Carrier-Mediated GABA Release Activates GABA Receptors on Hippocampal Neurons

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Gaspary, Heidi L., Wengang Wang, and George B. Richerson. Carrier-mediated GABA release activates GABA receptors on hippocampal neurons. J. Neurophysiol. 80: 270–281, 1998. γ-Aminobutyric acid (GABA) transporters are electrogenic and sodium-dependent and can operate in reverse when cells are depolarized or when there is reversal of the inward sodium gradient. However, the functional relevance of this phenomenon is unclear. We have examined whether depolarization induced by a physiologically relevant increase in extracellular [K+] leads to sufficient amounts of carrier-mediated GABA release to activate GABA_A receptors on neurons. Patch-clamp recordings were made from rat hippocampal neurons in culture with solutions designed to isolate chloride currents in the recorded neuron. Pressure microejection was used to increase extracellular [K+] from 3 to 12 mM. After blockade of vesicular GABA release by removal of extracellular calcium, this stimulus induced a large conductance increase in hippocampal neurons [18.9 ± 6.8 (SD) nS; n = 16]. This was blocked by the GABA_A receptor antagonists picrotoxin and bicuculline and had a reversal potential that followed the Nernst potential for chloride, indicating that it was mediated by GABA_A receptor activation. Similar responses occurred after block of vesicular neurotransmitter release by tetanus toxin. GABA_A receptors also were activated when an increase in extracellular [K+] (from 3 to 13 mM) was combined with a reduction in extracellular [Na+] or when cells were exposed to a decrease in extracellular [Na+] alone. These results indicate that depolarization and/or reversal of the Na+ gradient activated GABA receptors via release of GABA from neighboring cells. We found that the GABA transporter antagonists 1-(4,4-diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride (SKF89976A; 20–100 μM) and 1-2-[(diphenylmethylamine)oxo]ethyl-1,2,5,6-tetrahydro-3-pyridine-carboxylic acid hydrochloride (NO-711; 10 μM) both decreased the responses, indicating that the release of GABA resulted from reversal of the GABA transporter. We propose that carrier-mediated GABA release occurs in vivo during high-frequency neuronal firing and seizures, and dynamically modulates inhibitory tone.

INTRODUCTION

γ-Aminobutyric acid (GABA) transporters are sodium dependent and electrogenic (Kavanaugh et al. 1992; Mager et al. 1993; Malchow and Ripps 1990), and as with other passive transporters, they will operate in either direction depending on which is most thermodynamically favorable (Cammack et al. 1994; Levi and Raiteri 1993; Nicholls and Attwell 1990). Under normal conditions, GABA transporters remove GABA from the extracellular space. Antagonists of GABA transporters enhance the inhibitory action of exogenous GABA (Krogsgaard-Larsen 1980), prolong inhibitory postsynaptic currents (IPSCs) (Rekling et al. 1990; Thompson and Gahwiler 1992) and have anticonvulsant properties (Schousboe et al. 1991; Suzdak et al. 1992). However, under some conditions “reverse operation” of GABA transporters can result in nonvesicular GABA release. For example, depolarization in the absence of extracellular Ca^{2+} induces release of GABA from retinal horizontal cells of some fish (Cammack and Schwartz 1993; Schwartz 1987; Yassuda and Kleinschmidt 1983) and toads (Schwartz 1982) and from growth cones of neurons isolated from rat forebrain (Taylor and Gordon-Weeks 1991). GABA also is released from cultured striatal neurons in response to 56 mM [K+] and GABA also is released from cultured striatal neurons in response to 56 mM [K+] and veratridine, and glutamate receptor agonists in the absence of calcium or after treatment with tetanus toxin (Pin and Bockaert 1989) and in response to electrical stimulation in rat striatal slices in the absence of calcium (Bernath and Zigmond 1988). This nonvesicular GABA release is Na dependent and can be blocked by antagonists of GABA transporters (Belhage et al. 1993).

In most previous studies, carrier-mediated GABA release has been measured biochemically, in which case it is not clear whether the GABA that is released causes functional effects. Postsynaptic responses due to carrier-mediated release have been measured in response to depolarization in catfish retinal neurons (Schwartz 1987) and by nipecotate (NPA) application in rat brain slices (Honmou et al. 1995; Solis and Nicoll 1992). However, there is currently no evidence that carrier-mediated GABA release induced by physiologically relevant stimuli results in significant electrophysiological effects in mammalian tissue. Here we report that a brief increase in [K+] from 3 to only 12 mM and/or a decrease in [Na+] induced large GABA_A receptor–mediated responses in cultured rat hippocampal neurons as a result of carrier-mediated GABA release from neighboring cells. A preliminary report of this work has been published in abstract form (Gaspary and Richerson 1996).

METHODS

Sources of chemicals and reagents

Picrotoxin, bicuculline methiodide (–), GABA, tetrodotoxin (TTX), ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), EDTA, Na-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), cytosine β-D-arabino-furanoside hydrochloride (Ara C), tetraethyl ammonium (TEA) chloride, TEA hydroxide, C6H5Cl, choline, CsOH, CsCl, papain, cystine, trypsin inhibitor, bovine serum albumin (BSA), streptomycin, penicillin, and all salts and chemicals not otherwise listed were purchased from Sigma Chemical (St. Louis, MO). (+)-2-Amino-5-phosphonopentanoic acid (AP-5), 6-cyano-7-nitroquinoxaline-2,3-
dione (CNQX), RS(+)-nicotinic acid (NPA), and 1-(2-[(di-
phenylmethylene)amino]oxyl)ethyl)-1,2,5,6-tetrahydro-3-pyri-
dine-carboxylic acid hydrochloride (NO-711 or NNC-711) were purchased from Research Biochemicals (RBI, Natick, MA). N-
(2,6-dimethylphenylcarbamoylmethyl) triethylammonium chloride (QX-314) and tetanus toxin were purchased from Alomone Labs
(Jerusalem, Israel). 1-(4-DiPhenyl-3-butenyl)-3-piperindinecar-
boxylic acid hydrochloride (SKF89976A) is a generous gift of
SmithKline Beecham Pharmaceuticals (King of Prussia, PA). Fetal
bovine serum (FBS), Neurobasal medium, and B27 supplement
were purchased from Gibco BRL Products (Gaithersburg, MD).
Dulbecco’s modified Eagle’s medium (DMEM) and F12 supple-
ment were purchased from JRH Biosciences (Lenexa, KS).

Cell culture
Primary cultures of hippocampus were prepared from embryonic
or fetal (E19–P1) Sprague-Dawley rats using aseptic technique.
Hippocampi were dissected under direct microscopic visualization
(Stemi 2000-C; Carl Zeiss, Thornwood, NY). Tissue was placed
in oxygenated, HEPES-buffered Ringer solution, which contained
(in mM) 130 NaCl, 4 KCl, 1 MgCl₂, 1.5 CaCl₂, 10 HEPES, and
10 dextrose. pH 7.3, and incubated in digestion solution for 10
min (HEPES-buffered Ringer solution with 9 U/ml papain, 0.2
mg/ml cysteine, 1.5 mM extra CaCl₂, and 0.5 mM EDTA; pre-
activated for 30 min at 37°C). After digestion, tissue was washed
three times with culture medium (70% MEM/10% FBS, with 3.6
mg/ml glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin),
to which trypsin inhibitor (1.5 mg/ml) and BSA (1.5 mg/ml) were
added, then triturated five to eight times with a Pasteur pipette.
Hippocampal cells were plated at a density of 2.5–5×10⁴ cells/ml
on 12 mm round, poly-l-ornithine and laminin-covered coverslips
in 12-well culture dishes (Corning Glass Works, Corning, NY)
and incubated in culture medium at 37°C with 5% CO₂ in room
air. At 4 days, the medium was switched to Neurobasal medium
with B27 supplement (1:50). Although Neurobasal medium can
inhibit glial growth (Brewer et al. 1993), we found that Ara-C
also was required to control glial growth after ~7 days in vitro
(DIV). Cultures were fed with half-medium changes on day 7 and
then weekly. In most cases, cultures were grown for 10–21 days
before recording because most cells <10 DIV had undetectable
responses to elevated [K⁺]. These responses first developed be-
tween 10 and 16 DIV, possibly because of maturation of neurons,
increased expression of transporters, or formation of cell to cell
contacts.

Electrophysiology
For recording, coverslips were placed in a chamber on the stage of
an inverted light microscope (Axiovert 100, Carl Zeiss), and
superfused (at 3–4 ml/min) with artificial cerebrospinal fluid con-
taining (in mM) 128 NaCl, 3.0 KCl, 2.0 MgCl₂, 2.0 CaCl₂, 1.3
NaH₂PO₄, 22 NaHCO₃, and 10 dextrose. During recording, the
bath solution was changed to one of the solutions described below.
All bath solutions were bubbled with 5% CO₂-95% O₂ at pH 7.4.
All experiments were performed at room temperature (24°C).

Whole cell, patch-clamp recordings were performed in voltage clamp mode using a patch-clamp amplifier (Axopatch 1D, Axon
Instruments, Foster City, CA). Recording electrodes (2.5–4.0
MΩ) were fabricated from thin-walled, borosilicate glass tubing
(Diamond General, Ann Arbor, MI) with a micropipette puller
(Sutter Instruments, Model P-87, Novato, CA). Electrodes were
filled with one of the following solutions (in mM): 1) EC₅₀ = 0
mV: 94 CsCl, 40 TEA (chloride salt), 5 HEPES, 10 EGTA, and
15 CsOH; 2) EC₅₀ = −60 mV: 13.4 CsCl, 40 TEA-OH, 105 meth-
anesulfonic acid, 85 CsOH, 10 EGTA, and 10 HEPES; or 3) QX-
314: 114 CsCl, 20 QX-314 (chloride salt), 20 CsOH, 10 HEPES,
and 10 EGTA.

Electrode solutions were adjusted to pH 7.2 with CsOH and to
an osmolality of 270 ± 5 mOsm with ultrafiltered, deionized H₂O
(Milli-Q Plus Water System, Millipore, Bedford, MA). For all
recordings, initial seal resistance was ≥ 1 GΩ. Recordings were
included in analyses if initial access resistance was <20 MΩ
(mean ± SD = 9.6 ± 2.8, n = 90) and changed <30% during the
recording and if input resistance remained ≥100 MΩ.

Data were digitized and stored on computer using a commer-
cially available data-acquisition system (TL-1 DMA interface, and
cpac software, Axon Instruments) and on tape with a VCR
(SLV-441, Sony, Park Ridge, NJ) using a digitizing unit (Neuro-
corder DR-484, Neurodata Instruments, New York, NY).

Experimental paradigm
For each recording, holding current and/or whole cell conduc-
tance was measured in hippocampal pyramidal neurons, while the
extracellular solution was exchanged rapidly with a “test” solution
locally applied using a microejection pipette. Two approaches were
used. In the first, the test solution was used to fill a pressure ejection
micropipette (tip diameter ≈ 10 μm) that was connected to a pressure
microejector (Picospritzer II, General Valve, Fairfield, NJ) and was positioned close to the recorded neuron. In the second, the “micropuffer” technique for the application of multiple solu-
tions developed by Greenfeld and Macdonald (1996) was used to
systematically vary the bicuculline concentration. In both cases,
trypan blue (0.2 mM) was added to the test solution to allow visual-
ization of the system to ensure that the soma and proximal dendrites of recorded neurons were bathed completely by test solu-
tion (Oyehese et al. 1995). Application of Ringer solution alone,
with or without trypan blue, did not result in a change in conduc-
tance in recorded neurons (n = 6).

Solutions were designed to block all currents in the recorded
neuron except those mediated by GABA_A receptors. Thus the stan-
dard solutions blocked all nonchloride currents in the following
way (Table 1): 1) TTX was added to the bath solution to block
Na+ currents; 2) the bath solution contained no Ca²⁺ but did
contain EGTA; 3) Cs⁺ and TEA were included in the electrode
solution to block outward K⁺ currents; 4) TEA (2 mM) was in-
cluded in the bath solution to block inward K⁺ currents; and 5)
CNQX and AP-5 were included in the bath solution to block gluta-
mate receptors.

In some individual experiments (described in the figure leg-
ends), this recipe was modified in the following ways: 1) QX-314
(20 mM) was included in the electrode solution, in place of TTX
in the bath solution (Connors and Prince 1982); 2) Cd²⁺ was
included in the bath solution with 0.5 mM Ca²⁺ to block Ca²⁺-
influx (Cd²⁺ solution); 3) TEA was not included in the bath solu-
tion in those experiments that increased [K⁺], to 12 mM without
a change in [Na⁺]; or 4) in experiments using tetanus toxin
pretreatment, 2 mM CaCl₂ was included in the Ringer solution
(nl Ca²⁺). We assumed that these solutions would block GABA_A-
receptor–mediated responses because GABA_A receptors modulate
calcium and potassium conductances, which were blocked as just
described.

To block vesicular GABA release, parallel experiments were performed in the following ways: the bath solution contained no
Ca²⁺, but did contain EGTA (0 Ca²⁺ solution), cadmium (100
μM) was added to the bath solution (0.5 mM Ca²⁺), or cultured
eurons were incubated with tetanus toxin (1 μg/ml or ≥60 nM
in culture medium) for 18–30 h before recording (Albus and Ha-
bermann 1983; Monyer et al. 1992; Pin and Bockaert 1989).

Microejection (test) solutions were identical to the bath solution
at the time, except for an increase in [K⁺] and/or a decrease in
The concentrations of these chemicals were matched to those of the bath solution. In some experiments, voltage-clamp pulses to +60 mV were used to directly measure the reversal potential of the induced response. In other cases, reversal potential was determined by extrapolation of the \( I-V \) curve of the peak GABA-induced current. The 10–90% rise time was calculated for induced responses using plots of either slope conductance versus time or holding current versus time.

For all results, values are means \pm SD unless otherwise stated. Probability values were determined using the Mann-Whitney \( U \) test. Data were analyzed using commercially available software (Excel, Microsoft, Redmond, WA; Origin, Microcal, Northampton, MA).

**RESULTS**

**Nipeocotate acid induced currents in hippocampal cultures due to carrier-mediated GABA release**

In hippocampal slices, rapid application of NPA induces heteroexchange release of GABA via the GABA transporter (Szerb 1982), which then activates GABA receptors on neighboring neurons (Honmou et al. 1995; Solis and Nicoll 1992). Measurement of these responses requires the following conditions to be true: neurons must be present with GABA\(_A\) receptors, neurons with GABA\(_A\) receptors must lie in close proximity to other neurons and/or glia that possess GABA transporters, free cytosolic GABA, available for release, must be present in neurons and/or glia that have GABA transporters, and the extracellular space must be restricted enough to prevent rapid diffusion of released GABA so that it reaches a sufficient concentration to activate GABA\(_A\) receptors. These conditions exist in slices (Honmou et al. 1995; Solis and Nicoll 1992), but it was not clear whether they would exist in tissue culture. We wanted to use tissue culture to study carrier-mediated GABA release, because it permitted better control of extracellular ion con-
centrations, and neurons could be incubated with tetanus toxin for 24 h to block vesicular release. However, to use this approach, it was first necessary to demonstrate that the above conditions existed in culture.

In Ringer solution containing TTX, AP-5, and CNQX, there was spontaneous miniature synaptic activity. When QX-314 was used in the recording electrode in place of TTX in the bath, frequent, large postsynaptic potentials also were recorded. Both forms of spontaneous activity were blocked completely by bicuculline, 0 Ca\(^{2+}\) solution, or tetanus toxin pretreatment. When solutions were used with symmetric [Cl\(^-\)] (\(E_{Cl} = 0 \text{ mV}\)) and a holding potential of −60 mV, this activity was depolarizing. However, under physiological conditions in adult hippocampal neurons, this GABAergic input would be expected to be hyperpolarizing and inhibitory.

When GABA (100 \(\mu\text{M}\)) was applied by pressure microejection to cultured hippocampal neurons for 0.5 s in 0 Ca\(^{2+}\) solution, a large inward current was induced in neurons voltage clamped at −60 mV (\(E_{Cl} = 0 \text{ mV}\); \(n = 6\); Fig. 1A). This current resulted from a rapid increase in whole cell conductance of 36.4 ± 14 nS (10–90% rise time = 0.47 ± 0.26 s; \(n = 6\)), which quickly returned to baseline (Fig. 1B). Smaller currents were induced by application of GABA (10 \(\mu\text{M}\)) and were blocked reversibly by bicuculline (50 \(\mu\text{M}\); \(n = 2\)). The reversal potential of the response to GABA was approximately equal to the calculated Nernst potential for chloride (0 ± 1.8 mV; \(E_{Cl} = 0\); \(n = 5\); Fig. 1C).

Rapid application of NPA also activated GABA\(_A\) receptors. Pressure microejection of NPA (10 mM) for 1–3 seconds in either 0 Ca\(^{2+}\) (\(n = 6\)) or Cd\(^{2+}\) (\(n = 2\)) solution induced an inward current. This current was also due to an increase in whole cell conductance, but in this case it was slower in onset than with GABA (10–90% rise time = 3.37 ± 1.98 s, \(n = 6\); Fig. 1B) and had a more gradual return to baseline. The mean baseline conductance in these cells was 4.8 ± 2.7 nS, with a mean increase of 12.7 ± 5.7 nS in response to NPA application (\(n = 8\)). The response to NPA was reversibly blocked by picrotoxin (50 \(\mu\text{M}\), \(n = 6\)), and the reversal potential of the NPA response was close to the Nernst potential for chloride (Fig. 1D: 4.0 ± 8.5 mV when \(E_{Cl} = 0 \text{ mV}\); \(n = 2\); Fig. 1E: −56.2 ± 4.3 mV when \(E_{Cl} = −60 \text{ mV}\), \(n = 6\)). These responses were similar to those previously obtained in hippocampal slices using the same concentration of NPA (10 mM) (Honmou et al. 1995; Solis and Nicoll 1992). NPA-induced responses are due to activation of GABA\(_A\) receptors from heteroexchange release of GABA and not due to direct effects on the GABA\(_A\) receptor (Solis and Nicoll 1992). These results indicated that cell culture is a useful model system for detecting electrophysiological responses due to carrier-mediated GABA release.

**GABA\(_A\) receptors were activated in response to elevation of [K\(^+\)]\(_o\) in the absence of extracellular Ca\(^{2+}\)**

During high-frequency neuronal firing, extracellular potassium rises. Even modest firing rates can lead to an increase in [K\(^+\)]\(_o\) to >12 mM (Krnicjvic et al. 1980; Somjen and Giacchino 1985), which may contribute to the pathophysiology of seizures (Fisher et al. 1976). We hypothesized that depolarization of neurons and/or glia caused by a rise in [K\(^+\)]\(_o\) to 12 mM would be sufficient to induce significant amounts of carrier-mediated release of GABA.

When [K\(^+\)]\(_o\) was increased near the soma and proximal dendrites of a recorded neuron to 12 mM for 3 s with
microejection of test solution in the absence of extracellular calcium \((n = 16)\), a large inward current was induced of \(1.14 \pm 0.4\) nA (holding potential \(-60\) mV; \(E_{\text{Cl}} = 0\) mV; Fig. 2A, left). Assuming the reversal potential of this response was equal to the calculated Nernst potential for Cl\(^-\), the magnitude of the induced current would correspond to an increase in whole cell conductance of 18.9 ± 6.8 nS. Similar responses were obtained in the presence of 100 \(\mu\)M Cd\(^{2+}\) \((n = 12)\). In these cells, the rise time of the response to 12 mM [K\(^+\)]\(_o\) was slower than that produced by direct application of GABA (mean 10–90% rise time = 5.90 ± 2.80 s; \(n = 12\)) but was similar to that produced by NPA.

The response to increased [K\(^+\)]\(_o\) was blocked reversibly by bicuculline (1–50 \(\mu\)M) in a dose-dependent manner (Fig. 2, A and B). The reversal potential of the response induced by 12 mM [K\(^+\)]\(_o\) was approximately equal to the calculated Nernst potential for chloride \((-3.3 \pm 2.9\) mV when \(E_{\text{Cl}} = 0\) mV, \(n = 3\); -56.5 ± 4.2 mV when \(E_{\text{Cl}} = -60\) mV, \(n = 8\); Fig. 2C). These results indicated that elevated [K\(^+\)]\(_o\), in the absence of extracellular calcium or presence of cadmium, induced nonvesicular release of GABA, which then activated GABA\(_A\) receptors.

**Elevation of [K\(^+\)]\(_o\) also activated GABA\(_A\) receptors after tetanus toxin treatment**

Tetanus toxin blocks synaptic vesicle exocytosis due to proteolytic cleavage of synaptobrevin (Schiavo et al. 1992). To provide further evidence that GABA-receptor–mediated responses induced by 12 mM [K\(^+\)]\(_o\), were a result of nonvesicular GABA release, cultured cells were preincubated with tetanus toxin (1 \(\mu\)g/ml) for 18–30 h before recording (Albus and Habermann 1983; Monyer et al. 1992; Pearce et al. 1983; Pin and Bockaert 1989). This protocol resulted in complete elimination of spontaneous large and miniature IPSCs and excitatory postsynaptic currents (EPSCs; \(n = 3\); data not shown).

When we used tetanus toxin to block vesicular exocytosis, the responses were similar to those when Ca\(^{2+}\) influx was blocked. These experiments were performed using normal Ca\(^{2+}\) Ringer solution. In neurons preincubated with tetanus toxin, application of 12 mM [K\(^+\)]\(_o\) for 3 s induced large inward currents \((590 \pm 240\) pA, \(E_{\text{in}} = -60\) mV, \(E_{\text{Cl}} = 0\) mV, \(n = 12\); Fig. 3B). In contrast, currents induced by 12 mM [K\(^+\)]\(_o\) in neurons that had not been treated with tetanus toxin (Fig. 3A, sister culture dishes) were 2.4 times larger \((1.4 \pm 5.3\) nA, \(n = 5\)) than those in tetanus toxin-treated neurons. The response to 12 mM [K\(^+\)]\(_o\) was blocked by bicuculline (500 \(\mu\)M in bath only) in control \((n = 3\); Fig. 3A) and in tetanus toxin-treated \((n = 4\); Fig. 3B) neurons. Comparison of the responses after blockade of vesicular exocytosis with the responses resulting from both vesicular and nonvesicular release (Fig. 3C) suggested that nonvesicular release makes up a significant fraction of the total release induced by depolarization.

**GABA\(_A\) receptors were activated by reversal of the transmembrane sodium gradient**

Because the GABA transporter is both electrogenic and sodium dependent, we hypothesized that a change in the
transmembrane sodium gradient also would induce sufficient carrier-mediated GABA release to produce measurable post-synaptic responses. During high-frequency firing and seizures, [Na\(^+\)]\(_o\) increases while [Na\(^+\)]\(_i\) decreases. There is evidence that efflux of GABA through the transporter can be induced by a rise in [Na\(^+\)]\(_o\), (Cammack et al. 1994). However, using pressure microejection of solutions to the outside of cells, it is not possible to replicate experimentally the actual changes in transmembrane sodium gradient that occur because [Na\(^+\)]\(_o\) can not be rapidly changed. Therefore we instead applied a test solution of 0 mM [Na\(^+\)]\(_o\), and 13 mM [K\(^+\)]\(_o\), to produce the largest possible driving force for sodium efflux. When this solution was applied for 3 s in the presence of cadmium (100 \(\mu\)M), an inward current of 810 \pm 270 pA \((n = 23)\) was induced at a holding potential of -60 mV \((E_{Cl} = 0 \text{ mV})\). Similar responses were obtained in 0 Ca\(^{2+}\) solution (Fig. 4A). Typical of responses induced by application of 12 mM [K\(^+\)]\(_o\), alone, the response was relatively slow in onset (10-90\% rise time = 4.88 \pm 1.60 s). Assuming the reversal potential was equal to the Nernst potential for Cl\(^-\), the induced current would correspond to an increase in whole cell conductance of 13.0 \pm 4.8 nS.

The conductance change induced by 0 [Na\(^+\)]\(_o\), and increased [K\(^+\)]\(_o\), also was measured directly by using voltage-clamp pulses from -60 to -80 mV and -100 mV. When a solution with 0 mM [Na\(^+\)]\(_o\), and 13 mM [K\(^+\)]\(_o\), was applied by pressure microejection for 1 s in 0 Ca\(^{2+}\) bath solution \((n = 18)\), an increase in whole cell conductance of 7.08 \pm 6.74 nS occurred (Fig. 4B) from a baseline conductance of 6.27 \pm 3.70 nS. The increase in conductance was blocked reversibly by bath application of picrotoxin (50 \(\mu\)M; \(n = 4\)) or bicuculline (500 \(\mu\)M in the bath only; \(n = 2\); Fig. 4B). The reversal potential of the response to 0 [Na\(^+\)]\(_o\), and increased [K\(^+\)]\(_o\), was near the Nernst potential for Cl\(^-\) \((1.9 \pm 2.93 \text{ mV when } E_{Cl} = 0 \text{ mV}; n = 8; -56 \pm 3.5 \text{ mV when } E_{Cl} = -60 \text{ mV}; n = 8; \text{ Fig. 4C})\).

Responses also were induced when cells were exposed to 1.0 mM [Na\(^+\)]\(_o\), alone \((n = 5)\). The responses to 1.0 mM [Na\(^+\)]\(_o\), were smaller than those to 12 mM [K\(^+\)]\(_o\), and slower in onset (Fig. 4A). These results suggested that depolarization was a more potent stimulus for reversal of the GABA transporter than a decrease in [Na\(^+\)]\(_o\). In retrospect, because of the effect of a rise in [Na\(^+\)]\(_o\), on carrier-mediated GABA efflux, a decrease in [Na\(^+\)]\(_o\), to 0 mM may not be the maximal Na\(^+\) gradient stimulus. Instead, a sudden rise in [Na\(^+\)]\(_o\), might be more effective but could not be accomplished with the pressure microejection approach used here.

**GABA receptor activation was prevented by blockade of the GABA transporter**

The responses obtained with changes in [K\(^+\)]\(_o\), and [Na\(^+\)]\(_o\), were apparently due to release of GABA from neighboring neurons and/or glia with subsequent activation of GABA\(_A\) receptors on the recorded neuron. Thus the recorded neuron served as a sensitive, functionally relevant biological assay of GABA release (Fig. 5A). Vesicular release is calcium dependent and sensitive to tetanus toxin and would be blocked under the conditions of these experiments. To provide further evidence that the release of GABA was due to reversal of the GABA transporter, we examined the effect of GABA transporter antagonists on the evoked responses.

If the observed responses to changes in [K\(^+\)]\(_o\), and [Na\(^+\)]\(_o\), resulted from carrier-mediated GABA release, it would be predicted that application of GABA transporter antagonists would decrease the responses. Alternatively, if the observed responses resulted from residual, unblocked vesicular release, nonspecific leakage, or some other undefined form of GABA release, then blockade of the GABA transporter would lead to an increase in the response because the GABA transporter would clear the extracellular space of this released GABA (Fig. 5B).

SKF89976A is a highly specific, noncompetitive GABA transporter antagonist that previously has been used to study the electrophysiological response to carrier-mediated GABA release induced by NPA application (Solis and Nicoll 1992). SKF89976A previously has been used at concentrations from 10 to 100 \(\mu\)M (Belhage et al. 1993; Cammack et al. 1994; Mager et al. 1993; Solis and Nicoll 1992; Taylor and Gordon-Weeks 1991) to obtain block of GABA transport. SKF89976A and the other noncompetitive GABA transporter antagonists tiagabine and NO-711 are highly lipid-
soluble drugs and have a slow time to peak effect (Solis and Nicoll 1992) and are difficult to reverse after application (Thompson and Gahwiler 1992). Therefore, the following approach was used. Recordings were made in Ca²⁺-Ringer solution. A baseline response was induced by pressure microjection of 0 [Na⁺]₀, 13 mM [K⁺]₀, solution. Then the GABA transport inhibitor SKF89976A (20 μM, n = 1; 40 μM, n = 5; or 100 μM, n = 6) was applied for 8 min, and a second response was induced. When this was done, the area under the curve of the induced current response was reduced to 54% ± 15% of the initial response (n = 12; Fig. 5C and E). Rundown of GABAₐ receptors of approximately this amount can occur during whole cell recording but typically does not begin until >15 min of recording (Chen et al. 1990; Honmou et al. 1995). However, to be certain that the observed reduction of the response was not a result of rundown, we used paired recordings from different neurons in the same dishes as controls before using the same dish for recording from neurons to which SKF89976A was applied. When this was done, the response in these control recordings to the second application of 0 [Na⁺]₀, 13 mM [K⁺]₀ was 108% ± 28% (P > 0.1; n = 10) of the first response 8 min earlier (Fig. 5E). When the change in response over the first 8 min of each recording was compared between the SKF89976A-treated and control groups, the decrease of the response in the treated cells was significantly different from the control group (P = 0.005). The mean response at eight minutes for only those cells treated with 40 μM SKF89976A was 53.6 ± 28% of the first response (n = 5), which also was significantly different than for the control group (P = 0.004), as was the mean response for only those cells treated with 100 μM SKF89976A (58.2% ± 12%; n = 6; P = 0.004). The response decreased to 36% of control in one cell treated with 20 μM SKF89976A. There was no significant difference in the response between cells treated with 40 μM or 100 μM SKF89976A.

A different approach was used to verify these results using a second GABA transporter antagonist, NO-711. NO-711 is a non-competitive GABA transporter antagonist that has been suggested to be relatively selective for neuronal GABA transporters (Suzdak et al. 1992). Responses were induced by 12 mM [K⁺]₀ in tetanus toxin-treated cultures with normal Ringer solution with 2 mM Ca²⁺. The responses to 12 mM [K⁺]₀ were measured immediately after establishing stable whole cell recordings (n = 5). These responses were compared with those of neurons in the same culture dishes after NO-711 (10 μM) had been applied for ≈15 min before beginning whole cell recording (n = 6). In cells treated with tetanus toxin in which NO-711 had been applied, the response to 12 mM [K⁺]₀ was reduced significantly compared with controls (57.6 ± 4.6% of control; Fig. 5, D and F).

**Prolonged exposure to high potassium led to decay of the GABA response**

Brief exposure to rapid increases in [K⁺]₀ induced large, calcium-independent, GABAₐ-receptor-mediated responses in cultured hippocampal neurons, which peaked over ~5–6 s and gradually returned to baseline during the next 20–30 s. To characterize the response to sustained increases in [K⁺]₀, experiments were repeated with application of 12 mM [K⁺]₀ for 30 s to 1 min. This induced a peak response within 5–10 s, but despite continued exposure to 12 mM [K⁺]₀, the response would rapidly decrease before the end of the stimulus (n = 6; Fig. 6). Note the difference in holding current at the end of the pulse and the baseline at the end of the trace.

**DISCUSSION**

**Mechanism of response induced by elevated [K⁺]₀**

We have demonstrated that an increase in [K⁺]₀, with or without a decrease in [Na⁺]₀, induced a large increase in conductance in cultured hippocampal neurons with a reversal potential equal to the Nernst potential for chloride. Antago-
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FIG. 5. GABA-mediated responses induced by 12 mM [K+]o alone or 0 [Na+]o/13 mM [K+]o were blocked by the GABA transport inhibitors 1-((4,4-diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride (SKF89976A) and 1-(2-[[di-phenylmethylene]amino]oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridine-carboxylic acid hydrochloride (NO-711). A: schematic model for the mechanism of induced responses. Recorded neuron acted as a functionally relevant, biological assay of GABA released from neighboring neurons. B: schematic of the theoretical effect of GABA transporter blockade on alternative mechanisms of GABA release. If the response was due to carrier-mediated release, transporter antagonists would decrease synaptic GABA concentrations and postsynaptic responses. In contrast, if recorded GABA responses were due to incomplete blockade of vesicular release or nonspecific leakage, synaptic GABA levels and postsynaptic responses would increase in the presence of GABA transporter blockers. C: 2 responses to application of 0 [Na+]o/13 mM [K+]o Ringer solution obtained in the same neuron. Bottom: control response. Top: response in same cell after application of SKF89976A (40 μM) for 8 min. D: superimposed recordings from 2 different neurons from a single culture dish. Response to 12 mM [K+]o in a control neuron is compared with that in a neuron exposed to NO-711 for 15 min. E: SKF89976A decreased the response to 0 [Na+]o/13 mM [K+]o. Plotted is the ratio, expressed as a percentage, of the area under the curve (AUC) of the response at 8 min to the response at baseline (t = 0) for control cells (108 ± 28%; n = 10), and for SKF89976A-treated cells (54 ± 15%; n = 12). ***P = 0.005, Mann Whitney U test. F: mean response (AUC) in 5 control neurons (9.0 ± 1.0 nA·s; mean ± SE) vs. 6 cells pretreated with NO-711 (10 μM; 5.1 ± 0.7 nA·s; mean ± SE). Each recording was paired with a matched control neuron from the same culture dish. **P < 0.025, Mann Whitney U test. Experiments in C and E performed in Cd²⁺ Ringer solution. Data in D and F from tetanus toxin-treated cells using modified Ringer solution with 2 mM CaCl₂ and with QX-314 (20 mM) in the patch electrode instead of TTX in the bath.

nists of GABA_α receptors reversibly blocked the conductance, indicating that it was due to evoked release of GABA. The response occurred after blockade of vesicular release by removal of Ca²⁺, addition of Cd²⁺, or preincubation with tetanus toxin. Specific antagonists of the GABA transporter decreased the response. We conclude that this GABA_α-receptor–mediated response was due to carrier-mediated GABA release from neighboring neurons and/or glia induced by changing the electrochemical gradient to favor reverse operation of the GABA transporter.

Blockade of calcium influx (Del Castillo and Engbaek 1954; Pin and Bockaert 1989) and tetanus toxin (Albus and Habermann 1983; Monyer et al. 1992; Pin and Bockaert 1989) commonly are used to prevent vesicular release. In our hands, the loss of spontaneous IPSCs and EPSCs indicated that these manipulations were effective. However, one could interpret the incomplete block of responses by NO-711, and SKF89976A as consistent with a mixture of carrier-mediated release and residual vesicular GABA release. We considered this possibility, but others have shown incom-
complete block by these agents. For example, the GABA transport inhibitor SKF89976A (20 μM) only produces 50–60% inhibition of postsynaptic responses to carrier-mediated GABA release induced by NPA (Solis and Nicoll 1992). SKF89976A (10–100 μM) is also only partially effective in blocking calcium-independent release of GABA from rat forebrain synaptosomes (70–80% inhibition), and rat forebrain growth cones (60–75% inhibition) (Taylor and Gordon-Weeks 1991). Although SKF89976A can result in nearly complete blockade of GABA transporter currents mediated by GAT-1 expressed in Xenopus oocytes (Quick et al. 1997), hippocampal cultures contain multiple isoforms of the GABA transporter and multiple neuronal and glial cell types, which have different sensitivities to antagonists (Clark and Amara 1994; Clark et al. 1992; Larsson et al. 1988; Schousboe and Westergaard 1995). In addition, our electrophysiological assay detected GABA released near postsynaptic receptors and may be very sensitive to small amounts of GABA released locally. This portion of released GABA may be less sensitive to GABA transporter antagonists and not be easily detected by biochemical assays of GABA in the bulk bath solution.

If the responses we observed were a combination of carrier-mediated release and vesicular release, blockade of the transporter would lead to enhancement of the vesicular portion due to blockade of reuptake (Krogsgaard-Larsen 1980; Rekling et al. 1990; Thompson and Gahwiler 1992), and inhibition of the carrier-mediated portion due to blockade of reverse transport. The minimum possible contribution from carrier-mediated release then would be approximately half of the observed 7–19 nS responses, still a substantial effect. However, we favor the conclusion that the entire response to 12 mM [K+]o resulted from carrier-mediated release and that incomplete block by the GABA transporter antagonists SKF89976A and NO-711 was consistent with what is expected from these agents (Fichter et al. 1996; Solis and Nicoll 1992; Taylor and Gordon-Weeks 1991).

We also considered the possibility that the responses evoked by [K+]o resulted from direct measurement of transporter currents. The kinetics were similar to those of transporter currents during uptake of exogenously applied GABA in skate retinal horizontal cells (Malchow and Ripps 1990). However, our results were not consistent with direct measurement of transporter currents for the following reasons. 1) Bicuculline and picrotoxin do not block transporter currents (Malchow and Ripps 1990). 2) Although transporter currents are dependent on [Cl−], their I-V curve is usually not linear, and their reversal potential does not follow \( E_{Cl^-} \) (Cammack et al. 1994). 3) Transporter currents can be measured when present in high abundance, such as after transfection into HEK293 cells (Cammack et al. 1994) or in skate retinal horizontal cells (Malchow and Ripps 1990), but even in those cases the currents are small (<100 pA). The currents we measured were too large to be transporter currents in hippocampal neurons. 4) The recorded neuron was voltage clamped, making it unresponsive to the depolarizing effect of 12 mM [K+]o, and eliminating any change in driving force for the transporter. 5) The whole cell patch-clamp electrode solution contained no Na+ and no GABA, which are required for the GABA transporter to carry an outward current (Cammack et al. 1994). This was not consistent with our results showing reversal of the current. Thus the similarity of the kinetics of our responses and transporter currents likely reflect the fact that our responses were an indirect measure of GABA efflux via the transporter. This observation also provides further support for the hypothesis that these responses were due to carrier-mediated GABA release.

Source of carrier-mediated GABA release

The carrier-mediated GABA release occurred in the immediate vicinity of GABA_\text{A_4} receptors on the recorded neurons. It is theoretically possible that GABA released from a cell could stimulate GABA receptors on itself. However, for the reasons mentioned above, GABA would not have been released from the recorded neuron using our experimental approach. Thus under these experimental conditions, GABA came from neighboring cells. The current data do not differentiate between neurons, glia, or both as the source of this GABA release. Carrier-mediated GABA release can occur from glia (Gallo et al. 1991) as well as from neurons, so it is possible that either cell type is involved. The intracellular store of GABA is presumably a cytosolic pool, rather than the vesicular pool.

Magnitude of responses induced by carrier-mediated GABA release

The postsynaptic conductance change induced by 12 mM [K+]o was often >10 nS. These were very large responses for these neurons with mean resting conductance of 6.3 nS. In comparison, direct application of 100 μM GABA for 0.5 s resulted in an increase in conductance of 36.4 ± 14 nS, which is comparable with previous studies where application of 10 μM to 1 mM GABA for 0.5 s induced a conductance of 5–50 nS in hippocampal neurons (Huguenard and Alger 1986). In contrast to the conductance induced by direct application of GABA, the conductance change due to carrier-
mediated GABA release was much longer in duration. These large and long conductance changes would be expected to have major functional effects.

The response to 12 mM [K+]o in tetanus toxin-treated cells was 42% of the response in control cells. This indicates that nonvesicular GABA release was a significant fraction of total (vesicular + nonvesicular) release. Previous biochemical assays of calcium-independent and tetanus toxin-insensitive GABA release have indicated that ~25% of release induced by 56 mM [K+]o was nonvesicular (Pin and Bockaert 1989). Whereas those results were assumed to reflect GABA release diffusely, without necessarily having electrophysiological effects, the current results suggest that is not the case. Thus nonvesicular release may play a more important functional role in response to elevations in [K+]o than was recognized previously.

Threshold for reversal of the GABA transporter

It generally is accepted that GABA transporters carry two Na+ ions and one Cl− ion (Erecinska 1987; Lester et al. 1994; Mager et al. 1993) with each molecule of GABA. Because GABA is a zwitterion with no net charge at physiological pH, GABA transporters are electrogenic, carrying one net positive charge with each translocation cycle. Thus it would be predicted that GABA transporters would operate in reverse during depolarization or when the inward Na+ gradient is decreased. That this is the case has been demonstrated in a variety of preparations (Belhage et al. 1993; Bernath and Zigmond 1988; Cammack et al. 1994; Mager et al. 1993; Moscowitz and Cutler 1980; Pin and Bockaert 1989; Schwartz 1982; Yazulla and Kleinschmidt 1983) or direct measurement of GABA transporter currents (Cammack and Schwartz 1993; Cammack et al. 1994; Mager et al. 1993; Malchow and Rips 1990). However, assaying efflux of GABA or measuring transporter currents leaves two questions unresolved. Is GABA released at a site where it can activate GABA receptors? Is the concentration of GABA released near receptors sufficiently high to have an electrophysiological effect?

Physiologic relevance

Previous studies of carrier-mediated release have primarily used biochemical assays of GABA efflux (Belhage et al. 1993; Bernath and Zigmond 1988; Larsson et al. 1983; Moscowitz and Cutler 1980; Pin and Bockaert 1989; Schwartz 1982; Yazulla and Kleinschmidt 1983) or direct measurement of GABA transporter currents (Cammack and Schwartz 1993; Cammack et al. 1994; Mager et al. 1993; Malchow and Rips 1990). However, assaying efflux of GABA or measuring transporter currents leaves two questions unresolved. Is GABA released at a site where it can activate GABA receptors? Is the concentration of GABA released near receptors sufficiently high to have an electrophysiological effect?

Although release of GABA by reverse transport has been demonstrated clearly, it has not been widely recognized as a form of release that is physiologically relevant. Increases in [K+]o to levels of ~12 mM have been measured both during seizures, as well as high-frequency neuronal firing (Fisher et al. 1976; Krnjevic et al. 1980; Somjen and Giacchino 1985). Under these conditions, it would be predicted that an increase in [Na+]o also would occur, which would facilitate reverse transport. Such a mechanism for GABA release may offer advantages during periods of high energy utilization, such as burst firing or seizures, because it relies only on the Na+ and K+ gradients and not on a continuous supply of ATP. Under these conditions, GABAergic vesicular exocytosis might be reduced, whereas carrier-mediated GABA release would be enhanced, possibly serving an autoneuroprotective mechanism.

The mechanism of the decrease in GABA response with prolonged exposure to 12 mM [K+]o, (Fig. 6) is unclear but may be due to desensitization of the GABA_A receptor or depletion of the cytosolic pool of GABA available for carrier-mediated release. This result suggests that carrier-mediated release may be greatest during brief periods of depolarization, as in bursts of high-frequency firing. Thus carrier-mediated release may contribute less to GABA-mediated inhibition during conditions of prolonged depolarization, as in stroke or status epilepticus.

Carrier-mediated release potentially could occur under physiological conditions for other neurotransmitter transporters. Glutamate transporters are also electrogenic and sodium dependent, and have been shown to reverse in vitro (Eliasof and Werblin 1993; Nicholls and Attwell 1990). However, the stoichiometry is different for the two types of transporter, and there may be a differential threshold for reverse operation. Elucidation of the relative contributions of reverse transport of these two neurotransmitters is important because reversal of GABA transporters could play a protective role, whereas reversal of glutamate transporters could be neurotoxic.

In human patients with temporal lobe epilepsy, electrophysiological studies have suggested deficiencies in GABAer-
gic inhibition (Knowles et al. 1992; Williamson et al. 1995). Similarly, in vivo microdialysis studies have demonstrated that GABA levels are decreased during seizures in the abnormal hippocampus (During and Spencer 1993), possibly due to deficient carrier-mediated GABA release (During et al. 1995). The novel anticonvulsant gabapentin enhances carrier-mediated release of GABA induced by NPA in hippocampal slices (Honmou et al. 1995). This agent also increases brain GABA levels (Petroff et al. 1996), which, if cytosolic, would enhance the driving force for carrier-mediated GABA release. The data presented here are consistent with the hypothesis that carrier-mediated release of GABA plays an inhibitory role during pathophysiological conditions and may be enhanced by some anticonvulsants (Richerson and Gaspery 1997).

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