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GRAVIDIN, AN ENDOGENOUS INHIBITOR OF PHOSPHOLIPASE A2 ACTIVITY, IS A SECRETORY COMPONENT OF IgA

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Gravidin, a phospholipase inhibitor characterised previously from amniotic fluid, was partially sequenced at the N-terminal and found to be identical to secretory component of human IgA. Inhibition of antiphospholipase activity was observed after incubation of gravidin with monoclonal antibody to human secretory component. Secretory component isolated from human saliva and breast milk was found to inhibit arachidonic acid release from human lymphocytes. It was concluded that gravidin is secretory component of IgA.

Inflammation, preterm labour and thrombosis are some of the damaging consequences of abnormal eicosanoid synthesis. Pharmacological agents such as corticosteriods, aspirin and indomethacin inhibit prostaglandin (PG) synthesis in vitro but are sometimes ineffective in therapeutic inhibition. A major rate-limiting step in the synthesis of PGs, the release of arachidonic acid from membrane phospholipid, is controlled by the enzyme phospholipase A_2 (PLA_2)(EC3.1.1.4). Consequently, inhibition of PLA_2 is likely to be of fundamental importance in the control of eicosanoid synthesis.

Gravidin is a PLA_2 inhibitor thought to have a role in the onset of human labour[1]. In this paper we present evidence that gravidin is identical to the secretory component of IgA and therefore an inhibitor of PLA_2. This newly identified property of SC may assist in the understanding of its role in the inflammatory process.

Methods

a.Purification of gravidin from amniotic fluid

Lyophilised human amniotic fluid (4g) was reconstituted and dialysed against buffer A (Tris-HCl,50 mM pH 7.4, containing 1mM benzamidine and 1mM EGTA) at 4°C. The dialysate was passed through a DE32 column (10 x 2 cm) in Buffer A, the flow-through precipitated (100% ammonium sulfate) and eluted proteins separated on a calibrated Sephadex G100 column (in Buffer A). The 80 kDa fraction was fractionated with a Mono-S FPLC column equilibrated in 0.1mM HEPES (pH 7.0), washed with 0.1 mM HEPES and protein eluted with a gradient of 1mM NaCl in 0.1mM HEPES (pH 7.0). The gravidin peak was detected with antibody raised to gravidin prepared as described

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previously [1]and stored at -20°C in 50% glycerol. Gravidin was incubated with dithiothreitol (1mM) for 15 min at room temperature before assay of activity.

b. Purification of secretory component from human saliva

SC (secretory component) was prepared from human saliva by 100% ammonium sulfate precipitation, gel filtration, (Sephadex G100 in buffer B)(10mM PO_4 pH 7.6) and DEAE cellulose chromatography (buffer B). The flow through was lyophilised and stored as for gravidin. Approximately 14 µg protein was recovered.

c.Assay of arachidonate release from lymphocytes

Inhibition of PLA_2 activity was determined using freshly obtained human lymphocytes isolated from blood with anti-coagulant and Sepalymph then washed twice, counted and incubated with [^{3}H]-arachidonic acid ([^{3}H]-AA) for 45 mins (2µCi/10^7 cells). The cells were washed, then suspended in G199 media containing 6mg/ml BSA and test substances (10^5 cells/tube in a volume of 300 µl). After 3-4h in an atmosphere of 5% CO_2 triplicate aliquots of incubation medium were removed, the cells precipitated by light centrifugation (100g for 5 sec) and radioactivity in the supernatant measured. Inhibition of phospholipase activity was determined in the presence and absence of Ca ionophore (A23187, 10^{-5}M). Radioactivity present in the supernatant at the beginning of the experiment (background) was subtracted from that released. The average dpm released by control cells was 3128 dpm above background and release was linear over the time of the experiment.

d.Protein sequence determination

Gravidin was purified as above from human amniotic fluid taken before the onset of labour. After blotting from a 10% SDS PAGE gel onto polyvinyldifluoride membrane (2) sequence analysis of the gravidin band was determined using gas-phase sequencer (Applied Biosystems model 470A) equipped with an on-line phenylthiohydantoin amino acid analyser (120A). Chemicals and the program (03RPTH) were as supplied by Applied Biosystems. Protein sequence data was used to search the NBRF data base (version 28.0) using the University of Wisconsin software[3].

e.Elisa assay for secretory component

Antigen was dissolved in carbonate buffer (0.1M pH 9.6), 100 µl pipetted into each well of a 96-well Nunc Maxisorb plate and incubated for 16h, then with blocking buffer (Tris HCl 10 mM pH 7.4 containing 150 mM NaCl and 1% nonfat milk powder) for thirty minutes, washed and incubated with primary antibody suspended in blocking buffer. After two hours incubation followed by washing, 200µl of secondary antibody (peroxidase-linked) was added to the wells and incubated for a further 30 mins, before colour was developed with orthophenylene diamine. The monoclonal and polyclonal antibodies were obtained from Sigma Chemical Co.

f.E coli assay for phospholipase A2 activity.

This was peformed as described by Rothut et al. except that assay buffer contained 1mg/ml BSA.(4)

Results

Gravidin is a protein which has been shown to inhibit porcine pancreas PLA_2 activity and to reduce prostaglandin synthesis by human decidual cells by inhibiting the release of arachidonic acid [5]. In order to characterise gravidin, a large scale purification from human amniotic fluid was performed and sequence of the product determined. The preparation contained a major 80 KDa protein which was subjected to sequence analysis after electroblotting to polyvinyldifluoride membrane (Fig.1). Search of the NBRF data base showed that the NH_2 terminal 10 residues were identical with the NH_2-terminal sequence of the secretory component of human IgA [6].

The protein sequence was obtained following electroblotting of protein isolated from human amniotic fluid. Because contaminating proteins could have been responsible for the observed activity, it was important to obtain secretory component from another source to confirm its activity as a PLA_2 inhibitor. Accordingly, secretory component was purified

1	2	3	4	5	6	7	8	9	10
lys	ser	pro	ile	phe	gly	pro	glu	(glu)	(val)

Fig. 1.
N-Terminal sequence of gravidin.
Order of identified amino acids from gravidin. There was some uncertainty about the identity of the last two amino acids due to the small peak height.

from saliva and human breast milk by a published method [7]. The product was confirmed by gel electrophoresis, ELISA measurement with monoclonal and polyclonal antibody raised to secretory component, and lack of cross reactivity with antihuman IgA or antihuman IgG antibodies from commercial sources.

As shown in Fig. 2, 10^{-9}M gravidin or secretory component isolated from saliva significantly inhibited the release of incorporated arachidonic acid from human

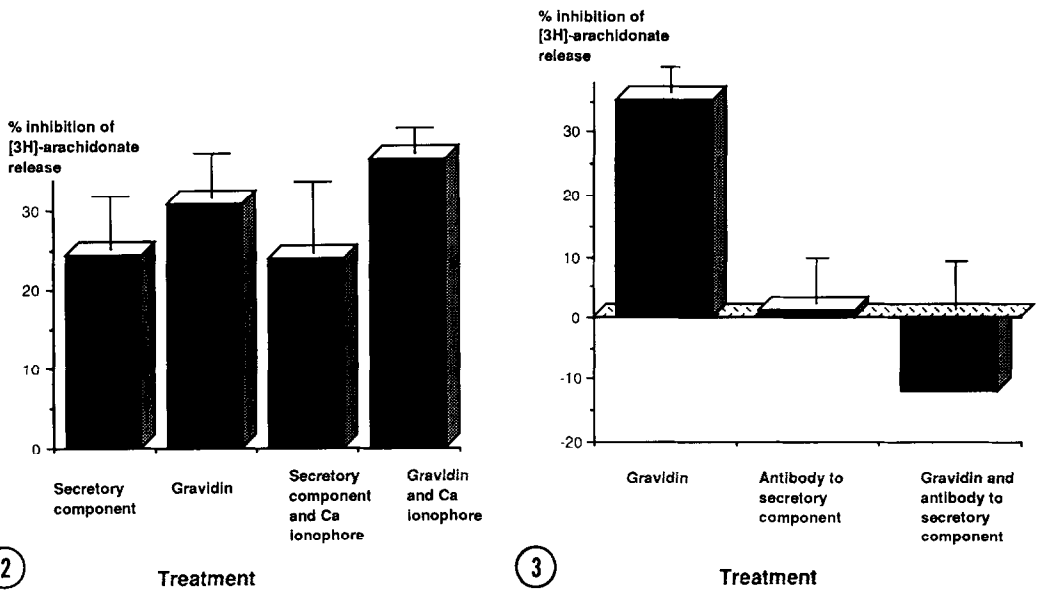

Fig.2.
Comparison of secretory component (10^{-9}M) and gravidin (10^{-9}M) on the inhibition of arachidonate release from human lymphocytes.
Both gravidin and secretory component (extracted from amniotic fluid and saliva respectively, as in the methods section) were preincubated with 1mM dithiothreitol for 15 min at room temperature before being added to the media. Results are presented as mean + SEM (n=3) of the released [^{3}H]-AA in the absence of treatment.

Fig. 3.
Incubation of gravidin with monoclonal antibody to human secretory component.
The assay for phospholipase was carried out as described in the Methods. Monoclonal antibody to human secretory component (obtained from Sigma) was preincubated with gravidin at a dilution of 1:10 for 30 min at room temperature before addition to the lymphocyte incubates. Final dilution of the antibody was 1:150. Gravidin was used at a concentration of 10^{-9}M. Results shown are the mean±SEM of three determinations.

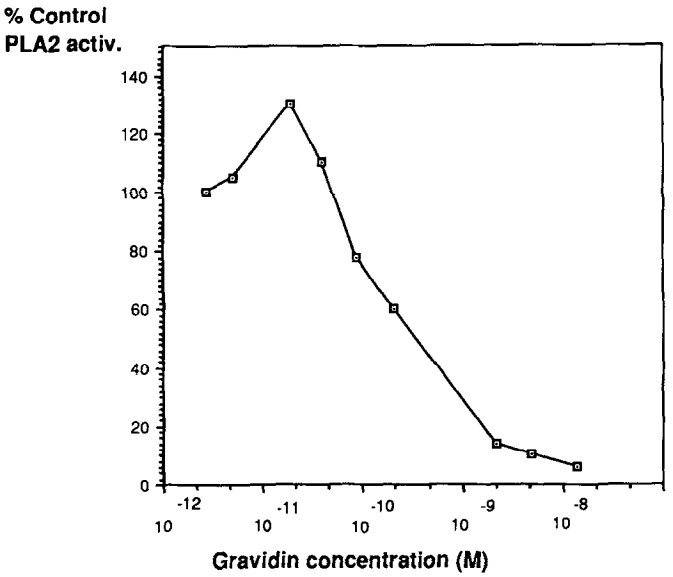

Fig.4.
Gravidin concentration and inhibition of PLA_2 activity.
Gravidin was extracted from amniotic fluid and used to determine the inhibition of PLA_2 activity in the E. coli assay described in the Methods section. The results are the mean of two experiments. Values differed by a maximum of 14.8 %.

lymphocytes. The inhibition of arachidonate release by secretory component isolated from saliva was less than that obtained by gravidin from amniotic fluid. Control experiments in which the activity of glycerol (10µl/300µl media) or dithiothreitol were tested showed no effect of these compounds. Secretory component extracted from from human milk (gift of Dr W. B. Watkins) was found to have inhibitory activity similar to that of salivary material (data not presented here).

In the presence of monoclonal antibody to SC gravidin was no longer inhibtory and became slightly stimulatory. By itself, the antibody had little effect on arachidonate release (Fig. 3).

Fig. 4 shows that the inhibitory effect of gravidin on PLA_2 activity increased with concentration; 10^{-8} M gravidin gave almost total inhibition in the *in vitro* assay. The K_i was calculated as 2.6×10^{-10}M.

The monoclonal antibody showed no cross-reactivity with IgG or IgM in ELISA. Gravidin isolated from amniotic fluid was detectable with monoclonal or polyclonal antibody to human secretory component, both giving a positive signal under the assay conditions.

Discussion

The data presented in this paper show that the protein previously called gravidin [1] is identical with secretory component of IgA. The evidence is that the N-terminal eight amino acids are identical (Fig. 1), monoclonal antibody against SC inhibits activity of the protein

(Fig.3), human secretory component from other sources has similar activity to gravidin in inhibiting the release of arachidonic acid from cells (Fig.2) and commercial monoclonal and polyclonal antibodies to secretory component gave a positive signal with gravidin.

The dose response curve of PLA_2 inhibition against gravidin concentration shows stimulation of activity with low concentrations of gravidin (Fig.4). As the assay buffer contained 1mg/ml BSA it is unlikely that stimulation by gravidin between 10^{-10}M and 10^{-11} M could have resulted from a nonsecific effect of protein such as removal of product inhibition.. It seems likely that the stimulation of arachidonate release observed in Fig 3 results from a low concentration of free gravidin.

A major group of endogenous regulators of PLA_2 that have been characterised are the lipocortins. It has been reported that the lipocortins are released from cells in response to corticosteroids[8] and inhibit PLA_2 in vivo [9]. A physiological role has not yet been rigorously defined for the lipocortins because they interact with substrate rather than enzyme [10]; activity is apparent only when the phospholipid substrate of PLA_2 is limiting, which seems an unlikely event *in vivo*. Attempts to inhibit nonstimulated arachidonic acid release with lipocortin in whole cells have not been successful and there appears to be little correlation with lipocortin levels and prostaglandin synthesis. The work described in this paper shows that gravidin inhibits arachidonate release in nonstimulated as well as stimulated lymphocytes and previous work showed that gravidin inhibited prostaglandin synthesis as well as arachidonate release in human decidual cells (5). Although we have not yet determined whether gravidin interacts with substrate or enzyme, the inhibition of arachidonic acid release by gravidin at nanomolar concentrations in the presence of 15 μM BSA would indicate a specific effect of gravidin.

Human secretory component is known for its role as a transport protein for dimeric IgA across epithelial membranes [11] but there are several reports that it is synthesised in the absence of IgA. In an early immunocytochemical study, secretory component in the fetal lung was detected at eight weeks of pregancy and in the placental membrane, gut and lung at 13-17 weeks [12] yet IgA is not normally synthesised by the fetus until term [12, 13]. A role for secretory component in the control of PLA_2 activity could explain its presence at the early stage of development.

Secretory component is bound to IgA through disulphide bridge formation and is difficult to displace from IgA (14), so it is unlikely that the action of the monoclonal antibody would be to inhibit binding to IgA. The binding of secretory component to IgA helps to prevent proteolysis of IgA thus prolonging its life [15]. Brandtzaeg [16], noted that secretory component appears to depend on a close association with an immunoglobulin polymer to remain intact in protease-containing external secretions. Thus a reciprocal protective effect may operate.

Reports of the concentration of secretory component in normal serum range from 2.3 μg/ml [17] to 11 μg/ml [16]. Most if not all secretory component in serum is bound to IgA or

IgM [17,18]. As the protein effectively inhibits PLA_2 activity in lymphocytes at a concentration of 10^{-9}M, the concentration in normal serum of around $3X10^{-8}$M seems inconsistent with a regulatory function. However it is possible that the antiphospholipase activity of the protein is reduced or inactivated by binding to IgA or IgM and this is currently under investigation.

Elevated levels of secretory component in serum have been reported during pregnancy [19]. The concentrations of secretory component concentrations are raised also in endometrial glandular epithelium [20] and in human uterine fluid [21] during the latter part of the menstrual cycle when progesterone production is high. The increased serum levels observed during pregnancy may be the result of induction by progesterone as Murdoch et al.[22] found that progesterone acts to increase the intra-epithelial content of secretory component in cervical epithelial cells. In short, secretory component may provide a link between progesterone and the maintenance of human pregnancy.

Acknowledgments

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