Oxidative Stress Alters Specific Membrane Currents in Isolated Cardiac Myocytes

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To evaluate the effects of oxidative stress on cardiac membrane currents, single cells from frog ventricle were exposed to tert-butyl hydroperoxide (t-BHP). Incubation of these cells with 2 mM t-BHP causes a rapid depletion of cellular glutathione, followed by a more gradual increase in the contents of malondialdehyde and conjugated dienes. Effects of this rapidly evolving oxidative stress were studied on sodium, calcium, and potassium currents of isolated ventricular cells. t-BHP caused a progressive decrease in the magnitude of sodium current obtained on depolarization from a holding potential of $-85 \text{ mV}$, which was accompanied by a shift in the reversal potential toward more negative potentials. The voltage dependence of the steady-state parameters for activation and inactivation was shifted, such that in peroxide-exposed cells, there was a greater overlap of activation and inactivation parameters, which would be expected to result in an increased window current. In addition, in the presence of t-BHP, the time constant for activation was decreased at most depolarizing potentials, whereas the time constant for inactivation was increased. The resultant sodium current transients were, therefore, slower in the presence of the peroxide because of slower inactivation. Prolonged exposure of the cells to t-BHP led to a complete and selective inhibition of the Na+ current. However, even when all the Na+ current was inhibited, the K+ and Ca2+ currents remained essentially unaltered. Also, no large outward currents were observed at this stage, indicating that ATP concentration was not drastically decreased. The barrier properties of plasma membrane remained intact, as it was possible to form gigohm seals between the patch pipette and the plasma membrane of cells treated with 2-14 mM t-BHP for up to 30 minutes. These results account for the proarrhythmic effects of free radicals and oxidative stress on cardiac tissues. (Circulation Research 1990;67:535–549)

Myocardial reperfusion after an ischemic episode is increasingly recognized as a critical event that leads to ventricular fibrillation and sudden cardiac death.$^{1-5}$ The ischemia-reperfusion trauma is associated with the release of cytoplasmic enzymes and changes in tissue morphology, calcium fluxes, contractility, and mitochondrial function. Although the most serious functional consequence of reperfusion injury is ventricular arrhythmia,$^4$ the precise sequence of events that leads to arrhythmia is not well understood.

Free-radical–initiated oxidative stress has been proposed to be a major cause of tissue damage during ischemia-reperfusion episodes because 1) reperfusion results in an increase in the electron spin resonance signal, indicating an increase in free-radical generation$^{6,7}$ and 2) the damage could be markedly attenuated by the administration of antioxidants.$^{8-10}$ A variety of biological reactions generate free radicals, particularly during univalent reduction of oxygen.$^{11,12}$ Free radicals alter the structural and functional integrity of the cell by a variety of mechanisms, including lipid peroxidation,$^{13}$ sulfhydryl oxidation,$^{14}$ proteolysis,$^{15-17}$ and shearing of the nuclear material.$^{18}$ Normally, free radicals are reduced by an intricate defense system consisting of a number of enzyme systems such as superoxide dismutase, catalase, selenium-dependent and -independent glutathione peroxidase (glutathione S-transferase), and glutathione reductase.$^{19}$ A number of these reactions require glutathione, which has a highly reactive sulfhydryl group.$^{20,21}$ Whenever the antioxidative capacity of the cell is compromised, either by way of decreased levels of glutathione and defense enzymes or increased levels of oxidants, oxidative damage occurs.

Heart cells have a high intracellular oxygen content (40 $\mu$M)$^{22}$ and because of increased electron...
transport chain activity and oxygen-dependent oxidation of myoglobin, these cells are constantly being exposed to oxidizing species. The increased levels of oxidants and decreased levels of reduced glutathione (GSH) and superoxide dismutase (approximately 75% of that present in the liver) and the absence of selenium-dependent peroxidase activity would render heart cells particularly susceptible to oxidative damage. In ischemic heart, the concentrations of GSH, GSH-peroxidase, and superoxide dismutase are further decreased. On reperfusion, the ischemic cells are exposed to a burst of free radicals. Because the defense capacity of cardiac myocytes is already compromised, reperfusion could lead to substantial cell damage.

Simulation of the ischemia-reperfusion condition has been achieved in situ by temporarily ligating the left anterior descending coronary artery and by controlled perfusion of isolated Langendorff preparations. Unfortunately, these multicellular models are complicated by limited access to the cell interior and by ion accumulation in intracellular spaces. To circumvent these difficulties, isolated myocytes have been used to study electrophysiological changes on exposure of cells to free-radical-generating systems. However, little is known about the way oxidative damage specifically affects ion channels in heart. In this paper, we report the effects of oxidative stress produced by tert-butyl hydroperoxide (t-BHP) on the ionic conductances of isolated frog ventricular myocytes, in which both extracellular and intracellular spaces are accessible within reasonable time frames and an effective voltage clamp is possible.

Materials and Methods

t-BHP, cyclic AMP, tetrodotoxin (TTX), protease (type XIV), and CdCl₂ were purchased from Sigma Chemical Co., St. Louis. Collagenase (type I, No. LS4196) was purchased from Worthington Biochemical Corp., Freehold, N.J. All other chemicals were of the highest purity available.

Ventricular myocytes were isolated from Rana catesbeiana by perfusion of the heart through the sinus venosus, according to the method of Breitwieser and Szabo. The heart was removed and rinsed in perfusion solution A, containing (mM) NaCl 100, KCl 5.4, MgCl₂ 1.0, and HEPES 20, pH 7.2 (equilibrated with 100% O₂). Cells were dissociated at 30°C using 230 units/ml collagenase and 0.5–1.0 units/ml protease. Ventricular cells were obtained after digestion for 30 minutes and subsequent shaking of the dissected ventricles in perfusion solution A containing 0.2 mM CaCl₂ and 10 mM lactic acid. The cells were stored in the modified solution A in slowly rotating vials at room temperature. Under these conditions, the myocytes did not show any spontaneous contractions, and more than 75% of the cells remained viable for 10–12 hours, as tested by trypan blue exclusion.

Solutions

The composition of the standard HEPES-buffered Ringer’s solution was (mM) NaCl 90, KCl 2.5, MgCl₂ 5.0, CaCl₂ 2.5, and HEPES 20, pH 7.4. No glucose was added to Ringer's solution unless otherwise stated. The 50% sodium solutions were prepared by replacing 45 mM NaCl by choline chloride or N-methylglucamine chloride. t-BHP solutions were prepared fresh daily. The standard internal solution used to fill the patch pipette contained (mM) K⁺ aspartate 60, KCl 50, EGTA 1, and Mg-ATP 1, pH 7.4 adjusted with KOH. In some experiments, the internal solution was supplemented with 10 mM NaCl. Cadmium chloride (0.5 mM) was routinely added in the solutions used to record Na⁺ current.

Estimation of Biochemical Parameters

Glutathione content of the myocytes was determined according to the method of Beutler. Briefly, the myocytes were pelleted in the modified perfusion solution A at 1,000g for 5 minutes and then resuspended in 1 ml Ringer’s solution with and without t-BHP. After the indicated incubation periods, the proteins were precipitated by metaphosphoric acid. The precipitate was removed by centrifugation, the supernatant was neutralized with 0.3 M Na₂CO₃, and an aliquot was used for GSH determination using 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB).

For malonaldehyde determinations, the cells were pelleted, resuspended in the Ringer’s solution with and without additives, and washed with normal Ringer’s solution. Malonaldehyde was determined using the thiobarbituric acid method by measuring the absorbance at 535 nm. For conjugated diene determinations, a 1-ml aliquot of cell suspension (100 mg protein/ml) was incubated with or without oxidants in the Ringer’s solution. At different time intervals, the reaction was stopped with 5 ml of 2:1 chloroform/methanol mixture. To facilitate extraction of charged lipids and phospholipids, 2 ml of 0.8% NaCl solution in water was added to the mixture, which was then filtered to remove particulates. The filtrate was allowed to separate into two phases (either by standing for 1 hour at 4°C or by centrifugation), and the lower organic layer was removed and evaporated under N₂ at 45°C. The dried lipid residue was then resuspended in 1 ml cyclohexane. In the absorbance spectrum of such a mixture of lipids, measured against a cyclohexane blank, the conjugated dienes stand out as a shoulder around 230–235 nm. The absorption peak for the conjugated dienes was calculated as the difference spectrum of partially peroxidized lipids relative to an equal quantity of the nonperoxidized control lipids. The approximate concentration of hydroperoxides produced was calculated using a molar extinction coefficient of 2.52×10⁴ M⁻¹ cm⁻¹. All absorbance measurements were made using a Gilford Response spectrophotometer (Ciba Corning Diagnostic Co., Oberlin, Ohio).
Electrophysiology

To study the membrane currents, the isolated ventricular cells were layered on a coverslip and superfused with extracellular solution at a rate of 0.5–1.0 ml/min. The whole-cell recording configuration of the patch-clamp technique was used. All experiments were done at room temperature (25°±1.0°C). Patch pipettes were made of square bore glass (Glass Co. of America, Millvile, N.J.) by a three-stage pull on a Flaming-Brown horizontal puller (model P80/PC, Sutter Instrument Co., San Rafael, Calif.). When filled with the standard internal solutions, the pipettes had resistances between 2 and 5 MΩ. The junction potentials of the pipette filled with the internal solution in the HEPES/Ringer’s solution were −2 to −4 mV. The data are not compensated for this effect. Ag/AgCl electrodes were used to establish contact with the pipette and the bathing solution.

Voltage-clamp pulses were designed on an IBM-compatible desktop computer and delivered to the bathing solution through an arbitrary waveform generator (model 75, Wavetek San Diego Inc., San Diego). Membrane currents passing through the pipette were recorded by a current-to-voltage converter consisting of an operational amplifier and a 100-MΩ feedback resistor. Currents were filtered with a low-pass 5-kHz Bessel filter (model 902-LPF, Frequency Devices Inc., Haverhill, Mass.) and recorded on FM magnetic tape (model 4D, Racal Recorders, Inc., Fullerton, Calif.) at 3½ IPS, corresponding to a bandwidth of 1,250 Hz. The data were either retracted on an X-Y plotter for analysis or digitized at 12-bit resolution for computer-assisted analysis.

The total series resistance was compensated before recording the membrane currents, using established techniques. The series resistance before compensation was estimated from the cell capacitance and the time constant for the decay of the capacitative transient. The initial capacitative current transient for small voltage displacements (5 mV) from rest (−85 mV) was well approximated by a single exponential (400–600 μsec) and was further reduced by series resistance compensation (to 100–150 μsec). The falling phase of the capacitative transient as well as the tail currents were smooth and without “notches,” indicating that the cells were adequately voltage clamped. Typically, the uncompensated series resistance while recording in the whole-cell mode was twice that of the electrode resistance in Ringer’s solution. Experiments in which abrupt decrease or increase in the series resistance was observed during the course of the experiment were discarded. A membrane capacitance of 110±21.5 pF (n=21) was determined and is similar to the values reported by earlier investigators.

Results

Effect of tert-Butyl Hydroperoxide on the Biochemical Parameters of Oxidative Stress

Frog cardiac cells were found to contain 2.8±0.5 μmol GSH per gram (wet weight) of cells (Table 1). This is about twice the amount of GSH reported for the mammalian heart (1.2 μmol/g), but is significantly lower than the GSH levels in mammalian liver (5–6 μmol/g). The concentration of cellular GSH in freshly isolated cells was comparable to that found in cells incubated for more than 5 hours at room temperature in perfusion solution A (containing 10 mM lactate and saturated with oxygen). Thus, storage alone does not cause significant oxidative stress. Incubation of isolated cells with 2 mM t-BHP in Ringer’s solution resulted in an almost complete (>95%) oxidation of GSH in 30–60 seconds, which is at the limit of the time resolution of these experiments. It is possible that cells incubated with t-BHP for more than 1 minute still retained subnanomolar quantities of GSH, which would be below the detection limit of the spectrophotometric method of estimation used.

Incubation of isolated frog myocytes with 2 mM t-BHP also caused a marked increase in the level of malonaldehyde, which tended to reach a plateau after 40 minutes, as shown in Table 1. Incubation with normal Ringer’s solution for up to 60 minutes, in contrast, did not alter malonaldehyde levels.

Lipids extracted from cells incubated with normal Ringer’s solution absorbed broadly between 210 and 280 nm. Lipids extracted from cells incubated with 2 mM t-BHP at room temperature for varying periods of time, however, exhibited a distinct shoulder between 230 and 260 nm (data not shown). The time course of the conjugated diene formation, measured by the increased absorbance at 238 nm, is shown in

Table 1. Effects of 2 mM tert-Butyl Hydroperoxide on Biochemical Parameters of Oxidative Stress on Isolated Frog Cardiac Myocytes

<table>
<thead>
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<th>Parameter</th>
<th>Time of incubation</th>
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<td>Malonaldehyde (absorbance at 535 nm)</td>
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<tr>
<td>Conjugated dienes (μM)</td>
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</tr>
<tr>
<td>Glutathione (μmol/g wet wt)</td>
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</tr>
</tbody>
</table>

Values are mean±SEM (n=3).

*No independent zero time values are present because the spectra of extracted lipids from tert-butyl hydroperoxide-treated cells were subtracted from the spectra of lipids from untreated cells.

†No glutathione was detected after 1 minute of incubation with peroxide.
Table 1. Unlike malonaldehyde formation, the increase in the conjugated diene continued for at least 60 minutes of incubation with the peroxide.

Effect of tert-Butyl Hydroperoxide on Membrane Currents

From the above experiments, it is evident that 2 mM t-BHP completely depletes cellular GSH, increases malonaldehyde, and causes lipid peroxidation. This concentration of t-BHP was chosen for all subsequent electrophysiological experiments, which examine changes in ionic currents resulting from progressive oxidative stress in the presence of a compromised cellular antioxidant capacity. The three main ionic currents studied were the fast inward sodium current (I\textsubscript{Na}), the slow inward calcium current (I\textsubscript{Ca}), and the background potassium current (I\textsubscript{Kl}).

Fast inward sodium current. The effect of t-BHP on I\textsubscript{Na} was studied using two experimental protocols. In the first protocol ("patched-cell protocol"), gigohm seals were made between the cell and the patch pipette, the patch was ruptured, and after an equilibration time of 3 minutes, the control data on the sodium channel activity of the cells were collected. Then the cells were superfused with t-BHP, and current changes were studied as a function of time. In the second experimental protocol ("incubated-cell protocol"), the cells were layered on the bottom of the recording chamber, equilibrated with normal Ringer's solution, and superfused with 2 mM t-BHP for varying lengths of time. While the cells were in t-BHP, seals were formed with the cells, and the patches were ruptured to record I\textsubscript{Na}. For this set of experiments, the control values were collected from a similar population of cells that was incubated for the same amount of time in normal Ringer's solution. The results from each of these protocols are described separately.

Patch-clamp protocol. The top panel of Figure 1 shows current traces obtained in response to a series of depolarizing steps (150 msec each) obtained from a holding potential of −85 mV. The current traces are similar to I\textsubscript{Na} obtained by whole-cell patch-clamp technique in a number of isolated cardiac myocytes.38–40 The peak inward current was measured as the maximum inward current obtained on depolarization relative to the current obtained at the end of the pulse. This procedure, which is similar to that used by other investigators,39 eliminates not only the leak current but also the steady-state component of the inward current and allows us to estimate only the fast inward I\textsubscript{Na}. The middle panel of Figure 1 shows the peak I\textsubscript{Na} and voltage (I-V) relation at different [Na]. The I-V curves are essentially symmetrical in shape with the peak I\textsubscript{Na} (1.6±0.4 nA, n=20) at [Na]=90 mM observed between 0 and 5 mV and a threshold around −50 mV. The I-V relation was essentially linear over the potential range from 0 to 50 mV and had an average slope conductance of 29±7.7 nS (n=20). The sodium currents shown in Figure 1 (top panel) and in all subsequent experiments were recorded in the presence of 0.5 mM Cd\textsuperscript{2+} to block I\textsubscript{Ca}. In a set of two preliminary experiments, this concentration of Cd\textsuperscript{2+} had no significant effect on I\textsubscript{Na}. Clark and Giles38 have also used 0.2-0.5 mM Cd\textsuperscript{2+} to pharmacologically isolate the frog atrial I\textsubscript{Na} from I\textsubscript{Ca}. Thus, the amphibian cardiac I\textsubscript{Na} seems to be relatively insensitive to Cd\textsuperscript{2+}, which effectively blocks half the mammalian cardiac I\textsubscript{Na} at a concentration of 0.18 mM and more than 90% I\textsubscript{Na} at 0.4 mM.41

The shape of the I-V relation shown in the middle panel of Figure 1 indicates that the cell was adequately voltage clamped.42 This is corroborated by the absence of "threshold phenomenon" near the voltage region of I\textsubscript{Na} activation and a gradual increase in the negative voltage limb of the I-V curve over a 30-mV range. Also, there is no change in either the potential of the peak current or threshold on varying [Na]. To further test the adequacy of the voltage clamp, peak I\textsubscript{Na} obtained on depolarization from a holding potential of −85 mV was compared with the current obtained in the presence of 0.75 and 1.0 µM TTX. The bottom panel of Figure 1 shows the I-V relation in the presence and absence of TTX. TTX reduced peak I\textsubscript{Na} but had no effect on the threshold of the fast inward current, the reversal potential (E\textsubscript{rev}), or the potential of peak I\textsubscript{Na}. Taken together, these observations indicate that the cells were adequately voltage clamped under the experimental conditions used.

Superfusion with 2 mM t-BHP initially (1–2 minutes) had no effect on I\textsubscript{Na} elicited on depolarization from a holding potential of −85 mV. Further superfusion caused a decrease in the peak I\textsubscript{Na}. Figure 2 shows current traces elicited on depolarization from a holding potential of −85 mV before (top panel) and after (middle panel) 10 minutes of perfusion with t-BHP, as well as the I-V relations for the current (bottom panel) before and after superfusion with 2 mM t-BHP for 6 and 10 minutes. The peroxide caused a sustained decrease in the peak I\textsubscript{Na}; there was a 40±5% decrease in I\textsubscript{Na} measured on depolarization to −5 mV in 15 minutes (n=12). This decrease was accompanied by a shift in E\textsubscript{rev} to more negative potentials. Also, as can be seen from the traces in the top and middle panels of Figure 2, the currents obtained in the presence of t-BHP appear "flattened," indicating that the peroxide alters both the rate of current activation and inactivation. Each of these effects of t-BHP was studied in greater detail.

Sodium currents are sensitive to both [Na] and [Na]\textsubscript{i}. For the experiments considered here, 10 mM Na\textsuperscript{+} was included in the pipette solution to prevent the sodium loss through the patch pipette and to stabilize [Na] near 10 mM. Although the use of low-resistance pipettes (2–5 MΩ) should lead to rapid equilibration of the cell interior with the pipette solution, the exact [Na] may be difficult to estimate as these cells are long and slender, and therefore, the [Na] at parts distal to the patch pipette may be different from the areas that are in closer contact with the pipette solution. Nevertheless, our results
FIGURE 1. Effect of varying [Na]₀ on sodium current of frog ventricular cell. Top panel: Current traces elicited by a series of voltage-clamp steps from a holding potential of -85 mV in Ringer's solution containing 90 mM [Na]₀. Depolarizing potentials were -30 (1), -20 (2), -10 (3), 20 (4), 30 (5), 40 (6), 50 (7), and 60 (8) mV. Calcium currents were blocked by 0.5 mM Cd²⁺. Middle panel: Current-voltage relations obtained at 90 (○), 60 (▲), and 45 (●) mM [Na]₀. Peak current at each membrane potential was measured relative to the current obtained at the end of the depolarizing pulse. Curves were drawn by eye. Bottom panel: Current-voltage relations in the absence (○) and presence (●) of 0.75 and 1 (▲) μM tetrodotoxin. Peak inward current was measured as above. Holding potential was -85 mV.

Indicate that E_rev depends on [Na]₀ when [Na]₀ is varied from 30 to 90 mM (Figure 3, upper panel), in a manner that was consistent with E_rev calculated using the Nernst equation with [Na]₀=10 mM (Figure 3, upper panel, solid line). Thus, the technique used for estimating E_rev is valid and closely reflects changes in the driving force of I_Na. The lower panel of Figure 3 shows the time course of the changes in E_rev of I_Na on exposure to t-BHP. In a set of control experiments (Figure 3, lower panel, upper trace), no significant change in E_rev was found on superfusion with normal Ringer's solution for up to 20 minutes. On addition of t-BHP, however, E_rev shifted to more negative values, indicating progressive sodium loading.
To study the effect of t-BHP on the voltage dependence of the sodium channel, a conventional double-pulse protocol was used. The membrane potential was held at –85 mV for 500 msec followed by depolarizing prepulses varying from –90 to –30 mV and finally a depolarizing pulse to +10 mV for 150 msec. The duration of the prepulse was 500 msec, as some investigators have reported a very slow inactivating component of the cardiac INa. The ratio of the amplitude of the inward current with and without the prepulse was plotted against the prepulse potential, and the inactivation parameter (h) was calculated at each potential. The data could be fitted by the following equation:

\[ h_a = \frac{1}{1 + \exp(V_h + V)/s} \]  

where \( V_h \) is the half inactivating voltage, and \( s \) is the slope factor. There was a shift in the steady-state inactivation toward hyperpolarizing direction soon after the patch electrode gained access to the cell interior. A similar shift has been observed by previous investigators, but the reason for such a shift is not known. Typically, the shift was 3–5 mV toward negative potentials within an observation period of 5 minutes. Therefore, the data reported for “control” values are after the first 5 minutes of stabilization period.

For seven ventricular myocytes in normal Ringer’s solution, the calculated \( V_h \) was –52.43 ± 1.80 mV,
and the slope factor was 6.64±0.74 mV. Steady-state activation (m) was calculated from the peak current elicited on depolarization from a holding potential of −85 mV. Curves for m were fitted to an equation similar to h with a negative exponential term. The fit yielded a slope factor of 9.44±0.89 mV and V₉ of −22.53±0.93 mV. The solid curves shown in the upper panel of Figure 4 represent a theoretical single Boltzmann distribution calculated using a sum of least-squares algorithm, indicating that both m and h could be adequately described as a single exponential. The m and h curves shown in Figure 4 overlap with a potential of maximum overlap near −41 mV.

Application of 2 mM t-BHP shifted the h curve to the right on the voltage axis; V₉ was shifted from −54.4 to −48 mV (Figure 4, upper panel, dotted line) with no significant change in the slope factor. This change was evident 1 minute after t-BHP addition and was accompanied by only a slight change in the m curve. Therefore, at this time there was a larger overlap of m and h curves, with a shift in the voltage of peak overlap from −41 to −38 mV. We consistently observe such a shift in V₉ of the h curve, and for a set of eight cells, an average shift of 6±1.5 mV was observed on superfusion of the cells with t-BHP for 5 minutes. Further superfusion of the cells with 2 mM t-BHP caused little change in h, but the m versus prepulse potential curve shifted to more negative values of the membrane potential (Figure 4, upper panel, dotted line). There was no change in maximal sodium conductance (Gₓₙₓ₉₉ₙ) per se on exposure to t-BHP. The half activation voltage shifted to more negative values. The data shown in the upper panel of Figure 4 were obtained from the same cell, which had been perfused with 2 mM t-BHP for 10 minutes. The V₉ value for the m curve was −30.1 mV, whereas the slope factor was 11.48 mV. A mean change in the slope factor of 3.4±0.6 mV and a shift of 5.7±0.9 mV were observed (n=11) for a perfusion time of 10 minutes. In the upper panel of Figure 4, the region of overlap of m and h that extended from −50 to 5 mV before the application of t-BHP was increased in the presence of the peroxide and extended from −70 to 10 mV. Thus, the net effect of this change would be an increase in the

**Figure 3.** Effect of tert-butyl hydroperoxide (t-BHP) on the reversal potential of sodium current (Iₓₙ₉). Upper panel: Iₓₙ₉ reversal potentials obtained on depolarizing from a holding potential of −85 mV. Reversal potentials were calculated at different [Na]ₒ from the current-voltage relations shown in Figure 1 and from two other cells stimulated by a similar pulse protocol at different [Na]ₒ. Values shown are mean (n=3); vertical bars represent SEM. Solid line was drawn using the Nernst equation, with [Na]ₒ=10 mM. Lower panel: Changes in Iₓₙ₉ reversal potential plotted as a function of time in normal Ringer’s solution (a, ○) and after exposure to 2 mM t-BHP (b, ●). Values are mean (n=5); vertical bars represent SEM. Na⁺ (10 mM) was included in the patch pipette.
window current. The magnitude of this current is proportional to $m_a^3 h_a$. The lower panel of Figure 4 shows the calculated window in the same cell as used to generate the data shown in the upper panel before and after a 10-minute exposure to t-BHP. The calculated window increased from $5.5 \times 10^{-2}$ to $7 \times 10^{-3}$. In the absence of the peroxide, the window has a peak at $-25$ mV. In the presence of the peroxide, the peak shifts to $-35$ mV.

To further characterize the effects of t-BHP on the characteristics of the channel, the voltage dependence of activation and inactivation kinetics was studied. Kinetic parameters were calculated using the following modified form of the Hodgkin and Huxley equation:

$$I(t) = \bar{I}\{1 - \exp[-(t - \delta)/\tau_m]\} \{\exp[-(t - \delta)/\tau_i] + b\}$$

at time $t$, $\bar{I}$ is the maximal sodium current, where $I$ is the total sodium current, and $\tau_m$ and $\tau_i$ are time constants for activation and inactivation, respectively. This equation assumes that initially, the steady-state parameter is zero, and $b$ represents the steady-state sodium conductance. An additional factor, $\delta$, has been included, which is the time delay and allows for the slowing of the voltage-clamp steps due to uncompensated series resistance. Data were fitted to Equation 2 using a multi-iterative nonlinear regression program using Marquardt algorithms. All standard errors and correlations calculated by this method are asymptotic and approximate. Both the rate of activation and inactivation were found to be adequately described by single exponentials as shown in Equation 2. In some studies (see Reference 38), inactivation has been described as a two expo-
nential process. On fitting our inactivation data with two exponentials, the residual sum of squares decreased slightly, but the error structure became nonrandom, and the standard errors on the calculated parameters increased. In a recent study on the frog ventricular $I_{Na}$, a single exponential equation was also found to adequately describe the time course of $I_{Na}$ inactivation. Figure 5 shows the initial phase of experimentally obtained $I_{Na}$ as discrete data points, before (lower trace) and after (upper trace) 10 minutes of superfusion with 2 mM t-BHP. These data points are overlaid with solid curves calculated using Equation 2. The parameters used are shown in Table 2. From this figure it is evident that a single exponential equation (Equation 2) adequately described the time course of $I_{Na}$ inactivation. Superfusion of the cardiac myocyte with 2 mM t-BHP affected both $\tau_{m}$ and $\tau_{h}$ (Figure 6, upper and lower panels). $\tau_{h}$ was increased by the peroxide at all depolarizing potentials except at $-25$ mV, in which case $\tau_{m}$ was decreased. However, $\tau_{m}$ was decreased and the effect was greater at negative potentials, whereas at more positive potentials, for example, 30 mV, $\tau_{m}$ was unaffected. The net result of these effects would be Na$^+$ currents inactivating relatively slowly in the presence of t-BHP. Although the changes appear to be slight, they account for the “flattening” of the inward current transients on exposure to the peroxide. The values of the two kinetic parameters in the absence and presence of the peroxide are given in Table 2. The peroxide did not have a significant effect on the steady-state component of the current elicited by the pulse protocol used. Thus, the values of the steady-state component b (shown in Table 2), whatever its source of origin, remained unaltered on superfusion with t-BHP.

**Incubated-cell protocol.** Because the whole-cell variant of the patch-clamp is an invasive technique and the cell membrane is ruptured to provide electrical continuity with the cell, it is likely that the normal “physiology” of the cell is altered by the method of study. In particular, the contents of the large patch pipette exchange with the cytoplasm and the defense capacity of the cell against oxidants may be altered because of loss of the intracellular antioxidants. To circumvent these problems, the incubated-cell protocol was used, in which $I_{Na}$ was recorded from cells preincubated with 2 mM t-BHP for varying lengths of time. The results from 13 such experiments are shown in Table 3. These results are similar to those obtained with the patch-clamp protocol, except that the alterations were more severe in this case as the cells could be incubated for longer periods of time with minimal alterations in their intracellular environment.

![Figure 5](image)

**Figure 5.** Effect of tert-butyl hydroperoxide (t-BHP) on the kinetics of sodium current. Current traces from a cell in normal Ringer’s solution (lower trace) and the same cell exposed to 2 mM t-BHP for 10 minutes. Continuous lines were drawn according to Equation 2 (see “Results”) using the parameters shown in Table 2, whereas the data are plotted as discrete points. The voltage-clamp protocol is shown at the top of the figure.

### Table 2. Effect of 2 mM tert-Butyl Hydroperoxide on Kinetic Parameters for Sodium Current in Frog Ventricular Cells

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<tr>
<th>Membrane potential (mV)</th>
<th>Maximal current (nA)</th>
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<th>Inactivation time constant (msec)</th>
<th>Steady-state sodium current</th>
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<td>-0.602±0.014</td>
<td>0.116±0.004</td>
<td>0.732±0.001</td>
<td>0.156±0.003</td>
</tr>
<tr>
<td>Plus 2 mM tert-butyl hydroperoxide (6 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-25</td>
<td>-0.203±0.003</td>
<td>0.457±0.004</td>
<td>25.101±0.619</td>
<td>1.161±0.028</td>
</tr>
<tr>
<td>-15</td>
<td>-0.699±0.013</td>
<td>0.352±0.001</td>
<td>14.917±0.628</td>
<td>0.382±0.024</td>
</tr>
<tr>
<td>0</td>
<td>-0.885±0.009</td>
<td>0.216±0.001</td>
<td>3.582±0.091</td>
<td>0.106±0.007</td>
</tr>
<tr>
<td>15</td>
<td>-0.754±0.006</td>
<td>0.147±0.004</td>
<td>2.504±0.023</td>
<td>0.141±0.001</td>
</tr>
<tr>
<td>25</td>
<td>-0.525±0.005</td>
<td>0.121±0.003</td>
<td>1.658±0.075</td>
<td>0.096±0.001</td>
</tr>
<tr>
<td>35</td>
<td>-0.251±0.007</td>
<td>0.099±0.001</td>
<td>1.089±0.029</td>
<td>0.032±0.002</td>
</tr>
</tbody>
</table>

Values are mean±SEM and were calculated using Equation 2 (see “Results”). All observations were made at room temperature. The inward current was obtained by depolarization for 150 msec to the indicated potentials from a holding potential of $-85$ mV.
milieu. A gradual time-dependent decrease in the peak $I_{Na}$ was accompanied by a shift in $E_{rev}$, which in some cases was as low as 15 mV, indicating that the cells were drastically Na⁺ loaded. For a set of five cells that were incubated for 15–17 minutes, the $h_{Na}$ value at −45 mV increased from a control value of 0.23±0.08 to 0.43±0.12, indicating that shifts in the inactivation produced were real and that the dialysis of the cytoplasm does not affect the response of the cell to oxidative stress. At longer incubations (>20 minutes), no $I_{Na}$ was observed at any depolarizing potential ranging from −45 to 65, from a holding potential of −85 mV. None of the effects of t-BHP could be reversed by washing the cell with normal Ringer’s solution for up to 30 minutes.

More significant, perhaps, is our observation that we could form gigohm seals with the cell membrane even when all the Na⁺ channel activity of the cell was irreversibly lost. Within our experimental observation period of 30 minutes, we could not find any evidence for large and nonspecific membrane leakage, even when the peroxide concentration was increased from 2 to 14 mM. This is to say the

**TABLE 3. Effect of Incubation With 2 mM tert-Butyl Hydroperoxide on Sodium Current of Frog Cardiac Cells**

<table>
<thead>
<tr>
<th>Cell No.</th>
<th>Incubation time (min)</th>
<th>Sodium current (nA)</th>
<th>Reversal potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>21</td>
<td>0.23</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>21</td>
<td>0.15</td>
<td>8</td>
</tr>
<tr>
<td>10</td>
<td>11</td>
<td>0.60</td>
<td>46</td>
</tr>
<tr>
<td>12</td>
<td>18</td>
<td>0.00</td>
<td>...</td>
</tr>
<tr>
<td>13</td>
<td>15</td>
<td>0.11</td>
<td>...</td>
</tr>
<tr>
<td>14</td>
<td>15</td>
<td>0.00</td>
<td>...</td>
</tr>
<tr>
<td>16</td>
<td>17</td>
<td>1.14</td>
<td>45</td>
</tr>
<tr>
<td>17</td>
<td>27</td>
<td>0.00</td>
<td>...</td>
</tr>
<tr>
<td>18</td>
<td>16</td>
<td>0.67</td>
<td>38</td>
</tr>
<tr>
<td>19</td>
<td>30</td>
<td>0.00</td>
<td>...</td>
</tr>
<tr>
<td>20</td>
<td>24</td>
<td>0.15</td>
<td>15</td>
</tr>
<tr>
<td>21</td>
<td>16</td>
<td>0.50</td>
<td>45</td>
</tr>
<tr>
<td>23</td>
<td>18</td>
<td>1.10</td>
<td>38</td>
</tr>
</tbody>
</table>

Cells were plated and equilibrated in normal Ringer’s solution for 5 minutes and were then superfused with tert-butyl hydroperoxide containing normal Ringer’s solution at a rate of 0.5 ml/min for the indicated time intervals, after which gigohm seals were made, and the cell membrane was ruptured to record sodium current.

**FIGURE 6. Effect of tert-butyl hydroperoxide (t-BHP) on the kinetics of sodium current.** Time constants for activation ($\tau_{m}$) (upper panel) and inactivation ($\tau_{h}$) (lower panel), plotted as a function of membrane potential in normal Ringer’s solution (○) and after 10 minutes of superfusion with 2 mM t-BHP (●). Values of $\tau_{m}$ and $\tau_{h}$ were calculated from Equation 2 (see “Results”). Continuous lines were drawn by eye; values of $\tau_{h}$ are plotted in a logarithmic scale.
membrane resistance ($r_m = 83\pm 9.5$ MΩ) remained within the range of values observed before incubation with t-BHP (79±12 MΩ). Cells in which the sodium channel activity was completely abolished still displayed normal K+ and Ca2+ conductance (see below). Moreover, these cells did not exhibit large outward potassium currents, which would be expected for drastically reduced ATP levels,47 suggesting that it is not a lack of ATP that produced the observed sodium load.

**Effects on the potassium currents.** Frog ventricular myocytes have a time-independent, inwardly rectifying background $I_{K1}$.48 Figure 7 shows the I-V relation for $I_{K1}$, determined for one cell by applying 250-msec test pulses from a holding potential of −85 mV. Ten micromolar TTX and 0.5 mM Cd2+ were used to block $I_{Na}$ and $I_{K1}$, respectively. The control $I_{K1}$-V relation exhibiting typical inward rectification is shown by open circles. These currents were totally inhibited by the addition of 5 mM Ba2+. Exposure of the cell to 2 mM t-BHP had no significant effect on the $I_{K1}$-V relation, when either the patched-cell ($n=10$) or the incubated-cell ($n=7$) protocol was used.

**Effects on the calcium currents.** The slow inward $I_{Ca}$ was examined using 450-msec depolarizing pulses from a holding potential of −45 mV in normal Ringer’s solution having 10 μM TTX to block $I_{Na}$. The upper panel of Figure 8 shows the pulse protocol used to obtain the results shown in the lower panels (a–d). $I_{Ca}$ was found to be completely inhibited by 0.5 mM Cd2+ and had a peak amplitude in the range of 100–125 pA, which increased to 450–500 pA when 50 μM cyclic AMP was included in the pipette.

Figure 8a shows $I_{Ca}$ obtained from a normal ventricular cell after 1 minute of rupturing the patch. The current was 100 pA, which increased to 450 pA as the cyclic AMP included in the patch pipette diffused to the cell interior. The observation that maximal summation of the current was evident in less than 1 minute indicates that perfusion of the cell by the pipette solution was rapid. This current, however, decayed (“rundown”) with an approximate half-life of 10 minutes. Figure 8b shows current traces from the same cell after 14 minutes of perfusion with normal Ringer’s solution. A marked decrease in the current magnitude is evident. In a set of seven cells, when perfused with 2 mM t-BHP containing Ringer’s solution soon after the seal was ruptured, the rate of rundown slowed (data not shown). In no case was there an increase in $I_{Ca}$ compared with the control values of the current before addition of t-BHP. It is possible that the effect of the peroxide, if slow, may have been masked by rundown. To rule out this possibility, the incubated-cell protocol was used. In this set of experiments, the cells were preincubated in 2 mM t-BHP for varying lengths of time before the seal was ruptured. Figure 8c shows $I_{Ca}$ for a cell incubated in 2 mM t-BHP for 17 minutes. The magnitude of $I_{Ca}$ obtained was comparable to that recorded from untreated cells; however, the rate of current rundown is faster than that observed for untreated cells. This is illustrated in Figure 8d, which shows that just 4 minutes after the patch was ruptured, the current was drastically reduced. The magnitude of $I_{Ca}$ recorded from five different cells was independent of the time of incubation (ranging from 10 to 35 minutes) with t-BHP. Furthermore, the peroxide had no effect on the kinetics of the current (data not shown). These observations indicate that there is no direct effect of t-BHP on the calcium channel and that the secondary effect on rundown did not mask an inhibitory effect of the peroxide on $I_{Ca}$.

**Discussion**

Exposing cells to free-radical–generating systems is the most direct way to study the physiological effects of oxidative stress. However, this simple approach has serious limitations in that oxidative damage could be counteracted by the native antioxidant capacity of the cell normally provided by free-radical–scavenging systems and high intracellular levels of GSH. We have used t-BHP, a substrate of glutathione peroxidase,49 to deplete the antioxidative capacity of the cell. Moreover, because t-BHP concentrations significantly higher than that of cellular GSH produce t-butoxy (RO) or t-butylperoxy (ROO) radicals,50,51 higher concentrations of t-BHP could be used to
initiate and sustain free-radical chain reactions. Depletion of cellular GSH at lower t-BHP concentrations and the initiation of oxidative damage at higher t-BHP concentrations are significant aspects of this work and provide a potentially useful model for the study of oxidative damage in cardiac cells.

Our results show that in dissociated cardiac myocytes, 2 mM t-BHP depletes more than 95% of cellular GSH within 1 minute. The efficacy of the process is corroborated by the fact that perfusion of rat heart with 100 μM t-BHP can oxidize more than 80% of cellular GSH in 5 minutes.25 In isolated myocytes, the absence of an unstirred extracellular space coupled with high concentration of t-BHP (2 mM) further accelerates GSH oxidation. As the cellular pool of GSH is depleted, excess t-BHP would initiate lipid peroxidation,52,53 protein sulphydryl oxidation, protein mixed disulfide formation with glutathione, and other oxidative processes. That oxidative stress is induced by t-BHP is evidenced in our experiments by a significant increase in the concentration of conjugated dienes and malonaldehyde. Thus, it seems reasonable to suppose that the electrophysiological changes observed in isolated frog ventricular cells during perfusion with t-BHP reflect the effects of progressive oxidative damage and lipid peroxidation.

The use of frog ventricular myocytes allowed us to study the whole-cell behavior of sodium currents in quantitative detail. Amphibian cardiac myocytes have poorly developed sarcoplasmic reticulum and practically no T tubule system, resulting in rather simple cable properties.40,54 Thus, it was possible to adequately voltage clamp these cells and to obtain reliable sodium transients, which is difficult to do with mammalian cardiac cells because of the complex ultrastructure and the high density of sodium channels, which prevent adequate voltage control at short times when using single microelectrodes.55 Frog ventricular I Na recorded by our experimental procedure is qualitatively similar to that described recently by Seyama and Yamaoka,40 although in our study, the time course of the currents is faster and the threshold potential and V h are shifted to more positive potentials. These differences probably are due to a higher temperature used in our study (25°C) as compared with the earlier report in which a temperature of 4°C was used.40 Although it is not implied that the metabolic pathways of amphibian and mammalian hearts are identical, it is quite likely that the mammalian cardiac myocytes respond similarly to oxidative stress, particularly the sodium channel, as this channel is a fairly well-conserved protein,56 and the electrophysiological properties of sodium channels of different species are similar.

The observations that frog ventricular I Na is affected by t-BHP without significant effects on the other major ionic membrane currents is an important new aspect of this study. Both the magnitude and the gating properties of the current are affected on exposure to the peroxide. The sodium channel has
been found to be sensitive to oxidants such as chloramine T,57 halazole, and hypochlorous acid, which drastically inhibit inactivation,58 whereas periodate, iodate, and hydrogen peroxide produce a parallel shift in the h, curve,58 with little or no effect on activation. However, the sensitivity of this channel to free-radical–generating systems and cellular oxidative stress, especially in relation to other ionic currents, has never been studied. In earlier studies on mammalian cardiac action potentials, the upstroke velocity \( V_{\text{max}} \) was found to decrease to 10% of the control values on exposure to 300 \( \mu \text{M} \) \( t\)-BHP for 30 minutes,59 and to 80% of the control on exposure to xanthisne-xanthine oxidase for 20–30 minutes,60 suggesting an inhibition of INa. Prolonged exposure of these cells to free-radical–generating systems led to depolarization of the cell membrane and a total loss of excitability.32,59 Our results, although obtained for the amphibian heart, are consistent with these earlier studies and suggest that direct inhibition of the cardiac INa could be responsible for the decrease in the upstroke velocity of the action potential. A decrease in INa on prolonged exposure to \( t\)-BHP was found when either the patched-cell protocol (Figure 2) or the incubated-cell protocol (Table 3) was used. Also, in cells incubated in \( t\)-BHP for more than 20 minutes, no INa was apparent, which could account for the loss of excitability of the cardiac cells on exposure to oxidative stress. The magnitude of INa in cardiac cells is very large as compared with other ionic currents; in bullfrog atria, peak INa is 30 times larger than peak INa.48 Therefore, even small changes in INa will drastically affect the action potential and the excitability of the cardiac cell. Moreover, an inhibition of the INa would lead to a reduced conduction velocity, which would increase the susceptibility of the cardiac tissue to reentrant arrhythmias.

At short times, exposure of cardiac cells to \( t\)-BHP alters the voltage dependence of the steady-state m, and h, parameters. In the absence of \( t\)-BHP, there was a distinct region of overlap of the m, and h, curves (Figure 4, upper panel), giving rise to a window current that extends from –50 to –5 mV (Figure 4, lower panel). Thus, at these potentials, a net steady-state inward INa should be present. However, the magnitude of this current is small. The peak m,-h, at –25 mV corresponds to \( 5.5 \times 10^{-4} \). When this value is used for the window and a value of 50 nS is used for \( G_{\text{Na},\text{max}} \), the maximal window current at –25 mV is calculated to be approximately equal to 2.2 pA \( (5.5 \times 10^{-4} \times 50 \text{nS} \times 80 \text{mV}) \), which is very small and would not contribute significantly to the repolarization phase of the action potential. Such a low window current is in excellent agreement with the value of 2.3 pA reported by Clark and Giles58 for frog atrial myocytes. At short times after the application of \( t\)-BHP, the peak of the window shifts to –35 mV, and its magnitude increases to \( 7 \times 10^{-3} \) (Figure 4, lower panel). At this stage, \( G_{\text{Na},\text{max}} \) is not altered and the window current induced by \( t\)-BHP is calculated to be near 31 pA \( (7 \times 10^{-3} \times 50 \times 90) \), which is a substan-
tial current. A steady-state inward current of this magnitude would depolarize the membrane (of approximately 100 pF capacitance) at a rate of 300 mV/sec. However, the repolarization rate during the action potential plateau depends on both sodium and potassium conductances. If the potassium conductance remains unaltered (as seen in our experiments), then the increased inward current would tend to drastically reduce the repolarization rate, and ultimately, the cell may fail to repolarize altogether. Indeed, exposure of isolated cardiac myocytes to free-radical–generating solutions has been shown to increase the action potential plateau, and prolonged exposures led to failure of some cells to repolarize beyond –40 mV.32

The primary observation of this study is that oxidative stress and lipid peroxidation cause marked changes in membrane currents without any nonspecific membrane disruption or leak. Unlike human erythrocytes,52 frog ventricular cells exposed to 2 mM \( t\)-BHP did not show any evidence of leakiness or colloid osmotic lysis for an observation period of 30 minutes. Even in the presence of 5–14 mM \( t\)-BHP, it was still possible to form gigohm seals and record ionic currents with the myocyte membrane remaining electrically tight. Thus, under the conditions and exposure times reported in this study, it seems rather unlikely that oxidative damage of the sarcolemma leads to any significant nonspecific loss of membrane permeability. Electrophysiological changes caused by lipid peroxides in the absence of nonspecific membrane disruption have also been observed by Nakaya et al,59 who did not find significant changes in intracellular \( K^+ \) activity in the presence of cumene hydroperoxide at concentrations that caused marked electrophysiological changes leading to a total loss of excitability.

In spite of the strong oxidative insult (i.e., incubation with 2 mM \( t\)-BHP for 15–30 minutes in the absence of glucose) the function of the \( K^+ \) and \( Ca^{2+} \) channels in the sarcolemma remained essentially unaltered. Interestingly, the squid axon potassium channel has also been reported to be relatively insensitive to oxidative, as exposure of the axon to chloramine T caused little or no change in the potassium channel at concentrations that abolished the inactivation of the sodium channel.57 In our study, even in cells with INa completely inhibited, the \( K^+ \) channel displayed practically normal characteristics, and the \( Ca^{2+} \) channel maintained normal function with or without added cyclic AMP. In cells preincubated with \( t\)-BHP, the calcium channel could still be activated by cyclic AMP, and large \( Ca^{2+} \) currents could be recorded that were not perceptibly different from those of untreated cells. The secondary effect of \( t\)-BHP on the rundown of the slow inward current is difficult to interpret, because the genesis of this phenomenon is not understood. In a recent study,61 \( t\)-BHP was found to augment and subsequently attenuate \( Ca^{2+} \) currents of rabbit sinoatrial node. Such a difference could be due to a
greater sensitivity of the mammalian pacemaker $I_{Ca}$ to oxidative stress.

Several investigators have reported that oxidative damage causes an increase in $[Ca^{2+}]_{o}$. Our results suggest that if there is a significant increase in $[Ca^{2+}]_{o}$, it could not be due to an increased $Ca^{2+}$ influx through the altered sarcoplasmal Ca$^{2+}$ channels. Of course, there remains the possibility that [Ca] may increase as a result of calcium release from intracellular stores. The sarcoplasmic reticulum or the mitochondria could be the major sources, especially as the latter have been shown to release calcium on oxidation of NADH or NADPH. Alternatively, a reduced calcium efflux via the sarcoplasmal pump or an influx of Ca$^{2+}$ through the Na$^+$-Ca$^{2+}$ exchanger may contribute to increased [Ca].

It has been known for more than 40 years that t-BHP causes a systolic standstill of the frog heart, which is preceded by arrhythmia and contracture. As the intracellular calcium stores in the frog heart are minimal, the contracture most probably results from an increased influx of extracellular calcium. Our data rule out a direct involvement of $I_{Ca}$ in this process and indicate that one possible route of entry could be sodium-calcium exchange. The data in the lower panel of Figure 3 clearly show that superfusion with t-BHP increases $[Na]$, which could result from either an increased Na$^+$ influx or a decreased Na$^+$ efflux. An increased influx could be mediated by the fast sodium channel, the Na$^+$-Ca$^{2+}$ exchange, or a steady-state sodium conductance. The fast $I_{Na}$ is decreased in the presence of t-BHP; therefore, it cannot account for the increased $[Na]$. However, it is possible that a decrease in Na$^+$ efflux via the Na$^+$-K$^+$ pump leads to an increase in [Na]. In vitro, the Na$^+$,K$^+$-ATPase is inactivated on alkylation of its sulfhydryl residues and thus may be sensitive to changes in oxidation-reduction state in vivo. Moreover, because the [Ca] was buffered by EGTA in the patch pipette, it is unlikely that the increase in [Na] could be due to alterations in [Ca]. Therefore, the hypothesis that the increase in [Na] is mediated by a progressive failure of the pump would be consistent with our data.

The high [Na], induced by t-BHP would exchange for extracellular calcium, causing an increase in the intracellular calcium, which would lead to contracture. Interestingly, an analogous situation seems to exist in ischemia superfusion injury, where imbalances in [Na], have been suggested to cause calcium overload via Na$^+$-Ca$^{2+}$ exchange. In our experiments, however, we did not observe contracture of the clamped cells because [Ca] was buffered by 1 mM EGTA. Nonclamped cells, in contrast, did show contracture and the formation of membrane “blebs,” suggesting that in the cells in which the [Ca], was not buffered, the free-calcium concentration was elevated significantly by t-BHP.

In conclusion, we have found that oxidative stress causes a selective and complete inhibition of $I_{Na}$ without significant alterations in the Ca$^{2+}$ and K$^+$ channels or the barrier properties of the plasma membrane. Before a total loss of $I_{Na}$, t-BHP causes changes in the gating properties of the channel. These changes would lead to depolarization of the sarclemma, inhibition of the upstroke velocity of the action potential, and failure of the cell to repolarize and will enhance “abnormal” automaticity, a condition that in the intact myocardium would be extremely arrhythmogenic.

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