

FORUM REVIEW ARTICLE

Oxidative Modulation of Voltage-Gated Potassium Channels

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Abstract

Significance: Voltage-gated K⁺ channels are a large family of K⁺-selective ion channel protein complexes that open on membrane depolarization. These K⁺ channels are expressed in diverse tissues and their function is vital for numerous physiological processes, in particular of neurons and muscle cells. Potentially reversible oxidative regulation of voltage-gated K⁺ channels by reactive species such as reactive oxygen species (ROS) represents a contributing mechanism of normal cellular plasticity and may play important roles in diverse pathologies including neurodegenerative diseases. **Recent Advances:** Studies using various protocols of oxidative modification, site-directed mutagenesis, and structural and kinetic modeling provide a broader phenomenology and emerging mechanistic insights. **Critical Issues:** Physicochemical mechanisms of the functional consequences of oxidative modifications of voltage-gated K⁺ channels are only beginning to be revealed. *In vivo* documentation of oxidative modifications of specific amino-acid residues of various voltage-gated K⁺ channel proteins, including the target specificity issue, is largely absent. **Future Directions:** High-resolution chemical and proteomic analysis of ion channel proteins with respect to oxidative modification combined with ongoing studies on channel structure and function will provide a better understanding of how the function of voltage-gated K⁺ channels is tuned by ROS and the corresponding reducing enzymes to meet cellular needs. *Antioxid. Redox Signal.* 21, 933–952.

Introduction

VOLTAGE-GATED POTASSIUM (K⁺) channels form a large family of ion channels with the common properties of strong selectivity for K⁺ and a dependence of their open probability (P_o) on the membrane voltage; at resting voltages of -70 to -90 mV such channels are typically closed, and they open when the membrane depolarizes. In excitable cells, these features enable voltage-gated K⁺ channels to counteract electrical excitation; they may contribute to setting and stabilizing the resting potential or they help to regulate action potential shape and frequency. In nonexcitable cells, such as in the epithelial cells of the inner ear or the kidney, they serve roles as K⁺ transport vehicles. To suit a wide range of physiological functions, voltage-gated K⁺ channels are diverse in their primary structure and the channel complexes often include auxiliary subunits as detailed below. In addition, through co- and post-translational protein modifications these channels are tuned to the specific cellular needs. A subgroup of such modifications is changes in channel function

induced by reactive oxygen species (ROS) or reactive oxygen intermediates. While various reviews focusing on potential roles of channel modification by ROS in diseases or degenerative phenomena have appeared [*e.g.*, (4, 86, 117)], we will focus here on mechanistic insights underlying the oxidative modulation of voltage-gated K⁺ channels.

The focus on molecular mechanism has become feasible because voltage-gated K⁺ channels have a long history of in-depth research. Stimulated by the first cloning of cDNAs of the Shaker channel from *Drosophila melanogaster* (55, 99, 101, 133), many other genes in various species were to follow. Numerous channel-related inherited diseases underscore a range of physiological functions [*e.g.*, (1)], and the progress in X-ray crystallography has provided us with detailed information on the atomic structure of some of these channels [*e.g.*, (74, 75)]. Last but not least, the functional properties of voltage-gated K⁺ channels can be evaluated with electrophysiological methods to a very high precision because their voltage-dependence allows the correction of leak and capacitive currents when measuring ionic currents in

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the voltage-clamp mode. Therefore, high-quality electrical signals combined with detailed kinetic modeling report on functional features of the channels and provide access to the investigation of even minute but potentially important modulating influences.

Voltage-gated K^+ channels are formed by α -subunits with six transmembrane segments (6TM, S1–S6; Fig. 1). Four α -subunits assemble to build a channel complex in which the narrow part is lined by the pore loops connecting S5 and S6. Some α -subunits can coassemble with different types of α -subunits thus creating diverse heterotetramers.

To become selective for K^+ , adjacent carbonyl oxygens located in the pore loops are directed into the center of the channel—a feature involving either G-Y-G or G-F-G in the sequence of the selectivity filter region (31). Projecting toward the cytosolic side, a water-filled cavity completes the channel pore. This is a site where various drugs bind to K^+ channels to occlude the pore or to interfere with the gating, that is, with the opening and closing of the ion conduction gate.

Voltage sensing is mediated by an action of the voltage-sensor domain (VSD, S1–S4), with S4 carrying the major component of the gating charge, and the pore/gate domain (PGD, S5–S6) (Fig. 1B, C). Conformational changes of the VSD induced by changes in the transmembrane electric field

are coupled by means of the cytosolic S4–S5 linker to the pore/gate module (58, 75). The latter then results in a movement of the S6 segments to open the pore at the intracellular side. This combination of VSD and PGD defines the fundamental features of voltage-gated K^+ channels. However, as described below, extended N- and C-terminal protein domains contribute many additional features such as the ability of the channels to undergo inactivation or to be modulated by second messengers such as Ca^{2+} or cyclic nucleotides.

Association of auxiliary protein subunits (β -subunits) further increases the diversity of K^+ channels. Some of them are cytosolic proteins that dock to the N-terminal domains of the α -subunits ($Kv\beta$) (74, 103) (Fig. 1A). Others are membrane proteins with one or two transmembrane segments like members of the KCNE or KCNMB family, respectively. In addition, Ca^{2+} -binding proteins such as calmodulin (115), K channel-interacting proteins (KChIPs) (3), or dipeptidyl aminopeptidase-like protein (DPPX) (87) may bind to the channels. In all cases, β -subunits equip the K^+ channel complexes with additional features.

Various nomenclatures exist to describe voltage-gated K^+ channels or the corresponding genes. Just concentrating on α -subunits, there is the HUGO nomenclature of human genes, the Kv notation according to the protein function, and a long

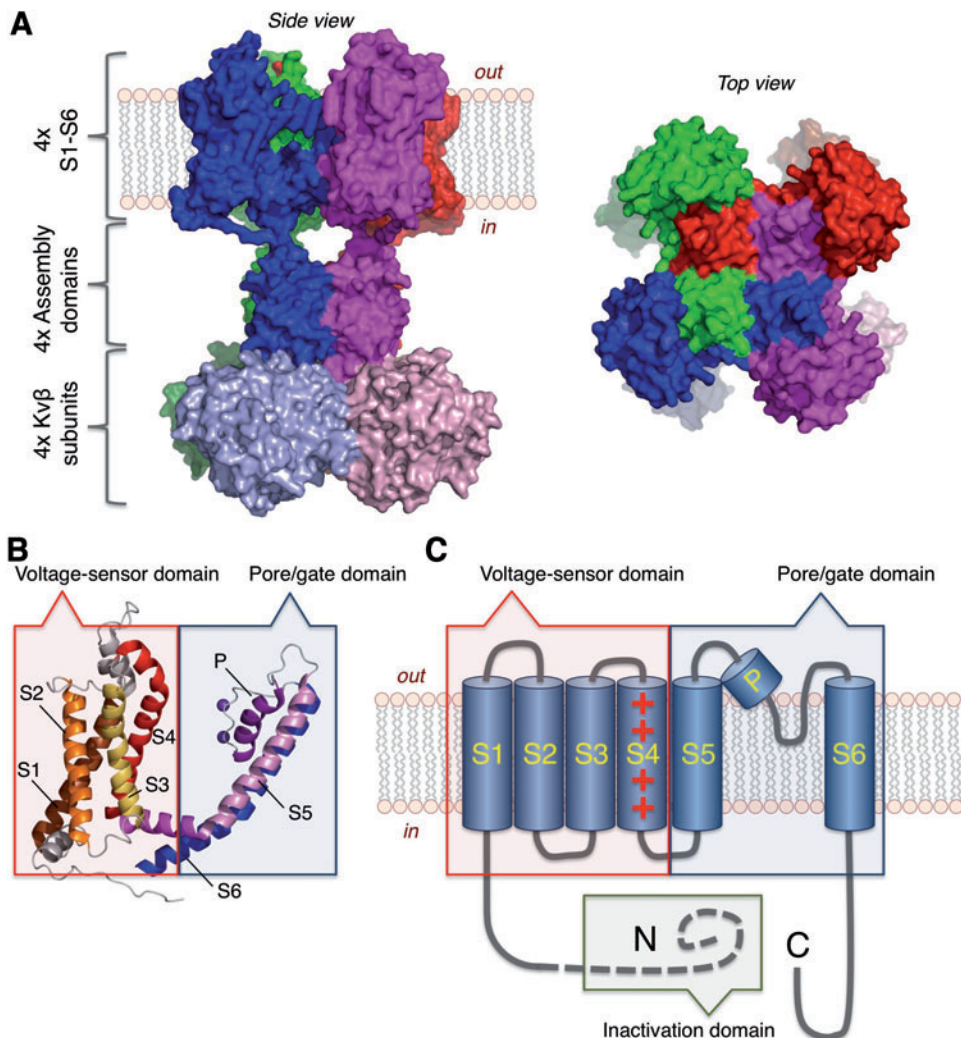


FIG. 1. Voltage-gated K^+ channels. (A) Structure of a Kv1.2/Kv2.1 chimeric channel according to Long *et al.* (74) (KCNA2/KCNB1; PDB 2R9R) with docked Kvβ2 subunits. (B) Structural elements covering S1–S6 of a single subunit of the Kv1.2/2.1 chimera. Two K^+ ions at the selectivity filter are also indicated. (C) Topological model of a 6TM α -subunit of voltage-gated K^+ channels illustrating the voltage-sensor domain (S1–S4), the pore/gate domain (S5–S6), and an N-terminal domain that functions in some channel types as inactivation “ball” domain. 6TM, six transmembrane segments. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

list of traditional names. In Figure 2A, a phylogenetic tree illustrates the relatedness of genes coding for α -subunits of voltage-gated K^+ channels using their HUGO names. The linear sequence is thus the basis for the formation of subfamilies within the family of voltage-gated K^+ channels (Fig. 2B). The largest one is the so-called "Kv family" (KCNA-KCND), which subdivides into Kv1 (*Shaker*, KCNA), Kv2 (*Shab*, KCNB), Kv3 (*Shaw*, KCNC), and Kv4 (*Shal*, KCND) channels. While coassembly within the subfamily is observed, subunits coded by these genes typically do not form heterooligomers with subunits from other subfamilies. There are more genes that belong to this subfamily (*e.g.*, Kv5-Kv9, KCNF, KCNG, KCNV, and KCNS) whose gene products can modulate the previously described subunits. Further subfamilies are *ether à go-go* (EAG) channels (Kv10-Kv12, KCNH) and KCNQ channels (Kv7, some of them also referred to as M-channels). Here, we focus on the Kv, EAG, and KCNQ subfamilies. In addition, we will discuss voltage- and Ca^{2+} -activated big conductance K^+ channels (BK channels, SLO1, KCa1.1, and KCNMA1); they are activated by cytosolic Ca^{2+} and possess an additional S0 transmembrane segment placing the N terminus to the extracellular side. Other members of this group of KCa or Slo channels also have a large conductance and are voltage dependent, but they are co-activated by Na^+ (KCNT1), Cl^- (KCNT2) or protons (KCNMA3).

Given the large number of K^+ channel complexes and considering that exposure to ROS often has an impact on

channel function—sometimes in a prominent manner, sometimes more subtle—the first step in analyzing the underlying molecular mechanisms is to identify the biochemical effector sites of ROS. On the level of gene transcription, ROS-dependent regulation of the expression of genes coding for K^+ channels can take place. Acute and direct ROS effects may be grouped into those affecting the channel protein itself, affecting some auxiliary subunits, or membrane components. If a known protein is affected, specific residues that are modified by ROS (typically cysteine, methionine, tyrosine, or histidine) may be identified to provide further insight. Some typical ROS molecules, their conversion, and select protein modifications often observed are presented in Figure 3. Rather than modifying the channel protein, alterations of the cellular ROS level may have an impact on the balance of redox couples such as Fe^{2+}/Fe^{3+} , GSH-red/GSH-ox, nicotinamide adenine dinucleotide phosphate (NADPH)/NADP⁺, *etc.* and those may be the molecules modulating channel function.

Finally, it needs to be assessed whether a ROS effect on a channel is the result of irreversible protein degradation (*e.g.*, *via* ubiquitination) or a reversible ROS-dependent regulation of channel function. As we will see below, in many cases a dependence of K^+ channels on exogenously applied oxidative modifiers has been demonstrated, but we often lack information on the physiological stimuli and the molecular mechanisms underlying the responses elicited by various ion channel types.

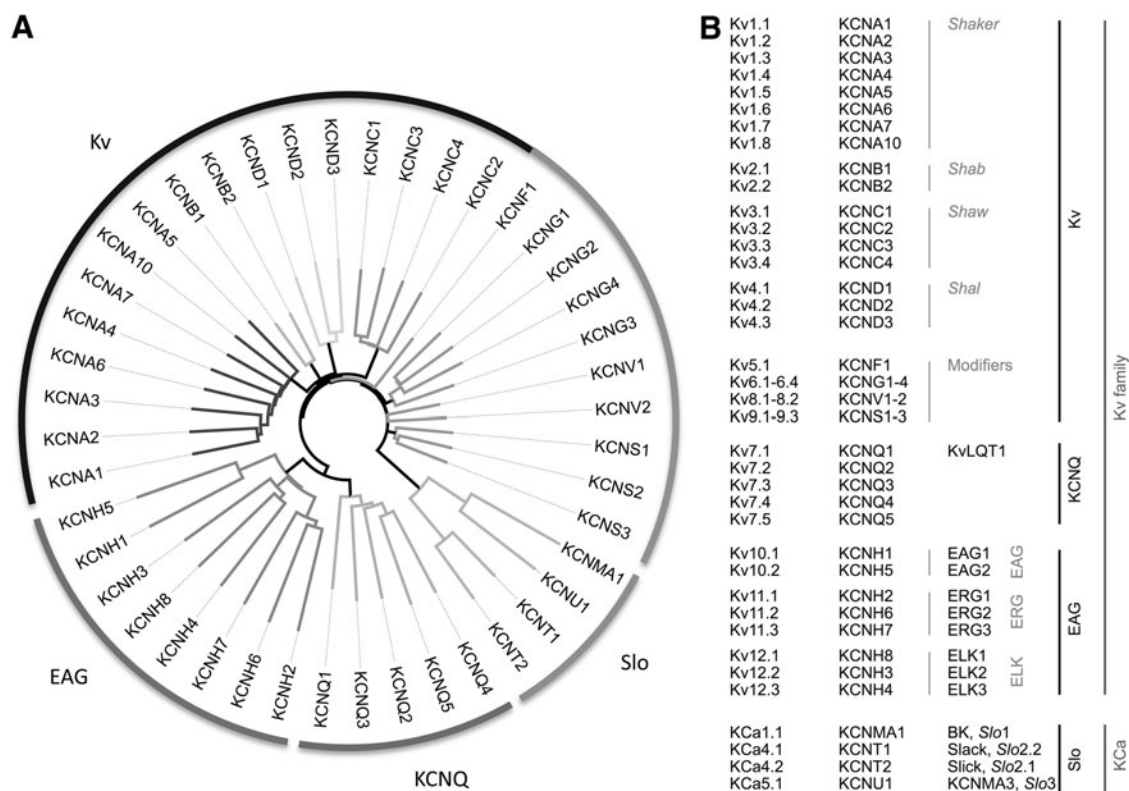


FIG. 2. Nomenclature of voltage-gated K^+ channels. (A) Phylogenetic tree of human genes coding for voltage-gated K^+ channel α -subunits of the 6TM family in HUGO nomenclature. The major subfamilies are indicated. (B) Names of proteins according to the Kv nomenclature, genes in HUGO nomenclature, and some traditional names of voltage-gated K^+ channels. Names of subfamilies (black) and groups (gray) are also indicated. Note that KCNQ1/Kv7.1 is also known as KCNA8, KCNA9, and Kv1.9.

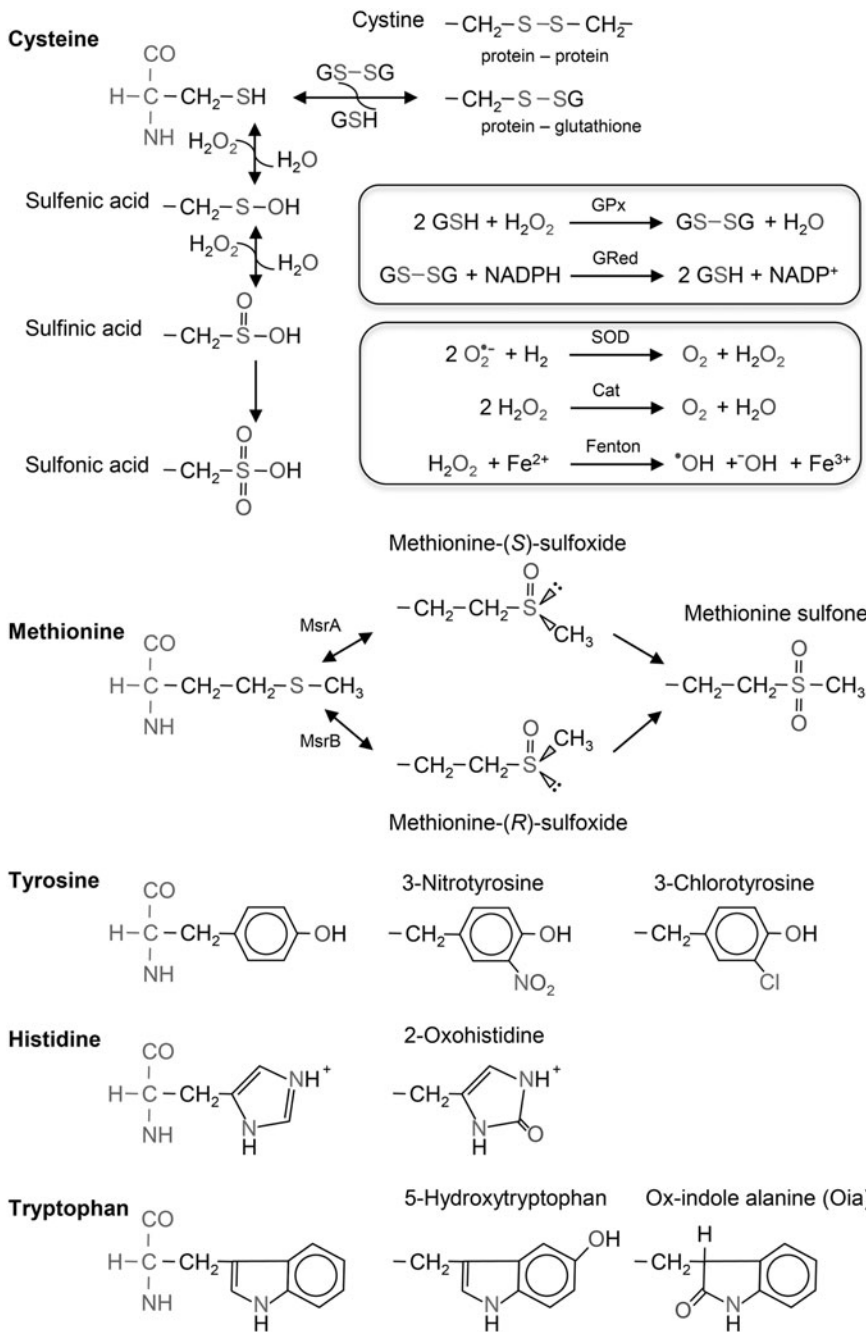


FIG. 3. ROS processing and protein modification. Modification of cysteine, methionine, tyrosine, histidine, and tryptophan often observed in proteins upon oxidative stress. *Top box:* Formation of glutathione dimers (GSSG), catalyzed by glutathione peroxidase (GPx) and recycling to reduced glutathione (GSH) via the NADPH system. *Bottom box:* Removal and formation of reactive species via superoxide dismutase (SOD), catalase (Cat), and the Fenton reaction involving transition metals (here: ferrous iron, Fe^{2+}). H_2O_2 , hydrogen peroxide; $\text{O}_2^{\bullet-}$, superoxide anion; OH^\bullet , hydroxyl radical; OH^- , hydroxyl anion; Fe^{3+} , ferric iron; MsrA/B, methionine sulfoxide reductase A/B; NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species.

The Kv Subfamily

Voltage-gated K^+ channels of the Kv subfamily activate on membrane depolarization and give rise to so-called delayed-rectifier currents. Some of them, in particular Kv1.4, Kv3, and Kv4 channels, can undergo rapid inactivation, an aspect leading to A-type currents, and this will be discussed below.

Kv1.1 and Kv1.2

Kv1.1 and Kv1.2 channels are found in the brain (77) and involved in regulation of neurotransmitter release (29, 54). Studying the impact of nitric oxide (NO) on paraventricular nucleus cells from rat slices, Yang *et al.* (150) observed that this

challenge increased spontaneous miniature inhibitory postsynaptic currents. Using specific Kv channel blockers, they further found that Kv1.1 and Kv1.2 serve as downstream effectors of NO-dependent GABA release in these neurons, possibly by a combination of NO and ROS forming very reactive but short-lived peroxynitrite (ONOO^-) (150).

An influence of peroxynitrite was also shown for Kv channels in small coronary arteries, where they play a role in vasodilation (70). An elevated glucose level attenuated the dilator function of Kv channels, possibly by nitration of a tyrosine residue. Peroxynitrite stress during hyperglycemia thus impairs Kv channel availability and may influence coronary blood flow and myocardial perfusion (70).

Conforti and Millhorn (22) showed that Kv1.2 channels could play a role in O₂ chemoreception in pheochromocytoma 12 (PC-12) cells derived from adrenal chromaffin cells. Following prolonged hypoxia (18 h, 10% O₂), the expression of Kv1.2 increased and the cells became more responsive to short-term hypoxia suggesting that Kv1.2 might modulate an O₂-sensitive signaling pathway (22, 23). While anoxia decreased the current through Kv1.2 channels, no impact on Kv2.1 was observed. A strategy based on this finding, involving Kv1.2/Kv2.1 channels and point mutations, revealed that a methionine in the S5 region of Kv1.2 (M380) is necessary for the response of Kv1.2 channels to anoxia (25).

Kv1.3

Kv1.3 channels are predominantly expressed in T- and B-lymphocytes and play a vital role in regulation of membrane potential and Ca²⁺ signaling (146). Inhibition of Kv1.3 channel activity leads to suppression of Ca²⁺ signaling, cytokine production, and T-lymphocyte activation and proliferation (16). Exposure of freshly isolated human T-lymphocytes and leukemic Jurkat T-cells to hypoxia inhibited Kv1.3 currents by up to 47% (24) and this effect could be attributed to a decreased Kv1.3 surface expression (19). This dependence of Kv1.3 on hypoxia may provide an explanation for the failure of T-cell activation in proliferating cancer tissue, which often provides hypoxic conditions.

Szigligeti *et al.* (126) provided a mechanistic link for the O₂ sensitivity of Kv1.3 channel in T-cells: Kv1.3 channels were resistant to hypoxia in Jurkat T-cells deficient in the src protein tyrosine kinase p56Lck and they gained O₂ sensitivity when constitutively active Lck (Y505FLck) was transfected to Lck-deficient cells (126).

Kv1.5

Kv1.5 channels contribute to the repolarizing ultra-rapid delayed-rectifier current I_{Kur} in the heart, and aberrations in Kv1.5 channels can lead to atrial fibrillation (91) and sudden cardiac death (90). Svoboda *et al.* (124) demonstrated that a global increase in sulfenic acid-modified proteins is associated with atrial fibrillation. Kv1.5 channels may be important players in this process because sulfenic acid modification appears to take place at a conserved cysteine at the C terminus (C581). Under prolonged oxidative stress, sulfenic acid modification diminished Kv1.5 channel surface expression. In freshly isolated rat hearts, acute sulfenic acid modification also inhibited Kv currents and induced sustained arrhythmia. This finding may provide a therapeutic clue for the treatment of atrial fibrillation (124).

Kv2.1

In neuronal tissue, Kv2.1 appears to be involved in modulation of neuronal apoptosis; using a model of experimentally induced apoptosis, Pal *et al.* (94) showed that cortical neurons expressing dominant-negative Kv2.1 became insensitive while Kv2.1-overexpressing CHO cells became susceptible to apoptosis induction. Since oxidative stress-induced apoptosis is a major etiology of neurodegenerative disorders and aging, suppression of Kv2.1 could be a protective strategy. A recent report by Dallas *et al.* (27) showed that carbon monoxide (CO), an endogenous signaling molecule typically

produced during the degradation of heme, inhibited native Kv2.1 channels in hippocampal neurons and recombinant Kv2.1 in HEK 293 cells. Application of the membrane-permeant oxidizing agent DTDP increased the surface expression of Kv2.1 and induced apoptosis while the CO donor CORM-2 diminished this vulnerability. This observation suggests a mechanism of neuroprotection, where CO provides resistance against oxidant-induced apoptosis *via* Kv2.1 suppression (27).

Kv2.1 expression has also been linked to Alzheimer's disease progression. A study by Cotella *et al.* (26) found that Kv2.1 forms oligomers in the presence of oxidizing agents, and old mice showed ~10-fold higher oligomer formation than young mice. Further, it was revealed that a conserved cysteine in the cytoplasmic side (C73) was the target of oxidation while mutant C73A was functionally indistinguishable from wild-type channels. In *Caenorhabditis elegans* it was found that β -amyloid facilitates oxidation of the Kv2.1 paralog KvS-1 and induces neuronal apoptosis while the mutant homolog to Kv2.1-C73A rendered the cells resistant to β -amyloid-induced oxidation and apoptosis. This study might provide a therapeutic clue for the treatment of age-related degenerative diseases (26). This hypothesis received support from the results of Yuan *et al.* (152) who reported that Donepezil, a commercially available drug that improves cognitive and global functions in Alzheimer's disease patients and for those suffering from vascular dementia (33), provides protection against oxygen/glucose deprivation-induced cell apoptosis by inhibiting Kv2.1 currents. Although the exact mechanism is not known, Donepezil may prevent the oxidation of Kv2.1 channels and thereby protect against apoptosis (152).

In pancreatic β -cells, Kv2.1 regulates glucose-dependent insulin secretion (81, 83), and insulin secretion also depends on the cytosolic status of the NADPH/NADP⁺ ratio (2). At body temperature, Kv2.1 undergoes some inactivation: the time course of inactivation was slowed down upon oxidation and depended on the NADPH/NADP⁺ ratio suggesting that oxidative control of Kv2.1 function is a key mechanism in the control of insulin release (82).

Oxygen sensing in the lung

Several Kv channels are involved in the regulation of the pulmonary circulation and implicated to be oxygen sensitive (86). A physiological response to hypoxia in the lung is hypoxic pulmonary vasoconstriction (HPV), which redirects the blood flow to better-ventilated areas of the lung, thus increasing the total area involved in gaseous exchange. Patients suffering from hypoxic pulmonary diseases and residents of high altitude have sustained HPV, which can lead to vascular remodeling, pulmonary hypertension, and possibly fatal heart failure (6, 86, 125). As illustrated in Figure 4A, the Kv-related redox regulation of HPV starts with a block of Kv channels because of ROS produced during hypoxia. K⁺ channel block results in cell depolarization that activates voltage-gated Ca²⁺ (Ca_v) channels, which are also directly activated by hypoxia (38). Finally, intracellular Ca²⁺ triggers contraction and induces HPV (6, 86, 125). It should be noted, though, that the extent of ROS production during acute and sustained hypoxia and reoxygenation remains controversial (39) and certainly further studies aiming at the quantitative ROS monitoring under such conditions are required.

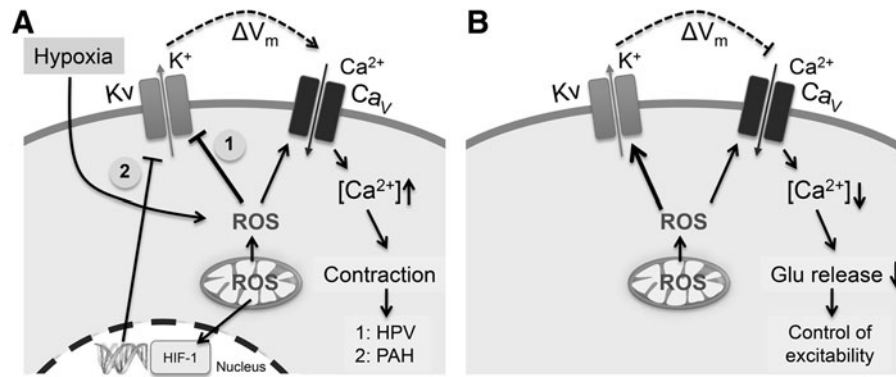


FIG. 4. Impact of ROS-dependent K^+ channel modulation on cell functions. (A) Examples of how redox-dependent inhibition of Kv channels in pulmonary smooth muscle cells may change cell function: inhibition of Kv channels directly by ROS [1] or downregulation *via* an impact on gene transcription [2] may lead to membrane depolarization, activation of Ca_V channels, influx of Ca^{2+} , and subsequent hypoxic pulmonary vasoconstriction (HPV, 1) or pulmonary arterial hypertension (PAH, 2), respectively. (B) In neurons, ROS-mediated activation of Kv channels, such as by removal of inactivation in A-type channels, may limit the influx of Ca^{2+} through depolarization-dependent Ca_V channels leading to decreased electrical excitability because of diminished transmitter release. Ca_V , voltage-gated Ca^{2+} ; HIF, hypoxia-inducible factor; Glu, glutamate.

Kv channels are also involved in vascular remodeling by modulating cell proliferation and apoptosis in pulmonary artery smooth muscle cells. Under chronic hypoxia, Kv channels in these cells, such as Kv1.5 and Kv2.1, are downregulated by the activation of transcription factors hypoxia-inducible factor-1 (HIF-1) and nuclear factor-activating T (NFT), which results in the pathogenesis of pulmonary arterial hypertension, a disease with 15% mortality rate (8). The loss of Kv channels causes membrane depolarization and a rise in intracellular $[Ca^{2+}]$ (Fig. 4A) that ultimately stimulates proliferation; the loss of Kv channels also leads to accumulation of intracellular K^+ , which inhibits caspases and reduces apoptosis, an event often related to the pathogenesis of cancer (7, 86).

Inactivating Kv Channels

The term “A-type currents” refers to depolarization-activated K^+ currents that rapidly inactivate. In neurons, these currents play a vital role in the control of excitability, regulation of presynaptic spike duration, Ca^{2+} entry, and neurotransmitter release (72). In cardiac muscle, the transient outward K^+ current (I_{to}) contributes to the initial repolarization phase of cardiac action potentials (138). Genetic analysis has revealed that A-type currents are mediated by voltage-gated K^+ channels of the Kv subfamily (Fig. 2), but the molecular mechanisms leading to inactivation are diverse.

Two inactivation processes, fast and slow, are readily recognized (50, 51). Fast inactivation follows the “ball-and-chain” mechanism in which one of the four N-terminal ends plug the channel cavity (50, 51, 155). This N-type inactivation is abolished by genetic deletion or enzymatic removal of the N terminus, and it can be restored by intracellular application of peptides derived from the N terminus (5, 155). Mammalian Kv channel α -subunits capable of generating A-type currents are divided into three major classes: (i) Kv1—*Shaker*-related: Kv1.4, (ii) Kv3—*Shaw*-related: Kv3.3, Kv3.4, and (iii) Kv4—*Shal*-related: Kv4.1-3. A special case is the *Drosophila* Shaker channel, for which various splice variants are equipped with N-terminal inactivation domains. Although only a limited

number of Kv α -subunits possess N-terminal structures capable of inducing N-type inactivation, some auxiliary cytoplasmic β -subunits of the Kv β family (Fig. 5) interact with Kv1 α -subunits to create N(β)-type inactivation by contributing their N-terminal inactivation domains (44, 45, 103).

For historical reasons, slow inactivation is termed “C-type inactivation” (50, 51). The molecular/atomic mechanism is not well understood but the pore structure and the permeating ions play a vital role [for review see Hoshi and Armstrong (47)]; therefore, the term C/P-type inactivation is also used (76). Nearly all voltage-gated K^+ channels can undergo C-type inactivation; typically this is slow but in some cases it can be on the order of 100 ms (98).

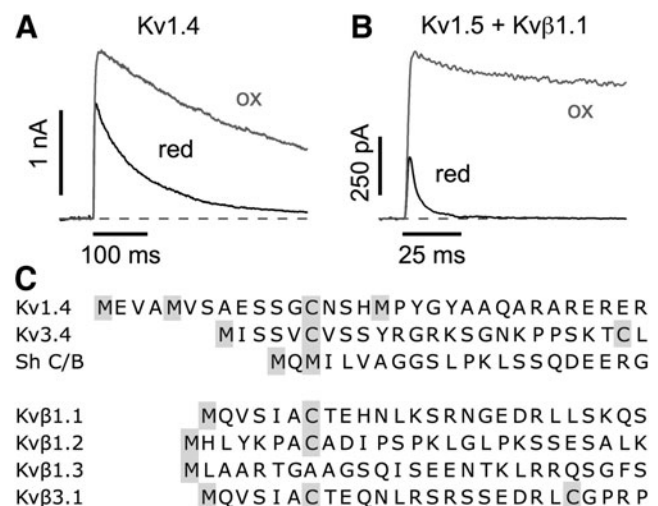


FIG. 5. N-type inactivation. (A, B) Current traces at 40 mV from *Xenopus* oocytes expressing Kv1.4 (A) and Kv1.5 + Kv β 1.1 (B) in the on-cell mode (black) and upon excision to the inside-out configuration (gray) indicating the redox dependence of fast inactivation. (C) Alignment of N-terminal sequences of α -subunits (top) and Kv β subunits (bottom) that induce N-type inactivation. Cysteine (C) and methionine (M) residues are highlighted.

In either case, regulation of A-type currents is a very powerful tool because even a minor slowdown of inactivation or an increased steady-state noninactivating component constitutes a gain of function phenomenon, which typically has a stronger impact on the electrical properties of a cell than a partial loss of function. Therefore, the kinetics of many A-type currents is subject to a tight control by the cellular redox milieu.

ROS regulation of N-type inactivation

ROS sensitivity of N-type inactivation can be observed easily when recording current from channels expressed in *Xenopus* oocytes; in the on-cell configuration of the patch-clamp method, inactivation is fast, but upon patch excision, inactivation gets progressively slower as the cytosolic side of the membrane patch is exposed to ambient oxygen-saturated bath solution (Fig. 5A). Reversibility is obtained by pushing the patch back into the reducing cytosol. Such experiments were first performed by Ruppertsberg *et al.* (106) who demonstrated the ROS sensitivity of rat Kv1.4 and Kv3.4 channels. Both channel types harbor N-terminal inactivation domains and both contain a cysteine residue (Fig. 5B). The mutation C13S in Kv1.4 rendered the inactivation insensitive to changes in the intracellular redox potential. Since Kv1.4 subunits can coassemble with other α -subunits of the Kv1 group and thereby can contribute the N-terminal domains for N-type inactivation, many Kv current components may exist under *in-vivo* conditions with ROS-dependent inactivation properties.

As simple as this regulation mechanism may seem, it is not unambiguously known what kind of oxidative modification actually occurs and why the inactivation is slowed down or eliminated altogether. A possible scenario is that the normally disordered N-terminal inactivation domain, which is supposed to enter the pore cavity to obstruct K⁺ current flow, is "immobilized" by forming a disulfide bridge with another protein-based thiol group. Such other group could be a cysteine of one of the other N-terminal domains, a cysteine elsewhere in the α -subunit that is accessible to the N terminus, or an auxiliary protein. Alternatively, covalent attachment of small soluble thiol-containing molecules such as glutathione may change the structure of the N-terminal inactivation domain in a manner sufficient to prevent inactivation. The cysteine itself can be oxidized by hydrogen peroxide (H₂O₂) to sulfenic (SOH) acid, to sulfinic (SO) acid, and finally to sulfonic (SO₂) acid (Fig. 3) (35). Depending on the extent of oxidation, such modified cysteine will then affect inactivation in a reversible or irreversible manner.

ROS-dependent regulation of N-type inactivation may be relevant in the action of Riluzole, which is used in the treatment of amyotrophic lateral sclerosis. Riluzole slowed down Kv1.4 N-type inactivation and the effect was diminished by dithiothreitol (DTT) and reduced glutathione, suggesting oxidation of C13 in the inactivation domain might be involved (148). The neuroprotective action of Riluzole could be accounted for controlling glutamate release at the axon terminal *via* a ROS-mediated increase in Kv1.4 current (148).

A-type currents, primarily mediated by Kv1.4 channels, are also found in small dorsal root ganglia neurons important for pain signaling. Since oxidative challenge removed fast inactivation of K⁺ channels in these cells, redox modulation of N-type inactivation could be an interesting target for the

physiology and pharmacology of pain signaling (53). A plausible scenario of how ROS-dependent removal of N-type inactivation may affect cellular excitability and glutamate release is illustrated in Fig. 4B.

Aging of *C. elegans*. A link of redox modulation of A-type currents to the process of aging was provided for KvS-1 (a paralog to Kv2.1) channels in *C. elegans* with a cysteine in the N terminus (C113) (15, 117). Endogenous ROS, which slow down inactivation of KvS-1 because of C113, are responsible for a loss of sensorial capacity during aging. Transgenic worms expressing wild-type and KvS-1-C113S channels revealed that the mutation leads to a preservation of chemotaxis during aging, suggesting that the process of ROS-mediated modulation of N-type inactivation could contribute to the age-associated neuronal degeneration (15, 117).

Kv β subunits. ROS-regulated N-type inactivation seems to be a potent and widespread phenomenon because several Kv β subunits also harbor N-terminal inactivation domains with ROS-sensitive cysteine residues (Fig. 5B, C). The first identified was Kv β 1.1, which harbors a cysteine at position 7 (103). Other examples are Kv β 3 [C7 and C22 (44)] and Kv β 1.2 (C8). Since such cytosolic Kv β subunits can dock to α -subunits of the Kv1 group, many delayed rectifier K⁺ channels can be transformed to A-type channels with rapid Kv β -induced inactivation. The occurrence of ROS-dependent and ROS-independent Kv β subunits suggests that, by means of gene transcription and alternative splicing, a cell can generate K⁺ current components with degrees of inactivation and sensitivities tailored to changes of the cellular redox status.

In addition to the ability of some Kv β subunits to induce N-type inactivation, they appear to have an enzymatic function as oxidoreductases, and this enzymatic activity can modulate fast inactivation *via* oxidation of the cofactor NADPH. Pan *et al.* (95, 96) showed that either enzymatic conversion of NADPH to NADP⁺ by a substrate or direct application of NADP⁺ to excised membrane patches with Kv1.1/Kv β 1.1 channels leads to changes in fast N-type inactivation. Interestingly, such results were also obtained with mutant Kv β 1.1-C7S, suggesting a unique mode of redox modulation without involving this critical cysteine residue (95, 96, 143). Further studies suggested that NADP⁺ binding to the Kv β 1.1 core domain influences the availability of the Kv β 1.1 N-terminal ball domain (96); likewise, the inactivation may in turn influence the catalytic activity.

Methionine oxidation. A special case of ROS-dependent inactivating Kv channels is the C/B splice variant of Shaker K⁺ channels in *Drosophila* (137). These channels (Sh C/B), when expressed in *Xenopus* oocytes, display a very noticeable variability in inactivation time course. Inspection of the N-terminal inactivation domain, however, does not reveal any cysteine residue. Application of the mild oxidant chloramine-T (ChT) potently removed inactivation suggesting that residues other than cysteine might be modified in an oxidative manner. Sh C/B channels contain a methionine at position 3 (Fig. 5C); when mutated to leucine, the variability of inactivation speed disappears (20).

Following cysteine, methionines, which also harbor a sulfur atom, are the residues that are the next most susceptible to

oxidation. In a first oxidative reaction, methionine is oxidized to methionine sulfoxide [Met(O)], in a second to methionine sulfone [Met(O₂)]. While Met(O₂) formation is physiologically irreversible, Met(O) can be reduced back to methionine by the aid of peptide methionine sulfoxide reductases (Msr) (Fig. 3) (48, 141). When mammalian variants of MsrA were coexpressed in *Xenopus* oocytes, inactivation of Sh C/B channels was fast and did not display a large scatter (20, 67). In addition, inactivation peptides with the Sh C/B sequence but with Met(O) instead of Met at position 3 did not induce inactivation in N-terminal-deficient Kv channels, while incubation of such peptides with MsrA and DTT partially restored their capability of mimicking inactivation (20). Methionine oxidation appears to be a relevant mechanism as even treatment of the oocytes with the antioxidant vitamin C had an influence on the inactivation time course of Sh C/B channels (21).

It is unclear whether oxidation of M3 in the N-terminal ends of Sh C/B channels is reversible under physiological conditions or whether the activity of MsrA in oocytes simply produces enough scavenging methionine groups to keep the cytosolic oxidative challenge low (48). Further, it must be considered that oxidation of methionine leads to two diastereomers of methionine sulfoxide, namely Met-S(O) and Met-R(O), which require different types of Msr: MsrA reduces Met-S(O) while MsrB reduces Met-R(O) (Fig. 3) (64, 69). In fact, coexpression of human MSRA and human MSRB2 with Sh C/B in *Xenopus* oocytes had a stronger effect than MSRA alone arguing that complete protection and/or reduction can be obtained when both diastereomers of Met(O) are addressed (62).

The mechanism by which methionine oxidation affects inactivation most likely is related to the change of the side-chain's physicochemical properties when an oxygen atom is attached to the sulfur. The residue will become less hydrophobic than methionine (12) and may thus eliminate a hydrophobic interaction of the critical methionine residue of the inactivation ball domain with the cavity of the channel. Aside from all N-terminal start methionines, mammalian sequences of N-terminal inactivation domains also contain methionine residues (e.g., M5 and M17 in Kv1.4, Fig. 5C; M29 in Kv3.4); methionine oxidation and enzymatically-driven reduction may be a regulatory mechanism existing beyond Sh C/B channels.

KCNA7 transcripts are found in the heart and skeletal muscle where the channels possibly play a role in repolarization of the action potential (36). In mouse, there are two isoforms of Kv1.7, where the long variant appears to be capable of undergoing N-type inactivation. However, in contrast with other N-type inactivating Kv channels, exposure to oxidants such as DTDP or H₂O₂ accelerated inactivation. Several redox-sensitive residues of the Kv1.7 protein (H21, C41, C43, and C44) were identified to take part in this process (36).

ROS regulation of C/P-type inactivation

While N-type inactivation is restricted to a few members of the Kv subfamily, almost all of them can undergo some sort of C/P-type inactivation. Thus, redox-mediated modulation of C/P-type inactivation may be a powerful tool to regulate the cellular availability of voltage-gated K⁺ channels. When exposed to oxidative challenge, inactivation of Shaker channels without N-terminal inactivation domains was acceler-

ated, and the mutation M440L in the pore-segment of the α -subunit abolished that effect. M440, strongly conserved among Kv channels, is located at the apex of the pore helix inside the cavity (17). However, it is unlikely that this site can be reduced by Msr and thus this represents an irreversible modification of M440. Likewise, exposure of Shaker channels to extracellular oxidants also accelerates inactivation and this effect was diminished by the mutation M448L (113). Again, this extracellularly accessible methionine at the pore entrance close to the selectivity filter is conserved and, hence, this kind of oxidative influence on P/C-type inactivation may also apply for many other Kv channels.

KCNQ Channels

Kv7 (KCNQ) channels are voltage-gated K⁺ channels known for their role in raising the threshold for action potential firing and stabilizing the resting membrane voltage in excitable cells. They are predominantly expressed in brain, heart, epithelia, and vestibular and auditory organs (52). In neurons, KCNQ underlies M-currents; the corresponding channels are mainly composed of Kv7.2/Kv7.3 heteromultimers and Kv7.4 homomultimers. M-currents were first recorded in frog neurons and later in mammalian peripheral and central neurons; stimulation of muscarinic acetylcholine receptors (hence the name "M") inhibited the currents, depolarized neurons, and increased neuronal excitability (14). The channels activate slowly at around -60 mV and do not inactivate at neuronal resting potentials. These properties enable Kv7 channels to stabilize the neuronal resting potential. They act as a "brake" on action potential firing when the neuron is exposed to an excitatory stimulus (42). In the heart, Kv7.1 contributes to the slowly activating K⁺ current *I_{Ks}*, which is essential for action potential termination. The underlying channel is a macromolecular complex composed of α - (Kv7.1) and modulatory β - (KCNE1) subunits. Mutations in either of them can give rise to congenital long QT syndrome and, hence, this channel was originally named KvLQT1 (59, 93).

Structurally, Kv7 channels are different from other channels of the Kv family with respect to a short cytoplasmic N terminus (~100 residues) and lack of a tetramerization (T1) domain. In addition, Kv7 channels harbor a large C-terminal domain (300–500 residues) with four α -helices, in which two proximal helices serve as a scaffold for cytoplasmic regulatory molecules and also facilitate oligomerization (52).

Kv7 channels have been reported to show oxidation sensitivity. H₂O₂ at physiological concentrations markedly increases M-currents formed by three of five Kv7 channels (40). Application of 5 μ M H₂O₂ to Kv7 channels expressed in Chinese hamster ovary cells increased currents mediated by Kv7.2/3 heterooligomers and Kv7.4 homooligomers while Kv7.1 and Kv7.3 homooligomers were not affected. A cysteine triplet in the S2–S3 linker of Kv7.4 (C112, C175, and C519) was identified as critical for this effect. These three cysteine residues are conserved in all Kv7 channels except for Kv7.1, suggesting that M-currents are specifically modulated by oxidation. Using a hypoxia-induced model of neurodegeneration by recording from hippocampal slice cultures exposed to oxygen/glucose deprivation, it was further found that enhanced M-currents induced by ROS provide protective effects on oxidative stress-related neurodegeneration. The

ROS-triggered enhancement of M-currents could possibly provide a mechanism protecting against oxidative stress-induced excitotoxicity caused by overactivation of ionotropic glutamate receptors (40).

In primary sensory (nociceptive) neurons, substance P enhanced M-type K^+ channel activity by releasing ROS from the mitochondria and thereby reduced excitability. The underlying mechanism involves activation of neurokinin receptor 1 and $G_{i/o}$ proteins and provides a ROS-related link between the neuromodulator and hyperalgesia (71).

NO has been shown to influence I_{Ks} in guinea-pig cardiomyocytes. Bai *et al.* (9) showed that the application of Ginsenoside Re, a traditional drug from *Panax ginseng*, generally known for its antiarrhythmic and vasorelaxing effect, caused an enhancement of I_{Ks} in isolated ventricular myocytes from guinea pig. The enhancement of I_{Ks} was abolished in the presence of inhibitors of NO synthase and an NO scavenger, suggesting that NO is responsible for the stimulation of I_{Ks} . Further, application of NO donor mimicked Ginsenoside Re and the effect was not mediated *via* the adenylate cyclase, cAMP-dependent protein kinase, and the serine–threonine phosphatase 2A pathway. The stimulatory effect of NO was reversed by DTT suggesting that NO possibly facilitates S-nitrosylation of a cysteine residue in Kv7.1 channels. This finding could explain the cardioprotective effect of Ginsenoside Re, which underlies NO-redox signaling (9).

In sensory neurons from dorsal root ganglia NO inhibited M-current and increased neuronal excitability (92). It further counteracted the stimulatory effect of ROS on Kv7.4 channels involving a triad of cysteine residues in the S2–S3 linker (C156–158 in Kv7.4). M-channels therefore appear to be regulated by NO and ROS in a reciprocal manner and possibly serve as dynamic redox sensors with a vital role in neuronal excitability (92).

EAG Channels

The EAG subfamily of voltage-gated K^+ channels was named according to its first member, the EAG channel, first identified in a mutant of *D. melanogaster* exhibiting leg-shaking behavior under ether anesthesia (63). Later, mammalian homologs were discovered and formed the EAG group within the EAG subfamily (10, 41). In humans, the EAG subfamily consists of three groups and comprises a total of eight channels (Fig. 2): (i) EAG—EAG1-2 (Kv10.1-2); (ii) EAG-related gene (ERG)—ERG1-3 (Kv11.1-3); (iii) EAG-like K^+ channel (ELK)—ELK1-3 (Kv12.1-3) (32, 80, 107, 110, 118, 119). The overall structure of EAG subfamily members is different from other voltage-gated K^+ channels in that they possess long N-terminal intracellular domains comprising a Per-Arnt-Sim (PAS) domain, a C-terminal cyclic nucleotide-binding homology domain (cNBHD), and a C-terminal C-linker (e.g., Fig. 6). The functions of PAS and cNBHD domains are not yet understood (10), but PAS domains are often involved in oxygen sensing (132). The C-linker seems to play an important role for EAG channels, and for Kv10.1 and Kv11.1 it has been shown that its function is subject to oxidative modulation (66, 108). It is noteworthy that the C-linker region has been postulated to be a critical component for gating of cyclic nucleotide-modulated channels such as HCN (156) and CNG (164), whose amino-acid sequences are similar to that of the EAG family of channels.

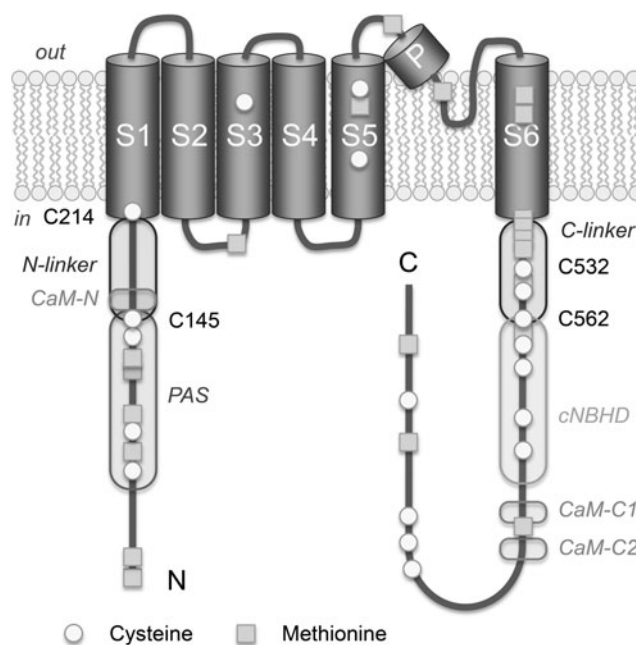


FIG. 6. Topological model of a human Kv10.1 (EAG1) subunit. Cysteine residues that are implicated in ROS-dependent changes of channel function (108) are indicated by number. CaM-N and CaM-C, N- and C-terminal-binding sites for calmodulin; PAS, Per-Arnt-Sim domain; cNBHD, cyclic nucleotide-binding homology domain.

The EAG group

EAG1 (Kv10.1) was the founding member of the EAG subfamily (142). It is characterized by (i) the dependence of its activation kinetics on the prepulse potential and extracellular Mg^{2+} ions (80, 134), and (ii) its inhibition by intracellular Ca^{2+} /calmodulin (115, 163). Kv10.1 is expressed in the brain where it presumably regulates neuronal excitability. However, Kv10.1-deficient mice display normal behavior, and no obvious histological or functional changes are observed (139). On the other hand, morpholino-mediated knockdown of *Kcnh1* in zebrafish indicates an important role in early development (122). Kv10.1 is abnormally overexpressed in many cancer cells where it facilitates cell cycle progression,

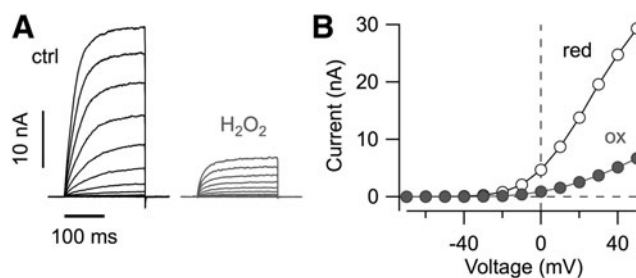


FIG. 7. Voltage-activated K^+ channels. (A) Whole-cell current recordings from HEK 293 cells expressing Kv10.1 channels with depolarizing voltage steps ranging from -70 to 50 mV before (ctrl) and after application of bath solution containing H_2O_2 . (B) Maximal currents from the data shown in (A) as a function of voltage.

proliferation, and migration, and it is considered as a potential cancer biomarker (100, 104).

Kv10.1 expression is linked to oxygen homeostasis as revealed by the following observations: (i) mild hypoxia (5% O₂, 4 h) induced a clear increase in HIF1 abundance in Kv10.1-expressing cells while no increase in HIF1 was observed under normoxia; (ii) Kv10.1-expressing tumors show increased vascularization and promotes VEGF secretion. This finding suggests that transformative properties of Kv10.1 are dependent on an interference with the cellular oxygen homeostasis system. Increased vascularization would augment oxygen and nutrient supply to tumor cells and that could provide growth advantage for Kv10.1-expressing cells (30).

Our studies have shown that Kv10.1 is exquisitely sensitive to changes in the intracellular redox conditions. Ionic currents through heterologously expressed Kv10.1 channels are progressively inhibited by treatment with cysteine modifiers such as DTNB, MTSES⁺, MTSET⁻, and H₂O₂ (Fig. 7). A systematic study of cysteine mutants and functional assays revealed that modification of Kv10.1 by cysteine-specific agents contains two components. A rapid component resulted in an alteration of gating parameters: (i) a right-shift of half-activation voltage, (ii) slowdown of activation kinetics, (iii) acceleration of deactivation kinetics, and (iv) a reduction of P_o. A slow component caused complete loss of function. While the latter component strongly depends on two cysteines in the C-linker structure (C532:C562, Fig. 6), the fast component is mediated by cysteine residues in the equivalent linker region of the N terminus (C145:C214), suggesting that those protein structures connecting the large cytosolic protein domains to the transmembrane voltage-sensor and pore/gate modules are important redox-dependent relay stations.

The ERG group

Kv11.1 (ERG1) is the most prominent member of the EAG subfamily. It is expressed in cardiac tissue, nervous tissues, smooth muscles, and abnormally overexpressed in tumor tissues (11, 43). The best known function is related to its appearance in the heart where it forms the basis for the rapid delayed-rectifier potassium current (I_{Kr}). This current component plays a vital role in the repolarization phase of cardiac action potentials (10, 116). This particular function can be achieved because, upon depolarization, Kv11.1 channels only yield a small steady-state outward current because of fast inactivation and slow activation, while, upon repolarization, rapid recovery from inactivation and slow deactivation give rise to large outward currents (114). Hereditary mutations in the *KCNH2* gene or drug-mediated interference with Kv11.1 channels therefore can lead to long QT syndrome and cardiac arrhythmia (68). In neurons, Kv11.1 regulates the spike frequency and controls the resting membrane potential (43, 68). The isoforms Kv11.2 (hERG2) and Kv11.3 (hERG3) are primarily expressed in the brain (119).

Taglialatela *et al.* (127) showed that ROS induced by iron/ascorbate (Fe/asc) increased Kv11.1-mediated outward current in *Xenopus* oocytes while the inward current was not affected. This augmentation of Kv11.1 current by ROS was caused by a 12-mV right-shift of the steady-state inactivation. ROS scavengers such as catalase, superoxide dismutase, and mannitol abolished the stimulatory effect of Fe/asc. Molecular analysis identified histidine residues at positions 578 and 587

within the S5–S6 linker to be responsible for the ROS-induced activation of Kv11.1 channels (Fig. 8) (97). This finding suggested that the increased liberation of Fe²⁺ from hearts during global ischemia followed by reperfusion might promote the Fenton reaction to yield high amounts of ROS; the subsequent oxidation of two histidine residues, presumably to 2-oxohistidine, then may facilitate the opening of Kv11.1 channels. The Fe/asc-induced Kv11.1 channel activation may give rise to an anti-arrhythmic influence by repolarizing the membrane potential and shortening the action potential duration. Further, the stimulatory effect of Fe/asc on the Kv11.1 channel was counteracted by NO⁻ produced by L-arginine (128). Of note, primarily neuronal Kv11.2 and Kv11.3 do not have the critical Fe/asc-sensitive histidines, suggesting that the histidine-directed redox regulation of I_{Kr} might be tissue specific.

Studying Kv11.1 channels expressed in HEK 293 cells, Kolbe *et al.* (66) demonstrated that Kv11.1 current was inhibited by the cysteine modifier MTSES⁺, MTSEA, and DTNB when applied from the cytosolic side while the modifiers remained ineffective when applied from the extracellular side. However, extracellular application of either H₂O₂ or tert-butyl hydroperoxide (tBHP) caused a small transient increase of tail currents at -40 mV, followed by a slower but potent inhibition of the currents. This observation suggested that the transient increase could be due to modification of the two extracellular histidines and the subsequent loss of function due to some intracellular sides of the channel protein. Detailed screening of critical cysteine residues revealed that C723, located in the C-linker region, is most prominently responsible for the channel's oxidation sensitivity; C828 and C740 contribute to a smaller extent (Fig. 8) (66). Mutation of H578:H587 diminished the transient current increase observed with H₂O₂/tBHP application (66).

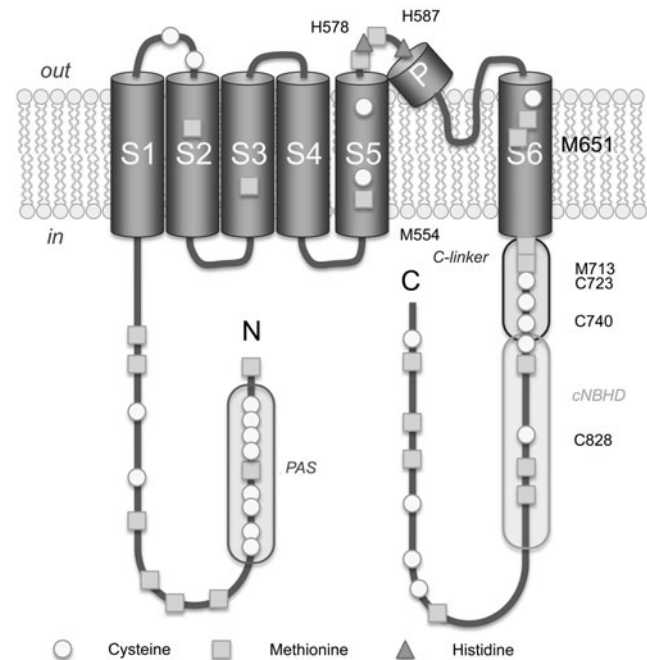


FIG. 8. Topological model of a human Kv11.1 (ERG1) subunit. Cysteine, methionine, and histidine residues that are implicated in ROS-dependent changes of channel function (66, 97) are indicated by number.

Interestingly, oxidative inhibition of Kv11.1 currents was strongly dependent on the deactivation kinetics. The Kv11.1 splice variant “Kv11.1b (hERG1b),” where the N terminus is truncated and deactivation is much faster than in Kv11.1a, was less sensitive toward thiol-directed modification. This finding suggests that the overall oxidation sensitivity of I_{Kr} in human cardiac myocytes, comprised of Kv11.1a and Kv11.1b (61), may depend on the composition of both Kv11.1 variants (66).

Methionines of Kv11.1 channels are also sensitive to oxidation. Su *et al.* (123) have shown that ChT, which preferentially oxidizes methionine, significantly decreased Kv11.1 current. The activation was slowed during depolarization to 30 mV but accelerated during depolarization to 0 or -10 mV and the deactivation was also accelerated upon repolarization. In the presence of recombinant bovine MsrA a diminished effect of ChT was observed (123). The result indicated that methionine oxidation is at least partially responsible for the Kv11.1 current inhibition. This finding was indeed confirmed by a mutagenesis study of Kolbe *et al.* (66) who observed that three methionine residues located in the C terminus (M554, M651, and M713) contribute to the inhibitory effect of ChT. The influence of ChT was significantly reduced by either mutation of the critical methionine triplet (M554:M651:M713) to leucines or by mutation (C723S), suggesting that the redox sensitivity of Kv11.1 channels is largely cysteine and methionine dependent (66). Collectively, the studies on the redox regulation of the Kv10.1 and Kv11.1 channels underscore the importance of the C-linker and cNBHD segment in gating of the channel and its coupling to oxidative processes (66, 108).

Abnormal QT prolongations were often observed in diabetic patients including type-1 insulin-dependent and type-2 noninsulin-dependent populations and the prolongations also are one of the major causes of sudden cardiac death in cardiac patients (105, 140, 158, 160). Under glucose stress, either by hyperglycemia or hypoglycemia, the Kv11.1 current was reduced, which is a likely cause of I_{Kr} dysfunction and QT prolongation. Moreover, under hyperglycemia Kv11.1 was downregulated at the protein level and insulin prevented QT prolongation and rescued I_{Kr} function (158). It was also shown for Kv11.1-expressing HEK 293 cells that treatment with the superoxide anion generating system xanthine/xanthine oxidase significantly diminished Kv11.1 current; co-treatment with vitamin E ameliorated that effect suggesting that the antioxidant vitamin E protects Kv11.1 from oxidative damage in diabetic conditions (161). To confirm that the observed Kv11.1 current reduction under hyperglycemia was due to modification of critical oxidation-sensitive residues, Kolbe *et al.* (66) compared the effect of low and high glucose conditions on the current densities obtained with wild-type and Kv11.1-C723S channels in HEK 293 cells; while the current density for wild-type was significantly smaller in high-glucose condition, Kv11.1-C723S channels were unaffected, strongly suggesting that C723 is a target of ROS produced during conditions of hyperglycemia.

Changes in the oxygen level also affects Kv11.1 channels. Long-term exposure of neuroblastoma (SH-SY5Y) cells to hypoxia (0.1% O_2) dramatically altered Kv11.1 currents by causing a 20-mV shift in the voltage dependence of the time constants of recovery from inactivation and deactivation kinetics, and a -19 mV left-shift in steady-state activation (37). Another study by Nanduri *et al.* (88) found that hypoxia (1% and 5% O_2) decreased the synthesis of recombinant Kv11.1 in

HEK 293 cells in a proteosomal degradation independent fashion. Moreover, the antioxidant N-acetyl cysteine (NAC) prevented the downregulation of Kv11.1 protein under hypoxia, suggesting that hypoxia enhances the ROS production in the mitochondria and, as a result, Kv11.1 translation was downregulated (88). Carotid bodies have been known to possess O_2 -sensitive K^+ channels that contribute to depolarization upon hypoxia. The O_2 sensitivity of dissociated type-I cells from rat carotid body changes with age until 14–16 days (102). Kim *et al.* (65) found an E-4031-sensitive K^+ current (E-4031 is a specific inhibitor of Kv11.1 channels) in 1-day-old rat carotid chemoreceptor cells, and that component was absent in 16-day-old rats. Moreover, E-4031 induced an increase in the cytosolic Ca^{2+} concentration in response to hypoxia in young cells but not in cells from 16-day-old rats, suggesting that the presence of Kv11.1 currents in the carotid body may dampen the ability of hypoxia to induce depolarization in an age-dependent manner.

Large-Conductance Voltage- and Ca^{2+} -Dependent K^+ Channels

Each Slo1 subunit in a tetrameric Slo1 voltage- and Ca^{2+} -dependent BK channel, depending on the alternative splicing process, has ~ 1100 residues (residue numbering according to human Slo1 AAB65837). The transmembrane domain of the Slo1 subunit is organized in a manner similar to that of other voltage-gated K^+ channels (Fig. 1C) except for the additional transmembrane domain S0 (Fig. 9A). The Slo1 subunit possesses a large cytoplasmic area containing two domains called RCK1 and RCK2 (60). Four sets of the RCK1-RCK2 pairs form the intracellular “gating ring” (145, 153, 154), which undergoes conformational changes on Ca^{2+} binding (57, 85) leading to opening of the ion conduction gate. Each of the four subunits has two distinct Ca^{2+} sensors with different functional properties: the RCK1 Ca^{2+} sensor and the RCK2 Ca^{2+} sensor (“ Ca^{2+} bowl”) [summarized in Hoshi *et al.* (49)].

Slo1 BK channels, made of four pore-forming Slo1 subunits and often with auxiliary β - or γ -subunits, are dualistically activated by membrane depolarization and an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (46, 49). The ion conduction gate in the PGD near the selectivity filter (135, 144, 162) typically has a finite but extremely low P_o in the absence of activation of the VSDs and of the Ca^{2+} sensors (Fig. 9A). However, operating in an allosteric manner, activation of all four VSDs by depolarization can increase P_o by $\sim 160,000$ -fold and binding of Ca^{2+} to all Ca^{2+} sensors in the channel can increase P_o by ~ 4000 -fold (46).

Any one of the allosteric gating components of the Slo1 BK channel, the PGDs, VSDs, and Ca^{2+} sensors, and the strengths of allosteric interactions among them could be subject to oxidative regulation. Numerous examples of changes in functional properties of native and heterologously expressed Slo1 BK channels by various experimental manipulations of the redox status have been reported. In some of the cases, the biophysical mechanisms and the molecular components responsible for the phenomena have been elucidated but the atomic structural and physicochemical bases remain largely unknown. The reported cases of oxidative modifications of Slo1 BK channels appear to involve modification of cysteine and/or methionine residues (Fig. 9). A typical human Slo1 protein (*e.g.*, AAB65837) has 29 cysteine and 30 methionine

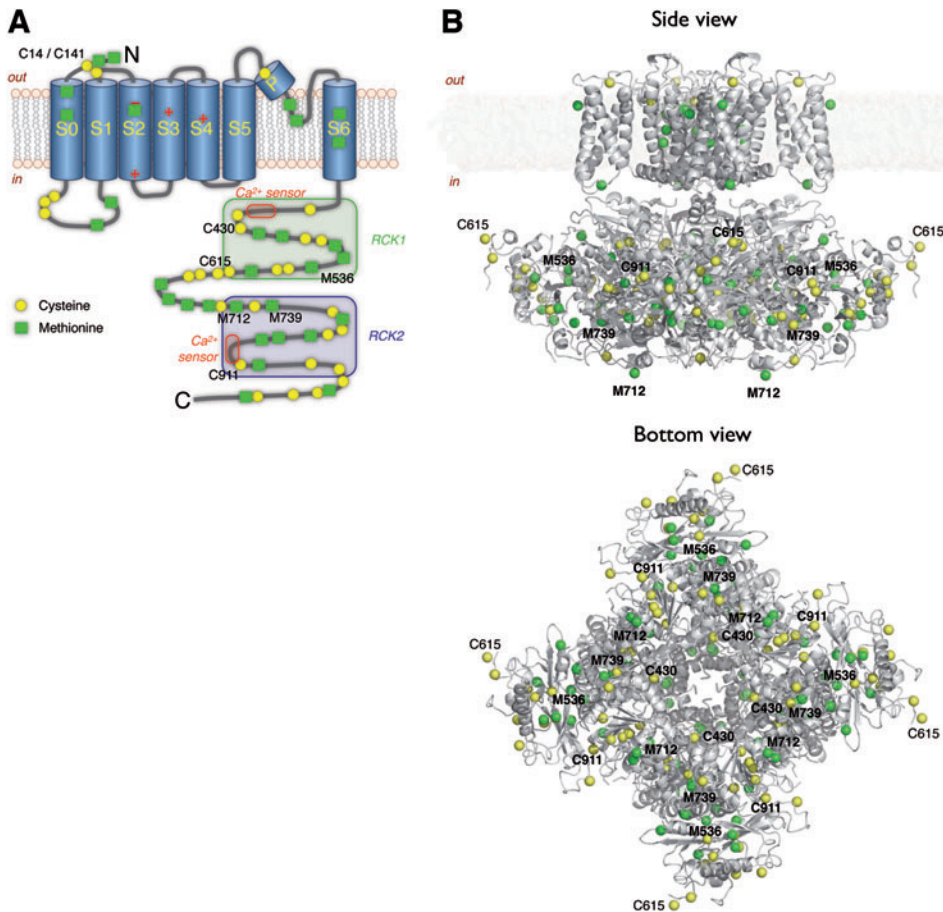


FIG. 9. Slo1 BK channels. (A) Topological model of a Slo1 BK α -subunit. Cysteine and methionine residues presumably important for the channel's oxidation sensitivity are indicated by residue number. RCK, regulator of conductance for K⁺ domains. (B) Plausible structure of a Slo1 BK channel complex. The transmembrane domain is a homology model based on the Kv1.2/2.1 structure (PDB 2R9R). The cytoplasmic domain is according to the structure of Wu *et al.* (PDB 3NAF) obtained in the absence of added Ca²⁺. S0 is not shown because Kv1.2/2.1 does not contain S0. Sulfur atoms of cysteines in the cytosolic domain are shown in yellow, methionines in green. Side view (*top*), bottom view (*bottom*). Numbers indicate important residues. BK, big conductance K⁺. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

residues, that is, 116 and 120, respectively, in one tetrameric complex. Oxidation of other amino acids, such as histidine and tryptophan, has not been explicitly implicated. Depending on their locations in the protein (*e.g.*, extracellular, transmembrane, intracellular) and reactivity (*e.g.*, adjacent residues and pH), any and many of the cysteine and methionine residues could be oxidatively modified. However, oxidative modifications of only a subset of the residues may lead to observable functional changes.

Oxidative status of cysteine in Slo1

Consistent with the oxidizing nature of the extracellular compartment, the two cysteine residues of Slo1 facing the extracellular side (C14 and C141) are disulfide linked under ambient conditions when heterologously expressed (73) (Fig. 9A). The remaining cysteine residues are more likely to remain reduced. Consequently, the side chains should contain a free SH or S⁻ group depending on the local environment, and they retain potential to be oxidized or modified. The atomic structures of the cytoplasmic domain (145, 153, 154) show that many cysteine residues reside near the protein surface (*e.g.*, C430, C612, C615, C695, C995, C1001, and C1028). Further, some cysteine residues are found in the close vicinity of each other; their S γ atoms are within 2.4 to 5.5 Å of each other (C348-C422, C722-C800, C797-C800, and C1011-C105). In comparison, the typical disulfide bond length is about ~2 Å. Assuming that the atomic structures are physiologically relevant, the aforementioned cysteine pairs have potential to be

oxidized to form disulfide bonds. The side chains of other cysteine residues may be modified to form sulfenic, sulfinic, or sulfonic groups (Fig. 3). It is possible that the sulfenic groups may react with glutathione under physiological conditions to form mix disulfides (35).

Functional consequences of cysteine oxidation in Slo1

Currents through heterologously expressed human Slo1 channels progressively decrease in size after patch excision with 1 or 100 μ M [Ca²⁺]_i (28). During this rundown process, the activation kinetics slows, and the macroscopic conductance-voltage curve (GV curve) becomes shallower and shifts to the positive direction without altering the maximal conductance (but see below) or the underlying unitary current size. These functional changes are readily reversed by the reducing agent DTT and mimicked by the oxidizing agent H₂O₂, leading to the idea that oxidation of cysteine caused the gating changes observed. The extent of rundown of Slo1 channels may depend on [Ca²⁺]_i. The rundown is less noticeable in the virtual absence of Ca²⁺ (129, 130, 159) and most prominent at intermediate concentrations of Ca²⁺ (159), indicating that the Ca²⁺-dependent activation mechanism of the channel is subject to oxidative regulation. Note that rundown may be observed in some patches without Ca²⁺, suggesting that multiple mechanisms and multiple targets may be operative (129). While one study (28) reported no change in the maximal macroscopic conductance, other studies documented a clear decrease in the maximal conductance and the

number of active channels during rundown, which is reversed by the reducing agent DTT (121, 159).

Cysteine 911 in the RCK2 Ca²⁺ sensor. Of the two Ca²⁺ sensors, the RCK2 Ca²⁺ sensor ("Ca²⁺ bowl") is influenced more readily by cysteine oxidation (130). In particular, C911 is located in close proximity of the RCK2 Ca²⁺ sensor; the distances between the Ca²⁺-ligating carbonyl oxygen atoms in the RCK2 Ca²⁺ sensor and the S_γ atom of C911 are ~7 to 13 Å. The oxidant H₂O₂ or the thiol modifier MTSEA impairs the contribution of the RCK2 Ca²⁺ bowl sensor, but not of the RCK1 sensor, to the overall Ca²⁺-dependent activation process of the channel. Mutation of C911 noticeably diminishes the propensity of Slo1 channels to rundown on patch excision (159) and impairs the effects of H₂O₂ and MTSEA at moderate [Ca²⁺]_i (127). The absence of a free SH/S⁻ group at position 911 thus disrupts the normal function of the RCK2 Ca²⁺ sensor and the Ca²⁺-dependent gating of the Slo1 channel.

Cysteine 430. Oxidation/modification of cysteine residues other than C911 may also lead to noticeable functional changes. Cysteine modifiers such as DTNB, NEM, MTSES⁻, and MTSET⁺ stimulate or inhibit currents through Slo1 channels (127, 158). A detailed biophysical analysis suggests that these thiol modifiers alter multiple components of the allosteric gating mechanism such as the intrinsic stability of the ion conduction gate and its coupling to the Ca²⁺ sensors and VSDs (158). One of the residues critical in mediating the channel's sensitivity to the cysteine modifiers is C430. This residue faces the inner central opening of the gating ring domain and the mutation C430A abolishes many of the effects observed (158). C430 is 25 to 30 Å away from the RCK1 Ca²⁺ sensor and the RCK2 Ca²⁺ sensor, and it is unlikely to participate directly in Ca²⁺ sensing of the channel. Comparison of the gating ring structures obtained under different conditions (PDB IDs 3NAF *vs.* 3U6N) suggests that the region containing C430 may exhibit a moderate conformational change (~4 Å) (49). This gating ring movement may be altered by modification of the side chain, affecting multiple gating characteristics. Mutation C430A, however, does not appreciably antagonize the inhibitory effect of H₂O₂ at 5 μM [Ca²⁺]_i (159), illustrating the distinct functional consequences of oxidation of different cysteine residues.

Cysteines 612 and 615. Free heme is thought to bind to the sequence CKACH located in the unstructured RCK1-RCK2 linker segment with nM affinities (56, 131, 151). The cysteine residues in this sequence, C612 and C615, modulate the affinity to the heme binding domain peptide to heme; the reduced peptide binds heme more tightly than the oxidized form (151). Electrophysiologically, the mutation C615S eliminates the effect of MTSET on the apparent Ca²⁺ affinity of the channel, suggesting that modification of the C615 side chain may occur. Because C615 is near the periphery of the gating ring domain and ≥30 Å away from the Ca²⁺ sensor sites, the effect is probably indirect or allosteric.

STREX insert. The *SLO1* transcript is extensively spliced to create many variants (18, 109). Some native Slo1 channels contain an insertion of 58 residues, of which 6 are cysteine, termed STREX (coded by the "stress regulated exon") between the RCK1 and RCK2 domains (136, 147). Select cysteine

residues in the STREX insert are palmitoylated and mediate the interaction of the STREX-containing channel with the plasma membrane in a phosphorylation-dependent manner (136). Application of the thiol modifier thimerosal, which is capable of creating ethylmercury adducts, decreases *P*_o of the STREX-containing Slo1 channel at moderate [Ca²⁺] and this inhibition is reversed by the reducing agent DTT (34). Inclusion of STREX may offer another mechanism of regulation of the Slo1 channel by cysteine oxidation. The STREX insert is also implicated in hypoxia sensing by the Slo1 channel but the mechanism is redox independent (84).

Other cysteine residues? While mutagenesis of C911, C430, and C615 diminishes many effects of application of H₂O₂ and select thiol modifiers, oxidation/modification of other cysteine residues could also contribute. The behavior of the ion conduction gate is intimately associated with the conformation changes of the gating ring domain (57, 85). Any change in the gating ring domain motion has potential to alter the channel gating and oxidative modification of the cysteine side chain at multiple positions may exert such an effect.

Functional consequences of methionine oxidation in Slo1

Of 30 methionine residues in Slo1, 11 of them are located in the transmembrane domain (N through S6; Fig. 9A) and the remainder in the cytoplasmic domain. As with cysteine, those facing the extracellular domain (M1, M10, and M21) are exposed to a more oxidizing condition. However, the exact oxidation status of these residues, whether their side chains exist as Met, Met(O), or Met(O₂) is unknown. The methionine residues within the membrane and the cytoplasmic domain are more likely to be reduced under basal conditions and retain potential to be oxidized. Some of them are clearly solvent exposed in the available structures (M442, M513, M663, M679, M712, and M1053). The remaining methionine residues face the protein interior, consistent with the notion that methionine is nonpolar. Since Met(O) is much more hydrophilic and comparable to aspartate, glutamate, and lysine (12), oxidation of buried methionine residues to Met(O), if it occurs, may have a greater impact on the channel function.

ChT increases Slo1 currents without any change in the single-channel current-voltage property (129). The current-enhancing effect was partially but not completely reversed by treatment with the enzyme MsrA and DTT together, implicating oxidation of methionine. In contrast with the current-enhancing effect of ChT, the cysteine modifiers DTNB, MTSEA, and PCMB all decreased currents. Biophysically, treatment with ChT moves the overall voltage dependence of activation to the negative direction by 30 mV, and this shift has been suggested to involve a change in the VSD function (129). An exhaustive mutagenesis study showed that contemporaneous substitution of three methionine residues (M536, M712, and M739) in the cytoplasmic gating ring domain eliminated the functional changes caused by ChT (112). M536 and M739 are located in nonpolar areas while M712 is solvent exposed toward the "bottom" of the gating ring domain (Fig. 9B). Presumably oxidation of the side chains of these methionine residues to Met(O) [or possibly to Met(O₂)] changes the conformation of the cytoplasmic gating ring domain, which is then transmitted to the transmembrane VSDs.

It is unknown whether treatment with ChT selectively oxidizes M536, M712, and M739 or that oxidation of other methionine residues has no functional sequence; biochemical or proteomic information is unavailable. The partial reversal of the effect of ChT by MsrA (129) may be mediated by its action on the solvent exposed M712. While rundown is attributed to cysteine oxidation, run-up is considered to be a consequence of methionine oxidation in Slo1, and both types of oxidation can occur so that the current size is sometimes unstable after patch excision (129).

Oxidation of β -subunits

Auxiliary β -subunits also contain multiple cysteine residues, many of which are well conserved among different β -variants and face the extracellular side (four to seven extracellular cysteine residues in human β -subunits). These cysteine residues are most probably disulfide linked. In particular, in Slo1 + β 3 channels, the reducing agent DTT markedly alters ion permeation properties of the channel complex (157). Whether these disulfide links are subject to physiological regulation remains unknown because the extracellular compartment is oxidizing.

Variable numbers of methionine residues are found in human β -subunits (from 3 to 11). Treatment of Slo1 + β 1 channels with ChT induces a larger shift in the voltage dependence (-75 mV) than that observed with Slo1 without any β -subunits (-30 mV) (108). The augmented response to ChT, however, does not require any methionine or cysteine within β 1 and it is thought that β 1 amplifies the functional consequence of oxidation of the aforementioned three methionine residues within Slo1 (111).

Physiological relevance

The physiological importance of Slo1 regulation by ROS remains to be elucidated. However, given the involvement of this channel in many physiological processes, fine-tuned adjustment of BK channel activity *via* reactive species is a likely scenario. It might be particularly relevant at sites of high ROS concentration. For example, H_2O_2 is generated from dismutation of superoxide anion (O_2^-), which is in turn created by the NADPH oxidase complex located in plasma membranes and organelles and also by the mitochondria. Thus, cellular metabolism *via* the NADPH oxidases and mitochondria could oxidatively influence Slo1 BK channels. Interestingly, some BK channels have been reported to exist in the inner mitochondria membrane (120, 149), where the channels may face greater concentrations of reactive species.

Oxidative modification of Slo1 channels is likely to be pathophysiologically important. One such example is found in dysfunction of Slo1 BK channels in type-2 diabetes. In Zucker diabetic fatty rats, a model system for type-2 diabetes, dilation of coronary artery vessels is impaired (79). Because Slo1 BK channels provide an important vasodilatory influence (13, 89), Lu *et al.* tested the hypothesis that dysfunction of Slo1 BK channels may contribute to the vascular dysfunction associated with type-2 diabetes by manipulating glucose levels in cell culture (78). A high level of glucose in the cell culture medium increased the overall ROS level and decreased P_o at $0.5 \mu M [Ca^{2+}]_i$. The high glucose medium failed to alter the C911A channel, suggesting that high glucose leads to oxida-

tive modification of C911 and impairs the Ca^{2+} -dependent activation of the Slo1 channel (78).

Conclusion and Perspectives

ROS regulation of voltage-gated K^+ channels is a multifaceted process ranging from protein degradation to "real" regulation in the sense that oxidative events are reversible. Thus, also with respect to the channels discussed here, ROS challenge is a double-edged sword, but several cases are reported in which oxidative modification of K^+ channels results in increased activity and, hence, a potential beneficial or protective influence.

Most of the studies examining oxidative regulation of Kv channels thus far utilized exogenous oxidants and modifiers, often in high concentrations. Some of these oxidants such as H_2O_2 are in fact found under physiological conditions, but the time- and spatially resolved quantification in cells remains a challenging task. Further, one ROS source alone may not be sufficient in many cases as, for example, the concurrent presence of transition metal ions often is required to catalyze ROS-related chemical reactions. Thus, a limiting factor of ROS-related research on ion channels is ROS monitoring in the appropriate physiological background.

Nevertheless, great progress was made in the understanding of how ion channels are regulated by reactive species. In many cases very detailed insight could be gained because the function of ion channels can be studied with very high precision—in many cases in inside-out and outside-out mode—providing access to both sides of the membrane. Voltage-gated K^+ channels bear the additional advantage of allowing automated leak correction and, by means of voltage-step protocols, the exact timing of channel activation and deactivation, such that detailed quantitative data sets with high time resolution can be generated. Using recombinant channel proteins and exogenous ROS sources together with site-directed mutagenesis enables identification of potential molecular targets (*e.g.*, side chains) and the biophysical mechanisms underlying the consequent functional changes. A technical limitation that still needs to be overcome is the ROS-related proteomics of membrane proteins—ideally in subcellular and temporal resolution—to better understand the chemical nature of oxidative protein modification. In addition, given the advent of many new 3D structures of ion channels, structural variants subjected to oxidative modulation may prove very insightful.

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Abbreviations Used

ΔV_m = change in membrane potential
 BK = big conductance K⁺
 Ca²⁺ = calcium ion
 [Ca²⁺]_i = cytosolic free Ca²⁺ concentrations
 Cat = catalase
 Cav = voltage-gated Ca²⁺
 ChT = chloramine-T
 cNBHD = cyclic nucleotide-binding homology domain
 CO = carbon monoxide
 DPPX = dipeptidyl aminopeptidase-like protein
 DTDP = 2,2'- or 4,4'- dithiodipyridine

Abbreviations Used (Cont.)

DTNB = 5,5'-dithiobis-(2-nitrobenzoic acid)
 DTT = dithiothreitol
 EAG = *ether à go-go*
 Fe/asc = iron/ascorbate
 Fe²⁺/Fe³⁺ = iron(II) ion/iron(III) ion
 Glu = glutamate
 GPx = glutathione peroxidase
 GRed = glutathione reductase
 GSH-red/GSH-ox = glutathione, reduced and oxidized
 GV curve = conductance-voltage curve
 H₂O₂ = hydrogen peroxide
 HIF-1 α = hypoxia-inducible factor-1 α
 HPV = hypoxic pulmonary vasoconstriction
 HUGO = nomenclature of human genes
 I_{Kr} = rapid delayed rectifier current
 I_{Ks} = slow delayed rectifier current
 I_{Kur} = repolarizing ultra-rapid delayed-rectifier current
 K⁺ = potassium ion
 KChiP = K channel interacting protein
 K_v = voltage-gated potassium (channel)
 K $\nu\beta$ = β -subunit of voltage-gated potassium channels
 Lck = lymphocyte-specific protein tyrosine kinase
 Met = methionine
 Met(O) = methionine sulfoxide
 Met(O₂) = methionine sulfone

Msr = methionine sulfoxide reductase
 MTSEA = 2-aminoethyl methanethiosulfonate hydrobromide
 MTSES⁺ = 2-sulfonatoethyl methanethiosulfonate
 MTSET⁻ = 2-(trimethylammonium)ethyl methanethiosulfonate
 NAC = N-acetyl cysteine
 NADH = nicotinamide adenine dinucleotide
 NADPH = nicotinamide adenine dinucleotide phosphate
 NEM = N-ethylmaleimide
 NFT = nuclear factor-activating T
 NO = nitric oxide
 O₂ = molecular oxygen
 ONOO⁻ = peroxynitrite
 PAH = pulmonary arterial hypertension
 PAS = Per-Arnt-Sim
 PC-12 = pheochromocytoma 12
 PCMB = *p*-chloromercuribenzoic acid
 PGD = pore/gate domain
 P_o = open probability
 RCK = regulator of conductance for K⁺
 ROS = reactive oxygen species
 src = family of nonreceptor tyrosine kinases
 SOD = superoxide dismutase
 STREX = stress regulated exon
 tBHP = tert-butyl hydroperoxide
 TM = transmembrane domain
 VSD = voltage-sensor domain
 WT = wildtype