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Differentiation of U937 Cells Enables a Phospholipase D-Dependent Pathway of Cytosolic Phospholipase A_2 Activation

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Treatment with dibutyryl cyclic AMP (dBcAMP) of the human, premonocytic U937 cell line results in differentiation toward a monocyte/granulocyte-like cell. This differentiation enables the cell to activate cytosolic phospholipase A_2 ($cPLA_2$) to release arachidonate upon stimulation. In contrast, undifferentiated cells are unable to release arachidonate even when stimulated with calcium ionophores. In the present research, a role for phospholipase D (PLD) in the regulation of $cPLA_2$ was shown based on a number of observations. First, the ionomycin- and fMLP-stimulated production of arachidonate in differentiated cells was sensitive to ethanol (2% (v/v)). Ethanol acts as an alternate substrate in place of water for PLD producing phosphatidylethanol (PEt) instead of phosphatidic acid. Indeed, ionomycin stimulation of differentiated cells produced a 14-fold increase in PEt levels. Further evidence for the involvement of PLD in the regulation of $cPLA_2$ came from the observation that the stimulated production of diacylglycerol (for which phosphatidic acid is a major source) was greatly diminished in undifferentiated cells as compared to differentiated cells. Moreover, the normally deficient activation of $cPLA_2$ in undifferentiated cells could be stimulated to release arachidonate if the cells were electroporated in the presence of GTP[γ]S and MgATP. This treatment stimulates phosphatidylinositol-4,5-bisphosphate (PIP_2) production which appears to activate PLD and $cPLA_2$ in subsequent steps. The phosphatidic acid (and diacylglycerol derived from phosphatidic acid) appears to greatly regulate the action of $cPLA_2$ by an unknown mechanism, and undifferentiated cells lack the ability to stimulate PLD activity due to a dysfunction of PIP_2 production.

The human, premonocytic U937 cell line can be differentiated toward a monocyte/granulocyte-like cell using a number of different agents including dibutyryl cyclic AMP (dBcAMP), DMSO, vitamin D3 analogs, and phorbol esters (for a review, see Ref. 1). Upon differentiation, these cells acquire the ability to release arachidonate in response to stimuli such as the *N*-formylated bacterial peptide fMLP as well as calcium ionophores [2, 3, 4, 5].

The hydrolysis of the *sn*-2 ester of arachidonoyl-containing phospholipids to produce arachidonate is catalyzed by a phospholipase A_2. While several different types of phospholipases A_2 have been identified, the group IV, or cytosolic, phospholipase A_2 ($cPLA_2$) is responsible for the production of the arachidonate in differentiated U937 cells. This conclusion is based on the observations that these cells contain large amounts of the $cPLA_2$ with no measurable secreted phospholipase A_2 ($sPLA_2$); and that selective $cPLA_2$ inhibitors, including an anti-$cPLA_2$ antibody, are able to block the stimulated release of arachidonate [3, 5, 6].

$cPLA_2$ is a calcium-dependent enzyme which is present in a number of different tissues and cells including monocytes, neutrophils, and platelets [7, 8, 9]. Unlike the $sPLA_2$, this enzyme has been shown to be selective for arachidonate-containing phospholipids [10, 11]. The $cPLA_2$ is normally located in the cytosol, but translocates to the membrane in response to sub-

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Abbreviations used: ARF, ADP-ribosylation factor; $cPLA_2$, cytosolic phospholipase A_2; dBcAMP, dibutyryl cyclic AMP; DAG, diacylglycerol; DMSO, dimethylsulfoxide; EtOH, ethanol; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; FPR, fMLP receptor; GTP[γ]S, guanosine 5′-O-(3-thiotriphosphate); HBSS, Hanks' buffered salt solution; $sPLA_2$, secreted phospholipase A_2; HSA, human serum albumin; IP_3, inositol 1,4,5-trisphosphate; MgATP, magnesium salt of adenosine 5′-triphosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PEt, phosphatidylethanol; $sPLA_2$, secreted phospholipase A_2.

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micromolar concentrations of calcium [12, 13]. Some evidence indicates that both phosphorylation of $cPLA_2$ at serine-505, which may be catalyzed by a MAP kinase [14, 15, 16], and an increase in the cytosolic calcium concentration are required to activate the enzyme to produce arachidonate [14, 15, 17]. Both PKC-dependent and PKC-independent pathways have been implicated in the receptor-mediated regulation of $cPLA_2$ [18, 19, 20]. In addition, it has been proposed that receptor-induced stimulation of $cPLA_2$ to produce arachidonate is dependent on the activation of PLC. The products of PLC, namely inositol trisphosphate and diacylglycerol, would then be expected to increase cytosolic calcium levels and activate the PKC- and/or MAP kinase-dependent phosphorylation of $cPLA_2$ (for a review, see Ref. 21).

However, it has recently been shown that phosphorylation of $cPLA_2$ and an influx of intracellular calcium are not sufficient to activate the enzyme to produce arachidonate in U937 cells [5]. Indeed, while undifferentiated U937 cells have large amounts of phosphorylated $cPLA_2$ and can be stimulated to induce a calcium influx and PLC activation, the cells are unable to produce arachidonate. There is some unrecognized mechanism in differentiated U937 cells as compared to undifferentiated cells which enables the $cPLA_2$ to function catalytically. This mechanism may be related to the observation that a pertussis toxin-sensitive G-protein of the $G_i\alpha$ class is required for activation of arachidonate production by $cPLA_2$ in a manner which is independent of adenylyl cyclase inhibition, intracellular calcium influx, and phosphorylation of $cPLA_2$ [5].

In this paper, we provide evidence that the differentiation of U937 cells allows for phospholipase D (PLD) to be maximally activated. The phosphatidic acid product of PLD is then converted into diacylglycerol, and the fMLP- or ionomycin-stimulated production of either phosphatidic acid or diacylglycerol potentiates the activity of $cPLA_2$ in differentiated cells. Inhibition of phosphatidic acid (and diacylglycerol) production attenuates the production of arachidonate catalyzed by $cPLA_2$.

EXPERIMENTAL PROCEDURES

Materials. Ionomycin (calcium salt) and fMLP were obtained from Calbiochem and Sigma, respectively. U937 cells were obtained from American Type Culture Collection. U937 cells which were stably transfected to express the reombinant human FPR were obtained from Dr. Eric Prossnitz (Scripps Research Institute) [22].

Cell culture. U937 cells were maintained in suspension culture in RPMI 1640 medium containing L-glutamine supplemented with 10% fetal calf serum and 50 μg/mL gentamycin in a 5% CO_2 incubator at 37°C. For differentiation experiments, cells were resuspended in fresh culture medium and grown for 24 h prior to the addition of 1 mM dBcAMP [23, 24]. Differentiation in the presence of dBcAMP was allowed to proceed for 48 h.

Determination of arachidonate mass produced in U937 cells. Cells were washed once in HBSS (without calcium or magnesium) and pelleted at 4°C. After resuspending at a concentration of 1×10^7 cells/ml in HBSS containing calcium, magnesium, and 0.2% HSA, the cells were incubated for five minutes at 37°C, and stimulated by the addition of either fMLP or ionomycin by adding 10 μL of DMSO solutions at the appropriate stimulator concentration to 1 mL of cells to give fMLP or ionomycin concentrations of 100 nM or 7 μM, respectively. Unless otherwise noted, stimulation was allowed to proceed for 5 mins before termination. Reactions were terminated in an ice bath and heneicosanoic acid (21:0) was added to the cells as an internal standard. In those experiments where the effect of EtOH was measured, a concentration of 2% (v/v) was used unless noted, and added 5 mins before stimulation of the cells. Extraction, derivatization, and GC-EC detection of the arachidonate produced was performed as previously described [5].

Measurement of diacylglycerol (DAG) levels in cells. Cells were stimulated as described above and the reactions (1 mL) were quenched by the addition of 3 mL of $CHCl_3$/methanol (1:2). After Bligh-Dyer extraction [25], the diacylglycerol (DAG) levels were measured using the DAG assay system from Amersham (RPN 200) in which DAG is converted into [^{33}P]phosphatidate for quantitation.

Phosphatidylethanol measurements. Using the procedure of Anthes *et al.* [26], the radiolabeled production of PEt was monitored by thin-layer chromatography. The data is represented as the percent of the total radioactivity that comigrates with PEt.

Electroporation of cells. For those experiments where the effect of GTP[g]S (100 μM) and MgATP (2 mM) in electropermeabilized cells was measured, the electroporation was carried out as described previously [5]. After electroporation, the cells were allowed to recover for 30 mins before stimulation with ionomycin.

RESULTS AND DISCUSSION

Inhibition of Stimulated Arachidonate Production in dBcAMP-Differentiated U937 Cells by Ethanol

Recent work has indicated that PLD may be involved in regulating the activity of $cPLA_2$ [27]. The phosphatidic acid product of PLD can be converted into diacylglycerol by the action of phosphatidate phosphorylase [26]. In fact, the production of diacylglycerol in rat mast cells and human neutrophils has been shown to result primarily from this pathway rather than by direct formation via PLC [28, 29, 30, 31]. In order to first determine whether PLD may be regulating the activation of $cPLA_2$ in U937 cells differentiated with dBcAMP, the cells were stimulated in both the presence and the absence of ethanol. PLD preferentially uses ethanol as a substrate in place of water to produce phosphatidylethanol (PEt) instead of phosphatidic acid [32]. Unlike phosphatidic acid, however, PEt cannot be used by phosphatidate phosphorylase to form diacylglycerol. As shown in Fig. 1, EtOH inhibited, albeit weakly, the fMLP-stimulated production of arachidonate (40% inhibition with 2% EtOH). This would suggest that PLD-dependent production of phosphatidic acid plays a role in regulating the fMLP-stimulated activation of $cPLA_2$. Ethanol was more effective at inhibiting the production of arachidonate stimulated by ionomycin treatment (70% inhibition with 2% EtOH).

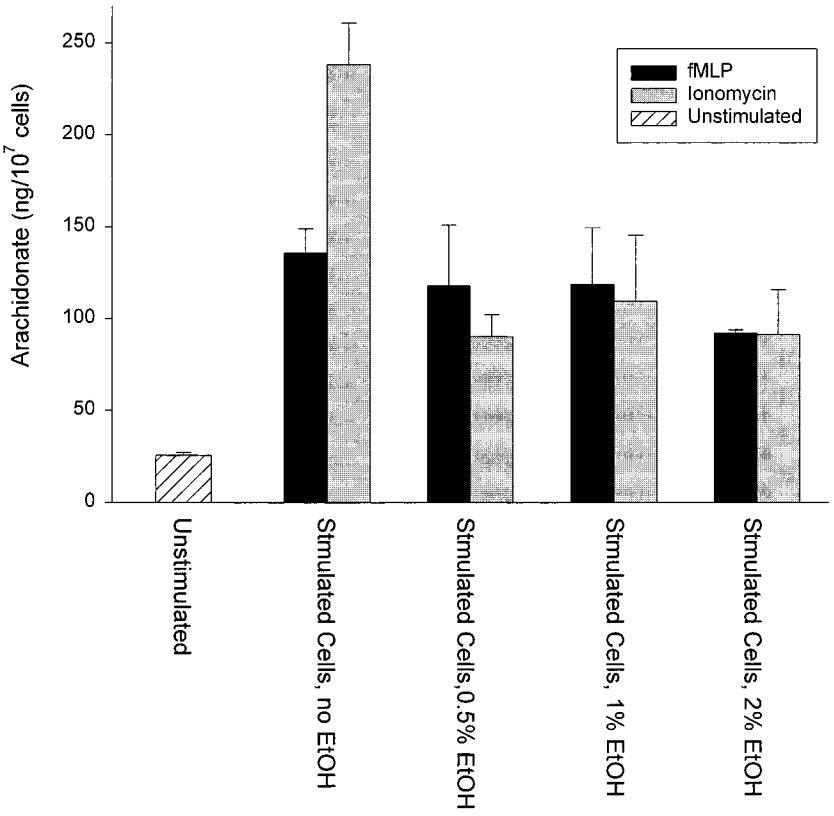

FIG. 1. Effect of ethanol on the stimulated production of arachidonate by either fMLP or ionomycin in dBcAMP-differentiated U937 cells.

Interestingly, ethanol (2%) inhibited the arachidonate production induced by both fMLP and ionomycion to the same absolute level (~65 ng/10^7 cells over unstimulated level). This indicates that the added levels of arachidonate normally stimulated by ionomycin over the levels measured upon fMLP stimulation are dependent on the activation of PLD, and that a small amount (~65 ng/10^7 cells) is independent of PLD.

PEt and DAG Measurements as a Measure of PLD and PLC Activation

Further evidence of selective activation of PLD by ionomycin in differentiated cells is detailed in Fig. 2. Here, stimulated production of PEt (in the presence of EtOH) was observed with ionomycin. It has previously been reported that fMLP stimulates PEt production in these cells [26].

Because diacylglycerol may result from either the direct action of PLC or from phosphatidate phosphorylase acting on phosphatidic acid liberated by the action of PLD, a direct measurement of the diacylglycerol produced in cells was made. Only ionomycin was able to stimulate, albeit modestly, the production of DAG in undifferentiated cells (see Fig. 3). This probably results from the direct action of PLC since the production of inositol 1,4,5-trisphosphate (IP_3), the other product of PLC, was shown to occur upon ionomycin stimulation in undifferentiated cells [5]. It is not surprising that

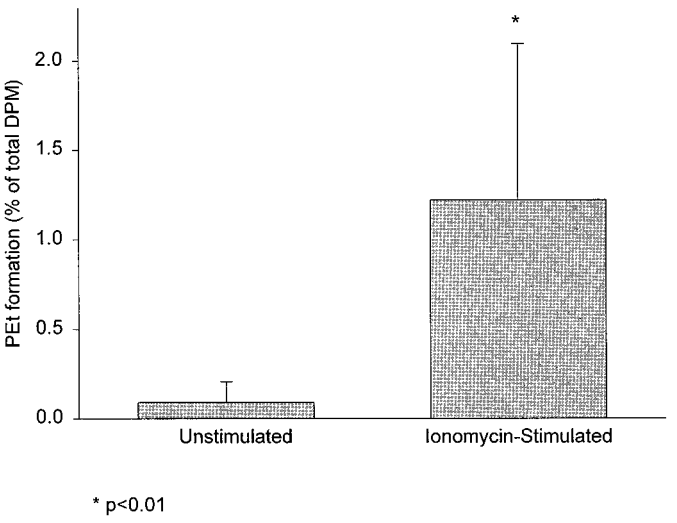

FIG. 2. Effect of ionomycin stimulation on the amount of phosphatidylethanol (PEt) in dBcAMP-differentiated U937 cells stimulated in the presence of ethanol (2% (v/v)).

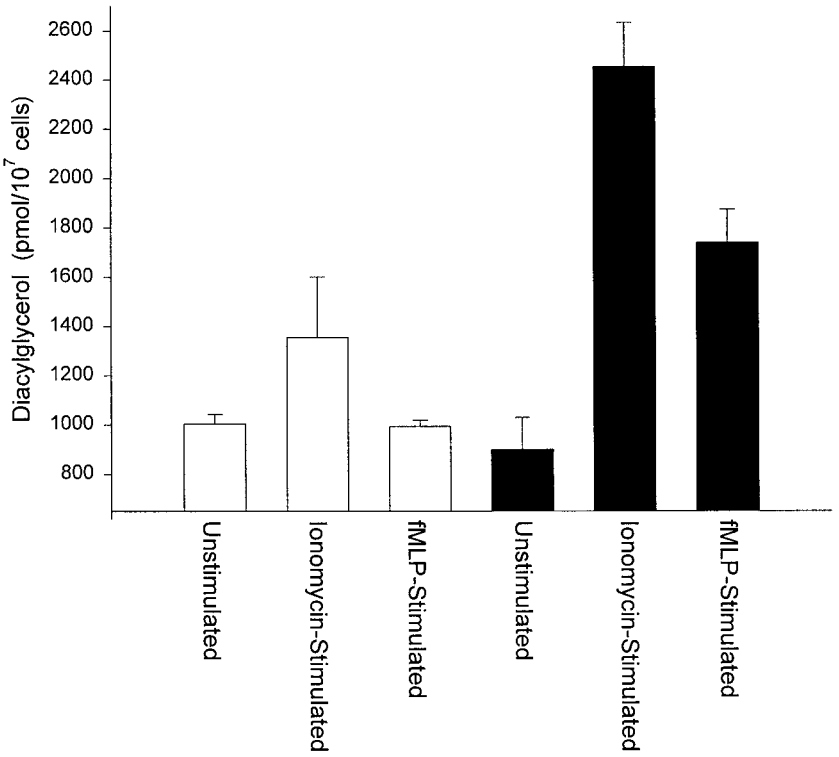

FIG. 3. Production of diacylglycerol in U937 cells stimulated with ionomycin or fMLP. *Open bars*, undifferentiated cells; *closed bars*, dBcAMP-differentiated cells.

fMLP failed to induce diacylglycerol production in undifferentiated cells since these cells do not express the receptor for fMLP and do not produce IP_3 when stimulated with fMLP [5, 22].

The results with differentiated cells, however, were very different. As shown in Fig. 3, both fMLP and ionomycin stimulated the production of diacylglycerol, with ionomycin stimulating diacylglycerol production nearly twice as effectively as that observed with fMLP stimulation. In a manner completely analogous to the effect of ethanol on arachidonate production, Fig. 4 shows that EtOH inhibited the production of diacylglycerol in both ionomycin- and fMLP-stimulated cells to the same absolute level (~450 pmol/10^7 cells over unstimulated levels). These results indicate that treatment with either fMLP or ionomycin stimulates a small amount of diacylglycerol production in differentiated cells via the action of PLC (~450 pmol/10^7 cells over unstimulated, not EtOH-sensitive). Indeed, both stimuli induced comparable amounts of IP_3 production in differentiated U937 cells [5]. Both stimuli also induce activation of PLD (EtOH-sensitive) to produce diacylglycerol, with ionomycin being more effective at doing so. This PLD activation appears to result in greater stimulation of $cPLA_2$ to release arachidonate. The degree of arachidonate production correlates well with diacylglycerol levels in differentiated cells.

Stimulation of Diacylglycerol and Arachidonate Production in FPR-Transfected, Undifferentiated Cells

We had previously shown that undifferentiated cells could be stimulated to release arachidonate if activation of a heterotrimeric G protein occurred [5]. Since this G protein appears to be of the $G_i\alpha$-type (probably $G_i2\alpha$), *undifferentiated* cells into which the fMLP receptor (FPR) had been transfected were used in order to investigate the role of the receptor versus cell differentiation on $cPLA_2$ regulation. The FPR has been shown to be a $G_i2\alpha$-coupled receptor [33, 34, 35]. Moreover, differentiated U937 cells express the FPR where undifferentiated cells do not [24].

As shown in Fig. 5, FPR transfection of *undifferentiated* U937 cells results in an ability of the cells to produce a small amount of arachidonate upon stimulation with either fMLP or ionomycin. Non-transfected cells are completely ineffective at producing arachidonate [5]. While it is not surprising that fMLP is now able to induce at least a small response in these cells since they now express the receptor, it is somewhat surprising that the cells are now sensitive to ionomycin which does not act through a receptor. This may suggest that a functional receptor is important in providing the proper architecture for a $G_i\alpha$-containing G pro-

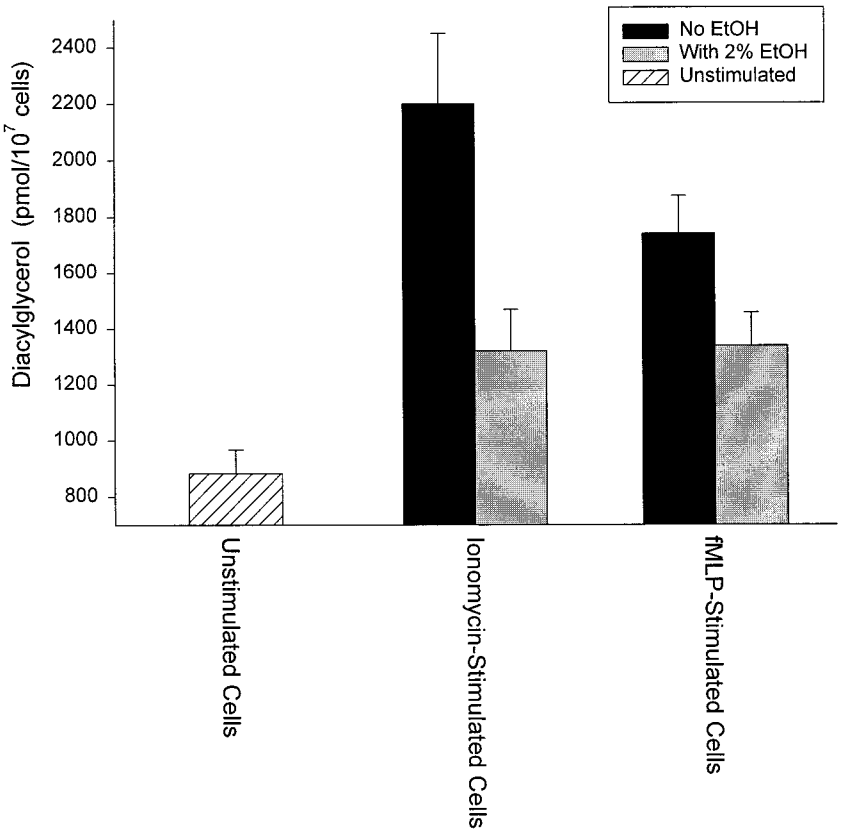

FIG. 4. Effect of ethanol (2% (v/v)) on the fMLP- and ionomycin-stimulated production of diacylglycerol in dBcAMP-differentiated U937 cells.

tein to function properly. This conclusion is also consistent with published results showing the activation of $cPLA_2$ in undifferentiated cells with mastoparan, a cationic peptide which selectively binds to and activates the receptor-recognition site of pertussis toxin-sensitive α-subunits of the G_i and G_o G-proteins [5].

Still, the amount of arachidonate produced by these FPR-transfected cells was far lower than that observed

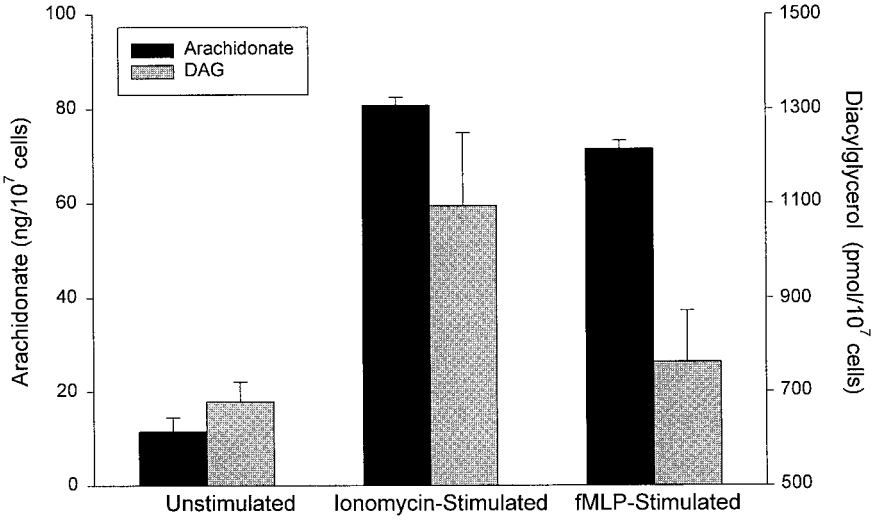

FIG. 5. Effect of ionomycin and fMLP stimulation on arachidonate and diacylglycerol production in FPR-transfected, undifferentiated U937 cells.

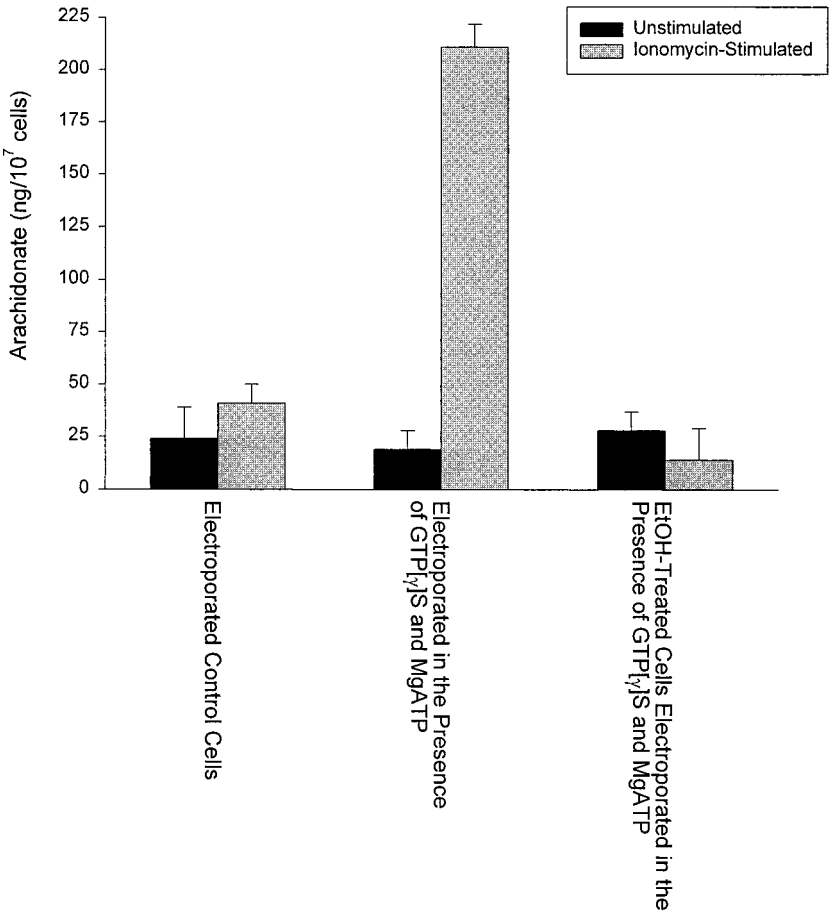

FIG. 6. Production of arachidonate by undifferentiated U937 cells electroporated in the presence of GTP[γ]S and MgATP.

in normal *differentiated* cells even though the FPR-transfected cells have somewhat greater levels of fMLP receptors expressed on the cell surface than do differentiated cells,[2] and a calcium influx can now be triggered with fMLP [5, 22]. Consistent with the role of diacylglycerol in the activation of $cPLA_2$, Fig. 5 shows that the FPR-transfected cells produced little diacylglycerol. In fact, the levels were no greater than in nontransfected, undifferentiated cells stimulated with ionomycin which probably only reflects PLC activation (see Fig. 3). The presence of the fMLP receptor alone is apparently not sufficient for PLD activation by either ionomycin or fMLP. The differentiation of these cells appears to be necessary to allow activation of PLD which in turn regulates $cPLA_2$. While differentiation of myeloid cell lines has been shown to increase PLD activity in cells, undifferentiated U937 cells still possess considerable PLD activity [36, 37].

Activation of PLD has been shown to occur upon stimulation with GTP[γ]S in many cells, including U937 cells [37, 38, 39]. Consistent with the role of PLD in the regulation of $cPLA_2$ in U937 cells, a small amount of arachidonate release in undifferentiated U937 cells has been accomplished by stimulating cells that had been electroporated in the presence of GTP[γ]S [5]. The fact that GTP[γ]S stimulates PLD and $cPLA_2$ indicates that the mechanism involves either a small GTP binding protein or a heterotrimeric G protein. Both types of G proteins have been shown to regulate the action of PLD [40, 41, 42].

While it is unknown what mechanism or component is lacking in undifferentiated cells as compared to differentiated cells which prevents activation of PLD (and of $cPLA_2$), recent publications suggest possible areas to explore. For instance, ADP-ridosylation factor (ARF) has been shown to regulate PLD by a direct interaction between the two proteins [42, 43], and is regulated by a heterotrimeric G protein of the G_i2/G_i3 class [44]. Interestingly, streptolysin-*O*-permeabilization of differentiated U937 cells and mast cells, which resulted in the cells losing the ability to produce arachidonate

[2] Unpublished results.

[45],[2] has also been reported to cause the loss of cytosolic ARF which then prevents PLD activation [44].

Another mechanism which may regulate PLD in U937 cells involves phosphatidylinositol-4,5-bisphosphate (PIP_2), which is produced by the actions of phosphatidylinositol 4-kinase and phosphatidylinositol-4-phosphate 5-kinase, both of which are ATP-dependent. PIP_2 greatly enhances the activity of PLD and has been shown to be critically important in PLD regulation [for a review see Ref. 46]. Indeed, PIP_2 activation in undifferentiated cells has been shown to be deficient, and synergistic activation of PIP_2 production in undifferentiated U937 cells can be induced by treating electropermeabilized cells with a combination of GTP[γ]S and MgATP [37]. This treatment also greatly stimulated PLD activity [37]. Consistent with the idea that PLD regulates the action of $cPLA_2$, Fig. 6 shows that electroporating undifferentiated U937 cells in the presence of a combination of GTP[γ]S and MgATP allows for the cells to be stimulated to produce arachidonate. Interestingly, the levels of arachidonate are equivalent to those achieved upon ionomycin stimulation of *differentiated* cells ($\sim$200 ng/mL over unstimulated levels), and were completely sensitive to ethanol treatment. These results support the conclusion that the stimulated production of PIP_2, normally dysfunctional in undifferentiated cells, regulates both PLD and $cPLA_2$ sequentially.

While the present research provides a mechanistic explanation as to what changes occur in U937 cells upon differentiation that allows $cPLA_2$ to function properly upon stimulation, there are still many aspects about PLD and $cPLA_2$ regulation that remain unclear. For instance, the mechanism by which differentiation allows the stimulated production PIP_2 (and subsequent PLD activation) in differentiated cells is not known. In addition, PLC is activated in undifferentiated cells to produce some diacylglycerol, but no $cPLA_2$ activation occurs even though PLC stimulation in *differentiated* cells appears to activate $cPLA_2$ to a small extent. Lastly, it is not clear how the products of PLD regulate $cPLA_2$ activity. Diacylglycerol may be acting through protein kinase C (PKC) to stimulate the signal transduction pathway leading to $cPLA_2$ activation [for a review, see Ref. 21]. Since it has been shown that ionomycin does not induce the phosphorylation of $cPLA_2$ in U937 cells [5], any PKC-dependent effects would have to be mediated through another, heretofore unrecognized factor which regulates $cPLA_2$. Indeed, it has been shown that some other mechanism, in addition to enzyme phosphorylation and an intracellular calcium influx, regulates the activation of $cPLA_2$ which is separate from enzyme phosphorylation [16, 47]. It may be that diacylglycerol directly affects the activity of $cPLA_2$ [48, 49]. Lastly, the phosphatidic acid product of PLD, rather than diacylglycerol resulting from phosphatidic acid, has been suggested to influence the activity of $cPLA_2$ [27, 42]. Work in our laboratory is ongoing in an effort to clarify these issues.

In conclusion, the diacylglycerol-dependent activation of $cPLA_2$ in differentiated U937 cells appears to be primarily regulated by both PLC and PLD, with PLD playing a larger role when stimulated with ionomycin. Activation of PLD in undifferentiated cells appears to be compromised due to the inability of the cells to generate PIP_2, unless electroporated in the presence of GTP[γ]S and MgATP. Because of the lack of activation of PIP_2 production in undifferentiated cells (and subsequent stimulation of PLD and $cPLA_2$), the U937 cell line represents an unusual opportunity to study the regulation of these enzymes.

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