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Apoptotic surface delivery of K^+ channels

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Abstract

Apoptosis in cortical neurons requires efflux of cytoplasmic potassium mediated by a surge in Kv2.1 channel activity. Pharmacological blockade or molecular disruption of these channels in neurons prevents apoptotic cell death, while ectopic expression of Kv2.1 channels promotes apoptosis in non-neuronal cells. Here, we use a cysteine-containing mutant of Kv2.1 and a thiol-reactive covalent inhibitor to demonstrate that the increase in K^+ current during apoptosis is due to *de novo* insertion of functional channels into the plasma membrane. Biotinylation experiments confirmed the delivery of additional Kv2.1 protein to the cell surface following an apoptotic stimulus. Finally, expression of botulinum neurotoxins that cleave syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) blocked upregulation of surface Kv2.1 channels in cortical neurons, suggesting that target soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins support proapoptotic delivery of K^+ channels. These data indicate that trafficking of Kv2.1 channels to the plasma membrane causes the apoptotic surge in K^+ current.
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Abbreviations: Kv, voltage-dependent potassium channel; DTDP, 2,2′-dithiodipyridine; CHO, Chinese hamster ovary; MTSET, (2-trimethylammoniumethyl) methanethiosulfate; DTT, dithiothreitol; GAPDH, glyceraldehyde phosphate dehydrogenase; BoNT, botulinum neurotoxin; t-SNARE, target soluble N-ethylmaleimide-sensitive factor attachment protein receptor; SNAP-25, synaptosome-associated protein of 25 kilodaltons; MAPK, mitogen-activated protein kinase; BAF, butoxy-carbonyl-aspartate-fluoromethyl ketone

Introduction

Cellular K^+ efflux is a critical and requisite step for the completion of the apoptotic program of numerous cell types following a broad range of apoptosis-inducing stimuli.[1,2] In cortical neurons, a delayed surge in voltage-gated potassium channel (Kv)-mediated electrical currents provides the likely exit route for this cation.[3] The potassium current enhancement, which is protein synthesis independent and precedes caspase activation,[4] can be triggered by classical apoptotic stimuli like staurosporine,[3] serum withdrawal,[3] and amyloid β,[5] or by oxidants such as 2,2′-dithiodipyridine (DTDP)[4,6] and peroxynitrite.[7,8] In oxidant-induced neuronal apoptosis, the enhancement of K^+ currents is dependent upon the liberation of intracellular Zn^{2+} from metal-containing proteins[6,7] and requires the activation of the mitogen-activated protein kinase (MAPK) pathway involving apoptosis signal-regulating kinase-1[8] and p38.[9]

We previously demonstrated that Kv2.1-encoded potassium channels mediate the enhanced K^+ currents observed in cortical neuron apoptosis.[10] Kv2.1 belongs to a family of voltage-activated potassium channels that is normally responsible for repolarizing the membrane potential of excitable tissue during electrical activity, thereby regulating cell firing. Dominant negative constructs of Kv2.1 not only prevent the current surge following an apoptotic stimulus in neurons, but are also neuroprotective.[10] Likewise, a novel high-affinity Kv2.1 blocker prevents oxidant-induced neuronal apoptosis.[11] Nonetheless, the molecular mechanism underlying the apoptotic increase in channel activity remains unknown. In the present study, we tested the hypothesis that *de novo* membrane insertion of Kv2.1-encoded channels, rather than a modification of previously resident surface channels, is responsible for the pronounced current surge observed during oxidant-induced apoptosis.

Results

Covalent modification of cysteine mutant Kv2.1 demonstrates membrane insertion of new channels following an apoptotic stimulus in CHO cells

We first investigated the apoptotic delivery of channels to the cell surface with Chinese Hamster Ovary (CHO) cells, which do not have any endogenous voltage-dependent K^+ channels.[12] These cells are rendered highly susceptible to dithiodipyridine (DTDP)-induced apoptosis following expression of Kv2.1, but not other potassium channels.[10] As in neuronal models,[3,4] we observed that Kv2.1-expressing CHO cells undergoing apoptosis have a similar surge in channel activity. Currents were measured with standard patch clamp electrodes after evoking them with a series of depolarizing membrane potential steps. As the cells can be of different sizes, current amplitudes were normalized to cell capacitance, which is an electrical measurement of membrane surface area. As shown in Figure 1a, Kv2.1-mediated current

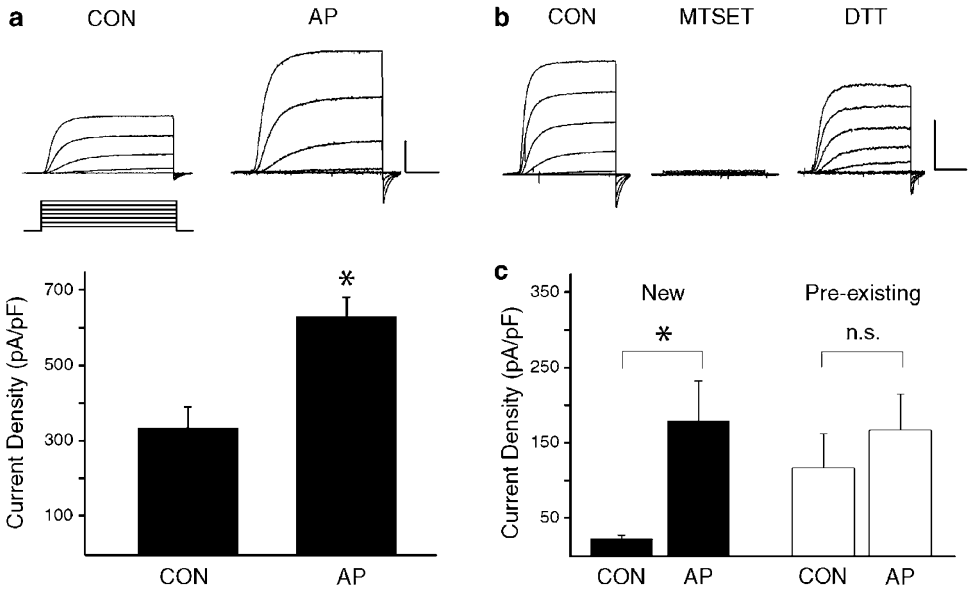

Figure 1 Apoptotic surface delivery of cysteine-mutant Kv2.1 channels in CHO cells. (**a**) Top: whole-cell K^+ currents from a Kv2.1-expressing control (CON) CHO cell or a cell triggered to undergo apoptosis (AP) by 25 μM DTDP (5 min) in the presence of the caspase inhibitor butoxy-carbonyl-aspartate-fluoromethyl ketone (BAF; 10 μM). Currents were evoked approximately 3 h after treatment by a series of voltage steps to $+35$ mV from a holding potential of -70 mV. Voltage steps are illustrated below the current traces. Scale bars: 5 nA, 20 ms. Bottom: mean $\pm$ S.E.M. current densities from control (CON) and apoptotic (AP) cells expressing Kv2.1. Current amplitudes were evoked with a voltage step to $+5$ mV from a holding potential of -70 mV and normalized to cell capacitance. ($n=19$; $^*P<0.05$). (**b**) Representative whole-cell recordings from a CHO cell expressing Kv2.1(I379C) before (left), after 5 min of perfusion with MTSET (middle; 4 mM), and after MTSET block was reversed with DTT (right; 1 mM). Currents were evoked by a series of voltage steps to $+35$ mV from a holding potential of –70 mV. Scale bars: 5 nA, 25 ms. (**c**) Left: mean $\pm$ S.E.M. current densities from CON and AP CHO cells expressing KV2.1(I379C) 3 h after initially silencing channels with MTSET (4 mM, 10 min). These currents represent the insertion of new channels on the cell surface. Current amplitudes were evoked with a voltage step to $+5$ mV from a holding potential of -70 mV and normalized to cell capacitance. Right: mean $\pm$ S.E.M. current densities of silenced channels in CON and AP CHO cells revealed by the change in current induced by reversal of MTSET block with DTT. Currents were evoked with a voltage step to $+5$ mV from -70 mV and represent the pre-existing channels; values were determined by subtracting the pre-DTT current amplitudes from the total, post-DTT responses ($n=9$; $^*P<0.05$)

densities were significantly enhanced in CHO cells undergoing apoptosis, when compared to vehicle-treated cells. This result, taken with our previous finding that this channel can promote apoptosis in these cells,[10] suggests that Kv2.1 closely interacts with cell death pathways even in cell types that normally do not express this channel.

Apoptosis-induced current enhancement was also seen in CHO cells after the expression of a Kv2.1(I379C) mutant channel (data not shown, but see below). The advantage of using this cysteine mutant is that the currents mediated by this channel can be covalently blocked by the thiol reagent (2-trimethylammoniumethyl) methanethiosulfate (MTSET),[13] with the block reversed by the introduction of dithiothreitol (DTT; Figure 1b). DTT reverses the block by reducing and thereby breaking the disulfide bond between MTSET and the engineered cysteine in the channel. The reversible silencing of pre-existing surface Kv2.1(I379C) channels by MTSET allowed us to address whether the upregulation in K^+ channel activity required for apoptosis was due to the insertion of new Kv2.1 channels or a modification of existing ones. CHO cells expressing Kv2.1(I379C) were treated with MTSET to block the pre-existing surface channels prior to induction of apoptosis with DTDP. At 3 h after DTDP treatment, the time it normally takes to observe a substantial current surge,[4] whole-cell recordings were performed. Vehicle-treated cells had very small currents, showing that few nascent channels arrived (Figure 1c, left). We reasoned that if apoptosis modified the gating of channels already in the plasma membrane, then this upregulation of channel activity would not be evident because these channels would still be covalently blocked by MTSET. However, DTDP-treated cells had large currents (Figure 1c, left), suggesting that new channels naïve to the MTSET block had appeared on the surface of cells undergoing apoptosis. All the cells were then treated with DTT to unmask the previously MTSET-silenced channels. We then digitally subtracted the pre-DTT current amplitudes from the post-DTT amplitudes to reveal the magnitude of the pre-existing Kv2.1-encoded currents. The current density of this pre-existing channel population (Figure 1c, right) was not significantly different in control and apoptotic cells, showing that MTSET-induced block remains stable and no change in the functionality of these channels occurred. These data suggest that the apoptotic increase in K^+ current in CHO cells arises from the insertion of new functional channels on the cell surface.

Biotinylation confirms membrane insertion of new Kv2.1 protein during apoptosis

A biochemical method was used to confirm that Kv2.1 protein increased in the plasma membrane in CHO cells undergoing apoptosis. Surface proteins in vehicle and DTDP-treated CHO cells expressing a poly myc-tagged wild-type Kv2.1

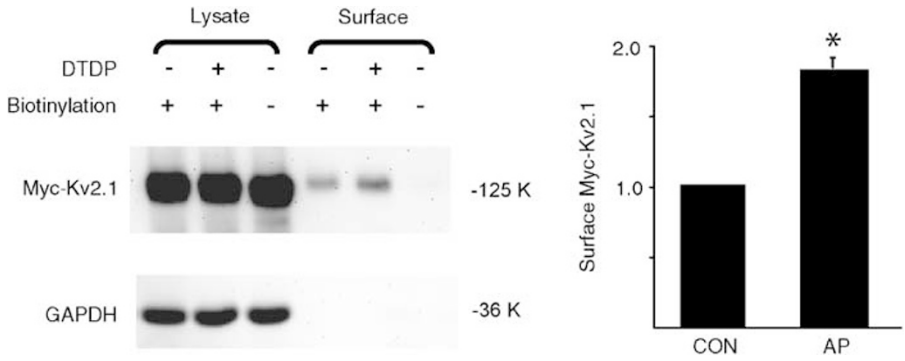

Figure 2 Surface biotinylation assay reveals the increase in plasma membrane Kv2.1 protein with apoptosis. Left: 3 h after treating CHO cells expressing Myc-Kv2.1 with vehicle or the apoptogen DTDP, cells were incubated with sulfo-NHS-SS-biotin and solubilized. Biotinylated proteins were isolated with immobilized neutravidin. Immunoblotting with anti-Myc and GAPDH antibodies were used to analyze protein abundance in the total lysate and isolated surface fractions. Right: mean ± S.E.M. of the normalized ratio of surface Kv2.1 to total Kv2.1 ($n = 3$; $^*P < 0.05$)

(myc-Kv2.1[14]) were isolated following biotinylation and assayed by immunoblot. Consistent with our electrophysiological data, induction of apoptosis nearly doubled the biotinylated fraction of myc-Kv2.1 without any change in total channel protein (Figure 2). Cytoplasmic glyceraldehyde phosphate dehydrogenase (GAPDH) was not biotinylated, showing that only surface proteins were labeled. Thus, induction of apoptosis increases wild-type Kv2.1 protein in the cell surface, in accordance with our functional studies using Kv2.1(I379C).

Increased channel surface expression in cortical neurons during apoptosis

We next evaluated whether channel trafficking could be observed in cortical neurons, in which the apoptotic surge in delayed rectifier current was originally discovered. For these studies, cerebrocortical cultures were transfected with Kv2.1(I379C). Initially, we confirmed that MTSET only inhibited channels in neurons expressing Kv2.1(I379C) and the thiol agent was inert against endogenous delayed-rectifier currents (Figure 3). As can be seen in this figure, potassium currents in cells not expressing Kv2.1(I379C) were insensitive to MTSET. In contrast, Kv2.1(I379C)-expressing neurons were partially sensitive to MTSET, likely reflecting the fraction of functional mutant channels on the cell surface. Kv2.1(I379C)-transfected cultures were then treated with MTSET prior to the induction of apoptosis with DTDP. After 3-h, electrophysiological measurements were obtained before and after a second MTSET treatment. Vehicle-treated neurons did not respond to the second MTSET challenge, although the existence of pre-existing MTSET-blocked channels could be revealed with DTT (Figure 4a, top; light gray trace reveals the previously MTSET-blocked channels revealed by DTT treatment). This demonstrates that the original population of MTSET-sensitive channels remained covalently modified and virtually no new MTSET-sensitive channels appeared in vehicle-treated cells. In contrast, neurons undergoing apoptosis had present on their surface a novel population of Kv2.1(I379C) channels that had not been blocked by the initial MTSET exposure, but that were

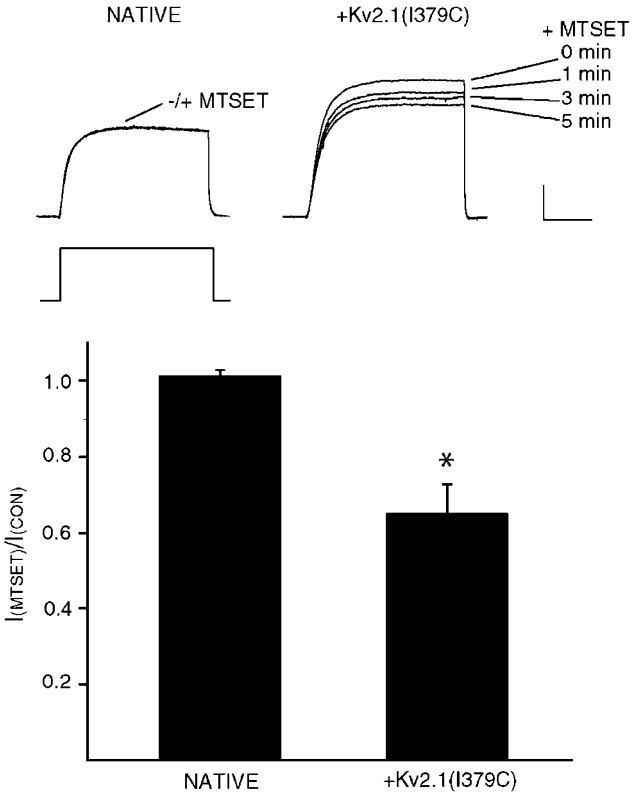

Figure 3 Functional expression of Kv2.1(I379C) in cortical neurons. Top: whole-cell K^+ currents from untransfected cortical neurons (left; native) or Kv2.1(I379C)-expressing neurons before, during and after a 5 min incubation with 4 mM MTSET. In the untransfected neuron, only the pre-treatment and 5 min MTSET treatment currents are show, which are virtually indistinguishable. Currents were evoked with a voltage step to + 35 mV from a holding potential of −70 mV, as illustrated below the trace. Scale bars: 2.5 nA, 20 ms. Bottom: MTSET produces a functional block in Kv2.1(I379C)-expressing cells ($n = 5$), without affecting the behavior of native channels ($n = 3$); $^*P < 0.005$. Currents were evoked with a voltage step to + 5 mV from a holding potential of −70 mV

sensitive to the second MTSET treatment (Figure 4a, bottom; Figure 4b). The MTSET-sensitive component was a delayed-rectifier current (Figure 4a, insert; this was obtained by digitally subtracting the second trace from the first one). These

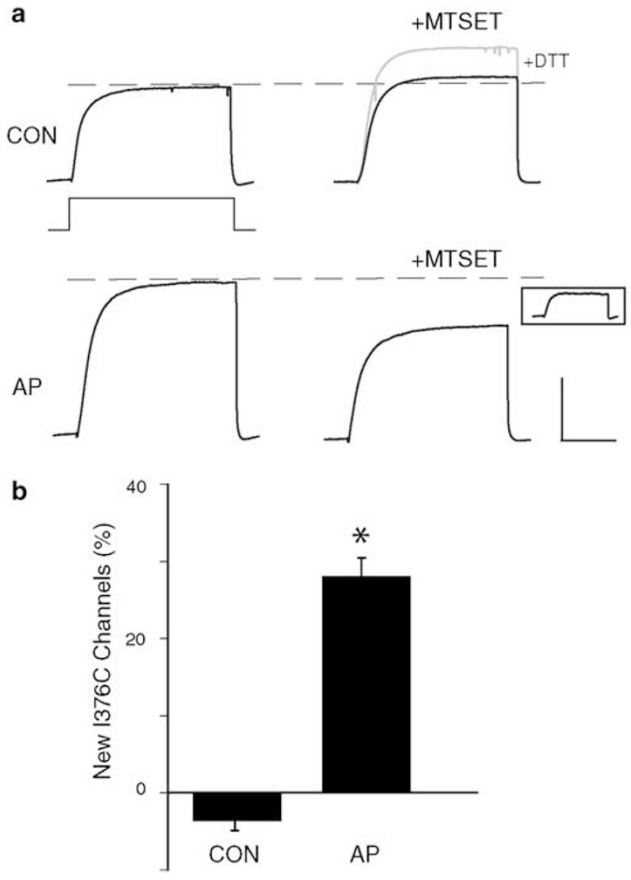

Figure 4 Surface delivery of Kv2.1(I376C) in cortical neurons during apoptosis. (**a**) Whole-cell K^+ currents from Kv2.1(I379C)-expressing control (CON) cortical neurons or neurons triggered to undergo apoptosis (AP) by 100 μM DTDP (10 min). Pre-existing surface Kv2.1(I379C) channels had been silenced with an initial MTSET treatment. Traces shown are single voltage steps from -75 to $+35$ mV before (left) and after 5 min of perfusion with a second MTSET treatment (right) 3 h after DTDP exposure. Voltage step is illustrated below the left current trace. Control cells were exposed to DTT following the second MTSET exposure to verify the presence of channels silenced by the first MTSET treatment. Scale bars: 5 nA, 25 ms. (**b**) Percent of new Kv2.1(I379C) channels present in CON and AP neurons, calculated from the ratio of current density before and after the second MTSET exposure ($n = 8$; $^*P < 0.05$)

data demonstrate that apoptotic injury induces *de novo* membrane insertion of Kv2.1(I379C) channels in cortical neurons.

Insertion of endogenous Kv2.1 channels in neurons requires functional t-SNARE proteins

Finally, we tested whether the disruption of exocytotic target soluble N-ethylmaleimide-sensitive factor attachment protein receptor (t-SNARE) proteins can prevent the apoptotic K^+ current surge. Neurons were transfected with plasmids encoding either botulinum neurotoxin (BoNT) C1 or E light chains together with a GFP-expressing plasmid. These toxins cleave the neuronal t-SNAREs exocytotic proteins syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25), respectively. In both cases, cells expressing the BoNTs showed no increase in K^+ currents following induction of apoptosis with DTDP. In contrast, the DTDP-induced current surge was readily measured in untransfected (i.e. GFP negative) neurons obtained from the same coverslips (Figure 5a–c), showing that the apoptotic stimulus had been effective. Prior experiments have shown that expression of GFP alone does not affect the apoptotic response.[9] Therefore, these findings indicate that the same t-SNARE proteins that support exocytotic transmitter release at nerve terminals mediate proapoptotic trafficking of native, endogenous neuronal K^+ channels.

Discussion

Apoptotic signaling pathways in several model systems converge upon a nearly ubiquitous requirement for cellular K^+ loss.[1,2] Although the mechanism underlying apoptotic K^+ efflux clearly depends on the repertoire of K^+ channels and pumps that are expressed in a given cell type,[2] Kv2.1 channels represent the principal, if not the sole, mediator of the enhanced potassium currents required for apoptosis in cortical neurons.[10] The experiments described here demonstrate that the apoptotic enhancement of Kv2.1 activity is due to a *de novo* insertion of these channels and not a modification of previous membrane-resident channels. We demonstrated this by utilizing a cysteine-containing mutant of Kv2.1 that can be covalently, but reversibly, blocked by a thiol-active agent.[13] This allowed us to silence channels residing in the plasma membrane prior to the apoptotic stimulus and detect the appearance of newly inserted channels. Control experiments demonstrated that the normal turnover of Kv2.1 channels in both CHO cells and neurons is very slow, compared to the apoptotic delivery of Kv2.1 channels. Furthermore, surface biotinylation verified that additional Kv2.1 protein was inserted into the plasma membrane following induction of apoptosis. Our findings thus represent the first demonstration that the apoptotic surge in K^+ current is a direct result of channel trafficking to the plasma membrane.

Our data strongly suggest that t-SNAREs are critical for the apoptotic insertion of endogenous Kv2.1 channels in apoptotic cortical neurons. To implicate neuronal t-SNAREs in apoptotic trafficking of neuronal Kv2.1 channels, we induced neuronal expression of botulinum neurotoxin light chains C1 and E. Each of these t-SNARE-specific proteases independently inhibited the apoptotic surge in neuronal delayed rectifier, suggesting a specific role for syntaxin and SNAP-25. Previous work had shown that these t-SNAREs can bind to and regulate the gating of Kv2.1.[15–18] However, since the gating of the delayed rectifier does not change with apoptosis,[2] the neurotoxin results, in concert with our trafficking experiments, are most consistent with the requirement of t-SNARE proteins for the membrane fusion required for insertion of new channels into the cell surface. This conclusion also is in accordance with the observations that overexpression of syntaxin in cell lines inhibits exocytosis[19] and Kv2.1 surface expression.[16] Of interest, t-SNARE proteins have also been implicated in the trafficking and/or surface expression of other plasma membrane channels,

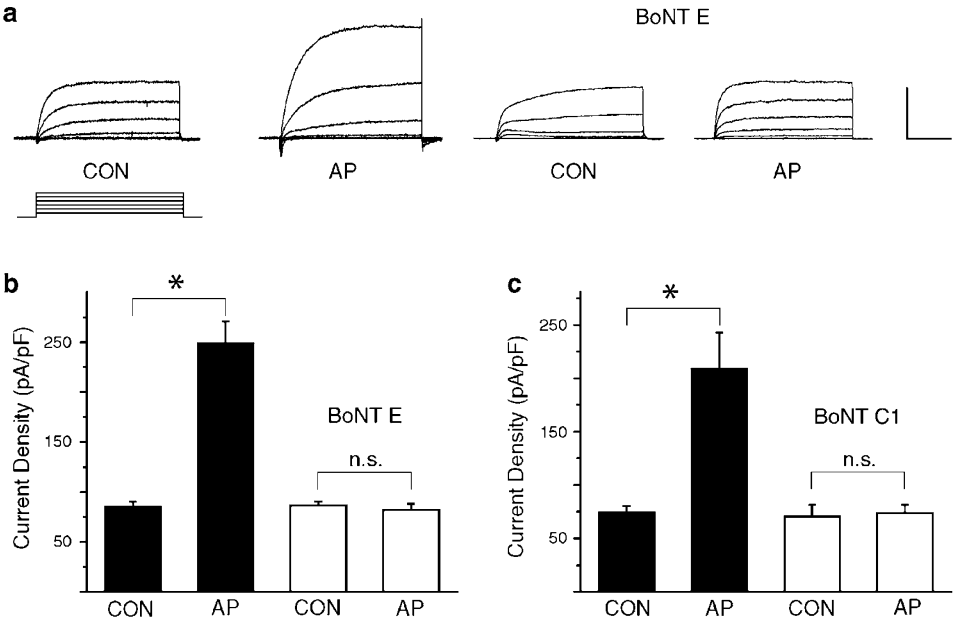

Figure 5 Botulinum neurotoxin-sensitive surface delivery of Kv2.1 channels after apoptotic injury in cortical neurons. **(a)** Neurons were co-transfected with the light-chain of Botulinum neurotoxin E and GFP. Traces represent whole-cell recordings obtained from GFP negative and GFP-positive neurons in a coverslip containing botulinum neurotoxin expressing cells that had been treated with either vehicle (Con) or 100 μM DTDP (AP) for 10 min to initiate apoptosis, approximately 3 h earlier. Scale bars: 5 nA, 25 ms. Currents were evoked with a series of voltage step to $+20$ mV from a holding potential of -70 mV and normalized to cell capacitance. Voltage steps are illustrated below the control cell. (**b**) Mean $\pm$ S.E.M. current densities experiments similar to that described above ($n=18$; $^*P<0.05$). (**c**) Summary of the identical experiment, but in neurons cotransfected with the light-chain of botulinum toxin C1 and GFP ($n=20$; $^*P<0.05$)

albeit all of them being ligand-gated ion channels.[20–23] Our results indicate that the same t-SNARE proteins that mediate neurotransmission at nerve terminals support exocytotic delivery of somatodendritic K^+ channels during neuronal apoptosis.

The location of the available pool of Kv2.1 for membrane insertion during apoptosis is not yet known. Immuno-electron microscopy has shown that endogenous Kv2.1 protein is present in intracellular membranes closely apposed to somatodendritic cell surface channel clusters.[24,25] Furthermore, a recent study utilizing fluorescence recovery after photobleaching and photoactivation[26] suggested that Kv2.1 channels move between intracellular compartments and the cell surface. Furthermore, a rapid and transient declustering of surface Kv2.1 channels has been described under certain pathophysiological conditions that are not necessarily apoptotic in nature (i.e. kainate-induced seizures *in vivo* and brief glutamate exposures *in vitro*).[27] This channel declustering appears to be correlated to calcium-dependent dephosphorylation of the channel and does not seem to affect surface expression. As the apoptotic current surge requires activation of the MAPK p38 pathway,[4] we believe that an initial phosphorylation event is responsible for the upregulation of Kv2.1 surface expression during apoptosis. Thus, it is unclear whether dispersion of surface clusters precedes or is related in any way to the apoptotic trafficking described in the present study. Nonetheless, the dynamic nature of Kv2.1 trafficking and declustering following injurious stimuli and the critical association of Kv2.1 with various apoptotic pathways places a unique role for this channel in the regulation of neuronal cell survival.

Materials and Methods

Tissue culture

Chinese hamster ovary (CHO) cells were seeded at 2.8×10^5 cells per well into six-well plates 24 h before transfection. Cells were transfected with either a Kv2.1 or a Kv2.1(I379C)-containing mammalian expression vector together with an enhanced green fluorescent protein expressing plasmid (pCMVIE-eGFP; Clontech, Palo Alto, CA, USA) at a 1 : 1 ratio in serum-free medium with 6 μl of Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA), and a total of 1.4 μg of DNA per well. Electrophysiological recordings from eGFP-positive CHO cells were performed approximately 24 h after transfection. Cortical cultures were prepared from embryonic day 16 Sprague–Dawley rats and grown on 12 mm cover glasses as described.[10] Pregnant rats were euthanized with CO_2 with the approval of the University of Pittsburgh School of Medicine and in accordance with National Institutes of Health protocols. Enhanced GFP was used as a marker for positively transfected neurons and was combined with the Kv2.1(I379C) expression vector or with plasmids encoding the BoNT light chain toxins E and C1 at a 1 : 1 ratio. Cortical cultures were transfected at 19–23 days *in vitro* using Lipofectamine 2000 (Invitrogen).[10] Briefly, 1.5 μg of cDNA was diluted in 50 μl Opti-Mem I medium and combined with 50 μl of Opti-Mem I medium containing 2 μl Lipofectamine 2000. Complexes were allowed to form for 30 min before addition to the cultures. Cortical cells were maintained for 48 h at 37°C 5% CO_2 before electrophysiological recordings.

Electrophysiological measurements

Recordings were performed using the whole-cell configuration of the patch-clamp technique as described earlier.[10] The extracellular solution

contained (in mM): 115 NaCl, 2.5 KCl, 2.0 $MgCl_2$, 10 HEPES, 10 D-glucose; pH was adjusted to 7.2 with concentrated KOH. Tetrodotoxin (0.25 μM) was added to inhibit voltage gated sodium channels. The electrode recording solution contained (in mM): 100 K-gluconate, 11 EGTA, 10 KCl, 1 $MgCl_2$, 1 $CaCl_2 \times 2H_2O$, 10 HEPES; pH was adjusted to 7.2 with concentrated KOH; 0.22 mM ATP was added and osmolarity was adjusted to 280 mOsm with sucrose. All measurements were obtained under voltage clamp with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA, USA) and pClamp software (Axon Instruments) using 2–3 MOhm electrodes. Partial compensation (80%) for series resistance was performed in all instances. Potassium currents, filtered at 2 kHz and digitized at 10 kHz (Digidata; Axon Instruments), were evoked with a series of 80 ms voltage steps from a holding potential of -70 mV to 20 or 35 mV, in 15 mV increments. Steady-state maximum amplitudes were measured relative to baseline and normalized to cell capacitance.

Induction of apoptosis

Kv2.1-expressing CHO cells were cotreated with DTDP (25 μM) and butoxy-carbonyl-aspartate-fluoromethyl ketone (BAF, 10 μM), a broad-spectrum cysteine protease inhibitor, for 5 min in 37°C 5% CO_2. Subsequently, CHO cells were maintained in BAF (10 μM) for 3 h. We have previously reported that BAF prevents Kv2.1-expressing CHO cells from dying following DTDP exposure,[10] and have found the caspase inhibitor necessary to maintain CHO cells sufficiently healthy for successful electrophysiological recordings.[9] Cortical neurons were treated with DTDP (100 μM) for 10 min at 37°C 5% CO_2, a condition that induces a well-characterized p38-dependent increase in K^+ currents approximately 3 h later, followed by caspase activation at 5–7 h and subsequent cell death.[4] All data are expressed as mean $\pm$ S.E.M. Current densities were analyzed using two-tailed *t* tests.

Biotinylation of membrane proteins

CHO cells were transfected with a plasmid encoding a Myc-tagged Kv2.1 by the procedure described earlier. At 3 h after treating Myc-Kv2.1-expressing CHO with either vehicle or DTDP (25 μM, 5 min), cells were incubated with 2 mg/ml sulfo-NHS-SS-biotin, and solubilized in 1% Triton, 0.1% SDS. Biotinylated proteins were isolated with immobilized neutravidin as described by Misonou *et al*.[27] Immunoblotting with anti-Myc and GAPDH antibodies were used to analyze protein abundance in the total lysate and isolated surface fractions.

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