Multiple Components of Synaptosomal [^{3}H]-γ-Aminobutyric Acid Release Resolved by a Rapid Superfusion System†

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ABSTRACT: Release of [^{3}H]-γ-aminobutyric acid ([^{3}H]GABA) from rat brain synaptosomes was studied with 60-ms time resolution, using a novel rapid superfusion method. Synaptosomes were prelabeled with [^{3}H]GABA via an associated GABA uptake system. KCl depolarization stimulated at least three distinct components of GABA release: (1) a phasic Ca-dependent component, which develops rapidly and decays with a time constant of at most 60 ms; (2) a tonic Ca-dependent component that persists after KCl depolarization is ended; (3) a Ca-independent component. The three components of GABA release are pharmacologically distinct. The phasic component was selectively blocked by 50 μM Cd^{2+}, while the tonic component was selectively blocked by 100 μM Ni^{2+}. The Ca-independent component was selectively blocked by nipecotic acid (IC_{50} = 21 μM), a known inhibitor of Na^+-dependent GABA uptake. The time course and amplitude of Ca-dependent GABA release evoked by the Ca^{2+} ionophore A23187 were nearly identical with Ca-dependent release evoked by depolarization. This result indicates that Ca-dependent GABA release depends primarily on Ca^{2+} entry into the nerve terminal, and not depolarization, per se. The properties of the phasic component suggest that it is normally initiated by a voltage-sensitive Ca^{2+} channel that is functionally and pharmacologically distinct from those previously described. The Ca-independent component of GABA release is probably mediated by reversal of the Na-dependent, electrogenic GABA uptake system. The ability to identify multiple components of GABA release on a physiologically relevant time scale may afford a more precise definition of the mechanism of action of drugs thought to affect neurotransmission in the brain.

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system and is found in abundance in most regions of the brain. Interest in GABAergic transmission has been intense due to the importance of GABA and to the availability of drugs that act by modifying the postsynaptic response to GABA, such as benzodiazepines and barbiturates. The kinetics of the postsynaptic response to GABA and mechanisms responsible for the action of drugs and toxins have been studied in detail (Barker & Owen, 1986). However, similar studies of the presynaptic release of GABA have been limited, and therefore the contribution of presynaptic release to the time course of postsynaptic events is uncertain. Studies of GABA release using preparations of presynaptic nerve terminals or brain slices have suffered from undefined temporal resolution, with release generally measured on a second to minute time scale (Cotman et al., 1976; Levi et al., 1981).

Postsynaptic events mediated by presynaptic release of GABA have been studied in a number of model systems, such as dorsal root ganglion (Dunlap, 1981) and in hippocampal slice preparations (Alger & Nicoll, 1982; Dingeldine & Korn, 1985) or primary cell culture (Segal & Barker, 1984). The postsynaptic potentials mediated by neurally evoked GABA release occur on a time scale of tens to hundreds of milliseconds. Assays of neurotransmitter release from brain slices or synaptosomes are generally performed on a second to minute time scale. Thus, there exists a gap in our knowledge of the time course of neurosecretory events and the postsynaptic currents that presumably reflect presynaptic release. To address this issue, we have developed a rapid superfusion system which can resolve release of [^{3}H]GABA on a 60-ms time scale. The kinetics of GABA release are complex; at least three distinct components of GABA release are observed that differ in their Ca dependence, voltage dependence, and sensitivity to various drugs and inorganic antagonists. A rapid, transient component of release has been identified that decays within 200 ms. This rapid component may be of particular relevance to GABA secretion responsible for mediating fast inhibitory postsynaptic potentials in central neurons.

Materials and Methods

Materials. The animals used for this study were male Sprague-Dawley rats, 50–200 g, obtained from Charles River Laboratories, Wilmington, MA. [^{3}H]GABA (72–93 Ci/mmol) was obtained from New England Nuclear, Boston, MA. Nimodipine was supplied by Dr. Alexander Scriabine of Miles Institute for Preclinical Pharmacology, New Haven, CT. ω-Conotoxin was obtained from the Peptide Institute or from Peninsula Labs. Nipecotic acid and aminooxyacetic acid were from Sigma Chemical Co. All other reagents were of the highest grade available.

Rapid Superfusion System. The rapid superfusion system is a device designed to measure release of neurotransmitters on a subsecond time scale. The apparatus employed here differs in two major respects from the superfusion system previously described (Pearce et al., 1988), which was used to detect intracellular ion channels in photoreceptors. First, in the current system, we employ a three-valve rather than a

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two-valve sample chamber. Second, the opening and closing of each of the three valves are computer controlled rather than controlled by the output of electrophysiological stimulators.

The specific details of the current device will be described elsewhere.[1] Briefly, it consists of three pressurized fluid reservoirs, each connected to a superfusion chamber configured with three coaxial solenoid-driven valves, and a rotating fraction collector. The three stainless-steel fluid reservoirs, containing solutions of various composition, are pressurized.

The superfusion chamber is recessed into the valve seat and accommodates the filter wafer assembly as previously described (Pearce et al., 1988). The filter wafer consists of a Millipore SC membrane filter (8-μm pore size), a GF/F glass fiber filter (1.2-μm pore size), and another SC filter. The preloaded vesicles are trapped on the filter wafer and are secured in the superfusion chamber with a Teflon outlet fitting. In addition, in the current version, a GF/B filter is placed directly above the wafer in the superfusion chamber to act as a baffle protecting the vesicles against shearing and turbulence. Finally, a Millipore RA membrane filter (1-μm pore size) is placed beneath the filter wafer to act as a "net", retaining any vesicles that may be dislodged from the filter wafer during the superfusion. The fraction collector is a circular platform with 50 15 × 45 mm vials (Atlantic Biomedical) juxtaposed on its perimeter. The platform rotates on a phonograph turntable placed directly beneath the outlet fitting.

The filter wafer loaded with radiolabeled synaptosomes is secured in the superfusion chamber with the Teflon outlet fitting. The superfusion protocol is triggered by the momentary closing of a reed switch as the permanent magnet attached to the turntable swings past. The valves are energized in the desired sequence, resulting in flow of one of three superfusion buffers into the superfusion chamber for a precise duration. An important design feature of the superfusion apparatus is that the "dead volume" ($\sim$30 μL) of the superfusion chamber is small relative to the flow rate (1.5 mL/s), assuring rapid exchange of solutions within the chamber and removal of small molecules from the filter wafer. The rate of solution change within the chamber can be estimated by introducing ^3H_2O into one of two superfusion buffers and measuring the rate of appearance and disappearance of ^3H_2O in the superfusion effluent. This process is exponential, with a time constant of 50–60 ms, which approximates an upper limit for temporal resolution of the system.

The superfusate is channeled through the narrow outlet fitting, exiting as a continuous, uninterrupted stream. The effluent stream is collected by the fraction collector, which effectively "cuts" the stream into 50 36-ms segments. One milliliter of scintillation cocktail (Hydrofluor, National Diagnostics, Somerville, NJ) is added to each vial, and each vial is counted in a β counter for 5–10 min. Because of the relatively small amounts of radioactivity collected in each fraction (50–1000 cpm/fraction) and the large number of fractions counted, the counting statistics were a major source of error in these experiments.

Synaptosome Preparation. Synaptosomes were prepared by differential centrifugation of rat brain homogenate followed by a sucrose velocity sedimentation step (Hajos, 1975). The 0.8 M sucrose velocity gradient layer was diluted 1:1 with the basal buffer [in mM: NaCl, 145; KCl, 5; $MgCl_2$, 1.2; D-glucose, 10; aminooxyacetate (a GABA transaminase inhibitor added to retard oxidation of GABA), 0.1; HEPES–Tris, 10; ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)[2]–Tris, 1; pH 7.5], and the vesicles were pelleted at 10000*g* for 10 min. The final pellet was resuspended in 1.5 mL of the basal buffer. A Ca^{2+}-selective electrode (Philips, Moller Glassblowers, Zurich, Switzerland) was used to determine the free [Ca^{2+}] in all buffers; solutions containing no EGTA and no added Ca^{2+} generally have a free [Ca^{2+}] of 3–5 μM (pCa 5.5). Tris–EGTA (1 mM) was added to the superfusion solutions to reduce the free [Ca^{2+}] to 10 nM (pCa 8), which is expected to be less than the resting free [Ca^{2+}] within synaptosomes (Brethes et al., 1987). Tris–EGTA was omitted from superfusion buffers containing Cd^{2+} or Ni^{2+} to eliminate the complex equilibria between EGTA and multiple divalent cations.

The synaptosomes were diluted with basal buffer to 0.5 mg of protein/mL and were gently agitated for 5 min prior to beginning the loading procedure. Loading was performed by combining 0.1 mL of the synaptosome suspension (50 μg of protein) with 5–10 μL of [^{3}H]GABA (75–93 Ci/mmol, 1 μCi/μL), giving an initial [[^{3}H]GABA] of 0.5–1.0 μM. Each 50-μg sample was incubated with radiolabel for 10 min at 19–22 °C; the loaded vesicles were then diluted to a volume of 0.8 mL and applied to the superfusion filter wafer. The filter wafer was held in place in a separate loading chamber, and a 30-mL syringe was used to gently push the solution through the filters. Approximately 10–20% of the [^{3}H]GABA was loaded into the vesicles at the end of the 10-min loading period. The loaded filter was secured in the superfusion chamber and given two 5-s washes to remove extravesicular radiolabel immediately prior to the superfusion experiment.

Standard Protocol. The synaptosomes were superfused with basal buffer with or without added $CaCl_2$ (Calcium Molarity Standard, Corning) for 0.65 s to establish a base-line level of GABA release. At that time (time = 0), the basal buffer is switched to a stimulation buffer. The synaptosomes are superfused with the stimulation buffer for 0.75 s, and the buffer is then switched back to the basal buffer for the final 0.40 s. GABA release is expressed as a percent of the total GABA content per fraction; this is defined as the quotient of the quantity of radioactivity in each individual fraction divided by the sum of radioactivity in all fractions plus radioactivity remaining on the filter at the end of the superfusion. The radioactivity remaining on the filter at the end of 1.8 s of superfusion is >95% of the total radioactivity; thus, the amount released in any given pulse is small relative to the total. Data are compiled using Lotus 1-2-3. Results for each curve in Figures 2–9 are plotted as the mean of at least three experiments performed on different days.

Radioactivity Released from Preloaded Synaptosome Cochromatographs with Authentic GABA. The identity of radioactivity released from the synaptosomes preloaded with [^{3}H]GABA was determined by thick-layer chromatography of the effluent collected during a superfusion experiment. The effluent from 10 fractions (50–70 μL) collected during the period of maximal evoked release was frozen and lyophilyzed. The residue from each fraction was resuspended in 10 mM HCl and was streaked onto a thick (1000 μm) layer silicic acid plate along with an unlabeled GABA standard (10 μmol) and a [^{3}H]GABA standard. A chromatogram was developed in 1-butanol/acetic acid/water (80:12:35); the plate was stained with ninhydrin to visualize GABA standards, and an R_f value of 0.16 was determined. The lanes containing [^{3}H]GABA (experimental and standards) were divided into 5-mm strips,

[1] Turner, Pearce, Calhoon, and Goldin, unpublished data.

[2] Abbreviations: EGTA; ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

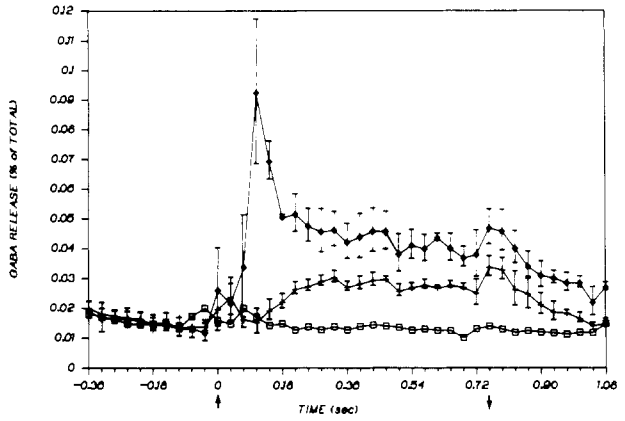

FIGURE 1: Subsecond kinetics of [^{3}H]GABA release are complex. Evoked release of GABA was measured under the following conditions: (□) basal + Ca^{2+} (in mM: Na^+, 145; K^+, 5; Ca^{2+}, 2.4); (+) depolarized (in mM: Na^+, 40; K^+, 110; EGTA, 1); (◇) depolarized + Ca^{2+} (in mM: Na^+, 40; K^+, 110; EGTA, 1; Ca^{2+}, 3.4). No significant increase in the rate of GABA release was observed when switching from the basal buffer to the basal buffer plus Ca^{2+} (error bars denote the standard deviation). Maximal rates of GABA release are observed within 100 ms of depolarization in the presence of physiological concentrations of Ca^{2+}.

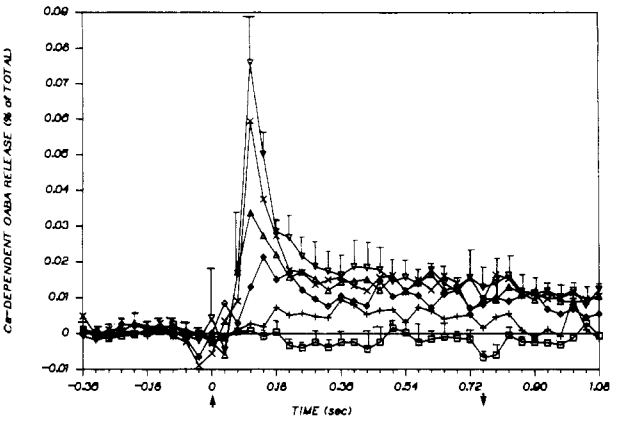

FIGURE 2: Two components of Ca-dependent [^{3}H]GABA release. Synaptosomes were superfused by using the standard protocol; the test solution contained 40 mM Na^+ and 110 mM K^+ plus the following free $[Ca^{2+}]$: (▽) 3.0 mM; (×) 1.0 mM; (△) 0.3 mM; (◇) 70 μM; (+) 25 μM; (□) 10 μM. Experiments performed at pCa 8.0 contain 1 mM EGTA and no added Ca^{2+}; the difference between release evoked under these conditions and basal conditions is defined as Ca-independent release since there is no inwardly directed Ca^{2+} concentration gradient and hence no Ca^{2+} entry. The net Ca-dependent component, after subtracting the Ca-independent component, is shown as a function of $[Ca^{2+}]_e$.

and the silica scraped from each strip was counted by liquid scintillation spectroscopy. All detectable radioactivity in the superfusion effluent comigrates with authentic GABA (based on the R_f value) and the [^{3}H]GABA standards. Recovery of radioactivity on the TLC plates was >90%, indicating that radioactivity in the effluent is primarily [^{3}H]GABA.

RESULTS

Three Distinct Components of GABA Release

The rapid superfusion device was initially used to measure the rates of synaptosomal GABA release under three conditions: basal (low K^+, low Ca^{2+}), depolarized (high K^+, low Ca^{2+}), and depolarized plus calcium (high K^+, high Ca^{2+}; Figure 1). When the synaptosomes are superfused with the basal buffer, a base-line rate of GABA release of 0.30%/s is observed. If the stimulating buffer contains 110 mM KCl substituted equivalently for NaCl, the rate of GABA release increases, in a manner related to the concentration of Ca^{2+} in the stimulating buffer. When external Ca^{2+} is maintained at pCa 8 by 1 mM EGTA, KCl depolarization results in an increase in the rate of GABA release to a new steady-state rate 2–3 times that of the base line. When the synaptosomes are depolarized by 110 mM KCl at a physiological $[Ca^{2+}]$ (2.4 mM), there is a rapid increase in the rate of release to a level 8–12 times the base-line rate. This peak rate decays rapidly over a period of 200 ms to a level that is initially 4–6 times base-line. When measured on a slow time scale, this remaining Ca-dependent release decays slowly over a period of 5–10 s (not shown). The time course of decay is similar when the superfusion buffer is switched to a basal 5K/pCa 8 buffer after 0.75 s of stimulation. Thus, depolarization-evoked GABA release is multiphasic and can be qualitatively described as follows: (1) a *Ca-independent component*; (2) a *phasic Ca-dependent component* that decays within 200 ms; and (3) a *tonic Ca-dependent component* that decays slowly but persists in the absence of Ca^{2+} entry.

To test the specificity of depolarization-stimulated GABA release, the synaptosomes were loaded with [^{35}S]methionine, an amino acid that would not be expected to be packaged by nerve endings into secretory vesicles. KCl depolarization stimulated [^{35}S]methionine release roughly 2-fold, but depolarization in the presence of millimolar $[Ca^{2+}]$ did not augment [^{35}S]methionine release (not shown).

The Ca^{2+} dependence of GABA release evoked by 110 mM KCl was measured as a function of external free $[Ca^{2+}]$ ranging between 10 nM and 3 mM (Figure 2). The time course of depolarization-evoked GABA release is similar at external $[Ca^{2+}]$ between 10 nM and 20 μM (not shown); the difference between basal release and release in 110 mM KCl is defined as the Ca-independent component. As the external $[Ca^{2+}]$ is elevated above 20 μM, there is an increase in the rate of GABA release that depends on depolarization and Ca^{2+}. At $[Ca^{2+}]$ of 25–70 μM, Ca-dependent release is dominated by the tonic component, indicating that the tonic component is qualitatively more sensitive to external $[Ca^{2+}]$. As the $[Ca^{2+}]$ is increased above 100 μM, the tonic component saturates, while the amplitude of the phasic component continues to increase.

The voltage dependence of the three components of GABA release (Figure 3) was examined by measuring the time course of release as a function of [KCl] in the stimulating buffer, at pCa 2.6 or pCa 8. In pCa 2.6 buffer, there is little increase in the rate of release at 15 mM KCl; increasing [KCl] to 30 mM results in a marked increase in the rate of GABA release (Figure 3A). When release is evoked in pCa 8 buffers, GABA release increases asymptotically to a level that is proportional to [KCl] in the stimulating buffer, without the presence of the transient component characteristic of release evoked in the presence of physiological Ca^{2+} concentrations (Figure 3B). When NaCl was replaced by *N*-methylglucamine sulfate or choline chloride instead of KCl, the rate of GABA release did not increase above base-line until the Na^+ concentration was reduced below 20 mM (not shown). Therefore, replacing NaCl with KCl evokes GABA release primarily by depolarizing the synaptosomes, not by altering the driving force on Na^+. The Ca-dependent release (the difference between release observed at pCa 8 and pCa 2.6; Figure 3C) shows a steep dependence on potassium concentration, since release is maximally activated between 15 and 30 mM K^+. On the basis of measurements with two electrochromic dyes [anionic bis-(oxonol) and cationic dicarbocyanine], we confirmed the observation (Blaustein & Goldring, 1975; Suszkiw, 1986) that synaptosomes behave as a K^+-selective electrode over the range

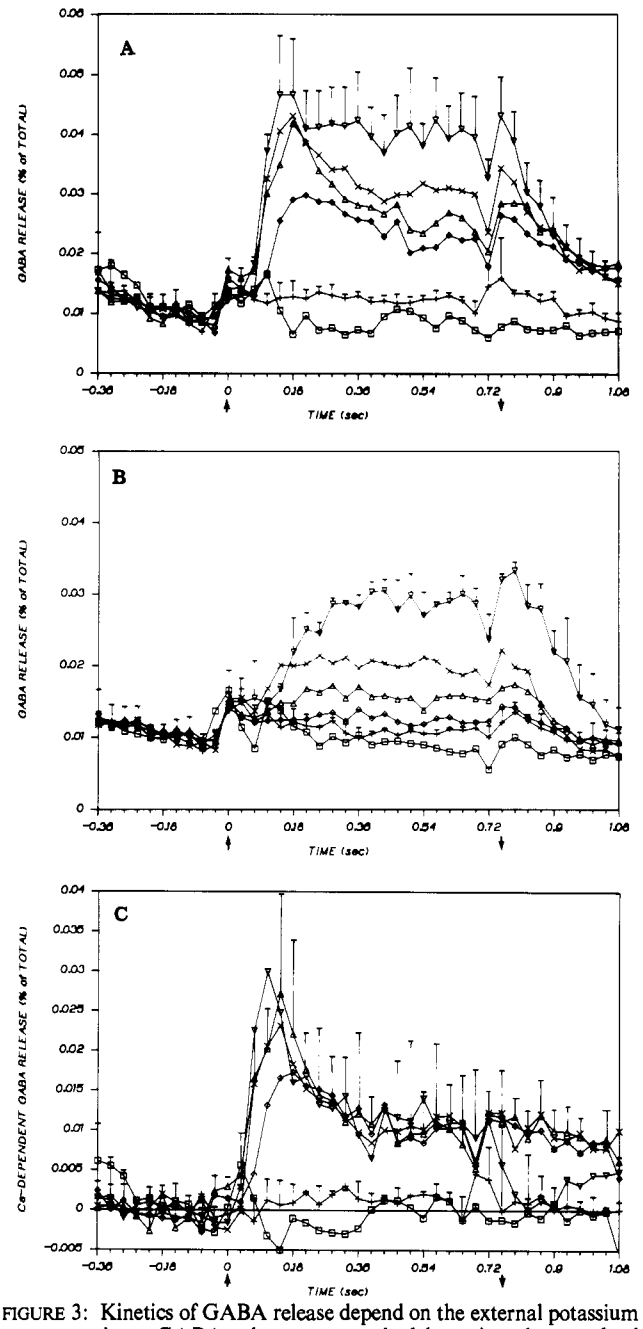

FIGURE 3: Kinetics of GABA release depend on the external potassium concentration. GABA release was evoked by using the standard protocol with a test buffer that contained 24 mM Ca^{2+} and the following [KCl] (in mM): (▽) 150; (×) 110; (△) 60; (◇) 30; (+) 15; (□) 5. KCl was substituted for an equivalent concentration of NaCl. (A) Time course of total GABA release with 2.4 mM Ca^{2+} in both basal and test buffers at the indicated [KCl] ($n = 4$). Symbols are used consistently in panels B and C. (B) The same experiment as in (A), with 1 mM EGTA and no added Ca^{2+}. (C) The Ca-dependent evoked release is the net difference between total release evoked by depolarization in the presence of Ca^{2+} (pCa 2.6) and depolarization in low Ca^{2+} (pCa 8) at each KCl concentration.

of 5–110 mM K^+, with a resting potential of ~−55 mV in 5 mM KCl basal buffer.

Pharmacological Characteristics of the Three Components

Nipecotic Acid Inhibits the Ca-Independent Component. GABA release evoked by 150 mM KCl, pCa 8, was measured in the presence of the GABA uptake system antagonist nipecotic acid (Figure 4) to assess the contribution of the GABA uptake system to Ca-independent release. Nipecotic acid is a cyclic analogue of GABA that is a weak antagonist of the

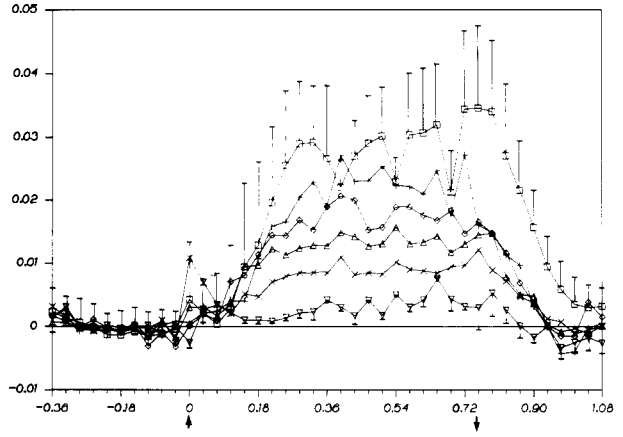

FIGURE 4: Nipecotic acid blocks the Ca-independent component. The sensitivity of the Ca-independent component to nipecotic acid, an inhibitor of neuronal GABA uptake, was tested by including nipecotate in both basal (5 mM K^+, pCa 8) and test (150 mM K^+, pCa 8) buffers, using the standard protocol. The nipecotate concentrations used were (□) 0 μM (control), (+) 0.3 μM, (◇) 3.0 μM, (△) 30 μM, (×) 0.3 mM, and (▽) 3.0 mM.

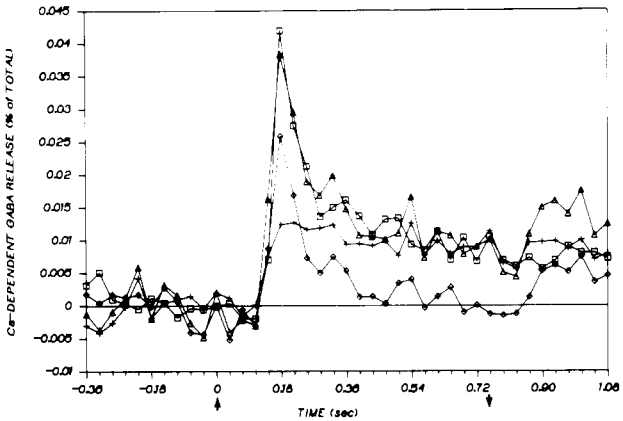

FIGURE 5: Inorganic antagonists Cd^{2+} and Ni^{2+} selectively block the two components of Ca-dependent release. Ca-dependent release was tested for sensitivity to two well-known inorganic blockers of Ca^{2+} entry: Ni^{2+} (100 μM) and Cd^{2+} (50 μM). GABA release evoked by 40 mM Na^+/110 mM K^+ was measured with 2.4 mM Ca^{2+} or with no added Ca^{2+}. When used, inorganic blockers were included in all solutions. The two phases of Ca-dependent release (□) are selectively blocked by Ni^{2+} (◇) and Cd^{2+} (+); Cd^{2+} blocks the *phasic* component, and Ni^{2+} blocks the *tonic* component. The sum of Cd^{2+}-sensitive Ca-dependent release plus Ni^{2+}-sensitive Ca-dependent release (△) is compared to the untreated control.

$GABA_A$ receptor but is a competitive inhibitor of (Na^+, Cl^-)-dependent GABA transport in synaptosomes, glial cells, and cultured neurons (Krogsgaard-Larson, 1980); 3 mM nipecotic acid inhibits the Ca-independent component by 80–90%; Ca-dependent release was not significantly affected at this concentration. Hill plots of the nipecotic acid effect (not shown) yield an IC_{50} value of 21 μM, with a Hill coefficient of $n_H = 0.34$, indicative of a broad dose–response relationship between nipecotate and block of GABA efflux.

Ca-Dependent Components Are Differentially Sensitive to Inorganic Antagonists. GABA release evoked by 110 mM KCl at pCa 5.5 or pCa 2.6 (no EGTA) was measured in the presence of 50 μM Cd^{2+} or 100 μM Ni^{2+} (Figure 5). These agents have been shown to selectively block three subtypes of Ca^{2+} channels found in sensory neuron cell bodies (Fox et al., 1987). Ni^{2+} had no significant effect on the Ca-independent component; Cd^{2+} reduces Ca-independent release by 40–60%. Ca-dependent release observed in the presence of Cd^{2+} and Ni^{2+} indicates that the two Ca-dependent components of GABA release are differentially sensitive to inorganic blockers.

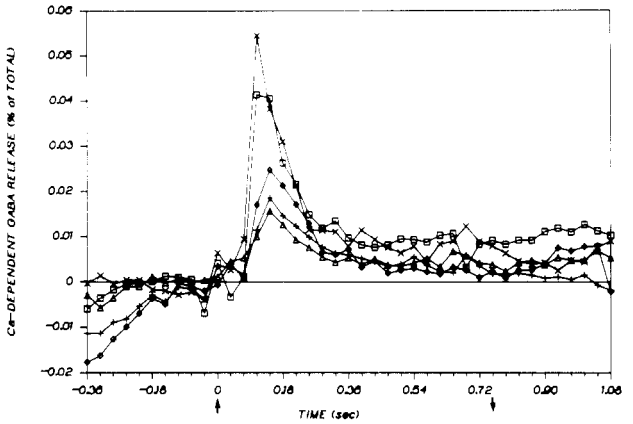

FIGURE 6: Ca-dependent GABA release can be evoked without depolarization by A23187. GABA efflux was measured by using a basal buffer containing 10 μM A23187 and no added Ca^{2+} (pCa 5.5), and Ca-dependent release was evoked by switching to an identical buffer with (pCa 2.6) or without (pCa 5.5) added Ca^{2+}. The difference between release measured in the presence and absence of Ca^{2+} in the stimulating buffer is defined as Ca-dependent release (□). On the same day, Ca-dependent release evoked by depolarization in 110 mM K^+ was also measured (×). Ionophore-evoked Ca-dependent release was also measured in the presence of 50 μM Cd^{2+} (+), 100 μM Ni^{2+} (◇), or 1 μM A23187 (△). Data shown are the average of three separate experiments.

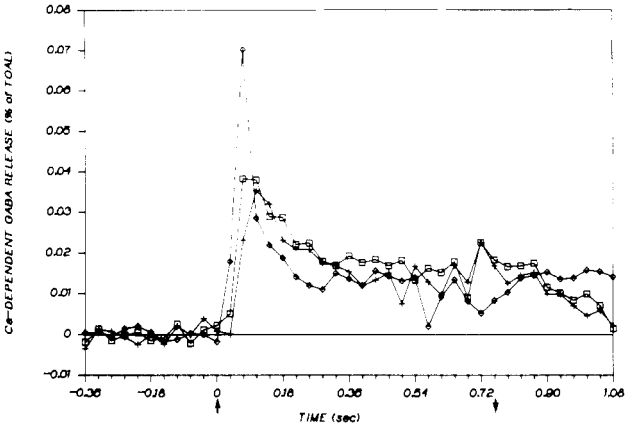

FIGURE 7: Prolonged depolarization of the vesicles does not inactivate Ca-dependent release or potentiate dihydropyridine effects. In order to assess any state-dependent effects of the dihydropyridines on GABA release, the vesicles were predepolarized in low Ca^{2+} buffer (110 mM K^+, pCa 8) prior to stimulation. The vesicles were depolarized for 4.35 s, and fractions containing released GABA were collected for an additional 0.65 s prior to the stimulating pulse, to establish a base line of GABA release. The vesicles were then superfused with the stimulating buffer (110 mM K^+, pCa 2.6) for 0.75 s and with basal buffer (5 mM K^+, pCa 8) for the final 0.40 s of collection. To measure Ca-independent release, the experiment was conducted in an identical fashion except for the use of a 110 mM KCl, pCa 8 stimulating buffer. The difference between release evoked by 110 mM KCl, pCa 2.6 and pCa 8, is defined as the Ca-dependent release. The effects of nimodipine on Ca-dependent GABA release were tested by comparing release in the absence of nimodipine (0.01% DMSO) (+) to release where all solutions contained 1 μM nimodipine/0.01% DMSO (□). The rates of release are also compared to release observed when the synaptosomes are "predepolarized" in 5 mM K^+, pCa 8 (◇). Data shown are the average of three independent experiments.

The phasic component is selectively blocked by Cd^{2+}, while the tonic component is selectively blocked by Ni^{2+}. When Ca-dependent release measured in the presence of Cd^{2+} is added to the Ca-dependent release measured in the presence of Ni^{2+} for each time point, the time course of the two-component release process in the absence of inorganic ions is reconstructed. The inorganic antagonists allow the two components to be dissected, indicating that the phasic and tonic components of GABA release are mediated by distinct Ca^{2+} entry pathways.

Ca-dependent GABA release can also be evoked by the Ca ionophore A23187 (Figure 6). Synaptosomes were superfused with a pCa 5.5 basal buffer containing A23187; release of GABA was evoked by switching to a pCa 2.6 basal buffer with A23187. The time course and amplitude of Ca-dependent GABA release evoked by Ca^{2+} plus 10 μM A23187 are nearly identical with the depolarization-evoked release. However, when 100 μM Ni^{2+} or 50 μM Cd^{2+} is added to the superfusion buffers, both the phasic and tonic components of Ca-dependent release are inhibited to a similar extent. The Ca-dependent release in the presence of either of the inorganic antagonists plus 10 μM A23187 is similar to the Ca-dependent release mediated by 1 μM ionophore alone. Cd^{2+} and Ni^{2+} block Ca-dependent GABA release evoked both by depolarization and by ionophore; both ions block the *ionophore-evoked* release, but the *depolarization-evoked* release is mediated by two separate pathways that are selectively blocked.

Voltage-Sensitive Ca^{2+} Entry Pathways Mediating GABA Release Are Pharmacologically Distinct from Previously Identified Ca^{2+} Channels. The three subclasses of Ca^{2+} channels that have been observed in sensory neuron cell bodies can be distinguished on the basis of a number of properties in addition to sensitivity to inorganic antagonists. The N-type neuronal Ca^{2+} channel is persistently blocked by the peptide toxin ω-conotoxin, is dihydropyridine resistant, and is sensitive to inactivation by prolonged depolarization (Tsein, 1987). The L-type neuronal Ca^{2+} channel is blocked by nimodipine under certain conditions. It has been shown in a number of systems that dihydropyridine blockade of L-type Ca^{2+} currents is a state-dependent effect (Bean, 1984; Cohen & McCarthy, 1987; Holz et al., 1987). The potency of the antagonist is much higher when the channel is in the open or inactive state, when drug binding stabilizes the inactive conformation. In order to observe potent dihydropyridine block of the channel, the membrane must be held at a relatively depolarized potential for prolonged periods.

We measured GABA release using a protocol that depolarizes the vesicles with 110 mM KCl, pCa 8, for 5 s prior to evoking Ca-dependent release at 110 mM KCl, pCa 2.6 or pCa 8 (Figure 7). This protocol should allow us to determine the relative contributions of N- and L-type neuronal Ca^{2+} channels, since prolonged depolarizations should inactivate the N-type Ca^{2+} channel and should potentiate dihydropyridine blockade of dihydropyridine-sensitive Ca^{2+} channels. The 5-s depolarization in the absence of an inwardly directed Ca^{2+} gradient had no significant effect on subsequent Ca-dependent GABA release. This indicates that the voltage-sensitive Ca^{2+} entry pathways show little inactivation during a 5-s depolarization in the absence of Ca^{2+} entry. Similar results were obtained using depolarizing pulses of KCl concentrations between 15 and 90 mM. Therefore, Ca-dependent release depends primarily on Ca^{2+} entry, not on membrane potential. Nimodipine (1 μM) does not significantly alter Ca-dependent GABA release regardless of the prepotential, indicating that there is no obvious state-dependent blockade of Ca-dependent GABA release.

A multiple-pulse protocol (Figure 8) was employed to test for possible use-dependent effects of nimodipine and ω-conotoxin on Ca-dependent release, and to demonstrate the ability to repetitively stimulate GABA release using the superfusion system. GABA release was evoked by a stimulating buffer containing 110 mM KCl, pCa 2.6 or pCa 8, for 360 ms every 20 s (0.05 Hz) 6–10 times. The greatest amount of Ca-dependent GABA release was observed in the first pulse; release

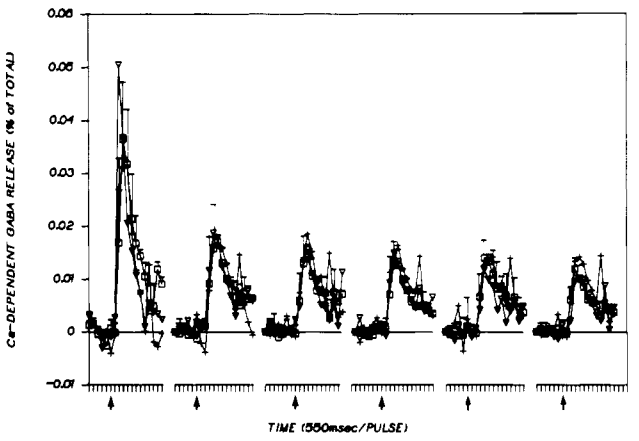

FIGURE 8: Ca-dependent GABA release is not sensitive to ω-conotoxin or dihydropyridine Ca^{2+} channel blockers. GABA release was measured in the presence of two classes of Ca^{2+} channel inhibitors, using a repetitive stimulation protocol. The vesicles were depolarized (as indicated by the arrows) at pCa 8 or pCa 2.6 for 360 ms and allowed to rest in basal buffer for 20 s. This regimen was repeated 6 times; the difference between release at pCa 2.6 and pCa 8 was defined as Ca-dependent release (□). GABA release was measured in the presence of nimodipine/0.01% DMSO at concentrations ranging from 0.1 to 3.0 μM [average of four experiments at 3 μM shown (▽)]. Vesicles were preincubated with 3.0 μM ω-conotoxin for up to 1 h prior to superfusion to allow sufficient time for the toxin to associate with its receptor. Ca-dependent release with 0.3 μM toxin included in the superfusate is shown (+) (averaged from five experiments).

declined to a level roughly half the original, which was maintained for at least 10 pulses. However, regardless of the preincubation duration (up to 1 h) or release protocol (standard or multiple pulse), ω-conotoxin (0.05–3.0 μM) had no significant effect on synaptosomal GABA release. The lack of effect could not be explained on the basis of inactive toxin, since a portion of the same batch of toxin inhibited Ca^{2+} currents in DRG neuron cell bodies potently and persistently (Holz et al., 1988). Likewise, nimodipine had no significant effect on Ca-dependent GABA release at doses between 0.1 and 3.0 μM in either the standard or the multiple-pulse protocol.

Discussion

We have used a novel superfusion device to measure neurosecretion of GABA from presynaptic nerve endings on a subsecond time scale. The process is multiphasic, and at least three distinct components now have been identified for the first time in an in vitro system. On the basis of these properties, it is apparent that experiments conducted on a second to minute time scale designed to measure release of GABA, and perhaps other neurotransmitters, lack the temporal resolution necessary to observe at least one of the major components.

The presence of two distinct components of Ca-dependent GABA release with different decay times could be important in determining the time course of inhibitory postsynaptic potentials (ipsps) observed at GABAergic synapses. In hippocampal slices, orthodromic stimulation results in both a fast ipsp associated with a $GABA_A$-mediated Cl^- flux and a slow ipsp associated with a $GABA_B$-mediated K^+ flux (Dutar & Nicoll, 1988). It is intriguing to hypothesize that the fast ipsp is mediated by the phasic component of GABA release and that the slow ipsp is mediated by the tonic component, since these processes occur on corresponding time scales. This hypothesis predicts that the fast ipsp would be selectively blocked by Cd^{2+} and the slow ipsp would be selectively inhibited by Ni^{2+}. While the estimate of a time constant for decay of the fast component is limited by the superfusion device, we can establish an upper limit of 60 ms, which is on the same time scale as the fast ipsp. Further refinements in the superfusion device should allow us to describe the time course of Ca-dependent GABA release quantitatively.

GABA Uptake Systems Are Involved in the Ca-Independent Component. The Ca-independent component mediates GABA release when the vesicles are depolarized in low external [Ca^{2+}]. The Ca-independent release is blocked by nipecotic acid, a selective blocker of the GABA uptake systems. These systems transport GABA, Na^+, and Cl^- in a ratio of 1:3:1 (Keynan & Kanner, 1988). Purification of this activity from rat brain synaptosomes has revealed two kinetically distinct forms of the transporter: a high-affinity form (K_m = 4 μM) and a low-affinity form (K_m = 400 μM). Under resting conditions, the electrochemical driving force for Na^+ entry (established by the Na/K-ATPase) is coupled to GABA uptake, concentrating extravesicular GABA in the nerve terminal. When the nerve terminal is depolarized, the driving force for GABA transport can reverse, leading to net efflux of GABA (Schwartz, 1987). Nipecotic acid, a cyclic analogue of GABA that is a weak GABA antagonist, is thought to act as a transition-state analogue, stabilizing the transporter and dissociating very slowly (Krogsgaard-Larson, 1980).

The broad dose–response relationship observed for blockade of the Ca-independent component is probably a result of a number of considerations. Two forms of GABA transporters with different affinities for GABA would be expected to contribute to a Hill coefficient of less than 1. Heterogeneity of the synaptosome preparation is another factor that could result in a broad dose–response relationship. Furthermore, nipecotic acid cannot be added to the vesicles until after they are loaded with [^{3}H]GABA, since this would block uptake of the tracer. The vesicles are exposed to drug for less than 1 min prior to the superfusion, which may be insufficient time for the drug to equilibrate with the transport system.

Ca-Dependent Release Is Composed of Two Components. The Ca-dependent release is of particular interest, due to the role of Ca^{2+} entry in stimulus–secretion coupling and the observation that neurotransmission requires millimolar external [Ca^{2+}] (Katz & Miledi, 1965; Miledi & Slater, 1966). The "Ca^{2+} hypothesis" (Katz, 1969) proposes that Ca^{2+} enters the nerve terminal in response to depolarization as a consequence of activating voltage-sensitive Ca^{2+} channels. The Ca^{2+} concentration within the presynaptic terminal, maintained at rest in the submicromolar range (Augustine et al., 1985; Brethes et al., 1987), increases as a result of Ca^{2+} entry. An intracellular Ca^{2+} receptor mediates fusion of secretory vesicles with the plasma membrane, spilling the contents of these vesicles into the synaptic cleft (quantal release).

When synaptosomes are depolarized in the presence of millimolar [Ca^{2+}], GABA release is increased over that observed in the absence of Ca^{2+}. The properties of Ca-dependent release suggest that there are two distinct components. The two components differ in time course, sensitivity to Ca^{2+}, and blocking by the inorganic antagonists Cd^{2+} and Ni^{2+}.

The Ca-dependent GABA release evoked by the calcium ionophore A23187 is similar in magnitude and time course to Ca-dependent release evoked by depolarization. Thus, any inactivation of Ca^{2+} entry does not limit Ca-dependent release, since flooding the intravesicular space with Ca^{2+} using A23187 evokes Ca-dependent GABA release which is similar in time course and magnitude to depolarization-evoked Ca-dependent release. Unlike Ca-dependent release evoked by depolarization, the inorganic antagonists Cd^{2+} and Ni^{2+} did not selectively block one or the other component. Cd^{2+} and Ni^{2+} each decreased the amplitude of both components of Ca-dependent

release evoked by 10 μM A23187 to a level similar to 1 μM A23187. Cd^{2+} and Ni^{2+} act as if they block ionophore-mediated release by binding tightly to the ionophore, thereby reducing the effective concentration of the ionophore available to mediate Ca^{2+} entry. The effects of the inorganic blockers on both depolarization-evoked and ionophore-evoked Ca-dependent release can be accounted for by altering the rate of Ca^{2+} entry without affecting subsequent steps of the release process. The selective block of depolarization-evoked phasic release by Cd^{2+} and tonic release by Ni^{2+} indicates that these two components are mediated by distinct voltage-sensitive pathways of Ca^{2+} entry.

Ca-dependent release is not significantly altered when the vesicles are depolarized for 5 s prior to exposure to Ca^{2+}. This observation, taken together with the result obtained with A23187, indicates that membrane depolarization per se is neither necessary nor sufficient to evoke Ca-dependent GABA release under the conditions used in this study. While depolarization is ordinarily required to admit Ca^{2+} to synaptosomes via voltage-sensitive pathways, we conclude that Ca-dependent GABA release is activated primarily as a result of Ca^{2+} entry, subsequently decays, and is not significantly influenced by membrane potential.

Identity of the Voltage-Sensitive Pathways for Ca^{2+} Entry. A major objective of these experiments is to identify the voltage-sensitive Ca^{2+} entry pathway(s) mediating Ca-dependent neurosecretion. We used three types of blockers of Ca^{2+} channels, inorganic antagonists (Cd^{2+} and Ni^{2+}), dihydropyridine antagonists (nimodipine), and the snail peptide toxin ω-conotoxin, in an effort to identify pharmacologically the voltage-sensitive pathways for Ca^{2+} entry leading to Ca-dependent release of GABA.

The selective block of the tonic component by 100 μM Ni^{2+} suggests that it is mediated by a T-type Ca^{2+} channel (Fox et al., 1987), based on the differential sensitivity to Ni^{2+}. However, predepolarization should lead to inactivation of a T-type channel, something that we did not observe. Further, it is somewhat paradoxical that a persistent component of release would be mediated by a transient influx of Ca^{2+}. The observation that, once triggered, the tonic component persists in the absence of an inwardly directed $[Ca^{2+}]$ gradient or depolarized potentials suggests that Ca^{2+} entry acts to initiate the tonic component and that it does not require continuous Ca^{2+} entry. The phasic component is distinguished from the tonic component on the basis of selective blockade by 50 μM Cd^{2+}. The lack of selectivity of inorganic ions in general, and Cd^{2+} in particular, makes it difficult to classify the Ca^{2+} entry pathway solely on the basis of Cd^{2+} sensitivity. However, block by inorganic ions does provide a useful tool to study the two components of Ca-dependent GABA release in isolation.

While dihydropyridine antagonists have been shown to block neuronal Ca^{2+} currents in DRG cell bodies (Holz et al., 1988), the effects of these drugs on presynaptic Ca^{2+} channels are a matter of considerable controversy. There are a number of reports that DHP blocks a portion of synaptosomal Ca^{2+} uptake (Turner & Goldin, 1985; Nordstrom et al., 1986; Dunn, 1988) or that they have no significant effect (Nachshen & Blaustein, 1979; Suszkiw et al., 1986; Reynolds et al., 1986). Likewise, ω-conotoxin has been shown to persistently and potently block N-type Ca^{2+} currents (McClesky et al., 1987), but there is some disagreement regarding the persistent block of neuronal dihydropyridine-sensitive Ca^{2+} channels (Kasai et al., 1987). ω-Conotoxin blocks a fraction of K^+-stimulated $^{45}Ca^{2+}$ uptake in synaptosomes (Reynolds et al., 1986), as well as a similar fraction of [^{3}H]norepinephrine release. Neither nimodipine nor ω-conotoxin had a significant effect on Ca-dependent release of GABA at concentrations up to 3 μM. The lack of effect of these drugs argues that the Ca^{2+} entry pathways mediating GABA release are pharmacologically distinct from Ca^{2+} channels previously identified in sensory neuron cell bodies. Furthermore, it is difficult to generalize about the properties of neuronal Ca^{2+} channels on the basis of observations made in any one particular preparation.

N- and T-type Ca^{2+} channels characteristically inactivate after prolonged depolarization. The Ca^{2+} entry pathways in synaptosomes mediating Ca-dependent GABA release are not significantly altered by a 5-s depolarizing pulse in low $[Ca^{2+}]$, further distinguishing this activity from transient Ca^{2+} channels observed previously. However, in our experiment, the depolarizing pulse was delivered under conditions where $[Ca^{2+}]_e$ <100 nM, as opposed to electrophysiological experiments where external $[Ca^{2+}]$ is millimolar but internal $[Ca^{2+}]$ is highly buffered. These two observations could be reconciled if the transient channels inactivate as a consequence of Ca^{2+} entry rather than changes in the transmembrane electric field.

The phasic component of GABA release must be mediated by a type of neuronal Ca^{2+} channel that is insensitive to Ni^{2+}, ω-conotoxin, nimodipine, and to prolonged depolarization, properties that are inconsistent with those previously described for Ca^{2+} channels in sensory neuron cell bodies. The tonic component is pharmacologically similar but is Cd^{2+} insensitive and kinetically distinct from the phasic component. These observations suggest that at least the phasic component of synaptosomal GABA release is mediated by a type of Ca^{2+} channel not found in cell bodies of sensory neurons. It is possible that a distinct type of Ca^{2+} channel found only in presynaptic nerve terminals is responsible for mediating transient neurosecretion of certain neurotransmitters. Alternatively, a wide variety of Ca^{2+} channel subtypes may exist that have specialized roles throughout the brain.

In summary, rapid superfusion of presynaptic nerve endings obtained from rat brain has revealed three components of depolarization-evoked GABA release. Although we cannot measure the activation rates of the three processes, they have been identified and characterized on a subsecond time scale for the first time in an in vitro system. While information on the activation rates of these processes is still out of reach, the amount of GABA release mediated by each component can be measured with a fair degree of certainty, and a protocol has been developed to selectively block each of them. This should enable us to study each component in isolation, allowing a more precise understanding of the regulation of neurotransmission and of the mechanisms of action of pharmacological agents which are thought to affect neurotransmission in the brain.

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Registry No. GABA, 56-12-2; Ca, 7440-70-2.

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^{1}H NMR Assignment and Secondary Structural Elements of Human Transforming Growth Factor α[†]

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ABSTRACT: The ^{1}H NMR spectrum of human transforming growth factor α (hTGF-α) has been completely assigned, and secondary structural elements have been identified as a preliminary step in determining the structure of this protein by distance geometry methods. Many of these structural elements closely correspond to those previously found in a truncated human EGF [Cooke et al. (1987) *Nature (London) 327*, 339-341] and murine EGF [Montelione et al. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5226-5230]. These include the presence of an antiparallel β-sheet between residues G19 and C34 with a type I β-turn at V25-D28, a type II β-turn at H35-Y38, and another short β-sheet between residues Y38-V39 and H45-A46.

Transforming growth factor α (TGF-α)[1] [see Derynk (1986) for a recent review] was originally discovered with TGF-β in the conditioned medium of virally transformed rat kidney fibroblasts (DeLarco et al., 1981). It was subsequently found in the conditioned medium of several human tumor cell lines (Todaro et al., 1980) and human tumor explants (Derynk et al., 1987) and has been detected in the urine of patients with disseminated cancers (Sherwin et al., 1983). In vitro addition of TGF-α to growth medium at nanomolar concentrations stimulates the growth of normal fibroblasts and epithelial cells (DeLarco & Todaro, 1978; Schultz et al., 1987), whereupon

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[1] Abbreviations: ^{1}H NMR, proton nuclear magnetic resonance; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; 2D, two dimensional; DQF-COSY, 2D double quantum filtered correlation spectroscopy; RELAY, 2D relayed coherence transfer spectroscopy; TOCSY(HOHAHA), 2D total correlation spectroscopy; 2Q-COSY, double quantum 2D correlation spectroscopy; NOESY, 2D NOE spectroscopy; hTGF-α, human transforming growth factor; th-EGF, truncated human epidermal growth factor; m-EGF, murine epidermal growth factor; EDTA, disodium ethylenediaminetetraacetate.

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