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γ-Aminobutyric Acid (GABA)- and Barbiturate-Mediated $^{36}Cl^-$ Uptake in Rat Brain Synaptoneurosomes: Evidence for Rapid Desensitization of the GABA Receptor-Coupled Chloride Ion Channel

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SUMMARY

"Desensitization" of the γ-aminobutyric acid (GABA) receptor-coupled chloride ion channel was studied using an *in vitro* method for measuring chloride (Cl^-) permeability in brain vesicles (synaptoneurosomes). Muscimol, a GABA agonist, stimulated $^{36}Cl^-$ uptake in rat cerebral cortical synaptoneurosomes in a concentration-dependent manner (EC_{50} 7.3 ± 0.5 μM), whereas pentobarbital stimulated $^{36}Cl^-$ uptake in a biphasic manner, indicated by a bell-shaped concentration-response relationship, with a maximal response at 500 μM (EC_{50} 271 ± 17 μM). Higher concentrations of pentobarbital led to progressively smaller stimulation of $^{36}Cl^-$ uptake and blocked muscimol-stimulated $^{36}Cl^-$ uptake. Lower concentrations of pentobarbital (100–200 μM), when added with muscimol, produced an additive effect in stimulating $^{36}Cl^-$ uptake, whereas even lower (subthreshold) concentrations of pentobarbital (50 μM) potentiated muscimol-stimulated $^{36}Cl^-$ uptake. Following continuous exposure of synaptoneurosomes (up to 20 min) to muscimol (50 μM) or pentobarbital (500 μM), the $^{36}Cl^-$ uptake response diminished to a new steady state level with a $t_{1/2}$ of ~6 sec and 30 sec, respectively. The decrement in response to these agonists was dependent on both concentration and length of exposure. No decrement was observed in the ability of subthreshold concentrations of pentobarbital to enhance muscimol-stimulated $^{36}Cl^-$ uptake following prolonged (20 min) incubation. "Heterologous desensitization" between muscimol and pentobarbital was observed in experiments where either muscimol or pentobarbital was added to the vesicles following pretreatment with the other. These findings suggest that "desensitization" of the GABA receptor/Cl^- ion channel may involve both the GABA and barbiturate recognition sites or a common effector component such as the ionophore itself.

GABA, one of the most ubiquitous inhibitory neurotransmitters in the central nervous system, decreases neuronal excitability by increasing membrane chloride (Cl^-) conductance (Refs. 1 and 2, for review see Ref. 3). Membrane Cl^- conductance is increased in response to the binding of GABA to recognition sites that are believed to be part of a supramolecular protein complex which contains distinct but interacting recognition sites for convulsants such as picrotoxin (4) and bicyclophosphorothionates (5, 6), depressants such as benzodiazepines (7, 8) and barbiturates (9–12), and a Cl^- ion channel (for reviews see Refs. 13–17). Although an actual recognition site for barbiturates has not yet been labeled, most likely because of its very low affinity (but see Ref. 18), receptor binding and electrophysiologic studies indicate that barbiturates bind to a site near the GABA-gated Cl^- ionophore (5, 6, 9–12, 19–21) and allosterically enhance GABA-activated increases in Cl^- conductance (19, 20). At higher concentrations, barbiturates also produce GABA-mimetic responses by increasing Cl^- conductance directly (19–21). The effects of barbiturates on GABA-activated Cl^- conductance to enhance GABAergic neurotransmission may represent a mechanism of action for their anesthetic, sedative/hypnotic, anxiolytic, and anticonvulsant properties.

The cellular actions of both GABA and barbiturates have been analyzed in great detail using electrophysiologic techniques. Early studies demonstrated a loss in the response of cortical neurons to GABA during prolonged GABA application, and it was suggested that this "fading" response might be due to receptor desensitization (1, 22). More recent studies have demonstrated a "desensitization" phenomenon associated with prolonged GABA or pentobarbital application to rat primary afferent neurons (23), frog sensory neurons (24), guinea pig hippocampal pyramidal cells (25), and rat hippocampal slices (26).

In the past, GABA- and barbiturate-mediated Cl^- conductance could only be studied electrophysiologically. However, biochemical methods for studying GABA and barbiturate receptor activity have now been described in cultured cerebral neurons (27), hippocampal slices (28), and, most recently, in subcellular brain preparations (29–32). We have used a novel

ABBREVIATIONS: GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

subcellular brain preparation, the "synaptoneurosome" (33), to measure GABA- and barbiturate-mediated $^{36}Cl^-$ efflux (29, 30) and uptake (31).

Previous reports by our laboratory (31, 34) and others (28, 32, 35) indicate that "desensitization" of muscimol, GABA, or ethanol-induced $^{36}Cl^-$ uptake or efflux in brain vesicles can be demonstrated. In the present study we have investigated in detail GABA- and barbiturate-induced "desensitization" of the GABA receptor/Cl^- ion channel. The results presented here are very similar to those obtained in electrophysiologic experiments (23–26), providing the means for more detailed examination of the molecular events involved in the desensitization of the GABA receptor complex. A preliminary report of these findings has been presented (36).

Materials and Methods

Preparation of synaptoneurosomes. Synaptoneurosomes were prepared from adult male Sprague-Dawley rats (175–200 g) as previously described (29, 30) using a modification of the preparation described by Hollingsworth *et al.* (33). Briefly, cerebral cortices were dissected free from white matter, and approximately 1 g of tissue was homogenized in 7 ml of buffer containing 20 mM HEPES-Tris, 118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, and 2.5 mM $CaCl_2$ (pH 7.4) using a glass-glass homogenizer (five strokes). The homogenate was transferred to a 40-ml tube, diluted with 30 ml of ice-cold (0–4°) buffer, and then filtered by gravity through three layers of nylon mesh (160 μm; TETKO Inc., Elmsford, NY) placed in a Millipore Swinex filter holder. The filtrate was then gently pushed through a 10-μM Mitex filter (LCWP 047, Millipore) using a 10-ml syringe. The filtered preparation was centrifuged at 1000 × *g* for 15 min. After discarding the supernatant, the pellet was gently resuspended in buffer and washed once more by centrifugation (1000 × *g*, 15 min). The final pellet was gently resuspended to a final protein concentration of approximately 20 mg/ml. The filtration steps described above effectively remove large cellular debris, oligodendrocytes, and intact neurons resulting in a relatively homogeneous population of both pre- and postsynaptic membrane vesicles (33, 37). The final tissue suspension was kept on ice until ready for assay (no longer than 30 min).

Measurement of $^{36}Cl^-$ uptake. $^{36}Cl^-$ uptake into synaptoneurosomes was studied as previously described (31). Aliquots of synaptoneurosomes (approximately 2 mg of protein) were preincubated at 30° for 20 min prior to the addition of 0.5 μCi of $^{36}Cl^-$ (specific activity 12.5 mCi/g; New England Nuclear, Boston, MA). Drugs were added simultaneously with the $^{36}Cl^-$ (0.5 ml total assay volume), unless otherwise noted. $^{36}Cl^-$ uptake was terminated 5 sec later (unless otherwise noted) by the addition of 5 ml of cold buffer (0°), followed by vacuum filtration through glass fiber filters (Whatman GF/C) that had been soaked with 0.05% polyethyleneimine to reduce nonspecific binding of $^{36}Cl^-$. The filters were washed two more times with 5 ml of ice-cold buffer and placed in scintillation vials containing 7 ml of Redi Solv (Beckman Instruments, Fullerton, CA). The filters were then counted using conventional liquid scintillation spectrometry at an efficiency of >95%.

Data analysis. Data are expressed as either net $^{36}Cl^-$ uptake [$^{36}Cl^-$ uptake in the presence minus that in the absence (basal) of drug] in cpm or nmol/mg of protein, or as per cent stimulation of $^{36}Cl^-$ uptake above basal levels. Two groups of means were compared using the Student's *t* test, and when more than two means were compared, a one-way analysis of variance, followed by Duncan's New Multiple Range Test (38), was used.

Results

Concentration-response relationships. The GABA agonist, muscimol, stimulated $^{36}Cl^-$ uptake into synaptoneurosomes in a concentration-dependent manner, with an EC_{50} of 7.3 ± 0.5 μM ($N = 12$) (Fig. 1A). A similar effect on $^{36}Cl^-$ uptake was observed with GABA (EC_{50} 10 μM, data not shown). Pentobarbital also stimulated $^{36}Cl^-$ uptake but exhibited a bell-shaped concentration-response relationship (see also Ref. 31). Maximal stimulation of $^{36}Cl^-$ uptake by pentobarbital occurred at a concentration of 500 μM (EC_{50} 271 ± 17 μM, $N = 8$) whereas, at concentrations greater than 500 μM, the stimulation of $^{36}Cl^-$ uptake by pentobarbital diminished (Fig. 1B). We have previously shown that ethanol also stimulates $^{36}Cl^-$ uptake in a biphasic manner (34). (Analysis of the effects of ethanol on $^{36}Cl^-$ uptake has been described in a separate study.[1]) In order to clarify further the nature of the diminished $^{36}Cl^-$ uptake observed with high concentrations of pentobarbital, we examined whether these high concentrations of pentobarbital would alter the response to a direct GABA receptor agonist. Simultaneous addition of 3 mM pentobarbital and 2 μM muscimol inhibited the muscimol response by 60% (Fig. 2), and this was due to a decrease in the maximal response to muscimol (data not shown). However, when supramaximally stimulating concentrations of muscimol (100 μM–1 mM) were added with a low concentration of pentobarbital (200 μM), the effects of $^{36}Cl^-$ uptake were nearly additive (Fig. 2). Similar effects were also observed with pentobarbital and supramaximally stimulating concentrations of GABA (10 mM) (data not shown). Finally, as expected, subthreshold concentrations of pentobarbital (50 μM) enhanced muscimol-stimulated $^{36}Cl^-$ uptake by decreasing the EC_{50} from 8.3 ± 0.7 to 4.0 ± 0.8 μM ($p < 0.05$, $N = 4$) (Fig. 3).

We have previously shown that the effects of muscimol and pentobarbital on $^{36}Cl^-$ uptake are blocked by the GABA antagonists, bicuculline and picrotoxin (31, 34, 39). Unlike their effects on basal $^{36}Cl^-$ efflux (29, 30), bicuculline and picrotoxin do not block basal $^{36}Cl^-$ uptake (measured up to 3 min).

Time-response relationships. The time course of the effects of muscimol and pentobarbital on $^{36}Cl^-$ uptake was studied in two ways. In the first set of experiments, the time course of $^{36}Cl^-$ uptake was measured in the absence (basal uptake) or presence of muscimol (20 μM) or pentobarbital (500 μM), which were added simultaneously with the $^{36}Cl^-$ (Fig. 4). During the first 20 sec, basal $^{36}Cl^-$ uptake was essentially linear. $^{36}Cl^-$ uptake in the presence of pentobarbital was linear until approximately 7–10 sec when a plateau was reached ("net uptake," Fig. 4B). The time course for muscimol-stimulated $^{36}Cl^-$ uptake was more complex since any linearity must have occurred prior to 1 sec, the earliest time point tested. For muscimol-stimulated $^{36}Cl^-$ uptake, a plateau was reached as early as 3–5 sec. (Fig. 4B).

In the second set of experiments, synaptoneurosomes were preincubated with agonists for various times *prior* to the addition of $^{36}Cl^-$. For all of these experiments $^{36}Cl^-$ uptake was terminated after 5 sec. Preincubation of brain vesicles with a maximally stimulating concentration of muscimol (50 μM) for as little as 3 sec resulted in a decreased response (Fig. 5A). This decrement in response reached a plateau at approximately 15 sec ($t_{1/2}$ ~6 sec) (Fig. 5A). The decrement in the response to a maximally stimulating concentration of pentobarbital (500 μM) was slower in onset and reached a plateau later (2 min) ($t_{1/2}$ ~30 sec) than was observed with muscimol (Fig. 5B). Similar results have been observed with ethanol (34), and the data have been

[1] P. D. Suzdak, R. D. Schwartz, and S. M. Paul, submitted for publication.

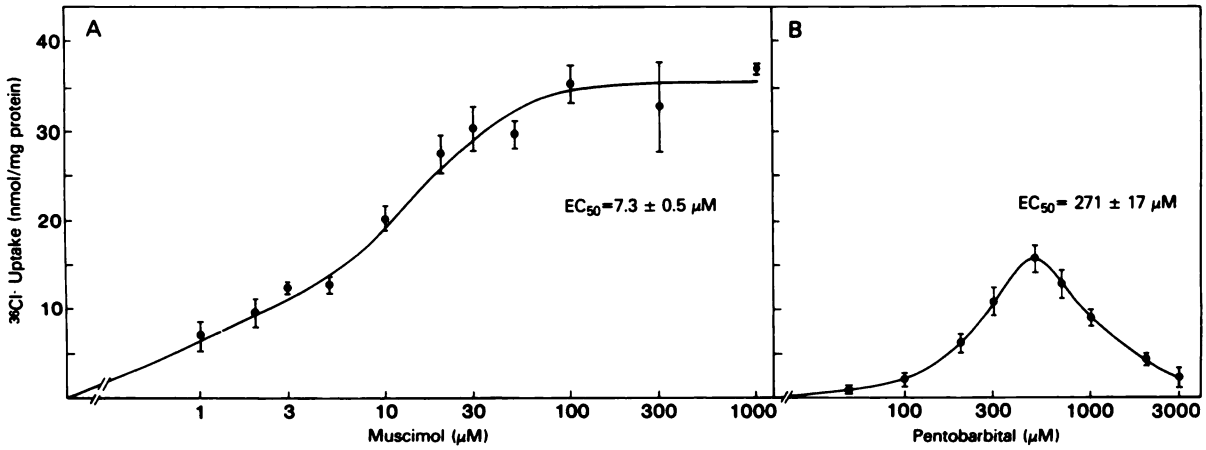

Fig. 1. Concentration-response curves for muscimol (A)- and pentobarbital-stimulated (B) $^{36}Cl^-$ uptake in cerebral cortical synaptoneurosomes. Synaptoneurosomes were incubated with various concentrations of muscimol or pentobarbital and $^{36}Cl^-$. Uptake of $^{36}Cl^-$ was terminated 5 sec later as described in Materials and Methods. Data are the means ± standard error from 12 and 8 experiments, respectively, performed in quadruplicate.

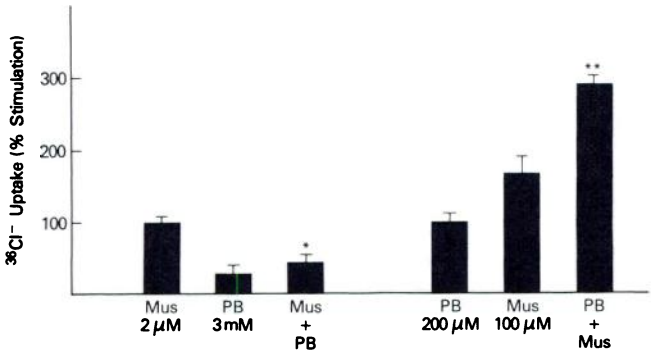

Fig. 2. Muscimol-stimulated $^{36}Cl^-$ uptake is blocked by a high concentration of pentobarbital. Synaptoneurosomes were incubated with $^{36}Cl^-$ and muscimol (*Mus*), pentobarbital (*PB*), or both. Uptake of $^{36}Cl^-$ was terminated 5 sec later as described in Materials and Methods. Data are the means ± standard errors of quadruplicate determinations from a single experiment and are representative of three such experiments. *, $p < 0.05$, compared to basal uptake; **, $p < 0.05$, compared to 200 μM pentobarbital and 100 μM muscimol. Basal uptake values were 10.1 ± 0.5 nmol/mg of protein.

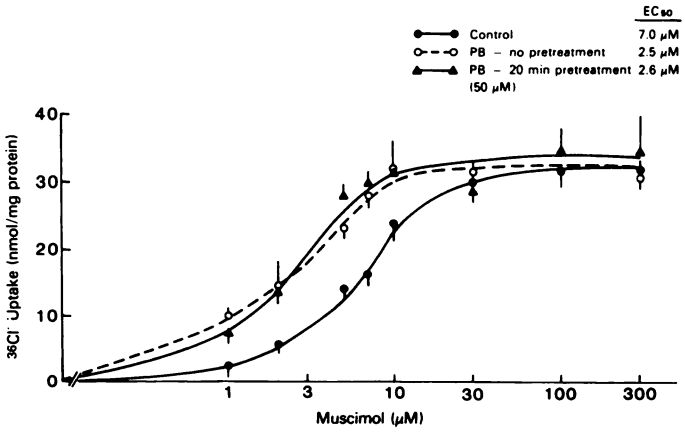

Fig. 3. Pentobarbital enhancement of muscimol-stimulated $^{36}Cl^-$ uptake. Synaptoneurosomes were either incubated simultaneously with $^{36}Cl^-$ and muscimol, EC_{50} = 7.0 μM (●); incubated simultaneously with $^{36}Cl^-$, muscimol, and pentobarbital (50 μM), EC_{50} = 2.5 μM (▲); or preincubated with pentobarbital (50 μM) prior to the addition of $^{36}Cl^-$ and muscimol, EC_{50} = 2.6 μM (○). Uptake of $^{36}Cl^-$ was terminated 5 sec later as described in Materials and Methods. Data are the means ± standard errors from a single experiment, performed in quadruplicate, and are representative of four such experiments (EC_{50} = 8.30 ± 0.75 versus 4.00 ± 0.82 and 4.35 ± 0.68, respectively, $N = 4$, $p < 0.05$).

included here for comparison (Fig. 5C). If the decrement in muscimol- or pentobarbital-induced $^{36}Cl^-$ uptake observed during preincubation were due to receptor desensitization, then it should be reversible. When the synaptoneurosomes were preincubated for 20 min with muscimol (50 μM) or pentobarbital (500 μM) and then washed two times by centrifugation, the subsequent addition of muscimol or pentobarbital, respectively, stimulated $^{36}Cl^-$ uptake to the same extent as in non-preincubated vesicles (Table 1). Thus, the decrement in response following preincubation with agonist appears to be reversible. Although it is also important to demonstrate that the decrement in response could be prevented with a GABA antagonist, this could not be performed since a washing step would be necessary to remove the antagonist prior to the addition of $^{36}Cl^-$. As shown in Table 1, the washing step itself reversed the decrement in response.

The decrement in response produced by preincubation of the synaptoneurosomes with muscimol and pentobarbital was also dependent on the concentration of agonist. Pretreatment of synaptoneurosomes with increasing concentrations of muscimol for 15 sec led to increasing decrements in response to muscimol, with an EC_{50} of approximately 4 μM (Fig. 6). Similarly, no decrement was observed with low concentrations of pentobarbital (100 μM) following a 20-min preincubation, whereas a decreased response (59%) to higher pentobarbital concentrations (500 μM) was evident by 1 min (Fig. 7). Preincubation with an intermediate concentration of pentobarbital (200 μM) for up to 2 min did not lead to a reduction in response. However, after 6 min of pretreatment (200 μM pentobarbital), the pentobarbital-induced response was decreased by 42% (data not shown). Pentobarbital, at subthreshold concentrations (50 μM), enhanced muscimol-stimulated $^{36}Cl^-$ uptake as demonstrated by the "left shift" of the muscimol concentration-response curve (Fig. 3). Pentobarbital was equally effective whether it was added simultaneously with muscimol and $^{36}Cl^-$ (i.e., no pretreatment) or added 20 min prior to muscimol and $^{36}Cl^-$ (Fig. 3). Thus, under these conditions, there was no decrement in the ability of low concentrations of pentobarbital to potentiate muscimol-stimulated $^{36}Cl^-$ uptake. In separate experiments in which a 20-min preincubation with a low concentration of pentobarbital (100 μM) did not result in a de-

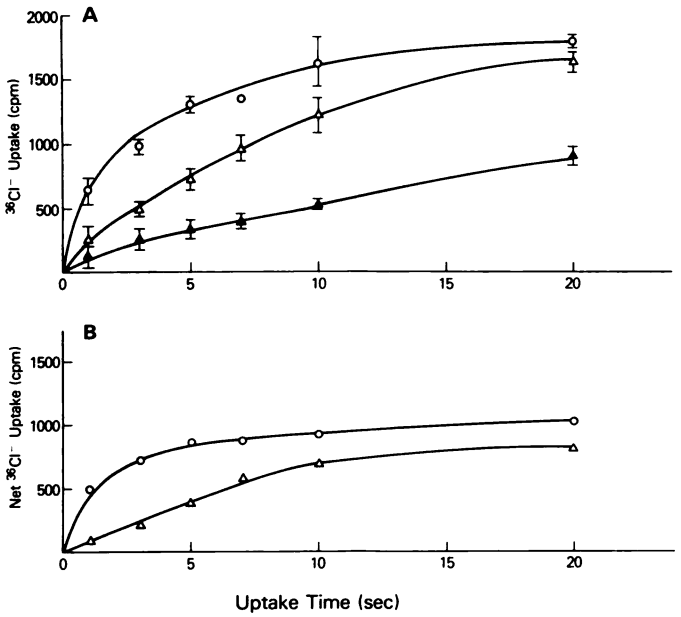

Fig. 4. Time course for muscimol- and pentobarbital-stimulated $^{36}Cl^-$ uptake. A. Synaptoneurosomes were incubated with $^{36}Cl^-$ and buffer (▲), 20 μM muscimol (○), or 500 μM pentobarbital (△) for various times before uptake of $^{36}Cl^-$ was terminated. B. Net uptake represents $^{36}Cl^-$ uptake (cpm) in the presence of drug minus $^{36}Cl^-$ uptake in the absence of drug. Data are the means ± standard errors of quadruplicate determinations from a single experiment and are representative of at least five such experiments.

creased response, a subsequent application of pentobarbital (100 μM) simultaneously with the $^{36}Cl^-$ produced an additive effect, and the latter response was equivalent to the stimulation by 200 μM pentobarbital in the absence of preincubation (data not shown).

Using this latter protocol, the phenomenon of "heterologous desensitization" could be studied. When synaptoneurosomes were preincubated for 20 min with maximally stimulating concentrations of pentobarbital (500 μM), there was an approximate 76% decrement in response (Fig. 8). The subsequent addition of either pentobarbital (500 μM) or muscimol (50 μM) failed to stimulate $^{36}Cl^-$ uptake beyond the level of the "desensitized" response (Fig. 8). The same was true when the vesicles were preincubated for 20 min with a maximally stimulating concentration of muscimol (50 μM). The subsequent addition of either muscimol (50 μM) or pentobarbital (500 μM) did not stimulate $^{36}Cl^-$ uptake above the "desensitized" level (Fig. 8). Similar heterologous desensitization has also been observed between pentobarbital and GABA (data not shown) and pentobarbital or muscimol and ethanol.[2]

Discussion

Various investigators have reported that the electrophysiologic responses of continuously applied GABA or barbiturates diminish over time (1, 23–26). We have observed a similar phenomenon using a method for measuring GABA-receptor-coupled $^{36}Cl^-$ flux *in vitro*. The stimulation of $^{36}Cl^-$ uptake into synaptoneurosomes by either the GABA agonist, muscimol, or pentobarbital, declined within seconds following incubation of the brain vesicles with these agonists. This was evident in

[2] P. D. Suzdak, R. D. Schwartz, and S. M. Paul, submitted for publication.

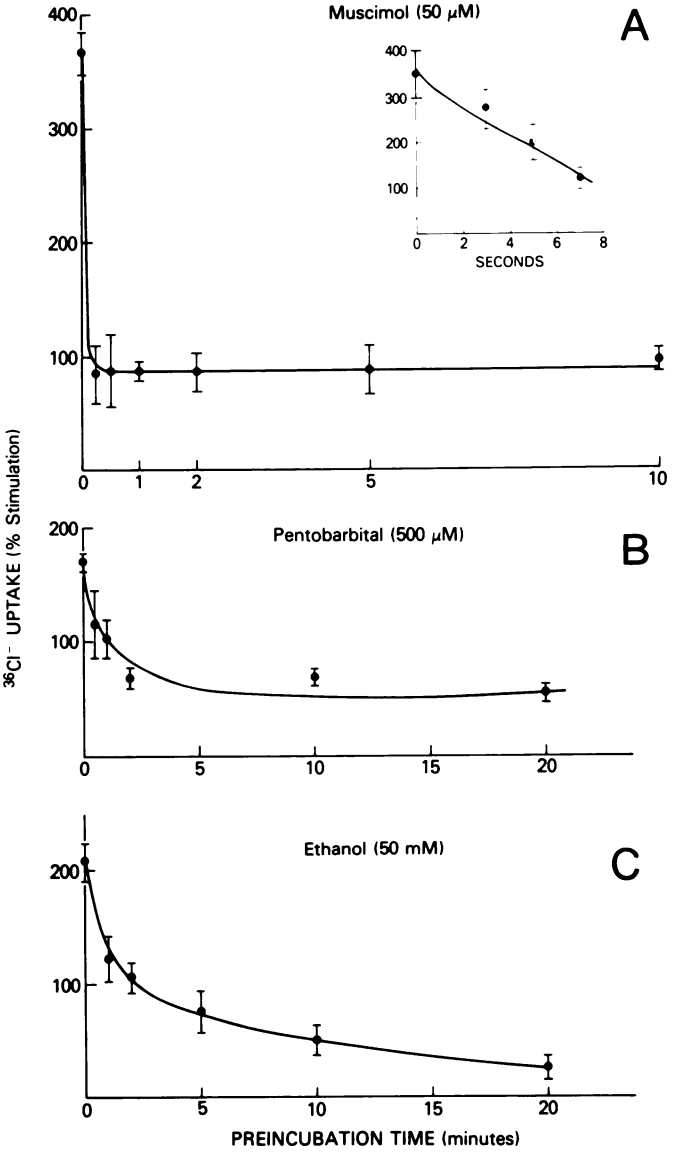

Fig. 5. Time course for decrement in response to continuous exposure to muscimol, pentobarbital, and ethanol. Synaptoneurosomes were preincubated with muscimol (A), pentobarbital (B), or ethanol (C) for various times before the addition of $^{36}Cl^-$. Uptake of $^{36}Cl^-$ was terminated 5 sec later as described in Materials and Methods. Note different time scales. Data are the means ± standard errors of quadruplicate determinations from a single experiment and are representative of at least three such experiments. Basal and drug-stimulated $^{36}Cl^-$ uptake values were: A, 9.5 ± 0.6 and 44.8 ± 2.2; B, 9.0 ± 1.7 and 24.1 ± 0.6; and C, 5.6 ± 1.1 and 15.7 ± 1.7 nmol/mg of protein, respectively.

experiments in which $^{36}Cl^-$ uptake subsequent to agonist preincubation was measured. Near-maximally effective concentrations of muscimol (which produced a greater increase in $^{36}Cl^-$ uptake than did maximally stimulating concentrations of pentobarbital) produced a faster onset and rate of decrement in response than did pentobarbital (see Fig. 5). The time course of the decrement in response to muscimol is similar to the time course of the decrement in response to GABA, observed in electrophysiologic studies of rat hippocampus (26). In the latter study the onset and decay ($t_{½}$) of the response decrement to GABA were 1.2 sec and 4.5 sec, respectively (compared to < 3 sec and 6 sec, respectively, in the present study). Although

TABLE 1

Decrement in response to muscimol and pentobarbital is reversed by washing

Synaptoneurosomes were preincubated for 20 min at 30° with buffer, muscimol (50 μM), or pentobarbital (500 μM) and were either washed twice by centrifugation or not washed prior to the addition of $^{36}Cl^-$, $^{36}Cl^-$ plus muscimol, or $^{36}Cl^-$ plus pentobarbital, respectively. Uptake of $^{36}Cl^-$ was terminated 5 sec later as described in Materials and Methods. Values are the means ± standard errors of three to five experiments, performed in quadruplicate.

Preincubation conditions	$^{36}Cl^-$ uptake
	% stimulation by muscimol (50 μM)
Buffer, 20 min	261 ± 45
Muscimol (50 μM), 20 min	103 ± 45[a]
Muscimol, 20 min, then washed	244 ± 51
	% stimulation by pentobarbital (500 μM)
Buffer, 20 min	141 ± 20
Pentobarbital (500 μM), 20 min	32 ± 12[a]
Pentobarbital, 20 min, then washed	118 ± 36

[a] $P < 0.05$ compared to the buffer and washed conditions.

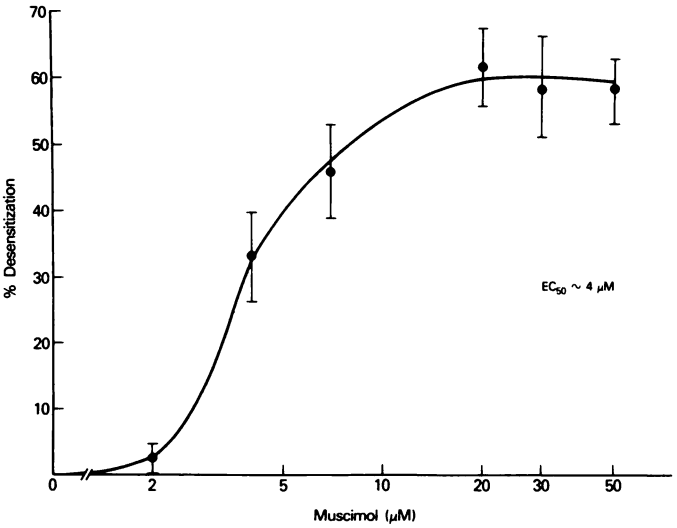

Fig. 6. Concentration dependence of the decrement in response to muscimol. Synaptoneurosomes were preincubated with various concentrations of muscimol for 0 sec or 15 sec before the addition of $^{36}Cl^-$. Uptake of $^{36}Cl^-$ was terminated 5 sec later as described in Materials and Methods. The decrement in response (% *desensitization*) was calculated as described in the text. Data are the means ± standard errors from three experiments, performed in quadruplicate.

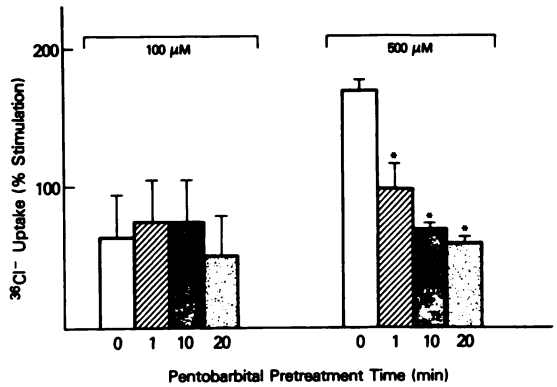

Fig. 7. Concentration dependence of the decrement in response to pentobarbital. Synaptoneurosomes were preincubated with pentobarbital (100 or 500 μM) for various times prior to the addition of $^{36}Cl^-$ or $^{36}Cl^-$ plus pentobarbital (500 μM). Data are the means ± standard errors of quadruplicate determinations from a single experiment and are representative of three such experiments. *, $p < 0.05$ compared to no preincubation. Basal uptake values were 9.0 ± 1.1 nmol/mg of protein.

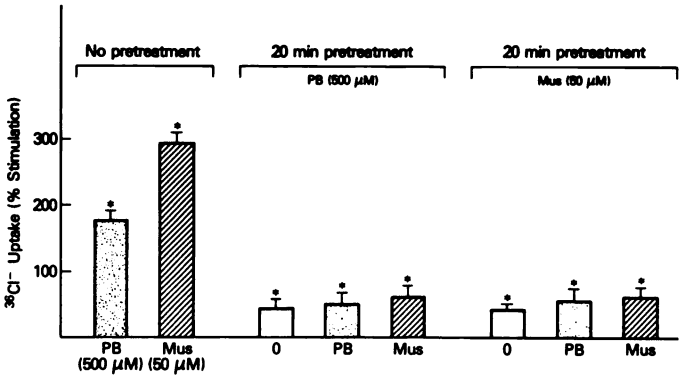

Fig. 8. "Heterologous desensitization" of pentobarbital- and muscimol-stimulated $^{36}Cl^-$ uptake. Synaptoneurosomes were preincubated with or without pentobarbital (*PB*) or muscimol (*Mus*) for 20 min prior to the subsequent addition of $^{36}Cl^-$ or $^{36}Cl^-$ plus pentobarbital or muscimol. Uptake of $^{36}Cl^-$ was terminated 5 sec later as described in Materials and Methods. Data are the means ± standard errors of quadruplicate determinations from a single experiment and are representative of at least four such experiments. *, $p < 0.05$, compared to basal uptake. Basal uptake values were 11.8 ± 1.1 nmol/mg of protein.

similar measurements were not made using barbiturates in the study by Thalmann and Herskowitz (26), the results obtained here are qualitatively similar to their findings: the more rapid response decrement (Fig. 5) is associated with the more rapid response development (Fig. 4) (i.e., muscimol > pentobarbital). In another study (24) in which electrophysiologic responses to GABA and pentobarbital were measured in frog sensory neurons, the onset and peak response to GABA were faster and of greater amplitude than those to pentobarbital. In addition, the decrement in response to GABA occurred more rapidly than that to pentobarbital (24), similar to the results presented here. Possible explanations for the different rates of development of both the peak response and the decrement in response to muscimol and pentobarbital include: 1) differences in the binding kinetics between these two agonists to their respective recognition sites, or 2) differences in the coupling of the respective recognition sites to the effector component. Despite the kinetic differences between these two drugs, the decrement in response to both muscimol and pentobarbital reached a plateau, so that stimulation of $^{36}Cl^-$ uptake was still measurable (Fig. 5). This has also been observed in electrophysiologic studies (26, 40) and could reflect the proportion of receptors resistant to "desensitization" at a given concentration of agonist (40). Alternatively, these phenomena might be related to the ability of receptors to adapt to prolonged stimulation (or inhibition) (41) resulting in a new steady state level of responsiveness.

Although recovery from the decrement in response could be measured indirectly (via washing), the kinetics of recovery could not be analyzed using the technique described here. Indeed, due to the rapid onset of GABA-mediated decrement in response, detailed kinetic analyses were not performed. However, Subbarao and Cash (35) have recently presented preliminary findings using a quench flow technique to measure GABA responses ($^{36}Cl^-$ uptake measured in the msec range) in a similar brain vesicle preparation. Presumably, this method would enable more detailed kinetic analyses of the character-

istics of GABA- and barbiturate-mediated desensitization and resensitization.

Various mechanisms for the decrement in response following incubation with GABA or pentobarbital have been proposed. These include: 1) removal of the drug via an uptake mechanism (26, 42–46), 2) accumulation of intracellular Cl^- (2, 47), thereby decreasing the inward driving force for Cl^-, and 3) desensitization of the GABA receptor-coupled ion channel (1, 23–26, 48). Electrophysiologic studies have shown that GABA uptake can account, in part, for the "fading" GABA response in certain areas of hippocampus since nipecotic acid, a GABA uptake inhibitor, prevented the fading or prolonged the duration of the GABA response (42, 43, 46). In contrast, other studies indicate that the contribution of an uptake mechanism in the response decrement to GABA in hippocampal slices is small (26). Nipecotic acid has also been reported to increase the potencies of GABA as well as muscimol [considered to be a poor substrate for the GABA uptake system (49)] in hippocampal (44) and olfactory cortical slices (45); however, we found no effect of nipecotic acid on the decrement in response to muscimol. In the present study, experiments in which a second addition of muscimol or pentobarbital failed to stimulate $^{36}Cl^-$ uptake beyond the "desensitized" level (Figs. 7 and 8), provide strong evidence that an uptake mechanism for muscimol or pentobarbital cannot account for the decrement in response to these agonists. It is more difficult to rule out the second possibility as a mechanism for the decrement in response to muscimol and pentobarbital. Although two recent electrophysiologic studies indicate that the accumulation of intracellular Cl^- does not play a major role in the response decrement to these agonists (25, 26), this cannot be determined from our experimental protocol. In order to determine whether the inward driving force for Cl^- is reduced during continuous application of the agonist, the membrane potential would have to be close to the equilibrium potential for Cl^-. This is more accurately measured in electrophysiologic experiments. Finally, the decrement in response to agonists could be due to desensitization of the GABA receptor and the barbiturate recognition sites coupled to the Cl^- ion channel. The data indicate that the response decrement is dependent upon both the concentration and length of exposure to muscimol and/or pentobarbital, which would be expected for a functional desensitization process. Furthermore, "cross-desensitization" between muscimol (or GABA) and pentobarbital was also evident, suggesting that "heterologous desensitization" of the GABA receptor/Cl^- ion channel occurs. This finding provides further evidence that pentobarbital, even at high concentrations (500 μM), probably has an allosteric interaction with the GABA recognition site to increase Cl^- conductance. Thus, desensitization of the barbiturate response could be produced allosterically via the GABA recognition site or via the barbiturate recognition site itself (also see below).

Another major difference in the response to muscimol (or GABA) and pentobarbital is the shape of the respective concentration-response curves. The biphasic concentration-response curve for pentobarbital is similar to that reported for pentobarbital stimulation of Cl^- conductance in frog sensory neurons (24), nicotinic receptor stimulation of ion flux in PC12 cells (50), and ethanol stimulation of $^{36}Cl^-$ uptake in synaptoneurosomes (34).

In our previous studies using the $^{36}Cl^-$ efflux assay (29, 30), pentobarbital stimulated $^{36}Cl^-$ efflux in a sigmoidal, concentration-dependent manner. Differences in assay conditions (i.e., temperature) probably result in a shift in the concentration at which a decrease in barbiturate-stimulated $^{36}Cl^-$ efflux might occur. This was difficult to test with higher pentobarbital concentrations (>3 mM) in the efflux assay due to limits in solubility. There are several possible factors underlying the attenuated response to high pentobarbital concentrations (>500 μM) observed here. The first involves blockade of the ion channel itself. There is sufficient evidence to suggest that high concentrations of barbiturates can block various ion conductances (51, 52), although electrophysiologic studies also indicate that blockade of Cl^- channels by high concentrations of barbiturates is unlikely (23). Second, barbiturates (as well as ethanol) alter membrane fluidity (53) and, at high concentrations, barbiturates would perturb the membrane microenvironment surrounding the GABA receptor/Cl^- ion channel, leading to a decrease in ion flux. In fact, the conductance-blocking action of barbiturates is reported to be due to their membrane-disordering properties (54). Third, it is possible that pentobarbital is acting indirectly (even at high concentrations) to increase $^{36}Cl^-$ flux by enhancing the actions of endogenous GABA. We have previously suggested that endogenous GABA is present in this preparation since the GABA antagonists, picrotoxin and bicuculline, decrease basal $^{36}Cl^-$ efflux (29, 30). Although this is not observed in the $^{36}Cl^-$ uptake experiments (probably due to differences in assay conditions), the presence of endogenous GABA can not be ruled out. However, it is the concentration of extravesicular GABA that is most important, and since subthreshold concentrations of pentobarbital (50 μM) enhance the action of muscimol (10 μM) yet have no effect on basal $^{36}Cl^-$ uptake, the concentrations of extravesicular GABA would have to be very small (<10 μM). Thus, the enhancement of subthreshold concentrations of endogenous GABA by pentobarbital may be maximal at pentobarbital concentrations near 500 μM and diminish at higher pentobarbital concentrations. In support of this hypothesis Willow and Johnston (55) have reported that pentobarbital enhances GABA receptor binding in brain membranes with a bell-shaped concentration-response relationship. The enhancement of GABA binding by pentobarbital is maximal at 125–500 μM, whereas at concentrations > 500 μM pentobarbital, the enhancement is attenuated (55). Electrophysiologic studies also indicate that pentobarbital enhances GABA-induced Cl^- currents in sensory neurons with a similar concentration-response relationship (24). Again, at concentrations of pentobarbital >400 μM, the enhancement of GABA responses declines (24). To explain this phenomenon, Higashi and Nishi (23) suggest that high concentrations of barbiturates may induce a conformational change in the receptor complex, thereby excluding GABA-activated responses, similar to a desensitization process. Thus, desensitization might also explain the attenuated responses to high concentrations of pentobarbital observed here. Functional desensitization has been proposed as the mechanism for similar attenuated responses to high concentrations of barbiturates (26) and carbachol (50). In this case a conformational change in the receptor complex leading to desensitization, could occur in the absence of agonist binding to the GABA recognition site (23). As discussed for the time-dependence relationships, desensitization of the barbiturate response could occur via allosteric interactions with the GABA recognition site or via the barbiturate

recognition site itself. Although a similar response to GABA agonists at high concentrations was not observed, it is possible that the mechanism for inducing a conformational change in the receptor by GABA agonists and barbiturates is different.

The pharmacologic significance of "desensitization" produced by GABA agonists and pentobarbital is unclear. Anesthetic concentrations of pentobarbital achieved in laboratory animals and humans (50–300 μM) (56) enhance GABA responses (19–21, 23, 24, 28, 32) and GABA receptor binding *in vitro* (11, 12, 55). In the present study the enhancement of muscimol-stimulated $^{36}Cl^-$ uptake by low (subanesthetic) concentrations of pentobarbital did not result in desensitization over a 20-min preincubation. However, it is possible that desensitization could develop from longer exposure to low pentobarbital concentrations. Continuous exposure to higher, yet clinically relevant concentrations of pentobarbital resulted in a desensitized response. As discussed above, the rapid onset of desensitization may lead to acute changes in the level of responsiveness of the GABA receptor. These changes may be important in the development of acute and chronic tolerance to drugs such as barbiturates and alcohol. It has been suggested that acute tolerance may develop quickly only to disappear when more long-lasting adaptive changes take place (57).

The rapid desensitization of the GABA receptor/Cl^- ion channel leading to acute changes in its level of responsiveness may also have physiologic significance. It is possible that desensitization of the GABA receptor might develop during normal brief flurries of GABA-activated inhibitory postsynaptic potentials (26). As a consequence, for example, disinhibition of hippocampal pyramidal cells (with a loss of the inhibitory postsynaptic potential) could occur, resulting in increased excitability and the development of seizures (58).

Our data suggest that the "desensitization" of the GABA receptor/Cl^- ion channel may involve not only the GABA recognition site but also a barbiturate recognition site located in a hydrophobic domain somewhere between the GABA agonist site and the Cl^- ion channel (23, 24). Although an endogenous ligand for the barbiturate site has not yet been isolated, the barbiturate-like activity of two endogenous steroids at the GABA receptor/Cl^- ion channel has been recently described (39). Further examination of the physiologic and pharmacologic role of the barbiturate recognition site in modulating GABA receptor activity will undoubtably lead to a greater understanding of the factors regulating the sensitivity and function of the GABA receptor/Cl^- ion channel.

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