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27. ACh-induced currents were recorded with a two-electrode voltage clamp at a holding potential of −40 mV. Electrodes were filled with 3 M KCl and had a resistance of less than 2 megaohms. The oocytes were perfused at 5 ml/min with Ca^{2+}-free Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $MgCl_2$, 1 μM atropine, 10 mM Hepes, pH 7.5 with NaOH), in which all reagents were applied.
28. In about 50% of the experiments, the second response after the application of the MTS derivative was greater than the first. We recorded the responses of L251C and of S248C for about 1 hour after the application of MTSEA. In oocytes in which we observed recovery from the inhibition, the half-time was about 40 min. This recovery was not due to reductive or hydrolytic cleavage of the disulfide bond because we observed the same rate of recovery from inhibition due to the reactions of 2 mM dithiothreitol followed by 1 μM 4-(*N*-maleimido)benzyltrimethylammonium (MBTA), in which the MBTA forms a stable thioether with Cys^{192} or Cys^{193} at the ACh binding site [P. N. Kao *et al.*, *J. Biol. Chem.* **259**, 11662 (1984)]. We conclude that the receptor at the oocyte surface is turning over with a half-time of about 40 min.
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38. We thank H. Andrews, P. Brehm, T. Claudio, C. Czajkowski, S. Goff, W. Hendrickson, J. Hubbard, J. Javitch, H. Lester, I. Lowy, J. Messing, J. Murphy, J. Pittman, M. Reitman, L. Role, B. Sakmann, and S. Siegelbaum for invaluable advice and materials, E. Silva-Herzog for early experiments, and G. Salazar-Jimenez and C. Kaufmann for technical assistance. Supported in part by research grants from the NIH (NS07065) and the Muscular Dystrophy Association. Supported by NIH training grant NS07258 and an MDA Research Fellowship (D.A.S.).

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Calcium Channels Coupled to Glutamate Release Identified by ω-Aga-IVA

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Presynaptic calcium channels are crucial elements of neuronal excitation-secretion coupling. In mammalian brain, they have been difficult to characterize because most presynaptic terminals are too small to probe with electrodes, and available pharmacological tools such as dihydropyridines and ω-conotoxin are largely ineffective. Subsecond measurements of synaptosomal glutamate release have now been used to assess presynaptic calcium channel activity in order to study the action of peptide toxins from the venom of the funnel web spider *Agelenopsis aperta*, which is known to inhibit dihydropyridine and ω-conotoxin–resistant neuronal calcium currents. A presynaptic calcium channel important in glutamate release is shown to be ω-Aga-IVA sensitive and ω-conotoxin resistant.

The key step in excitation-secretion coupling in presynaptic nerve terminals is the influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels (*1*). Analysis of Ca^{2+} currents in neuronal soma (*2–5*) and in certain nerve terminal preparations (*6–8*) has provided evidence for multiple types of Ca^{2+} channels. The sensitivity of presynaptic Ca^{2+} channels at amphibian (*9*), reptilian (*8*), and avian synapses (*10, 11*) to ω-conotoxin (ω-CgTx) has led to widespread acceptance of a predominant role for N-type channels in neuronal excitation-secretion coupling. However, in mammalian systems, Ca^{2+} entry, neurosecretion, and synaptic transmission are only partially inhibited (*12–15*) or largely resistant to ω-CgTx and dihydropyridines (*16–18*), suggesting that the exocytotic Ca^{2+} channel at most mammalian brain synapses is a distinct subtype.

Venom of the funnel web spider *Agelenopsis aperta* contains toxins that have been shown to inhibit Ca^{2+} channels in vertebrates. These toxins include funnel toxin (FTX) (*19, 20*), a low molecular weight polyamine, and the peptides ω-Aga-IIIA and ω-Aga-IVA, two members of a family of at least four ω-agatoxins (*21*). In mammalian neuronal somata, high-voltage–activated Ca^{2+} channels of the L- and N-types (*22*), as well as those of the P-type (*23*), are blocked by ω-Aga-IIIA. In contrast, ω-Aga-IVA has been reported to be more selective, targeting the P-type currents of cerebellar Purkinje cells and rat sensory neurons (*18*). The potent block of $^{45}Ca^{2+}$ uptake into rat brain synaptosomes by ω-Aga-IVA (*18*) suggests that presynaptic P-type channels may trigger neurosecretion in mammalian brain. We report that ω-Aga-IIIA and ω-Aga-IVA partially (but potently) block [^{3}H]glutamate release from rat brain synaptosomes. These results provide additional pharmacological criteria that can be used to identify presynaptic Ca^{2+} channels involved in excitation-secretion coupling on the basis of sensitivity to ω-Aga-IVA and resistance to ω-CgTx.

The effect of the peptide toxins on presynaptic Ca^{2+} channels was assayed on the basis of the ability to block [^{3}H]glutamate release from synaptosomes (a preparation enriched in intact presynaptic nerve terminals), obtained by homogenizing rat frontal cortex in isotonic sucrose. The terminals were incubated in a solution containing [^{3}H]glutamate to metabolically label an exocytotic pool that can be released in a Ca^{2+}-dependent manner when depolarized by an increase in the external K^+ concentration. The loaded synaptosomes were immobilized on a glass fiber filter and secured in a chamber where they were superfused with solutions of defined composition. The effluent stream containing released radioactivity was collected in 70-ms segments in vials juxtaposed on the perimeter of a phonograph turntable. The combination of superfusion flow rates of 1 to 2

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ml s^{-1} through a relatively small chamber enables delivery of depolarizing stimuli for precisely defined durations and for resolving the resulting neurosecretion on a 70-ms time scale (*16*, *24*).

To assess the sensitivity of presynaptic Ca^{2+} channels to ω-agatoxins, we examined the effect of 200 nM ω-Aga-IVA on [^{3}H]glutamate release as a function of depolarization by varying the KCl concentration in the stimulating buffers. We evoked release by superfusing radiolabeled synaptosomes with the desired concentration of KCl, in the presence of 0.1 mM EGTA with or without 1.3 mM added Ca^{2+}, resulting in stimulating buffers with free Ca^{2+} concentrations of 1.2 mM or less than 1 μM, respectively (Fig. 1, A and C). The arithmetic difference between the release rates under these two conditions is defined as Ca^{2+}-dependent release (Fig. 1, B and D). The kinetics of Ca^{2+}-dependent release vary according to the stimulus intensity: at low stimulus intensity (15 mM KCl) (Fig. 1B), the release rate was roughly constant throughout the depolarizing pulse. As the stimulus intensity was increased (60 mM KCl) (Fig. 1D), an initial rapid, transient rate of Ca^{2+}-dependent release appeared that decayed to a sustained plateau. Under control conditions, Ca^{2+}-dependent release persisted for at least 1 s after the stimulating solution had been washed away. This presumably reflects the slow rate of return of intraterminal Ca^{2+} concentration to basal levels. Release rates were not affected the Ca^{2+}-independent component. An identical stimulating buffer containing 1.3 mM Ca^{2+} produces a Ca^{2+}-dependent enhancement of the rate of release (▼). The difference between release in the presence and absence of the added Ca^{2+} is defined as Ca^{2+}-dependent release (▼) (B and D). (E) Cumulative Ca^{2+}-dependent release is determined over time as the sum of release in all fractions up to the indicated time point. Such results are plotted for control, ω-CgTx, ω-Aga-IIIA, and ω-Aga-IVA. (F) Cumulative release at t = 2.10 s plotted as a function of the [K^+] in the stimulating buffer for control (▼) and 200 nM ω-Aga-IVA (◇). Synaptosomes were prepared from rat frontal cortex as described (*16*). The synaptosomes were suspended in basal buffer [145 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.1 mM EGTA-tris, 10 mM D-glucose, and 10 mM Hepes-tris (pH 7.4)] and incubated on ice in the presence of the indicated concentrations of toxins. Fifty micrograms of synaptosomal protein (1 mg ml^{-1}) were incubated with 10 μCi of [^{3}H]glutamate (46 Ci $mmol^{-1}$) for 15 min in basal buffer to label the exocytotic pool of glutamate. The loaded synaptosomes were trapped on a filter sandwich and transferred to the superfusion device, where the sandwich was washed with basal buffer for two 5-s periods. We determined a baseline rate of release by superfusing for 1.05 s with basal buffer; at t = 0, the superfusion solution was switched to the stimulation buffer containing 1.3 mM $CaCl_2$, and the indicated concentration of KCl was substituted on an equimolar basis for NaCl. The synaptosomes were stimulated for 1.05 s, and the superfusion solution was then switched back to the original basal buffer. The superfusate containing the released radioactivity was collected with a fraction collector rotating at 16 rpm, and 50 consecutive 70-ms fractions were collected. The amount of radioactivity in each fraction and remaining on the filter at the end of the experiment was determined by liquid scintillation counting in a Beckman LS-7000 scintillation counter. Results are expressed as a percentage of the total radioactivity (sample per sample plus filter) contained in each fraction. Data are the average of four to six experiments for each condition performed on synaptosomes prepared for each such experiment (*30*).

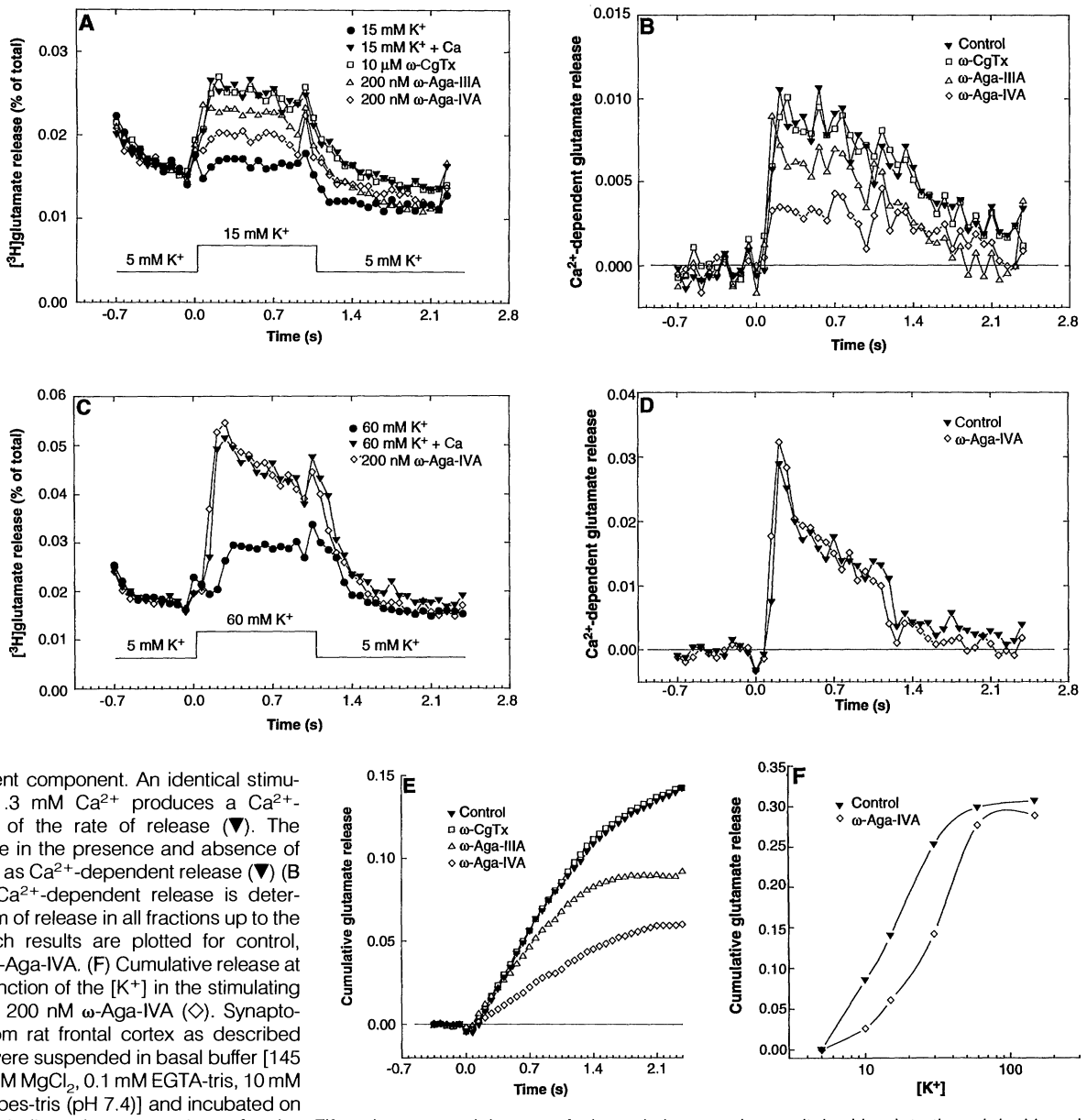

Fig. 1. ω-Agatoxins block Ca^{2+}-dependent glutamate release from rat synaptosomes. Synaptosomes were preloaded with [^{3}H]glutamate in the presence of 10 μM ω-CgTx (□), 200 nM ω-Aga-IIIA (△), or 200 nM ω-Aga-IVA (◇). The synaptosomes were immobilized on a filter support, and glutamate release was measured by superfusion (*24*). (**A** and **C**) [^{3}H]glutamate release is stimulated by an increase in the [K^+] in the stimulating buffer. In the absence of added Ca^{2+} (●), the release is defined as Ca^{2+}-independent, a result of efflux from a cytoplasmic glutamate pool produced by the voltage-dependent reversal of the Na^+- and K^+-dependent glutamate transporter of the plasma membrane (*29*). None of the toxins used al-

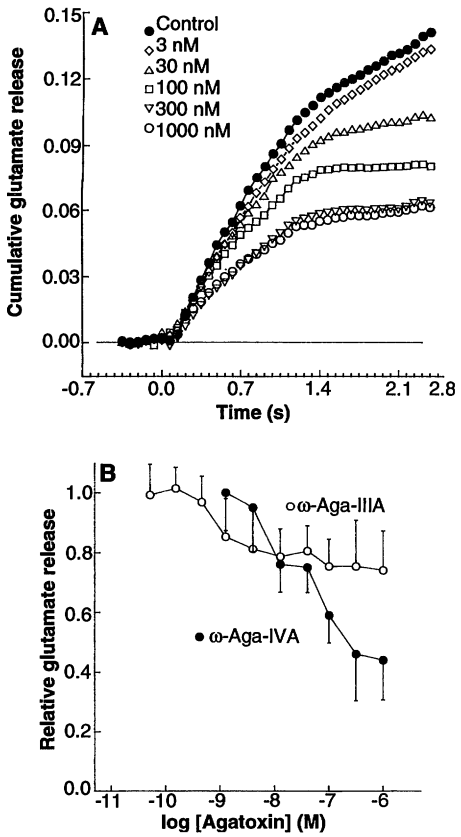

Fig. 2. Concentration-response relation for ω-Aga-IIIA and ω-Aga-IVA. (**A**) The effect of ω-Aga-IVA on Ca^{2+}-dependent [^{3}H]glutamate release was measured with a 1.05-s depolarizing pulse with 15 mM KCl. The synaptosomes were pretreated with 0 [control (●)], 3 nM (◇), 30 nM (△), 100 nM (□), 300 nM (▽), or 1000 nM (○) ω-Aga-IVA for 30 to 45 min before superfusion. Cumulative [^{3}H]glutamate release was calculated as in Fig. 1C. (**B**) Cumulative [^{3}H]glutamate release measured at t = 2.10 s was normalized to control values in the absence of toxin and is plotted against the logarithm of ω-Aga-IIIA (○) and ω-Aga-IVA (●) concentrations. In both (A) and (B), the data represent the average of six independent determinations for each condition; error bars in (B) represent SEM.

fected under any condition by saturating concentrations (10 μM) of ω-CgTx (*25*). In contrast, ω-Aga-IVA preferentially blocked Ca^{2+}-dependent release at lower stimulus intensities. Although ω-Aga-IVA blocked about 50% of Ca^{2+}-dependent glutamate release at 15 mM KCl, the extent of block decreased as the stimulus intensity increased until there was very little effect when K^+ was elevated above 30 mM (Fig. 1F).

The concentration-response relation was determined for ω-Aga-IIIA and ω-Aga-IVA (Fig. 2). Both ω-Aga-IIIA and ω-Aga-IVA produced a potent, saturable but partial block of Ca^{2+}-dependent glutamate release. Although ω-Aga-IIIA [IC_{50} (concentration that produced half-maximal block) = 0.74 nM)] was more potent than ω-Aga-IVA (IC_{50} = 30 nM), it was less efficacious (maximum inhibition = 23% versus 56%) (*26*). In order to determine whether the effects of the two toxins were additive, we measured release in the presence of high concentrations of both peptides (Fig. 3). The concentrations chosen were 100 nM ω-Aga-IIIA (about 100 times its IC_{50}) and 300 nM ω-Aga-IVA (about 10 times its IC_{50}). About 80% of glutamate release would be blocked under these conditions if the effects of the two toxins were additive. When applied together, the two toxins were less effective than ω-Aga-IVA alone, producing an amount of release similar to that seen with ω-Aga-IIIA.

We have shown that ω-Aga-IIIA and ω-Aga-IVA, peptides known to block neuronal Ca^{2+} currents, inhibit a component of the ω-CgTx–resistant, Ca^{2+}-dependent release of glutamate from rat brain synaptosomes. We conclude that P-type channels contribute a large component of presynaptic Ca^{2+} current that is coupled to glutamate release in the mammalian brain, on the basis of the sensitivity of this process to ω-Aga-IVA. Alternatively, the presynaptic Ca^{2+} channel of mammalian brain may be a distinct though uncharacterized subtype related to P-type channels on the basis of its sensitivity to ω-Aga-IVA. There are significant species differences in the properties of Ca^{2+} channels involved in neurosecretion: $^{45}Ca^{2+}$ uptake and glutamate release processes of rat brain are ω-agatoxin–sensitive and ω-CgTx–resistant, whereas those of chick brain are the pharmacological converse (*17, 18*). Similarly, ω-CgTx blocks neuromuscular transmission in frog but not in mouse (*9, 20*).

Although the data support the conclusion that a P-type channel directly mediates glutamatergic transmission, we cannot eliminate the possibility that Ca^{2+} entry through these channels over a period of 70 ms or longer can lead to transmitter release indirectly, as a result of overflow of Ca^{2+} into the active zones. However, the efficacy of the toxins was independent of the Ca^{2+} concentration in the stimulating buffers (*25*). If diffusion of Ca^{2+} into the active zone was involved in triggering release, it would be expected that the toxin would be more effective at lower Ca^{2+} concentrations. In an effort to limit the duration of the depolarizing pulse, we have developed a biochemical approach combining the Na^+ channel activator batrachotoxin and the pore blocker tetrodotoxin to depolarize synaptosomes for 10 to 30 ms. Using this stimulation paradigm (*27*), we have shown in preliminary results that the toxin blocks ~50% of Ca^{2+}-dependent [^{3}H]glutamate release.

The ω-agatoxins tested produced saturable but partial block of glutamate release. The combination of ω-Aga-IIIA and ω-Aga-IVA produced less block than that observed with ω-Aga-IVA alone. This suggests that block by ω-Aga-IIIA occludes that of ω-Aga-IVA in a manner similar to its occlusion of ω-CgTx block of avian synaptosomal Ca^{2+} uptake reported previously (*11*). Because the experiment was designed to favor the binding of ω-Aga-IIIA, the lack of additivity suggests that ω-Aga-IIIA and ω-Aga-IVA act on a single target at the same or at allosterically coupled sites, in a manner predicted by the law of mass action. The diminished efficacy of block by ω-Aga-IIIA suggests that it does not completely occlude the ion permeation pathway of a Ca^{2+} channel in the same manner as charybdotoxin blocks the Ca^{2+}-activated K^+ channel (*28*). Instead, this toxin may inhibit Ca^{2+} entry by partially occluding the channel.

In cerebellar Purkinje somata (*18*), ω-Aga-IVA completely abolishes P-type Ca^{2+} currents. If the toxin blocks synaptosomal Ca^{2+} channels in a similar way, our data indicate that glutamate release is coupled to P-type channels together with some pharmacologically distinct channel type (or

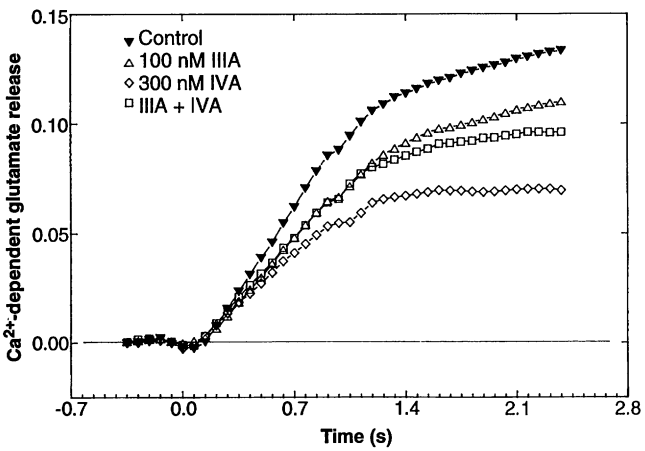

Fig. 3. Effects of ω-Aga-IIIA and ω-Aga-IVA are not additive. Ca^{2+}-dependent glutamate release evoked by a 1.05-s depolarizing pulse with 15 mM KCl was measured as in Fig. 1 for the untreated control (▼) and for synaptosomes pretreated with 100 nM ω-Aga-IIIA (△), 300 nM ω-Aga-IVA (◇), or both toxins together (□); ω-CgTx (10 μM) had no measurable effect in any combination with the ω-agatoxins (not shown). Results are the average of eight independent determinations for each condition.

types) resistant to dihydropyridines, ω-CgTx, and ω-Aga-IVA. The P-type channels and the resistant channels must reside together in glutamatergic terminals because strong depolarizations are able to overcome block by ω-Aga-IVA to maximally stimulate secretion. Thus L-, N-, and P-type channels may account for only part of the Ca^{2+} current in mammalian brain.

In conclusion, we have shown that ω-Aga-IIIA and ω-Aga-IVA block glutamate release from rat brain synaptosomes, a process resistant to ω-CgTx. This pharmacological evidence suggests that in rat brain, a P-type Ca^{2+} channel has a prominent role in neurotransmission at glutamatergic synapses and that the role for N-type channels in this process remains obscure. These results show that ω-agatoxins are important tools for identifying and characterizing presynaptic Ca^{2+} channels involved in neurotransmitter release and thus will prove useful in efforts to understand the ways in which neurosecretion and synaptic transmission are regulated.

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26. The effects of ω-Aga-IIIA and ω-Aga-IVA on cumulative, Ca^{2+}-dependent glutamate release were analyzed with the use of both double-reciprocal and Hill plots of the average values for release at each concentration of toxin tested. The IC_{50} value reported for ω-Aga-IIIA was obtained from double-reciprocal plots. We determined the potency of ω-Aga-IVA both by double-reciprocal and Hill analyses, using the I_{max} (maximal inhibition) value derived from the double-reciprocal plot for the Hill analysis. The value reported is that for the double-reciprocal analysis; the Hill analysis indicated an apparent K_i (inhibition constant) of 28.5 nM.
27. We have used the Na^+ channel activator batrachotoxin in Na^+-free solutions to increase the Na^+ permeability of synaptosomes, which are then depolarized by superfusion with a stimulating buffer containing 30 to 145 mM NaCl. The duration of the depolarization is limited by inclusion of the Na^+ channel blocker tetrodotoxin. The binding of tetrodotoxin to the Na^+ channel is a simple second-order reaction, so that the duration of the depolarization can be estimated from the product of the tetrodotoxin concentration and the forward rate constant for binding, estimated to be 10 to 30 $\mu M^{-1}\ s^{-1}$. At a concentration of 30 μM tetrodotoxin, we calculate that the depolarization will last ~10 ms [T. J. Tuner and K. Dunlap, *Soc. Neurosci. Abstr.* **17**, 577 (1991); *J. Cell. Biochem.* **16**, 225 (1992)].
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29. Reversal of the Na^+- and K^+-dependent glutamate transporter in the synaptic plasma membrane mediated the Ca^{2+}-independent glutamate release, as confirmed by the ability of transport inhibitors such as dihydrokainic acid and *threo*-β-hydroxyaspartic acid to completely block this component (T. J. Turner, unpublished results).
30. We performed release experiments in groups of 16 with at least one run for each experimental condition, using synaptosomes prepared immediately before each experiment. To minimize any time-dependent variability in the release rates, we randomized the order of runs for each experimental condition from day to day. Error bars for release rates, which were omitted for clarity, were generally less than 10% of the average values and did not exceed 20%.

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Effects of Kinesin Mutations on Neuronal Functions

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Kinesin is believed to generate force for the movement of organelles in anterograde axonal transport. The identification of genes that encode kinesin-like proteins suggests that other motors may provide anterograde force instead of or in addition to kinesin. To gain insight into the specific functions of kinesin, the effects of mutations in the kinesin heavy chain gene (*khc*) on the physiology and ultrastructure of *Drosophila* larval neurons were studied. Mutations in *khc* impair both action potential propagation in axons and neurotransmitter release at nerve terminals but have no apparent effect on the concentration of synaptic vesicles in nerve terminal cytoplasm. Thus kinesin is required in vivo for normal neuronal function and may be active in the transport of ion channels and components of the synaptic release machinery to their appropriate cellular locations. Kinesin appears not to be required for the anterograde transport of synaptic vesicles or their components.

Kinesin is a ubiquitous mechanochemical adenosine triphosphatase (ATPase) that can move toward the plus ends of microtubules in vitro (*1*, *2*). The native molecule is a heterotetramer consisting of two identical heavy chains and two light chains (*2*). The kinesin heavy chain, and more specifically the NH_2-terminal head of the heavy chain, appears to contain all of the mechanochemical elements necessary for generating microtubule-based movements (*3*). The light chains and the COOH-terminal tail of the heavy chain are thought to be involved in binding kinesin to vesicles or other cargo destined for microtubule-based transport (*2*, *4*).

On the basis of the polarity of kinesin's movement and its presence in neural cells, it was originally suggested that kinesin might act as a motor for anterograde axonal transport (*1*). It since has been shown that vesicle movement in axoplasm (both anterograde and retrograde) can be inhibited in vitro by an antibody that binds to the kinesin heavy chain (*5*). It also has been shown that kinesin heavy chain (*khc*) mutations in *Drosophila* cause behavioral defects that suggest a requirement for kinesin in normal neuromuscular functions (*6*). Although these data provide a persuasive argument that kinesin acts as a motor for axonal transport, the argument remains indirect.

Kinesin is a member of a superfamily of kinesin-like proteins that share similar mechanochemical domains (*7*), and multiple members of the kinesin superfamily are co-expressed in neural and other tissues (*8*). Thus, if kinesin is a motor for anterograde axonal transport, it is likely to function in the context of a complex set of anterograde motors. Consequently, understanding anterograde axonal transport and other micro-

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