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CHLORPROMAZINE PROTECTION AGAINST Ca^{2+}-DEPENDENT AND OXIDATIVE CELL INJURY

LIMITATIONS DUE TO DEPRESSED MITOCHONDRIAL FUNCTION

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Abstract—Chlorpromazine (CPZ), a phenothiazine, demonstrated both cytoprotective and toxic effects on cardiomyocytes. CPZ markedly reduced cytotoxicity caused by two toxic challenges, each with a distinct cytotoxic mechanism. Lethal cell injury was induced in cultured neonatal cardiomyocytes by either: (1) ionomycin, a Ca^{2+} ionophore that caused Ca^{2+}-dependent cell injury; or (2) ethacrynic acid (EA), a glutathione (GSH) depletor that killed cells primarily via peroxidative damage. Pretreatment with 50 μM CPZ reduced the extent of ionomycin-induced cell death, as measured by lactate dehydrogenase (LDH) leakage, but enhanced the loss of intracellular ATP and collapsed the mitochondrial transmembrane potential ($\Delta\Psi$). In EA-treated cultures, 50 μM CPZ also lowered LDH leakage and diminished the peroxidative damage responsible for the cytotoxicity, but again enhanced the loss of intracellular ATP and collapsed the $\Delta\Psi$. CPZ protection was incomplete and limited to a narrow concentration range that was essentially identical for both toxic challenges. Maximum protection was observed with 50 μM CPZ, yet the amount of residual damage was similar to the degree of injury caused by a mitochondrial uncoupler, carbonylcyanide-*m*-chlorophenylhydrazone alone. In the absence of either challenge, 50 μM CPZ did not affect cellular energy status or kill the cells, but a higher concentration of CPZ (150 μM) did deenergize unchallenged cardiomyocytes. These data demonstrate that CPZ can reduce cytotoxicity caused by either Ca^{2+}-dependent events or oxidative stress. However, even at an optimally protective level, CPZ in combination with either ionomycin or EA deenergized the cells, although neither toxic challenge nor 50 μM CPZ alone seriously affected $\Delta\Psi$. It would appear that intracellular perturbations induced by either challenge promote a depression of mitochondrial function by CPZ, which limits the protective action of the drug. Since both of the challenges used contain toxicologic features exhibited by a wide variety of toxic insults, results of this study have both mechanistic and clinical implications.

Key words: chlorpromazine; calcium; oxidative stress; cell death; mitochondria; cardiomyocytes

CPZ† is an important member of the phenothiazines, a widely used class of antipsychotic agents. In addition to clinical applications, CPZ has been used to examine the mechanisms of lethal cell injury in various experimental systems. Early studies demonstrated a protective role for the phenothiazine promethazine during chemical intoxication [1–3] and for CPZ during ischemic injury [4–6]. Results of these studies fostered the proposal that the protective effect of CPZ was due to the antagonism of Ca^{2+}-related events, specifically, the inhibition of Ca^{2+}-dependent phospholipase A_2 [6]. This and many other biochemical effects of CPZ are believed to be based on the ability of this drug to bind to calmodulin and inhibit its action on a variety of important enzymes [7, 8]. A number of subsequent studies have used CPZ as a calmodulin antagonist to examine the role of Ca^{2+} in the onset of lethal cell injury [9–15]. Results of these studies support the proposal that the excessive stimulation of Ca^{2+}-activated processes, such as nonlysosomal proteases, endonucleases and phospholipases, may be the predominant cytotoxic event for a variety of toxic insults [16].

It is also known, however, that many toxic challenges alter intracellular thiol status, as well as Ca^{2+} homeostasis, and peroxidative damage often accompanies the loss of cellular thiols [16–18]. In fact, it has been argued that under these circumstances oxidative damage is primarily responsible for the cell death observed and the elevated Ca^{2+} is an epiphenomenon [18]. In addition to being a calmodulin antagonist, CPZ also has antioxidant properties [19–25]. Therefore, under certain circumstances, CPZ protection may be due to more than simply the antagonism of Ca^{2+}-mediated events and thus the use of CPZ as a tool to study mechanisms of cell death is less than straightforward. Indeed, this agent has been shown to lower peroxidative damage *in vivo* [26, 27] and to protect cells from

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† Abbreviations: CPZ, chlorpromazine; EA, ethacrynic acid; HBSS, Hanks' balanced salt solution; LDH, lactate dehydrogenase; $[Ca^{2+}]_i$, cytosolic free calcium; $\Delta\Psi$, mitochondrial transmembrane potential; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; $TPMP^+$, triphenylmethylphosphonium cation; DPPD, *N,N'*-diphenyl-*p*-phenylenediamine; GSH, glutathione; TBARS, thiobarbituric acid reactive substances; and TCA, trichloroacetic acid.

lethal injury presumably by acting as an antioxidant [28–30]. The antagonistic effect of this drug toward both oxidative stress and Ca^{2+}-mediated toxicity point to the possible clinical use of CPZ to reduce the toxicity of certain pathologic conditions such as stroke and myocardial infarction. However, the clinical utility of this drug and its use as a mechanistic probe of cell death may be compromised by the fact that CPZ may also exacerbate toxicity [11, 31], possibly through an effect on energy metabolism [32–34].

The current study was prompted by preliminary findings demonstrating that CPZ protected cultured cardiomyocytes from Ca^{2+}-dependent cell death [35]. This initial investigation was widened, in order to gain a clearer understanding of the effects of CPZ on compromised cardiomyocytes. Toward this end, the action of CPZ on two distinct toxic challenges was examined. The first challenge kills cardiomyocytes by a Ca^{2+}-dependent mechanism induced by the Ca^{2+} ionophore ionomycin, and the second by oxidative damage initiated by thiol depletion [36]. Results described herein show that CPZ decreased the extent of injury to cardiomyocytes caused by both Ca^{2+}-dependent and oxidative insults. However, it appears that intracellular perturbations initiated by either challenge promote an action of CPZ upon mitochondrial function, which partially offsets the protective effects of the drug.

MATERIALS AND METHODS

Isolation and culturing of cardiomyocytes. Myocardial cells from 1- to 3-day-old Sprague–Dawley rat pups were isolated and cultured essentially according to the method of Bollon *et al.* [37] with several modifications aimed at increasing the yield of beating myocytes over that of non-muscle cells [38]. Cultures contained predominantly myocytes (>85%) with average beating rates of 150 beats/min and were used on the seventh day after initial plating.

Experimental protocol. Prior to treatment, culture medium was removed with two rinses of HBSS containing 25 mM HEPES, pH 7.4. All EA incubations were performed in this buffered HBSS at 37°, and ionomycin treatments were performed in modified HBSS that contained 5 mM Ca^{2+} and 25 mM HEPES at pH 7.4 at 37°. Ionomycin (Calbiochem, La Jolla, CA) and EA (Sigma, St. Louis, MO) were added to the incubations in absolute ethanol. Solvent controls confirmed that there were no vehicle effects on the parameters measured. CPZ was dissolved directly in HBBS, added to cultures either 15 min prior to or at 0 hr, for ionomycin and EA incubations, respectively. At the end of each incubation period, incubation solutions were removed and retained for subsequent analysis. Adherent cells were lysed with a buffer containing 100 mM NaF, 20 mM EDTA, 50 mM glycylglycine, 0.5% Triton X-100, and 0.5% glycogen, pH 7.4 [39]. Cells were scraped from plates and centrifuged at 13,500 *g* for 5 min; the resultant supernatant, termed cell lysate, was removed and reserved for biochemical measurements.

Determination of lethal cell injury. Lethal cell injury was based on the loss of plasma membrane integrity as determined by LDH leakage. LDH activity in both incubate and cell lysate was assayed spectrophotometrically [38], and the results were reported as percent of the total cellular LDH (incubate activity ÷ [incubate activity + lysate activity]). The effect of all treatments on total LDH activity was analyzed (data not shown). The results indicated that ionomycin, CPZ, CCCP or combinations of these agents had no effect on total LDH over the time courses and at the cell densities used. EA did inactivate approximately 12% of total LDH after 4 hr as compared with solvent controls ($P < 0.05$). However, no significant difference in total LDH activity was detectable for up to 4 hr between incubations containing EA alone and EA in combination with CPZ. The accuracy of LDH leakage as an indicator of lethal cell injury was assessed for both toxic insults by comparison with viability determinations using trypan blue exclusion and measurements of [^{14}C]adenine nucleotide leakage [40].

Measurements of cardiomyocyte energy status. Energy status was assessed by measuring cardiomyocyte ATP content and mitochondrial transmembrane potential ($\Delta\Psi$). Cell lysate ATP levels were measured with a bioluminescent assay kit (Sigma) using a Beckman LS5000TD scintillation counter configured as a photon counter. Cardiomyocyte values were obtained from standard curves where bioluminescence was plotted versus moles of ATP standards over a linear range of 10^{-12} to 10^{-15} mol/assay. The distribution of [^{3}H]TPMP$^+$ between cardiomyocytes and the incubation solutions was used to determine $\Delta\Psi$ status according to the procedure of Hoek *et al.* [41]. The relative contribution of the plasma membrane to the uptake

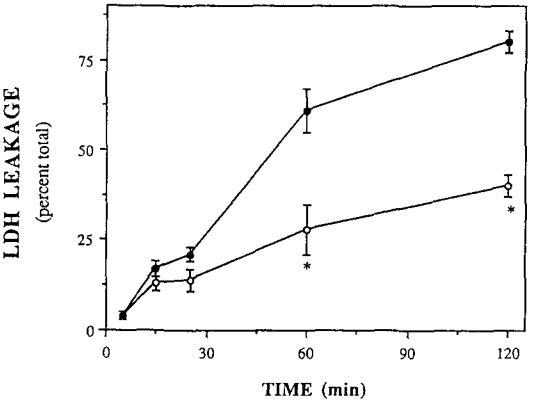

Fig. 1. Effect of CPZ on ionomycin-induced lethal cell injury to cardiomyocytes. Cardiomyocytes were preincubated with or without 50 μM CPZ for 15 min in HBSS. Cultures were then rinsed and exposed to 4 μM ionomycin and modified HBSS, in the absence (●) or presence (○) of 50 μM CPZ. LDH leakage at 2 hr in control cultures exposed to 50 μM CPZ in HBSS was 2 ± 1%. Values are the means ± SEM of measurements from 3–7 separate myocyte preparations. Key: (*) Significantly different from cultures treated with ionomycin alone ($P < 0.01$).

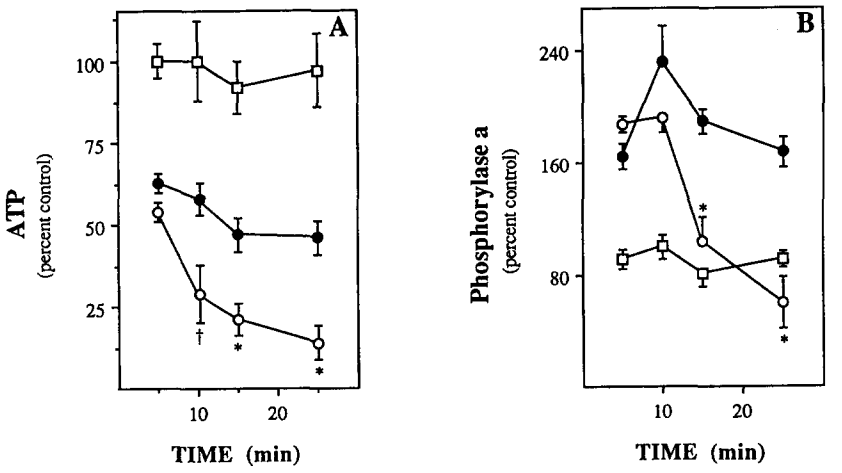

Fig. 2. Effect of CPZ on ionomycin-induced cardiomyocyte ATP depletion (A) and phosphorylase *a* activation (B). Cardiomyocytes were preincubated with or without 50 μM CPZ for 15 min in HBSS. Cultures were then rinsed and exposed to 4 μM ionomycin and modified HBSS, in the absence (●) or presence (○) of 50 μM CPZ, or with 50 μM CPZ in the absence of ionomycin (□). (A) At the times indicated, ATP was measured and is expressed as a percentage of the ATP content of vehicle controls (38 ± 3 nmol/mg protein). Values are the means ± SEM of measurements from 3–10 separate myocyte preparations. Differences between cultures treated with ionomycin alone and those treated with ionomycin plus CPZ are significant (†$P < 0.05$; *$P < 0.01$). (B) At the times indicated, phosphorylase *a* activity was measured and is expressed as a percentage of the phosphorylase *a* activity of vehicle controls (64 ± 3 nmol P_i/(min · mg protein). Values are the means ± SEM of measurements from at least 4–8 separate myocyte preparations. Differences between cultures treated with ionomycin alone and those treated with ionomycin plus CPZ are significant (*$P < 0.01$).

and retention of [^{3}H]TPMP$^+$ was assessed by depolarizing the plasma membrane with 30 mM KCl. Approximately 90% of the [^{3}H]TPMP$^+$ uptake was dependent on the $\Delta\Psi$, a value that is in close agreement with results of hepatocyte studies [42, 43].

Other biochemical measurements. The Ca^{2+}-dependent conversion of phosphorylase *b* to phosphorylase *a* was used as an indirect measurement of $[Ca^{2+}]_i$ [39, 44]. Phosphorylase *a* activity in cell lysates was determined by measuring phosphate release from glucose-1-phosphate, by the method of Gilboe *et al.* [45], as modified by Starke *et al.* [42]. Protein content was determined by the method of Bradford [46]. GSH analyses were performed according to the HPLC method of Reed *et al.* [47] after lysing cells with 6.5% TCA. Lipid peroxidation was assessed by measuring TBARS, using the method of Stacey and Klaassen [48] after terminating incubations by the addition of 100% TCA to a final concentration of 12%.

Statistical analysis. Significant differences between two groups were determined by a two-tailed unpaired *t*-test, and multiple comparisons were made using analysis of variance with the Tukey followup test.

RESULTS

The effect of CPZ on Ca^{2+}-ionomycin-induced toxicity in cardiomyocyte cultures over a 120-min time course is shown in Fig. 1. The Ca^{2+}-ionomycin challenge caused substantial LDH leakage by 120 min, and, as an indicator of lethal cell injury, LDH leakage was in close agreement with a loss of cell viability assessed at 120 min by both trypan blue staining (84 ± 3%) and [^{14}C]adenine nucleotide leakage (89 ± 9%). A survey of the literature, together with results of a preliminary study [35], indicated that 50 μM CPZ was an appropriate concentration with which to begin a series of experiments focussed on CPZ protection. The presence of 50 μM CPZ (Fig. 1) decreased the extent of Ca^{2+}-ionomycin induced damage by approximately 50% over the 120-min time course, and 50 μM CPZ alone caused no LDH leakage. When incubations were conducted with HBBS prepared without Ca^{2+}, LDH leakage after a 120-min exposure to either ionomycin, ionomycin plus 50 μM CPZ, or 50 μM CPZ alone, was only 2 ± 1, 6 ± 1, or 3 ± 1%, respectively.

The Ca^{2+}-ionomycin challenge decreased cardiomyocyte ATP to approximately 50% of control values well before the onset of any significant LDH leakage (Fig. 2A). While 50 μM CPZ alone had no detectable effect on cardiomyocyte ATP levels, it caused a marked enhancement of the ionomycin-induced ATP depletion. For example, by 15 min ATP levels in ionomycin-treated cultures dropped to 18 ± 2 nmol/mg protein, while ionomycin plus 50 μM CPZ reduced intracellular ATP to 8 ± 2 nmol/mg protein. ATP content in cardiomyocytes treated with 50 μM CPZ alone remained at levels similar to those of vehicle controls (38 ± 3 nmol/mg protein). The effect of CPZ on ATP depletion also occurred prior to any substantial LDH leakage.

As expected, ionomycin exposure led to a rapid increase of $[Ca^{2+}]_i$, as determined indirectly by the

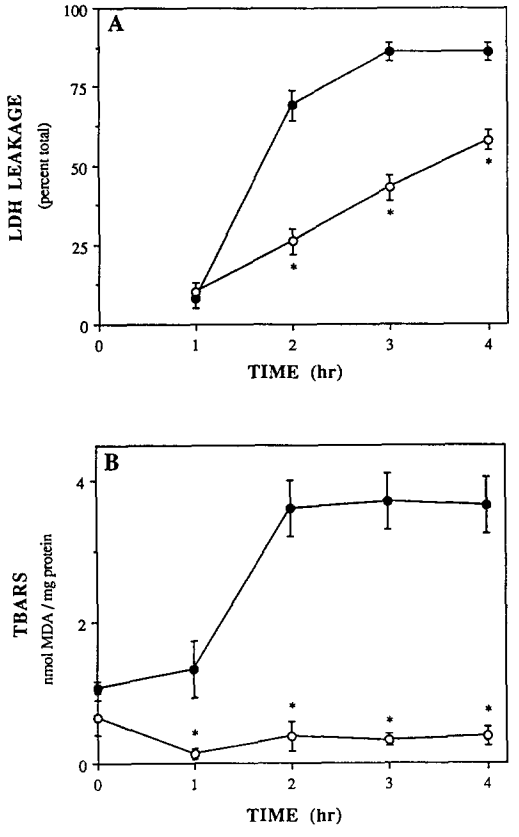

Fig. 3. Effect of CPZ on EA-induced oxidative damage to cardiomyocytes. Cardiomyocytes were incubated with 150 μM EA in the absence (●) or presence (○) of 50 μM CPZ. (A) At the times indicated, cell injury was determined by the release of LDH into the incubation medium. LDH leakage at 4 hr in control cultures exposed to 50 μM CPZ in HBSS was 3 ± 1%. Values are the means ± SEM of measurements from 5 separate cardiomyocyte preparations. Key: (*) Significantly different from cultures incubated with EA alone ($P < 0.01$). (B) At the times indicated, lipid peroxidation was determined by measuring TBARS. Values are the means ± SEM of measurements from 5 separate cardiomyocyte preparations. Key: (*) Significantly different from cultures incubated with EA alone ($P < 0.01$).

Ca^{2+}-dependent activation of phosphorylase *a* (Fig. 2B). The extent of the phosphorylase *a* activation observed was less than 50% of total cardiomyocyte activity and of a magnitude similar to that in cardiomyocytes treated for 1 hr with 150 μM EA [36]. Interestingly, the presence of 50 μM CPZ had no effect on the initial burst of phosphorylase *a* activation, but eventually caused a sharp decrease in the activity of this calmodulin-dependent enzyme to levels of unstimulated controls before any substantial LDH leakage had occurred (Fig. 2B). CPZ alone did not stimulate the activation of phosphorylase *a*.

CPZ also partially protected cardiomyocytes from EA toxicity. The onset of LDH leakage caused by 150 μM EA was delayed compared with that induced by ionomycin (Fig. 3A). The degree of LDH leakage observed at 2 hr was similar to the extent of toxicity measured at this time by trypan blue staining (80 ± 3%) and [^{14}C]adenine nucleotide leakage (80 ± 4%), as compared with control values of 4 ± 1, 9 ± 4, and 8 ± 1%, respectively. CPZ (50 μM) markedly delayed the onset of EA-induced LDH leakage and afforded a significant degree of protection for the entire 4-hr incubation (Fig. 3A). Since 150 μM EA kills cardiomyocytes primarily as a result of peroxidative damage triggered by GSH depletion [36], the effect of CPZ on EA-induced lipid peroxidation and GSH depletion was determined. EA (150 μM) caused an increase in TBARS, a measure of lipid peroxidation, that closely paralleled the loss of cardiomyocyte viability (Fig. 3B). The antioxidant activity of 50 μM CPZ was evident by a decrease in EA-induced TBARS formation (Fig. 3B), that occurred without any significant effect of CPZ on the extent of GSH depletion caused by EA (data not shown). However, although CPZ completely blocked EA-induced TBARS production, it only partially prevented the LDH leakage (Fig. 3A). In contrast, at 2 hr the antioxidant DPPD decreased TBARS to undetectable levels and lowered LDH leakage to 6 ± 1%, a value similar to the control and significantly lower than EA plus CPZ ($P < 0.02$).

To understand further the limits of CPZ protection, the effect of CPZ concentration on cardiomyocyte injury caused by both toxic challenges was determined. At 2 hr, when the toxicity of each challenge was substantial, CPZ offered a rather narrow window of protection (Fig. 4). In both cases 50 μM CPZ yielded the most protection, whereas at higher concentrations the protective effect of the drug diminished. The highest concentration used was 150 μM CPZ, and this caused only modest LDH leakage (7 ± 2%) in unchallenged cultures. Interestingly, although CPZ decreased both EA-induced LDH leakage and TBARS, at concentrations of 50 μM CPZ and above TBARS formation was eliminated, yet LDH leakage actually increased with higher CPZ concentrations (Fig. 4B).

The concentration-dependent profile of CPZ protection shown in Fig. 4 suggests that some additional cytotoxic action of the drug partially offsets the protective effects. One such potentially cytotoxic action of CPZ is the exacerbation of ionomycin-induced ATP depletion (Fig. 2). CPZ is known to uncouple oxidative phosphorylation [32–34], and such an effect could explain the enhanced ATP depletion. To test this possibility, $\Delta\Psi$ of challenged cells was measured in the presence and absence of CPZ prior to the onset of any significant LDH leakage and at times when phosphorylase *a* activation, an indicator of $[Ca^{2+}]_i$, had peaked for both ionomycin (Fig. 2) and EA [36] treatments (20 and 60 min, respectively). The integrity of the cardiomyocyte $\Delta\Psi$ was determined by measuring the retention of [^{3}H]TPMP$^+$. While neither 50 μM CPZ nor ionomycin alone had any substantial effect on $\Delta\Psi$, the combination of these two agents rapidly collapsed $\Delta\Psi$ to an extent observed with the mitochondrial uncoupler CCCP (Table 1). Thus, it appears that this combination brings forth an

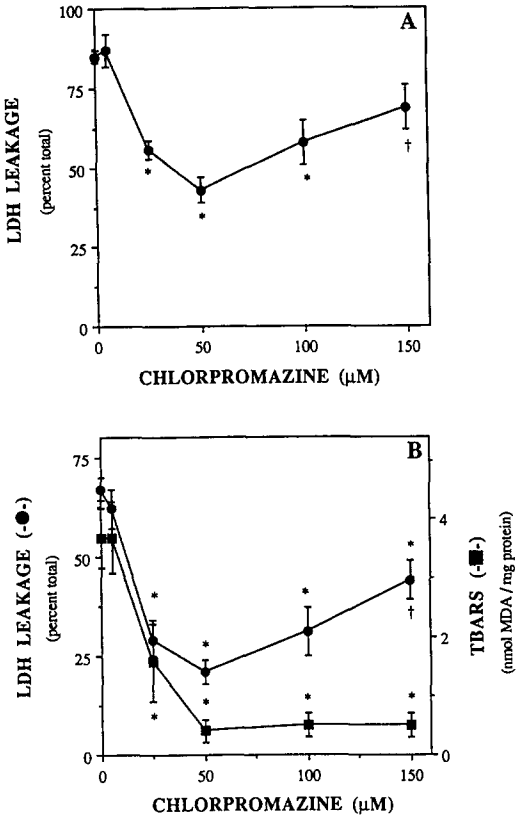

Fig. 4. Concentration-dependent effects of CPZ on ionomycin-induced and EA-induced lethal cell injury to cardiomyocytes. Cardiomyocytes were incubated with either: (A) ionomycin in modified HBSS (●) in the presence of 0–150 μM CPZ. Cell injury was determined at 2 hr by the release of LDH into the incubation medium. Key: (*) Significantly different from cultures incubated with ionomycin alone ($P < 0.01$), or (†) significantly higher than incubations with ionomycin plus 50 μM CPZ ($P < 0.01$), or (B) EA (150 μM) in the presence of 0–150 μM CPZ. Cell injury was determined at 2 hr by the release of LDH into the incubation medium (●). Lipid peroxidation was also determined by measuring TBARS (■). Values are the means ± SEM of measurements from 6–10 separate cardiomyocyte preparations. Key: (*) Significantly different from cultures incubated with EA alone ($P < 0.01$), or (†) significantly higher than incubations with EA plus 50 μM CPZ ($P < 0.01$).

Table 1. Effect of CPZ on [^{3}H]TPMP$^+$ retention during EA and ionomycin treatments

Treatment	% of Total [^{3}H]TPMP$^+$ 20 min	60 min
Control	66 ± 9	69 ± 11
Ionomycin	58 ± 9	
Ionomycin + CPZ	25 ± 6*	
EA		52 ± 11*
EA + CPZ		26 ± 8*
CPZ 50 μM	67 ± 13	58 ± 9
150 μM	27 ± 4*	
CCCP 25 μM	25 ± 4*	

Cardiomyocytes were incubated in culture medium containing [^{3}H]TPMP$^+$ for 60 min, rinsed twice with HBSS, and then treated with either 4 μM ionomycin or 150 μM EA in the absence or presence of 50 μM CPZ, or with CPZ alone. An additional 15 min incubation in HBSS with or without CPZ preceded the 20 min control, ionomycin, and CPZ treatments. At the times indicated, the percentage of the total [^{3}H]TPMP$^+$ retained was determined, as described in the text, and adjusted for LDH leakage. Values are means ±SD (N = 6–21).

* Significantly different from respective EA, ionomycin or control treatments at $P < 0.01$.

Table 2. Effect of CPZ on ATP content of EA-treated cardiomyocytes

Treatment	(nmol/ATP mg protein)
Control	30 ± 8
150 μM EA	26 ± 6
50 μM CPZ	25 ± 3
150 μM EA + 50 μM CPZ	12 ± 4*

Cardiomyocytes were incubated with either 150 μM EA in the absence or presence of 50 μM CPZ, or with 50 μM CPZ alone. At 60 min, the cardiomyocyte ATP content was determined, as described in the text. Values are means ± SD (N = 7–14).

* Significantly different from vehicle control at $P < 0.01$.

uncoupling activity that could limit the protective action of CPZ.

CPZ also affected the $\Delta\Psi$ of EA-treated cardiomyocytes (Table 1). After a 60 min treatment with either 50 μM CPZ or 150 μM EA, [^{3}H]TPMP$^+$ retention was close to that of vehicle controls. CPZ in combination with EA, however, reduced TPMP$^+$ retention to levels seen with the uncoupler CCCP (Table 1). The effect of CPZ together with EA on $\Delta\Psi$ was accompanied by a corresponding effect on cardiomyocyte ATP content. After a 60 min exposure to either 150 μM EA or 50 μM CPZ, cellular ATP levels were not significantly different ($P > 0.1$) from controls (Table 2). In combination, however, these agents decreased cardiomyocyte ATP levels by 54%. Thus, while 50 μM CPZ alone did not affect $\Delta\Psi$ or ATP levels markedly, in concert with either toxic challenge, 50 μM CPZ did compromise cardiomyocyte energy metabolism. Such a direct effect on the $\Delta\Psi$ might explain the residual toxicity seen with optimally protective CPZ concentration.

Since an uncoupling effect could explain the limited nature of CPZ protection, the toxicity of an uncoupler was examined for comparison. The kill curves for incubations containing 1 and 25 μM CCCP are plotted in Fig. 5, along with those from incubations containing either ionomycin plus 50 μM CPZ or EA plus 50 μM CPZ. It is interesting to note that although uncoupling was complete by 20 min after treatment with 25 μM CCCP, it took roughly 1 hr before maximal LDH leakage occurred. More importantly, both the onset and magnitude of cell

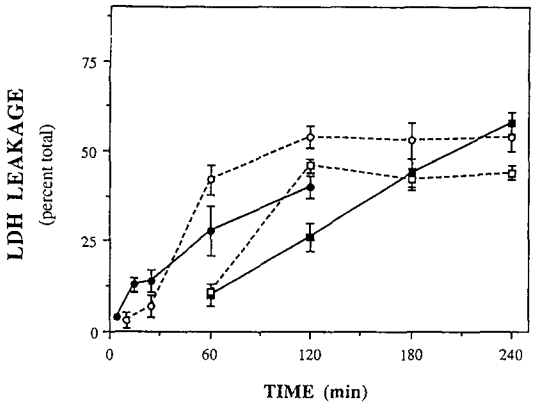

Fig. 5. Comparison of lethal cell injury induced by ionomycin plus CPZ, EA plus CPZ, or the uncoupler CCCP. Cardiomyocytes were incubated with either 4 μM ionomycin plus 50 μM CPZ (●), 150 μM EA plus 50 μM CPZ (■), 1 μM CCCP alone (□), or 25 μM CCCP alone (○). Lethal cell injury was determined at the times indicated based on LDH leakage. Values are the means ± SEM of measurements from 3–10 separate cardiomyocyte preparations.

killing seen with either 25 μM CCCP or ionomycin plus 50 μM CPZ were essentially identical. In addition, while kill curves for both 1 μM CCCP or EA plus 50 μM CPZ were similar, in each case maximal LDH leakage was observed approximately 1 hr after that seen with either 25 μM CCCP or ionomycin plus CPZ. Since CPZ in combination with either challenge collapsed $\Delta\Psi$ to the same extent as did CCCP (Table 1), these results suggest that uncoupling is sufficient to account for both the timing and magnitude of residual cell killing observed with CPZ in combination with either challenge.

DISCUSSION

In the current study, the action of CPZ on two distinct toxic challenges, one involving the elevation of $[Ca^{2+}]_i$ by ionomycin and the other the induction of endogenous oxidative stress with the GSH depletor EA, was examined in cultured cardiomyocytes. While CPZ reduced the cytotoxicity of both challenges, the protective action of CPZ appeared to be offset by a CPZ-induced collapse of the mitochondrial $\Delta\Psi$ and a subsequent reduction of cardiomyocyte ATP content.

Earlier studies suggested that CPZ blocked the toxic effects of elevated $[Ca^{2+}]_i$ by inhibiting the Ca^{2+}-dependent phospholipase A_2 [4–6, 42]. Subsequent work demonstrated that the Ca^{2+}-antagonism of CPZ was based on the inhibition of calmodulin-mediated processes [7, 8], prompting the use of the drug to examine mechanisms of Ca^{2+}-dependent cell death [9–13, 15]. The Ca^{2+}-dependent toxic challenge used in the current study has been examined recently in cardiomyocytes.* Briefly, conditions were selected that elevated $[Ca^{2+}]_i$ to cytotoxic levels directly with ionomycin, yet did not seriously compromise mitochondrial function. Although the challenge caused a partial depletion of ATP, this was reversible and apparently due to enhanced ATP utilization by Ca^{2+} extrusion pumps, rather than to mitochondrial impairment. Furthermore, results of inhibitor studies indicated that the primary cause of cardiomyocyte injury was Ca^{2+}-dependent phospholipase C activation, and not ATP depletion or oxidative stress.* A preliminary study indicated that 50 μM CPZ reduces the toxicity of this challenge significantly [35], and the results described herein reveal several actions of CPZ on challenged cardiomyocytes. For example, the termination of ionomycin-induced cardiomyocyte phosphorylase *a* activation by CPZ is consistent with its role as a calmodulin inhibitor. Such an inhibition of degradative enzymes regulated by calmodulin may explain the protective action of CPZ toward Ca^{2+}-dependent cell death. However, ionomycin-induced ATP depletion was enhanced by a CPZ concentration that was nontoxic from the standpoint of both LDH leakage and energy metabolism. In addition, while neither ionomycin nor CPZ alone had any substantial effect on $\Delta\Psi$, in combination these agents rapidly collapsed the $\Delta\Psi$. In light of these findings it is not surprising that CPZ did not eliminate completely ionomycin toxicity. To gain a better understanding of the overall significance of these results, the effect of CPZ was examined on a different type of challenge, one where oxidative stress is the predominant toxic event [36].

A variety of studies have provided evidence that lends support to the proposal that CPZ protection may also be due to an antioxidant effect of the drug [19–30]. It is not clear, however, whether inhibition of lipid peroxidation is due to a direct, radical chain-breaking action of CPZ [23], or to physio-chemical alterations of membrane lipids induced by the drug [49]. The cardiomyocyte model of oxidative stress used in the current study has been defined recently [36]. In this model, the nonoxidizing alkylating agent EA, at a concentration of 150 μM, effectively eliminates cardiomyocyte GSH within 1 hr and induces substantial cell injury by 2 hr. The resulting cell death is due primarily to the endogenous oxidative stress that originates at the mitochondrial respiratory chain. Although $[Ca^{2+}]_i$ is elevated during this challenge, any toxic contribution of Ca^{2+} is considered to be secondary to the oxidative damage. As with earlier studies of oxidative insult, CPZ demonstrated antioxidant properties in the EA model. However, while the drug totally eliminated the peroxidative damage, it only partially ameliorated the cytotoxicity of EA. This finding is in contrast to results obtained with the antioxidant DPPD, which completely eliminated both the EA-induced lipid peroxidation and cytotoxicity. It appears that, in addition to the protective antioxidant effect, a toxic action of CPZ surfaces during the EA challenge and, as with the ionomycin challenge, this toxic action appears to be directed toward cardiomyocyte energy metabolism. For example, while EA alone at 60 min had little effect on energy metabolism, the combination of EA and CPZ markedly decreased

* Babson *et al.*, Manuscript submitted for publication.

cardiomyocyte ATP content and collapsed $\Delta\Psi$ (Tables 1 and 2).

It has been known for a considerable time that CPZ can uncouple oxidative phosphorylation [32–34], and this effect is consistent with the deenergization of cardiomyocytes observed in the current study. The data reveal two somewhat different situations, each of which would be expected to counteract the protective effects of CPZ. In the first and most obvious situation, a high concentration of CPZ (150 μM) deenergized unchallenged cardiomyocytes (Table 1). The second and more interesting situation is where the combination of CPZ at an optimally protective level of 50 μM and either of two toxic challenges collapsed cardiomyocyte $\Delta\Psi$, even though neither of the toxic challenges nor 50 μM CPZ alone seriously affected $\Delta\Psi$ (Table 1). Here, intracellular perturbations, possibly common to both challenges, appear to promote uncoupling by CPZ. One possible example is Ca^{2+} which has the potential to disrupt mitochondrial function [42] and was increased by both challenges. Although it appears that neither challenge elevated $[Ca^{2+}]_i$ to levels that alone markedly affected $\Delta\Psi$ (Table 1), it is possible that CPZ sensitized the mitochondria to these levels of $[Ca^{2+}]_i$, or, conversely, that elevated $[Ca^{2+}]_i$ sensitized the mitochondria to a lower concentration of CPZ. The temporal relationships between $[Ca^{2+}]_i$ elevation, depressed energy metabolism, and cell injury are also consistent with a role for Ca^{2+}. Although this is an interesting possibility, others exist, and additional studies will be required to gain a clearer understanding of what intracellular conditions do contribute to the residual injury observed with CPZ.

While the results of this study suggest that it is the uncoupling activity of CPZ that limits the protective action of the drug against two important types of toxic challenges, other possible interpretations warrant consideration. For example, the observed limits of CPZ protection could be imposed by a toxic action of CPZ unrelated to uncoupling. This seems less likely for two reasons. First, 50 μM CPZ in combination with either ionomycin or EA uncoupled cardiomyocyte mitochondria as effectively as a known uncoupler, CCCP (Table 1). Second, both the timing of the onset and the degree of cell injury seen with CPZ in challenged cells were comparable to that observed with an uncoupler alone (Fig. 5). Thus, the residual cell injury observed with CPZ in combination with either challenge can be explained entirely on the basis of an uncoupling action of the drug. Indeed, earlier results suggested that the protective effects of phenothazines may be limited by morphologic alterations of cardiomyocyte mitochondria caused by these drugs [11]. Therefore, the observed CPZ-induced collapse of $\Delta\Psi$ (Table 1) may reflect such morphologic alterations. It also can be argued that the residual cell injury results because CPZ only partially suppresses the primary toxic mechanism of each challenge. Attempts to test this possibility with the ionomycin challenge were stymied by the fact that CPZ interfered with the assay of the Ca^{2+}-dependent phospholipase C activation believed to be the predominant toxic event for this challenge in cardiomyocytes. However, the validity of this alternative interpretation was evaluated for the EA challenge, and it does not appear to be a likely explanation. EA toxicity is caused by oxidative injury associated with TBARS formation [36]. The antioxidant DPPD prevented TBARS formation and lowered LDH leakage to control levels. CPZ also completely suppressed TBARS formation (Fig. 4B), yet only partially decreased cell injury to a level observed with the uncoupler CCCP (Fig. 5). These considerations, taken together with other results of this study, suggest that the limited protection afforded by CPZ is less likely to be due to a partial suppression of either toxic mechanism or some toxic action of CPZ unrelated to uncoupling, but rather to a direct action of CPZ on mitochondrial function that is unmasked in the challenged cardiomyocytes.

The clinical importance of CPZ is underscored by its widespread use as an antipsychotic agent. In addition, results of numerous studies cited herein support the logic of an expanded clinical role for CPZ directed toward the prevention of cellular damage resulting from certain pathologic conditions and chemical challenges. However, data from the current study point to a biochemical basis for caution in both the current and expanded clinical use of this drug. A key reason for this caution is the potentially toxic action of CPZ toward mitochondrial function that can be enhanced by intracellular conditions accompanying perturbations of intracellular thiol status and Ca^{2+} homeostasis. The fact that these two toxic events are central to a wide variety of chemical and pathologic insults [16–18] suggests that the results of the current study may have broad clinical implications.

In sum, the results of this study demonstrate that CPZ can antagonize two fundamental mechanisms of lethal cell injury in cardiomyocytes, one where the primary cytotoxic event is Ca^{2+}-dependent and another where the toxicity is due primarily to oxidative stress. However, a CPZ-induced depression of mitochondrial function is promoted by intracellular perturbations accompanying either toxic challenge, and this counteracts the protective effects of the drug. The data illustrate the complexity of using a nonspecific inhibitor such as CPZ to explore mechanisms of cell death. These data also suggest that the clinical use of CPZ to alleviate toxicity based on mechanisms that involve elevated $[Ca^{2+}]_i$ or oxidative damage may have limitations and could, under certain circumstances, actually exacerbate an existing toxic situation.

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