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Responses of γ-Aminobutyrate Receptor from Rat Brain: Similarity of Different Preparation Methods; Muscimol Induced Desensitization and Chloride Exchange

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Summary. Chloride-36 exchange into three different membrane vesicle preparations from rat brain homogenate was followed. The different preparations all contained the same sealed vesicular components characterized by their rates of chloride exchange. The GABA-mediated $^{36}Cl^-$ exchange in all the preparations occurred in two phases shown to be mediated by two distinguishable receptors present in the activity ratio of 5 : 1 as previously described (Cash, D.J., Subbarao, K. 1987. *Biochemistry* **26:**7556, 7562). Reported differences do not result from differences in the membrane preparations used or from the use of a GABA-mimetic instead of GABA, but from experimental differences. The preparations compared were made with mild or vigorous homogenization and with different extents of purification from solutes or membrane components: (*i*) a synaptoneurosome preparation, (*ii*) a Ficoll gradient preparation, and (*iii*) a washed P_2 preparation. In each preparation the same four populations of membrane vesicles were characterized by their $^{36}Cl^-$ influx rates: (*i*) a major population (40–50%) ($t_{1/2}$ = 1.4 min), (*ii*) a slower exchanging major population (40–55%) ($t_{1/2}$ = 24 min), (*iii*) a minor population (5–12%) containing active GABA receptor and having the GABA-independent permeability of the slower exchanging population, and (*iv*) a very small exchange (~2%) ($t_{1/2}$ ~ 0.2 sec). The GABA-independent $^{36}Cl^-$ exchange processes were kinetically first order. The relative quantities of the different vesicle populations varied slightly with the preparation and purification technique. The identity of these components, observed in the different preparations, was attributed to the vesicle formation being dependent on the morphology and properties of the membrane rather than the preparation method. The soluble brain extract was GABA-mimetic with the two observed receptors, causing channel opening and desensitization. But little washing of the membrane was required to observe the function of both receptors. Muscimol was GABA-mimetic with both receptors. With muscimol, channel opening occurred at 2.6-fold lower concentrations while desensitization was unaltered relative to GABA. This is additional evidence that these responses are mediated by different pairs of binding sites. The dependence of desensitization rate on muscimol concentration indicated that there are two binding sites mediating desensitization, as described with GABA.

Key Words membrane vesicles · quench flow · GABA receptor · chloride exchange · muscimol · rat brain

Introduction

Transmembrane chloride exchange, dependent on GABA receptor agonists and effectors, has been observed with preparations from brain homogenate containing sealed vesicles (Sánchez, Toledo & González, 1984; Schwartz, Skolnick & Hollingsworth, 1984; Allan et al., 1985; Subbarao & Cash, 1985). These observations provided an opportunity for the study of the responses of the GABA receptor by direct measurement of $^{36}Cl^-$ translocation in membrane preparations, which can be mixed rapidly with solutions of known and controlled composition. Measurements of the function of the GABA receptor could be made, which are complementary to the information from electrophysiological techniques (for reviews *see* Krnjevik, 1981; Enna & Gallagher, 1983) and measurements of high affinity ligand binding (for reviews *see* Olsen, 1982*a,b*; Snodgrass, 1983; DeFeudis, 1984*a,b;* Johnston, Allan & Skerritt, 1984). The whole progress of the $^{36}Cl^-$ exchange could be followed by making measurements in short times. Rate constants of $^{36}Cl^-$ exchange and receptor desensitization, which together control the extent of $^{36}Cl^-$ exchange, could be determined (Cash & Subbarao, 1987*a–d*).

These measurements of efflux (Schwartz et al., 1984; Schwartz et al., 1985) or of influx (Sánchez et al., 1984; Allan et al., 1985; Harris & Allan, 1985; Subbarao & Cash, 1985) of $^{36}Cl^-$ radiotracer were made with membrane preparations prepared in dif-

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ferent ways. Some differences in results were apparent. For example, a "Synaptoneurosome" preparation exhibited chloride exchange stimulated by pentobarbital (Schwartz et al., 1984; Schwartz, et al., 1985), which was not appreciable with other preparations (Allan & Harris, 1986*b;* Cash & Subbarao, 1988). This was also observed in influx measurements (Schwartz, Suzdak & Paul, 1986*b*) and was thought to indicate the presence of some endogenous GABA (Schwartz et al., 1984, 1985, 1986*a,b*) rather than a property of the membrane itself.

Desensitization of the receptor was followed by measuring the decrease in GABA-mediated influx of $^{36}Cl^-$, due to prior incubation of the membrane with neurotransmitter (Subbarao & Cash, 1985) using a preparation homogenized in sucrose solution, purified on a Ficoll gradient, and washed and resuspended in a solution containing glucose. In these experiments, two phases of desensitization were seen with rates increasing with GABA concentration. A slower phase with a half time of ~600 msec at saturation with GABA was preceded by a fast phase with a half time of ~40 msec at saturation. Subsequently, a similar experiment was made with the "Synaptoneurosome" preparation and muscimol (Schwartz et al., 1986*a,b*). In that experiment, desensitization was seen in several seconds without the initial fast phase. Although GABA-mediated $^{36}Cl^-$ influx progressed in two phases with the former preparation (Subbarao & Cash, 1985; Cash & Subbarao, 1987*a–d*), no initial rapid phase was reported with muscimol or pentobarbital with the "Synaptoneurosome" preparation (Schwartz et al., 1986*a,b*).

The fundamental properties of individual receptors in native membrane should be independent of the experimental procedure. It was important to establish which results are general properties of these membrane preparations and which are peculiar to particular experimental methods. Variations in procedure might conceivably have caused differences in the observations. (*i*) Differences in the specific membrane vesicles or in the state of the receptor(s) might have been caused by the different preparation techniques. (*ii*) Differences in the solution composition, including the presence of solutes extracted from the brain might have resulted from different preparation and washing procedures. (*iii*) Differences in the $^{36}Cl^-$ exchange might have resulted from differences in experimental design or technique, or from the use of difference agonists. The suitability of different preparations has been the subject of discussion (Harris & Allan, 1985; Schwartz et al., 1985; Allan & Harris, 1986*a;* Harris & Allan, 1986; Paul et al., 1986).

In this paper, we report an investigation of the effect of various experimental differences on the results observed, (*i*) differences in homogenization procedure, (*ii*) the presence of active substances in the brain extract, and (*iii*) the use of the GABA-mimetic, muscimol, instead of GABA. With three different membrane preparations, we compared the GABA receptor-mediated chloride exchange, as well as the unspecific (GABA-independent) chloride exchange, which reflects the total vesicular volume. (*i*) The "Synaptoneurosome" preparation (Hollingsworth et al., 1985), in which unbroken cells are removed by filtration after a mild homogenization, was considered suitable (Schwartz et al., 1985; Paul et al., 1986) because of the demonstrated presence of sealed postsynaptic vesicles said to result from the gentle treatment. A similar "Microsac" vesicle preparation (Harris & Allan, 1985; Allan & Harris, 1986*a,b*), also derived from the same preparation (Daly et al., 1980) has been used. These preparations are both homogenized by hand in buffer solution without sucrose, but differ by the presence of the filtration step in the former. (*ii*) A "Ficoll gradient" preparation (Subbarao & Cash, 1985; Cash & Subbarao, 1987*c*), purified after a vigorous, short homogenization in sucrose solution followed by subsequent removal of heavy components by centrifugation, was shown to exhibit GABA-mediated chloride exchange in two phases, each described by first order processes of $^{36}Cl^-$ exchange and desensitization (Cash & Subbarao, 1987*a–d*). (*iii*) A "Washed P_2" preparation prepared as (*ii*) but not purified on a Ficoll gradient, exhibited the same properties of GABA-mediated chloride exchange as (*ii*) (Cash & Subbarao, 1987*a–d*).

We found that these different preparations contained the same vesicular components. Two major components of approximately equal volume undergo GABA-independent chloride exchange. A small fraction of the internal volume undergoes GABA receptor-mediated chloride exchange. This occurs in two phases, due to two distinguishable receptors with muscimol as well as GABA.

Materials and Methods

Washed P_2 Fraction

Male Sprague-Dawley rats, four to seven weeks old, were killed by decapitation with a guillotine. The cerebral cortex, quickly removed, was rinsed with cold saline, cut into 1-mm slices and suspended in 30 ml of solution A (0.32 M sucrose, 10 mM N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.5, containing the protease inhibitors, leupeptin (5 μg/ml), antipain (5 μg/ml), pepstatin A (5 μg/ml), aprotinin (10 μl/ml)

(Sigma Chemical) phenyl methyl sulfonyl fluoride (1 mM) and the antioxidant, butylated hydroxytoluene (20 μM). All solutions were at 0–4°C and the manipulations were performed on ice. The mixture was homogenized with spinning double-sided knives using a Virtis 45 homogenizer (setting 30, 5 sec). To this homogenate, 30 ml of solution B (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.5), was added with gentle stirring. The mixture was centrifuged for 4 min, 270 $\times$ g. The supernatant (S_1) was centrifuged for 30 min, 23,640 $\times$ g. The supernatant (S_2) was used as the cerebral cortex extract in some experiments (*see* Fig. 2). In this case, the volumes of solutions A and B used prior to this step were halved (to 15 ml). The pellet (P_2) was resuspended in 10 ml of solution B using a glass-Teflon homogenizer by hand (eight strokes), diluted to 30 ml with solution B and centrifuged for 30 min, 23,640 $\times$ g. The pellet (washed P_2) was resuspended in 10 ml of solution B and diluted to 750 μg protein/ml. Protein concentration was assayed by the bicinchoninic acid method (Smith et al., 1985).

Ficoll Gradient Preparation

The resuspended P_2 fraction was layered onto a 4–12% Ficoll gradient (20 ml) in solution A and centrifuged 1 hr, 110,000 $\times$ g. The middle band (8–10% Ficoll) was diluted with twice the volume of solution B with gentle stirring, allowed to stand for 20 min and centrifuged for 30 min, 35,000 $\times$ g. The pellets were resuspended in 8 ml of solution B and made up to 750 μg protein/ml.

Synaptoneurosome Preparation (Hollingsworth et al., 1985)

The sliced cerebral cortex (*see above*) in 7 ml of solution C (118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, 10 mM HEPES, pH 7.4) containing the protease inhibitors and antioxidant present in solution B (*see above*) was homogenized using a glass-Teflon homogenizer by hand (seven strokes). The suspension was diluted with 30 ml of solution C and passed twice through three layers of 100 μm Nylon mesh. The filtrate was passed through a 10-μm Millipore filter (LCWP 047) and centrifuged for 15 min, 1100 $\times$ g. The pellet was suspended in 10 ml of solution C using a glass-glass homogenizer by hand made up to 30 ml with solution C and centrifuged for 15 min, 1100 $\times$ g. The pellet was resuspended in 8 ml of solution C and adjusted to a concentration of 750 μg protein/ml. In various experiments described, this preparation was followed with solution B, or the wash of the membrane was omitted, or the protease inhibitors and antioxidant were omitted. These modifications did not cause significant differences in the chloride exchange observations.

Unspecific (GABA-Independent) Chloride Exchange

The membrane preparation was kept on ice. A sample (225 μl) was withdrawn and incubated in a test tube at 30°C for 3 min in a water bath. The isotope influx was initiated by the addition of $^{36}Cl^-$ (225 μl) (7.5 μCi/ml) in the buffer solution to the briskly vortexed membrane suspension. Incubation at 30°C was continued. In some cases, GABA (2 mM) was added in the $^{36}Cl^-$ solution to determine the GABA-specific $^{36}Cl^-$ influx. At the end of the reaction time, a solution containing bicuculline methiodide (3 mM) was added to the vortexed membrane suspension, which was immediately passed through a Whatman GF/C filter disc. The filter was washed three times with 10 ml buffer at 0°C, dried at 100°C, and counted with toluene-based scintillation fluid.

GABA- or Muscimol-Mediated Chloride Exchange

The $^{36}Cl^-$ influx was measured at 30°C, pH 7.5 using the quench flow apparatus as described previously (Cash & Subbarao, 1987*b*). The membrane (225 μl) was mixed with solution containing $^{36}Cl^-$ (15 μCi/ml) in the absence or presence of GABA or muscimol (225 μl) and the specific influx was terminated by mixing with a solution containing 3 mM bicuculline methiodide (225 μl). Each time point is derived by subtracting the mean of triplicate background determinations from the mean of triplicate measurements in the presence of agonist. The background measurement was unaffected by the presence of bicuculline methiodide (1 mM) showing that the concentration of free endogenous GABA in solution did not significantly affect the measurements. When bicuculline methiodide (1 mM) was present with the GABA (1 mM), the measurement was equal to the background indicating that all the GABA-specific chloride exchange was due to the $GABA_A$ receptor.

GABA- or Muscimol-Mediated Receptor Desensitization

Desensitization was followed at 30°C, pH 7.5, by measuring the decrease in the GABA- or muscimol-mediated $^{36}Cl^-$ influx, due to preincubation with GABA or muscimol, using the quench flow apparatus as described previously (Aoshima, Cash & Hess, 1981; Cash & Subbarao, 1987*c*). The equilibrium value (M_∞) was determined by omitting GABA from the first incubation and extending the second (assay) incubation time to 4 sec.

Results

Measurements of the transmembrane equilibration of $^{36}Cl^-$ in the three membrane preparations are summarized in Fig. 1 and the Table. For all the preparations, the GABA-independent $^{36}Cl^-$ exchange progressed in two major phases with half times of $t_{1/2}$ = 1.4 min and $t_{1/2}$ = 24 min. These two phases correspond to transmembrane equilibration of chloride into two populations of vesicles with approximately equal total internal volume. In each case, the $^{36}Cl^-$ exchange was kinetically first order. These two major phases were preceded by a very small, rapid phase of $^{36}Cl^-$ uptake equivalent to circa 2% of the total internal volume. Equation (1) describes the progress of $^{36}Cl^-$ influx into two main compartments following a small, rapid uptake of $^{36}Cl^-$,

$$M_t' = M_a + M_b\,(1 - \exp - k_b t) + M_c\,(1 - \exp - k_c t) \quad (1)$$

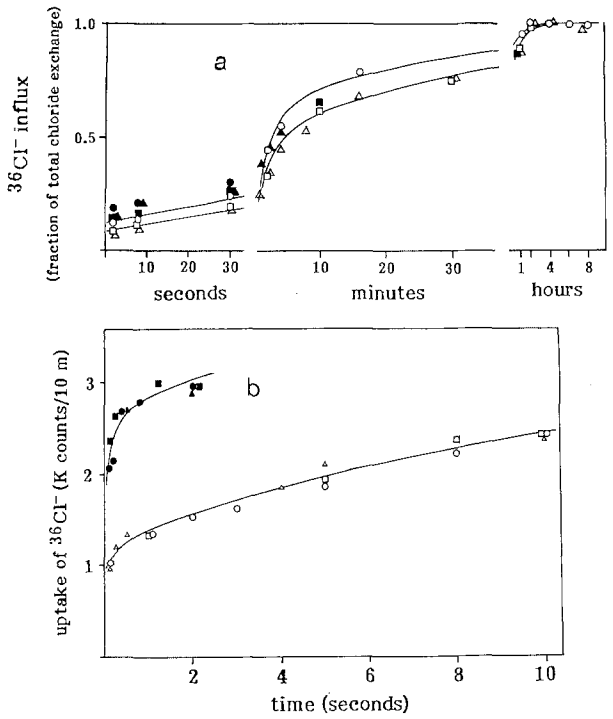

Fig. 1. Unspecific (GABA-independent) and GABA-mediated chloride exchange with the membrane preparations described in the Table. Influx of $^{36}Cl^-$ at 30°C, pH 7.5 measured as described in Materials and Methods. Measurements were made in the absence (open symbols) and the presence (filled symbols) of 1000 μM GABA. The two major phases of unspecific influx with half times of 1.4 and 24 min are shown as well as the GABA-mediated influx. (*a*) The "Ficoll gradient" preparation (□,■) and the "Washed P_2" fraction (○,●) in solution B. The "Synaptoneurosome" preparation in solution C (△,▲). The filter blank is subtracted. The lines are computed from Eq. (1) and the values given in the Table. (*b*) the "Synaptoneurosome" preparation in solution C measured with the quench flow machine. The counts include the assay background. The different symbols refer to different "Synaptoneurosome" preparations

where M_t' is the fraction of the total unspecific $^{36}Cl^-$ influx, which has occurred at time t, M_a, M_b and M_c are the fractions of the total chloride exchange equilibrated in three phases and k_b and k_c are first order rate constants for chloride exchange in the two major phases. The value given for M_a (Table) includes counts occluded in the membrane on the filter. For the different preparations, the $^{36}Cl^-$ equilibration in each phase proceeds with the *same* first order rate constant. The relative quantity of each phase varies slightly in the different preparations (Table). The "Washed P_2" fraction contains somewhat more of the faster exchanging fraction, but purification on the Ficoll gradient enriches the preparation in total internal volume (Table) and increases the proportion of the slower exchanging fraction to the same value as that in the "Synaptoneurosome" preparation (Fig. 1*a*).

The GABA-mediated $^{36}Cl^-$ influx (Fig. 1, filled symbols) was determined in the same experiments as the GABA-independent $^{36}Cl^-$ exchange. On average it was larger in the "Synaptoneurosome" preparation than in the P_2 fraction and it was not significantly enriched by the Ficoll gradient purification (Table). The GABA-independent $^{36}Cl^-$ influx into the volume, which undergoes GABA-mediated $^{36}Cl^-$ exchange, took place mainly in the period 5 min to 1 hr (Fig. 1*a*). Thus, the GABA receptor-accessed internal volume consists of the slower exchanging membrane fraction. Figure 1*b* shows measurements of GABA-mediated $^{36}Cl^-$ influx to the "Synaptoneurosome" preparation made with the quench flow machine. These results are similar to those made with the washed P_2 preparation (Cash & Subbarao, 1987*b*).

Previously, we reported that the chloride exchange measured with the "Ficoll gradient" and the "Washed P_2" preparations progressed in two phases described by Eq. (2) (Cash & Subbarao, 1987 *b,c*)

$$\frac{[Mt]}{[M_\infty]} = 1 - \exp - \left[J_A\left(\frac{1-\exp-\alpha t}{\alpha}\right) + J_B\left(\frac{1-\exp-\beta t}{\beta}\right)\right] \quad (2)$$

where M_t/M_∞ is the fraction of equilibration of the GABA-specific $^{36}Cl^-$ influx into the vesicles, J_A and J_B are initial first order rate constants for chloride exchange in the two phases of $^{36}Cl^-$ influx, and α and β are the first order rate constants for desensitization (depletion of J_A and J_B) of those phases, respectively. The progress of the GABA-mediated $^{36}Cl^-$ influx with the "Synaptoneurosome" preparation was followed with different concentrations of GABA (Fig. 3). With this preparation also, the measurements could not be fitted by the equation for a single phase of chloride exchange (Eq. (2) where $J_A = 0$), but the progress of ion exchange was described by Eq. (2). The $^{36}Cl^-$ influx mediated by muscimol with the "Synaptoneurosome" preparation also proceeded in two phases, following Eq. (2) as illustrated in Fig. 4.

Desensitization of the receptor by GABA in the "Synaptoneurosome" preparation was followed by measuring the decrease in chloride exchange mediated by GABA, caused by preincubation of the membrane with GABA (Aoshima et al., 1981; Subbarao & Cash, 1985; Cash & Subbarao, 1987*c*). The loss of activity could not be fitted by the expression for a single phase of desensitization (Eq. (3) where $A = 0$). The activity decreased in two phases (Fig. 5)

Table. Properties of the internal compartments undergoing chloride exchange in membrane preparations made from rat cerebral cortex by different methods[a]

	Washed P_2	Ficoll gradient	Synaptoneurosome
Total internal volume · $[Cl_I^-]/[Cl_O^-]$[b]	3.16 μl/mg protein	4.01 μl/mg protein	2.68 μl/mg protein
Specific (GABAR accessed) internal volume · $[Cl_I^-]/[Cl_O^-]$[b]	0.21 μl/mg protein	0.21 μl/mg protein	0.29 μl/mg protein
Fraction of chloride exchange			
(A) mediated by GABA	0.064	0.053	0.109
(B) in the rapid GABA-independent phase,[c] M_a	0.12	0.08	0.06
(C) in the second GABA-independent phase, M_b	0.49	0.40	0.39
(D) in the third GABA-independent phase,[d] M_c	0.39	0.52	0.55
Rate constants for chloride exchange			
(A) in the second GABA-independent phase, k_b	7.5×10^{-3} sec^{-1}	8.5×10^{-3} sec^{-1}	9.0×10^{-3} sec^{-1}
(B) in the third GABA-independent phase, k_c	5.5×10^{-4} sec^{-1}	4.5×10^{-4} sec^{-1}	4.5×10^{-4} sec^{-1}

[a] The preparations were incubated with 3.75 μCi ^{36}Cl /ml with membrane concentrations of 375 μg protein/ml in solution B (250 μg protein/ml in solution C for the synaptoneurosome preparation) at 30°, pH 7.5.

[b] Since the equilibration measured is of specific activity, the actual volume depends on the ratio of the concentrations inside $[Cl_I^-]$ and outside $[Cl_O^-]$ the vesicles.

[c] The value of M_a includes $^{36}Cl^-$ occluded in the membrane on the filter.

[d] The value for M_c includes the GABA receptor-specific internal volume given in (A).

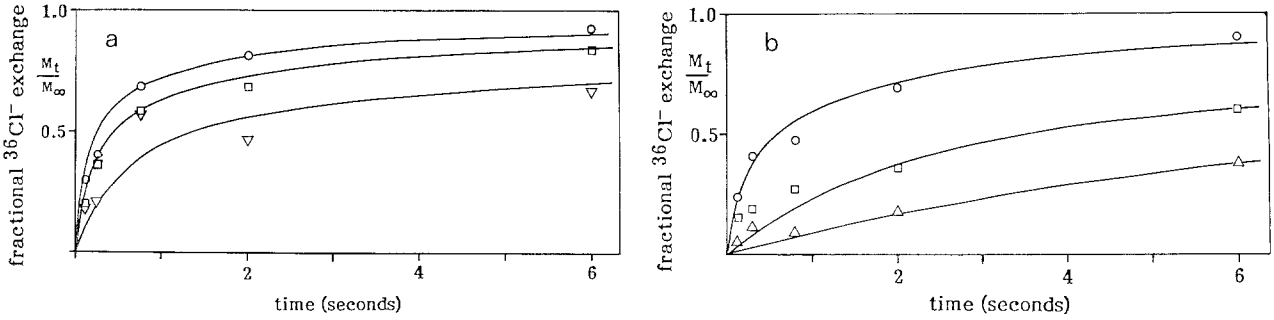

Fig. 2. Chloride exchange mediated by the cerebral cortex extract measured with the "Washed P_2" preparation. The $^{36}Cl^-$ influx progresses in two phases described by Eq. (2) (described in the text). The preparation used was a "Washed P_2" fraction shown not to contain a GABA concentration in solution which significantly affects chloride exchange (Cash & Subbarao, 1987*b,c*). (The background $^{36}Cl^-$ influx, with no added GABA, was not decreased by the addition of bicuculline or picrotoxin.) (*a*) The membrane in solution B was mixed with 1 volume of solution D (50% solution A, 50% solution B) or solution S_2 containing $^{36}Cl^-$ in the presence or absence of GABA, and the progress of $^{36}Cl^-$ influx (7.5 μCi/ml) was measured as described in Materials and Methods. The lines were computed with Eq. (2) (*see* text for definitions of parameters) with the values of the parameters as follows: (□) 40 μM GABA in solution B, J_A = 2.2 sec^{-1}, α = 3.5 sec^{-1}, J_B = 0.44 sec^{-1}, β = 0.3 sec^{-1}; (○) 40 μM GABA in solution S_2, J_A = 3.5 sec^{-1}, α = 5.0 sec^{-1}, J_B = 0.7 sec^{-1}, β = 0.4 sec^{-1}; (▽) solution S_2 (no added GABA), J_A = 0.8 sec^{-1}, α = 1.4 sec^{-1}, J_B = 0.16 sec^{-1}, β = 0.14 sec^{-1}. Solution S_2 is seen to mediate chloride exchange. (*b*) The membrane in solution B was mixed with 1 volume of solution D or solution S_2. After incubation for 6 sec, 1 volume of solution B containing $^{36}Cl^-$ in the presence or absence of GABA was added and the progress of $^{36}Cl^-$ influx (5 μCi/ml) was measured. Values of the parameters were as follows: (○) 40 μM GABA after preincubation with solution D, J_A = 1.7 sec^{-1}, α = 3.5 sec^{-1}, J_B = 0.50 sec^{-1}, β = 0.25 sec^{-1}; (□) 40 μM GABA after preincubation with solution S_2, $J_A \simeq 0$, J_B = 0.3 sec^{-1}, β = 0.25 sec^{-1}; (△) solution B (no added GABA) after preincubation with solution S_2, $J_A \simeq 0$, J_B = 0.1 sec^{-1}, β = 0.08 sec^{-1}. The chloride exchange is seen to have been desensitized relative to (*a*) by preincubation with solution S_2. For these experiments, the volumes of solution used in the preparation were halved (to give 30 ml S_2 per cerebral cortex)

following Eq. (3) (Cash & Subbarao, 1987*c,d*)

$$-\ln\left(1 - \frac{M_t}{M_\infty}\right) = A \exp - \alpha_p t_p + B \exp -\beta_p t_p \quad (3)$$

where the subscript p denotes the preincubation, t_p is the preincubation time and α_p and β_p are first order rate constants for desensitization during the preincubation in the first and second phases, respectively. A and B are complex constants depending on the assay ($^{36}Cl^-$ influx) incubation conditions (Cash & Subbarao, 1987*c*). The fast phase corresponded to the loss of 80% of the channel opening activity. Similar observations have been made with the "Washed P_2" and "Ficoll gradient" preparations (Cash & Subbarao, 1987*c*).

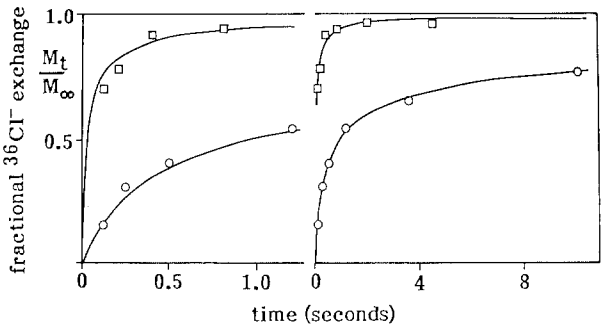

Fig. 3. Chloride exchange mediated by GABA with the "Synaptoneurosome" preparation. The membrane suspension was mixed with 1 volume of solution C containing $^{36}Cl^-$ in the presence or absence of GABA and the progress of $^{36}Cl^-$ influx was followed as described in Materials and Methods. The lines are computed with Eq. (2) with the values of the parameters as follows: (○) 20 μM GABA, $J_A = 1.3$ sec^{-1}, $\alpha = 2.1$ sec^{-1}, $J_B = 0.18$ sec^{-1}, $\beta = 0.18$ sec^{-1}; (□) 1000 μM GABA, $J_A = 23$ sec^{-1}, $\alpha = 18$ sec^{-1}, $J_B = 2.6$ sec^{-1}, $\beta = 1$ sec^{-1}. Two phases of $^{36}Cl^-$ influx can be seen. The fast phase alone would give 47% (with 20 μM GABA) or 72% (with 1000 μM GABA) of the equilibrated influx

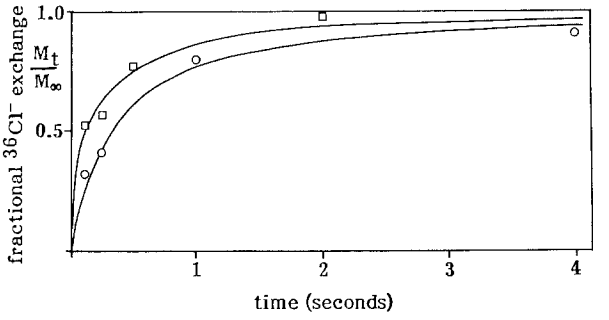

Fig. 4. Chloride exchange mediated by muscimol with the "Synaptoneurosome" preparation in solution B, as described for GABA in Fig. 3. Values of the parameters were as follows: (○) 20 μM muscimol, $J_A = 2.0$ sec^{-1}, $\alpha = 1.5$ sec^{-1}, $J_B = 0.45$ sec^{-1}, $\beta = 0.15$ sec^{-1}; (□) 100 μM muscimol, $J_A = 8.0$ sec^{-1}, $\alpha = 15$ sec^{-1}, $J_B = 2.0$ sec^{-1}, $\beta = 0.7$ sec^{-1}. Two phases of $^{36}Cl^-$ influx can be seen. The fast phase alone would give 73% (20 μM muscimol) and 44% (100 μM muscimol) of the equilibrated influx. M_∞ was determined with 1000 μM GABA incubated for 4 sec.

Desensitization of the GABA receptor in the "Synaptoneurosome" preparation during incubation with muscimol was followed in the same way (Fig. 6*a*). As with GABA, the desensitization proceeded in two phases. The ordinate intercept of the second phase of desensitization was independent of the muscimol concentration, showing that the second phase is due to a second, distinguishable receptor (Cash & Subbarao, 1987*c*). The value of this intercept indicated that 80% of the initial activity was lost in the first phase, the same fraction of activity as in the experiments with GABA.

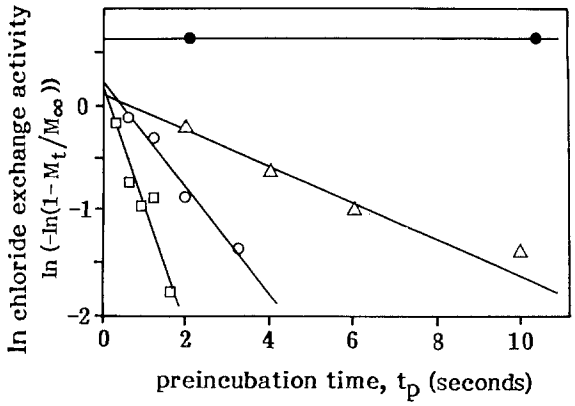

Fig. 5. Desensitization of GABA receptor in the "Synaptoneurosome" preparation by GABA in solution C. The $^{36}Cl^-$ influx in the presence of 1000 μM GABA for 400 msec was measured following preincubation for time, t_p with GABA as described in Materials and Methods. The value for M_∞ (3213 counts/10 min) was determined by incubation with 1000 μM GABA for 4 sec. In the first order plot, the chloride exchange activity is seen to have decreased in a fast phase preceding the measurements. The ordinate intercept does not decrease with increasing [GABA] below saturation. The lines are calculated with Eq. (3) with the values of β_p given (assay conditions, $J_A = 15$ sec^{-1}, $\alpha = 19$ sec^{-1}, $J_B = 3.7$ sec^{-1}, $\beta = 1.3$ sec^{-1}); (●) no GABA, (△) 40 μM GABA, $\beta_p = 0.18$ sec^{-1}; (○) 100 μM GABA, $\beta_p = 0.5$ sec^{-1}; (□) 1000 μM GABA, $\beta_p = 1.2$ sec^{-1}

The dependence on muscimol concentration of the rate of desensitization of the GABA receptor, measured with the synaptoneurosome preparation, is shown in Fig. 7. The steepness of the response curve requires the binding of muscimol at two sites on the receptor to cause desensitization, in agreement with the results with GABA (Cash & Subbarao, 1987*c*).

To investigate the effect of solutes in the brain extract on the chloride exchange process, the supernatant solution from the centrifugation after the Virtis homogenization was added back to the membrane in the quench flow machine. The soluble brain extract-mediated $^{36}Cl^-$ influx in a manner equivalent to circa 20 μM GABA in the reaction solution (Fig. 2*a*). When added with 40 μM GABA, the brain extract caused only a small change in the progress of $^{36}Cl^-$ influx as would be predicted (Fig. 2*a*). When the brain extract was added 6 sec before 40 μM GABA and the $^{36}Cl^-$ tracer, the progress of the $^{36}Cl^-$ influx was retarded by an extent equivalent to desensitization by circa 20 μM GABA in the reaction solution (Fig. 2*b*). The $^{36}Cl^-$ influx due to the brain extract alone also was slowed by preincubation for 6 sec with the extract (Fig. 2*b*). Because of this effect of the brain extract on densensitization and channel opening, the importance of thorough

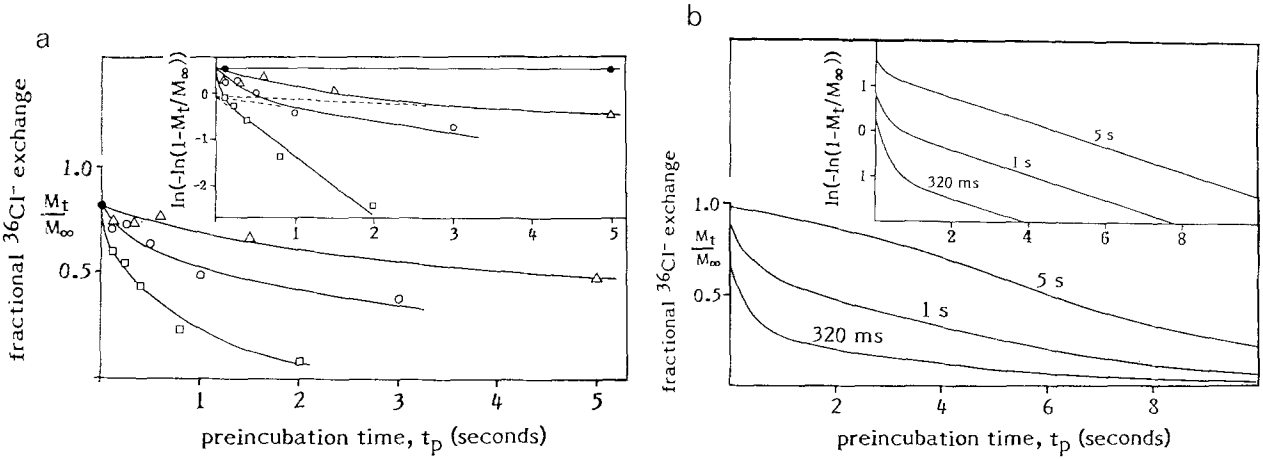

Fig. 6. Desensitization of the GABA receptor in the "Synaptoneurosome" preparation by muscimol in solution B. (*a*) The $^{36}Cl^-$ influx in the presence of 1000 μM muscimol for 320 msec was measured following preincubation for time t_p with muscimol as described in Materials and Methods. The lines are calculated with Eq. (3) using the values of the parameters given (assay conditions, $J_A = 15$ sec^{-1}, $\alpha = 20$ sec^{-1}, $J_B = 3.6$ sec^{-1}, $\beta = 1.3$ sec^{-1}); (●) no muscimol, (△) 20 μM muscimol, $\alpha_p = 1$ sec^{-1}, $\beta_p = 0.08$ sec^{-1}; (○) 40 μM muscimol, $\alpha_p = 4$ sec^{-1}, $\beta_p = 0.27$ sec^{-1}; (□) 1000 μM muscimol, $\beta_p = 1.3$ sec^{-1}. Inset: First order plot of the activity decrease. The initial fast phase corresponds to the loss of 80% of the activity. The ordinate intercept of the second phase is independent of muscimol concentration. (*b*) Computer simulation of an experiment with 50 μM muscimol in both incubations. The lines correspond to assay (second incubation) times of 5 sec, 1 sec and 320 msec. The values used are, in the assay, $J_A = 5$ sec^{-1}, $\alpha = 4$ sec^{-1}, $J_B = 1.3$ sec^{-1}, $\beta = 0.27$ sec^{-1} and in the preincubation, $\alpha_p = 4$ sec^{-1}, $\beta_p = 0.27$ sec^{-1}. The fast phase is not seen with a long assay time in which the $^{36}Cl^-$ exchange is equilibrated in a time less than the assay time

washing of the membrane on the reproducibility of the measurements and the observation of two phases was investigated. The progress of the chloride exchange as well as the desensitization was followed using the "Synaptoneurosome" preparation in which the membrane was not washed between the decanting of the homogenization solution and resuspension of the membrane in the reaction solution, as originally described (Schwartz et al., 1984, 1985, 1986*a*; Hollingsworth et al., 1985). The results of these experiments were no different from usual, showing both the progress of $^{36}Cl^-$ influx and desensitization in two phases with both GABA and muscimol (Figs. 3-6). These measurements (with a membrane concentration of 750 μg protein/ml) were made with a solution diluted 50-fold relative to the original cerebral cortex extract (or relative to the experiments with the extract added back). This compares with a circa four-fold dilution in measurements with 10 mg protein/ml and no washing stage.

The possibility of removal of endogenous GABA from solution by its uptake into vesicles was investigated. After the first centrifugation in the "Synaptoneurosome" preparation, half of the preparation was resuspended in solution C containing the GABA-uptake inhibitors, guvacine (500 μl) and nipecotic acid (500 μM) and half in solution C as usual. With no further washing of the membrane, the progress of $^{36}Cl^-$ uptake with 100 μM GABA was measured after standing for 4 hr at 4°C. Both

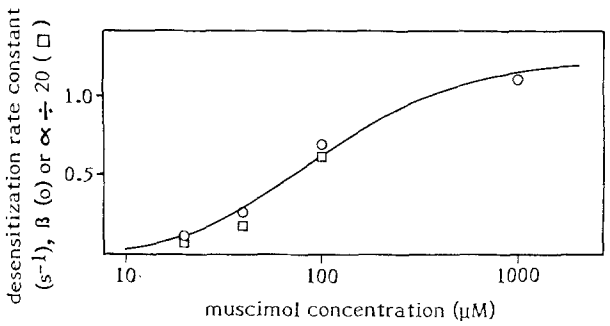

Fig. 7. Dependence of the rate of desensitization of the GABA receptor mediated by muscimol on muscimol concentration. (○) the second phase rate constant, β; (□) the first phase rate constant, α (on ordinate scale × 20). The line is calculated from the expression, $\beta = k_2/(1 + K/[L])^2$, where the maximal rate, $k_2 = 1.25$ sec^{-1}, the dissociation constant, $K = 40$ μM, and $[L]$ is the concentration of muscimol. The rate of desensitization from the singly liganded receptor is relatively small (*cf.* Cash & Subbarao, 1987*b*) and is neglected in this treatment. This dependence on muscimol concentration is similar to that for GABA (Cash & Subbarao, 1987*b*,*c*)

phases of $^{36}Cl^-$ exchange were observed in each case, but the sample containing the GABA-uptake inhibitors had its chloride exchange activity decreased about 2.4-fold ($J_A = 1.5$ sec^{-1}, $J_B = 0.5$ sec^{-1} compared with $J_A = 3.0$ sec^{-1}, $J_B = 1.2$ sec^{-1}). The "unspecific", baseline up to 10 sec was unaffected by the GABA-uptake inhibitors indicating

that the concentration of GABA that caused this desensitization did not give significant $^{36}Cl^-$ exchange. These concentrations of the uptake inhibitors do not affect the responses of the receptor to GABA (Cash & Subbarao, 1987*b*).

Discussion

Unspecific (GABA-Independent) Chloride Exchanging Membrane

The different preparations studied were remarkably similar. The GABA-independent transmembrane chloride exchange occurred in two main phases corresponding to two major populations of internal volume (Fig. 1). The relative quantities (total volume) of the different populations can vary slightly with preparation method, and can be changed by purification procedures (Table). The different populations of vesicle each exchanged chloride with the solution with first order kinetics. The *rates* of chloride exchange were the same for the corresponding populations in the different preparations regardless of the preparation procedure. This identity of rates requires not only membrane of the same permeability, but internal compartments of the same surface/volume ratio. Very likely the vesicles from the different preparations are the same size. A similar, biphasic chloride exchange has been observed with crayfish muscle strips (Ticku & Olsen, 1977).

GABA Receptor-Mediated Chloride Exchanging Membrane

The different preparations also contained a minor vesicular component (Table), which gave chloride exchange mediated by GABA, attributed to the GABA receptor (Figs. 1 and 3). These GABA-specific vesicles underwent unspecific chloride exchange at the rate of the slower exchanging major population. This fraction, containing functional GABA receptor, cannot at present be unambiguously identified with a visualized population of membrane particles because of its relatively low internal volume (6–12% of the total).

Measurements of GABA receptor-mediated $^{36}Cl^-$ influx (Fig. 3) and receptor desensitization (Fig. 5) with the "Synaptoeneurosome" preparation gave the same results as with the other preparations (Cash & Subbarao, 1987*b,c*). The chloride exchange progressed in two phases, each progressively attenuated by desensitization. Each phase is described in terms of a first order rate constant for ion exchange and a first order rate constant for desensitization. The ordinate intercept ($t_p = 0$) of the second phase in the first order plot of chloride exchange activity (Fig. 5) was independent of GABA concentration and corresponded to circa 20% of the initial chloride exchange activity. Thus, the chloride exchange is mediated by two distinguishable receptors with different desensitization rates present in the activity ratio of 5 : 1 with this preparation as has been explained elsewhere (Cash & Subbarao, 1987*c*).

Comparison of Muscimol with GABA

With muscimol, the $^{36}Cl^-$ influx (Fig. 4) and receptor desensitization (Fig. 6) proceeded in the same way as with GABA. Two phases were evident and the measured activities in each phase were similar to those with GABA. The ordinate intercept of the slower phase was independent of muscimol concentration. Thus, muscimol is GABA-mimetic with both receptors, which are present in an activity ratio of circa 5 : 1 at saturation with muscimol. The dependence of desensitization rate on muscimol concentration (Fig. 7) is the same as on GABA concentration, with a half-response concentration near 100 μM muscimol. This dependence requires two muscimol binding sites mediating desensitization (Fig. 7, legend) as found previously with GABA (Cash & Subbarao, 1987*b,c*). For both receptors, the half-response concentration for chloride exchange with muscimol (35 μM) is 2.6-fold lower than that with GABA, while the concentration dependence of desensitization is not significantly different for these two ligands. This supports previous evidence that these different responses are mediated by different pairs of binding sites (Cash & Subbarao, 1987*b*).

Saturation Curves for Receptor Responses and Chloride Exchange

The $^{36}Cl^-$ influx will cease when, (*i*) equilibration of $^{36}Cl^-$ across the membrane is reached (a function of internal volume), or (*ii*) all the receptor is desensitized (a function of the ratio of initial ion-exchange rates to desensitization rates, $(J_A/\alpha + J_B/\beta)$ (Eq. (1) and internal volume), or (*iii*) the $^{36}Cl^-$ exchange is first inhibited by receptor antagonists (a function of the *rates* of ion-exchange and desensitization and internal volume). The half-response concentration (or EC_{50}) for $^{36}Cl^-$ influx in the finite time is a complex function of the above-mentioned properties (Eq. (1)). The concentration giving maximal $^{36}Cl^-$ influx is reached before that for maximal response of receptor. Hence, the same is true for the half-

response concentration. These measured concentrations decrease with increasing ion-influx assay time. For example, the determined half-response concentrations for GABA receptor in a rat brain preparation were 105 and 82 μM (chloride exchange rate) and 115 and 114 μM (desensitization rate) for the two receptors, respectively. The EC_{50} value for $^{36}Cl^-$ influx, observed in the *same* experiments (Cash & Subbarao, 1987*d*), which varied with the assay time was 4 μM (50-sec assay), 9 μM (5-sec assay), 12 μM (3-sec assay) and 20 μM GABA (1-sec assay), as predicted from the determined values of the parameters. Only determinations of *initial* rate give a half-response concentration for response of the receptor. There is no discrepency between the high dissociation constants of the receptor (Cash & Subbarao, 1987*b,c*) and low EC_{50} values for $^{36}Cl^-$ influx (for example, *see* Allan & Harris, 1986*b*; Yang & Olsen, 1987; Schwartz & Mindlin, 1988).

Some values reported for the concentration of response of the GABA receptor have been reviewed briefly (Cash & Subbarao, 1987*b,c*; Yang & Olsen, 1987). Values given for the half-response concentration vary over the range circa 1–100 μM GABA. The extent to which these differences depend on the experimental technique or reflect real differences in the receptors is not fully known at this time. Recently, different GABA receptors cloned from different cDNAs from bovine brain and expressed in *Xenopus* oocytes have been reported to differ in their response to increasing GABA concentration (Levitan et al., 1988). Thus, it is possible that differences in dissociation constant might result from the expression of different receptor subunits.

Endogenous Solute Activity

The solution from the brain homogenate contained active factor(s), equivalent to circa 40 μM GABA in a volume of 30 ml per cerebral cortex, which caused channel opening and desensitization of the GABA receptor (Fig. 2). When this was added back to purified membrane (1 : 1), it removed the fast phase in less than 6 sec, and the slow phase more slowly. After removal of this supernatant solution and resuspension of the membrane in the final solution with no washing, a procedure, which diluted the active contaminant by only circa 40-fold, the $^{36}Cl^-$ exchange and receptor desensitization were seen to take place in two phases as normally observed when the membrane was washed thoroughly. Thus, there is no very active (high affinity) contaminant in solution in these preparations that might prevent the observation of the two receptors.

The sodium-independent binding of GABA to membrane preparations (Peck, Schaefer & Clark, 1973; Zukin, Young & Snyder, 1974; Enna & Snyder, 1975) is believed to be a measure of GABA receptor. Measurements of GABA and muscimol binding to membrane prepared with tissue grinders (e.g., Potter Elvehjem type) were affected by endogenous GABA (*see* Fisher, Tuchek & Johnson, 1986), which apparently was slowly released from the membrane internal volume (Greenlee, Van Ness & Olsen, 1978). With washed membrane, binding to two types of site was observed (Beaumont et al., 1978; Skerritt, Chow & Johnston, 1982). GABA binding to three different sites characterized by different dissociation constants, the highest of which is circa 1 μM, has been reported (Falch & Krogsgaard-Larsen, 1982; Olsen, 1982*a*). That the GABA binding measurement is made after conformational changes, of which the equilibrium is shifted by GABA, is supported by (*i*) the long time (minutes) of the progress of GABA binding and (*ii*) the higher affinity of GABA in binding measurements than in measurements of initial rates of chloride exchange or desensitization. These conformational changes may result from or constitute receptor desensitization.

Apparently, quantities of endogenous GABA approaching 1 μM (overall concentration) are present in these membrane preparations. At this concentration, desensitization was seen in chloride exchange measurements (Cash & Subbarao, 1987*c*) and conformational changes were involved in measurements of GABA binding (*see above*). Yet, added GABA mediated substantial $^{36}Cl^-$ influx. An explanation of this paradox in that in these experiments the endogenous GABA has been removed from the outside solution by being pumped into an internal compartment by the GABA-uptake mechanism, which remains active in the solution conditions used (containing glucose, oxygen, sodium and a low concentration of potassium outside the vesicles). When a membrane preparation was allowed to stand in the presence of GABA-uptake inhibitors, the chloride exchange activity was reduced 2.4-fold by an endogenous substance (presumably GABA) at a concentration, which did not mediate $^{36}Cl^-$ influx detectable in 10 sec. Apparently studies of the *function* of the *active site* of the receptor can be made with these preparations when the solution conditions allow continued functioning of the neurotransmitter-uptake mechanism. Our results show that relatively concentrated membrane preparations (10 mg protein/ml prepared with no additional washing stages) could contain endogenous activity equivalent to circa 7 μM GABA (overall concentration).

Comparison of Receptor-Mediated Measurements with Other Reports

In measurements of the progress of $^{36}Cl^-$ influx, the fast phase should be apparent as a positive ordinate intercept from extrapolation of the measurements during the slower, second phase. In the examples given with muscimol (Schwartz et al., 1986*b*; Schwartz & Mindlin, 1988) this could be the case. With pentobarbital, a slow phase of $^{36}Cl^-$ exchange, but no significant fast phase, was reported (Schwartz et al., 1986*b*). Pentobarbital increases the amplitude of the GABA-mediated $^{36}Cl^-$ exchange due to the slower desensitizing receptor, but not that due to the faster desensitizing receptor (Cash & Subbarao, 1988). Thus, this observation is consistent with channel opening mediated by pentobarbital together with endogenous GABA. The reactivation of inhibited receptor, which is expected in these conditions (Cash & Subbarao, 1988), is evident from the shape of the $^{36}Cl^-$ influx curve (Schwartz et al., 1986*b*).

In the measurement of desensitization of the receptor by muscimol, no fast phase was apparent before the slower phase proceeded in several seconds (Schwartz et al., 1986*b*). The reason why the faster phase, which reflects the activity of most (4/5) of the receptor, was not observed is demonstrated by the simulation of this experiment with various assay times shown in Fig. 6*b*. In the assay (second incubation) conditions, the $^{36}Cl^-$ exchange is equilibrated in a time that is less than the incubation time (5 sec). To follow a desensitization process by measuring the decrease of ion exchange due to a preincubation, the assay conditions must give less than complete ion-exchange. The simulation shows that a significant loss of $^{36}Cl^-$ influx is not observed with a 5-sec assay until after the first phase of desensitization is complete. With a 320-msec incubation, the faster phase would be seen.

Other Studies of Receptor-Containing Membrane Vesicles

With the membrane preparation from the electric organ of *Electrophorus electricus*, the measurement of transmembrane flux of $^{86}Rb^+$ or $^{22}Na^+$ indicated that 15–25% of the total ion exchange was mediated by acetylcholine receptor (Hess et al., 1975; Kim & Hess, 1981; Sachs et al., 1982). A total internal volume of 1.9 μl/mg protein and an acetylcholine receptor-accessed internal volume of 0.5 μl/mg protein were reported (Sachs et al., 1982). The purified fraction of vesicles containing functional receptor has an internal volume of 2.0–2.4 μl/mg protein (Hess, Cash & Aoshima, 1980; Hess et al., 1981; Sachs et al., 1982). With membrane preparations from the electric organ of *Torpedo sp.* (Jeng, St. John & Cohen, 1981), 68% of the total $^{22}Na^+$ exchange was mediated by acetylcholine receptor. The estimated internal volume was 0.26 μl/mg protein, and for the purified preparation, 0.68 μl/mg protein, 90% of which was accessed by acetylcholine receptor. As the authors pointed out (Jeng et al., 1981), these estimates appeared to be low, possibly because of the efflux assay method employed.

Membrane Vesicle Formation

The nature of the sealed vesicles in brain homogenate was independent of the method of preparation. This suggests that vesicle formation is characteristic of the morphology and properties of the membrane rather than on the method of homogenization. Particular types of vesicles are formed from a particular membrane. A similar conclusion was suggested by a consideration of the precise first order kinetics of ion-exchange and receptor desensitization observed with acetylcholine receptor as well as GABA receptor (Cash et al., 1988). The high value of internal volume/mg protein in the brain preparations (which are rich in membrane protein) relative to those from the electric organs of fish might result from a larger proportion of membrane, which has a morphology favorable for resealing after homogenization. The shape of the presynaptic terminal and postsynaptic boutons favors the formation of synaptosomes and neurosomes.

Conclusion

Three different types of vesicles in membrane preparations from rate cerebral cortex homogenate were characterized. These vesicles had quantitatively the same properties when the membrane was prepared in different ways. The vesicles containing active GABA receptor displayed the properties of the two distinguishable receptors previously described.

Apparent differences in various reports are not due to different membrane preparation methods or to the use of the GABA-mimetic, muscimol. Discrepencies could arise due to the following. (*i*) The use of solutions, which do not support the continued functioning of a GABA-uptake system in the suspension will give rise to GABA in the external solution. (*ii*) In the absence of an active GABA-uptake system different degrees of removal of GABA from the external solution by washing the membrane lead to different receptor activities. (*iii*) The choice of different $^{36}Cl^-$ influx assay times leads to different

half-response concentrations for $^{36}Cl^-$ exchange. (The concentration dependence for receptor itself requires the determination of initial rates.) (*iv*) The choice of a too large $^{36}Cl^-$ influx assay time leads to the eclipse of the faster phase of desensitization.

In appropriate conditions, native membrane preparations exhibit kinetically homogeneous GABA receptor activity, which can be followed in times from milliseconds to minutes in a system suitable for biochemical manipulation. The measurements are suitable for quantitative analysis.

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