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Mechanisms underlying the modulation of the L-type Ca\textsuperscript{2+} channel by hydrogen peroxide in guinea-pig ventricular myocytes

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Running title: H\textsubscript{2}O\textsubscript{2}-mediated facilitation of Ca\textsubscript{v}1.2 channels

Contribution:
L. Yang carried our most of the experiments and wrote the manuscript.
J. Xu, E. Minobe, L. Yu and R. Feng did a part of the experiments and discussed on the data.
A. Kameyama and K. Yazawa contributed to the planning of this study and discussed on the data and the manuscript.
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Abstract
Although Cav1.2 Ca\(^{2+}\) channels are modulated by reactive oxygen species (ROS), the underlying mechanisms are not fully understood. In this study, we investigated effects of hydrogen peroxide (H\(_2\)O\(_2\)) on the Ca\(^{2+}\) channel using a patch-clamp technique in guinea-pig ventricular myocytes. Externally applied H\(_2\)O\(_2\) (1 mM) increased Ca\(^{2+}\) channel activity in the cell-attached mode. A specific inhibitor of Ca\(^{2+}\)/calmodulin-dependent protein kinase II, (CaMKII) KN-93 (10 \(\mu\)M), partially attenuated the H\(_2\)O\(_2\)-mediated facilitation of the channel, suggesting both CaMKII-dependent and independent pathways. However, in the inside-out mode, 1 mM H\(_2\)O\(_2\) increased channel activity in a KN-93-resistant manner. Since H\(_2\)O\(_2\)-pretreated calmodulin did not reproduce the H\(_2\)O\(_2\) effect, the H\(_2\)O\(_2\) target was presumably assigned to the Ca\(^{2+}\) channel itself. A thiol-specific oxidizing agent mimicked and occluded the H\(_2\)O\(_2\) effect. These results suggest that H\(_2\)O\(_2\) facilitates the Ca\(^{2+}\) channel through oxidation of cysteine residue(s) in the channel as well as via the CaMKII-dependent pathway.

**Keywords** calcium channel, reactive oxygen species, H\(_2\)O\(_2\), calmodulin, cardiac myocytes
Introduction
An alteration in the cell’s redox state such as increased reactive oxygen species (ROS) production is associated with pathology [1-3]. In the heart, ROS as highly reactive compounds accumulate in tissues during myocardial ischemia/reperfusion and cause peroxidation of lipids and proteins [4], which play an important role in the pathogenesis of ischemia/reperfusion abnormalities, including myocardial stunning, irreversible injury, and reperfusion arrhythmias [5]. ROS-induced Ca$^{2+}$ overload is one of the major causes of cardiomyocyte injury during ischemia/reperfusion [6]. Ca$^{2+}$ overload induced by oxidation is thought to be mediated by increased Ca$^{2+}$ release from the sarcoplasmic reticulum (SR) through the Ca$^{2+}$ release channel (ryanodine receptor 2, RyR2) and decreased Ca$^{2+}$ uptake by inhibiting Ca$^{2+}$-ATPase (SERCA) activity [7].

L-type (Cav1.2) Ca$^{2+}$ channels (LTCCs) in the myocardium sarcolemma are the main route for Ca$^{2+}$ influx into cells. Different from skeletal muscle, in cardiac myocytes, Ca$^{2+}$ influx through LTCCs triggers Ca$^{2+}$ release, determining the Ca$^{2+}$ dynamics in the cardiac myocytes. Accumulating evidence shows that basal activity of LTCCs is modulated by cytoplasmic factors including protein kinase-mediated phosphorylation [8, 9], phosphatase-mediated dephosphorylation (8-9) and the interaction with Ca$^{2+}$ and Mg$^{2+}$ [10], lipids [11] and proteins [12, 13]. Recent studies suggest that the function of LTCC is crucially modulated by ROS during ischemia/reperfusion [14, 15]. Exposure of myocytes to a high concentration of H$_2$O$_2$ results in alteration of Ca$^{2+}$ channel activity and cellular Ca$^{2+}$ homeostasis [16]. However, the mechanism underlying the effects of oxidation is so far controversial since both inhibition and facilitation of LTCCs by oxidation have been suggested [17-20]. The mechanism of the ROS effect on LTCCs also remains elusive. For example, H$_2$O$_2$ has been suggested to facilitate LTCCs by activation of Ca$^{2+}$/CaM-dependent protein kinase II (CaMKII) through oxidation of methionine residues in CaMKII or increasing Ca$^{2+}$ release through RyR (20). On the other hand, Tang et al. [19] have reported that H$_2$O$_2$-induced facilitation of LTCCs is mediated by glutathionylation of LTCCs.
To explore the effect of oxidation on cardiac LTCCs and the underlying mechanisms, the inside-out mode of patch-clamp technique is beneficial since the internal side of LTCC can be well controlled. We have previously found that LTCC activity is maintained with calmodulin (CaM) and ATP without run-down of the channel in the inside-out patches [12, 21-26]. In this study, using this method we have investigated the effect of H$_2$O$_2$ on the current through LTCCs. H$_2$O$_2$ was found to increase Ca$^{2+}$ channel activity both in the cell-attached mode and the inside-out mode. The specific CaMKII inhibitor KN-93 partially attenuates the facilitation effect in the cell-attached mode, but had no effect in the inside-out mode. Our results suggest H$_2$O$_2$ facilitates LTCCs through CaMKII-dependent and independent pathways.

**Materials and methods**

**Materials**

MgATP, hydrogen peroxide (H$_2$O$_2$) tablet and 5,5’-dithiobis (2-nitrobenzoic acid) (DNTB) (cysteine residue oxidation reagent) were purchased from Sigma-Aldrich (St. Louis, MO, USA), KN-93 (CaMKII inhibitor) and KN-92 (inactive analog of KN-93) were from Calbiochem (San Diego, CA, USA) and Bay K 8644 (Ca$^{2+}$ channel agonist) was from Wako (Osaka, Japan).

**Preparation of single cardiac myocytes**

Single ventricular myocytes were isolated from adult guinea-pig hearts by collagenase dissociation as described previously [27]. In brief, a female guinea pig (weight 300-500 g) was anesthetized with pentobarbital sodium (30 mg/kg, i.p.), and the aorta was cannulated in situ under artificial respiration. The dissected heart was mounted on a Langendorff apparatus and perfused with Tyrode solution for 3 min, then with nominally Ca$^{2+}$-free Tyrode solution for 5 min, and finally with Ca$^{2+}$-free Tyrode solution containing collagenase (0.08 mg/ml; Yakult) for 7–15 min. The collagenase was washed away with a high K$^+$, low Ca$^{2+}$ solution (storage solution). The single ventricular myocytes were dispersed and filtered through a stainless steel
mesh (105 µm). 0.05 mg/ml protease (Type XIV, Sigma) and 0.02 mg/ml DNase I (Type IV, Sigma) were incubated with the myocytes to improve the success rate in attaining a gigaohm seal. The enzyme-treated myocytes were then washed twice by centrifugation (800 rpm for 3 min) and stored at 4°C.

The experiments were carried out under the approval of the Committee of Animal Experimental, Kagoshima University.

Solutions
Tyrode solution contained (in mM) 135 NaCl, 5.4 KCl, 0.33 NaH₂PO₄, 1.0 MgCl₂, 5.5 glucose, 1.8 CaCl₂, and 10 HEPES-NaOH buffer (pH 7.4). The storage solution was composed of (in mM) 70 KOH, 50 glutamic acid, 40 KCl, 20 KH₂PO₄, 20 taurine, 3 MgCl₂, 10 glucose, 10 HEPES, and 0.5 EGTA and the pH was adjusted to 7.4 with KOH. The pipette solution contained (in mM) 50 BaCl₂, 70 tetraethylammonium chloride, 0.5 EGTA, 0.003 BAY K 8644, and 10 HEPES-CsOH buffer (pH 7.4). The basic internal solution consisted of (in mM) 90 potassium aspartate, 30 KCl, 10 KH₂PO₄, 1 EGTA, 0.5 MgCl₂, 0.5 CaCl₂, and 10 HEPES-KOH buffer (pH 7.4; free Ca²⁺ 80 nM, pCa 7.1). CaM and MgATP were dissolved in basic internal solution in the inside-out patch mode.

Preparation of CaM
The cDNA of human CaM cloned into the pGEX6P-3 vector (GE Healthcare Bioscience, Uppsala, Sweden) was expressed as a glutathione-S-transferase (GST) fusion protein in Escherichia coli BL21 and purified using glutathione–Sepharose 4B (GE Healthcare). The GST region was cleaved by PreScission Protease (GE Healthcare). The purity of CaM was confirmed by SDS-PAGE and quantified by the Bradford method (Thermo Fisher Scientific, Rockford, IL, USA) with bovine serum albumin as the standard and a correction factor of 1.69.

Patch clamp and data analysis
Barium current through LTCCs was recorded in the cell-attached and inside-out mode using the patch-clamp technique. For the recording in the cell-attached mode, the myocytes were perfused with the basic internal solution at 31–35°C using a patch pipette (2–4 MΩ) containing 50 mM Ba²⁺ and 3 µM Bay K8644. Bay K8644, a Ca²⁺ channel agonist, was used to prolong the open time of the channel to facilitate the experiments. Barium currents through LTCCs were elicited by depolarizing pulses from -70 to 0 mV for a 200 ms duration at a rate of 0.5 Hz. The currents were recorded with a patch-clamp amplifier (EPC-7; List, Darmstadt, Germany) and fed to a computer at a sampling rate of 3.3 kHz. The mean current (I) was measured and divided by the unitary current amplitude (i) to yield NPo, based on the equation 

\[ I = N \times P_o \times i, \]

where N is the number of channels in the patch and Po is the time-averaged open-state probability of the channels. Data are presented as the mean ± S.E. Student’s t-test or ANOVA with post-hoc Tukey HSD test was used to estimate statistical significance and values of P < 0.05 were considered as significant.

RESULTS

H₂O₂ facilitated Ca²⁺ channel in the cell-attached mode via CaMKII-dependent and independent pathways

We first examined the effect of H₂O₂ on the current through LTCCs in the cell-attached mode in guinea-pig ventricular myocytes. After recording the current for 2 minutes as a control, 1 mM H₂O₂ was applied in the perfusion solution. As shown in Fig. 1A and B, Ca²⁺ channel activity was rapidly increased without a change in the unitary current amplitude. Taking an average of six patches, the Ca²⁺ channel activity was increased to 206 ± 32 % of the control (Fig. 1D). This result confirmed the facilitating effect of H₂O₂ on LTCCs.

Facilitation of LTCCs by glutathionylation [19] or phosphorylation mediated by activated CaMKII [20] during oxidative stress has been proposed. To evaluate the possible CaMKII-dependent effect of H₂O₂, we incubated the cardiomyocytes with 10 µM KN-93, a specific CaMKII inhibitor, for 10 minutes before recording the current. Under this condition, it has
been reported that activity of CaMKII is nearly completely inhibited by KN-93 [28, 29]. As shown in Fig. 1C, KN-93 significantly attenuated H₂O₂-mediated facilitation (132 ± 15 % (n=9) vs 206 ± 32 % with no drug, P<0.05), while KN-92, an inactive analog of KN-93, partially attenuated the facilitation but it was not statistically significant (156 ± 13 % (n=5) vs 206 ± 32 % with no drug, P=0.30). These results suggested that not only CaMKII-dependent but also independent pathways were involved in H₂O₂-mediated facilitation of LTCCs.

**H₂O₂ facilitated Ca²⁺ channel in the inside-out mode independently of CaMKII**

To explore the mechanism of CaMKII-independent facilitation of LTCCs produced by H₂O₂, we investigated the H₂O₂ effect on LTCCs in the inside-out patches in which the Ca²⁺ channel activity was maintained by application of 1 μM CaM together with 3 mM ATP [12, 21-26]. In the inside-out patch mode, LTCCs were disconnected with cytoplasmic factors and perfused with an artificial solution with known composition. This was quite beneficial to examine direct effects of external reagents on LTCC. After the patch was excised and moved to a small inlet in the perfusion chamber which was connected to a microinjection system, CaM/ATP was immediately applied to induce Ca²⁺ channel activity, the single channel current was recorded for 3 minutes as a control current, then 1 mM H₂O₂ was added into the CaM/ATP solution. As shown in Fig. 2A, H₂O₂ significantly increased the CaM-induced Ca²⁺ channel activity in the inside-out mode. This facilitatory effect of H₂O₂ was concentration dependent up to 1-2 mM, and higher concentrations of H₂O₂ conversely inhibited Ca²⁺ channel activity presumably because of a non-specific deteriorating effect of H₂O₂ (Fig. 2B). These results suggested that H₂O₂ (<2 mM) facilitated Ca²⁺ channel activity in the inside-out patches via a direct modification of LTCC and/or its closely-located proteins such as CaM and CaMKII. To assess the possibility that CaMKII was still located near the channel and modulated channel activity in the excised patches, we examined the effect of KN-93 in the inside-out patches. As shown in Fig. 2C, KN-93 had only a small effect on the H₂O₂-mediated facilitation of LTCCs in the inside-out mode. In summary, channel activity was modulated by H₂O₂ from 144 ± 32 %
(control) to 272 ± 70 % in the absence of KN-93 (n=5), and from 139 ± 21 % to 231 ± 29 % in the presence of KN-93 (n=6). Although KN-93 seemed to slightly attenuate the increasing effect of H$_2$O$_2$ on channel activity, this difference was statistically insignificant. Thus KN93 did not significantly affect the H$_2$O$_2$-mediated facilitation of the Ca$^{2+}$ channel in the inside-out mode.

H$_2$O$_2$ may also be able to oxidize CaM and modulate the effect of CaM on Ca$^{2+}$ channel activity. To assess this possibility, we examined the effect of oxidized CaM pretreated with 1 mM H$_2$O$_2$ at room temperature for 30 minutes. As shown in Fig. 3A and B, after Ca$^{2+}$ channel activity was maintained by intact (untreated with H$_2$O$_2$) CaM + ATP in the inside-out patches, we substituted the oxidized CaM for the untreated CaM. Channel activity did not change significantly, suggesting that CaM was not oxidized or oxidized CaM, if any, had similar potency as the untreated CaM on activity of LTCC (Fig. 3C). This result suggested that oxidation of CaM was not involved in the H$_2$O$_2$-mediated facilitation of LTCCs, and thus a direct oxidation of LTCCs might be a possible mechanism for the facilitation in the inside-out mode.

**Cysteine residues in the Ca$^{2+}$ channel are involved in H$_2$O$_2$-mediated facilitation**

The α1C subunit of LTCC contains 38 cysteine residues and 36 methionine residues in the cytoplasmic chains, which are potentially subject to oxidation modification. To identify the amino acid residue which was oxidized by H$_2$O$_2$ and responsible for the H$_2$O$_2$-mediated facilitation of LTCCs, a specific oxidizing agent of cysteine residues 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was applied in place of H$_2$O$_2$. As shown in Fig. 4, 1 mM DTNB significantly increased Ca$^{2+}$ channel activity maintained by CaM (from 129 ± 22 % up to 184 ± 21 %, n=5), suggesting that oxidation of cysteine residue(s) was responsible, at least partially, for the H$_2$O$_2$-mediated facilitation of LTCCs. Since there is no specific oxidizing agent of methionine residues available, we examined the effects of H$_2$O$_2$ on LTCC after application of DTNB. Application of H$_2$O$_2$ + CaM after DTNB + CaM only slightly increased
Ca\textsuperscript{2+} channel activity and was statistically insignificant (Fig. 5). These results suggested that oxidation of cysteine residue(s) was the major cause of the H\textsubscript{2}O\textsubscript{2}-mediated facilitation of LTCCs in the inside-out patches.

**Discussion**

In the present study, we investigated the effect of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) on the L-type Ca\textsuperscript{2+} channel (LTCC) of cardiac myocytes in the cell-attached and the inside-out mode. We found that H\textsubscript{2}O\textsubscript{2} facilitates cardiac LTCCs through two pathways: 1) direct oxidation of cysteine residue(s) of the channel; 2) indirect pathways via activation of CaMKII.

Changes of the redox state in the cardiac myocytes play an important role in heart diseases. LTCC as a major regulator of cardiac function is subject to redox modification. Although there is accumulating evidence supporting that reactive oxygen species (ROS) modulate the function of LTCCs, the results of these studies are controversial: Oxidizing agents are reported to inhibit the human and rabbit cardiac LTCC expressed in HEK293 cells [17, 18]. In isolated guinea-pig ventricular myocytes, oxidation decreased the current through LTCC [30]. On the other hand, a decrease in cellular superoxide and H\textsubscript{2}O\textsubscript{2} is associated with a decrease in the current of native [31, 32] and expressed LTCCs [17, 33], while the thiol-specific oxidizing agent DNTB increases the LTCC current [34]. The complicated interactions between LTCC and uncontrolled cytoplasmic factors may be partially responsible for the uncertainty surrounding the effects of oxidation on LTCCs. The present study took advantage of the inside-out mode of patch-clamp technique in which most of the cytoplasmic factors were washed out [12, 21-26]. Our results show that H\textsubscript{2}O\textsubscript{2} facilitates LTCC at concentrations up to 2 mM and inhibits the channel at higher concentrations. This finding may partly account for the diverse results in the previous studies.

The underlying mechanisms of modulation of the Ca\textsuperscript{2+} channel by H\textsubscript{2}O\textsubscript{2} are not completely clear. Song et al. [20] have reported that H\textsubscript{2}O\textsubscript{2}-mediated facilitation of the Ca\textsuperscript{2+} channel through activation of CaMKII can be activated either by Ca\textsuperscript{2+}/CaM or oxidation of methionine.
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residues in CaMKII. Thus, H$_2$O$_2$ is involved in both activation processes: 1) H$_2$O$_2$ enhances Ca$^{2+}$ release from SR by increasing RYR activity; 2) Oxidation of methionine residues in CaMKII protein (amino acid numbers: 281 and 282 in mouse) sustains the kinase activity independent of Ca$^{2+}$/CaM [3]. However, Tang et al. [19] suggest that H$_2$O$_2$ facilitates the Ca$^{2+}$ channel through direct glutathionylation of the channel protein. It is difficult to distinguish the direct effect of H$_2$O$_2$ from an indirect one when examining whole cells. In the present study, we employed a method to record LTCC current in the inside-out mode in which channel activity was maintained by CaM/ATP in the internal solution [12, 21-26]. Our results show that the CaMKII specific inhibitor KN-93 did not completely attenuate H$_2$O$_2$-mediated facilitation of LTCC in the cell-attached mode, suggesting that H$_2$O$_2$-mediated facilitation is mediated by not only a CaMKII-dependent pathway but also CaMKII-independent pathways. The finding that KN-93 does not inhibit the H$_2$O$_2$ effect in the inside-out mode indicated that the CaMKII-mediated facilitation is absent in the inside-out mode, and hence implies that CaMKII might not be attached to LTCC or, if present, it might not be in a state sensitive to oxidation in our inside-out patches.

Thus our results indicate that, in addition to the CaMKII-dependent pathway, there is an additional CaMKII-independent pathway for the H$_2$O$_2$-mediated facilitation of LTCC. Since most intracellular proteins are washed out in the inside-out patches, it is likely that direct oxidation of LTCC or its associated proteins by H$_2$O$_2$ might be involved in the facilitation of LTCC. Since H$_2$O$_2$-pretreated CaM does not mimic the facilitatory effect of H$_2$O$_2$, oxidation of CaM does not account for the mechanism of facilitation. This is consistent with the fact that human CaM does not contain any cysteine residues. Thus, it seems most likely that the Ca$^{2+}$ channel protein itself undergoes direct oxidation by H$_2$O$_2$ as the CaMII-independent pathway of the LTCC facilitation.

Both cysteine and methionine residues are subject to oxidation by H$_2$O$_2$. The pore-forming subunit $\alpha 1C$ of cardiac LTCC is rich in cysteine and methionine residues in the cytoplasmic chains [35]. Our findings that the specific cysteine oxidizing agent DTNB mimics
the H₂O₂ effect and that the effect of subsequently applied H₂O₂ is largely occluded suggests that cysteine residue(s) are involved in the H₂O₂-mediated facilitation. However, this does not exclude a possible involvement of a methionine residue(s). Future work should determine the oxidation sites responsible for the H₂O₂-mediated modulation of LTCC. In conclusion, LTCC may undergo ROS-mediated modification via the direct oxidation of LTCC as well as the indirect pathways involving CaMKII activation. This would be relevant for the understanding of ROS-mediated regulation of ion channels and Ca²⁺ overload and arrhythmogenesis during oxidation stress on the heart.

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Conflict of interest
The authors declare that they have no conflict of interest.
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Figure legends

**Fig. 1** H$_2$O$_2$ facilitates L-type Ca$^{2+}$ channel activity in cell-attached mode.  
A Time course of channel activity ($NPo$) recorded in cell-attached mode before and after application of 1 mM H$_2$O$_2$.  
B Examples of current traces of the Ca$^{2+}$ channels before (a) and after (b) application of H$_2$O$_2$ taken at the times indicated in A.  
C Effect of 1 mM H$_2$O$_2$ on channel activity ($NPo$) recorded in the cell-attached mode in the presence of 10 µM KN-93.  
D Summary of the normalized activity of the Ca$^{2+}$ channel treated with H$_2$O$_2$ with no drug (n=6), and with 10 µM KN-93 (a specific CaMKII inhibitor) (n=9) or KN-92 (an inactive form of KN-93) (n=5).  
Mean channel activity (60 traces) in each patch was normalized to the corresponding control value, averaged in the same group, and shown as mean ± S.E. * P<0.05 and ** P<0.01 vs. control (Student’s t-test), and # P<0.05 and NS not significant vs. H$_2$O$_2$ without drug (ANOVA and Tukey HSD test).

**Fig. 2** H$_2$O$_2$-mediated facilitation of Ca$^{2+}$ channel activity in inside-out mode.  
A and C Time course of channel activity ($NPo$) recorded in cell-attached (c.a.) mode followed by inside-out (i.o.) mode, in which channel activity was maintained with 1 µM CaM + 3 mM ATP, and then 1 mM H$_2$O$_2$ without (A) or with (C) 10 µM KN-93 was additionally applied as indicated by the boxes in each graph. ATP was included throughout the experiments in the inside-out mode.  
B Concentration-dependent effect of H$_2$O$_2$. Normalized channel activity was plotted against concentration of H$_2$O$_2$. Data (n=4-6) were fitted with a combined Hill’s equation as:

$$
(100 + A \cdot [H_2O_2]^{n_f}/(K_{d_f}^{n_f} + [H_2O_2]^{n_f})) \cdot (K_{d_i}^{n_i}/(K_{d_i}^{n_i} + [H_2O_2]^{n_i})),
$$

where [H$_2$O$_2$] is the concentration of H$_2$O$_2$, A the extent of facilitation, K$_{d_f}$ and K$_{d_i}$ apparent dissociation constants, and $n_f$ and $n_i$ Hill’s numbers for facilitation and inhibition, respectively. The fitted curve was drawn with $A=147$, K$_{d_f}$ =0.68 mM, $n_f$ =2, K$_{d_i}$ =3.11 mM and $n_i$=3 ($r^2=0.983$).  
D Summary of the normalized channel activity in the presence of CaM + ATP in...
i.o. mode before and after addition of H$_2$O$_2$ ± KN-93 (n=5-6). Data are shown as mean ± S.E.

* P<0.05 vs. CaM (t-test), NS not significant (ANOVA).

**Fig. 3** Facilitation of Ca$^{2+}$ channel by H$_2$O$_2$ is not because of oxidation of CaM. CaM was pre-incubated with 1 mM H$_2$O$_2$ for 30 min and then applied to the Ca$^{2+}$ channels. A Time course of channel activity recorded first in cell-attached (c.a.) mode followed by inside-out (i.o.) mode with 1 µM non-treated (intact) CaM + 3 mM ATP, followed by substitution with H$_2$O$_2$-treated CaM (1 µM). B Example of current traces for the control in c.a. mode (a), with CaM + ATP in i.o mode (b) and H$_2$O$_2$-treated CaM + ATP (c), at the time period indicated in A. C Summary of normalized channel activity induced by CaM + ATP (n=7) and H$_2$O$_2$-treated CaM + ATP (n=6). Data are shown as mean ± S.E. *** P<0.001 vs. control (c.a.), NS not significant (ANOVA and Tukey HSD test).

**Fig. 4** DTNB, a specific oxidant of cysteine residues, facilitates Ca$^{2+}$ channel activity in inside-out mode. A Time course of channel activity recorded first in cell-attached (c.a.) mode followed by inside-out (i.o.) mode. After patch excision, 1 µM CaM + 3 mM ATP was applied to maintain channel activity, and then DTNB (1 mM) was additionally applied. B Example of current traces for the control in c.a. mode (a), with CaM + ATP in i.o mode (b) and with CaM + ATP + 1 mM DTNB (c) at the time period indicated in A. C Summary of normalized channel activity in the presence of CaM + ATP and CaM + ATP + DTNB (n=5). Data are shown as mean ± S.E. * P<0.05 vs. control (t-test).

**Fig. 5** A Time course of channel activity recorded first in cell-attached (c.a.) mode followed by inside-out (i.o.) mode. After patch excision, 1 µM CaM + 3 mM ATP was applied to maintain channel activity, then DTNB (1 mM) was added, and finally H$_2$O$_2$ (1 mM) was additionally applied. B Summary of channel activity induced by CaM + ATP + DTNB (n=5) and CaM + ATP + H$_2$O$_2$ (n=6), normalized to the activity values obtained for conditions of
CaM + ATP. Data are shown as mean ± S.E. NS not significant (t-test).
Fig. 1

Panel A: Graph showing changes in NPo over time with exposure to H2O2 (1 mM).

Panel B: Electrical recordings comparing control (a) and exposure to H2O2 (b).

Panel C: Graph showing changes in NPo over time with exposure to H2O2 and KN-93.

Panel D: Bar graph showing channel activity (%), with symbols indicating statistical significance:
- **: p < 0.01
- *: p < 0.05
- #: p < 0.01 with Knoll modification
- NS: Non-significant

Exposure conditions:
- H2O2: - +
- KN-93: - - + +
- KN-92: - - - - + +
Fig. 3
Fig. 4

A

Time (min)

NPo

0 2 4 6 8

c.a. CaM CaM + DTNB

i.o.

0 2

B

100 ms

2 pA

C

Channel activity (%)

0 100 200 300

c.a. CaM CaM + DTNB

**
Fig. 5

A

Time (min)

[B] Channel activity (%)