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21. A cDNA fragment common to all the alternatively spliced cDNAs was excised by digestion with Xho I and Sma I, subcloned into the vector pQE-31 (Qiagen), and expressed as a hexahistidine-tagged protein in bacteria (*29*). The expressed protein contained an additional 18 vector–encoded amino acids (MRGSHHHHHHTAPHASSV) at the NH_2-terminus and 9 amino acids (VDLEPSLIS) at the COOH-terminus of the sequence indicated between the arrowheads in Fig. 3. The recombinant protein was purified by chromatography on a nickel-chelate column in 8 M urea, and elution was with 250 mM imidazole. After dialysis against buffer A (*18*), the protein was assayed for DNA binding and association with Fos and Jun as described for NFATp purified from T cells (*6*). To generate antisera, we immunized rabbits with the purified recombinant NFATp.
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31. In vitro transcription reactions were carried out in Namalwa nuclear extracts as described (*24*). Fos and Jun proteins and truncated NFATp (NFATpXS) were used at 500 μM, and plasmid templates linearized by Eco RI were used at 80 μg/ml. Transcripts purified from the reactions were analyzed by polyacrylamide gel electrophoresis (PAGE) and quantitated with the use of a phosphorimager.
32. We thank R. Robinson and M. Gordy for technical assistance and S. Harrison and L. Glimcher for critical reading of the manuscript. Supported by NIH grants CA42471 and GM46227 and a grant from Hoffmann–La Roche, Inc. (to A.R.), NIH grant NS25078 (to P.G.H.), and a grant from the Institute of Chemistry and Medicine funded by Hoffmann–La Roche, Inc. (to G.L.V.). P.G.M. is a Special Fellow of the Leukemia Society of America, J.J. is a Fellow of the Medical Foundation, and T.K.K. is a Fellow of the Helen Hay Whitney Foundation.

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Nonuniform Probability of Glutamate Release at a Hippocampal Synapse

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A change in the probability of neurotransmitter release (P_r) is an important mechanism underlying synaptic plasticity. Although P_r is often assumed to be the same for all terminals at a single synapse, this assumption is difficult to reconcile with the nonuniform size and structure of synaptic terminals in the central nervous system. Release probability was measured at excitatory synapses on cultured hippocampal neurons by analysis of the progressive block of *N*-methyl-D-aspartate receptor–mediated synaptic currents by the irreversible open channel blocker MK-801. Release probability was nonuniform (range of 0.09 to 0.54) for terminals arising from a single axon, the majority of which had a low P_r. However, terminals with high P_r are more likely to be affected by the activity-dependent modulation that occurs in long-term potentiation.

The probability of transmitter release (P_r) from individual synaptic terminals can be estimated from excitatory postsynaptic current (EPSC) amplitude fluctuations by the use of a statistical model of the release process (quantal analysis) (*1, 2*). This approach is complicated if P_r is not the same for all terminals (*2*). It is difficult to estimate P_r at central synapses because miniature EPSC amplitudes are close to the intrinsic recording noise level and are highly variable (*2, 3*). Furthermore, the assumptions underlying statistical models of transmitter release may not always be appropriate at central synapses (*2, 4*). For instance, the standard binomial model assumes that P_r is uniform at all synaptic terminals. To test directly for nonuniform P_r, we developed an alternative to quantal analysis.

Whole-cell recordings were made from single cultured rat hippocampal neurons that formed recurrent (autaptic) synapses (*5*). Recordings of *N*-methyl-D-aspartate (NMDA) receptor–mediated EPSCs were made before and during exposure to the NMDA open channel blocker, MK-801 (5 to 20 μM) (*6*). Channels were irreversibly blocked under our recording conditions (*7*). The MK-801 exposure increased the decay rate of the EPSC (Fig. 1, A and B) and, with repeated stimuli, progressively reduced its amplitude (Fig. 1A). The progressive block rate was measured by the fitting of a single exponential to the EPSC peak amplitude plotted against stimulation number. The rate of progressive block reflects, in part, P_r. If P_r is high then more terminals will release transmitter, more postsynaptic NMDA channels will open, and the progressive block should be more rapid. Consistent with this hypothesis, the progressive block rate was proportional to P_r (Fig. 1, C and D). Raising the calcium concentration increased the EPSC amplitude in the absence of MK-801 (Fig. 1C), and the progressive block rate in MK-801 (5 μM) increased proportionally (Fig. 1D) (*8*). Thus, the progressive block rate provides a relative measure of P_r. To obtain a quantitative measure of P_r, estimates of the time course of glutamate in the synaptic cleft, NMDA channel open probability (P_o) and MK-801 binding rate are also required. All these parameters have been measured (*6, 9–11*), but P_o was obtained from outside-out patch or whole-cell recordings that include extrasynaptic channels (*6, 11*). Therefore, we examined the P_o of synaptically activated NMDA channels.

Channel open probability has been calculated from the progressive block of NMDA currents by MK-801 (*11*), but this approach cannot be applied to synaptic currents because progressive block is also influenced by P_r. However, the faster decay rate of NMDA receptor–mediated EPSCs in the presence of MK-801 (Fig. 1B) can be used to estimate P_o. This rate can be used because the irreversible block of open channels early in the synaptic response prevents reopenings later in the response and thus accelerates the EPSC decay (*11*). This acceleration increases with increasing P_o. A chemical kinetic model (*9*) (Fig. 2A inset) was used to fit the time course of the synaptic current recorded in the absence and presence of MK-801 (5 μM) (Fig. 2A) (*12*). The channel opening rate was the only free parameter in the kinetic model that affected the change in decay rate produced by MK-801, and it had an optimum value of $12.4 \pm 0.7\ s^{-1}$ (mean ± SEM, $n = 11$). Channel open probability was then calculated from the opening and closing rates (r_o and r_c, respectively) with the equation $P_o = r_o/(r_o + r_c)$; P_o was 0.053 ± 0.003 ($n = 11$). The open probability of an NMDA channel at the peak of a synaptic response (P_o*) was also calculated from the optimum kinetic model to be 0.041 ± 0.003 ($n = 11$). This probability is less than P_o because some desensitization and agonist dissociation occur during the rising phase of the response. Our estimate of P_o* was significantly lower than that for channels in outside-out patches (P_o* = 0.27) (*11*). This discrepancy was not due to differences in the analysis procedures (*13*). The lower

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value of P_o* indicates that 60 NMDA channels are required at an individual terminal to produce a miniature EPSC with a peak amplitude of 5 pA (*14*). Control experiments suggested that the binding rate of MK-801 was accurate and consistently reached the synaptic cleft (*15*). In addition, MK-801 had no presynaptic effect and acted on a population of NMDA receptors with uniform P_o (*16*).

The estimate of P_o can be combined with the progressive block rate to estimate P_r quantitatively. The fraction of channels that are irreversibly blocked per stimulus determines the progressive block rate. At terminals where transmitter is released, the fraction of channels that are blocked can be estimated from the optimal kinetic model parameters (33 ± 3% at 5 μM MK-801; n = 11) (*17*). Thus, if all terminals released transmitter (P_r = 1.0), the predicted progressive block rate would be 33% per stimulus. Release probability can be calculated as the measured progressive block rate divided by the predicted progressive block rate, on the assumption that P_r = 1.0. In calcium (2.7 mM) and MK-801 (5 μM), the initial progressive block rate was 11.9 ± 0.7% per stimulus (n = 11) and P_r = 0.38 ± 0.07. A uniform P_r should lead to a single exponential progressive block. However, the data could not be fitted with a single exponential function because the sum of squared errors was 11 ± 2 (n = 6) times greater than for a double exponential fit (Fig. 2B). The two time constants were 5.5 ± 0.4 and 40 ± 5 stimuli (n = 6), consistent with two synaptic terminal types, one with P_r = 0.54 ± 0.05 and the other with P_r = 0.09 ± 0.02. The amplitude ratio of the fast to slow exponential components was 1.1:5.9 (n = 6), which indicates that high P_r terminals contributed more to synaptic transmission than low P_r terminals, although the latter were from 0.7 to 6.4 times more numerous (*18*). The progressive block rate remained proportional to EPSC amplitude as calcium concentration was varied (Fig. 1, C and D), suggesting that P_r was uniformly modulated by calcium and that terminals did not simply switch between high and low P_r states. Although the data were well fitted by two exponentials, the possibility of a continuous P_r distribution is not excluded. The finding of nonuniform P_r is not dependent on the kinetic model of the NMDA receptor or the estimate of P_o. These are only needed for a quantitative measurement of P_r.

The nonuniformity of P_r raised the possibility that its modulation by presynaptic receptors might also be nonuniform. We tested for nonuniform modulation using baclofen, which reduces P_r through presynaptic γ-aminobutyric acid ($GABA_B$) receptors (*19*). The EPSC amplitude reduction produced by baclofen (50 μM) was measured before and after a period of stimulation in the presence of MK-801 (5 μM) plus baclofen (50 μM) (Fig. 3). If baclofen reduces P_r more effectively at some terminals than at others, then NMDA receptors at baclofen-sensitive terminals should be partially protected from block during exposure to MK-801 plus baclofen. Thus, baclofen would be expected to reduce EPSC amplitude more effectively after block in MK-801 plus baclofen. Baclofen reduced EPSC amplitude by 64 ± 8% before block and by 84 ± 3% after block (n = 7, paired t test, P < 0.01) (Fig. 3, A and C), consistent with nonuniform modulation. The first time constant of progressive block in MK-801 plus baclofen was not significantly changed (4.8 ± 0.5 stimuli; n = 5) (Fig. 3B) relative to that of the control in MK-801 alone (5.5 ± 0.4 stimuli; n = 7), suggesting that a subpopulation of high-P_r terminals was insensitive to baclofen. In contrast, the first time constant of progressive block was slowed (13 ± 3 stimuli; n = 5) in low calcium (1.0 mM) (Fig. 1D), suggesting a uniform reduction of P_r at all high P_r terminals. A fast component of progressive block reappeared during continued block in MK-801 alone (Fig. 3D), consistent with a subpopulation of high P_r terminals that had been protected during the earlier period of MK-801 plus baclofen. Thus, baclofen powerfully depressed trans-

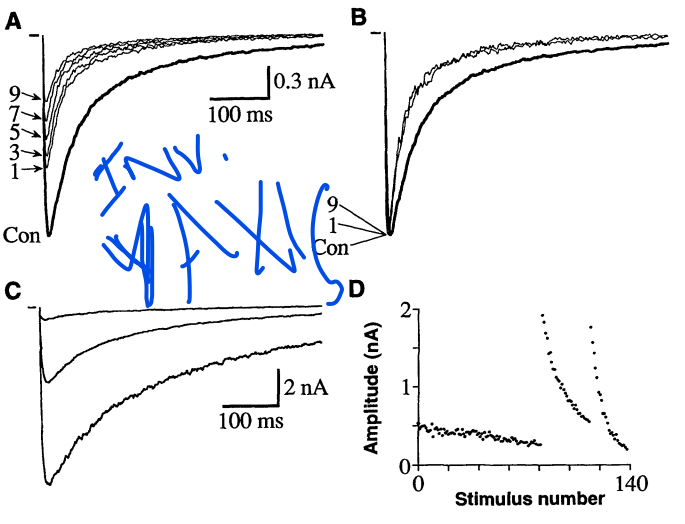

Fig. 1. (**A**) An NMDA receptor–mediated EPSC was recorded in the (thick trace, Con) absence and (thin traces) presence of MK-801 (5 μM). The first, third, fifth, seventh, and ninth EPSCs after the step into MK-801 demonstrate a progressive block of the response. (**B**) The first and ninth MK-801 EPSCs from (A) normalized to the EPSC in the absence of MK-801; scale as in (A). (**C**) The raising of calcium concentration increased the amplitude of an EPSC. The EPSC was recorded in the presence of (top trace) 0.5, (middle trace) 1.0, and (bottom trace) 2.7 mM calcium. (**D**) In the presence of MK-801 (5 μM) the progressive block rate of the EPSC became faster as the calcium concentration increased (0.5 mM, lower left trace; 1.0 mM, middle trace; 2.7 mM, far right trace). Data are from the same EPSC as in (C). For this synapse, the ratio of EPSC amplitudes in the absence of MK-801 was 1:6.0:14.2, and the ratio of the progressive block rates was 1:6.3:14.5, respectively. In other neurons, the ratio of the averaged progressive block rates was 1:5.6:14.3 (n = 4, 5, and 7 for calcium concentrations of 0.5, 1, and 2.7 mM, respectively).

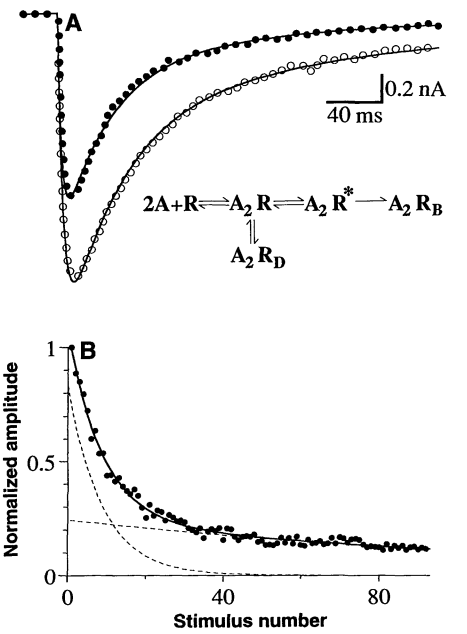

Fig. 2. (**A**) The NMDA P_o was obtained by fitting of the EPSC time course with a five-state kinetic model. An NMDA receptor–mediated EPSC recorded under (open circles) control conditions and (filled circles) in the presence of MK-801 (5 μM) is shown, together with optimally fitted transients from the kinetic model (solid traces). The P_o for this EPSC was 0.052. The inset shows the reaction scheme used to model the chemical kinetic properties of the NMDA receptor and its interactions with glutamate and MK-801: A, molecule of glutamate; R, NMDA receptor; R*, open state; R_D, desensitized state; and R_B, irreversibly blocked state. (**B**) Nonuniform P_r. An EPSC was recorded in the continuous presence of MK-801 (5 μM). Amplitude (filled circles) was plotted against stimulus number. The time course was well fitted with a double exponential function (solid trace). The fast and slow components of the exponential fit are shown as dashed traces.

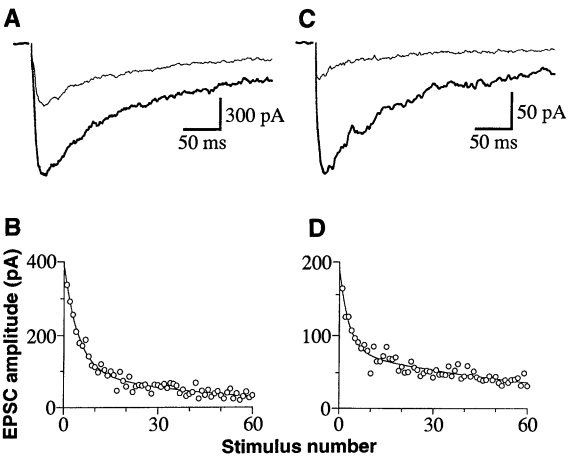

Fig. 3. (**A**) An EPSC recorded in the (thick trace) absence and (thin trace) presence of baclofen (50 μM). Baclofen reduced the EPSC amplitude by 55%. (**B**) Progressive block by MK-801 (5 μM) plus baclofen (50 μM) [same EPSC as in (A)]. EPSC amplitudes (open circles) were plotted against stimulus number and fitted with a double exponential function (solid trace). (**C**) After block by MK-801 plus baclofen, exposure again to baclofen (50 μM; thin trace) reduced the EPSC amplitude by 75% (thick trace). Data are from the same EPSC as in (A). (**D**) Continued progressive block by MK-801 (5 μM) alone. EPSC amplitudes (open circles) were fitted with a double exponential function (solid trace).

mitter release (>84%) at some terminals, whereas other terminals were unaffected.

The nonuniform P_r and nonuniform modulation observed in our experiments have important implications for studies of synaptic plasticity. Autaptic synapses are not present in the intact hippocampus but appear to be functionally indistinguishable from synapses between cultured neurons that have served as useful models of central excitatory synapses (*3, 10, 20*). The progressive block technique may be complicated by the poor diffusion of lipophilic MK-801 in hippocampal slices, because MK-801 (80 μM) did not initially reduce the EPSC amplitude (*21*) although this MK-801 concentration should produce a 90% amplitude reduction (*15*). The low P_r terminals we observed may correspond to the "silent" terminals postulated at spinal cord synapses (*22*). However, most studies have either not considered nonuniform P_r or have assumed uniform P_r (*23*).

The ~10-fold range of P_r values from the present study is comparable to the ~10-fold range of pre- and postsynaptic membrane specialization areas observed in the CA1 region of rat hippocampus (*24*). A larger terminal could have more vesicle docking and release sites, leading to higher P_r. If vesicle docking sites at an individual terminal function independently, multivesicular release will occur (*25*). Our results predict that 54% of release events from high P_r terminals and 9% of release events from low P_r terminals would be multivesicular (*26*). Nonuniform P_r may also influence the synaptic enhancement produced by different stimulation patterns. For example, long-term potentiation (LTP) can be induced by tetanic stimulation or by the pairing of one or a few presynaptic stimuli with postsynaptic depolarization. Tetanic stimulation would produce transmitter release from almost all terminals, whereas pairing would favor high P_r terminals because low P_r terminals are unlikely to be active. A selective enhancement of postsynaptic responsiveness at high P_r terminals would lead to an increased EPSC amplitude coefficient of variation, as observed after LTP (*23, 27*). A selective enhancement of release from large, high P_r terminals with large quantal amplitudes would lead to an increased average miniature EPSC amplitude (*28*).

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5. Hippocampal neurons from neonatal rats were dissociated and grown in cell culture on "microdots" similar to those described by M. M. Segal [*J. Neurophysiol.* **65**, 761 (1991)]. Whole-cell recordings were obtained with a patch-clamp amplifier (Axon Instruments) after the cells had been maintained for 1 to 2 weeks in culture. Currents were digitally sampled at 4 kHz and low-pass filtered at 2 kHz. Pipettes contained 150 mM potassium gluconate, 10 mM Hepes, 10 mM EGTA, 1 mM $CaCl_2$, 3 mM $MgCl_2$, 1 mM adenosine triphosphate, and 0.3 mM guanosine triphosphate (pH 7.25; 315 mosmol). Control extracellular solution contained 165 mM NaCl, 2.4 mM KCl, 0.5 to 3 mM $CaCl_2$, 10 mM Hepes, 10 mM D-glucose, 0.01 mM glycine, and 0.1 mM picrotoxin (pH 7.25; 325 mosmol). Reservoirs of media were connected by solenoid latching valves (General Valve, Fairfield, NJ) to a series of quartz flow pipes (inside diameter of 400 μm).
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7. An isolated neuron was voltage clamped (−70 to −80 mV), and brief (1 to 2 ms) depolarizing voltage steps were applied at 5- to 10-s intervals, evoking autaptic EPSCs in the majority of neurons. EPSC amplitudes were measured by averaging over the range 20 to 400 ms after stimulus onset, and the measurements were scaled so that the average EPSC amplitude equaled the peak amplitude of the ensemble average EPSC. Except where stated, all solutions contained 6-cyano-7-nitroquinoxaline-2,3-dione (5 μM) to block α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor–mediated EPSCs.
8. The analysis of progressive block was based on the assumptions that, for periods up to 30 min, P_r for a given terminal was constant and that MK-801 unbinding was negligible. In the absence of MK-801, EPSC amplitude declined during the first 20 stimuli but then stabilized. A regression line fitted from stimulus 20 to 90 had a slope not significantly different from zero ($P > 0.05$, $n = 4$). Progressive block experiments were performed after the EPSC amplitude had stabilized. The reversibility of MK-801 block was tested by the recording of EPSCs evoked for 80 to 200 stimuli after nearly complete (80 to 90%) MK-801 block. The slope of the EPSC amplitude regression line was not significantly different from zero ($P > 0.05$, $n = 4$), consistent with negligible MK-801 unbinding.
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12. A chemical kinetic model of glutamate and MK-801 binding at the NMDA channel was constructed. The parameters of the model were adjusted to fit simultaneously EPSCs recorded in the absence and presence of MK-801. Inaccuracies resulting from trial-to-trial amplitude fluctuations were reduced by the averaging of EPSCs (5 to 25). In the presence of MK-801, an exponential was fitted to EPSC amplitudes as a function of stimulus number, and the amplitude of the fitted exponential at the first stimulus was used to normalize the ensemble averaged EPSC. The kinetic model was based on the assumption that glutamate peaked at 1 mM and decayed exponentially with a 1-ms time constant (*10*). This time course results in 97% saturation of postsynaptic NMDA receptors (*10*). Glutamate binding and unbinding rates were fixed at previously determined values (*9*). A simplex optimization procedure was used to adjust the opening rate, the desensitization and resensitization rates, and the number of channels to give the best fit to the averaged EPSCs. The goodness of fit was assessed by means of a weighted sum of squared errors between the data and model transients. For each data point, the error was divided by the normalized amplitude of the model transient at that point. The closing rate was fixed at 220 s^{-1}, corresponding to the experimentally observed ~5-ms synaptic current rise time (20 to 80%) (*9*), and the MK-801 binding rate was fixed at 24 μM^{-1} s^{-1} (*6, 11*).
13. The estimate of P_o* was based, in part, on a 49 ± 8% ($n = 5$) reduction in the first time constant of EPSC decay in the presence of MK-801 (20 μM). This value contrasts with the reduction in decay time constant (86 ± 2%; $n = 8$) seen in outside-out patches after a brief pulse of glutamate (1 to 4 ms) (C. E. Jahr, personal communication). The larger effect of MK-801 on outside-out patches is consistent with a higher P_o.
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15. The reduction in the NMDA EPSC peak amplitude produced by a given concentration of MK-801 is primarily dependent on the MK-801 binding rate. This rate can be independently predicted from the reduction in NMDA channel mean open time (MOT) that results from MK-801 block. This prediction is possible because EPSC amplitude is proportional to P_o = MOT/(MOT + MCT), where MCT equals the mean closed time. A $P_o << 1$ indicates that MCT >> MOT, and EPSC amplitude is approximately proportional to MOT. In control conditions, MOT is limited by the closing rate and equals $1/r_c$. When MK-801 is added, $MOT_{mk} = 1/\{r_c + [MK\text{-}801]\ r_{bind}\}$, where r_{bind} equals the MK-801 binding rate to open channels. Thus, EPSC amplitude reduction in MK-801 ≈ $1 - (MOT_{mk}/MOT)$. Setting $r_c = 220\ s^{-1}$, $r_{bind} = 24\ \mu M^{-1}\ s^{-1}$, and [MK-801] = 5 μM gives an amplitude reduction of about 35%. This value is slightly

different from the kinetic model prediction (32%) because of non-equilibrium considerations. At 80 μM MK-801, amplitude reduction ≈90%.

16. In the presence of DL-2-amino-5-phosphonovalerate [DL-AP5 (100 μM)] to block NMDA receptors, the AMPA receptor–mediated EPSC was unaffected by MK-801 (5 μM; $n = 6$), demonstrating that MK-801 did not have a presynaptic action. The assumption that all NMDA channels have the same P_o was tested as follows: If glutamate activates a mixed population of NMDA channels, then those with higher values of P_o will be preferentially blocked. This relation will bias the population toward lower P_o channels as more channels are blocked, and the decay of the EPSC should be slowed. However, the EPSC decay remained constant, even after >50% block by MK-801 (Fig. 1B), suggesting that P_o was similar for all synaptic NMDA channels.
17. With the optimum kinetic model parameters for each EPSC, the model simulated two EPSCs that were evoked 10 s apart in the continuous presence of 5 μM MK-801. The relative amplitude reduction of the second response gave the proportion of channels blocked during the first response.
18. The ratio of the number of high to low P_r terminals is equal to the ratio of the area under the fast to that under the slow exponential component of progressive block. These ratios are equal because the time constant of an exponential component is proportional to P_r and because its amplitude is proportional to the number of terminals and inversely proportional to P_r. Thus, the exponential amplitude multiplied by the time constant (or the area under the exponential) is proportional to the number of terminals and is independent of P_r. The ratio of the efficacy of high to low P_r terminals is equal to the ratio of the amplitude of the fast to that of the slow exponential component.
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26. At a single presynaptic terminal, the probability that a given release event consists of two or more vesicles is equal to the conditional probability that a second vesicle is released, given that the first is released. If vesicle docking and release sites function independently and if there are many sites per terminal, then the release of one vesicle will not significantly affect the probability that a second vesicle will be released. Thus, the probability that a given release event is multivesicular is approximately equal to P_r.
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Functional Stoichiometry of Shaker Potassium Channel Inactivation

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Shaker potassium channels from *Drosophila* are composed of four identical subunits. The contribution of a single subunit to the inactivation gating transition was investigated. Channels carrying a specific mutation in a single subunit can be labeled in a heterogeneous population and studied quantitatively with scorpion toxin sensitivity as a selection tag. Linkage within a single subunit of a mutation that removes the inactivation gate to a second mutation that affects scorpion toxin sensitivity demonstrates that only a single gate is necessary to produce inactivation. The inactivation rate constant for channels with a single gate was one-fourth that of channels with four gates. In contrast, the rate of recovery from inactivation was independent of the number of gates. It appears that each of the four open inactivation gates in a Shaker potassium channel is independent, but only one of the four gates closes in a mutually exclusive manner.

In the first quantitative theory of Na^+ and K^+ channel gating, Hodgkin and Huxley postulated that multiple gating particles existed and the overall conductivity depended on the positions of all the particles (*1*). Now, with our growing understanding of the proteins that make ion channels, we can begin to attach physical meaning to the notion of multiple gating particles. We asked how inactivation gating in Shaker K^+ channels depends on the movements of individual inactivation gates contributed by each of the four identical subunits.

The best understood gating transition is N-type inactivation in Shaker K^+ channels, the spontaneous closing that occurs after voltage-dependent channel opening. The NH_2-terminus of the Shaker channel causes inactivation by forming a cytoplasmic gate, like the "ball and chain" proposed by Armstrong and Bezanilla to explain inactivation in Na^+ channels (*2–4*). The Shaker K^+ channel consists of four identical subunits. It is not known whether the channel has four separate inactivation gates, one from each subunit, and if so, whether more than one gate is required to produce inactivation. Peptides corresponding to part of the NH_2-terminus of a Shaker K^+ channel can aggregate to form multimers (*5*), raising the possibility that a single gate could be formed through the coassembly of four NH_2-termini.

We studied the number and independence of inactivation gates by exploiting the channel's susceptibility to a scorpion toxin (*6*). A mutation of Asp^{431} to Asn (D431N) affects toxin sensitivity in a recessive manner: the mutation must be present in all four subunits to render the channel insensitive (*7*). Thus, if wild-type and D431N mutant subunits are coexpressed, channels with four mutant subunits can be distinguished from channels containing at least one wild-type subunit because a single wild-type subunit confers toxin sensitivity.

Scorpion toxin inhibits by binding at the extracellular face of the channel, whereas the inactivation gate, formed by the NH_2-terminus, is located on the intracellular side of the membrane. Toxin sensitivity and inactivation gating are independent; mutations affecting one property do not affect the other. We therefore used the recessive nature of the mutation at position 431 to ask if only a single gate can cause inactivation. The approach is illustrated in Fig. 1A. The sketch shows the heteromultimeric channels that can result from the coexpression of two different subunits. If an inactivation gate is present only on the toxin-sensitive subunit, then by applying toxin to the population of channels we can determine whether a single gate is sufficient for inactivation. Channels with at least one gate will be blocked because the gate is linked to the toxin-sensitive subunit. Therefore, if a single gate produces inactivation, then all of the toxin-sensitive channels will inactivate and the insensitive channels will not. In such an experiment, some of the channels in the mixed population inactivated and some did not (Fig. 1B). When toxin was applied, the entire inactivating component was blocked and the sustained component was insensitive (Fig. 1C). This outcome exactly matches our expectation if a single gate causes inactivation.

If subunits without an intact inactivation gate were unable to coassemble with the wild-type subunits, then the channels expressed in the experiment described above would have either four gates or no gates; the experiment of Fig. 1 (A through C) does not exclude this possibility. By

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