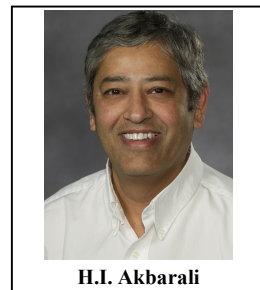


Postranslational Modification of Ion Channels in Colonic Inflammation

Hamid I. Akbarali* and Minhong Kang

Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA 23298, USA



H.I. Akbarali

Abstract: Voltage-gated ion channels are key regulators of cell excitability. There is significant evidence that these channels are subject to modulation by redox status of the cells. Here we review the post-translational modifications of ion channels that occur in colonic inflammation. The redox mechanisms involve tyrosine nitration, covalent modification of cysteine residues and sulfhydration by hydrogen sulfide in experimental colitis. In the setting of colonic inflammation, modifications of cysteine and tyrosine are likely to occur at several sites within the same channel complex. In this review we describe alterations in channel function due to specific modifications of tyrosine and cysteine residues by reactive nitrogen, oxygen and hydrogen-sulfide resulting in altered motility.

Keywords: Calcium channel, hydrogen sulfide, oxidative stress, tyrosine nitration.

1. INTRODUCTION

It is now well recognized that the gating properties of many ion channels are modulated by the redox status of the cells [1]. Reactive oxygen (ROS) and reactive nitrogen (RNS) species modify specific amino acids within the ion channel protein, largely cysteine and/or tyrosine residues thus affecting the functional activity of these channels. The cysteine residues can be modified by nitrosylation, palmitoylation, and/or sulfhydration [2]. Tyrosine residues can be nitrated by peroxynitrite thus altering channel function. Pro-oxidant reactive oxygen species include superoxide, hydrogen peroxide, singlet oxygen and hydroxyl ions. The physiological generation of reactive species are generally highly regulated, with its detoxification by antioxidant scavengers such as glutathione, catalase, and superoxide dismutase which can readily detoxify their active intermediates on biological systems. In inflammatory bowel diseases that include ulcerative colitis and Crohn's disease, an imbalance may affect gastrointestinal motility. In mouse models of experimental colitis, enhanced oxidative stress results in decreased contractility as a result of reduced cell excitability. In this review, we focus on the modulation of some of the ion channels by free radicals in colonic inflammation.

In conditions of gastrointestinal inflammation, altered motility may be accompanied by enhanced peripheral sensitization of sensory neurons. The sensitization of the neurons may be at the level of spinal, supraspinal or within intrinsic primary afferents of the gastrointestinal tract. For example, in rat experimental colitis model, colorectal distension results in increased excitability and responsiveness of the lumbosacral spinal neurons [3].

Acute colonic inflammation also enhances the excitability of colon projecting neurons within the dorsal root ganglia in the lumbosacral region [4]. The changes in neuronal (that affect peripheral sensitization) and muscle excitability (affecting gastrointestinal motility) may occur as a result of altered ion channel function. In the dorsal root ganglia the cell bodies of neurons projecting from the inflamed colon demonstrate significantly larger sodium currents mainly Nav1.8 tetrodotoxin-resistant Na⁺ channels. These changes can be attributed to oxidative stress.

Oxidative stress has been extensively studied in gastrointestinal inflammation [5, 6]. Although, initially considered as mainly due to the production of superoxide (O₂⁻) and its conversion to oxidants such as hydrogen peroxide (H₂O₂) by superoxide dismutase, it is now recognized that generation of the reactive hydroxy radical (OH) *via* Fenton chemistry further enhances tissue damage. Reactive oxygen species (ROS) have been demonstrated in the inflamed intestine in clinical and experimental models with findings of altered transcription of endogenous antioxidants such as glutathione peroxidase [7]. Differences in the levels of antioxidants in inflammatory bowel disease patients has also been noted implicating an imbalance in colitis [8].

1.1. Tyrosine Nitration of Calcium Channels in Colonic Inflammation

Studies of calcium channels in the context of pathological insults such colonic inflammation have shown significant attenuation of the calcium influx in smooth muscle cells leading to decreased muscle contraction. Calcium channels are critical for mediating gastrointestinal contractions as demonstrated by selective inactivation of the L-type voltage gated calcium channel, Cav1.2 in smooth muscle. Global knock-out of Cav1.2 is embryonic lethal. In a tamoxifen-inducible Cre-lox based strategy, exons 14 and 15 of Cav1.2 were "floxed" during recombination resulting in

*Address correspondence to this author at the Department of Pharmacology and Toxicology, Virginia Commonwealth University, 1112 East Clay Street, McGuire Hall Rm# 317, Richmond, VA 23298, USA Country; Tel: 804-828-9688; E-mails: hiakbarali@vcu.edu

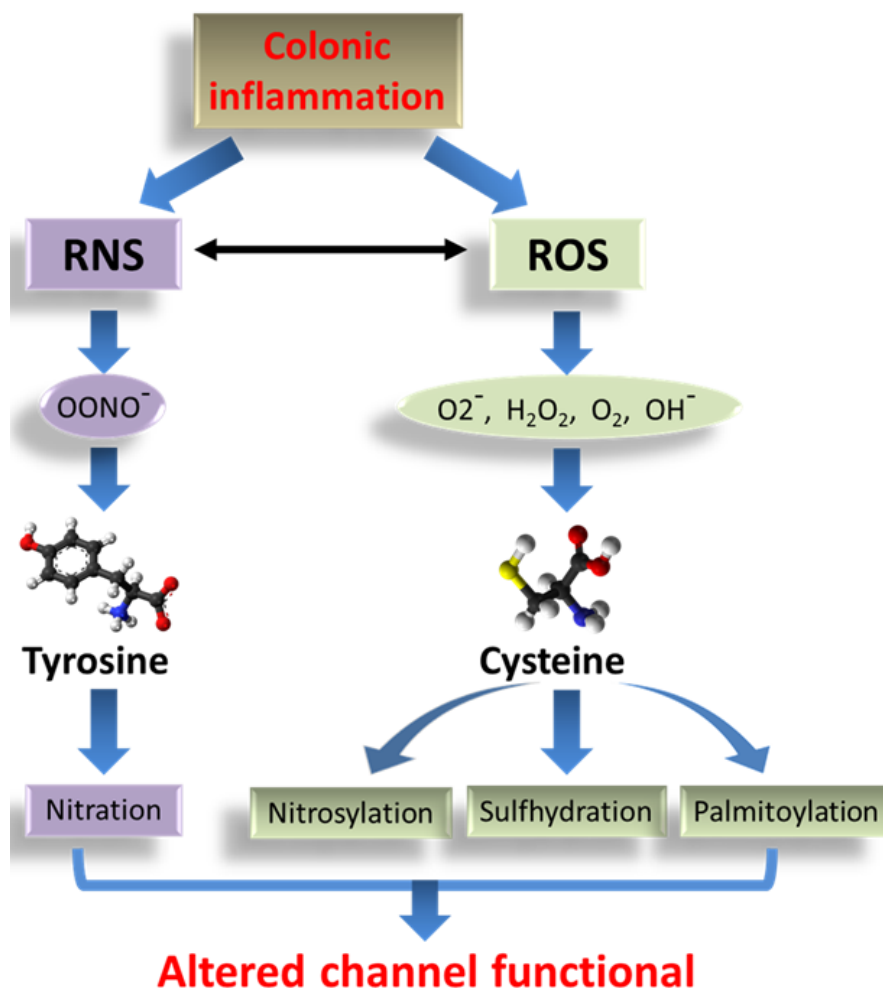


Fig. (1). A schematic representation of reactive nitrogen and oxygen species and their modification of specific amino acids within an ion channel complex.

an inactivated calcium channel in vascular, urinary and gastrointestinal smooth muscle [9]. Loss of smooth muscle Cav1.2 resulted in death due to paralytic ileus in ~ 4 weeks after initiation of gene inactivation with tamoxifen. This was accompanied by the loss of Cav1.2 protein expression after 14 days resulting in decreased amplitude of spontaneous contractions and smooth muscle contractions mediated by muscarinic receptor activation. These studies establish the importance of the voltage-dependent calcium channel in colonic motility.

Reduced calcium channel function has also been observed in experimental colitis. Calcium currents in single smooth muscle cells in the dog, rat and mouse colon demonstrated a 50–70% decrease in calcium currents following inflammation [10-12]. This correlates with decreased contractions of colonic muscle strips in both animal models of colitis as well as in humans in response to depolarizing K⁺ solutions. Although the calcium currents are decreased, both the protein and gene expression of calcium channel isoforms are not altered in the murine colon following inflammation [13]. One possible explanation for the decrease in current amplitude may be due to altered regulation for these channels. Calcium channels in colonic

smooth muscle are phosphorylated by the tyrosine kinase, c-src kinase. Direct association of c-src kinase with the calcium channel has been demonstrated by coimmunoprecipitation of the pore forming alpha subunit of Cav1.2b with anti-src and anti-phosphotyrosine antibodies [14]. Furthermore, src-kinase inhibitors significantly attenuate calcium currents, while tyrosine phosphatase inhibitors enhance the amplitude of the calcium current. Kang *et al.*, showed that inflammation resulted in reduced inhibition of the calcium currents by the src kinase inhibitors [13]. This was attributed to decrease in the ability of c-src kinase to phosphorylate the channel in inflamed tissues. Studies in our laboratory further established that the carboxy terminus of the calcium channel is the major site for src regulation. The protein-protein interaction between c-src and the calcium channel involves binding of the SH3 domain of c-src to proline rich regions in the COOH terminus of the hCav1.2b and the binding of SH2 domain of c-src to phosphorylated tyrosine Y2134 and Y1837.

Peroxynitrite is a reactive nitrogen species that is produced during inflammation and has been implicated in the nitration of tyrosine residues. Nitration prevents tyrosine phosphorylation and thus results in reduced src-kinase binding to the calcium channel. Kang *et al.*, showed that

mutation or nitration of Y2134 and Y1837 prevented src binding to the carboxy terminus of the calcium channels [15]. Ross *et al.*, [16] further showed that in inflamed colon, increased expression of tyrosine nitrated calcium channels prevented the binding of c-src kinase. In contrast to the modifications of cysteine residues by ROS and S-nitrosylation by NO, more pathological conditions are created when peroxynitrite, formed due to enhanced production of NO, overwhelms the dismutations of O_2^- by superoxide dismutase. Nakayama and colleagues have examined the kinetics and mechanism by which tyrosine kinase affects calcium channel [17]. They showed that Cav1.2b undergoes state transitions to a second open state, termed O₂, upon depolarization. In this state, the channels exhibit minimal inactivation during depolarization. This sustained calcium influx promotes tonic contractions. Ross *et al.*, [18] showed that colonic inflammation decreased the shift to O₂ state and that mutations of c-terminal tyrosine or nitration decreased the availability of the channels in the O₂ state. These findings provide a mechanistic insight as to how tyrosine nitration during oxidative stress alters the gating kinetics of single calcium channels resulting in decreased calcium influx.

The altered gating of the calcium channels also affects excitation-transcriptional coupling. Calcium-mediated gene transcription is coupled to L-type calcium channel activation by the transcription factor, cyclic AMP response element binding protein (CREB). Nuclear CREB is phosphorylated at Ser133 upon cell depolarization. Translocation of calmodulin to the nucleus acts as a primary mechanism for activation of calmodulin dependent kinase(s) and phosphorylation of CREB [19]. The open probability of L-type calcium channel was shown to be coupled to the CREB phosphorylation [20]. During colonic inflammation, depolarization-mediated expression of phospho-CREB is reduced due to tyrosine nitration of the calcium channel [21]. Thus, not only excitation-contraction but also excitation-transcription coupling is altered by oxidative stress in colonic inflammation.

The type of posttranslational modification is critical whether the effect is to be either inhibitory or stimulatory. Unlike tyrosine nitration, S-nitrosylation by S-nitrosothiols, which donate NO, stimulate L-type Ca^{2+} currents in ventricular myocytes [22]. While there are significant numbers of cysteine residues that may be potential thiol redox-sensitive sites of the channel, not all cysteines may be accessible to reactive nitrogen species or ROS [23]. It is noteworthy that S-nitrosylation may be important in S-nitrosothiols – signaling mechanism while tyrosine nitration may occur primarily in pathological conditions. We have recently [24] demonstrated that denitration of Cav1.2b by cell lysates from activated macrophages may be present indicative of potential enzymatic mechanisms for reversing nitrated proteins.

1.2. Thiol Modification in Colonic Inflammation

Hydrogen peroxide can covalently modify cysteine residues resulting in formation of disulphide bridges between apposing thiols. In studies of colonic smooth muscle, H_2O_2 was found to partially decrease the transient outward K^+ current in mouse colonic smooth muscle cells [25]. The

cysteine-modifying membrane impermeable DTNB applied at the extracellular surface mimicked this effect. This was reversed by the intracellular dialysis with the reducing agent, dithiothreitol (DTT) indicating a complex interplay between extracellular and intracellular cysteine residues in the modulation of the K^+ channel. The effects of H_2O_2 were specific for the transient outward K^+ currents and did not affect the delayed rectifier K^+ currents.

Monochloramine is a highly potent lipophilic oxidant produced during colitis as a result of the disruption of the integrity of epithelial barrier that results in accumulation of ammonia within the submucosa. Monochloramine is produced when hypochlorous acid (produced by the reaction of chloride and myeloperoxidase) reacts with ammonia. Ammonia is produced under physiological conditions by bacteria that ferment nitrogen-containing compounds in the lumen of the colon. Monochloramine increases the activity of the large-conductance BKCa channel and shifts the steady-state voltage dependence of activation to the left by -22 mV [26]. Alkylation of the sulfhydryl groups by N-ethylmaleimide (NEM) prevented the effects of monochloramine. Monochloramine abolished the transient outward K^+ currents in murine colon smooth muscle cells, and increased the delayed rectifier K^+ currents. This effect may be through modification of cysteine and possibly methionine residues [25, 27]. The presence of increased amounts of covalently modified ion channels may contribute to altered excitability in gastrointestinal inflammation similar to the accumulation of oxidized proteins in other diseases. Determining the relative sensitivity of the various cysteine modifications to endogenous reducing agents will be important in developing therapeutic strategies in treating altered excitability.

1.3. Sulfhydration in Colonic Inflammation

Hydrogen sulfide (H_2S) is now emerging as an important cell-signaling molecule, similar to nitric oxide and carbon monoxide. In colonic inflammation, H_2S synthesis is enhanced and may play a protective role due to modulation of ATP-sensitive potassium channels. Inhibition of the synthesizing enzymes appears to exaggerate experimental colitis [22]. ATP-sensitive K^+ (K_{ATP}) channels are expressed in a many cell types [28, 29]. K_{ATP} channels are hetero-octomers composed of four regulatory sulfonylurea subunits (SUR1, SUR2A or SUR2B) and four ATP-sensitive pore forming inwardly rectifying potassium channel subunits (K_{ir} 6.1 or K_{ir} 6.2). The function of the K_{ATP} channels is regulated by physiological or pathophysiological stimuli, including hypoxia, hyperglycemia, ischemia, and oxidative stress, thereby regulating the cellular excitability depending on the metabolic state [30]. Reactive oxygen species lower the K_{ATP} channel activity in cerebral arterioles [31] and coronary arteries [32] while facilitate its opening in cardiac myocytes [33] and pancreatic β -cells [34]. The differential responses of K_{ATP} channels to ROS between tissues could be due to differential expression of K_{ATP} channel isoforms. In cloned channels, mutation of Cys⁴² in the N-terminus of Kir6.2 rendered the channel insensitive to sulfhydryl reactive agents indicative of Kir subunit as the potential site for redox regulation [35]. Cui and Fan [28] suggested that sulfhydryl modification of Cys⁴² allosterically modulates the channel

and not direct pore blockage. Nitric oxide was also found to activate K_{ATP} channels in DRG neurons due to direct S-nitrosylation of SUR1 subunit. Mutation of Cys⁷¹⁷ in the nucleotide binding domain 1 (NBD1) of SUR1 resulted in decreased currents by NO donors [36]. S-glutathionylation also inhibits the Kir6.1/SUR2B channel by targeting mainly Cys¹⁷⁶ of the Kir6.1 subunit, which is likely to structurally prevent the pore-forming inner helix from undergoing necessary conformational change for channel gating, thus retaining the channel in its closed state [2]. It has to be noted that S-glutathionylation is a post-translational modification of proteins in several physiological or pathophysiological conditions [29], and often associated with the adverse effects of oxidative stress.

More recently, K_{ATP} channels have been established as the potential targets for hydrogen sulfide in colonic inflammation. The mechanism for activation of the channel involves sulfhydration of cysteine residues either within the Kir 6.1 subunit [37] or the sulfonylurea receptor [38]. An increase in the bursting activity of single ATP-sensitive K channels was noted in the DSS model of colitis [39] which may be due to increased sulfhydration by H₂S. Gade *et al.*, [40] found that the SUR2B subunit but not Kir6.1 of the K_{ATP} channel was sulfhydrated by NaHS. The sulfhydration was also observed in colonic inflammation. These studies also identified an allosteric modulation of the channel in mouse colon by H₂S. The effect of the K_{ATP} channel opener, levcromakalim is markedly enhanced by low concentrations of H₂S as well as in the setting of colonic inflammation suggesting an allosteric modulation. The activation of the K_{ATP} currents by NaHS and levcromakalim were inhibited by alkylation of cysteine residues with N-ethylmaleamide.

While many studies have focused on the effects of free radicals on ion channels, there is less information on how the various pro-oxidants affect ion channel function in combination. For example, it is not clear whether the same cysteine residues are s-nitrosylated, or subject to disulfide bond formation or whether ion channels that are nitrated can be sulfhydrated and how these different redox modulations affect channel function. Recent studies are beginning to provide some mechanistic insights into the interaction between the effects of various free radicals on individual proteins and ion channels. For example, Kang *et al.*, [41] examined the interaction between hydrogen-sulfide mediated sulfhydration and tyrosine nitration within the K_{ATP} channel complex. These studies show that sulfhydration of cysteine residues C²⁴ and C¹⁴⁵⁵ of SUR2B prevent the nitration of tyrosine residues in the Kir6.1 subunit. This may provide a mechanism by which H₂S imparts a protective effect in colonic inflammation. Further work in this area is necessary to delineate the physiological implication of oxidative stresses on single protein complexes.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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