Ischemia-Induced Alterations in Myocardial

$(Na^+ + K^+)$-ATPase and Cardiac Glycoside Binding

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A B S T R A C T The effects of ischemia on the canine myocardial $(Na^+ + K^+)$-ATPase complex were examined in terms of alterations in cardiac glycoside binding and enzymatic activity. Ability of the myocardial cell to bind tritiated ouabain in vivo was assessed after 1, 2, and 6 h of coronary occlusion followed by 45 min of reperfusion, and correlated with measurements of in vitro $(Na^+ + K^+)$-ATPase activity and in vitro [³H]-ouabain binding after similar periods of ischemia. Regional blood flow alterations during occlusion and reperfusion were simultaneously determined utilizing 15-μm radioactive microspheres to determine the degree to which altered binding of ouabain might be flow related.

Anterior wall infarction was produced in 34 dogs by snaring of confluent branches of the left coronary system. Epicardial electrograms delineated ischemic and border zone areas. Coronary reperfusion after 2 and 6 h of occlusion was associated with impaired reflow of blood and markedly impaired uptake of [³H]ouabain in ischemic myocardium. In both groups, in vivo [³H]-ouabain binding by ischemic tissue was reduced out of proportion to the reduction in flow. Despite near-complete restoration of flow in seven dogs occluded for 1 h and reperfused, [³H]ouabain remained significantly reduced to 58±9% of nonischemic uptake in subendocardial layers of the central zone of ischemia. Thus, when coronary flow was restored to areas of myocardium rendered acutely ischemic for 1 or more hours, ischemic zones demonstrated progressively diminished ability to bind ouabain. To determine whether ischemia-induced alteration in myocardial $(Na^+ + K^+)$-ATPase might un-
derlie these changes, $(Na^+ + K^+)$-ATPase activity and [³H]ouabain binding were measured in microsomal fractions from ischemic myocardium after 1, 2, and 6 h of coronary occlusion. In animals occluded for 6 h, $(Na^+ + K^+)$-ATPase activity was significantly reduced by 40% in epicardial and by 35% in endocardial layers compared with nonischemic myocardium. Comparable reductions in in vitro [³H]ouabain binding were also demonstrated. Reperfusion for 45 min after occlusion for 6 h resulted in no significant restoration of enzyme activity when compared to the nonreperfused animals. In six animals occluded for 2 h, a time at which myocardial creatine phosphokinase activity remains unchanged, $(Na^+ + K^+)$-ATPase activity was reduced by 25% compared with nonischemic enzyme activity. In five dogs occluded for 1 h, $(Na^+ + K^+)$-ATPase activity in ischemic myocardium was unchanged from control levels.

We conclude that reduced regional myocardial blood flow, local alterations in cellular milieu, and altered glycoside-binding properties of $(Na^+ + K^+)$-ATPase all participate in the reduction in cardiac glycoside binding observed after reperfusion of ischemic myocardium. In addition, after 2 or more hours of severe ischemia, myocardial $(Na^+ + K^+)$-ATPase catalytic activity is significantly reduced despite incubation in the presence of optimal substrate concentrations.

INTRODUCTION

A substantial body of information has accumulated in recent years concerning the metabolic alterations associated with acute myocardial ischemia and hypoxia (1, 2), and the role of these changes in myocardial cell injury. After experimental myocardial infarction, there is an efflux of potassium from the intracellular to the extracellular space (3) and a net influx of sodium and water (4). There is convincing evidence that the chief

molecular mediator of the active transport of sodium and potassium is the Na^+ and K^+-activated ATP phosphohydrolase enzyme complex known as $(Na^+ + K^+)$-ATPase (5). This enzyme system binds digitalis glycosides with high affinity and specificity, and may well constitute the receptor for the pharmacologic and toxic effects of digitalis (5-10). Limited information concerning ischemia-induced alterations in the $(Na^+ + K^+)$-ATPase system of myocardium is available, and the extent to which the functional integrity of $(Na^+ + K^+)$-ATPase limits the potential viability of ischemic myocardium remains unknown. We have previously shown that 1 h after acute myocardial infarction in dogs, there is a marked alteration of early digoxin uptake in ischemic myocardial tissue (11). Further studies showed that reperfusion after 2 h of coronary occlusion was associated with impaired recovery of in vivo digoxin binding in severely ischemic myocardium (12). These findings suggested possible ischemia-induced alterations in myocardial cardiac glycoside-binding capacity, but left open the possibility that the glycoside might be unable to reach membrane receptor sites because of regional flow limitations.

The purpose of the present studies was to determine the extent to which the integrity of the myocardial membrane $(Na^+ + K^+)$-ATPase system is affected by ischemia by, firstly, assessing the ability of the myocardial cell to bind tritiated ouabain in vivo after varying periods of coronary occlusion followed by reperfusion; and secondly, by measurement of in vitro $(Na^+ + K^+)$-ATPase activity and in vitro [^{3}H]ouabain binding after similar periods of ischemia with or without reflow. Regional blood flow alterations during occlusion and reperfusion were simultaneously determined to ascertain the degree to which altered binding of ouabain might be flow related.

METHODS

Experimental model. An open chest canine model was employed in these studies, as previously described (11). Animals were anesthetized with intravenous pentobarbital (30 mg/kg) after which a left thoracotomy was performed. Respiration was maintained with a Harvard respirator (Harvard Apparatus Co., Inc., Millis, Mass.) and all animals breathed 40% oxygen throughout the experiment. All animals maintained a Po_2 of 80 mm Hg or greater (range: 80–155) during the course of these experiments. Arterial pH ranged from 7.37 to 7.42. The heart was suspended in a pericardial cradle, after which 2-0 Mersilene ligatures (Ethicon Inc., Somerville, N. J.) were placed upon multiple confluent branches of the left anterior descending and circumflex coronary arteries supplying an area of the apex and/or anterior wall of the left ventricle. Coronary venous branches were left intact.

In each experiment, 10–12 electrogram-mapping sites were designated on the anterior and lateral surface of the left ventricle (Fig. 1). Electrocardiographic epicardial-surface mapping at each site was performed utilizing a
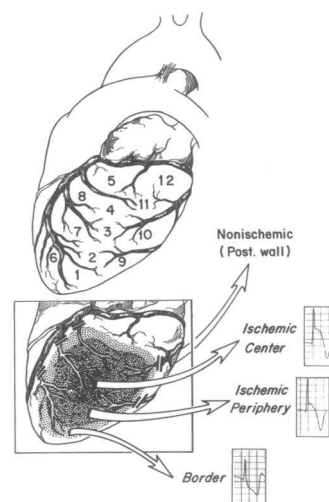
FIGURE 1 Diagrammatic representation of the canine heart in the left lateral projection with 12 epicardial mapping sites delineated. Anterior wall infarction (lower panel) is produced by snaring of confluent branches of the left coronary system. Central and peripheral zones of S-T segment elevation and the border zone of S-T segment depression are shown.

stainless steel cylinder 8 cm long and 2.5 mm in diameter, the tip of which was smoothly ground and polished. The cylinder was attached to the exploring electrode of the conventional electrocardiographic lead system. This electrode was held lightly at a right angle to the epicardium during the surface-mapping procedure. The tip of the electrode was dipped into normal saline at room temperature before recording at each site. In no instance was there evidence of myocardial ischemia or injury demonstrable in the control epicardial electrocardiograms, which were standardized at 1 mm/mV stylus deflection.

A femoral artery was isolated and a catheter passed into the central aorta. Arterial pressure was monitored with a Statham P23Db pressure transducer (Statham Instruments, Inc., Oxnard, Calif.). Both arterial pressure and electrocardiographic recordings were recorded at a paper speed of 50 mm/s on a Gould brush recorder (Gould Inc., Instrument Systems Div., Cleveland, Ohio). In the experiments in which regional myocardial blood flow was assessed, an 8-inch polyethylene catheter with a flared end was inserted into the left atrium through a stab wound in the left atrial appendage and held in place with a purse string suture.

After control epicardial electrograms and pressure measurements were obtained, each coronary arterial branch previously isolated was snared sequentially (5 min apart) until an area of cyanosis comprising about 50% of the anterolateral wall was observed. This usually required four ligations. The incidence of ventricular fibrillation in this experimental infarct model is about 10%. Any dogs developing ventricular fibrillation during occlusion or reperfusion were not included in the study. Epicardial electrograms were then recorded at the same sites mapped previously in the control period. Four zones were delineated: (a) a central zone of S-T segment elevation greater than 1 mV; (b) a peripheral zone of ischemia showing a lesser degree of S-T elevation; (c) a border zone of ischemic S-T de-

pression greater than 1 mV; and (d) a nonischemic zone within which S-T segments remained isoelectric. In three animals only S-T depression was observed in the region of myocardium supplied by the occluded coronary arterial branches. Reperfusion was accomplished after 1, 2, or 6 h of occlusion by release of snares in the same sequence as in the occlusion phase. At the end of each experiment, the heart was rapidly excised, and full-thickness myocardial samples weighing about 2 g were obtained from each of the four zones. Each sample was then divided into endocardial and epicardial halves for the determinations subsequently described.

Effects of ischemia and reperfusion on [³H]ouabain uptake and regional blood flow. In 19 dogs, regional myocardial blood flow and [³H]ouabain uptake were measured after varying periods of coronary occlusion and after 45 min of reperfusion. Distribution of regional myocardial blood flow was determined by bolus injections of ⁸⁵Sr-(12.0 mCi/g) or ¹⁴¹Ce-(9.30 mCi/g) labeled carbonized microspheres of 15±5 μm diameter (3M Co., St. Paul, Minn.), suspended in 10% dextran, into the left atrium over a period of 1 s. Approximately 4 × 10⁶ microspheres were used in each injection. It has previously been demonstrated that this dose of microspheres does not result in hemodynamic alterations (13). In the present experiments, no significant changes in mean arterial blood pressure or heart rate were observed after injection of the spheres.

Aggregation of the spheres was minimized by mixing for 30 s in a Vortex mixer (Scientific Industries, Inc., Queens Village, N. Y.), followed by sonication for 10 min in an ultrasonic bath (Nuclear Associates, Inc., Westbury, N. Y.).

At the end of 1 (n = 7), 2 (n = 6), or 6 (n = 7) h of occlusion, a bolus of ⁸⁵Sr-labeled microspheres was injected. Epicardial electrograms were recorded before and 5 min after microsphere injection. No changes in the epicardial electrogram were noted as a result of the injection of microspheres. All snares on coronary vessels were then released in the order in which they were occluded. After 15 min of reperfusion, 0.5 mg of [³H]ouabain [New England Nuclear, Boston, Mass.], diluted with unlabeled ouabain U.S.P. [Eli Lilly and Co., Indianapolis, Ind.] was administered intravenously in a foreleg vein. 30 min later (45 min after onset of reperfusion), another bolus of 4 × 10⁶ microspheres, this time labeled with ¹⁴¹Ce, was injected into the left atrium. Microspheres were injected 30 min after ouabain was administered to allow for the delay in the attainment of maximum myocardial uptake of the glycoside. An epicardial electrogram was recorded 5 min later, after which the heart was rapidly excised and myocardial samples obtained as noted above. Multiple samples from ischemic, border, and nonischemic zones were analyzed for microsphere and [³H]ouabain content in the following manner. The weighed tissue samples were placed in plastic vials and counted in a two-channel gamma well spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.) at appropriate energy windows. Disintegrations per minute per gram wet weight for ⁸⁵Sr and ¹⁴¹Ce were calculated. The samples were then cut into 1-2-mm³ fragments, placed in 15 ml of the liquid scintillation medium described by Bray (14), and shaken in a water bath at 37°C for 48 h. The myocardial fragments were then sedimented by a 15 min centrifugation at 12,000 g and the supernate was counted for tritium activity. [³H]-Ouabain concentrations (nanograms per gram wet weight) were calculated after correction for quenching by the use of internal standards of [³H]ouabain. In no instance was the supernatant phase contaminated with detectable gamma activity. Greater than 97% complete elution of ³H counts

was documented by comparison with duplicate samples fully digested with NCS (Amersham/Searle Corp., Arlington Heights, Ill.) before counting.

Effects of ischemia and reperfusion on myocardial (Na⁺ + K⁺)-ATPase. The integrity of the myocardial cell membrane (Na⁺ + K⁺)-ATPase system after varying periods of ischemia was further assessed by in vitro measurement of enzymatic activity and ability to bind [³H]ouabain. The effect on enzyme activity of reperfusion of ischemic myocardium after 6 h of coronary occlusion was also studied. 20 dogs underwent acute infarction in the manner described previously. Delineation of ischemic myocardium was again accomplished by epicardial electrode mapping. Nine animals underwent coronary occlusion for 6 h; three of these were reperfused for 45 min. 11 additional dogs underwent occlusion for a period of either 1 or 2 h after which they were sacrificed.

At the end of each occlusion (or occlusion and reperfusion) period, the hearts were rapidly excised and frozen at −20°C. Initial experiments demonstrated no significant difference in enzyme activity in fresh myocardium or in hearts frozen by immediate immersion in liquid nitrogen compared with samples immediately frozen at −20°C. 7 days later the hearts were thawed at 4°C and the left ventricular myocardium divided into four 10-g samples comprising endocardial and epicardial halves of ischemic (zone of S-T segment elevation) and nonischemic regions. The excess fat and coronary vessels were dissected free from epicardial surfaces.

Partial purification of (Na⁺ + K⁺)-ATPase was carried out with minor modifications of the method of Akera et al. (15). All procedures were carried out at 4°C. Samples were cut into 1-2-mm³ portions and homogenized in 0.25 M sucrose, 5 mM histidine, 5 mM EDTA, 0.15% sodium desoxycholate, 10 mM dithiothreitol, adjusted with Tris base to pH 6.8. Homogenization was carried out with a Virtis 23 homogenizer (VirTis Co., Inc., Gardiner, N. Y.) set at full speed for a total time of 10 s. After removal of an aliquot, the crude homogenate was centrifuged at 12,000 g for 30 min. The supernate was then centrifuged at 100,000 g for 60 min and the pellet resuspended in 17 ml of 0.25 M sucrose, 5 mM histidine, 1 mM EDTA, 10 mM dithiothreitol, adjusted with Tris base to pH 7.0. The suspension was centrifuged again at 100,000 g for 60 min. The resulting pellets were resuspended in 8.5 ml of the same solution and 8.5 ml of 2.0 M lithium bromide was slowly added. This mixture was stirred slowly for a period of 30 min, then centrifuged at 100,000 g for 30 min. The final pellet was then resuspended in 5-7 ml of 0.25 M sucrose, 5 mM histidine, 1 mM EDTA, adjusted to pH 7.0 with Tris base, in preparation for assay procedures. Each resuspension of the pellet was accompanied by three manual strokes with an ice-cold, ground glass homogenizer.

Because of the possibility that homogenization of ischemic myocardium might result in alteration in the yield of (Na⁺ + K⁺)-ATPase relative to other tissue components, parallel experiments were performed in which (Na⁺ + K⁺)-ATPase activity was assayed in crude homogenates of ischemic and nonischemic myocardium. In these experiments, further homogenization steps were carried out to obtain a more uniform suspension. 3 ml of the initial crude homogenate was added to 15 ml of 0.15% sodium desoxycholate solution containing 0.25 M sucrose, 5 mM histidine, 1 mM EDTA, and 10 mM dithiothreitol. This was homogenized at full speed for 1 min, employing 15-s periods of homogenization alternating with 30 s of cooling. The final uniform suspension was then assayed for ATPase activity.

Protein concentrations of purified pellets and crude homogenates were determined by the Lowry et al. procedure (16) using bovine serum albumin as standard. $(Na^+ + K^+)$-ATPase assays were carried out in a final volume of 1 ml containing 50 mM Tris HCl buffer, pH 7.4, 100 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, 5 mM Tris-ATP, and 50–75 μg of enzyme protein. Identical tubes, save for absence of NaCl and KCl, were assayed for Mg^{++}-dependent ATPase activity and blank tubes lacking enzyme were assayed to permit correction for nonenzymatic hydrolysis of ATP. All assays were performed in duplicate. The reaction was begun by adding Tris-ATP. After incubation for 15 min at 37°C, the reaction was terminated by addition of 1.5 ml of ice-cold 8.3% trichloroacetic acid. The mixture was centrifuged at 5,000 g for 3 min and 2-ml aliquots were withdrawn for inorganic phosphate analysis by the method of Fiske and Subbarow (17). $(Na^+ + K^+)$-ATPase activity in micromoles Pi per milligram of protein per hour was calculated as the difference between total ATPase activity in the presence of Na^+ and K^+ and the base-line activity observed with Mg^{++} alone.

In all animals [³H]ouabain binding to $(Na^+ + K^+)$-ATPase was studied in vitro in specimens from ischemic and nonischemic zones essentially according to the method of Schwartz et al. (18). The reaction mixture contained 5 mM $MgCl_2$, 4 mM Tris-ATP, 100 mM NaCl, 1 mM EDTA, and 50 mM Tris-HCl adjusted to pH 7.4. Tritiated ouabain (sp act 14.6 Ci/mmol), 0.2 μM, was present with or without addition of 0.2 mM unlabeled ouabain to permit correction for nonspecific binding. Each tube contained 0.2–0.3 mg enzyme protein, in a total volume of 2.0 ml. The reaction was initiated with addition of the enzyme protein and proceeded for 10 min at 37°C before transferring the tubes to an ice bath and then centrifuging at 100,000 g for 30 min. After discarding the supernate, pellets were dissolved in 0.5 ml of 2% sodium dodecyl sulfate and aliquots were counted for ³H activity in a liquid scintillation spectrometer using toluene detergent-based liquid scintillation fluid (Instagel, Packard Instrument Co., Inc., Downers Grove, Ill.). [³H]Ouabain binding (picomoles ouabain per milligram enzyme protein) was calculated after correction for quenching by the use of internal standards. Specific binding to the enzyme was corrected for nonspecific binding in the presence of 0.2 mM unlabeled ouabain. Ratios of [³H]-ouabain binding to enzymatic activity for partially purified microsomal enzyme samples were in the 4–5 pmol [³H]-ouabain/μmol P_1 cleaved per h range, higher than ratios 1.2–1.4 for crude myocardial homogenates, apparently reflecting decreased turnover numbers for the partially purified enzyme.

RESULTS

Regional flow and [³H]ouabain uptake in nonischemic myocardium. Regional blood flow was homogeneous in nonischemic posterior wall myocardium. The mean percent difference in microsphere distribution between duplicate samples was 10%. Transmural flow in normal myocardium was unchanged after reperfusion compared to values observed at the end of the occlusion period. The mean endocardial/epicardial ratios of microsphere and [³H]ouabain distribution in nonischemic posterior wall myocardium for animals undergoing coronary occlusion for 1, 2, and 6 h followed by reperfusion are shown in Table I. In 1- and 2-h occluded and reperfused

TABLE I
Endocardial/Epicardial Ratios of Distribution of Microspheres and [³H]Ouabain in Nonischemic Myocardium

Duration of occlusion	⁸⁵Sr (Occlusion)	¹⁴¹Ce (Reflow)	[³H]Ouabain (Reflow)
h			
1 (n = 7)	1.23±0.08	1.22±0.09	1.21±0.05
2 (n = 5)	1.22±0.16	1.24±0.19	1.25±0.09
6 (n = 7)	0.93±0.02*	0.94±0.04*	1.14±0.03*

* $P < 0.05$ compared to 1-h occlusion values.

animals, flow to endocardial layers of nonischemic myocardium was significantly greater than flow to epicardial layers during both occlusion and reperfusion periods. In 6-h occluded and reperfused animals regional flow was slightly but not significantly increased in epicardial layers. The endocardial/epicardial flow ratios in nonischemic myocardium during occlusion and reperfusion were significantly lower when compared to 1-h occluded and reperfused animals. Mean endocardial/epicardial ratio of [³H]ouabain in nonischemic myocardium was similar to the transmural flow ratios in all three groups.

1-h Occlusion group. In seven dogs occluded for 1 h, reperfusion resulted in almost complete reflow to ischemic periphery and border zone areas (Fig. 2). In the center of the ischemic zone, at the end of 1 h of occlusion and before reflow, regional flow was reduced to 6±3% (SE) of nonischemic flow in endocardial and 29±8% in epicardial layers $(P < 0.001)$. After 45 min
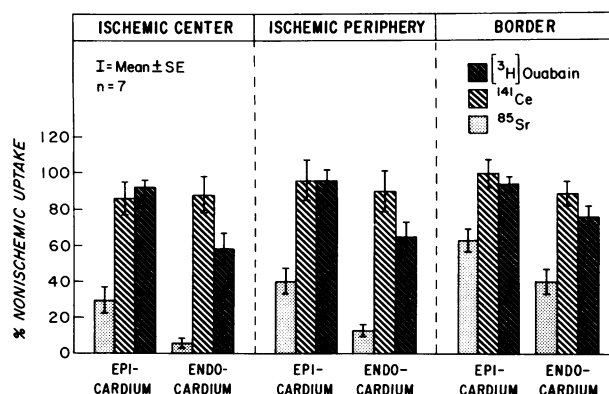

FIGURE 2 Regional blood flow and ouabain uptake in ischemic myocardium in 1-h occluded and reperfused dogs. The first bar of each triad (stippled) represents regional flow (⁸⁵Sr microspheres) as percent of nonischemic flow at the end of 1 h of coronary occlusion. The second bar of each triad (light cross-hatched) represents regional flow (¹⁴¹Ce microspheres) as percent of nonischemic flow after 45 min of reperfusion. The third bar of each triad (dark cross-hatched) represents [³H]ouabain uptake during reperfusion.

of reperfusion, flow was 88±10% of nonischemic flow in endocardial and 86±9% in epicardial regions. These reductions were not statistically significant at the $P = 0.05$ level. Mean endocardial and epicardial ouabain concentrations in nonischemic myocardium were 187±20 and 156±19 ng/g wet weight, respectively. Despite near-normal flow, [³H]ouabain uptake remained significantly reduced to 58±9% in endocardial layers of this zone as compared to the mean nonischemic endocardial concentration ($P < 0.001$). Mean [³H]ouabain concentration in epicardial layers after reperfusion was 92±8% of nonischemic uptake. Mean endocardial/epicardial flow ratio in the central ischemic zone was 0.20± 0.04 at the end of the 1-h occlusion period and significantly increased to 1.28±0.18 after 45 min reperfusion ($P < 0.001$). The endocardial/epicardial ratio of [³H]-ouabain uptake after reperfusion was 0.73±0.08, significantly lower than the transmural gradient of [³H]-ouabain in nonischemic tissue (1.21±0.05).

In the periphery of the ischemic zone, regional flow as judged by microsphere distribution was markedly reduced (Fig. 2). After reperfusion of this zone, nutrient blood flow was 90±11 and 96±11% of nonischemic flow in endocardial and epicardial layers, respectively. As was observed in the center of the ischemic zone, [³H]ouabain uptake during reperfusion remained significantly reduced to 65±8% in endocardial tissue as compared to nonischemic ouabain concentrations ($P < 0.05$).

Border zone flow was also significantly reduced at 1 h of occlusion to 40±7% of nonischemic flow in endocardium and 63±6% in epicardium ($P < 0.05$). Reperfusion of this zone resulted in complete restoration of flow, similar to that observed in the periphery of the ischemic region. The border zone endocardial/epicardial flow ratio was 0.69±0.06 during the occlusion phase and significantly increased to 1.09±0.01 after reperfusion in this group of animals ($P < 0.01$). Subendocardial ouabain uptake, however, remained depressed in six of the seven dogs reperfused after 1 h of coronary occlusion and ranged from 61 to 84% of nonischemic uptake.

6-h Occlusion group. Regional flow and [³H]ouabain uptake values for dogs occluded for 6 h and reperfused are shown in Fig. 3. In seven dogs occluded for 6 h, regional blood flow at the end of the occlusion period, just before reperfusion, was reduced to 4±1% of nonischemic flow in endocardial and to 15±4% in epicardial layers of the center of the ischemic zone ($P < 0.001$). The endocardial/epicardial flow ratio was markedly reduced to 0.20±0.06 as compared to 0.93±0.02 in nonischemic myocardium. This reduction in flow at the end of 6-h occlusion was similar to the reduction in flow observed in the group occluded for 1 h. After 45 min of reperfusion, regional blood flow was only 53±11%

of nonischemic flow in endocardial ($P < 0.05$), but was 108±20% in epicardial layers. This finding is in contrast to that observed in the 1-h occluded group of dogs in which flow to ischemic areas after 45 min of reperfusion was not significantly reduced in comparison to nonischemic flow values. The endocardial/epicardial flow ratio during reperfusion in the 6-h group was only 0.51±0.09, compared to the nonischemic endocardial/epicardial transmural flow ratio of 0.94±0.04 ($P < 0.05$). Mean (SE) [³H]ouabain concentrations in the nonischemic posterior wall samples were 183±13 ng/g in endocardial and 161±9 ng/g in epicardial layers. This difference was statistically significant ($P < 0.05$). After reperfusion, [³H]ouabain uptake was significantly reduced to 29±7% of nonischemic uptake in endocardial layers and to 62±10% in epicardial layers of the center of the ischemic zone ($P < 0.001$). This impairment in ouabain binding after reperfusion could not entirely be explained by the reduction in regional flow, the reduction of glycoside uptake being significantly greater than the reduction in flow. In epicardial layers of the central ischemic region, flow returned to nonischemic levels after reperfusion while the mean ouabain concentration remained decreased by 38% as compared to nonischemic glycoside concentrations.

In the periphery of the ischemic zone, regional flow was also markedly reduced at 6 h of occlusion to 15±7% of nonischemic flow in endocardial and to 24±7% in epicardial regions ($P < 0.001$). After reperfusion, flow was variable in endocardial layers of this zone and ranged from 34 to 174% of control (mean 82±20%). In
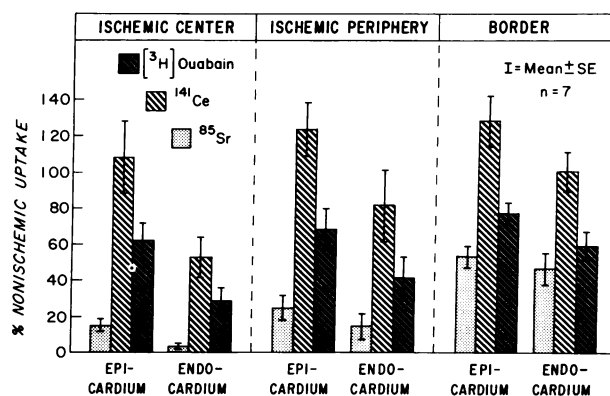

FIGURE 3 Regional blood flow and [³H]ouabain uptake in ischemic myocardium in 6-h occluded and reperfused dogs. The first bar of each triad (stippled) represents regional flow (⁸⁵Sr microspheres) as percent of nonischemic flow, at the end of 6 h of coronary occlusion. The second bar of each triad (light cross-hatched) represents regional flow (¹⁴¹Ce microspheres) as percent of nonischemic flow, after 45 min of reperfusion. The third bar of each triad (dark cross-hatched) represents [³H]ouabain uptake during reperfusion.

three of the seven animals, flow remained below 45% of nonischemic values. Mean flow in the epicardial region was 124±14% of nonischemic flow (range: 78-191%), suggesting some reactive hyperemia in this region. [³H]-ouabain uptake during reperfusion in the ischemic periphery was significantly reduced to 42±12% in endocardial and to 68±10% in epicardial regions ($P < 0.05$). As seen in the central ischemic zone, the decrease in ouabain uptake was again significantly greater than the concomitant reduction in flow after reperfusion.

Regional flow in the border zone after reperfusion in the 6-h occluded animals was slightly greater than nonischemic flow, again reflecting reactive hyperemia in this region. [³H]ouabain uptake in the border zone, however, remained significantly reduced after reperfusion when compared with nonischemic uptake.

In six of the seven dogs in this group, subendocardial hemorrhage and intramyocardial edema were grossly evident at the time of sacrifice in the central and peripheral zones of ischemia. There was no evidence of thrombi in branches of the left anterior descending or circumflex coronary arteries at the time of sectioning of the heart.

2-h Occlusion group. To estimate more accurately the duration of occlusion beyond which reperfusion no longer restored normal flow, similar experiments were carried out in another group of five dogs occluded for 2 h and reperfused. As shown in Fig. 4, the findings were intermediate between those observed in the 1- and 6-h occluded and reperfused groups. The initial decrease in flow during occlusion was comparable with that seen in the other two groups. In three of the five animals, endo-
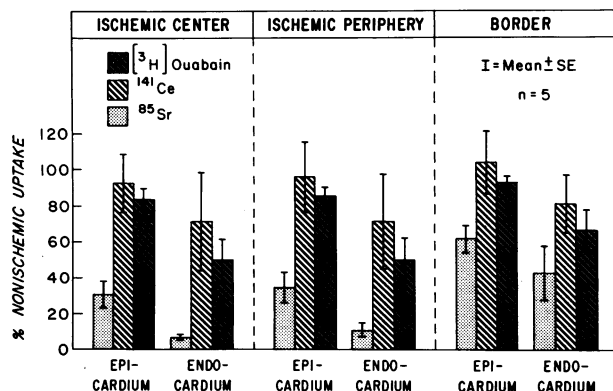

FIGURE 4 Regional blood flow and [³H]ouabain uptake in ischemic myocardium in 2-h occluded and reperfused dogs. The first bar of each triad (stippled) represents regional flow (⁸⁵Sr microspheres), as percent of nonischemic flow, at the end of 2 h of coronary occlusion. The second bar of each triad (light cross-hatched) represents regional flow (¹⁴¹Ce microspheres), as percent of nonischemic flow, after 45 min of reperfusion. The third bar of each triad (dark cross-hatched) represents [³H]ouabain uptake during reperfusion.
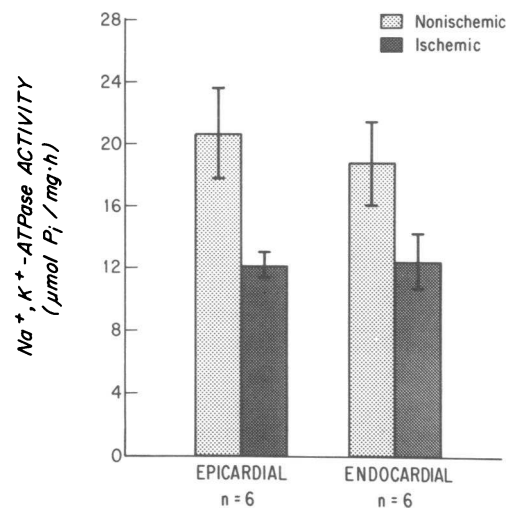

FIGURE 5 (Na⁺ + K⁺)-ATPase activity in myocardial microsomal fractions from epicardial and endocardial samples in dogs occluded for 6 h. Bars represent mean values±1 SE. Differences between nonischemic and ischemic values are statistically significant ($P < 0.001$ for both).

cardial flow after reperfusion remained reduced to less than 40% of nonischemic flow in the center and periphery of the ischemic zone. In the remaining two animals, transmural flow patterns in ischemic zones were similar to or greater than that observed in nonischemic regions. [³H]ouabain uptake during reperfusion, however, was significantly impaired in all regions except for epicardial layers of the border zone.

(Na⁺ + K⁺)-ATPase activity and in vitro [³H]ouabain binding. The above results, together with our prior studies of digoxin binding to ischemic myocardium (11, 12), indicate that reduction in blood flow alone cannot account for the observed decrement in myocardial cardiac glycoside binding after ischemia and reperfusion. To test the hypothesis that ischemia-induced alteration in (Na⁺ + K⁺)-ATPase might contribute to the observed changes, in vitro studies of myocardial preparations were performed in which optimal ionic milieu and substrate (ATP) concentrations were provided for (Na⁺ + K⁺)-ATPase activity or for [³H]ouabain binding. (Na⁺ + K⁺)-ATPase activity and in vitro [³H]ouabain binding of myocardial microsomal fractions from subepicardial and subendocardial samples in six dogs occluded for 6 h are shown in Figs. 5 and 6. Both (Na⁺ + K⁺)-ATPase activity and [³H]ouabain binding were uniform in all nonischemic areas of left ventricular myocardium. In epicardial samples of ischemic myocardium from these animals, (Na⁺ + K⁺)-ATPase activity was significantly reduced to 61.3±4.6% of nonischemic enzyme activity ($P < 0.001$). Subendocardial enzyme activity was also significantly reduced to 66.8±4.9% of nonischemic ac-
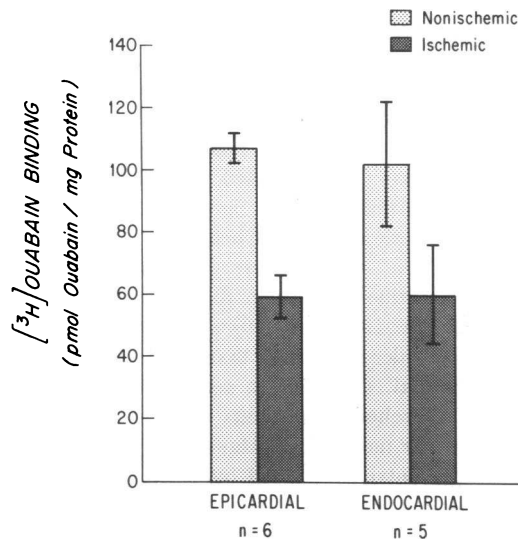
FIGURE 6 In vitro [³H]ouabain binding in microsomal fractions from epicardial and endocardial samples in dogs occluded for 6 h. Differences between nonischemic and ischemic values are statistically significant (epicardial: $P < 0.001$; endocardial: $P < 0.05$).

FIGURE 7 $(Na^+ + K^+)$-ATPase activity in myocardial microsomal fractions from epicardial and endocardial samples in dogs occluded for 2 h. Bars represent mean values±1 SE. Differences between nonischemic and ischemic values are statistically significant ($P < 0.05$ for both).

tivity ($P < 0.001$). As shown in Fig. 6, in vitro [³H]-ouabain binding was also significantly impaired in microsomal fractions from ischemic epicardial and endocardial samples, by a magnitude comparable to the reduction in $(Na^+ + K^+)$-ATPase activity ($P < 0.001$). In the two dogs from this group so studied, $(Na^+ + K^+)$-ATPase activity in the crude homogenates of myocardial samples were reduced in the ischemic zone to a degree closely similar to that observed in microsomal fractions from the same samples (32 and 33% reductions in crude homogenate $(Na^+ + K^+)$-ATPase activities in epicardial and endocardial samples compared with 39 and 29% reductions in microsomal activities).

In three dogs occluded for 6 h and reperfused for 45 min, $(Na^+ + K^+)$-ATPase activity remained significantly reduced to 69±9% of nonischemic activity in epicardial and 55±6% in endocardial layers of the ischemic zone. The average reduction in enzyme activity was similar to that observed in 6-h occluded dogs in which reperfusion was not carried out.

Fig. 7 shows the values for $(Na^+ + K^+)$-ATPase activity in ischemic and nonischemic myocardium in six animals occluded for 2 h. Enzyme activity was significantly reduced to 75±9% of nonischemic activity ($P < 0.05$) in epicardial layers and to 76±8% in endocardial layers ($P < 0.05$). The reduction of ATPase activity in the ischemic zone (endocardial and epicardial) was significantly less than that observed in the 6-h occluded group. As shown in Fig. 8, [³H]ouabain binding to the microsomal enzyme preparation was also significantly
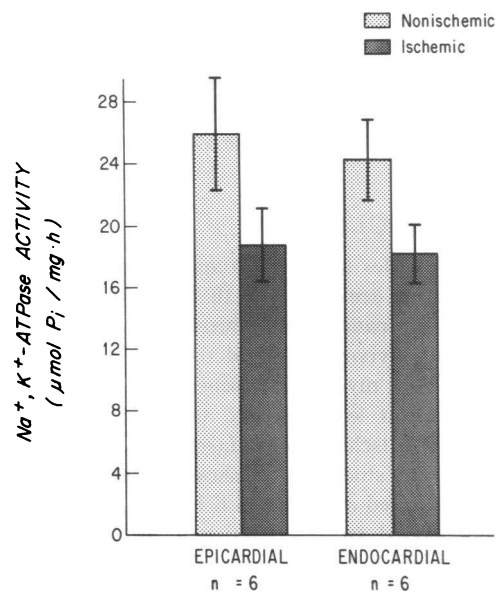
impaired in this group. The mean percent decrease in binding was again comparable to the percent reduction in enzyme activity. In the two animals in this group so studied, $(Na^+ + K^+)$-ATPase activity in crude homogenates of ischemic tissue samples also showed a reduction in activity closely similar to that measured in the microsomal pellets of these same samples. Crude homogenate $(Na^+ + K^+)$-ATPase activities were reduced by an average of 23% compared to 26.5% reduction in microsomal activities. In contrast, we found no decrease in
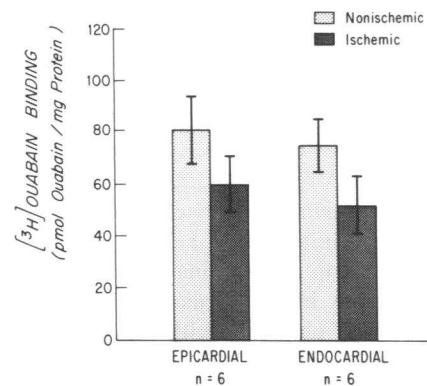

FIGURE 8 In vitro [³H]ouabain binding in microsomal fractions from epicardial and endocardial samples in dogs occluded for 2 h. Differences between nonischemic and ischemic values are statistically significant by paired t test ($P < 0.025$ for both).

myocardial creatine phosphokinase activity in samples from the ischemic center after 2 h of occlusion.

In five dogs occluded for 1 h, no significant decrease in $(Na^+ + K^+)$-ATPase activity in either subendocardial or subepicardial layers of the ischemic zone was demonstrable.

DISCUSSION

Previous studies in our own and other laboratories (11, 19, 20) have demonstrated that after coronary arterial occlusion in canine experimental models, marked reduction in in vivo binding of tritiated digoxin occurs in the region of myocardial ischemia. These findings are attributable, at least in part, to alterations in regional blood flow to ischemic or infarcted myocardium (20).

Subsequent studies (12) showed that coronary reperfusion instituted after 2 and 6 h of myocardial ischemia was associated with a persistent marked reduction in digoxin uptake in regions perfused by the previously occluded coronary arteries. Impaired binding of the glycoside in vivo after reperfusion was more severe with longer ischemic periods before flow restoration (12).

At least three mechanisms can be postulated for impairment of left ventricular cardiac glycoside uptake in experimental, and presumably clinical, myocardial ischemia. First, the decrement in in vivo glycoside binding is almost certainly related in part to reduced regional blood flow to ischemic tissue. Alterations in cardiac glycoside uptake with administration after reperfusion might still result from persistent reductions in regional flow due to the no reflow phenomenon, previously demonstrated after similar periods of experimental coronary occlusion (21, 22). Second, impaired digoxin binding could result from ischemia-induced alterations in the local milieu preventing optimum binding of the glycoside. Local increases in extracellular potassium concentration, known to occur with acute myocardial ischemia (3), would tend to decrease cardiac glycoside binding (23, 24). Depletion of cellular ATP stores could be an additional factor in reduced glycoside binding by ischemic myocardium. ATP, required for optimal binding of cardiac glycosides to $(Na^+ + K^+)$-ATPase (6), has been shown to be rapidly depleted during acute ischemia (2). In addition, reperfusion instituted after 1 h of coronary occlusion has been shown in an experimental model to result in a greater decrease in myocardial ATP and ATP/ADP ratio than was observed with occlusion alone (25). Third, acute myocardial ischemia might cause reduced cardiac glycoside-binding activity of $(Na^+ + K^+)$-ATPase due to structural and/or functional damage to the transport enzyme complex that would be demonstrable even in the presence of optimal binding conditions.

Only limited data have been available previously concerning primary alterations in myocardial $(Na^+ + K^+)$-ATPase catalytic activity resulting from myocardial ischemia or hypoxia. In an isolated rat heart model, severe hypoxia has been shown to result in a reversible depression in $(Na^+ + K^+)$-ATPase activity (26). Schwartz et al. (27) reported that in dogs undergoing circumflex coronary artery ligation, $(Na^+ + K^+)$-ATPase activity was reduced in hearts of dogs 7 days after ligation. These investigators did not observe a decrease in enzyme activity in the first 24 h of ischemia. In the present study, the canine infarct model employed differed considerably from that used in the studies of Schwartz et al. (27) and it seems likely that differing severity of ischemia accounts for the differences observed. In our model, a substantial area of anterior wall of the left ventricle, defined by both epicardial electrogram mapping and radioactive microsphere distribution, was rendered severely ischemic, with flow in the center of the ischemic region reduced by more than 95% compared to nonischemic areas.

The present experiments were undertaken to define which of the mechanisms just postulated account for the reduced cardiac glycoside finding observed after acute myocardial ischemia, and to determine whether, and to what extent, acute myocardial ischemia of varying durations alters myocardial $(Na^+ + K^+)$-ATPase catalytic activity as measured in vitro. [³H]ouabain was used in the present studies in preference to less polar glycosides such as digoxin and digitoxin that tend to bind nonspecifically to myocardium to a substantially greater extent than ouabain (28).

In the first group of experiments described, regional myocardial blood flow during occlusion and reperfusion and in vivo [³H]ouabain binding were simultaneously assessed to determine the degree to which altered cardiac glycoside binding was flow related. It has been shown previously that distribution of 8-15 μm radioactively labeled carbonized microspheres around the circumference of the normal canine left ventricle is homogeneous, and that there is a small but significant preferential deposition of these particles in endocardial regions of nonischemic myocardium (29-37). The findings of the present study are similar in this regard. After coronary occlusion, regional blood flow was markedly reduced in ischemic zones characterized by S-T segment elevation or depression in the epicardial electrogram, and a striking reversal in endocardial/epicardial flow ratios was observed. Regional flow was reduced in subendocardial layers of the center of the ischemic zone by 94-96%, and the endocardial/epicardial flow ratio was reduced to values in the 0.2-0.3 range. The marked reduction in flow to ischemic myocardium in the present study is presumably due to the fact that visible vessels which might have served as sources of collateral blood flow were ligated.

In the experimental ischemia model used in these studies, coronary reperfusion after 1 h of occlusion was associated with a restoration of myocardial blood flow to nonischemic values. However, in vivo [³H]ouabain uptake after reperfusion remained significantly diminished (by 42%) in subendocardial layers of the central zone of ischemia. Coronary reperfusion after 2 and 6 h of occlusion resulted in impaired reflow of blood in addition to impaired uptake of [³H]ouabain in severely ischemic myocardium. In these groups of animals, [³H]ouabain uptake by ischemic myocardium was reduced to a substantially greater extent than the reduction in flow. In addition, at the time [³H]ouabain was injected (15 min postreperfusion) the degree of reduced reflow is actually less than that observed after 45 min of reperfusion (12). Thus, after 2 and 6 h of coronary occlusion, the reduced reflow phenomenon is a contributory, but not the sole factor responsible for impaired glycoside binding observed after reperfusion of ischemic myocardium.

It is apparent from the data presented that coronary occlusion of 1- or more h duration also resulted in ischemia-induced structural or functional alterations in ouabain-binding components of the myocardium. Impairment of in vivo ouabain uptake in ischemic myocardium after 2 and 6 h of coronary occlusion was accompanied by significantly reduced in vitro (Na⁺ + K⁺)-ATPase catalytic activity and ouabain binding in microsomal fractions and in crude homogenates from ischemic areas, despite incubation in the presence of optimal concentrations of Na⁺, K⁺, Mg⁺⁺, and ATP. Comparable reductions in (Na⁺ + K⁺)-ATPase in subendocardial and subepicardial halves of the center of the ischemic zone may be related to the severe reduction in flow (> 70%) produced in both regions during the occlusion phase of the experiment.

Diminished (Na⁺ + K⁺)-ATPase activity after 2 h of coronary occlusion was not accompanied by creatine phosphokinase depletion in ischemic myocardium and other investigators have also found that creatine phosphokinase depletion occurs only after more prolonged durations of ischemia (37, 38). This difference in early ischemia-induced reductions in activity of these two enzyme systems is not surprising in view of their known differences in cellular localization and fractionation behavior (39, 40).

After only 1 h of myocardial ischemia, (Na⁺ + K⁺)-ATPase activity was not significantly reduced when compared to nonischemic control enzyme activity from the same heart. Since reduced [³H]ouabain binding in vivo in the central ischemic zone was observed with 1 h of ischemia followed by reperfusion, despite complete restoration of blood flow as judged by radioactive microsphere distribution, it appears that alteration in the local milieu also plays a part in ischemia-induced impairment of cardiac glycoside binding.

Thus, all three postulated mechanisms (reduced regional myocardial blood flow, local alterations in the milieu of cellular cardiac glycoside-binding sites, and altered glycoside-binding properties of (Na⁺ + K⁺)-ATPase) appear to participate in the reduction of cardiac glycoside binding observed after reperfusion of ischemia myocardium.

The significant reduction in in vitro myocardial (Na⁺ + K⁺)-ATPase activity observed in the present studies would be expected to be reflected in reduced active transmembrane transport of Na⁺ and K⁺. Furthermore, in view of ischemia-induced substrate limitations, the magnitude of reduction in monovalent cation transport in the in vivo situation would be expected to be considerably in excess of the reductions in (Na⁺ + K⁺)-ATPase observed in vitro in the presence of optimum Na⁺, K⁺ Mg⁺⁺, and ATP concentrations. The effects of ischemia on active monovalent cation transport systems in myocardium deserve further study.

ACKNOWLEDGMENTS

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