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[^{3}H]ADENOSINE UPTAKE AND RELEASE FROM SYNAPTOSOMES

ALTERATIONS BY BARBITURATES

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Abstract—The effects of barbiturates on adenosine movements across the synaptic plasma membrane have been investigated using rodent whole brain synaptosomes. The hypothesis tested was that some of the depressant actions of these drugs may be mediated through interference with an endogenous adenosine system. Adenosine uptake was studied using synaptosomes prepared from Swiss–Webster mice. After preincubation at 37°, [^{3}H]adenosine was added to the synaptosomes in the presence or absence of pentobarbital, methohexital, phenobarbital, or 5-(2-cyclohexylideneethyl)-5-ethyl barbituric acid (CHEB) at various concentrations and times. All four compounds significantly inhibited [^{3}H]-adenosine uptake at concentrations of 100–300 μM. Pentobarbital did not affect the distribution of synaptosomal adenosine metabolites. Release of [^{3}H]adenosine was studied using the P_2 pellet from male CD-1 mice. Addition of 50 mM KCl caused an enhancement of ^{3}H-efflux mainly due to increased release of adenosine and inosine. This effect was abolished in the presence of 250 μM ethylene glycol bis(β-aminoethyl-ether)-N,N^2-tetraacetic acid (EGTA). Pentobarbital, 0.3 mM, caused a significant increase in the net potassium-induced release of [^{3}H]adenosine. These results suggest that some of the depressant effects of barbiturates may be due to inhibition of adenosine reuptake and enhancement of release resulting in elevated synaptic adenosine levels.

Adenosine and related purines are thought to function as endogenous neuromodulators in the CNS [1]. Biochemical, electrophysiological, and behavioral evidence point to a depressant action of endogenous adenosine in brain tissue. Adenosine has been shown to inhibit the release of various neurotransmitters from synaptosomes [2], brain slices [3–5], and *in vivo* [6]. The uptake and release of adenosine have also been characterized in synaptosomes [7] and cortical slices [8]. In electrophysiological experiments, adenosine depresses both spontaneous [9] and evoked neuronal activity in the CNS [10]. These results correlate well with the behavioral actions of adenosine. Sedative, hypnotic, and anticonvulsant effects have been reported after adenosine administration [11, 12]. These effects of adenosine are proposed to be due to an interaction with putative receptor sites [13–15].

Certain exogenously administered compounds may exert their pharmacological effects by interfering with the neuronal adenosine system. Several studies have suggested that the excitatory effects of methylxanthines, such as caffeine, are related to their abilities to block adenosine receptors. Benzodiazepines have been shown to inhibit adenosine uptake into rat brain synaptosomes in therapeutically effective concentrations, suggesting that this inhibition may be an important factor in the central actions of these compounds [16]. However, the actual concentrations obtained in brain during therapy may produce minimal effects on adenosine uptake [17]. Other drugs with sedative properties, such as phenothiazines, have also been reported to block adenosine uptake in synaptosomes [18]. The enhancement by opiates of adenosine release from cortical slices [19] and *in vivo* rat cerebral cortex [20] suggests that adenosine may mediate some of the central effects of morphine.

Because of the correlation between the effects of these drugs on adenosine uptake and release and their depressant effects, we were interested in investigating the effects of the barbiturate class of depressant drugs on adenosine uptake and release in synaptosomes. Our results give further confirmation to the suggestion that the action of some CNS depressants may be mediated through an interaction with endogenous adenosine in the brain.

MATERIALS AND METHODS

Subjects were Swiss–Webster mice (20–35 g) obtained from Southern Animal Farms (Alabama) or bred and maintained at the University of Texas (Austin, TX). All animals were adapted to a 12/12 hr light–dark cycle with food and water *ad lib.* for several days before use. 5-(2-Cyclohexylideneethyl)-5-ethyl barbituric acid (CHEB†) was a gift from Dr. Hall Downes (University of Oregon Medical School, Portland, OR). Tritiated adenosine was purchased

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† Abbreviations: CHEB, 5-(2-cyclohexylideneethyl)-5-ethyl barbituric acid; EGTA, ethylene glycol bis(β-aminoethyl-ether)-N,N^2-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; and GABA, γ-aminobutyric acid.

from New England Nuclear, Boston, MA ([2,8,5′-^{3}H], 50 Ci/mmole; [2,8-^{3}H], 30 Ci/mmole) and Amersham, Arlington Heights, IL ([2-^{3}H], 21 Ci/mmole). These stock solutions varied in purity from 80 to 90% [^{3}H]adenosine monitored by TLC and were used without further purification. Other chemicals were obtained from the Sigma Chemical Co., St. Louis, MO.

Adenosine uptake method. Synaptosomes were isolated from whole brains by the method of Cotman [21] using differential and Ficoll gradient separation techniques. Sucrose solutions (0.32 M) used to prepare the Ficoll gradients were buffered with 5 mM Tris–HCl or Hepes-NaOH, pH 7.4. Final pellets were resuspended in incubation medium (NaCl, 136 mM; KCl, 5 mM; $CaCl_2$, 1.2 mM; $MgCl_2$, 1.3 mM; glucose, 10 mM; Tris base, 20 mM; pH 7.4 adjusted with HCl) to give a protein concentration of 1 to 2.5 mg/ml. Protein content of synaptosomes was assayed by the method of Oyama and Eagle [22]. [^{3}H]Adenosine uptake was assayed by a slight modification of the method of Bender *et al.* [7]. Incubation medium (0.5 ml) with and without barbiturate was warmed to 37° before adding a 0.5-ml aliquot of synaptosomes. After 5 min, [^{3}H]adenosine (1.5 nmoles/μCi) was added in another 0.5 ml volume to give a usual final concentration of 1.0 μM. After the appropriate time periods, 5 ml of washing solution (sucrose, 260 mM; $CaCl_2$, 2 mM; $MgCl_2$, 2 mM; Tris–HCl, 20 mM; pH 7.5) at room temperature was added to the tube, followed by rapid filtration of the solution through Whatman GF/B glass microfibre filters on a Millipore sampling manifold. Excess ^{3}H was washed away by two 5-ml washes with washing solution. Lysed synaptosomes were prepared by resuspending half of the final pellet in lysing buffer (10 mM Tris–HCl, pH 7.4) for at least 30 min. [^{3}H]Adenosine uptake by lysed synaptosomes was studied as described above for intact synaptosomes. After the final wash, filters were transferred to scintillation vials, 10 ml of scintillation mixture was added, and ^{3}H counted in a Beckman LS-7500 scintillation counting system. Counting efficiency was approximately 55–60%. The net uptake of adenosine was determined by subtracting the lysed values from the total ^{3}H accumulated by the intact synaptosomes. In initial experiments, 1 mM adenosine was added to intact synaptosomes along with [^{3}H]adenosine to determine nonspecific binding of [^{3}H]adenosine. Net values obtained by the two methods were approximately the same.

Adenosine release method. After preparation of the P_2 crude synaptosomal pellet [21], the synaptosomes were resuspended in incubation medium to give a protein concentration of 2.5 to 3.5 mg/ml. A 0.5-ml aliquot of the suspension was added to 0.5 ml of incubation medium at 37° for 5 min. [^{3}H]Adenosine (1.5 to 2.0 μCi) was added to the sample to load the synaptosomes for 15 min. At this time, 5 ml of washing solution was added, and the synaptosomes were filtered through Whatman GF/B filters and washed with two 5-ml portions of washing solution. The filters were immediately transferred to glass scintillation vials, 1.7 ml of incubation medium was added, and the vials were placed in a shaking incubator at 37°. At the end of the first minute of incubation, all of the medium was removed by aspiration and 0.85 ml of fresh medium was added. Every minute thereafter the buffer bathing the synaptosomes was aspirated and replaced with 0.85 ml of fresh medium. After 20 min, the efflux of ^{3}H was relatively constant. Depolarization of the synaptosomes was achieved by adding a solution containing 50 mM KCl substituted isotonically for NaCl. The ^{3}H in the effluent was counted by adding 10 ml scintillation mixture to the aspirated sample in a scintillation vial and counting as before. At the end of the experiment, the filter containing the synaptosomes was also counted. Results are expressed as a ratio of the amount of ^{3}H released into the medium to the total synaptosomal ^{3}H present per minute. Drugs were added after 15 min of the initial washout period.

Separation of adenosine metabolites. The characterization of synaptosomal adenosine metabolites was determined using the method of Bender *et al.* [7]. Quadruplicate samples were extracted with three 1-ml aliquots of boiling distilled water. The extracts were combined, and the ^{3}H-purines were concentrated by the method of Pull and McIlwain [8]. Charcoal (5 mg) was added to the extract and the tube was shaken on ice for 30 min. The suspension was centrifuged, the supernatant fraction was discarded, and 2 ml of 20% 0.3 N NH_4OH in ethanol (v/v) was added to the pelleted charcoal. After 30 min of shaking on ice, the suspension was centrifuged and the supernatant fraction was carefully transferred to another tube. Solvent was removed by warming under a stream of N_2. The residue was reconstituted in 100 μl distilled water. Duplicate 20 μl samples were then spotted on a Silica Gel G thin-layer plate (250 μm, Analtech, Pittsburgh, PA) along with marker compounds adenosine, adenine, hypoxanthine, cyclic AMP, inosine, and nucleotides. The TLC plate was developed in *n*-butanol–ethylacetate–methanol–ammonium hydroxide (7:4:3:4, by vol.) after the method of Shimizu *et al.* [23]. Spots were visualized under shortwave ultraviolet light, scraped into vials, and counted as previously described.

Determination of released adenosine metabolites was also performed. Synaptosomes were loaded with 10 μCi [^{3}H]adenosine (sp. act. 21 Ci/mmole) as described before. After the initial 20-min washout period, five consecutive samples were collected and combined. Depolarization with KCl, 50 mM, proceeded for 3 min and, again, five consecutive samples of the depolarized effluent were collected. Duplicate experiments were combined, and the ^{3}H was concentrated and analyzed as described above. Recovery of purines extracted by charcoal adsorption varied from 37% for adenosine, 41% for nucleotides, 44% for hypoxanthine, 50% for inosine, to 59% for cyclic AMP monitored by u.v. absorption of eluates of standards that had been run through the extraction procedure. Approximately 90% of the radioactivity applied to the TLC plates was recovered. All values in this report are uncorrected for losses incurred during the extraction and recovery procedures.

RESULTS

Properties of synaptosomal adenosine uptake and the effects of pentobarbital. Indicative of a carrier-

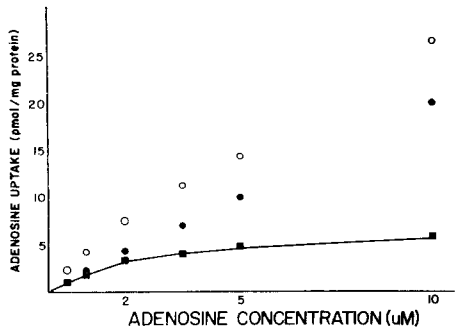

Fig. 1. Effect of substrate concentration on [^{3}H]adenosine uptake in mouse whole brain synaptosomes. Synaptosomes, prepared as described under Materials and Methods, were preincubated for 5 min at 37°. [^{3}H]Adenosine was added to give a final concentration of 0.5 to 10.0 μM. After 30 sec, samples were diluted with washing solution and filtered through fiberglass filters. After two additional 5-ml washes, the radioactivity remaining on the filter was counted. Open circles indicate total [^{3}H]adenosine uptake by intact synaptosomes. Closed circles denote nonspecific binding of [^{3}H]adenosine to filters and lysed synaptosomes. Net [^{3}H]adenosine uptake (squares) was obtained by subtracting lysed uptake from total uptake. Points represent mean of six to eleven experiments.

mediated transport process, Fig. 1 shows that net [^{3}H]adenosine uptake by mouse whole brain synaptosomes was saturable. Nonlinear regression analysis of the data by the method of Wilkinson [24] revealed an apparent K_m of 2.8 μM and a V_{max} of 7.4 pmoles/mg protein for 30 sec. These results are in agreement with those obtained previously with rat cortical synaptosomes [7]. Time course determination of [^{3}H]adenosine uptake in mouse whole brain synaptosomes showed that the uptake process was linear through 5 min (Fig. 2). After 5 min, the rate of

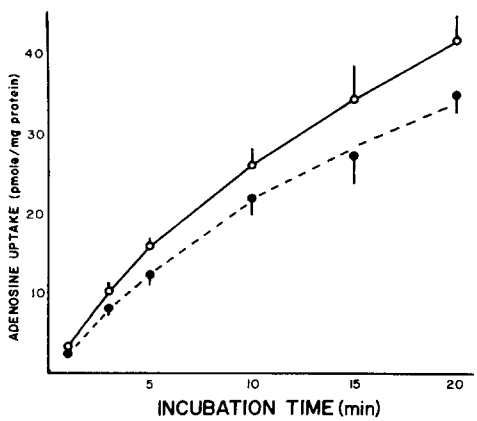

Fig. 2. Pentobarbital effect on the time course of net [^{3}H]-adenosine uptake in mouse whole brain synaptosomes. Synaptosomal preparation and experimental procedures were carried out as described in the legend of Fig. 1 except that the time of incubation with 1 μM [^{3}H]adenosine varied from 1 to 20 min. Only net uptake is presented for controls (○) and 0.2 mM pentobarbital (●) which was present during preincubation and [^{3}H]adenosine challenge. All drug-treated points are significantly lower than controls ($P < 0.05$, paired t-test). Each value represents the mean ± S.E.M. for four preparations.

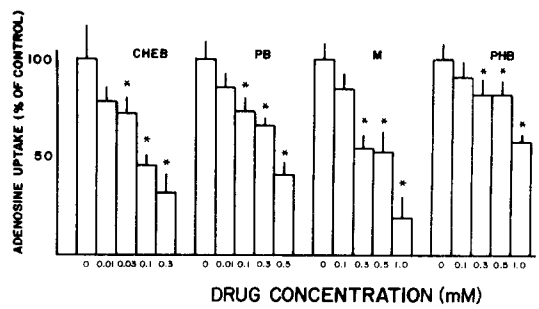

Fig. 3. Concentration-dependent inhibition of net [^{3}H]-adenosine uptake by barbiturates. [^{3}H]Adenosine uptake was determined in mouse whole brain synaptosomes for 30 sec as described in the legend of Fig. 1. Final concentration of [^{3}H]adenosine was 1.0 μM. Asterisks indicate a significant difference from their respective controls ($P < 0.05$, two-way ANOVA using Newman–Keuls as the post hoc test). Control values (mean ± S.E.M.) were: CHEB, 2.19 ± 0.38; pentobarbital (PB), 2.88 ± 0.26; methohexital (M), 2.51 ± 0.19; and phenobarbital (PHB), 1.59 ± 0.13 pmoles/mg protein. Each bar represents the mean ± S.E.M. of five to nine experiments.

uptake began to fall slightly. All drug studies reported here were performed during the linear phase (30 sec of uptake). Several barbiturates were tested at different concentrations for their effects on synaptosomal adenosine uptake. As shown in Fig. 3, CHEB, pentobarbital, methohexital, and phenobarbital inhibited net [^{3}H]adenosine uptake in a concentration-dependent manner. CHEB exhibited the most potent effect with significant inhibition occurring at a concentration of 30 μM. Pentobarbital and methohexital did not show any statistically significant reduction of net [^{3}H]adenosine uptake until the concentration was raised to 100 and 300 μM respectively. Phenobarbital also caused a significant inhibition of adenosine uptake; however, the magnitude of the depression was smaller than that seen with the other barbiturates at similar concentrations.

Several kinetic experiments were done to analyze the nature of the inhibitory effect of pentobarbital [^{3}H]adenosine uptake. Our results suggested that pentobarbital inhibited the uptake process in a non-classical, noncompetitive manner in that both V_{max} and apparent K_m were reduced by 0.2 mM pentobarbital (data not shown).

Pentobarbital effect on metabolism of adenosine. Control and pentobarbital- (0.2 mM) treated synaptosomes were extracted after 30 sec of [^{3}H]adenosine uptake to determine if barbiturate treatment affected the metabolism of the accumulated [^{3}H]adenosine. Table 1 shows that [^{3}H]adenosine was rapidly metabolized in control intact synaptosomes. Only 54% of the original [^{3}H]adenosine remained after 30 sec with the major metabolites being nucleotides and inosine. Previous studies have reported similar results although our work showed a slightly more active metabolism [7, 25]. Hypoxanthine was also found in small amounts as well as trace amounts of adenine and cyclic AMP. Lysed synaptosomes also retained some of the metabolic machinery for metabolizing adenosine. Again, in the lysed synaptosomes, the major pathway involved adenosine kinase (EC 2.7.1.20) as seen by the amount of ^{3}H-nucleotides

Table 1. Metabolism of [^{3}H]adenosine taken up by control and drug-treated mouse whole brain synaptosomes*

	% of Radioactivity recovered			
	Control		0.2 mM Pentobarbital	
Derivative	Intact	Lysed	Intact	Lysed
Adenine	1.6 ± 0.5	1.6 ± 0.4	2.0 ± 0.3	2.5 ± 0.8
Adenosine	54.2 ± 4.8	71.7 ± 3.1	55.4 ± 1.9	68.3 ± 6.3
Hypoxanthine	5.3 ± 1.0	5.1 ± 1.2	4.8 ± 0.8	5.1 ± 1.1
Cyclic AMP	2.6 ± 0.6	2.9 ± 0.8	2.7 ± 0.7	2.3 ± 0.4
Inosine	9.0 ± 0.4	3.7 ± 0.2	9.0 ± 0.7	4.3 ± 0.6
Nucleotides	27.3 ± 5.1	15.1 ± 1.5	26.1 ± 1.3	17.4 ± 4.6

* Synaptosomes were incubated with [^{3}H]adenosine for 30 sec, and uptake was halted by dilution, filtration, and washing. ^{3}H-Purines taken up by the synaptosomes were extracted with boiling water and concentrated by charcoal adsorption. Individual purines were separated by TLC of 20 μl of the redissolved residue (see Materials and Methods). Results represent the means ± S.E.M. of four experiments.

detected. The amount of metabolic activity in lysed preparations was lower as would be expected following cellular disruption. The observed metabolism of [^{3}H]adenosine by lysed synaptosomes may have resulted from the release of cytoplasmic adenosine-metabolizing enzymes during hypoosmotic treatment or, alternatively, to regained viability of lysed synaptosomes reexposed to incubation medium during the 5-min preincubation and 30-sec ^{3}H-challenge. Pentobarbital (0.2 mM) did not alter the metabolic profile of any of the [^{3}H]adenosine metabolites analyzed in either intact or lysed synaptosomes.

Characteristics of ^{3}H release from [^{3}H]adenosine-labeled synaptosomes and the effects of pentobarbital. Figure 4 shows that synaptosomes, previously labeled with [^{3}H]adenosine, which had been incubated in calcium-free medium released a small amount of ^{3}H under resting conditions. Changing the medium bathing the synaptosomes from one containing 5 mM KCl and no calcium to one containing 50 mM KCl and 1.2 mM calcium resulted in an increased efflux of ^{3}H. After 5 min of exposure to the depolarizing medium in the presence of calcium, the rate of ^{3}H-efflux changed from 1.5 to 2.5% per min or about 0.9% per min. When calcium-free incubation medium replaced the depolarizing medium, the ^{3}H-efflux slowly returned to pre-stimulus levels. As also shown in Fig. 4, the omission of calcium from the depolarizing medium still allowed a significant increase in the rate of ^{3}H-efflux from the synaptosomes. However, the magnitude of the depolarization-induced release was slightly reduced under calcium-free conditions compared to stimulated release with calcium. Addition of 250 μM EGTA to the depolarizing medium resulted in a complete inhibition of the KCl enhancement of ^{3}H-efflux seen when calcium was present (Fig. 5). However, when the EGTA concentration was reduced by the addition of calcium-free medium, the release of ^{3}H increased to about the peak attained during exposure to 50 mM KCl and 1.2 mM $CaCl_2$.

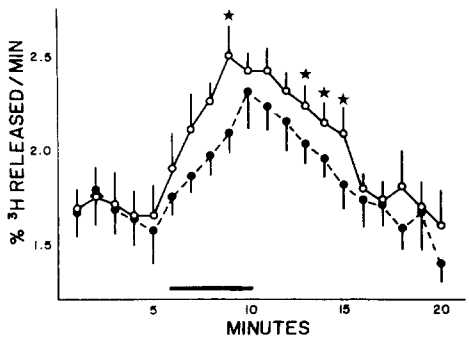

Fig. 4. Calcium dependence of ^{3}H-efflux from [^{3}H]adenosine-labeled synaptosomes. A crude (P_2) pellet was prepared from mouse whole brain. After 15 min of incubation with 1.5 to 2.0 μCi of [^{3}H]adenosine (final concentration 4.8 to 6.4 nM), the labeled synaptosomes were collected on a glass microfiber filter, washed, and exposed to fresh calcium-free medium every minute for 20 min. Thereafter, the effluent was collected into scintillation vials and the amount of ^{3}H-efflux was determined by liquid scintillation spectrometry. The time indicated by the bar on the abscissa indicates exposure to medium containing 50 mM KCl (substituted isoosmotically for NaCl). The open circles represent samples with 1.2 mM $CaCl_2$ added to the depolarizing medium while the closed circles were calcium free throughout. Values represent mean ± S.E.M. of seven experiments. The effect of 1.2 mM $CaCl_2$ on depolarized release of ^{3}H is significant at 9, 13, 14, and 15 min ($P < 0.05$, paired t-test) as indicated by stars.

Inclusion of 0.3 mM pentobarbital in the depolarizing medium caused no change in the pattern of the fractional ^{3}H-release rate over the entire stimulation period. Preexposure of the synaptosomes for 5 min with 0.3 mM pentobarbital likewise had no effect on the release of ^{3}H from either resting or K^+-stimulated synaptosomes (data not shown).

Extraction and separation of labeled synaptosomal purines released in the absence of depolarization showed that adenosine and inosine accounted for most of the released tritium in a ratio of about 3:2 (Table 2). Stimulation of the synaptosomes with 50 mM KCl resulted in an increased release of adenosine, inosine, nucleotides, and another minor metabolite which cochromatographed with 3′,5′-cyclic AMP. Under resting conditions, pentobarbital, 0.3 mM, did not alter the release of [^{3}H]adenosine or its metabolites. Adenosine and inosine again accounted for approximately 90% of the ^{3}H released from the barbiturate-treated synaptosomes after

Table 2. Effect of pentobarbital on K^+-stimulated release of ^{3}H-purines from synaptosomes*

	Control (N = 6)		0.3 mM Pentobarbital (N = 6)	
Derivative	50 mM K^+	5 mM K^+	50 mM K^+	5 mM K^+
Adenine	0.124 ± 0.022	0.148 ± 0.088	0.151 ± 0.049	0.066 ± 0.006
Adenosine	5.87 ± 0.67†	4.26 ± 0.73	6.72 ± 0.70†‡	4.12 ± 0.65
Hypoxanthine	0.363 ± 0.056	0.435 ± 0.096	0.443 ± 0.074	0.354 ± 0.086
Cyclic AMP	0.207 ± 0.030†	0.109 ± 0.008	0.220 ± 0.030†	0.078 ± 0.012
Inosine	4.78 ± 0.122†	2.89 ± 0.78	4.11 ± 0.74†‡	2.69 ± 0.70
Nucleotides	0.396 ± 0.122†	0.248 ± 0.039	0.466 ± 0.118†‡	0.231 ± 0.054

* Values represent ^{3}H-purines released in 5 min from mouse brain synaptosomes. ^{3}H-Purines were extracted using charcoal adsorption, and metabolites were separated using TLC on silica gel G plates as described in the legend of Table 1. Data, given as means ± S.E.M., were analyzed by two-way ANOVA with Newman–Keuls as the post hoc test.

† $P < 0.05$ compared to release under nondepolarizing conditions.

‡ $P < 0.05$ compared to the depolarized release of the ^{3}H-purine in the absence of pentobarbital.

extraction and analysis. Depolarization of pentobarbital-treated synaptosomes with 50 mM KCl caused a significant increase in the release of ^{3}H-nucleotides as well as an enhanced release of adenosine and inosine. The data indicate that the net potassium-induced release of [^{3}H]adenosine and ^{3}H-nucleotides was enhanced significantly by 0.3 mM pentobarbital. In addition, pentobarbital caused a significant reduction in the net K^+-induced release of inosine. Thus, although pentobarbital did not alter the apparent release of the total tritium label, analysis of [^{3}H]-adenosine metabolites showed that 0.3 mM pentobarbital increased K^+-stimulated [^{3}H]-adenosine release but inhibited the evoked release of [^{3}H]-inosine.

DISCUSSION

The biochemical and electrical activities elicited by adenosine in brain tissue are characteristically inhibitory [1, 26]. These findings in conjunction with studies demonstrating the release and reuptake of adenosine by nerve ends [7] and the existence of several types of adenosine receptors [14] strongly suggest that adenosine plays an important role as a regulator of neuronal function [26].

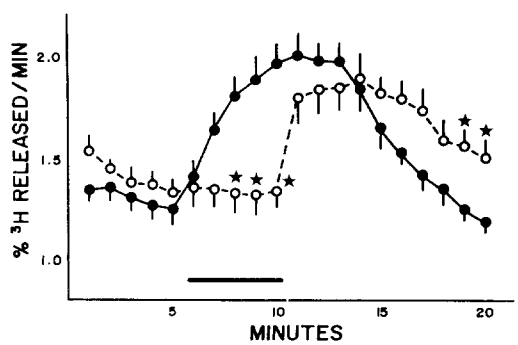

Fig. 5. Effect of EGTA on ^{3}H-release from [^{3}H]adenosine-labeled synaptosomes. Values were derived as described in the legend of Fig. 4. The initial 5-min period was calcium free for both sets. Exposure to 50 mM KCl indicated by the bar on the abscissa included 1.2 mM $CaCl_2$ for the closed and 250 μM EGTA for the open circles. After the period of depolarization, both sets were again washed with calcium-free medium. Data represent mean ± S.E.M. of four experiments. Stars indicate that the values obtained with EGTA-treated samples are significantly different from controls ($P < 0.05$, paired t-test). A 250 μM concentration of EGTA had no effect on nondepolarized release of ^{3}H.

Several classes of psychotropic agents with sedative or hypnotic properties have been shown to alter adenosine uptake or release in the central nervous system in a manner consistent with a pharmacological mechanism of action [27]. In agreement, the results of the present investigation suggest that the depressant effects of barbiturates may, at least in part, result from the elevation of synaptic levels of adenosine in the brain accomplished through inhibition of uptake and facilitation of release. Our results demonstrate that the anesthetic potencies of pentobarbital, methohexital, and phenobarbital correlate well with their abilities to inhibit adenosine uptake. Anesthetic concentrations of pentobarbital and methohexital (300 μM) [28] reduced net synaptosomal adenosine uptake by 33 and 45%, respectively, while the same concentration of phenobarbital inhibited uptake by 18%. Interestingly, CHEB, a convulsant barbiturate, significantly depressed net [^{3}H]adenosine uptake into synaptosomes at a lower concentration than pentobarbital, methohexital, or phenobarbital. Although this finding may appear to be contradictory to our above suggestions, we have shown recently that CHEB also exhibits depressant properties if convulsions are blocked with phenobarbital [29].

Pentobarbital did not change the metabolic profile of the accumulated [^{3}H]adenosine which may suggest that barbiturates act at a plasma membrane site associated with the adenosine carrier to inhibit uptake. In addition, the barbiturate concentrations which caused significant reductions in [^{3}H]adenosine uptake are lower than those necessary to produce inhibition of GABA or norepinephrine uptake *in vitro* [30, 31]. This observation suggests that the membrane bound adenosine transporter may be more sensitive to barbiturates than other neurotransmitter uptake systems.

As stated earlier, in addition to inhibiting adenosine uptake, barbiturates may further increase syn-

aptic levels of adenosine by increasing the depolarization-induced release of adenosine from nerve ends, an action of pentobarbital that was seen only when adenosine was separated from other ^{3}H-purines which were also released from prelabeled synaptosomes. This observation illustrates the importance of measuring not only the quantities of adenosine released, but also its metabolites when studying drug effects on purine release from presynaptic nerve endings. Earlier studies which investigated the action of amobarbital on release of radiolabeled purines reported either an inhibition [32] or no change [33]. However, these studies did not separate the individual purines released, so comparison to the present results is difficult. The enhancement of depolarization-induced release of [^{3}H]-adenosine by 0.3 mM pentobarbital is probably not attributable to inhibition of the reuptake process or drug-induced changes in the activity of adenosine-metabolizing enzymes. The absence of any significant barbiturate effect on the resting release of any of the adenosine metabolites analyzed precludes such conclusions. Pentobarbital also inhibited the K^+-induced release of inosine. Adenosine and inosine accounted for the major portion of the ^{3}H released by the synaptosomes. The opposite changes in adenosine and inosine release caused by pentobarbital may have resulted in no apparent change in the total efflux of ^{3}H-label.

The depolarization-induced release of radiolabeled adenosine from cerebral tissues has been demonstrated previously, and our experiments confirm these results in mouse synaptosomes [8, 34, 35]. Several investigators have obtained conflicting results concerning the calcium dependence of potassium-induced adenosine release from synaptosomes. Kuroda and McIlwain [36] reported that changing the superfusion medium from calcium containing to calcium deficient inhibits release induced by high potassium. Fredholm and Vernet [35] showed that removal of calcium has a small inhibiting effect on adenosine release from hypothalamic synaptosomes. Bender *et al.* [7] reported that the inclusion of 250 μM EGTA in their releasing medium actually increases the release of adenosine. In the present study, removal of calcium from the depolarizing medium led to a small but significant depression of the release of [^{3}H]adenosine derivatives from mouse whole brain synaptosomes. Inclusion of 250 μM EGTA completely abolished potassium-induced release. However, release was than augmented when EGTA was washed out. After several minutes, the ^{3}H-release rate remained elevated in the EGTA-treated synaptosomes in contrast to control synaptosomes which showed the expected decline in ^{3}H-efflux while the potassium concentration was restored to pre-stimulus levels. The stimulated release of ^{3}H observed when calcium was omitted from the depolarizing medium (Fig. 4) may indicate that the extracellular calcium concentration plays only a small role in the release process. The K^+-induced release of [^{3}H]adenosine derivatives from synaptosomes may be primarily dependent on an EGTA accessible membrane bound pool of calcium. The extracellular concentration of calcium may serve to maintain this level of membrane bound calcium.

Bender *et al.* [37] have reported previously that pentobarbital at concentrations of 10 and 50 μM does not affect [^{3}H]adenosine uptake into rat cerebral cortical synaptosomes. However, these concentrations are subanesthetic [28, 38] and probably correlate better with antianxiety actions of barbiturates [1]. A recent study by Phillis and Wu [27] has shown that high concentrations of pentobarbital and phenobarbital inhibit [^{3}H]adenosine uptake into rat cortex synaptosomes, which is in agreement with our results. The concentration of pentobarbital and methohexital which caused significant reductions in [^{3}H]adenosine uptake in the present study are those at which strong sedative or anesthetic effects are seen. Thus, our results obtained with high concentrations of barbiturates are in line with the suggestion that anxiolytic effects of barbiturates are probably not related to an interaction with adenosine [1].

The combined effects of barbiturates on adenosine uptake and release demonstrated in this investigation would lead to increased tonic inhibitory influence of adenosine in the CNS. Recent studies suggest that methylxanthines block the effects of adenosine through an interaction with adenosine receptors [14]. Caffeine has been shown to shorten barbiturate sleeptime [39]. This effect may be due to a blockade of barbiturate-enhanced adenosine activity in the CNS. Furthermore, theophylline, an adenosine receptor antagonist, inhibited barbiturate-induced enhancement of the inhibitory postsynaptic potential in an isolated olfactory cortex slice preparation [40]. This result may also be partly explained by theophylline antagonism of increased synaptic adenosine levels produced by pentobarbital. Enhancement of adenosine influence in the CNS may also contribute to some of the observed actions of pentobarbital on the biochemistry of nerve ends such as inhibition of neurotransmitter release [38, 41]. Adenosine has been shown to modulate the release of neurotransmitters in brain [2–5], and this action may be due to an inhibitory action of adenosine on calcium influx into the nerve end [42, 43]. However, we have not observed any inhibition of potassium-induced $^{45}Ca^{2+}$ influx in synaptosomes by adenosine in our laboratory (unpublished observations).

Barbiturates may produce their depressant effects through a variety of actions on neuronal function. Barker and Gainer [44] have shown that pentobarbital depresses excitatory postsynaptic potentials. Other proposed mechanisms of barbiturate anesthesia include interactions with the GABA-activated chloride ionophore [45] and inhibition of calcium influx [46–48] with a subsequent reduction in neurotransmitter release [41, 49]. In addition, we have shown that barbiturates decreased adenosine uptake and increased depolarization-induced adenosine release in nerve ends. Thus, drug-induced enhancement of adenosine activity in the synapse may contribute to some of the depressant effects of barbiturates.

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