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Ryanodine Receptor-Mediated Rapid Increase in Intracellular Calcium Induced by 7,8-Benzo(a)Pyrene Quinone in Human and Murine Leukocytes

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Benzo(a)pyrene (BaP) is an environmentally prevalent polycyclic aromatic hydrocarbon (PAH) known to produce immunotoxicity in murine and human lymphocytes. Previous studies by our lab have shown that certain BaP metabolites increase intracellular Ca^{2+} in human and murine lymphocytes. The mechanism by which these BaP metabolites increase Ca^{2+} may involve src kinase activation and mitochondrial oxidative stress. We have implicated a new pathway of Ca^{2+} elevation in lymphocytes produced by a novel BaP metabolite, BaP-7,8-dione (7,8-BPQ). This ortho quinone is produced from BaP-7,8-dihydrodiol by aldoketoreductase 1C1 (AKR1C) isoforms in human cells. We have previously shown that 7,8-BPQ increases Ca^{2+} levels in an *in vitro* rabbit skeletal muscle sarcoplasmic reticulum (SR) vesicle model via interaction with ryanodine receptors (RyR). In the present study, we found that 7,8-BPQ produced a RyR-dependent rapid increase in intracellular Ca^{2+} in the Daudi human B cell line. However, other BP-diones including 1,6-, 3,6-, and 6,12-BPQs failed to produce a rapid increase in Ca^{2+}. Instead they produced a late increase in intracellular Ca^{2+}, presumably via a redox-cycling-dependent loss of Ca^{2+} buffering capacity by mitochondria. Functional RyR were detected in Daudi using a ^{3}H-ryanodine binding assay. The studies were extended to normal human peripheral blood and murine spleen cells, where it was found that 7,8-BPQ rapidly elevated intracellular Ca^{2+} in B cells and T cells in both species. The Ca^{2+}-elevating effect of 7,8-BPQ was prevented by pretreatment with a high concentration of ryanodine (500 μM). Collectively, these results demonstrate a novel mechanism of Ca^{2+} elevation by an environmentally relevant metabolite of BaP in murine and human lymphocytes.

Key Words: **ryanodine receptor; calcium; 7,8-benzo(a)pyrene quinone.**

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants known to be carcinogenic and immunotoxic in humans and animals (Burchiel and Luster, 2001). The immunotoxicity of PAHs is likely due to a combination of genotoxic and nongenotoxic mechanisms, such as alterations in signaling and oxidative stress pathways. Benzo(a)pyrene (BaP), a prototypic PAH, has been widely investigated for many years. Certain metabolites of BaP, most notably BaP-7,8-diol, 9,10-epoxide (BPDE), formed by BaP bioactivation with cytochrome P450 1B1/1A1 (CYP1B1/1A1) and microsomal epoxide hydrolase (EPHX1), appear to be important in immunotoxicity. Our previous studies have shown that BPDE alters cell signaling and Ca^{2+} homeostasis in lymphoid and nonlymphoid cells (Burchiel and Luster, 2001; Davila *et al*., 1995; Mounho *et al*., 1997; Tannheimer *et al*., 1997, 1999; Zhao *et al*., 1996). BPDE and other PAHs may act via direct or indirect activation of protein tyrosine kinases that are associated with cell signaling through T- and B-cell antigen receptors (Archuleta *et al*., 1993; Davila *et al*., 1999; Mounho and Burchiel, 1998).

Calcium is an important second messenger that regulates various biological functions in cells (Berridge, 2001). Calcium signaling in lymphocytes and other cells is influenced by such properties as the duration, intensity, or frequency of oscillations (Lewis, 2003). We have found that several BaP metabolites alter Ca^{2+} signaling in Daudi human B cells, albeit by different mechanisms. Cytochrome P450 activation of BaP appears to be critical for immunotoxicity to human peripheral blood cells (Davila *et al*., 1996). Our previous studies also found that BaP-7,8-diol alters intracellular calcium levels after several hours of treatment of normal human peripheral blood mononuclear cells (HPBMC) (Mounho and Burchiel, 1998). Using Daudi human B cells as an *in vitro* model, we found increases in Ca^{2+} levels through activation of Src-like protein tyrosine kinases (Mounho and Burchiel, 1998). Recently, we found that quinones of BaP (BPQs) also signal Ca^{2+}, but through several different pathways. BP-quinones, including 1,6-BPQ, 3,6-BPQ, and 6,12-BPQ, are formed through cytochrome P450,

The authors certify that all research involving human subjects was done under full compliance with all government policies and the Helsinki Declaration.

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peroxidase, and photochemical (UV) reactions (Reed *et al*., 2003). BPQs are known to redox-cycle, leading to oxidative stress and energy depletion in mitochondria followed by loss of Ca^{2+} buffering capacity in cells (Zhu *et al*., 1995). More recently we discovered another potential mechanism of Ca^{2+} elevation in cells due to a unique interaction of an ortho-BPQ (7,8-BPQ) with ryanodine receptors (RyR) (Pessah *et al*., 2001). In these studies, we found that 7,8-BPQ causes Ca^{2+} release in rabbit skeletal muscle sarcoplasmic reticulum (SR) vesicles through a selective interaction with RyR.

RyR are homotetrameric calcium release channels of intracellular stores, which were first described on the sarcoplasmic reticulum (SR) in mammalian skeletal muscle cells and cardiac muscle cells (Fill and Copello, 2002; McPherson and Campbell, 1993). More recently, RyR have been found on the endoplasmic reticulum of nonexcitable cells (Sei *et al*., 1999). Mammalian RyRs are expressed as three widely distributed isoforms: RyR1, RyR2, and RyR3. Recent studies have shown that RyR1 and RyR2 are expressed in nonexcitable cells, such as lymphocytes (Sei *et al*., 1999). RyR3 is widely expressed in many types of cells (Ogawa *et al*., 2000). To investigate the role of RyR, ryanodine binding, which occurs only with the open channel, is frequently used as a probe of RyR function (Pessah *et al*., 1986). Low concentrations of ryanodine (~1 μM) activate RyRs, whereas high concentrations (~100 μM) irreversibly inhibit RyR calcium release.

Based on previous studies of calcium responses produced by BPQs and other BaP metabolites in lymphoid and nonlymphoid cells, as well as rabbit muscle SR vesicles, we hypothesized that 7,8-BPQ would selectively (as compared to other BPQs) elevate intracellular Ca^{2+} in human and murine B and T cells via a RyR-dependent mechanism. In this report, we utilized Daudi human B cells, HPBMC, and murine spleen cells to analyze the immediate and delayed Ca^{2+} responses following exposure to 7,8-BPQ and various other BPQ and BaP metabolites. Our data show that only 7,8-BPQ produces a significant rapid increase in intracellular Ca^{2+}. We found that this increase in Ca^{2+} was blocked by high concentrations of ryanodine. Therefore, we conclude that Ca^{2+} elevation by 7,8-BPQ in human and murine lymphoid cells is due to activation of RyR.

MATERIALS AND METHODS

Reagents and antibodies. Benzo(a)pyrene (BaP, Sigma Cat. No. B1760) (purity >97%) and ryanodine (purity >95%) (Sigma Cat. No. R6017) were obtained from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO). All BaP metabolites used in this paper were purchased from Midwestern Research Institute (Kansas City, MO), with the exception of 7,8-beno(a)pyrene quinone (7,8-BPQ) that was provided by the Penning laboratory. Tissue-culture grade (purity >95%) dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO) was used to dissolve and dilute BaP and its metabolites, and the final concentration of DMSO used was adjusted to 0.1% (v/v). Fluo-3/AM was purchased from Molecular Probes (Eugene, OR). Fico/Lite LymphoH (Cat. No. 140150) was purchased from Atlanta Biological (Lawrenceville, GA). Phycoerythrin (PE)-conjugated mouse anti-human CD3 and mouse anti-human CD19 monoclonal antibodies were from BD Pharmingen (San Diego, CA).

Cell line. The Daudi human B cell line was obtained from American Type Culture Collection (Rockville, MD) and was grown in a humidified, 37°C, 5% CO_2 incubator in complete RPMI 1640 medium containing 20% fetal bovine serum (Hyclone, Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin (Cambrex), and 2 mM L-glutamine.

Preparation of human peripheral blood mononuclear cells (HPBMC). Human mononuclear cells were isolated from healthy blood donors under an approved IRB protocol using Fico/Lite LymphoH density gradient centrifugation method. Briefly, collected peripheral blood was placed in 6 × 10 ml Vacutainer® plasma tubes with heparin (VWR Cat. No.VT6480). Heparinized blood was diluted with equal volume of sterile phosphate-buffered saline (PBS) (without calcium and magnesium salts) (Sigma Chemical Co., St. Louis, MO), and then 30 ml of this mixture was overlayed on 15 ml Fico/Lite LymphoH at room temperature (RT). After density gradient centrifugation for 30 min at 200 × *g* without brake at RT, peripheral blood mononuclear cells were collected from the white layer between the plasma fraction and the Fico/Lite LymphoH fraction. The HPBMC were washed twice with PBS, and the cell pellet was then resuspended in 3 ml complete RPMI 1640 medium containing 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin (Cambrex), and 2 mM L-glutamine. Cells were counted and adjusted to 20×10^6 cells/ml.

Preparation of mouse spleen cells. Spleens from female wild-type C57BL/6N mice (13–14 weeks old) (Harlan laboratories, Indianapolis, IN) were aseptically removed and were dissociated in RPMI 1640 complete medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 unit/ml penicillin, and 2 mM L-glutamine. Cells were centrifuged at 280 × *g* for 10 min, and pellets were resuspended and maintained in 2 ml RPMI 1640 complete medium on ice. The nucleated spleen cells were counted using the trypan blue (Sigma Chemical Co., St. Louis, MO) exclusion method.

Intracellular calcium measurement using flow cytometry. The analysis of intracellular Ca^{2+} levels in Daudi human B cells, HPBMC, and mouse spleen cells was performed by flow cytometric methods (Burchiel *et al.*, 1999, 2000; Rethi *et al.*, 2002). For all experiments, cells were loaded with in 200 μl of 2 μM Fluo-3/AM (Molecular Probes) in FACS tubes and incubated at 37°C, 5% CO_2 for 1 h, with cells being resuspended approximately every 20 min to ensure even dye loading. Then 800 μl of Dulbecco's phosphate buffered saline (DPBS) (with calcium and magnesium) were added to yield a 1 ml solution. Ca^{2+} responses were observed in two ways: (1) For rapid real-time analysis, Fluo-3 loaded cells were treated with various PAHs and continuously monitored for up to 30 min. (2) For longer incubations periods (1 h or more), after treatment with PAHs, cells were loaded with Fluo-3, and the fluorescence intensity was measured by flow cytometry. For Daudi human B cell experiments, cells were resuspended in complete RPMI medium at 2×10^5 cells/ml, and 1-ml cell aliquots were placed into three 12 × 75 mm tubes. After the centrifugation and aspiration, the cells were loaded with Fluo3. For HPBMC studies, 10 μl of 20×10^6 cells/ml HPBMC were loaded with Fluo-3 and stained with PE-conjugated mouse anti-human CD3 or mouse anti-human CD19 to label T or B cells, respectively. For murine spleen cell studies, 10 μl of 20×10^6 cells/ml murine spleen cells were put in 12 × 75 mm tubes and loaded with the addition of 200 μl of Fluo-3AM. After 1 h, 800 μl of DPBS solution was added to yield 2×10^5 cells/ml. Tubes were capped and covered with aluminum foil to transport to Flow Cytometry Facility for analysis. Fluorescence intensities were measured in the resuspended cells by FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) to obtain baseline readings. Mean channel fluorescence intensities were calculated using CellQuest software.

Measurement of ^{3}H-ryanodine binding. High affinity binding of ^{3}H-ryanodine (57 Ci/mmol; New England Nuclear, Boston, MA) to crude microsomal membrane extracts (0–360 μg/ml) from cultured Daudi cells was performed in the presence of 500 mM KCl, 20 mM HEPES, 100μM $CaCl_2$, 1 mM DTT, 100 μM PMSF, 100 μg/ml BSA, 1 μg/ml leupeptin, pH 7.4, and

10 nM ^{3}H-ryanodine (Pessah *et al.*, 1987). The binding reaction was initiated by the addition of microsomal membranes to the medium, and the mixture was permitted to equilibrate at 37°C for 2 h. Nonspecific binding was assessed in the presence of 10 μM unlabeled ryanodine. Separation of bound and free ligand was performed by rapid filtration through Whatman GF/B glass fiber filters using a Brandel cell harvester (Gaithersburg, MD). Filters were washed with two volumes of 0.5 ml ice-cold wash buffer containing 20 mM Tris–HCl, 250 mM KCl, 15 mM NaCl, 50 μM $CaCl_2$, pH 7.1, and placed into vials with 5 ml scintillation cocktail (Ready Safe; Beckman Instruments, Inc., Fullerton, CA). The ^{3}H-ryanodine remaining on the filters was quantified by liquid scintillation spectrometry.

Statistical design and analysis. All of the data reported in this paper were analyzed by SigmaStat software (SPSS, Point Richmond, CA). The statistical differences were determined by one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test, Dunnett's test and Student's *t*-test. A *p*-value of ≤ 0.05 between DMSO control and PAH-treated samples was considered significant.

RESULTS

Immediate Ca^{2+} Response Produced by 7,8-BPQ in Daudi Human B Cells

The immediate calcium responses produced by BaP and various metabolites were evaluated using real-time flow cytometry. As shown in Figure 1A, 7,8-BPQ produced a statistically significant increase in intracellular Ca^{2+} in Daudi human B cells within 6 min. Neither BaP nor any of the other BaP metabolites studied produced such rapid increase in Ca^{2+}, including 1,6-BPQ, 3,6-BPQ, 6,12-BPQ, and BPDE. The Ca^{2+} response produced by 7,8-BPQ in Daudi was continually measured for 30 min (Figure 1B). A 1 μM concentration of 7,8-BPQ increased intracellular Ca^{2+} levels with a time to peak of 10 min. The Ca^{2+} level then declined during the next 20 min. DMSO control samples did not produce any measurable effect on intracellular Ca^{2+} response. This result shows that 7,8-BPQ produces a rapid increase of intracellular free Ca^{2+} in Daudi human B cells.

BaP Metabolites Induce Intracellular Ca^{2+} Elevation at Different Time Points

To further investigate the Ca^{2+} effect by BaP and various metabolites, Daudi human B cells were treated with 1 μM of various PAHs for 30 min, 2, 4, 8, and 18 h. Treated cells were loaded with Fluo-3/AM and incubated at 37°C for 1 h. 3,6-BPQ increased Ca^{2+} within 2 h, BPDE increased Ca^{2+} within 4 h, whereas, 1,6-BPQ and 6,12-BPQ increased Ca^{2+} at later time points (8–18 h) (Fig. 2A). 7,8-BPQ did not show any Ca^{2+} effect at these late time points. These results suggest that increases in intracellular Ca^{2+} by BaP metabolites may be associated with different metabolic pathways and mechanisms. BaP itself does not possess significant Ca^{2+}-elevating activity at early time points in Daudi human B Cells. After an 18 h exposure to 1 μM PAH treatments, BPDE, 1,6-BPQ, 3,6-BPQ, and 6,12-BPQ significantly increased intracellular Ca^{2+} in Daudi cells (Fig. 2B). We noted that 3,6-BPQ fluorescence (no Fluo-3 loading) in the green channel was significantly higher than DMSO controls, and therefore controls must be run with and without the Ca^{2+} dye to correct for the fluorescence produced by the test compound; however, none of the other PAHs demonstrated significant fluorescence at the wavelength pairs used to measure Fluo-3 (not shown). Therefore, only background fluorescence associated with 3,6-BPQ needed to be corrected for analysis with Fluo-3.

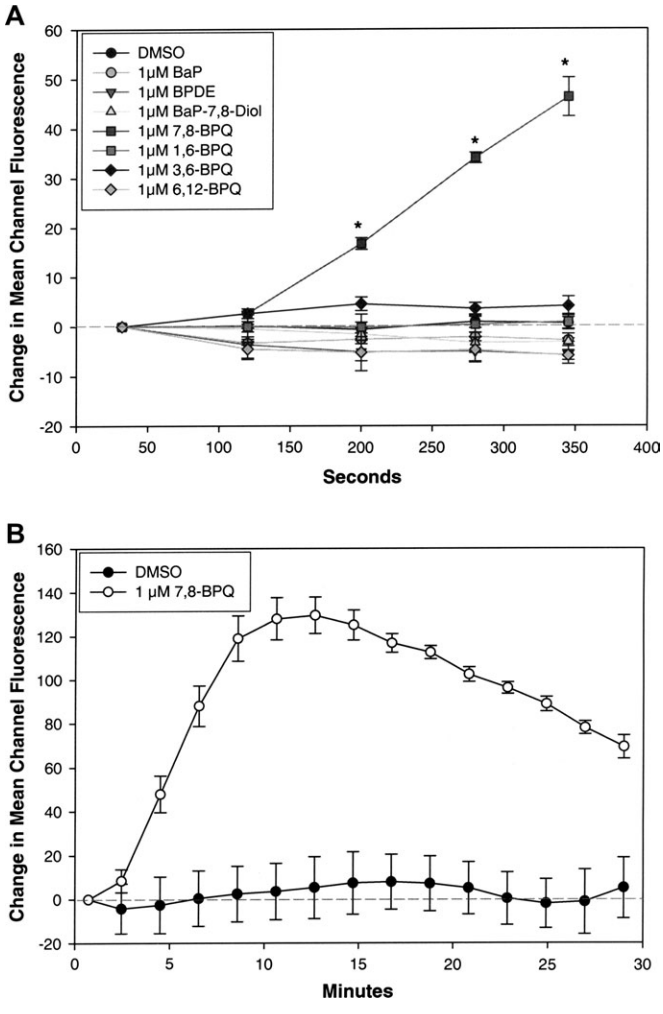

FIG. 1. Flow cytometric analysis of the rapid calcium release by BaP-quinones in Daudi Human B cells. Daudi human B cells (2×10^5 cells) were loaded in 200 μl, 2 μM Fluo-3/AM at 37°C, 5% CO_2 for 1 h; 800 μl complete RPMI medium was then added to yield 2×10^5 cells/ml. Fluorescence intensities were measured in real-time using a FACSCalibur. (A) After loading with Fluo-3, Daudi cells were dosed with 1 μM PAHs, and the fluorescence intensities were monitored for 6 continuous min. (B) after the baseline measurement, Daudi cells were treated with 1 μM BaP-7,8-quinone (7,8-BPQ), and measurement of the change of fluorescence intensities continued for 30 min. Mean channel fluorescence intensities were calculated by CellQuest software. The plots represent the mean ± standard error of triplicate measurements. Asterisks (*) indicate statistically significant differences from the DMSO vehicle control ($p < 0.05$ by Dunnett's test).

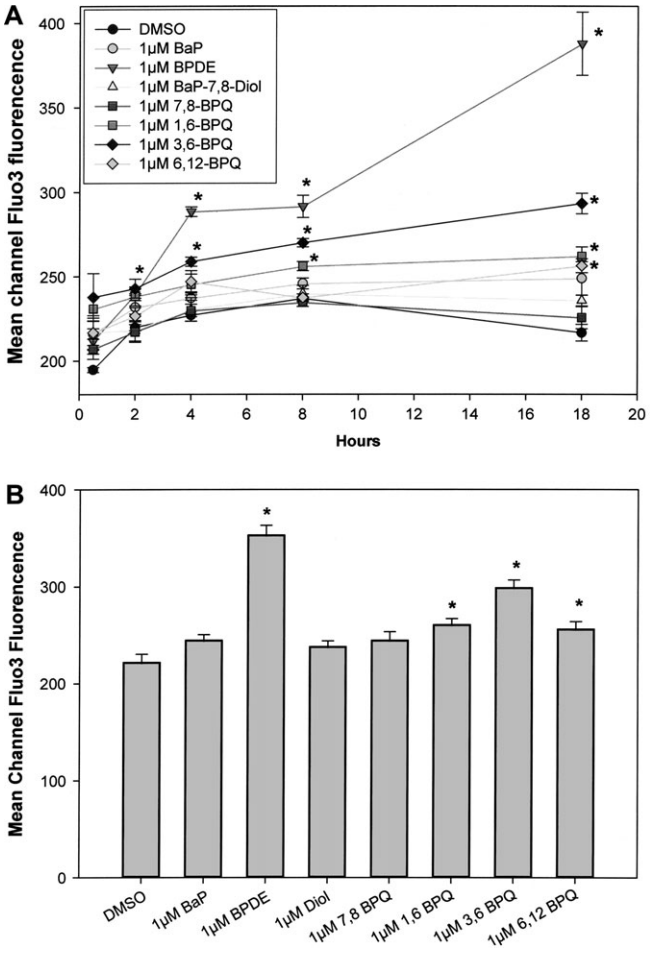

FIG. 2. Effect of PAHs on Ca^{2+} responses in Daudi cells at later time points. Changes in intracellular Ca^{2+} measured at different time points after treatment with PAHs. (A) Daudi B cells were treated with 1 μM various PAHs for 30 min, 2, 4, 8, and 18 h. Treated cells were then pelleted and resuspended in complete medium containing 2 μM Fluo-3 and incubated at 37°C for 1h. The flow cytometric method was used to measure the change of Fluorescence. (B) Change in intracellular Ca^{2+} produced by BaP and its various metabolites measured at 18 h by flow cytometry. There was no significant cell death, as assessed using propidium iodide, following 18 h treatment of Daudi human B cells with 1 μM BPQs.

Detection of RyRs in Daudi Human B Cells

Because ryanodine specifically binds RyR in the open state, high-affinity binding of ^{3}H-ryanodine can be used to verify the expression of functional RyR (Pessah *et al*., 1986). Figure 3 shows the specific binding of ^{3}H-ryanodine to Daudi microsomal membranes. RyRs are large (~560 kDa) homotetrameric receptors that mediate Ca^{2+} release from the endoplasmic reticulum stores. Our previous studies show that 7,8-BPQ induces RyR-mediated Ca^{2+} release from microsomal fractions (Pessah *et al*., 2001). Thus, this result, coupled with our Ca^{2+} studies above, demonstrates that functional RyRs are expressed in human lymphocytes.

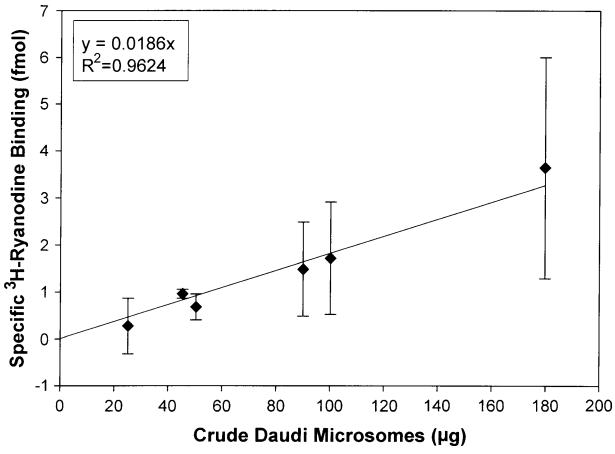

FIG. 3. ^{3}H-Ryanodine binding to Daudi microsomes. Daudi microsomes were incubated with ^{3}H-ryanodine as described in Materials and Methods. Data were fit by linear regression ($r^2 = 0.9624$).

The Immediate Calcium Response Induced by 7,8-BPQ Is Inhibited by Ryanodine in Daudi Cells

Previous investigators found that RyRs are expressed in Jurkat T-lymphocytes (Hohenegger *et al*., 1999) and play an important role in the regulation of intracellular Ca^{2+}. To test whether RyRs are responsible for 7,8-BPQ-induced Ca^{2+} elevation, we used ryanodine (50 and 100 μM) to specifically block the RyRs. As reported earlier, high concentrations of ryanodine (>100 μM) irreversibly inhibit RyR-mediated Ca^{2+} release (Zimanyi *et al*., 1992). Daudi human B cells were pretreated with 100 μM ryanodine and loaded with the Fluo-3 dye for 1h. PAH treatments were added after the baseline reading, and samples were monitored for 20 min by flow cytometry (Fig. 4). The intracellular Ca^{2+} level in the 100 μM

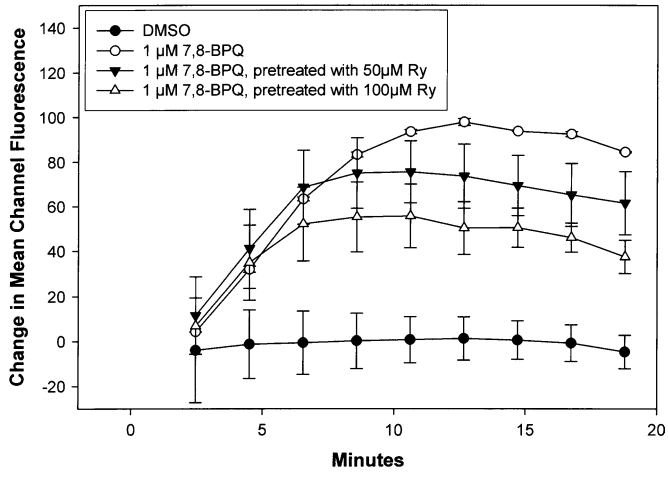

FIG. 4. The effect of ryanodine on Daudi human B cells. Daudi cells were pretreated for 1 h with 50 μM or 100 μM ryanodine (Ry) and were then loaded with Fluo-3/AM dye. Immediate intracellular Ca^{2+} was measured by flow cytometry as described in Materials and Methods. Results are shown as the change in Mean Channel Fluorescence for triplicate samples ± SEM.

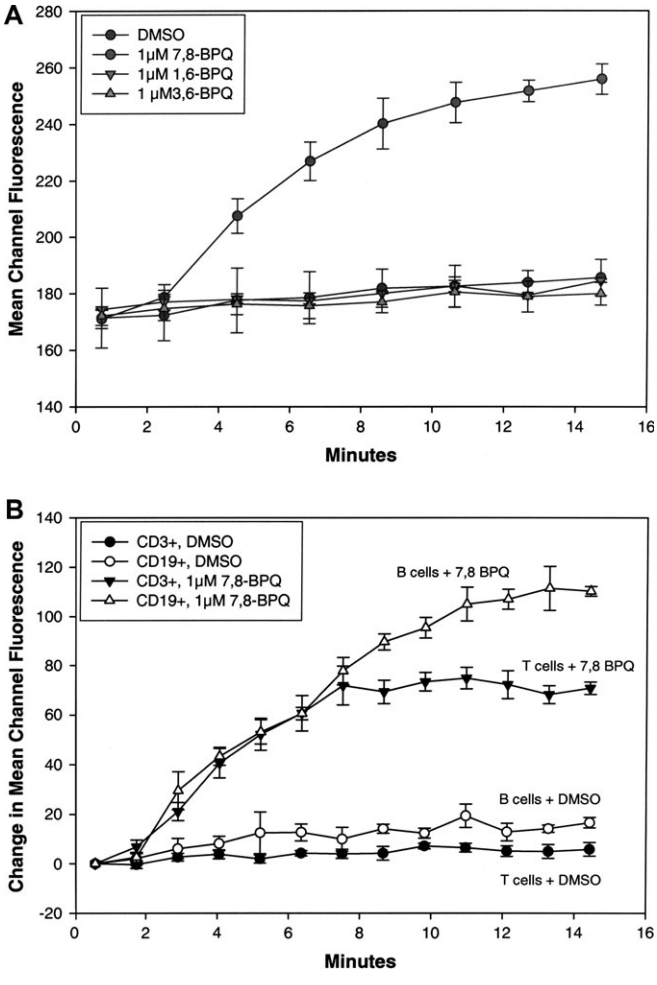

FIG. 5. Rapid intracellular Ca^{2+} response in HPBMC. (A) HPBMC were isolated from healthy donors by Ficoll-hypaque method as described in Material and Methods. Cells were loaded with Fluo-3/AM dye for 1 h. After the baseline reading, HPBMC were treated by 7,8-BPQ, 1,6BPQ, 3,6BPQ, and DMSO control. Results are shown as the Mean Channel Fluorescence. (B) Surface-marker-defined T cells, B cells, and monocytes in HPBMC were treated with 7,8-BPQ and DMSO control. The immediate intracellular Ca^{2+} response was continuously monitored for 15 min. Results are shown as the change in Mean Channel Fluorescence ± SEM. The numbers shown in this figure were the averages of triplicate determinants.

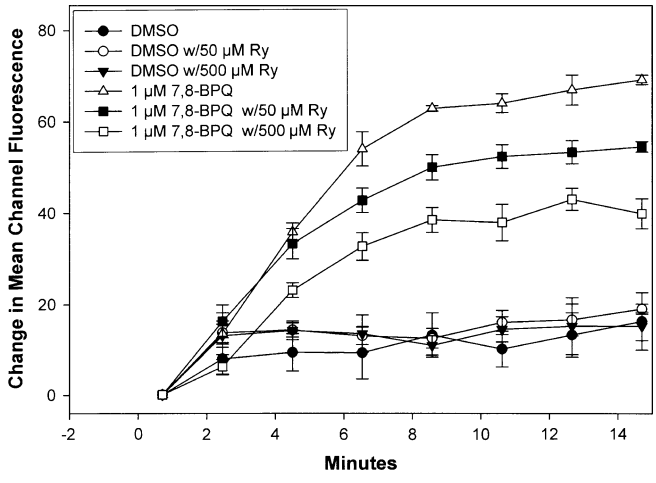

FIG. 6. HPBMC pretreatment with ryanodine to block RyRs. HPBMC were pretreated for 1 h with 50 μM or 500 μM ryanodine (Ry) and loaded with Fluo-3/AM dye at 37°C in a 5% CO_2 incubator. Immediate intracellular Ca^{2+} responses were monitored in real time by flow cytometry as described in Methods.

ryanodine-treated group was significantly decreased compared with non-ryanodine-treated control group. This finding suggests that RyRs regulate the rapid Ca^{2+} response of Daudi to 7,8-BPQ.

Immediate Calcium Response in HPBMC

To investigate the immediate Ca^{2+} response in normal HPBMC, freshly isolated HPBMC from healthy blood donors were treated with 1,6-BPQ, 3,6-BPQ, 7,8-BPQ, or DMSO *in vitro*. We observed that the 7,8-BPQ increase in intracellular Ca^{2+} was maximal at about 10 min, which was a result quite similar to that obtained with Daudi (Fig. 5A). Unlike Daudi, however, the rise in intracellular Ca^{2+} in HPBMC was maintained for much longer periods of times (data not shown). Because HPBMC are composed of both T and B lymphocytes, we wondered whether these cells have the same intracellular Ca^{2+} response to 7,8-BPQ. Mouse anti-human CD3 and CD19 monoclonal antibodies were used to study the human T- and B-cell subsets. Results showed that 7,8-BPQ significantly increased intracellular Ca^{2+} in both cell populations (Fig. 5B). Interestingly, the Ca^{2+} response to 7,8-BPQ in B cells appeared to be higher than in T cells.

To examine the role of the RyR in the 7,8-BPQ-induced immediate Ca^{2+} response, HPBMC were pretreated with 50 μM or 500 μM ryanodine for 1 h. We observed that both concentrations of ryanodine reduced the Ca^{2+} response produced by 7,8-BPQ (Fig. 6). Inhibition of the Ca^{2+} response was not complete, however, suggesting that other mechanism may also be involved in the response to 7,8-BPQ.

7,8-BPQ Produces Immediate Ca^{2+} Response in Mouse Splenocytes

To further extend our findings in HPBMC to murine lymphocytes, we investigated the response of mouse spleen cells to 7,8-BPQ. As shown in Figure 7A, the immediate Ca^{2+} increase was only observed in the 7,8-BPQ-treated group of spleen cells. The mouse spleen cell response (~80 channel fluorescence shift) was somewhat lower than that seen in HPBMC (over 120 channel fluorescence shift). 1,6-BPQ and 3,6-BPQ treatments did not produce significant changes in intracellular Ca^{2+} in spleen cells compared with DMSO control. To determine if this Ca^{2+} increase was mediated by RyR, we pretreated murine spleen cells with different concentrations of ryanodine (50 μM or 500 μM) for 1 h. At the 500 μM

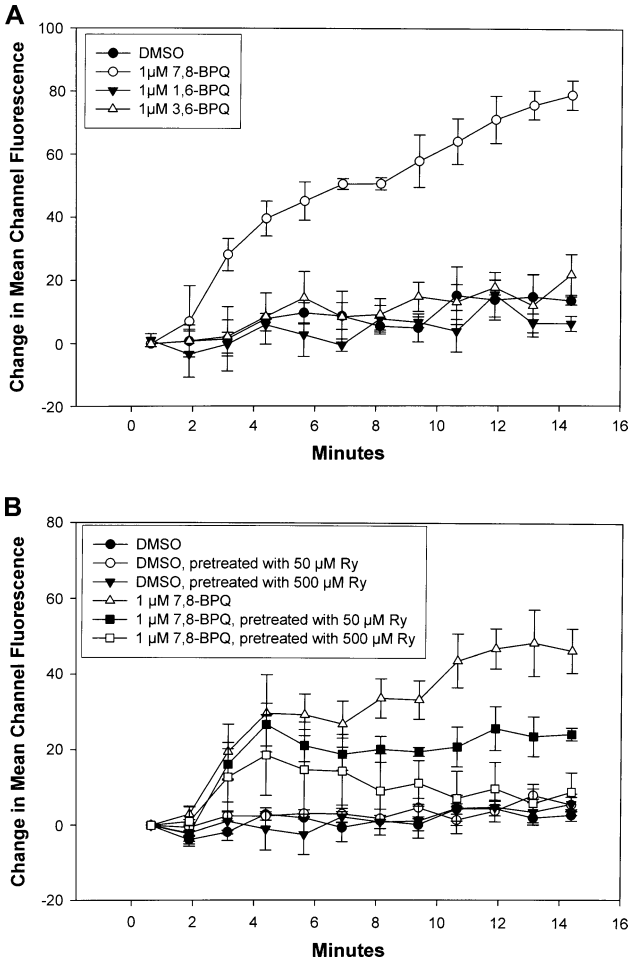

FIG. 7. The immediate intracellular Ca^{2+} elevation in murine splenocytes. Mouse spleens were aseptically removed and single cell suspensions were prepared in complete RPMI 1640 medium as described under Material and Methods. Cells were loaded with Fluo-3/AM dye for 1 h. After the baseline reading, murine splenocytes were treated with 7,8-BPQ in DMSO, with DMSO vehicle serving as a control. (A) The rapid Ca^{2+} response to 7,8-BPQ, 1,6-BPQ, and 3,6-BPQ was measured continuously for 15 min. (B) Experiments were performed as in (A), except cells were pretreated with 50 or 500 μM ryanodine (Ry). Results are shown as the change in Mean Channel Fluorescence ± SEM for triplicate samples.

concentration of ryanodine, we found that Ca^{2+} elevation was significantly reduced in mouse spleen cells (Fig. 7B). These results strongly suggest that murine splenocytes also utilize RyR in response to 7,8-BPQ to cause a rapid Ca^{2+} signal.

DISCUSSION

Our laboratory is interested in the mechanisms by which BaP and other environmental polycyclic aromatic hydrocarbons (PAHs) produce immunomodulation and immunotoxicity. It is known that BaP can be metabolized by cytochrome P450 and other enzymes to produce bioactive metabolites (Burchiel and Luster, 2001). These bioactive metabolites include 1,6-BPQ, 3,6-BPQ, 6,12-BPQ, 7,8-BPQ, BaP-7,8-Diol, and BaP-7,8-Diol-9,10-epoxide (BPDE). In the present study we evaluated BaP and six metabolites for their ability to alter Ca^{2+} levels in Daudi human B cells, human peripheral blood mononuclear cells (HPBMC), and murine splenocytes using flow cytometry. 7,8-BPQ is a particularly interesting BaP metabolite that is derived from the metabolism of BaP-7,8-dihydrodiol under the influence of a dihydrodiol dehydrogenase (DD1), recently identified as AKR1C1 (Burczynski *et al*., 1998, 1999). We have previously shown that 7,8-BPQ produces a rapid increase in Ca^{2+} by activation of ryanodine receptors (RyR) in a rabbit skeletal muscle SR vesicle model system (Pessah *et al*., 2001). Concentrations of 0.1–1 μM 7,8-BPQ were required in our studies to elevate intracellular Ca^{2+}, which may be at the high end of environmentally relevant concentrations. However, we have no accurate way to determine the actual *in vitro* concentration of 7,8-BPQ, and it is well known that this agent extensively binds to proteins and other macromolecules, suggesting that the effective concentration may be far less.

Our previous studies demonstrated that two P450-derived metabolites of BPDE and BaP-7,8-Diol increased tyrosine phosphorylation of Lyn and Syk (Mounho and Burchiel, 1998). This phosphorylation is known to result in the activation of $PLC_{\gamma 1}$ and $PLC_{\gamma 2}$ leading to cleavage of PIP_2 and release of inositol-1,4,5-trisphosphate (IP_3) (Winslow *et al*., 2003). IP_3-sensitive Ca^{2+} release receptors are located on the ER in lymphocytes. The current studies also demonstrated the presence of ryanodine receptors on microsomes (ER fraction) obtained from Daudi human B cells. Thus, there are apparently two closely related Ca^{2+} channels that control Ca^{2+} release from the ER of lymphoid cells, the IP_3R and RyR (Grafton and Thwaite, 2001). It has been proposed that IP_3 induces a small amount of Ca^{2+} release from ER, which then triggers a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism (Fill and Copello, 2002). RyR are also activated by increased cytosolic Ca^{2+} (Pessah *et al*., 2001). Therefore, RyR may play an important role in lymphocytes to induce or maintain Ca^{2+} signaling responses.

The present study demonstrated that 7,8-BPQ produces a rapid increase in intracellular Ca^{2+} in Daudi human B cells, human peripheral blood B and T cells, and murine spleen cells. By contrast, the Ca^{2+} response of other BaP metabolites, including 1,6-BPQ, 3,6-BPQ, and BPDE, occurred at later time points (2–18 h). Therefore, our data suggest that 7,8-BPQ utilizes different signaling pathways to elicit early Ca^{2+} effects. Because we detected the presence of RyRs in Daudi microsomes, and we found that high concentrations of ryanodine prevented the 7,8-BPQ Ca^{2+} response in Daudi human B cells, human peripheral blood B and T cells, and murine spleen cells, we believe that RyR are responsible for the Ca^{2+} response of lymphoid cells to 7,8-BPQ. In our previous work discussed above with BaP-7,8-Diol, we presumed that BaP-7,8-diol was converted to BPDE to produce its effects on src kinases.

However, we now know that Daudi and other human lymphocytes express the AKR1C1, and thus they have the ability to make the 7,8-BPQ. Other BPQs also increase intracellular Ca^{2+}, but we believe that these much later effects are caused by redox-cycling and loss of Ca^{2+} buffering capacity by mitochondria due to ATP depletion, although this hypothesis was not tested in our current studies (Zhu *et al.*, 1995). Because we found that 7,8-BPQ induces rapid increases in intracellular Ca^{2+} levels in human and murine B and T cells, we are now examining pathways for endogenous 7,8-BPQ formation, as well as the regulation of the human AKR1C1 gene in human cells and its homologue in mice.

In summary, we found that 7,8-BPQ is a potent and specific activator of RyR in the present studies leading to a rapid rise in intracellular Ca^{2+} in human and murine B and T cells. We believe that the early and rapid Ca^{2+} response produced by 7,8-BPQ may be immunomodulatory and may play a role in the immunotoxicity induced by BaP. BPQs have previously been found to activate murine splenic T cells following *in vitro* treatment at concentrations of 0.1 and 1 μM (Burchiel *et al*., 2004), although human leukocytes are inhibited following *in vitro* treatment with BPQs. Ca^{2+} elevation by 7,8-BPQ may also play a role in immune activation, in that many Ca^{2+}-sensitive pathways regulate cell activation, such as adhesion molecular activation, enzymatic activation, exocytosis, migration and response to other ion channels activation (Panyi *et al*., 2004). Changes in intracellular Ca^{2+} are also likely important in specific gene regulation, perhaps through PKCs (Lewis, 2003). Pomorski *et al*. (2004) found that intracellular calcium changes regulate cytokine expression, such as IL-1β, and IL8, and effect the monocytes migration. However, this calcium-regulated specific gene expression occurs at relatively late time points. Thus, the early and rapid increase in Ca^{2+} produced by 7,8-BPQ suggests that RyR may play an important role in lymphocyte activation and may be a target of environmental agents.

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REFERENCES

Archuleta, M. M., Schieven, G. G., Ledbetter, J. J., Deanin, G. G., and Burchiel, S. S. (1993). 7,12-Dimethyl-benz[a]anthracene activates protein-tyrosine kinases Fyn and Lck in the HPB-ALL human T-cell line and increases tyrosine phosphorylation of phospholipase C-gamma 1, formation of inositol 1,4,5-trisphosphate, and mobilization of intracellular calcium. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 6105–6109.

Berridge, M. J. (2001). The versatility and complexity of calcium signalling. *Novartis Found. Symp.* **239,** 52–64; discussion 64–67, 150–159.

Burchiel, S. W., Edwards, B. S., Kuckuck, F. W., Lauer, F. T., Prossnitz, E. R., Ransom, J. T., and Sklar, L. A. (2000). Analysis of free intracellular calcium by flow cytometry: Multiparameter and pharmacologic applications. *Methods* **21,** 221–230.

Burchiel, S. W., Lauer, F. T., Gurulé, D., Mounho, B. J., and Salas, V. M. (1999). Uses and future applications of flow cytometry in immunotoxicity testing. *Methods* **19,** 28–35.

Burchiel, S. S., Lauer, F. F., McDonald, J. J., and Reed, M. M. (2004). Systemic immunotoxicity in AJ mice following 6-month whole body inhalation exposure to diesel exhaust. *Toxicol Appl. Pharmacol.* **196,** 337–345.

Burchiel, S. W., and Luster, M. I. (2001). Signaling by environmental polycyclic aromatic hydrocarbons in human lymphocytes. *Clin. Immunol.* **98,** 2–10.

Burczynski, M. M., Harvey, R. R., and Penning, T. T. (1998). Expression and characterization of four recombinant human dihydrodiol dehydrogenase isoforms: Oxidation of trans-7, 8-dihydroxy-7,8-dihydrobenzo[a]pyrene to the activated o-quinone metabolite benzo[a]pyrene-7,8-dione. *Biochemistry* **37,** 6781–6790.

Burczynski, M. M., Lin, H. H., and Penning, T. T. (1999). Isoform-specific induction of a human aldo-keto reductase by polycyclic aromatic hydrocarbons (PAHs), electrophiles, and oxidative stress: Implications for the alternative pathway of PAH activation catalyzed by human dihydrodiol dehydrogenase. *Cancer Res.* **59,** 607–614.

Davila, D. D., Davis, D. D., Campbell, K., Cambier, J. J., Zigmond, L. L., and Burchiel, S. S. (1995). Role of alterations in Ca(2+)-associated signaling pathways in the immunotoxicity of polycyclic aromatic hydrocarbons. *J. Toxicol. Environ. Health* **45,** 101–126.

Davila, D. D., Lane, J. J., Lauer, F. F., and Burchiel, S. S. (1999). Protein tyrosine kinase activation by polycyclic aromatic hydrocarbons in human HPB-ALL T cells. *J. Toxicol. Environ. Health* **56,** 249–261.

Davila, D. D., Romero, D. D., and Burchiel, S. S. (1996). Human T cells are highly sensitive to suppression of mitogenesis by polycyclic aromatic hydrocarbons and this effect is differentially reversed by alpha-naphthoflavone. *Toxicol. Appl. Pharmacol.* **139,** 333–341.

Fill, M., and Copello, J. A. (2002). Ryanodine receptor calcium release channels. *Physiol. Rev.* **82,** 893–922.

Grafton, G., and Thwaite, L. (2001). Calcium channels in lymphocytes. *Immunology* **104,** 119–126.

Hohenegger, M., Berg, I., Weigl, L., Mayr, G. W., Potter, B. V., and Guse, A. H. (1999). Pharmacological activation of the ryanodine receptor in Jurkat T-lymphocytes. *Br. J. Pharmacol.* **128,** 1235–1240.

Lewis, R. S. (2003). Calcium oscillations in T-cells: Mechanisms and consequences for gene expression. *Biochem. Soc. Trans.* **31,** 925–929.

McPherson, P. S., and Campbell, K. P. (1993). The ryanodine receptor/Ca^{2+} release channel. *J. Biol. Chem.* **268,** 13765–13768.

Mounho, B. J., and Burchiel, S. W. (1998). Alterations in human B cell calcium homeostasis by polycyclic aromatic hydrocarbons: Possible associations with cytochrome P450 metabolism and increased protein tyrosine phosphorylation. *Toxicol. Appl. Pharmacol.* **149,** 80–89.

Mounho, B. J., Davila, D. R., and Burchiel, S. W. (1997). Characterization of intracellular calcium responses produced by polycyclic aromatic hydrocarbons in surface marker-defined human peripheral blood mononuclear cells. *Toxicol. Appl. Pharmacol.* **145,** 323–330.

Ogawa, Y., Kurebayashi, N., and Murayama, T. (2000). Putative roles of type 3 ryanodine receptor isoforms (RyR3). *Trends Cardiovasc. Med.* **10,** 65–70.

Panyi, G., Varga, Z., and Gaspar, R. (2004). Ion channels and lymphocyte activation. *Immunol. Lett.* **92,** 55–66.

Pessah, I. N., Beltzner, C., Burchiel, S. W., Sridhar, G., Penning, T., and Feng, W. (2001). A bioactive metabolite of benzo[a]pyrene, benzo[a]pyrene-7,8-dione, selectively alters microsomal Ca^{2+} transport and ryanodine receptor function. *Mol. Pharmacol.* **59,** 506–513.

Pessah, I. N., Francini, A. O., Scales, D. J., Waterhouse, A. L., and Casida, J. E. (1986). Calcium-ryanodine receptor complex. Solubilization and partial characterization from skeletal muscle junctional sarcoplasmic reticulum vesicles. *J. Biol. Chem.* **261,** 8643–8648.

Pessah, I. N., Stambuk, R. A., and Casida, J. E. (1987). Ca^{2+}-activated ryanodine binding: Mechanism of sensitivity and intensity modulation by Mg^{2+}, caffeine, and adenine nucleotides. *Mol. Pharmacol.* **31,** 232–238.

Pomorski, P., Watson, J. M., Haskill, S., and Jacobson, K. A. (2004). How adhesion, migration, and cytoplasmic calcium transients influence interleukin-1beta mRNA stabilization in human monocytes. *Cell Motil. Cytoskeleton* **57,** 143–157.

Reed, M., Monske, M., Lauer, F., Meserole, S., Born, J., and Burchiel, S. (2003). Benzo[a]pyrene diones are produced by photochemical and enzymatic oxidation and induce concentration-dependent decreases in the proliferative state of human pulmonary epithelial cells. *J. Toxicol. Environ. Health* **66,** 1189–1205.

Rethi, B., Detre, C., Gogolak, P., Kolonics, A., Magocsi, M., and Rajnavolgyi, E. (2002). Flow cytometry used for the analysis of calcium signaling induced by antigen-specific T-cell activation. *Cytometry* **47,** 207–216.

Sei, Y., Gallagher, K. L., and Basile, A. S. (1999). Skeletal muscle type ryanodine receptor is involved in calcium signaling in human B lymphocytes. *J. Biol. Chem.* **274,** 5995–6002.

Tannheimer, S. L., Barton, S. L., Ethier, S. P., and Burchiel, S. W. (1997). Carcinogenic polycyclic aromatic hydrocarbons increase intracellular Ca^{2+} and cell proliferation in primary human mammary epithelial cells. *Carcinogenesis* **18,** 1177–1182.

Tannheimer, S. L., Lauer, F. T., Lane, J., and Burchiel, S. W. (1999). Factors influencing elevation of intracellular Ca^{2+} in the MCF-10A human mammary epithelial cell line by carcinogenic polycyclic aromatic hydrocarbons. *Mol. Carcinog.* **25,** 48–54.

Winslow, M. M., Neilson, J. R., and Crabtree, G. R. (2003). Calcium signaling in lymphocytes. *Curr. Opin. Immunol.* **15,** 299–307.

Zhao, M., Lytton, J., and Burchiel, S. W. (1996). Inhibition of sarcoendoplasmic reticulum calcium ATPases (SERCA) by polycyclic aromatic hydrocarbons: Lack of evidence for direct effects on cloned rat enzymes. *Int. J. Immunopharmacol.* **18,** 589–598.

Zhu, H., Li, Y., and Trush, M. A. (1995). Characterization of benzo[a]pyrene quinone-induced toxicity to primary cultured bone marrow stromal cells from DBA/2 mice: Potential role of mitochondrial dysfunction. *Toxicol. Appl. Pharmacol.* **130,** 108–120.

Zimanyi, I., Buck, E., Abramson, J. J., Mack, M. M., and Pessah, I. N. (1992). Ryanodine induces persistent inactivation of the Ca^{2+} release channel from skeletal muscle sarcoplasmic reticulum. *Molec. Pharmacol.* **42,** 1049–1057.