Alteration of Synaptic Membrane Cholesterol/Phospholipid Ratio Using a Lipid Transfer Protein

EFFECT ON γ-AMINOBUTYRIC ACID UPTAKE*

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A procedure was developed to vary the cholesterol-to-phospholipid (Ch/PL) ratio of synaptic plasma membranes and synaptosomes using a nonspecific lipid transfer protein so that membrane lipid composition could be correlated with presynaptic function. In synaptic plasma membranes, Ch/PL molar ratios from 0.21 to 1.19 were produced from a normal value of 0.52 ± 0.01 by incubation with the transfer protein and an excess of either phosphatidylcholine or cholesterol/phosphatidylcholine liposomes for 60 min at 32°C. In synaptosomes, Ch/PL ratios from 0.16 to 0.81 were similarly produced in the normal value of 0.38 ± 0.04. Cholesterol loading or depletion of the membranes was accompanied by a decrease or increase, respectively, in the phospholipid-to-protein ratio. The fluidity of the synaptic plasma membrane, as estimated by 1,6-diphenylhexatriene anisotropy measurements, was increased by lowering the Ch/PL ratio and decreased by raising the Ch/PL ratio.

Decreasing the Ch/PL ratio of synaptosomes and synaptic plasma membrane vesicles resulted in loss of sodium-dependent γ-aminobutyric acid (GABA) uptake (70-100% loss at Ch/PL ratios decreased to 40% of normal) and reduction in the number of accessible GABA-binding sites. Choline uptake was not affected in these same preparations. GABA uptake was restored by reinserting cholesterol into the membrane. Synaptosomal membrane potential and synaptic plasma membrane sodium permeability were not affected by changing the Ch/PL ratio. Increase in the Ch/PL ratio above normal had no effect on either choline or GABA uptake. Both the decrease in the Ch/PL ratio and the increase in the lipid-to-protein ratio increase membrane "fluidity," which may modulate the vertical displacement and motional characteristics of the GABA transporter.

The ratio of Ch/PL in mammalian plasma membranes is a major determinant of membrane viscosity (1). The influence of the Ch/PL ratio (and therefore membrane viscosity) on membrane functions has been studied by manipulation of membrane cholesterol content in specific cells or organelles, either by dietary means (2) or by incubation of membranes in vitro with PC or cholesterol/PC liposomes. The latter method relies on steroid partitioning between liposomes and membranes without significant transfer of phospholipid (3). These techniques have been used to study cholesterol effects on membrane transport (4-6), receptor mobility or signaling (7, 8), and membrane-associated enzymatic activities (9-11). In addition, malignant transformation (12) and spur cell formation in liver disease (13) have been correlated with altered membrane cholesterol content. However, these methods, used in the past for altering membrane cholesterol content, have serious disadvantages. Dietary manipulation (in vivo or in cell culture) is not effective for all cell types and results in fatty acid changes as well (14, 15). The liposome sterol partitioning method has been effective for cells of the circulatory system, but changes in other types of membranes are difficult to accomplish by this method. We have found that several hours of incubation achieve only minor changes (10-20%) in the Ch/PL ratio of synaptic plasma membranes when sticking of liposomes to the membranes is accounted for by using a nonexchangeable marker. Shapiro and Barchi (16) have reported larger changes in the synaptic plasma membrane Ch/PL ratio with much longer (24-h) incubations. However, degradative changes which can occur within this time span are a serious problem. Because of these limitations, we have explored the use of a lipid transfer protein to facilitate cholesterol transfer, and have achieved large changes in membrane lipid composition within a relatively brief time span. This study is concerned with evaluating the role of cholesterol in synaptosomal function using a nonspecific lipid transfer protein to vary membrane Ch/PL ratio over a wide range.

MATERIALS AND METHODS

Lipids

Egg yolk (Type V-E) and dioleoyl (98%, synthetic) 1-α-phosphatidylcholine were obtained from Sigma and cholesterol (+99%) was from Eastman. The phosphatidylcholines and cholesterol were pure as judged by their migration from Eastman. The phosphatidylcholines and cholesterol were pure as judged by their migration from Silica Gel H plates in chloroform-methanol-water (65:25:4, v/v) and n-hexane-diethyl ether-acetic acid (70:30:1, v/v), respectively, [2-14C]glycerol trioleate (triolein), 2 Ci/mmol, from ICN Pharmaceuticals (Irvine, CA) and phenyl-1,3,5-hexatriene; GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KH medium, 130 mM NaCl, 5 mM KCl, 1.5 mM MgCl₂, 1.2 mM Na phosphate, 10 mM glucose, 0.5 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 10 mM HEPES, pH 7.4; KHS medium, KH medium + 0.2 M sucrose; PC, phosphatidylcholine; SH medium, 0.32 M sucrose, 2.5 mM HEPES, pH 7.4; SPM, synaptic plasma membranes; TPP⁺, tetraphenylphosphonium ion.

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3 The abbreviations used are: Ch/PL, cholesterol/phospholipid (molar ratio); CC5, 3,3'-dipentyl 2,2'-oxacarbocyanine; DPH, 1,6-di-
Phosphorus was measured by the method of Chen et al. (25) as described in our laboratory (17-19). Equimolar cholesterol/egg PC liposomes containing [14C]cholesterol and a trace of [3H]triolein were prepared for use in assay of reconstituted enzyme activity. The lipids (1-2 mg of lipid) and proteins were mixed in chloroform and evaporated under a stream of N2. The lipid films were dispersed in 0.25 M sucrose, 50 mM HEPES, 1 mM EDTA, pH 7.4, at 1 mg of PC/ml and sonicated to clarity in a bath sonicator under N2 atmosphere.

For use in the cholesterol depletion or loading experiments, PC (egg or dioleoyl) with or without 1.7-1.9 mol of cholesterol/mol of PC was mixed in chloroform with a trace (2,000-10,000 dpm/pg of P) of [3H]triolein or [14C]cholesterol (and sometimes [3H]-labeled PC). After evaporation of the solvent under a stream of N2, the lipids were further dried under vacuum in a desiccator prefilled with N2, and then dispersed in 0.22 M sucrose, 10 mM HEPES, pH 7.4, at PC concentrations of 5-10 mg/ml for the PC liposomes and 3-5 mg/ml for the cholesterol/PC liposomes. The PC liposomes were sonicated under N2 until translucent in the bath sonicator. Liposomes containing cholesterol were sonicated using a Branson sonicator with a 1/2-inch tumbled horn under a stream of N2. These dispersions were then centrifuged at g0 × 45 min at 20 °C to remove titanium fragments and undispersed lipid.

Other Chemicals and Isotopes

γ-[2,3-3H]Aminobutyric acid (25-43 Ci/mmol), [methyl-14C]choline chloride (49.5 mCi/mmol), [14C]methanol (3.4 mCi/mmol), [carboxyl-14C]cholesterol (0.026 mCi/mg), [14C]starch (0.22 mCi/g), [14C]glycerol (0.01 mCi/g). [14C]triolein (95 mCi/mmol) from New England Nuclear. γ-Aminobutyric acid was from Sigma, and DPH was from ICN K & K Laboratories (Plainview, NY). [3H]HPPD Br and [14C]cholesterol were generous gifts of Dr. R. Ronald Kabanoff (Roche Institute, Nutley, NJ) and Dr. Alan S. Waggoner (Center for Chemical Analysis, Amherst College, Amherst, MA), respectively.

Preparation of Synaptosomes and Synaptic Plasma Membranes

Rat forebrain synaptosomes were prepared from a washed crude mitochondrial pellet essentially by the method of Gray and Whittaker (20) as modified by Hajos (21). In the final step of the procedure, the synaptosomes were centrifuged into a layer of 0.8 M sucrose, 10 mM HEPES, pH 7.4. This layer was collected and diluted over a 1-h period with 3 volumes of KH medium with periodic swirling. The diluted synaptosomes were recovered by centrifugation at 28.5-348 (w/w) sucrose interface and then diluted synaptosomes were recovered by centrifugation at 9900 g, pellets.

Synaptic plasma membranes were prepared from the washed crude mitochondrial pellet in a manner similar to that of Jones and Matus (22). As described by Jones and Matus, the plasma membrane band was collected at the 28.5-348 (w/w) sucrose interface and then diluted 2-fold with cold water. The synaptic plasma membranes were recovered by centrifugation at 87,000 × g0, 120 min, forming a pellet with a white outer rim and a slightly darker center. The outer rim (Type I SPM) and the center (Type II SPM) were separated, with a spatula and suspended in SH medium at 10-30 mg of protein/ml. The purer SPM fraction (Type I) had a Ch/PL molar ratio of 3.52 ± 0.01, a phosphorus-to-protein ratio of 33-37 pg/mg, and a sodium-dependent GABA uptake activity of 25-45 pmol/min/mg of protein at 0.15 M NaCl. This fraction was used for the lipid exchanges unless stated otherwise. The crude SPM fraction (Type II) had somewhat lower Ch/PL and PL/protein ratios and a GABA uptake activity of 10-15 pmol/min/mg. This fraction was also suitable for lipid exchange and gave results qualitatively similar to those obtained with the purer SPM fraction. If not used immediately, the membranes were quick-frozen in liquid nitrogen and stored at -85 °C. Sodium-dependent GABA uptake was stable for at least 1 month of frozen storage.

GABA Uptake and Membrane Lipids

Measurement of GABA Uptake by Synaptic Plasma Membrane Vesicles

Sodium gradient-dependent uptake of [2,3-3H]GABA by synaptic plasma vesicles at 25 °C was studied by the filtration method of Kanner (29). Measurements were performed directly following lipid transfer experiments. A 15-min incubation was used to load the vesicles with potassium phosphate as described by Kanner (see Fig. 4). The loading of the vesicles (normal and cholesterol-altered) with potassium phosphate by this procedure was found, by following passive 42K uptake, to be virtually complete after 10 min. The membranes were collected on Millipore HAMK, 0.45-μ filters. These filters quantitatively trapped liposomes present in the samples, allowing reliable subtraction of their contribution to the total 42K radioactivity. This contribution was less than 10% of the total radioactive tracer on the filter.

Measurement of Synaptosomal Uptake of Choline and GABA

[3H]GABA and [14C]choline uptake by synaptosomes was measured in a single assay mixture by a centrifugation method described by Simon and Kubar (30) for [3H]choline. The incubation medium was KHS containing 1 μM [2,3-3H]GABA and 2 μM [methyl-14C]choline (37 °C). Sample blanks without radioactive GABA and choline were used to subtract the contribution of liposomal 14C and/or [3H]-labeled triolein to the total radioactivity of the synaptosomal pellets.

Measurement of GABA Binding

Specific binding of [2,3-3H]GABA to synaptic plasma membrane vesicles was measured in 0.1 M NaCl at 0-4 °C in the absence of ion gradients across the vesicle membrane. Specific and nonspecific binding was differentiated by observing the difference between binding in the presence and absence of a large excess of unlabeled GABA. The membranes were loaded with 0.1 M NaCl, 1 mm MgCl2, 25 mM HEPES, pH 7.4, by incubation at 37 °C for 15 min in that solution.
and then collected by centrifugation as described previously for the GABA uptake assay. The samples were then incubated at 0–4 °C for 60 min (to binding equilibrium) in the same solution containing 0.15–10.0 μM (2,3-[3H]GABA (2.5–25 Ci/mmol) ±1 mM unlabeled GABA. The equilibrated samples (20 μl in volume) were transferred directly to Amicon 0.22-μm cellulose ester filters and filtered under vacuum. The filters were then washed with two 4-ml portions of cold buffer without isotope, air-dried on filter paper, dissolved in 1 ml of ethylene glycol monooethyl ether, and counted.

Permeability Measurements

Light Scattering—Light-scattering intensity was measured at a wavelength of 350 nm 90° to the incident beam using a Durrum D-110A monochromator fluorescence spectrophotometer. The light-scattering probe protein was albumin, the excitation wavelength was 280 nm, and the emission wavelength was 350 nm, both at 0–4 °C. The increase in fluorescence intensity of the dye in a Perkin-Elmer MPF-44B fluorometer at 30 °C. Excitation and emission were set at 353 and 456 nm, respectively. The increase in fluorescence observed upon addition of the synaptosomes to the dye in 5 mM K+ medium, and ΔF∞ was calculated, where ΔF∞ is the change in fluorescence upon addition of the synaptosomes to the dye in 5 mM K+ medium, and ΔF is the change in fluorescence upon depolarization with 65 mM KCl.

Preparation and Assay of the Lipid Transfer Protein

The nonspecific lipid transfer protein was partially purified from beef liver by the method of Crain and Zilversmit (35), omitting the heat treatment and octylglycerol column chromatography. In the final stage of purification, the lipid transfer activity was eluted from a CM-cellulose column with 25 mM sodium phosphate, 45 mM NaCl, 5 mM β-mercaptoethanol, 0.02% sodium azide, pH 7.4.

The transfer protein was dialyzed against elution buffer with little loss of activity for 1–2 months. Prior to use, an aliquot was concentrated 5–10-fold without appreciable loss of activity in an Amicon ultrafiltration cell with a PM-10 membrane under N2 pressure. When applied to synaptosomes, the transfer protein was dialyzed against elution buffer without azide directly before or after concentration. Finally, the concentrate was centrifuged to sediment any aggregated material.

For estimation of the amount of transfer activity needed in lipid transfer experiments, liposomes containing egg PC, [14C]cholesterol, and [14C]triolein (5.8 μg of phosphorus and 1.1 μg of cholesterol) were added to a mixture of heat-treated mitochondria (69 μg of phosphorus prepared according to Crain and Zilversmit (35)) and 1.0–2.0 unit of transfer protein in 0.25 mM sucrose, 50 mM HEPES, pH 7.4 (32 °C), in a final volume of 1.0 ml. At time intervals during which a linear rate of [14C]cholesterol transfer could be observed, the reactions were interrupted by 5-fold dilution with ice-cold buffer. The samples were immediately centrifuged through a 2-ml layer of 0.65 M sucrose at 100,000 × g for 20 min. The pellet was solubilized in 1 ml of Protosol (New England Nuclear), and then assayed for [14C]cholesterol and [3H]triolein by double isotope liquid scintillation counting. The counts of [3H]triolein transferred to the mitochondria through the exchange were corrected for the presence of co-sedimented liposomes by subtracting the counts of [3H]triolein in the pellet multiplied by the 14C/3H ratio in the starting liposomes. The specific activity of the transfer protein preparation used in these studies, in units of nanomoles of cholesterol transferred to the mitochondria/min at 32 °C, was about 30 units/mg of protein.

Membrane Potential Measurements

The synapticosomal membrane potential was measured using [3H]TPP+ and [14C]methanol (less than 5 ppm) in KHS medium based on the principles described by Rottenberg (33). The internal vesicular volume was also determined in parallel measurements (32) from the relative distributions of [3H]extran and [14C]H2O. The synaptosomes were preincubated in KHS medium containing 2.5 μg/ml oligomycin, 1.0 μg/ml antimycin A, and either 5 or 65 mM KC1 for 37 °C for 30 min before addition of the isotope. After equilibration with the isotope for 10 min at 37 °C, the synaptosomes were collected by centrifugation in a Beckman Microfuge. A sample of the synaptosomes which had previously been lysed in deionized water at 37 °C was also run under similar conditions and was used to determine nonspecific binding of [3H]TPP+ in the absence of a membrane potential. After correcting for nonspecific binding, the ratio of [3H]TPP+ inside and outside the synaptosomes was calculated and inserted into the Nernst equation to yield the membrane potential.

The possibility that the isolated synaptic plasma membrane vesicles have a membrane potential in the presence of the ionic gradients imposed for the GABA uptake assay was investigated by the [3H]TPP+ method. The synaptic plasma membranes were loaded with calcium and then resuspended in 0.1 M potassium phosphate, 1 mM MgCl2, pH 7.0, as described earlier for the uptake measurements. Aliquots were diluted 10-fold in either 0.1 M NaCl, 1 mM MgCl2 or 0.1 M potassium phosphate, 1 mM MgCl2 containing [3H]TPP+ (0.25 μM) and [14C]methanol, or [3H]TPP+ and [14C]H2O. After 2 min, the samples were centrifuged in a Beckman Airfuge at 50,000 × g for 5 min. The supernatants and pellets were separated and used to calculate membrane potential essentially as described for the synaptosomes, except that the vesicles with potassium phosphate both inside and outside were used as blanks for [3H]TPP+ binding in the absence of a membrane potential.

The synapticosomal membrane potential was also measured qualitatively using the permeant fluorescent cation Co2+, by a method based on that of Sims et al. (33) as applied to synaptosomes by Blaaustein and Goldring (34). Synaptosomes in KHS medium were incubated at 37 °C for 10 min, and then added (4.2 μg of total phospholipid) to 2 ml of KHS medium containing 2 μM Co2+, while continuously recording the fluorescence intensity of the dye in a Perkin-Elmer MPP-44B fluorescence spectrophotometer at about 30 °C. Excitation and emission were set at 400 and 550 nm, respectively. After the increase in fluorescence observed upon addition of the synaptosomes, 60 μl of 3 M KCl were added with rapid mixing to depolarize the synaptosomes. Further addition of KCl caused no further increase in fluorescence. As an index of the membrane potential, ΔF∞ = ΔF/ΔF∞ KCl was calculated, where ΔF∞ KCl is the change in fluorescence upon addition of the synaptosomes to the dye in 5 mM K+ medium, and ΔF is the change in fluorescence upon depolarization with 65 mM KCl.

Lipid Transfer Procedures

Synaptic Plasma Membranes—A mixture of synaptic plasma membranes (about 1 mg of protein/ml) and lipid transfer protein (0–46 units/ml) in 0.32 M sucrose was preincubated for 10 min at 32 °C. The PC or cholesterol/PC liposomes, for cholesterol depletion or loading, respectively, were added to start the exchange. For cholesterol depletion, the ratio of [3H]triolein to [14C]cholesterol was about 10. The exchange was terminated by centrifugation of the membranes through a layer of 0.5 M sucrose at 2 °C for 40 min at 100,000 × g. The pellets were suspended in 0.32 M sucrose at 2-4 mg of protein/ml. Sodium gradient-dependent uptake of [3H]GABA and the membrane potential were assayed immediately. Several small aliquots were frozen in liquid nitrogen and stored at −85 °C for subsequent permeability, DPH fluorescence polarization, and GABA-binding measurements. The [14C]triolein content of the pellet was used to correct the cholesterol and phospholipid (and in some cases [3H]PC) content of the pellet for sticking of liposomes. This was done using the PC, cholesterol, and [3H]PC ratios of the starting liposomes. [3H]Trioelain was used in some cases as the nonexchangeable marker in the absence of other labeled lipids.

To reverse induced alterations in the membrane Cl/PC ratio, a solution of 3.0 M KCl was added. The ratio of [3H]GABA uptake was then measured for 2 min. The incubation buffer was immediately centrifuged through a 2-ml layer of 0.65 M sucrose at 100,000 × g for 20 min. The pellet was solubilized in 1 ml of Protosol (New England Nuclear), and then assayed for [14C]cholesterol and [3H]triolein by double isotope liquid scintillation counting. The counts of [3H]triolein transferred to the mitochondria through the exchange were corrected for the presence of co-sedimented liposomes by subtracting the counts of [3H]triolein in the pellet multiplied by the 14C/3H ratio in the starting liposomes. The specific activity of the transfer protein preparation used in these studies, in units of nanomoles of cholesterol transferred to the mitochondrion/min at 32 °C, was about 30 units/mg of protein.
Cholesterol depletion and loading of synaptic plasma membranes

Synaptic plasma membranes (63 μg of P, 0.4 mg of cholesterol, 1.9 mg of protein) were incubated with or without egg PC liposomes (267 μg of P) or cholesterol/egg PC liposomes (3.41 mg of cholesterol, 146 μg of P) in the presence of varying amounts of transfer protein for 60 min at 32 °C as described under "Materials and Methods" (the final volume was 1.75 ml). The membranes were separated from the liposomes and transfer protein as described under "Materials and Methods." Correction was made for liposome sticking using a nonexchangeable marker, [14C]triolein. The total sample phosphorus referable to sticking of liposomes was 18-25% in the case of cholesterol depletion and 10-15% in the case of cholesterol loading.

The lipid transfer protein was found to be an effective means of varying the Ch/PL ratio in synaptic plasma membranes and synaptosomes. In synaptic plasma membranes, the Ch/PL ratio could be varied from 0.81 to 0.21-1.19 by incubation with cholesterol/egg PC liposomes (molar ratio = 1.9) contained 214 dpm of [3H]PC and 134 dpm of [14C]triolein/nmol of PC. The cholesterol/egg PC liposomes (267 μg of P) or cholesterol/egg PC liposomes (3.41 mg of cholesterol, 146 μg of P) in the presence of transfer protein for 60 min at 32 °C as described under "Materials and Methods." Correction was made for liposome sticking using a nonexchangeable marker, [14C]triolein. The total sample phosphorus referable to sticking of liposomes was 18-25% in the case of cholesterol depletion and 10-15% in the case of cholesterol loading.

RESULTS

The lipid transfer protein was found to be an effective means of varying the Ch/PL ratio in synaptic plasma membranes and synaptosomes. In synaptic plasma membranes, Ch/PL molar ratios were varied over a range of 0.21-1.19 from a normal value of 0.52 ± 0.01 by incubation with transfer protein in the presence of an excess of either egg PC or cholesterol/egg PC liposomes for 60 min at 32 °C (Table I). In synaptosomes, the Ch/PL ratio could be varied from 0.16 to 0.81 compared with a normal value of 0.38 ± 0.04. In the latter case, changes in the Ch/PL ratio of the synaptosomal plasma membrane were similar to the changes observed in the whole synaptosome (Table II). The degree of cholesterol loading or depletion was controlled by varying the amount of transfer activity present and was minimal (cf. Table I) in the absence of transfer protein.

Induced changes in cholesterol and phospholipid content of

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**Table I**

| Type of liposome used* | Transfer units | Cholesterol/ protein | P/protein | Ch/PL | PL originating from liposomes | Δ cholesterol† | Δ PL† |
|-----------------------|----------------|----------------------|-----------|-------|-------------------------------|----------------|-------|
| None                  | 26.1           | 214                  | 33.0      | 0.52  | Undetectable                  | −34            | −71   |
| Egg PC                | 0              | 200                  | 31.1      | 0.52  | 1.7                           | +10            | +16   |
| Egg PC                | 1.9            | 175                  | 35.7      | 0.39  | 17.8                         | −98            | +77   |
| Egg PC                | 3.7            | 166                  | 37.1      | 0.36  | 24.0                         | −121           | +123  |
| Egg PC                | 11.2           | 146                  | 37.4      | 0.31  | 36.0                         | −173           | +132  |
| Egg PC                | 26.1           | 143                  | 49.2      | 0.23  | 46.3                         | −181           | +513  |
| Egg PC                | 80.7           | 149                  | 58.0      | 0.21  | 54.6                         | −165           | +797  |
| Ch/egg PC             | 0              | 217                  | 33.6      | 0.52  | 1.7                           | +10            | +10   |
| Ch/egg PC             | 0.8            | 244                  | 31.3      | 0.62  | 0.1                           | +60            | +60   |
| Ch/egg PC             | 1.9            | 259                  | 29.3      | 0.71  | 3.5                           | +119           | +293  |
| Ch/egg PC             | 3.7            | 250                  | 27.0      | 0.74  | 3.1                           | +96            | +203  |
| Ch/egg PC             | 11.2           | 318                  | 25.7      | 0.99  | 10.0                         | +271           | −245  |
| Ch/egg PC             | 26.1           | 352                  | 25.5      | 1.11  | 14.5                         | +359           | −252  |
| Ch/egg PC             | 80.7           | 371                  | 24.9      | 1.19  | 19.3                         | +408           | −271  |

* The egg PC liposomes contained 187.5 dpm of [3H]PC (1-palmitoyl-2-oleoyl) and 218 dpm of [14C]triolein/nmol of PC. The cholesterol/egg PC liposomes (molar ratio = 1.9) contained 214 dpm of [3H]PC and 134 dpm of [14C]triolein/nmol of PC.

† Nanomoles of 3H-labeled PC/nmol of total membrane phospholipid.

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**Table II**

| Membranes       | Type of liposomes | Time of incubation | Ch/PL mol/mol |
|-----------------|-------------------|--------------------|---------------|
| Synaptosomes    | None              | 3 h                | 0.40          |
| Plasma membranes|                   |                    | 0.51          |
| Synaptosomes    | Egg PC            | 45 min             | 0.23          |
| Plasma membranes|                   |                    | 0.25          |
| Synaptosomes    |                   | 3 h                | 0.12          |
| Plasma membranes|                   |                    | 0.11          |
| Synaptosomes    |                   | 45 min             | 0.68          |
| Plasma membranes|                   |                    | 0.67          |
| Synaptosomes    |                   | 3 h                | 0.98          |
| Plasma membranes|                   |                    | 1.10          |
GABA Uptake and Membrane Lipids

Synaptosomal membranes could be reversed by a second incubation with fresh liposomes and transfer protein (Fig. 1). When \(^{14}\text{C}\)cholesterol which had been incorporated into synaptic plasma membranes by a previous incubation was transferred back out into egg PC liposomes, the specific activity of \(^{14}\text{C}\)cholesterol in the membrane did not change. Therefore, the previously introduced cholesterol had equilibrated with the bulk pool of membrane cholesterol (Table III).

Cholesterol loading or depletion of synaptosomes and synaptic plasma membranes was accompanied by a decrease or increase, respectively, in the phospholipid-to-protein ratio.

### Table III

\(^{14}\text{C}\)Cholesterol loaded into synaptic plasma membranes equilibrates with endogenous membrane cholesterol

| Sample | Cholesterol/protein (\(\mu\)g/mg) | \(^{14}\text{C}\)Cholesterol/cholesterol (dpm/\(\mu\)g cholesterol) | Ch/PL |
|--------|----------------|----------------|------|
| 1. Untreated SPM | 214 | 0 | 0.53 |
| 2. \(^{14}\text{C}\)Cholesterol-loaded SPM | 478 | 403 | 1.29 |
| 3. Unloaded SPM (sample 2 was re-exchanged to lower the Ch/PL ratio.) | 288 | 410 | \(a\) |

*The Ch/PL ratio of the “unloaded” membranes could not be corrected for liposome sticking (since these membranes were incubated with unlabeled liposomes), and so was not reported.*

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**Fig. 1.** Reversal or enhancement of cholesterol depletion and loading by a second incubation. Synaptosomes (0.75 mg of protein/ml) were incubated with dioleoyl PC liposomes (237 \(\mu\)g of P) (\(\square\)) or cholesterol-dioleoyl PC liposomes (215 \(\mu\)g of cholesterol; 9.6 \(\mu\)g of P) (\(\bullet\)) and 25 units/ml of transfer protein at 32 °C for 9 h. During the incubation, aliquots were taken for lipid analysis. Liposome sticking, which increased the phosphorus content of the samples by 12-20%, was corrected for using \(^{3}\text{H}\)triolein. After 2 h of loading or depletion, aliquots were centrifuged as described under “Materials and Methods” and incubated a second time with transfer protein and either the same type of liposomes as during the first incubation (\(\square\) and \(\bigcirc\)) or with the other type of liposomes (\(\triangle\) and \(\bigtriangleup\)).

**Fig. 2.** Freeze-fracture electron micrographs of normal, cholesterol-depleted, and cholesterol-loaded synaptic plasma membranes. A, control membranes (Ch/PL ratio = 0.51); B, cholesterol-depleted membranes (Ch/PL = 0.21); C, cholesterol-loaded membranes (Ch/PL = 1.12). The synaptic plasma membranes (1.75 mg of protein, 63 \(\mu\)g of P, 400 \(\mu\)g of cholesterol) were incubated in the presence of approximately 40 units of transfer protein and either egg PC liposomes (270 \(\mu\)g of P) or cholesterol/egg PC liposomes (3.41 mg of cholesterol, 146 \(\mu\)g of P) for 60 min at 32 °C in order to alter the Ch/PL ratio. The membranes were re-isolated after the lipid transfer for lipid analysis and freeze-fracture electron microscopy. Control membranes were incubated under similar conditions except that transfer protein and liposomes were omitted. The membranes were fixed overnight in 2.5% glutaraldehyde, 0.32 M sucrose (buffered at pH 7.4) at 0–4 °C before preparation for freeze-fracture as described by Saito et al. (36).
for each component, whose \( r_c \) values could be determined separately, the observed anisotropy, \( r \), is given by the dotted line.

The normal Ch/PL ratio (for this preparation of membranes) is marked of phospholipid plus cholesterol associated with that component. The corrections in anisotropy due to the presence of liposomes were empirically determined to be equivalent to the fractional weight corrections in anisotropy due to the presence of liposomes. Correction was made on the spectrophotometrically determined \( \Delta \lambda \) and \( \Delta \lambda \) values, respectively.

The reported anisotropies of DPH in the synaptic plasma membranes are more than 10% for cholesterol depletion and 2% for cholesterol loading. The corrections in anisotropy due to liposome contamination were less than 10% for cholesterol depletion and 2% for cholesterol loading. The corrected anisotropy of DPH in propylene glycol at -50°C, 0.362 (28). The standard deviations are for two separate determinations on the same samples. When no standard deviation is given, only one measurement was performed.

Fig. 3. DPH fluorescence anisotropy in synaptic plasma membranes at 4, 25, and 37°C as a function of the Ch/PL ratio. Synaptic plasma membranes (223 µg of cholesterol and 35 µg of P/ ml) were incubated in the presence of varying amounts of transfer protein (2-45 units/ml) with egg PC liposomes (150 µg of P/ml) or cholesterol/egg PC liposomes (2105 µg of cholesterol and 91 µg of P/ml) for 90 min at 32°C in order to alter the Ch/PL ratio. The membranes were re-isolated after the lipid transfer for lipid analysis and DPH anisotropy measurements as described under “Materials and Methods.” Liposome sticking, which increased the phosphorus content of the samples by 8 to 23%, was corrected for using \(^{14} \text{C} \) dextran and \(^{3} \text{H} \) water as described under “Materials and Methods.” The internal volumes of normal and lipid-altered synaptosomes and synaptic plasma membranes are shown in Figs. 3 and 8.

TABLE IV
Internal volumes of normal and lipid-altered synaptosomes and synaptic plasma membrane vesicles

| Membrane       | Treatment          | Ch/PL mol/mol | Membrane PL/mg protein mol/mol | Membrane cholesterol/mg protein mol/mol | Internal volume µl/mg protein |
|----------------|--------------------|---------------|-------------------------------|-----------------------------------------|-------------------------------|
| SPM            | No transfer protein or liposomes | 0.53          | 0                             | 0                                        | 6.5 ± 0.5                     |
|                 | Transfer protein only | 0.52          | +22                           | 0                                        | 6.9                          |
|                 | Transfer protein + egg PC liposomes | 0.19          | +1016                         | -202                                     | 11.4                         |
|                 | Transfer protein + cholesterol/PC liposomes | 0.90          | -78                           | +385                                     | 9.0                          |
| Synaptosomes   | No transfer protein or liposomes | 0.43          | 0                             | 0                                        | 4.1 ± 0.8                     |
|                 | Transfer protein only | 0.44          | -7                            | +1.9                                     | 4.2 ± 0.6                    |
|                 | Transfer protein + egg PC liposomes | 0.15          | +507                          | -42                                      | 5.6 ± 0.6                    |

* Synaptic plasma membranes and synaptosomes were incubated with transfer protein and liposomes as described in Figs. 3 and 8.

** Nanomoles of cholesterol and phospholipid gained or lost/mg of protein during the lipid exchange, compared to the membranes incubated without the transfer protein or liposomes.

The internal volumes were measured using \(^{14} \text{C} \) dextran and \(^{3} \text{H} \) water as described under “Materials and Methods.” The standard deviations are for two separate determinations on the same samples. When no standard deviation is given, only one measurement was performed.

Fig. 4. Sodium gradient-dependent GABA uptake by synaptic plasma membrane vesicles. Uptake of [2,3-\(^{3} \text{H} \)]GABA (0.15 µM) by synaptic plasma membrane vesicles (0.19 mg of protein/ml) was measured as described under “Materials and Methods” under three sets of conditions: at 25°C with 0.1 M NaCl, 1 mM MgCl\(_2\) outside and 0.1 M potassium phosphate, 1 mM MgCl\(_2\) inside (O); at 25°C with 0.1 M NaCl, 1 mM MgCl\(_2\) inside as well as outside (O); and at 25°C with 0.1 M KCl, 1 mM MgCl\(_2\) outside and 0.1 M NaCl, 1 mM MgCl\(_2\) inside (X). Based on the observed uptake capacity of approximately 85 pmol of GABA/mg of protein, an intravesicular volume of 6.7 µl/mg of protein (Table IV), this represents, at an external GABA concentration of 0.15 µM, a GABA concentration gradient of 85-fold across the vesicle membranes.

The membranes could be accounted for largely by the increase in PL/protein ratio (not shown). Therefore, exchange of membrane and liposomal phospholipid was not a major factor. Phospholipid analysis of the cholesterol-depleted membranes indicated little decrease in phospholipids other than PC (less than 17% on a per milligram of protein basis), although due to PC transfer from the liposomes, the membrane PC content increased from a normal value of 40% to as much as 76% of the total phospholipid (not shown).

Freeze-fracture electron micrographs of control and cholesterol-altered synaptic plasma membranes showed vesicles of similar morphology (Fig. 2). The internal volumes of synaptic plasma membrane vesicles and synaptosomes increased with both cholesterol loading and cholesterol depletion (up to 35
and 70%, respectively), in accord with a net transfer of lipid from the liposomes to the membranes in both cases (Table IV).

The fluorescence anisotropy of DPH was measured in synaptic plasma membranes and found to increase with increase in the Ch/PL ratio (Fig. 3). An increase in DPH anisotropy indicates a decrease in membrane fluidity. Decreasing temperature (between 4 and 37 °C), like increasing Ch/PL ratio, decreased the fluidity of synaptic plasma membranes.

Sodium gradient-dependent uptake of [3H]GABA by synaptic plasma membrane vesicles became saturated at 45-90 pmol of GABA/mg of protein, depending on the membrane preparation, at 25 °C and an external GABA concentration of 0.15 μM (Fig. 4). Uptake was dependent on the presence of a downhill sodium concentration gradient. The measured GABA uptake capacity was therefore limited in part by the rate at which the imposed sodium gradient dissipated. The uptake rate was temperature dependent, at 4 °C being 5% of that at 25 °C. Kanner (29) has reported a Km of 2.5 μM for GABA uptake in a similar preparation of synaptic plasma membranes. This value agrees with that reported for intact synaptosomes (36) and is approximately the same as the dissociation constant for GABA binding (in 0.1 M NaCl) presented later (Fig. 9).

The correlation of GABA uptake with the Ch/PL ratio in synaptic plasma membranes of modified lipid composition is shown in Fig. 5. The uptake rate and capacity were progressively lowered by depletion of membrane cholesterol. This pattern was consistently observed, with a 70-100% loss of uptake rate at Ch/PL ratios near 0.2. GABA uptake by synaptic plasma membranes depleted of cholesterol could be restored to 80% of the control rate by reloading the vesicles with cholesterol (Fig. 6). Incubation with transfer protein or liposomes alone did not affect GABA uptake, nor was any change seen in the membranes excessively loaded with cholesterol (Fig. 5).

Another type of reversal experiment (Fig. 7) was conducted in order to separate the effects of changes in the lipid/protein ratio from the effects of changes in the Ch/PL ratio on GABA uptake by synaptic plasma membrane vesicles. This separation was achieved by determining whether an increasing lipid/protein ratio correlated with loss of uptake even at Ch/PL ratios above normal. Synaptic plasma membrane vesicles previously loaded with cholesterol were depleted of cholesterol by a second incubation with fresh transfer protein and egg PC liposomes. A parallel sample, incubated the first time with transfer protein only, was similarly depleted of cholesterol. For both, increases in the lipid-to-protein ratio correlated with

![Fig. 5. Sodium gradient-dependent GABA uptake by synaptic plasma membranes as a function of Ch/PL ratio. Synaptic plasma membranes were altered in their Ch/PL ratio by incubation with transfer protein and egg PC (○) or cholesterol/egg PC (●) liposomes as described in Fig. 3. Control membranes were incubated with transfer protein only (□) or with neither transfer protein nor liposomes (△). GABA uptake was measured at 25 °C in 0.15 μM [3H]GABA, 0.16 mg of membrane protein/ml in the presence of a sodium concentration gradient (0.1 M K phosphate, 1 mM MgCl2 inside and 0.1 M NaCl, 1 mM MgCl2 outside) immediately following re-isolation of the membranes after lipid transfer. The uptake rate was approximately linear at 1 min and was saturated at 5 min. The phosphorus/protein ratios (micrograms/mg) for the 12 samples shown, reading from lowest to highest Ch/PL ratio, were 68.6, 67.2, 60.1, 43.4, 40.3, 36.7 (△), 36.0 (△), 35.6, 36.4, 32.8, 34.4, and 32.2. The horizontal and vertical lines denote the Ch/PL ratio and GABA uptake rate and capacity in the control synaptic plasma membranes.

![Fig. 6. Reversal of the changes in cholesterol-depleted synaptic plasma membranes by reinsertion of cholesterol. Synaptic plasma membrane vesicles (Type II), 1.0 mg of protein/ml, were submitted to two consecutive incubations. The first accomplished cholesterol depletion, and the second one reloading, each with appropriate controls. The samples shown were incubated as follows: 1, both incubations with transfer protein but no liposomes; 2, incubation first with egg PC liposomes (156 μg of P/ml) and transfer protein, then with transfer protein only; 3, 4, and 5 were portions of 2 taken after the first incubation, and then incubated with cholesterol/egg PC liposomes (4.18 mg of cholesterol and 181 μg of P/ml) only (3) or cholesterol/egg PC liposomes and transfer protein (4 and 5). The transfer protein concentration in each incubation was 9 units/ml. Both incubations were at 22 °C, the first for 90 min and the second for 75 min. Liposome sticking increased the phosphorus content of the samples by less than 22% and was corrected for using [3H]triolein. Sodium gradient-dependent GABA uptake and DPH fluorescence anisotropy measurements were conducted at 25 °C as described under "Materials and Methods." The numbers in parentheses in A are the phosphorus/protein ratios (μg of P/mg of protein) for each sample.
Fig. 7. Loss of sodium gradient-dependent GABA uptake with cholesterol depletion as a function of the lipid/protein ratio. Type II synaptic plasma membranes (43.4 μg of P, 0.24 mg of cholesterol, and 1.6 mg of protein/ml) were incubated with transfer protein (12 units/ml) ± cholesterol/egg PC liposomes (2.35 mg of cholesterol and 10 μg of P/ml) for 70 min at 32 °C as described under "Materials and Methods." The control membranes (open symbols), incubated first with transfer protein only, and the cholesterol-loaded membranes (solid symbols) were then reincubated for 40 min at 1.5 mg of membrane protein/ml with either transfer protein (12 units/ml) only (○, ●) or with egg PC liposomes (165 μg of P/ml) and 0.5 (□, △), 3 (△, △), or 12 (□, ●) units of transfer protein/ml. Liposome sticking increased the phosphorus content of the samples by 15-20% and was corrected for using \[^{1}H\]triolein and \[^{14}C\]triolein (see "Materials and Methods"). Sodium gradient-dependent GABA uptake was measured for each sample after the second incubation at 25 °C in 0.15 μM \[^{2,3}H\]GABA as described under "Materials and Methods." The lipid/protein ratio is the total weight of membrane phospholipid and cholesterol/mg of membrane protein, after correcting for liposome sticking using \(^{1}H\) and \(^{14}C\)-labeled triolein. The numbers in parentheses are the Ch/PL ratios (mole/mole) for each point.

Fig. 8. Comparison of GABA and choline uptake in cholesterol-depleted synaptosomes. Synaptosomes (1 mg of protein/ml) were incubated at 32 °C for 60 min as described under "Materials and Methods" with (A) no transfer protein or liposomes, (B) transfer protein only (40 units/ml), (C) egg PC liposomes (101 μg of P/ml) and 20 units/ml of transfer protein, or (D) egg PC liposomes (101 μg of P/ml) and 40 units/ml of transfer protein. Liposome sticking was corrected for using \[^{1}H\]triolein. Less than 16% of the sample phosphorus was referable to stuck liposomes in all cases. Uptake of \[^{1}C\] choline (2 μM) and \[^{1}H\]GABA (1 μM) was measured at 37 °C following re-isolation of the synaptosomes after the lipid transfer as described under "Materials and Methods." The rates were measured while uptake was linear (at 2 min). The error bars indicate the standard error of two determinations.

The effect of alteration in the Ch/PL ratio on function was also studied in intact synaptosomes. Whereas the isolated synaptic plasma membrane vesicles are more readily studied in terms of membrane permeability and fluidity (DPH anisotropy), the synaptosomes retain more functions which may be studied and correlated. For instance, they have a large, measurable membrane potential which is dependent on energization and is therefore a good indicator of viability. Synaptosomal GABA uptake decreased with cholesterol depletion (Fig. 8), but was not affected by the transfer protein alone or by cholesterol loading (the latter not shown). Hence, synaptosomes and synaptic plasma membranes responded similarly with regard to GABA uptake upon alteration of the membrane Ch/PL ratio. Uptake of choline by the synaptosomes was not significantly affected by cholesterol depletion (Fig. 8) or loading (not shown). Choline uptake is apparently inoperative in isolated synaptic plasma membranes and therefore could not be studied in that system.

The loss of GABA uptake observed upon lowering of the membrane Ch/PL ratio was not due to a more rapid dissipation of the required sodium concentration gradient. Light-scattering measurements of passive NaCl permeability in control and lipid-modified synaptic plasma membranes showed that the half-times of the fast and slow components of the Na⁺ ion movement, as well as their relative proportions, did not change significantly with cholesterol depletion (Table V). \(^{22}Na\) permeability also was unchanged in cholesterol-depleted synaptic plasma membrane vesicles assayed with equal internal and external Na⁺ concentrations, confirming that the loss of sodium gradient-dependent GABA uptake was not due to a change in membrane permeability to sodium.

The transmembrane potential of the synaptosomal membrane, as measured by \[^{1}H\]TPP⁺ in the presence of oligomycin and antimycin A, was not significantly affected by lowering the Ch/PL ratio to 0.15 (Table VI). Increasing the external potassium concentration from 5 to 65 mM depolarized the synaptosomes, as did 0.2 mM veratridine (not shown), confirming the measured potential as referable to the synaptosomal plasma membrane. The membrane potential of normal and lipid-altered synaptosomes was also estanated using Cö fluorescent. There was no change in membrane potential, as measured by this method, in cholesterol-depleted (Table VI) or cholesterol-loaded (not shown) synaptosomes. Furthermore, no transmembrane potential was detectable with \[^{1}H\] TPP⁺ in isolated synaptic plasma membrane vesicles, regardless of previous exposure to transfer protein or liposomes. This was true even within 2 min of imposition of ionic gradients for GABA uptake measurements. GABA uptake was diminishing although still active in this time frame (cf. Fig. 4).

Specific binding of \[^{1}H\]GABA to synaptic plasma membranes of lowered Ch/PL ratio was decreased relative to controls and cholesterol-loaded membranes, paralleling the loss of GABA uptake. The binding was studied under conditions minimizing GABA uptake: 9-4 °C in 0.1 M NaCl, but in the absence of a sodium gradient across the vesicle membrane. Specific binding of \[^{1}H\]GABA was 1.71 ± 0.01 pmol of GABA/mg of protein at 0.15 μM GABA in normal synaptic plasma membranes (Ch/PL ratio = 0.51) which had gone through a
GABA Uptake and Membrane Lipids

TABLE V
Sodium permeability and GABA uptake rate of control and cholesterol-depleted synaptic plasma membrane vesicles

| Treatment                        | Ch/PL | P/protein | GABA uptake | Light scattering | Rate constants | "Na permeability " |
|----------------------------------|-------|-----------|-------------|-----------------|----------------|-------------------|
|                                  | mol/mol| mg/mg | pmol/min/mg |                 | min | min^-1 | min |
| No transfer protein or liposomes | 0.53  | 36.7   | 28          | 5.8 ± 0.7       | 0.51 ± 0.07  | 0.97 ± 0.01 | 2.6 |
| Transfer protein only            | 0.52  | 36.7   | 30          | 5.6 ± 0.7       | 0.53 ± 0.10  | 0.06 ± 0.01 | 2.0 |
| Transfer protein + egg PC liposomes | 0.19  | 67.2   | 9           | 8.0 ± 1.8       | 0.68 ± 0.11  | 0.07 ± 0.02 | 3.2 |

* Uptake was measured at 25 °C after the lipid exchange as described in Fig. 5 (in the presence of a downhill Na+ gradient).

" The synaptic plasma membranes, 0.05-0.10 mg of protein/ml in SH medium, were diluted with an equal volume of 0.3 M NaCl in SH medium. The decay in light scattering upon reswelling (due to salt entry) was fitted to an equation which is the sum of two exponential decays (fast and slow) as described under "Materials and Methods." The half-time for the overall decay was read from the fitted decay curve. The numbers in parentheses under the rate constants are the percentages by which the fast and slow components are weighted in determining the overall decay. For a decay of the form Y = A.exp(-kfast t) + B.exp(-kslow t), the percentage weight of the fast component is A/(A + B) × 100. The expressed errors are the standard deviations of duplicate or triplicate measurements made on each sample.

" Escape of 125Na from preloaded membrane vesicles was measured at 25 °C as described under "Materials and Methods."

TABLE VI
Membrane potential of control and cholesterol-depleted synaptic synaptosomes

| Synaptosomes incubated with or without egg PC liposomes and either 20 or 40 units/ml of transfer protein as described under "Materials and Methods" and in Fig. 8. The synaptic plasma membrane potential was measured directly following the exchange, 30-60 min after lipid transfer, before measurement of binding. Specific GABA binding in the presence of 0.1 M NaCl, 1 mM MgCl2, pH 7.4, was measured at 0-4 °C in the absence of ionic gradients (see "Materials and Methods"). Sample blanks, without [3H]GABA, were used to subtract nonspecific [3H]GABA trapping on the filter, respectively. These estimates apply to measurements of 0.15 pmol [3H]GABA concentration for the cholesterol-depleted membranes. The Ch/PL molar ratios of the control and cholesterol-depleted membranes were 0.51 and 0.18, and their respective GABA uptake rates (see Fig. 51 were 29.9 and 5.0 pmol/min/mg. The total heights of the vertical bars are twice the standard error for three separate binding measurements at each GABA concentration. The lines were drawn by linear regression without statistical weighting. Bmax (binding capacity) and KD were determined from the y- and x-intercepts, respectively. For the control, Bmax and KD were 2.56 ± 0.5 pmol and 31 ± 5.6 pmol of GABA/mg of protein. The cholesterol-depleted membranes had a KD of 2.00 ± 0.6 pmol/mg and a Bmax of 16 ± 4.2 pmol of GABA/mg of protein.

control incubation without transfer protein or liposomes. Untreated membranes bound 1.68 pmol/mg of protein. The cholesterol-loaded membranes (Ch/PL ratio = 0.97) and the cholesterol-depleted membranes (Ch/PL ratio = 0.18) bound 1.72 ± 0.13 and 1.14 ± 0.20 pmol of GABA/mg of protein, respectively. In a single experiment, depletion of the synaptic plasma membrane Ch/PL ratio to 45% of normal followed by

repletion to 73% was accompanied by a decrease in the number of binding sites (at 0.15 pmol GABA) to 46% of control and recovery to 86% of control. Binding to control and cholesterol-depleted membranes over a range of GABA concentrations...
lipid/protein ratio. However, it is clear from Fig. 7 that, at a 1:1 ratio, cholesterol can be correlated directly with loss and recovery of membrane function. In contrast, synaptosomal uptake of GABA was not affected by alteration of the membrane Ch/PL ratio. GABA uptake could be restored by reinserting cholesterol into the membrane, suggesting that the loss of function was referable at least in part to the removal of cholesterol.

In isolated synaptic plasma membranes, sodium gradient-dependent uptake of GABA has been shown to be enhanced by agents which would be expected to create or increase a negative interior membrane potential, such as valinomycin in the presence of higher intravesicular potassium (29). In synaptic states, a membrane potential may also influence sodium-dependent GABA uptake (40). The relative influence of the sodium concentration gradient and transmembrane potential on GABA uptake has not been determined. The membrane potential of the intact synaptosomes was not affected by protein-mediated cholesterol depletion (Table VI), and GABA uptake was retained in synaptic plasma membrane vesicles in the apparent absence of a transmembrane potential. Hence, the membrane potential is not sensitive to changes in membrane cholesterol content, nor does it seem to be required for GABA transport. Our data do not rule out the possibility that the presence of a negative electrical potential might stimulate GABA uptake. Despite the apparent absence of a membrane potential, the synaptic plasma membrane vesicles were able to generate a GABA concentration gradient of about 80-fold, based on an internal volume of 6.7 μl/mg of protein and a capacity of 80 pmol of GABA/mg of protein (cf. Fig. 4).

There is a complication in the correlation of loss of GABA uptake with decrease in membrane cholesterol content that the decrease in the Ch/PL ratio is attended by an increase in the lipid/protein ratio during protein-mediated lipid transfer experiments. We have been able to sort out the effects referable to changes in Ch/PL and lipid/protein ratios on GABA uptake (Fig. 7). A decrease in GABA uptake correlates with both a decrease in the Ch/PL ratio and an increase in the lipid/protein ratio. However, it is clear from Figs. 7 and 8 that, at any given lipid/protein ratio, GABA uptake is inhibited with a decrease in cholesterol content. Removal and reinsertion of cholesterol can be correlated directly with loss and recovery of GABA uptake (Fig. 6): GABA uptake by cholesterol-depleted synaptic plasma membranes was restored to 80% of the control by restoring the original Ch/PL ratio, even though very little of the phospholipid incorporated into the membrane during cholesterol depletion was removed during reinsertion of cholesterol.

Although the influence of the lipid/protein ratio on GABA uptake is evident, a specific effect on GABA transport by egg PC, introduced into the membrane during cholesterol depletion, appears unlikely for several reasons. First, the fatty acid composition of the egg PC incorporated into the membrane was similar to that of the synaptic plasma membrane PC: both are relatively high in palmitic acid and low in long chain polyunsaturated fatty acids (41, 42). By contrast, the fatty acid compositions of phosphatidylserine and phosphatidylethanolamine in the synaptosomal plasma membrane are much higher in long chain polyunsaturated fatty acids. Second, the loss of GABA uptake could be reversed by treatment with transfer protein and cholesterol/egg PC liposomes, a process which itself results in transfer of liposomal egg PC to the membrane (cf. Table I). Third, in other studies, we found that exchange of up to 50% of the synaptosomal PC pool with several classes of synthetic PCs, including dimyristoyl PC, dioleoyl PC, and dielaidoyl PC, resulted in no change in synaptic uptake of GABA. These latter experiments made use of a specific PC exchange protein from beef liver. Loss of GABA uptake also does not appear to be referable to loss of specific membrane phospholipids since little of the original membrane phospholipid was lost during cholesterol depletion experiments. The major lipid alterations were exchange of liposomal egg PC with membrane cholesterol and addition of liposomal PC to the membranes without back exchange.

The decrease in the Ch/PL ratio and increase in the lipid/protein ratio which occur concomitantly during transfer protein-mediated cholesterol depletion can both be correlated with increased membrane fluidity. Rigidification of phospholipids in their liquid-crystalline state by the presence of cholesterol has been demonstrated in liposomes using several techniques, including fluorescence (1, 43), EPR (44), and NMR (45). The incorporation of protein into phospholipid vesicles has been found to result in a small decrease in the rate of intramolecular phospholipid motions as compared to the phospholipid vesicles alone (46). In this study, we find that loss of GABA uptake by synaptic plasma membrane vesicles correlates with reduced DPH fluorescence anisotropy, and therefore increased membrane fluidity (see Figs. 3 and 6). Thus, it is plausible that GABA uptake is lost during protein-mediated cholesterol depletion directly in response to increased membrane fluidity, resulting from both a decrease in the Ch/PL ratio and, to a lesser degree, increase in the lipid/protein ratio. We cannot rule out the possibility of a specific requirement for cholesterol by the GABA transporter. In tissue culture studies, Goldstein et al. (47) have shown that cholesterol is essential for cell growth. The molecular basis for this essentially has not been established.

There have been several reports of reduced surface accessibility, and decreased rotational mobility, of proteins in membranes fluidized by a variety of means, including sterol exchange into PC liposomes (48-51), cholesterol extraction with polyvinyl pyrrolidone dispersions of PC or linoleic acid (52, 53), phospholipid enrichment of cells by liposome fusion (54), and malignant transformation (1). The response of a particular protein receptor to membrane fluidity changes would reflect particular characteristics and orientation in the membrane and, perhaps, the method used to alter the membrane's fluid state. Four different receptors have been shown to be reduced in surface accessibility by increased fluidity of the membrane. These include β-adrenergic receptors in Chang liver cells (64),
serotonin (52) and opiate (53) receptors in mouse brain membranes, and the glucose carrier protein in human erythrocytes (51). Other receptors, in contrast, did not respond to changes in membrane fluidity. These include $\beta$-adrenergic receptors in turkey erythrocytes fluidized by insertion of cis-vaccenic acid (55) and $\alpha$-adrenergic receptors in cholesterol-depleted human platelets (8). Dependence of carrier-mediated glucose transport on membrane fluidity has also been reported in erythrocytes (51) and adipocytes (66).

Our $[^{3}H]$GABA-binding analysis correlates loss of sodium gradient-dependent GABA uptake with decreased number of available GABA-binding sites (Fig. 9). The lost sites were not expelled from the membrane, since uptake could be restored by reinserting cholesterol into the membrane. (Released sites would have been removed from the system during centrifugation of the membranes through sucrose before the cholesterol-reloading experiment.) Sodium-dependent GABA-binding sites have been shown to be referable to the sodium-dependent GABA uptake system found in synaptosomal preparations (57). A decrease in sodium-dependent GABA receptors would be expected to decrease GABA transport. High affinity sodium-independent GABA binding ($K_d \approx 0.2 \mu M$) to what are probably postsynaptic GABA receptors (58, 59) may also be included in the data presented in Fig. 9. However, the contribution of these sites in synaptic plasma membrane preparations (about 5 pmol/mg of protein) is comparatively small (57, 58). Loss of GABA uptake therefore correlates with increased membrane fluidity and a reduction in the number of available sodium-dependent GABA-binding sites.

The observed loss of GABA receptor accessibility upon membrane fluidization is consistent with the model of Shinitzky and co-workers (48–50) that the vertical displacement of membrane proteins is controlled by the lateral pressure exerted by surrounding membrane lipids. Such lateral pressure would be decreased by membrane fluidization. The vertical displacement model is based largely on studies which have correlated increased membrane fluidity with decreased accessibility of membrane proteins to impermeant reagents. According to this model, the lowering of specific GABA binding and therefore decreased GABA uptake rate which we observe with decreased Ch/PL ratio in synaptic plasma membranes would be due to an inward vertical displacement of the GABA transporter protein. That is to say, the GABA-binding sites normally exposed at the surface of the membrane become buried and inaccessible. The decrease in GABA binding is not directly proportional to the loss of uptake. For the cholesterol-depleted membranes shown in Fig. 9, the rate of GABA uptake was reduced to 16.7% of the control, whereas binding capacity was reduced to only about 50% of the control. Therefore, additional factors are apparently involved. The GABA transporter protein has been solubilized and reconstituted into lipid vesicles by Kanner (60). Study of the motional characteristics of the transporter protein in a reconstituted system of defined composition could provide more definitive interpretation of the effect of membrane lipid composition on the GABA uptake mechanism.

As yet, the application of lipid transfer proteins to modification of the lipid composition of membranes has been limited. This study illustrates the power of such application. Crain and Zilversmit (61) found using the nonspecific transfer protein from beef liver that in microsomal membranes a 27% depletion of phosphatidylethanolamine produced a 37% inhibition of glucose-6-phosphate phosphohydrolase activity. Voelker and Kennedy (62) used the PC exchange protein to provide lipid substrate to an isolated plasma membrane fraction for sphingomyelin synthesis, with the implication that transfer proteins may serve in this role physiologically.

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REFERENCES
1. Shinitzky, M., and Inbar, M. (1976) Biochim. Biophys. Acta 433, 133–140
2. Kress, J., and Ostwald, R. (1971) Biochim. Biophys. Acta 249, 647–650
3. Bruckdorfer, K. R., Edwards, P. A., and Green, C. (1968) Eur. J. Biochem. 4, 506–511
4. Wiley, J. S., and Cooper, R. A. (1975) Biochim. Biophys. Acta 413, 425–431
5. Deuticke, B., and Ruska, C. (1976) Biochim. Biophys. Acta 433, 638–653
6. Labelle, E. F. (1979) Biochim. Biophys. Acta 555, 259–269
7. Shinitzky, M., and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367–394
8. Insel, P. A., Nirenberg, P., Turnbull, J., and Shattil, S. J. (1978) Biochemistry 17, 5269–5274
9. Kielmier, H. K. (1975) Biochim. Biophys. Acta 413, 143–156
10. Klein, L, Moore, L., and Pastan, I. (1978) Biochim. Biophys. Acta 506, 42–53
11. Sinha, A. K., Shattil, S. J., and Colman, R. W. (1977) J. Biol. Chem. 252, 3100–3114
12. Inbar, M., and Shinitzky, M. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 2188–2190
13. Cooper, R. A., Arner, E. C., Wiley, J. S., and Shattil, S. J. (1975) J. Clin. Invest. 55, 115–126
14. Ostwald, R., Yamanaka, W., and Light, M. (1970) Proc. Soc. Exp. Biol. Med. 134, 814–820
15. Baldassare, J. J., Saito, Y., and Silbert, D. F. (1979) J. Biol. Chem. 254, 1108–1113
16. Shapiro, H. K., and Barchi, R. L. (1981) J. Neurochem. 36, 1813–1818
17. Elbli, H. (1980) Chem. Phys. Lipids 26, 239–247
18. Elbl, H. (1980) Chem. Phys. Lipids 26, 405–409
19. Elbl, H., and Kovatchev, S. (1981) Methods Enzymol. 72, 632–639
20. Gray, E. G., and Whitaker, V. P. (1963) J. Anat. 96, 79–87
21. Hajes, F. (1975) Brain Res. 93, 485–489
22. Jones, D. H., and Matus, A. I. (1974) Biochim. Biophys. Acta 356, 276–287
23. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
24. Ross, E., and Schatz, G. (1973) Anal. Chem. 54, 304–306
25. Chen, P. S., Toribara, T., and Warner, H. (1956) Anal. Chem. 28, 1756–1758
26. Rouser, G., and Fleischer, S. (1967) Methods Enzymol. 10, 385–406.
27. Shinitzky, M., and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652–2657
28. Shinitzky, M., and Inbar, M. (1974) J. Mol. Biol. 85, 603–615
29. Kanner, B. L. (1976) Biochemistry 15, 1207–1211
30. Simon, J. R., and Kubar, M. J. (1976) J. Neurochem. 27, 93–99
31. Bevington, R. B. (1989) Data Reduction and Error Analysis for the Physical Sciences, McGraw-Hill Book Company, New York
32. Rottenberg, H. (1979) Methods Enzymol. 55, 547–569
33. Sims, P. J., Waggoner, A. S., Wang, C. H., and Hoffman, J. F. (1974) Biochemistry 13, 3310–3314
34. Baulastein, M. P., and Goldring, J. M. (1975) J. Physiol. 247, 590–615
35. Crain, R. C., and Zilversmit, D. B. (1980) Biochemistry 19, 1433–1439
36. Saito, A., Wang, C.-T., and Fleischer, S. (1978) J. Cell Biol. 79, 601–616
37. Weber, G. (1952) Biochem. J. 51, 140–155
38. Martin, D. L. (1973) J. Neurochem. 21, 345–356
39. Iversen, L. L. (1973) in Perspectives in Neuropharmacology (Snyder, S. H., ed) pp. 75–111, Oxford University Press, New York
40. Baulastein, M. P., and King, A. C. (1976) J. Membr. Biol. 30, 155–173
41. Breckenridge, W. C., Gombar, G., and Morgan, I. G. (1972) Biochim. Biophys. Acta 266, 895–907
42. Mason, J. T., and Huang, C. (1978) Ann. N. Y. Acad. Sci. 308, 29–49
43. Vanderkooi, J., Fischkoff, S., Chance, B., and Cooper, R. A. (1974) Biochemistry 13, 1589–1595
44. Hubbell, W. L., and McConnell, H. M. (1971) J. Am. Chem. Soc. 93, 314–326
45. Chapman, D., and Penkett, S. A. (1966) Nature (Lond.) 211, 1304–1305
46. Seelig, J., Tamm, L., Hymel, L., and Fleischer, S. (1981) Biochemistry 20, 3922–3932
47. Goldstein, J. L., Helgeson, J. A. S., and Brown, M. S. (1979) J. Biol. Chem. 254, 5403–5409
48. Borochov, H., Shinitzky, M. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 4526–4530
49. Borochov, H., Abbot, R. E., Schachter, D., and Shinitzky, M. (1979) Biochemistry 18, 251–255
50. Shinitzky, M., and Rivnay, B. (1977) Biochemistry 16, 982–986
51. Yuli, I., Wilbrandt, W., and Shinitzky, M. (1981) Biochemistry 20, 4250–4256
52. Heron, D. S., Shinitzky, M., Hershkowitz, M., and Samuel, D. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 7463–7467
53. Heron, D., Israeli, M., Hershkowitz, M., Samuel, D., and Shinitzky, M. (1981) Eur. J. Pharmacol. 72, 361–364
54. Bakardjieva, A., Gilla, H. J., and Helmreich, E. J. M. (1979) Biochemistry 18, 3016–3023
55. Hanski, E., Rimon, G., and Levitzki, A. (1979) Biochemistry 18, 846–853
56. Pilch, P. F., Thompson, P. A., and Czech, M. P. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 915–918
57. Lester, B. R., and Peck, E. J., Jr. (1979) Brain Res. 161, 79–97
58. Lester, B. R., Miller, A. L., and Peck, E. J., Jr. (1981) J. Neurochem. 36, 154–164
59. Zukin, S. R., Young, A. B., and Snyder, S. H. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 4802–4807
60. Kanner, B. I. (1978) FEBS Lett. 89, 47–50
61. Crain, R. C., and Zilversmit, D. B. (1981) Biochemistry 20, 5320–5326
62. Voelker, D. R., and Kennedy, E. P. (1982) Biochemistry 21, 2753–2759