Effect of Pentobarbital on Neurones in the Reticular Formation of the Brain Stem: Ionophoretic Study in the Rat

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Abstract. The effect of ionophoretically applied pentobarbital (PB) upon neurones in the nucleus reticularis gigantocellularis of the rat was studied. PB applied through a micropipette depressed the spontaneous activity of 81% of the neurones tested; the remaining neurones did not change their firing rates. Regardless of current intensities used for PB ejection (5—60 nA) there was no increase in the firing rate during PB administration. The depression was dependent upon both the control firing rate and the PB dose; a total depression of activity was observed at currents between 40 and 60 nA. EC_{50} (15.5 nA, about 5×10^{-5} mol.l^{-1} — the drug concentration was approximated theoretically) was assessed from the dose-response curve. Repeated application resulted in a shift of EC_{50} towards higher current values (desensitization). The Hill coefficient was calculated in conformity with the classical theory. From its value (1.4), it may be assumed that the occupation of only one subunit of the binding site is enough to give a response. Possible mechanisms of action of PB upon neurones are discussed.

Key words: Pentobarbital — Ionophoresis — Rat reticular neurones — Nucleus gigantocellularis

Introduction

Pentobarbital (PB) [5-ethyl-5-(1-methylbutyl) barbiturate sodium] is frequently used in both clinical practice and experimental work for its anaesthetic effects. Regardless of the widespread use of PB, opinions on mechanisms of its action remain diverging (Ho and Harris 1981; Richards 1972).

In vertebrates, general anaesthesia appears at concentrations of PB in the extracellular fluid higher than 0.2 mmol.l^{-1} (Fisher et al. 1948), i. e. approximately at doses of 20—30 mg.kg^{-1} (Barnes and Eltherington 1966); general anaesthesia vanishes at concentrations between 0.02 and 0.04 mmol.l^{-1} (Fisher et al. 1948). The anaesthesia lasts several tens of minutes, depending on both, the dose and the route of administration (Mason et al. 1983).

Appreciable changes in activity of thalamic neurones (Albe-Fessard and Besson 1973; King et al. 1957) and of some cortical functions (Swank and Watson

1949) could be induced by relatively small doses of PB (about 10 mg.kg^{-1}). The reticular formation (RF) is extremely sensitive to the action of PB (Bradley and Key 1958; Frank and Ohta 1971); depending on the dose, (3 – 8 mg.kg^{-1}) different degrees of activity depression was observed in the RF (Paul-David et al. 1960; Bradley and Key 1958). After systemic administration, PB first binds to plasma proteins, being continuously released thereafter (Goldstein and Aronow 1960); this makes the determination of the time course of the PB action on individual neurones difficult. Ionophoretic application allows an analysis of the direct action of PB on single neurones. So far, only few data on the effect of ionophoretic application of PB on the nerve cells have been available; most of them were obtained in in vitro experiments (Barker and Ransom 1978).

The aim of the present paper was to test the effect of PB on single neurones — in the nucleus reticularis gigantocellularis (NRG) in the RF — in in vivo experiments to characterize the action of PB and its time course, to quantify this action by constructing the dose-response curve (DRC), and to assess PB-neurone binding through determination of the Hill coefficient.

Materials and Methods

Experiments were carried out on 12 albino rats (Wistar) of either sex, weighing 200—250 g. Animals were fixed in a stereotaxic apparatus under chloralose anaesthesia (10 mg/100 g i. p., 1% solution in saline), paralysed by Tricuran (DHW Rodleben, 4—5 mg, 1% solution i. p., repeated doses) and arteficially ventilated (tracheotomia).

Threebarreled micropipettes (Simax glass) for extracellular recording (total tip diameter of 3—6 μm) were inserted through an opening in the dura mater and intact cerebellum into the RF (NRG region — stereotaxic coordinates AP + 10, L 1, V 10 according to the atlas by Fifková and Maršala 1967). The recording barrel was filled with 3 mol.l^{-1} NaCl (tip resistance 1—3 MΩ). The second barrel was filled with the same solution as the recording one, it was used either to reduce electrical effects of the ejecting current, or to evaluate possible effects of electric current alone; the current alone had no effect. The third barrel contained 0.5 mol.l^{-1} solution of PB (Spofa) in distilled water (pH = 10, solution conductance 19 ± 2 mS, tip resistance 80—150 MΩ). Release of PB ions from the micropipette between the applications was supressed by retaining current (+ polarity, 10 nA).

Single unit activity was displayed on an oscilloscope, integrated by an F/U converter, and recorded on a line recorder. All data were evaluated from rate meter records.

Results

Responses of the reticular neurones to PB application

The spontaneous activity of the investigated neurones varied between 5—110 spikes.s^{-1} (see Fig. 3). The majority of neurones (41) showed tonic continuous firing, in the remaining part of the neurones (26), discharges were grouped into bursts.

Application of PB (if not indicated otherwise, the applications lasted 30 s

Table 1. Some characteristics of the responses of neurones in nucleus reticularis gigantocellularis to 30 s application of PB with different current intensities (mean values ±SD): $n = 17$ (the same population as in Fig. 2).

Response characteristics		5 nA	10 nA	20 nA	40 nA
Latency	s	6.90±5.13	5.50±5.03	5.60±4.49	4.40±4.05
Slope	$10^{-2}\,s^{-1}$	−1.36±0.77	−1.63±0.96	−1.99±0.89	−2.25±0.71
Recovery time	s	33.10±18.80	34.10±13.50	46.10±19.50	52.01±17.30

each) caused a depression of the spontaneous activity in 54 out of 67 neurones tested (81%), i. e. 13 neurones (19%) remained unaffected. Units unaffected by PB application exhibited a type of spontaneous activity comparable to that seen in depreesed neurones (firing rate ranged from 7—80 spikes . s^{-1}; 3 of them with bursting activity). After ceasing the application, the firing rate returned to its previous level in 32 neurones (59%); in 15 (28%) units, it remained lower (decrease by 7—45% of the control values) and in 7 (13%) neurones, an increase in their firing rate up to 58% of the control values was observed. Other parameters, which characterize the action of PB upon the neuronal population investigated are shown in Table 1.

An increase in the ejecting current resulted in a shortening of the latency (time period from the start of the ejection till the onset of the response); however, in the range of ejecting current used (5—40 nA), this effect was not clear-cut. The increase in current intensity had a stronger effect on the rate of depression of the discharge activity; this is documented by the slope k, defined as $-\frac{\Delta f/f}{\Delta T}$ (Fig. 1C, Table 1); f is the initial firing rate, Δf is the difference between the initial firing rate and that at the moment of the maximal response, ΔT is the time interval between the start of application and the maximal response. Duration of the recovery time of the response, the third parameter analyzed, increased with the increasing current values. The total duration of the response can be derived as time of application plus recovery time minus latency; its values ranged from 48—117 s.

Dose-response curve (DRC)

After having determined the level of the spontaneous activity for 1—3 min, PB was ejected with increasing current intensities (5; 10; 15; 20; 40 nA — Fig. 1A). Ninety seconds were let to elapse between individual applications to control

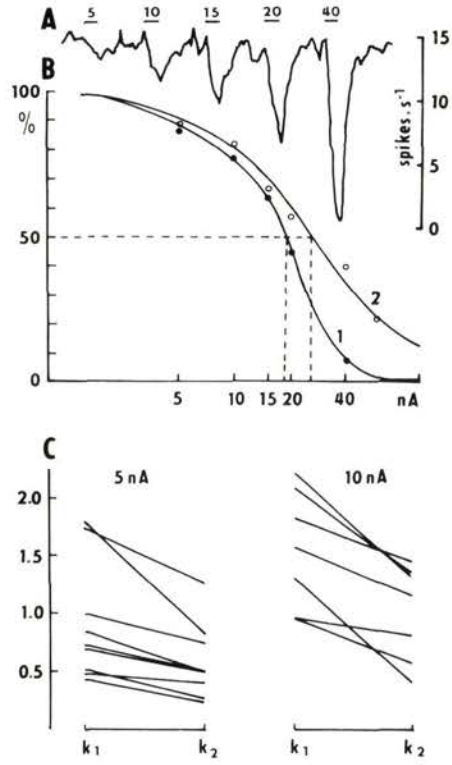

Fig. 1. Inhibitory effect of pentobarbital (PB) on neurones in the reticular formation. *A*: Rate meter records document spontaneous activity changes (ordinate, spikes.s^{-1}) in nucleus reticularis gigantocellularis (NRG), (PB, 30 s application — horizontal bars; numbers indicate ejecting current in nA). *B*: Curves 1, 2 illustrate dose-response dependence of the reticular neurone (ordinate — decrease of firing rate in % of the baseline firing; abscissa — ejecting currents, log scale) at repeated applications of PB (interval between the 1 st and 2 nd application was 210 s). Vertical dashed lines represent EC_{50} change between the 1 st and 2nd application. *C*: Comparison of the slopes — k, see text (ordinate, values in $10^{-2}.s^{-1}$) at the first (k_1) and second (k_2) application of PB (abscissa) lasting 30 s by 5 nA (9 neurones) and 10 nA current (7 neurones), respectively.

spontaneous activity; each application lasted 30 s. The relationship between the activity decrease (in %) and the dose of PB (in log scale) is represented by a typical sigmoidal curve (Fig. 1*B*, 2*A*). It can be seen that an almost total depression of neuronal activity in the NRG resulted at 40—60 nA currents (1.2—1.8μC).

Repeated applications of PB (Fig. 1*B*) — tested for each value of the ejecting current — resulted in a shift of the DRC towards higher current values (to the right); the decrease of PB efficiency was expressed by a lowering of the slope values — Fig. 1*C* (the time interval between the first and the second application of

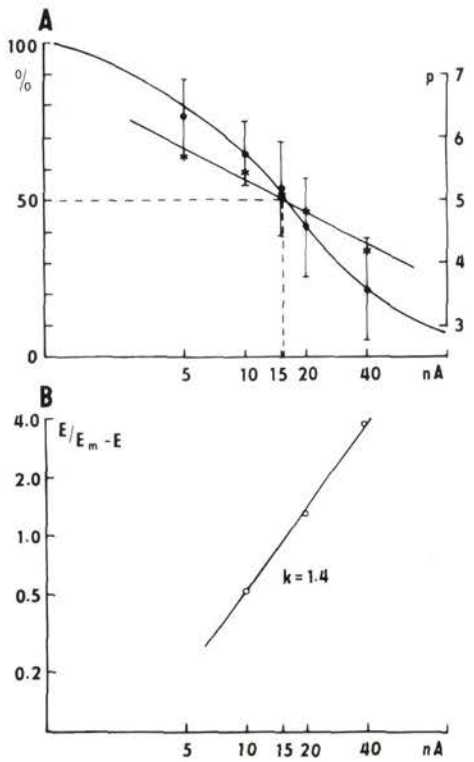

Fig. 2. DRC and Hill plot for the investigated population of neurones. *A*: DRC (points) and the respective probit (asterisks) for 17 neurones in NRG (PB, 30 s applications). Abscissa: ejecting currents (in log scale) for PB, ordinates: response (% — mean values ± SD) and probit (p). The current which produced a 50% (p = 5) decrease from the control firing was 15.5 nA (0.465 μC), (vertical broken line). *B*: Log-log dependence $\frac{E}{E_m - E}$ (E — firing decrease in %, E_m — maximal decrease) upon ejecting currents of PB (in nA, abscissa). The slope (k) of this relationship, i. e. the Hill coefficient was 1.4.

the same current was about 10 min). An increase in slope values was observed only in 3 cases (9%, not shown).

The dose-dependent inhibitory effect of PB on a population of 17 neurones is shown in Fig. 2*A*. Each point on the DRC was converted to its corresponding probit (p) value; its linear relationship ($r = 0.98$) to the logarithm of the ejecting currents refers to a lognormal distribution of the population investigated.

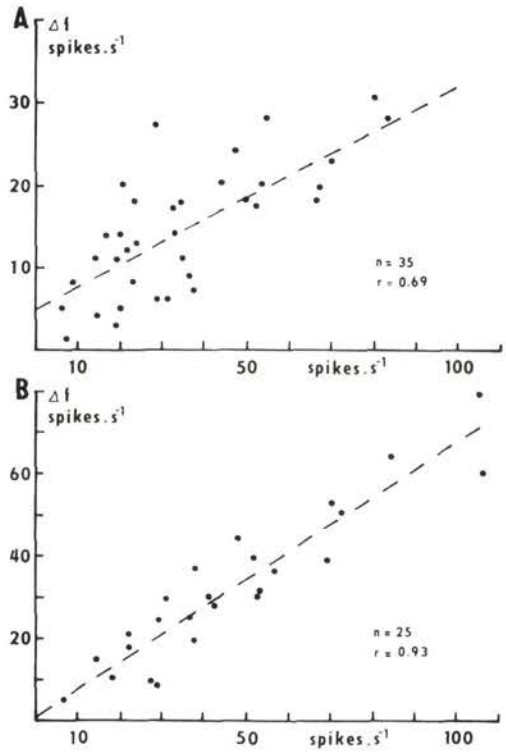

Fig. 3. Dependence of the effect of PB application upon the level of spontaneous activity. Abscissa: control firing rate of neurones (spikes.s^{-1}). Ordinate: maximal frequency change (depression, Δf, spikes . s^{-1}) evoked by a 30 s application of PB (A — 10 nA, B — 40 nA). Each point represents the result of one application; linear regression lines are shown, n — number of neurones tested, r — correlation coefficient.

The Hill coefficient for the PB action and the dependence of the effect upon the level of the spontaneous firing rate

In order to characterize interaction of PB with binding sites, the Hill coefficient (the slope of the Hill plot) was calculated. The dependence of the ratio $\frac{E}{E_m - E}$ upon currents of 10; 20; and 40 nA was tested in 17 neurones (Fig. 2*B*). E represents decrease of the firing rate in %, E_m is the maximal (100%) decrease. From this relationship, the value of 1.4 was obtained (Fig. 2*B*). It is interesting that, when lower currents (5; 10; 15; 20 nA) were used, the value of the Hill coefficient was close to 1.0 ($k = 0.99$). The increase in the Hill coefficient at higher ejecting currents was probably due to mechanisms of negative cooperation. Both

values of the Hill coefficient indicate that the occupation of only one subunit of the binding site by PB might be enough to give a response.

With regard to different levels of spontaneous firing rates in the population investigated (5—110 spikes.s^{-1}) it was reasonable to determine the relationship between the effect and the control firing rate. Individual points on Fig. 3 represent frequency decreases after PB application (ordinate) in dependence upon the respective control firing rates (abscissa). The correlation coefficient (linear regression) revealed a better correlation for higher values of the current: 20 nA and 40 nA ($r = 0.88$ and $r = 0.93$ respectively); for 5 nA and 10 nA, the correlation was worse ($r = 0.67$ and $r = 0.69$ respectively). Consequently, at higher current values, the decrease in the firing rate was proportional to the control level. In the less pronounced dependence at lower current values, diffusion may have a more important role.

Discussion

PB appears to cause depression, facilitation or modulation of neuronal activity through multiple mechanisms (Valdman 1967; Morris 1978; Nanobashvili and Narikashvili 1982). The final effect depends on the location, concentration or mode of administration of PB. Neurones in reticular and ventrolateral thalamic nuclei showed burst activity or tonic continuous discharges after intravenous application of PB (Nanobashvili and Narikashvili 1982). Slow high amplitude waves and periodic spindle volleys were observed in the cerebral cortex after systemic PB administration (Valdman 1967).

Mechanisms of depression can act either in the presynaptic or postsynaptic part of the neuronal junction. In the presynaptic endings, this effect may result from a decrease in transmitter release (Eccles et al. 1963; Nicoll 1975b). The explanation of this effect on subcellular level is based on the presumption that PB reduces Ca^{2+} entry into the presynaptic ending (Blaustein and Ector 1975; Haycock et al. 1977). The depression of the neuronal activity on the postsynaptic membrane is probably due to the depression of $Na^+ - K^+$-dependent conductance mechanisms coupled to postsynaptic receptors (Barker 1975; Barker and Gainer 1973; Nicoll 1975a) and also to depression caused by the GABA receptor activation (Nicoll 1975a, b). An opposite mode of action — enhancement of activity — was observed at low PB concentrations (0.01—0.02mmol.l^{-1}) with an increase in acetylcholine release from the brain tissue (Richter and Waller 1977), and facilitation of the synaptic transmission in the cuneate nucleus (Morris 1978).

The only effect during the ionophoretic application of PB was a depression of the activity of reticular neurones. No facilitation appeared even when low ejecting current (5 nA) was used (an approximate PB concentration of 10^{-5} mol.l^{-1} — see Appendix). A certain proportion of units investigated (19%) remained unaffected

by PB — in this case, it was the activity of axons that may have been recorded, although the existence of neurones resistent to PB cannot be ruled out.

There were only small differences among latencies in correlation to different intensities of ejecting currents. The time course of the onset phase of the depression (slope) was much more dependent on the extracellular PB concentration. The slope rised with the increasing current intensities, and this corresponded to a more rapid rate of the depression.

At the end of the PB application, the firing rate returned to its previous level in the majority of the neurones investigated (59%), i. e. in those cases the binding of PB to its binding site was fairly labile. A smaller proportion of neurones (28%) did not return to their initial level of firing (the influence of PB leakage from the micropipette was eliminated by the retaining current). The existence of reticular neurones forming more stable binding with PB may be suggested. The rest of the neurones (13%) exceeded their baseline firing level at the end of PB application; a similar phenomenon was observed during ionophoresis of GABA (Reader 1980).

The ejecting current producing a 50% decrease of firing (EC_{50}), as derived from DRC, was 15.5 nA (about 5×10^{-5} mol.l^{-1}). This indicates an appreciable sensitivity of RF neurones to PB; the obtained value is much lower than EC_{50} for spinal neurones (2.8×10^{-4} mol.l^{-1}), (Shulz and Macdonald 1981). The shift of DRC towards higher current values (a shift of EC_{50} within 7 nA) at repeated doses of PB may have been due to prolonged changes of the binding site for PB (desensitization).

The value of the Hill coefficient was calculated in conformity with the classical theory. From its value (1.4) and assuming PB acting at the binding site of the GABA receptor complex („barbiturate receptor" — Olsen 1981; Nicoll 1975a, b), it may be speculated that the occupation of one subunit of this complex by PB might be enough to give a response.

Appendix

Estimation of the drug concentration in the extracellular space

For the estimation of the drug concentration in the extracellular space, the tip of the micropipette was considered as a point source within a uniform medium, where drug ions move only by diffusion. Then, in the case of linear diffusion of the substance supplied at a constant rate, m, per unit time, with the diffusion coefficient D, a distance r, and time, t, the concentration is given by the expression (Jaeger 1965):

$$c = \frac{m}{4\pi Dr} \operatorname{erfc} \frac{r}{2(Dt)^{1/2}} \qquad (1)$$

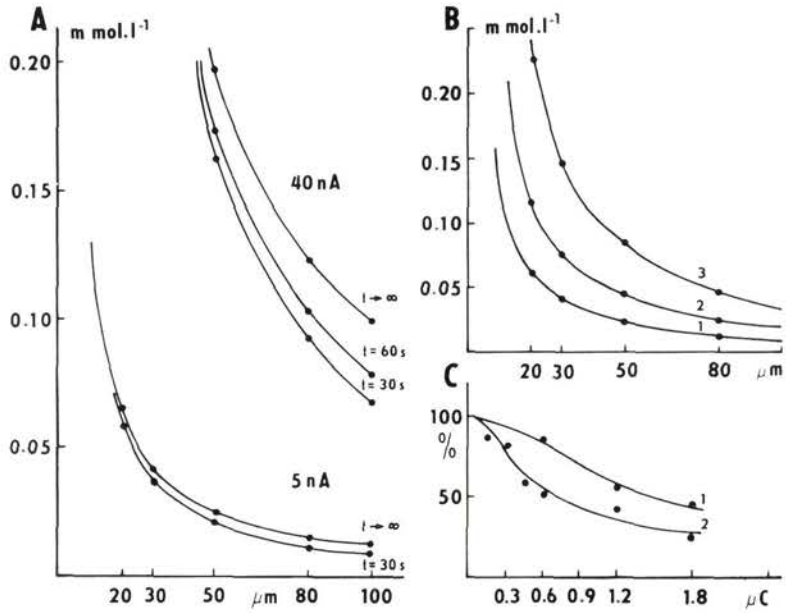

Fig. 4 *A*: Theoretical dependence of the drug concentration (ordinate) in the vicinity of the neurone upon the distance of the tip of the micropipette (abscissa), time of application (t), and current intensity (nA) — see text. *B*: Curves representing the concentration (ordinate) — distance (abscissa) relationship) for the same charge passed through the micropipette. Curve 1: application 120 s, 5 nA (0.6μC); curve 2: aplication 60 s, 10 nA (0.6μC); curve 3: application 30 s, 20 nA (0.6μC). *C*: DRC recorded in the same neurone. Curve 1: application 120 s (first application), curve 2: application 30 s (second application); the time interval between the first and the second application was 4 min. Abscissa: values of the charges passed through the micropipette; ordinate: activity decrease in %.

where $m = \frac{in}{zF}$ and

$$\operatorname{erfc} y = 1 - \frac{2}{\pi^{1/2}} \int_o^y e^{-x^2} dx$$

i is the ejecting current; n is the transport number; z is the ion valency and F is Faraday's connstant.

The time of application varied between 30 and 120 s. The distance between the neurone and the tip of the micropipette was tested at the end of the registration by a vertical shift of the micropipette; the distance was about 50 μm (cessation of the activity; damaging of the neurone). For a diffusion coefficient of $D = 1$ and a transport number of $n = 0.3$ (values of D and n were estimated — Krnjević et al.

1963; Reader 1980; Zieglgänsberger et al. 1974), the concentrations ranged from 1×10^{-5} to 1.5×10^{-4} mol.l^{-1} for the current intensities used (5 – 40 nA), (Fig. 4*A*).

In accordance with the Faraday law the same charge carrying the same amount of the substance should have the same effect. When the concentration-distance relationship for different currents were plotted (meeting the condition of the same charge, e. g. 0.6 μC — Fig. 4*B*), different concentrations were derived from equation (1) for the defined distance. Also, different dose-response curves were plotted in dependence upon the passed charge (Fig. 4*C*). Curve 1 represents the dose-response relationship for the 120 s application, curve 2 for the 30 s application. The shift of DRC corresponding to different degrees of depression was probably due to different concentrations of the drug (Fig. 4*B*), although the same charge was used.

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