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Effect of hydrogen peroxide on persistent sodium current in guinea pig ventricular myocytes[1]

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Key words

hydrogen peroxide; myocardium; patch-clamp techniques; sodium channels

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Abstract

Aim: To study the effect of hydrogen peroxide (H_2O_2) on persistent sodium current ($I_{Na.P}$) in guinea pig ventricular myocytes. **Methods:** The whole-cell, cell-attached, and inside-out patch-clamp techniques were applied on isolated ventricular myocytes from guinea pig. **Results:** H_2O_2 (0.1 mmol/L, 0.5 mmol/L and 1.0 mmol/L) increased the amplitude of whole-cell $I_{Na.P}$ in a concentration-dependent manner, and glutathione (GSH 1 mmol/L) reversed the increased $I_{Na.P}$. H_2O_2 (1 mmol/L) increased persistent sodium channel activity in cell-attached and inside-out patches. The mean open probability was increased from control values of 0.015±0.004 and 0.012±0.003 to 0.106±0.011 and 0.136±0.010, respectively ($P<0.01$ *vs* control). They were then decreased to 0.039±0.024 and 0.027±0.006, respectively, after the addition of 1 mmol/L GSH ($P<0.01$ *vs* H_2O_2). The time when open probability began to increase and reached a maximum was shorter in inside-out patches than that in cell-attached patches (4.8±1.0 min *vs* 11.5±3.9 min, $P<0.01$; 9.6±1.6 min *vs* 18.7±4.7 min, $P<0.01$). **Conclusion:** H_2O_2 increased the $I_{Na.P}$ of guinea pig ventricular myocytes in a concentration-dependent manner, possibly by directly oxidating the cell membrane.

Introduction

The persistent sodium current ($I_{Na.P}$) in ventricular myocytes results from inactivate-resistant sodium channels continuing to open for long periods during prolonged depolarization. It plays an important role in maintaining the plateau of the action potential (AP), determining AP duration and transmural dispersion of repolarization, and development of cardiac arrhythmias[1]. Some studies suggest that hypoxia can increase cardiomyocyte $I_{Na.P}$ and the potentiated $I_{Na.P}$ can induce intracellular calcium overload and ischemic arrhythmias[2,3]. Recently, we studied the mechanisms of $I_{Na.P}$ generation and augmentation in normoxic and hypoxic conditions. The results suggest that excess nitric oxide (NO) produced during hypoxia can increase ventricular myocyte $I_{Na.P}$ by oxidizing cell membrane sodium channel protein, and the reducing agent dithiothreitiol (DTT) can block the potentiated $I_{Na.P}$ completely. This implies that oxidants can increase cardiac $I_{Na.P}$, and that reducing and the antioxidation agents can reduce $I_{Na.P}$[4]. A burst of hydrogen peroxide (H_2O_2) is generated in the myocardium during ischemia. Its effects on cell damage and membrane lipid peroxidation have been reported widely. However, reports on the effect of H_2O_2 on $I_{Na.P}$ in ventricular myocytes are scarce and contradictory[5,6]. In the present study, we examined the effects of H_2O_2 on $I_{Na.P}$ in guinea pig ventricular myocytes and explored the possible mechanisms underlying them.

Materials and methods

Isolation of guinea pig ventricular myocytes Adult guinea pigs (250 g–300 g, of either sex, Grade II, Certificate No 19-023, the Experiment Animal Center of Wuhan University of Science and Technology, Wuhan, China) were anesthetized with pentobarbital sodium (30 mg/kg, ip) 20 min after an intraperitoneal injection of 2000 units of heparin. Hearts were excised rapidly and perfused retrogradely on a langendorff apparatus (with a Ca^{2+}-free Tyrode's solution for 5 min), before the perfusate was switched to an enzyme-

containing solution [0.1 g/L collagenase type I, 0.01 g/L protease E, 0.5 g/L bovine serum albumin (BSA) in the same solution] for 8 min–10 min. The perfusate was finally changed to KB solution containing: 70 mmol/L KOH, 20 mmol/L taurine,50 mmol/L glutamic acid, 40 mmol/L KCl, 20 mmol/L KH_2PO_4, 3 mmol/L $MgCl_2$, 0.5 mmol/L egtazic acid, 10 mmol/L HEPES, and 10 mmol/L glucose, pH 7.4, for a 5 min period. These perfusates were bubbled with 100% O_2 and maintained at 37 °C. The ventricles were cut into small chunks and gently agitated in KB solution. The cells were filtered through nylon mesh and stored in KB solution at 4 °C.

Electrical recordings Myocytes were transferred to a chamber mounted on the mechanical stage of an inverted microscope (XDS-1, Chongqing, China), and perfused with normal Tyrode's solution containing: 135 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 0.33 mmol/L NaH_2PO_4, 10 mmol/L HEPES, 10 mmol/L glucose, pH7.4. Patch electrodes were pulled with a 2-stage puller (PP-830, Narishige Group, Tokyo, Japan). For whole-cell recordings, their resistances were in the range of 1.5 MΩ–3 MΩ when filled with a pipetted solution containing: 120 mmol/L CsCl, 1.0 mmol/L $CaCl_2$, 5 mmol/L $MgCl_2$, 5 mmol/L Na_2ATP, 10 mmol/L TEACl, 11 mmol/L egtazic acid, and 10 mmol/L HEPES, pH 7.3. The external solution was Tyrode's solution with $CdCl_2$ (200 μmol/L). For single-channel recordings, the shanks of pipettes with resistance of 6 MΩ–10 MΩ were coated with Sylgard and the tips were heat polished. The pipettes were filled with a solution composed of 180 mmol/L NaCl, 1.3 mmol/L KCl, 1. 5 mmol/L $CaCl_2$, 0.5 mmol/L $MgCl_2$, 5 mmol/L Na_2ATP, 3. 0 mmol/L $CoCl_2$, 10 mmol/L TEACl, 10 mmol/L 4-AP, 10 mmol/ L CsCl, 5 mmol/L HEPES, and 5 mmol/L glucose, pH 7.4. For single-channel cell-attached recordings, the myocytes were perfused with normal Tyrode's solution. For single-channel inside-out recordings, the perfusate was composed of 120 mmol/L KCl, 0.1 mmol/L $CaCl_2$, 2.0 mmol/L $MgCl_2$, 0. 1 mmol/L egtazic acid, and 10 mmol/L HEPES, pH 7.4. These perfusates were bubbled with 100% O_2. All experiments were carried out at room temperature (21±2 °C). Currents were obtained with a patch-clamp amplifier (EPC-9, Heka Electronic, Lambrecht, Pfalz, Germany), filtered at 2 kHz, digitized at 10 kHz, and stored on a computer hard disk for further analysis.

Drugs and reagents Collagenase type I and CsCl were obtained from Gibco (GIBCO TM, Invitrogen Co., Paisley, UK). Protease E, 4-AP, TEACl, Na_2ATP, and egtazic acid were purchased from Sigma Chemical Co (Saint Louis, Missouri, USA). Bovine serum albumin, HEPES, and taurine were obtained from Roche(Basel, Switzerland). Tetrodotoxin (TTX) was purchased from Hebei Fisheries Research Institute (Qinhuangdao, China). H_2O_2 was a production of Wuhan Zhongnan Chemical Reagent Co (Wuhan, China). Reduced GSH was a product of Shanghai Bio Life Science and Technology (Shanghai, China).

Data analysis Whole-cell recordings were analyzed using PulseFit (V8.65, HEKA). Current density was calculated by dividing the current amplitude by the cell capacitance. Single-channel recordings were analyzed using TAC+TACFit (X4.0.9, Bruxton, Seattle, Washington, USA). Capacitance transients and leakage currents were nullified by off-line subtracting fits of average blunt traces. The channel activity after a 50 ms depolarization pulse was calculated as persistent sodium channel. Open probability was calculated from the total open times of 50 sweeps divided by the total sweep duration. Histograms of channel open time distribution were fitted to single exponentials using TACFit. All data were expressed as mean±SD. Student's *t*-test was used for simple comparisons and ANOVA was used when appropriate. $P<0.05$ was considered to be statistically significant.

Results

H_2O_2 increases the amplitude of $I_{Na.P}$ in a concentration-dependent manner The experiments were carried out in the whole-cell configuration of the patch-clamp techniques. Currents were evoked by a 500 ms pulse to -30 mV from a holding potential of -120 mV. TTX (1.5 μmol/L) blocked completely the smaller persistent inward current, but had less effect on the transient sodium current ($I_{Na.T}$), (n=7 cells from 4 guinea pigs; Figure 1). The recorded current was proved to be $I_{Na.P}$.

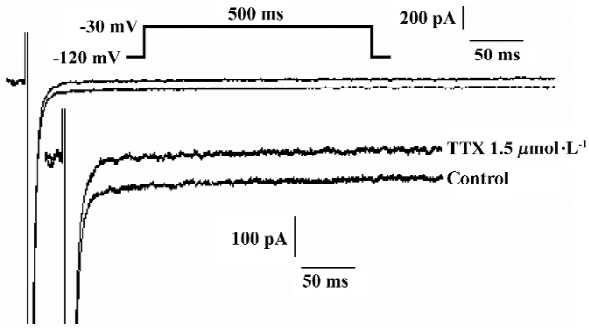

Figure 1. Effect of tetrodotoxin (TTX) on persistent sodium current ($I_{Na.P}$) in guinea pig ventricular myocytes. The figure shows the current curves before and after exposure to TTX (1.5 μmol/L).

Using the pulse protocol described above, 4 cells were perfused with H_2O_2 (0.5 mmol/L) after the $I_{Na.P}$ had stabilized. $I_{Na.P}$ began to increase at approximately 5 min and reached a

maximum at 10–12 min after perfusion with H_2O_2. The current is able to last for another 30 min at this level. In another 8 cells, H_2O_2 was added into solution in a cumulative manner after the control $I_{Na.P}$ values were recorded. Cells were perfused with H_2O_2 (0.1 mmol/L, 0.5 mmol/L and 1.0 mmol/L) and GSH (1.0 mmol/L) at 10 min intervals, and the currents were recorded in the same cell. H_2O_2 increased the amplitude of $I_{Na.P}$ in a concentration-dependent manner, while GSH reversed the increased $I_{Na.P}$ (Figure 2). The amplitude of $I_{Na.P}$ was recorded at 200 ms of the pulse to eliminate the effect of $I_{Na.T}$. H_2O_2 (0.1 mmol/L, 0.5 mmol/L, and 1.0 mmol/L) increased the mean current density of $I_{Na.P}$ from the control value 0.385±0.032 pA/pF to 0.578±0.080 pA/pF , 0.763±0.094 pA/pF, and 1.007±0.179 pA/pF, respectively (n=8, P<0.01 *vs* control). The mean current density was decreased to 0.329± 0.063 pA/pF after the application of 1.0 mmol/L GSH (n=8, P<0.05 *vs* control).

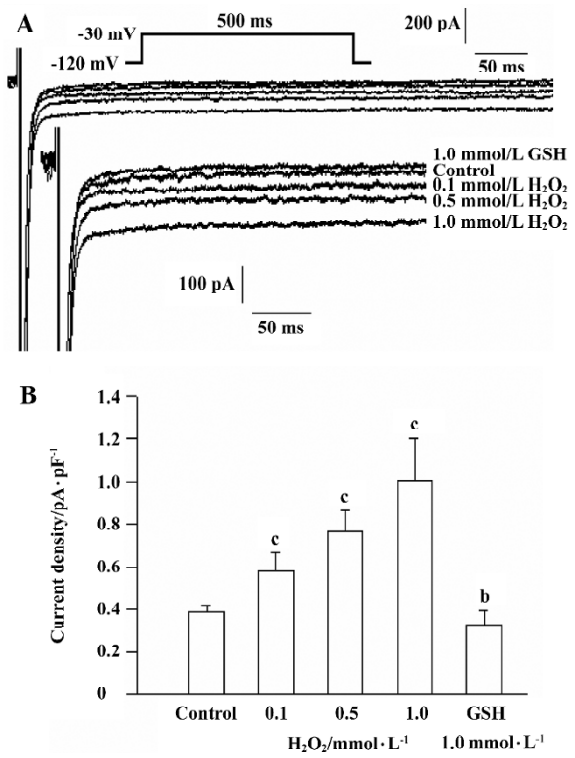

Figure 2. Effect of H_2O_2 on persistent sodium current ($I_{Na.P}$) in guinea pig ventricular myocytes. (A) H_2O_2 (0.1 mmol/L, 0.5 mmol/L and 1.0 mmol/L) increases $I_{Na.P}$ in a concentration-dependent manner, and glutathione (GSH; 1.0 mmol/L) reverses the increased $I_{Na.P}$ induced by H_2O_2. (B) The mean current densities of $I_{Na.P}$ under different conditions. n=8 cells. Mean±SD. ^bP<0.05; ^cP<0.01 *vs* control.

Effect of glutathione on persistent sodium channel activity induced by H_2O_2 The experiments were carried out in cell-attached and inside-out patches. Currents were activated by a 700 ms voltage pulse to -50 mV from a holding potential of -120 mV.

In cell-attached recording (the resting membrane potential was -74.6±7.7 mV, n=12, which was measured in current-clamp mode under identical conditions), bath solution with 1 mmol/L H_2O_2 was used to perfuse the cell. In 17 cell-attached patches, persistent sodium channel activity increased remarkably at 11.5±3.9 min. The current type was changed from background currents to burst currents (flaring up rapidly but subsiding over a very long period). At 18.7 ± 4.7 min the channel activity reached a maximum. In 6 of the 17 patches, the channel activity could maintain for another 30 min at this level (Figure 3).

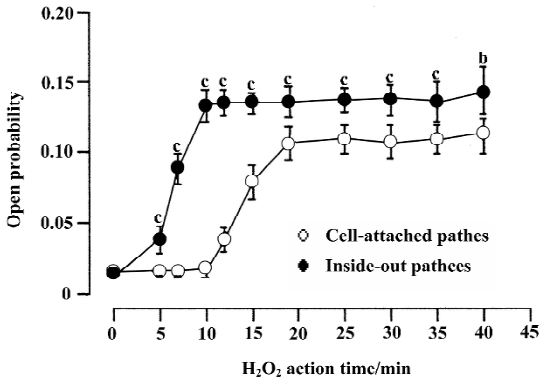

Figure 3. H_2O_2 increases persistent sodium channel activity in a time-dependent manner. The figure shows the mean open probability changing course with time in cell-attached (n=6) and inside-out (n=5) patches after the application of 1 mmol/L H_2O_2. The abscissa shows the action time of H_2O_2. The ordinate shows the mean open probability. n=5–6 patches. Mean±SD. ^bP<0.05; ^cP<0.01 *vs* cell-attached patches group.

In 5 of the 17 patches, lidocaine (100 μmol/L) was added to the bath solution and the increased $I_{Na.P}$ was blocked completely at approximately 5 min (Figure 4).

We applied 1 mmol/L GSH in 6 other patches. The presentation traces are shown in Figure 5. Before exposure to H_2O_2, channel activity was very low or often absent (Figure 5A). After 10-min treatment with H_2O_2, persistent sodium channel activity increased markedly (Figure 5B) and reached a maximum at 20 min (Figure 5C). GSH (1 mmol/L) reversed the increase in sodium channel activity caused by H_2O_2 (Figure 5D). H_2O_2 (1 mmol/L) increased the mean open probability and mean open time from control values of 0.015± 0.004 and 0.744±0.190 ms to 0.106±0.011 and 1.966±0.539 ms, respectively (n=6, both P<0.01 *vs* control). They were then decreased to 0.039± 0.024 and 1.137±0.153 ms, respectively,

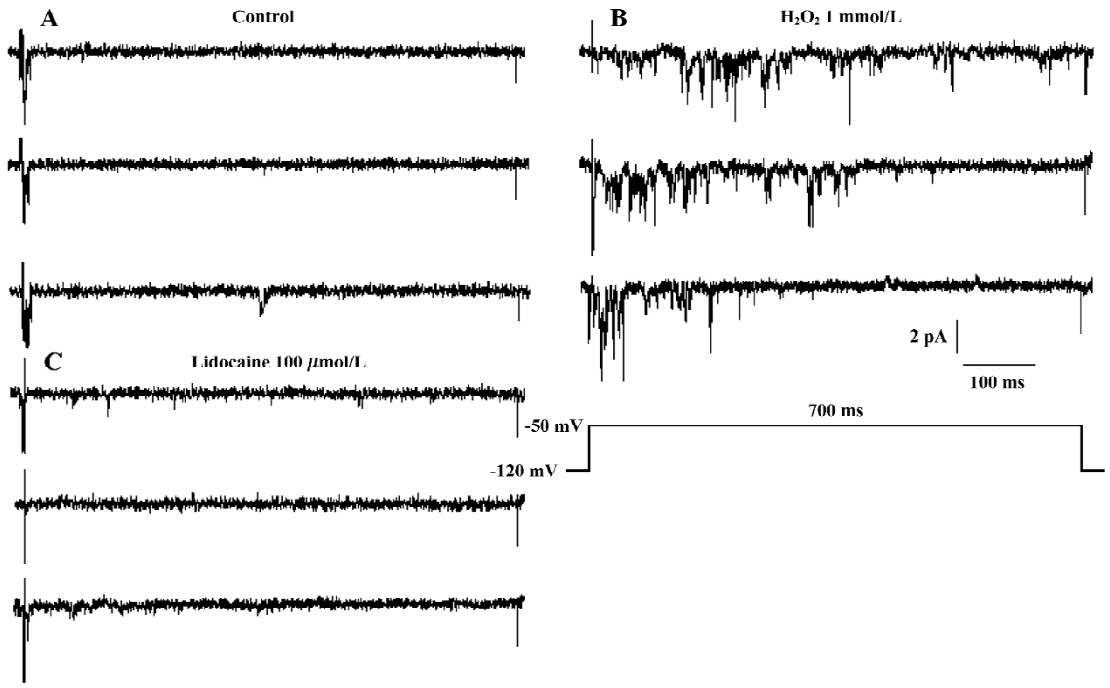

Figure 4. Lidocaine blocks persistent sodium channel activity induced by H_2O_2. The individual current traces were evoked by a voltage step to -50 mV from a holding potential of -120 mV in a cell-attached patch. (A) Control, (B) H_2O_2 (1 mmol/L) and (C) lidocaine (100 μmol/L).

after the application of 1 mmol/L GSH (n=6, both P<0.01 *vs* H_2O_2). Figure 6 gives an example of corresponding all-time histograms and mean open-time histograms from another cell-attached patch.

The results for inside-out recordings were similar to those for cell-attached recordings. In the 10 inside-out patches, persistent sodium channel activity increased markedly at 4.8±1.0 min and reached maximum at 9.6±1.6 min after the application of 1 mmol/L H_2O_2. Compared with the cell-attached recordings, the times when the sodium channel activity in inside-out recordings began to increase (4.8±1.0 min *vs* 11.5±3.9 min, P<0.01) and reached a maximum (9.6±1.6 min *vs* 18.7±4.7 min, P<0.01) were shorter. In 5 of the 10 patches, the channel activity could be maintained for another 30 min at this level (Figure 3). In the other 5 patches, 1 mmol/L GSH was applied. H_2O_2 (1 mmol/L) increased the mean open probability and mean open time from the control value 0.012±0.003 and 0.537±0.015 ms,respectively, to 0.136±0.010 and 0.966±0.130 ms, respectively (n=5, both P<0.01 *vs* control). They were decreased to 0.027±0.006 and 0.672±0.042 ms, respectively, after the application of 1 mmol/L GSH (n=5, both P<0.01 *vs* H_2O_2).

Discussion

A large number of studies have reported that H_2O_2 in cardiomyocytes is increased during ischemia. The excessive amount of H_2O_2 leads to intracellular Ca^{2+} overload and cell damage. Recent reports show that hypoxia can increase $I_{Na.P}$ in ventricular myocytes, induces intracellular sodium overload, which promotes Ca^{2+} overload via reverse Na^+-Ca^{2+} exchange, and prolongs the duration of action potential (AP) after-depolarization[2,3]. Thus, the present study on the effect of H_2O_2 on $I_{Na.P}$ is very important to further understand the mechanisms of cardiomyocytes injury induced by H_2O_2, and the nature of $I_{Na.P}$.

In whole-cell patch-clamp recordings, 1.5 μmol/L TTX blocked completely the smaller inactivation-resistant inward current (Figure 1). Similarly, in cell-attached recordings, 100 μmol/L lidocaine blocked the potentiated inward currents induced by H_2O_2 (Figure 4). These results affirm that the recorded currents are I_{NaP}. Barrington *et al* reported that there were no effects from a 30-min exposure to 1 mmol/L H_2O_2 on the slowly inactivating sodium currents of feline ventricular myocytes, so hydroperoxide could not induce an intracellular sodium overload[5]. However, our study shows that H_2O_2 increases the amplitude of whole-cell $I_{Na.P}$ of guinea pig ventricular myocytes in a concentration-dependent manner (Figure 2) and the activity of persistent sodium channel in both single-channel recordings (Figures 3–6). These results confirm that H_2O_2 can increase $I_{Na.P}$ of guinea pig

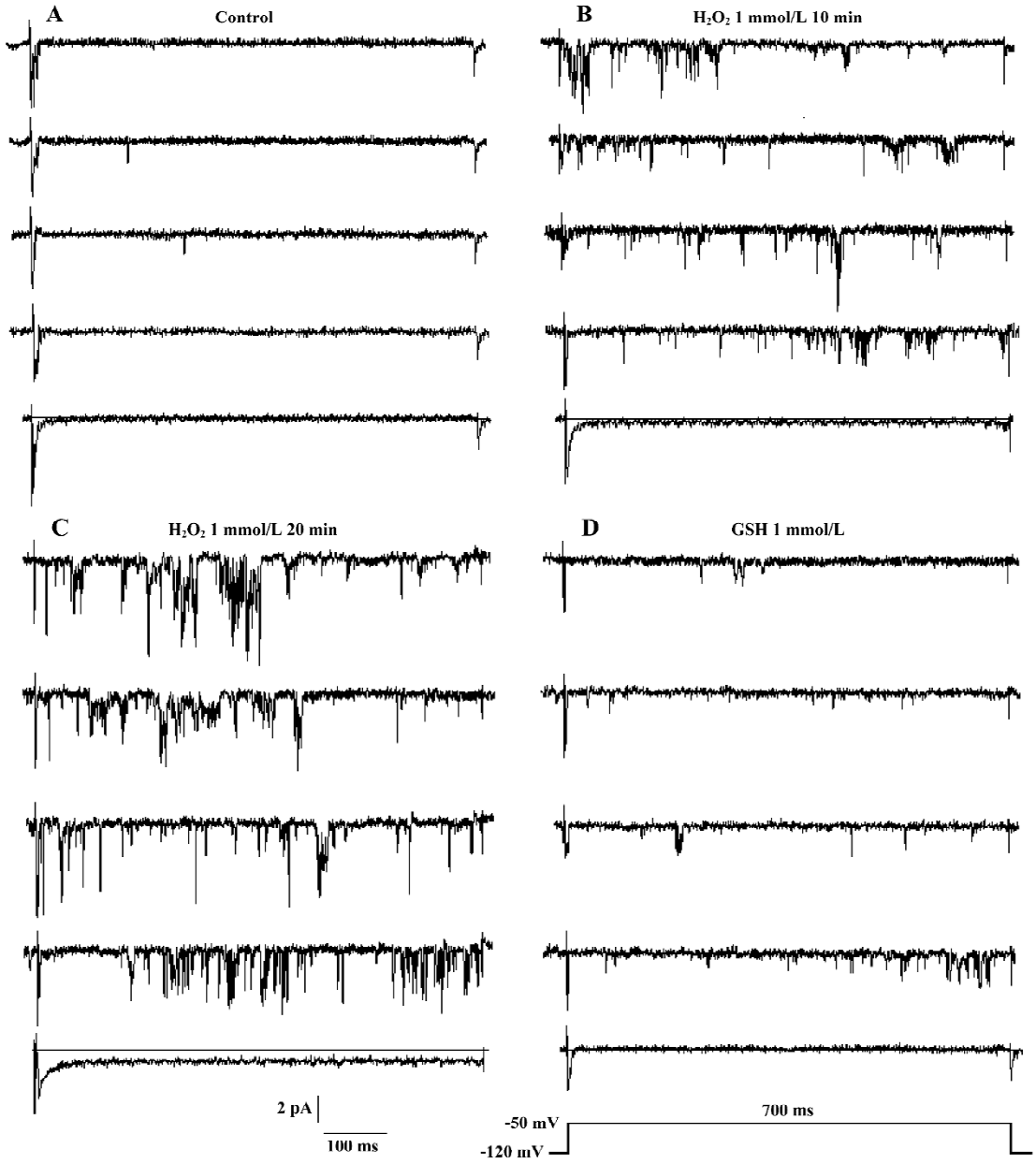

Figure 5. Effect of glutathione (GSH) on persistent sodium channel activity induced by H_2O_2. The individual current traces were evoked by a voltage step to -50 mV from a holding potential of -120 mV in a cell-attached patch. The upper four traces in each of parts (A–D) are original current records whereas the fifth trace in each panel shows the average of 50 sweeps. (A) Control, (B) 10 min after perfusion with 1 mmol/L H_2O_2, (C) 20 min after perfusion with 1 mmol/L H_2O_2 and (D) GSH (1 mmol/L).

ventricular myocytes, which is different from the report of Barrington *et al*. The disparity may be attributable to species differences. The result that H_2O_2 increases $I_{Na.P}$ in a concentration-dependent manner is similar to our previous report that hypoxia increases the $I_{Na.P}$ of guinea pig ventricular myocytes in a time-dependent manner[4], further suggesting that the increase in $I_{Na.P}$ is closely associated with reactive oxygen species. Ward and Giles observed that H_2O_2 produced a marked prolongation of the AP by slowing inactivation of the TTX-sensitive sodium current, which was verified by the single-channel cell-attached recording results that late opening events were enhanced when 200 μmol/L H_2O_2 was included in the recording pipette[6]. Bisindolylamaleimide, the protein kinase C (PKC) blocker, significantly

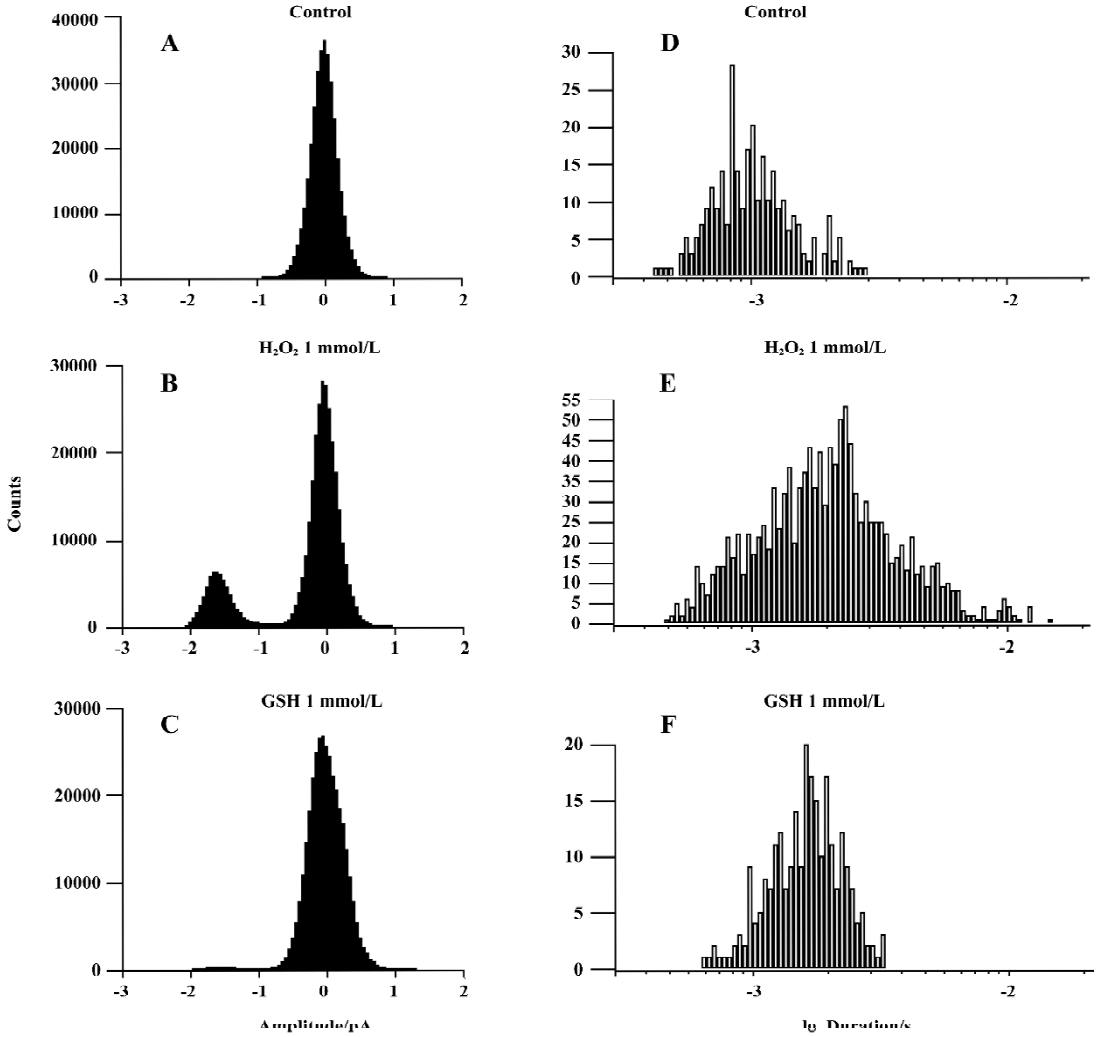

Figure 6. Effect of glutathione (GSH) on mean open probability and mean open time of persistent sodium channels enhanced by H_2O_2. Each figure comes from 50 individual current traces in a cell-attached patch. Parts (A–C) show the all-point histograms, and (D–F) show the mean open time histograms. (A,D) Control, (B,E) H_2O_2 (1 mmol/L) and (C,F) GSH (1 mmol/L). The fitted mean open times were 0.750 ms, 2.076 ms and 1.338 ms in (D–F), respectively.

delayed and attenuated the development of AP prolongation, which indicated involvement of an intracellular second messenger-PKC pathway[6]. In our study, H_2O_2 increased the persistent sodium channel activity in both the inside-out and cell-attached patches, which suggests that H_2O_2 may take effect by directly oxidizing the cell membrane in addition to its involvement as an intracellular second messenger. These results are consistent with the findings of Hammarström and Gage who reported that hypoxia, NO, and sodium cyanide could increase persistent sodium channel activity in rat hippocampal neurons in inside-out recordings, which could then be reversed by DTT[7,8].

H_2O_2 increased the persistent sodium channel activity in both cell-attached and inside-out patches (Figure 3). However, the time when the persistent sodium channel activity began to increase and reached a maximum was significantly shorter in inside-out patches compared with cell-attached patches. One possible explanation is that the inside-out patches, which were directly exposured to H_2O_2, lost the protection of intracellular antioxidant enzymes and antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, ascorbic acid, α-tocopherol and glutathione.

Glutathione inhibited the H_2O_2-elicited persistent sodium channel activity (Figures 2,5,6), which suggested that H_2O_2 could take effect by oxidation. It is similar to our previous

report, which shows that excessive NO produced during hypoxia can increase the $I_{Na.P}$ of ventricular myocytes by oxidizing cell membrane sodium channel proteins and generating I_{NaP} under normoxic conditions, probably in association with the oxidation state of channel proteins[4]. H_2O_2 is a cysteine-specific oxidant[9] and GSH is an important antioxidant that can protect the protein's thiol group from oxidation[10]. The present study shows that GSH can reverse the persistent sodium channel activity caused by H_2O_2. Therefore, we think that persistent sodium channel activity may be associated with oxidation state, and oxidants can increase activity by oxidizing the channel protein. H_2O_2 as an oxidant may increase persistent sodium channel activity by oxidizing the thiol group of proteins in cell membranes.

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