

The rate of aldehyde fixation of the exocytotic machinery in cultured hippocampal synapses

Christian Rosenmund^{a,*}, Charles F. Stevens^b

^a Max-Planck-Institut für Biophysikalische Chemie, AG Zelluläre Neurobiologie, 37070 Göttingen, Germany

^b Howard Hughes Medical Institute, The Salk Institute Molecular Neurobiology Laboratory, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA

Received 13 January 1997; accepted 11 March 1997

Abstract

The rate at which the transmitter release machinery is fixed by 2% glutaraldehyde at hippocampal synapses and the amount of release evoked by the fixative have been investigated. We recorded from hippocampal cells while fixative was applied with a rapid flow system. Release is blocked in less than a second and fixative-produced exocytosis is at most a few percent of what would be caused by a hypertonic stimulus that completely depletes the readily releasable pool of vesicles. The postsynaptic receptors for glutamate cease to respond to agonist with a time constant of approximately 3 s when fixative is applied. We conclude that some essential component of the exocytotic apparatus is fixed in less than a second and that the fixative does not significantly deplete the readily releasable pool. © 1997 Elsevier Science B.V.

Keywords: Synapse; Culture; Glutaraldehyde; Fixation; Exocytosis and hippocampus

1. Introduction

Current views of synapse function hold that vesicles must be 'docked and cocked' in order to undergo exocytosis (see Südhof, 1995). Because release occurs so quickly after the arrival of a nerve impulse, these readily releasable vesicles must already be in position for membrane fusion (Almers and Tse, 1990) and would thus be a subset of the vesicle pool that is immediately adjacent to the active zone. Recent physiological studies of hippocampal synapses have defined a 'readily releasable' pool of vesicles that must itself correspond, at least in large part, to the release competent pool of vesicles (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996). In order to correlate the functionally defined and morphologically defined readily releasable pools, combined physiological/morphological studies are required.

Questions about the relation between electron micrograph images and structures in the living cell are always difficult, but the effect of fixation on the morphologically defined docked vesicle pool (i.e. those vesicles that are immediately adjacent to the active zone) is especially problematic: Smith and Reese (1980) have shown that, at the frog neuromuscular junction, fixatives can cause the release of neurotransmitter, and thus could deplete, to an unknown extent, the store of vesicles docked to the active zone. Because fixatives have been found to cause release, this source of artifact needs to be evaluated for any quantitative study of presynaptic structure.

Although Smith and Reese have investigated this question for the frog neuromuscular junction, extrapolation of their findings to central synapses in culture is of uncertain validity. Fixation of excitatory synapses in hippocampal neuronal cultures may be more or less rapid than fixation of a nerve-muscle preparation, access of the fixative to the synapses is different in the two

* Corresponding author.

cases, and the different morphology of the active zones between the two synaptic types could limit range of validity for the Smith and Reese conclusions.

For this reason, we have reinvestigated this question for synapses made by rodent hippocampal neurons in culture. We find that fixation interrupts release before a significant depletion can occur. Thus, the quantitative evaluation of the docked vesicle pool might be inaccurate for other reasons, but it appears that depletion of this pool by the fixation procedure is not a dominant source of error.

2. Methods

The methods used here are the same as those described in Rosenmund and Stevens (1996). Briefly, conventional or microisland cultures were prepared with a modification of the Bekkers and Stevens (1991) method, and hippocampal neurons were studied with whole cell recording after 5–14 days in culture; the microisland studies used microislands with only a single neuron. The solution application was made with the fast-flow system described by Rosenmund et al. (1995) which provides changes in the local environment over the entire dendritic tree in less than about 50 ms (exchange time constant 18 ms).

The extracellular medium normally contained 167 mM NaCl, 2.4 mM KCl, 10 mM HEPES, 10 mM glucose, 3 mM CaCl₂ and 1 mM MgCl₂ together with 100 μM Picrotoxin and 2 μM strychnine. For the study of calcium channels 5 mM BaCl₂, 10 mM tetraethyl ammonium and 1 μM tetrodotoxin (TTX) were added to the standard extracellular solution. Solutions were made hypertonic by adding the specified quantity of sucrose to this standard extracellular solution and cells were fixed by rapid superfusion with the same solution to which 2% glutaraldehyde was added. Hypertonicity is specified in terms of the number of mOsm of solute added above the tonicity of the standard extracellular solution.

3. Results

Our initial experiment was to measure the response of hippocampal neurons in culture to application of fixative (2% glutaraldehyde, a solution that is hypertonic by 200 mOsm). Using a rapid superfusion system (Rosenmund et al., 1995) we first applied a solution made hypertonic with sucrose and then compared the response to a solution made hypertonic (to the same extent) with glutaraldehyde. As usual the sucrose solution produced release (Fig. 1A, top trace) from synapses through a calcium independent mechanism (Rosenmund and Stevens, 1996) but the glutaraldehyde

solution produced no detectable release at the onset of superfusion (Fig. 1A, middle trace). Fixation was satisfactory in that the hypertonic solution no longer produced synaptic currents following the application of glutaraldehyde (Fig. 1A, bottom trace). The effect of fixative application is seen more clearly in Fig. 1B where the derivative of the recorded currents has been taken to emphasize the transient synaptic currents. Synaptic currents are produced by the hypertonic solution (Fig. 1B, top trace) but are not visible with application of the fixative (Fig. 1B, bottom trace).

After the onset of fixative superfusion an inward current develops that reaches a plateau of about 1 nA in magnitude and is then maintained after the fixative is removed (Fig. 1A, middle trace). This maintained current does not appear to be of synaptic origin (compare the noise produced by miniature synaptic currents in the top trace of Fig. 1A with the response to glutaraldehyde in the middle trace). To confirm this impression, the fixative was applied to synapses whose receptors were blocked with Bicuculline and NBQX. As anticipated, the inward current persisted unchanged (Fig. 1D, top trace) and no rapid transients are seen with the onset of superfusion (bottom trace).

The preceding results show that 2% glutaraldehyde does not produce significant release from hippocampal synapses in culture, but does not answer the question of how rapidly some essential component of the exocytotic machinery is fixed. To determine how quickly transmitter release is blocked by fixative, we switched rapidly from an hypertonic solution to the same hypertonic solution to which 2% glutaraldehyde was added. Hypertonic solution produces release by a calcium independent mechanism (Rosenmund and Stevens, 1996) and appears to circumvent certain key parts of the release machinery because an hypertonic environment still produces release in the usual way in mutant mice that lack synaptotagmin 1, a gene product required for normal synaptic transmission (Geppert et al., 1994). Therefore, fixation of the response to hypertonic solution should not reflect the inactivation of proteins required for the earlier steps in the release process (calcium channels, synaptotagmin) but should relate to later activated parts of exocytotic machinery. As is apparent from Fig. 1C (top trace), the release produced by hypertonic solution was eliminated in less than a second after the switch to the glutaraldehyde containing solution. This is better seen in Fig. 1C (bottom trace) where the derivative of the current is presented to emphasize rapid transients.

Although responses evoked by hypertonic solution decrease rapidly with fixation, this does not necessarily mean that release was blocked; perhaps release continued, but the postsynaptic receptors were fixed so that they could no longer respond to the released glutamate. To investigate this question, we compared block of

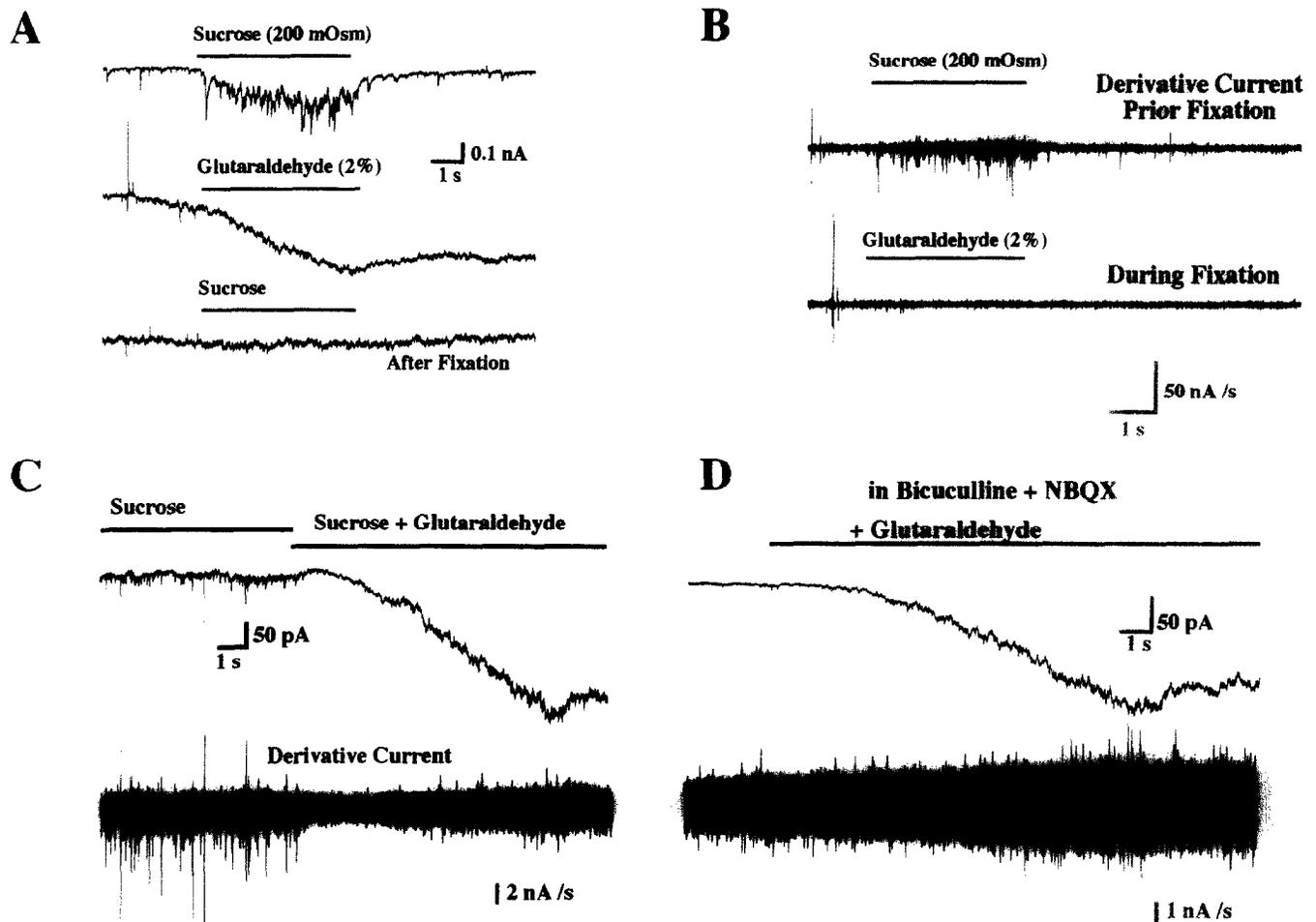


Fig. 1. (A) Current traces as a function of time for a neuron grown in conventional culture. Superfusion of 200 mOsm solution (standard extracellular solution + 200 mOsm of sucrose) produces neurotransmitter release (upper trace). Superfusion with 2% glutaraldehyde causes no obvious release at the start of the application but produces a slowly increasing and persistent inward current (middle trace). After application of the fixative, application of hypertonic solution no longer produced synaptic currents (bottom trace). The 0.1 nA and 1 s scale bars apply to the three traces. The extracellular solution contained $1 \mu\text{M}$ TTX. Similar results were seen in six other cells. (B) First derivatives with respect to time of the current traces from A as a function of time during application of hypertonic solution. The hypertonic solution was applied at the indicated time (top trace) and 2% glutaraldehyde at the corresponding time as shown (bottom trace). The vertical scale (50 nA/s) and time scale (1 s) apply to both traces. (C) Release caused by hypertonic solution is rapidly blocked by 2% glutaraldehyde. Superfusion by 200 mOsm hypertonic solution was rapidly switched to the same hypertonic solution with 2% glutaraldehyde added (top trace). The currents produced are shown in the middle trace where it can be seen that synaptic currents (evident as rapid noise produced by miniature synaptic currents during hypertonic solution application) vanish in less than a second after the switch to fixative (middle trace). When the current trace is differentiated with respect to time to emphasize rapid transients (miniature synaptic currents), it can be seen that synaptic currents disappear in less than a second (bottom trace). (D) The inward current produced by fixative is independent of activation of postsynaptic GABA_A and non-NMDA glutamate receptors. This inward current (top trace) persisted in the presence of $10 \mu\text{M}$ Bicuculline and $10 \mu\text{M}$ NBQX. The bottom trace shows the temporal derivative of the top trace to reveal rapidly changing currents. The 1 s time bar applies to both traces. The 50 pA current calibration applies to the top trace and the 1 nA/s calibration bar to the bottom trace. Similar results were obtained in seven similar experiments.

synaptic transmission with the rate at which responses to kainate vanish during fixation and with the disappearance rates of calcium currents and sodium currents. Fig. 2 displays the time course of these effects after the onset of fixation (again 2% glutaraldehyde added to our standard extracellular solution); note that the solution exchange occurs in less than 50 ms (Rosenmund et al., 1995). Synaptic currents evoked by nerve impulses (produced by a brief depolarizing change in voltage in cells that made only autapses) decrease approximately exponentially with a time constant of 1.4 s, whereas the

responsiveness of the postsynaptic membrane, assayed by kainate application, declines three times more slowly (time constant = 4.4 s). Fixation of sodium and calcium channels, as measured by the decrease of sodium and calcium channel currents, occurs at an intermediate rate (2.2 s for barium currents and 4.6 s for sodium currents). Since appreciable kainate responsiveness persists after synaptic transmission is blocked (Figs. 1 and 2), we conclude that some essential component of the release cascade is fixed rapidly, and that no significant release is produced by the fixative itself under the conditions of these experiments.

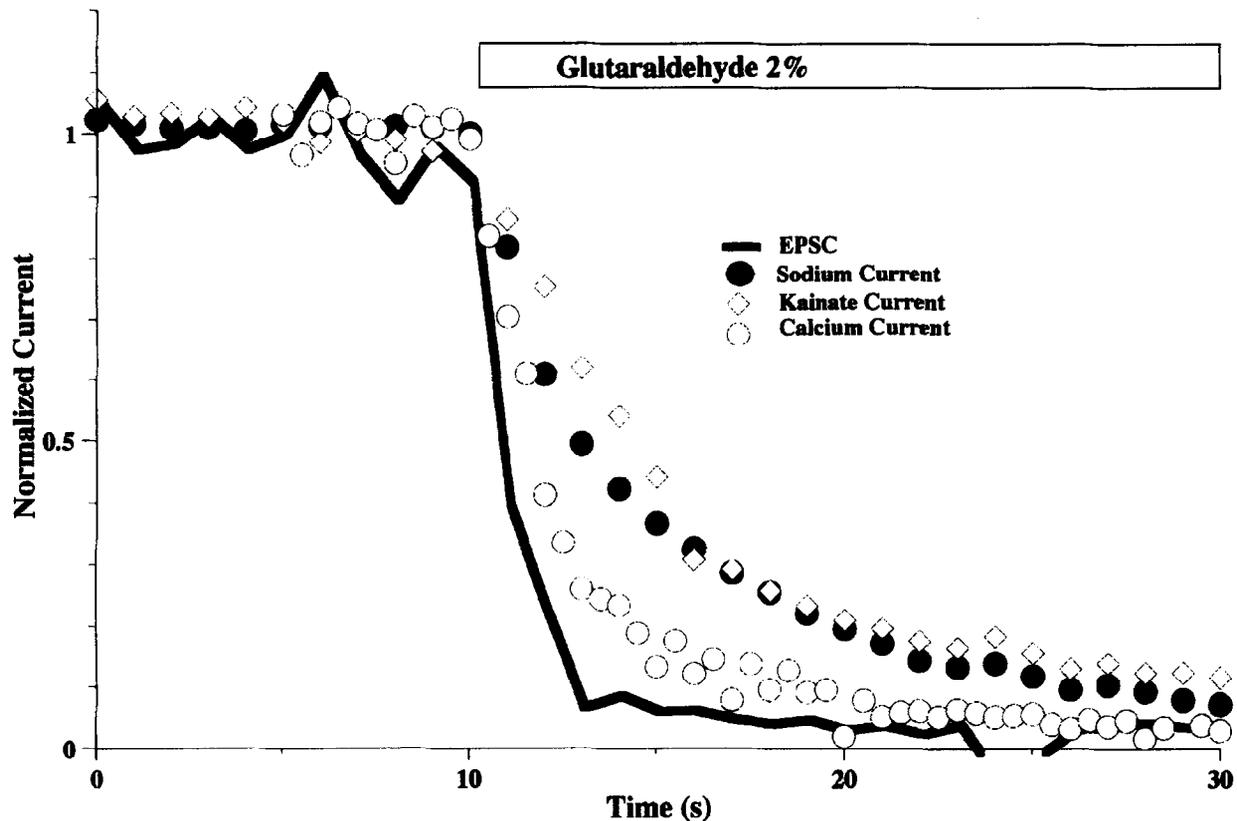


Fig. 2. Time course with which fixation blocks nerve evoked synaptic currents, sodium currents, calcium currents and the response to kainate. Rat hippocampal neurons grown in 'microdot' cultures were held at -60 mV. Repetitive (1 Hz) brief depolarizations to $+10$ mV caused action potentials which in turn produce synaptic currents. The amplitude of the autaptic current is plotted as a function of time after the rapid application of 2% glutaraldehyde. The autaptic currents are blocked rapidly (time constant = 1.4 s; $n = 8$ cells). Calcium currents (measured as barium influx with $1 \mu\text{M}$ TTX present) are blocked with a time constant of 2.2 s ($n = 4$ cells), and sodium currents disappear more slowly (time constant = 4.6 s; $n = 5$ cells). The response to kainate application is also blocked, but more slowly than synaptic transmission (time constant = 4.4 s; $n = 5$ cells).

4. Discussion

The experiments described here demonstrate that fixative evoked release is unlikely to deplete the pool of docked vesicles. This conclusion is a plausible one because exocytosis involves the interactions of many proteins (see Südhof, 1995) so a fixative has many targets, any one of which could shut the process down. We wish to stress how little release occurs in the second before fixation blocks transmission. From Fig. 1 of Rosenmund and Stevens (1996), it can be seen that the release produced by 200 mOsm is much less than the maximal release than can be produced (with, for example, a 500 mOsm solution), and the release produced by 2% glutaraldehyde is very much less than what is seen with 200 mOsm solution (compare the first second of solution applications in Fig. 1). The depletion of the readily releasable pool by fixative is thus well less than 10%.

The source of the difference between the current results and those reported earlier for the frog neuromuscular junction is unclear. Obvious differences are (1) synaptic structure, (2) accessibility of synapses to solu-

tion and (3) rapidity of fixative application. The large and highly organized active zones of the neuromuscular junction might render this synapse less susceptible to fixative. For example, if mechanical factors at one location in the active zone impinge on release at another, then a small number of fixed locations might inhibit release at neighboring docking sites for central synapses whereas the same fraction of fixed locations might have a smaller effect on the extended linear frog active zones. Alternatively, conditions in culture might make synapses more accessible to fixative than the larger synaptic areas with their ensheathing Schwann cell that are found at the frog neuromuscular junction. Studies with rapid application of solution (Rosenmund et al., 1995) reveal that the solution reaches the synaptic membrane in less than 50 ms, implying the absence of significant mixing barriers in culture. Finally, the rapid application of fixative with the rapid solution change system, as opposed to the more gradual ramping up of fixative concentration that results from standard bath solution changing, might account for part of the difference. We feel that all three explanations are likely to contribute.

Although caution in relating structural data, revealed by electron microscopy, to synaptic function is always appropriate, depletion of docked vesicles at active zones by fixative seems to be a minor source of artifact for central synapses in culture.

References

- Almers W, Tse FW. Transmitter release from synapses: does a preassembled fusion pore initiate exocytosis. *Neuron* 1990;4:813–8.
- Bekkers JM, Stevens CF. Excitatory and inhibitory autaptic currents in isolated hippocampal neurons maintained in cell culture. *Proc Natl Acad Sci USA* 1991;88:7834–8.
- Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF, Südhof TC. Synaptotagmin I: A major Ca^{2+} sensor for transmitter release at a central synapse. *Cell* 1994;79:717–27.
- Rosenmund C, Feltz A, Westbrook GL. Synaptic NMDA receptor channels have a low open probability. *J Neurosci* 1995;15:2788–95.
- Rosenmund C, Stevens CF. Definition of the readily releasable pool of vesicles at hippocampal synapses. *Neuron* 1996;16:1–20.
- Smith JE, Reese TS. Use of aldehyde fixatives to determine the rate of synaptic transmitter release. *J Exp Biol* 1980;89:19–29.
- Stevens CF, Tsujimoto T. Estimates for the pool size of releasable quanta at a single central synapse and for the time required to refill a pool. *Proc Natl Acad Sci USA* 1995;92:846–9.
- Südhof TC. The synaptic vesicle cycle: A cascade of protein-protein interactions. *Nature* 1995;375:645–53.