Glycolytic inhibition causes spontaneous ventricular fibrillation in aged hearts


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Morita N, Lee JH, Bapat A, Fishbein MC, Mandel WJ, Chen P, Weiss JN, Karagueuzian HS. Glycolytic inhibition causes spontaneous ventricular fibrillation in aged hearts. Am J Physiol Heart Circ Physiol 301: H180–H191, 2011.—Selective glycolytic inhibition (GI) promotes electromechanical alternans and triggered beats in isolated cardiac myocytes. We sought to determine whether GI promotes triggered activity by early afterdepolarization (EAD) or delayed afterdepolarizations in intact hearts isolated from adult and aged rats. Dual voltage and intracellular calcium ion ([Ca^2+]) fluorescence optical maps and single cell glass microelectrode recordings were made from the left ventricular (LV) epicardium of isolated Langendorff-perfused adult (~4 mo) and aged (~24 mo) rat hearts. GI was induced by replacing glucose with 10 mM pyruvate in oxygenated Tyrode’s. Within 20 min, GI slowed [Ca^2+] transient decline rate and shortened action potential duration in both groups. These changes were associated with ventricular fibrillation (VF) in the aged hearts (64 out of 66) but not in adult hearts (0 out of 18; P < 0.001). VF was preceded by a transient period of focal ventricular tachycardia caused by EAD-mediated triggered activity leading to VF within seconds. The VF was suppressed by the ATP-sensitive K (K_ATP) channel blocker glibenclamide (1 μM) but not (0 out of 7) by mitochondrial K_ATP block. The Ca-calmodulin-dependent protein kinase II (CaM-KII) blocker KN-93 (1 μM) prevented GI-mediated VF (P < 0.05). Block of Na-Ca exchanger (NCX) by SEAM0400 (2 μM) prevented GI-mediated VF (3 out of 5), provided significant bradycardia did not occur. Aged hearts had significantly greater LV fibrosis and reduced connectin 43 than adult hearts (P < 0.05). We conclude that in aged fibrotic unlike in adult rat hearts, GI promotes EADs, triggered activity, and VF by activation of K_ATP channels CaMII and NCX. Fibrosis; aging; early afterdepolarizations; triggered activity; calcium cycling; optical mapping

GLYCOLYSIS ACCOUNTS FOR ~5% of total cellular ATP production in the beating heart under aerobic conditions (19, 52). However, studies in isolated cardiac myocytes have shown that selectively inhibiting glycolytic ATP production (12, 20), without affecting mitochondrial ATP production or redox state (2, 21), can lead to electromechanical alternans and triggered action potentials. This is consistent with other experimental evidence supporting a functional coupling between glycolytic ATP production and membrane protein function, including intracellular calcium ion concentration ([Ca^2+]2+) transport by the sarco(endo)plasmic reticulum Ca^2+-ATPase (SERCA2a) (52), Na^+ transport by Na-K-ATPase (42), and regulation of sarcolemmal ATP-sensitive K (K_ATP) channels (48).

For each glucose molecule, glycolysis generates two pyruvate, two ATP, and two reduced NADH molecules, according to the reaction: glucose + 2[NAD]^+ + 2[ADP] + 2[P] → 2pyruvate + 2NADH + 2ATP + 2H_2O. Replacing glucose with pyruvate inhibits forward glycolytic flux while providing mitochondria with abundant substrate (pyruvate) for oxidative phosphorylation, resulting in selective glycolytic inhibition (GI) without impairing overall cellular energy production (47).

Reduction of glycolytic ATP supply to the SERCA2a pump and other membrane proteins could lead to 1) reduced Ca^2+ transient decline rate, 2) shortening of the action potential duration (APD) due to activation of ATP-sensitive potassium (K_ATP) channels (37, 48), and 3) a shift toward cellular pro-oxidant state (4, 14, 15, 52) events that together may promote phase three early afterdepolarizations (EADs) initiated by forward mode Na-Ca exchanger (NCX) during late systole (38). Furthermore, reduced SERCA2a activity and the associated elevation of cytosolic Ca^2+ coupled with a pro-oxidant shift may promote activation of Ca-calmodulin kinase II (CaM-KII), which increases the L-type calcium current and the late Na current (1, 8, 24) to facilitate EADs (40, 46, 50). Unlike isolated myocytes, intact ventricular muscle may be shielded from the arrhythmogenic consequences of these changes by the sink effect of adjacent well-coupled myocytes, which prevent a single myocyte from manifesting an afterdepolarization unless a large number of its neighbors are also similarly inclined (51). Consistent with this scenario, we recently showed in experimental and simulation studies that oxidative stress sufficient to initiate EADs and triggered activity in isolated myocytes (50) failed to cause arrhythmias in young adult rat ventricles (32). However, a high incidence of EADs, triggered activity, and ventricular fibrillation (VF) occurred in aged fibrotic rat ven-
tricles, in which fibrosis and other aging-related changes alter the source-sink relationship in a manner facilitating emergence of EAD-mediated arrhythmias (31, 32). These findings led us to hypothesize that the arrhythmogenic effects of GI observed at the isolated myocyte level (2, 20) might not manifest as arrhythmias in normal well-coupled ventricular tissue from young adult rat hearts, but might emerge in aged rat hearts in which the source-sink relationship has been altered by fibrosis and other aging-related factors. We show that in aged, but not young adult, rat hearts, GI promotes EADs, triggered activity, and VF by a mechanism involving activation of $K_{ATP}$ channels, CaMKII, and NCX.

METHODS AND MATERIALS

The research protocol was approved by the Institutional Animal Care and Use Committee and followed the guidelines of the American Heart Association (AHA). Langendorff preparation. Male Fisher 344 young 3- to 4-mo-old ($N = 18$) and aged 24- to 30-mo-old ($N = 66$) rats were used in this study. The hearts of the anesthetized rats were removed, and the ascending aorta was cannulated for retrograde perfusion through the coronary ostia as we previously described (32, 36). Briefly, the isolated hearts were immersed in cold oxygenated Tyrode’s and the entire duration of the cannulation of the aorta and securing the perfusion tubing in place took 3 to 4 min. The cannulated hearts were then mounted in the tissue bath, and oxygenated Tyrode’s perfusion continued at 37 ± 0.5 degrees Celsius. The composition of the Tyrode’s solution (in mM) was: 125 NaCl, 4.5 KCl, 1.8 NaH$_2$PO$_4$, 24 NaHCO$_3$, 1.8 CaCl$_2$, 0.5 MgCl$_2$, 5.5 glucose, and 100 mg/l albumin.

Optical mapping. The hearts were stained with RH237 and Rhod-2 AM for simultaneous dual voltage (V) and Ca$_{2+}$ fluorescent optical imaging, respectively, as we described previously (32). Cytochalasin D (5 μmol/l) was added to the perfusate to eliminate motion artifact during optical recordings (32). Ca$_{2+}$ transient decay rate constant was determined by a monoexponential fit during the coronary ostia of each heart we previously described (32, 36). Briefly, the isolated hearts were immersed in cold oxygenated Tyrode’s and the entire duration of the cannulation of the aorta and securing the perfusion tubing in place took 3 to 4 min. The cannulated hearts were then mounted in the tissue bath, and oxygenated Tyrode’s perfusion continued at 37 ± 0.5 degrees Celsius. The composition of the Tyrode’s solution (in mM) was: 125 NaCl, 4.5 KCl, 1.8 NaH$_2$PO$_4$, 24 NaHCO$_3$, 1.8 CaCl$_2$, 0.5 MgCl$_2$, 5.5 glucose, and 100 mg/l albumin.

Dynamic APD restitution. In 10 aged and 10 adult hearts, we recorded with a glass microelectrode from 4 to 6 epicardial cells in each heart at the base and the midwall on the LV epicardial surface during pacing at a cycle length (CL) of 250 ms before and 30 min after GI. The pacing electrode was located at the base of the LV epicardial surface. The results on AP data were pooled in each age group, since there were no significant base versus midwall differences in AP properties. We constructed dynamic APD restitution curves for 90 percent repolarization (APD$_{90}$) before and after GI using single cell glass microelectrode. Pacing started at a CL of 250 ms and then decreased by 20 ms until 160 and then by 10 ms (29, 30, 32). Conduction velocity (CV) was estimated in both age groups before and after GI ($N = 8$ in each group) during optical AP recordings while pacing from the base of the LV epicardial surface at a CL of 250 ms. CV was calculated by dividing the conduction time from LV base to LV apex by the distance from LV base to the LV apex.

Metabolic and pharmacological interventions. In this portion of the study we used an additional 35 aged rats to test the efficacy of drugs against GI-mediated VF. We did not test adult rats in this part of the study, since no spontaneous VF occurred during GI in the adult rat hearts (see below). Selective GI was induced by substituting 5.5 mM glucose with 10 mM pyruvate in oxygenated, otherwise normal Tyrode’s solution (12). To determine whether GI-induced VF was mediated through alteration of cellular redox state, the reducing agent N-acetylcysteine (NAC; 2 mM) was tested in eight aged rats. In four hearts, NAC antioxidant therapy was initiated 15 min before and continuing throughout the GI period (preventive therapy). In an additional four hearts, NAC was administered after the onset of GI-mediated arrhythmias (suppressive therapy, $N = 4$). Because elevated cytosolic Ca$_{2+}$ levels activating CaMKII (8, 32) could potentially mediate GI effects, we also examined the preventive ($N = 5$) and suppressive ($N = 4$) effects of CaMKII inhibitor KN-93 (1 μM). The preventive effect of the inactive form (KN-92, 1 μM) (8) on GI-mediated VT/VF was tested in four aged hearts, respectively. In six aged hearts after the conclusion of GI-mediated initiation of VF and optical mapping studies, the hearts were perfused for 90 min with normal Tyrode’s solution containing glucose present but no pyruvate to determine reversibility of GI-induced VF in the aged hearts.

Because a decrease in cellular ATP level and the associated shortening of the APD after GI (48) may result either from sarcoclemmal K$_{ATP}$ channel or mitochondrial K$_{ATP}$ channel (23, 27), we used the selective mitochondrial K$_{ATP}$ channel blocker 5-hydroxydecanoic acid (5-HD; 1 μM; $N = 6$ for preventive and $N = 3$ for suppressive $N = 3$) (13) and the potent sarcoclemmal and mitochondrial K$_{ATP}$ channel blocker glybenclamide (1 μM; $N = 6$) (23, 27) to determine the site of K$_{ATP}$ channel activation after GI.

Histological analysis. Percent tissue fibrosis was determined from routinely prepared histological sections stained by Masson’s trichrome method for collagen as we previously described (28). Briefly, using a grid that divided the field of view into 100 squares, the number of collagenous tissue (blue stain) at the 100 intersection points in the grid was scored as 1 (present) or 0 (absent). Results are expressed as percent occupied by fibrosis to the total area examined. Four to five longitudinal sections were made from the LV anterior base and midwall and the posterior LV and two to three longitudinal sections in the RV in five aged and five adult hearts. Necrotic tissue in cryoablated hearts was visualized grossly using 1% triphenyl tetrazolium chloride (TTC) that stains viable myocardium red, whereas necrotic tissue fails to stain (32). Distribution of gap junction connexin (Cx43) was evaluated by immunohistochemical staining for Cx43 as described previously (35). Briefly, after the conclusion of the electrophysiological studies, the hearts were fixed in 4% neutral buffered formalin. Five transmural sections (5 micrometer thick) were
made through the base and the midwall of the LV epicardium in each of the four aged and four adult hearts stained for Cx43. The paraffin embedded sections were stained with Cx43 using a modified immunocytochemical ABC method. The slides were then incubated with the primary Cx43 antibody overnight at 4 degrees centigrade. The secondary antibody, biotinylated mouse IgG, was then applied for 10 min, followed by horseradish peroxidase for 30 min and then the chromagen diaminobenzidine for 10 min. Finally, the slides were counterstained in weak hematoxylin for 5 min and coverslipped. Immunostaining was performed in pairs, with adult and aged tissues isolated from identical LV sites. Each sample was examined under an Olympus microscope (BX60). Ten to 12 fields of longitudinally sectioned fibers from each animal were then analyzed using Image-Pro Plus (Media Cybernetics, Silver Spring, MD) to quantify Cx43 positive spots. The amount of Cx43 was expressed as a percentage of the total cellular and extracellular area.

Statistical analyses. Significant differences in the incidence of VF (dichotomous comparisons) were determined using Fisher exact test. Likelihood ratio test was used to determine significance of site-specific origination of focal activity in the LV. Changes in $\frac{d}[d_{2,100}]$ decline rate constant, $\tau$, and AP properties were determined using repeated-measures ANOVA. Differences among individual means were verified subsequently by Newman-Keuls post hoc tests. Since normality of $\frac{d}{2,100}$ distribution cannot be assumed to exist, we therefore used the bootstrapping methods to detect significant differences (7, 25). To compare the ratios, we therefore used bootstrap methods with random resampling (1,000 times) with replacement. If the actual value was greater than 975 of the 1,000 values of the random re-sampling, we concluded that the $F$ value was statistically significant at the 0.025 level (2-sided test for $\alpha = 0.05$) (7, 25). $P \leq 0.05$ was considered significant. All data are presented as means ± SD.

RESULTS

Effects of GI on cardiac rhythm in intact aged and adult hearts. In control experiments, no spontaneous arrhythmias occurred in four aged and four adult hearts perfused for up to 3 h with oxygenated Tyrode's perfusion containing glucose. When glycolysis was inhibited by replacing glucose with pyruvate in the arterial perfusate, however, VF occurred spontaneously during sinus rhythm (CL = 374 ± 120 ms) after a mean of 22 ± 8 min in 29 out of 31 aged rat hearts, but in none of the 18 young adult hearts exposed to GI for up to 2 h ($P < 0.001$). VF was preceded by a transient period of VT at a CL = 71 ± 25 that arose suddenly from sinus rhythm, as shown in Fig. 1A. Optical mapping (Fig. 1B) showed that the VT had a
focal mechanism, originating from the base of the LV epicardium in 24 out of 35 episodes (69%) in 10 mapped hearts. The focal VT propagated repeatedly as single wavefront from base to apex (Fig. 1B) but within 2 s degenerated to VF (mean CL, 48 ± 12 ms; Fig. 1, A and B). Transition from VT to VF was characterized by a wavebreak midway between the base and the apex (Fig. 1, B and C). The two ends of the wavefronts then propagated laterally to join together at the base of the heart and re-enter through the site of the initial block inscribing a figure 8 type reentry (Fig. 1B, snapshot 128 ms). Wavebreak resulted from spatially discordant APD alternans when a site with short APD propagated to a site with long APD as shown in Fig. 1D optical AP recordings. In the remaining 31% of the VF episodes, the origin of the VT preceding the VF could not be determined. In these episodes a single wavefront entered the mapped region either from the apex (7 episodes, 20%) or the lateral LV (4 episodes, 11%). The effects of GI were reversible. Replacing pyruvate with glucose suppressed the spontaneous episodes of VF within 30 min (N = 10).

**Cellular mechanism of the focal VT leading to VF.** To gain insight into the cellular mechanism of focal VT preceding VF in aged hearts, VF was terminated with an electrical shock. Since focal VT typically originated at the base of the LV epicardium, a myocyte in this region was impaled with the roving glass microelectrode to capture the onset of the next VT/VF episode (N = 18). As shown in Fig. 2A, EADs arose initially and caused single triggered beats (12 min) registering as ventricular ectopic beats on the ECG, which after a mean of 22 ± 6 min led to short runs of triggered activity (nonsustained VT) and then VF, respectively (Fig. 2A). EADs arose from a mean membrane potential of −56 ± 12 mV (N = 18) at a time when the ECG, LV, and left atrial electrograms were isoel-

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**Fig. 2.** Time course of emergence of epicardial early afterdepolarizations (EADs), triggered activity, and VF in an aged heart exposed to GI. A: simultaneous microelectrode (top) and pseudo-ECG (bottom) recordings at baseline and at increasing time (18 min) after GI. EADs emerge 10 min after GI after APD shortening (8 min post-GI); EAD-mediated single triggered APs arise after 12 min causing premature ventricular depolarization as seen on the pseudo-ECG, which then evolve into short runs of triggered activity causing VT (15 min), which then degenerates to VF (18 min post-GI). B: epicardial EAD emerges when the ECG and the right ventricular (RV Beg) and left atrial (LA Beg) electrograms manifest isoelectric interval, indicating absence of electrical activity elsewhere in the heart.
tric, indicating absence of an outside electrical source to drive the impaled cell exhibiting the EADs (Fig. 2B).

To exclude the possibility that breakthrough excitation from underlying tissue might be the cause of epicardial EADs, we cryoablated the endo- and midmyocardial tissues in five aged hearts, sparing only a thin rim of LV surviving epicardial tissue as shown in Fig. 3. GI still caused epicardial cells to manifest EADs, triggered activity, and VF in all of five cryoablated hearts studied, indicating that the endo- and midmyocardial cells including endocardial Purkinje fibers are not the sole source of EADs and that epicardial cells have an intrinsic ability to generate EADs. However, unlike noncryoablated hearts, VF in cryoablated aged hearts was not sustained beyond 10 s and self-terminated spontaneously, perhaps due to reduced tissue mass (17).

Table 1. Effects of GI on action potential & Ca^{2+} transient in aged and adult rat hearts

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Adult</th>
<th>Aged</th>
<th>P</th>
<th>Adult</th>
<th>Aged</th>
<th>P</th>
</tr>
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<tr>
<td>RMP, mV</td>
<td>−83 ± 0.1</td>
<td>−83 ± 13</td>
<td>NS</td>
<td>−82.6 ± 0.7</td>
<td>−83 ± 0.8</td>
<td>NS</td>
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<td>APA, mV</td>
<td>108 ± 9</td>
<td>104 ± 13</td>
<td>NS</td>
<td>102 ± 8</td>
<td>108 ± 12</td>
<td>NS</td>
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<td>APD_{90}, ms</td>
<td>92 ± 10</td>
<td>76 ± 4</td>
<td>&lt; 0.05</td>
<td>98 ± 11</td>
<td>81 ± 12</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>APD_{50}, ms</td>
<td>28 ± 11</td>
<td>24 ± 8</td>
<td>NS</td>
<td>30 ± 9</td>
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<td>NS</td>
</tr>
<tr>
<td>dV/dt_{max}, V/s</td>
<td>168 ± 20</td>
<td>176 ± 21</td>
<td>NS</td>
<td>170 ± 26</td>
<td>172 ± 16</td>
<td>NS</td>
</tr>
<tr>
<td>Slope APD_{90}, R</td>
<td>1.1 ± 0.2</td>
<td>1.1 ± 0.5</td>
<td>NS</td>
<td>1.3 ± 0.5</td>
<td>1.3 ± 0.6</td>
<td>NS</td>
</tr>
<tr>
<td>Ca^{2+}, τ, ms</td>
<td>71 ± 2</td>
<td>80 ± 3</td>
<td>P &lt; 0.05</td>
<td>82 ± 6</td>
<td>101 ± 8*</td>
<td>P &lt; 0.05</td>
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<tr>
<td>τ/ADP_{90}</td>
<td>0.71 ± 0.07</td>
<td>0.98 ± 0.08*</td>
<td>P &lt; 0.05</td>
<td>0.82 ± 0.09</td>
<td>1.28 ± 0.19*†</td>
<td>P &lt; 0.05</td>
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<tr>
<td>CV, cm/s</td>
<td>40 ± 12</td>
<td>41 ± 13</td>
<td>NS</td>
<td>28 ± 10</td>
<td>26 ± 14‡</td>
<td>NS</td>
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</table>

Values are means ± SD. GI, glycolytic inhibition; Ca^{2+}, intracellular calcium ion concentration; RMP, resting membrane potential; APA, action potential amplitude (in mV); APD_{90} and APD_{50}, action potential duration to 90% and 50% repolarization, respectively; dV/dt_{max}, maximum slope of phase zero AP; τ, calcium transient decline rate constant; CV, conduction velocity; NS, not significant. *Significantly (P < 0.05) larger than baseline in both groups; †significantly (P < 0.05) larger than adult GI; ‡significantly (P < 0.05) larger than adult GI. All measurements were made at a PCL of 250 ms. APD_{90} restitution curves were constructed during dynamic pacing starting at PCL of 250 ms to 120 ms in 20- to 10-ms decrements.
**Effects of GI on APD, Ca\(^{2+}\) cycling, and CV.** GI shortened APD\(_{90}\) in both age groups but had no effect on the other AP parameters (Table 1). GI also prolonged the Ca\(^{2+}\) transient decay time constant (\(\tau_{\text{Ca}}\); Table 1), with greater slowing in the aged compared with young adult hearts (\(P < 0.01\); Table 1). In aged hearts, \(\tau_{\text{Ca}}\) also exhibited significant heterogeneity over the LV epicardial surface (base vs. apex) both before and after GI (Fig. 4A). Overall, the ratio of \(\tau_{\text{Ca}}\) to APD\(_{90}\) was significantly (\(P < 0.01\)) greater in aged compared with young adult hearts (Fig. 4E and Table 1). CV from LV epicardial base to apex and measured during pacing at a CL of 250 ms was significantly slower in the aged compared with adult hearts both before and after GI.

The combined effect of shortened APD\(_{90}\) and prolonged \(\tau_{\text{Ca}}\) during GI led to Ca\(^{2+}\) being much more elevated over the voltage range at which EADs arose (around \(-60\) mV). In aged hearts, Ca\(^{2+}\) remained at 70 ± 13% of the peak Ca\(^{2+}\) transient value, compared with 48 ± 4% in young adult hearts (\(P < 0.05\); Fig. 4E). Elevated Ca\(^{2+}\) during repolarization may facilitate EAD formation (32, 36, 44) by stimulating forward mode of NCX (34, 38). Consistent with this idea, the emergence of EADs coincided in time with a mean Ca\(^{2+}\) level of 70 ± 13% of the peak systolic Ca\(^{2+}\) transient amplitude (\(N = 6\)) as shown in Fig. 5A by triple microelectrode and dual optical V-Ca\(^{2+}\) mapping. In contrast, when the APs were not associated with EADs, the Ca\(^{2+}\) level at \(-60\) mV (mean EAD takeoff voltage) was 35 ± 5% (\(P < 0.01\); Fig. 5, A and B).

The effects of K\(_{\text{ATP}}\) channel block of GI-mediated VF. The K\(_{\text{ATP}}\) channel blocker glibenclamide (1 \(\mu\)M) reversed APD shortening by GI and suppressed the VF in five out of seven aged hearts (\(P < 0.05\); Tables 2 and 3).
selective mitochondrial K<sub>ATP</sub> channel blocker 5-HD (1 μM) had no effect on GI-mediated VF in all seven aged hearts studied and failed to reverse APD shortening caused by GI (Tables 2 and 3).

The influence of CaMKII inhibition on GI-mediated VF. To evaluate whether CaMKII, activated by the Ca<sup>2+</sup> cycling alterations induced by GI, contributed to EAD-mediated arrhythmias and VT/VF (8), we tested the effects of the CaMKII inhibitor KN-93 (45) on GI-mediated VF, using its inactive form KN-92 as a control. As shown in Table 3, KN-93 (1 μM) suppressed GI-mediated EADs in five out of nine aged hearts studied (√<i>P</i> < 0.05) without affecting Ca<sup>2+</sup> transient decline rate constant or APD (data not shown). In contrast, KN-92 (1 μM) had no effect on GI-mediated VF (Table 3) or on Ca<sup>2+</sup> transient or APD in any of the six aged hearts studied (data not shown). These findings suggest that CaMKII activation may contribute to GI-mediated EADs and VT/VF in aged hearts, but its effects are not overriding or absolutely necessary.

Redox state and GI-mediated VT/VF. Although GI shifts the cellular redox balance to a pro-oxidant state (21), we
investigated the role of the reducing agent NAC (16) on GI-mediated VF. As shown in Table 3, NAC (2 mM) had no effect on GI-mediated VF either when given before (15 min) GI onset (N = 4) or after GI had already induced VF (N = 4).

**Effects of SEA0400 in preventing GI-mediated VF.** The influence of SEA0400 (2 μM) was evaluated in six aged hearts. In three hearts, SAE0400 had no effect on the sinus heart rate, but in three hearts it slowed heart rate from a mean of 180 ± 16 beats/min to 95 ± 14 beats/min. SEA0400 prevented GI-induced VF in all three hearts with no sinus slowing, but failed to protect against GI-induced VF in the three hearts with severe sinus bradycardia. Pacing of the latter hearts at a CL of 350 ms restored the protective effect of SEA0400 against GI-induced VF.

**Tissue fibrosis and Cx43 immunostaining.** Quantitative analysis using Masson’s trichrome stain showed significantly greater fibrosis in the aged compared with young adult hearts (averaging 35 ± 10% vs. 3.4 ± 1%; P < 0.001). Fibrosis in aged hearts was heterogeneous in distribution, with dense subendocardial fibrosis and intermediate levels of fibrosis in the midventricular and subepicardial myocardium (Fig. 6), consistent with our previous findings (32). Quantitative analysis of Cx43 immunostaining at the base and the midwall of the LV showed significantly less Cx43 positive spots in the aged compared with adult ventricles (0.8 ± 0.02% vs. 1.7 ± 0.4%; P < 0.05), as shown in Fig. 7.

**Body and heart weight.** The body and hearts weights of the aged rats were significantly (P < 0.01) heavier than adults rats (421 ± 41 vs. 331 ± 59 g and 1.4 ± 0.3 and 0.9 ± 0.1 g, respectively). Ratio of heart to body weight in milligrams per gram was 3.32 ± 0.8 in the aged versus 2.71 ± 0.64 (P < 0.05), indicating mild to moderate myocyte hypertrophy as shown in Figs. 6 and 7.

**DISCUSSION**

**Major findings.** The predisposition of aged, but not young adult, hearts to EAD-mediated triggered activity, VT and VF during the relatively mild metabolic stress of GI constitutes the major finding of this study. GI caused simultaneous shortening of the APD and slowing of Ca$^{2+}$ decline rate, resulting in maintained elevation of Ca$^{2+}$ during phase 3 repolarization, a condition that has been previously shown to promote late phase 3 EADs in fibrotic rabbit ventricles (34) and in canine pulmonary veins (38) and atria (3).

Although we have observed EADs to occur both during phase 2 and during late phase 3, the phase 2 EADs did not cause triggered beats. However, as the period of GI prolonged, the highly arrhythmogenic late phase 3 EADs predominated, leading to triggered activity, VT and VF.

Maintained elevation of Ca$^{2+}$ enhances the forward mode NCX, generating a net depolarizing inward current (26, 38). The ability of NCX inhibitor SEA0400 to prevent GI-mediated EADs supports this scenario. However, the protective effect of NCX inhibition was rate dependent and was lost when the SEA0400 significantly slowed the sinus rate. This may indicate that the EAD-mediated arrhythmias during bradycardia were less dependent on NCX and more dependent on the reactivation of the L-type Ca current and reduced repolarization reserve associated with bradycardia (56).

Although GI had qualitatively similar influences on APD and Ca$^{2+}$ in both age groups, the slowing of the Ca$^{2+}$ transient decline rate was quantitatively greater and more heterogeneous in aged hearts. However, this difference in Ca$^{2+}$ decline rate could not fully explain the differential arrhythmic response to GI in the aged versus young adult hearts, because slowing of the Ca$^{2+}$ decline rate with the SERCA2a inhibitor thapsigargin (5 μM) failed to promote VF in the absence (N = 4) and presence (N = 4) of GI all eight adult hearts studied (data not shown). Similarly, the difference between the two age groups could not be explained by differences in APD restitution, since the slopes of the restitution curves were not different between two age groups both at baseline and after GI. However, it is possible that other aging-related electrophysiological remodeling factors contributed to the greater susceptibility of aged hearts to EAD-mediated arrhythmias during GI. For example, GI caused significantly greater disparity in APD$_{90}$ and the calcium transient duration in the aged compared with young hearts.

**Table 2. Effects of glybenclamide, 5-HD, and SEA0400 on GI-induced shortening of APD in aged hearts**

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<th>GI</th>
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<th>GI + SEA0400</th>
<th>GI + Glybenclamide</th>
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<td>APD$_{50}$, ms</td>
<td>30.4 ± 4.3</td>
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<td>APD$_{90}$, ms</td>
<td>84.6 ± 8.7</td>
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<td>APA, mV</td>
<td>100 ± 7.5</td>
<td>97.6 ± 5.8</td>
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<td>dV/dt$_{max}$, V/s</td>
<td>176 ± 11.4</td>
<td>170 ± 15.3</td>
<td>180 ± 27.7</td>
<td>171.6 ± 7.7</td>
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Values are means ± SD. 5-HD, 5-hydroxydecanoate. *P < 0.05 compared with GI.

**Table 3. Effects of pharmacological agents on GI-induced VF in aged hearts**

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<tr>
<th>Glybenclamide</th>
<th>5-HD</th>
<th>KN-93</th>
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<td>P</td>
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n, number of hearts; VF, ventricular fibrillation; NAC, N-acetylcysteine. *In 3 hearts, SEA0400-induced severe sinus bradycardia; ventricular pacing at a cycle length of 350 ms restored the protective effect of SEA0400 against GI-mediated VF in all 3 hearts. Inclusion of these 3 hearts rendered the protective effect of SEA0400 against GI-induced VF (6 out of 6 hearts) significant (P < 0.01).
prevented by the sarcolemmal KATP channel blocker glibenclamide, implicating activation of sarcolemmal KATP channels. We also tested the effects of selective mitochondrial KATP channel blocker 5-HD, which did not reproduce the effects of glibenclamide at preventing either APD shortening or EAD-mediated arrhythmias. Despite preventing EAD-mediated arrhythmias in aged hearts, glibenclamide had no effect on Ca\textsuperscript{2+} dynamics (22, 54), strongly suggesting that APD shortening plays a critical role in GI-mediated EADs and VF.

The role of fibrosis. The most striking histological difference between the aged and the adult rat ventricles was the presence of extensive fibrosis in the aged hearts, consistent with previous reports (10, 36). Aging is also associated with reduced gap junctional Cx43, as shown previously by Spach and Dolber (41) and observed in aged rat hearts (Fig. 6). Both increased fibrosis and gap junction loss reduce the electrical coupling between myocytes, making it more likely that the relatively weak currents producing EADs can overcome the source-to-sink mismatch imposed by adjacent normally repolarizing tissue (11, 55). It is of interest to note the relative preferential origination of EADs (69% of the episodes) from epicardial tissue (11, 55). It is of interest to note the relative preferential origination of EADs (69% of the episodes) from epicardial cells located at the base of the LV, which had an intermediate level of fibrosis (i.e., ∼35%) compared with other regions. In a previous study (32), simulations suggested that intermediate fibrosis provides a more favorable sink-source relationship for EAD formation and propagation at the tissue level, since with too much fibrosis (e.g., LV endocardium) EAD-mediated triggered beats fail to propagate, whereas with too little fibrosis (e.g., right ventricle, LV apex, septum), the source-sink mismatch remains too great for EADs to form.

The role of KATP channels. APD shortening during GI was prevented by the sarcolemmal K\textsubscript{ATP} channel blocker glibenclamide, implicating activation of sarcolemmal K\textsubscript{ATP} channels as the mechanism of APD shortening. This finding is consistent with previous studies showing that glycolytically derived ATP preferentially regulates sarcolemmal K\textsubscript{ATP} channels (48). Since glibenclamide blocks mitochondrial as well as sarcolemmal K\textsubscript{ATP} channels, we also tested the effects of selective mitochondrial K\textsubscript{ATP} channel blocker 5-HD, which did not reproduce the effects of glibenclamide at preventing either APD shortening or EAD-mediated arrhythmias. Despite preventing EAD-mediated arrhythmias in aged hearts, glibenclamide had no effect on Ca\textsuperscript{2+} dynamics (22, 54), strongly suggesting that APD shortening plays a critical role in GI-mediated EADs and VF.

The roles of CaMKII and oxidative stress. Consistent with studies in isolated cardiac myocytes subjected to GI (21), the reducing agent NAC did not prevent EADs, suggesting that changes in the redox state of the cell did not play a major role, even though oxidative stress is known to induce EAD-mediated VT/VF in aged rat hearts via oxidative activation of CaMKII (32). Nevertheless, CaMKII activation, which promotes EADs by altering L-type Ca\textsuperscript{2+} current and enhancing late Na\textsuperscript{+} current (9, 45, 49, 50), did appear to contribute to EAD-mediated arrhythmias during GI, since CaMKII inhibition with KN-93 (but not its inactive form KN-92) was partially effective in preventing GI-mediated EADs and VF in five of nine aged hearts.

Clinical implications. VF is the most common cause of sudden cardiac death, which prematurely claims the lives of ∼300,000 persons every year in the US (39). Since animal models of spontaneous VF arising from otherwise stable background cardiac rhythms are rare, the isolated aged rat heart model may be of value in evaluating how altered metabolic, oxidative, and ionic factors predispose to spontaneous VF, which would result in sudden cardiac death in an intact animal.

Although the clinical relevance of GI may be questioned, we point out that replacement of glucose with pyruvate is a relatively mild form of GI. Normally the heart prefers fatty acids over glucose, and a variety of diseased cardiac conditions, including hypertrophy, heart failure, and diabetes, are characterized by a reduction in glucose oxidation (5, 18, 33). Importantly, a major consequence of chronic oxidative stress in the heart is GI (4), which may amplify the alterations in Ca\textsuperscript{2+} cycling and APD shortening to exert adverse arrhythmic consequences. The evidence linking oxidative stress to GI may be relevant to the increased risk of atrial fibrillation (AF) as well as shown by us (36) and others in animal models and in humans (6, 43). Strategies to enhance glycolysis and/or reverse...
tissue fibrosis may be new therapeutic targets to reduce the risk of VF and AF in diseased and aged hearts.

Limitations. When the EADs were recorded at the LV base using an intracellular microelectrode, the proximity to the site of origin of focal VT is only approximate. However, because the tissue is a syncytium, the membrane voltage cannot vary tremendously over a length scale shorter than the tissue electrical space constant, typically 1 to 2 mm, which includes many thousands of myocytes. Therefore, even though the microelectrode is impaling a single myocyte, it reflects the voltage of thousands of adjacent myocytes as well (51). As the EAD propagates further away from its site of origin into surrounding tissue, the take-off potential becomes lower and lower as the impulse encounters progressively more repolarized tissue. Therefore, the EAD take-off potential may be more positive than our estimate of $-56 \text{ mV}$, if the recording site was several millimeters or more away from the true site of origin. However, because we observed a similar EAD take-off potential in the cryoablated hearts, which excludes a site of origin more than 1 mm below the epicardial surface, we do not think that the error is likely to be too large. As indicated in results, we cannot exclude the possibility that EADs could also originate in intact hearts from deeper myocardial cells and endocardial Purkinje fiber network. In fact 31% of the VF episodes in the intact hearts originated from the outside of the mapped region. The origin of these triggers could have been the endocardial Purkinje fiber network. However, our cryoablation studies destroying the entire mid- and endocardial layers indicate that epicardial cells are capable of generating EAD-mediated triggered activity and VF. The contribution of epicardial cells in the genesis of EADs is further suggested by the presence of isoelectric interval on the ECG during the emergence of epicardial EADs, indicating the absence of electrical activity elsewhere in the heart preceding the emergence of epicardial EADs. It may be argued that the observed differences in the arrhythmic outcomes between the adult and aged groups could be an artifact of the use of the excitation-contraction uncoupler CytoD, which have been shown to have different effects on APD in failing versus normal myocytes (53). However, the fact that GI caused APD shortening in both groups suggests that the observed differences in the two groups were not the result of a preferential effect of CytoD on aged compared with young/adult hearts. CytoD also reduces energy demand, which makes the energetic consequences of GI less severe. This suggests that the effects of GI might be further exacerbated in a working heart preparation, although previous studies have shown global ATP and creatine phosphate levels are well maintained when glucose is replaced by pyruvate in this setting (47).
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