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13. ABSTRACT *(Maximum 200 words)*

The growth of mammary epithelial cells in response to growth factors is augmented by linoleic or arachidonic acid. This may explain the association between dietary fatty acid intake and increased risk of breast cancer. We are exploring the mechanism by which these fatty acids exert this growth-promoting effect with the central hypothesis that oxidative metabolism is a crucial step. Moreover, we are testing the hypothesis that induction of prostaglandin H synthase is required. In the first year we have shown that this enzyme is induced in breast epithelial cells in response to growth factors, and have found that the growth-promoting effect of fatty acids largely, but not completely, requires conversion to prostaglandins. The additional effect appears to be mediated via activation of specific isotypes of protein kinase C. We are in the process of additional testing of the role of key metabolic enzymes with antisense knockout of individual candidates.

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Stephen M. Prescott Aug. 29, 1995

PI - Signature Date

Table of Contents

Introduction

Nature of the problem

There is substantial evidence that lipid second messengers, including prostaglandins, participate in normal growth responses and in abnormal growth, including breast carcinogenesis (1-4). Most such studies have been carried out in animals – where cellular and molecular events are difficult to dissect precisely – or in experiments that compare tumor cell lines with their normal counterpart. This project will use human cells that are thought to reflect the earliest pathogenic events (5,6).

Background

Many cells respond to specific stimuli by synthesizing and releasing prostaglandins and related compounds, all of which are oxidized derivatives of arachidonic acid, and are collectively termed eicosanoids. These potent mediators elicit effects locally, often stimulating the tissue of production. Eicosanoids have been implicated as second messengers in many physiological and pathological responses including regulation of growth (7-10).

Epidemiological studies have implicated dietary fat – in particular, linoleic aid – as a risk factor for the development of breast cancer. This has been controversial, but the negative studies might have lacked sufficient statistical power if the key event is conversion of the linoleate to a metabolite such as a prostaglandin – *i.e.* the downstream events might not correlate well with dietary intake. The association observed in humans is strengthened by the results from dietary studies in animals. Moreover, epidemiological studies in humans have shown that intake of compounds that inhibit prostaglandin synthesis is associated with decreased risk of breast cancer and animal models have yielded the same result.

Purpose of the present work

Our original plan of research had five specific aims although the scientific review group recommended that aim five not be pursued in this funding period as it was premature. Thus, we have focused on the first four, which are:

1. Determine the Metabolic Fate of Linoleic Acid that Stimulates the Growth of Breast Epithelial Cells
2. Determine Whether Prostaglandin H Synthase is Induced During Breast Tumorigenesis
3. Test the Hypothesis That Metabolites of Arachidonic Acid are Essential Mediators of the Responses of Breast Epithelial Cells to EGF and Phorbol Esters
4. Determine the Molecular Basis for Regulation of Expression of PHS II

In particular, we have made the most progress on aims 1 and 3, which are described in detail below.

Methods of approach

Cell strains: The 184 cell strains were obtained from Dr. Martha Stampfer who developed them originally (5). There are three related strains of cells: a primary cell type known as 184 cells, and two clonally derived immortalized lines, the 184A1s and the 184B5s. The 184A1s, although immortalized, share many of the characteristics of the non-immortalized 184 cells. The 184B5 cells, on the other hand, are less normal and possess some of the chromosomal aberrations typically associated with breast cancer. These cells are used in our laboratory as a model of the progression of breast cells from a totally normal cell, the 184 cells, to a slightly less normal cell (the 184A1s), to the 184B5 cells which may be representative of a cell that has one "hit", and has begun down the pathway towards carcinogenesis.

Body

Characterization of the growth response of the 184 cell strains
Growth of the 184 cell strains is known to be dependent on epidermal growth factor (EGF). Under normal culture conditions, these cells produce TGFα, which interacts with the EGF receptor. However, these cells are not capable of making sufficient TGFα to support their own growth, so EGF must be supplemented to the medium. To characterize the growth response of the 184 cell strains, they were made quiescent by treating with EGF-free medium in the presence of a monoclonal antibody to the EGF receptor. 48 hours later the cells were quiescent. Growth was stimulated by the addition of a supramaximal concentration of EGF. The response of the cells was determined by a thymidine incorporation time course. Every 3 or 6 hours following the addition of EGF, cells were pulsed for one hour with ^{3}H-thymidine. The cells were then harvested and thymidine incorporation measured. The maximum rate of DNA synthesis occurred at 18 hours following EGF stimulation for the 184 cells (Fig.1A), at 24 hours for the 184A1 cells (Fig.1B) and at 15 hours for the 184B5 cells (Fig.1C).

Linoleic acid stimulates the growth of the 184 cell lines
184 (Fig.2A), 184A1 (Fig.2B), and 184B5 (Fig.2C) cells were stimulated with linoleic acid (LA) in the presence or absence of epidermal growth factor (EGF). Linoleic acid did not stimulate the growth of 184 cells in the presence or absence of EGF. 184A1 cells were stimulated by LA in the presence, but not the absence of EGF. The concentration of linoleic acid that maximally stimulated growth in these cells was 17.8 μM. 184B5 cells were even more sensitive to the growth-stimulating effects of LA. In these cells, LA stimulated growth in the presence or absence of EGF, with a maximally effective concentration between 3.6-10.7 μM. Because linoleic acid stimulated the growth of 184B5 cells so strongly, we decided to further characterize the mechanism responsible for this growth stimulation.

Linoleic acid does not stimulate growth by the formation of EGF/TGFα
Because EGF/TGFα is the most potent stimulator of growth of the 184 cell strains, one potential mechanism by which linoleic acid worked was through causing the production of TGFα by these cells. The TGFα would then interact with the EGF receptor and stimulate cell growth. If linoleic acid was acting by this mechanism to stimulate cell growth, there should be a lag between the time of maximum growth stimulation seen with EGF as a stimulus and linoleic acid as a stimulus. To test this, we performed a time course of growth stimulation of the 184B5 cells using either EGF or linoleic acid as a stimulus. We found that the time of maximal growth stimulation was the same whether EGF or linoleic acid was used as a stimulus, suggesting that linoleic acid stimulated growth of the 184B5 cells through a mechanism that did not involve TGFα formation (Fig.3).

Linoleic acid may stimulate growth through prostaglandin H synthase type 2
Linoleic acid may be metabolized to form a variety of biologically active products. The best known pathway for product formation involves the conversion of linoleic acid (18:2) to arachidonic acid (20:4). Arachidonic acid is the precursor for a wide range of biologically active compounds catalyzed by the actions of various prostaglandin synthases and lipoxygenases. Experiments in the past few years have revealed the presence of an inducible isoform of prostaglandin synthase (11), termed prostaglandin synthase type 2 (PHS-2). PHS-2 is induced by a variety of cytokines, mitogens and

tumor promoters. Dysregulation of PHS-2 expression has been demonstrated in colon carcinoma. Experiments were designed to determine whether PHS-2 might be responsible for the increased growth of 184B5 cells in response to linoleic acid. Messenger RNA was isolated from 184A1 and 184B5 cells that were either quiescent or had been stimulated with EGF or PMA. cDNA was prepared from these samples and reverse transcriptase PCR performed. We found that both the 184A1s and the 184B5 cells contained PHS-2 mRNA when stimulated. Interestingly, we found that the 184B5 cells also contained high levels of PHS-2 mRNA when they were quiescent (Fig.4). This suggested that these cells might be primed for the increase in substrate that linoleic acid would provide for them, and might be able to quickly metabolize it to a growth-stimulatory metabolic product.

184B5 cells make PGE_2 in the absence of stimulation

Prostaglandin E_2 (PGE_2) is the predominant prostaglandin product of epithelial cells including the 184 cell strains. To determine if the prostaglandin synthase mRNA constitutively present in the 184B5 cells corresponded to constitutively active PHS-2 protein, we measured the formation of PGE_2 in quiescent 184A1 and 184B5 cells and in those which were stimulated with EGF or PMA. Quantitation of PGE_2 was performed by radioimmunoassay. We found that both 184B5 cells made much higher levels of PGE_2 than did 184A1 cells (Fig.5). Furthermore, the 184B5 cells produced high levels of PGE_2 whether they were quiescent or stimulated. This further suggested that these cells were primed to make a biologically active metabolite from linoleic acid that would stimulate cell growth.

Inhibitors of prostaglandin synthase attenuate linoleic acid -stimulated growth of 184B5 cells

If PHS-2 is responsible for the formation of a biologically active growth-stimulatory compound, it should be possible to inhibit linoleic acid stimulated growth by treating the 184B5 cells with an inhibitor of prostaglandin synthase. We used two inhibitors of prostaglandin synthase in an attempt to inhibit linoleic acid stimulated growth. We found that flurbiprofen (Fig.6A) and indomethacin (Fig.6B) inhibited linoleic acid-stimulated growth. However, the concentrations of these compounds necessary to inhibit growth was high, indicating that there may have been effects other than specific inhibition of prostaglandin synthase.

Products of PHS-2 can partially account for linoleic acid-stimulated growth

If linoleic acid is stimulating growth by causing the formation of a biologically active product, we should be able to identify this product and add it to the 184B5 cells to stimulate their growth in a manner analogous to that seen with linoleic acid. We initially tried PGE_2, which, as mentioned above, is the major prostaglandin product of 184B5 cells. PGE_2 did not stimulate the growth of 184B5 cells at any concentration tested (Fig.7). PHS-2 is also able to catalyze the formation of 15-hydroxyeicosatetraenoic acid (15-HETE) from arachidonic acid. We tested the ability of 15-HETE to stimulate the growth of 184B5 cells and found that it did stimulate the growth of the cells, but not to the same extent as did linoleic acid. PHS-2 has been demonstrated to oxygenate fatty acids other than arachidonic acid, and may be able to utilize linoleic acid directly as a substrate. If linoleic acid was a substrate for PHS-2, the products formed would be 9- and 13-hydroxyoctadecadienoic acids (9- and 13-HODE). The HODEs have been demonstrated to have a variety of biological activities and may be able to stimulate the growth of some cells. To determine if HODEs could be responsible for the growth stimulation seen in response to linoleic acid, cells were stimulated with HODEs in a manner equivalent to LA stimulation. As found for 15-HETE, 9-HODE, 13-HODE or a combination of the two, was partially able to stimulate the growth of 184B5 cells (Fig.8).

Linoleic acid may activate protein kinase C to stimulate the growth of 184B5 cells
Reverse transcriptase PCR was used to examine the isoforms of protein kinase C present in 184A1 and 184B5 cells. mRNA was isolated from unstimulated cells and cDNA prepared by reverse transcription (12). This cDNA was then amplified by PCR with primers specific for the classical PKC isoforms α, β and γ, the novel isoforms δ, ε, η and ϑ, and the atypical isoforms ζ, λ and μ (Fig.9). 184A1 and 184B5 were found to contain the same isomers: α, δ, ε, η, ϑ, ζ, λ and μ. Of these isoforms, α and ζ are known to be activated by fatty acids, including linoleic acid. It is likely that other isoforms will also be found to be activated by fatty acids.

Other unsaturated fatty acids stimulate growth of 184B5 cells
When other investigators examined the ability of fatty acids to activate protein kinase C, they found that a wide range of unsaturated fatty acids, but not saturated fatty acids, were able to activate PKC. We hypothesized that if linoleic acid was stimulating growth through the activation of protein kinase C, then other unsaturated fatty acids should also stimulate cell growth. We tested the ability of a panel of unsaturated and saturated fatty acids to stimulate the growth of 184B5 cells. We found that all of the unsaturated fatty acids that we tested were able to stimulate the growth of 184B5 cells, although generally not to the same extent as did linoleic acid (Fig.10). Saturated fatty acids, on the other hand, were unable to stimulate cell growth. This suggested that linoleic acid may be stimulating the growth of 184B5 cells in part through the activation of protein kinase C. The abbreviations used are: LA=linoleic acid, GLA=gamma linolenic acid, DHLA=dihomolinolenic acid, AA=arachidonic acid, PA=palmitic acid, and SA=stearic acid.

Inhibitors of protein kinase C attenuate the linoleic acid stimulated growth of 184B5 cells
If linoleic acid stimulates the growth of 184B5 cells through the activation of protein kinase C, this stimulation of growth should be inhibitable by blocking protein kinase C activity. We used inhibitors of protein kinase C to block its activity in 184B5 cells. When staurosporine was used to inhibit protein kinase C activity, we found that the growth response to linoleic acid was greatly attenuated (Fig.11A). When calphostin C was used to block protein kinase C activity, the growth response to linoleic acid was only partially inhibited (Fig.11B). Calphostin C blocks protein kinase C activity through a rather complex, light-dependent mechanism and is thought to inhibit only the classical group of PKCs (α, β and γ). These results suggested that linoleic acid was not stimulating growth through the activation of one of the classical PKCs, but rather through a novel or atypical isoform. As further evidence that linoleic acid was not stimulating the growth of 184B5 cells through the activation of a classical PKC, we downregulated the classical and novel isoforms of PKC using PMA. Pretreatment of the cells with PMA for 48 hours totally blocked the growth response of the 184B5 cells to PMA, but did not alter the growth response to linoleic acid (Fig.11C). This suggested that linoleic acid may stimulate the growth of 184B5 cells through the activation of an atypical protein kinase C, such as PKCζ. Experiments to further test this hypothesis are currently underway. These experiments include knocking out PKCζ with antisense, and blocking its activity by treating cells with peptides directed at the pseudosubstrate site. These experiments should allow unequivocal determination of the involvement of PKC in the growth response of 184B5 cells to linoleic acid.

Conclusions

Our results continue to indicate a prominent role for prostaglandins and related compounds in the growth augmentation by linoleic acid. To allow unequivocal determination of the role of PHS-2 in the growth response of 184B5 cells to linoleic acid, experiments are currently being performed to knock out PHS-2 in these cells by treating with antisense oligonucleotides designed specifically against PHS-2. This should eliminate the uncertainty regarding the specificity of the actions observed.

There also clearly is an effect that is independent of prostaglandins, and our evidence to date suggests that it is mediated via protein kinase C. Experiments are underway to examine the functional relevance of this in PMA and EGF-stimulated growth using isotype-specific inhibitors and antisense to individual isotypes.

The other aspects of the project will proceed on the original timetable as the results thus far have supported their relevance. No change in focus or general approach is anticipated.

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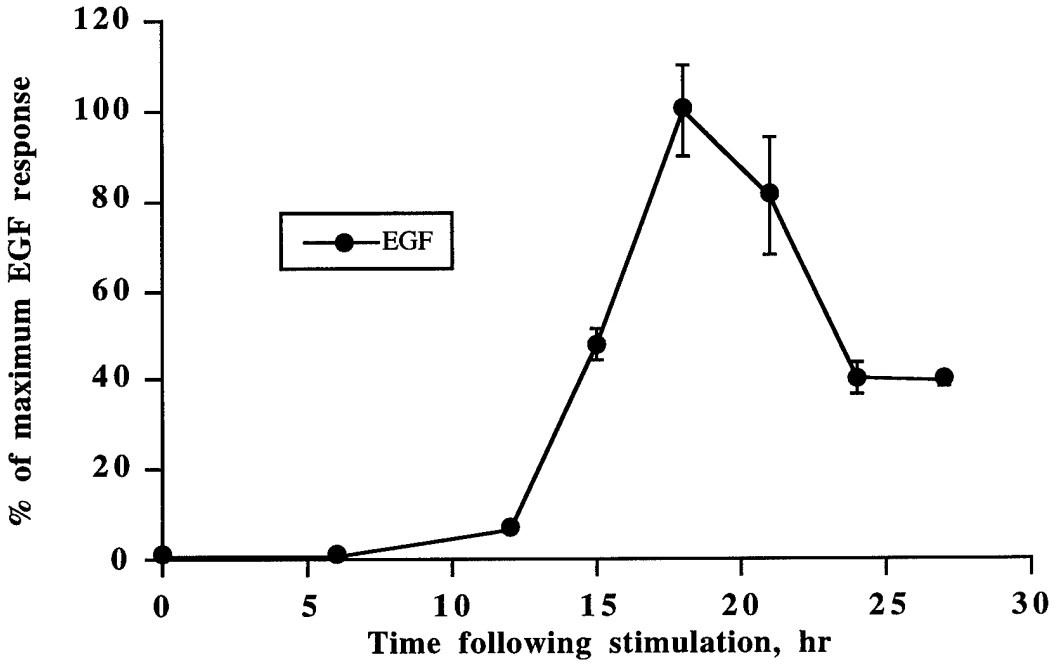
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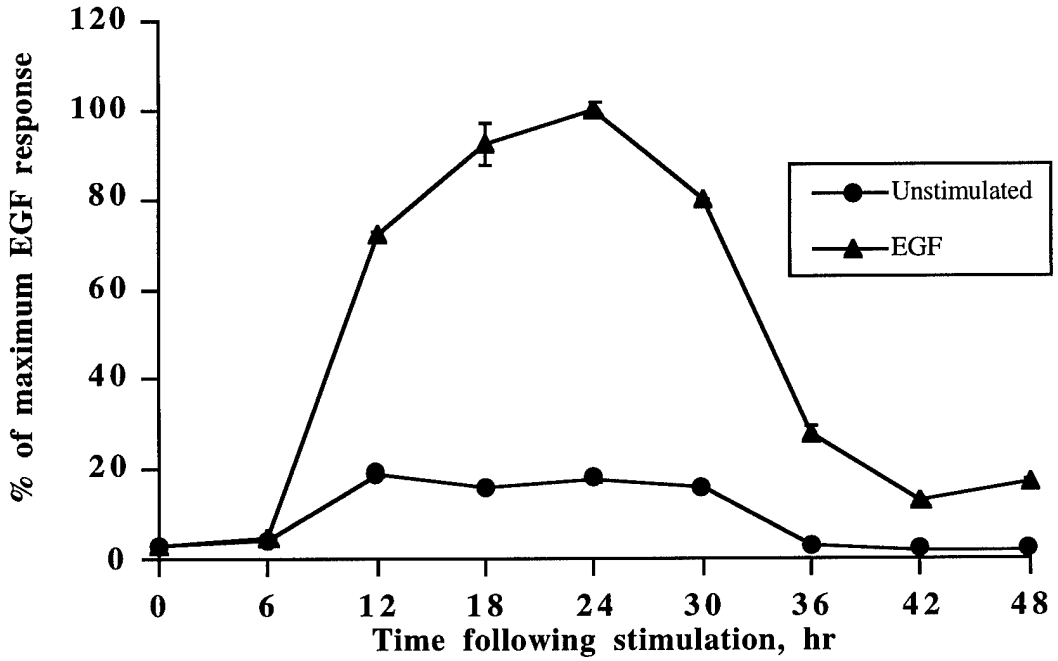
Time following stimulation, hr

Figure 1A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Unstimulated
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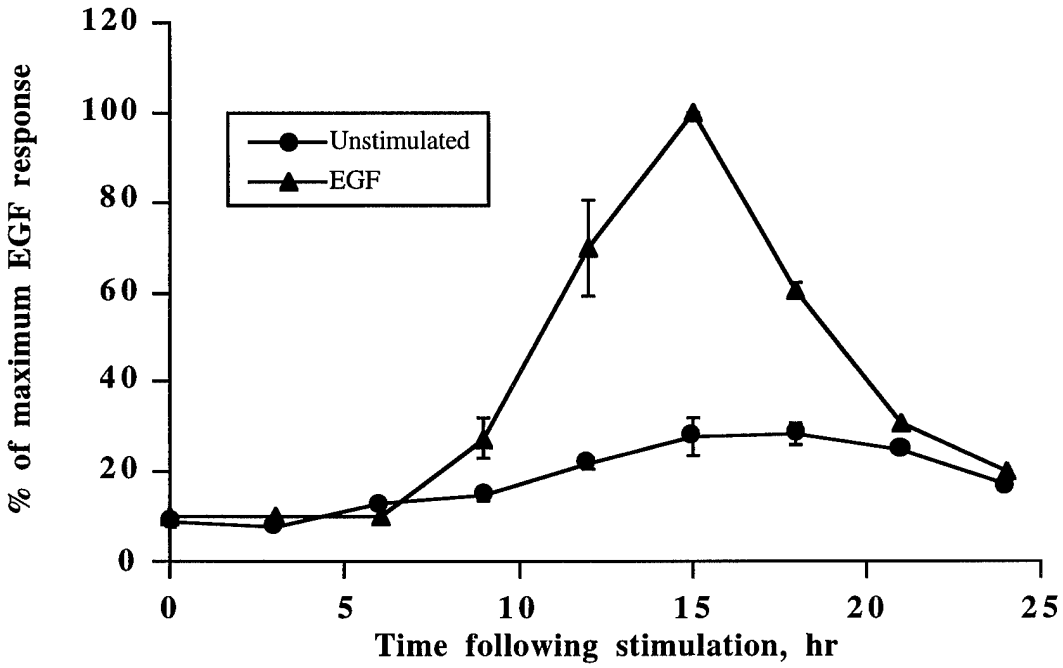
Figure 1B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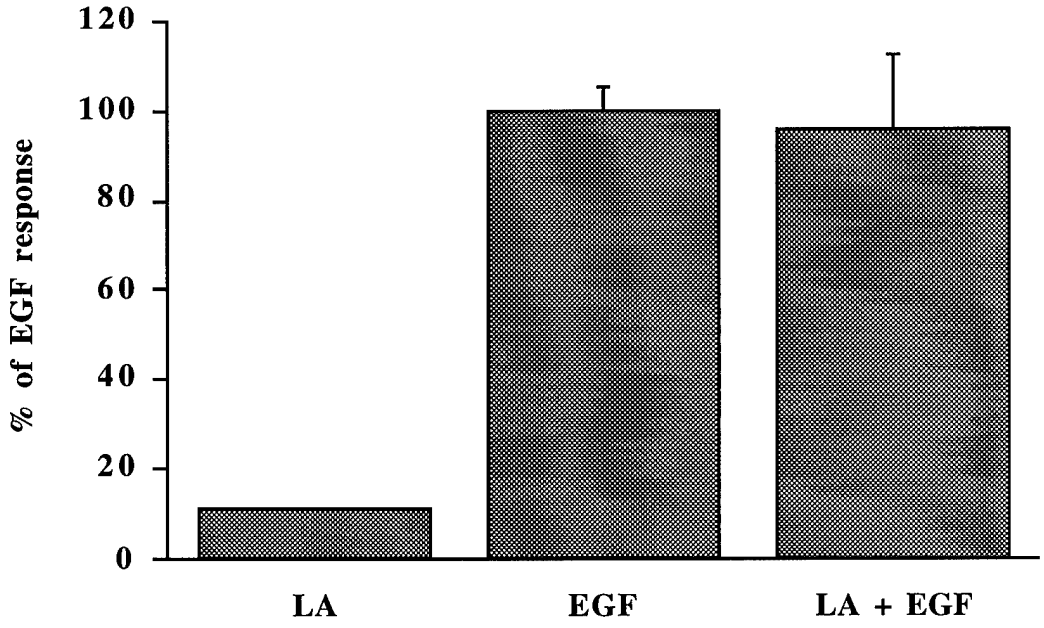
Time following stimulation, hr

Figure 1C



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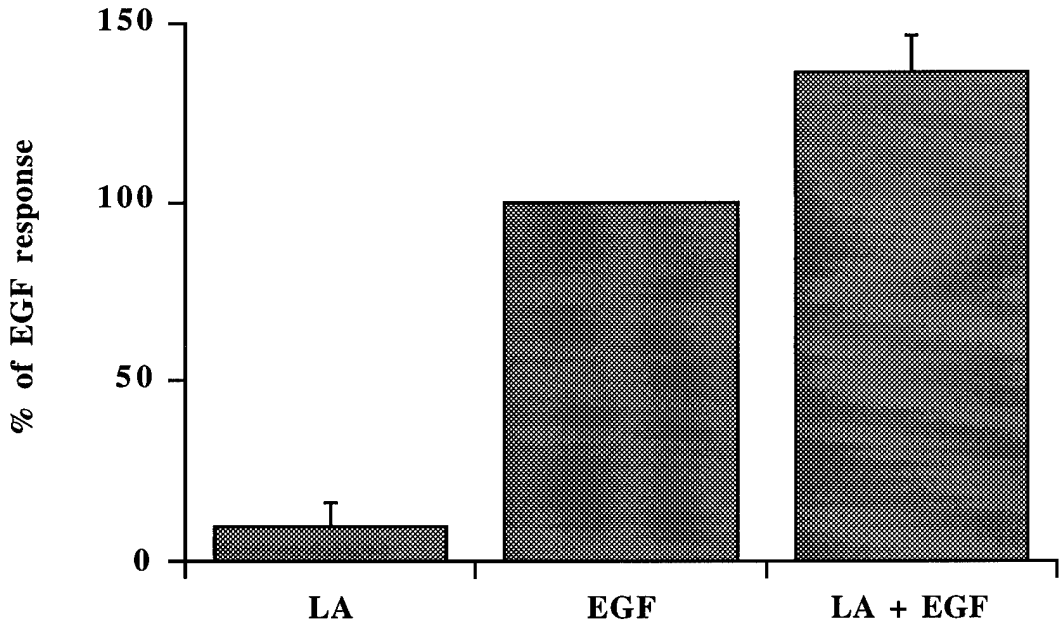

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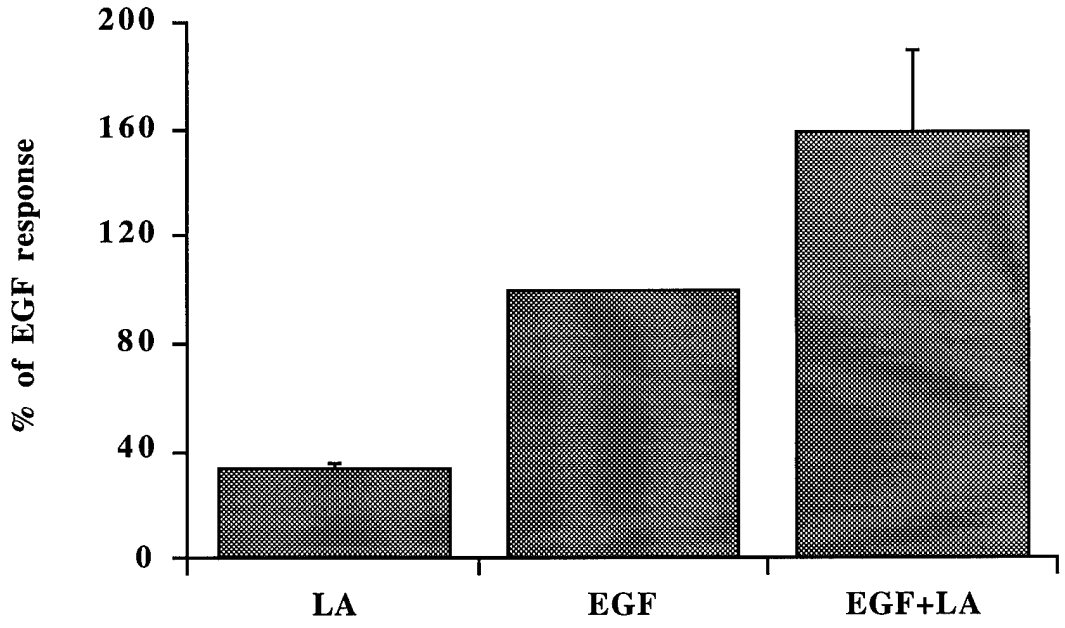

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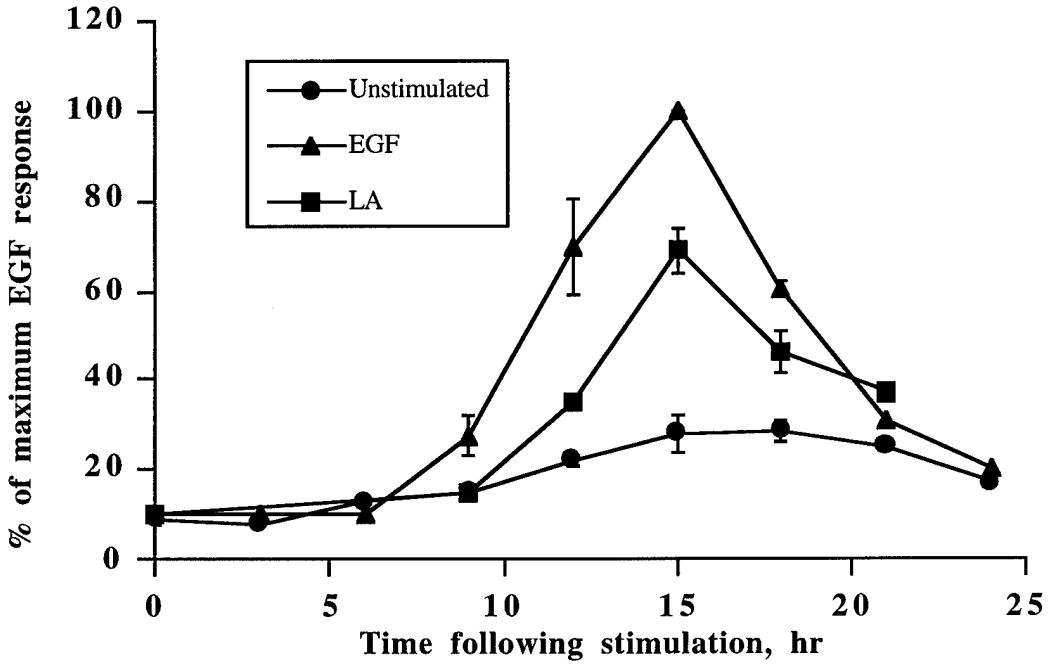
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Unstimulated
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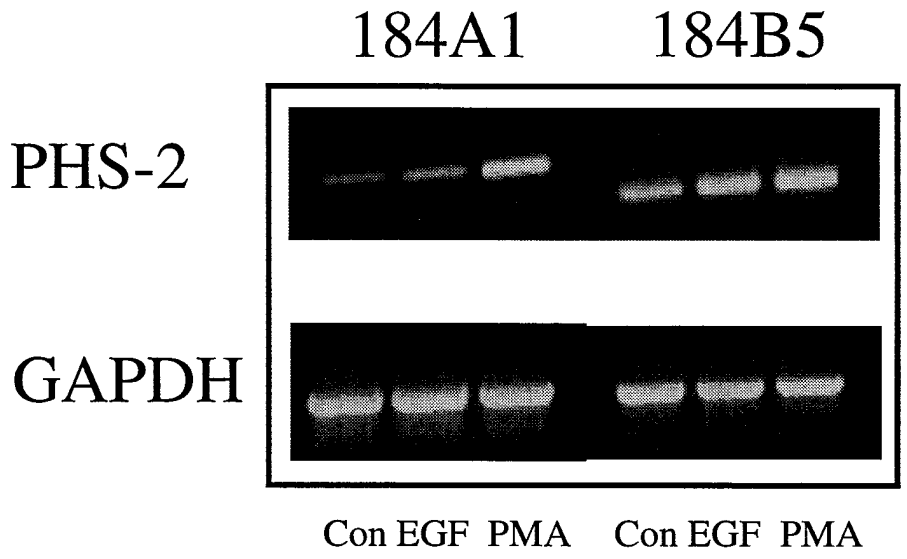
Time following stimulation, hr

Figure 3

184B5 cells constitutively express PHS-2

Reverse-transcriptase PCR



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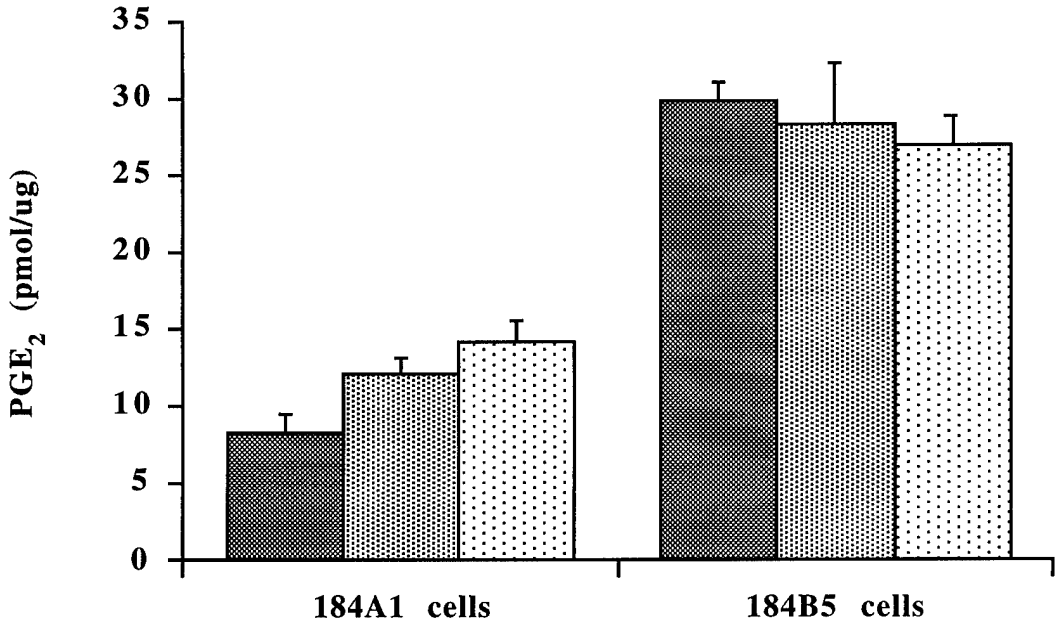

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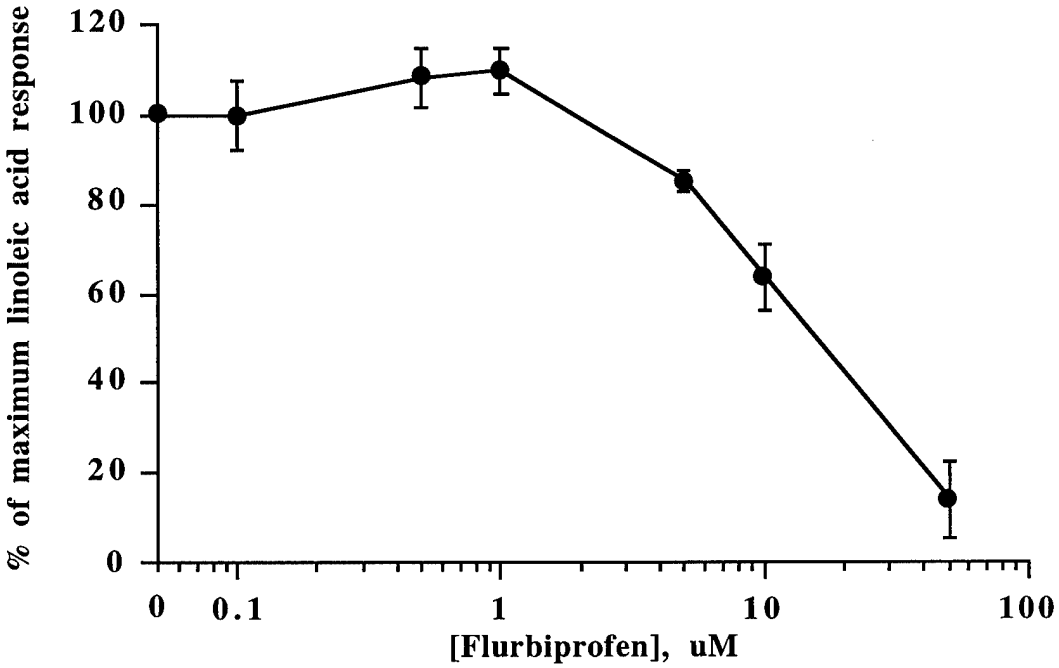
% of maximum linoleic acid response
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[Flurbiprofen], uM

Figure 6A

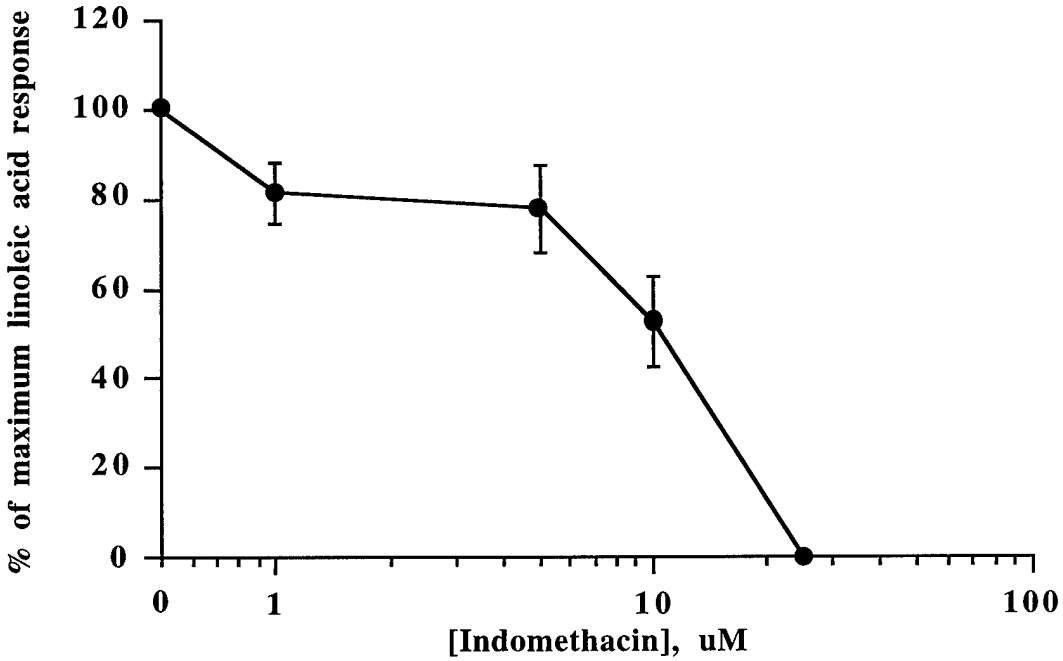
% of maximum linoleic acid response
120
100
80
60
40
20
0
0
1
10
100
[Indomethacin], uM

Figure 6B

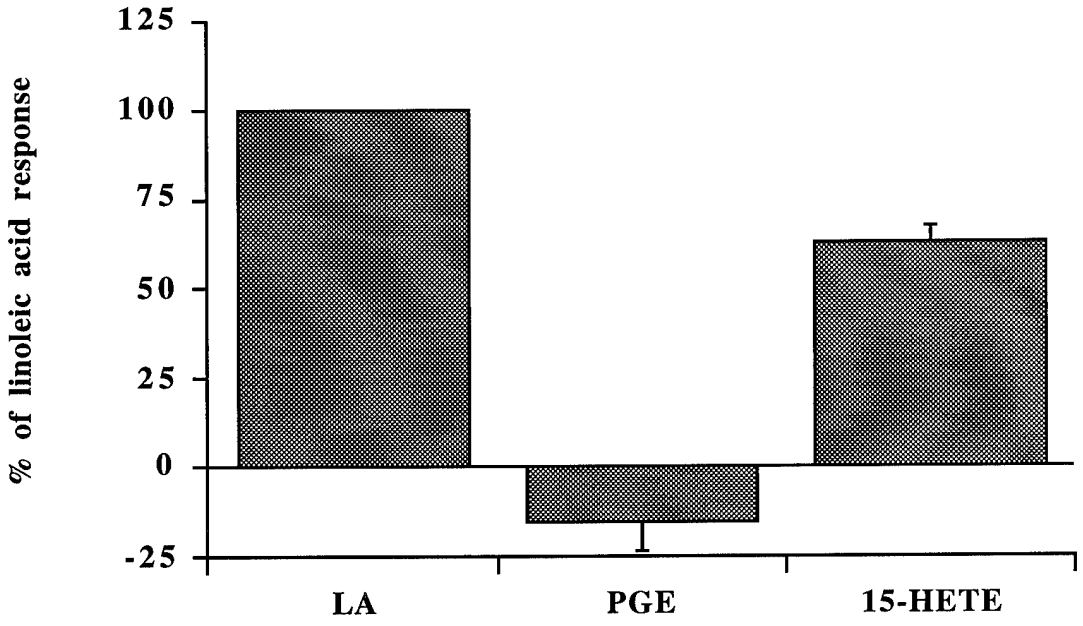

Figure 7

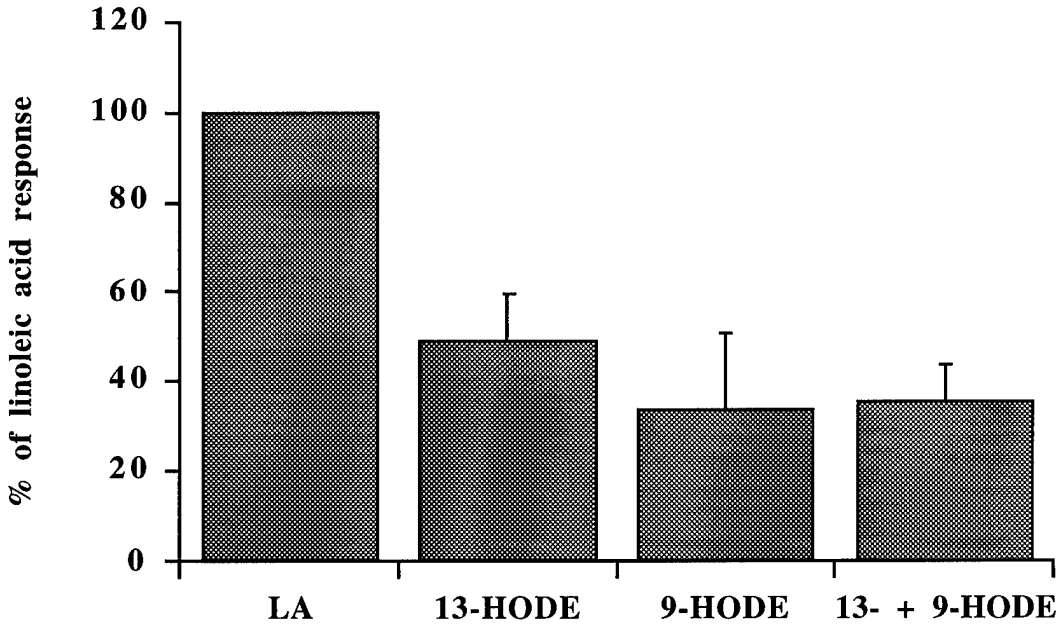
% of linoleic acid response
120
100
80
60
40
20
0
LA
13-HODE
9-HODE
13- + 9-HODE

Figure 8

184B5 cells express many isoforms of PKC

Reverse-transcriptase PCR

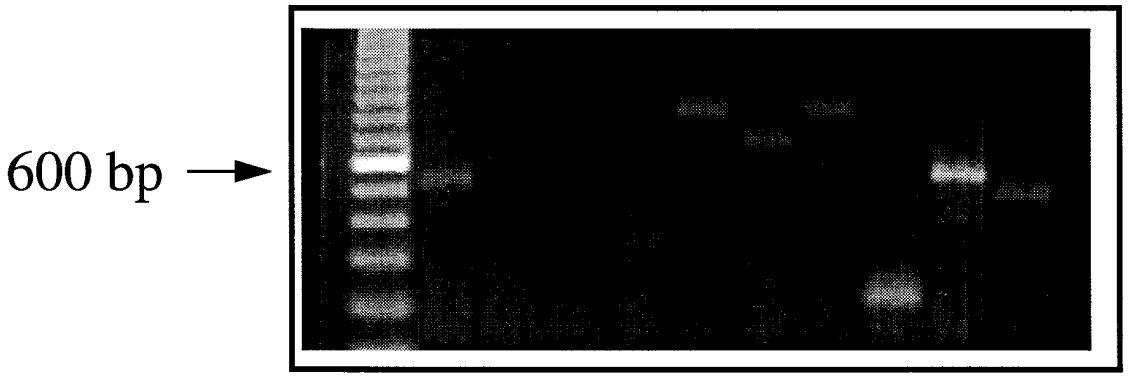

Figure 9

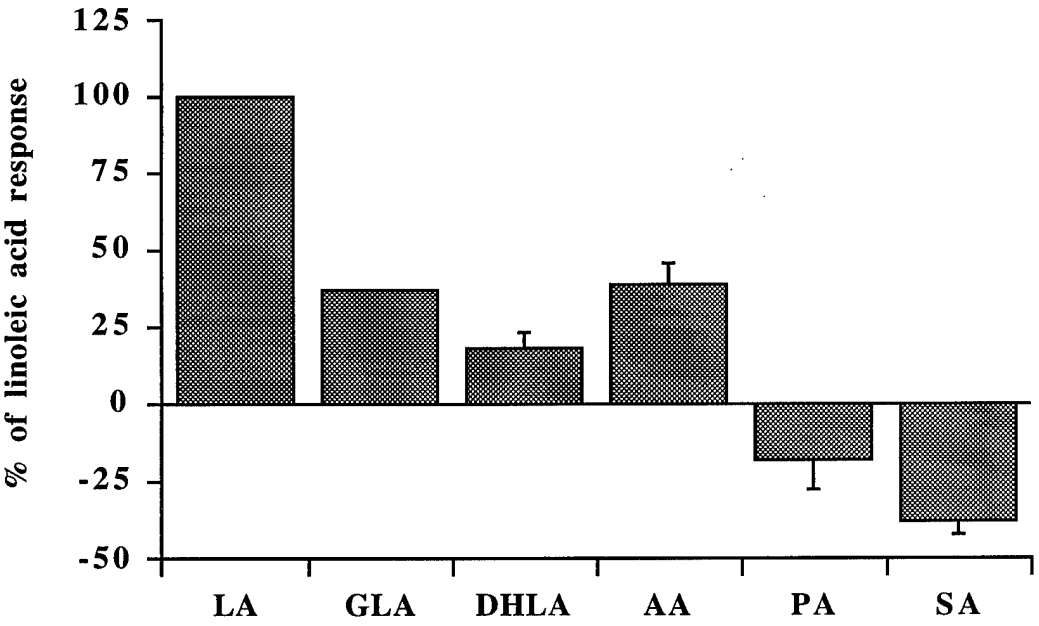
% of linoleic acid response
125
100
75
50
25
0
-25
-50
LA
GLA
DHLA
AA
PA
SA

Figure 10

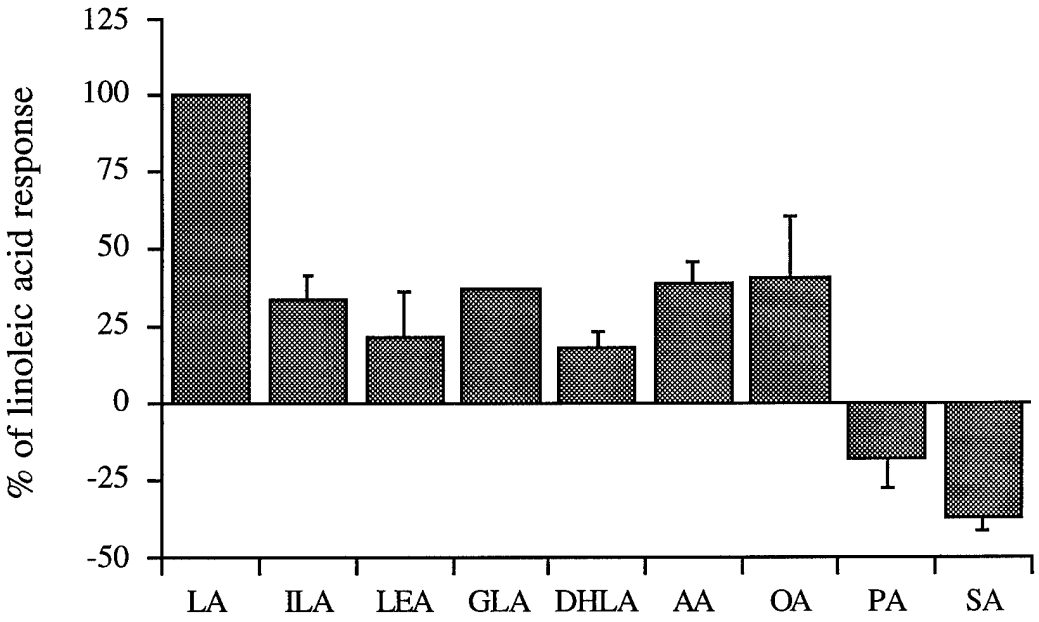

Figure 10 cont.

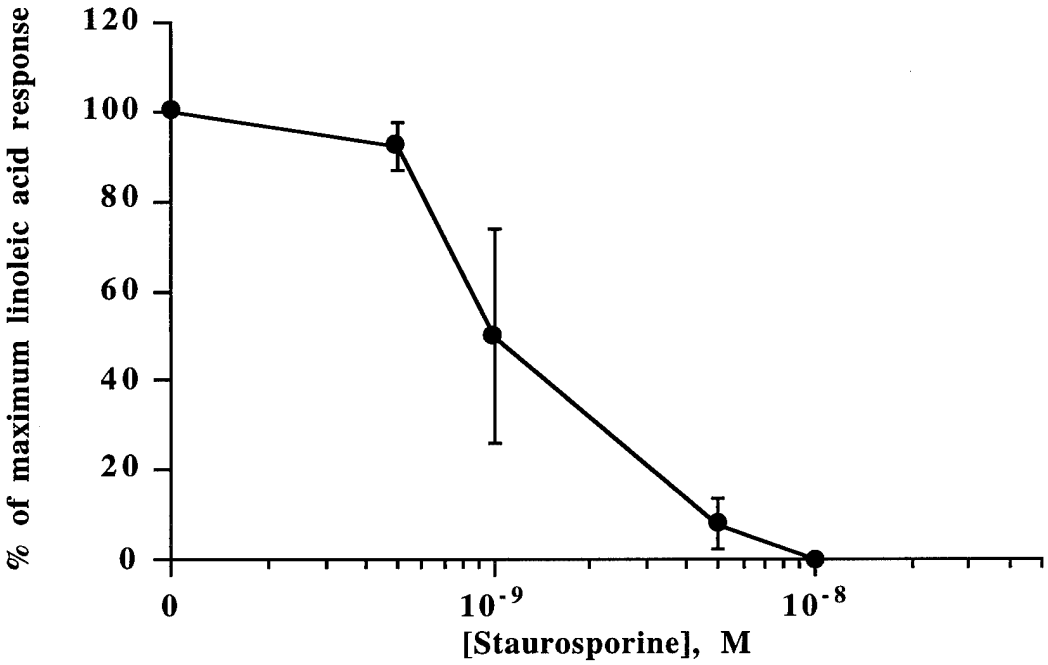
% of maximum linoleic acid response
120
100
80
60
40
20
0
0
10-9
10-8
[Staurosporine], M

Figure 11A

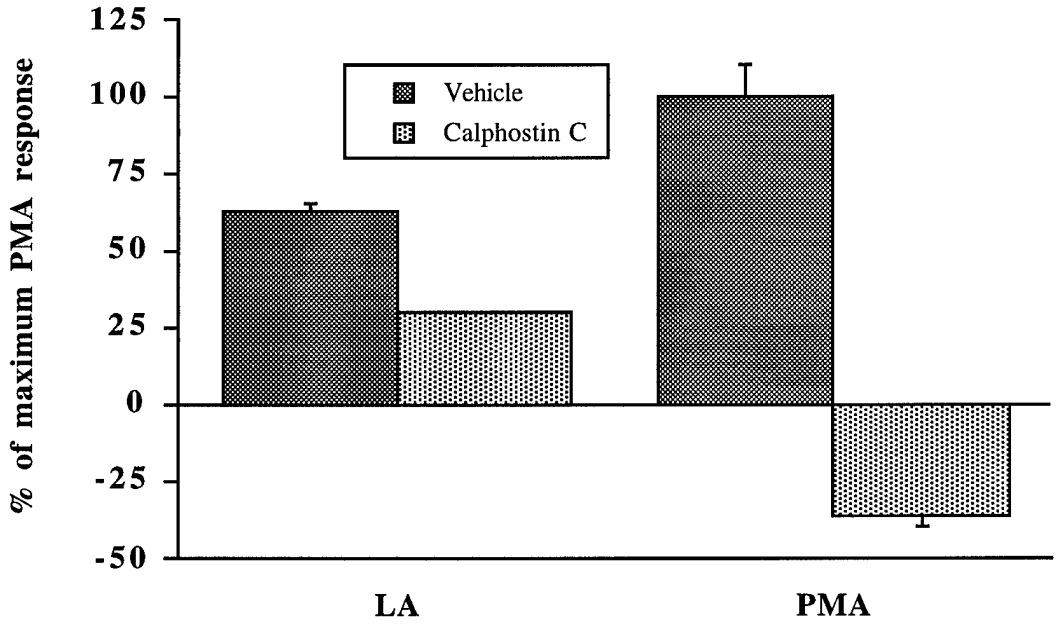
125
100
75
50
25
0
-25
-50
% of maximum PMA response
Vehicle
Calphostin C
LA
PMA

Figure 11B

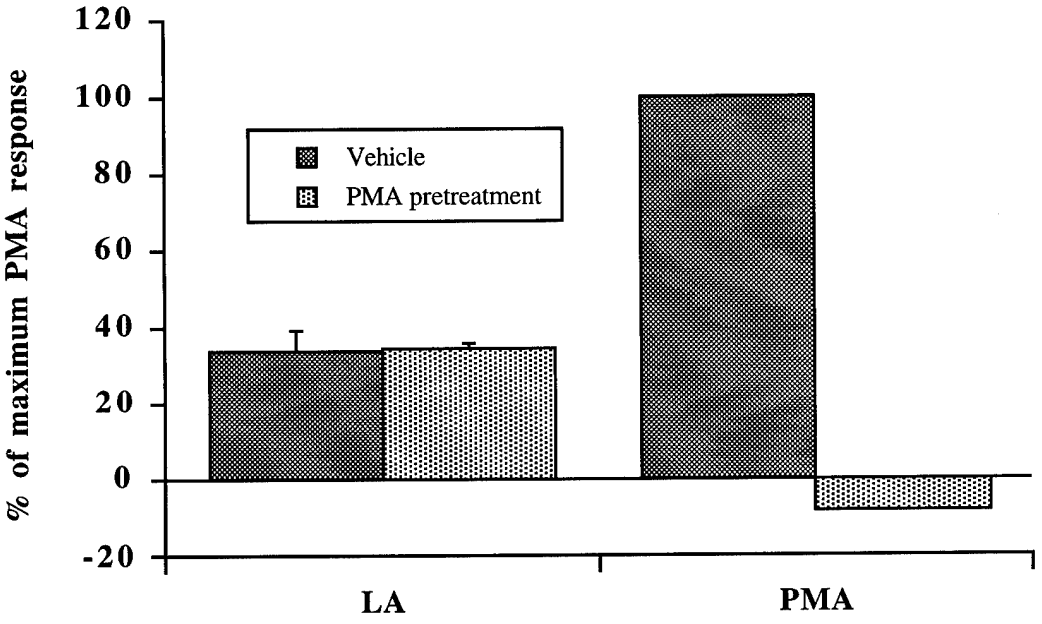
120
100
80
60
40
20
0
-20
% of maximum PMA response
Vehicle
PMA pretreatment
LA
PMA

Figure 11C