Journal of Cellular Biochemistry 83:401–413 (2001)

High Dose of Dexamethasone Upregulates TCR/CD3-Induced Calcium Response Independent of TCR ζ Chain Expression in Human T Lymphocytes

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Abstract Glucocorticoids are very potent anti-inflammatory and immunosuppressive agents that modulate cellular immune responses, although, the molecular mechanisms that impart their complex effects have not been completely defined. We have previously demonstrated that dexamethasone (Dex), a synthetic glucocorticoid, biphasically modulates the expression of TCR (T cell receptor) ζ chain in human T cells. At 10 nM, it induced the expression of TCR ζ chain whereas at 100 nM, it inhibited its expression. In parallel to the upregulation of TCR ζ chain, the TCR/CD3-mediated $[Ca^{2+}]_i$ response was enhanced in 10 nM Dex-treated cells. However, at 100 nM, Dex treatment enhanced TCR/CD3-mediated $[Ca^{2+}]_i$ response without the induction of TCR ζ chain expression. Because the classical transcriptional model of glucocorticoid action cannot account for the effects of high dose of Dex, here we studied alternative mechanisms of action. We show that, increased and more sustained TCR/CD3-mediated $[Ca^{2+}]_i$ response was also observed in 100 nM Dex-treated cells in the presence of actinomycin D or cycloheximide suggesting that cellular transcription and/or de novo protein synthesis are not required for the induction. The TCR/CD3-mediated hyper $[Ca^{2+}]_i$ response in 100 nM Dex-treated cells was readily reversible by short-term culture in steroid-free medium. RU-486, a competitive antagonist of Dex, inhibited the increase in $[Ca^{2+}]_i$ response suggesting that the effect of Dex is mediated through the glucocorticoid receptor. Although the lipid-raft association of the TCR ζ chain was not significantly increased, high-dose of Dex increased the amount of ubiquitinated form of the TCR ζ chain in the cell membrane along with increased levels of actin. Fluorescence microscopy showed that high-dose of Dex alters the distribution of the TCR ζ chain and form more distinct clusters upon TCR/CD3 stimulation. These results suggest that high dose of Dex perturbs the membrane distribution of TCR ζ chain leading to more functional signaling clusters that result in increased TCR/CD3-mediated $[Ca^{2+}]_i$ response independent of TCR ζ chain expression. J. Cell. Biochem. 83: 401–413, 2001.

Key words: human T lymphocytes; T cell receptor; T cell signaling; Dexamethasone; $[Ca^{2+}]_i$ response; TCR ζ chain; lipid rafts

Signaling upon the engagement of the T cell receptor complex (TCR) results in a cascade of events requiring the recruitment and activation of non-receptor tyrosine kinases [Samelson, 1985]. TCR ζ chain plays a critical role in the surface expression and signal transduction via TCR [Weiss and Littman, 1994]. Upon TCR activation, tyrosine residues within the three immunoreceptor tyrosine based activation motifs (ITAMs) of the TCR ζ chain become phosphorylated by Lck and Fyn leading to the association and activation of ZAP-70. Once activated, Fyn, Lck, Syk, and ZAP-70 cooperate in

The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

Grant sponsor: NIH; Grant number: RO1 AI42269; Grant sponsor: STOC of the Medical Research and Material Command.

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Received 26 April 2001; Accepted 15 June 2001

DOI 10.1002/jcb.1238

the tyrosine phosphorylation, activation, and juxtaposition of downstream signal tranducers that contribute to the initiation of MAP-kinase cascades, PI3-kinase and Ca^{2+} flux [Irving et al., 1993; Zenner et al., 1996; Kersh et al., 1998]. The mobilization of calcium from intracellular stores occurs with widely different spatial and temporal profiles, that ultimately regulates diverse cellular processes and activation of T cells.

The TCR ζ subunit exists in multiple molecular forms and populations [Caplan and Baniyash, 2000]. Unphosphorylated TCR ζ chain monomers migrate on SDS–PAGE at a molecular weight of 16 kDa. It is believed that, partially and fully tyrosine-phosphorylated forms of the TCR ζ chain undergo conformational changes and migrate at 21 and 23 kDa, respectively [Baniyash et al., 1988]. Van Oers et al. [2000] has recently shown that the degree of phosphorylation of the tyrosine residues in the three different ITAMs results in different molecular weight forms of TCR ζ chain. Following phosphorylation, the TCR ζ chain undergoes ubiquitination and ultimately degradation by the proteasomes [Cenciarelli et al., 1996; Valitutti et al., 1997].

Multiple evidence also suggests that TCR ζ chain in T cells is attached to the actin-cytoskeleton. This has been reviewed recently by Caplan and Baniyash [2000]. Interestingly, unlike the phosphorylated forms of TCR ζ chain migrating at 21 and 23 kDa, the phosphorylated membrane bound TCR ζ chain migrates at 16 kDa. Although it has been suggested that this difference is the consequence of molecular structural variation of the actin bound form to the soluble form, the precise nature of the changes is not known. The TCR ζ chain associated with the detergent-insoluble fraction is also distributed between the lipid-rich microdomains of membranes known as lipid-rafts [Simons and Ikonen, 1997]. Distinct forms and fractions of the TCR ζ chain had been implicated in different signal transduction functions in T cells. It has been reported that after activation, the soluble fraction of TCR ζ chain translocates to the lipid-rafts in membranes [Kosugi et al., 1999]. These findings suggest that there is a dynamic equilibrium between the various molecular forms and fractions of TCR ζ chain in resting T cells and changing the distribution of these forms could alter TCR/CD3 mediated signaling.

Glucocorticoid hormones are potent anti-inflammatory and immunosuppressive drugs widely used to treat patients with inflammatory and autoimmune diseases [Hoffman, 1993]. Glucocorticoid hormones exert their immunosuppressive/anti-proliferative effects largely through the modulation of gene transcription [Beato, 1989]. They block the expression of a number of cytokines and also enhance the expression of various cytokine receptors [Zacharchuk et al., 1990; Wiegers et al., 1995]. Dexamethasone (Dex), a synthetic glucocorticoid, modulates immune function, although, the complete mechanism that underlie these immune regulations is not clear. Recently, we demonstrated that a low-dose of Dex (10 nM) induces the expression of multiple forms of TCR ζ chain including detergent-soluble, detergent-insoluble, and ubiquitinated forms, in human T cells [Nambiar et al., 2001]. Consistent with the increase in the expression of TCR ζ chain, the antigen-receptor mediated phosphorylation of cellular protein substrates or $[Ca^{2+}]_i$ responses were also enhanced in 10 nM Dex-treated T cells. However, at high dose (100 nM), Dex failed to induce the expression of TCR ζ chain, and rather exhibited an inhibitory effect on TCR ζ chain expression. Interestingly, in 100 nM Dex-treated cells the TCR/CD3-induced $[Ca^{2+}]_i$ response was significantly high compared to the untreated control cells. These results suggest that high dose of Dex has a different effect(s) on TCR/CD3 induced signaling in T cells, that is independent of the modulation of TCR ζ chain. Since the therapeutic doses of Dex are broad, herein, we sought to study the alternative mechanism(s) of induction of TCR/CD3 induced $[Ca^{2+}]_i$ response in 100 nM Dex-treated human T cells.

In the present study, we show that Dex at 100 nM induces the TCR/CD3-mediated $[Ca^{2+}]_i$ response in the presence of actinomycin D or cycloheximide, suggesting that the transcription process or de novo protein synthesis is not required for the induction of $[Ca^{2+}]_i$ response. The enhanced $[Ca^{2+}]_i$ response was readily reversible by short-time culture in steroid-free medium and inhibited by RU486, an antagonist of Dex suggesting that it is mediated by glucocorticoid receptors. We further show that Dex affects the membrane distribution of the TCR ζ chain. Together, these results suggest that the enhanced TCR/CD3-mediated $[Ca^{2+}]_i$ response in 100 nM Dex-treated cells is the

consequence of receptor-specific intercalation of Dex with the plasma membrane that indirectly alters the fluidity, mobility, and function of signaling proteins that favors the cascade of TCR/CD3 mediated signaling.

MATERIALS AND METHODS

Cells and Antibodies

Monocyte and macrophage depleted peripheral blood mononuclear cells (PBMCs) were obtained by elutriation of the leukopheresed blood samples of normal donors of 18–38 years age. Written informed consent was obtained from all participating subjects and the protocol of study was approved by the Health Use Committee of the Walter Reed Army Institute of Research. T lymphocytes were isolated from the PBMCs by depletion of non-T cells using a cocktail of hapten-conjugated antibodies and MicroBeads coupled to anti-hapten mAb and magnetic separation on MACS column (Miltenyi Biotec, Auburn, CA). In all cases, the percentage purity of T-cells in the isolated population was >96% by anti-CD3 ϵ staining and FACS analysis using an Epics Altra flow cytometer (Coulter, Hialeah, FL). Anti-TCR ζ mAb (clone 1D4) was from Pharmingen (Becton Dickinson Co., San Diego, CA) and anti-CD3 ϵ antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-CD3 mAb, OKT3, was from Ortho Biotech, Inc. (Raritan, NJ). Horseradish peroxidase (HRP) conjugated anti-phosphotyrosine mAb 4G10 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Other biochemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO).

Dex Treatment

Cells were suspended at a concentration of 5×10^6 cells/5 ml in RPMI medium containing 5% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin in tissue culture flasks. Indicated concentrations of Dex (Sigma Chemical Co., St. Louis, MO) were added and the cells were then transferred to an incubator and rested at 37°C for various time intervals and harvested for the study. Control T lymphocytes were incubated at 37°C in the same medium.

Immunoblotting of TCR ζ Chain and Other Signaling Proteins

Five million control or Dex-treated T cells were lysed in 1% Nonidet P-40 (NP-40) (Sigma Chemical Co., St. Louis, MO) lysis buffer as described previously [Liossis et al., 1998]. After centrifugation, the protein content of the supernatant was determined using a protein assay reagent from Bio-Rad (Hercules, CA). Proteins were separated on a 16% SDS–PAGE (Invitrogen Novex, Carlsbad, CA) (10 μg/lane) and transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were blocked with 3% non-fat dry milk and subsequently immunoblotted with TCR ζ chain C-terminal antibody, clone 1D4 (Pharmingen) or N-terminal murine mAb 6B10.2. The blots were incubated with HRP-conjugated goat anti-mouse and developed using the ECL chemiluminescent kit from Amersham Pharmacia Biotec (Piscataway, NJ). Membranes were stripped in Immunopure (Pierce, Chicago, IL) solution for 1.5 h, reblocked and reprobed with other antibodies of interest and control mAbs along with respective HRP-conjugated secondary antibodies.

SDS–PAGE and Immunoblotting of the Detergent-Insoluble Fraction

Five million T cells treated with or without Dex for the indicated period of time were lysed in 1% NP-40 lysis buffer as described previously [Liossis et al., 1998]. The lysate was centrifuged at 15,000 rpm for 10 min at 4°C. The pellet was solubilized by mechanical agitation in the presence of 4% SDS. The mixture was boiled for 20 min in sample buffer containing 4% SDS and electrophoretically separated in a 16% gel and transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were immunoblotted with various antibodies and developed as described above.

Fluorescence Microscopy

Five million cells were treated with 100 nM Dex for 24 h. The cells were activated with 10 μg/ml OKT3 for 2 min. Cells were fixed with 0.25% paraformaldehyde and permeabilized with digitonin. Cells were incubated with TCR ζ chain C-terminal antibody and LAT antibody for 1 h and then washed in staining buffer. Rhodamine-labeled goat anti-mouse IgG and FITC-labeled donkey anti-rabbit IgG secondary

antibodies were added and incubated for 1 h. The cells were washed, and mounted on slides along with appropriate secondary antibody controls. The samples were visualized in an Olympus fluorescence microscope at a magnification of 100×.

Estimation of the Lipid Raft-Associated Fraction of TCR ζ Chain

We measured the raft-associated fraction of TCR ζ chain in T cells by treating with methyl-β-cyclodextrin, which solubilizes membrane cholesterol and disrupts lipid-rafts [Moran and Miceli, 1998]. Disruption of the lipid rafts leads to the translocation of the lipid-raft associated form of the TCR ζ chain from detergent-insoluble membrane fraction to detergent-soluble fraction. Cells were treated with 30 μg/ml methyl-β-cyclodextrin for 30 min at 25°C and lysed as described earlier. The detergent-insoluble membrane fraction was washed and solubilized in sample loading buffer. The detergent-soluble fraction and the detergent-insoluble membrane fractions were electrophoresed as described above and immunoblotted with TCR ζ chain C-terminal mAb and other relevant antibodies.

Measurement of Free Cytoplasmic Ca^{2+} Concentration

Free cytoplasmic Ca^{2+} concentrations were estimated in INDO-1 loaded cells as previously described [Liossis et al., 1998]. Briefly, 5×10^6 cells were treated with indicated concentrations of Dex for indicated periods of time in 5 ml culture medium. One milliliter of cell suspension was removed from the flask and the cells were loaded with the fluorescent dye acetoxymethylester INDO-1 (Molecular Probes, Eugene, OR) (1 μg/ml) for 15 min at 37°C. Cells were analyzed using an Epics Altra (Coulter Corp., Hialeah, FL) flow cytometer equipped with a high power dual wavelength (365 and 488 nm) argon laser. In each run, first the cells were run unstimulated to record the baseline fluorescence ratio which represents the resting $[Ca^{2+}]_i$ levels. After 40 s either antibody OKT3 (10 μg/ml), or the isotype control mIgG2a, was added to the tube and the ratio of the fluorescence, which is directly proportional to the free cytosolic Ca^{2+}, was recorded for a total of 10 min. The recorded signals were statistically analyzed using MultiTime (version 3, Phoenix Flow Systems, San Diego, CA).

Ionomycin or Thapsigargin Mediated $[Ca^{2+}]_i$ Response in Dex-Treated Cells

T cells were preincubated with or without Dex (100 nM) for 24 h and loaded with INDO-1 for 40 min in the incubator. After recording the baseline for 40 s, ionomycin was added at 1 μM final concentration and the Ca^{2+} flux was recorded up to 10 min.

Thapsigargin, is an inhibitor of Ca^{2+} ATPase pump, is responsible for the reuptake of Ca^{2+} to the endoplasmic reticulum. Measurement of thapsigargin pool of Ca^{2+} requires complete depletion of the extracellular Ca^{2+}. Therefore, before measuring the effect of thapsigargin on the T cells, EGTA was added to the cell suspension at 5 mM. The final concentration of thapsigargin in the cell suspension was 10 μM. Great care was taken to clean the sample line between each individual run with ethanol and filtered RPMI medium.

RESULTS

Time Course Study of Dex-Induced TCR/CD3-Mediated $[Ca^{2+}]_i$ Response in Human T cells

Previously we have demonstrated that 100 nM Dex treatment for 24 h enhances the TCR/CD3-induced $[Ca^{2+}]_i$ response in human T cells [Nambiar et al., 2001]. At first, we performed a time course study of the effect of Dex on the TCR/CD3-induced $[Ca^{2+}]_i$ response. T cells were treated with 100 nM Dex for various time periods and then the TCR/CD3-induced $[Ca^{2+}]_i$ response was measured as described in Materials and Methods. The results indicate that in 100 nM Dex-treated cells the TCR/CD3-induced $[Ca^{2+}]_i$ response was enhanced significantly as early as 7 h after treatement with Dex (Fig. 1). Optimum induction of the TCR/CD3-induced $[Ca^{2+}]_i$ response was observed at 33 h post-treatment with 100 nM Dex. Under these conditions the percentage cell survival was similar in both 100 nM Dex-treated and untreated cells. A further increase in the time of incubation of T cells with 100 nM Dex resulted in both lower peak and sustained $[Ca^{2+}]_i$ response than that of cells treated for 33 h. Although the enhancement of TCR/CD3-induced $[Ca^{2+}]_i$ response remained high for more than 48 h in 100 nM Dex-treated cells, the magnitude of the response diminished gradually. A similar pattern of time-course effects of Dex on the $[Ca^{2+}]_i$

response was observed in 10 nM Dex-treated cells although the magnitude of the response was lower when compared to that of the 100 nM Dex-treated cells (data not shown).

Effect of Ionomycin on the $[Ca^{2+}]_i$ Response in 100 nM Dex-Treated Cells

There are several factors which influence the magnitude and pattern of a $[Ca^{2+}]_i$ response after TCR/CD3-stimulation. A rise in TCR/CD3-mediated $[Ca^{2+}]_i$ signal after T cell activation is derived from both intra- and extra-cellular sources. Intracellular Ca^{2+} stores are also critically involved in the control of Ca^{2+} influx across the plasma membrane. The intracellular pool of Ca^{2+} is mainly stored in the endoplasmic reticulum (ER) and is important in determining the magnitude of a $[Ca^{2+}]_i$ signal. Assessment of the ionomycin pool of Ca^{2+} in Dex-treated cells, permits the indication of gross differences in membrane integrity and/or permeability. To examine the effect of Dex on the ionomycin pool of Ca^{2+}, we treated T cells with 100 nM Dex for 24 h and then measured the ionomycin-mediated $[Ca^{2+}]_i$ response. As shown in Figure 2A, 100 nM Dex-treatment moderately inhibits the ionomycin induced $[Ca^{2+}]_i$ response compared to untreated cells. The time required to reach the optimum response was also slightly delayed in Dex-treated cells. These results suggest that high dose of Dex affects the cell membrane integrity in human T cells.

Thapsigargin Pool of Ca^{2+} in Dex-Treated Cells

Thapsigargin inhibits the Ca^{2+} pump ATPase, which is required for the reabsorption of the calcium released during cell activation to the ER [Thastrup et al., 1990]. Inhibition of the

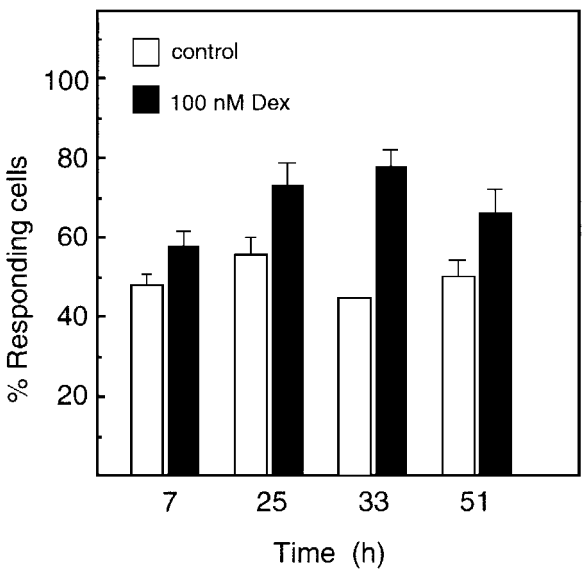

Fig. 1. Time course studies on the effect of dexamethasone on TCR/CD3 induced $[Ca^{2+}]_i$ responses in human T cells. Five million cells were treated with 100 nM dexamethasone for indicated periods of time followed by INDO-1 loading for 15 min at 37°C. Cells were analyzed using an Epics Altra flow cytometer as described in Materials and Methods. In each run, first the cells were run unstimulated to record the baseline fluorescence which represents the resting $[Ca^{2+}]_i$ levels. After 40 s, 10 μg/ml OKT3 antibody or the isotype control mIgG2a (base line) was added to the tube and the fluorescence was recorded for a total of 10 min. Histogram depicts the percentage of responding cells at 7 min after OKT3 stimulation in untreated controls and 100 nM dexamethasone-treated cells at various time periods (mean±SEM, n = 4). A positive responding cell is one whose $[Ca^{2+}]_i$ is increased by two standard deviations above the mean background levels.

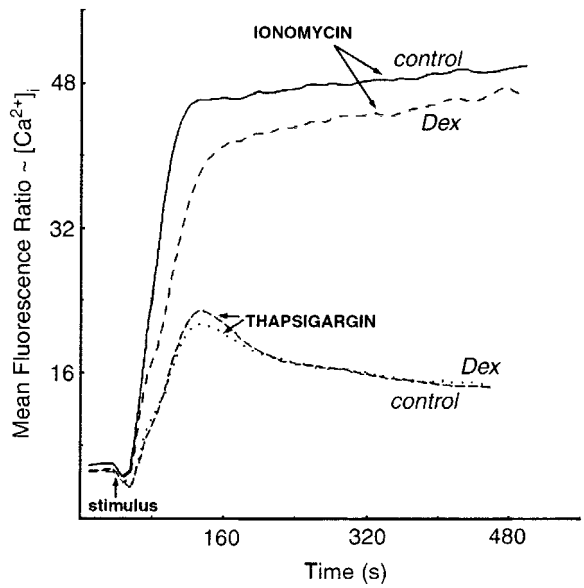

Fig. 2. The effect of high-dose of Dexamethasone on ionomycin and thapsigargin induced calcium responses. Five million cells were treated with or without 100 nM dexamethasone for 24 h followed by INDO-1 loading for 40 min at 37°C. Cells were analyzed using an Epics Altra flow cytometer as described in Materials and Methods. After recording the baseline for 40 s, ionomycin was added at 1 μM final concentration and the Ca^{2+} flux was recorded up to 10 min. Thapsigargin, is an inhibitor of Ca^{2+} ATPase pump, responsible for the reuptake of Ca^{2+} to the endoplasmic reticulum. Measurement of thapsigargin pool of Ca^{2+} was done depleting the extracellular Ca^{2+} by adding excess EGTA to the cell suspension (5 mM). The final concentration of thapsigargin in the cell suspension was 10 μM. A positive responding cell is one whose $[Ca^{2+}]_i$ is increased by two standard deviations above the mean background levels. A representative of three different experiments with very similar results is shown.

Ca^{2+} ATPase results in a pool of calcium known as 'thapsigargin pool'. The thapsigargin pool of Ca^{2+} also contributes to the intracellular $[Ca^{2+}]_i$ response and is important in regulating the plasma membrane Ca^{2+} entry. T cells were treated with 100 nM Dex for 24 h and the thapsigargin-mediated $[Ca^{2+}]_i$ response was measured after depleting the extracellular Ca^{2+} by excess EGTA as described in Material and Methods. In Dex-treated cells the thapsigargin pool of Ca^{2+} remained very similar to that of untreated cells (Fig. 2B). This result indicates that the upregulation of TCR/CD3-induced $[Ca^{2+}]_i$ response by high dose of Dex is not the consequence of any unknown affect of Dex on the ER membranes thereby preventing the uptake of released Ca^{2+} to the ER. Indirectly, this finding also suggests that the decreased Ca^{2+} entry in Dex-treated cells by ionomycin is not the consequence of a defective regulatory effect on Ca^{2+} entry by the thapsigargin-induced pool of Ca^{2+}.

Culture in Steroid-Free Medium Reverse the TCR/CD3-Mediated Hyper-$[Ca^{2+}]_i$ Response in 100 nM Dex-Treated Cells

To ascertain that the effect of 100 nM Dex is reversible, we treated the cells with 100 nM Dex for 24 h and then the medium was removed and the cells were incubated in steroid-free medium. After 8 h incubation in steroid-free medium the TCR/CD3-induced $[Ca^{2+}]_i$ response was measured. The data show that the induction of TCR/CD3-induced $[Ca^{2+}]_i$ response in 100 nM Dex-treated cells was readily reversed by the removal of Dex from the culture medium (Fig. 3A). This suggests that the effects of 100 nM Dex is reversible and not very drastic on human T cells.

Induction of TCR/CD3-Mediated $[Ca^{2+}]_i$ Response by 100 nM Dex Requires Binding to the Glucocorticoid-Receptor

To determine whether the induction of TCR/CD3-mediated $[Ca^{2+}]_i$ response by 100 nM Dex requires binding to its intracellular receptor, we evaluated the effects of steroid hormone antagonist RU486. Pre-incubation of T cells with 100 nM RU486 did not affect the ability of the cells to mobilize intracellular calcium in response to TCR/CD3 triggering (Fig. 3B). However, pre-incubation with 100 nM RU486 inhibited the enhanced TCR/CD3-induced $[Ca^{2+}]_i$ response observed in 100 nM Dex-treated cells, indicating the induction of TCR/CD3-induced $[Ca^{2+}]_i$ response requires the formation of a Dex-glucocorticoid receptor complex.

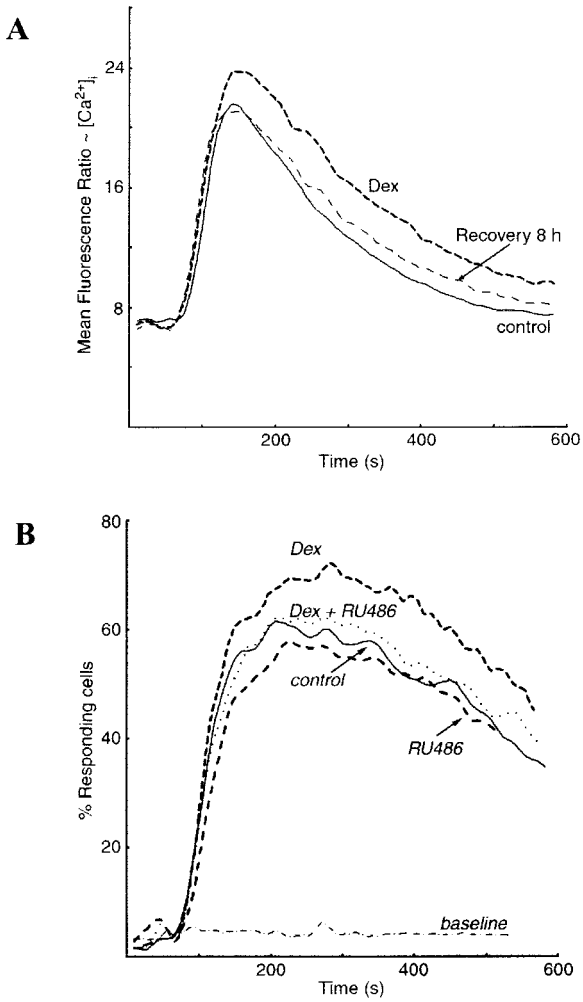

Fig. 3. Short-time culture in steroid-free medium or incubation with glucocorticoid receptor antagonist, RU486 readily reverses the enhanced TCR/CD3-mediated $[Ca^{2+}]_i$ responses in 100 nM dexamethasone treated cells. **A**: Five million cells were treated with 100 nM dexamethasone for 24 h. The medium was replaced with steroid-free RPMI medium and further incubated for 8 h followed by INDO-1 loading for 30 min at 37°C. Cells were analyzed using an Epics Altra flow cytometer as described in Materials and Methods. In each run, first the cells were run unstimulated to record the baseline fluorescence which represents the resting $[Ca^{2+}]_i$ levels. After 40 s, 10 μg/ml OKT3 antibody or the isotype control mIgG2a (base line) was added to the tube and the fluorescence was recorded for a total of 10 min. **B**: Cells were pretreated with 100 nM glucocorticoid receptor antagonist RU486 for 1 h and then incubated with 100 nM dexamethasone for 24 h. OKT3-induced $[Ca^{2+}]_i$ response of cells pretreated with RU486 in the presence or absence of 100 nM dexamethasone was measured as described above. A positive responding cell is one whose $[Ca^{2+}]_i$ is increased by two standard deviations above the mean background levels. A representative of three different experiments with very similar results is shown.

One-Hundred Nanomoles Dex-Induces TCR/CD3-Mediated $[Ca^{2+}]_i$ Response in the Presence of Actinomycin D

Transcription assays using TCR ζ chain-promoter-luciferase gene constructs have suggested that the mechanism of induction of TCR ζ chain in low-dose Dex-treated cells involves increased rate of transcription. The rate of transcription of the TCR ζ chain gene was also increased marginally in 100 nM Dex-treated cells [Nambiar et al., 2001]. To test whether induction of TCR ζ chain gene or other unknown genes are associated with the enhanced TCR/CD3-induced $[Ca^{2+}]_i$ response, the cells were pre-incubated with transcription inhibitor actinomycin D (0.5 μg/ml) and then treated with 100 nM Dex for 24 h. The TCR/CD3-induced $[Ca^{2+}]_i$ response was measured in 100 nM Dex-treated and untreated cells in the presence and absence of actinomycin D. The TCR/CD3-induced $[Ca^{2+}]_i$ response data is shown in Figure 4A. Increased and more sustained TCR/CD3-induced $[Ca^{2+}]_i$ response was observed in 100 nM Dex-treated cells in the presence of actinomycin D suggesting that the well established transcription activation activity of Dex does not appear to be involved in the induction of TCR/CD3-induced $[Ca^{2+}]_i$ response. This is also consistent with the rapid time-course of the induction of TCR/CD3-induced $[Ca^{2+}]_i$ response by 100 nM Dex. Apparently, 100 nM Dex has a receptor-mediated direct effect on cells that causes the induction of TCR/CD3-induced $[Ca^{2+}]_i$ response process.

De Novo Protein Synthesis is not Required for the Induction of TCR/CD3-Mediated $[Ca^{2+}]_i$ Response in 100 nM Dex-Treated Cells

To examine if de novo protein synthesis plays a role in the induction of TCR/CD3-induced $[Ca^{2+}]_i$ response in 100 nM Dex-treated cells, we pre-treated the cells with and without cycloheximide (1 μg/ml) and then incubated them with 100 nM Dex for 24 h. TCR/CD3-induced $[Ca^{2+}]_i$ response was measured in cells treated with and without 100 nM Dex in the presence and absence of cycloheximide. The results show that the potentiation of TCR/CD3-induced $[Ca^{2+}]_i$ response was observed in 100 nM Dex-treated cells in the presence of cycloheximide indicating that the effect of 100 nM Dex in not a consequence of increased protein synthesis (Fig. 4B). Treatment with mitomycin C (0.5 μg/ml), an

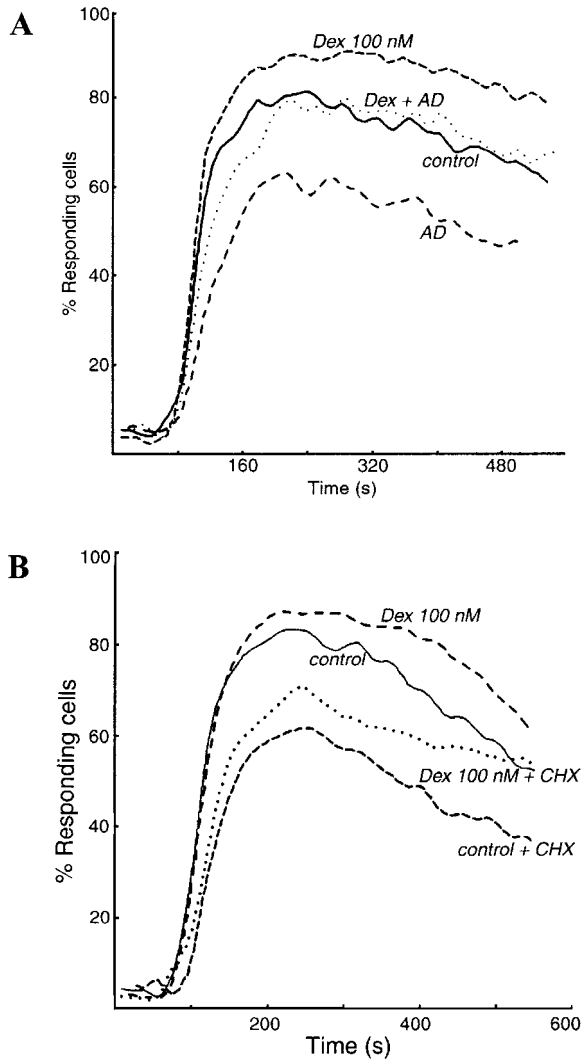

Fig. 4. One-hundred nanomoles dexamethasone induces TCR/CD3-mediated $[Ca^{2+}]_i$ responses in the presence of actinomycin D (**A**) or cycloheximide (**B**) in human T cells. Five million cells were treated with 10 μg/ml of cycloheximide (CHX) or 0.5 μg/ml actinomycin D (AD) for 1 h. One-hundred nanomoles dexamethasone was added and incubated for 24 h followed by INDO-1 loading for 30 min at 37°C. Cells were analyzed using an Epics Altra flow cytometer as described in Materials and Methods. In each run, first the cells were run unstimulated to record the baseline fluorescence which represents the resting $[Ca^{2+}]_i$ levels. After 40 s, 10 μg/ml OKT3 antibody or the isotype control mIgG2a (base line) was added to the tube and the fluorescence was recorded for a total of 10 min. A: OKT3-induced $[Ca^{2+}]_i$ response in cells treated and untreated with 10 μg/ml cycloheximide in the presence or absence of 100 nM dexamethasone. B: OKT3-induced $[Ca^{2+}]_i$ response of cells treated with 0.5 μg/ml actinomycin D in the presence or absence of 100 nM dexamethasone. A positive responding cell is one whose $[Ca^{2+}]_i$ is increased by two standard deviations above the mean background levels. A representative of three different experiments with very similar results is shown.

inhibitor of DNA replication and cell proliferation, resulted in a very similar increase in the OKT3-mediated $[Ca^{2+}]_i$ response in 100 nM Dex-treated cells (data not shown).

Analysis of the Lipid Raft Associated Fraction of TCR ζ Chain in 100 nM Dex-Treated T Cells

Several studies have shown that TCR ζ chain is localized to cholesterol and sphingomyelin enriched membrane microdomains known as lipid-rafts [Simons and Ikonen, 1997; Montixi et al., 1998; Ilangumaran et al., 2000; Yasuda and Kosugi, 2000]. Upon TCR/CD3 activation the TCR ζ chain has been shown to be recruited to these distinct membrane microdomains, suggesting that the lipid-raft association of the TCR ζ chain is important for T cell signaling [Kosugi et al., 1999]. Different lines of evidence indicate that derivatives of glucocorticoids associate with the cell membrane and modify the membrane fluidity and affect integral protein activity [van Bommel et al., 1987; Golden et al., 1999]. To further assess whether the increase in the TCR/CD3-induced $[Ca^{2+}]_i$ response in 100 nM Dex-treated cells is due to its intercalation with the membrane and alteration of the distribution of membrane-associated signaling molecules leading to increased association with membrane lipid rafts and thereby increasing TCR/CD3 mediated signal transduction, we measured the raft-associated fraction of TCR ζ chain. T cells were incubated with methyl-β-cyclodextrin to disrupt the lipid-rafts and thus solubilize the lipid raft-associated form of the TCR ζ chain from detergent-insoluble membrane fragments. Cells were treated with 100 nM Dex for 24 h and then with methyl-β-cyclodextrin for 30 min at 25°C followed by lysis. The detergent-insoluble membrane pellet was washed and solubilized in sample loading buffer and electrophoresed along with detergent-soluble fraction. The increase in the amount of TCR ζ chain in the detergent-soluble sample after treatment with methyl-β-cyclodextrin was considered as lipid-raft associated fraction. Immunoblotting using a TCR ζ chain C-terminal mAb showed that the amount of lipid-raft associated form of the unphosphorylated TCR ζ chain was not significantly increased in 100 nM Dex-treated cells compared to control T cells (Fig. 5). Interestingly, we noted that, in 100 nM Dex-treated cells the ubiquitinated form of the TCR ζ chain was increased in the detergent-insoluble membrane fraction (Fig. 5A). Immunobloting of CD3 ϵ chain show that the increase in the protein expression is specific to ubiquitinated TCR ζ chain and also indicate equal loading of proteins in different samples in the gel (Fig. 5B). Anti-ubiquitin antibody immunoblotting show that the 38 kDa band obtained with the TCR ζ chain antibody is ubiquitinated form of the TCR ζ chain (Fig. 5C). Similarly, the level of β-actin was also increased in the detergent insoluble membrane fraction in 100 nM Dex-treated cells compared to untreated samples (Fig. 5D). Although methyl-β-cyclodextrin solubilized some of the ubiquitinated form of the TCR ζ chain in controls, it failed to solubilize the ubiquitinated TCR ζ chain from the detergent-insoluble fraction of 100 nM Dex-treated cells.

One-Hundred Nanomoles Dex Treatment Alters the Distribution of TCR ζ Chain in Human T Lymphocytes

TCR ζ chain is essential for the assembly, transport, and surface expression of the TCR complex. Surface expressed TCR ζ chain is associated with the actin-cytoskeleton and also with lipid rafts [Rozdzial et al., 1995; Simons and Ikonen, 1997; Montixi et al., 1998; Caplan and Baniyash, 2000; Ilangumaran et al., 2000; Yasuda and Kosugi, 2000]. Upon ligation, T cell receptors cluster with an inherent affinity greater than unligated T cell receptors. The stability of these clusters is critical for intracellular signaling [Reich et al., 1997]. It has also been shown that the signaling molecules involved in T cell activation segregate into distinct areas forming supramolecular activation clusters [Monks et al., 1998]. Recently, it has been demonstrated that TCR ζ chain clustering coincides with the increase in intracellular calcium [Krummel et al., 2000]. To determine whether treatment with 100 nM Dex indeed changes the redistribution of membrane associated TCR ζ chain, and thereby promotes clustering, we examined the immunostaining of rhodamine-labeled TCR ζ chain in the presence and absence of 100 nM Dex by fluorescence microscopy. Permeabilized cells were double-labeled with rhodamine-labeled anti TCR ζ chain antibody and FITC-labeled LAT antibody, a marker for lipid-rafts. In control cells, the rhodamine-labeled TCR ζ chain was uniformly distributed (Fig. 6). In 100 nM Dex-treated cells, the TCR ζ chain staining was altered with more clustering compared to control sample (Fig. 6).

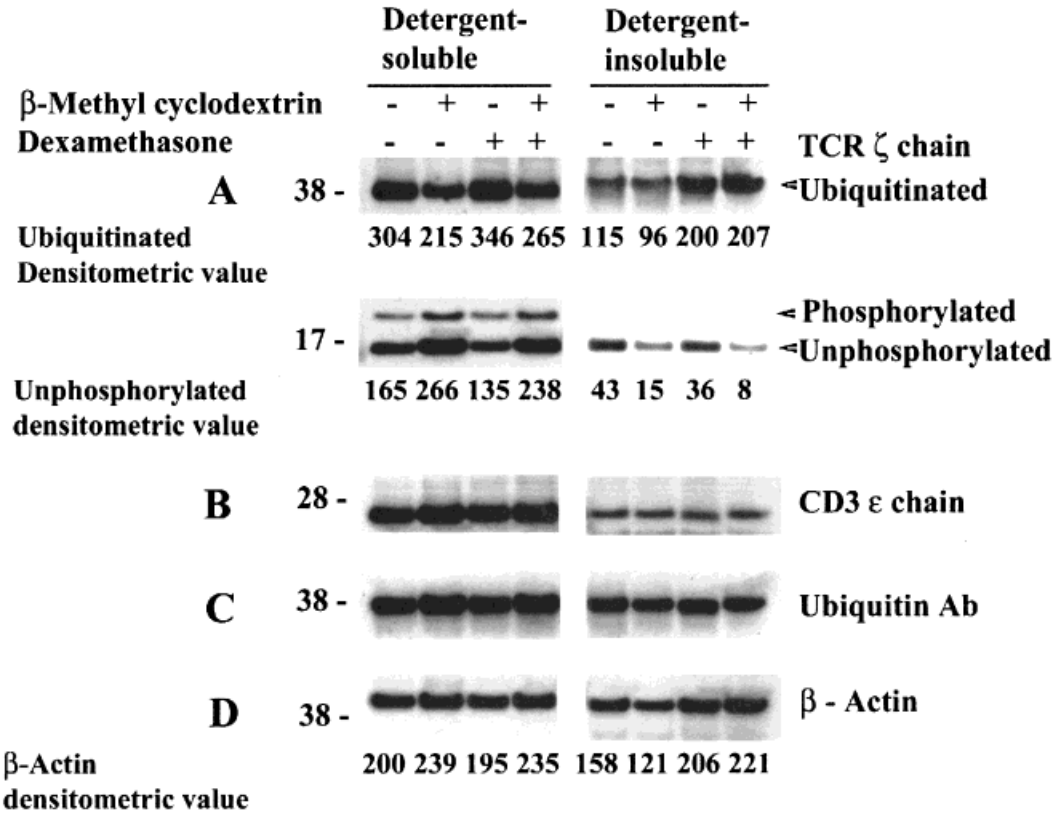

Fig. 5. Analysis of lipid raft-associated form of the TCR ζ chain in dexamethasone treated cells. Five million cells were treated with or without 100 nM dexamethasone for 24 h at 37°C. Cells were then incubated with and without the lipid-raft disrupting agent, β-methyl cyclodextrin (10 μg/ml) for 30 min at room temperature. The cells were lysed and the detergent-insoluble and insoluble fractions were collected by centrifugation, separated by SDS–PAGE, transferred and blotted with the TCR ζ chain C-terminal mAb as described in Materials and Methods. **A**: Upon treatment with methyl-β-cyclodextrin, the levels of the p16 and phosphorylated p21, p23 forms of the TCR ζ chain were increased in the detergent-soluble fraction. Dex-treated cells showed increased levels of ubiquitinated form of the TCR ζ chain in the detergent-insoluble membrane that was not changed by methyl-β-cyclodextrin treatment. **B**: The blots were stripped and reprobed with CD3 ϵ antibody to confirm equal loading of β methyl cyclodextrin-treated and untreated samples. **C**: Immunoblots were also reprobed with anti-ubiquitin antibody to confirm ubiquitination of the TCR ζ chain. **D**: The blots were probed with β-actin antibody. A representative result of three different experiments is shown. Densitometric values indicated for the ubiquitinated and p16 kDa form of the TCR ζ chain and β-actin are in arbitrary units.

Upon activation of TCR/CD3 with OKT3, the TCR ζ chain clustering became more prominent in 100 nM Dex treated cells. The bright immunostained TCR ζ chain clusters superimpose with FITC-labeled LAT staining suggesting that they are colocalized in lipid-rafts. Thus, treatment with 100 nM Dex causes a marked perturbation in the distribution of TCR ζ chain in human T cells and increased TCR clustering upon activation. Lower incubation time (7 h) with 100 nM Dex also induced a change in the distribution of the TCR ζ chain although less in magnitude compared to 24 h treated cells. Similarly, the TCR/CD3-induced $[Ca^{2+}]_i$ response is also low in 7 h Dex treated cells compared to 24 h suggesting that there is a correlation between the membrane effect and TCR/CD3-induced $[Ca^{2+}]_i$ response in high-dose Dex-treated cells. Lowe-dose of Dex (10 nM), that upregulate the TCR ζ chain expression did not induce any change in the membrane distribution of the TCR ζ chain (data not shown).

DISCUSSION

Here, we demonstrate that Dex at a high dose upregulates TCR/CD3-induced $[Ca^{2+}]_i$ response by membrane-mediated mechanisms in human T cells. There are several factors which influence the magnitude and pattern of $[Ca^{2+}]_i$ response after TCR/CD3-stimulation. A rise in TCR/CD3-mediated $[Ca^{2+}]_i$ signal after T cell activation is derived from both intra- and

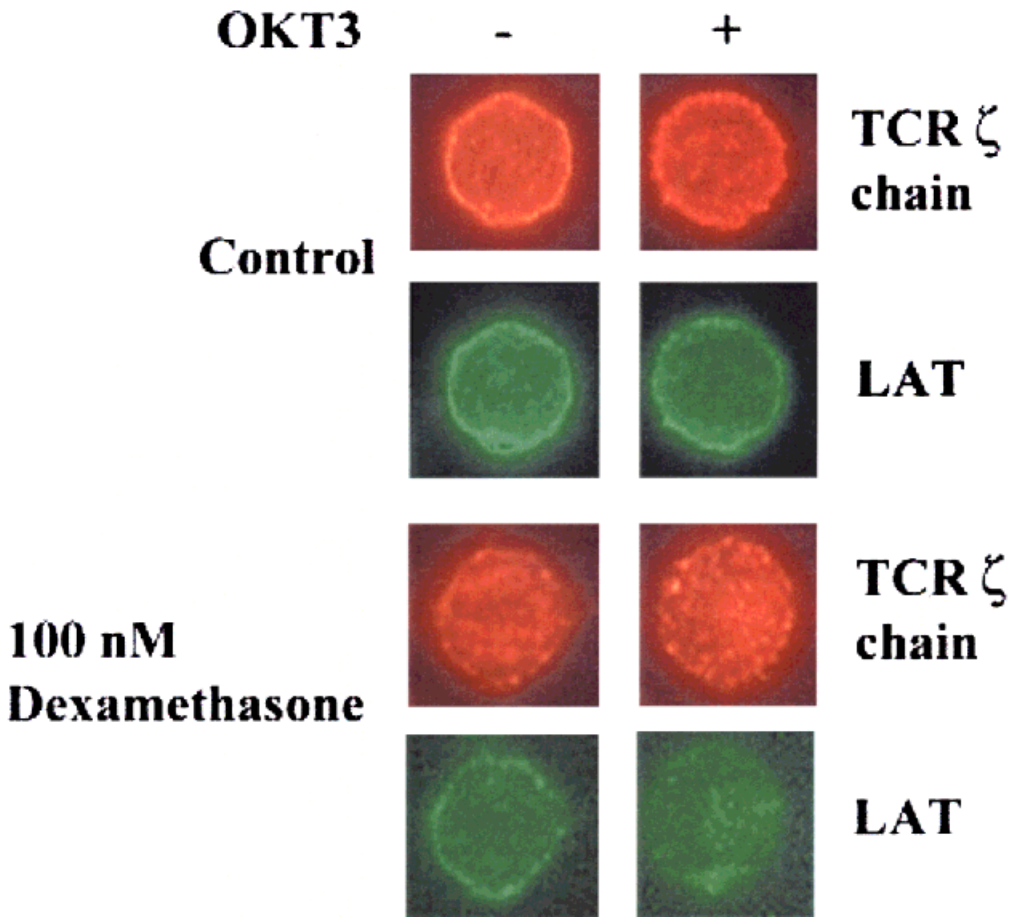

Fig. 6. One-hundred nanomoles dexamethasone alters the distribution of TCR ζ chain in human T cells. Five million cells were treated with 100 nM dexamethasone for 24 h. The cells were activated with 10 μg/ml of OKT3 antibody for 2 min. Cells were fixed with 0.25% paraformaldehyde and permeabilized with digitonin. Cells were incubated with anti-mouse TCR ζ chain C-terminal monoclonal antibody and anti-rabbit LAT antibody (lipid raft marker) for 1 h and then washed in staining buffer. Rhodamine-labeled anti-mouse goat IgG and FITC labeled anti-rabbit donkey IgG secondary antibodies were added and incubated for 1 h. The cells were washed, mounted on slides, and the samples were visualized in an Olympus fluorescence microscope at a magnification of 100×. In 100 nM Dex-treated cells, the TCR ζ chain appeared to be more clustered compared to untreated control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

extra-cellular sources. Intracellular Ca^{2+} store in the ER is critically involved in the control of Ca^{2+} influx across the plasma membrane. In high dose Dex-treated cells, the ionomycin induced $[Ca^{2+}]_i$ response was lower than control cells, suggesting that Dex affects the membrane integrity. On the other hand, this result also suggests that the hyper $[Ca^{2+}]_i$ response in Dex-treated cells is not the consequence of increased permeability of the cells to Ca^{2+}. Similar amounts of 'thapsigargin pool' of Ca^{2+} in Dex-treated and untreated cells suggest that the intracellular stores of Ca^{2+} is not altered in Dex-treated cells. It has been proposed that the ER is physically linked to the influx channels at the plasma membrane. A depletion of intracellular Ca^{2+} stores induces conformational changes in the ER that are transmitted to the plasma membrane Ca^{2+} influx channels via the cytoskeleton, leading to its opening [Berridge, 1995]. However, lack of difference in the thapsigargin pool suggests that the increased TCR/CD3-induced $[Ca^{2+}]_i$ response in Dex-treated cells is not the consequence of altered regulation of the membrane channels by ER Ca^{2+} storage. TCR/CD3-mediated $[Ca^{2+}]_i$ response does not involve Ca^{2+} stored in the mitochondria and nuclear envelope, thus excluding the possibility of contributions for the $[Ca^{2+}]_i$ signaling from these sources.

The increased TCR/CD3 induced $[Ca^{2+}]_i$ response was suppressed by the glucocorticoid

antagonist, RU486. RU486, at 100 nM completely inhibited the enhanced TCR/CD3-mediated $[Ca^{2+}]_i$ response in 100 nM M Dex-treated cells suggesting that the effect of Dex is specific and mediated by the glucocorticoid receptor. It has been previously reported that Dex does not mobilize steroid receptors in T cell hybridomas [Zacharchuk et al., 1990]. The cell surface expression of TCR associated molecules was also not affected by high-dose Dex treatment. The enhancing effect of Dex on TCR/CD3-induced $[Ca^{2+}]_i$ response was found to be readily reversible upon short-term culture in steroid-free medium, indicating that high-dose Dex-treated cells were not committed to apoptosis under these experimental conditions. Of note, cells treated with 100 nM Dex were found to be viable by trypan blue exclusion similar to untreated cells. Although the stimulation of TCR/CD3 induced $[Ca^{2+}]_i$ response by 100 nM Dex required glucocorticoid receptors, de novo protein synthesis or transcription was not required. We believe that Dex binding to glucocorticoid receptor may cause more profound and different changes in the membrane integrity and distribution of TCR ζ chain than direct intercalation with the membrane. High-dose Dex binding to glucocrticoid receptor may cause membrane changes more effectively by altering the receptor structure than direct intercalation with the membrane. Also it is not clear at this time whether the effect is caused by both direct intercalation of Dex to membrane and binding of the Dex to glucocorticoid receptor that may prime the membrane changes.

Because de novo protein synthesis or RNA synthesis is not required for the induction of TCR/CD3-mediated $[Ca^{2+}]_i$ response in high dose Dex-treated cells, we reasoned that Dex may act by modulating the membrane distribution and orientation of the signaling molecules. Non-genomic effects of several steroid hormones on the plasma membrane lipid mobility have been reported [van Bommel et al., 1987]. Dex decreases the membrane fluidity of Leukemia cells [Kiss et al., 1990; Golden et al., 1999]. The lipid-bilayer fluidity or lateral mobility modulates the structure and function of biomembranes and potentially favors the supramolecular clustering of the signaling molecules and thereby alters T cell signaling. Intracellular mediators have been shown to regulate CD2 lateral diffusion and cytoplasmic Ca^{2+} mobilization upon CD2-mediated T cell activation [Chan et al., 1991; Liu et al., 1995; Liu and Golan, 1999]. Our fluorescence microscopy data show that indeed Dex at high-dose alters the distribution of existing TCR ζ chain resulting in preformed clusters.

The ubiquitin-mediated degradation of proteins plays an important role in receptor down-regulation and in the control of a variety of signal transduction processes [Hershko and Ciechanover, 1998]. The ubiquitin system has been implicated in the immune response and abnormalities in ubiquitin-mediated processes have been shown to cause pathological conditions. Ligation to ubiquitin also may affect the function by altering signal transduction properties and associations of modified proteins. Both phosphorylated and non-phosphorylated ζ molecules were found to undergo ubiquitination [Cenciarelli et al., 1992; Hou et al., 1994]. Clearly more work is required to explain how increased ubiquitinated form of the TCR ζ chain in the detergent insoluble membrane fraction of Dex-treated cells contributes to enhanced TCR/CD3 mediated $[Ca^{2+}]_i$ response.

It has been shown that 10–40% of the TCR ζ chain is associated with the detergent-insoluble membrane fraction [Caplan and Baniyash, 1996, 2000]. In addition, T cell activation causes tyrosine phosphorylation of TCR ζ chain and the phosphorylated form binds to the actin-cytoskeleton and thus translocates to the membrane [Caplan et al., 1995; Rozdzial et al., 1995]. The actin scaffold recruits or stabilizes specialized membrane domains enriched in glycolipids and signaling molecules that are implicated in T cell activation [Grakoui et al., 1999]. The supramolecular assembly of the signaling complexes is critical for T cell activation. Imaging of antigen recognition has shown that compulsory cytoskeletal alterations are required for the triggering of an intracellular calcium response [Delon et al., 1998]. Increased detergent-insoluble membrane associated actin in Dex-treated cells may stabilize the lipid-rafts and supramolecular assembly as well as promote clustering and signaling, enhancing the TCR/CD3 mediated signaling and $[Ca^{2+}]_i$ mobilization in Dex-treated cells.

The data presented here clearly suggest that modulation of membrane structure and distribution of TCR ζ chain has a profound effect on TCR/CD3 mediated signal transduction. An increase in TCR/CD3 mediated signaling was also identified in a *N*-acetylglucosaminyl

transferase gene knockout mice, that alters the molecular lattice of glycoprotein and galectin on the cell surface [Demetriou et al., 2001]. How the changes in membrane biophysical characteristics affect the lateral mobility of signaling molecules and subsequently the TCR/CD3-mediated $[Ca^{2+}]_i$ response in high-dose of Dex-treated cells? Although at this time the data is insufficient to suggest one particular model, a number of interesting possibilities may be considered. It is possible that the change in the membrane dynamics caused by intercalation of Dex could bring phospholipase C γ to close proximity leading to more easy and rapid activation. Intercalation of the Dex may exclude the silencing signaling phosphatases from the antigen-recognition site in the plasma membrane. Altered distribution of membrane signaling molecules may prevent segregation of the kinases and thus making them more available for phosphorylation. Increased level of actin association with the cell membrane may facilitate clustering and stability of signaling molecules and formation of supramolecular assembly. Altering the T cell signal transduction by modulating the membrane structure offers another quick mode to activate T cells and decrease tolerance.

In conclusion, Dex at high concentrations upregulates the TCR/CD3 induced $[Ca^{2+}]_i$ response in human T cells. This enhancing property of the Dex was neither dependent on transcription nor on de novo protein synthesis. The enhancing effect was readily reversible and mediated through the glucocorticoid receptor. The increased $[Ca^{2+}]_i$ response in high dose Dex-treated cells seems to be caused by membrane-mediated mechanisms that change the distribution of signaling molecules leading to more functional signaling clusters. Together, these experiments produce a broader picture of the mechanism action of Dex on $[Ca^{2+}]_i$ signaling and should help further define the mechanism(s) of steroid-membrane interactions that mediate biological effects in human T cells.

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