Activation of Neurotransmitter Release in Hippocampal Nerve Terminals During Recovery From Intracellular Acidification

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Trudeau, Louis-Eric, Vladimir Parpura, and Philip G. Haydon. Activation of neurotransmitter release in hippocampal nerve terminals during recovery from intracellular acidification. *J. Neurophysiol.* 81: 2627–2635, 1999. Intracellular pH may be an important variable regulating neurotransmitter release. A number of pathological conditions, such as anoxia and ischemia, are known to influence intracellular pH, causing acidification of brain cells and excitotoxicity. We examined the effect of acidification on quantal glutamate release. Although acidification caused only modest changes in release, recovery from acidification was associated with a very large (60-fold) increase in the frequency of miniature excitatory postsynaptic currents (mEPSCs) in cultured hippocampal neurons. This was accompanied by a block of evoked EPSCs and a rise in intracellular free Ca^{2+} ($[Ca^{2+}]_i$). The rise in mEPSC frequency required extracellular Ca^{2+}, but influx did not occur through voltage-operated channels. Because acidic pH is known to activate the Na^+/H^+ antiporter, we hypothesized that a resulting Na^+ load could drive Ca^{2+} influx through the Na^+/Ca^{2+} exchanger during recovery from acidification. This hypothesis is supported by three observations. First, intracellular Na^+ rises during acidification. Second, the elevation in $[Ca^{2+}]_i$ and mEPSC frequency during recovery from acidification is prevented by the Na^+/H^+ antiporter blocker EIPA applied during the acidification step. Third, the rise in free Ca^{2+} and mEPSC frequency is blocked by the Na^+/Ca^{2+} exchanger blocker dimethylbenzamil. We thus propose that during recovery from intracellular acidification a massive activation of neurotransmitter release occurs because the successive activation of the Na^+/H^+ and Na^+/Ca^{2+} exchangers in nerve terminals leads to an elevation of intracellular calcium. Our results suggest that changes in intracellular pH and especially recovery from acidification have extensive consequences for the release process in nerve terminals. Excessive release of glutamate through the proposed mechanism could be implicated in excitotoxic insults after anoxic or ischemic episodes.

INTRODUCTION

The final cascade of events leading to neurotransmitter release from synaptic vesicles is known to be initiated by a rise in intracellular calcium ($[Ca^{2+}]_i$) (Dodge and Rahamimoff 1967). Other intracellular ions have also been proposed to play a critical role in the release process. Among others, a role has been suggested for intracellular sodium (Adam-Vizi et al. 1993; Nordmann and Stuenkel 1991) and hydrogen ions. Intracellular pH may be an important variable influencing neurotransmitter release in a number of ways such as *1*) influencing the uptake of some neurotransmitters in synaptic vesicles (Fykse and Fonnum 1996), *2*) regulating synaptic vesicle endocytosis (Lindgren et al. 1997; Wang et al. 1995), and *3*) possibly influencing some of the multiple protein–protein interactions involved in the process of synaptic vesicle exocytosis.

Neurotransmitter release could also be affected by intraterminal pH through an indirect effect on $[Ca^{2+}]_i$. A number of possible mechanisms may be proposed. First, intracellular pH may affect the ability of endogenous buffers to bind calcium ions (Zucker 1981). Second, calcium currents can be pH sensitive (Mironov and Lux 1991; Takahashi et al. 1993), directly impacting neurotransmitter release (Barnes et al. 1993). Finally, the activity of ionic pumps and exchangers may be pH sensitive. The sodium–hydrogen antiporter, for example, is activated by intracellular acidification, leading to sodium uptake from the extracellular medium and extrusion of intracellular protons (Jean et al. 1985; Raley-Susman et al. 1991). This is one mechanism through which many cell types attempt to compensate for deviations from normal intracellular pH levels. Because some synaptic terminals are known to express particularly high levels of the sodium–calcium exchanger (Fontana et al. 1995; Luther et al. 1992; Michaelis et al. 1994; Reuter and Porzig 1995), one possible consequence of intracellular acidification and such sodium loading may be to cause an increase in $[Ca^{2+}]_i$ through reverse activity of the Na^+/Ca^{2+} exchanger or diminished rate of calcium efflux. In support of this type of mechanism, it has recently been demonstrated that veratridine-mediated sodium loading may activate neurotransmitter release by inducing calcium uptake independently of the activation of voltage-gated calcium channels (Bouron and Reuter 1996). Evidence has also been presented to suggest that under some conditions activation of Na^+/Ca^{2+} exchange can trigger noradrenaline release from cultured sympathetic neurons (Wakade et al. 1993) and dopamine release from tuberoinfundibular hypothalamic neurons (Annunziato et al. 1992). Functional coupling of Na^+/H^+ and Na^+/Ca^{2+} exchangers has also been demonstrated in non-neuronal systems (Urcelay et al. 1994). One potential problem with the hypothesis that acidification may initiate such a sequential activation of the Na^+/H^+ and Na^+/Ca^{2+} exchangers is that the activity of the latter is actually inhibited by acidic pH (and activated by alkaline pH) (Doering and Lederer 1993). However, because intracellular acidification is often followed by rebound alkalinization on cessation of the acidic challenge, this may provide ideal conditions for sodium loading and reverse activation of the Na^+/Ca^{2+} exchanger.

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A better understanding of the influence of intracellular pH on neurotransmitter release may provide data helpful in understanding neurotoxicity associated with conditions such as cerebral ischemia, anoxia, and hypoglycemia. Indeed these pathological conditions are known to be associated with perturbations of intracellular pH in brain cells. Ischemia and anoxia cause intracellular acidification and sodium loading, which can be followed by rebound alkalinization during recovery (Chen et al. 1992; Chopp et al. 1990; Friedman and Haddad 1994; Pirttila and Kauppinen 1992). In the heart, evidence has been presented to suggest that acidification during ischemia may be associated with a rise in intracellular sodium and a subsequent activation of Na^+/Ca^{2+} exchange (Murphy et al. 1991). Reverse operation of the Na^+/Ca^{2+} exchanger has also been shown to occur in response to sodium accumulation during anoxia in myelinated axons (Lehning et al. 1996; Stys et al. 1992). Hypoglycemia may also be associated with intracellular alkalinization and is associated with excessive release of excitatory amino acids and excitotoxic damage (Auer and Siesjo 1993).

To determine more directly how intracellular acidification affects neurotransmitter release, we have recorded quantal glutamate release from cultured hippocampal neurons under such conditions. We find that recovery from acidification is associated with a substantial activation of the spontaneous release process under conditions where action potential-evoked release is mostly blocked.

METHODS

Cell culture

Primary cultures of neonatal rat hippocampal neurons were prepared as described previously (Trudeau et al. 1996). Briefly, the hippocampus was dissected from newborn rats and dissociated with papain. Neurons and astrocytes were plated onto collagen/poly-L-lysine–coated glass coverslips (0.1 mg/ml). Culture medium contained 5% fetal calf serum (GIBCO BRL; Grand Island, NY) and Mito + serum additives (Collaborative Biomedical Products; Bedford, MA). A mitotic inhibitor was added after the 4th day in culture to halt glial proliferation (5-fluoro-2-deoxyuridine and uridine, 5 μM). One-third of the medium was replaced with fresh medium twice a week. Neurons were used in physiological experiments after 3–4 wk in culture. In preliminary experiments we noted that the major observations reported in this paper appeared to be less robust in 2-wk-old cultures. In some experiments neuron-enriched cultures were prepared, as previously described (Goslin and Banker 1991), to permit better visualization of neuronal processes.

Electrophysiology

Whole cell recordings were performed on the stage of a Nikon Diaphot inverted microscope equipped with phase contrast optics. Signals were recorded through Axopatch-1D patch amplifiers (Axon Instruments; Foster City, CA), digitized at 5 kHz and acquired in pClamp software (version 6.0) (Axon Instruments). Miniature synaptic currents were further analyzed with ACSPLOUF software (Dr. P. Vincent, UCSD, CA). In most experiments it was possible to adequately resolve all unitary synaptic events. However, in a subset of experiments the frequency of miniature excitatory synaptic currents (mEPSCs) rose to very high levels, and it is possible that some proportion of elementary events failed to be detected. This may have caused a slight underestimation of maximal mEPSC frequencies but should not affect any of the conclusions reached in this paper. Normal extracellular saline contained (in mM) 140 NaCl, 2 $MgCl_2$, 2 $CaCl_2$, 5 KCl, 10 HEPES, 6 sucrose, and 10 glucose (pH 7.35). High-divalent saline was used to decrease spontaneous synaptic activity and to facilitate recording of monosynaptic action potential-evoked EPSCs. It contained (in mM) 135.5 NaCl, 7 $MgCl_2$, 3 $CaCl_2$, 5 KCl, 10 HEPES, and 8 glucose (pH 7.35). Propionate saline used to acidify neurons was prepared by completely replacing NaCl with Na propionate in either normal or high divalent salines. Nominally calcium-free saline contained (in mM) 140 NaCl, 5 KCl, 4 $MgCl_2$, 1 EGTA, 10 HEPES, 4 sucrose, and 10 glucose (pH 7.35). To record action potential-evoked EPSCs, 10 μM SR-95531 (RBI; Natick, MA) was added to the saline to block $GABA_A$ receptors. To record mEPSCs, normal saline was supplemented with 10 μM SR-95531 to block $GABA_A$ receptors and 0.5 μM TTX (Calbiochem) to block sodium channels. The internal patch pipette solution used to record action potential-evoked synaptic currents contained (in mM) 140 K gluconate, 10 EGTA, 4 Mg-ATP, 0.2 Tris-GTP, and 10 HEPES (pH 7.35). To record mEPSCs, the internal patch pipette solution consisted of (in mM) 117.5 Cs gluconate, 10 NaCl, 4 $MgCl_2$, 5 EGTA, 2 Mg-ATP, 0.2 Tris-GTP, and 15 HEPES (pH 7.35). Statistical comparisons were performed with Student's *t*-test, unless otherwise indicated.

Imaging

$[Ca^{2+}]_i$, pH, and sodium levels were monitored by fluorescence imaging with a silicon-intensified target camera (Hamamatsu; Bridgewater, NJ), IC-300 intensified charge-coupled device (CCD) camera (Photon Technology International; Monmouth Junction, NJ), or CH-250 cooled CCD camera (Photometrics; Tucson, AZ). Images were acquired with Metafluor (Universal Imaging; West Chester, PA) or NeD_{LC} software (Prairie Technologies; Waunakee, WI). Signals were measured from regions representing cell bodies and/or major neurites. Fluorescent dyes {fura-2AM, fluo-3AM, 2′,7′-bis-(2-carboxyethyl)-5-carboxyfluorescein–AM (BCECF-AM), and 1,3-benzenedicarboxylic acid, 4,4′-[1,4,10-trioxa-7,13-diazacyclopentadecane-7,13-diylbis(5-methoxy-6,12-benzofurandiyl)]–AM (SBFI-AM)} were acquired from Molecular Probes (Eugene, OR). Calcium levels were estimated according to Basarsky et al. (1995). Calibration was performed in situ (Thomas and Delaville 1991) with the Ca^{2+} ionophore 4-BrA-23187 (Molecular Probes) at the end of each experiment. Although it is known that the affinity for calcium of fluorescent indicators such as fluo-3 may be sensitive to acidic pH, this is not likely to have significantly affected the results reported because intracellular pH never reached the critical level of 5.5 (Lattanzio and Bartschat 1991). Furthermore, the experiments performed in this study investigated calcium changes during alkalinization of the cytosol. Alkalinization by itself has not been reported to have a major effect on fluo-3 fluorescence. This is experimentally supported by data in Fig. 5, where, in the absence of external calcium, alkalinization had no effect on fluo-3 fluorescence.

RESULTS

To produce intracellular acidification, cultured hippocampal neurons were exposed to saline in which NaCl was replaced with the weak acid Na propionate. This approach has been previously shown to produce reliable intracellular acidification in neurons and other cell types (Chen et al. 1998; Lindgren et al. 1997; Stuenkel and Nordmann 1993). An advantage of this approach is that intracellular alkalinization can be produced on propionate saline washout, under conditions where the extracellular solution is again normal, bypassing problems related to the extracellular effects of alkalinizing agents such as ammonium chloride, which is known, for example, to affect glutamate receptors (Fan and Szerb 1993; Fan et al. 1990). Imaging experiments were performed with the fluorescent pH indicator BCECF. We found that,

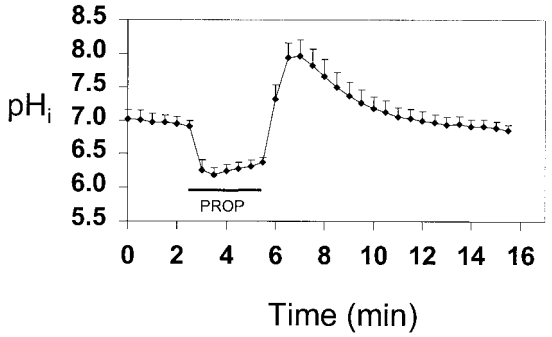

FIG. 1. Intracellular acidification and rebound alkalinization produced by propionate. Intracellular pH in cultured hippocampal neurons was measured by imaging of BCECF fluorescence. The cells were exposed to propionate-containing saline for 3 min (—). Propionate (PROP) caused a rapid intracellular acidification followed by rebound alkalinization on washout of propionate with normal saline. Cells then gradually recovered to basal pH. pH recovery in the presence of propionate was slow, and full recovery on washout required >10 min. Points represent mean ± SE ($n = 10$).

as expected, propionate produced a rapid intracellular acidification followed by partial and gradual recovery (Fig. 1) ($n = 10$). Washout with normal saline caused a rapid rebound intracellular alkalinization within 1 min. This was followed by a gradual recovery to resting intracellular pH (Fig. 1).

Effect on action potential-evoked and spontaneous neurotransmitter release

Dual whole cell recording experiments were performed to monitor action potential-evoked neurotransmitter release. The presynaptic neuron was maintained at its resting potential, whereas the postsynaptic neuron was voltage clamped at −60 mV. Action potentials evoked rapid EPSCs (Fig. 2*A*), which were blocked by the glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (not shown). Intracellular acidification produced by propionate-containing saline produced only transient and variable decreases in the amplitude of EPSCs (Fig. 2, *A* and *B*) (68 ± 22% of control, mean ± SE, $P > 0.05$; $n = 7$). The amplitude of EPSCs recovered completely within 3 min in the continued presence of propionate (Fig. 2, *A* and *B*). Washout of propionate rapidly produced an almost complete block of evoked EPSCs (Fig. 2, *A* and *B*) (11 ± 5% of control, $P < 0.05$, $n = 7$). This block of evoked release occurred within 30 s after perfusion with normal saline (Fig. 2*C*), in parallel to the timing of rebound alkalinization (Fig. 1). Block of release was not caused by irreversible damage to nerve terminals, as reintroduction of propionate-containing saline allowed an immediate and almost complete recovery (Fig. 2, *A* and *B*) (74 ± 5% of control, $n = 7$). In the absence of such re-acidification, EPSC amplitude was found to recover gradually but very slowly (to 15% of basal level within 10 min of washout).

Propionate-induced intracellular acidification was associated with a modest but reliable increase in the frequency of spontaneous mEPSCs (600 ± 136% of control, $P < 0.05$, $n = 8$) (Fig. 3, *A* and *B*). This was short lasting, similar to the observed decrease in evoked EPSC amplitude during acidification. Considering the powerful block of action potential-evoked glutamate release after removal of propionate saline, it was some-

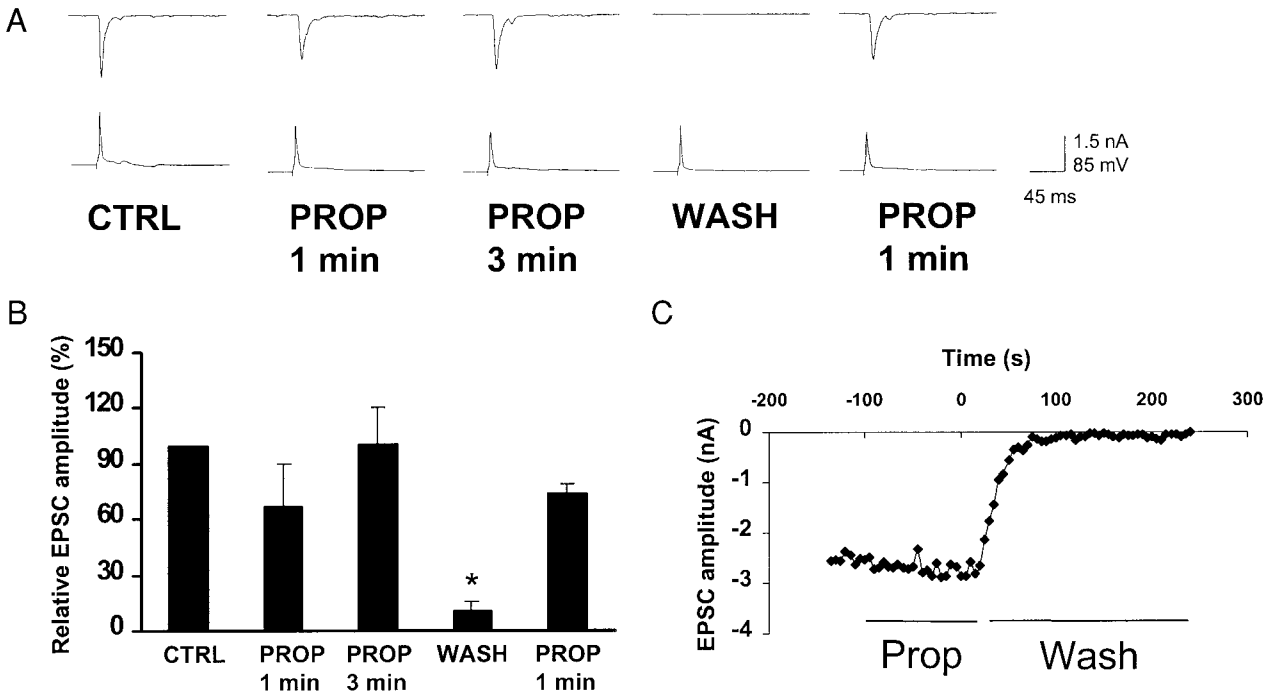

FIG. 2. Block of action potential-evoked excitatory postsynaptic currents (EPSCs) during recovery from acidification. Dual whole cell recordings from pairs of cultured hippocampal neurons. The presynaptic neuron (*A*, *bottom traces*) was briefly depolarized to trigger an action potential. This produced an EPSC in the voltage-clamped postsynaptic neuron (*A*, *top traces*). Although propionate caused only a brief and modest reduction in EPSC amplitude (not significant), an almost complete block was observed on washout of the propionate-containing saline (WASH). Rapid recovery of the EPSC amplitude was observed when the neurons were re-acidified by a second application of propionate saline. Data from 7 experiments are summarized in *B*. Bars represent mean ± SE ($n = 7$; $^*P < 0.05$). Time course of the block of evoked EPSCs on propionate saline washout is illustrated in *C* for the experiment shown in *A*. EPSC amplitude started to decline as soon as 10 s after the beginning of the washout, and a maximal block was obtained by 60–70 s.

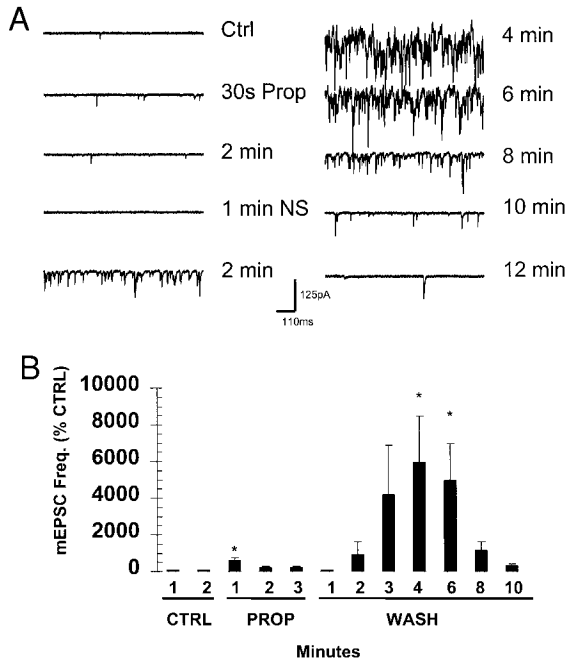

FIG. 3. Delayed stimulation of mEPSC frequency. Whole cell recordings of mEPSCs in the presence of TTX (*A*). Propionate saline produced a small, short-lasting, and rapid increase in mEPSC frequency (30 s Prop). Washout of the acidifying propionate-saline with normal saline (NS) was also accompanied by an enhancement of mEPSC frequency, but this was delayed by 2–4 min after the beginning of the washout period and was much more substantial (4 min). The effect then gradually declined with time. Summary data from 8 such experiments are shown in *B* (*$P < 0.05$).

what unexpected to observe that the mEPSC frequency rose to very high levels ≤2–4 min after washout (Fig. 3, *A* and *B*). This increase in mEPSC frequency was not immediate, such as for the block of evoked EPSCs, and was delayed in relation to the timing of the peak of intracellular alkalinization, suggesting that alkalinization per se did not stimulate exocytosis. After 4 min of propionate washout, the frequency of mEPSCs was increased ~60-fold above baseline (5,940 ± 2,530% of control, $P < 0.05$, $n = 8$) (Fig. 3, *A* and *B*). Recovery was gradual and reached levels close to baseline by 10 min (310 ± 131% of baseline, $P > 0.05$, $n = 8$) (Fig. 3, *A* and *B*). This massive increase in release was obtained without any significant depolarization of neuronal cell bodies. On average, resting potential during the control period was −55 ± 2 mV, whereas it was −52 ± 3 mV after 4 min of propionate saline washout, which is at the peak of the increase in mEPSC frequency.

Role of $[Ca^{2+}]_i$

To determine whether the substantial rise in mEPSC frequency was caused by a rise in $[Ca^{2+}]_i$ in neurons, imaging experiments were performed with the fluorescent calcium indicators fluo-3 and fura-2. In experiments performed with fluo-3, propionate-containing saline sometimes caused a small and unreliable increase in $[Ca^{2+}]_i$ (Fig. 4). However, washout of propionate was associated with a delayed and significant rise in $[Ca^{2+}]_i$ ($n = 8$; ANOVA and Tukey's post hoc test, $P < 0.01$) (Fig. 4). The time course of this rise in $[Ca^{2+}]_i$ was very similar to the time course of the elevation in mEPSC frequency observed on propionate saline washout (Fig. 3*B*). By using recorded fluorescence changes in cells and standard calibration curves for fluo-3 (Minta et al. 1989), we estimated a $[Ca^{2+}]_i$ accumulation of ~700 nM caused by rebound alkalinization. In parallel experiments with fura-2, we confirmed that similar $[Ca^{2+}]_i$ accumulations (~800 nM) occur in neuronal processes ($n = 2$).

To determine whether extracellular calcium was required for the rebound rise in $[Ca^{2+}]_i$ and mEPSC frequency, propionate saline was washed out with nominally calcium-free saline instead of normal saline. We found that, although rebound alkalinization was unimpaired under such conditions (Fig. 5*B*), both the rebound rise in $[Ca^{2+}]_i$ (Fig. 5*C*) and mEPSC frequency (Fig. 5*A*) were prevented. Reintroduction of normal saline after calcium-free saline still caused a substantial rise in $[Ca^{2+}]_i$ (Fig. 5*C*) and mEPSC frequency (2,606 ± 924% of control after 1 min, $P < 0.05$, $n = 4$) (Fig. 5*A*), thereby showing that the driving force behind the rise in mEPSC frequency was not dissipated by the absence of external calcium during rebound alkalinization. These results suggest that calcium influx is important for the rebound increase in mEPSC frequency. The observation that the rise in mEPSC frequency is blocked under conditions where the rebound intracellular alkalinization still occurs suggests that the latter phenomenon is not sufficient by itself to cause the rise in mEPSC frequency. Further support for this hypothesis comes from the observation that direct intracellular alkalinization with 15 mM ammonium chloride fails to cause a rise in mEPSC frequency ($n = 2$, mEPSC frequency at 60% of baseline after 3 min; results not shown).

Because the delayed rise in $[Ca^{2+}]_i$ and mEPSC frequency requires extracellular calcium, it may be hypothesized that this occurs through the activation of voltage-gated calcium channels. Alternately, calcium influx could occur through a carrier such as the Na^+/Ca^{2+} exchanger. The role of calcium channels was tested by determining whether washing out propionate-containing saline with saline containing 100 μM cadmium would prevent the rebound rise in mEPSC frequency. This concentration of cadmium is sufficient to block most high-voltage-activated calcium channels (Bouron and Reuter 1996) (results not shown) while sparing the activity of the Na^+/Ca^{2+} exchanger (Bouron and Reuter 1996). We found that the re-

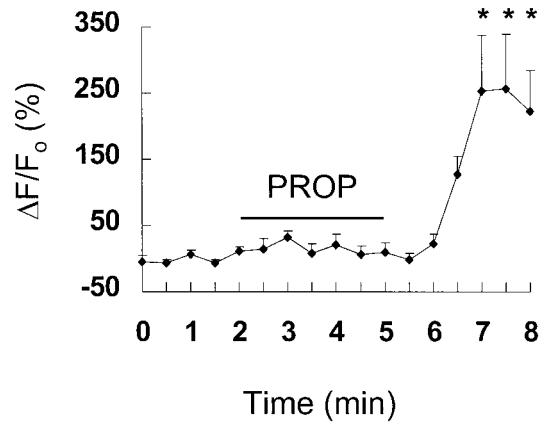

FIG. 4. Delayed increase in intracellular free calcium ($[Ca^{2+}]_i$). Calcium measurements with fluo-3 fluorescence imaging. Propionate saline (PROP) produced a small and variable increase in free calcium in neuronal cell bodies and major neurites. Washout of propionate saline was accompanied by a delayed and substantial rise in calcium. Summary data from 8 experiments are shown. Points represent mean ± SE ($n = 8$; *$P < 0.01$, ANOVA and post hoc Tukey's test).

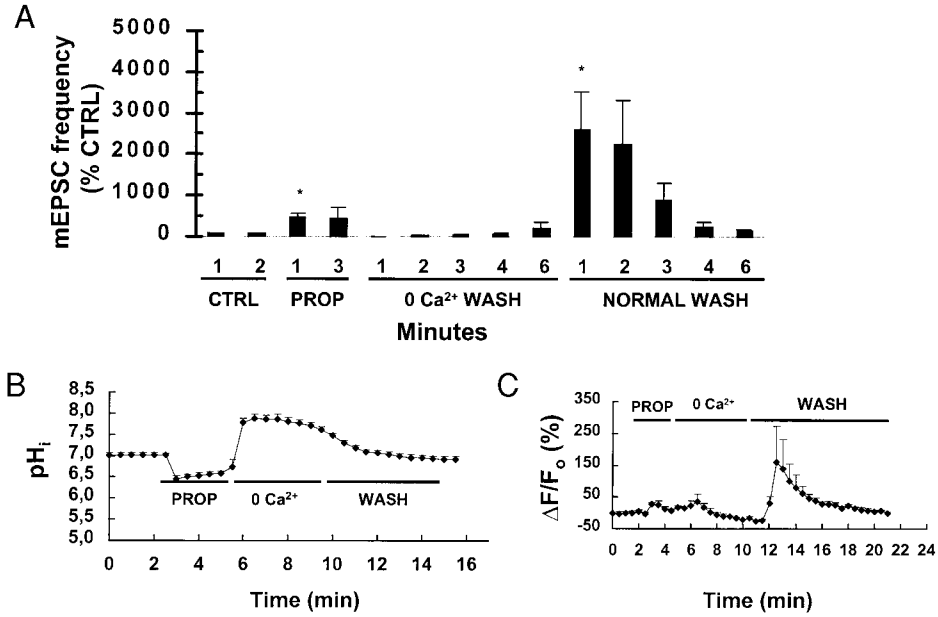

FIG. 5. Requirement for extracellular calcium. The frequency of mEPSCs was measured in the presence of TTX (*A*). Perfusion of propionate-containing saline (PROP) for 3 min was followed by washout with nominally calcium-free saline (0 Ca^{2+} WASH). This fully prevented the rise in the frequency of mEPSCs seen in control preparations. Substitution of calcium-free saline for normal saline (NORMAL WASH) allowed the rebound rise in mEPSC frequency to be expressed. Although it was able to block the rise in mEPSC, washout with calcium-free saline did not prevent the rebound rise in intracellular pH measured by BCECF imaging (*B*). However, this treatment (0 Ca^{2+}) was effective at blocking the delayed rise in $[Ca^{2+}]_i$ measured by fluo-3 imaging (*C*). Reintroduction of normal saline then still permitted calcium to rise (WASH) (C).

bound rise in mEPSC frequency that follows intracellular acidification was still clearly present under such conditions (Fig. 6*A*). Additionally, the delayed rise in $[Ca^{2+}]_i$ that parallels rebound alkalinization was unaffected by cadmium (Fig. 6*B*) ($n = 9$). Both the rise in mEPSC frequency and the rise in $[Ca^{2+}]_i$ were not significantly different from those obtained under control conditions ($P > 0.05$). These results suggest that, although calcium influx may trigger the rise in mEPSC frequency, this does not occur through voltage-gated calcium channels.

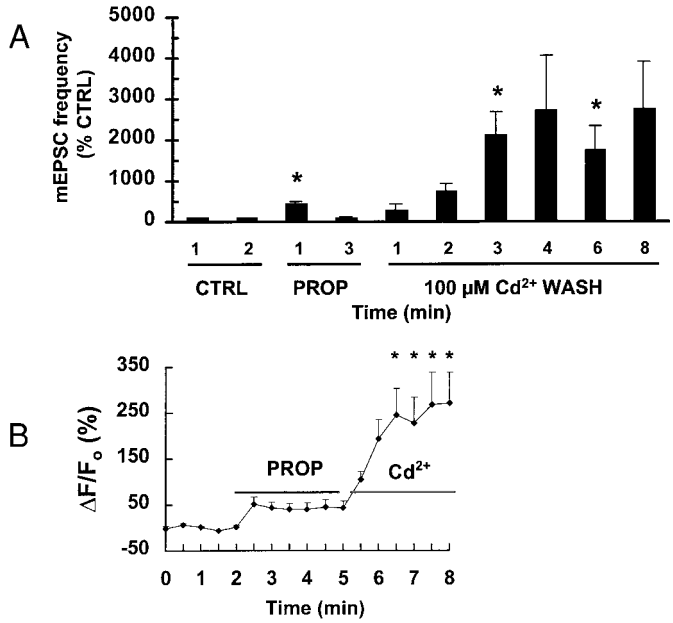

FIG. 6. Calcium channel blockade fails to prevent the rebound rise in mEPSC frequency. *A*: perfusion of propionate-containing saline (PROP) for 3 min was followed by washout with saline containing 100 μM cadmium (100 μM Cd^{2+} WASH). This failed to prevent the rise in the frequency of mEPSCs seen in control preparations. It was also ineffective at preventing the delayed rise in $[Ca^{2+}]_i$, measured with fluo-3 (*B*). Bars and points represent mean ± SE (*$P < 0.01$).

Role of ion exchangers

One hypothesis that may be proposed is that intracellular acidification activates the Na^+/H^+ exchanger, thereby driving Na^+ accumulation in the cytoplasm. This sodium load may then provide a driving force, allowing reverse activation of the Na^+/Ca^{2+} exchanger to cause an accumulation of calcium in synaptic terminals. Such a hypothesis finds support from the recent observation that synaptic terminals are richly endowed with the Na^+/Ca^{2+} exchanger (Luther et al. 1992; Michaelis et al. 1994; Reuter and Porzig 1995). The contribution of the Na^+/H^+ exchanger was tested in preliminary experiments with the classical blocker amiloride. We found that application of amiloride (1 mM) together with propionate-containing saline prevented the rise in mEPSC frequency during propionate washout ($n = 3$) (data not shown). Because amiloride is known to have significant affinity not only for the Na^+/H^+ exchanger but also for the Na^+/Ca^{2+} exchanger as well as other pumps (Murata et al. 1995), we performed a second series of experiments with the more specific amiloride derivative 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA). This compound can be used at much lower concentrations and is known to have close to one order of magnitude higher affinity for the Na^+/H^+ exchanger than for the Na^+/Ca^{2+} exchanger (Murata et al. 1995). We found that 10 μM EIPA prevented the ability of propionate-containing saline to induce the rebound rise in mEPSC frequency (Fig. 7*A*) ($n = 3$). As expected for a block of Na^+/H^+ exchange, this antagonist was also effective at blocking the induction of rebound alkalinization (Fig. 7*B*) ($n = 5$). The delayed rise in $[Ca^{2+}]_i$ was also completely prevented by EIPA (Fig. 7*C*) ($n = 6$). To provide additional support for the hypothesis that a sodium load may be induced by intracellular acidification, we used SBFI, a fluorescent ratiometric sodium indicator to directly monitor intracellular sodium levels (Minta and Tsien 1989; Rose and Ransom 1997). Propionate-containing saline was found to produce a gradual and long-lasting rise in intracellular sodium in neurons (Fig. 8) ($n = 6$), compatible with our results suggesting that acidification activates Na^+/H^+

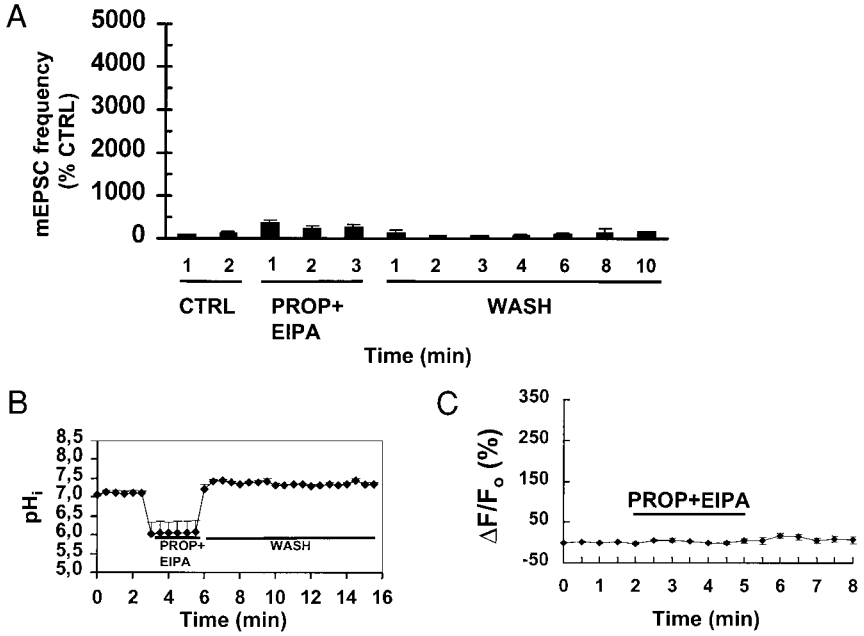

FIG. 7. The Na^+/H^+ antiporter blocker 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) prevents the action of propionate. *A*: co-perfusion of propionate-containing saline together with EIPA (10 μM) prevented the occurrence of the rise in the frequency of mEPSCs seen in control preparations during the washout period. It also blocked most of the alkalinization that follows propionate washout without preventing the propionate-induced acidification (*B*). Delayed rise in $[Ca^{2+}]_i$ seen in control preparations was also completely prevented (*C*). Bars and points represent mean ± SE ($^*P < 0.05$).

exchange that consequently leads to a loading of neurons with Na^+.

If the delayed rise in $[Ca^{2+}]_i$ and mEPSC frequency is caused by activation of Na^+/Ca^{2+} exchange during the rebound alkalinization phase, then blocking this exchanger during the propionate saline washout period should block the rise in calcium and mEPSC frequency. This was tested with 2′,4′-dimethylbenzamil (DMB), a Na^+/Ca^{2+} exchanger blocker that is not fully specific for the Na^+/Ca^{2+} exchanger at all concentrations but that is known to have close to one order of magnitude more affinity for this exchanger than for the Na^+/H^+ exchanger or the sodium ATPase (Murata et al. 1995). At a concentration of 10 μM, DMB was found to produce a complete block of the delayed rise in mEPSC frequency (Fig. 9*A*) and $[Ca^{2+}]_i$ elevation ($n = 7$) (Fig. 9*B*).

DISCUSSION

Our results demonstrate that acidification and rebound alkalinization may have considerable effects on quantal neurotransmitter release from synaptic terminals. Although acidification itself produces some modification of neurotransmitter release (Figs. 2 and 3), removal of acidification has a more significant impact on glutamate release. One major consequence of recovery from temporary acidification is a pronounced but reversible block of action potential-evoked neurotransmitter release (Fig. 2). This effect is not caused by a block of postsynaptic CNQX-sensitive glutamate receptors because mEPSCs are still easily detectable during this period (Fig. 3). The block of release is also not caused by a toxic reaction to rebound alkalinization

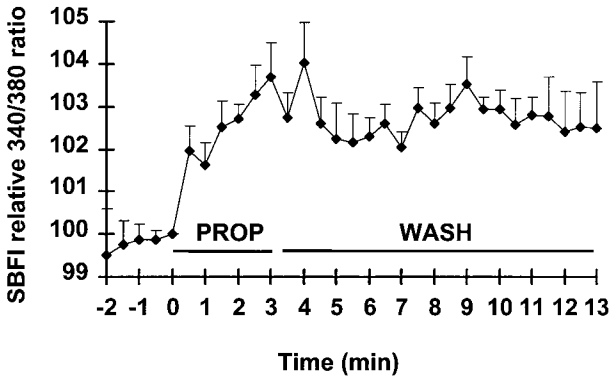

FIG. 8. Propionate-induced acidification is accompanied by a rise in sodium. Intracellular sodium was measured with SBFI. A gradual rise in sodium was induced by propionate-containing saline. Recovery during the washout period was very slow. Points represent mean ± SE ($^*P < 0.05$).

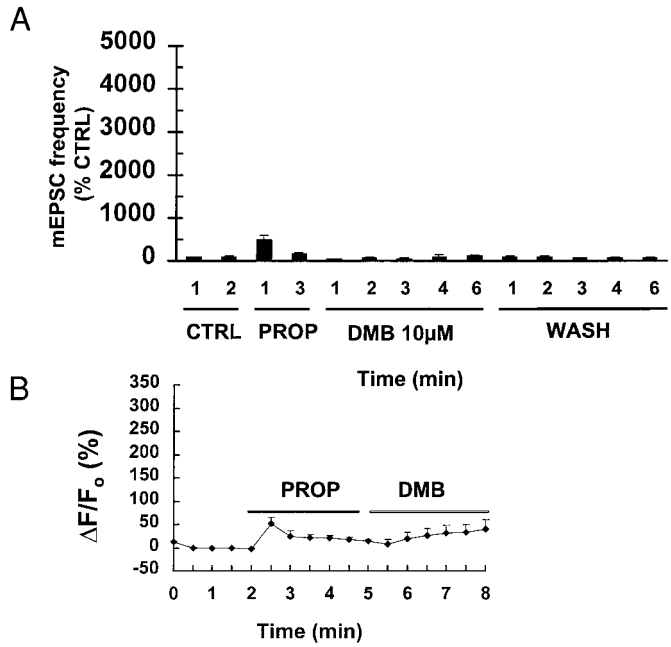

FIG. 9. The Na^+/Ca^{2+} exchanger blocker DMB prevents the rebound rise in mEPSC frequency. Frequency of mEPSCs was measured in the presence of TTX (*A*). Perfusion of propionate-containing saline (PROP) for 3 min was followed by washout with saline containing 10 μM DMB. This fully prevented the rise in the frequency of mEPSCs seen in control preparations. The delayed rise in $[Ca^{2+}]_i$ was also prevented (*B*). Bars and points represent mean ± SE ($^*P < 0.05$).

because the effect is rapidly reversible on re-acidification of neurons (Fig. 2). Although we have not identified the specific mechanism responsible for the block of action potential-evoked EPSCs, the time course of this block follows that of rebound alkalinization (Fig. 2*C*). One possibility is that intracellular alkalinization of synaptic terminals leads to a block or inactivation of voltage-dependent calcium channels. However, as mentioned previously, the opposite has been reported; alkalinization is known to facilitate calcium currents (Mironov and Lux 1991; Takahashi et al. 1993). Although other mechanisms may be proposed, two possibilities are that calcium channel coupling to synaptic vesicle exocytosis is perturbed or that the synaptic protein complex responsible for the release of neurotransmitter is directly inactivated.

A second major consequence of recovery from acidification is a delayed but massive increase in the frequency of mEPSCs (Fig. 3). In many preparations, the stimulation of quantal secretion was large enough to be reminiscent of the effects of powerful secretagogues such as α-latrotoxin (Capogna et al. 1996; Krasnoperov et al. 1997). This effect was notably delayed in all neurons (Fig. 3), in contrast to the block of action potential-evoked EPSCs (Fig. 2*C*). This is compatible with the idea that the two phenomena are mediated by distinct mechanisms. It also suggests that the increase in mEPSC frequency is not induced solely by the intracellular alkalinization that follows washout of propionate saline. This increase in mEPSC frequency was reversible over time, suggesting that the effect was also not caused by an irreversible toxic reaction, although a partial depletion of readily releasable vesicles could have occurred. Rather our calcium imaging experiments suggest that the stimulation of spontaneous exocytosis may be caused by an intracellular accumulation of free calcium (Fig. 4). Perfusion of propionate-containing saline was initially associated with a brief and relatively small rise in $[Ca^{2+}]_i$ (Fig. 4*B*). This occurred in parallel to intracellular acidification and could be caused by the previously described decrease in the calcium-buffering power of cellular cytoplasm induced by acidification (Zucker 1981) or by release of calcium from intracellular stores (Chen et al. 1998), although other mechanisms cannot be rejected. This modest and short-lasting rise was followed by a more substantial and delayed increase in $[Ca^{2+}]_i$ on washout of propionate-containing saline. The delay was usually between 2 and 4 min (Fig. 4*B*), in close parallel to the delay observed for the rise in mEPSC frequency (Fig. 3). Our observation that the delayed rise in mEPSC frequency does not occur if propionate-containing saline is washed out with calcium-free saline (Fig. 5) suggests that the rise in $[Ca^{2+}]_i$ is due to influx or transport of calcium across the cellular membrane. The finding that re-introduction of normal calcium-containing saline still causes a rise in mEPCS frequency after exposure of cells to calcium-free saline (Fig. 5*A*) suggests that the driving force for the calcium entry, whatever its nature, was still present after this delay. Washout of propionate-containing saline with saline containing 100 μM cadmium was also not effective at blocking the delayed rise in mEPSC frequency (Fig. 6). Because this concentration of cadmium is sufficient to block most high-voltage-activated calcium currents in neurons, this may be taken as evidence that the intracellular accumulation of calcium is not occurring through activation of voltage-dependent calcium channels. This was important to determine because previous reports have suggested that some classes of calcium channels may be facilitated by alkalinization (Mironov and Lux 1991; Takahashi et al. 1993).

If calcium channels do not cause the delayed rise in $[Ca^{2+}]_i$ and mEPSC frequency, then an alternate hypothesis is that the rise in calcium is mediated by a transporter-based mechanism. One hypothesis is that acidification causes the activation of the Na^+/H^+ antiporter and thus a rise in intracellular sodium. On washout of propionate-containing saline, this rise in sodium may provide a driving force sufficient to cause a reverse activation of the Na^+/Ca^{2+} exchanger. Because this exchanger is otherwise known to be highly concentrated in nerve terminals, it is conceivable that such a mechanism may be responsible for the observed delayed rise in mEPSC frequency. Alkalinization that follows washout of propionate saline may play a permissive role in this process because it has been shown that the Na^+/Ca^{2+} exchanger can be activated under alkaline conditions and inhibited under acidic conditions (Doering and Lederer 1993). Nonetheless, the current set of data does not allow us to conclude that alkalinization per se is required for the activation of Na^+/Ca^{2+} exchange.

Our data provide four independent pieces of evidence that support the hypothesis that elevated exocytosis results from calcium influx driven by Na^+/Ca^{2+} exchange that is activated by elevated intracellular Na that accumulates as a result of Na^+/H^+ antiporter activity during acid conditions. First, we show that two blockers of the Na^+/H^+ exchanger, amiloride and EIPA, block rebound alkalinization and the delayed rise in mEPSC frequency (Fig. 7) when applied simultaneously with the acidifying saline. Second, EIPA also blocks the delayed rise in calcium (Fig. 7*C*). Third, propionate-containing saline causes the predicted rise in intracellular sodium (Fig. 8). This rise is relatively long lasting, compatible with the observation that the driving force for the increase in calcium and mEPSC frequency is also relatively long lasting (Fig. 5). Fourth, a blocker of the Na^+/Ca^{2+} exchanger, DMB, is able to block the rise in mEPSC frequency and $[Ca^{2+}]_i$, even when applied after the propionate-containing saline (Fig. 9), under conditions where the rise in sodium has already occurred. Finally, we have found that direct intracellular alkalinization with ammonium chloride does not cause an increase in spontaneous release. This argues in favor of the idea that an initial intracellular acidification is specifically required to obtain the observed increase in secretion and that alkalinization is not a sufficient condition. This is compatible with the hypothesis that sodium loading of neurons during acidification provides a driving force for the subsequent rise in mEPSC frequency during the recovery period. Although DMB has a ~10-fold higher affinity for the Na^+/Ca^{2+} exchanger than for other targets such as the Na^+/H^+ antiporter or the sodium ATPase, it is not fully selective for the Na^+/Ca^{2+} exchanger (Murata et al. 1995). However, because the block of Na^+/H^+ exchange by DMB displays an IC50 above 60 μM (Murata et al. 1995), it is likely that at 10 μM DMB will not produce a substantial block of the Na^+/H^+ exchanger. Nonetheless, taken by itself the block of the rise in mEPSC frequency by DMB does not prove the involvement of Na^+/Ca^{2+} exchange. However, taken with the rest of our results this observation provides support for this hypothesis and can be considered the simplest explanation of our findings.

It should be noted that because our experiments were performed in nominally CO_2 and HCO_3-free medium we have not evaluated the role of sodium-dependent Cl/HCO_3 exchange in rebound alkalinization. Such an exchange mechanism could

possibly contribute to rebound alkalinization in vivo (Schwiening and Boron 1994).

Our results demonstrate a substantial influence of intracellular pH on quantal glutamate release in hippocampal neurons. We find that intracellular acidification has only modest effects on release, even under conditions where pH decreases by more than one-half a unit (Fig. 2). However, recovery from acidification is associated with both a powerful block of action potential-evoked EPSCs and a delayed but substantial rise in mEPSC frequency. Although our experimental conditions do not specifically mimic pathological conditions such as ischemia and hypoxia, it is noteworthy that such conditions may be associated with intracellular acidification and possibly with a sequential acidification, alkalinization sequence (Chen et al. 1992; Pirttila and Kauppinen 1992). It will be important for future studies to examine the influence of in vitro models of ischemic or hypoxic episodes on intracellular pH, calcium, and quantal glutamate release. A combined block and stimulation of action potential-evoked and spontaneous glutamate release, respectively, could have dramatic influences of brain functions and play some role in the observed deficits associated with conditions such as ischemia or hypoxia. It is of interest to note that it has been hypothesized that neuronal death in brain ischemia may be caused by two phases of excessive glutamate release. The first may occur during the ischemic episode and be caused by reverse operation of the glutamate transporter. The second may occur after ischemia and be caused by glutamate released from vesicular stores (Szatkowski and Attwell 1994). One exciting possibility is that this second phase is caused by reversed activation of the Na^+/Ca^{2+} exchanger in synaptic terminals during recovery from the acidic challenge.

We thank Drs. Harald Reuter and Alexandre Bouron for helpful comments on this manuscript.

This work was supported in part by National Institute of Neurological Disorders and Stroke Grants NS-26650 and NS-24233 to P. G. Haydon and by postdoctoral fellowships from the Human Frontier Science Program and the Medical Research Council (MRC) of Canada to L.-E. Trudeau. L.-E. Trudeau is now a Michael Smith scholar of the MRC of Canada and is supported by grants from the MRC, the Fonds de la Recherche en Santé du Québec, and the EJLB Foundation. V. Parpura is supported by funds from the Whitehall Foundation.

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Received 9 November 1998; accepted in final form 8 February 1999.

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