

Barbiturate and picrotoxin-sensitive chloride efflux in rat cerebral cortical synaptoneuroosomes

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The effects of various barbiturates and picrotoxin in modifying the efflux of chloride (³⁶Cl⁻) was studied in a novel subcellular preparation from rat cerebral cortex, the 'synaptoneurosome'. Dilution of synaptoneuroosomes pre-loaded with ³⁶Cl⁻ resulted in rapid efflux of ³⁶Cl⁻ that could be measured as early as 10 s following dilution. In the presence of barbiturates such as pentobarbital and hexobarbital there was a significant increase in ³⁶Cl⁻ efflux which was not observed with the pharmacologically-inactive barbiturate, barbital. The effect of barbiturates in enhancing ³⁶Cl⁻ efflux was also stereospecific [(-)-DMBB > (+)-DMBB] and reversed by picrotoxin. By contrast, picrotoxin alone significantly inhibited ³⁶Cl⁻ efflux. These data demonstrate pharmacologically relevant Cl⁻ transport for the first time in a subcellular brain preparation.

Chloride efflux Brain synaptoneurosome Barbiturate Picrotoxin GABA

1. INTRODUCTION

γ -Aminobutyric acid (GABA), the principal inhibitory neurotransmitter in brain, is thought to exert its physiologic actions by increasing membrane permeability to Cl⁻. A number of sedative/hypnotic drugs like the barbiturates have also been shown to enhance GABA-mediated Cl⁻ permeability as well as to activate directly Cl⁻ flux [1-3]. These phenomena have been studied extensively using electrophysiologic techniques [1,2] but because of methodologic limitations, biochemical studies of Cl⁻ transport in brain have generally been unsuccessful. Recently, GABA-mediated transport of ³⁶Cl⁻ has been reported in both rat hippocampal slices [4] and in intact cultured embryonic chick cerebral neurons [5]. However, there have been no reports using subcellular preparations from rat brain, possibly because such preparations are too permeable for measurement of neurotransmitter-gated or voltage-dependent Cl⁻ flux. Recently, we have examined ³⁶Cl⁻ transport in a novel subcellular preparation from

rat brain, the 'synaptoneurosome' [6,7] and now report both barbiturate and picrotoxin-sensitive ³⁶Cl⁻ efflux from this preparation.

2. MATERIALS AND METHODS

2.1. Preparation of synaptoneuroosomes

Synaptoneuroosomes were prepared from cerebral cortices from adult, male Sprague-Dawley rats (200-250 g) as described and characterized in [6,7]. Cerebral cortices were dissected free from white matter and homogenized in buffer containing 20 mM Hepes-Tris, 118 mM NaCl, 4.7 mM KCl, 1.18 mM MgSO₄ and 2.5 mM CaCl₂ (pH 7.4) using a glass-homogenizer (5 strokes). The homogenate was filtered through 3 layers of nylon mesh and then through a 10 μ m Millipore filter. The filtered preparation was centrifuged at 1000 \times g for 15 min. After discarding the supernatant the pellet was resuspended in buffer to a final protein concentration of 10 mg/ml.

2.2. Chloride efflux

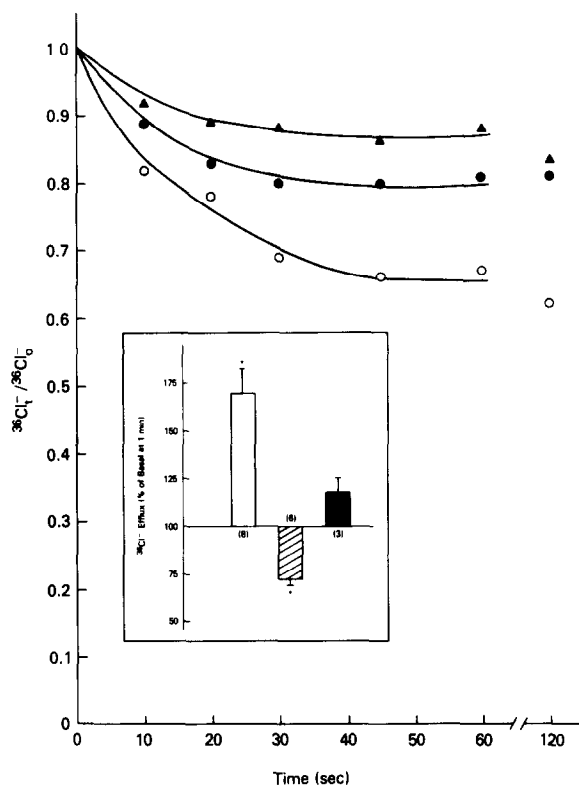
³⁶Cl⁻ efflux was studied using a modification of the method in [8] for measurement of cation flux

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in *Torpedo* microsacs and that in [9] for measurement of $^{36}\text{Cl}^-$ efflux in *Torpedo* membrane vesicles. Synaptoneurosomes (10 mg protein/ml) were incubated with $^{36}\text{Cl}^-$ ($5 \mu\text{Ci/ml}$, spec. act. 12.5 mCi/g, NEN, Boston, MA) for 60 min at 0°C . Aliquots of synaptoneurosomes equivalent to approx. 4 mg protein were diluted 50-fold with buffer at 25°C in the presence or absence of drug. After rapid mixing, the diluted suspension was filtered at various time intervals under vacuum through Whatman GF/C filters which were immediately washed with 5 ml cold buffer. The filters were placed in scintillation fluid and counted at an efficiency of $>90\%$. The loss or efflux of $^{36}\text{Cl}^-$ from the pre-loaded synaptoneurosomes was determined by measuring the amount of radioactivity retained by the filters.

3. RESULTS

A rapid efflux of $^{36}\text{Cl}^-$ was observed and could be reliably measured within the first 10 s after dilution of the $^{36}\text{Cl}^-$ -preloaded synaptoneurosomes (fig.1). A slower phase of $^{36}\text{Cl}^-$ efflux followed the initial rapid phase. In the presence of pentobarbital there was a significant increase in $^{36}\text{Cl}^-$ efflux compared to the basal (no drug) condition (fig.1). By contrast, in the presence of picrotoxin there was a significant inhibition of $^{36}\text{Cl}^-$ efflux compared to basal conditions (fig.1). Further, the pentobarbital-induced efflux of $^{36}\text{Cl}^-$ was significantly inhibited by picrotoxin (fig.1, inset). In preliminary experiments where the concentration of pentobarbital was varied in the presence and absence of picrotoxin this inhibition appeared to be competitive in nature (in preparation). Pentobarbital was shown to stimulate $^{36}\text{Cl}^-$ efflux with an EC_{50} of approx. $200 \mu\text{M}$ (fig.2). The effects of several other barbiturates on $^{36}\text{Cl}^-$ efflux were also studied. As shown in table 1, a number of pharmacologically active barbiturates decreased the amount of $^{36}\text{Cl}^-$ retained by the synaptoneuro-



in buffer containing 1 mM picrotoxin (\blacktriangle), or 1 mM pentobarbital (\circ) as described in the text. (Initially these experiments were performed at the temperatures described above and yielded results qualitatively similar to those obtained when synaptoneurosomes were loaded at 0°C and diluted at 25°C . Based on temperature-dependence experiments, the latter procedure was chosen for all subsequent experiments.) $^{36}\text{Cl}^-$ efflux is expressed as a ratio of the amount of $^{36}\text{Cl}^-$ retained by the synaptoneurosomes at various time intervals ($^{36}\text{Cl}_t$) relative to that at time zero ($^{36}\text{Cl}_0$). Time zero represents the amount of $^{36}\text{Cl}^-$ retained by an equivalent amount of undiluted synaptoneurosomes which were filtered in the same manner as the diluted preparation. Data are from a single experiment performed in triplicate and are representative of 8 such experiments. Inset: $^{36}\text{Cl}^-$ efflux expressed as the percent of $^{36}\text{Cl}^-$ efflux relative to basal (no drug) conditions at 1 min. Basal efflux at 1 min was $21.8 \pm 1.0\%$ of that at time zero, whereas efflux at 1 min in the presence of pentobarbital (open bar) picrotoxin (hatched bar), and picrotoxin plus pentobarbital (closed bar) was $36.9 \pm 2.4\%$ ($p < 0.001$ vs basal), $16.4 \pm 1.3\%$ ($p < 0.01$ vs basal), and $26.6 \pm 3.5\%$ ($p = \text{NS}$ vs basal), respectively. Data represent the mean \pm SE of the number of experiments indicated in parentheses. * $p < 0.05$ using Student's t -test.

Fig.1. Effects of pentobarbital, picrotoxin, and pentobarbital plus picrotoxin on $^{36}\text{Cl}^-$ efflux from synaptoneurosomes. Synaptoneurosomes from rat cerebral cortex were preloaded with $^{36}\text{Cl}^-$ for 60 min (25°C) and efflux was measured by diluting the synaptoneurosomes 50-fold (0°C) in buffer alone (\bullet) or

somes at 2 min. (-)-DMBB was the most potent barbiturate tested and this effect was stereospecific since (-)-DMBB was almost twice as potent as (+)-DMBB ($p < 0.01$). Phenobarbital was the least potent barbiturate while barbital itself was inactive. Similar effects were also observed in synaptoneurosomes from hippocampus and cerebellum (not shown). Interestingly, the relative order of potency of the various barbiturates in enhancing $^{36}\text{Cl}^-$ efflux correlates very well ($r = 0.93$, $p < 0.01$) with their ability to enhance [^3H]diazepam binding to rat brain membranes [10].

4. DISCUSSION

Morphologic studies show that synaptoneurosomes contain both pre- and post-synaptic membranes which form closed vesicles and biochemical studies reveal that these membrane vesicles retain functional receptor-mediated activities [6,7]. The synaptoneurosome preparation is a further purification of the vesicular preparation from guinea pig cortex originally described by authors in [11] and used in the study of cAMP accumulation [12] and membrane depolarization by batrachotoxin [13].

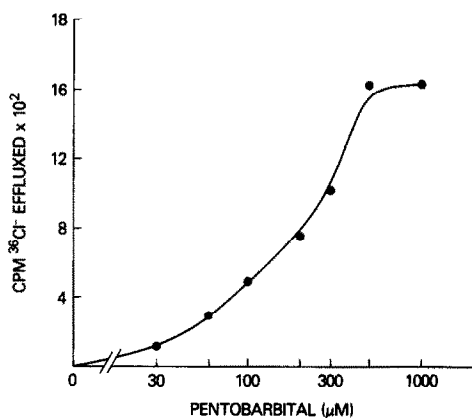


Fig. 2. The effects of pentobarbital (30 μM –1 mM) on $^{36}\text{Cl}^-$ efflux from cerebral cortical synaptoneurosomes. Efflux of $^{36}\text{Cl}^-$ from preloaded synaptoneurosomes was measured at 2 min as described in table 1. $^{36}\text{Cl}^-$ efflux is expressed as the difference between the amount of $^{36}\text{Cl}^-$ retained by the synaptoneurosomes in the absence and presence of pentobarbital. Data represent the mean of quadruplicate determinations and are from one experiment which is representative of 7 experiments performed in quadruplicate.

The vesicles which are essentially derived from the P1 fraction appear to maintain a suitable permeability barrier to $^{36}\text{Cl}^-$ so that its transport may be reliably measured. In other experiments we have observed that the pentobarbital induced efflux of $^{36}\text{Cl}^-$ from synaptoneurosomes is inhibited by 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) (in preparation), a specific inhibitor of chloride transport in red blood cells [14]. It is unlikely that binding of $^{36}\text{Cl}^-$ to membranes is being measured since dilution of the synaptoneurosomes in hypotonic buffer or in 1% SDS results in an immediate loss of $^{36}\text{Cl}^-$ from the preloaded vesicles (not shown).

The effects of pentobarbital on $^{36}\text{Cl}^-$ transport observed here are similar to those reported in [4] using rat hippocampal slices, although in the latter study the EC_{50} value for pentobarbital was about 10-fold higher. Pentobarbital and picrotoxin are believed to act at a site on the GABA/ Cl^- ionophore receptor complex, distinct from the GABA recognition site, but associated with the Cl^- ionophore itself [15]. In electrophysiologic

Table 1

Effects of various barbiturates on $^{36}\text{Cl}^-$ efflux from cerebral cortical synaptoneurosomes

Drug (300 μM)	$^{36}\text{Cl}^-$ retained by synaptoneurosomes (% of control)
None	100.0 \pm 1.9
(-)-DMBB	67.7 \pm 1.6 ^a
Pentobarbital	70.0 \pm 3.1 ^a
Hexobarbital	75.3 \pm 3.9 ^a
(+)-DMBB	82.0 \pm 1.4 ^a
Phenobarbital	89.7 \pm 1.9 ^a
Barbital	95.0 \pm 2.3

Efflux of $^{36}\text{Cl}^-$ was measured after a 50-fold dilution of the preloaded synaptoneurosomes (600 μg protein to a final volume of 3 ml). The amount of radioactivity retained by the synaptoneurosomes was measured 2 min later by filtering the entire mixture as described in section 2. The ratio of $^{36}\text{Cl}_t/^{36}\text{Cl}_0$ at 2 min in the absence of drug was 0.26 ± 0.01 . Data are expressed as the mean \pm SE for 3 experiments performed in triplicate. ^a $p < 0.05$ using Student's t -test. The percent $^{36}\text{Cl}^-$ retained in the presence of (-)-DMBB was significantly less than that retained in the presence of (+)-DMBB ($p < 0.01$ using Student's t -test)

studies, pentobarbital not only enhances GABA-activated increases in Cl^- conductance, but also directly increases Cl^- conductance, resulting in decreased neuronal excitability [1,2]. These effects are also reversed by picrotoxin [1,2]. Our results demonstrating that the pentobarbital-induced increase in $^{36}\text{Cl}^-$ efflux in synaptoneurosomes is reversed by picrotoxin, suggest that we are measuring pharmacologically relevant Cl^- transport associated with the GABA/ Cl^- ionophore receptor complex. The inhibition of basal $^{36}\text{Cl}^-$ efflux by picrotoxin suggests the presence of endogenous GABA. More recent studies in our laboratory have revealed that the GABA agonist, muscimol, also stimulates efflux of $^{36}\text{Cl}^-$ from preloaded synaptoneurosomes with an EC_{50} of approx. $8 \mu\text{M}$ (in preparation). Thus, Cl^- channel activation by endogenous GABA may contribute to basal $^{36}\text{Cl}^-$ efflux which is inhibited by picrotoxin. To our knowledge this is the first report of barbiturate and picrotoxin sensitive $^{36}\text{Cl}^-$ efflux in a subcellular preparation from brain. This preparation may prove valuable in studying the actions of other drugs thought to regulate GABA-mediated Cl^- permeability (e.g., the benzodiazepines) [16,17] as well as to define the biochemical and molecular mechanisms mediating Cl^- conductance in the central nervous system.

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