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SHORT COMMUNICATIONS

Effects of doxorubicin and verapamil on calcium uptake in primary cultures of rat myocardial cells

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Doxorubicin is a very effective antineoplastic agent, but its clinical use is limited by cardiotoxicity [1, 2]. Although the mechanism for the myocardial injury is not clear, the toxicity has been related to oxidative stress leading to peroxidative injury [3], to disturbances in energetics [4–6], and to disturbances in calcium homeostatis [7]. Calcium overload can result in depletion of high energy phosphates and cause cellular injury [8].

We have shown that prolonged exposures (≥12 hr) of cultured heart cells to doxorubicin causes arrhythmias and morphological changes, some of which are preventable by ubiquinone [9]. Lowe and Smallwood [10] also demonstrated a toxic effect of doxorubicin on isolated rat myocites. Dasdia *et al.* [11] studied the effect of doxorubicin on calcium exchange in cultured mouse heart cells. The present study was designed to evaluate calcium uptake after brief exposures of cultured heart cells to low concentrations of doxorubicin. The effect of verapamil on this system also was evaluated.

Materials and methods

Cell culture technique. Hearts from 3 to 5-day-old Sprague–Dawley rats were used to prepare primary cultures according to a previously described method [12].

Drug treatments. Experiments were conducted 4–5 days after initial plating of the cells. Doxorubicin and verapamil were freshly prepared by dissolution in medium specifically designed for uptake studies [12]. Levels of doxorubicin tested were 5×10^{-7}, 2×10^{-7}, and 5×10^{-8} M which are similar to levels attained clinically [18]. Verapamil was tested at 1×10^{-6} and 1×10^{-5} M.

^{45}Calcium uptake determinations. The method of Fosset *et al.* [13] was modified and used to measure ^{45}calcium uptake [12].

Results and discussion

The data in Table 1 show that doxorubicin induced a dose-dependent increase in calcium accumulation in cultured rat cardiac myocytes. This increase was biphasic in that it appeared within a minute, was gone within 1.5 min, and then started to increase again, approaching significance by 7 min and reaching it in 10 min. Table 2 shows that both phases of the increased calcium uptake were blocked in a dose-dependent manner by verapamil, indicating that the uptake involves the voltage-dependent calcium channels [14]. The return to control levels following the initial increase in calcium was probably due to normal intracellular calcium removal processes triggered by the calcium influx [14]. These conclusions are compatible with the work of Lazarus *et al.* [15] who showed that doxorubicin-induced alteration in calcium conductance leads to a prolonged action potential duration in rat papillary muscles. These conclusions also are consistent with the work of Azuma *et al.* [16] who showed that doxorubicin increases slow channel calcium influx into chick hearts. In addition, a weak and transient positive inotropic effect of doxorubicin has been reported [17] that apparently does not involve the sodium pump [18]. The increase in channel-mediated calcium influx may explain the positive inotropic effect.

Table 1. Effect of doxorubicin on calcium uptake in cultured rat heart cells*

Duration of treatment (min)	Calcium uptake (nmoles/mg protein)			
	Treatment (molar concentration of doxorubicin):			
	Controls	5×10^{-8}	2×10^{-7}	5×10^{-7}
1.0	1.45† (±0.02)	4.09‡ (±0.91)	4.92§‖ (±0.72)	6.94¶ (±0.48)
1.5	1.46† (±0.10)	1.11** (±0.12)	1.63†† (±0.35)	1.77‡‡ (±0.19)
2.5	1.73† (±0.20)	1.15** (±0.09)	1.44†† (±0.08)	1.57‡‡ (±0.17)
3.0	1.66† (±0.22)	1.42‡** (±0.15)	1.19†† (±0.18)	1.24‡‡ (±0.11)
5.0	1.62† (±0.13)	1.24‡** (±0.15)	1.13†† (±0.24)	1.35‡‡ (±0.29)
7.0	1.54† (±0.32)	2.43‡** (±0.43)	2.54‖†† (±0.54)	3.83¶‡‡ (±0.63)
10.0	1.55† (±0.30)	4.03‡** (±0.70)	5.62§ (±0.75)	11.02§§ (±1.41)

* Values represent the mean ± S.E. (N = 4–6). Statistical comparisons were done by ANOVA, followed by Scheffe's method of multiple contrasts. The horizontal lines under rows are inclusive of groups that do not differ significantly from each other ($P \leq 0.01$). In the columns, groups superscripted with the same symbol do not differ significantly from each other ($P \leq 0.01$).

The second phase of increased calcium accumulation was reduced by verapamil, indicating that a calcium channel dependent process was involved in this uptake, also. Doxorubicin has several deleterious actions on the cell, including membrane peroxidation [3] and reduced energy availability [4–6]. Intracellular calcium removal and sequestration are intact membrane-dependent and energy-dependent processes [14] which may have become ineffective with time due to accumulated doxorubicin injury. Continued calcium influx would predominate, giving rise to the second phase of calcium uptake which could explain the increased cardiac calcium associated with doxorubicin toxicity [7].

Table 2. Effect of verapamil and doxorubicin on calcium uptake in cultured rat heart cells*

Duration of treatment (min)	Calcium uptake (nmoles/mg protein)					
	Controls (no drugs added)	Controls + Verapamil (10^{-6} M)	Controls + Verapamil (10^{-5} M)	Doxorubicin (5×10^{-7} M) + Verapamil (10^{-6} M)	Doxorubicin (5×10^{-7} M) + Verapamil (10^{-5} M)	Doxorubicin by itself (5×10^{-7} M)
1.0	1.54† (±0.06)	1.75‡ (±0.09)	1.48§ (±0.06)	4.07‖ (±0.26)	2.93¶ (±0.06)	6.94 (±0.48)
1.5	1.54† (±0.09)	1.28‡ (±0.09)	1.36§ (±0.14)	2.06** (±0.25)	1.60¶ (±0.28)	1.77 (±0.19)
10.0	1.46† (±0.16)	1.54‡ (±0.02)	1.55§ (±0.12)	2.36** (±0.23)	2.03¶ (±0.29)	11.02 (±1.41)

* The data for doxorubicin by itself were taken from Table 1 (these data were not used in the statistical calculations). The values represent the mean ± S.E. (N = 4–6). Statistical comparisons were done by ANOVA, followed by Scheffe's method of multiple contrasts. The horizontal lines under rows are inclusive of groups that do not differ significantly from each other ($P \leq 0.01$). In the columns, groups superscripted with the same symbol do not differ significantly from each other ($P \leq 0.01$).

The hope that calcium channel blocking agents might prevent the clinical toxicity of doporubicin appears to be unlikely. The combination of verapamil or nifedipine with doxorubicin is associated with *enhanced* toxicity [19, 20]. A possible explanation for this may be that the negative inotropic effect produced by such calcium blockers may have been additive with the cardiac failure induced by doxorubicin. In contrast, Lenzhofer *et al.* [21] reported protection with a regimen of nifedipine and tocopherol. It is difficult to draw conclusions from this study because tocopherol, by itself, has a protective action against doxorubicin toxicity [22].

In summary, doxorubicin caused a biphasic, dose-dependent increase in calcium entry into the cells at concentrations of 5×10^{-8}, 2×10^{-7}, and 5×10^{-7} M. The uptake was measurable at 1 min of exposure to the doxorubicin, was gone at 1.5 min, and was measurable again after 10 min of exposure. Both of these phases were reduced by verapamil. Doxorubicin appears to cause channel mediated influx of calcium. This process, acting in conjunction with other actions leading to reduced intracellular calcium removal, may explain the myocardial calcium accumulation that occurs with doxorubicin.

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