TOXICOLOGY AND APPLIED PHARMACOLOGY **145,** 202–210 (1997)
ARTICLE NO. TO978175

The Role of Intracellular Calcium in Antimony-Induced Toxicity in Cultured Cardiac Myocytes[1]

H. E. Wey,[2] D. Richards, M. A. Tirmenstein,[3] P. I. Mathias, and M. Toraason

Cellular Toxicology Section, Experimental Toxicology Branch, Division of Biomedical and Behavioral Science, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, 4676 Columbia Parkway, Cincinnati, Ohio 45226

Received October 28, 1996; accepted March 21, 1997

The Role of Intracellular Calcium in Antimony-Induced Toxicity in Cultured Cardiac Myocytes. Wey, H. E., Richards, D., Tirmenstein, M. A., Mathias, P. I., and Toraason, M. (1997). *Toxicol. Appl. Pharmacol.* **145, 202–210.**

Trivalent antimony, delivered as potassium antimonyl tartrate (PAT), has been previously shown to induce an oxidative stress and toxicity in cultured neonatal rat cardiac myocytes. The present study investigates the effect of PAT on intracellular free calcium ($[Ca^{2+}]_i$), which has been implicated in the toxicity of agents inducing oxidative stress, and explores its role in PAT toxicity. Exposure to 50 or 200 μM PAT led to progressive elevation in diastolic or resting $[Ca^{2+}]_i$ and eventually a complete loss of $[Ca^{2+}]_i$ transients that occurred well before cell death as assessed by LDH release. Prior loading of myocytes with the intracellular calcium chelator BAPTA (10 to 40 μM), protected against PAT toxicity in the presence and absence of extracellular calcium, and demonstrated a crucial role for $[Ca^{2+}]_i$ in PAT toxicity. Exposure to 200 μM PAT in the absence of extracellular calcium slightly elevated $[Ca^{2+}]_i$, but only to levels comparable to resting $[Ca^{2+}]_i$ for cells in 1.8 mM extracellular calcium. This demonstrated that although PAT toxicity was dependent on $[Ca^{2+}]_i$, a large increase above resting levels was not needed, and also that some calcium was mobilized from intracellular stores. However, the caffeine-releasable pool of sarcoplasmic reticulum calcium was increased, not depleted, by exposure to 200 μM PAT. These results demonstrate that PAT disrupts $[Ca^{2+}]_i$ handling and support a role for a calcium-dependent event, but do not support the necessity of events in PAT-induced cell death that are mediated by a large elevation in $[Ca^{2+}]_i$.

Exposure to antimony compounds has long been associated with cardiotoxicity through both clinical observations and animal studies. Early animal studies noted cardiac pathology following acute and subchronic exposure to trivalent antimony-containing compounds by intraperitoneal (Bradley and Fredrick, 1941) or inhalation (Brieger *et al.*, 1954) routes of administration. The National Institute for Occupational Safety and Health (NIOSH, 1988–1990) estimates that over 250,000 individuals are potentially exposed to antimony compounds in the workplace. Because of the importance of antimonial salts in the treatment of parasitic disease, there are numerous reports on their effects in humans. A recent report of the treatment with pentavalent antimonial salts of otherwise healthy soldiers for cutaneous leishmaniasis described reversible changes in electrocardiographic T-wave amplitude, systolic and diastolic blood pressure, and heart rate (Hepburn *et al.*, 1994). Studies involving human exposure to trivalent forms of antimony have been associated with hypotension, bradycardia, ventricular tachycardia, syncope, electrocardiographic changes, and sudden death (Winship, 1987; Honey, 1960; Hepburn *et al.*, 1994). Despite this evidence from animal and human data indicating potential cardiotoxic effects of antimony-containing compounds, little is known regarding the cellular toxicity of antimony.

We recently described the occurrence of oxidative stress and toxicity in cultured rat neonatal myocytes following exposure to a water soluble form of trivalent antimony, potassium antimonyl tartrate (PAT) (Tirmenstein *et al.*, 1995). Considerable research has implicated roles for oxidative stress and altered intracellular cytosolic free calcium ($[Ca^{2+}]_i$) homeostasis in ischemic myocardial injury and links between the two have been described (for review see Silverman and Stern, 1994). A large body of work on ischemic myocardial injury involves the use of cultured adult or neonatal cardiac myocytes. These cultured cells exhibit beating activity (myocyte contraction) that is spontaneous or can be induced by electric field stimulation. Myocyte contraction is accompanied by a transient increase in $[Ca^{2+}]_i$ (hereafter referred to as the $[Ca^{2+}]_i$ transient) that is initiated by flux of ionic calcium (Ca^{2+}) across the sarcolemma and sustained by release of Ca^{2+} from the sarcoplasmic reticulum (SR). The $[Ca^{2+}]_i$ transient is characterized by a rapid rise in $[Ca^{2+}]_i$ to a peak (systolic $[Ca^{2+}]_i$), and a return to resting levels (diastolic $[Ca^{2+}]_i$) that is mediated by extrusion of

[1] Presented in part at the Society of Toxicology meeting at Baltimore, MD, March 5–9, 1995. Mention of company names or products does not constitute an endorsement by NIOSH.

[2] To whom correspondence should be addressed.

[3] Current address: College of Pharmacy, Washington State University, Pullman, WA 99164-6510.

0041-008X/97 $25.00

Ca^{2+} across the sarcolemma and reuptake of Ca^{2+} by the SR. The $[Ca^{2+}]_i$ transient can be monitored using Ca^{2+}-binding fluorescent dyes (i.e., fura-2), and is a direct reflection of beating activity. Agents inducing oxidative stress in cardiac myocytes have been shown to cause inhibition of $[Ca^{2+}]_i$ transients and an elevation in diastolic or resting $[Ca^{2+}]_i$ that led to a $[Ca^{2+}]_i$ overload—a condition where resting $[Ca^{2+}]_i$ was higher than peak systolic $[Ca^{2+}]_i$ of beating cells (Oe *et al.,* 1994; Toraason *et al.,* 1994). The elevated $[Ca^{2+}]_i$ that follows exposure of cardiac myocytes to agents inducing oxidative stress has been shown to be an early prelethal event (Eley *et al.,* 1991; Oe *et al.,* 1994; Persoon-Rothert *et al.,* 1994; Josephson *et al.,* 1991; Clague *et al.,* 1993), and has been suggested to be a key element of oxidative stress-induced toxicity. Sustained elevation in $[Ca^{2+}]_i$ can lead to mitochondrial dysfunction, activation of catabolic enzymes, or induction of apoptosis (Nicotera *et al.,* 1992) and subsequent cell death. These observations suggest that alterations in $[Ca^{2+}]_i$ handling could also be linked to the oxidative stress in PAT intoxicated myocytes. Furthermore, PAT-induced effects on $[Ca^{2+}]_i$ handling could be responsible for the observed inhibition of beating activity in PAT-exposed myocytes (Tirmenstein *et al.,* 1995).

There is considerable evidence that agents that bind to or affect the redox status of thiol groups can alter the function of calcium regulatory proteins. For example, heavy metals (Abramson *et al.,* 1983) and hydrogen peroxide (Boraso and Williams, 1994) have been shown to effect release of calcium from isolated sarcoplasmic reticulum through binding or oxidation of protein thiol groups. Eley *et al.* (1991) demonstrated in isolated rabbit ventricular myocytes that hypochlorous acid induced the release of Ca^{2+} from internal stores through alteration of protein thiol redox status. The activity of myocyte sarcolemmal Na^+-Ca^{2+} exchange protein (Coetzee *et al.,* 1994) and L-type Ca^{2+} channel protein (Murphy *et al.,* 1990; Chiamvimonvat *et al.,* 1995) have been shown to be sensitive to sulfhydryl modification. Treatment of neonatal cardiac myocytes with ethacrynic acid has been shown to deplete glutathione and result in oxidative stress that was preceded by elevated $[Ca^{2+}]_i$ (Dhanbhoora and Babson, 1992). These observations are of interest given that trivalent antimony has been shown to react with thiol groups (Stemmer, 1976; Basinger and Jones, 1981) and decrease glutathione in neonatal myocytes (Tirmenstein *et al.,* 1995). Thus, the reduction in cellular glutathione by PAT exposure may render the cell susceptible to oxidative stress and might also involve alteration in $[Ca^{2+}]_i$. In addition, direct binding of antimony to protein thiols could alter the function of calcium regulatory proteins and subsequently $[Ca^{2+}]_i$. Therefore, we undertook studies to determine the effect of PAT on neonatal rat cardiac myocyte $[Ca^{2+}]_i$, and conducted investigations on its potential role in ensuing toxicity.

MATERIALS AND METHODS

Chemicals. Newborn calf serum (NCS) was purchased from Hyclone Laboratories (Logan, UT). PAT was obtained from Aldrich Chemical Company (Milwaukee, WI). The acetoxy methyl (AM) esters of fura-2 and BAPTA (1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid), fura-2 pentapotassium salt, BAPTA pentapotassium salt, and TPEN (*N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl)ethylenediamine) were purchased from Molecular Probes (Eugene, OR). All other chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific Company (Fair Lawn, NJ).

Preparation of myocytes. Cardiac ventricular cells were isolated from 2- to 4-day-old Sprague–Dawley rat pups obtained from a breeding colony maintained in the NIOSH animal quarters which is accredited by the American Association for Accreditation of Laboratory Animal Care. Briefly, neonatal hearts were minced and subjected to an overnight cold trypsin digestion as previously described (Toraason *et al.,* 1990). A single cell suspension in M199 medium with 10% NCS was prepared from the digested hearts. Viability was above 90% as determined by trypan blue exclusion. A preplating procedure was used to reduce the percentage of fibroblasts and the resulting myocyte-enriched suspension was used for preparing cultures for experiments. Coverslip cultures were prepared by placing five drops of a cell suspension ($7-8 \times 10^5$ cells/ml) as a meniscus on a sterile 25-mm quartz coverslip (approximately 2×10^5 cells/cm^2) contained in a 35-mm culture dish. The cultures were incubated overnight to allow cell attachment before addition of 2 ml of M199 medium with 10% NCS, and used for experiments 24 hr later. For toxicity assays, 1 ml of a diluted suspension (4×10^5 cells/ml) was added to wells of 12-well plates (approximately 2×10^5 cells/cm^2), and cultured for 48 hr before use in toxicity assays. These procedures resulted in subconfluent cultures of spontaneously beating myocytes that appeared primarily as clusters of dense, stellate cells in which the peripheral regions of cells often overlapped.

Intracellular calcium. Changes in $[Ca^{2+}]_i$ of neonatal cardiac myocytes were monitored using the fluorescent calcium-binding dye fura-2. The myocytes cultures on glass coverslips were loaded with fura-2 by incubation with 3 μM fura-2 acetoxymethyl ester in M199 containing 10% NCS at 37°C for 20 min. The coverslip was mounted in a temperature-controlled (30°C) suffusion chamber (Medical Systems Corp., Great Neck, NY) on the stage of an inverted fluorescence microscope (Nikon Diaphot). The suffusion chamber held approximately 1 ml of buffer and was open to room air. Buffer was continuously pumped (Rabbit-Plus, Rainin, Inst. Co., Emeryville, CA) into the suffusion chamber and removed by vacuum aspiration. Cells were alternately illuminated (20 Hz) at 340 and 380 nm via a fiber optics bundle coupled to a dual excitation spectrofluorometer (Deltascan, Photon Technology International Inc., South Brunswick, NJ). Emitted light passed through a barrier filter (Nikon 515IF filter) was detected by a photomultiplier tube, and expressed as counts per second. Background fluorescence at 340 and 380 nm excitation was obtained for each experiment from a representative cell not containing fura-2, and was subtracted from the fluorescence signals for all cells studied that day. Data were analyzed as the ratio of background-corrected fluorescence at 340 nm excitation to 380 nm excitation (F_{340}/F_{380} ratio). F_{340}/F_{380} ratios for an individual cell would be proportional to $[Ca^{2+}]_i$, as described by Grynkiewicz *et al.* (1985), but converting F_{340}/F_{380} ratio to $[Ca^{2+}]_i$ would require internal calibration to estimate the binding affinity of Ca^{2+} to fura-2 in the cell environment. In this study, F_{340}/F_{380} ratios were not converted to $[Ca^{2+}]_i$ (nM) because PAT treatment resulted in hypercontracture and compromised cell integrity; this precluded obtaining an internal calibration as viable cells are required for this procedure. Because fura-2 primarily would be localized in the cytosol, changes in F_{340}/F_{380} ratios were assumed to primarily represent changes in cytosolic free calcium.

Experiments were performed at 30°C and included a 30-min equilibration by suffusing at 2 ml/min either with HBSS (Hank's Balanced Salt Solution) buffer, modified to contain 1.8 mM calcium (in mM: NaCl, 137; KCl, 5.4; $CaCl_2$-$2H_2O$, 1.8; MgSO4, 0.8; KH_2PO_4, 0.44; Na_2HPO_4, 0.34; Na_2HCO_3,

4.2; glucose, 5.5; pH 7.4), or with Ca^{2+}-free HBSS containing 1 mM EGTA (ethylene glycol bis(β-aminoethyl ether) *N,N′*-tetraacetic acid). Immediately following the equilibration period, cultures were suffused with the appropriate buffer containing PAT or sodium potassium tartrate (the sodium salt of the antimony ligand in PAT) for 3 min at 2 ml/min to quickly change the medium in the suffusion chamber before reducing the flow to 0.25 ml/min to prevent dessication. Individual cells were chosen for study and contributions of overlapping and adjoining cells were minimized by an adjustable aperture. Myocytes were identified by their distinctive morphology and only attached, non-hypercontracted, cells were chosen. Unless otherwise stated, fluorescence data were collected for 11 sec, and illumination of cells was blocked by a shutter when data were not being collected. To monitor time-dependent changes in $[Ca^{2+}]_i$ during PAT exposure, a new cell was selected every 3 min and 11 sec of fluorescence data were collected. A single experiment involved data collection from up to 44 cells in the same culture. In a cell-free cuvette system, concentrations of PAT up to 6 mM had no effect on the fluorescence of fura-2 pentapotassium salt (5 μM) in a buffer containing 10 mM MOPS (3-(*N*-morpholinepropanesulfonic acid), 100 mM KCl, and 10 mM EGTA. For studies of fura-2 content, the change in fluorescence at the isosbestic (calcium-insensitive) excitation wavelength was followed. The isosbestic wavelength for our experimental setup was determined to be 358 nm by monitoring fluorescence of fura-2 loaded beating myocytes.

Caffeine-induced calcium release from the SR was assessed by production of a caffeine-induced $[Ca^{2+}]_i$ transient as described by Bassani *et al.* (1994). A single myocyte was selected for study and the culture was suffused with calcium- and sodium-free HBSS (in mM: choline chloride, 141; KCl, 0.8; $MgSO_4$, 0.8, KH_2PO_4, 0.44; K_2HPO_4-$3H_2O$, 0.34; $KHCO_3$, 4.2; glucose, 5.5; pH 7.4, with KOH) for 2 min at 4 ml/min to rapidly inhibit spontaneous calcium transients. Next, the culture was rapidly suffused with 10 mM caffeine in Ca^{2+}-free HBSS for 2 min at 4 ml/min. Ca^{2+}-free HBSS was used to prevent contributions to $[Ca^{2+}]_i$ from flux across the plasmalemma. After the appearance of the caffeine-induced $[Ca^{2+}]_i$ transient, the cell was suffused with HBSS for 10 min to replenish SR calcium stores before exposure to 200 μM PAT in 1.8 mM extracellular calcium. PAT exposure was continued until $[Ca^{2+}]_i$ became elevated and $[Ca^{2+}]_i$ transients were completely inhibited. Finally, the same cell was tested again for caffeine-induced calcium release from the SR using the same procedure described above.

Assessment of cell killing. Cell killing was determined as the loss of fura-2 or lactate dehydrogenase (LDH) as a result of nonspecific and irreversible leakage across the plasma membrane. For assessment of toxicity by LDH release, myocytes were exposed to PAT dissolved in HBSS or calcium-free HBSS and incubated at 37°C. Previous studies have shown that sodium potassium tartrate is not toxic at the concentrations employed here (Tirmenstein *et al.*, 1995). LDH activity was determined spectrophotometrically at 340 nm as the reduction of nicotinamide adenine dinucleotide coupled to LDH-mediated oxidation of lactate using a kit from Sigma (Procedure No. 228-UV; Sigma Chemical Co.), and was expressed as units/ml. At the end of the exposure period, the treatment buffer was removed and 1 ml 1% Triton X-100 in HBSS was added and incubated for 10 min at 37°C to lyse attached cells. LDH release was calculated as percentage of LDH activity in the treatment buffer relative to the sum of LDH activity in the treatment buffer and lysed cell solution.

Statistical analysis. The Student *t* test or one-way analysis of variance was performed using the Statgraphics (Version 5, STSC, Inc., Rockville, MD) statistical package. Following one-way analysis of variance, differences between controls and specific treatments were determined using Scheffe's test for multiple comparisons.

RESULTS

PAT exposure inhibits calcium transients and increases resting calcium. Neonatal rat cardiac myocytes exhibit beating activity of which the $[Ca^{2+}]_i$ transient is an integral component. Figure 1 illustrates the effect of 200 μM PAT for different durations of exposure on $[Ca^{2+}]_i$ transients as monitored by changes in the F_{340}/F_{380} ratio. The first observable change was an elevation in diastolic $[Ca^{2+}]_i$ (Fig. 1, 40 min of PAT exposure). The frequency of $[Ca^{2+}]_i$ transients and cell contractions became rapid and irregular as the diastolic $[Ca^{2+}]_i$ continued to rise (Fig. 1, 60 min of PAT exposure). Eventually, $[Ca^{2+}]_i$ transients and cell contractions were completely inhibited (Fig. 1, 80 min of PAT exposure). Finally, the cell experienced $[Ca^{2+}]_i$ overload (Fig. 1, 108 min of PAT exposure), a sustained F_{340}/F_{380} ratio greater than peak F_{340}/F_{380} ratio achieved during a normal $[Ca^{2+}]_i$ transient (F_{340}/F_{380} ratio $>$ 1.5 to 2.0). $[Ca^{2+}]_i$ overload was closely followed by hypercontracture and loss of adherence to the culture dish. Selected cells with $[Ca^{2+}]_i$ overload were not hypercontracted and still retained fura-2, but this state was irreversible as demonstrated by hypercontracture and loss of adherence even when PAT exposure was removed (data not shown). Figure 2 shows the average diastolic $[Ca^{2+}]_i$ for cells in consecutive 9-min intervals. This analysis of data demonstrated a progressive increase in diastolic $[Ca^{2+}]_i$ that became statistically significant ($p < 0.05$) following approximately 36 min of exposure to 200 μM PAT. The larger variability in the F_{340}/F_{380} ratio of PAT-exposed cells reflects the variability in progression of the effect of PAT on $[Ca^{2+}]_i$ for individual cells. Some variability also results from differences between cells in the calibration of F_{340}/F_{380} ratios to $[Ca^{2+}]_i$. However, similar results were obtained when a single cell was repeatedly sampled over time (data not shown). Following the F_{340}/F_{380} ratio over time on the same cell would faithfully reflect $[Ca^{2+}]_i$ because

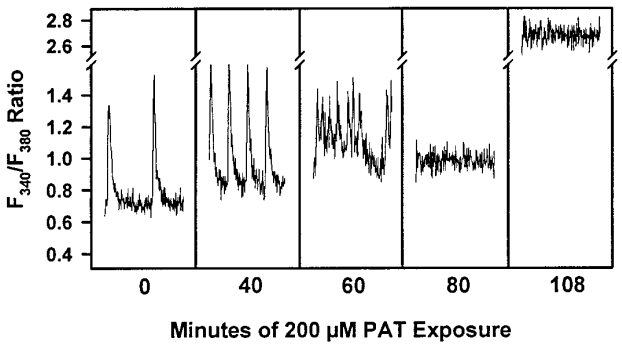

FIG. 1. Effect of exposure to 200 μM PAT on $[Ca^{2+}]_i$ transients of spontaneously beating neonatal rat cardiac myocytes. Each panel contains a representative tracing of fluorescence data (F_{340}/F_{380}) acquired at 20 Hz for 11 sec. F_{340}/F_{380} is the ratio of fluorescence counts at 340 nm excitation to 380 nm excitation and is proportional to $[Ca^{2+}]_i$. Each tracing was taken using a different myocyte in the same experiment at the time specified at the bottom of that panel. The sequence from left to right presents the effect of suffusion with 200 μM PAT in 1.8 mM extracellular calcium on $[Ca^{2+}]_i$ transients and resting $[Ca^{2+}]_i$ at the times indicated.

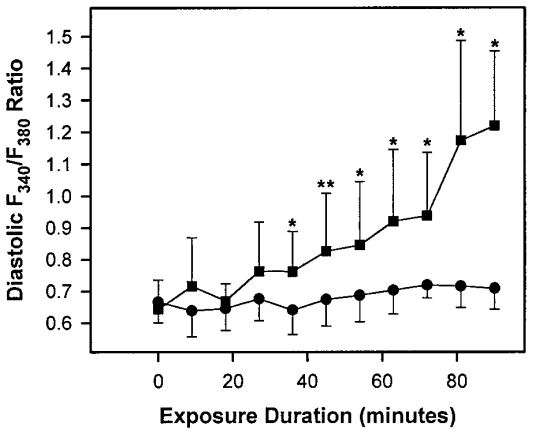

FIG. 2. Effect of exposure to 200 μM PAT (■) on diastolic F_{340}/F_{380} ratios in neonatal rat cardiac myocytes. Controls (●) were exposed to 200 μM Na-K-tartrate. The extracellular calcium concentration of the buffer was 1.8 mM. F_{340}/F_{380} is the ratio of fluorescence counts at 340 nm excitation to 380 nm excitation and is proportional to $[Ca^{2+}]_i$. An average F_{340}/F_{380} ratio was determined for the diastolic period of calcium transients, and means (±SD) calculated from values in successive 9-min periods. Cells exhibiting calcium overload (F_{340}/F_{380} ratio > 2.0) were excluded from the mean. All comparisons were to control values at the same time period using the *t* test (five experiments for PAT exposure and three experiments for control). Significant differences at $^*p < 0.05$ and $^{**}p = 0.05$.

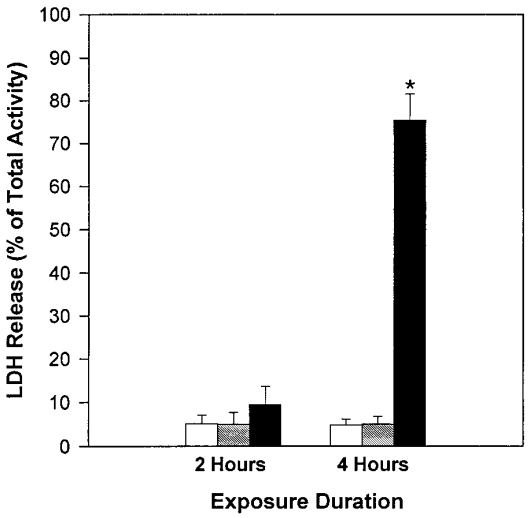

FIG. 3. LDH release by neonatal rat cardiac myocytes exposed to 50 (▨) and 200 μM (■) PAT. Myocyte cultures were exposed to 50 or 200 μM PAT for the length of time indicated on the abscissa, and then LDH activity (units/ml) was determined in the treatment buffer and in the remaining adherent cells to obtain the total LDH activity in the culture. Control cultures (□) were incubated in HBSS alone. LDH release was calculated as percentage of total activity. All values are expressed as the means ± SD, $n = 3$ experiments. *A significant difference ($p < 0.01$) from controls at the same time period.

the calibration of F_{340}/F_{380} ratios to $[Ca^{2+}]_i$ would remain constant.

Myocytes were also exposed to 50 μM PAT and fura-2 fluorescence examined at two time periods: 50 to 70 min and 100 to 120 min (Table 1). Myocytes exhibited a small but significant elevation of diastolic $[Ca^{2+}]_i$ after 50 to 70 min of exposure compared to that prior to PAT exposure in the same cultures. After 100 to 120 min of exposure, most myocytes had ceased to beat spontaneously and $[Ca^{2+}]_i$ was significantly elevated. Exposure to 50 μM PAT rarely resulted in $[Ca^{2+}]_i$ overload during a 2-hr exposure. The dose-dependent nature of the effect of PAT on $[Ca^{2+}]_i$ was demonstrated by the later occurrence and reduced magnitude of $[Ca^{2+}]_i$ change for 50 μM PAT compared to 200 μM PAT.

TABLE 1
Diastolic F_{340}/F_{380} Ratios of Neonatal Rat Cardiac Myocytes during Exposure to 50 μM PAT

Minutes of exposure	Diastolic F_{340}/F_{380}
Control	0.59 ± 0.09 (38)
50–70	0.68 ± 0.09 (22)*
100–120	0.97 ± 0.09 (21)*

Note. Fluorescence data (F_{340}/F_{380} ratios) were taken on randomly selected myocytes before suffusion with 50 μM PAT in HBSS (control), and also within the intervals listed for minutes of exposure to 50 μM PAT. Values are expressed as mean ± SD, and in parentheses are the total number of myocytes sampled in five experiments.

* Significant difference from control at $p < 0.01$.

LDH release in myocyte cultures exposed to 50 and 200 μM PAT are presented in Fig. 3. LDH release during exposure to 200 μM PAT was slightly, but not significantly, elevated after 2 hr and greatly elevated after 4 hr. LDH release during exposure to 50 μM PAT remained at control levels for up to 4 hr. We have shown previously that exposure to 50 μM PAT results in significant cell killing after an 18-hr exposure (Tirmenstein *et al.,* 1995). Diastolic $[Ca^{2+}]_i$ was significantly elevated after exposure to 200 μM PAT for approximately 36 min and 50 μM PAT for approximately 60 min, and in both cases occurred well before significant increases in LDH leakage. These results clearly show that the effect on $[Ca^{2+}]_i$ was not due to nonspecific membrane leakiness that occurs with cell death because elevated $[Ca^{2+}]_i$ occurs well before significant cell killing.

Although PAT was found to increase the F_{340}/F_{380} ratio, it remained necessary to confirm the change was due to Ca^{2+} and not other metals. In addition to Ca^{2+}, intracellular mobilization of Zn^{2+} has been demonstrated in adult myocytes exposed to hypochlorous acid (Tatsumi and Fliss, 1994) and neuron cells exposed to methyl mercury (Hare *et al.,* 1993). The ability of Zn^{2+} to bind to fura-2 and alter its fluorescence characteristics has been described (Grynkiewicz *et al.,* 1985; Hechtenberg and Beyersmann, 1993). In this regard, an increase in fluorescence at the isosbestic wavelength and reversal of the change following treatment

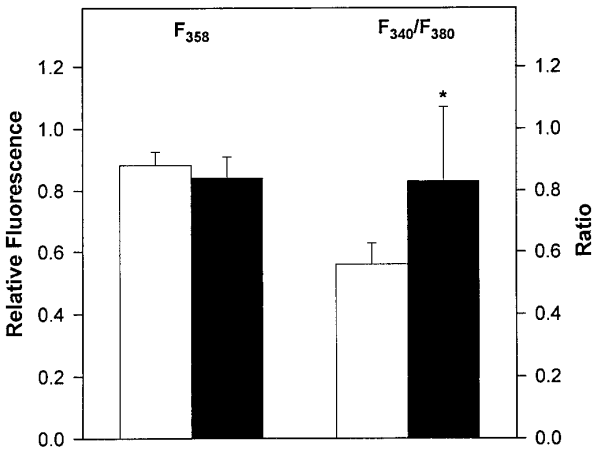

FIG. 4. Effect of exposure to 200 μM PAT for 60 min on calcium-insensitive fluorescence of fura-2 in neonatal rat cardiac myocytes. For each experiment, a single myocyte was selected and fura-2 fluorescence at the calcium-insensitive excitation wavelength (F_{358}) and the F_{340}/F_{380} ratio prior to and after suffusion for 60 min with 200 μM PAT (■) in HBSS or Na-K-tartrate (□) was measured. F_{340}/F_{380} is the ratio of fluorescence counts at 340 nm excitation to 380 nm excitation and is proportional to $[Ca^{2+}]_i$. F_{358} is the fluorescence counts at 358 nm excitation and is insensitive to $[Ca^{2+}]_i$ but is altered by Zn^{2+} binding. In the left panel, F_{358} is expressed as fluorescence at 60 min relative to the initial fluorescence. The right panel shows the F_{340}/F_{380} ratio at 60 min. All values are expressed as the means ± SD, $n = 5$ experiments. Asterisk denotes a significant difference ($p < 0.05$) from controls at the same time period.

with the heavy metal chelator, TPEN, have been used to distinguish Zn^{2+} from Ca^{2+} mobilization in cells (Hare *et al.,* 1993). To investigate this possibility in PAT-exposed myocytes, fluorescence at the isosbestic excitation wavelength (F_{358}), as well as the F_{340}/F_{380} ratio, was monitored on the same myocyte exposed to 200 μM PAT for 60 min. The fluorescence values at 358 nm excitation gradually decreased for both tartrate- and PAT-treated cells, but after 60 min were not significantly different (Fig. 4). Concurrently, the F_{340}/F_{380} ratio of PAT-exposed cells was significantly increased compared to tartrate controls after 60 min. Furthermore, when PAT-treated cells with elevated $[Ca^{2+}]_i$ were treated with TPEN (10 μM) to chelate intracellular heavy metals, there was an increase in fluorescence at 358 nm excitation, exactly the opposite direction expected for Zn^{2+} mobilization (data not shown). These data support the interpretation that the PAT-induced increase in F_{340}/F_{380} ratio reflects increased $[Ca^{2+}]_i$.

The role of $[Ca^{2+}]_i$ *in PAT-induced toxicity.* The role of calcium was further defined by assessing the toxicity of PAT under conditions of reduced extracellular or intracellular calcium. Cell killing was assessed by the release of LDH into the incubation buffer. Removal of extracellular calcium did not prevent the toxicity of a 4-hr exposure to 200 μM PAT. In fact, just the omission of extracellular calcium for 4 hr (no PAT treatment) resulted in a small, but significantly higher, release of LDH (Fig. 5). Thus, flux of extracellular calcium across the plasmalemma was not a necessary event in PAT toxicity. To prevent a rise in $[Ca^{2+}]_i$, myocytes were loaded with the intracellular calcium chelator BAPTA-AM (10 to 40 μM). BAPTA provided significant protection in a dose-dependent fashion against cell killing by a 4-hr exposure to 200 μM PAT in 0 or 1.8 mM extracellular calcium (Fig. 5). BAPTA alone did not have a significant effect on LDH release, and the small cytotoxic effect of extracellular calcium omission was not prevented by BAPTA pretreatment. Also, it is unlikely that the protective effect of BAPTA was due to binding of antimony since BAPTA was completely effective at 1/10th of the antimony concentration. These results clearly demonstrate that PAT toxicity was dependent on $[Ca^{2+}]_i$.

In the absence of extracellular calcium, changes in $[Ca^{2+}]_i$ are due to mobilization from intracellular pools. Therefore, the effect of PAT on myocyte $[Ca^{2+}]_i$ in the absence of extracellular calcium was investigated to determine if such an increase could be detected. Although a 4-hr incubation in 0 mM extracellular calcium resulted in some toxicity (Fig. 5), no adverse effects were evident by microscopic examination following the 30-min equilibration in 0 mM extracellular calcium, and only cells retaining fura-2 were selected to be

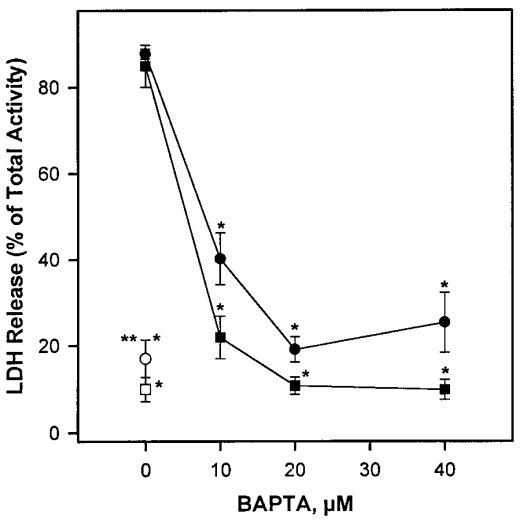

FIG. 5. Effect of BAPTA on release of LDH from neonatal cardiac myocytes following exposure to 200 μM PAT in 0 (●) or 1.8 mM (■) extracellular calcium for 4 hr. Cultures were supplemented with 10 to 40 μM BAPTA for 1 hr prior to the addition of 200 μM PAT in HBSS containing 1.8 mM extracellular calcium or calcium-free HBSS with 1 mM EGTA. Control cultures were exposed to HBSS alone (□) or calcium-free HBSS with 1 mM EGTA (○). LDH activity (units/ml) was determined in the treatment buffer and in the remaining adherent cells to obtain the total LDH activity in the culture. LDH release was calculated as percentage of total activity. All values are expressed as the mean ± SD, $n = 3$ experiments. *Significant difference ($p < 0.01$) from the mean of cultures treated with 200 μM PAT in 0 or 1.8 mM extracellular calcium; and **significant difference ($p < 0.05$) from control cultures in HBSS.

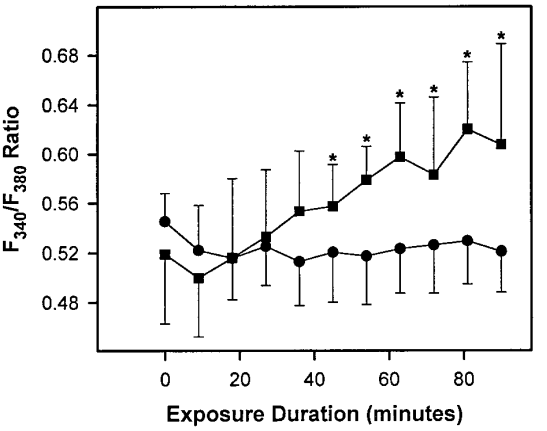

FIG. 6. Effect of 200 μM PAT exposure (■) in 0 mM extracellular calcium on F_{340}/F_{380} ratios of neonatal cardiac myocytes. Controls (●) were exposed to 200 μM Na-K-tartrate in 0 mM extracellular calcium. F_{340}/F_{380} is the ratio of fluorescence counts at 340 nm excitation to 380 nm excitation and is proportional to $[Ca^{2+}]_i$. An average F_{340}/F_{380} ratio was determined for each cell, and means (±SD) were calculated from values in successive 9-min periods. All comparisons were to control values at the same time period using the *t* test (three experiments for both PAT exposure and control). *Significant difference at $p < 0.05$.

examined during PAT exposure. As shown in Fig. 6, $[Ca^{2+}]_i$ was significantly elevated after 45 min of exposure to 200 μM PAT in calcium-free buffer. The time course for PAT-induced elevation of $[Ca^{2+}]_i$ was similar for cells exposed in 0 mM extracellular calcium or 1.8 mM extracellular calcium. Despite the fact that F_{340}/F_{380} ratios could not be converted to calcium concentration, the magnitude of the change in $[Ca^{2+}]_i$ was clearly greater for PAT exposure in 1.8 mM compared to 0 mM extracellular calcium (compare Figs. 2 and 6). This result demonstrates that although PAT toxicity was dependent on $[Ca^{2+}]_i$, a large increase in $[Ca^{2+}]_i$ above resting levels was not a requirement for PAT toxicity.

Effect of PAT on sarcoplasmic reticulum calcium. A major intracellular calcium storage site in the cardiac myocyte, and a source of calcium for $[Ca^{2+}]_i$ transients, is the SR. Oxidative stress induced in rabbit adult myocytes by hypochlorous acid has been shown to increase diastolic $[Ca^{2+}]_i$ presumably through release of calcium from the caffeine-releasable SR pool (Eley *et al.*, 1991). Thus, the SR may be a contributing source to $[Ca^{2+}]_i$ during PAT-induced oxidative stress and toxicity. Treatment with caffeine has been used to assess the calcium content in myocyte SR (Bassani *et al.*, 1994), and therefore, this procedure was used in PAT-treated myocytes to assess the state of the caffeine-releasable SR calcium pool.

Exposure to a maximally effective concentration of caffeine in the absence of extracellular calcium results in the release of calcium into the cytosolic space that transiently elevates $[Ca^{2+}]_i$ (caffeine-induced $[Ca^{2+}]_i$ transient) and depletes the caffeine-sensitive calcium pool of the SR. The size of the resulting caffeine-induced $[Ca^{2+}]_i$ transient can be used to estimate the size of the caffeine-sensitive SR calcium pool. A myocyte was challenged with caffeine in 0 mM extracellular calcium to obtain a baseline caffeine-induced $[Ca^{2+}]_i$ transient, suffused with HBSS to replenish SR calcium pools, and then exposed to 200 μM PAT in 1.8 mM extracellular calcium until diastolic $[Ca^{2+}]_i$ was elevated as shown in Fig. 1 (80 min of 200 μM PAT exposure). The same myocyte was then rechallenged with caffeine in 0 mM extracellular calcium. As shown for a representative experiment in Fig. 7, there was a robust caffeine-induced $[Ca^{2+}]_i$ transient in PAT-treated cells. In 4 of 5 cells, the caffeine-induced $[Ca^{2+}]_i$ transient following PAT exposure was larger (168 to 336%) than before exposure. This result suggests that there was a loading of the SR with calcium, presumably due to the elevated $[Ca^{2+}]_i$ that occurred during PAT exposure. It is of interest to note in Fig. 7 that when cells exposed to PAT in 1.8 mM extracellular calcium were switched to the calcium-, sodium-free buffer just before treatment with caffeine, $[Ca^{2+}]_i$ was elevated when compared to the level before PAT exposure. This result demonstrates that the large elevations in diastolic $[Ca^{2+}]_i$ resulting from PAT exposure were readily reversible and due to the presence of extracellular calcium. However, the small changes in diastolic $[Ca^{2+}]_i$ associated with PAT exposure in 0 mM extracellular calcium

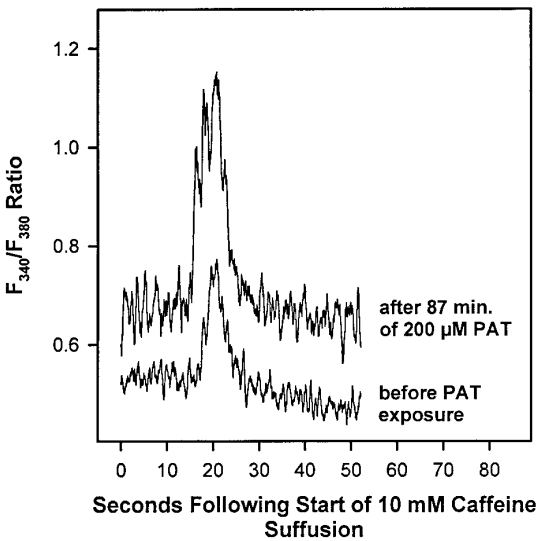

FIG. 7. Representative tracings of the caffeine-induced $[Ca^{2+}]_i$ transient in the same neonatal cardiac myocyte before and after exposure to 200 μM PAT. For each experiment, a single cell was rapidly exposed to 10 mM caffeine in 0 mM extracellular calcium (bottom tracing), and then returned to HBSS for 10 min before exposure to 200 μM PAT in 1.8 mM extracellular calcium. PAT exposure continued until the F_{340}/F_{380} ratio was elevated and spontaneous $[Ca^{2+}]_i$ transients were completely inhibited (87 min for this representative cell). The same cell was then treated again with 10 mM caffeine in 0 mM extracellular calcium (upper tracing). In both tracings, the culture was rapidly suffused with Ca^{2+}, Na^+-free HBSS before treatment with caffeine to inhibit spontaneous $[Ca^{2+}]_i$ transients.

were not reversible and occurred in the absence of SR calcium depletion. Attempts to conduct the caffeine experiment with exposure to PAT in 0 mM extracellular calcium were unsuccessful because caffeine peaks could not be obtained in control cells exposed to 0 mM extracellular calcium for 60 min.

DISCUSSION

Increased $[Ca^{2+}]_i$ has often been found during the prelethal phase of toxic cell injury and has been implicated in mechanisms of cell death for a variety of cell types and toxicant exposures (Trump and Berezesky, 1995; Nicotera *et al.,* 1992). This study describes an early effect of antimony on rat neonatal myocyte $[Ca^{2+}]_i$. Exposure to antimony, delivered as PAT, led to a progressive elevation in the resting or diastolic $[Ca^{2+}]_i$ and eventual cessation of beating activity that preceded cell death. Similar effects on $[Ca^{2+}]_i$ transients to those described here have also been reported for H_2O_2-exposed rat neonatal myocytes (Toraason *et al.,* 1994), and for adult rat myocytes exposed to a mixture of arachidonic acid and lipoxygenase to generate lipid hydroperoxides (Oe *et al.,* 1994). However, conflicting evidence exists regarding a causal role for elevated $[Ca^{2+}]_i$ in myocardial cell death (Herman *et al.,* 1990; Bond *et al.,* 1994). Cell death caused by metabolic inhibitors or reduced oxygen tension leads to hypoxia and elevated $[Ca^{2+}]_i$ that is a relatively late event, and the elevated $[Ca^{2+}]_i$ appears to result from reversal of sarcolemmal Na^+-Ca^{2+} exchange as a result of ATP depletion (Silverman and Stern, 1994). In this case, elevated $[Ca^{2+}]_i$ may contribute to irreversible injury through enhanced activity of hydrolytic enzymes, but has not been considered a necessary event in cell death (Herman *et al.,* 1990; Bond *et al.,* 1994). Conversely, exposure to agents inducing oxidative stress results in elevated $[Ca^{2+}]_i$ that occurs well before loss of cell viability (Eley *et al.,* 1991; Oe *et al.,* 1994; Persoon-Rothert *et al.,* 1994; Josephson *et al.,* 1991; Clague *et al.,* 1993), and before significant reduction in cellular ATP (Josephson *et al.,* 1991; Wang *et al.,* 1995). The present study demonstrated that $[Ca^{2+}]_i$ was significantly elevated well before cell death following exposure to both 50 and 200 μM PAT. Thus, for cultured myocytes, the profile of biochemical abnormalities following antimony exposure resembled the profile reported for agents inducing oxidative stress and was consistent with a role for altered $[Ca^{2+}]_i$ handling in PAT-induced cell death.

To support a causal role for elevated $[Ca^{2+}]_i$ in PAT-induced cell death it was also necessary to determine if preventing changes in $[Ca^{2+}]_i$ would protect against cell death. To this end, two approaches have been used; the first has been to conduct exposures in the absence of extracellular calcium, and the second has been to increase the intracellular calcium buffering capacity with specific calcium chelators. Using the former approach, Persoon-Rothert *et al.* (1994) found that oxidative stress, induced in cultured neonatal myocytes by exposure to cumene hydroperoxide, led to increased $[Ca^{2+}]_i$ prior to cell death, but exposure in the absence of extracellular calcium resulted in cell death without a rise in $[Ca^{2+}]_i$. Based on this result Persoon-Rothert *et al.* suggested that elevated $[Ca^{2+}]_i$ was not a causal event in cumene hydroperoxide toxicity. In contrast, a role for elevated $[Ca^{2+}]_i$ in toxicant-induced cell death has been suggested by the use of intracellular calcium chelators in adult rat cardiomyocytes exposured to *tert*-butylhydroperoxide (Castro and Bhatnagar, 1993) and neonatal rat cardiomyocytes exposed to ethacrynic acid (Dhanbhoora and Babson, 1992). In the present study, removal of extracellular calcium did not offer protection from PAT-induced toxicity; however, a small rise in $[Ca^{2+}]_i$ was observed that preceded cell death. The maximum $[Ca^{2+}]_i$ attained by cells exposed to 200 μM PAT in 0 mM extracellular calcium was similar in magnitude to that of unexposed cells in 1.8 mM extracellular calcium and substantially less than the levels in cells exposed to PAT in 1.8 mM extracellular calcium. These results were consistent with a requirement for $[Ca^{2+}]_i$ in PAT toxicity. Additional evidence supporting the necessity for $[Ca^{2+}]_i$ in PAT toxicity was demonstrated by the ability of the intracellular calcium chelator BAPTA to protect myocytes from toxicity during a 4-hr exposure to 200 μM PAT in the presence or absence of extracellular calcium. This result suggests that the contribution of calcium mobilization from intracellular pools to the total $[Ca^{2+}]_i$ burden in cells exposed to PAT in 1.8 mM extracellular calcium was relatively small, and that flux of calcium across the plasmalemma was the source for most of the increased $[Ca^{2+}]_i$. These results also demonstrate that large increases in $[Ca^{2+}]_i$ were not necessary for PAT-induced toxicity, but some level of $[Ca^{2+}]_i$ was required to permit the progression of toxicity. Furthermore, the needed Ca^{2+} was provided by intracellular stores.

Increased $[Ca^{2+}]_i$ in the absence of extracellular calcium must involve the release of bound or compartmentalized calcium from intracellular pools such as the SR and mitochondria. However, the absence of SR calcium depletion was demonstrated by a caffeine-induced $[Ca^{2+}]_i$ transient following exposure of neonatal myocytes to PAT. Furthermore, the caffeine-induced $[Ca^{2+}]_i$ transient following PAT treatment was significantly greater than that observed prior to PAT treatment. This result is consistent with calcium loading of the SR as a result of PAT-induced elevated $[Ca^{2+}]_i$, and in conflict with this organelle contributing to the PAT-induced rise in $[Ca^{2+}]_i$. Despite this finding, the SR might still be a source of calcium for the PAT-induced increased $[Ca^{2+}]_i$ occurring in the absence of extracellular calcium. This possibility remains because of the observation that suffusion of neonatal myocytes with calcium-free buffer for 60 min results in a loss of caffeine-releasable calcium.

This was likely due to leakage of calcium from the SR into the cytosol and subsequent movement out of the cell, which involves active pumping and passive exchange of calcium across the sarcolemma. Therefore, inhibition of calcium extrusion across the sarcolemma in conjunction with a slow leak of calcium out of the SR could account for the elevated $[Ca^{2+}]_i$ in neonatal myocytes exposed to PAT in the absence of extracellular calcium. Known calcium extrusion mechanisms include the sarcolemmal Ca^{2+}-ATPase and Na^+-Ca^{2+} exchange (Silverman and Stern, 1994). Na^+-Ca^{2+} exchange appears to account for the majority of Ca^{2+} extrusion during the $[Ca^{2+}]_i$ transient of neonatal myocytes (Bassani *et al.,* 1994). It is of interest, therefore, that oxidative stress has been shown to cause a sulfhydryl group-mediated decrease in Na^+-Ca^{2+} exchange activity of guinea pig cardiac myocytes (Coetzee *et al.,* 1994). The ability of trivalent antimony to form thioantimonites (Stemmer, 1976) and reduce cellular glutathione (Tirmenstein *et al.,* 1995) supports possible effects on Na^+-Ca^{2+} exchange activity. However, antimony may also affect calcium influx across the sarcolemma. In this regard, there is evidence for sulfhydryl group involvement in control of activity of the L-type calcium channel (Murphy *et al.,* 1990; Chiamvimonvat *et al.,* 1995) and calcium leak channel activity (Wang *et al.,* 1995; Jabr and Cole, 1995). A role for mitochondria as a source of calcium must also be considered. Release of calcium from mitochondria following exposure to agents inducing oxidative stress has been well documented (Nicotera *et al.,* 1993; Richter and Schlegel, 1993) and may also contribute to the rise in $[Ca^{2+}]_i$ observed.

Oxidative stress has been shown to be a component of PAT-induced cell death in cultured neonatal rat myocytes by virtue of the induction of lipid peroxidation and protection by antioxidants (Tirmenstein *et al.,* 1995). Antimony, unlike transition metals that directly reduce molecular oxygen and generate reactive oxygen species, likely induces oxidative stress indirectly. For example, oxidative stress can be brought about by agents that abruptly deplete glutathione (Miccadei *et al.,* 1988; Dhanbhoora and Babson, 1992), which can lead to a reduced ability to detoxify endogenously generated hydrogen peroxide. The present study examined $[Ca^{2+}]_i$ changes prior to PAT-induced myocyte cell death, and demonstrated the ability of PAT to disrupt $[Ca^{2+}]_i$ handling in the cardiomyocyte and lead to elevated $[Ca^{2+}]_i$, as has been found for other agents inducing oxidative stress in isolated cardiomyocytes (Eley *et al.,* 1991; Oe *et al.,* 1994; Persoon-Rothert *et al.,* 1994; Josephson *et al.,* 1991; Clague *et al.,* 1993). Furthermore, the results support the existence of a calcium-dependent event, but do not support the necessity for events in PAT-induced cell death that are mediated by a large elevation in $[Ca^{2+}]_i$. The role of $[Ca^{2+}]_i$ in a calcium-dependent event may be of a passive, or permissive, nature. In contrast, elevated $[Ca^{2+}]_i$ presumably plays an active role in initiating injury through activation of phospholipases, endonucleases, and proteases (Trump and Berezesky, 1995; Nicotera *et al.,* 1992), and have been referred to as calcium-mediated events in chemically induced cell death (Nicotera *et al.,* 1992). It is not clear what event in the pathway to PAT-induced cell death might be calcium-dependent. However, one possibility may be mitochondrial calcium cycling (Nicotera *et al.,* 1992) that can be initiated by agents inducing oxidative stress and can lead to collapse of mitochondrial membrane potential and mitochondrial damage. Calcium chelators have been shown to trap calcium released from mitochondria and thereby block calcium cycling and protect against mitochondrial damage (Nicotera *et al.,* 1992).

In addition to the antimony-induced alterations in $[Ca^{2+}]_i$ that are associated with cell death, other effects of antimony on $[Ca^{2+}]_i$ handling would be of interest due to the importance of the $[Ca^{2+}]_i$ transient for normal function of the beating cardiomyocyte. In this regard, the elevated $[Ca^{2+}]_i$ that occurred in the presence of extracellular calcium did have a deleterious effect on the $[Ca^{2+}]_i$ transient and consequently myocyte contractility. Therefore, it is of interest that an increase in diastolic $[Ca^{2+}]_i$ appears to be a feature of myocytes from diseased hearts, and has been demonstrated in myocytes isolated from rats (Capasso *et al.,* 1993) and human patients with heart failure (Beuckelmann *et al.,* 1992). Also, a range of irreversible pathophysiologic and reversible physiologic changes have been described in association with exposure to antimony compounds (Bradley and Fredrick, 1941; Brieger *et al.,* 1954; Winship, 1987; Honey, 1960; Hepburn *et al.,* 1994). Nonlethal effects on myocyte $[Ca^{2+}]_i$ may be involved in the observed reversible changes in heart function associated with antimony exposure (Hepburn *et al.,* 1994), and pathophysiological changes not resulting from myocyte cell death.

REFERENCES

Abramson, J. J., Trimm, J. L., Weden, L., and Salama, G. (1983). Heavy metals induce rapid calcium release from sarcoplasmic reticulum isolated from skeletal muscle. *Proc. Natl. Acad. Sci. USA* **80,** 1526–1530.

Basinger, M. A., and Jones, M. M. (1981). Structural requirements for chelate antidotal efficacy in acute antimony(III) intoxication. *Res. Commun. Chem. Pathol. Pharmacol.* **32,** 355–363.

Bassani, J. W. M., Qi, M., Samarel, A. M., and Bers, D. M. (1994). Contractile arrest increases sarcoplasmic reticulum calcium uptake and SARCA gene expression in cultured neonatal rat heart cells. *Circ. Res.* **74,** 991–997.

Beuckelmann, D. J., Näbauer, M., and Erdmann, E. (1992). Intracellular calcium handling in isolated ventricular myocytes from patients with terminal heart failure. *Circulation* **85,** 1046–1055.

Bond, J. M., Harper, I. S., Chacon, E., Reece, J. M., Herman, B., and Lemasters, J. J. (1994). The pH paradox in the reperfusion injury to rat neonatal cardiac myocytes. *Ann. NY Acad. Sci.* **723,** 25–37.

Boraso, A., and Williams, A. J. (1994). Modification of the gating of the cardiac sarcoplasmic reticulum Ca^{2+}-release channel by H_2O_2 and dithiothreitol. *Am. J. Physiol.* **267,** H1010–H1016.

Bradley, W. R., and Fredrick, W. G. (1941). The toxicity of antimony. *Ind. Med. (Ind. Hyg. Sec.)* **2,** 15–22.

Brieger, H., Semisch, C. W., Stasney, J., and Piatnek, D. A. (1954). Industrial antimony poisoning. *Ind. Med. Surg.* **23,** 521–523.

Capasso, J. M., Li, P., and Anversa, P. (1993). Cytosolic calcium transients in myocytes isolated from rats with ischemic heart failure. *Am. J. Physiol.* **265,** H1953–H1964.

Castro, G. J., and Bhatnagar, A. (1993). Effect of extracellular ions and modulators of calcium transport on survival of tert-butyl hydroperoxide exposed cardiac myocytes. *Cardiovascular Res.* **27,** 1873–1881.

Chiamvimonvat, N., O'Rourke, B., Kamp, T. J., Hofmann, F., Flockerzi, V., and Marban, E. (1995). Functional consequences of sulfhydryl modification in the pore-forming subunits of cardiovascular Ca^{2+} and Na^{2+} channels. *Circ. Res.* **76,** 325–334.

Clague, J. R., Harvey, R., and Langer, G. A. (1993). Protamine and other polycationic drugs inhibit calcium leak in cardiac cells during metabolic inhibition and free radical exposure. *J. Pharmacol. Exp. Ther.* **267,** 1349–1354.

Coetzee, W. A., Ichikawa, H., and Hearse, D. J. (1994). Oxidant stress inhibits Na-Ca-exchange current in cardiac myocytes by sulfhydryl groups? *Am. J. Physiol.* **266,** H909–H919.

Dhanbhoora, C. M., and Babson, J. R. (1992). Thiol depletion induces lethal cell injury in cultured cardiomyocytes. *Arch. Biochem. Biophys.* **293,** 130–139.

Eley, D. W., Korecky, B., Fliss, H., and Desilets, M. (1991). Calcium homeostasis in rabbit ventricular myocytes. Disruption by hypochlorous acid and restoration by dithiothreitol. *Circ. Res.* **69,** 1132–1138.

Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260,** 3440–3450.

Hare, M. F., McGinnis, K. M., and Atchison, W. D. (1993). Methylmercury increases intracellular concentrations of Ca^{++} and heavy metals in NG108-15 cells. *J. Pharmacol. Exp. Ther.* **266,** 1626–1635.

Hechtenberg, S., and Beyersmann, D. (1993). Differential control of free calcium and free zinc levels in isolated bovine liver nuclei. *Biochem. J.* **289,** 757–760.

Hepburn, N. C., Nolan, J., Fenn, L., Herd, R. M., Neilson, J. M. M., Sutherland, G. R., and Fox, K. A. A. (1994). Cardiac effects of sodium stilbogluconate: Myocardial, electrophysiological and biochemical studies. *Q. J. Med.* **87,** 465–472.

Herman, B., Gores, G. J., Nieminen, A-L., Kawanishi, T., Harman, A., and Lemasters, J. J. (1990). Calcium and pH in anoxic and toxic cell injury. *C.R.C. Crit. Rev. Toxicol.* **21,** 127–148.

Hoffmann, P., Heinroth, K., Richards, D., Plews, P., and Toraason, M. (1994). Depression of calcium dynamics in cardiac myocytes-a common mechanism of halogenated hydrocarbon anesthetics and solvents. *J. Mol. Cell. Cardiol.* **26,** 579–589.

Honey, M. (1960). The effects of sodium antimony tartrate on the myocardium. *Br. Heart J.* **22,** 601–616.

Jabr, R. I., and Cole, W. C. (1995). Oxygen-derived free radical stress activates nonselective cation current in guinea pig ventricular myocytes. *Circ. Res.* **76,** 812–824.

Josephson, R. A., Silverman, H. S., Lakatta, E. G., Stern, M. D., and Zweier, J. L. (1991). Study of the mechanisms of hydrogen peroxide and hydroxyl free radical-induced cellular injury and calcium overload in cardiac myocytes. *J. Biol. Chem.* **266,** 2354–2361.

Miccadei, S., Kyle, M. E., Gilfor, D., and Farbre, J. L. (1988). Toxic consequences of the abrupt depletion of glutathione in cultured rat hepatocytes. *Arch. Biochem. Biophys.* **265,** 311–320.

Murphy, B. J., Washkurak, A. W., and Tuana, B. S. (1990). Dihydropyridine binding to the L-type Ca^{2+} channel in rabbit heart sarcolemma and skeletal muscle transverse-tubules: Role of disulfide, sulfhydryl and phosphate groups. *Biochim. Biophys. Acta* **1052,** 333–339.

National Institute for Occupational Safety and Health (1988–1990). National Occupational Exposure Survey, DHHS (NIOSH) Publications 88-106, 89-102, 89-103. U.S. Government Printing Office, Washington, DC.

Nicotera, P., Bellomo, G., and Orrenius, S. (1992). Calcium-mediated mechanisms in chemically induced cell death. *Annu. Rev. Pharmacol. Toxicol.* **32,** 449–470.

Oe, H., Kuzuya, T., Hoshida, S., Nishida, M., Hori, M., Kamada, T., and Tada, M. (1994). Calcium overload and cardiac myocyte cell damage induced by arachidonate lipoxygenation. *Am. J. Physiol.* **267,** H1396–H1402.

Persoon-Rothert, M., Egas-Kenniphaas, J. M., van der Valk-Kokshoorn, E. J. M., Buys, J. P., and van der Laarse, A. (1994). Oxidative stress-induced perturbations of calcium homeostasis and cell death in cultured myocytes: Role of extracellular calcium. *Mol. Cell. Biochem.* **136,** 1–9.

Richter, C., and Schlegel, J. (1993). Mitochondrial calcium release induced by prooxidants. *Toxicol. Lett.* **67,** 119–127.

Silverman, H. S., and Stern, M. D. (1994). Ionic basis of ischaemic cardiac injury: Insights from cellular studies. *Cardiovasc. Res.* **28,** 581–597.

Stemmer, K. L. (1976). Pharmacology and toxicology of heavy metals: Antimony. *Pharmacol. Ther. A.* **1,** 157–160.

Tatsumi, T., and Fliss, H. (1994). Hypochlorous acid mobilizes intracellular zinc in isolated rat heart myocytes. *J. Mol. Cell. Cardiol.* **26,** 471–479.

Tirmenstein, M. A., Plews, P. I., Walker, C. V., Woolery, M. D., Wey, H. E., and Toraason, M. A. (1995). Antimony-induced oxidative stress and toxicity in cultured cardiac myocytes. *Toxicol. Appl. Pharmacol.* **130,** 41–47.

Toraason, M., Heinroth-Hoffmann, I., Richards, D., Woolery, M., and Hoffmann, P. (1994). H_2O_2-induced oxidative injury in rat cardiac myocytes is not potentiated by 1,1,1-trichloroethane, carbontetrachloride, or halothane. *J. Toxicol. Environ. Health* **41,** 489–507.

Toraason, M., Krueger, J. A., Breitenstein, M. J., and Swearengin, T. F. (1990). Depression of contractility in cultured cardiac myocytes from neonatal rat by carbon tetrachloride and 1,1,1-trichloroethane. *Toxicol. In Vitro* **4,** 363–368.

Trump, B. F., and Berezesky, I. K. (1995). Calcium-mediated cell injury and cell death. *FASEB J.* **9,** 219–228.

Wang, S-Y., Clague, J. R., and Langer, G. A. (1995). Increase in calcium leak channel activity by metabolic inhibition or hydrogen peroxide in ret ventricular myocytes and its inhibition by polycation. *J. Mol. Cell. Cardiol.* **27,** 211–222.

Winship, K. A. (1987). Toxicity of antimony and its compounds. *Adv. Drug React. Ac. Pois. Rev.* **2,** 67–90.