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IMPROVEMENT OF ISCHEMIA-REPERFUSION-INDUCED MYOCARDIAL DYSFUNCTION BY MODULATING CALCIUM-OVERLOAD USING A NOVEL, SPECIFIC CALMODULIN ANTAGONIST, CGS 9343B

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Abstract—The present paper explores the mechanism of calcium-overloaded cardiac cell exocytosis during reperfusion of ischemic myocardium. A novel specific inhibitor of calmodulin, CGS 9343B, was used to pretreat an ischemic heart in an effort to enhance myocardial preservation. The experimental model employed an isolated *in situ* pig heart subjected to 120 min of ischemic insult by reversibly occluding the left anterior descending coronary artery, the last 60 min being superimposed with global hypothermic cardioplegic arrest. This ischemic episode was followed by 60 min of revascularization. CGS 9343B enhanced post-ischemic myocardial recovery, as judged by improved regional as well as global myocardial functions, better preservation of high-energy phosphate compounds, and reduced release of creatine kinase. Since this compound blocks calmodulin without inhibiting protein kinase C, the results of this study suggest that calmodulin-dependent kinase, rather than protein kinase C, is primarily involved in expressing calcium-overloaded cell exocytosis, and a specific calmodulin antagonist such as CGS 9343B can be used to salvage an ischemic heart from reperfusion injury.

Uncontrolled calcium influx into the myocardial cell leading to the genesis of severe myocardial dysfunction has been observed during reperfusion of ischemic myocardium [1, 2]. The calcium overload occurs as soon as ischemic hearts are exposed to calcium-containing blood during revascularization. Recent studies have emphasized the role of calmodulin in Ca^{2+}-mediated changes in membrane integrity [3]. Calmodulin is abundant in heart, and it is reasonable to assume that many intracellular calcium activities are mediated by this calcium-receptor protein. For example, *N*-(6-amino-hexyl)-5-chloro-1-naphthalene-sulfonamide (W-7) has been shown to provide some protection of ischemic heart from reperfusion injury [4]. Other calmodulin antagonists such as chlorpromazine and trifluoperazine have also been found to be effective in reducing myocardial damage associated with calcium overload [5–7].

Although the above studies suggest a role of calmodulin in myocardial ischemic-reperfusion injury, a definitive mechanism of calmodulin-mediated transmembrane calcium movement cannot be established from the above studies because of the non-specific effects of these calmodulin antagonists [8, 9]. Although Ca^{2+}-calmodulin can activate certain calmodulin-dependent protein kinases resulting in improved phosphorylation [10], the role of protein kinase C cannot be ruled out from these studies primarily because these calmodulin blockers also inhibit protein kinase C [8, 9]. Recently, a novel, potent, and selective inhibitor of calmodulin activity has been described. This compound, CGS 9343B†, can specifically inhibit calmodulin activity without affecting protein kinase C [11]. In this study, we have used this compound to protect ischemic myocardium from reperfusion injury. The results of our study suggest that blocking of calmodulin may be an important regulatory step in relieving an ischemic-reperfused heart from calcium overload.

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† ΔL, systolic segment shortening; CGS 9343B, 1,3-dihydro-1[1-[(4-methyl-4*H*-pyrrolo[1,2-*α*][4,1]benzoxazepin-4-yl)methyl]-4-piperidinyl]-2*H*-benzimidazol-2-one (1:1)meleate; CK, creatine kinase; CP, creatine phosphate; EDL, end-diastolic length; ESL, end-systolic length; LAD, left anterior descending; LV max dp/dt, left ventricular maximum dp/dt; LVDP, left ventricular developed pressure; and LVEDP, left ventricular end-diastolic pressure.

MATERIALS AND METHODS

All chemicals used in this experiment were of the highest purity available. The CK assay kit, ATP, and CP were purchased from the Sigma Chemical Co., St. Louis, MO. CGS 9343B was a gift from the CIBA-Geigy Corp., Summit, New Jersey.

Animal preparation. The *in situ* isolated pig heart model used in this study was described previously [12, 13]. Forty Yorkshire pigs weighing between 15 and 20 kg underwent cardiopulmonary bypass surgery, such that the heart was isolated in its own perfusion circuit without perfusion of the rest of the

Table 1. Effects of 10 μM CGS 9343B on regional myocardial function

	Systolic segment shortening (ΔL) (% of baseline)		End-diastolic length (EDL) (mm)	
	Control	CGS 9343B	Control	CGS 9343B
Control				
LAD	100	100	10.0 ± 0.01	10.1 ± 0.01
Non-LAD	100	100	10.1 ± 0.01	10.1 ± 0.02
15 min CGS 9343B (pre-occlusion)				
LAD	100.6 ± 1.8	115.5 ± 4.8	10.0 ± 0.0	10.4 ± 0.06
Non-LAD	102.6 ± 1.5	109 ± 5.7	10.0 ± 0.02	10.7 ± 0.09
30 min LAD-occlusion				
LAD	14.2 ± 5.9	20.3 ± 5.2	11.1 ± 0.27	10.6 ± 0.06
Non-LAD	112.8 ± 4.1	96.5 ± 6.4	10.3 ± 0.01	10.7 ± 0.08
60 min LAD-occlusion				
LAD	2.2 ± 1.4	14.4 ± 3.1*	11.1 ± 0.27	10.6 ± 0.05
Non-LAD	117.4 ± 8.3	102.6 ± 7.4	10.2 ± 0.07	10.6 ± 0.08
15 min Reperfusion				
LAD	20.4 ± 4.2	50.3 ± 5.1†	10.2 ± 0.27	10.3 ± 0.08
Non-LAD	101.8 ± 6.4	95.8 ± 7.1	10.0 ± 0.06	10.5 ± 0.09
60 min Reperfusion				
LAD	11.8 ± 2.6	42.3 ± 4.5†	10.4 ± 0.22	10.7 ± 0.11
Non-LAD	99.6 ± 3.7	112.3 ± 8.8	10.1 ± 0.04	10.2 ± 0.08

Results are means ± SEM of six to nine experiments in each group. Baseline values for control: LAD, 3.28 to 9.38 mm; non-LAD, 2.38 to 7.78 mm. Baseline values for CGS 9343B: LAD, 3.04 to 7.5 mm; non-LAD, 2.4 to 8.33 mm.

* $P < 0.05$ compared to control.

† $P < 0.01$ compared to control.

animal. The systemic circulatory blood was drained into an oxygenator, and superior and inferior vena cava were ligated. The coronary perfusion pressure and temperature were carefully regulated at 75 mm Hg and at 37° respectively. Hearts were paced at 120 beats/min by ventricular pacing.

After allowing 15 min for stabilization of the isolated heart preparations, hearts were perfused for a further period of 15 min in the presence (experimental) or absence (control) of 10 μM CGS 9343B. The LAD coronary artery was then reversibly occluded distal to the first diagonal branch for 120 min, the first 60 min being at normothermia and the remaining 60 min superimposed with global hypothermic cardioplegic arrest induced by high potassium (35 mEq/L) crystalloid cardioplegic solution containing hydroxyethyl starch to mimic surgical revascularization in a clinical setting. The hearts were then reperfused for an additional period of 60 min at normothermia.

During the experiments, myocardial biopsies were withdrawn from the left ventricle in the LAD as well as non-LAD-severed regions using a dental drill. Global and regional functions were determined only from the hearts whose biopsies were not taken for biochemical studies. Coronary effluents were also withdrawn from this latter group for evaluation of biochemical parameters.

Measurements of myocardial functions. Indicators of global myocardial functions such as measurements of isovolumetric LVDP, maximum first derivative of LV max dp/dt, and LVEDP were measured by inserting a Miller Micro Tip Catheter transducer (Miller Instruments, Inc., TX) within a 10-ml compliant balloon into the left ventricle as described elsewhere [13]. The left ventricular segment functions were measured with ultrasonic crystals implanted in a circumferential plane at segments of LAD and non-LAD distributions in the left ventricle. The transducers were placed near the endocardium through small epicardial punctures, and the two crystals of each pair were separated by 1 cm. Tracings of segmental length were recorded on a Honeywell AR-6 recorder (Honeywell, Inc., NY), interfaced with an oscilloscope (Tektronix, Inc., OR), and coupled to a Sonomicrometer (Triton Technology, CA). End-diastolic and end-systolic segment lengths were identified on the recordings. The calculated values were normalized by dividing the observed segment length by the control and end-diastolic segment length, and multiplying by 10. The systolic change was calculated as the difference between the EDL and the ESL. Coronary blood flow, myocardial oxygen extraction, and oxygen consumption were measured as described previously [12, 13].

Assay of high energy phosphate compounds and serum CK. Adenosine triphosphate and creatine phosphate were determined from the myocardial biopsies according to the method described by Cordis *et al.* [14] with some modifications. Neutralized myocardial extracts were injected onto a Waters (Milford, MA) NOVA-PAK C-18 Radial-PAK cartridge (5 mm × 10 cm, 4 μm particle size) in a Z-module. The effluent was monitored at 210 nm for 4 min and then monitored at 259 nm for 7 min. The initial mobile phase of 48 nM monobasic potassium phosphate, 1 mM tetrabutylammonium phosphate (pH 5.8) was used for 4 min followed by a step gradient to 20% acetonitrile in the initial buffer. The retention times of ATP and CP were 9.1 and 2.2 min respectively. CK was assayed in perfusate using a CK assay kit (Sigma Chemical Co.) [12].

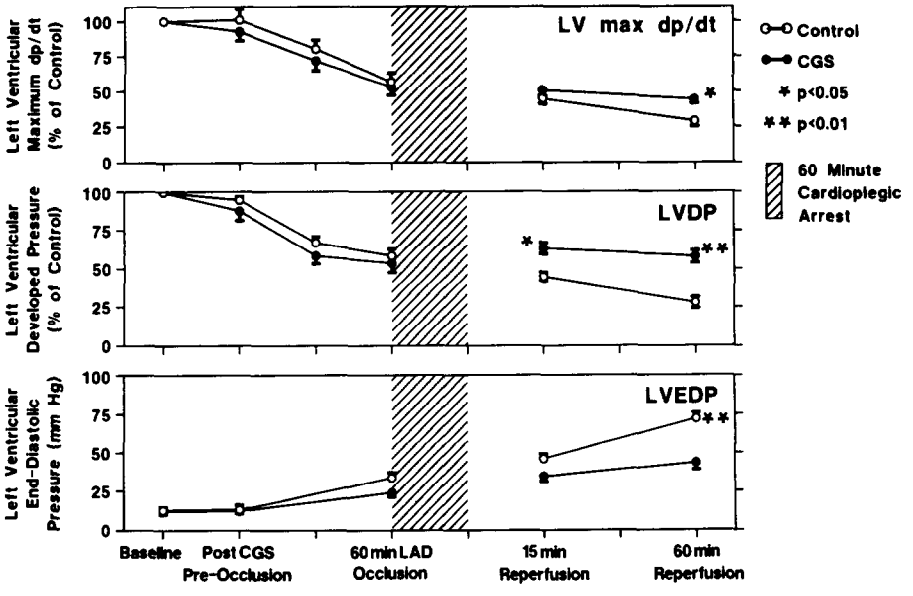

Fig. 1. Effects of CGS 9343B on left ventricular maximum dp/dt (top), left ventricular developed pressure (middle), and left ventricular end-diastolic pressure (bottom) as a function of duration of ischemia and reperfusion. Control values for left ventricular maximum dp/dt: for non-CGS, 1000 to 2500 mm Hg/sec; for CGS, 1112 to 2000 mm Hg/sec. Control values for left ventricular developed pressure: for non-CGS, 113.3 to 170.1 mm Hg; for CGS, 124.4 to 163.5 mm Hg.

RESULTS

Regional and global functions of heart. LAD occlusion was consistently accompanied by ΔL with late systolic elongation (Table 1). After 30 min of normothermic LAD occlusion, ΔL decreased by 85% of the baseline values and decreased further by 98% after 60 min of occlusion. EDL was increased by only 10% after LAD occlusion, which was not significantly different from the baseline values, and returned to the preischemic level upon reperfusion. CGS 9343B enhanced the values of ΔL during the reperfusion compared to the nontreated group. Within 15 min of reperfusion, the CGS 9343B-mediated increase in ΔL was 250% compared to control. This improvement in ΔL persisted throughout the reperfusion phase. EDL was restored to normal levels in all groups. Control experiments were performed simultaneously in non-LAD distribution regions. ΔL and EDL levels were never altered significantly when measured in the perfused region in any of these groups.

CGS 9343B also improved global myocardial contractility and compliance during reperfusion of ischemic myocardium (Fig. 1). In all groups, irrespective of treatment, LV max dp/dt decreased markedly during ischemia, but dropped further during reperfusion only in the control group. CGS 9343B prevented this further reduction of LV max dp/dt during reperfusion. LVDP was also dropped markedly and to the same extent during 60 min of LAD occlusion in both groups. In the control group, LVDP dropped further during reperfusion, and treatment with CGS 9343B inhibited further reduction of LVDP during reperfusion. LVEDP, on the other hand, increased during LAD occlusion and during reperfusion. Treatment with CGS 9343B inhibited the rise of LVEDP during these periods.

Coronary blood flow, myocardial oxygen extraction, and oxygen consumption. The effects of CGS 9343B on total coronary blood flow and myocardial oxygen extraction and oxygen consumption as a function of ischemia and reperfusion are shown in Table 2. Treatment with CGS 9343B for 15 min prior to LAD occlusion increased total coronary blood flow as well as myocardial oxygen consumption during reperfusion. In the control group, myocardial reperfusion deteriorated rather than improved these two variables, which is compatible with the observation that global myocardial function decreased during reperfusion from the levels observed at 60 min after LAD occlusion. Treatment with CGS 9343B prevented further decline of coronary blood flow and moderated the decrease of oxygen consumption during reperfusion significantly.

High energy phosphate compounds in heart and release of creatine kinase. Levels of both ATP and CP decreased appreciably during LAD occlusion and cardioplegic arrest, as expected. Treatment with CGS 9343B could not prevent the decrease in CP (Fig. 2). Upon reperfusion, CP increased in both groups, but CGS 9343B was able to enhance the values of CP significantly after 60 min of reperfusion compared to untreated controls. The levels of ATP, on the other hand, remained at the same low level after the reperfusion phase. However, significantly

Table 2. Effects of 10 μM CGS 9343B on coronary blood flow, myocardial oxygen, extraction, and oxygen consumption as a function of ischemia and reperfusion

	Coronary blood flow (% of baseline control)		Oxygen extraction (% of baseline control)		Oxygen consumption (% of baseline control	
	Control	CGS 9343B	Control	CGS 9343B	Control	CGS 9343B
Pre-occlusion	96.1 ± 5.6	99.1 ± 7.5	102.2 ± 5.0	80 ± 5.3	98.8 ± 5.4	101.2 ± 16.45
30 min LAD-occlusion	80.1 ± 7.7	81.0 ± 8.5	78.9 ± 6.7	75 ± 5.7	62.6 ± 5.3	76.7 ± 20.60
60 min LAD-occlusion	69.5 ± 6.9	65 ± 6.0	81.2 ± 5.5	83 ± 6.9	53.4 ± 6.3	77.8 ± 17.43
15 min Reperfusion	53.0 ± 6.6	71 ± 5.3*	85.9 ± 9.5	62 ± 4.6*	41.9 ± 2.9	85.7 ± 23.6*
60 min Reperfusion	37.0 ± 4.9	77 ± 6.2†	95.6 ± 13.4	78 ± 6.9*	25.7 ± 3.2	39.1 ± 8.4*

Results are means ± SEM of six to nine experiments in each group. Baseline values: coronary blood flow: control, 126.77 to 151.35 ml/min/100 g wet wt, and CGS 9343B, 97.6 to 174.2 ml/min/100 g wet wt; oxygen extraction: control, 23.81 to 47.17, and CGS 9343B, 29.2 to 59.72; and oxygen consumption: control, 2.39 to 3.42 μl/min/g wet wt, and CGS 9343B, 2.59 to 5.97 μl/min/g wet wt.

* $P < 0.05$.

† $P < 0.01$.

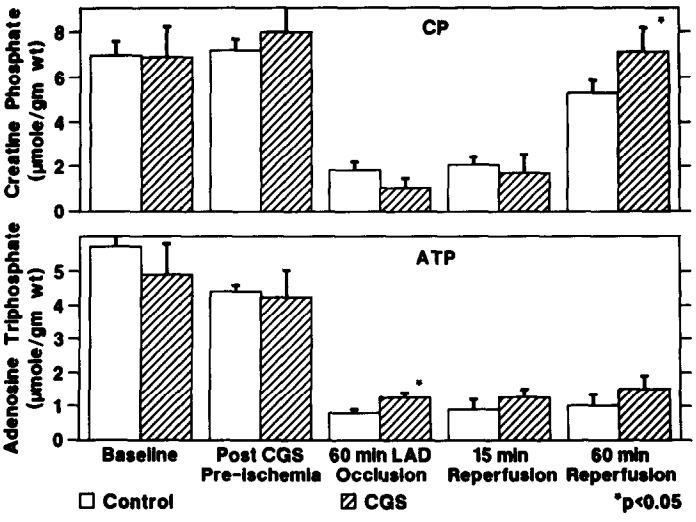

Fig. 2. Effects of CGS 9343B on creatine phosphate (top) and adenosine triphosphate (bottom) levels in heart as a function of duration of ischemia and reperfusion.

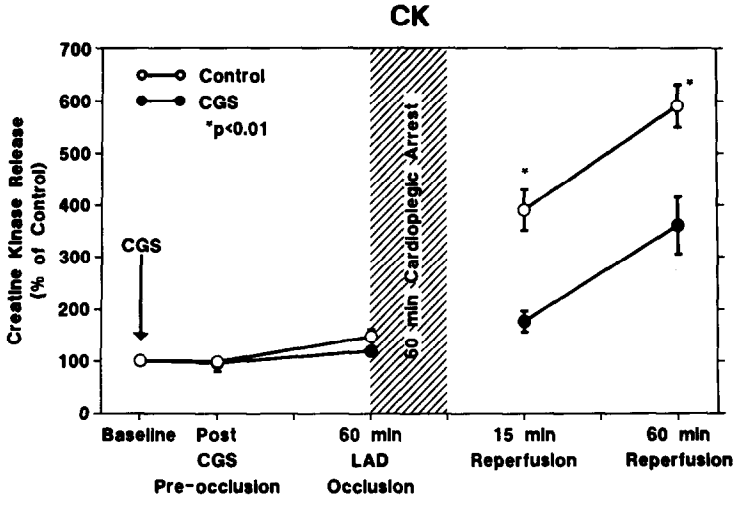

Fig. 3. Effects of CGS 9343B on creatine kinase release as a function of duration of ischemia and reperfusion. Control values for non-CGS were: 50–105 I.U./liter; control values for CGS were: 58–97 I.U./liter.

higher levels of ATP were noticed in the CGS 9343B group after 60 min of LAD occlusion. The release of CK from the ischemic heart was minimal in both groups (Fig. 3). During reperfusion, these values increased significantly. However, the increase in CK release during reperfusion was blocked significantly by CGS 9343B.

DISCUSSION

Reperfusion of ischemic myocardium is associated with the accumulation of massive amounts of Ca^{2+} in the cardiac muscle cells [15, 16]. Reversibly injured cells can be revived during reperfusion, provided the cells can maintain homeostasis with respect to Ca^{2+}. If, on the other hand, cells are injured irreversibly, reperfusion may not only provide no beneficial effects, but it may also be deleterious.

Calcium overloading occurs during postischemic reperfusion by three major routes: Ca^{2+}-selective voltage-activated channels, Na^{+}/Ca^{2+} exchanger, and uncontrolled entry through disrupted sarcolemma. Current knowledge suggests that intracellular accumulation of calcium is responsible, either directly or indirectly, for a number of detrimental pathological events such as breakdown of membrane phospholipids or accumulations of lysoglycerophospholipids and free fatty acids, which occur during reperfusion of ischemic myocardium. These phenomena are under the direct control of the Ca^{2+} messenger system: Ca^{2+}-binding protein, calmodulin and/or protein kinase C [17, 18]. Theoretically, therefore, inhibitors of these Ca^{2+} messenger systems should provide an ischemic heart adequate protection from the reperfusion injury. Indeed, calmodulin antagonists such as trifluo-

perazine [6, 7], W-7 [4], and chlorpromazine [5] all provide certain beneficial effects in cardiac performance during reperfusion after acute myocardial infarction. However, since these calmodulin blockers are also inhibitors of protein kinase C [8, 9], it has not been possible to demonstrate which of these two Ca^{2+} messenger systems is involved in processing the observed exocytosis functions of Ca^{2+} during ischemia and reperfusion. The calmodulin blocker CGS 9343B, used in our study, has been shown to be ineffective on protein kinase C up to a 100 μM concentration [11]. CGS 9343B has provided significant myocardial improvement during reperfusion as judged by improved recovery of global as well as regional cardiac functions and better preservation of high energy phosphate compounds and lower release of CK, which compares favorably with the degree of protection provided by other calmodulin blockers such as trifluoperazine (TFP) [7]. This would tend to suggest that the unique function of Ca^{2+} in the process of exocytosis may be expressed principally by calmodulin. However, our study does not exclude the role of protein kinase C in transmembrane calcium movement during the revascularization process.

Revascularization of ischemic myocardium is associated with destabilization of cell membrane, leading to the formation of membrane blebs and ultimately causing increased membrane permeability. Consequently, a massive amount of calcium accumulates intracellularly, causing extensive damage to the myocardium. Calmodulin has been thought to play an important role in calcium-mediated changes in membrane permeability by modulating calcium-dependent cellular processes. The biological regulation of Ca^{2+} can be expressed in two ways: by binding with proteins such as calmodulin followed by activation of various enzymes, including certain protein kinases, or by directly activating protein kinase C. The results of our study suggest the validity of the former pathway in the Ca^{2+}-mediated regulation of exocytosis processes occurring during the reperfusion of ischemic myocardium, because CGS 9343B can inhibit only calmodulin and not protein kinase C. Thus, CGS 9343B probably inhibited kinases other than protein kinase C, resulting in subsequent modification of the phosphorylation in heart. Several other reports also point out the validity of such a hypothesis. Calmidazolium, a potent inhibitor of calmodulin, has been shown to inhibit protein phosphorylation and ATP-dependent Ca^{2+} uptake, but not Ca^{2+}-mediated ATPase activity in sarcoplasmic reticulum [19]. Okabe and his coworkers also suggested that calmodulin-dependent protein kinase might be involved in the regulation of calcium release channel [20]. Jett and her coworkers have identified a membrane-bound calcium-calmodulin-dependent protein kinase II in canine heart [21]. Our study supports these previous reports and further demonstrates that Ca^{2+}-loaded cell exocytosis during the reperfusion of ischemic myocardium may be regulated by calmodulin-dependent kinase.

Apart from the demonstration that Ca^{2+} entry during reperfusion may be regulated by calmodulin-dependent kinase, this report clearly indicates that inhibition of calmodulin alone, without the inhibition of protein kinase C, should provide significant beneficial effects during cardiac recovery after an ischemic episode. This study also warrants the use of this new compound, CGS 9343B, to protect an ischemic heart from reperfusion injury.

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