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Definition of the Readily Releasable Pool of Vesicles at Hippocampal Synapses

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Summary

A readily releasable pool of quanta, tentatively identified with docked synaptic vesicles, has been defined by analysis of the neurotransmitter release caused by application of hypertonic solutions. The goal of this work is to determine the relationship of this functionally defined readily releasable pool to the one drawn upon by action potential-evoked release. We find that hypertonic solutions do not act through changes in intracellular calcium. Since the release produced by action potentials and hypertonic solutions varies in parallel as the pool size is changed, we conclude that the same pool is shared by both mechanisms. This conclusion, taken together with other observations in the literature, means that the synaptic release probability depends on the size of the readily releasable pool.

Introduction

A pool of readily releasable quanta has been defined for hippocampal synapses (Stevens and Tsujimoto, 1995) and retinal amacrine cell synapses (Borges et al., 1995) in culture. This pool consists of about 12 quanta per synapse, and when the pool has been completely depleted, replenishing it takes about 10 s in hippocampal synapses. One method used for defining the readily releasable pool (Stevens and Tsujimoto, 1995) was to apply hypertonic solution (Bekkers and Stevens, 1992) to a small population of identified hippocampal synapses for several seconds, and to count every quantum that was released. When a hypertonic solution is first applied, the quantal release rate jumps rapidly to a relatively high level (about 10 quanta per second per synapse) and then declines approximately exponentially to a low, steady level. The readily releasable pool is defined as those quanta that are released during the transient burst of exocytotic activity following application of hypertonic solution. When pairs of hypertonic solution applications are used, then the initial peak release rate is depressed at short reapplication intervals and returns exponentially to its high, resting level (10 quanta per second per synapse) with a time constant on the order of 10 s. Stevens and Tsujimoto (1995) interpreted each quantal response as exocytosis of a single synaptic vesicle, and tentatively identified the readily releasable pool with the population of docked and fusion competent ("cocked") vesicles (Südhof, 1995); the recovery after depletion of the pool was interpreted as a refilling of empty docking sites with vesicles ready to undergo exocytosis.

Since the stimulation method used to define the readily releasable pool involved the local application of hypertonic solution rather than action potentials, the relation of the pool thus defined to the population of quanta usually drawn upon by action potentials is unknown. The main goal of the work reported here is to establish this relationship between the readily releasable pools identified by these distinct means of synaptic activation (action potentials versus hypertonic solution). We shall conclude that the pool of quanta defined with stimulation by hypertonic solution is the same as that employed when release is evoked by action potentials. This conclusion has an important corollary: if an active zone can release at most one quantum (Zucker, 1973; Redman, 1990; Korn and Faber, 1991; Stevens and Wang, 1995), then the probability that a quantum is released is related to the size of the readily releasable pool (Zucker, 1973).

Results

Experiments were carried out using whole-cell recording from rat hippocampal neurons grown in tissue culture for 1–2 weeks. Autaptic connections, which express the same physiological properties as usual synapses (Bekkers and Stevens, 1991), were studied in most cases to insure that the same population of synapses was investigated whether synapses were activated by action potentials or by hypertonic solution. We have applied solutions made hypertonic by adding sucrose. For convenience, we shall refer to these solutions in terms of the degree of hypertonicity. For example, "100 mOsm" would indicate normal extracellular saline solution, to which 100 mOsm/liter of sucrose has been added; this solution would be hypertonic by 100 mOsm.

MEPSC Rate Depends on Tonicity

To use hypertonic solutions as a tool for investigating release mechanisms and the vesicle pool from which they draw, we need to establish a dose/response curve, the relationship between miniature excitatory postsynaptic current (MEPSC) occurrence rate, and the hypertonicity of the superfused solution. When hypertonic solution is applied to the boutons on the entire dendritic tree of a neuron, an initial high rate of quantal release induced by the solution declines exponentially to a low, steady level in the continued presence of the hypertonic solution (Figure 1A). As illustrated in this figure, increasing the hypertonicity from 100 mOsm to 500 mOsm has a profound effect on release rate. At the highest hypertonicity, 500 mOsm, individual MEPSCs are poorly resolved, and one simply sees a current transient with superimposed MEPSCs. Stevens and Tsujimoto (1995) demonstrated that this current was composed of superimposed MEPSCs, but the region of application in this earlier study was much smaller than the area here where

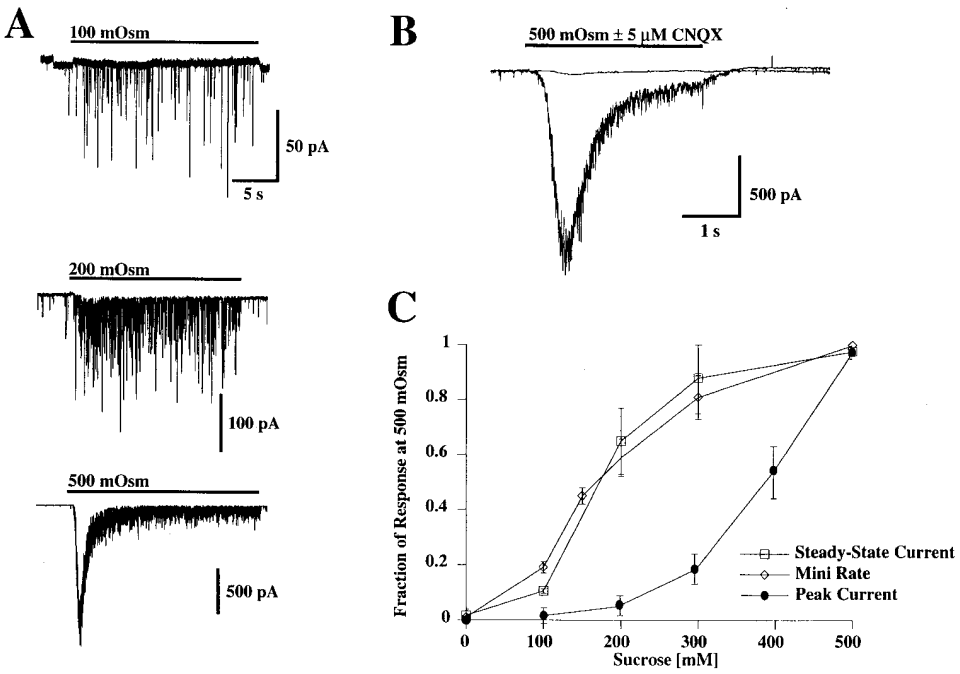

Figure 1. Release of MEPSCs by Application of Hypertonic Solution

(A) Whole-cell responses from a cultured rat hippocampal neuron (10 days in vitro) evoked by applications of 100, 200, and 500 mM sucrose added to the saline bathing the cells. Holding potential −60 mV.

(B) Responses evoked in another cell by application of 500 mM sucrose (thick lower trace) are blocked in presence of CNQX (upper thin trace).

(C) Dose-response relationship of miniature EPSC rate (open circles), steady-state current (open squares), and peak response (closed circles) normalized to the response at 500 mM. The peak response was estimated by averaging the synaptic current for a period of 50 ms around the time of the maximal initial response.

most of the dendritic tree was superfused with the hypertonic saline. To confirm that the currents illustrated in Figure 1A are indeed of entirely synaptic origin, we applied 5 μM 6- Cyano-7-nitroquinoxaline-2,3-dione (CNQX) to block the responses of the non-NMDA receptors. As shown in Figure 1B, this treatment abolished the current produced by the 500 mOsm solution. Results like those illustrated in Figure 1B were found in five cells.

In Figure 1C, the initial peak synaptic current, the steady-state MEPSC rate, and the steady-state average current, all produced by application of the hypertonic solution, are plotted as a function of hypertonicity of the "release" solution. The data in this figure were derived from 5–10 measurements per point for 16 cells. The S-shaped dose response relationship that results (Figure 1C) provides a standard to be used in later parts of this investigation. Note that the peak release rate is shifted to the right of the steady-state release curve so that half-maximal steady-state release requires about 175 mOsm, whereas the half-maximal peak release rate occurs at greater than 350 mOsm (the response appears not to be saturated at 500 mOsm). Similar curves for the steady state release as a function of osmolarity have been reported by Blioch et al. (1968) and Hubbard et al. (1968).

Hypertonic Solution-Evoked Release Is Not Calcium Mediated

For two reasons, we suspected that calcium ions might not be involved in the exocytosis caused by an hypertonic environment. First, a number of authors (Hubbard et al., 1968; Blioch et al., 1968; Quastel et al., 1971; Shimoni et al., 1977) have found that hypertonic solution-produced release is not dependent on extracellular calcium concentration at the neuromuscular junction. Second, Geppart et al. (1994) observed that neurotransmitter release produced by hypertonic solution is normal in mutant mice lacking a functional synaptotagmin 1, whereas calcium-mediated release is severely attenuated in these animals. On the basis of the experiments described below, we conclude that hypertonic solution does not act through a calcium-dependent mechanism.

As might be expected from the earlier work cited above, we found that application of hypertonic solutions very effectively evoked release even with low extracellular calcium concentrations (100 mM; n = 6, data not shown). Since the synaptotagmin mutant animals still release normally at low levels of intracellular calcium (it is the rapid release to higher levels of intracellular calcium that is impaired in these animals [Geppert et al., 1994]), we needed to determine if influx of very small amounts of calcium could mediate the release we observe, or if neurotransmitter release might depend on liberation of calcium ions from intracellular stores.

If calcium influx is involved in the release produced by hypertonic solutions, then this release should be prevented, or at least diminished, by blocking calcium channels. Therefore, we examined the effect of 0.2 mM Cd ions on the release produced by hypertonic solution, and found in experiments on five cells that release is unchanged when calcium channels are blocked (Figure 2C).

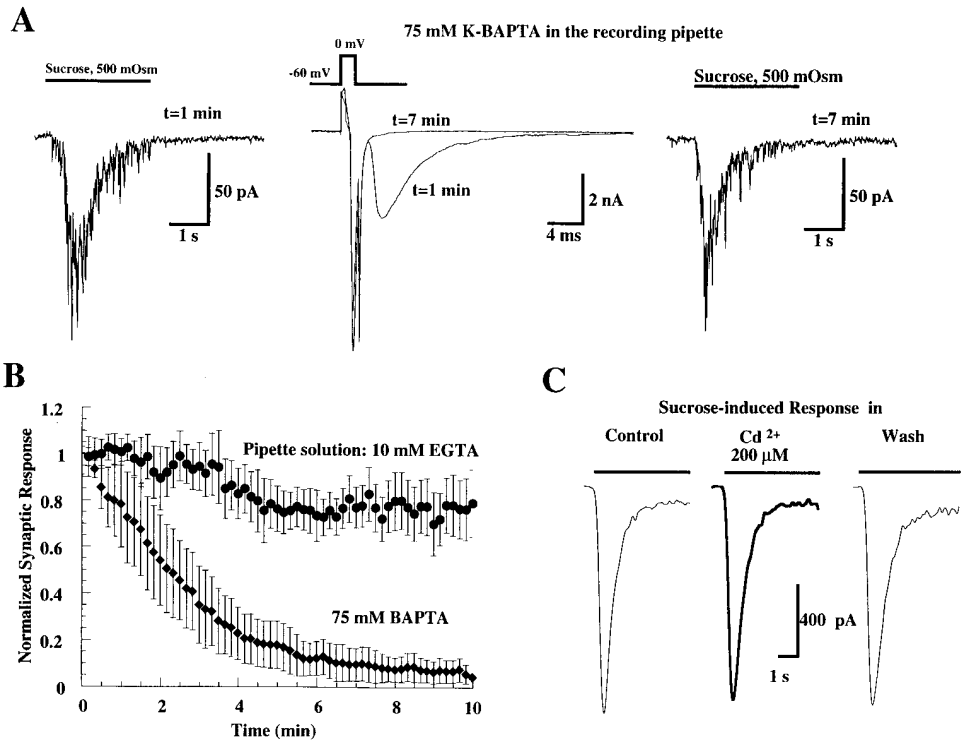

Figure 2. Release Activated by Application of Hypertonic Solution Is Independent of Intra- and Extracellular Calcium

(A) Left and right panels show responses to hypertonic solution (500 mM) at 1 and 7 min of whole-cell recording. K-gluconate was replaced by 75 mM K-BAPTA in the pipette solution. The action potential-evoked autaptic EPSC (middle panel) was blocked, whereas the response to hypertonic solution (left and right trace) was hardly affected.

(B) Time course for decline of the action potential-evoked synaptic response for dialysis with 10 mM EGTA (closed circles, data from eight cells), or 75 mM BAPTA (closed diamonds, data from five cells).

(C) Responses to hypertonic solutions are not inhibited when $CdCl_2$ (200 μM) was added to control and hypertonic solution. Compare the middle record with the control records at the left and right. Note that the higher frequency components of the signal, above 100 Hz, were attenuated for clarity.

Since neither removing extracellular calcium nor blocking calcium channels altered the exocytosis rate in the experiments just described, we conclude that the calcium influx mechanism plays a negligible role in the release produced by hypertonic solution. The alternative calcium- mediated mechanism for hypertonic solution evoked exocytosis involves calcium release from internal stores. We have used two approaches to investigate this possibility.

First, we have added sufficient quantities of BAPTA (75 mM) to the whole-cell recording pipette solution (experiments on five cells) to block almost all action potential-evoked synaptic transmission after the BAPTA diffuses to the axon terminals, a process that takes about 10 min (Figure 2B). As can be seen in the sample records in Figure 2A (left and right), when action potential-evoked release is almost completely blocked, the response to hypertonic solution (500 mOsm) is hardly affected.

Second, we have employed various pharmacological agents that should modify effects of internal calcium stores by depleting these stores or preventing release from them. As illustrated in Figure 3B, caffeine, 2,5-Di (t-butyl)-1, 4-hydrochinone (BHQ), ryanodine, and thapsigargin all were without appreciable effect on the release produced by hypertonic solution, although caffeine should cause release from internal stores, and BHQ, which should block release from internal stores, both produced a modest decrease in the size of the action potential-evoked EPSC, an effect illustrated for BHQ in Figure 3A. The mechanism of these effects is unclear, but may indicate a minor action of these drugs on calcium channels.

Various neuromodulatory agents modify transmitter release, possibly through effects on calcium channels. For example, baclofen, a $GABA_B$ agonist, decreases transmitter release at excitatory synapses and also downregulates calcium channels (Nicoll and Alger, 1979; Augustine and Charlton, 1986), as does the activation of presynaptic metabotropic glutamate receptors (Forsythe and Clements, 1990; Lester and Jahr, 1990). Thus, we also checked to see if agents that modulate transmitter release modify the response to hypertonic solutions. Baclofen (50 μM) decreased the action potential-evoked release to 34.4% ± 6.3% ($p < 0.01$) of its initial value, while the response to hypertonic solution was unaffected (98.0% ± 4.5% of its initial value; $n = 8$). Trans-(1S,3R)-ACPD (10 μM) gave similar results ($n = 4$).

Since hypertonic solution appears not to act through a calcium-mediated release mechanism, how does it work? Application of hypertonic solution produces a visible decrease in the volume of neuron cell bodies and processes in the region of application. The increase in

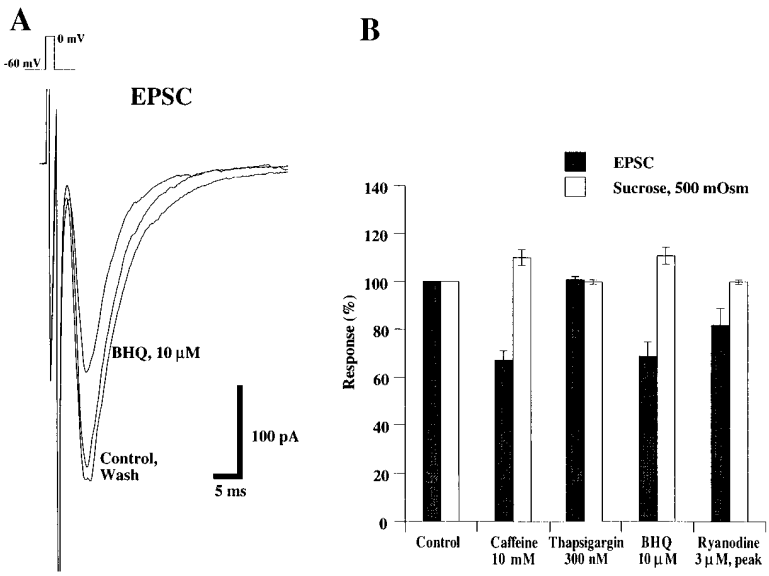

Figure 3. The Effect of Various Modulators of Intracellular Calcium Stores on Action Potential and Hypertonic-Evoked Responses

(A) 10 μM BHQ produces a reversible inhibition in the amplitude of synaptic currents.

(B) Summary of effects produced by various chemical modulators on the responses evoked by action potentials (closed bars) and hypertonic solution (open bars). The inhibition of the EPSCs by BHQ and caffeine are statistically significant ($p < 0.01$). Responses were normalized to the control response obtained before application of the indicated pharmacological agent. Each data set is from 5–9 measurements. Error bars represent SEM.

exocytosis rate might therefore simply have a mechanical cause: the energy barrier that must be surmounted for a fusion event to occur could be lowered by the deformation of the active zone that attends hypertonic induced volume changes (see Discussion).

Hypertonic Solution Modifies Nerve Impulse-Evoked Neurotransmitter Release

The overall goal of the experiments reported here is to determine the extent to which the readily releasable pool of quanta defined by hypertonic solution-evoked release coincides with the pool of quanta (that we assume are individual synaptic vesicles) available for action potential-produced release. The general strategy has been to deplete the pool of readily releasable vesicles by application of hypertonic solution and then to assay the pool size and recovery time course by action potential-evoked release. If the vesicular pools drawn upon by the two release mechanisms coincide, then depletion of the pool defined by application of hypertonic solution should produce a parallel depletion and refilling of the pool used by action potential-evoked release. Of course, the reverse should also be true: depletion of the readily releasable pool by repeated action potentials should simultaneously exhaust the pool of vesicles needed for release by hypertonic solution. The experiments that are described below examine the extent to which the pools drawn upon by the two modes of synaptic activation (hypertonic solution and action potentials) are coextensive, and we conclude that they mostly are.

An initial test of the notion that both modes of stimulation draw on the same pool of readily releasable vesicles is to combine simultaneous nerve stimulation and application of hypertonic solution. Since we know that hypertonic solution increases release probability, if action potentials and hypertonic solution are acting at the same release sites, one might expect that the number of quanta released by an action potential would be increased when hypertonic solution is present, at least for a period of time before significant depletion of the available quanta has a chance to occur. This is indeed what is observed as had previously been reported for the neuromuscular junction (Hubbard et al., 1968; Blioch et al., 1968). We start with a description of such an experiment.

When a 300 mOsm solution is rapidly superfused over synapses on the dendritic tree of a neuron, and the same synapses (autapses) are activated during the superfusion by producing action potentials in soma of a neuron, the size of the action potential-evoked postsynaptic response increases transiently. This is illustrated in Figure 4A (and in Figure 4B, on a faster time base for a different cell) with specimen-autaptic currents evoked at various times before and after the onset of the hypertonic solution superfusion. Note that in Figure 4A, the first autaptic current after the hypertonic solution application onset is nearly the same size as the control current, but that the second synaptic current (elicited about 400 ms after the onset of superfusion) is increased by about 40%. After the maximum synaptic current is reached, the size of the evoked EPSC systematically declines (Figure 4A), a phenomenon that we shall ascribe to depletion of the readily releasable pool. In Figure 4C, the average facilitation and depression produced by 300 mOsm and 500 mOsm solutions is illustrated for a population of cells. As hypertonic solutions have no effect on MEPSC amplitude (Stevens and Tsujimoto, 1995) and a single hippocampal synapse releases at most one quantum (Stevens and Wang, 1995), we interpret these response size changes as modifications of release probability. The 300 ms to 400 ms delay from the onset of superfusion to the increased transmitter release is probably the result of the time it takes the hypertonic solution to act; we note that changes in cell volume do not occur instantly, but rather with a noticeable delay.

Our initial interpretation of the experiments illustrated in this figure is that the increase in release probability reflects a synergistic action of hypertonic solution and calcium (from influx through voltage-activated calcium channels) on the exocytotic apparatus, and that the subsequent decline in release probability signals depletion

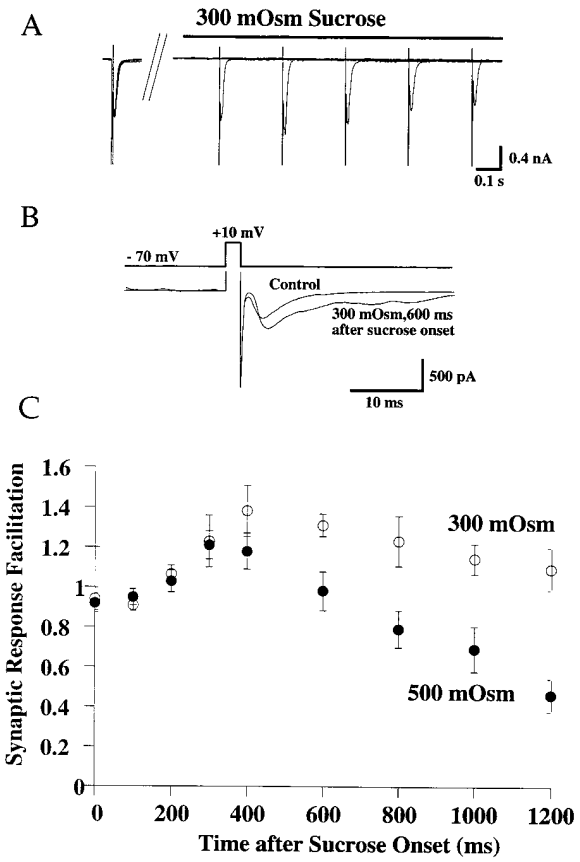

Figure 4. Transient Potentiation and Depression of Hypertonic Solution on Transmitter Release Evoked by Action Potentials

(A) Superimposed specimen records from a cell illustrating the facilitation and depression of action potential-produced release caused by hypertonic solution. The times that each record was taken relative to the onset of superfusion with hypertonic solution is indicated by the position of the record. Note that the large, initial downward transient is the capacitance current produced by the stimulating voltage change and that the autaptic current is the second downward (inward) current.

(B) Superimposed specimen records taken before and 600 ms after the onset of superfusion by a 300 mOsm solution with expanded time base (as illustrated by the calibration bars).

(C) Averaged data from a population of cells illustrating the facilitation and depression of action potential-produced release at various times after the application of hypertonic solution. Action potentials were produced by brief soma depolarization at times from 0–1200 ms after the start of application of 300 mOsm (open circles) or 500 mOsm (closed circles) solution, and the amplitude of the synaptic current was normalized to the size of the response obtained 3 s before the application of the hyperosmotic solution. Data points are averages of 4–7 responses from eight cells for the 300 mOsm solution and 4–9 responses from nine cells for the 500 mOsm solution; error bars represent the SEM. The baseline was determined from the average current 15 ms before the EPSC and the EPSC magnitude was estimated by averaging the first 15 ms of the EPSC.

of available vesicles. We interpret the smaller increase in action potential-evoked release produced by the 500 mOsm solution to the more rapid depletion produced by this solution. We now turn to a more detailed examination of the increase and subsequent decline of response size.

Although hypertonic solution does not cause release by a calcium-mediated mechanism, application of hypertonic solutions might alter calcium channel properties so that the increase or decrease in release probability described in Figure 4 could reflect effects on calcium channels (and consequently on calcium influx) rather than on the release process itself. To investigate this possibility, we examined somatic calcium currents before, during, and after application of a 300 mOsm solution. As seen in Figure 5C, the hypertonic solution decreases the calcium current magnitude moderately (about 30%). The voltage steps from −60 to +10 mV used to evoke calcium currents were presented at 3 Hz, so the time course with which calcium currents decrease can be followed on a time scale that is short, compared with the changes the release probability illustrated in Figure 4. The time course of hypertonic solution effects on calcium current magnitude is shown in Figure 5D, where it is seen that the decrease in calcium current occurs mostly within 333 ms and that the current amplitude then remains constant through a 5 s period of hypertonic solution application. Insofar as the somatic calcium channels are typical of those in the presynaptic membrane, we can conclude that the increase in action potential-evoked release produced by hypertonic solution is not a calcium channel effect because the observed inhibition in calcium currents would predict a decrease rather than an increase in the release probability.

Furthermore, the continuous decrease in release probability seen in Figure 4 cannot be mainly due to the inhibition of calcium currents (assuming that somatic currents are typical), because the time course for decline in release is slower than the effect on calcium currents. Of course, the facilitation of release caused by hypertonic solutions may be underestimated in Figure 4 because of a competing decrease in calcium current.

We turn now to a more detailed examination of the inhibition of transmitter release produced by hypertonic solutions applied for periods longer than the duration of the facilitatory effect just described. In Figure 5A, the size of the action potential-evoked synaptic current is plotted as a function of time, during an experiment in which hypertonic solutions were applied at various times as indicated in the figure. When hypertonic solution is present (with steady-state release), the action potential-evoked neurotransmitter release is diminished in a dose-dependent manner, with about a 30% decrease with a 100 mOsm solution, and a 80% decrease with a 300 mOsm solution. As seen in Figure 5B, the MEPSC release rate, the steady-state average synaptic current produced by the hypertonic solution, and the inhibition of the action potential-evoked synaptic current all vary in parallel as the amount of hypertonicity is changed; this parallel change is most easily interpreted by assuming that inhibition of action potential-evoked release and hypertonic solution- produced exocytosis employ the same final common path.

If the same pool of releasable vesicles is shared by the two release mechanisms (hypertonic solution-and action potential-evoked), the release should recover in parallel for both after the depletion of the releasable pool. As seen for the specimen records in Figure 6A, when a depolarization/hypertonic solution pulse pair is

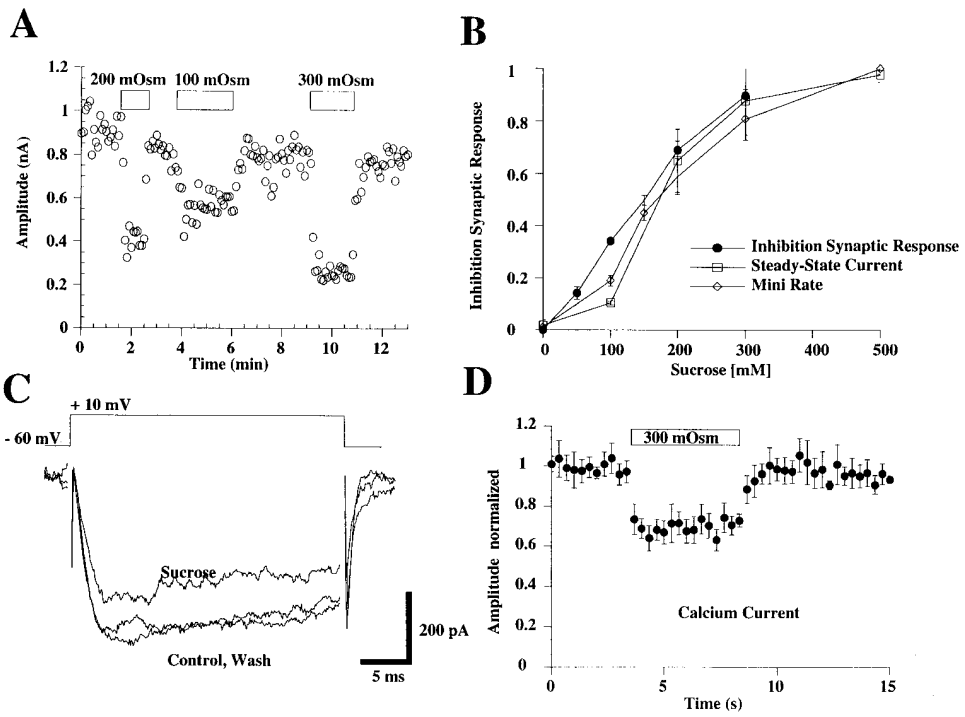

Figure 5. Steady-State Effects of Application of Hypertonic Solution on Transmitter Release

(A) Amplitude of autaptic postsynaptic currents evoked at a frequency of 0.2Hz. Open bars indicate the time of application of the hypertonic solution with the indicated hypertonicity.

(B) Dose-response relationship for the inhibition of synaptic release by sucrose. Filled circles give the relative inhibition of the response to an action potential generated in the soma. For comparison, steady-state current (open squares) and MEPSC rate (open circles) from Figure 1C are plotted. Note the slight left shift of the synaptic inhibition curve.

(C) Calcium currents are inhibited by hypertonic solutions. Depolarization from −60 to +10 mV evoked an inward current in solutions containing 5 mM $BaCl_2$, 3 mM $CaCl_2$, 10 mM TEA, 1 μM TTX. These currents were blocked by 200 μM $CdCl_2$ (data not shown).

(D) Time course of inhibition of calcium currents when 300 mOsm hypertonic solution is applied. Currents were evoked every at 3 Hz. Data averaged for six cells. Points give SEM. Neurons were 5–7 days in culture.

followed by a second pair 5 s later, the second pair of responses are both decreased. The time course of this phenomenon is shown in Figure 6B; the relative size of the response to voltage stimulation and to an application of 500 mOsm solution are plotted as a function of time. As is apparent from this figure, both responses recover in parallel, the expected result for a shared pool of readily releasable quanta.

Repeated action potential-evoked releases also produces apparent depletion, as illustrated in Figure 7A, where the EPSC amplitude is plotted as a function of time during 20 Hz stimulation. As can be seen in the figure, the EPSC amplitude approaches a low value after about 10 stimuli. Specimen records in Figure 7B, taken at 3 s and 60 s during the recovery from such a action potential-produced depletion, document that the recovery of responses to voltage stimulation (left) and application of hypertonic solution (right) proceed in parallel. The average time course of this effect (from experiments on 10 neurons with 3–7 observations for each data point) is shown in Figure 7C where it can be seen that when the readily releasable pool is depleted by rapid stimulation of the soma, both the action potential- and hypertonic solution-evoked release recover with the same time course. Pooled data from 25 experiments are shown in Figure 7D, where the inhibition of the action potential-evoked response is shown to be proportional to the inhibition of the hyperosmotic-evoked response, as the cell recovers from partial depletion by voltage stimulation.

Experiments of the type illustrated in Figure 7 offer an additional test of the notion that the readily releasable pool is shared by hypertonic and the usual release mechanisms. With the action potential-evoked release illustrated in Figure 7A, the EPSC amplitude declines with repeated stimuli owing to depletion. If each synaptic current is integrated to give the charge transferred, and if this charge transfer is added up for all of the stimuli, we should find a number that is proportional to the pool size (the postsynaptic charge transfer produced by the entire pool). If the pool drawn upon by hypertonic solution is the same one, then the total charge transfer produced by the application of hypertonic solution should be the same as that from action potential-produced depletion. When the charge transfer during depletion caused by the two methods is compared, we find that the total postsynaptic charge transfer produced during action potential-produced depletion is 77% ± 15% of that for hypertonic solution-produced depletion ($n = 6$). A single action potential does not deplete very

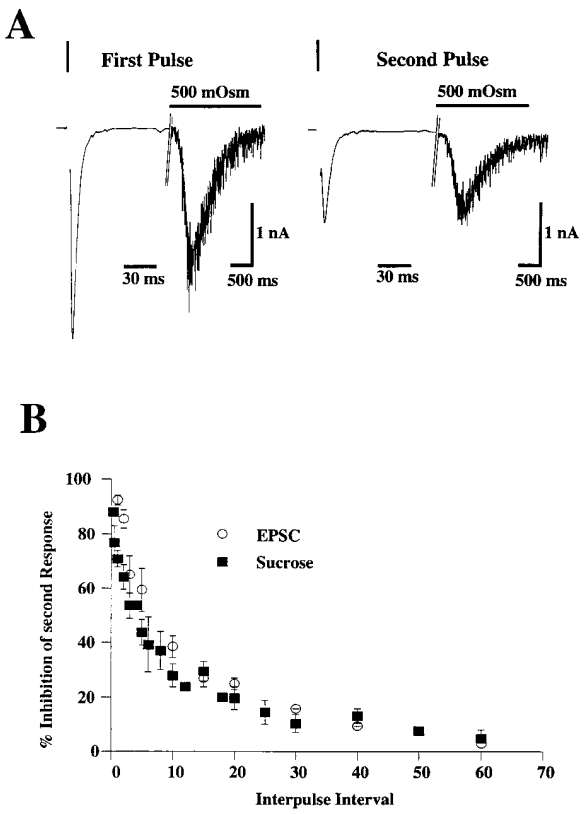

Figure 6. Time Course of Paired-Pulse Inhibition of Action Potential- and Hypertonic Solution-Evoked Responses

(A) A pair of autaptic responses evoked by brief depolarization of the soma immediately followed by a 2 s application of 500 mOsm solution at a given (5 s) interval. Note that the EPSC and the response to hypertonic solution application are shown on a different time scale.

(B) Plot of the time course of recovery from inhibition produced by hypertonic solution as in (A) of the EPSC (open circles) and response to hypertonic solution (closed squares) as a function of the time between applications of hypertonic solution (measured in seconds). Peak synaptic currents were measured by averaging currents 15 ms (action potential-evoked) and 50 ms (hypertonic solution-evoked) around the maximal response. Error bars represent SEM. Each data point consists of 3–12 recordings. Multiple measurements on one cell were done in an interval of 2 min.

much of the readily releasable pool: the charge transfer for an isolated EPSC (3 mM Ca, 1 mM Mg) is 4.3% ± 2.3% of the total pool charge (n = 8) defined by hypertonic solution. These measurements are subject to a number of errors: charge transfer is strongly influenced by errors in determining the baseline and also in nonsynaptic currents, such as the capacitative currents associated with the stimulation of action potentials. Also, the amount of refilling during depletion can be different during a train of action potentials than during a application of hypertonic solution of a similar duration. Since these errors are difficult to measure, we believe our estimates of charge transfer can be in error by perhaps a factor of two. Within the uncertainty of our measurements, the pool sizes estimated in this way agree.

If the readily releasable pool is shared by both release processes, release should occur at the same sites for both. And, if the same release sites are used, so should be the same postsynaptic receptors. To test this notion, we blocked NMDA receptors by producing action potential-evoked release in the presence of Dizocilpine maleate (MK-801), and then tested the extent to which the response of NMDA receptors to hypertonic solution (in the presence of CNQX) had also been blocked. As would be expected, the EPSC recorded with a nominally zero extracellular concentration of magnesium has two components, a rapid CNQX-sensitive one and a slow component that reveals the NMDA receptor channel function (Figure 8A, left). When action potential-evoked release occurs in the presence of 10 μM MK-801, the CNQX sensitive component is unchanged, but the NMDA receptor component declined steadily to a low level as MK-801 blocked the NMDA receptors. The time course of this block is illustrated in Figure 8B, and a sample record after block is seen in Figure 8C (left); compare this record with the control EPSC shown in Figure 8A (left). When the response to hypertonic solution is tested in the presence of CNQX before and after block of NMDA receptors by the MK-801, we find a parallel inhibition of responses to both action potential- and hypertonic solution-evoked release, as illustrated in Figure 8D for 11 experiments of this type. Note that although the release produced by both methods is highly correlated and the data are rather scattered, inhibition with action potential-evoked release tends to be slightly greater.

Discussion

Our main conclusions are that hypertonic solution produces neurotransmitter release by a mechanism that is not mediated by calcium, and that this release mechanism and the usual calcium-dependent one share a common pool of readily releasable quanta. We first examine the possible interpretations of readily releasable pool and then propose a picture of where hypertonic solutions act.

The decline of EPSC amplitude during repeated stimulation and its recovery after the cessation of stimulation have long been interpreted as indicating the existence of a readily available pool from which released quanta are drawn. The size of the evoked response (the EPSC amplitude) measures the available pool size. Early evidence for this interpretation came from Thies (1965), who discovered that variations in the quantity of transmitter released correlated in the expected way with the extent to which the hypothetical pool is decreased. More recently, Liu and Tsien (1995) have carried out experiments that activate the Ca-mediated release pathway by potassium produced depolarizations, and they have also interpreted their results in terms of a readily releasable pool that is slowly refilled, once depleted. Although the qualitative conclusions of the Liu and Tsien (1995) and the Stevens and Tsujimoto (1995) studies are similar, the quantitative estimates of pool size and refilling rate are significantly different between the two laboratories. Owing to important differences in the methods used, the source of the divergent quantitative estimates is unclear. Liu and Tsien used 1 s-long applications of hyperkalemic solution that were repeated every 5 s for

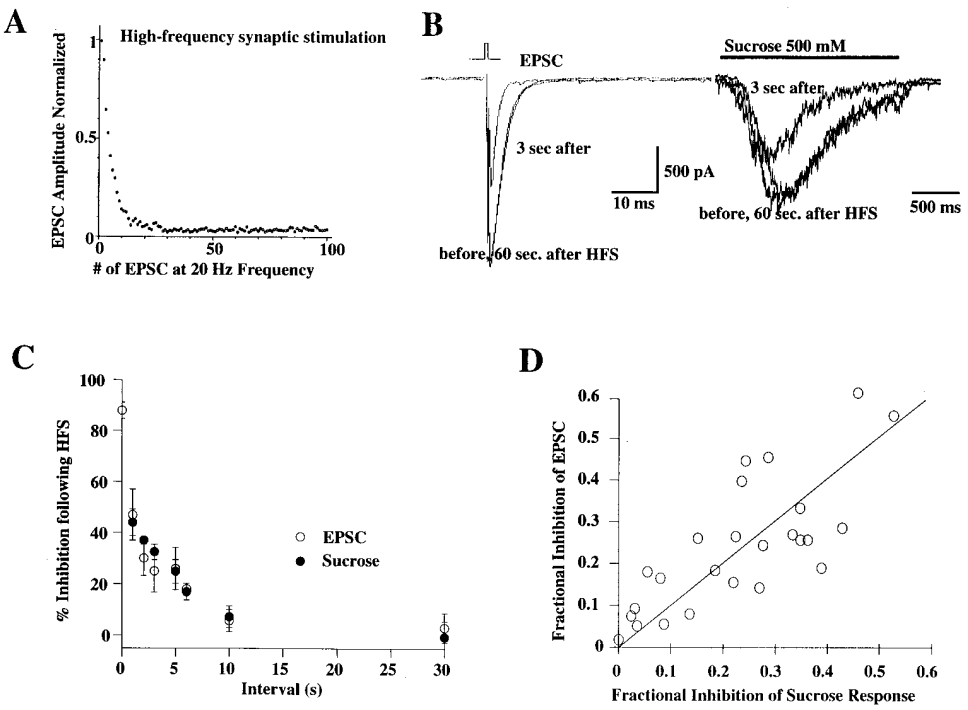

Figure 7. High Frequency Stimulation of Autapses (20 Hz, 100 Stimuli) Reduces Action Potential- and Hypertonic-Evoked Responses in a Similar Manner

(A) Peak autaptic EPSC as a function of time during 20 Hz stimulation. Amplitude of EPSC was normalized to the size of the first response. Extracellular calcium was 3 mM, magnesium 1 mM.

(B) Current traces show autaptic EPSCs (left superimposed traces) and responses to hypertonic solution application (right superimposed traces) before, and 3 and 60 s after high frequency stimulation.

(C) Time course of recovery from inhibition of autaptic sEPSC (open circles) and hypertonic solution evoked responses (closed circles) produced by high frequency (20 Hz) action potentials.

(D) Correlation of changes of the amplitude of action potential- and hypertonic solution-evoked responses after high frequency action potentials for 25 experiments.

1.5 min (rather than a single episode of continuous superfusion with hypertonic solution for 5 s) and measured the total quantity of release produced by each application. Thus, they would have been examining a different pool than the one defined by Stevens and Tsujimoto. In any case, to the extent that the standard interpretation used made in these earlier studies is correct, our evidence for a shared readily releasable pool seems quite compelling.

Our main uncertainty is that the existence of a morphologically defined readily releasable pool has not yet been demonstrated, although the docked vesicles offer an obvious candidate for this pool. However, one could imagine that with continued release, some critical factor might become depleted and recover with a 10 s time constant. As Stevens and Tsujimoto (1995) and Liu and Tsien (1995) have pointed out previously, such a mechanism, or a similar one that involves the buildup of an inhibitory factor, would make quantitatively indistinguishable predictions from the depletion model. Our conclusion would in this case be modified to suppose that both methods for producing release deplete the same essential substance. The definitive interpretation of our observations will have to await an independent morphological means for defining the readily releasable pool. In the meantime, we shall continue to consider our results in terms of what we view as the most likely mechanism, a readily releasable pool that we provisionally identify with the population of morphologically docked vesicles.

Implicit in our conclusions is a very important mechanistic implication: if the decline in release rate that occurs with application of hypertonic solution actually does represent decrease in the size of a pool of readily releasable quanta, and if the action potential-produced exocytosis does draw on this same pool, then release probability varies with the size of the pool of readily releasable quanta. This is true because, as Stevens and Wang (1995) have shown, hippocampal synapses behave unreliably and release at most a single quantum; the probability of that release is governed by pool size, together with other factors like the magnitude of the local calcium concentration. The idea that release probability is related to the size of the releasable pool was first proposed by Zucker (1973), on the basis of his results from quantal analysis at the crayfish neuromuscular junction and anatomical data on that synapse type, but this notion has attracted little attention in the intervening years.

Stevens and Tsujimoto (1995) reported that after the readily releasable pool is depleted, it recovers with a time constant of about 8 s, and we have confirmed this

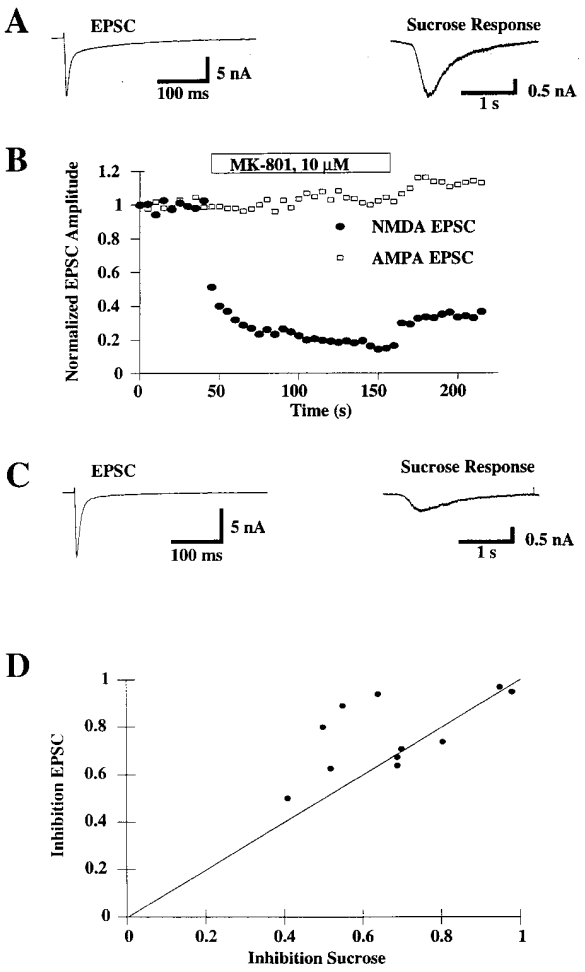

Figure 8. Use-Dependent Block by MK-801 Decreases the Amplitude of Autaptic EPSCs and Responses to Hypertonic Solution in Parallel

(A) Left trace shows typical dual component autaptic EPSC. Right trace shows the response evoked by application of 500 mM sucrose in presence of CNQX, glycine and absence of magnesium from the same cell. This response represents the macroscopic NMDA hypertonic response, and was blocked by D-(-)-2-Amino-5-phosphonovaleric acid (AP5, 50 μM, data not shown).

(B) AMPA (open squares) and NMDA (closed squares) autaptic EPSC amplitudes as a function of time after application of 10 μM MK-801. AMPA component was estimated by simply measuring the peak synaptic current; we estimate that there is a contamination of this measurement by the presence of the NMDA component of approximately 10%. NMDA component amplitude was measured by integrating the current between 30 and 100 ms, following the onset of the EPSC. Note that the NMDA component gradually decreases in the presence of MK-801.

(C) Example for the autaptic EPSC and response to hypertonic solution application after MK-801 block of synaptic NMDA receptors.

(D) Correlation between inhibition produced by MK-801 of autaptic EPSC and the response to hypertonic solution in 11 experiments.

recovery rate here (see Figure 6B). One might expect that such an important property as refilling rate would be regulated, possibly by activity. In this connection, note that the refilling rate after tetanic action potential stimulation is several fold more rapid (Figure 7C). This difference may reflect a rise in calcium that resulted, in the second case, from a series of action potentials and produced an increase mobilization rate of the reserve pool of vesicles.

Several authors have proposed that hypertonic solutions could act to produce release by increasing intracellular calcium concentration, either by concentration of calcium that is present through water removal or by release from internal stores (Cohen and Van der Kloot, 1985; Delaney et al., 1991; Brosius et al., 1992; Yu and Miller, 1995). Brosius et al. reported that the increased release associated with ethanol exposure does not have an accompanying increase in intracellular calcium concentration (as measured by Indo-1 fluorescence changes), but that fluorescence signals interpreted as calcium concentration changes could be detected with application of hypertonic solution. However, these authors could not determine what proportion of the increased release produced by hypertonic solution resulted from attendant changes in calcium concentration. Changes in dye concentration due to osmotically induced shrinkage could have given an artifactual signal, or the preparation used (chick ciliary ganglion somas) might well release calcium from internal stores in response to hypertonic stimulation. Yu and Miller (1995) reported that the response to hypertonic solutions in retina was markedly diminished by cadium and cobalt, but this effect could have in part reflected the behavior of neural circuits, rather than properties of individual synapses. Additional experiments will be required to determine the extent to which calcium changes play a role for various preparations.

We noted earlier that the response to hypertonic solution tended to be slightly larger than that to nerve stimulation at synapses whose NMDA receptors had been blocked by MK-801. MK-801 can block channels only if the NMDA receptors are activated, that is, only if glutamate has bound to them and induced an opening. Analysis of the kinetics of MK-801 block has led to the conclusion that some synapses in cultured neurons release transmitter with lower probability than others (Rosenmund et al., 1993) so that, after a period of repeated stimulation in the presence of MK-801, the higher probability synapses would be almost completely blocked, but the lower probability synapses would be still only partially blocked. To release neurotransmitter very rapidly when a nerve impulse produces a rapid transient change in the local calcium concentration, vesicles must be docked and cocked. That is, they must be attached to the exocytotic machinery, and the various biochemical steps that prepare the vesicles for release must have been completed (Südhof 1995). For example, at the frog neuromuscular junction, most release can be estimated to occur within an approximately 100 μs- long window, following nerve impulse arrival (Barrett and Stevens [1972]; Figure 3, extrapolated to room temperature). The calcium concentration transient produced by a nerve impulse must then be transduced into exocytosis, presumably by a calcium-produced conformational change of some protein (such as synaptotagmin) associated with the exocytotic machinery. The pool of readily releasable vesicles should include these docked and cocked vesicles, and is plausibly postulated to be identical with them. On this view, hypertonic solution would

produce a change in the exocytotic machinery that substituted for the calcium-induced conformation change that produces release. Since hypertonic solution visibly distorts the neuronal membranes by causing shrinkage, the most likely mechanism of action is simply a mechanical one somewhat like a stress-induced change in enzyme activity (Zenchenko and Morozov, 1995) or channel state (Yang and Sachs, 1989). Thus, hypertonic solution-produced release would engage the exocytotic machinery at some step in the sequence of molecular events after the point at which calcium acts.

Experimental Procedures

Microisland cultures preparation was modified from Bekkers and Stevens (1991). The extracellular medium contained 167 mM NaCl, 2.4 mM KCl, 10 mM HEPES, 10 mM glucose, 3 mM $CaCl_2$, 1 mM $MgCl_2$. The osmolarity was 325 mOsm (pH 7.2). Picrotoxin (100 μM) and strychnine (2 μM) were added to inhibit spontaneous synaptic activity and GABA/glycine chloride channels. Osmolarity in the hypertonic solutions was varied by adding either sucrose or sorbitol to regular extracellular medium. Tetrodotoxin (500 nM) was regularly added to this solution, except when action potential-evoked release was being studied. For calcium current measurement, the extracellular medium also contained 5 mM BaCl2, 10 mM tetraethylammonium (TEA), and 1 μM TTX. Fast-flow application of solutions followed procedures as described in Rosenmund et al. (1995). Briefly, solutions were applied using an array of quartz flow pipes (Polymicro Technology, USA, 430 μM ID) positioned within 100–200 μM of the neuron and connected to gravity-fed reservoirs. Each flow pipe was controlled by a solenoid valve, and the pipe array was moved with a piezoelectric bimorph (Vernitron, USA) under the control of the computer software.

Experiments were performed using hippocampal neurons after 5–14 days in culture. To examine synaptically-activated currents, experiments were performed using recurrent excitatory synapses (autapses) on microisland cultures. Currents were recorded using an Axopatch 200A amplifier (Axon Instruments, USA). Patch pipettes were fabricated from borosilicate glass (TWF 150, Word Precision Instruments, USA) pulled with a two-step puller (Narishige). Pipettes had "bubble numbers" ranging from 7.2–8.0. After firepolishing, the pipette tip diameter was 2 μm–3 μm and the resistance was 1 MΩ–2.5 MΩ. Pipette solutions included 135 mM Cs gluconate, 10 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, 5 mM Mg-ATP (pH 7.3). The osmolarity was 310 mOsm. For synaptic experiments, potassium salts were substituted for cesium salts in the pipette solution. This substitution is used to give brief action potentials in the axon and to minimize noise by providing a resting potential near the desired holding potential. For the experiments illustrated in Figures 2A and 2B, potassium gluconate was entirely replaced by 75 mM K_4BAPTA. The series resistance was 60%–90% compensated; only recordings with access resistance below 10 MΩ were included in the analysis. Cell capacitance (5 pF–25 pF) was compensated. Data were acquired on a IBM 486 clone using software written in AXOBASIC (Axon Instruments, USA), and analyzed on a Macintosh computer using AXOGRAPH software (Axon Instruments, USA). Acquisition rate was 2 kHz–5 kHz, and data were filtered at half the acquisition rate with an 8-pole Bessel filter (Frequency Devices, USA). Data are expressed as percent of control ± SE. Statistical significance was tested using one-way analysis of variance with the Bonferroni-Dunn procedure for multiple comparisons. P levels are given for significance levels <5%. Drugs were obtained from Sigma, except t-ACPD and CNQX from Tocris Cookson, England; BHQ, Ryanodine, and Thapsigargine from Alomone Laboratories, Israel; MK-801 and R ± Baclofen from Research Biochemicals International, USA.

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