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Fluorescence Measurements of Anion Transport by the $GABA_A$ Receptor in Reconstituted Membrane Preparations†

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ABSTRACT: A fluorescence assay for measuring the functional properties of the $GABA_A$ receptor in reconstituted membrane vesicles is described. This assay is based on a method previously described to measure monovalent cation transport mediated by the nicotinic acetylcholine receptor in membranes from *Torpedo* electric organ [Moore, H.-P. H., & Raftery, M. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4509–4513]. The $GABA_A$ receptor has been solubilized from bovine brain membranes and reconstituted into phospholipid vesicles. Influx of chloride or iodide into the vesicles has been measured in stopped-flow experiments by monitoring the fluorescence quench of an anion-sensitive fluorophore trapped within the vesicles. Muscimol, a $GABA_A$ receptor agonist, stimulated a rapid uptake of either chloride or iodide. Stimulation of chloride influx was dependent on the concentration of muscimol, and the midpoint of the dose–response curve occurred at approximately 0.3 μM. Agonist-stimulated uptake was enhanced by diazepam and blocked by desensitization and by the antagonists bicuculline and picrotoxin. These receptor-mediated effects are shown to be qualitatively similar to measurements of $^{36}Cl^-$ and $^{125}I^-$ efflux using synaptoneurosomes prepared from rat cerebral cortex. The advantages of the fluorescence method in terms of its improved time resolution, sensitivity, and suitability for quantitating $GABA_A$ receptor function are discussed.

The interaction of γ-aminobutyric acid (GABA)[1] with its receptor results in the opening of a chloride ion channel which appears to be an integral part of the receptor–protein complex (Schofield et al., 1987). GABA is the major inhibitory neurotransmitter in mammalian brain, and modulation of GABAergic transmission has a profound effect on cellular excitability. Many pharmacological agents act on the $GABA_A$ receptor and alter its chloride-transporting properties. In addition to binding sites for GABA and its analogues, the receptor also has distinct sites for benzodiazepines, for barbiturates, and for the alkaloid picrotoxin (Olsen, 1982). Both benzodiazepines and barbiturates potentiate the actions of GABA but apparently by different mechanisms. Benzodiazepines have been shown to increase the affinity of the receptor for GABA (Tallman et al., 1978; Wastek et al., 1978; Briley & Langer, 1978; Martin & Candy, 1978; Karobath & Sperk, 1979) whereas barbiturates appear to prolong channel opening time [reviewed by Olsen (1981) and Tallman and Gallager (1985)].

Although electrophysiological experiments suggested that the interaction of GABA with its receptor results in an increase in chloride ion conductance (Krnjevic, 1974; McBurney & Barker, 1978; Nistri et al., 1980), only recently has this been demonstrated to occur in cell-free membrane preparations from brain. Synaptoneurosomes have been used to measure $^{36}Cl^-$ uptake or release in response to added GABA (Harris & Allan,

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[1] Abbreviations: GABA, γ-aminobutyric acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MSQ, 6-methoxy-*N*-(3-sulfopropyl)quinolinium; β-octyl glucoside, *n*-octyl β-D-glucopyranoside; Tris, tris(hydroxymethyl)aminomethane.

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1985), barbiturates (Schwartz et al., 1984; Allan & Harris, 1986a), ethanol (Suzdak et al., 1986; Allan & Harris, 1986b), anesthetics (Huidobro-Toro et al., 1987), and benzodiazepines (Morrow & Paul, 1988). The synaptoneurosome preparations consist of membranes of both pre- and postsynaptic origin and were originally described for studying the coupling between brain receptors and adenylate cyclase (Daly et al., 1980). While these preparations have been useful in providing a qualitative description of drug effects on chloride permeability changes, the heterogeneity of the membrane population and the unsuitability of the preparation for biochemical manipulation preclude quantitative analysis. Furthermore, most studies have lacked the necessary time resolution to study the kinetics of chloride flux responses. Using a quench-flow technique, Cash and Subbarao (1987a,b) have shown that the GABA receptor in a similar brain homogenate preparation undergoes a rapid desensitization upon exposure to agonist. It is therefore necessary to measure ion flux on millisecond time scales in order to obtain detailed information on the ion gating effect.

The necessity of using rapid kinetic techniques to study functional properties of ion channels in isolated membrane vesicles has previously been recognized in the study of the nicotinic acetylcholine receptor. Moore and Raftery (1980) first introduced a fluorescence quenching technique to measure receptor-mediated cation transport on time accessible to stopped-flow spectroscopy. In this method, membrane vesicles were first loaded with a water-soluble fluorophore, and extravesicular dye was removed by gel filtration. The influx of thallous ion, which mimics Na^+ in this system, was then monitored by the quenching of the fluorescence of the entrapped fluorophore. In addition to its superior time resolution, a major advantage of this spectroscopic method over quench-flow techniques is its high information content due to the continuous monitoring of the influx process.

In this paper, we describe the development of a similar fluorescence quenching method to study chloride and iodide transport mediated by the $GABA_A$ receptor in reconstituted membrane vesicles. We demonstrate that the reconstituted receptor retains many of the functional properties that may be measured by $^{36}Cl^-$ and $^{125}I^-$ efflux assays using synaptoneurosome preparations. This rapid kinetic technique will facilitate quantitative analysis of the ion-transporting properties of the $GABA_A$ receptor and studies of channel activation and modulation.

Materials and Methods

Preparation of Synaptoneurosomes from Rat Cerebral Cortex. Synaptoneurosomes were prepared from the cerebral cortex of 150–200-g male Sprague-Dawley rats (Biolabs Inc.) as described by Schwartz et al. (1985). All operations were carried out close to 0 °C. Cerebral cortex was dissected free of white matter on ice and was homogenized in 10 volumes of ice-cold buffer (20 mM Hepes–Tris, pH 7.4, 118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, and 2.5 mM $CaCl_2$) using five strokes of a glass–glass homogenizer (B. Braun Instruments) operating at 400 rpm. The homogenate was diluted with 3 volumes of ice-cold buffer, and the mixture was filtered by gravity through three layers of 160-μm nylon mesh (Tetko Inc., Elmsford, NY) mounted in a Millipore Swinnex Filter holder. The filtrate was filtered with slight pressure through a 10-μm Millipore filter, and the filtrate was centrifuged for 15 min at $1000g_{max}$. The supernatant was discarded, and the pellet was resuspended at a concentration of approximately 10 mg of protein/mL in the same buffer using five strokes of the glass–glass homogenizer as above.

^{36}Cl Efflux Measurements of Synaptoneurosome Preparations. Synaptoneurosomes were first loaded with ^{36}Cl (New England Nuclear) by incubation on ice for 1 h with ^{36}Cl at a final concentration of 5 μCi/mL. Efflux was initiated by 50-fold dilution of the synaptoneurosomes into ice-cold buffer, and the mixture was constantly stirred during the assay. At time intervals of 5, 15, 30, 45, 60, and 90 s after dilution, 3-mL aliquots were rapidly filtered under vacuum through GF/C filters (Whatman) mounted in a Hoefer filtration apparatus, and the filters were washed with two 5-mL volumes of ice-cold buffer containing 30 mM sucrose. The filters were extracted in 5 mL of 3a70 scintillation fluid (Research Products International) before being counted for ^{36}Cl. In experiments in which it was desired to preincubate the synaptoneurosomes with drugs, the loaded vesicles were incubated with the appropriate drug for 30 min on ice prior to initiation of the efflux assay by dilution.

^{125}I Efflux Measurements of Synaptoneurosomes. In experiments of ^{125}I efflux, the synaptoneurosomes were prepared as described above except that the buffer used throughout was 20 mM Hepes–Tris, pH 7.4, 100 mM NaCl, 18 mM NaI, 4.7 mM KCl, 1.18 mM $MgSo_4$, and 2.5 mM $CaCl_2$. The same buffer was used in the flux experiments which were otherwise identical with those described for ^{36}Cl above except that the synaptoneurosomes were first loaded with ^{125}I (ICN) at a final concentration of 0.2 μCi/mL in the incubation mixture. Aliquots were filtered through GF/C filters as described above, and the filters were counted for ^{125}I in a Beckman 8000 γ counter.

Preparation of Bovine Brain Membranes. Bovine brains were obtained from a local slaughterhouse and were immediately frozen on dry ice and stored at −80 °C. Membranes were prepared from cerebral cortex as described elsewhere (Dunn et al., 1989).

Binding of Radiolabeled Ligands to Membrane Preparations. The binding of [^3H]muscimol and [^3H]flunitrazepam (New England Nuclear) to membrane preparations was measured by using filtration assays (Dunn et al., 1989).

Solubilization and Reconstitution of the GABA Receptor. The $GABA_A$ receptor was solubilized from bovine brain membranes using 1% β-octyl glucoside and reconstituted using a 4:1 mixture of asolectin (Associated Concentrates, New York) and bovine brain phospholipids as described elsewhere (Dunn et al., 1989). Following removal of detergent by gel filtration on a Sephadex G-50-80 (1.5 × 90 cm) column equilibrated in 10 mM Hepes–Tris, pH 7.4, and 150 mM NaCl, the vesicles which eluted in the void volume were dialyzed against two 4-L changes of the same buffer over a 16-h period. Reconstituted membranes were diluted in 10 mM Hepes–Tris/mM $NaNO_3$, pH 7.4, and collected by centrifugation for 60 min at 18 000 rpm in a Sorvall SS34 rotor. Following resuspension, the vesicles were washed at least once by dilution in a large volume of the $NaNO_3$ buffer followed by centrifugation and resuspension in approximately 1 mL of the same buffer.

Loading of Reconstituted $GABA_A$ Receptor Containing Vesicles with Fluorophore. The anion-sensitive fluorophore 6-methoxy-*N*-(3-sulfopropyl)quinolinium (MSQ) was obtained from Molecular Probes Inc. (Eugene, OR). This was loaded into reconstituted vesicles by the freeze–thaw procedure previously described (Moore & Raftery, 1980). One volume (1–1.5 mL) of reconstituted vesicles in 10 mM Hepes–Tris, pH 7.4, and 150 mM $NaNO_3$ was mixed with an equal volume of 20 mM MSQ in 10 mM Hepes–Tris, pH 7.4, and 130 mM $NaNO_3$. The mixture was frozen in liquid nitrogen and al-

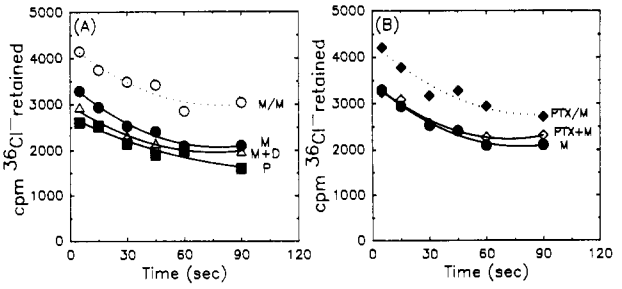

FIGURE 1: Effects of $GABA_A$ receptor ligands on $^{36}Cl^-$ efflux from rat synaptoneurosome preparations. Synaptoneurosomes were loaded with $^{36}Cl^-$ as described in the text. Efflux was initiated by dilution into buffer containing the appropriate drug(s), and at the indicated times after dilution, aliquots were filtered, and the filters were counted for residual entrapped isotope. (A) Effects of 10 μM muscimol (●), 10 μM muscimol plus 10 μM diazepam (△), or 500 μM pentobarbital (■) on $^{36}Cl^-$ efflux. In (○), the synaptoneurosomes were preincubated with 10 μM muscimol for 30 min on ice prior to dilution into buffer containing 10 μM miscimol. (B) Slow onset of action of picrotoxin. Synaptoneurosomes were diluted into buffer containing 10 μM muscimol alone (●) or 10 μM muscimol plus 100 μM picrotoxin (◇). In (◆), the membranes were preincubated with 100 μM picrotoxin for 30 min on ice prior to dilution into muscimol plus picrotoxin as in (◇).

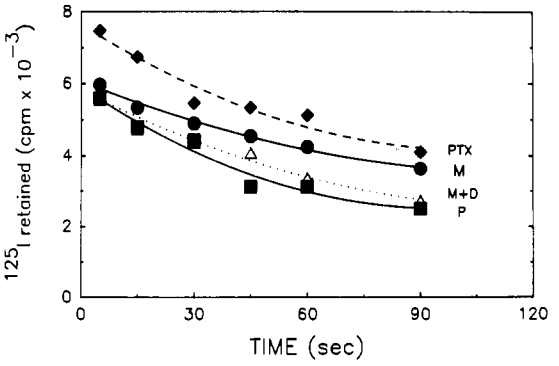

FIGURE 2: Effects of $GABA_A$ receptor ligands on $^{125}I^-$ efflux in rat synaptoneurosome preparations. Experimental procedures were as in the legend to Figure 1 except that the synaptoneurosomes were first loaded with ^{125}I. Efflux was initiated by dilution into buffer containing 10 μM muscimol (●), 10 μM muscimol plus 10 μM diazepam (△), or 500 μM pentobarbital (■) or were preincubated with 100 μM picrotoxin (◆) before dilution into buffer containing 10 μM miscimol.

lowed to thaw slowly in an iced water bath. The freeze–thaw procedure was repeated once, and the thawed mixture was applied to a Sepadex G-25-300 column (1.5 × 35 cm) equilibrated in 10 mM Hepes–Tris, pH 7.4, and 150 mM $NaNO_3$. The fluorophore-containing vesicles, which eluted in the void volume, were diluted approximately 2-fold in the same buffer, shielded from light, and kept on ice until use, which was within 3 h of column separation.

Equilibrium Fluorescence Measurements. Fluorescence measurements were made by using a Perkin-Elmer MPF44A fluorometer thermostated at 25 °C.

Stopped-Flow Measurements of Anion Transport into Reconstituted Preparations. Stopped-flow fluorescence measurements were carried out with a Durrum D-110 stopped-flow fluorometer as previously described (Dunn et al., 1980) with an excitation wavelength of 355 nm and measuring fluorescence emission with a GG420 Schott glass filter (Melles Griot, Irvine, CA). All kinetic experiments were carried out at 25 °C. Changes in fluorescence with time were fit by nonlinear regression techniques to

$$F(t) = A_0 + A_1/[1 + KC_\infty(1 - e^{-k_1 t})] + k_0 t$$

where $F(t)$ is the fluorescence level at time t, A_0 is the residual fluorescence after completion of the rapid phase of quench, A_1 is the amplitude of the rapid quench, k_1 is the corresponding rate constant, and the term $k_0 t$ was used to correst for subsequent slower reactions that approximate a linear quench on these time scales. When chloride was used as the quenching anion, the term KC_∞ was fixed at 5.985 by using the measured Stern–Volmer constant (K) of 79.8 M^{-1} (see below) and the known final chloride concentration (C_∞) of 75 mM. Similarly, when iodide was the quenching anion, this term was fixed at 15.375 by using the Stern–Volmer constant for iodide of 205 M^{-1} (see below) and the final concentration of 75 mM.

Drugs. Muscimol, pentobarbital (Na^+ salt), diazepam, and picrotoxin were obtained from Sigma Chemical Co. Bicuculline methochloride was obtained from Cambridge Research Biochemicals.

Results

Chloride and Iodide Efflux Measurements in Rat Brain Synaptoneurosomes. Figure 1 shows the results of a $^{36}Cl^-$ efflux experiment using synaptoneurosomes prepared from rat cerebral cortex. After loading of the membranes by incubation with the isotope for 1 h on ice, efflux was initiated by dilution into buffer containing the appropriate drug. At various times after dilution, the amount of residual entrapped $^{36}Cl^-$ was measured in filtration assays. Efflux was stimulated by pentobarbital and by diazepam when this was added at the same time as muscimol. Preincubation of the loaded synaptoneurosomes with muscimol blocked the response, presumably due to desensitization of the receptor upon exposure to agonist. The effects of picrotoxin are illustrated in Figure 1B. Picrotoxin inhibited the efflux when it was preincubated with the membranes prior to dilution, but not when it was present only in the dilution buffer. This suggests that picrotoxin has a rather slow onset of action, i.e., greater than the time period of these assays.

In electrophysiological experiments, it has previously been shown that iodide is at least as permeable as chloride through the GABA receptor ion channel (Gallagher et al., 1978). It was of interest to investigate receptor-mediated permeability changes of ^{125}I in synaptoneurosome preparations, both because of the higher energy of this isotope and because, as discussed below in the development of a spectroscopic flux assay, iodide is a more efficient quencher of dye fluorescence than chloride. The results in Figure 2 show that many of the properties of iodide efflux are similar to those discussed above for $^{36}Cl^-$ flux, including potentiation of the muscimol response by diazepam, an increase in efflux by pentobarbital, and block by preincubation with picrotoxin.

The above results demonstrate that the GABA receptor and its associated ion channel are, at least to some extent, functional in synaptoneurosomes and that ion flux is modulated with the pharmacological specificity expected of drugs such as benzodiazepines, barbiturates, and picrotoxin. However, during the course of these studies, we became acutely aware of the dangers inherent in drawing quantitative conclusions about the effects of ligands on receptor function using such heterogeneous membrane preparations and assays that are less than optimal. In many preparations, we have found that an apparent chloride efflux may be measured in the absence of added agonist and muscimol does not increase the response. These results suggest that synaptoneurosomes may contain high levels of endogenous GABA which cause channel activation. This is not in the least surprising considering the crudeness of the preparations and the difficulty of reducing endogenous GABA in even well-washed membranes (Enna & Snyder, 1977; Greenlee et al., 1978). However, the present

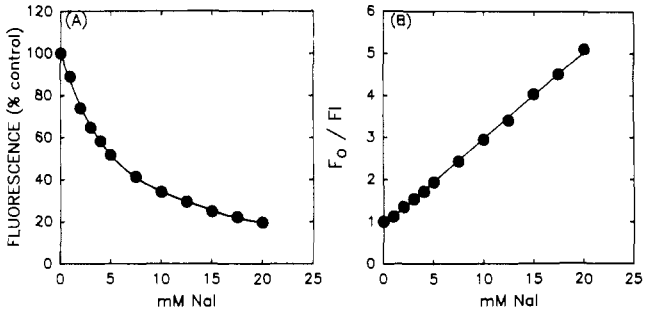

FIGURE 3: Quenching of MSQ fluorescence by iodide. (A) The fluorescence of 100 μM MSQ was measured in the presence of increasing concentrations of NaI using excitation and emission wavelengths of 355 and 440 nm, respectively. The osmotic strength of the solutions was kept constant by isoosmotic substitution of $NaNO_3$ by NaI (final concentration 100 mM), and the solutions were buffered at pH 7.4 with 10 mM Hepes–Tris. (B) Stern–Volmer plot of the data in (A). F_0 and Fl are the fluorescence intensities in the absence and presence of the indicated concentrations of I^-, respectively, and the solid line is a linear-squares fit giving a slope of 205 M^{-1}.

findings, which are consistent with several previous reports (Schwartz et al., 1984, 1985), are disturbing in view of the possibility that the presence of endogenous GABA desensitizes the receptor. This raises questions about whether the observed actions of drugs may in reality be effects on the desensitized state of the $GABA_A$ receptor. Furthermore, the rate of leakage of $^{36}Cl^-$ out of the vesicles is fast even when the GABA receptor is blocked by drugs (Figure 1). Most of the specific efflux is complete within the first 5 s, i.e., the earliest time point in these filtration assays, and thus much of the flux response is unobservably fast. These, and other considerations discussed below (see Discussion), reinforced the necessity of using quantitative methods to study ion flux responses of the $GABA_A$ receptor in a more defined and controllable environment. We have therefore developed a spectroscopic method to measure the kinetics of anion flux in reconstituted membrane preparations containing the $GABA_A$ receptor.

Choice of Fluorophore and Quenching by Anions. As previously discussed by Moore and Raftery (1980), a fluorescent probe that is suitable for monitoring ion flux in membrane vesicles is one that is water soluble, is impermeable to the vesicle membrane, and does not bind to or insert significantly into the membrane bilayer. A fluorescence probe that meets these requirements is 6-methoxy-*N*-(3-sulfopropyl)-quinolinium (MSQ), which, being a zwitterion and having a negatively charged sulfonic acid group at neutral pH, is not appreciably membrane permeant. This fluorophore is a pH-insensitive fluorescent standard that has previously been used for determination of halide ions (Wolfbeis & Urbano, 1983; Urbano et al., 1984).

MSQ exhibits broad excitation and emission spectra with maxima at 355 and 440 nm, respectively. Both chloride and iodide ions effectively quenched the fluorescence of MSQ, as illustrated for iodide in Figure 3. From these data, the Stern–Volmer constant was determined to be 205 M^{-1}. Similar experiments using chloride as the quenching anion gave a Stern–Volmer constant of 79.8 M^{-1}. These values compare favorably with that of 96 M^{-1} for thallous quenching of 8-aminonaphthalene-1,3,6-trisulfonic acid previously used successfully in a similar assay (Moore & Raftery, 1980).

Effects of $NaNO_3$ on the Binding of [^{3}H]Flunitrazepam to the Membrane-Bound $GABA_A$ Receptor. The basis of the fluorescence quenching approach to measure receptor-mediated anion transport is that, prior to flux measurements, the probe trapped within the membrane vesicles must remain fluorescent. Membrane preparations are therefore equilibrated in a non-

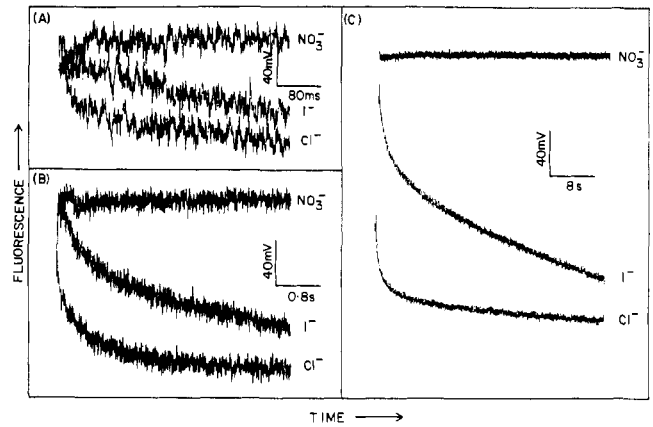

FIGURE 4: Kinetics of chloride and iodide influx using protein-free vesicles monitored by changes in the fluorescence of entrapped MSQ. Liposomes were prepared from a 4:1 mixture of asolectin and bovine brain phospholipids in the absence of solubilized protein as described in the text. The vesicles were equilibrated in 10 mM Hepes–Tris, pH 7.4, and 150 mM $NaNO_3$ and loaded with 10 mM MSQ by freeze–thaw cycles, and extravesicular dye was removed by gel filtration. The dye-loaded vesicles were rapidly mixed in a stopped-flow apparatus with an equal volume of the same buffer containing 150 mM $NaNO_3$, 150 mM NaCl, or 150 mM NaI as indicated in the figure. The changes in fluorescence of MSQ (measured in millivolts) were recorded on rapid (A), intermediate (B), and slow (C) time scales. Mixing with nitrate did not produce significant changes in fluorescence, but both chloride and iodide quenched the fluorescence of the entrapped fluorophore. For the fast phase, data fitting gave for chloride A_1 = 135 mV and k_1 = 17.1 s^{-1} and for iodide A_1 = 58 mV and k_1 = 0.38 s^{-1}. On all time scales, the apparent rate of quench was observed to be greater with chloride than with iodide. However, on slower time scales, the data could not be adequately described by the equations given in the text since the fluorescence quench took place in several phases which were not well separated.

quenching buffer (10 mM Hepes–Tris, pH 7.4, and 150 mM $NaNO_3$) prior to, and during, the fluorophore loading procedures. Since it has previously been reported that the binding of benzodiazepines to the GABA receptor is anion dependent (Squires, 1981), we have investigated the effects of buffer composition on the binding of [^{3}H]flunitrazepam to bovine brain membrane preparations. In equilibrium binding experiments, there was no change in the number of binding sites measured in buffer containing NaCl or $NaNO_3$, but the affinity for [^{3}H]flunitrazepan was reduced 2–3-fold in the nitrate buffer. Dissociation constants obtained from measurements using buffer containing 150 mM NaCl or 150 mM $NaNO_3$ were 2.2 and 5.1 nM, respectively.

Permeability of Protein-Free Vesicles to Chloride and Iodide. A prerequisite for measuring specific anion transport through the $GABA_A$ receptor ion channel is that the rate of nonspecific leakage of the ion through the lipid bilayer is sufficiently slow that this does not obscure specific effects. In preliminary experiments, protein-free liposomes were prepared by excluding solubilized protein during the reconstitution procedures. MSQ was loaded inside the vesicles by freeze–thaw cycles as described under Materials and Methods, and extravesicular dye was removed gel filtration. Figure 4 shows the results of experiments in which the MSQ-loaded vesicles in buffer containing 150 mM $NaNO_3$ were rapidly mixed with a buffer in which the $NaNO_3$ was replaced by either NaCl or NaI. In each case, there ws a quench of MSQ fluorescence, but this was not unobservably fast and was readily measurable on stopped-flow time scales. In contrast, mixing of MSQ in solution (i.e., not trapped within membrane vesicles) with either chloride or iodide leads to a very rapid, submillisecond quench that cannot be measured by the stopped-flow technique (data not shown).

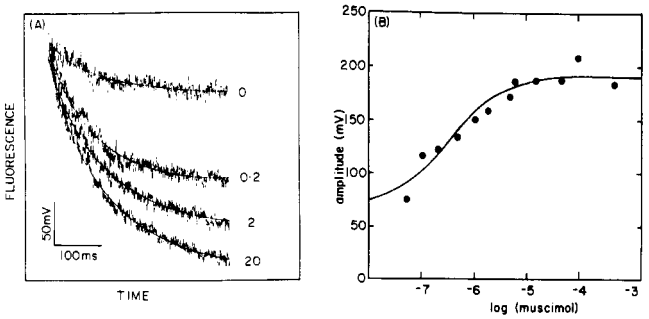

FIGURE 5: Effect of muscimol concentration on chloride uptake by reconstituted membrane preparations. Reconstituted vesicles containing the $GABA_A$ receptor were loaded with MSQ in 10 mM Hepes–Tris, pH 7.4, and 150 mM $NaNO_3$, and excess dye was removed by gel filtration. (A) The MSQ-loaded vesicles were mixed in a stopped-flow apparatus with an equal volume of 10 mM Hepes–Tris, pH 7.4, and 150 mM NaCl containing muscimol to give final concentrations of 0, 0.2, 2, or 20 μM as indicated in the figure. Uptake of chloride was monitored by recording the changes in fluorescence of the entrapped fluorophore. Although the rate of the fluorescence quench was not significantly dependent on muscimol concentration, the amplitude of the fast quench increased with concentration. Data were fit by the equation given under Materials and Methods, and on these time scales, the contribution of k_0t to the fluorescence quench was negligible. Traces shown are representative of at least four determinations, and for each trace, the best-fit parameters are given with the average values ± the standard deviation for these replicates given in parentheses. No muscimol: A_1 = 66 mV (80 ± 21 mV); k_1 = 1.4 s^{-1} (1.1 ± 0.4 s^{-1}). 0.2 μM muscimol: A_1 = 122 mV (122 ± 17 mV); k_1 = 1.5 s^{-1} (1.2 ± 0.3 s^{-1}). 2 μM muscimol: A_1 = 158 mV (156 ± 8 mV); k_1 = 1.0 s^{-1} (1.1 ± 0.2 s^{-1}). 20 μM muscimol: A_1 = 188 mV (184 ± 14 mV); k_1 = 1.1 ± 0.1 s^{-1} (1.2 ± 0.3 s^{-1}). (B) Effect of muscimol concentration on the amplitude (A_1) of the rapid fluorescence quench. Data were obtained from experiments as in (A). The effects of muscimol concentration on the mean value of A_1 were fit by the equation $A_1 = Amp_m[L]/(K_d + [L] + Amp_0$ where A_1 is the measured amplitude of the fluorescence quench and Amp_m and Amp_0 are the maximum stimulation of the fluorescence quench by high concentrations of muscimol and the amplitude of the leak rate measured in the absence of agonist, respectively. Nonlinear regression fitting gave values of Amp_0 = 72 mV, Amp_m = 155 mV, and K_d = 0.28 μM.

The quench in fluorescence of entrapped MSQ upon mixing with NaCl or NaI took place in several phases as illustrated by the different time courses in Figure 4. The results demonstrate that the fluorescence changes are considerably faster using chloride than those observed using iodide, suggesting that chloride leaks more rapidly through the vesicle membrane. Since ion transport through the $GABA_A$ receptor channel is likely to occur on a millisecond time scale, it is obviously important to reduce, as much as possible, potential nonspecific leakages in this time region. In this respect, it appears from the results shown in Figure 4A that iodide may offer some advantage over chloride for measuring functional responses of the receptor. Although it is not possible to directly correlate leak rates of protein-free and protein-containing membranes [see, e.g., Tanaka et al. (1986)], results described below suggest that iodide also leaks more slowly through protein-containing vesicles.

Effect of Muscimol Concentration on Chloride Uptake by Reconstituted Vesicles. When MSQ-loaded vesicles were mixed with muscimol and chloride, there was a stimulation of chloride uptake as monitored by the fluorescence quench of the entrapped dye on millisecond time scales. This rapid uptake was followed by a slower quench resulting from the equilibration of chloride with the interior of the vesicles. The rapid stimulation of ion transport was dependent on the concentration of muscimol as illustrated in Figure 5. An interesting result is that the rate of chloride influx was not significantly increased by the presence of muscimol but rather

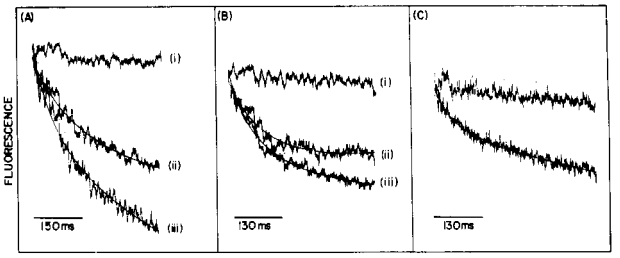

FIGURE 6: Kinetics of anion transport by the reconstituted $GABA_A$ receptor. The traces shown are representative of quadruplicate measurements, and the kinetic parameters listed are for the traces illustrated with average values ± the standard deviation given in parentheses. (A) Potentiation of chloride uptake by diazepam. MSQ-loaded vesicles in 10 mM Hepes–Tris/150 mM $NaNO_3$, pH 7.4, were mixed with the same buffer containing 150 mM NaCl and final concentrations of 10 μM diazepam alone (i), 10 μM muscimol (ii), or 10 μM muscimol plus 10 μM diazepam (iii). Each trace is the average of three recordings. Best-fit parameters for muscimol were A_1 = 89 mV (109 ± 21 mV) and k_1 = 1.14 s^{-1} (1.0 ± 0.2 s^{-1}) and for muscimol plus diazepam A_1 = 152 mV (194 ± 31 mV) and k_1 = 1.24 s^{-1} s^{-1} (1.0 ± 0.3 s^{-1}). (B) Slow onset of action of picrotoxin. In (i), MSQ-loaded vesicles were preincubated with 50 μM picrotoxin for 10 min before being mixed with 50 μM picrotoxin and 10 μM muscimol (final concentrations). An identical experiment was carried out in (ii) except that the membranes were not preincubated with picrotoxin. In (iii), Membranes were mixed with 10 μM muscimol alone. Each trace is the average of four determinations. Kinetic parameters for (ii) were A_1 = 105 mV (114 ± 10 mV) and k_1 = 1.3 s^{-1} (1.2 ± 0.1 s^{-1}) and for (iii) A_1 = 130 mV (121 ± 8 mV) and k_1 = 1.9 s^{-1} (1.7 ± 0.5 s^{-1}). (C) Agonist-mediated iodide uptake in reconstituted vesicles. MSQ-loaded vesicles were mixed with 150 mM NaI in the absence (upper trace) or presence (lower trace) of 10 μM muscimol. Virtually no fluorescence quench on these time scales was observed in the absence of agonist, but for the lower trace, values for the kinetic parameters were estimated to be A_1 = 53 mV (55 ± 5 mV) and k_1 = 1.1 s^{-1} (1.0 ± 0.3 s^{-1}).

the amplitude of the fluorescence quench was increased in a concentration-dependent fashion. These results may readily by interpreted by the likelihood that, because of the low density of $GABA_A$ receptors in brain membranes, only a small fraction of the reconstituted vesicles will contain even one $GABA_A$ receptor (see Discussion). Under these circumstances, increasing occupancy of the receptor by muscimol will be reflected in an increasing number of vesicles having one chloride channel opened. If the rate of chloride transport through each activated channel is constant, the result will be an increase in the amplitude of chloride uptake since this is a measure of the number of responsive vesicles, but not the rate which reflects the number of activated channels per vesicles (Moore & Raftery, 1980). A study of the concentration dependence of chloride uptake is shown in Figure 5B. The midpoint of the flux response occurred at 0.28 μM muscimol. Similar experiments using four different preparations have given K_d's in the range of 0.2–1.0 μM.

Benzodiazepine Enhancement of Muscimol-Stimulated Chloride Flux. Figure 6A shows the result of an experiment in which MSQ-loaded vesicles were mixed with muscimol alone or muscimol plus diazepam. There is a clear potentiation of the flux response in the presence of diazepam which is consistent with previous electrophysiological studies of the potentiation of muscimol action by benzodiazepines (Choi et al., 1978; MacDonald & Barker, 1978).

Effects of Antagonists on Chloride Flux Responses of Reconstituted Membranes. Using synaptoneurosome preparations, it was found that picrotoxin inhibition of ^{36}Cl efflux was facilitated by preincubation of the membranes with this drug (Figure 1). It was therefore suggested that picrotoxin may have a relatively slow onset of action. A similar effect was observed in the fluorescence experiments. Figure 6B shows

that preincubation of the vesicles with picrotoxin inhibited the muscimol-induced chloride uptake but there was much less inhibition when picrotoxin was added only at the same time as muscimol. Such a dependence on time of exposure to antagonist was not found with bicuculline which produced almost complete inhibition even when added at the same time as muscimol (data not shown).

Uptake of Iodide by Reconstituted Preparations. When MSQ-loaded vesicles were mixed with buffer containing NaI, there was virtually no observable quench of the dye fluorescence on rapid time scales (Figure 6C), suggesting that, as found using protein-free vesicles, the rate of leakage of iodide across the vesicles membrane is relatively slow. Addition of muscimol led to a stimulation of iodide, uptake (Figure 6C), which, like the chloride response, was blocked by desensitization, bicuculline, and preincubation with picrotoxin (data not shown).

Discussion

A fluorescence assay has been developed to measure anion transport mediated by the $GABA_A$ receptor in reconstituted membrane vesicles. This assay is adapted from one previously described for measuring acetylcholine receptor function (Moore & Raftery, 1980) and is based on the fluorescence quenching of an optical probe loaded within the vesicles. With this technique, ion transport may be measured on millisecond time scales that are physiologically relevant. This approach offers much improved time resolution over the previous methods that have been used to measure $GABA_A$ receptor function in vitro. Also, the use of reconstituted preparations provides the opportunity to study structure–function relationships of the receptor under defined conditions.

Chloride transport mediated by the $GABA_A$ receptor has previously been studied using synaptoneurosome preparations (Schwartz et al., 1984, 1985; Harris & Allan, 1985; Allan & Harris, 1986a,b; Suzdak et al., 1986; Huidobro-Toro et al., 1987; Morrow & Paul, 1988). Characterization of these preparations (Daly et al., 1980; Hollingsworth et al., 1985) has been of great importance in the study of brain transport processes. Previously, it was not possible to measure such functions in vitro due to the lack of a sealed brain membrane preparation suitable for ion flux studies. In the present study, rat brain synaptoneurosomes have been used to study radiotracer anion effluxes in response to GABA receptor ligands. The results obtained for $^{36}Cl^-$ efflux responses are similar to those reported by other investigators including observations of desensitization resulting from exposure to muscimol, enhancement of efflux by diazepam and pentobarbital, and block by picrotoxin. These experiments have been extended to demonstrate that receptor-mediated $^{125}I^-$ efflux may also be measured in these preparations. These findings are in agreement with electrophysiological studies of the anion selectivity of the $GABA_A$ receptor channel (Gallagher et al., 1978).

Synaptoneurosomes may, therefore, be used to study some of the properties of GABA receptor function. However, the nature of the preparations leads to serious experimental limitations. These include the heterogeneity of the membrane species and their fragility to experimental manipulation. They appear to contain considerable endogenous GABA that may confound the interpretation of ion flux results, and because of high levels of nonspecific binding, it is virtually impossible to measure specific ligand binding in these preparations (unpublished observations). Synaptoneurosomes also display a high resting permeability to chloride ions, and this makes the quantitation of ion flux using manual filtration techniques very difficult. Furthermore, in a quench-flow study of the kinetics of $^{36}Cl^-$ influx in a similar membrane preparation, it was found that the $GABA_A$ receptor undergoes two phases of desensitization, the more rapid of which was complete within 200 ms (Cash & Subbarao, 1987). Thus, rapid kinetic techniques are required to study the properties of ion transport through the activated receptor channel.

The above considerations suggested that rigorous investigation of the functional properties of the GABA receptor and its complex drug interactions necessitates the availability of a defined membrane system that is more amenable to biochemical manipulation. Most ligand binding studies have been carried out using well-washed membrane preparations from mammalian brain. Electron microscopic investigation of such bovine brain membranes has demonstrated that they are unsuitable for ion flux experiments since the membranes are fragmented and contain few, if any, intact vesicles (unpublished observations). The approach we have therefore taken is to solubilize the receptor from bovine brain membranes and reconstitute it into phospholipid vesicles (Dunn et al., 1989). In reconstituted membranes, the $GABA_A$ receptor remains functionally active, and these preparations may be used for both ligand binding and ion flux studies.

Muscimol stimulated the uptake of chloride and iodide by reconstituted vesicles. This effect was concentration dependent, and, to a first approximation, the amplitude of the fluorescence quench increased hyperbolically with muscimol concentration with an apparent K_d of about 0.3 μM (Figure 5). The rate of quench did not show a significant dependence on muscimol concentration. As discussed under Results, this is likely to be a consequence of the low density of $GABA_A$ receptors in native and reconstituted preparations which makes it unlikely that there is more than one receptor per vesicle. The number of receptors per vesicle may be roughly estimated if it is a assumed that the vesicles in the present preparation have a diameter of 1000 Å, similar to measurements of the size of the vesicles prepared by using a similar reconstitution protocol for the solubilized insulin receptor (Gould et al., 1982). For a vesicle 1000 Å in diameter and 50 Å in bilayer thickness, it may be estimated [see Wu and Raftery (1982)] that each vesicle contains 1.03×10^5 phospholipid molecules. From measurements of total phosphate, the reconstituted membranes used in the present study contain approximately 18 μmol of phospholipid/mg of protein (S. M. J. Dunn, unpublished results). It has also been shown that the density of high-affinity binding sites for [^{3}H]muscimol in these preparations is about 1.1 pmol/mg of protein (Dunn et al., 1989). If it is assumed that there is one high-affinity site for $GABA_A$ receptor [although this is likely to be an underestimate; see Casalotti et al. (1986)], the mole ratio of phospholipid to $GABA_A$ receptor is $(1.6 \times 10^7):1$. Thus, it may readily be estimated that only about 0.6% of the vesicles will contain even one $GABA_A$ receptor. Further investigation of this phenomenon will require quantitation of the number of receptors per vesicles after reconstitution of the purified protein.

The stimulation of anion uptake by muscimol was enhanced by diazepam. Investigation of the concentration dependence of this effect will reveal whether this enhancement is the result of a stimulation of the maximum rate of influx or a shift in the dose dependency of the muscimol response to lower concentrations as previously suggested (Choi et al., 1981; Morrow & Paul, 1988). Anion flux was flocked by desensitization, bicuculline, and pretreatment with picrotoxin, suggesting that the reconstituted $GABA_A$ receptor retains many of the functional properties of the native receptor. It should be noted

that one aspect of receptor function that has not yet been successfully reconstituted is the stimulation of chloride flux by barbiturates. Pentobarbital neither stimulated ion flux nor affected the muscimol-induced uptake. In previous studies of receptor solubilization (Stephenson & Olsen, 1982), it has been shown that barbiturate enhancement of benzodiazepine binding (Leeg-Lundberg et al., 1980) is unstable after solubilization. One possible explanation for the lack of barbiturate effects on ion transport is, therefore, that our present solubilization conditions result in the loss of this binding component.

In conclusion, the $GABA_A$ receptor has been reconstituted into phospholipid vesicles with retention of many of its functional properties, and a fluorescence assay has been developed to measure the kinetics of receptor-mediated ion transport in these preparations. This will facilitate studies of structure–function relationships of the receptor and will help in the elucidation of the molecular basis for the complex effects of drugs on channel activity.

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