Effect of hydrogen peroxide on the membrane currents of sinoatrial node cells from rabbit heart

JIQING GUO, WAYNE R. GILES, AND CHRISTOPHER A. WARD

1Department of Physiology and Biophysics, University of Calgary, Calgary, Alberta T2N 4N1; and 2Department of Physiology, Queen's University, Kingston, Ontario, Canada K7L 3N6

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Guo, Jiqing, Wayne R. Giles, and Christopher A. Ward. Effect of hydrogen peroxide on the membrane currents of sinoatrial node cells from rabbit heart. Am J Physiol Heart Circ Physiol 279: H992–H999, 2000.—The effects of H2O2 on pacemaker activity and underlying membrane currents were studied in isolated rabbit sinoatrial (SA) node cells using perforated patch current- and voltage-clamp methods. Short-term exposure (<10 min) of the nodal cells to H2O2 (200 μM) resulted in an initial shortening of spontaneous action potential cycle length (from 445 ± 60 to 398 ± 56 ms; P < 0.05) and a prolongation of action potential duration. H2O2 (100 μM) significantly increased peak L-type Ca2+ current (I_{Ca,L}) from −384 ± 77 to −439 ± 84 pA (116 ± 2%, n = 6). Additionally, the persistent or non-inactivating component of I_{Ca,L} was increased from −52 ± 3 to −88 ± 14 pA (174 ± 19%, n = 6). The hyperpolarization-activated current (I_h) was decreased from −228 ± 62 to −161 ± 72 pA after exposure to H2O2 (n = 7). There were no changes in the delayed rectifier K+ current (I_K) (n = 7). H2O2-induced Ca2+ currents were blocked by 2 μM nicardipine (n = 6), 2 mM Ni2+ (n = 2), and the protein kinase C (PKC) inhibitor bisindolylmaleimide (10−7 M; n = 4) but not by 20 μM tetrodotoxin. These results suggest that H2O2 can increase the spontaneous pacing rate in rabbit SA node cells by enhancing I_{Ca,L} and that this effect is mediated by a PKC-dependent pathway.

H2O2 causes a biphasic effect on the spontaneous pacemaker rate: an initial increase is followed by a decrease (36). Our results show that the increase in pacemaker rate is associated with an increase in I_{Ca,L}. There is a remarkable correlation between the tissue content of glutathione peroxidase, which detoxifies H2O2, and the severity of reperfusion injury (17). Direct application of H2O2 has also been shown to initiate proarrhythmic activity (1, 6, 36).

Although some aspects of the underlying mechanisms by which H2O2 generates rhythm disturbances have been studied in some detail, there is little information and no consensus regarding the specific effects of H2O2 on individual transmembrane currents (19). H2O2 has been reported to increase several different currents including the ATP-sensitive K+ current (12) and inward-rectifying K+ current (24); but apparently it can also decrease the delayed rectifier K+ current (3). In addition, in mammalian ventricle, H2O2 appears to have a number of different effects on L-type Ca2+ currents (I_{Ca,L}) with increases, decreases, or no change being reported (3, 28, 33, 34). Based on our previous study (36) and other findings, it is likely that the differing effects of the actions of H2O2 on I_{Ca,L} in cardiac tissue can be attributed to recording conditions, although variations in animal species and tissue should be considered.

Recently, we confirmed that the effects of H2O2 on rat ventricular action potential waveforms were dependent upon recording conditions, with measurable effects observed only when the myoplasm was not dialyzed (36). Our results showed that in rat ventricular myocytes, H2O2 prolonged action potential duration (APD) by selectively slowing the rate of fast inactivation of the tetrodotoxin (TTX)-sensitive sodium current (I_{Na}). These effects of H2O2 appear to be mediated by the intracellular second messenger, protein kinase C (PKC).

To date, however, there have been few studies aimed at determining the effects of H2O2 on cells derived from the primary pacemaker tissue, the sinoatrial node (SAN). In the present study, we examined the effects of H2O2 on the spontaneous action potential generation and underlying ionic currents in isolated SAN cells. In the pacemaker myocytes from the adult rabbit heart, H2O2 causes a biphasic effect on the spontaneous pacemaker rate: an initial increase is followed by a decrease of the spontaneous cycle length with prolonged exposure. Our results show that the increase in pacemaker rate is associated with an increase in I_{Ca,L}. There is a...
slight decrease the hyperpolarization-activated current (I_B) but no measurable effect on the delayed rectifier K⁺ current (I_K).

METHODS

Cell isolation. Rabbit sinoatrial cells were isolated using a method described previously by Tanaka et al. (32) in 1996 with the following modifications. New Zealand White rabbits (1.5–2.0 kg) were anesthetized with pentobarbital sodium (40 mg/kg). The chest was quickly opened, and the aorta was cannulated to allow perfusion of the coronary vessels with a solution (KB) (14), and individual myocytes were obtained by gentle trituration. The isolated cells were stored at 4°C until used in experiments.

Isolated SAN cells had a fusiform shape and a smooth surface with no discernible cross-striations. Cell size was in the range of 5–10 μm width and 50–100 μm length. The capacitance of an isolated myocyte averaged 38.3 ± 9.2 pF (n = 24).

Solution and drugs. The Tyrode solution contained (in mM) 145 NaCl, 5.4 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 1.0 Na₂HPO₄, 5.0 HEPES, and 5.5 glucose (pH 7.4, adjusted with NaOH). The KB solution contained (in mM) 90 potassium glutamate, 4.7 KCl, 10 KH₂PO₄, 20 taourine, 0.5 EGTA, 5 HEPES, 1.0 MgCl₂, and 10 glucose (pH 7.3, adjusted with KOH). For action potential and whole cell current recordings, the pipette solution contained (in mM) 110 potassium aspartate, 20 KCl, 5.0 MgCl₂, 1 CaCl₂, 5 disodium ATP, 10 HEPES, and 10 EGTA (pH 7.2, adjusted with KOH). In some experiments, Cs⁺ was substituted for K⁺ on an equimolar basis.

H₂O₂ stock solution (0.5 M) was prepared each day by diluting 30% H₂O₂ (Fisher Scientific) with distilled water and protected from light to minimize photodegradation. H₂O₂ was added to the superfusion solution to a final concentration of either 100 or 200 μM and applied to the cells at a rate of 2–3 ml/min. Both concentrations of H₂O₂ resulted in similar changes of membrane potential and whole cell currents, indicating that there is little concentration dependence over this range. Bisindolylmaleimide (BIS) was obtained from Calbiochem (San Diego, CA). All the other chemicals were obtained from Sigma.

Recording methods. The amphotericin B (0.2 mg/ml) perforated-patch clamp technique, as described previously by Ward and Giles (36) in 1997, was used in both action potential and whole cell membrane current recordings. Total “pipette” resistance was less than 20 MΩ for action potential recordings and was less than 10 MΩ for voltage-clamp measurements. Aspartate-containing pipette solutions resulted in a liquid junction potential of ~10 mV, which was corrected. All the experiments were carried out at 34 ± 0.5°C. Data acquisition and analysis was performed using the customized software Cellsoft (D. Bergman, University of Calgary).

Statistical data are given as mean ± SE. Statistical significance was taken to be P < 0.05 as evaluated by Student’s paired t-test.

RESULTS

Effect of H₂O₂ on spontaneous pacemaker activity and action potentials in SAN cells. Rabbit SAN cell pacemaker activity and action potentials were recorded continuously. These spontaneous action potential waveforms, recorded in control conditions (Fig. 1; Table 1), were very similar to those reported in previous studies (13). In each experiment, data was acquired only after the access resistance had stabilized and the cells generated stable rhythmic action potentials for at least 5 min.

Addition of H₂O₂ to the superfusion solution resulted in time-dependent, biphasic alterations of spontaneous

<table>
<thead>
<tr>
<th>H₂O₂</th>
<th>APD 50, ms</th>
<th>CL, ms</th>
<th>dV/dt max, V/s</th>
<th>MDP, mV</th>
<th>OS, mV</th>
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<tr>
<td>0 μM</td>
<td>104 ± 16</td>
<td>445 ± 60</td>
<td>10.4 ± 2.1</td>
<td>−70.6 ± 1.7</td>
<td>33.4 ± 3.8</td>
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<tr>
<td>100 μM</td>
<td>113 ± 17</td>
<td>398 ± 56</td>
<td>10.4 ± 2.1</td>
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Values are means ± SE; n = 5. SA, sinoatrial; MDP, maximum diastolic potential; CL, cycle length; dV/dt max, maximum rate of depolarization; APD 50, action potential duration at 50% of repolarization; OS, overshoot. *P < 0.05 compared with control, paired t-test.
Fig. 2. Changes of L-type Ca\(^{2+}\) current (I_{Ca,L}), delayed rectifier K\(^{-}\)current (I_{K}), and hyperpolarization-activated current (I_{f}) after exposure to H\(_2\)O\(_2\). A: current changes in response to a depolarization in a SAN cell before exposure to H\(_2\)O\(_2\) (trace a), following early exposure to H\(_2\)O\(_2\) (trace b), and after long-term exposure to H\(_2\)O\(_2\) (trace c) are superimposed. Both I_{Ca,L} and I_{K} were activated by a 500-ms depolarizing step to 0 mV from a holding potential of −60 mV. Na\(^{+}\) current (I_{Na}) was inactivated by a 100-ms conditioning pulse to −35 mV immediately preceding the test pulse. The membrane potential was then repolarized to −40 mV to record tail currents due to deactivation of I_{K}; B: superimposed current traces of I_{f} were activated by a hyperpolarization test pulse to −100 mV. Prepulse was the same as that in A. C: representative plots of the time course for the changes in I_{Ca,L}, I_{f}, and I_{K} following exposure to H\(_2\)O\(_2\). I_{Ca,L} was measured as the peak inward current during the depolarization to 0 mV, I_{f} was measured as the initial amplitude of the outward tail current at −40 mV. I_{K} was measured as the maximum inward current during the hyperpolarization to −100 mV. The current records in A and B were obtained at the times indicated by corresponding letters.

Firing rate and action potential waveforms. Both 100 and 200 μM H\(_2\)O\(_2\) gradually increased the spontaneous firing rate of SAN cells (over a period of 5 to 10 min) with no apparent differences between these H\(_2\)O\(_2\) concentrations. A representative effect of 200 μM H\(_2\)O\(_2\) on spontaneous pacemaker activity and action potential is illustrated in Fig. 1. In all cells examined, H\(_2\)O\(_2\) resulted in a shift of the action potential threshold to more negative membrane potentials (Fig. 1A) and a simultaneous prolongation of APD. The overshoot of the action potentials was also increased in five of seven cells examined. No measurable change was found in maximum diastolic potentials (MDP) or in the initial one-third of diastolic depolarization of pacemaker potential. The effects of 200 μM H\(_2\)O\(_2\) on these action potential parameters are summarized in Table 1.

Prolonged superfusion of SAN cells with H\(_2\)O\(_2\)-containing solution (for more than 20–30 min) resulted in a progressive decrease of firing rate in SAN cells. This was accompanied by a depolarizing shift of the MDP, a decrease of the action potential amplitude, and a marked change of the action potential waveform (Fig. 1B). To minimize this variability and address the underlying ionic mechanisms for the remainder of these experiments, we focused on the early effects of H\(_2\)O\(_2\), i.e., those due to H\(_2\)O\(_2\) exposures of less than 10 min.

Effect of H\(_2\)O\(_2\) on the whole cell membrane currents. To examine the ionic mechanism(s) responsible for the positive chronotropic effect of H\(_2\)O\(_2\), transmembrane ion currents were recorded using the same conditions as were used for action potential measurements. In each experiment the membrane potential was held at −60 mV to mimic the normal MDP and avoid activation of I_{f}. I_{Ca,L} was recorded by applying a voltage step to 0 mV for 500 ms immediately following a 100-ms conditioning step to −35 mV to inactivate Na\(^{+}\) (I_{Na}) and T-type Ca\(^{2+}\) (I_{Ca,T}) currents. The membrane potential was then repolarized to −40 mV for 1 s to record the tail current due to I_{K}. This clamp protocol was repeated every 20 s during the experiment. After exposure to H\(_2\)O\(_2\), the peak inward current at the onset of the test (P2) depolarization increased gradually (over 5 to 10 min; Fig. 2, A and C), as predicted from the increased firing rate of the spontaneous action potentials (Fig. 1A). In five cells, 200 μM H\(_2\)O\(_2\) increased the peak inward current at 0 mV from −232 ± 91 to −344 ± 107 pA (P < 0.05). In each experiment a persistent inward current was also observed (Fig. 2, A and B). The H\(_2\)O\(_2\)-induced increases in both peak and persistent current components were strongly but not completely inhibited by 2 μM nicardipine (n = 6; see also Fig. 3). Subsequent experiments with 2 mM Ni\(^{2+}\) (n = 2; data not shown), which does completely block I_{Ca,L}, resulted in findings similar to those with nicardipine. Together, these findings suggest that I_{Ca,L} is responsible for the increase in inward current. Currents were fit to a double exponential equation to quantify the effects of H\(_2\)O\(_2\) on current inactivation. The fast time constant was accelerated from 13.0 ± 1.6 to 9.9 ± 1.2 ms (n = 6), whereas the slow time constant was significantly slowed from 33.0 ± 7.6 to 157.1 ± 69.9 ms (n = 6) following exposure to H\(_2\)O\(_2\), respectively.

In SAN cells, activation of I_{K} initiates and controls action potential repolarization, and its deactivation is an important contributor to the rate of pacemaker depolarization (13). Possible effects of H\(_2\)O\(_2\) on I_{K} were evaluated by monitoring changes of the tail current of I_{K} at −40 mV. The I_{K} tail current (which is a gradual decay of outward current after repolarization from 0 to −40 mV) showed no significant change in either amplitude or decay rate during exposure to H\(_2\)O\(_2\) (Fig. 2C). Peak tail currents, determined prior to and at 5 min after exposure to H\(_2\)O\(_2\) were 39 ± 8 and 44 ± 10 pA, respectively (P > 0.05).

I_{f} in many pacemaker cells, contributes to the MDP and modulates the last one-third of diastolic depolarization (5). In principle, an increase in I_{f} could augment the spontaneous firing rate of SAN cells. To examine this possibility, I_{f} was recorded by hyperpolarizing the membrane potential to −100 mV for 0.5 s. A slow but progressive decrease of I_{f} was observed following expo-
sure to H$_2$O$_2$ (Fig. 2, B and C). It should be noted that this gradual decrease occurred in the same cells and over an identical time period for which the increasing $I_{\text{Ca,L}}$ was observed (Fig. 2C), indicating that this decrease is not likely due to a general rundown of membrane currents. In seven cells observed, $I_f$ at $-100$ mV was decreased from $-228 \pm 62$ pA in control conditions to $-161 \pm 72$ pA following 5-min exposure to H$_2$O$_2$.

**Effect of intracellular dialysis.** Previously, we and others have demonstrated that, in ventricular myocytes, the effects of H$_2$O$_2$ are mediated by second messengers and therefore are strongly dependent upon the recording conditions utilized (36). To evaluate this phenomenon in SAN cells, we repeated some experimental protocols using standard ruptured patch recording techniques (Fig. 3). Under these experimental conditions, H$_2$O$_2$ caused no changes in spontaneous rate or action potential waveform. As well, H$_2$O$_2$ had no effect on $I_{\text{Na}}$ under these conditions other than a gradual decline in both $I_{\text{Ca,L}}$ and $I_f$ ($n = 5$), consistent with current rundown associated with this recording method.

H$_2$O$_2$ increases $I_{\text{Ca,L}}$: To further examine whether changes in $I_{\text{Ca,L}}$ may be responsible for the positive chronotropic effect of H$_2$O$_2$, more detailed voltage-clamp experiments were performed. $I_{\text{Ca,L}}$ was isolated using Cs$^+$-rich pipette solutions, and 5 mM Cs$^+$ was added to the superfusate. These experimental conditions block both K$^+$ currents. However, complete block of outward K$^+$ currents with Cs$^+$ is very difficult to achieve under perforated patch conditions. From a holding potential of $-80$ mV, $I_{\text{Ca,L}}$ was activated by depolarizing steps to selected test membrane potentials, following a conditioning pulse to $-35$ mV for 100 ms to inactivate $I_{\text{Na}}$ and $I_{\text{Ca,T}}$. Figure 4A shows a typical record of $I_{\text{Ca,L}}$ at $-10$ mV. H$_2$O$_2$ (100 $\mu$M) induced an increase in inward current similar to that observed when using K$^+$-rich pipette solutions. Current-voltage (I-V) relationships measured using the peak inward current or the current at the end of the step showed that H$_2$O$_2$ increased both parameters over a broad voltage range (Fig. 4B), with no significant shift of the I-V relationship. All of this inward current was completely blocked by 2 $\mu$M nicardipine, suggesting that the H$_2$O$_2$-induced current can be attributed to $I_{\text{Ca,L}}$. In six cells, $I_{\text{Ca,L}}$ measured as the nicardipine-sensitive current at $-10$ mV was increased from $-384 \pm 77$ to $-439 \pm 84$ pA ($116 \pm 2\%$, $P < 0.05$) at the peak and from $-52 \pm 3$ to $-88 \pm 14$ pA ($174 \pm 19\%$, $P < 0.05$) near the end of 500-ms depolarizing pulses.

To gain more insight into the effect of H$_2$O$_2$ on $I_{\text{Ca,L}}$, the current traces recorded before and after H$_2$O$_2$ exposure were subtracted. The resulting difference in current defines the H$_2$O$_2$-induced effect (Fig. 4C). Although there was some variation in rate and extent of the decay of the H$_2$O$_2$-induced current component among cells, a consistent finding was that this difference current was much slower compared with the $I_{\text{Ca,L}}$ recorded under control conditions. These results demonstrate that H$_2$O$_2$ can augment the peak current and may slow the inactivation rate of $I_{\text{Ca,L}}$ as well (see DISCUSSION).

**Effect of TTX on H$_2$O$_2$-induced inward current(s).** It has been reported previously that H$_2$O$_2$ slows the rate of $I_{\text{Na}}$ inactivation in rat ventricular myocytes (2, 36). We therefore examined the possible involvement of the TTX-sensitive $I_{\text{Na}}$ in H$_2$O$_2$-induced current in SAN cells. This was done by applying the selective inhibitor TTX. In the presence of 20 $\mu$M TTX, H$_2$O$_2$ still increased the inward current activated by a depolarizing step to 0 mV (Fig. 5). $I_{\text{Ca,L}}$ was increased from $-333 \pm 96$ to $-385 \pm 107$ pA ($117 \pm 2\%$) at the peak and from $-38 \pm 4$ to $-72 \pm 14$ pA ($174 \pm 19\%$) near the end of 500-ms depolarizing pulses ($n = 4$). This finding confirms that TTX had no significant effect on the H$_2$O$_2$-induced current and that the initial effects (1–5 min) of H$_2$O$_2$ in SAN cells is mediated primarily by $I_{\text{Ca,L}}$.

**Involvement of PKC in the effect of H$_2$O$_2$ on $I_{\text{Ca,L}}$.** Previously, it has been demonstrated that inhibition of PKC attenuates the effects of H$_2$O$_2$ on action potential waveform in rat ventricular myocytes (36). Since the negative findings using ruptured patch recordings in this study (Fig. 3) suggest that second messengers are also involved in H$_2$O$_2$-induced changes in $I_{\text{Ca,L}}$, we examined the potential role of PKC as a modulator of
the effects of H$_2$O$_2$ on SAN cells. In these experiments, SAN cells were incubated with BIS, a potent PKC inhibitor, for at least 3 h to ensure complete PKC inhibition. Under these conditions, the ability of 100 µM H$_2$O$_2$ to increase $I_{\text{Ca,L}}$ was almost completely abolished (Fig. 6A). In each of four cells examined, the peak and the persistent $I_{\text{Ca,L}}$ currents in the presence of H$_2$O$_2$ were compared with the control levels (102.8 ± 0.5% and 102.6 ± 3.4% of the control, respectively). Neither value was significantly different from those without BIS incubation (Fig. 6B). Thus PKC inhibition strongly decreases the effect of H$_2$O$_2$ on $I_{\text{Ca,L}}$ in SAN cells.

DISCUSSION

Previous reports have shown that t-butyl hydroperoxide, a reactive oxygen agent, produced an initial increase followed by a decrease in spontaneous pacemaker rate in a multicellular SAN preparation (27, 28). These findings were interpreted in terms of a corresponding biphasic change in $I_{\text{Ca,L}}$, $I_p$, and $I_K$ (27). Our results from single cell recordings showed a similar biphasic rate change induced by H$_2$O$_2$. We found that the initial positive chronotropic effect of H$_2$O$_2$ is primary due to an increase in the amplitude and/or slowing of the inactivation rate of $I_{\text{Ca,L}}$. A slow decrease in $I_p$ and no change in $I_K$ were observed in same period after exposure to H$_2$O$_2$. Our study focused on the initial positive chronotropic effect of H$_2$O$_2$ on SAN cells, since these effects are likely to be produced by a changes in specific ionic currents as opposed to being due to general rundown of the cell.

$I_{\text{Ca,L}}$ is one of the transmembrane ionic currents that generates pacemaker activity in adult rabbit SAN cells.
sugestion that \( \text{H}_2\text{O}_2 \) can slow the inactivation of this \( \text{Ca}^{2+} \) current and/or shift the voltage dependence of its inactivation to more negative potentials. Further study is needed to identify the mechanisms of \( \text{H}_2\text{O}_2 \) on \( I_{\text{Ca,L}} \) inactivation, as this decrease in inactivation appears to be the major mechanism by which \( \text{H}_2\text{O}_2 \) enhances both peak and persistent \( I_{\text{Ca,L}} \). Consistent with our experimental data, mathematical modeling of the SAN pacemaker action potential suggests that an incomplete inactivating component of \( I_{\text{Ca,L}} \) could alter the plateau phase and the APD (4). Although \( I_{\text{Ca,L}} \), inactivation kinetics alterations were not reported following oxidant exposure in multicellular SAN preparations (27), this may be due to altered intercellular coupling between the individual SAN cells, which can change the spatial uniformity under voltage-clamp conditions, making it difficult to record small current changes. The interaction between heterogeneous cells in SAN tissue (18) in the presence of reactive oxygen species may also contribute to the reported differences in results obtained using single myocytes vs. those from multicellular preparations.

Prolongation of APD following \( \text{H}_2\text{O}_2 \) exposure has been reported previously in other cardiac myocytes (3, 24, 36). When coupled with depolarization of the resting membrane potential, this is thought to be one cause of reperfusion arrhythmias (7, 19). Other ionic current changes, e.g., a slowing of the rate of fast inactivation of \( I_{\text{Na}} \), a decrease in \( I_{\text{K}} \), and/or an altered inward-rectifying \( K^+ \) current \( (I_{\text{Kf}}) \), have also been suggested to be responsible for the observed changes in APD in the heart (3, 24, 36). Pacemaker cells from the SAN express very little \( I_{\text{Kf}} \) and those from the central region of the SAN have no detectable \( I_{\text{Na}} \) (13, 18) (see also Fig. 4, at conditional pulse at \(-35 \text{ mV} \) in nicardipine). Our experiments demonstrated that the \( \text{H}_2\text{O}_2 \)-induced current was not sensitive to TTX pretreatment. Thus the mechanism of prolongation of APD in SAN cells is different from that previously identified in ventricular myocytes (36).

In multicellular SAN preparations, biphasic changes in \( I_{\text{f}} \) and \( I_{\text{K}} \) have been reported after exposure to \( \text{t}-\text{butyl hydroperoxide} \) (27). However, we found no enhancement of these currents by \( \text{H}_2\text{O}_2 \). This difference may be explained, in part, by differences in study techniques and/or reactive oxygen agents we used in this study. \( I_{\text{f}} \) (measured at \(-100 \text{ mV} \)) was consistently decreased after exposure to \( \text{H}_2\text{O}_2 \). It is difficult to ascertain whether this decrease is attributed to a rundown of the current during the experiment. Nevertheless, we can conclude that \( \text{H}_2\text{O}_2 \)-induced alterations in this current are not responsible for the observed effects on action potential waveform: a decrease of \( I_{\text{f}} \) would have been expected to decrease the automaticity (5, 35). However, it should be noted that when electrically coupled to other SAN cells, as in multicellular preparations, it is possible that the small effects that we report on \( I_{\text{f}} \) could be an important mediator for the effects of \( \text{H}_2\text{O}_2 \).

In previous reports examining the effects of reactive oxygen species in guinea pig ventricular and frog atrial cells, the \( I_{\text{K}} \) was decreased (3, 33). Deactivation of \( I_{\text{K}} \) is
an important contributor to the pacemaker depolarization in SAN cells. A decrease of $I_K$ would result in a prolongation of the APD. Therefore, it was important to examine the effects of $H_2O_2$ on this current. We failed to find any changes in $I_K$ tail current following exposure to $H_2O_2$. This lack of effect might be explained by a difference in channel subtypes in this cell. In rabbit SAN cells, $I_K$ has been reported to be mainly due to the rapid delayed rectifier current (21, 25). In contrast, in guinea pig ventricular and frog atrial myocytes, in which the previous studies were done, the slow delayed rectifier current predominates (10, 26).

The possible involvement of second messenger systems in the observed effects of $H_2O_2$ was examined in the final part of this study. Previous studies have shown that $H_2O_2$ exposure leads to alterations in intracellular calcium homeostasis (11, 31, 37). This could alter the activity of ionic currents regulated by intracellular calcium. PKC has also been identified as a mediator for the effects of $H_2O_2$ (34, 36, 37). Our results suggest that a second messenger mediates the effects of $H_2O_2$ on SAN cells as well. Thus, under experimental conditions in which the myoplasm was dialyzed with the pipette solution, the initial positive chronotropic effects of $H_2O_2$ were blunted or abolished. PKC is implicated as this soluble mediator, since pretreatment of cells with the PKC inhibitor BIS significantly attenuated the effects of $H_2O_2$. This is somewhat similar to our previous findings (36, 37).

However, the direct effects of PKC activation on $I_{Ca,L}$ are somewhat controversial. $I_{Ca,L}$ has been reported to be increased (and then decrease) (8, 20, 30), decrease (29, 31), or remain unchanged (16). These discrepancies might be explained by variations of endogenous PKC levels and different PKC isoforms being expressed in different cell types (9, 15, 16, 22). In addition to PKC, it has also been shown that glutathione is a necessary cofactor for $H_2O_2$-induced effects (31). However, when glutathione was present, $H_2O_2$ decreased $I_{Ca,L}$ (31), whereas we report an increased current in the present study.

In summary, our results demonstrate a significant positive chronotropic effect followed by a negative chronotropic effect of $H_2O_2$ in cells isolated from the rabbit SAN. As well, $H_2O_2$ prolonged the duration of the SAN action potentials. Voltage-clamp measurements established that $H_2O_2$ induced a nicardipine- and Ni$^{2+}$-sensitive and TTX-insensitive inward current, $I_{Ca,L}$. Pretreating the cells with an inhibitor of PKC prevented this increase of $I_{Ca,L}$. These findings suggest that reactive oxygen species generated during reperfusion of previously ischemic myocardium can alter heart rate by enhancing pacemaker activity of SAN cells.

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