How To Give a Cell a Heart Attack
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Circ Res. 2006;99:111-112
doi: 10.1161/01.RES.0000234908.21102.f9

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How To Give a Cell a Heart Attack

Michael D. Stern

Cardiac ischemia is the leading killer in the developed world. It is a complex pathology, with myriad factors—electrical, chemical, metabolic, mechanical, and immunological—manifesting on scales ranging from the molecular to the whole organism. So it is not surprising that, despite decades of study, we cannot succinctly identify the injury that is central to this disorder. But, ultimately, the fate of millions of people comes down to what happens to a cardiac myocyte when it is deprived of its blood supply. It can die—by necrosis, apoptosis, or mechanical self-destruction. It isn’t always easy to decide when it is “dead.” It can lose its contractile function, its relaxation function, and/or its electrical function. It can hibernate and, if provoked by earlier nonlethal episodes of ischemia, it can “learn” to protect itself on time scales from minutes to weeks.

It would make sense, then, to study cardiac ischemia at the single-cell level. This turns out to be surprisingly difficult to do. The isolated cardiac myocyte in the laboratory is normally bathed in an ocean of moving fluid. In contrast, even under the best of conditions, the myocyte in its native habitat is secluded from its sources of nutrition. The PO2 at the myocyte surface is normally no more than 20 torr. The extracellular space, in which the cell must dispose of its metabolic waste, is tiny. In the heart, it is very easy to make a myocyte ischemic. In a petri dish, it is almost impossible. When studying cardiac myocytes on a patch-clamp apparatus, it is customary to bubble the perfusing solution with oxygen. If you use nitrogen instead, you will see no change in physiology! To make matters worse, present-day methods of measuring the electrical activity of a myocyte require that it be freely accessible from above by a long micropipette.

Some 18 years ago, Howard Silverman and I developed a crude fix for this problem.1 By establishing an upward laminar flow of a heavier-than-air inert gas (argon is the only practical choice) in a conical well, it was possible to exclude oxygen from cells in a dish at the bottom of the well, even while allowing open access above for instrumentation. The practical problems didn’t end there. It was necessary to perfuse the dish with anoxic buffer. Because all plastics are practical problems didn’t end there. It was necessary to allow open access above for instrumentation. The oxygen from cells in a dish at the bottom of the well, even if you use nitrogen instead, you will see no change in physiology! To make matters worse, present-day methods of measuring the electrical activity of a myocyte require that it be freely accessible from above by a long micropipette.

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For all these reasons, the technique was cumbersome, and only a few other intrepid investigators adopted it. But its greatest problem was that it was a model of hypoxia, not ischemia. The accumulation of metabolites could not be emulated in the “vast” volume of the 5-mm-wide, 1-mm-deep dish. While it was possible to investigate the effects of externally applied CO2, acidosis and elevated potassium—by means of simultaneous solution and gas switching whose awkwardness can be imagined—this was still not “genuine” ischemia.

In this issue of Circulation Research, Ganitkevich et al2 describe a quantum improvement in technique, which overcomes most of the above problems. Using modern nanofabrication methods, they replaced the primitive glass dish at the bottom of the argon well with a glass “chip” on which microscopic rectangular wells have been etched into a hydrophobic photore sist coating. Each 192-pL well can snugly accommodate a single ventricular myocyte. Once a myocyte has been captured on the end of a patch pipette, it is lowered into a well, and the bulk fluid in the chamber is removed, leaving the myocyte isolated in its own tiny volume of extracellular fluid trapped within the well by surface tension. In this way, Ganitkevich et al have cleverly simulated the claustrophobic environment of the myocyte in its native tissue, and the model becomes quite similar to ischemia rather than simply hypoxia.

In a further improvement over previous methods, Ganitkevich et al have cleverly noted that, instead of the slow and awkward mixing of gases in a manifold, it is possible to achieve graded hypoxia by the simple expedient of a controlled reduction of the argon flow rate, allowing a small amount of oxygen to back-diffuse against the laminar flow. The actual oxygen tension at the bottom of the well is measured with a miniature Clark electrode. The exact PO2 numbers should probably not be taken too seriously, because these electrodes are notoriously inaccurate at low tensions. But, because the admixture of oxygen is controlled by basic physical laws of transport and diffusion, the resulting PO2 at a given location should be highly reproducible as long as the

DOI: 10.1161/01.RES.0000234908.21102.f9

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flow remains laminar. With modern computer power, it would be possible to simulate the flow and calculate the true pO2 from first principles, if it were needed. This method of grading hypoxia allows dynamic alteration of the pO2 in 2 seconds, a vast improvement over the cumbersome manual gas and solution switching required previously. And, of course, because there is no perfusion, the whole issue of protecting the perfusate from oxygen contamination disappears. Instead, there is now the new option of measuring the ionic composition of the extracellular fluid during ischemia, using ion-selective microelectrodes.

There is every reason to believe that this new technique will reopen the field of single-cell ischemia research. Already, Ganitkevich et al have presented some interesting early mechanistic results. They have demonstrated the graded nature of the ATP-inhibited K+ current as a function of pO2, and the dynamic response of mitochondrial membrane potential during small reoxygenation steps. They were even able to resolve the 4.8-second kinetic delay between mitochondrial polarization and cytosolic [ATP]. Interestingly, the response of mitochondrial polarization to step-wise reoxygenation was biphasic, with a depolarizing phase at pO2 greater than 1 torr. The authors plausibly attribute this to mitochondrial permeabilization by the action of reactive oxygen species (ROS), which are produced during reoxygenation beginning even at 0.3 torr. This is an important mechanism of reperfusion injury, and also plays a signaling role in preconditioning and probably other processes.

Ganitkevich et al were able to measure the ROS involved, more-or-less. I say more-or-less because ROS were measured by means of a dichlorofluorescein derivative, whose limitations for this purpose are more than evident in this article. In most studies, fluorescence signals from DCF increase monotonically, at a rate that measures ROS, reflecting the effectively irreversible oxidation of the nonfluorescent dye-precursor. In addition, we found that DCF was progressively photo-oxidized by atmospheric oxygen, at a rate proportional to pO2 and illumination intensity. However, Ganitkevich et al found that DCF fluorescence actually fell during periods of anoxia. They attribute this to photo-reduction of the dye by cellular reducing agents, which obviously depends on species and condition. Clearly, DCF can be considered no more than a semiquantitative guide to the production of ROS. In view of the great importance of ROS in ischemia and other forms of cell injury, there is an urgent need for better methods to measure them in single cells (dye chemists take note!).

The technique of Ganitkevich et al comes much closer to a single-cell ischemia model than any previous work, but it is not yet perfect. The reported studies were performed with pH controlled by a HEPES buffer, which markedly alters cellular acid–base regulation (during both normoxia and hypoxia) compared with the physiological bicarbonate buffer system. Future studies of a bicarbonate-buffered system will require adding CO2 to the argon—easily done. However, unlike other metabolic byproducts of ischemia, volatile CO2 will not remain confined to the tiny volume of extracellular fluid. So pCO2 will have to be controlled by the investigators, at levels believed to be representative of ischemic conditions—which means a return to gas-switching. Because the 192 pL volume in which the myocyte is confined will equilibrate rapidly with the gas phase, this will offer an unparalleled opportunity to study the dynamics of the cellular pH-regulation machinery, during normoxia as well as ischemia. Another major limitation, which will not be so easy to overcome, is that isolated myocytes are mechanically unloaded, which greatly alters the energy supply/demand balance during ischemia—requiring most of the experiments to be done in the absence of extracellular glucose.

I am hopeful that this improved method will lead to a resurgence of studies of ischemia at the single cell level. This will create a demand for improvements in the basic chemical measurements on single cells. Not only the DCF method for measuring ROS, but also dye methods for measuring mitochondrial membrane potential, intracellular calcium, pH, and intracellular sodium still have significant calibration issues—as do ion-selective microelectrodes used to monitor the extracellular space. Despite the simplicity and reliability of the new method, investigators entering the field will be well-advised to keep in mind the basic physics involved in rigorous oxygen exclusion and to take note of the meticulous experimental details that are so thoroughly described in the Appendix of Ganitkevich et al.

**Source of Funding**

This work was wholly supported by the Intramural Research Program of the National Institute on Aging, National Institutes of Health.

**Disclosures**

None.

**References**


**Key Words:** ischemia □ myocyte □ hypoxia □ reperfusion □ mitochondria