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Molecular events in the induction of a nonresponsive state in interleukin 2-producing helper T-lymphocyte clones

(T-cell tolerance/antigen presentation/T-cell activation/inositol phosphates/calcium ionophore)

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ABSTRACT **Exposure of normal interleukin 2 (IL-2)-producing helper T-cell clones to antigen and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-treated antigen-presenting cells results in proliferative unresponsiveness to subsequent stimulation with antigen and normal antigen-presenting cells. In the present study, we have examined the molecular events that accompany the induction of this unresponsive state. T cells stimulated in this manner failed to produce IL-2, but interleukin 3, interferon-γ, and IL-2 receptors were partially induced and T-cell receptor β mRNA was fully induced. Although T-cell unresponsiveness correlated with an IL-2 production defect, addition of IL-2 during the induction phase failed to prevent development of the unresponsive state. The critical biochemical event appeared to be an increase in intracellular calcium. Removal of calcium from the medium prevented induction of the unresponsive state, whereas addition of the calcium ionophore ionomycin induced unresponsiveness as well as all of the related partial activation events. Thus, an increase in intracellular calcium under nonmitogenic conditions appears to initiate an alternative activation program that prevents the T cell from producing IL-2 in response to subsequent normal activation signals. The significance of this *in vitro* model for tolerance induction *in vivo* is discussed.**

The ability of an organism to discriminate self from nonself molecules is a learned biological event (reviewed in ref. 1). Clones of lymphocytes that are capable of reacting to self components are deleted or suppressed through an unknown *in vivo* mechanism. Recently, however, several *in vitro* model systems have been described for functionally inactivating interleukin 2 (IL-2)-producing helper T-cell clones. Exposure to antigen in association with class II (Ia) molecules on either T cells (2), chemically fixed antigen-presenting cells (APC) (3, 4), or planar lipid membranes (5), all of which are incapable of providing the additional signals required for full T-cell activation, induced a state of proliferative unresponsiveness to foreign antigen. The unresponsiveness lasted for >1 week *in vitro*, although the cells remained viable and responsive to the growth hormone IL-2. In addition, chemically fixed APC could induce antigen-specific T-cell unresponsiveness *in vivo* (3, 4). In the present report, we describe the underlying molecular events associated with this unresponsive state *in vitro* and show that the state can be induced chemically by treating the cells with a calcium ionophore.

MATERIALS AND METHODS

Animals, Reagents, and Cell Lines. B10.A/SgSn and C57BL/10Sn mice, antigens, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (ECDI) were obtained or prepared as described (3). Ionomycin was purchased from Calbiochem. Recombinant IL-2 was kindly provided by Cetus (Mountain View, CA). Normal T-cell clones F1.A.2 and A.E7 were derived and maintained as described (3, 6).

Induction of T-Cell Unresponsiveness *in Vitro*. Splenocytes or resting B cells (7) used as APC were treated with ECDI as described (3). To induce unresponsiveness, normal T-cell clones were preincubated for 24–48 hr with various ECDI-treated or normal APC populations, with or without antigen. The next day, supernatants were removed for lymphokine assays, and 2×10^4 cells were transferred to a microtiter plate, pulsed with [^{3}H]thymidine, and harvested 16 hr later. The remaining cloned T cells were purified on Ficoll/Hypaque (Pharmacia) density gradients, washed, and stained for IL-2 receptor expression, spun in a cytocentrifuge (5×10^3 to 3×10^5) for *in situ* hybridization, or restimulated (2×10^4) as described (3) at various later times with 5×10^5 irradiated (3000 R; 1 R = 2.58×10^{-4} C/kg) B10.A splenocytes with or without pigeon fragment 81-104 or with IL-2 alone (100 units/ml).

Lymphokine Assays. IL-2 activity was assayed as described on the IL-2-dependent CTLL line (8). Interleukin 3 (IL-3) activity was assessed by measuring the ability of supernatants to support the growth of 5×10^4 IL-3-dependent DA-1 cells (9) for 24 hr, the last 6–8 hr of which contained 1 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine. Interferon-γ was measured by Biofluids (Rockville, MD) as described in a viral protection assay (10). A.E7 produces only interferon-γ, and not -α or -β (6).

IL-2 Receptor Expression. A.E7 cells were purified from preincubation cultures and stained with biotinylated monoclonal anti-IL-2 receptor antibody (7D4) (11) followed by fluorescein isothiocyanate-avidin. The fluorescence intensities of 50,000 cells were quantitated on a FACS II (Becton Dickinson Immunocytometry System, Sunnyvale, CA) as described (12).

***In Situ* Hybridization.** *In situ* hybridization was performed by a modification (13) of the procedure of Harper *et al.* (14). The sensitivity and specificity of this technique has been described in detail elsewhere (13).

Intracellular Calcium Measurements. A.E7 cells (4×10^6 cells per ml) were loaded with 1 μM indo-1/AM (15) by 45-min incubation at 37°C. Either normal or ECDI-treated resting B cells were incubated at 37°C for 1 hr at 2×10^8 cells per ml with or without pigeon fragment 81-104. The reaction was then initiated by mixing 0.2 ml of T cells (4×10^6) with

Abbreviations: APC, antigen-presenting cell(s); $[Ca^{2+}]_i$, intracellular calcium concentration; ECDI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; InsP_1, inositol monophosphate; InsP_2, inositol bisphosphate; InsP_3, inositol trisphosphate; IL-2, interleukin 2; IL-3, interleukin 3; PKC, protein kinase C.

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0.2 ml of B cells (4×10^7) and antigen or ionomycin alone. Cells were analyzed as described (16) over a 10- to 20-min period using a FACS II equipped with argon and krypton ion lasers (Spectra Physics, Mountain View, CA). The ratio of 404 nm to 485 nm fluorescence was used as a measure of the intracellular calcium concentration ($[Ca^{2+}]_i$). The actual $[Ca^{2+}]_i$ was derived from an *in situ* flow cytometric calibration curve (16).

Inositol Phosphate Accumulation. A.E7 cells were incubated overnight at 10^7 cells per ml in inositol-free Dulbecco's minimal essential medium (Biofluids) containing 20–50 μCi of *myo*-[2-^{3}H]inositol per ml (17.1 Ci/mmol; New England Nuclear). The cells were washed twice and resuspended in RPMI 1640 medium 5% fetal calf serum containing 10 mM LiCl (which inhibits inositol-1-phosphate). A.E7 cells ($0.5–2 \times 10^6$) were incubated for 20 or 90 min at 37°C with $3 \times 10^6–10^7$ normal or ECDI-treated B cells with or without pigeon fragment 81-104. The accumulation of water-soluble inositol monophosphate ($InsP_1$) plus inositol bisphosphate ($InsP_2$) or total inositol phosphates was determined using AG1-X8 formate columns and 0.4 M ammonium formate or 1 M sodium formate/0.1 M formic acid, respectively, for the elution buffers as described (17, 18). After elution of $InsP_1$ plus $InsP_2$, the columns were washed extensively with 0.4 M ammonium formate until cpm were reduced to background levels. Inositol trisphosphates ($InsP_3$) were then eluted with 1 M ammonium formate. Radiolabeled inositol phosphates were quantitated by liquid scintillation counting using Beckman Ready-Solv HP as the scintillant. cpm were converted to dpm by correcting for counter efficiency and quenching.

RESULTS

Lymphokine and IL-2 Receptor Induction. Our previous studies demonstrated that *in vitro* preincubation of normal IL-2-producing helper T-cell clones with antigen and ECDI-treated APC (3, 4) or Ia-molecule-containing planar lipid membranes (5) resulted in unresponsiveness to subsequent stimulation with antigen and untreated APC. To further dissect this phenomenon, we examined the activation state of the T-cell clones under conditions that result in unresponsiveness (Table 1). The normal T-cell clone A.E7 proliferated in response to pigeon cytochrome *c* fragment 81-104 and untreated B10.A APC (Exp. 1, group A). Proliferation correlated with the presence of IL-2 in the culture supernatant and a marked induction of surface IL-2 receptor (Exps. 1 and 2, group A). Interferon-γ and the colony-stimulating factor IL-3 could also be detected in the culture supernatant (Exps. 1 and 2, group A). These normal activation conditions did not cause unresponsiveness to restimulation with antigen and untreated APC (Exp. 2, group A), although the dose–response curve was shifted 3- to 10-fold to higher antigen concentrations (data not shown) and the maximum response was 2-fold higher (compare to Exp. 2, group B). In contrast, when ECDI-treated B10.A APC were used, the same T-cell clone did not proliferate (Exp. 1, group C) and produced little or no IL-2 in response to pigeon fragment 81-104 or the cross-reactive peptide moth 86-89; 93-103(93E) (Exps. 1 and 2, group C), although partial induction of surface IL-2 receptors, interferon-γ, and IL-3 activity (Exps. 1 and 3, group C) was observed. In agreement with our earlier results (3), these partial activation conditions resulted in T-cell

Table 1. Lymphokine production by a normal T-cell clone stimulated with ECDI-treated APC and antigen

Group	APC	Antigen	Proliferation,* cpm	IL-2,† units	IFN-γ,‡ units	IL-3,§ units	IL-2R,¶ MFI	Restimulation response, Δcpm‖	
								A + 81-104	IL-2
				Exp. 1					
A	A	81-104	118,356	90	250	315	357**	ND	ND
B	A-ECDI	—	1,041	<1	<5	<2	26**	62,050	3,446
C	A-ECDI	81-104	2,358	<1	125	60	48**	8,889	19,218
D	B10-ECDI	—	1,348	<1	<5	<2	ND	73,267	3,754
E	B10-ECDI	81-104	1,292	<1	<5	<2	28**	64,516	4,023
				Exp. 2					
A	A	Peptide††	ND	17	630**	158	310	65,752	122,952
B	A-ECDI	—	ND	<1	<5	<2	20	34,843	44,367
C	A-ECDI	Peptide	ND	1	200	69	63	855	96,471
D	—	Ionomycin	ND	<1	50	20	32	774	53,220
				Exp. 3					
A	A-ECDI	—	584	ND	ND	ND	20**	27,696	108,425
B	A-ECDI	81-104	2,876	ND	ND	ND	107**	2,332	174,675
C	A-ECDI	IL-2	72,088	ND	ND	ND	52**	69,065	271,495
D	A-ECDI	81-104 + IL-2	88,196	ND	ND	ND	587**	713	180,388

Cultures (24 hr, Exps. 1 and 3; 40 hr, Exp. 2) contained 7.5×10^5 A.E7 cells and 5×10^6 of the indicated splenocytes with or without pigeon fragment 81-104 (0.5 μM in Exp. 1; 1.0 μM in Exp. 3) or moth peptide 86-89;93-103(93E) (5 μM in Exp. 2) or ionomycin (1 μM in Exp. 2). Where indicated in Exp. 3, preincubation cultures contained 5 units of recombinant IL-2 per ml. After preincubation, cloned T cells were reisolated on density gradients. IFN-γ, interferon-γ; ND, not determined.

*Results are expressed as thymidine incorporation (cpm) of 2×10^4 A.E7 cells.

†IL-2 units were calculated as the reciprocal of the supernatant dilution that yielded 50% of maximal CTLL proliferation. The very low level (5.9% of control) of IL-2 observed in Exp. 2 (group C) may be related to inadequate ECDI fixation.

‡IFN-γ units were calculated as the reciprocal of the supernatant dilution that yielded 50% protection of L929 fibroblasts infected with vesicular stomatitis virus.

§IL-3 units were calculated as the reciprocal of the supernatant dilution that yielded 50% of the maximal DA-1 response. Fresh medium containing ionomycin did not stimulate DA-1 proliferation.

¶Results expressed as mean fluorescence intensity (MFI) of cells precultured for 40 hr and stained with anti-IL-2 receptor antibody as described in *Materials and Methods*.

‖A.E7 cells (2×10^4) were restimulated (immediately after preincubation) with 5×10^5 B10.A splenocytes and pigeon fragment 81-104 (0.1 μM in Exps. 1 and 2; 0.001 μM in Exp. 3) or with IL-2 alone (100 units/ml). Results are expressed as Δcpm (cpm of antigen-stimulated cultures minus cpm of cultures without antigen).

**Results obtained from cells cultured under similar conditions but on a different day.

††Moth peptide 86-89;93-103(93E).

unresponsiveness to subsequent restimulation with untreated APC and antigen (Exps. 1 and 2, group C; Exp. 3, group B). This proliferative unresponsiveness was caused by an inability of the T cells to produce IL-2 when stimulated with antigen and normal APC (4, 5) and not to an inability of the cells to respond to IL-2 (Exps. 1 and 2, group C; Exp. 3, group B). In fact, T cells preincubated with antigen and ECDI-treated APC were more responsive to exogenous IL-2 than control T cells (e.g., compare groups B and C in Exp. 1), probably because of the partially increased surface IL-2 receptor expression. Partial IL-2 receptor induction, interferon-γ production, IL-3 activity, and unresponsiveness were ligand-specific events because they were not induced by ECDI-treated B10.A APC without antigen (Exps. 1 and 2, group B; Exp. 3, group A) or allogeneic ECDI-treated B10 APC (lacking the appropriate Ia molecule) with (Exp. 1, group E) or without (Exp. 1, group D) antigen.

These results were extended to the mRNA level using the technique of *in situ* hybridization, which allows quantitation of mRNA production on a single-cell basis (Fig. 1). Incubation of a normal T-cell clone with antigen and ECDI-treated B10.A, but not B10, APC resulted in partial induction of IL-2 receptor mRNA, but no detectable IL-2 mRNA. Interestingly, full induction of T-cell receptor β mRNA was observed.

Others have reported that IL-2 production and proliferation require not only T-cell receptor occupancy, but also second signals provided by the APC (19–21). Our results suggest that ECDI-treated APC present foreign antigen to T cells in association with Ia molecules but do not provide the accessory signals necessary for full activation. Without these signals, T-cell receptor occupancy results in a distinct pattern of activation events that culminate in an inability to produce IL-2 in response to normal antigen stimulation (4, 5). Alternatively, this state is induced because of a qualitative difference in receptor occupancy by antigen and Ia molecules on ECDI-treated APC—e.g., an inability to effectively cross-link receptors.

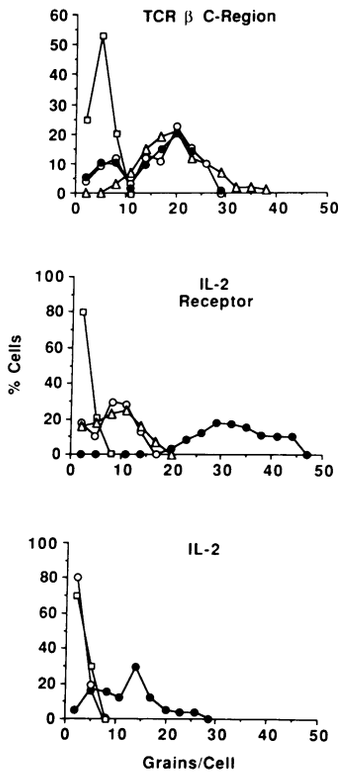

FIG. 1. Analysis of T-cell activation by *in situ* hybridization. A.E7 cells were cultured as described in Table 1 (Exp. 1) with ECDI-treated B10 splenocytes and 0.5 μM pigeon fragment 81-104 (□), ECDI-treated B10.A splenocytes and 0.5 μM pigeon fragment 81-104 (○), irradiated B10.A splenocytes and 0.5 μM pigeon fragment 81-104 (●), or 1 μM ionomycin alone (△). IL-2 mRNA expression (detected with the PMIL-2-20 probe) was assessed after 6 hr of preincubation, IL-2 receptor (IL2R8 probe) and T-cell receptor (TCR) β chain constant (C) region (86T5 probe) mRNAs after 16 hr of preincubation. Results are expressed as percentage of cells containing a given number of silver grains.

Effects of IL-2 Addition During Stimulation with ECDI-Treated APC. Recent reports have demonstrated that IL-2 increases the cell-surface expression of its own receptor (22). We therefore considered the possibility that the low level of IL-2 receptor induction observed after stimulation with ECDI-treated APC and antigen was related to the IL-2 production defect (Table 1, Exp. 3). Incubation of a normal T-cell clone with exogenous IL-2 (group C) or with ECDI-treated APC and antigen (group B) resulted in partial increases in surface IL-2 receptor expression above the resting level (group A). However, the combination of IL-2, ECDI-treated APC, and antigen synergized to give high levels of expression of surface IL-2 receptors (group D). Therefore, we conclude that the IL-2 receptor induction pathway is intact in T cells stimulated with ECDI-treated APC and antigen. This pathway appears to be suboptimally induced only because of the failure to produce IL-2.

Since IL-2, ECDI-treated APC, and antigen could induce IL-2 receptor expression to maximal levels, we next examined whether exogenous IL-2 could prevent the induction of the unresponsive state (Table 1, Exp. 3). Addition of IL-2 during the preculture with ECDI-treated APC and antigen failed to prevent the induction of unresponsiveness despite the fact that the cells divided in response to the IL-2 (group D). The augmented restimulation response of T cells incubated with IL-2 (compare groups A and C) may be related to increased IL-2 receptor expression or more rapid progression through the cell cycle. In any case, the relative degree of unresponsiveness induced was not changed by the presence of IL-2 in the preculture. These results show that the absence of IL-2 during the induction of the unresponsive state is not a prerequisite for inducing that state. Once induced, however, the failure to proliferate in response to normal signals is due to an IL-2 production defect (4, 5).

Early Activation Events for IL-2 Production. Weiss *et al.* have demonstrated that, as for other growth-related receptors, T-cell receptor occupancy and subsequent IL-2 production are associated with increases in inositol phospholipid metabolism (reviewed in ref. 20). T-cell receptor perturbation causes the cleavage of phosphatidylinositol-4,5-bisphosphate to 1,4,5-inositol trisphosphate and diacylglycerol (23). 1,4,5-Inositol trisphosphate is then thought to cause increases in the $[Ca^{2+}]_i$ due to the release of calcium from intracellular stores, while diacylglycerol activates protein kinase C (PKC). These two events then synergize via unknown pathways to initiate transcription of the IL-2 gene (20). Since normal T-cell clones failed to produce IL-2 under the conditions that resulted in unresponsiveness, we examined these early activation events.

Kinetic experiments (4) revealed that both inositol phosphate accumulation and $[Ca^{2+}]_i$ increase were significantly slower (e.g., inositol phosphates were first detectable at 10–15 min) in response to APC and antigen compared to soluble stimulants such as anti-T3 antibodies. This difference may be related to the time required to form conjugates between the T-cell clone and the APC. Therefore, early activation events were measured at 20 min or later. As shown in Fig. 2, 20-min incubation of a normal T-cell clone with untreated APC and antigen resulted in dose-dependent increases in inositol phosphates and $[Ca^{2+}]_i$. Maximum responses were achieved at 10–30 μM antigen. Half-maximal responses for inositol phosphates were achieved at $\approx$0.3 μM

and for $[Ca^{2+}]_i$ at 0.03 μM. In a second experiment, after 90 min of incubation, the half-maximal total inositol phosphate response was achieved at 0.03 μM (Fig. 2*a*). In sharp contrast, following incubation of the clone with ECDI-treated APC and high antigen concentrations, only very low, but significant, total inositol phosphate production was detected (Fig. 2 *a* and *b*). Surprisingly, the increases in $[Ca^{2+}]_i$ (Fig. 2*c*) in response to antigen and ECDI-treated APC were greater than expected based on the phosphatidylinositol turnover. For example, at 30 μM antigen the $[Ca^{2+}]_i$ was 600 nM, (equivalent to the half-maximal response under normal activation conditions), but InsP_3 was not detectable (Fig. 2*b*) and total inositol phosphates were 5% of the maximal response achieved with normal activation (Fig. 2*a*). These results suggested the possibility that a nonmitogenic increase in $[Ca^{2+}]_i$ induced by ECDI-treated APC might be responsible for the onset of the unresponsive state.

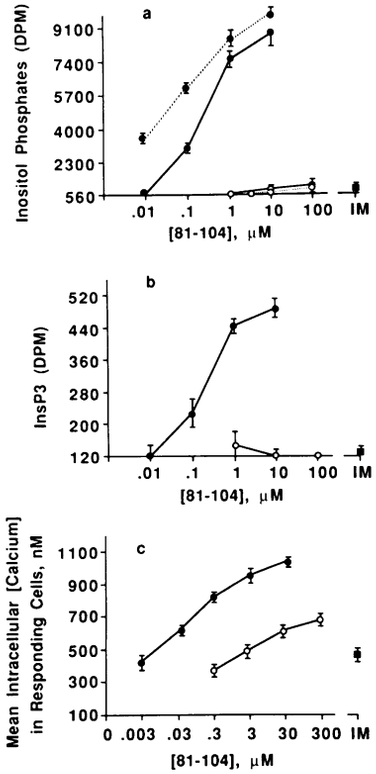

FIG. 2. Inositol phosphate accumulation and intracellular calcium increases after stimulation of a normal T-cell clone with ECDI-treated APC and antigen. (*a*) *myo*-[2-^{3}H]Inositol-labeled A.E7 cells (····, 5 × 10^5; ——, 2 × 10^6), were incubated with the indicated antigen concentrations and 3 × 10^6 (····) or 10^7 (——) ECDI-treated (○) or normal (●) B10.A B cells or with 1.5 μM ionomycin (IM) alone (■). InsP_1 plus InsP_2 (——) or total inositol phosphates (····) were measured after 20 or 90 min of incubation, respectively. The results are expressed as dpm ± SEM; dpm from cultures without antigen were 566 ± 26. (*b*) InsP_3 was measured after 20 min under the incubation conditions described for *a*; dpm from cultures without antigen were 112 ± 20. (*c*) Indo-1/AM-labeled A.E7 cells (4 × 10^6) were mixed with the indicated antigen concentrations and 4 × 10^7 ECDI-treated (○) or normal (●) B cells or with 1 μM ionomycin alone (■) and analyzed for 10–20 min on the FACS II. By the end of the sampling period, ≈60% of the A.E7 cells incubated with normal APC, 35% of the cells incubated with ECDI-treated APC, and 100% of the cells incubated with ionomycin had an intracellular calcium concentration greater than resting levels (100 nM). The percentage of cells responding to either ECDI-treated or normal APC reached these levels at all antigen concentrations tested (data not shown). However, as shown in *c*, the actual steady-state levels of intracellular calcium achieved by responding cells was antigen dose dependent with both ECDI-treated and normal APC. The results are expressed as the mean $[Ca^{2+}]_i$ ± SEM sampled in responding cells at ≈60-sec intervals 10–20 min after initiating the reaction.

Essential Role of Increased Intracellular Calcium in Induction of the Unresponsive State. To test this hypothesis, a normal T-cell clone was first incubated with ECDI-treated APC and antigen in the presence or absence of EGTA. Removal of free calcium from the medium by the addition of EGTA during the time that the cells were exposed to ECDI-treated APC and antigen completely prevented the clone from becoming unresponsive (antigen restimulation response of 78,840 cpm with EGTA, 371 cpm without). This effect was reversed by the addition of $CaCl_2$ (antigen restimulation response of 963 cpm). These results demonstrate that calcium is required to induce the unresponsive state.

Next, we examined the effects of the calcium ionophore ionomycin. Ionomycin caused an influx of external calcium (Fig. 2*c*) without inducing inositol phospholipid metabolism (Fig. 2 *a* and *b*), proliferation (data not shown), or IL-2 production (Table 1, Exp. 2, group D). However, as in the case of stimulation with ECDI-treated APC and antigen, incubation with ionomycin induced the T-cell clone to produce low levels of IL-3 and interferon-γ activity (Exp. 2, group D), to partially increase IL-2 receptor mRNA (Fig. 1) and surface expression (Table 1, Exp. 2, group D), and to fully increase levels of T-cell receptor β mRNA (Fig. 1). Most importantly, ionomycin treatment rendered the normal T-cell clone unresponsive to subsequent stimulation with untreated APC and antigen (Exp. 2, group D). The level of T-cell unresponsiveness induced by ionomycin was closely correlated with the $[Ca^{2+}]_i$ achieved (Fig. 3). Unresponsiveness could not be explained by toxicity, because ionomycin-treated T cells responded normally to exogenous IL-2 (Fig. 3; Table 1, Exp. 2, group D). Therefore, for every parameter tested, ionomycin mimicked the effects of ECDI-treated APC

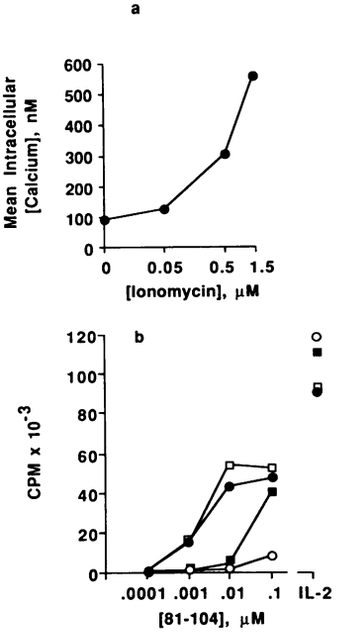

FIG. 3. Dose–response relationship between ionomycin-induced $[Ca^{2+}]_i$ increase and subsequent unresponsiveness. (*a*) Increases in $[Ca^{2+}]_i$ in response to the indicated ionomycin concentrations were determined as described in Fig. 2 legend. The relatively low levels of $[Ca^{2+}]_i$ achieved at these ionomycin concentrations are probably related to the high serum concentration (10%) in the medium. (*b*) A.E7 cells (5 × 10^5) were preincubated overnight with medium (●) or with 1.5 μM (○), 0.5 μM (■), or 0.05 μM (□) ionomycin, washed, and stimulated with normal APC and the indicated antigen concentrations or with IL-2 alone (100 units/ml).

and antigen on normal T-cell clones. These results demonstrate that an increase in $[Ca^{2+}]_i$ under conditions of undetectable inositol phospholipid metabolism can lead to unresponsiveness in IL-2-producing T-cell clones.

DISCUSSION

Our earlier observations, that the induction of T-cell unresponsiveness and normal activation have identical antigen- and Ia-molecule fine specificity (3–5), as well as the current observation that both stimuli result in comparable induction of T-cell receptor β mRNA, suggest that receptor occupancy by antigen and Ia molecules is similar on ECDI-treated and untreated APC. However, ECDI treatment either inactivates an APC accessory signal or alters T-cell receptor cross-linking, perhaps by affecting Ia-molecule mobility, such that full T-cell activation does not occur. Receptor occupancy under these conditions results in increases in $[Ca^{2+}]_i$ but very little phosphatidylinositol turnover. As a consequence of these biochemical events an alternative activation state is induced, manifested by defective IL-2 production but normal IL-2 receptor surface expression and signal transduction. Thus, this type of nonmitogenic T-cell receptor occupancy effectively dissociates the IL-2 production and IL-2 receptor expression pathways. Similar conclusions about the distinctiveness of these two pathways have been reached from studies of nonmitogenic lectins (24). Once the unresponsive state has been fully induced, the failure to proliferate to subsequent normal antigen stimulation correlates with an IL-2 production defect that we have shown cannot be attributed to decreased T-cell receptor expression (4, 5).

The cause of the increase in $[Ca^{2+}]_i$ in response to antigen and ECDI-treated APC is currently unclear. Recent evidence suggests that intracellular calcium stores are extremely sensitive to $InsP_3$ (20, 25) and that conversion of $InsP_3$ to 1,3,4,5-inositol tetrakisphosphate might bring extracellular calcium into the cell (26). Thus, the low levels of inositol phosphates produced under the conditions that induced unresponsiveness may be sufficient to cause the increases in $[Ca^{2+}]_i$. Alternatively, the $[Ca^{2+}]_i$ increase may be independent of inositolphospholipid metabolism and occur by a different mechanism for activating calcium channels (27). Receptor-mediated increases in $[Ca^{2+}]_i$ under conditions of minimal inositolphospholipid metabolism (28) or PKC activation (29) have been reported in other systems. No matter what the basis is for the increase in $[Ca^{2+}]_i$, the failure of ECDI-treated APC or ionomycin to stimulate normal phosphatidylinositol turnover suggests that little diacylglycerol would be produced and consequently PKC may not be fully activated. Preliminary results in our system suggest that antigen-specific phosphorylation events are significantly reduced in T cells activated with ECDI-treated APC (D. Mueller, M.K.J., and R.H.S., unpublished data). Thus, it is possible that an imbalance between increased $[Ca^{2+}]_i$ and insufficient PKC activation or some other signal not induced by ECDI-treated APC is involved in initiating subsequent biochemical events that eventually lead to the unresponsive state. Since our previous results (5) demonstrated that the induction of unresponsiveness could be blocked by cycloheximide, it is likely that those events involve synthesis of new proteins that ultimately prevent IL-2 production.

Although the studies presented in this paper have used a mature T-cell clone, these results may be applicable to self-tolerance induction in thymocytes. Thus, thymocytes may become self-tolerant when confronted with thymic APC that are deficient in accessory signaling or that do not properly cross-link T-cell receptors. Thymic epithelial cells, which express surface Ia molecules but do not stimulate T-cell clones to proliferate (30), fit this description, although *in vivo* experiments with deoxyguanosine-treated thymocytes suggest that epithelial cells do not induce tolerance (31). Alternatively, immature thymocytes may at some stage of development become tolerant as a consequence of T-cell receptor occupancy concurrent with an inability to receive or respond to APC accessory signals. Consistent with this idea, Ramarli *et al.* (32) have reported that anti-T3 treatment of medullary thymocytes (conditions that stimulate mature T cells) results in unresponsiveness to anti-T11 stimulation due to an IL-2 production defect. In either case, unresponsiveness may be explained by nonmitogenic T-cell receptor occupancy and the related increase in $[Ca^{2+}]_i$ as described here for mature T cells. If so, then this biochemical mechanism could be the molecular basis for T-cell tolerance induction *in vivo*.

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