SA1

Histamine-Stimulated Calcium Oscillations in Human Endothelial Cells: Possible Role of Reactive Oxygen Species and Redox Sensitivity of the IP_3 Receptor

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The activities of several known Ca^{2+} entry and release channels are affected by oxidant stress and therefore may link cellular redox state and Ca^{2+} homeostasis. Redox sensitivity of Ca^{2+} signaling pathways is not only important pathophysiologically, but also during membrane receptor stimulation, when reactive oxygen species (ROS) function as intracellular second messengers. Previous work has shown that a vascular NADPH oxidase plays an important role in agonist-stimulated ROS production. We have studied whether ROS affect agonist-
stimulated Ca^{2+} signaling in human aortic endothelial cells (HAEC) stimulated by histamine [Hu Q, *et. al.* (2002) *J Biol Chem* **277,**32546-32551]. Histamine (1 μM) increased the fluorescence of 2',7'-dihydrodichlorofluorescin diacetate in HAEC, an indicator of ROS production. This was partially inhibited by the NADPH oxidase inhibitor diphenyleneiodonium (DPI, 10 μM), by the farnesyltransferase inhibitor H-Ampamb-Phe-Met-OH (2 μM), and in HAEC transiently expressing $Rac1^{N17}$, a dominant negative allele of the protein Rac1, which is essential for NADPH oxidase activity. In indo 1-loaded HAEC, 1 μM histamine triggered oscillations of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that were blocked by DPI or by H-Ampamb-Phe-Met-OH. Histamine-stimulated ($[Ca^{2+}]_i$) oscillations were not observed in HAEC lacking functional Rac1 protein but were observed when transfected cells were simultaneously exposed to a low concentration of hydrogen peroxide (H_2O_2, 10 μM), which by itself did not alter either $[Ca^{2+}]_i$ or levels of inositol 1,4,5-trisphosphate (IP_3). Stimulation of the NADPH oxidase by NADPH stimulated a time- and concentration-dependent increase in superoxide and H_2O_2 production by HAEC that was blocked by DPI and was significantly attenuated in cells transiently expressing $Rac1^{N17}$. In permeabilized Mag-indo 1-loaded cells, NADPH and H_2O_2 each decreased the threshold concentration of IP_3 required to release intracellularly stored Ca^{2+} and shifted the IP_3-Ca^{2+} release dose-response curve to the left [Hu Q, *et. al.* (2002) *J Biol Chem* **275,**15749-15757]. Concentrations of H_2O_2 as low as 3 μM increased the sensitivity of intracellular Ca^{2+} stores to IP_3 and decreased the IP_3 EC_{50} from 423.2 ± 54.9 to 276.9 ± 14.4 nM (mean ± SD, n = 6, $p < 0.05$). At a concentration of 100 μM, NADPH decreased the EC_{50} in response to IP_3 by more than 50% to 170.6 ± 23.2 nM, n = 3, $p < 0.05$). The effect of NADPH on IP_3-stimulated Ca^{2+} release was blocked by catalase and by DPI and was not observed in cells lacking functional Rac1 protein. These data suggest that histamine generates ROS in HAEC at least partially via NADPH oxidase activation. NADPH oxidase-derived ROS are critical to the generation of $[Ca^{2+}]_i$ oscillations in HAEC during histamine stimulation, perhaps by increasing the sensitivity of the endoplasmic reticulum to IP_3. The sensitivity of the IP_3 receptor to oxidative stress may play an important role in cell signaling during membrane receptor stimulation and may be crucial to the downstream activation of Ca^{2+}-sensitive transcription factors like NF-κB when the vascular endothelium responds to inflammatory stimuli.

SA2

Redox Modifications of RyR Channels Affect Calcium Release in Muscle and Neurons

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Calcium release mediated by ryanodine receptors/calcium release channels (RyR channels) is required to elicit the contraction of skeletal and cardiac muscle and to induce neuronal plasticity in the hippocampus. In particular, RyR-mediated calcium-induced calcium release (CICR) is a powerful mechanism for the amplification and propagation of calcium signals initially generated by calcium entry into cells. RyR channels are especially susceptible to redox modifications by a variety of non-physiological or endogenous redox molecules, which produce significant changes on channel activity. Consequently, we have investigated the effects of redox modification of RyR channels on calcium release in skeletal and cardiac muscle and on the activation of signaling cascades and transcription factors in neurons. All experiments were done in compliance with the protocols for animal care, handling and experimentation approved by the Facultad de Medicina, Universidad de Chile.

Using SR vesicles enriched in RyR1 channels isolated from rabbit skeletal muscle (Aracena et al 2003), we studied the effects of S-nitrosylation and S-glutathionylation by two endogenous redox-active agents - glutathione disulfide (GSSG) and S-nitrosoglutathione (GSNO) - on CICR. We found that S-glutathionylation of 3 cysteine residues per RyR1 channel monomer diminishes channel inhibition by magnesium while S-nitrosylation of different cysteines enhances the activation of the channel by calcium. Furthermore, we found that the transverse tubules (plasma membrane invaginations) of skeletal muscle cells possess an endogenous NAD(P)H oxidase that on incubation with NAD(P)H generates superoxide anion, which is rapidly converted into hydrogen peroxide. Activation of this enzyme significantly stimulates RyR1 channels in vitro and in skeletal muscle cells in culture.

In SR vesicles isolated from dog heart muscle (Domenech et al 1998), we found that 0.1 mM NADPH enhances calcium release kinetics; addition of superoxide dismutase or catalase prevented these effects. All four NAD(P)H oxidase subunits probed (gp91, p22, p47 and p67) were found in cardiac SR vesicles, suggesting that reactive oxygen species generated by the NAD(P)H oxidase activate RyR2-mediated release. We also found that brief periods of tachycardia - which prior to prolonged coronary artery occlusion reduce infarct size (Sanchez et al 2003) - enhanced the synthesis de novo of RyR2 channels, stimulated calcium release from isolated SR vesicles and increased 2-fold the rates of NAD(P)H-dependent superoxide production compared to the controls. Noteworthy,

tachycardia also increased 2-fold the association of the p47 subunit to SR vesicles. Accordingly, we propose that tachycardia enhances calcium release by promoting oxidative activation of RyR2 channels and by stimulating de novo RyR2 synthesis.

In neurons, we have investigated whether oxidative activation of RyR channels promotes the phosphorylation of ERKs and CREB, which is required for the calcium-dependent gene expression associated with long lasting synaptic plasticity in the hippocampus. To induce changes in cell redox state, we added hydrogen peroxide to neurons in culture (N2a or hippocampal neurons) or to mouse hippocampal slices, or increased the Fe content of PC12 cells in culture. In all cases, hydrogen peroxide addition significantly enhanced phosphorylation of CREB and ERKs, whereas increasing cell Fe content increased cytoplasmic calcium concentration and stimulated the phosphorylation and the nuclear translocation of phospho-ERK1/2. Ryanodine (50-100 μM) significantly inhibited all these effects, suggesting that oxidative activation of RyR-mediated calcium release from intracellular stores mediates the observed enhancement of ERK/CREB phosphorylation.

In summary, the present results suggest that oxidative activation of RyR channels enhances calcium release in skeletal or cardiac muscle cells and neurons. The resulting increase in cellular calcium concentration may sustain calcium-dependent gene expression during normal cellular function, as shown in neurons. Furthermore, oxidative stress, through excessive stimulation of RyR-mediated calcium release, may lead to the development of pathological conditions.

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SA3

Roles of intracellular calcium stores and mitochondria in cell death induced by mitochondrial oxidative stress.

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The mitochondrial respiratory chain is a major site at which oxygen free radicals are generated, especially under conditions in which the respiratory chain is inhibited in the presence of oxygen. Increased mitochondrial radical production is thought to play a major part in the pathway to cell death seen at reperfusion of anoxic heart and brain.

We have modelled mitochondrial-specific oxidative stress using the lipophilic cationic fluorescent dye tetramethyl rhodamine methyl ester (TMRM), which accumulates within mitochondria in response to mitochondrial membrane potential. The dye is widely used as a potential sensitive probe, but it is also highly phototoxic as illumination generates increased singlet oxygen (Duchen, 2000; Jacobson and Duchen, 2002). As the dye is localised to the mitochondria, so is the source of radical species. Illumination of astrocytes or cardiomyocytes loaded with TMRM reveals a stereotypical progression of events consisting initially of an increase in spontaneous focal transient and reversible mitochondrial depolarisations. After a period of time that varies between cell types and illumination intensity, this is followed by a global mitochondrial depolarisation. In freshly isolated ventricular cardiomyocytes this is seen as a wave that progresses along the length of the myocyte over a period of minutes. This global mitochondrial depolarisation is followed by ATP depletion, seen in myocytes as the onset of rigor, and eventually by cell death. We have found that cells can be protected by i) depleting intracellular calcium stores with thapsigargin or by buffering intracellular calcium with EGTA-AM or BAPTA-AM or ii) by cyclosporine A or sanglifehrin, that inhibit opening of the mitochondrial permeability transition pore (mPTP).

Measurements of intracellular and intramitochondrial calcium concentrations suggest that oxidative stress increases the probability of SR calcium release, seen as an increased frequency of calcium sparks and waves in cardiac myocytes, followed by an increase in mitochondrial calcium loading, measured using dyes such as rhod-2. Both ryanodine and IP3 gated channels have been shown previously to show an increased probability of calcium release in response to oxidative stress. We therefore propose that these events can be readily explained by a scheme in which oxidative stress increases local ER/SR calcium release. The calcium is taken up by mitochondria which become progressively calcium loaded. The combination of mitochondrial calcium loading and mitochondrial oxidative stress increases the probability of permeability transition (Jacobson and Duchen, 2002). This may initially be transient and reversible, but eventually becomes irreversible, causing global mitochondrial depolarisation. This is followed by ATP depletion and an inevitable progression to cell death. We suggest that the basis for this response is provided by the proximity of SR/ER and mitochondria, by the mitochondrial capacity to accumulate calcium, and by the sensitivity of three channel types: the ryanodine receptor, the IP3 gated channel and the mPTP - to ROS (see Missiaen et al., 1991; Kawakami et al., 1998; Petronilli et al., 1994), largely reflecting the roles of critical thiol groups in regulating the opening probability of each class of channel.

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SA4

Oxygen Sensing, Mitochondria, Reactive Oxygen Species, and Hypoxic Pulmonary Vasoconstriction

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Mammalian systems activate a number of adaptive responses during hypoxia that protect the organism from the consequences of severe oxygen deprivation. These responses are evident at the organismal level, the tissue level, and at the cellular level. They include transcriptional activation, and Hypoxia Inducible Factors-1 and -2 (HIF-1 and -2) are critical for the activation of genes encoding glycolytic enzymes, membrane glucose transporters, vascular growth factors, erythropoietin, and nitric oxide synthase. Post-translational responses to hypoxia include neurotransmitter release, alterations in membrane ion transport, and changes in vascular smooth muscle tone. In the lung, hypoxic pulmonary vasoconstriction (HPV) helps to optimize gas exchange but it also contributes to the development of pulmonary hypertension in hypoxic lung diseases. Much is known about the signaling pathways mediating the molecular responses to hypoxia, but the mechanisms of oxygen sensing underlying transcriptional and post-translational responses are not well understood. Putative models that may act as cellular oxygen sensors mediating HPV include the NAD(P)H oxidase family, which should decrease the production of reactive oxygen species (ROS) as the cellular O_2 tension decreases, O_2-sensitive ion channels, and the mitochondrial electron transport chain (ETC). We have proposed that hypoxia paradoxically stimulates ROS release from the mitochondrial ETC, producing an oxidant signal capable of triggering increases in intracellular Ca^{2+} concentrations during hypoxia. This response appears to be initiated by the release of Ca^{2+} from intracellular stores, followed by the entry of extracellular calcium via L-type Ca^{2+} channels and/or through capacitative calcium entry via store operated calcium channels. The increase in cellular oxidant stress during hypoxia can be detected using ROS-sensitive probes such as dichlorofluorescein or dihydrorhodamine, by novel FRET-based redox sensors or redox-sensitive fluorescent proteins, and by EPR spectroscopy. The primary site of ROS generation during hypoxia appears to be Complex III, although increased oxidant generation at other sites including Complex II may be sufficient to activate the response under some conditions. The exogenous addition or over-expression of antioxidant enzymes abolishes the cytosolic ROS signal and the increase in intracellular calcium in pulmonary artery smooth muscle cells. By contrast, increases in oxidant generation, or administration of exogenous oxidants during normoxia can activate the hypoxia response pathway under normoxic conditions. Much controversy still exists regarding the cellular mechanisms of oxygen sensing. However, there is good agreement that this mechanism plays an important role in survival at stages ranging from development through adulthood.

SA5

Modulation of neuronal voltage-gated potassium channels by oxidative stress during β amyloid-induced neurotoxicity

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Intracellular K^+ plays a strategic role in cell survival acting as a negative regulator of some key enzymes involved in apoptosis, such as caspases and endonucleases, and by inhibiting cytochrome c-dependent apoptosome formation; furthermore blockade of K^+ efflux inhibits cell proliferation and apoptotic cell death. As a matter of fact, an increased activity of plasma membrane K^+ channels, leading to a decreased cytoplasmic K^+ concentrations, occurs in different neurotoxicity models, such as during serum deprivation, staurosporine, ceramide or NMDA exposure, as well as upon lowering extracellular K^+ concentrations ($[K^+]_e$). Furthermore, alteration of K^+ channel function in brain cells seems to play a relevant role also in the pathophysiology of Alzheimer Disease (AD); in fact, treatment for 3-24 hours with neurotoxic Aβ fragments enhances voltage-gated K^+(VGK) channel activity in mouse SN-56 hybrid septal-neuroblastoma cells, in primary rat cerebellar granule cells, in cortical astrocytes and in microglial cells. Interestingly, the inhibition of K^+ efflux, either by pharmacological tools or by increasing $[K^+]_e$, fully prevented cell death induced by Aβ.

Despite the large number of studies documenting an involvement of K^+ channels in AD-related neuronal cell death, the molecular steps linking the exposure to neurotoxic events to the changes in K^+ channel function remain controversial and poorly understood. Therefore, in the present study, we have evaluated the molecular mechanisms by which neurotoxic Aβs ($A\beta_{1-42}$, $A\beta_{25-35}$) enhance the expression of VGK currents in PC-12 cells differentiated by nerve growth factor (NGF) treatment and in primary rat hippocampal neurons. To this aim, we have evaluated the dependence of the enhanced K^+ channel activity on extracellular Ca^{2+} availability, time-related production of ROS, protein synthesis, and transcriptional induction of nuclear factor-kappa B (NF-kB).

The neurotoxic β-amyloid peptide $A\beta_{25-35}$ caused a dose- (0.1-10 μM) and time-dependent (>12 hours) enhancement of VGK currents in PC-12 cells and primary rat hippocampal neurons. Similar effects were exerted by $A\beta_{1-42}$, but not by the non-neurotoxic $A\beta_{25-35}$ peptide. Both inactivating and non-inactivating K^+ currents components were potentiated upon neurotoxic Aβs treatment. $A\beta_{25-35}$ also caused an increased production of reactive oxygen species (ROS) which started at 20 minutes, peaked at 3 hours and lasted for 24 hours; this $A\beta_{25-35}$-induced ROS production was abolished upon the removal of extracellular Ca^{2+} from the incubation medium. Interestingly, ROS production seems to trigger VGK current increase since vitamin E (50 μM) completely abolished not only ROS production, but also $A\beta_{25-35}$-induced VGK currents enhancement. The protein synthesis inhibitor cycloheximide (1 μg/ml) and the transcription inhibitor actinomycin-D (50 ng/ml) blocked $A\beta_{25-35}$-induced VGK current enhancement, suggesting that this potentiation is mediated by transcriptional activation induced by ROS. Interestingly, the specific NF-kB inhibitor SN-50 (5 μM), but not its inactive

analogue SN-50M (5 μM), fully counteracted the $A\beta_{25-35}$-induced enhancement of VGK currents, providing evidence for a role of this family of transcription factors in regulating neuronal K^+ channel function during Aβ exposure.
Altogether, the present results suggest that cell death which follows neurotoxic Aβ peptides exposure of clonal and primary neurons involves an highly-coordinated sequence of events which include a Ca^{2+}-dependent increased availability of ROS which, in turn, activates NF-kB transcription factors thus leading to the enhanced expression of VGK channels in the neuronal membrane. Given that an increased K^+ channel function is likely to deplete intracellular K^+, and that a decreased cytoplasmic K^+ concentration represents an important prerequisite for cell death progression and execution mechanisms during exposure to several neurotoxic insults, the present result may be of considerable pathophysiological relevance for AD, as well as for other neurodegenerative disorders.

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SA6

Hypoxic up-regulation of L-type Ca^{2+} channels: involvement of reactive oxygen species

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Prolonged hypoxia can lead to remodeling of tissue functions, in part via the altered expression of cellular proteins. Of these, we have shown that numerous ion channel types display altered functional expression (Peers, 2002), which can have marked effects on cell function. The mechanisms underlying hypoxic modulation of channels are poorly understood, but we have provided evidence that hypoxic up-regulation of L-type Ca^{2+} channels is mediated by amyloid peptides of Alzheimer's disease in both PC12 cells and cerebellar granule neurones (Taylor et al., 1999) and unpublished observations). Thus we have established a potential mechanism to account for the known increased incidence of Alzheimer's disease following hypoxic / ischemic episodes (Moroney et al., 1996). Here, we describe the up-regulation by chronic hypoxia (CH) of L-type Ca^{2+} channels in HEK 293 cells stably expressing the α_{1C} subunit of the human cardiac L-type Ca^{2+} channel (Scragg et al., 2004).
Whole-cell patch clamp recordings revealed that 24h exposure to 2.5% O_2 dramatically increased Ca^{2+} current density in this recombinant expression system. CH also increased the levels of endogenous Alzheimer's amyloid β peptides (AβPs), determined immunocytochemically. Pharmacological prevention of AβP production (via exposure to inhibitors of secretase enzymes that are required to cleave AβP from its precursor protein) prevented hypoxic augmentation of currents, as did inhibition of vesicular trafficking with bafilomycin A1, whereas application of exogenous AβPs mimicked the effects of CH. The enhancing effect of exogenous AβPs or CH were abolished following incubation with the monoclonal 3D6 antibody, raised against the extracellular N' terminus of AβP. Immunolocalization and immunoprecipitation studies provided compelling evidence that AβPs physically associated with the α_{1C} subunit, and this association was promoted by hypoxia.
Current augmentation in response to CH was absent in cells in which the mitochondrial electron transport chain (ETC) was depleted following treatment with ethidium bromide (ρ^0 cells) or in control (ρ^+) cells in the presence of 1μM rotenone. Hypoxic augmentation of currents could be mimicked in ρ^0 cells by the exogenous production of O_2^- by xanthine / xanthine oxidase. In ρ^0 cells, exogenously applied AβPs enhanced currents by a similar degree to that seen in cells with an intact ETC. The antioxidants ascorbate (200 μM) and TROLOX (500 μM) ablated the effect of CH in ρ^+ cells, but were without effect on AβP mediated augmentation of Ca^{2+} current in ρ^0 cells. Thus oxidant production in complex I of the mitochondrial ETC is a critical factor, acting upstream of AβP production in the up-regulation of Ca^{2+} channels in response to chronic hypoxia.
These data suggest an important role for AβPs in mediating the increase in Ca^{2+} channel activity following CH, and show that AβPs act post-transcriptionally to promote L-type Ca^{2+} channel insertion into (and / or retention within) the plasma membrane. Furthermore, they indicate that mitochondrially-derived reactive oxygen species are intimately involved in this process. Such an action will likely contribute to the Ca^{2+} dyshomeostasis of Alzheimer's disease (LaFerla, 2002).

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SA7

Oxidation activates anion channels in malaria-infected human erythrocytes

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Intraerythrocytic growth of the malaria parasite Plasmodium falciparum activates yet unidentified organic osmolyte and anion channels in the membrane of the human host erythrocyte. These channels deliver nutrients to the parasite, dispose of waste products, and counter-regulate the infection-induced swelling of the host cell (1). The parasite has been demonstrated to impose oxidative stress on its host erythrocyte which is produced during the digestion of the host hemoglobin (2). This oxidative stress seems to be involved in channel activation since reduced glutathione (but not oxidized glutathione) applied during whole-cell recording to the intracellular membrane face inactivates the channels in parasitized human erythrocytes. In addition, oxidation (1 mM t-butylhydroperoxide) of non-infected cells induces activation of identical channels (3).
The channel activation upon oxidation or during infection is accompanied by ATP release from the erythrocyte. Moreover, purinoceptor antagonists or degradation of extracellular ATP by

apyrase inhibit channel activation in oxidized or parasitized erythrocytes indicating autocrine purinergic signaling through P2Y1 receptor and further metabotropic purinoceptor subtypes (4). Accordingly, the organic osmolyte and anion channels activate delayed in oxidized or P. berghei-infected erythrocytes from P2Y1 knockout mice as compared to wildtype cells. In sharp contrast to oxidized or parasitized erythrocytes, extracellular ATP has no effect on channel activity in untreated control erythrocytes (4). The activation of the organic osmolyte and anion channels in oxidized or parasitized erythrocytes is paralleled by the activation of ClC-2 Cl-selective channels which contribute to the cell volume regulation of the parasitized erythrocyte. In addition, oxidation (1 mM t-butylhydroperoxide) and reduction (5 mM dithiothreitol) activates and inactivates ClC-2 heterologously expressed in Xenopus laevis oocytes, respectively (5).
In summary, the increase in membrane permeability of malaria-infected human erythrocytes is probably induced by parasite-derived oxidative stress. Oxidation triggers ATP release-mediated purinergic signaling and activation of at least two different types of anion channels in the erythrocyte membrane.

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SA8

Redox modulation of a non-selective cation channel: Possible implication in epithelial cell necrosis

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Intracellular accumulation of ROS, such as hydrogen peroxide, superoxide anion and hydroxyl radical results from normal metabolic processes or toxic insults. The degree of oxidative stress is determined by the balance between free radical synthesis and degradation. Alterations of this balance have been associated to aging and several pathological processes such as inflammation and ischemia-reperfusion. High, long-standing levels of ROS can cause necrosis and direct treatment of cells with oxidants has been demonstrated to induce necrosis, which appears to be the result of acute cellular dysfunction in response to severe stress conditions or after exposure to toxic agents. Intracellular calcium and sodium overload has been recognised as a critical step in necrosis. Previously, the involvement of a fenamate-sensitive Ca^{2+}-activated nonselective cation channel (NSCC) in free radical-induced rat liver cell necrosis was demonstrated (1). Based on that observation, the effect of radical oxygen species (ROS) and oxidizing agents on the gating behavior of a NSCC in a liver-derived epithelial cell line (HTC) has been now addressed. Single channel currents were recorded in HTC cells using the excised inside-out configuration of the patch-clamp technique. In this cell line, a 19 pS Ca^{2+}-activated, ATP- and fenamate-sensitive NSCC nearly equally permeable to monovalent cations is characterised. In the presence of Fe^{2+}, exposure of the intracellular side of NSCC to H_2O_2 increased their open probability (Po) by $\backsim$40% without affecting the unitary conductance. Desferroxamine as well as the hydroxyl radicals (·OH) scavenger MCI-186 inhibited the effect of H_2O_2, indicating that the increase in Po was mediated by ·OH. Exposure of the patch membrane to the oxidizing agent 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) had a similar effect to ·OH. The increase in Po induced by ·OH or DTNB was not reverted by preventing ·OH formation or by DTNB washout, respectively. However, the reducing agent dithiothreitol (DTT) completely reversed the effects on Po of both ·OH and DTNB. A similar increase in Po was observed by applying the physiological oxidizing molecule GSSG. Moreover, GSSG-oxidized channels showed enhanced sensitivity to Ca^{2+}. The effect of GSSG was fully reversed by GSH (2). These results suggest an intracellular site(s) of action of oxidizing agents on cysteine targets on the fenamate-sensitive NSCC protein implicated in epithelial cell necrosis.

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