J. Membrane Biol. 145, 257–266 (1995)

The Journal of
Membrane
Biology

Use of $^{82}Br^-$ Radiotracer to Study Transmembrane Halide Flux: The Effect of a Tranquilizing Drug, Chlordiazepoxide on Channel Opening of a $GABA_A$ Receptor

D.J. Cash, P. Serfözö, K. Zinn

Department of Biochemistry, School of Medicine, University of Missouri, Columbia MO 65211

Received: 19 October 1994/Revised: 9 February 1995

Abstract. We used the short-lived radionuclide, $^{82}Br^-$ to follow γ-aminobutyrate (GABA) receptor-mediated halide exchange into membrane vesicles from rat cerebral cortex in millisecond and second time regions using quench-flow technique. The radioisotope was prepared by neutron capture [$^{81}Br^-(n,\gamma)^{82}Br^-$] on irradiation of a natural isotope of bromine, $^{81}Br^-$ in a neutron flux. $^{82}Br^-$ decays by β-emission with secondary γ-emission. Possible advantages of $^{82}Br^-$ over $^{36}Cl^-$ in anion tracer measurements include, (a) a short lifetime ($t_{1/2}$ = 35.3 hr), which alleviates contamination and disposal problems, (b) high counting efficiency (1.54) due to the secondary radiation, (c) measurement with a γ-counter as well as a β-counter, (d) a simple preparation not requiring subsequent purification steps giving a specific activity depending on the irradiation time. With 6 hr irradiation time the specific activity was sufficient to make measurements with <1 mM Br^-, which is less than the bromide concentration known to affect the properties of $GABA_A$ receptor. The radiotracers, $^{82}Br^-$ and $^{36}Cl^-$ could be compared with the same solution composition. In conditions where a direct effect of binding of halide to receptor does not contribute to a difference in measured ion-flux, $^{82}Br^-$ was translocated only marginally faster than $^{36}Cl^-$. The effect of chlordiazepoxide (CDPX) (2-250 μM) on the progress of GABA (10 μM)-mediated $^{82}Br^-$ uptake was measured in a time range of 200 msec to 20 sec using quench-flow technique. The two phases of anion exchange previously reported in this experimental model with GABA alone were observed. The rate of $^{82}Br^-$ exchange was increased 2.3-fold at 30–60 μM CDPX and was not further increased with increasing [CDPX]. The rate of halide exchange is a measure of open channel concentration. The isotope exchange rate constant, J, in a membrane vesicle preparation, is a measure of the membrane permeability per internal volume/surface area, $J = P_mA/V$. Receptor desensitization rate was also increased by CDPX, but unlike the isotope exchange rate, it continued to increase up to at least 250 μM CDPX.

Key words: Membrane vesicles — Quench flow — Kinetics — Bromide permeability — Ion flux — GABA receptor

Introduction

γ-Aminobutyrate ($GABA_A$) receptors (Schwartz, 1988; Stephenson, 1988; Haefely, 1990; Knapp, Malatynska & Yamamura, 1990; Olsen & Tobin, 1990; Whiting, McKernan & Iversen, 1990; Kofuji et al. 1991; Kardos, 1993) function by forming an open channel across cell membrane, through which chloride ion can diffuse, in response to the binding of GABA. An additional response to GABA binding is the progressive loss of channel opening activity during exposure to GABA (desensitization), which occurs much more slowly than channel opening and closing. $GABA_A$ receptors are the major class of inhibitory receptors in the brain. They are involved in numerous types of processing and their modulation by pharmacological or physiological factors can have a variety of effects.

$GABA_A$ receptor responses are modulated by the benzodiazepine class of drugs. In particular the tranquilizer, chlordiazepoxide (Librium, CDPX) increased transmembrane chloride conductance mediated by GABA (Macdonald & Barker, 1978; Choi, Farb & Fischbach, 1981) and $GABA_A$ receptor desensitization in electophysiological measurements (Mierlak & Farb, 1988; Farrant, Gibbs & Farb, 1990). Enhancement of GABA receptor mediated responses is believed to be the basis of the pharmacological activity of this drug. Benzodiaz-

Correspondence to: D.J. Cash

epine derivatives which are anxiolytic, including CDPX, increased the GABA-mediated transmembrane flux of $^{36}Cl^-$ in membrane vesicles (Lehoullier & Ticku, 1987; Allan et al., 1985; Harris & Allan, 1985; Malatynska et al., 1989; Taguchi & Kuriyama, 1990; Serfozo & Cash, 1992) as well as the chloride conductance (Macdonald & Barker, 1978; Choi, Farb & Fischbach, 1981; Chan & Farb, 1985; Bormann & Kettenmann, 1988). The response curve for channel opening is shifted to lower GABA concentrations. Behavioral syndromes which involve the action of $GABA_A$ receptors and are affected by these anxiolytic drugs include the effects of alcohol, stress and epilepsy. For example, the effect of certain stress protocols on animals caused a rapid change in GABA receptor mediated $^{36}Cl^-$ flux and also a decrease in binding of benzodiazepine derivatives (Drugan et al., 1989): however the enhancement of binding of diazepines by chloride ion was increased (Havoundjian, Paul & Skolnick, 1986*a,b*). These effects paralleled the increase in the number and affinity of binding sites for t-butylbicyclophosphorothionate (^{35}S-TBPS), a $GABA_A$ receptor inhibitor and convulsant which binds at a site that changes on channel opening (Havoundjian, Paul & Skolnick, 1986; Havoundjian & Skolnick, 1986). Stress decreased the antiseizure activity of the benzodiazepine, flunitrazepam (Deutsch, Park & Hitri, 1994).

Ethanol enhanced GABA-mediated $^{36}Cl^-$ flux with vesicles, at the concentrations of its behavioral effects (Suzdak et al., 1986; Allan & Harris, 1987; Glowa et al., 1988; Mehta & Ticku, 1988; Ticku, 1989; Harris, 1990). Ethanol decreased the effect of flunitrazepam to increase GABA stimulated $^{36}Cl^-$ flux and increased the inhibition of chloride flux by drugs which had the opposite (inhibitory) effect (Buck & Harris, 1990). After chronic administration of alcohol to rats, tolerance and withdrawal symptoms were observed. The withdrawal symptoms could be decreased by a single injection of flunitrazepam, which apparently reset the mechanism for tolerance and dependence (Buck, Heim & Harris, 1991). The GABA-mediated $^{36}Cl^-$ flux remained unaltered (Mihic et al., 1992), but the binding of derivatives which reduce the effects of alcohol was altered (Suzdak et al., 1986; Lister, 1988; Mhatre, Mehta & Ticku, 1988; Durcan & Lister, 1989).

Experimental approaches to how these proteins function at the chemical level include; (i) electrophysiological measurements of changes in the electrical properties of cell membrane; (ii) measurements of binding to the receptor, of natural and pharmacological ligands, and (iii) measurements of changes in membrane permeability using $^{36}Cl^-$ radiotracer. Rapid mixing, chemical kinetic techniques with reaction times of a few milliseconds and above allow the study of the responses of active (undesensitized) receptor, which, in the presence of GABA, is in rapid equilibrium with its open-channel state. GABA-mediated influx of $^{36}Cl^-$ radiotracer into membrane vesicles containing $GABA_A$ receptor has been used as a measure of receptor-mediated transmembrane halide exchange, the rate of which is proportional to open channel concentration. Measurements following the whole time course of the isotope equilibration and desenzitization allow the independent determination of channel opening equilibrium and desenzitization rates.

The use of $^{36}Cl^-$ as an isotope tracer for transmembrane chloride transport is relatively expensive and requires disposal of radioactive waste. Moreover this isotope requires purification and has sometimes been supplied in a condition not giving a satisfactory filter disc assay. In this work, we investigate the use of $^{82}Br^-$ as a radiotracer[1] for transmembrane chloride transport. This isotope is as permeable through $GABA_A$ receptor channel as chloride and has the advantages of relatively low cost, short lifetime and high counting efficiency. Bromide is more like chloride than is iodide and, unlike iodide, is not oxidized in solution.

Because of interest in the effects on a protein mechanism, of various molecules, binding at several interacting sites on the protein complex, as well as the physiological and pharmacological corollaries, the effect of a benzodiazepine, over the whole concentration range of its response, is investigated with an experimental model previously characterized (Cash & Subbarao, 1987*a,b*). Using $^{82}Br^-$, the effect of a tranquilizing drug of the benzodiazepine family, CDPX on the response of a rapidly desensitizing GABA receptor ($t_{1/2}$ = 33 msec at saturation with GABA) in native membrane freshly prepared from rat cerebral cortex is examined.

Materials and Methods

Membrane Vesicle Suspension

Male Sprague Dawley rats, 4–6 weeks old, were decapitated by guillotine. The brain was immersed in solution B (in mM): 145 NaCl, 5.0 KCl, 1.0 $MgCl_2$, 1.2 $CaCl_2$, 10 glucose, 10 HEPES[1], pH 7.5 and the cerebral cortex was dissected. 1 mm slices of cerebral cortex were suspended in 30 ml solution A (320 mM sucrose, 10 ml HEPES, pH 7.5) containing the protease inhibitors, phenylmethylsulfonyl fluoride (1 mM), aprotinin (10 μg/ml), antipain (5 μg/ml), leupeptin (5 μg/ml), pepstatin A (5 μg/ml) and the antioxidant, butylated hydroxytoluene (20 μM). All manipulations were performed at 0–4°. The mixture was homogenized with a Virtis 45 homogenizer (setting 30, 5sec). An equal volume of solution B was added and the mixture was centrifuged at 270 × *g* for 4 min. The supernatant was centrifuged at 6500 × *g* for 20 min. The pellet was resuspended in 8 ml solution B using a glass-Teflon hand homogenizer and centrifuged at 4000 × *g* for 15 min. The pellet was resuspended in solution B and adjusted to 750 μg protein/ml. Protein concentration was measured with the bicinchoninic acid method (Smith et al., 1985).

[1] $^{82}Br^-$ can be obtained from the Missouri University Research Reactor Center (MURR), Research Park, Columbia, Missouri, 65211.

Progress of $^{82}Br^-$ Influx

Rapid mixing and short reaction times were achieved by quench-flow technique (Cash & Hess, 1981) in continuous flow or pulsed mode (Fersht & Jakes, 1975) with an in-line filter disk assay (Cash et al., 1991). The experiments were performed at 30°C, pH, 7.5. The membrane vesicle preparation was warmed from 0°C within two min after loading into the machine and was held at 30°C for an additional minute before actuation. The ion flux was initiated (receptor channels opened) by mixing the membrane suspension (225 μl) with an equal volume of solution B containing $^{82}Br^-$ (25 μCi/ml) (this made the solution 0.18–0.65 mM Br^-, depending on the specific activity of the radioisotope) and GABA (and CDPX where stated). Channel opening was terminated by mixing with the same volume of solution B containing 3mM bicuculline methiodide (Olsen et al., 1975; Pong & Graham, 1972) and the mixture was rapidly passed through a glass fiber filter disk (Sleicher & Schuell No. 31) using a low vacuum (100 mm Hg below atmosphere). *It was found that glass fiber disks supplied by Whatman or Fisher since 1989 are no longer satisfactory for this assay due to inaccurate, as well as very imprecise results (this also applies to the $^{36}Cl^-$ flux assay).* The membrane, retained on the disk, was washed with solution B (10 ml) three times so that the total contact time with the wash solution was about 5 sec. The disks were dried and counted with a scintillation coctail (Bio-Safe NA, Research Products) for 10 min. The measured counts were corrected for $^{82}Br^-$ decay ($t_{1/2}$ = 35.3 hr) by normalizing to the first count (minus counter background) by the equation;

cpm (corr.) = (cpm − counter background)/(exp(−ln2 × time elapsed (hr)/35.3)).

The (−)bicuculline methiodide (Simonyi et al., 1989) was synthesized by methylation of (+) bicuculline (Sigma Chemical) in methylene dichloride (Cash & Subbarao, 1987). It has previously been demonstrated that this quenching of halide flux is sufficiently rapid (Cash & Subbarao, 1987). The baseline was not decreased in the presence of 50 μM bicuculline methiodide. For all points, the total flux including the GABA-mediated influx and the unspecific background (baseline) were each determined in triplicate. In experiments with less than 10 μM GABA, the GABA uptake inhibitor, nipecotic acid (1 mM) was added to the solution.

The baseline count was typically 1620 c/10 min at short times, rising to 6280 c/10 min at 10 sec. The maximal GABA-mediated influx was 4700 c/10 min. For comparison, when $^{36}Cl^-$ was used (mixing the vesicle suspension with 10 μCi/ml $^{36}Cl^-$ (New England Nuclear)), the background count was 820 c/10 min rising in 10 sec to 2810 c/10 min and the maximal GABA mediated influx was 1300 c/10 min. The precision of the $^{82}Br^-$ uptake measurements was normally 2.7% (e.g., 1257 ± 35 c/10 min) to 4.6%, hence the precision of the GABA-mediated flux was approx. 7%.

Preparation of ^{82}Br Isotope [1]

High purity ammonium bromide (99.999%, Puratronic, Johnson Matthey) (4.0 mg, 0.0414 m mol.) sealed in a quartz vial in an aluminum can was irradiated at a thermal neutron flux of 6.5×10^{13} neutrons cm^{-2} s^{-1} and resonance flux of 2.0×10^{12} neutrons cm^{-2} s^{-1} for 6 hr in the reflector, behind additional moderators (University of Missouri Research Reactor). The samples were allowed to decay for 15 hr to reduce radiation from other short-lived bromine radionuclides and from induced activity in the quartz vial. These short-lived bromine radionuclides included $^{82m}Br^-$ ($t_{1/2}$ = 6.13 min), $^{80}Br^-$ ($t_{1/2}$ = 17.68 min) and $^{80m}Br^-$ ($t_{1/2}$ = 4.42 hr). The target was dissolved in deionized water (600 μl). The $^{82}Br^-$ was allowed to stand for a further 36 hr to ensure that $^{80}Br^-$ was less than 5% of the $^{82}Br^-$ radioactivity. Recoveries averaged 65–70% based on the predicted radioactivity from the measured neutron fluxes.

From 4.0 mg ammonium bromide irradiated for 6 hr, 3–4 mCi $^{82}Br^-$ was obtained. After 36 hr decay, the specific activity of the $^{82}Br^-$ was 1.5 mCi/mg Br^-. High resolution gamma-ray spectroscopy (quality control on 10 runs) revealed no gamma-emitting impurities (measured using a Nuclear Data 6700 instrument, with an intrinsic Ge detector and the emission at 5543 or 776 keV as the reference). The radioactive decay of samples from three different runs (subsequent to the $^{80}Br^-$ decay) was followed by repeated liquid scintillation counting, until reaching the background counts and was consistent with a single exponential decay of $^{82}Br^-$. The final solution was 4.9–8.2 mCi/ml (0.59 ml 70.17 mM Br^-).

$^{82}Br^-$ decays by β emission, with multiple secondary γ-ray emissions. These effectively made the scintillation counting efficiency equivalent to 1.54. Gamma-rays of interest include (with abundances in parenthesis); 554.3 keV (70.76%), 619.1 keV (43.4%), 698.4 keV (28.5%), 776.5 keV (83.5%), 827.8 KeV (24.0%), 1044.1 keV (27.2%), 1317.5 keV (26.5%), 1474.9 keV (16.3%).

Materials

All water was purified to a resistance of 17 megohm cm^{-1} and passed through a 0.22 μm Millipore filter. GABA, butylated hydroxytoluene, (+)bicucculine, chlordiazepoxide, and the protease inhibitors were from Sigma Chemical. HEPES was from Calbiochem.

Abbreviations

GABA, γ-aminobutyrate; CDPX, chlordiazepoxide; TBPS, t-butylbicyclophosphorothionate; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonate.

Results and Discussion

Radionuclide

The radionuclide, ^{82}Br can be produced from the naturally abundant bromine isotope, ^{81}Br by capture of a neutron according to the reaction, $^{81}Br(n,\gamma)^{82}Br$. (Naturally occurring bromine contains 50.69% ^{79}Br and 49.31% ^{81}Br.) This preparation requires irradiation of the sample in a neutron flux and subsequent dissolution and dilution to the required strength. The conversion increases with the duration of irradiation. In the present work, a specific activity sufficient to measure halide exchange or influx into membrane vesicles, while maintaining a low total bromide concentration (<0.5 mM) was obtained with six hours exposure to the neutron flux.

Advantages of ^{82}Br as a halogen radioactive tracer are its relatively low cost or ease of preparation, its short lifetime ($t_{1/2}$ = 35.3 hr), eliminating problems and the expense of long-term contamination and disposal, its detection in gamma counters as well as beta counters, and its greater efficiency of counting (apparently 1.54 in the

scintillation counter). The chloride radioisotope ^{36}Cl in contrast must be purified, has a relatively high cost, a long lifetime and is not detected in gamma counters. Other alternative halide tracers are isotopes of iodine. Iodide is chemically less like chloride than is bromide and, unlike bromide, iodide is relatively easily oxidized to iodine with the possibility of increasing the background measurement by labeling the membrane.

Bromide is an analogue of chloride in transport through many anion channels, including those of $GABA_A$ receptors. When experiments can be performed within a few days after its provision, $^{82}Br^-$ is a suitable radiotracer to use in studies of anion transport.

GABA-Mediated Halide Exchange

After homogenizing a region of brain, a preparation can be obtained, containing sealed vesicles, formed from cell membrane with active (capable of forming open channel) GABA receptor (Sanchez, Toledo & Gonzalez, 1984; Allan et al., 1985; Harris & Allan, 1985; Subbarao & Cash, 1985; Schwartz et al., 1986). By using $^{36}Cl^-$ as the receptor-permeant radioisotope tracer, the concentration of open channel could be measured as well as the attenuation of channel opening activity (desensitization) which takes place on exposure of the membrane to GABA (Cash & Subbarao, 1987). The whole time course of isotope influx could be measured using rapid reaction techniques.

When the $GABA_A$ receptor channels are opened, the rapid adjustment of membrane potential, to the value determined by the chloride concentrations on each side of the membrane, which occurs if the membrane potential was not previously solely controlled by the chloride concentrations, corresponds to a very small net transfer of halide. The radioisotope influx associated with this is negligible relative to the subsequent stoichiometric transmembrane radiotracer exchange, at a constant membrane potential, the rate of which is proportional to the open channel concentration, $[R']f_o$ (Appendix 2). When the progress of $^{36}Cl^-$ influx is followed (e.g., Fig. 1), the transmembrane anion exchange rate constant, J is given by the slope of the influx curve (isotope exchange rate) divided by the uncompleted fraction of the equilibrium isotope exchange (Eq. 1):

$$J = (d[^*X^-]/dt)/(1 - [^*X^-]_t/[^*X^-]_\infty) \quad (1)$$

where the symbols are defined as in Appendix 1. In practice the initial value of J, before its attenuation by desensitization, is determined by fitting Eq. A1 (Appendix 1) to measurements of the whole time course of the radiotracer influx (Cash & Hess, 1980). It was found previously, with this preparation, that GABA-mediated chloride flux takes place in two phases, each of which is

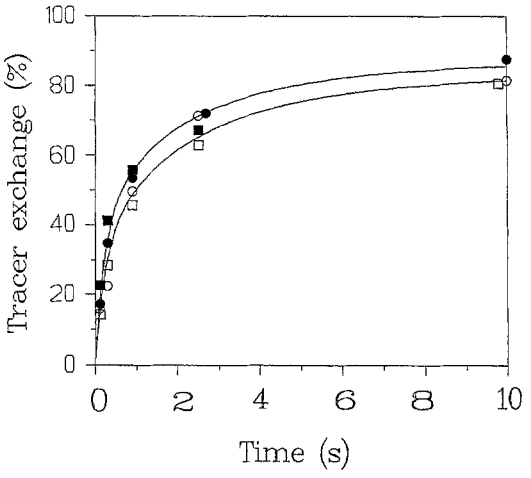

Fig. 1. Influx of $^{82}Br^-$ (closed symbols) and $^{36}Cl^-$ (open symbols) mediated by GABA (40 μM). Two experiments (squares and circles), with different membrane preparations from different rats, are shown. In each experiment $^{82}Br^-$ influx and $^{36}Cl^-$ influx were measured separately with the same membrane preparation in the same buffer solution (soln. B). The lines are computed with Eq A1, using the values; for $^{82}Br^-$; $J_A = 1.8\ s^{-1}$; $\alpha = 3.5\ s^{-1}$; $J_B = 0.39\ s^{-1}$; $\beta = 0.25\ s^{-1}$; and for $^{36}Cl^-$; $J_A = 1.4\ s^{-1}$; $\alpha = 3.5\ s^{-1}$; $J_B = 0.32\ s^{-1}$; $\beta = 0.25\ s^{-1}$.

attenuated by a desensitization process. These two phases have been attributed to two distinguishable receptors with different rates of desensitization (Cash & Subbarao, 1987 *a,b*). The faster desensitizing receptor present has, initially, 4/5 of the channel opening activity and is desensitized about sixteen times faster than the next preponderant $GABA_A$ receptor.

Effect of Chlordiazepoxide on GABA Receptor-mediated Halide Exchange

The effect of various concentrations of CDPX on GABA-mediated halide exchange was studied by following $^{82}Br^-$ uptake as a measure of transmembrane halide exchange. Influx of $^{82}Br^-$ mediated by 10 μM GABA is shown in Fig. 2. This concentration of GABA is at the foot of the receptor response curve of J_A/[GABA] (Cash & Subbarao, 1987) where the percentage increase in halide exchange rate by CDPX is greatest (Serfozo & Cash, 1992). The initial rate with 10 μM GABA was approximately doubled by the presence of 30 μM CDPX. The initial rate constant, J_A increased from 0.51 s^{-1} to 1.14 s^{-1} and was not significantly altered by further increases in CDPX concentration up to 250 μM. although desensitization rates measured from the progress of $^{82}Br^-$ exchange continued to increase up to 250 μM GABA (Fig. 2).

In the experimental model studied in this work, the two phases of halide exchange, observed with GABA alone, are retained in the presence of CDPX (Fig. 3).

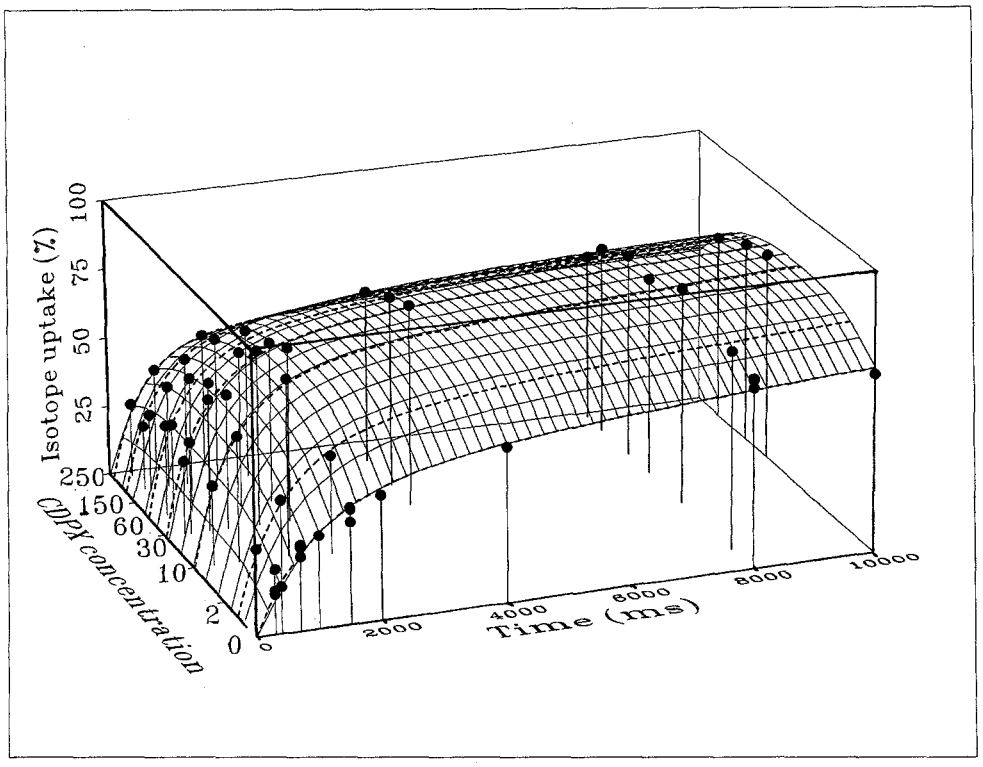

Fig. 2. $^{82}Br^-$ uptake mediated by 10 μM GABA with varying CDPX concentration. At the front is the progress of isotope uptake with GABA alone. Towards the back the CDPX concentration is increased (on a logarithmic scale). The broken lines are calculated from Eq. A1 fitted to the measurements. The initial rate of $^{82}Br^-$ uptake was doubled by 30 μM CDPX. At the highest CDPX concentrations, the final isotope exchange is decreased by increased desensitization rates. Values of the parameters of the dashed lines are; (0 μM CDPX) $J_A = 0.51\ s^{-1}$; $\alpha = 0.75\ s^{-1}$; $J_B = 0.045\ s^{-1}$; $\beta = 0.030\ s^{-1}$; (2 μM CDPX) $J_A = 0.80\ s^{-1}$; $\alpha = 0.82\ s^{-1}$; $J_B = 0.050\ s^{-1}$; $\beta = 0.030\ s^{-1}$; (10 μM CDPX) $J_A = 1.1\ s^{-1}$; $\alpha = 1.0\ s^{-1}$; $J_B = 0.069\ s^{-1}$; $\beta = 0.060\ s^{-1}$; (30 μM CDPX) $J_A = 1.1\ s^{-1}$; $\alpha = 1.1\ s^{-1}$; $J_B = 0.085\ s^{-1}$; $\beta = 0.11\ s^{-1}$; (60 μM CDPX) $J_A = 1.1\ s^{-1}$; $\alpha = 1.3\ s^{-1}$; $J_B = 0.11\ s^{-1}$; $\beta = 0.17\ s^{-1}$; (150 μM CDPX) $J_A = 1.0\ s^{-1}$; $\alpha = 1.5\ s^{-1}$; $J_B = 0.10\ s^{-1}$; $\beta = 0.17\ s^{-1}$; (250 μM CDPX) $J_A = 1.0\ s^{-1}$; $\alpha = 1.6\ s^{-1}$; $J_B = 0.080\ s^{-1}$; $\beta = 0.30\ s^{-1}$.

In the presence of this drug, the halide exchange is faster in both phases and is desensitized faster in both phases.

Ion-flux Rate and Permeability

The membrane permeability is linearly related to the ion flux rate (Hess et al., 1984), for a given vesicle size and shape. The measured ion-flux rate constant, J, which would vary with vesicle size (Cash et al., 1988) is a measure of membrane permeability (P_m) per vesicle volume/surface ratio (Appendix 2).

Tracer exchange, in a system in quasi-equilibrium, will be kinetically first order, regardless of the mechanism (McKay, 1943). In principle, the rate of equilibration of specific activity between compartments depends on the rate constants in both directions (Appendix 3). However the values are weighted in favor of the solution undergoing the greater concentration (specific activity) change (Eq. A5), and since the internal volume of the vesicles is very much smaller than the external volume, the rate depends on the efflux rate constant (Takeyasu, Udgaonkar & Hess, 1983). If the tracer ion is translocated at a different rate from the pertinent permeant ion, the appropriate exchange rate constant can be obtained by dividing the measured rate constant by the relative permeability of the radiotracer (Appendix 3).

Comparison of GABA-mediated $^{82}Br^-$ Flux with $^{36}Cl^-$ Flux

The progress of GABA-mediated influx of $^{82}Br^-$ was compared with that of $^{36}Cl^-$ with the same membrane preparation, in the same experimental session and in the same solution (154 mM Cl^- containing 0.3 mM Br^-). The $^{82}Br^-$ uptake was only marginally higher than that of $^{36}Cl^-$ (Fig. 1). The initial flux rate constants were obtained by fitting Eq. A1 (Appendix 1) to the experimental points. The relative permeability calculated from the radiotracer exchange rate constants was $^{82}Br^-/^{36}Cl^- = 1.19 \pm 0.26$ for transport through this $GABA_A$ receptor channel. The precision of the measurements of $^{82}Br^-$ uptake was ± 3–5% (standard deviation) varying slightly

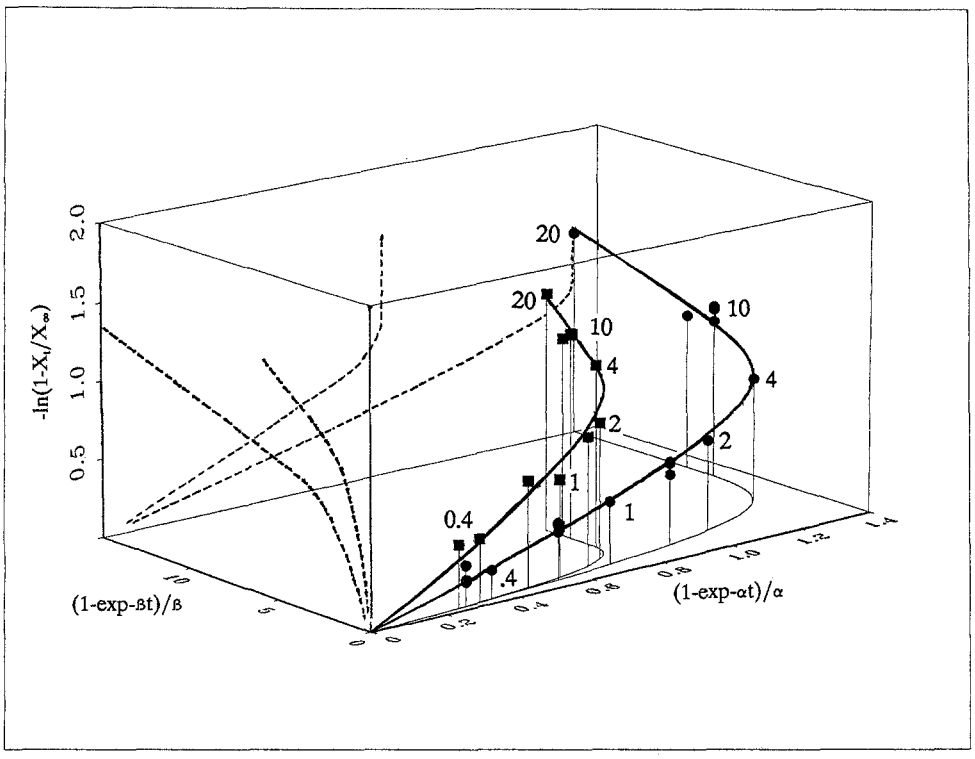

Fig. 3. Progress of $^{82}Br^-$ isotope exchange with 10 μM GABA in the absence (●) and presence (■) of CDPX (60 μM). This plot, according to Eq. A1., demonstrates the biphasic nature of the $^{82}Br^-$ influx. The fitted lines correspond to the values given in Fig. 2 legend. The reaction times (seconds) are indicated on the lines. Each line lies in a plane (*not shown* for simplicity) corresponding to its values of J_A and J_B and passing through the origin. Drop lines from the data points extend to a projection of the line on the bottom plane. The broken lines are projections of the fitted lines onto the plane surfaces corresponding to plots of $-\ln(1 - X_t/X_\infty)$ against $(1 - \exp - \alpha t)/\alpha$ and $(1 - \exp - \beta t)/\beta$ respectively.

with the membrane preparation and filter disk batch. Thus the precision of the GABA specific $^{82}Br^-$ influx was ≈7%. The precision of the rate constants was approx. 20%. The composition of the solution in the $^{82}Br^-$ experiment differed from that in the $^{36}Cl^-$ experiment only by the presence of less than 1 mM bromide ion (0.2 mM in a typical experiment). Bromide concentration could be further reduced by increasing the specific activity of $^{82}Br^-$ by increasing the duration of neutron irradiation during preparation.

In aqueous solution, the diffusion coefficients, limiting conductances and mobilities of chloride and bromide are similar ($Br^-/Cl^- = 1.025$). The Pauling crystal radius of bromide is slightly larger than chloride ($Br^-/Cl^- = 1.09$) and the estimated hydrated, Stokes' radius is slightly smaller than chloride ($Br^-/Cl^- = 0.98$) (Araki, Ito & Oscarsson, 1961; Ito, Kostyuk & Oshima, 1962; Wright & Diamond, 1977; Edwards, 1982). However, on the basis of electrophysiological measurements of GABA receptor-mediated halide transport in different systems, bromide has appeared more permeable than chloride (Gallagher, Higashi & Nishi, 1978). Reported values for the relative permeability determined from reversal potentials have been, $Br^-/Cl^- = 2.1$ in frog dorsal root ganglia (Inomata et al., 1986), 1.7 (Hamill, Bormann & Sakmann, 1983) and 1.5 (Bormann, Hamill & Sakmann, 1987) in cultured mammalian spinal neurons, 1.29 in crayfish neuromuscular junction (Takeuchi & Takeuchi, 1969) and 1.21 in mammalian dorsal root ganglia (Takeuchi & Takeuchi, 1971). It was inferred that receptor-mediated transport is a property of the channel as well as the anion, on the basis of the lack of correspondence of $GABA_A$ receptor channel permeability and of receptor mediated conductance (Ito, Kostyuk & Oshima, 1962; Edwards, 1982; Hamill, Bormann & Sakmann, 1983; Inomata et al., 1986; Bormann, Hamill, & Sakmann, 1987; Robertson, 1989) with the properties of the permeating ions in bulk aqueous solution. Different sequences of anion permeability through various channels could be explained by the binding of anion at the channel with a binding energy including different contributions with different dependencies on anion size.

In general, bromide has been found to bind to globular proteins more strongly than chloride and has higher permeability than chloride through many biological channels. The free energy of hydration of chloride is larger than that of bromide, although the entropy decrease is larger (Wright & Diamond, 1977; Edsall &

McKenzie, 1978). The lower energy of dehydration of bromide may contribute to its stronger binding at charged and/or hydrophobic sites.

Effect of Bromide on GABA Receptor

Comparison of the permeability of bromide ion, through $GABA_A$ receptor channel, with that of chloride has been obscured by evidence that the binding of anions, including chloride and bromide, alters the properties of the receptor. The electophysiological measurements mentioned above and some isotope tracer flux measurements (Tehrani et al., 1986; Luu et al., 1987) were made by the complete or partial substitution of bromide for chloride on the extracellular side of the receptor. Anions, including bromide, which can permeate the receptor channels (Araki, Ito & Oscarsson, 1961; Mohler & Okada, 1978) can alter the properties of $GABA_A$ receptor (Brookes & Werman, 1973). For example, binding affinities for various ligands were altered by bromide more than by chloride (Enna & Snyder, 1977; Mackerer & Kochman, 1978; Martin & Candy, 1978; Milbrath et al. 1979; Costa, Rodbard & Pert, 1979; Olsen, 1981; Olsen & Snowman, 1982; Supavilai et al. 1982; Squires et al. 1983; Supavilai & Karobath, 1984; Havoundjian, Paul, & Skolnick, 1986*a;* Maksay & Simonyi, 1986; Garrett, Blume & Abel, 1989).

$GABA_A$ receptor-mediated flux of chloride was increased by external bromide. For example the influx of $^{36}Cl^-$ in 5 sec, mediated by the GABA mimetic, muscimol (Luu et al., 1987) and the GABA-mediated efflux of chloride ion measured electophysiologically (Robertson, 1989) were increased when chloride ion outside was replaced by bromide. The inhibitory postsynaptic potential due to open $GABA_A$ receptor channel was prolonged with bromide ion substituted for chloride (Takeuchi & Takeuchi, 1971; Wright & Diamond, 1977; Onodera & Takeuchi, 1979; Adams, Gage, & Hamill, 1982; Robertson, 1989).

These effects of permeant anions, on the extracellular side of the receptor, became significant at concentrations above 10 mM bromide. In the experiments described herein, the total concentration of bromide ion was less than 1 mM in the buffer solution which contained 154 mM chloride as usual. Therefore the effect on receptor mechanism due to binding of bromide at an external site is not a significant contribution to an apparent difference between the transmembrane rates of $^{82}Br^-$ and $^{36}Cl^-$ radiotracers in these conditions. Any difference observed depends on a difference in permeability.

The authors thank the staff of the University of Missouri Research Reactor Center, Columbia (MURR) for their encouragement and help and for provision of $[^{82}Br]NH_4Br$. This work was supported in part by a grant from the Research Council of the University of Missouri Medical School and in part by the Missouri Agricultural Experiment Station (No. BCHB0307). P.S. held a Missouri Institute of Psychiatry fellowship.

References

Adams, D.J., Gage, P.W., Hamill, O.P. 1982. Inhibitory postsynaptic currents at Aplysia cholinergic synapses: effects of permeant anions and depressant drugs. *Proc. R. Soc. Lond.* **214:**2–50

Allan, A.M., Harris, R.A., Subbarao, K., Cash, D.J. 1985. Demonstration of GABA-stimulated $^{36}Cl^-$ flux with isolated brain membranes. *Fed. Pros.* **44:**1634

Allan, A.M., Gallaher, E.J., Gionet, S.E., Harris, R.A. 1988. Genetic selection for benzodiazepine ataxia produces functional changes in the gamma-aminobutyric acid receptor chloride channel complex. *Brain Res.* **452:**118–126

Allan, A.M. Harris, R.A. 1987. Acute and chronic ethanol treatments alter GABA receptor-operated chloride channels. *Pharmacology, Biochemistry & Behavior* **27:**665–670

Araki T., Ito, M., Oscarsson, O. 1961. Anion permeability of the synaptic and nonsynaptic motoneurone membrane. *J. Physiol.* **159:**410–435

Bormann, J., Hamill, O.P., Sakmann, B. 1987. Mechanisms of anion permeation through channels gated by glycine and gamma-aminobutyric acid in mouse cultured spinal neurones. *J. Physiol.* **385:**243–286

Bormann, J., Kettenmann, H. 1988. Patch-clamp study of gamma-aminobutyric acid receptor Cl-channels in cultured astrocytes. *Proc. Natl. Acad. Sci. USA* **85:**9336–9340

Brookes, N., Werman, R. 1973. The cooperativity of gamma-aminobutyric acid action on the membrane of locust muscle fibers. *Mol. Pharmacol.* **9:**571–579

Buck, K.J., Harris, R.A. 1990. Benzodiazepine agonist and inverse agonist actions on GABAA receptor-operated chloride channels. II. Chronic effects of ethanol. *J. Pharmacol. Exp. Ther.* **253:**713–719

Buck, K.J., Heim, H., Harris, R.A. 1991. Reversal of alcohol dependence and tolerance by a single administration of flumazenil. *J. Pharmacol. Exp. Ther.* **257:**984–989

Cash, D.J., Hess, G.P. 1980. Molecular mechanism of acetylcholine receptor-controlled ion translocation across cell membranes. *Proc. Natl. Acad. Sci. USA* **77:**842–846

Cash, D.J., Hess, G.P. 1981. Quenched flow technique with plasma membrane vesicles: acetylcholine receptor-mediated transmembrane ion flux. *Anal. Biochem.* **112:**39–51

Cash, D.J., Subbarao, K. 1987*a*. Desensitization of gamma-aminobutyric acid receptor from rat brain: two distinguishable receptors on the same membrane. *Biochemistry* **26:**7556–7562

Cash, D.J., Subbarao, K. 1987*b*. Channel opening of gamma-aminobutyric acid receptor from rat brain: molecular mechanisms of the receptor responses. *Biochemistry* **26:**7562–7570

Cash, D.J., Langer, R.M., Subbarao, K., Bradbury, J.R. 1988. Transmembrane flux and receptor desensitization measured with vesicles: Homogeneity of vesicles investigated by computer simulation. *Biophys. J.* **54:**909–919

Cash, D.J., Subbarao, K., Bradbury, J.R., Mayes, G.G. 1991. Filter assay technique and quench-flow experiments: examples of receptor-mediated transmembrane ion-exchange measured with membrane vesicles. *J. Biochem. & Biophys. Methods* **23:**151–161

Chan, C.Y., Farb, D.H. 1985. Modulation of neurotransmitter action: control of the gamma-aminobutyric acid response through the benzodiazepine receptor. *J. Neurosci.* **5:**2365–2373

Choi, D.W., Farb, D.H., Fischbach, G.D. 1981. Chlordiazepoxide se-

lectively potentiates GABA conductance of spinal cord and sensory neurons in cell culture. *J. Neurophysiol.* **45:**621–631

Costa, T., Rodbard, D., Pert, C.B. 1979. Is the benzodiazepine receptor coupled to a chloride anion channel? *Nature* **277:**315–317

Deutsch, S.I., Park, C.H., Hitri, A. 1994. Allosteric effects of a GABA receptor-active steroid are altered by stress. *Pharmacology, Biochemistry & Behavior* **47:**913–917

Drugan, R.C., Morrow, A.L., Weizman, R., Weizman, A., Deutsch, S.I., Crawley, J.N., Paul, S.M. 1989. Stress-induced behavioral depression in the rat is associated with a decrease in GABA receptor-mediated chloride ion flux and brain benzodiazepine receptor occupancy. *Brain Res.* **487:**45–51

Durcan, M.J., Lister, R.G. 1989. Reduction of the intoxicating effects of ethanol by drugs acting at the benzodiazepine-GABA receptor complex. *Pharmacology, Biochemistry & Behavior* **32:**667–670

Edsall, J.T., McKenzie, H.A. 1978. Water and proteins. I. The significance and structure of water; its interaction with electrolytes and non-electrolytes. *Adv. Biophys.* **10:**137–207

Edwards, C. 1982. The selectivity of ion channels in nerve and muscle. *Neurosci.* **7:**1335–1366

Enna, S.J., Snyder, S.H. 1977. Influences ions, enzymes, and detergents on gamma-aminobutyric acid-receptor binding in synaptic membranes of rat brain. *Mol. Pharmacol.* **13:**442–453

Farrant, M., Gibbs, T.T., Farb, D.H. 1990. Molecular and cellular mechanisms of GABA/benzodiazepine-receptor regulation: electrophysiological and biochemical studies. *Neurochem Res.* **15:**175–191

Fersht, A.R., Jakes, R. 1975. Demonstration of two reaction pathways for the aminoacylation of tRNA. Application of the pulsed quenched flow techniques. *Biochemistry* **14:**3350–3356

Gallagher, J.P., Higashi, H., Nishi, S. 1978. Characterization and ionic basis of GABA-induced depolarizations recorded in vitro from cat primary afferent neurones. *J. Physiol.* **275:**263–282

Garrett, K.M., Blume, A.J., Abel, M.S. 1989. Effect of halide ions on [35S]butylbicyclophosphorothionate binding. *J. Neurochem.* **53:**935–939

Glowa, J.R., Crawley, J., Suzdak, P.D., Paul, S.M. 1988. Ethanol and the GABA receptor complex: studies with the partial inverse benzodiazepine receptor agonist Ro 15-4513. *Pharmacology, Biochemistry & Behavior* **31:**767–772

Haefely, W. 1990. The GABA-benzodiazepine interaction fifteen years later. *Neurochem. Res.* **15:**169–174

Hamill, O.P., Bormann, J., Sakmann, B. 1983. Activation of multiple-conductance state chloride channels in spinal neurones by glycine and GABA. *Nature* **305:**805–808

Harris, R.A. 1990. distinct actions of alcohols, barbiturates and benzodiazepines on GABA-activated chloride channels. *Alcohol* **7:**273–275

Harris, R.A., Allan, A.M. 1985. Functional coupling of gamma-aminobutyric acid receptors to chloride channels in brain membranes. *Science* **228:**1108–1110

Harris, R.A., Allan, A.M., Daniell, L.C., Nixon, C. 1988. Antagonism of ethanol and pentobarbital actions by benzodiazepine inverse agonists: neurochemical studies. *J. Pharmacol. Exp. Ther.* **247:**1012–1017

Havoundjian, H., Paul, S.M., Skolnick, P. 1986*a*. Rapid, stress-induced modification of the benzodiazepine receptor-coupled chloride ionophore. *Brain Res.* **375:**401–406

Havoundjian, H., Paul, S.M., Skolnick, P. 1986*b*. The permeability of gamma-aminobutyric acid-gated chloride channels is described by the binding of a "cage" convulsant, t-butylbicyclophosphoro[35S]thionate. *Proc. Natl. Acad. Sci. USA* **83:**9241–9244

Havoundjian, H., Skolnick, P. 1986. A quantitative relationship between Cl-enhanced [3H]flunitrazepam and [35S]t-butylbicyclophosphorothionate binding to the benzodiazepine/GABA receptor chloride ionophore complex. *Brain Res.* **387:**281–287

Hess, G.P., Aoshima, H., Cash, D.J., Lenchitz, B. 1981. Specific reaction rate of acetylcholine receptor-controlled ion translocation: a comparison of measurements with membrane vesicles and with muscle cells. *Proc. Natl. Acad. Sci.* USA **78:**1361–1365

Hess, G.P., Kolb, H.A., Lauger, P., Schoffeniels, E., Schwarze, W. 1984. Acetylcholine receptor (from Electrophorsus electricus): a comparison of single-channel current recordings and chemical kinetic measurements. *Proc. Natl. Acad. Sci. USA* **81:**5281–5285

Inomata, N., Oomura, Y., Akaike, N., Edwards, C. 1986. The anion selectivity of the gamma-aminobutyric acid controlled chloride channel in the perfused spinal ganglion cell of frog. *Neurosci. Res.* **3:**371–383

Ito, M., Kostyuk, P.G., Oshima, T. 1962. Further study on anion permeability of inhibitory postsynaptic membrane of cat motoneurones. *J. Physiol.* **164:**150–156

Kardos, J. 1993. The $GABA_A$ receptor channel mediated chloride ion translocation through the plasma membrane: new insights from 36Cl- ion flux measurements. *Synapse* **13:**74–93

Knapp, R.J., Malatynska, E., Yamamura, H.I. 1990. From binding studies to the molecular biology of GABA receptors. *Neurochem. Res.* **15:**105–112

Kofuji, P., Wang, J.B., Moss, S.J., Huganir, R.L., Burt, D.R. 1991. Generation of two forms of the gamma-aminobutyric acidA receptor gamma 2-subunit in mice by alternative splicing. *J. Neurochem.* **56:**713–715

Lehoullier, P.F., Ticku, M.K. 1987. Benzodiazepine and beta-carboline modulation of GABA-stimulated 36Cl-influx in cultured spinal cord neurons. *Eur. J. Pharmacol.* **135:**235–238

Lister, R.G. 1988. Interactions of ethanol with benzodiazepine receptor ligands in tests of exploration, locomotion and anxiety. *Pharmacology, Biochemistry & Behavior* **31:**761–765

Luu, M.D., Morrow, A.L., Paul, S.M., Schwartz, R.D. 1987. Characterization of $GABA_A$ receptor-mediated 36chloride uptake in rat brain synaptoneurosomes. *Life Sci.* **41:**1277–1287

Macdonald R., Barker, J.L. 1978. Benzodiazepines specifically modulate GABA-mediated postsynaptic inhibition in cultured mammalian neurones. *Nature* **271:**563–564

Mackerer, C.R., Kochman, R.L. 1978. Effects of cations and anions on the binding of 3H-diazepam to rat brain. *Proc. Soc. Exp. Biol. & Med.* **158:**393–397

Maksay, G., Simonyi, M. 1986. Kinetic regulation of convulsant (TBPS) binding by GABAergic agents. *Mol. Pharmacol.* **30:**321–328

Martin, I.L., Candy, J.M. 1978. Facilitation of benzodiazepine binding by sodium chloride and GABA. *Neuropharmacol.* **17:**993–998

Malatynska, E., Knapp, R., Ikeda, M., Yamamura, H.I. 1989. Beta-carboline interactions at the BZ-GABA receptor chloride-ionophore complex in the rat cerebral cortex. *Brain Research Bulletin* **22:**845–848

McKay, H.A.C. 1943. Kinetics of some exchange reactions of the type, RI + I^{*-} to RI* + I^- in alcoholic solution. *J. Am. Chem. Soc.* **65:**702–706

Mehta, A.K., Ticku, M.K. 1988. Ethanol potentiation of GABAergic transmission in cultured spinal cord neurons involves gamma-aminobutyric acidA-gated chloride channels. *J. Pharmacol. Exp. Ther.* **246:**558–564

Mhatre, M., Mehta, A.K., Ticku, M.K. 1988. Chronic ethanol administration increases the binding of the benzodiazepine inverse agonist and alcohol antagonist [3H]RO15-4513 in rat brain. *Eur. J. Pharmacol.* **153:**141–145

Mihic, S.J., Kalant, H., Liu, J.F., Wu, P.H. 1992. Role of the gamma-

aminobutyric acid receptor/chloride channel complex in tolerance to ethanol and cross-tolerance to diazepam and pentobarbital. *J. Pharmacol. Exp. Ther.* **261:**108–113

Mierlak, D., Farb, D.H. 1988. Modulation of neurotransmitter receptor desensitization: chlordiazepoxide stimulates fading of the GABA response. *J. Neurosci.* **8:**814–820

Milbrath, D.S., Engel, J.L., Verkade, J.G., Casida, J.E. 1979. Structure-toxicity relationships of 1-substituted-4-alkyl-2,6,7-trioxabicyclo[2.2.2.]octanes. *Toxicol. & Appl. Pharmacol.* **47:**287–293

Mohler, H., Okada, T. 1978. Properties of gamma-aminobutyric acid receptor binding with (+)-[3H]bicuculline methiodide in rat cerebellum. *Mol. Pharmacol.* **14:**256–265

Olsen, R.W. 1981. The GABA postsynaptic membrane receptor-ionophore complex. Site of action of convulsant and anticonvulsant drugs. *Molecular & Cellular Biochemistry* **39:**261–279

Olsen, R.W., Ban, M., Miller, T., Johnston, G.A. 1975. Chemical instability of the GABA antagonist bicuculline under physiological conditions. *Brain Res.* **98:**383–387

Olsen, R.W., Snowman, A.M. 1982. Chloride-dependent enhancement by barbiturates of gamma-aminobutyric acid receptor binding. *J. Neurosci.* **2:**1812–1823

Olsen, R.W., Tobin, A. J. 1990. Molecular biology of $GABA_A$ receptors. *FASEB J.* **4:**1469–1480

Onodera, K., Takeuchi, A. 1979. An analysis of the inhibitory postsynaptic current in the voltage-clamped crayfish muscle. *J. Physiol.* **286:**265–282

Pong, S.F., Graham, L.T. 1972. N-methyl bicuculline, a convulsant more potent than bicuculline. *Brain Res.* **42:**486–490

Robertson, B. 1989. Characteristics of GABA-activated chloride channels in mammalian dorsal root ganglion neurones. *J. Physiol.* **411:**285–300

Sanchez, C.M., Toledo, M.C., Gonzalez, M.P. 1984. The chloride channel opening by GABA as an energy dependent process. *Rev. Esp. Fisiol.* **40:**375–379

Schwartz, R.D. 1988. The $GABA_A$ receptor-gated ion channel: biochemical and pharmacological studies of structure and function. *Biochem. Pharmacol.* **37:**3369–3375

Schwartz, R.D., Skolnick, P., Seale, T.W., Paul, S.M. 1986. Demonstration of GABA/barbiturate-receptor-mediated chloride transport in rat brain synaptoneurosomes: a functional assay of GABA receptor-effector coupling. *Adv. Biochem. Psychopharmacol.* **41:**33–49

Serfozo, P., Cash, D.J. 1992. Effect of a benzodiazepine (chlordiazepoxide) on a $GABA_A$ receptor from rat brain. Requirement of only one bound GABA molecule for channel opening. *FEBS Lett.* **310:**55–59

Simonyi, M., Blasko, G., Kardos, J., Kajtar, M. 1989. The GABA antagonist (+)-bicuculline is levorotatory. *Chirality* **1:**178–179

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150:**76–85. **163:**279.

Squires, R.F., Casida, J.E., Richardson, M., Saederup, E. 1983. [35S]t-butylbicyclophosphorothionate binds with high affinity to brain-specific sites coupled to gamma-aminobutyric acid-A and ion recognition sites. *Mol. Pharmacol.* **23:**326–336

Stephenson, F.A. 1988. Understanding the $GABA_A$ receptor: a chemically gated ion channel. *Biochemical. J.* **249:**21–32

Subbarao, K., Cash, D.J. 1985. Functional responses of the gamma-aminobutyrate receptor from brain. *Soc. Neurosci. Abstr.* **11:**275

Supavilai, P., Karobath, M. 1984. [35S]-t-butylbicyclophosphorothionate binding sites are constituents of the gamma-aminobutyric acid benzodiazepine receptor complex. *J. Neurosci.* **4:**1193–1200

Supavilai, P., Mannonen, A., Collins, J.F., Karobath, M. 1982. Anion-dependent modulation of [3H]muscimol binding and of GABA-stimulated [3H]flunitrazepam binding by picrotoxin and related CNS convulsants. *Eur. J. Pharmacol.* **81:**687–691

Suzdak, P.D., Glowa, J.R., Crawley, J.N., Schwartz, R.D., Skolnick, P., Paul. S.M. 1986. A selective imidazobenzodiazepine antagonist of ethanol in the rat. *Science* **234:**1243–1247

Takeuchi, A., Takeuchi, N. 1969. A study of the action of picrotoxin on the inhibitory neuromuscular junction of the crayfish. *J. Physiol.* **205:**377–391

Takeuchi, A., Takeuchi, N. 1971. Variations in the permeability properties of the inhibitory post-synaptic membrane of the crayfish neuromuscular junction when activated by different concentrations of GABA. *J. Physiol.* **217:**341–358

Takeyasu, K., Udgaonkar, J.B., Hess, G.P. 1983. Acetylcholine receptor: evidence for a voltage-dependent regulatory site for acetylcholine. Chemical kinetic measurements in membrane vesicles using a voltage clamp. *Biochemistry* **22:**5973–5978

Taguchi, J., Kuriyama K. 1990. Functional modulation of cerebral gamma-aminobutyric acidA receptor/benzodiazepine receptor/chloride ion channel complex with ethyl beta-carboline-3-carboxylate: presence of independent binding site for ethyl beta-carboline-3-carboxylate. *J. Pharmacol. Exp. Ther.* **253:**558–566

Tehrani, M.H.J., Vaidyanathaswamy, R., Verkade, J.G., Barnes, E.M. 1986. Interaction of t-butylbicyclophosphorothionate with gamma-aminobutyric acid-gated chloride channels in cultured neurons. *J. Neurochem.* **46:**1542–1547

Ticku, M.K. 1989. Ethanol and the benzodiazepine-GABA receptor-ionophore complex. *Experientia* **45:**413–418

Whiting, P., McKernan, R.M., Iversen, L.L. 1990. Another mechanism for creating diversity in gamma-aminobutyrate type A receptors: RNA splicing directs expression of two forms of gamma 2 phosphorylation site. *Proc. Natl. Acad. Sci. USA* **87:**9966–9970

Wright, E.M., Diamond, J.M. 1977. Anion selectivity in biological systems. *Physiol. Rev.* **57:**109–156

Appendix 1

Progress of Transmembrane Isotope Exchange

The $GABA_A$ receptor-mediated transmembrane halide exchange (Figs. 1 and 2) has previously been shown to proceed in two phases due to two distinguishable types of receptor, with a 10- to 16-fold difference in rates of desensitization (Cash & Subbarao, 1987*b*). Equation A1 describes the receptor-mediated radiotracer-influx, with initial ion exchange rates given by the first order rate constants, J_A and J_B which are attenuated by desensitization processes with first order rate constants α and β respectively. $[^*X^-]_t$ and $[^*X^-]_\infty$ are the isotope concentrations inside the vesicles at time, t and at equilibrium respectively.

$$-ln\left(1-\frac{[^*X^-]_t}{[^*X^-]_\infty}\right)=J_A\left(\frac{1-\exp-\alpha t}{\alpha}\right)+J_B\left(\frac{1-\exp-\beta t}{\beta}\right) \qquad \text{(A1)}$$

This equation is a simple transformation of a form given previously (Cash & Subbarao, 1987). It illustrates that a plot of $-ln(1-[^*X^-]_t/[^*X^-]_\infty)$ against $(1-\exp-\alpha t)/\alpha$ and $(1-\exp-\beta t)/\beta$ describes a plane with slopes of J_A and J_B in the two time dimensions respectively (Fig. 3). A single phase of flux (e.g. Eq. A1 with $J_B = 0$,) would give a linear plot of $-ln((1-[^*X^-]_t/[^*X^-]_\infty)$ against $(1-\exp-\alpha t)/\alpha$.

Appendix 2

Ion-flux rate and Permeability

The initial rate of isotope exchange, mediated by a single type of receptor is,

$$\frac{d[*X^-]}{dt} = \bar{J}[R']f_0[*X^-] = J[*X^-] \tag{A2}$$

where $[*X^-]$ is the specific activity gradient, $[R']$ is the molar concentration of receptor with respect to volume inside the vesicles, f_o is the fraction of receptor in the open-channel state and J is the rate coefficient for ion translocation through open channel (Hess et al., 1984; Hess et al., 1981). $\bar{J}$ $(M^{-1}s^{-1})$ is a permeability coefficient, P_m $(cm\ s^{-1})$ per receptor density in the membrane $(mol\ cm^{-2})$.

The measured 1st order rate constant, J is,

$$J = \bar{J}[R']f_0\ s^{-1} \qquad [R'] = \frac{nA}{NV}M \tag{A3}$$

where N is Avogadro's number. There are n receptor molecules per unit surface area, A which encloses internal volume V. Hence,

$$J = P_m\frac{A}{V} = P_m\frac{N}{n}[R']\ s^{-1} \tag{A4}$$

which shows that the rate coefficient, from transmembrane ion-exchange measurements, is related to the membrane permeability coefficient by the surface/volume ratio. J is a permeability coefficient, P_m $(cm\ s^{-1})$ per mol receptor/receptor concentration (or per internal vol/surface area).

Appendix 3

Comparison of Different Permeabilities

Derivation of the rates of exchange in systems at equilibrium or quasi-equilibrium (McKay, 1943), adapted for exchange between two different volumes, predicts that relaxation to equalization of specific activity of tracer isotope in each volume will be kinetically 1st order. If the tracer ion is transferred at a rate different from the pertinent permeant ion, the progress of tracer influx is described by,

$$\frac{[*X^-]_t}{[*X^-]_\infty} = 1 - \exp - P_r(k_1\frac{V_I}{V_0} + k_0)t \tag{A5}$$

where $P_r = k_{Br}/k_{Cl}$ is relative permeability; k_I and k_o are translocation of chloride ion into and out of the vesicles respectively. $[*X^-]_t$ and $[*X^-]_\infty$ have the meanings described in Appendix 1. In the present experiments, with a suspension of vesicles, $V_I \ll V_o$ and the expression simplifies to,

$$\frac{[*X^-]_t}{[*X^-]_\infty} = 1 - \exp - Jt \tag{A6}$$

where $J = P_rk_o$. Thus the isotope exchange rate is determined by the rate constant for efflux from the vesicles (Takeyasu, Udgaonkar & Hess, 1983).