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Diacylglycerol Mediates the T-Cell Receptor-Driven Ca^{2+} Influx in T Cells by a Novel Mechanism Independent of Protein Kinase C Activation

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Abstract The mechanism of Ca^{2+} influx in nonexcitable cells is not known yet. According to the capacitative hypothesis, Ca^{2+} influx is triggered by IP_3-mediated Ca^{2+} release from the intracellular Ca^{2+} stores. Conversely, many workers have reported a lack of association between release and influx. In this work, the role of diacylglycerol (DAG) as the mediator of T-cell receptor (TCR)-driven Ca^{2+} influx in T cells was investigated. Stimulation of mouse splenic T cells with naturally occurring DAG caused Ca^{2+} entry in a dose- and time-dependent manner. Such stimulation was blocked by Ni^{2+}, a divalent cation known to block Ca^{2+} channels. Inhibition of protein kinase C (PKC) by calphostin C did not inhibit, but slightly enhanced, the DAG-stimulated Ca^{2+} entry. However, inhibition of DAG metabolism by DAG kinase and lipase inhibitors enhanced the DAG-stimulated Ca^{2+} entry. DAG lipase and kinase inhibitors also enhanced the Ca^{2+} entry in T cells stimulated through TCR/CD3 complex with anti-CD3 antibody. Calphostin C did not affect the anti-CD3-stimulated Ca^{2+} entry. These results showed that TCR-driven Ca^{2+} influx in T cells is mediated by DAG through a novel mechanism(s) independent of PKC activation. J. Cell. Biochem. 78:222–230, 2000.

Key words: Ca^{2+} influx; diacylglycerol; T cells

One of the challenging issues in cell biology is the mechanism of agonist-stimulated Ca^{2+} influx in nonexcitable cells. In these cells, activation of phosphoinositide signaling pathway by many agonists results in the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). This is followed by a biphasic increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), a transient phase caused by IP_3-stimulated release of Ca^{2+} from the intracellular stores followed by a sustained phase caused by Ca^{2+} influx into the cell [for review see Berridge, 1993].

Although the mechanism of IP_3-mediated Ca^{2+} release is relatively better understood, enigma remains regarding the mechanism of Ca^{2+} influx. Intense research is going on to understand this. The widely accepted capacitative hypothesis states that IP_3-mediated emptying of Ca^{2+} store triggers the Ca^{2+} influx [Putney, 1990]. A serious weakness of this model is that the mechanism whereby empty store triggers Ca^{2+} influx has not been identified and characterized yet. As opposed to this hypothesis, it has been shown that the blocking of IP_3-mediated Ca^{2+} release by heparin in vascular smooth-muscle cells, Xenopus oocyte, and Chinese hamster ovary cells [Huang et al., 1991; Lupu-Meiri et al., 1994; Mathias et al., 1997], and by IP_3 receptor blocker TMB-8 in T cells [Chakrabarti et al., 1995] did not inhibit Ca^{2+} influx. However, inhibition of PIP_2 hydrolysis suppressed Ca^{2+} influx in these cells [Huang et al., 1991; Chakrabarti et al., 1995]. Furthermore, it has been shown that bradykinin stimulated Ca^{2+} influx in NG108-15 cells independent of the state of IP_3-sensitive Ca^{2+} store [Tak-Man and Thayer, 1993]. More recently, it has been shown that emptying of the ryanodine-sensitive Ca^{2+} stores (overlapping with IP_3-sensitive stores) did not induce Ca^{2+} influx in endothelium cells [Sasajima et al., 1997]. In addition, it has been clearly shown

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that thapsigargin, a microsomal Ca^{2+}-ATPase inhibitor commonly used to demonstrate capacitative Ca^{2+} entry:

1. stimulated Ca^{2+} influx in NG108-15 and T cells by mechanism independent of emptying of the Ca^{2+} stores [Tak-Man and Thayer, 1993; Chakrabarti et al., 1995].
2. failed to stimulate Ca^{2+} entry in many cells [Jackson et al., 1988; Ghosh et al., 1991; Bian et al., 1991; Palmer et al., 1994]. and
3. involved a functionally distinct entity in inducing Ca^{2+} influx as compared to that induced by receptor activation in T cells [Sei et al., 1995].

Lack of dependence of Ca^{2+} influx on IP_3-mediated emptying of Ca^{2+} stores, but on PIP_2 hydrolysis, indicated the direct involvement of one of the second messengers in Ca^{2+} influx. In view of this, as stated in the second messenger hypothesis [for review see Irvine, 1992], inositol phosphates may be considered as the likely candidates. However, no clear-cut and consistent experimental evidence, in terms of identity of the compounds and their mode of operation, exists to support this hypothesis. This leaves the other second messenger DAG as the likely candidate for the Ca^{2+} influx factor. It has been reported that a DAG-like molecule, 1-*O*-alkyl-2-acetyl-*sn*-glycerol, can induce Ca^{2+} entry in bronchial epithelial cells [Stoll et al., 1994], and a DAG analogue dioctanoylglycerol caused Ca^{2+} release from the intracellular store and influx from outside [Ebanks et al., 1989]. These findings strengthen the possibility that endogenous DAG may function as a Ca^{2+} influx factor. However, to date, a physiological role of endogenously produced DAG as the mediator of agonist-stimulated Ca^{2+} influx has not been documented.

In the present study, we explored the Ca^{2+} influxing effect of endogenous second-messenger DAG and its involvement in agonist-stimulated Ca^{2+} influx in T cells. Our results showed that DAG mediated the T-cell receptor (TCR)-driven Ca^{2+} influx in T cells by a novel mechanism independent of protein kinase C (PKC) activation.

MATERIALS AND METHODS

Materials

Diacylglycerols 1-stearoyl-2-arachidonoyl-*sn*-glycerol (SAG) and 1-stearoyl-2-linoleoyl-*sn*-glycerol (SLG), DAG kinase inhibitor R59022, DAG lipase inhibitor RHC80267, and PKC inhibitor calphostin C were purchased from Calbiochem-Novabiochem Intl., San Diego, CA. $^{45}Ca^{2+}$ (specific activity 64.8 mCi/g) was from Bhabha Atomic Research Center, Bombay, India. Antibody to the epsilon (ε) subunit of mouse CD3 complex anti-CD3 (αCD3) was bought from Boehringer Mannheim, Indianapolis, IN. All other materials were purchased from Sigma-Aldrich, St. Louis, MO. Hanks balanced salt solution (HBSS), used in this study, was buffered with 25 mM HEPES and 0.035% $NaHCO_3$.

Measurement of $^{45}Ca^{2+}$ Entry in T Cells

Splenic T cells were isolated in HBSS from balb/c mice by plastic adherence and nylon wool column method as described before [Chakrabarti et al., 1995]. Cells (1×10^6) were aliquoted in each well of a 96-well microtiter plate and were incubated for 1 min at 37°C in a water bath. Reaction started by adding prewarmed reagents along with $^{45}Ca^{2+}$ ($\sim 8 \times 10^6$ cpm/well) to a final concentration of 1.3 mM. After incubation for the appropriate periods, intact cells were harvested and washed with saline on glass-fiber filter by PHD cell harvester. The filter discs were dried, and uptake of radioactivity by the cells was measured by a scintillation counter. Ca^{2+} influx into the cells was indicated from $^{45}Ca^{2+}$ entry and expressed as Ca^{2+} entry (pmol per 10^6 cells), which was calculated as cpm per 10^6 cells/cpm per pmol Ca^{2+}.

Determination of Cell Viability

The viability of T cells, after various treatments, was measured by MTT assay [Mosmann, 1983]. Cells were incubated with 2.5 mg/ml MTT in RPMI (supplemented with 10% heat inactivated fetal calf serum) for 4 h at 37°C. After the cells were washed with normal saline, the formazone crystals were solubilized in acidic isopropanol (with 0.04 N HCl). The absorbance of the solution was measured at 490 nm in an automated enzyme-linked immunosorbent assay reader (Molecular device). The viability was calculated as % viability = 100 × absorbance of treated sample/absorbance of untreated sample.

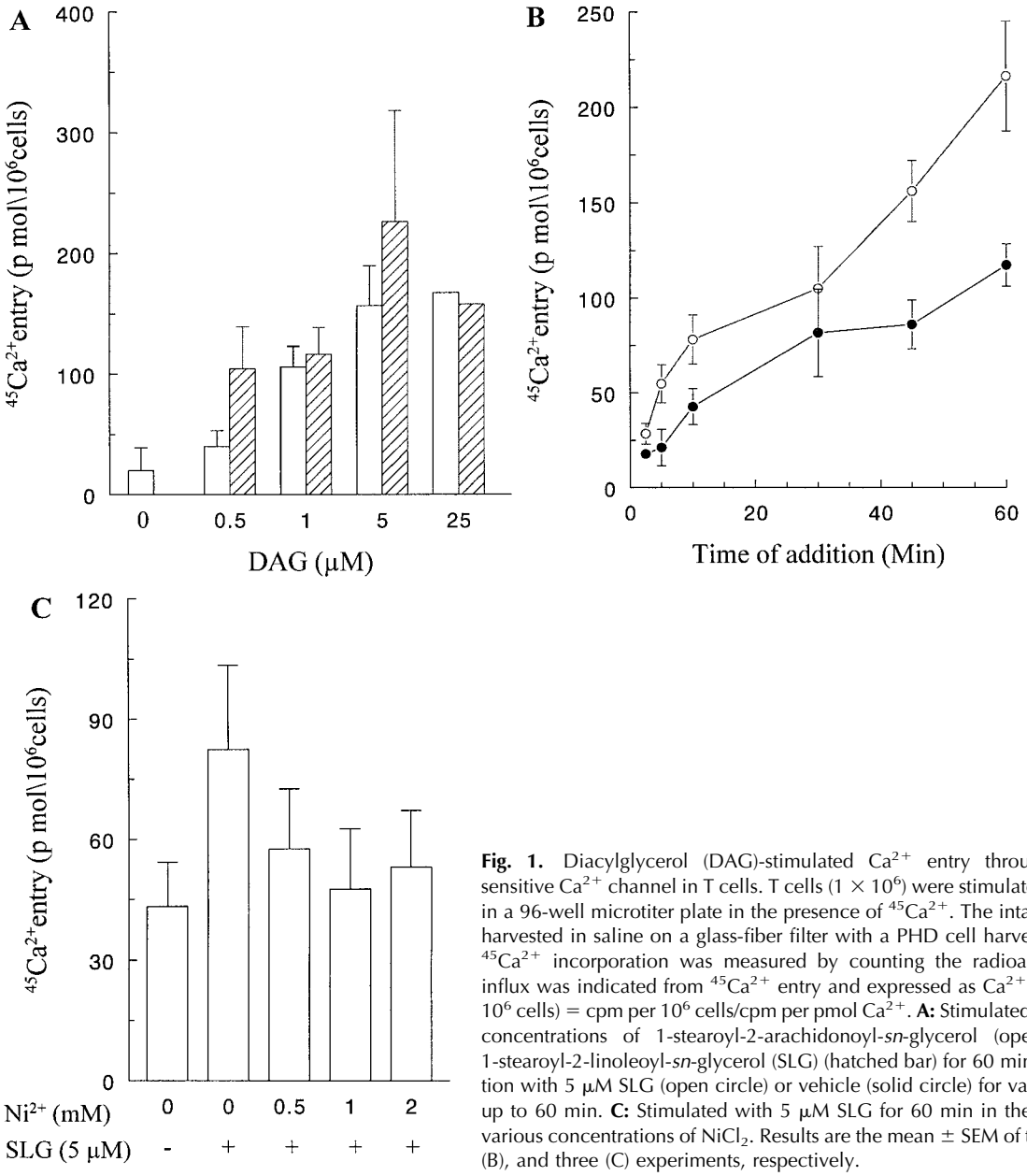

Fig. 1. Diacylglycerol (DAG)-stimulated Ca^{2+} entry through a Ni^{2+}-sensitive Ca^{2+} channel in T cells. T cells (1×10^6) were stimulated with DAG in a 96-well microtiter plate in the presence of $^{45}Ca^{2+}$. The intact cells were harvested in saline on a glass-fiber filter with a PHD cell harvester, and the $^{45}Ca^{2+}$ incorporation was measured by counting the radioactivity. Ca^{2+} influx was indicated from $^{45}Ca^{2+}$ entry and expressed as Ca^{2+} entry (pmol/10^6 cells) = cpm per 10^6 cells/cpm per pmol Ca^{2+}. **A:** Stimulated with various concentrations of 1-stearoyl-2-arachidonoyl-*sn*-glycerol (open bar) and 1-stearoyl-2-linoleoyl-*sn*-glycerol (SLG) (hatched bar) for 60 min. **B:** Stimulation with 5 μM SLG (open circle) or vehicle (solid circle) for various periods up to 60 min. **C:** Stimulated with 5 μM SLG for 60 min in the presence of various concentrations of $NiCl_2$. Results are the mean ± SEM of three (A), five (B), and three (C) experiments, respectively.

RESULTS

Diacylglycerol Stimulated Ca^{2+} Entry Through a Ni^{2+}-Sensitive Channel in T Cells

The stimulatory effect of DAG on Ca^{2+} influx in T cells was investigated using two naturally occurring DAGs, SAG and SLG. We used $^{45}Ca^{2+}$ specifically, instead of fluorescence Ca^{2+} indicator, to ascertain that any increase in intracellular Ca^{2+} is caused by the Ca^{2+} influx from outside but not caused by Ca^{2+} mobilization from any other sources. T cells were stimulated for 60 min with various concentrations of DAG in the presence of $^{45}Ca^{2+}$. Both of the compounds induced Ca^{2+} entry into the cells in a dose-dependent manner (Fig. 1A). The maximum influx occurred with 5 μM of both the compounds, 156.7 ± 33 and 226 ± 92 pmol/10^6 cells with SAG and SLG, respectively. However, at higher concentrations of DAG, the response started to decline (Fig. 1A), without any change in cell viability (data not shown). Because SLG was more potent than SAG and later has the potential to liberate arachidonic acid generating several lipid mediators, known

to be involved in lymphocyte activation through the activation or inhibition of different signaling pathways [for review see Hadden, 1988], only SLG was used in all subsequent experiments. Study of the time course of stimulation revealed that DAG-stimulated Ca^{2+} entry was detectable significantly over background entry as early as by 5 min, which increased steadily up to 60 min of stimulation (Fig. 1B). In many other experiments, we found that stimulation for more than 60 min resulted in the gradual decline in the Ca^{2+} entry relative to the background without affecting the cell viability (data not shown).

The authenticity of Ca^{2+} influx pathway was ascertained by testing the effect of Ni^{2+} on the DAG-stimulated $^{45}Ca^{2+}$ entry. That cation is known to block Ca^{2+} channel in various cells including T cells [Lewis and Cahalan, 1989; Chow and Jondal, 1990; Haverstick and Gray, 1993; Premack et al., 1994; Chakrabarti et al., 1995]. As shown in Figure 1C, Ni^{2+} inhibited the DAG-stimulated $^{45}Ca^{2+}$ entry in T cells in a dose-dependent manner without affecting the cell viability. Ni^{2+} did not affect the basal entry of Ca^{2+} in cells (data not shown).

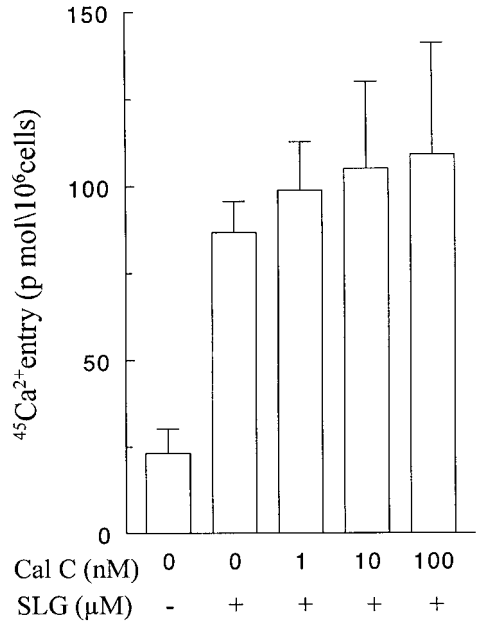

Fig. 2. Effect of Calphostin C on diacylglycerol-stimulated Ca^{2+} entry in T cells. T cells were stimulated with 5 μM 1-stearoyl-2-linoleoyl-*sn*-glycerol (SLG) for 60 min in the presence of various concentrations of calphostin C (Cal C) and Ca^{2+} entry measured as in Fig. 1. The results are the mean ± SEM of five experiments.

Effect of PKC Inhibitor on DAG-Stimulated Ca^{2+} Entry in T Cells

Because DAG normally functions to activate PKC, the involvement of this kinase in DAG-stimulated Ca^{2+} entry was determined using a specific PKC inhibitor, calphostin C (Cal C). T cells were stimulated with 5 μM DAG in the presence or absence of Cal C in ambient light, because light is required to activate Cal C. The inhibitor did not inhibit the DAG-stimulated Ca^{2+} entry, but only a slight enhancement at higher dose (1.3-folds at 100 nM) was obtained (Fig. 2). The effectiveness of Cal C on T cell was evident from its light-dependent inhibitory effect on mitogen-induced T cell proliferation (data not shown). This result showed that DAG-stimulated Ca^{2+} entry in T cells is independent of activation of PKC.

Effect of DAG Lipase Inhibitor on DAG-Stimulated Ca^{2+} Entry

Because DAG stimulated Ca^{2+} entry independent of PKC activation (Fig. 2), the involvement of other DAG metabolites was explored in an effort to understand how DAG might have stimulated Ca^{2+} influx. One of the two major metabolic pathways of DAG in the cell is its degradation to monoacylglycerol (MAG) and subsequently to glycerol catalyzed by the enzyme DAG lipase and MAG lipase sequentially [Ford and Gross, 1990; Asaoka et al., 1991; Florin-Christensen et al., 1992; Konard et al., 1994]. To assess the involvement of this pathway in DAG-stimulated Ca^{2+} influx, a specific DAG lipase inhibitor RHC80267 was used. The inhibitor enhanced the DAG-stimulated Ca^{2+} entry in a dose-dependent manner (Fig. 3A). A significant enhancement (1.5-fold) was detectable at a concentration of 1 nM, which became maximum (~2-fold) at 5 nM concentration of the inhibitor and remained steady thereafter. The inhibitor itself had no effect on the $^{45}Ca^{2+}$ level in the cells (data not shown).

Effect of DAG Kinase Inhibitor on DAG-Stimulated Ca^{2+} Entry

The other major metabolic pathway of DAG is its conversion to phosphatidic acid, catalyzed by the enzyme DAG kinase [Billah et al., 1989; Ford and Gross, 1990; Asaoka et al., 1991; Lee et al., 1991; Florin-Christensen et al., 1992; Rubin et al., 1992]. Involvement of this path-

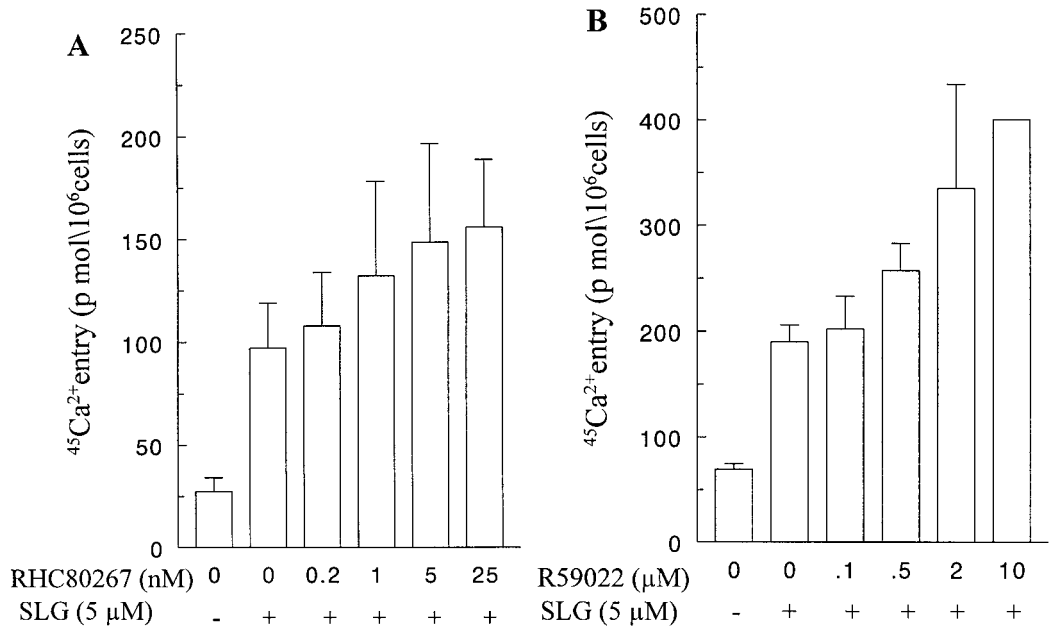

Fig. 3. Enhancement of diacylglycerol (DAG)-stimulated Ca^{2+} influx in T cells by DAG lipase and kinase inhibitors. T cells were stimulated with 5 μM 1-stearoyl-2-linoleoyl-*sn*-glycerol (SLG) for 60 min in the presence of various concentrations of DAG lipase inhibitor RHC80267 (**A**) and DAG kinase inhibitor R59022 (**B**) and Ca^{2+} entry measured as in Fig. 1. The results are the mean ± SEM of seven (A) and three (B) experiments, respectively.

way in DAG-stimulated Ca^{2+} influx was probed using the specific DAG kinase inhibitor R59022. The inhibitor drastically enhanced the DAG-stimulated Ca^{2+} entry in T cells in a dose-dependent manner (Fig. 3B). The enhancing effect was significantly detectable (1.6-fold) at 0.5 μM and became maximum (~3-fold) at 10 μM concentration of the inhibitor. R59022 itself did not cause any Ca^{2+} influx in the cells (data not shown).

DAG-Mediated TCR-Driven Ca^{2+} Influx in T Cells

The TCR is an antigen receptor on the T-cell surface in close physical association with multimeric protein complex CD3 (TCR/CD3), which is required to send the activation signal into the cell on occupancy of TCR with antigen. The action of the antigen can be mimicked by antibodies to CD3 or TCR. Thus, in this study, T cells were stimulated with a monoclonal antibody to mouse CD3ε subunit (αCD3), known to induce Ca^{2+} release and influx, DNA synthesis, and cytotoxic function in mouse T cells [Leo et al., 1987; Chakrabarti et al., 1995]. The antibody caused a net maximum Ca^{2+} entry (~2.5-fold over unstimulated control) at 1 μg/ml by 10 min of stimulation, beyond which it started to decline (Fig. 4A,B, respectively), perhaps because of known agonist-induced rapid internalization of TCR/CD3 complex. To determine the involvement of endogenous DAG in the αCD3-stimulated Ca^{2+} influx, we investigated whether RHC80267 and R59022 can affect the αCD3-stimulated Ca^{2+} entry the same way as they affected the DAG-stimulated Ca^{2+} entry. Our results showed that both RHC80267 and R59022 enhanced the αCD3-stimulated Ca^{2+} entry in T cells (Fig. 4C). RHC80267 caused 1.9-fold and 2.2-fold enhancement at 1 nM and 5 nM concentrations, respectively, which is almost the same as the enhancement of DAG-stimulated Ca^{2+} entry. With R59022, enhancement (2.3-fold) was observed at 10 μM but not at 2 μM concentration. As expected, the PKC inhibitor Cal C neither inhibited nor enhanced the αCD3-stimulated Ca^{2+} influx (Fig. 4C).

DISCUSSION

In the present study, we provided a simple but clear insight into the nature of the factor mediating the TCR-driven Ca^{2+} influx in T cells. We have presented results showing

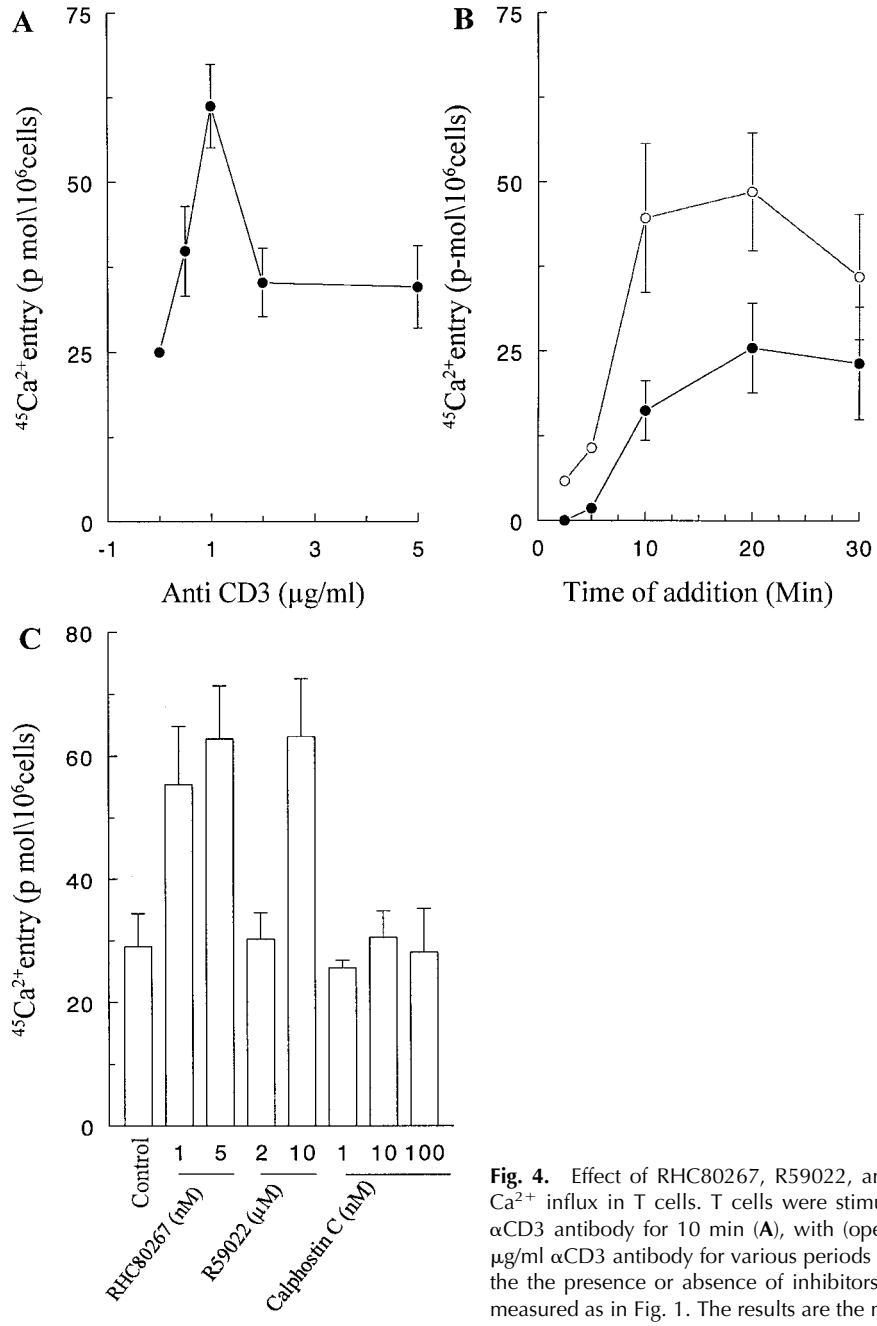

Fig. 4. Effect of RHC80267, R59022, and Calphostin C on αCD3-stimulated Ca^{2+} influx in T cells. T cells were stimulated with various concentrations of αCD3 antibody for 10 min (**A**), with (open circle) and without (solid circle) 1 μg/ml αCD3 antibody for various periods (**B**) or with 1 μg/ml αCD3 antibody in the the presence or absence of inhibitors (**C**) for 10 min and with Ca^{2+} entry measured as in Fig. 1. The results are the mean ± SEM of the three experiments.

that DAG stimulated Ca^{2+} influx in T cells in a dose- and time-dependent manner. The possibility of a nonspecific action of DAG was ruled out from our observations that the DAG-stimulated Ca^{2+} entry was blocked by Ni^{2+} and was attenuated at higher concentration of DAG (25 μM) without any change in the cell viability. A nonspecific Ca^{2+} entry is not expected to be blocked by a Ca^{2+} channel blocker and attenuated with increasing concentration of the stimulant. Thus, these results clearly established that naturally occurring DAG can stimulate Ca^{2+} influx in T cells, specifically through an authentic Ca^{2+} channel. The concentration of DAG in the cytosol to be attained by 5 μM exogenous DAG (causing maximum Ca^{2+} influx) would be much less than the physiological level of DAG required for PKC activation, because at least a 30 μM and 100 μM exogenous DAG

are required for significant activation of PKC and interleukin-2 receptor expression, respectively, in T cells [Szamel et al., 1989; Asaoka et al., 1991]. This indicates that DAG-stimulated Ca^{2+} influx in T cells is independent of PKC activation, which is further supported by our finding that Cal C (specific PKC inhibitor) did not block DAG-induced Ca^{2+} influx. The decline in Ca^{2+} entry at higher concentrations of DAG may be caused by the intense activation of PKC, because it has been reported that intense PKC activation by pharmacological agents (phorbol esters) suppressed agonist-stimulated Ca^{2+} influx in T cells and neutrophils [Konard et al., 1994; Tuominen et al., 1994; Sei et al., 1995; Haverstick et al., 1997].

In the present study, we observed Ca^{2+} entry as early as 5 min of stimulation with DAG, but for a very strong signal we have to allow sufficient time (60 min) for Ca^{2+} entry in a maximum number of T cells in the population. That was because, unlike using an intracellular fluorescent Ca^{2+} indicator, which instantly and continuously records every change in $[Ca^{2+}]_i$, $^{45}Ca^{2+}$ entry can be measured only after washing the cells at given time points after stimulation. Thus, as soon as the stimulus is removed during washing, the $[Ca^{2+}]_i$ tends to go back to the basal level rapidly by virtue of the presence of a highly active Ca^{2+} pump in the plasma membrane and Ca^{2+} stores, resulting in the weakening of the signal. It is also possible that during prolonged stimulation (1) the activated PKC caused the secretion of some autocrine factors that acted on the T cells to stimulate Ca^{2+} influx; or (2) DAG did not stimulate Ca^{2+} influx into cytosol, but stimulated the uptake of $^{45}Ca^{2+}$ by endoplasmic reticulum from cytosol. The first possibility was ruled out based on our finding that PKC inhibitor did not affect the DAG-stimulated Ca^{2+} influx, and the second one was ruled out based on the finding that Ni^{2+} inhibited only the DAG-stimulated but not the basal increase in intracellular $^{45}Ca^{2+}$.

DAG is known to function through the activation of PKC. However, our results showed that DAG-stimulated Ca^{2+} influx was independent of PKC activation. Thus, we explored whether metabolic conversion of DAG through two major metabolic pathways, DAG kinase and lipase pathways, was linked to Ca^{2+} influx. We found that both the DAG kinase and DAG lipase inhibitors enhanced the DAG-stimulated Ca^{2+} influx in T cells, showing that conversion of DAG through neither of these pathways was essential in stimulating Ca^{2+} influx. Rather, inhibition of these metabolic pathways made DAG more available to the mechanism regulating Ca^{2+} influx.

Thus far, all the results showed that DAG stimulated Ca^{2+} influx in T cells by a novel mechanism, independent of PKC activation and DAG metabolism through the major metabolic pathways. To establish the role of endogenous DAG as the mediator of receptor-driven Ca^{2+} influx, we examined whether DAG kinase and DAG lipase can modulate the αCD3-stimulated Ca^{2+} influx the same way as they modulated the DAG-stimulated Ca^{2+} influx. Our results clearly showed that both the DAG kinase (R59022) and DAG lipase (RHC80267) inhibitor enhanced the αCD3-stimulated Ca^{2+} influx. Unlike RHC80267, the dose of R59022 required to enhance αCD3- and DAG-stimulated Ca^{2+} entry was different. Although 2 μM R59022 caused almost maximum enhancement of DAG-stimulated Ca^{2+} entry, a dose of 10 μM was required to cause significant enhancement of αCD3-stimulated Ca^{2+} influx. This difference is attributed to the fact that stimulation of T cells through the TCR/CD3 complex increases the DAG kinase activity [van der Bend et al., 1994]. Conceivably, a higher concentration of R59022 will be required for the significant inhibition of the increased activity of DAG kinase. That is why more R59022 was required to enhance αCD3-stimulated Ca^{2+} influx as compared to DAG-stimulated influx. The PKC inhibitor Cal C did not have any inhibitory or enhancing effect, indicating the noninvolvement of PKC in αCD3-stimulated Ca^{2+} entry. This is supported by the finding that PKC inhibitor did not affect the agonist-stimulated Ca^{2+} influx in neutrophils [Tuominen et al., 1994]. Based on these results, we propose that TCR-driven Ca^{2+} influx in T cells is mediated by DAG through a novel mechanism independent of PKC activation and metabolism through DAG kinase and lipase pathways.

The results presented here have clearly established, for the first time, the second-messenger DAG as a potential Ca^{2+} influx factor or a precursor for that influx factor in T

cells, and thus opened a new definitive direction to study the mechanism of Ca^{2+} influx in nonexcitable cells.

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