Br. J. Pharmac. (1986), **87**, 57–62

A comparison of the acute inflammatory response in adrenalectomised and sham-operated rats

R.J. Flower[1], L. Parente[2], Paola Persico* & J.A. Salmon

The Wellcome Research Laboratories, Department of Mediator Pharmacology, Langley Court, Beckenham, Kent BR3 3BS and Dipartimento di Farmacologia Sperimentale*, Via Leopoldo Rodino 22, 80138 Napoli, Italy

1 Carrageenin pleurisy was induced in adrenalectomised (ADX) and sham-operated (SHO) rats.

2 The magnitude and duration of inflammation, as estimated by fluid exudation and cell migration, was greatly increased (approximately doubled) in ADX rats compared with that in their SHO controls.

3 The content of eicosanoids (6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), thromboxane B_2 (TXB_2), and leukotriene B_4 (LTB_4)) in inflammatory exudates from ADX rats was significantly (2–4 fold) greater than that of their SHO controls.

4 Resident macrophages obtained from ADX rats produced more eicosanoids per cell per unit time when stimulated *in vitro* with zymosan, than did cells from the SHO controls.

5 Administration of glucocorticoids blocked the inflammatory response and reduced the release of eicosanoids both *in vitro* and *in vivo* in both groups of rats.

6 These data are consistent with the notion that physiological amounts of glucocorticoids exert a tonic inhibitory action on phospholipase activity in normal animals and that the increased secretion of these hormones during the inflammatory response serves to check and control the development of inflammation.

Introduction

When healthy mammals are exposed to stress, injury or infection, they respond with an increased secretion of ACTH and consequently, a rise in blood glucocorticoids. This physiological response is essential for survival, but after almost forty years of discussion and research the reason for this remains a mystery (Ganong, 1979).

In a recent and very provocative review, Munck and his colleagues (Munck *et al.*, 1984) have proposed the following hypothesis: (a) that the physiological function of the stress-induced increases in glucocorticoid levels is to protect not against the source of stress itself, but against the normal defence reactions that are activated within the host by exposure to these noxious stimuli; and that (b) the glucocorticoids accomplish this function by turning off those defence reactions thus preventing them from over-shooting and themselves threatening homeostasis.

When the organism is injured or infected, one of its most important defence mechanisms is the inflammatory response. This response has as its main objectives the neutralization and removal of pathogens, the rebuilding and restructuring of diseased or injured tissue, and ultimately the restoration of normal structure and function. A biological response of the complexity of inflammation requires close control and integration, and it seems highly likely that these events are regulated primarily by the release of potent chemicals from the participating tissues and cells. In particular, eicosanoids such as the prostaglandins and leukotrienes seem to be of particular importance in controlling the local vascular events and cell migration associated with the inflammatory response.

It is now well established that an important component of the anti-inflammatory action of glucocorticoids is their ability to inhibit the enzyme phospholipase A_2 (PLA_2) thereby decreasing the formation of pro-inflammatory eicosanoids and preventing other membrane events linked to the activation of this enzyme (for a recent review on this see Flower, 1984). We and others have demonstrated that the anti-PLA_2 effect is exerted by second messenger proteins synthesized *in vitro* and *in vivo* by target cells under steroid stimulation (Blackwell *et al.*, 1980; Hirata *et al.*, 1980;

[1]Present address: The Department of Pharmacology, University of Bath, Claverton Down, Bath BA2 7AT
[2]Author for correspondence.

Cloix *et al.*, 1983; Gupta *et al.*, 1984). These proteins are able to inhibit both lysosomal and membrane bound PLA_2 (Ghiara *et al.*, 1984) and exert anti-inflammatory effects (Blackwell *et al.*, 1982; Parente *et al.*, 1984). This family of proteins has recently been renamed the lipocortins (Di Rosa *et al.*, 1984).

Adrenalectomised (ADX) rats have very small amounts of lipocortin in their body fluids, presumably because of the absence of glucocorticoids, and ACTH is unable to induce the formation of these proteins in ADX animals although it is most effective in normal rats (Blackwell *et al.*, 1982).

In this paper we describe experiments demonstrating that the magnitude and duration of the carrageenin-induced acute inflammatory response, and the formation of pro-inflammatory eicosanoids is much greater in ADX rats than in sham-operated (SHO) controls. We believe that these data support the hypothesis of Munck, and suggest a physiological role for the lipocortin proteins in the homeostatic response of animals to noxious stimuli.

Methods

Surgically prepared ADX and SHO rats (250 ± 10 g body weight) were obtained 48 h after surgery from Hacking and Churchill (Huntingdon, U.K.) and used for the experiments two weeks later. ADX rats were given 0.9% w/v NaCl solution (saline) to drink, while SHO rats were given tap water. The animals were fed on standard chow pellets *ad libitum*, and their body weight was checked every day; it showed a mean increment of 4 ± 1 g per day in both groups. Adrenalectomy was always checked macroscopically following cell collection and in some animals the serum levels of corticosterone were measured by fluorimetry (SHO 278.6 ± 38.3 ng ml^{-1}, $n = 16$; ADX 26.9 ± 4.1 ng ml^{-1}, $n = 16$). Data from ADX animals in which there was macroscopic evidence of adrenal tissue remaining were rejected.

Measurement of eicosanoid biosynthesis in vitro

Rat peritoneal leucocytes were harvested as previously described (Blackwell *et al.*, 1980): 10^6 cells were incubated at 37°C with opsonized zymosan (0.1 mg ml^{-1}) to induce eicosanoid biosynthesis. In some samples hydrocortisone (2 μM) was added at the beginning of the incubation. After 90 min the amounts of eicosanoids in the cell-free incubation medium were measured by the specific radioimmunoassays described by Salmon *et al.* (1982) and Higgs *et al.* (1983). Thromboxane B_2 (TXB_2), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and leukotriene B_4 (LTB_4) were measured in order to have an index of both cyclo-oxygenase and lipoxygenase activities.

Carrageenin pleurisy

The animals were lightly anaesthetized with ether and pleurisy was initiated by injection into the pleural cavity of 0.2 ml of a 1% w/v carrageenin suspension in saline. After 2, 4, 8 and 24 h the rats were killed by exposure to carbon dioxide and the exudates collected. The volumes were measured and the numbers of migrating leucocytes estimated with a Neubauer chamber under light microscopy (Parente *et al.*, 1979; Blackwell *et al.*, 1982). The concentration of eicosanoids in the cell-free exudates were also measured (see previous section). In those experiments in which the effect of dexamethasone was investigated, the steroid (0.1 mg kg^{-1} s.c.) was administered 30 min before carrageenin, control animals received a s.c. injection of saline (1 ml kg^{-1}), and the exudates were collected 4 h after carrageenin.

Materials

The following drugs and chemicals were used: hydrocortisone sodium succinate, zymosan A from *S. cerevisiae* yeast, lambda-carrageenin type IV (Sigma); and dexamethasone sodium phosphate (Decadron MSD). The reagents used in the eicosanoid radioimmunoassays are detailed in the papers quoted in the previous section. All other solvents, buffer salts and general reagents were of Analar grade, or the highest purity attainable. Statistical analysis was performed using paired and unpaired Student's *t* tests.

Results

Eicosanoid biosynthesis in vitro

The results of these experiments are shown in Table 1. After 90 min of incubation with opsonized zymosan leucocytes from both SHO and ADX rats released substantial amounts of eicosanoids. However, the amounts released by leucocytes from ADX animals were much greater, showing a mean net increase of 124% for TXB_2, 122% for 6-keto-$PGF_{1\alpha}$ and 167% for LTB_4. The differences in the release between SHO and ADX leucocytes were statistically highly significant. Hydrocortisone (2 μM) reduced the release of both cyclo-oxygenase and lipoxygenase metabolites significantly. The steroid had a greater effect against the leucocytes collected from ADX animals.

Carrageenin pleurisy

Figure 1 shows the pattern of the inflammation induced by carrageenin in the rat pleural cavity of the two groups of rats. The magnitude and duration of the inflammatory response in ADX rats was much greater

Table 1 Biosynthesis of eicosanoids by leucocytes from adrenalectomised (ADX) and sham-operated (SHO) rats

	Biosynthesis (ng/10^6 cells)		*% inhibition by 2 μM hydrocortisone*	
Eicosanoid	*SHO*	*ADX*	*SHO*	*ADX*
TXB_2	3.3 ± 0.1	7.4 ± 0.9*	45.5 ± 2.5†	72.2 ± 8.2††
6-keto-$PGF_{1\alpha}$	6.9 ± 0.5	15.3 ± 0.7*	ND	ND
LTB_4	0.15 ± 0.1	0.4 ± 0.01*	50.7 ± 4.1†	70.9 ± 4.4††

Cells were incubated for 90 min at 37°C with opsonized zymosan (0.1 mg ml^{-1}) (see Methods). Results represent the mean ± s.e.mean of 5 experiments.
*$P < 0.001$ significantly different from SHO values. †$P < 0.01$; ††$P < 0.001$ significantly different from control values.
ND: not measured. TXB_2 = thromboxane B_2, 6-keto-$PGF_{1\alpha}$ = 6-keto-prostaglandin $F_{1\alpha}$ and LTB_4 = leukotriene B_4.

than in SHO animals. In the latter group the response reached its peak at 8 h after the injection of carrageenin (1.5 ± 0.18 ml exudate and 141.0 ± 15 × 10^6 leucocytes per rat). In ADX rats the migration of leucocytes was maximum at 8 h (257.7 ± 32.5 × 10^6 per rat) however, the exudation peaked at 24 h (4.67 ± 0.47 ml per rat). At 4,8 and 24 h after the injection of carrageenin the differences in

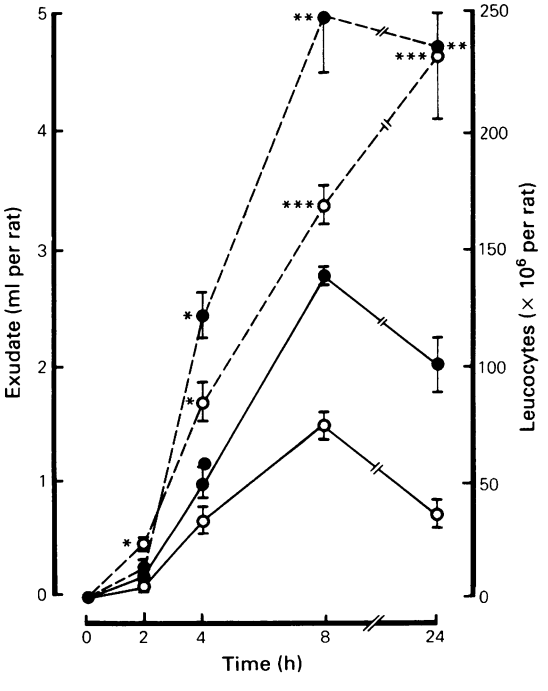

Figure 1 Exudate formation (○) and leucocyte migration (●) in pleural inflammatory exudates, collected 2, 4, 8 and 24 h after carrageenin injection, from sham-operated (SHO; continuous lines) and adrenalectomised rats (ADX; broken lines). The results are the mean of 4 experiments; vertical lines show s.e.mean. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ADX vs SHO group.

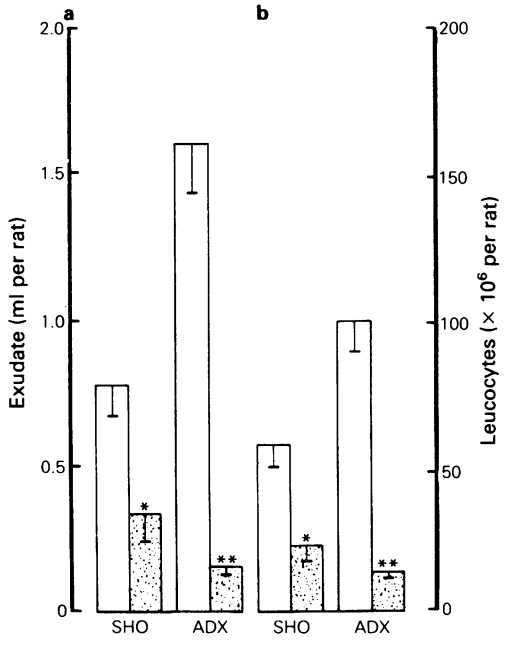

Figure 2 (a) Exudate formation and (b) leucocyte migration in pleural inflammatory exudates collected 4 h after carrageenin injection from sham-operated (SHO) and adrenalectomised (ADX) rats. Open columns: control animals receiving saline (1 ml kg^{-1} s.c.); stippled columns: animals receiving dexamethasone (0.1 mg kg^{-1} s.c. 30 min before carrageenin). The results represent the mean of 10 experiments; vertical lines show s.e.mean. *$P < 0.01$; **$P < 0.001$; treated vs control animals.

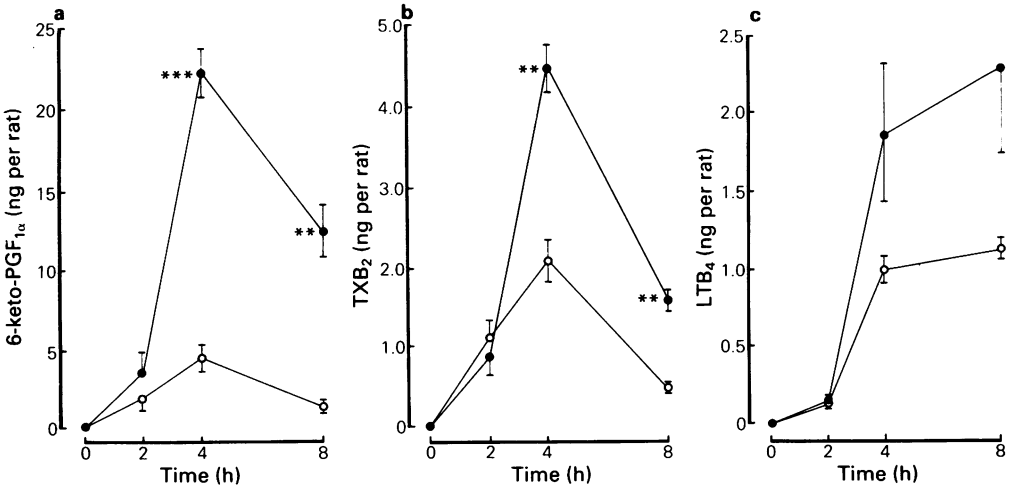

Figure 3 The 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-PGF$_{1\alpha}$) (a), thromboxane B_2 (TXB$_2$) (b) and leukotriene B_4 (LTB$_4$) (c) content of cell-free exudates, collected 2, 4 and 8 h after carrageenin injection, from sham-operated (SHO) (○) and adrenalectomised (ADX) rats (●). The results are the mean of 4 experiments; vertical lines show s.e.mean. $^{**}P<0.01$; $^{***}P<0.001$; ADX vs SHO group.

exudation and leucocyte migration between ADX and SHO animals were statistically highly significant. Dexamethasone (0.1 mg kg^{-1} s.c. 30 min before carrageenin) inhibited the exudation and leucocyte migration in both SHO and ADX rats (Figure 2). The steroid reduced the exudate formation in SHO and ADX animals by 46.4% and 91.3%, respectively. The inhibition of cell migration by the steroid in SHO and ADX animals was 60.3% and 87.0%, respectively.

Eicosanoid content of inflammatory exudates

6-keto-PGF$_{1\alpha}$, TXB$_2$ and LTB$_4$ were detected in cell-free exudates collected 2, 4 and 8 h after the administration of carrageenin. No eicosanoids were detected in 24 h exudates. The highest concentration of 6-keto-PGF$_{1\alpha}$ was observed in the 4 h exudates from both groups of animals. The amount was significantly increased in the ADX (22.3 ± 2.3 ng per rat) compared to SHO rats (4.4 ± 1.5 ng per rat $P<0.001$) (Figure 3a). The amount of TXB$_2$ detected was smaller; however, its release followed a similar pattern to that of 6-keto-PGF$_{1\alpha}$ showing a peak at 4 h and a significant difference between ADX (4.5 ± 0.5 ng per rat) and SHO animals (2.1 ± 0.5 ng per rat $P<0.01$) (Figure 3b). The release of LTB$_4$ showed a peak at 8 h after carrageenin and again a difference between ADX (2.3 ± 0.6 ng per rat) and SHO rats (1.5 ± 0.5 ng per rat) was observed but this did not achieve statistical

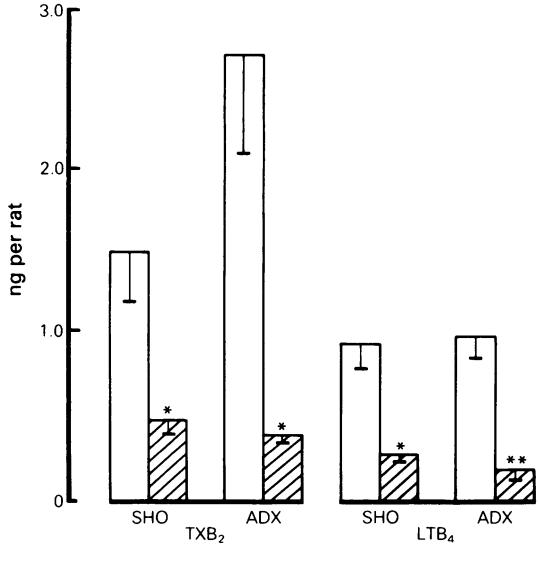

Figure 4 The thromboxane B_2 (TXB$_2$) and leukotriene B_4 (LTB$_4$) content of cell-free exudates collected 4 h after carrageenin injection from sham-operated (SHO) and adrenalectomised (ADX) rats. Open columns: control animals receiving saline 1 ml kg^{-1} s.c. Hatched columns: treated animals receiving dexamethasone 0.1 mg kg^{-1} s.c. 30 min before carrageenin. The results are the mean of 10 experiments; vertical lines show s.e.mean. $^{*}P<0.01$; $^{**}P<0.001$; treated vs control animals.

significance (Figure 3c). The release of TXB_2 and LTB_4 was inhibited by the administration of dexamethasone (Figure 4) and this inhibitory effect was more pronounced in ADX animals compared to that in the SHO group.

Discussion

Phospholipase A_2, which is presumed to initiate the release of fatty acid substrates for eicosanoid synthesis, occurs in many forms within the cell. In the case of stimuli which release eicosanoids by intereacting with surface receptors on the macrophage, it is probably the plasma membrane bound enzyme which is responsible for the hydrolysis of phosphatides. Phospholipase A_2 is a powerful hydrolytic enzyme and the notion that it is simply embedded into the membrane *per se* raises serious theoretical objections in that this would quickly lead to degradation of the lipid layer. Hirata (1981) has proposed that the plasma membrane enzyme is permanently complexed with lipocortin and that 'activation' of the phospholipase is preceded by the phosphorylation of lipocortin which inactivates this inhibitor. Reactivation of lipocortin by endogenous membrane alkaline phosphatase allows termination of phospholipase activity when the receptor phosphorylation system ceases to operate. Phosphorylation of this protein was observed in cells undergoing stimulation and coincides with periods of phospholipase A_2 activation and eicosanoid release.

Although there may be a small constitutive component, the synthesis and release of lipocortin is greatly stimulated by glucocorticosteroids and the concentrations of the protein in body fluids of adrenalectomised animals is small (Blackwell *et al.*, 1982). In 'resting' cells there is probably a fairly low level of phospholipase activity associated perhaps with changes in membrane biochemistry and this is reflected in the relatively small release of eicosanoids seen in the urine of 'normal' animals or from carefully prepared cultured cells. However, when cells are exposed to activating stimuli, such as occurs in carrageenin inflammation, there is a dramatic increase in the activity of this enzyme and a corresponding rise in the synthesis of eicosanoids.

We reasoned that when cells which could no longer make lipocortin were activated they should respond with an exaggerated eicosanoid synthesis consistent with the lack of control over phospholipase activity and, to translate these observations into an *in vivo* context, that the inflammatory reponse should also be heightened.

Both these effects were seen in the experiments described in this paper. In many cases the differences were very marked. Of course, adrenalectomy is a fairly drastic procedure and it could be argued that it was the removal of some other factor, such as mineralocorticoids or catecholamines, which was responsible for the biological effect observed.

Whilst we have not yet specifically excluded these factors it should be recognized that mineralocorticoids are without effect on phospholipase activity or inflammation (Blackwell *et al.*, 1978). Also (although they could alter the cardiovascular aspects of inflammation) it is difficult to envisage a mechanism by which circulating catecholamines could so radically alter the level of eicosanoid mediator release, especially since similar effects to those found *in vivo* were observed *in vitro* under conditions in which neither group of cells was exposed to catecholamines. Finally, the phenomenon can be completely reversed *in vivo* and *in vitro* by the administration of glucocorticoids.

Although most of the experimental evidence indicates that phospholipase A_2 is the enzyme responsible for eicosanoid release in the macrophage (at least in the circumstances described here), there is a possibility that some other cascade of enzymes (possibly including phospholipase C) was involved. During the course of this work we attempted to measure phospholipase A_2 activity in cell membrane fractions directly, but although some results indicated differences between the two groups, the data were generally difficult to reproduce and this part of the project foundered largely on technical grounds.

Elsewhere we have reported that levels of the Paf-acether precursor, lyso-Paf-acether, are elevated in inflammatory exudates in adrenalectomised rats relative to those in controls and also *in vitro* (Parente & Flower, 1985). The route by which this material is generated is much less ambiguous and almost certainly represents a straightforward phospholipase A_2 action. We therefore feel confident that the results we report are truly the result of changes in phospholipase activity.

The notion of Munck (Munck *et al.*, 1984) that the endogenous glucocorticoids act to control the intensity of our 'defence reactions' (in this case inflammation) is obviously a very powerful concept and one which can accommodate many of the experimental findings in this area. Clearly the results we have observed are also congruent with this notion, and reinforce data published elsewhere which demonstrate that the monoclonal antibody to lipocortin can prevent steroids from exerting their full local anti-inflammatory action (Flower *et al.*, 1984). The data presented here also suggest that this protein exerts an important tonic inhibitory influence upon leucocyte function.

There is an interesting parallel between the increased susceptibility of the adrenalectomised rats to inflammation and the condition of patients from whom steroids are suddenly withdrawn. Because of adrenal suppression induced by the exogenous hor-

mone, these patients are unable to produce their own glucocorticoids (and are thus temporarily 'adrenalectomised') and suffer a 'rebound' period during which the pain and inflamation appears greatly exacerbated. The molecular mechanism which is responsible for this well known clinical condition may well involve the temporary absence of lipocortin in the plasma and body fluids of these people.

We are very grateful to Dr D. Riddall for performing the corticosterone estimations and to Mrs E. Betts for skilful technical assistance.

References

BLACKWELL, G.J., CARNUCCIO, R., DI ROSA, M., FLOWER, R.J., LANGHAM, C.S.J., PARENTE, L., PERSICO, P., RUSSELL-SMITH, N.C. & STONE, D. (1982). Glucocorticoids induce the formation and release of anti-inflammatory and anti-phospholipase proteins into the peritoneal cavity of the rat. *Br. J. Pharmac.*, **76,** 185–195.

BLACKWELL, G.J., CARNUCCIO, R., DI ROSA, M., FLOWER, R.J., PARENTE, L. & PERSICO. P. (1980). Macrocortin: a polypeptide causing the anti-phospholipase effect of glucocorticoids. *Nature*, **287,** 147–149.

BLACKWELL, G.J., FLOWER, R.J., NIJKAMP, F.P. & VANE, J.R. (1978). Phospholipase A_2 activity of guinea-pig isolated perfused lungs: stimulation and inhibition by anti-inflammatory steroids. *Br. J. Pharmac.*, **62,** 79–89.

CLOIX, J.F., COLARD, O., ROTHHUT, B. & RUSSO-MARIE, F. (1983). Characterisation and partial purification of 'renocortins', two polypeptides formed in renal cells causing the anti-phospholipase-like action of glucocorticoids. *Br. J. Pharmac.*, **79,** 313–321.

DI ROSA, M., FLOWER, R.J., HIRATA, F., PARENTE, L. & RUSSO-MARIE, F. (1984). Nomenclature announcement, Anti-phospholipase proteins. *Prostaglandins*, **28,** 411–442.

FLOWER, R.J. (1984). Macrocortin and the anti-phospholipase proteins. In *Advances in Inflammation Research*, Vol. 8, ed. Weissmann, G., pp. 1–34. New York: Raven Press.

FLOWER, R.J., WOOD, J.N. & PARENTE, L. (1984). Macrocortin and the Mechanism of Action of the Glucocorticoids. In *Advances in Inflammation Research*. Vol. 7, ed. Otterness, I., Capetola, R. & Wong, S. pp. 61–78. New York: Raven Press.

GANONG, W.F. (1979). The adrenal medulla and adrenal cortex. In *Review of Medical Physiology*, 9th Edition. Ch. 20. pp. 277–300. Los Altos, U.S.A.: Lange Medical Publications.

GHIARA, P., MELI, R., PARENTE, L. & PERSICO, P. (1984). Distinct inhibition of membrane bound and lysosomal phospholipase A_2 by glucocorticoid induced proteins. *Biochem. Pharmac.*, **33,** 1445–1450.

GUPTA, C., KATSUMATA, M., GOLDMAN, A.S., HEROLD, R. & PIDDINGTON, R. (1984). Glucocorticoid-induced phospholipase A_2 inhibitory proteins mediate glucocorticoid teratogenicity *in vitro*. *Proc. natn. Acad. Sci. U.S.A.*, **81,** 1140–1143.

HIGGS, G.A., MONCADA, S., SALMON, J.A. & SEAGER, K. (1983). The source of thromboxane and prostaglandin in experimental inflammation. *Br. J. Pharmac.*, **79,** 863–868.

HIRATA, F. (1981). The regulation of lipomodulin, a phospholipase inhibitory protein, in rabbit neutrophils by phosphorylation. *J. biol. Chem.*, **256,** 7730–7733.

HIRATA, F., SCHIFFMANN, E., VENKATASUBRAMANIAN, K., SALOMON, D. & AXELROD, J. (1980). A phospholipase A_2 inhibitory protein in rabbit neutrophils induced by glucocorticoids. *Proc. natn. Acad. Sci.. U.S.A.*, **77,** 2533–2536.

MUNCK, A., GUYRE, P.M. & HOLBROOK, N.J. (1984). Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocrine Rev.*, **5,** 25–44.

PARENTE, L., DI ROSA, M., FLOWER, R.J., GHIARA, P., MELI, R., PERSICO, P., SALMON, J.A. & WOOD, J.N. (1984). Relationship between the anti-phospholipase and anti-inflammatory effects of glucocorticoid induced proteins. *Eur. J. Pharmac.*, **99,** 233–239.

PARENTE, L. & FLOWER, R.J. (1985). The generation of lyso-PAF in experimental inflammation. In *Inflammatory Mediators*. ed. Higgs, G.A. &. Williams, T.J. pp. 65–71. Basingstoke: Macmillan Press.

PARENTE, L., KOH, M.S., WILLOUGHBY, D.A. & KITCHEN, A. (1979). Studies on cell motility in inflammation. I. The chemotactic activity of experimental inflammatory exudates. *Agents Actions*, **9,** 196–200.

SALMON, J.A., SIMMONS, P.M. & PALMER, R.M.J. (1982). A radio-immunoassay for leukotriene B_4. *Prostaglandins*, **24,** 225–235.

(Received August 9, 1985.
Revised October 8, 1985.
Accepted October 10, 1985.)