(n = 24). An answering female, in a separate opaque cage 1 m from the male on the same wooden bench, increased male tremulation production to 12.25 ± 3.09 per 5-minute period (n = 24; U = 569, t = 5.83, $P < 0.0005$; median value, 5.5 tremulations). Males also produced 21.42 ± 13.34 airborne calls per 5-minute period without an answering female (n = 24), and no airborne calls when a tremulating female answered.
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24. Supported by predoctoral fellowships from the Smithsonian Institution, NSF grant DEB-8211975, and the American Association of University Women; the Smithsonian Tropical Research Institute (STRI), Florida Entomological Society, National Academy of Sciences, Sigma Xi, and the American Society of Mammalogists (J.J.B.); and by the Natural Sciences and Engineering Research Council of Canada operating grant 4946 (G.K.M.). Facilities and logistical support provided by STRI. M. D. Tuttle allowed use of his flight cages and J. W. Hardy use of his audiospectrograph. R. Abbey, A. Cabrera, R. Cambra, and E. Stockwell helped in the field. R. M. R. Barclay, M. B. Fenton, J. E. Lloyd, T. J. Walker, and J. H. Fullard read the manuscript. Voucher specimens are in the National Museum of Natural History. Florida Agricultural Experiment Station Journal Series 8369.

4 March 1987; accepted 6 July 1987

Glycolysis Preferentially Inhibits ATP-Sensitive K^+ Channels in Isolated Guinea Pig Cardiac Myocytes

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In heart, glycolysis may be a preferential source of adenosine triphosphate (ATP) for membrane functions. In this study the patch-clamp technique was used to study potassium channels sensitive to intracellular ATP levels in permeabilized ventricular myocytes. Activation of these K^+ channels has been implicated in marked cellular K^+ loss leading to electrophysiological abnormalities and arrhythmias during myocardial ischemia. The results showed that glycolysis was more effective than oxidative phosphorylation in preventing ATP-sensitive K^+ channels from opening. Experiments in excised inside-out patches suggested that key glycolytic enzymes located in the membrane or adjacent cytoskeleton near the channels may account for their preference for glycolytic ATP.

STUDIES IN INTACT ISOLATED HEART and cultured myocytes have suggested that glycolysis has a special role in maintaining membrane function during myocardial ischemia and metabolic inhibition (*1–5*). In the isolated rabbit ventricle, selective inhibition of glycolysis resulted in much greater extracellular K^+ accumulation than selective inhibition of oxidative (mitochondrial) metabolism despite similar total cellular levels of high-energy phosphates (*5*). Extracellular K^+ accumulation during early myocardial ischemia results from increased K^+ efflux (*6, 7*) and activation of adenosine triphosphate (ATP)–sensitive K^+ channels has been implicated as a possible cause of this efflux (*8–10*). These channels open only when the concentration of ATP falls below a critical level. However, the critical level of ATP that completely suppresses ATP-sensitive K^+ channels in excised membrane patches from heart is much lower (approximately 0.2 m*M*) than the concentration of ATP during ischemia except at very late stages (*8*). Unless either the sensitivity of ATP-sensitive K^+ channels to ATP concentration is markedly altered by other sequelae of metabolic inhibition or ATP stores are compartmentalized in the cardiac cell, it seems unlikely that activation of ATP-sensitive K^+ channels could cause marked K^+ loss beginning after 30 seconds of ischemia (*6, 7*). The results of this study show that under conditions in which cellular ATP consumption is intrinsically high, glycolytic (anaerobic) metabolism is more effective than oxidative (aerobic) metabolism in suppressing ATP-sensitive K^+ channels in patch-clamped single ventricular myocytes. The preference of ATP-sensitive K^+ channels for glycolytically generated ATP may be due to the presence of key glycolytic enzymes strategically located near K^+ channels.

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Single ventricular myocytes were isolated enzymatically from hearts of guinea pigs (300 to 400 g) (*11*) and studied at room temperature by means of the gigaseal patch-clamp technique (*12*). Single-channel recordings were made with fire-polished patch electrodes (tip diameter 1 to 4 μm, resistance 1 to 3 megohms) mounted to the headstage of List EPC-7 patch-clamp amplifier. Data were recorded on a modified videocassette recorder and chart recorder and analyzed on a PDP 11-23 computer. The experimental chamber (0.5 ml) was mounted on the stage of an inverted microscope and was continuously perfused (1 to 4 ml/min) throughout the experiment. The standard filling solution of the patch electrode contained (millimolar concentration): KCl, 150; Hepes, 5; and KOH to bring the *p*H to 7.3. The standard bath solution contained: KCl, 138 to 147 (total K^+ concentration 150); Hepes, 5; EGTA, 2; $CaCl_2$, 0.5; $MgCl_2$, 2; and KOH to bring the *p*H to 7.1. Various substrates were added as described later. The free Ca^{2+} concentrations of the patch electrode and bath solutions were 4 to 6 μ*M* and <0.1 μ*M*, respectively. For single-channel recordings during multiple interventions the patch electrode was arbitrarily held at +40 mV relative to the bath. Results are expressed as mean ± standard deviation.

Most single-channel recordings were made from "open" cell-attached membrane patches (*10*) in which first a gigaseal was formed at one end of a cell with the patch electrode. The other end of the cell was then exposed for 10 to 30 seconds to a stream of bath solution containing 0.1% saponin delivered by positive pressure through a second electrode positioned just over the surface membrane. As soon as the membrane in this region was locally disrupted (detected by a slight swelling of the cell easily visible at ×800 magnification), the saponin-containing electrode was quickly withdrawn. Under these conditions, openings of inwardly rectifying K^+ channels were commonly observed as long as 2 m*M* ATP was present in the bath solution. The single-channel conductance of these channels determined from the linear portion of the current-voltage (*I-V*) curve was 39 ± 4 picosiemens (pS). Removal of ATP from the bath solution that perfused the cell reversibly activated ATP-sensitive K^+ channels in the membrane patch distinguishable from inwardly rectifying K^+ channels by their larger single-channel conductance (76 ± 5 pS). Permeabilized cells were capable of generating ATP endogenously by a variety of metabolic pathways when provided with the appropriate substrates (Fig. 1). Washout of 2 m*M* ATP from the bath caused multiple ATP-sensitive

K^+ channels to open (Fig. 1A). Addition of mitochondrial substrates to the bath promptly caused the channels to close. ATP-sensitive K^+ channels remained closed in the presence of mitochondrial substrates until 1 μM FCCP [carbonyl cyanide(4-trifluoromethoxy) phenyl hydrazone, a mitochondrial uncoupler] was added (Fig. 1A, second tracing). Either of two combinations of substrates for the ATP-producing steps of glycolysis (GS1 or GS2) also caused ATP-sensitive K^+ channels to close in the absence of exogenous ATP (Fig. 1B). Adenosine diphosphate (ADP; 0.5 mM) alone or other individual glycolytic substrate components did not suppress channel openings. Glycolytic substrates remained effective in the presence of 1 μM FCCP (Fig. 1C). Creatine phosphate (2 mM) alone had no effect unless 0.5 mM ADP was added (as substrate for creatine kinase). In typical permeabilized cells ATP-sensitive K^+ channels could be activated and suppressed repeatedly over as long as 45 minutes by transiently removing ATP or various substrates.

These observations indicate that myocytes permeabilized at one end by saponin remain metabolically intact and are capable of generating sufficient ATP to prevent ATP-sensitive K^+ channels from opening via several metabolic processes. To determine whether the responsiveness of ATP-sensitive K^+ channels to ATP differed depending on the metabolic source of ATP, we compared the effects of glycolytic and mitochondrial substrates in the presence of an exogenous ATP-consuming system, hexokinase plus 2-deoxyglucose (Fig. 2). Hexokinase catalyzes the phosphorylation of 2-deoxyglucose to 2-deoxyglucose-6-phosphate (nonmetabolizable) and in the process degrades ATP to ADP. Exposure of a permeabilized cell to this ATP-consuming system was intended to simulate the competition for ATP by the variety of ATP-consuming processes in the working myocardium.

In Fig. 2, at the beginning of the continuous trace, openings of a single inwardly rectifying K^+ channel were occasionally observed during exposure to standard bath solution containing mitochondrial substrates and hexokinase (10 IU/ml). Removal of mitochondrial substrates and addition of 10 mM 2-deoxyglucose to the hexokinase in the bath solution (first arrow) caused many ATP-sensitive K^+ channels to open. Reexposure to mitochondrial substrates (second arrow) in the presence of both hexokinase and 2-deoxyglucose now resulted in only a partial transient suppression of ATP-sensitive K^+ channel activity, presumably because ATP generated by mitochondria was being degraded by the reaction with hexokinase and 2-deoxyglucose before it could reach the ATP-sensitive K^+ channels in the patch. However, glycolytic substrates (third arrow) continued to effectively suppress ATP-sensitive K^+ channels in the presence of hexokinase plus 2-deoxyglucose, even after 1 μM FCCP (fourth arrow) was added to inhibit mitochondrial ATP production. The inwardly-rectifying K^+ channel remained active with glycolytic substrates present. The findings were reproducible (second and third tracing), and mitochondrial substrates remained effective in the presence of hexokinase when 2-deoxyglucose was omitted (eighth arrow), although transient openings of ATP-sensitive K^+ channels were observed until the effects of FCCP from the previous intervention wore off. ATP (2 mM, without hexokinase plus 2-deoxyglucose) also was effective (twelfth arrow). In 15 permeabilized cells the average current through ATP-sensitive K^+ channels in the patch with hexokinase plus 2-deoxyglucose present fell to 81 ± 35% of the control value when mitochondrial substrates were added and to 34 ± 29% when glycolytic substrates and FCCP were added ($P < 0.005$ by paired t test). In eleven of these cells 2 mM creatine was included with mitochondrial substrates to facilitate high-energy phosphate transfer from the mitochondria to the cytoplasm via the phosphocreatine shuttle. The reduction in average current through the ATP-sensitive K^+ channels was similar to

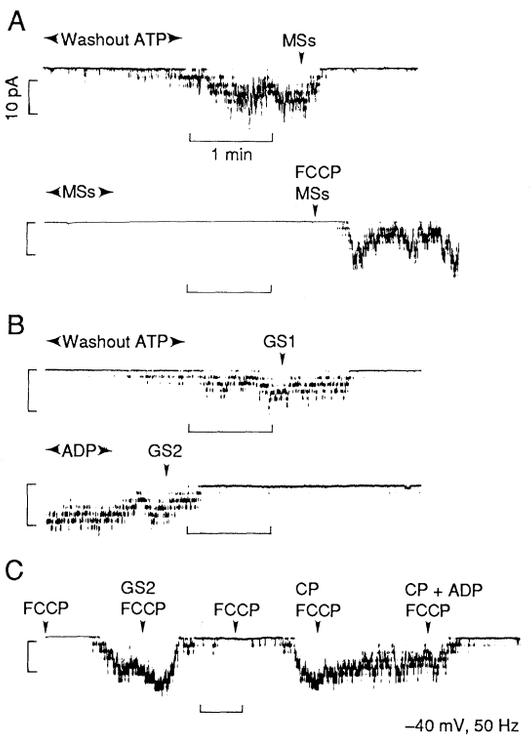

Fig. 1. The effect of various metabolic substrates on ATP-sensitive K^+ channels in open cell-attached patches. See text for details of each trace. Mitochondrial substrates (MSs) included 2 mM pyruvate, glutamate, and creatine, 1 mM K_2HPO_4, and 0.5 mM ADP. Glycolytic substrates included either 2 mM fructose-1,6-diphosphate, 1 mM NAD, 1 mM K_2HPO_4 and 0.5 mM ADP (GS1), or 2 mM PEP and 0.5 mM ADP (GS2). CP is creatine phosphate (2 mM). The patch electrode was held at +40 mV relative to the bath (equivalent to a membrane potential of −40 mV) throughout. Patch electrode contained 150 mM KCl and 5 mM Hepes, and the bath contained standard solution plus various substrates as indicated. Inward current is downward. Filter setting, 50 Hz.

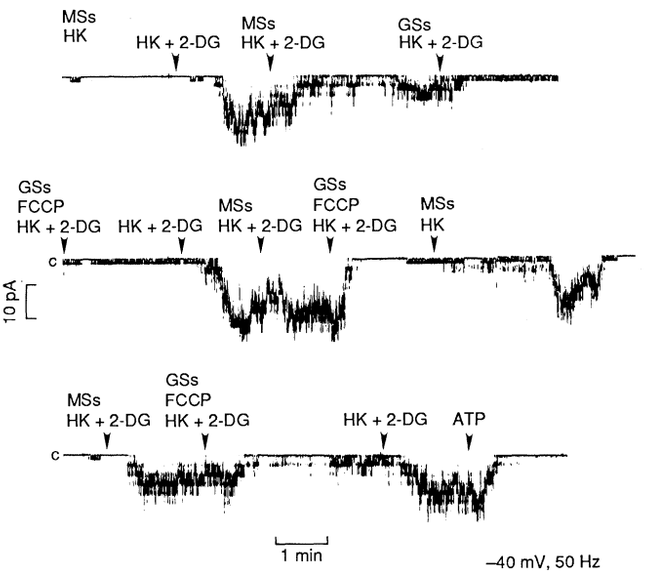

Fig. 2. Effects of mitochondrial versus glycolytic substrates on ATP-sensitive K^+ channels in an open cell-attached patch with hexokinase (HK) plus 2-deoxyglucose (2-DG) present. See text for details. Mitochondrial substrates (MSs) consisted of 2 mM pyruvate and glutamate, 1 mM K_2HPO_4, and 0.5 mM ADP, pH 7.1. Glycolytic substrates (GSs) consisted of 2 mM fructose-1,6-diphosphate and PEP, 1 mM NAD and K_2HPO_4, and 0.5 mM ADP, pH 7.1.

those experiments without creatine present (82 ± 34 and 81 ± 43%). In all of these cells mitochondrial substrates had completely suppressed ATP-sensitive K^+ channels in the presence of hexokinase without 2-deoxyglucose.

The greater effectiveness of glycolytic than of mitochondrial substrates at suppressing ATP-sensitive K^+ channels when hexokinase plus 2-deoxyglucose was present could be due to several factors. For example, an intermediate of glycolytic metabolism might directly block the channels or increase their sensitivity to ATP, or glycolytic enzymes located in close proximity to the channels might generate a higher local ATP concentration than mitochondria. Therefore, the effects of various glycolytic intermediates on ATP-sensitive K^+ channels were studied in excised (cell-free) inside-out patches. In the absence of exogenous ATP none of the following compounds (1 to 2 m*M*) alone or in various combinations significantly affected the average current through ATP-sensitive K^+ channels: fructose-1,6-diphosphate, glyceraldehyde-3-phosphate, glyceraldehyde-3-phosphate plus glyceraldehyde-3-phosphate dehydrogenase (2 to 10 IU/ml) plus nicotinamide adenine dinucleotide (NAD) plus K_2HPO_4 (to generate glycerate-1,3-diphosphate), NAD or its reduced form (NADH), glycerate-3-phosphate, glycerate-2-phosphate, phosphoenolpyruvate (PEP), pyruvate, or ADP (0.5 m*M*). However, in 5 of 27 inside-out patches, a combination of all the necessary substrates for the ATP-producing steps of glycolysis (mediated by the enzymes phosphoglycerokinase and pyruvate kinase) reversibly and reproducibly suppressed ATP-sensitive K^+ channels and reduced the average current through the channels (relative to the control value with 0.5 m*M* ADP present) to 1.6, 3.3, 7.1, 13.6, and 64%, respectively. In 1 of 11 inside-out patches, substrates for the second ATP-producing step alone (involving pyruvate kinase) reduced the average current through ATP-sensitive K^+ channels to zero. The effects of 2 m*M* glyceraldehyde-3-phosphate and PEP, 2 IU glyceraldehyde-3-phosphate dehydrogenase per milliliter, 1 m*M* NAD and K_2HPO_4, and 0.5 m*M* ADP on ATP-sensitive K^+ channels in one of these excised inside-out patches are shown (Fig. 3). Replacement of 2 m*M* ATP with 0.5 m*M* ADP in the bath solution (first arrow) caused multiple ATP-sensitive K^+ channels in the patch to open. Addition of glycolytic substrates for the ATP-producing steps of glycolysis (second arrow) promptly shut the ATP-sensitive channels. The sequence was then repeated.

The ability of the appropriate combination of glycolytic substrates to suppress ATP-sensitive K^+ channels in some excised patches is consistent with the hypothesis that phosphoglycerate kinase or pyruvate kinase, or both,

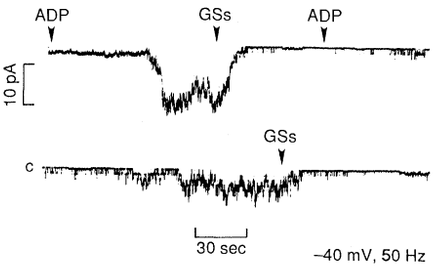

Fig. 3. Effect of glycolytic substrates (GSs) on ATP-sensitive K^+ channels in an excised inside-out patch. See text for details. Glycolytic substrates included 2 m*M* glyceraldehyde-3-phosphate and PEP, 2 IU of glyceraldehyde-3-phosphate dehydrogenase per milliliter, 1 m*M* NAD and K_2HPO_4, and 0.5 m*M* ADP.

are located in the sarcolemma or cytoskeleton in the immediate vicinity of K^+ channels. The ineffectiveness of the substrates in the majority of patches may be related to damage or loss of enzymes during patch excision. Alternatively, the geometry of individual patches may determine whether ATP generated locally by glycolytic enzymes can accumulate sufficiently to suppress ATP-sensitive K^+ channel activity, since electronmicrographs of excised patches have shown variability in the amount of cytoskeleton and cytoplasm retained (*13*).

These findings suggest that under conditions of high intrinsic ATP consumption glycolysis is more effective than mitochondrial metabolism in suppressing ATP-sensitive K^+ channels in permeabilized myocytes. Several limitations of this interpretation should be mentioned. We cannot exclude the possibility that saponin damaged the mitochondria, although exposure to saponin was brief and was localized to one end of the cell and mitochondrial membranes are considered more resistant to the detergent effects of saponin than is sarcolemma. Furthermore, the extent of mitochondrial damage would have to be severe for mitochondrial ATP-generating capacity to fall below that of glycolysis, since one molecule of mitochondrial substrate generates approximately 17 ATP molecules, compared to only 2 ATP for each molecule of glycolytic substrate. It is also conceivable that the exogenous ATP-consuming system, hexokinase plus 2-deoxyglucose, was distributed inhomogeneously throughout the cell in a manner that favored access to mitochondrial ATP. Although we cannot exclude this possibility, the results with excised inside-out patches suggest that a close proximity between key glycolytic enzymes and ATP-sensitive K^+ channels is a plausible explanation for their preference for glycolytic ATP. We have not systematically tested the possibility that certain glycolytic intermediates may alter the sensitivity of ATP-sensitive K^+ channels to ATP concentration. None of these intermediates directly blocked the channels in the absence of exogenous ATP in excised inside-out patches, and in open-cell attached patches substrates for the last step of glycolysis alone (PEP and ADP) effectively suppressed ATP-sensitive K^+ channels together but not individually, which suggests that no glycolytic intermediates were necessary.

Our findings are consistent with several observations in intact isolated beating heart (*5*), including the marked cellular K^+ loss caused by selective inhibition of glycolysis despite normal total cellular levels of ATP and creatine phosphate, and the rise in extracellular K^+ concentration when glucose was replaced with pyruvate or acetate as myocardial substrate. Glycolysis may play a special role in maintaining membrane functions in other tissues (*14, 15*). This is a particularly intriguing possibility in the pancreas, in which insulin release by beta islet cells is regulated by ATP-sensitive K^+ channels (*16–18*). In heart, activation of ATP-sensitive K^+ channels during ischemia depresses excitability and probably shortens the time window during which the heart is susceptible to lethal arrhythmias. A preferential role of glycolysis in regulating the activity of these channels and perhaps other membrane functions may have important implications for attempts to improve myocardial function during ischemia and reperfusion.

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19. Supported by NIH grants HL27845, HL36729, and HL01890 (Research Career Development Award to J.N.W.), American Heart Association grant-in-aid 83-626, and the Laubisch Endowment. We thank R. E. Weiss, M. Morad, K. I. Shine, G. A. Langer, and A. J. Brady for advice, encouragement, and support.

13 April 1987; accepted 6 July 1987