Decreased inhibition by gravidin of arachidonate release from transformed compared to nontransformed cells

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1 Gravidin (a phospholipase A₂ inhibitor) reduced the release of arachidonic acid from human lymphocytes by 51% at 10⁻⁸ M.

2 Under normal culture conditions, nanomolar gravidin caused a significant reduction in the release of free arachidonic acid from human lymphocytes or nontransformed fibroblasts but in transformed cells, nanomolar gravidin was ineffective.

3 Inhibition of arachidonate release appeared to be related to rate of growth as inhibitory effects of gravidin on Jurkat cells and HL-29 cells could be observed if the cells were cultured under conditions where DNA synthesis was low.

4 The reported disparate effects of lipocortin on cell phospholipase A₂ activity may be reconciled if DNA synthesis is investigated.

Keywords: Gravidin; phospholipase A₂; DNA synthesis; arachidonic acid; cell growth; lymphocytes; lymphoma

Introduction

Regulation of phospholipase A₂ (PLA₂) (EC3.1.1.4) activity holds the key to the control of the arachidonic acid (AA) cascade and therefore inflammation and other disorders. Glucocorticoids are well characterized anti-inflammatory agents that are reported to inhibit PLA₂ through induction and release of lipocortins (Hirata *et al.*, 1980; Blackwell *et al.*, 1982). However, the antiphospholipase activity of lipocortins is controversial as in some systems, synthesis of lipocortins is coincidental with no change or an increase in phospholipid hydrolysis (Duval *et al.*, 1986; Northup *et al.*, 1988; Mitchell *et al.*, 1988). We isolated and characterized a PLA₂ inhibitor, gravidin (Wilson & Christie, 1991) that has been linked with maintenance of human pregnancy (Wilson *et al.*, 1989). At low concentrations gravidin inhibited human endometrial cell prostaglandin synthesis and decidual cell and lymphocyte [³H]-AA release (Wilson *et al.*, 1985; Wilson & Christie, 1991) but it was noted that the active concentration varied according to the cell type used, suggesting that cells may differ in sensitivity to gravidin as for lipocortin. We have now extended these studies to human cells from other tissues and confirm that the effect of gravidin is selective in that it differs according to the type of cell tested and is markedly reduced in cells derived from tumours. Furthermore, for a given cell type, the inhibitory response to gravidin only occurs when cells are synthesizing DNA slowly.

Methods

Cell culture

Lymphocytes Lymphocytes were obtained with informed consent from healthy volunteers and one lymphoma patient diagnosed with a malignant lymphoma (nodular, poorly differentiated). Blood was collected in citrate anticoagulant and centrifuged in Sepalymph at 1000 *g* for 30 min. The layer of lymphocytes was removed carefully, washed twice in medium G199 with Earle's salts pre-equilibrated with 5% CO₂ and immediately labelled with [³H]-AA (180 Ci mmol⁻¹) as described below.

Fibroblasts Human foreskin fibroblasts were obtained from primary cell cultures that had been stored in liquid N₂ (–196°C) and grown before treatment for at least two cell cycles in RPMI 1640 medium containing 5% foetal calf serum (FCS), 50 mg ml⁻¹ penicillin and 50 U ml⁻¹ streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Cells were detached from their dishes by treatment with 0.05% trypsin for 2 min before labelling with [³H]-AA.

Measurement of [³H]-arachidonic acid release

Radiolabelled AA in ethanol was dissolved in G199 media; the ethanol solvent constituted less than 0.1% of the final volume. Cells washed free from FCS were incubated for 45 min in 0.5 ml medium containing [³H]-AA (1 µCi/10⁶ cells). The radiolabelled cells were washed twice in G199 medium containing 1 mg ml⁻¹ BSA and resuspended in G199 medium to a final concentration of approximately 4 × 10⁶ cells per ml. Aliquots (50 µl) of the cell suspension were placed in Eppendorf tubes containing test substances in a final volume of 350 µl and incubated at 37°C in an atmosphere of 5% CO₂. Each treatment was performed in triplicate.

Following incubation, 100 µl of the cell suspension was removed from each incubation tube and the cells centrifuged lightly into a pellet before 75 µl of the supernatant was removed and its associated radioactivity was measured in a liquid scintillation spectrometer [³H]-AA release which was linear over 5 h, was measured at time 0, 3 and 4 h. The mean of the 3 and 4 h values was taken for all data. Released radioactivity in the presence of gravidin was expressed as a percentage of the radioactivity released in the absence of gravidin. The average amount of [³H]-AA incorporated by an aliquot containing 10⁵ cells was 6118 ± 1010 d.p.m. During incubation, 3280 ± 390 d.p.m. were released and the d.p.m. in the supernatant at zero time averaged 29.5% of the total d.p.m. released during the experiment (mean ± s.e., *n* = 27 for all data above).

Preparation of gravidin

Gravidin was extracted from amniotic fluid as previously described (Wilson & Christie, 1991) and stored at –20°C in HEPES buffer (100 mM, pH 7.0) containing 50% glycerol. Purity was judged to be 98% on a Coomassie Blue stained SDS gel. Before its addition to test solutions, gravidin was incubated with 1 mM dithiothreitol (DTT) for 15 min at room temperature. Neither glycerol nor DTT affected the release of

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[³H]-AA from the cells at the concentrations in the test solutions.

Thymidine incorporation

Cells were washed free of FCS and incubated with [³H]-methyl-thymidine (1 µCi/0.5 ml cells; 25 Ci mmol⁻¹) for 3.5 h. The cells were pelleted and washed with SSC (0.15 M NaCl, 0.017 M Na citrate pH 7.00) resuspended in SSC containing 0.3 mg ml⁻¹ BSA and left on ice for 30 min. DNA was precipitated with HCl-sodium pyrophosphate (1 ml 12% w/v in 1.5 M HCl) at 0°C for 15 min and then washed three times by centrifugation in 0.5% w/v Na pyrophosphate in 0.5 M HCl before radioactivity in the precipitate was measured.

Results

The effect of different doses of gravidin on [³H]-AA release from normal unstimulated lymphocytes is shown in Figure 1. There was no change in [³H]-AA release at 10⁻¹¹ M gravidin but a statistically insignificant increase in PLA₂ activity occurred at 10⁻¹⁰ M. Inhibition reached a maximum of 51% between 3 and 10 nM gravidin.

To determine whether gravidin inhibits [³H]-AA release in other cells, investigations were extended to confluent cell lines and cells from a variety of other sources. Gravidin was used at 10⁻⁹ M because at this concentration half the maximum response was obtained with lymphocytes. A comparison of cell types showed that gravidin was substantially more inhibitory to nontransformed than transformed cells (Table 1). Gravidin at a higher concentration (5 × 10⁻⁹ M) caused insignificant inhibition of arachidonate release from HT-29 or HL-60 cells (data not shown).

To determine if the different response of transformed and nontransformed cells could be attributed to differences in rate of growth, DNA synthesis was measured in cells of the same type from different sources (Table 2) and it was observed that DNA synthesis was significantly lower in normal lymphocytes that were inhibited by gravidin compared to lymphoma lymphocytes that were not.

To test further the hypothesis that the action of gravidin was related to the rate of cell growth, cultures of normal fibroblast, Jurkat and HL-60 cells were harvested and assayed for gravidin effects at intervals after subculture. DNA synthesis

Figure 1 The effect of increasing gravidin concentration on lymphocyte [³H]-arachidonic acid ([³H]-AA) release. Lymphocytes were collected from healthy volunteers, washed and preincubated with [³H]-AA as described in the Methods section. Nonstimulated release of [³H]-AA was measured in the absence and presence of gravidin at different concentrations. Values given are the means and range of two experimental results and are expressed as a percentage of the control value in the absence of gravidin.

Figure 2 Effect of gravidin with cells with different rates of growth. Cells were treated with or without gravidin (10⁻⁹ M) at different stages of growth and thymidine incorporation/10⁵ cells measured at the same time as inhibition of [³H]-AA release by gravidin. Values shown are pooled from at least two subcultures and are expressed as a percentage of the control value in the absence of gravidin: (■) fibroblasts; (●) HT-29 cells; (○) Jurkat cells.

Table 1 The effect of gravidin on [³H]-arachidonic acid ([³H]-AA) release from different cultured human cell types

Cell type	Source	Normal or transformed	% Inhib	s.e.mean	n
HCT-8	Ileocaecal cancer	Transformed	-4	3.5	3
HT-29	Colon cancer	Transformed	-4	3.5	3
HL-60	Leukemia	Transformed	2	1.0	3
Jurkat	T cell	Transformed	11	5.7	3
Lymphoma	Lymphocytes	Transformed	2	6.4	3
	Lymphocytes	Normal	27	6.4	9
Fibroblasts	Foreskin	Normal	24	5	3

Cells were treated with gravidin (10⁻⁹ M) as described in Methods. Non-stimulated release of [³H]-AA was measured in the absence and presence of gravidin at different concentrations. The lymphoma patient donated cells three times; 9 healthy donors contributed cells. Values given are the means of the experimental results and are expressed as a percentage of the control value in the absence of gravidin.

Table 2 Incorporation of thymidine by lymphocytes from different sources

Cell source	Normal or transformed	Thymidine (d.p.m. incorporated/10 ⁵ cells)	s.e.mean	n
Jurkat	Transformed	1221	257	3
Lymphoma cells	Transformed	971	281	2
Lymphocytes	Normal	247	107	3

Lymphocytes were obtained from healthy donors, one lymphoma patient and T cells established in culture and assayed for thymidine incorporation as described in the Methods section.

was measured at the same times and the assays were made every two or three days until the cell number no longer increased and thymidine incorporation fell to below 1500 d.p.m./ 10^5 cells. Both thymidine incorporation and gravidin inhibition of arachidonate release were measured from the same cultures so that the two parameters could be correlated. The inhibitory effect of gravidin was found to be inversely related to the amount of DNA synthesized by the cells (Figure 2) with measurable inhibition only in cells with low rates of DNA synthesis. Gravidin stimulated [^3H]-AA release from fibroblasts with very high rates of DNA synthesis, while at all rates of DNA synthesis there was reduced sensitivity to gravidin in HT-29 cells and Jurkat cells compared to normal fibroblasts.

Discussion

Gravidin, an inhibitor of PLA_2 has recently been identified as secretory component of IgA (Wilson & Christie, 1991). Although gravidin is a potent inhibitor of PLA_2 in human endometrial cells, (Wilson *et al.*, 1985), it was found to have variable or little effect on transformed cells investigated in this study whereas [^3H]-AA release from normal lymphocytes and fibroblasts was significantly inhibited by gravidin.

A comparison of normal versus transformed lymphocytes from different sources showed that they responded differently to gravidin. Transformation appeared to make the cells much less sensitive to gravidin as [^3H]-AA release from both Jurkat cells (a T cell line) and lymphocytes obtained from an untreated lymphoma patient was inhibited less than [^3H]-AA release from normal cells.

To test whether the effect of gravidin on cells varied with their rate of growth, thymidine incorporation into DNA was compared with gravidin effect on [^3H]-AA release (Figure 2). With both normal and transformed cells, gravidin inhibition was greatest when thymidine incorporation was low indicating that the effect of gravidin depended on the rate of cell growth. However, the rate of DNA synthesis at which the cells began to respond to gravidin varied according to the cell type and the effects on transformed cells occurred only at very low rates of growth.

Another phospholipase inhibitor, lipocortin, is synthesized in response to glucocorticoid or dexamethasone treatment of cells (Hirata *et al.*, 1980; Blackwell *et al.*, 1982) and dexamethasone also has different effects on confluent and growing cells. In one study, Mitchell *et al.* (1988) demonstrated

enhanced lipocortin and prostaglandin synthesis in cultured cells from human amnion. However, Gibb & Lavoie (1990) in a later study found that the inhibitory effect of dexamethasone on prostaglandin synthesis by human amnion cells depended on whether or not the cells were freshly cultured or had been cultured for several days. Glucocorticoids inhibited freshly cultured cells but became stimulatory after several days of culture. Such inconsistencies in the anti-phospholipase effect of lipocortin have impeded its acceptance as an inhibitor of PLA_2 (Duval *et al.*, 1986; Northup *et al.*, 1988). From our data and the other results, we suggest that the susceptibility of phospholipase to inhibitors changes with growth rate.

The reason for the decreased sensitivity of transformed cells to gravidin is not known. The lack of effect was not due to the protease treatment used to detach the cells from their dishes as cultured fibroblasts which responded to gravidin were detached from the dishes in the same way as the transformed cell lines (Figure 2). Increased PLA_2 activity has been associated with cell transformation (Alonso & Santos, 1990) and appears to be necessary for high growth rates (Palombella & Vacek, 1989). Possibly, acquired resistance to inhibition of phospholipase is an early event in cell transformation.

A selective effect of gravidin could be advantageous under some circumstances. Gravidin has been shown to inhibit decidual cell prostaglandin synthesis and thus may play a role in the maintenance of human pregnancy (Wilson *et al.*, 1985). Indomethacin, a cyclo-oxygenase inhibitor is sometimes used to delay preterm labour but may have adverse effects due to inhibition of prostaglandin synthesis in the foetus (Moise *et al.*, 1988; Mari *et al.*, 1989; Eronen *et al.*, 1991). If gravidin inhibits phospholipase only in non-growing cells, it would have an advantage over indomethacin in that foetal prostaglandin production would not be compromised.

In summary, our results show that cells have different sensitivity to gravidin depending on their stage of growth; actively growing cells are less affected by gravidin than freshly cultured or confluent cells. An *in vivo* consequence of this finding could be a selective effect of gravidin on cell types at different stages of growth (e.g. foetal vs. maternal cells during pregnancy). Transformed cells may be less sensitive to gravidin than growth controlled cells because of altered control of PLA_2 . It seems possible that reported variable responses of other cells to lipocortins may also be explained by different rates of cell growth.

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