Calcium as Mediator of Isoproterenol-Induced Myocardial Necrosis

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Isoproterenol (ISO), a drug which causes an increased strength of myocardial contraction when administered to animals in μg/kg doses, causes myocardial necrosis when given mg/kg doses. Previous studies suggested that necrosis might be due to flooding of the heart muscle cells by calcium. To determine if this is true, and to distinguish between flooding due to release of Ca from sequestered intramyocardial compartments and increased influx from the blood, we have measured total myocardial calcium ($[Ca]_m$) after ISO administration. The concentration of myocardial calcium, measured by atomic absorption spectroscopy after dry ashing, increased within 10 minutes of the intraperitoneal injection of ISO in rats. After 10 minutes the $[Ca]_m$ remained constant at its new level for at least 50 minutes if the dose of ISO was 10^2 μg/kg or less but continued to rise at a slower rate than noted during the first 10 minutes if the ISO dose was 10^3 μg/kg or more. As measured 1 hour after ISO administration, the increase in $[Ca]_m$ was proportional to the dose, up to 10^2 μg/kg. At higher doses there was no further increase until the dose exceeded 2×10^3 μg ISO/kg. Since the amount of necrosis is proportional to dose from about 10^2 to 10^5 μg/kg, while the changes in $[Ca]_m$ are not proportional to dose over this entire range, it is concluded that ISO-induced myocardial necrosis is not mediated exclusively by flooding of heart muscle with plasma-derived calcium, although this is undoubtedly an important factor. This conclusion was further supported by experiments showing that propranolol, at doses which completely suppressed the increase in $[Ca]_m$ due to ISO, did not completely prevent necrosis. (Am J Pathol 69:459–470, 1972)

WITHIN MINUTES of the administration of large doses of isoproterenol (ISO) to rats, a sequence of ultrastructural changes occurs in the myocardium which culminates in overt necrosis.[1] These changes begin 2 minutes after the intraperitioneal injection of ISO with hypercontraction and mitochondrial swelling of afflicted cells. By 8 minutes the hypercontraction has advanced to the point of fusion of adjacent sarcomeres, forming "contraction bands." The swollen mitochondria now contain doughnut-shaped dense granules which are indistinguishable from calcium deposits. By 8 hours the

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Supported by Grant HL 13731 from the US Public Health Service and a grant from the Montana Heart Association.

Presented in part at the 1972 meeting of the International Study Group for Research in Cardiac Metabolism, Winnipeg, Canada.

Accepted for publication Sept 5, 1972.

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afflicted cells begin to fragment and phagocytosis can be observed. The early changes after ISO injection are quite different from those seen in necrosis due to ischemia, where relaxation rather than hypercontraction is the rule.[2] The sequence of ultrastructural events observed within 8 minutes of ISO administration suggests that necrosis is actually caused by excessive levels of Ca within the heart muscle cells. This could occur through a reduction in cellular ATP levels brought about by calcium activation of actomyosin ATPase [3] and uncoupling of oxidative phosphorylation.[4] The uncoupling of oxidative phosphorylation occurs as calcium is taken up by mitochondria and deposited within them in the morphologically characterisitc form described above.[5] Changes in myocardial calcium concentration and influx rates of calcium into myocardium of ISO-treated animals have already been reported [6,7] and both values were found to be elevated. These determinations, however, were made serveral hours after ISO administration, so that the observed changes could well be due to passive equilibration of necrotic myocardium with plasma calcium. Presumably passive influx of calcium has been noted when coronary perfusion is reestablished after a period of ischemia sufficient to produce necrosis.[8] Since changes in $[Ca]_m$ would be expected late in the evolution of necrosis, because of the much higher calcium concentration in plasma than in heart muscle, we have analyzed changes in the concentration of myocardial calcium after ISO administration as a function of time, in an attempt to distinguish ISO-related changes from nonspecific changes attendant on necrosis per se.

Materials and Methods

Male rats, weighing 200 to 250 g, obtained from Sprague-Dawley Company were used throughout. Isoproterenol (Sigma Chemical Co) and propranolol (gift of Ayerst Laboratories) were dissolved in isotonic saline and injected intraperitoneally. When both drugs were administered to the same animal, the propranolol was given 10 to 30 seconds before the ISO. Control animals were injected with 1 ml of isotonic sodium chloride solution, until it was found that the $[Ca]_m$ of saline-injected animals did not change with time from injection, and was indistinguishable from the $[Ca]_m$ of noninjected animals. All injections were given between 8:00 and 11:30 AM. At various times after injection, animals were anesthetized with ether, and the beating heart removed. The ventricles were dissected free of the pericardium, atria and great vessels, washed briefly in isotonic sodium chloride solution, blotted dry and placed in preweighed platinum crucibles. The crucibles were placed in a cool muffle furnace, the temperature of which was raised to 600 C over a 2-hour period. The crucibles were then allowed to cool slowly. The resulting ash was dissolved in 2 N HCl containing 3% lanthanum (w v) to a final volume of 10 ml in glass volumetric flasks. The calcium content of these samples was determined in a Perkin-Elmer model 303 atomic absorption spectrometer by comparing their absorption

values at 422.7 mμ to those obtained with a set of standard solutions. All glass and plasticware were washed in concentrated nitric acid. The only exposure of the samples to glass was the brief period in the 10-ml volumetric flasks. Standard calcium solutions were prepared in the same concentration of HCl and lanthanum as the unknowns. The standard solutions were stored in polyethylene containers, and were checked periodically against freshly prepared standard. All hydrochloric acid was distilled into hydrochloric acid-washed polyethlene bottles. Values for $[Ca]_m$ were expressed as mmoles of calcium per kg of wet weight ventricular myocardium. Recovery was assessed by comparing the calcium content per unit wet weight of myocardium of 3 or more hearts to that of 3 or more similar hearts to each of which 1 μmole of Ca had been added before addition of HCl to the crucible.

For estimation of the extent of myocardial necrosis after various doses of ISO and propranolol, the animals were returned to their cages after injection. They fed and drank *ad libitum*, as before injection. Forty-eight hours after injection they were killed by an overdose of ether. The hearts were removed and sectioned transversely with razor blades, so that three or four sections from the apex to the base were obtained. All sections were fixed in 10% neutral formalin. Dehydration, paraffin infiltration, sectioning at 5 μ, and hematoxylin and eosin staining were carried out by routine procedures. The slides were labeled with random numbers, so that the source of tissue was known only to a person not involved in the histologic analysis. The extent of the myocardial necrosis was evaluated according to the ad hoc criteria shown in Table 1.

Results

The $[Ca]_m$ of control animals varied somewhat among different shipments of rats. This variation was not sufficient to interfere significantly with the results. The overall mean $[Ca]_m$ of 77 animals, representing the control animals of all experiments reported here, was 0.850 ± 0.006 mmoles calcium/kg myocardium (wet weight). The extremes of $[Ca]_m$ among all shipments studied were 0.906 ± 0.035 (mean of 3 animals ± SEM) and 0.773 ± 0.006 (mean of 3 animals ± SEM). The overall recovery was 100.4% ± 1.7% (mean of 14 separate determinations involving 102 animals ± SEM). It is possible that such variations in the

Table 1—Light Microscopic Grading of ISO Lesions*

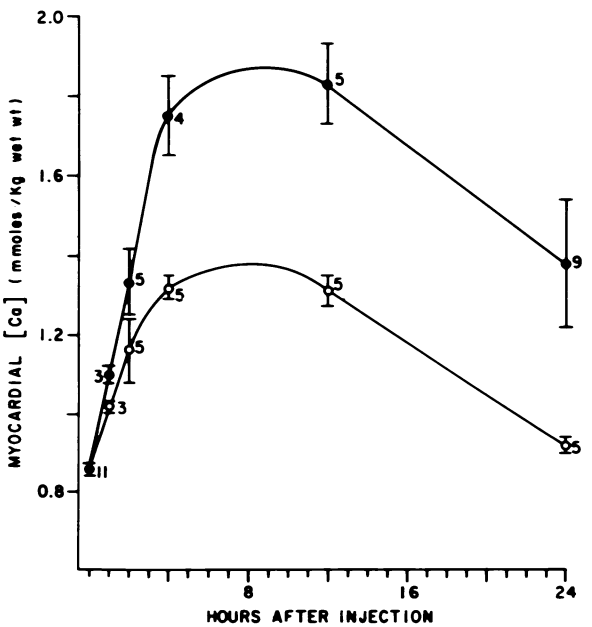
Grade	Morphology
0	No lesions noted
1	Possible rare single-cell necrosis
2	Rare but undisputed necrotic lesions
3	Common necrosis
4	Abundant necrosis
5	Confluent massive necrosis

* At least three transverse sections of each heart were examined. One of these was always from the apex, one from the base and one approximately half-way between. All slides were coded and then graded by a single observer who did not know the significance of the code.

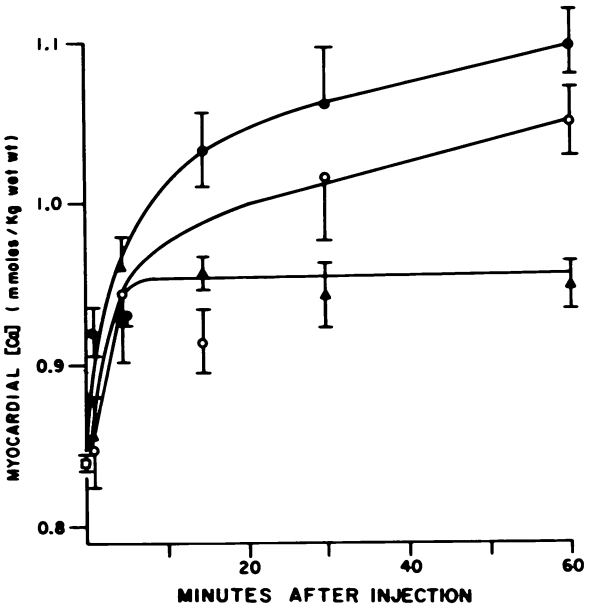
"normal" $[Ca]_m$ account for the descrepancy between our values and the higher control values reported by Lehr.[6]

The changes in $[Ca]_m$ after 10^3 and 10^4 μg ISO/kg are shown as a function of time in Text-figure 1. Both of these doses produce myocardial necrosis,[9] although only a small amount occurs after 10^3 μg/kg. The $[Ca]_m$ begins to rise immediately after drug administration at either dose, attains a dose-dependent maximum between 4 and 12 hours, and then begins to return toward control values. Both of these curves peak at a time corresponding to the phase of early myocardial cell fragmentation and phagocytosis.[1] The changes in $[Ca]_m$ demonstrated in Text-figure 1 could, therefore, be reasonably attributed to the movement of Ca from the plasma, where its concentration in mammals is about 2.5 mM,[10] into dead heart muscle cells which are no longer able to maintain the normal low intracellular levels. With this line of reasoning, it is difficult to attribute necrosis to controlled Ca influx, as has been theorized.[1,7] However, the time scale in Text-figure 1 is too large to allow visualization of initial changes in $[Ca]_m$ after ISO administration.

To demonstrate changes in $[Ca]_m$ as early as possible after ISO administration, we carried out the series of experiments partially illustrated in Text-figure 2. Here it is seen that, at the three doses tested, a



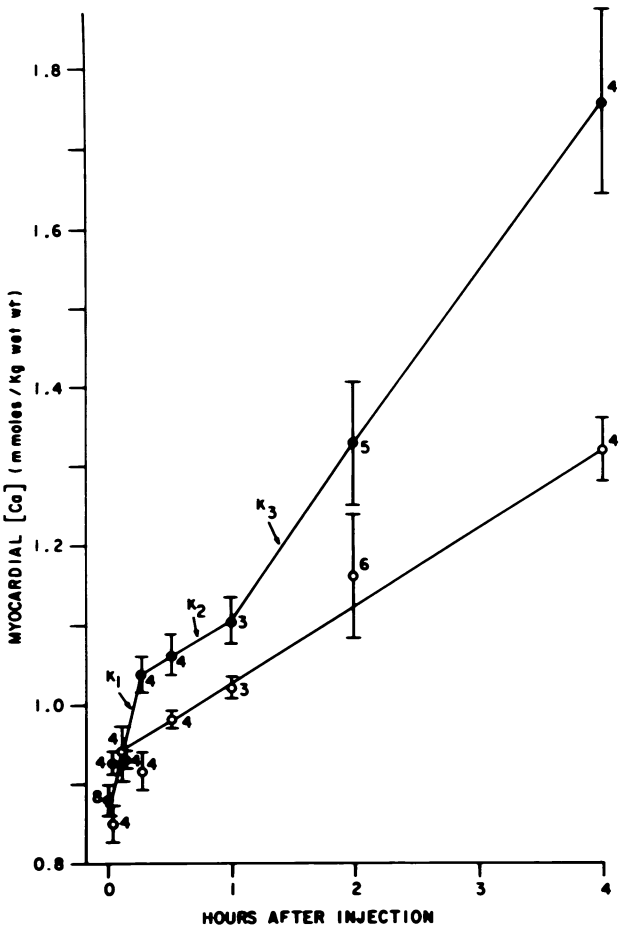
TEXT-FIG 1—Myocardial calcium as a function of time after ISO administration. Animals were sacrified at the indicated times after the intraperitoneal injection of ISO, and the Ca content of the ventricular muscle determined. Open circles indicate myocardial calcium after 10^3 μg ISO/kg. *Solid circles* indicate [Ca]m after 10^4 μg ISO/kg. The number next to each data point reflects the number of animals in that group.



TEXT-FIG 2—Myocardial calcium as a function of time after ISO administration. The dose of ISO was either 10^2 μg/kg (*triangles*), 10^3 μg/kg (*open circles*) or 10^4 μg/kg (*solid circles*). Each data point represents the mean of 4 animals, except for the 60-minute points, which are based on 3 animals and 0 time, which is based on 51 control animals; *brackets* indicate SEM.

rapid increase in $[Ca]_m$ occurs within 10 minutes after drug administration. At 10^2 μg/kg, the $[Ca]_m$ remains constant at this new value for the 1-hour duration of the experiment. A curve of similar shape was obtained at 10^1 μg ISO/kg, but this leveled off at a lower $[Ca]_m$, not clearly higher than the control. At 10^3 and 10^4 μg/kg there is also a rapid increase in $[Ca]_m$. The response at these doses differs from that found at lower doses in the occurrence of a continued slower increase in $[Ca]_m$ after the initial rapid increase. The response after 10^5 μg ISO/kg was similar to that seen after 10^4 μg/kg, in terms of the curve shape and magnitude of the response. These data indicate that the increase in $[Ca]_m$ after ISO can be resolved into more than one kinetic phase, raising the possibility that while, there is an increase in $[Ca]_m$ due to equilibration of necrotic myocardium with plasma, an earlier phase of the increase might be a controlled process which is etiologically related to the subsequent necrosis.

In order to simplify discussion, we have divided the increase in $[Ca]_m$ into three kinetic phases (Text-figure 3). The change in $[Ca]_m$ per minute occurring within the first 10 minutes of ISO administration is referred to as K_1, that occurring between 10 and 60 minutes as K_2, and that occurring between 60 and 240 minutes as K_3. At all doses of ISO between 10^1 and 10^5 μg/Kg, K_1 has a value of approximately 20 μmoles Ca/kg myocardium/min. K_2 is zero for 10^2 μg ISO/kg or less, but is



TEXT-FIG 3—Myocardial calcium as a function of time after ISO administration. This figure is composed of data points taken from Text-figures 1 and 2, and plotted on a time scale which spans the three apparent kinetic phases of increase in myocardial calcium. *Open* and *solid circles* represent, respectively, myocardial calcium after 10^3 and 10^4 μg ISO/kg. Brackets show SEM; the number of animals in each group is indicated.

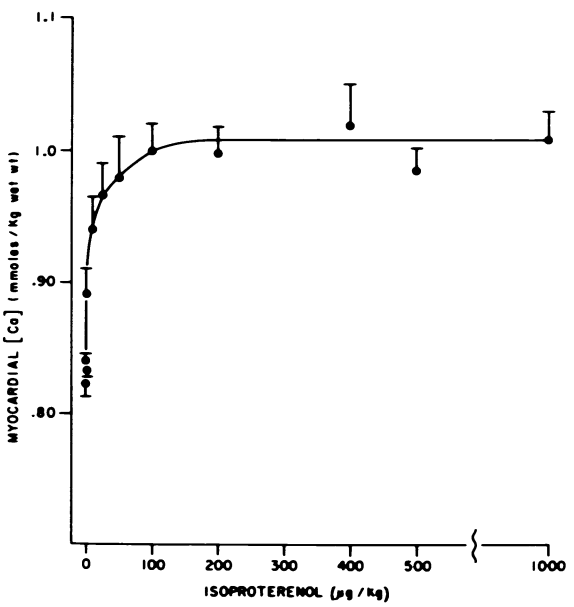
approximately 1.3 μmoles Ca/kg myocardium/min for 10^3, 10^4 or 10^5 μg ISO/kg. K_3 is the same as K_2 for 10^3 μg ISO/kg, but increases to 3.6 μmoles Ca/kg myocardium/min at 10^4 μg ISO/kg.

Although the data presented here do not establish the mechanism of the three kinetic phases, consideration of electron microscopic findings [1] suggests that K_3 is related to the breakdown of sarcolemmal functions which limit influx of plasma calcium into "normal" heart muscle cells. This conclusion is compatible with the observation that a definite K_3 phase occurs in animals treated with 10^4 μg ISO/kg but is not recognizable after treatment with 10^3 μg ISO/kg. At the former dose there is extensive myocardial necrosis, while at the latter dose there is minimal necrosis.[9] In the absence of definitive data at the present time, we postulate that K_1 represents Ca influx stimulated by the release of endogenous

norepinephrine from myocardial storage sites [11,12] and that K_2 reflects the direct effect of ISO on sarcolemmal Ca uptake.

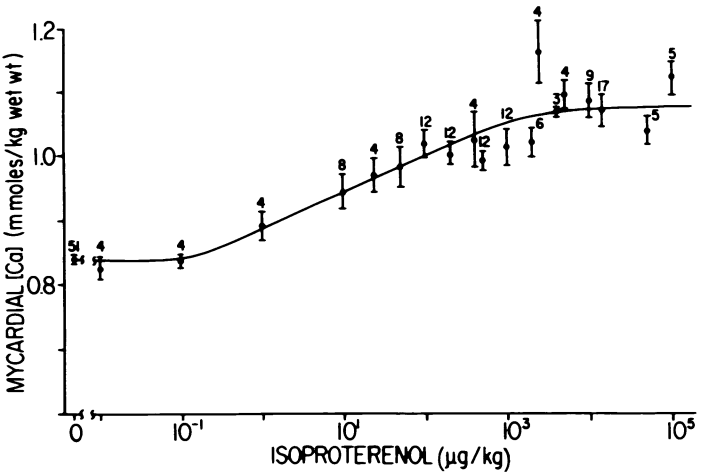
The kinetic data, described above, indicate that the response of $[Ca]_m$ up to 1 hour after ISO is the same at 10^4 and 10^5 μg/kg. This suggests that influx of Ca from the plasma into heart muscle cells is not the exclusive determinant of myocardial necrosis. For this reason, an accurate dose-response curve, including the changes due to both the K_1 and K_2 phases, is especially important. Such a curve, which is based on data from many separate experiments in order to minimize differences in responsiveness among groups of animals, is illustrated in Text-figure 4. This shows that, 1 hour after ISO administration, a dramatic, dose-dependent increase in $[Ca]_m$ occurs over the range of 1 to 100 μg/kg, but no further increase in $[Ca]_m$ occurs as the dose is increased to 10^3 μg/kg. In Text-figure 5 the same data are shown again, together with the response at doses up to 10^5 μg ISO/kg. Use of a logarithmic scale for the abscissa of this graph demonstrates a linear relationship between the log(ISO) and the 1-hour increment in $[Ca]_m$ between 10^{-1} and 10^2 μg ISO/kg. The plateau between 10^2 and approximately 10^3 μg ISO/kg is again noted, but the $[Ca]_m$ at doses above this is increased, although erratic.

To increase the objectivity of evaluation, we divided the data shown in Text-figure 5 into three dose ranges and calculated the regression of $[Ca]_m$ on log(ISO) for each of these ranges, using the

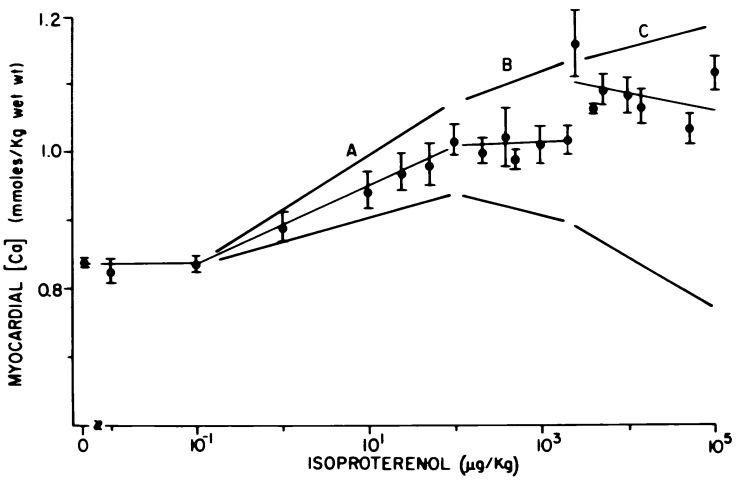


TEXT-FIG 4—Myocardial calcium as a function of ISO dose. Animals were killed 1 hour after the indicated dose of ISO. These same animals were used for part of the data in Text-figure 5, where the number of animals in each dose group is indicated. Brackets show SEM.

Hewlett-Packard Company program for two variable regression lines. The computations were carried out on a Hewlett-Packard model 9810A calculator. The calculated regression lines are shown in Text-figure 6, together with the 95% confidence limits of the slope. The



TEXT-FIG 5—Myocardial calcium as a function of ISO dose. Animals were killed one hour after the indicated dose of ISO. Use of a log scale for the abscissa allows coverage of a large dose range, and shows that a linear relationship exists between log(ISO) and the increment in myocardial calcium from 10^{-1} to 10^{2} μg/kg. Brackets show SEM; numbers over brackets show number of rats in each group.



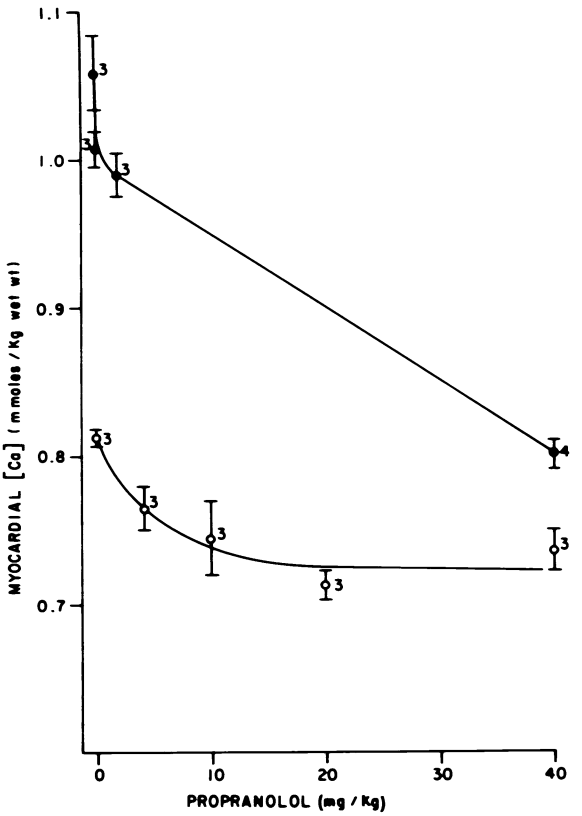
TEXT-FIG 6—Same data illustrated in Text-figure 5, with calculated slopes for data encompassed by line segments *A*, *B* and *C*. The upper and lower dark lines represent the 95% confidence limits of the slope of the encompassed data points, but have no relevance to the vertical position of the line segment. Although the difference in slope between segments A and B was significant at the 0.025 level, there was no significant difference in slope between segments B and C.

significance of the difference in slope between the three regression lines was calculated using the t distribution for differences in regression lines.[13] This indicated that the difference in slope between the regression lines of segments A and B of Text-figure 6 is significant at the 0.025 level. The difference in slope between sections B and C was not significant. The difference in mean $[Ca]_m$ of all data points included in segment B was significantly different from those in segment C at the 0.001 level (Student's t test). The results of these analyses are compatible with the dose-response curve as illustrated in Text-figure 5.

Effect of β-Adrenergic Blockade

If the myocardial necrosis associated with ISO were due to a specific effect of ISO on myocardial Ca metabolism, then we would predict that propranolol would influence changes in $[Ca]_m$ and necrosis to a similar extent. It has been shown that β-adrenergic blockading agents do decrease the influx of Ca into heart muscle of ISO-stimulated preparations.[7] It has also been reported, however, that propranolol does not influence myocardial necrosis after ISO.[14] To test this further, we injected various doses of propranolol into rats, followed immediately by either no further treatment or by 1.5×10^4 μg ISO/kg. The changes in $[Ca]_m$ 1 hour later are illustrated in Text-figure 7. It is clear from these data that a dose of 40 mg/kg of propranolol completely prevents the increase in $[Ca]_m$ due to 1.5×10^4 μg ISO/kg. Propranolol also causes a reduction of the $[Ca]_m$ of "normal" animals. It is not at all clear that the $[Ca]_m$ reduction involves the same calcium compartment or control mechanism in both cases.

On the basis of the data shown in Text-figure 7, we would predict that if ISO-induced myocardial necrosis was mediated by an increase in influx of Ca from plasma, then 40 mg/kg of propranolol should completely prevent the necrosis due to 1.5×10^4 μg ISO/kg. In order to judge the amount of necrosis after different drug treatments, we adopted the criteria described in Table 1. These criteria provide a better index for minimum than for maximum lesion severity. Since the experimental objective was to demonstrate the degree to which lesion severity approached zero, this greater sensitivity in the minimal lesion range was desirable. The results of this experiment, indicated in Table 2, show that, while propranolol does decrease the extent of myocardial necrosis, it does not completely prevent its occurrence, even at doses which completely prevent the increase in $[Ca]_m$ at 1 hour after ISO administration.



TEXT-FIG 7—Myocardial calcium as a function of propranolol dose. Animals were injected with indicated dose of propranolol and then immediately given 1.5×10^4 μg ISO/kg (*upper curve*) or no further treatment (*lower curve*). All animals were sacrificed 1 hour after injection. Brackets show SEM; the number of animals in each group is indicated.

Table 2—Effect of Propranolol on Grade of Myocardial Lesion Produced by ISO*

ISO (mg/kg)	Propranolol (mg/kg)		
	0	4	40
0	0.2 ± 0.2	0.6 ± 0.6	0.1 ± 0.1*
15	3.5 ± 0.6	2.4 ± 0.2	1.2 ± 0.2
	P < 0.001	**P** < 0.027	**P** < 0.001
50	3.6 ± 0.3*	2.6 ± 0.7	1.8 ± 0.4
	P < 0.001	**P** < 0.06	**P** < 0.001

* Lesion grades were assigned on the basis of a 0 to 5 scale. All values are means of 5 animals ± SEM, except those indicated by an asterisk, which are means of 10 animals ± SEM. **P** values are based on a comparison (Student's **t** test) of the experimental mean values with the corresponding group which received the same dose of proporanolol, but no ISO.

Discussion

The work presented here shows that the total myocardial calicum increases after administration of ISO, and that this increase is governed

by more than one mechanism. The dose-response curve and the kinetic analysis indicate a relationship between myocardial Ca uptake and necrosis, but there are imperfections in this relationship. The fact that the isoproterenol dose-$[Ca]_m$ response curve levels off over an ISO dose range in which the amount of necrosis is proportional to dose suggests that it is not exclusively the increase in total myocardial calcium which is responsible for necrosis. Since mitochondria show evidence of Ca flooding within minutes after the administration of a necrogenic dose of ISO, it is plausible to postulate that ISO exerts an effect on the proportional distribution of Ca among the subcellular organelles. If this resulted in a disproportionate increase in mitochondrial Ca, through a direct effect of ISO on the subcellular organelles, then a decreased rate of mitochondrial ATP synthesis would be expected, and necrosis could ensue. A decreased myocardial ATP level after necrogenic doses of ISO has been reported,[15] and preliminary experiments in this laboratory indicate that ISO has a direct stimulatory effect on mitochondrial Ca uptake. The occurrence of some myocardial necrosis after combined treatment with ISO and propranolol also suggests that altered myocardial uptake of Ca from the plasma is not the exclusive basis for necrosis. Although it is possible that factors other than Ca and its effect on mitochondria could be etiologically related to myocardial necrosis after ISO, the electron microscopic data combined with the observations described here certainly indicate that Ca does play a prominent role.

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