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# Excitatory and inhibitory autaptic currents in isolated hippocampal neurons maintained in cell culture

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ABSTRACT **Individual rat hippocampal neurons, grown in isolation from other neurons on small spots of permissive substrate, were studied in order to characterize the electrical properties of the synapses that such cells formed with themselves (autapses). Excitatory (probably glutamatergic) or inhibitory (probably type A γ-aminobutyratergic) autapses were frequently found. Excitatory autaptic currents reversed near the potential expected for monovalent cations were blocked by the glutamatergic antagonist kynurenic acid, and possessed a slow component with the pharmacological profile of *N*-methyl-D-aspartate-type channels. These currents also exhibited trial-to-trial statistical fluctuations in their amplitudes, this being well-described by quantal analysis. Inhibitory autaptic currents reversed at hyperpolarized potentials, as expected for chloride-permeable pores and were blocked by picrotoxin, a type A γ-aminobutyric receptor antagonist. It is concluded that autaptic currents in culture are identical to those found at synapses.**

Although neurons have only infrequently been reported to form synapses on their own processes (1–6), such "autaptic" connections—to use the term coined by van der Loos and Glaser (3)—may not be uncommon. For example, van der Loos and Glaser (3) reported that, in Golgi preparations, 6 of 12 well-impregnated occipital cortical pyramidal cells exhibited autapses, and Karabelas and Purpura (6) found that 2 of 14 horseradish peroxidase-filled neurons in substantia nigra formed synapses on themselves, one with at least 20 boutons. Furthermore, these estimates of autapse frequency could be underestimates because part of the dendritic trees of the neurons examined was outside the plane of the section.

The possible functional significance of such autaptic loops remains obscure because the operation of autapses has received even less study than their existence. van der Loos and Glaser (3) speculated that autapses might serve a self-inhibitory function. Physiological evidence for the existence of autapses has also been reported in cultured chicken spinal ganglion cells (2) and in sympathetic ganglion cells cocultured with myocytes (4). However, it is not known whether glutamatergic and γ-aminobutyratergic (GABAergic) central neurons can form functional autapses or how the operation of functional autapses might compare with that of synapses on the same cell type.

In the course of experiments designed for other purposes, we have had the opportunity to study autaptic circuits formed by rat hippocampal neurons maintained in culture. We find that when a neuron's axon is constrained to grow within the region of the cell's own dendritic tree, abundant autaptic connections typically form. The operation of these autapses, both excitatory and inhibitory, seems indistinguishable from that of synapses in the same culture system. We note that such autaptic circuits offer an unusually homogeneous population of synapses for physiological investigations.

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## MATERIALS AND METHODS

**Cell Culture.** The method of Furshpan and collaborators (4, 7, 8) was used to grow single, isolated hippocampal neurons on small "islands" of substrate. Briefly, the procedure was as follows. A 0.15% agarose solution was spread thinly on 12-mm round coverslips in 24-well culture plates and allowed to dry. A glass microatomizer (Fisher) was then used to spray onto the agarose a fine mist of substrate solution containing rat tail collagen (Biomedical Technologies, Stoughton, MA) at 0.25 mg/ml and poly(D-lysine) (Collaborative Research) at 0.4 mg/ml; this yielded randomly distributed spots of substrate 100–1000 μm in diameter. Neurons from the CA1–CA3 regions of hippocampi of newborn Long–Evans rats were dissociated as described (9), and 0.5 ml of the cell suspension, diluted to 5–8 × $10^4$ cells per ml, was added to each well. At this density, after about a week in culture each well usually contained several "islands" that were each occupied by a single neuron.

**Electrophysiology.** Conventional whole-cell patch clamping was employed, using an Axopatch 1-A controlled by a PC/AT computer. Series resistance compensation was set at 80–100%. The usual bath solution was 137 mM NaCl/5 mM KCl/3 mM $CaCl_2$/0, 1, or 10 mM $MgCl_2$/10 mM glucose/5 mM Hepes, pH 7.3, with osmolarity adjusted to 310–315 mOsm with sorbitol. The pipette solution was 150 mM potassium methyl sulfate/5 mM KCl/5 mM tetrapotassium bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid ($K_4$BAPTA)/10 mM Hepes, pH 7.2, 305–310 mOsm. Experiments were performed at room temperature (22–25°C). Immunohistochemistry was done as described (9).

## RESULTS

We have studied autaptic circuits on 30 islands inhabited by single neurons. Fig. 1*A* shows a phase-contrast micrograph of an isolated neuron growing on a monolayer of glial cells on a spot of collagen/poly(D-lysine) substrate. At this level of magnification, no neuronal processes are visible in the region surrounding the island, suggesting that this neuron is electrically isolated from other neurons in the culture. This conclusion is supported by other evidence provided by Segal and Furshpan (8).

Fig. 1*B* shows the same neuron under brightfield illumination after immunohistochemical staining for synapsin I, which is a protein that is localized at presynaptic terminals and which therefore provides a marker for the presence of synapses (10). Punctate staining is visible at many of the points of axo–dendritic contact. This staining pattern is similar to that which is observed in hippocampal cultures where neurites are not spatially constrained and in which

Abbreviations: GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate; mepac, miniature excitatory autaptic current.

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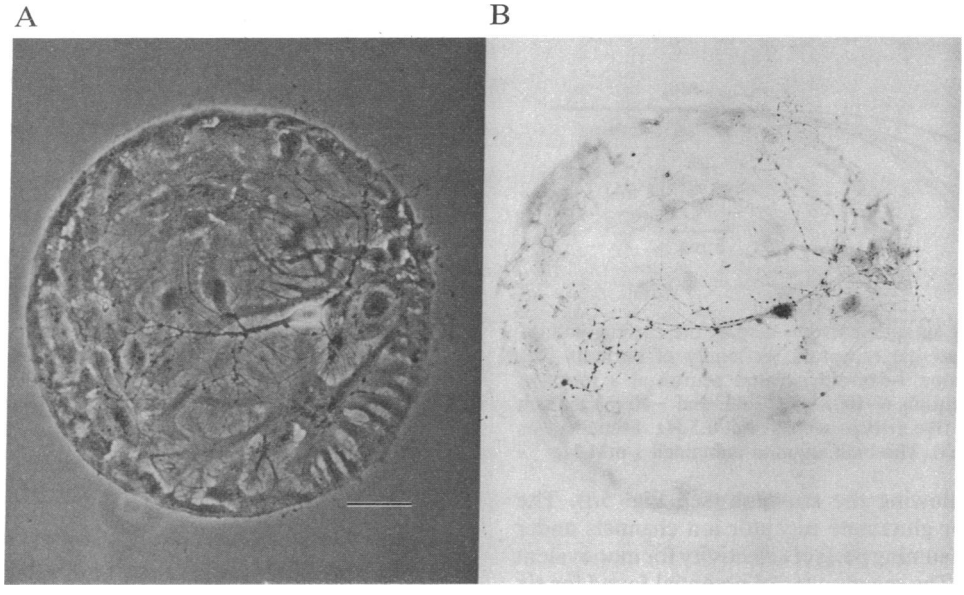


FIG. 1. Anti-synapsin I labeling reveals the presence of self-synapses (autapses) on an isolated hippocampal neuron in culture. (*A*) Phase-contrast micrograph of a neuron that was grown for 9 days on an "island" of glial cells on a spot of collagen/poly(D-lysine). (*B*) The same neuron viewed in brightfield illumination after fixation and staining for synapsin I. Punctate staining demonstrates the presence of autapses. (Bar = 50 μm.)

heterosynapses presumably predominate (e.g., ref. 9). A cell on an island typically makes dozens to hundreds of synapses with itself, depending on the island's size and the length of time in culture. After 6 days in culture, a neuron would usually have perhaps a dozen autapses, and this number would increase to the hundreds after an additional week of growth.

Electrophysiological investigations (described below) have revealed that these neurons do indeed make functional autaptic connections, either excitatory or inhibitory. Of the 30 autaptic circuits studied, 14 were excitatory and 16 inhibitory; we have never found a single cell that made both inhibitory and excitatory connections. Examples of excitatory and inhibitory autaptic circuits are shown in Figs. 3 and 5. Our impression is that neurons with simpler dendritic trees form inhibitory connections and that cells whose dendritic trees are more richly endowed with fine branches are excitatory.

When a neuron is briefly stimulated to produce an action potential, synaptic potentials can be seen just after the spike, and when the recording is switched from current clamp to voltage clamp, clear autaptic currents are revealed (Fig. 2). In the instance illustrated, which is excitatory, the peak autaptic current is about 1 nA and the autaptic depolarization is >15 mV. As might be expected, the autaptic potentials occur only when the depolarization is sufficient to evoke an action potential, and they occur with fixed latency after the spike when the latter exhibits temporal jitter (data not shown). In the voltage-clamp mode, the test pulse must be sufficiently large to activate $Na^+$ current. Presumably, then, this initiates an action potential in the unclamped axonal processes, leading to autaptic currents that are recorded at the soma under reasonable voltage control. Excitatory and inhibitory autaptic currents and potentials are all blocked by removing external $Ca^{2+}$ (data not shown).

Figs. 3 and 4 provide further evidence that excitatory autaptic currents like those in Fig. 2 are glutamatergic. Fig. 3 shows a family of autaptic currents recorded in an isolated cell while the voltage was stepped to a fixed test potential (10 mV) from a range of holding potentials (−100 mV to −40 mV). The extrapolated reversal potential in this case was around −9 mV, although this may have been distorted by a

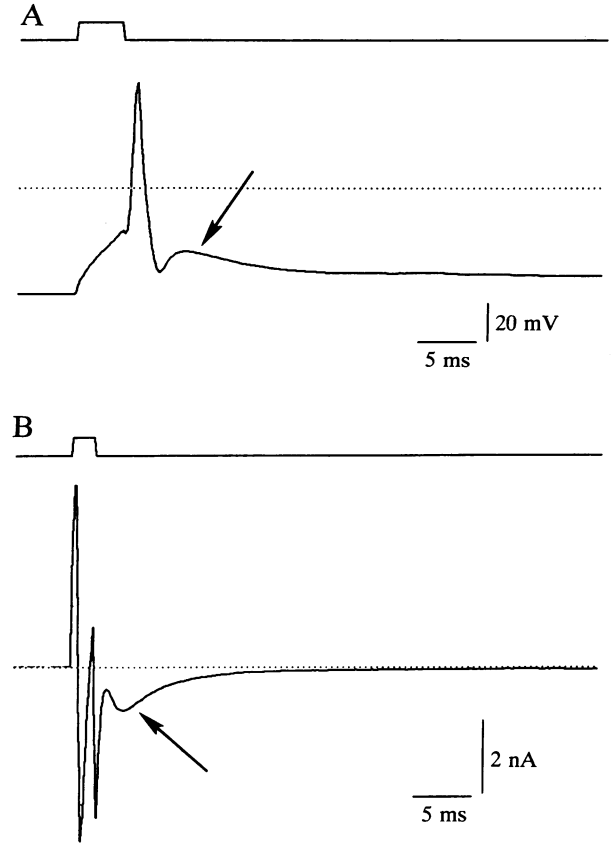


FIG. 2. Autaptic transmission (arrow) is visible in current-clamp (*A*) and voltage-clamp (*B*) recording modes. (*A*) Excitatory postautaptic potential in an isolated 9-day-old neuron following an action potential in the neuron. The cell was hyperpolarized to −60 mV from its resting potential of −55 mV. The stimulus was a 4-ms, 0.6-nA current step. (*B*) The same cell voltage-clamped at −60 mV. The stimulus was a 2-ms step to 20 mV. The initial outward spike is a capacity transient as the cell is depolarized, followed by an inward $Na^+$ current, followed by an inward capacity transient as the cell is repolarized. The bath solution contained no added $Mg^{2+}$.

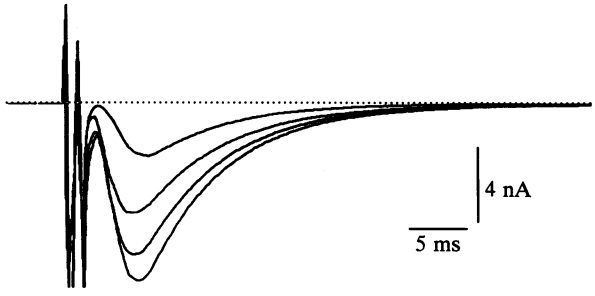


FIG. 3. Excitatory autaptic currents display the current–voltage properties of glutamatergic receptors. A family of currents was recorded from the same 9-day-old isolated neuron at a range of different holding potentials (−100, −80, −60, and −40 mV). Each trace is an average of five sweeps recorded at 0.5 Hz. Stimulus was a 1.4-ms step to 20 mV. The bath solution contained 1 mM $Mg^{2+}$.

$K^+$ tail current following the stimulus (see Fig. 5*B*). The Nernst potential for glutamate receptor ion channels under these conditions, assuming perfect selectivity for monovalent cations, is −6 mV. The mean reversal potential found for six cells was −4 ± 4 mV (± SD).

Like their synaptic counterparts (11), the excitatory autaptic currents are blocked by external kynurenic acid (1 mM) in a reversible manner (Fig. 4*A*). Some excitatory autaptic transmission, like some excitatory synaptic transmission, is dual-functional: in addition to the early, rapid "non-NMDA" component, autaptic currents may exhibit a prolonged component arising from *N*-methyl-D-aspartate (NMDA) receptor channels. Such a slow component was observed in all five experiments for which conditions were appropriate; an example is shown in Fig. 4*B*. This component is revealed when $Mg^{2+}$ is excluded from the bath solution and can be reversibly blocked by external 50 μM D-2-amino-5-phosphonovaleric acid (Fig. 4*B*) or, at hyperpolarized potentials, high external $Mg^{2+}$ concentration (data not shown). The non-NMDA autaptic current was selectively blocked by 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (data not shown). All of these properties are identical to those found for synaptic excitatory currents in culture (9, 12).

Some neurons make inhibitory autapses. A family of traces of inhibitory autaptic currents, recorded at different holding potentials (−100 mV to −20 mV), is presented in Fig. 5*A*. These currents have a reversal potential of around −60 mV, determined by measuring the current amplitude 50 ms after the stimulus, by which time the contaminating $K^+$ tail current has mostly relaxed to the baseline (Fig. 5*B*). The mean reversal potential found for eight cells was −63 ± 3 mV (± SD). The Nernst potential for perfectly $Cl^-$-selective $GABA_A$-type channels under these conditions is −86 mV; however, the current–voltage behavior of these currents was markedly nonlinear under our conditions (data not shown), suggesting that a simple Nernst treatment is inappropriate. These currents are also slower than the non-NMDA excitatory autaptic currents (compare time scales in Figs. 3 and 5). Their time course is unaffected by high external $Mg^{2+}$ concentration (data not shown), an observation that rules out the

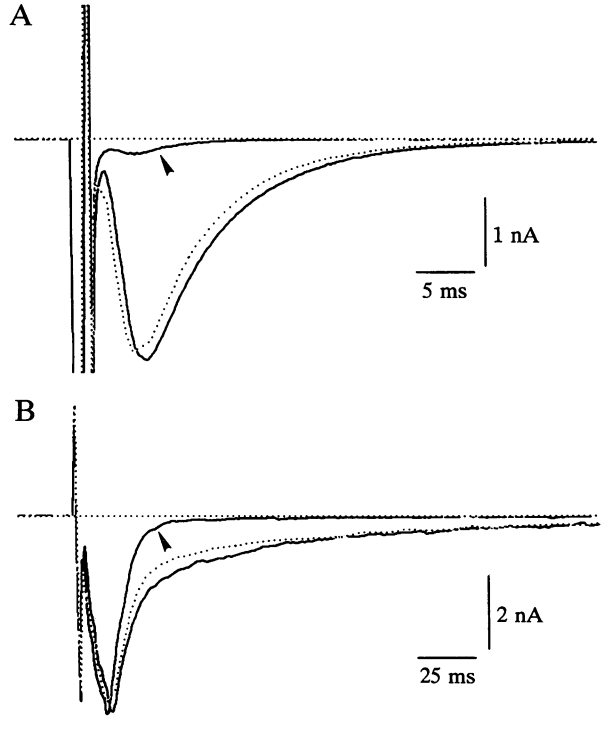


FIG. 4. Excitatory autaptic currents display some pharmacological properties of glutamatergic receptors. (*A*) Effect of 1 mM kynurenic acid in the bath. Trace indicated with arrowhead was obtained in the presence of kynurenate; the dotted trace was obtained after the kynurenate was washed out. Stimulus was a 1.4-ms step to 20 mV; holding potential was −60 mV; cell was 9 days in culture; 1 mM $Mg^{2+}$ was in the bath. (*B*) Effect of 50 μM D-2-amino-5-phosphonovalerate (D-APV) in the bath. Arrowed trace was obtained in the presence of D-APV, dotted trace after washing out the D-APV. In this experiment the bath solution lacked $Mg^{2+}$ and contained 1 μM glycine and 1 μM strychnine. Stimulus was a 2-ms step to −10 mV; holding potential was −60 mV; cell was 20 days in culture.

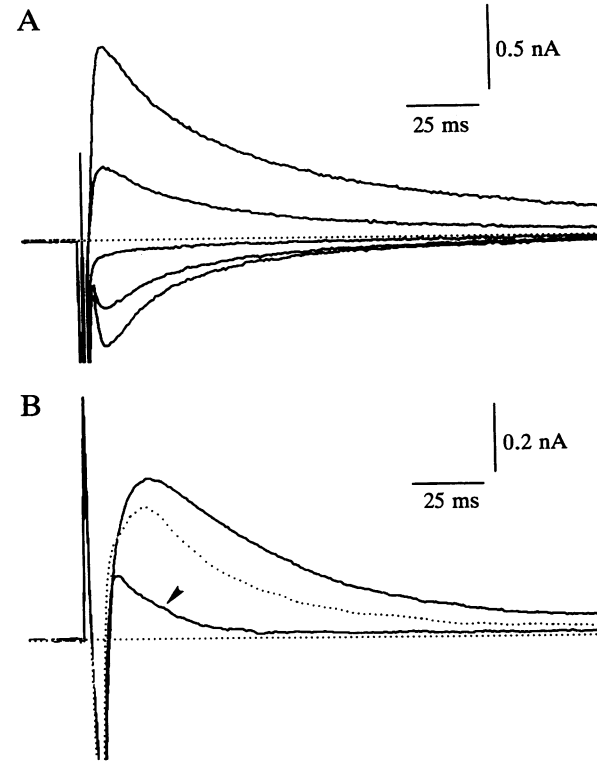


FIG. 5. Inhibitory autaptic currents display some properties of $GABA_A$ receptors. (*A*) A family of currents recorded from the same 18-day-old isolated neuron at a range of different holding potentials (−100, −80, −60, −40, and −20 mV). Each trace is an average of five sweeps recorded at 0.5 Hz. Stimulus was a 1.6-ms step to 20 mV. The bath solution contained 1 mM $Mg^{2+}$. (*B*) Effect of 100 μM picrotoxin in the bath. Trace indicated with arrowhead was obtained in the presence of picrotoxin, dotted trace after washing out the picrotoxin. Note the $K^+$ tail current that is revealed in the presence of picrotoxin. Stimulus was a 4-ms step to 10 mV; holding potential was −30 mV; cell was 18 days in culture; bath solution lacked $Mg^{2+}$.

participation of NMDA-type channels. Like their synaptic counterparts, the hippocampal inhibitory autaptic currents are blocked reversibly by 100 $\mu$M picrotoxin (Fig. 5*B*; ref. 13).

Autaptic transmission, like synaptic transmission, is statistical in nature. In 10 mM $Mg^{2+}$ and reduced (0.75 mM) $Ca^{2+}$ concentration, what would usually be a smooth autaptic current with a quite constant amplitude (for example, see Fig. 3) is resolved into probabilistically occurring units (Fig. 6*A*). In the tail of the main release, spontaneous mepacs can be seen. Spontaneous mepacs have a variable amplitude and are quantitatively similar to their synaptic counterparts (Fig. 6*B*; cf. refs. 9, 14, and 15). As one finds for excitatory synaptic transmission, the probability of recording an autaptic current (or charge transfer) of a particular size was well-described in five experiments by the quantal theory of transmitter release (9, 16), when account was taken of the observed size variations in the individual mepacs (Fig. 6*C*). The histogram illustrated is well-fitted by a Poisson distribution with a mean of 11.3.

## DISCUSSION

Autaptic transmission appears indistinguishable from synaptic transmission. Thus, autapses in our culture system not only function, they do so just like their synaptic counterparts: (*i*) both excitatory transmission (with NMDA and non-NMDA components) and inhibitory transmission are seen, with similar permeation, kinetic, and pharmacological properties; (*ii*) mepacs occur and have mean size and size distributions that are similar to those of synaptic excitatory miniatures; and (*iii*) excitatory autaptic transmission appears to be quantal.

Apart from their intrinsic interest, autaptic connections in culture may be of value in physiological studies of synaptic transmission. Unlike conventional approaches, in which pairs of neurons and electrodes are required (one presynaptic, the other postsynaptic), autaptic experiments require only one electrode, and, provided the island is sufficiently small, connectivity of the cell to itself is virtually assured. Furthermore, autaptic transmission is necessarily monosynaptic and the origin of all synapses on the neuron is known. The latter may be important in quantal analyses. Often, such analyses associate spontaneous miniatures, which arise from all synapses on a cell, with evoked synaptic currents, which may arise from only a subset of those synapses—namely, those that are postsynaptic to the axon being stimulated (9, 16). This may cause errors if synapses are heterogeneous. In the case of autapses, however, both spontaneous and evoked currents originate in the same population. This would reduce the effect of synaptic heterogeneity.

Because all our experiments have been done in culture, and more particularly on neurons completely isolated from other neurons but in contact with glia, we cannot confidently extrapolate our conclusions to autapses in the brain. Nevertheless, neither excitatory nor inhibitory neurons exhibited any reluctance to form autapses, and those autapses appeared to operate entirely like normal synapses. Thus, we anticipate that autapses in brain, when techniques are developed to study them, will also be found to be fully functional.

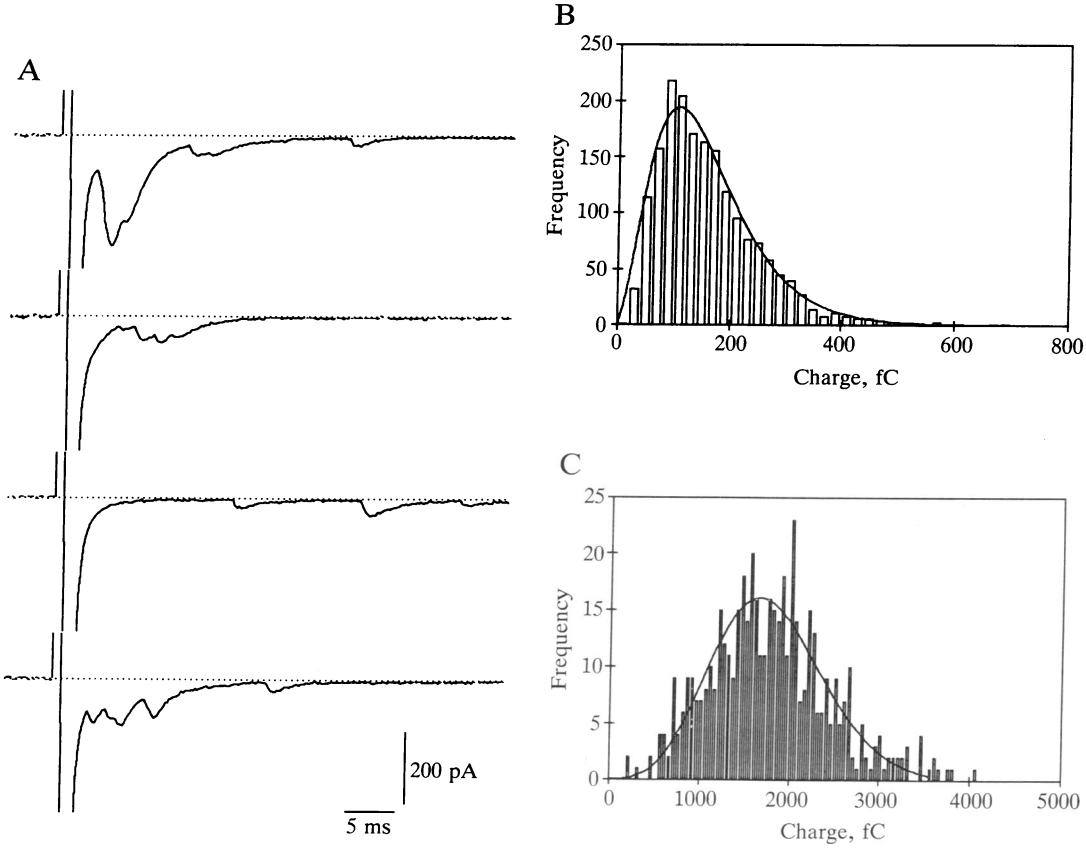


FIG. 6. Excitatory autaptic currents exhibit quantal fluctuations and are well-described by standard quantal theory. (*A*) Consecutive sweeps from an 11-day-old cell, stimulated at 1 Hz with a constant 2.8-ms step to 0 mV from a holding potential of −60 mV. The bath solution contained 10 mM $MgCl_2$ and 0.75 mM $CaCl_2$. (*B*) Distribution of the charges carried by spontaneous miniature excitatory autaptic currents (mepacs) recorded in the same cell as for *A*. The superimposed smooth curve is the function given in ref. 14 and was fitted by eye. (*C*) Histogram of charges carried by evoked autaptic currents like those in *A*. The currents were integrated over 80 ms, starting just after the stimulus; the stimulus artifact charge was subtracted by subtracting the mean charge found for sweeps in which autaptic transmission did not occur ("failures"), recorded in the same cell in 0.5 mM $CaCl_2$. The superimposed smooth curve is a Poisson distribution that was fitted to the histogram as described elsewhere (9).

Their significance, then, would be determined completely by their placement and abundance; a single synapse among the thousands typically present might mean little, but cases of multiple autaptic connections, like the example given by Karabelas and Purpura (6), might well have some computational significance.

**Note Added in Proof.** After this work was completed, Segal (17) reported excitatory and inhibitory autaptic connections in solitary cultured hippocampal neurons. He showed that excitatory and inhibitory autaptic potentials are blocked by kynurenic acid and bicuculline, respectively, confirming the basic pharmacological separation reported here. He also noted the presence of spontaneous miniature excitatory autaptic potentials in solitary neurons that had been identified as excitatory.

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