A small phospholipase inhibitory factor released by cultured cell lines

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Cells of the mouse macrophage-like cell line RAW_{264} release a dialysable inhibitor of phospholipase activity into their culture medium. This inhibitor can be detected in saline solution, Hanks solution and a variety of tissue culture media in the presence or absence of serum. The inhibitor is stable at 4°C, unaffected by trypsin, nucleases, or boiling, and partially extractable with chloroform/methanol. The release of both arachidonic acid and prostaglandins from mouse macrophages or human monocytes is inhibited by this material. A variety of other cell types release the inhibitor, which is effective against stimulation of arachidonic acid release from cultured macrophages by zymosan, serum, immune complexes and the calcium ionophore A23187.

Arachidonic acid *Prostaglandin* *Phospholipase* *Macrophage*

1. INTRODUCTION

The provision of free arachidonic acid is a rate limiting step in the production of prostaglandins and leukotrienes by macrophages presented with inflammatory stimuli [1]. Arachidonic acid is present at high concentrations in the cell membrane and lipid bodies of macrophages, mainly in the form of phospholipids [2]. These reserves can be mobilised by the action of phospholipase A2 (PLA_2), by either addition of exogenous enzyme or stimulation of one of the two macrophage PLA_2 activities [3,4], as well as by phospholipase C and diacylglycerol lipase action. Addition of PLA_2 inhibitors such as mepacrine to stimulated macrophages lowers the amount of arachidonic acid released, with a consequent reduction in the amount of prostaglandins produced. PLA_2 may thus play a pivotal role in the control of arachidonic acid metabolism and has been invoked as the site of action of various glucocorticoid hormone induced anti-inflammatory second messengers [5,6].

We have used primary macrophage cultures and the macrophage-like cell line RAW_{264} [7] to investigate the control of macrophage PLA_2 activity in vitro. The release of ^{14}C metabolites from macrophages pre-labelled with [^{14}C]arachidonic acid has been used as a measure of phospholipase activity. In addition, we have used a prostaglandin E2 (PGE_2) radioimmunoassay as an indirect measure of changes in phospholipase activity. By means of these 2 assays, we have detected a small phospholipase inhibitory factor (SPLIF) in culture medium conditioned by the RAW_{264} cell line. Here we describe some of the properties of SPLIF, which is released by a variety of cell lines and is active in both murine macrophages and human monocytes.

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2. MATERIALS AND METHODS

BALB/c female mouse macrophages were prepared by culturing adherent cells from peritoneal lavages in RPMI 1640, 10% FCS (Flow) supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml) for 24 h in 24 well plates (Linbro). RAW_{264} cells were cultured in the same medium. All incubations were carried out at 37°C in a 5% CO_2 humidified atmosphere. Human monocytes were prepared by Ficoll/triosil gradient centrifugation from peripheral blood and cultured for 24 h in the same medium [8].

Phospholipase activity in cultured cells was assessed by incubating each well of a 24 well plate containing 2.5×10^5 BALB/c peritoneal macrophages with 0.5 ml of 0.2 μCi/ml [^{14}C]-arachidonic acid (Amersham, 60 mCi/mM) in medium for 2 h. Cells were then washed 3 times with serum free RPMI, the test sample added, and counts released measured 45 min later by sampling, microfuging and counting aliquots in PCS scintillant (Amersham). All assays were carried out in duplicate or triplicate. ^{14}C counts released were linear up to this time, and cultures stimulated with 10% foetal calf serum (Flow) or zymosan (100 μg/ml, Sigma) gave 4–10-times background release.

PGE_2 radioimmunoassay [9] was carried out on supernatant samples from cells plated into 48 well plates (Costar) (10^5 macrophages/well) after overnight incubation with test samples. All assays were carried out in triplicate. Stimulation of counts released correlates with increased PGE_2 production by zymosan or FCS treated cultures.

3. RESULTS

3.1. *SPLIF production in vitro*

We studied the appearance of SPLIF from confluent RAW_{264} cells incubated with fresh medium in the presence or absence of hydrocortisone hemisuccinate (fig.1). A maximal inhibition of [^{14}C]arachidonic acid metabolite release of about 50% was observed after 4 h of culture when supernatants were assayed on test macrophages. This effect was also apparent in the presence of steroid. Thereafter, we used overnight culture supernatants from confluent RAW_{264} cells. Comparing conditioned SPLIF containing medium with unconditioned controls, we found that the inhibition of ^{14}C release was mirrored in a diminution of PGE_2 release, measured by immunoassay. Using 10% FCS stimulated macrophages, we found an inhibition of overnight PGE_2 production from 31.6 ng/ml (SE1.4) to 19.4 ng/ml (SE4) comparing conditioned with control RPMI.

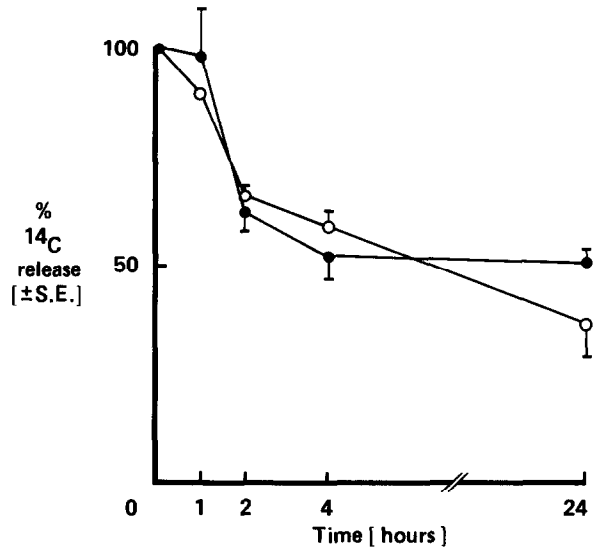

Fig.1. Release of ^{14}C-labelled arachidonic acid metabolites from pre-labelled macrophages, treated for 45 min with tissue culture supernatants (RPMI 1640, 10% FCS), conditioned by RAW_{264} cells for various times in the presence (●) and absence (○) of hydrocortisone (1 μg/ml).

To determine whether we were measuring the appearance of an inhibitory activity or merely monitoring the exhaustion of a medium component essential for phospholipase activity, we prepared RAW_{264} conditioned solutions of serum free RPMI, Hanks solution and simple saline, and compared them with controls. Fig.2 demonstrates that SPLIF appears in the simplest saline solutions and we can therefore be confident that we are dealing with the appearance of an inhibitor.

3.2. *SPLIF inhibits the action of distinct inflammatory stimuli*

A variety of unrelated stimuli induce arachidonic acid release from macrophages [10]. The metabolites produced are dependent upon the stimulus, perhaps because of distinct metabolic pools of arachidonic acid and pathways of

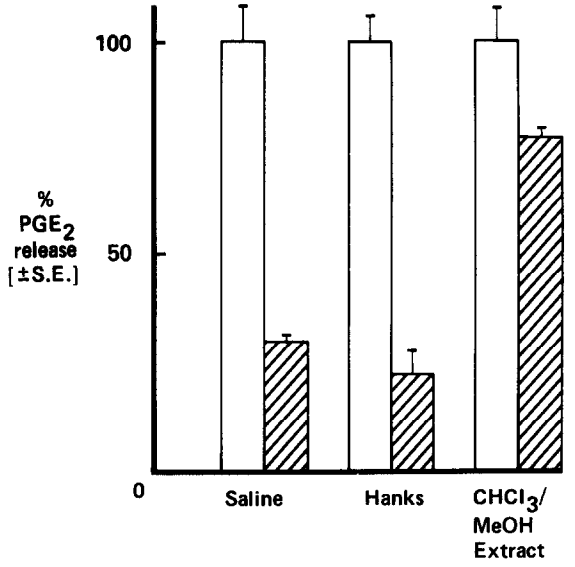

Fig.2. PGE_2 production by macrophages incubated overnight with RAW_{264} conditioned media (hatched) and controls. Conditioned saline (0.85% NaCl) was diluted 1:3 (v/v) with RPMI 1640, and chloroform/methanol extracts re-suspended in an equivalent volume of RPMI 1640 after lyophilisation. FCS was then added to a final concentration of 10%.

mobilisation [11]. The phagocytosis of zymosan has been shown to lead to the release of lysosomal PLA_2 with concomitant PGE_2 production which is steroid inhibitable [12]. On the other hand, the calcium ionophore A23187 which stimulates arachidonic acid mobilisation into lipoxygenase products is not inhibited by steroids [16]. We tested a number of inflammatory stimuli on macrophages in the presence or absence of RAW_{264} derived SPLIF. Table 1 shows that SPLIF lowers the release of arachidonic acid derivatives by all stimuli investigated, including the calcium ionophore A23187.

3.3. *SPLIF is released by, and acts upon, various cell types*

We tested the species specifically of RAW_{264} SPLIF by measuring the release of [^{14}C]arachidonic acid release from human monocytes. Fig.3 demonstrates that RAW_{264} SPLIF acts upon human monocytes to the same extent as mouse macrophages or RAW_{264} cells.

We next investigated medium conditioned by a

Table 1

Inhibition of [^{14}C]arachidonic acid metabolite release from macrophages by RAW_{264} conditioned RPMI 1640, using a variety of stimuli to elicit arachidonic acid release

Stimulus	% inhibition
10% FCS	55
100 μg/ml zymosan	41
Monoclonal IgG2b ascites fluid 10 μg/ml	33
A23187 calcium ionophore 2.5 μg/ml	42

variety of cell lines for SPLIF activity. Using the [^{14}C]arachidonic acid release assay, we demonstrated SPLIF release from cultured macrophages. Endogenous PGE_2 production by the generating cells did not allow us to test macrophage SPLIF on PGE_2 production by target macrophages, but using other cell lines which do not synthesize PGE_2 [13,15], we found non-adherent and non-macrophage-like cells also release SPLIF into their

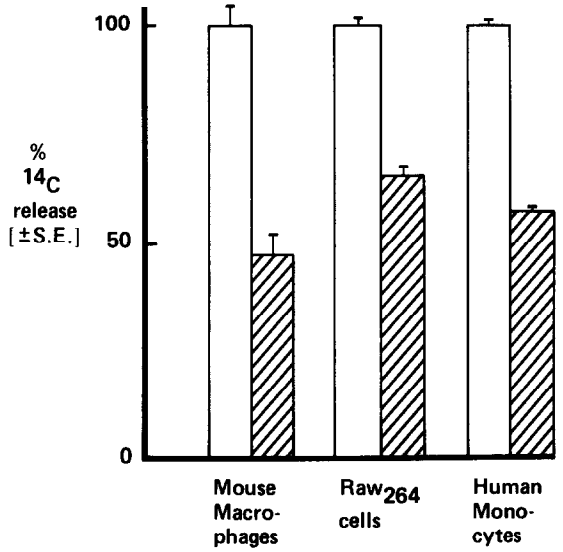

Fig.3. Release of [^{14}C]arachidonic acid metabolites from pre-labelled mouse peritoneal macrophages, RAW_{264} cells [7], and human monocytes [8], stimulated for 45 min with 10% FCS containing control and RAW_{264} conditioned RPMI 1640 (hatched).

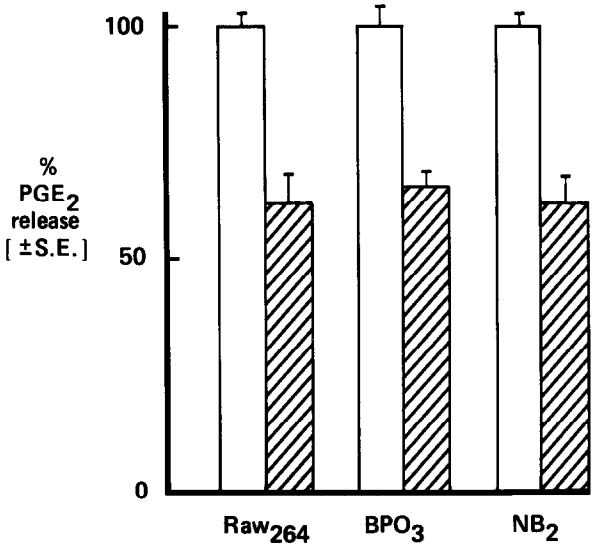

Fig.4. Macrophage PGE_2 production in the presence of RPMI 1640, conditioned overnight (hatched) with either RAW_{264} cells, BP03 mouse hybridoma cells ([15] and Baxter, D., Coote, P., Wood, J., unpublished), or NB2 lymphoma cells [13].

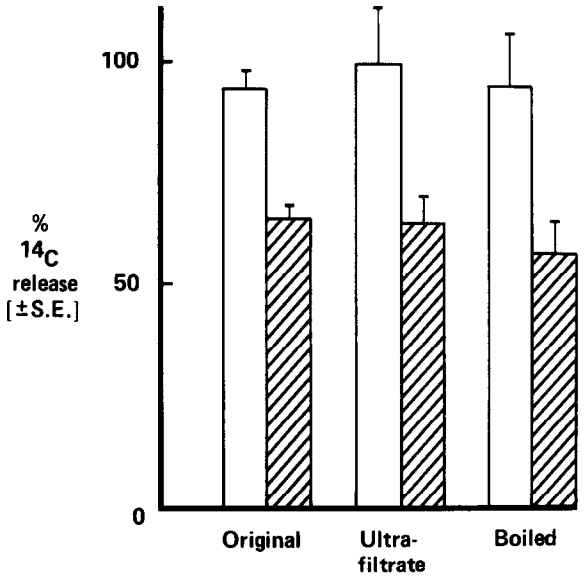

Fig.5. RAW_{264} conditioned RPMI 1640 (hatched) after XM-10 ultrafiltration and boiling. Effect on macrophage [^{14}C]arachidonic acid metabolite release after 45 min stimulation with 10% FCS.

culture medium (fig.4). All the cell-lines tested proved positive for SPLIF release.

3.4. *Characterisation of SPLIF activity*

SPLIF activity is lost from dialysates of conditioned medium. We prepared ultra-filtrates of control and SPLIF containing RAW_{264} conditioned serum-free medium using Diaflo XM-10 (Amicon) filters. Fig.5 shows that the ultrafiltrate retains its SPLIF activity implying that the molecular mass is less than 10 kDa. These ultrafiltrates can be boiled for 5 min without loss of activity. Ultrafiltrates of conditioned medium, resterilised by millipore filtration have been stored for weeks at 4°C with no loss of activity. We attempted to identify the nature of SPLIF by enzymic degradation. Incubation with trypsin (1 mg/ml), RNase A (0.1 mg/ml) or DNase I (0.1 mg/ml) (Sigma) for 90 min at 37°C followed by boiling to inactivate the enzymes led to no loss of SPLIF activity, measured using the ^{14}C release assay. We next tried chloroform/methanol extraction of control and conditioned medium, and found (fig.2) a partial extraction of inhibitory activity, although less than that present in the original material.

Ballou and Cheung have recently reported the pressure of an endogenous phospholipase inhibitory activity in platelets [14]. This activity appears to be associated with a mixture of unsaturated fatty acids [17]. We tested the hypothesis that SPLIF might also be such a mixture. When RAW_{264} conditioned Hanks solution is analysed by a thin layer chromatography on Whatman LKDF plates, using a solvent system of pentane:diethylether:acetic acid (80:20:1, v/v), a major band of inhibitory activity was found with an R_f of 0.61, running between palmitoleic acid and oleic acid standards (30% inhibitor of ^{14}C release). It thus seems plausible that the activity described here is associated with the appearance of unsaturated fatty acids in conditioned medium.

4. DISCUSSION

We have demonstrated the presence of SPLIF in medium conditioned by a variety of cell lines. Endogenous modulators of phospholipase activity are of considerable interest, not only for our understanding of the mechanism of arachidonic acid mobilisation into a variety of biologically ac-

tive oxidation products, but also in the search for novel anti-inflammatory agents. The properties of SPLIF show similarities with those of an inhibitor recently identified in platelets [14] and subsequently found to comprise a mixture of unsaturated fatty acids. The fact that SPLIF is released in simple salt solutions from many different cell types suggests that it may be a passively shed normal membrane constituent of a lipidic nature. TLC analysis confirms an association between the appearance of inhibitory activity and the presence of material comigrating with authentic unsaturated fatty acid markers. It thus appears likely that SPLIF and the inhibitory fraction isolated from platelets are closely related. Ballou and Cheung have shown that unsaturated fatty acids, including arachidonic acid, are potent non-competitive inhibitors of phospholipase activity [17]. Our experiments show that such inhibition also results in lowered prostaglandin synthesis, suggesting that arachidonic acid is not a component of SPLIF, and that unsaturated fatty acids may play an anti-inflammatory role by limiting the release of arachidonic acid.

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