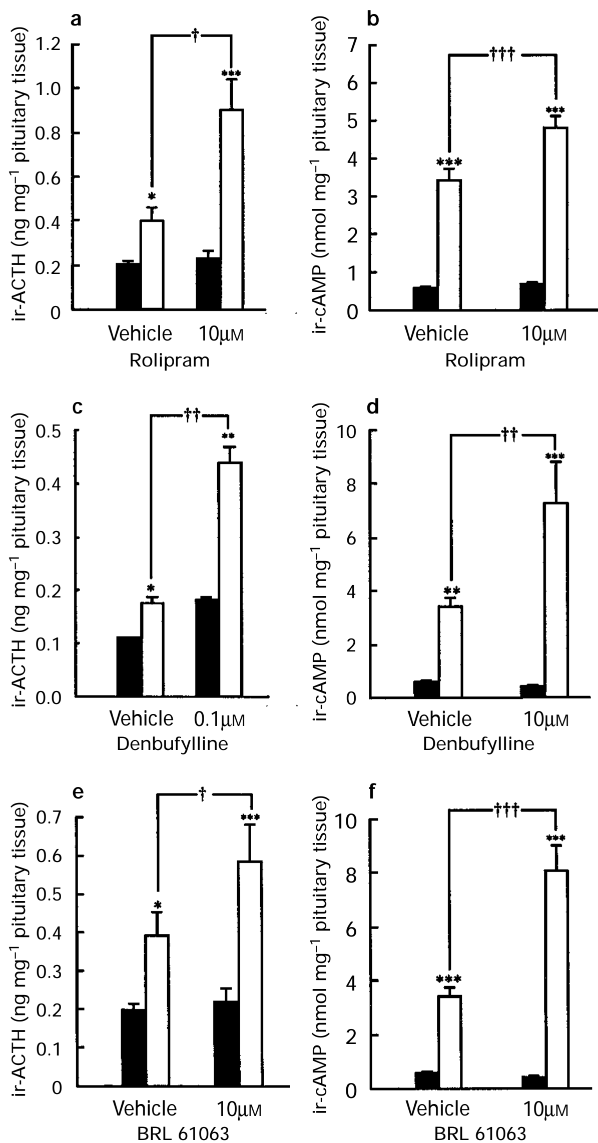

Figure 3 Effects of rolipram (10 μM, a and b), denbufylline (0.1 μM, c; 10 μM, d) and BRL 61063 (10 μM, e and f) in the presence (□) and absence (■) of forskolin (100 μM) on the release of ir-ACTH (a, c and e) from anterior pituitary tissue *in vitro* and on the cyclic AMP content of the tissue (b, d and f). Values represent the mean ± s.e.mean ($n=5-6$). *$P<0.05$, **$P<0.01$, ***$P<0.005$ vs vehicle control. †$P<0.05$, ††$P<0.01$, †††$P<0.005$ forskolin vs forskolin + drug (ANOVA + Fischer's test).

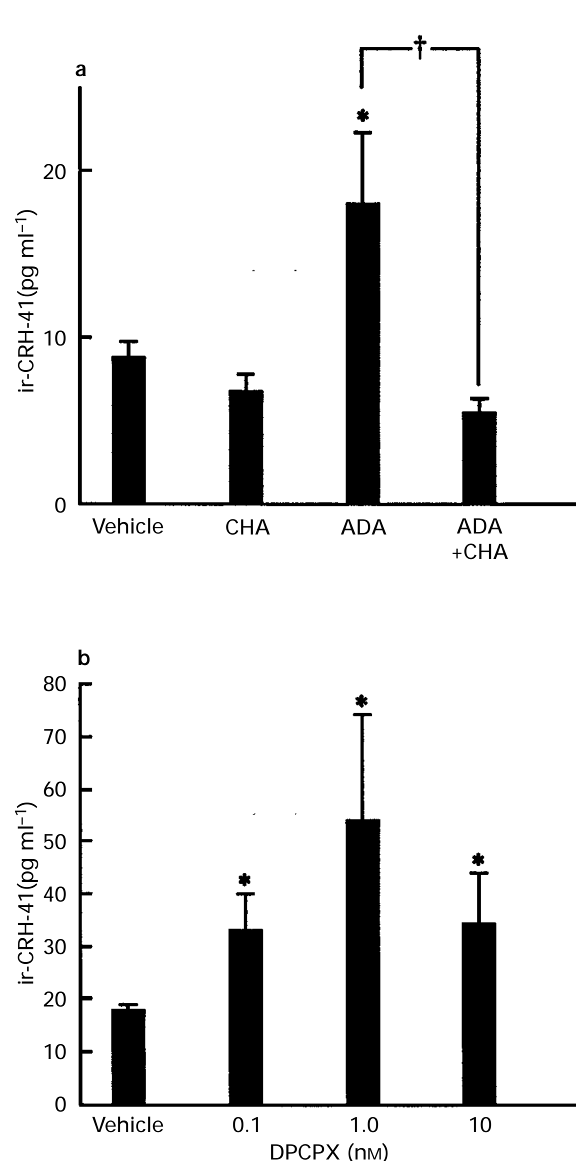

Figure 4 Effects of (a) adenosine deaminase (ADA, 5 u ml^{-1}) and N^6-cyclohexyladenosine (CHA, 0.1 μM) and (b) 1,3 dicyclopropyl-8-cyclopentylxanthine (DPCPX) on the release *in vitro* of corticotrophin releasing hormone (CRH-41) from isolated hypothalami. Values represent the mean ± s.e.mean ($n=5-6$), *$P<0.05$, (a) ADA vs ADA + CHA (†$P<0.05$ Student's *t* test). (b) *$P<0.05$, vs vehicle control (ANOVA + Fischer's test).

ir-CRH-41 by the hypothalamus *in vitro*. ADA (5 u ml^{-1}) caused a significant ($P<0.05$) increase in ir-CRH-41 release; its effects were abolished by CHA (10 μM, $P<0.05$) which alone had no significant effect on ir-CRH-41 release (Figure 4a). DPCPX (0.1 – 10 nM) also stimulated the release of ir-CRH-41 ($P<0.05$) although at the highest concentration tested its effects were reduced (Figure 4b). Parallel measurements showed that the release of ir-AVP from the hypothalamus was unaffected by ADA and the two adenosine receptor ligands (data not shown). DPCPX (1 μM) and ADA (0.1 – 10 u ml^{-1}) had weak inhibitory and stimulant effects, respectively, on the release *in vitro* of ir-ACTH from the pituitary gland while CHA (0.1 – 10 nM) was without effect (data not shown). Ligand binding studies with [^{3}H]-DPCPX as a probe demonstrated the presence of specific high affinity A_1 binding sites in the hypothalamus ($K_d=0.7$ nM; $B_{max}=367\pm32$ fmol mg^{-1} protein and in the hippocampus ($K_d=1$ nM; $B_{max}=1165\pm145$ fmol mg^{-1} protein). Binding of the ligand in both the hypothalamus (Figure 5a) and the hippocampus (Figure 5b) was displaced by CHA ($IC_{50}=1$ nM (hypothalamus) and 2 nM (hippocampus)), BRL 61063 ($IC_{50}=80$ nM (hypothalamus) and 100 nM (hippocampus)) and denbufylline ($IC_{50}=5$ μM (hypothalamus) and 9 μM (hippocampus)) but not by rolipram.

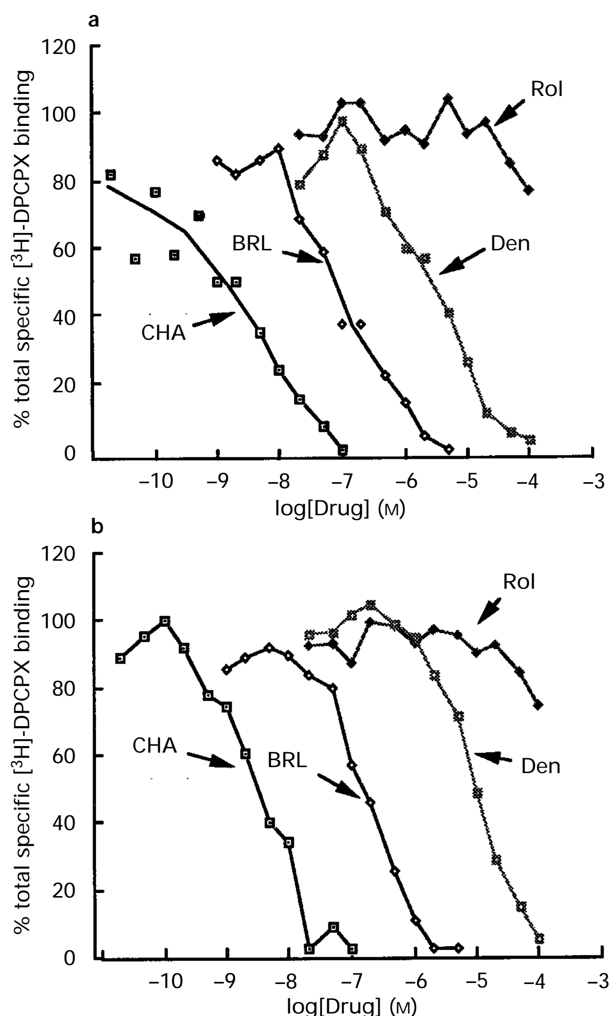

Figure 5 Displacement of specific [^{3}H]-1,3 dicyclopropyl-8-cyclopentyl-xanthine ([^{3}H]-DPCPX) binding in membrane preparations from (a) hypothalamus and (b) hippocampus by N^6-cyclohexyladenosine (CHA), rolipram (Rol), denbufylline (Den) and BRL 61063 (BRL). Values represent the mean ± s.e.mean ($n=3$). A concentration of 1 nM [^{3}H]-DPCPX was used. The calculated IC_{50}s were denbufylline = 5 μM (hypothalamus) and 9 μM (hippocampus), BRL 61063 = 80 nM (hypothalamus) and 100 nM (hippocampus).

Discussion

The results presented confirm our previous observations that acute administration of denbufylline causes a marked activation of the HPA axis in the rat (Hadley *et al*., 1996). They also show for the first time that two other PDE-4 inhibitors, rolipram and BRL 61063, are effective in this regard through mechanisms which involve actions at the levels of both the hypothalamus and the anterior pituitary gland.

It is evident from our *in vivo* study that peripheral administration of rolipram, denbufylline or BRL 61063 produces a pronounced increase in serum corticosterone. It is unlikely that this reflects direct actions of the drugs on the adrenal cortex since this tissue expresses only very small amounts of PDE-4 (Beavo, 1988). Moreover, we have previously shown that the adrenocortical responses to denbufylline are preceded by a hypersecretion of ACTH (Hadley *et al*., 1996) as also are those to rolipram (vehicle (1 ml kg^{-1}) = 2.9 ± 0.3 μiu ml^{-1}; rolipram (40 μg kg^{-1}) = 5.3 ± 0.7 μiu ml^{-1}, $P<0.01$, rolipram (200 μg kg^{-1}) = 5.0 ± 0.3 μiu ml^{-1}, $P<0.01$, $n=5-6$). Furthermore, the stimulating effects of denbufylline on corticosterone secretion are abolished by pretreatment of the animals with dexamethasone, a steroid which suppresses the centrally mediated HPA response to stressful stimuli but fails to modify the adrenal response to exogenous ACTH (Hadley *et al*., 1996). In principle, it could be argued that the hypersecretion of corticosterone induced by the three PDE-4 inhibitors used in the present study is merely a consequence of non-specific ‘stressful’ actions of the drugs. While we cannot exclude the possibility that such actions contribute to the overall response, two important lines of evidence argue against this being the primary mechanism of HPA activation. First, our *in vitro* studies showed clearly that all three enzyme inhibitors exerted significant direct actions on both the hypothalamus and the pituitary gland which augmented the secretion CRH-41 and ACTH. Secondly, stress normally causes a fall in serum LH (Euker *et al*., 1975; Du Ruisseau *et al*., 1979; Cover *et al*., 1991) but denbufylline stimulated the secretion of the gonadotrophin while rolipram and BRL 61063 were without effect.

Previous workers have advocated that the stimulant actions of non-selective xanthine PDE inhibitors (e.g. caffeine, IBMX) on HPA function *in vivo* are exerted principally at the hypothalamus or possibly elsewhere in the CNS (Spindel *et al*., 1980a,b; 1983; Nicholson, 1989). In contrast, we have argued on two bases that the adrenocortical responses to denbufylline are affected primarily at the pituitary level. First, *in vitro* studies have shown that denbufylline stimulates the release of ir-ACTH and potentiates the secretory responses to other ACTH-secretagogues which act via cyclic AMP-dependent mechanisms (Hadley *et al*., 1993; 1996); by contrast, this xanthine has relatively low efficacy at the hypothalamic level as a CRH-41 secretagogue (Hadley *et al*., 1996). Secondly, although the adenocortical responses to denbufylline *in vivo* are ablated by pharmacological lesions of the hypothalamus, they are largely reinstated when the tonic drive to the corticotrophs is restored by administration of corticotrophin releasing factors (Hadley *et al*., 1996). Our finding that central injections of rolipram or denbufylline fail to raise the serum corticosterone and that BRL 61063 is effective in this regard only at a high dose level, also militate strongly against a principal action of the drugs in the CNS as too do our observations that all three drugs augment the release of ACTH from the pituitary gland *in vitro*. However, an action at the hypothalamic level cannot be excluded entirely on these bases for the significant rise in serum ir-corticosterone induced by the i.c.v. injection of the saline vehicle may have masked the effects of the drugs. Moreover, our *in vitro* data demonstrated clear positive effects of the three PDE-4 inhibitors on the secretion of ir-CRH-41 by the hypothalamus, although it may be argued these responses could reflect actions on the terminals of the CRH-41 neurones which lie outside the blood brain barrier and, thus, may not be accessible to drugs administered centrally *in vivo*. Such an argument is indeed tenable for, if PDE-4 plays a critical role in

the regulation of cyclic AMP-dependent CRH-41 release, it is likely to be localized in the nerve terminals so as to effect local control of cyclic AMP turnover. Equally, adenosine A_1 receptors, which provide further targets for denbufylline and BRL 61063 in the hypothalamus (see below), are likely to be located on nerve terminals.

Our findings that rolipram, denbufylline and BRL 61063 stimulate the release of ir-ACTH from the pituitary gland *in vitro* confirm and extend previous studies with rolipram (Hadley *et al*., 1993; Koch & Lutz-Bucher, 1995) and denbufylline (Hadley *et al*., 1996). Several lines of evidence lead us to believe that these effects are due primarily, although not necessarily exclusively, to blockade of PDE-4. Firstly, although alone the three drugs produced at best only modest effects on basal ir-ACTH release and tissue cyclic AMP content, they each potentiated markedly the rises in ir-ACTH release and cyclic AMP accumulation induced by forskolin. These findings accord with views from studies on neutrophils (Nielson *et al*., 1986; Keuhl *et al*., 1987) and pituitary tissue (Hadley *et al*., 1996) that the effects of PDE-4 inhibitors are more pronounced in cells in which the cyclic AMP concentration is raised by pharmacological stimulation than in resting cells. Thus, in resting pituitary cells where cyclic AMP turnover is low, blockade of PDE has little effect on nucleotide levels, and hence on ir-ACTH release, due to low substrate availability. However, in cells in which cyclic AMP generation is raised by forskolin, PDE blockade greatly augments the intracellular content of the nucleotide and thus potentiates ir-ACTH release. Secondly, in accord with their equipotency as PDE-4 inhibitors ($K_i \sim 1$ μM, Beavo, 1988; Wilke *et al*., 1989; Buckle *et al*., 1994), rolipram, denbufylline and BRL 61063 appeared to be equipotent in our *in vitro* system in respect of both basal ir-ACTH release and pituitary cyclic AMP accumulation. Moreover, although BRL 61063 and denbufylline, like other xanthines such as caffeine and IBMX, possess other pharmacological properties which include significant adenosine receptor blocking activity (Nicholson *et al*., 1989; Buckle *et al*., 1994), rolipram is more specific in its actions, although it may exert some effects via a distinct 'rolipram binding site' (Schmiechen *et al*., 1990). It thus seems unlikely that blockade of the adenosine receptors present in the anterior pituitary gland (Anand-Srivastava *et al*., 1989; Hadley *et al*., 1990) contributes to the stimulant actions of BRL 61063 and denbyfylline on ir-ACTH release. Such a view is further supported by our finding that treatment of the pituitary segments with adenosine deaminase and/or various selective adenosine receptor ligands had only minimal effects on ir-ACTH release, suggesting that adenosine is not an important factor in the regulation of the secretion of this pituitary hormone.

The mechanisms underlying the stimulating effects of rolipram, denbufylline and BRL 61063 on the secretion *in vitro* of ir-CRH-41 from the hypothalamus are more difficult to discern. The finding that all three compounds potentiated the rise in intracellular cyclic AMP accumulation induced by forskolin suggests PDE-4 is present in the tissue preparation. However, while the increases in CRH-41 release induced by rolipram in the presence and absence of forskolin favour a PDE-4-dependent action, the observation that BRL 61063 is more potent in eliciting basal CRH-41 release raises the possibility that the actions of the xanthines may involve blockade of adenosine receptors. Surprisingly, there is a paucity of data in the literature regarding the role of adenosine in the regulation of the release of CRH-41 and AVP from the hypothalamus. The present study demonstrated the presence of a small population (vs hippocampus) of specific high affinity adenosine A_1 binding sites in the hypothalamus with a K_d for DPCPX similar to that obtained here and by others in the hippocampus and other CNS tissues (Ebersolt *et al*., 1981; Lee & Reddington, 1986; Fastbom *et al*., 1987; Weber *et al*., 1988; Bisserbe *et al*., 1992). Moreover, BRL 61063 and denbufylline bound readily to both the hypothalamic and the hippocampal receptors with IC_{50} values close to those obtained in other tissues (Nicholson *et al*., 1989; Buckle *et al*., 1994). Our functional studies which showed that adenosine deaminase produces an increase in CRH-41 release *in vitro* which is overcome by the A_1-selective agonist, CHA, provide novel evidence that the hypothalamic A_1 receptors exert a tonic inhibitory influence on CRH-41 release, as also does our finding that A_1 receptor blockade stimulates the release of this neuropeptide. While much remains to be learnt about the role of adenosine in the hypothalamus, these data are consistent with the hypothesis that the stimulant actions of BRL 61063 and denbufylline on CRH-41 release are effected in part by blockade of A_1 receptors.

Although the secretion of ir-CRH-41 *in vitro* was influenced readily by PDE-4 inhibitors (rolipram, denbufylline and BRL 61063) and adenosine receptor ligands, parallel changes in ir-AVP release were not observed. This perhaps is surprising, since CRH-41 and AVP are co-released from the parvocellular neurones of the parventricular-median eminence (PV-ME) tract which control the release of ACTH (Plotsky *et al*., 1985; Sawchenko, 1987). However, although adrenalectomy augments the amount of AVP (and CRH-41) available for release from these neurones, the bulk of AVP in the hypothalamus is derived from the magnocellular neurones which project to the posterior pituitary gland while a cohort of neurones originating in the suprachiasmatic nucleus provides a further source (Loxley *et al*., 1993b). Thus, small drug-induced changes in ir-AVP release from CRH-41 neurones of the PV-ME tract may go undetected. Indeed, had any overt changes in ir-AVP release been observed in this preparation they would have been likely to reflect changes in the activity of the magnocellular system (Loxley *et al*., 1993a,b; Taylor *et al*., 1995).

The effects of rolipram, denbufylline and BRL 61063 on LH release were less marked and more variable than those on the HPA axis. Indeed, only denbufylline produced a significant increase in LH release *in vivo* when given either peripherally or centrally. The failure of rolipram to increase ir-LH release suggests that mechanisms other than PDE-4 inhibition may mediate the increases in LH release produced by denbufylline *in vivo*. An obvious possibility is blockade of adenosine A_1 receptors, although the apparent failure of an adenosine A_1 receptor agonist to influence serum ir-LH when injected into the lateral ventricle (Ondo *et al*., 1989) does not accord with this view; nor does the inability of BRL 61063 to augment the secretion of the gonadotrophin.

In conclusion, the results show clearly that the PDE-4 inhibitors rolipram, denbufylline and BRL 61063 activate the HPA axis in the rat through actions on the hypothalamus and the anterior pituitary gland which lead to increased secretion of CRH-41 and ACTH. The actions of the drugs on the pituitary gland may be explained largely by inhibition of PDE-4. Blockade of PDE-4 may also underlie the actions of rolipram on the hypothalamus, but the rises in CRH-41 release provoked by denbufylline and BRL 61063 may also involve other mechanisms, in particular blockade of adenosine A_1 receptors. Whether these compounds exert similar effects on HPA function 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 cortisol in man. Nonetheless, our finding that three PDE-4 inhibitors cause marked activation of the HPA axis in the rat supports our premise (Hadley *et al*., 1996) that this area warrants careful and thorough investigation.

This work was generously supported by SmithKline Beecham Pharmaceuticals. We are also grateful to Professor P.J. Lowry (University of Reading, U.K.), Professor L.H. Rees (St Bartholomew's Hospital, London) and the W.H.O. (South Mimms, U.K.) for reagents for the ACTH assay, to NIADDK (U.S.A.) for reagents for the LH radioimmunoassay, to Professor E.W. Hillhouse (University of Warwick, U.K.), Dr F. Sancho-Franco (Madrid, Spain) and Dr Brent Williams (University of Edinburgh, U.K.) and Professor

C.R.W. Edwards (Imperial College School of Medicine, U.K.) for antisera to CRH-41, AVP and corticosterone, respectively, to Dr I. Kitchen (University of Surrey, U.K.) for advice on the adenosine receptor binding assay, to Schering AG (Berlin, Germany) for rolipram and to Mr C. Rantle and Mr J. Philip for technical assistance.

References

AGUILERA, G., HARWOOD, J.P., WILSON, J.X., MORELL, J., BROWN, J.H. & CATT, K.J. (1983). Mechanisms of action of corticotropin-releasing factor and other regulators of corticotropin release in rat pituitary cells. *J. Biol. Chem.*, **258,** 8039–8045.

AL-DUJAILI, E.A.S., WILLIAMS, B.C. & EDWARDS, C.R.W. (1981). The development and application of a direct radioimmunoassay for corticosterone. *Steroids*, **37,** 157–176.

ANAND-SRIVASTAVA, M.B., CONTIN, M. & GUTKOWSKA, J. (1989). Adenosine regulates the release of adrenocorticotrophic hormone (ACTH) from cultured pituitary cells. *Mol. Cell. Biochem.*, **89,** 21–28.

BEAVO, J.A. (1988). Multiple isozymes of cyclic nucleotide phosphodiesterase. *Adv. Second Messenger Phosphorylation Res.*, **22,** 1–30.

BEAVO, J.A. & REIFSNYDER, D.H. (1990). Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. *Trends Pharmacol. Sci.*, **11,** 150–155.

BISSERBE, J.S., PASCAL, O., DECKERT, J. & MAZIERE, B. (1992). Potential use of DPCPX as probe for *in vivo* localisation of brain A_1 adenosine receptors. *Brain Res.*, **599,** 6–12.

BOLGER, G., MICHAELI, T., MARTINS, T., JOHN, T.S., STEINER, B., RODGERS, L., RIGGS, S., WIGGLER, M. & FERGUSON, K. (1993). A family of human phosphodiesterases homologous to the dunce learning and memory gene product of Drosophila melangaster are potential targets for antidepressant drugs. *Mol. Cell. Biol.*, **13,** 6558–6571.

BRADBURY, M.W.B., BURDEN, J., HILLHOUSE, E.W. & JONES, M.T. (1974). Stimulation electrically and by acetylcholine of the rat hypothalamus *in vitro*. *J. Physiol.*, **239,** 269–283.

BUCKINGHAM, J.C. & HODGES, J.R. (1977a). The use of corticotrophin production by adenohypophysial tissue *in vitro* for the detection and estimation of potential corticotrophin releasing factors. *J. Endocrinol.*, **72,** 187–193.

BUCKINGHAM, J.C. & HODGES, J.R. (1977b). Production of corticotrophin releasing hormone by the isolated hypothalamus of the rat. *J. Physiol.*, **272,** 467–479.

BUCKLE, D.R., ARCH, J.R.S., CONNOLLY, B.J., FENWICK, A.E., FOSTER, K.A., MURRAY, K.J., READSHAW, S.A., SMALLBRIDGE, M. & SMITH, D.G. (1994). Inhibition of cyclic nucleotide phosphodiesterase by derivatives of 1,3-bis(cyclopropylmethyl)-xanthine. *J. Med. Chem.*, **37,** 476–485.

BUTCHER, R.W. & SUTHERLAND, E.W. (1962). Adenosine 3′,5-phosphate in biological materials. 1. Purification and properties of cyclic 3′,5-nucleotide phosphodiesterase and use of this enzyme to characterise adenosine 3′,5-phosphate in human urine. *J. Biol. Chem.*, **237,** 1244–1250.

CONTI, M., NEMOZ, G., SETTE, C. & VICINI, E. (1995). Recent progress in understanding the hormonal regulation of phosphodiesterases. *Endocrine Rev.*, **16,** 370–389.

COVER, P.O., LAYCOCK, J.F., GARTSIDE, I.B. & BUCKINGHAM, J.C. (1991). A role for vasopressin in the stress-induced inhibition of gonadotrophin secretion: Studies in the Brattleboro rat. *J. Neuroendocrinol.*, **3,** 413–417.

DU RUISSEAU, P., TACHE, Y., BRAZEAU P. & COLLU, R. (1979). Effects of chronic immobilization stress on pituitary hormone secretion, on hypothalamic factor levels, and on pituitary responsiveness to LHRH and TRH in female rats. *Neuroendocrinology*, **29,** 90–99.

EBERSOLT, C., PREMONT, J., PROCHIANTZ, A., PEREZ, M. & BOCKAERT, J. (1981). Inhibition of brain adenylate cyclase by A1 adenosine receptors: Pharmacological characteristics and locations. *Brain Res.*, **267,** 123–129.

EMANUEL R.L., GIRARD, D.M., THULL, D.C. & MAJZOUB, J.A. (1990). Second messengers involved in the regulation of corticotropin-releasing hormone mRNA and peptide in cultured rat fetal hypothalamic primary cultures. *Endocrinology*, **126,** 3016–3021.

EUKER, J.S., MEITES, Y. & RIEGLE, G.D. (1975). Effects of acute stress on serum LH and prolactin in intact, castrate and dexamethasone-treated male rats. *Endocrinology*, **96,** 85–92.

FASTBOM, J., PAZOS, A. & PALACIOS, J.M. (1987). The distribution of adenosine A1 receptors and 5′ nucleotidase in the brain of some commonly used experimental animals. *Neuroscience*, **22,** 813–826.

GARSIDE, D.A. & HARVEY, P.W. (1992). Endocrine toxicology of male reproductive system. In *Endocrine Toxicology*, ed. Atterwill, C.K. & Flack, J.D., pp. 285–312, Cambridge: University Press.

GIGUERE, V. & LABRIE, F. (1982). Vasopressin potentiates cyclic AMP accumulation and ACTH release induced by corticotrophin-releasing factor in rat anterior pituitary cells in culture. *Endocrinology*, **111,** 1752–1753.

GRAHAME-SMITH, D.G., BUTCHER, R.W., NEY, R.L. & SUTHERLAND, E.W. (1967). Adenosine 3′,5 monophosphate as the intracellular mediator of the action of adrenocorticotrophic hormone on the adrenal cortex. *J. Biol. Chem.*, **242,** 5535–5541.

HADLEY, A.J., FLACK, J.D. & BUCKINGHAM, J.C. (1990). A role for adenosine receptors in the regulation of ACTH and LH secretion. *J. Endocrinol.*, **127,** 50.

HADLEY, A.J., FLACK, J.D. & BUCKINGHAM, J.C. (1993). Effects of selective phosphodiesterase inhibitors on the release of ACTH and LH from the rat anterior pituitary gland *in vitro*. *Pharmacol. Communications*, **3,** 283–295.

HADLEY, A.J., KUMARI, M., COVER, P.O., OSBORNE, J., FLACK, J.D. & BUCKINGHAM, J.C. (1996). Stimulation of the hypothalamo-pituitary-adrenal axis in the rat by the type 4 phosphodiesterase (PDE-4) inhibitor, denbufylline. *Br. J. Pharmacol.*, **119,** 463–470.

HILLHOUSE, E.W. & MILTON, N.G.N. (1989). Effect of noradrenaline and GABA on the secretion of CRF-41 and AVP from rat hypothalamus *in vitro*. *J. Endocrinol.*, **122,** 719–723.

HU, S-B., TANNAHILL, L.A., BISWAS, S. & LIGHTMAN, S.L. (1992). Release of corticotropin-releasing factor-41, arginine vasopressin and oxytocin from fetal hypothalamic cells in culutre: response to activation of intracellular second messengers and corticosteroids. *J. Endocrinol.*, **132,** 57–65.

KEUHL, F.A., ZANETTI, M.E., SODERMAN, D.D., MILLER, D.K. & HAM, E.A. (1987). Cyclic AMP-dependent regulation of lipid mediators in white cells - a unifying concept for explaining the efficacy of theophylline in asthma. *Am. Rev. Resp. Dis.*, **136,** 210–213.

KILPATRICK, M.J., COLLINS, W.P. & NEWTON, J.R. (1976). Studies on the release of gonadotrophins during the superfusion of isolated rat pituitaries in a continuous flow system. *J. Reprod. Fertil.*, **46,** 25–30.

KOCH, B. & LUTZ-BUCHER, B. (1995). Multifactorial regulation of pituitary adenylate cyclase-activating polypeptide (PACAP)-induced production of cyclic AMP in AtT-20 corticotrophs: major involvement of rolipram-sensitive and insensitive phosphodiesterases. *Mol. Cell. Endocrinol.*, **112,** 27–34.

LABRIE, F., GAGNE, B. & LEFEVRE, G. (1982a). Corticotropin-releasing factor stimulates adenylate cyclase activity in anterior pituitary gland. *Life Sci.*, **31,** 1117–1121.

LABRIE, F., VIELLEUX, R., LEFEVRE, G., COY, D.H., SUERIAS-DIAZ, J. & SCHALLY, A.V. (1982b). CRF stimulates accumulation of adenoisine 3′5-monophosphate in rat pituitary corticotrophs. *Science*, **216,** 1007–1008.

LEE, K.S. & REDDINGTON, M. (1986). 1, 3 dipropyl-8-cyclopentyl-xanthine (DPCPX) inhibition of [^{3}H] N-ethyl-carboxamidoadenosine (NECA) binding allows the visualisation of putative non-A1 adenosine receptors. *Brain Res.*, **368,** 394–398.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.D. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193,** 265–275.

LOXLEY, H.D., COWELL, A.M., FLOWER, R.J. & BUCKINGHAM, J.C. (1993a). Modulation of the hypothalamo-pituitary adrenocortical responses to cytokines in the rat by lipocortin 1 and glucocorticoids: a role for lipocortin 1 in the feedback inhibition of CRF-41 release? *Neuroendocrinology*, **57,** 801–814.

LOXLEY, H.D., COWELL, A.M., FLOWER, R.J. & BUCKINGHAM, J.C. (1993b). Effects of lipocortin 1 and dexamethasone on the secretion of corticotrophin releasing factors in the rat: *in vitro* and *in vivo* studies. *J. Neuroendocrinol.*, **5,** 51 – 61.

NEGRO-VILAR, A., SANCHO-FRANCO, F., KWAITKOWSKI, M. & SAMSON, W.K. (1979). Failure to detect immunoassayable arginine vasopressin in mammalian pineals. *Brain Res. Bull.*, **4,** 789 – 792.

NICHOLSON, C.D., CHALLISS, R.A.J. & SHAHID, M. (1991). Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. *Trends Pharmacol. Sci.*, **12,** 19 – 27.

NICHOLSON, C.D., JACKMAN, S.A. & WILKE, R. (1989). The ability of denbufylline to inhibit cyclic nucleotide phosphodiesterase and its affinity for adenosine receptors and the adenosine reuptake site. *Br. J. Pharmacol.*, **97,** 889 – 897.

NICHOLSON, C.D. & SHAHID, M. (1994). Inhibitors of cyclic nucleotide phosphodiesterase isoenzymes - their potential utility in the therapy of asthma. *Pulm. Pharmacol.*, **7,** 1 – 17.

NICHOLSON, S.A. (1987). The effect of caffeine on plasma corticosterone and pituitary adrenocorticotrophin (ACTH) release in the rat is antagonised by adenosine. *J. Physiol.*, **39,** 124P.

NICHOLSON, S.A. (1989). Stimulatory effect of caffeine on plasma corticosterone and pituitary-adrenocortical axis in the rat. *J. Endocrinol.*, **122,** 535 – 543.

NIELSON, C.P., CROWLEY, J.J., CUSACK, B.J. & VESTAL, R.E. (1986). Therapeutic concentrations of theophylline and enprofylline potentiate catecholamine effects and inhibit leukocyte activation. *J. Allergy Clin. Immunol.*, **78,** 660 – 667.

ONDO, J.G., WALKER, M.W. & WHEELER, D.D. (1989). Central actions of adenosine on pituitary secretions of prolactin, luteinising hormone and thyrotropin. *Neuroendocrinology*, **49,** 654 – 658.

PLOTSKY, P.M., BRUHN, T.O. & VALE, W. (1985). Evidence for multifactor regulation of the adrenocorticotropin secretory response to hemodybnamic stimuli. *Endocrinology*, **116,** 633 – 639.

REES, L.H., COOKE, D.M., KENDALL, J.W., ALLEN, C.F., KRAMER, R.M., RATCLIFFE, J.G. & KNIGHT, R.A. (1971). A radioimmunoassay for rat plasma ACTH. *Endocrinology*, **89,** 254 – 261.

SAWCHENKO, P.E. (1987). Adrenalectomy-induced enhancement of CRF-41 and vasopressin immunoreactivity in parvocellular neurones: Anatomic, peptide and steroid specificity. *J. Neurosci.*, **7,** 1093 – 1096.

SCHMIECHEN, R., SCHNEIDER, H.H. & WACHTEL, H. (1990). Close correlation between behavioural response and binding *in vivo* for inhibitors of the rolipram-sensitive phosphodiesterase. *Psychopharmacology*, **102,** 17 – 20.

SEASHOLTZ, A.F., THOMPSON, R.C. & DOUGLAS, J.O. (1988). Identification of a cyclic adenosine monophosphate-responsive element in the rat corticopropin-releasing hormone gene. *Mol. Endocrinol.*, **2,** 1311 – 1319.

SETTE, C., VICINI, E. & CONTI, M. (1994). Modulation of cellular responses by hormones: a role of cAMP specific, rolipram-sensitive phosphodiesterases. *Mol. Cell. Endocrinol.*, **100,** 75 – 79.

SPINDEL, E.R., ARNOLD, M., CUSACK, A.B. & WURTMAN, R.J. (1980a). Effects of caffeine on anterior pituitary and thyroid function in the rat. *J. Pharmacol. Exp. Ther.*, **214,** 58 – 62.

SPINDEL, E.R., MCCALL, A.J. & WURTMAN, R.J. (1980b). Lack of endocrine effects of a low dose of caffeine in man. In *Proceedings of the 3rd International Caffeine Workshop*, International Life Sciences Institute, Washington DC.

SPINDEL, E.R., GRIFFITH, I. & WURTMAN, R.J. (1983). Neuroendocrine effects of caffeine. II. Effects on thyrotropin and corticosterone secretion. *J. Pharmacol. Exp. Ther.*, **225,** 346 – 350.

SPINDEL, E.R. & WURTMAN, R.J. (1984). Neuroendocrine effects of caffeine in rat and man. In *Caffeine-Perspectives from Recent Reasearch*. ed. Dews, P.B. pp. 119 – 128. Berlin: Springer-Verlag.

SUDA, T., YAJIMOTO, F., TOMORI, N., DEMURA, H. & SHIZUME, K. (1985). *In vitro* study of immunoreactive corticotropin-releasing factor release from the rat hypothalamus. *Life Sci.*, **37,** 1499 – 1505.

SULLIVAN, P., BEKIR, S., JAFFAR, Z., PAGE, C., JEFFERY, P. & COSTELLO, J. (1994). The anti-inflammatory effects of low dose theophylline in atopic asthma. *Lancet*, **343,** 1006 – 1008.

TAYLOR, A.D., COWELL, A.M., FLOWER, R.J. & BUCKINGHAM, J.C. (1993). Lipocortin 1 mediates an early inhibition action of glucocorticoids on the secretion of ACTH by the rat anterior pituitary gland *in vitro*. *Neuroendocrinology*, **58,** 430 – 439.

TAYLOR, A.D., LOXLEY, H.D., FLOWER, R.J. & BUCKINGHAM, J.C. (1995). Immunoneutralization of lipocortin 1 reverses the acute inhibitory effects of dexamethasone on the hypothalamo-pituitary-adrenocortical responses to cytokines in the rat *in vitro* and *in vivo*. *Neuroendocrinology*, **62,** 19 – 31.

WEBER, R.G., JONES, R., PALACIOS, J.M. & LOHSE, M.J. (1988). Autoradiographic visualisation of A_1-adenosine receptors in brain and peripheral tissues of rat and guinea-pig using ^{125}I-HPIA. *Neurosci. Lett.*, **87,** 215 – 220.

WIDMAIER, E.P., LIM. A.T. & VALE, W. (1989). Secretion of corticotropin-releasing factor from cultured rat hypothalamic cells. Effects of catecholamines. *Endocrinology*, **124,** 583 – 590.

WILKE, R., ARCH, J.R.S. & NICHOLSON, C.D. (1989). Tissue selective inhibition of cyclic nucleotide phosphodiesterase by denbufylline. *Arzneim-Forsch/Drug Res.*, **39,** 665 – 667.

(Received January 7, 1997
Revised February 15, 1997
Accepted February 26, 1997)