Influence of Hypoxia on Adrenergic Modulation of Triggered Activity in Isolated Adult Canine Myocytes

Silvia G. Priori, MD; Kathryn A. Yamada, PhD; and Peter B. Corr, PhD

Although findings from several reports suggest that nonreentrant or focal mechanisms contribute to the genesis of arrhythmias during early ischemia, the contribution of triggered activity arising from early or delayed afterdepolarizations has not been resolved. We have previously demonstrated that β- but not α-adrenergic stimulation induces afterdepolarizations and triggered activity in isolated normoxic myocytes. In the present study, the influence of the extent of cellular derangements as well as increases in [K+]0 on α- and β-adrenergic–mediated afterdepolarizations and triggered activity was evaluated. Adult canine myocytes were exposed to one of the following experimental conditions with simultaneous intracellular transmembrane action potential recordings: 1) low P0₂ (less than 10 mm Hg, obtained using a specially designed hypoxic chamber) and low (6.8) pH; 2) low P0₂, low pH, and high extracellular potassium ([K+]0) (10 mM); or 3) severe metabolic inhibition with cyanide (10⁻⁶ M). Cells from each group were superfused with either the α-agonist phenylephrine (10⁻⁵ or 10⁻⁷ M, with 10⁻⁵ M nadolol) or the β-agonist isoproterenol (10⁻⁶ M). Moderate changes in the action potentials were observed under conditions 1 and 2 (moderate hypoxia), whereas marked but reversible changes were observed with cyanide (severe metabolic inhibition). During moderate hypoxia in normal [K+]0, delayed afterdepolarizations or triggered activity were elicited by both α- (12 of 13 cells) and β-adrenergic (five of five cells) stimulation. Increasing [K+]0 during moderate hypoxia completely abolished the afterdepolarizations induced by α-adrenergic stimulation and prevented the occurrence of triggered activity. In contrast, the influence of β-adrenergic stimulation was only attenuated by an increase in [K+]0. Exposure to cyanide completely prevented the induction of afterdepolarizations and triggered activity by both α- and β-adrenergic stimulation. Our findings indicate that moderate hypoxia in normal [K+]0 is associated with the development of adrenergic-mediated afterdepolarizations and triggered activity. In contrast, accumulation of [K+]0, or severe impairment of cellular metabolism is accompanied by inhibition of adrenergic-mediated afterdepolarizations and triggered activity. (*Circulation* 1991;83:248–259)

Results from several reports indicate that nonreentrant or focal mechanisms contribute to the genesis of arrhythmias during early ischemia as well as during subsequent reperfusion.¹⁻⁶ We have shown using three-dimensional cardiac mapping that nonreentrant or focal mechanisms can account for approximately 25% of the spontaneous arrhythmias during early ischemia² and 75% of arrhythmic episodes during subsequent reperfusion.⁶ These studies also demonstrated that automatic or focal activity during ischemia or reperfusion can originate not only from the endocardium but also from epicardial regions in which Purkinje fibers are not present. These findings suggest that this nonreentrant or focal activity must occur not only in Purkinje cells but also in ventricular muscle cells.

The observation that the occurrence of delayed afterdepolarizations (DADs) leading to triggered rhythms is reduced markedly in ventricular muscle in the presence of metabolic inhibitors has suggested that triggered rhythms originating in ventricular muscle are unlikely to contribute to arrhythmogenesis during early ischemia.⁷,⁸ However, the extent of ischemia in the intact heart is heterogeneous, and the
level of cellular depression as well as the concentrations of intracellular and extracellular ions varies. Accordingly, triggered rhythms may occur in regions at the periphery of the ischemic zone in which cellular depression or the accumulation of extracellular potassium (\( [K^+]_o \)) is less pronounced.

Myocardial ischemia may also alter the arrhythmogenic response to adrenergic stimulation, particularly the relative influence of \( \alpha \)- versus \( \beta \)-adrenergic receptor stimulation. For example, exposure of isolated adult myocytes to 10 minutes of hypoxia results in a threefold increase in the number of \( \alpha \)-adrenergic receptors\(^9\) reflecting events that occur in the in situ heart in response to ischemia.\(^{10}\) Likewise, hypoxia of 10 minutes' duration results in an eightfold reduction in the concentration of norepinephrine required to produce a comparable increase in the second messenger, 1,4,5-inositol trisphosphate (IP\(_3\)).\(^{11}\) Because IP\(_3\) has been shown to elicit an increase in intracellular calcium, an event recognized to be critical to the development of afterdepolarizations,\(^{1}\) it is possible that \( \alpha \)-adrenergic stimulation performed under hypoxic conditions may enhance the propensity for the development of triggered rhythms.

The present study was performed to determine whether arrhythmogenic mechanisms mediated by \( \alpha \)-adrenergic stimulation develop in hypoxic myocytes, whether increases in \( [K^+]_o \) modify triggered activity induced by \( \alpha \)- or \( \beta \)-adrenergic stimulation, and whether different levels of cellular metabolic impairment have different effects on the occurrence of afterdepolarizations and triggered activity. An isolated ventricular myocyte preparation was chosen to obviate electrotonic or pacemaker influences mediated by adjacent cells as occurs in multicellular preparations. In addition, the isolated myocytes permitted evaluation in ventricular muscle compared with Purkinje cells.\(^{12}\)

**Methods**

**Preparation of Adult Canine Myocytes**

Dogs were anesthetized with thiopental (30 mg/kg i.v.), a left thoracotomy was performed, and the hearts were excised rapidly. Epicardial strips were removed from the left ventricle and placed immediately into Krebs-Henseleit buffer and gassed with 95% \( O_2 \)-5% \( CO_2 \) (pH 7.4). The tissue strips were then trimmed into pieces measuring approximately \( 6 \times 8 \times 4 \) mm for the cell isolation procedure. The tissue pieces were rinsed once in Krebs-Henseleit buffer and twice in \( Ca^{2+} \)-free HEPES buffer (115 mM NaCl, 5 mM KCl, 35 mM sucrose, 10 mM glucose, 20 mM HEPES, and 4 mM taurine; pH 7.0 with 1 N NaOH; bubbled with 100% \( O_2 \)). Pieces of tissue were placed in \( 125 \) ml polyethylene wide-mouth bottles with 22 ml of \( Ca^{2+} \)-free HEPES buffer for a 5-minute washout period. The solution was discarded and replaced with 10 \( \mu \)M \( Ca^{2+} \)-HEPES buffer containing 0.05% collagenase (type CLS II, Worthington, Freehold, N.J.) plus 0.28 mg/ml protease (type XIV; Sigma Chemical, St. Louis, Mo.), and each bottle was placed into a 35°C water bath. A Harvard respirator (model 607; Harvard Apparatus, South Natick, Mass.) was connected to the needle end of 10-ml glass syringes (without plungers), and the wide end of each syringe was placed into the polyethylene bottles such that the solution was drawn into the syringe with each inspiratory cycle of the respirator. The respirator was adjusted to permit the HEPES-collagenase solution plus tissue to be drawn up to seven eighths of the syringe height at a rate of 18 times a minute. A stream of \( O_2 \) was applied continuously to each bottle during the isolation procedure. The tissue was agitated for 30 minutes after which the HEPES-collagenase-protease solution was decanted and replaced with fresh collagenase solution without protease. A second 30-minute incubation was followed by six additional 15-minute incubation periods. A fresh solution of HEPES-collagenase was used for each of the six subsequent incubation steps. The two initial 30-minute harvests contained primarily red blood cells and contracted myocytes and were therefore discarded. Harvests from the subsequent 15-minute incubation steps were centrifuged at 45g for 2 minutes, and the myocyte pellets were resuspended in 2 ml of 50-\( \mu \)M \( Ca^{2+} \)-HEPES (pH 7.2).

The percentage of elongated myocytes was increased by separation from contracted and rounded cells using a percoll gradient. Myocytes were layered over 6 ml of 50-\( \mu \)M \( Ca^{2+} \)-HEPES with 1.4% bovine serum albumin (pH 7.2), mixed with 4 ml isotonic percoll solution (3.64 ml percoll, 360 \( \mu \)l 9% NaCl, 20 \( \mu \)l of 1 N acetic acid; pH 7.3), and centrifuged at 120g for 5 minutes. The pellet was resuspended in 50-\( \mu \)M \( Ca^{2+} \)-HEPES and washed once in the same medium. The \( Ca^{2+} \) concentration of the myocyte preparation was brought up slowly by steps to 100, 200, 350, and finally 500 \( \mu \)M by addition of appropriate amounts of 10-mM \( Ca^{2+} \)-HEPES every 30 minutes. Overall, recovery of the myocyte preparation was 30–40% of the wet weight of the tissue. This technique provided elongated quiescent myocytes whose morphological and electrophysiological properties have been reported previously.\(^9\)

**Electrophysiological Recordings**

A drop of the suspension of myocytes was placed on the bottom of a plastic chamber positioned on the stage of a Nikon Diaphot inverted microscope. The myocytes were superfused with a solution of the following composition (mM): 133.5 NaCl, 4.8 KCl, 1.2 MgCl\(_2\), 1.2 NaH\(_2\)PO\(_4\), 10 HEPES, 10 glucose, 1.2 CaCl\(_2\), pH adjusted to pH 7.25–7.35 with 1 N NaOH. This will be referred to as the "normoxic solution." The solution was passed through a thermostatically controlled heating ring surrounding the bath before superfusion of the cells to permit precise temperature regulation between 35°C and 36°C. The superfusate flow rate was adjusted to 2–3 ml/min to preclude movement of the unattached myocytes and maintain stability of the intracellular impalement.
A hydraulic micromanipulator (Narishige, model MO-103) was used to position a glass capillary microelectrode (tip resistance, 40–80 MΩ) with which cells were intracellularly impaled. The electrical signal was passed through an electrometer with capacitance neutralization (Axoclamp 2-A, Axon Instruments, Foster City, Calif.), displayed on an oscilloscope (Tektronix 5111A), and stored on electromagnetic tape (Vetter recorder model A) for subsequent off-line computer analysis. On-line computer acquisition was performed through an A/D conversion board (T-1 DMA interface, Scientific Solutions, Solon, Ohio) connected to a computer (High Performance Working Station, Zenith, St. Joseph, Mich.) with the Axotape 1.2 (Axon Instruments, Foster City, Calif.) acquisition software. Myocytes were electrically stimulated by intracellular current injection through the microelectrode using depolarizing pulses (1–5-msec duration) of small amplitude (0.5–4 nA). Impalement was considered successful when it resulted in a stable resting membrane potential of more than ~80 mV, and the cell could be paced up to 3.5 Hz. Occasionally, cells presented with a prolonged plateau and/or spontaneous depolarizations. These cells were always excluded from study. Before any treatment or pacing protocol was initiated, continuous and stable intracellular recordings were collected for a control interval of 20 minutes. The pacing protocols, without intervention, were then performed at frequencies ranging from 1 to 4 Hz to determine the characteristics of the transmembrane action potential recordings including amplitude and duration at 95% of full repolarization and to exclude that either early or delayed afterdepolarizations could be elicited by the pacing protocol. Drug interventions were performed only after both the control recording interval and the initial pacing protocol were completed.

**Experimental Protocols**

**Hypoxia.** Previous studies from our laboratory have indicated that to simulate ischemia in vivo with hypoxia in vitro requires reduction in PO₂ to less than 15 mm Hg to adequately inhibit mitochondrial β-oxidation of fatty acids, leading to an increase in cellular long-chain acylcarnitines. In the present study, this was achieved by using a chamber constructed of Plexiglas that formed a tight seal on the stage of the microscope and into which the microelectrode holder was positioned and surrounded by flexible rubber to permit movement of the electrode but prevent air exchange. The solution was made hypoxic by continuous bubbling with 100% N₂ for more than 3 hours before superfusion into the bath. Stainless-steel tubing was used throughout, except for the small portion connected to the peristaltic pump. A stream of nitrogen flowed continuously through the chamber surrounding the bath, and a nitrogen atmosphere was maintained by a vacuum line connected to the opposite side of the chamber. The ionic composition of the hypoxic solution was identical to the normoxic solution except no glucose was present and 2-deoxyglucose (10 mM) was added to inhibit anaerobic glycolysis to simulate the inhibition induced by accumulating lactate during in vivo ischemia. The pH of the hypoxic solution was 6.8. In experiments evaluating the influence of an increase in [K⁺], the same hypoxic solution was used with a K⁺ concentration of 10 mM.

After 20 minutes of control recordings in the normoxic solution, during which cells were paced at 1–4 Hz to assess that no spontaneous automaticity or triggered activity developed under control conditions, individual protocols were begun. In the first series of studies, three groups of experiments were performed. In the first group (n=6), cells were exposed to hypoxia for 15 minutes during which pacing protocols were performed continuously at frequencies of 1–4 Hz. Each pacing sequence lasted for 20–30 beats and was followed by an observation time of 10 seconds during which the occurrence of afterdepolarizations or automatic beats was observed. After the 15-minute hypoxic interval, reoxygenation was initiated abruptly by superfusion with the normoxic solution. Intracellular recordings were maintained for as long as 30 minutes after reoxygenation during which the pacing protocols were repeated. In the second group (n=5), cells were exposed to 10 minutes of hypoxia; then, β-adrenergic stimulation with isoproterenol (10⁻⁶ M) was begun while hypoxia was continued for 5 additional minutes before reoxygenation. Pacing protocols were performed throughout as described above. In the third group (n=13), cells were exposed to hypoxia for 10 minutes followed by α-adrenergic stimulation with phenylephrine 10⁻⁷ M (n=7) or 10⁻⁵ M (n=6) during 5 additional minutes of hypoxia before reoxygenation. In all experiments with phenylephrine, nadolol (10⁻⁵ M) was infused simultaneously to inhibit any β-adrenergic component of phenylephrine. Pacing protocols were performed throughout as described above.

**Elevated [K⁺]ᵢ.** In nine additional experiments, the influence of increasing [K⁺]ᵢ, was evaluated during hypoxia, with and without α- or β-adrenergic stimulation, to simulate conditions occurring during ischemia in vivo. Three cells were exposed to combined hypoxia and increased [K⁺]ᵢ (10 mM) for 15 minutes and are referred to as “hypoxia plus high [K⁺]ᵢ” control. Six cells were exposed for 10 minutes to hypoxia and elevated [K⁺]ᵢ, and were then exposed to either α- (phenylephrine, 10⁻⁷ M, plus nadolol, 10⁻⁵ M) or β- (isoproterenol, 10⁻⁶ M) adrenergic stimulation in the same hypoxic solution with elevated [K⁺]ᵢ. Reoxygenation was performed in all nine cells at normal [K⁺]ᵢ (4 mM), without adrenergic agonists.

**Metabolic inhibition.** Metabolic inhibition was achieved by adding carbonyl cyanide (10⁻⁶ M) to the normoxic solution. Six cells were exposed to metabolic inhibition for 10 minutes followed by a return to the normoxic solution without cyanide. Twelve cells were exposed to metabolic inhibition for 5 minutes
followed by an additional 5 minutes in the presence of isoproterenol (10⁻⁶ M, n=6) or phenylephrine (10⁻⁷ M, n=6) plus nadolol (10⁻⁵ M). After a cumulative interval of 10 minutes of exposure to metabolic inhibition, cells were then exposed to the normoxic solution without cyanide and washout was maintained as long as 30 minutes.

Biochemical assays. The levels of long-chain acylcarnitines were measured in cells (n=6) exposed to 15 minutes of hypoxia in the presence of 10 mM deoxyglucose after a 20-minute normoxic control interval. Aliquots of cells were taken from the bath during the control interval and after exposure of the cells to hypoxia. Details of the assay procedures for long-chain acylcarnitines in this cell system have been described in a previous publication from our laboratory.15

Terminology. We defined afterpotentials and rhythmic activity according to criteria described previously in the literature.1,16 Terms used were as follows. Early afterdepolarization (EAD) is a depolarizing afterpotential interrupting the plateau or the phase 3 repolarization.

DAD is a depolarizing afterpotential occurring after termination of repolarization when the resting membrane potential has returned to the diastolic value. It is enhanced by fast pacing rates that cause an increase in amplitude and a shortening of the coupling interval of the DAD to the preceding paced beat.

Triggered activity is activity in which nondriven action potentials originate from DADs. This form of activity has to be strictly dependent on the existence of driven beats preceding the nondriven ones. It cannot occur in an unstimulated preparation. Triggered activity shows the same dependency on faster pacing frequency as described for DADs and is always preceded or followed by DADs.

Automatic activity is activity in which a nondriven action potential does not originate from a DAD. It may lack a prominent phase 4 depolarization and therefore can arise from a normally polarized membrane potential.17

Data Analysis and Chemicals

Action potential recordings were stored on-line on 5¼-in. floppy disks and were analyzed off-line with the Axotape analysis program. Data were transferred to an Asystant file for statistical analysis. Data are expressed as mean±SD. Normal distribution of values was assessed by the Kolmogorov-Smirnov test to permit the use of parametric tests. Paired t tests were used for analysis of the effect of hypoxia and metabolic inhibition on the action potential parameters in each group and to evaluate changes in long-chain acylcarnitine during hypoxia. One-way analysis of variance and Tukey tests were used for multiple comparisons, χ² test with Yates’ correction was used for comparing the incidence of DADs in different experimental groups. For all comparisons, differences with a probability value of less than 0.05 were considered significant.

Drugs and chemicals used in the study were phenylephrine, isoproterenol, carbonyl cyanide, and 2-deoxyglucose (Sigma Chemical) and nadolol (kindly supplied by Squibb, Inc., Princeton, N.J.).

Results

Inhibition of Glycolysis in Normoxic Cells

Treatment of normoxic cells (n=4) with 2-deoxyglucose (10 mM) over 40 minutes did not alter action potential parameters or induce EADs or DADs when paced at 1–4 Hz.

Influence of Hypoxia

Twenty-four cells were exposed to hypoxia for 10 minutes. In six cells, the hypoxic interval was continued for an additional 5 minutes without agonists. In the remaining experiments, α-adrenergic (n=13) or β-adrenergic (n=5) stimulation was begun during the last 5 minutes of the 15-minute hypoxic interval. Action potential duration at 95% repolarization shortened after 10 minutes of hypoxia from 294±35 to 243±73 msec (p<0.005), and total action potential amplitude was reduced from 101±11 to 81±12 mV (p<0.001), with no difference among the various groups of cells (NS for hypoxia alone versus hypoxia plus α-adrenergic stimulation versus hypoxia plus β-adrenergic stimulation). Modifications in the resting membrane potential were sometimes difficult to evaluate because of changes in DC offset over time; therefore, these data are not included. Long-chain acylcarnitine content increased during hypoxia from 54.4±0.7 to 199.4±96.4 pmol/mg protein (p<0.01), confirming that β-oxidation was inhibited when cells were exposed to hypoxia, which is analogous to what occurs within 5 minutes of ischemia in vivo.15 During hypoxia, cells required an increase in the magnitude of intracellular current to elicit an action potential coincident with the development of alternating shorter and longer action potential durations, even at the lower pacing frequencies. Of the six cells exposed to 15 minutes’ hypoxia alone, only one developed multiple DADs with a mean amplitude of 4 mV and a coupling interval of the last paced beat to the first DAD of 600 msec. None of the cells exposed to hypoxia alone developed triggered activity or DADs during reoxygenation. In the cell exhibiting DADs during hypoxia alone, the DADs abated after 15 minutes of reoxygenation.

Hypoxia and α-Adrenergic Stimulation

Thirteen cells were exposed to α-adrenergic stimulation during hypoxia. Exposure to the initial 10-minute hypoxia interval elicited DADs in only two of 13 cells; the incidence of afterdepolarizations, however, increased (p<0.01 versus hypoxia alone) when phenylephrine stimulation was superimposed. Two concentrations of phenylephrine were used in the group: 10⁻³ M (n=6) and 10⁻⁷ M (n=7). DADs during hypoxia with phenylephrine occurred in five of six cells exposed to the highest concentration (10⁻³ M) and six of seven cells exposed to the lowest
concentration (10⁻⁷ M) (Figures 1 and 2), indicating that phenylephrine at 10⁻⁷ M elicited the maximal response for the induction of DADs in this setting. In both groups, DADs occurred only when pacing was performed at a frequency of 2 Hz or more. The mean amplitude of the DADs was 3±1.7 mV at a 2-Hz pacing frequency and 5.8±3.3 mV at a 3-Hz pacing frequency (p<0.05 versus 2 Hz), and the coupling interval of the first DAD was 3,100±1,863 msec at a 2-Hz pacing frequency and 1,940±1,600 msec at 3-Hz pacing frequency (p<0.05 versus 2 Hz). The relation between the coupling interval or the amplitude of DADs and the frequency of pacing is illustrated with an example in Figure 3, and the data are summarized in Figure 4. The coupling interval decreased and the amplitude of DADs increased with increasing pacing frequency, similar to that described for DADs in other in vitro studies and in vivo preparations. Spontaneous oscillations to voltages more positive than the resting membrane potential, similar to those defined as DADs, never occurred when the cells were not stimulated.

Triggered activity occurred in only one of 13 cells exposed to α-adrenergic stimulation with pacing at 3 Hz and consisted of single action potentials that never initiated a sustained triggered rhythm. In this cell, the amplitude of the DADs was not larger than that observed in the 12 preparations that did not develop triggered activity. At the moment of reoxygenation, two of the cells that had developed DADs during hypoxia and α-adrenergic stimulation developed sustained triggered rhythms after pacing at 3 Hz (Figure 5). In the remaining cells, DADs that developed during hypoxia persisted during reoxygenation for a mean of 15±3 minutes. In two cells, reoxygenation in the presence of phenylephrine also induced automatic activity that did not originate from a DAD but from a resting membrane potential of -70 and -72 mV, respectively. One of these examples is shown in Figure 6. In both cases, after several spontaneous beats, the membrane potential arrested at the plateau level (Figure 6A).

**Hypoxia and β-Adrenergic Stimulation**

Five cells were exposed to β-adrenergic stimulation. During the 10 minutes of hypoxia that preceded β-adrenergic stimulation, DADs developed in one of five cells. After stimulation with isoproterenol (10⁻⁶ M), DADs and triggered activity developed in all cells when pacing at frequencies of 1 Hz or more. The amplitude of DADs increased significantly with increasing pacing frequency, whereas coupling intervals of the DADs significantly decreased (Figure 3B and Figure 4). Mean amplitudes of DADs were 2.4±1.1, 6.4±1.7, and 10.2±3.5 mV at 1-, 2-, and 3-Hz pacing frequencies, respectively, whereas coupling intervals were 2,266±802 msec, 1,700±1,077, and 1,180±766 msec, respectively. Note that at each
whereas the hypoxic cell, after termination of pacing at 2 Hz (left panel), two triggered action potentials develop; after termination of pacing at 3 Hz (right panel), three triggered action potentials develop, followed by subthreshold delayed afterdepolarizations. Note that coupling intervals of triggered beats developing after pacing at 3 Hz are shorter than those of triggered beats developing after pacing at 2 Hz.

frequency of stimulation, the coupling interval of DADs induced by β-adrenergic stimulation was slightly lower (p=0.1) compared with DADs induced by α-adrenergic stimulation, whereas the amplitude response was higher (p<0.01 at the 2- and 3-Hz frequencies). Pacing at 1–2 Hz during isoproterenol and hypoxia also elicited EADs in three of five cells. At the moment of reoxygenation, triggered activity was present in all five cells and spontaneously terminated within a few minutes in two cases (Figure 7), whereas it lasted throughout the 30-minute reoxygenation interval in the remaining three cells.

Influence of Elevated Extracellular K⁺

This group included nine cells—three cells exposed to hypoxia in the presence of 10 mM [K⁺]o, for 15 minutes and six cells in which after 10 minutes of hypoxia either α- or β-adrenergic stimulation was performed for the subsequent 5-minute hypoxic interval, always in the presence of 10 mM [K⁺]o. In this group, the amplitude of action potential decreased to an extent similar to that observed during hypoxia in normal [K⁺]o (from 102±8 to 79±6 mV, p<0.001 versus control, NS versus hypoxia alone), whereas action potential duration decreased more markedly (from 257±41 to 166±51 msec, p<0.001 versus control, p<0.02 versus hypoxia alone). These changes were reversible after reoxygenation in normal [K⁺]o. Neither DADs nor triggered activity developed in any of the cells exposed to hypoxia in the absence of adrenergic stimulation. Reoxygenation with normal [K⁺], did not elicit DADs in any of the cells in this group. Of the cells exposed to phenylephrine during high [K⁺], plus hypoxia, none developed DADs. However, with reoxygenation in normal [K⁺], all three cells developed DADs with a mean time of onset of 40±15 seconds. The amplitude of the DADs was 4±0.5 mV (range, 3–5 mV), and the coupling interval ranged from 300 to 1,800 msec (Figure 8). In addition, one of these cells developed automatic activity during reoxygenation.

All three cells exposed to β-adrenergic stimulation still developed DADs during hypoxia plus high [K⁺], while pacing at frequencies of 2 Hz or more with an amplitude of 8±2 mV (range, 6–10 mV) and a coupling interval of 150–600 msec. However, no triggered activity was observed, contrary to what has been observed with β-adrenergic receptor activation during hypoxia at normal [K⁺], (p<0.05). Thus, high [K⁺], prevented the occurrence of sustained rhythms during β-adrenergic stimulation. Indeed, after reoxygenation with normal [K⁺], all three cells developed triggered activity and EADs within 3–5 minutes (Figure 9). Triggered rhythms, as well as afterdepolarization...
DADs are afterdepolarizations (DADs), which develop at a pacing frequency of 3 Hz, but they never reach threshold for activation of an action potential. Bottom tracing: After reoxygenation, delayed afterdepolarizations lead to development of triggered activity. This triggered activity terminates with a subthreshold delayed afterdepolarization (not shown).

**Figure 5.** Top tracing: Control recording demonstrates that delayed afterdepolarizations are not present during normoxic interval. Middle tracing: After 15 minutes of hypoxia and 5 minutes of α-adrenergic stimulation, multiple oscillations develop at a pacing frequency of 3 Hz, but they never reach threshold for activation of an action potential. Bottom tracing: After reoxygenation, delayed afterdepolarizations lead to development of triggered activity. This triggered activity terminates with a subthreshold delayed afterdepolarization (not shown).

Influence of Severe Metabolic Inhibition

Nine cells were exposed to cyanide (10^{-6} M). The action potential duration at 95% repolarization shortened from 257±13 to 82±11 msec (p<0.001), and amplitude was decreased from 92±6 to 64±4 (p<0.01). These changes in action potential parameters were completely reversible after 10–20 minutes of washout of the cyanide (Figure 10). α-Adrenergic stimulation (phenylephrine 10^{-7} M plus nadolol 10^{-5} M, n=3) or β-adrenergic stimulation (isoproterenol 10^{-8} M, n=3) 5 minutes after exposure to cyanide failed to elicit any form of afterdepolarizations or triggered activity. Therefore, severe metabolic inhibition prevented the induction of triggered activity by either α- or β-adrenergic stimulation. This inhibition of DADs was reversible with washout of cyanide. To further assess the inhibitory effect of cyanide on the induction of DADs by adrenergic agonists, in three additional cells cyanide was infused after inducing DADs and triggered activity in the

**Figure 6.** Example of automatic beats developing after reoxygenation. Panel A: After 60 seconds of reoxygenation, action potential duration progressively prolongs; when pacing is terminated, automatic beats appear (*) that are independent of delayed afterdepolarizations (DADs), even if a DAD is present just before the last automatic beat that stops at the plateau level. Panel B: Twenty seconds after reoxygenation following hypoxia and administration of isoproterenol in presence of elevated [K+]o, several DADs are present, but they do not reach the threshold for activation. Note that DADs do not follow each paced beat and that they have a variable coupling to the preceding action potential. This behavior is rather atypical of DADs and is probably related to washout of inhibitory effect of high [K+]o on the development of DADs that creates a rapidly evolving setting in which a stable pattern cannot be achieved. One automatic action potential (*) originates between two paced beats during the slow rate of pacing.
presence of \(\beta\)-adrenergic stimulation in normoxic cells. In all cases, treatment with cyanide suppressed triggered activity (Figure 11).

**Discussion**

Single isolated cardiac cells have a very low oxygen consumption,\(^{14,19}\) primarily due to the lack of attachment to other contracting cells and thereby a minimal load. They have been shown to be remarkably resistant to oxygen deprivation.\(^{19}\) Our goal was to achieve a \(\text{P}_{O_2}\) level as low as possible in the solution superfusing the cells. To obtain this, a closed system was necessary to avoid contamination of the solution from room air. In our hypoxic chamber, the \(\text{P}_{O_2}\) was consistently less than 10 mm Hg and allowed us to observe electrophysiological and biochemical alterations over the 10-minute hypoxic interval.

To simulate ischemia in vivo, during hypoxia the cells were exposed to glucose free medium, thereby promoting glycogen as a source of intracellular glucose to be used via anaerobic glycolysis. Although a similar shift in metabolism occurs in vivo in response to ischemia,\(^{20}\) the rapid accumulation of lactate normally inhibits anaerobic glycolysis. Because isolated cells lack an extracellular restricted space that serves as an obstacle for diffusion of lactic acid in vivo,\(^{14}\) it is likely that in our preparation the free diffusion of lactate precludes its intracellular accumulation; therefore, anaerobic glycolysis is sufficient to maintain, to a large extent, cellular metabolism. Thus, to more accurately mimic ischemia in vivo, 2-deoxyglucose, which blocks glycolysis,\(^{13,14}\) was used. Administration of 2-deoxyglucose to fully oxygenated cells failed to elicit any electrophysiological effects, indicating that metabolism was dependent on \(\beta\)-oxidation of fatty acids and maintenance of normal action potentials when oxygen was available. No afterdepolarizations were induced by 2-deoxyglucose, excluding any direct effect of the compound that could favor the development of afterdepolarizations. This system permitted evaluation of the effects of catecholamines in single cells exposed to hypoxic conditions that were analogous to ischemia in vivo.

A 15-minute hypoxic interval was selected because previous findings from our laboratory indicated that this interval is sufficient to inhibit \(\beta\)-oxidation of fatty acids in isolated myocytes leading to an accumulation of long-chain acylcarnitines.\(^{9}\) Indeed, in the present study, we observed a more-than-threefold increase in long-chain acylcarnitines when cells were exposed to hypoxia within the chamber, analogous to that observed in vivo in the ischemic area.\(^{15}\) These findings confirm that inhibition of \(\beta\)-oxidation of fatty acids
was achieved. The accumulation of long-chain acylcarnitine within the sarcolemma has been shown to mediate the threefold increase in surface α₁-adrenergic receptors as well as enhance coupling to IP₃. Cells exposed to 10 minutes of hypoxia exhibited a consistent depression of electrophysiological parameters; in 12–15% of the preparations, DADs occurred in the absence of adrenergic stimulation. This latter finding indicates that there is a stage of moderate cellular depression in which DADs can occur in the absence of adrenergic stimulation. The fact that a moderate degree of metabolic impairment favors rather than impairs the development of DADs most likely is a consequence of an increase in calcium in response to hypoxia analogous to the increase in intracellular Ca²⁺ during early ischemia in intact tissue. This moderate cellular depression did not modify the influence of β-adrenergic stimulation reported in normoxic cells, but it did unmask an arrhythmogenic effect of α-adrenergic stimulation. Recent findings from our laboratory using the same-cell system have shown that α-adrenergic activation does not induce DADs or EADs under normoxic conditions. This α-adrenergic-mediated effect, which appeared exclusively in the presence of hypoxia, is likely to be mediated by the enhanced increase in intracellular IP₃ known to occur under these conditions. Because IP₃ has been reported to induce calcium release from the sarcoplasmic reticulum, it could be argued that the voltage oscillations that we observed and considered to be DADs might be due to localized release of calcium from the sarcoplasmic reticulum that can occur in a resting cell and therefore cannot be called DADs. However, the voltage deflections that we observed never occurred in a quiescent cell. The dependence of these membrane voltage oscillations on a preceding action potential strongly suggests that these spontaneous depolarizations are DADs. When the effect of altering pacing frequency was studied, a typical frequency dependency indicative of DADs was observed (Figure 4). When cells were paced at a faster rate, the amplitude of the DADs increased, and the coupling interval decreased. It is interesting to note that the coupling interval of β-adrenergic agonist-induced DADs is slightly shorter than that of α-adrenergic receptor-mediated DADs but is usually longer compared with what is observed in the same experimental preparation during normoxia. Whether this prolongation of the coupling interval of the DADs from the preceding action potential occurs as a direct result of the influence of hypoxia and its underlying cellular mechanism will require future investigation.

Triggered activity also appeared at the more rapid rates of stimulation (Figure 7). This feature distinguishes these events from automatic activity recorded in ventricular muscle that is enhanced by slow pacing frequencies. Triggered rhythms always terminated with subthreshold DADs, as shown in Figure 7.

![Figure 9](image_url)

**Figure 9.** Top traces: From left to right, shown are a control recording during superfusion with the normoxic solution, a recording after exposure to hypoxia in the presence of 10 mM [K⁺]₀, and a recording after administration of isoproterenol (10⁻⁶ M) during hypoxia and high [K⁺]₀. β-Adrenergic stimulation induced delayed afterdepolarizations (arrow) but not triggered activity, at variance with what was consistently observed when β-adrenergic stimulation was performed in hypoxic cells in the presence of normal [K⁺]₀. Lower tracing: At reoxygenation with normal [K⁺]₀, after termination of pacing, a triggered beat with early afterdepolarizations developed.
It has been reported in other cellular preparations that an increase in $[K^+]_o$ is one component of ischemia that suppresses the development of afterdepolarizations as well as triggered activity. This observation was confirmed in the present study because afterdepolarizations induced by $\alpha$-adrenergic stimulation during hypoxia were precluded when $[K^+]_o$ was elevated. The effect of $\beta$-adrenergic stimulation was also markedly attenuated by high $[K^+]_o$ because triggered activity was never observed, whereas isoproterenol induced triggered activity in 100% of cells during hypoxia when $[K^+]_o$ was normal. Because the accumulation of $[K^+]_o$ during ischemia in vivo has been shown to have marked regional variations, it is likely that the development of triggered rhythms during ischemia would be more prone to occur in areas in which $[K^+]_o$ is not markedly augmented, for example, in areas bordering the ischemic zone. This is also the region in which nonreentrant or focal mechanisms usually occur in response to ischemia or reperfusion in vivo.

Reoxygenation with normoxic buffer at normal $[K^+]_o$, after exposure to hypoxia and high $[K^+]_o$, elicited DADs and triggered activity only in the cells that were exposed to either an $\alpha$-adrenergic or $\beta$-adrenergic agonist during the hypoxia interval. This observation suggests that an increase in $[K^+]_o$ prevents development of afterdepolarizations but does not prevent the intracellular changes resulting from the interaction between hypoxia and adrenergic activation that become manifest upon reoxygenation when a normal level of $[K^+]_o$ is restored.

Automatic firing independent of DADs was observed in 15% of the cells evaluated during reoxygenation. In three cases, it developed in cells subjected to $\alpha$-adrenergic stimulation, and in one case, it developed in a cell subjected to $\beta$-adrenergic stimulation in the presence of high $[K^+]_o$, but it never occurred in the absence of catecholamines. Automatic firing appeared not to originate from DADs even if DADs were present concomitantly, and were not preceded by slow diastolic depolarization, but rather originated from a relatively negative membrane potential of $-70$ mV. Ventricular muscle cells normally do not exhibit spontaneous automaticity. However, the possibility that a pacemaker current ($I_t$ or $I_f$-like) can be present in myocytes has been suggested. Spontaneous activity in ventricular muscle has been described as differing from automaticity in Purkinje cells or sinus nodal cells in which a prominent diastolic depolarization precedes the automatic firing. In ventricular tissue, automatic activity has been described to occur with a minimal slow diastolic depolarization and at relatively negative membrane potentials. Our findings in isolated myocytes are in agreement with those observations made in epicardial tissue since in the present study the automatic beats appeared at a normally polarized more negative membrane potential. The conditions under which automaticity in ventricular muscle were described previously are rather unphysiological and include exposure to Ba$^{2+}$ or amantadine, whereas the present data indicate that reoxygenation in the presence of adrenergic agonists are a novel stimulus for the induction of spontaneous automaticity in ventricular muscle.

Severe cellular depression was attained by administration of cyanide, which consistently induced marked depression of electrophysiological indexes and prevented the development of afterdepolarizations and triggered activity by either $\alpha$- or $\beta$-adrenergic agonists. For example, exposure to cyanide consistently abolished or prevented the development of afterdepolarizations induced by $\beta$-adrenergic stimulation. These findings are in agreement with previous observations that severe cellular depression, even in the absence of additional components of ischemia, can prevent the development of afterdepolarizations and triggered activity. Although the mech-
anisms responsible for this effect are unknown, it is likely that under conditions of cellular metabolic impairment, the ATP-dependent uptake and release of Ca\textsuperscript{2+} from the sarcoplasmic reticulum would be attenuated. Therefore, during early ischemia or hypoxia, before metabolic impairment is sufficiently severe, both DADs and triggered activity could be elicited, whereas with a more severe metabolic derangement during prolonged ischemia, analogous to treatment with cyanide, even catecholamine-induced triggered activity would be precluded.

The finding that reoxygenation of cells exposed to cyanide always elicits DADs independent of adrenergic activation suggests that intracellular changes that occur after severe, but not moderate, cellular depression favor the development of triggered rhythms during reoxygenation.\textsuperscript{29} It is likely that only when severe metabolic derangements occur, together with a massive increase in intracellular calcium, cells reach the calcium overload state typical of reperfusion.\textsuperscript{30} Under these conditions, the occurrence of triggered activity is not dependent on adrenergic stimulation because the increase in intracellular calcium may already be maximal as a consequence of reoxygenation in severely impaired tissue.

In conclusion, the present study demonstrated that afterdepolarizations and triggered activity can develop during moderate hypoxia, in the absence of an increase in [K\textsuperscript{+}].\textsuperscript{22} During this stage of hypoxia, \( \beta \)-adrenergic induction of afterdepolarizations is unchanged from what was observed under normoxic conditions.\textsuperscript{22} However, moderate hypoxia is associated with an increase in \( \alpha \)-adrenergic responsivity shown by the development of \( \alpha \)-adrenergic-mediated DADs that cannot be induced in normoxic cells. An increase in [K\textsuperscript{+}], during moderate hypoxia blunts the development of afterdepolarizations and triggered activity during both \( \alpha \) and \( \beta \)-adrenergic activation. Exposure to severe metabolic impairment is associated with a complete inhibition of the development of afterdepolarizations and triggered activity, but reversal of this metabolic impairment can elicit triggered activity, even in the absence of adrenergic stimulation. These data indicate that during the progression of ischemia in vivo, there is a transient phase that might be prolonged in the border zone of the ischemic area in which afterdepolarizations can be induced by \( \alpha \) as well as \( \beta \)-adrenergic mechanisms.

**Acknowledgments**

We express our gratitude to Jane McHowat, PhD, for performing the long-chain acylcarnitine assays, to Evelyn Kanter for preparing the myocytes, and to Ava Ysaguirre for preparation of the manuscript.

**References**

23. Fabiato A: Inositol (1,4,5)-trisphosphate-induced release of \( Ca^{++} \) from the sarcoplasmic reticulum of skinned cardiac cells. Biophys J 1986:49:190a

KEY WORDS - afterdepolarizations • hypoxia • arrhythmias • \( \alpha \)-adrenergic receptors • \( \beta \)-adrenergic receptors
Influence of hypoxia on adrenergic modulation of triggered activity in isolated adult canine myocytes.
S G Priori, K A Yamada and P B Corr

Circulation. 1991;83:248-259
doi: 10.1161/01.CIR.83.1.248

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1991 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/83/1/248

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/