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Specific Glycine-Accumulating Synaptosomes in the Spinal Cord of Rats

(neurotransmitters/glutamate/neutral inhibition/γ-aminobutyric acid)

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ABSTRACT **Subcellular fractionation of rat spinal cord on continuous sucrose density gradients provides evidence for the existence of a specific synaptosomal fraction (enriched in pinched-off nerve endings) that accumulates glycine selectively by way of a high-affinity transport system. The particles in this fraction sediment to a less-dense portion of sucrose gradients than do particles that accumulate neutral, basic, aromatic, and acidic amino acids. Particles accumulating γ-aminobutyric acid are even less-dense than those storing exogenous glycine. The glycine-specific synaptosomal fraction also exists in the brain stem but not in the cerebral cortex. These findings provide neurochemical support for the suggestion that glycine has a specialized synaptic function, perhaps as a neurotransmitter, in mammalian spinal cord.**

Several lines of evidence favor a synaptic role for glycine in mammalian spinal cord. Most importantly, in neurophysiologic studies, glycine mimics the behavior of the natural inhibitory transmitter in its conductance effects and blockade by strychnine (1–3). The regional localization of glycine within the spinal cord is consistent with that of the natural inhibitory transmitter (4–7). Electron microscopic autoradiography reveals a localization of accumulated radioactive glycine predominantly in nerve terminals in the spinal cord (8, 9). Accumulated radioactive glycine can be released from the spinal cord by electrical depolarization of spinal cord slices (10, 11).

Recently a high-affinity uptake system for glycine into slices (12, 13) or synaptosomal preparations (13–15) of rat spinal cord was described that was not manifested by most other amino acids (14, 15) and that was present in spinal cord (13–15) and brain stem (13), but not in the cerebral cortex (13–15).

Previously we reported a synaptosomal fraction with distinct sedimentation characteristics on density gradient centrifugation of cerebral cortexes that accumulated glutamic and aspartic acids (16) by means of a high-affinity transport system. Nerve terminals in the spinal cord that accumulate radioactive glycine differ in their morphology from those that did not selectively take up this amino acid (9). Accordingly, it is conceivable that glycine-accumulating synaptosomes may differ in their physical properties from the general population of synaptosomes in the spinal cord. We report here that radiolabeled glycine is accumulated by homogenates of rat spinal cord into a unique synaptosomal fraction that can be distinguished from the particles that store other amino acids.

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METHODS AND MATERIALS

Sprague-Dawley male rats (150–200 g) were decapitated, and their spinal cords, medulla-pons, or cerebral cortexes rapidly removed and placed on dental wax on ice. All subsequent procedures were conducted at 0–4°. After they were weighed, the tissues were homogenized in 20:1 (v/w) 0.32 M sucrose with a glass homogenizer with a motor-driven Teflon pestle (0.09–0.11 mm clearance). The homogenates were centrifuged at 1000 × *g* for 10 min to remove nuclei and cellular debris. 1.5-ml Aliquots of the supernatant fluid were added to 50-ml beakers containing 20 ml of Krebs–Ringer–Tris buffer (pH 7.4 at 37°) and also containing 11 mM glucose, 1.25 mM calcium, and labeled amino acids (^{3}H, 0.1 μM; ^{14}C, 1.0 μM). The beakers were incubated in a Dubnoff metabolic shaker at 37° for 4 min. Under these conditions paper chromatographic analysis in three solvents showed that 90% of the radioactive compounds were unmetabolized (14, 15). After incubation, the contents of the beakers were transferred to 40-ml centrifuge tubes containing 10 ml of ice-cold 0.32 M sucrose and centrifuged at 27,000 × *g* for 5 min at 0–4° to form a crude mitochondrial pellet. This pellet was rinsed with 10 ml of cold 0.32 M sucrose and suspended in 1.1 ml of 0.32 M sucrose. In order to disperse the pellet uniformly, it was necessary to agitate it for 30 sec on a Variwhirl mixer, pass the suspension through a 1-ml volumetric pipette 10–15 times, and again agitate vigorously for 45 sec. The consistency of the resultant suspension was checked visually for the presence of particle aggregates, and agitation was continued if necessary. The final suspension was layered onto a continuous sucrose density gradient prepared by linear dilution of 7.0 ml of 1.5 M sucrose with 7.0 ml of 0.5 M sucrose with a triple-outlet mixer (Buchler 2-5014). Gradients were maintained at 4° for 12 hr before use. The gradients were centrifuged at 100,000 × *g* for 15 min with a SW 50.1 rotor in a Spinco L2-65B ultracentrifuge, a condition of "incomplete sedimentation equilibrium" (16, 17). Fractions were obtained by piercing the bottoms of the tubes and collecting 10-drop aliquots. 32–34 Fractions were obtained and assayed for double-label radioactivity in vials containing 10 ml of Bray's phosphor (18) with a model 3375 Packard Tri Carb liquid scintillation spectrometer.

Monoamine oxidase activity of the fractions was assayed by a modification of the method of Wurtman and Axelrod (19) as described (17). Monoamine oxidase was used as a marker for mitochondria because the sensitivity of the available radiometric assay (19) enabled the detection of this enzymatic activity in discrete, small gradient fractions. Protein was measured by the method of Lowry *et al.* (20), with bovine serum albumin as a standard. For determination of the potassium concentration in the fractions of the gradient, each

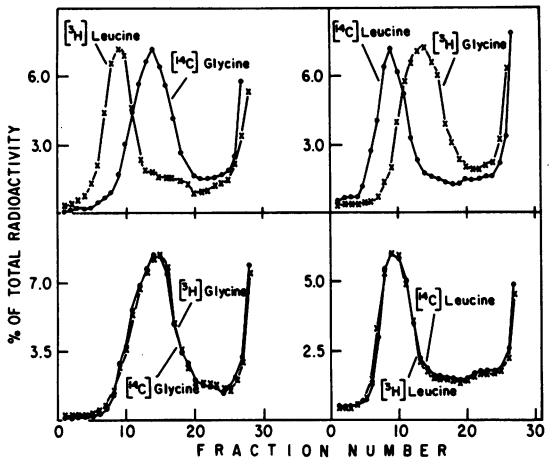

FIG. 1. Comparison of the subcellular localization in spinal cord of labeled glycine and leucine in linear, continuous sucrose density gradients (1.5–0.5 M). Homogenates were prepared from rat spinal cords and incubated with two amino acids at a time. A pellet formed from the homogenates was suspended in 0.32 M sucrose, layered onto continuous sucrose density gradients, and centrifuged at 100,000 × *g* for 15 min. Each experiment was repeated 3–6 times.

fraction was diluted to a final concentration of 0.32 M sucrose and centrifuged at 27,000 × *g* for 15 min. The supernatant fluid was discarded, and the remaining pellets were suspended by mechanical agitation in 2 ml of 0.4 N perchloric acid and maintained at 4° for 2 hr. The supernatant fluid obtained from each of these samples was analyzed for K^+ content on a Techtron atomic absorption spectrophotometer.

All labeled compounds were obtained from New England Nuclear Corporation. Specific activities of the ^3H compounds were between 2 and 40 Ci/mmol, while specific activities for the ^{14}C compounds were between 20 and 250 Ci/mol.

RESULTS

In order to differentiate populations of synaptosomal particles storing various putative neurotransmitters, in the present and previous studies (16, 17, 21–24) we labeled various transmitters with different radioactive isotopes and compared their distribution in sucrose density gradients centrifuged by "incomplete equilibrium sedimentation," a procedure that enhances the separation of different populations of synaptosomes (16, 17). By labeling one putative transmitter with tritium and another with carbon, subtle differences in sedimentation properties can be detected reliably.

When tissue preparations from rat spinal cord were centrifuged on sucrose gradients, glycine-accumulating particles sedimented in a less-dense region than did those accumulating leucine (Fig. 1). Similar results were obtained with whole spinal cord and with the lumbosacral section, which contains relatively less myelin than other portions of the cord. Accordingly, it seems that the myelin content of cord preparations did not markedly alter synaptosomal sedimentation. Experiments with reverse labeling of these amino acids and experiments in which spinal cord preparations were incubated with both tritiated and [^{14}C]leucine all ensured that the separations between leucine- and glycine-storing particles were not simply isotopic effects.

During 8 months of experimentation, it became evident that optimal resolution of glycine-accumulating synaptosomes

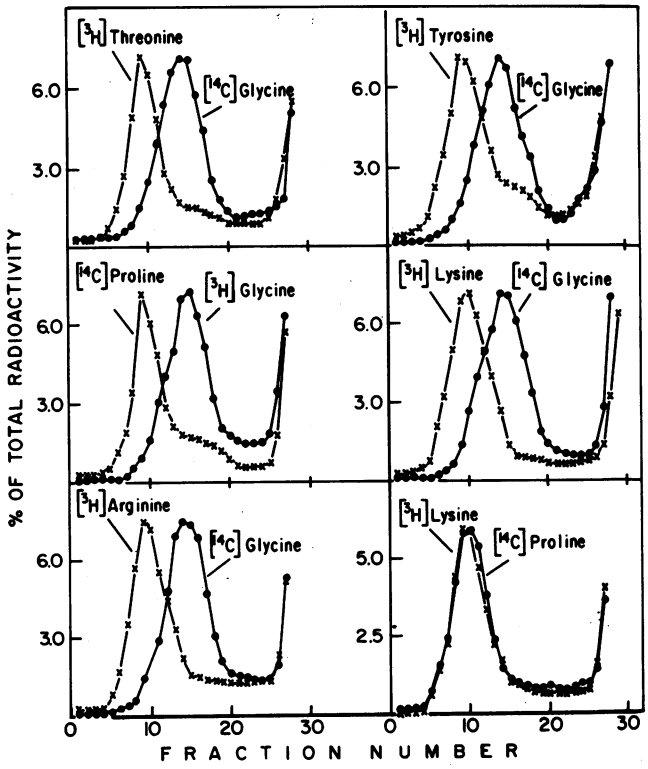

FIG. 2. Comparison of the subcellular localization in spinal cord of representatives of neutral, basic, and aromatic amino acids with glycine in linear, continuous sucrose density gradients. Experimental details are as in Fig. 1.

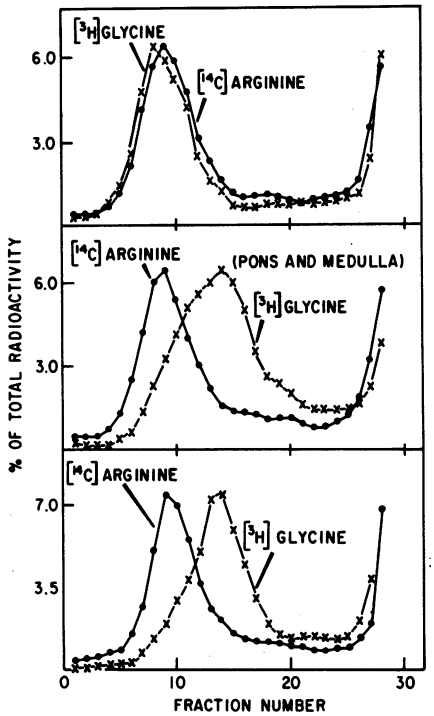

FIG. 3. Comparison of the distribution profiles of [^3H]glycine and [^{14}C]arginine in linear, continuous sucrose density gradients with homogenates from cerebral cortex (*top*), brain stem (*middle*), and spinal cord (*bottom*). Experimental details as in Fig. 1.

from those storing other amino acids depended on certain factors, some of which were indeterminate. Separation of glycine-storing particles was not obtained reproducibly if the clearance between pestle and homogenizer exceeded 0.11 mm. Sometimes, even with the appropriate pestle–homogenizer clearances, optimal separations were not obtained for several weeks, at which time the separations again appeared for no apparent reason. Accordingly, we systematically investigated factors that might contribute to the separation of the various synaptosomal particles. Sedimentation properties of the particles were essentially the same with reagent grade sucrose (Mallinkrodt) as with ultra-pure ribonuclease-free sucrose (Mann). Similar results were obtained when all solutions were prepared with demineralized, glass-distilled H_2O as when demineralized H_2O (Hydroservices Co.) or singly-distilled tap H_2O were used. Addition of 1–5 mM NaCl, KCl, $MgSO_4$, $ZnSO_4$, or $CaCl_2$ to the gradients did not affect the separation of glycine-storing particles from other synaptosomes. At 1 mM, KCl, $MgSO_4$, $ZnSO_4$, and $CaCl_2$ caused all synaptosomal particles to sediment in less-dense portions of the gradient, but did not affect any type of synaptosome differentially. NaCl at 1–5 mM had no effect on synaptosomal sedimentation properties. At concentrations in excess of 5 mM, NaCl caused an apparent clumping of synaptosomal particles, which then failed to sediment under our experimental conditions.

Leucine is a general metabolic amino acid and might be expected to be accumulated to a similar extent by most synaptosomes. We also compared the subcellular localization of glycine in spinal cord with that of other representatives of neutral, basic, and aromatic amino acids (Fig. 2). Radiolabeled threonine, tyrosine, proline, lysine, and arginine showed essentially the same profiles as did leucine, all of which were readily distinguished from that of the accumulated glycine.

A distinctive feature of the synaptic action of glycine is that it mimics the behavior of a natural inhibitory transmitter, presumably of inhibitor neurons activated by certain IA afferents, in the spinal cord. By contrast, cerebral cortical

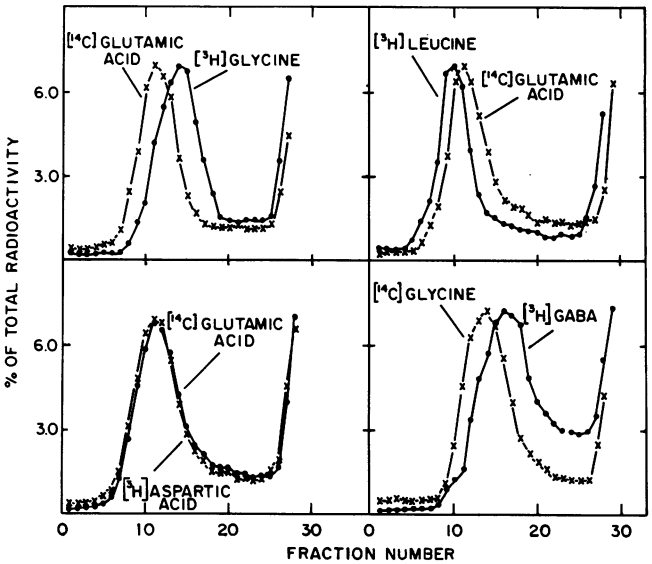

FIG. 4. Comparison of the subcellular distribution in spinal cord of labeled glutamic and aspartic acids, glycine, and γ-aminobutyric acid (GABA). Experimental details as in Fig. 1.

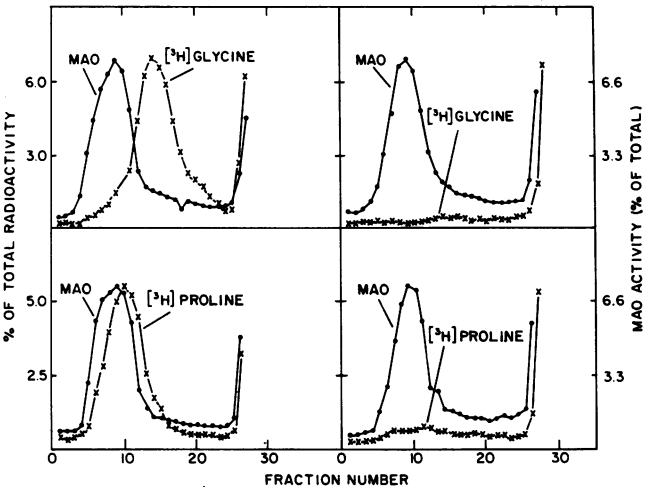

FIG. 5. Comparison of the subcellular distribution in spinal cord of tritiated glycine and proline and monoamine oxidase (MAO) activity in linear, continuous sucrose density gradients. Hypotonic shock (*right, top* and *bottom*) was achieved by resuspending pellets in distilled water instead of 0.32 M sucrose. MAO assay is described in *Methods*. Other experimental details are as in Fig. 1.

units are far less responsive to iontophoresed glycine. Interestingly, the unique high-affinity uptake of glycine by central nervous synaptosomes is demonstrable in spinal cord (13–15) and brain stem (13), but not in rat cerebral cortex (13–15). Accordingly, the subcellular localization of radiolabeled glycine was compared with that of arginine in spinal cord, brain stem, and cerebral cortex (Fig. 3). The profiles of glycine and arginine were separated in the brain stem, just as in spinal cord, but were superimposable in the cerebral cortex.

Previously we reported that radiolabeled glutamic and aspartic acids were accumulated in cerebral cortex into a unique population of synaptosomes that sedimented in a less-dense region of sucrose gradients than did synaptosomes accumulating other amino acids, including glycine (16). In our spinal cord preparations, as observed earlier in cerebral cortex, radiolabeled glutamic acid sedimented in a less-dense region than did leucine (Fig. 4). Profiles for glutamic and aspartic acids were superimposable, a finding that corresponds well with the identical subcellular localization of these two radiolabeled compounds in cerebral cortex (16) and their accumulation by similar, if not identical, high-affinity transport systems in cerebral cortex and spinal cord synaptosomes (14–15).

Although glutamic acid-accumulating particles sedimented in a less-dense portion of the gradient than did those taking up leucine, glycine-accumulating particles were still less dense than those that stored glutamic acid (Fig. 4). γ-Aminobutyric acid-accumulating particles localized in an even less-dense portion of the gradients than did those that stored glycine, a finding reminiscent of the results of Iversen and Johnston (25) using different centrifugation techniques.

Synaptosomal fractions isolated from cerebral cortex contain several subcellular components (26, 28). By contrast with isopycnic centrifugation, in which free mitochondria sediment in a more-dense region of sucrose gradients than do synaptosomes (17, 23, 26, 27), under conditions of incomplete equilibrium sedimentation, free mitochondria contaminate the syn-

aptosomal fraction (16, 17). In our spinal cord preparations the profile for monoamine oxidase activity, a marker for free mitochondria as well as those contained within synaptosomes, was not separable from the profile of [^{3}H]proline (Fig. 5). However, glycine-accumulating particles were well separated from the profile of monoamine oxidase activity. Synaptosomes are thought to be the major osmotically sensitive subcellular constituents of homogenates of mammalian central nervous tissue. Hypotonic shock completely liberated accumulated glycine and proline without altering the profile of monoamine oxidase activity (Fig. 5). In other experiments the distribution of potassium and protein within these gradients was compared with that of accumulated glycine and leucine. Potassium was used as a marker for cytoplasm occluded within synaptosomes, since synaptosomes constitute the major store of occluded cytoplasm within brain homogenates; however, it is possible that small dendrites and glia not ruptured by homogenization would contain some occluded cytoplasm. Potassium and protein profiles resembled that of radiolabeled leucine and were more dense than that of glycine. Taken together, these findings are consistent with a localization of glycine within synaptosomal particles.

In both spinal cord and cerebral cortex, glutamic acid is accumulated by a unique high-affinity uptake system, as well as a low-affinity transport similar to that which exists for other amino acids and presumably subserves general metabolic functions (14, 29, 30). A unique population of synaptosomes could be demonstrated to accumulate low concentrations of radiolabeled glutamic acid (16), which predominantly enter the high-affinity transport. At high concentrations, the subcellular profile of radiolabeled glutamic acid was less well separated from those of other amino acids. The fact that the high-affinity synaptosomal uptake of glycine is demonstrable in spinal cord and brain stem but not in cerebral cortex parallels the regional distribution of the unique glycine-accumulating synaptosomes. This observation suggests that the high-affinity glycine uptake is responsible for transport of glycine into the unique population of synaptosomes. Thus, at high concentrations of glycine, entry is primarily by the low-affinity transport, and the unique subcellular profile of glycine should no longer be apparent. Accordingly, experiments were performed in which homogenates were incubated with 2.0 mM glycine of one radiolabel while other spinal cord homogenates were incubated with 1.0 μM glycine of the other label. At the high concentration, the profile of labeled glycine was in a more-dense region of the gradient, whose peak differed by 4 fractions from that obtained at the low concentration of this amino acid.

DISCUSSION

The major finding of this study is that exogenous glycine is accumulated into a unique population of synaptosomes in spinal cord that can be separated reliably from synaptosomes accumulating other amino acids. The degree of separation of glycine synaptosomes from those accumulating other amino acids was more marked in this study than was the separation of unique glutamic acid-accumulating synaptosomes described previously (16).

Because synaptosomal fractions are often contaminated by other tissue components, the question arises as to whether the glycine-accumulating particles are indeed synaptosomes. Several items of evidence favor a synaptosomal localization for the accumulated glycine. Although free mitochondria can contaminate synaptosomal fractions prepared by incomplete equilibrium sedimentation, in our studies the profile of glycine was readily distinguished from that of monoamine oxidase activity, a marker for mitochondria. The osmotic sensitivity of glycine-accumulating particles is also consistent with the known osmotic sensitivity of synaptosomes. Moreover, recent electron microscopic autoradiographic evidence indicates that low concentrations of radiolabeled glycine accumulated by rat spinal cord tend to be highly localized to nerve terminals (8, 9).

If glycine serves as a transmitter in the spinal cord (31–33), it is probable that only a portion of the endogenous glycine has this property, while a major proportion serves more general metabolic functions. To investigate this question it would be valuable to determine the localization of endogenous glycine on our sucrose gradients. Unfortunately, in preliminary experiments the sensitivity of available assays for endogenous glycine was not sufficient for its localization within our sucrose gradients.

Unique high-affinity uptake systems by synaptosomes for glycine have been described in spinal cord (13–15) and brain stem (13), but not in cerebral cortex (13–15). Similarly, the unique glycine-accumulating synaptosomal fraction can be demonstrated in spinal cord and brain stem but not in cerebral cortex. Our experiments showing that high concentrations of glycine distribute like the general population of synaptosomes suggest that the high-affinity transport of glycine occurs into the glycine-accumulating synaptosomes described here. Recently we found an absolute sodium requirement for the high-affinity glycine uptake system that is not manifested by the low-affinity uptake systems for glycine and other amino acids (34, 35). If high-affinity glycine transport labels the unique glycine synaptosomes, then glycine should not enter these particles in the absence of sodium. When spinal cord homogenates were incubated with radiolabeled glycine in the absence of sodium, the subcellular distribution of accumulated glycine was the same as that of leucine and unlike that of particles that had accumulated glycine in the presence of sodium (34, 35). These experiments indicate that the high-affinity glycine transport is mediated by the unique synaptosomal fraction described in this study.

The fact that glycine mimics the natural inhibitory transmitter in spinal cord but not in cerebral cortex coincides elegantly with the demonstration of a high-affinity uptake into unique synaptosomes for glycine in spinal cord but not in cerebral cortex. Although the neurophysiologic activity of applied glycine suggests a transmitter role, it does not prove that endogenous glycine has any specialized synaptic behavior. The existence of a high-affinity uptake of glycine into unique synaptosomes in spinal cord clearly establishes that this amino acid possesses a specialized synaptic function different from that of other amino acids. It is possible that the high-affinity uptake of glycine into unique nerve terminals may serve as a mechanism for inactivation of this compound's postulated synaptic activities. It may also provide a tool to label these nerve terminals and examine the disposition of the hypothesized transmitter pool of glycine under various physiological and pharmacological conditions.

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