Activation of phospholipase A₂ and stimulation of prostaglandin E₂ production by transforming growth factor- α in rat thymic epithelial cells requires influx of calcium

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The stimulation of both phospholipase A₂ (PLA₂) enzymic activity and the production of prostaglandin E₂ (PGE₂) by transforming growth factor- α (TGF- α) and Ca²⁺ ionophore A23187 in TEA3A1 rat thymic epithelial cells were studied. TGF- α by itself at various concentrations (5–200 ng/ml) had no effect on the stimulation of PGE₂ production. A23187 (1 μ g/ml) by itself stimulated PGE₂ production on average by 18-fold over the control. When TGF- α (50 ng/ml) was added to the cells in the presence of A23187, a synergistic stimulation (on average 45-fold) of PGE₂ production was observed. Synergistic stimulation was also observed at the level of arachidonic acid released from phospholipid pools, suggesting the activation of PLA₂ enzymic activity. We have found that this synergistic activation of PLA₂ enzymic activity and subsequent stimulation of PGE₂ production required the activation of epidermal growth factor (EGF) receptor tyrosine kinase and Ca²⁺ influx. This was shown by the fact that genistein, an inhibitor of tyrosine kinase, blocks the

synergistic stimulation by TGF- α and A23187 and by the fact that the stimulation of PGE₂ production by TGF- α and A23187 is dependent on the culture-medium Ca²⁺ concentrations. The requirement for Ca²⁺ influx instead of intracellular mobilization of Ca²⁺ was shown by the fact that PGE₂ production was not stimulated when cells were treated with TGF- α and thapsigargin. Moreover, the synergistic stimulation of PGE₂ production by TGF- α and A23187 was not affected in protein kinase C down-modulated cells. In addition, the synergistic stimulation was not observed in cells treated with either phorbol 12-myristate 13-acetate (PMA) and TGF- α or PMA and A23187, and in cells treated with TGF- α and thapsigargin. The requirement for the activation of receptor tyrosine kinase seems to be specific to the EGF receptor, since a synergistic stimulation of PGE₂ production was not observed when cells are treated with either insulin-like growth factor-I or fibroblast growth factor-I in the presence of A23187.

INTRODUCTION

Prostaglandin plays an important role in the development of thymocytes. The importance of prostaglandin production in the thymus has been implicated by the fact that the level of prostaglandin in the thymus is 4–10 times that in the plasma [1,2]. Conversely, it has been shown that the levels of prostaglandins E and F (PGE and PGF) in the thymus of young lupus-prone NZB and NZB/W mice are abnormally low. This low level of prostaglandin is related to thymocyte hyperplasia in thymus cortex [1–3]. Injection of PGE₁ into NZB/W mice prevented the onset of this early-phase thymocyte hyperplasia in the thymus of these animals [2,3]. Recent studies on fetal thymus organ culture also have shown that the arachidonate (AA) metabolites effectively affect the development, growth and function of T lymphoid cells in the thymus [4].

The thymic epithelial cells, among other cells of the thymus, are considered to be responsible for the constitutive production and the maintenance of high levels of prostaglandins in the thymus [5–7]. It is therefore important to study the regulatory mechanism responsible for sustaining active AA metabolism in thymic epithelial cells. In our laboratory, we have developed rat thymic epithelial cell line, TEA3A1 [8]. Using this cell line, we have been studying the regulation of phospholipase A₂ (PLA₂) enzymic activity and prostaglandin production [7,9,10]. In TEA3A1 cells, PLA₂ is the key enzyme involved in the release of

free AA from phospholipid pools. Moreover, PGE₂ is the major AA metabolite produced by the cells after stimulation of PLA₂ enzymic activity [7,10]. In this study, we report that transforming growth factor- α (TGF- α) stimulates PLA₂ activity and PGE₂ production in TEA3A1 cells, provided that cells are stimulated concomitantly with Ca²⁺ ionophore A23187.

MATERIALS AND METHODS

Reagents

Ca²⁺-free WAJC404 medium used in the experiment was a gift from Kyokuto Pharmaceutical Co., Tokyo, Japan. High-glucose Dulbecco's modified Eagle's medium (DME) was purchased from GIBCO BRL (Grand Island, NY, U.S.A.). [³H]PGE₂ (185 Ci/mmol) and [³H]AA (76 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Unlabelled PGE₂ was obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.). Bovine insulin, human transferrin, dexamethasone, A23187, thapsigargin and fatty-acid-free BSA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Iron-supplemented calf serum (sCS) was obtained from Hyclone Laboratory (Logan, UT, U.S.A.). Phorbol 12-myristate 13-acetate (PMA) was purchased from Calbiochem (La Jolla, CA, U.S.A.). Anti-PGE₂ antibody was raised in our laboratory. TGF- α , fibroblast growth factor-I (FGF-I) and insulin-like growth factor-I (IGF-I) were

Abbreviations used: PLA₂, phospholipase A₂; PGE, prostaglandin E; TGF- α , transforming growth factor- α ; AA, arachidonic acid; DME, Dulbecco's modified Eagle's medium; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF-I, insulin-like growth factor-I; r.i.a., radioimmunoassay.

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purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.).

Cell culture

TEA3A1 cells were cultured as described elsewhere [8]. Briefly, the stock cells were cultured in WAJC404A medium (low- Ca^{2+} medium) supplemented with 10 $\mu\text{g}/\text{ml}$ insulin, 10 $\mu\text{g}/\text{ml}$ transferrin, 10 nM dexamethasone and 2% sCS. All of the experimental cells were cultured in a 1:1 mixture of DME and WAJC404A medium (DW medium; high- Ca^{2+} medium) supplemented with 10 $\mu\text{g}/\text{ml}$ insulin, 10 $\mu\text{g}/\text{ml}$ transferrin, 10 nM dexamethasone and 2% sCS in 6-well culture plates (Costar). At confluence, cells were washed three times with DME containing 10 mg/ml fatty-acid-free BSA and incubated in 1 ml of DME in the presence or absence of indicated amounts of A23187, TGF- α , PMA and thapsigargin for 15 min unless stated otherwise.

Radioimmunoassay (r.i.a.) for PGE_2

Culture media of experimental cells were harvested after the incubation period, centrifuged at 12000 g for 1 min, and the supernatants were collected and stored at -20°C until assayed for PGE_2 by r.i.a., carried out as previously described [10].

AA release assay

TEA3A1 cells cultured to confluence were prelabelled with 3 $\mu\text{Ci}/\text{ml}$ [^3H]AA for 6 h. AA release assays were carried out as previously described [10]. The levels of [^3H]AA released from the cells were determined by t.l.c. using silica gel G plates. T.l.c. plates were developed in the organic phase of a mixture of ethyl acetate/heptane/acetic acid/water (38:12:17:33, by vol.) and spots corresponding to unlabelled AA standard were scraped and counted for radioactivity in a liquid scintillation counter.

Down-modulation of protein kinase C (PKC)

TEA3A1 cells were treated as previously described in order to down-modulate PKC [10]. Briefly TEA3A1 cells cultured to

confluence were washed once with DME, and then incubated with DME containing 500 ng/ml PMA in 100 $\mu\text{g}/\text{ml}$ BSA for 24 h at 37°C . After the incubation period, cells were washed for 3×30 min, each with 1 ml/well DME containing 10 mg/ml fatty-acid-free BSA at 37°C . Control cells were also treated the same way, except that they did not receive PMA. An extensive washing with DME containing BSA is necessary to remove excess PMA from the culture.

RESULTS

Synergistic stimulation of PGE_2 production and activation of PLA_2 enzymic activity by TGF- α and A23187

TEA3A1 cells were treated for 15 min with increasing concentrations of TGF- α (5–200 ng/ml) added either alone or in the presence of 1 μg of the Ca^{2+} ionophore A23187/ml. At the end of the incubation period, medium was collected to measure PGE_2 production. As shown in Figure 1(a), TGF- α alone did not have any effect on PGE_2 production by TEA3A1 cells [controls 0.11 ± 0.01 ng/ml; TGF- α $(0.08\text{--}0.13) \pm 0.01$ ng/ml]. However, when added in the presence of A23187, TGF- α stimulated PGE_2 production in a dose-dependent fashion which reached a plateau at 100 ng/ml. At this concentration, TGF- α stimulated PGE_2 production by 65-fold (7.18 ± 0.38 ng/ml), whereas A23187 alone stimulated PGE_2 production by 15-fold (1.66 ± 0.04 ng/ml). There are some variations in fold stimulations between experiments, probably due to the difference in the passage numbers of the cells used between experiments. Cells with lower passage numbers tend to respond well to stimulatory agents, compared with cells with higher passage numbers. In order to minimize such variations, experiments were carried out on cells under 40 passages. Variations in PGE_2 production between experiments that we have observed are as follows: 0.11 ± 0.03 ng/ml (maximum 0.19 ng, minimum 0.08 ng, $n = 27$) for controls; 0.10 ± 0.02 ng/ml (maximum 0.14 ng, minimum 0.07 ng, $n = 33$) for TGF- α (50 ng/ml); 2.06 ± 0.37 ng/ml (maximum 2.74 ng, minimum 1.61 ng, $n = 15$) for A23187 (1 $\mu\text{g}/\text{ml}$); and 5.00 ± 0.73 ng/ml (maximum 6.1 ng, minimum 3.69 ng, $n = 15$) for TGF- α (50 ng/ml) + A23187 (1 $\mu\text{g}/\text{ml}$). The difference between control and TGF- α values is not significant ($P > 0.1$, unpaired t test,

Figure 1 Dose- and time-dependent increase in PGE_2 production by TGF- α in the presence of A23187

TEA3A1 cells were cultured in 6-well culture clusters to confluence. Cells were then washed as described in the Materials and methods section and were incubated in 1 ml of DME containing indicated doses of TGF- α with or without 1 $\mu\text{g}/\text{ml}$ A23187 for 15 min (a). Production of PGE_2 by TEA3A1 cells cultured for various lengths of time in the presence of A23187, TGF- α or A23187 and TGF- α was also determined (b). Cells were incubated for the indicated lengths of time in 1 ml of DME containing 1 $\mu\text{g}/\text{ml}$ A23187, 50 ng/ml TGF- α or A23187 and TGF- α . Addition of an identical amount of vehicle solution served as a control. Media were harvested and levels of PGE_2 produced by the cells were determined by r.i.a. Each point represents the mean $\pm$ S.D. ($n = 3$).

Table 1 [³H]AA release stimulated by TGF- α and A23187

Release of [³H]AA from prelabelled cells in response to TGF- α , A23187 or TGF- α and A23187 was quantified. Confluent TEA3A1 cells were prelabelled for 6 h with [³H]AA and washed thoroughly as described. Cells were incubated in 1 ml of DME containing vehicle control, 50 ng/ml TGF- α , 1 μ g/ml A23187 or 50 ng/ml TGF- α and 1 μ g/ml A23187 for 15 min. Media were harvested and the levels of [³H]AA released from the cells were determined after isolation of AA by t.l.c. Values are means $\pm$ S.D. ($n = 3$).

	[³ H]AA released (c.p.m.)
Control	153 $\pm$ 44
TGF- α	177 $\pm$ 72
A23187	268 $\pm$ 59
TGF- α + A23187	497 $\pm$ 103

Table 2 Stimulation of PGE₂ production by TGF- α , A23187 and PMA

Production of PGE₂ by TEA3A1 cells in response to PMA and A23187 (a) or TGF- α and PMA (b) was examined. Experiments were performed as previously described. Cells were cultured in 1 ml of DME containing 50 ng/ml TGF- α , 1 μ g/ml A23187, 1 μ g/ml PMA or combination of two factors as described for 15 min. Media were harvested and the levels of PGE₂ produced were determined by r.i.a. Values are means $\pm$ S.D. ($n = 3$).

PGE ₂ (ng/ml)				
(a)	Control	A23187	PMA	PMA + A23187
	0.18 $\pm$ 0.02	2.08 $\pm$ 0.10	2.96 $\pm$ 0.12	3.96 $\pm$ 0.28
(b)	Control	TGF- α	PMA	TGF- α + PMA
	0.10 $\pm$ 0.02	0.08 $\pm$ 0.01	2.67 $\pm$ 0.31	2.77 $\pm$ 0.2

two-tailed P value); however, the difference between A23187 and TGF- α + A23187 values is highly significant ($P < 0.0001$, unpaired t test, two-tailed P value). The average fold stimulation for 1 μ g/ml A23187 was 20-fold, and that for 50 ng/ml TGF- α + 1 μ g/ml A23187 was 50-fold, over the control and TGF- α -only values. It is clear from these results that TGF- α and A23187 act in synergy to stimulate the production of PGE₂ in TEA3A1 cells. Time course studies of the effect of TGF- α and A23187 added either individually or together indicate that, in all cases, the stimulation of PGE₂ production is rapid and reached a plateau within 15 min of incubation (Figure 1b).

In general, the production of free AA is the rate-limiting step in PGE₂ production. Since PLA₂ is usually the key enzyme responsible for AA release from phospholipid pools, we have investigated whether TGF- α and A23187 affected PLA₂ activity in TEA3A1 cells. PLA₂ enzymic activity can be indirectly estimated by a [³H]AA-release assay [11]. When [³H]AA-prelabelled cells were incubated for 15 min in the presence of TGF- α and A23187, a synergistic stimulation of [³H]AA release was observed (Table 1), indicating that they stimulated PLA₂ enzymic activity.

Stimulation of PLA₂ enzymic activity by TGF- α and A23187 is independent of PKC activation

We have reported previously that, in TEA3A1 cells, PMA at

Table 3 TGF- α - and A23187-mediated stimulation of PGE₂ production in control and PKC-down-modulated cells

Synergistic stimulation of PGE₂ production by TGF- α and A23187 was examined in control and PKC-down-modulated cells. Experimental cells were prepared as previously described. PKC-down-modulation was carried out as described in the Materials and methods section. Both control and PKC-down-modulated cells were cultured in 1 ml of DME containing 1 μ g/ml PMA, 1 μ g/ml A23187, 50 ng/ml TGF- α or 1 μ g/ml A23187 + 50 ng/ml TGF- α for 15 min. Media were harvested and the levels of PGE₂ production were determined by r.i.a. Values are means $\pm$ S.D. ($n = 3$).

Treatment	Cells ...	PGE ₂ (ng/ml)	
		Control	PKC-down-modulated
Control		0.10 $\pm$ 0.03	0.10 $\pm$ 0.01
PMA		1.62 $\pm$ 0.12	0.09 $\pm$ 0.01
A23187		1.96 $\pm$ 0.17	1.92 $\pm$ 0.08
TGF- α		0.13 $\pm$ 0.01	0.09 $\pm$ 0.00
TGF- α + A23187		5.06 $\pm$ 0.18	5.66 $\pm$ 0.37

concentrations above 10 ng/ml stimulates PLA₂ enzymic activity through activation of PKC [10]. Therefore, experiments were carried out to examine whether synergistic stimulation by A23187 of PGE₂ production can be observed in PKC-activated TEA3A1 cells. Cells were treated with 1 μ g/ml PMA and A23187 for 15 min. As shown in Table 2(a), when PMA and A23187 were added individually, the production of PGE₂ was stimulated by 30-fold and 20-fold respectively. However, when they were added together, a stimulation of only 40-fold was observed, indicating a less than additive effect. Alternatively, since treatment of cells with A23187 leads to an influx of Ca²⁺ and subsequent activation of PKC [12], experiments were carried out to examine whether the treatment of cells with TGF- α in the presence of PMA resulted in a synergistic stimulation of PGE₂ production. As shown in Table 2(b), synergistic stimulation of PGE₂ production was not observed when cells were treated with 50 ng/ml TGF- α in the presence of 1 μ g/ml PMA. Moreover, in order to show that PKC activation was not involved in the TGF- α - and A23187-mediated stimulation of PGE₂ production, experiments were carried out to examine whether the synergistic stimulation of PGE₂ production by TGF- α and A23187 could occur in PKC-down-modulated cells. TEA3A1 cells were treated with 500 ng/ml PMA for 24 h to down-modulate PKC as described previously [10]. After extensive washings to remove excess PMA, cells were treated with 1 μ g/ml PMA, 1 μ g/ml A23187, 50 ng/ml TGF- α , or 50 ng/ml TGF- α in the presence of 1 μ g/ml A23187. As shown in Table 3, in PKC-down-modulated cells, PMA-mediated stimulation of PGE₂ production was completely blocked, whereas stimulation of PGE₂ production by A23187 alone or in the presence of TGF- α remained unaffected.

Effect of TGF- α on PGE₂ production requires the presence of Ca²⁺ in the medium

It is clear from the above studies that, in order for TGF- α to stimulate AA metabolism, Ca²⁺ ionophore must be added to the culture of TEA3A1 cells. This indicates that Ca²⁺ is required for the activation of PLA₂ by TGF- α . Additional evidence comes from experiments carried out to examine the prostaglandin production in cells incubated in Ca²⁺-free medium or in medium containing 1.4 mM Ca²⁺. When cells were incubated for 15 min with A23187 and TGF- α in a Ca²⁺-free medium, stimulation of

Table 4 TGF- α - and A23187-mediated stimulation of PGE₂ production is dependent on extracellular Ca²⁺ concentration

Production of PGE₂ in response to TGF- α and A23187 in cells cultured in Ca²⁺-free medium and medium containing 1.4 mM Ca²⁺ was examined. Confluent cells were washed three times with Ca²⁺-free WAJC404 medium and incubated in 1 ml of either Ca²⁺-free or 1.4 mM Ca²⁺-containing WAJC404 medium for 15 min at 37 °C in the presence of either 1 μ g/ml A23187 or 1 μ g/ml A23187 plus 50 ng/ml TGF- α . At the end of the incubation period, media were harvested and the levels of PGE₂ produced by the cells were determined by r.i.a. Values are means $\pm$ S.D. ($n = 3$).

Medium ...	PGE ₂ (ng/ml)	
	Ca ²⁺ -free	With 1.4 mM Ca ²⁺
A23187	0.37 $\pm$ 0.07	5.18 $\pm$ 0.20
TGF- α + A23187	0.54 $\pm$ 0.02	10.17 $\pm$ 1.53

PGE₂ production was not observed (Table 4). When Ca²⁺ (1.4 mM) was added back to the Ca²⁺-free medium, A23187-mediated stimulation as well as A23187- and TGF- α -mediated synergistic stimulation of PGE₂ production was restored (Table 4). Thus the presence of Ca²⁺ in the culture medium is essential for A23187-mediated as well as A23187- and TGF- α -mediated stimulation of PGE₂ production.

Effect of thapsigargin on TGF- α mediated stimulation of PGE₂ production

The data presented above suggest that the stimulation of PGE₂ production by TGF- α requires Ca²⁺ influx. To examine this point further, experiments were also carried out to examine whether the stimulation of intracellular Ca²⁺ discharge could stimulate PGE₂ production in a synergistic manner with TGF- α . For this purpose, PKC-down-modulated TEA3A1 cells were treated with thapsigargin in order to stimulate the intracellular Ca²⁺ discharge in the absence or presence of TGF- α . PKC-down-modulated cells were used in this experiment because thapsigargin is known to stimulate PKC-mediated pathways. We have previously reported that the activation of PKC leads to the stimulation of PGE₂ production [10]. In PKC-down-modulated cells, an increase in PGE₂ production by thapsigargin treatment does not take place. Moreover, we have shown that synergistic stimulation of PGE₂ production by TGF- α and A23187 is not affected in PKC-down-modulated cells. Thus, if intracellular Ca²⁺ discharge in the presence of TGF- α results in a synergistic stimulation of PGE₂ production, it should be readily observed in PKC-down-modulated cells. We have found that the treatment of cells with up to 100 nM thapsigargin had no effect on PGE₂ production in the absence or presence of TGF- α . A higher dose of thapsigargin (200 nM) stimulated PGE₂ production by the cells; however, synergism was not observed in the presence of TGF- α (results not shown).

Activation of PLA₂ occurs with TGF- α , but not with FGF-I and IGF-I, in the presence of A23187

TGF- α is considered to exert its action through binding to the epidermal growth factor (EGF) receptor [13]. The EGF receptor, like receptors for FGFs and IGF-I, have intrinsic tyrosine kinase activity [14]. In order to examine whether the activation of any type of receptor tyrosine kinase with concomitant influx of Ca²⁺ may lead to the synergistic activation of PGE₂ production, cells

were treated with FGF-I and IGF-I in the presence of A23187. TEA3A1 cells have receptors for these growth factors. Both FGF-I and IGF-I stimulate growth of TEA3A1 cells; however, neither FGF-I nor IGF-I had an effect on the stimulation of PGE₂ production (results not shown). Moreover, although synergistic stimulation of PGE₂ production was observed with TGF- α and A23187, it was not observed with FGF-I and IGF-I in the presence of A23187 (results not shown).

DISCUSSION

It is known that EGF stimulates prostaglandin synthesis in many types of cells. The EGF receptor initiates a variety of immediate responses that activate distinct signal-transduction pathways upon ligand binding. These responses include, but are not limited to, transient increase in intracellular Ca²⁺ [15], possibly through inositol trisphosphate (InsP₃) formation by activation of phospholipase C γ [16], Ca²⁺ influx [17], and AA release through PLA₂ activation [18]. Since some of these responses are related and lead to the activation of PKC, and since PKC is known to activate PLA₂ in several experimental systems, several studies have been carried out to examine whether PKC is involved in the activation of PLA₂ by EGF [15]. Previously, we have described the presence of EGF receptors expressed on TEA3A1 thymic epithelial cells [19]. TGF- α shares sequence similarity with EGF and is known to exert its biological activity through binding to EGF receptor [14]. We have found that thymic epithelial cells are expressing TGF- α mRNA and producing TGF- α (results not shown). Since prostaglandin production is an important physiological function of thymic epithelial cells, the elucidation of the role of TGF- α in AA metabolism of thymic epithelial cells is of importance. In the present study, using intact TEA3A1 cells in culture, we were able to show that TGF- α stimulates PLA₂ enzymic activity only under an experimental condition where A23187 was added concomitantly. Treatment of cells with TGF- α alone (doses between 0.5 and 200 ng/ml, incubation time between 5 and 60 min) had no effect on PGE₂ production. Our results indicate that influx of Ca²⁺ is required for the activation of PLA₂ by TGF- α . The fact that stimulation of PGE₂ production by TGF- α and A23187 was dependent on the presence of Ca²⁺ in the culture medium support the idea that influx of Ca²⁺ is required for the activation of PLA₂. On the other hand, our results indicate that the transient as well as the sustained increase in cytosolic Ca²⁺ by InsP₃ is not sufficient to activate PLA₂ enzymic activity. The fact that a synergistic stimulation of PGE₂ production did not take place in PKC-down-modulated cells treated with TGF- α and thapsigargin supports this idea. Thapsigargin is known to increase cytosolic Ca²⁺ levels by inhibiting Ca²⁺-ATPase of endoplasmic reticulum without affecting plasma-membrane Ca²⁺-ATPase [20]. Thus, thapsigargin is used to modulate the cytosolic Ca²⁺ levels targeted by the agonist-induced InsP₃ [21]. Results from our experiments with thapsigargin indicate that PLA₂ cannot be activated by TGF- α in cells where a rise in cytosolic Ca²⁺ is sustained. At present, in TEA3A1 cells we do not know if the activation of EGF receptor leads to InsP₃ generation, as shown in other cell lines [16,22]. However, even if that is the case, since TGF- α and FGF-I had no effect on PGE₂ production when added alone, a transient mobilization of Ca²⁺ by InsP₃ is not sufficient for the activation of PLA₂ by TGF- α . Nevertheless, it is still possible that a sustained rise in cytosolic Ca²⁺ above a certain threshold may be necessary in this activation process.

Results from experiments using PKC-down-modulated cells, together with the fact that synergy was not observed in cells treated with either PMA plus A23187 or PMA plus TGF- α ,

strongly suggest that the activation of PKC is not required in this process. Moreover, the fact that genistein could completely block this effect (results not shown) indicates that tyrosine kinase of the EGF receptor plays an important role in the activation of PLA₂. Although there are many receptor tyrosine kinases, the activation of PLA₂ through a tyrosine-phosphorylation process appears to be limited to the action of the EGF receptor. Tyrosine kinases of other growth-factor receptors that are present in TEA3A1 cells are not capable of activating PLA₂ enzymic activity even in the presence of A23187. This observation may indicate that the subcellular distribution of this particular PLA₂ is limited to a specific location in the cell, possibly at the plasma membrane closely associated with EGF receptors. Taking these two observations together, the activation process of this particular PLA₂ enzymic activity requires a tyrosine-phosphorylation step by EGF receptor tyrosine kinase with concomitant influx of Ca²⁺. We do not know at present if the Ca²⁺ ionophore A23187 is mimicking the function of a physiological factor. Experiments are being carried out to identify the natural agonist that is responsible for stimulating Ca²⁺ influx and PGE₂ production.

Our observation is in agreement with a previous study by Goldberg et al. [11] showing that PLA₂ activation did not involve a PKC-mediated pathway and did not take place in transfected NIH 3T3 cells expressing a mutant (tyrosine kinase-defective) EGF receptor. However, our observations differ slightly from studies by Margolis et al. [23] using glomerular mesangial cells. In glomerular mesangial cells, EGF was shown to enhance PLA₂ activity in vasopressin-treated cells. Vasopressin alone can stimulate PLA₂ enzymic activity and PGE₂ production in these cells via activation of PKC. Thus, synergy was observed in these cells when EGF was added to vasopressin- or PMA-treated cells. The activation of PLA₂ independently of the phospholipase-C/PKC pathway has been reported in a number of experimental systems [12,24] and is not unique to the EGF receptor. Bradykinin-mediated activation of PLA₂ in pig aorta endothelial cells [25], in transformed BALB/3T3 cells [26] and in MDCK cells [27] are a few examples.

This study, together with our previous study which described the existence of PKC-mediated pathway leading to the activation of PLA₂ enzymic activity in TEA3A1 cells [10], indicates the existence of two distinct activation pathways for PLA₂ in TEA3A1 cells. However, it is not clear at present whether there are two distinct PLA₂s that are regulated differently or whether there is only one type of PLA₂ that is regulated by two separate

pathways. It is tempting to think that, although the activation of PLA₂ by both pathways leads to an increase in PGE₂ production, each pathway may lead to a distinct biological effect in TEA3A1 cells.

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