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Barbiturate Inhibition of Calcium Uptake by Depolarized Nerve Terminals *in Vitro*

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SUMMARY

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The effect of barbiturates on ^{45}Ca uptake by pinched-off presynaptic terminals (synaptosomes) from rat brain has been examined. Calcium uptake is stimulated by depolarizing agents (potassium, veratridine, or gramicidin D), and this extra uptake is inhibited by sodium pentobarbital; 0.4–0.5 mM pentobarbital reduces the extra uptake by 50%. Sodium thiopental is also effective and, at a concentration of 0.2 mM, reduces the potassium-stimulated calcium uptake by about 50%. In one experiment, 0.9 mM sodium phenobarbital exhibited little inhibitory effect on calcium uptake. Two other central depressants, ethanol (100 mM) and chloroform (3–12 mM), had no significant effect on the potassium-stimulated calcium uptake. The inhibitory action of pentobarbital did not appear to involve competition between the barbiturate and external calcium. Also, the calcium entry which was promoted by raising internal and lowering external sodium was only slightly reduced by pentobarbital; this indicates that the inhibitory action of pentobarbital is limited primarily to the calcium entry triggered by depolarizing agents. The data are compatible with the idea that pentobarbital and thiopental may interfere with transmitter release and synaptic transmission by reducing the depolarization-triggered calcium entry in presynaptic terminals.

INTRODUCTION

The cellular mechanisms responsible for the sedative and anesthetic actions of barbiturates have been the subject of numerous investigations during the past few decades, but most of the details have remained elusive. At rather high concentrations (1–10 mM), barbiturates have a local anesthetic action (1) which can be accounted for by inhibition of the sodium conductance increase in the axon (2). Similarly high concentrations are known to interfere with mitochondrial respiration (3, 4). Nevertheless, these concentrations are considerably in excess of pharmacologically useful concentrations; furthermore, synaptic transmission is much more sensitive to the action of barbiturates than is axonal conduction (5). Thus it seems possible that the central depressant action of barbiturates may be a consequence of interference with synaptic transmission.

Both pre- and postsynaptic actions of barbiturates have been described (6). However, available evidence indicates that inhibition of the monosynaptic spinal reflex may be due exclusively to an effect on the presynaptic terminals (7, 8), since moto-

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neuron excitability was unchanged, or even increased, by depressant doses of barbiturates. Løyning and his colleagues (7) suggested that the observed reduction in the excitatory postsynaptic potential might be the result of decreased transmitter release. This supposition was verified by Weakly (9), whose quantal analysis indicated that subanesthetic intravenous doses (10 mg/kg) of pentobarbital and thiopental reduced, by about 25%, the average amount of transmitter released by group Ia afferent impulses. Furthermore, Matthews and Quilliam (10) found that amylobarbital significantly inhibited acetylcholine release from superior cervical ganglia.

Recent electrophysiological studies (11) have helped to elucidate some of the steps in the transmitter release process. A central feature of this process is a depolarization-induced calcium conductance increase. The subsequent entry of calcium, moving into the terminal down its electrochemical gradient, is believed to trigger transmitter release as a result of the increase in the intracellular ionized Ca^{2+} concentration (12, 13). Although the mechanism by which barbiturates block transmitter release is unknown, Quastel *et al.* (14) have suggested that pentobarbital may act by "suppression of a depolarization-sensitive Ca permeability system."

Pinched-off presynaptic terminals (synaptosomes), prepared from brain homogenates, retain considerable functional integrity (15, 16) and have been used to study some of the steps in the transmitter release process *in vitro* (17–19). As in intact nerve terminals, there is a depolarization-induced increase in calcium permeability, and calcium entry appears to trigger transmitter release. The present report describes the effect of barbiturates on this sequence of events in synaptosomes. The main observation, in support of the hypothesis of Quastel *et al.* (14), is that 100–200 μM pentobarbital significantly inhibits the depolarization-induced calcium conductance increase. This effect may be sufficient to account for the inhibition of synaptic transmission by low doses of barbiturates *in vivo*.

TABLE 1

Composition of representative solutions

In addition to the components listed, all solutions contained the following millimolar concentrations: $MgCl_2$, 1.3; NaH_2PO_4, 1.2; glucose, 10; mannitol, 5; and Tris base, 20. The solutions were buffered to pH 7.65 (at 25°) by titration with maleic acid.

Solution	NaCl	KCl	LiCl	$CaCl_2$
	mM	*mM*	*mM*	*mM*
Na + 5K	132	5	0	1.2
Ca-free Na + 5K	132	5	0	0
Na + 0K	137	0	0	1.2
137 mM K saline	0	137	0	1.2
Li + 0K	0	0	137	1.2

MATERIALS AND METHODS

Solutions. The composition and nomenclature of representative solutions are shown in Table 1. Intermediate concentrations of alkali metal ions (see RESULTS) were obtained by mixing the appropriate solutions (e.g., Na + 5K and 137 mM K saline). The EGTA[1] stopping solution was similar to Ca-free Na + 5K, but contained 30 mM Tris-EGTA in place of the glucose and mannitol.

Drugs. The solutions containing barbiturates and/or veratridine were titrated to pH 7.65 (at 25°) with Tris base or maleic acid. Veratridine was dissolved in a few drops of 1 N HCl, neutralized, and made up in Na + 5K. The (+)-isomer of 5-(1,3-dimethylbutyl)-5-ethylbarbituric acid was dissolved in a few drops of 1 N NaOH, neutralized, and made up in either Na + 5K or 137 mM K saline. Gramicidin D was dissolved in ethanol and diluted with Na + 5K to give a final concentration of 7 mM ethanol in the incubation solutions; the controls for the gramicidin D experiment also contained 7 mM ethanol. Details regarding incubation of synaptosomes with drugs are given under RESULTS. Sodium pentobarbital and sodium thiopental were supplied by Abbott Laboratories; (+)-DMBB was supplied by Eli Lilly and Company. Veratridine was purchased from

[1] The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; (+)-DMBB, (+)-isomer of 5-(1,3-dimethylbutyl)-5-ethylbarbituric acid.

K & K Laboratories, and gramicidin D and ouabain, from Sigma Chemical Company. Radioactive isotopes were obtained from New England Nuclear Corporation.

Preparation of synaptosomes. Synaptosomes were prepared from whole rat brain homogenates (four brains per experiment) by the differential and sucrose gradient centrifugation procedure of Gray and Whittaker (20). The material from the 0.8–1.2 M sucrose interface (synaptosomes) was diluted with approximately 12 volumes of ice-cold Ca-free Na + 5K over a 20-min period. Aliquots of this synaptosome suspension were then centrifuged at 9000 × *g* for 4 min at 2°; all subsequent centrifugations were performed at the same speed and temperature.

Experimental protocol. The synaptosome pellets (1.0–1.2 mg of protein) were resuspended in 1.0 ml of physiological salt solution ± drug (usually Na + 5K, but see RESULTS for details of individual experiments). The suspensions were incubated for 15 min at 30° to bring the synaptosomes to a physiological steady state. Additional aliquots (usually 0.5 or 1.0 ml) of solutions containing ^{45}Ca (specific activity, 1 μCi/μmole of calcium in final solution) and [^{3}H]mannitol (specific activity, 0.8 μCi/μmole of mannitol in final solution) were then added. The [^{3}H]mannitol was used to correct for extracellular calcium. Appropriate samples also contained depolarizing agents, i.e., increased potassium concentrations, or veratridine or gramicidin D. The suspensions were incubated with the tracer-containing solutions for 1 min at 30° [the ^{45}Ca uptake was linear during this period (19)]. Calcium uptake was halted by the addition of 0.5 ml of ice-cold EGTA stopping solution and 6.0 ml of ice-cold Ca-free Na + 5K. The suspensions were centrifuged, the supernatant was discarded, and the pellets were rinsed with 7.0 ml of ice-cold Na + 5K, which was also discarded. The pellets were resuspended in 8.0 ml of ice-cold Na + 5K and again centrifuged. The rinse-wash-rinse cycle was repeated once more. The synaptosome pellets were dissolved in 2.0 ml of 1 N NaOH for 40 min at 50°. A 1.0-ml aliquot was transferred to a plastic counting vial and neutralized with HCl, and 10 ml of toluene–Triton X-100 scintillation mixture (21) were added. The samples were counted in a three-channel Packard liquid scintillation counter; ^{45}Ca and ^{3}H counts were separated by the channels-ratio method. Specific activities of ^{45}Ca and [^{3}H]mannitol were computed from the counts in the incubation fluids. All samples were corrected for quenching. Synaptosome digests were autoanalyzed for protein by the method of Lowry *et al.* (22), using bovine serum albumin as a standard.

Statistics. Standard errors for the mean potassium-stimulated calcium uptake (SE_Δ) values were calculated according to the formula

$$SE_\Delta = \sqrt{(SE_{5K})^2 + (SE_{K^+})^2}$$

where SE_{5K} = standard error of the mean calcium uptake from Na + 5K, and SE_{K^+} = standard error of the mean calcium uptake from potassium-rich medium (or other stimulating medium). Two-way analysis of variance was used to determine the significance of the difference (*p* values) between the potassium-stimulated (or veratridine-stimulated, etc.) calcium uptake in the absence and presence of the barbiturates.

RESULTS

Effect of barbiturates on calcium uptake triggered by increased external potassium. Voltage-sensitive fluorescent dye studies indicate that synaptosomes have membrane potentials, and that they may be depolarized by increasing external potassium (16). The rate of calcium accumulation by synaptosomes is dramatically increased by raising the external potassium concentration, $[K]_o$ (17–19), as shown in Fig. 1.

Figure 1 also shows the effects of sodium pentobarbital and sodium thiopental on ^{45}Ca uptake from Na + 5K and potassium-rich media. In this experiment the uptake from Na + 5K was reduced below control levels (inhibition not significant) by both barbiturates at concentrations of 0.1 or 0.3 mM; in numerous other experiments these

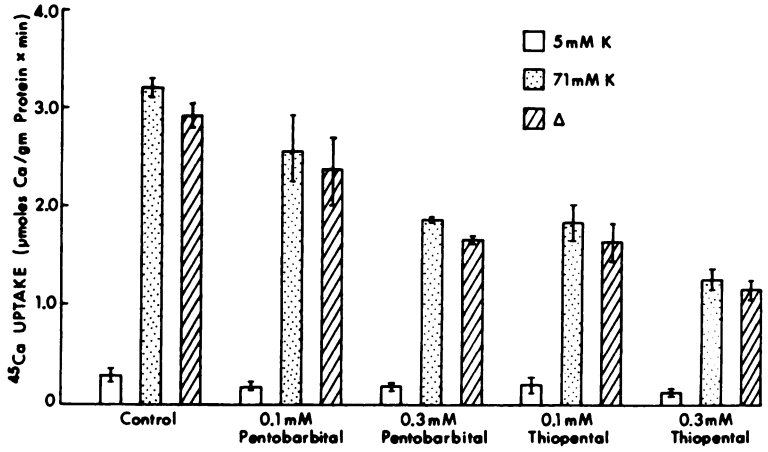

FIG. 1. *Effects of sodium pentobarbital and sodium thiopental on ^{45}Ca uptake by synaptosomes*

Synaptosomes were incubated for 15 min at 30° in 1.0 ml of Na + 5K with or without barbiturate. Then 1.0 ml of ^{45}C-containing Na + 5K with or without barbiturate or 137 mM K saline with or without barbiturate was added, and after a further 1-min incubation ^{45}Ca uptake was terminated with EGTA stopping solution and ice-cold calcium free Na + 5K. In this experiment the suspensions were centrifuged, and the pellets were rinsed and immediately prepared for counting (without washing). [^{3}H]Mannitol was used to correct for extrasynaptosomal ^{45}Ca. Open bars, ^{45}Ca uptake from Na + 5K; stippled bars, uptake from 71 mM K saline; hatched bars, potassium-stimulated ^{45}Ca upatke (Δ). The barbiturate concentration present during the initial and final incubations is indicated under each set of bars. Each bar represents the mean of four determinations ± standard error.

agents had little or no effect on the calcium uptake from Na + 5K.

The ^{45}Ca uptake from potassium-rich medium was markedly inhibited by both sodium pentobarbital and sodium thiopental. Thiopental (0.1 mM) reduced the potassium-stimulated uptake by nearly 50%, and a comparable effect was observed with 0.3 mM pentobarbital.

The results of a number of experiments are summarized in Fig. 2. This graph shows the percentage inhibition of the potassium-stimulated calcium uptake (○ and ●) plotted as a function of the pentobarbital concentration; the potassium-stimulated uptake is the difference between the uptake from potassium-rich medium and from Na + 5K. The pentobarbital data fit a rectangular hyperbola with a half-saturation (or 50% inhibition) value of about 0.47 mM. The more limited thiopental data from the experiment of Fig. 1 and one other experiment indicate that this agent is somewhat more potent than pentobarbital; 0.2 mM thiopental inhibited potassium-stimulated calcium uptake by about 50%.

In most experiments the barbiturates were present during preliminary incubation of appropriate samples. It was therefore important to determine whether or not the inhibition of potassium-stimulated calcium uptake was an indirect result of an altered monovalent cation distribution. Three types of experiments were made to test this possibility. In one experiment the accumulation of ^{86}Rb [which is handled like ^{42}K (17)] was determined in control synaptosomes and in synaptosomes poisoned with 1 mM ouabain. Pentobarbital (1 mM) had no significant effect on the steady ^{86}Rb content of either the control or poisoned synaptosomes.

Tests with a membrane potential-sensitive fluorochrome (16) provided further evidence that the internal potassium concentration was not markedly affected by pentobarbital. When external potassium was increased, synaptosomes incubated with 1 mM pentobarbital showed about the same fluorescence changes (presumably indicative of membrane potential changes) as control synaptosomes.

A third type of experiment was designed to determine whether or not the duration of

prior exposure would influence the inhibitory action of the barbiturates on potassium-stimulated calcium uptake. Presumably a very short prior incubation with barbiturate would minimize the extent of any potential alteration in internal ion concentration. The results of such an experiment are shown in Table 2. The potassium-stimulated calcium uptake was inhibited by about 50% whether 0.4 mM

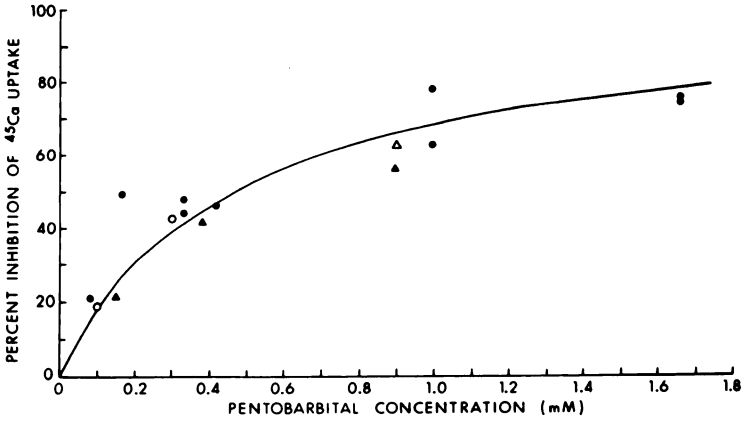

FIG. 2. *Effect of sodium pentobarbital concentration on ^{45}Ca uptake triggered by depolarizing agents*

● and O, potassium-stimulated calcium uptake; ▲, veratridine-stimulated calcium uptake; Δ, gramicidin D-stimulated calcium uptake (see Table 2). O, pentobarbital data from the experiment of Fig. 1; in all other instances the synaptosomes were washed twice as described in MATERIALS AND METHODS. Each symbol on the graph represents the difference between the means of three or four determinations. The curve was drawn to fit the equation

$$\% \text{ inhibition of } ^{45}\text{Ca uptake} = \frac{100}{1 + K_b/[B]}$$

where $[B]$ is the sodium pentobarbitol concentration in the medium, and K_b is the pentobarbital concentration at which the depolarizing agent-stimulated ^{45}Ca uptake is reduced by 50% (0.47 mM sodium pentobarbital).

TABLE 2

Effect of duration of prior incubation with 0.4 mM sodium thiopental on inhibition of potassium-stimulated ^{45}Ca uptake

Following a 12-min incubation at 30°, each 1.0-ml synaptosome suspension was diluted with 1.0 ml of Na + 5K or 1.0 ml of 137 mM K saline containing ^{45}Ca. The ^{45}Ca solutions used for the synaptosomes treated with thiopental during the incubation only (line 4) contained 0.4 mM thiopental. Thus, in all samples treated with thiopental (lines 2–4), the drug concentration during the 1-min ^{45}Ca incubation period was only 0.2 mM.

Conditions	Prior incubation with 0.4 mM thiopental	^{45}Ca uptake[a] from		Δ_K	Inhibition	P[b]
		Na + 5K	71mM K			
	min	*μmoles Ca/g protein × min*			%	
1. Control	0	0.41 ± 0.06	2.66 ± 0.17	2.25 ± 0.18		
2. +Thiopental	12	0.45 ± 0.02	1.52 ± 0.03	1.07 ± 0.04	53	<0.01
3. +Thiopental	2	0.49 ± 0.03	1.69 ± 0.07	1.20 ± 0.08	47	<0.01
4. +Thiopental (0.2 mM only during incubation with ^{45}Ca)	0	0.50 ± 0.09	2.05 ± 0.16	1.55 ± 0.18	31	<0.05

[a] Each value is the mean of three determinations ± standard error.

[b] Significance of the difference between the potassium-stimulated calcium uptake in the absence and in the presence of thiopental.

sodium thiopental was present throughout the 12-min prior incubation (line 2) or only for the last 2 min of it (line 3); in both cases the thiopental concentration during ^{45}Ca incubation was only 0.2 mM. Even when thiopental (0.2 mM) was present only during the 1-min period of incubation with ^{45}Ca (line 4), the potassium-stimulated ^{45}Ca uptake was inhibited by 31%. These data, and the results of the ^{86}Rb and fluorescent dye experiments, all indicate that the inhibitory effect of the barbiturates does not require a long preliminary incubation, and is probably not due to an alteration in the intrasynaptosomal ion content.

Effect of pentobarbital on calcium uptake triggered by other depolarizing agents. Fluorescent dye data (16) indicate that veratridine and gramicidin D may also depolarize synaptosomes, although their mode of action is different from that of increased $[K]_o$ (23, 24). When added to ^{45}Ca-containing Na + 5K, these agents also stimulate calcium uptake (19) (Table 3). The data in Table 3 show that the veratridine-stimulated uptake and the gramicidin-stimulated uptake can be inhibited by sodium pentobarbital. These data fit the curve for inhibition of potassium-stimulated calcium uptake (Fig. 2, Δ and ▲) and provide further evidence (see ref. 19) that the calcium permeability increase triggered by veratridine (and gramicidin D) may be very similar (or identical) to the one triggered by increased $[K]_o$.

Effect of pentobarbital on internal sodium-dependent calcium uptake. If synaptosomes are depleted of internal potassium and loaded with sodium by incubation in calcium-free Na + 0K containing 1 mM ouabain, they accumulate calcium at a rapid rate when incubated in calcium-containing, sodium-free medium (25, 26). Under these circumstances the influx of ^{45}Ca (Na_i-dependent calcium uptake) may involve a carrier-mediated exchange of sodium for calcium; the properties of this calcium entry mechanism are different from those of the potassium-stimulated or veratridine-stimulated calcium uptake (19, 26). A further distinction may be the difference in response to pentobarbital (Table 4). The Na_i-dependent calcium uptake is only slightly inhibited (by about 18%) by 0.6 mM pentobarbital—a concentration sufficient to inhibit the potassium-stimulated uptake by approximately 57% (Fig. 2). Thus pentobarbital does not indiscriminately block calcium uptake by syn-

TABLE 3

Effect of sodium pentobarbital on ^{45}Ca uptake stimulated by veratridine or gramicidin D

Synaptosomes were incubated for 15 min at 30° in 1.0 ml of Na + 5K with or without pentobarbital at the concentration indicated below. Then 0.5 ml of ^{45}Ca-containing Na + 5K was added, and the suspensions were incubated for 1 min. In some instances the added tracer-labeled solutions also contained 75 μM veratridine (experiment 1) or 10 μg/ml of gramicidin D (experiment 2) to stimulate ^{45}Ca uptake.

Expt.	Pentobarbital concentration[a]	^{45}Ca uptake[b]		Δ	Inhibition	P[c]
		Na + 5K	Stimulated			
	mM	*μmoles Ca/g protein × min*			%	
1	0	0.18 ± 0.01	0.66 ± 0.02	0.48 ± 0.02		
	0.15	0.16 ± 0.01	0.54 ± 0.01	0.37 ± 0.01	23	<0.01
	0.38	0.14 ± 0.01	0.42 ± 0.03	0.27 ± 0.03	44	<0.01
	0.9	0.12 ± 0.01	0.33 ± 0.01	0.21 ± 0.01	57	<0.01
2	0	0.33 ± 0.01	1.12 ± 0.01	0.78 ± 0.01		
	0.9	0.40 ± 0.01	0.70 ± 0.01	0.29 ± 0.01	63	<0.01

[a] In the prior incubation medium; concentration in the medium during ^{45}Ca uptake was two-thirds of this value.

[b] Each value is the mean of three determinations ± standard error.

[c] Significance of the Δ value relative to the Δ value in the absence of pentobarbital.

TABLE 4

Effect of sodium pentobarbital on internal sodium-dependent ^{45}Ca uptake by synaptosomes

Synaptosomes were incubated in 1.0 ml of calcium-free Na + 0K with 1 mM ouabain ("Na-loaded") or calcium-free Li + 0K with 1 mM ouabain ("Li-loaded") for 15 min at 30°. As indicated below, some of these solutions also contained 0.2 or 0.6 mM sodium pentobarbital. Following the first incubation the suspensions were centrifuged, and the pellets were resuspended in 1.0 ml of ^{45}Ca-containing Li + 0K with or without 0.2 or 0.6 mM pentobarbital and incubated for 1 min at 30°.

Pentobarbital concentration	^{45}Ca uptake[a]		Δ	Inhibition	P[b]
	Na-loaded	Li-loaded			
mM	*μmoles Ca/g protein × min*			%	
0	3.91 ± 0.07	1.14 ± 0.03	2.77 ± 0.08		
0.2	3.30 ± 0.29	0.88 ± 0.04	2.41 ± 0.29	13	NS
0.6	3.22 ± 0.05	0.93 ± 0.02	2.28 ± 0.05	18	<0.01

[a] Each uptake value is the mean of four determinations ± standard error.

[b] Significance of the Δ value relative to Δ in the absence of pentobarbital. NS = not significant ($p > 0.05$).

TABLE 5

Effects of sodium phenobarbital and (+)-DMBB on potassium-stimulated ^{45}Ca uptake by synaptosomes

Synaptosomes were incubated in 1.0 ml of Na + 5K with or without phenobarbital or (+)-DMBB for 15 min at 30°. Then 0.5 ml of ^{45}Ca-containing Na + 5K or 137 mM K saline (final $[K]_o$ = 49 mM) was added, and the incubation was continued for 1 min. The data are from two different synaptosome preparations (experiments 1 and 2, respectively).

Expt.	Barbiturate	Concentration[a]	^{45}Ca uptake[b] from		Δ_K	Inhibition	P[c]
			Na + 5K	49 mM K			
		mM	*μmoles Ca/g protein × min*			%	
1	Control	0	0.45 ± 0.01	1.39 ± 0.24	0.93 ± 0.24		
	Phenobarbital	0.3	0.51 ± 0.01	1.44 ± 0.17	0.92 ± 0.17	1	NS
	Phenobarbital	0.9	0.42 ± 0.05	1.21 ± 0.14	0.79 ± 0.15	15	NS
2	Control	0	0.61 ± 0.03	1.97 ± 0.16	1.35 ± 0.16		
	(+)-DMBB	1.0	0.45 ± 0.05	0.88 ± 0.09	0.43 ± 0.10	68	<0.01

[a] In the prior incubation medium; concentration during ^{45}Ca incubation was two-thirds of this value.

[b] Each value is the mean of three determinations ± standard error.

[c] Significance of the Δ value relative to Δ in the absence of barbiturate. NS = not significant ($p > 0.5$).

aptosomes; its effect appears to be limited primarily to the extra uptake triggered by depolarizing agents.

Effects of other barbiturates on potassium-stimulated ^{45}Ca uptake. The effects of two other barbiturates, phenobarbital and (+)-DMBB, on calcium uptake were also examined, and the results are shown in Table 5. Even at a concentration of 0.9 mM, inhibition of the potassium-stimulated calcium uptake by sodium phenobarbital was minimal, and not statistically significant. By comparison, 1.0 mM (+)-DMBB markedly inhibited the potassium-stimulated calcium uptake. This was at first a somewhat puzzling observation, because (+)-DMBB is a convulsant barbiturate with no apparent sedative action in intact animals (27), although the racemic mixture has only a depressant effect when applied directly to cortical neurons (28). However, subsequent electrophysiological experiments[2] on synaptic transmission in isolated rat superior cervical ganglia showed that (+)-DMBB inhibits synaptic transmission in a manner which is indistinguishable from that of pentobarbital (5) or thiopental.

[2] M. P. Blaustein, unpublished observations.

TABLE 6

Effects of chloroform and ethanol on potassium-stimulated ^{45}Ca uptake by synaptosomes

Synaptosomes were incubated for 15 min at 30° in 0.9 ml Na + 5K with or without chloroform (experiment 1) or 1.0 ml of Na + 5K with or without ethanol (experiment 2). Then 0.6 ml (experiment 1) or 0.5 ml (experiment 2) of ^{45}Ca-containing Na + 5K or 137 mM K saline was added, and the suspensions were incubated for 1 min at 30°.

Expt.	Drug	Concentration[a]	^{45}Ca uptake[b] from		Δ_K[c]	Inhibition
			Na + 5K	K-rich media		
		mM	*μmoles Ca/g protein × min*			%
1 (58 mM K)	Control	0	0.37 ± 0.01	2.38 ± 0.13	2.01 ± 0.13	
	Chloroform	3.0	0.35 ± 0.02	2.42 ± 0.14	2.07 ± 0.14	
	Chloroform	6.0	0.40 ± 0.02	2.31 ± 0.17	1.91 ± 0.17	5
	Chloroform	12.0	0.35 ± 0.05	2.33 ± 0.09	1.98 ± 0.10	1.5
2 (49 mM K)	Control	0	0.61 ± 0.03	1.97 ± 0.16	1.36 ± 0.16	
	Ethanol	100	0.31 ± 0.01	1.82 ± 0.35	1.50 ± 0.35	

[a] Drug concentration in the ^{45}Ca incubation solution.

[b] Each value is the mean of three determinations ± standard error.

[c] None of the Δ_K values in the presence of chloroform or ethanol was significantly different from the respective control ($p > 0.05$ in all cases).

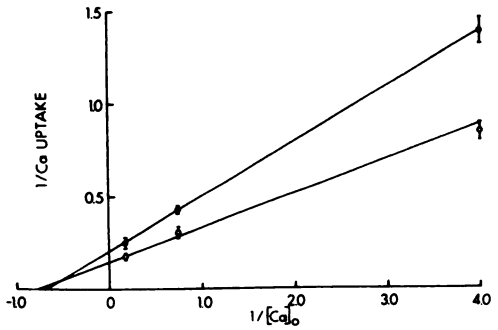

FIG. 3. *Double-reciprocal plot of effect of external calcium concentration on potassium-stimulated calcium uptake in the absence* (○) *and presence of* (●) *of sodium pentobarbital*

Synaptosomes were incubated for 15 min at 30° in 1.0 ml of Na + 5K containing 0.25 mM $CaCl_2$, either without (○) or with (●) 0.5 mM sodium pentobarbital. Then 1.0 ml of barbiturate-free Na + 5K or 137 mM K saline was added; these solutions contained ^{45}Ca and sufficient ^{40}Ca to give the $[Ca]_o$ (in the 2.0-ml suspension) shown on the abscissa. The samples were incubated for 1 min at 30° before terminating the ^{45}Ca uptake with EGTA. Three determinations of calcium uptake were made for each condition ($[Ca]_o$, $[K]_o$, pentobarbital). The ordinate shows the reciprocal of potassium-stimulated calcium uptake; abscissa, $1/[Ca]_o$. The bars indicate the standard errors. The lines were drawn by eye.

Effects of ethanol and chloroform on potassium-stimulated calcium uptake. Two other anesthetic agents, ethanol and chloroform, were tested for their effects on ^{45}Ca uptake by synaptosomes. As shown in Table 6, these agents had no significant inhibitory effect on potassium-stimulated calcium uptake, even at concentrations in excess of anesthetic levels (5). Thus not all anesthetics necessarily block the depolarization-triggered calcium permeability increase in presynaptic terminals.

Effect of external calcium concentration on inhibition by pentobarbital. Since the potassium-stimulated uptake of calcium was inhibited by pentobarbital, it seemed worthwhile to test this effect at several different external calcium concentrations ($[Ca]_o$) to determine whether or not the barbiturate acts as a competitive inhibitor of calcium. Data from one experiment are shown in Fig. 3; in this double-reciprocal plot the lines do not extrapolate to the same calcium uptake at infinite $[Ca]_o$. Similar results were obtained in two other experiments. The tentative conclusion is that calcium and pentobarbital do not compete for common sites.

DISCUSSION

Inhibition of calcium entry should block synaptic transmission. The data described above clearly show that pharmacologically useful concentrations of sodium pentobarbital and sodium thiopental significantly reduce the calcium uptake which is in-

duced by depolarizing agents in synaptosomes. This calcium entry mechanism is strikingly similar to one which has been described in intact presynaptic terminals (11, 13, 29) and which, even in synaptosomes, is associated with transmitter release (18, 19). Available evidence indicates that an increase in the internal ionized Ca^{2+} concentration (12), which may be expected to occur when calcium enters the terminals (13), may actually be involved in triggering transmitter release (29). If the entry of calcium is the rate-limiting step, then inhibition of calcium entry during depolarization should result in decreased transmitter release, especially if there is a nonlinear relationship between $[Ca]_o$ (or calcium entry) and transmitter release (29, 30). According to this interpretation (see ref. 14), the evidence that barbiturates reduce transmitter release at central (9) and peripheral (10) synapses could be accounted for by the inhibition of presynaptic calcium permeability increase reported here. This view is also consistent with recent observations[2] that the stimulation-induced uptake of calcium by rat superior cervical ganglia (31) is inhibited by pentobarbital and thiopental.

Implications for a mechanism of barbiturate action. Since pentobarbital and thiopental can be employed as general anesthetics, a relevant question is whether or not the inhibition of presynaptic calcium entry, reported here, may contribute to the anesthetic action of these barbiturates. Although an unqualified answer cannot be given, it seems reasonable to suggest that the reduced calcium entry may cause sufficient inhibition of transmitter release at critical central synapses (e.g., in the reticular activating system) to induce a state of anesthesia. However, it is important to emphasize that the cellular mechanism proposed here may not apply to all "general" anesthetics. As shown above, ethanol and chloroform do not appear to block the calcium permeability increase (but see ref. 14). This may be compatible with electrophysiological data (8) which indicate that some anesthetics may affect primarily presynaptic elements, and others, postsynaptic neurons.

What are the molecular mechanisms involved? It is readily apparent that further study will be required to resolve the molecular mechanisms responsible for the pentobarbital and thiopental inhibition of calcium accumulation. Nevertheless, the limited data available do help to rule out certain possibilities. For one, the lack of competition between calcium and pentobarbital (Fig. 3) implies that the barbiturate probably does not interact specifically with the "calcium selectivity" site of the calcium conductance "channel." Preliminary data on the relationship between calcium uptake and the external potassium concentration (or membrane potential; cf. refs. 16, 19) indicate that the percentage inhibition by pentobarbital remains approximately constant when $[K]_o$ is increased progressively from 20 to 70 mM. This may mean that the barbiturate does not simply shift the calcium permeability curve on the voltage axis (19). One possibility is that the lipid-soluble barbiturate molecules may be inserted in the presynaptic membrane at, or close to, hypothetical "calcium channels" (perhaps at specific "receptor" sites) so that the consequent constriction of the calcium channels from lateral pressure could cause the reduction of calcium entry observed.

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