Dioctanoylglycerol Regulation of Cytosolic Ca^{2+} by Protein Kinase C–Independent Mechanism in HIT T-15 Islet Cells

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The effect of activators of protein kinase C (PKC) on cytosolic concentration of free Ca^{2+} ($[Ca^{2+}]_i$) was assessed in insulin-secreting islet cell line HIT T-15. Dioctanoylglycerol (DiC8) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) evoked activation of PKC. Basal $[Ca^{2+}]_i$ was 65–160 nM. DiC8 induced triphasic increases in $[Ca^{2+}]_i$; phase 2 was the most prominent and consistent one. With 25–150 μM DiC8, $[Ca^{2+}]_i$ increased in a dose-dependent manner during phase 2; half-maximal stimulatory dose was 53 μM. TPA did not evoke any increase in $[Ca^{2+}]_i$. Staurosporine, sphingosine, and H7, which are inhibitors of PKC, did not block DiC8-induced rise in $[Ca^{2+}]_i$. DiC8-induced rise in $[Ca^{2+}]_i$ was also seen in cells that had been depleted of PKC by prior exposure to TPA. DiC8-induced rise in $[Ca^{2+}]_i$ still occurred in the presence of the Ca^{2+}-channel blocker verapamil or when the extracellular Ca^{2+} had been reduced from 2.5 mM to 30 nM by EGTA. Three immediate metabolites of DiC8, monooctanoylglycerol, octanoate, and glycerol, did not evoke any change in $[Ca^{2+}]_i$. Monooleoylglycerol and R59022, which induce increases in endogenous diacylglycerol (DAG) by inhibiting DAG kinase, evoked increases in $[Ca^{2+}]_i$. DiC8 did not cause any change in inositol 1,4,5-trisphosphate levels. DiC8 evoked biphasic increases in insulin release; the second-phase increase in $[Ca^{2+}]_i$ preceded the late phase of insulin secretion. Exogenous DAGs should be used with caution in assessing PKC function. Changes in the generation of DAGs must be included among the mechanisms by which Ca^{2+} homeostasis is regulated in islet cells. Endogenous DAGs may amplify insulin secretion not only via activation of PKC but also via increases in $[Ca^{2+}]_i$. *Diabetes* 40:621–27, 1991

In islet cells, activation of phospholipid metabolism leads to formation of diacylglycerol (DAG;1,2). The significance of the increases in the production of DAG, which occurs in islet cells in response to certain nutrient secretagogues, remains controversial (3–5). DAG is considered to exert its biological effects by activating protein kinase C (PKC). Although exogenous activators of PKC such as phorbol esters or synthetic analogues of DAG stimulate insulin secretion, decreased availability and inhibition of activation of PKC seem to have little or no influence on the secretory responses to nutrients (6–13). However, in several types of cells, besides activating PKC, phorbol esters and DAG analogues were found to induce increases in cytosolic concentrations of free Ca^{2+} ($[Ca^{2+}]_i$). Increases in $[Ca^{2+}]_i$ were evoked by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in platelets (14), rat adrenal gland (15), *Aplysia* neurons (16), rat pituitary cells (17), and RINm5F insulin-secreting islet cells (18). Cell-permeable dioctanoylglycerol (DiC8) and 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) induced increases in $[Ca^{2+}]_i$ in rat pituitary cells (19). Information is limited on the role of PKC activation in the increases in $[Ca^{2+}]_i$ induced by phorbol esters or diglycerides. In promyelocytic HL60 cells (20) and T lymphocytes (21), DiC8 seemed to evoke increases in $[Ca^{2+}]_i$ independent of the activation of PKC.

The purpose of this study was to define the role of DAG in the regulation of Ca^{2+} homeostasis in islet cells and the mediation of this role by the activation of PKC. We found that DiC8 evokes major increases in $[Ca^{2+}]_i$ by a mechanism different from the activation of PKC.

RESEARCH DESIGN AND METHODS

The vendors of the reagents were as follows: DiC8, TPA, ATP, phenylmethylsulfonyl fluoride (PMSF), histone type III-S, leupeptin, caprylic (octanoic) acid, monooctanoylglycerol, monooleoylglycerol, OAG, verapamil, and sphingosine were from Sigma (St. Louis, MO); H7 was from Seikagaku (St. Petersburg, FL); staurosporine was from Fluka (Ronkonkoma, NY); [γ-^{32}P]ATP was from ICN (Lisle, IL); fura 2 acetoxymethyl ester (fura 2-AM) was from Molecular Probes

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(Junction City, OR); bovine serum albumin (BSA) was from Miles (Elkhart, IN) or ICN; R59022 was from Research Diagnostics (Flanders, NJ); and tissue-culture reagents were from Gibco (Grand Island, NY).

The insulin-secreting glucose-sensitive HIT T-15 islet cell line (22), passage 65, was generously supplied by A.E. Boyd III (Baylor College of Medicine, Houston, TX). The cells were cultured in RPMI-1640 growth medium containing 2 mM glutamine, 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO_2/95% air at 37°C. The media were changed every 48 h, and the cells were passaged every 7 days. The cells used in this study were from passages 68 to 80. During these passages, the insulin secretory response of the cells to glucose remained intact.

The subcellular redistribution of PKC was assessed with a method reported earlier (23). On reaching confluency, HIT T-15 cells were detached in a solution of 0.05% trypsin and 0.02% EDTA; the solution was quenched with excess serum-enriched growth medium. The detached cells were centrifuged at 90 × *g* for 4 min, rinsed, and preincubated for 45 min in Krebs-HEPES buffer (KHB) containing 20 mM HEPES, 25 mM $NaHCO_3$, 120 mM NaCl, 4.7 mM KCl, 0.81 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.7 mM glucose, and 0.1% fatty acid–free BSA (pH 7.4). Then, the cells were incubated for various periods in solutions containing DiC8 or TPA; BSA was excluded because the increase in $[Ca^{2+}]_i$ induced by DiC8 is blunted in its presence. After incubations, the cells were harvested by centrifugation and permeabilized in a buffer containing 50 μM digitonin. The cytosol and membrane fractions were separated by centrifugation at 11,000 × *g* for 1 min. The PKC activity in the cytosol and membrane fractions was determined (24).

Measurement of $[Ca^{2+}]_i$ was performed as follows. The cells were trypsinized and rinsed twice in modified KHB (containing 1.6 mM $CaCl_2$, 0.1 mM EGTA, and 5 mg/ml BSA) as described above. The cells (2 × 10^6/ml) were incubated with 0.75 μM fura 2-AM for 40 min at 37°C while being mixed gently. Control cells were incubated with the vehicle for fura 2-AM (0.038% dimethyl sulfoxide [DMSO]). The cells were rinsed twice and resuspended in modified KHB at a density of 4 × 10^6 cells/ml. For the measurement of $[Ca^{2+}]_i$, 0.5 ml of the cell suspension was microcentrifuged at 350 × *g* for 10 s, resuspended in BSA-free KHB, and transferred to a cuvette. The fluorescence in the samples was measured at 37°C with a computer-driven dual-beam spectrofluorometer (Spex Fluorolog CM1). Excitation wavelengths were set at 335 and 380 nm, with 2.5-mm slit widths. The ratio of the fluorescence at these wavelengths was monitored at the emission wavelength of 505 nm with a slit width of 1.25 mm. The concentration of $[Ca^{2+}]_i$ was calculated with the formula

$$[Ca^{2+}]_i = \frac{K_d(R - R_{min})Sf2}{(R_{max} - R)Sb2}$$

where K_d is the dissociation constant for fura 2-AM at 37°C (224 nM), R is the observed ratio of 335:380 fluorescence, R_{min} is the same ratio observed in the absence of Ca^{2+}, R_{max} the same ratio in the presence of excess Ca^{2+}, and $Sf2$ and $Sb2$ are the fluorescence intensities of the free- and Ca^{2+}-bound forms of fura 2-AM at 380 and 335 nm, respectively (25).

At the end of the experiments, the viability of the cells was ~95%, as determined by their ability to exclude trypan blue dye. Possible interference by autofluorescence of any of the test agents was checked by loading the cells with DMSO rather than fura 2-AM and then adding the individual test agents. None of the test agents emitted detectable autofluorescence.

For measurement of inositol 1,4,5-trisphosphate (IP_3) generation, HIT T-15 cells were grown in six-well tissue-culture plates at a density of 10^6 cells · ml^{-1} · $well^{-1}$. The growth medium was removed, and the cells were rinsed twice with KHB and preincubated in KHB at 37°C for 45 min. The preincubation medium was removed, then the cells were incubated at 37°C for different time intervals in fresh BSA-free medium containing DiC8 or vehicle (0.1% ethanol) while the plates were being rocked gently. The incubation medium was removed; 1 ml ice-cold 0.5 M trichloroacetic acid was added quickly to the cells. The cells were scraped, transferred into centrifuge tubes, and microfuged at 11,000 × *g*. The supernatant was extracted with ether and dried in a rotary evaporator (Speed-Vac, Savant). The levels of IP_3 in the samples were measured by a radioreceptor assay with partially purified receptors prepared from bovine adrenal cortex, as described (26). The total protein content of each batch of cells was determined as described (27); the tissue content of IP_3 was expressed as picomoles per milligram of islet cell protein.

For measurement of insulin secretion, HIT T-15 cells were grown in wells of culture plates and processed as described above for IP_3 measurements. After exposure to DiC8 or vehicle for different periods, the media were removed, enriched with BSA to a final concentration of 0.1%, and stored at −20°C, to be assayed subsequently for insulin. Insulin levels in the media were measured by a double-antibody radioimmunoassay (28), and the amounts of insulin secreted were expressed as picomoles per milligram of islet cell protein.

The results are given as means ± SE for *n* observations in duplicates as indicated. The statistical significance of the differences between mean values was assessed by two-tailed Student's *t* test; $P < 0.05$ was considered significant.

RESULTS

The integrity of PKC activation in our line of HIT T-15 cells was ascertained by determining the redistribution of the enzyme from the cytosol to the membrane fraction. DiC8 activates PKC in intact and broken cell preparations (29,30). Within seconds, in detached intact HIT T-15 cells, DiC8 induced stable association of the PKC activity with the membrane fraction (Fig. 1). TPA also activated the PKC. When 100 μM DiC8 and 100 nM TPA were administered individually, the maximal increases above basal levels in membrane-associated PKC in 30 s were 56 ± 16% (n = 5) and 136 ± 34% (n = 2), respectively.

Next, we monitored the $[Ca^{2+}]_i$ in detached intact HIT T-15 cells in the presence of the individual activators of PKC. The mean resting $[Ca^{2+}]_i$ was 102 ± 25 nM (n = 50). On addition of DiC8, multiphasic increases in $[Ca^{2+}]_i$ occurred in a dose-related manner (Fig. 2). At concentrations <50 μM, the $[Ca^{2+}]_i$ response to DiC8 was biphasic. Phase 1 of

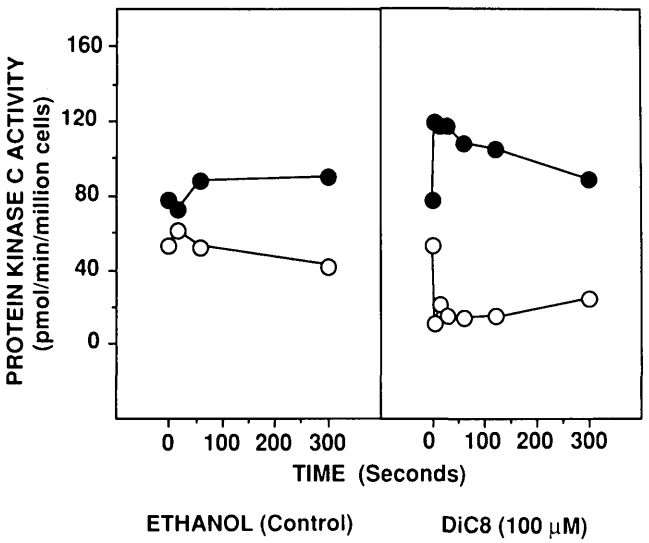

FIG. 1. Time kinetics of changes in membrane (●) and cytosolic (○) protein kinase C (PKC) activity evoked by 100 μM dioctanoylglycerol (DiC8) or vehicle (1% ethanol). DiC8 or vehicle was added to cells suspended in Krebs-HEPES buffer. Cytosol and membrane fractions were prepared at different time intervals, and PKC activity was determined as in METHODS. Values are representative of 2 experiments performed in duplicate.

the increase occurred within 5 s of addition of the agonist and dissipated within several seconds; the magnitude of the increases was 7–12% above the basal levels. Phase 2 started ~20 s after the addition of the stimulus, reached a maximum around 80 s, and subsided by 120 s. Above 50–75 μM, DiC8 also evoked a third-phase increase in $[Ca^{2+}]_i$, which reached a plateau in 15–20 min. Among the three phases, the second phase was the most prominent and consistent component; during this phase, the maximal increases above basal levels in response to 25, 50, 75, 100, and 150 μM DiC8 were 25 ± 2, 35 ± 5, 58 ± 7, 52 ± 6, and 50 ± 8%, respectively (n = 4–7). For the second phase, the estimated half-maximal stimulatory concentration of DiC8 was 53 μM. Ethanol, which had been used at concentrations up to 1% as a vehicle for DiC8, did not evoke any change in $[Ca^{2+}]_i$ even at a concentration of 4%.

The role of PKC activation in the observed changes in $[Ca^{2+}]_i$ was investigated with three different strategies. First, the effect of TPA, which we documented to activate PKC in the HIT T-15 cells, was tested. Unlike DiC8, 2, 50, 100, 200, or 600 nM TPA did not evoke any increase in $[Ca^{2+}]_i$, as exemplified for 200 and 600 nM TPA in Fig. 3. Second, the cells were pretreated for 4–5 min with the individual chemically unrelated inhibitors of PKC staurosporine (100 nM), sphingosine (10 μM), or H7 (50 μM) (31); DiC8 was then added in the presence of the inhibitor. The increases in $[Ca^{2+}]_i$ induced by 50 μM DiC8 in the presence of these inhibitors were 32 ± 7, 41 ± 7, and 37 ± 6%, respectively; these increases were similar to those observed in the absence of any inhibitor. Third, 2 μM TPA was added to the growth medium 18 h before harvesting the cells for the experiments. Prolonged exposure to TPA results in the depletion of PKC activity (32–34); indeed, when tested just before the fluorometric studies, PKC activity was found to be depleted by ~95%. In these PKC-depleted cells, the increase in $[Ca^{2+}]_i$ in response to 75 μM DiC8 still occurred; during the second phase, the maximal increment above basal levels was 51%, which is a magnitude similar to that observed in cells not pretreated with TPA (58%).

Experiments were carried out to determine whether the increases in $[Ca^{2+}]_i$ induced by DiC8 represented influx versus intracellular mobilization of Ca^{2+}. As a positive control for influx-associated increases in Ca^{2+}, the model of K^+-induced depolarization of the plasma membrane and the consequent opening of voltage-dependent Ca^{2+} channels was used (35). In detached intact HIT T-15 cells in response to 40 mM KCl, $[Ca^{2+}]_i$ increased promptly from a basal value of 106 ± 9 to 267 ± 16 nM (n = 7). Next, extracellular Ca^{2+}

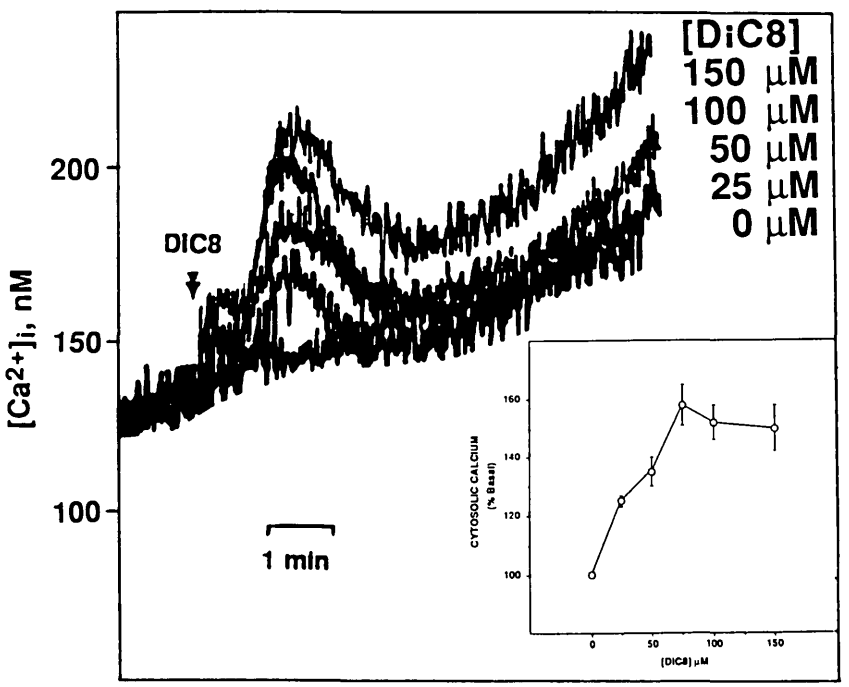

FIG. 2. Effect of various concentrations of dioctanoylglycerol (DiC8) on cytosolic concentrations of free Ca^{2+} ($[Ca^{2+}]_i$) in HIT T-15 cells. Ratio of fura 2-AM fluorescence was measured at excitation wavelengths 335 and 380 nm, and emission was monitored at 505 nm. Values obtained were converted to concentration of ionic Ca^{2+} as explained in METHODS. Representative individual spectrofluorometric tracings, from bottom to top, display changes in $[Ca^{2+}]_i$ evoked by vehicle (1% ethanol) and 25, 50, 100, and 150 μM DiC8, respectively (final concentrations). *Arrow*, time point reagents were added. *Inset*, mean ± SE maximal increments above basal levels in $[Ca^{2+}]_i$ during 2nd-phase increase (n = 4–7).

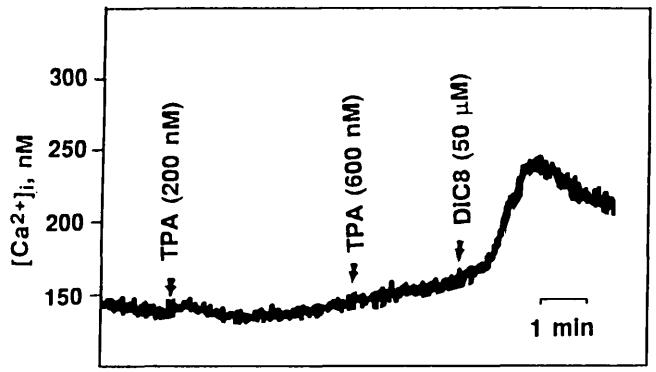

FIG. 3. Representative spectrofluorometric tracing of differential effects of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and dioctanoylglycerol (DiC8) on changes in cytosolic concentrations of free Ca^{2+} ($[Ca^{2+}]_i$). *Arrows*, time points TPA and DiC8 were added. Final concentrations are given.

was depleted by adding 10 mM EGTA to the cuvette; as a result, $[Ca^{2+}]_i$ decreased to 43% of the basal level and reached a new steady state. In the presence of EGTA, 40 mM KCl failed to induce any rise in $[Ca^{2+}]_i$ above this new basal level. Similarly, the response to KCl was completely abolished in the presence of the Ca^{2+}-channel antagonist verapamil (100 μM). On the other hand, in the presence of EGTA or 100 μM verapamil, the second-phase increase in $[Ca^{2+}]_i$ in response to DiC8 still occurred at a magnitude similar to that seen in control experiments (Fig. 4).

To determine whether DiC8 was unique among DAGs in its ability to evoke increases in $[Ca^{2+}]_i$, the effect of OAG, another cell-permeable DAG, was explored. Fifty micromolar OAG induced a rapid monophasic increase in $[Ca^{2+}]_i$, which reached a maximum in 1 min and remained elevated thereafter at a level 68 ± 11% above baseline. As was the case with DiC8, the increases in $[Ca^{2+}]_i$ in response to OAG were not affected by verapamil (Fig. 4). On the other hand, the increases in $[Ca^{2+}]_i$ with OAG did not occur in the presence of EGTA, unlike those with DiC8.

We considered the possibility that the increases in $[Ca^{2+}]_i$ observed with DiC8 may have been induced by one or more of its metabolites. In detached, intact HIT T-15 cells, an immediate metabolite of DiC8, monooctanoylglycerol (200 μM), failed to evoke an increase in $[Ca^{2+}]_i$ (Fig. 5). Similarly, neither octanoic acid (100 μM) nor glycerol (10 mM) had an effect (data not shown).

To determine whether endogenously generated DAGs may act like DiC8 in inducing increases in $[Ca^{2+}]_i$, detached intact HIT T-15 cells were treated individually with two inhibitors of DAG kinase, monooleoylglycerol (30) and R59022 (36). Thus, the conversion of DAG to phosphatidic acid would be prevented, and endogenous DAG would accumulate. Monooleoylglycerol (100 μM) evoked a sustained increase in $[Ca^{2+}]_i$ from a basal level of 85 to 250 nM (Fig. 5); this effect lasted as long as 10 min. When 50 μM DiC8 was added several minutes after the addition of monooleoylglycerol, no further increases in $[Ca^{2+}]_i$ occurred (data not shown). R59022 (10 μM) evoked biphasic increases in $[Ca^{2+}]_i$ from a basal value of 84 to 218 nM within 5 s, which decreased to 118 nM in 10 s and then remained elevated for as long as 3 min. As observed with OAG, the rise in $[Ca^{2+}]_i$ induced by monooleoylglycerol or R59022 was abolished by EGTA.

Because the increases in $[Ca^{2+}]_i$ induced by DiC8 appeared to be the result of mobilization of Ca^{2+} from an intracellular pool, the possibility was considered that DiC8 may have induced the breakdown of endogenous phosphatidylinositol, thus liberating IP_3, which then could have translocated Ca^{2+} from the endoplasmic reticulum (37–40). Anchored cells were incubated for 15 s to 5 min in the presence of 100 μM DiC8 or its vehicle, 0.1% ethanol. In the radioreceptor assay used, the cell extracts contained readily detectable levels of IP_3; DiC8 did not evoke any discernible change (Fig. 6).

A possible relationship between DiC8-induced increases in $[Ca^{2+}]_i$ and stimulation of insulin secretion was considered. Anchored cells were incubated for 0.5, 1, 2, and 5 min in

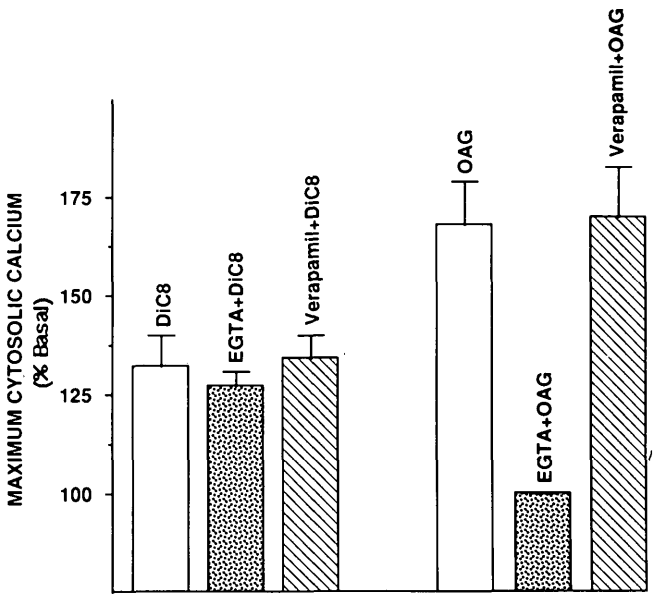

FIG. 4. Effects of EGTA and verapamil on 2nd-phase increase in cytosolic concentrations of free Ca^{2+} ($[Ca^{+2}]_i$) induced by dioctanoylglycerol (DiC8) and 1-oleoyl-2-acetyl-*sn*-glycerol (OAG). Cells were incubated with 10 mM EGTA (under these conditions, final concentration of ionized Ca^{2+} in extracellular media was 30 nM) or 100 μM verapamil and were stimulated with 50 μM DiC8 or 50 μM OAG. Values for $[Ca^{2+}]_i$ represent means ± SE of maximal increments above basal levels during 2nd phase (n = 3–5).

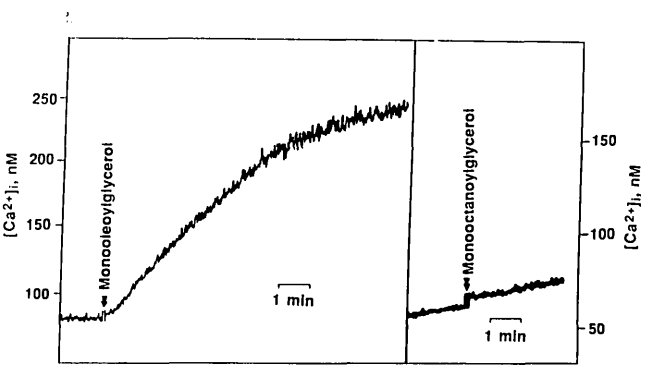

FIG. 5. Representative spectrofluorometric tracings of effects of monooctanoylglycerol (200 μM) and monooleoylglycerol (100 μM) on cytosolic concentrations of free Ca^{2+} ($[Ca^{2+}]_i$). Other details are as explained in Fig. 2.

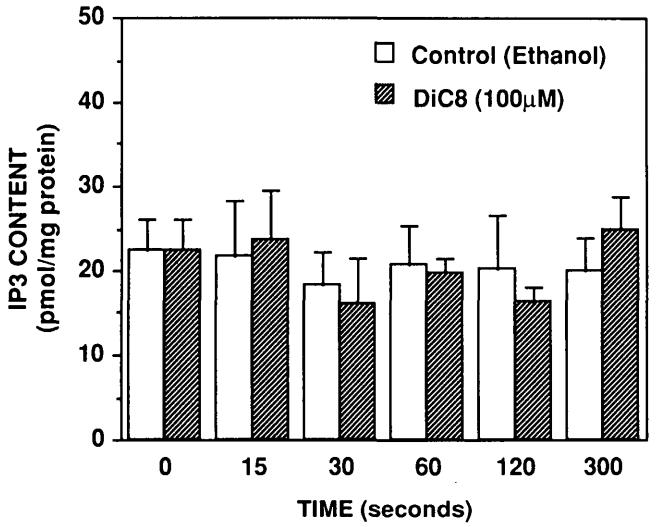

FIG. 6. Inositol 1,4,5-trisphosphate (IP3) content in HIT T-15 cells incubated with 100 μM dioctanoylglycerol (DiC8) or vehicle (0.1% ethanol) for several periods. For details, see METHODS.

the presence of 100 μM DiC8 or its vehicle, 0.1% ethanol; immunoreactive insulin levels were measured in the incubation media (Fig. 7). After a 10-min preincubation in the presence of 1.7 mM glucose at time 0, the basal level of insulin was 0.13 ± 0.02 pmol/mg protein. In the control experiments, where the vehicle was added at time 0 over the ensuing 5 min, insulin levels increased to 0.25 ± 0.03 pmol/mg protein. In the 30-s samples, DiC8 induced a 40% increase in insulin levels compared with the control (vehicle) samples. The levels in the 1- and 2-min samples were similar to those in the 30-s samples. In the 5-min samples, insulin levels were significantly higher than those in the 2-min samples, suggesting that a second-phase release had occurred.

In the same batches of cells in suspension, in response to 100 μM DiC8, a small but significant increase in $[Ca^{2+}]_i$ was already apparent at 5 s, which remained at a plateau until 30 s (Fig. 7). This was followed by a second-phase increase, which was maximal at 60 and 80 s and returned toward the basal level by 120 s. A large third-phase increase followed.

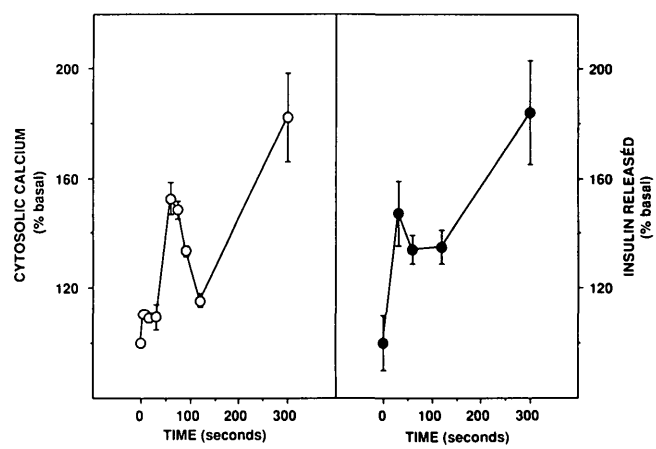

FIG. 7. Time course of changes in cytosolic concentrations of free Ca^{2+} ($[Ca^{2+}]_i$) and release of insulin during static incubations in response to 100 μM dioctanoylglycerol (DiC8; n = 3–4). For details, see METHODS.

To assess the contribution of the activation of PKC to DiC8-induced insulin secretion, three experiments were conducted in which anchored cells were incubated in KHB containing 100 nM staurosporine or vehicle (0.1% DMSO) for 5 min and then stimulated with 100 μM DiC8 for an additional 5 min. In the presence of staurosporine, DiC8-induced insulin release was similar to that observed in the presence of the vehicle alone (the increments above basal were 86 ± 19 and 78 ± 17%, respectively).

DISCUSSION

Our results suggest that, in insulin-secreting islet cells, DAG evokes multiphasic increases in $[Ca^{2+}]_i$ and that mechanisms in addition to the activation of PKC are involved in this action.

The pattern of DiC8-induced increases in $[Ca^{2+}]_i$ in the HIT T-15 cells was complex. The rapid first phase was small and frequently masked by the prominent second phase. The third phase occurred at high concentrations of DiC8 and persisted for the duration of our observations, suggesting that this last phase may represent a nonspecific irreversible alteration in the cell membrane induced by DiC8. In this study, we focused on the second phase of the DiC8-induced increase in $[Ca^{2+}]_i$, because it occurred consistently in a wide range of concentrations, was detectable fluorometrically with confidence even at a concentration of 10 μM, was reproducible in the same batch of cells on repetitive administration in a dose-dependent manner, and displayed a pattern consistent with a biological phenomenon.

By convention, the increases in $[Ca^{2+}]_i$ seen in conjunction with stimulation of phosphoinositide turnover have been attributed to the generation of IP_3. Our observation that exogenous DiC8 evokes consistent increases in $[Ca^{2+}]_i$ in HIT T-15 islet cells invites a reassessment of the mechanisms of action of the individual biologically potent metabolites of phospholipids. DAG generated concurrently with IP_3 could be responsible in part for the increases in $[Ca^{2+}]_i$, which occur on stimulation of phosphoinositide turnover. The increases in $[Ca^{2+}]_i$ in response to analogues of DAG, which had been observed in different cell types (10,19–21), indicate that our findings are not merely aberrant responses occurring in an abnormal cell line.

We observed increases in $[Ca^{2+}]_i$ in response to DiC8 within a concentration range in which the activation of PKC also occurred. Seemingly, a DAG can activate both signal pathways simultaneously.

In studying the contribution of PKC to cell regulation, phorbol esters such as TPA have been used as a convenient substitute for DAG, the endogenous activator of PKC. In our study, TPA and DiC8 induced the translocation of PKC to the cell membrane, indicative of the activation of PKC. Because DiC8 induced the activation of PKC within 5 s and before the second-phase increase in $[Ca^{2+}]_i$, we considered the possibility that the activation of PKC may have been responsible for the rise in $[Ca^{2+}]_i$. The following lines of evidence indicate that the activation of PKC did not play a mediating role in the increases in $[Ca^{2+}]_i$ induced by DiC8: *1*) TPA did not evoke any increase in $[Ca^{2+}]_i$. *2*) Inhibitors of PKC did not inhibit the DiC8-induced increases in

$[Ca^{2+}]_i$. 3) DiC8-induced increases in $[Ca^{2+}]_i$ also occurred in PKC-depleted cells.

An increase in $[Ca^{2+}]_i$ could represent either influx from the extracellular space or mobilization from an intracellular organelle. The second-phase increase in $[Ca^{2+}]_i$, which we observed in HIT T-15 cells in response to DiC8, is likely to be due to intracellular mobilization, because it still occurred when extracellular Ca^{2+} had been depleted with EGTA or when voltage-dependent Ca^{2+} channels had been inhibited by verapamil. On the other hand, OAG-induced rise in $[Ca^{2+}]_i$ was inhibitable by EGTA but not by verapamil, thus representing Ca^{2+} influx via a verapamil-insensitive channel. Thus, DAGs appear to induce increases in $[Ca^{2+}]_i$ by several mechanisms in HIT T-15 cells. The differences in the patterns of the increases in $[Ca^{2+}]_i$ in response to DiC8 and OAG are consistent with this interpretation.

Three immediate metabolites of DiC8, namely octanoic acid, monooctanoylglycerol, and glycerol, did not evoke any increase in $[Ca^{2+}]_i$, even at concentrations higher than those likely to be formed from DiC8. This observation reduces the likelihood that the effect of DiC8 was mediated by one of its metabolites. DAG kinase facilitates the metabolism of DAGs to phosphatidic acid, an endogenous Ca^{2+} ionophore (41,42). We do not believe that the increases in $[Ca^{2+}]_i$ we observed could be attributed to generation of phosphatidic acid from DiC8, because two chemically unrelated inhibitors of DAG kinase, monooleoylglycerol and R59022, evoked increases rather than decreases in $[Ca^{2+}]_i$. In fact, the rise in $[Ca^{2+}]_i$ observed with these agents could be due to accumulation of endogenous DAG resulting from inhibition of DAG kinase. In any case, the increase in $[Ca^{2+}]_i$ observed with these compounds represented Ca^{2+} influx rather than intracellular mobilization, thus resembling mechanistically OAG and not DiC8.

DAG activates phospholipases (43–45). Thus, the rise in $[Ca^{2+}]_i$ observed in response to DiC8 could have been mediated by IP_3 derived from endogenous phospholipid pools. If so, levels of endogenous IP_3 should have increased rapidly on administration of DiC8. The fact that we did not observe any significant change in the IP_3 levels after monitoring them over a period long enough for such a rise to occur weakens this likelihood.

DiC8 induced biphasic release of insulin. Our findings are not sufficiently conclusive regarding the roles of the accompanying increases in PKC activity and in $[Ca^{2+}]_i$ in either phase of insulin release. The fact that staurosporine, an inhibitor of the activation of PKC, was without effect weakens the likelihood that PKC was involved. The phase-1 increase in $[Ca^{2+}]_i$, although sufficiently rapid to have contributed to the early phase of insulin secretion, was minor. On the other hand, the major phase-2 increase in $[Ca^{2+}]_i$ coincided with the transition from the early into the late phase of insulin secretion; it may have contributed to the late-phase release. This interpretation is confounded by the fact that the data on insulin secretion were obtained under static incubation conditions with anchored cells, whereas detached cells had to be used for the PKC and $[Ca^{2+}]_i$ assays.

We conclude that, in the HIT T-15 islet cell line, the DAG analogue DiC8 evokes multiphasic increases in $[Ca^{2+}]_i$ in addition to and independent of PKC activation. The second and major phase of the increase is due to mobilization of Ca^{2+} from an intracellular compartment into the cytosol by a PKC-independent mechanism. Whether the first and third phases of DiC8-induced increases in $[Ca^{2+}]_i$ represent influx or mobilization of Ca^{2+} is indeterminate; in the case of OAG, the increase in $[Ca^{2+}]_i$ was clearly due to influx. Our results invite caution in the use of exogenous synthetic DAGs as tools for assessing PKC function. Changes in the generation of DAGs must be included among the mechanisms by which Ca^{2+} homeostasis is regulated in islet cells. Endogenous DAGs may amplify insulin secretion not only via the activation of PKC but also via increases in $[Ca^{2+}]_i$.

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