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SHORT COMMUNICATIONS

Pentobarbital depression of stimulus-secretion coupling in brain— Selective inhibition of depolarization-induced calcium-dependent release*

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A synaptic locus of action is favored for the actions of barbiturates in producing anesthesia [1–10]. Most investigators agree that the local anesthetic action of barbiturates upon axonal conduction is not required for their general anesthetic action [cf. Ref. 1]. However, both pre- and post-synaptic effects have been described for anesthetic concentrations of barbiturates that could result in the commonly observed decrement in synaptic excitability [11–14].

A selective barbiturate inhibition of excitatory (depolarizing) post-synaptic conductance changes appears to constitute the bases for barbiturate depression of synaptic transmission in vertebrate neuromuscular and invertebrate systems [1, 5, 11]. But in the mammalian central nervous system, barbiturates inhibit excitatory synaptic transmission by a predominantly pre-synaptic mechanism which results in a decrease in the amount of transmitter released [3, 6, 8–10]. On the other hand, inhibitory synaptic transmission appears to be enhanced by pentobarbital [5, 15].

We investigated the effects of pentobarbital (PB, 20–200 μM) upon Ca-dependent secretion of norepinephrine (NE) and γ-aminobutyric acid (GABA), two presumably inhibitory transmitters in brain [16, 17], from synaptosomes isolated from mammalian brain. Isolated synaptosomal fractions provide an ideal preparation to investigate drug effects upon transmitter secretion. Isolation procedures, in addition to eliminating direct interneuronal interactions, allow rapid and accurate control of extracellular fluid composition by minimizing diffusional barriers.

Mouse forebrain synaptosomal fractions were prepared and incubated with [^{3}H](-)-NE (3.7 Ci/m-mole, NEN), [^{14}C]GABA (232 mCi/m-mole, Amersham) or both as previously described [18]. After loading, tissue aliquots were washed with a Ca-deficient, HEPES†-Ringer solution (see Fig. 1 legend) every 30 sec in a negative pressure filtration system [19, 20]. Release was stimulated by 1.5 mM $CaCl_2$, added to the seventh-wash solutions. Depolarizing agents (KCl and veratridine [21, 22]) and the ionophore A23187‡ [23], known to increase Ca permeability in excitable tissue [22–25], were added either during the seventh wash (stimulation period) or during washout and stimulation (maximum exposure 210 sec) to facilitate Ca-dependent release. PB was also added either during stimulation or during prior washout and stimulation.

Efflux is expressed as the percentage of the dis./min in the seventh wash with respect to the total dis./min present in the tissue immediately prior to the seventh wash. Release is expressed as the Ca-induced increase in per cent efflux of tissue treated equivalently with the exception of Ca addition [18, 20].

Blaustein and Ector [13] have recently suggested that a primary pentobarbital action in brain is the depression of depolarization-dependent Ca influx into nerve terminals. In that Ca influx provides the trigger for transmitter secretion [18, 26–29], a reduction of depolarization-triggered Ca entry by pentobarbital might be sufficient to result in a decrease in transmitter secretion. Depolarizing conditions were studied to determine whether the PB-induced depression of Ca uptake reported by Blaustein and Ector [13] might be sufficient to result in a decrease in transmitter release. Non-depolarizing conditions that promote Ca influx were also studied to determine whether the actions of PB upon depolarization-sensitive Ca ionophores were selective.

Pentobarbital depression of K-facilitated, Ca-dependent release. As shown in Fig. 1, pretreatment of K^+-depolarized synaptosomal tissue with PB for 180 sec (200 μM, 210 sec total washout and stimulation exposure) decreased the efflux of both [^{3}H]NE and [^{14}C]GABA in the presence of Ca. Efflux in the absence of Ca was, however, unaffected by the prior exposure to PB. In other similar experiments, 20 μM PB pretreatment significantly decreased Ca-dependent efflux of both [^{3}H]NE and [^{14}C]GABA, but the depression was much smaller than the approximately

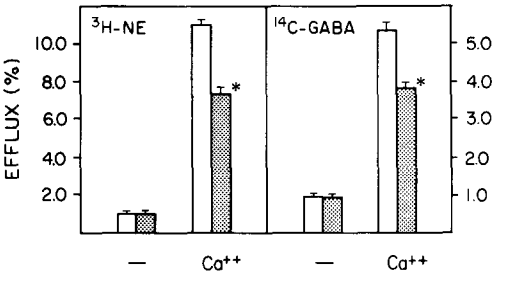

Fig. 1. Effects of pentobarbital pretreatment upon [^{3}H]NE and [^{14}C]GABA efflux. Synaptosomal fractions were resuspended in a HEPES–Ringer medium (150 mM NaCl, 20 mM HEPES, 10 mM D-glucose, 5 mM KOH, 1.2 mM $MgSO_4$, 1.2 mM Na_2HPO_4, 1 mM L-ascorbic acid, 1 mM aminooxy acetic acid, and 0.1 mM pargyline HCl; pH 7.4 with Tris). Tissue suspensions were incubated with 0.26 μM [^{3}H]NE and 0.43 μm [^{14}C]GABA for 10 min at 37°. After incubation, tissue was aliquoted onto filter units and washed with elevated KCl (+50 mM) HEPES–Ringer media either with or without 200 μM pentobarbital added. After six consecutive washes at room temperature (22–24°) over 3 min, a seventh 20-sec wash was administered which, for half of the samples in each condition, contained added $CaCl_2$ (1.5 mM). Radioactivity in the filters and seventh-wash filtrates was determined by liquid scintillation spectroscopy, and the per cent efflux from each sample was determined [dis./min (filtrate)/dis./min(filter + filtrate)]. Open bars indicate non-drug efflux; stipled bars indicate efflux in the presence of 200 μM pentobarbital. Values represent the means and S.E.M. for four determinations. The asterisk (*) indicates $P < 0.05$, two-tailed t-test.

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† *N*-2-hydroxyethylpiperzine-*N'*-2-ethanesulfonic acid.

‡ A gift from Dr. Robert Hamill, Eli Lilly.

35 per cent inhibition observed in Fig. 1. Thus, the depression of Ca-dependent release of NE and GABA was dose-dependent and, in contrast to previous reports for PB effects upon transmitter release from brain [30, 31], the dose range for the effect in the present study is within the range for general anesthesia [6], yet below local anesthetic concentrations for pentobarbital [6, 7, 9, 32].

Selectivity of the PB effect upon Ca-dependent efflux, but not the "K-dependent" efflux, suggests an interference by PB at some reaction within the depolarization–secretion sequence subsequent to depolarization. (Note: Cutler *et al.* [33] report a PB-induced decrease in K-dependent [^{3}H]GABA release from brain slices in the presence of Ca, but do not distinguish Ca- vs K-dependent effects.) One possibility, as supported by Blaustein and Ector [13] is a selective PB inhibition of Ca permeation through the depolarization-sensitive pre-synaptic Ca ionophore generally associated with transmitter secretion systems [18, 26, 34, 35]. Another possibility, previously untested, is that PB depresses one of the several subsequent reactions in the secretion processes (e.g. transmitter availability or transmitter extrusion) thus leading to a decrease in transmitter released.

Selectivity of pentobarbital depression of Ca-dependent release. Generality of PB depression of Ca-dependent transmitter release to other facilitating depolarizers, such as veratridine [22], would support the interpretation that PB exerts its inhibitory actions subsequent to depolarization. However, use of an artificial Ca ionophore such as A23187 [23] should allow distinction between Ca permeation and some other reaction (subsequent) as a locus for PB effects. Conceptually, A23187 (which over time periods investigated in these studies would not be expected to be depolarizing [36]) provides a bypass to the normal membrane selectivity against Ca permeation. Thus, if PB acts at some step subsequent to the depolarization-triggered Ca influx, PB should depress Ca-dependent release in the presence of the artificial ionophore.

Thus, we investigated the ability of PB to influence Ca-dependent release in the presence of either elevated KCl, veratridine or A23187. In these experiments, the duration of exposure to both PB and the facilitating agents was decreased to minimize the possibility of influencing intraterminal mitochondrial Ca metabolism. After incubation with labeled transmitter, synaptosomal tissue was washed in HEPES–Ringer medium (low K^+, Ca-deficient). Facilitating agents were administered either during the stimulation period alone or during the stimulation period and 60 sec preceding stimulation. PB was administered only during the stimulation period (20 sec). In order to determine the interactions of PB with the ability of the facilitating agents alone to promote Ca-dependent release, parallel samples were run in the absence of the facilitating agent, and the resulting per cent release values were substracted as appropriate (see Tables 1 and 2).

As demonstrated in Table 1, a 20-sec exposure to PB (200 μM) depressed the Ca-dependent release of both [^{3}H]NE and [^{14}C]GABA in the presence of both 50 mM KCl and 100 μM veratridine. Thus, the ability of PB to depress Ca-dependent release was general to depolarization-facilitated release in that KCl and veratridine depolarize in characteristically different fashions [22]. Further, these data demonstrate that pretreatment with PB is not necessary to antagonize the effects of Ca and that PB fails to antagonize K-dependent efflux (Ca-independent) even when K is pulsed.

In a separate set of experiments, we also compared the effects of PB upon KCl- and A23187-facilitated, Ca-dependent release of [^{3}H]NE. Facilitating agents were administered 60 sec prior to Ca stimulation in order to allow time for A23187 to take effect. PB (200 μM) and Ca were administered only during the stimulation period. As before, PB depressed Ca-dependent release in the presence of elevated KCl (Table 2). More importantly, A23187-facilitated Ca-dependent release was unaffected by PB. And, in other experiments (not presented), 200 μM PB failed to inhibit Ca-dependent release of [^{3}H]NE and [^{14}C]GABA in the presence of 2 μM A23187 even when the PB was administered during both washout and stimulation.

The results of these experiments provide demonstration that PB inhibits the depolarization-triggered secretion of accumulated neurotransmitters from brain at PB concentrations (20–200 μM) within anesthetic ranges. The depression of Ca-dependent [^{3}H]NE and [^{14}C]GABA release was somewhat surprising following the findings of Nicoll *et al.* [5, 15] that inhibitory synaptic transmission in brain is enhanced and prolonged by PB. This discrepancy is not trivially resolved, however, in that: (1) cortical slices also demonstrate a similar decrease in [^{3}H]GABA release [33], and (2) the Ca-dependent release of [^{3}H]NE and [^{14}C]GABA from synaptosomes closely mimics the secretion of endogenous transmitter in similar situations [18].

The depression of release by PB was restricted to situa-

Table 1. Effects of pentobarbital upon calcium-dependent release facilitated by depolarizing agents*

Agent	Agent-specific calcium-dependent release			
	Per cent [^{3}H]NE release		Per cent [^{14}C]GABA release	
	Without PB	With PB (200 μM)	Without PB	With PB (200 μM)
KCl (50 mM)	5.22 ± 0.48	3.46 ± 0.70†	3.78 ± 0.32	2.05 ± 0.13†
Veratridine (100 μM)	4.93 ± 0.29	3.44 ± 0.40†	4.09 ± 0.44	2.32 ± 0.18†

* Synaptosomal suspensions were incubated and plated onto filter units as in Fig. 1. After six washes with HEPES Ringer medium (3 mM K^+, Ca-deficient), the seventh wash, or stimulation period, consisted of one of the following media:

[HEPES–Ringer / HEPES–Ringer + KCl (50 mM) / HEPES–Ringer + veratridine (100 μM)] × [with or without $CaCl_2$ 1.5 mM] × [with or without PB 200 μM]

Ca-dependent efflux (release) was calculated as the difference between per cent efflux in the presence of Ca and per cent efflux in the absence of Ca for otherwise identically treated tissue. To obtain Ca-dependent release resulting specifically from the addition of KCl or veratridine, per cent release in the absence of the depolarizing agent was subtracted from release in the presence of agent. The release values in HEPES–Ringer alone were as follows: [^{3}H]NE, 3.50 ± 0.28 and 2.85 ± 0.18 per cent with PB; [^{14}C]GABA, 1.04 ± 0.36 and 0.92 ± 0.10% with PB. All values represent the mean ±S.E.M. of four determinations.

† $P < 0.05$, two-tailed *t*-test for inherently paired samples, compared to release in the absence of PB.

Table 2. Comparison of pentobarbital effects upon depolarization- and ionophore-facilitated calcium-dependent release*

Agent	Agent-specific calcium-dependent release (per cent [^{3}H]NE release) Without PB	With PB (200 μM)
KCl (50 mM)	8.04 ± 0.86	5.62 ± 0.40†
A23187 (10 μM)	7.14 ± 0.66	7.60 ± 0.64

* Synaptosomal tissue was prepared and treated essentially as in Table 1. After incubation with [^{3}H]NE, tissue was washed in either HEPES–Ringer or HEPES–Ringer with 0.1% dimethylsulfoxide (DMSO). Facilitating agents were administered where appropriate during the 5–7 washes (90-sec total exposure). Ca and PB were added only during the seventh, or stimulation, wash as in Table 1. Ca-dependent release resulting specifically from the addition of KCl or A23187 was obtained by subtracting per cent release in the absence of the facilitating agent (respectively, HEPES–Ringer with or without 0.1% DMSO). The release values without agent were as follows: HEPES–Ringer, 3.10 ± 0.12 and 2.54 ± 0.28 per cent with PB; HEPES–Ringer with 0.1% DMSO, 3.73 ± 0.36 and 2.38 ± 0.16 per cent with PB. All values represent the mean and S.E.M. of four determinations.

† $P < 0.05$, two-tailed t-test for inherently paired samples, compared to release in the absence of PB.

tions in which depolarizing agents (KCl and veratridine) were used to facilitate Ca influx, delimiting a selective locus of action for this PB effect: the voltage-sensitive pre-synaptic Ca ionophore associated with transmitter secretion. The failure of PB to modify Ca-dependent release in the presence of A23187 demonstrates the viability of secretion processes subsequent to Ca permeation. And, in combination with PB inhibition of release in the presence of elevated KCl or veratridine, this brackets the locus of action sequentially within the postulated sequence of stimulus-secretion coupling processes to the depolarization-triggered Ca permeation step.

General membrane effects of PB (e.g. alteration of surface charge or fluidity) seem unlikely as a mechanism, since such general effects would be expected to alter the efficacy of A23187 for facilitating Ca-dependent release as well. The ability of PB to affect the coupling of $[Ca]_0$ to release specifically by depolarizing agents could, however, result from a selective PB effect on membrane fluidity or surface charge at specialized regions of the pre-synaptic membrane (i.e. the voltage-sensitive, or "late," Ca ionophore [34, 35]).

The present experiments suggest that investigation of PB effects at the level of such "late" Ca ionophores may be useful for further distinguishing molecular mechanisms contributing to PB action. That is, PB may inhibit the ability of depolarization to activate the ionophores, perhaps by reducing the number of activated ionophores or the duration of activation, or it may inhibit the permeability offered by the ionophores by decreasing the mobility of Ca through the ionophores or the concentration of Ca in the ionophores.

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