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# The Calcium Signal for Balb/MK Keratinocyte Terminal Differentiation Induces Sustained Alterations in Phosphoinositide Metabolism without Detectable Protein Kinase C Activation*

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**Balb/MK keratinocytes require epidermal growth factor for proliferation and terminally differentiate in response to elevated extracellular $Ca^{2+}$ concentrations. The molecular pathways controlling cell differentiation in this system have yet to be established. We show that a dramatic and sustained activation of phosphoinositide metabolism is produced upon addition of $Ca^{2+}$ to Balb/MK cultures. The pattern of inositol trisphosphate isomers released in response to $Ca^{2+}$ challenge appeared to be atypical. Inositol 1,3,4-trisphosphate release was observed by 30 s and was produced earlier than any alteration in inositol 1,4,5-trisphosphate levels. Concomitant with the liberation of inositol phosphates, an increased production of diacylglycerol was observed. Despite a 3-fold increase in diacylglycerol levels detected even at 12 h after $Ca^{2+}$ addition, no evidence of functional activation or down-regulation of protein kinase C was found. This was established by measuring p80 phosphorylation, epidermal growth factor binding, and protein kinase C levels by immunoblotting. Analysis of the diacylglycerol generated following $Ca^{2+}$ addition to Balb/MK cells revealed that a significant proportion of that lipid was an alkyl ether glyceride molecular species. Therefore, it is possible that this diacylglycerol molecular species may play a role in the $Ca^{2+}$-induced differentiation program of Balb/MK cells through mechanisms other than stimulation of classical protein kinase C.**

The study of normal epithelial cell growth and differentiation has been aided in recent years by the development of tissue culture systems for propagation of such cells. We have reported the development of a cloned mouse epidermal keratinocyte (Balb/MK) cell line, which requires epidermal growth factor (EGF)[1] for growth and terminally differentiates in response to extracellular $Ca^{2+}$ concentrations greater than 1 mM (1, 2). The molecular pathways controlling cell differentiation in this system have yet to be established.

Phosphoinositide (PI) metabolism is a ubiquitous transducing mechanism that has been proposed to be involved in the control of a number of cellular functions (3). For example, in the promyelocytic leukemia cell line HL-60, which can differentiate along either the granulocytic or monocytic pathway, phorbol myristate acetate (PMA) is a potent inducer of monocytic differentiation (4, 5). PMA is the pharmacological analogue of diacylglycerol (DAG), a product of phosphodiesteratic hydrolysis of inositol phospholipids and an important cofactor for protein kinase C (3, 6). Thus, experimental evidence suggests a possible role for inositol phospholipid metabolism in cell differentiation (7-9). Evidence that $Ca^{2+}$ is a potent activator of phospholipase C-mediated hydrolysis of inositol phospholipids (10) as well as the trigger of terminal differentiation in Balb/MK cells prompted us to investigate the possible role of PI turnover in the control of the differentiation program of these cells.

## MATERIALS AND METHODS

Balb/MK cells were grown on plastic culture dishes in low calcium (0.05 mM $Ca^{2+}$) containing Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal calf serum and 5 ng/ml EGF. In some experiments, cells were maintained in a chemically defined medium consisting of low calcium minimum Eagle's medium supplemented with transferrin (5 ng/ml), selenium (1 μM), insulin (10 μg/ml), and EGF (5 ng/ml) (2).

*Extraction and Analysis of Inositol Phosphates (InsPs)*—Cells grown to confluence in 30-mm culture dishes were incubated for 60 h in the presence of 15 μCi/ml *myo*-[$^3$H]inositol. After labeling, cultures were either washed twice with serum-free minimum Eagle's medium containing 5 μg/ml transferrin and selenium (1 μM) and incubated for 1 h in this medium or challenged immediately with $Ca^{2+}$. Lithium chloride (LiCl, 10 mM) was included 15 min prior to $Ca^{2+}$ (2 mM) additions. Reactions were stopped with trichloroacetic acid, and InsPs were extracted as described (11). In some experiments, total InsPs were separated from free [$^3$H]inositol and glycerophosphoinositol by using Dowex 1-X8 anion-exchange columns (11). For the separation of distinct species of InsPs, an HPLC method was followed. Samples were loaded in an Ultrasil-AX column (4.6 × 250 mm; Beckman) and eluted with an ammonium formate gradient similar to that described previously (11). Samples were coinjected with 20 μl of 1 mM ATP and 200 cpm of [$^{32}$P]Ins-1,4,5-$P_3$ as elution standards for the identification of the different $InsP_3$ isomers. InsP, Ins-1,4-$P_2$, Ins-1,4,5-$P_3$, Ins-1,3,4-$P_3$, and Ins-1,3,4,5-$P_4$ were identified by using appropriate standards.

*Analysis of Diacylglycerol Release*—Confluent cultures were incubated with 30 μCi/well [U-$^{14}$C]glycerol for 60 h. After this labeling period, cells were exposed to $Ca^{2+}$ (2 mM) for various times. Cellular lipids were extracted with 0.4 ml of methanol and then processed by a method published previously (12). Lipids were fractionated by TLC with a solvent system containing hexane/diethylether/acetic acid

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[1] The abbreviations used are: EGF, epidermal growth factor; PI, phosphoinositide; InsP(s), inositol phosphate(s); Ins-1,4-$P_2$, inositol 1,4-bisphosphate; Ins-1,4,5-$P_3$, inositol 1,4,5-trisphosphate; Ins-1,3,4-$P_3$, inositol 1,3,4-trisphosphate; Ins-1,3,4,5-$P_4$, inositol 1,3,4,5-tetrakisphosphate; PtdIns, phosphatidylinositol; PMA, phorbol myristate acetate; DAG, diacylglycerol; HPLC, high pressure liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.



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(40:60:1) (vol/vol/vol). DAG was visualized by exposing plates to iodine vapor and identified by using a mixture of 1-stearoyl-2-arachidonyl glycerol and 1,2-dipalmitoylglycerol. Spots were scraped off, and radioactivity was estimated by liquid scintillation counting. In some experiments, DAG from either unstimulated or $Ca^{2+}$-activated cells was treated for 15 min at room temperature with 2 N KOH in methanol. Afterwards, reaction products were extracted and fractionated by TLC as described above. This experimental protocol was followed to detect the presence of an alkyl ether glyceride molecular species.

*Estimation of the Release of Phosphorylcholine and Phosphorylethanolamine*—Cells grown to confluence in 30-mm culture dishes were incubated for 60 h in medium supplemented with either 10 μCi/ml [*methyl*-$^{14}C$]choline or [1,2-$^{14}C$]ethanolamine. After labeling, cultures were incubated in the presence of $Ca^{2+}$ (2 mM) for different times. Reactions were stopped by a method published previously (12). Water-soluble choline and ethanolamine metabolites were fractionated by thin layer chromatography with the following solvent system: methanol/NaCl (0.5%, w/v)/$NH_4OH$ (100:100:2, v/v/v). After autoradiography, spots corresponding to phosphorylcholine and phosphorylethanolamine, respectively, were identified by using purified standards. These were scraped off, and radioactivity was estimated by liquid scintillation counting.

*Estimation of Protein Kinase C Activation by Analysis of Endogenous 80-kDa Protein Phosphorylation*—Phosphorylation of endogenous proteins was performed by slight modification of the method described by Rodriguez-Peña and Rozengurt (13). Briefly, confluent cultures in 60-mm dishes were rinsed with Tris-buffered saline (0.15 M NaCl, 20 mM Tris-HCl, pH 7.4) and then incubated in low calcium minimum Eagle's medium (phosphate free) supplemented with 0.25 mCi/ml [$^{32}P$]orthophosphate (Du Pont-New England Nuclear, 9,000 Ci/mmol). After 4 h, $Ca^{2+}$ (2 mM), PMA (200 ng/ml), or both were added to the cells for different times. The reactions were stopped by removing the medium and rapidly rinsing cultures with ice-cold Tris-buffered saline. Cells were then extracted with 10% (w/v) trichloroacetic acid at 4 °C for 30 min. The acid-soluble material was discarded, and the precipitated proteins were rinsed twice with Tris-buffered saline and solubilized in 0.3 ml of lysis buffer consisting of 9.5 M urea, 2% (w/v) Nonidet P-40, 5% (v/v) β-mercaptoethanol, and 2% LKB (Pharmacia LKB Biotechnology Inc.) Ampholines (pH range, 3.5–10). The resulting lysates were stored at −70 °C until use. Before electrophoresis, samples were thawed and clarified by centrifugation (12,000 × *g*).

Two-dimensional polyacrylamide gel electrophoresis was performed essentially as described by O'Farrell (14) using isoelectric focusing in the first dimension followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide) in the second dimension. Isoelectric focusing gels contained 1.4% LKB ampholytes (pH 3.5–5) plus 0.6% LKB ampholytes (pH 3.5–10) to resolve highly acidic polypeptides. After electrophoresis, fixed gels were dried, and radiolabeled proteins were visualized by autoradiography using Kodak XAR-2 film exposed to the gels at −70 °C. Radioactivity in individual proteins was determined by cutting spots from dried gels, eluting radioactivity using Protosol (Du Pont-New England Nuclear), and counting in a liquid scintillation counter. Phosphorylation of the protein kinase C substrate (p80) was normalized by a ratio method in which the radioactive incorporation of the p80 spot was compared with reference phosphoproteins in the same gel. Phosphorylation of these internal reference proteins was found to be unaffected by treatment of the cells with either PMA or $Ca^{2+}$.

*Identification of Protein Kinase C by Immunoblotting*—Cell extracts containing 100 μg of total cell protein were obtained from confluent cultures of Balb/MK cells treated as described in the legend to Fig. 6. Following denaturation in SDS sample buffer, proteins were resolved in 10% SDS-polyacrylamide gels and then transferred electrophoretically onto a polyvinylidene difluoride membrane (Immobilon, Millipore Continental Water Systems, Bedford, MA).

To visualize protein kinase C, the membrane was first incubated for 2 h in 50 mM Tris-HCl (pH 7.5) containing 2% nonfat dry milk, 1% Triton X-100, and 10 mM EDTA (Blotto; ABI, Columbia, MD) to block nonspecific binding sites. The membrane was rinsed three times in the same solution and then incubated for 2 h at 24 °C with a monoclonal anti-protein kinase C antibody (clone MC5, Amersham Corp.) at a concentration of 1 μg/ml in Blotto. After several rinses, bound antibodies were detected by incubation of the membrane for 1 h in Blotto containing 2 × $10^5$ cpm/ml $^{125}I$-labeled protein A. The blot was rinsed and exposed to Kodak XAR-2 film for 5–7 days at −70 °C.

*$^{125}I$-EGF-binding Experiments*—Balb/MK cells were seeded in 12-well COSTAR dishes at a density of 2 × $10^5$ cells/well 2 days prior to the binding experiments. Cells were washed two times in phosphate-buffered saline and placed in 1 ml of binding buffer (118 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8.8 mM dextrose, 0.5% bovine serum albumin, 100 mM Hepes, pH 7.4) at 15 °C for 3 h supplemented with 50,000 cpm $^{125}I$-EGF (specific activity, 91 μCi/ng). Cells were washed four times with ice-cold binding buffer, solubilized with 0.5 ml of NaOH (0.25 M), and quantified by scintillation counting. Nonspecific binding was determined by parallel incubation of each condition with 500 ng of EGF (receptor grade, Collaborative Research, Bedford, MA). Both $Ca^{2+}$ and PMA were added for 1 h at 37 °C prior to the beginning of the binding experiment, and these conditions were maintained during the binding period.

## RESULTS

*Elevation of Extracellular $Ca^{2+}$ Activates PI Metabolism in Balb/MK Cells*—Phospholipase C attack on inositol phospholipids generates a putative second messenger, Ins-1,4,5-$P_3$ (15). This is the substrate of a 3-kinase that yields Ins-1,3,4,5-$P_4$ (11). The latter appears to be involved in the control of entry of $Ca^{2+}$ from the extracellular milieu after the discharge of intracellular Ins-1,4,5-$P_3$-sensitive pools (16). Ins-1,3,4,5-$P_4$ is further metabolized by means of a 5-phosphomonoesterase yielding Ins-1,3,4-$P_3$ whose activities, if any, as a second messenger are still unknown.

Because increased $Ca^{2+}$ has been shown to activate PI metabolism in some cell systems (10), we sought to determine whether the $Ca^{2+}$ signal for terminal differentiation of Balb/MK cells led to alterations in PI metabolism. Thus, [$^3H$]inositol-labeled Balb/MK cells were incubated either in the absence or in the presence of $Ca^{2+}$ (2 mM). Water-soluble InsPs were then extracted and analyzed by anion-exchange HPLC. Fig. 1 shows representative profiles of InsPs from either untreated (Fig. 1*a*) or $Ca^{2+}$-treated Balb/MK cells (Fig. 1, *b* and *c*). It can be seen that the individual isomers of $InsP_3$ (namely, Ins-1,4,5-$P_3$ and Ins-1,3,4-$P_3$) and $InsP_4$ were well resolved and that Ins-1,3,4-$P_3$ was the most abundant $InsP_3$ isomer at both times after $Ca^{2+}$ challenge (Fig. 1, *b* and *c*). Increased levels of Ins-1,3,4,5-$P_4$ were observed at both 2 min and 2 h after $Ca^{2+}$ addition (Fig. 1, *b* and *c*).

In order to determine the more detailed kinetics of $Ca^{2+}$-induced release of different InsPs, [$^3H$]inositol-labeled Balb/MK cells were treated with $Ca^{2+}$ (2 mM) for different times and InsPs analyzed as above. Ins-1,3,4-$P_3$ was the earliest to show an increased level with a significant elevation at 30 min following $Ca^{2+}$ addition (Fig. 2*a*). Fig. 2*a* shows that after an initial reduction measured at 30 min, Ins-1,4,5-$P_3$ increased over basal values by 1 h after $Ca^{2+}$ challenge. Significantly increased levels in $InsP_4$ were also detected by 30 min after $Ca^{2+}$ addition (Fig. 2*a*). $Ca^{2+}$-induced changes in $InsP_2$ were observed by 1–1.5 h (Fig. 2*b*), whereas an increase in InsP was detected only after a 2-h incubation in the presence of $Ca^{2+}$ (Fig. 2*c*).

This appeared to be an unusual pattern of InsPs release, since normally, after agonist challenge, Ins-1,3,4-$P_3$ levels increase only after a previous rise of Ins-1,4,5-$P_3$ (11). Thus, we examined the early kinetics of the release of these $InsP_3$ isomers. As can be seen in Fig. 3*a*, the earliest detectable change in Ins-1,4,5-$P_3$ was after 5 min at which point a decrease was observed. Ins-1,4,5-$P_3$ levels remained below that of the control during the first 20 min following $Ca^{2+}$ exposure. In contrast, Ins-1,3,4-$P_3$ and Ins-1,3,4,5-$P_4$ increased over basal values by 30 s, the earliest time point measured. Fig. 3*b* shows the results obtained when labeled inositol was included during the entire period of $Ca^+$ exposure to ensure isotopic equilibrium. As can be observed, the pattern of $InsP_3$ isomers and $InsP_4$ released was similar by using this protocol. Finally,

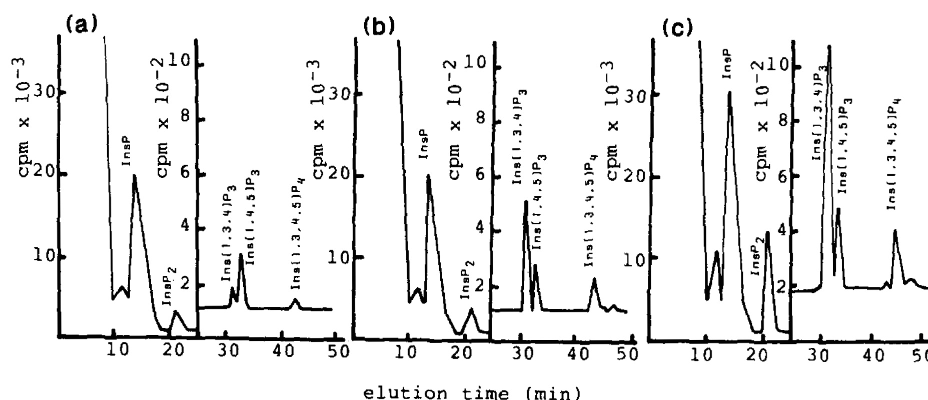


FIG. 1. **Representative profiles of $Ca^{2+}$-induced release of InsPs.** [$^3$H]Inositol-labeled Balb/MK cells were stimulated with $Ca^{2+}$ (2 mM) for 0 (*a*), 2 min (*b*), or 2 h (*c*). InsPs were then extracted and fractionated by anion-exchange HPLC by using a 0–1.7 M ammonium formate gradient as described under "Materials and Methods." The figure is representative of at least four experiments.

to exclude effects of LiCl on the observed pattern of InsP metabolites, we also performed incubations in its absence. Under these conditions, $Ca^{2+}$-induced release of InsPs was still observed, although the response was somewhat reduced. Furthermore, the pattern of $InsP_3$ isomers released was unchanged (Table I).

The atypical kinetics observed with respect to production of the two $InsP_3$s after $Ca^{2+}$ addition could result from an effect of the $Ca^{2+}$ signal on enzymes that regulate the conversion of Ins-1,4,5-$P_3$ into Ins-1,3,4-$P_3$. Such a conversion could hypothetically occur without phospholipase C-catalyzed generation of Ins-1,4,5-$P_3$ during the early time period after $Ca^{2+}$ challenge. Alternatively, $Ca^{2+}$-induced alterations in $InsP_3$ levels could accompany phospholipase C-catalyzed generation of DAG. To address these possibilities, [$^{14}$C]glycerol-labeled Balb/MK cells were stimulated with $Ca^{2+}$ (2 mM) for different times, after which DAG levels were determined. Fig. 4 shows that DAG levels increased significantly over basal values by 30 s after $Ca^{2+}$ challenge and remained markedly elevated for at least 12 h. Considering the specific activities of [$^3$H]inositol and [$^{14}$C]glycerol utilized as well as relative counting efficiences for the two isotopes and assuming that isotopic equilibrium was achieved, the levels of Ins-1,3,4-$P_3$ and DAG produced during the first 2 min after $Ca^{2+}$ challenge were roughly comparable on a molar basis. Thus, the results were consistent with the hypothesis that both of these metabolites arise from phosphoinositide metabolism.

In order to exclude sources of DAG other than PtdInsPs, in separate experiments we measured the effect of $Ca^{2+}$ on the release of phosphorylcholine or phosphorylethanolamine as a possible consequence of phosphatidylcholine or phosphatidylethanolamine hydrolysis, respectively. We observed no effect of $Ca^{2+}$ stimulation on either of these metabolites in Balb/MK cells prelabeled with [$^3$H]choline or [$^3$H]ethanolamine (Table II). Furthermore, the levels of triacylglycerol were not diminished after the addition of $Ca^{2+}$, excluding the possibility that activation of a triacylglycerol lipase was the cause of the increased DAG levels described above. Taken together, our results indicated that the $Ca^{2+}$ signal for terminal differentiation was a potent stimulus for the phosphodiesteratic breakdown of polyphosphoinositides in Balb/MK keratinocytes.

*Lack of Effect of Inhibitors of Cyclooxygenase, Lipoxygenase, and Proteinases on $Ca^{2+}$-induced Release of InsPs in Balb/MK Cells*—In some cell systems, phospholipase C activation by $Ca^{2+}$ ionophores has been shown to be due to the action of arachidonic acid metabolites. These are produced as consequence of the release of arachidonic acid by a $Ca^{2+}$-activatable phospholipase $A_2$ (17, 18). Therefore, it was possible that the $Ca^{2+}$-induced release of InsPs in Balb/MK cells was not the result of a direct action of $Ca^{2+}$ on phospholipase C but was due to the effect of arachidonic acid metabolites released in response to $Ca^{2+}$. In order to investigate this possibility, [$^3$H]inositol-labeled Balb/MK cells were stimulated with $Ca^{2+}$ (2 mM) for 30 min after a 20-min preincubation with indomethacin (20 $\mu$M) and nordihydroguaiaretic acid (20 $\mu$M), potent inhibitors of cyclooxygenase and lipoxygenase, respectively. At these inhibitor concentrations, arachidonic acid metabolism was completely abolished (data not shown) in accordance with results published previously (19). Despite this inhibition of arachidonic acid metabolism, $Ca^{2+}$ retained its ability to induce InsPs release.

Another possible indirect mechanism for phospholipase C activation by $Ca^{2+}$ involves the action of proteases. $Ca^{2+}$-dependent proteases have been isolated from a number of tissues (20), and there have been reports of a protease-dependent pathway for activation of inositol phospholipid metabolism (21). In order to address the potential relevance of such a pathway in $Ca^{2+}$-induced release of InsPs, [$^3$H]inositol-labeled Balb/MK cells were preincubated for 20 min either with leupeptin (100 $\mu$M) or aprotinin (10 $\mu$g/ml) prior to $Ca^{2+}$ exposure. $Ca^{2+}$-induced InsPs release was not affected by these protease inhibitors. Thus, we conclude that the effect of $Ca^{2+}$ on InsPs release was unlikely to result from a protease-activated pathway. Taken together, our findings suggest that $Ca^{2+}$-induced InsPs release in Balb/MK cells was due to the action of the $Ca^{2+}$-terminal differentiation signal on the enzymatic machinery controlling phosphodiesteratic hydrolysis of PtdInsPs.

*Specificity of the $Ca^{2+}$-induced Pattern of InsPs Release in Balb/MK Cells*—In order to investigate the specificity of the $Ca^{2+}$-induced alterations in phosphoinositide metabolism, Swiss 3T3 fibroblasts or porcine aortic endothelial cells were grown to confluence in medium containing [$^3$H]inositol and then switched to low calcium medium for 4 h. Subsequent treatment with a $Ca^{2+}$ concentration of 2 mM failed to cause any significant release of InsPs (data not shown, see also Refs. 10 and 28). As a further test, we measured the appearance of phosphoinositide metabolites in response to the calcium ionophore A23187 in Balb/MK cells. Although the toxicity of this compound precluded measurement of terminal differentiation, short term treatment (3-h) using 10 $\mu$M A23187 led to the rapid accumulation of Ins-1,3,4-$P_3$, (185% of control at 30 min, 280% at 3 h), which was accompanied by an initial decrease (67% of control at 30 min) followed by a delayed increase (140% of control at 3 h) of Ins-1,4,5-$P_3$. This pattern of InsPs release was comparable to that elicited by extracellular $Ca^{2+}$ alone (Figs. 2 and 3).

*$Ca^{2+}$ Signal for Balb/MK Differentiation Is Not Associated with Protein Kinase C Activation*—The rapid and sustained

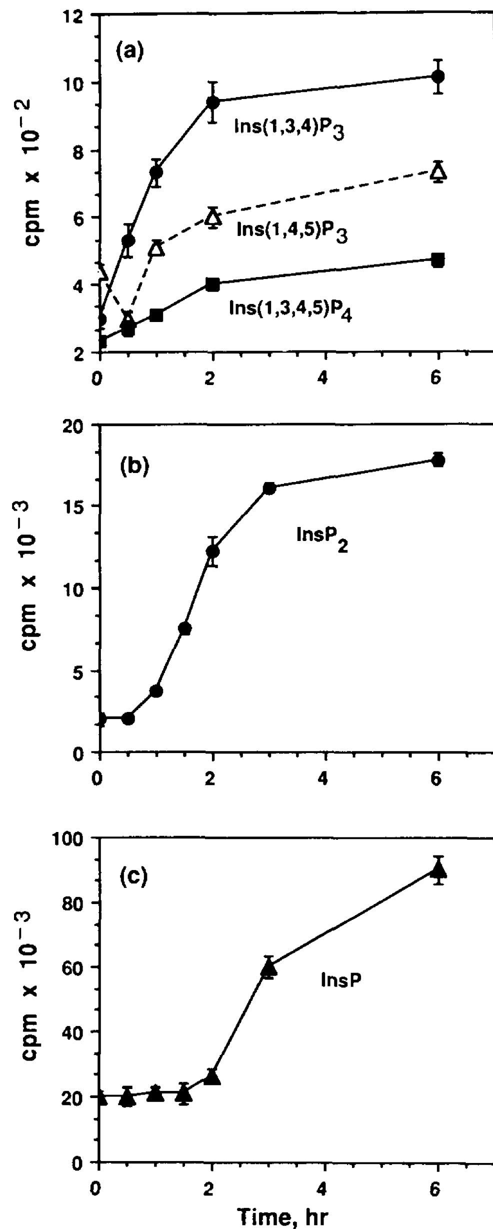


FIG. 2. **Time course of the $Ca^{2+}$ effect on release of different InsPs.** Cells were labeled with 15 μCi/ml [$^3$H]inositol for 60 h, after which cultures were either washed twice with serum-free medium and stimulated with 2 mM $Ca^{2+}$, or cells were challenged with $Ca^{2+}$ (2 mM) in the presence of labeled inositol. In both cases, LiCl was added 10 min before $Ca^{2+}$ stimulation. InsPs were extracted and separated by anion-exchange HPLC as described under "Materials and Methods." The data shown in this figure represent experiments in which the label was removed prior to $Ca^{2+}$ challenge. A similar pattern and kinetics of InsPs release were observed when labeled inositol was present throughout $Ca^{2+}$ stimulation. Results are expressed as means ± S.D. of three independent experiments with incubations in duplicate.

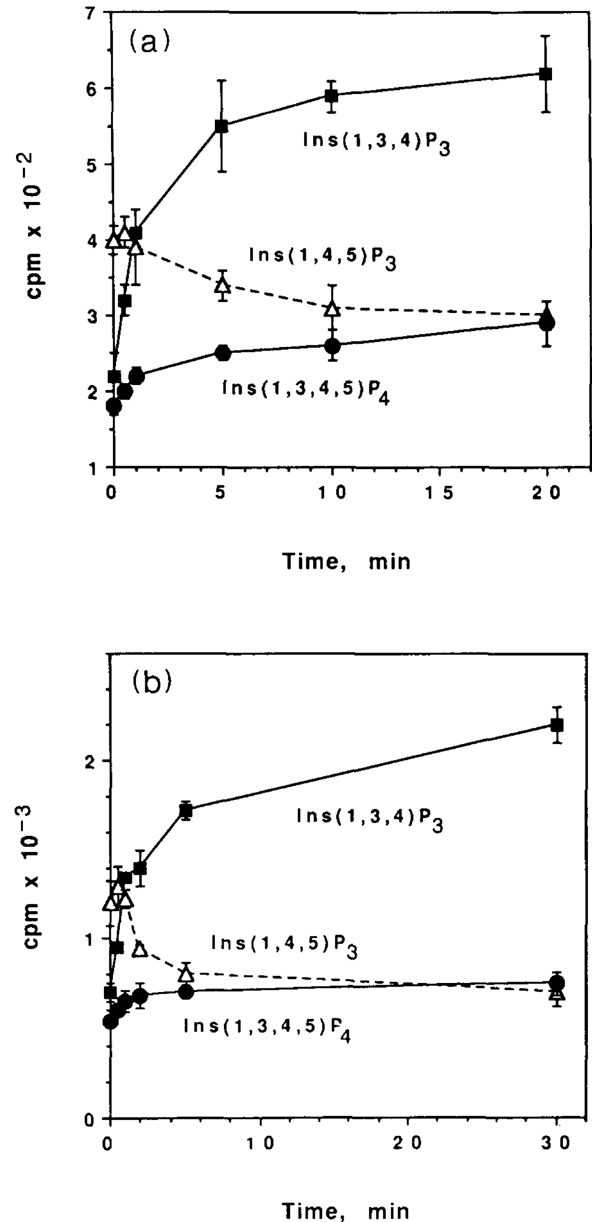


FIG. 3. **Time course of the effect of $Ca^{2+}$ on the release of different $InsP_3$ isomers.** Balb/MK keratinocytes were incubated with [$^3$H]inositol as described in Fig. 2 and then stimulated with $Ca^{2+}$ (2 mM) either in the absence (*a*) or in the continuous presence (*b*) of radiolabel. Afterward, InsPs were extracted and separated as described under "Materials and Methods." Results are expressed as the means ± S.D. of three independent experiments with each incubation performed in duplicate.

increase in DAG observed in response to $Ca^{2+}$ suggested that protein kinase C activation might be involved in $Ca^{2+}$-induced terminal differentiation of Balb/MK keratinocytes. To investigate the effect of the DAG generated by $Ca^{2+}$, we examined several parameters of protein kinase C activity. It has been shown previously that the phosphorylation of an 80-kDa acidic protein (p80) is a good marker for DAG-mediated protein kinase C activation *in vivo* in a number of cell systems, including Balb/MK cells (22). p80 phosphorylation was estimated after fractionation of trichloroacetic acid-insoluble material from [$^{32}$P]orthophosphate-labeled Balb/MK by two-dimensional gels. As shown in Fig. 5*e*, PMA, a potent protein kinase C stimulant (6), induced a dramatic increase in p80 phosphorylation. In contrast, we observed no increase in p80 phosphorylation following $Ca^{2+}$ challenge for up to 24 h (Fig. 5, *b–d*). These results suggested that the DAG induced by $Ca^{2+}$ addition to Balb/MK cells was incapable of activating protein kinase C as measured by this parameter. Two test whether $Ca^{2+}$ addition in some way altered protein kinase C sensitivity to DAG, we also tested the effect of PMA on p80 phosphorylation in $Ca^{2+}$-treated Balb/MK cells. As shown in Fig 5, *f–h*, PMA induced a pronounced phosphorylation of p80 comparable to that observed in the absence of $Ca^{2+}$ (Fig. 5*e*).

Table I

*Effect of LiCl on the levels of different InsPs in $Ca^{2+}$-activated Balb/MK cells*

[³H]Inositol-labeled Balb/MK cells either untreated or preincubated in the presence of LiCl (10 mM) for 15 min were stimulated with $Ca^{2+}$ (2 mM) for different times. Afterward, InsPs were extracted and fractionated by anion-exchange HPLC as described.

| Treatment | InsP | $InsP_2$ | Ins-1,3,4-$P_3$ | Ins-1,4,5-$P_3$ |
|---|---|---|---|---|
| | *cpm × 10⁻³* | | *cpm × 10⁻²* | |
| None | | | | |
| 30 min | 18.5 ± 0.8 | 1.9 ± 0.2 | 2.0 ± 0.1 | 4.0 ± 0.1 |
| 3 h | 19.0 ± 0.5 | 2.2 ± 0.2 | 2.2 ± 0.2 | 4.1 ± 0.2 |
| LiCl | | | | |
| 30 min | 18.6 ± 1.2 | 2.2 ± 0.6 | 2.0 ± 0.2 | 4.0 ± 0.1 |
| 3 h | 20.1 ± 0.8 | 2.5 ± 0.4 | 2.4 ± 0.2 | 4.3 ± 0.1 |
| $Ca^{2+}$ | | | | |
| 30 min | 18.2 ± 0.3 | 2.2 ± 0.5 | 4.1 ± 0.2 | 2.8 ± 0.2 |
| 3 h | 35.1 ± 1.2 | 16.5 ± 0.6 | 6.5 ± 0.5 | 6.2 ± 0.3 |
| LiCl + $Ca^{2+}$ | | | | |
| 30 min | 20.2 ± 0.9 | 2.3 ± 0.6 | 5.3 ± 0.2 | 2.9 ± 0.2 |
| 3 h | 59.0 ± 4.5 | 16.6 ± 0.8 | 9.2 ± 1.0 | 6.0 ± 0.3 |

Table II

*Lack of effect of $Ca^{2+}$ on the release of phosphorylcholine and phosphorylethanolamine in Balb/MK keratinocytes*

[³H]Choline- or [³H]ethanolamine-labeled Balb/MK cells were incubated in the presence of $Ca^{2+}$ (2 mM) for different times, after which water-soluble choline and ethanolamine metabolites were extracted and fractionated by thin layer chromatography as described under "Materials and Methods." Spots corresponding to phosphorylcholine and phosphorylethanolamine were scraped off and radioactivity content estimated by liquid scintillation counting. Results are expressed as means ± S.D. of three independent experiments with incubations performed in duplicate.

| Time after $Ca^{2+}$ addition | Phosphorylcholine | Phosphorylethanolamine |
|---|---|---|
| *h* | *cpm × 10⁻³* | *cpm × 10⁻³* |
| 0 | 23.2 ± 0.5 | 22.8 ± 0.4 |
| 0.25 | 24.6 ± 0.3 | 23.1 ± 0.6 |
| 0.5 | 22.8 ± 0.2 | 25.2 ± 0.9 |
| 1 | 24.4 ± 0.3 | 24.1 ± 0.6 |
| 2 | 25.0 ± 0.6 | 23.9 ± 0.2 |
| 6 | 22.9 ± 0.5 | 22.9 ± 0.3 |
| 12 | 24.8 ± 0.6 | 23.7 ± 0.4 |

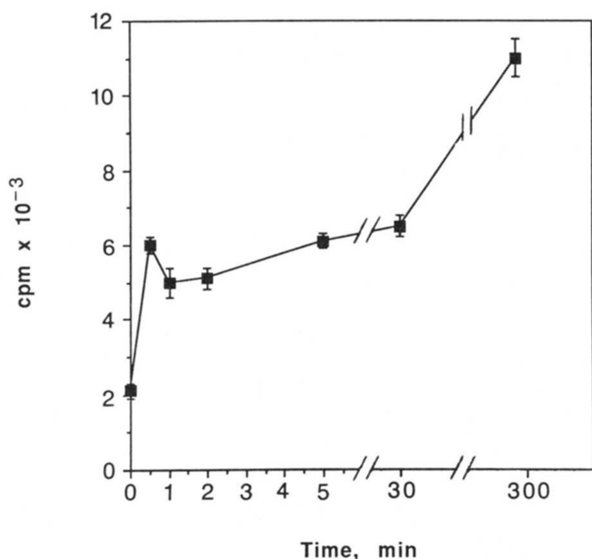


Fig. 4. **$Ca^{2+}$-induced formation of diacylglycerol.** Balb/MK cells were labeled with 30 μCi/ml [U-¹⁴C]glycerol for 60 h to achieve isotopic equilibrium. The cells were then exposed to $Ca^{2+}$ (2 mM) for various times in the continued presence of label. Cellular lipids were extracted, and diacylglycerol was fractionated by thin layer chromatography as described under "Materials and Methods." Results are expressed as the means ± S.D. of three independent experiments with incubations in duplicate.

Another sensitive measure of protein kinase C activation *in vivo* is the down-regulation of EGF receptors (23), which can be quantitated by a decrease in ¹²⁵I-EGF binding. As shown in Table III, Balb/MK cells exposed to $Ca^{2+}$ showed no detectable changes in ¹²⁵I-EGF binding. In contrast, PMA induced a dramatic decrease in EGF receptors both in the absence and in the presence of $Ca^{2+}$ challenge. These results confirmed that DAG produced in $Ca^{2+}$-triggered Balb/MK cells was unable to activate protein kinase C.

We also measured protein kinase C protein levels by immunoblotting with a specific monoclonal antibody. As shown in Fig. 6, PMA induced a profound down-regulation of protein kinase C protein levels after a 24-h treatment. However, $Ca^{2+}$ challenge led to no detectable changes in protein kinase C basal levels or in the ability of PMA to down-regulate this protein.

*Evidence for the Release of an Alkyl Ether Glyceride Molecular Species in $Ca^{2+}$-activated Balb/MK Keratinocytes*—It has

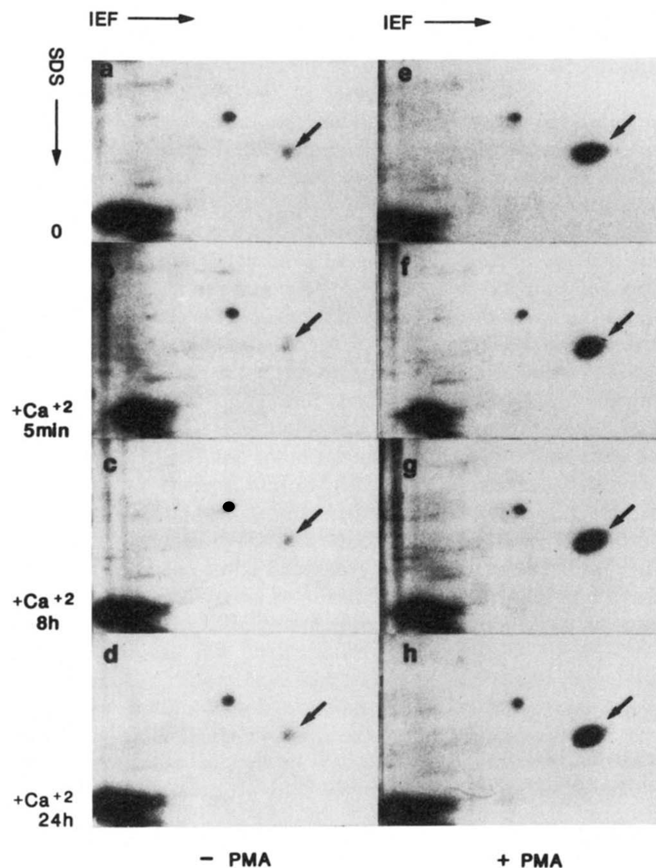


Fig. 5. **Resolution of acidic phosphoproteins in intact Balb/MK cells by two-dimensional polyacrylamide gel electrophoresis.** Cells were labeled for 4 h with [³²P]orthophosphate and then stimulated with either dimethyl sulfoxide (control solvent) (*a*) or $Ca^{2+}$ (2 mM) for 5 min (*b*), 8 h (*c*), or 24 h (*d*). Cells cultured in parallel were exposed to PMA (200 ng/ml) for 5 min after treatment with control solvent (*e*) or $Ca^{2+}$ for 5 min (*f*), 8 h (*g*), or 24 h (*h*). Phosphoproteins were separated by isoelectric focusing (*IEF*) (pH 3.5–5) in the first dimension followed by 10% SDS-polyacrylamide gel electrophoresis in the second dimension. The position of the 80-kDa phosphoprotein is indicated by an *arrow*. The figure is representative of duplicate experiments.

TABLE III

*Effect of $Ca^{2+}$ on PMA-induced down-regulation of $^{125}$I-EGF binding to Balb/MK keratinocytes*

Cells were incubated with 50,000 cpm of $^{125}$I-EGF at 15 °C for 3 h after 1-h preincubation with different concentrations of PMA or $Ca^{2+}$ (2 mM) or both. Afterward, unbound $^{125}$I-EGF was washed off with ice-cold buffer, and cells were hydrolyzed with 0.25 M NaOH. EGF binding was estimated by scintillation counting. Results expressed as cpm are means ± S.D. of three independent experiments with incubations in duplicate.

| | $^{125}$I-EGF bound | | | |
|---|---|---|---|---|
| | No PMA | PMA | | |
| | | 1 ng/ml | 3 ng/ml | 50 ng/ml |
| | | *cpm/well* | | |
| $-Ca^{2+}$ | 1223 ± 169 | 1021 ± 196 | 796 ± 21 | 380 ± 22 |
| $+Ca^{2+}$ | 1254 ± 53 | 950 ± 57 | 707 ± 31 | 255 ± 24 |

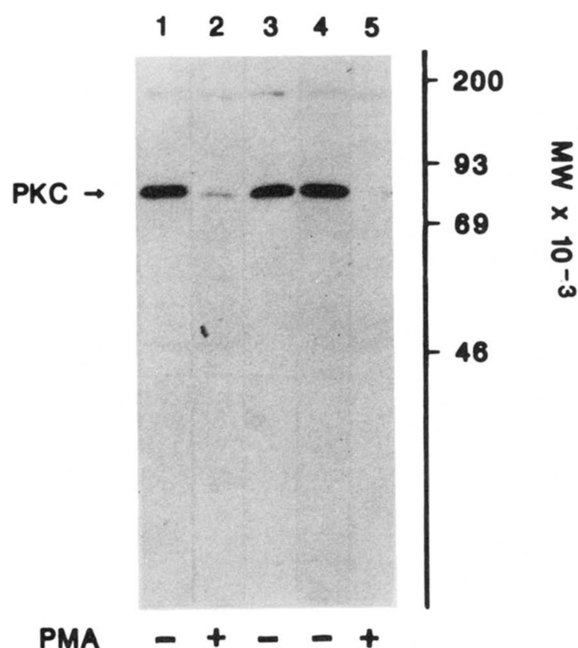


FIG. 6. **Effect of $Ca^{2+}$ and PMA on protein kinase C protein levels in Balb/MK keratinocytes.** Lysates containing 100 μg of total cellular protein were resolved by electroblotting as described under "Materials and Methods." Protein kinase C (*PKC*) was visualized using a monoclonal anti-protein kinase C antibody (clone MC5) followed by $^{125}$I-labeled protein A. Immunoglobulin-protein A complexes were detected by autoradiography. *Lane 1*, normal Balb/MK cells in low $Ca^{2+}$ medium; *lane 2*, Balb/MK treated with PMA (500 ng/ml) for 24 h; *lane 3*, $Ca^{2+}$ (2 mM) for 4 h; *lane 4*, $Ca^{2+}$ (2 mM) for 24 h; *lane 5*, $Ca^{2+}$ (2 mM) + PMA (500 ng/ml) for 24 h. Molecular weight markers are shown on the *right*.

been reported that the addition of 1-hexadecyl-2-acetylglyceride, the 1-*O*-alkyl-2-acylglycerol analogue of 1-oleoyl-2-acetylglyceride, induces HL-60 cells to enter the myeloid differentiation program in the absence of detectable protein kinase C activation (24, 25). Furthermore, 1-oleoyl-2-acetylglyceride was unable to induce differentiation in this cell system despite a pronounced activation of protein kinase C (26). Therefore, it was of interest to investigate whether the DAG produced in $Ca^{2+}$-stimulated Balb/MK cells contained an alkyl ether glyceride molecular species. To test this possibility, [$^{14}$C] glycerol-labeled Balb/MK cells were stimulated with $Ca^{2+}$, after which lipids were extracted, DAG isolated and subjected to KOH hydrolysis.

Fig. 7, *a* and *b*, shows that the DAG present under basal conditions was completely degraded by KOH treatment, indicating that all the molecular species that composed this DAG were of the 1,2-diacylglycerol type. It can also be seen in Fig. 7, *c* and *d*, that although the DAG produced after 2 h of $Ca^{2+}$ stimulation in Balb/MK cells was completely degraded by KOH, a molecular species that migrated at the monoacylglycerol position arose after the alkali treatment. This species represented around 30% of the total DAG. Thus, a considerable portion of the DAG produced by 2 h after $Ca^{2+}$ addition to Balb/MK consists of a form in which one of the fatty acids was linked to the glycerol backbone through an alkyl ether bond. This structurally resembles 1-hexadecyl-2-acetylglyceride which, upon exogenous addition, induced differentiation in HL-60 cells (24, 25).

## DISCUSSION

In this report, we show that the $Ca^{2+}$ signal for terminal differentiation in Balb/MK cells leads to a sustained activation of inositol phospholipid metabolism. The pattern of InsPs release was found to be atypical and similar to that observed in ionophore-stimulated HL-60 cells (27). Shortly after the addition of $Ca^{2+}$, we observed a very rapid increase in Ins-1,3,4-$P_3$ followed by a decrease of Ins-1,4,5-$P_3$. Concomitant with the production of Ins-1,3,4-$P_3$, a rapid and sustained increase in DAG was observed. It should be noted that the concentration of extracellular $Ca^{2+}$ required to trigger these responses in Balb/MK cells is present in normal cell culture medium (1.2–1.4 mM). Moreover, in other cell systems, exposure to increasing $Ca^{2+}$ concentration was not found to have any effect on phosphoinositide metabolism. All of these findings argue that the $Ca^{2+}$-elicited changes in phosphoinositide metabolism demonstrated in our studies may be important in mediating the differentiation program in Balb/MK keratinocytes.

The rapid increase in Ins-1,3,4-$P_3$ levels observed following $Ca^{2+}$ stimulation of Balb/MK cells could theoretically be accounted for by $Ca^{2+}$-activated phosphodiesteratic hydrolysis of a hypothetical PtdIns-3,4-$P_2$. Recently, Whitman *et al.* (29) have identified a novel phosphatidylinositol 3-kinase that could provide the first step in such a pathway leading to biosynthesis of PtdIns-3,4-$P_2$. Studies in other systems, however, have failed to identify PtdIns-3,4-$P_4$ (30, 31). We were unable to detect a phosphatidylinositol bisphosphate species distinct from PtdIns-4,5-$P_2$ in Balb/MK cells,[2] although its transient production cannot be excluded. Another possible source of Ins-1,3,4-$P_3$ could arise from 5-phosphomonoesterase action on Ins-1,3,4,5-$P_4$ derived from $Ca^{2+}$-stimulated hydrolysis of PtdIns-3,4,5-$P_3$ (30–32). Thus, phosphodiesteratic degradation of either PtdIns-3,4-$P_2$ or PtdIns-3,4,5-$P_3$ could theoretically account for the rapid and sustained production of both Ins-1,3,4-$P_3$ and DAG identified in $Ca^{2+}$-activated Balb/MK cells.

The above potential pathways would not require alterations in Ins-1,4,5-$P_3$, a well defined metabolite of PtdIns-4,5-$P_2$. Therefore, neither readily explains the initial decrease that we observed in Ins-1,4,5-$P_3$ during the first 20 min after $Ca^{2+}$ addition to Balb/MK cells. In this regard, there is experimental evidence that $Ca^{2+}$, through calmodulin, activates the metabolism of Ins-1,4,5-$P_3$ through the 3-kinase/5-phosphomonoesterase pathway (33, 34) and that $Ca^{2+}$ potently activates PI-specific phospholipase C *in vitro* (10). Thus, a plausible model consistent with our data suggests that $Ca^{2+}$ activates phospholipase C attack on PtdIns-4,5-$P_2$, generating DAG and Ins-1,4,5-$P_3$. The latter would then be rapidly converted to Ins-1,3,4-$P_3$.

[2] J. Moscat, unpublished observations.

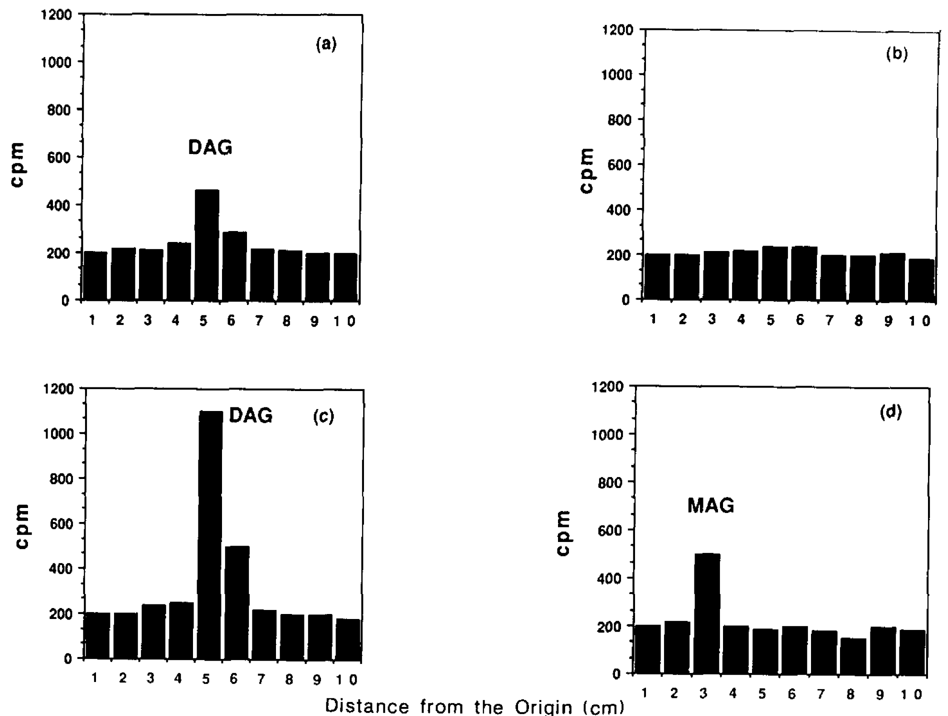


FIG. 7. **Effect of alkali treatment on the diacylglycerol produced by $Ca^{2+}$-activated Balb/MK.** [$^{14}$C]Glycerol-labeled Balb/MK cells were either untreated or stimulated with $Ca^{2+}$ (2 mM) for 2 h. Lipids were extracted and [$^{14}$C]diacylglycerol isolated after TLC. DAG samples were either treated with methanol or with KOH-containing methanol. Afterward, lipids were reextracted and fractionated by TLC. Regions of 1 cm were scraped and radioactivity content of each band estimated by liquid scintillation counting. (*a*) DAG from unstimulated cells; (*b*) DAG from unstimulated cells after alkali treatment; (*c*) DAG from $Ca^{2+}$-activated cells; (*d*) DAG from $Ca^{2+}$-activated cells after alkali treatment. A monoacylglycerol species (*MAG*) was observed in this sample. The figure is representative of at least three independent experiments.

The dramatic and sustained production of DAG in $Ca^{2+}$-treated Balb/MK cells was not associated with any detectable evidence of functional activation or down-regulation of protein kinase C. We also demonstrated that the same cells had the capacity to respond to a classical stimulus to protein kinase C in the absence or presence of the $Ca^{2+}$ challenge. Furthermore, prolonged treatment of Balb/MK cells with PMA did not induce the appearance of terminal differentiation markers.[3] Taken together, these findings imply that classical protein kinase C activation is neither necessary nor sufficient for differentiation in this keratinocyte system.

1-Hexadecyl-2-acetylglyceride, a 1-*O*-alkyl-2-acylglycerol, has been shown to be a potent inducer of differentiation in HL-60 cells (24, 25). Our present results indicate that part of the DAG generated following $Ca^{2+}$ addition to Balb/MK cells is an alkyl ether-linked glyceride, consistent with the structure of 1-hexadecyl-2-acetylglyceride. Thus, this DAG molecular species may play a role in the $Ca^{2+}$-induced differentiation program of Balb/MK cells through mechanisms other than stimulation of classical protein kinase C activity. Recently, protein kinase C has been shown to consist of a family of isozymes whose physiological activation remains to be elucidated (35–37). Therefore, it is conceivable that the unusual DAG molecular species generated following $Ca^{2+}$ addition to Balb/MK cells may activate distinct protein kinase C isozymes not detected by our biochemical or immunological assays. Further characterization of this $Ca^{2+}$-induced DAG species will allow a direct test of its effects on keratinocyte differentiation as well as its possible role in the activation of distinct protein kinase C isozymes that may be expressed in Balb/MK cells.

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[3] T. Fleming, unpublished observations.

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