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Apoptotic proteins Reaper and Grim induce stable inactivation in voltage-gated K^+ channels

(apoptosis/ion channel/*Drosophila*/programmed cell death)

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ABSTRACT ***Drosophila* genes *reaper*, *grim*, and *head-involution-defective* (*hid*) induce apoptosis in several cellular contexts. N-terminal sequences of these proteins are highly conserved and are similar to N-terminal inactivation domains of voltage-gated potassium (K^+) channels. Synthetic Reaper and Grim N terminus peptides induced fast inactivation of Shaker-type K^+ channels when applied to the cytoplasmic side of the channel that was qualitatively similar to the inactivation produced by other K^+ channel inactivation particles. Mutations that reduce the apoptotic activity of Reaper also reduced the synthetic peptide's ability to induce channel inactivation, indicating that K^+ channel inactivation correlated with apoptotic activity. Coexpression of Reaper RNA or direct injection of full length Reaper protein caused near irreversible block of the K^+ channels. These results suggest that Reaper and Grim may participate in initiating apoptosis by stably blocking K^+ channels.**

Programmed cell death or apoptosis is a mechanism for removing superfluous cells during development (1). Dysregulation of apoptosis occurs in cancer, reperfusion injury after ischemic episodes, neurodegenerative disorders, and other pathological conditions (2). The stereotypic apoptotic program involves activation of caspases (3) and a caspase-activated DNase (4). In *Drosophila*, *reaper*, *grim*, or *hid* gene expression triggers apoptosis in a caspase-dependent manner (5–12). Proteins encoded by these genes have highly conserved amino-terminal sequences (5, 6, 9), site-directed mutagenesis of which showed some of the conserved residues in the N termini to be important for induction of apoptosis (10, 13, 14). The 65-residue Reaper protein, localized to cytoplasmic regions contiguous with the plasma membrane (12), shows a transient marked increase before caspase activation (8, 15). The exact mechanism by which the Reaper, Grim, and Hid proteins trigger the apoptotic program had not yet been identified, the functional significance of their conserved N-terminal sequences remaining unknown (16).

Ion channels in the plasma membrane are involved in many cellular functions. For example, opening of Na^+ channels promotes membrane depolarization whereas K^+ channel opening results in hyperpolarization. Recent evidence indicates a role for ion channels in apoptosis (17–24). Membrane depolarization, resulting either from increased Na^+ influx or decreased K^+ efflux, is associated with pro-apoptotic activity in many cell types. *Lurcher* and *Weaver* mutant mice exhibit severe ataxia resulting from extensive apoptotic neuronal cell death (17, 18). *Lurcher* produces a constitutively active δ2 glutamate receptor (19) whereas *Weaver* changes the ionic selectivity of a G-protein coupled inward rectifier K^+ channel (20). Although different channel types are involved, both mutations give rise to increased Na^+ influx and significant membrane depolarization (18, 21). Binding of Fas-ligand to the Fas (APO-1/CD95) receptor stimulates apoptosis of Jurkat T lymphocytes that is related directly to reduced Kv1.3 channel activity (22). Application of 4-aminopyridine induces apoptosis of malignant astrocytoma cells by blocking outward K^+ currents (23). Chronic depolarization may be linked closely with initiation of apoptosis rather than with the cell death program itself. For example, depolarization may facilitate opening of other voltage-activated channels, such as plasma membrane- or organelle membrane-associated Ca^{2+} channels, and pulsatile increases in intracellular free Ca^{2+} have been implicated in activation of the caspase-dependent cell death cascade (24, 25). Severe and prolonged membrane depolarization may be sufficient to tip the delicate balance of pro- and antiapoptotic forces in favor of initiation of the cell-death program.

Voltage-dependent Shaker (Sh) K^+ channels open in response to depolarization and subsequently undergo N-type inactivation by a "ball and chain" mechanism (26, 27). The 20 N-terminal residues of the ShB channel form the inactivation "ball," which is tethered to membrane-spanning channel domains by the following ≈200-residue "chain." Inactivation occurs when the N-terminal inactivation ball physically occludes the inner pore of the channel from the cytoplasmic side (26–28). Stability of the inactivated state is enhanced by the hydrophobicity of approximately the first 10 residues of the inactivation ball whereas positively charged amino acids within the following 10 residues promote entry into the inactivated state via electrostatic interactions (26, 29, 30). Deletions in the distal N terminus of the channel disrupt inactivation, which can be reversed by application of a 20-residue synthetic peptide corresponding to the initial N-terminal sequence of the channel (26, 27). Ancillary β subunits in some K^+ channel complexes serve to produce N-type inactivation by a similar mechanism (31).

The conserved N-terminal sequences of Reaper, Grim, and Hid resemble those N-terminal Sh K^+ channel domains that are involved in inactivation (Fig. 1*A*; refs. 5, 6, 9, and 32). This sequence similarity led to the hypothesis that Reaper, Grim, and Hid facilitate initiation of apoptosis by inducing inactivation of K^+ channels. Sustained inactivation of K^+ channels will result in chronic membrane depolarization that may lead to the initiation of the caspase-dependent apoptotic program, perhaps by increasing the level of cytosolic free Ca^{2+}. To test this hypothesis, we examined the effects of Reaper, Grim, and Hid on inactivation of voltage-dependent K^+ channels heterologously expressed in *Xenopus* oocytes. The results suggest that

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these proteins may help trigger apoptosis by causing stable inactivation of K^+ channels.

MATERIALS AND METHODS

Electrophysiology. ShBΔ6–46:T449V and Kv1.3 K^+ channels were expressed in *Xenopus* oocytes by injecting the RNAs as described (26). All procedures conformed to an animal use protocol approved by the University of Iowa Animal Care and Use Committee. Whole-oocyte currents were recorded with a Warner 725C amplifier (Warner Instruments, Hamden, CT). The electrodes had a typical initial input resistance of 0.5 to 0.8 megaohms when filled with 3 M KCl. Patch-clamp recordings were made by using an AxoPatch 200A (Axon Instruments, Foster City, CA). The patch-clamp output signal was low-pass filtered through an 8-pole Bessel filter (Frequency Devices, Haverhill, MA) and was digitized by using an ITC-16 AD/DA board (Instrutech, Port Washington, NY). Data were collected and analyzed by using PULSE (HEKA Electronics, Lambrecht/Pfalz, Germany), IGORPRO (WaveMetrics, Lake Oswego, OR), and DATADESK (DataDescriptions, Ithaca, NY) running on Apple Macintosh computers. The external solution contained (in mM): 140 NaCl, 2 $MgCl_2$, 2 KCl, 10 Hepes (*N*-methyl-D-glucamine) (pH 7.2). The internal solution contained (in mM): 140 KCl, 11 EGTA, 2 $MgCl_2$, 10 Hepes (*N*-methyl-D-glucamine) (pH 7.2). Macroscopic currents recorded at +50 mV in the presence of synthetic peptide (100 μM) were fitted with the simple bimolecular reaction scheme presented later in the text, including the kinetic states and parameters described in Zagotta *et al.* (33) using k_{ON} and k_{OFF} as the only free parameters. Parameters were optimized by using a customized routine implemented in IGORPRO.

Peptide Synthesis. Peptide synthesis was carried out on a custom-built synthesizer and was HPLC-purified at the City of Hope Peptide Synthesis Facility (34). All peptides were characterized by mass spectroscopy. Different batches of the peptides produced identical physiological results. Peptides, including full length Reaper, synthesized and purified by the University of Iowa Peptide Synthesis Facility, gave identical results.

Reaper Expression. Reaper cDNA was obtained from B. Hay (California Institute of Technology, Pasadena, CA) and was used as the template DNA in a PCR amplification reaction by using the following sense and antisense primers to introduce *Afl*II and *Hin*dIII restriction enzyme cleavage sites at the 5′ and 3′ ends, respectively (restriction sites are italicized):

sense primer:

TACAGTG*CTTAAG*CAACCAAAGATATTAAACTA-ACCATGGCAGTGGCATTCTACAT

antisense primer:

CCCCAGATC*AAGCTT*AAAAACCAGCTTTTCATTG-CGATGGCTTGCGATATTTGCCGG.

Amplified DNA was digested with *Afl*II and *Hin*dIII and was subcloned directly into the pGEMHE expression vector followed by sequencing of the insert DNA. Reaper protein expression in *Xenopus* oocytes injected with RNA was confirmed by Western blot analysis by using an affinity-purified rabbit polyclonal anti-Reaper peptide antibody provided by G. Pronk (Chiron; ref. 8). Cells expressing K^+ channels without Reaper typically remained viable and suitable for electrophysiological recording for 1 week or more whereas those expressing Reaper lasted only for 1 or 2 days.

RESULTS

Synthetic peptides corresponding to the N-terminal inactivation domains of various K^+ channels induce inactivation in a concentration-dependent manner when applied to the cytoplasmic side of the membrane (27, 29, 30, 35). If the *Drosophila* apoptosis triggering proteins induce inactivation of K^+ channels, synthetic peptides corresponding to their distal N-terminal sequences should act similarly. We tested the effects of short synthetic peptides corresponding to the N-terminal sequences of Reaper, Grim, and Hid on the ShBΔ6–46:T449V channel, which shows neither N- or C-type inactivation in *Xenopus* oocytes (26, 36). Application of the Reaper or Grim peptide (100 μM) induced a time-dependent decline of the voltage-dependent K^+ currents (Fig. 1*B*). Reaper and Grim peptides gave qualitatively similar results to those produced by the same concentration of the ShB N-terminal inactivation peptide (Fig. 1*B*). The synthetic Hid peptide was not effective in inducing inactivation during the 80-ms timescale shown but produced noticeable reductions in K^+ current amplitude on a 0.4-s timescale (data not shown). Concentration-dependent reductions of K^+ channel currents by Reaper and Grim synthetic peptides were noted even at low peptide concentrations, with ≈50% reduction in amplitude of the steady-state current occurring at 1 μM (Fig. 1*C*).

Reaper and Grim peptides reduced the mean open time of the K^+ channel, similar to that observed for the ShB peptide (27). Representative openings and open time histograms obtained from a single ShBΔ6–46:T449V channel in an inside-out patch before and after application of the Reaper peptide are shown in Fig. 1*D*. The Reaper peptide reduced the mean open time in a concentration-dependent manner, the reciprocal of the mean open time being linearly related to peptide concentration (Fig. 1*E*). Similar results were obtained with the Grim peptide (data not shown). Such a linear relationship is consistent with a simple bimolecular reaction scheme in which a single peptide molecule binding to an open channel blocks the current flow as indicated below, where C represents multiple closed states as described in Zagotta *et al.* (33), O represents the open state, and B represents the peptide-bound nonconducting stat

$$C \rightleftharpoons O \underset{k_{OFF}}{\overset{[\text{Peptide}]\,k_{ON}}{\rightleftharpoons}} B$$

Based on the concentration dependence of the mean open time, the value of k_{ON} for the Reaper peptide was estimated to be 1.6 s^{-1} μM^{-1}, similar to the value previously determined for the ShB peptide (27). Rate constants of entry into the blocked state and of recovery from the blocked state also were estimated from the time courses of the macroscopic currents recorded in the presence of the peptides (Fig. 1*F*). The estimated k_{ON} value for the ShB peptide was noticeably greater than that for the Reaper, Grim, or Hid peptide, consistent with the greater content of positively charged residues in the ShB peptide (26, 29, 30). The estimated k_{OFF} values for the Reaper, Grim, and Hid peptides were significantly less than that for the ShB peptide (Fig. 1*F*), indicating that Reaper and Grim peptides produce more stable inactivation of the ShBΔ6–46:T449V channels than does the ShB peptide. The *Drosophila* ShB inactivation peptide inactivates both vertebrate and invertebrate Sh-type K^+ channels (27, 37). If Reaper and Grim peptides act by the same mechanism to inactivate K^+ channels, then they also should induce inactivation in mammalian Sh-type K^+ channels expressed in *Xenopus* oocytes. Application of Reaper and Grim N-terminal peptides to the cytoplasmic side of mammalian Kv1.3 K^+ channels, which have been shown to be responsible for the major outward K^+ current in lymphocytes (38), produced results identical to those obtained by using *Drosophila* Sh K^+ channels (data not shown).

If the Reaper action on K^+ channels were physiologically relevant to apoptosis, those Reaper mutations that decrease Reaper's cell killing activity should decrease the N-terminal peptide's ability to inactivate the K^+ channel. Some amino acid substitutions in the Reaper N-terminal domain have pronounced effects on apoptotic activity in transfected cells.

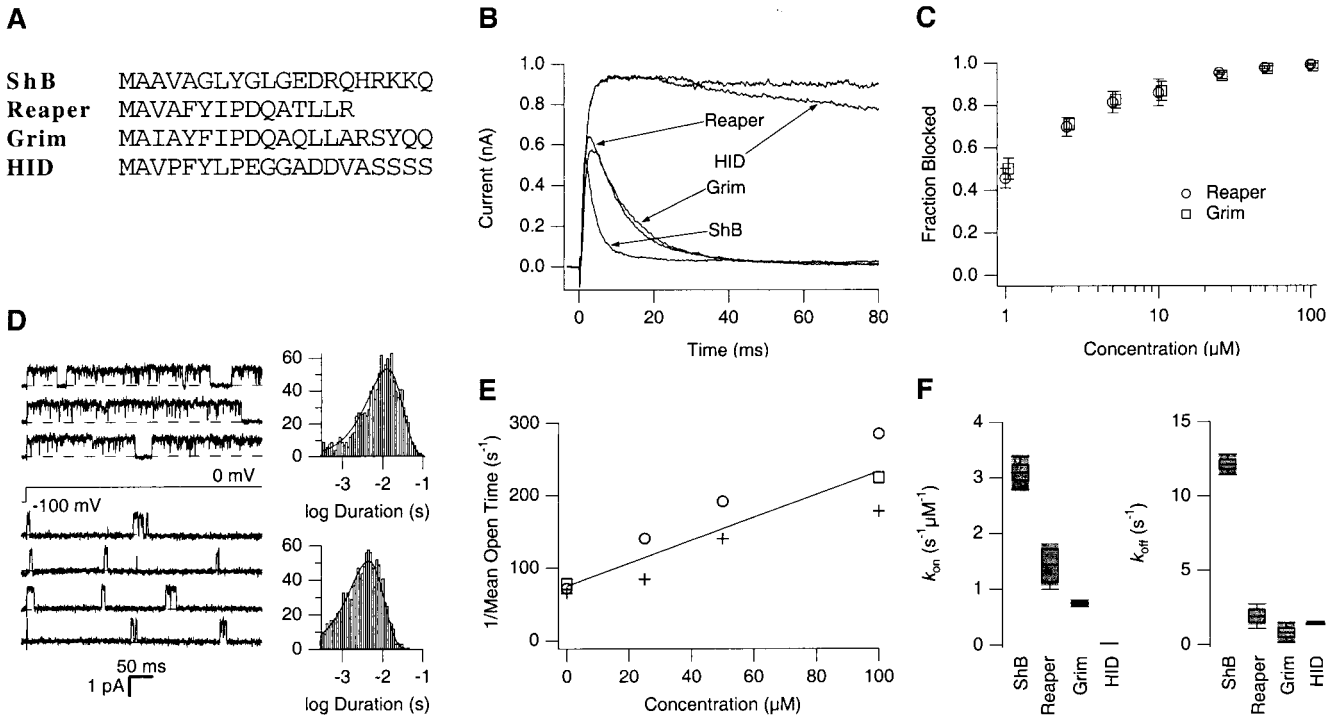

FIG. 1. Short synthetic apoptosis peptides induce inactivation in voltage-dependent ShBΔ6–46:T449V K^+ channels. (*A*) N-terminal sequences of the ShB K^+ channel, Reaper, Grim, and Hid. The number of amino acids shown corresponds to the length of synthetic peptides used in the experiments. Peptides were synthesized as described in *Materials and Methods*. (*B*) Representative macroscopic currents recorded from an inside-out patch at +50 mV in the presence of the ShB, Reaper, Grim, or Hid peptide. Peptides were applied to the cytoplasmic side of the membrane patch at 100 μM concentration. (*C*) Concentration dependence of the fractional steady-state block of the ShBΔ6–46:T449V current by the Reaper and Grim N-terminal peptides (mean ± SD, $n = 5$). Steady-state currents were measured in response to pulses to +50 mV for 200 ms to 1.5 s in duration. Fraction of the steady-state current blocked is plotted as a function of the peptide concentration. (*D*) Effect of the Reaper N terminus peptide on openings of a single ShBΔ6–46:T449V channel. Representative openings at 0 mV with and without the Reaper peptide are shown. The upper three sweeps were recorded in the absence of and the lower four sweeps were recorded in the presence of Reaper peptide (100 μM). The open time histograms generated from the openings without peptide (upper) and with peptide (lower) also are shown. Solid lines in the histograms represent single exponential fits to the data. The mean open times for the control and peptide data were 12.8 ms and 4.4 ms, respectively. (*E*) Concentration dependence of the reciprocal of the mean open time. The reciprocals of the mean open times, measured in the presence of different concentrations of the Reaper N-terminal peptide, are plotted as a function of the peptide concentration. The different symbols show the results obtained from three different experiments. The straight line shows the concentration dependence as predicted from the open channel scheme discussed in the text. The slope of the line, which represents k_{ON}, was 1.6 s^{-1} μM^{-1} and the *y* intercept was 75 s^{-1}. (*F*) Rate constants (k_{ON} and k_{OFF}) of the block of ShBΔ6–46:T449V currents by the synthetic N-terminal peptides. Rate constants were estimated from macroscopic current recordings as described in *Materials and Methods*. The estimated values of blocking and unblocking rate constants, k_{ON} and k_{OFF}, respectively, are compared by using boxplots.

Reaper F5A and Y6A mutants, which had 50% less cell killing activity than wild-type Reaper (10, 13), also were less effective in inactivating the ShBΔ6–46:T449V currents, leaving greater steady-state residual currents (Fig. 2*A*). The F5A and Y6A peptides were particularly less effective in blocking K^+ channel currents at low concentrations (Fig. 2*B*). At 5 μM, the wild-type Reaper peptide blocked ≈80% of the current whereas the mutant peptides blocked only 20–40%. Analysis of the inactivation entry and recovery rate constants showed that the F5A and Y6A mutations increased the rate constant of recovery from inactivation (k_{OFF}) by 3- to 7-fold without markedly affecting the rate constant of entry into the inactivated state (k_{ON}) (Fig. 2*C*). These changes signify destabilization of the inactivated state by ≈0.64 and 1.1 kcal/mol by the F5A and Y6A mutations, respectively. That substitution of a bulky phenylalanine or tyrosine residue with an alanine in the N-terminal Reaper peptides destabilized the inactivated state is consistent with the previous finding that N-type inactivation is reduced by decreased hydrophobic content of the initial 10 residues of the inactivation ball (26, 29, 30). Recovery from the Reaper F5A-induced inactivation was also significantly faster than that induced by wild-type Reaper as measured by a standard double pulse paradigm (Fig. 2*D*). Reaper A11N and P8A mutations have been shown to have no effect on cell-killing activity (10). We found that the A11N substitution, which does not alter the hydrophobicity or the charge on the peptide, did not affect the Reaper peptide's ability to inactivate the K^+ channels (Fig. 2*E*). The Reaper P8A mutant peptide was insoluble in the recording solution and, therefore, was not tested. Overall, the results show a strong positive correlation between Reaper's cell killing and channel inactivating abilities.

The ShB N-terminal inactivation ball normally is tethered to the channel itself. In voltage-gated K^+ channel complexes in which ancillary β subunits provide the inactivation domain, the β subunits are located in close proximity to the channel in the plasma membrane. If Reaper promotes apoptosis by inactivating K^+ channels, the full length Reaper protein, as well as its N terminus peptide, should inactivate the channels when coexpressed in the same cell. We examined how coexpressed Reaper affects ShBΔ6–46:T449V channels in *Xenopus* oocytes. One day after injection of Reaper and ShBΔ6–46:T449V RNAs, mean K^+ current amplitude in oocytes injected with both Reaper and ShBΔ6–46:T449V RNAs was significantly less than that recorded in oocytes injected with channel RNA alone (Fig. 3*A*). Unlike the N terminus peptide, full length Reaper did not induce marked time-dependent inactivation of ShBΔ6–46:T449V K^+ channels. The observed reduction in the K^+ channel

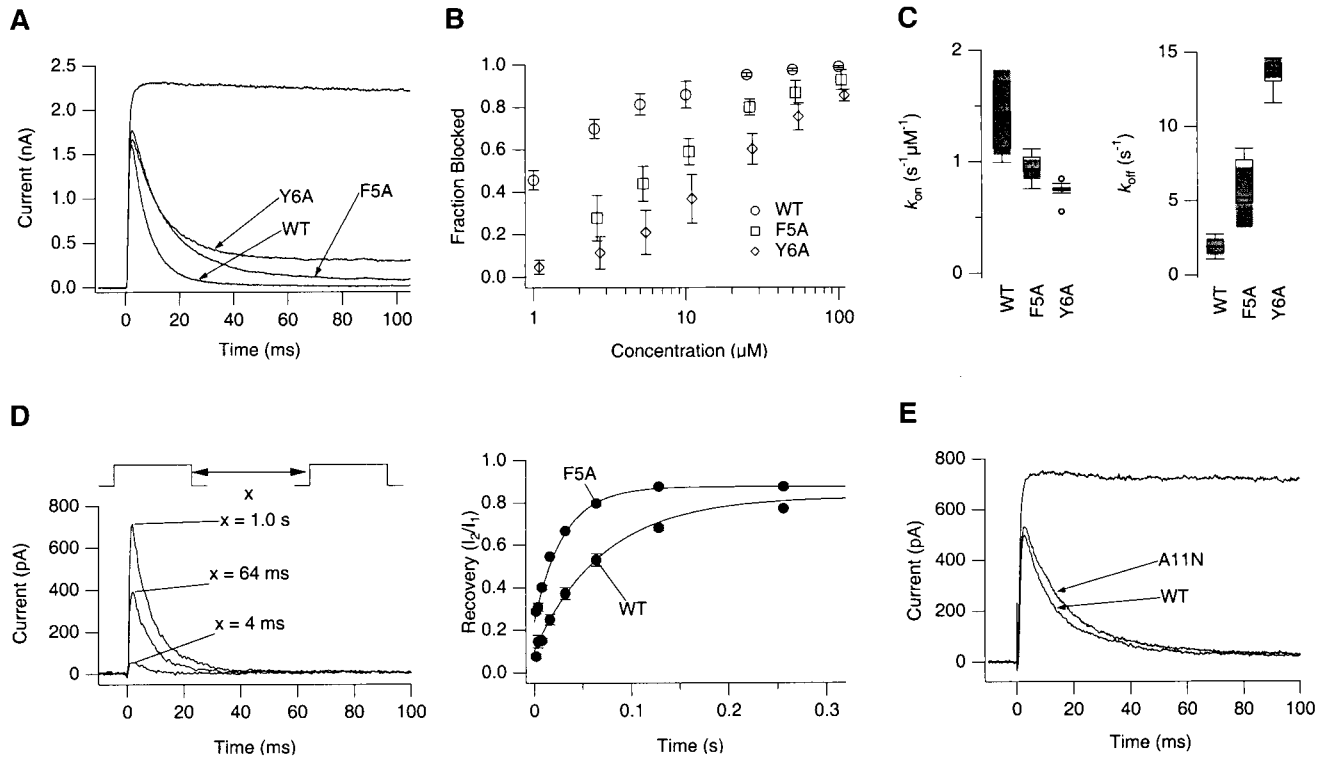

FIG. 2. Mutations known to affect Reaper's cell killing ability also affect K^+ channel blocking ability. (*A*) Blocking effects of Reaper N terminus peptides (wild-type, F5A, and Y6A) on ShBΔ6–46:T449V currents. Currents recorded from an inside-out patch at +50 mV are shown. Peptides were applied at 100 μM. (*B*) Concentration dependence of the channel block by the Reaper wild-type, F5A, and Y6A N terminus peptides (mean ± SD, $n = 5$). Macroscopic ShBΔ6–46:T449V currents were measured in response to depolarization to +50 mV for 200 ms to 1.5 s in duration. Normalized steady-state current amplitudes are plotted as a function of the peptide concentration. (*C*) Rate constants (k_{ON} and k_{OFF}) of the block by the Reaper wild-type, F5A, and Y6A N terminus peptides. Rate constants were estimated by fitting the macroscopic current time course in the presence of the peptides at 100 μM as described in *Materials and Methods*. Values of the rate constants are compared by using boxplots. (*D*) Recovery from the Reaper F5A peptide-induced block is faster. Time course of recovery from peptide-induced block was estimated by using a standard double-pulse protocol. Two depolarizing pulses to +50 mV, separated by various intervals as indicated, were applied. Relative current amplitudes, amplitude elicited by the second pulse divided by that from the first pulse, are plotted. Representative ShBΔ6–46:T449V currents recorded in the presence of the Reaper F5A peptide during the second pulses to +50 mV are shown (left). Recovery time courses observed with wild-type and F5A Reaper peptides (100 μM) are compared (right; mean ± SD, $n = 3$). Smooth lines represent single exponential fits to the data. Recovery time constants for wild-type and F5A Reaper were 70 ms and 30 ms, respectively. (*E*) The A11N mutation does not affect the Reaper N terminus peptide's ability to block the channel. Reaper wild-type and A11N peptides were applied at 100 μM. The time courses of the currents recorded in the presence of the two peptides were similar at all concentrations examined (1 to 100 μM, data not shown).

current amplitude in oocytes coinjected with K^+ channel and Reaper RNAs might result from saturation of the oocyte translational machinery or Reaper-induced apoptosis that is unrelated to direct interaction of Reaper with the channel. Three lines of evidence argue against this possibility. Coinjection of oocytes with RNAs encoding the K^+ channel and a voltage-dependent Ca^{2+} channel β3 subunit did not reduce the K^+ current amplitude (data not shown), indicating that the translational capacity of the oocytes had not been saturated. Sequential injection of Reaper RNA into oocytes expressing ShBΔ6–46:T449V K^+ channels resulted in a significant (>75%) reduction in the K+ current amplitude within 1 day (Fig. 3*B*), indicating that full length Reaper reduces K^+ current amplitude by blocking K^+ channels already in the membrane. Pressure-injection of synthetic full length (65 residues) Reaper protein into oocytes expressing the K^+ channels resulted in a >50% reduction in mean K^+ current amplitude within 2 hr (Fig. 3*C*), suggesting that full length Reaper protein is targeted rapidly to, and directly blocks, functional K^+ channels present in the plasma membrane.

Application of synthetic full length Reaper (100 μM) to the cytoplasmic side of a membrane patch from a cell expressing ShBΔ6–46:T449V K^+ channels markedly reduced K^+ current amplitude (Fig. 3*D*). Peak K^+ current amplitude in the presence of full length Reaper remained reduced relative to control amplitude even after wash and the current time course showed little inactivation. Significant reductions in K^+ current amplitude also were observed in some patches after application of full length synthetic Reaper at a concentration of 100 nM (data not shown). The effects of full length Reaper were distinct from those of the short N-terminal peptide in two important aspects. Channel block by the full length protein was use-dependent, and K^+ current amplitude became progressively smaller with repeated depolarization (Fig. 3*E*). The effect of the full length Reaper protein was essentially irreversible (Fig. 3 *D* and *E*); Reaper-induced K^+ channel block persisted even after a 20-min wash with peptide-free solution. Hyperpolarization to −140 mV, which should facilitate recovery from N-type inactivation, resulted in a small increase in K^+ channel current amplitude, indicating that the channels remained functional throughout the experiment (Fig. 3*E*). In the absence of conditioning hyperpolarization, a progressive increase in channel block again was observed. Similar results were obtained with Kv1.3 channels (data not shown). Full length Reaper reduced the mean open time of the ShBΔ6–46:T449V channel without affecting the single channel current amplitude and also increased the number of depolarizing epochs that failed to elicit a channel opening (data not shown). These results show that full length Reaper induces an extremely stable and near irreversible block of the K^+ channels.

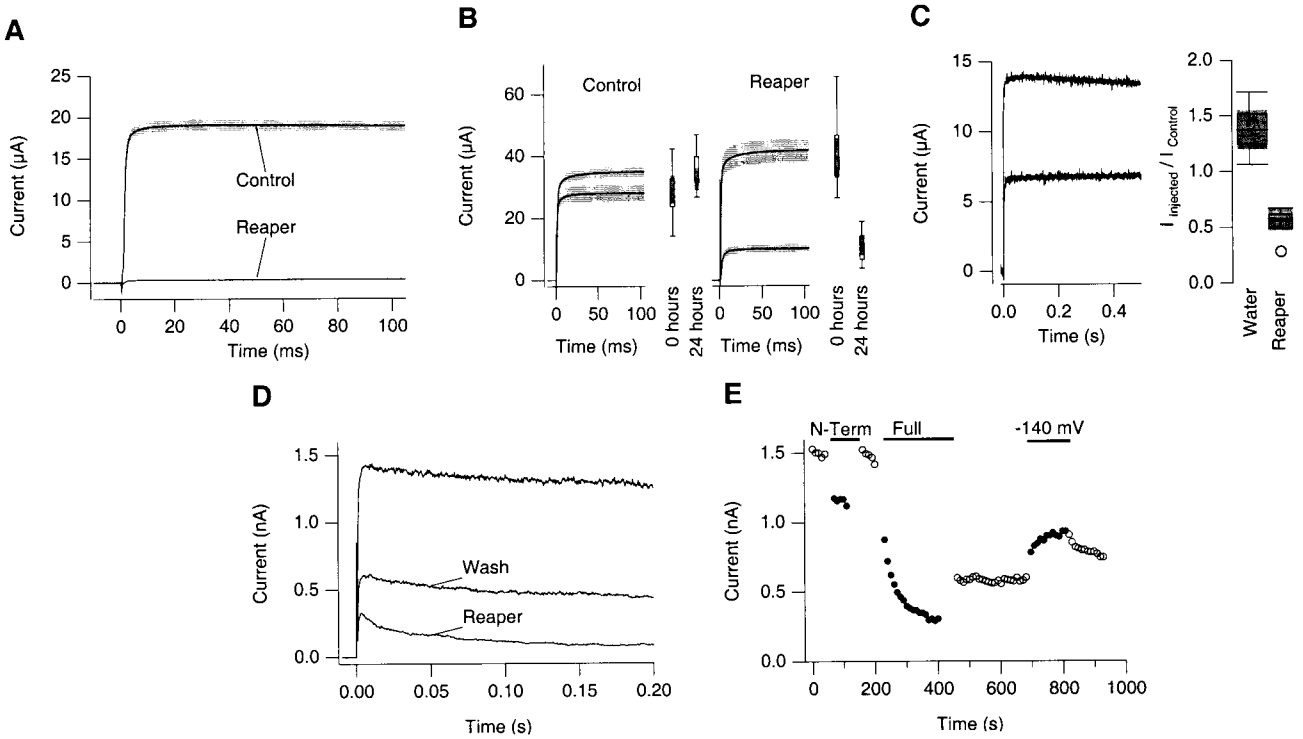

FIG. 3. Effects of full length Reaper protein on the K^+ channel. (*A*) Coinjection of Reaper and ShBΔ6–46:T449V RNAs into *Xenopus laevis* oocytes inhibits functional expression of the channel. Two-electrode voltage-clamp recordings at +50 mV were made 1 day after injection of the RNAs. Solid lines represent the averaged currents from 13 cells. Shaded areas represent the respective SEM. (*B*) Reaper RNA injection reduces K^+ currents through the ShBΔ6–46:T449V channels already in the membrane. Oocytes were injected with ShBΔ6–46:T449V channel RNA on Day 0. On Day 1, the cells were divided into two groups, and control recordings were made (current amplitudes at 0 hr). Cells then were injected with either Reaper RNA or H_2O (control). Currents again were recorded 24 hr later (current amplitude values at 24 hr). Injection of Reaper RNA resulted in significant reduction in current amplitude (right) whereas injection of H_2O had no effect (left). Solid lines represent mean currents recorded by using a two-electrode voltage clamp from 10 to 15 cells. Shaded areas represent the respective SEM. (*C*) Direct injection of synthetic full length Reaper peptide decreases the ShBΔ6–46:T449V current amplitude. Control currents were recorded by using a two-electrode voltage-clamp, and the cells then were injected immediately with either H_2O (40 nl) or full length synthetic Reaper protein (40 nl, 1 mM). Cells were allowed to recover for 2 hr after injection, and currents then were recorded from the same cells. Representative currents before and after peptide injection are shown (left). Relative changes in the current amplitudes are compared by using boxplots (right). (*D*) Synthetic full length Reaper peptide induces inactivation in the ShBΔ6–46:T449V K^+ channel. Macroscopic currents recorded from one patch are shown. The control current, the current recorded in response to the 18th pulse in the presence of Reaper, and the current after wash-out are shown. Depolarizing pulses to +50 mV were applied every 10 s. (*E*) The effect of synthetic full length Reaper is near irreversible. Peak macroscopic ShBΔ6–46:T449V current amplitudes recorded from one inside-out patch in response to repeated depolarizing pulses to +50 mV from a holding potential of −90 mV at 10-s intervals are shown. Synthetic Reaper N terminus peptide (100 μM) and full length Reaper synthetic peptide (100 μM) were applied to the intracellular side of the patch. The block induced by the short N terminus peptide (N-term) was completely reversible by washing the chamber with peptide-free solution. In contrast, channel block induced by full length Reaper peptide (Full) was only partially reversible. Hyperpolarization to −140 mV resulted in only a slight increase in peak current amplitude.

DISCUSSION

We show that short synthetic peptides corresponding to the conserved N-terminal sequences of two *Drosophila* apoptosis-trigger proteins, Reaper and Grim, act by a mechanism similar to naturally occurring inactivation particles to block K^+ flux through pores of voltage-dependent K^+ channels (Fig. 1). The positive correlation between channel blocking ability and apoptotic activity of N-terminal Reaper mutants (Fig. 2) suggests that the K^+ channel blocking ability of the Reaper peptide may be physiologically relevant to apoptosis. The observation that the K^+ channel block induced by full length Reaper is essentially irreversible (Fig. 3) whereas that induced by the N terminus peptide is readily reversible suggests that Reaper may interact with the K^+ channel protein through multiple domains. These results suggest that Reaper acts as a very stable inactivation particle or an intracellular K^+ channel toxin and that K^+ channel block is physiologically relevant to initiation of apoptosis.

Our results indicate that voltage-dependent K^+ channels may be one of the targets of Reaper and suggest the following model of Reaper action. Reaper protein, induced by a variety of stimuli, almost irreversibly blocks the K^+ channels and leads to sustained depolarization of the cell. Depolarization has been associated with increased cell death and may be linked closely with the initiation of apoptosis, perhaps by facilitating opening of other depolarization-activated ion channels, such as Na^+ and Ca^{2+} channels (however, see ref. 39). Excess intracellular Ca^{2+} has been implicated in apoptosis (24), and opening of voltage-dependent Ca^{2+} channels in the plasma membrane or release of Ca^{2+} from internal stores results in activation of cell-death associated caspases (25). Chronic Reaper-induced membrane depolarization may result in intracellular free Ca^{2+} oscillations that are sufficient to trigger the cascade leading to activation of the caspase-dependent DNase. This proposed model for Reaper action is consistent with the following observations: (*i*) High-level Reaper expression is observed in the cytoplasm 1–2 hr before caspase activation, persists only for a few hours, and then is degraded rapidly (8, 12, 15); (*ii*) inhibitors of apoptosis act to prevent Reaper-induced apoptosis (7, 11) by physically interacting with the Reaper protein and sequestering it to a perinuclear location away from its cytoplasmic and/or plasma membrane associated sites of action (12); and (*iii*) the region of sequence

similarity shared by Reaper, Grim, and Hid, the N-terminal 14 amino acids of each protein, is required for the induction of apoptosis (14).

The lack of a stringent amino acid sequence requirement for a K^+ inactivation particle suggests that other proteins may be capable of producing K^+ channel inactivation and, perhaps, explains why mammalian homologues of Reaper, Grim, or Hid have not yet been identified (16). One attractive hypothesis is that Reaper, Grim, and Hid may alter different subsets of ion channels to induce severe and rapid depolarization. This possibility is consistent with the observation that Reaper, Grim, and Hid exhibit different efficiencies in inducing apoptosis in specific cell types (5–7, 9, 16). Potential targets of Reaper, Grim, and Hid might include both cation- and anion-selective channels that are localized to either plasma or organelle membranes. Our results also suggest that the presence of a large number of K^+ channels, particularly those resistant to block by Reaper and Grim, may have antiapoptotic effects. This relationship between K^+ channel block and apoptosis may lead to novel therapeutic interventions for the treatment of several human diseases including cancer, stroke, heart disease, and neurodegenerative disorders.

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