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Nitrosative stress and potassium channel-mediated neuronal apoptosis: is zinc the link?

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Abstract

Nitrosative stress has been implicated in a large number of neurological disorders. The molecular mechanisms underlying the neuronal injury associated with this stimulus, however, are not clearly understood. Emerging evidence suggests that the liberation of intracellular zinc as well as over-activation of potassium channels may be two important components of nitrosative stress-induced neuronal death.

Keywords

Nitric oxide; Peroxynitrite; Metallothionein; Potassium channel; Zinc; p38; Apoptosis; Kv2.1

Introduction

Nitric oxide (NO) has long been known to have a multitude of contrasting effects on biological systems and is an important neuronal cellular messenger (Garthwaite et al. 1988; Garthwaite and Boulton 1995). NO has also been associated with *N*-methyl-D-aspartate (NMDA) neurotoxicity, neuronal death in stroke, as well as being implicated in a variety of neurological disorders (Dawson and Dawson 1995; Aizenman et al. 1998). Emerging evidence indicates, however, that many of the toxic effects of NO may, in fact, be mediated by peroxynitrite (Estevez and Jordan 2002). Peroxynitrite can have a number of deleterious effects in cells, including oxidation of protein and non-protein sulfhydryls, induction of membrane lipid peroxidation, as well as inhibition of the electron transport chain (Radi et al. 1991, 1994). In addition to these effects, peroxynitrite can react with proteins to form nitrotyrosines, inhibiting the phosphorylation of tyrosine residues and thereby interfering with intracellular signal transduction pathways (Brito et al. 1999). Indeed, elevated levels of nitrotyrosine have been detected in a variety of disease states, including amyotrophic lateral sclerosis, Parkinson's disease and Alzheimer's disease (Groves et al. 1999). In the present review we discuss the possible mechanism by which nitrosative stress induces neuronal death, and summarize findings pertaining to NO-derived-species-induced release of intracellular zinc, and the link between this process and the role of potassium channels in neuronal apoptosis. We conclude by describing the effects of peroxynitrite on neuronal voltage-gated potassium channel function.

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Zinc toxicity in the CNS

Zinc has been shown to be directly toxic to neurons (Yokoyama et al. 1986; Choi et al. 1988; Koh 2001). In nearly all previous studies, Zn^{2+}-mediated neuronal cell death was dependent upon influx of the cation into cells, a process facilitated by concurrent membrane depolarization (Weiss et al. 1993). Indeed, Zn^{2+}-induced injury can be blocked by cell-impermeant metal chelators (Koh et al. 1996), and by antagonists of Ca^{2+}-permeable voltage- and glutamate-gated ion channels, which are potential entry routes for this ion (Weiss et al. 1993; Koh and Choi 1994; Sensi et al. 1997). As a substrate for the Na^{+}-Ca^{2+} exchanger, Zn^{2+} may also enter neurons via this route during periods of energetic dysfunction and collapsing ion gradients (Sensi et al. 1997). Cellular influx of synaptically released Zn^{2+} has been suggested to contribute to the neuronal damage associated with ischemia and epilepsy, as "chelatable," and presumably free Zn^{2+} is present in synaptic vesicles (Frederickson 1989). In turn, vesicular Zn^{2+} can be released from neurons upon depolarization (Assaf and Chung 1984; Howell et al. 1984; Aniksztejn et al. 1987; Vogt et al. 2000) and translocate from presynaptic release sites into postsynaptic neurons (Sloviter 1985; Koh et al. 1996; Frederickson et al. 1988; Tonder et al. 1990; Koh et al. 1996; Lee et al. 2000; but see Kay 2003).

The mechanism by which Zn^{2+} induces cell death is unresolved. Zn^{2+} is redox inactive under most physiological conditions (Berg and Shi 1996), and, as such, is thought to be relatively non-toxic in and of itself (Cohen and Duke 1984). Unlike iron or copper, Zn^{2+} does not catalyze directly the formation of reactive oxygen species. Hence, several possible mechanisms have been proposed to account for the toxic actions of Zn^{2+} following its translocation from the extracellular space into the cytoplasm. Manev et al. (1997) and Sensi et al. (1999, 2000, 2003) have suggested that Zn^{2+} can enter mitochondria and induce the release of oxygen-derived free radicals by disrupting mitochondrial integrity. In contrast, Dineley et al (2003) have suggested that the effects of Zn^{2+} on mitochondrial membrane potential and the ensuing dysfunction do not require influx of the metal into the mitochondria. Nonetheless, Zn^{2+} has been shown to affect several key components of mitochondrial function (Kleiner 1974), including inhibition of complex III (Link and von Jagow 1995) and α-ketoglutarate dehydrogenase (Brown et al. 2000), in addition to inducing permeability transition (Wudarczyk et al. 1999). Furthermore, recent work by Sheline et al. (2000) has suggested that Zn^{2+} can inhibit glycolysis and deplete NAD^{+}. Each of these studies points to a critical role for mitochondrial dysfunction in Zn^{2+} toxicity, yet the specific cell signaling pathways that contribute to Zn^{2+}-induced injury remain ill defined.

Zn^{2+}-induced cellular injury has been associated with both necrosis and apoptosis (Kim et al. 1999a,1999b). The activation of these apparently disparate cell death pathways most likely correlates to the intensity of Zn^{2+} exposure (Manev et al. 1997; Kim et al. 1999a,1999b). Other factors, such as activation of Zn^{2+}-dependent transcription factors (Atar et al. 1995;Park and Koh 1999) and protein kinases (Csermely et al. 1988;Noh et al. 1999;McLaughlin et al. 2001), as well as the release of apoptogens such as ceramide (Spence et al. 1989;Hannun 1994;Brugg et al. 1996;Schissel et al. 1996), or activation of death induction proteins (Park et al. 2000;Kim and Koh 2002), probably influence the process by which neurons die following Zn^{2+} exposure. Interestingly, in some systems Zn^{2+} can also inhibit apoptosis (Zalewski et al. 1993;Fraker and Telford 1997;Perry et al. 1997;Chai et al. 1999;Ho et al. 2000;Schrantz et al. 2001), and cellular depletion of this metal can be deleterious to cells (Ahn et al. 1998;Adler et al. 1999;Virag and Szabo 1999). As such, the balance between the physiological and pathophysiological actions of Zn^{2+} closely resembles that of another important ion, calcium (Lipton and Kater 1989;Toescu 1998). Indeed, Zn^{2+} may be sequestered by intracellular organelles (Manev et al. 1997; Sensi et al. 1999,2000), bound to metal chaperone proteins such as metallothionein (MT; Aschner et al. 1996), and regulated by plasma membrane transporters (McMahon and Cousins 1998), all of which contribute to the maintenance intracellular Zn^{2+}

homeostasis in a manner akin to that observed with intracellular Ca^{2+} (Colvin et al. 2003). Additionally, as has been suggested for Ca^{2+} (Choi 1992), Zn^{2+} is likely to trigger a variety of molecular signaling pathways depending upon the intensity and sub-cellular localization of the stimulus, as well as the energetic status of the cell (Weiss et al. 2000).

Liberation of intracellular zinc

Zinc is the second most abundant metal in the body, after iron (Takeda 2000). Of the total zinc content in the brain, only 10% is free or "chelatable," and contained within synaptic vesicles (Frederickson 1989). Rather than existing as a free ion in the cytoplasm, non-vesicular cellular Zn^{2+} is complexed tightly to cysteine residues of proteins such as MT, and Zn^{2+} finger-containing transcription factors (Frederickson 1989; Berg and Shi 1996). It has been shown, however, that Zn^{2+} can be liberated from these stores by oxidative stimuli, NO-related species, as well as other metals such as Cd^{2+} (Fliss and Menard 1991; Kroncke et al. 1994; Berendji et al. 1997; Cuajungco and Lees 1998; Maret and Vallee 1998; Jiang et al. 1998; Aravindakumar et al. 1999; Aizenman et al. 2000; Pearce et al. 2000; Katakai et al. 2001; St Croix et al. 2002; Lee et al. 2003; Zhang et al. 2003; Hara and Aizenman 2004). Maret and Vallee (1998) have argued that MT-bound zinc can be released readily by mild thiol oxidants, in particular by disulfides such as oxidized glutathione (Maret 1994, 1995). This is due to the fact that MT itself has a relatively low redox potential (−366 mV; Maret and Vallee 1998). Hence, even though MT has a relatively high affinity for Zn^{2+} (K_d 1.4×10^{-13} M; Kagi 1993), thiol oxidants may act as cellular signals that induce the transfer of Zn^{2+} from MT to other proteins with lower affinities for this metal (Jiang et al. 1998).

Incubation of MT with the thiol oxidant 2,2′-dithiodipyridine (DTDP) results in the oxidation of all 20 cysteines contained in the protein and release of all 7 zinc atoms in cell-free assays (Maret and Vallee 1998). We have utilized DTDP to induce the release of zinc from intracellular metalloproteins in cortical neurons in vitro (Aizenman et al. 2000). Importantly, DTDP-liberated Zn^{2+} induces neuronal apoptosis via a p38 kinase-dependent process (McLaughlin et al. 2001). In that process, the intracellular release of Zn^{2+} is followed, sequentially, by the production of reactive oxygen species, p38 activation, enhancement of K^+ currents, and, finally, caspase activation (McLaughlin et al. 2001). This enhancement of K^+ currents has been shown to be a crucial element in several apoptotic paradigms (Yu 2003).

Potassium channel-mediated neuronal apoptosis

The elevation of extracellular potassium blocks apoptosis in neurons originating from a variety of central and peripheral locations. In mouse neocortical neurons, apoptosis induced by serum deprivation, or exposure to staurosporine is accompanied by amplification of a voltage-gated potassium current (Yu et al. 1997). Surprisingly, apoptosis can be prevented by the potassium channel blocker tetraethylamonium (TEA) (Yu et al. 1997). The same study also showed that exposure to the K^+ ionophore valinomycin, or the potassium channel opener cromakalin can induce apoptosis. Electrophysiological measurements from cortical neurons undergoing apoptosis in other settings have also shown enhancement of K^+ currents. Neuron cultures exposed to ceramide have enhanced delayed rectifier K^+ currents before subsequent caspase activation and apoptosis (Yu et al. 1999). The application of TEA is neuroprotective against ceramide-induced death. In a model for amyloid β-peptide (Aβ) toxicity, a cholinergic septal cell line (SN56) exposed to Aβ exhibited a sizeable increase in TEA-sensitive outward potassium currents (Colom et al. 1998). In a separate study, cultured murine cortical neurons showed an enhancement in delayed-rectifier K^+ currents after Aβ exposure (Yu et al. 1998). Co-treatment with TEA proved to be protective in both of these studies. Alterations in potassium channel activity have also been seen in transient cerebral ischemia models. Whole-

cell voltage-clamp recordings from rat hippocampal slices have demonstrated that a significant increase in potassium currents occurs in CA1 neurons 6–8 h after carotid artery occlusion (Chi and Xu 2000). We have shown that primary neuronal cultures exposed to the sulfhydryl oxidizing agent DTDP also have augmented potassium currents and that this enhancement is required for the ensuing apoptotic cascade (McLaughlin et al. 2001). Thus it seems that neuronal injury provoked by various apoptotic stimuli including oxidative stress is associated with amplified potassium currents. However, the role of potassium currents in the apoptotic cascade as well as the mechanism for their augmentation has yet to be defined clearly.

It has been proposed that the enhancement of potassium currents during the apoptotic process leads to a decrease in the concentration of this cation in the cytoplasm (Yu et al. 1999) and it is the decrease of intracellular potassium that acts as an apoptotic signal. Indeed, K^+ is the predominant intracellular cation and it is conceivable that the cell shrinkage associated with apoptosis is due to the loss of cellular K^+. Evidence for this hypothesis can be seen in a variety of non-neuronal models of apoptosis. Walev et al. (1995) have demonstrated that the K^+ depletion in monocytes results in the activation of caspases. Cidlowski and colleagues have estimated that intracellular [K^+] falls from 140 mM to 35–50 mM in lymphocytes undergoing apoptosis (Bortner and Cidlowski 1999). They also determined that the activation of caspase-3 as well as the activation of the apoptotic enzyme nuclease is facilitated by such a reduction in [K^+]. Conversely, apoptotic nuclease activity is completely inhibited at the normal physiological concentration of K^+ (150 mM). The relevance of potassium-dependent inhibition of apoptotic enzymes was also shown in the same study by sorting apoptotic thymocytes into normal and shrunken subpopulations by flow cytometry. This sorting demonstrated that caspase activation and nuclease activity were restricted to shrunken cells with low potassium content. A similar observation was made in an earlier study using dexamethasone-treated mouse lymphoma cell line (S49 Neo) cultures (Bortner and Cidlowski 1996). This study as well demonstrated that only shrunken apoptotic cells with a low [K^+] have caspase activity and degraded DNA. Even though these studies show possible implications of intracellular potassium loss in promoting cell death, they have yet to be demonstrated in neuronal models of apoptosis.

Another issue is the identity of the potassium channel or channels that are involved in this process. A number of functionally distinct voltage-gated potassium channel types have been identified in mammalian neurons, including low- and high-conductance, Ca^{2+}-dependent channels, inward rectifiers, transient A-type channels, and the delayed rectifier. We, as well as others, have observed that the enhanced currents are outward, have slow kinetics, and are sensitive to TEA, consistent with a delayed rectifier channel. However, certain studies have also described alterations in a transient outward current that is sensitive to 4-aminopyridine (4AP). Yu et al. (1997) have observed an increase in this fast inactivating current after serum deprivation and a decrease in this current after staurosporine treatment in cortical cultures. Chi and Xu (2000) also have shown an increase in amplitude for the transient current in CA1 pyramidal neurons after transient forebrain ischemia. This reported alteration is consistent with the observation that the excitability of CA1 neurons progressively decreases after an ischemic insult. It does not seem likely, however, that changes in the rapidly inactivating K current are involved in the apoptotic death pathway. Blocking this current with 4AP during any neuronal death paradigm associated with enhanced K currents has been reported to be ineffective as a protective strategy. Other studies have reported that M-type potassium channel blockers, such as linopiridine, promote survival in rat sympathetic neurons deprived of nerve growth factor (Xia et al. 2002). This effect, as well as the antiapoptotic effect of elevated extracellular potassium in sympathetic neurons, seems to be mediated by an increase in intracellular Ca^{2+} through L-type voltage-dependent Ca^{2+} channels (Galli et al. 1995). Work in central neurons has shown that TEA and elevated extracellular potassium is protective independently of changes in intracellular Ca^{2+} (Yu et al. 1997). Given these results, it seems that the alteration

of a delayed rectifier channel is what is crucial in a variety of apoptotic pathways. Recently, the specific channel mediating the enhanced potassium currents associated with neuronal apoptosis has been identified by our group (Pal et al. 2003). We have shown that disruption of Kv2.1-encoded potassium channels prevents the current enhancements after treatment with toxic doses of DTDP or staurosporine. Disruption of these channels also precludes the ensuing cell death after treatment with these apoptogens. It thus seems that Kv2.1-encoded potassium channels provide the main exit route for K^+ in cortical neurons undergoing apoptosis.

Peroxynitrite mediated enhancement of potassium currents

3-Morpholinosyndnomine (SIN-1) decomposes in solution to release both NO and superoxide (Holm et al. 1998). Three h after treatment of cortical neurons with 1 mM SIN-1, a robust potentiation occurs in potassium currents with relatively slow kinetics, consistent with that of delayed rectifier channels (Fig. 1a). This increase in steady-state amplitude is not accompanied by any change in voltage dependence (Fig. 1b), as reported previously (Yu et al. 1997; Pal et al. 2003), or by any significant change in the activation time constant (step to +5 mV from a holding potential of −70 mV: 9.6±2.7 ms *n*=9, control; 13.5±1.5 ms *n*=14, SIN-1; means ±SEM). On the basis of the extremely fast reaction rate between NO and superoxide (2×10^{10} M^{-1} s^{-1}; Kissner et al. 1998) we assumed that the effect of SIN-1 is mediated by peroxynitrite. To confirm this, we co-treated neurons with 1 mM SIN-1 and 25 μM 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron [III] (FeTPPS), a ferric porphyrin complex that can isomerize peroxynitrite to nitrate very rapidly (Misko et al. 1998). Co-treatment with FeTPPS completely blocked any current enhancement produced by SIN-1 (Fig. 1c). Treatment with FeTPPS alone had no effect on potassium. We also determined whether the direct application of peroxynitrite itself could mimic the effects of SIN-1 on potassium currents. Cultures were thus treated continuously with 150 μM peroxynitrite and whole-cell recordings obtained 3 h after initiation of treatment. Peroxynitrite produced a consistent and sizeable potentiation in potassium currents (Fig. 2), indistinguishable from the effects produced by SIN-1. These results show that neuronal exposure to peroxynitrite can result in sizeable changes in delayed rectifier potassium currents.

To establish whether zinc release is also involved in the potassium current changes mediated by peroxynitrite, we determined whether the zinc chelator *N*,*N*,*N*',*N*'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) inhibits the current enhancements induced by SIN-1. Co-exposure to 1 mM SIN-1 and 10 μM TPEN completely prevented the current enhancement (Fig. 3), indicating that zinc chelation does in fact prevent the signal transduction pathway involved in the peroxynitrite-induced amplification of potassium currents. To rule out the possibility that the origin of the liberated zinc was extracellular, we repeated these experiments with a zinc chelator that is not membrane permeable. Tricine, at a concentration previously shown to be able to buffer high levels of extracellular zinc (10 mM; Paoletti et al 1997), was ineffective in blocking the SIN-1 induced enhancement of potassium currents (Fig. 3). This indicates that the source of the zinc mediating this effect is, in fact, intracellular.

Recently, we have also tested a highly selective and neuroprotective inhibitor of p38, SB 239063 (Underwood et al. 2000; McLaughlin et al. 2001; Legos et al. 2002), to determine whether p38 activation is required for SIN-1 induced changes in potassium channel activity, similar to the cascade initiated by DTDP (McLaughlin et al. 2001). Neurons co-treated for 3 h with SIN-1 and SB 239063 (20 μM) showed no enhancement in whole cell potassium currents (Fig. 3). To confirm these results, we disrupted endogenous p38*α* function with the use of a dominant-negative (DN) expression construct. Recordings were obtained from neurons expressing the p38 DN and green fluorescent protein (GFP) and treated with SIN-1. Disruption of p38 function with this method was sufficient to prevent the current enhancement produced by SIN-1 (Fig. 3). In contrast, non-transfected neurons on the same cover-slip (Pal et al.

2003) still displayed increases in current density. To test for the possibility that the overexpression of p38 protein itself may have interfered with potassium channel conductance, we recorded from neurons transfected with wild-type p38 and GFP. Under these circumstances we observed no changes in current density. Thus, p38 function appears to be necessary for changes in potassium channel activity stimulated by peroxynitrite.

These results demonstrate that peroxynitrite can up-regulate voltage-gated potassium channel activity in cultured cortical neurons. This enhancement occurs through the participation of intracellular zinc, and p38. Similar results were reported very recently by Bossy-Wetzel et al. (2004), confirming the existence of the zinc-p38-K channel neuronal cell death axis described in our earlier work with thiol oxidants (Aizenman et al. 2000; McLaughlin et al. 2001). The mechanism by which zinc activates p38 after peroxynitrite exposure still needs further elucidation, although recent reports have shown that mobilized intracellular zinc can affect mitochondrial function (Dineley et al. 2003; Sensi et al. 2003), which may in turn lead to p38 activation (Tobiume et al. 2001; McLaughlin et al. 2001). One can thus envision a scenario in which peroxynitrite triggers the release of intracellular zinc, causing mitochondrial impairment and p38 activation, leading to the enhancement of voltage-gated potassium currents; a pathway that is virtually identical to that induced by oxidants such as DTDP (Aizenman et al. 2000; McLaughlin et al. 2001).

Perspectives

The enhancement of potassium channel activity following an apoptotic stimulus may prove to be a widespread phenomenon in neuronal injury. Following the identification of Kv2.1-encoded channels as the species responsible for the potentiation of delayed rectification following oxidative injury (Pal et al. 2003), a large-scale, high-throughput screening in a yeast system very recently identified a novel lead neuroprotective compound that blocks Kv2.1-mediated currents and neuronal apoptosis in the low micromolar range (Zaks-Makhina et al. 2004). Hence, selective pharmacological targeting of the potassium channels responsible for the enhanced currents may provide a novel therapeutic strategy in a wide number of neurodegenerative disorders.

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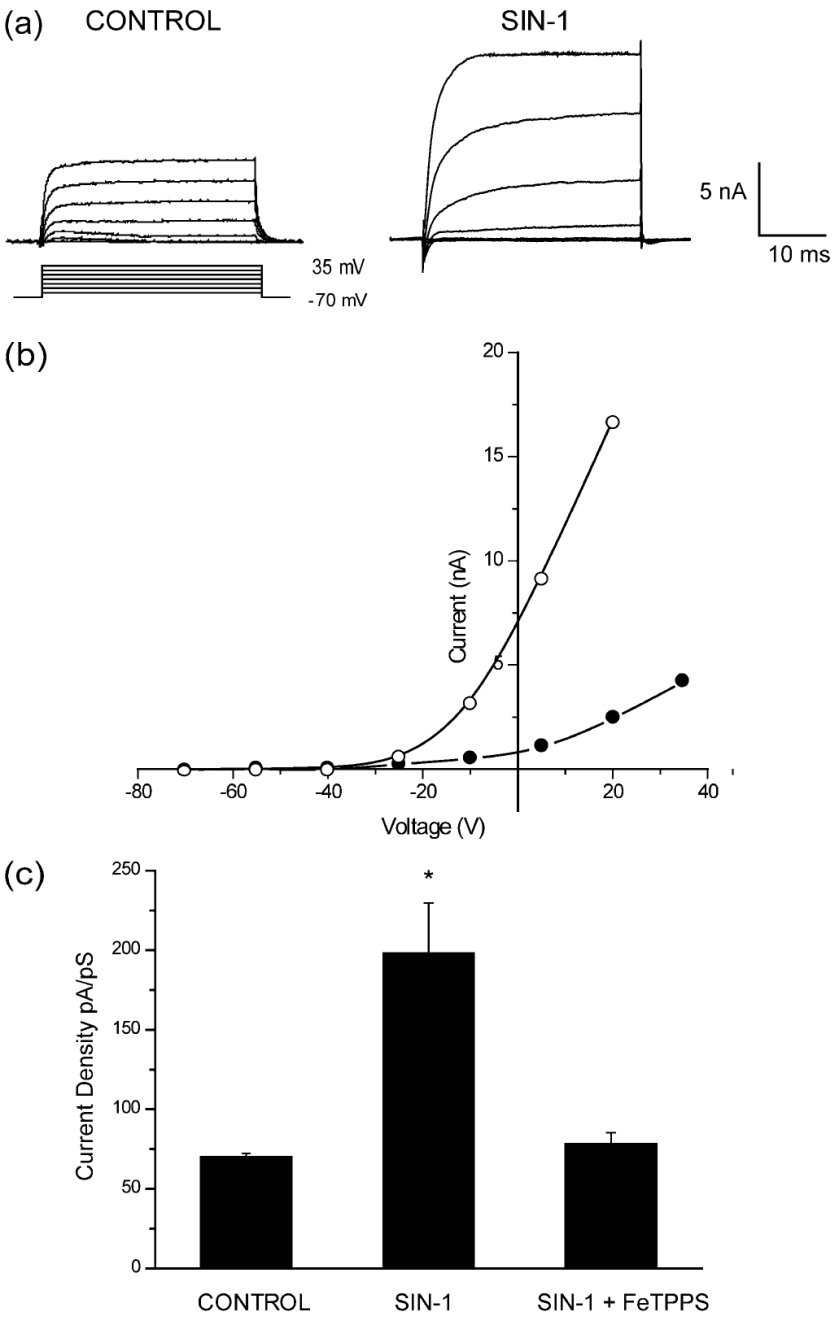

Fig. 1a–c.

3-Morpholinosydnonimine (*SIN-1*)-induced changes in potassium channel function. **a** Whole-cell potassium currents obtained in two separate cortical neurons after 180 min exposure to either vehicle (*control*) or 1 mM SIN-1. Currents were evoked by a series of voltage steps to +35 mV from a holding potential of −70 mV. **b** Current/voltage relationship of a single vehicle-treated neuron (*closed circles*) and a neuron exposed to 1 mM SIN-1 (*open circles*). **c** Whole-cell potassium currents were obtained from cortical neurons following 180 min exposure to vehicle (*n*=4), 1 mM SIN-1 alone (*n*=7), or SIN-1 in the presence of 25 μM 5,10,15,20-tetrakis (4-sulfonatophenyl)porphyrinato iron [III] (*FeTPPS*, *n*=6). Potassium currents were evoked by a voltage step to +5 mV from a holding potential of −70 mV. Current amplitudes were

normalized to cell capacitance. Mean±SEM; *P<0.001 vs. control (ANOVA followed by a Dunnett multiple comparison test)

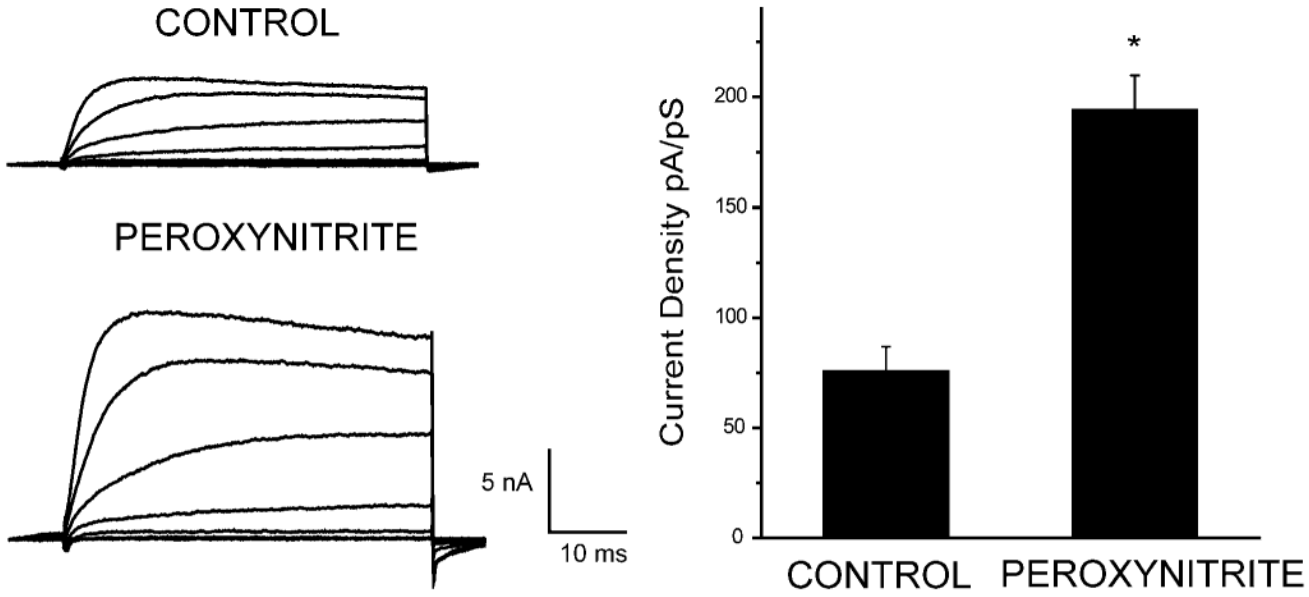

Fig. 2.

Peroxynitrite enhances potassium channel currents. Whole-cell potassium currents were obtained from cortical neurons following 180 min exposure to vehicle (*n*=4), or peroxynitrite (100 μM, *n*=6). Current amplitudes were normalized to cell capacitance. Potassium currents were evoked with a series of voltage step to +35 mV from a holding potential of −70 mV. Bars represent mean (±SEM) potassium current density (to +5 mV) for both treatment groups; **P*<0.001 vs. control (two-tailed Student's *t*-test)

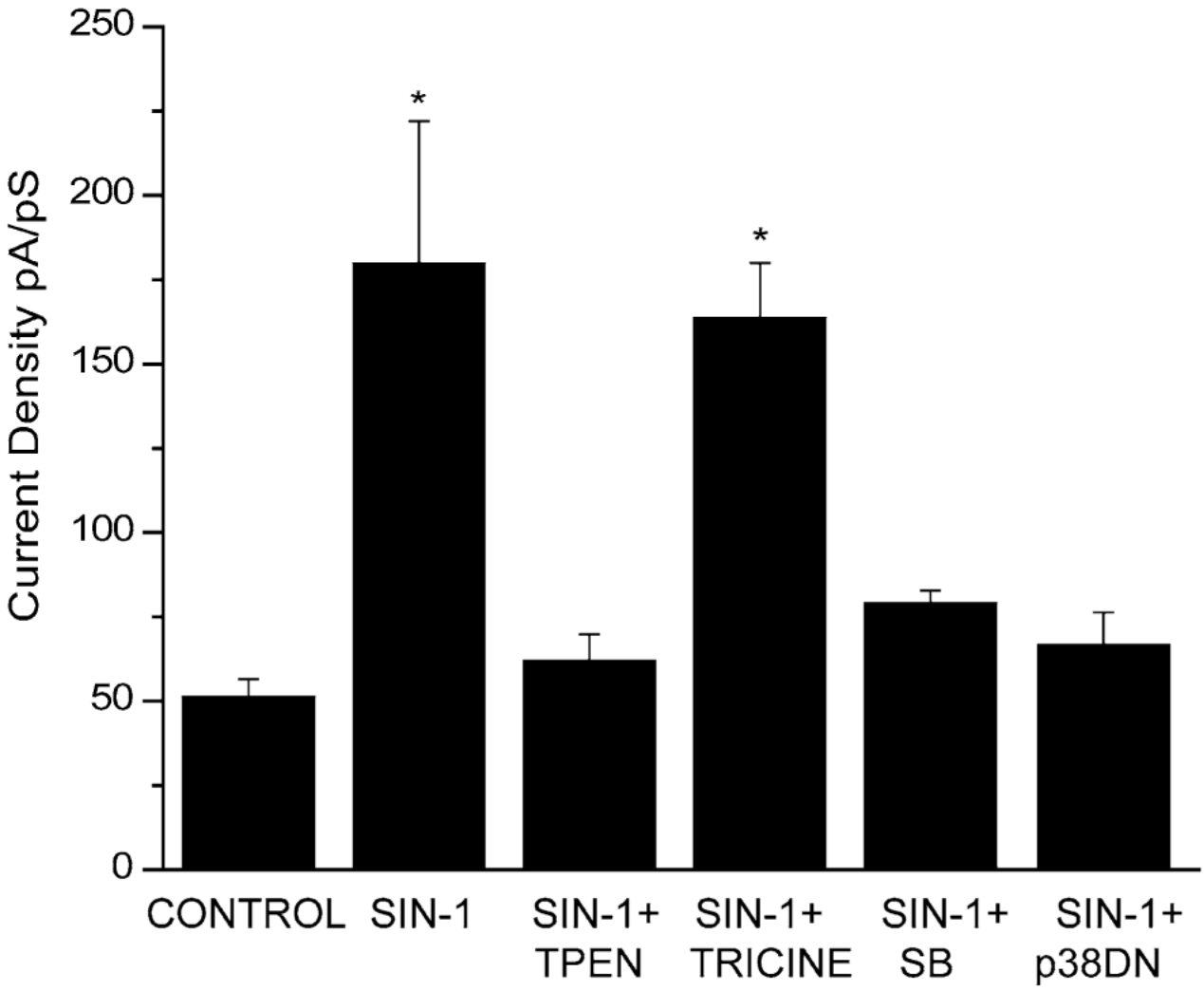

Fig. 3.

Role of Zn^{2+} and p38 and in peroxynitrite-induced current enhancement. Neurons were exposed for 180 min to vehicle (*n*=4), 1 mM SIN-1 (*n*=6), and 1 mM SIN-1 in the presence of the zinc chelators *N*,*N*,*N*',*N*'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN, 10 μM, *n*=6), tricine (10 mM, *n*=6), or the p38 inhibitor SB 239063 (20 μM, *n*=8). Recordings were also made in neurons expressing a p38 dominant-negative construct (*p38DN*). Potassium currents were evoked by voltage steps to +5 mV from a holding potential of −70 mV. Current amplitudes were normalized to cell capacitance. Means±SEM; **P*<0.005 vs. control (ANOVA followed by a Dunnett multiple comparisons test)