Oxidative modification of M-type K⁺ channels as a mechanism of cytoprotective neuronal silencing

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Voltage-gated K⁺ channels of the Kv7 family underlie the neuronal M current that regulates action potential firing. Suppression of M current increases excitability and its enhancement can silence neurons. We here show that three of five Kv7 channels undergo strong enhancement of their activity by oxidative modification induced by physiological concentrations of hydrogen peroxide. A triple cysteine pocket in the channel S2–S3 linker is critical for this effect. Oxidation-induced enhancement of M current produced a hyperpolarization and a dramatic reduction of action potential firing frequency in rat sympathetic neurons. As hydrogen peroxide is robustly produced during hypoxia-induced oxidative stress, we used an oxygen/glucose deprivation neurodegeneration model that showed neuronal death to be severely accelerated by M current blockade. Such blockade had no effect on survival of normoxic neurons. This work describes a novel pathway of M-channel regulation and suggests a role for M channels in protective neuronal silencing during oxidative stress.

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Introduction

Reactive oxygen species (ROS) are the cytotoxic forms of oxygen that are abundantly produced during oxidative stress (Fiskum, 2000; Starkov and Fiskum, 2003), a condition accompanying ischemia and many disorders such as heart failure, stroke, brain trauma, diabetes, Alzheimer's disease and hypertension (Behl, 1999; Fiskum, 2000; Vincent *et al*, 2002; Poulet *et al*, 2006). Here we report the novel phenomenon of ROS-induced oxidative modification of neuronal

voltage-gated Kv7 K⁺ channels and augmentation of their currents, and suggest a role of such enhancement in cytoprotection against ROS-induced neuronal damage.

Kv7 (M-type) channels are encoded by the *KCNQ* family of genes (Wang *et al*, 1998). Kv7 channels have a relatively depolarized threshold voltage for activation and slow kinetics, permitting a significant fraction of channels to be open at the resting membrane potential of neurons (Brown and Adams, 1980; Constanti and Brown, 1981). Consequently, inhibition of M currents dramatically enhances neuronal excitability (Brown, 1988; Marrion, 1997), whereas their augmentation has a 'silencing' effect (Hetka *et al*, 1999; Gu *et al*, 2005). Correspondingly, loss-of-function mutations in Kv7.2 and 7.3 result in an inherited form of epilepsy (Biervert *et al*, 1998; Singh *et al*, 1998) and the Kv7 opener retigabine has potent anticonvulsant activity (Rostock *et al*, 1996).

Many neuronal ion channels and receptors can be modulated by oxidative modifications. Thus, BK channels (Tang *et al*, 2004), heteromeric I_{Ks}-like K⁺ channels (Busch *et al*, 1995), ASIC channels (Andrey *et al*, 2005) and NMDA receptors (Aizenman *et al*, 1989) are inhibited by millimolar concentrations of oxidizing agents, and GIRK K⁺ channels are activated by reducing agents (Zeidner *et al*, 2001). Effects of oxidation/reduction on the kinetics of some Kv channels (Liu and Gutterman, 2002; Caouette *et al*, 2003) and HERG channels (Berube *et al*, 2001) have also been reported. Probably the most relevant to this work is a study showing that inwardly rectifying K_{ATP} channels assembled from the pore-forming Kir6.2 and auxiliary SUR subunits (Inagaki *et al*, 1995; Sakura *et al*, 1995) are augmented by endogenous hydrogen peroxide (H₂O₂) in dorsal striatal neurons (Avshalumov and Rice, 2003) and the resulting hyperpolarization of neurons have been suggested as a mechanism of neuroprotective silencing in hypoxia (Guatteo *et al*, 1998; Trauner and Kramer, 2004). Thus, this work puts forward the concept of neuroprotection conferred by the ROS-induced augmentation of neuronal potassium current.

Here, we use molecular, electrophysiological and cell biology methods to show that physiological concentrations of the ROS H₂O₂ induce a strong enhancement of M-channel activity. We characterize the biophysics of this oxidative action, its locus on the channel proteins and the resulting effects on neuronal discharge properties and on cell viability during hypoxic conditions. We hypothesize that oxidative modification of Kv7 channels represents an important mechanism to protect neurons against ROS-induced damage.

Results

H₂O₂ potently augments currents of Kv7.2, 7.4 and 7.5

We expressed Kv7.1–7.5 homomultimers and Kv7.2/7.3 heteromultimers in Chinese hamster ovary (CHO) cells and studied the effects of H₂O₂ (0.5–500 μM) on the currents produced by these channels under perforated-patch voltage

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clamp. Tonic intracellular H_2O_2 concentration in mammalian cells can be as high as $0.7 \mu M$ (Stone, 2004) and H_2O_2 production can increase in ischemic brain by more than 10-fold (Starkov and Fiskum, 2003). As extracellular H_2O_2 application results in an intracellular concentration some 7–10-fold below that of extracellular (Stone, 2004), we estimated that intracellular $[H_2O_2]$ in our experiments covered the range reachable *in vivo*. We first examined the effect of H_2O_2 at 0 mV, which is a saturating voltage for Kv7 channels (Gamper *et al*, 2003). Bath-application of H_2O_2 induced a sharp augmentation of steady-state currents of Kv7.2, 7.4 and 7.5 channels (Figure 1) in a concentration-dependent manner

Figure 1 Effect of H_2O_2 on whole-cell currents of Kv7 channels. (A) Typical whole-cell current recorded from a CHO cell expressing Kv7.5 using the voltage protocol depicted below. The steady-state current at 0 mV deactivates slowly when the voltage step to -60 mV is applied and the voltage step back to 0 mV reactivates current back to the steady-state level. (B–G) Individual Kv7.1–5 homomultimers (B–D, F, G) or Kv7.2/7.3 heteromultimers (E) were expressed in CHO cells. Whole-cell currents were elicited by repetitive application of the voltage protocol shown in (A) with a 3 s interval. H_2O_2 (300 μM), DTT (2 mM) and the M-channel blockers, linopiridine or XE991 (both 50 μM), were applied during the periods indicated by the bars. Plotted are normalized current amplitudes versus time during the experiment. Representative current traces from each experiment (not normalized) are depicted in the insets. (H) Summarized data for experiments shown in (B–G).

(Figure 2). The mean current augmentation (300 μM H_2O_2) was 1.95 ± 0.18 ($n=11$), 2.98 ± 0.37 ($n=15$) and 2.90 ± 0.43 ($n=9$)-fold, respectively. At 300 μM , the H_2O_2 effect usually reached a plateau within 5 min of application (Figure 2B). Currents from Kv7.1 and 7.3 channels were not affected by H_2O_2 (Figure 1B, D, H). Currents from Kv7.2/7.3 channels (which underlie most neuronal M currents) were augmented by H_2O_2 by 1.8 ± 0.1 -fold ($n=10$; Figure 1E). Importantly, H_2O_2 effects on all affected channels were completely reversed by the reducing agent, dithiothreitol (DTT, 2 mM; Figure 1C, E–G), suggesting that these effects are due to a reversible oxidative modification. Notably, DTT reduced Kv7 currents in H_2O_2 -treated cells back to the resting level within several minutes, but did not inhibit them further (Figure 1C, E–G). For example, application of DTT returned the current amplitude of Kv7.2/7.3 currents to $113 \pm 17\%$ ($n=5$) of the level observed before H_2O_2 treatment. Also, DTT had no effect on Kv7 currents in non- H_2O_2 -treated cells (data not shown). These data suggest that channels are not tonically modified. We studied the concentration dependence of H_2O_2 action on Kv7.4 (Figure 2). Notably, H_2O_2 concentrations as low as 50 μM produced a strong effect and even at 5 μM , H_2O_2 induced significant augmentation of Kv7.4 currents. At an isochronal point 5 min after H_2O_2 application, half-maximal effect was achieved at a concentration of $23 \pm 3 \mu M$.

Figure 2 Concentration dependence of the effect of H_2O_2 on whole-cell Kv7.4 currents. H_2O_2 at 0.5, 5, 50, 300 or 500 μM were applied to CHO cells expressing Kv7.4 channels under perforated-patch current recording. The H_2O_2 effect was quantified isochronally at 5 min after H_2O_2 application (gray line in B). (A) Summarized data were fit by a Hill equation of the form: $I_{H_2O_2}/I_0 = 1 + a * [H_2O_2]^n / (EC_{50}^n + [H_2O_2]^n)$, where a is the maximal current augmentation and n is the Hill coefficient. The fit gave n and EC_{50} values of 1.3 ± 0.16 and $22.5 \pm 2.9 \mu M$, respectively, with R_{sq} value of 0.999. For each H_2O_2 concentration except for 300 μM , $n=5$; for 300 μM , $n=15$. (B) Superimposed are individual experiments with different H_2O_2 concentrations.

Figure 3 Oxidative modification of Kv7 channels induces changes in current kinetics. **(A)** The effect of H_2O_2 (300 μ M) on the voltage dependence of activation of Kv7.5 was measured by plotting the amplitudes of tail currents (deactivating currents upon return of the voltage to a hyperpolarized voltage) against the voltage. Tail currents were recorded at -60 mV following a family of voltage pulses between -100 and 40 mV. On the left, the voltage protocol used is depicted (top) to record currents shown below and on the right two panels. Encircled are scaled-up tail currents. **(B)** Pooled tail current amplitudes from the experiments similar to that shown in (A) are plotted against voltage and fit by Boltzmann equations (left). On the right are plotted the results of similar experiments for Kv7.2/7.3. **(C)** The effect of H_2O_2 (300 μ M) on the kinetics of Kv7.5 and Kv7.2/7.3 current activation and deactivation are shown. On the lower panel, activating and deactivating currents are scaled to match each other for better comparison. Statistics are given in the Supplementary Table 1.

Besides its effect on current amplitudes at saturating voltages, H_2O_2 also induced a negative shift in channel voltage dependence, accelerated the kinetics of activation and slowed deactivation (Figure 3; Supplementary Table 1). These effects were also reversed by DTT. The observed voltage-shifts were not enough to account for the several-fold increase of current amplitudes at saturating voltages. The synergistic effects of a strong increase in current amplitudes at saturating voltages (reflecting a voltage-independent augmentation) and the left-shift in voltage dependence predict a dramatic increase in M-channel activity at typical neuronal threshold voltages and a strong inhibition of neuronal discharge.

We then tested if the effect of H_2O_2 on whole-cell currents is due to an increase in channel open probability (P_o) using single-channel patch recordings. For these experiments, we chose Kv7.5 as the H_2O_2 effect for this channel was especially robust (Figure 1), and patches containing a single Kv7.5 channel are much easier to obtain than for Kv7.4 (Li *et al*, 2004). Single-channel experiments at the saturating voltage

Figure 4 Oxidative modification of Kv7.5 increases its open probability. **(A)** Single-channel recording at 0 mV in cell-attached configuration from a CHO cell expressing Kv7.5 before (left) and 4 min after 300 μ M H_2O_2 application. **(B)** Multichannel cell-attached 'macropatch' recording at 0 mV showing the effect of H_2O_2 and DTT (2 mM) on Kv7.5 channels.

of 0 mV indeed revealed a dramatic H_2O_2 -induced increase in the maximal P_o of Kv7.5 (Figure 4A). In cell-attached patches containing a single channel, bath-application of 300 μ M H_2O_2 strongly increased the P_o of Kv7.5 at 0 mV from 0.07 ± 0.03 to 0.51 ± 0.09 ($n = 5$), whereas the single-channel conductance remained unchanged (2.34 ± 0.28 versus 2.20 ± 0.25 pS). From this result for Kv7.5, we suggest that the voltage-independent increase in macroscopic currents induced by H_2O_2 is due to an increase in P_o . Figure 4B shows a cell-attached experiment with many Kv7.5 channels in the patch that also shows remarkable current augmentation by H_2O_2 application.

As the H_2O_2 action is to make the opening conformation of the channels more favorable, one might expect the H_2O_2 effect to be state dependent, such that open channels are modified more rapidly than closed ones. However, the effect of H_2O_2 did not show an obvious state dependence as application of H_2O_2 (300 μ M, 5 min) to closed Kv7.4 channels (kept closed using a holding potential of -60 mV) resulted in augmentation of the currents nearly as strong and as rapid as when using our normal voltage-clamp protocol in which cells are held at 0 mV. Thus, for cells held at -60 mV, H_2O_2 treatment augmented the Kv7.4 currents by 2.2 ± 0.3 -fold ($n = 4$, data not shown), only slightly less than when using our usual voltage protocol (Figure 1F, H).

A triplet of cysteines in the channel S2-S3 linker mediates oxidative sensitivity of Kv7

We then sought to investigate the site of H_2O_2 action, with our working hypothesis being the direct oxidative modification of channel amino acids. Although several types of residues (e.g. methionines, arginines and aromatic amino acids) can be modified by oxidizers, by far the most susceptible to oxidation are the sulfhydryl groups of cysteines (Stadtman, 2001). H_2O_2 -sensitive Kv7 channels share six common cysteines at positions equivalent to C112, C156, C157, C158, C175 and C519 in Kv7.4 (Figure 5A). The residues C156, C157 and C158 form a triple-cysteine pocket in the cytoplasmic S2-S3 linker. We substituted alanines for single cysteines at positions C112, C175 and C519 in Kv7.4 and also generated a triple cysteine mutant in which C156, C157 and C158 were all substituted by alanines ('triple-Cys mutant'). We chose Kv7.4 as a backbone for our mutagenesis, as among homomeric Kv7 channels it displays by far the most

Figure 5 A triple-cysteine pocket is the putative site of oxidative modification of Kv7 channels. **(A)** Schematic alignment of conserved cysteines in Kv7.1–5 channels. Coloured grey are the H₂O₂-insensitive channels. **(B)** Whole-cell experiments show that C112A, C75A and C519A mutants of Kv7.4 retain their H₂O₂ sensitivity, whereas triple C-to-A substitutions at positions 156, 157 and 158 (CCC) abolished such sensitivity. **(C)** Summary of the H₂O₂ effect on the four Kv7.4 mutants (top). Bottom diagram summarizes further screening of the ‘triple-Cys’ region. ** $P \leq 0.01$; *** $P \leq 0.001$.

reliable and robust expression in CHO cells (Gamper *et al*, 2003). Figure 5B and C shows experiments using these four Kv7.4 mutants (see also Supplementary Table II and Supplementary Figure 1 for detailed data on the expression and properties of these mutants). The single mutants C112A, C175A and C519A showed unaltered H₂O₂ sensitivity, with currents augmented by H₂O₂ (300 μ M) by 2.4 ± 0.2 ($n = 8$), 2.1 ± 0.3 ($n = 6$)- and 3.0 ± 0.4 ($n = 6$)-fold, respectively. In contrast, the triple-Cys mutant appeared to be totally insensitive to H₂O₂ (Figure 5B, C); thus, after 5 min of H₂O₂ application, its currents were $89.2 \pm 8.5\%$ of control ($n = 11$). There was also no effect of H₂O₂ on the kinetic properties of currents from the triple-Cys mutant (Supplementary Table II). To exclude the possibility that the mutant channel is insensitive to oxidation owing to a high

intrinsic maximal P_o (that could thus not be increased further), we performed single-channel analysis of its maximal P_o in cell-attached patches. These experiments revealed the maximal P_o of the triple-Cys mutant to be 0.16 ± 0.05 ($n = 8$; Supplementary Figure 2). Although this is higher than the maximal P_o of wt Kv7.4 (0.07, (Li *et al*, 2004); present study), it would still be low enough to permit a several-fold augmentation. Thus, from these results using Kv7.4 mutagenesis, we conclude the triple-cysteine pocket at C156, 157, 158 to be the site of H₂O₂ action.

Although Kv7.3 does possess this triplet of cysteines (Figure 5A), its maximal P_o is near unity (Li *et al*, 2004), predicting little possible augmentation of its whole-cell currents at saturating voltage, as was seen (Figure 1). In contrast to other Kv7 subunits, Kv7.1 has only one cysteine within the S2–S3 linker region (Figure 5A). To provide further evidence that this triple-Cys pocket is the locus of H₂O₂ action, we introduced two additional cysteines in this region to create the homologous triple-Cys pocket as in the other Kv7 channels, and tested whether this mutagenesis bestowed sensitivity to oxidation to Kv7.1. The double-mutant channel in which R86 and S87 (the positions homologous to C157 and C158 in Kv7.4) were both substituted by cysteines expressed weakly (Supplementary Figure 1), but nevertheless responded to H₂O₂ application with substantial augmentation of the current (Supplementary Figure 3). At 0 mV, current augmentation (300 μ M H₂O₂, 5 min) for the mutant was 1.55 ± 0.1 ($n = 7$). Although significant, the H₂O₂ sensitivity of this Kv7.1 mutant was considerably weaker than that of the other H₂O₂-sensitive Kv7 channels. One possible reason for this fact is that Kv7.1 has an intrinsically high maximal P_o . Unfortunately, there is no reliable information on the P_o of Kv7.1, as its single-channel conductance (as calculated from noise analysis) is well below 1 pS (Yang and Sigworth, 1998); nevertheless, such a high maximal P_o for Kv7.1 has been predicted based upon structural-functional similarities with Kv7.3 (Gamper *et al*, 2005).

Oxidation of cysteines can promote formation of intra- or intermolecular disulfide bonds and we wondered if the effect were owing to promotion of intersubunit disulfide bonds. However, nonreducing PAGE analysis did not reveal H₂O₂-induced multimerization of Kv7.4 specific to the triple cysteine region (data not shown), suggesting that formation of intersubunit or intermolecular disulfide bonds is unlikely to underlie the observed effects of oxidation on Kv7 currents. To study the possibility of intrasubunit disulfide bond formation, we created three single mutants: C156A, C157A and C158A, and a double mutant C156A, C157A. Interestingly, all four mutants showed considerably reduced, but still significant, augmentation by H₂O₂ (Figure 5C, Supplementary Table II). Currents of the C156A, C157A, C158A and C156A, C157A mutants were augmented by 1.4 ± 0.1 ($n = 7$)-, 1.6 ± 0.1 ($n = 7$)-, 1.5 ± 0.2 ($n = 7$)- and 1.2 ± 0.1 ($n = 8$)-fold, respectively. These values are lower than the augmentation of wild-type Kv7.4 (3.0 ± 0.4 , $P \leq 0.01$), but still greater than unity ($P \leq 0.05$). Although the double mutant displays only weak sensitivity to H₂O₂, it is still significantly greater than that of the triple-cysteine mutant described above ($P \leq 0.05$). Thus, even one cysteine in the triplet suffices for noticeable augmentation by H₂O₂. These data argue against fixed or random disulfide bound formation within the cysteine triplet. Instead, it is more likely that the cumulative

oxidation of all three cysteines has an additive effect (see Discussion).

Oxidation augments native M currents in superior cervical ganglion (SCG) neurons and reduces their excitability

The powerful effects of H_2O_2 on cloned Kv7 channels predicted a strong effect on native neuronal M current, and on neuronal excitability. To test these questions, we probed the effect of oxidative modification of native M channels in neurons. Consistent with the augmentation of Kv7.2/7.3 currents in CHO cells, M current in superior cervical ganglion (SCG) sympathetic neurons was augmented by 1.5 ± 0.1 -fold ($n = 6$, $P \leq 0.01$) by H_2O_2 (300 μ M, 0 mV), and this effect was also reversed by DTT (Figure 6A). Pharmacological augmentation of M current in SCG neurons (Zaika *et al*, 2006), and in hippocampal and sensory neurons has been shown to strongly reduce somatic excitability (Passmore *et al*, 2003; Yue and Yaari, 2004). Thus, we tested the effect of H_2O_2 on the excitability of SCG cells in response to current injection under perforated-patch current clamp. We found that H_2O_2 indeed strongly reduced neuronal excitability (Figure 6B). As M channels are partially open at the resting potential (V_{rest}), and H_2O_2 causes a left-shift in Kv7.2/7.3 voltage dependence (Figure 3), we expected H_2O_2 to make V_{rest} more negative, and this was the case. In control neurons, V_{rest} was -55 ± 1 mV ($n = 25$), and H_2O_2 induced a hyperpolarization of V_{rest} to -59 ± 0.3 mV ($n = 17$, $P \leq 0.01$). H_2O_2 also reduced neuronal discharge in response to depolarizing currents. Thus, in control, the action potential firing frequency (APF) during injection of a 100 pA depolarizing current was 8.6 ± 0.4 AP/s ($n = 25$), whereas after H_2O_2 , it was 5.1 ± 0.3 AP/s ($n = 17$, $P \leq 0.01$). Correspondingly, the current required to reach threshold for action potential firing (AP_{thresh}) was changed from 29 ± 4 pA (control) to 40 ± 9 pA (H_2O_2 ; $n = 11$, $P \leq 0.05$). All of these changes are consistent with a decrease in somatic excitability caused by the oxidative modification-dependent enhancement of M current. As a control, we applied the M-channel blockers, linopirdine or XE991 (both at 10 μ M), after H_2O_2 application. XE991 caused the expected depolarization of V_{rest} and a dramatic increase in somatic excitability. After XE991, V_{rest} was shifted to -50 ± 1 mV ($n = 15$, $P \leq 0.01$), and APF and AP_{thresh} were altered to 10.2 ± 0.8 AP/s and 21 ± 1 pA ($n = 15$, $P \leq 0.05$ for both). There were no H_2O_2 - or XE991-induced changes in the responses to hyperpolarizing currents (Figure 6B), indicating that hyperpolarization-activated currents (such as I_H) were not affected by these treatments. We also tested the effect of the M-channel opener, retigabine (10 μ M), as a comparison. After retigabine treatment, V_{rest} , APF and AP_{thresh} were -65 ± 1 mV ($n = 10$, $P \leq 0.01$), 0.8 ± 0.3 AP/s ($P \leq 0.001$) and 67 ± 7 pA ($P \leq 0.05$), respectively, qualitatively similar to the effects of H_2O_2 . These experiments support our hypothesis that augmentation of M channels by ROS induces silencing of neurons.

Enhancement of M current exerts a cytoprotective effect in a hypoxia-induced neurodegeneration model

Increased mitochondrial H_2O_2 (and other ROS) generation is one of the triggers of neuronal death associated with acute ischemic brain injury and with chronic neurodegenerative diseases (Sun and Chen, 1998; Nicholls and Budd, 2000;

Figure 6 H_2O_2 enhances native M currents and decreases somatic excitability of SCG neurons. (A) Summarized time course of H_2O_2 -induced augmentation of M current in SCG neurons ($n = 6$). Inset shows current traces from an exemplar experiment before any treatment and during the consecutive application of 300 μ M H_2O_2 , 2 mM DTT and 50 μ M linopirdine (as labelled). The voltage protocol is shown underneath current traces. (B) SCG neurons were studied under current clamp, and action potentials recorded from a trio of current pulses (current protocol is shown in the inset at the bottom). The voltage records before (top) or after 5 min of bath-application of 300 μ M H_2O_2 (middle) and finally after bath-application of 50 μ M XE991 (bottom) are shown.

Fiskum, 2004). To investigate a possible neuroprotective role of M currents under conditions that simulate cerebral ischemia–reperfusion, we studied neurodegeneration in hippocampal slice cultures exposed to oxygen/glucose deprivation (OGD; Pringle *et al*, 1997; Noraberg *et al*, 1999). Hippocampal pyramidal neurons express M currents and

Figure 7 M currents protect hippocampal neurons against oxygen/glucose deprivation (OGD)-induced neurodegeneration. **(A)** Four groups of organotypic hippocampal slices were used. The control group was without any treatment (Control); the second group was without OGD but was incubated (30 min) in the presence of 10 μ M XE991 (Control + XE). The third group was exposed to OGD (no oxygen, no glucose) for 30 min (OGD). The fourth group was 30 min OGD-treated in the presence of 10 μ M XE991 (OGD + XE). PI uptake was monitored optically 3, 8 and 24 h after OGD (PI only enters dead/dying cells and only fluoresces when bound to DNA). Inset on the top shows fluorescent images of slices in each experimental condition 24 h after OGD. **(B)** Experiment similar to that shown in (A) but the M-channel blocker XE991 was replaced with the M channel opener, retigabine (RT, 10 μ M). Number of experiments for each data point is given in the text.

their enhancement has been shown to suppress excitability (Gu *et al*, 2005). In the first experiment, four groups of organotypic slices were used (Figure 7A). The first group was without any treatment (Control); the second group was without OGD but was incubated in the presence of XE991 (10 μ M) for 30 min (Control + XE). The third group was challenged with OGD by substitution of Neurobasal medium for that lacking glucose and bubbled with a gas mixture of 5% CO_2 /95% N_2 . After 30 min of OGD, normal medium was returned to the slices (OGD). In the fourth group, OGD media was supplemented with XE991 (10 μ M) (OGD + XE). Neuronal death was optically monitored by propidium iodide (PI) uptake (Norberg *et al*, 1999), at 3, 8 and 24 h after OGD, OGD + XE991 or XE991 exposure.

We found that blockade of M current with XE991 dramatically increased OGD-induced neuronal death, but did not affect survival of normoxic slices. Normalized PI uptake 24 h after treatments in Control, Control + XE, OGD and OGD + XE

slices were 0.2 ± 0.03 ($n = 17$), 0.21 ± 0.02 ($n = 16$), 0.48 ± 0.09 ($n = 13$, $P \leq 0.05$) and 0.64 ± 0.04 ($n = 15$, $P \leq 0.001$), respectively. These data suggest that the activation of M channels during OGD is cytoprotective. To further probe the possible cytoprotective action of M channels, we tested whether retigabine would indeed limit neuronal death. The results shown in Figure 7B support this hypothesis. Indeed, normalized PI uptake in OGD/retigabine-treated slices (OGD + RT) was only 0.22 ± 0.02 ($n = 13$) versus 0.33 ± 0.03 ($n = 11$, $P \leq 0.05$) in OGD-only treated slices. Taken together, these experiments are consistent with a neuroprotective role for M current in an oxidative stress-related model of neurodegeneration.

Discussion

In this work, we demonstrate a strong enhancement of Kv7 K^+ currents induced by physiological concentrations of H_2O_2 . The enhancement is due to the oxidative modification of cysteines in a triple cysteine pocket within the cytosolic S2–S3 linker. The modification induced a voltage-independent increase in channel maximal P_o and a left-shift in channel voltage dependence. Accordingly, H_2O_2 treatment of SCG neurons induced a hyperpolarization and reduction of action potential firing, thus producing a ‘silencing’ effect. Furthermore, our experiments on the oxidative-stress-induced neurodegeneration of organotypic hippocampal slices suggest a cytoprotective role for such M-channel-mediated silencing.

We find that Kv7.2, 7.4 and 7.5 are potentially augmented by H_2O_2 , whereas Kv7.1 and 7.3 are not. The most prominent effect of H_2O_2 on Kv7 channels is the increase of channel maximal P_o . Kv7 channels have highly divergent tonic maximal P_o values; thus, Kv7.2, 7.4 and Kv7.5 have a rather low P_o (in the range of 0.1–0.2), whereas Kv7.3 has a maximal P_o near unity (Selyanko *et al*, 2001; Li *et al*, 2004). As H_2O_2 acts by increasing channel P_o , it is, therefore, not surprising that currents of Kv7.3 are not augmented, even though Kv7.3 has the necessary triplet of cysteines. Kv7.1 has only one cysteine in the ‘triple C’ region. Reconstitution of the complete CCC sequence in Kv7.1 by substitution of R86 and S87 by cysteines bestowed moderate H_2O_2 sensitivity to this mutant, further supporting our conclusion that this triplet of cysteines mediates the H_2O_2 sensitivity of Kv7 channels. The fact that the effect of oxidation on the Kv7.1 mutant is weaker than on the Kv7.2, 7.4 and 7.5 is consistent with our previous prediction that Kv7.1 has high maximal P_o (Gamper *et al*, 2005).

Among amino-acid side chains, sulphhydryl groups of cysteines are most susceptible to oxidation (Stadtman, 2001). Cysteine residues can be reversibly oxidized to cysteine sulfenic acids (Cys-SOH), which can then be further oxidized to produce irreversible derivatives, sulfinic (Cys-SO₂H) and sulfonic (Cys-SO₃H) acids (Paget and Buttner, 2003) or form intra- or intermolecular disulfide bonds with another protein or low molecular-weight thiol such as glutathione (Paget and Buttner, 2003). Cys-SO₂H and Cys-SO₃H cannot be reduced by DTT (Poole *et al*, 2004) and, thus, unlikely to be involved in oxidative modification of Kv7 channels. We were also unable to detect formation of intermolecular disulfide bonds specific to the triple cysteine region under nonreducing PAGE. Formation of intramolecular disulfide bonds within the

cysteine triplet also seems unlikely to be necessary as neither single nor double cysteine substitutions within the triplet were sufficient to completely abolish the H₂O₂ effect. Thus, we believe that the most probable mechanism of Kv7 oxidative modification is cumulative oxidation of cysteines in the triplet to cysteine sulfenic acids. The growing literature suggests that Cys-SOH can be stabilized within the protein (Poole *et al*, 2004) and underlie a number of regulatory modifications of proteins (Poole *et al*, 2004). The possibility of secondary disulfide bond formation within the cysteine triplet or glutathionylation cannot be ruled out at this time; however, we were unable to see H₂O₂-induced glutathionylation of Kv7.4 in co-immunoprecipitation experiments (data not shown).

Kv7 current augmentation could be induced by an external H₂O₂ concentration as low as 5 μ M, suggesting an exquisite sensitivity of Kv7 channels to oxidation. Interestingly, extracellular release of up to 4 μ M H₂O₂ due to oxidation of dopamine in rat brain has been measured experimentally (Kulagina and Michael, 2003). Such high H₂O₂-sensitivity places Kv7 channels among the candidate proteins that are first to respond to ROS production. Thus, we hypothesize that ROS generated under oxidative stress induces oxidative modification and augmentation of M currents in neurons. The resulting reduction in neuronal discharge could thus provide a mechanism of protective silencing against oxidative-stress-induced excitotoxicity caused by overactivation of ionotropic glutamate receptors and excessive influx of Ca²⁺ and Na⁺ (Won *et al*, 2002). We used the model of OGD-induced neurodegeneration of organotypic hippocampal slices to mimic conditions occurring in the brain during acute ischemia/reperfusion (Pringle *et al*, 1997). In good agreement with our hypothesis, OGD-induced neurodegeneration of hippocampal slices was dramatically increased when OGD was combined with M-current blockade, which however had no effect on the survival of normoxic slices. In addition, OGD-induced neurodegeneration was reduced when OGD was combined with retigabine. Thus, our data support the hypothesis that neuronal silencing conferred by M-channel augmentation can be a powerful mechanism for neuronal viability.

Kv7.2–7.5 subunit are widely expressed throughout the central and peripheral nervous systems, producing homomeric and heteromeric channels of differential composition in different neuronal types (for an overview of Kv7 subunit distribution in the mammalian CNS, see Dalby-Brown *et al*, 2006). While it is generally accepted that the classical M currents of ganglia cells are mostly Kv7.2/7.3 heteromultimers (Wang *et al*, 1998; Delmas and Brown, 2005), it is also recognized that Kv7.3 can form heteromeric channels with Kv7.4 and 7.5. Moreover, all Kv7 subunits, when assembled as homomultimers, can form functional M-type channels (Delmas and Brown, 2005), thus providing a mechanism for spatial diversity of native M currents. We here report that the sensitivity of neuronal Kv7 channels to oxidation-induced modification is not uniform: Kv7.3 is virtually insensitive to such modification. Kv7.2 have an intermediate sensitivity while Kv7.4 and 7.5 express robust sensitivity. We therefore hypothesize that the strength of neuroprotection via oxidative M-current augmentation will strongly depend on the M-channel expression profile at the site of oxidative damage.

Besides ischemia/reperfusion, ROS-induced neurodegeneration is a condition of many pathologies, such as diabetic polyneuropathy (Vincent *et al*, 2002) or Alzheimer's disease (Behl, 1999); highly elevated levels of ROS have also been found in the aging brain (Serrano and Klann, 2004). Thus, neuronal silencing by the oxidative modification of M channels can represent a fundamental mechanism of neuroprotection. Such a role for M current in the case of stroke is particularly intriguing. The disruption of oxygen and glucose to cerebral tissue following a stroke can result in necrotic cell death and the formation of an infarct. In a focal infarct, the 'penumbral zone,' surrounding nonviable tissue may retain viability upon reperfusion. However, such reperfusion is associated with large generation of ROS, which is potentially cytotoxic. Cell viability in this large region is thus not fixed, but depends on neuronal rescue mechanisms, with cell death in the penumbra typically well delayed after the onset of the hypoxic episode (Sweeney *et al*, 1995). Thus, we suggest neuronal silencing during ROS generation by M-channel augmentation can be a powerful mechanism for maximizing neuronal viability in the large penumbral zone, consistent with accumulating evidence showing ROS to induce a number of death/survival signaling pathways in the ischemic brain (Sugawara *et al*, 2004).

Materials and methods

Cell culture, transfection and SCG isolation

CHO cells were handled as recently described (Gamper *et al*, 2003). Mutations in Kv7.4 were made by QuikChange (Stratagene) and verified by sequencing. Transfections were made using Polyfect reagent (Qiagen, Hilden, Germany) and enhanced green fluorescent protein was used as a reporter. Rat SCG neurons were cultured from juvenile rats as previously described (Gamper *et al*, 2003).

Electrophysiology

Amphotericin B perforated patch-clamp experiments were performed and analyzed as previously described (Gamper *et al*, 2005). In this variant of whole-cell recording, amphotericin B antibiotic added to the pipette solution forms pores in the patch of membrane in the tip of the pipette, enabling electrical continuity between the recording pipette and the intracellular compartment, without dialysis of the intracellular milieu. Currents were recorded using an EPC-9 amplifier and PULSE/PULSEFIT software (HEKA Elektronik). To evaluate Kv7 current amplitudes, CHO cells were held at 0 mV, and 500 ms steps to –60 mV, followed by 600 ms pulses back to 0 mV, were applied. The amplitude of the current was defined as the difference between the holding current at 0 mV and the current at the beginning of the 600 ms pulse back to 0 mV or, in some cells, as the XE991- or linopirdine-sensitive current at 0 mV. Current-clamp experiments on SCG neurons were carried out in perforated-patch configuration. Action potentials were evoked and recorded with an EPC-9 amplifier by injection of depolarizing currents. The sampling frequency was 2.5 kHz. Single-channel and macro-patch cell-attached recordings were performed and analyzed as previously described (Li *et al*, 2004). Briefly, pipettes had resistances of 7–15 M Ω when filled with pipette solution; the bath was a high K⁺ solution (see DNA constructs, solutions and materials), and the intracellular potential was assumed to be 0 mV. Currents were recorded using an Axopatch 1-D amplifier (Molecular Devices, Union City, CA), sampled at 5 kHz, and filtered at 0.5–1 kHz. Single-channel data were analyzed using PulseFit and TAC (Bruxton, Seattle, WA) software. Open and closed events were analyzed by using the '50% threshold criterion.' At a given potential, the single-channel amplitude (*i*) was calculated by fitting all-point histograms with single or multi-Gaussian curves. The difference between the fitted 'closed' and 'open' peaks was taken as *i*. When superimposed openings were observed, the number of channels in the patch was estimated from the maximal number of superimposed openings.

The apparent NP₀ was estimated as follows:

$$NP_0 = \left(\sum_{j=1}^N t_{ij} \right) / T$$

where t_j is the time spent at each current level corresponding to $j=0, 1, 2, \dots, N$, T is the duration of the recording and N is the number of current levels (minimum number of active channels).

Organotypic slices and OGD

Organotypic hippocampal cultures were prepared as previously described (Stoppini *et al*, 1991; Norberg *et al*, 1999). Briefly, P7 Sprague-Dawley rat pups (Harlan) were anesthetized with isoflurane, decapitated and the brains removed into cold (4°C) Gey's balanced salt solution (Sigma) supplemented with 25 mM glucose and 30 mM KCl. Each hippocampus was dissected from the brain, sliced with a tissue chopper (400 µm), and each slice was transferred to a membrane insert (0.4 µm, 30 mm diameter, Millipore). Inserts were placed in a six-well culture tray (Corning) with 1 ml of modified OPTI-MEM culture medium (50% Opti-MEM, 25% horse serum, 25% Hank's BSS; all from GIBCO), supplemented by glucose to a final concentration of 25 mM. Cultures were incubated at 36°C (5% CO₂). After 3 days in culture, the medium was replaced with Neurobasal medium supplemented with 25 mM glucose, 1 mM L-glutamine and 2% B27 supplement (GIBCO), changed every 3 days. After 2 weeks in culture, PI was added to each well (2 µM). All subsequent solutions contained the same concentration of PI. PI is a polar compound that only enters cells with damaged cell membranes; inside the cell it binds to DNA and exhibits bright red fluorescence. Thus, PI uptake has been extensively used as an indicator of neuronal integrity and cell viability (Norberg *et al*, 1999). OGD was achieved by exposing slices for 30 min to the glucose-free BSS (120 mM NaCl, 1.25 mM NaH₂PO₄, 5 mM KCl, 2 mM MgSO₄, 25 mM NaHCO₃, 20 mM HEPES, 2 mM CaCl₂) bubbled with a gas mixture of 5% CO₂/95% N₂. After OGD, normal Neurobasal medium was replaced in each well. For monitoring of the PI uptake, culture wells were placed on an inverted Nikon Diaphot 200 microscope (4× objective) equipped with epifluorescence illumination (Rhodamine filter set). Images were acquired using a CCD camera (MTI 3CCD, Dage). Slices were imaged immediately before OGD and then 3, 8 and 24 h after OGD. Culture wells were then placed in a refrigerator (4°C) for another 24 h and then imaged. Mean fluorescence was calculated using Image-J software (National Institutes of Health); pooled values from multiple images (3–5) were quantified for each slice. Uptake index (UI) was calculated as the difference in fluorescence following treatment (drug, OGD or a combination of both) and before OGD

(pretreatment), normalized to the maximal change in fluorescence (the difference between fluorescence of a dead slice and that before pretreatment):

$$UI = \frac{F_{\text{treatment}} - F_{\text{pretreatment}}}{F_{\text{death}} - F_{\text{pretreatment}}}$$

DNA constructs, solutions and materials

Plasmids encoding human Kv7.1, human Kv7.2, rat Kv7.3, human Kv7.4 and human Kv7.5 (GenBank accession numbers NM_000218, AF110020, AF091247, AF105202, and AF249278, respectively) were given to us by Michael Sanguinetti (University of Utah, Salt Lake City, UT; Kv7.1), David McKinnon (State University of New York, Stony Brook, NY; Kv7.2 and Kv7.3), Thomas Jentsch (Zentrum für Molekulare Neurobiologie, Hamburg, Germany; Kv7.4) and Klaus Steinmeyer (Aventis Pharma, Frankfurt am Main, Germany; Kv7.5) and subcloned into pcDNA3 or pcDNA3.1 (Invitrogen).

To record Kv7 currents in perforated patch experiments, we used an external solution containing (mM): 160 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES (pH 7.4 with NaOH) in combination with a pipette solution containing (mM): 160 KCl, 2 MgCl₂, 5 HEPES, 0.5 mg/ml amphotericin B. For cell-attached recordings, the external solution contained (mM): 175 KCl, 4 MgCl₂, 10 HEPES (pH 7.4 with KOH) and the pipette solution contained (mM): 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM HEPES (pH 7.4 with NaOH). For current-clamp perforated-patch experiments on SCG neurons, the pipette solution contained (mM): 90 K⁺-acetate, 20 KCl, 40 HEPES, 3 MgCl₂, 3 EGTA, 1 CaCl₂ (pH 7.4 with KOH) and the bath solution contained (mM): 140 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose (pH 7.4 with NaOH). Reagents were obtained as follows: H₂O₂ (HUMCO); DTT, linopirdine, PI (Sigma); DMEM, fetal bovine serum, Penicillin/Streptomycin, L-glutamine (Gibco); XE991 was a kind gift from Michael E Schnee (Dupont Pharmaceuticals).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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