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# Ca-Transporting System of the Left Ventricular Sarcoplasmic Reticulum in the Rat Heart and Damage to Its Membrane During Ischemia and Reperfusion

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Ischemia and reperfusion of various duration are shown to result in a nonlinear increase in the level of free Ca in myocardial homogenates. A striking dissociation has been observed in the effect of ischemia and reperfusion on the rate of Ca transport in the sarcoplasmic reticulum, on the one hand, and the permeability of its membranes on the other.

**Key Words**: *sarcoplasmic reticulum; ischemia; reperfusion*

A considerable increase in the concentration of free Ca in ischemia is one of the most important mechanisms by which ischemia and reperfusion exert their damaging effects, because excess Ca can not only induce the development of contracture but also lead to the activation of destructive cellular enzymes, namely proteases [13,26], lipases, and phospholipases [1,3,5,26]. This aggravates the damage to intracellular, including membrane, structures. Ca accumulation in cardiomyocytes in ischemia and especially during reperfusion is the overall result of several processes, the prime ones being damage to the sarcolemma and the entry of Ca

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into the cell [3,7,22], damage to the intracellular membrane Ca depots - mitochondria and sarcoplasmic reticulum (SPR) - and the exit of Ca from these. Impaired Ca transport may be due both to a reduction in Ca-ATPase activity and to a pathological increase in the permeability of Ca depot membranes. In considering these two possibilities in relation to one other, it should be remembered that Ca-ATPase and Ca transport remain highly stable during ischemia and reperfusion [19,20,23], as do mitochondrial function [11] and the mitochondrial membranes [4]. However, the question of how ischemia and subsequent reperfusion might affect permeability of the SPR membrane to Ca has remained unexplored. In view of this, the purpose of the present study was to assess the impact of ischemia produced by coronary artery ligation and of subsequent reperfusion on the permeability of SPR membranes and on Ca uptake by SPR vesicles, which depends on Ca-ATPase activity. For this, we compared the effects of ischemia/reperfusion on the permeability of SPR membranes and on Ca transport.

## MATERIALS AND METHODS

*Myocardial ischemia and reperfusion.* The experiments were conducted on male Wistar rats (body weight 250 g) with open chest under Nembutal anesthesia (50 mg/kg) and artificial ventilation. Local ischemia was produced by ligating the descending branch of the left coronary artery at its very base, while reperfusion was instituted by removing the ligature. The duration of ischemia ranged from 20 to 40 min and that of reperfusion, from 5 to 15 min. Concurrently, a one-lead ECG was recorded on a Mingograph-34 (Siemens-Elema, Sweden) and the frequency and duration of ventricular tachycardia and fibrillation were estimated. For subsequent biochemical studies, hearts with maximally pronounced ischemia were used. The two criteria by which the severity of ischemia was judged were, first, the maximal area of cyanosis involving the anterior wall and apex of the left ventricle (LV) and, second, segment ST elevation on the ECG. The free LV wall was cut out, in which the ischemic zone 30 min after coronary artery ligation constituted 36.8% of the LV volume according to morphometric data of our laboratory [2]. (According to other authorities, this zone accounted for 37% of the LV mass 40 min after ligation and 2 h of reperfusion [12] and 40% after 48 h [9].) In estimating the mass of an ischemic myocardium one should bear in mind that the ischemic zone involves mainly the free LV wall and virtually does not extend to the septum,

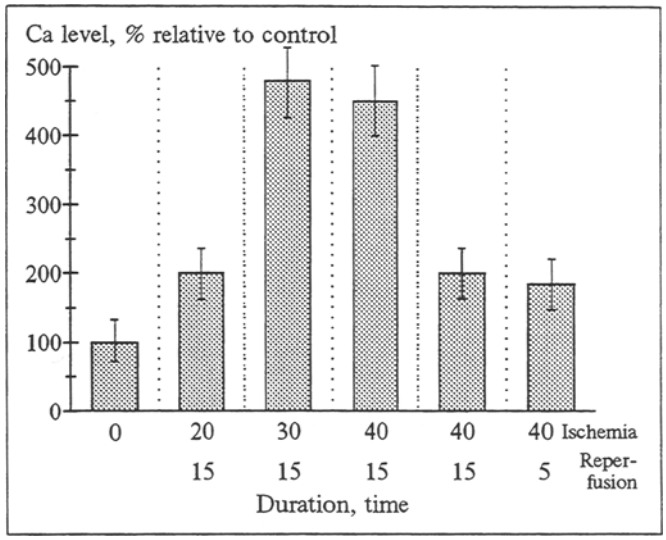


**Fig. 1.** Levels of free Ca in rat myocardial homogenates in the control and after ischemia and reperfusion of various duration. The values are means and their scatters for 7 samples in each of the two series.

whose mass makes up about 50% of the LV mass. For this reason, in our experiments involving prolonged ischemia and perfusion, the ischemic myocardial zone constituted some 37% of the LV mass, or 70-80% of the mass of the free LV wall.

*Homogenization of hearts.* Free LV walls of the hearts from control and test rats were cut out on ice, washed in physiological saline, and frozen in liquid nitrogen. The hearts were then disintegrated with an Ultra-Turrox homogenizer in a medium containing 100 mM KCl, 20 mM imidazole (pH 7.8), and 25% glycerol, at a tissue to medium ratio of 1:4.

*Ca transport in the SPR.* $Ca^{2+}$ transport was measured in an Orion EA 940 ionometer with a Ca-selective electrode by the rate at which added $Ca^{2+}$ was taken up by SPR vesicles. $Ca^{2+}$ accumulation by mitochondria was prevented by $NaN_3$ and $Ca^{2+}$ absorption by sarcolemmal vesicles was prevented by added $K^+$ oxalate, which does not pass into these vesicles. The rate of Ca transport in the SPR was determined in thermostatically controlled cells with agitation for 5 min, adding 50-200 µl of the homogenate to 5 ml of a medium containing 10 mM KCl, 15 mM $K^+$ oxalate, 20 mM HEPES, pH 7.0, 4 mM $MgCl_2$, and 5 mM $NaN_3$. ATP and Ca were added before measurements to concentrations of 4 mM and 2-20 µM, respectively. The level of unbound Ca was determined in the same medium by the initial (at 20 sec) response of the electrode.

*Autolysis of homogenates.* Autolysis of heart homogenates was carried out at 0° and 37°C. Its purpose was twofold: first, to detect those slight injuries sustained by the enzyme-membrane com-

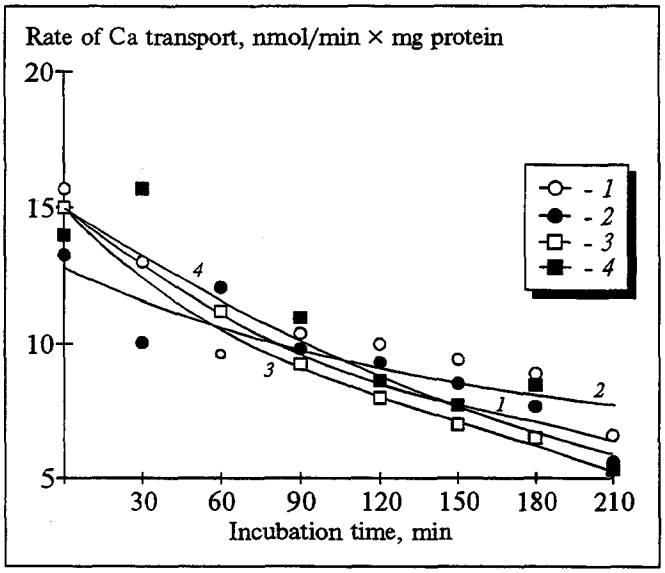


Fig. 2. Autolysis at 37°C with constant agitation in the incubation medium in the control (*1*) and after 40 min of ischemia followed by reperfusion for 5 (*2*), 10 (*3*), and 15 (*4*) min.

plex of Ca transport in the myocardial SPR that do not affect Ca transport activity (the possibility of such injuries was demonstrated by us previously [6,17]), and, second, to assess the state of the SPR membrane by recording the level of Ca leakage from the SPR in the course of autolysis. It is important to note that while the results obtained for a slow autolytic process (at 0°C) are qualitatively similar to those obtainable when autolysis is rapid (at 37°C), quantitative differences between these results are appreciable, so that even slight injuries suffered by the enzyme system or membranes of the SPR can be detected. Rapid autolysis was effected in thermostatically controlled (at

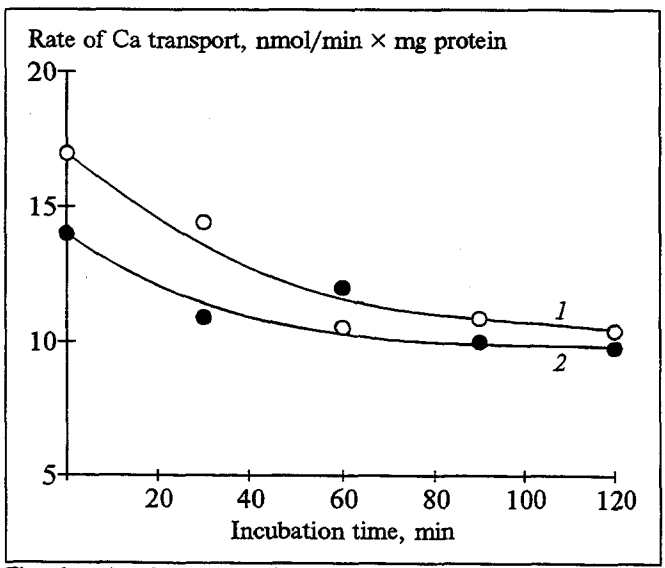


Fig. 3. Autolysis at 37°C in the control (*1*) and after 30 min of ischemia and 15 min of reperfusion (*2*).

37°C) cells in a medium that was of the same composition as the incubation medium used for measuring the rate of Ca transport in the SPR except that Ca and ATP were not added; the homogenate dilution was 1:2. Slow autolysis was conducted without diluting the homogenate, in the homogenization medium at 0°C; the low temperature and the presence of glycerol in the medium had a stabilizing effect on the membrane system of Ca transport in the SPR and considerably slowed the process of its autolytic damage.

## RESULTS

In the first stage of the study, in which the impact of ischemia and reperfusion on the accumulation of free Ca in heart homogenates was evaluated, the degree of Ca accumulation was found to vary considerably with the duration of ischemia and reperfusion. As shown in Fig. 1, the outflow of Ca was greatest, exceeding 4.5-fold the control level, after 30-40 min of ischemia and 15 min of reperfusion. After 20 min of ischemia and 15 min of reperfusion, the concentration of free Ca was also higher than in the control, though only by a factor of 2. A similar effect - not only qualitatively, but also quantitatively - was observed when the duration of reperfusion was shortened to 5 or 10 min while that of ischemia was prolonged (30-40 min): the Ca level was double that in the control. Interestingly enough, increasing the duration of reperfusion twofold from 5 to 10 min without altering that of ischemia (40 min) (Fig. 1) did not alter significantly the accumulation of free Ca in the sarcoplasm, whereas increasing the time of reperfusion 1.5-fold from 10 to 15 min led to a sharp rise in the Ca level. Similarly, the level of free Ca rose 2.5 times when the duration of ischemia was increased by only 10 min (from 20 to 30 min), although in this case the Ca level rose only 2-fold over the first 20 min of ischemia and virtually the same increase was observed when ischemia was prolonged to 40 min. These results indicate that the relationship between Ca levels and the duration of ischemia and reperfusion was nonlinear, and that there were certain critical points (an increase from 20 to 30 min for ischemia and from 10 to 15 min for reperfusion) at which a qualitative change in the process and a greatly accelerated outflow of free, unbound Ca to myocardial homogenates occurred. Such a considerable Ca accumulation in the homogenates may result both from damage to the SPR membranes and Ca leakage and from defective functioning of the Ca-transporting system.

The second stage of the study was therefore aimed at evaluating the Ca-transporting system of the myocardial SPR. It was found that after ischemia and reperfusions of various duration - even after a 40 min-ischemia and 15-min reperfusion when, as shown above, Ca accumulation in the myocardial homogenates was maximal - the rate of Ca uptake by SPR vesicles did not change significantly. Such a high resistance of the Ca-transporting system to the damaging actions of ischemia and reperfusion suggests that this system makes no contribution to the accumulation of free Ca during ischemia. To check this, homogenates of control and test hearts were subjected to autolysis in order to detect slight changes in the Ca-transporting system that do not affect Ca transport activity. As can be seen in Figs. 2 and 3, the rapid autolysis at 37°C after 30 to 40 min of ischemia and 15 min of reperfusion did not significantly alter the rate of Ca transport in the SPR as compared to its control value. We then carried out autolysis at 0°C for reasons mentioned above (see *Autolysis of homogenates*). The results are shown in Fig. 4. It will be seen that the rate of Ca transport in the SPR after 40-min ischemia and 15-min reperfusion declined during autolysis much faster than in the control. Indeed, after 48 h of autolysis the rate of Ca transport had decreased by only one-third in the control while being inhibited almost completely after 40-min ischemia and 15-min reperfusion. Thus, it was only by resorting to slow autolysis that evidence of minor changes in the state of the Ca-transporting system of cardiac myocytes could be obtained - its reduced resistance to endogenous injurious factors that are activated in the processes of ischemia and reperfusion and that come into play during autolysis. It is clear, though, that such changes in the state of this system could not have contributed significantly to the manifold increases in free Ca concentration we had recorded after prolonged ischemia and reperfusion.

Accordingly, our next task was to evaluate the state of the SPR membranes after ischemia and reperfusion. To this end, we examined the time course of Ca accumulation in the homogenates during autolysis, which reflects the extent of damage sustained by SPR membranes since the endogenous factors of degradation activated during ischemia and reperfusion can damage the membrane Ca depots in the process of autolysis, whereas the mitochondrial membranes are spared in ischemia [4]. As shown in Fig. 5, whereas no significant Ca accumulation occurred in the control samples during autolysis, the latter did lead to

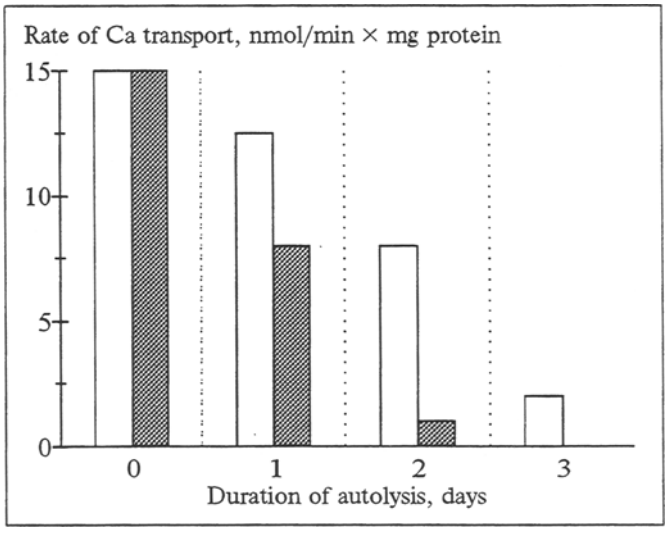


Fig. 4. Fall in Ca-transport rate during autolysis at 0°C in the control (white bars) and after 40 min of ischemia and 15 min of reperfusion (dark bars).

significant rises in Ca concentration after ischemia and reperfusion. The fact that the Ca concentration did not rise significantly in the control indicates that a 120-min incubation at 37°C did not result in damage to the SPR membranes by endogenous factors. In contrast, the level of free Ca in heart homogenates after 30-40 min of ischemia and 15 min of reperfusion was 6.5 times higher than in the control (Fig. 5). It should be stressed that after prolonged ischemia and reperfusion the Ca level was 4.5 times higher than in the control even before autolysis, and the impression gained is that although the membranes were damaged, the damage was slight: after 120 min of autolysis, when the Ca level in the control remained virtually the same as before it, the Ca level after ischemia had risen by only one-third, and the major parameter characterizing the extent of membrane damage is the rate at which the concentration of free Ca rises, for it reflects how much Ca is leaking from the SPR vesicles during autolysis. However, when the kinetics of Ca accumulation during autolysis is considered (Fig. 5, *a* and *b*), it can be seen that there is a threshold concentration of unbound Ca in the homogenates, a certain equilibrium level of free Ca, above which its concentration cannot rise even with considerable prolongation of autolysis (to 120 min in our case). In the homogenates of hearts that had suffered ischemia for 30-40 min and reperfusion for 15 min, the maximal level was attained as early as after 30 min of autolysis and was maintained thereafter, whereas the concentration of free Ca in the control samples was 6.5-times lower than that maximum even after 120 min of autolysis.

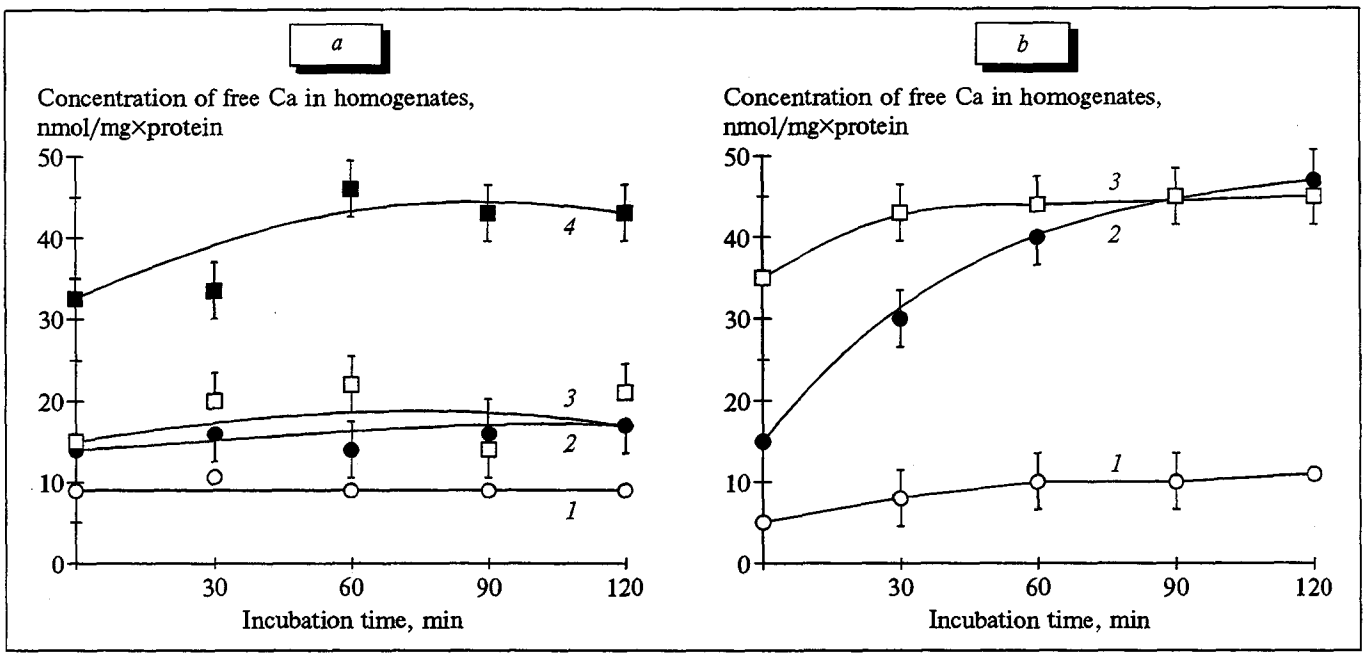


Fig. 5. Accumulation of free Ca during autolysis at 37°C, *a*) in the control (*1*) and after 5 (*2*), 10 (*3*), and 15 (*4*) min of reperfusion and the longest duration of ischemia (40 min). *b*) in the control (*1*) and after 20 (*2*) and 30 (*3*) min of ischemia and the longest duration of reperfusion (15 min).

In order to confirm that the SPR membranes sustained catastrophic injuries caused during autolysis by the factors accumulated in the course of prolonged ischemia and reperfusion, we shortened the duration of ischemia to 20 min while keeping the reperfusion time unchanged (15 min) and then subjected the myocardial homogenates to the same autolysis as before (Fig. 5, *b*). It can be seen that in this case the level of free Ca before autolysis was much lower than after 30- or 40-min ischemia, being only 1.8 times higher than in the control (vs. 6.5 times after 30-40 of ischemia). This means that more Ca should issue from the SPR after 20-min ischemia than after 30-40-min ischemia in order that the maximum Ca level be attained. In fact, the maximal Ca level had already been reached by minute 60 and was 3 times as high as before autolysis, whereas in the control samples the Ca concentration did not rise even by 10 nmol/mg protein.

Finally, one more aspect of this study deserves special mention, namely the evaluation of the role played by the damaging effect of reperfusion on Ca outflow from the SPR. It follows from Fig. 5, *b* that reducing the reperfusion time did not just lower the level of Ca outflow (as could be expected) but also elicited a disproportionate response. Indeed, after 40-min ischemia and 5-min or 10-min reperfusion (Fig. 5, *a*), the initial Ca level (before autolysis) was only 1.9 times higher than in the control, similarly to what was observed after 20-min ischemia and 15-min reperfusion. However, in contrast to the latter situation (15-min perfusion), in which the rate of Ca loss was high (Fig. 5, *b*), the rate of Ca loss after shorter reperfusion times was much lower, and the Ca level had risen by only 45% (Fig. 5, *a*).

Thus, the above analysis of the kinetic curves describing Ca accumulation in the course of autolysis and reflecting Ca leakage from the SPR furnishes evidence of catastrophic damage sustained by SPR membranes during ischemia and reperfusion. The resistance of these membranes to endogenous destructive factors activated during ischemia and reperfusion is now drastically reduced.

The present study warrants a discussion of at least three aspects. First, the data on Ca accumulation indicate a nonlinear rise in Ca levels during ischemia and reperfusion. In the literature, this aspect has not been dealt with in detail - there are only a few studies concerned with certain temporal points. It has been reported, for example, that no increase in Ca occurs early during ischemia [24, 25], that the concentration of free Ca rises to 2.7 mM after just 11-15 min of ischemia [25], and that Ca returns to a normal level over 20 min of subsequent reperfusion [8]. Ca accumulation was also shown to be maximal after 15-20 min of reperfusion following 60-min ischemia and after 6-16 min of reperfusion following 30-min ischemia [21]. Our results identify those durations of ischemia and reperfusion (30-40 min and 15 min, respectively) that are conducive to the greatest release of unbound Ca in heart homogenates, which is indicative of damage to the myocardial SPR membranes. More-

over, our results show that the amount of Ca released is related to the duration of ischemia and reperfusion in a nonlinear manner. The time intervals between 20 and 30 min of ischemia and 10 and 15 min of reperfusion are those critical points at which the gradual destruction of SPR membranes and progressive rise in free Ca concentration in the homogenates may be said to reach a qualitatively new level at which the SPR is damaged to a much greater extent.

Second, our findings attest to a high stability of the Ca-transporting system in the SPR during ischemia and reperfusion. Thus, neither Ca transport activity nor even the resistance to endogenous injurious factors that exert their damaging effects during rapid autolysis was altered after prolonged ischemia and reperfusion, and it was only by resorting to slow autolysis that evidence of slight injuries sustained by the Ca-transporting system could be obtained. Other authorities have demonstrated the stability of ATPase activity in the SPR Ca-ATPase and of the Ca uptake rate even during longer-lasting ischemias. For example, no depression of oxalate-supported Ca accumulation in SPR from various regions of the heart was found by Nayler *et al.* following 60-min and even 120-min ischemia [20]; a similar finding was made by Lee *et al.* using oxalate (but not phosphate) to support Ca transport [16], while Hess *et al.* were able to register only a 10-30% reduction in Ca transport activity in SPR for various regions of the heart after prolonged ischemia and reperfusion [14]. Furthermore, when morphological lesions resulting from ischemias of various duration were recorded in dogs, there was no evidence of damaged SPR function [18,19]. When ischemia lasted less than 30 min, the SPR function was restored during reperfusion [10,23]. Very recently, Kaplan *et al.* reported that the ischemia and reperfusion resulting from coronary occlusion did not lead to substantial changes in either the dissociation constant for Ca or the rate of Ca release from myocardial SPR [15]. In our study, no significant changes in Ca transport activity in the SPR could be detected after prolonged ischemia followed by reperfusion and, moreover, neither ischemia nor reperfusion was found to have appreciably affected the enzyme's intrinsic properties, whose impairment could be detected by estimating the resistance of the membrane-enzyme complex to endogenous injurious factors. Here, it is important to note that it was the duration of reperfusion that played a crucial role in the damage to SPR membranes (Fig. 5, *a* and *b*). Although reperfusion arrhythmias are known to arise effectively during the first few seconds of reperfusion, in our study even a 10-min reperfusion after a 40-min ischemia failed to lead to substantial membrane damage: evidence of such damage appeared only after 15 min of reperfusion. The late onset of membrane damage can probably be explained, in part at least, by temporal differences in how cellular structures are affected by injurious factors, in particular lipid peroxidation (LPO), which has been shown to be activated during reperfusion. Thus, the ion channels which are responsible for the generation of impulses and their conduction into the heart, and whose damage occurring early in the course of reperfusion leads to arrhythmias, are the first target for the injurious factors, whereas with continued reperfusion damage to intracellular membrane structures, Ca outflow from the SPR, and labilization of lysosomal enzymes also occur. Since the released Ca reactivates LPO and since, in addition, Ca-dependent proteases are activated as well even at 3 mM Ca (a concentration attained after 15-20 min of ischemia [13]), it may be concluded that by the time autolysis is initiated the homogenates already contain endogenous injurious factors at levels high enough to enable them to manifest their activity in the process of autolysis.

The third and main conclusion to be drawn from the present results is that a striking dissociation exists between the action of ischemia and reperfusion on Ca-transport activity and on the permeability of SPR membranes. Indeed, while prolonged ischemias and reperfusions had only a marginal effect on the state of the SPR's Ca-transporting system and did not significantly alter the Ca transport rate, they did decrease the stability of the SPR membranes drastically. The concept of the stability of Ca-ATPase and Ca transport shown previously in the literature and our own studies should now be supplemented with one of catastrophic damage suffered by SPR membranes, which, along with the damage sustained by the sarcolemma, is conducive to the buildup of free Ca during ischemia and reperfusion and to the development of intracellular lesions. One may therefore speak of a new marker of ischemia- and reperfusion-induced injuries - namely reduced stability or resistance of SPM membranes to endogenous injurious factors which are activated to different degrees during ischemias and reperfusions of different duration, as can be judged by Ca leakage from the SPR in the course of autolysis. This criterion may be used to advantage in future searches for effective protective agents and interventions that could be usefully employed in cases of prolonged ischemia and reperfusion.

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# Binding of $^3$H-Diazepam in Rat Brain Eleven Months After Termination of Corazole Kindling

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Binding of $^3$H-diazepam in rat cerebellum decreases by 14% ($p<0.05$) 11 months after termination of kindling and one day after injection of a test dose of corazole (30 mg/kg), while it increases by 19.5% after a single injection of a convulsive dose of corazole (50-75 mg/kg). No changes are found in the cortex.

**Key Words:** *cerebellum; brain cortex; corazole kindling, diazepam; benzodiazepine receptors*

Kindling, produced by chronic electrical stimulation of brain structures [5,8] or chronic administration of convulsants [1,4,9,], induces a state of seizure predisposition (SP), due to which earlier subconvulsive agents evoke seizures. One of the features of this kindling-induced pathological state is that predisposition to seizures persists for a long time (weeks or months) after the termination of electrical stimulation or the discontinuation of con-

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