Biochemical Pharmacology, Vol. 33, No. 12, pp. 1989–1992, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00
Pergamon Press Ltd.

ROLE OF CALCIUM IN ISOPROTERENOL CYTOTOXICITY TO CULTURED MYOCARDIAL CELLS

KENNETH RAMOS, ALAN B. COMBS and DANIEL ACOSTA*
Division of Pharmacology and Toxicology, College of Pharmacy, University of Texas, Austin, TX 78712, U.S.A.

(*Received* 17 *June* 1983; *accepted* 18 *November* 1983)

Abstract—Primary cultures of rat myocardial cells were used to evaluate the cellular dynamics of calcium accumulation after exposure to isoproterenol (ISO). Non-toxic concentrations of ISO (2.4×10^{-7} M) caused a gradual increase in myocyte calcium uptake. These effects peaked 3 min after exposure and returned to control levels within 2 min. Toxic concentrations of ISO caused a biphasic increase in calcium uptake. The initial phase peaked 1 min after exposure and returned to control levels by 3 min. A second phase was characterized by a progressive increase in calcium uptake that plateaued 10 min after exposure. Ascorbic acid (AA, 5×10^{-3} M) and sodium bisulfite (SB, 9.6×10^{-4} M) did not modify the calcium uptake of the initial phase, whereas propranolol (1×10^{-6} M) and verapamil (1×10^{-5} M) prevented the initial rise in calcium uptake. In contrast, the antioxidants prevented the second phase of ISO-induced calcium uptake, whereas verapamil and propranolol did not. The toxic accumulation of calcium induced by ISO may be due to oxidative damage of the sarcolemma. Antioxidants may prevent the formation of oxidative metabolites from ISO and the subsequent calcium overload. Our results show that agents which modify slow calcium-channel transport do not prevent ISO-induced calcium overload in our cell culture system.

Myocardial cell injury is observed after exposure of the heart to high concentrations of ISO† and related catecholamines. The mechanism by which these compounds damage the heart is not clear. Several investigators suggest that cardiotoxicity results from excessive cardiac stimulation causing abnormal calcium accumulation within myocardial cells [1–4]. More recently, however, oxidative metabolites of catecholamines have been implicated in the production of myocardial cell damage [5, 6].

The major metabolic pathways for the degradation of catecholamines involve two enzyme systems, catechol-*O*-methyl transferase and monoamine oxidase. Catechol-*O*-methyl transferase catalyzes the formation of *O*-methyl derivatives. Monoamine oxidase activity results in the formation of products in which the ethanolamine side chain of the molecule undergoes oxidative deamination. Singal and coworkers [6] have proposed that high levels of catecholamines may saturate these enzyme systems and allow alternative pathways to catalyze the formation of aminochromes and other oxidative products. These products produce cellular damage and contractile failure in the isolated perfused heart [5–7].

In the present study, primary cultures of rat heart myocytes were used as a model to evaluate temporal changes in myocyte calcium uptake induced by isoproterenol. *In vitro* models of cardiotoxicity offer a number of advantages over *in vivo* testing. Changes which occur shortly after exposure to cardiotoxins can be easily examined, and alterations in cell function can be detected before severe damage occurs. Various pharmacological agents were tested to study the mechanism by which ISO alters sarcolemmal calcium permeability. Antioxidants were evaluated to determine if agents which prevent the oxidation of catecholamines reduce ISO-induced calcium accumulation. Agents which block or alter slow calcium-channel transport were also examined.

Our results suggest that calcium accumulation in cultured myocardial cells after exposure to toxic levels of isoproterenol is not related to calcium-channel mediated transport. Accumulation of calcium is most likely the result of non-specific processes of calcium uptake. Oxidative degeneration of the sarcolemma induced by oxidative metabolites of ISO may be responsible for the altered sarcolemmal permeability to calcium.

MATERIALS AND METHODS

Cell culture technique. Hearts from 3- to 5-day-old Sprague–Dawley rats (N = 25) were minced into small fragments and dissociated with 0.15% trypsin (Difco 1:250) in Earle's balanced salt solution. Two million cells were plated in 35 mm culture dishes (Falcon). Muscle cells were separated from non-muscle cells by a pour-off technique based on the rate of attachment of the cells to the culture dishes [8]. Myocyte cultures were maintained in 2 ml of Eagle's minimum essential medium (MEM) with 5% newborn calf serum (GIBCO). The medium was replaced 4 days after initial plating. All solutions were sterilized by passage through a Millipore filter (pore size 0.22 μm). Cells were grown in a humidified

* Author to whom correspondence should be addressed.
† Abbreviations: ISO, isoproterenol; AA, ascorbic acid; and SB, sodium bisulfite.

BP 33:12-K

environment (37°) of 5% CO_2/95% air (about 20% O_2) to maintain a pH of 7.2 to 7.4.

Drug treatments. Experiments were conducted 4–5 days after initial plating of the cells. The following pharmacological agents were tested: ISO, AA, SB, propranolol and verapamil. These agents were dissolved in medium designed for uptake studies (see $^{45}Ca^{2+}$ uptake determinations). In the case of ISO, precautions were taken to avoid contact of the drug with metallic surfaces to minimize the degree of autooxidation.

Drug stock solutions were freshly prepared and stored in light-resistant containers. Serum was excluded from these solutions to prevent binding of the drug to macromolecules present in the serum. Aliquots of drug stock solutions were added to the culture dishes to give the desired final concentration. Addition of antioxidants did not cause significant pH changes in the culture medium as indicated by phenol red and pH measurements. Concentrations of ISO tested were: 2.4×10^{-7}, 2.4×10^{-5}, 1×10^{-4} and 5×10^{-4} M. Ascorbic acid and SB were added to the cultures to give a final concentration of 5×10^{-4} and 9.6×10^{-4} M respectively. Verapamil was tested in the following concentrations: 1×10^{-5}, 1×10^{-6} and 1×10^{-7} M, whereas the concentrations of propranolol tested were: 1×10^{-4}, 1×10^{-5} and 1×10^{-6} M.

$^{45}Ca^{2+}$ uptake determinations. A method reported by Fosset *et al.* [9] was modified and used to measure $^{45}Ca^{2+}$ uptake. The culture medium was removed and replaced with 2 ml of medium designed for uptake studies. This standard medium consisted of a 25 mM Hepes [4-(2-hydroxyethyl)-1-piperazinethanesulfonate] buffer adjusted to pH 7.4 with Tris–HCl. Other constituents of the medium included NaCl (140 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), $MgSO_4$ (0.8 mM), and glucose (5 mM). Cells were equilibrated for 30 min after which an aliquot of $^{45}Ca^{2+}$ (New England Nuclear) stock solution alone or in combination with test agents was added to the dishes. The final concentration of $^{45}Ca^{2+}$ in the dishes was 0.6 μCi/ml. The radioactive medium was removed at the end of the desired exposure periods. Cells were washed three times with 1.5 ml of washing medium. This medium contained 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.5 mM lanthanum and 25 mM Tris buffer (pH 7.4, 20°). The washing time was about 20 sec. This short period was sufficient time to remove extracellular radioactive ions without loss of intracellular $^{45}Ca^{2+}$ [9]. The cells were suspended in 1.5 N NaOH, and an aliquot was taken for protein determinations. The final suspension was combined with 10 ml of cocktail solution (Ready-Solv EP), and the radioactivity was measured in a liquid scintillation spectrometer (Beckman model LS 1800). A "zero time" assay was conducted by adding $^{45}Ca^{2+}$ to the cultures and immediately (~10 sec) washing the cells by the standard procedure. The zero time value was used to normalize the uptake measurements of control and treated cultures.

Statistical analysis. Analysis of variance and Scheffe's comparisons were used to assess the statistical significance of the differences observed, $P < 0.001$. Values always represent mean ± S.E.M.

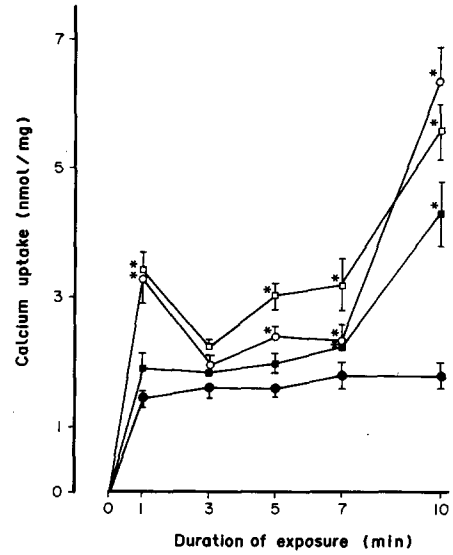

Fig. 1. Myocyte calcium uptake after exposure to isoproterenol for various time periods. Values represent the mean ± S.E.M. Key: (*) significantly different from their respective controls ($P < 0.01$), N = 8–10 replicate cultures; (●) untreated control; (■) 2.4×10^{-5} M ISO; (○) 1×10^{-4} M ISO; and (□) 5×10^{-4} M ISO.

RESULTS

The calcium uptake of cultured myocytes after exposure to toxic levels of ISO for various times is shown in Fig. 1. Isoproterenol induced a biphasic increase in myocyte calcium uptake. The initial phase peaked 1 min after exposure and returned to control levels within 3 min. Subsequently, a second phase was identified. This phase was characterized by a progressive, dose-dependent increase in calcium uptake that plateaued 10 min after exposure and was maintained for at least 30 min.

A comparison between calcium uptake in cultures exposed to a toxic dose of ISO (5×10^{-4} M) and a non-toxic dose (2.4×10^{-7} M) is presented in Fig. 2.

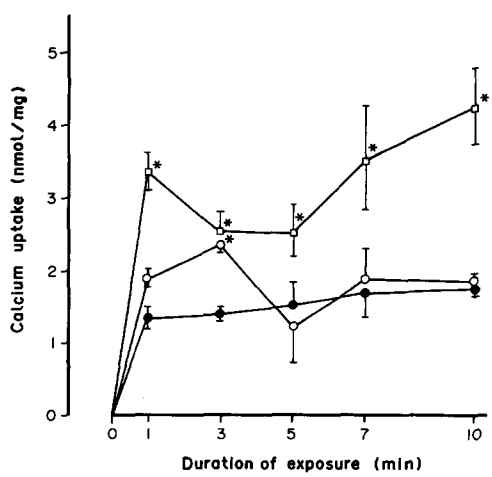

Fig. 2. Myocyte calcium uptake after exposure to 2.4×10^{-7} M and 5×10^{-4} M isoproterenol for various time periods. Values represent the mean ± S.E.M. Key: (*) significantly different from their respective controls ($P < 0.01$), N = 4 replicate cultures; (●) control; (○) 2.4×10^{-7} M ISO; and (□) 5×10^{-4} M ISO.

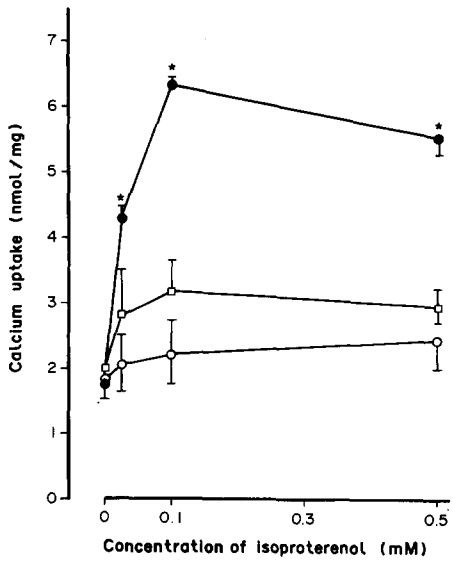

Fig. 3. Myocyte calcium uptake induced by isoproterenol alone or in the presence of ascorbic acid (5×10^{-3} M) or sodium bisulfite (9.6×10^{-4} M) 10 min after exposure. Cultures concurrently exposed to isoproterenol and ascorbic acid or sodium bisulfite were compared to untreated controls and respective antioxidant controls. Values represent the mean ± S.E.M. Key: (*) significantly different from their respective controls ($P < 0.01$), N = 5–10 replicate cultures; (●) ISO alone; (□) ISO + AA; and (○) ISO + SB.

A gradual increase in myocyte calcium uptake was observed with the non-toxic dose of ISO. This effect peaked 3 min after exposure and returned to control levels within 2 min. As observed in Figs. 1 and 2, a biphasic response was observed in myocyte cultures exposed to toxic levels of ISO. There was an initial increase in calcium uptake which was higher than that observed after non-toxic exposure to 2.4×10^{-7} M ISO. A second phase was evident 7 min after toxic exposure to ISO. There was a progressive increase in calcium uptake which never returned to control levels as was observed with the non-toxic exposure to ISO.

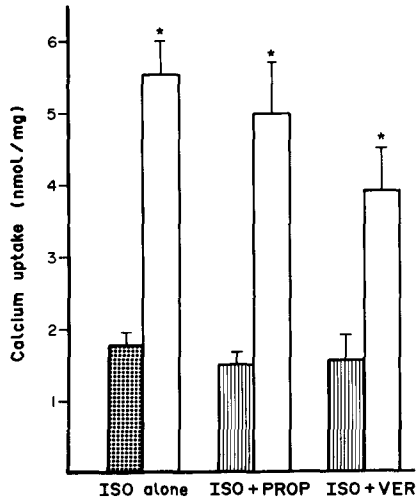

Fig. 4. Effects of propranolol (1×10^{-6} M) and verapamil (1×10^{-5} M) on myocyte calcium uptake induced by 5×10^{-4} M ISO 10 min after exposure. Values represent the mean ± S.E.M. Key: (*) significantly different from their respective controls ($P < 0.01$), N = 5–10 replicate cultures; (▦) untreated control; (▥) respective antagonist control; and (□) 5×10^{-4} M ISO.

Table 1. Myocyte calcium uptake induced by isoproterenol alone and in the presence of various pharmacological agents 1 min after exposure

Treatment	Calcium uptake* (nmoles/mg)
Untreated control†	1.45 ± 0.3
ISO alone (5×10^{-4} M)	3.4 ± 0.31‡
ISO + AA (5×10^{-3} M)	3.34 ± 0.5‡
ISO + SB (9.6×10^{-4} M)	4.31 ± 0.94‡
ISO + verapamil (1×10^{-5} M)	1.97 ± 0.3
ISO + propranolol (1×10^{-6} M)	2.05 ± 0.5

* Values represent the mean ± S.E.M., N = 5 replicate cultures.

† Values of antioxidant, propranolol and verapamil controls were similar to those of untreated controls.

‡ Significantly different from their respective controls ($P < 0.01$).

The calcium uptake induced by ISO alone or in the presence of AA or SB after a 10-min exposure is shown in Fig. 3. The uptake observed in cultures concurrently treated with ISO and AA or SB was lower than that of cultures treated with ISO alone. The effects of propranolol (1×10^{-6} M) and verapamil (1×10^{-5} M) on the calcium uptake observed 10 min after exposure to 5×10^{-4} M ISO are shown in Fig. 4. Verapamil and propranolol slightly reduced the calcium uptake induced by ISO. However, these effects were not statistically significant. Similar results were obtained when other concentrations of propranolol (1×10^{-5} and 1×10^{-7} M) or verapamil (1×10^{-4} and 1×10^{-6} M) were tested (data not shown).

In contrast to the data presented in Fig. 4, Table 1 shows the calcium uptake observed after exposure of cultured heart cells to ISO alone (5×10^{-4} M) and in the presence of various agents for 1 min. Verapamil (1×10^{-5} M) and propranolol (1×10^{-6} M) considerably reduced ISO-induced calcium uptake, whereas AA and SB did not.

DISCUSSION

In previous studies we have shown that high concentrations of ISO cause sarcolemmal and mitochondrial injury to cultured myocytes [10, 11]. Injury to the inner mitochondrial membrane was observed 1.5 hr after exposure to concentrations ≥ than 2.4×10^{-5} M ISO. Leakage of intracellular enzymes and ions was observed only after prolonged exposures (≥4 hr). Ascorbic acid (5×10^{-3} M) and SB (9.6×10^{-4} M) prevented the loss of cell viability, mitochondrial damage, and leakage of potassium and cytosolic enzymes produced by toxic concentrations of ISO [10, 11]. This cytotoxicity study on ISO and the antioxidants suggests that the formation of oxidative intermediates may be a significant factor in the cardiotoxicity of ISO.

The calcium uptake patterns induced by non-toxic and toxic concentrations of ISO in cultured myocardial cells were markedly different. Non-toxic concentrations of ISO caused a gradual increase in calcium uptake that lasted for several minutes, whereas toxic concentrations induced a biphasic response. The initial phase of calcium uptake observed after exposure of cultured cells to toxic concentrations of ISO may represent beta-adrenoceptor-mediated calcium-channel transport. Propranolol, a beta-receptor antagonist, blocked this phase. This observation is in agreement with previous reports by other investigators which showed that propranolol (10^{-6} M) completely prevented the rise of cyclic AMP induced by 10^{-4} M ISO [12]. In addition, agents which prevent slow calcium-channel mediated transport (such as verapamil) prevented its occurrence. The initial phase of calcium uptake was not observed in cultures exposed to 2.4×10^{-5} M ISO. The magnitude of this phase may be determined by the interaction of unoxidized ISO with beta-adrenoceptors. The residual amount of unoxidized ISO in cultures exposed to 2.4×10^{-5} M may not be sufficient to cause a significant increase in calcium uptake. The effects of propranolol and verapamil on the initial phase of calcium uptake induced by ISO suggest that this phase depends on beta-receptor-mediated calcium transport. This concept is supported by preliminary studies which showed that both compounds antagonized the increased calcium uptake induced by non-toxic concentrations of ISO (2.4×10^{-7} M) in a dose-dependent manner (data not shown).

The second phase of calcium uptake, observed only after exposure to toxic concentrations of ISO, was characterized by progressive calcium accumulation. This phase was not antagonized by propranolol or verapamil, but AA and SB prevented its occurrence. These observations suggest that the second phase of the uptake curve is not a beta-receptor-mediated calcium-channel response.

Accumulation of large amounts of calcium within myocardial cells may alter the integrity and function of several membrane systems. In the case of mitochondria, accumulation of calcium results in deficient energy production leading to contracture and activation of proteinases and phospholipases [13, 14]. Activation of these degradative enzymes may inhibit membrane-bound enzymes such as Na^+/K^+-ATPase. As a result, there is an increase in Na^+ levels and loss of cytoplasmic K^+. The increased Na^+ content would further enhance calcium accumulation through the Na^+–Ca^{2+} exchange system. The summation of these alterations leads to cellular dysfunction and cardiotoxicity.

Recently, it has been suggested that oxidative degeneration of membrane lipids results in increased calcium permeability [15]. One could speculate that oxidative metabolites of ISO interact with the lipid bilayer, cause sarcolemmal injury, and alter calcium regulatory mechanisms. Additional data are required to further clarify this concept. Such an approach may help unify current theories of chemical-induced cytotoxicity in which calcium ions mediate cellular injury [16, 17].

Usually, metabolism serves to increase the water solubility and elimination of foreign substances. However, drug metabolism does not always result in detoxication. Many compounds are metabolically activated to reactive products that are highly cytotoxic. Acetaminophen and cyclophosphamide serve as good examples. The concept of metabolic activation by cardiovascular tissues has not received much attention. Cardiac enzymes and mitochondria may catalyze the oxidation of ISO to form reactive intermediates [18–20]. These reactive metabolites may participate in ISO-induced cardiotoxicity. Antioxidants may prevent the oxidation of ISO and, thus, the formation of reactive intermediates that cause cytotoxicity. This is supported by previous studies in which (1) antioxidants suppressed free radical formation induced by ionizing radiation and carcinogenic compounds [21], and (2) AA prevented acetaminophen-induced hepatotoxicity [22]. These observations suggest that antioxidants may prove useful in preventing various forms of toxic injury in which reactive intermediates may be involved. Finally, the present study suggests that many concepts in the area of drug-induced cardiomyopathies may require re-evaluation.

REFERENCES

1. A. Fleckenstein, in *Calcium and the Heart* (Eds. P. Harris and L. Opie), p. 135. Academic Press, London (1971).
2. T. Balazs and V. J. Ferrans, *Environ. Hlth Perspect.* **26**, 181 (1978).
3. H. Reuter, *J. Physiol., Lond.* **242**, 429 (1974).
4. L. H. Opie and F. T. Thandroyen, *Fedn Proc.* **42**, 2465 (1983).
5. J. C. Yates and N. S. Dhalla, *J. molec. cell. Cardiol.* **7**, 807 (1975).
6. P. K. Singal, K. S. Dhillon, R. E. Beamish, N. Kapar and N. S. Dhalla, *Br. J. exp. Path.* **63**, 167 (1982).
7. N. S. Dhalla, J. C. Yates, S. L. Lee and A. Singh, *J. molec. cell. Cardiol.* **10**, 31 (1978).
8. D. G. Wenzel, J. W. Wheatley and G. D. Byrd, *Toxic. appl. Pharmac.* **17**, 77 (1978).
9. J. Fosset, J. DeBarry, M-C. Lenoir and M. J. Lazdunski, *J. biol. Chem.* **252**, 6112 (1977).
10. K. Ramos and D. Acosta, *Toxicology* **26**, 81 (1983).
11. K. Ramos, A. B. Combs and D. Acosta, *Toxic. appl. Pharmac.* **70**, 317 (1983).
12. T. J. C. Higgins, D. Allsopp and P. J. Bailey, *J. molec. cell. Cardiol.* **11**, 101 (1979).
13. N. G. Nayler, *Am. J. Path.* **102**, 262 (1981).
14. J. B. Bassingthwaighte and H. Reuter, in *Electrical Phenomena in the Heart* (Ed. W. C. De Mello), p. 353. Academic Press, New York (1972).
15. A. V. Lebedev, D. O. Levitsky, V. A. Loginov and V. N. Smirnov, *J. molec. cell. Cardiol.* **14**, 99 (1982).
16. J. L. Farber, K. R. Chien and S. Mittnacht, *Am. J. Path.* **102**, 271 (1981).
17. F. A. X. Schanne, A. B. Kane, E. E. Young and J. L. Farber, *Science* **206**, 700 (1979).
18. S. Gubbjarnason, B. Doell and G. Oskarsdottir, *Acta biol. med. germ.* **37**, 777 (1978).
19. M. D. Hawley, S. B. Tatawawadi, S. Pckarski and R. N. Adams, *J. Am. chem. Soc.* **89**, 447 (1967).
20. H. P. Misra and I. Fridovich, *J. biol. Chem.* **247**, 3170 (1972).
21. J. A. Hinson, S. D. Nelson and J. R. Gillette, *Molec. Pharmac.* **15**, 419 (1979).
22. R. J. Hargreaves, J. G. Evans, D. Pelling and K. R. Butterworth, *Toxic. appl. Pharmac.* **64**, 380 (1982).