

## ELEVENTH GADDUM MEMORIAL LECTURE

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# Lipocortin and the mechanism of action of the glucocorticoids

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### Pharmacologists have neglected the glucocorticoids

Because of a curious division of labour amongst life scientists, the hormones of the adrenal medulla have traditionally been considered the preserve of pharmacologists, whilst those of the adrenal cortex have been studied almost exclusively by endocrinologists or (more recently) by molecular biologists.

This state of affairs is especially strange when one considers the importance of steroids and their synthetic congeners in clinical medicine: the glucocorticoids are by far the most potent and effective anti-inflammatory agents we possess and unlike the aspirin-like drugs, they are active against virtually every type of inflammatory disease. Asthma, psoriasis, multiple sclerosis and the arthritides are all susceptible to glucocorticoid therapy – indeed it is often the only form of therapy that is effective in such diseases. Because of this unusually wide spectrum of activity these drugs are extensively used with some 15 million prescriptions being issued each year in the U.K. alone.

Another feature of the glucocorticoids which should endear them to pharmacologists is that they initiate their biological effects by combining with a soluble receptor, whose structure and function are well understood (indeed it is probably the best characterized of all receptors), for which a complete range of agonists, antagonists and partial agonists is available, and which at the very least could surely teach us a very great deal about the basic biology of drug-receptor interactions.

How is it then that the field of glucocorticoid pharmacology has fallen into such neglect? The reason, as we shall see, seems to be connected with early ideas about the function of these hormones, and to a certain extent, with misconceptions about the nature of the inflammatory response itself. What-

ever the reason however, Sir John Gaddum would surely not have approved. In 1954 he wrote,

‘Pharmacology is not only the handmaid of therapeutics; it is a science in its own right, and has many other tasks to perform. The most important one is to find out how drugs act’.

In this lecture I will review work by myself and my colleagues, as well as other scientists, which suggests that one mechanism by which these drugs act is by the induction and release of a regulatory protein called lipocortin. The work which led up to the discovery of lipocortin was surely very much in the Gaddum tradition for he always championed the use of bioassay techniques as sensitive tools for the detection of new substances. But before describing these observations in detail let us first glance at the history of the field.

### Inflammation is a defence mechanism

Unlike machines, living beings are able to recover spontaneously from most of the damage sustained during their lifetime. Injury to, or infection of, the host evokes a well-tested repertoire of responses collectively known as ‘inflammation’. This sophisticated and singularly effective process has as its main objectives the neutralisation and removal of pathogens, the rebuilding and restructuring of diseased or injured tissue, and ultimately the restoration of normal structure and function.

The complexity of inflammation rivals that of any other physiological system and, as may be imagined, is closely controlled and regulated. Phagocytic cells

must be summoned to the inflammatory site, the immune system must be primed, there must be an increased access of certain plasma proteins to the site of injury and many other concomitant local and systemic changes must also occur.

Normally, the inflammatory response is a spectacularly successful 'defence mechanism'; invading organisms are neutralised (and in many cases can never regain access to the host), injury resolves spontaneously with healing occurring and complete restoration of function. Like all other biological systems however, the inflammatory response occasionally goes awry and under these circumstances the body's response to disease may assume the character of a 'disease' in itself. Chronic inflammatory conditions such as rheumatoid arthritis probably fall into this category. Although the agent which triggers the original inflammation is seldom identified, the resulting reaction causes severe local tissue damage; this exacerbates and perpetuates the inflammation which in turn causes more damage and so on. The inflammatory response never terminates under these circumstances. Little if any healing occurs, and a continuing cycle of inflammation-tissue destruction-further inflammation is established.

It is upon such occasions that the physician intervenes to quench the inflammation with drugs and other treatment. We know from the work of Vane and his colleagues (see below) that the most common anti-inflammatory drugs, the aspirin-like drugs, seem to work by preventing the generation of the pro-inflammatory prostaglandins thus relieving the symptoms of the illness, but the mechanism of action of the more potent glucocorticoids, has always been unclear.

The anti-inflammatory steroids are analogues of the endogenous steroid hydrocortisone. All healthy mammals have this hormone (or a closely related compound) in their blood and there is a marked diurnal variation in secretion. For many years our ideas about the function of the glucocorticoids in the regulation of normal physiology were hazy and unclear. It had been known since the 1930s that when stressed, injured or infected, man and other animals invariably responded with a rise in blood steroid levels. Hans Selye, the Austrian-born physiologist, regarded this response as a defensive manoeuvre and believed that steroids were somehow responsible for the mounting of inflammatory response. Selye listed many other physiological events which occurred in response to stress and noted that these tended to be stereotyped in nature. He called these responses 'The General Adaptation Syndrome' (Selye, 1946).

For a long while Selye's ideas about steroid physiology held sway (even today they continue to exert a considerable influence on our thinking), but an unex-

pected finding made shortly after the Second World War cast doubt upon this notion. Phillip Hench was an American physician who was struck by the fact that arthritic women who developed jaundice or became pregnant, frequently experienced a dramatic remission of their symptoms. During pregnancy, blood glucocorticoids levels rise dramatically and Hench surmised that these hormones were responsible for the anti-inflammatory action. Cortisol had just been isolated and was shortly to be totally synthesized, so Hench was able to test the hormone in patients with rheumatoid arthritis and other chronic inflammatory diseases. He found it to be dramatically effective (Hench *et al.*, 1949). This observation laid the foundation for modern day glucocorticoid therapy for it was soon found that these drugs are exceedingly effective against almost any type of inflammation, regardless of its cause.

This demonstration that the glucocorticoids were actually *anti-inflammatory* caused consternation because it was not in accordance with contemporary notions of their normal function, largely based on Selye's work. To circumvent this problem the scientific community adopted an odd compromise: the idea that in 'physiological' amounts these hormones had one type of effect, whereas in 'pharmacological' amounts they had another, often opposing, action (cf. Gaunt, 1974).

Recently a new proposal has been put forward by Munck and his colleagues (Munck *et al.*, 1984) which suggests that the physiological function of the glucocorticoids secreted during stress or injury is *not to protect against the source of the stress itself* (as was originally believed) *but to protect the organism against the normal defence mechanisms which are activated by stress*. In other words, glucocorticoid secretion is one arm of a homeostatic mechanism which checks and controls the activity of the inflammatory (and other) responses. This hypothesis is, I believe, of great explanatory power. It reconciles a great many, apparently contradictory, observations and may well herald a renaissance of interest in glucocorticoid physiology and pharmacology.

One of the most important and fundamental findings in the field of glucocorticoid biology was the discovery of the glucocorticoid receptor (Munck & Brinck-Johnson, 1967; 1968; Schaumburg & Bojesen, 1968) and the elucidation of the mechanism whereby occupation of the intracellular receptor protein leads to an increase or decrease in the transcription of certain genes, thus bringing about the biological action of the steroids (Buller & O'Malley, 1976; Chan & O'Malley, 1976; Baxter 1976; Munck & Leung, 1977). This is sometimes known as the 'classical pathway of steroid action'.

It is now clear that these receptors are present in most, if not all, cells in the mammalian body and

that the overwhelming majority of the biological action of the glucocorticoids are brought about by steroid-receptor interactions and their sequelae. In the face of this knowledge, the concept that there are 'physiological' and 'pharmacological effects' is vanishing and is being replaced with the unifying notion that 'Glucocorticoid effects can consequently be defined succinctly as those which are mediated by glucocorticoid receptors' (Munck *et al.*, 1984).

This generalisation extends to inflammation – at least acute inflammation – for Tsurufji and his colleagues (1979) have demonstrated that the anti-inflammatory action of dexamethasone in rats can be blocked by the administration of inhibitors of protein and RNA synthesis. Additionally, the author and his colleagues have shown that glucocorticoid receptor antagonists can block the anti-inflammatory actions of these drugs providing evidence for the 'classical pathway of steroid action in acute inflammation' (Peers *et al.*, 1988).

#### **Many aspects of the inflammatory response are regulated by lipid mediators**

I have argued that inflammation is a biological response of great sophistication and complexity requiring integration and tight regulation. How is it controlled?

The most plausible mechanistic explanation yet to emerge is the 'chemical theory' and during the last decade this has dominated the attention of most biomedical researchers. According to this idea, chemicals released from dead, dying, 'activated' or injured cells are responsible for initiating the familiar signs and symptoms of inflammation together with many of the underlying pathological changes and systemic effects which are also seen. Histamine, 5-hydroxytryptamine and bradykinin, are amongst the local hormones that have been implicated in the development of inflammation, but it is the lipid mediators such as the prostaglandins, leukotrienes and platelet activating factor (Paf) which have currently captured the imagination of the research community and which have been the focus of intense study.

It is not my intention to review the compelling evidence that suggests a role for these mediators in the inflammatory response (cf. Ferreira & Vane, 1979; Piper, 1983; Bray, 1983 for a detailed guide to the bibliography covering this section) but certain aspects of their biosynthesis have to be mentioned here so that the arguments made later in this paper will appear clear.

Both the prostaglandins and the leukotrienes (collectively known as the eicosanoids) are biosynthesized from polyunsaturated fatty acids, pre-eminent amongst which in land-dwelling mammals,

is arachidonic acid. This fatty acid (as well as some other related compounds) can be transformed by the fatty acid cyclo-oxygenase into the cyclic endoperoxides and thence prostaglandins, thromboxanes and prostacyclin, and by other enzymes into leukotrienes. The first of these transformations may occur in practically any cell in the body (with the exception of the erythrocyte) whilst the latter series of reactions occur mainly in leukocytes (cf. Hamberg *et al.*, 1974; 1975; Murphy *et al.*, 1979; Moncada, 1982).

Paf is probably biosynthesized in a two-stage process in which a specific ether phosphatide precursor is catalytically hydrolysed to lyso-Paf by phospholipase A<sub>2</sub> and then acetylated by a specific acetyl transferase (Albert & Snyder, 1983). It has been observed that a high proportion of this ether phosphatide precursor also contains arachidonic acid esterified to the 2'-position such that cleavage by phospholipase liberates both the precursors for the eicosanoids, and that for Paf simultaneously (Albert & Snyder, 1984).

Biologically active lipids are, of course, not the only chemicals involved in inflammation. For example, complement plays a role in cell migration and oedema formation, and fever – a common sequel to inflammation is regulated by the release from macrophages of yet another type of chemical, the polypeptide interleukine 1. Other, less well characterized factors regulating immune and other functions are also generated by lymphocytes and influence other cells such as monocytes. There is evidence for substantial interactions between inflammatory mediators (see for example Williams, 1983).

Finally, there must be some mechanism, as yet unidentified, but presumably also dependent on the release of chemicals, which terminates the inflammatory response and initiates the healing process.

#### **Early work suggested glucocorticoids were without effects on prostaglandin synthesis**

When the inhibitory action of aspirin on the prostaglandin forming cyclo-oxygenase was discovered, many other types of drugs were tested as putative inhibitors (Vane 1971; Ferreira *et al.*, 1971; Smith & Willis, 1971; Flower *et al.*, 1972). Amongst inactive compounds in this test were the narcotic analgesics and the anti-inflammatory glucocorticoids. Actually some inhibition was seen at high concentrations by some other workers (Greaves & Macdonald Gibson, 1972) but it is important to note that these experiments were all performed with broken cell preparations. The negative effects observed with the glucocorticoids were puzzling, because although the steroidal drugs were many times more active in experimental models of inflammation, they were all

**Table 1** Glucocorticoids have potent anti-inflammatory actions but do not inhibit the prostaglandin-forming cyclo-oxygenase directly

Class	Drug	Cyclo-oxygenase (IC <sub>50</sub> µg ml <sup>-1</sup> )	Rat paw edema (ED <sub>50</sub> mg kg <sup>-1</sup> )
Aspirin-like	Meclofenamic acid	0.03	15.0
	Niflumic acid	0.03	47.0
	Indomethacin	0.06	6.0
	Mefenamic acid	0.17	68.0
Glucocorticoid	Dexamethasone	≥ 100	0.01
	Triamcinolone	≥ 100	0.08
	Hydrocortisone	≥ 100	13.0

Data from Flower *et al.* (1972).

inactive against the cyclo-oxygenase enzyme, and thus, by implication on prostaglandin synthesis (see Table 1).

Whilst these findings suggested that the glucocorticoids had no effect on the prostaglandin system, a number of observations soon began to appear in the literature which apparently contradicted this conclusion. In 1974, Herbaczynska-Cedro & Staszewska-Barczak demonstrated that the release of a prostaglandin-like substance into the venous blood of exercising dogs was blocked by hydrocortisone. Shortly after this came a report by Lewis & Piper in which it was observed that steroids could antagonize the release from isolated fat pads of prostaglandin E<sub>2</sub> which accompanies ACTH-induced vasodilatation (Lewis & Piper, 1975). The suggestion made at that time to explain these findings was that the glucocorticoids were preventing the release of prostaglandins from the adipocytes, rather than their biosynthesis but results of other experiments by different groups suggested another interpretation. In 1975, Gryglewski and his colleagues found that two glucocorticoids, hydrocortisone and dexamethasone, prevented the noradrenaline-induced release of prostaglandins from rabbit perfused mesenteric vascular bed as well as the immunologically-induced release of prostaglandins from guinea-pig lungs. The direct conversion of arachidonic acid into prostaglandins in these preparations was not blocked by the glucocorticoids indicating that they had no inhibitory activity on the cyclo-oxygenase itself.

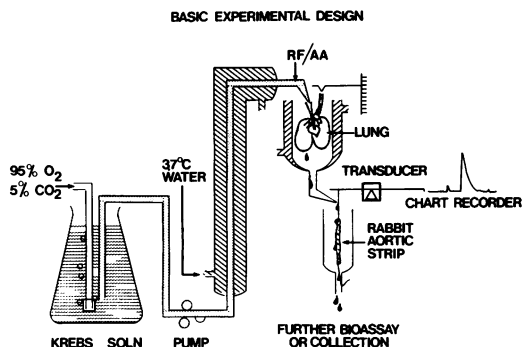
Because the rate-limiting step in prostaglandin synthesis seems to be the release of arachidonic acid from some intracellular lipid pool, probably the phosphatide pool, it was suggested that the glucocorticoids were interfering with substrate release from membrane phospholipids. This conclusion was supported by some elegant experiments from Levine's group published in 1976. Using cultured fibroblasts labelled with tritiated arachidonic acid, this group was able to demonstrate that the anti-inflammatory

glucocorticoids prevented phospholipid deacylation and arachidonic acid release which accompanied stimulation by various agents (Hong & Levine, 1976). Levine's group proposed that the mechanism of action of these drugs was to prevent substrate release and suggested that this might underlie their inflammatory action. Several other groups also reported that steroids could prevent synthesis of prostaglandins by intact cells and in each case the mechanism was consistent with an action on the supply of substrate.

#### The site of glucocorticoid action was examined by use of the guinea-pig perfused lung

In addition to the studies cited above the author and his colleagues, then at the Wellcome Foundation, were also studying the effect of steroids upon prostaglandin synthesis and release in the light of some early work implicating the enzyme phospholipase A<sub>2</sub> in this event (Flower & Blackwell, 1976). The system used was the guinea-pig perfused isolated lung, a simple technique which has proved extremely useful to workers in the prostaglandin field (Piper & Vane, 1969).

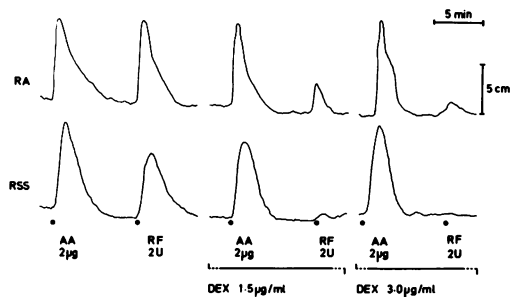
The perfused lungs can be induced to release thromboxane and other prostaglandin endoperoxide derivatives in two ways: firstly, by the injection or infusion into the lungs of the substrate arachidonic acid, and secondly by the injection of 'releasing factors' such as histamine, bradykinin, SRS-A (as it used to be known) or antigen. Figure 1 shows the basic experimental set up used for this work. All these substances apparently release eicosanoids (as detected by superfusion bioassay) by liberating arachidonic acid within the lung tissue by some ill-defined mechanism. Under investigation at that time was another releasing factor, RCS-RF (an acronym of Rabbit Aorta Contracting Substance - Releasing Factor), a low molecular weight peptide found in



**Figure 1** The guinea-pig perfused isolated lung was used for many experiments on steroid hormone action. The procedure was adapted from that described by Piper & Vane (1969): Krebs solution gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> was pumped at 10 ml min<sup>-1</sup> through a catheter inserted into the pulmonary artery into the pulmonary circulation of a guinea-pig isolated lung. The left heart was cut away allowing the perfusate to escape and this was then diverted over a series of bioassay tissues selected for their sensitivity to eicosanoids. The rabbit aortic strip is particularly sensitive to prostaglandin endoperoxides and thromboxane A<sub>2</sub>. The release of these substances can be elicited by the injection of arachidonic acid substrate (AA) or releasing factors (RF) into the pulmonary artery catheter. The isolated smooth muscles used for the bioassay are rendered insensitive to the direct action of AA or RF by the continuous infusion over the tissues of a mixture of antagonists to histamine, 5-hydroxytryptamine, catecholamines and acetylcholine together with indomethacin to prevent the endogenous synthesis of prostaglandins.

immunologically-shocked lung effluent (Piper & Vane, 1969). RCS-RF had no direct effect upon the assay tissues but when injected into the lung it caused a release of biologically active substances identified as a mixture containing chiefly thromboxane and prostaglandin endoperoxides. Arachidonic acid injections induced a similar effect.

When a glucocorticoid such as dexamethasone was infused into this preparation, it was observed that the generation of biologically active substances elicited by the releasing factor was blocked whereas the conversion of arachidonic acid was unimpaired (Nijkamp *et al.*, 1976). There was always a time delay with the first effects of steroid being observed after about 30 min of infusion. To produce greater than 50% inhibition it was often necessary to continue the infusion for 45–60 min or even longer. Of course, when indomethacin was given to the lung preparation, the generation of products in response to both arachidonic acid and RCS-RF was blocked.



**Figure 2** The glucocorticoid dexamethasone (DEX) inhibits the release of thromboxane A<sub>2</sub> (TXA<sub>2</sub>) caused by the injection into the guinea-pig perfused lung of RCS-RF (RF) but not arachidonic acid (AA). The release of TXA<sub>2</sub> was detected by a rabbit aorta strip (RA) and rat stomach strip (RSS) superfused in series. Contractions were recorded isotonically. Injections of AA or RF were made into the pulmonary artery catheter. Dexamethasone was infused for 30 min (indicated by the dotted lines) in the concentrations indicated before TXA<sub>2</sub> release was elicited by AA or RF. Reprinted by permission from Blackwell *et al.* (1978) *Br. J. Pharmacol.*, 62, 79–89.

Figure 2 shows the results of an experiment done at that time.

Dexamethasone was not the only steroid able to produce this effect: all the common glucocorticoids shared this activity (see Table 2). Mineralocorticoids and most sex steroids were inactive in this test (although in some experiments oestradiol seemed to have a similar action). Further experiments revealed that the releasing activity of some other agents such as 5-HT and histamine was also blocked by the steroids, although curiously, the action of bradykinin was not (see Figure 3). The steroid-induced block of eicosanoid release was not irreversible. If the infusion of the drug was stopped the inhibition gradually faded with (in most cases) a complete return to control values within 60–90 min (see Figure 4).

Studies in which the arachidonic acid content of the perfusate was measured by gas chromatography indicated that when releasing factors were injected into the perfused lung there was a transient release of arachidonic acid into the effluent, and that during steroid infusion, this release was inhibited or reduced. This inhibition was easily reversed when the steroid infusion was terminated (Nijkamp *et al.*, 1976).

#### Phospholipase A<sub>2</sub> is activated by releasing factors

The most widely accepted idea at this time was that the major store of polyunsaturated fatty acid precursors in the cell were the phospholipids, and that it

**Table 2** All glucocorticoids tested inhibited the release of thromboxane A<sub>2</sub> (TXA<sub>2</sub>) from guinea-pig perfused lungs and their rank order of potency strongly resembles that of their anti-inflammatory activity

Class	Steroid	Inhibition of TXA <sub>2</sub> release (IC <sub>50</sub> , µg ml <sup>-1</sup> )	Relative potency*	Relative anti-inflammatory potency
Glucocorticoid	Dexamethasone	1.4	31.2	25.0
	Betamethasone	1.4	31.2	25.0
	Triamcinolone	4.2	10.4	5.0
	Fludrocortisone	5.1	8.5	10.0
	Prednisolone	6.4	6.8	4.0
	Corticosterone	33.6	1.3	0.4
	Cortisone	43.5	1.0	0.8
	Hydrocortisone	43.8	1.0	1.0
Sex steroid	Oestradiol 17-β	1.9	23.1	?
	Progesterone	≥ 100	≤ 0.01	?
	Testosterone	≥ 100	≤ 0.01	Nil
Mineralocorticoid	Aldosterone	≥ 80.0	≤ 0.5	Nil

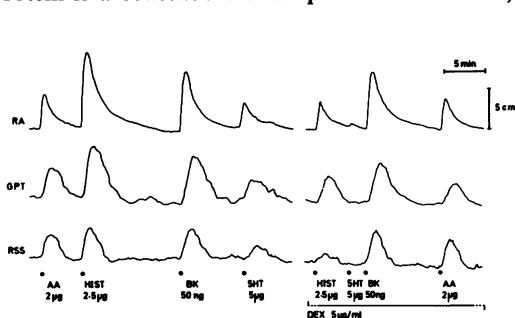
Data compiled mainly from Blackwell *et al.* (1978).

\* Hydrocortisone = 1

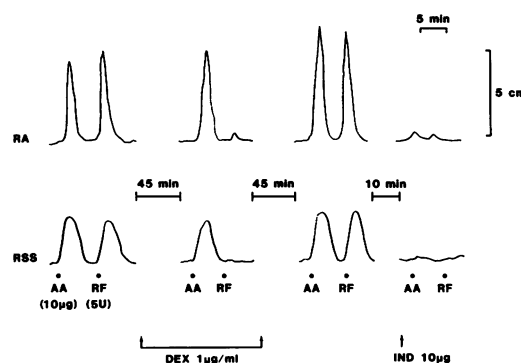
was the liberation of arachidonic acid from this pool under the influence of the enzyme phospholipase A<sub>2</sub> which was the first step in the generation of prostaglandins. Virtually all mammalian cells contain phospholipases A<sub>2</sub> and there are several types which differ in their pH optima, calcium requirement and subcellular location (see review by Van den Bosch, 1980), but it seemed highly likely that the enzyme most relevant to the generation of arachidonic acid was the plasma membrane phospholipase A<sub>2</sub>. This protein is a constituent of all plasma membranes, it

has a requirement for calcium, and a neutral or alkaline pH optimum. If such a potent hydrolytic enzyme were simply embedded in the membrane it would digest the membrane phospholipids very rapidly. Since this obviously does not happen, the enzyme must be present as some functionally inactive complex which can be 'switched on' in some way by stimuli known to release arachidonic acid.

To test if this concept obtained in the lung, a spe-



**Figure 3** The glucocorticoid dexamethasone (DEX) inhibits the release of thromboxane A<sub>2</sub> (TXA<sub>2</sub>) caused by injection into a perfused guinea-pig lung of histamine (HIST) and 5-hydroxytryptamine (5-HT) but not bradykinin (BK). Three isolated smooth muscles were used to detect the release of TXA<sub>2</sub>: the rabbit aortic strip (RA) the guinea-pig trachea (GPT) and the rat stomach strip (RSS). The left hand panel shows the control responses and the right hand panel the responses obtained following a 30 min infusion of dexamethasone. Note that the release of TXA<sub>2</sub> by histamine or 5-HT is diminished but not that induced by bradykinin or arachidonic acid. Reprinted by permission, from Blackwell *et al.* (1978) *Br. J. Pharmacol.*, 62, 79–89.



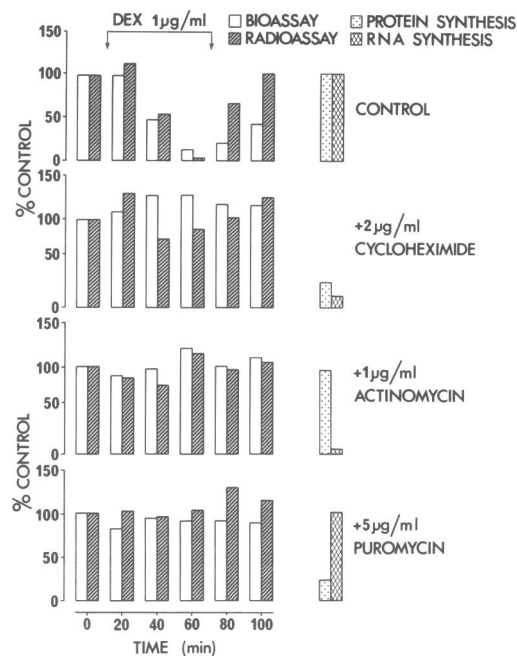
**Figure 4** The dexamethasone (DEX) inhibition of RCS-RF (RF)-induced thromboxane A<sub>2</sub> (TXA<sub>2</sub>) release from the guinea-pig perfused lung is reversible. The isolated tissues used in this experiment were the rabbit aortic strip (RA) and the rat stomach strip (RSS). The figure shows the record obtained in response to arachidonic acid (AA) or RCS-RF (RF) injections given 45 min before, during, or 45 min after the termination of an infusion of dexamethasone (1 µg/ml). The right hand panel shows that a single injection of indomethacin (IND) depresses the release of TXA<sub>2</sub> caused by both agonists.

cifically labelled phospholipid substrate of phospholipase  $A_2$  was synthesized with a radioactive fatty acid in the 2'-position. The author and his colleagues reasoned that if a cell membrane phospholipase was attached to the surface of some cell-type in the lung (as it is with several other cell types) then it should be revealed by examining the hydrolysis of the radioactive fatty acid.

When aliquots of the labelled isotope (suspended as micellae in buffer) were injected into the pulmonary circulation of the lung we found that there was a 'background' hydrolysis of the labelled phospholipid and that this was strongly inhibited by infusions of glucocorticoids such as dexamethasone (Blackwell *et al.*, 1978). Again, the onset of the steroid effect occurred after a time delay and was reversible upon the termination of the infusion. Experiments of a similar nature also determined that phospholipase  $A_2$ -like activity was increased by the injection of releasing factors such as RCS-RF, histamine and SRS-A and that this elevated hydrolytic activity was also blocked by glucocorticoids.

The obvious question was whether or not the glucocorticoids caused the inhibition of phospholipase activity by the 'classical pathway' of steroid action. We found that there was indeed a high affinity glucocorticoid binding protein in the cell-free supernatants of the guinea-pig lung (Flower & Blackwell, 1979). The receptor was detected by use of tritiated dexamethasone as a ligand, and this was displaced from the receptor with unlabelled dexamethasone, hydrocortisone or the glucocorticoid receptor antagonist corticosterone. Interestingly, this latter compound also partially inhibited the effect of the steroid in the perfused lung preparation. Further experiments established that the anti-phospholipase effect of steroids in the lungs could be reversed by inhibition of RNA synthesis (by agents such as actinomycin D) and of protein synthesis (by agents such as cycloheximide and puromycin): Figure 5 illustrates this point well. Danon & Assouline (1978) had also observed that the glucocorticoid effects on prostaglandin production by renal interstitial cells depended upon unimpaired RNA and protein synthesis and going even further, Russo-Marie and her colleagues (1979) demonstrated unequivocally that the inhibitory action of corticosteroids on prostaglandin synthesis by rat renomedullary cells is mediated through receptor occupancy and requires ongoing RNA and protein synthesis.

All these experiments strongly suggested that steroids inhibited phospholipase  $A_2$  by a mechanism depending upon glucocorticoid receptor occupancy, followed by *de novo* RNA and protein synthesis and begged the question of whether the glucocorticoids were inducing the synthesis or release of an inhibitor of phospholipase  $A_2$ .



**Figure 5** Inhibition of protein or RNA synthesis by cycloheximide, actinomycin or puromycin prevents the inhibition by dexamethasone (DEX) of phospholipase-dependent events in the guinea-pig perfused lung. Phospholipase  $A_2$ -activity was measured in two ways: by a bioassay technique (open columns) in which the inhibition of RCS-RF-induced thromboxane  $A_2$  release was measured relative to that induced by arachidonic acid and by a radiochemical assay (shaded columns) in which the hydrolysis of labelled phosphatidylcholine was measured after passage through the pulmonary circulation. All the results from these assays were normalised so that they could be represented on a single axis.

The cross-hatched and stippled columns on the right indicate protein and RNA synthesis in the lung measured as a percentage of control synthesis. Assessment of these parameters was made by measuring the incorporation of labelled precursors into macromolecules.

The figure shows that dexamethasone reversibly depresses phospholipase  $A_2$  activity in the perfused lung with a latent period of 30–40 min and that this is prevented by treatment of the organ with agents which depress RNA or protein synthesis. Reprinted, by permission, from Flower & Blackwell (1979) *Nature*, 278, 456–459.

#### A 'second messenger' of steroid action detected in the lung and macrophage

To search for an inhibitor of phospholipase we devised a bioassay experiment in which two guinea-pig isolated lungs were perfused in series (Flower &

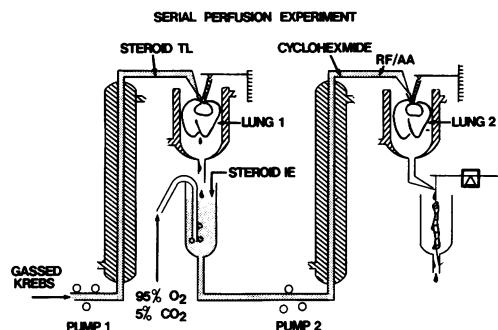


Figure 6 A 'second-messenger' of steroid action was detected using an experimental design in which two guinea-pig isolated lungs were perfused in series. The effluent from the first lung was re-oxygenated and pumped into the second lung. The latter organ was used for the measurement of phospholipase  $A_2$  activity using a bioassay or radiochemical assay technique. This lung also received a constant infusion of cycloheximide to render it insensitive to the direct actions of glucocorticoids. Dexamethasone was infused either through the pulmonary circulation of the first lung (TL) or into the effluent draining the tissue (IE).

If dexamethasone given TL induced a soluble inhibitor of phospholipase  $A_2$  activity then it should pass from the first ('generator') lung to the second ('target') lung and there cause an effect on phospholipase-dependent events. Infusions of dexamethasone IE produce no effect because of the presence of cycloheximide, thus the only difference between TL or IE infusions must be caused by contact between the glucocorticoid and normal lung tissue.

Blackwell, 1979). The design of the experiment (see Figure 6) was such that the outflow from one lung (generator lung) was pumped into a second lung (target lung) in which phospholipase activity was assessed by the radiochemical assay or bioassay (i.e. release of prostaglandins). The second lung was rendered insensitive to steroids by the continuous infusion of cycloheximide. The design of this experiment meant that when a steroid was infused directly into the second lung, there was little or no effect on phospholipase activity. However, when the drug was infused into the first lung, we observed that the phospholipase activity of the second test lung declined after a short lag period (see Figure 7). The most obvious interpretation of these experiments was that a soluble inhibitor of phospholipase was being secreted from the first lung under steroid stimulation and was being transported in the perfusate into the second lung.

In another type of experiment which also reinforced this idea, two lungs were independently perfused: one of these received steroid, the other, vehicle (see Figure 8). The perfusates were collected and

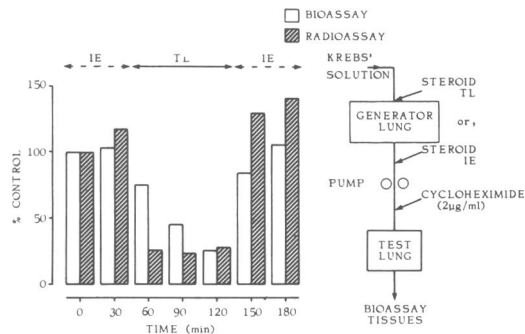
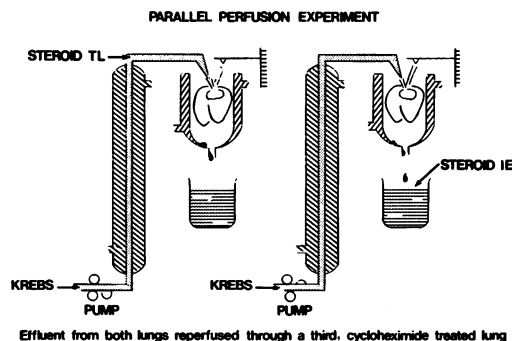


Figure 7 Infusion of dexamethasone into guinea-pig perfused lungs causes the release of a second messenger which depresses phospholipase activity. The experimental design was that outlined in Figure 6. When dexamethasone was infused IE there was no change in the phospholipase  $A_2$  activity of the 'target' lung as estimated by a bioassay (open columns) or a radiochemical (shaded column) technique. When the dexamethasone was infused TL however there was a prompt fall in the phospholipase activity in the target lung which was reversed when the infusion was once again changed to the IE route. This was taken to indicate that a 'second messenger' appeared in the perfusate when steroids were in contact with the cells in the untreated lung. Reprinted, by permission, from Flower & Blackwell (1979) *Nature*, 278, 456-459.

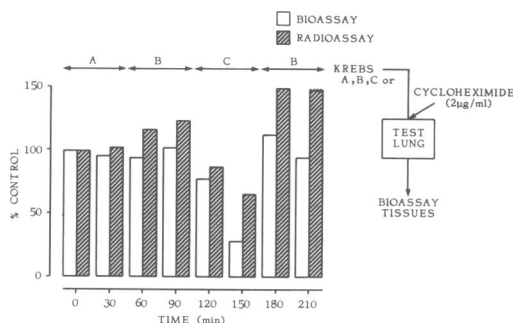
later tested on a third cycloheximide-treated lung. The perfusate from the steroid-treated lung blocked phospholipase activity in the cycloheximide-treated lung but the perfusate from the control lung was



Effluent from both lungs reperfused through a third, cycloheximide treated lung

Figure 8 A different type of experimental design used to search for a steroid-induced second messenger. In this scheme, two guinea-pig isolated lungs were perfused in parallel, one received dexamethasone as a constant infusion through the pulmonary artery (TL) whilst the other received an equivalent amount directly into the effluent (IE). After collecting the perfusate from each lung for 1 h it was re-oxygenated and tested on a fresh cycloheximide-treated organ. Although both aliquots of perfusate contain equivalent amounts of steroid, only one should contain induced second messengers.



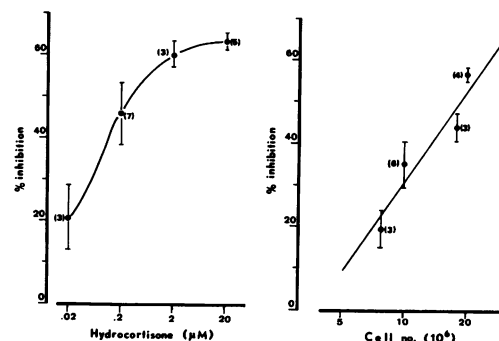


**Figure 9** Effluent from steroid-treated lungs contains a 'second messenger' which depresses phospholipase  $A_2$  activity in a cycloheximide-treated test lung. The experiments design was that outlined in Figure 8. Phospholipase  $A_2$  activity was estimated by bioassay (open column) or radiochemical techniques (shaded columns) already described. The test lung received Krebs solution only (A), Krebs effluent containing steroid (B) or Krebs effluent from a lung-treated with steroids (C). Only the latter contained inhibitory activity. Reprinted by permission, from Flower & Blackwell (1979) *Nature*, **278**, 456-459.

inactive (see Figure 9). If the perfusate containing the anti-phospholipase factor were boiled or treated with proteolytic enzymes, then the biological activity was quickly and completely lost. This experiment strongly suggested that the inhibitory factor was a protein.

In other systems too, the action of glucocorticoids was found to be more complex than was originally envisaged. Bray & Gordon had found in 1976 that corticosteroids blocked prostaglandin synthesis by guinea-pig macrophages and in 1979 Di Rosa and his colleagues at the University of Naples demonstrated that rat peritoneal lavage cells (about 80% macrophages) were also highly sensitive to the prostaglandin inhibitory effect of steroids and that this effect too was dependent on *de novo* RNA and protein synthesis. They obtained evidence that this effect was also caused by the release from the cells of a phospholipase inhibitory protein (Carnuccio *et al.*, 1980). Further experiments revealed that the release of this inhibitor was dependent upon the concentration of the inducing steroid and that the amount of the protein present depended upon the cell number as well as the drug concentration (see Figure 10).

When the biological profile of the lung and macrophage-derived inhibitors were compared it was determined that they were both proteins, and had similar molecular weights (about 15 k) as judged by a gel exclusion chromatography. Both inhibitors were also interchangeable in their actions, that is to say the macrophage-derived material was active in the guinea-pig lung system and *vice versa*. Finally, both proteins shared a similar resistance to heating being



**Figure 10** The release from rat peritoneal lavage macrophages of 'macroscortin' (since renamed 'lipocortin') is dependent upon the concentration of steroid (hydrocortisone) and upon the number of target cells present. In these experiments lavage macrophages were incubated in Krebs solution (37°C) *in vitro* for 90 min in the presence of hydrocortisone and the cells removed by centrifugation. The supernatants were dialysed and the inhibitory activity tested by measuring the reduction in eicosanoid synthesis achieved when the samples were mixed with fresh zymosan-stimulated cells. Left hand panel; the release inhibitory activity from lavage macrophages ( $10^7$ ) is dependent upon the concentration of hydrocortisone. Right hand panel; the release of inhibitory material in the presence of fixed concentration of hydrocortisone (20  $\mu$ M) is dependent upon the number of macrophages in solution. Reprinted by permission, from Blackwell *et al.* (1980) *Nature*, **287**, 147-149.

stable for up to 10 min at 70°C but being destroyed at higher temperatures.

These and other results encouraged the author and his colleagues to believe that we were dealing with a single protein which we christened 'macroscortin' (Blackwell *et al.*, 1980): 'cortin' to indicate that the protein was induced (or released) specifically by the glucocorticoids and mimicked their action in the two systems, while the prefix 'macro' indicated the most likely cellular origin (macrophages) also that their molecular weight (15 k) was considerably in excess of that of the steroids themselves.

#### Other protein inhibitors of phospholipase $A_2$ activity are discovered

Two other groups had independently arrived at a similar conclusion concerning the mechanism of action of steroids on phospholipase. At the National Institute of Health in Bethesda, Hirata and his colleagues had been investigating the mechanism of neutrophil chemotaxis and observed that when neutrophils were stimulated with chemoattractants such as f-Met-Leu-Phe there were rapid changes in membrane biochemistry which included phospholipid methylation and arachidonic acid release (Hirata *et*

*al.*, 1979). These authors demonstrated that both events were preceded by an activation of phospholipase A<sub>2</sub> in the cell membrane, and that drugs which inhibited this enzyme such as the anti-malarial, mepacrine, blocked both the phospholipase A<sub>2</sub> activation and the chemotactic response.

When steroids were tested as inhibitors in the chemotaxis assay, both cell movement and phospholipase A<sub>2</sub> were blocked. Only glucocorticoids (dexamethasone, fluocinolone, hydrocortisone, and cortisone) were effective, oestradiol 17-B, testosterone and progesterone were without activity. As in the guinea-pig lung experiments, steroid receptors were demonstrated in the neutrophils and displacement of labelled dexamethasone from the receptor was observed in the presence of inducing steroids. The inhibitory activity of the steroids was also abrogated by inhibitors of protein and RNA synthesis.

When the particulate fractions of the steroid-treated neutrophils were solubilised with the non-ionic detergent NP40, it was found to contain a phospholipase inhibitory protein which chromatographed on Sephadex with an apparent molecular weight of 40 k (Hirata *et al.*, 1980). If neutrophils were first incubated with labelled lysine the Sephadex fractions containing the 40 k protein were found to have a greater incorporation of the isotope when prepared from steroid treated cells than did similar fractions derived from untreated cells. The conclusion from these experiments was that glucocorticoids blocked chemotaxis by inhibiting phospholipase A<sub>2</sub> and this was the result of an induction (or at least an increase) of protein inhibitor of this enzyme. This protein was subsequently christened 'lipomodulin' by the N.I.H. group.

Another laboratory interested in the question of steroid inhibition of prostaglandin synthesis, was that of Russo-Marie and her colleagues at the Necker Hospital in Paris (Russo-Marie *et al.*, 1979; Russo-Marie & Duval, 1982). Rat renomedullary interstitial cells in culture produce large amounts of prostaglandins, and both non-steroidal and steroidal drugs have inhibitory effects on the generation of these lipids. In a series of beautiful experiments they were able to demonstrate that the glucocorticoids dexamethasone and corticosterone, inhibited prostaglandin synthesis and that the concentrations required to do this generally correlated well with those required to occupy glucocorticoid binding sites. Once again, only steroids with the glucocorticoid properties prevented prostaglandin secretion, this effect was not caused by a depression of cell growth or cyclo-oxygenase activity and was blocked by inhibitors of macromolecule synthesis.

Later experiments by this group indicated that the inhibition of prostaglandin synthesis was secondary to phospholipase inhibition and this in turn was

caused by the generation of a heat-labile non-dialysable factor subsequently dubbed 'renocortin'. Analysis of renocortin demonstrated that it was a mixture of two proteins, one having a molecular weight of 15 k and the other a molecular weight of 30 k (Cloix *et al.*, 1983).

At the Childrens Hospital of Philadelphia, Gupta and his colleagues demonstrated that dexamethasone induces the formation of phospholipase inhibitory proteins ('PLIP') in cultures of embryonic palate cells and thymocytes. The molecular weight species observed (55 k, 40 k, 28 k and 15 k) are very similar to those observed by the other groups (Gupta *et al.*, 1984; Gupta & Goldman, 1985).

Longenecker and his colleagues (1987) have also isolated from human peritoneal dialysis fluid, a 40 k phospholipase inhibitor. This protein displays many properties common with the other inhibitors referred to above in that it inhibits eicosanoid release from cells. No information is available on the steroid inducibility and the available evidence tends to suggest that it is not identical to the protein now named 'lipocortin 1' (see below) although it may well be related in some way.

#### A change in nomenclature

Initially, it seemed that macrocortin, lipomodulin and renocortin were three different proteins induced by the glucocorticoids, but a comparison of the conditions of their generation and a more rigorous examination of the distribution of molecular weights, their anti-enzyme and immunological properties led the Wellcome group, the N.I.H. group and the Necker Hospital group to the conclusion that all these proteins were functionally identical and all active fragments of the same precursor. A unified nomenclature was agreed and the name 'lipocortin' proposed (Di Rosa *et al.*, 1984). It was suggested that the disparity in molecular weights was a consequence of proteolysis.

#### Lipocortin has been isolated and purified

Once established as a distinct entity, several groups expended considerable energy in an attempt to purify and sequence lipocortin. Whilst the highly purified protein was soon available for biological work, it was some time before the structural work on the molecule was completed.

In some early work Hirata used primary cultures of rabbit neutrophils stimulated for 16 h with glucocorticoids as the main source of lipocortin (Hirata *et al.*, 1980; Hirata, 1983). Using the concentrated conditioned media as a starting material, purification to

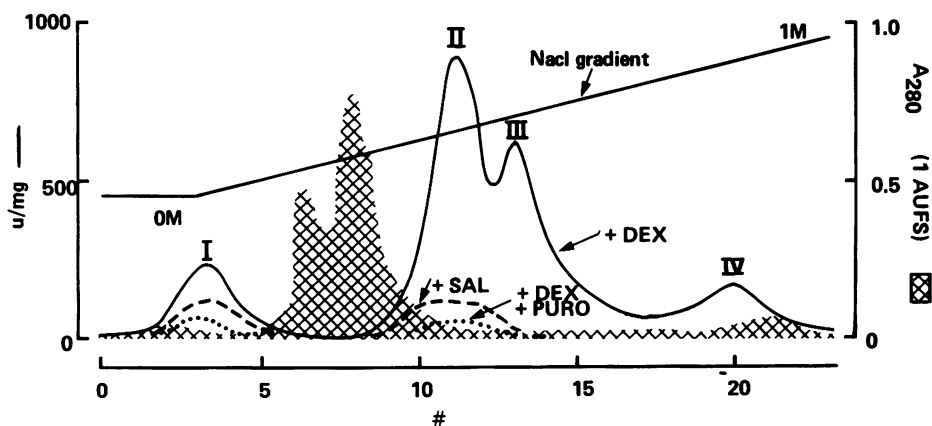
near homogeneity (as judged by SDS-PAGE) was achieved by sequential chromatography on DEAE ion exchange resins and Sephadex G-75. The purified protein seemed to be a glycoprotein (estimated at 10% sugar residues w/w) in as much as it was retained by Con-A affinity columns. The predominant species detected in these studies had a molecular weight ( $M_r$ ) of 40k although other species were seen including a 15k and 25–30k species. The latter were apparently formed by a proteolysis of the 40k form since the relative proportions changed when protease inhibitors were omitted from the mixture.

Lipocortin was partially purified by Russo-Marie and her colleagues (Cloix *et al.*, 1983) using supernatants of cultured rat renomedullary cells which had been treated for 24h with dexamethasone. Chromato-focussing and gel filtration techniques revealed two species with anti-phospholipase activity having isoelectric points of 8.3 and 5.8 (an isoelectric point of 9.4 for the 40k protein had been suggested by Hirata). After chromatography of the crude mixture on Ultrogel columns, two species of 15 and 30k were recovered but it was not clear which species related to each isoelectric point. In more recent experiments in which serum-free medium was used, an additional species of 40k was detected. Apparently foetal calf serum contains a protease which cleaves the 40k species to the 30k species. It is

not clear whether the 30 to 15k transformation is catalysed by a specific enzyme or a general cellular protease. The 15k form was substantially phosphorylated and was only fully active after treatment with alkaline phosphatase (see below).

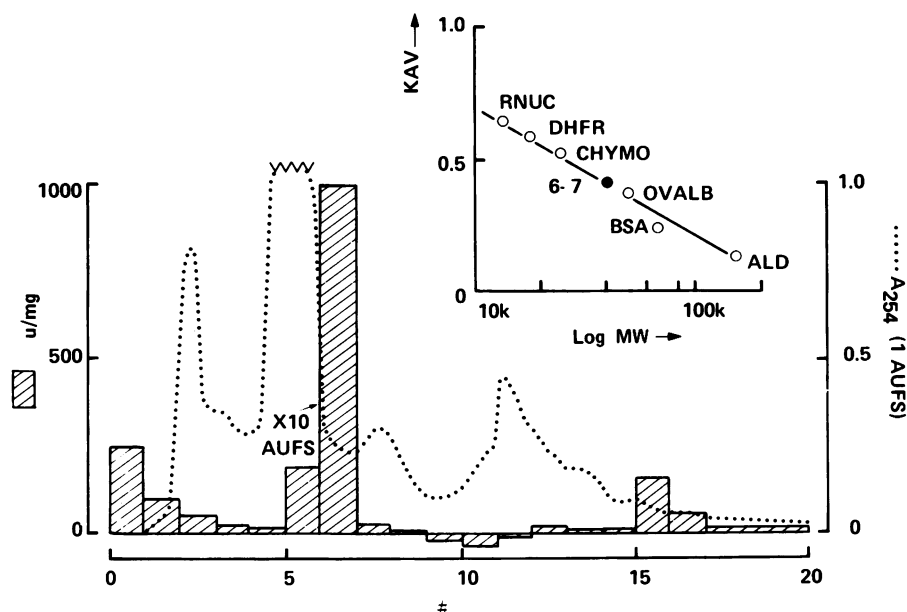
In contrast to the *in vitro* systems described above, the author's group used rat peritoneal lavage fluid from steroid-treated rats as starting material (Blackwell *et al.*, 1982). This material contains a substantial amount of anti-phospholipase activity although it is a more complex mixture of proteins than that generated by either of the other techniques. The work of Hirata *et al.* (1982) has revealed that a substantial proportion of the anti-phospholipase proteins in this mixture is phosphorylated and that they are not inhibitory in the *in vitro* phospholipase assay system until treated with alkaline phosphatase. Because of this it is likely that early estimates of the anti-phospholipase content of peritoneal fluid underestimated the actual amounts present.

Partially purified anti-phospholipase proteins from rat lavage fluid was prepared by DEAE ion exchange chromatography and gel filtration. A complex pattern of active peptides was often seen eluting from DEAE columns and several proteins eluting at different ionic strengths appeared, on subsequent analysis, to have the same molecular weight (see Figure 11). In the first experiments Sephadex

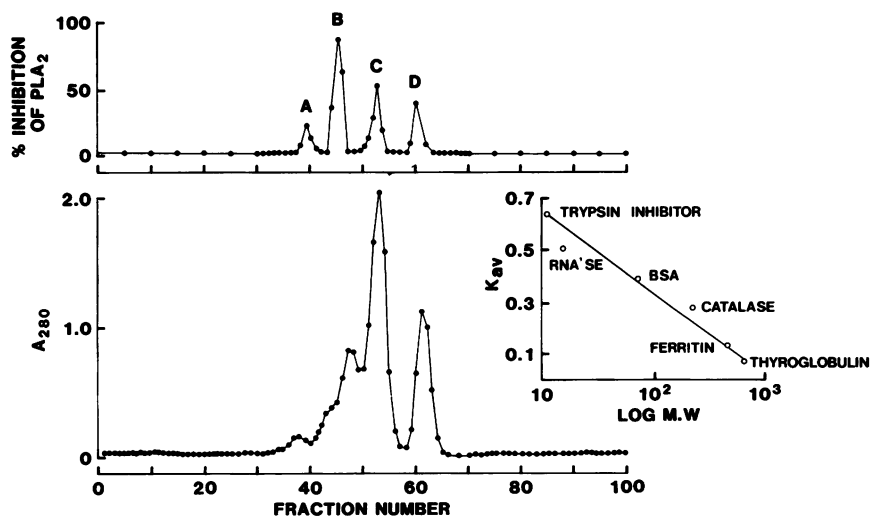


**Figure 11** The phospholipase inhibitory activity in lavage fluid from steroid-treated rats can be resolved into discrete components by DEAE ion exchange chromatography. Peritoneal lavage fluid was collected from rats which had received dexamethasone ( $1 \text{ mg kg}^{-1}$ ), saline alone, or dexamethasone and puromycin (to prevent protein synthesis). After dialysis and concentration the proteins were applied to a DEAE ion exchange column and eluted with a  $0\text{--}1 \text{ M NaCl}$  salt gradient. Fractions were collected and tested for anti-phospholipase activity in a cell-free enzyme assay. The figure shows the anti-phospholipase activity expressed as  $\text{u mg}^{-1}$  protein (left hand scale) in steroid-treated (continuous line), saline-treated (broken line) and dexamethasone and puromycin-treated (dotted line) samples. The cross-hatched area represents the protein concentration estimated by u.v. absorbance at 280 nm (right hand scale).

The anti-phospholipase activity was resolved into four separate components labelled as polypeptides I–IV which eluted at different salt concentrations. Polypeptides I and II appeared to have very comparable molecular weights and were used most often for biological experiments. Reprinted, by permission, from Blackwell *et al.* (1982) *Br. J. Pharmacol.*, 76, 185–194.



**Figure 12** The molecular weight of lipocortin can be ascertained by h.p.l.c. gel exclusion techniques. In this experiment polypeptide II (see Figure 11) was collected following DEAE chromatography concentrated and subjected to h.p.l.c. gel exclusion chromatography. Each fraction was tested for anti-phospholipase activity (cross hatched columns, left hand scale) and an estimate of the protein concentration was obtained by continuous monitoring at 254 nm (dotted line, right hand scale). The vast majority of the anti-enzyme activity was eluted in fraction 7. Since the column was calibrated with marker proteins (ribonuclease (RNUC), dihydrofolate reductase (DHFR), chymotrypsin (CHYMO), bovine serum albumin (BSA) and aldolase (ALD), see inset) it was possible to assign an apparent mol. wt. of 39–40 k to polypeptide II. Experiments with polypeptide I gave similar results. Reprinted, by permission, from Blackwell *et al.* (1982) *Br. J. Pharmacol.*, 76, 185–194.



**Figure 13** Crude peritoneal lavage fluid from steroid-treated rats contains anti-phospholipase proteins with different molecular weights.

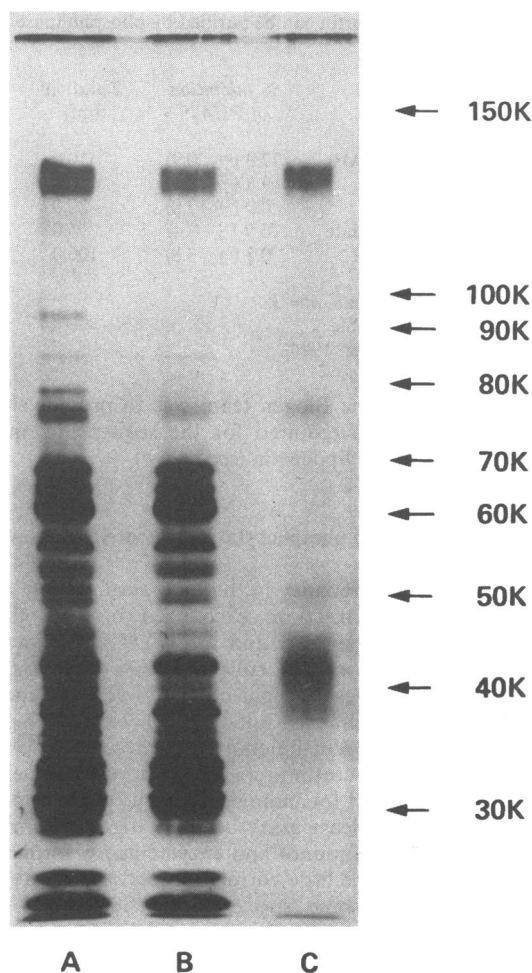
After collection, lavage fluid was collected, dialysed, concentrated and applied to a sephadex S 300 gel exclusion column. The anti-phospholipase activity in each fraction was estimated (top record) as was the protein ( $A_{280}$ , bottom record). The column was calibrated with marker proteins (see inset) and the four peaks were assigned apparent molecular weights of ~200 k (A), 125 k (B), 40 k (C) and 15 k (D).

G-75 was used as gel filtration medium but later gel exclusion chromatography was performed by high performance liquid chromatography (h.p.l.c.) using a TDK 200 SW column. With these methods the most prominent species detected was the 40 k protein although a 15 k species was also often found (see Figure 12). In other experiments Sephacryl SF300 was employed and led to the detection of more anti-phospholipase proteins with much higher weights (principal species 125 k, see Coote *et al.* 1983 and Figure 13). The phospholipase inhibitory activity of these large proteins was neutralised by the monoclonal RM23 (see below) in the same way as lower molecular weight forms were. These proteins also reduced prostaglandin production by cells and seemed to behave in every way as the lower molecular weight species. It could be that these molecules are in fact precursors of the 40 k species or simply related proteins, but direct evidence for this will have to await structural elucidation of all peptides.

Although considerable purification can be achieved by combination of ion exchange and gel filtration chromatography this was not sufficient to give homogeneous protein bands on SDS gel electrophoresis when rat peritoneal lavage fluid is used as a starting material. The monoclonal antibody RM 23 proved difficult to use when coupled to an affinity matrix, but another technique, phospholipase A<sub>2</sub> affinity chromatography, substantially improved the procedure. Typically, ex-DEAE fractions containing a mixture of proteins were passed through the column phospholipase A<sub>2</sub> linked to agarose beads) whereupon 80–90% of the biological activity was retained by the bound ligand. This could be subsequently eluted with 2 M KCl and a considerable purification now achieved (see Figure 14 and Table 3). The phospholipase A<sub>2</sub> affinity column is also very effective for purifying the high molecular weight inhibitor.

The 40 k protein had the same molecular weight on SDS-PAGE in the presence or absence of a reducing agent implying that it is a single polypeptide chain (Hirata, 1983).

At the time of writing the most complete analysis of the phospholipase inhibitory activity in rat peritoneal lavage fluid has been furnished by Pepinsky and his colleagues at the Biogen Research Corporation (Pepinsky *et al.*, 1986). These workers purified the predominant phospholipase inhibitory protein from lavage fluid and characterized it as a 37 kDa protein which was also present in a series of lower molecular weight species including a 30 k, 24 k and 15 k form. This inhibitor, which probably corresponded to 'polypeptide 1' according to the authors purification scheme (see Figure 11), was purified to homogeneity using a combination of ion exchange and gel exclusion chromatography and submitted to partial sequence analysis. The distribution of the inhibitory



**Figure 14** Lipocortin can be highly purified by phospholipase A<sub>2</sub>-affinity chromatography. The figure shows a silver stained 10% SDS-PAGE chromatogram. Band A represents a crude and complex mixture of proteins collected from the active fraction eluting from a DEAE column. Most of these proteins passed through the column (porcine pancreatic phospholipase A<sub>2</sub> linked onto agarose beads) although the biological activity was retained on the column (see Table 3). After elution of the column with 2 M KCl virtually all the biological activity was recovered from the column and was subsequently identified as the ~41 k protein in band C. The other proteins on this band (~75 k and ~130 k) are probably contaminants. Reprinted, by permission, from Parente & Flower (1984) *Eur. J. Pharmacol.*, 99, 233–239.

protein was investigated between organs and amongst certain cell lines. Interestingly the protein was found to cross react with an antibody raised against an inhibitor of phospholipase found in Cobra (*Naja naja*) venom. The thorough and pains-

**Table 3** Lipocortin can be purified by phospholipase A<sub>2</sub> (PLA<sub>2</sub>) affinity chromatography

Fraction	% inhibition of PLA <sub>2</sub> *	Total vol (ml)	Total (units)	Total protein (μg)	Specific activity (u mg <sup>-1</sup> )	Purification factor	Yield (%)
Original (ex-DEAE)	77.9 (± 0.9)	10.0	217.6	1740	125.1	—	—
Run through	9.8 (± 3.0)	10.0	25.1	1020	24.6	—	—
PBS wash	0.2 (± 13.0)	~20.0	0.0	UD	—	—	—
Dialysed KCl eluate	73.9 (± 2.4)	5.0	103.2	26.8	3850.7	30.8	47.4
Dialysate control	0.5 (± 3.8)	100.0	—	UD	—	—	—

\* mean ± s.e. mean, *n* = 3.

UD = undetectable

Data from Flower (1984).

taking work of the Biogen team was to provide the basic information required for the sequencing and cloning of human lipocortin (see below).

#### Lipocortin may be phosphorylated by protein kinases

An apparent discrepancy in the biological activity of some lipocortin fractions exchanged between the author, his collaborators and the N.I.H. group was settled by Hirata and his colleagues in a way which shed further interesting light on the biology of lipocortin and which has initiated an entirely new approach to oncogene function.

Samples of lipocortin prepared from rat peritoneal fluid were assayed for biological activity on a whole cell eicosanoid-release assay before being sent to the N.I.H. for radioimmuno- and enzyme assay. Initially there seemed to be little correlation between the two types of assay some species being detectable by radioimmunoassay (RIA) but not by the enzyme assay. Hirata surmised that lipocortin might be phosphorylated and demonstrated that when treated with alkaline phosphatase the samples were active in the enzyme assay as well as being detectable by radioimmunoassay (Table 4) (Hirata *et al.*, 1982). Presumably the samples were active in the original cell assay because leucocytes contain some alkaline phosphatase associated with the cell membrane.

**Table 4** Treatment with alkaline phosphatase increases the inhibitory potency of lipocortin in a phospholipase A<sub>2</sub> assay system

Mol wt. fraction	Inhibition of phospholipase A <sub>2</sub> activity (u mg <sup>-1</sup> )	
	before treatment	after treatment
40 k	150	480
30 k	118	260
15 k	0	160

Table from Hirata *et al.* (1982).

By using a cell-free kinase system, Hirata (1981) was able to confirm that phosphorylation of lipocortin renders the protein incapable of inhibiting phospholipase A<sub>2</sub>. When beef heart cyclic AMP-dependent protein kinase was added together with cyclic AMP and ATP the protein was almost completely inactivated and this corresponded with an incorporation of radioactive phosphate into the lipocortin molecule if radioactive ATP was used. The inactivated protein could be easily reactivated by alkaline phosphatase treatment. The exact type of protein kinase responsible for this reaction in living cells has not been determined but the acceptor site on the molecule seems to be a tyrosine or a serine (Hirata, 1983).

That lipocortin 1 is a substrate for protein kinases has now been confirmed by several groups. Indeed it is likely to be identical to several previously described, but uncharacterized, proteins which have long been known to be substrates for various oncogene kinases (see section below). This may possibly explain why certain tumours generate large amounts of eicosanoids.

#### Lipocortin has biological properties consistent with phospholipase inhibitory activity

##### (a) Anti-enzyme activity in vitro

A basic property of all species of the lipocortin family is their ability to inhibit phospholipase A<sub>2</sub> activity. In many of the original experiments the evidence that phospholipase was directly inhibited was compelling, but ultimately only circumstantial, the site of action of the protein being deduced from its behaviour in complex cellular systems.

The problem was that the most likely target for these proteins was the membrane-bound phospholipase, an enzyme notoriously difficult to assay. A partial solution was achieved by using the more readily available soluble pancreatic enzyme as a

model 'target'. This was very much a 'second best' option because there are obviously differences between the membrane bound enzyme and the pancreatic protein. Indeed, there are considerable problems in assaying even this latter species since its behaviour does not comply with the tenets of classical Michaelis-Menten kinetics and exhibits a number of anomalies (cf. Chang *et al.*, 1987). The area is a treacherous one with many pitfalls and the behaviour of lipocortin in such assays is unexpectedly complex.

The inhibition of phospholipase A<sub>2</sub> activity in a cell-free system was first demonstrated by Hirata (1981). Lipocortin was found to exert a concentration-dependent inhibition of pancreatic phospholipase as assessed in a micellar assay using labelled phosphatidylcholine as a substrate.

The pancreatic phospholipase A<sub>2</sub> was not the only phospholipase enzyme to be inhibited by lipocortin: Hirata (1981) observed that phospholipase A<sub>2</sub> from the snake (*Naja naja*) and bee venom were also inhibited to comparable degrees. Phospholipase C, from two bacterial sources was also blocked, but the inhibition was not so pronounced. Phospholipase D (from cabbage), was also inhibited but again it was not so susceptible to inhibition as the A<sub>2</sub> enzymes. Equilibrium dialysis experiments suggested that protein might bind Ca<sup>2+</sup> ions and Hirata suggested that this action underlay its ability to inhibit phospholipase. Recently, the Ca<sup>2+</sup> properties of lipocortin have been confirmed. According to Schlaepfer & Haigler (1987) the molecule contains four Ca<sup>2+</sup> binding sites although it has little affinity for the cation in the absence of phosphatidylserine. The ability of lipocortin to form a ternary complex with Ca<sup>2+</sup> and phospholipids is extremely interesting and may be important from the mechanistic viewpoint.

Further studies by Hirata (1983) suggested that lipocortin reduced the  $V_{\max}$  of the enzyme-catalysed reaction but not the  $K_m$  value for the substrate, and it was suggested that a stoichiometric complex was formed between the protein and the inhibitor. This complex was disrupted by some detergents suggesting that hydrophobic binding was important for the inhibitory action.

The author's group (Blackwell *et al.*, 1982) studied the release of anti-phospholipase proteins into peritoneal lavage fluid of the rat also using the pancreatic enzyme as a target and micellar phosphatidylcholine as a substrate and used the assay as a basis for estimating the amount of lipocortin present. The lavage fluid from 'normal' rats was found to contain abundant anti-phospholipase activity as estimated in the *in vitro* assay, but this was greatly reduced in the lavage fluid from adrenalectomised animals. Administration of steroids to both groups caused an increase within 1 h. In addition to these studies, there

are several other reports of proteins present in plasma or cell (especially leukocyte) extracts which have antiphospholipase activity in *in vitro* assay systems (Bartolf & Franson, 1987; Authi *et al.*, 1982).

The results obtained by Hirata and his colleagues and by the author's group suggested that lipocortin bound to phospholipase itself brought about inhibition of enzyme activity, and indeed this property was sometimes utilized as a means of purifying the protein (see above). Recently however the mechanism of action of these proteins in bringing about an inhibition of the enzyme has been disputed.

Davidson and her colleagues (1987) studied the inhibition of pancreatic and snake venom phospholipases by preparations of calpactin II (probably identical to lipocortin 1; see below) and were unable to demonstrate inhibition of the enzyme at saturating concentrations of substrate. Inhibition was seen only when the substrate were present in rate-limiting amounts and the inhibition could be overcome by adding substrate, but not more enzyme. These authors suggested that lipocortin produced inhibition of phospholipase activity by sequestering substrate and that it did so by binding to it in a Ca<sup>2+</sup> dependent manner.

Published work from some other groups also support their contention: Aarsman *et al.* (1987) observed that the inhibition by a human monocyte lipocortin of phosphatidylethanolamine hydrolysis by the pancreatic enzyme or by partially purified intracellular phospholipases was substrate-dependent and the same group (Rothhut *et al.*, 1987) found that this inhibitor blocked hydrolysis of a negatively charged, but not zwitterionic phospholipid substrate. Haigler *et al.* (1987) also failed to find an interaction between lipocortin and phospholipase A<sub>2</sub> and suggested that the inhibitory action of the protein was caused by an effect on the substrate.

Lipocortin is indeed able to bind to certain phosphatides such as the substrates in the assays used by many of these groups (phosphatidylserine and phosphatidylethanolamine) but since it does not apparently bind to phosphatidylcholine vesicles (Schlaepfer & Haigler, 1987), the substrate used in all the early studies, it is difficult to envisage how this notion explains the anti-phospholipase action on this substrate and in any case could not explain the phospholipase affinity column data.

It is certainly true however that the Ca<sup>2+</sup> and lipid binding properties of this molecule could give rise to spurious results when the substrate concentration is low as it is in the widely used *E. coli* assay. It is evident that we still do not fully understand the behaviour of this protein in the apparently simple assay systems used for estimating phospholipase activity and perhaps the simplest explanation which would accommodate both sets of data is that lipo-

cortin can have effects both on the enzyme and its substrate.

(b) *Suppression of mediator production by cells in vitro*

It was the steroid-induced inhibition of prostaglandin production by cells that originally provoked much of the interest in the mechanism of steroid action. The anti-phospholipase proteins mimic the action of steroids in many systems quite closely, although little latency of action is observed and their effect is resistant to the action of protein/RNA synthesis inhibitors. The inhibition of cellular prostaglandin synthesis by these proteins is dose-dependent, leukotriene production is also blocked as one would expect (Parente *et al.*, 1984), and the inhibitory effect of these proteins, like that of the

steroids themselves, is readily reversed by the addition of exogenous arachidonic acid (see Table 5). The inhibitory effects of the aspirin-like drugs are, of course, not reversed by supplying the substrate, a fact that is entirely consistent with the differing sites of action of the two types of agent.

Another putative inflammatory mediator formed by the action of phospholipase  $A_2$  is Paf. Parente & Flower (1985) demonstrated that affinity-purified lipocortin blocked the release of lyso-Paf (the precursor of Paf) from rat resident macrophages stimulated with zymosan, and that this action was prevented by the neutralising monoclonal RM 23 when this was present in the mixture (see Table 6). Interestingly the latter monoclonal can also inhibit the blocking action of hydrocortisone itself providing additional evidence that the inhibitory action of glucocorticoids is mediated by lipocortin (see Table 7).

**Table 5** Partially-purified lipocortin preparations inhibit the production by leucocytes both of cyclo-oxygenase and lipoxygenase products

Treatment	Concentration ( $\mu\text{g ml}^{-1}$ )	$\text{PGE}_2$	% inhibition* P	$\text{LTB}_4$	P
Hydrocortisone	10	$41.2 \pm 3.4$	<0.01	$45.0 \pm 5.8$	<0.01
Lipocortin fraction	100	$42.4 \pm 1.9$	<0.01	$52.4 \pm 1.5$	<0.01

\* Values are mean  $\pm$  s.e. mean,  $n = 5$ .  
Data from Parente *et al.* (1984).

**Table 6** Affinity purified lipocortin inhibits the release by leukocytes of lyso-Paf

Treatment	*Lyso-Paf (pg per $10^6$ cells)	% inhibition	P
None	$1459.6 \pm 132.1$	—	—
Lipocortin 10 $\mu\text{g}$	$770.4 \pm 73.0$	$47.2 \pm 4.5$	<0.01

\* Values are means  $\pm$  s.e. mean,  $n = 5$ .  
Data from Parente & Flower (1985).

**Table 7** A monoclonal antibody (RM23) recognising lipocortin can reverse the inhibitory effect of hydrocortisone on prostaglandin release from macrophages

Hydrocortisone ( $\mu\text{g ml}^{-1}$ )	Neutralising antibody diln	% inhibition of $\text{PGE}_2$ synthesis
1.0	—	88.5
1.0	1:300	60.7
1.0	1:100	11.0
1.0	1:50	0.0

A 'control' antibody of the same type and subclass was without effect at any concentration.  
Data from Flower *et al.* (1984).

Several experiments suggest that lipocortin inactivates membrane phospholipases by interacting with the enzyme from *outside* the cell. In the guinea-pig perfused lung system for example, phospholipase activity is inhibited almost as soon as lipocortin infusions are begun, and declines shortly after the infusions are terminated (Blackwell *et al.*, 1982). It seems inherently improbable that a protein of a minimum size 15 k could so rapidly gain access to the interior of cells as it would have to do if the target enzyme was cytosolic. Again, Hirata *et al.* (1980) observed that the steroid inhibition of neutrophil chemotaxis (which was associated with an inhibition of phospholipase  $A_2$ ) was completely reversed if the neu-



trophils were exposed to the proteolytic enzyme pronase. This experiment suggests that at least part of the inhibitory protein is accessible to the (extracellular) hydrolytic activity of enzyme. A further observation which tends to support the notion that lipocortins are cell-membrane proteins was made using the anti-lipocortin monoclonal 4-4C3. This antibody was observed to stain proteins on cell surfaces, such as murine thymocyte subsets (Hirata, 1983; Hirata & Iwata, 1983). Again, the experiment with the monoclonal RM23 cited above also supports this idea.

Cirino & Flower (1987a,b) observed that lipocortin-like activity was easily dislodged from cells when they were washed in low  $\text{Ca}^{2+}$  media and that the *ex vivo* inhibition of eicosanoid generation by steroids could be abrogated if cells were subjected to this procedure. Authentic recombinant human lipocortin 1 (see below) also required the presence of  $\text{Ca}^{2+}$  before it produced an inhibition of eicosanoid synthesis in macrophages. Even repetitive washing of cells by vigorous centrifugation in  $\text{Ca}^{2+}$  containing solutions is often sufficient to remove lipocortin (Carnuccio *et al.*, 1981).

An important conclusion to be derived from all this work is that the *ex vivo* actions of steroids may be missed if the protocol involves a step where cells are exposed to low calcium environments or to multiple washing procedures. This could explain many discrepancies in the literature.

### (c) Anti-inflammatory activity *in vivo*

Clearly, these induced proteins have biochemical properties consistent with anti-inflammatory activity,

but is there any evidence that they actually produce an anti-inflammatory effect *in vivo*? The author's group used crude protein extracts from peritoneal lavage fluid from dexamethasone- or saline-treated rats to test the general hypothesis that there is a soluble anti-inflammatory substance produced in the body fluids of steroid-treated animals (Blackwell *et al.*, 1982). The proteins were concentrated, dialysed to remove steroid and injected together with carrageenin into the pleural cavity of anaesthetized rats. Four hours later the animals were killed and the extent of the inflammation assessed by measuring the fluid exudation and leukocyte infiltration. Protein extracts from rats treated with saline alone did not alter the pleurisy when compared to that produced by carrageenin alone, but a substantial inhibition occurred when proteins from dexamethasone-treated rats were used. This was caused by the presence of an induced protein in the exudate because boiling destroyed the activity, and treatment of the rats in which the protein was raised with actinomycin D prevented the anti-inflammatory principle from appearing (see Table 8). Fractionation of the active extracts on DEAE ion exchange cellulose indicated that the peaks of anti-inflammatory activity coincided with anti-phospholipase activity.

In later studies partially purified (ex-DEAE and Sephadex G-75) anti-phospholipase proteins were used as anti-inflammatory agents in the carrageenin paw oedema model (Parente *et al.*, 1984). Again this test showed that lipocortin-containing fractions suppressed the oedema with a duration of action of about 3 h, and that the effect was reversed by a supra-injection of arachidonic acid.

Hirata (1983) has also demonstrated inhibition of raw paw oedema by lipocortin.

**Table 8** A heat-labile, non-dialysable protein(s) induced in rats by the administration of dexamethasone (Dex) exerts anti-inflammatory effects in the rat pleurisy model

Expt	Treatment	†Exudate vol (ml per rat)		†Leukocytes ( $10^6$ per rat)	
			% change		% change
1	Saline	0.84 ± 0.08 (11)	—	90.8 ± 9.1 (11)	—
	Proteins from control rats	0.89 ± 0.13 (14)	+5.9	95.7 ± 12.5 (15)	+5.4
	Proteins from Dex-treated rats	0.41 ± 0.09 (14)	-57.2**	53.6 ± 6.9 (15)	-43.9***
2	Saline	0.59 ± 0.02 (3)	—	35.4 ± 3.7 (3)	—
	Proteins from Dex-treated rats	0.30 ± 0.13 (3)	-49.2**	10.6 ± 3.3 (3)	-70.1***
	as above, boiled	0.62 ± 0.07 (3)	+5.1	28.7 ± 2.6 (3)	-18.9
3	Proteins from control rats	0.84 ± 0.06 (4)	—	55.2 ± 8.7 (4)	—
	Proteins from Dex-treated rats	0.27 ± 0.08 (3)	-67.9*	32.5 ± 5.1 (4)	-41.1*
	Proteins from Dex + actinomycin D-treated rats	0.77 ± 0.04 (4)	-8.3	50.1 ± 1.8 (4)	-9.2

† Values are mean ± s.e. mean with *n* in parentheses.

Data from Blackwell *et al.* (1982).

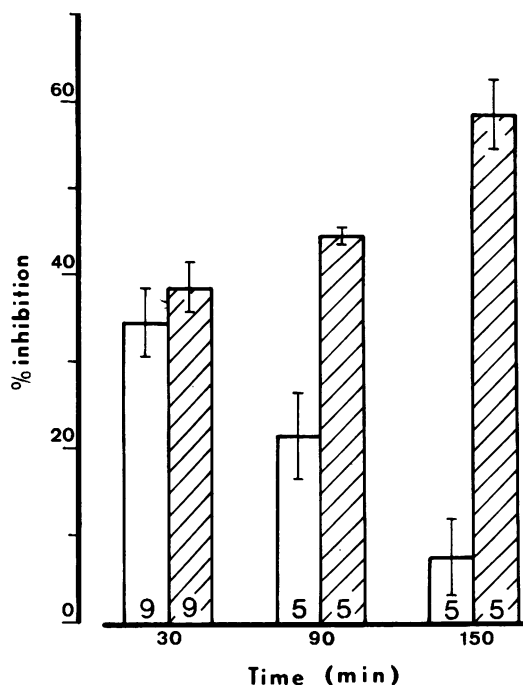
\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

**Both the synthesis and the release of lipocortin appear to be controlled by glucocorticoids**

In the first studies of steroid-induced inhibition of prostaglandin synthesis it was assumed that the glucocorticoids promoted the generation of anti-phospholipase proteins by straightforward induction of protein synthesis. In some cells this may be true, but in the macrophage the situation seems more complex. Blackwell *et al.* (1980), noticed that untreated macrophages contained a substantial amount of lipocortin which could be recovered from the supernatant phase if the cells were lysed by freezing and thawing. When intact macrophages were treated with steroids there was a 'secretion' or release of the preformed proteins into the medium: after 30 min incubation with the steroid, for example approximately half the lipocortin was released into the extracellular fluid. After 90 min approximately two thirds, and after 150 min, virtually all the lipocortin stores was externalised with only a small amount of bioactivity remaining inside the cells themselves (see Figure 15). When these depleted cells were washed and resuspended in fresh medium they were found to be unresponsive to the inhibitory actions of steroids and this lack of responsiveness persisted until fresh lipocortin was synthesized within the cells, an event which required a further 2–2.5 h *in vitro*, although it was much faster *in vivo* (Carnuccio *et al.*, 1981; Blackwell, 1983).

This experiment suggests that in the macrophage at least, the steroid-induced response can be divided into an acute secretion or release phase which begins about 30 min after the addition of steroid and a further synthesis of lipocortin some 4 to 5 h after the initial contact with the steroid. Both events seem to be blocked by RNA/protein synthesis inhibitors and may both therefore depend on the 'classical pathway' of steroid action. The latter resynthesis phase could be caused by actual *de novo* synthesis of the protein or it might be secondary to the catabolism of a high molecular weight precursor catalysed by some steroid-induced processing enzyme. The trigger for the resynthesis phase could be either the steroid itself, or the fact that intracellular levels of lipocortin have fallen below a set point. At the moment the evidence suggests that the steroid itself increases lipocortin resynthesis.

Steroids induce cells to synthesize and release anti-phospholipase proteins *in vitro* and presumably should do so *in vivo* too. In the healthy living animal, of course, there is a constant steroid background with a superimposed circadian rhythm. Obviously the genes which code for these anti-phospholipase proteins were designed to respond to fluctuations in endogenous steroid secretion and indeed the dose-response curve suggests that the system can respond



**Figure 15** The initial release of lipocortin from cells is caused by the exteriorization of the protein from cells. In this experiment the inhibitory activity (on eicosanoid generation) was measured both within peritoneal lavage macrophages (following cell lysis) and in the supernatant phase after incubation with hydrocortisone  $20 \mu\text{M}$  for various times. At time '0' (data not shown) inhibitory activity was detected in cell lysates (open columns) but not in the supernatant (cross hatched columns) but by 30 min about half the total inhibitory activity was extracellular. By 150 min little activity remained in the cells and the vast majority was extracellular. Reprinted, by permission, from Blackwell *et al.* (1980) *Nature*, 287, 147–149.

within the physiological range (Blackwell *et al.*, 1980). One would therefore anticipate a certain lipocortin background in normal animals and that this would *quantitatively* rather than *qualitatively* be changed by steroid administration.

Rat peritoneal macrophages generate lipocortin *in vivo* and peritoneal lavage fluid from untreated rats also contained abundant amounts of lipocortin-like activity (Blackwell *et al.*, 1982). This was increased several fold by the injection of glucocorticosteroids but not if rats were first treated with inhibitors of RNA/protein synthesis. An increase in peritoneal lipocortin levels was observed within 30 min of steroid injection and reached a peak at about 60 min returning to control levels within 3 to 4 hours of giving the drug. Interestingly, peritoneal fluid from

**Table 9** Glucocorticoids and ACTH release lipocortin *in vivo*

Type of rat	Background	†Inhibition of phospholipase activity (units) Dexamethasone (1 mg kg <sup>-1</sup> )	Hydrocortisone (10 mg kg <sup>-1</sup> )	ACTH (300 µg kg <sup>-1</sup> )
Normal (or sham-operated)	81.0 ± 5.3 (11)	193.4 ± 10.0 (25)**	144.4 ± 1.1 (2)**	153.9 ± 1.3 (2)**
Adrenalectomised	29.9 ± 11.3 (6) <sup>1</sup>	129.3 ± 14.9 (6)**	68.8 ± 0.48 (2)	15.5 ± 0.9 (2)

Values are mean ± s.e. mean, *n* in parentheses.

\*\* Significantly greater than background levels ( $P < 0.001$ ).

<sup>1</sup> Significantly less than sham operated controls ( $P < 0.05$ ).

Data from Blackwell *et al.* (1982).

untreated adrenalectomised animals were found to contain considerably less biologically active lipocortin than peritoneal fluid from normal (or sham-operated) rats although the injection of steroid led to an immediate release. This of course is consistent with the absence of glucocorticosteroids in the adrenalectomised rats. ACTH and mild stress released lipocortin into lavage fluid in normal, but not adrenalectomised, animals (see Table 9).

Lipocortin may therefore exert a tonic regulatory tone on phospholipase A<sub>2</sub> activity *in vivo* (see below).

### Lipocortin is widely distributed

The peritoneal macrophage was used as a source of lipocortin in many of the early experiments and this remains one of the richest known cellular sources of the protein, although there is also a great deal in placenta (Huang *et al.*, 1986). It is possible that the lipocortin generated by the guinea-pig perfused lung was actually derived from alveolar macrophages and indeed in some unpublished experiments Parente & Flower (1984) observed that alveolar macrophages (obtained by bronchial lavage) from several species release lipocortin in response to glucocorticoids. That other types of cell can also generate this protein was illustrated by studies already referred to such as Hirata's early work on lipocortin in the neutrophil, and the work of Russo-Marie and her colleagues with kidney interstitial cells. Endothelial and mesothelial cells also generate lipocortin-like substances (De Caterina & Wekslar, 1986; Rosenbaum *et al.*, 1986; Van de Helde *et al.*, 1986a, b) as do skin fibroblasts (Errasfa *et al.*, 1985) and human endometrium (Gurpide *et al.*, 1986) in response to glucocorticoids.

Coote, Flower and Wood (unpublished data cited in Flower, 1984) studied the distribution of lipocortin in the rat using a labelled antibody and a solid phase assay system and noted that it was widely distributed and could be detected in every organ tested.

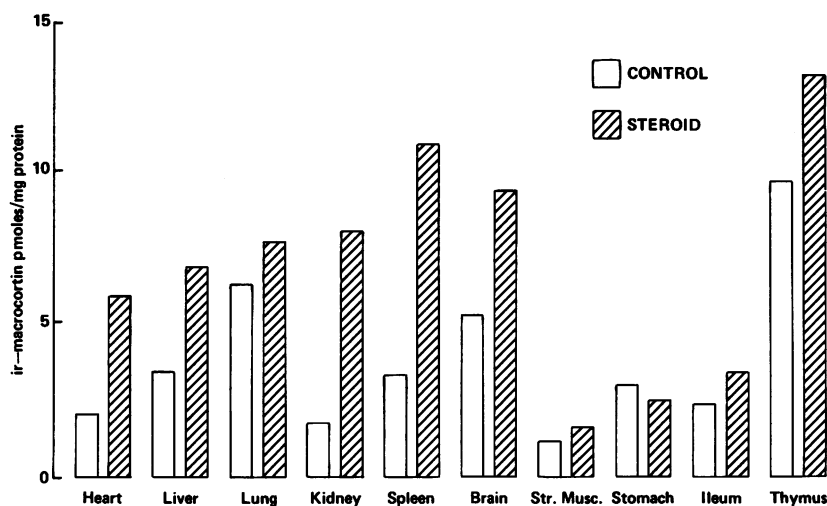
Thymus, spleen, lung and brain had especially high amounts of the protein under 'resting' conditions (i.e. with no exogenous steroids). When treated with dexamethasone the immunoreactivity of all organ extracts (except some gastrointestinal tissues) was increased within 2 h in some cases by 4–5 fold (see Figure 16). Similar results were obtained in the guinea-pig.

### Lipocortin appears to exert a constant regulatory effect *in vivo*

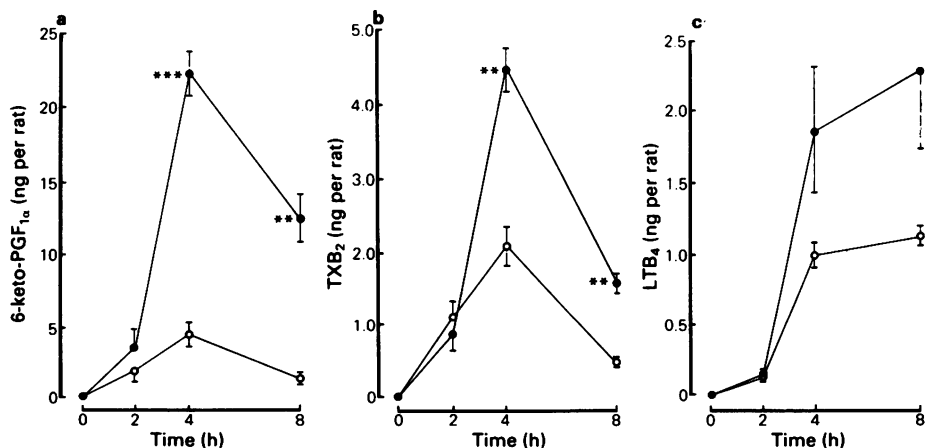
The blood of all healthy mammals contains circulating glucocorticoids and the toe of the dose-response curve of steroid-stimulated lipocortin release lies within this physiological range (Blackwell *et al.*, 1980; see Figure 10) it is therefore not surprising that lipocortin is present in normal cells and tissues, although it is apparently washed off cells rather rapidly when they are re-suspended *ex vivo* particularly in the absence of calcium (which may be the reason why such preparations are prone to generate a large amount of prostaglandins).

To discover how an absence of steroids, and by implication, lipocortin would alter the nature of the inflammatory response *in vivo*, the author and his colleagues compared the acute inflammatory response to carrageenin in the adrenalectomised and sham-operated rat (Flower *et al.*, 1986). In a carefully matched experiment the irritant was introduced into the pleural cavity of the two groups of rats and the extent of the inflammation assessed at various time points.

The magnitude and duration of the inflammation as estimated by fluid exudation and cell migration was greatly increased (approximately doubled) in adrenalectomised animals compared with their sham-operated controls. The eicosanoid and lyso-Paf (Parente & Flower, 1985) content of the inflammatory fluid obtained from the adrenalectomised animals was also greatly increased (2–4 fold) compared with the controls (see Figure 17). The adminis-



**Figure 16** Steroid treatment of rats increases tissue lipocortin content. Rats were injected with dexamethasone ( $1 \text{ mg kg}^{-1}$ ) or saline and killed 2 h later. Organs were removed, homogenized in buffer and adjusted to a standard protein concentration. Samples were spotted onto a nitrocellulose sheet and the presence of lipocortin revealed with an iodinated monoclonal antibody thus enabling a semiquantitative assessment of the lipocortin concentration to be made. A 'control' antibody also displayed low binding to these organ extracts but this did not change with steroid treatment. Reprinted, by permission, from Flower (1984) In *Advances in Inflammation Research* Vol. 8, pp. 1–33.



**Figure 17** Glucocorticoids, and probably therefore lipocortin, seem to exert a continuous inhibitory tone on cellular eicosanoid production *in vivo*. In this experiment an inflammation was induced in adrenalectomised and sham operated rats by injection of carrageenin into the pleural cavity. The inflammatory fluid was collected at various times and the eicosanoid (6-keto prostaglandin F<sub>1α</sub> (6-keto PGF<sub>1α</sub>), thromboxane B<sub>2</sub> (TXB<sub>2</sub>) and leukotriene B<sub>4</sub> (LTB<sub>4</sub>)) content estimated by radioimmunoassay. Inflammatory fluid from adrenalectomised rats (●) invariably contained more eicosanoids than inflammatory fluid from sham-operated controls (○) suggesting that glucocorticoids exert a tonic inhibitory influence on eicosanoid production *in vivo*. \*P < 0.01; \*\*\*P < 0.001. Reprinted, by permission, from Flower *et al.* (1986) *Br. J. Pharmacol.*, 87, 57–62.

tration of glucocorticoids to both groups of rats reversed the inflammatory process, the adrenalectomised animals being more sensitive.

Interestingly, even when resident macrophages were collected from adrenalectomised rats and stimulated with zymosan *ex vivo*, the eicosanoid production per cell in unit time was greater than with cells from control animals.

The latter data were confirmed by Vincent and his colleagues (1986) who found a striking increase in leukotriene B<sub>4</sub> (LTB<sub>4</sub>) production by peritoneal macrophages from adrenalectomised animals (interestingly the amount in splenic tissue was decreased). In addition to the enhanced synthesis and release of mediators, produced from endogenous arachidonic acid, Vincent and his colleagues found that the production of products from exogenous arachidonic acid also seemed to be increased. Possibly steroids also provide a long-term suppressive influence on the levels of cyclo-oxygenase and lipoxygenase enzymes as well as acute effect on phospholipase A<sub>2</sub>.

There is a considerable body of largely anecdotal evidence suggesting that adrenalectomy enhances the effects of pro-inflammatory substances. Undoubtedly the loss of the adrenal medulla is also important, however treatment of animals with the glucocorticoid receptor antagonist RU38486 leads to a qualitatively similar effect on inflammation and lyso-Paf release *ex vivo* (Peers *et al.*, 1988) so the adrenal cortex and therefore lipocortin is certainly of considerable significance in this phenomenon. In view of the fact that steroids increase  $\beta$ -adrenoceptor numbers it may well be that there is a very important interaction between the hormones of the adrenal medulla and the cortex.

All these data suggest that if the amount of lipocortin becomes limiting then the organism/cell is unable to maintain control over the development of the inflammatory response or phospholipase activity. It implies that lipocortin or a similar protein provides a tonic inhibitory influence on cellular phospholipase activity and that one of the ways in which steroids act is by increasing the available amount of this inhibitor.

#### Human lipocortin 1 has been sequenced and cloned

In a study already referred to, Pepinsky *et al.* (1986) purified a protein from rat peritoneal lavage fluid which inhibited phospholipase A<sub>2</sub> and subjected it to partial sequence analysis. This protein, which represented the major inhibitor present in lavage fluid, had a mass of 37 kDa, but gave rise to a series of proteolytic fragments including species at 30, 24 and 15 kDa: these corresponded very closely to the

**Table 10** The amino acid composition of human recombinant lipocortin 1

Amino acid	Mol %
Asp	39
Thr	22
Ser	19
Gln	39
Gly	20
Ala	33
Cys	4
Val	19
Met	9
Iso	21
Leu	34
Pro	8
Tyr	11
Phe	11
His	5
Lys	32
Trp	1
Arg	19

Data from Wallner *et al.* (1986).

observed distribution of anti-phospholipase proteins in the systems described above.

The sequence of several tryptic fragments of the rat peritoneal lavage protein were determined and this information was used to synthesize chemically pools of oligonucleotide probes. With these probes it was possible to locate, in a U937 cDNA library, the human gene. From this it was possible to deduce the complete amino acid sequence, and thus the primary structure of the protein (Wallner *et al.*, 1986). Human lipocortin is very closely related to the rat protein isolated by Pepinsky and his colleagues suggesting that it is highly conserved throughout evolution. It is a very polar molecule, with approximately a third of its amino acids being charged (see Table 10). These are distributed throughout the molecule separated by short stretches of hydrophobic residues. There are 4 cysteines close to the C-terminal (263, 270, 324 and 343) which presumably can form disulphide bridges.

Recombinant human lipocortin does not apparently contain a leader peptide, and although it is clearly recovered as an extracellular protein, the mechanism whereby its release from cells is effected is unclear at the moment. Lipocortin contains a single potential glycosylation site (Asn-43-Ser45) and also contains the consensus sequences for both tyrosine and threonine phosphorylation sites (Tyr 21 and Thr 212). The latter point is of a special significance since it had already been demonstrated that the naturally occurring molecule could be phosphorylated, and that the phosphorylated form was inactive as a phospholipase A<sub>2</sub> inhibitor (Hirata, 1981).

To confirm that human lipocortin was a phospholipase inhibitory protein, a full length coding sequence was expressed in *E. coli* and crude extracts of these *E. coli* organisms, or highly purified fractions containing the recombinant protein were found to be highly inhibitory in a popular conventional cell-free phospholipase A<sub>2</sub> enzyme assay (but see earlier section on assay techniques).

Using Northern blot analysis, it was possible to demonstrate that the lipocortin gene was indeed glucocorticoid-sensitive: mRNA levels in peritoneal lavage cells were increased by approximately 6 fold 2 h after the injection of an inducing steroid.

Huang *et al.* (1986) observed that human placental extracts contained two types of lipocortin. One species (subsequently christened lipocortin 1) was identical to the recombinant protein previously cloned but the other (called lipocortin 2) was a different protein. Like lipocortin 1, lipocortin 2 does not contain a signal sequence: both proteins are very polar molecules with approximately one third of their total amino acids being charged. Overall there was approximately 50% sequence homology suggesting that the genes for the two proteins arose by gene duplication. It was within the central region of lipocortin 2 that the greatest homology with lipocortin 1 was observed.

Interestingly the sequences near the N-terminus of the two proteins were substantially different although both contained a sequence which can be phosphorylated by tyrosine kinases.

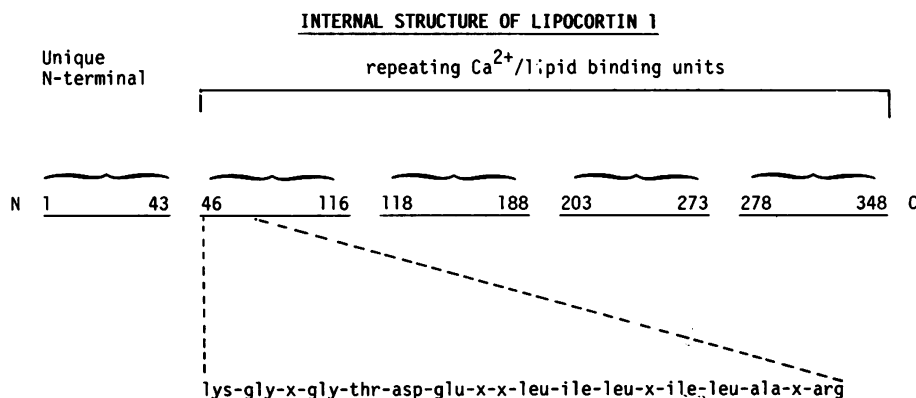
Both proteins have very comparable molecular weight and similar pI values (7.9) both are inhibitors of phospholipase activity and require the presence of calcium before association with membranes can occur.

We do not know anything about the biological activity of lipocortin 2, other than the fact it has approximately the same antiphospholipase activity as lipocortin 1. However, the protein has now been cloned and it will presumably only be a matter of time before such data are available.

#### Lipocortin 1 and 2 are related to other membrane-associated proteins

Kretsinger & Creutz (1986) observed the presence of three repeating consensus sequences in lipocortin 1, and Munn & Mues (quoted in Huang *et al.*, 1986) have gone further and suggested that, except for the first 43 amino acids the primary structure of lipocortin 1 is built from 4 repeats of a single unit (see Figure 18). The same basic structure has been observed in lipocortin 2 and similar sequences are also seen in some other calcium (see Table 11) and lipid binding proteins such as calpactin (see Table 11). In fact, some very recent work has demonstrated that the cytoskeletal protein previously referred to as 'calpactin 2', is in all probability, identical to lipocortin 1 (Glenney *et al.*, 1987).

It is likely that lipocortin 1 and lipocortin 2 are related, or identical to, some other hitherto unsequenced proteins too and, indeed, may belong to a super family of membrane-associated proteins. For example, a very important discovery made by the Biogen group (Pepinsky & Sinclair, 1986 and Huang *et al.*, 1986) is that lipocortin 1 is identical to the substrate for the EGF receptor kinase and that lipocortin 2 is identical to the PP60 src kinase sub-



**Figure 18** Lipocortin 1 contains a unique N-terminal and four-fold repeat sequences with Ca<sup>2+</sup> and lipid binding properties. The lipocortin 1 molecule may be considered to possess 5 domains: a unique N-terminal and four regions 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<sup>2+</sup> and certain phospholipids and may be the mechanism by which lipocortin attaches to cell membranes. Adapted from data in Saris *et al.* (1986) *Cell*, **46**, 201-202.

**Table 11** Relationships between the Ca<sup>2+</sup>/phospholipid binding proteins

67–73 kDa	35–40 kDa	34–39 kDa	28–32.5 kDa
Intestinal (73 kDa)	P35 Calpactin II Lipocortin 1	P36 (P34, P39) Calpactin I Lipocortin 2 Protein I	Protein II
anti-PLA <sub>2</sub> (70 kDa)			Endonexin
Calelectrin (70 kDa)		Calelectrin (36 kDa)	Calelectrin 32.5 kDa)
Lymphocyte (68 kDa)		Lymphocyte (33 kDa)	Lymphocyte (28 kDa)
Calcimedlin (67 kDa)	Calcimedlin (35 kDa) Chromobindin IX EGF-receptor Kinase substrate	Calcimedlin (33 kDa) Chromobindin VIII pp60 <sup>src</sup> kinase substrate	

Modified, with permission from Saris *et al.* (1986).

strate. These results are important, for whilst the molecular weight of the major cellular substrates for these kinases has been known for a long time their precise function was a mystery. The fact that lipocortin 1 and 2 are inhibitors of phospholipase activity may provide a valuable clue as to the role of these proteins in the regulation of cell growth and division, and promises to be a fruitful and rewarding area of future research, particularly in the light of some recent work showing that microinjection of the *ras* oncogene into fibroblasts leads to phospholipase A<sub>2</sub> activation, membrane ruffling (Bar-Sagi & Feramisco, 1986) and proliferative responses (Stacey & Kung, 1984).

#### Recombinant human lipocortin 1 shares many of the actions of 'macrocortin' and the glucocorticoids

Cloning of the human lipocortin 1 gene was a major breakthrough because it enabled large amounts of the protein to be produced for biological testing.

Because there were several, apparently distinct, antiphospholipase components in peritoneal lavage fluid, an obvious question was whether lipocortin 1 could account for the properties observed in the early experiments with 'macrocortin' containing extracts. At the time of writing, apart from the *in vitro* anti-enzyme activity, the activity of lipocortin 1 has been checked in three systems known to be sensitive to 'macrocortin' they are, the guinea-pig perfused lung system, the release of eicosanoids by endothelial cells and the carageenin-induced paw oedema in the rat.

Cirino & Flower (1987b) demonstrated that recombinant human lipocortin 1 (ex *E. coli*) strongly inhibited the release of prostacyclin by human umbilical arterial endothelial cells, with complete suppression occurring in concentrations above 0.1  $\mu$ M (see Table 12).

When dealing with recombinant proteins, particularly those derived from *E. coli* (the most popular vector), the perennial problem of purity arises. Even when the protein is (as in the case of lipocortin 1)

**Table 12** Human recombinant lipocortin 1 inhibits prostacyclin generation by human umbilical artery

Sample	†PGI <sub>2</sub> (ng per 100 mg tissue)	% inhibition
Control	37.0 ± 2.85 (4)	—
Lipocortin 0.01 $\mu$ M	28.6 ± 2.15 (2)	22.7
0.02	9.3 ± 0.95 (2)	74.9
0.025	8.5 ± 2.89 (4)	77.0
0.05	6.9 ± 3.0 (4)	81.4
0.1	0.8 ± 0.1 (2)	97.8
Sham lipocortin 0.1 $\mu$ M	37.0 ± 1.0 (2)	—
Lipocortin (0.1 $\mu$ M) + trypsin	33.0 ± 2.0 (2)	10.8
Indomethacin 10 $\mu$ M	8.5 ± 1.5 (2)	77.0

† Values are mean ± s.e. mean, *n* in parentheses.  
Data from Cirino & Flower (1987).

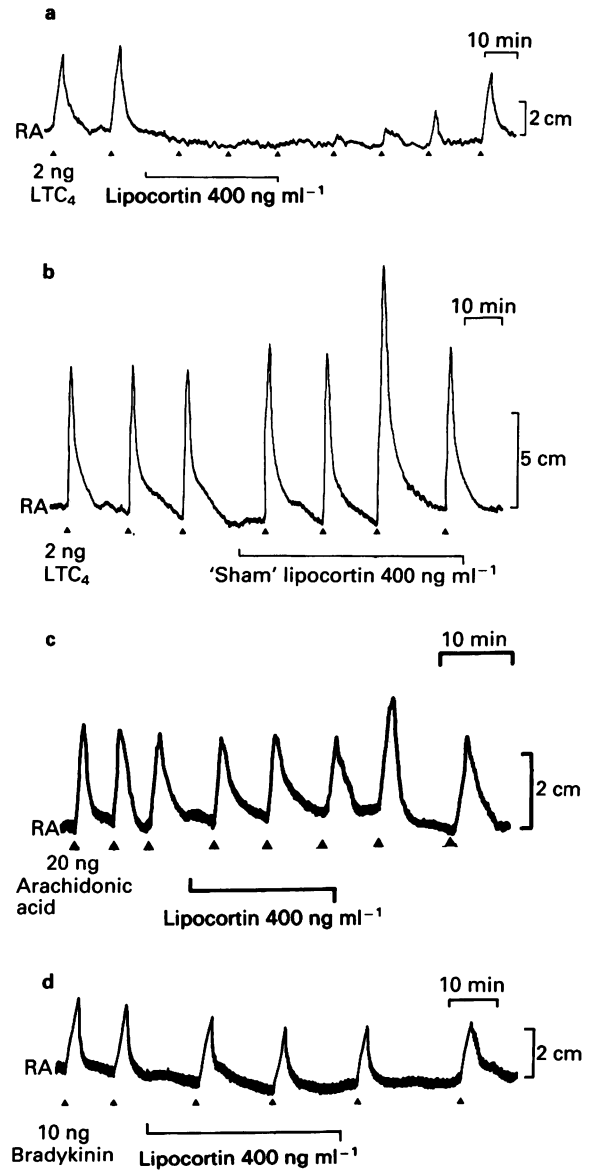
greater than 99% pure protein, traces of *E. coli* proteins, endotoxin or DNA can cause false positives and negatives in many experiments, particularly those involving isolated leucocytes or models of inflammation. To circumvent this type of problem a particular type of control was devised and tested. 'Sham' lipocortin was a preparation produced by extracting *E. coli* organisms, which did not contain the lipocortin plasmid, and processing it in an identical fashion to the preparations containing lipocortin. In this way, one obtains a sample which contains both qualitatively and quantitatively the same contaminants as the lipocortin preparations but without the active protein. This was routinely employed as a control in all our early studies with this protein.

Cirino and his colleagues Cirino, Peers, Flower, Browning, Sinclair & Pepinsky, unpublished observations also tested recombinant lipocortin 1 in the guinea-pig perfused lung preparation. Like macrocortin, and the glucocorticoids themselves, lipocortin was able to block the release of thromboxane  $A_2$  from the perfused lung when this was elicited by leukotriene  $C_4$  or FMLP but not when elicited by bradykinin or arachidonic acid (see Figure 19). Unlike steroids; but like macrocortin itself, the action of lipocortin was very rapid and was easily reversed when the infusion was stopped. As with the steroids and macrocortin, the effect was cumulative with time and the actual amount of inhibition was dependent upon the concentration of the agonist as well as the concentration of lipocortin itself.

Experiments in which lipocortin 1 has been shown to block eicosanoid release provide useful evidence that this protein can mimic some actions of steroids but the real field of interest is inflammation. To what extent can this protein reproduce the potent anti-inflammatory effect of these drugs?

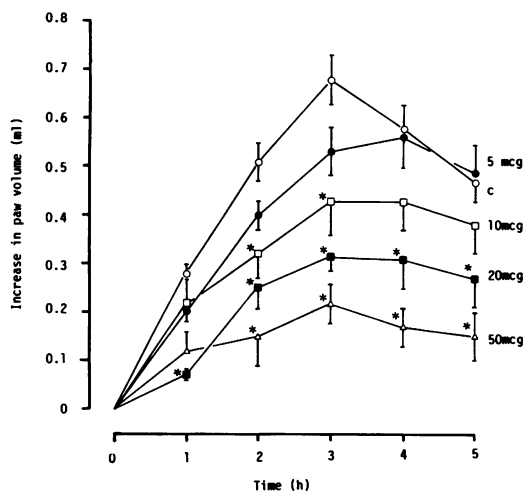
It is too early to answer this question fully yet: at the time of writing only one study has been completed, but this had proved most interesting. Cirino *et al.* (unpublished observations) studied the action of lipocortin 1 given locally in the rat paw oedema assay, a very well characterized model of inflammation sensitive both to steroidal and non-steroidal drugs.

The swelling of the paw was substantially inhibited by lipocortin, when the agent inducing inflammation was carrageenin (see Figure 20) which acts principally by releasing eicosanoids, but not when Paf or dextran were used. The latter two irritants probably act by releasing mast cell amines and although they are sensitive to steroid they are not sensitive to inhibition by the non-steroidal, 'aspirin-like', drugs. Another agent which also works by liberating mast cell amines is the enzyme phospholipase  $A_2$  itself. In this particular case the purified phospholipase  $A_2$  from the venom of *Naja naja mocam-*



**Figure 19** Human recombinant lipocortin 1 mimics the action of 'macroscortin' and glucocorticoids in the guinea-pig perfused isolated lung system. In these experiments leukotriene  $C_4$  ( $LTC_4$ ), bradykinin and arachidonic acid were used as the releasing agents. The release of thromboxane  $A_2$  ( $TXA_2$ ) was detected using the superfused rabbit aortic strip. Trace (a): inhibition by lipocortin of the release of  $TXA_2$  induced by 2 ng  $LTC_4$ . Trace (b): lack of effect of 'sham' preparation of lipocortin (see text for details) on the release of  $TXA_2$  induced by 2 ng  $LTC_4$ . Traces (c) and (d): lack of effect of lipocortin on the release of  $TXA_2$  induced by bradykinin or arachidonic acid. Reprinted, by permission, from Cirino *et al.* (1987) *Nature*, 328, 270-272.





**Figure 20** Human recombinant lipocortin 1 inhibits carrageenin-induced oedema of the rat paw. Lipocortin was injected locally into the paw at the same time as the carrageenin solution. Measurements of paw volumes were made each hour using conventional plethysmographic techniques. For technical reasons no more than 50  $\mu$ g lipocortin could be given.

The relationship between the log dose and the inhibition was linear and the  $ED_{50}$  10–20  $\mu$ g per paw. On a molar basis, lipocortin is over 100 times more effective than indomethacin in this model.

*bique* was employed. The pro-inflammatory action was greatly reduced when this agent was premixed with lipocortin 1 before injection, presumably because the protein was binding to, and inactivating the inhibitor.

These experiments suggest that lipocortin 1 duplicates those actions of steroids which are mediated by the release of eicosanoids or by the direct action of phospholipase itself but not those caused by the direct action of substances such as Paf, histamine or 5-HT. Since the glucocorticoids can inhibit *all* these types of oedema it would strongly suggest that another protein (or mechanism) is involved. Di Rosa and his colleagues have indeed suggested that such a protein exists and have named it 'vasocortin'.

#### There are autoantibodies to lipocortin 1 in the sera of patients with chronic inflammatory diseases

Hirata and his colleagues (1981) made the important and original observation that a high proportion of patients with chronic rheumatic diseases have detectable autoantibodies to lipocortin in their sera, whereas disease-free people did not. The titres of antibody were particularly high in patients with systemic lupus erythematosus and rheumatoid arthritis,

diseases in which there are frequently autoantibodies to a variety of cell surface and other proteins.

This work led the N.I.H. group to the concept that the presence of these autoantibodies may contribute to the aetiology of these diseases by removing 'protective' proteins which regulate eicosanoid generation.

This observation was followed up by the author's colleagues at the Bath Institute of Rheumatic Diseases (Podgorski *et al.*, 1987). Using recombinant human lipocortin 1 as a 'target' they devised an ELISA assay for measuring anti-lipocortin autoantibodies and examined the sera of selected patients attending the Royal National Hospital for Rheumatic Diseases as well as a large number of controls.

These authors confirmed the presence of both 1 gG and 1 gM anti-lipocortin autoantibodies in sera from patients with systemic lupus and rheumatoid arthritis but also made two striking and original observations. In patients with systemic lupus the antibodies seemed present whether or not the patients had already received any steroid therapy but in the group with rheumatoid arthritis, significantly elevated titres were only seen in patients who were receiving oral glucocorticoid therapy. In both groups the presence of a high titre of autoantibodies correlated strongly with the clinical phenomenon of steroid 'resistance'. Many other patients with other inflammatory diseases were also investigated but no autoantibodies were detected, whether or not they were receiving steroids or were 'steroid resistant'.

This notion, that the administration of glucocorticoids may encourage the formation of autoantibodies to lipocortin in certain susceptible groups and that if present to excess these autoantibodies may lead to steroid resistance, or at least some manifestation of it, is obviously a radical one with important implications for a correct understanding of glucocorticoid therapy.

#### Final reflections

The glucocorticoids inhibit our 'defence reactions' at many levels. One way in which they achieve this is by inhibiting the synthesis of chemicals involved in the promotion of the inflammatory response. The production of many mediators involved in the response to infection, injury, haemorrhage or metabolic disturbances are under glucocorticoid control such that elevated levels of hormone in the blood suppresses their formation. In many cases the action of these mediators is blocked as well.

It might be thought that the glucocorticoids act simply by decreasing the synthesis rate of these protein regulators of inflammation such as the lymphokines, or of the enzymes which make prostaglan-

dins. Whilst this undoubtedly does occur, another mechanism is also employed: that is, the glucocorticoid-induced synthesis of inhibitory proteins.

Lipocortin (and possibly other related proteins) then is a sort of 'second messenger' of the glucocorticoids. It is only one of many such regulatory proteins but it is an important one, controlling as it does the mediators which promote development of the symptoms of the inflammatory response. It is undoubtedly the significant component of the inbuilt mechanism for terminating the inflammatory response which the physician exploits, for when he gives his patients relatively large doses of steroids to control an inflammatory response, he is in reality increasing the synthesis of these 'second messenger' proteins such as lipocortin to a near maximum.

All the early studies on lipocortin were performed *in vitro*, that is under conditions in which steroids were not normally present. Under these circumstances the generation and appearance of lipocortin seemed absolutely dependent upon the presence of glucocorticoids in the perfusing medium. These findings have led some to the erroneous notion that lipocortin was only present following treatment with exogenous steroids.

Of course, all healthy mammals have circulating glucocorticoids and thus it is more rational to expect that lipocortin is a normal constituent of plasma and tissues (as indeed it appears to be), although the amount present in the cells can be increased by raising the concentration of endogenous or exogenous steroids.

There has been a corresponding change in our appreciation of the function of lipocortin. Originally, it was regarded mainly as an 'anti-inflammatory protein' but today it seems more likely that this protein is present in most cells, and that its function is to control phospholipase A<sub>2</sub> activity and to allow lipid hydrolysis only under strictly defined circumstances. This reversible inhibitory function of lipocortin could well be controlled by the

phosphorylation and dephosphorylation cycle described above. Naturally, during inflammation, phospholipase is substantially activated and thus there is a requirement for a greater than normal supply of the inhibitory protein, hence the relationship between the rate of synthesis and the release of steroid hormones.

The discovery, characterisation, isolation, sequencing and cloning of lipocortin has opened up an entirely new and exciting chapter in cell biology and also holds out a strong promise for the future of anti-inflammatory therapy. In addition to their beneficial clinical effects, steroids produce a wide spectrum of side effects which preclude the use of these drugs for long periods of time except in the very seriously ill. These side effects are caused by changes in the transcription of specific genes in the same way as the anti-inflammatory effects. It has long been an article of a faith of scientists working in this area that if we could identify and isolate the 'second messengers' of steroid action that are responsible for the anti-inflammatory effects, it should be possible to produce drugs which possess many of the beneficial action of steroids without incurring the heavy penalty of side effects. The real value of this work is that it enables us to take our first steps in that direction.

#### Dedication

This manuscript is dedicated to all my colleagues who shared the excitement and frustration of researching this area and to John Vane, who taught me his own particular version of Gaddum's 'Technique of Superfusion'.

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#### References

- AARSMAN, A.J., MYNBEEK, G., VAN DEN BOSCH, H., ROTHHUT, B., PRIEUR, B., COMERA, C., JORDON, L. & RUSSO-MARIE, F. (1987). Lipocortin inhibition of extracellular and intracellular phospholipases A<sub>2</sub> is substrate concentration dependent. *FEBS Letters*, **219**, 176-180.
- ALBERT, D.H. & SNYDER, F. (1983). Biosynthesis of 1-Alkyl-2-acetyl-Sn-glycero-3-phosphocholine (Platelet-Activating factor) from 1-alkyl-2-acyl-3n-glycerophosphocholine by rat alveolar macrophages. Phospholipase A<sub>2</sub> and acetyltransferase activities during phagocytosis and ionophore stimulation. *J. Biol. Chem.*, **258**, 97-102.
- ALBERT, D.H. & SNYDER, F. (1984). Release of arachidonic acid from 1-alkyl-2-acyl-sn-glycero-3-phosphocholine, a precursor of platelet-activating factor, in rat alveolar macrophages. *Biochem. Biophys. Acta*, **796**, 92-101.
- AUTHI, K.S., SOLANKY, A. & TRAYNOR, J.R. (1982). Inhibition of an inflammatory exudate phospholipase A<sub>2</sub> by an endogenous inhibitor of polymorphonuclear leucocytes. *Pharmacol. Res. Commun.*, **14**, 401-407.
- BAR-SAGI, D. & FERAMISCO, J.R. (1986). Induction of membrane ruffling and fluid phase pinocytosis in quiescent fibroblasts by *ras* proteins. *Science*, **233**, 1061-1068.
- BARTOLF, M. & FRANSON, R.C. (1987). Modulation by cytosol and commercial proteins of acid-active phos-

- pholipase A<sub>2</sub> from adrenal medulla. *Biochem. Biophys. Acta*, **917**, 308–317.
- BAXTER, J.D. (1976). Glucocorticoid hormone action. In *Pharmacology and Therapeutics Part B*. ed. Gill, G.N. Vol. 2, pp. 605–659. Oxford: Pergamon Press.
- BLACKWELL, G.J. (1983). Specificity and inhibition of glucocorticoid-induced macrocortin secretion from rat peritoneal macrophages. *Br. J. Pharmacol.*, **79**, 587–594.
- 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., 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. Pharmacol.*, **76**, 185–194.
- BLACKWELL, G.J., FLOWER, R.J., NIJKAMP, F.P. & VANE, J.R. (1978). Phospholipase A<sub>2</sub> activity of guinea-pig isolated perfused lungs: stimulation and inhibition by anti-inflammatory steroids. *Br. J. Pharmacol.*, **62**, 79–89.
- BRAY, M.A. (1983). Pharmacology and pathophysiology of leukotriene B<sub>4</sub>. In *Prostacyclin, Thromboxane and Leukotrienes*. ed. Moncada, S. *Br. Med. Bull.*, Vol. 39, pp. 249–254.
- BRAY, M.A. & GORDON, D. (1976). Effects of anti-inflammatory drugs on macrophage prostaglandin biosynthesis. *Br. J. Pharmacol. Chemother.*, **57**, 466P.
- BULLER, R.E. & O'MALLEY, B.W. (1976). The biology and mechanism of steroid hormone receptor interaction with the eukaryotic nucleus. *Biochem. Pharmacol.*, **25**, 1–12.
- CARNUCCIO, R., DI ROSA, M. & PERSICO, P. (1980). Hydrocortisone induced inhibitor of prostaglandin biosynthesis in rat leucocytes. *Br. J. Pharmacol.*, **68**, 14–16.
- CARNUCCIO, R., DI ROSA, M., FLOWER, R.J. & PINTO, A. (1981). The inhibition by hydrocortisone of prostaglandin biosynthesis in rat peritoneal leucocytes is correlated with intracellular macrocortin levels. *Br. J. Pharmacol.*, **74**, 322–324.
- CHAN, L. & O'MALLEY, B.W. (1976). Mechanism of action of the sex steroid hormones. *New Engl. J. Med.*, **294**, 1372–1379.
- CHANG, J., MUSSER, J.H. & MCGREGOR, H. (1987). Phospholipase A<sub>2</sub>: function and pharmacological regulation. *Biochem. Pharmacol.*, **36**, 2429–2436.
- CIRINO, G. & FLOWER, R.J. (1987a). The inhibitory effect of lipocortin on eicosanoid synthesis is dependent upon Ca<sup>2+</sup> ions. *Br. J. Pharmacol.*, **92**, 521P.
- CIRINO, G. & FLOWER, R.J. (1987b). Human recombinant lipocortin 1 inhibits prostacyclin production by human umbilical artery in vitro. *Prostaglandins*, **34**, 59–62.
- CIRINO, C., FLOWER, R.J., BROWNING, J.L., SINCLAIR, L.K. & PEPINSKY, R.B. (1987). Recombinant human lipocortin inhibits thromboxane release from guinea-pig isolated perfused lung. *Nature*, **328**, 270–272.
- CLOIX, J.F., COLARD, O., ROTHUT, 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. Pharmacol.*, **79**, 313–321.
- COOTE, P.R., DI ROSA, M., FLOWER, R.J., PARENTE, L., MERRETT, M. & WOOD, J.N. (1983). Detection and isolation of a steroid-induced antiphospholipase protein of high molecular weight. *Br. J. Pharmacol.*, **80**, 597P.
- DANON, A. & ASSOULINE, G. (1978). Inhibition of prostaglandin biosynthesis by corticosteroids requires RNA and protein synthesis. *Nature*, **273**, 552–554.
- DAVIDSON, F.F., DENNIS, E.A., POWELL, M. & GLENNEY, J.R. (1987). Inhibition of phospholipase A<sub>2</sub> by lipocortins and calpactins – an effect of binding to substrate phospholipids. *J. Biol. Chem.*, **262**, 1698–1705.
- DE CATERINA, R. & WEKSLER, B.B. (1986). Modulation of arachidonic acid metabolism in human endothelial cells by glucocorticoids. *Thrombosis Haemostasis*, **55**, 369–374.
- DI ROSA, M., FLOWER, F.J., HIRATA, F., PARENTE, L. & RUSSO-MARIE, F. (1984). Nomenclature announcement. Anti-phospholipase proteins. *Prostaglandins*, **28**, 441–442.
- DI ROSA, M. & PERSICO, P. (1979). Mechanism of inhibition of prostaglandin biosynthesis by hydrocortisone in rat leucocytes. *Br. J. Pharmacol.*, **66**, 161–163.
- ERRASFA, M., ROTHUT, B., FRADIN, A., BILLARDON, C., JUNIEN, J.L., BURE, J. & RUSSO-MARIE, F. (1985). The presence of lipocortin in human embryonic skin fibroblasts and its regulation by anti-inflammatory steroids. *Biochim. Biophys. Acta*, **847**, 247–254.
- FERREIRA, S.H., MONCADA, S. & VANE, J.R. (1971). Indomethacin and aspirin abolish prostaglandin release from the spleen. *Nature (New Biol.)*, **231**, 237–239.
- FERREIRA, S.H. & VANE, J.R. (1979). The mode of action of anti-inflammatory agents which are prostaglandin synthetase inhibitors. In *Handbook of Experimental Pharmacology*, Vol. 50 (II), *Anti-inflammatory Drugs*. ed. Vane, J.R. & Ferreira, S.H. Berlin: Springer-Verlag.
- FLOWER, R.J. (1984). Macrocortin and the anti-phospholipase proteins. In *Advances in Inflammation Research*, Vol. 8, ed. Weissmann, G. pp. 1–33. New York: Raven Press.
- FLOWER, R.J. & BLACKWELL, G.J. (1976). The importance of phospholipase A<sub>2</sub> in prostaglandin biosynthesis. *Biochem. Pharmacol.*, **25**, 285–291.
- FLOWER, R.J. & BLACKWELL, G.J. (1979). Anti-inflammatory steroids induce biosynthesis of a phospholipase A<sub>2</sub> inhibitor which prevents prostaglandin generation. *Nature*, **278**, 456–459.
- FLOWER, R.J., GRYGLEWSKI, R., HERBACYNSKACEDRO, K. & VANE, J.R. (1972). The effects of anti-inflammatory drugs on prostaglandin biosynthesis. *Nature (New Biol.)*, **238**, 104–106.
- FLOWER, R.J., PARENTE, L., PERSICO, P. & SALMON, J.A. (1986). A comparison of the acute inflammatory response in adrenalectomised and sham operated rats. *Br. J. Pharmacol.*, **87**, 57–62.
- GADDUM, J.H. (1954). The science of pharmacology. *Nature*, **173**, 14–15.
- GAUNT, R. (1974). History of the adrenal cortex. In *Handbook of Physiology* (7). ed. Blaschko, H., Sayers, G. & Smith, A.D. Washington D.C.: American Physiological Society.
- GLENNEY, J.R., TACK, B. & POWELL, M.A. (1987). Calpactins: Two distinct Ca<sup>2+</sup>-regulated phospholipid- and actin-binding proteins isolated from lung and placenta. *J. Cell. Biol.*, **104**, 503–511.
- GREAVES, M.W. & McDONALD-GIBSON, W. (1972). Inhibi-

- tion of Prostaglandin biosynthesis by corticosteroids. *Br. Med. J.*, **2**, 83-84.
- GRYGLEWSKI, R.J., PANCZENKO, B., KORBUT, R., GRODZINSKA, L. & OCETKIEWICZ, A. (1975). Corticosteroids inhibit prostaglandin release from perfused mesenteric blood vessels of rabbit and from perfused lungs of sensitized guinea-pigs. *Prostaglandins*, **10**, 343-355.
- GUPTA, C. & GOLDMAN, A.S. (1985). Dexamethasone-induced phospholipase A<sub>2</sub> inhibitory proteins (PLIP) influenced by the H-2 histocompatibility region (41980). *Proc. Soc. Exp. Biol. Med.*, **178**, 29-35.
- GUPTA, C., KATSUMATA, M., GOLDMAN, A.S., PIDDINGTON, R. & HEROLD, R. (1984). Glucocorticoid-induced phospholipase A<sub>2</sub> inhibitory proteins mediate glucocorticoid teratogenicity in vitro. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 1140-1143.
- GURPIDE, E., MARKIEWICZ, L., SCHATZ, F. & HIRATA, F. (1986). Lipocortin output by human endometrium in vitro. *J. Clin. Endocrinol. Metab.*, **63**, 162-166.
- HAIGLER, H.T., SCHLAEPFER, D.D. & BURGESS, W.H. (1987). Characterisation of lipocortin 1 and an immunologically unrelated 33 kDa protein as epidermal growth factor receptor/kinase substrates and phospholipase A<sub>2</sub> inhibitors. *J. Biol. Chem.*, **262**, 6921-6930.
- HAMBERG, M., SVENSSON, J. & SAMUELSSON, B. (1974). Prostaglandin endoperoxides. A new concept concerning the mode of action and release of prostaglandins. *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 3824-3828.
- HAMBERG, M., SVENSSON, J. & SAMUELSSON, B. (1975). Thromboxanes: A new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 2994-2998.
- HENCH, P.S., KENDALL, E.C., SLOCUMB, C.H. & POLLEY, H.F. (1949). The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone: compound E) and of pituitary adrenocorticotrophic hormone on rheumatoid arthritis. *Proc. Staff Meet. Mayo Clin., Rochester*, **25**, 81.
- HERBACZYNSKA-CEDRO, K. & STASZEWSKA-BARCZAK, J. (1974). Adrenocortical hormones and the release of prostaglandin-like substances (PLS). *Abstracts of II Congress of Hungarian Pharmacological Society*, p. 19, Budapest.
- HIRATA, F. (1981). The regulation of lipomodulin, a phospholipase inhibitory protein, in rabbit neutrophils by phosphorylation. *J. Biol. Chem.*, **256**, 7730-7733.
- HIRATA, F. (1983). Lipomodulin: a possible mediator of the action of glucocorticoids. In *Advances in Prostaglandin, Thromboxane and Leukotriene Res.* ed. Samuelsson, B., Paoletti, R. & Ramwell, P. Vol. 11, pp. 73-78. New York: Raven Press.
- HIRATA, F., CORCORAN, B.A., VENKATASUBRAMANIAN, K., SCHIFFMANN, E. & AXELROD, J. (1979). Chemoattractants simulate degradation of methylated phospholipids and release of arachidonic acid in rabbit leukocytes. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 2640-2643.
- HIRATA, F., DEL CARMINE, R., NELSON, C.A., AXELROD, J., SCHIFFMANN, E., WARABI, A., DE BLAS, A.L., NIRENBERG, M., MANGANIELLO, V., VAUGHAN, M., KUMAGAI, S., GREEN, I., DECKER, J.L. & STEINBERG, A.D. (1981). Presence of autoantibody for phospholipase inhibitory protein, lipomodulin, in patients with rheumatic diseases. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 3190-3194.
- HIRATA, F. & IWATA, M. (1983). Role of lipomodulin, a phospholipase inhibitory protein, in immunoregulation by thymocytes. *J. Immunol.*, **130**, 1930-1936.
- HIRATA, F., NOTSU, Y., IWATA, M., PARENTE, L., DI ROSA, M. & FLOWER, R.J. (1982). Identification of several species of phospholipase inhibitory proteins by radioimmunoassay for lipomodulin. *Biochem. Res. Commun.*, **109**, 223-230.
- HIRATA, F., SCHIFFMANN, E., VENKATASUBRAMANIAN, K., SALOMON, D. & AXELROD, J. (1980). A phospholipase A<sub>2</sub> inhibitory protein in rabbit neutrophils induced by glucocorticoids. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 2533-2536.
- HONG, S.C. & LEVINE, L. (1976). Inhibition of arachidonic acid release from cells as the biochemical action of anti-inflammatory steroids. *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 1730-1734.
- HUANG, K.-S., WALLNER, B.P., MATTALIANO, R.J., TIZARD, R., BURNE, C., FREY, A., HESSION, C., McGRAY, P., SINCLAIR, L.K., PINGCHONG CHOW, E., BROWNING, J.L., RAMACHANDRAN, K.L., TANG, J., SMART, J.E. & PEPINSKY, R.B. (1986). Two human 35 kD inhibitors of phospholipase A<sub>2</sub> are related to substrates of pp60 v-SRC and of the epidermal growth factor receptor/kinase. *Cell*, **46**, 191-199.
- KRETSINGER, R.J. & CREUTZ, C.E. (1986). Consensus in exocytosis. *Nature*, **320**, 573.
- LEWIS, G.P. & PIPER, P.J. (1975). Inhibition of release of prostaglandins as an explanation of some of the actions of anti-inflammatory corticosteroids. *Nature (Lond.)*, **254**, 308-311.
- LONGENECKER, J.P., ROSE, J.W., GIFFIN, K., SHEPARD, D. & JOHNSON, L.K. (1987). Isolation of a human, endogenous, phospholipase A<sub>2</sub> inhibitory, anti-inflammatory protein. In *Advances in Prostaglandin, Thromboxane and Leukotriene Res.* ed. Samuelsson, B., Paoletti, R. & Ramwell, P.W. Vol. 17, pp. 581-586. New York: Raven Press.
- MONCADA, S. (1982). Biological Importance of Prostacyclin. VIIIth Gaddum Lecture. *Br. J. Pharmacol.*, **76**, 3-31.
- MUNCK, A. & BRINCK-JOHNSON, T. (1967). Specific metabolic and physicochemical interactions of glucocorticoids in vivo and in vitro with rat adipose tissue and thymus cells. *Excerpta Med. Intern. Congr. Ser.*, 132-472.
- MUNCK, A. & BRINCK-JOHNSON, T. (1968). Specific and non-specific physicochemical interactions of glucocorticoids and related steroids with rat thymus cells in vitro. *J. Biol. Chem.*, **243**, 5556-5560.
- 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.
- MUNCK, A. & LEUNG, K. (1977). Glucocorticoid receptors and mechanisms of action. In *Receptors and Mechanism of Action of Steroid Hormones*. Part II. ed. Pasqualini, J.R. pp. 311-319. New York: Marcel Dekker.
- MURPHY, R.C., HAMMARSTROM, S. & SAMUELSSON, B. (1979). Leukotriene C<sub>4</sub>. A slow-reacting substance from murine mastocytoma cells. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4275-4279.
- NIJKAMP, F.P., FLOWER, R.J., MONCADA, S. & VANE, J.R. (1976). Partial purification of RCS-RF (rabbit aorta contracting substance-releasing factor) and inhibition of

- its activity by anti-inflammatory steroids. *Nature*, **263**, 479–482.
- 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. Pharmacol.*, **99**, 233–239.
- PARENTE, L. & FLOWER, R.J. (1985). Hydrocortisone and macrocortin inhibit the zymosan-induced release of lyso-PAF from rat peritoneal leucocytes. *Life Sci.*, **36**, 1225–1231.
- PEERS, S.H., MOON, D. & FLOWER, R.J. (1988). Reversal of the anti-inflammatory effects of dexamethasone by the glucocorticoid antagonist RU 38486. *Biochem. Pharmacol.*, **37**, 556–557.
- PEPINSKY, R.B., SINCLAIR, L.K., BROWNING, J.L., MATTALIANO, R.J., SMART, J.E., CHOW, E.P., FALBEL, T., RIBOLINI, A., GARWIN, J. & WALLNER, B.P. (1986). Purification and partial sequence analysis of a 37 kDa protein that inhibits phospholipase A<sub>2</sub> activity from rat peritoneal exudates. *J. Biol. Chem.*, **261**, 4239–4246.
- PEPINSKY, R.B. & SINCLAIR, L.K. (1986). Epidermal growth factor – dependent phosphorylation of lipocortin. *Nature*, **321**, 81–84.
- PIPER, P.J. & VANE, J.R. (1969). Release of additional factors in anaphylaxis and its antagonism by anti-inflammatory drugs. *Nature (Lond.)*, **223**, 29–35.
- PIPER, P.J. (1983). Pharmacology of leukotrienes. In *Prostaglandin, Thromboxane and Leukotrienes*, ed. Moncada, S. *Br. Med. Bull.*, Vol. 39, pp. 255–259.
- PODGORSKI, M.R., GOULDING, N.J., HALL, N.D., FLOWER, R.J., MADDISON, P.J. & PEPINSKY, R.B. (1987). Autoantibodies to recombinant lipocortin in RA and SLE. *Br. J. Rheumatol.*, **26**, Suppl. 1, 54–55.
- ROSENBAUM, R.M., CHELI, C.D. & GERRITSEN, M.E. (1986). Dexamethasone inhibits prostaglandin release from rabbit coronary microvessel endothelium. *Am. J. Physiol.*, **250**, C970–C977.
- ROTHHUT, B., CAMERA, C., PRIEUR, B., ERRASFA, M., MINASSIAN, G. & RUSSO-MARIE, F. (1987). Purification and characterisation of a 32 kDa phospholipase A<sub>2</sub> inhibitory protein (lipocortin) from human peripheral blood mononuclear cells. *FEBS Letters*, **219**, 169–175.
- RUSSO-MARIE, F. & DUVAL, D. (1982). Dexamethasone-induced inhibition of prostaglandin production does not result from a direct action on phospholipase activities but is mediated through a steroid-inducible factor. *Biochim. Biophys. Acta*, **712**, 177–185.
- RUSSO-MARIE, F., PAING, M. & DUVAL, D. (1979). Involvement of glucocorticoid receptors in steroid-induced inhibition of prostaglandin secretion. *J. Biol. Chem.*, **254**, 8498–8504.
- SARIS, C.J.M., TACK, B.F., KRISTENSEN, T., GLENNEY, Jr. J.R. & HUNTER, T. (1986). The cDNA sequence for the protein-tyrosine kinase substrate P36 (calpactin 1 heavy chain) reveals a multidomain protein with internal repeats. *Cell*, **46**, 201–212.
- SCHAUMBURG, B.P. & BOJESEN, E. (1968). Specificity and thermodynamic properties of the corticosteroid binding to a receptor of rat thymocytes *in vitro*. *Biochim. Biophys. Acta*, **170**, 172.
- SCHLAEPFER, D.D. & HAIGLER, H.T. (1987). Characterisation of Ca<sup>2+</sup>-dependent phospholipid binding and phosphorylation of lipocortin 1. *J. Biol. Chem.*, **262**, 6931–6937.
- SELYE, H. (1946). The general adaptation syndrome and the diseases of adaptation. *J. Clin. Endocrinol. Metab.*, **6**, 117.
- SMITH, J.B. & WILLIS, A.L. (1971). Aspirin selectivity inhibits prostaglandin production in human platelets. *Nature (New Biol.)*, **231**, 235–237.
- STACEY, D.W. & KUNG, H.-F. (1984). Transformation of NIH 3T3 cells by micro injection of H-ras P21 protein. *Nature*, **310**, 508–511.
- TSURUFUJI, S., SUGIO, K. & TAKEMASA, F. (1979). The role of glucocorticoid receptors and gene expression in the anti-inflammatory action of dexamethasone. *Nature*, **280**, 408–410.
- VAN DE VELDE, V.J., BULT, H. & HERMAN, A.G. (1986a). Dexamethasone and prostacyclin biosynthesis by serosal membranes of the rabbit peritoneal cavity. *Agents & Actions*, **17**, 308–309.
- VAN DE VELDE, V.J., HERMAN, A.G. & BULT, H. (1986b). Effects of dexamethasone on prostacyclin biosynthesis in rabbit mesothelial cells. *Prostaglandins*, **32**, 169–178.
- VAN DEN BOSCH, H. (1980). Intracellular phospholipases A. *Biochem. Biophys. Res. Commun.*, **604**, 191–246.
- VANE, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action of aspirin-like drugs. *Nature (New Biol.)*, **231**, 232–235.
- VINCENT, J.E., ZIJLSTRA, F.J., VAN DEN BROCK, A.M. & GEZEL, T.E. (1986). Opposite effects of adrenalectomy on eicosanoid release in rat peritoneal macrophages and spleen. *Prostaglandins*, **32**, 132–136.
- WALLNER, B.P., MATTALIANO, R.J., HESSION, C., CATE, R.L., TIZARD, R., SINCLAIR, L.K., FOELLER, C., PINGCHONG CHOW, E., BROWNING, J.L., RAMACHANDRAN, K.L. & PEPINSKY, R.B. (1986). Cloning and expression of human lipocortin, a phospholipase A<sub>2</sub> inhibitor with potential anti-inflammatory activity. *Nature*, **320**, 77–81, 1986.
- WILLIAMS, T.J. (1983). Interactions between prostaglandins, leukotrienes and other mediators of inflammation. In *Prostaglandin, Thromboxane and Leukotrienes*, ed. Moncada, S. *Br. Med. Bull.*, Vol. 39, pp. 239–242.

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