Convergent Ca^{2+} and Zn^{2+} signaling regulates apoptotic Kv2.1 K^+ currents

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A simultaneous increase in cytosolic Zn^{2+} and Ca^{2+} accompanies the initiation of neuronal cell death signaling cascades. However, the molecular convergence points of cellular processes activated by these cations are poorly understood. Here, we show that Ca^{2+} -dependent activation of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) is required for a cell death-enabling process previously shown to also depend on Zn^{2+} . We have reported that oxidant-induced intraneuronal Zn^{2+} liberation triggers a syntaxin-dependent incorporation of Kv2.1 voltage-gated potassium channels into the plasma membrane. This channel insertion can be detected as a marked enhancement of delayed rectifier K^+ currents in voltage clamp measurements observed at least 3 h following a short exposure to an apoptogenic stimulus. This current increase is the process responsible for the cytoplasmic loss of K^+ that enables protease and nuclease activation during apoptosis. In the present study, we demonstrate that an oxidative stimulus also promotes intracellular Ca^{2+} release and activation of CaMKII, which, in turn, modulates the ability of syntaxin to interact with Kv2.1. Pharmacological or molecular inhibition of CaMKII prevents the K^+ current enhancement observed following oxidative injury and, importantly, significantly increases neuronal viability. These findings reveal a previously unrecognized cooperative convergence of Ca^{2+} - and Zn^{2+} -mediated injurious signaling pathways, providing a potentially unique target for therapeutic intervention in neurodegenerative conditions associated with oxidative stress.

Calcium has long been recognized as a critical component of neuronal cell death pathways triggered by oxidative, ischemic, and other forms of injury (1). Indeed, Ca^{2+} deregulation has been associated with a variety of detrimental processes in neurons, including mitochondrial dysfunction (2), generation of reactive oxygen species (3), and activation of apoptotic signaling cascades (4). More recently, zinc, a metal crucial for proper cellular functioning (5), has been found to be closely linked to many of the injurious conditions in which Ca^{2+} had been thought to play a prominent role (6–10). In fact, it has been suggested that a number of deleterious properties initially attributed to Ca^{2+} may have significant Zn^{2+} -mediated components (11, 12). Although it is virtually impossible to chelate, or remove, Ca^{2+} without disrupting Zn^{2+} levels (13), the introduction of techniques to monitor Ca^{2+} and Zn^{2+} simultaneously in cells (14) has made it increasingly apparent that both cations have important yet possibly distinct roles in neuronal cell death (12, 15–18). However, the relationship between the cell death signaling pathways activated by the cations is unclear, and possible molecular points of convergence between these signaling cascades have yet to be identified.

Injurious oxidative and nitrosative stimuli lead to the liberation of intracellular Zn^{2+} from metal binding proteins (19). The released Zn^{2+} , in turn, triggers p38 MAPK- and Src-dependent Kv2.1 channel insertion into the plasma membrane, resulting in a prominent increase in delayed rectifier K^+ currents in dying neurons, with no change in activation voltage, ~3 h following a brief exposure to the stimulus (20–26). The increase in Kv2.1 channels present in the membrane mediates a pronounced loss of intracellular K^+ , likely accompanied by Cl^- (27, 28), that facilitates apoptosome assembly and caspase activation (20, 29–34).

Indeed, K^+ efflux appears to be a requisite event for the completion of many apoptotic programs, including oxidant-induced, Zn^{2+} -mediated neuronal death (21).

Ca^{2+} has been suggested to regulate the p38 MAPK signaling cascade via Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII)-mediated activation of the MAP3K apoptosis signaling kinase-1 (ASK-1) (35). Because ASK-1 is also required for p38-dependent manifestation of the Zn^{2+} -triggered, Kv2.1-mediated enhancement of K^+ currents (36), we hypothesized that the p38 activation cascade may provide a point of convergence between Ca^{2+} and Zn^{2+} signals following oxidative injury. Here, we report that Ca^{2+} and Zn^{2+} signals do, in fact, converge on a cellular event critical for the K^+ current enhancement, and that CaMKII is required for this process. However, CaMKII does not act upstream of p38 activation as originally hypothesized, but instead interacts with the *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein syntaxin, which we showed to be necessary for the insertion of Kv2.1-encoded K^+ channels following an apoptotic stimulus (23).

Results

Injurious Oxidant Exposure Leads to CaMKII Activation in Neurons.

Exposure to the thiol oxidant 2,2'-dithiodipyridine (DTDP) liberates intracellular Zn^{2+} and enables neuronal injury via a p38- and Kv2.1-dependent process (19, 21). However, DTDP has also been shown to induce intracellular Ca^{2+} release in cardiomyocytes through cysteine oxidation of sarcoplasmic reticulum ryanodine receptors (37). Thus, the first step in uncovering a possible role for Ca^{2+} in Zn^{2+} -dependent oxidative injury was to determine whether DTDP caused a similar increase in Ca^{2+} in neurons. First, we confirmed the effects of DTDP on intraneuronal Zn^{2+} release by using the specific fluorescent Zn^{2+} indicator FluoZin-3 AM (38). As expected (19), we observed a pronounced increase in fluorescent signal in cultured rat cortical neurons following 30 μM DTDP exposure, which was abolished by concurrent treatment with the Zn^{2+} chelator *N,N,N',N'*-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN; 3 μM). TPEN was also sufficient to decrease basal FluoZin-3 fluorescence, presumably by effectively competing with the fluorescent indicator for the cation (Fig. 1A).

To monitor DTDP-induced changes in intraneuronal Ca^{2+} , we used the fluorescent Ca^{2+} indicator Fura-2 AM. Cortical neurons were exposed to 30 μM DTDP in the presence of 3 μM TPEN to chelate any liberated Zn^{2+} that would otherwise interfere with a measurable Ca^{2+} signal, because Fura-2 also detects free Zn^{2+} (39). Exposure to DTDP plus TPEN led to a significant increase in Fura-2 fluorescence, demonstrating that, similar to cardiomyocytes, the oxidizing agent also induces a Ca^{2+} response in neurons. To identify the source of the released Ca^{2+} , we depleted endoplasmic

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Fig. 1. Oxidant exposure leads to intracellular Zn^{2+} and Ca^{2+} release and CaMKII activation. (A) Neurons loaded with the Zn^{2+} indicator FluoZin-3 AM were exposed to DTDP (30 μ M) with or without TPEN (3 μ M). A subset of cells was exposed to CaMKIIntide (1 μ M) before DTDP treatment. (Left) Representative responses averaged from ~15 cells from a single coverslip. Arrow indicates start of drug application (calibration: 15 absolute units or a.u., 2 min; FluoZin-3 emission measured at 520 nm). (Right) Quantified results, with ΔF equal to the difference between the average baseline fluorescence just before DTDP treatment and the average maximal fluorescence during treatment (mean $\pm$ SEM, $n = 5-7$; $***P < 0.001$, paired t test). (B) Neurons loaded with the Ca^{2+} indicator Fura-2 AM were exposed to DTDP (30 μ M) in the presence of TPEN (3 μ M); some coverslips were pretreated with thapsigargin (TG; 1 μ M, 30 min) to deplete ER Ca^{2+} . (Left) Representative responses averaged from ~15 cells in a single coverslip (calibration: 10 a.u., 2 min; Fura-2 emission measured at 510 nm). (Right) Quantified results ($n = 5$; $*P < 0.05$, paired t test). (C) Representative immunoblot and quantified results from neuronal samples obtained under control conditions or after 10-min exposure to 30 μ M DTDP in the absence or presence of either 3 μ M TPEN or 100 μ M BAPTA-AM. Blots were probed with antibodies specific for p-CaMKII or CaMKII. Data are expressed as a ratio of mean $\pm$ SEM signal of phospho to total CaMKII ($n = 5$; $***P < 0.01$, $*P < 0.05$; ANOVA/Bonferroni). Neither TPEN nor BAPTA-AM alone significantly decreased CaMKII protein levels, when averaged across all experiments. (D) Representative immunoblot and quantified results from neuronal samples obtained under control conditions or after 10-min exposure to 30 μ M DTDP with or without 3 μ M TPEN. Blots were probed with antibodies specific for oxidized M281/282 CaMKII or total CaMKII. Data are expressed as a ratio of mean $\pm$ SEM signal of oxidized to total CaMKII ($n = 3$).

reticulum (ER) Ca^{2+} stores with the ER Ca^{2+} -ATPase inhibitor thapsigargin (TG; 1 μ M) before exposure to DTDP plus TPEN. Under these conditions, exposure to the thiol-oxidizing agent did not generate a change in fluorescence, confirming that the released Ca^{2+} originates from the ER (Fig. 1B). Of note, ryanodine receptors, which as mentioned earlier can be gated by DTDP (37), spatially colocalize with Kv2.1 channel clusters in neurons (40).

To determine whether DTDP-induced Ca^{2+} release led to activation of CaMKII, immunoblotting was performed on protein samples obtained from neuronal cultures treated with 30 μ M DTDP alone or in combination with either 3 μ M TPEN or the acetoxymethyl ester of the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid tetrakis (BAPTA-AM; 100 μ M). DTDP exposure caused a significant increase in T286-phosphorylated (active) CaMKII, which was unaffected by Zn^{2+} chelation but blocked by BAPTA-AM (Fig. 1C). These results indicate that DTDP-liberated Ca^{2+} , but not Zn^{2+} , is responsible for CaMKII phosphorylation. In addition to Ca^{2+} -dependent activation of CaMKII, sustained activation of the kinase can be achieved via the oxidation of its M281/282 residues (41). However, we observed no difference in immunoblots obtained from CaMKII-immunoprecipitated protein samples from vehicle- and DTDP-treated neurons by using an antibody directed toward the oxidized forms of M281/282 (Fig. 1D; 100 μ M H_2O_2 serving as a positive control, Fig. S1). Therefore, it is unlikely that oxidative CaMKII activation appreciably contributes to the signaling cascades triggered by coordinated intracellular release of Ca^{2+} and Zn^{2+} . Lastly, we found that activation of CaMKII, in and of itself, does not influence Zn^{2+} release, because treatment with the myristoylated (myr) inhibitory CaMKII peptide CaMKIIntide (1 μ M) before DTDP exposure had no effect on FluoZin-3 fluorescence (Fig. 1A). Taken together, these results demonstrate that a thiol oxidative insult, previously shown to initiate a Zn^{2+} -mediated cell death pathway in neurons, can also trigger Ca^{2+} -dependent activation of CaMKII.

CaMKII Acts Independently of p38 Activation. ASK-1, an upstream kinase in the p38 MAPK signaling pathway, has been reported to

be activated by CaMKII in a Ca^{2+} -dependent fashion (35). Because Zn^{2+} -triggered activation of p38 also requires ASK-1 (21, 36), we hypothesized that a convergence of Ca^{2+} and Zn^{2+} signaling processes may occur at the level of p38 phosphorylation. Immunoblots were obtained from protein samples of cortical neurons previously exposed to DTDP (30 μ M) alone or in the presence of either myr-CaMKIIntide (1 μ M) or TPEN (3 μ M). As expected (21), TPEN significantly prevented the effect of DTDP on p38 phosphorylation. In contrast, myr-CaMKIIntide was unable to block this effect (Fig. 2A). Thus, although DTDP-triggered Ca^{2+} release leads to CaMKII activation in neurons, the kinase does not appear to be involved in the phosphorylation of p38 following Zn^{2+} liberation.

We next investigated whether activity of the kinase was in fact required for an oxidant-induced increase in K^+ currents. To explore this possibility, we used Chinese hamster ovary (CHO) cells, a recombinant expression system that lacks endogenous voltage-gated K^+ channels (22, 42), yet has all of the cell signaling components required to produce an apoptotic current increase in Kv2.1-transfected cells (22). CHO cells expressing Kv2.1 with or without a kinase inactive CaMKIIK42R mutant were subjected to a 10-min exposure to 30 μ M DTDP followed by 3-h maintenance in butoxy-carbonyl-aspartate-fluoromethyl ketone (BAF; 10 μ M), a broad-spectrum protease inhibitor used during recording for its ability to prevent apoptosis downstream of the K^+ current enhancement (21). We found that the DTDP-induced increase in K^+ currents seen in Kv2.1-expressing CHO cells was absent in cells coexpressing CaMKIIK42R (Fig. 2B), strongly suggesting that CaMKII activity is necessary for this process.

Kv2.1-expressing CHO cells were also used to confirm that the involvement of CaMKII in the K^+ current enhancement occurs independently of p38 activation. Our group has shown that p38 directly phosphorylates Kv2.1 at residue S800, which is required for the apoptotic K^+ current enhancement to commence (24). Moreover, CHO cells expressing a pseudophosphorylated mutant, Kv2.1S800E, exhibit an innate, p38-independent increase in K^+ current densities that mimics the enhanced currents observed

Fig. 2. CaMKII modulates Kv2.1-mediated K^+ currents independently of p38 activation. (A) Zn^{2+} chelation, but not CaMKII inhibition, prevents DTDP-induced p38 activation. Representative immunoblot and quantified results from neuronal samples obtained under control conditions or after 10-min exposure to 30 μ M DTDP in the absence or presence of 1 μ M CaMKIIntide or 3 μ M TPEN. Blots were probed with antibodies specific for p-p38 or p38. Data are expressed as a ratio of mean $\pm$ SEM signal of phospho to total p38 ($n = 7$ for control and $n = 6$ for CaMKIIntide and TPEN groups; * $P < 0.05$; ANOVA/Bonferroni). Note that there was no significant effect of either treatment on basal p38 protein levels across all experiments. (B) Representative whole-cell K^+ currents and pooled mean $\pm$ SEM current densities recorded from Kv2.1-expressing CHO cells treated (10 min) with either vehicle ($n = 15$) or 30 μ M DTDP ($n = 10$), and CHO cells coexpressing Kv2.1 and CaMKI42R treated with vehicle ($n = 9$) or 30 μ M DTDP ($n = 11$). Recordings were performed 3 h following exposure. (C) CaMKI42R blocks the K^+ current enhancement induced by the Kv2.1S800E mutation. Representative whole-cell K^+ currents and corresponding mean $\pm$ SEM current densities recorded from CHO cells expressing either Kv2.1 ($n = 9$), Kv2.1S800E ($n = 26$), or Kv2.1S800E + CaMKI42R ($n = 10$). Currents were evoked by a series voltage steps from -80 mV to $+80$ mV in 10-mV increments. To determine current density values, steady-state current amplitudes were measured 180 ms after the initiation of the $+10$ mV step and normalized to cell capacitance. Representative traces shown were selected because they match the current densities shown on the bar graphs. ** $P < 0.01$; ANOVA/Dunnett. (Scale bars: 5 nA, 25 ms.)

in apoptogen-exposed, wild-type Kv2.1-expressing cells (24). Here, we observed that the increased current densities seen in Kv2.1S800E-expressing CHO cells were absent in cells that also expressed CaMKI42R (Fig. 2C). These observations support our biochemical results obtained in neurons (Fig. 2A) and strongly argue that although CaMKII is necessary for the increase in K^+ currents, its site of action is distinct from the events leading to phosphorylation of Kv2.1 residue S800 by p38.

CaMKII Interacts with Syntaxin. CaMKII has been shown to regulate exocytosis via a specific interaction with syntaxin (43–46). In addition, syntaxin is known to bind to the most proximal region of the Kv2.1 C terminus, termed C1a, during Ca^{2+} -facilitated exocytosis in pancreatic and other nonneuronal cells (47–49). Importantly, we showed that syntaxin is also required for the membrane insertion of Kv2.1 channels during apoptosis (23). Based on these observations, and because CaMKII is also necessary for the K^+ current increase, we hypothesized that an interaction between CaMKII and syntaxin would be detected in our system. Syntaxin immunoprecipitates of protein samples obtained from CHO cells transfected with a syntaxin-expressing plasmid, with or without Kv2.1, were probed for CaMKII. Results revealed that endogenous CaMKII coimmunoprecipitates with syntaxin, and that this interaction is enhanced by Kv2.1 coexpression (Fig. 3A). In these experiments, we also confirmed the described association of syntaxin and Kv2.1 (Fig. 3A) (47–49). The increased CaMKII signal in Kv2.1-expressing cells could be the result of a direct interaction of the kinase with the K^+ channel. Although we detected a measurable, albeit weak, Kv2.1 signal in immunoblots obtained from CaMKII-immunoprecipitated protein samples, this interaction was disrupted in cells overexpressing the C1a region of Kv2.1 (Fig. 3B). As mentioned (48), C1a contains the channel's syntaxin-binding site and can

displace native Kv2.1 from syntaxin. Thus, we suggest that syntaxin provides the link between CaMKII and Kv2.1. Nonetheless, we tested whether CaMKII overexpression in CHO cells could lead to phosphorylation of Kv2.1, because a low-stringency scan of possible phosphorylation sites of the channel sequence (50) revealed up to seven putative serine targets for CaMKII. In contrast to a pronounced shift in the molecular weight of the channel following phosphorylation by cyclin-dependent kinase 5 (CDK5), a known multiple-residue kinase of Kv2.1 (51), CaMKII had no effect (Fig. S2). Furthermore, the specific actions of CDK5 on Kv2.1 residue S603 phosphorylation were confirmed with the use of a phosphospecific antibody (51), again with CaMKII generating no signal (Fig. S2). This result is altogether not surprising, however, given that Ca^{2+} -dependent changes in Kv2.1 phosphorylation status have been primarily linked to calcineurin phosphatase activity and dephosphorylation of the channel (52).

Next, we tested whether phosphorylation of the Kv2.1 residues involved in the apoptotic K^+ current enhancement led to an increased association of the channel and syntaxin. We also investigated whether interfering with CaMKII function disrupted this interaction. In addition to p38-dependent phosphorylation of Kv2.1 S800, an N-terminal Src kinase-targeted tyrosine (Y124) (53) is critical for the expression of the apoptotic current enhancement (25). We have found that overexpression of Src in CHO cells is sufficient to trigger maximal phosphorylation of both Y124 and S800, the latter likely occurring via activation of endogenous p38 (Fig. S3) (52). Here, we found that Src overexpression in CHO cells significantly enhanced the ability of syntaxin to bind Kv2.1, and inclusion of the kinase inactive CaMKI42R mutant completely abolished this effect (Fig. 3C). These results indicate that changes in the phosphorylation profile of Kv2.1 that normally lead to the apoptotic K^+ current

Fig. 3. CaMKII regulates Kv2.1/syntaxin binding. (A) Coimmunoprecipitation of CaMKII and syntaxin is enhanced in the presence of Kv2.1. Representative coimmunoprecipitation and quantification of blots derived from CHO cells transiently expressing syntaxin with or without Kv2.1. Following syntaxin immunoprecipitation, blots were probed with antibodies against Kv2.1, CaMKII, or syntaxin (mean $\pm$ SEM, $n = 4$; * $P < 0.05$; paired t test). (B) Coimmunoprecipitation of Kv2.1 and CaMKII is disrupted by overexpression of Kv2.1 C1a region, which contains the syntaxin binding site. Representative coimmunoprecipitation and quantified results from CHO cells transfected with Kv2.1 or Kv2.1 + C1a. Following CaMKII immunoprecipitation, blots were probed with antibodies against Kv2.1 or CaMKII ($n = 4$; * $P < 0.05$; paired t test). (C) CaMKII inhibition prevents the increase in Src-mediated Kv2.1/syntaxin binding. Representative coimmunoprecipitation and quantified results of protein samples from syntaxin-expressing CHO cells cotransfected with either Kv2.1, Kv2.1 + Src, Kv2.1 + CaMKIIK42R, or Kv2.1 + CaMKIIK42R + Src. Syntaxin was immunoprecipitated and blots were probed with antibodies against Kv2.1 or syntaxin ($n = 5$; * $P < 0.05$; paired t test).

enhancement are accompanied by an increased interaction of the channel with syntaxin, and that a functional CaMKII is necessary for this process.

CaMKII Is Required for Oxidant-Induced K^+ Current Enhancement and Cell Death in Neurons. Lastly, we evaluated whether disruption of CaMKII activity could prevent the endogenous apoptotic K^+ current increase and be neuroprotective in oxidant-exposed cortical neurons. We observed that both myr-CaMKIINtide (1 μ M) and transfection with CaMKIIK42R blocked the increased K^+ current densities normally seen in neurons 3 h after DTDP exposure (30 μ M, 10 min; Fig. 4*A* and *B*). In a separate set of studies, we confirmed that the Zn^{2+} chelator TPEN (3 μ M) also effectively inhibited the increase in K^+ currents induced by DTDP exposure (Fig. S4). Next, to determine whether inhibition of CaMKII was neuroprotective, we transfected neurons with a plasmid encoding CaMKIIK42R (or empty vector) in addition to a luciferase-expressing plasmid used to assess viability in transfected cells (54). Neurons were later exposed to activated rat microglia (55), which we showed to be a powerful inducer of Zn^{2+} -dependent, Kv2.1-mediated injury via peroxynitrite production (56). We observed that neurons expressing CaMKIIK42R were significantly protected from microglia toxicity compared with vector-expressing cells (Fig. 4*C*). Moreover, neurons expressing this mutant were also protected from DTDP toxicity (60 μ M, 10 min; Fig. S5). From these data, we conclude that a CaMKII activity is necessary for both the K^+ current enhancement and subsequent lethal injury in neurons.

Discussion

Defining the specific contributions of Ca^{2+} and Zn^{2+} to neuronal injury pathways has been hindered by the fact that most, if not all, Ca^{2+} -binding agents and indicators also effectively interact with Zn^{2+} (11, 13, 39, 57). Although Zn^{2+} probes that do not appreciably interfere with Ca^{2+} -mediated signals exist (58–60), and simultaneous changes in Ca^{2+} and Zn^{2+} have been detected in injured neurons (15–18), identifying the specific and separate downstream signaling cascades activated by each cation has remained elusive. Here, the demonstration that intraneuronal Ca^{2+} rises independently of Zn^{2+} following an oxidative stimulus, and

that Ca^{2+} -dependent CaMKII activity is necessary for the syntaxin-mediated regulation of apoptotic Kv2.1-mediated K^+ currents, provides a glimpse into the molecular underpinning of what are likely to be widespread dual cation regulatory processes in cells.

CaMKII has been implicated in neuronal cell death processes, because inhibition of this kinase is neuroprotective in both in vitro and in vivo injury models (61–64). Nonetheless, as is the case for many other signaling kinase systems, CaMKII is associated with the regulation of both neuroprotective and neurodestructive cellular signaling pathways (65, 66). As such, inhibiting kinase activity is likely to be a poor candidate approach for translational therapeutic studies. We show here, however, that CaMKII activity is necessary for the syntaxin-dependent, apoptosis-enabling enhancement of Kv2.1-mediated K^+ channel activity following oxidative injury. This process is likely mediated via the direct interaction of CaMKII with syntaxin, a mechanism that may be amenable to manipulation as a novel therapeutic strategy.

Exactly how CaMKII participates in the syntaxin-dependent, apoptotic exocytotic insertion of Kv2.1 channels into the plasma membrane has yet to be resolved. During exocytosis, syntaxin transitions from a closed to an open conformation, which can only occur after the linker domain of the protein is released from its SNARE-binding H3 domain (67, 68). Interestingly, not only has CaMKII been shown to bind to the linker domain of syntaxin during neuronal exocytosis, but it is also the only known protein to bind to this region (43). Based on our coimmunoprecipitation results, we suggest that the enhanced binding of syntaxin to the dual-phosphorylated Kv2.1 is facilitated by the direct interaction of functional CaMKII with syntaxin. Moreover, this process may be further enabled by the recruitment of additional CaMKII to the Kv2.1/syntaxin complex, as our results also suggest.

Most Kv2.1 channels are typically found in clusters on the somatodendritic plasma membrane (69). Although the clustered channels may conduct current poorly (70), these aggregates appear to be the site of surface delivery of additional Kv2.1 protein (71). Therefore, it will be important to establish in future work whether stimuli that affect channel clustering (72, 73) can regulate the SNARE-dependent insertion of new channels during apoptosis, as was evaluated for a stromal cell-derived chemokine (26). To aid in the resolution of these issues, the results

Fig. 4. CaMKII inhibition blocks apoptotic K^+ current increase and subsequent cell death in cortical neurons. (A) Representative whole-cell K^+ currents and corresponding mean $\pm$ SEM current densities recorded under control conditions ($n = 15$) or 3 h after exposure (10 min) to 30 μ M DTPD ($n = 13$), 1 μ M CaMKIIntide ($n = 12$), or 30 μ M DTPD + 1 μ M CaMKIIntide ($n = 14$). (B) Representative whole-cell K^+ currents and corresponding mean $\pm$ SEM current densities recorded from untransfected cells without ($n = 7$) or with 30 μ M DTPD ($n = 8$), and CaMKIIK42R-expressing cells without ($n = 10$) or with 30 μ M DTPD ($n = 9$). Currents were evoked by a series of voltage steps from -80 mV to $+80$ mV in 10-mV increments. To determine current density values, steady-state current amplitudes were measured 180 ms after the initiation of the $+10$ mV step and normalized to cell capacitance. $**P < 0.01$; ANOVA/Dunnett. (Scale bars: 10 nA, 25 ms.) (C) CaMKIIK42R-expressing neurons are resistant to an overnight exposure to activated microglia (AMG) compared with vector-expressing cells. Kainic acid (KA; 1 mM overnight) was used as an index for nonapoptotic, total neuronal kill (mean $\pm$ SEM of a single representative experiment performed in quadruplicate). $**P < 0.01$, $***P < 0.001$, compared with untreated cells from each group; ANOVA/Bonferroni. (Inset) Percent toxicity from either AMG or KA exposure for vector-expressing (green) or CaMKIIK42R-expressing (blue) neurons (mean $\pm$ SEM, $n = 7$; $**P < 0.01$; paired t test). (D) Pathway illustrating the convergence of Zn^{2+} - and Ca^{2+} -mediated signaling pathways during oxidative neuronal injury. Oxidant exposure leads to simultaneous release of Ca^{2+} and Zn^{2+} , with consequent Ca^{2+} -dependent activation of CaMKII, and Zn^{2+} -dependent phosphorylation of p38 via a 12-lipoxygenase (12-LOX)-associated activation of ASK-1 (74, 75). These parallel pathways then converge to facilitate the association of syntaxin with Kv2.1.

presented in this study establish Ca^{2+} and CaMKII as critical regulators of the injurious, Kv2.1-dependent K^+ current increase in neurons. Importantly, we also identify an unprecedented co-operative convergence of Zn^{2+} - and Ca^{2+} -mediated signaling pathways during oxidative neuronal cell death.

Materials and Methods

Details on all procedures used in this study can be found in the *SI Materials and Methods*. Methods described include cell culture procedures, imaging and electrophysiological measurements, as well as biochemical assays. The

University of Pittsburgh's Institutional Animal Care and Use Committee reviewed and approved the animal protocol utilized in this study.

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