Hydrogen Peroxide-Induced Stimulation of L-Type Calcium Current in Guinea Pig Ventricular Myocytes and Its Inhibition by Adenosine A1 Receptor Activation

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ABSTRACT

Hydrogen peroxide (H₂O₂) produces complex cardiac effects that may involve altered calcium homeostasis. The cardiotoxic effects of H₂O₂ can be attenuated by adenosine A1 receptor agonists. The present study examined the effect of H₂O₂ on L-type Ca⁹⁺ current (I_{Ca,L}) in guinea pig ventricular myocytes under two different recording conditions and the influence of adenosine receptor agonists. H₂O₂ (100 μM) did not have any significant effect on L_{Ca,L} under conventional whole cell patch configuration. However, when recorded under nystatin perforated patch configuration, H₂O₂ caused a gradual and significant increase (84 ± 14%) in I_{Ca,L} compared to control values. N°-cyclopentyladenosine (CPA), an adenosine A1 receptor agonist, significantly attenuated the effect of H₂O₂. The inhibitory effect of N°-cyclopentyladenosine was antagonized by 8-cyclopentyl-1,3-dipropylxanthine, an adenosine A1 receptor antagonist. The A2A and A3 receptor agonists, 2-p-(2-carboxyethyl)phenethylamino-5°-N-ethylcarboxamidoadenosine (CGS-21680) and 1-deoxy-1-[6-[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]N-methyl-β-D-ribofuranuronamide, respectively, did not modulate the enhancement of I_{Ca,L} by H₂O₂. Moreover, the effects of N°-cyclopentyladenosine were mimicked by the protein kinase C inhibitor bisindolylmaleimide. Thus, our results demonstrate a potent stimulatory effect of H₂O₂ on I_{Ca,L} in guinea pig ventricular myocytes. We further demonstrate that adenosine A1 receptor activation attenuates this effect. Our results suggest a potential basis for altered calcium homeostasis in response to H₂O₂ as well as the salutary effects of A1 receptor activation against H₂O₂-induced cardiotoxicity.

Reperfusion of previously ischemic myocardium is associated with the generation of large amounts of reactive oxygen species (Slezak et al., 1995). H₂O₂, a small, uncharged and relatively stable molecule that diffuses easily through tissue (Hoffman et al., 1984; Welsh et al., 1985), is considered to be an important mediator of reperfusion-induced abnormalities (Beresewicz and Horackova, 1991; Duan and Moffat, 1992). H₂O₂ is formed in mitochondria as a dismutation product of the superoxide radical (O₂⁻) under physiological conditions. However, under ischemic conditions, there is proteolytic modification of xanthine dehydrogenase to xanthine oxidase (McCord and Roy, 1982) which may produce a burst of O₂⁻ and H₂O₂ when oxygen is reintroduced during reperfusion.

H₂O₂ has several adverse effects on the myocardium including induction of cardiac arrhythmias (Beresewicz and Horackova, 1991; Duan and Moffat, 1992). These effects may be the result of its ability to induce lipid peroxidation (Rubin and Farber, 1984), enzyme activation (Mekhfi et al., 1996), altered energy metabolism (Spragg et al., 1985), protein oxidation (Fliss et al., 1988) or changes in intracellular calcium concentration (Hyslop et al., 1986; Ward and Moffat, 1995) singly or in combinations of these factors. It has been postulated that the calcium overload observed following exposure to H₂O₂ may contribute to oxidant-induced cellular damage (Kaneko et al., 1994). However, the mechanism by which H₂O₂ increases [Ca⁺⁺]_i concentration is not clear. It is possible that H₂O₂ increases [Ca⁺⁺], by altering the activity of ion channels and/or transport proteins, either directly or through effects on other systems that modulate their activity. Several studies have reported the effects of H₂O₂ on various ion channels and exchangers. H₂O₂ has been reported to alter the function of delayed rectifier K⁺ current (Goldhaber et al., 1989), inward rectifier K⁺ current (Matsuura and Shattock, 1991), adenosine triphosphate-sensitive K⁺ current (Goldhaber and

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ABBREVIATIONS: H₂O₂, hydrogen peroxide; I_{Ca,L}, L-type calcium current; [Ca⁺⁺]_i, cytosolic calcium; PKC, protein kinase C; BIS, bisindolylmaleimide; EGTA, ethylene glycol-bis(β-aminooethyl ether)-N,N,N',N''-tetra acetic acid; HEPES, N-[2-hydroxyethyl]piperazine-N''-[2-ethanesulfonic acid]; CPA, N°-cyclopentyladenosine.
Materials and Methods

Animals. Male albino guinea pigs (300–350 g), obtained from Charles River (St. Constant, Quebec, Canada), were maintained in the Health Sciences Animal Care facility of The University of Western Ontario, in accordance with the guidelines of the Canadian Council on Animal Care (Ottawa, Ontario, Canada).

Cell isolation. The method used for the isolation of guinea pig myocytes is similar to that described previously (Thomas et al., 1997). Briefly, heparinized guinea pigs were decapitated and the heart was mounted on a Langendorff apparatus and perfused retrogradely through the aorta (10 ml/min) with oxygenated (100% O2) calcium-free solution of the following composition (mM): 120 NaCl, 3.8 KCl, 1.2 KH2PO4, 1.2 MgSO4, 10 HEPES, 11 glucose (pH was adjusted to 7.4 with NaOH) at 37°C. The hearts were then perfused with calcium-free solution containing collagenase type-2 (Worthington Biochemical Corporation, Freehold, NJ) and protease (Sigma Chemical Co., St. Louis, MO). After enzymatic digestion and subsequent attenuation of Ca2+ currents are unaltered by H2O2 (Beresewicz and Horackova, 1991; Cerbai et al., 1991). One possible explanation for this variability could be related to different recording conditions used in these studies. This is especially important when the marked differences in the effects of H2O2 on action potentials under different conditions are considered (Ward and Giles, 1997; Barrington, 1994). In our study, we examined the effect of micromolar concentrations (comparable to the levels of H2O2 under conditions of ischemia/reperfusion) on ICa,L, under conventional whole cell and nystatin perforated patch configurations. The former rendered the interior of the cells vulnerable to dialysis although the latter maintained a more physiological intracellular milieu for a considerably longer period. Adenosine, through the activation of adenosine A1 receptors, has been shown to exert significant cardioprotective effects (Thorton et al., 1992). Previous work from our laboratory has shown that A1 receptor activation protects against the deleterious effects of H2O2 in terms of attenuation of cardiodepression produced by this oxidative stressor (Karmazyn and Cook, 1992). In addition, ischemic preconditioning confers protection against H2O2 via an adenosine-dependent mechanism (Gan et al., 1996). Accordingly, we also examined the influence of adenosine A1 receptor activation on the effects of H2O2 in terms of potential ability to modulate changes in ICa,L.

Results

Step depolarization of myocytes from -40 to 10 mV elicited a time- and voltage-dependent inward current which had all the characteristics of ICa,L. These currents were completely blocked by verapamil and enhanced by Bay K-8644 and showed characteristic current voltage relationships.

Effect of H2O2 on ICa,L under conventional whole cell patch configuration. Guinea pig ventricular myocytes were superfused with H2O2 (100 μM) and ICa,L was recorded under whole cell configuration. Figure 1A shows recordings from a typical experiment under conventional whole cell patch configuration, before and after the myocyte was exposed to H2O2. Very little changes were observed in the peak ICa,L when the myocyte was exposed to H2O2 for 25 min. Figure 2 depicts the effect of H2O2 on ICa,L under conventional whole cell configuration (n = 5). Figure 2A shows the current voltage relationship of ICa,L, under whole cell configuration. H2O2 did not significantly change the magnitude of the peak or cause any significant voltage shift. The mean results obtained from five myocytes exposed to H2O2 for a period of 25 min (fig. 2B) revealed that H2O2 has no signifi-
Effect of \( \text{H}_2\text{O}_2 \) under conventional whole cell configuration. In contrast to its failure to alter \( I_{\text{Ca,L}} \) when using whole cell recording, \( \text{H}_2\text{O}_2 \) caused a large increase in peak \( I_{\text{Ca,L}} \) in ventricular myocytes when perforated patch configuration was used. Figure 1B shows tracings of \( I_{\text{Ca,L}} \) from an experiment in which the \( I_{\text{Ca,L}} \) was recorded under nystatin perforated patch and the results of six experiments in which the myocytes were exposed to \( \text{H}_2\text{O}_2 \) are shown in figure 3. Figure 3A shows the current-voltage relationships indicating an \( \text{H}_2\text{O}_2 \)-induced increase in \( I_{\text{Ca,L}} \), and a shift in the peak current to slightly more negative values, measured after 25-min exposure to \( \text{H}_2\text{O}_2 \). The \( \text{H}_2\text{O}_2 \) effect observed in six ventricular myocytes is summarized in figure 3B, showing a time-dependent increase in \( I_{\text{Ca,L}} \) during exposure to \( \text{H}_2\text{O}_2 \).

**Effect of adenosine A1 receptor activation.** We previously reported an inhibitory effect of adenosine A1 receptor activation on the cardiac effects of \( \text{H}_2\text{O}_2 \) (Karmazyn and Cook, 1992). Consequently, we next examined the effect of CPA, an adenosine A1 agonist on the \( \text{H}_2\text{O}_2 \)-induced enhancement of \( I_{\text{Ca,L}} \). Figure 4A shows typical data obtained in the presence of CPA (5 \( \mu \text{M} \)) and demonstrates an almost complete inhibition of \( \text{H}_2\text{O}_2 \)-induced activation of \( I_{\text{Ca,L}} \). The data for the inhibitory effect of CPA, observed in six ventricular myocytes are summarized in figure 4B. CPA by itself was without effect on basal \( I_{\text{Ca,L}} \). However, as figure 4B demonstrates, CPA significantly reduced the effects of \( \text{H}_2\text{O}_2 \) on \( I_{\text{Ca,L}} \).

We further confirmed A1 receptor specificity by examining the effect of 1 \( \mu \text{M} \) DPCPX (added 5 min before CPA), an adenosine A1 receptor antagonist on this response. Figure 5 shows the individual tracings (A) and the time course (B) obtained from a typical experiment. DPCPX completely abolished the inhibitory effect of CPA against the \( \text{H}_2\text{O}_2 \) induced enhancement of \( I_{\text{Ca,L}} \). Three other experiments showed identical results (not shown).

**Effect of PKC inhibition.** There is evidence that PKC may modulate some of the effects of \( \text{H}_2\text{O}_2 \) in cardiac tissues (Ward and Moffat, 1995). Accordingly, the last set of experiments were done to determine the effect of PKC inhibitor BIS. In these experiments, BIS (20 nM) was added to the superfusion solution, 5 min before \( \text{H}_2\text{O}_2 \) administration and was without effect on its own on \( I_{\text{Ca,L}} \). As shown in figure 7, BIS attenuated the \( \text{H}_2\text{O}_2 \) effect on \( I_{\text{Ca,L}} \) significantly. Figure 7A shows the current voltage relationship obtained from four ventricular myocytes in which BIS was administered before \( \text{H}_2\text{O}_2 \). Figure 7B represents the peak \( I_{\text{Ca,L}} \) data from five myocytes demonstrating the inhibition by BIS of \( \text{H}_2\text{O}_2 \)-induced \( I_{\text{Ca,L}} \) enhancement. We further examined whether BIS can modulate the effects of CPA on the \( \text{H}_2\text{O}_2 \) effects. In two experiments (not shown) addition of CPA to BIS-treated cells resulted in an almost 100% inhibition in the ability of \( \text{H}_2\text{O}_2 \) to activate \( I_{\text{Ca,L}} \).
Our results demonstrate for the first time, to our knowledge, a pronounced elevation in $I_{Ca,L}$ by $H_2O_2$, an effect dependent on recording configurations. Thus the increase in $I_{Ca,L}$ observed with $H_2O_2$ under nystatin perforated patch configuration was not observed using conventional whole cell ruptured patch technique. Because calcium channel function depends substantially on cytoplasmic factors, in the conventional ruptured patch method calcium currents can “run down” significantly (Belles et al., 1988; Kameyama et al., 1988). This is mainly due to the loss of low molecular weight cellular components by dialysis to the pipette solution. However, some of the alterations in drug response could be due to the constituents of pipette solution interfering with the signal transduction pathways. The perforated patch configuration minimizes the washout or dilution of cytoplasmic constituents that either modulate or are required for ion channel activity and avoids the disruption of normal intracellular $Ca^{2+}$ buffering mechanisms (Korn et al., 1991). Previous reports have shown that $H_2O_2$ can exert divergent effects under different recording conditions. For example, Barrington (1994) demonstrated the differences in the effect of $H_2O_2$ on action potentials in the absence and presence of EGTA in the pipette. This study also demonstrated that $H_2O_2$ prolonged action potential duration when recorded using high resistance standard microelectrodes where the dialysis of internal cytoplasm does not occur. Similarly, in an-

![Fig. 3. $H_2O_2$ stimulated $I_{Ca,L}$ recorded under nystatin perforated patch configuration. A, Peak current voltage relationship from 6 myocytes. Voltage clamp protocols were as in figure 2. $H_2O_2$ (100 $\mu$M) induced a large increase in $I_{Ca,L}$ and this was associated with a slight shift in the $I$–$V$ relationship to more negative values (25-min exposure time). Values are expressed as mean ± S.E.M. B, Effect of $H_2O_2$ (100 $\mu$M) on peak $I_{Ca,L}$ in six ventricular myocytes. $H_2O_2$ caused a significant increase in $I_{Ca,L}$. The enhancement was significant from 5-min exposure and continued to increase with time. Values are expressed as % ± S.E.M. with control values taken as 100%.

![Fig. 4. $H_2O_2$-induced $I_{Ca,L}$ stimulation is inhibited by adenosine A1 agonist CPA. A, Individual traces obtained from a representative experiment demonstrating the inhibition of $H_2O_2$ effect by CPA. a, Control $I_{Ca,L}$ recorded in a ventricular myocyte in the presence of 5 $\mu$M of CPA. b, $I_{Ca,L}$ from the same myocyte after 25 min exposure to $H_2O_2$. CPA was present in the solution throughout the experiment. B, Overall effect of CPA on $H_2O_2$ stimulation of $I_{Ca,L}$ (n = 6). Values are expressed as % of control levels ± S.E.M. *P < .05 compared to $H_2O_2$ (analysis of variance with Student-Newman-Keuls test).

![Fig. 5. Adenosine A1 antagonist DPCPX blocks the effect of CPA on $H_2O_2$ stimulation of $I_{Ca,L}$. A, Individual traces were obtained at times marked a, b, c, d and e in B. CPA did not inhibit the $I_{Ca,L}$ increase caused by $H_2O_2$ in the presence of DPCPX. B, Time course of the experiment shown in A. CPA was added 5 min after DPCPX and 5 min before $H_2O_2$.}
other study, H2O2 did not exert any significant effects on action potentials or cell shortening under whole cell recording conditions, but induced marked prolongation of the action potential duration and an increase in cell shortening under perforated patch techniques have reported an increase in cell shortening but the same concentrations failed to show any significant effects on action potential duration under whole cell ruptured patch configuration. Even though any generalized comparison of the data available on the effect of H2O2 may not be totally accurate, it is important to note that almost all of the studies using high resistance electrodes or perforated patch techniques have reported an increase in the action potential duration by H2O2 (Berszewicz and Horackova, 1991; Duan and Moffat, 1992; Barrington, 1994; Ward and Giles, 1997; Satoh and Matsui, 1994) whereas using whole cell ruptured patch have shown a decrease or no change in action potential duration with H2O2 (Goldhaber et al., 1989; Barrington, 1994; Ward and Giles, 1997). Increased action potential duration could reflect one or several of the many mechanisms that regulate action potential duration, such as outward K+ current, Na+-Ca++ exchange, sarcolemmal Ca++ channels and [Ca++]i. Our results clearly indicate that a facilitated entry of Ca++ influx through voltage-dependent Ca++ channels may contribute to Ca++ overload induced by H2O2.

It is interesting to note that an inhibitory effect of H2O2 on action potential duration has also been reported (Goldhaber et al., 1989). However, Barrington (1994) has shown that the recording mode and the composition of the pipette solution can affect H2O2 response on the action potential. Recently, Ward and Giles (1997) have demonstrated that under amphotericin patch configuration, H2O2 (50–200 μM) induced a marked prolongation of action potential duration and an increase in cell shortening but the same concentrations failed to show any significant effects on action potential duration under whole cell ruptured patch configuration. Even though any generalized comparison of the data available on the effect of H2O2 may not be totally accurate, it is important to note that almost all of the studies using high resistance electrodes or perforated patch techniques have reported an increase in the action potential duration by H2O2 (Berszewicz and Horackova, 1991; Duan and Moffat, 1992; Barrington, 1994; Ward and Giles, 1997; Satoh and Matsui, 1994) whereas using whole cell ruptured patch have shown a decrease or no change in action potential duration with H2O2 (Goldhaber et al., 1989; Barrington, 1994; Ward and Giles, 1997). Increased action potential duration could reflect one or several of the many mechanisms that regulate action potential duration, such as outward K+ current, Na+-Ca++ exchange, sarcolemmal Ca++ channels and [Ca++]i. Our results clearly indicate that an augmented Ca++ influx through voltage-dependent Ca++ channels may contribute to Ca++ overload induced by H2O2.

The selective effect of H2O2 on ICa,L under nystatin configuration also suggests that an intracellular moiety may be involved in this action of H2O2. It is possible that this messenger becomes inactivated or diluted by some components of...
the pipette solution under whole cell patch configuration. Activation of PKC by H$_{2}$O$_{2}$ directly (Larsson and Cerutti, 1989) and indirectly through activation of phospholipase D (Natarajan et al., 1993) has been demonstrated in noncardiac tissues, although to our knowledge this has not as yet been demonstrated in the heart. Nonetheless, it has also been reported that PKC activation mediates the H$_{2}$O$_{2}$-induced elevation in cytosolic calcium in ventricular myocytes (Ward and Moffat, 1995). Moreover, PKC-activating phorbol esters have been shown to stimulate the calcium current in neonatal rat cardiac myocytes (Dosemecli et al., 1988). Taken together, these studies support our contention that the stimulatory effect of H$_{2}$O$_{2}$ on I$_{Ca,L}$ is, at least partly, mediated via PKC. The exact mechanisms for PKC-mediated H$_{2}$O$_{2}$-induced activation of I$_{Ca,L}$ requires further studies although it likely involves a phosphorylation-dependent process. There is also evidence that some of the salutary effects of A1 receptor activation, particularly its involvement in ischemic preconditioning, may be mediated by PKC. Moreover, A1 receptor activation stimulates PKCβ in rat ventricular myocytes (Henry et al., 1996). The ability of BIS, a PKC inhibitor, to mimic the effect of CPA as well as its inability to prevent the inhibitory effects of CPA were therefore of some surprise although the results may suggest that distinct PKC isoforms may be involved in regulating the modulatory role of H$_{2}$O$_{2}$ on I$_{Ca,L}$. Further studies are necessary to delineate the potential role of this family of isozymes either with respect to H$_{2}$O$_{2}$-induced effects on the calcium current or the inhibitory effects of A1 receptor activation. The inability of BIS to completely prevent I$_{Ca,L}$ activation is suggestive of additional cellular mechanisms for H$_{2}$O$_{2}$-induced effects. These intracellular events may also explain the basis for the time-dependent effects of H$_{2}$O$_{2}$ observed in our study as well reported by other investigators (Hayashi et al., 1989; Kimura et al., 1992).

Adenosine A1 receptor agonists have been shown in various studies to protect the ischemic and reperfused myocardium (Thornton et al., 1992). Although the precise mechanism for this protection is not known, we previously reported that CPA inhibits the cardiotoxic effects of H$_{2}$O$_{2}$ (Karmazyn and Cook, 1992) at least suggesting that this could be a contributory factor. To determine the possible mechanisms for these effects, we examined the effect of CPA, a selective adenosine A1 receptor agonist, on the enhancement of I$_{Ca,L}$ induced by H$_{2}$O$_{2}$. Our results show that CPA significantly inhibits the H$_{2}$O$_{2}$-induced stimulation of I$_{Ca,L}$. The A1 receptor specificity of this effect was further confirmed by the ability of DPCPX to reverse the effects of CPA. However, it should also be stated that although A1 receptors are the predominant adenosine receptor subtype found in the ventricular myocardium, A2 and A3 receptors are also found in the ventricular myocyte [see Cook and Karmazyn (1996) for review]. A2 receptor agonists have been reported to have minimal protective influence on the heart although A2 receptor-mediated cardioprotective actions of adenosine are observed under in vivo conditions, where the antineutrophil and antiplatelet actions of A2 receptor activation can induce protection (Schlack et al., 1993). However, because it has been reported that A2 receptors are modulated under ischemic conditions (Zucchi et al., 1992), we also examined the influence of the A2A receptor-selective agonist CGS-21680 on the effect of H$_{2}$O$_{2}$. Our results suggest a lack of A2A receptor involvement on this effect. Furthermore, it is unlikely that A3 receptors are involved in the inability of IB-MECA to affect H$_{2}$O$_{2}$ actions.

**Summary and Conclusion**

In summary, our study shows that H$_{2}$O$_{2}$ can activate I$_{Ca,L}$ in guinea pig ventricular myocytes when using perforated patch configuration. This effect of H$_{2}$O$_{2}$ appears to be at least partially dependent on PKC activity. Our study also demonstrates a potent ability of A1 receptor activation to inhibit the effects of H$_{2}$O$_{2}$ which is not shared by either A2A or A3 receptor agonists. These findings suggest a novel and potentially important role of A1 receptors in the regulation of the cardiac effects of H$_{2}$O$_{2}$, particularly under pathological conditions.

**References**


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