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Redox sensitivity of store-operated calcium entry in human lymphocytes

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**REDOX SENSITIVITY OF STORE-OPERATED CALCIUM ENTRY IN HUMAN
LYMPHOCYTES**

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Pamela Lynn Killoran

August 1998

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ABSTRACT

REDOX SENSITIVITY OF STORE-OPERATED CALCIUM ENTRY IN HUMAN LYMPHOCYTES

by Pamela Killoran

The influence of cellular redox state on calcium regulation in resting compared to activated human lymphocytes was investigated. A new quantitative fluorescence technique termed Differential Ratiometric Fluorescence Spectroscopy was employed. Reducing conditions induced by the elevation of intracellular glutathione enhanced, while oxidation induced by glutathione depletion inhibited activation-induced store-operated calcium influx in Jurkat T-cells. Complementary changes in the intracellular calcium concentration were observed. Oxidation induced by exposure to ionizing radiation inhibited store-operated calcium influx but not basal calcium influx in Jurkat T-cells and human peripheral blood lymphocytes. Elevation of the intracellular concentration of glutathione prevented the inhibition of calcium influx by γ -irradiation. Similar to γ -irradiation, both the superoxide-generating xanthine/xanthine oxidase system and hydrogen peroxide inhibited activation-induced calcium influx in Jurkat T-cells. In conclusion, (1) reductive interventions stimulated, whereas oxidative perturbations inhibited calcium signaling in human T-lymphocytes and (2) the redox sensitivity of calcium regulation was primarily dependent on cell activation.

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1. INTRODUCTION

1.1 Store-operated Calcium Entry

The process by which cells relay information from the extracellular environment across the plasma membrane into the intracellular environment is called signal transduction or cell signaling. Signals carried by first messengers such as hormones, growth factors, neurotransmitters, and cytokines are relayed to their targets by the various components of signal transduction including receptors, second messengers, protein kinases, and phosphoproteins. The cellular response to signal transduction is the induction of biological activities including gene transcription, cell proliferation and neurotransmission. The second messenger calcium (Ca^{2+}) is a key component of signal transduction in virtually all cell types (for reviews see refs. 1, 2). Under resting conditions, the intracellular free calcium concentration ($[\text{Ca}^{2+}]_i$), which is tightly regulated by the cell, is 10,000-fold lower than the extracellular calcium concentration $[\text{Ca}^{2+}]$, resulting in a large concentration gradient across the plasma membrane. Intracellular Ca^{2+} is compartmentalized within two primary stores, the mitochondria and the endoplasmic reticulum. Activation of cell surface receptors by a number of first messengers results in the elevation of $[\text{Ca}^{2+}]_i$ due to the release of Ca^{2+} from intracellular stores and/or the influx of extracellular Ca^{2+} . In excitable cells like neurons, endocrine and muscle cells, extracellular Ca^{2+} entry is controlled by voltage-operated Ca^{2+} channels which become activated during action potentials, generating large inward Ca^{2+} currents.

In non-excitabile cells like endothelial cells, hepatocytes, and cells of the immune system, voltage operated Ca^{2+} channels are not expressed. In these cells, extracellular Ca^{2+} entry is controlled by the filling state of the intracellular Ca^{2+} stores (3, 4). The Ca^{2+} signal in response to cell activation is biphasic, consisting of the release of Ca^{2+} from the endoplasmic reticulum store into the cytoplasm, followed by the entry of extracellular Ca^{2+} across the plasma membrane. The first phase, or release of Ca^{2+} from the endoplasmic reticulum store, leads to a transient elevation in $[\text{Ca}^{2+}]_i$, and is mediated by the binding of inositol-1,4,5-trisphosphate to receptors on the endoplasmic reticulum surface (2, 5). The second phase, store-operated Ca^{2+} entry, is initiated by Ca^{2+} store depletion and facilitated by the opening of store-operated Ca^{2+} channels in the cell membrane (6, 7). Store-operated Ca^{2+} entry, also known as capacitative Ca^{2+} entry (8, 9), is necessary for the refilling of Ca^{2+} stores as well as the sustained elevation of $[\text{Ca}^{2+}]_i$, which is required for induction of biological activities including secretion, differentiation, gene expression, and proliferation (for review see ref. 10). Although a number of mechanisms for store-operated Ca^{2+} entry have been proposed including the release of a putative second messenger from depleted Ca^{2+} stores, kinase/phosphatase activity, and a direct coupling of the store with plasma membrane Ca^{2+} channels, a definitive model has not been accepted (11).

1.2 Cellular Redox Balance

The intracellular balance between oxidants and antioxidants or “redox state” is an

important factor in cell homeostasis. The cell has developed a number of antioxidant defense systems in order to maintain a reducing intracellular environment and protect against oxidative damage generated by free radicals from both external and internal sources (for reviews see refs. 12-14). Free radicals are ions or molecules containing one or more unpaired electrons making them highly reactive towards other cellular components. Examples are the superoxide radical ($O_2^{\bullet}$), the hydroxyl radical ($^{\bullet}OH$) and nitric oxide ($NO^{\bullet}$). Free radicals and other reactive oxygen species (ROS) are produced during oxidative phosphorylation in the mitochondria (15), and generated during environmental exposure to UV or ionizing radiation (16). At high concentrations, ROS may be cytotoxic causing membrane lipid peroxidation, and damage to proteins and DNA (15, 17).

The cellular redox state, similar to Ca^{2+} metabolism, is tightly controlled by the cell. The primary intracellular redox regulator is the glutathione/glutathione reductase system. Glutathione (GSH), which is present in mM-concentrations within the cell, spontaneously reacts with ROS reducing them to non-reactive compounds (18). Other antioxidant defense systems include superoxide dismutase, catalase, and glutathione peroxidase which catalyze the enzymatic dismutation of $O_2^{\bullet}$, hydrogen peroxide (H_2O_2), and the reduction of H_2O_2 and lipid peroxides, respectively (13). Recent evidence suggests that the cellular oxidation/reduction balance may be an important modulator of signal transduction events including Ca^{2+} regulation, phosphorylation activity, transcription factor activation, gene transcription, and apoptosis (for reviews see refs. 19-23). A few

specific redox-responsive targets have been identified in signal transduction pathways, including the ras proto-oncogene product p21 (24) and the Ca^{2+} -dependent serine/threonine phosphatase calcineurin (25). Membrane currents are also reported to be redox sensitive. Potassium channel activity, for example, is affected by oxidative and reducing interventions in a number of cell systems (26-28). There exists some evidence that transmembrane Ca^{2+} influx is modulated by oxidative interventions in non-excitabile cells; both inhibitory and stimulatory effects of oxidation on Ca^{2+} influx have been reported (29-32).

1.3 Ca^{2+} and Redox Signaling in T-lymphocytes

Ca^{2+} signaling in T-lymphocytes, may be especially responsive to changes in redox potential. For instance, activation of T-lymphocytes by antigenic or mitogenic stimulation results in the generation of oxidative products (33-35) and these products may play a role in the mediation of cell signaling events (36). In T-lymphocytes, redox changes have been reported to modulate a number of activities including NF- κ B activation (36), IL-2 gene expression (37, 38), proliferation (39, 40), and apoptosis (41, 42). T-lymphocytes are a good model system for studying store-operated Ca^{2+} entry since it is the primary Ca^{2+} influx pathway in these cells (6). In addition, the activation response of T-lymphocytes following anitgenic or mitogenic stimulation is distinctly dependent on Ca^{2+} influx. Store-operated Ca^{2+} entry is required, for example, for NF-AT-dependent gene transcription (43, 44), cell proliferation (45, 46), and for maintaining

oscillations in $[Ca^{2+}]_i$ (47, 48). The importance of store-operated Ca^{2+} entry in regards to overall immune activity is noted by some clinical cases of immunodeficiency (49, 50). In these cases, peripheral blood lymphocytes from immunodeficient patients maintained normal mobilization of Ca^{2+} from intracellular stores, but exhibited a reduced Ca^{2+} influx response following antigenic stimulation.

1.4 Study Goals and Summary

Store-operated Ca^{2+} entry is a key component of T-lymphocyte activation. The detailed steps involved in its regulation, however, have not been determined. Although the intracellular redox balance has been reported to modulate a number of cellular signal transduction pathways, knowledge regarding the redox sensitivity of store-operated Ca^{2+} entry is limited. The goal of this thesis was to investigate the influence of the cellular redox environment on Ca^{2+} regulation in resting compared to activated human T-lymphocytes. Reducing and oxidative changes in the cellular redox state were facilitated by the elevation or depletion, respectively, of the physiological intracellular redox regulator, GSH. The sensitivity of Ca^{2+} signaling to external oxidative interventions including, ionizing radiation, H_2O_2 , and $O_2^{\bullet -}$ was also examined. The influence of redox changes on both resting (basal) Ca^{2+} homeostasis and activation-induced Ca^{2+} signaling were determined. Changes in Ca^{2+} influx and $[Ca^{2+}]_i$ following redox interventions were monitored with the fluorescent Ca^{2+} indicator, Fura-2. A new method was employed for measuring differences in Ca^{2+} influx and $[Ca^{2+}]_i$ called

Differential Ratiometric Fluorescence Spectroscopy (DRFS). In DRFS, experimental and control cell samples are measured simultaneously in real time, thus eliminating the influence of time-dependent cell state changes on experimental outcome. With this method, due to its increased resolution compared to conventional Ca^{2+} spectrofluorimetry, even modest changes in Ca^{2+} (Mn^{2+}) influx or $[\text{Ca}^{2+}]_i$ ($\cong 10\text{-}30\%$) were reliably resolved following oxidant or antioxidant interventions.

This study presents evidence for the redox sensitivity of Ca^{2+} influx and $[\text{Ca}^{2+}]_i$ in human T-cells. In section 2, the response of Ca^{2+} influx and $[\text{Ca}^{2+}]_i$ to acute and long-term redox changes facilitated by the modulation of intracellular GSH are described. In section 3, the acute cellular response of Ca^{2+} influx to external oxidizing interventions including ionizing radiation, H_2O_2 , and $\text{O}_2^{\bullet-}$ are reported. The ability of GSH to protect against oxidant-induced changes in Ca^{2+} influx is also described. In summary, redox changes towards reduction were found to enhance, while oxidation was found to inhibit store-operated Ca^{2+} entry in T-lymphocytes. In addition, the redox sensitivity of Ca^{2+} regulation was determined to be dependent on cell activation; store-operated Ca^{2+} influx was highly sensitive to redox modulation, whereas basal Ca^{2+} influx was mostly insensitive to redox interventions. Furthermore, elevation of the intracellular concentration of GSH ($[\text{GSH}]$) protected against the inhibition of store-operated Ca^{2+} influx by ionizing-radiation.

2. REDOX MODULATION OF CALCIUM INFLUX BY ELEVATION AND DEPLETION OF INTRACELLULAR GLUTATHIONE

2.1 Overview

GSH is the primary regulator of the intracellular redox state and one of the major cellular defenses against reactive oxygen species and free radicals (for overview see refs. 12, 15, 18). GSH is synthesized intracellularly by the consecutive actions of γ -glutamylcysteine synthetase and GSH synthetase. It is a tripeptide consisting of the amino acids γ -glutamate, cysteine, and glycine, and is found ubiquitously in eukaryotic cells at a concentration of between 1-10 mM. GSH eliminates ROS through a reduction/oxidation reaction by donating a hydrogen from the thiol group on its cysteine moiety. Reaction of GSH with free radicals occurs spontaneously while its reaction with other ROS including H_2O_2 and other peroxides is catalyzed by the enzyme GSH peroxidase. Both reactions result in the oxidation of GSH. Oxidized GSH (GSSG) is converted back to reduced GSH by GSH reductase and NADPH, maintaining a GSH:GSSG ratio of approximately 100:1. GSH, is also involved in the detoxification and elimination of certain drugs through GSH transferase catalyzed reactions. Besides its role in the protection against ROS and cytotoxic compounds, GSH is important for a number of biological activities including protein synthesis, DNA synthesis, and enzyme activity.

A number of methods have been used experimentally to modulate [GSH] both

in vitro and *in vivo*. Methods for depleting cellular GSH include the use of GSH-conjugating agents, limiting the supply of cysteine, and inhibiting the *de novo* synthesis of GSH. L-Buthionine-(SR)-sulfoximine (BSO) is a highly specific and irreversible inhibitor of the enzyme, γ -glutamylcysteine synthetase, that catalyzes the reaction of cysteine with glutamate (the rate-limiting step in GSH synthesis) (40). Incubation of cells with μM -concentrations of BSO leads to complete depletion of GSH within 24 h. The rapid depletion of [GSH] can be achieved by irreversible conjugation with diethylmaleate (DEM) (51). Methods for increasing [GSH] include enhancing GSH synthesis by gene transfer, increasing cellular levels of cysteine (the limiting substrate for GSH synthesis), and administration of extracellular GSH (18). Elevation of intracellular GSH by the addition of extracellular GSH is limited since it is not permeable to the plasma membrane, and in some cell types no transport system for the uptake of extracellular GSH exists (12). Increasing [GSH] by supplying extracellular cysteine may also be limited. Although the reduced form of cysteine is actively transported into the cell, more than 90% of extracellular cysteine exists in its oxidized form as cystine, and in some cells, including lymphocytes, no transport system for cystine exists (52). α -Lipoic-acid, which is a component of certain mitochondrial dehydrogenase complexes and an essential cofactor in oxidative metabolism (53), has been shown to increase [GSH] in human T-lymphocytes *in vitro* when added extracellularly in μM -concentrations (54). Elevation of [GSH] by α -lipoic-acid involves its ability to reduce extracellular cystine to cysteine promoting its uptake and availability for the synthesis of GSH (55). N-acetyl-L-

cysteine (L-NAC) is a membrane permeable form of cysteine that rapidly elevates [GSH] when supplied in mM-concentrations (56). Both L-NAC and α -lipoic-acid have been used to treat pathological conditions *in vivo* including, HIV infection (57), and diabetes (53), respectively.

In the following section, the influence of oxidation and reduction on basal Ca^{2+} influx and activation-induced Ca^{2+} influx in human T-lymphocytes is reported. Oxidative and reducing intracellular conditions were facilitated by the depletion and elevation of the endogenous cellular redox regulator GSH. The Ca^{2+} response to both acute (10 min) and long term (24 h) redox changes was examined.

2.2 Experimental Procedures

Cell Handling. The human leukemic cell line Jurkat clone E6-1 (American Type Culture Collection, Rockville, MD) was maintained in RPMI 1640 cell culture media with 10% fetal calf serum. All experiments and incubations were performed at 37 °C in buffer containing in [mM]: 145 NaCl, 5 KCl, 1 CaCl_2 , 1 Na_2HPO_4 , 0.5 MgSO_4 , 5 glucose, 10 HEPES and 0.225 sulfinpyrazone (pH 7.4). Store-operated Ca^{2+} influx was activated by the microsomal Ca^{2+} -ATPase inhibitor thapsigargin. Thapsigargin rapidly depletes the endoplasmic reticulum Ca^{2+} store by inhibiting the endoplasmic reticulum Ca^{2+} -ATPase pump and thereby preventing the uptake of cytoplasmic Ca^{2+} . In some cases, store-operated Ca^{2+} influx was activated by an antibody to the T-cell receptor. Either thapsigargin (0.1 μM ; Sigma Chemical Co., St. Louis, MO) or anti-CD3 monoclonal

antibody (α -CD3-mAb, 0.66 μ g/ml, UCHT-1; Bio-Design International, Kennebunkport, ME) was injected into the cell suspension during Ca^{2+} measurements to initiate store-operated Ca^{2+} entry.

Ca^{2+} Influx and $[\text{Ca}^{2+}]_i$ Determinations. Ca^{2+} influx and $[\text{Ca}^{2+}]_i$ were measured in real time using the fluorescent Ca^{2+} indicator Fura-2 AM. The acetoxymethyl ester (AM) of Fura-2 is cell-membrane-permeable by passive diffusion. Once inside the cell the AM group is cleaved by intracellular esterases yielding the cell-membrane-impermeant dye. Upon binding Ca^{2+} , Fura-2 exhibits a shift in the excitation spectra from 340 to 380 nm that is observable by monitoring the emission at 510 nm. Monitoring the 340/380 nm excitation ratio of Fura-2 provides a measurement of $[\text{Ca}^{2+}]_i$ that is relatively independent of dye concentration (58). $[\text{Ca}^{2+}]_i$ was calculated from the ratio of fluorescence signals as previously described (58). Ca^{2+} influx measurements were facilitated by the surrogate divalent cation manganese (Mn^{2+}). Mn^{2+} , which is not normally present within the cytoplasm, is permeable to Ca^{2+} channels, binds Fura-2 with greater affinity than Ca^{2+} , and quenches Fura-2 fluorescence upon binding. Excitation of Fura-2 at 360 nm (the isobestic wavelength for Fura-2), results in a fluorescence emission (510 nm) which is dependent on Fura-2 concentration and independent of $[\text{Ca}^{2+}]_i$. Using Mn^{2+} as a tracer for Ca^{2+} , Ca^{2+} influx was measured by monitoring the quenching of Fura-2 fluorescence as previously described (59).

Prior to Ca^{2+} (Mn^{2+}) influx and $[\text{Ca}^{2+}]_i$ measurements, cells (10^7 /ml) were loaded with

3 μM Fura-2 (Molecular Probes, Eugene, OR) for 35 min at 37 °C, rinsed twice in buffer and stored at room temperature. All experiments (6-8 per day) were completed within 3 h of Fura-2 loading. For Ca^{2+} influx experiments, Mn^{2+} (100 μM) was added to the cell suspension, and quenching of Fura-2 fluorescence was monitored by DRFS (360 nm excitation, 510 nm emission) as described under Differential Ratiometric Fluorescence Spectroscopy. The signal was calibrated for dynamic range (total quenchable intracellular Fura-2) and autofluorescence by the injection of a Ca^{2+} ionophore (5 μM ionomycin or 50 μM digitonin) at the end of each experiment, allowing the complete quenching of Fura-2 fluorescence by extracellular Mn^{2+} . The data from each recording were corrected for autofluorescence and normalized to dynamic range by the relation $y_c = [(y_t - y_i)/(y_m - y_i)]$; where y_c and y_t represent corrected and raw data values at time t , respectively, y_m represents a raw data value 10 s after Mn^{2+} addition, and y_i represents a raw data value 60 s after ionomycin or digitonin addition. Ca^{2+} influx, expressed in %/min, was determined from the linear regression of the normalized data over a 2-min period. $[\text{Ca}^{2+}]_i$ was monitored by DRFS (340/380 nm excitation, 510 nm emission) as described under Differential Ratiometric Fluorescence Spectroscopy. Signals were calibrated by the sequential addition of the Ca^{2+} ionophore digitonin (50 μM) and the Ca^{2+} chelator EGTA (5 mM), allowing the saturation and subsequent quenching of Fura-2 fluorescence. Calculations were performed using a previously described data analysis software package (60). Statistical significance was determined by paired or unpaired t-test using SigmaStat statistical software (Jandel Corp., San Rafael, CA).

Differential Ratiometric Fluorescence Spectroscopy (DRFS). A diagram of the DRFS instrument employed in the detection of Ca^{2+} (Mn^{2+}) influx and $[\text{Ca}^{2+}]_i$ is shown in Fig. 2.1 (61). A dual-wavelength spectrofluorimeter was modified to allow differential ratiometric measurements in real-time (Photon Technologies International, Monmouth Jct., NJ). The fluorescence signals from experimental and control cell samples were recorded simultaneously in real time at a sampling frequency of 4 Hz. Cell samples (3 ml, $10^6/\text{ml}$) were pre-incubated at 37 °C for 10 min prior to Ca^{2+} measurements, maintained at 37 °C in thermostatically-controlled cuvette holders, and continuously stirred throughout fluorescence recording (cuvette A and B in Fig. 2.1). DRFS experiments were alternated with matching DRFS sham vs. control experiments. Such experiments consisted of two identically-treated control cell samples, and were performed to quantify possible differences in fluorescence recordings due to any factor other than the experimental intervention of interest. Examples of sham vs. control and experimental vs. control recordings are shown in Fig. 2.2. Experimental vs. control recordings were normalized to sham vs. control recordings for Ca^{2+} (Mn^{2+}) influx and $[\text{Ca}^{2+}]_i$ experiments.

Modulation and Determination of Intracellular Glutathione Concentration [GSH]. For short-term modulation of [GSH], Fura-2 loaded cells were incubated for 10 min with 1 mM DEM (Aldrich Chemical Co., Milwaukee, WI) or 10 mM L-NAC (Sigma), to decrease or increase [GSH], respectively. For long-term modulation of [GSH], cells were cultured for 24 h in the presence of either 0.5 mM BSO (Sigma) to deplete [GSH], or

Fig. 2.1. Differential Ratiometric Fluorescence Spectroscopy (DRFS). DRFS instrument used to detect Ca^{2+} (Mn^{2+}) influx and $[\text{Ca}^{2+}]_i$. The interconnecting lines represent electrical pathways; dotted lines represent excitation and emission light paths. Interference filters (F) are described by bandpass (340, 360, 380 or 510 nm). Ca^{2+} (Mn^{2+}) influx was detected by single wavelength excitation (360nm); $[\text{Ca}^{2+}]_i$ was detected by ratiometric dual wavelength excitation (340/380). A thermostatted cuvette holder is located in the center of sample compartments A and B. In each experiment, the fluorescence signal from the experimentally treated cell sample (cuvette A) and the control cell sample (cuvette B) were recorded simultaneously. In sham vs. control experiments both cuvette A and cuvette B contained identically treated cell samples.

0.2 mM α -lipoic-acid (Sigma) to increase [GSH]. To replicate osmolality and sodium concentration changes associated with L-NAC treatment, control cells were incubated with the stereoisomer of L-NAC, N-acetyl-D-cysteine (D-NAC; Research Organics,

Fig. 2.2. Example of DRFS recording under sham and experimental conditions.

Refer to Fig. 2.1 for diagram of DRFS instrument. A, Recording of $[Ca^{2+}]_i$ from a single sham vs. control experiment. Identically treated sham (cuvette A) and control (cuvette B) cell samples were measured *simultaneously* and injected with equal amounts of thapsigargin at $t = 500$ s. B, Recording of $[Ca^{2+}]_i$ from a single experiment. BSO-treated and control cell samples were measured *simultaneously* and injected with equal amounts of thapsigargin at $t = 500$ s. C, Recording of Ca^{2+} (Mn^{2+}) influx from a single sham vs. control experiment. Identically treated sham (cuvette A) and control (cuvette B) cell samples were measured *simultaneously* and injected with equal amounts of Mn^{2+} ($t = 60$ s), thapsigargin (TG, $t = 300$ s) and ionomycin (IM, $t = 600$ s). Ca^{2+} (Mn^{2+}) influx in sham treated vs. control cells, respectively, was 2.1 %/min vs. 2.4 %/min for basal influx ($t = 80$ -200 s) and 10.5 %/min vs. 10.6 %/min following thapsigargin stimulation ($t = 310$ -430 s). D, Recording of Ca^{2+} (Mn^{2+}) influx from a single experiment. γ -Irradiated and control cell samples were measured *simultaneously* and injected with equal amounts of Mn^{2+} ($t = 60$ s), thapsigargin (TG, $t = 300$ s) and ionomycin (IM, $t = 600$ s). Ca^{2+} (Mn^{2+}) influx in irradiated vs. control cells, respectively, was 2.3 %/min vs. 2.4 %/min for basal influx ($t = 80$ -200 s) and 9.9 %/min vs. 11.2 %/min following thapsigargin stimulation ($t = 310$ -430 s).

Cleveland, OH). All other control cells were treated with suitable solvent (< 1%).

Total [GSH] was determined by the GSH-reductase recycling assay (62). This assay measures the chromogenic product (thio-nitrobenzoic acid, TNB) formed from the oxidation of GSH by dithiobis-2-nitrobenzoic acid (DTNB). Reduced GSH is enzymatically regenerated from oxidized GSH by GSH-reductase and NADPH, and the rate of TNB formation is proportional to the concentration of GSH. The assay was performed in microtiter plates as described previously (63, 64). Briefly, cell samples (4×10^6 cells) were rinsed in buffer (100 mM sodium phosphate, 1 mM EDTA, pH 7.5), resuspended in 0.4 ml HCl (10 mM) and sonicated on ice using a Microson-XL cell disrupter with microprobe (Misonix, Farmingdale, NY). Proteins were precipitated on ice for 15 min by addition of 0.1 ml sulfosalicylic acid (6.5 %) and centrifuged at $2,000 \times g$ for 15 min. Supernatant was stored at -70°C for later analysis. GSH standards (0.5-35 μM reduced GSH equivalents) were prepared from a stock of 500 μM oxidized GSH (Sigma). Solutions containing GSH, DTNB (1mM) and NADPH (1mM) were prepared fresh on each day of use. The final reaction mix per well contained 0.02 ml sample or standard, 0.15 mM DTNB, 0.25 mM NADPH, and 1 U/ml GSH-reductase (Type IV, bakers yeast, Sigma) in a total volume of 0.28 ml. Absorbance (405 nm) was recorded at 30-s intervals for 6 min using a Bio-Tek ES-311 microplate reader (Bio-Tek Instruments, Winooski, VT). Total [GSH] expressed in $\text{nmol}/10^7$ cells was determined by comparing the absorbance slope from each sample with that of the standard curve.

2.3 Results

Responses to Short-term Redox Perturbations of Store-operated Ca^{2+} (Mn^{2+}) Influx

Compared to Basal Ca^{2+} (Mn^{2+}) Influx in Jurkat T-cells. Cells were exposed to reducing conditions by a 10-min pre-incubation with L-NAC to increase [GSH]. Control cells were pre-incubated with D-NAC which did not increase [GSH] but duplicated the changes in sodium concentration and osmolality associated with L-NAC treatment.

Store-operated Ca^{2+} entry was induced by either the endoplasmic reticulum Ca^{2+} -ATPase inhibitor thapsigargin or α -CD3-mAb. An example of a DRFS recording of Ca^{2+} (Mn^{2+}) influx measured simultaneously in L-NAC- and D-NAC-treated cells is shown in Fig. 2.3A. The traces are fluorescence recordings (360 nm excitation, 510 nm emission) from L-NAC- and D-NAC-treated cell samples loaded with Fura-2. Equal amounts of Mn^{2+} were injected simultaneously into both cell samples at $t = 60$ s to initiate the detection of basal Ca^{2+} (Mn^{2+}) influx. Basal Ca^{2+} (Mn^{2+}) influx was determined by the slope of each trace over the time period 70-190 s. Thapsigargin was injected at $t = 300$ s to deplete the endoplasmic reticulum Ca^{2+} store and initiate detection of store-operated Ca^{2+} (Mn^{2+}) influx (310-430 s). Ionomycin was injected at the end of the experiment ($t = 600$ s) to determine the amount of Mn^{2+} -quenchable Fura-2 in each sample (for detailed description of Mn^{2+} influx calculations see Experimental Procedures). As illustrated by the DRFS recording, basal Ca^{2+} (Mn^{2+}) influx was only slightly elevated in the L-NAC- compared to D-NAC-treated cells, while store-operated Ca^{2+} (Mn^{2+}) influx was substantially enhanced in the L-NAC-treated cell sample. An identical experiment

Fig. 2.3. Short-term (10 min) redox modulation of Ca^{2+} (Mn^{2+}) influx in Jurkat T-Cells. A, DFRS recording of Ca^{2+} (Mn^{2+}) influx measured *simultaneously* in L-NAC- and D-NAC-treated cell samples. Cell samples were injected simultaneously with equal amounts of Mn^{2+} (t = 60 s), thapsigargin (TG, t = 300 s) and ionomycin (IM, t = 600 s). Ca^{2+} (Mn^{2+}) influx in L-NAC and D-NAC (control) treated cells, respectively, was 2.6 %/min vs. 1.8 %/min for basal influx (t = 70-190 s), and 11.9 %/min vs. 8.9 %/min following thapsigargin stimulation (t = 310-430 s). B, Pooled results of repeated experiments (detailed in A) for L-NAC vs. D-NAC (open bar) and DEM vs. control (hatched bar) conditions. Data are expressed as difference from control (mean $\pm$ S. E.) for basal influx (L-NAC, n = 34; DEM, n = 13), and α -CD3-mAb-activated influx (L-NAC, n = 12; DEM, n = 4) or thapsigargin-activated influx (L-NAC, n = 22; DEM, n = 9). C, Modulation of [GSH] by 10-min treatment with L-NAC (open bar, n = 18) or DEM (hatched bar, n = 4). Data are expressed as percent difference from control (mean $\pm$ S. E.). ** P < 0.001, * P < 0.01 (paired t-test; treated vs. control).

was performed for cells exposed to an oxidative condition, following 10-min incubation with the GSH-conjugating agent DEM. The pooled results of repeated experiments for reducing and oxidative conditions are compared in Fig. 2.3B. An increase in [GSH] induced by L-NAC treatment (open bars), enhanced both basal Ca^{2+} (Mn^{2+}) influx (0.6 ± 0.1 %/min; $n = 34$, $P < 0.001$) and store-operated Ca^{2+} (Mn^{2+}) influx following stimulation with thapsigargin (1.9 ± 0.4 %/min; $n = 22$, $P < 0.001$) or α -CD3-mAb (1.5 ± 0.3 %/min; $n = 12$, $P < 0.001$) compared to D-NAC-treated controls. In contrast, a decrease in [GSH] induced by DEM treatment (hatched bars) inhibited store-operated Ca^{2+} (Mn^{2+}) influx following stimulation with thapsigargin (-1.4 ± 0.3 %/min; $n = 9$, $P < 0.01$) or α -CD3-mAb (-0.7 ± 0.05 %/min; $n = 4$, $P < 0.001$). Basal Ca^{2+} (Mn^{2+}) influx, however was not affected by DEM treatment (0.1 ± 0.1 %/min; $n = 13$, $P > 0.05$). As illustrated by Fig. 2.3C, [GSH] following L-NAC treatment (open bar) was increased by 15.5 ± 4.0 % ($n = 18$, $P < 0.001$) compared to D-NAC, while DEM (hatched bar) decreased [GSH] by -66.8 ± 4.1 % ($n = 4$, $P < 0.001$) compared to control. [GSH] (nmol/ 10^7 cells) was 25.9 ± 1.3 and 22.9 ± 1.4 in L-NAC and D-NAC-treated cells, and 9.4 ± 1.8 and 28.0 ± 2.1 in DEM-treated and control cells, respectively.

Responses to Long-term Redox Perturbations of Store-operated Ca^{2+} (Mn^{2+}) Influx Compared to Basal Ca^{2+} (Mn^{2+}) Influx in Jurkat T-cells. To assess whether long-term exposure to reducing or oxidative conditions modulated store-operated Ca^{2+} (Mn^{2+}) influx in a manner consistent with short-term (10 min) redox interventions, Ca^{2+} (Mn^{2+}) influx

was measured following 24 h culture of cells with the GSH-elevating agent α -lipoic-acid or the GSH-depleting agent BSO. An example of a DRFS recording of Ca^{2+} (Mn^{2+}) influx measured simultaneously in BSO-treated and control cells is shown in Fig. 2.4A. As illustrated by the traces, basal Ca^{2+} (Mn^{2+}) influx was only slightly reduced in the BSO-treated compared to control cells, while store-operated Ca^{2+} (Mn^{2+}) influx was substantially inhibited in the BSO-treated cell sample. An identical experiment was performed for α -lipoic-acid-treated cells. The pooled results of repeated experiments for each condition are compared in Fig. 2.4B. Consistent with short-term redox interventions, a long-term reducing condition induced by α -lipoic-acid treatment (open bars) enhanced store-operated Ca^{2+} (Mn^{2+}) influx following thapsigargin stimulation (0.8 ± 0.2 %/min; $n = 6$, $P < 0.01$), while an oxidative condition induced by BSO treatment (hatched bars) inhibited store-operated Ca^{2+} (Mn^{2+}) influx (-6.6 ± 1.4 %/min; $n = 10$, $P < 0.001$). The effects of long-term (24 h) redox changes on basal Ca^{2+} (Mn^{2+}) influx are opposite to those of short-term (10 min) redox interventions (Fig. 2.4B vs. Fig. 2.3B). Basal Ca^{2+} (Mn^{2+}) influx was not significantly affected by long-term reduction (0.3 ± 0.2 %/min; $n = 6$, $P > 0.05$) but was inhibited by long-term oxidation (-2.0 ± 0.4 %/min; $n = 15$, $P < 0.001$). As shown in Fig 2.4C, α -lipoic-acid (open bar) increased [GSH] by 98.7 ± 13.7 % ($n = 3$, $P < 0.001$), while BSO (hatched bar) decreased [GSH] by -98.8 ± 0.7 % ($n = 3$, $P < 0.05$) compared to untreated controls. [GSH] (nmol/ 10^7 cells) was 50.9 ± 1.2 and 25.8 ± 1.6 in α -lipoic-acid-treated and control cells, and 0.3 ± 0.2 and 24.5 ± 2.8 in BSO-treated and control cells, respectively.

Fig. 2.4. Long-term (24 h) redox modulation of Ca^{2+} (Mn^{2+}) influx in Jurkat T-cells. A, DRFS recording of Ca^{2+} (Mn^{2+}) influx measured *simultaneously* in BSO-treated and control cell samples. Cell samples were injected simultaneously with equal amounts of Mn^{2+} (t = 60 s), thapsigargin (TG, t = 200 s) and ionomycin (IM, t = 400 s). Ca^{2+} (Mn^{2+}) influx in BSO treated vs. control cells, respectively, was 2.9 %/min vs. 4.0 %/min for basal influx (t = 70-190 s) and 13.6 %/min vs. 16.5 %/min following thapsigargin stimulation (t = 220-340 s). B, Pooled results of repeated experiments (detailed in A) for α -lipoic-acid (α -LA) vs. control (open bar) and BSO vs. control (hatched bar) conditions. Data are expressed as difference from control (mean $\pm$ S. E.) for basal influx (α -LA, n = 6; BSO, n = 15) and thapsigargin-activated influx (α -LA, n = 6; BSO, n = 10). C, Modulation of [GSH] by 24 h treatment with α -LA (open bar, n = 3) or BSO (hatched bar, n = 3). Data are expressed as percent difference from control (mean $\pm$ S. E.). *** P < 0.001, ** P < 0.01, * P < 0.05 (paired t-test; treated vs. control).

Response to Redox Perturbation of Basal $[Ca^{2+}]_i$ Compared to Stimulated $[Ca^{2+}]_i$ in Jurkat T-cells. In order to determine whether redox-induced changes in Ca^{2+} influx (described above) were accompanied by corresponding changes in $[Ca^{2+}]_i$, $[Ca^{2+}]_i$ was monitored before and after stimulation with thapsigargin under reducing and oxidative conditions. Short-term (10 min) reduction by L-NAC and long-term (24 h) oxidation by BSO were chosen for these experiments since these two redox interventions had modulated both store-operated Ca^{2+} (Mn^{2+}) influx and basal Ca^{2+} (Mn^{2+}) influx (compare Figs. 2.3 and 2.4). Cells were treated with L-NAC or BSO as described above. An example of a DRFS recording of $[Ca^{2+}]_i$ measured simultaneously in L-NAC- and D-NAC-treated cells is shown in Fig. 2.5A. Cell samples were injected simultaneously with equal amounts of thapsigargin at $t = 500$ s to deplete the endoplasmic reticulum Ca^{2+} store. $[Ca^{2+}]_i$ was calculated from fluorescence recordings (340/380 nm excitation, 510 nm emission) as described in Experimental Procedures. As illustrated by the traces, basal $[Ca^{2+}]_i$ was similar in L-NAC- and D-NAC-treated cells ($t = 450$ -500 s). However, both peak $[Ca^{2+}]_i$ following the release of Ca^{2+} from stores, and the plateau phase representing store-operated Ca^{2+} influx ($t = 1450$ -1500 s) were substantially elevated in L-NAC- compared to D-NAC-treated cells. An identical experiment was performed for BSO-treated cells (see Fig. 2.2A, B). The pooled results from repeated experiments for each condition are shown in Fig. 2.5B. Consistent with the observed responses in store-operated Ca^{2+} (Mn^{2+}) influx (see Figs. 2.3 and 2.4), reduction (L-NAC, open bars) significantly enhanced the peak $[Ca^{2+}]_i$ (67 ± 15 nM; $n = 4$, $P < 0.05$) and the plateau

Fig. 2.5. Redox modulation of $[Ca^{2+}]_i$ in Jurkat T-lymphocytes. *A*, DRFS recording of $[Ca^{2+}]_i$ measured *simultaneously* in L-NAC and D-NAC treated cell samples. Cell samples were injected simultaneously with equal amounts of thapsigargin (TG) at $t = 500$ s. *B*, Pooled results of repeated experiments (detailed in *A*) for L-NAC vs. D-NAC (open bar, $n = 4$) and BSO vs. control (hatched bar, $n = 5$) conditions. Data are expressed as difference from control (mean $\pm$ S. E.) for basal, peak, and plateau phase $[Ca^{2+}]_i$. * $P < 0.05$ (paired t-test; treated vs. control).

phase (44 ± 8 nM; $n = 4$, $P < 0.05$), whereas oxidation (BSO, hatched bars) significantly inhibited the peak $[Ca^{2+}]_i$ (-46 ± 11 nM; $n = 5$, $P < 0.05$) and the plateau phase (-21 ± 6 nM; $n = 5$, $P < 0.05$) compared to control. On the other hand, in contrast to the observed responses in basal Ca^{2+} (Mn^{2+}) influx (see Figs. 2.3 and 2.4), basal $[Ca^{2+}]_i$ was

not significantly affected by treatment with either L-NAC (-1 ± 3 nM; $n = 4$, $P > 0.05$) or BSO (-8 ± 4 nM; $n = 5$, $P > 0.05$).

2.4 Discussion

L-NAC is a commonly used agent for elevating [GSH] *in vitro*. L-NAC was used in this study to rapidly elevate [GSH] in Jurkat T-cells. However, during initial experiments, artifacts associated with the use of L-NAC were encountered. By using D-NAC (the stereoisomer of L-NAC) as a control, it was discovered that Ca^{2+} (Mn^{2+}) influx and $[\text{Ca}^{2+}]_i$ are particularly sensitive to the changes in osmolality and sodium concentration associated with L-NAC and D-NAC treatment (data not shown). Therefore, treatment of control cells with D-NAC was necessary to ensure that the observed changes in Ca^{2+} following L-NAC treatment were indeed due to elevated [GSH]. Although the physico-chemical properties of L-NAC and D-NAC are similar, D-NAC cannot be utilized by the cellular enzyme γ -glutamylcysteine synthetase as a substrate for GSH synthesis (65). Surprisingly, in preliminary experiments, [GSH] in Jurkat T-cells was elevated by both D-NAC and L-NAC treatment. When cells were treated in cystine-free buffer, however, D-NAC did not elevate [GSH]. The ability of D-NAC to increase [GSH] in cystine containing media was most likely due to enhanced cellular uptake of the reduced form of cystine (cysteine), facilitated by the extracellular reducing properties of D-NAC (53). Prevention of [GSH] elevation in control cells was achieved by washing the cells with cystine-free buffer prior to D-NAC and L-NAC treatment. The above findings

indicate that use of L-NAC without a proper control would make it difficult to distinguish between the effect of GSH elevation and the effect of uncontrolled changes associated with the physico-chemical properties of L-NAC.

A summary of the reported responses in Ca^{2+} (Mn^{2+}) influx and $[\text{Ca}^{2+}]_i$ to the tested redox interventions is presented in percent effect for comparison (see Table 2.1). A shift in the cellular redox state towards reduction, induced by GSH elevation, enhanced store-operated Ca^{2+} (Mn^{2+}) influx (Figs. 2.3 and 2.4) and the activation-induced increase in $[\text{Ca}^{2+}]_i$ (Fig. 2.5) in Jurkat T-cells. The effect of GSH elevation on basal Ca^{2+} influx was not as pronounced compared to its effect on store-operated Ca^{2+} influx. Although basal influx was increased to a small degree following short-term GSH elevation (Fig. 2.3), a change in basal $[\text{Ca}^{2+}]_i$ was not detected (Fig. 2.5). In addition, basal influx was not perturbed by long-term GSH elevation (Fig. 2.4). These results indicate that activation-induced Ca^{2+} signaling is more sensitive than resting Ca^{2+} homeostasis to redox changes induced by the elevation of [GSH]. Based on these results, T-cell activation under reducing conditions will potentiate both store-operated Ca^{2+} influx and activation-induced increases in $[\text{Ca}^{2+}]_i$. An increase in Ca^{2+} signaling may lead to an increase in Ca^{2+} -dependent activities including gene transcription, cytokine secretion, and cell proliferation. In independent studies, the elevation of [GSH] with L-NAC was reported to lead to a three-fold increase in IL-2 dependent splenocyte proliferation (66) and the enhancement of cell proliferation and IL-2 secretion in T-lymphocytes (67). The potentiation of Ca^{2+} signaling by GSH elevation in non-excitable cells has not previously

Table 2.1. **Redox modulation of Ca^{2+} (Mn^{2+}) influx and $[\text{Ca}^{2+}]_i$ in Jurkat T-cells and HPBL.**

	L-NAC (10 min)	α-Lipoic-acid (24h)	DEM (10 min)	BSO (24 h)
[GSH]	16 $\pm$ 4 (18)	99 $\pm$ 14 (3)	- 67 $\pm$ 4 (4)	- 99 $\pm$ 1 (3)
Ca^{2+} (Mn^{2+}) Influx				
Basal	13 $\pm$ 4 (34)	n. s. (6)	n. s. (13)	-33 $\pm$ 4 (15)
Thapsigargin	23 $\pm$ 6 (22)	8 $\pm$ 2 (6)	-12 $\pm$ 3 (9)	-29 $\pm$ 5 (10)
α-CD3-mAb	17 $\pm$ 4 (12)		-10 $\pm$ 1 (4)	-16 $\pm$ 4 (5)
$[\text{Ca}^{2+}]_i$				
Basal	n. s. (4)			n. s. (5)
Peak	23 $\pm$ 5 (4)			-10 $\pm$ 2 (5)
Plateau	25 $\pm$ 5 (4)			-8 $\pm$ 2 (5)

Notes. Summary of pooled results from experiments presented in Figs. 2.3 - 2.5. Data are expressed as percent difference from control (means $\pm$ S. E.). Minimum significance level is $P < 0.05$ (n. s. indicates no significance). Number of replicates are indicated in parentheses.

been reported. However, consistent findings were reported in the nervous system where GSH enhanced $^{45}\text{Ca}^{2+}$ uptake in rat cerebellar granule cells following cell activation (68).

In contrast to reducing conditions, oxidative perturbations inhibited Ca^{2+} signaling in Jurkat T-cells. As shown in Figs. 2.3 and 2.4, depletion of [GSH] inhibited store-

operated Ca^{2+} (Mn^{2+}) influx and the activation-induced increase in $[\text{Ca}^{2+}]_i$ (Fig. 2.5). Consistent with reducing interventions, the effect of oxidation on store-operated Ca^{2+} influx was more pronounced compared to its effect on basal Ca^{2+} influx (Figs 2.3 and 2.4). Basal influx was not perturbed by short term GSH depletion, and although it was inhibited following long-term GSH depletion, no change in basal $[\text{Ca}^{2+}]_i$ was detected (Fig 2.5). Activation-dependent Ca^{2+} signaling, therefore, was more sensitive than resting Ca^{2+} homeostasis to oxidative changes. Based on these results, T-cell activation under oxidative conditions will inhibit Ca^{2+} signaling, and possibly result in the inhibition of Ca^{2+} -dependent activities. In fact, in human peripheral blood lymphocytes, a reduction in [GSH] was reported to cause a decrease in mitogen- or IL-2-dependent cell proliferation (69).

This study found that GSH depletion inhibits activation-induced increases in $[\text{Ca}^{2+}]_i$ following thapsigargin stimulation (Fig. 2.5). Other studies have reported that GSH depletion inhibits α -CD3-mAb-induced increases in $[\text{Ca}^{2+}]_i$ in T-cells (70, 71). However, when thapsigargin was used to initiate Ca^{2+} store depletion, in order to bypass signaling pathways upstream of Ca^{2+} store release, $[\text{Ca}^{2+}]_i$ was reported to be insensitive to the effects of GSH depletion (71), which is opposite to the finding of this study. This discrepancy might be explained by differences in the experimental methods used for measuring changes in $[\text{Ca}^{2+}]_i$. For example, preliminary experiments for this study revealed that Ca^{2+} regulation can be very sensitive to changes in cell state and cell handling which can result in significant variability between sequentially measured

samples. With DRFS biological variability is eliminated by monitoring experimental and control samples simultaneously, which is not possible using conventional cuvette-based spectroscopy. The conclusions of the study by Staal *et al* (71) regarding the redox regulation of Ca^{2+} , are significantly different from the results of this study. While Staal *et al* concluded that store-operated Ca^{2+} influx (thapsigargin-stimulated) is not redox sensitive, the results reported here demonstrate that store-operated Ca^{2+} influx stimulated by thapsigargin is in fact redox sensitive to a moderate degree.

Short-term (10 min) compared to long-term (24 h) exposure to redox perturbations induced qualitatively and quantitatively different responses. As shown in Table 2.1, store-operated Ca^{2+} (Mn^{2+}) influx was more responsive to a 16% elevation in [GSH] by 10 min incubation with L-NAC than to a 99% elevation in [GSH] by 24 h incubation with α -lipoic-acid. In addition, basal Ca^{2+} (Mn^{2+}) influx was perturbed by short-term GSH elevation but was not affected by long term GSH elevation (Table 2.1). It appears that T-cells have the ability to adapt to reducing conditions, especially in the case of resting Ca^{2+} (Mn^{2+}) homeostasis. Alternatively, it cannot be ruled out that the different response magnitudes may be the result of using different agents for increasing [GSH] in the short-term (L-NAC) and the long-term (α -lipoic-acid) conditions. No adaptive response was observed in the case of oxidative perturbations. Ca^{2+} influx was more sensitive to long term (24 h) than to short-term (10 min) GSH depletion, possibly due to a greater reduction in [GSH] (99% vs. 67%, Table 2.1).

Further studies are required to identify the specific redox-responsive elements that are

involved in modulating Ca^{2+} regulation. Store-operated Ca^{2+} (Mn^{2+}) influx induced by both thapsigargin and α -CD3-mAb was modulated by redox interventions in a qualitatively similar manner. Because thapsigargin effectively depletes Ca^{2+} stores without inducing upstream signaling events associated with T-cell-receptor activation (3), the primary redox-sensitive targets involved in the modulation of store-operated Ca^{2+} (Mn^{2+}) influx are most likely located downstream of Ca^{2+} store release. One plausible candidate mechanism is the direct redox interaction with plasma membrane Ca^{2+} channels. For non-voltage-gated Ca^{2+} channel activity, no evidence is yet available in support of this idea. On the other hand, several reports describing the redox modulation of other kinds of ion channel activity, including membrane K^{+} channels (26, 27, 72), Ca^{2+} -dependent chloride channels (73), and voltage-gated L-type Ca^{2+} channels have appeared (74, 75). For example, oxidative conditions inhibited currents in L-type Ca^{2+} channel pore-forming subunits from rabbit smooth muscle, and this inhibition could be reversed with a reducing agent (75). The findings described here, showing that reductants stimulate whereas oxidants inhibit store-operated Ca^{2+} (Mn^{2+}) influx, are consistent with the above mentioned study. Thus, it appears possible that direct redox modulation of store-operated Ca^{2+} channels might be responsible for the effects reported here. Another potential target concerns redox sensitive enzymes that have been reported to modulate Ca^{2+} influx, such as protein tyrosine kinase (PTK) (76, 77), sphingomyelinase (78, 79), and protein kinase C (PKC) (80, 81). In Jurkat T-cells, for example, low concentrations of H_2O_2 were shown to stimulate PKC activity (78), and

PKC activation was reported to inhibit store-regulated Ca^{2+} influx in human lymphocytes (79). Another possible redox sensitive target may be located in mitochondria.

Mitochondrial Ca^{2+} cycling, which is a redox sensitive process (19, 82), was recently reported to modulate the rate of store-operated Ca^{2+} entry in Jurkat T-cells (83).

Identification of the redox-sensitive elements that mediate the observed redox-modulation of Ca^{2+} signaling may help to identify the mechanisms involved in regulating store-operated Ca^{2+} entry.

The exact consequences of redox-dependent changes in store-operated Ca^{2+} influx, on the order of 10-30 %, for overall cell function are not known. However, store-operated Ca^{2+} influx is an essential component of signal transduction pathways in many cell types and even moderate flux changes may lead to significant effects on cellular processes. For example, regulation of cAMP synthesis by Ca^{2+} -sensitive adenylyl cyclases, in non-excitabile cells, was strictly dependent on store-operated Ca^{2+} influx rather than Ca^{2+} store release or the global increase in $[\text{Ca}^{2+}]_i$ following cell activation (84, 85). In T-cells, store-operated Ca^{2+} influx is essential for inducing NF-AT activation (43), IL-2 gene expression (86) and cell proliferation (45) *in vitro*. In addition, clinical cases of immunodeficiency have been identified that are associated with defective capacitative Ca^{2+} influx in T-cells (49, 50). T-cells from such immunodeficient patients exhibited severely reduced proliferation following activation. Although Ca^{2+} mobilization from the intracellular store following T-cell activation was intact, store-operated Ca^{2+} influx was either severely reduced or absent depending on the stimulus. Even modest changes in

store-operated Ca^{2+} influx may alter activation-dependent gene transcription and proliferation in T-cells. In a group of Jurkat T-cell mutants, for example, capacitative Ca^{2+} influx was reduced to levels of 0 to 40% of parental cells and this reduction correlated with a corresponding reduction in the magnitude of NF-AT-dependent gene expression (44).

In summary, Ca^{2+} signaling in Jurkat T-cells is sensitive to redox modulation by elevation and depletion of [GSH]. Reduction was found to enhance while oxidation was found to inhibit store-operated Ca^{2+} entry and the increase in $[\text{Ca}^{2+}]_i$ following thapsigargin stimulation. Redox modulation of Ca^{2+} influx was primarily dependent on cell activation; activation-induced Ca^{2+} signaling was more sensitive compared to resting Ca^{2+} homeostasis to changes in the intracellular redox balance. Based on these results it can be proposed that oxidative conditions, due to the inhibition of Ca^{2+} signaling, might lead to depressed immune function, and may be a factor in certain disease states, and in immunosclerosis during aging *in vivo*. On the other hand, a reducing environment may enhance immune function by restoring or potentiating Ca^{2+} signaling in T-lymphocytes. In combination with the knowledge that modulation of $[\text{Ca}^{2+}]_i$ alone can be responsible for changes in lymphocyte proliferation (45, 46), the results of this study suggest that redox modulation of Ca^{2+} signaling may be a sufficient cellular mechanism for explaining opposing effects of elevated and reduced [GSH] on lymphocyte proliferation.

3. IONIZING RADIATION INHIBITS STORE-OPERATED CALCIUM ENTRY IN HUMAN LYMPHOCYTES: PROTECTION BY GLUTATHIONE

3.1 Overview

Ionizing radiation exerts its primary effect on the cell by the generation of free radicals through water radiolysis (for overview see ref. 87). The products of water radiolysis, hydroxyl radical ($\cdot\text{OH}$), hydrogen peroxide (H_2O_2) and aqueous electron, undergo chemical reactions with oxygen and other cellular components resulting in the formation of additional reactive oxygen species (ROS) and organic radicals. Although the DNA damaging effects of ionizing radiation have been extensively studied, the response of cell signaling to radiation exposure is not nearly as well characterized. A few of the cellular responses to ionizing radiation, including changes in gene expression and the induction of apoptosis, are thought to be mediated through signal transduction pathways (88-90). The effects of radiation exposure on components of signal transduction include ceramide generation (88), tyrosine kinase activation (91), and NF- κ B activation (92). Some of the responses to ionizing radiation such as gene transcription and apoptosis are Ca^{2+} dependent activities (10). Although several studies have looked at the acute effects of radiation on Ca^{2+} regulation directly (93-97), in the majority of these studies only effects on basal Ca^{2+} regulation were examined (93-96). The effect of ionizing radiation on activation-dependent store-operated Ca^{2+} entry has not yet been investigated. Therefore, the influence of γ -irradiation on both basal and store-operated Ca^{2+} entry in human

T-lymphocytes was studied. The ability of GSH to protect against radiation induced changes in Ca^{2+} regulation was also examined, since GSH is the primary endogenous cellular defense against ROS. The mechanisms of radioprotection by GSH include the restoration of damaged molecules by hydrogen donation, as well as the direct scavenging of free radicals. Although the protective action of GSH with respect to the damaging effects of ionizing radiation has been studied in great detail (for review see ref. 98), no information is as yet available regarding its potential radioprotective activity during Ca^{2+} signaling.

3.2 Experimental Procedures

Cell Handling, Ca^{2+} Influx Measurements and DRFS. Human peripheral blood lymphocytes (HPBL) were isolated daily by Histopaque-1077 (Sigma) density centrifugation from whole blood buffy coat (Stanford University Blood Bank). To inhibit store-operated Ca^{2+} influx, cells were incubated for 10 min (37° C) with the store-operated Ca^{2+} channel inhibitor SKF-96365 (Calbiochem, San Diego, CA). In order to inhibit PTK, control and experimental cell samples were pre-treated with 25 μM genistein (Calbiochem) for 10-min (37° C). All other procedures are identical to those described in section 2.2.

Exposure to Ionizing Radiation. Cells were exposed to γ -irradiation ($^{137}\text{cesium}$) at a dose rate of 1.8 Gy/min for 5.5 min, resulting in a total dose of 10 Gy. To determine the

dose-response relationship for γ -irradiation, cells were exposed to doses of 5, 10, 20 or 40 Gy at a dose rate of 10.9 Gy/min. In order to maintain the cells at 37 °C during radiation exposure, an insulated cuvette holder was designed consisting of a small removable water bath inserted into a styrofoam block. The cell samples were incubated at 37 °C for 10 min and placed in the styrofoam block for radiation exposure. The temperature drop in the cell suspension during the transfer process was less than 1 °C. Simultaneously handled control cells were treated identically but placed outside the radiation chamber. Ca^{2+} (Mn^{2+}) influx was measured 5 min after radiation exposure.

Exposures to $\text{O}_2^{\bullet-}$ and H_2O_2

The $\text{O}_2^{\bullet-}$ -generating xanthine/xanthine oxidase system was used to expose cells to $\text{O}_2^{\bullet-}$. Xanthine oxidase is one isoform of the mammalian enzyme xanthine oxoreductase, which is present in liver, intestine, plasma and lung cells (for reviews see refs. 99-101). Xanthine oxoreductase is involved in the metabolism of purines from genomic material resulting in the end product uric acid. Xanthine oxidase requires oxygen to degrade xanthine to uric acid, producing $\text{O}_2^{\bullet-}$ as a by product while the other isoform, xanthine dehydrogenase, requires NAD to catalyze the degradation of xanthine to uric acid, producing NADH as a by product. For exposure to $\text{O}_2^{\bullet-}$, cells were incubated for 10 min at 37 °C with 100 mU/ml xanthine oxidase and 1 mM xanthine prior to Ca^{2+} (Mn^{2+}) influx recording. Control cells were treated with xanthine oxidase alone because xanthine oxidase was found to inhibit Ca^{2+} (Mn^{2+}) influx to a small degree in the absence of added

substrate (data not shown). Xanthine alone did not affect Ca^{2+} (Mn^{2+}) influx (data not shown). In some experiments, experimental and control cells were pre-treated with the antioxidant L-NAC for 10-min prior to $\text{O}_2^{\bullet-}$ exposure. For exposure to H_2O_2 , cells were pre-incubated with 50 μM H_2O_2 for 10 min at 37 °C prior to Ca^{2+} (Mn^{2+}) influx recording. H_2O_2 was diluted daily from a fresh 3% stock solution (Aldrich).

3.3 Results

Response to Ionizing Radiation of Store-operated Ca^{2+} (Mn^{2+}) Influx Compared to Basal Ca^{2+} (Mn^{2+}) Influx in Jurkat T-cells and HPBL. The acute effect of 10-Gy γ -irradiation, on both basal and store-operated Ca^{2+} entry in Jurkat T-cells compared to HPBL was examined. An example of a DRFS recording of Ca^{2+} (Mn^{2+}) influx in γ -irradiated and control HPBL is shown in Fig. 3.1A. As illustrated by the traces, basal Ca^{2+} (Mn^{2+}) influx was similar in irradiated and control cells samples, however store-operated Ca^{2+} (Mn^{2+}) influx following stimulation with thapsigargin was substantially inhibited by radiation. Identical experiments were performed with Jurkat T-cells and the pooled results of repeated experiments for normal and transformed cells are compared in Fig. 3.1B. Radiation exposure did not significantly alter basal Ca^{2+} (Mn^{2+}) influx in either Jurkat T-cells (-0.04 ± 0.1 %/min; $n = 13$, $P > 0.05$) or HPBL (-0.8 ± 0.3 %/min; $n = 3$, $P > 0.05$). In contrast, store-operated Ca^{2+} (Mn^{2+}) influx following stimulation with thapsigargin was inhibited by γ -irradiation in both Jurkat T-cells (-1.0 ± 0.1 %/min; $n = 10$, $P < 0.001$) and HPBL (-3.6 ± 0.6 %/min; $n = 3$, $P < 0.05$). The inhibition of

Fig. 3.1. Activation-dependent inhibition of Ca^{2+} (Mn^{2+}) influx by γ -irradiation in Jurkat T-cells and HPBLs. A, DRFS recording of Ca^{2+} (Mn^{2+}) influx in HPBLs measured *simultaneously* in γ -irradiated (1.8 Gy/min) and control cell samples 5 min after radiation exposure. Cell samples were injected simultaneously with equal amounts of Mn^{2+} ($t = 100$ s), thapsigargin (TG, $t = 350$ s) and ionomycin (IM, $t = 650$ s). Ca^{2+} (Mn^{2+}) influx in irradiated and control cells, respectively, was 1.6 %/min vs. 2.0 %/min for basal influx ($t = 100$ -220 s), and 13.6 %/min vs. 17.5 %/min following thapsigargin stimulation ($t = 380$ -500 s). B, Pooled results of repeated experiments (detailed in A) in HPBLs and Jurkat T-cells. Data are expressed as difference from control (mean $\pm$ S. E.) for thapsigargin-activated influx (hatched bars; HPBLs, $n = 3$, Jurkat $n = 10$), and basal influx (solid bars; HPBLs $n = 3$, Jurkat $n = 13$). ** $P < 0.001$; * $P < 0.05$ (paired t-test; irradiated vs. control).

store-operated Ca^{2+} influx by γ -irradiation was significantly larger in HPBL compared to Jurkat T-cells ($P < 0.05$). In order to determine the dose-response relationship for

radiation exposure, further experiments were performed in Jurkat T-cells at radiation doses of 5, 10, 20 and 40 Gy. As shown in Fig. 3.2, γ -irradiation significantly inhibited store-operated Ca^{2+} (Mn^{2+}) influx after thapsigargin stimulation in a dose-dependent manner (5-40 Gy, $P < 0.01$). The reduction in store-operated Ca^{2+} (Mn^{2+}) influx at a radiation dose of 20 Gy (-1.7 ± 0.2 , $n = 13$) and 40 Gy (-2.2 ± 0.2 , $n = 9$), respectively, was significantly larger than at 5 Gy (-0.7 ± 0.2 , $n = 16$) and 10 Gy (-0.8 ± 0.3 , $n = 15$), $P < 0.05$. In contrast to store-operated Ca^{2+} (Mn^{2+}) influx, basal Ca^{2+} (Mn^{2+}) influx did not respond to γ -irradiation even at doses of up to 40 Gy. These results demonstrate that the sensitivity of Ca^{2+} influx to radiation exposure is dependent on cell activation.

The Inhibition of Ca^{2+} (Mn^{2+}) Influx by Ionizing Radiation Involves the SKF-96365-Inhibitable Store-operated- Ca^{2+} -channel Pathway. In order to test whether the inhibition of activation-induced Ca^{2+} (Mn^{2+}) influx by γ -irradiation was via the store-operated- Ca^{2+} -channel pathway, Jurkat T-cells were pre-incubated with SKF-96365 prior to radiation exposure. SKF-96365 has been characterized as an inhibitor of store-operated Ca^{2+} influx in T-lymphocytes (102). For these experiments cells were exposed to a radiation dose of 40 Gy, which in previous experiments inhibited Ca^{2+} (Mn^{2+}) influx to the largest degree (Fig. 3.2). This dose was chosen to give the widest dynamic range for characterizing the effect of SKF-96365. As shown in Fig. 3.3, in the absence of SKF-96365, 40 Gy γ -irradiation inhibited thapsigargin-stimulated Ca^{2+} (Mn^{2+}) influx (-2.3 ± 0.1 ; $n = 5$, $P < 0.01$) to a degree similar in magnitude to that observed in previous

Fig. 3.2. Dose-response for the activation-dependent inhibition of store-operated Ca^{2+} (Mn^{2+}) influx by γ -irradiation in Jurkat T-cells. Ca^{2+} (Mn^{2+}) influx measurements were performed as described in Fig. 3.1A following radiation doses of 0 , 5 , 10 , 20 and 40 Gy (n = 17, 16, 15, 13, and 9, respectively). Data are expressed as difference from control (mean $\pm$ S. E.) for thapsigargin stimulated influx (hatched bars) and basal influx (solid bars). * p < 0.001 (paired t-test; irradiated vs. control).

experiments (see Fig. 3.2). In the presence of 5 and 10 μM SKF-96365, however, the response to radiation was reduced in a concentration-dependent manner, and was significantly inhibited in the presence of 10 μM SKF-96365 (n = 5, P < 0.01).

Fig. 3.3. SKF-96365 inhibits the reduction in store-operated Ca^{2+} (Mn^{2+}) influx following γ -irradiation. Cells were pre-incubated with 0, 5 or 10 μM SKF-96365 prior to radiation exposure (40 Gy, 10.9 Gy/min). Ca^{2+} (Mn^{2+}) influx measurements were performed as described in Fig. 3.1. Data are expressed as difference from control (mean $\pm$ S. E.) in thapsigargin-stimulated Ca^{2+} (Mn^{2+}) influx for control cells (open bar), and cells treated with 5 μM (grey bar) or 10 μM (black bar) SKF-96365, $n = 5$ for each group. * $p < 0.001$ (t-test; SKF-96365-treated vs. 0 μM SKF-96365 control).

Elevation of Intracellular Glutathione by α -lipoic-acid Reduces the Response of Store-operated Ca^{2+} (Mn^{2+}) Influx to Ionizing Radiation. GSH is the primary antioxidant within the cell. One mechanism by which GSH exerts its radioprotective effects is by the direct scavenging of ROS. To determine whether elevated intracellular GSH could prevent the reduction in store-operated Ca^{2+} (Mn^{2+}) influx following γ -irradiation, cell

preparations were cultured in the absence or presence of the GSH elevating agent α -lipoic-acid (200 μ M) for 24 h prior to radiation exposure. In order to be able to clearly resolve an α -lipoic-acid-mediated reduction in the Ca^{2+} influx response to γ -irradiation, cell preparations that were found to exhibit a moderate to high level of radiosensitivity were examined. This group included cell preparations in which the response to radiation in the control cell sample was equal to or greater than the mean radiation response for all control cell samples (see Fig. 3.2 for mean radiation responses in untreated cells). This was necessary because it was not possible to observe a reduction in the radiation effect by α -lipoic-acid when the corresponding control cell sample exhibited a low sensitivity to radiation. As shown in Fig. 3.4, elevation of [GSH] with α -lipoic-acid ($127 \pm 11\%$, $n = 14$, $P < 0.01$) protected cells against the radiation-induced inhibition of store-operated Ca^{2+} (Mn^{2+}) influx. The protective action of GSH was dependent on radiation dose. At radiation doses of 5, 20, and 40 Gy, α -lipoic-acid treatment significantly reduced the response of store-operated Ca^{2+} (Mn^{2+}) influx to γ -irradiation by 62%, 48% and 22%, respectively ($n = 9, 6$, and 5 , respectively; $P < 0.05$). α -Lipoic-acid treatment also reduced the radiation response to 10-Gy irradiation, although not significantly (31%, $n = 7$, $P > 0.05$).

Response to $\text{O}_2^{\bullet-}$ and H_2O_2 of Store-operated Ca^{2+} (Mn^{2+}) Influx Compared to Basal Ca^{2+} (Mn^{2+}) Influx in Jurkat T-cells. Exposure to ionizing radiation generates ROS in the form of $\cdot\text{OH}$, $\text{O}_2^{\bullet-}$ and H_2O_2 . In order to test whether exposure to chemical oxidizing

Fig. 3.4. GSH Protects against radiation-induced inhibition of Ca²⁺ (Mn²⁺) influx. Cells were cultured for 24 h in the presence or absence of 200 μM α-lipoic-acid. Ca²⁺ (Mn²⁺) influx measurements were performed as described in Fig. 3.1A, following radiation doses of 5, 10, 20, and 40 Gy (n = 9, 7, 6, and 5, respectively). Data are expressed as difference from control (mean ± S. E.) for thapsigargin-stimulated Ca²⁺ (Mn²⁺) influx in control cells (hatched bars) and α-lipoic-acid-treated cells (solid bars). Figure insert, [GSH] in control cells (hatched bar) and α-lipoic-acid-treated cells (solid bar), n = 14. ** P < 0.01, * P < 0.05 (paired t-test; α-lipoic-acid-treated vs. control).

agents would inhibit Ca²⁺ (Mn²⁺) influx in a manner consistent with γ-irradiation, cells were pre-incubated for 10-min with either H₂O₂ (50 μM) or the O₂^{•-}-generating xanthine/xanthine oxidase system (1 mM/100 μM). As illustrated by the DRFS recording in Fig. 3.5, store-operated Ca²⁺ (Mn²⁺) influx following thapsigargin addition

Fig. 3.5. Activation-dependent inhibition of Ca^{2+} (Mn^{2+}) influx by H_2O_2 in Jurkat T-cells. DRFS recording of Ca^{2+} (Mn^{2+}) influx measured *simultaneously* in H_2O_2 -treated ($50\ \mu\text{M}$) and control cell samples 10 min after H_2O_2 exposure. Cell samples were injected simultaneously with equal amounts of Mn^{2+} ($t = 60\ \text{s}$), thapsigargin (TG, $t = 300\ \text{s}$) and digitonin (DG, $t = 500\ \text{s}$). Ca^{2+} (Mn^{2+}) influx in H_2O_2 treated and control cells, respectively, was $3.0\ \%/ \text{min}$ vs. $3.2\ \%/ \text{min}$ for basal influx ($t = 100\text{--}220\ \text{s}$), and $10.6\ \%/ \text{min}$ vs. $14.7\ \%/ \text{min}$ following thapsigargin stimulation ($t = 310\text{--}430\ \text{s}$). Figure insert, pooled results of repeated experiments expressed as difference from control (mean $\pm$ S. E.) for thapsigargin-activated influx (hatched bar) and steady-state influx (solid bar), $n = 4$. * $P < 0.05$ (paired t-test; H_2O_2 -treated vs. control) .

was substantially inhibited in the H_2O_2 -treated cell sample compared to control, while basal Ca^{2+} (Mn^{2+}) influx was similar in H_2O_2 -treated and control celis. The pooled results from repeated experiments with H_2O_2 (insert to Fig. 3.5) are consistent with those of

γ -irradiation (Figs. 3.1 and 3.2). Store-operated Ca^{2+} (Mn^{2+}) influx was significantly inhibited by H_2O_2 (-3.5 ± 0.9 %/min; $n = 4$, $P < 0.05$), while basal Ca^{2+} (Mn^{2+}) influx was not affected (0.0 ± 0.2 %/min; $n = 4$, $P > 0.05$). The effects of xanthine/xanthine oxidase treatment on Ca^{2+} (Mn^{2+}) influx are also consistent with the effects of γ -irradiation and H_2O_2 described above. As illustrated by the DRFS recording in Fig. 3.6, store-operated Ca^{2+} (Mn^{2+}) influx was reduced in xanthine/xanthine oxidase-treated cells compared to control while basal Ca^{2+} (Mn^{2+}) influx was not affected (0.7% /min vs. 0.8% /min). The pooled results show (insert to Fig. 3.6) that $\text{O}_2^{\bullet-}$ inhibited store-operated Ca^{2+} (Mn^{2+}) influx (-2.5 ± 0.4 %/min; $n = 4$, $P < 0.01$), while no significant change in basal Ca^{2+} (Mn^{2+}) influx was observed (0.2 ± 0.4 %/min; $n = 4$, $P > 0.05$). The influence of the anti-oxidant and GSH-elevating agent, L-NAC, on the $\text{O}_2^{\bullet-}$ -induced inhibition of Ca^{2+} (Mn^{2+}) influx, was also examined. As shown in Fig. 3.7A-C, pretreatment with L-NAC (10-min; 1, 10 and 20 mM) prevented the inhibition of store-operated Ca^{2+} (Mn^{2+}) influx by $\text{O}_2^{\bullet-}$ in a concentration-dependent manner. All of the tested oxidizing agents including ionizing radiation, H_2O_2 treatment, and $\text{O}_2^{\bullet-}$ generation inhibited store-operated Ca^{2+} (Mn^{2+}) influx but not basal Ca^{2+} (Mn^{2+}) influx, in a consistent manner. In addition, GSH elevation prevented the inhibition of store-operated Ca^{2+} (Mn^{2+}) influx by both chemical intervention and γ -irradiation.

Ionizing Radiation-induced Inhibition of Store-operated Ca^{2+} Influx in Jurkat T-cells is Independent of Tyrosine Kinase Activation. PTK activation is thought to mediate early

Fig. 3.6. Activation-dependent inhibition of Ca^{2+} (Mn^{2+}) influx by $\text{O}_2^{\cdot -}$ in Jurkat T-cells. DRFS recording of Ca^{2+} (Mn^{2+}) influx measured *simultaneously* in cell samples treated for 10 min with xanthine plus xanthine oxidase (X/XO; 1 mM/100 μM) and xanthine oxidase alone (XO, control). Cell samples were injected simultaneously with equal amounts of Mn^{2+} ($t = 60$ s), thapsigargin (TG, $t = 300$ s) and ionomycin (IM, $t = 600$ s). Ca^{2+} (Mn^{2+}) influx in cell samples treated with xanthine plus xanthine oxidase and xanthine oxidase alone, respectively, was 1.2 %/min vs. 1.4 %/min for steady-state influx ($t = 100$ -220 s), and 4.9 %/min vs. 7.6 %/min following thapsigargin activation ($t = 310$ -430 s). Figure insert, pooled results of repeated experiments are expressed as difference from control (mean $\pm$ S. E.) for thapsigargin-activated influx (hatched bar) and basal influx (solid bar), $n = 4$. * $P < 0.01$ (paired t-test; X/XO-treated vs. control).

cellular responses to ionizing radiation exposure. Therefore, the role of radiation-induced PTK activation in the inhibition of store-operated Ca^{2+} influx by γ -irradiation was examined. Fig. 3.8A-C shows DRFS recordings of store-operated Ca^{2+} (Mn^{2+}) influx

Fig. 3.7. L-NAC protects against superoxide-induced inhibition of store-operated Ca^{2+} (Mn^{2+}) influx. A-C, DRFS recording of Ca^{2+} (Mn^{2+}) influx measured *simultaneously* in cell samples treated for 10-min with xanthine plus xanthine oxidase (X/XO; 1 mM/100 μM) and xanthine oxidase alone (control) in the presence of (A) 1 mM, (B) 10 mM or (C) 20 mM L-NAC. Cell samples were injected simultaneously with equal amounts of Mn^{2+} ($t = 60\text{s}$, not shown), thapsigargin (TG, $t = 300\text{ s}$) and digitonin ($t = 500\text{ s}$, not shown).

following thapsigargin addition, measured simultaneously in (A) sham-treated vs. control cells, (B) radiation-treated vs. control cells and (C) radiation-treated vs. control cells pre-treated with 25 μM genistein to inhibit PTK. Store-operated Ca^{2+} (Mn^{2+}) influx was determined by the slope of each recording over the time period 310-420 s. As

Fig. 3.8. Radiation-induced inhibition of store-operated calcium influx in Jurkat T-cells in the absence or presence of the tyrosine kinase inhibitor genistein. DRFS recordings of thapsigargin-activated Ca²⁺ (Mn²⁺) influx measured *simultaneously* in (A) sham-treated and control cell samples, (B) irradiated and control cell samples in the absence of genistein and (C) irradiated and control cell samples in the presence of genistein (25 μ M). Cell samples were injected simultaneously with equal amounts of Mn²⁺ (t = 100 s, not shown), thapsigargin (TG, t = 300 s) and ionomycin (t = 600 s, not shown). Thapsigargin-activated Ca²⁺ (Mn²⁺) influx was (A) 10.5 %/min vs. 10.6 %/min in sham-treated and control cells, (B) 9.9 %/min vs. 11.2 %/min in irradiated and control cells and (C) 8.9 %/min vs. 10.0 %/min in irradiated and control cells following genistein treatment, respectively. D, Pooled results of repeated experiments (detailed above) for the conditions: sham vs. control (filled bar, n = 3), irradiated vs. control in the absence of genistein (hatched bar, n = 3) and irradiated vs. control in the presence of genistein (open bar, n = 3). Data are expressed as difference from control in thapsigargin stimulated Ca²⁺ (Mn²⁺) influx (mean $\pm$ S. E.). ** p < 0.01, * p < 0.05 (paired t-test; irradiated vs. control).

illustrated by the traces, store-operated Ca^{2+} (Mn^{2+}) influx was (A) identical in sham-treated compared to control cells, (B) inhibited in irradiated compared to control cells, and (C) similarly inhibited in irradiated compared to control cells pre-treated with the PTK inhibitor genistein. The pooled results of repeated experiments for each condition are summarized in Fig. 3.8D. As expected, there was no significant difference in store-operated Ca^{2+} (Mn^{2+}) influx between sham-treated and control cells (0.2 ± 0.2 %/min; $n = 3$, $P > 0.05$), demonstrating the ability of DRFS to reliably resolve small differences in Ca^{2+} (Mn^{2+}) influx. Consistent with previous results (Fig. 3.1B), γ -irradiation inhibited store-operated Ca^{2+} (Mn^{2+}) influx in Jurkat T-cells (-1.1 ± 0.1 %/min; $n = 3$, $P < 0.05$). In addition, γ -irradiation exposure in cells pre-treated with genistein inhibited store-operated Ca^{2+} (Mn^{2+}) influx to a degree similar to γ -irradiation alone (-1.0 ± 0.0 %/min; $n = 3$, $P < 0.01$). These results suggest the lack of involvement of PTK activity in mediating the acute effects of 10-Gy γ -irradiation on store-operated Ca^{2+} influx.

3.4 Discussion

A summary of the reported responses in Ca^{2+} influx to radiation and other oxidizing agents is presented in percent effect for comparison (Table 3.1). Radiation was found to inhibit store-operated Ca^{2+} (Mn^{2+}) influx in both Jurkat T-cells and HPBL (Fig. 3.1). The response in Ca^{2+} (Mn^{2+}) influx to radiation was dose-dependent for the tested doses, as determined with Jurkat T-cells (Fig 3.2). Importantly, modulation of Ca^{2+} (Mn^{2+}) influx by γ -irradiation was dependent on T-cell activation. This is illustrated by the finding that

Table 3.1. Oxidant-induced modulation of Ca^{2+} (Mn^{2+}) influx in Jurkat T-cells and HPBL.

Treatment	Ca^{2+} (Mn^{2+}) Influx		
	Basal	Thapsigargin	α -CD3 mAb
γ -Irradiation, Jurkat (10 Gy)	n. s. (13)	-8.2 ± 1.0 (10)	-8.0 ± 1.6 (3)
γ -Irradiation, Jurkat (40 Gy)	n. s. (9)	-14.6 ± 1.4 (9)	
γ -Irradiation, HPBL (10 Gy)	n. s. (3)	-18.2 ± 2.3 (3)	
H_2O_2 , Jurkat (50 μM)	n. s. (4)	-22.6 ± 6.3 (4)	
Xanthine/xanthine oxidase, Jurkat	n. s. (4)	-33.9 ± 6.2 (4)	

Notes. Summary of pooled results from experiments presented in Figs. 1, 5, and 6. Data are expressed as percent difference from control (mean $\pm$ S. E.). Minimum significance level is $P < 0.05$ (n. s. indicates no significance). Number of replicates are indicated in parentheses.

basal Ca^{2+} (Mn^{2+}) influx was not affected by 10-Gy γ -irradiation in HPBL, nor was it perturbed by substantially higher doses (up to 40 Gy) in Jurkat T-cells (Figs. 3.1B, 3.3). The fact that radiation exposure modified Ca^{2+} signaling but did not affect resting Ca^{2+} homeostasis also demonstrates that the response to radiation described above is not the result of non-specific effects, such as membrane damage from lipid peroxidation or a radiation-related change in the fluorescence properties of Fura-2, for example. The effect of radiation exposure was significantly reduced by SKF-96365 treatment in a

concentration-dependent manner (Fig. 3.3), suggesting that radiation specifically inhibits the SKF-96365-inhibitable store-operated Ca^{2+} influx pathway (102). The inhibition of Ca^{2+} signaling by ionizing radiation has not been previously reported in non-excitabile cells, however ionizing radiation at doses of 3-10 Gy inhibited voltage-dependent $^{45}\text{Ca}^{2+}$ uptake in rat brain synaptosomes (97). In addition voltage-dependent Ca^{2+} channels in mouse oocytes were inhibited by whole-body X-ray irradiation at doses of 10-100 Gy (96). Although in the present study basal Ca^{2+} influx was found to be insensitive to γ -irradiation in human T-lymphocytes (Fig. 3.1), in HeLa cells γ -irradiation at a dose of 4 Gy was reported to increase basal Ca^{2+} (Mn^{2+}) influx (95), suggesting that such effects might be cell-type-dependent.

The xanthine/xanthine oxidase system and H_2O_2 generate free radicals similar to those produced during ionizing radiation exposure. Exposure to these chemical oxidants were found to inhibit Ca^{2+} influx in a manner consistent with ROS generation by γ -irradiation (Table 3.1). In addition, the inhibition of Ca^{2+} influx by xanthine/xanthine oxidase was reduced by pre-incubation with the anti-oxidant L-NAC, in a concentration-dependent manner (Fig. 3.7). These results suggest that the effect of radiation exposure on store-operated Ca^{2+} influx may be mediated by ROS. The activation-dependent inhibition of Ca^{2+} influx by H_2O_2 , described above (Fig. 3.5), is in apparent contrast to previously reported findings. In independent studies, H_2O_2 was reported to elevate basal Ca^{2+} influx (32) and $[\text{Ca}^{2+}]_i$ (103) in Jurkat T-cells. However, the H_2O_2 -mediated changes in $[\text{Ca}^{2+}]_i$ were transient, with the return of $[\text{Ca}^{2+}]_i$ to pre-treatment levels within 15-20 min (103).

A transient increase in basal Ca^{2+} influx and $[\text{Ca}^{2+}]_i$ immediately following addition of H_2O_2 , which returned to pre-treatment levels within 10 min, could also be observed under the conditions used in this study (data not shown). In the present study, the response of Ca^{2+} signaling to H_2O_2 , however, was determined subsequent to these transient Ca^{2+} elevations. This is substantiated by the fact that basal Ca^{2+} influx in H_2O_2 -treated cells was identical to control cells at the time of measurement. This observation also indicated that the filling state of the Ca^{2+} stores was similar in H_2O_2 -treated and control cells at the time of measurement, since a change in the state of the Ca^{2+} store would have become apparent as a difference in basal Ca^{2+} influx between treated and control cells (104). Therefore, it appears that in resting T-cells the initial response to H_2O_2 may be a transient elevation in basal $[\text{Ca}^{2+}]_i$ and Ca^{2+} influx, whereas Ca^{2+} signaling, which is critical for T-cell activation, is reduced under oxidative conditions.

GSH is one of the major cellular defenses against free radicals and other ROS and has been extensively studied for its radioprotective properties (98). A 2-fold elevation in [GSH] by α -lipoic-acid significantly prevented the inhibition of store-operated Ca^{2+} (Mn^{2+}) influx by radiation in Jurkat T-cells (Fig. 3.4). The degree of protection was dose-dependent with the greatest protection observed at the lowest radiation dose of 5 Gy (67%). Protection was significant but less pronounced at the highest radiation dose of 40 Gy (22%). These results demonstrate the ability of GSH to protect against the radiation-induced inhibition of Ca^{2+} signaling and point to the dose-dependent involvement of ROS in the inhibition of store-operated Ca^{2+} (Mn^{2+}) influx by γ -irradiation.

The role of PTK in the inhibition of store-operated Ca^{2+} (Mn^{2+}) influx by ionizing radiation was also examined since ionizing radiation at doses of 0.5-10 Gy has been reported to initiate cellular signaling processes through PTK activation (88, 92, 105, 106). The results showed, however, that γ -irradiation inhibited store-operated Ca^{2+} (Mn^{2+}) influx even when PTK activity was chemically inhibited (see Fig. 3.8A-D). Because PTK inhibition did not modulate the observed response to radiation exposure, it appears that PTK activation is not required for the acute reduction in store-operated Ca^{2+} (Mn^{2+}) influx following γ -irradiation. In addition, it appears unlikely that PTK activation would result in the inhibition of Ca^{2+} influx since the reduction of PTK activity was previously found to inhibit Ca^{2+} (Mn^{2+}) influx (107, 108, 109). PKC is another potential mediator of the effect of γ -irradiation on Ca^{2+} entry. For example, PKC is activated within seconds following X-irradiation (93), and PKC activation inhibits store-operated Ca^{2+} influx in human lymphocytes (81). Further studies are needed to directly test the possible link between PKC and the radiation-induced inhibition of store-operated Ca^{2+} entry.

Ceramide, the key messenger in the sphingomyelinase signaling pathway, is a reported mediator of radiation-induced apoptosis (110, 90). Ceramide generation, which can be initiated by a number of external signals including ionizing radiation, tumor-necrosis-factor- α , and IL-1 β , involves the hydrolysis of sphingomyelin by plasma membrane-associated sphingomyelinase (111). There exists evidence suggesting that ceramide may be involved in mediating the reduction in store-operated Ca^{2+} influx following γ -irradiation. For example, (i) the concentration of ceramide increases within seconds

following ionizing radiation exposure in endothelial cells (88, 111), and *(ii)* ceramide was reported to inhibit activation-dependent Ca^{2+} (Mn^{2+}) influx in both human neutrophils and rat leukemia cells (79, 112). Based on these reports and the results of this study, the sphingomyelinase signaling pathway is one plausible mediator for the inhibition of store-operated Ca^{2+} (Mn^{2+}) influx by ionizing radiation in Jurkat T-cells. However, specific inhibitors for sphingomyelinase, which are required to test this hypothesis, are not yet available.

Recent reports indicate that GSH is an endogenous inhibitor of plasma-membrane-associated sphingomyelinase (78, 113). For example, *(i)* GSH was found to inhibit sphingomyelinase in isolated enzyme preparations (78), *(ii)* GSH depletion by BSO resulted in the elevation of ceramide levels in intact human leukemia cells (78), and *(iii)* GSH inhibited the generation of ceramide induced by tumor-necrosis-factor- α in mammary carcinoma cells (113). Based on these findings, elevation of [GSH] prior to radiation exposure would be expected to inhibit radiation-induced activation of sphingomyelinase, and the subsequent generation of ceramide. Therefore, elevated GSH might prevent the ceramide-mediated inhibition of store-operated Ca^{2+} entry proposed above. The above results describing the protective action of GSH on radiation-induced inhibition of Ca^{2+} signaling (Fig. 3.4) are consistent with this hypothesis.

In summary, exposure to ionizing radiation inhibited activation-dependent Ca^{2+} signaling through store-operated Ca^{2+} channels in human T-lymphocytes in a dose-dependent manner. Resting Ca^{2+} homeostasis, however, was not perturbed by radiation,

even at a dose of 40 Gy. The effects of γ -irradiation on Ca^{2+} signaling appear to be mediated by ROS since (i) the Ca^{2+} response to H_2O_2 and $\text{O}_2^{\bullet-}$ was consistent with the response to γ -irradiation, and (ii) elevation of [GSH] protected against the inhibition of Ca^{2+} influx by γ -irradiation. In combination these results demonstrate that exposure to oxidizing interventions including ionizing radiation reduces activation-induced Ca^{2+} signaling in human T-lymphocytes. Possible consequences could be the inhibition of Ca^{2+} influx-dependent activities including gene expression and cell proliferation. Indeed, H_2O_2 treatment suppressed mitogen-induced NF-AT and IL-2 reporter activity in Jurkat T-cells (114). It is therefore possible that the suppression of store-operated Ca^{2+} entry by ROS may, at least in part, mediate the observed depression in immune function associated with *in vivo* exposure to ionizing radiation of doses of 1-7 Gy, for example (115, 116).

4. SUMMARY

The purpose of this thesis was to conduct a systematic investigation of the influence of redox perturbations from oxidant and antioxidant treatments on store-operated Ca^{2+} entry in human T-cells. A new method for monitoring Ca^{2+} homeostasis was employed, called DRFS, wherein experimental and control cell samples are measured simultaneously, allowing the rapid and accurate real-time detection of even potentially small fluorescent changes between two cell samples. This technique was able to reliably resolve redox-induced changes in Ca^{2+} (Mn^{2+}) influx or $[\text{Ca}^{2+}]_i$ on the order of $\cong 10\text{-}30\%$, which is not easily accomplished, particularly with online Ca^{2+} (Mn^{2+}) flux measurements, with conventional single cuvette-based fluorescence spectroscopy (117). The findings of this study demonstrate that the sensitivity of Ca^{2+} influx and $[\text{Ca}^{2+}]_i$ to redox perturbations critically depends on the state of the cell: non-stimulated and resting lymphocytes were mostly insensitive to the tested redox interventions, whereas in stimulated cells redox perturbations induced significant changes in Ca^{2+} influx and $[\text{Ca}^{2+}]_i$. Specifically, reduction enhanced while oxidation inhibited both store-operated Ca^{2+} influx and the activation-dependent increase in $[\text{Ca}^{2+}]_i$ in human T-cells.

Based on these results and on those of other studies, one may expect that oxidative conditions will inhibit store-operated Ca^{2+} influx in human T-lymphocytes, and suppress Ca^{2+} -dependent biological activities including cytokine secretion and cell proliferation. Thus oxidation-induced reduction in store-operated Ca^{2+} entry following T-cell activation might contribute to decreases in immune function for certain oxidative conditions such as

induced by ionizing radiation exposure, certain disease states, and immunosclerosis during aging. Conversely, reducing conditions will enhance store-operated Ca^{2+} influx leading to the enhancement of Ca^{2+} -dependent cellular activities. From a clinical view point, interventions that can effectively shift the redox equilibrium towards reduction may enhance store-operated Ca^{2+} influx, possibly leading to the stimulation of immune cell activity.

Further studies are necessary to identify the mechanisms involved in the redox modulation of store-operated Ca^{2+} entry. A number of potential candidates exist including, the regulation of sphingomyelinase activity and ceramide generation by GSH, the direct modulation of Ca^{2+} channel proteins, and redox modulation of the putative Ca^{2+} influx factor or other mediators of store-operated Ca^{2+} entry. The identification of key mediators involved in the regulation of store-operated Ca^{2+} entry may be aided by the knowledge that store-operated Ca^{2+} influx is redox sensitive. The direct testing of Ca^{2+} channel redox sensitivity must await cloning and expression of the store-operated Ca^{2+} channel in a suitable test system. Regulation of sphingomyelinase activity and ceramide generation by GSH is another plausible mechanism for explaining the oxidant induced redox modulation of Ca^{2+} entry. For example, (i) ceramide is elevated under oxidative conditions including GSH depletion and ionizing radiation, (ii) ceramide inhibits activation-induced Ca^{2+} signaling and (iii) GSH inhibits ceramide generation. Future experiments might include the measurement of ceramide levels in Jurkat T-cells by high

performance liquid chromatography before and after exposure to redox interventions, to determine whether changes in activation-induced Ca^{2+} influx correspond with redox-induced changes in ceramide concentration.

ABBREVIATIONS

BSO	Buthionine sulfoximine
Ca ²⁺	Calcium
[Ca ²⁺] _i	Intracellular free calcium concentration
α-CD3-mAb	Monoclonal antibody to the CD3 epitope of the T-cell receptor
DEM	Diethylmaleate
DRFS	Differential ratiometric fluorescence spectroscopy
DTNB	Dithiobis-2-nitrobenzoic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether) tetraacetic acid
GSH	Glutathione
[GSH]	Intracellular concentration of total glutathione
Gy	Gray
H ₂ O ₂	Hydrogen peroxide
HPBL	Human peripheral blood lymphocytes
Mn ²⁺	Manganese
D-NAC	N-acetyl-D-cysteine
L-NAC	N-acetyl-L-cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
O ₂ ^{•-}	Superoxide anion
•OH	Hydroxyl radical
PKC	Protein kinase C
PTK	Protein tyrosine kinase
ROS	Reactive oxygen species
TNB	Thio-nitrobenzoic acid

REFERENCES

1. Putney, Jr., J. W., and G. St. J. Bird. 1993. The inositol phosphate-calcium signaling system in nonexcitable cells. *Endocrine Rev.* 14:610.
2. Berridge, M. J. 1993. Inositol trisphosphate and calcium signalling. *Nature* 361:315.
3. Takemura, H., A. R. Hughes, O. Thastrup, and J. W. Putney, Jr. 1989. Activation of calcium entry by the tumor promoter thapsigargin in parotid acinar cells. *J. Biol. Chem.* 264:12266.
4. Gouy, H., D. Cefai, S. B. Christensen, P. Debré, and G. Bismuth. 1990. Ca^{2+} influx in human T lymphocytes is induced independently of inositol phosphate production by mobilization of intracellular Ca^{2+} stores: a study with the Ca^{2+} endoplasmic reticulum-ATPase inhibitor thapsigargin. *Eur. J. Immunol.* 20:2269.
5. Streb, H., R. F. Irvine, M. J. Berridge, and I. Schulz. 1983. Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature* 306:67.
6. Zweifach, A., and R. S. Lewis. 1993. Mitogen-regulated Ca^{2+} current of T lymphocytes is activated by depletion of intracellular Ca^{2+} stores. *Proc. Natl. Acad. Sci. USA* 90:6295.
7. Hoth, M., and R. Penner, 1992. Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* 355:353.
8. Putney, Jr., J. W. 1986. A model for receptor-regulated calcium entry. *Cell Calcium* 7:1.
9. Putney, Jr., J. W. 1990. Capacitative calcium entry revisited. *Cell Calcium* 11:611.
10. Parekh, A. B., and R. Penner. 1997. Store depletion and calcium influx. *Physiol. Rev.* 77:901.
11. Berridge, M. J. 1995. Capacitative calcium entry. *Biochem. J.* 312:1.
12. Meister, A., and M. E. Anderson. 1983. Glutathione. *Ann. Rev. Biochem.* 52:711.
13. Meister, A. 1988. Glutathione metabolism and its selective modification. *J. Biol. Chem.* 263:17205.

14. Harris, E. D. 1992. Regulation of antioxidant enzymes. *FASEB J.* 6:2675.
15. Reed, D. J. 1990. Glutathione: toxicological implications. *Annu. Rev. Pharmacol. Toxicol.* 30:603.
16. Bensasson, R. V., E. J. Land, and T. G. Truscott. 1993. *Excited States and Free Radicals in Biology and Medicine*. Oxford University Press, New York.
17. Stadtman, E. R. 1993. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu. Rev. Biochem.* 62:797.
18. Packer, L. 1995. Glutathione metabolism. *Methods Enzym.* 251:3.
19. Suzuki, Y. J., H. J. Forman, and A. Sevanian. 1997. Oxidants as stimulators of signal transduction. *Free Rad. Biol. Med.* 22:269.
20. Monteiro, H. P., and A. Stern. 1996. Redox modulation of tyrosine phosphorylation-dependent signal transduction. *Free Rad. Biol. Med.* 21:323.
21. Sun, Y., and L. W. Oberley. 1996. Redox regulation of transcriptional activators. *Free Rad. Biol. Med.* 21:335.
22. Sen, C. K., and L. Packer. 1996. Antioxidant and redox regulation of gene transcription. *FASEB J.* 10:709.
23. Slater, A. F. G., C. S. I. Nobel, and S. Orrenius. 1995. The role of intracellular oxidants in apoptosis. *Biochim. Biophys. Acta* 1271:59.
24. Lander, H. M., D. P. Hajjar, B. L. Hempstead, U. A. Mirza, B. T. Chait, S. Campbell, and L. A. Quilliam,. 1997. A molecular switch on p21^{ras}. *J. Biol. Chem.* 272:4323.
25. Wang, X., V. C. Culotta, and C. B. Klee. 1996. Superoxide dismutase protects calcineurin from inactivation. *Nature* 383:434.
26. Ruppersberg, J. P., M. Stocker, O., Pongs, S. H., Heinemann, R. Frank, and M. Koenen, 1991. Regulation of fast inactivation of cloned mammalian I_K(A) channels by cysteine oxidation. *Nature* 352:711.

27. Vega-Saenz de Miera, E., and B. Rudy. 1992. Modulation of K⁺ channels by hydrogen peroxide. *Biochem. Biophys. Res. Comm.* 186:1681.
28. Kuo, S. S., A. H. Saad, A. C. Koong, G. M. Hahn, A. J. Giaccia. 1993. Potassium-channel activation in response to low doses of γ -irradiation involves reactive oxygen intermediates in nonexcitatory cells. *Proc. Natl. Acad. Sci. USA* 90:908.
29. Elliott, S. J., J. G. Meszaros, and W. P. Schilling. 1992. Effect of oxidant stress on calcium signaling in vascular endothelial cells. *Free Rad. Biol. Med.* 13:635.
30. Doan, T. N., D. L. Gentry, A. A. Taylor, and S. J. Elliott. 1994. Hydrogen peroxide activates agonist-sensitive Ca²⁺-flux pathways in canine venous endothelial cells. *Biochem. J.* 297:209.
31. Rojanasakul, Y., L. Wang, A. H. Hoffman, X. Shi, N. S. Dalal, D. E. Banks, and J. K. H. Ma. 1993. Mechanisms of hydroxyl free radical-induced cellular injury and calcium overloading in alveolar macrophages. *Am. J. Respir. Cell Mol. Biol.* 8:377.
32. Beiqing, L., K. V. Hackshaw, and R. L. Whisler. 1996. Calcium signals and protein tyrosine kinases are required for the induction of c-jun in Jurkat cells stimulated by the T cell-receptor complex and oxidative signals. *J. Interferon Cytokine Res.* 16:77.
33. Sekkat, C., J. Dornand, and M. Gerber. 1988. Oxidative phenomena are implicated in human T-cell stimulation. *Immunology* 63:431.
34. Rouahi, N., C. Levallois, F. Favier, J.-L. Balmes, and J.-C. Mani. 1989. Flow cytometric analysis of oxidative product formation in phytohemagglutinin-stimulated ethanol-treated immune mononuclear cells. *Drug Alcohol Dependence* 23:55.
35. Benichou, G., J. M. Kanellopoulos, F. Mitene, P. Galanaud, and G. Leca. 1989. T-cell chemiluminescence: a novel aspect of T-cell membrane activation studied with a Jurkat tumour cell line. *Scand. J. Immunol.* 30:265.
36. Schreck, R., P. Rieber, P. A. Baeuerle. 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF- κ B transcription factor and HIV-1. *EMBO J.* 10:2247.
37. Gmünder, H., S. Roth, H.-P. Eck, H. Gallas, S. Mihm, and W. Dröge. 1990. Interleukin-2 mRNA expression, lymphokine production and DNA synthesis in glutathione-depleted T cells. *Cell. Immunol.* 130:520.

38. Freed, B. M., N. Lempert, and D. A. Lawrence. 1991. Inhibition of interleukin-2 production in the human T cell line JURKAT by nonpolar maleimides. *Toxicol. Appl. Pharmacol.* 107:173.
39. Hamilos, D. L., P., Zelarney, and J. J. Mascali. 1989. Lymphocyte proliferation in glutathione-depleted lymphocytes: direct relationship between glutathione availability and the proliferative response. *Immunopharmacology* 18:223.
40. Suthanthiran, M., M. E. Anderson, V. K. Sharma, and A. Meister. 1990. Glutathione regulates activation-dependent DNA synthesis in highly purified normal human T lymphocytes stimulated via the CD2 and CD3 antigens. *Proc. Natl. Acad. Sci. USA* 87:3343.
41. Sandstrom, P. A., M. D. Mannie, and T. M. Buttke. 1994. Inhibition of activation-induced death in T cell hybridomas by thiol antioxidants: oxidative stress as a mediator of apoptosis. *J. Leukocyte Biol.* 55:221.
42. Fernandez, A., J. Kiefer, L. Fosdick, and D. J. McConkey. 1995. Oxygen radical production and thiol depletion are required for Ca^{2+} -mediated endogenous endonuclease activation in apoptotic thymocytes. *J. Immunol.* 155:5133.
43. Serafini, A. T., R. S. Lewis, N. A. Clipstone, R. J. Bram, C. Fanger, S. Fiering, L. A. Herzenberg, and G. R. Crabtree. 1995. Isolation of mutant T lymphocytes with defects in capacitative calcium entry. *Immunity* 3:239.
44. Fanger, C. M., M. Hoth, G. R. Crabtree, and R. S. Lewis. 1995. Characterization of T cell mutants with defects in capacitative calcium entry: genetic evidence for the physiological roles of CRAC channels. *J. Cell Biol.* 131: 655.
45. Lichtman, A. H., G. B. Segel, and M. A. Lichtman. 1983. The role of calcium in lymphocyte proliferation (an interpretive review). *Blood* 61:413.
46. Birx, D. L., M. Berger, and T. A. Fleisher. 1984. The interference of T cell activation by calcium channel blocking agents. *J. Immunol.* 133:2904.
47. Lewis, R. S., and M. D. Cahalan. 1989. Mitogen-induced oscillations of cytosolic Ca^{2+} and transmembrane Ca^{2+} current in human leukemic T cells. *Cell Regulation* 1:99.
48. Donnadieu, E., G. Bismuth, and A. Trautmann. 1992. Calcium fluxes in T lymphocytes. *J. Biol. Chem.* 267:25864.

49. Partiseti, M., F. Le Deist, C. Hivroz, A. Fischer, H. Korn, and D. Choquet. 1994. The calcium current activated by T cell receptor and store depletion in human lymphocytes is absent in a primary immunodeficiency. *J. Biol. Chem.* 269:32327.
50. Le Deist, F., C. Hivroz, M. Partiseti, C. Thomas, H. A. Buc, M. Oleastro, B. Belohradsky, D. Choquet, and A. Fischer. 1995. A primary T-cell immunodeficiency associated with defective transmembrane calcium influx. *Blood* 85:1053.
51. Buchmüller-Rouiller, Y., S. B. Corradin, J. Smith, P. Schneider, A. Ransijn, C. V. Jongeneel, and J. Mauël. 1995. Role of glutathione in macrophage activation: effect of cellular glutathione depletion on nitrite production and leishmanicidal activity. *Cell. Immunol.* 164:73.
52. Meier, T., and R. D. Issels. 1995. Promotion of cyst(e)ine uptake. *Methods Enzymol.* 252:103.
53. Packer, L., S. Roy, and C. K. Sen. 1997. α -Lipoic acid: a metabolic antioxidant and potential redox modulator of transcription. *Adv. Pharmacol.* 38:79.
54. Han, D., H. J. Tritschler, and L. Packer. 1995. α -Lipoic acid increases intracellular glutathione in a human T-lymphocyte Jurkat cell line. *Biochem. Biophys. Res. Comm.* 207:258.
55. Packer, L., E. H. Witt, and H. J. Tritschler. 1995. Alpha-lipoic as a biological antioxidant. *Free Rad. Biol. Med.* 19:227.
56. Anderson, M. E., and A. Meister. 1987. Intracellular delivery of cysteine. *Methods Enzymol.* 143: 313.
57. Dröge, W., R. Kinscherf, S. Mihm, D. Galter, S. Roth, H. Gmünder, T. Fischbach, and M. Bockstette. 1996. Thiols and the immune system: effect of N-acetylcysteine on T cell system in human subjects. *Methods Immunol.* 251:255.
58. Grynkiewicz, G., M., Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440.
59. Merritt, J. E., R. Jacob, and T. J. Hallam. 1989. Use of manganese to discriminate between calcium influx and mobilization from internal stores in stimulated human neutrophils. *J. Biol. Chem.* 264:1522.

60. Carson, J. J. L., and F. S. Prato. 1996. Fluorescence spectrophotometer for the real time detection of cytosolic free calcium from cell suspensions during exposure to extremely low frequency magnetic fields. *Rev. Sci. Instrum.* 67:4336.
61. Walieczek, J., Differential Fluorescence Ratiometric Spectroscopy. 1995. US-patent 5,383,023.
62. Tietz, F. 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* 27:502.
63. Baker, M. A., G. J. Cerniglia, and A. Zaman. 1990. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. *Anal. Biochem.* 190:360.
64. Vandeputte, C., I. Guizon, I. Genestie-Denis, B. Vannier, and G. Lorenzon. 1994. A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. *Cell Biol. Toxicol.* 10:415.
65. Sjödin, K., E. Nilsson, A. Hallberg, and A. Tunek. 1989. Metabolism of N-acetyl-L-cysteine: some structural requirements for the deacetylation and consequences for the oral bioavailability. *Biochem. Pharmacol.* 38:3981.
66. Yim, C.-Y, J. B. Hibbs, Jr., J. R. McGregor, R. E. Galinsky, and W. E. Samlowski. 1994. Use of N-acetyl cysteine to increase intracellular glutathione during the induction of antitumor responses by IL-2. *J. Immunol.* 152:5796.
67. Eylar, E., C. Rivera-Quinones, C. Molina, I. Báez, F. Molina, and C. M. Mercado. 1993. N-acetylcysteine enhances T cell functions and T cell growth in culture. *Inter. Immunol.* 5:97.
68. Janáky, R., V. Varga, P. Saransaari, and S. S. Oja. 1993. Glutathione modulates the N-methyl-D-aspartate receptor-activated calcium influx into cultured rat cerebellar granule cells. *Neurosci. Lett.* 156:153.
69. Iwata, S., T. Hori, N. Sato, Y. Ueda-Taniguchi, T. Yamabe, H. Nakamura, H. Masutani, and J. Yodoi. 1994. Thiol-mediated redox regulation of lymphocyte proliferation. *Immunol.* 152:5633.

70. Kavanagh, T. J., A. Grossmann, J. C. Jinneman, S. B. Kanner, C. C. White, D. L. Eaton, J. A. Ledbetter, and P. S. Rabinovitch. 1993. The effect of 1-Chloro-2,4-dinitrobenzene exposure on antigen receptor (CD3)-stimulated transmembrane signal transduction in purified subsets of human peripheral blood lymphocytes. *Toxicol. Appl. Pharmacol.* 119:91.
71. Staal, F. J. T., M. T. Anderson, G. E. J. Staal, L. A. Herzenberg, C. Gitler, and L. A. Herzenberg. 1994. Redox regulation of signal transduction: tyrosine phosphorylation and calcium influx. *Proc. Natl. Acad. Sci. USA* 91:3619.
72. Duprat, F., E. Guillemare, G. Romey, M. Fink, F. Lesage, M. Lazdunski, and E. Honore. 1995. Susceptibility of cloned K⁺ channels to reactive oxygen species. *Proc. Natl. Acad. Sci. USA* 92:11796.
73. Kourie, J. I. 1997. A redox O₂ sensor modulates the SR Ca²⁺ countercurrent through voltage-and Ca²⁺-dependent Cl⁻ channels. *Am. J. Physiol.* 272:C324.
74. Chiamvimonvat, N., B. O'Rourke, T. J. Kamp, R. G. Kallen, F. Hofmann, V. Flockerzi, and E. Marban. 1995. Functional consequences of sulfhydryl modification in the pore-forming subunits of cardiovascular Ca²⁺ and Na²⁺ channels. *Circ. Res.* 76:325.
75. Campbell, D. L., J. S. Stamler, and H. C. Strauss. 1996. Redox modulation of L-type calcium channels in ferret ventricular myocytes. *J. Gen. Physiol.* 108:277.
76. Abe, J., M. Kusuvara, R. J. Ulevitch, B. C. Berk, and J-D. Lee. 1996. Big mitogen-activated protein kinase 1 (BMK1) is a redox-sensitive kinase. *J. Biol. Chem.* 271:16586.
77. Tepel, M., S. Kühnapfel, G. Theilmeier, C. Teupe, R. Schlotmann, and W. Zidek. 1994. Filling state of intracellular Ca²⁺ pools triggers trans plasma membrane Na²⁺ and Ca²⁺ influx by a tyrosine kinase-dependent pathway. *J. Biol. Chem.* 269:26239.
78. Liu, B., and Y. A. Hannun. 1997. Inhibition of the neutral magnesium-dependent sphingomyelinase by glutathione. *J. Biol. Chem.* 272:16281.
79. Wong, K., X.-B. Li, and N. Hunchuk. 1995. N-acetylsphingosine (C₂-ceramide) inhibited neutrophil superoxide formation and calcium influx. *J. Biol. Chem.* 270:3056.

80. Whisler, R. L., Y. G. Newhouse, L. Beiqing, B. K. Karanfilov, M. A. Goyette, and K. V. Hackshaw. 1994. Regulation of protein kinase enzymatic activity in Jurkat T cells during oxidative stress uncoupled from protein kinases: role of oxidative changes in protein kinase activation requirements and generation of second messengers. *Lymphokine Cytokine Res.* 13:399.
81. Balasubramanyam, M., M. Kimura, A. Aviv, and J. P. Gardner. 1993. Kinetics of calcium transport across the lymphocyte plasma membrane. *Am. J. Physiol.* 65:C321.
82. Lehninger, A. L., A. Vercesi, and E. A. Bababunmi. 1978. Regulation of Ca^{2+} release from mitochondria by the oxidation-reduction state of pyridine nucleotides. *Proc. Natl. Acad. Sci. USA* 75:1690.
83. Hoth, M., C. M. Fanger, and R. S. Lewis. 1997. Mitochondrial regulation of store-operated calcium signaling in T lymphocytes. *J. Cell Biol.* 137:633.
82. Chiono, M., R. Mahey, G. Tate, and D. M. F. Cooper. 1995. Capacitative Ca^{2+} entry exclusively inhibits cAMP synthesis in C6-2B glioma cells. *J. Biol. Chem.* 270:1149.
85. Fagan, K. A., R. Mahey, and D. M. F. Cooper. 1996. Functional co-localization of transfected Ca^{2+} -stimulable adenylyl cyclases with capacitative Ca^{2+} entry sites. *J. Biol. Chem.* 271:12438.
86. Gardner, P., A. Alcover, M. Kuno, P. Moingeon, C. M. Weyand, J. Goronzy, and E. L. Reinherz. 1989. Triggering of T-lymphocytes via either T3-Ti or T11 surface structures opens a voltage-insensitive plasma membrane calcium-permeable channel: requirement for interleukin-2 gene function. *J. Biol. Chem.* 264:1068.
87. Alpin, E. L. 1998. *Radiation Biophysics*, 2nd edition. Academic Press, San Diego, CA.
88. Prasad, A. V., N. Mohan, B. Chandrasekar, and M. L. Meltz. 1995. Induction of transcription of "immediate early genes" by low-dose ionizing radiation. *Radiat. Res.* 143:263.
89. Lu-Hesselmann, J., G. Messer, D. van Beuningen, P. Kind, and R. U. Peter. 1997. Transcriptional regulation of the human IL5 gene by ionizing radiation in Jurkat T cells: evidence for repression by an NF-AT-like element. *Radiat. Res.* 148:531.

90. Haimovitz-Friedman, A., C.-C. Kan, D. Ehleiter, R. S. Persaud, M. McLoughlin, Z. Fuks, and R. N. Kolesnick. 1994. Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. *J. Exp. Med.* 180:525.
91. Kharbanda, S., Z.-M. Yuan, E. Rubin, R. Weichselbaum, and D. Kufe. 1994. Activation of Src-like p56/p53^{lyn} tyrosine kinase by ionizing radiation. *J. Biol. Chem.* 269:20739.
92. Schieven, G. L., J. M. Kiriara, D. E. Myers, J. A. Ledbetter, and F. M. Uckun. 1993. Reactive oxygen intermediates activate NF- κ B in a tyrosine kinase-dependent mechanism and in combination with vanadate activate the p56^{lck} and p59^{lyn} tyrosine kinases in human lymphocytes. *Blood* 82:1212.
93. Szumiel, I., B. Sochanowicz, and I. Buraczewska. 1990. Ca²⁺ mobilization is related to the lethal effect of X-irradiation in L5178Y cells. *Int. J. Radiat. Biol.* 58:125.
94. Hallahan, D. E., D. Bleakman, S. Virudachalam, D. Lee, D. Grdina, D. W. Kufe, and R. R. Weichselbaum. 1994. The role of intracellular calcium in the cellular response to ionizing radiation. *Radiat. Res.* 138:392.
95. Todd, D. G., and R. B. Mikkelsen. 1994. Ionizing radiation induces a transient increase in cytosolic free [Ca²⁺] in human epithelial tumor cells. *Cancer Res.* 54:5224.
96. Yoshida, S. 1997. Effects of X-irradiation on the calcium channel of the mouse oocyte. *Life Sci.* 60:1377.
97. Kandasamy, S. B., and A. H. Harris. 1992. Effects of prostaglandins, inositol 1,4,5-trisphosphate, and phorbol esters on radiation-induced decreases in calcium influx in rat brain synaptosomes. *Radiat. Res.* 131:43.
98. Bump, E. A., and J. M. Brown. 1990. Role of glutathione in the radiation response of mammalian cells *in vitro* and *in vivo*. *Pharmac. Ther.* 47:117.
99. Many, A., C. A. Hubel, and J. M. Roberts. 1996. Hyperuricemia and xanthine oxidase in preeclampsia, revisited. *Am. J. Obstet. Gynecol.* 174:288.
100. Saugstad, O. D. 1996. Role of xanthine oxidase and its inhibitor in hypoxia: reoxygenation injury. *Pediatrics* 98:103.

101. Hille, R., and T. Nishino. (1995) Xanthine oxidase and xanthine dehydrogenase. *FASEB J.* 9:995.
102. Chung, S. C., T. V. McDonald, and P. Gardner. 1994. Inhibition by SK&F 96365 of Ca^{2+} current, IL-2 production and activation in T lymphocytes. *British J. Pharmacol.* 113:861.
103. Sen, C. K., S. Roy, and L. Packer. 1996. Involvement of intracellular Ca^{2+} in oxidant-induced NF- κ B activation. *FEBS Lett.* 385:58.
104. Hofer, A. M., C. Fasolato, and T. Pozzan. 1998. Capacitative Ca^{2+} entry is closely linked to the filling state of the internal Ca^{2+} stores: a study using simultaneous measurements of I_{CRAC} and intraluminal $[\text{Ca}^{2+}]$. *J. Cell Biol.* 140:325.
105. Kasid, U., S. Suy, P. Dent, S. Ray, T. L. Whiteside, and T. W. Sturgill. 1996. Activation of Raf by ionizing radiation. *Nature* 382:813.
106. Schmidt-Ullrich, R. K., K. Valerie, P. B. Fogleman, and J. Walters. 1996. Radiation-induced autophosphorylation of epidermal growth factor receptor in human malignant mammary and squamous epithelial cells. *Radiat. Res.* 145:81.
107. Sargeant, P., R. W. Farndale, and S. O. Sage. 1993. ADP- and thapsigargin-evoked Ca^{2+} entry and protein-tyrosine phosphorylation are inhibited by the tyrosine kinase inhibitors genistein and methyl-2,5-dihydroxycinnamate in Fura-2-loaded human platelets. *J. Biol. Chem.* 268: 18151.
108. Lee, K.-M., K. Toscas, and M. L. Villereal. 1993. Inhibition of bradykinin- and thapsigargin-induced Ca^{2+} entry by tyrosine kinase inhibitors. *J. Biol. Chem.* 268:9945.
109. Grönroos, E., A. Schippert, M. Engström, A. Sjölander. 1995. The regulation of leukotriene D_4 -induced calcium influx in human epithelial cells involves protein tyrosine phosphorylation. *Cell Calcium* 17:177.
110. Santana, P., L. A. Peña, A. Haimovitz-Friedman, S. Martin, D. Green, M. McLoughlin, C. Cordon-Cardo, E. H. Schuchman, Z. Fuks, and R. Kolesnick. 1996. Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation -induced apoptosis. *Cell* 86:189.
111. Hannun, Y. A. 1996. Functions of ceramide in coordinating cellular responses to stress. *Science* 274:1855.

112. Nakamura, Y., S. Nakashima, K. Ojio, Y. Banno, H. Miyata, and Y. Nozawa. 1996. Ceramide inhibits IgE-mediated activation of phospholipase D, but not of phospholipase C, in rat basophilic leukemia (RBL-2H3) cells. *J. Immunol.* 156:256.
113. Liu, B., N. Andrieu-Abadie, T., Levade, P. Zhang, L. M. Obeid, and Y. A. Hannun. 1998. Glutathione regulation of neutral sphingomyelinase in tumor necrosis factor- α -induced cell death. *J. Biol. Chem.* 273:11313.
114. Beiqing, L., M. Chen, and R. L. Whisler. 1996. Sublethal levels of oxidative stress stimulate transcriptional activation of c-jun and suppress IL-2 promoter activation in Jurkat T cells. *J. Immunol.* 157:160.
115. Stewart, C. C. and A. P. Carlos. 1976. Effect of irradiation on immune responses. *Radiology.* 118:201.
116. Harrington, N. P., K. A. Chambers, W. M. Ross and L. G. Fillion. 1997. Radiation damage and immune suppression in splenic mononuclear cell populations. *Clin. Exp. Immunol.* 107:417.
117. Walleczek, J. 1994. Immune cell interactions with extremely low frequency magnetic fields: experimental verification and free radical mechanisms. In *On The Nature of Electromagnetic Field Interactions with Biological Systems*, Frey, A., ed. Landes, Austin, TX, pp. 167-180.

IMAGE EVALUATION TEST TARGET (QA-3)

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