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STUDIES ON THE ROLES AND INTERACTIONS OF
PHOSPHOLIPID METABOLITES, CYTOKINES, STEROIDS AND
LIPOCORTIN 1 IN THE CONTROL OF HYPOTHALAMO-
PITUITARY-ADRENOCORTICAL FUNCTION IN THE RAT.

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Abstract.

Prolonged treatment with high doses of anti-inflammatory corticosteroids results invariably in a spectrum of undesirable effects, the most hazardous of which is severe suppression of hypothalamo-pituitary-adrenocortical (HPA) function. Despite many studies, the mechanisms by which the steroids exert their regulatory effects are unknown. The beneficial anti-inflammatory actions of the corticosteroids have been attributed in part to a family of Ca^{2+} and phospholipid binding proteins, the lipocortins, whose biological properties encompass the capacity to block the activity of the enzyme phospholipase A_2 (PLA_2) and thus the generation of pro-inflammatory eicosanoids. In the present study in vivo and in vitro methods were used in an attempt to determine whether the regulatory effects of the steroids on the HPA axis are effected by a similar mechanism.

The influence of phospholipid metabolites on the release of ir-corticotrophin releasing factor (ir-CRF-41) and ir-arginine vasopressin (ir-AVP) from isolated rat hypothalami were studied in vitro . Phospholipase A_2 and the PLA_2 activator, melittin, stimulated the release of both peptides from hypothalami from intact and chronically adrenalectomized animals. Their effects were antagonised invariably by the PLA_2 inhibitor, quinacrine. Short term (<24h) exposure to dexamethasone readily blocked the AVP response to PLA_2 in hypothalami from adrenalectomized rats although it had no effect on the responses of tissue from intact animals. The effects of the steroid treatment on the PLA_2 induced secretion of CRF-41 were more variable and less pronounced. In contrast, more prolonged steroid treatment (6 and 14 days) readily inhibited the secretion of CRF-41 but had a weaker effect on AVP. The CRF-41 responses to PLA_2 were unaffected or potentiated by inhibitors of cytochrome P450 and 5-lipoxygenase enzymes and attenuated by a dual lipoxygenase/cyclo-oxygenase inhibitor. They were however reduced or abolished by inhibition of cyclo-oxygenase enzymes. Cyclo-oxygenase blockade did not impair AVP responses to PLA_2. A cytochrome P450 inhibitor and a dual lipoxygenase/cyclo-oxygenase inhibitor were also ineffective in this respect, but in one experiment a 5-lipoxygenase inhibitor, abolished the response.

Both in vivo and in vitro studies were performed to examine the influence of lipocortin on HPA function. Human recombinant lipocortin 1 (hr-LC1, i.c.v.) had no affect on

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the resting plasma corticosterone concentration in conscious rats. The lowest dose (0.3μg) tested potentiated the HPA response to histamine stress but higher doses (0.6-1.2μg) were without effect. Neither the resting nor the stress-induced secretion of corticosterone was affected by LC1 antisera (i.c.v.). In vitro, hr-LC1 failed to affect basal ACTH release from the pituitary gland but significantly inhibited HE or K^+-induced release. In contrast, 1-188 LC-1 elevated basal ACTH release but had no effect on either HE or K^+-stimulated release. At the hypothalamic level, hr-LC1 failed to influence the spontaneous release of CRF-41 or the release evoked by PLA_2 or K^+. By contrast, it stimulated the release of AVP and potentiated the AVP responses to PLA_2 but not to K^+.

The effects of a number of cytokines on the secretion in vitro of ir-CRF-41 and ir-AVP by the hypothalamus and of ir-adrenocorticotrophic hormone (ir-ACTH) by the pituitary gland were investigated. A cytokine-rich medium, derived from macrophages challenged with endotoxin, produced pronounced concentration-dependent increases in the release in vitro of all three peptides. None of the purified cytokines tested exhibited the same degree of activity. Thus, tumour necrosis factor α (TNFα), interleukins 1α & 1β, 6 and 8 had no affect on basal ACTH secretion but in some instances a depression of the secretory responses to HE was apparent. At the hypothalamic level, IL-1α, IL-1β and TNFα produced weak stimulatory effects on the secretion of both CRF-41 and AVP. IL-6 and IL-8 were more active in this respect. The responses to both IL-6 and IL-8 were readily inhibited by dexamethasone. The steroid also reduced IL-1α-induced ir-AVP release although it failed to affect the ir-CRF-41 response. Lipocortin 1 significantly inhibited ir-CRF-41 release initiated by IL-6 and IL-8 stimulation but potentiated IL-6-induced ir-AVP release.

The results indicate that both phospholipid metabolites generated by PLA_2 and cytokines may play a role in the regulation of CRF-41 and AVP release from the hypothalamus. They also provide evidence for differential control of the release of the two peptides and suggest that only the CRF-41 responses to PLA_2 are effected by prostanoids. Further studies are required to identify the arachidonic acid metabolites effecting the release of AVP. The actions of lipocortin on ir-CRF-41 release closely resembled the effects of steroids. Thus inhibition which was evident following cytokine but not K^+ or PLA_2 stimulation, was stimulus specific. It is therefore possible that lipocortins may modulate HPA responses to cytokine stimulation through an action on CRF-41 and not AVP release.

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The Hypothalamo-Pituitary-Adrenal axis (HPA).

Glucocorticoids play a key role in the maintenance of homeostasis. They exert widespread, primarily catabolic actions on protein, carbohydrate and lipid metabolism and are essential for protecting the organism from stress. In addition they possess anti-inflammatory and immunosuppressive properties. The secretion of the glucocorticoids is normally maintained within narrow limits. Excessive production, which may result from primary adrenal disease or excessive ACTH release from the pituitary gland or from an ectopic tumour, manifests itself as Cushings syndrome, while impairment of glucocorticoid secretion may result in Addison's disease or, in the case of specific inherited enzyme deficiency, the adrenogenital syndrome. The characteristics of these conditions are described in a number of texts (*Drury & Rees, 1987*).

Cortisol and the semi-synthetic glucocorticoids (eg prednisolone) provide an essential means of replacement therapy in subjects with adrenocortical insufficiency. They are also exploited clinically to control inflammatory diseases such as asthma and rheumatoid arthritis and to produce immunosuppression in autoimmune diseases (eg myaesthenia gravis). However, the high doses required to produce beneficial therapeutic effects in these conditions inevitably result in the manifestation of a spectrum of undesirable features not least of which is the potentially hazardous suppression of adrenocortical function which may persist for a considerable period after the drug is withdrawn.

The glucocorticoids, cortisol in man (*Bush & Sandberg, 1953*), and corticosterone in the rat (*Bush, 1953*), are synthesised in the zona fasiculata of the adrenal cortex from cholesterol. The newly synthesised steroid is released directly into the bloodstream where it is transported to its target cells in association with a carrier protein transcortin and, to a lesser extent, with plasma albumin. The pattern of secretion varies according to a circadian rhythmn which is linked to the sleep/wake cycle. Thus, in the rat glucocorticoid levels are lowest in the morning while in man the minimum levels occur in the evening. Glucocorticoids are also released in substantial additional quantities during stress. The steroids are inactivated in the liver primarily by reduction of the A-ring. The resultant tetra-hydro compounds may undergo further modification before being conjugated and excreted in the urine and bile.

The synthesis of the glucocorticoids is largely dependent on the pituitary hormone

corticotrophin (ACTH) which acts on membrane bound receptors in the zona fasiculata of the cortex and, by a process which involves activation of adenylate cyclase and Ca^{2+} influx (*Grahame-Smith, Butcher, Ney & Sutherland, 1967; Birmingham & Bartanova, 1973*), activates the rate-limiting enzyme of the steroidogenic pathway and thus facilitates the conversion of cholesterol to pregnenolone and the subsequent generation of biologically active steroids.

Adrenocorticotrophic hormone (ACTH) and related peptides.

Corticotrophin is a straight chain peptide comprising 39 amino acids (*Shepherd, Willson, Howard, Bell, Davis, Davis, Eigher & Shakespear, 1956*). It is synthesised from a large 30K precursor molecule pro-opiomelanocortin (POMC) in specialised cells, the corticotrophs, in the adenohypophysis. POMC also contains the sequences for a number of other peptides (Fig 1) which are co-released with ACTH (*Guillemin, Vargo; Rossier, Minick, Ling, Rivier, Vale & Bloom, 1977; Vale, Rivier, Yang, Minick & Guillemin, 1978; Proulx-Ferland, Labrie, Dumont & Côté, 1982*). These include β-lipotrophin (β-LPH) which itself contains the sequence for γ– and β-endorphin, α-melanocyte-stimulating hormone (α-MSH) and pro-γ-MSH, the precursor of N-POC peptides (1-76) and (1-48) and γ-MSH (*Mains, Eipper & Ling, 1977; Nakanishi, Inoue, Kita, Nakamura, Chang, Cohen & Numa, 1979; Hale, Besser & Rees, 1986*). Pro-γ-MSH potentiates the steroidogenic response to ACTH (*Al-Dujaili, Hope, Estivariz, Lowry & Edwards, 1981*) while N-terminal POMC peptides promote adrenal mitogenesis (*Estivariz, Carino, Lowry & Jackson, 1988*). The role of the opioid peptides co-secreted with ACTH is unknown.

Corticotrophin releasing factors.

ACTH secretion is controlled by a 'neurohormonal factor' termed the corticotrophin releasing factor, CRF (*Saffran, Schally & Benfey, 1955*), which is released from the hypothalamus and transported in the hypophyseal portal vessels to the anterior pituitary gland where it stimulates ACTH release (*DeGroot & Harris, 1950; Ganong & Hume, 1954*).

The exact nature of CRF has been the subject of much controversy. For many years it was thought to be a single substance but it is now known to comprise a number of factors which act synergistically to stimulate ACTH release (*Gillies, Linton & Lowry,*
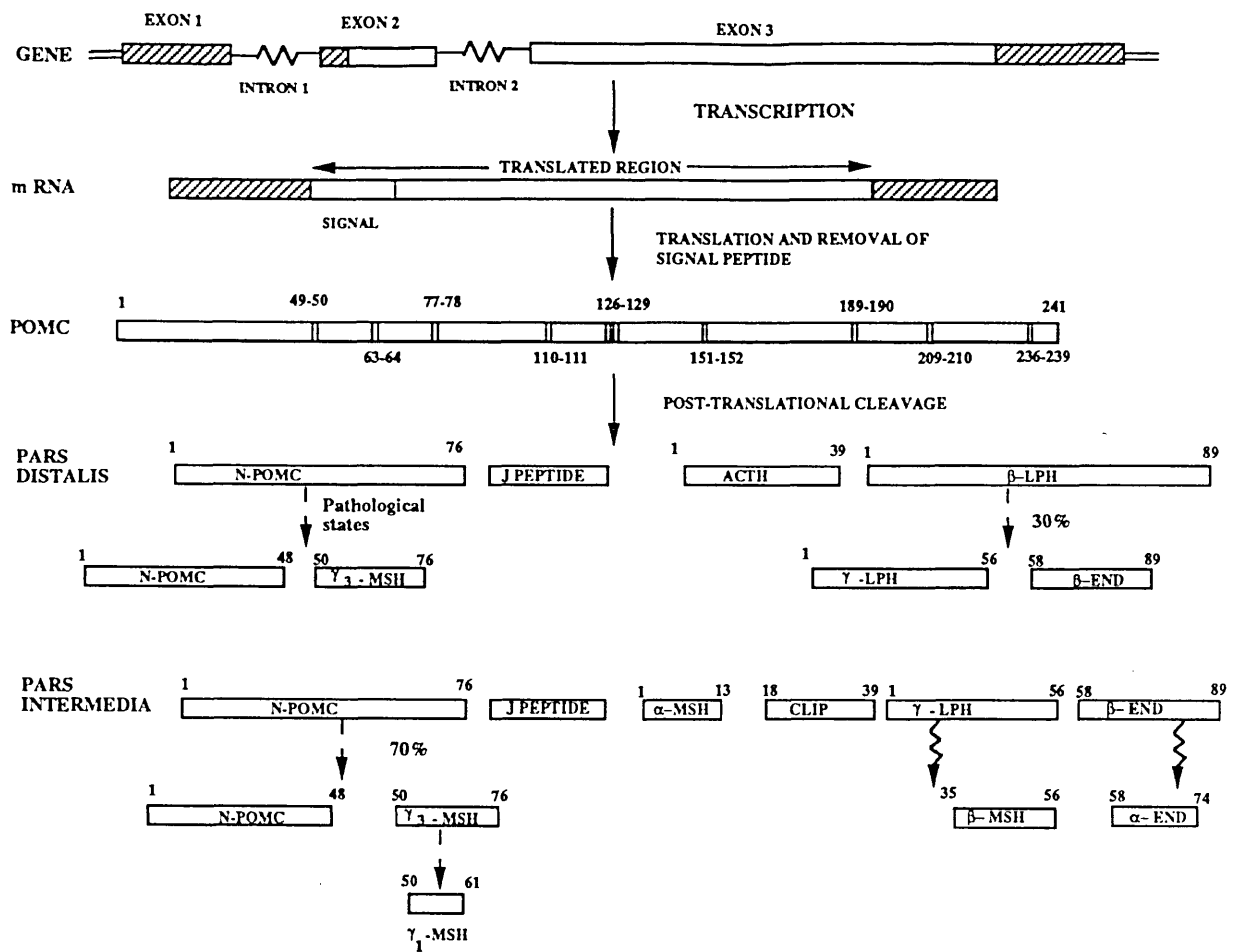
Fig. 1: Synthesis and post-translational processing of pro-opiomelanocortin in the corticotrophs of the pars distalis and pars intermedia.

▨▨▨	= untranslated nucleic acid sequences
‖	= sites of paired basic amino acids (i.e. potential cleavage sites) in the pro-hormone
– – ►	= incomplete processing
∿∿►	= species specific incomplete processing

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1982). The most important of these appear to be the 41-amino acid corticotrophin releasing factor, CRF-41 (*Vale, Spiess, Rivier & Rivier, 1981*), and arginine vasopressin (AVP).

(i) Corticotrophin releasing factor (CRF-41).

Ovine CRF-41 was first isolated and characterised in 1981 (*Vale et al., 1981*). The natural and synthetic peptides were shown to increase the release of ACTH from cultured pituitary cells (*Vale et al., 1981; Vale, Vaughan, Smith, Yamamoto, Rivier & Rivier, 1983*), with 15-41 being the minimal sequence for full activity (*Vale et al., 1981*). The sequences of rat and human CRF-41, which are identical (*Rivier, Speiss & Vale, 1983; Shibahara, Morimoto, Furutani, Notake, Takahashi, Shimizu, Horikawa & Numa, 1983; Patthy, Horváth, Mason-Garcia, Szöke, Schlesinger & Schally, 1985; Rivier & Plotsky, 1986*) were subsequently shown to differ from the ovine molecule by 7 amino acids and the carboxyl terminus was found to be important for biological activity.

Ovine and rat CRF-41 produce concentration-related increases in ACTH release in vitro from pituitary segments (*Antoni, Holmes & Jones, 1983; Buckingham, 1985*) perifused pituitary cells (*Gillies et al., 1982; Watanabe & Orth, 1988*), primary anterior pituitary cell cultures (*Rivier, Rivier, Lederis & Vale, 1983; Vale et al., 1983*) and At-T-20 mouse pituitary tumour cells (*Hook, Heisler, Sabol & Axelrod, 1982*). CRF-41 is also effective in vivo and, when administered intravenously, it produces dose-related increases in the plasma concentrations of ACTH and POMC related peptides in pharmacologically blocked rats (*Rivier, Brownstein, Spiess, Rivier & Vale, 1982; Buckingham, 1984*), in sheep (*Donald, Redekopp, Cameron, Nicolls, Bolton, Livesey, Espiner, Rivier &Vale , 1983; Kalin, Gonder & Shelton, 1983*) and in man (*Grossman, Nieuwenhuyzen-Kruseman, Perry, Tomlin, Schally, Coy, Rees, Comaru-Schally & Besser, 1982; Conaglen, Donald, Espiner, Livesey & Nicolls, 1984*).

The actions of CRF-41 on the anterior pituitary gland are mediated by specific high affinity receptors (*Wynn, Aguilera, Morell & Catt, 1983; De Souza, Perrin, Rivier, Vale & Kuhar, 1984; Perrin, Haas, Rivier & Vale, 1986*) which are positively coupled to adenylate cyclase (*Labrie, Veilleux, Lefèvre, Coy, Sueiras-Diaz & Schally, 1981; Giguère, Labrie, Côté, Coy, Sueiras-Diaz & Schally, 1982; Labrie, Gagné & Lefèvre, 1982; Aguilera, Harwood, Wilson, Morell, Brown & Catt, 1983;*

Miyazaki, Reisine & Kebabian, 1984; Reisine, Rougon, Barbet & Affolter, 1985; Reisine, 1989). The peptide not only initiates the release of ACTH and other POMC peptides from corticotrophs (*Vale, Rivier, Brown, Spiess, Koob, Swanson, Bilezikjian, Bloom & Rivier, 1983; Rivier & Plotsky, 1986*) but also stimulates the synthesis of POMC mRNA and, hence, POMC (*Bruhn, Sutton, Rivier & Vale, 1984; Reisine et al., 1985; Suda, Tozawa, Yamada, Ushiyama, Tomori, Sumitomo, Nakagami & Shizume, 1988; Suda, Tozawa, Ushiyama, Tomori, Sumitomo, Nakagami, Yamada, Demura & Shizume, 1989*).

CRF-41 is synthesised by neurones in several hypothalamic regions which project mainly to the external lamina of the median eminence (ME) from a precursor molecule prepro-CRF (*Furutani, Morimoto, Shibahara, Noda, Takahashi, Hirose, Asai, Inayama, Hayashida, Miyata & Numa, 1983; Jingami, Mizuno, Takahashi, Shibahara, Furutani, Imura & Numa, 1985*). Immunohistochemical studies have revealed that the majority of CRF-41 containing perikarya are in the parvocellular subdivisions of the hypothalamic paraventricular nucleus (PVN) (*Antoni, Palkovits, Makara, Linton, Lowry & Kiss, 1983; Swanson, Sawchenko, Rivier & Vale, 1983; Bruhn, Plotsky & Vale, 1984; Silverman, Hou-Yu & Chen, 1989*) and the expression of CRF in the parvocellular neurosecretory neurons is enhanced by adrenalectomy (*Sawchenko, 1987*). This area is also rich in the mRNA which encodes for the CRF-41 precursor (*Liposits, Görcs, Sétáló, Lengvári, Flerkó, Vigh & Schally, 1983; Léránth, Antoni, & Palkovits, 1983*). CRF-41 is also expressed in certain magnocellular neurons eg those projecting from the PVN, the supraoptic nucleus (SON) and the accessory magnocellular nuclei of the hypothalamus to the ME and oxytocinergic neurons which project to the neural lobe of the pituitary gland (*Burlet, Tonon, Tankosic, Coy & Vandry, 1983; Antoni, Palkovits, Makara, Kiss, Linton, Lowry & Léránth, 1984; Sawchenko, Swanson & Vale 1984a*). There is also evidence that the axons of CRF-41 containing neurons may establish axo-somatic synapses with CRF-41 perikarya in the PVN (*Liposits, Paull, Sétáló & Vigh, 1985*) together with axo-dendritic synapses with CRF-41 containing magnocellular neurons in the PVN and in the SON (*Shioda, Nakau, Kitazawa & Sunayama, 1985*). In addition, the CRF-41 cells in the PVN may synapse with magnocellular oxytocinergic or vasopressinergic neurons (*Léránth et al., 1983*).

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(ii) Arginine vasopressin (AVP).

AVP and related peptides stimulate ACTH secretion both in vivo (*McCann & Fruit, 1957; De Wied, Bouman & Smelik, 1958*) and in vitro (*Pearlmutter, Rapino & Saffran, 1975; Buckingham & Hodges, 1977a; Gillies, VanWimersma Griedanus & Lowry, 1978*). The corticotrophic response requires extracellular calcium (Ca^{2+}) (*Zimmerman & Fleischer, 1970*) and is mediated by specific V_1-like receptors (*Rivier, Rivier, Mormede & Vale, 1984; Buckingham, 1987*) which are positively coupled to phospholipase C (*Raymond, Leung, Veilleux & Labrie, 1985*).

The early claims, based on observations that stress often resulted in the secretion of vasopressin and ACTH (*Mirsky, Stein & Paulisch, 1954*), that AVP was the CRF, (*McCann & Brobeck, 1954; Martini & Morpurgo, 1955; McCann & Fruit, 1957*) have not been substantiated . Although AVP stimulates ACTH release it is only weakly active and, in a number of preparations, the slopes of its dose response lines differ significantly from those of hypothalamic extract alone demonstrating that the CRF in hypothalamic extract is not identical to AVP (*Portanova & Sayers, 1973; Buckingham & Hodges 1977a; Krieger, Liotta & Brownstein, 1977; Gillies et al., 1978; Gillies & Lowry, 1978*). Nevertheless, observations that hypothalamic extracts from rats which congenitally lack vasopressin (Brattleboro strain) possess relatively weak corticotrophin releasing activity (*Krieger et al., 1977; Buckingham & Leach, 1979; Gillies, Estivariz & Lowry, 1979*) together with reports that the adrenocortial response to stress is attenuated in vasopressin deficient animals (*Arimura, Saito, Bowers & Schally, 1967; Wiley, Pearlmutter & Miller, 1974*) suggest that AVP may be important in this respect.

The possibility that CRF-induced ACTH release could be potentiated by vasopressin (*Yates, Russell, Dallman, Hedge, McCann & Dhariwal, 1971*) was examined by two groups. Gillies and Lowry (1979) proposed that AVP was the major CRF, but it required the presence of a synergistic factor for full biological activity. Their claim was based on observations (i) that the corticotrophin releasing activity of stalk median eminence (SME) extracts was abolished by pre-incubation with vasopressin antiserum and (ii) chromatographic separation of the SME extract resulted in two peaks of CRF activity; the major peak eluted in the expected position for vasopressin and recombination of the two fractions produced a complex exhibiting strong CRF-like activity, similar to that of SME extract (*Gillies et al., 1979*). By contrast, another group reported that treatment of SME extracts from normal rats with vasopressin

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antiserum did not abolish its ACTH releasing activity but attenuated it in such a way that its biological potency and slope of its dose response lines closely resembled those of Brattleboro extracts (*Buckingham, 1981*).These workers also found that the attenuated corticotrophin releasing activity of extracts from Brattleboro rats could be 'normalized' by the addition of vasopressin at concentrations well below those required to elicit ACTH release (*Buckingham & Leach, 1979; Buckingham, 1981*). From these findings it was suggested that vasopressin facilitates the corticotrophin releasing activities of other more potent CRF's (*Buckingham, 1981*).

Following the isolation of CRF-41 in 1981 several workers demonstrated the ability of AVP to potentiate the actions of CRF-41 in vivo (*Rivier & Vale, 1983; Graf, Kastin & Fischman, 1985*) and in vitro (*Turkelson, Thomas, Arimura, Chang, Chang & Shimizu, 1982; Bény & Baertschi, 1982; Gillies et al., 1982; Giguère & Labrie, 1982; Antoni et al., 1983; Vale, Vaughan, et al., 1983; Buckingham, 1985*) and it is now generally agreed that the two peptides act synergistically to promote ACTH release. The mechanism by which the synergistic response is achieved is not clear. It appears to involve enhanced CRF-41 induced cAMP formation (*Giguère & Labrie, 1982; Lutz-Bucher & Koch, 1985*) and may involve actions of AVP on a yet unclassified vasopressin receptor distinct from the V_1-like site responsible for the direct secretory actions of the peptide (*Baertschi & Friedli, 1985; Buckingham, 1987*).

The importance of the combined actions of AVP and CRF-41 on ACTH release has been emphasised further by functional and anatomical studies. Both AVP and CRF-41 are present in hypophyseal portal blood in concentrations greater than those in the peripheral blood (*Gibbs & Vale, 1982; Plotsky, Bruhn & Vale, 1985*). The pituitary adrenocortical response to specific stressors is blocked more effectively by combined administration of antibodies against both peptides than either antibody alone.(*Linton, Tilders, Hodgkinson, Berkenbosch, Vermes & Lowry, 1985*). Immunohistochemical studies have revealed that vasopressin is present in the parvicellular neurones passing from the PVN to the median eminence and its expression is increased following adrenalectomy (*Davis, Arentzen, Reid, Manning, Wolfson, Lawrence & Baldino, 1986*) as too is its secretion into the portal blood (*Plotsky et al., 1985; Fink, Robinson & Tannahill, 1988*). It is colocalised with CRF-41 in the parvicellular neurones in both adrenalectomized (*Tramu, Croix & Pillez, 1983; Kiss, Mezey & Skirboll, 1984; Sawchenko, Swanson & Vale, 1984b; Whithall, Mezey & Gainer, 1985*) and intact rats (*Whithall et al., 1985*). AVP is also conveyed to the ME via the

magnocellular neurosecretory pathway and is found in preterminal magnocellular neurons (*Whithall et al., 1985*).

(iii) Other ACTH secretagogues.

The actions of CRF-41 and AVP together do not account for all the corticotrophin releasing activity of the hypothalamus, since ACTH release induced by a combination of the two peptides is less than that secreted on stimulation with hypothalamic extract (*Gillies et al., 1982; Buckingham, 1985*). Furthermore, incubation of the hypothalamic extract from normal rats with antisera to CRF-41 and AVP does not completely block the corticotrophin releasing properties of the extract (*Linton, Gillies & Lowry, 1983*). It is now thought that, in addition to CRF-41 and AVP, the CRF complex includes at least one other factor (*Hashimoto, Murakami, Ohno, Kageyama, Hattori, Aoki & Takahara, 1983; Gillies, Puri, Linton & Lowry, 1984*). Its identity is not known but several substances have been shown to possess ACTH releasing properties such as adrenaline, noradrenaline, oxytocin, angiotensin II and vasoactive intestinal peptide (VIP).

Considerable controversy surrounds the role of adrenaline and noradrenaline in the regulation of ACTH secretion by the pituitary gland. Adrenergic and noradrenergic nerve fibres originating in the brain stem (*Palkovits, 1981*) and cervical sympathetic plexus (*Gallardo, Chiocchio & Tramezzani, 1984*) terminate in the median eminence and there is evidence to suggest that adrenaline can be released into the portal circulation (*Gibbs, 1985a*). However, although some workers have claimed that the catecholamines stimulate ACTH release in vivo (*Mezey, Resine, Palkovits, Brownstein & Axelrod, 1983; Mezey, Resine, Brownstein, Palkovits & Axelrod, 1984; Takao, Hashimoto & Ota, 1988*) and in vitro (*Vale & Rivier, 1977; Bény & Baertschi, 1981; Giguère, Côté & Labrie, 1981; Giguère & Labrie, 1983; Perkins, Evans, Thorner, Gibbs & Cronin, 1985; Tilders, Berkenbosch, Venues, Linton & Smelik, 1985*) others have found them to be without effect both in vivo (*Buckingham & Hodges, 1983; Tilders, Berkenbosch & Smelik, 1986*) and in vitro (*Buckingham & Hodges, 1977a, 1983; Gillies & Lowry, 1978*)

Oxytocin, angiotensin II and VIP, all of which are located in pathways terminating in the median eminence (*Hoffman, Krupp, Schrag, Nilaver, Valiquette, Kilcoyne & Zimmerman, 1982; Burlet et al., 1983; Sawchenko et al., 1984a b; Lind, Swanson,*

Bruhn & Ganten, 1985; Mezey & Kiss, 1985), have also been shown to stimulate the release of ACTH both in vivo (*Maran & Yates, 1977; Rivier & Vale, 1985*) and in vitro (*Gailiard, Grossman, Gillies, Rees & Besser, 1981; Capponi, Favrod-Coune, Gailliard & Muller, 1982; Antoni et al., 1983; Vale, Vaughan, et al., 1983; Westendorf, Phillips & Schonbrunn, 1983; Gibbs, Vale, Rivier & Yen, 1984; Baertschi & Friedli, 1985*). Oxytocin potentiates the responses to CRF-41. Its actions thus resemble those of AVP and may involve the same intracellular mechanisms (*Gibbs et al., 1984*). By contrast, the effects of angiotensin II are generally additive with those of CRF-41 (*Aguilera et al., 1983; Abou-Samra, Catt & Aguilera, 1986a*), although potentiation has been reported by other groups (*Aguilera, Wynn, Harwood, Hanger, Millan, Crewe & Catt, 1986; Gaillard, Riondel, Ling & Muller, 1988*)

Other substances which have been shown to facilitate ACTH release in vitro include atrial naturitic factor (ANF), (*Horváth, Ertl & Schally, 1986; Takao, Hashimoto & Ota, 1988b*) γ-aminobutyric acid receptor agonists, (*Anderson & Mitchell, 1986*) cholecystokinin, (*Reisine & Jensen, 1986*) gastrin releasing peptide (*Hale, Price, Ackland, Doniach, Ratter Besser & Rees, 1984*) and serotonin (*Spinedi & Negro-Vilar, 1983*). Their physiological role in this context is obscure.

Corticotrophin release inhibiting factors (CRIFs).

Although the hypothalamus exerts a predominantly stimulatory influence on ACTH secretion, evidence has accumulated that CRIFs may also be produced (*Grossman & Tsagarakis, 1989*). The first such candidate was substance P (*Jones, Gillham, Holmes, Hodges & Buckingham, 1978*) but more recently attention has focussed on the atriopeptins. Although ANF has been shown to stimulate ACTH release (*Horváth et al., 1986*), atriopeptins 1-28 and 5-28 inhibit CRF-41/AVP stimulated ACTH release in vitro. Their inhibitory actions are mediated by the atriopeptin receptor domain of the membrane bound guanylyl cyclase protein (*Leitman, Andersen, Kuno, Kamisaki, Chang & Murad, 1986; Scarborough, Schenk, McEnroe, Arfsten, Kang, Schwartz & Lewicki, 1986; Chinkers, Garbers, Chang, Lowe, Chin, Goeddel & Schulz, 1989*) and thus resemble those of the cyclic GMP derivatives which permeate the cell membrane (*Antoni & Dayanithi, 1989*).

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Neural control of CRF-41 and AVP release.

The secretion of CRF-41 and AVP into the hypophyseal portal blood is controlled primarily by a complex of intra- and extra-hypothalamic neural pathways which relay essential information concerning the circadian periodicity (*David-Nelson & Brodish, 1969; Takebe, Sakakura, Horiuchi & Mashimo, 1971; Kiss, 1988*), metabolic and osmotic status, environmental changes, physical and emotional trauma etc. Attempts to characterize the neural pathways have involved a variety of experimental approaches including anatomical investigations at the light and electron microscope level, biochemical correlates of transmitter turnover with hypothalamo-pituitary activity, functional studies following electrical stimulation or lesioning of 'specific' pathways or pharmacological manipulation of neurochemical transmission and in vitro studies utilising acutely removed or cultured hypothalamic tissue. In vivo studies have relied primarily on the use of indirect indices of hypothalamic function (i.e. estimates of circulating ACTH and/or corticosterone) although in some recent studies the ir-CRF-41 and ir-AVP contents in portal blood have been determined (*Horn, Robinson & Fink, 1985; Plotsky, Otto & Sutton, 1987*). Early in vitro studies involved the measurement of total bioactive CRF but the advent of sensitive radioimmunoassays now permits direct parallel measurements of the two peptides.

From the evidence available it appears that many of the numerous ascending and descending pathways which converge on the hypothalamus are involved. Of particular importance appear to be fibres originating in the brain stem (*Cunningham & Sawchenko, 1988*), ràphe nucleus, hippocampus and amygdala (*Mason, 1959; Knigge, 1961; Beaulieu, Pelletier, Vaudry & Barden, 1989*) but other pathways originating for example in the cortex probably also play a significant role as too do interneurones within the hypothalamus (*Silverman et al., 1989*). Not surprisingly a spectrum of neurotransmitter substances and neuropeptides has been implicated in these mechanisms. Although there are some reports to the contrary (*Vermes & Telegdy, 1972; 1976; Telegdy & Vermes, 1973; Berger, Barchas & Vernikos-Danellis, 1974*), it is generally agreed that cholinergic (*Abe & Hiroshige, 1974; Bradbury, Burden, Hillhouse & Jones, 1974; Hillhouse, Burden & Jones, 1975; Buckingham & Hodges 1977b; 1979; Suda, Yajima, Tomori, Sumitomo, Nakagami, Ushiyama, Demura & Shizume, 1987; Calogero, Gallucci, Bernardini, Saoutis, Gold & Chrousos 1988b; Clarke & Gillies, 1988; Hillhouse & Milton 1989a*) and 5-hydroxytryptaminergic (*Abe & Hiroshige, 1974; Chambers & Brown, 1976; Jones,*

Hillhouse & Burden, 1976; Buckingham & Hodges 1977b; 1979; Holmes, Di Renzo, Beckford, Gillham & Jones, 1982; Hillhouse & Milton 1989a) pathways are stimulatory to CRF-41/AVP release while GABA-ergic neurones exert a powerful inhibitory tone (Makara & Stark, 1974; Jones et al., 1976; Buckingham & Hodges 1977b; 1979; Plotsky et al., 1987; Hillhouse & Milton 1989b). The role of catecholamines is less clear cut. Data from a variety of in vivo studies (Van Loon, Scapagnini, Cohen & Ganong, 1971; Van Loon, Hilger, King, Boryczka & Ganong, 1971; Scapagnini, Annunziato, Lombardi, Olivier & Preziosi, 1975; Ganong, 1980; Steiner & Grahame-Smith, 1980) and from in vitro experiments in which 'bioactive' CRF was determined (Jones et al., 1976; Buckingham & Hodges, 1979) suggested that (nor)adrenergic pathways inhibit the secretion of the releasing factor. By contrast, other studies, including those in which CRF-41 and AVP were measured directly by immunoassay in the portal blood (Plotsky, 1987) and in vitro (Jones, Gillham, Campbell, Al-Taher, Chuang & Di Sciullo, 1987; Besser, Grossman, Rees & Tsagarakis, 1988; Calogero, Bernardini, Gold & Chrousos, 1988a; Hillhouse & Milton, 1989b; Widmaier, Lim & Vale, 1989), favour a stimulatory role for the catecholamines. Interestingly, a similar controversy surrounds the role of opioids which, in vivo and in vitro, stimulate the release of bioactive CRF in the rat (Lotti, Kokka & George, 1969; Gibson, Ginsburg, Hall & Hart, 1979b; Buckingham, 1982a; 1984; Buckingham & Cooper, 1984a b; 1986) but depress the secretion of immunoreactive CRF-41 (Plotsky, 1986; Tsagarakis, Navara, Rees, Besser, & Grossman, 1989). The reason for these discrepancies is not clear but may reflect the ability of noradrenaline and opioids to elicit the release of a potent corticotrophin release inhibitory factor (Cover & Buckingham, 1989b; Tsagarakis et al., 1989). Less attention has focussed on the roles of the other transmitter substances but, from the evidence available, it appears that substances such as excitatory amino acids (Hokfelt, Fahrenkrug, Ju, Ceccatelli, Tsuruo, Meister, Mutt, Rundgren, Brodin & Terenius et al., 1987; Ceccatelli, Eriksson & Hokfelt, 1989), ANP (Takao et al., 1988b), neuropeptide Y (Liposits, Sievers & Paull, 1988), angiotensin II (Klingbeil, Keil, Chang & Reid, 1988) and VIP (Hokfelt et al., 1987; Ceccatelli et al., 1989) may also be important in this respect.

The role of cytokines in the regulation of Hypothalamo-pituitary-adrenocorticotrophic function.

Cytokines, including the interleukins I-VI and tumour necrosis factor (TNF) are immunoregulatory proteins synthesised by a wide variety of cells in response to infection or inflammation (*Oppenheim, Kovács, Matsushima & Durum, 1986*) and play key roles in the pathophysiological responses. For example Interleukin I, which is produced by macrophages, keratinocytes and brain glial cells, has immunological effects including the control of differentiation and activation of lymphocytes and the stimmulation of lymphokine production (*Rosenwasser, Dinarello & Rosenthal, 1979; Maizel, Mehta, Ford, & Lachman, 1981; Oppenheim, Stadler, Siraganian, Mage & Mathieson, 1982; Mizel, 1982*). In addition it also mediates several components of the acute phase reaction, including the febrile response and synthesis of acute phase proteins (*Dinarello, 1984; Breder, Dinarello & Saper, 1988*). The production and the action of several cytokines including IL-1 and other mediators of inflammation are inhibited by glucocorticoids (*Wahl, Altman & Rosenstreich, 1975; Del Rey, Besedovsky & Sorkin, 1985*) reflecting the anti-inflammatory properties of the steroids. The communication between the immune and neuroendocrine systems appears to be two-way (*Cross, Markesbury, Brooks & Roszman, 1980; Guillemin, Colin & Melnechuck, 1985*) and there is now substantial evidence that certain cytokines stimulate the HPA axis. This provides a mechanism whereby adrenocortical activity could be stimulated following immune recognition of infection and thus rapidly precipitate the stress response. Indeed during antigenic challenge glucocorticoid secretion is enhanced in parallel with the intensity of the immune response (*Berczi, Nagy, Olschowka & Livnat, 1985; Grossman, 1985; Plotnikoff, Murgo, Miller, Corder & Faith, 1985*)

Recently IL-1 (*Besedovsky, Del Rey, Sorkin & Dinarello, 1986; Besedovsky & Del Rey, 1987; Del Rey, Besedovsky, Sorkin & Dinarello, 1987; Katsuura, Gottschal, Dahl & Arimura, 1988; Sharp, Matta, Peterson, Chao & McAllen, 1989*), IL-6 (*Naitoh, Fukata, Tominaga, Nakai, Tamai, Mori & Imura, 1985*) and TNF (*Sharp et al., 1989*) have been shown to stimulate the secretion of ACTH and corticosterone but their mechanism and site of action of HPA activation is not yet clear. IL-1 however does not appear to act directly at the level of the adrenal cortex to stimulate the secretion of corticosterone (*Woloski, Smith, Meyer, Fuller & Blalock, 1985*). Evidence for a direct effect of IL-1 at the level of the pituitary remains contradictory. Several workers have shown IL-1 induces the release of ACTH <u>in vitro</u> (*Woloski et*

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al., 1985; Bernton, Breach, Holaday, Smallridge & Fein, 1987) whereas others have found it to be without effect (Berkenbosch, Van Oers, Del Rey, Tilders & Besedovsky, 1987; Sapolsky, Rivier, Yamamoto, Plotsky & Vale, 1987; Tsagarakis, Gillies, Rees, Besser & Grossman, 1989) as also is TNF (Sharp et al 1989). More recent in vivo studies suggest that IL-1 does not act at the pituitary level but at a site in the brain (Katsuura et al., 1988; Ovadia, Abramsky, Barak, Conferti, Saphier & Weidenfeld, 1989) and that its central action may involve prostaglandins (PG) since IL-1 induced ACTH release is blocked by the administration of indomethacin (Katsuura et al., 1988). The exact site of action of the cytokines within the brain remains to be determined, however recent reports that IL-1 (Sapolsky et al., 1987; Tsagarakis et al., 1989) and IL-6 (Naitoh et al., 1988) stimulate CRF-41 secretion suggest that the PVN of the hypothalamus may be the target site.

The role of corticosteroids in the regulation of Hypothalamo-pituitary-adrenocorticotrophic function.

Corticosteroids modulate the activity of the hypothalamo-pituitary-adrenal axis by inhibiting directly and indirectly the secretion of corticotrophin releasing factors and ACTH. Thus, a reduction in circulating corticosteroids produced by e.g. adrenalectomy results in a rise in the serum ACTH concentration and exaggeration of the hypothalamo-pituitary-adrenocorticotrophic response to stress (*Buckingham & Hodges, 1974; 1975; Buckingham, 1979*). Conversly administration of corticosteroids depresses both the resting and stress-induced release of corticotrophin. Several mechanisms have been postulated to explain this phenomenon which is referred to as 'negative feedback'.

(i) Sayers and Sayers' hypothesis.

Sayers and Sayers (1947) were the first to show that stress induced secretion of ACTH was inhibited by exogenous steroids and that the degree of inhibition depended on the dose of corticosteroid administered. They suggested (i) that stress increases the corticosteroid requirement of peripheral tissue and that the resultant decrease in blood steroid concentration stimulated the pituitary to release ACTH and (ii) that the degree of peripheral steroid utilization was proportional to the intensity of the stress applied. A number of subsequent studies did not support this hypothesis (*Moya & Selye, 1948; Hall, Finerty, Hall & Hess, 1951; Fortier, Yrarrazaval & Selye, 1951; Cox, Hodges, & Vernikos, 1958*) which was finally rejected when it became possible to estimate corticosteroid levels directly and it became apparent that stress always produces a rise not a fall in circulating glucocorticoids (*Herbst, Yates, Glenister & Urquhart, 1960*).

(ii) The variable set-point hypothesis

On the basis of studies in which corticosteroids were administered intravenously, Yates, Leeman, Glenister & Dallman (1961) suggested that, under conditions of stress, the 'set-point' of the negative feedback control mechanism was reset at a higher level so as to permit ACTH release until such time that the circulating levels of corticosteroids attained the new set-point. In this event, the pituitary adrenocorticotrophic response to a specific stress would be prevented by

administration of a single dose of corticosterone in a dose sufficient to raise the serum concentrations of corticosterone at the time of the stress to levels normally precipitated by the stress. Other findings failed to substantiate this hypothesis and it was shown that although chronic changes in the level of circulating corticosteroids, such as those induced by adrenalectomy or prolonged corticosteroid therapy, influenced markedly the ACTH response to stress (*Hodges & Mitchley, 1970a b; Buckingham & Hodges, 1974; 1976; Buckingham, 1979*) acute manipulation of the steroid level was not effective in this respect. Thus application of a stressor 15 or 30 min after a single intra-peritoneal or subcutaneous injection of corticosterone (i.e. at a time when the serum steroid levels were maximal) failed to suppress the pituitary-adrenocortical response to stress (*Hodges & Jones, 1963*). Suppression of ACTH release was however observed several hours later when the steroid level had declined (*Smelik, 1963; Hodges & Jones, 1964; Hodges & Sadow, 1967; Jones, Brush & Neame, 1972*). Thus it was proposed that the ACTH response to stress was largely independent of the concentration of the steroid in the blood at the time of stress and that the regulatory effects of the steroids required time to develop i.e. the feedback mechanism was delayed. (*Hodges & Jones, 1963; Smelik, 1963; Hodges & Sadow, 1967; Jones et al., 1972*).

(iii) Fast and delayed feedback

Further studies on the negative feedback mechanism showed that the control of ACTH secretion occurred in two temporally distinct phases (*Dallman & Yates, 1969; Jones et al., 1972*). Thus inhibition of the stress response was observed following i.p. infusion of corticosterone either 5 min (fast feedback) or 2 h (delayed feedback) before stress. The period between these two phases was termed the 'silent period' during which no reduction of the stress response could be seen. Delayed feedback was further subdivided into intermediate feedback (2-10h) and slow feedback (over hours to days).

Fast feedback was found to depend on the rate of increase of corticosterone in the blood and to be saturated at supra-physiological concentrations of corticosterone (*Jones et al., 1972; Abe & Critchlow, 1977; Kaneko & Hiroshige, 1978a; Fehm, Voigt, Kummer, Lang & Pfeiffer, 1979*). Delayed feedback, on the other hand, was found to depend on the concentration of steroid previously reached in the plasma and was therefore termed 'proportional feedback' (*Dallman & Yates, 1969*).

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Sites of feedback action:

The actual or predominant site of feedback action of the corticosteroids has been the subject of much controversy. The adenohypophysis (*Fleisher & Rawls, 1970a b c*), the hypothalamus (*Hillhouse & Jones, 1976; Liposits, Uht, Harrison, Gibbs, Paull & Bohn, 1987; Uht, McKehry, Harrison & Bohn, 1988*) (notably in the paraventricular region) and the hippocampus (*McEwen, de Kloet & Rostene, 1986*) (CA1 & CA2 regions) are rich in type II corticosterone receptors (i.e. glucocorticoid receptors, GR) (*Fuxe, Wilkström, Okret, Agnati, Härfstrand, Yu, Granholm, Zoli, Vale & Gustafsson, 1985; Sutanto & de Kloet, 1987; Kiss, Van Eekelen, Reul, Westphal & deKloet, 1988; Sutanto, Van Eekelen, Reul & de Kloet, 1988*) and type II GR mRNA (*Magarinos, Ferrini & De Nicola, 1989*) and each of these areas may contribute to the regulatory actions of the steroids on HPA function.

(i) Pituitary site

Early evidence that corticosteroids may act at the level of the corticotrophs was provided by studies which demonstrated the ability of the steroids to suppress the pituitary adrenocorticotrophic response to AVP and 'CRF' in intact animals (*De Wied & Mirsky, 1959; Arimura, Bowers, Schally, Saito & Miller, 1969; Gonzalez-Luque, L'Age, Dhariwal & Yates, 1970*). Subsequently, dexamethasone was shown to reduce the pituitary ACTH content and adrenal weight (*Chowers, Conforti & Feldman, 1967*) and to attenuate the ACTH response to stress (*Russell, Dhariwal, McCann, & Yates, 1969*) when injected into the pituitary gland. In addition, when given systemically, the steroids were found to reduce the plasma corticosterone concentrations in intact and hypophysectomised rats bearing heterotopic pituitary glands (*Kendall & Allen, 1968*). Other workers failed to find any inhibition of ACTH release by intra-pituitary infusions of steroid (*Kendall, 1962; Stark, Gyevai, Acs, Szalay & Varga, 1968*), possibly because the exposure of the pituitary corticotrophs to the steroid was inadequate (*Gonzalez-Luque et al., 1970*).

Since then, both fast and delayed feedback effects have been demonstrated at the pituitary level utilising in vivo and in vitro techniques. In vivo corticosteroid pre-treatment inhibits CRF-induced ACTH release in intact (*Keller-Wood, Leeman, Shinsako & Dallman, 1988*) and hypothalamic lesioned animals (*Jones, Hillhouse & Burden, 1977*). In vitro glucocorticoids inhibit the release of ACTH from pituitary

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segments (*Arimura et al., 1969; Buckingham & Hodges, 1977a; Widmaier & Dallman, 1984*), isolated anterior pituitary cells (*Sayers & Portanova, 1974; Buckingham, Cowell & Flower, 1987*), cultured anterior pituitary cells (*Fleisher & Rawls, 1970a b c; Abou-Samra, Catt & Aguilera, 1986b*) and cultured mouse pituitary tumour cells (*Herbert, Allen & Paquette, 1978*).

The rapid effects appear to involve inhibition of release only and are not associated with alterations in pituitary ACTH content (*Buckingham & Hodges, 1977a*). By contrast, the delayed effects involve inhibition of both the synthesis and release of the peptide (*Buckingham, 1979*). Thus, in vitro, delayed feedback is associated with a reduction in ACTH content (*Buckingham, 1979; 1982b; Abou-Samra et al., 1986b*) and in POMC mRNA (*Nakamura, Nakamishi, Sueoka, Imura & Numa, 1978*) while in vivo pituitary POMC mRNA and ACTH content are reduced by pro-longed corticosteroid treatment (*Koch, Bucher & Mailhe, 1974; Buckingham & Hodges, 1977c*) but increased by adrenalectomy or adrenal enucleation (*Fortier & De Groot, 1959; Hodges & Jones, 1964; Nakanishi, Kita, Taii, Imura & Numa, 1977; Schachter, Johnson, Baxter & Roberts, 1982*).

(ii) Hypothalamic site

Early evidence for an action at the hypothalamic level was derived from studies in which implantation of corticosteroids in the hypothalamus was shown to inhibit ACTH release (*Smelik & Sawyer, 1962; Chowers, Feldman & Davidson, 1963; Corbin, Mangili, Motta & Martini, 1965; Grimm & Kendall, 1968*). This approach however was criticized as the possibility existed that the implanted steroid diffused from the median eminence to the hypophyseal portal system and thereby acted at the level of the adenohypophysis (*Grimm & Kendall, 1968*).

As techniques improved and it became possible to measure hypothalamic CRF activity using a bioassay technique it became evident that adrenalectomy produced an increase in CRF bioactivity which could be reversed by corticosteroid replacement (*Vernikos-Danellis, 1965; Sato, Sato, Shinsako & Dallman, 1975; Buckingham, 1979*) and that, in intact rats, corticosteroids not only reduce the hypothalamic CRF content (*Buckingham & Hodges 1977c; Buckingham, 1979*) but also prevent the rise which normally occurs in response to stress (*Buckingham, 1979*). These results suggested that the delayed feedback effect of corticosteroids on resting and stress-induced ACTH release was effected in part by inhibition of the synthesis and release of CRF bioactivity (*Buckingham, 1979*). Further evidence to support this concept was

provided by in vitro studies which also demonstrated the equisite sensitivity of the hypothalamus to the regulatory effects of the steroids. Thus in vitro, a delayed feedback effect, involving inhibition of CRF release and a reduction in CRF content, was readily demonstrated with very low concentrations of steroid (*Jones & Hillhouse, 1976; Jones et al., 1977, Buckingham, 1982b*). Rapid feedback, which was also sensitive to low levels of steroids, was associated with a small increase in CRF content suggesting an effect on release comparable to that observed at the pituitary level (*Buckingham 1982b*).

More recently RNA blot hybridisation, immunocytochemistry and in situ hybridisation histochemistry (*Lightman & Young, 1989*) together with direct measurements of CRF-41 and AVP in portal blood and in vitro have been used to study the effects of corticosteroids on the synthesis and release of the two peptides. Reports that adrenalectomy selectively initiates dexamethasone reversible expression of AVP mRNA and AVP in the parvocellular neurones of the PVN (*Davis et al., 1986; Kovács, Kiss, Makara, 1986*) led to suggestions that the delayed regulatory actions of glucocorticoids may involve primarily alterations in AVP. This concept was supported by the observations of Fink, Robinson and Tannahill (1988) who found marked changes in AVP but not the CRF-41 content of portal blood following adrenalectomy and dexamethasone treatment. Other data however strongly implicate alterations in CRF-41 and, hence changes in CRF-41 message and immunoreactive CRF-41 have been reported in animals in which the steroid milieu has been manipulated (*Plotsky & Vale, 1984; Jingami, Matsukura, Numa & Imura, 1985; Kovács et al., 1986; Plotsky, Otto & Sapolsky, 1986; Kovács & Mezey, 1987*)

(iii) Extrahypothalamic sites

Early studies indicated that corticosteroids may exert their inhibitory effects at sites in the median eminence (*Smelik & Sawyer, 1962; Chowers et al., 1963; Chowers et al., 1967; Grimm & Kendall, 1968*) and at a variety of extrahypothalamic sites such as regions of the mesencephalic relicular formation (*Corbin et al., 1965*), amydala (*Davidson & Feldman, 1967*) and in the septum or thalamus (*Bohus, Nyakas & Lissak, 1968; Dallman & Yates, 1968*). From the evidence available it now appears that the hippocampus is the most important in this respect (*Kovács et al., 1986*).

Mechanism of action of the steroids.

The fast feedback effects of glucocorticoids are too rapid to involve classical steroid-receptor mediated de novo protein synthesis and may be due to the action of the steroid on a receptor close to or in the membrane, causing membrane stabilisation which may involve calcium (Ca^{2+}) flux (*Jones & Hillhouse, 1976*). However, delayed feedback effects appear to be mediated by the type II glucocorticoid receptor and to involve protein synthesis. Certainly pre-incubation of pituitary tissue with actinomycin D or cycloheximide prior to dexamethasone treatment, prevents the suppressive effect of the steroids (*Arimura et al., 1969; Portanova & Sayers, 1974; Brattin & Portanova, 1977; Phillips & Tashjian, 1982; Abou-Samra et al., 1986b*). Similarly, at the hypothalamic level alterations in protein synthesis are involved (*Jingami et al., 1985; Davis et al., 1986; Kovács & Mezey, 1987*).

The nature of the second messenger protein effecting the type II responses in these tissues is not known. Considerable progress has however been made in the characterisation of second messenger effecting the actions of glucocorticoids in other tissue types notably in macrophages (*Di Rosa & Persico, 1979; Blackwell, Carnuccio, Di Rosa, Flower, Langham, Parente, Persico, Russell-Smith & Stone, 1982*). Such proteins may be important in the manifestation of the anti-inflammatory actions of steroids (*Flower & Blackwell, 1979; Carnuccio, Di Rosa & Persico, 1980*) but their role, if any, in the expression of the steroid-induced suppression of HPA function is unknown.

The anti-inflammatory action of steroids

The anti-inflammatory actions of the corticosteroids are mediated primarily by specific intracellular receptors and involve the generation of second messenger proteins. Thus, they are blocked by the glucocorticoid antagonist, RU 38486 (*Peers, Moon & Flower, 1988*) and by inhibitors of RNA/protein synthesis (*Tsurufuji, Sugio & Takemasa, 1979*). Two putative second messenger proteins have been identified, lipocortin (*DiRosa, Flower, Hirata, Parente & Russo-Marie, 1984*) and vasocortin (*Carnuccio, DiRosa, Guerrassio, Iuvone & Sartebin, 1987*). The anti-inflammatory actions of the former have been largely attributed to its capacity to block the activity of the enzyme phospholipase A_2 (PLA_2) and hence to prevent the generation of pro-inflammatory eicosanoids (*Flower & Blackwell, 1979; Carnuccio et al., 1980*). Several lines of evidence support this concept.

Glucocorticoids inhibit stimulus evoked prostaglandin liberation from rabbit mesenteric blood vessels (*Gryglewski, Panczenko, Korbut, Grodzinska & Ocetkiewicz, 1975*), cultured fibroblasts (*Hong & Levine, 1976*), and guinea-pig lungs in vitro (*Piper & Vane, 1969; Nijkamp, Flower, Moncada & Vane, 1976*). Their actions are overcome by the addition of arachidonic acid suggesting that the steroids interfere with the release of the fatty acid from membrane phospholipids, possibly by inhibiting the activity of the membrane associated protein, PLA_2. The steroid induced inhibition of arachidonate release is also dependent on the de novo generation of protein for, in a variety of preparations including the perfused lung preparation (*Flower & Blackwell, 1979*), renal interstitial cells (*Danon & Assouline, 1978*), rat renomedullary cells (*Russo-Marie, Paing & Duval, 1979*) and rat peritoneal lavage cells (*Di Rosa & Persico, 1979*) the responses to steroids are also readily reversed by mRNA and protein synthesis inhibitors. Moreover, glucocorticoid treated lungs produce a soluble factor which blocks stimulated prostaglandin release from parallel lungs rendered insensitive to steroids by the administration of a protein synthesis inhibitor (*Flower & Blackwell, 1979*). The bioactivity of this factor is destroyed by exposure to heat or proteolytic enzymes (*Flower & Blackwell, 1979*).

The protein second messenger isolated from rat peritoneal leucocytes was termed macrocortin (*Blackwell, Carnuccio, Di Rosa, Flower, Parente & Persico, 1980*). Its activity was found to be indistinguishable from those of two steroid inducible proteins, lipomodulin and renocortin isolated from neutrophils (*Hirata, Schiffmann,*

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Venkatasubramanian, Salomon & Axelrod, 1980; Hirata, 1981) and rat renomedullary interstitial cells *(Cloix, Colard, Rothhut & Russo-Marie, 1983)* respectively. The proteins were thus collectively renamed 'lipocortin' *(Di Rosa et al., 1984)*. Following purification, the amino-acid sequence of Lipocortin I was determined in 1986 *(Wallner, Mattaliano, Hession, Cate, Tizard, Sinclair, Foeller, Pingchang Chow, Browning, Ramachandran & Pepinsky, 1986)*.

A number of studies have emphasised the importance of corticosteroids in the induction of lipocortin. For example, adrenalectomy decreases and steroid-treatment increases the 'anti-PLA$_2$' activity of rat peritoneal lavage fluid *(Blackwell et al., 1982)*. In addition, several other groups have reported the presence of proteins in plasma, serum or cell extracts which possess anti-PLA$_2$ activity in vitro *(Authi, Solanky & Traynor, 1982; Bartolf & Franson, 1987; Sato, Miyahara & Utsumi, 1988; Sorenson, Kelly, Murray & Nelson, 1988)*. In vivo and in vitro experiments suggest that the steroid induced release of lipocortin in macrophages is divided into phases both of which are dependent on protein synthesis. An 'active release' phase which begins after 30 min of exposure, and a 'synthesis' phase which occurs 4 to 5 hours following steroid administration. In vitro lipocortin is released from intact macrophages within 30 mins of steroid treatment, with only a small amount remaining in the cells after 150 mins *(Blackwell et al., 1980)*. Following resuspension of the cells in fresh media, a lag period of approx. 2-2 $\frac{1}{2}$ hours occurs during which the macrophages are unresponsive to inhibitory actions of steroids, before fresh lipocortin was re-synthesised in the cells *(Carnuccio, Di Rosa, Flower & Pinto, 1981)*. Similarly in vivo, rat peritoneal macrophages generate lipocortin in response to steroids *(Blackwell et al., 1982)*. Release occurs within 30 min. of steroid injection, reaches a peak at 60 min, and returns to control levels within 3 to 4 hours.

Purified and recombinant lipocortin inhibit, in a concentration dependant manner, the activity of PLA$_2$ in a cell free system *(Hirata, 1981)*. Lipocortins have been implicated in the anti-PLA$_2$ activity exhibited by dexamethasone which inhibits eicosanoid production by rat polymorphonuclear neutrophils *(Fradin, Rothhut, Poincelot-Canton, Errasfa & Russo-Marie, 1988)*. Recombinant human lipocortin 1 has been shown to possess anti-PLA$_2$ activity when infused into guinea-pig lung preparations *(Cirino, Flower, Browning, Sinclair & Pepinsky, 1987)*. In addition it is effective against pancreatic PLA$_2$ in vitro *(Northup, Valentine-Braun, Johnson, Severson & Hollenberg, 1988)* but is unable to inhibit arachidonate release from cultured zymosan-stimulated mouse peritoneal macrophages, or inflammation in a rat paw

odema test (*Northup et al., 1988*).

More recently human recombinant lipocortin 1 has been shown to inhibit PLA_2 from various sources including P388D1 macrophage-like cell line (*Davidson, Lister & Dennis, 1990*)

Like the corticosteroids, lipocortin also inhibits the PLA_2-dependant generation of leukotrienes, PGE_2 (*Parente, Di Rosa, Flower, Ghiara, Meli, Persico, Salmon & Wood, 1984*) and lyso-PAF (*Parente & Flower, 1985*) from zymosan activated macrophages. Its effects are abolished by co-incubation with a neutralising antibody, RM23, as too are those of hydrocortisone (*Parente & Flower, 1985*).

Lipocortin is also effective in vivo. Thus protein extracts from peritoneal lavage fluid from dexamethasone or saline treated animals (*Blackwell et al., 1982*), lipocortin (*Hirata, 1983*) and anti-phospholipase proteins with lipocortin-containing fractions (*Parente et al., 1984*) have all been shown to possess anti-inflammatory properties in the carrageenin rat paw odema model. In contrast, in the adrenalectomized animal in which the level of steroids and, thus, lipocortin are reduced the acute inflammatory response to carrageenin is greatly increased compared with that of sham operated controls (*Flower, Parente, Persico & Salmon, 1986*). Furthermore, the eicosanoid and lyso-PAF content of inflammatory fluid from adrenalectomized rats is increased compared with that from control or adrenalectomized-steroid treated animals (*Parente & Flower, 1985*). Similarly macrophages from adrenalectomized rats release greater quantities of eicosanoids ($PGF_{1\alpha}$, TXB_2 and LTB_4) (*Flower et al., 1986*) and LTB_4 (*Vincent, Zijlstra, Van den Brock & Gezel, 1986*) when stimulated in vitro compared with those from control animals. It is possible that the effects may be attributable to the capacity of adrenalectomy to enhance the effects of pro-inflammatory substances. However Peers and colleagues (1988) showed that administration of the glucocorticoid receptor antagonist RU 38486 produced a qualitatively similar effect on inflammation and lyso-Paf release suggesting that the adrenal cortex and lipocortin are important for the development of anti-inflammatory effects.

The biological activity of lipocortin is Ca^{2+} dependent (*Schlaepfer & Haigler, 1987*) and is readily destroyed by phosphorylation (*Hirata, 1981; 1983*); indeed, in some cells it may provide a substrate for the epidermal growth factor- and insulin-receptor tyrosine kinases (*Pepinsky & Sinclair, 1986; Karasik, Pepinsky & Kahn, 1988; Karasik, Pepinsky, Shoelson & Kahn, 1988*). Recently doubts have arisen over its ability to inhibit the activity of PLA_2 and the bulk of the evidence available now

indicates that the apparent inhibition of PLA_2 is the consequence of the protein binding, in a Ca^{2+} dependent manner, to the phospholipid substrate thereby reducing its availability (*Aarsman, Mynbeek, Van den Bosch, Rothhut, Prieur, Comera, Jordon & Russo-Marie, 1987; Davidson, Dennis, Powell & Glenney, 1987; Haigler, Schlaepfer & Burgess, 1987; Rothhut, Comera, Prieur, Errasfa, Minassian & Russo-Marie, 1987*).

Controversy has arisen over the site of lipocortin action. Early work indicated that lipocortin is a cell-membrane protein which inactivates membrane phospholipases through an extracellular action. Hirata et al. (1980) showed that exposure of neutrophils to the proteolytic enzyme, pronase, prevented the steroid inhibition of neutrophil chemotaxis and suggested that the inhibitory protein was therefore accessible to the hydrolytic activity of the enzyme from the outside of the cell. Further evidence came from work by Flower and Blackwell (1979) who showed that PLA_2 inhibition in the guinea-pig perfused lung system occurred rapidly following lipocortin infusion, and declined once the perfusion had stopped. These workers thought that it was unlikely that such a large molecular weight compound could gain easy access to the cytosol and therefore must act extracellularly. Finally, an anti-lipocortin monoclonal (4-4C3) was found to stain proteins on cell surfaces (*Hirata, 1983; Hirata & Iwata, 1983*). However, these findings do not preclude the possibility that lipocortins also act intracellulary to attenuate the production of eicosanoid second messengers (*Dartois & Bouton, 1988*) or to act as substrates for receptor tyrosine kinases (*Karasik, Pepinsky, Shoelson et al., 1988; Varicovski, Chahwala, Whitman, Cantley, Schindler, Chow, Sinclair & Pepinsky, 1988; Johnsson, Marriott & Weber, 1988*).

(i) Purification of Lipocortin

In early attempts to purify and sequence lipocortin, peritoneal lavage fluid from steroid-treated rats (*Blackwell et al., 1982*) and media from glucocoritcoid stimulated cultured neutrophils (*Hirata et al., 1980; Hirata, 1983*) or supernatants from dexamethasone treated rat renomedullary cells (*Cloix et al., 1983*) were used as sources of the protein. Initially a combination of sequential chromatography on DEAE-ion exchange resins and sephadex G-75 was used. Subsequently high performance liquid chromatography (HPLC) (*Blackwell et al., 1982*) together with PLA_2 affinity columns were incorporated into the purification procedure (*Parente &*

Flower, 1985). The purified protein was found to exist predominantly as a 37KDa species. However, smaller molecular weight 30K, 24K and 15K forms were also found to exhibit similar inhibitory properties (*Pepinsky, Sinclair, Browning, Mattaliano, Smart, Chow, Falbel, Ribolini, Garwin & Wallner, 1986*).

In order to sequence lipocortin, a purified sample of the protein was subjected to proleolytic cleavage and the amino acid sequence of several tryptic fragments determined (*Pepinsky et al., 1986*). Appropriate oligonucleotide probes were synthesised and used to locate the human gene and to determine the complete amino acid sequence and the primary structure of lipocortin (*Wallner et al., 1986*).

In 1986, the genes encoding two structurally related 38kDa proteins, lipocortins I & II, were cloned. More recently the human genes which code lipocortin I & II have been regionally localised in the human genome (*Huebner, Cannizzaro, Frey, Hecht, Hecht, Croce & Wallner, 1988*). The peptides were shown to be highly polar (approximately one third of their amino acids were charged) and to exhibit 50% sequence homology. The C-terminus of each included four repeat regions of 70 amino acids each containing a virtually identical (90% homology) 17 amino acid sequence. The N-terminal sequence of the two proteins differed markedly but both contained areas susceptible to proteolysis. Functionally, the C-terminal repeat sequences appear to be important for the Ca^{2+}/phospholipid binding properties of the molecule while the N-terminal, which contains potential phosphorylation sites (*Karasik et al., 1988; Karasik, Pepinsky, Shoelson et al., 1988; Varticovski et al., 1988*) appears to confer biological specificity (Fig 2.).

Several other Ca^{2+}/phospholipid binding proteins have since been characterized. These include protein II (*Weber, Johnsson, Plessmann, Van Soling, Ampe & Vandekerchove, 1987*), lipocortin III and V (*Pepinsky, Tizard, Mattaliano, Sinclair, Miller, Browning, Pingchang, Chow, Burne, Huang, Pratt, Wachter, Hession, Frey & Wallner, 1988*) and P68/67kDa Calelectrin (*Crompton, Owens, Totty, Moss, Waterfield & Crumpton, 1988; Südhof, Slaughter, Leznicki, Barjon & Reynolds, 1988*), endonexin-II (*Schlaepfer, Mehlman, Burgess & Haigler, 1987*), human placenta anticoagulant protein (*Funakoshi, Henimark, Hendrickson, McMullen & Fujikawa, 1987; Funakoshi, Hendrickson, McMullen & Fujikawa, 1987*) lipocortin II/ P36 (*Erikson, Tomasiewicz & Erikson, 1984; Huang, Wallner, Mattaliano, TTizard, Burne, Frey, Hession, McGray, Sinclair, Pingchang Chow, Browning, Ramachandran, Tang, Smart & Pepinsky, 1986; Glenney, 1986; Saris, Tack, Kristensen, Glenney & Hunter, 1986; Hom, Sudhof, Lozano, Haindl & Rocha,*

1988), calpactins I & II (*Glenney, 1986; Glenney, Tack & Powell, 1987; Hom et al., 1988*) and chromobindins (*Creutz, Zaks, Hamman, Crane, Martin, Gould, Oddie & Parsons, 1987*). Some of these proteins have proved to be closely related or identical with one another and, hence, much confusion has arisen over their terminology which is summarised in Table 1.

Unique
N-terminal

repeating Ca^{2+}/ lipid binding units

N   1   43   46   116   118   188   203   273   278   348   C

lys - gly - x - gly - thr - asp - glu - x - x - leu - ile - leu - x - ile - leu - ala - x - arg

37
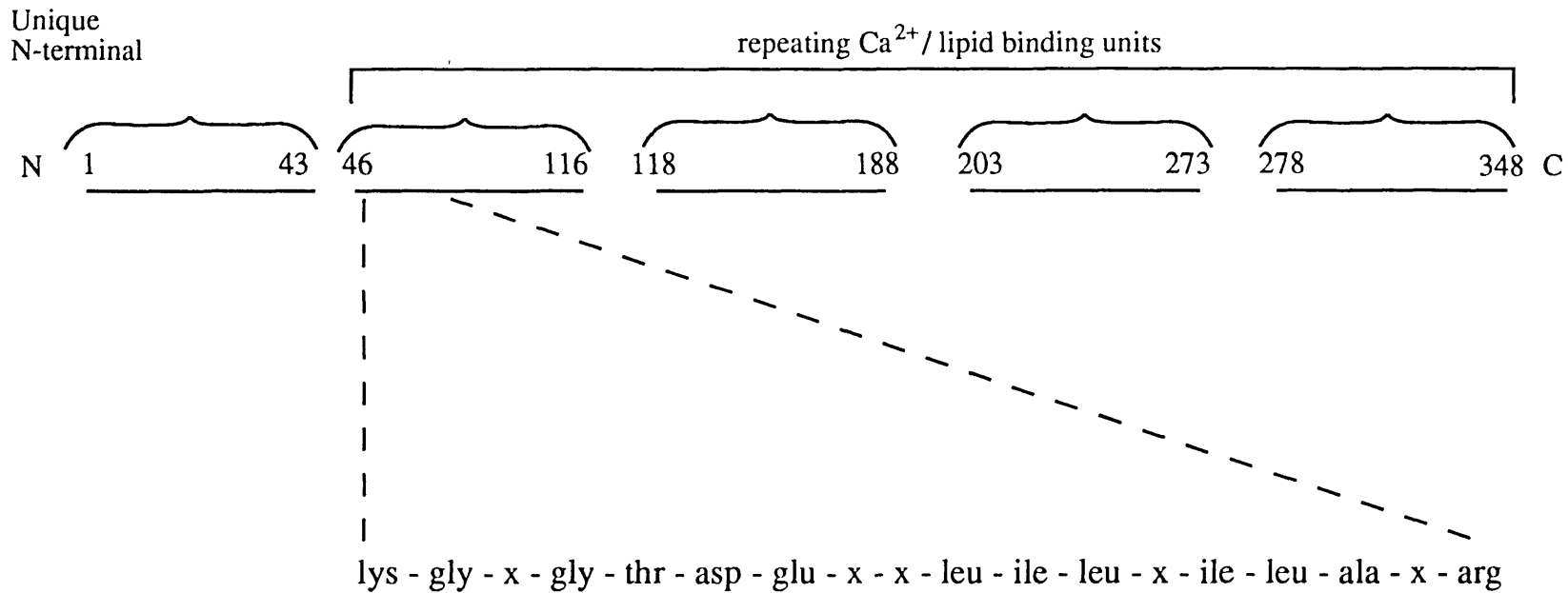
Fig. 2 : Internal structure of lipocortin 1 . The lipocortin 1 molecule possesses five domains : a unique N-terminus and four reigions each of which begin with the same 17 amino acid sequence. This type of molecular architecture seems to be common in proteins which bind Ca^{2+}and certain phospholipids and may be the mechanism by which lipocortin attaches to cell membranes. Adapted from Flower (1988).

Lipocortin	Mass kDa	Apparent identity
Lipocortin I	38	Lipocortin I[a,t,u,w,x,z]
		p35[b-e]
		Chromobindin-9[f]
		Calpactin II[e,w]
Lipocortin II	38	Lipocortin II[a,z]
		p35[a,e,g,h]
		Chromobindin-8[f]
		Calpactin I[e,g,v]
		Protein I[i,j]
Lipocortins III and IV	35	Endonexin[i,k,l,t,v,y]
		Chromobindin-4[f,y]
		35-kDa-Calcimedin[m]
		p32.5 Calelectrin[k]
		Protein II[i,j,v]
Lipocortin V	35	Lipocortin/(Renocortin)[n]
		Chromobindin-5[f]
		Endonexin-II[o,u]
		Anticoagulant protein (PAP)[p]
Lipocortin VI	68	Protein III[q]
		p68[l,r,β]
		Chromobindin-20[f]
		67-kDa-Calcimedin[m,α]
		67-kDa-Calelectrin[l,s,u,v]

a Huang et al., 1986	b Fava and Cohen, 1984
c Pepinsky and Sinclair, 1986	d De et al., 1986
e Glenney, 1986	f Creutz et al., 1987
g Saris et al., 1986	h Erikson et al., 1984
i Weber et al., 1987	j Gerke and Weber, 1984
k Geisow et al., 1986	l Geisow and Walker, 1986
m Smith and Dedman, 1986	n Rothhut et al., 1987
o Schlaepfer et al., 1987	p Funakoshi et al., 1987b
q Shadle et al., 1985	r Davies and Crumpton, 1985
s Sudhof et al., 1988	t Antonicelli et al , 1988
u Ahn et al , 1988	v Hom et al , 1988
w Shimizu et al , 1988	x Errasfa & Russo-Marie, 1988
y Hamman et al , 1988	z Comera et al , 1990
α Kobayashi & Tasima 1990	β Crompton et al ,1988.

Table 1 : Different members of the lipocortin family and their apparent identities.

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(ii) Distribution of lipocortin

Lipocortins are found in numerous tissues including the placenta (*Huang et al., 1986*) and a number of cells generate these proteins including peritoneal macrophages (*Blackwell et al., 1982*), alveolar macrophages (*Parente & Flower, 1984*), neutrophils (*Hirata et al., 1980; Hirata, 1981*), kidney interstitial cells (*Cloix et al., 1983*), endothelial and mesothelial cells (*De Caterina & Weksler, 1986; Rosenbaum, Cheli & Gerritsen, 1986; Van de Velde, Bult & Herman, 1986a b*), skin fibroblasts (*Errasfa, Rothhut, Fradin, Billardon, Junien, Bure & Russo-Marie, 1985*) and human endometrium (*Gurpide, Markiewicz, Schatz & Hirata, 1986*) in response to glucocorticoids. Lipocortin has also been shown under resting conditions in the thymus, spleen, lung and brain but the extent to which it is 'steroid' inducible in these tissues is not yet known.

The role of eicosanoids in the regulation of the Hypothalamo-pituitary-adrenocorticotrophic function.

Arachidonic acid and its metabolites have been implicated in the control of neuroendocrine function (*Cowell & Buckingham, 1989*). They may act as intracellular second messengers in calcium mediated processes or stimulate or amplify other second messenger systems such as cAMP and cGMP (*Axelrod, Burch & Jelsema, 1988*). Alternatively they may be released from cells and, by activating cell surface receptors, serve as a primary messenger exerting either autocrine or paracrine effects (*Axelrod et al., 1988*).

Biologically active eicosanoids are formed within the hypothalamus and pituitary gland from AA, by the action of three separate enzyme systems (i) Cyclo-oxygenase which leads to the formation of prostanoids, i.e. prostaglandins (PG's), thromboxanes (TX's) and prostacyclin (PG I_2) (*Wolfe, 1982*) (ii) Lipoxygenase producing leukotrienes (LT's) and hydroxyeicosatetraenoic acids (HETE's) (*Wolfe, 1982*) and (iii) Cytochrome P450 dependent epoxygenase giving rise to the epoxides (EET's) (*Chacos, Falck, Wixtrom & Capdevila, 1982*). Their production is dependent on the liberation of AA substrate which occurs as a consequence of the stimulation of specific cell-surface receptors. Arachidonic acid may be derived from membrane bound phospholipids by the direct action of phospholipase A_2 (PLA_2) (*Axelrod et al., 1988*). Alternatively it may also be produced from arachidonyl diglyceride as a result of the action of phospholipase C (PLC) on membrane bound phosphoinositides or phosphatidylcholine. In this event the fatty acid is generated either due to the action diglyceride lipase on the diglyceride, or following phosphorylation, through the action of PLA_2 (*Axelrod et al., 1988*).

Several studies have shown that eicosanoids may be involved in the regulation of hypothalamo-pituitary-adrenocorticotrophic activity, but their role remains unclear. Some groups have shown that systemic administration of prostaglandins stimulates HPA activity and thus ACTH release (*DeWied, Witter, Versteeg & Mulder, 1969; Hedge & Hanson, 1972; Coudert & Faiman, 1973*). The site of action of the eicosanoids was thought to be at the pituitary level since systemic administration of prostaglandins of the E, but not the F series, enhanced ACTH release in both normal rats and animals in which endogenous CRF release had been blocked pharmacologically (*DeWied et al., 1969*). Other groups however, have shown that

prostanoids may also attenuate the secretory activity of corticotrophs. Systemic administration (*Wiedenfeld, Siegel, Conforti & Chowers, 1980*) or injection into the pituitary gland (*Hedge, 1977; Thompson & Hedge, 1978*) of the cyclo-oxygenase inhibitor, indomethacin, stimulates the secretion of ACTH and causes an exaggeration of the HPA response to stress. Again, its actions appear to be effected at the pituitary level since implantation of indomethacin into the median eminence of the hypothalamus suppresses HPA activity (*Hedge, 1977; Thompson & Hedge, 1978*). Moreover, systemic injections of indomethacin not only enhance resting and stress-induced activity of the pituitary gland in intact rats, but also raise serum ACTH concentrations in animals in which endogenous CRF release has been prevented surgically (*Wiedenfeld, Siegel, Feldman & Chowers, 1981*).

Apart from two reports to the contrary (*Vale, Rivier & Guillemin, 1971; Buckingham & Hodges, 1977a*), in vitro studies have shown that prostanoids do not influence the spontaneous release of ACTH or the co-secreted β-endorphin. Indeed although prostaglandins and prostacyclin do not influence the resting secretion of ACTH (*DeWied et al., 1969; Heisler, 1984; Buckingham, Cowell & Flower, 1987a*) or β-endorphin (*Vlaskovska & Knepel, 1984; Vlaskovska, Hertting & Knepel, 1984*) by a variety of in vitro preparations, CRF-41 induced ACTH release and CRF-41 and AVP stimulated β-endorphin release are attenuated in the presence of PGE_2 (*Vlaskovska & Knepel, 1984; Vlaskovska et al., 1984; Sobel, 1987*). Furthermore indomethacin potentiates CRF-41 (*Vlaskovska et al., 1984; Abou-Samra, Catt & Aguilera, 1986c*) and AVP (*Vlaskovska & Knepel, 1984; Vlaskovska et al., 1984; Okajima & Hertting, 1986*) induced corticotrophic responses of pituitary tissue in vitro.

Interestingly phospholipase A_2 and the phospholipase A_2 activator melittin, both of which promote the formation of arachidonic acid, enhance corticotrophin secretion by a number of in vitro preparations including mouse pituitary tumour cells (*Heisler, Resine, Hook & Axelrod, 1982*), pituitary segments (*Knepel & Meyen, 1986; Nicholson, Gillham & Jones, 1987*) and dispersed (*Buckingham, Cowell & Flower 1987a b; Cowell, Buckingham & Flower 1989*) and cultured (*Abou-Samra et al., 1986c*) rat pituitary cells. Similarly ACTH release is also stimulated by arachidonic acid (*Abou-Samra et al., 1986c; Buckingham, et al., 1987a b; Stone, Carey, Cowell & Buckingham, 1989*) although platelet activating factor (PAF) which can also be liberated from phospholipids by PLA_2 (*Braquet, Touqui, Shen & Vargaftig, 1987*) is ineffective in this respect (*Cowell, Flower & Buckingham, 1991*). Furthermore in vitro studies have shown that corticotrophin release is also enhanced by phorbol esters which increase intracellular arachidonate (*Heisler, 1984; Abou-Samra et al., 1986a b;*

Dartois & Bouton, 1986; 1988).

Evidence for the role of lipoxygenase metabolites in the regulation of pituitary hormone secretion is conflicting, possibly due to the lack of selectivity and specificity of the lipoxygenase inhibitors available. Of the lipoxygenase metabolites, none of the 5-lipoxygenase products (Leukotrienes B_4, C_4,D_4, & 5-HETE methylester) or the selective 5-lipoxygenase inhibitor AA861, influenced basal or stimulated β-endorphin or ACTH secretion in vitro (*Vlaskovska & Knepel, 1984; Knepel & Meyen, 1986; Buckingham, Cowell & Flower 1988; Stone et al., 1989).* The role of the 12 and 15-lipoxygenase products is unclear. The non-selective lipoxygenase inhibitor NDGA and the dual inhibitor of cycloxygenase/lipoxygenase enzymes, ETYA, attenuate AVP and CRF-41 induced β-endorphin (*Vlaskovska & Knepel, 1984*) and CRF-41 induced ACTH release in vitro (*Luini & Axelrod, 1985; Stone et al., 1989*). Conversely, other workers have shown that NDGA and the dual cycloxygenase inhibitors BW755C and phenidone fail to affect stimulated ACTH secretion in vitro while ACTH release in response to hypothalamic extract and CRF -41 is attenuated in the presence of ETYA. Finally, cytochrome P450 inhibitors, which inhibit epoxyeicosatrienoic acid production, have also been shown to inhibit corticotrophin secretion. CRF-41 induced ACTH release is inhibited by SKF525A and piperonyl butoxide but not metyrapone (*Luini & Axelrod, 1985*) whereas AVP-induced ACTH release is inhibited by both metyrapone and piperonyl butoxide. Furthermore SKF525A has also been shown to inhibit arachidonic acid, phospholipase A_2, CRF-41 or hypothalamic extract induced ACTH release from acutely dispersed pituitary cells (*Buckingham, Cowell & Flower, 1988*).

The role of eicosanoids in the regulation of the secretion of CRF's is less well understood. There is some evidence that prostanoids may facilitate the release of corticotrophin releasing factors. Prostaglandins E1, $F_{1\alpha}$, $F_{2\alpha}$, A and B all elicit corticotrophin release when injected into the median eminence, but not the lateral hypothalamus, pituitary gland or the tail vein of the rat (*Hedge & Hanson, 1972; Hedge, 1976*). Similarly implantation of indomethacin into the hypothalamus, attenuates stress-induced ACTH release (*Hedge & Thompson, 1975; Thompson & Hedge, 1978*). Their site and mode of action are not known. Thus it is not clear whether they act on CRF-producing cells or on the neurones which modulate their activity nor is it known whether their actions involve alterations in the release of CRF-41, AVP or other regulatory factors. Relatively little is known of the roles of leukotrienes and platelet activating factor (PAF) or epoxides in the control of CRF's.

Aims of project

Despite the reports which have claimed that the anti-inflammatory actions of the glucocorticoids are effected in part by the induction of proteins (lipocortins) which block the production of eicosanoids it is not yet known whether the powerful inhibitory action of steroids on the HPA axis are effected through a similar mechanism. Accordingly, the aims of the experiments described in this thesis were to investigate further the effect of products of PLA2 activation and cytokines on CRF-41 and AVP release from the hypothalamus in vitro, to determine if these responses could be inhibited by steroids and whether this effect was possibly via the induction of lipocortins.

MATERIALS AND METHODS.

Animals.

Adult male Sprague-Dawley rats weighing approximately 190-210g were used. They were bred in the Comparative Biology Unit at Charing Cross and Westminster Medical School and housed, 5 per cage, in a room with controlled lighting (lights on 08.00-20.00h) in which the temperature was maintained at 21-23°C. Food and water were available ad libitum. In one experiment rats congenitally lacking hypothalamic vasopressin (Brattleboro strain) were used together with the parent (Long Evans) control strain. These animals were bred and housed as above.

Adrenalectomy.

Bilateral adrenalectomy was performed by the dorsal approach using sodium pentobarbitone (Sagital, 6mg/100g body weight, i.p. in a volume of 0.2ml/100g, May and Baker Ltd.), as the anaesthetic. After surgery the rats were given 0.9% sodium chloride in place of drinking water. They were kept for 7-12 days after the operation, and then killed by decapitation usually between 08.00-10.00h to avoid changes associated with circadian rhythm. Completeness of adrenalectomy was verified post-mortem. Sham operated control animals were anaesthetized and operated on as above but the adrenal glands were not removed.

Drug treatments.

Dexamethasone sodium phosphate (Organon) was administered to intact and adrenalectomized rats in the drinking water/saline at a concentration of $20\mu g/ml$ for 24h and at a concentration of $2\mu g/ml$ for 6 and 14 days prior to removal of the hypothalami for in vitro studies. In all cases the steroid was added to the drinking water in the morning and the experiments commenced at the same time either the following morning or 6 or 14 days later.

Static incubation of whole hypothalami.

The static incubation system used was a modification of the method described by Bradbury, Burden, Hillhouse & Jones (1974) and Buckingham & Hodges (1977b, 1979).

Adult male Sprague-Dawley rats, some of which were adrenalectomized 7-21 days previously, were killed by stunning and decapitation. The skull cap was removed and the frontal lobes were lifted to expose the optic nerves which were cut before the whole brain was reflected. The hypothalamus was dissected out, handling via the optic nerves. The 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 at a depth of approximately 2mm. The tissue block weighing 40mg (wet weight) was immediately transferred to a tube containing 1ml Earles balanced salt solution containing 0.25% w/v bovine serum albumin (BSA),(Sigma Chemical Company Ltd.), together with 100K.I.U./ml aprotinin proteinase inhibitor (Bayer UK Ltd.) and 0.0176% w/v ascorbic acid (BDH. Chemicals Ltd.) in order to maintain the stability of the peptides released (Gillies et al., 1982). The tubes were placed on a shaking platform in a water bath maintained at 37°C and pre-incubated for 60 min while gassed constantly with $95\%O_2/5\%CO_2$. During this period the medium was replaced twice, once after 30 min and again after 45 min. The hypothalami were then subjected to three successive 15 min incubation periods (I-III). Where appropriate, secretagogues were present in the incubation medium during the final incubation period while antagonists were present throughout both the pre-incubation and the subsequent three successive 15 min periods. 1ml samples of medium from all the incubation periods I-III were collected. One aliquot (500μl) was snap frozen and stored at -20°C for AVP estimation. The remainder was used for CRF-41 determination which was done immediately since the peptide is not resistant to the freeze/thaw process. In later experiments aliquots of 600μl were taken and freeze dried. The residue was then reconstituted in 450μl of assay buffer to concentrate the sample and allow the CRF-41 content to be determined by radioimmunoassay.

Static incubation of anterior pituitary segments.

The static incubation system used was a modification of the method described by Buckingham & Hodges (1977a).

Adult male Sprague-Dawley rats, adrenalectomised 7-21 days previously, were stunned and decapitated. The skull cap was removed and the brain deflected to expose the pituitary gland which was removed. The neurointermediate lobe was separated and discarded. The remaining tissue was cut with a scalpel blade into four pieces. Each quarter was transferred to a tube containing 1ml of Earles balanced salt solution with 100K.I.U./ml aprotinin proteinase inhibitor (Bayer UK Ltd.) in order to maintain the stability of the peptides released (Gillies et al., 1982). The tubes were placed on a shaking platform in a water bath maintained at 37°C and pre-incubated for 120 min while gassed constantly with $95\%O_2/5\%CO_2$.

The pituitary segments were then subjected to a single 30-60 min incubation period. Where appropriate, secretagogues were present in the incubation medium during this incubation period. Samples of medium from the incubation period were collected, snap frozen and stored at -20°C for ACTH assay. The pituitary segments were weighed on a torsion balance. Posterior pituitary glands from intact rats were incubated as a whole using the same technique and samples of medium assayed for AVP content.

Static incubation of dispersed pituitary cells.

The dispersed pituitary cell system used in the studies with media collected from macrophages stimulated with endotoxin, was a modification of that described by Negro-Vilar and Lapetina (1985).

(i) Preparation of dispersed pituitary cells.

Adrenalectomized adult male Sprague-Dawley rats were decapitated, the pituitary glands were removed and the neurointermediate and anterior lobes separated. The former were discarded. Each anterior lobe was cut into approximately 10 pieces with a scalpel blade. The combined fragments of 5-8 anterior pituitary glands were transferred to an incubation pot containing 10ml Earles balanced salt solution containing 0.1% (w/v) collagenase (Boehringer Mannheim) through which 95% $O_2/5\%$ CO_2 gas was bubbled continuously, and placed in a water bath maintained at

37°C. During the 30 min incubation the pot was shaken constantly. In addition, periodic gentle repetitive pipetting was used to aid the dispersion. The pituitary pieces were then allowed to settle and the supernatant fluid was poured into a plastic tube which was stoppered and stored at room temperature. A further 10ml aliquot of medium containing collagenase was added to pituitary fragments and the dispersion procedure repeated. Three such harvests were collected and centrifuged at 800-1000 rpm for 10 min to recover the cells. The supernatant fluids were discarded and each cell pellet was then resuspended in 10ml incubation medium consisting of Earles balanced salt solution containing BSA (0.25% w/v), Trasylol (100 K.I.U./ml) benzyl penicillin (15μg/ml) and streptomycin (25μg/ml) and this medium was used for all further incubations. The centrifugation procedure was repeated and the cells from each harvest were resuspended in 1ml incubation medium, pooled and filtered through 100μm nylon gauze to remove any remaining connective tissue or clumps of cells. An aliquot (10μl) of this cell suspension was taken to determine the cell count. Cell viability was also verified using the trypan blue exclusion test and usually found to be greater than 90%, and never less that 85%.

(ii) Incubation of anterior pituitary cells.

The cells were diluted to a known concentration in incubation medium (1×10^6 cells/ml) and pre-incubated for 1h on a shaking platform in a water bath maintained at 37°C while gassed constantly with 95% O_2/5% CO_2. At the end of the pre-incubation period the cell suspension was centrifuged at 800-1000 rpm for 10 min at 4°C. The supernatant fluid was discarded and the cell pellet resuspended in incubation medium. Aliquots of the cell suspension (3×10^5 cells/0.3ml) were then taken and 0.3 mls of either incubation medium with endotoxin (25μg/ml) 0.4 HE/ml, or media collected from macrophages (2,4 and 8×10^6 cell/ml) incubated with endotoxin were added to each tube. The cell suspension 5 $\times 10^5$ cells/ml) were then incubated for a further 1h under the same conditions as in the pre-incubation period and then centrifuged at 800-1000 rpm for 10 min at 4°C. The cell pellets were discarded and the supernatant fluids snap frozen and stored at -20°C for ACTH assay.

Method for harvesting macrophages.

Adult male Sprague-Dawley rats were stunned and decapitated. The skin on the underside of the rat was gently lifted and cut to expose the body wall of the peritoneal cavity, care was taken at this stage to ensure the cut did not penetrate into the body wall. 40ml of ice cold heparinised saline (0.9% NaCl+25iU/ml heparin) was then injected into the peritoneal cavity and the body wall gently massaged for approximately 3min. A small hole was subsequently cut through the body wall, the fluid sucked out with the aid of a plastic pipette and transferred to a centrifuge tube which was kept on ice. When macrophage harvests from 10 rats had been collected the fluid was pooled and centrifuged for 15min at 800-1000 rpm. The supernatant fluid was decanted off and the pellet was resuspended in 2ml of heparinised saline. An aliquot (10μl) of the cell suspension was taken to determine the cell count with the aid of a haemocytometer.

Incubation of macrophages with endotoxin.

The macrophage yield was usually between 35-40x10^6 cells/ml. The cells were diluted in Earles balanced salt solution containing 0.25% w/v bovine serum albumin, together with 100K.I.U./ml aprotinin proteinase inhibitor and 0.0176% w/v ascorbic acid to give concentrations ranging from 1-4x10^6 cells/ml. Aliquots (5ml) of each cell suspension were then incubated for 1h at 37°C in the presence of 250ng/ml of endotoxin K235 (Sigma Chemical Company Ltd.) while gassed constantly with 95%O$_2$/5%CO$_2$. After 1h the cell suspensions were centrifuged for 15min at 800-1000 rpm and the supernatant fluids collected and applied to either pituitary or hypothalamic tissue in vitro.

Drugs and antisera.

Adrenocorticotrophic hormone   (*National institute for biological standards*)
Adrenocorticotrophin antiserum   (*Dr. T. Yeo gift*)
^{125}I-Adrenocorticotrophic hormone   (*Prof. L. Rees gift*)
Arginine vasopressin   (*National institute for biological standards*)
Arginine vasopressin antiserum   (*Prof. S. Lightman ; Dr. E. Hillhouse gift*)
^{125}I-Arginine vasopressin   (*Amersham International plc.*)
BW755C   (*Burroughs Wellcome*)
Corticosterone   (*Sigma chemical company Ltd.*)
Corticosterone antiserum   (*Dr Brent Williams gift*)
^{125}I-Corticosterone   (*Immunodiagnostics Ltd*)
Corticotrophin releasing factor (CRF-41)   (*Penninsula laboratories Inc.*)
Corticotrophin releasing factor antiserum   (*Dr. E. Hillhouse gift*)
^{125}I-Corticotrophin releasing factor   (*Dr. S. Tsagarakis gift*)
Dexamethasone sodium phosphate   (*Organon laboatories Ltd.*)
Endotoxin K235   (*Sigma chemical company Ltd.*)
Ibuprofen   (*Sigma chemical company Ltd.*)
ICI 207968   (*ICI plc.*)
Indomethacin   (*Sigma chemical company Ltd.*)
Interleukin 1α (IL-1α)   (*World Health Organisation WHO.*)
Interleukin 1β (IL-1β)   (*World Health Organisation WHO.*)
Interleukin 6 (IL-6)   (*World Health Organisation WHO.*)
Interleukin 8 (IL-8)   (*World Health Organisation WHO.*)
Lipocortin   (*Biogen-J. Browning gift*)
Lipocortin 1-188   (*ICI plc.-F. Carey & R. Forder gift*)
Lipocortin antibody 1-188   (*ICI plc.-F. Carey & R. Forder gift*)
Luteinizing hormone (LH)   (*National Institute of Diabetes & Digestive & Kidney diseases*)

Luteinizing hormone antiserum   (*National Hormone & Pituitary Programme*)
^{125}I-Luteinizing hormone   (*iodinated at CXWMS*)
Melittin   (*Sigma chemical company Ltd.*)
Phospholipase A$_2$ (*Naja Naja*)   (*Sigma chemical company Ltd.*)
r-Phospholipase A$_2$ (*Biogen-J. Browning gift*)
Quinacrine   (*Sigma chemical company Ltd.*)
SKF 525A   (*Smith Kline & French laboratories Ltd*)
Tumour necrosis factor (TNF)   (*World Health Organisation WHO.*)

Chemical name of drugs used.

BW755C   3-amino-1-[m-(trifluoromethyl)-phenyl]-2-pyrazoline
SKF525A   2-diethyl-aminoethyl-2,2-diphenylvalerate
ICI207968   2-3-pyridylmethyl-indazolinone

Where possible the drugs were dissolved in the incubation medium (Earles balanced salt solution-BDH Chemicals Ltd).
BW755C, ibuprofen, ICI 207928 and indomethacin were all dissolved in absolute ethanol (BDH Chemicals Ltd) and then diluted in the incubation medium so that the final ethanol concentration never exceeded 0.2%.

Cannulation of the third ventricle.

Rats were anaesthetized with sodium pentobarbitone (Sagatal, 6mg/100g body weight, i.p. in a volume of 0.2ml/100g, May and Baker Ltd.) and placed in a stereotaxic frame. The ear bars were positioned in the external auditory meatae, and the incisor bar positioned 5mm above the level of the ear bars. The head was shaved to facilitate subsequent closure of the wound and an incision was made in an anterio-posterior direction from just behind the eyes, back to the level of the ears. The skin was retracted from the skull and the periosteum scraped clear to expose the skull. A guide cannula, made from a 21 gauge (0.8x50mm) syringe needle (BD, Microlance) trimmed to 8mm in length with the end filed smooth to ensure a clear hole, was positioned on bregma and then raised clear of the head. Two small holes were drilled through the skull, approximately 5mm anterior and posterior of bregma and slightly lateral to the midline, using a size 1.5mm dental burr. A larger hole was drilled directly on bregma and extended 1mm laterally leaving just a thin layer of skull. The dura was then punctured with a sharp needle. Care was taken at this point to avoid perforating the saggital sinus. Any bleeding was stopped by applying pressure with a cotton bud, until the flow ceased. A size 10 B.A. stainless steel, self tapping screw was then fixed into each of the two smaller holes. The guide cannula was lowered into position until its tip was just at the level of the dura. To bypass the saggital sinus the cannula was then moved 1mm laterally , lowered about 2mm and returned to its original position

before lowering to its full extent. Acrylic dental cement (Simplex Rapid, Austenal Dental, Harrow) was applied around the cannula and screws in order to secure the cannula, in position. The cement was allowed to harden and the wound closed using Mersilk sutures before removing the animal from the stereotaxic frame. A stopper made from a 15mm length of wire was placed in the guide cannula to ensure that it remained open. The animals were kept warm on a heated pad until they regained consciousness.

The animals were weighed each day for one week before the cannulation so as to acustom them to handling, and for 3-4 days after the operation. The animals were allowed at least 5 days to recover from the trauma of the operation and any animal which did not gain weight normally, or appeared unhealthy, was immediately humanely killed, although this was rarely necessary.

Intra-cerebro-ventricular (ICV) injections.

Solutions (2-5μl) were injected into the brain through an injection cannula which protruded 1mm beyond the guide cannula, and was fixed via polypropylene tubing, to a 10μl Hamilton microsyringe. Lipocortin 1 and the antibody against lipocortin 1 were made up in sterile saline for injection and the appropriate vehicles were used for the control injections. The rats were lightly restrained (hand-held) and the stopper removed from the cannula. A fine needle similar to the one being used for the injections was first inserted through the cannula, to check that the guide cannula was still open and had not sealed over. The injection cannula was then inserted into the guide cannula and the solution slowly injected over about 30-60s before the injection cannula was removed. Five minutes after the i.c.v. injection some of the rats were given an i.p. injection of histamine (0.6mg/100g/0.2ml) ('quantitative stress'). All the rats were killed by decapitation 25min following the i.c.v. injection and the trunk blood collected into chilled heparinised tubes. Samples of blood were then spun at 2000g and the serum collected and stored at -20°C for corticosterone estimation.

In a number of animals the position of the cannula in the brain was verified by injecting trypan blue dye (Sigma Chemical Company Ltd.) through the cannula. The brain was then sectioned and the track of the cannula examined.

Radioimmunoassays (RIA's).

(i) Arginine Vasopressin (AVP).

Arginine vasopressin (AVP) was measured by radioimmunoassay (RIA) as described by Eckland, Todd, & Lightman (1988). The antibody (Ab) was kindly donated by Prof. S. Lightman, Medical Unit Westminster Hospital and was raised in rabbit against AVP_{1-9}. It was found to have negligble cross reactivity with oxytocin (<0.1%) and DD-AVP (<0.01%).

Standard solutions of synthetic AVP (National Institute for Biological Standards and Control) were prepared by serial dilution of a stock solution (100ng/ml AVP in phosphate buffer with 0.5% w/v bovine serum albumin (BSA Sigma Chemical Company Ltd.) stored at -20°C) in assay buffer, to produce solutions ranging in concentration from 0.0039-2ng/ml. The assay buffer used was 0.1M Tris HCl (Sigma Chemical Company Ltd.) with 0.2% w/v bovine serum albumin at pH 7.4. Test samples were also diluted (1:2) in assay buffer. The final volume of the assay tubes was 250μl.

Tubes containing 150μl aliquots of standard or test solution with 50μl AVP Ab (1:8000 initial dilution) were prepared in duplicate. Tubes containing 200μl assay buffer (50μl in place of AVP Ab) and 150μl assay buffer with 50μl AVP Ab respectively, were also set up to determine non-specific and the total binding. The tubes were shaken on a vortex mixer and incubated at 4°C for 24h after which 50μl ^{125}I-AVP (Amersham International plc.), diluted in assay buffer to give aproximately (4,000 counts/min/50μl), were added to each tube. Tubes were also set up containing 50μl of ^{125}I-AVP to determine the total counts. The tubes were vortexed and incubated for a further 24h at 4°C. The 'Ab bound' and free radioactivity were separated by adding 750μl of absolute ethanol (James Burrough (F.A.D.) Ltd., kept at -20°C) to each of the assay tubes except those measuring total counts. The tubes were then vortexed and centrifuged at 4000g for 20min at 4°C. The supernatant fluid was decanted off and the radioactivity of the pellet was counted on an automatic gamma counter (LKB, Turku, Finland) using a 1-3 min count time.

In later experiments arginine vasopressin was determined by radioimmunoassay (RIA) as described by Hillhouse and Milton (1989a). The antibody (Ab) was kindly donated by Dr. E. Hillhouse Department of Medicine, King's College, Denmark Hill and was

raised in rabbit against AVP1-9 with defined specificity (*Hillhouse & Milton 1989a*).

Standard solutions of synthetic AVP (National Institute for Biological Standards and Control) were again prepared by serial dilution of a stock solution (100ng/ml AVP in phosphate buffer with 0.5% w/v bovine serum albumin stored at -20°C) in assay buffer, to produce solutions ranging in concentration from 3.9-2000pg/ml. The assay buffer used was 0.05M phosphate substituted with 0.25% w/v bovine serum albumin at pH 7.4. The final volume of the assay tubes was 800µl.

Tubes containing 100µl aliquots of standard or test solution with 500µl assay buffer and 100µl AVP Ab (1:45000 initial dilution) were prepared in duplicate. Tubes containing 700µl assay buffer (100µl in place of AVP Ab) and 600µl assay buffer with 100µl AVP Ab respectively, were also set up to determine non-specific and the total binding. The tubes were shaken on a vortex mixer and incubated at 4°C for 24h after which 100µl ^{125}I-AVP (Amersham International plc.), diluted in assay buffer to give aproximately (10,000 counts/min/100µl), were added to each tube. Tubes were also set up containing 100µl of ^{125}I-AVP to determine the total counts. The tubes were vortexed and incubated for a further 24h at 4°C. The 'Ab bound' and free radioactivity were separated using a double antibody method. 50µl anti-rabbit precipitating serum (IDS Washington, England) diluted 1:24 (v/v) together with 50µl non-immune rabbit serum diluted 1:200, and 500µl 4% polyethylene glycol PEG (BDH Chemicals Ltd.) diluted in 0.05M phosphate buffer, were added to each of the assay tubes except those for total counts. The tubes were then vortexed and centrifuged at 4000g for 1h at 4°C. The supernatant fluid was aspirated off and the radioactivity of the pellet was counted on an automatic gamma counter (LKB, Turku, Finland) using a 1 min count time.

(ii) Corticotrophin Releasing Factor-41 (CRF-41)

Corticotrophin releasing factor-41 (CRF-41) was measured by the radioimmunoassay (RIA) described by Hillhouse & Milton (1989a). The antibody (Ab) was kindly donated by Dr. E. Hillhouse Department of Medicine, King's College, Denmark Hill and was raised in rabbit conjugated with β–γ–globulin (BGG) against rat CRF. The antibody cross reactivity was 100% with rat and human CRF-41, 35% with human CRF21-49 fragment, 0.1% with human CRF1-20, 0.02% with human CRF6-33 fragment, 75% with oxidised CRF, 9% with ovine CRF, and 0.03% with sauvagine. It was found to have no cross reactivity with AVP, somatostatin, LHRH or TRH.

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Standard solutions of human CRF-41 (Penninsula Laboratories) were prepared by serial dilution of a stock solution (10μg/ml CRF-41 in 0.1N acetic acid with 0.1% w/v bovine serum albumin stored at -20°C) in assay buffer, to produce solutions ranging in concentration from 5-2500pg/ml. The assay buffer used was 0.05M phosphate buffer with 0.25% w/v bovine serum albumin, 100μl/100ml Triton X-100 (BDH Chemicals Ltd.) and 100μl/100ml 2-mercaptoethanol (Sigma Chemical Company Ltd.) at pH 7.4. Test samples were diluted (1:2) in assay buffer or used neat. The final volume of the assay tubes was 300μl.

Tubes containing 200μl aliquots of standard or test solution, with 50μl CRF-41 Ab (1:10,000 initial dilution) were prepared in duplicate. Tubes containing 250μl assay buffer (50μl in place of CRF-41 Ab), or 200μl assay buffer and 50μl CRF-41 Ab, were also set up to determine non-specific and total binding respectively. The tubes were shaken on a vortex mixer and incubated at 4°C for 24h after which 50μl ^{125}I-CRF (provided by Dr S. Tsagarakis at St. Bartholomew's Hospital) diluted in assay buffer to give approximately (5,000 counts/min/50μl) was added to each tube. Tubes were also set up containing 50μl ^{125}I-CRF to determine the total counts. The tubes were vortexed and incubated for a further 24h. at 4°C. The 'Ab bound' and free radioactivity were separated using a double antibody method. 50μl anti-rabbit precipitating serum (IDS Washington, England) diluted 1:24 (v/v) together with 50μl non-immune rabbit serum diluted 1:200, and 500μl 4% polyethylene glycol PEG (BDH Chemicals Ltd.) diluted in 0.05M phosphate buffer, were added to each of the assay tubes except those for total counts. The tubes were then vortexed and centrifuged at 4000g for 1h at 4°C. The supernatant fluid was aspirated off and the radioactivity of the pellet was counted on an automatic gamma counter (LKB, Turku, Finland) using a 1 min count time.

(iii) Adrenocorticotrophic hormone (ACTH).

Adrenocorticotrophic hormone (ACTH) was measured by the radioimmunoassay (RIA) described by Rees, Cook, Kendall, Allen, Kramer, Ratcliffe & Knight (1971). The antibody (Ab) was kindly donated by Dr. T. Yeo, Hammersmith Hospital and was raised in rabbit against ACTH$_{1-24}$. It was found to have negligible cross reactivity with β–endorphin, β–LPH, CLIP, γ3-MSH and α-MSH, but bound readily with ACTH$_{1-24}$ and ACTH$_{1-39}$.

Standard solutions of human ACTH (National Institute for Biological Standards and Control) were prepared by serial dilution of a stock solution (1μg/ml ACTH in 0.1M HCl containing 10^{-3}M ascorbic acid stored at -20°C) in assay buffer, to produce solutions ranging in concentration from 78-2500pg/ml. The assay buffer used was 0.01M Phosphate buffer with 1.25% v/v human serum albumin (Blood Products Laboratory, Elstree) and 0.5% v/v 2-mercaptoethanol (Sigma Chemical Company Ltd.) at pH 7.4. Test samples were also diluted in assay buffer. The final volume of the assay tubes was 400μl.

Tubes containing 100μl aliquots of standard or test solution with 200μl assay buffer and 100μl ACTH Ab (1:3000 dilution in a solution of non-immune rabbit serum (Wellcome Reagents Ltd.) diluted1:200 in assay buffer) were prepared in duplicate. Additional tubes containing 300μl assay buffer and 100μl non-immune rabbit serum (1:200 dilution) or 300μl assay buffer with 100μl ACTH Ab were also set up to determine non-specific and total binding respectively. The tubes were shaken on a vortex mixer and incubated at 4°C for 24h after which 100μl ^{125}I-ACTH (provided by Prof. L. Rees, St. Bartholomew's Hospital) diluted in assay buffer to give approximately 10,000 counts/min/50μl, were added to each tube. Tubes were also set up containing 100μl ^{125}I-ACTH to determine the total counts. The tubes were vortexed and incubated for a further 72h at 4°C. The 'Ab bound' and free radioactivity were separated using a double antibody method. A solution of anti-rabbit precipitating serum (IDS Washington, England) diluted 1:24 v/v in assay buffer was prepared. 100μl of the solution were added to each of the assay tubes except those for total counts. The tubes were vortexed and incubated for a further 24h at 4°C. The tubes were then centrifuged at 3000g for 20min at 4°C. The supernatant fluid was aspirated off and the radioactivity of the pellet was counted on an automatic gamma counter (LKB, Turku, Finland) using a 1 min count time.

(iv) Corticosterone.

Corticosterone was measured by the radioimmunoassay (RIA) described by Al-Dujaili, Williams & Edwards (1981).

This direct method does not require preliminary extraction of the plasma. Standard solutions of corticosterone were prepared (0.78-200 ng/ml) and plasma samples diluted 1:10 in phosphate/citrate buffer pH 3.0. Assay tubes containing 25μl aliquots of a standard or test solution, 100μl corticosterone antibody (kindly donated by Dr.

Brent Williams Royal Infirmary Edinburgh coded RI3, diluted 1:36,000) and 100μl 5,000cpm (^{125}I)-corticosterone added sequentially, were prepared. The contents of the tubes were mixed and incubated at 4°C for 24h. After the addition of a dextran-coated charcoal suspension (500μl/tube) the tubes left at 4°C for 15min and then centrifuged at 4000g for 20min. The supernatant fluid was aspirated and the free-fraction counted on an automatic gamma counter (LKB, Turku, Finland) using a 1 min count time.

(v) Luteinizing hormone (LH).

Luteinizing hormone was determined by radioimmunoassay (RIA) as described by Kilpatrick, Collins and Newton (1976).The reagents were supplied by the National Hormone and Pituitary Programme.

Standard solutions of standard LH (NIADDK-rat LH-RP-2) were prepared by serial dilution of a stock solution (5μg/ml LH in distilled water stored at -20°C) in assay buffer, to produce solutions ranging in concentration from 0.125-128ng/ml. The assay buffer used was 0.05M phosphate substituted with 0.5% w/v bovine serum albumin at pH 7.4. The final volume of the assay tubes was 600μl.

Tubes containing 100μl aliquots of standard or test solution with 400μl assay buffer and 50μl LH Ab (NIADDK-anti-rat LH-S-10 1:15000 initial dilution) were prepared in duplicate. Tubes containing 550μl assay buffer (50μl in place of LH Ab) and 500μl assay buffer with 50μl LH Ab respectively, were also set up to determine non-specific and the total binding after which 50μl ^{125}I-LH, diluted in assay buffer to give approximately 20,000 counts/min/50μl, were added to each tube. Tubes were also set up containing 50μl of ^{125}I-LH to determine the total counts. The tubes were vortexed and incubated for 24h at room temperature. The 'Ab bound' and free radioactivity were separated using a double antibody method. 200μl sheep anti-rabbit precipitating serum (SARFc) diluted 1:20 (v/v in 4% polyethylene glycol PEG) were added to each of the assay tubes, except those for total counts, which were then left at room temperature for 30min. A further 1ml 2% PEG (BDH Chemicals Ltd.) diluted in 0.05M phosphate buffer with 1% sodium azide, was then added to each tube, except those for total counts and the tubes were vortexed and centrifuged at 4000g for 30min at 4°C. The supernatant fluid was aspirated off and the radioactivity of the pellet was counted on an automatic gamma counter (LKB, Turku, Finland) using a 1 min count time.

Scintillation proximity assay for Prostaglandin E_2 (PGE_2)

Prostaglandin E_2 was measured using a Scintillation proximity assay (SPA). This is a novel technique applicable to radiobinding assays which eliminates the need for a separation step and addition of liquid scintillant. In common with other radioimmunoassay systems, the scintillation proximity assay is based on the competition between unlabelled ligand and a fixed quantity of labelled ligand for a limited number of binding sites on a specific antibody (anti-PGE_2). In the SPA assay the antibody bound ligand is reacted with a scintillation proximity reagent, which contains flumicrospheres (fluorescent beads containing scintilant and coated with second antibody). Labelled and unlabelled antibody bound ligand compete for the binding sites on the beads and labelled ligand will make the beads fluoresce. Measurement in a β scintillation counter enables the amount of labelled ligand to be calculated. The greater the concentration of unlabelled ligand the lower the counts per minute. The concentration of unlabelled ligand in the sample can then be determined from the standard curve.

These assays were carried out at the Hammersmith Hospital and all the reagents were kindly donated by Dr. Mark Sullivan.

Standard solutions of standard PGE_2 were prepared in duplicate by serial dilution of a stock solution ($10\mu g/ml$ PGE_2 in absolute ethanol) in ethanol, to produce solutions ranging in concentration from 10-1000pg/$50\mu l$. The ethanol was then evaporated off and $50\mu l$ assay buffer (phosphate buffered saline with 0.1% w/v bovine serum albumin at pH 7.4) added to each tube.

Tubes containing $50\mu l$ aliquots of standard or test solution with $50\mu l$ PGE_2 Ab (ICN-immunobiologicals, raised in rabbit against synthetic PGE_2 congugated with BSA and diluted to standard concentration recommended by source company) were prepared in duplicate. The final volume of the assay tubes was $300\mu l$. Tubes containing $100\mu l$ assay buffer ($50\mu l$ in place of PGE_2 Ab) and $50\mu l$ assay buffer with $50\mu l$ PGE_2 Ab respectively, were also set up to determine non-specific and the total binding, after which $100\mu l$ ^3H-PGE_2, diluted in assay buffer to give aproximately $0.1\mu Ci/ml$-2.2×10^4 disintegrations/min/tube and $100\mu l$ SPA reagent were added to each tube. Tubes were also set up containing $100\mu l$ ^3H-PGE_2 with 2ml of scintillant to determine the total counts. The tubes were vortexed and incubated for 24h at $4°C$. No separation process was necessary and the radioactivity was counted on an automatic beta counter (LKB, Turku, Finland) using a 3 min count time.

Competitive enzyme-linked immunosorbant assay for estimation of rat lipocortin 1.

Liporcortin 1 was measured in samples from selected experiments using the method described by Smith, Smith & Buckingham (1990). The wells of a Ninc Immulon type 1 96 well microtitre plate (Gibco, Paisley, UK) were each coated with 2.5 mg purified recombinant human lipocortin 1 (Biogen Research Corp. Cambridge, MA, USA) in 100μl phosphate buffered saline (PBS; Oxoid) pH 7.4, incubated at 4°C for 24h and washed fived times with 200μl PBS containing 0.05% (v/v) Tween 20 (Sigma Chemical Co., Dorset UK). Aliquots (200μl) of foetal bovine serum albumin (2% v/v in pBS) were added to each well, the incubation continued at 37°C for 60min and the wells washed throughly (x 5, PBS-Tween). Serial dilutions of the standard preparation (recombinant human lipocortin 1, 0-25ng/well) or tissue protein extracts (homogenised in 10mM HEPES, 5mM EDTA, 100mM sodium chloride, 200mM phenyl methyl suphonyl fluoride; Sigma Chemical Co. and total protein measured [15]), were prepared in PBS-Tween and dispensed in volumes of 50μl into wells together with 50μl rabbit polyclonal lipocortin 1 antibody (842, 1:5000 dilution, Biogen Research Corp.). The plate was incubated at 4°C for 24h, washed (x 5, PBS-Tween) and, following the addition of 100μl/well goat anti rabbit IgG conjugated to horseradish peroxidase (1:2000 dilution in PBS-Tween; Sigma Chemical Co.), incubated for 60min at 37°C. After a final cycle of five washes in PBS-Tween, 100μl freshly prepared peroxidase substrate, o-phynylene diamine (0.04% w/v in PBS with 0.4 μl/ml H_2O_2; Sigma Chemical Co.) was added to each well. The plate was developed at room temperature in the dark for 20min at which point the reaction was stopped abruptly by the addition of 50μl H_2SO_4 (2.5M). The optical densities were read at 492nm (Uniskan, Labsystems, UK). The results for unknown samples were calculated from the standard curve and expressed as ng lipocortin 1/mg protein.

Statistical analysis.

The results were analysed using the Mann Whitney U test. This non-parametric test was used instead of the students t-test (a parametric test) since the 'n values' were quite small (4-7) and therefore this equivalent non-parametric test was thought to be more appropriate. Differences were considered to be significant if the probability was less than 5% ($P<0.05$).

RESULTS

(i) Incubation of hypothalamic tissue *in vitro*.

(a) Peptide estimation.

Figs. 3i-iii show typical standard curves for the radioimmunoassays of ir-AVP and ir-CRF-41. With respect to AVP, using the antibody donated by Prof. S. Lightman, the sensitivity of the assay, that is the smallest dose giving a depression of binding significantly different from the reference, was 0.0156ng/ml (P<0.01, t-test n=10) with inter-assay and intra-assay coefficients of variance of 16.22% and 8.8% respectively. Antibody specificity was not tested as it was described fully elsewhere (Eckland, Todd & Lightman 1988). However, further verification was provided by failure of the assay to detect ir-AVP in medium in which hypothalamic tissue from rats congenitally lacking hypothalamic AVP (Brattleboro strain) had been incubated (0.004ng/ml vs.0.4ng/ml in Long Evans controls). Using another antibody of defined specificity (*Hillhouse & Milton 1989a*-provided by Dr. E. Hillhouse) assay sensitivity was 7.8pg/ml (P<0.01 t-test n=10) with inter and intra-assay coefficients of variation of 3.64% and 11.9%. Both AVP assay methods were sufficiently sensitive to detect the peptide in medium in which the hypothalamic tissue from normal rats hand been incubated and, in both instances, the dilution curves of the medium were parallel with those of the standard preparation (synthetic AVP1-9) (Fig. 3i & ii).

The sensitivity of the CRF-41 assay, of which the inter and intra-assay coefficients of variation were 4.36% and 9.2% respectively, was 20pg/ml (P=0.001, t-Test, n=9) and was thus only just sufficiently sensitive to detect the basal peptide release. Thus, when equipment became available, samples were freeze dried and concentrated prior to assay. As with the AVP assays, dilution curves of the medium in which the hypothalamic tissue had been incubated, were parallel with those of the standard (Fig. 3iii).

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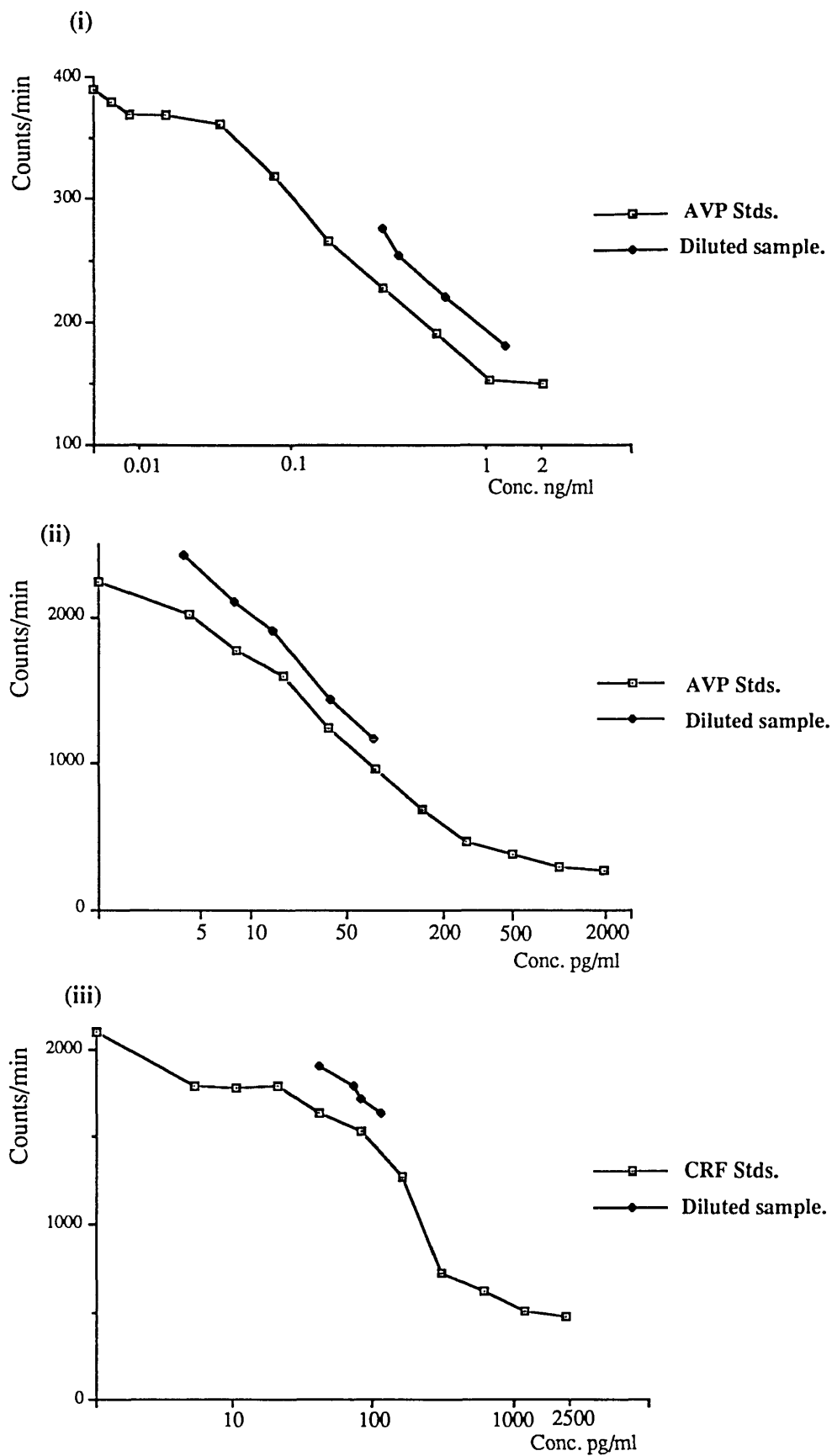
Fig. 3 : Typical standard curves from (i) AVP radioimmunoassay -Prof. S. Lightmans antiserum (ii) AVP radioimmunoassay and (iii) CRF radioimmunoassay-Dr. E. Hillhouse antisera showing parallelism of diluted samples.

(b) Secretion of ir-CRF-41 and ir-AVP in response to a non-specific depolarizing stimulus (56mM K⁺).

Following an initial pre-incubation period (60-90min), two successive 15 min incubations were necessary to stabilize the resting release of ir-AVP and ir-CRF-41 from rat hypothalamic tissue in vitro. The tissue then responded readily to a non-depolarizing stimulus (56mM K^+) with significant (P=0.005 B1 vs. S1, Mann Whitney U test) increases in the release of the two peptides (Fig. 4). When challenged repeatedly with K^+ (56mM) the AVP responses of the tissue declined progressively (Fig. 4). Thus, although basal release did not alter significantly with time (P=0.350 B1 vs. B2 and P=0.409 B1 vs B3 for ir-AVP, Mann Whitney U test), peptide release in reponse to the second and third stimulations with K^+ were significantly reduced when compared with the release following the initial stimulation (P= 0.032 S1 vs. S2 and P=0.005 S1 vs. S3 for ir-AVP, Mann Whitney U test). In contrast, ir-CRF-41 release was consistent, in terms of peptide output, following two successive K^+ stimulations.

(i)

(ii)
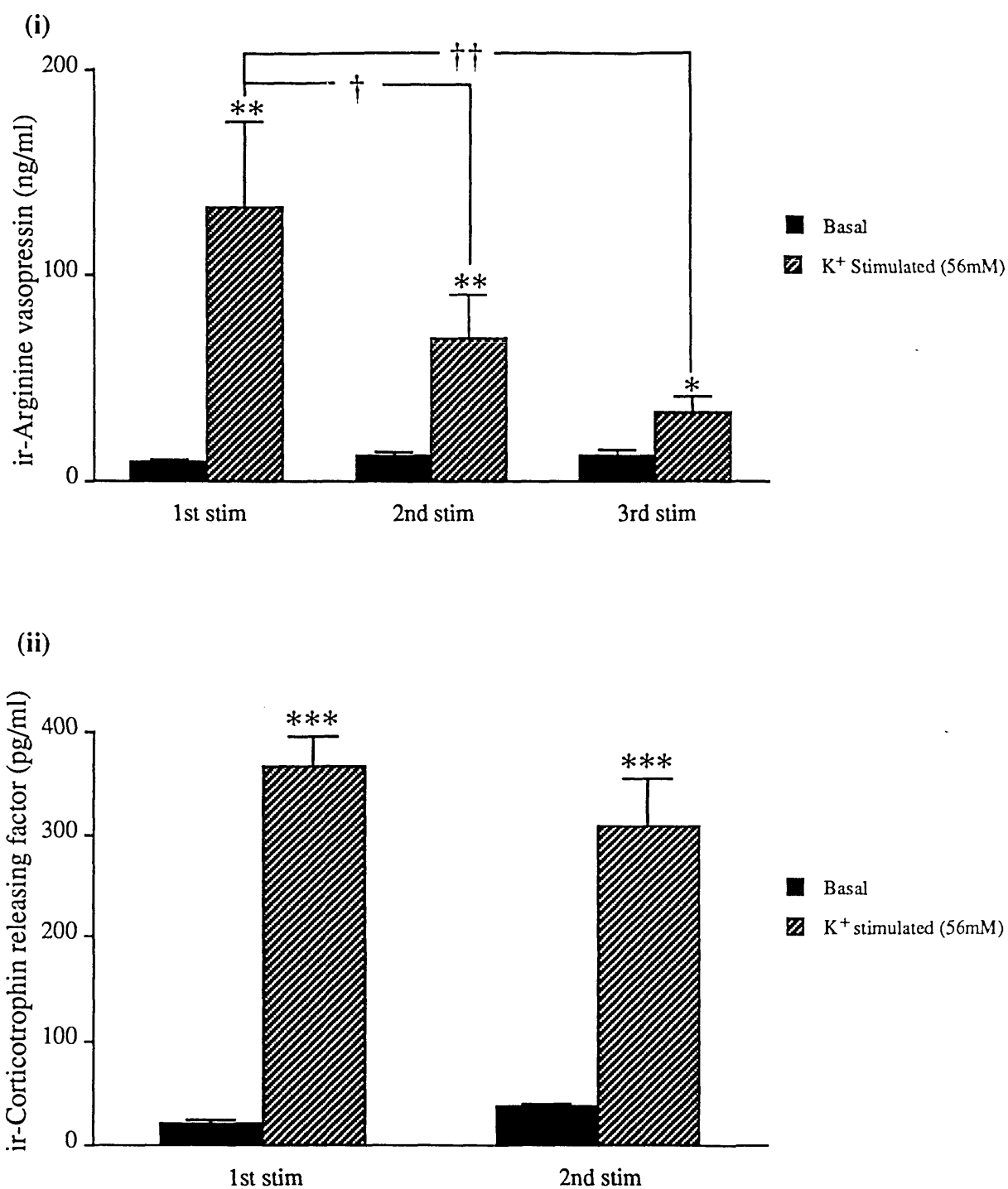
Fig. 4 : The effect of repeated stimulation with K^+ (56mM) on **(i)** ir-arginine vasopressin and **(ii)** ir-corticotrophin releasing factor release from hypothalami from adrenalectomized rats. Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01; ***P<0.001 basal vs. K^+ stimulated; †P<0.05; ††P<0.01, Mann Whitney U test.

(ii) Effects of Phospholipase A_2 (PLA$_2$), melittin and the phospholipase A2 inhibitor, quinacrine on the secretion of ir-AVP and ir-CRF-41 by isolated hypothalamic tissue in vitro.

Figs. 5-8 illustrate the effects of graded concentrations of phospholipase A_2 and melittin on the release in vitro of ir-AVP and ir-CRF-41 from hypothalami from intact and adrenalectomized rats.

Phospholipase A_2 (25-50U/ml) stimulated the release of ir-AVP and, at the highest concentration tested (50U/ml), it caused a 3.5 fold increase in peptide release from tissue from both intact ($P<0.05$, Mann Whitney U test) and adrenalectomized ($P<0.01$, Mann Whitney U test) rats (Fig. 5). The enzyme also caused significant ($P<0.05$, Mann Whitney U test) increases in ir-CRF-41 release from both groups of tissue; its effect on tissue from intact rats was maximal at a concentration of 25U/ml (Fig. 6i).

The effects of PLA$_2$ on both tissues were mimicked by melittin (0.5-2.0μg/ml) which thus produced significant ($P<0.05$, Mann Whitney U test) increases in the release of ir-AVP (Fig. 7i & ii) and ir-CRF-41 (Fig 8i & ii).

Figs. 9-11 show the effect of the PLA$_2$-inhibitor, quinacrine (10^{-4}M) on the PLA$_2$ and melittin stimulated release of ir-AVP and ir-CRF-41 from hypothalami from intact and adrenalectomized rats. Corresponding data from sham-operated controls are shown in Fig. 27.

Addition of quinacrine to the pre-incubation medium had no effect on the basal release of ir-CRF-41 from hypothalami from intact or adrenalectomized rats (Fig. 11). However, it tended to facilitate the spontaneous secretion of ir-AVP particularly in tissue from intact and sham adrenalectomized animals (Figs. 9, 10 & 27). The marked increment in ir-AVP secretion elicited by PLA$_2$ in tissue from intact, adrenalectomized and sham adrenalectomized ($P<0.01$, Mann Whitney U test) was effectively inhibited ($P<0.01$, intact & adrenalectomized, $P<0.05$, sham-operated, Mann Whitney U test) by pre-incubation with quinacrine (10^{-4}M). Similarly, quinacrine abolished the ir-AVP response to melittin in tissue from intact animals ($P=0.014$, Mann Whitney U test). Its effect on stimulated ir-AVP release in tissue from adrenalectomized animals although apparent was less marked, possibly because the drug increased the basal release of the peptide. Thus, there were no significant differences between the absolute values in ir-

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AVP concentration following melittin stimulation in drug treated and control groups but the increment in ir-AVP release was reduced significantly (P=0.014, Mann Whitney U test). Surprisingly, quinacrine (10^{-4}M) had no effect on the PLA_2-induced secretion of ir-CRF-41 from tissue from intact (Fig. 11i) or sham-adrenalectomized (Fig. 27ii) rats but reduced significantly (P<0.05, Mann Whitney U test) the secretory response in tissue from adrenalectomized rats (Fig. 11ii).

(i)

(ii)
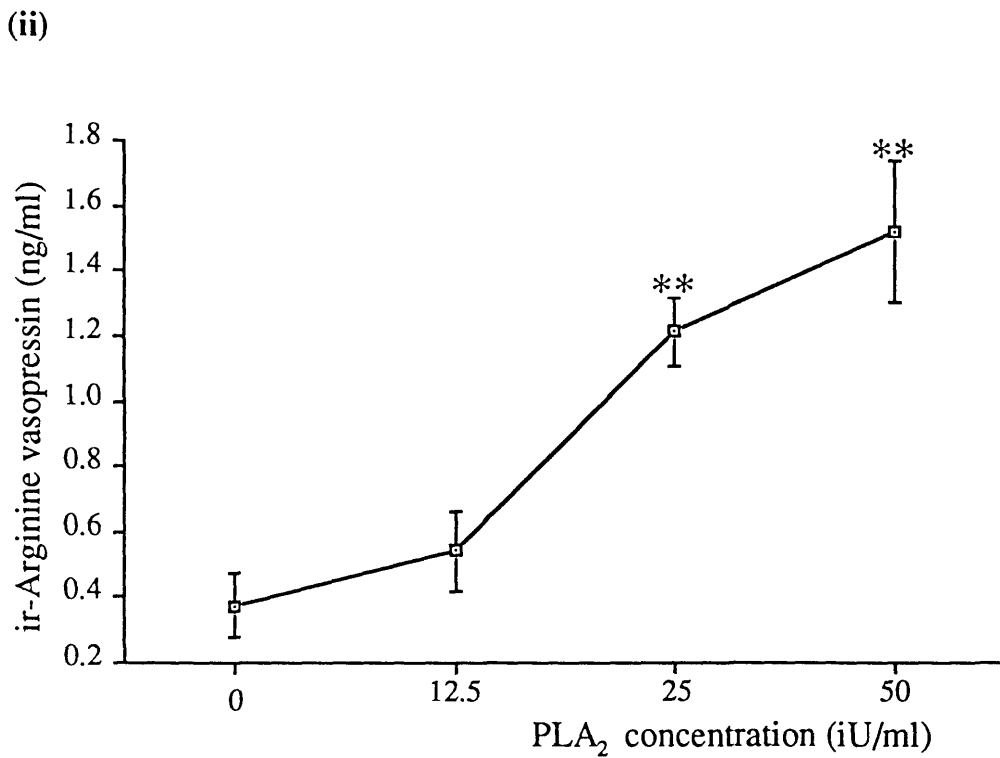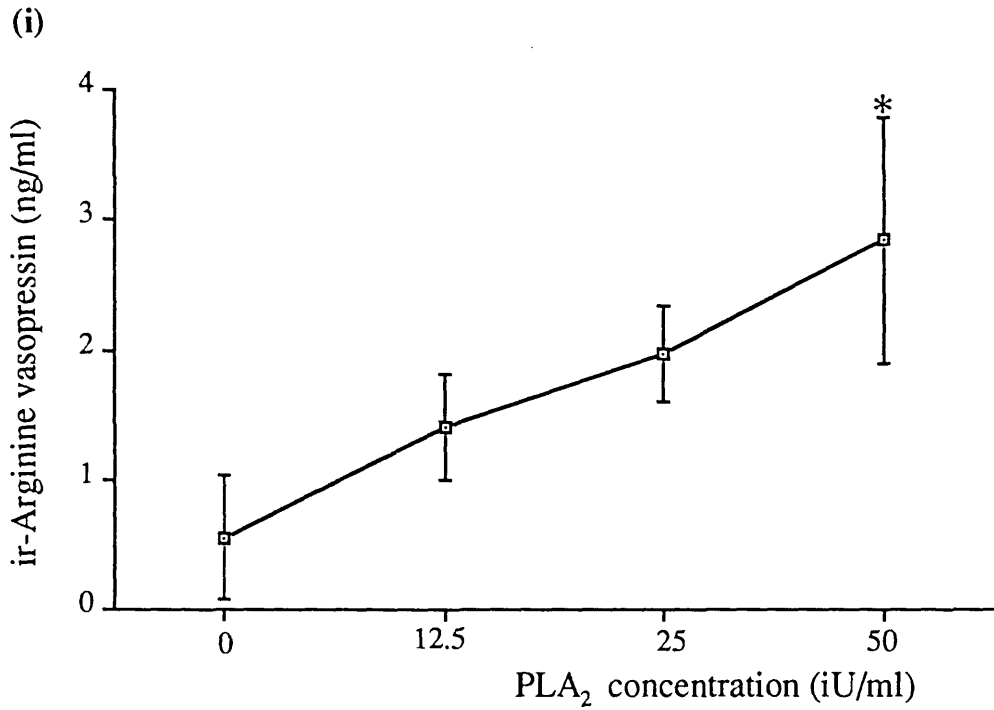
Fig. 5: The effect of graded doses of phospholipase A_2 (PLA$_2$) on ir-arginine vasopressin release from hypothalami from **(i)** intact (n=3-5) and **(ii)** adrenalectomized rats (n=4-6). Each point represents the mean and is shown with its standard error (*P<0.05 ; **P<0.01, Mann Whitney U test).

Fig. 6 : The effect of graded doses of phospholipase A$_2$ (PLA$_2$) on ir-corticotrophin releasing factor release from hypothalami from (i) intact (n=3-5) and (ii) adrenalectomized rats (n=3-5). Each point represents the mean and is shown with its standard error (*P<0.05, Mann Whitney U test).
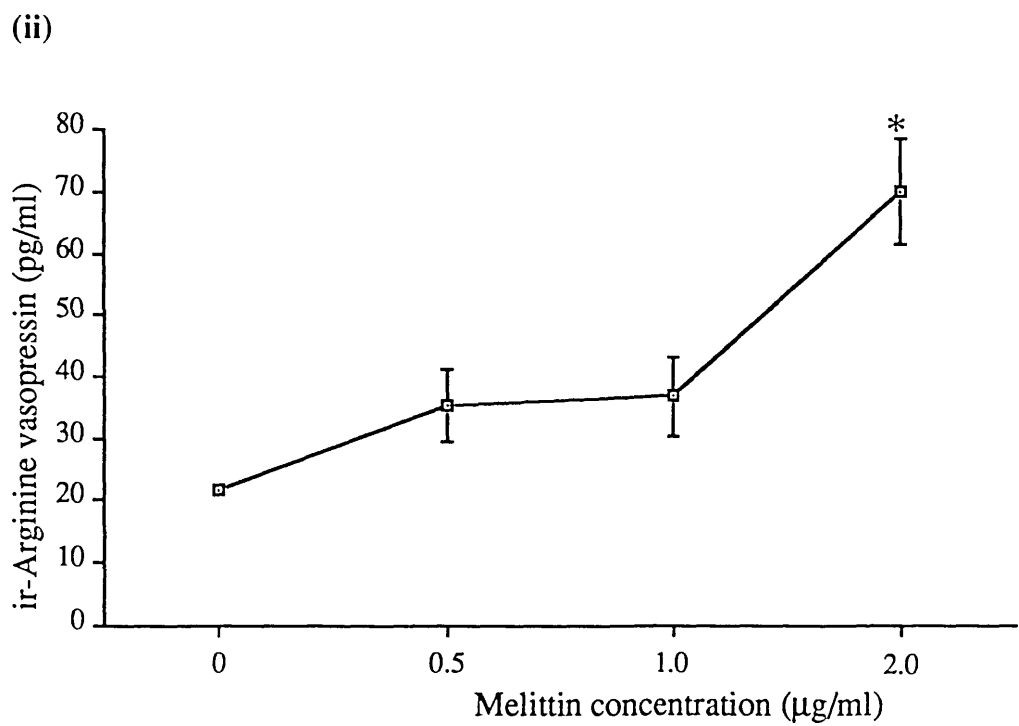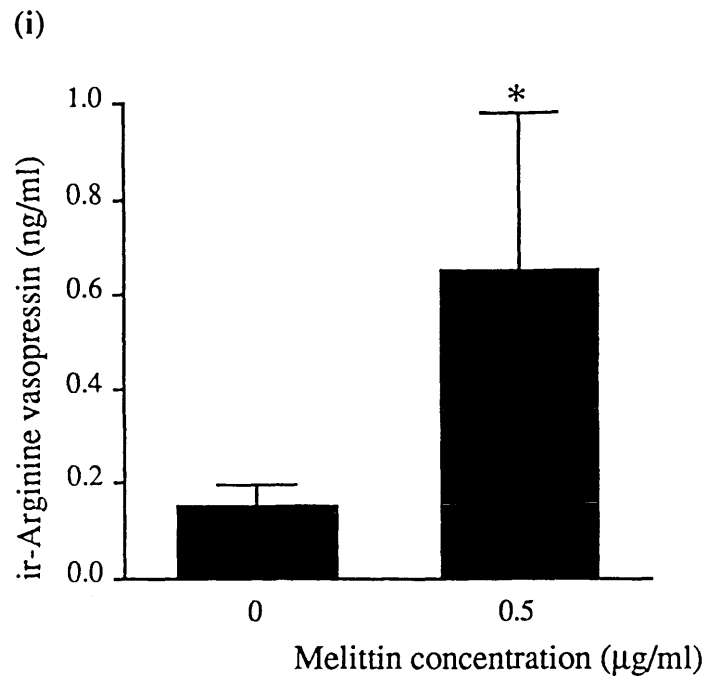
Fig. 7 : The effect of graded doses of melittin on ir-arginine vasopressin release from hypothalami from **(i)** intact (n=3-4) and **(ii)** adrenalectomized rats (n=3-5). Each point represents the mean and is shown with its standard error (*P<0.05, Mann Whitney U test).

(i)

(ii)
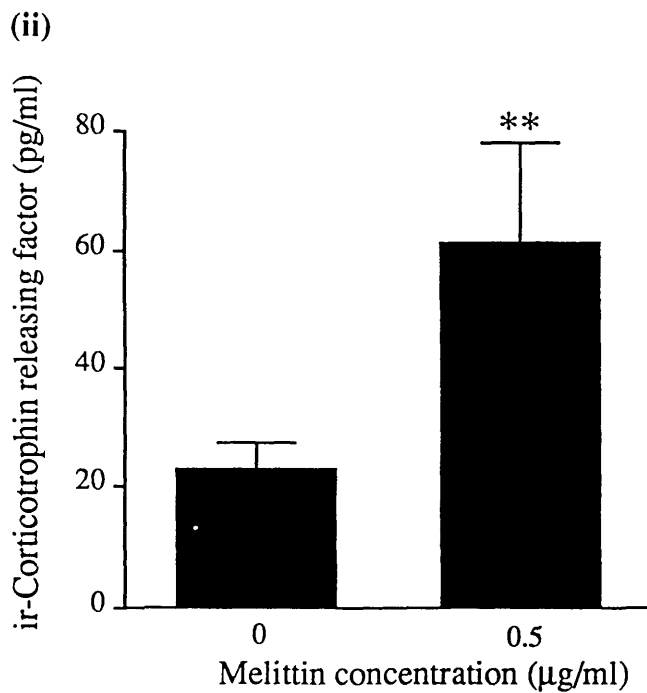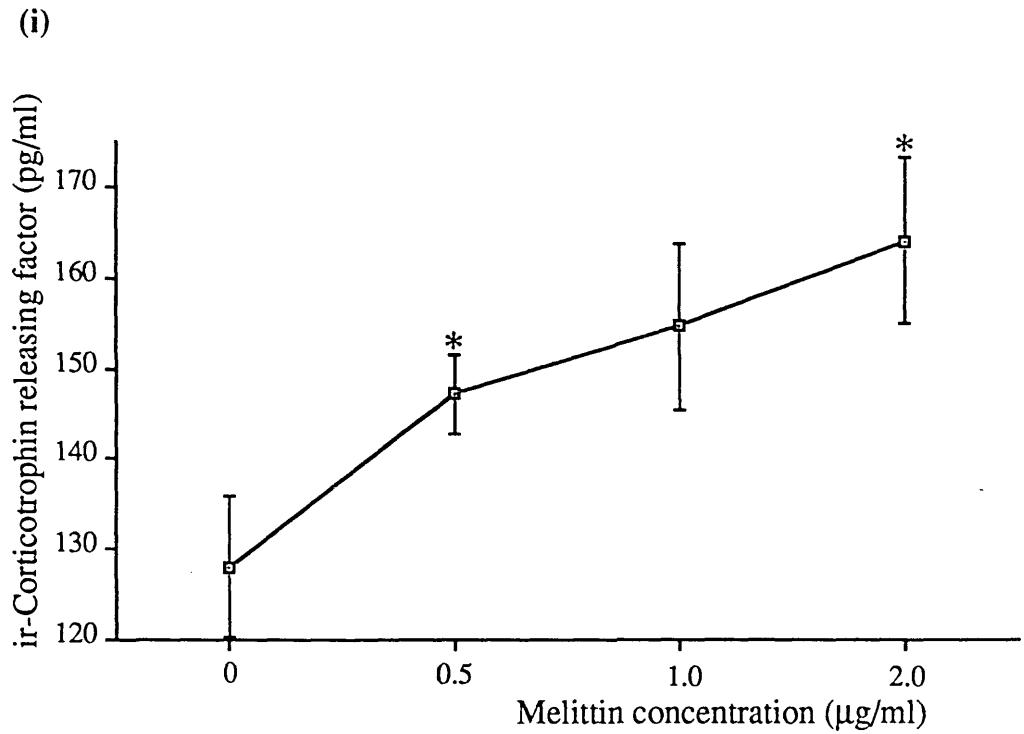
Fig. 8 : The effect of graded doses of melittin on ir-corticotrophin releasing
factor release from hypothalami from (i) intact (n=3-5) and (ii)
adrenalectomized rats (n=4-5). Each point represents the mean and is
shown with its standard error (*P<0.05 ;**P<0.01, Mann Whitney U
test).
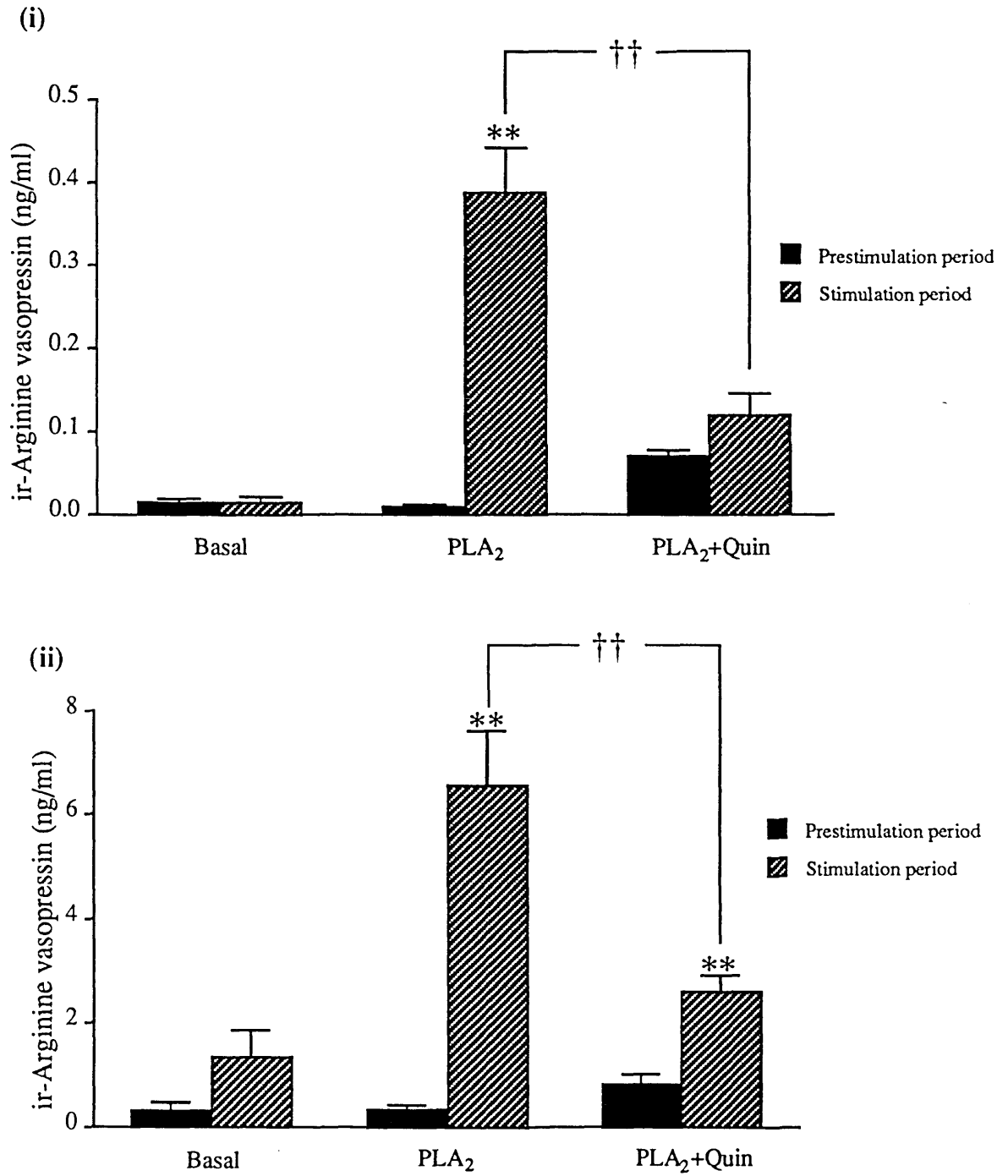
Fig. 9 : The effect of quinacrine (Quin, 10^{-4}M) on phospholipase A_2 (PLA$_2$ 25U/ml) induced ir-arginine vasopressin release from hypothalami from **(i)** intact (n=5) and **(ii)** adrenalectomized rats (n=4-5). Each column represents the mean and is shown with its standard error (**P<0.01 prestimulation period vs. stimulation period; ††P<0.01, Mann Whitney U test).

(i)

(ii)
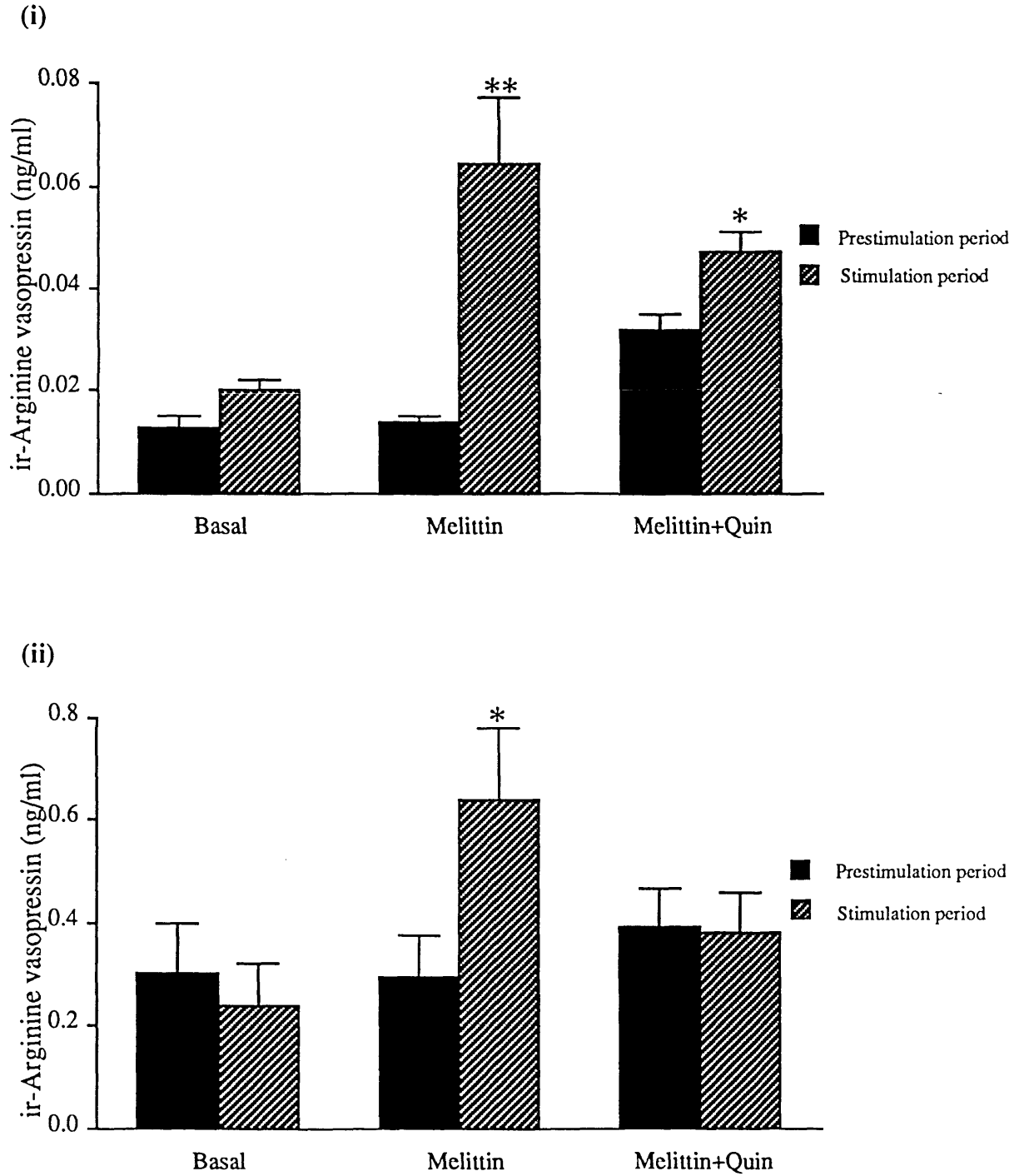
Fig. 10 : The effect of quinacrine (Quin, 10^{-4} M) on melittin (1μg/ml) induced ir-arginine vasopressin release from hypothalami from (i) intact (n=4-5) and (ii) adrenalectomized rats (n=3-5). Each column represents the mean and is shown with its standard error (*P<0.05 ; **P<0.01 prestimulation period vs. stimulation period, Mann Whitney U test).
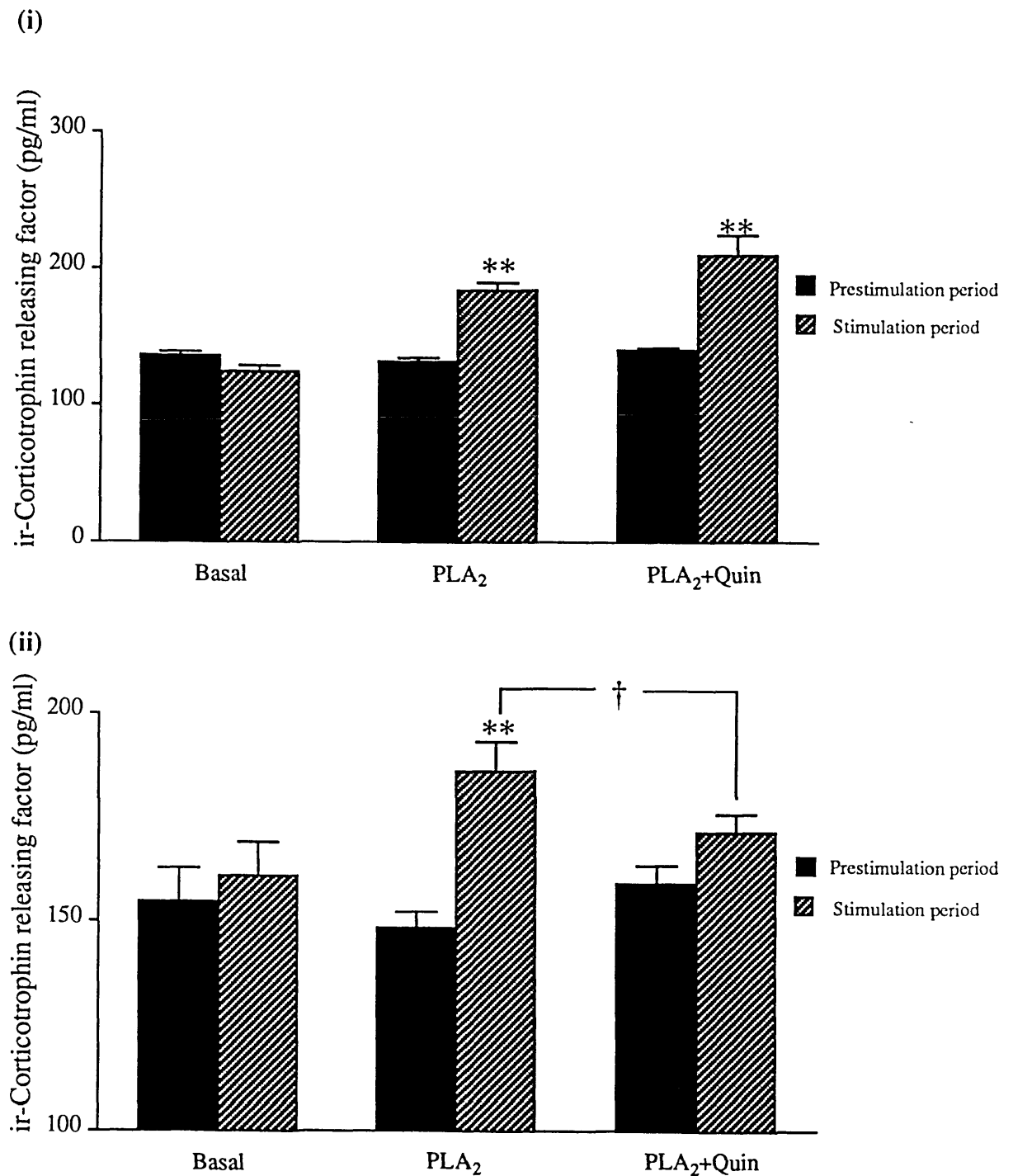
Fig. 11 : The effect of quinacrine (Quin, 10^{-4} M) on phospholipase A_2 (PLA_2 25U/ml) induced ir-corticotrophin releasing factor release from hypothalami from (i) intact (n=5) and (ii) adrenalectomized rats (n=5). Each column represents the mean and is shown with its standard error (**$P<0.01$ prestimulation period vs. stimulation period; †$P<0.05$, Mann Whitney U test).

iii) The effects of arachidonic acid and inhibitors of arachidonic acid metabolism on the secretion of ir-AVP and ir-CRF-41 by isolated hypothalamic tissue in vitro.

Arachidonic acid ($10^{-4}M$) had no effect on the release of ir-AVP from hypothalami from adrenalectomized rats when bovine serum albumin (BSA, 0.25%) was included in the incubation medium (Fig. 12ii). In the absence of BSA the fatty acid appeared to stimulate AVP release but the responses were highly variable and did not reach significance (P=0.200) probably due to the low n values (Fig. 12i).

Figs. 13-17 show the effects of various inhibitors of cyclo-oxygenase, lipoxygenase and epoxygenase enzymes on PLA_2-induced ir-AVP release. In these studies PLA_2 (25U/ml) produced the anticipated significant (P<0.05, Mann Whitney U test) increases in ir-AVP release from hypothalami from intact and adrenalectomized rats. The alcohol vehicle, employed for ibuprofen, ICI 207968 and BW755C, was generally without effect on ir-AVP release although in one experiment (Fig. 16) it attenuated the response to PLA_2 and in another (Fig. 13i) the basal release of the peptide was surprisingly high, an effect which was reversed by the cyclo-oxygenase inhibitor, ibuprofen (10^{-5} & $10^{-4}M$).

Neither the cyclo-oxygenase inhibitors, ibuprofen (10^{-5} & $10^{-4}M$, Figs. 13i & ii) and indomethacin ($10^{-4}M$, Figs. 14i & ii), nor the dual cyclo-oxygenase/lipoxygenase inhibitor, BW755C ($10^{-4}M$, Figs. 15i & ii), attenuated the AVP-response to PLA_2 stimulation in tissue from intact or adrenalectomized animals. To the contrary, indomethacin appeared to potentiate the response to this secretagogue (Figs. 14i & ii). In the studies with the 5-lipoxygenase inhibitor, ICI 207968 ($10^{-4}M$), the AVP response of tissue from adrenalectomized rats to PLA_2 was attenuated by the ethanol vehicle. Furthermore no response to PLA_2 was evident in the presence of the enzyme inhibitor in tissue from adrenalectomized rats (Fig. 16). Blockade of epoxygenase enzymes with SKF 525A ($10^{-4}M$) inhibited (P<0.05, Mann Whitney U test) the AVP response to PLA_2 in hypothalamic tissue from intact (Fig. 17i) but not adrenalectomized rats (Fig. 17ii). In both tissues the basal release of the peptide was unaffected by the presence of SKF 525A in the medium.
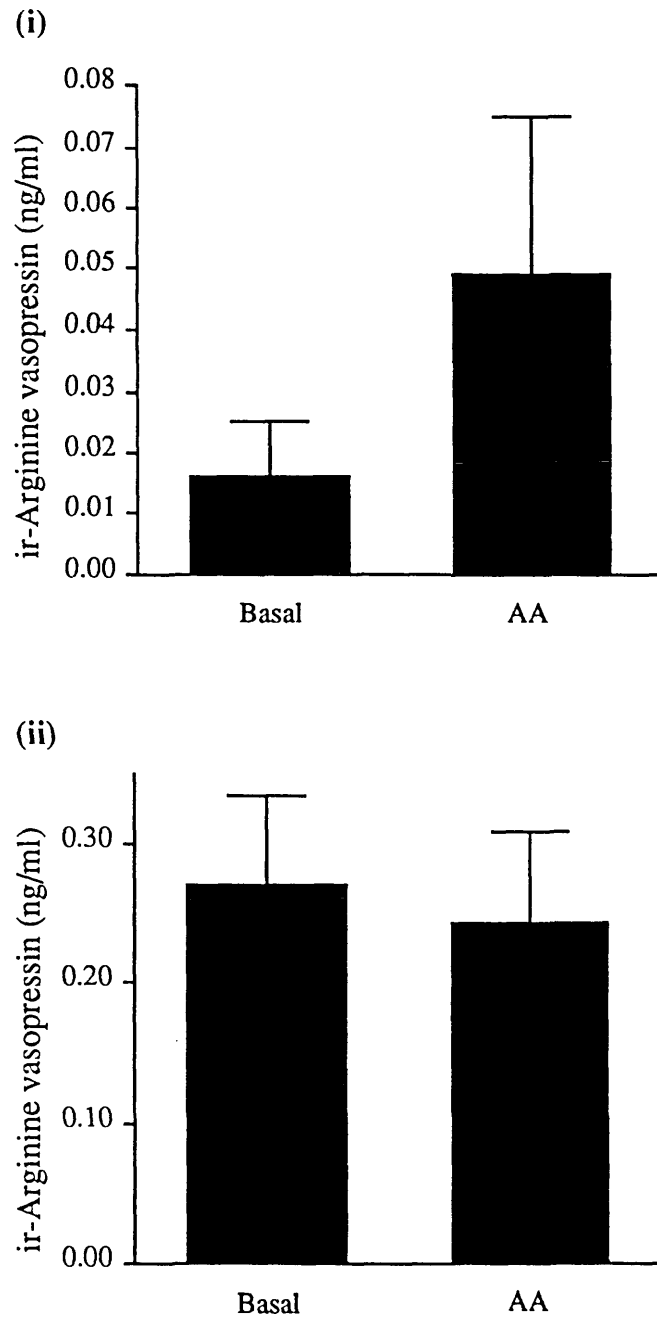
Fig. 12 : The effect of arachidonic acid (AA, 10^{-4}M) on ir-arginine vasopressin release from hypothalami from adrenalectomized rats (**i**) without (n=3) and (**ii**) with (n=5) bovine serum albumin (0.25%) in the incubation medium. Each column represents the mean and is shown with its standard error.
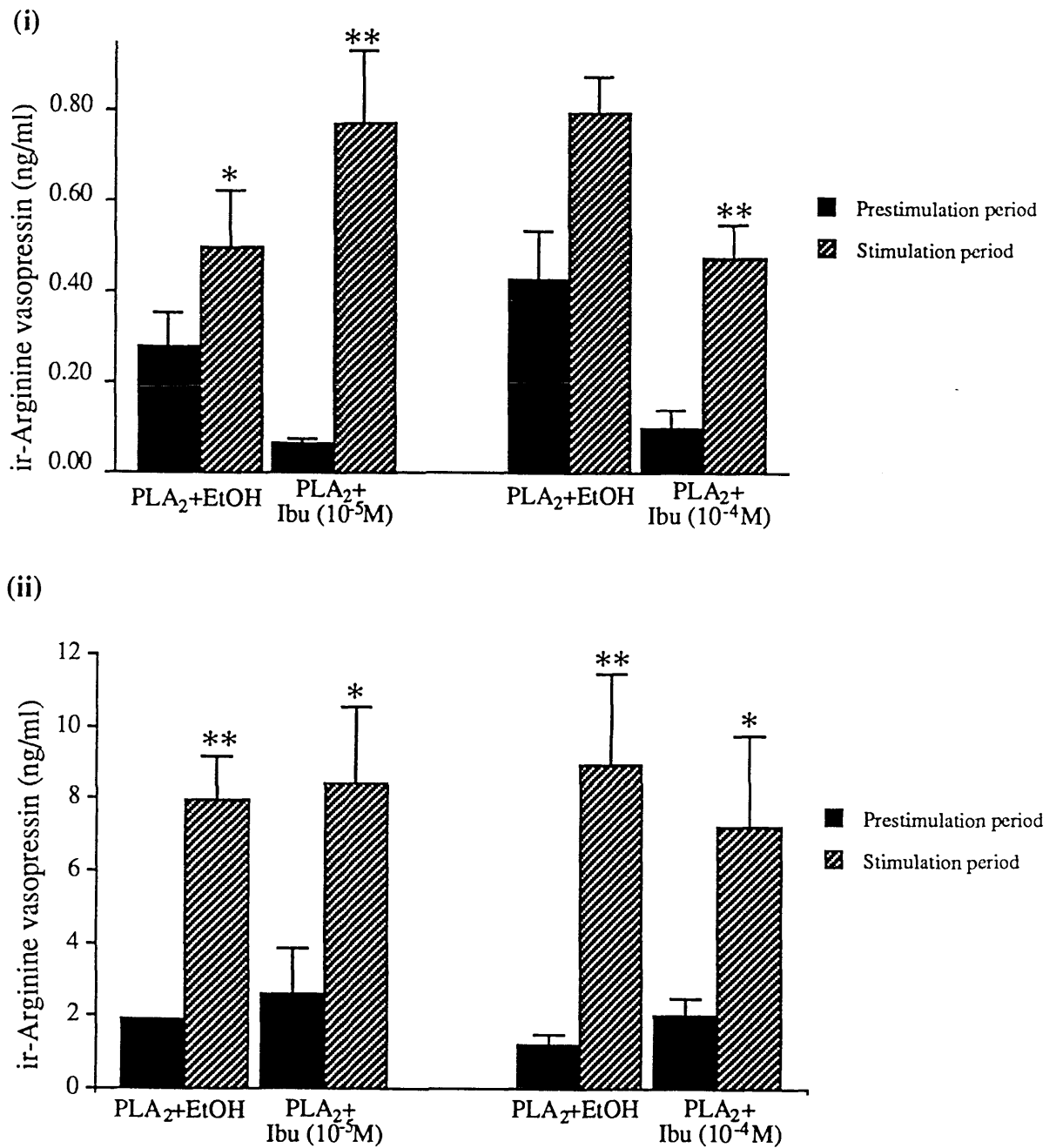
Fig. 13 : The effect of ibuprofen (Ibu) or the ethanol vehicle (EtOH) on phospholipase A_2 (PLA$_2$ 25U/ml) stimulated ir-arginine vasopressin release from hypothalami from (i) intact (n=4-5) and (ii) adrenalectomized rats (n=4-5). Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01 prestimulation period vs. stimulation period, Mann Whitney U test).

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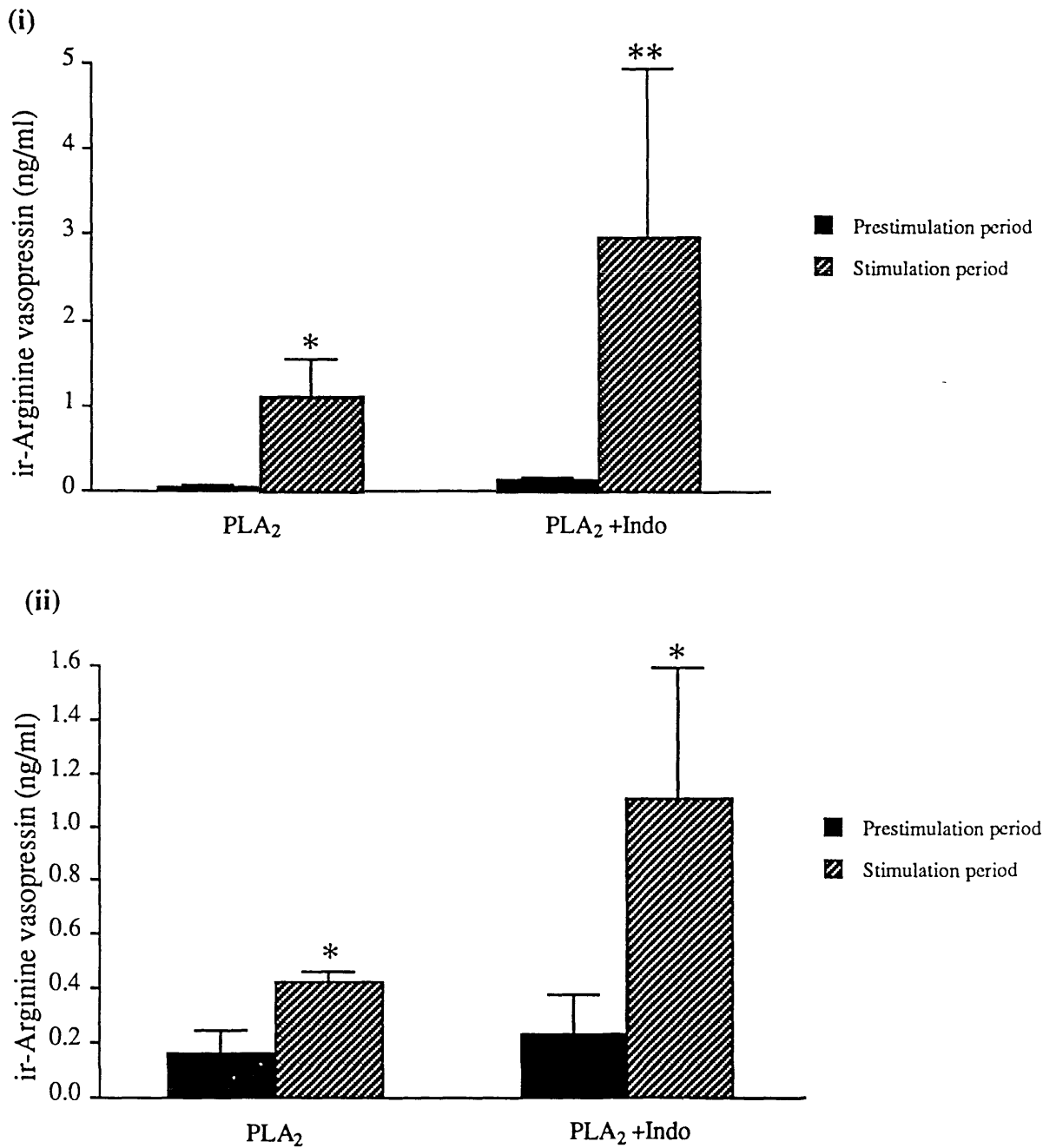
Fig. 14 : The effect of indomethacin (Indo, 10^{-4}M) on phospholipase A_2 (PLA$_2$ 25U/ml) stimulated ir-arginine vasopressin release from hypothalami from **(i)** intact (n=3-4) and **(ii)** adrenalectomized rats (n=4-5). Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01 prestimulation period vs. stimulation period, Mann Whitney U test).

75

(i)

(ii)

Fig. 15 : The effect of the dual cycloxygenase/lipoxygenase inhibitor (BW755C, $10^{-4}M$) or the ethanol vehicle (EtOH) on phospholipase A_2 (PLA_2 25U/ml) stimulated ir-arginine vasopressin release from hypothalami from (i) intact (n=4-5) and (ii) adrenalectomized rats (n=4-5). Each column represents the mean and is shown with its standard error (**$P<0.01$ prestimulation period vs. stimulation period, Mann Whitney U test).
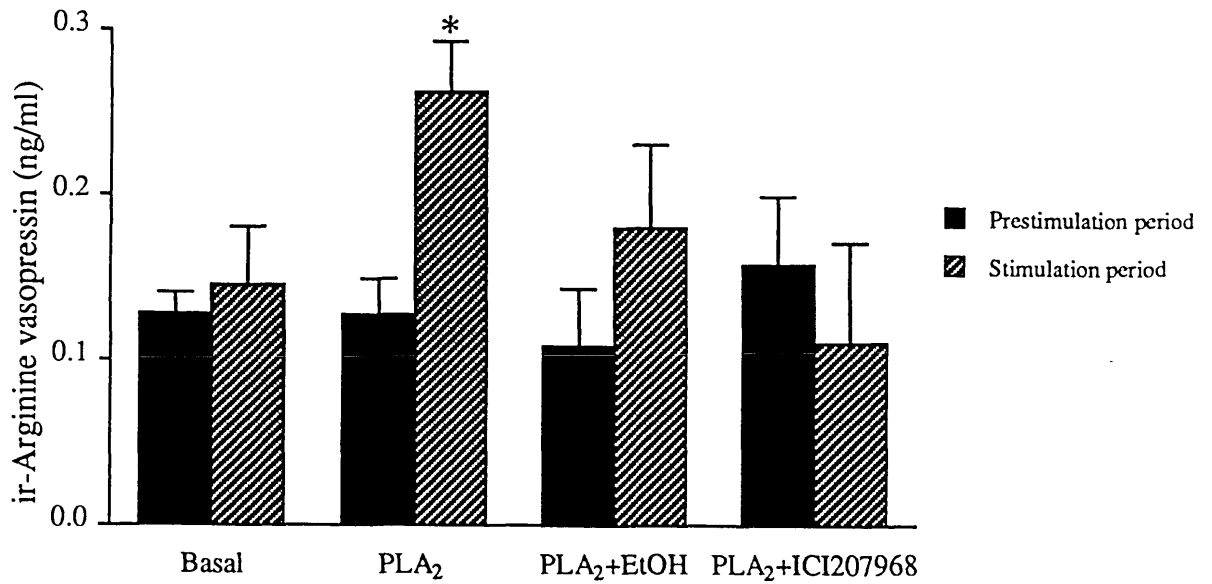
Fig. 16 : The effect of the 5-lipoxygenase inhibitor (ICI 207968, $10^{-4}M$) or the ethanol vehicle (EtOH) on phospholipase A_2 (PLA$_2$ 25U/ml) stimulated ir-arginine vasopressin release from hypothalami from adrenalectomized rats (n=3-5). Each column represents the mean and is shown with its standard error (*P<0.05 prestimulation period vs. stimulation period, Mann Whitney U test).
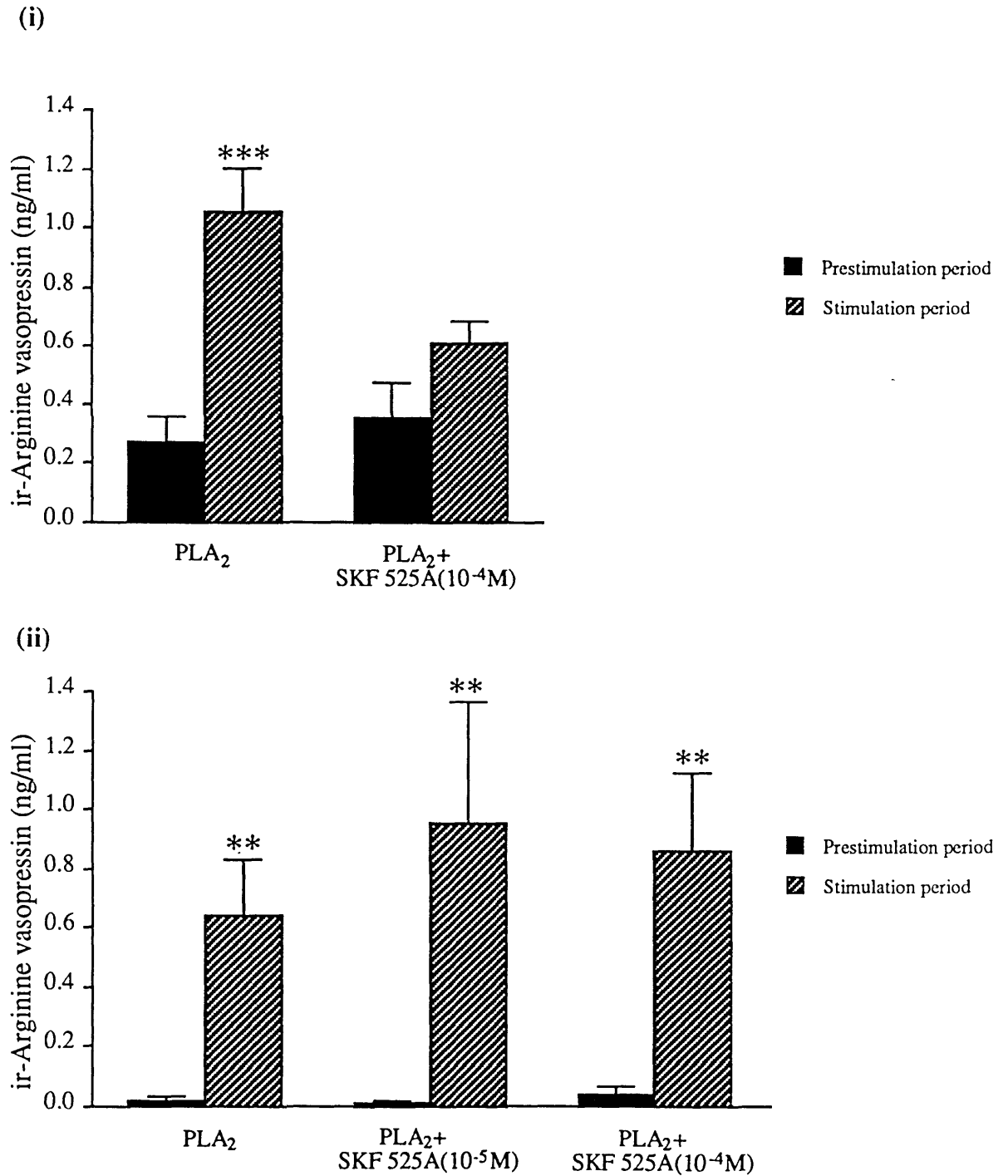
Fig. 17 : The effect of the epoxygenase inhibitor (SKF 525A) on phospholipase A_2 (PLA$_2$ 25U/ml) stimulated ir-arginine vasopressin release from hypothalami from (i) intact (n=3-5) and (ii) adrenalectomized rats (n=5). Each column represents the mean and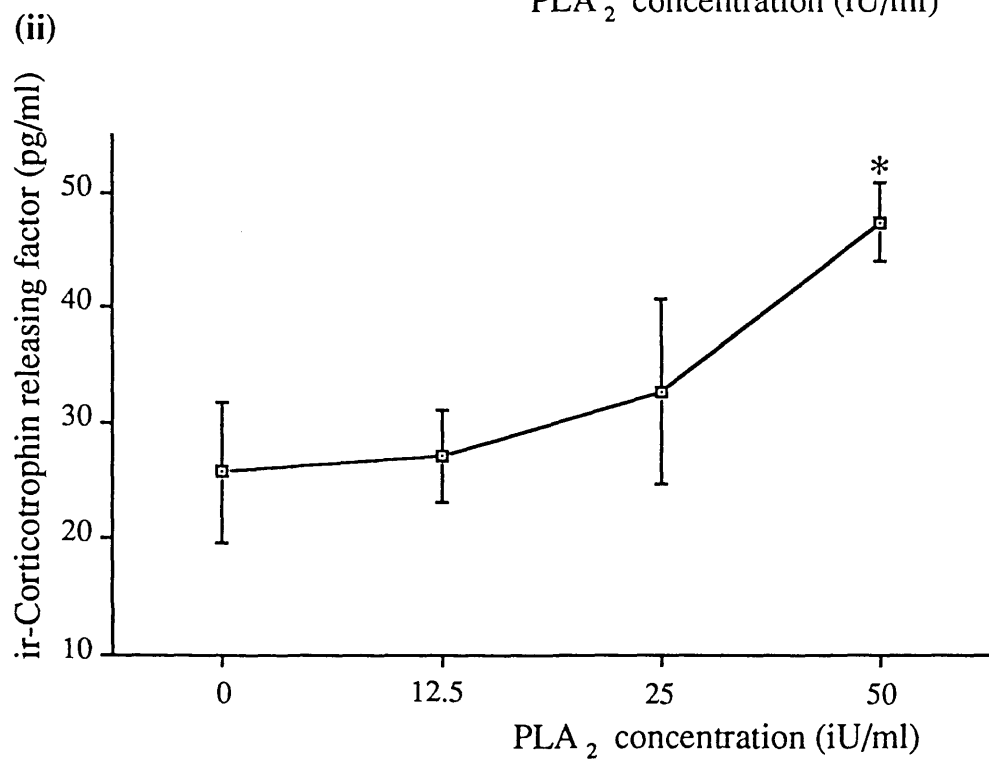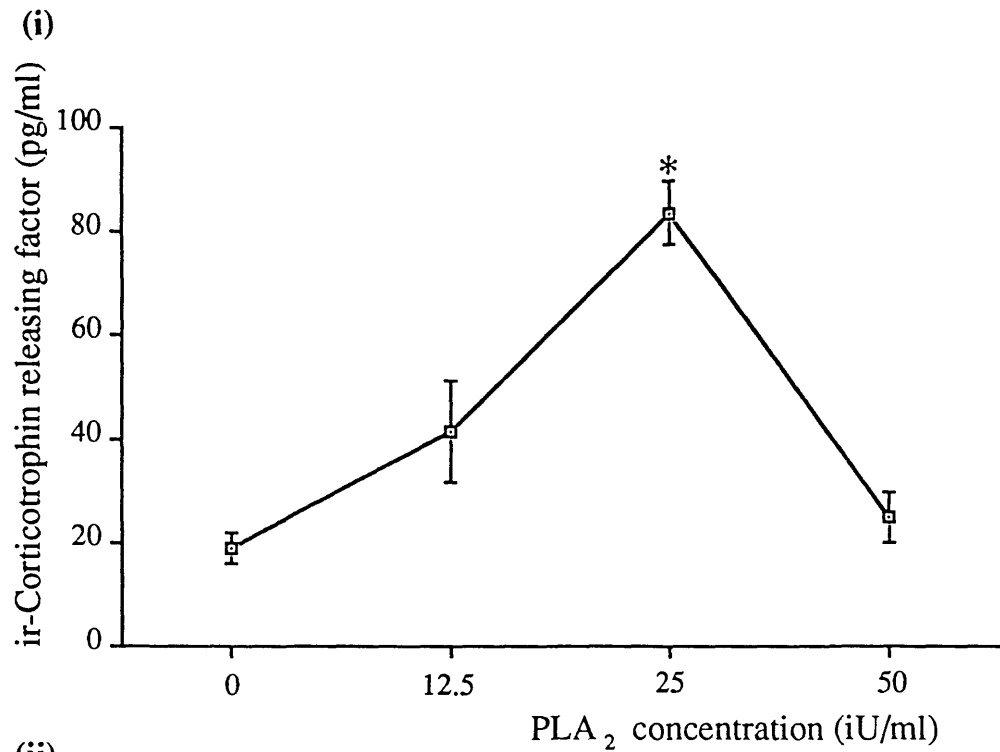 is shown with its standard error (**P<0.01;***P<0.001 prestimulation period vs. stimulation period, Mann Whitney U test).

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Figs. 18-22 show the results from parallel studies in which the effects of the various inhibitors of arachidonic acid metabolism on PLA_2-induced CRF-41 release were examined. In all cases, PLA_2 (25U/ml) produced the anticipated increase (P<0.05, Mann Whitney U Test) in CRF-41 release from hypothalami from intact and adrenalectomized rats. The alcohol vehicle had variable effects on the release of the peptide and while in some experiments it was without effect in certain instances it elevated the basal release (Fig. 21) and potentiated (Fig 18ii & 21) or attenuated (Fig. 19ii) the secretory responses to PLA_2.

Neither ibuprofen ($10^{-4}M$) nor indomethacin ($10^{-4}M$) influenced the resting release of CRF-41 from hypothalami from intact or adrenalectomized animals. Both compounds however reduced the secretory responses to PLA_2. Thus, although in tissue from intact rats, ibuprofen had no significant effect on the absolute concentrations of ir-CRF-41 following stimulation with PLA_2, it reduced significantly (P<0.05, Mann Whitney U test) the increment in peptide release (Fig. 18i). Furthermore, it also reduced significantly (P<0.01, Mann Whitney U test) the pronounced hypersecretion of CRF-41 elicited by the combination of PLA_2 and the alcohol vehicle in tissue from adrenalectomized rats. Like ibuprofen, indomethacin ($10^{-4}M$) also appeared to reduce the increment in CRF-41 release evoked by PLA_2 in tissue from intact rats but, the standard errors were large and the effects were not significant (P= 0.206, Mann Whitney U test, Fig. 19i). Nevertheless, in the presence of the inhibitor, PLA_2 failed to produce a significant increase in ir-CRF-41 release. The effects of indomethacin in tissue from adrenalectomized rats were difficult to interpret as the alcohol vehicle attenuated the response to PLA_2. However, when compared to PLA_2 alone, the responses to the enzyme were reduced significantly (P<0.05, Mann Whitney U test) in the presence of indomethacin (Fig. 19ii).

The dual cyclo-oxygenase/lipoxygenase inhibitor BW755C ($10^{-4}M$) also appeared to attenuate the CRF-41 responses to PLA_2 in tissue from intact and adrenalectomized rats. However, these data were complicated by the fact that the drug itself produced a significant elevation in the basal release of CRF-41 from tissue from intact rats while, in tissue from adrenalectomized rats, the responses to PLA_2 in the presence of the ethanol vehicle were highly variable (Figs. 20i & ii). However, when compared to PLA_2 alone, the responses in the presence of BW755C were reduced significantly (P<0.05, Mann Whitney U test) (Fig. 20ii).

In the study with the 5-lipoxygenase inhibitor ICI 207968 (10^{-4}M), the alcohol vehicle produced highly significant (P<0.01, Mann Whitney U test) increases in resting and PLA$_2$ stimulated ir-CRF-41 release in tissue from adrenalectomized rats which were fully reversed (P<0.01, Mann Whitney U test) by the enzyme inhibitor. The drug however failed to affect the significant (P<0.01, Mann Whitney U test) secretory response to PLA$_2$ (Fig. 21).

Blockade of epoxygenase enzymes with SKF 525A (10^{-4}M) did not reduce PLA$_2$-induced ir-CRF-41 release from hypothalamic tissue from intact or adrenalectomized rats. On the contrary, in the former the peptide release was enhanced by the drug (P<0.05, Mann Whitney U test) while in the latter it was unaffected (Figs. 22i & ii).

Table 2 shows the ratios of PLA$_2$-induced ir-AVP: ir-CRF-41 release from hypothalamic tissue from intact and adrenalectomized rats. The mean (χ) of the five ratios was higher in tissue from adrenalectomized animals.
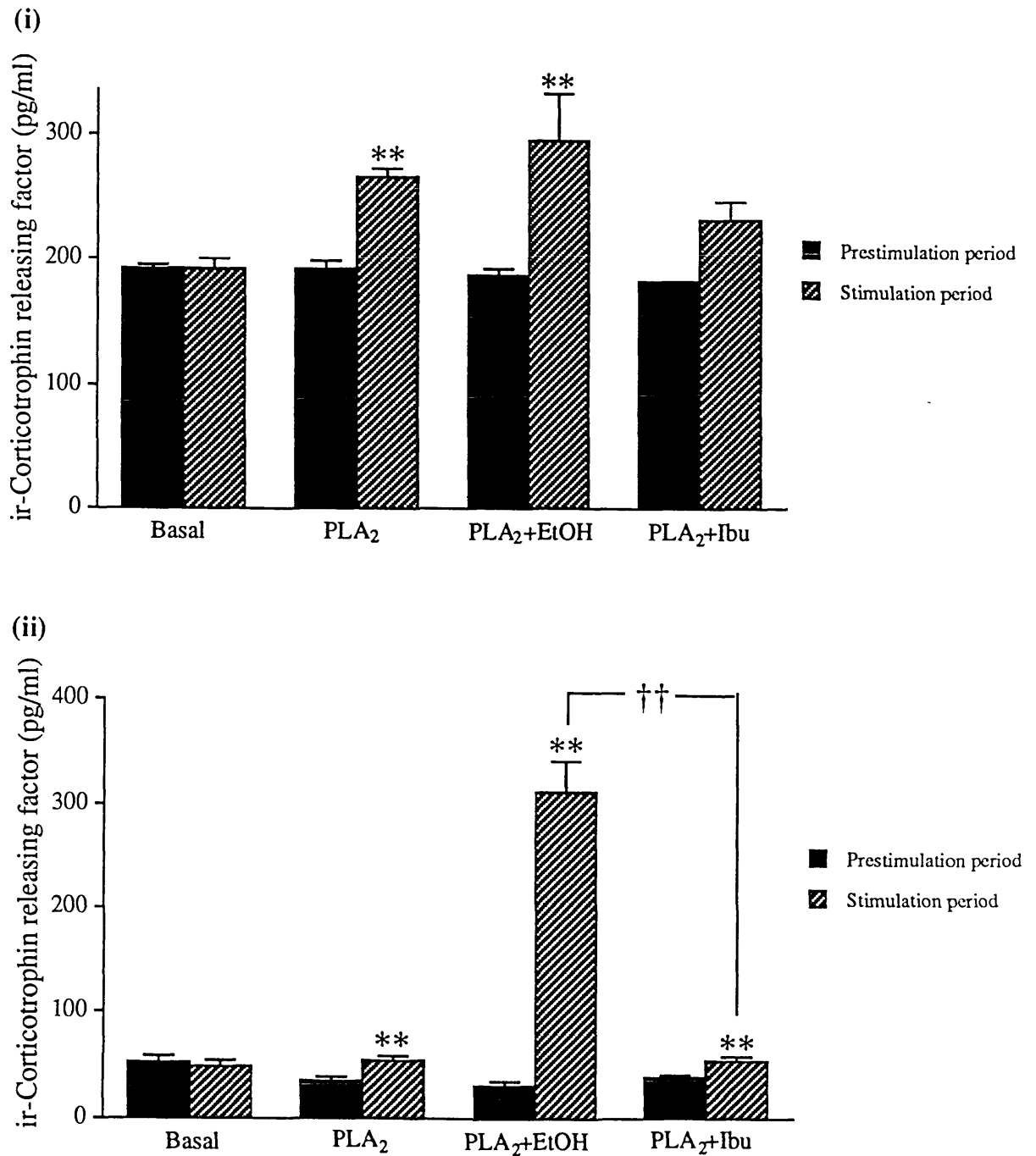
Fig. 18 : The effect of ibuprofen (Ibu, $10^{-4}M$) or the ethanol vehicle (EtOH) on phospholipase A_2 (PLA$_2$ 25U/ml) stimulated ir-corticotrophin releasing factor release from hypothalami from (i) intact (n=4-5) and (ii) adrenalectomized rats (n=5). Each column represents the mean and is shown with its standard error (**P<0.01 prestimulation period vs. stimulation period; ††P<0.01, Mann Whitney U test).
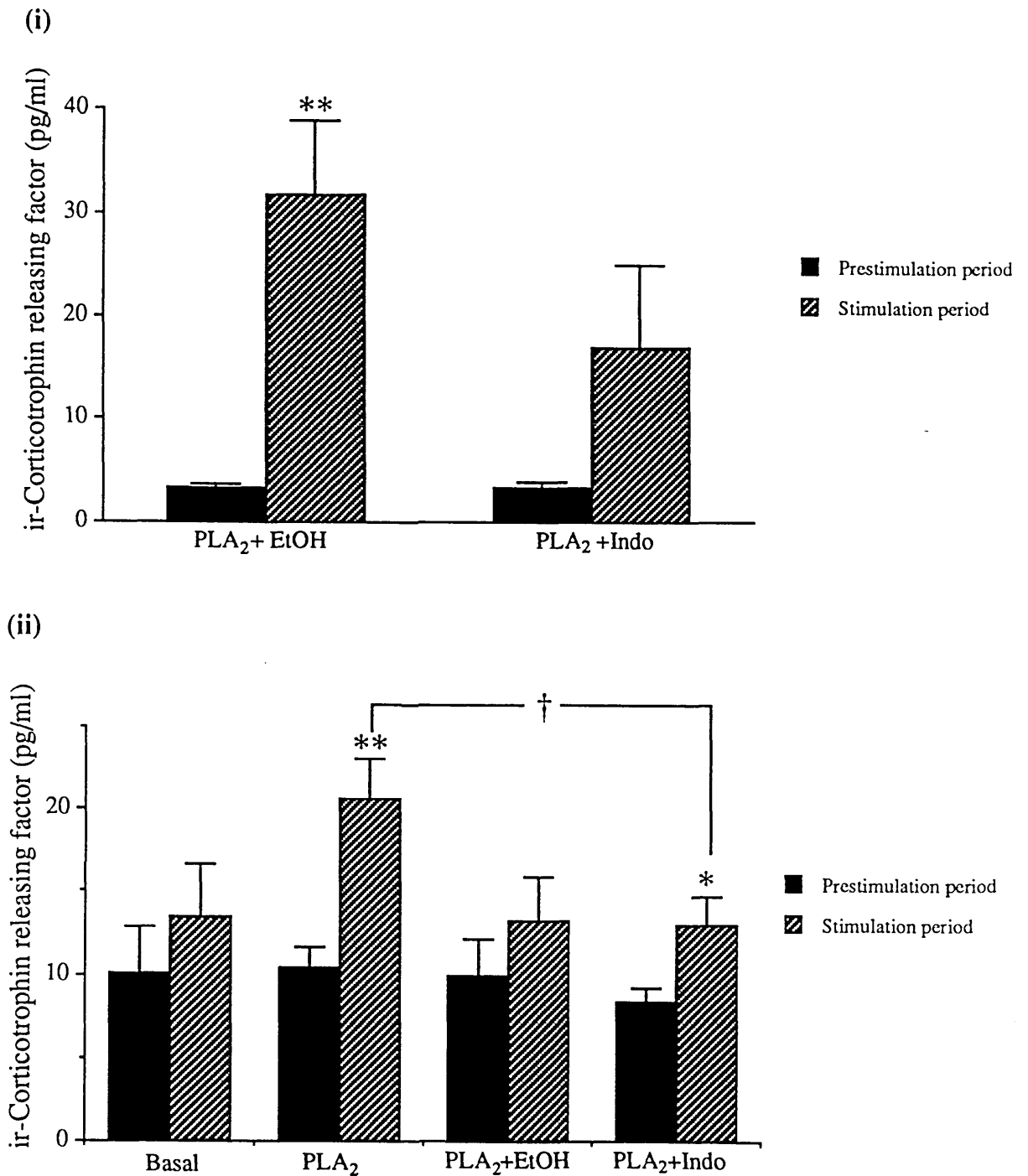
Fig. 19 : The effect of indomethacin (Indo, 10^{-4}M) or the ethanol vehicle (EtOH) on phospholipase A_2 (PLA_2 25U/ml) stimulated ir-corticotrophin releasing factor release from hypothalami from (i) intact (n=4-5) and (ii) adrenalectomized rats (n=4-5). Each column represents the and is shown with its standard error ($*$P<0.05; $**$P<0.01 prestimulation period vs. stimulation period; $†$P<0.05, Mann Whitney U test).
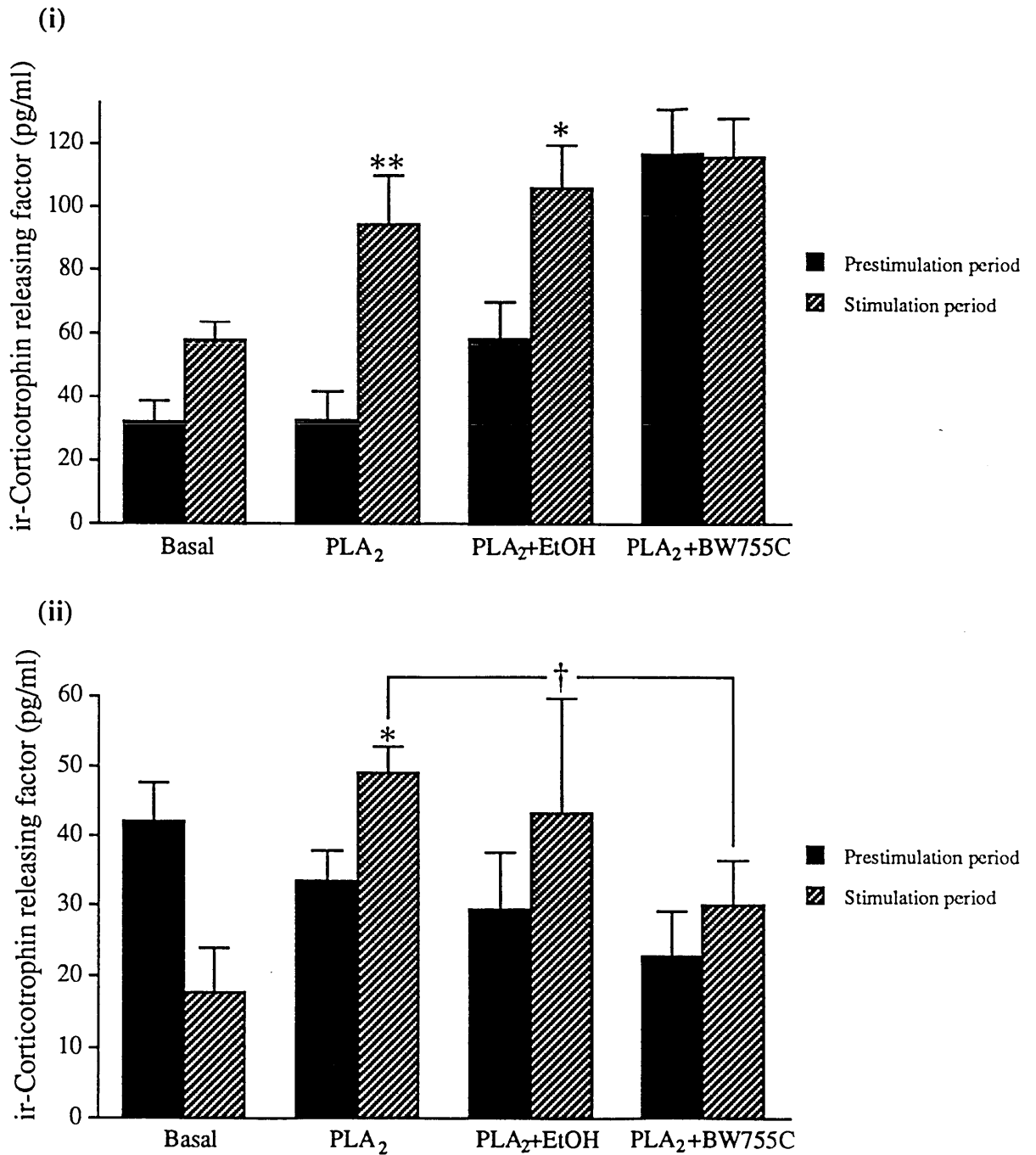
Fig. 20 : The effect of BW755C (10^{-4} M) or the ethanol vehicle (EtOH) on phospholipase A_2 (PLA$_2$ 25U/ml) stimulated corticotrophin releasing factor release from hypothalami from (i) intact (n=5) and (ii) adrenalectomized rats (n=4-5). Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01 prestimulation period vs. stimulation period; †P<0.05, Mann Whitney U test).
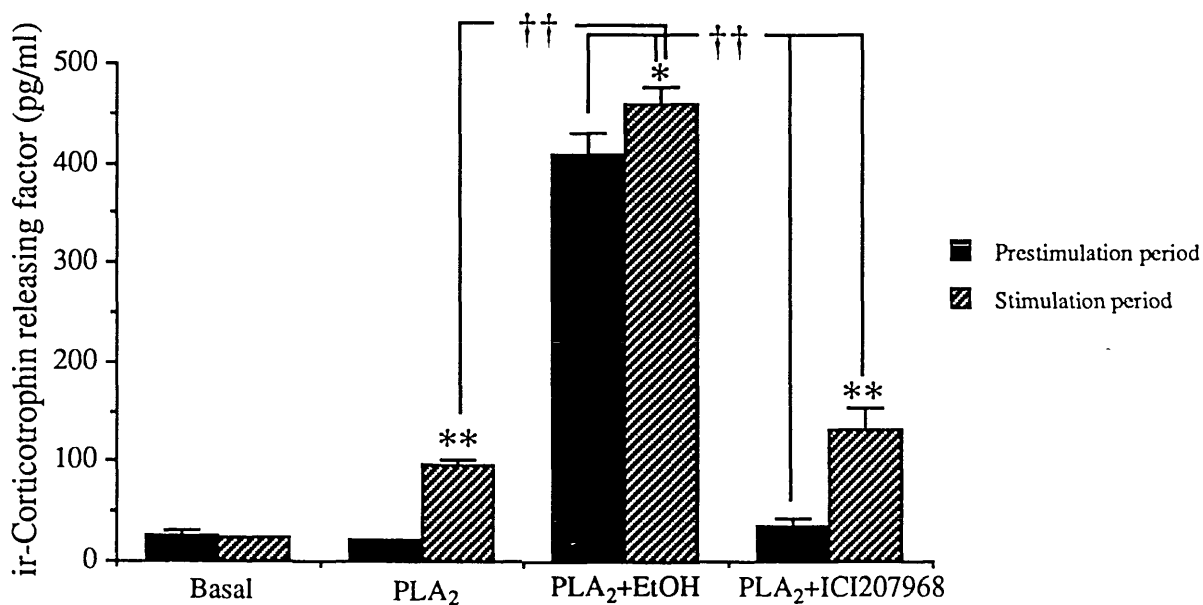
Fig. 21 : The effect of the 5-lipoxygenase inhibitor (ICI 207968, 10^{-4}M) or the ethanol vehicle (EtOH) on phospholipase A_2 (PLA_2 25U/ml) stimulated ir-corticotrophin releasing factor release from hypothalami from adrenalectomized rats (n=5). Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01 prestimulation period vs. stimulation period; ††P<0.01, Mann Whitney U test).

(i)
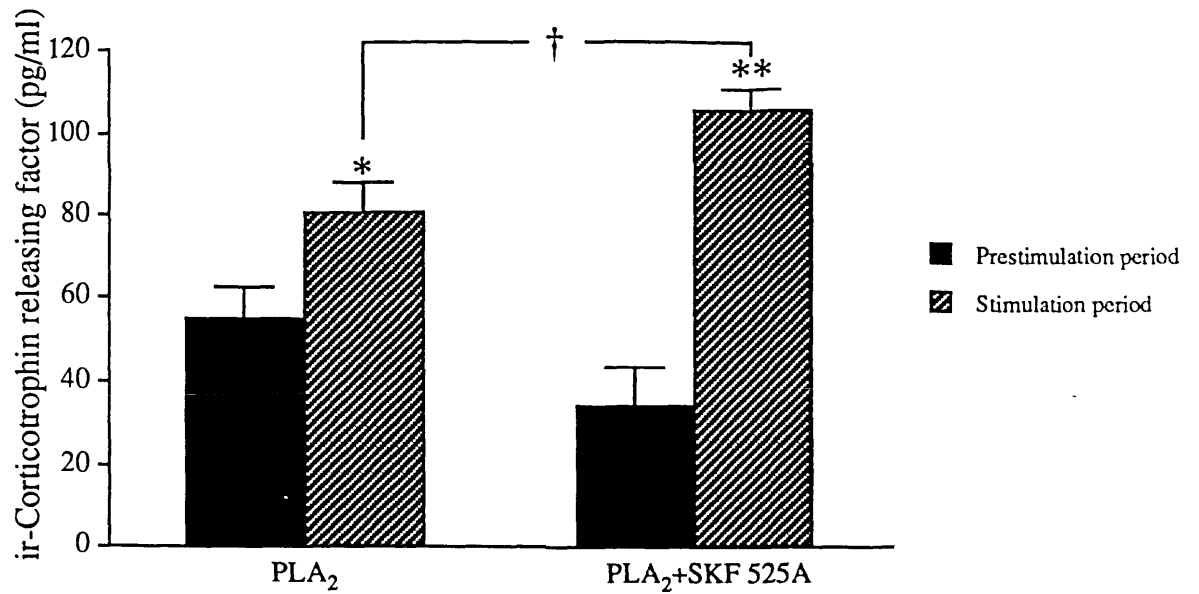
(ii)
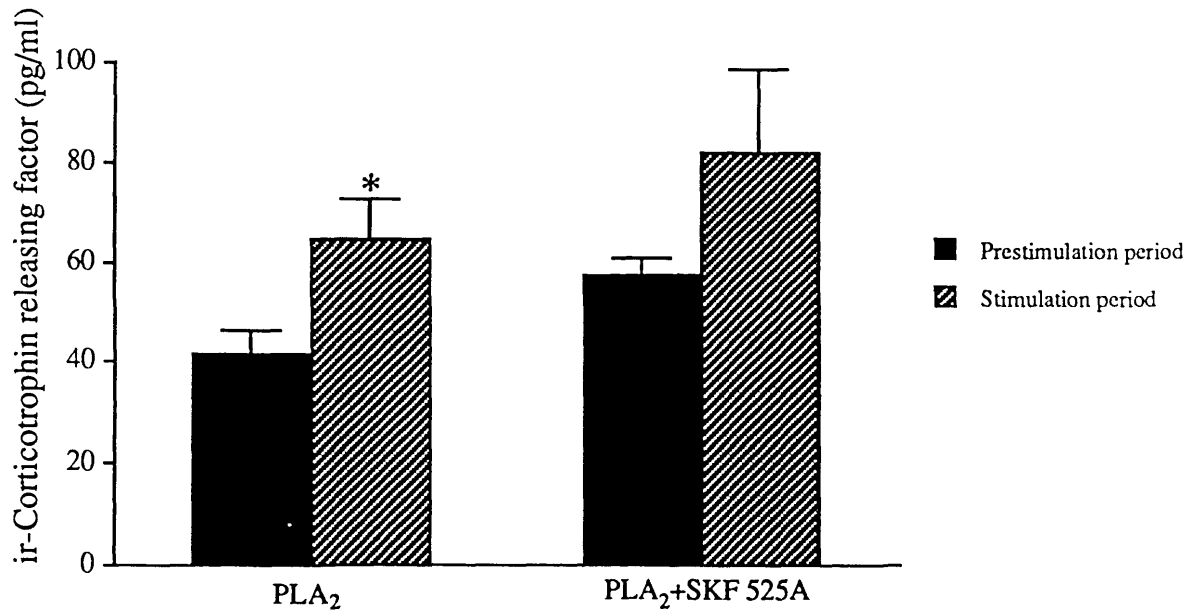
Fig. 22 : The effect of the epoxygenase inhibitor (SKF 525A, 10^{-4} M) on phospholipase A_2 (PLA_2 25U/ml) stimulated ir-corticotrophin releasing factor release from hypothalami from (i) intact (n=3-5) and (ii) adrenalectomized rats (n=3-5). Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01 prestimulation period vs. stimulation period; †P<0.05, Mann Whitney U test).

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Ratio of PLA_2-induced ir-AVP : ir-CRF-41 release

	Intact	Adrenalectomized
	3	40
	12	10
	2	33
	20	72
	7	10
χ	8.8	33
se	3.3	11.5

Table. 2 : The ratio of Phospholipase A_2-induced ir-AVP:ir-CRF-41 release from hypothalamic tissue from intact and adrenalectomized rats. The mean (χ) and standard error (se) of the five ratios are also shown.

iv) The effects of corticosteroids in vivo and in vitro on stimulus induced peptide release from isolated hypothalamic tissue.

(a) The effects of dexamethasone in vitro on PLA$_2$-induced secretion of ir-AVP and ir-CRF-41.

The effects of exposure of the hypothalamic tissue to dexamethasone (10^{-6}M) throughout the pre-incubation period on the subsequent secretory responses to PLA$_2$ and melittin are shown in Figs. 23-27.

Dexamethasone (10^{-6}M) had no significant effect on the release of ir-AVP from tissue from intact (Fig. 23i) or sham adrenalectomized (Fig. 27i) rats following challenge with PLA$_2$ (25U/ml). Thus, there were no significant differences following stimulation between either the absolute values of peptide release or the increments in peptide release in the steroid and non-steroid treated groups. By contrast, dexamethasone readily suppressed the ir-AVP responses to PLA$_2$ in the tissue from adrenalectomized animals (Fig. 23ii), effectively reducing both absolute concentrations of the peptide in the medium ($P<0.05$, Mann Whitney U test) and the increment in peptide release ($P<0.05$, Mann Whitney U test). Dexamethasone (10^{-6}M) also effectively inhibited ($P<0.01$, Mann Whitney U test) the melittin induced ($0.5\mu g$/ml) increment in ir-AVP release from tissue from adrenalectomized rats. However, its effects on the melittin induced release of the neuropeptide from hypothalami from intact rats were less clear cut possibly because the response to melittin in the control group was so variable. Thus, dexamethasone had no significant effect on the absolute levels of ir-AVP following melittin stimulation although it reduced significantly ($P<0.05$, Mann Whitney U test) the increment in peptide release.

With respect to the secretion of ir-CRF-41, dexamethasone (10^{-6}M) had no effect on the PLA$_2$-induced release of the neuropeptide from hypothalami from adrenalectomized (Fig. 25ii) or sham adrenalectomized (Fig. 28ii) rats but reduced significantly ($P<0.01$, Mann Whitney U test) the secretory responses of the tissue from intact animals. By contrast, the steroid had no effect on the ir-CRF-41 reponses of the tissues from intact rats to melittin (although the increment in peptide release appeared reduced this was not significant $P=0.056$, Mann Whitney U test) but reduced significantly ($P<0.05$, Mann Whitney U test) the responses of the tissues from adrenalectomized rats.
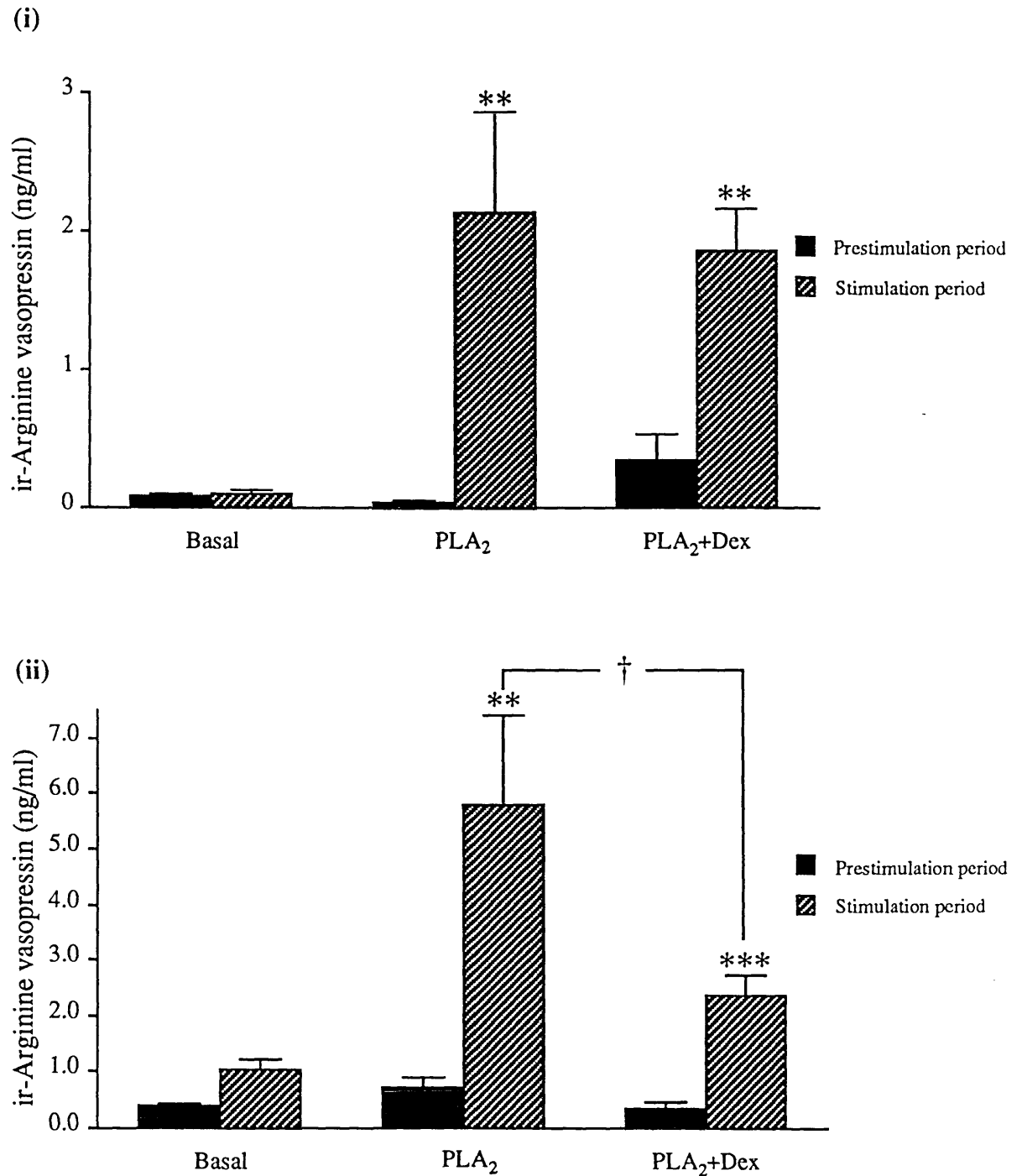
Fig. 23 : The effect of dexamethasone (Dex, $10^{-6}M$) on phospholipase A_2 (PLA$_2$ 25U/ml) induced ir-Arginine vasopressin release from hypothalami from (i) intact (n=5) and (ii) adrenalectomized rats (n=4-6). Each column represents the mean and is shown with its standard error (**P<0.01; ***P<0.001 prestimulation period vs. stimulation period; †P<0.05, Mann Whitney U test).
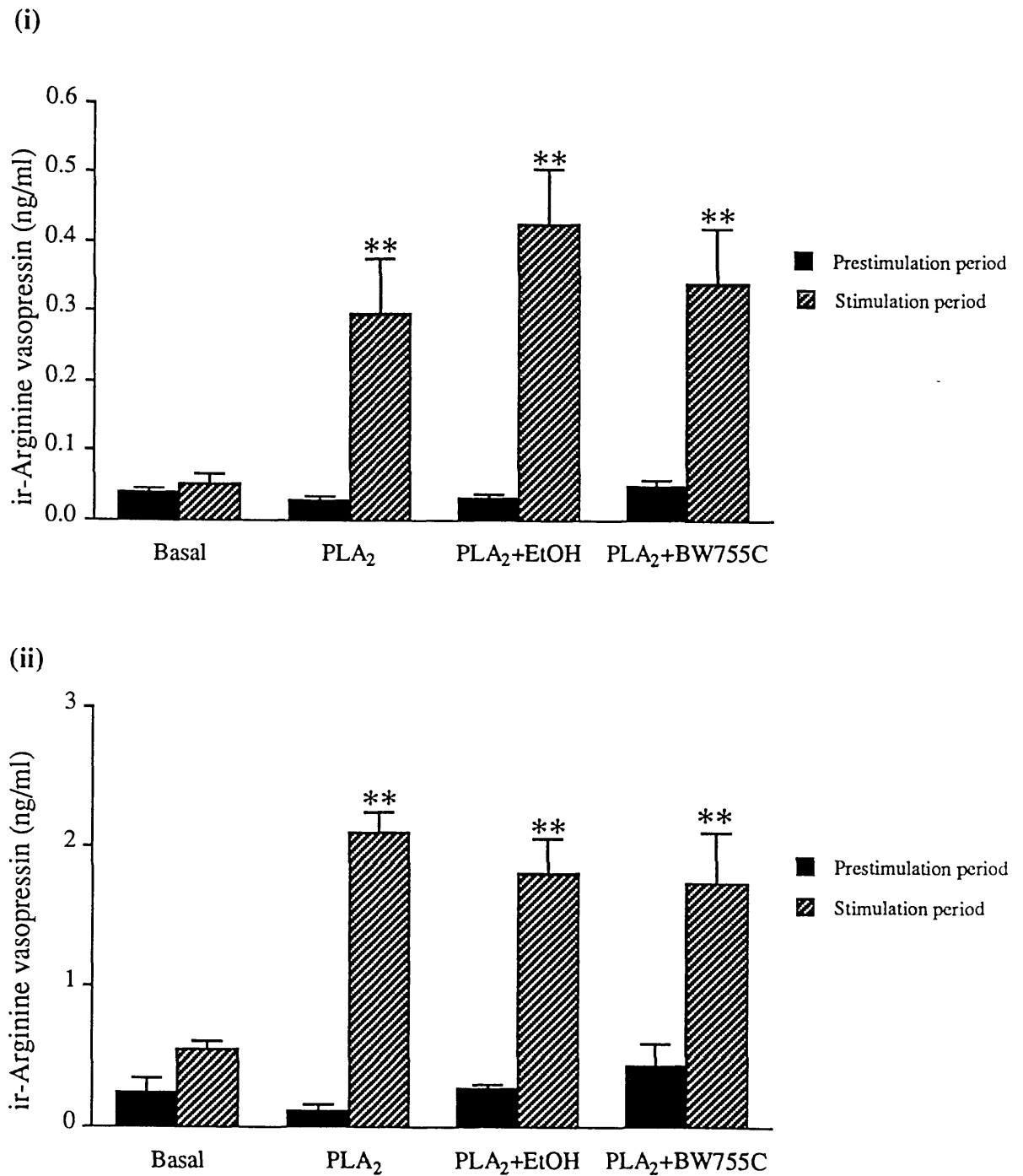
(i)

(ii)
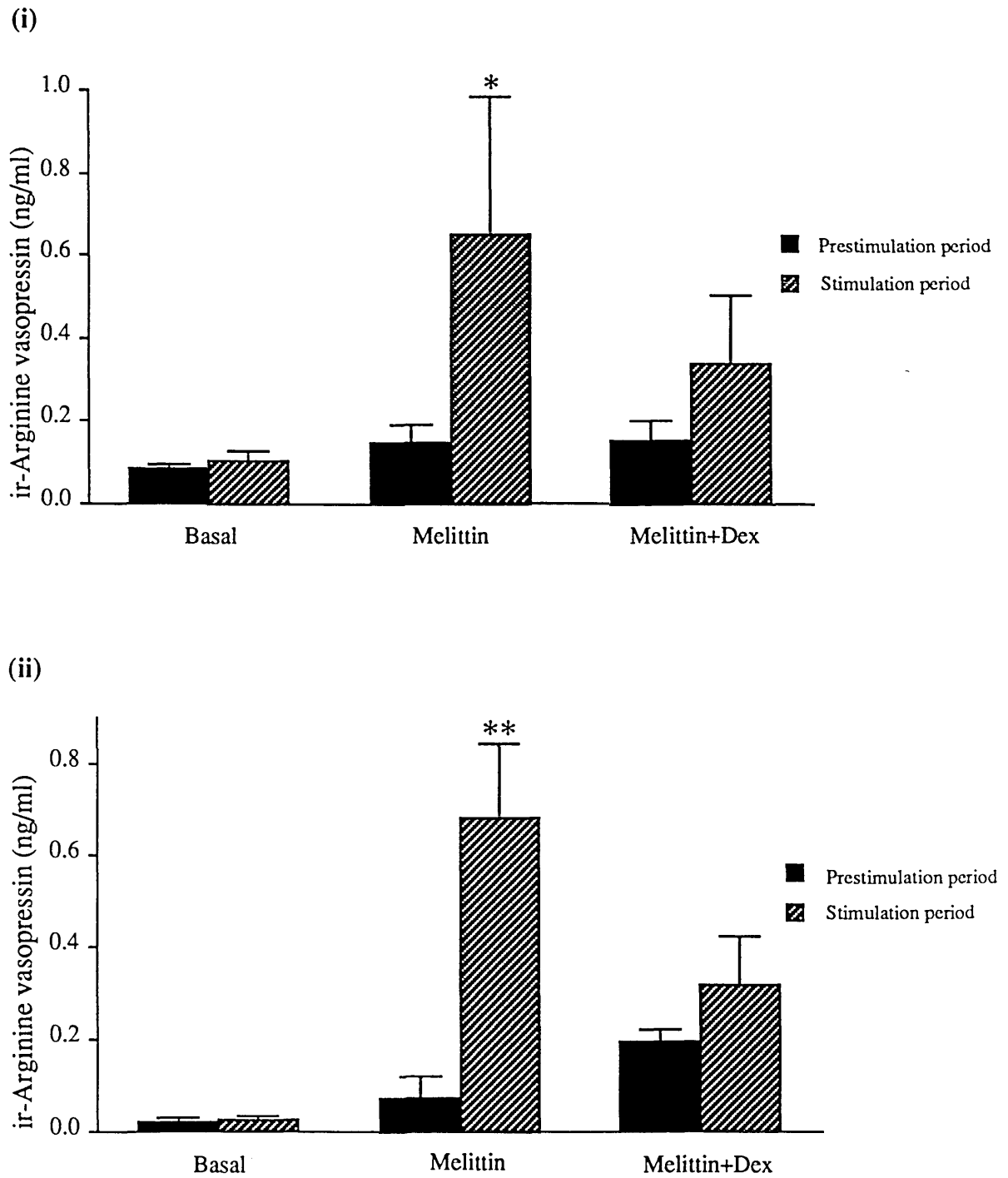
Fig. 24 : The effect of dexamethasone (Dex, 10^{-6}M) on melittin ($0.5\mu g/ml$) induced ir-arginine vasopressin release from hypothalami from **(i)** intact (n=4-5) and **(ii)** adrenalectomized rats (n=4-5). Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01 prestimulation period vs. stimulation period, Mann Whitney U test).

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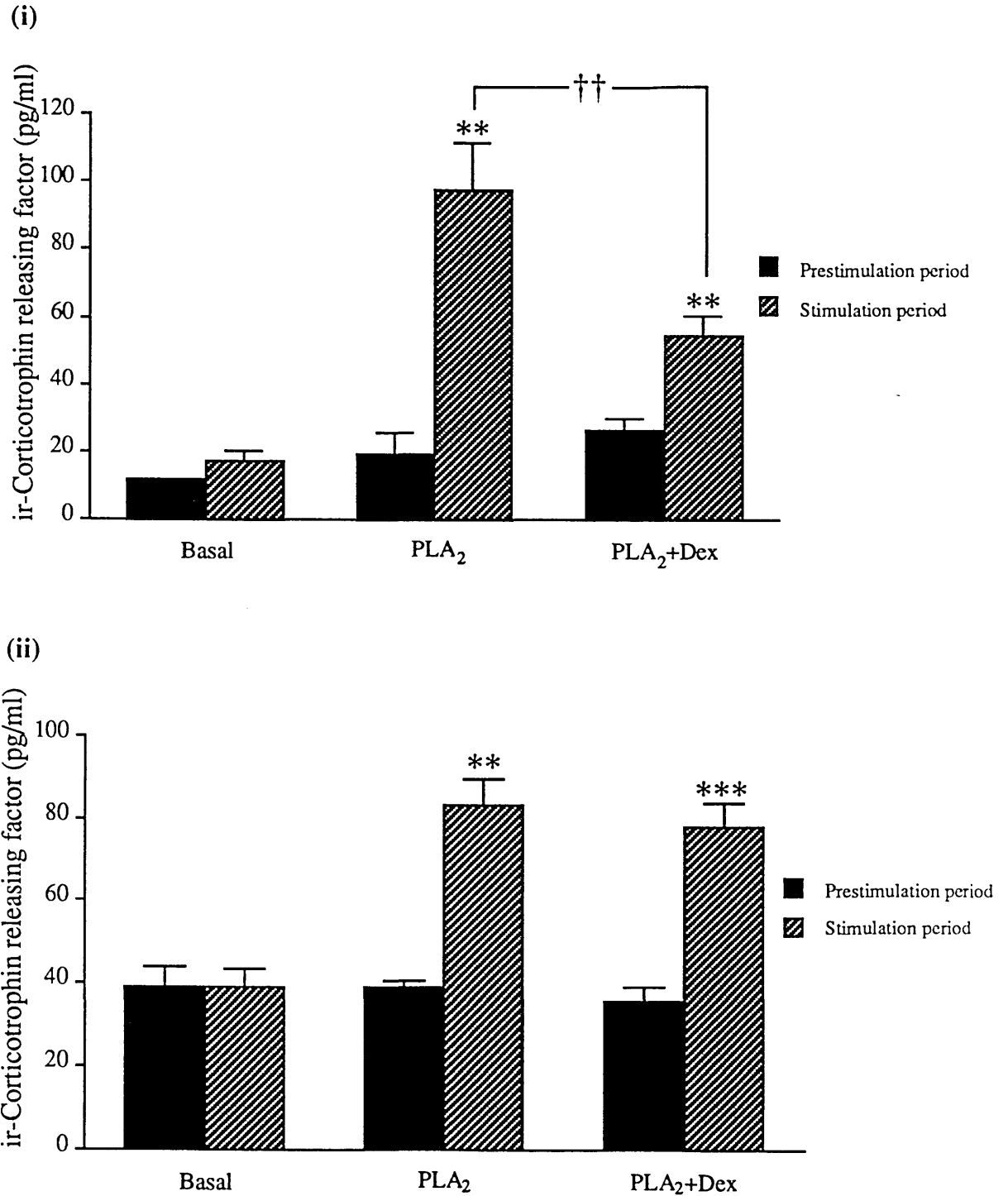
Fig. 25 : The effect of dexamethasone (Dex, 10^{-6} M) on phospholipase A_2 (PLA$_2$ 25U/ml) induced ir-corticotrophin releasing factor release from hypothalami from (i) intact (n=4-5) and (ii) adrenalectomized rats (n=4-5). Each column represents the mean and is shown with its standard error (**P<0.01 ***P<0.001 prestimulation period vs. stimulation period; ††P<0.01, Mann Whitney U test).

Fig. 26 : The effect of dexamethasone (Dex, 10^{-6} M) on melittin (0.5μg/ml) induced ir-corticotrophin releasing factor release from hypothalami from (i) intact (n=4-5) and (ii) adrenalectomized rats (n=4-5). Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01 prestimulation period vs. stimulation period; †P<0.05, Mann Whitney U test).

(i)

(ii)
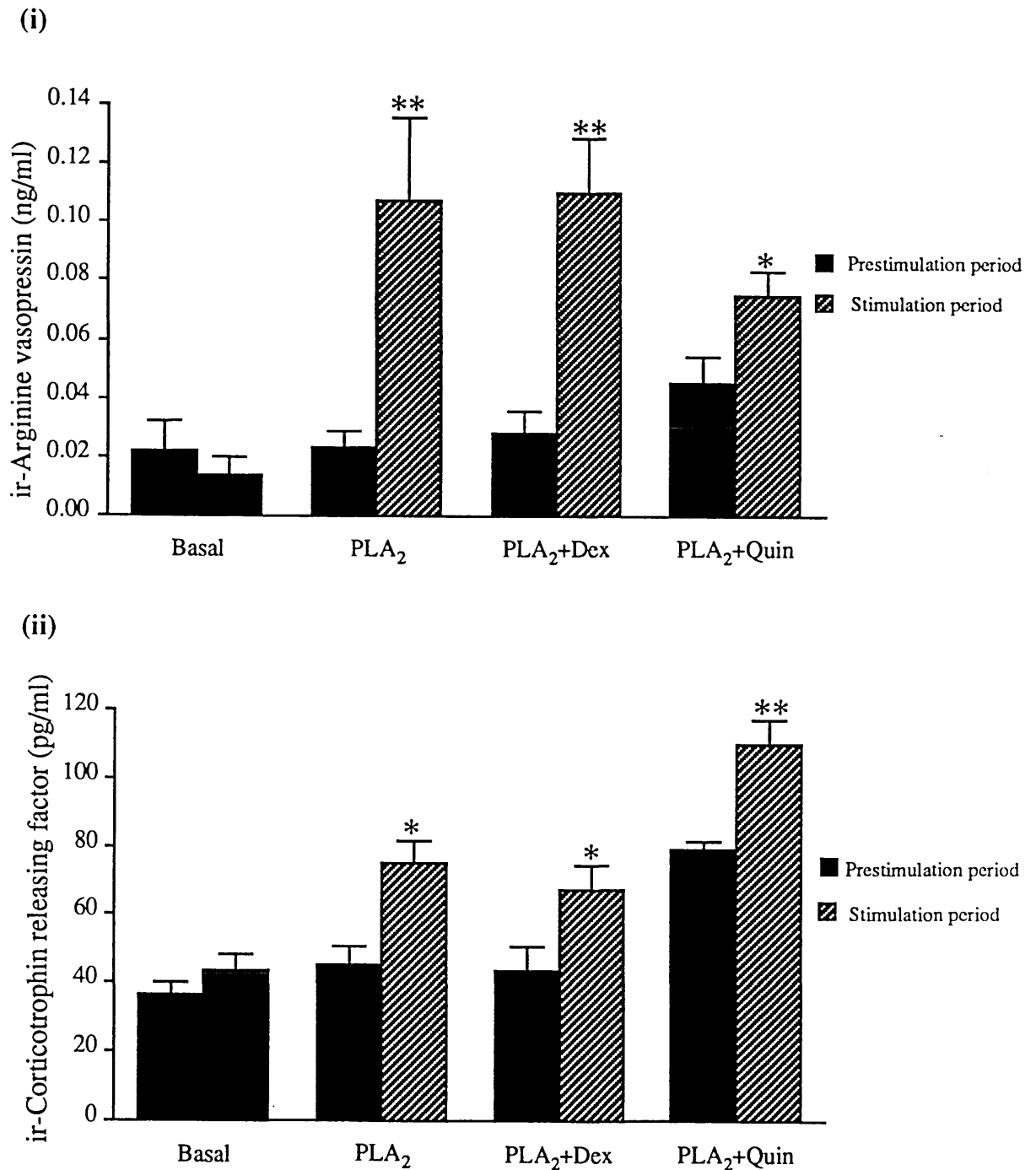
Fig. 27 : The effect of dexamethasone (Dex, 10^{-6}M) or quinacrine (Quin, 10^{-4}M) on phospholipase A_2 (PLA_2 25U/ml) induced (**i**) ir-arginine vasopressin (n=4-5) and (**ii**) ir-corticotrophin releasing factor (n=4-5) release from hypothalami from sham operated animals. Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01 prestimulation period vs. stimulation period, Mann Whitney U test).

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(b) The effects of pre-treatment with dexamethasone in the drinking water on PLA$_2$-induced secretion of ir-AVP and ir-CRF-41 in vitro.

Figs. 28-32 illustrate the effects of PLA$_2$ on the secretion of ir-AVP and ir-CRF-41 from hypothalami from intact and adrenalectomized rats treated, immediately prior to autopsy, with dexamethasone (included in the drinking water/saline at concentrations of 20µg/ml for 24h or 2µg/ml for 6 or 14 days).

PLA$_2$ (25U/ml) produced the expected significant (P<0.01, Mann Whitney U test) increases in ir-AVP release from hypothalamic tissue from control animals (intact and adrenalectomized) (Figs. 28-30). Overnight treatment with the steroid had no effect on the ir-AVP response of hypothalami from intact rats to PLA$_2$ (Fig. 28i) but reduced significantly (P<0.01, Mann Whitney U test) the effects of the enzyme on hypothalami from adrenalectomized rats (Fig. 28ii). By contrast, the PLA$_2$-induced secretion of ir-AVP by hypothalami from intact rats was reduced (P<0.05, Mann Whitney U test) by the 6-day steroid treatment (Fig 29i) but that from tissue from adrenalectomized animals was not (Fig. 29ii). When given for 14 days prior to autopsy (2µg/ml in the drinking water/saline), dexamethasone appeared to elevate the spontaneous release of ir-AVP from hypothalami from intact and adrenalectomized animals. No further increase in peptide release was apparent following stimulation with PLA$_2$ (Fig. 30).

Phospholipase A$_2$ (25U/ml) also produced the anticipated significant (P<0.05, Mann Whitney U test) increase in ir-CRF-41 release in the tissue from intact and adrenalectomized rats (Figs. 31 & 32). Overnight treatment with the steroid failed to affect PLA$_2$-stimulated ir-CRF-41 release from either tissue (Fig. 31). Phospholipase A$_2$ induced ir-CRF-41 release from tissue from adrenalectomized rats was unaffected by prolonged steroid treatment (2µg/ml in the drinking water/saline for 6 days) but that from tissue from intact rats was reduced significantly (P<0.01, Mann Whitney U test) (Fig. 32i).
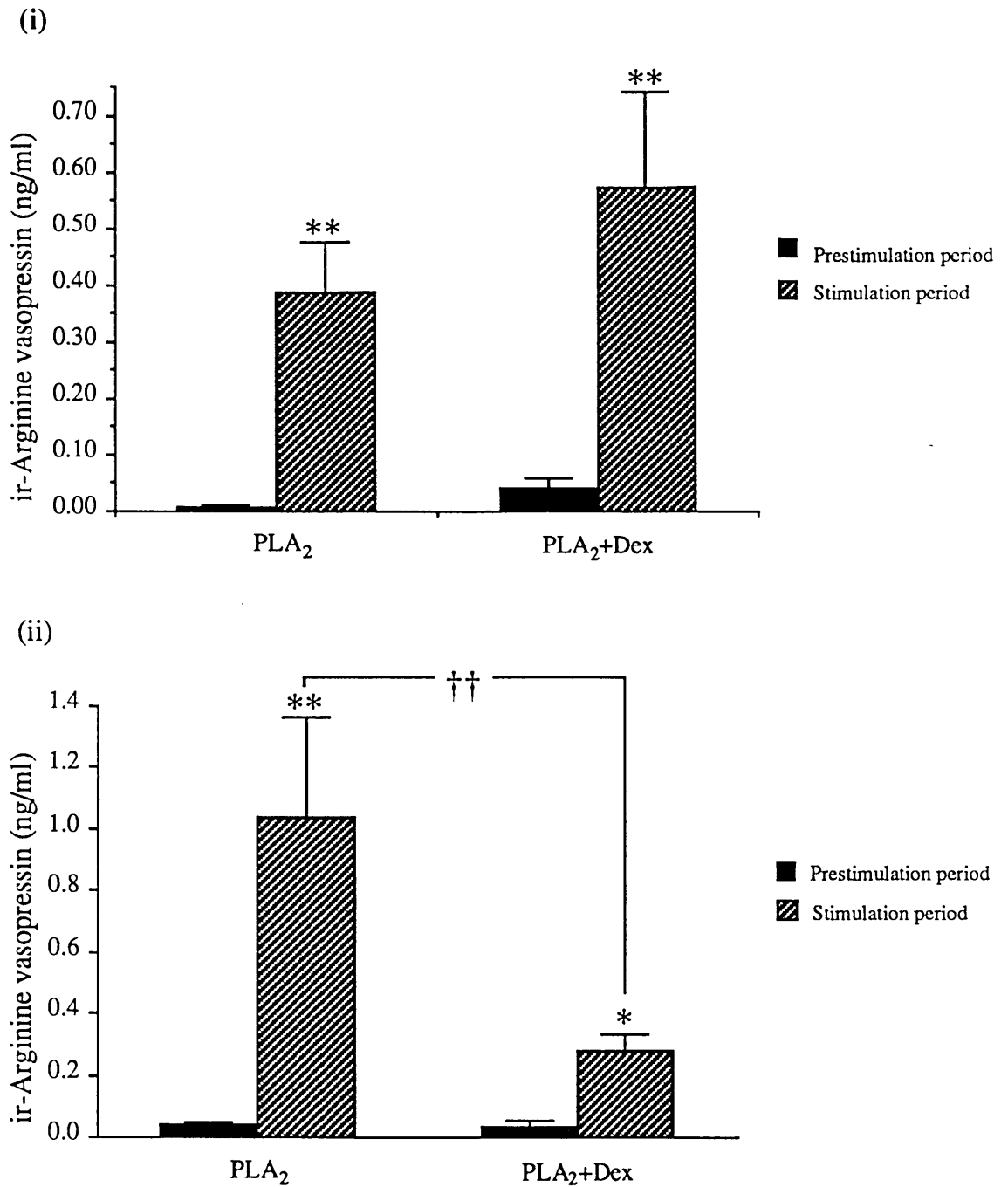
Fig. 28 : The effect of administration of dexamethasone (Dex, 20μg/ml in drinking water/saline for 24h prior to autopsy) on phospholipase A_2 (PLA$_2$ 25U/ml) induced ir-arginine vasopressin release from hypothalami from (i) intact (n=5) and (ii) adrenalectomized rats (n=3-4). Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01 prestimulation period vs. stimulation period; †† p<0.01, Mann Whitney U test).

(total dose of steroid ingested (i) intacts ≈ 704μg/rats, (ii) adrenalectomized ≈1000μg/rat)
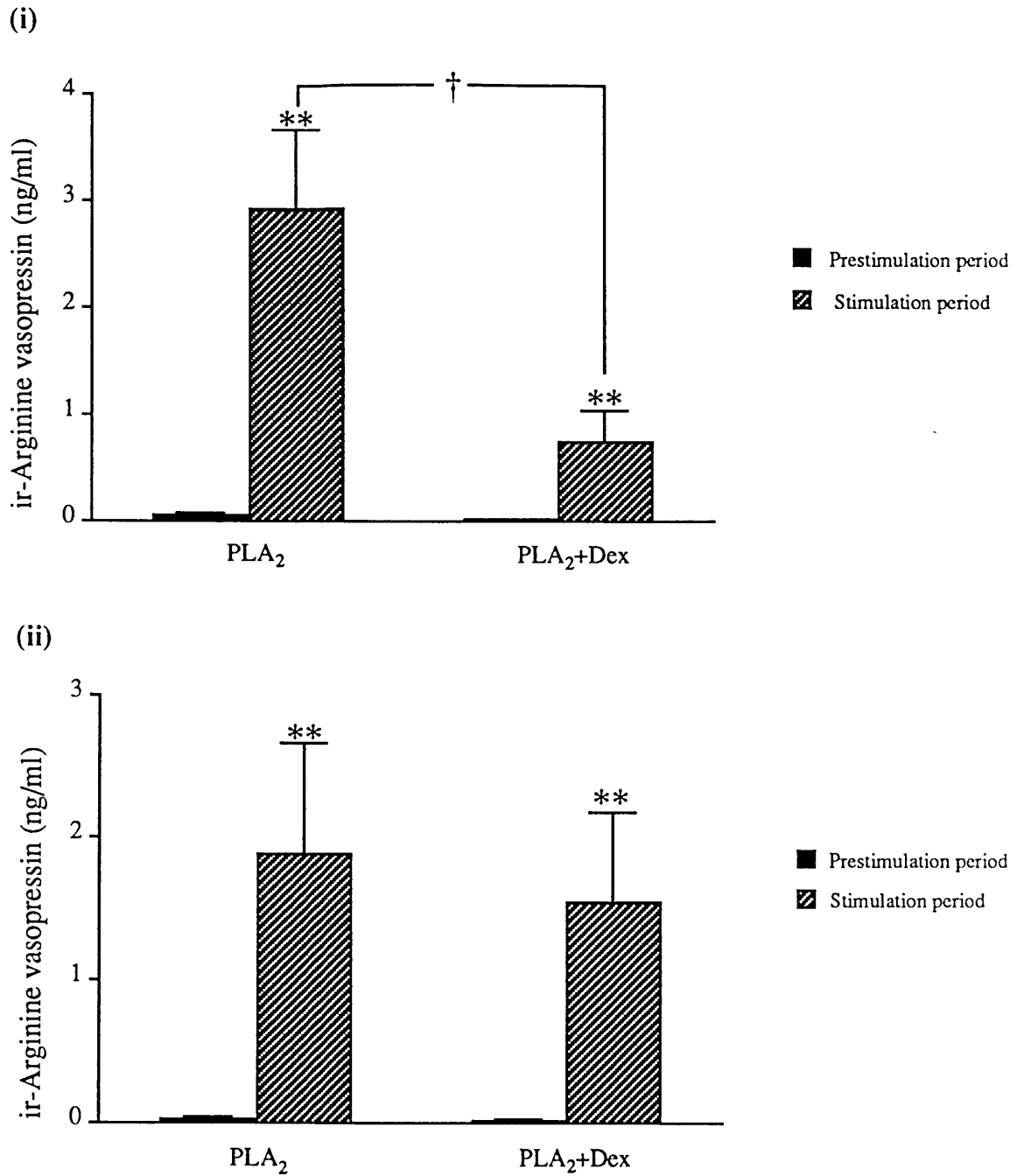
Fig. 29 : The effect of administration of dexamethasone (Dex, $2\mu g/ml$ in the drinking water/saline for 6 days prior to autopsy) on phospholipase A_2 (PLA_2 25U/ml) induced ir-arginine vasopressin release from hypothalami from (i) intact (4-5) and (ii) adrenalectomized rats (n=4-5). Each column represents the mean and is shown with its standard error (**$P<0.01$ prestimulation period vs. stimulation period; †$P<0.05$, Mann Whitney U test).

(total dose of steroid ingested (i) intacts $\approx 462\mu g$/rats, (ii) adrenalectomized $\approx 602\mu g$/rat)

(i)

(ii)
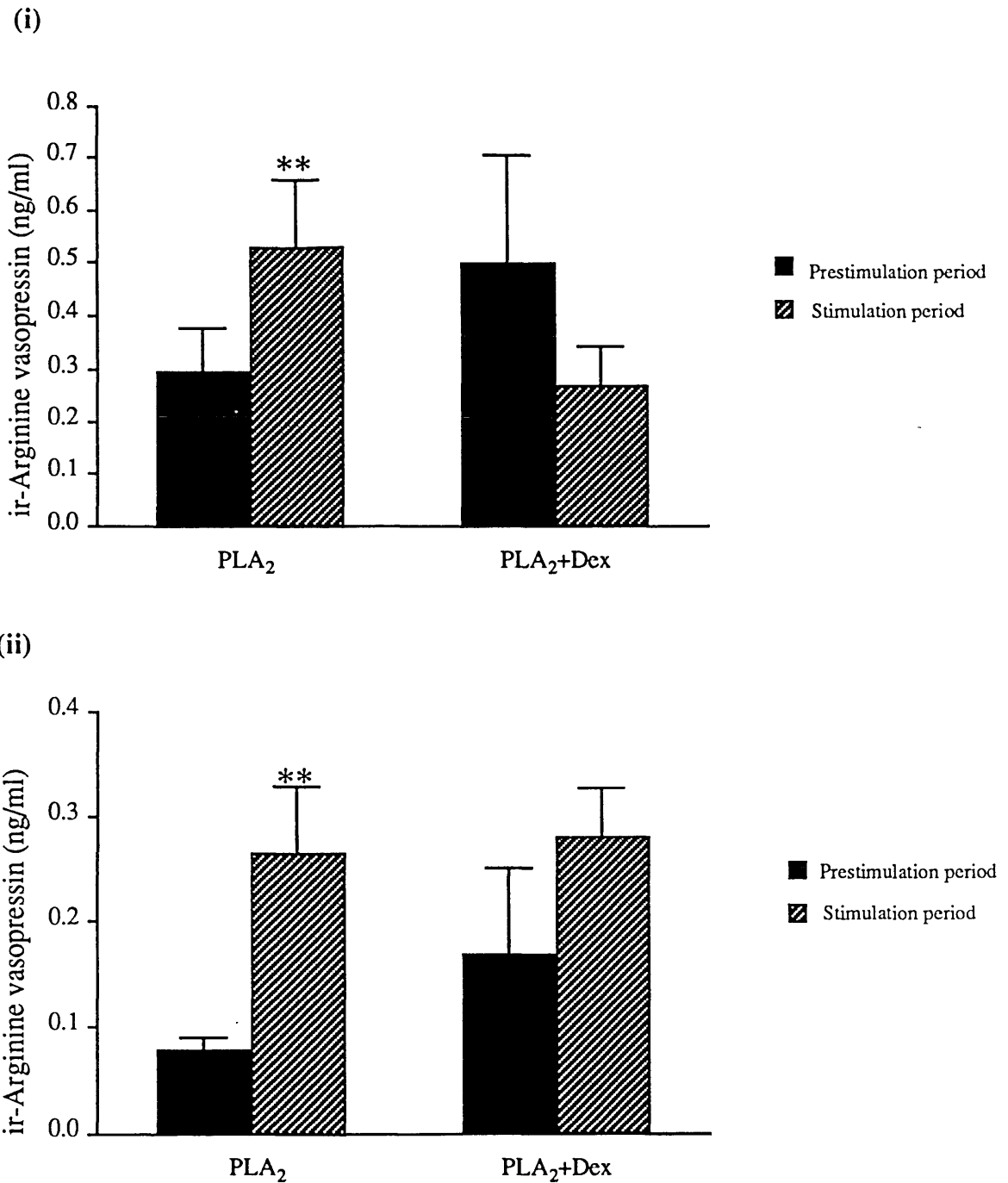
Fig. 30 : The effect of administration of dexamethasone (Dex, $2\mu g/ml$ in the drinking water/saline for 14 days prior to autopsy) on phospholipase A_2 (PLA_2 25U/ml) induced ir-arginine vasopressin release from hypothalami from (i) intact (n=4-5) and (ii) adrenalectomized rats (n=4-5). Each column represents the mean and is shown with its standard error (**P<0.01 prestimulation period vs. stimulation period, Mann Whitney U test).

(total dose of steroid ingested (i) intacts $\approx$ $1052\mu g/rats$, (ii) adrenalectomized $\approx 2512\mu g/rat$)
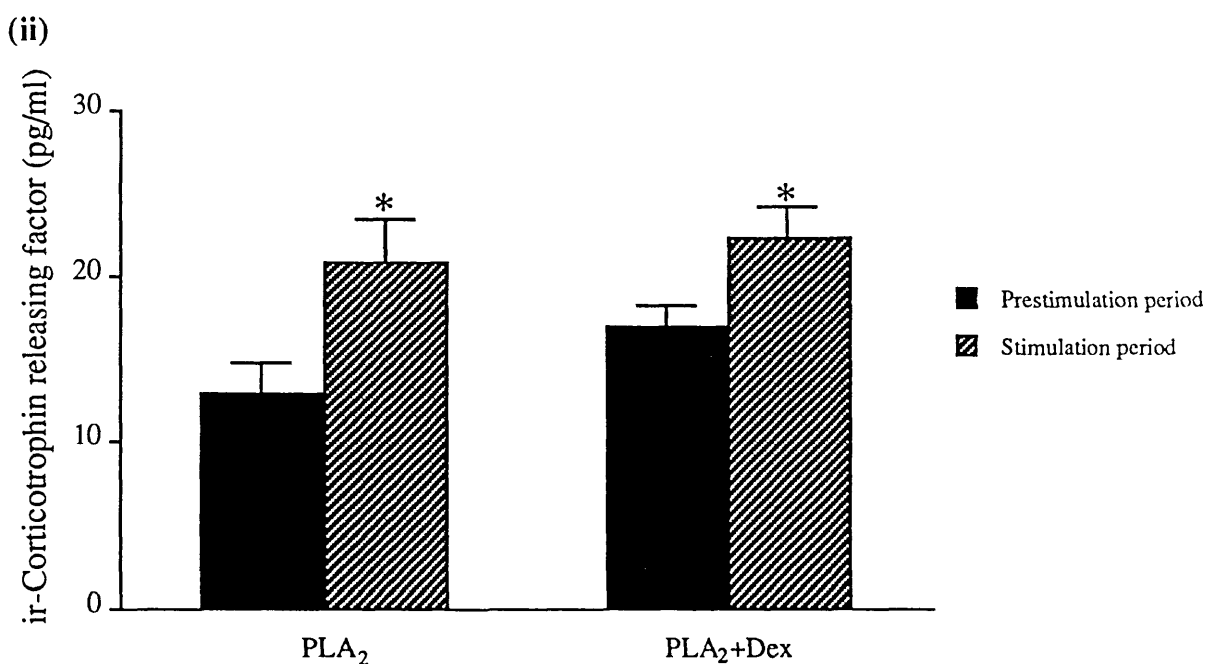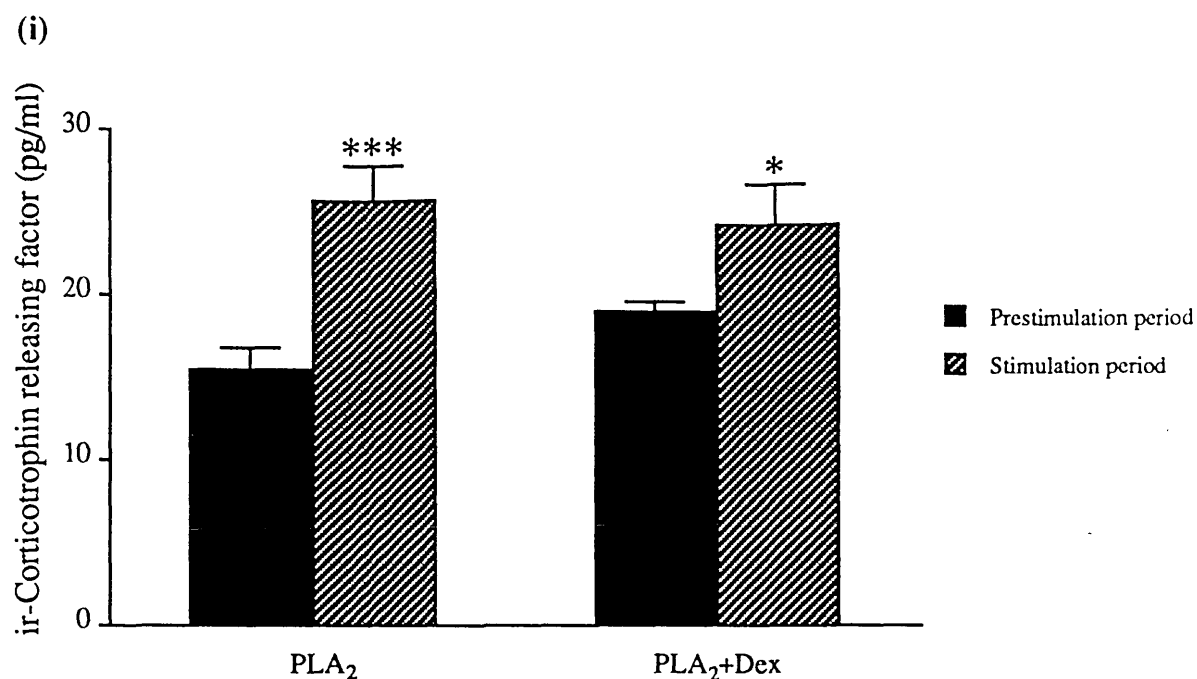
Fig. 31 : The effect of administration of dexamethasone (Dex, 20μg/ml in drinking water/saline for 24h prior to autopsy) on phospholipase A_2 (PLA₂ 25U/ml) induced ir-corticotrophin releasing factor release from hypothalami from (i) intact (n=5) and (ii) adrenalectomized rats (n=5). Each column represents the mean and is shown with its standard error (*P<0.05; ***P<0.001 prestimulation period vs. stimulation period, Mann Whitney U test).

(total dose of steroid ingested (i) intacts ≈ 704μg/rats, (ii) adrenalectomized ≈1000μg/rat)

Fig. 32 : The effect of administration of dexamethasone (Dex, 2μg/ml in the drinking water/saline for 6 days prior to autopsy) on phospholipase A_2 (PLA_2 25U/ml) induced ir-corticotrophin releasing factor release from hypothalami from **(i)** intact (n=4-5) and **(ii)** adrenalectomized rats (n=5). Each column represents the mean and is shown with its standard error (**P<0.01 prestimulation period vs. stimulation period; ††P<0.01, Mann Whitney U test).

(total dose of steroid ingested (i) intacts ≈ 462μg/rats, (ii) adrenalectomized ≈602μg/rat)

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(v) The effects of lipocortin 1 on the functional activity of the hypothalamo-pituitary-adrenal axis.

(a) The effects of lipocortin 1 on the secretion of ir-AVP, ir-CRF-41 and ir-PGE$_2$ by isolated hypothalamic tissue in vitro.

Fig. 33 demonstrates the effects of graded concentrations (1-100ng/ml) of human recombinant lipocortin 1 on ir-AVP and ir-CRF-41 secretion in vitro by isolated hypothalamic tissue from adrenalectomized rats. At a concentration of 10ng/ml, lipocortin 1 significantly ($P<0.05$, Mann Whitney U test.) stimulated ir-AVP release. A higher concentration (100ng/ml) appeared to be without effect, possibly because the basal release was elevated markedly. Lipocortin 1 failed to stimulate ir-CRF-41 secretion at all the concentrations tested (Fig 33ii).

Figs 34-36 illustrate the effects of lipocortin 1 on PLA$_2$ induced ir-AVP; ir-CRF and ir-PGE$_2$ release from hypothalami from adrenalectomized rats. PLA$_2$ produced the anticipated significant ($P<0.01$, Mann Whitney U test) increases in ir-AVP (Figs 34i & 35i) and ir-CRF-41 (Figs 34ii & 36i) secretion. It also caused significant ($P<0.05$, Mann Whitney U test) increases in ir-PGE$_2$ release (Figs 35ii & 36ii).

Lipocortin 1 (10ng/ml) significantly ($P<0.05$, Mann Whitney U test) elevated basal ir-AVP release although the lower concentration (5ng/ml) was without effect in this respect. The lower concentration (5ng/ml) also failed to affect the response to PLA$_2$ but the higher concentration (10ng/ml) potentiated markedly the secretory response to this secretagogue ($P<0.01$, Mann Whitney U test) (Fig 34i). In a second experiment, 10ng/ml lipocortin was without effect but a higher concentration (1 µg/ml) produced a marked potentiation of PLA$_2$-induced ir-AVP release ($P<0.01$, Mann Whitney U test, Fig 35i).

In contrast, lipocortin 1 failed to affect basal or PLA$_2$-induced ir-CRF-41 release (Figs 34ii & 36i). Low concentrations of the protein (5-20ng/ml) caused significant ($P<0.05$, Mann Whitney U test) elevations in basal PGE$_2$ levels and potentiated the PLA$_2$-induced increments in PGE$_2$ output ($P<0.01$ Mann Whitney U test, Figs 35ii & 36ii). A high concentration (1µg/ml) was without effect.

The effects of lipocortin 1 on ir-AVP and ir-CRF-41 release in response to 56mM K$^+$ stimulation were also investigated (Fig 37i & ii). As expected K$^+$ stimulation (56mM) caused significant ($P<0.001$, Mann Whitney U test) increases in the release of both ir-AVP and ir-CRF-41. The presence of lipocortin 1 (10ng/ml) however failed to affect basal or K$^+$-induced secretion of either peptide.

(i)

(ii)
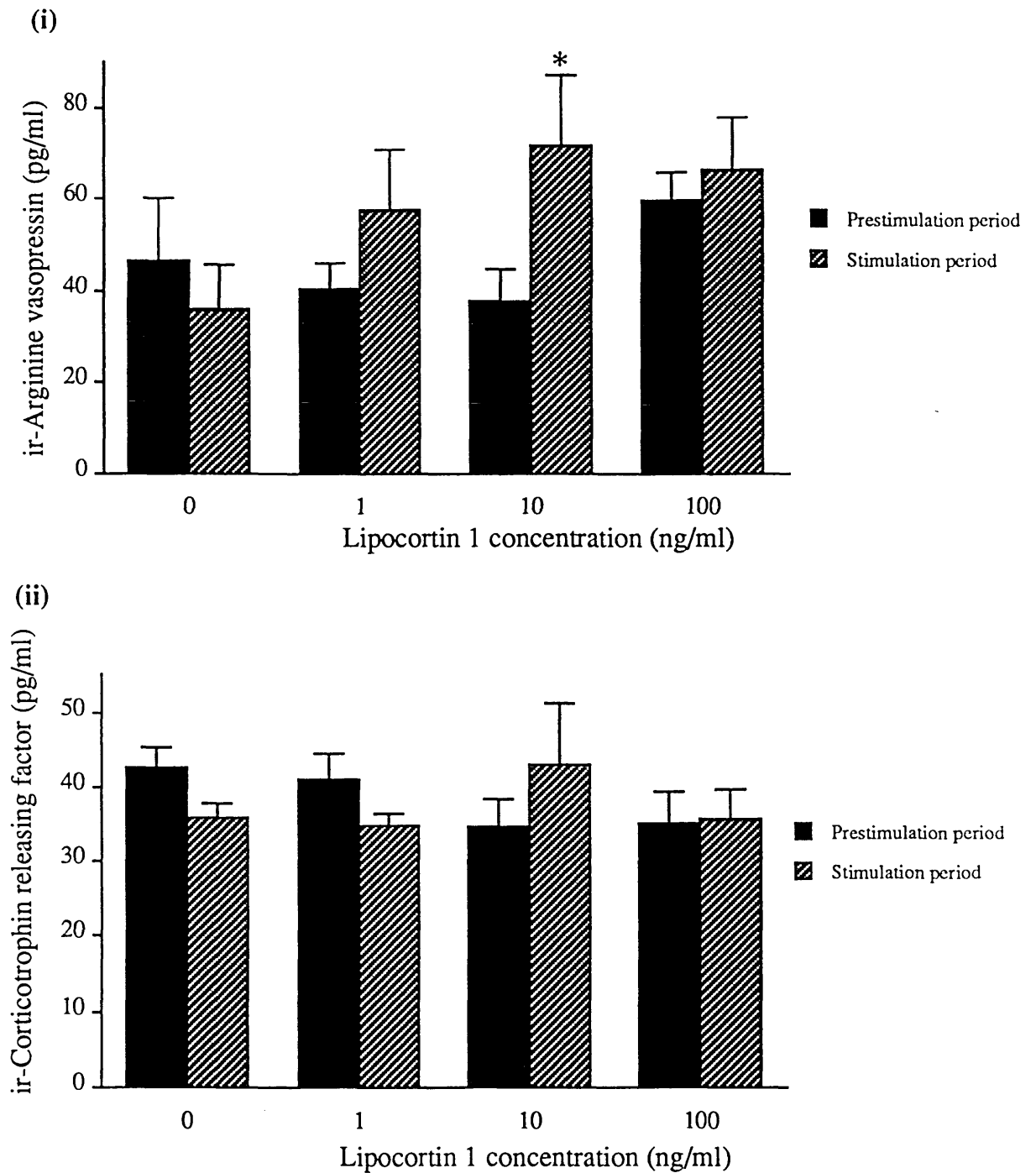
Fig. 33 : The effect of graded concentrations of human recombinant lipocortin 1 on **(i)** ir-arginine vasopressin (n=5-6) and **(ii)** ir-corticotrophin releasing factor (n=5-6) release from hypothalami from adrenalectomized rats . Each column represents the mean and is shown with its standard error (*P<0.05 prestimulation period vs. stimulation period, Mann Whitney U test).

(i)

(ii)

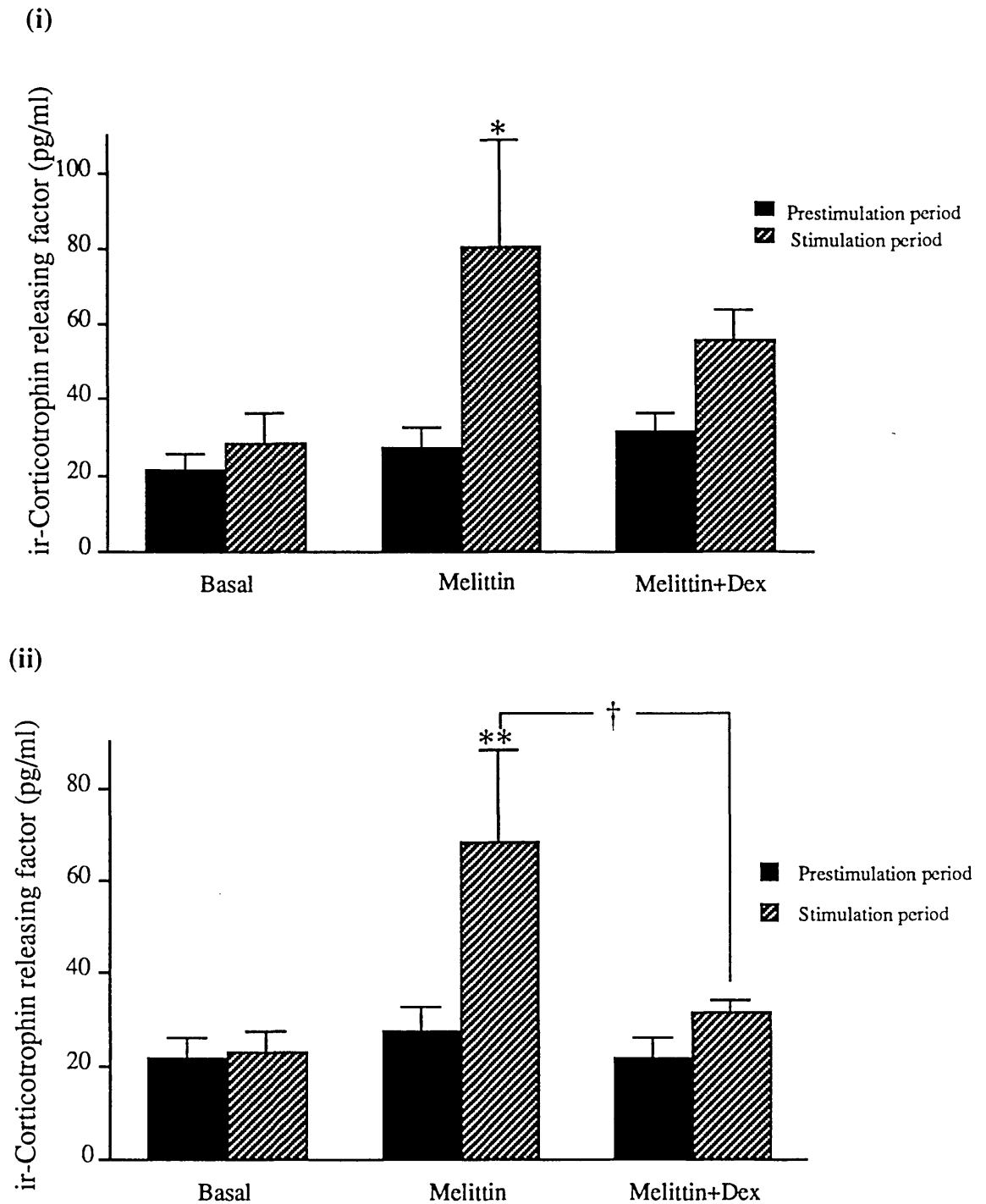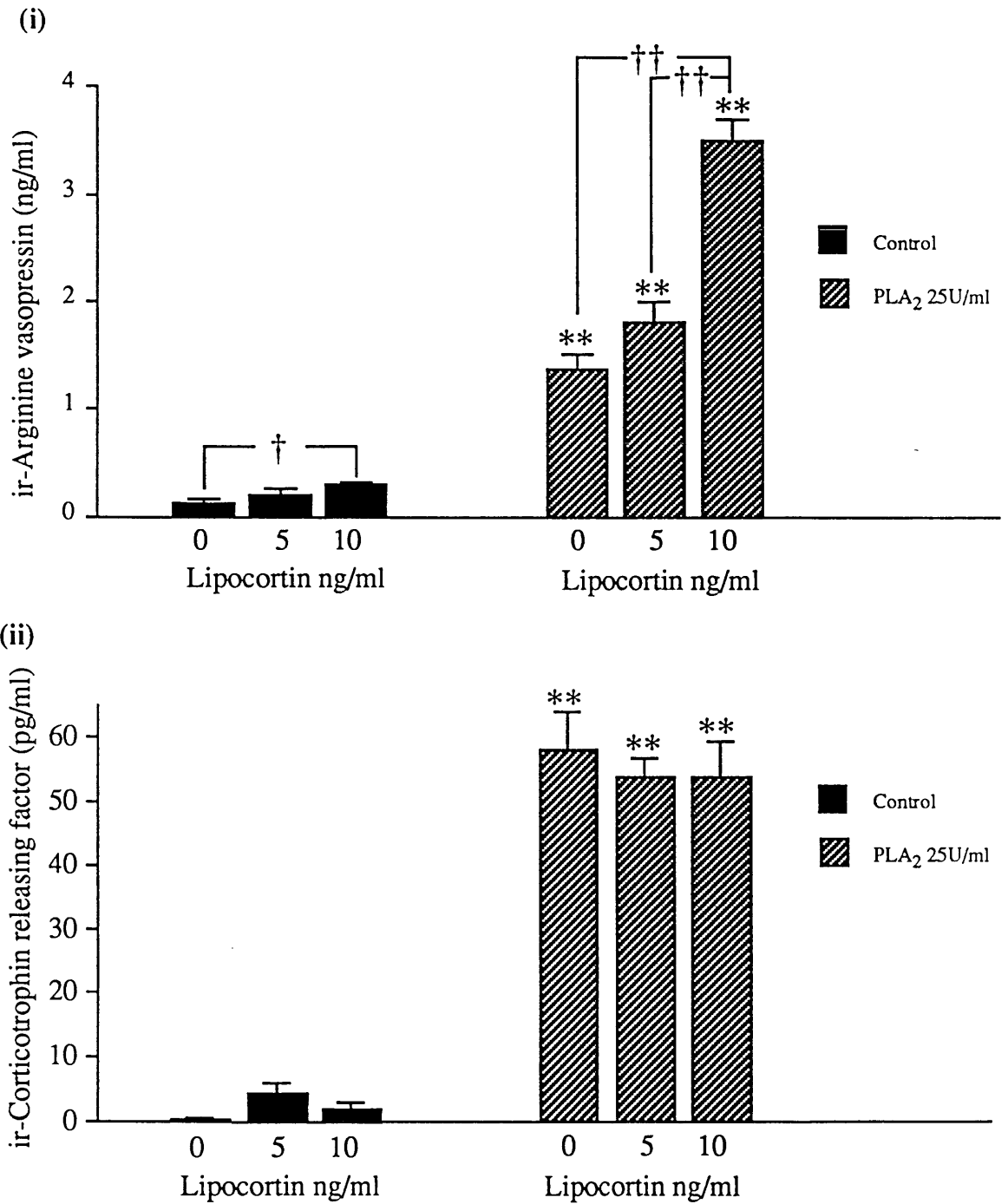Fig. 34 : The effect of graded concentrations of human recombinant lipocortin on phospholipase A_2 (PLA_2 25U/ml) induced (i) ir-arginine vasopressin and (ii) ir-corticotrophin releasing factor release from hypothalami from adrenalectomized rats (n=5). Each column represents the mean and is shown with its standard error (**P<0.01 control vs. PLA_2 stimulated; †P<0.05; ††P<0.01, Mann Whitney U test).

(i)

(ii)
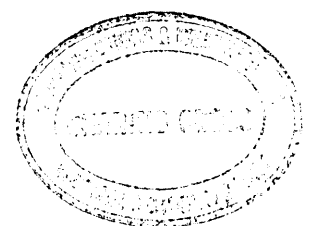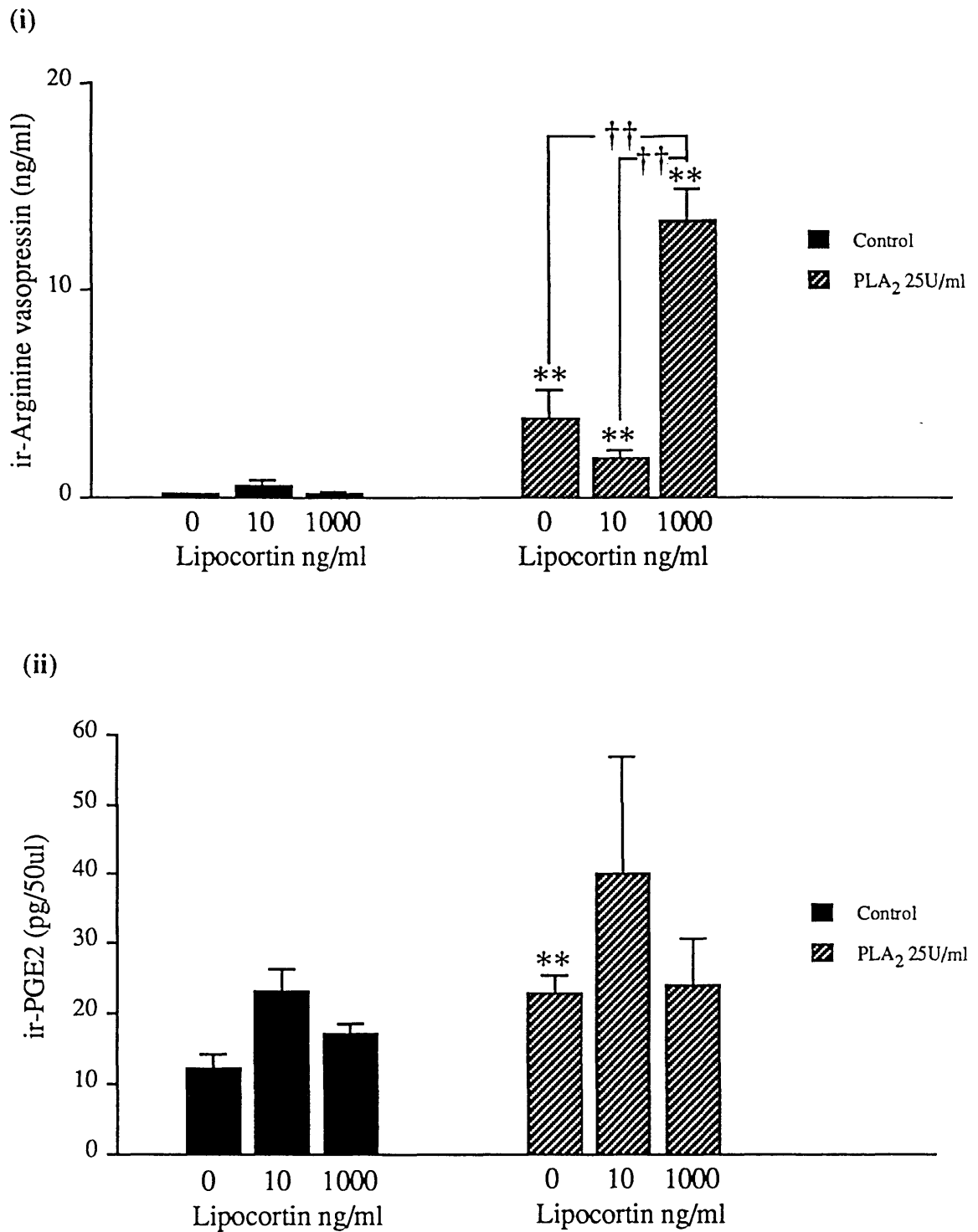
Fig. 35 : The effect of graded concentrations of lipocortin 1 on phospholipase A_2 (PLA_2 25U/ml) induced (i) ir-arginine vasopressin (n=5) and (ii) ir-prostaglandin E_2 (ir-PGE_2) (n=4-5) release from hypothalami from adrenalectomized rats. Each column represents the mean and is shown with its standard error (**$P<0.01$ control vs. PLA_2 stimulated; ††$P<0.01$, Mann Whitney U test).
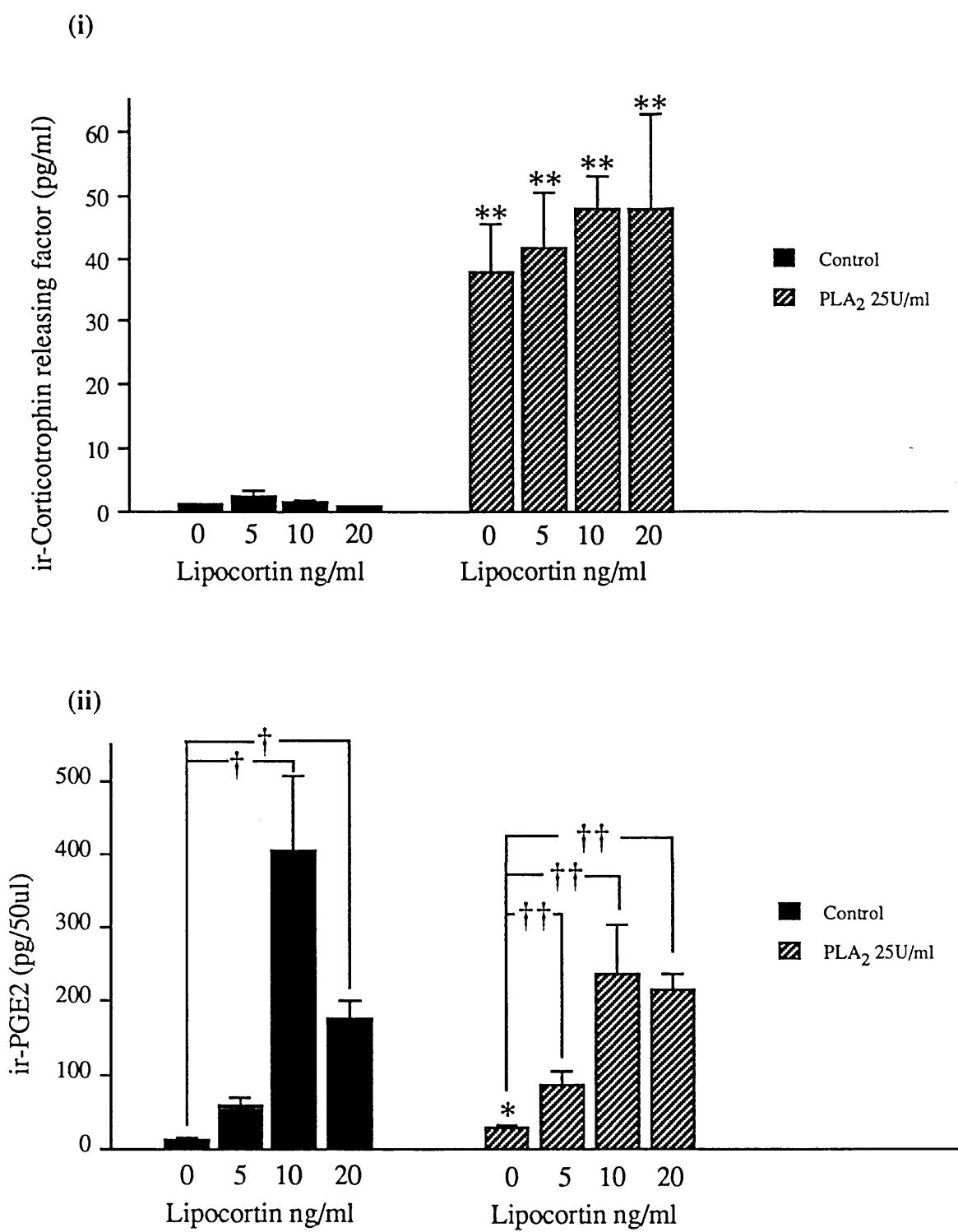
Fig. 36 : The effect of graded concentrations of lipocortin 1 on phospholipase A_2 (PLA$_2$ 25U/ml) induced (i) ir-corticotrophin releasing factor (n=4-6) and (ii) ir-prostaglandin E$_2$ (ir-PGE$_2$) (n=4-6) release from hypothalami from adrenalectomized rats. Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01; control vs. PLA$_2$ stimulated; †P<0.05; ††P<0.01, Mann Whitney U test).

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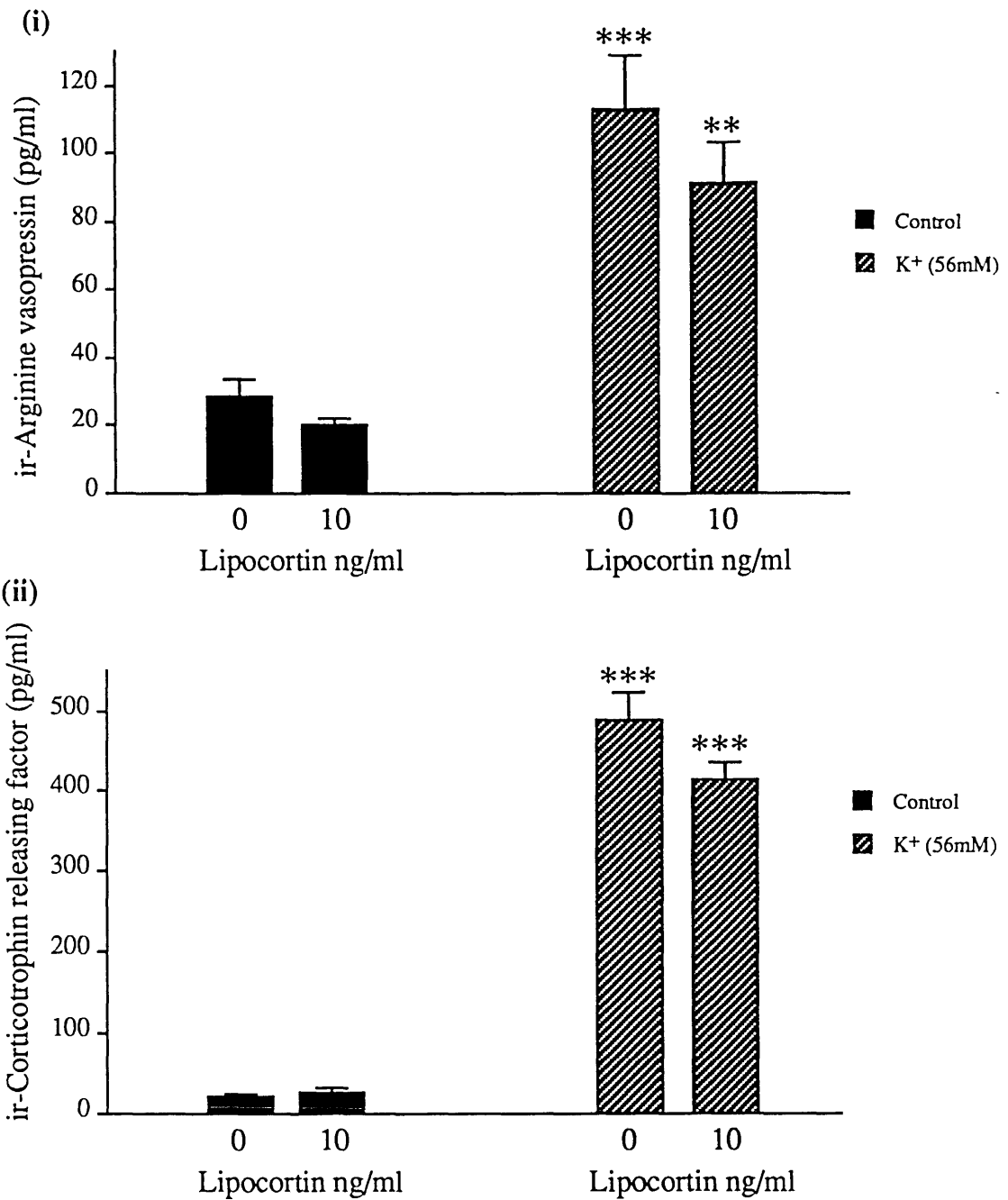
Fig. 37 : The effect of lipocortin on potassium (K^+ 56mM) induced (i) ir-arginine vasopressin (n=6) and (ii) ir-corticotrophin releasing factor (n=6) release from hypothalami from adrenalectomized rats. Each column represents the mean and is shown with its standard error (**P<0.01; ***P<0.001 control vs. K^+ stimulated, Mann Whitney U test).

(b) The effects of lipocortin neutralizing antibody on ir-AVP and ir-CRF-41 secretion in vitro.

Fig. 38 illustrates the effect of a lipocortin 1 neutralizing antibody (ICI plc) on PLA_2-induced ir-AVP and ir-CRF-41 release in the presence and absence of dexamethasone. Again PLA_2 produced the anticipated significant (P<0.01, Mann Whitney U test) increases in the secretion of both peptides. Dexamethasone ($10^{-6}M$) significantly (P<0.05, Mann Whitney U test) attenuated PLA_2-induced ir-AVP release but failed to affect the ir-CRF-41 response to the same secretagogue. The effect of the lipocortin 1 neutralizing antibody was difficult to determine due to the varying effects of the non-immune rabbit serum (NRS) control. Thus with respect to ir-AVP release, the lipocortin antibody caused a significant (P<0.05, Mann Whitney U test) reduction in the PLA_2-induced reponse. However, since NRS alone also reduced ir-AVP release in response to PLA_2, there was no significant difference between PLA_2-stimulated ir-AVP release in the presence of the lipocortin antibody or its control (NRS). The inhibitory effect of dexamethasone ($10^{-6}M$) on PLA_2-induced ir-AVP release was not affected by NRS or the lipocortin neutralizing antibody (Fig. 38i).

With respect to ir-CRF-41 release, dexamethasone, the lipocortin antibody and its control NRS, all failed to affect PLA_2-induced CRF-41 secretion. However, in the presence of NRS the response to PLA_2 was significantly (P<0.01, Mann Whitney U test) potentiated by dexamethasone. This potentiation was not apparent in the presence of the lipocortin antibody (Fig. 38ii).
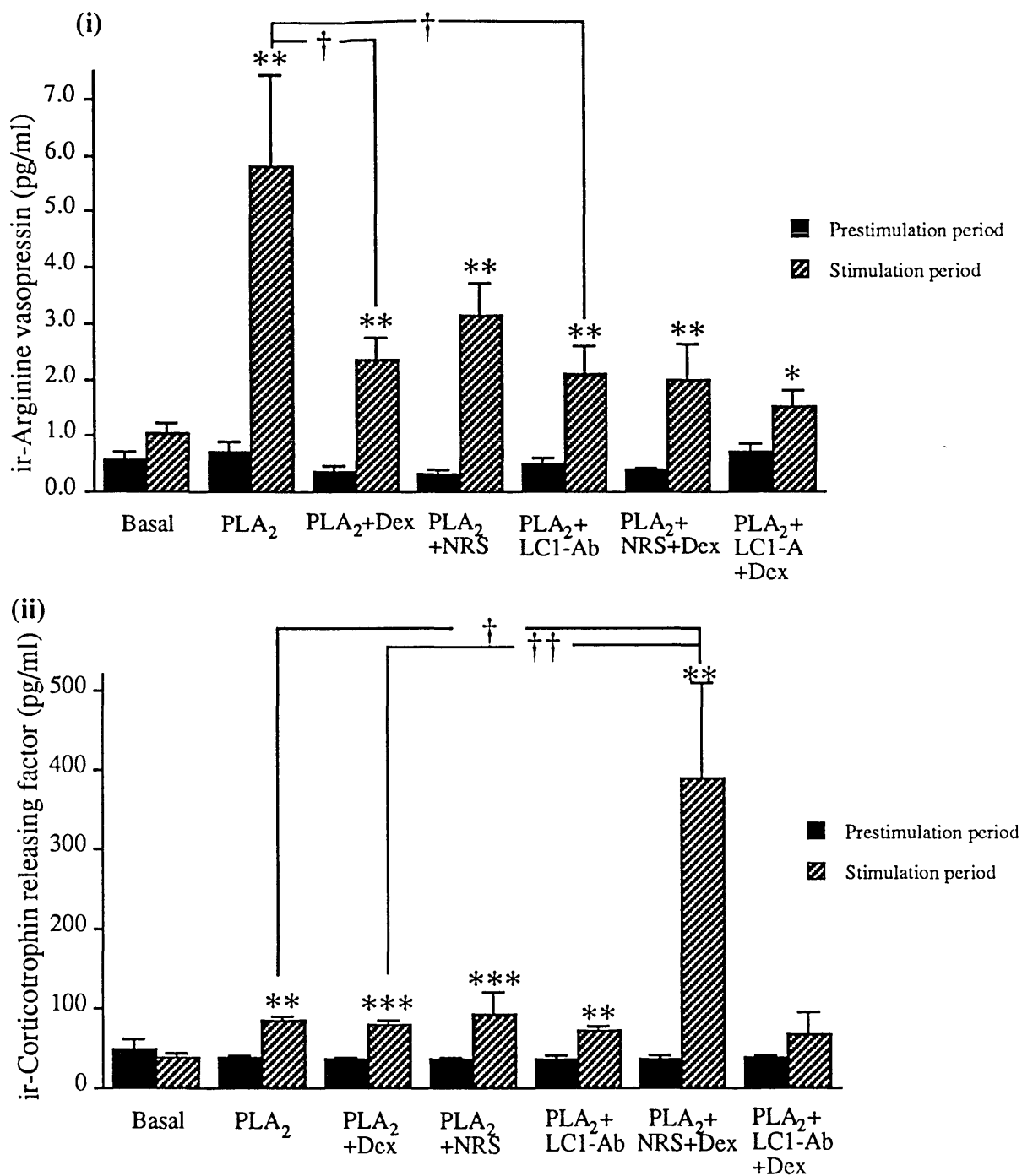
Fig. 38 : The effects of a lipocortin 1 neutralizing antibody (LC1-Ab, 1:500), non-immune rabbit serum (NRS) and dexamethasone (Dex, 10^{-6} M) on phospholipase A_2(PLA_2 25U/ml) induced (i) ir-arginine vasopressin (n=4-6) and (ii) ir-corticotrophin releasing factor (n=4-6) release from hypothalami from adrenalectomized rats. Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01; ***P<0.001 prestimulation period vs. stimulation period; †P<0.05; ††P<0.01, Mann Whitney U test).

(c) The effects of lipocortin 1 on the secretion of ir-Corticosterone and ir-LH in vivo.

Figs. 39 & 40 illustrate the effects of i.c.v. administration of the lipocortin neutralizing antibody and lipocortin 1 on serum ir-corticosterone and ir-luteinizing hormone levels before and 20min after stress. Neither the NRS nor LC1-antibody influenced the pre-stress serum concentrations of corticosterone. Histamine stress (0.6mg/100g/0.2ml) caused a significant (P<0.01, Mann Whitney U test) increase in ir-corticosterone concentrations in the NRS-treated controls. This response was unaffected by the lipocortin neutralizing antibody (Fig. 39i). In contrast the serum levels of ir-luteinizing hormone (ir-LH) were unaffected by stress, however the presence of the lipocortin antibody caused a slight elevation (P<0.01, Mann Whitney U test) in ir-LH concentrations of the serum samples from both stressed and unstressed animals.

Lipocortin 1 (0.3, 0.6, & 1.2μg) had no effect on the resting serum ir-corticosterone concentrations. The two higher doses also failed to affect the significant (P<0.05, Mann Whitney U test) increase in ir-corticosterone levels induced by histamine stress. In contrast the lowest dose tested (0.3μg) caused a significant (P<0.05, Mann Whitney U test) potentiation of the stress-induced release of ir-corticosterone (Fig. 40i).

In control and lipocortin 1-treated (0.3, 0.6 & 1.2μg i.c.v.) rats, lipocortin 1 had no effect on the basal serum ir-LH concentrations. ir-Luteinizing hormone concentrations were also unaffected by histamine stress, however the highest dose of lipocortin 1 tested (1.2μg) caused a significant (P<0.05, Mann Whitney U test) reduction in serum ir-LH following exposure to histamine (Fig. 40ii).

Fig. 41 illustrates the hypothalamic content of lipocortin 1 from intact rats or from animals bearing cannulae placed in the third ventricle. Hypothalamic lipocortin 1 content was significantly elevated in animals which received saline i.c.v. followed by histamine (0.6mg/100g/0.2ml) i.p. when compared with the untreated intact controls and the untreated cannulated animals (P<0.01 and P<0.05 respectively, Mann Whitney U test).

(i)

(ii)
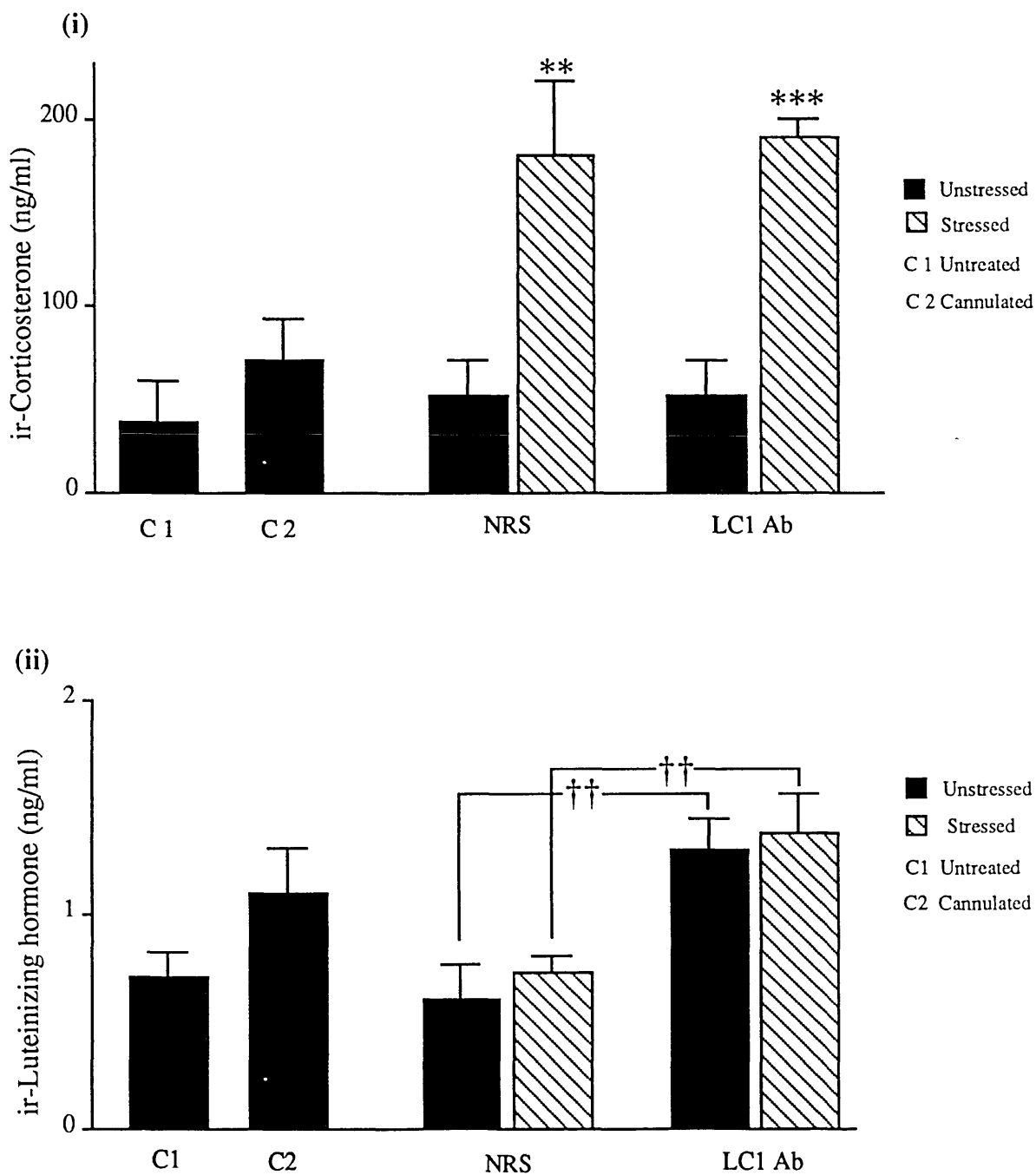
Fig. 39 : The effect of lipocortin 1 neutralizing antibody (LC1-Ab, 1:200, 3μl) or non-immune rabbit serum (NRS, 1:200, 3μl) on (i) ir-corticosterone and (ii) ir-luteinizing hormone concentrations in serum samples from control (unstressed) and histamine treated (0.6mg/100g/0.2ml i.p. stressed) rats. Each column represents the mean and is shown with its standard error (**P<0.01; ***P<0.001 unstressed vs. stressed; ††P<0.01, Mann Whitney U test).

108
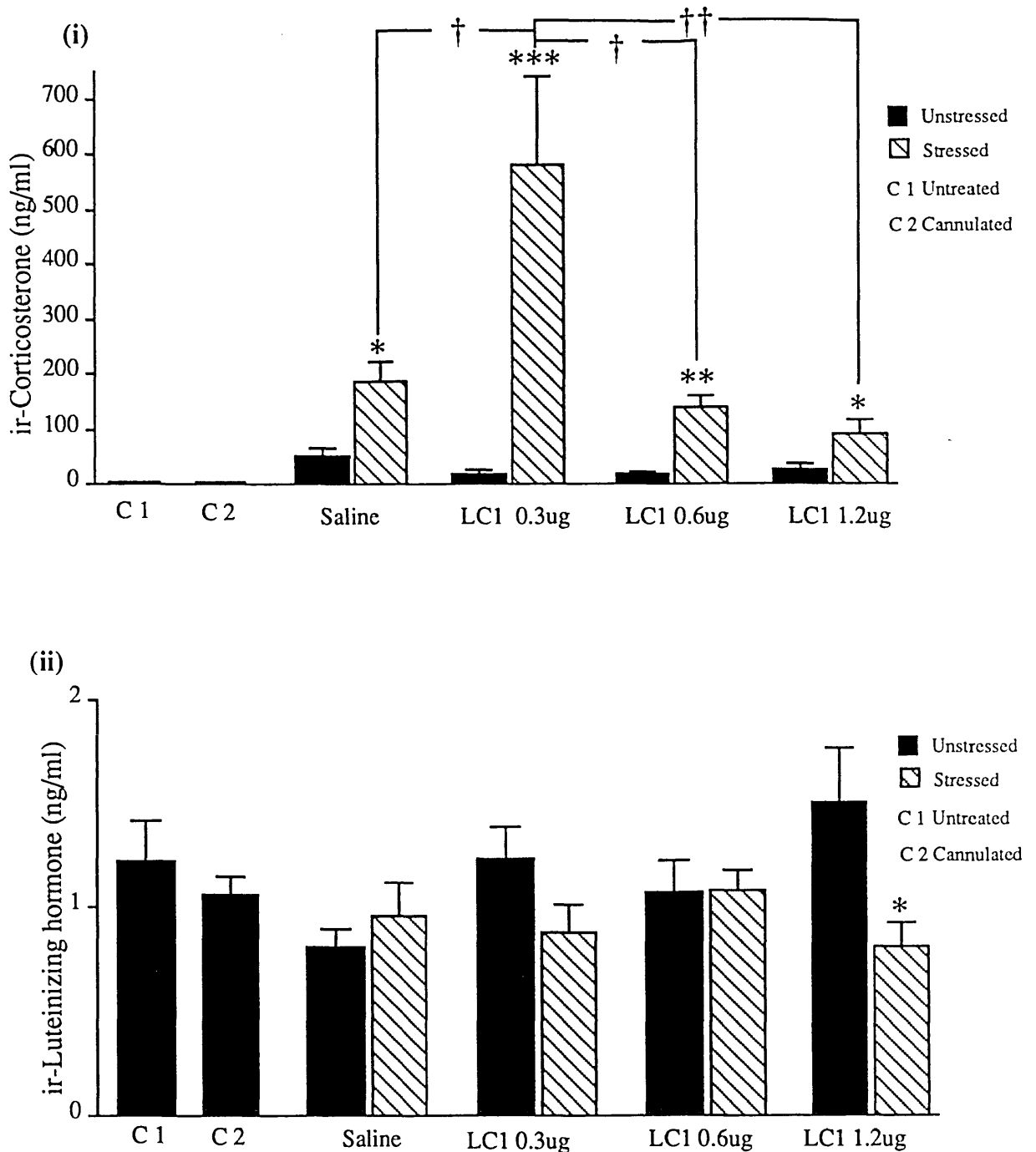
Fig. 40 : The effects of lipocortin 1 (LC1, 3μl) or saline on (i) ir-corticosterone and (ii) ir-luteinizing hormone concentrations in serum samples from control (unstressed) and histamine treated (0.6mg/100g/0.2ml, stressed) rats. Each column represents the mean and is shown with its standard error (n=6; *P<0.05; **P<0.01; ***P<0.001 unstressed vs. stressed; †P<0.05; ††P<0.01, Mann Whitney U test).
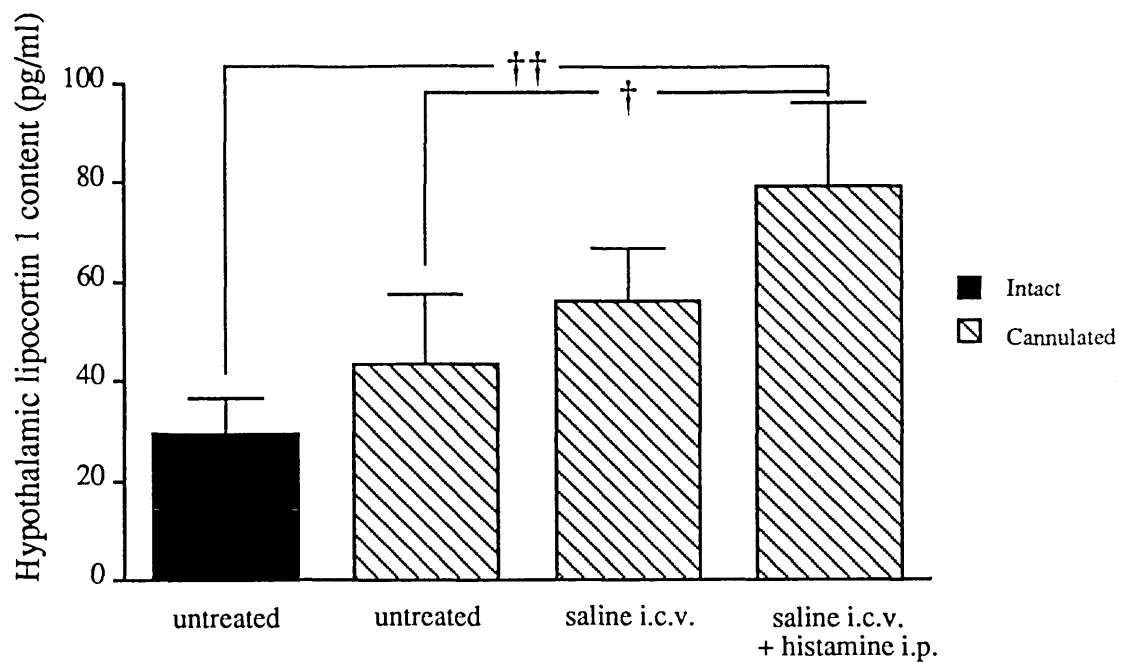
Fig. 41 : The hypothalamic content of lipocortin 1 from intact rats and rats bearing cannulae placed in the third venticle which were either untreated or given an i.c.v. injection of saline (0.9%/3µl) alone or stressed (i.p. injection of histamine 0.6mg/100g/0.2ml). Each column represents the mean (n=5-6) and is shown with its standard error ($\dagger$P<0.05;$\dagger\dagger$P<0.01, Mann Whitney U test).

(d) The effects of lipocortin 1 on the secretion of ir-AVP and ir-ACTH by isolated pituitary tissue in vitro.

Figs. 42 & 43 illustrate the effects of lipocortin 1 on hormone secretion by the pituitary gland. Lipocortin 1 (100ng/ml) had no significant effect on the release of ir-AVP from posterior pituitary tissue but it reduced significantly (P<0.05, Mann Whitney U test) the marked increase in ir-AVP secretion induced by 56mM K^+ (Fig.42).

Fig. 43 demonstrates the effect of Biogen recombinant lipocortin 1 and the ICI lipocortin fragment (1-188) on basal and hypothalamic extract (HE) or K^+-stimulated ir-ACTH release. As expected both HE and K^+ (56mM) induced significant (P<0.01 & P<0.001, Mann Whitney U test) increases in ir-ACTH secretion from adenohypophysial tissue in vitro. Recombinant human lipocortin 1 failed to affect basal ir-ACTH release, however the lowest dose (1ng/ml) caused a significant (P<0.05, Mann Whitney U test) reduction in HE-induced ir-ACTH release. In addition, the response to 56mM K^+ was also significantly (P<0.05, Mann Whitney U test) attenuated in the presence of 100ng/ml recombinant lipocortin (Fig. 43i).

In contrast, the ICI N-terminal fragment lipocortin 1-188 (10 & 100ng/ml) produced significant (P<0.001 & P<0.01 respectively, Mann Whitney U test) increases in basal corticotrophin secretion. However, the fragment (1, 10 & 100ng/ml) failed to affect ir-ACTH release in response to either HE (0.1/ml) or K^+ (56mM) (Fig. 43ii).

111
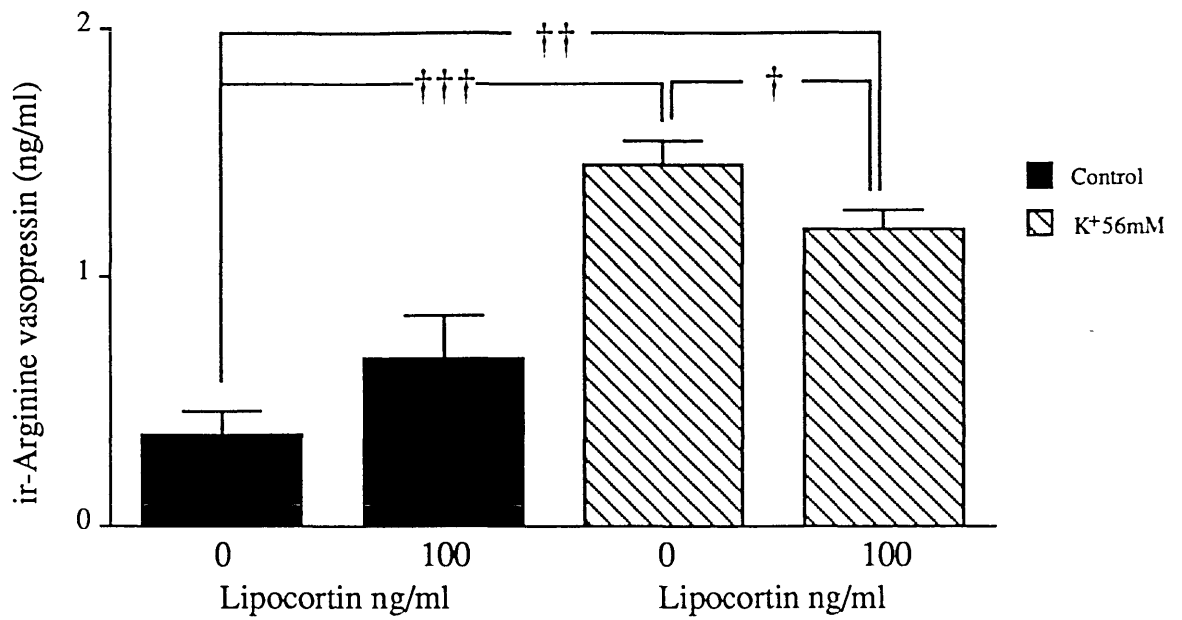
Fig. 42 : The effect of human recombinant lipocortin 1 on basal and potassium (K$^+$56mM) induced ir-arginine vasopressin release from isolated posterior pituitary tissue from intact rats. Each column represents the mean and is shown with its standard error (n=5-6; †P<0.05, ††P<0.01, †††P<0.001, Mann Whitney U test).
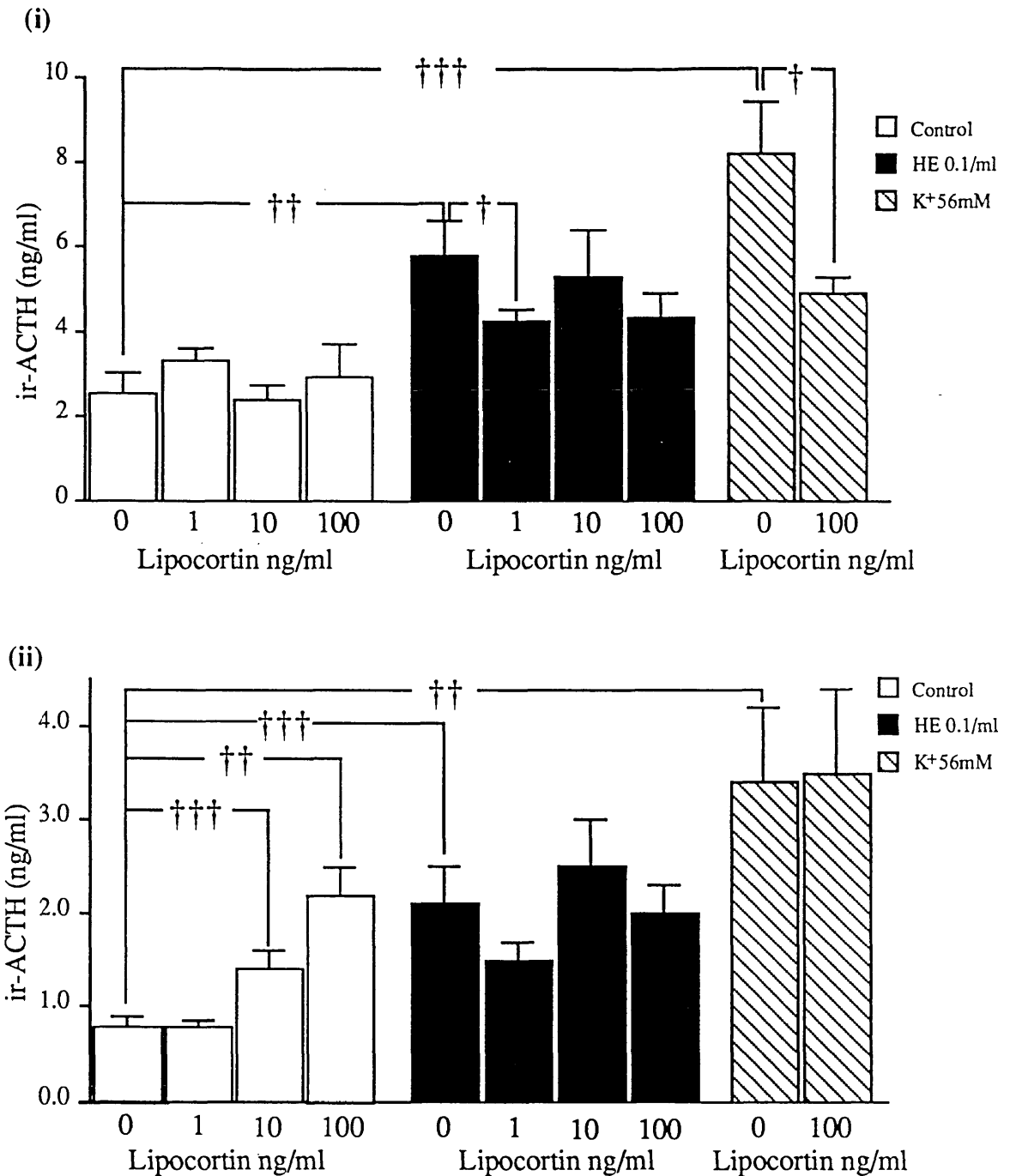
Fig. 43 : The effects of (i) Biogen human recombinant lipocortin 1 (n=6-7) and (ii) ICI lipocortin 1-188 (n=5-6) on basal and hypothalamic extract (HE 0.1/ml) or potassium (K^+56mM) induced ir-corticotrophin (ir-ACTH) release from anterior pituitary tissue from intact rats. Each column represents the mean and is shown with its standard error (†P<0.05; ††P<0.01; †††P<0.001, Mann Whitney U test).

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*(vi)* *The effect of cytokines on hormone secretion by the hypothalamo-pituitary-adrenal axis.*

Table 3 shows the relationship between peptide weight and the international unitage for the cytokines used in these studies. Figs. 44 & 45 show the effects of medium collected from macrophages stimulated in vitro with endotoxin (K235, 25µg/ml) on the release, in vitro of ir-ACTH by pituitary tissue and irAVP and ir-CRF by hypothalamic tissue. Medium from the stimulated macrophages readily elicited (P<0.01, Mann Whitney U test) increases in ir-ACTH; ir-AVP and ir-CRF-41 release (Figs. 44 & 45i, ii). With respect to ir-ACTH and ir-CRF-41 the magnitude of the responses depended on the concentration of macrophages stimulated (Fig. 44 & 45ii) and medium collected from the two highest concentrations (2 x 10^6 & 4 x 10^6 cells/ml) of macrophages produced significant (P<0.01 & P<0.001 respectively, Mann Whitney U test) increases in ir-ACTH release which were greater than those induced by hypothalamic extract alone. In contrast, only medium collected from the highest concentration (4 x 10^6 cells/ml) of stimulated macrophages caused a significant (P<0.01, Mann Whitney U test) increase in ir-AVP secretion (Fig. 45i).

Figs. 46-49 demonstrate the effects of graded concentrations of the interleukins, 1L-1α; 1L-1β; 1L-6; and 1L-8 on the secretion in vitro of ir-AVP and ir-CRF-41 by isolated hypothalami from adrenalectomized rats. All the interleukins tested produced significant (P<0.05, Mann Whitney U test) increases in ir-AVP release. Furthermore the responses to 1L-β and 1L-8 appeared to be concentration related (Fig. 47i & 49i) The maximal release occurring at concentrations of 50pg/ml for 1L-1β and 500pg/ml for 1L-8. The variable basal release made it difficult to determine whether the responses to 1L-α and 1L-6 were also concentration related. Thus although the increment in peptide release differed between doses, the absolute levels were unchanged (Fig. 46i & 48i).

Similarly, all the interleukins produced significant (P<0.05, Mann Whitney U test) increases in ir-CRF release. The ir-CRF 41 release in response to 1L-6 and 1L-8 but not 1L-1α appeared to be concentration related with maximal release (P<0.01, Mann Whitney U test) at 20µg/ml for 1L-6 and 1000pg/ml for 1L-8 (Figs. 48ii & 49ii). Again the responses to 1L-1α were difficult to interpret because of the variation in basal release and therefore, although there were differences in the increment in peptide release between doses, the absolute levels were unchanged (Fig. 46ii). Of all the concentrations of 1L-1β tested however, only the highest (1000pg/ml) caused a significant (P<0.01, Mann Whitney U test) increase in ir-CRF-41 secretion (Fig 47ii).

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CYTOKINE	PEPTIDE WEIGHT
IL-1α	10pg $\equiv$ 1iU
IL-1β	10pg $\equiv$ 1iU
IL-6	200pg $\equiv$ 1iU
IL-8	Not standardised
TNFα	25pg $\equiv$ 1iU

Table. 3 : The relationship between the peptide weight and the international unitage for the cytokines used in these studies.
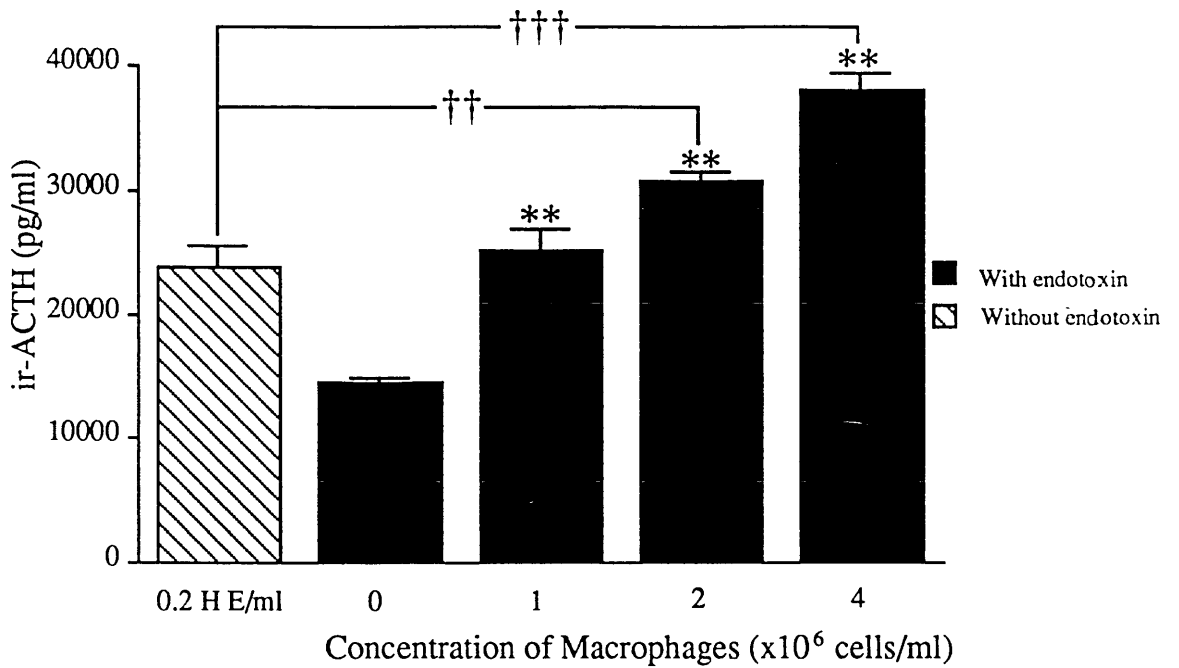
Fig. 44 : The effects of hypothalamic extract (0.2 HE/ml) and medium collected from macrophages stimulated with endotoxin (K235, 25µg/ml) on ir-corticotrophin (ir-ACTH) release from dispersed anterior pituitary cells in vitro. Each column represents the mean and is shown with its standard error (n=8; **P<0.01; ***P<0.001 endotoxin alone vs. endotoxin+macrophages; ††P<0.01; †††P<0.001, Mann Whitney U test).

(i)

(ii)
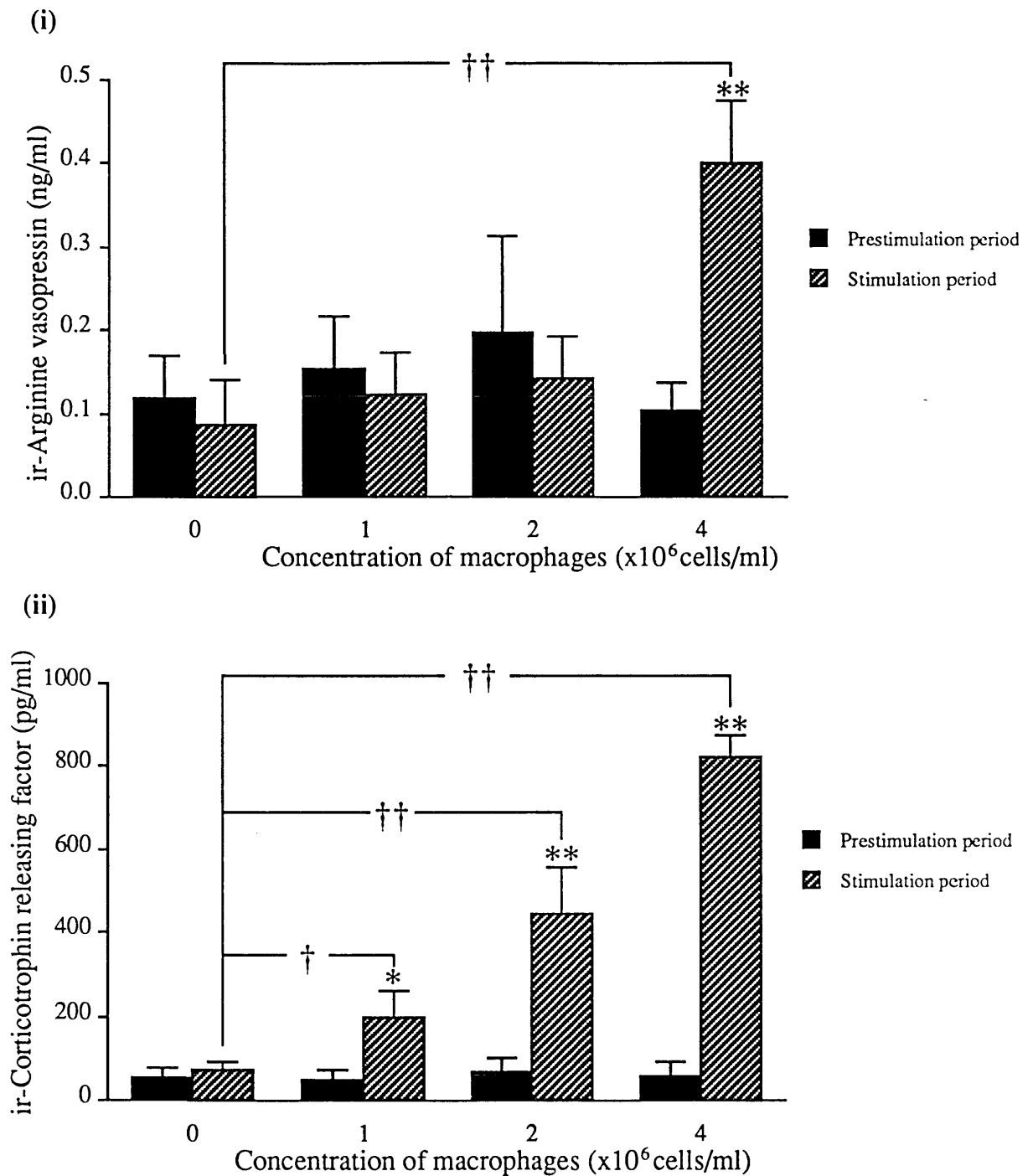
Fig. 45 : The effect of medium collected from macrophages stimulated with endotoxin (K235 25µg/ml) on (i) ir-arginine vasopressin (n=4-5) and (ii) ir-corticotrophin releasing factor (n=4-5) release from hypothalamic tissue from intact rats in vitro. Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01 prestimulation period vs. stimulation period; †p<0.05; ††p<0.01, Mann Whitney U test).

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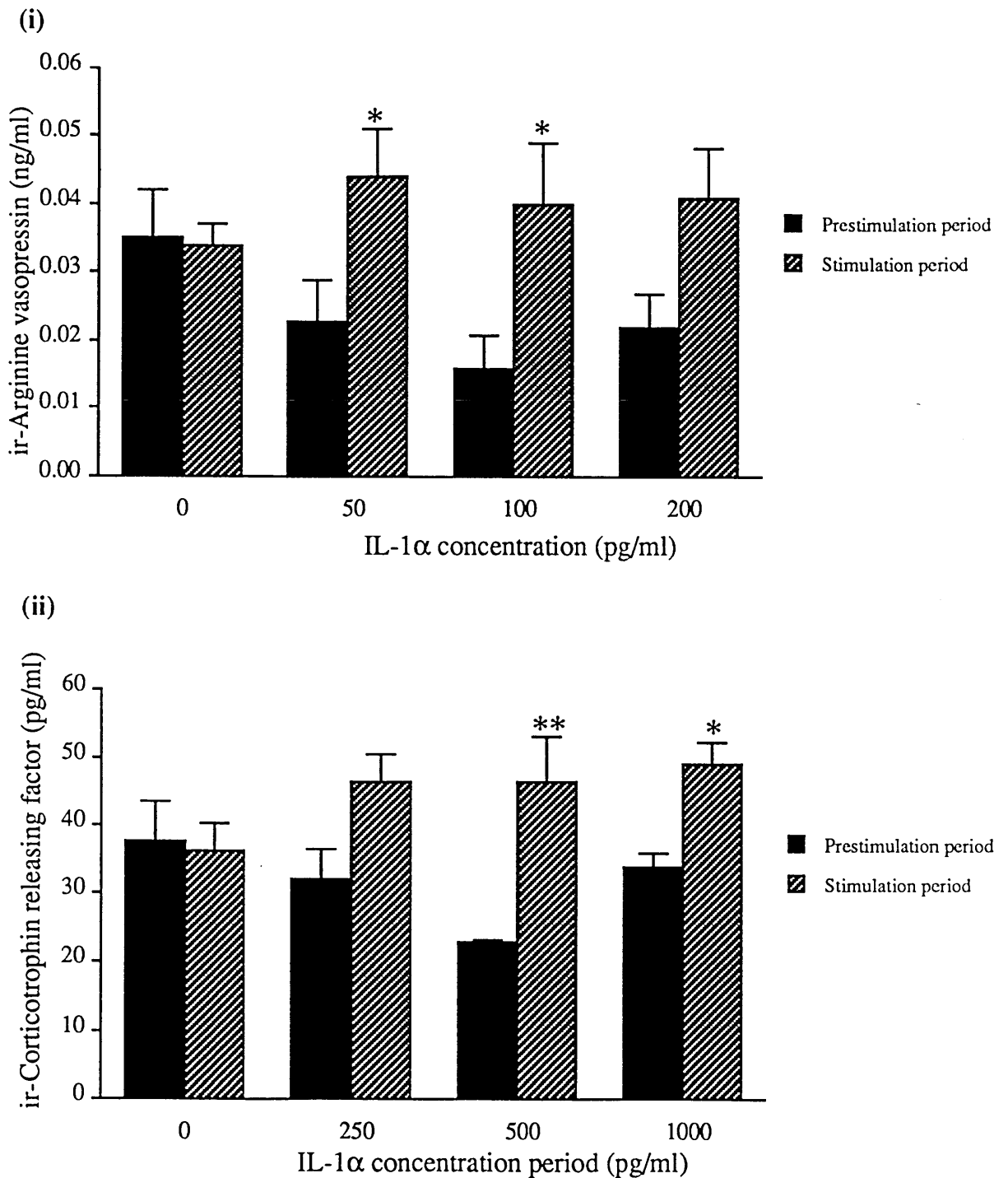
Fig. 46 : The effect of graded doses of interleukin 1α (IL-1α recombinant human -W.H.O.) on (i) ir-arginine vasopressin (n=3-5) and (ii) ir-corticotrophin releasing factor (n=3-4) release from hypothalami from adrenalectomized rats. Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01 prestimulation period vs. stimulation period, Mann Whitney U test).

(i)

(ii)
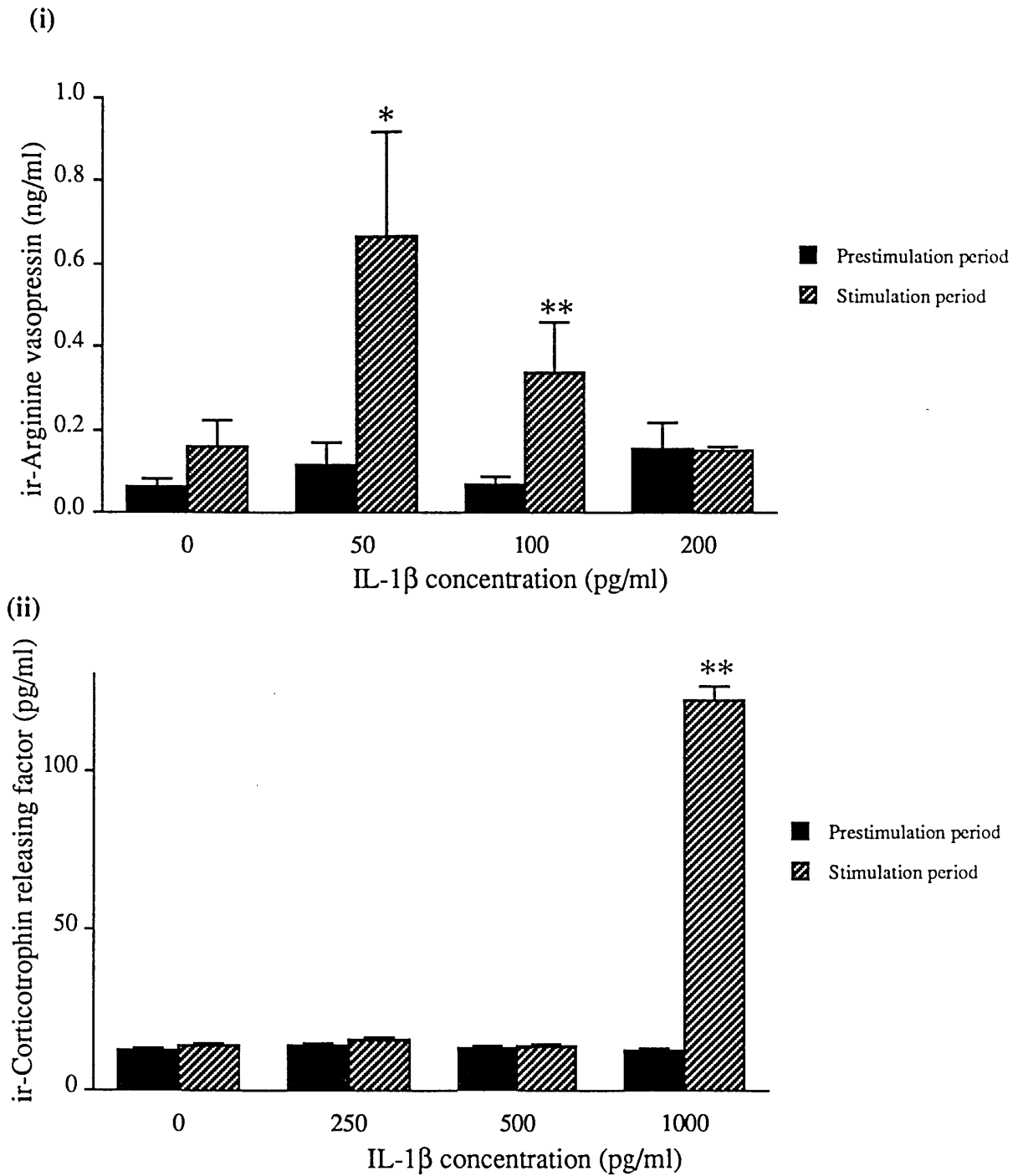
Fig. 47 : The effect of graded doses of interleukin 1β (IL-1β (i) recombinant human-W.H.O. (ii) recombinant murine-Glaxo Geneva) on (i) ir-arginine vasopressin (n=4-5) and (ii) ir-corticotrophin releasing factor (n=4-6) release from hypothalami from adrenalectomized rats. Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01 prestimulation period vs. stimulation period, Mann Whitney U test).

(An example from a responding tissue)

119
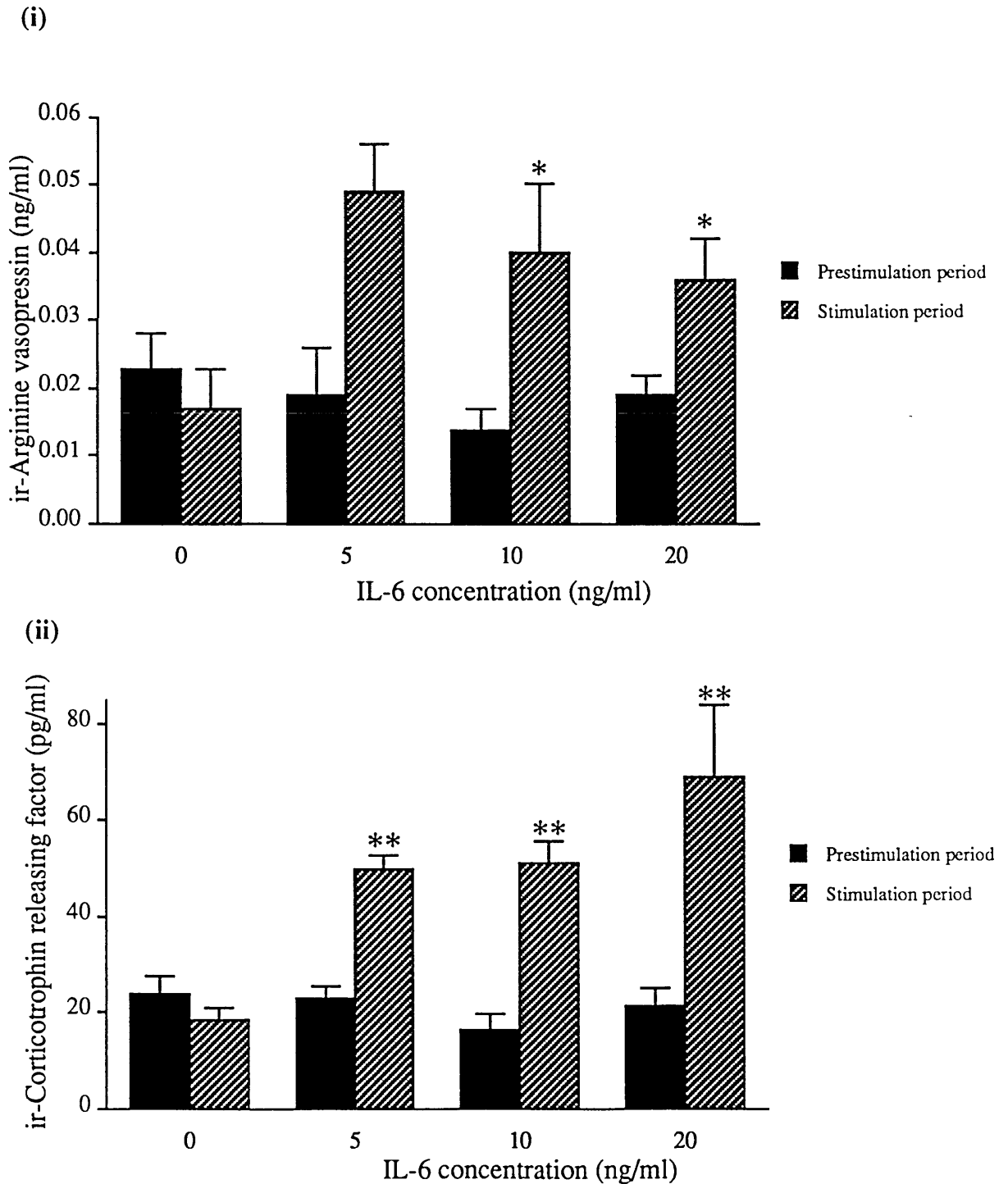
Fig. 48 : The effect of graded doses of interleukin 6 (IL-6 recombinant human-W.H.O.) on (i) ir-arginine vasopressin (n=3-5) and (ii) ir-corticotrophin releasing factor (n=4-5) release from hypothalami from adrenalectomized rats. Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01 prestimulation period vs. stimulation period, Mann Whitney U test).
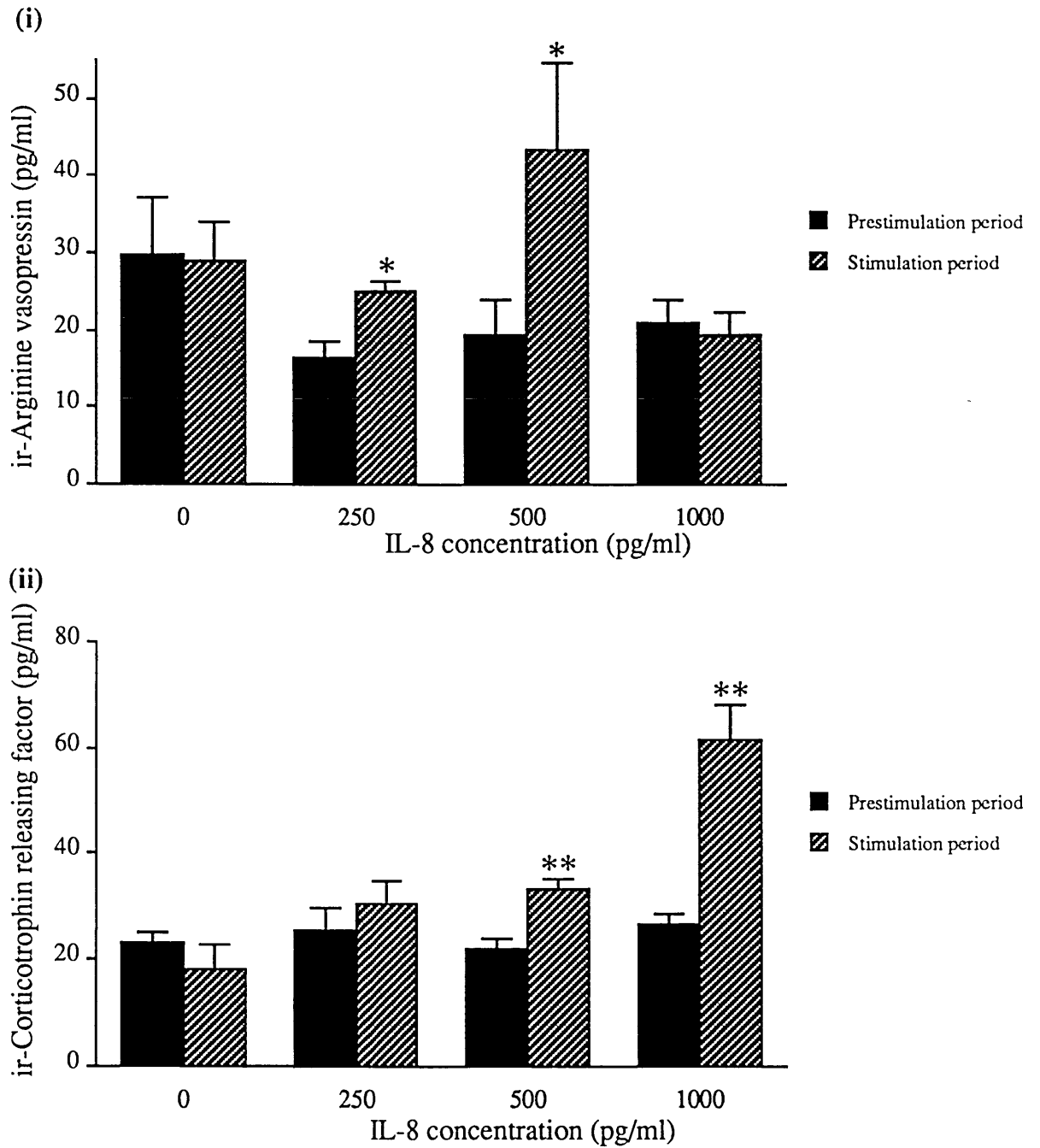
Fig. 49 : The effect of graded doses of interleukin 8 (IL-8 recombinant human -W.H.O.) on **(i)** ir-arginine vasopressin (n=3-6) and **(ii)** ir-corticotrophin releasing factor (n=4-6) release from hypothalami from adrenalectomized rats. Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01 prestimulation period vs. stimulation period, Mann Whitney U test).

Fig. 50 illustrates the effect of tumour necrosis factor (TNF) either alone, or in combination with IL-1β (50pg/ml), on ir-AVP secretion. Although both concentrations of TNF tested appeared to stimulate ir-AVP release (Fig. 50i), the effects were significant (P<0.05, Mann Whitney U test) only with the high one (2500pg/ml). When the lower concentration (625pg/ml) was applied in combination with IL-1β (50pg/ml) a significant potentiation of the ir-AVP response was observed (P<0.05, Mann Whitney U test) (Fig. 50ii).

Figures 51-53 illustrate the effect of dexamethasone (10^{-6}M) included in the medium throughout the pre-stimulation and stimulation periods, on interleukin induced ir-AVP and ir-CRF-41 secretion from hypothalamic tissue from adrenalectomized rats incubated in vitro.

All of the interleukins tested produced significant (P<0.05, Mann Whitney U test) increases in ir-AVP release.

The ir-AVP release in response to 100 pg/ml of IL-1α was significantly (P<0.05, Mann Whitney U test) reduced by the presence of the steroid. The steroid also reduced (P=0.057, Mann Whitney U test), the smaller increment in peptide release evoked by the higher dose of interleukin (1000 pg/ml) (Fig. 51i). Similarly, dexamethasone (10^{-6}M) also reduced significantly (P<0.05, Mann Whitney U test) the increments in ir-AVP release induced by IL-6 (10ng/ml) and IL-8 (1ng/ml) (Figs. 52i & 53i).

With respect to ir-CRF-41 release, all the interleukins tested produced the anticipated significant (P<0.05, Mann Whitney U test) increases in ir-CRF-41 secretion (Figs. 51ii -53ii). The responses were affected variably by the presence of dexamethasone. Thus, dexamethasone failed to inhibit the ir-CRF-41 release induced by 500pg/ml IL-1α; on the contrary, it appeared to potentiate the response (Fig. 51ii). The effects of the steroid on the response to the lower concentration of IL-1α (100pg/ml) were more difficult to interpret as basal ir-AVP release was increased in the presence of dexamethasone. No further increase in peptide release was apparent in the presence of the interleukin. In contrast, ir-CRF-41 secretion in response to both IL-6 and IL-8 was reduced by the steroid. Thus dexamethasone caused a significant (P<0.05, Mann Whitney U test) reduction in both the absolute amount of ir-CRF-41 released in reponse to IL-6 and the increment in peptide release induced by IL-8 (P<0.05, Mann Whitney U test) (Figs. 52ii & 53ii).
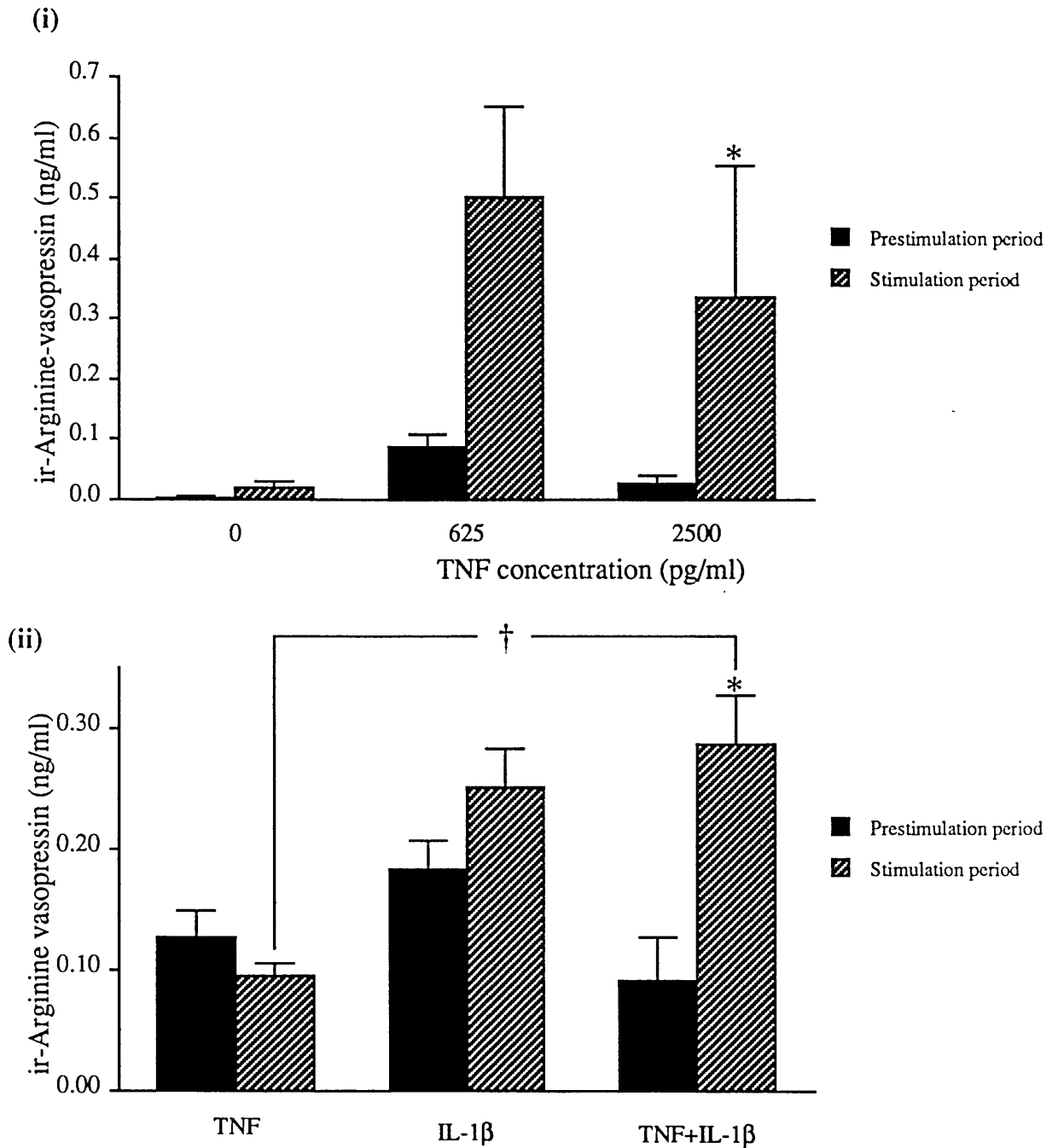
Fig. 50 : The effects of (i) graded doses of tumour necrosis factor (TNF recombinant human-W.H.O.) and (ii) tumour necrosis factor (TNF 625pg/ml) and interleukin 1β (IL-1β 50pg/ml recombinant human-W.H.O.) alone and in combination on ir-arginine vasopressin (n=4-5) release from hypothalami from adrenalectomized rats. Each column represents the mean and is shown with its standard error (*P<0.05 prestimulation period vs. stimulation period; †P<0.05, Mann Whitney U test).

(i)

Prestimulation period

Stimulation period

†

**

ir-Arginine vasopressin (ng/ml)

0.50
0.40
0.30
0.20
0.10
0.00

IL-1α
100pg/ml

IL-1α 100pg/ml
+Dex

IL-1α
1000pg/ml

IL-1α 1000pg/ml
+Dex

(ii)

**

**

*

Prestimulation period

Stimulation period

ir-Corticotrophin releasing factor (pg/ml)

60
50
40
30
20
10
0

IL-1α
250pg/ml

IL-1α 250pg/ml
+Dex

IL-1α
500pg/ml

IL-1α 500pg/ml
+Dex
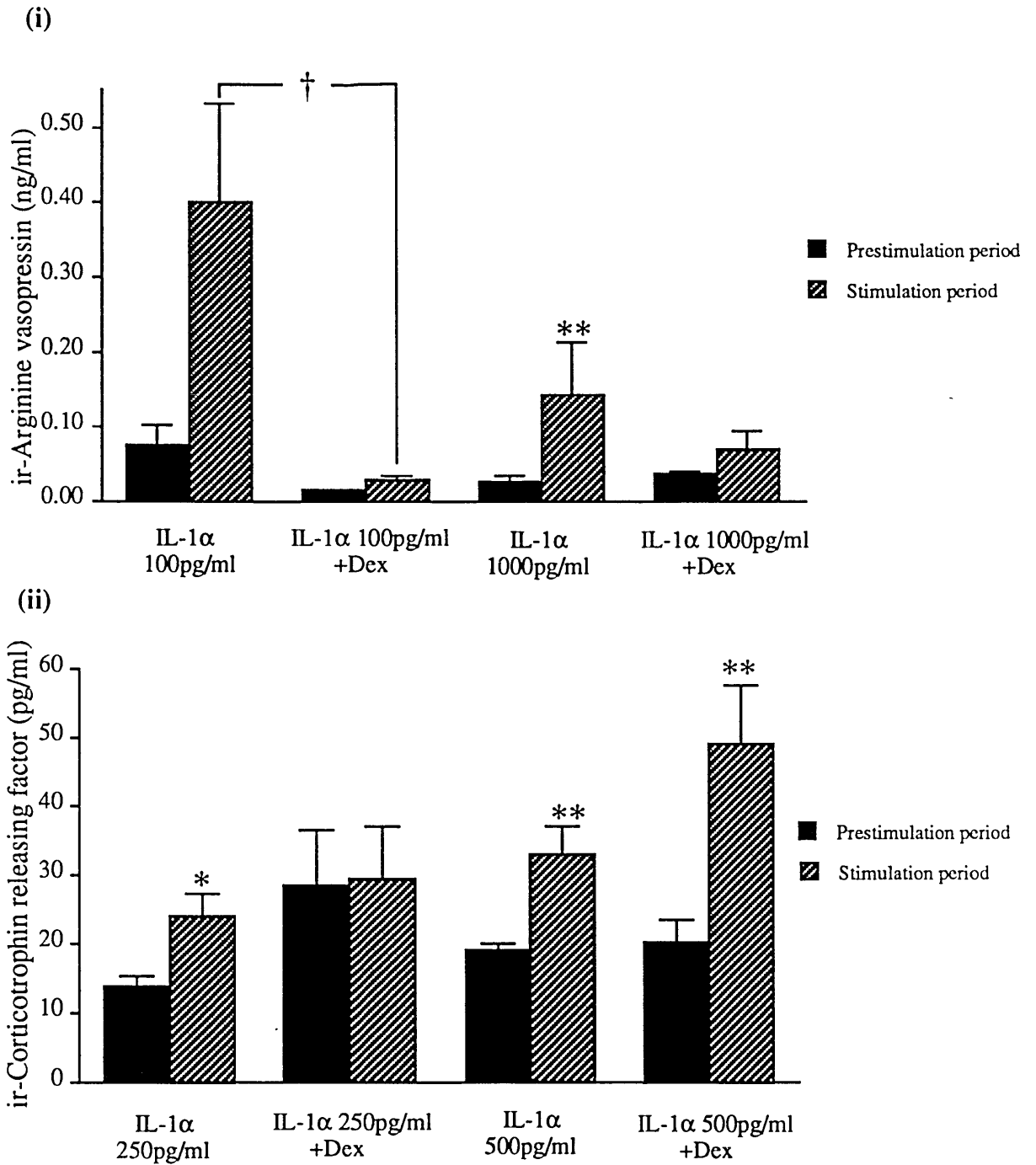
Fig. 51 : The effect of dexamethasone (Dex 10^{-6}M) on interleukin 1α (IL-1α recombinant human-W.H.O.) induced (i) ir-arginine vasopressin (n=2-5) and (ii) ir-corticotrophin releasing factor (n=3-5) release from hypothalami from adrenalectomized rats. Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01 prestimulation period vs. stimulation period; †P<0.05, Mann Whitney U test).
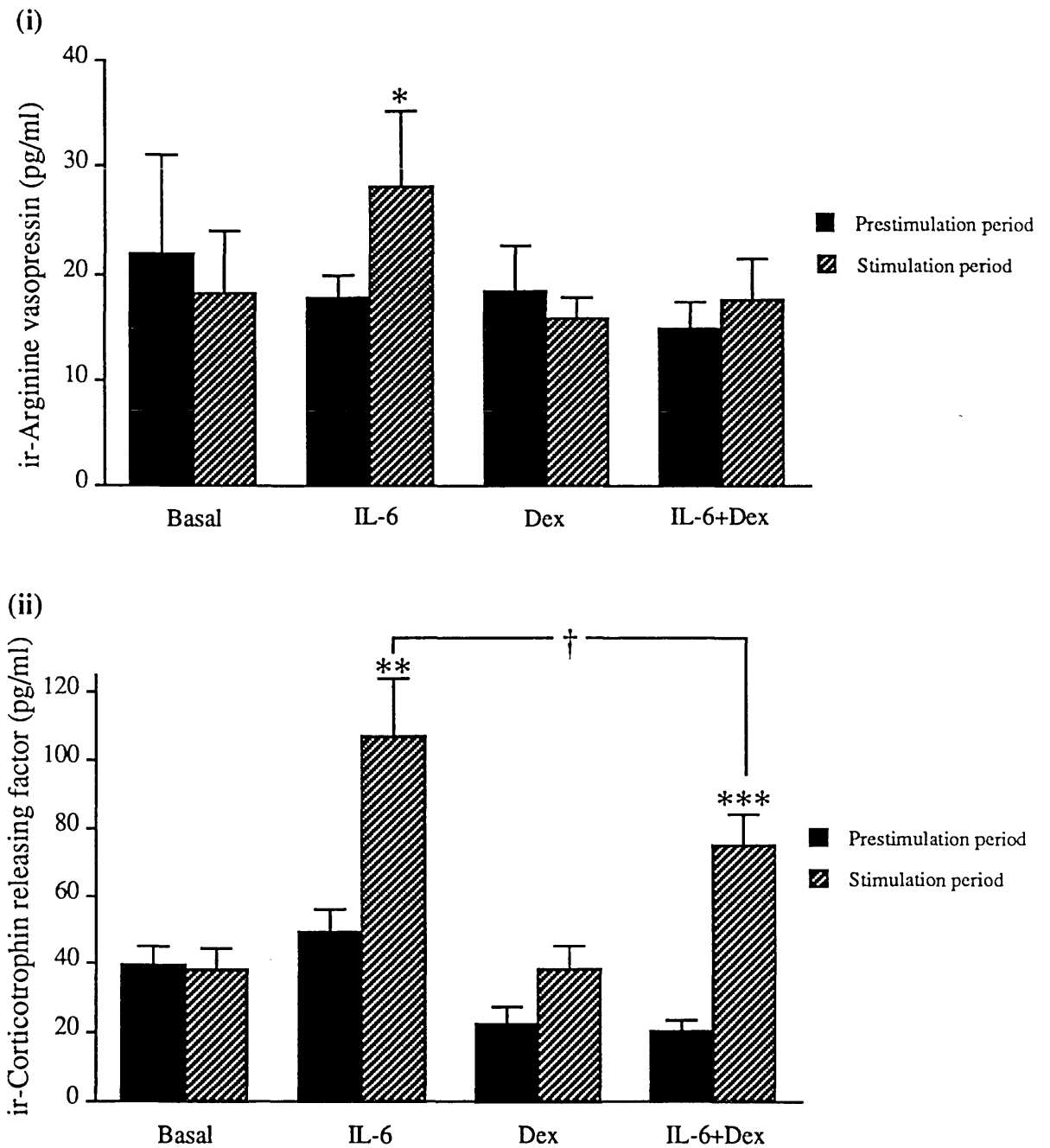
Fig. 52 : The effect of dexamethasone (Dex $10^{-6}M$) on interleukin 6
(IL-6 10ng/ml recombinant human-W.H.O.) induced (i) ir-arginine
vasopressin (n=5-6) and (ii) ir-corticotrophin releasing factor
(n=5-6) release from hypothalami from adrenalectomized rats. Each
column represents the mean and is shown with its standard error
(*P<0.05; **P<0.01; ***P<0.001 prestimulation period vs. stimulation
period; †P<0.05, Mann Whitney U test).

(i)

(ii)
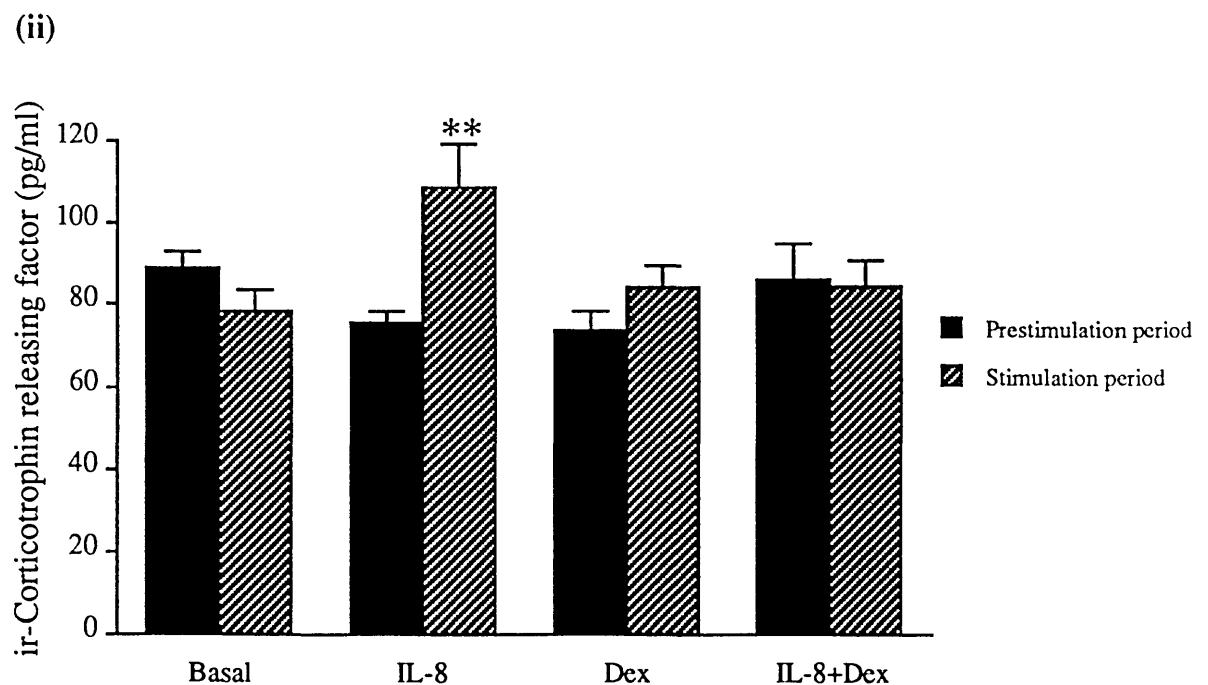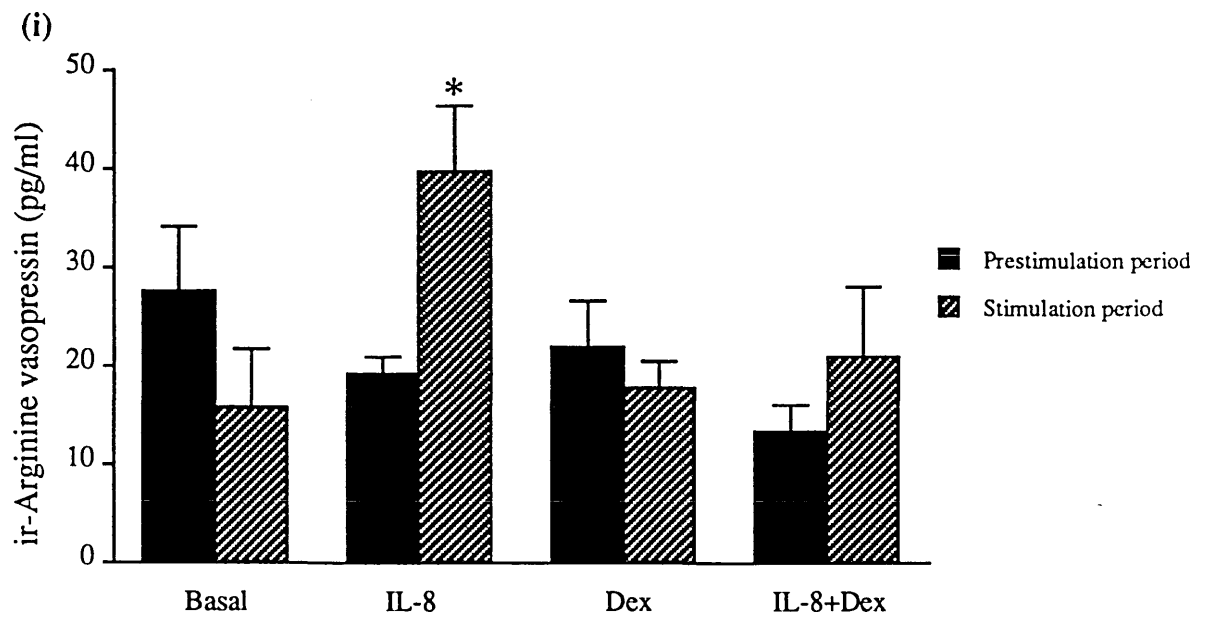
Fig. 53 : The effect of dexamethasone (Dex 10^{-6} M) on interleukin 8 (IL-8 1.0 ng/ml
recombinant human-W.H.O.) induced (i) ir-arginine vasopressin (n=4-6) and
(ii) ir-corticotrophin releasing factor (n=3-6) release from hypothalami from
adrenalectomized rats. Each column represents the mean and is shown with
its standard error (*P<0.05; **P<0.01 prestimulation period vs. stimulation
period, Mann Whitney U test).

Figs. 54 & 55 illustrate the effects of lipocortin on IL-6 and IL-8 induced ir-AVP and ir-CRF-41 secretion from hypothalamic tissue from adrenalectomized rats. Significant increases in ir-AVP release were apparent following stimulation with the higher (5ng/ml) but not the lower (3.4ng/ml) concentration of IL-6. Addition of lipocortin (5 & 10ng/ml) which in this experiment had no effect on resting ir-AVP, potentiated the secretory responses to IL-6 (3.4 & 5ng/ml). The effects were highly significant (P<0.01, Mann Whitney U test) (Fig. 54i).

Unfortunately, in the study with IL-8, the concentration of the cytokine tested (1000pg/ml) failed to produce a significant increase in ir-AVP release. Similarly lipocortin was without effect (Fig. 55i).

In complete contrast, lipocortin reduced markedly the ir-CRF-41 response to IL-6. Thus, both concentrations of the peptide (5 & 10ng/ml) significantly attenuated (P<0.01, Mann Whitney U test) the responses to IL-6 (3.4 & 5ng/ml) (Fig. 54ii). Similarly the significant increase in ir-CRF-41 release evoked by IL-8 (P<0.05, Mann Whitney U test) was significantly (P<0.05, Mann Whitney U test) reduced by lipocortin (10ng/ml) (Fig. 55ii).

Table 4 illustrates the effects of the cytokines (IL-1α, IL-1β, IL-6 and IL-8) alone or in combination with TNFα on basal or hypothalamic extract (HE 0.1/ml) induced ir-ACTH release from isolated pituitary segments in vitro.

None of the interleukins tested when applied alone or in combination with TNFα (1250pg/ml) influenced the basal release of ir-ACTH from the pituitary segments. In all cases, hypothalamic extract (HE, 0.1/ml) elicited significant (P<0.01, Mann Whitney U test) increases in ir-ACTH release. In two of four experiments this response was significantly (P<0.05, Mann Whitney U test) reduced by TNFα (1250pg/ml). Interleukin 1α (500pg/ml), IL-β (200pg/ml) and IL-8 (500 & 1000pg/ml) alone or in combination with TNFα (1250pg/ml), failed to affect HE-induced ir-ACTH release. However, the lowest concentration of IL-8 tested (250pg/ml) caused a significant (P<0.01, Mann Whitney U test) reduction in stimulated ir-ACTH release. Similar inhibitory effects were also apparent with IL-6 (5 & 10ng/ml) when given alone (P<0.05 & P<0.001 respectively, Mann Whitney U test) or together with TNFα (1250pg/ml) (P<0.05, Mann Whitney U test).

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(i)

ir-Arginine vasopressin (ng/ml)

12 — 10 — 8 — 6 — 4 — 2 — 0

†† †† ††

** ** * **

■ Prestimulation period
▨ Stimulation period

Basal | Lipo 10ng/ml | IL-6 3.4ng/ml | IL-6 5ng/ml | IL-6 3.4ng/ml +Lipo 5ng/ml | IL-6 5ng/ml +Lipo 5ng/ml | IL-6 3.4ng/ml +Lipo 10ng/ml | IL-6 5ng/ml +Lipo 10ng/ml

(ii)

ir-Corticotrophin releasing factor (pg/ml)

100 — 80 — 60 — 40 — 20 — 0

†† †† †† ††

** *

■ Prestimulation period
▨ Stimulation period

Basal | Lipo 10ng/ml | IL-6 3.4ng/ml | IL-6 5ng/ml | IL-6 3.4ng/ml +Lipo 5ng/ml | IL-6 5ng/ml +Lipo 5ng/ml | IL-6 3.4ng/ml +Lipo 10ng/ml | IL-6 5ng/ml +Lipo 10ng/ml

Fig. 54 : The effect of human recombinant lipocortin 1 (Lipo 5-10ng/ml) on interleukin 6 (IL-6 3.4-5ng/ml recombinant human-W.H.O.) induced (i) ir-arginine vasopressin (n=3-6) and (ii) ir-corticotrophin releasing factor (n=3-6) release from hypothalami from adrenalectomized rats. Each column represents the mean and is shown with its standard error (*P<0.05; **P<0.01 prestimulation period vs. stimulation period; ††P<0.01, Mann Whitney U test).
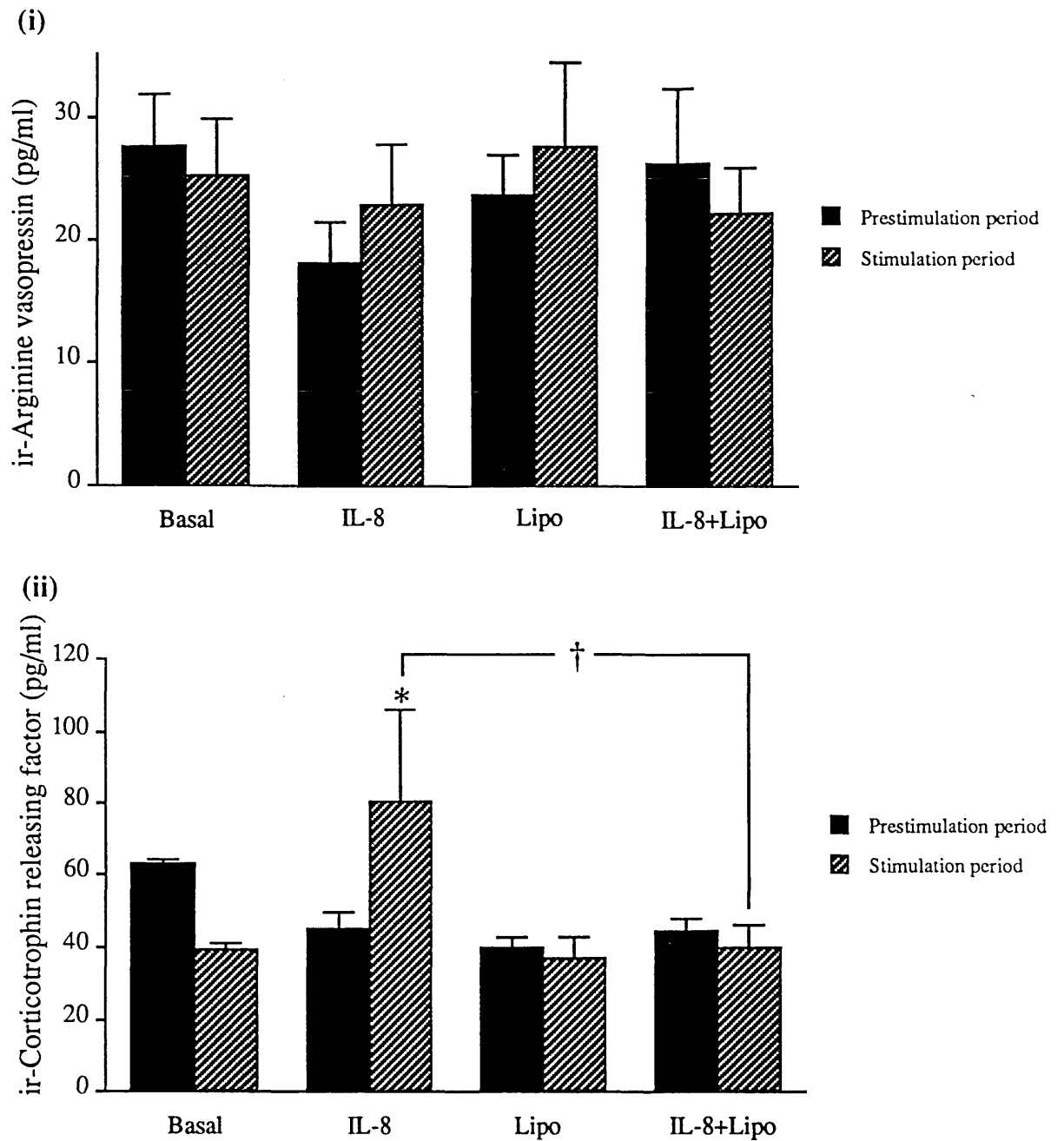
Fig. 55 : The effect of human recombinant lipocortin 1 (Lipo 10ng/ml) on interleukin 8 (IL-8 1.0ng/ml recombinant human-W.H.O.) induced (i) ir-argininevasopressin (n=5-6) and (ii) ir-corticotrophin releasing factor (n=4-6) release from hypothalami from adrenalectomized rats. Each column represents the mean and is shown with its standard error (*P<0.05 prestimulation period vs. stimulation period; †P<0.05, Mann Whitney U test).

|	ACTH Release (ug/mg)	
	Basal	0.1 H.E./ml
Control	1.9 ± 0.2	6.7 ± 0.7**
1L-1α (500pg/ml)	2.8 ± 0.5	7.4 ± 0.6
TNFα (1250 pg/ml)	2.1 ± 0.4	5.4 ± 0.8†
1L-1α (500pg/ml)+TNFα (1250pg/ml)	2.9 ± 0.7	5.3 ± 0.9
Control	1.6 ± 0.2	3.6 ± 0.3***
1L-1ß (200pg/ml)	1.9 ± 0.3	4.0 ± 0.2
TNFα (1250pg/ml)	1.9 ± 0.1	3.6 ± 0.4
1L-1ß (200pg/ml)+TNFα (1250pg/ml)	1.9 ± 0.4	4.6 ± 0.5
Control	2.3 ± 0.3	7.4 ± 0.8***
1L-6 (5ng/ml)	2.3 ± 0.4	5.2 ± 0.7†
1L-6 (10ng/ml)	2.3 ± 0.5	4.3 ± 0.2†††
TNFα (1250pg/ml)	2.3 ± 0.6	5.6 ± 0.5†
1L-6 (10ng/ml)+TNFα (1250pg/ml)	2.4 ± 0.5	5.1 ± 0.6†
Control	2.2 ± 0.5	6.9 ± 0.9**
1L-8 (250pg/ml)	2.2 ± 0.3	4.8 ± 0.4†
1L-8 (500pg/ml)	3.3 ± 0.3	6.1 ± 1.0
1L-8 (1000pg/ml)	3.1 ± 0.4	7.6 ± 0.8
TNFα (1250pg/ml)	2.1 ± 0.2	6.6 ± 0.9
1L-8(1000pg/ml)+TNFα (1250 pg/ml)	2.2 ± 0.2	5.3 ± 0.1

Table. 4 : The effects of several interleukins (IL's) alone or in combination with tumour necrosis factor α (TNFα) on basal and hypothalamic extract (H.E.) induced ir-adrenocorticotrophic hormone (ir-ACTH) release from isolated pituitary segments from adrenalectomized rats. Each value represents the mean and is shown with it standard error (n=6-7; **P<0.01;***P<0.001 basal vs. H.E.-stimulated; †P<0.05; †††P<0.001 control vs. cytokine stimulated, Mann Whitney U test).

DISCUSSION

(i) Incubation of hypothalamic tissue in vitro

Investigation at the cellular level of the wide array of mechanisms regulating the secretion of the hypothalamic hormones in vivo is complicated by the inaccessibility of the hypothalamus to experimental manipulation and by the difficulties of monitoring directly the secretion of the various neurohormones. These problems have led to the widespread use of in vitro models which, unlike their in vivo counterparts, permit detailed examination of the actions and interactions of putative controlling factors in a precisely controlled environment. Various preparations have been developed utilising whole hypothalami (Bradbury et al., 1974; Buckingham & Hodges, 1977b; Leposavic, Cover & Buckingham, 1988; Leposavic, Dashwood, Ginsberg & Buckingham, 1990) or hypothalamic fragments in which the organ is either bisected (Shimatsu, Kato, Matsushita, Katahami, Yanaihara & Imura, 1982), quartered (Maeda & Frohman, 1980) or sliced (Iversen, Iverson, Bloom, Douglas, Brown & Vale, 1978) and maintained under static or dynamic conditions.

Much of the work described in this thesis involved the use of static incubates of whole hypothalamic tissue coupled with radioimmunoassay for the determination of CRF-41 and AVP. This preparation was first developed by Bradbury et al. (1974) who demonstrated the ability of the tissue to retain its functional integrity in vitro and to respond to electrical and pharmacological stimuli with changes in the degree of its secretory activity. It has since been successfully exploited in a number of laboratories for studies on the mechanisms controlling the secretion of a number of hypothalamic hormones including CRF (Buckingham & Hodges, 1977b) and GnRH(Leposavic et al., 1990). Obviously the validity of comparing results from in vitro experiments to in vivo situations is questionable. However, there has been a good correlation between data obtained using the isolated hypothalamus in vitro (Bradbury et al., 1974; Jones et al., 1976; Buckingham & Hodges, 1977b & 1979; Buckingham & Cooper, 1986) with the findings of in vivo studies (Abe & Hiroshige, 1974; Chambers & Brown, 1976; Chihara, Kalo, Maeda, Matsukura & Inmura, 1976; Hayes & Stewart, 1985; Cover & Buckingham, 1989). This in vitro preparation has the advantage over those employing fragments or slices in that it retains the 3-dimensional structure and therefore closely resembles the in vivo situation. Thus, the releasing factor neurones and a large proportion of the neuronal connections and connections between neurones

and non-neuronal supporting cells remain intact. It also, however, has the disadvantage that the tissue viability is limited. The tissue block used (5x3x2mm) is considerably greater than the maximum volume of brain tissue (1mm^3) reported to permit adequate diffusion of nutrients and metabolites to and from the centre of the tissue (*Lumsden, 1968*). Therefore, although cells located near the edge of the hypothalamic block should retain their cellular integrity, the cells in the centre of the tissue may undergo necrosis. Metabolic and histological studies indicate that the viability of such preparations is limited to 3h, during which the O_2 consumption is linear (*Bradbury et al., 1974; Berelowitz, Kronheim, Pimstone & Sheppard, 1978*) and following which signs of deterioration with varying degrees of perineuronal odema occur although no indications of neuronal death are apparent (*Bradbury et al., 1974*). Thus, although some workers have recently reported to successfully maintain hypothalami in vitro for more than 24h (*Calogero et al., 1988b*), maintenance beyond the 3h period utilized in the present study may give rise to misleading results.

Viability of the tissue may be prolonged by the utilization of hypothalamic fragments (*Iverson et al., 1978; Maeda & Frohman, 1980; Terry, Rorstad & Martin, 1980; Shimatsu et al., 1982*) and/or perifusion systems (*Gallardo & Ramirez, 1977; Kao & Weisz, 1977; Terry et al., 1980; Shimatsu et al., 1982; Yajima, Suda, Tomori, Sumitomo, Nakagami, Ushiyama, Denmura & Shizume, 1986; Redei, Branch, Gholami, Lin & Taylor, 1988; Turkleson, 1988*) which improve the diffusion of nutrients and metabolites to and from the tissue. However, the improved viability is obtained at the expense of the maintenance of cellular integrity and 3-D structure and the small amounts of hormone released from perifused tissue make detection a major problem. An effective compromise of the bisected organ has however been successfully exploited by a number of groups (*Maeda & Frohman, 1980; Kalra Crowley & Kalra, 1987; Tsagarakis, Holly, Rees, Besser & Grossman, 1988; Tsagarakis, Ge, Rees, Besser & Grossman, 1989*).

In the present study the hypothalami were subjected to a 60-90min pre-incubation period, to enable the tissue to recover from the trauma of excision and to minimise leakage from several neuronal surfaces. This was followed by two 15min washes, to establish an acceptable level of basal release, and a maximum of two 15-30min successive stimulations. Several groups have claimed that hypothalamic tissue, incubated in vitro, may respond consistently, in terms of peptide output, to two or more successive stimulations (*Bradbury et al., 1974, Tsagarakis et al., 1988*). Our findings suggest that this is not the case for ir-AVP release, since when challenged

repeatedly (x3) with 56mMK$^+$, there was a successive reduction in the output of AVP but not CRF-41 (Fig. 4). In view of these findings and the limited viability (<3h) of the preparation, the tissue was subjected to only one test stimulus (15-30min duration as appropriate), followed by a non-specific depolarising stimulus (56mMK$^+$) to check viability.

Obviously the success of the preparation is largely dependent on the stability of the neurohormones released into the medium and on the methods employed for their detection. Since hypothalamic tissue is rich in proteolytic enzymes (*Griffiths, Jeffcoate & Holland, 1977*) and certain peptides are readily oxidized (eg CRF-41) or adsorbed (*Gillies et al., 1982*), incubations were performed in medium supplemented with the protease inhibitor, aprotinin (0.5%), a reducing agent, ascorbic acid (10^{-3}M) and bovine serum albumin (0.25%) to minimize peptide degradation, reduction and adsorption of the released hormones, although the necessity of these additives has been disputed (*Berelowitz et al., 1978*). Radioimmunoassay methods were used for the detection of AVP, CRF-41 and, in some studies, prostaglandin E$_2$ in the incubation medium.

In the initial experiments, the AVP antibody employed (generously donated by Prof. S Lightman) was raised in rabbit against AVP$_{1-9}$. In subsequent studies another antibody (generously donated by Dr E Hillhouse), also raised in rabbit against AVP, was used to detect AVP as described by Hillhouse and Milton (*1989a*). The antiserum donated by Prof. Lightman showed negligible cross-reactivity with oxytocin (<0.1%) and desamino-D-arginine vasopressin (vasopressin V$_2$ receptor agonist) DD-AVP (<0.01%) (*Eckland, Todd & Lightman, 1988*) while the antiserum donated by Dr Hillhouse showed significant cross-reactivity with arginine vasotocin (*Negro-Vilar, Sanchez-Franco, Kwiatkowski & Samson, 1979*). Further evidence for the specificity of the first antibody employed was provided by studies on incubates of hypothalamic tissue from Brattleboro rats in which little or no AVP immunoreactivity was detected. In addition, with both antisera, serial dilutions of medium in which hypothalamic tissue from normal rats had been incubated were parallel with the dilution curves of the standard (Fig. 3i, ii). Attempts to separate AVP from the medium by FPLC were unsuccessful due to the small size of the peptide and therefore it was not possible to demonstrate a single peak of activity which co-eluted with the standard. However both assays had the advantage that they were sufficiently sensitive to detect AVP in the medium without the need for prior concentration and within assay variation was good (8.8% and 11.9%). Since samples from any one experiment were always in one assay, the inter-assay variation was not important but, nevertheless it

was 16.22% and 13.5%.

The CRF-41 antibody used (generously donated by Dr E Hillhouse) was raised in rabbit against rat CRF-41 conjugated with β-γ-globulin. The antiserum showed 100% cross reactivity with rat and human CRF-41, 35% with human CRF21-41 fragment, 9% with ovine CRF but negligible cross-reactivity with biologically inactive fragments human CRF1-20 (0.1%), human CRF6-33 (0.02%) and 0.03% with sauvagine. It also showed no cross-reactivity up to 1μg/ml with AVP, somatostatin, LHRH, TRH and angiotensin II. Furthermore, serial dilutions of medium in which hypothalamic tissue from normal rats had been incubated were parallel with the dilution curves of the standard, suggesting that the immunoreactivity of the test medium was identical to that of the assay standard (Fig. 3iii). In addition, although chromatographic separation of the medium by FPLC was again unsuccessful, HPLC resulted in a single peak of activity which co-eluted with the standard. The sensitivity of this assay was not sufficient to enable basal CRF-41 release to be detected in all experiments and thus some data from early experiments were lost. The necessity of concentrating the incubation medium was therefore recognised and when facilities became available this was achieved by freeze drying the sample and reconstituting the residue in a smaller volume. Within assay variation was good (9.2%) since the samples from any one experiment were again always contained within one assay. The inter-assay variation was not important but nevertheless it was 12.5%. Although the assays employed for AVP and CRF-41 were both precise and highly specific, the results were quoted in terms of immunoreactive AVP (ir-AVP) and CRF-41 (ir-CRF-41) release.

In a few studies, PGE_2 in the incubation medium was detected using a scintillation proximity assay. This is a novel technique applicable to radiobinding assays which eliminates the need for a separation step and addition of liquid scintillant. It therefore has the advantage of being, both quick and precise. This assay was performed on site at the Hammersmith Hospital and all the reagents necessary were kindly donated by Dr M Sullivan.

In the studies described in this thesis, hypothalamic tissue from intact and chronically (7-14 days) adrenalectomized rats was used to study the mechanisms controlling the release of ir-AVP and ir-CRF-41. Both preparations provide useful models but it is important to recognise the obvious differences between them.

In tissue from intact animals the CRF-41 released will originate largely from the

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parvocellular neurones originating in the paraventricular nucleus (PVN) and terminating in the median eminence (*Antoni, Palkovits et al., 1983; Swanson et al., 1983; Bruhn, Plotsky et al., 1984; Silverman et al., 1989*). The AVP by contrast will be derived primarily from the magnocellular neurones of the supra optic and paraventricular nuclei. Since the axons of these neurones extend primarily to extrahypothalamic sites, namely the posterior pituitary gland (*Rhodes, Morrell & Pfaff, 1981; Sawchenko, 1982*) and brain stem (*Sawchenko, 1983*) they were inevitably severed during the initial dissection procedure and the pre-incubation period was thus regarded as essential to permit the resealling of the nerve endings. Additional AVP release may also have occurred from neurones originating in the suprachiasmatic nucleus (*Robinson, 1986*) and from the parvocellular neurones of the PVN. However, with respect to the latter, this would be minimal since only small quantities of the peptide are expressed in these cells in the intact rat (*Tramu et al., 1983; Kiss et al., 1984; Sawchenko, et al., 1984a, b*).

Adrenalectomy, which eliminates the regulatory effects of the glucocorticoids on the hypothalamus, has important effects on the secretion of both peptides and not only increases CRF-41 turnover but also facilitates the synthesis and release of AVP in the parvocellular division of the PVN. Indeed the removal of the adrenal glands enhances the immunoreactive content of and the immunostaining for both peptides in the parvocellular division of the paraventricular nucleus (PVN) (*Robinson, Self, Verbalis & Brownstein, 1983; Sawchenko et al., 1984a, b; Liposits & Paull, 1985; Alonso, Szafaczyk & Assenmacher, 1986*). Moreover, AVP immunoreactivity, usually confined primarily to the magnocellular subdivision, is co-expressed with CRF-41 in a high percentage of CRF-41 cells in the parvocellular neurosecretory neurones following adrenalectomy (*Tramu et al., 1983; Kiss et al., 1984; Sawchenko et al., 1984b; Whitnall et al., 1985*). In addition, adrenalectomy also results in increased gene transcription and production of both CRF-41 and AVP pro-hormones in the parvocellular PVN perikarya (*Jingami et al., 1985; Wolfson, Manning, Davis, Arentzen & Baldino, 1985; Davis et al., 1986*). Furthermore, in vivo studies have shown that CRF-41 and AVP release into the hypophysial portal plasma is increased following surgical and pharmacological adrenalectomy (*Plotsky & Sawchenko, 1987; Fink et al., 1988*). Thus, these reports strongly suggest that a proportion of the ir-AVP released by hypothalamic tissue from adrenalectomized rats incubated in vitro may be derived from the parvocellular neurones of the PVN. This concept is supported by the work of Holmes and colleagues. Following in vivo studies using lesioning techniques these workers confirmed that in the intact rat AVP secretion occurs from two types of nerve fibre in the median eminence; a small quantity from the

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parvocellular but the majority from the magnocellular neurones (*Holmes, Antoni, Aguilera & Catt, 1986*). They also showed that the ratio of AVP:CRF-41 release from isolated median eminence tissue incubated in vitro is increased following both short and long term adrenalectomy (*Holmes, Antoni, Catt & Aguilera, 1986*). A marked augmentation by adrenalectomy of AVP release from the median eminence incubated in vitro has also been demonstrated by other workers (*Knepel, Nutto, Meyer & Vlaskovska, 1984; Verbalis, Baldwin, Ronnekleiv & Robinson, 1986*). Furthermore, in vitro studies have shown that increasing the ratio of AVP:CRF-41 without changing the concentration of CRF-41, stimulates ACTH release when both peptides are added to rat anterior pituitary tissue (*Antoni, Holmes, Makara, Kárteszi & László, 1984; Baertschi & Friedli, 1985; Buckingham, 1985*). From these results, Holmes et al. (1986) suggested that AVP was the major stimulus of ACTH release in adrenalectomized rats and that unlike in the intact rat, the AVP released originated from parvocellular neurones where the peptide was co-localised with CRF since in the rats with lesions of the PVN, adrenalectomy failed to enhance the release of AVP by median eminence tissue (*Knepel et al., 1984*) and there was no detectable release of CRF-41.

Interestingly in the present work, if the ratio of PLA_2-stimulated AVP:CRF-41 release is considered (Table 2), then in the majority of experiments it is increased in tissue from adrenalectomized rats, which is in accord with the published literature. However, the anticipated differences between tissue from intact and adrenalectomized rats in absolute quantities of the peptides released following stimulation with various secretagogues, were not always apparent and in some cases peptide release was higher in tissue taken from intact rats. The reasons for this discrepancy are not clear. However since in the present study the experiments on tissue from intact and adrenalectomized animals were invariably performed on different days such comparisons are not necessarily valid as the absolute peptide release may have been influenced by a variety of external factors. For example, factors such as temperature, time of dissection, or the level of stress of the rats at autopsy may have contributed to the differences observed. In fact, since the magnitude of basal and stimulated peptide release varied considerably between experiments comparison of absolute hormone secretion between experiments performed on different days, was avoided.

ii) The effects of Phospholipase A_2 (PLA$_2$), Melittin and the PLA$_2$ inhibitor, quinacrine, on the secretion of ir-AVP and ir-CRF-41 by isolated hypothalamic tissue _in vitro_

The results presented in this thesis show clearly that both phospholipase A_2 (PLA$_2$) and the PLA$_2$ activator, melittin, readily initiate concentration dependent increases in the release of CRF-41 and AVP from hypothalami from intact and chronically adrenalectomized rats. Since both secretagogues are reported to exert harsh effects on cell membranes and, in certain instances to produce cell lysis (_Habermann, 1972_), it is important to consider the specificity of the observed responses. Phospholipase A_2, which belongs to a family of enzymes responsible for the catabolism of phospholipids (_Blackwell & Flower, 1983; Chang, Musser & McGregor, 1987_), cleaves the Sn-2-acyl bond of phospholipids producing equimolar amounts of lysophospholipids and free fatty acids; it is thought to play a key role in the production of various eicosanoids (_Flower & Blackwell, 1976_). Phospholipase A_2 enzymes are ubiquitously distributed throughout eukaryolic cells as well as in bacteria and protozoa. Over forty PLA$_2$ enzymes, derived principally from snake venoms and mammalian pancreas, have been sequenced and structurally defined (_Verheij, Slotboom & de Haas, 1981_). Each contains a high degree of disulphide cross-linking and is extremely stable to heat and acid treatment (_Elsbach, Weiss, Franson, Becherdite-Quagliata, Schneider & Harris, 1979_).

In situ PLA$_2$ enzymes exist as membrane bound or soluble forms. The former are strongly bound to plasma, golgi or mitochondial membranes and require the presence of calcium ions for optimal catalytic activity; most are optimally active at neutral to alkaline pH. The latter, which may form monomers or dimers of varying stability in solution, have been isolated from lysosomes and usually have acidic pH optima, with no requirement for calcium ions (_Blackwell & Flower, 1983_). However soluble PLA$_2$ exhibiting a neutral pH optimum and Ca^{2+} dependence has been demonstrated during activation of phagocytic cells (_Elsbach et al., 1979; Wightman, Dahlgren, Davies & Bonney, 1981_). In addition, PLA$_2$ can exist in either an activated or inactivated state, for example stimulated neutrophils release active PLA$_2$ (_Franson, Dobrow, Weiss, Elsbach & Weglichi, 1978; Vadas, Wasi, Movat & Hay, 1981; Lanni & Becker, 1983_) whereas pancreatic PLA$_2$ is secreted as the inactive zymogen and requires enzymatic cleavage by proteolytic enzymes for activation.(_De Haas, Postema, Nieuwenhuiizen & Van Deenen, 1969_). The PLA$_2$ enzyme used in the majority of the present studies was isolated from a snake venom (_Naja Naja-_ Sigma

Chemical Co.) which is highly active over a wide pH range but optimal at neutral pH and is Ca^{2+} dependent. In addition, recombinant human PLA_2 (Biogen Inc., USA) was also used in one study.

Phospholipase A_2 activity is facilitated by a variety of peptides derived from the venoms of various bees, wasps and hornets (*Mollay & Kreil, 1974; Argiolas & Pisano, 1983*). An example of one such peptide is melittin, isolated from bee venom (*Habermann, 1972*) and demonstrating a remarkable synergism with PLA_2 in its action on cell membranes of lecithin liposomes (*Mollay & Kreil, 1974*). It is a hydrophobic 26 amino acid peptide which is strongly surface active and cytolytic (*Habermann, 1972*). It produces a number of biological effects only some of which may be attributable to its actions on PLA_2. For example it damages enzyme systems bound to cell membranes, diminishes electron transport in mitochondria and uncouples oxidative-phosphorylation (*Habermann, 1972*). Melittin also causes depolarisation and shortening of skeletal and cardiac muscle, constriction or dilation of blood vessels (depending on dose or organ) and increases vascular permeability on local application (*Habermann, 1972*).

For a number of reasons it seems unlikely that the responses to both PLA_2 and melittin reported here were due solely to cell lysis. Firstly the increases in both ir-AVP and ir-CRF-41 release were dose related. Secondly, the amount of peptide release was relatively small compared with that released by a non-specific stimulus. Thirdly the subsequent responsiveness of the tissue to K^+ was intact. Fourthly the effects of the venoms were mimicked by recombinant human PLA_2. Finally, and perhaps most importantly, in almost all the cases, PLA_2 and melittin-induced peptide release were readily blocked by the presence of the PLA_2 inhibitor quinacrine in the incubation medium (Fig. 9-11).

Quinacrine appears to produce its effects by affecting the substrate-enzyme interface rather than by directly inhibiting enzyme activity. Phospholipase A_2 activity is greatly influenced by the physicochemical state of the phospholipid substrate and therefore any agent that disturbs the lipid-water interface would diminish enzyme activity and could physically prevent PLA_2 from hydrolysing the phospholipid substrate (*Bonsen, de Haas, Pieterson & Van Deenen, 1972*). Quinacrine, a cationic amphiphilic compound, has the ability to form complexes with the phospholipid substrate and thereby prevent enzymatic attack (*Lüllmann & Wehling, 1979*). It would, therefore, be expected to inhibit PLA_2 and melittin stimulated peptide release in all cases. Its inability to inhibit PLA_2-stimulated ir-CRF-41 release from hypothalamic tissue from

intact rats (Fig. 11i) may be due to an inadequate period of exposure of the inhibitor to the phospholipid substrate, or possibly, due to an insufficient concentration of the inhibitor to block all the PLA_2 binding sites.

Interestingly, both phospholipase A_2 and melittin have been shown to activate the HPA system in vivo in experimental animals (*Dunn & Killion, 1988*) and the rise in cortisol secretion evident in man in conditions of septic shock is associated with a pronounced elevation in circulating PLA_2 (*Vadas, Pruzanski, Stefanski, Sternby, Mustard, Bohnen, Fraser, Farewell & Bombardier, 1988*). Of course such responses may reflect non-specific stressful effects of the drugs (eg hypotension). However, in vitro studies have revealed that both melittin and PLA_2 (derived from pancreas, snake or bee venoms) exert direct effects on the pituitary gland and stimulate the secretion of ACTH apparently by increasing the formation of pro-secretory eicosanoids rather than by virtue of non-specific actions (*Abou-Samra et al., 1986c; Knepel & Meyen 1986; Cowell, Flower & Buckingham, 1991*). The present study, which illustrates the ability of PLA_2 and melittin to facilitate the release of the major corticotrophin releasing factors, CRF-41 and AVP, suggest that an action at the hypothalamic level may also be important in this respect.

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iii) The influence of arachidonic acid and its metabolites on the release of ir-AVP and ir-CRF-41 from isolated hypothalamic tissue in vitro.

Phospholipase A_2 (PLA_2) acts on membrane bound phospholipids to liberate arachidonic acid (*Axelrod et al., 1988*) and, if the membrane phospholipid is an alkylacylglycerophosphocholine, lyso-PAF from which platelet activating factor (PAF) is generated by the action of acetyl-Co-A: 1-alkyl-2 lyso-sn-glycero-3-phosphorylcholine acetyltransferase (*Braquet et al., 1987*). It is therefore possible that the increases in ir-AVP and ir-CRF-41 release elicited by PLA2 and melittin are effected by either arachidonic acid, products of its metabolism or PAF.

The possibility that PAF may be important in this respect was not addressed in this study. Nevertheless its involvement cannot be excluded as there is considerable evidence to suggest that PAF is a potential mediator in the central nervous system. Endogenous PAF has been identified in bovine cerebral tissue (*Tokumura, Kamiyasu, Takauchi & Tsukatani, 1987*) and relatively high levels of the enzyme involved in the synthesis and metabolism of PAF have been found in rat brain (*Blank, Lee, Fitzgerald & Synder, 1981; Francescangeli & Gorraci, 1989*). Furthermore, specific PAF binding sites have been localised in gerbil and rat brains (*Domingo, Spinnewyn, Chabrier & Braquet, 1988; Junier, Tiberghien, Rougeot, Fafeur & Dray, 1988*) and PAF receptor sites have been characterized in the rat hypothalamus (*Junier et al., 1988*). In vitro PAF inhibits the release of luteinizing hormone releasing hormone (LHRH) and somatostatin (*Junier et al., 1988*) stimulates the release of β-endorphin (*Rougeot, Junier, Everaere, Braquet & Dray, 1988*) but has no effect on the spontaneous or K^+-induced release of α-MSH from perifused hypothalamic slices (*Blasquez, Jégou, Delarue, Delbende, Trauchand Bunel, Braquet & Vaudry, 1990*).

The limited data available on its actions on the HPA axis are conflicting. Thus, although PAF has been shown to elicit the release of CRF-41 in vitro (*Rougeot et al., 1988*), in vivo it reduces the concentrations of ACTH in the adenohypophysis and of corticosterone in the plasma and adrenal gland, effects which are reversed by concomitant administration of the selective PAF antagonist BN 52021 (*Blasquez et al., 1990*). It seems unlikely that these effects are the consequence of powerful actions of PAF at the pituitary level for, in vitro, neither PAF (*Blasquez et al., 1990; Cowell et al., 1991*) nor the PAF antagonist (*Cowell et al., 1991*) influence the resting or neurochemically evoked secretion of ACTH by rat pituitary tissue. Despite the apparent increases in CRF-41 release observed in vitro by Rougeot et al. (*1988*), it is still possible that the responses to PAF observed in vivo may reflect actions at the

hypothalamic level involving inhibition of the release of AVP or other ACTH regulating hypothalamic hormones. Alternatively they may be due to extra-hypothalamic, possibly peripheral actions of PAF. Obviously this area merits further investigation.

Several workers have suggested that eicosanoids may contribute to the complex mechanisms regulating the secretion of corticotrophin releasing factors by the hypothalamus (*Cowell & Buckingham, 1989*). The present study, in which the effects of various inhibitors of arachidonic acid metabolism on the PLA_2-induced release of ir-AVP and ir-CRF-41 from the hypothalamus were examined, are largely in accord with this view. The results obtained with arachidonic acid itself were however less convincing for hypothalamic tissue, maintained under normal incubation conditions, failed to release ir-AVP when challenged with arachidonic acid ($10^{-4}M$) (Fig 15ii). This may be explained by the high affinity of the fatty acid for the bovine serum albumin (BSA, 0.25% w/v) present in the medium. Certainly in studies on pituitary tissue, removal of albumin from the medium resulted in a ten fold decrease in the ED50 of arachidonic acid on luteinizing hormone release (*Naor, Kiesel, Vanderhoek & Catt, 1985*). Similarly, the sensitivity of the corticotrophs to arachidonic acid is inversely related to the albumin content of the medium (*Naor et al., 1985*). A preliminary study performed in the absence of BSA (Fig 15i) produced variable data (n=3) which, although not significant (P=0.200) suggested that arachidonic acid may facilitate the release of ir-AVP. More extensive studies involving examination of the effects of graded doses of arachidonic acid on the secretion of the neuropeptide by larger groups of hypothalami may have yielded more positive data. However, this line of investigation was not pursued firstly because BSA is generally regarded as being necessary to reduce variation attributable to neuropeptides 'sticking' to the incubation tubes and secondly because higher doses of arachidonic acid may have effectively lysed the cells. Subsequent experiments therefore involved examination of the effects of various inhibitors of arachidonic acid metabolism on PLA_2-induced ir-AVP and ir-CRF-41 release.

Like the study with arachidonic acid, these experiments were not without problems as several of the enzyme inhibitors employed (namely ibuprofen, indomethacin, BW 755C and ICI 207968) were not water soluble and were therefore dissolved initially in a small volume of alcohol (final concentration <0.2%). In all cases appropriate ethanol vehicle controls were included and in the majority of experiments the vehicle was without effect. However, on two occasions elevations in the basal release of ir-AVP (Fig. 16i) and ir-CRF-41 (Fig. 24) were apparent. The effects on ir-CRF-41 release were not surprising since acute ethanol exposure has been shown to activate the HPA

axis resulting in the release of CRF-41 and ACTH (*Redei et al., 1988a*) and to stimulate the release of various other adenohypophysial hormones including prolactin (*Seilicovich, Duvilanshi, Gimeno, Franchi, del-Carmen-Diaz & Lasaga, 1988*). Furthermore, according to Redei et al. (*1988*), ir-CRF-41 release from superfused hypothalami in vitro is increased by acute exposure to ethanol. However, the potentiation by ethanol of spontaneous AVP release was surprising since ethanol is well known to inhibit the release of arginine vasopressin (*Rang & Dale, 1987a*). The actions of ethanol on AVP secretion at the hypothalamic level may simply have reflected a non-specific/toxic effect of the ethanol on the cell membrane. This is highlighted by the finding that ethanol also potentiated PLA_2-induced ir-CRF-41 in one experiment (Fig. 21ii). However, overall the effects of the alcohol vehicle on PLA_2-induced peptide release were variable and in other experiments reductions in the ir-AVP (Fig. 19) and ir-CRF-41 (Figs. 22ii & 23ii) responses to the enzyme were apparent. One explanation for these effects is that PLA_2 activity is greatly influenced by the physicochemical state of the phospholipid substrate such that any further perturbation of the lipid-water interface diminishes enzyme activity (*Bonsen et al., 1972*). Thus, since n-alkanols such as ethanol interfere with the substrate-enzyme interface (*Chang et al., 1987*), the alcohol may have inhibited PLA_2 activity by preventing the enzymatic attack of the phospholipid substrate.

In the present study the effects of various inhibitors of the three enzyme systems of arachidonic acid metabolism on ir-AVP and ir-CRF-41 release from hypothalamic tissue were examined.

Products of the cyclo-oxygenase metabolism (ie prostanoids) have been strongly implicated in the regulation of the secretion of corticotrophin releasing factors by the hypothalamus. Indeed, stimulatory effects of exogenous prostaglandins on HPA activity were first reported as early as 1969 (*DeWied et al., 1969*). Since then several workers have demonstrated potentiation of corticotrophin release following systemic administration of prostaglandins (*Hedge & Hanson, 1972; Coudert & Faiman, 1973*). These effects appear to be exerted at the hypothalamic level since injection of several prostaglandins, including PGE_1, into the median eminence but not the lateral hypothalamus, the pituitary gland or the tail vein of the rat initiates ACTH secretion (*Hedge & Hanson, 1972; Hedge, 1976*). However PGE_1 fails to elicit ACTH release in animals in which hypothalamo-pituitary communication has been impaired by surgical lesioning or treatment with morphine (*DeWied et al., 1969; Peng, Six & Munson, 1970*). Furthermore, implantation of the cyclo-oxygenase inhibitor indomethacin, into the hypothalamus but not the pituitary gland attenuates stress-

induced HPA activity (*Hedge & Thompson, 1975; Thompson & Hedge, 1978*). The present study, in which the ability of ibuprofen, indomethacin and BW 755C to attenuate PLA_2-induced ir-CRF-41 and ir-AVP release was examined, is in accord with this concept. The data also suggest that the actions of the prostanoids are mediated selectively by changes in the secretion of ir-CRF-41 and not ir-AVP since these inhibitors effectively blocked ir-CRF-41 without affecting ir-AVP release. Several other lines of evidence support this view. Prostaglandins have been localised in a wide variety of tissues including the hypothalamus and the pituitary gland (*Wolfe, 1982; Fafeur, Gouin & Dray, 1985*). More recently the capacity of the medial basal hypothalamus to produce PGE_2, $PGF_{2\alpha}$, TxB_2 and 6-keto $PGF_{1\alpha}$ from [^{14}C] arachidonate in the rat has been reported (*Franchi, Gimeno, Cardinali & Vacas, 1987*) as too has the presence of PGD_2 synthetase and PGD_2 in the brain (*Ujikara, Urade, Eguchi, Hayashi, Ikai & Hayaishi, 1988*). In vitro studies have demonstrated that $PGF_{2\alpha}$ stimulates CRF-41 secretion (*Bernardini, Chiarenza, Calogero, Gold & Chrousos, 1989*). Furthermore, the HPA response to interleukin 1 (IL-1), which according to several groups are effected primarily by an increased release of CRF-41 (*Uehara, Gottschall, Dahl & Arimura, 1987b; Berkenbosch, deGoeij, Del Rey & Besedovsky, 1989*), are attenuated by indomethacin (*Katsuura et al., 1988*).

From the present study it was not possible to identify either the cyclo-oxygenase metabolite(s) or the prostanoid receptor responsible for effecting the response to PLA_2. Ojeda, Naor & McCann (*1978*) strongly favoured a role for PGE's (PGE_1/PGE_2) on the grounds that the PGE content of the median eminence is higher than that of the medial basal hypothalamus or the anterior pituitary gland. On the other hand, reports that $PGF_{2\alpha}$ is an effective CRF-41 secretagogue together with the observation that the effects of $PGF_{2\alpha}$ are inhibited by a thromboxane A_2 receptor antagonist suggest that the response may be effected not only by prostaglandins but also by thromboxanes and that it may be mediated by thromboxane A_2 receptors (*Bernardini et al., 1989*). Interestingly, not all cyclo-oxygenase products appear to exert a stimulatory effect on CRF-41 release and, according to Bernardini and colleagues (*1989*) PGE_2 is ineffective in this respect in vitro. It is of course possible that the failure of these workers to demonstrate a response to PGE_2 reflects the poor state of the hypothalamic tissue which was maintained in vitro for 24h prior to stimulation. However PGE_2 and $PGF_{2\alpha}$ have been shown to act at different receptors to produce opposing effects in other tissues (*Rang & Dale, 1987b*). Furthermore, in the one experiment it was tested (data not shown), PGE_2 was also ineffective in our hypothalamic preparation. Moreover, direct measurements of PGE_2 indicated that a

rise in the hypothalamic content of the prostaglandin was not associated with increased CRF-41 release (Fig. 36   ii). Other reports also indicate that the role of prostanoids may be more complex. For example, according to Redei et al. (*1988b*) exogenous arachidonic acid increases CRF pulse amplitude from superfused hypothalamic blocks in vitro when indomethacin is included in the medium (*Redei, Branch, Lin & Taylor, 1988b*). Since indomethacin also inhibits 5-lipoxygenase enzymes (*Siegel, McConnell, Porter & Cuatrecasas, 1980; Siegel, McConnell, Porter, Selph, Truax, Vinegar & Cuatrecasas, 1980*), it is possible that this potentiation reflects a reduction in the generation of leukotrienes rather than modulation of prostanoid synthesis. Certainly the hypothalamus has the capacity to generate a number of arachidonic acid metabolites other than prostanoids. These include leukotriene C_4 (LTC_4) (*Lindgren, Hokfelt, Dahlen, Patrono & Samuelsson, 1984*), 12-HETE and to a lesser extent 5-HETE & 15-HETE (*Gerozissis, Vulliez, Saavedra, Murphy & Dray, 1985*). High activities of the enzymes 5-lipoxygenase and LTA_4 hydrolase involved in LTB_4 synthesis have been detected in brain tissues including the hypothalamus (*Shimizu, Takusagawa, Izumi, Ohishi & Seyama, 1987*). In addition cytochrome P450 enzymes have also been localised to a small extent in the brain (*Paul, Axelrod & Diliberto, 1977*). Furthermore, as might be expected from their distribution, lipoxygenase products have been implicated in a number of neuroendocrine effects. Thus 12-HETE readily induces the secretion of LHRH in vitro while 5-HETE is only weakly active in this respect (*Gerozissis et al., 1985*). In other studies LTC_4 is a potent LHRH secretagogue (*Gerozissis, Saadi & Dray, 1987*). Leukotrienes generated by the 5-lipoxygenase pathway have also been shown to stimulate gonadotrophin release (*Hulting, Lindgren, Hokfelt, Eneroth, Werner, Patrono & Samuelsson, 1985; Kiesel, Przylipiak, Rabe & Runnebaum, 1987*), while 15-lipoxygenase products are potent stimulators of prolactin secretion (*Rabier, Chavis, Crastes-de-Paulet & Damon, 1988*) in vitro. In addition, 5,6-epoxyeicosatrienoic acid (5,6-EET) has also been shown to elicit LHRH release in vitro (*Capdevila, Chacos, Falck, Manna, Negro-Vilar & Ojeda, 1983*) and EETs have been implicated in the regulation of somatostatin (*Capdevila et al., 1983; Junier et al., 1988*) and corticotrophin (*Cowell et al., 1991*) release. Furthermore epoxy derivatives of arachidonic acid are potent stimulators of prolactin secretion in vitro (*Cashman, Hanks & Weiner, 1987*).

The results presented here, provide little evidence to suggest that products of the 5-lipoxygenase pathway facilitate CRF-41 release. BW 755C is non-selective and thus, in view of the earlier discussion it seems likely that its effects are attributable to blockade of the cyclo-oxygenase rather than the lipoxygenase enzymes. Moreover, ICI

207968, a selective 5-lipoxygenase inhibitor, failed to inhibit PLA_2-stimulated peptide release. On the contrary, the inhibitor appeared to cause a small, although not significant, potentiation of stimulated ir-CRF-41 secretion. This finding contrasts with observations of Bernardini et al. (*1989*) that the 5-lipoxygenase products LTB_4 and LTC_4 but not LTD_4 stimulate the secretion of CRF-41 in vitro. However, as discussed previously there may be problems with their preparation due to deterioration of the hypothalamic tissue. Indeed electron microscopy studies of the hypothalamic explants which have been maintained in vitro for beyond 24h have revealed degrees of demyelination of the nerve fibres (*Calogero, Bernardini, Margioris, Bragdy, Gallucci, Munson, Tamarkin, Tomai, Brady, Gold & Chrousos, 1989*). Further studies involving the use of graded concentrations of selective 5-lipoxygenase inhibitors and leukotriene receptor agonists and antagonists are necessary to unravel this controversy.

The possibility that products of 12- or 15-lipoxygenase metabolism or the lipoxins, which are produced as a result of a unique interaction of the 15- and 5-lipoxygenase enzymes (*Serhan, Hamberg & Samuelsson 1984; Serhan, Hamberg, Samuelsson, Morris & Wishka, 1986*) was not addressed in this study and, although 12-HETE has been implicated in the control of other hypothalamic hormones (*Gerozissis et al., 1987*) to our knowledge the potential roles of these eicosanoids in the control of CRF-41/AVP secretion has not been investigated by other workers.

A role for the epoxygenase products in eliciting CRF-41 release seems unlikely since blockade of the cytochrome P450 epoxygenase pathway potentiated PLA_2-induced release of the neuropeptide suggesting that epoxygenase products may be inhibitory and/or the consequent shift in arachidonic acid metabolism favours the generation of pro-secretory substances i.e. the prostanoids.

As with ir-CRF-41 release it is difficult to determine which arachidonic acid metabolites are responsible for effecting the PLA_2-induced increase in ir-AVP release. However, from the results presented here, it seems unlikely that the responses observed in tissue from either intact or adrenalectomized rats were effected by products of the cyclo-oxygenase pathway (prostaglandins, prostacyclin or thromboxanes) since both the cyclo-oxygenase inhibitors, ibuprofen and indomethacin and the dual cyclo-oxygenase/lipoxygenase inhibitor BW 755C failed to inhibit PLA_2-stimulated peptide release. On the contrary indomethacin appeared to potentiate ir-AVP release from tissue from both intact and adrenalectomized rats. Thus, these results are in complete contrast to those for PLA_2-stimulated ir-CRF-41 release and provide firm evidence for the differential control of ir-CRF-41 and AVP release by prostanoids. The response to

indomethacin may be taken to suggest either that prostanoids are inhibitory to AVP release or that the consequent shift in arachidonic acid metabolism favours the formation of more potent secretagogues. In this context it is interesting to note that inhibitory roles have been ascribed to cyclo-oxygenase products in other areas of the endocrine system eg. in the control of insulin (*Fujimoto & Metz, 1987*) and ACTH (*Hedge, 1976; 1977*) secretion. Furthermore PGE_2 inhibits 5-HT release by brain tissue in vitro (*Schlicker, Fink & Göthert, 1987*). However, there is as yet little evidence to suggest an inhibitory role of prostanoids in the control of AVP release. On the contrary, the limited evidence available suggests that prostanoids facilitate the release of AVP from magnocellular neurones since endotoxin-induced increases in circulating AVP levels in humans are prevented by pre-treatment with ibuprofen (*Michie, Majzoub, O'Dwyer, Revhaug & Wilmore, 1990*). Furthermore, i.c.v. injection of PGE_2 but not PGD_2 has been shown to increase plasma levels of AVP (*Hashimoto, Noto & Nahajima, 1989*) suggesting their actions are at the hypothalamic level.

The present study has shed little light on the roles, if any, of products of lipoxygenase and epoxygenase enzymes in the control of AVP release. As mentioned above, the dual cyclo-oxygenase/lipoxygenase blocker (BW 755C) was without effect. The results with the 5-lipoxygenase inhibitor were difficult to interpret for although the apparent inhibition of the AVP response to PLA_2 stimulation in tissue from adrenalectomized rats may be taken to indicate that products of the 5-lipoxygenase pathway are important in this context, the pronounced drug-induced rise in basal peptide release makes the data inconclusive. Similarly, the data from the studies involving the epoxygenase inhibitor SKF 525A were not clear cut. Thus, although in tissue from intact rats, ir-AVP release in response to PLA_2 was reduced by the inhibitor, this was not true for tissue from adrenalectomized animals where the inhibitor was consistently without effect. Since adrenalectomy increases the formation of AVP in the parvocellular neurones of the PVN (see earlier discussion), this raises the possibility that epoxides may initiate the release of vasopressin from magnocellular but not from parvocellular neurones.

The apparent inability of inhibitors of 5-lipoxygenase, epoxygenase and cyclo-oxygenase enzymes to block completely the AVP response to PLA_2 raises the possibility that metabolites generated by 12 or 15-lipoxygenase enzymes or the lipoxins may be important in this respect; clearly this is worthy of further investigation. In addition it is of course possible that arachidonic acid itself may be

partly responsible for effecting the increase in ir-AVP release observed following stimulation with PLA_2 since in other cell types it has been shown to activate protein kinase C (*Murakami & Routtenberg, 1985*), elevate cytosolic Ca^{2+} (*Axelrod et al., 1988*) and facilitate the fusion of secretory vesicles and thus exocytosis (*Drust & Creutz, 1988*).

Thus, the results presented here consistently indicate that PLA_2-induced ir-CRF-41 release is effected by one or more products of the cyclo-oxygenase pathway. The eicosanoids involved in the mediation of PLA_2-stimulated ir-AVP release remain to be determined. The present data suggests that cyclo-oxygenase products are not important in this respect but raise the possibility that lipoxygenase and/or epoxygenase products may be involved as too may arachidonic acid itself.

iv) The effects of corticosteroids in vivo and in vitro on stimulus induced peptide release from isolated hypothalamic tissue.

The regulatory effects of the corticosteroids on HPA activity are mediated by at least three distinct negative feedback mechanisms which are effective over different time courses. These include (i) fast or rate sensitive feedback which occurs within seconds to minutes of corticosterone administration, (ii) intermediate feedback which is effective within 1 or 2h of steroid administration and maximally effective within 2-4h and (iii) slow delayed feedback, the inhibitory effects of which develop over a period of hours to days in response to repeated or continuous administration of corticosterone (*Kellerwood & Dallman, 1984; Jones & Gillham, 1988*).

In the experiments described in this thesis both 'intermediate' and 'slow delayed' inhibitory actions of the steroids were examined. For the former dexamethasone was included in the incubation medium for 1.5h prior to stimulation of the hypothalamic tissue with PLA_2 or melittin while for the latter animals were pre-treated with dexamethasone in the drinking water for 6 or 14 days prior to autopsy. A further group in which the steroid was included in the drinking water for 24h prior to autopsy was also included. In theory this treatment should be sufficient to permit the development of a delayed feedback response. However, since rats drink primarily at night and some delay will ensue between ingestion and absorption, it is unlikely that elevated levels of dexamethasone would be achieved for a sufficently long period to induce a delayed effect. This is highlighted by the fact that the effects of the 24h steroid treatment on PLA_2-induced ir-AVP release were indistinguishable from those obtained when dexamethasone was included in vitro in the incubation medium but differed in several respects from those of the 6 and 14 day treatments.

The steroid used in these studies was dexamethasone. This is a synthetic analogue of the naturally occuring corticosteroid which has a methyl group attached to C_{16} that decreases its mineralocorticoid activity without affecting its glucocorticoid activity. The preparation employed for both the in vitro and the in vivo studies was dexamethasone sodium phosphate, a soluble, orally active salt which is hydrolysed to the free base within 3 min of its injection in vivo (*Miyabo, Nakamura, Kuwazima & Kishida, 1981*) and therefore should gain easyaccess to its site of action. Administration of dexamethasone in the drinking water provides a non-stressful method of maintaining the plasma concentration of the steroid at a physiological level that avoids the transient

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phase of high, non-physiological concentration which occurs immediately following an injection (*Buckingham & Hodges, 1975*). Although dexamethasone is not a natural steroid, it has been used extensively in studies of glucocorticoid feedback (*Birnberg, Lissitzky, Hinman & Herbert, 1983*) and it binds with high affinity to glucocorticoid receptors (type II) in the brain and pituitary gland which are thought to be involved in the feedback control of ACTH secretion (*Reul, Van den Bosch & de Kloet, 1987*). It should therefore exert negative feedback effects similar to corticosterone, the naturally occurring glucocorticoid in the rat.

The precise mechanisms whereby steroids exert their feedback effects on the HPA axis are unknown. However, intermediate effects appear to involve de novo protein synthesis (*Arimura et al., 1969*) while slow delayed feedback effects involve suppression of POMC, AVP and CRF-41 genes (*Jingami et al., 1985*). Since the well known anti-inflammatory actions of dexamethasone have been partially attributed to its capacity to induce the synthesis of a calcium dependent protein 'lipocortin' (de novo protein synthesis) which inhibits the activity of the enzyme PLA_2 (*Fradin et al., 1988*) these studies addressed the possibility that the regulatory actions of steroids on the hypothalamus may be attributed, in part, to blockade of this enzyme.

The in vitro model used in the present study to examine the effects of dexamethasone on PLA_2 and melittin stimulated ir-AVP and ir-CRF-41 release has been used previously to study the three phases of steroid action (*Bradbury et al., 1974; Jones & Hillhouse, 1976; Buckingham & Hodges, 1977b; Jones et al., 1977; Buckingham, 1979*). In these early studies the experimenters were unaware of the existence of multiple CRF's and examined only the effect of steroids on total bioactive CRF release. Nevertheless, they were able to determine (i) that the rapid effects involve the inhibition of release and not synthesis of the releasing factor while the delayed effects involve the inhibition of synthesis and release, (ii) that both intermediate and delayed effects involve interaction with specific type II receptors and (iii) the intermediate effect involves de novo protein synthesis. Unlike these earlier studies, the present study examined the intermediate and delayed effects of steroids on ir-AVP and ir-CRF-41 release and demonstrated clearly that dexamethasone produces differential effects not only on PLA_2 and melittin induced ir-AVP and ir-CRF-41 release but also on peptide release from hypothalamic tissue from intact and adrenalectomized rats. The data thus provide evidence for differential steroid control of AVP and CRF-41 release. In addition, they indicate strongly that the feedback effects of dexamethasone on the hypothalamus can not be attributed entirely to the induction of a PLA_2 inhibitor for in

this event it should block the secretory responses to exogenous PLA_2 in all conditions studied. Nevertheless, the finding that the ir-AVP and ir-CRF-41 responses to cytokine-stimulation (which appear to be mediated by activation of PLA_2, *Katsuura et al., 1988*) were inhibited by dexamethasone coupled with the observation that the steroids block certain aspects of the hypothalamic response to exogenous PLA_2 tend to support the concept that induction of a PLA_2 inhibitor may contribute to the feedback effects of the steroids.

With respect to ir-AVP release, the results consistently show that stimulus-induced peptide release from hypothalamic tissue from adrenalectomized, but not intact, animals is readily inhibited by inclusion of dexamethasone either in the drinking water for 24h prior to autopsy or in the incubation medium. This differential effect is interesting and is probably associated with the increased appearence of AVP in the parvocellular neurones of the paraventricular nucleus in adrenalectomized rats (*Tramu et al., 1983; Kiss et al., 1984; Sawchenko et al., 1984b; Whitnall et al., 1985*). As discussed previously, in tissue from adrenalectomized rats ir-AVP released in response to the various secretagogues will have originated not only from magnocellular but also from parvocellular neurosecretory neurones whereas in intact tissue the majority of ir-AVP released is probably derived from magnocellular neurones. As only the parvocellular AVP neurones appear to be susceptible to glucocorticoid feedback (*Sawchenko, 1987*), the selective effect of dexamethasone on peptide release from adrenalectomized tissue may be expected. The apparent failure of dexamethasone to inhibit stimulus-induced ir-AVP release from hypothalamic tissue from intact animals may simply reflect the fact that so little AVP is released in vitro from the parvocellular neurones in the PVN that an inhibitory action could not be detected.

The effects of dexamethasone on the release of CRF-41 were less consistent. Thus PLA_2-induced CRF-41 release from hypothalamic tissue from intact rats (Fig.25i) but not sham-operated control animals (Fig. 27ii) was significantly reduced by dexamethasone in vitro. This is surprising since both tissues originated from animals in which the adrenal glands had been left intact and therefore the steroid may have been expected to have had similar effects on peptide release from both tissues. The inconsistent results were further highlighted by the fact that PLA_2- but not melittin-induced ir-CRF-41 release from tissue from intact animals was inhibited by dexamethasone whereas in the tissue from adrenalectomized rats the opposite was true. One possible explanation for these inconsistent data is that intermediate feedback actions are exerted primarily on the release of AVP and not CRF-41. Similar

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conclusions were drawn by Fink and colleagues (*1988*) who observed that an intermediate feedback signal in adrenalectomized rats affected AVP but not CRF-41 release into the portal blood and proposed that the intermediate feedback effects of glucocorticoids were mediated by a reduction in AVP but not CRF-41 secretion into the portal blood coupled with a marked reduction in pituitary responsiveness to CRF-41 (*Fink et al., 1988*). However, in contrast Plotsky et al. (*1986*) demonstrated that in animals in which glucocorticoid synthesis was blocked (pharmacological adrenalectomy), a 2h corticosterone infusion reduced both basal and stress-induced (nitroprusside-induced hypotension) CRF-41 secretion but failed to alter AVP or oxytocin output (*Plotsky, Otto & Sapolsky, 1986*) suggesting that intermediate feedback affects CRF-41 and not AVP secretion. Interestingly the rats employed by Plotsky et al. (*1986*) were anaesthetized with urethane which has been shown to greatly increase ACTH secretion (*Fink et al., 1988*). Since the CRF-41 concentrations in the portal blood of urethane anaesthetized intact rats are 3-4 times higher than those of corresponding sodium pentobarbitone anaesthetized animals (*Sherward & Fink, 1991*) it is possible that the relative tone of CRF-41 and AVP to the corticotrophs may vary according to the anaesthetic used (*Sherward & Fink, 1991*), a phenomenon which obviously complicates the interpretation of the data. It is also possible that the relative effects of steroid feedback on AVP and CRF-41 release are stimulus specific since the roles of CRF-41 and AVP in controlling ACTH release appear to vary with different stresses. For example there is a decrease in the secretion of AVP but not CRF-41 into the portal blood together with a decrease in pituitary responsiveness to CRF-41 following hypothermia (*Gibbs, 1985b*). In contrast haemorrhage stress results in an increased secretion of CRF-41 which can be blocked by dexamethasone administration (*Plotsky & Vale, 1984*). The present results also indicate that the intermediate mechanism of steroid feedback on the HPA axis is stimulus specific since although dexamethasone failed to inhibit PLA_2-induced ir-CRF-41 release from hypothalamic tissue from adrenalectomized rats, the steroid readily inhibited IL-6 and IL-8 stimulated peptide release from equivalent tissues (see discussion later).

Delayed feedback, which is characterized by a sensitivity to the plasma concentration and the total dose of steroid exposure (*Kellerwood & Dallman, 1984*) results in a decrease in pituitary ACTH content, the expression of POMC and an inhibition of ACTH secretion (*Kellerwood & Dallman, 1984*). Immunohistochemical studies have shown that its effects may be exerted via modifications of both AVP and CRF-41 release as well as by direct actions on the pituitary gland. Indeed both AVP and CRF-41 immunostaining in the PVN are increased following adrenalectomy or

hypophysectomy (*Stillman, Recht, Seif, Robinson & Zimmerman, 1977; Sawchenko et al., 1984b; Sawchenko, 1987*). Furthermore, local implantation of dexamethasone in the vicinity of the PVN prevents the increased immunostaining of these peptides in the parvocellular division of the PVN following adrenalectomy (*Kovács et al., 1986*). Steroid treatment also reduces the rise in pre-pro-CRF mRNA (*Jingami et al., 1985*), CRF mRNA (*Kovács & Mezey, 1987*) and AVP mRNA (*Davis et al., 1986*) in the adrenalectomized rat. Moreover, in such animals corticosterone treatment depresses both AVP and CRF mRNA in parvocellular neurones while in magnocellular neurones CRF mRNA is increased but AVP mRNA is unaffected (*Swanson & Simmons, 1989*).

More recent studies have provided further evidence for a strong central component of the delayed feedback process which is mediated primarily by modulation of ir-CRF-41 secretion (*Plotsky & Vale, 1984; Plotsky et al., 1986*). Although delayed effects of dexamethasone on the content of both AVP and CRF-41 in the PVN have been reported (*Kovács et al., 1986*), several lines of evidence indicate that slow delayed feedback effects are mediated principally by an inhibition of ir-CRF-41 secretion which is coupled with a lesser reduction in AVP release (*Fink et al., 1988; Sherward & Fink, 1991*). Certainly in long term hypophysectomized rats the marked inhibition of CRF-41 secretion into the portal blood induced by continuous corticosterone administration from a corticosterone pellet implanted s.c. 5 days prior to blood collection (*Sherward & Fink, 1991*) is accompanied by only a small reduction in portal blood AVP. Moreover, in the sheep, a 17h glucocorticoid infusion lowers basal ACTH release by inhibiting basal hypothalamo-portal concentration of ir-CRF-41 but not ir-AVP (*Canny, Funder & Clarke, 1989*).

At first sight, the data from the present study on intact rats is in accord with this hypothesis. Thus the 6 day treatment with the steroid effectively abolished the ir-CRF-41 response to PLA_2 but only partially reduced the concomitant release of ir-AVP. The results of the 14 day treatment were more difficult to interpret as firstly the CRF-41 samples were lost and secondly the steroid treatment also elevated the basal release of ir-AVP to a level equivalent to that attained following PLA_2 stimulation. Thus, in the presence of the steroid there was no significant difference between ir-AVP release from the prestimulation and stimulation periods. As discussed earlier, the AVP produced by hypothalami from intact animals is derived primarily from magnocellular not parvocellular neurones. Such neurones may well be sensitive to the delayed regulatory actions of steroids, a concept which is supported by recent reports that

glucocorticoid receptors have been identified in the magnocellular neurosecretory cells (*Kiss et al., 1988*). Thus, it is possible that although delayed feedback effects within the parvocellular system are exerted predominantly on CRF-41 secreting neurones, magnocellular AVP neurosecretory neurones may also be sensitive to delayed glucocorticoid feedback.

The failure of the extended steroid treatment to block PLA_2-stimulated ir-CRF-41 and ir-AVP release from hypothalamic tissue from adrenalectomized rats is interesting and may reflect the fact that the dose of the steroid employed ($2\mu g/ml$) was not sufficient to overcome the increased expression of the peptides which would have occurred in the 7 days which elapsed between the surgical removal of the adrenal glands and the start of the steroid treatment. The dose employed has been used in previous studies with intact rats (*Buckingham & Hodges, 1977c*) but, to my knowledge has not been used in adrenalectomized animals. Comparison of the ratio of ir-AVP: ir-CRF-41 release from hypothalami from adrenalectomized rats in control and steroid treated groups demonstrated that this is in fact the case since the dose employed for the 6 day treatment failed to affect the increased ratio of ir-AVP to ir-CRF-41 observed.

If lipocortin is involved in mediating the intermediate feedback effects of the steroids on the HPA axis it would be expected to have similar effects on both ir-AVP and ir-CRF-41 release. Thus the present results suggest that it is not involved. However, to investigate this further the effect of lipocortin on peptide release by the HPA axis was examined.

v) The effects of lipocortin 1 on the functional activity of the HPA axis.

Various properties have been ascribed to the lipocortins including the ability to regulate aspects of the immune response (*Uede, Hirata, Hirashima & Ishizaka, 1983*), blood coagulation (*Funakoshi, Heimark et al., 1987; Funakoshi et al., 1987*), differentiation (*Gupta, Katsumata, Goldman, Herold & Piddington, 1984*) and inflammation (*Flower, Wood & Parente, 1984; DiRosa, 1985*). More recently these proteins have also been implicated in the process of exocytosis and fusion of secretory granules (*Hamman, Gaffey, Lynch & Creutz, 1988; Pollard, Burns & Rojas, 1988; Blackwood & Ernst, 1990*). DiRosa et al. (*1984*) and Flower (*1988*) showed that lipocortin 1 is steroid inducible in macrophages and proposed that it may serve as a second messenger protein for the anti-inflammatory actions of steroids and, by inhibiting the activity of PLA_2, prevent the generation of pro-inflammatory eicosanoids (*Cirino & Flower, 1987; Cirino et al., 1987*). Other workers have challenged these concepts and hence the role of lipocortin 1 as a steroid second messenger (*Bienkowski, Petro & Robinson, 1989; Beyaert, Sunffys, Van Roy & Fiers, 1990*), its steroid inducibility (*Brönnegård, Andersson, Edwall, Lund, Norstedt & Carlstedt-Duke, 1988; Hullin, Raynal, Ragab-Thomas, Fauvel & Chap, 1989*) and its ability to block the activity of PLA_2 in macrophages in vivo (*Northup et al., 1988*) and other tissues (*Bienkowski et al., 1989; Hullin et al., 1989; Beyaert et al., 1990*) have become a matter of controversey.

The present study represents one of the first attempts to examine the potential role of lipocortin 1 as a steroid second messenger within the HPA axis. While the work has been underway, conflicting reports as to the steroid-inducibility of lipocortin 1 in the brain have arisen. Recent studies utilizing western blotting and ELISA techniques indicate that the distribution of lipocortin 1 in the brain does not parallel that of steroid receptors (*Smith, Flower & Buckingham, 1990*). Furthermore, using the same techniques, Smith et al. (*1991-unpublished data*) have shown that lipocortin 1 content of discrete brain areas is unaffected by chronic (14 days) adrenalectomy, steroid treatment or the chronic stress of an i.p. injection of endotoxin (endotoxin causes a prolonged increase in endogenous glucocorticoids (*Suzuki, Oh & Nakano, 1986*)). Conversely, recent immunohistochemical studies have demonstrated the presence of lipocortin 1 in several areas of the brain including the cell bodies of pyramidal and granular cell layers in the gyrus dentatus and CA_1, CA_2 and CA_3 areas of the hippocampus, the pattern of distribution of lipocortin 1 in these hippocampal areas

matching that of glucocorticoid receptors (*Strijbos, Tilders, Carey, Forder & Rothwell, 1991*). Furthermore, in the same study, chronic (14 days) adrenalectomy was shown to cause a reduction of the staining intensity of pyramidal and granular cells in the hippocampal reigons when compared to intact or sham operated control animals (*Strijbos et al., 1991*).

The present study provides little evidence to suggest that lipocortin 1 contributes to the regulatory actions of steroids on the HPA axis at the hypothalamic level. Attempts to neutralize the inhibitory effects of the steroids on CRF-41/AVP release in vitro with a lipocortin 1 antibody (LC-1 Ab) were unsuccessful since the non-immune rabbit serum (NRS) control itself had marked effects on the ir-AVP response to PLA_2 and appeared to potentiate ir-CRF-41 release in the presence of PLA_2 and dexamethasone (Figs. 38i, ii). This complication was not apparent in vivo for the NRS had no effect on resting corticosterone levels. However, although endogenous glucocorticoids are well known to tonically attenuate the HPA response to stress, an i.c.v. injection of a lipocortin 1 neutralizing antibody was without effect on either the resting or stress-induced release of ir-corticosterone (Fig. 39i). In addition, i.c.v. administration of lipocortin 1 (0.6 & 1.2μg) failed to inhibit the stress-induced hypersecretion of ir-corticosterone while the lowest dose tested (0.3μg) caused a marked potentiation of ir-corticosterone secretion (Fig. 40i).

It is of course possible that in these in vivo experiments neither the antisera nor the recombinant protein reached their appropriate target sites. If, as the evidence available suggests (*Arimura et al., 1969*), the intermediate feedback actions of the glucocorticoids involve the generation of a protein second messenger, it is highly probable that the messenger will act within the cell in which it is produced. In this event it is unlikely that either the antibody or the recombinant protein would reach their site of action. On the other hand there is evidence that lipocortin 1 may be released from cells and exert actions on the outer surface of the cells (*Flower, 1988*). This may also be true within the brain for lipocortin antiserum given i.c.v. has been shown to partially reverse the inhibition by dexamethasone of the IL-1β-induced thermogenic response (*Carey, Forder, Edge, Greene, Horan, Strijbos & Rothwell, 1990*). Furthermore, lipocortin 1-188 (LC-1-188) but not the parent protein, hr-LC1, have been shown to inhibit centrally-induced fever and thermogenesis when given i.c.v. (*Strijbos, Browning, Ward, Forder, Carey, Horan & Rothwell 1990*).

Our finding that the lowest dose of LC-1 potentiated the HPA response to stress coupled with the observation that stress precipitates an increase in the lipocortin 1

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content of the hypothalamus raises the possibility that lipocortin 1 may contribute to rather than inhibit the hypothalamic response to trauma. The mechanism by which this is achieved remains to be elucidated. Our in vitro data indicate that AVP may be important in this respect for, although lipocortin 1 had no effect on K^+-induced ir-AVP release (possibly because the tissue was already maximally stimulated), it stimulated the basal release of ir-AVP and potentiated the secretory responses to PLA_2 or IL-6. Furthermore, lipocortin 1, like AVP is present in relatively high concentrations in the SON, paraventricular nucleus and median eminence (Smith et al., 1990).

To what extent the hypersecretion of AVP observed in vitro reflects an action of lipocortin 1 on the parvocellular neurones is not clear. Certainly an action on the magnocellular neurones cannot be excluded at this stage although interestingly, in posterior pituitary tissue the release of ir-AVP (which originates solely from magnocellular neurones) elicited by K^+ was inhibited by lipocortin 1 (Fig. 42). Further studies utilizing hypothalami from intact rats are necessary to determine whether lipocortin 1 exerts different effects on the magnocellular and parvocellular neurones at the hypothalamic level.

The increased hypothalamic AVP release observed in vitro clearly can not be attributed to the anti-PLA_2 properties of the protein described by DiRosa et al. (1984). On the contrary, the protein appeared to potentiate the actions of PLA_2 and increase the formation of PGE_2. It is unlikely that PGE_2 was responsible for the increase since in vitro, in our hands the release of the peptide was not affected either by cyclo-oxygenase inhibitors or by PGE_2 itself (data not shown). However, as discussed earlier other phospholipid metabolites released concomitantly (eg. PAF, products of the lipoxygenase or epoxygenase metabolic pathways or arachidonic acid) could be involved. Despite the apparent increase in cyclo-oxygenase activity CRF-41 release was unaffected by lipocortin 1. This appears to contradict our previous results which indicated that prostanoids facilitate the secretion of this neuropeptide. However, since we and other workers (Bernardini et al.,1989) have failed to demonstrate ir-CRF-41 release in response to PGE_2 stimulation, it is possible that the cyclo-oxygenase product(s) responsible for mediating ir-CRF-41 release was not increased by lipocortin 1. Other reports have also suggested that lipocortins do not exert anti-PLA_2 activity in certain tissues. For example, in human amnion cells, dexamethasone raises intracellular lipocortin 1 concentrations and this is associated with an increase in prostaglandin production (Mitchell, Lytton & Varticovski, 1988).

A more likely explanation for the actions of lipocortin on hypothalamic AVP release is a membrane associated effect involving Ca^{2+}-dependent exocytosis of neurosecretory

granules containing this peptide. In view of the considerable amount of evidence available, there can be no doubt that lipocortin related proteins bind to membrane phospholipids in a Ca^{2+}-dependent manner (*Davidson, Lister & Dennis, 1990; Ernst, Hoye, Blackwood & Jaye, 1990*). Indeed this is one property associated with the family of proteins and lipocortin 1 can be purified by taking advantage of this quality by a process involving reversible Ca^{2+}-dependent association with cellular particulate fractions (*Schlaepfer & Haigler, 1987*). Lipocortin related proteins are found in numerous secretory tissues including mammary glands, in the ductal epithelium (*Lozano, Haindl & Rocha, 1989*) and microvilli of adenocarcinomas (*Liv, Brew, Carraway & Carraway, 1987*), in the syncytiotrophoblast microvillus cytoskeleton of the placenta (*Webb & Mahadevan, 1987*) and in both the tail and the head region of human spermatozoa (*Berruti, 1988*). Thus they may be associated with many processes involving membrane-membrane interactions namely secretion by, transport across and fusion of cell membranes.

Several members of the lipocortin family have been implicated in the process of exocytosis. Lipocortins III and IV (endonexin, 32kDa) are both Ca^{2+}-dependent proteins which bind to chromaffin granule membranes and thus may regulate membrane fusion events which occur during exocytosis (*Hamman et al., 1988*). Furthermore, the 47kDa protein, lipocortin VII (annexin VII/synexin) has been shown to act as a cytosolic mediator of Ca^{2+} action during exocytosis (*Pollard et al., 1988*). Interestingly, the localisation of lipocortin 1 in certain cell types is strongly influenced by intracellular Ca^{2+}. Thus lipocortin 1, which has been located both in the cytosol and in the particulate fraction of cells (*William, Mroczkowski, Cohen & Kraft, 1988; Campos-Gonzalez, Kanemitsu & Boynton, 1989*), can be removed from the particulate fraction by incubation with Ca^{2+} chelators or by treatment with PLA_2 or phospholipase C (*William et al., 1988*). Furthermore translocation of the protein from the cytoplasm to the cell membrane in guinea pig neutrophils is coupled with an increase in intracellular Ca^{2+} (*Sato, Okimasu, Takakashi, Miyahara, Matsuno & Utsumi, 1988*) and addition of the Ca^{2+} ionophore, A23187, to a human myelocytic cell line causes lipocortin 1 to associate with the cell membrane (*William et al., 1988*). More recent studies involving simultaneous measurements of cytosolic Ca^{2+} and secretion in single bovine adrenal chromaffin cells have confirmed the role of Ca^{2+} in exocytosis (*Cheek, Jackson, O'Sullivan, Moreton, Berridge & Burgoyne, 1989*) and suggested that the triggering of exocytosis from these cells requires a specific spatial distribution of Ca^{2+} (*Cheek, O'Sullivan, Moreton, Berridge & Burgoyne, 1989*). Furthermore using permeabilised chromaffin cells in which secretion has been reduced as a result of leakage of cellular components, Ali and colleagues (*1989*) have

demonstrated that lipocortin I & II (calpactin and p36) are essential for exocytosis from these cells since these proteins are able to reconstitute secretion from the permeabilised cells, an effect which is blocked by p36 antiserum (*Ali, Geisow & Burgoyne, 1989*). Therefore it is possible that lipocortin 1 could promote exocytosis of AVP containing vesicles and thereby increase ir-AVP release.

The actions of lipocortin 1 on CRF-41 release in vitro, unlike its effects on AVP secretion, closely resembled those of steroids and appeared to be stimulus specific. Thus, like dexamethasone, lipocortin 1 readily inhibited ir-CRF-41 release following IL-6 stimulation (Fig. 54ii) but failed to block peptide release from hypothalamic tissue from adrenalectomized rats in response to PLA_2 or K^+ stimulation (Figs. 36i & 37ii). The failure of lipocortin 1 to affect peptide release initiated by K^+ may reflect the fact that the amount of peptide released in response to this powerful depolarising stimulus was so great compared with that evoked by other secretagogues (eg PLA_2 and IL-6) that any small inhibition caused by lipocortin 1 would have been masked. Obviously the fact that lipocortin 1 exerts effects similar to dexamethasone on CRF-41 release does not necessarily indicate that these proteins mediate the effects of steroids. On the contrary, the data merely indicate that lipocortin shares with the steroids the ability to inhibit the CRF-41 response to specific stimuli (i.e. cytokines).

Interestingly, lipocortin 1 has been shown to inhibit cytokine actions in other preparations. For example, Carey et al. (*1990*) showed that i.c.v. injection of a lipocortin 1 fragment (1-188) into the third ventricle blocked the thermogenic response to IL-1β. The effects of the protein appeared to be exerted at a point before PG synthesis since it did not inhibit PGE_2-induced thermogenesis. Since thermogenic actions of CRF-41 have been described previously (*Morley & Levine, 1982; LeFeuvre et al., 1987*) and CRF-41 is thought to mediate the thermogenic responses to IL-1β (*Rothwell, 1989*), it is possible that the inhibitory actions of lipocortin on IL-1β-induced thermogenic responses are due to the protein acting on selective populations of CRF-41 containing neurones.

Our attempts to determine whether lipocortin 1 contributes to the regulatory actions of glucocorticoids at the pituitary level generated conflicting data for the two forms of lipocortin employed namely recombinant lipocortin 1 protein (Biogen Inc. USA) and a lipocortin 1-188 fragment (ICI plc.) produced widely differing results. The actions of the recombinant protein largely resemble those of glucocorticoids described by others.

Thus, although the recombinant lipocortin 1 failed to affect basal ir-ACTH release, it did cause a significant reduction in the ACTH response to hypothalamic extract (HE) or K^+-stimulation suggesting that lipocortin may contribute to the inhibitory effects of steroids on HPA activity at the pituitary level. Further evidence to support this concept is provided by reports that lipocortin 1 is steroid inducible in the pituitary gland of adrenalectomized rats following short but not long term dexamethasone treatment (*Smith et al., 1991 unpublished data*). Moreover in intact rats lipocortin 1 turnover is increased in rats treated overnight with dexamethasone (*Smith et al., 1991 unpublished data*). In addition, endotoxin treatment which elevates endogenous glucocorticoids, stimulates lipocortin formation in pituitary tissue from intact but not adrenalectomized animals. An inhibitory role for lipocortin 1 at the pituitary level is not supported by the data with the N-terminal fragment 1-188 for unlike the parent protein, it caused marked dose dependent increases in basal ir-ACTH secretion. This inevitably complicated interpretation of the data following stimulation with HE or K^+ for the apparent failure to elicit a further response may reflect the fact that the tissue was already maximally stimulated.

Recombinant lipocortin 1 and a lipocortin 1 fragment have recently been reported to exert differential effects on central IL-β-induced fever and thermogenesis following i.c.v. administration (*Strijbos et al., 1990*). These workers suggested that the ability of the lipocortin fragment to inhibit centrally-induced fever and thermogenesis reflected its smaller molecular size which enabled it to reach target sites inaccessible to the larger parent protein. Differential pharmacokinetic properties may also explain the ability of the lipocortin fragment but not the recombinant protein to produce dose dependent increases in basal ir-ACTH release as thesmaller molecule may gain access to the intracellular compartment more readily and thus promote exocytosis of ACTH containing vesicles. In this event, it is possible that lipocortin and lipocortin-like proteins may exert widely differing effects on ir-ACTH release at the level of the pituitary, depending on their accessibility to target sites which may be intracellular.

Although the present study concentrated on the influence of lipocortin 1 on the HPA axis, ir-luteinizing hormone (ir-LH) levels were also measured, as a control, in the in vivo experiments. Our findings that the LC-1 antibody, given i.c.v., increased LH release while the recombinant protein (1.2μg i.c.v.) reduced the post-stress serum LH concentration were interesting and, coupled with the observation that stress elevates the LC-1 content of the hypothalamus raise the possibility that LC-1 may inhibit the release of LHRH and thus contribute to the inhibition of LH secretion observed in stress (*Cover et al., 1991*).

vi) The effects of cytokines on hormone secretion by the HPA axis.

Infection, injury or antigenic challenge all trigger the inflammatory response. During conditions of bacteraemia, endotoxaemia or septic shock the activity of the HPA axis increases markedly. In addition plasma concentrations of PLA_2 (*Vadas et al., 1988*) and of various cytokines including interleukin 1 (IL-1) (*Damas, Reuter, Gysen, Demonty & Lann, 1989*) and interleukin 6 (IL-6) (*Fong, Moldawer, Marano, Wei, Tatter, Clarick, Santhanam, Sherris, May, Sehgal & Lowry, 1989; Hack, DeGroot, Felt-Bersma, Nuijens, Strack van Schijndel, Eerenberg-Belmer, Thijs & Aarden, 1989*) are raised. Since we have already provided evidence that PLA_2 and the products of arachidonic acid metabolism may stimulate HPA activity at the level of both the hypothalamus and the pituitary gland (*Cowell et al., 1991*), the potential roles of cytokines in this respect was of obvious interest.

Macrophages are the primary source of IL-1, IL-6, TNF and other pro-inflammatory mediators (*Roitt, Brostoff & Male, 1989*). However, IL-1 is also produced by epidermal, epithelial, lymphoid and vascular tissues (*Dinarello, 1988*) while IL-6 may be released by monocytes, endothelial cells and fibroblasts (*Aarden, 1989*). Interleukin-1 and IL-6 are found in certain brain tissues. For example, IL-1 is produced by glial cells in the CNS (*Fontana, Kristensen, Dubs, Gemsa & Weber, 1982; Fontana, Weber & Dayer, 1984*) and is present in the human hypothalamus (*Breder, Dinarello & Saper, 1988*). In addition, small amounts of IL-6 are produced by the basal medial hypothalamus in vitro and IL-6 production is stimulated by endotoxin treatment (*Spangelo, Judd, MacLeod, Goodman & Isakson, 1990*). The brain cell types responsible for the synthesis of IL-6 appear to be astrocytes and microglia which are present in the basal medial hypothalamus and are stimulated by endotoxin or other stress-related factors to enhance IL-6 production (*Spangelo & MacLeod, 1990*). Interleukin 1α and IL-1β share a common receptor which has been localised in the brain (*Haour, Ban, Milon, Baran & Fillion, 1990; Farrar, Kilian, Ruff, Hill & Pert, 1987*). High density IL-1 binding is present in typically neurone-rich areas (i.e., the granule cell layer and the pyramidal cell layer of the dentate gyrus, the pyramidal cell layer of the hippocampus and the granule cell layer of the cerebellum), moderately high binding is present in the anterior dorsal thalamus and ventromedial hypothalamus while low density binding occurs in the median eminence, the nucleus accumbens, the anterior hypothalamus, the mamillary and pontine nuclei and the deep layers of the olfactory tubercle (*Farrar et al., 1987*). The IL-6 receptor has also been identified in many different types of cells (*Taga, Kawanishi, Hardy, Hirano & Kishimoto, 1987*).

Endotoxin stimulates the release of pro-inflammatory mediators including cytokines from macrophages (*Bakouche, Koff, Brown & Lachman, 1987; Cavaillon, Fitting, Caroff & Haeffner-Cavaillon, 1989*). The results from our initial studies, in which products released by endotoxin-stimulated macrophages were found to stimulate the release of not only ir-ACTH from the pituitary gland but also ir-CRF-41 and, to a lesser extent, ir-AVP from the hypothalamus, are in accord with the hypothesis that macrophage products may contribute to the activation of the HPA axis in conditions such as septic shock. It is unlikely that the observed responses reflect direct actions of endotoxin on the hypothalamus or pituitary gland for endotoxin alone did not affect the secretory activity of these tissues. Furthermore, both the ir-ACTH and ir-CRF-41 responses appear to be proportional to the number of macrophages previously incubated in the medium. The fact that stimulation of AVP release was evident only when the hypothalamic tissue was challenged with medium collected from the highest number of macrophages appears to conflict with our later in vitro results where low concentrations of a number of individual cytokines were found to readily induce AVP release. However, from the initial studies with medium from macrophages it was not possible to identify which macrophage products were responsible for mediating the observed effects since the incubation medium was not assayed for individual cytokine contents. Nevertheless, there is evidence to suggest that macrophages release IL-1 in response to endotoxin and that endotoxin may stimulate the HPA axis through a mechanism involving activation of IL-1 receptors (*Rivier, Chizzonite & Vale 1989*). Thus, we decided to investigate these actions further by examining the effects of several cytokines, namely IL-1α, IL-1β, TNF, IL-6 and IL-8, on hormone release from the HPA axis.

There are reports that IL-1 induces ACTH release in vitro through a direct action on the pituitary gland (*Bernton et al., 1987*). Furthermore, IL-6 has been shown to stimulate prolactin, growth hormone and luteinizing hormone secretion by anterior pituitary cells in vitro (*Spangelo, Judd, Isakson & MacLeod, 1989*) and is itself produced by these cells where it may function as an intrapituitary releasing factor (*Spangelo, MacLeod & Isakson, 1990*). However, although medium collected from endotoxin stimulated macrophages readily induced ir-ACTH release, none of the individual interleukins tested in these studies stimulated the basal release of ir-ACTH directly. On the contrary, any apparent effects on hypothalamic extract-stimulated ir-ACTH were inhibitory rather than stimulatory. Thus IL-6 and, to a lesser extent, single doses of TNFα and IL-8 caused significant reductions in HE-induced ir-ACTH release.

Interestingly, inhibitory effects of TNFα on the ACTH responses to CRF-41, AVP and angiotensin II have been described recently (*Gaillard, Turnill, Sappino & Muller, 1990*). These workers suggested that the inhibitory effects of TNFα on the secretion of ACTH may contribute to the increased mortality observed in cases of severe septic shock with high circulating levels of TNFα.

Our findings with IL-1 are also in accord with several other studies which have shown that the cytokine has no effect on ACTH release from pituitary tissue in vitro (*Berkenbosch et al., 1987; Sapolsky et al., 1987; Tsagarakis, Gillies et al., 1989*). Other reports however, have indicated that several cytokines, including IL-1β, stimulate ACTH release directly from pituitary cell cultures but their effects are apparent only after a latent period. Thus, IL-1α, IL-1β, and IL-6 stimulate the release and synthesis of ACTH following a 4h contact period (*Fukata, Usui, Naitoh, Nakai & Imura, 1989*) while IL-1α and IL-1β stimulate ACTH release from cultured anterior pituitary cells following chronic (>15h contact period) but not acute (3h) exposure of the cells to the cytokines (*Suda, Tozawa, Ushiyama, Tomori, Sumitomo, Nakagami, Yamada, Demura & Shizume, 1989*). Therefore, it is possible that the present study failed to demonstrate any stimulatory effects of the cytokines because the contact time of the cytokine with the tissue was too short. Thus it is possible that the model employed in these studies was not suitable in this instance.

In the light of these results, the data from the macrophage studies are difficult to explain and raise the possibility that the increase in ACTH release initiated by medium collected from endotoxin-stimulated macrophages was due to substances other than interleukins 1, 6, and 8 or TNFα, released concomitantly. Thus, the ACTH responses may involve the combined synergistic actions of many cytokines such as macrophage/granulocyte colony stimulating factors (M-CSF/G-CSF) released by macrophages following an antigenic challenge.

The bulk of the evidence available suggests that cytokines such as interleukin 1 stimulate HPA activity through an action at the level of the hypothalamus, possibly via CRF-41 release (*Uehara et al., 1987b; Berkenbosch et al., 1989*). The data from the macrophage studies support this hypothesis as too do the findings that interleukins 1α, 1β, 6 and 8 all cause significant increases in ir-CRF-41 release.
Interleukin-1α (*Sapolsky et al., 1987*), IL-1β (*Uehara et al., 1987a, b*) and IL-6 (*Naitoh, Fukata, Tominaga, Nakai, Tamai, Mori & Imura, 1988*) have each been shown to stimulate HPA activity in vivo when given i.p. but to our knowledge the

actions of IL-8 on this axis have not been investigated previously. The actions of IL-1 and IL-6 have been largely attributed to increased CRF-41 release since the responses to these cytokines are inhibited by immunoneutralization with a CRF-41 antiserum administered prior to the cytokine injection. Several other studies have emphasized the role of cytokines in the initiation of CRF-41 secretion. Thus, although Uehara and colleagues found that i.v. administration of IL-1α failed to increase plasma levels of ACTH, IL-1β was effective in this respect (*Uehara et al., 1987a*). In addition, IL-1α and IL-1β have been shown to stimulate CRF-41 gene expression in the rat hypothalamus in vivo (*Suda, Tozawa, Ushiyama, Sumitomo, Yamada & Demura, 1990*) and to elicit the release of the neuropeptide from isolated rat hypothalami in vitro (*Tsagarakis, Gillies et al., 1989*).

In the present study, interleukins 1α, 1β, 6 and 8 and TNFα also caused significant increases in ir-AVP release. Interestingly, the ir-AVP responses did not parallel those of ir-CRF-41 in that the ir-AVP response to the majority of cytokines tested followed a 'bell-shaped' curve with the maximal peptide release occurring at relatively low doses of the cytokines. By contrast, in the majority of experiments, ir-CRF-41 release was greatest in the presence of the higher concentrations of the interleukins. The AVP responses are interesting since several workers have suggested that IL-1 effects on the HPA axis are mediated by CRF-41 released from neurones which do not contain AVP (*Berkenbosch et al., 1989*). Unfortunately, because of this, many of the in vitro studies have concentrated on CRF-41 responses and have not measured AVP release concomitantly (*Tsagarakis, Gillies et al., 1989*). However, according to Jarvis (*1990*), Gaillard and colleagues have shown that AVP release from cultured hypothalami is not affected by IL-1β, IL-6, TNFα and thymosin fraction 5 whereas these cytokines all increase CRF-41 release. The present data contradict these reports and suggest that AVP does play a role in mediating the effects of cytokines on the HPA axis. The apparent discrepancy between these reports and the present data may be due to a number of factors. Firstly, there may be a fundamental difference between the distribution of cytokine receptors on cultured hypothalamic neurones and freshly removed tissue. Secondly, few workers have performed extensive dose-response studies and, as the present data illustrate, the doses required to elicit AVP and CRF-41 release differ markedly. Thirdly, although CRF-41 antisera block the ACTH response to cytokines in vivo, this does not necessarily preclude a role for AVP for, since CRF-41 and AVP act synergistically to stimulate corticotrophin release and AVP alone is a very weak secretagogue, it is possible that the immunoneutralization of CRF-41 may

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also have masked the pituitary response to any increase in AVP release. In support of a role for AVP is the finding that IL-1β stimulates the release of AVP from rat neurohypophysis indicating that IL-1β is involved in the control of AVP release from magnocellular neurones (*Christensen, Hansen & Fjalland, 1989*). Thus, one explanation for this controversy may be that the AVP released in our preparation in response to cytokines is derived from magnocellular rather than parvocellular neurones. Obviously further experiments are necessary utilizing hypothalami from intact rats before this can be resolved.

Although IL-1α and IL-1β are potent activators of the HPA axis in vivo (*Besedovsky, Del Rey, Sorkin & Dinarello, 1986; Uehara et al., 1987a, b*) the present study indicates that their actions on the hypothalamus in vitro are invariably

when effective
weak compared with those observed following IL-6 and IL-8 stimulation. The reason for this is unclear. However, there is evidence to suggest that IL-6 exhibits a synergistic action with IL-1 (*Elias, Trinchieri, Beck, Simon, Sehgal, May & Kern, 1988*) and reports that IL-1 stimulates the production of IL-6 by endothelial cells (*Sironi, Breviario, Proserpio, Biondi, Vecchi, Van Damme, Dejana & Mantovani, 1989*) and IL-8 (neutrophil chemotactic factor) by extravascular non-circulating cells such as dermal fibroblasts (*Larsen, Zachariae, Mukaida, Anderson, Yamada, Oppenheim & Matsushima, 1990*). Thus, it is possible that the effects of IL-1 (α & β) on the HPA axis are mediated or potentiated by locally produced IL-6 and IL-8. The relatively small effects of IL-1α and β on CRF-41 release observed in vitro in these experiments may therefore reflect the fact that IL-6 and/or IL-8 are required as cofactors/mediators for IL-1-induced hormone release. Thus, although there is evidence to show that IL-6 (*Spangelo et al., 1990*) is formed in the hypothalamus it is possible that there was insufficient time for this to occur in the present preparation since the tissue was in contact with the cytokine for only 30min.

The idea for the temporal appearance of other cytokines post induction of an initial mediating cytokine has also been described for tumour necrosis factor (TNF). This cytokine is involved in the initiation of the inflammatory response and also mediates repair and remodelling processes which occur during inflammation. Since TNF appears to be a proximal mediator during the initial phase of inflammation, its effects must be maintained via the expression of other cytokines subsequent to its production. Thus, generation of colony stimulating factor, IL-1, IL-6, IL-8 and monocyte chemotactic factor following TNF induction has been observed (*Kunkel, Strieter, Chensue, Basha, Standiford, Ham & Remick, 1990*). The present results support the

hypothesis that TNFα requires the presence of other cytokines to exert its effects since, although TNFα (625pg/ml) alone failed to induce a significant increase in ir-AVP release, in the presence of IL-1β a pronounced secretory response was apparent. These findings however contrast with data from recent in vivo studies which suggest that TNFα exerts its stimulatory effect on HPA activity either directly or via stimulation of other cytokines which elicit the secretion of CRF-41 but not AVP (*Bernardini, Kamilaris, Calogero, Johnson, Gomez, Gold & Chrousos, 1990*). However, this conclusion was based on the use of an antibody to CRF-41 which, as previously discussed, may also have masked the pituitary response to AVP. Obviously this area merits further investigation.

In addition to their effects on hormone secretion within the HPA axis, cytokines have also been implicated in the intrahypothalamic pyrogenic and thermogenic response apparently by eliciting the release of CRF-41 (*Rothwell, 1989*). Several workers have shown that IL-1 acts centrally, probably within the hypothalamus, to stimulate thermogenesis and thus increase body temperature (*Dinarello, 1984; Busbridge, Dascombe, Tilders, Van Oers, Linton & Rothwell, 1989; Dascombe, LeFeuvre, Sagey, Rothwell & Stock, 1989*). Since the pyrogenic response appears to involve the stimulation of prostaglandin production by IL-1 (*Fontana et al., 1984*), it is possible that the activation of the HPA axis induced by cytokines also involves prostaglandin synthesis. This concept is supported by a number of reports which indicate that the ACTH response to IL-1β (administered i.v. or i.c.v.) is readily inhibited by pre-treatment with indomethacin (*Katsuura et al., 1988; Morimoto, Murakami, Nakamori, Sakata & Watanabe, 1989; Murakami & Watanabe, 1989; Watanabe, Morimoto, Sakata & Murakami, 1990*). According to Murakami and Watanabe (*1989*) and Morimoto et al. (*1989*) the cyclo-oxygenase product responsible for mediating these effects is PGE_2 since microinjection of PGE_2 induced ACTH responses similar to those seen following i.v. injection of IL-1α. Furthermore, systemic pre-treatment with a CRF-41 antiserum significantly reduced the ACTH response induced by intrahypothalamic injection of PGE_2 (*Watanabe et al., 1990*). However, other workers have demonstrated that ir-CRF-41 release is initiated by $PGF_{2\alpha}$ but not by PGE_2 (*Bernardini et al., 1989*) and in the present study PGE_2 was without effect on ir-CRF-41 secretion (data not shown). The reason for these contrasting results is unclear. However, since cytokines act via stimulation of PLA_2 (*Katsuura et al., 1988*), it is highly likely that a spectrum of eicosanoids was produced in the hypothalamus. Further studies are necessary to identify not only the

prostanoid metabolites responsible for the increase in CRF-41 release but also the non-prostanoid metabolites (arachidonic acid, lipoxygenase and epoxygenase products) which may be responsible for the increase in AVP release. From the present study it is not possible to identify the site of action of the cytokines. They may act directly on the releasing factor neurones as suggested by Berkenbosch et al. (*1989*). Alternatively, they may act on cytokine receptors on the surrounding cells. Interleukin-1β has been shown to stimulate PGE_2 production by rat astrocyte cultures suggesting that the astrocytes may be the target cells for IL-1β in the CNS (*Katsuura, Gottschall, Dahl & Arimura, 1989*). Alternatively, it is possible that the cytokine response may involve the population of noradrenergic neurones in the hypothalamus concerned with the initiation of CRF-41 release. Indeed IL-1 administration has been shown to stimulate hypothalamic noradrenaline metabolism probably reflecting an increased activity of noradrenergic neurones (*Dunn, 1988*).

The existence of a physiological interaction between immune and HPA activities is well recognised. During an antigenic challenge, blood glucocorticoid levels increase in proportion to the magnitude of the immune response (*Besedovsky, Sorkin, Keller & Muller, 1975*). Several lines of evidence suggest that the corticosteroids liberated attenuate the production/activity of cytokines. Early studies illustrated the ability of corticosteroids to inhibit IL-1 production by macrophages (*Snyder & Ueanue, 1982*). Other workers have also suggested that the glucocorticoids produced during the course of the immune response suppress IL-1 production (*Besedovsky et al., 1986; Lee, Tsou, Chan, Thomas, Petrie, Eugui & Allison 1988*). It is thus possible that the actions of cytokines on the HPA axis may be subject to negative feedback regulation by steroids. The present data support this hypothesis. Dexamethasone readily inhibited the neuroendocrine responses to both IL-6 and IL-8, a finding which could reflect inhibition of the PLA_2 coupled signal transduction mechanism. The steroid also blocked the AVP but not the CRF-41 response to IL-1α. The reason for this differential effect, which could involve blockade of signal transduction and the generation of 'mediator' cytokines (eg. IL-6), is not clear. However, it is possible that a longer contact time with the steroid may also have blocked the CRF-41 response for, according to Cambronero, Borrell and Guaza (*1989*) pre-treatment of perifused rat hypothalami with dexamethasone in vitro or in vivo (prior to autopsy) attenuates IL-1β-induced CRF-41 secretion.

Since the inhibitory effects of dexamethasone on cytokine-induced ir-CRF-41 secretion are mimicked by lipocortin, it is possible that the two are acting by a common

mechanism, perhaps via blockade of PLA_2 as prostanoids are thought to be responsible for mediating the effects of IL-1. However, the fact that both steroids and lipocortin exert similar effects on cytokine induced ir-CRF-41 release does not necessarily indicate that their actions are exerted at the same point in the sequence of events effecting stimulus-secretion coupling. Indeed a common mechanism could not explain the actions of lipocortin and steroids on ir-AVP release, for dexamethasone clearly inhibits the secretory responses to both exogenous PLA_2 and interleukins while lipocortin potentiates the responses to these secretagogues. The mechanisms underlying these striking differential effects remain to be elucidated.

Conclusions.

The results presented here provide evidence that PLA_2 activates the HPA axis by acting at the level of the hypothalamus to initiate the secretion of the two major corticotrophic releasing factors, CRF-41 and AVP. This finding may be of both physiological and pathophysiological importance for, in conditions such as septic shock, high concentrations of PLA_2 are found in the circulation. Furthermore, cytokines which are also released in septicaemia activate the HPA axis by acting on specific receptors within the hypothalamus which are positively coupled to PLA_2. Thus, PLA_2 may provide an important link between the immune and neuroendocrine systems. Although PLA_2 stimulates the release of both CRF-41 and AVP, the responses appear to be differentially mediated and while prostanoids appear to effect the rise in CRF-41 release they do not account for the increase in AVP release.

The finding that the secretory responses to PLA_2 and the PLA_2 activator, melittin, are not consistently blocked by dexamethasone suggests that the regulatory action of the steroids at the hypothalamic level can not be attributed entirely to blockade of this enzyme by a mechanism analogous to their anti-inflammatory effects seen in macrophages (*Flower et al., 1984*). It is unlikely that lipocortins which have been implicated as mediators of the anti-inflammatory action of steroids play a major role in the intermediate feedback effects of steroids on the hypothalamus. The studies with lipocortin 1 and the lipocortin 1 antibody led to similar conclusions for although lipocortin 1 blocked cytokine induced CRF-41 release, it had no effect on the CRF-41 response to PLA_2 itself and potentiated the AVP response to these secretagogues.

Our finding that lipocortin 1, unlike dexamethasone, is not a powerful suppressor of HPA function raises the possibility that lipocortin-like compounds may ultimately provide a new approach to the control of inflammation devoid of the potential hazard of HPA suppression.

Summary.

1. Phospholipase A_2 (25-100U/ml) and the PLA_2 activator, melittin (0.5-1.0µg/ml), caused dose dependent increases in the release in vitro of both CRF-41 and AVP from hypothalami from intact and chronically adrenalectomized animals. Phospholipase A_2 also readily induced ir-AVP and ir-CRF-41 release from hypothalami from sham operated animals.

2. Phospholipase A_2 and melittin induced ir-AVP release in vitro from hypothalamic tissue from intact, adrenalectomized and sham-operated animals was readily inhibited by quinacrine (10^{-4}M). Quinacrine also blocked PLA_2-stimulated ir-CRF-41 release from hypothalamic tissue from adrenalectomized rats but failed to influence the release of the peptide from tissue from intact or sham operated control animals.

3. Neither the cyclo-oxygenase inhibitors, ibuprofen (10^{-5} & 10^{-4}M) and indomethacin (10^{-4}M), nor the dual cyclo-oxygenase/lipoxygenase inhibitor BW 755C (10^{-4}M), attenuated the AVP response to PLA_2 stimulation in tissue from intact or adrenalectomized animals.The cytochrome P450 inhibitor SKF 525A (10^{-4}M) was also ineffective in this respect but in one experiment the 5-lipoxygenase inhibitor, ICI 207968 (10^{-4}), abolished the response.

4. Ibuprofen (10^{-4}M) and indomethacin (10^{-4}M) failed to affect the resting release of CRF-41 but inhibited the secretory responses to PLA_2 from tissues from intact and adrenalectomized animals. CRF-41 release induced by PLA_2 was unaffected or potentiated by inhibitors of cytochrome P450 (SKF 525A, 10^{-4}M) and 5-lipoxygenase (ICI 207968, 10^{-4}M) enzymes and attenuated by the dual lipoxygenase/cyclo-oxygenase inhibitor (BW 755C, 10^{-4}M).

5. Inclusion of dexamethasone (10^{-6}M) in the medium had no significant effect on ir-AVP release from hypothalamic tissue from intact or sham operated rats following PLA_2 stimulation but readily suppressed the ir-AVP response of tissue from adrenalectomized animals. Dexamethasone inhibited melittin induced ir-AVP release from tissue from adrenalectomized rats. The steroid had no effect on PLA_2-induced ir-CRF-41 release from hypothalami from adrenalectomized or sham operated rats but significantly reduced the secretory responses of tissue from intact animals. It also

inhibited melittin-stimulated ir-CRF-41 release from hypothalamic tissue from adrenalectomized but not intact rats. Similar data were obtained when the steroid was included in the drinking water for 24h prior to autopsy.

6. Pre-treatment with dexamethasone for 6 days prior to autopsy readily inhibited PLA$_2$-induced ir-CRF-41 and to a lesser extent ir-AVP release from hypothalamic tissue from intact but not adrenalectomized rats.

7. In vivo, human recombinant lipocortin 1 (hr-LC1) administered i.c.v. had no affect on the resting plasma corticosterone concentration in conscious rats. The lowest dose. (0.3µg) tested potentiated the HPA response to histamine stress although higher doses (0.6-1.2µg) were without effect. Neither the resting nor the stress-induced secretion of corticosterone was affected by i.c.v. administration of LC1 antisera. hr-Lipocortin 1 (1.2µg) also reduced ir-LH levels during stress and both resting and stress-induced ir-LH levels were increased in the presence of hr-LC1 antisera.

8. In vitro, hr-LC1 failed to influence the spontaneous release of CRF-41 or the release evoked by PLA$_2$ or K$^+$. By contrast, the peptide stimulated the release of AVP and potentiated the AVP responses to PLA$_2$ but not to K$^+$. It also caused dose dependent increases in basal and PLA$_2$-stimulated ir-PGE$_2$ release. Attempts to neutralize the inhibitory effects of dexamethasone on PLA$_2$-induced CRF-41 and AVP release from isolated hypothalami were not successful as the normal rabbit serum control interfered with the response to PLA$_2$.

9. Human recombinant lipocortin 1 failed to affect basal but significantly reduced K$^+$-stimulated ir-AVP release from isolated posterior pituitary tissue in vitro. It also failed to affect basal ACTH release from anterior pituitary tissue in vitro but significantly inhibited HE or K$^+$-induced release. In contrast the synthetic 1-188 N-terminal fragment appeared to elevate basal ACTH release but was without effect on either hypothalamic extract or K$^+$-stimulated release.

10. Medium collected from macrophages stimulated with endotoxin in vitro readily induced the release of ir-ACTH from anterior pituitary tissue and, ir-CRF-41 and to a lesser extent ir-AVP from hypothalamic tissue in vitro. Interleukin-1α, IL-1β and TNF produced weak stimulatory effects on the secretion of both ir-CRF-41 and ir-AVP. Interleukin-6 and IL-8 were more active in this respect and initiated highly significant dose dependent increases in peptide release. The peptide release in response

to both IL-6 and IL-8 was readily inhibited by dexamethasone (10^{-6}M) which also reduced IL-1α-induced ir-AVP release, although it failed to affect the ir-CRF-41 response.

11. Lipocortin 1 significantly inhibited ir-CRF-41 release in response to both IL-6 and IL-8 stimulation but caused a marked potentiation of IL-6-induced ir-AVP release.

12. Interleukin-1α and IL-1β, alone or in combination with tumour necrosis factor (TNFα 1250pg/ml) had no effect on either resting or HE-induced ACTH secretion. In a number of experiments TNFα alone produced a small but significant decrease in HE-induced peptide release. IL-6 and IL-8 also failed to affect basal ACTH release but IL-6 (5 & 10ng/ml) and IL-8 (250pg/ml) attenuated the secretory responses to hypothalamic extracts.

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Abbreviations.

AA	= Arachidonic acid.
Ab	= Antibody.
ACTH	= Adrenocorticotrophic hormone.
ANF/P	= Atrial naturitic factor/peptide.
AVP	= Arginine vasopressin.
(α/β)-End.	= (α/β)-endorphin.
(α/γ)-MSH	= (α/γ) Melanocyte-stimulating hormone.
(β/γ)-LPH	= (β/γ) Lipotrophin.
BSA	= Bovine serum albumin.
cAMP	= Cyclic adenosine monophosphate.
cGMP	= Cyclic guanylate monophosphate.
CLIP	= Corticotrophin-like intermediate lobe peptide.
CNS	= Central nervous system.
CRF-41	= Corticotrophin releasing hormone-41.
DD-AVP	= Desamino-D-arginine vasopressin.
DEAE	= Diethylaminoethyl.
Dex	= Dexamethasone.
EDTA	= Ethylene diaminetehra-acetic acid.
EET	= Epoxyeicosatetraenoic acid.
ELISA	= Enzyme-linked immunosorbant assay.
EtOH	= Ethanol.
ETYA	= Eicosatetraynoic acid.
FPLC	= Fast pressure liquid chromatography.
GnRH	= Gonadotrophin releasing hormone.
GR	= Glucocorticoid receptor.
HE	= Hypothalamic extract.
HETE	= Hydroxyeicosatetraenoic acid.
HPA	= Hypothalamo-pituitary-adrenal axis.
HPLC	= High performance liquid chromatography.
hr-LC1	= Human recombinant lipocortin 1.
Ibu	= Ibuprofen.
i.c.v.	= Intra-cerebro-ventricular.
IgG	= Immunoglobulin G.
IL (1α, 1β, 6, 8)	= Interleukin (1α, 1β, 6, 8).
Indo	= Indomethacin.
i.p.	= Intra-peritoneal.
ir	= Immunoreactive.
iU.	= International units.
KIU	= Kallikrein inactivator units.
LC1-188	= Lipocortin 1-188 fragment.
LH	= Luteinizing hormone.
LHRH	= Luteinizing hormone releasing hormone.
LT (B4, C4)	= Leukotriene (B4, C4).
M-CSF/G-CSF	= Macrophage/ granulocyte-colony stimulating factor.
ME	= Median eminence.
mRNA	= Messenger ribonucleic acid.
NDGA	= Nordihydroguaiaruetic acid.
N-POMC	= Pro-opiomelanocortin.
NRS	= Non-immune rabbit serum.
PAF	= Platelet activating factor.
PBS	= Phosphate buffered saline.
PEG	= Polyethylene glycol.
PG (D2, E2, F1α, F2α)	= Prostaglandins (D2, E2, F1α, F2α).
PG I2	= Prostacyclin.
PLA2	= Phospholipase A2.
PLC	= Phospholipase C.
PVN	= Paraventricular nucleus.
Quin	= Quinacrine.
RIA	= Radio-immunoassay.
SARFc	= Sheep anti-rabbit Fc.
SME	= Stalk median eminence.
SON	= Supraoptic nucleus.
SPA	= Scintillation proximity assay.
TNFα	= Tumour necrosis factorα.
TRH	= Thyrotrophin releasing hormone.
TxB2	= Thromboxane B2.
VIP	= Vasoactive intestinal peptide.
v/v	= volume/volume.
w/v	= weight/volume.