Ryanodine Receptor Signaling Is Required for Anti-CD3-Induced T Cell Proliferation, Interleukin-2 Synthesis, and Interleukin-2 Receptor Signaling

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Abstract   Ryanodine receptors (RyR) are involved in regulating intracellular Ca^{++} mobilization in T lymphocytes. However, the importance of RyR signaling during T cell activation has not yet been determined. In this study, we have used the RyR-selective antagonists, ruthenium red and dantrolene, to determine the effect of RyR blockade on T cell receptor-mediated activation events and cytokine-dependent T cell proliferation. Both ruthenium red and dantrolene inhibited DNA synthesis and cell division, as well as the synthesis of interleukin (IL)-2 by T lymphocytes responding to mitogenic anti-CD3 antibody. Blockade of RyR at initiation of culture or as late as 24 h after T cell receptor stimulation inhibited T cell proliferation, suggesting a requirement for sustained RyR signaling during cell cycle progression. Although flow cytometry revealed that RyR blockade had little effect on activation-induced expression of the α chain (CD25) of the high affinity IL-2 receptor, the inhibitory effect of RyR antagonists could not be reversed by the addition of exogenous IL-2 at initiation of culture. In addition, both ruthenium red and dantrolene had a strong inhibitory effect on IL-2-dependent proliferation of CTLL-2 T cells. These data indicate that RyR are involved in regulating IL-2 receptor signaling that drives T cell progression through the cell cycle. We conclude that RyR-associated Ca^{++} signaling regulates T cell proliferation by promoting both IL-2 synthesis and IL-2-dependent cell cycle progression. J. Cell. Biochem. 92: 387–399, 2004.   © 2004 Wiley-Liss, Inc.

Key words: T lymphocyte; calcium; interleukin-2; ryanodine receptor

The release of Ca^{++} from intracellular stores and the consequent sustained influx of extracellular Ca^{++} through store-operated Ca^{++} channels lead to a dramatic rise in free intracellular Ca^{++} levels that constitutes an early and critical signal for antigen-induced T cell activation [Lewis, 2001]. For example, Ca^{++} is essential for activation of the Ca^{++}/calmodulin-dependent serine/threonine phosphatase, calcineurin, which promotes nuclear translocation of nuclear factor of activated T cells (NFAT) and NFAT-dependent transcription of cytokine genes such as interleukin (IL)-2 [Wesselborg et al., 1996]. Ca^{++} is also required for activation of calpain, a protease that regulates lymphocyte function-associated antigen-1 (LFA-1)-mediated adhesive contact between T lymphocytes and antigen-presenting cells [Stewart et al., 1998]. Signaling through inositol 1,4,5-trisphosphate (IP_3) receptors and ryanodine receptors (RyR) located on the endoplasmic reticulum constitute the principal means of inducing the regulated release of Ca^{++} from intracellular stores, although the recently described nicotinic acid adenine dinucleotide

phosphate (NAADP) receptor may also contribute to intracellular Ca^{++} mobilization [Grafton and Thwaite, 2001].

Three distinct types of RyR have been identified in mammalian cells. Type 1 and type 2 RyR were originally described in skeletal and cardiac muscle, respectively [Takeshima et al., 1989; Otsu et al., 1990], whereas type 3 RyR expression was first reported in the brain [Takeshima et al., 1993]. However, type 3 RyR mRNA has more recently also been identified in skeletal and cardiac muscle, as well as non-muscle tissues, while mRNA transcripts coding for all three RyR isoforms are expressed throughout the brain [Ledbetter et al., 1994]. Although an initial study that examined Jurkat T cells suggested that only type 3 RyR are expressed by T lymphocytes [Hakamata et al., 1994], a more recent study has revealed that primary peripheral human T cells express both type 1 and type 2 RyR but, surprisingly, lack type 3 RyR expression [Hosoi et al., 2001]. In any case, type 3 RyR are not likely to be critical for T lymphocyte Ca^{++} signaling since T cells from type 3 RyR-deficient mice proliferate normally in response to a mitogenic stimulus [Takeshima et al., 1996]. The sustained phase of Ca^{++} signaling that is mediated by RyR in mouse and human T cells is stimulated by cyclic ADP-ribose (cADPR) that is formed following ligation of the T cell receptor [Guse et al., 1993, 1999; Bourguignon et al., 1995]. In addition, transient tyrosine phosphorylation of RyR by the tyrosine kinase p59^{fyn} has been suggested to regulate intracellular Ca^{++} mobilization via RyR [Guse et al., 2001].

Although the role of IP_3 in mediating Ca^{++} release from internal stores during T cell activation has been comparatively well studied, little is known about the importance of RyR signaling for T cell activation [Grafton and Thwaite, 2001; Lewis, 2001]. Ruthenium red and dantrolene are potent and selective inhibitors of RyR-mediated release of Ca^{++} from intracellular stores [Fruen et al., 1997; Xu et al., 1999; Beutner et al., 2001; Lendvai et al., 2001]. In this study, we have tested the hypothesis that RyR signaling is required for T cell activation by using the RyR antagonists ruthenium red and dantrolene to inhibit RyR function in mouse T lymphocytes stimulated with mitogenic anti-CD3 monoclonal antibody (mAb). Our results show that RyR signaling is necessary for T cell proliferation and IL-2

synthesis, as well as for IL-2 receptor signal transduction.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice were purchased from Charles River Canada (Lasalle, Que.) and housed in the Carleton Animal Care Facility at Dalhousie University. Mice were maintained on standard mouse chow and water supplied ad libitum. Mice were routinely used at 8–12 weeks of age.

Reagents and Cell Lines

RPMI 1640 medium was supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (all ICN Biomedicals Canada, Ltd., Mississauga, Ont.), 5 mM HEPES buffer (pH 7.4; Sigma-Aldrich, Oakville, Ont.), and 5% fetal calf serum (heat-inactivated at 56°C for 30 min; Invitrogen Life Technologies, Burlington, Ont.). Hamster anti-mouse CD3 mAb was from hybridoma clone 145-2C11, which was kindly provided by Dr. J. Bluestone (University of Chicago, Chicago, IL). Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD25 mAb (IgG_1) and FITC-conjugated rat IgG_1 were from Cedarlane Laboratories (Hornby, Ont.). Fluo-4-AM and 5-(and-6)-carboxyfluroescein diacetate succinimidyl ester (CSFE) were from Molecular Probes, Inc. (Eugene, OR). Recombinant mouse IL-2 was obtained from Peprotech Canada, Inc. (Ottawa, Ont.). Ruthenium red, dantrolene, and probenecid were purchased from Sigma-Aldrich. EL-4 T lymphoma cells, A20 B lymphoma cells, and IL-2-dependent CTLL-2 T cells were obtained from the American Type Culture Collection (Manassas, VA). CTLL-2 cells were maintained by in vitro passage in RPMI 1640 medium supplemented with 10% fetal calf serum and 2.5 ng/ml IL-2.

T Lymphocyte Isolation and Activation

Mouse spleens were removed under aseptic conditions and spleen cell suspensions were prepared in cold phosphate buffered saline (PBS; pH 7.2). Erythrocytes were removed by osmotic shock and the remaining lymphocytes were washed, resuspended in RPMI 1640 medium, and passaged through a nylon wool column (Polysciences, Inc., Warrington, PA) to deplete B cells and macrophages. The resulting T cell-rich preparation (>95% viable by trypan

blue dye exclusion test) was added to 96-well round-bottom microtitre plates or to 24-well flat-bottom tissue culture plates. Each microtitre well contained 2.5×10^5 cells in a final volume of 0.2 ml while each flat-bottom well contained 8×10^6 cells in a final volume of 2 ml. All microtitre cultures were performed in quadruplicate. T cells were activated by the addition of anti-CD3 mAb in the form of hybridoma culture supernatant at a final dilution of 1:20 (previously determined to induce an optimal T cell proliferative response). T cell cultures were maintained in a 5% CO_2 humidified atmosphere at 37°C. T cell proliferation in microtitre plates was measured by pulsing each well with 0.5 µCi of tritiated thymidine ([^{3}H]TdR; Sp. Act. 65 Ci/mmol; ICN Biomedicals Canada) for 6 h prior to termination of culture. T cells were then harvested with a multiple sample harvester onto glass fiber mats and [^{3}H]TdR incorporation was measured by scintillation counting. For cell cycle analysis, T cells were stained for 10 min at 37°C with 2.5 µM CSFE. Immediately after staining, T cells were thoroughly washed and used in experiments. Fluorescence was determined by flow cytometry after 72 h of culture. After 24 h of culture, T cells and supernatants were harvested from flat-bottom plates for flow cytometry and ELISA measurements, respectively.

IL-2-Dependent Proliferation Assay

IL-2-dependent CTLL-2 cells were washed with PBS, resuspended in complete RPMI 1640 medium containing 10% fetal calf serum, and plated at 1×10^4 cells/well in quadruplicate wells of a 96-well microtitre plate. All cultures received 2.5 ng/ml IL-2 without or with various concentrations of ruthenium red, with the exception of the negative control which received only medium. Cultures were incubated for 24 h, and during the final 6 h of culture DNA synthesis was assessed by [^{3}H]TdR incorporation as previously described for primary T cell cultures.

Real-Time Ca^{++} Flux Analysis

T cells were washed and resuspended at 5×10^6 cells/ml in Hanks buffered salt solution (HBSS) containing 20 mM $CaCl_2$, 20 mM $MgCl_2$, and 4 mM probenecid. T cells were then loaded with 4 µM Fluo-4-AM by incubation at 37°C for 45 min. Following two washes with HBSS containing 5% fetal calf serum, T cells were resuspended at 2×10^5 cells/ml in ice-cold HBSS

containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Aliquots of 4×10^5 T cells were then warmed to 37°C for 5 min and placed in a 37°C thermostated quartz cuvette with magnetic stirring in a RF-1501 spectrofluorophotometer (Shimadzu, Tokyo, Japan). T cells were treated with medium alone or with ruthenium red (50 µg/ml) followed by stimulation with anti-CD3 mAb (1:100 ascites). T cells were subsequently exposed to 10% Triton-X 100 (positive control) and 0.5 M EGTA (negative control) in a sequential fashion. Fluorescence was measured at 520 nm after excitation at 485 nm.

Flow Cytometry

T cells were washed with PBS, resuspended in immunofluorescence buffer (1% bovine serum albumin, 0.2% sodium azide in PBS) containing either FITC-conjugated anti-CD25 mAb or FITC-conjugated rat IgG$_1$ (both at 5 µg/ml), and incubated for 30 min at 4°C. T cells were then washed twice with immunofluorescence buffer and resuspended in PBS containing 1% paraformaldehyde prior to analysis with a Becton-Dickinson FACScan.

ELISA

IL-2 concentrations in cell-free supernatants from 24-h cultures of anti-CD3-activated T cells were measured by sandwich ELISA using paired mAbs, recombinant cytokines, and protocols supplied by BD Pharmingen.

Statistical Analysis

Statistical comparisons of data were performed using the Instat statistics program (GraphPad Software, San Diego, CA). One way analysis of variance (ANOVA) and the Tukey–Kramer test were used for multiple comparisons while Student's t-test was used to compare differences between two different sample groups. P values less than 0.05 were considered to be statistically significant.

RESULTS

RyR Blockade Inhibits DNA Synthesis and Cell Division in T Cells

Ruthenium red is a polycationic dye that is a selective inhibitor of RyR signaling [Xu et al., 1999; Beutner et al., 2001]. To confirm that ruthenium red prevented Ca^{++} mobilization in T cells, we loaded freshly isolated mouse T cells with Fluo-4-AM and measured intracellular

Ca^{++} fluxes in control T cells and ruthenium red-treated T cells in response to anti-CD3 mAb, which mimics antigen-induced crosslinking of the T cell receptor/CD3 complex. As expected, Ca^{++} mobilization was abrogated when T cells were stimulated with anti-CD3 mAb in the presence of ruthenium red (Fig. 1).

To determine the importance of RyR signaling for T cell activation, mouse T cells were stimulated with anti-CD3 mAb in the absence or presence of various concentrations of ruthenium red, and T cell proliferation was assessed by [^{3}H]TdR incorporation into newly synthesized DNA following 24, 48, and 72 h of culture. Figure 2A shows that anti-CD3-induced T cell proliferation was inhibited in a dose-dependent fashion by ruthenium red at all time points that were examined. Similar results were obtained when dantrolene, a muscle relaxant that is a potent and specific RyR antagonist [Fruen et al., 1997; Lendvai et al., 2001], was added to T cell cultures containing anti-CD3 mAb (Fig. 2B). Anti-CD3-induced T cell proliferation in the presence of 0.2% DMSO, which was the vehicle

for dantrolene, was not significantly different from the proliferation of T lymphocytes activated with anti-CD3 mAb in the presence of medium. A 24-h exposure to high concentrations of ruthenium red or dantrolene did not have any detrimental effect on T lymphocyte viability, as assessed by trypan blue dye exclusion test (medium control, 92% viable, vs. medium + 50 µg/ml ruthenium red, 91% viable; vehicle control, 82% viable, vs. medium + 10 µg/ml dantrolene, 81% viable). In addition, concentrations of ruthenium red or dantrolene that strongly inhibited DNA synthesis by anti-CD3-activated T cells did not affect the uptake of [^{3}H]TdR over a 4-h period by mouse T cells (5×10^4 EL-4 cells/culture-medium control, $11,185 \pm 304$ cpm, vs. medium + 25 µg/ml ruthenium red, $10,845 \pm 312$ cpm; vehicle control, $12,381 \pm 1,615$ cpm, vs. medium + 10 µg/ml dantrolene, $11,923 \pm 395$ cpm) or mouse B cells (5×10^4 A20 cells/culture-medium control, $45,555 \pm 2,465$ cpm, vs. medium + 25 µg/ml ruthenium red, $42,111 \pm 4,147$ cpm; vehicle control, $42,629 \pm 7,959$ cpm, vs. medium +
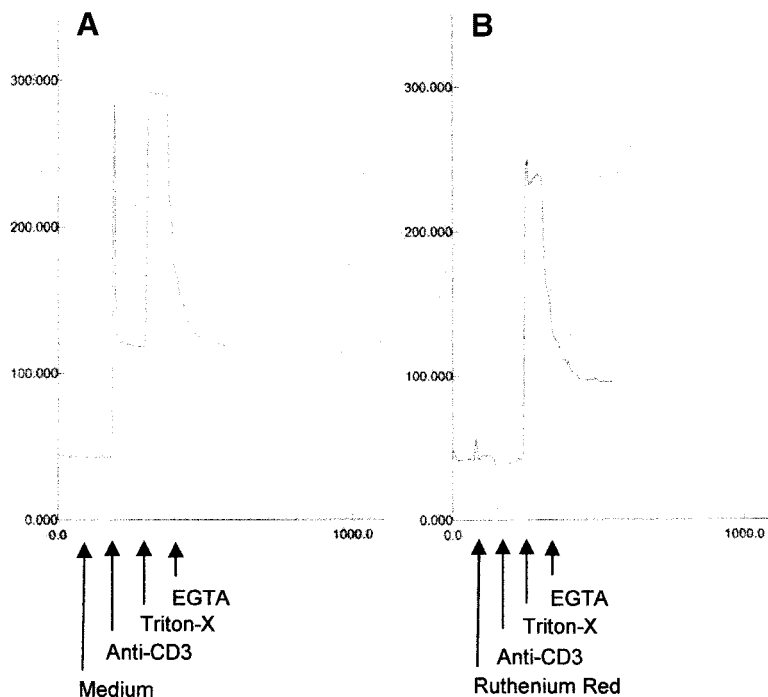

Fig. 1. Ruthenium red prevents intracellular Ca^{++} mobilization by T cells in response to anti-CD3 monoclonal antibody (mAb). T cells were loaded with 4 µM Fluo-4-AM and placed in a 37°C thermostated quartz cuvette with magnetic stirring. Following treatment with medium alone (**panel A**) or with 50 µg/ml ruthenium red (**panel B**), T cells were stimulated with anti-CD3 mAb (1:100 ascites). T cells were subsequently exposed to 10% Triton-X 100, as a positive control, and 0.5 M EGTA, as a negative control, in a sequential fashion. Fluorescence was measured at 520 nm after excitation at 485 nm. Values on the y-axis denote relative intensity while values on the x-axis denote time in seconds.
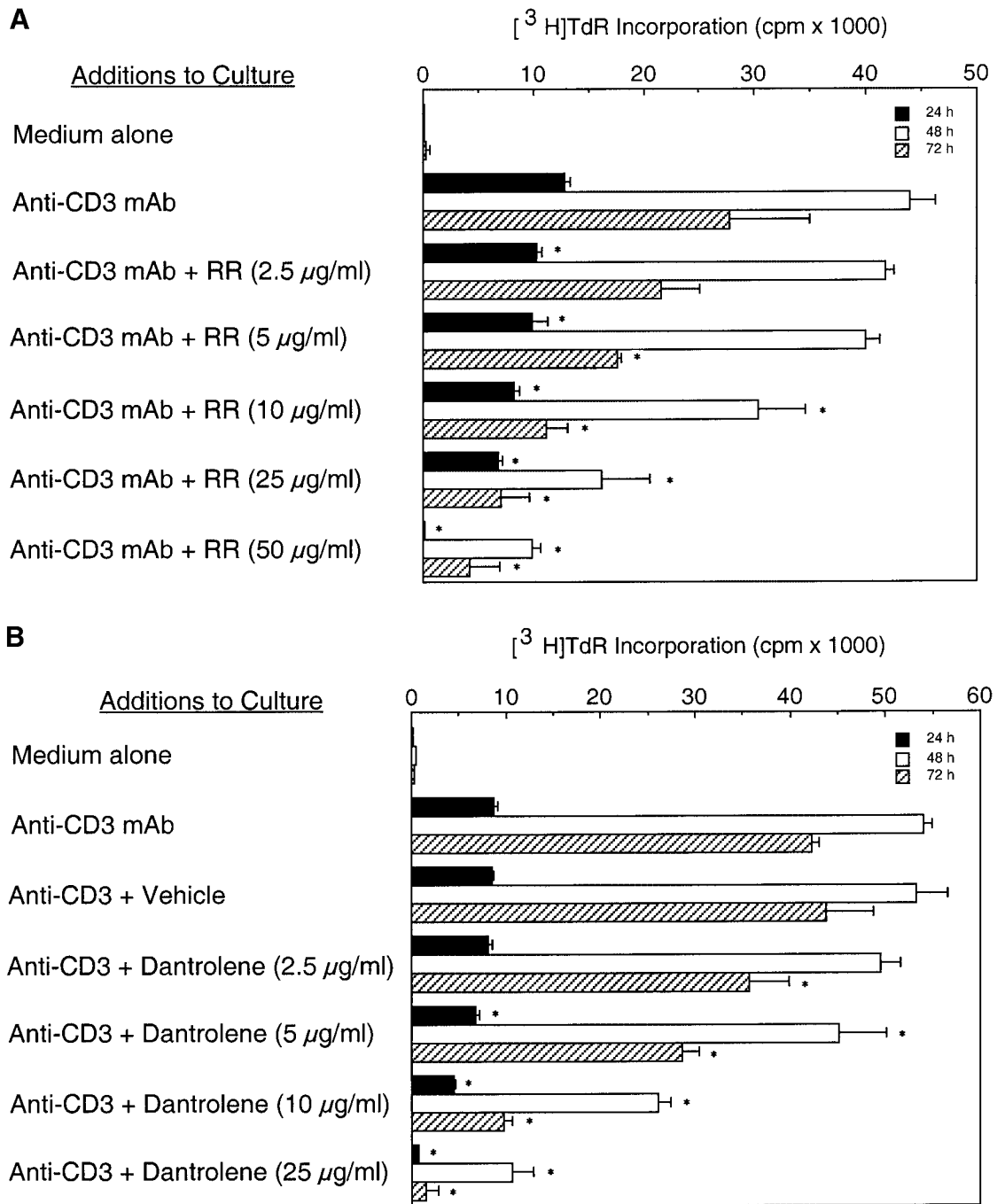
A

B

Fig. 2. Inhibition of T cell proliferation by ryanodine receptor (RyR) blockade. T cells were cultured in medium alone or stimulated with anti-CD3 mAb in the absence or presence of the indicated concentrations of ruthenium red (**panel A**) or dantrolene (**panel B**). After 24, 48, or 72 h of culture, T cell proliferation was measured by [^{3}H]TdR incorporation. Results are expressed as mean cpm ± SD of quadruplicate samples. Asterisk indicates statistical significance by ANOVA and Tukey–Kramer multiple comparisons test.

10 μg/ml dantrolene, 36,805 ± 2,268 cpm). The inhibitory effect of RyR antagonists on anti-CD3-induced T cell proliferation was, therefore, not the result of a nonspecific toxic effect or blockade of [^{3}H]TdR uptake by the drugs.

We also employed CSFE labeling to monitor cell division following T cell activation in the absence or presence of ruthenium red or dantrolene. Whereas T cells stimulated with anti-CD3 mAb underwent approximately four

rounds of division over a 72-h period, in the presence of ruthenium red (50 µg/ml) or dantrolene (25 µg/ml) there was a clear failure of anti-CD3-stimulated T cells to progress through the cell cycle (Fig. 3). This result was consistent with the profound inhibitory effect that RyR antagonists had on DNA synthesis by activated T cells (Fig. 2). Interestingly, Figure 4 shows that both ruthenium red and dantrolene strongly inhibited DNA synthesis even when
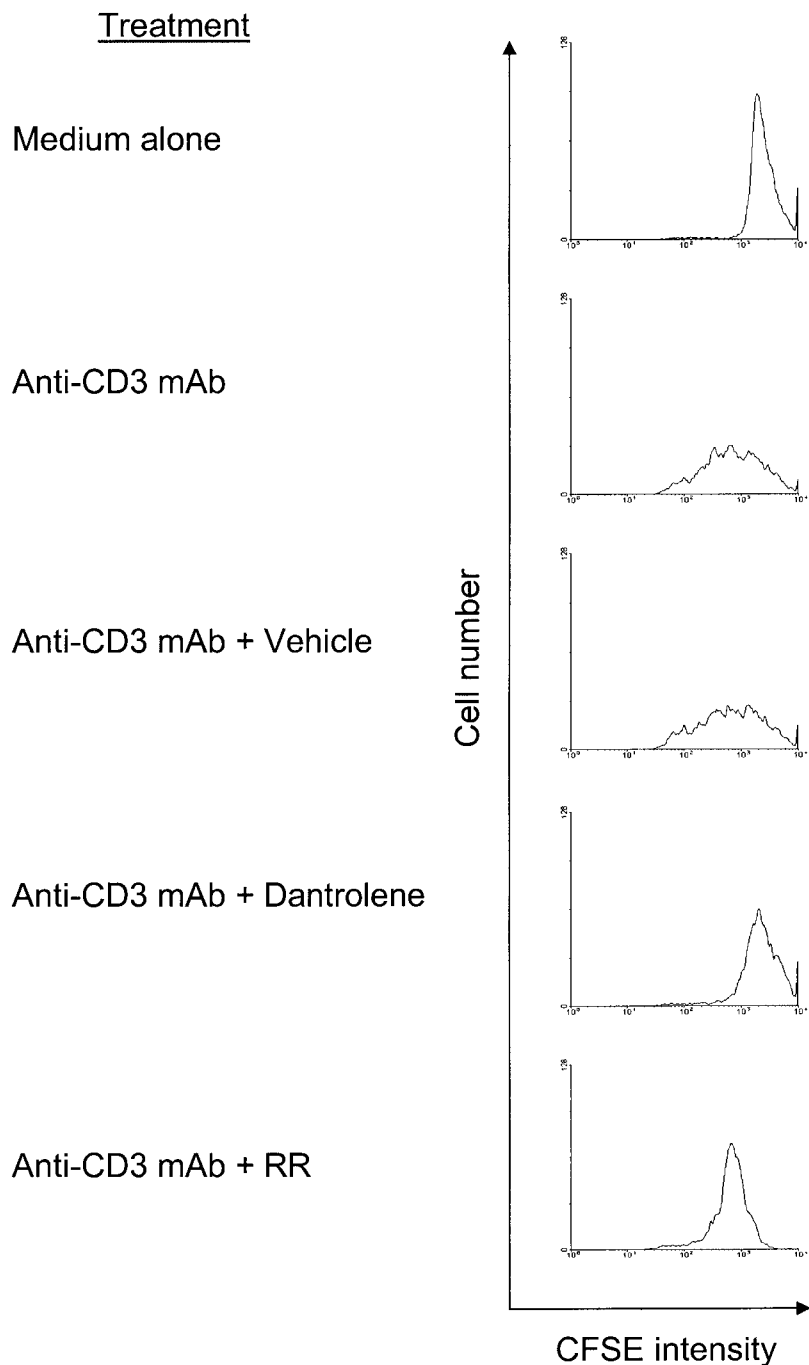
Treatment

Medium alone

Anti-CD3 mAb

Anti-CD3 mAb + Vehicle

Anti-CD3 mAb + Dantrolene

Anti-CD3 mAb + RR

CFSE intensity

Fig. 3. RyR blockade prevents cell division following T cell stimulation with anti-CD3 mAb. Active T cell division was monitored by labeling T cells with 5-(and-6)-carboxyfluroescein diacetate succinimidyl ester (CSFE) prior to culture for 72 h in medium alone, medium plus anti-CD3 mAb, or medium plus anti-CD3 mAb and ruthenium red (50 µg/ml) or dantrolene (25 µg/ml). Cell cycle progression of CSFE-labeled T cells was measured by flow cytometry.

A

[^{3}H]TdR Incorporation (cpm x 1000)

Additions to Culture

Medium alone

Anti-CD3 mAb

Anti-CD3 mAb + RR, 0 h

Anti-CD3 mAb + RR, 6 h

Anti-CD3 mAb + RR, 12 h

Anti-CD3 mAb + RR, 24 h

B

[^{3}H]TdR Incorporation (cpm x 1000)

Additions to Culture

Medium alone

Anti-CD3 mAb

Anti-CD3 mAb + Dantrolene, 0 h

Anti-CD3 mAb + Dantrolene, 6 h

Anti-CD3 mAb + Dantrolene, 12 h
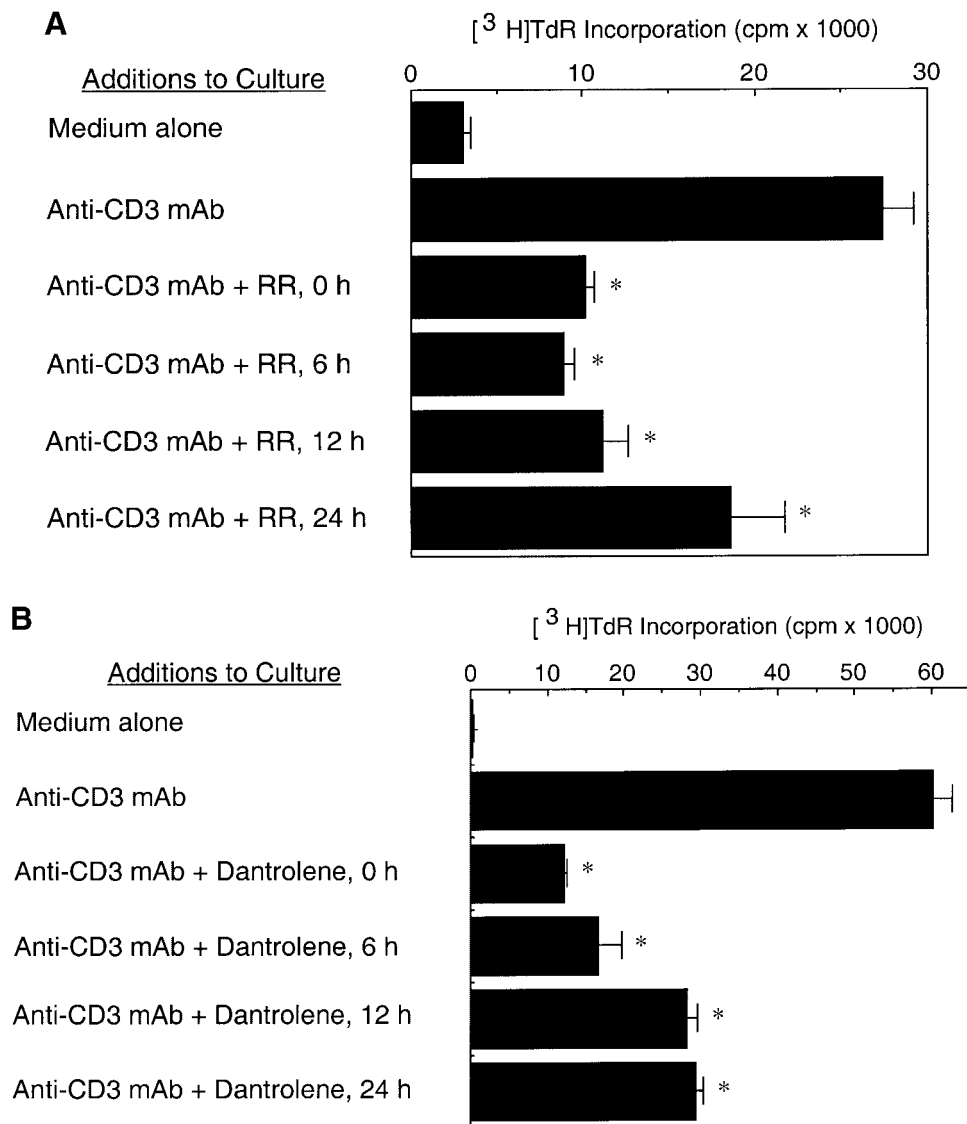
Anti-CD3 mAb + Dantrolene, 24 h

Fig. 4. Time course of RyR antagonist addition to anti-CD3-activated T cell cultures. T cells were cultured in medium alone or stimulated with anti-CD3 mAb in the absence or presence of 25 μg/ml ruthenium red (**panel A**) or dantrolene (**panel B**) added at the indicated times. After 48 h of culture, T cell proliferation was measured by [^{3}H]TdR incorporation. Results are expressed as mean cpm ± SD of quadruplicate samples. Asterisk indicates statistical significance by ANOVA and Tukey–Kramer multiple comparisons test.

added to culture as late as 24 h after T cell stimulation with anti-CD3 mAb. These data suggest that RyR signaling must be sustained for optimal T cell expansion.

RyR Signaling Is Required for IL-2 Synthesis

Because T cell proliferation in response to T cell receptor stimulation is largely an IL-2-dependent process [Smith, 1988], we next examined the effect of RyR blockade on IL-2 synthesis by anti-CD3-activated T cells. Figure 5 shows that the addition of either ruthenium

red or dantrolene at initiation of culture had a dramatic inhibitory effect on the subsequent ability of anti-CD3-activated T cells to synthesize IL-2, as determined by ELISA measurements of IL-2 levels in 24-h culture supernatants. In contrast, flow cytometric analysis revealed that anti-CD3-induced expression of the α chain (CD25) of the high affinity IL-2 receptor was not substantially affected when RyR signaling was antagonized (Fig. 6). These data indicate that RyR signaling is important for IL-2 gene expression but
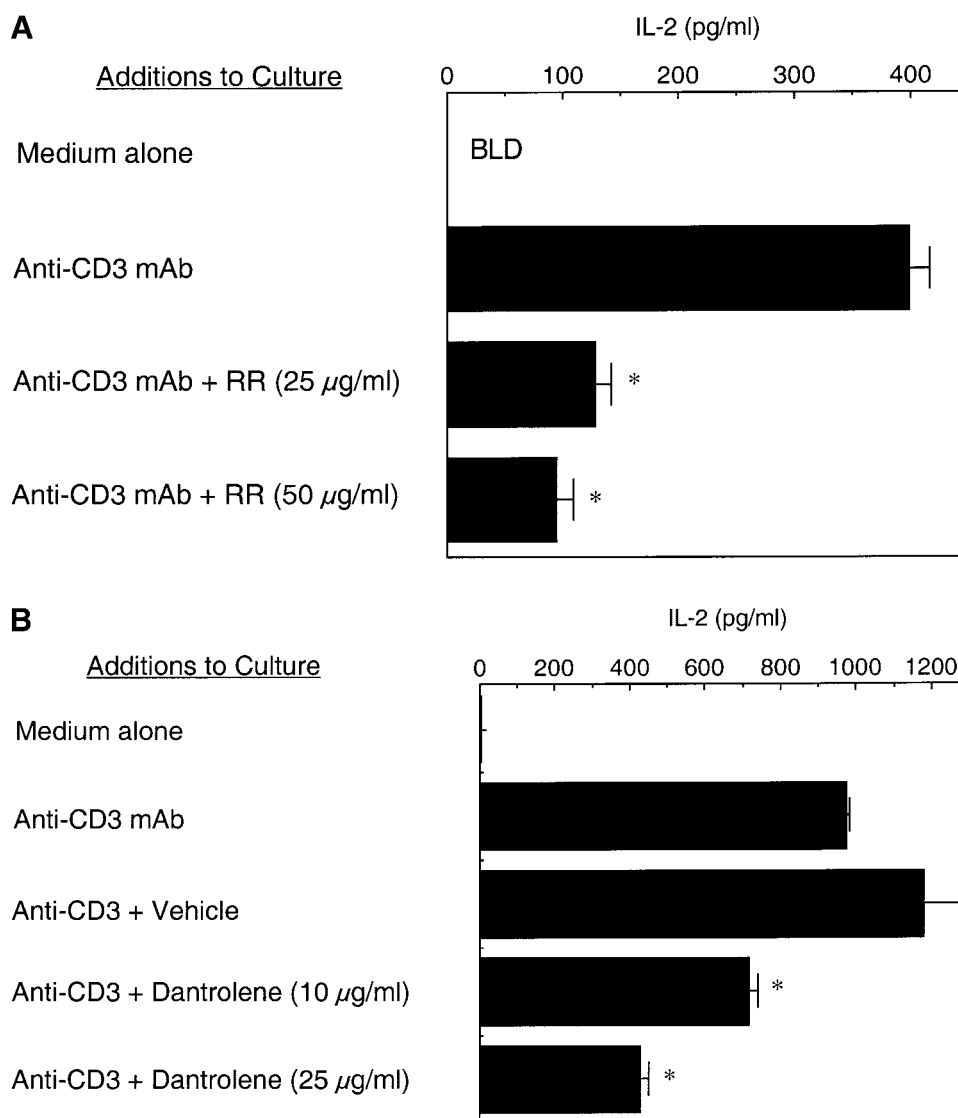
A

B

Fig. 5. RyR blockade inhibits interleukin (IL)-2 synthesis by anti-CD3-activated T cells. T cells were cultured in medium alone or stimulated with anti-CD3 mAb in the absence or presence of the indicated concentrations of ruthenium red (**panel A**) or dantrolene (**panel B**). IL-2 in 24-h cell-free culture supernatants was measured by ELISA. Results are expressed as mean pg/ml $\pm$ SD of quadruplicate samples. Asterisk indicates statistical significance by ANOVA and Tukey–Kramer multiple comparisons test.

not CD25 synthesis following T cell receptor stimulation.

RyR Blockade Interferes With IL-2-Dependent T Cell Proliferation

Since anti-CD3-induced IL-2 synthesis was impaired but CD25 expression remained intact following treatment of T cells with RyR antagonists, we next tested whether the addition of exogenous IL-2 might be able to overcome the inhibitory effect of RyR blockade on anti-CD3-induced T cell proliferation. Figure 7 shows that

the addition of exogenous IL-2 resulted in enhanced T cell proliferation in response to anti-CD3 mAb but failed to stimulate the proliferation of anti-CD3-activated T cells in the presence of ruthenium red. This finding suggested that RyR blockade also interfered with one or more steps in IL-2 receptor signaling. To confirm that RyR signaling is involved in IL-2-driven T cell proliferation, we measured the IL-2-dependent proliferation of CTLL-2 T cells in the absence or presence of RyR antagonists. As shown in Figure 8, both ruthenium red
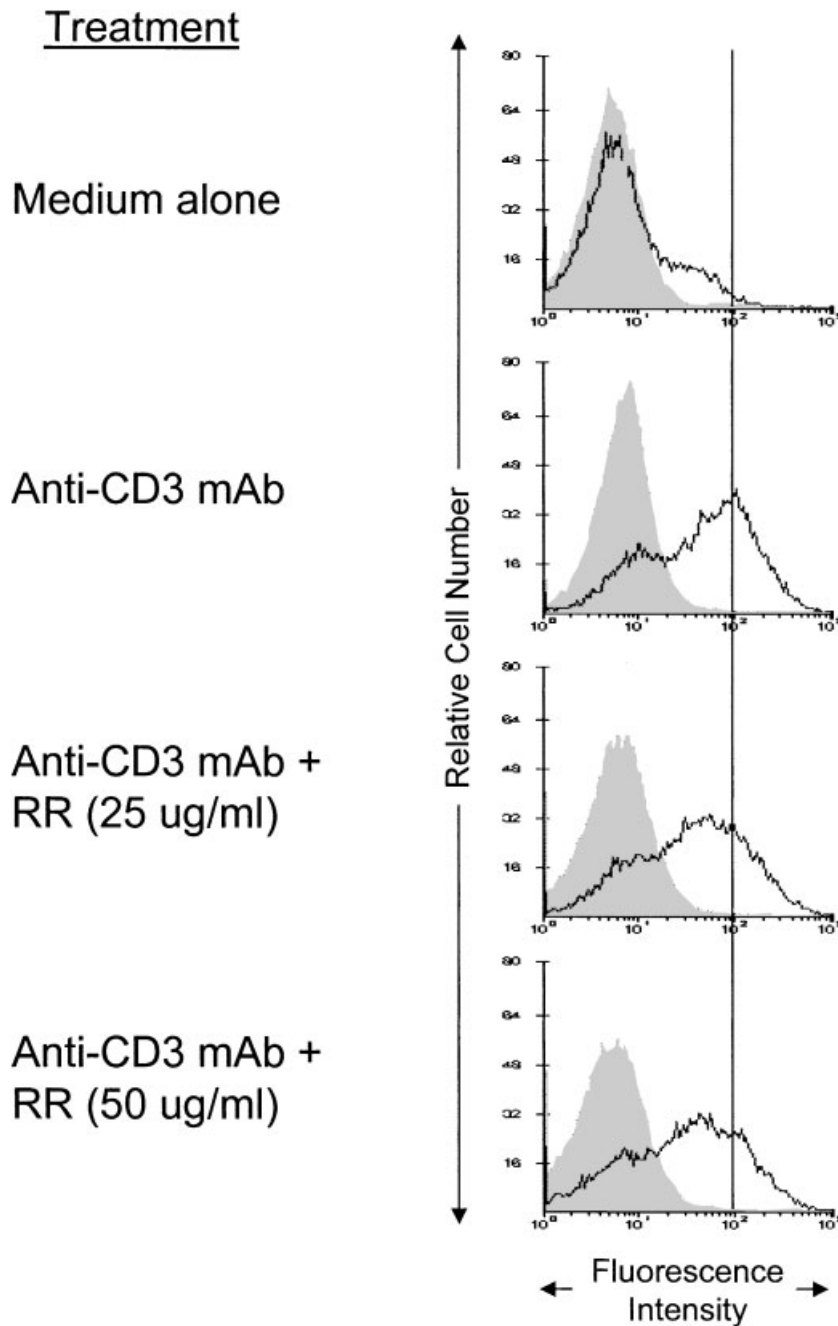
Treatment

Medium alone

Anti-CD3 mAb

Anti-CD3 mAb +
RR (25 ug/ml)

Anti-CD3 mAb +
RR (50 ug/ml)

Fig. 6. CD25 expression is not affected by RyR blockade. T cells were cultured in medium alone or stimulated with anti-CD3 mAb in the absence or presence of the indicated concentrations of ruthenium red. After 24 h of culture, CD25 expression was measured by flow cytometry. Cytofluorimetric profiles for unstained isotype controls (filled peaks) and for T cells stained with anti-CD25 mAb (open peaks) are shown.

and dantrolene inhibited the IL-2-dependent proliferative response of CTLL-2 cells in a dose-dependent manner. These data are consistent with a role for RyR in the signal transduction processes that drive T cell proliferation following stimulation of the IL-2 receptor.

DISCUSSION

Intracellular Ca^{++} levels in lymphocytes are predominantly regulated by signaling through IP_3 receptors and RyR that occurs after antigen receptor triggering [Grafton and Thwaite,

[³H]TdR Incorporation (cpm x 1000)

Additions to Culture

0   10   20   30   40

Medium alone

Anti-CD3 mAb

Anti-CD3 mAb + IL-2   *

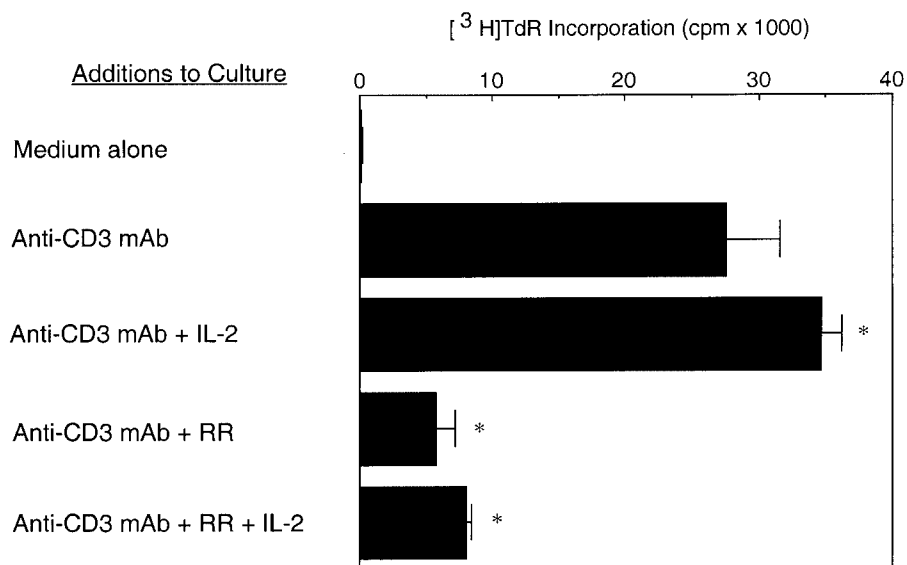Anti-CD3 mAb + RR   *

Anti-CD3 mAb + RR + IL-2   *

Fig. 7. Addition of exogenous IL-2 fails to rescue T cells from the inhibitory effect of RyR blockade. T cells were cultured in medium alone or stimulated with anti-CD3 mAb in the absence or presence of 50 µg/ml ruthenium red and/or 400 pg/ml IL-2. After 48 h of culture, T cell proliferation was measured by [³H]TdR incorporation. Results are expressed as mean cpm ± SD of quadruplicate samples. Asterisk indicates statistical significance by Student's t-test in comparison to T cells stimulated with anti-CD3 mAb alone.

2001]. Three IP$_3$ receptor subtypes have been described, all of which are expressed to varying degrees by T lymphocytes [Sugiyama et al., 1994]. T cell receptor mediated recruitment of tyrosine kinases results in phospholipase Cγ activation and the hydrolysis of phosphatidylinositol 4,5-bisphosphate, yielding the second messenger IP$_3$ that mobilizes Ca^{++} from intracellular stores via interactions with IP$_3$ receptors on endoplasmic reticulum, and causes the initial transient intracellular Ca^{++} peak [Guse et al., 1993]. The subsequent sustained rise in intracellular Ca^{++} concentration requires T cell receptor-induced production of the second messenger cADPR and the influx of extracellular Ca^{++} through store-operated Ca^{++} channels [Guse et al., 1999]. cADPR has been shown to promote a sustained Ca^{++} flux in T lymphocytes by activating RyR on the endoplasmic reticulum [Bourguignon et al., 1995; Guse et al., 1999].

In this study, we have used ruthenium red and dantrolene, which are potent and selective inhibitors of RyR signaling [Fruen et al., 1997; Xu et al., 1999; Beutner et al., 2001; Lendvai et al., 2001], to determine the effect of RyR blockade on T cell activation. Our data suggest an important role for RyR-mediated Ca^{++} mobilization during T cell activation since RyR blockade with either ruthenium red or dantrolene at the time of T cell receptor stimulation

had a marked inhibitory effect on DNA synthesis, cell division, and IL-2 production by T lymphocytes. It is noteworthy that type 3 RyR are unlikely to be involved in mouse T cell activation because T lymphocytes from type 3 RyR-knockout mice proliferate normally in response to mitogenic concanavalin A [Takeshima et al., 1996]. Moreover, peripheral human T lymphocytes have been reported to lack type 3 RyR [Hosoi et al., 2001]. Furthermore, the ability of dantrolene to inhibit mouse T cell activation suggests that signaling through type 1 RyR rather than type 2 RyR is required during this process, since dantrolene has been reported to inhibit type 1 RyR but not type 2 RyR [Fruen et al., 1997]. The transcription factor NFAT, which is critical for IL-2 gene expression that in turn drives T cell proliferation [Wesselborg et al., 1996], requires a sustained increase in intracellular Ca^{++} in order to remain localized within the nucleus [Dolmetsch et al., 1997]. Failure of NFAT to persist in the T cell nucleus in the face of RyR blockade may account for the inhibitory effects of ruthenium red and dantrolene on T cell proliferation and IL-2 synthesis. Moreover, the fact that T cell activation could be inhibited when RyR were blocked with ruthenium red or dantrolene as long as 24 h after T cell receptor stimulation suggests a requirement for sus-
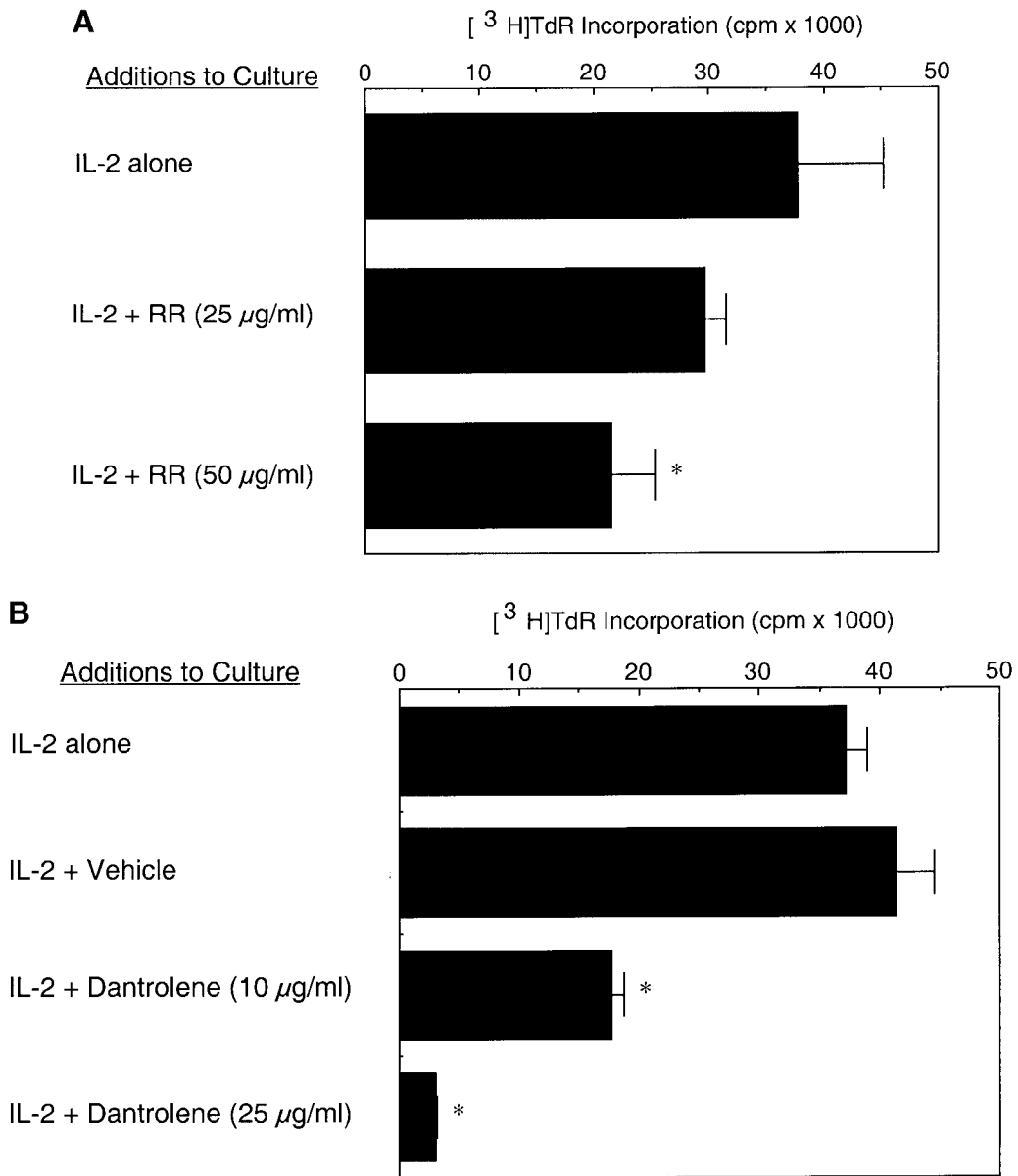
A

[^{3}H]TdR Incorporation (cpm x 1000)

Additions to Culture

IL-2 alone

IL-2 + RR (25 μg/ml)

IL-2 + RR (50 μg/ml)

B

[^{3}H]TdR Incorporation (cpm x 1000)

Additions to Culture

IL-2 alone

IL-2 + Vehicle

IL-2 + Dantrolene (10 μg/ml)

IL-2 + Dantrolene (25 μg/ml)

Fig. 8. RyR blockade inhibits IL-2-dependent T cell proliferation. IL-2-dependent CTLL-2 T cells were stimulated with 2.5 ng/ml IL-2 in the absence or presence of the indicated concentrations of ruthenium red (**panel A**) or dantrolene (**panel B**). After 18 h of culture, proliferation was measured by [^{3}H]TdR incorporation over 6 h. Results are expressed as mean cpm ± SD of quadruplicate samples. Asterisk indicates statistical significance by ANOVA and Tukey–Kramer multiple comparisons test.

tained RyR signaling during T cell receptor-stimulated progression of T cells through the cell cycle. This is consistent with the observation that intracellular Ca^{++} levels in T lymphocytes remain well above baseline for at least 1 h after T cell receptor triggering by antigen [Donnadieu et al., 1992].

IL-2 and CD25 are both early activation genes that are transcribed within 45 min and 2 h, respectively, of T cell receptor stimulation [Ull-

man et al., 1990]. It was, therefore, surprising that RyR blockade inhibited IL-2 expression by activated T cells without having any appreciable effect on CD25 expression. This finding may be explained by differential activation of transcription factors in response to Ca^{++} signals of differing amplitude and duration. Studies conducted with B lymphocytes have revealed that a low, sustained intracellular Ca^{++} plateau is required for NFAT activation

whereas NF-κB activation results from the initial transient intracellular Ca^{++} peak [Dolmetsch et al., 1997]. Although differential activation of transcription factors by intracellular Ca^{++} responses with different kinetics and magnitude has not yet been examined in T lymphocytes, it is reasonable to expect that transcription factors in B and T lymphocytes respond similarly to Ca^{++} signals that arise from antigen receptor stimulation. Several different transcription factors, including NFAT and NF-κB, regulate early activation genes in T cells [Ullman et al., 1990]. NFAT is particularly important for IL-2 gene transcription since cyclosporin A, which prevents calcineurin-dependent translocation of NFAT to the T cell nucleus, virtually abrogates IL-2 synthesis by T cells [Bickel et al., 1987; Henderson et al., 1991]. In contrast, T cell expression of CD25 is only partially inhibited by cyclosporin A [Reed et al., 1986; Kaiser et al., 1993], implying a less important role for NFAT in CD25 gene transcription. On the other hand, NF-κB binding to an enhancer-like element in the CD25 gene is important for CD25 expression [Lin et al., 1990]. Since RyR signaling is responsible for maintaining a long-lasting Ca^{++} flux in T lymphocytes [Guse et al., 1999], RyR blockade would be expected to interfere with NFAT activation and thereby severely impair IL-2 gene expression. Conversely, activation of NF-κB that occurs in response to the transient peak of intracellular Ca^{++} release caused by IP_3 receptor signaling, in combination with residual NFAT activity due to incomplete blockade of RyR, may account for near normal CD25 gene transcription in the presence of ruthenium red. This is in line with the observation that activation-induced CD25 expression is impaired but not absent in T cells from NFATp-deficient mice [Schuh et al., 1998].

Whether or not IL-2-induced T lymphocyte proliferation requires an intracellular Ca^{++} flux has been the focus of some controversy. Two different laboratories have reported that exposure to IL-2 causes an increase in intracellular Ca^{++} in IL-2-dependent mouse T cell lines [Gearing et al., 1985; Utsunomiya et al., 1986]. In contrast, other investigators have failed to find evidence for an intracellular Ca^{++} flux when cultures of IL-2-sensitive mouse or human T lymphocytes were induced to proliferate by the addition of IL-2 [Mills et al., 1985]. Our own data are consistent with a requirement

for sustained RyR-mediated intracellular Ca^{++} mobilization by T cells in response to IL-2 receptor signaling since (1) anti-CD3-activated T cells that had normal CD25 expression failed to proliferate in response to exogenous IL-2 when RyR were blocked by ruthenium red and (2) IL-2-dependent mouse CTLL-2 cells exhibited a reduced capacity to proliferate in response to IL-2 in the presence of RyR antagonists. In this regard, it is noteworthy that glycine, which blunts anti-CD3-induced increases in intracellular Ca^{++} concentration in rat T cells, also inhibits the IL-2-dependent proliferation of mouse CTLL-2 cells [Stachlewitz et al., 2000]. One possibility is that RyR-mediated increases in intracellular Ca^{++} levels are required for the activation of kinases that regulate T cell proliferation in response to IL-2 receptor signaling. Alternatively, RyR signaling may be involved in mediating the changes in intracellular Ca^{++} concentration that are known to regulate cell cycle progression [Santella, 1998]. However, this seems unlikely because both ruthenium red and dantrolene failed to inhibit the cytokine-independent proliferation of EL-4 T lymphoma cells and A20 B lymphoma cells, as determined in MTT dye assays (data not shown).

In summary, we have shown that the RyR antagonists ruthenium red and dantrolene inhibited T cell proliferation and IL-2 synthesis stimulated by mitogenic anti-CD3 mAb, as well as interfering with IL-2-dependent T cell proliferation without substantially affecting activation-induced expression of the high affinity IL-2 receptor. These findings imply an important role for RyR in the Ca^{++}-dependent signal transduction processes that are involved in T cell receptor-mediated T cell activation and IL-2-driven T cell proliferation.

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