The diacylglycerol analogue, 1,2-*sn*-dioctanoylglycerol, induces an increase in cytosolic free Ca^{2+} and cytosolic acidification of T lymphocytes through a protein kinase C-independent process

Roger EBANKS,* Chaim ROIFMAN,† Alan MELLORS* and Gordon B. MILLS‡§

*Guelph–Waterloo Center for Graduate Work in Chemistry, Department of Chemistry and Biochemistry, University of Guelph, Ontario, Canada N1G 2W1, †Division of Immunology and Rheumatology, Research Institute, Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8, and ‡Oncology Research, Toronto General Hospital, 200 Elizabeth Street, Toronto, Ontario, Canada M5G 2C4

In this paper, we demonstrate that low concentrations (0.5–2.5 μM) of 1,2-*sn*-dioctanoylglycerol (DiC_8), a potent diacylglycerol used in many previous studies to probe the role of protein kinase C (PKC) in cell activation, cause cytosolic alkalinization of human, mouse and pig T lymphocytes through PKC-mediated activation of the Na^+/H^+ antiport. However, at higher concentrations ($\geq 12.5 \mu\text{M}$), the effect on cytosolic pH (pH_i) is reversed, resulting in a marked cytosolic acidification, followed by a gradual return of pH_i to baseline values. DiC_8 also induces marked changes in cytosolic free calcium concentrations ($[\text{Ca}^{2+}]_i$), initially by releasing calcium from intracellular stores, followed by a net transmembrane influx of calcium. The DiC_8 -induced cytosolic acidification, the resultant return to baseline pH and the increase in $[\text{Ca}^{2+}]_i$ are independent of activation of PKC. Unlike many other agents which increase $[\text{Ca}^{2+}]_i$, DiC_8 does not induce phosphatidylinositol hydrolysis with the resultant production of inositol phosphates. Other compounds known to activate PKC, including the closely related diacylglycerol analogues, 1,2-*sn*-dihexanoylglycerol and 1,2-*sn*-didecanoylglycerol, phorbol esters and mezerein, did not induce changes in $[\text{Ca}^{2+}]_i$ or cytosolic acidification in T lymphocytes. Thus the action of DiC_8 on intact lymphocytes is different from that of phorbol esters and other diacylglycerols, and is specific to the length of the acyl chains. Because changes in $[\text{Ca}^{2+}]_i$ are often associated with cell proliferation and cell differentiation, some effects of DiC_8 on intact cells may be a consequence of changes in $[\text{Ca}^{2+}]_i$.

INTRODUCTION

Activation of the Ca^{2+} - and lipid-dependent phosphotransferase protein kinase C (PKC) by phorbol esters, 1,2-*sn*-diacylglycerols (DAG) or growth factors has been associated with cell differentiation and proliferation (reviewed in [1–3]). Since a variety of structurally unrelated activators of PKC are also potent tumour promoters, activation of PKC is postulated to be a contributing factor in tumour initiation and progression [1–3]. This possibility is strengthened by the observations that interaction of growth factors with their receptors activates PKC, and that introduction of oncogenes into cells also alters the localization and degree of activation of PKC [1–3].

The mechanisms by which activation of PKC leads to stimulation of intracellular processes, including secretion, differentiation and proliferation, have been probed in many systems by measuring the effects of activators of PKC, including phorbol esters, mezerein, teleocidin and cell-membrane-permeant DAG analogues, on these processes [1–15]. Studies with semi-purified PKC and in intact cells demonstrate that DAGs with chain lengths

from 4 to 18 are potent activators of PKC, those with chain lengths of 8–12 being the most active [4–10].

In this report, we demonstrate in mouse, human and pig lymphocytes that 1,2-*sn*-dioctanoylglycerol (DiC_8), a DAG analogue commonly used to activate PKC [1–7], activates the Na^+/H^+ antiport, presumably through stimulation of PKC. However, DiC_8 also has effects which do not involve PKC. In particular, DiC_8 stimulates cytosolic acidification, release of Ca^{2+} from intracellular stores, and a net transmembrane influx of Ca^{2+} . These actions are markedly different from the effects of other PKC activators, including DAG analogues, mezerein and phorbol esters. DiC_8 -induced changes in $[\text{Ca}^{2+}]_i$ may, in part, be responsible for many of the reported effects of DiC_8 .

MATERIALS AND METHODS

Media and stock solutions

Complete culture medium was RPMI 1640 (Gibco, Grand Island, NY, U.S.A.) supplemented with 2 mM-glutamine (Gibco), 50 μM -2-mercaptoethanol (Sigma

Abbreviations used: AM, acetoxymethyl ester derivative; BAPTA, 1,2-bis(*O*-aminophenoxy)ethane-*NNN'*-tetra-acetic acid; BCECF, 2',7'-bis(2-carboxyethyl)-5-(and 6)-carboxyfluorescein; $[\text{Ca}^{2+}]_i$, cytosolic free calcium concentration; DAG, diacylglycerol; DiC_6 , 1,2-*sn*-dihexanoylglycerol; DiC_8 , 1,2-*sn*-dioctanoylglycerol; DiC_{10} , 1,2-*sn*-didecanoylglycerol; EPA, 5-(*N*-ethyl-*N*-propylamino)amiloride; Indo-1, 1-[2-amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2'-amino-5'-methylphenoxy)ethane-*NNN'*-tetra-acetic acid; IP, *myo*-inositol 1-phosphate; IP_3 , *myo*-inositol 1,4-bisphosphate; IP_3 , *myo*-inositol 1,4,5-trisphosphate; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PDB, phorbol 12,13-dibutyrate; PDD, phorbol 12,13-didecanoate; 4 α -PDD, 4 α -phorbol 12,13-didecanoate; PDZ, phorbol 12,13-dibenzoate; PHA, phytohaemagglutinin; pH_i , intracellular pH; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

§ To whom reprint requests should be addressed.

Chemical Co., St. Louis, MO, U.S.A.), gentamycin (Sigma) and 10 % (v/v) fetal-calf serum (Sterile Systems Inc., Logan, UT, U.S.A.). Phytohaemagglutinin-P (PHA) was obtained from Difco Laboratories (Detroit, MI, U.S.A.). The acetoxymethyl derivatives of 2',7'-bis(2-carboxyethyl)-5-(and 6)-carboxyfluorescein (BCECF-AM), 1-[2-amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2'-amino-5'-methylphenoxy)ethane-*NNN'*-tetra-acetic acid (Indo-1 AM) and 1,2-bis(*O*-aminophenoxy)ethane-*NNN'*-tetra-acetic acid (BAPTA-AM) were from Molecular Probes (Eugene, OR, U.S.A.). Ionomycin was from Calbiochem (San Diego, CA, U.S.A.). Buffer A was (in mM): NaCl 140, KCl 1, MgCl₂ 1, glucose 10, CaCl₂ 1 and Hepes (Sigma) 20, pH 7.23. The amiloride analogue 5-(*N*-ethyl-*N*-propylamino)amiloride (EPA), a gift from Dr. E. J. Cragoe (Merck, Sharp and Dohme Research Laboratories, West Point, PA, U.S.A.; see refs. [16,17] for proof of inhibition of the antiport in lymphocytes), was dissolved at 5 mM in dimethyl sulfoxide and used at 10 μ M final concentration. DiC₈ was obtained from Serdary Research Laboratories Inc. (London, Ont., Canada), Life Sciences Resources (Milwaukee, WI, U.S.A.), and Molecular Probes. Most of the experiments reported herein are with DiC₈ from Serdary Research Laboratories. The essential details were confirmed with DiC₈ from the other sources. By n.m.r. at 400 MHz, the DiC₈ from Serdary Research Laboratories was confirmed to be the 1,2-*sn*-isomer. 1,2-*sn*-Dihexanoylglycerol (DiC₆) and 1,2-*sn*-didecanoylglycerol (DiC₁₀) were from Serdary Research Laboratories. 1-Oleoyl-2-acetyl-*sn*-glycerol (OAG) was from Life Sciences Resources. Phorbol 12,13-dibutyrate (PDB), phorbol 12,13-didecanoate (PDD), 4 α -phorbol 12,13-didecanoate (4 α -PDD), 4 α -phorbol, phorbol 12,13-dibenzoate (PDZ), 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA), 1-myristoyl-2-acetyl-*sn*-glycerol, 1-decanoyl-2-acetyl-*sn*-glycerol, mezerein and nigericin were from Calbiochem (San Diego, CA, U.S.A.). DAG analogues were dried under N₂, resuspended in dimethyl sulfoxide and sonicated. Stock solutions of phorbol esters, mezerein and DAG analogues were dissolved in dimethyl sulfoxide at concentrations which allowed addition of 0.2 % solvent. This amount of solvent was without effect in each assay. In addition, DiC₈ or solvent did not show significant effects on cell viability as measured by Trypan Blue permeability or by cell function (either proliferation or interleukin-2 production; see [16–21] for techniques). All other reagents were from Sigma unless otherwise indicated.

Cells

Fresh peripheral blood mononuclear cells from human pig and murine spleen cells were isolated on Ficoll Hypaque (Pharmacia, Uppsala, Sweden). The murine lymphoma cell line LBRM 331A5 [18] was carried in complete medium and subcultured every 3 days. The murine cell line P1, which lacks PKC [19], was carried in complete medium supplemented with 100 units of recombinant interleukin-2 (Cetus)/ml.

[Ca²⁺]_i measurements

[Ca²⁺]_i was measured by fluorescence of intracellularly trapped dye Indo-1 as described [20–22]. Each assay was calibrated by addition of 5 μ M-ionomycin followed by 1 mM-Mn²⁺. [Ca²⁺]_i was determined as described [20–23].

Inositol phosphate production

Inositol phosphate production was measured essentially as described [24–28] except that LiCl (20 mM) was added to prevent breakdown of *myo*-inositol 1-phosphate (IP) and *myo*-inositol 1,4-bisphosphate (IP₂) [24–28]. The Dowex 1X8 technique has been confirmed by h.p.l.c. over a Mono Q column [28].

Cytosolic pH

Intracellular pH (pH_i) was measured by fluorescence with BCECF and calibrated exactly as previously described [16,17,19].

Depletion of PKC

LBRM-331A5 cells were incubated with 1 μ M-TPA for 18 h, a standard technique for removal of PKC [19,29–31]. Lack of PKC was confirmed by measuring phosphotransferase activity *in vitro* and phorbol-ester-stimulated Na⁺/H⁺ exchange as previously described [19,29], except that PKC was partially purified by binding and elution from DEAE-cellulose DE52 columns [19,29–31].

RESULTS

DiC₈ induces cytosolic acidification

Addition of 50 μ M-DiC₈, which is similar to concentrations of DiC₈ used in previous studies [1–15], to peripheral blood lymphocytes from human (Fig. 1a) and pig (Fig. 1b), mouse spleen cells (results not shown) and murine LBRM 331A5 cells (Fig. 1c) induced a marked decrease in pH_i. After this cytosolic acidification, pH_i gradually returned to baseline values. In contrast, as has been reported with other PKC agonists (reviewed in [1–3] and [32]), TPA induced a cytosolic alkalization of approx. 0.2 pH unit in each cell type (Figs. 1d–1f). The pH_i was increased by agents which activate semi-purified PKC in phosphotransferase assays (PDB, PDD, PDZ, OAG, 1-myristoyl-2-acetyl-*sn*-glycerol, 1-decanoyl-2-acetyl-*sn*-glycerol, DiC₆, DiC₁₀ and mezerein; results not shown); it was not affected by agents which do not activate semi-purified PKC in phosphotransferase assays (α -phorbol, 4 α -PDD and phorbol triacetate; results not shown). Therefore cytosolic alkalization was induced by agents which activate PKC, but not by those that do not. Remarkably, DAG analogues with 6 (DiC₆) or 10 (DiC₁₀) carbon atoms (up to 50 μ M) induced cytosolic alkalization (results not shown), whereas that with 8 carbon atoms (DiC₈) induced marked acidification (Fig. 1).

DiC₈ induces marked changes in [Ca²⁺]_i

Incubation of peripheral blood mononuclear cells from human (Fig. 2a) and pig (Fig. 2b), mouse spleen cells (results not shown) and murine LBRM 331A5 cells (Fig. 2c) with DiC₈ (50 μ M) resulted in a rapid increase in [Ca²⁺]_i. In freshly isolated cells, this increase was followed by a slight decrease and then a further marked rise in [Ca²⁺]_i. In the LBRM 331A5 cell line, after the initial increase, [Ca²⁺]_i returned to near-normal values. [Ca²⁺]_i then increased gradually to a value similar to that observed after incubation of freshly isolated lymphocytes with DiC₈. In contrast, TPA had no effect on [Ca²⁺]_i in any of the cell types (Figs. 2d–2f).

Fig. 1. Effect of DiC₈ on pH_i

Fresh Ficoll–Hypaque-selected peripheral blood lymphocytes from human or pig or LBRM 331A5 cells were loaded with BCECF and added to a stirred heated cuvette as described in the Materials and methods section. After equilibration, agents were added as indicated. Tracings are for: (a) human cells, DiC₈ (50 μM); (b) pig cells, DiC₈ (50 μM); (c) LBRM 331A5 murine cells, DiC₈ (50 μM); (d) human cells, TPA (10 nM); (e) pig cells, TPA (10 nM); (f) LBRM 331A5 murine cells, TPA (10 nM). The results represent one tracing of at least three of each type. pH_i was calibrated as described in the Materials and methods section. The scales for (d), (e) and (f) are identical.

Low concentrations of DiC₈ increase pH_i

Although DiC₈ at 50 μM gave acidification (Fig. 1), DiC₈ at 0.5–2.5 μM induced a cytosolic alkalization of LBRM 331A5 cells (Figs. 3a–3c). Similar to the cytosolic alkalization induced by TPA (Figs. 3f and 3g) and each of the PKC activators listed above, the increase in pH_i was inhibited by amiloride analogues (Fig. 3f) and was dependent on the presence of extracellular Na⁺ (Fig. 3g), which indicated that the increase in pH_i was due to activation of the Na⁺/H⁺ antiport.

Recovery from cytosolic acidification is not a consequence of activation of the Na⁺/H⁺ antiport

Higher concentrations of DiC₈ induced a marked cytosolic acidification (Figs. 3d, 3e and 1). Amiloride analogues slightly decreased the degree of cytosolic acidification, but had no effect on the subsequent pH_i recovery (Fig. 3h). Although the pH_i decrease was blunted when cells were incubated in medium in which extracellular Na⁺ was replaced iso-osmotically with cations which are not transported by the antiport (choline, Fig. 3i; K⁺, Fig. 4a; or *N*-methylglucamine, results not shown), the subsequent pH_i recovery proceeded normally. TPA-induced cytosolic alkalization was effectively blocked by the amiloride analogues or in Na⁺-free medium (Figs. 3f and 3g), which indicates that the Na⁺/H⁺ antiport was blocked under these incubation conditions. Therefore the effects of high concentrations of DiC₈ on pH_i are not dependent on activation of the Na⁺/H⁺ antiport.

Confirmation that DiC₈ induces cytosolic acidification

To eliminate the possibility that the changes in fluorescence of BCECF were due to interaction of DiC₈ with BCECF, we utilized the ability of nigericin to equilibrate intracellular and extracellular pH for cells incubated in K⁺-rich medium (in which K⁺ was substituted iso-osmotically for Na⁺) [16,17,32]. In cells incubated with nigericin in K⁺-rich medium, the transmembrane pH gradient is abolished and any change in fluorescence after addition of DiC₈ would be due to an interaction of DiC₈ with the BCECF dye. When cells were incubated in K⁺-rich medium, DiC₈ (50 μM) induced changes in the fluorescent signal which were similar to those in Na⁺-rich medium (compare Fig. 4a with Figs. 3e and 1c). In contrast, when DiC₈ was added to cells which had been preincubated with nigericin, the change in the fluorescent signal was almost completely blocked (Fig. 4b). This shows that the changes in the fluorescent signal after addition of DiC₈ were not due to an interaction of DiC₈ with the BCECF dye.

Null-point calibration of changes in intracellular pH

We utilized the ability of nigericin to equalize intracellular and extracellular pH in K⁺-rich medium as a 'null point' technique to determine the magnitude of cytosolic acidification. The technique is based on the observation that in K⁺-rich medium, when the extracellular pH equals pH_i, addition of nigericin will not result in a change in the fluorescent signal. When nigericin was added to K⁺-rich medium (pH 7.23) after the addi-

Fig. 2. Effect of DiC_8 on $[\text{Ca}^{2+}]_i$

Fresh Ficoll–Hypaque-selected peripheral blood lymphocytes from human or pig or LBRM 331A5 cells were loaded with Indo-1 as described in the Materials and methods section and added to a stirred heated cuvette in a Hitachi F4000 spectrofluorimeter. After equilibration, agents were added as indicated. Tracings are for: (a) human cells, DiC_8 ($50 \mu\text{M}$); (b) pig cells, DiC_8 ($50 \mu\text{M}$); (c) LBRM 331A5 murine cells, DiC_8 ($50 \mu\text{M}$); (d) human cells, TPA (10 nM); (e) pig cells, TPA (10 nM); (f) LBRM 331A5 murine cells, TPA (10 nM). Each assay was calibrated by addition of ionomycin ($5 \mu\text{M}$) and Mn^{2+} (1 mM). The calibration curve was the same for all tracings. The results represent one tracing of at least three of each type.

tion of DiC_8 (Fig. 4c), the fluorescent signal increased, owing to equilibration of pH_i with the external pH. This indicated that the pH_i after incubation with DiC_8 was lower than the pH of the K^+ -rich medium (pH 7.23). However, when the extracellular pH was adjusted to 6.8 (Fig. 4d), nigericin did not induce a change in the fluorescent signal, which indicates that, after incubation with DiC_8 , pH_i declined to about 6.8.

Is pH_i recovery a result of permeabilization?

The apparent pH_i recovery after acidification induced by DiC_8 may simply reflect permeabilization of the cells to H^+ ions and loss of H^+ ions into the medium. If pH_i recovery was the result of permeabilization of the cells to H^+ ions, then pH_i would not recover after cytosolic acidification at an extracellular pH equal to the minimum pH_i (6.8). When the extracellular pH was decreased to 6.8, the magnitude and kinetics of the response to $50 \mu\text{M}$ - DiC_8 were not altered (Fig. 4e). These results indicate that pH_i recovery after cytosolic acidification was not due to permeabilization of the cells to H^+ ions, and that H^+ ions could be exported against a concentration gradient.

Changes in cytosolic free Ca^{2+} concentrations

In LBRM 331A5 cells, agents which activate PKC did not alter $[\text{Ca}^{2+}]_i$. The agents tested included TPA (Figs 2d–2f), PDB, PDD, PDZ, OAG, 1-decanoyl-2-acetyl-sn-glycerol, mezerein, DiC_8 and DiC_{10} (results not shown). At very low concentrations ($0.5 \mu\text{M}$), DiC_8 had no effect on intracellular $[\text{Ca}^{2+}]_i$ (Fig. 5a), but, at 1.25 – $2.5 \mu\text{M}$,

DiC_8 induced an increase in $[\text{Ca}^{2+}]_i$ which reached a plateau at values above baseline (Figs. 5b and 5c). Higher concentrations of DiC_8 (12.5 – $50 \mu\text{M}$; Figs. 5d and 5e) induced, within seconds, a marked increase in $[\text{Ca}^{2+}]_i$, followed, within minutes, by a return to near-normal values. $[\text{Ca}^{2+}]_i$ then increased to very high values (Figs. 5d and 5e), which were maintained for at least 1 h. DiC_8 -induced changes in $[\text{Ca}^{2+}]_i$ did not parallel either DiC_8 -induced acidification or alkalinization. Concentrations that induced alkalinization (1.25 – $2.5 \mu\text{M}$) and those that induced acidification (12.5 – $50 \mu\text{M}$) induced increases in $[\text{Ca}^{2+}]_i$. It therefore appears that the increase in $[\text{Ca}^{2+}]_i$ did not result solely from cytosolic alkalinization or cytosolic acidification.

Source of Ca^{2+}

Incubation of LBRM 331A5 cells in nominally Ca^{2+} -free medium in the presence of $0.5 \mu\text{M}$ -EGTA blunted, but did not prevent, the initial increase in $[\text{Ca}^{2+}]_i$ (Fig. 5f). In contrast, in Ca^{2+} -free medium, the delayed increase in $[\text{Ca}^{2+}]_i$ was totally blocked (Fig. 5f). Addition of sufficient free Ca^{2+} to the Ca^{2+} -free medium to increase free Ca^{2+} to 1 mM resulted in a rapid increase in $[\text{Ca}^{2+}]_i$ (Fig. 5f). Therefore, in LBRM 331A5 cells, DiC_8 -induced increases in $[\text{Ca}^{2+}]_i$ can be readily dissected into two components. The first event is a rapid change in $[\text{Ca}^{2+}]_i$, which is not dependent on extracellular Ca^{2+} and is likely to be due to release from intracellular stores. The second event, the delayed increase in $[\text{Ca}^{2+}]_i$, seems to be a consequence of increased membrane permeability to Ca^{2+} .

Fig. 3. Titration of the effect of DiC_8 on pH_i in LBRM 331A5 cells

BCECF-loaded LBRM 331A5 cells were prepared as described in Fig. 1. After equilibration, cells were incubated in Buffer A with DiC_8 at (a) $0.1 \mu\text{M}$, (b) $0.5 \mu\text{M}$, (c) $2.5 \mu\text{M}$, (d) $12.5 \mu\text{M}$ or (e) $50 \mu\text{M}$. In each tracing, TPA (10 nM) was added at the second arrow. To determine the requirements for activation of the Na^+/H^+ antiport, cells were incubated with $10 \mu\text{M}$ of the amiloride analogue EPA, or in choline $^+$ -rich medium, which is Buffer A in which Na^+ has been iso-osmotically replaced with choline $^+$. The additions were as follows: (f) Na^+ -rich Buffer A, EPA, DiC_8 ($2.5 \mu\text{M}$), TPA (10 nM); (g) choline-rich Buffer A, DiC_8 ($2.5 \mu\text{M}$), TPA (10 nM); (h) Na^+ -rich Buffer A, EPA, DiC_8 ($50 \mu\text{M}$), TPA (10 nM); and (i) choline-rich Buffer A, DiC_8 ($50 \mu\text{M}$), TPA (10 nM). pH_i was calibrated as described in Fig. 1. The calibration curve was the same for all tracings.

Fig. 4. Determination of changes in pH_i with a 'null-point' technique

LBRM 331A5 cells were loaded with BCECF as described in Fig. 1. For tracings (a)–(d), cells were washed immediately before use and suspended in warm K^+ -rich medium, which is Buffer A in which Na^+ has been replaced iso-osmotically with K^+ . For tracing (e) cells were suspended in Buffer A, pH 6.8. The additions at the arrows are: (a) K^+ -rich Buffer A, pH 7.23, DiC_8 ($50 \mu\text{M}$); (b) K^+ -rich Buffer A, pH 7.23, nigericin ($0.2 \mu\text{g/ml}$), DiC_8 ($50 \mu\text{M}$); (c) K^+ -rich Buffer A, pH 7.23, DiC_8 ($50 \mu\text{M}$), nigericin ($0.2 \mu\text{g/ml}$); (d) K^+ -rich Buffer A, pH 6.8, DiC_8 ($50 \mu\text{M}$), nigericin ($0.2 \mu\text{g/ml}$); (e) Na^+ -rich Buffer A, pH 6.8, DiC_8 ($50 \mu\text{M}$). The calibration curve was the same for tracings (a) and (b).

Fig. 5. Effect of DiC_8 on $[\text{Ca}^{2+}]_i$.

LBRM 331A5 cells (5×10^7) were loaded with Indo-1 as described in Fig. 2. For BAPTA-loaded cells, cells (2.5×10^7) were incubated with $50 \mu\text{M}$ of the acetoxymethyl derivative for 1 h. Cells were washed and re-suspended in a stirred heated cuvette. After equilibration, DiC_8 was added in Ca^{2+} -containing buffer A at (a) $0.5 \mu\text{M}$, (b) $1.25 \mu\text{M}$, (c) $2.5 \mu\text{M}$, (d) $12.5 \mu\text{M}$, (e) $50 \mu\text{M}$. To determine the effect of chelation of Ca^{2+} , cells were incubated with the following additions: (f) nominally Ca^{2+} -free medium, EGTA ($0.5 \mu\text{M}$) medium, DiC_8 ($50 \mu\text{M}$) and Ca^{2+} (1.5 mM) were added where indicated; (g) BAPTA-loaded cells, Ca^{2+} -containing medium, DiC_8 ($50 \mu\text{M}$); (h) BAPTA-loaded cells, Ca^{2+} -free medium, EGTA ($0.5 \mu\text{M}$) and DiC_8 ($50 \mu\text{M}$) added where indicated. Each run was calibrated by addition of ionomycin ($5 \mu\text{M}$) and Mn^{2+} (1 mM). The calibration curve was the same for tracings (a)–(d) and (e)–(h). The results represent one tracing of at least three of each type.

Table 1. Inositol phosphate production by DiC_8

Inositol phosphate production was measured as described in the Materials and methods section. Results (c.p.m.) are corrected for background radioactivity, which did not exceed 35 c.p.m. in any sample. The results represent means $\pm$ S.E.M. for four replicates from two different experiments of four experiments performed.

DiC_8 concn. (μM)	Radioactivity (c.p.m.)		
	IP_3	IP_2	IP
0	40 ± 16	10 ± 7	351 ± 5
0.5	42 ± 15	6 ± 5	326 ± 15
2.5	34 ± 17	7 ± 5	326 ± 3
12.5	53 ± 1	16 ± 9	364 ± 15
50	22 ± 7	20 ± 2	345 ± 18
PHA ($20 \mu\text{g/ml}$)	99 ± 22	64 ± 26	861 ± 20

Since intracellular stores of Ca^{2+} are limited, changes in $[\text{Ca}^{2+}]_i$ owing to release of Ca^{2+} from intracellular stores are decreased or completely abolished in cells in which intracellular Ca^{2+} is chelated with BAPTA [23,27]. In BAPTA-loaded cells, DiC_8 induced a slower prolonged increase in $[\text{Ca}^{2+}]_i$ (Fig. 5g). As indicated in Fig. 5(h), when both intracellular and extracellular Ca^{2+} were chelated by incubating BAPTA-loaded cells in Ca^{2+} -free medium (with EGTA), DiC_8 -induced changes in $[\text{Ca}^{2+}]_i$ were abrogated. This indicates that the cells were loaded with sufficient BAPTA to prevent changes in $[\text{Ca}^{2+}]_i$

owing to release of Ca^{2+} from intracellular stores. These data suggest that the initial increase in $[\text{Ca}^{2+}]_i$ is normally due to release of Ca^{2+} from intracellular stores. There is, in BAPTA-loaded cells, a delayed DiC_8 -induced increase in $[\text{Ca}^{2+}]_i$ which is probably due to an influx of Ca^{2+} from extracellular sources. It also indicates that the delayed influx of $[\text{Ca}^{2+}]_i$ is not dependent on an increase in $[\text{Ca}^{2+}]_i$ owing to the initial release of Ca^{2+} from intracellular stores, and thus is not likely to be due to opening of Ca^{2+} -activated Ca^{2+} channels.

The ability to chelate both intracellular and extracellular Ca^{2+} was used to demonstrate that the apparent changes in $[\text{Ca}^{2+}]_i$ were not due to an interaction of DiC_8 with Indo-1 or to intrinsic fluorescence of DiC_8 . When BAPTA-loaded cells were incubated in Ca^{2+} -free medium (with EGTA), DiC_8 did not induce an alteration in the fluorescent signal (Fig. 5h). This indicates that the previously observed changes in the fluorescent signal were due to changes in $[\text{Ca}^{2+}]_i$ rather than to interaction of DiC_8 with Indo-1 or to fluorescence of DiC_8 .

Production of inositol phosphates

Inositol 1,4,5-trisphosphate (IP_3), a product of membrane phosphatidylinositol hydrolysis by phospholipase C, has been reported to induce the release of Ca^{2+} from intracellular stores [24,25,28,33]. As indicated in Table 1, under conditions where DiC_8 induced changes in $[\text{Ca}^{2+}]_i$ and pH_i , we did not detect production of IP_3 or its metabolites, IP_2 or IP. DiC_8 ($50 \mu\text{M}$) failed to induce inositol phosphate production over a time course from 1 to 60 min (results not shown). As indicated in Table 1 and as reported previously [34], PHA increased produc-

Fig. 6. Effect of DiC_8 on pH_i and $[\text{Ca}^{2+}]_i$ in cells deficient in PKC

LBRM 331A5 cells or P1 cells were loaded with BCECF or Indo-1 as described in Figs. 1 and 2. Where indicated, LBRM 331A5 cells were depleted of PKC by incubation for 18 h with $1 \mu\text{M}$ -TPA (depletion of PKC was confirmed by measuring Ca^{2+} , TPA and phosphatidylserine-dependent phosphotransferase activity with semi-purified PKC). Additions were as follows: (a) BCECF-loaded normal LBRM 331A5, TPA (10 nM), DiC_8 (50 μM), (b) BCECF-loaded PKC-depleted LBRM 331A5, TPA (10 nM), DiC_8 (50 μM), (c) BCECF-loaded P1 cells, TPA (10 nM), DiC_8 (1.25 μM), (d) BCECF-loaded P1 cells, TPA (10 nM), DiC_8 (50 μM), (e) Indo-1-loaded PKC-depleted LBRM 331A5 cells, DiC_8 (50 μM), (f) Indo-1-loaded P1 cells, DiC_8 (50 μM). pH_i was calibrated as described in Fig. 1. $[\text{Ca}^{2+}]_i$ was calibrated as described in Fig. 2. The calibration curve was the same for tracings (a)–(d). Note that the scales are different in tracings (e) and (f). The results represent one tracing of at least three of each type.

tion of each of the inositol phosphates in LBRM 331A5 cells. This response to PHA demonstrated that the cells were sensitive to appropriate stimulation and that the assay system was sufficiently sensitive to detect changes in inositol phosphate production. The data suggest that the release of Ca^{2+} from intracellular stores induced by DiC_8 is not a consequence of production of IP_3 .

Activation of PKC is not required for DiC_8 -induced cytosolic acidification or increase in $[\text{Ca}^{2+}]_i$

When LBRM 331A5 cells were incubated with $1 \mu\text{M}$ -TPA for 18 h, which depletes many different cell types, including lymphocytes, of functional PKC [19,29–31], they lacked PKC as defined by phosphotransferase activity of semi-purified cell lysates. In a typical experiment, DE52-purified lysates of LBRM-331A5 cells incubated with TPA (1 μM) for 18 h had 1520 ± 2100 c.p.m. of PKC-specific phosphotransferase activity (defined as radioactivity in the presence of TPA and phosphatidylserine minus that in the absence of TPA and phosphatidylserine), whereas untreated cells had 17450 ± 1430 c.p.m. (see refs. [19,31] for details).

In normal LBRM 331A5 cells, addition of TPA induced an increase in pH_i (Fig. 6a). In these cells, subsequent addition of DiC_8 still induced cytosolic acidification (Fig. 6a). Although the magnitude of the change in pH_i was similar to that in cells which had not been preincubated with TPA, the intracellular pH did not reach as low a value in the TPA-treated cells as in cells which had not been incubated with TPA. This suggested that prior activation of the Na^+/H^+ antiport by TPA tended to compensate for the acidifying process.

When similar experiments were performed in LBRM

331A5 cells which had been depleted of PKC by prolonged incubation with high concentrations of TPA, TPA did not induce a change in pH_i (cf. Figs. 6a and 6b). This confirmed the phosphotransferase assays, which indicated that these cells were depleted of functional PKC. However, when these PKC-depleted cells were incubated with 50 μM - DiC_8 , the effect on cytosolic pH was indistinguishable from the effect of a similar concentration of DiC_8 on LBRM 331A5 cells which had not been depleted of PKC (cf. Fig. 6b with Fig. 3b). In these cells, low concentrations of DiC_8 (0.5 μM) did not alter pH_i (results not shown). This indicates that DiC_8 induces two processes, cytosolic alkalization through activation of PKC and cytosolic acidification followed by a recovery of pH_i which is independent of the activation of PKC.

In murine P1 cells, which appear to lack functional PKC [19], low concentrations of DiC_8 (0.5 μM) did not induce cytosolic alkalization (Fig. 6c). TPA also did not induce an increase in pH_i in P1 cells (Fig. 6d). However, in P1 cells higher concentrations of DiC_8 (50 μM) induced cytosolic acidification with similar kinetics and magnitude to those induced in LBRM-331A5 cells (Fig. 6d). This indicates that low concentrations of DiC_8 induced cytosolic alkalization as a result of activation of the Na^+/H^+ antiport by PKC. In addition, it confirms that the cytosolic acidification and recovery of pH_i after incubation with high concentrations of DiC_8 do not require activation of PKC.

To determine if activation of PKC is required for DiC_8 -induced changes in $[\text{Ca}^{2+}]_i$, we also utilized PKC-depleted LBRM 331A5 cells and P1 cells. Both types of cells showed DiC_8 -induced changes in $[\text{Ca}^{2+}]_i$, which had

Fig. 7. Effect of preventing changes in $[Ca^{2+}]_i$ on changes in pH_i .

Cells were loaded as described in Fig. 1 with BCECF or with BCECF and BAPTA as described in Fig. 5. Cells were incubated with the following additions: (a) BCECF-loaded cells, Ca^{2+} -containing Buffer A, 30 nM-ionomycin; (b) BCECF- and BAPTA-loaded cells, Ca^{2+} -free Buffer A, EGTA 0.5 μM , and 30 nM-ionomycin where indicated; (c) BCECF- and BAPTA-loaded cells, Ca^{2+} -free Buffer A, EGTA 0.5 μM and DiC_8 (50 μM) where indicated; (d) BCECF- and BAPTA-loaded cells, Ca^{2+} -free Buffer A, EGTA 0.5 μM and DiC_8 (1.25 μM) where indicated. pH_i was calibrated as described in Fig. 1. The calibration curve was the same for all tracings. The results represent one tracing of at least three of each type.

similar magnitude and kinetics to those in normal LBRM 331A5 cells (cf. Figs. 6e and 6f with Fig. 3e). Therefore, DiC_8 -induced changes in $[Ca^{2+}]_i$ were not secondary to activation of PKC.

Relationship between change in $[Ca^{2+}]_i$ and pH_i

In some cell types, cytosolic acidification has been reported to be a consequence of increases in $[Ca^{2+}]_i$ [35]. In other cell types, changes in pH_i have been reported to induce increases in $[Ca^{2+}]_i$ [36]. In LBRM 331A5 cells, the cation ionophore ionomycin, at concentrations (40 nM) which induce similar increases in $[Ca^{2+}]_i$ to DiC_8 (50 μM), induced cytosolic acidification, followed by recovery of pH_i to near baseline values (Fig. 7a). The similarity of this pattern to that induced by DiC_8 suggested that the effect of DiC_8 on pH_i may have been secondary to DiC_8 -induced changes in $[Ca^{2+}]_i$.

We tested this possibility by measuring pH_i when changes in $[Ca^{2+}]_i$ were blocked through chelation of both intracellular and extracellular Ca^{2+} (Fig. 5h). When ionomycin-induced changes in $[Ca^{2+}]_i$ were prevented by incubating BAPTA-loaded cells in Ca^{2+} -free medium, ionomycin-induced changes in pH_i were also inhibited (Fig. 7b). This indicated that ionomycin-induced changes in pH_i were indeed secondary to changes in $[Ca^{2+}]_i$. When similar experiments were performed with DiC_8 , the changes in pH_i induced by either high (50 μM ; Fig. 7c) or low (0.5 μM , Fig. 7d) concentrations of DiC_8 were not significantly altered. As we had previously demonstrated (Fig. 5h), DiC_8 does not increase $[Ca^{2+}]_i$ in BAPTA-loaded cells in Ca^{2+} -free medium. This indicates that DiC_8 -induced changes in $[Ca^{2+}]_i$ are not obligatory for the changes in pH_i . Nevertheless, since changes in $[Ca^{2+}]_i$ can induce cytosolic acidification, the cytosolic acidification induced by DiC_8 may have been the result of a combination of $[Ca^{2+}]_i$ -dependent and $[Ca^{2+}]_i$ -independent effects.

We also tested the effect of inhibition of the Na^+/H^+ antiport on DiC_8 -induced changes in $[Ca^{2+}]_i$. When the antiport was inhibited by the addition of amiloride analogues (not presented) or by replacement of extracellular Na^+ with choline, *N*-methylglucamine or K^+

(results not shown), DiC_8 -induced changes in $[Ca^{2+}]_i$ were not altered. This indicated that changes in $[Ca^{2+}]_i$ were not secondary to activation of the antiport, and activation of the antiport was not an obligatory event for DiC_8 -induced changes in $[Ca^{2+}]_i$.

DISCUSSION

Many recent studies have utilized DiC_8 as a specific activator of PKC in intact cells [1–3, 7–15]. Although it is clear that DiC_8 activates PKC in intact cells, the studies reported herein show that DiC_8 , at the concentrations used in previous studies, induces marked cytosolic acidification and an increase in $[Ca^{2+}]_i$. Since they were not induced by other DAG analogues, mezerein, or phorbol esters, and they occurred in cells depleted of PKC and in mutant cells which lack PKC, these changes were not due to activation of PKC, and activation of PKC was not required. Even the closely related analogues DiC_6 and DiC_{10} did not induce similar changes, so that C_8 acyl chains at the 1- and 2-*sn* positions appear to be critical for the process which leads to cytosolic acidification and increases in $[Ca^{2+}]_i$.

The cellular effects of the DiC_8 preparations utilized do not appear to be caused by contaminants. Four different lots of DiC_8 from three different suppliers induced similar changes in pH_i and $[Ca^{2+}]_i$. In addition, n.m.r. failed to detect any major contaminants in DiC_8 from Serdary Research Laboratories, and confirmed that the DiC_8 was in the 1,2-*sn* configuration. Therefore, the effects on pH_i and $[Ca^{2+}]_i$ are probably due to an effect of DiC_8 rather than to a contaminant in the preparations.

The pH_i recovery after the cytosolic acidification was not a consequence of activation of the Na^+/H^+ antiport. This is an unexpected finding, as pH_i recovery after intracellular acidification in other systems, including T cells, has been reported by ourselves and others to be dependent on activation of the Na^+/H^+ antiport, and is inhibited by amiloride analogues or by Na^+ -free medium [16, 32]. Similarly, increases in $[Ca^{2+}]_i$ were not obligatory for DiC_8 -induced cytosolic acidification.

By chelating either extracellular or intracellular Ca^{2+} ,

we were able to separate DiC_8 -induced changes in $[\text{Ca}^{2+}]_i$ into two processes. The early increase in $[\text{Ca}^{2+}]_i$ was dependent on release of Ca^{2+} from intracellular stores (not blocked in Ca^{2+} -free medium), whereas the delayed increase in $[\text{Ca}^{2+}]_i$ was dependent on transmembrane uptake of Ca^{2+} (blocked in Ca^{2+} -free medium). The rapid increase in $[\text{Ca}^{2+}]_i$ induced by DiC_8 was quite different from that which has been reported for PHA-stimulated LBRM 331A5 T cells or other human and murine T cells [27,34,37–39] in that it was not associated with membrane phospholipid turnover and the resultant production of IP_3 . The delayed uptake of Ca^{2+} was not due to a generalized increase in cell permeability, as the cells were not permeable to H^+ . This suggested that Ca^{2+} entered the cell through a relatively specific pathway.

Previous reports [14,40–42] indicated that diacylglycerols, including DiC_8 , could increase $[\text{Ca}^{2+}]_i$ in rat pituitary cells, platelets and human HL60 cells. However, those reports, in contrast with the present (Fig. 2) and previously reported results [1–3,31,32], indicated that TPA and OAG as well as DiC_8 increased $[\text{Ca}^{2+}]_i$ in rat pituitary cells, and OAG, but not TPA, increased $[\text{Ca}^{2+}]_i$ in HL60 cells and platelets. In HL60 cells, DiC_8 induces increases in phospholipid turnover, which could explain the changes in $[\text{Ca}^{2+}]_i$ observed [40]. However, in T lymphocytes, DiC_8 did not induce detectable changes in inositol phosphate metabolism, and DAG analogues, phorbol esters and mezerein did not alter $[\text{Ca}^{2+}]_i$. Therefore, some other mechanism appears to be involved in DiC_8 -induced changes in $[\text{Ca}^{2+}]_i$.

In addition, similarly to DiC_8 -induced changes in $[\text{Ca}^{2+}]_i$ and cytosolic pH, phorbol esters and DAG analogues have been demonstrated to have different effects on cells which have been attributed to non-PKC-mediated effects of one or the other agent [12,43–49]. Whether the same pathways are involved in each of these effects remains to be determined.

The studies reported herein show that DiC_8 induces marked cytosolic acidification and an increase in $[\text{Ca}^{2+}]_i$ at least in T lymphocytes. Since these effects were not shared by other activators of PKC, and functional PKC is not required for these processes, they are clearly not a consequence of activation of PKC. Therefore, the efficacy of DiC_8 in intact cells, which has made this lipid a frequently used tool in cell studies, may be dependent on the ability of DiC_8 to increase $[\text{Ca}^{2+}]_i$ rather than to activate PKC.

This work was supported by the Medical Research Council of Canada, the Natural Sciences and Engineering Research Council of Canada and the National Cancer Institute of Canada. G.B.M. is a Medical Research Council of Canada Scholar and the recipient of McLaughlin Scientist Award.

REFERENCES

- Kikkawa, U. & Nishizuka, Y. (1986) *Annu. Rev. Cell. Biol.* **2**, 149–178
- Rozengurt, E. (1986) *Science* **234**, 161–166
- Bell, R. M. (1986) *Cell* **45**, 631–632
- Hunнан, Y. A., Loomis, C. R. & Bell, R. M. (1986) *J. Biol. Chem.* **261**, 7184–7190
- Hunнан, Y. A., Loomis, C. R. & Bell, R. M. (1985) *J. Biol. Chem.* **260**, 10039–10043
- Ganong, B. R., Loomis, C. R., Hunнан, Y. A. & Bell, R. M. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1184–1188
- Jetten, A. M., Ganong, B. R., Vandenbark, G. R., Shirley, J. E. & Bell, R. M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1941–1945
- Davis, R. J., Ganong, B. R., Bell, R. M. & Czech, M. P. (1985) *J. Biol. Chem.* **260**, 1562–1566
- Davis, R. J., Ganong, B. R., Bell, R. M. & Czech, M. P. (1985) *J. Biol. Chem.* **260**, 5315–5322
- Lapetina, E. G., Reep, B., Ganong, B. R. & Bell, R. M. (1985) *J. Biol. Chem.* **260**, 1358–1361
- Kiss, Z., Deli, E., Shoji, M., Koeffler, H. P., Pettit, G. R., Vogler, W. R. & Kuo, J. K. (1987) *Cancer Res.* **47**, 1302–1307
- Morin, M. J., Kreutter, D., Rasmussen, H. & Sartorelli, A. C. (1987) *J. Biol. Chem.* **262**, 11758–11763
- Galizzi, J.-P., Qar, J., Fosset, M., Renterghem, C. V. & Lazdunski, M. (1987) *J. Biol. Chem.* **262**, 6947–6950
- Albert, P. R., Wolfson, G. & Tashuian, A. H. (1987) *J. Biol. Chem.* **262**, 6577–6581
- Seghal, P. B., Walther, Z. & Tamm, I. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3663–3667
- Mills, G. B., Cragoe, E. J., Gelfand, E. W. & Grinstein, S. (1985) *J. Biol. Chem.* **260**, 12500–12507
- Mills, G. B., Cheung, R. J., Cragoe, E. J., Grinstein, S. & Gelfand, E. W. (1986) *J. Immunol.* **136**, 1150–1154
- Gillis, S. & Mizel, S. B. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1133–1137
- Mills, G. B., Benedict, S., Mellors, A., Grinstein, S. & Gelfand, E. W. (1988) in *Interleukin 2* (Smith, K. A., ed.), pp. 113–135, Academic Press, San Diego
- Mills, G. B., Cheung, R. K., Grinstein, S. & Gelfand, E. W. (1985) *J. Immunol.* **134**, 2431–2435
- Gelfand, E. W., Cheung, R. K., Mills, G. B. & Grinstein, S. (1987) *J. Immunol.* **138**, 527–531
- Gryniewicz, G., Poenie, M. & Tsien, R. Y. (1986) *J. Biol. Chem.* **260**, 3440–3448
- Tsien, R. Y. (1980) *Biochemistry* **19**, 2396–2404
- Majerus, P. W., Connolly, T. M., Deckmyn, H., Ross, T. S., Bross, T. E., Ishii, H., Bansal, V. S. & Wilson, D. B. (1986) *Science* **234**, 1519–1526
- Moolenaar, W. H., Defize, L. H. K., Van Der Saag, P. T. & De Laat, S. W. (1986) *Curr. Top. Membr. Transp.* **26**, 137–156
- Mills, G. B., Stewart, D., Mellors, A. & Gelfand, E. W. (1986) *J. Immunol.* **136**, 3019–3025
- Gelfand, E. W., Mills, G. B., Cheung, R. K., Lee, J. W. & Grinstein, S. (1987) *Immunol. Rev.* **95**, 59–87
- Bansal, V. S., Inhorn, R. C. & Majerus, P. W. (1987) *J. Biol. Chem.* **262**, 9444–9451
- Blackshear, P. J., Stumpo, D. J., Huang, J.-K., Nemenoff, R. A. & Spach, D. H. (1987) *J. Biol. Chem.* **262**, 7774–7781
- Rozengurt, E., Rodriguez-Pena, A., Coombs, M. & Sinnett-Smith, J. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5748–5752
- Grinstein, S., Mack, E. & Mills, G. B. (1986) *Biochem. Biophys. Res. Commun.* **134**, 8–13
- Grinstein, S. & Rothstein, A. (1986) *J. Membr. Biol.* **90**, 1–12
- Berridge, M. J. (1986) *Sci. Am.* **255**, 142–152
- Abraham, R. T., Ho, S. N., Barna, T. J. & McKean, D. J. (1987) *J. Biol. Chem.* **262**, 2719–2728
- Rosoff, P. M. & Cantley, L. C. (1985) *J. Biol. Chem.* **260**, 14053–14061
- Siffert, W. & Akkerman, J. W. N. (1987) *Nature (London)* **325**, 456–458
- Kuno, M. & Gardner, P. (1987) *Nature (London)* **326**, 301–304
- Gray, L. S., Gnarr, J. S., Russel, J. H. & Englehard, V. H. (1987) *Cell* **50**, 119–130
- Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *Nature (London)* **295**, 68–71

40. Restrepo, D. & Knauf, P. A. (1986) *J. Gen. Physiol.* **88**, 49a
41. Brass, L. F. & Laposta, M. (1987) *Biochem. Biophys. Res. Commun.* **142**, 7–14
42. Bruns, C., Schachtele, C. & Marme, D. (1987) *FEBS Lett.* **221**, 23–27
43. Welsh, C. J. & Cabot M. C. (1987) *J. Cell. Biochem.* **35**, 231–237
44. May, W. S., Lapetina, E. G. & Cuatrecasas, P. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1281–1286
45. Muir, J. G. & Murray, A. W. (1986) *Biochim. Biophys. Acta* **885**, 176–180
46. Ways, D. K., Dodd, R. C. & Earp, H. S. (1987) *Cancer Res.* **47**, 3344–3347
47. Malviya, A. N., Louis, J. C. & Zwiller, J. (1986) *FEBS Lett.* **199**, 213–217
48. Kolesnick, R. N. & Paley, A. E. (1987) *J. Biol. Chem.* **262**, 9204–9209
49. Bijveld, C., Geelan, M. J. H., Houweling, M. & Vaartjes, W. J. (1988) *Biochem. Biophys. Res. Commun.* **151**, 193–197

Received 3 June 1988/10 October 1988; accepted 14 November 1988