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[8-(Diethylamino)Octyl-3,4,5-Trimethoxybenzoate, HCl], the Inhibitor of Intracellular Calcium Mobilization, Blocked Mitogen-Induced T Cell Proliferation by Interfering With the Sustained Phase of Protein Kinase C Activation

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Abstract The physiological role of IP_3-dependent Ca^{2+} release in T cell activation was in question due to the contradictory findings that [8-(Diethylamino)octyl-3,4,5-trimethoxybenzoate, HCl] (TMB-8), an inhibitor of intracellular Ca^{2+} mobilization, blocked T cell proliferation, curtailing specifically the level of released Ca^{2+} did not affect T cell activation and T cell line lacking IP_3 receptor was defective in IL-2 production in response to TCR/CD3 ligand. In the present study we found that TMB-8 inhibited Concanavalin A (Con A)- but not PMA/Ionomycin-induced T cell proliferation in a reversible and dose-dependent manner. The kinetic study revealed that TMB-8 exerted the inhibitory effect at a very early step of T cell activation. The Ca^{2+} ionophore ionomycin augmented instead of overcoming the inhibitory effect of TMB-8, although the same doses of ionomycin alone had no effect on Con A-induced T cell proliferation. PMA the metabolically stable, but not diacylglycerol (DAG) the metabolically labile, activator of protein Kinase C (PKC) completely overcome the antiproliferative effect of TMB-8. A specific DAG lipase inhibitor RHC80267 also overcome the effect of TMB-8. Taken together, these results showed that the process of Ca^{2+} release through IP_3 receptor, not the released Ca^{2+}, is essential for the sustained phase of PKC activation during T cell proliferation. J. Cell. Biochem. 76:539–547, 2000.

Key words: Ca^{2+} release; IP_3; PKC; TMB-8; T cell proliferation

T cells possess on their surface clonotypic T cell receptor (TCR) in physical association with an invariant protein complex, CD3. In the TCR/CD3 complex, TCR recognizes the antigen and CD3 sends that signal into the cell, triggering a cascade of biochemical events leading to T cell activation and proliferation [for review see Imboden, 1988; Premack and Gardner, 1992]. The landmarks of that signaling cascade are the activation of protein kinase C (PKC) and a biphasic rise of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), due to inositol trisphosphate (IP_3)-dependent release from the intracellular stores followed by an influx from outside [Imboden, 1988; Premack and Gardner, 1992]. The effect of antigen on the T cell can be mimicked by mitogenic lectins and antibodies to TCR/CD3 complex [Premack and Gardner, 1992]. The absolute necessity of increase in $[Ca^{2+}]_i$ for T cell activation was evident from several findings, such as extracellular Ca^{2+}-dependent mitogenic effect of Ca^{2+} ionophore on lymphocytes [Luckasen et al., 1974; Maino et al., 1974], activation or expression of several proteins involved in T cell activation by an increase in $[Ca^{2+}]_i$ [Premack and Gardner, 1992], and inhibition of IL-2 secretion and T cell proliferation by preventing Ca^{2+} influx with extracellular Ca^{2+} chelators or Ca^{2+} channel blockers [Mills et al., 1985; Gelfand et al., 1986].

While the studies noted above unequivocally document the necessity of increase in $[Ca^{2+}]_i$, due to Ca^{2+} influx in T cell activation, a question remains regarding the importance of Ca^{2+} release in the process. According to the capacitative model of Ca^{2+} influx [Putney, 1990], it may be argued that Ca^{2+} release is required to trigger Ca^{2+} influx. However, it has been clearly demonstrated in many cells, including T cells,

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that Ca^{2+} influx is not triggered by Ca^{2+} release [Huang et al., 1991; Chakrabarti et al., 1995; Mathias et al., 1997]. In one study, it has been shown that curtailing specifically the transient peak but not the sustained level of increased $[Ca^{2+}]_i$, by loading the cells with appropriate concentration of intracellular Ca^{2+} chelator BAPTA, did not suppress IL-2 production, IL-2 receptor (IL2R) expression, and proliferation in T cells [Gelfand et al., 1988]. These results were interpreted to suggest that the Ca^{2+} entry from outside but not the release from intracellular Ca^{2+} stores is essential for T cell activation. On the contrary, it has been shown that an inhibitor of intracellular Ca^{2+} mobilization (TMB-8) blocked mitogen-induced T cell proliferation and T cells lacking type1 IP_3 receptor were defective in IL2 production after TCR stimulation [Grier and Mastro, 1985; Jayaraman et al., 1995].

In this report we attempted to decipher the role of IP_3-dependent Ca^{2+} release in T cell activation. Our results showed that the process of the Ca^{2+} release through the IP_3-receptor, not the released Ca^{2+}, is essential for the sustained phase of PKC activation required for T cell proliferation.

MATERIALS AND METHODS

Reagents

Concanavalin A (Con A) was purchased from Pharmacia AB (Uppsala, Sweden). RPMI-1640 was from HiMedia Laboratories Limited (Mumbai, India). Diacylglycerol (1-stearoyl-2-linoleoyl-*sn*-glycerol), DAG lipase inhibitor RHC80267, Calphostin C, Ionomycin, and TMB-8 ([8-(Diethylamino)octyl-3,4,5-trimethoxybenzoate, HCl]) were purchased from Calbiochem-Novabiochem Intl. (San Diego, CA). [^{3}H]thymidine (specific activity 18,000 mCi/mmole) was from Bhabha Atomic Research Center (Mumbai, India). PMA (Phorbol 12-Myristate 13-Acetate), L-glutamine, and MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal Bovine Serum was purchased from Gibco BRL Life Technologies, Inc. (Grand Island, NY).

Cells and Culture Medium

T cells were isolated from the spleen of balb/C mice in RPMI-1640 medium supplemented with 2 mM L-glutamine, streptomycin (0.1 mg/ml), and Gentamycin (0.2 mg/ml) as described before [Chakrabarti et al., 1995]. Unless otherwise mentioned, isolated T cells were maintained and cultured in above RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (complete medium) at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cell Proliferation Assay

Proliferation of T cells was measured by the incorporation of [^{3}H]thymidine in the replicating DNA. T cells (1×10^5 cells/well in 0.1 ml) were stimulated with Con A or a combination of PMA and Ionomycin in a round bottom 96-well plate. The cells were pulsed with [^{3}H]thymidine (1 μCi/ml) at 24 h, harvested with a PHD cell harvester at 60 h of culture, and [^{3}H]thymidine incorporation was measured by a liquid scintillation counter. The effect of various agents on proliferation was expressed as the percentage of control proliferation = 100 × proliferation in the presence of the agent/proliferation in the absence of the agent (control proliferation).

Determination of Cell Viability

The viability of T cells, after treatment with various agents, was measured by MTT assay [Mosmann, 1983]. T cells were washed and incubated with 2.5 mg/ml MTT in complete RPMI medium for 4 h at 37°C in 5% CO_2 incubator. After washing the cells with normal saline, the formazone crystals formed were solubilized in acidic isopropanol (with 0.04 N HCl). The absorbance of the solution was measured at 492 nm in E^{max} automated ELISA reader (molecular device).

RESULTS

TMB-8 Inhibited Concanavalin A-Stimulated T Cell Proliferation

TMB-8 ([8-(Diethylamino)octyl-3,4,5-trimethoxybenzoate, HCl]) is an intracellular Ca^{2+} antagonist. It has been shown to specifically block agonist-stimulated Ca^{2+} release from intracellular Ca^{2+} stores without affecting Ca^{2+} influx in many cells including T cells [Chiou and Malagodi, 1975; Mix et al., 1984; Clapper and Lee, 1985; Erne and Pletscher, 1985; Donowitz et al., 1986; Chakrabarti et al., 1995]. It prevents Ca^{2+} mobilization (release) by stabilizing Ca^{2+} binding to the store [Chiou and Malagodi, 1975]. To determine the role of Ca^{2+} release in T cell proliferation, first the effect of TMB-8 on this process was examined. Freshly isolated T cells were pretreated with TMB-8 for

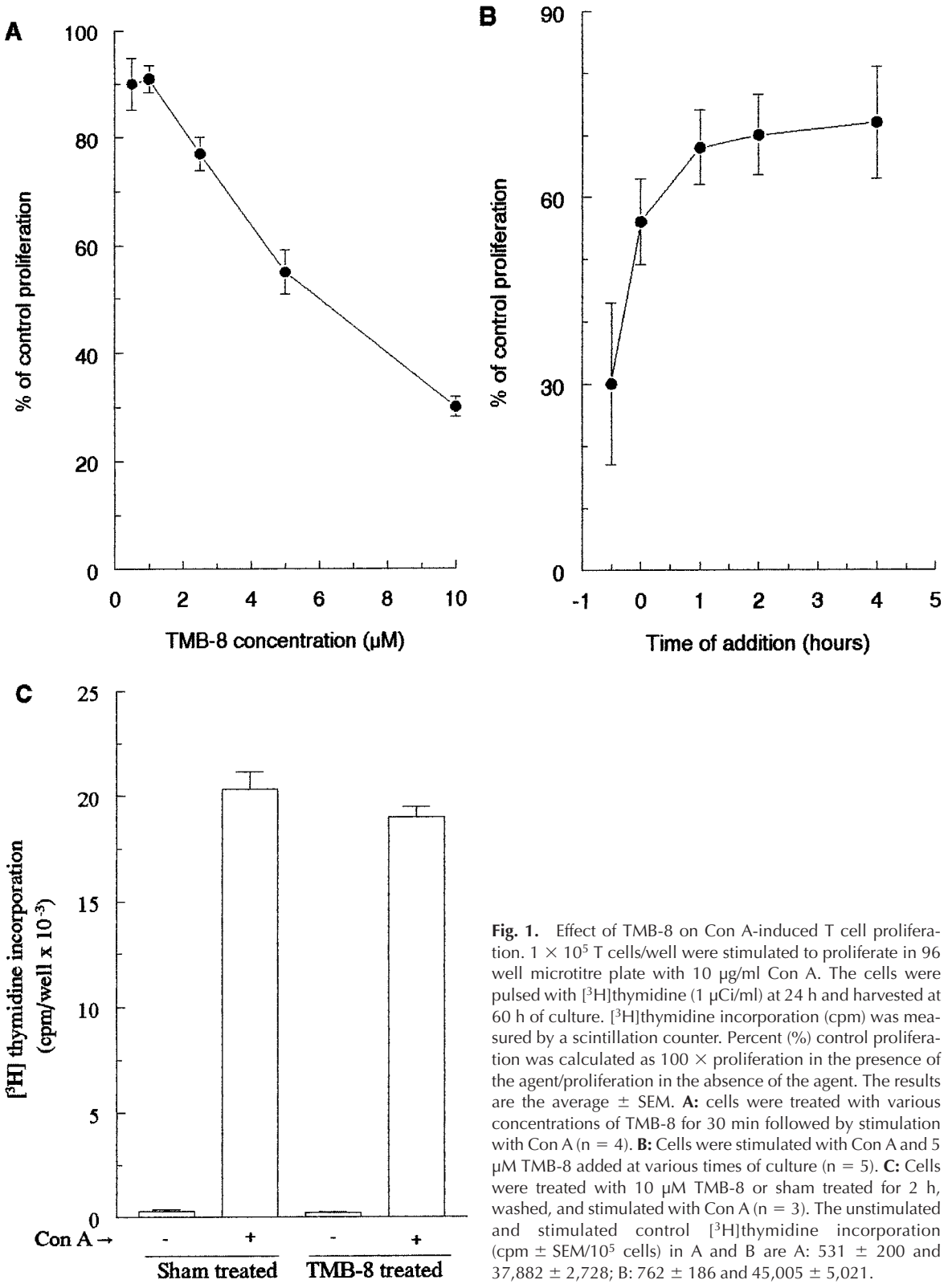

Fig. 1. Effect of TMB-8 on Con A-induced T cell proliferation. 1×10^5 T cells/well were stimulated to proliferate in 96 well microtitre plate with 10 μg/ml Con A. The cells were pulsed with [^{3}H]thymidine (1 μCi/ml) at 24 h and harvested at 60 h of culture. [^{3}H]thymidine incorporation (cpm) was measured by a scintillation counter. Percent (%) control proliferation was calculated as 100 × proliferation in the presence of the agent/proliferation in the absence of the agent. The results are the average ± SEM. **A:** cells were treated with various concentrations of TMB-8 for 30 min followed by stimulation with Con A (n = 4). **B:** Cells were stimulated with Con A and 5 μM TMB-8 added at various times of culture (n = 5). **C:** Cells were treated with 10 μM TMB-8 or sham treated for 2 h, washed, and stimulated with Con A (n = 3). The unstimulated and stimulated control [^{3}H]thymidine incorporation (cpm ± SEM/10^5 cells) in A and B are A: 531 ± 200 and 37,882 ± 2,728; B: 762 ± 186 and 45,005 ± 5,021.

30 min and then stimulated to proliferate with Concanavalin A (Con A). Our results showed that TMB-8 supressed Con A-stimulated proliferation in a dose-dependent manner, with a maximum inhibition (70%) at 10 μM and about 50% inhibition at 5 μM (Fig. 1A). The cell viability was not affected at these concentrations of TMB-8, but at concentrations greater than 10 μM (data not shown). Although 10 μM TMB-8 did not affect the cell viability, in rare occasions a combination of the same concentration of the inhibitor and certain other agents affected the cell viability slightly. Thus, in most of the subsequent experiments 5 μM TMB-8 was used, though 10 μM TMB-8 caused the maximum inhibition of proliferation.

To determine which stage of T cell activation was affected by TMB-8, we studied the temporal pattern of proliferation inhibition by the inhibitor. Results in Figure 1B show that a significant inhibition (70% inhibition) occurred if TMB-8 was added to the culture 30 min before stimulation. Addition of TMB-8 at the same time of Con A addition (0 h) resulted in about 48% inhibition. From 60 min onward cell proliferation became largely resistant to TMB-8 inhibition. These results suggested that a very early and short-lived step of signaling pathway was affected by TMB-8 in a non-toxic way. The non-toxicity of TMB-8 was further ascertained by examining the reversibility of its effect. T cells were incubated for 2 h with the highest inhibitory concentration of TMB-8 (10 μM), washed, and stimulated with Con A. Results in Figure 1C show that washing of the cells, after TMB-8 treatment, can completely remove the inhibitory effect of TMB-8, indicating that its action is reversible and hence non-toxic.

TMB-8 Did Not Inhibit PMA/Ionomycin-Induced T Cell Proliferation

In T cells TMB-8 inhibits Ca^{2+} release specifically without affecting Ca^{2+} influx [Chakrabarti et al., 1995]. Thus, to ascertain further the specificity of TMB-8 effect on Con A-induced proliferation and to determine which step of TCR signaling is affected, we examined the effect of TMB-8 on T cell proliferation stimulated with a combination of phorbol-12-myristate-13-acetate (PMA) and Ca^{2+} ionophore ionomycin. PMA activates PKC, and ionomycin increases $[Ca^{2+}]_i$, thus bypassing the requirement of all TCR/CD3-mediated signaling events proximal to those two events to induce T cell proliferation. Our results showed that 5 μM TMB-8 did not affect PMA/Ionomycin-induced proliferation of T cells significantly (Fig. 2). This result indicates that TMB-8 suppressed Con A-induced T cell proliferation specifically by blocking some event(s) proximal to PKC activation and increase in $[Ca^{2+}]_i$.

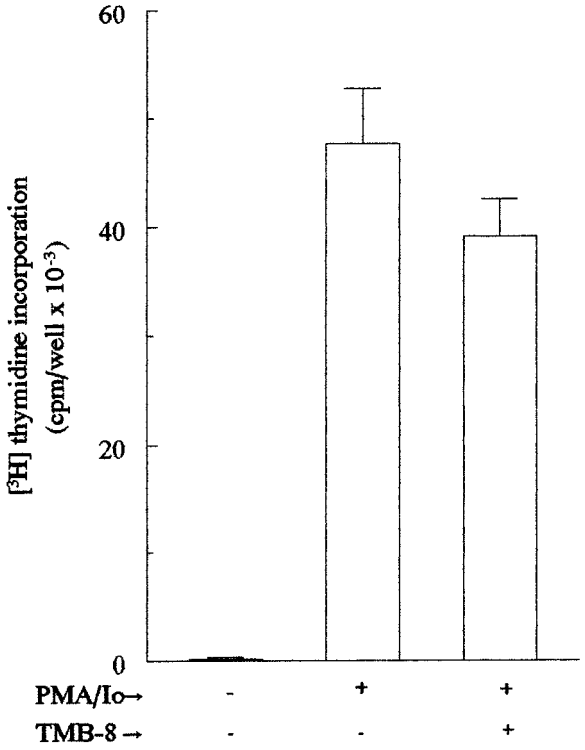

Fig. 2. TMB-8 did not affect PMA/ionomycin-induced T cell proliferation. T cells were treated for 30 min with 5 μM TMB-8 and then stimulated with a combination of 0.25 nM PMA and 2.0 μM ionomycin (Io) as in Figure 1. Results are the mean ± SEM of three experiments.

Effect of Ionomycin on the Anti-Proliferative Effect of TMB-8

As TMB-8 inhibits Ca^{2+} release [Chakrabarti et al., 1995] and did not inhibit PMA/Ionomycin-induced proliferation (Fig. 2), we explored whether Ca^{2+} ionophore ionomycin, which increases $[Ca^{2+}]_i$ through entry from outside and release from intracellular store, can overcome the effect of TMB-8 on T cell proliferation. T cells were pretreated with 5 μM TMB-8 followed by stimulation with Con A in the presence or absence of different doses of ionomycin. TMB-8 caused about 50% inhibition of proliferation as expected and 0.5 μM ionomycin did not alter this (Fig. 3A). However, 2 μM ionomycin enhanced instead of overcoming the inhibitory effect of TMB-8; the inhibition of proliferation was about 50% and 80% in the absence and presence of ionomycin, respectively (Fig. 3A).

That dose of ionomycin was not toxic to the cells, as it (1) did not affect cell viability alone or in combination with TMB-8 and Con A (data not shown) and (2) induced T cell proliferation in combination with PMA (Fig. 2). Interestingly, ionomycin alone did not affect Con A-induced T cell proliferation (Fig. 3B). The above results indicate a synergism between TMB-8 and ionomycin in inhibiting T cell proliferation.

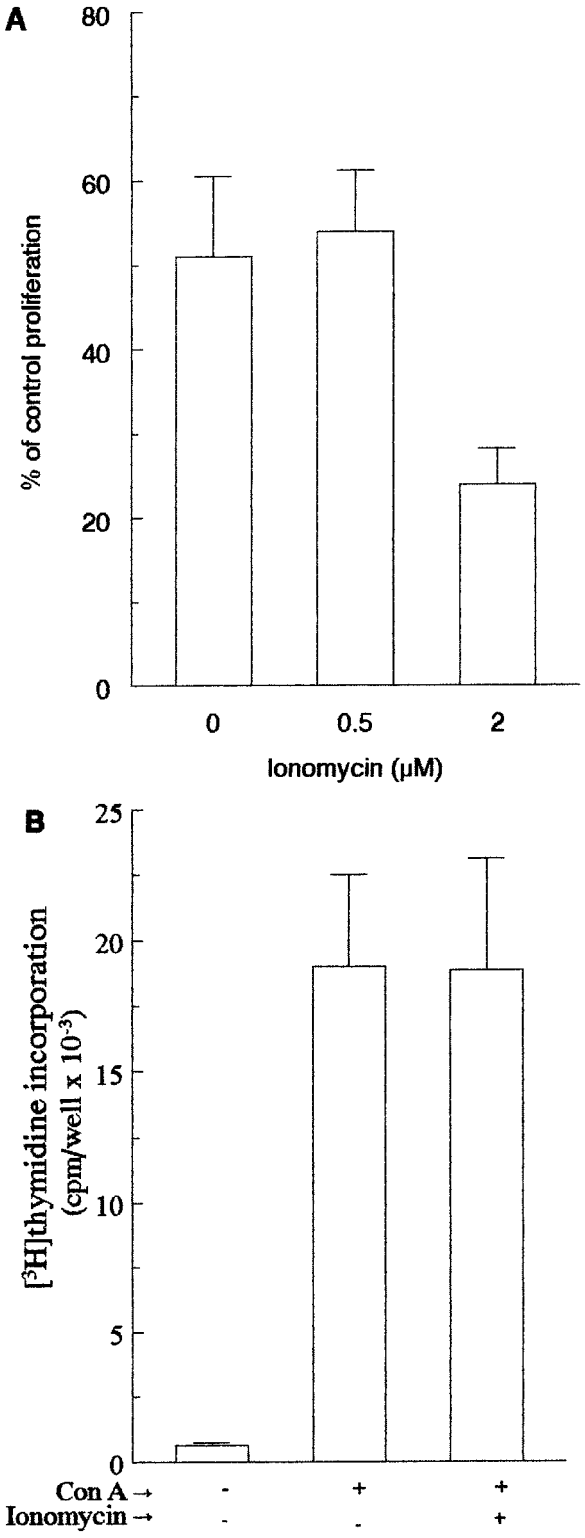

Fig. 3. Effect of ionomycin on anti-proliferative effect of TMB-8. T cells were stimulated to proliferate with 10 μg/ml Con A as in Figure 1. **A:** Stimulation in the presence or absence of TMB-8 alone or in combination with different doses of ionomycin (n = 5). Percentage of control proliferation was calculated as in Figure 1. **B:** Stimulation in the presence or absence of 2 μM ionomycin (n = 9). The unstimulated and stimulated [^{3}H]thymidine incorporation (cpm ± SEM/10^5 cells) in A are 520 ± 153 and 37,257 ± 2,552, respectively.

Effect of PKC Activator on the Anti-Proliferative Effect of TMB-8

Our results showed that TMB-8 affected T cell activation at a step proximal to the activation of PKC and increase in $[Ca^{2+}]_i$, but ionomycin failed to overcome this effect. Thus we explored whether an activator of PKC can overcome the effect of TMB-8 on Con A-induced T cell proliferation. Our initial study showed that the natural activator of PKC diacylglycerol (DAG), which is metabolically labile in all cells studied, including T cells [Asaoka et al., 1991], failed to reverse the anti-proliferative effect of TMB-8 (data not shown). Following this result we tested the ability of PMA, a metabolically stable activator of PKC [Rando, 1988], to overcome the TMB-8 effect. We found that PMA overcomes the inhibitory effect of TMB-8 on Con A-stimulated proliferation of T cells in a dose dependent manner with a complete reversal at 0.05 nM PMA (Fig. 4A). However, the same concentrations of PMA alone do not induce any T cell proliferation (data not shown). These results show that a relatively sustained activation of PKC can overcome the antiproliferative effect of TMB-8.

Temporal Pattern of Proliferation Inhibition by PKC Inhibitor

As PMA reversed the antiproliferative effect of TMB-8 (Fig. 4A), we explored whether TMB-8 affected T cell proliferation by directly inhibiting PKC. To that end, the kinetics of inhibition of T cell proliferation by Calphostin C, which specifically inhibits PKC by competing for DAG and phorbol ester binding site [Bruns et al., 1991], was examined and compared with that of TMB-8. Our results showed that 0.5 μM Calphostin C completely inhibited Con A-induced T cell proliferation even when added at 12 h of culture (Table I). This is in

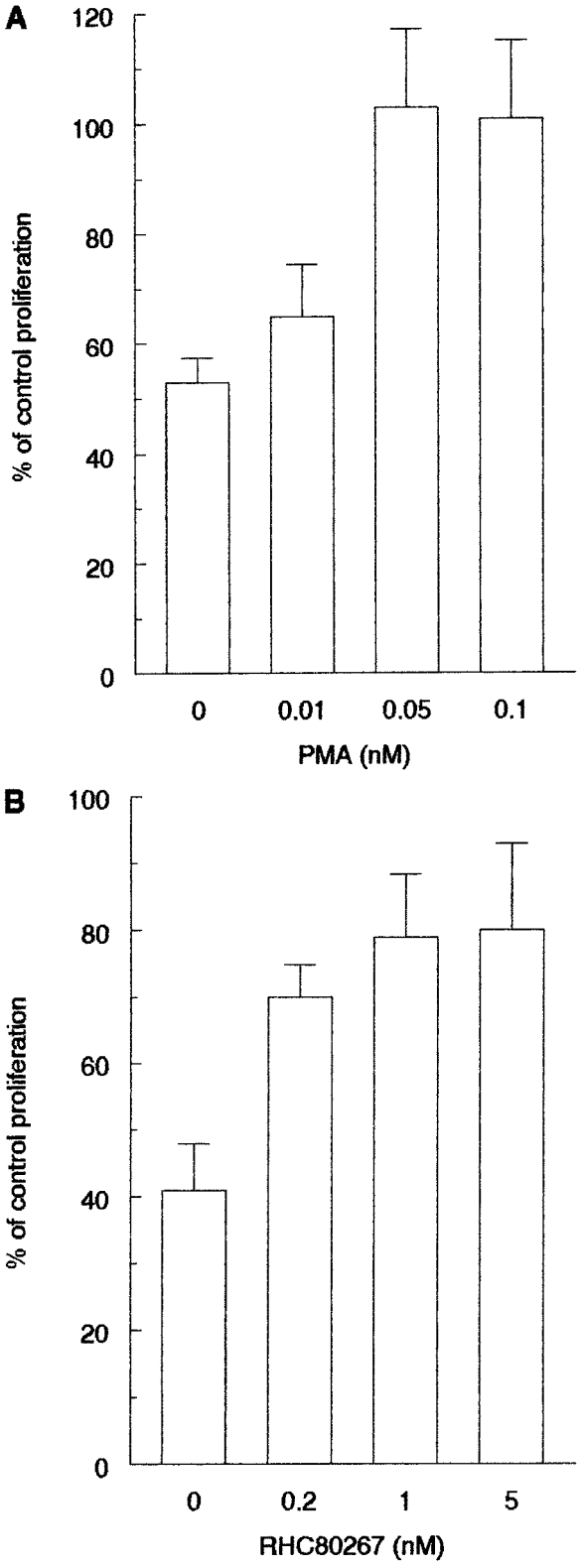

TABLE I. Temporal Pattern of Calphostin C Inhibition of Con A-Induced T Cell Proliferation[a]

Calphostin C addition time (hr)	Con A (10 μg/ml)	[3H]thymidine incorporation (cpm/well) Experiment 1	Experiment 2
−	−	487	408
−	+	40660	45695
0	+	462	507
6	+	553	638
12	+	532	493

[a]T cells (1×10^5/well) were stimulated to proliferate with 10 μg/ml Con A, as described in Materials and Methods, and 0.5 μM Calphostin C was added at different times of culture. Results of two experiments are presented. −, no addition; +, addition.

sharp contrast with TMB-8, which did not cause a significant inhibition of Con A-induced T cell proliferation if added just after 1 h of stimulation (Fig. 1B). This effect of Calphostin C is specific, because that can be competed out by PMA (data not shown). These results clearly showed that TMB-8 did not inhibit T cell proliferation by directly inhibiting PKC. Kinetics of Calphostin C inhibition of proliferation also showed that a prolonged PKC activation at least for 12 h of stimulation is required for Con A-induced T cell proliferation and thus confirmed the previous findings that a sustained activation of PKC is required for T cell proliferation [Manger et al., 1987; Davis and Lipsky, 1989; Szamel et al., 1989; Xu et al., 1989; Berry et al., 1990; Asoaka et al., 1991; Nishizuka, 1995].

DAG Lipase Inhibitor Reversed the Anti-Proliferation Effect of TMB-8

Based on the ability of PMA, but not of DAG, to overcome the inhibitory effect of TMB-8, we explored whether maintaining the stimulated level of endogenous DAG can have the same effect as PMA. Inside the cells DAG is produced upon agonist stimulation and rapidly degraded

Fig. 4. Overcoming the inhibitory effect of TMB-8 by PMA and RHC80267. T cells were stimulated to proliferate with 10 μg/ml Con A as in Figure 1 in the absence or presence of TMB-8 alone or in combination with different doses of PMA (**A,** n = 3) or DAG lipase inhibitor RHC80267 (**B,** n = 4). Percentage of control proliferation was calculated as in Figure 1. The unstimulated and stimulated [3H]thymidine incorporation (cpm ± SEM/10^5 cells) are A: 547 ± 141 and 40,781 ± 1,810; B: 196 ± 147 and 28,304 ± 9,069.

TABLE II. Effect of RHC80267 on Con A-Induced T Cell Proliferation[a]

RHC80267 (nM)	[^{3}H]thymidine incorporation cpm/well ($\bar{x}$ ± SEM) No Con A	10 μg/ml Con A
0.0	214 ± 137	24067 ± 155
0.2	116 ± 23	24159 ± 666
1.0	88 ± 37	23981 ± 58

[a]T cells (1×10^5/well) were stimulated to proliferate with or without 10 μg/ml Con A in the presence or absence of different doses of RHC80267 and proliferation was measured as in Figure 1. The results are the mean ± SEM of three experiments.

to glycerol and free fatty acids by sequential action of DAG lipase and MAG lipase, and this degradation can be prevented by a specific DAG lipase inhibitor RHC80267 [Konrad et al., 1994; Nishizuka, 1995]. We found that RHC80267 overcome the inhibitory effect of TMB-8 on Con A-induced T cell proliferation in a dose dependent manner with almost complete overcome at 1.0 nM of RHC80267 (Fig. 4B). However, this maximum overcoming dose of RHC80267 alone did not induce any T cell proliferation, nor did it affect Con A-induced proliferation (Table II). This indicated that stabilizing the endogenous DAG, which is produced after TCR stimulation [Asoaka et al., 1991], can overcome the inhibitory effect of TMB-8 on Con A-induced T cell proliferation.

DISCUSSION

In this study attempts were made to decipher the role of IP_3-stimulated Ca^{2+} release in T cell activation. Our results showed that the specific inhibition of IP_3-stimulated Ca^{2+} release by TMB-8 blocked the Con A-induced T cell proliferation without affecting the cell viability. Kinetics and reversibility study revealed that TMB-8 inhibited T cell proliferation by blocking an early step of TCR/CD3 signaling pathway in a non-toxic way. Inability of TMB-8 to inhibit PMA/ionomycin-stimulated proliferation indicated that the early step of signaling pathway blocked by TMB-8 is located proximal to PKC activation and increase in $[Ca^{2+}]_i$. Based on these results and previous findings that TMB-8 inhibited agonist (TCR/CD3 ligand)-induced Ca^{2+} release without affecting influx in mouse T cells [Chakrabarti et al., 1995], we suggest that TMB-8 inhibits TCR/CD3-dependent T cell proliferation by blocking the IP_3-mediated Ca^{2+} release.

As Ca^{2+} release increases the $[Ca^{2+}]_i$, we explored whether ionomycin, which increases $[Ca^{2+}]_i$ through entry from outside and release from the intracellular Ca^{2+} stores, can overcome TMB-8 effect. However, the ionophore failed to overcome the inhibitory effect of TMB-8 on Con A-induced T cell proliferation. Ionomycin not only failed to overcome, but also enhanced the inhibitory effect of TMB-8 on T cell proliferation. This effect of ionomycin may be due to partial emptying of the Ca^{2+} store. Since ionomycin also causes the emptying of Ca^{2+} stores, it has the potential to interfere with the IP_3-dependent Ca^{2+} release, as it has been shown that a fully charged IP_3-sensitive Ca^{2+} store is required for IP_3 to bind to its receptor strongly and causes significant Ca^{2+} release [Missiaen et al., 1992; Oldershaw and Taylor, 1993]. Slight inhibition of IP_3-mediated Ca^{2+} release by ionomycin may not have an effect on T cell activation, but will synergestically facilitate the TMB-8 effect. That was the reason that ionomycin itself did not inhibit T cell proliferation, but enhanced the inhibitory effect of TMB-8. The above results clearly showed that Ca^{2+} release specifically through IP_3 receptor, not by any other means, plays a vital role in T cell activation. That role does not appear just to increase $[Ca^{2+}]_i$ or empty the Ca^{2+} stores, but to confer some changes in the IP_3 receptor or other component of the store which might be essential for T cell proliferation. If the role of IP_3 was executed through the increase in $[Ca^{2+}]_i$ or emptying the Ca^{2+} store, ionomycin should have overcome the effect of TMB-8.

Next we explored whether direct activation of PKC by a PKC activator can reverse the effect of TMB-8 on Con A-induced T cell proliferation. We found that DAG, the degradable natural activator of PKC, failed to counteract the effect of TMB-8. However, PMA, a long-lasting PKC activator, completely overcame the inhibitory effect of TMB-8, although PMA alone could not induce T cell proliferation in our system. These results suggested that blocking of Ca^{2+} release arrested PKC activation, leaving other signaling events intact. It is possible that TMB-8 directly inhibited PKC. However, our findings that the temporal pattern of TMB-8 inhibition of proliferation was very different from that of Calphostin C ruled out that possibility. If both supressed T cell proliferation by directly inhib-

iting PKC, the temporal pattern of their inhibitory effect should have been the same. Also, it has been reported that TMB-8 did not inhibit PKC directly in leukocytes [Christiansen et al., 1986]. These findings showed that TMB-8 did not inhibit T cell proliferation by inhibiting PKC directly but by blocking the machinery necessary for sustained activation of PKC.

It is well established that there are two phases of DAG production, one initial transient phase followed by a prolonged sustained phase from two different phospholipid precursors PIP_2 and phosphatidylcholine, catalyzed by the enzyme PLC and PLD, respectively [Nishizuka, 1995]. The consequence is the sustained activation of PKC, which is required for a long-term cellular response such as proliferation of T cells [Manger et al., 1987; Davis and Lipsky, 1989; Szamel et al., 1989; Xu et al., 1989; Berry et al., 1990; Asoaka et al., 1991; Nishizuka, 1995]. Our present observation that T cell proliferation is susceptible to inhibition by PKC inhibitor even after 12 h of stimulation confirmed this report. The ability of PMA, but not DAG, to overcome the effect of TMB-8 suggested that IP_3 receptor is essential for the sustained phase of DAG production and PKC activation. If this is true, the anti-proliferative effect of TMB-8 can be overcome by preventing the stimulated level of DAG from declining. In support of this, we found that a specific DAG lipase inhibitor, RHC80267, which prevents cellular degradation of DAG, overcomes the effect of TMB-8 on Con A-induced T cell proliferation. However, RHC80267 alone did not have any effect on Con A-induced proliferation. These results clearly showed that Ca^{2+} release through IP_3 receptor plays a vital role in sustaining the DAG level and PKC activation. How it may be related to the sustained phase of DAG is not clear. Of note, it has been shown that in neuroblastoma TMB-8 inhibited phosphatidylcholine synthesis by preventing choline uptake [Palmer et al., 1992].

Based on the present work we suggest that not the released Ca^{2+} but the process of Ca^{2+} release through the IP_3 receptor is essential for sustaining PKC activation which is required for T cell proliferation. What kind of changes Ca^{2+} release causes to the IP_3-dependent Ca^{2+} stores and how that may be linked to sustained PKC activation is not clear at this moment. For the first time we have identified a definitive functional role of IP_3-dependent Ca^{2+} release in T cell activation.

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