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Effects of sodium pentobarbital on the components of electroretinogram in the isolated rat retina

Natalia V. Kapousta-Bruneau *

Department of Ophthalmology, Neuroscience Building, University of Michigan, 1103 E. Huron Street, Ann Arbor, MI 48104-1687, USA

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Abstract

Photovoltages, the fast $P_3(t)$ component of electroretinogram (ERG), were registered between two microelectrodes across the rod outer segments. The $P_2(t)$ component, obtained by subtracting the ERGs measured before the application of 50 μM APB from those measured after the application of 50 μM APB, was used as an indicator of depolarizing bipolar cell activity. Measurements of the scotopic threshold response (STR) and the oscillatory potentials (OPs) were used as indicators of third order neuron activity. The slow $P_3^*(t)$ component, obtained by subtracting the photovoltages from the transretinal recording in the APB-treated retina was used as an indicator of Müller cell activity. The components of the ERG obtained in normal superfusate medium were compared with those obtained in the presence of 100 μM sodium pentobarbital. We found that sodium pentobarbital slowed the kinetics of the $P_2(t)$ component and increased its latency. The fast $P_3(t)$ component was not affected by pentobarbital. The slow $P_3^*(t)$ component was slightly reduced in the presence of pentobarbital. The minor components of the ERG, the STR and the OPs, were strongly suppressed by pentobarbital. These results suggest that in rat retina pentobarbital does not affect photoreceptors, but it does affect bipolar cells and Müller cells, and it suppresses activity of third order neurons.

Keywords: Retina; Electroretinogram; Pentobarbital; Rat

1. Introduction

Sodium pentobarbital is commonly used as a general anesthetic for laboratory animals (Frederiksen, Henthorn, Ruo & Atkinson, 1983; Erhardt, Hebestedt, Aschenbrenner, Pichotka & Blumel, 1984; Flecknell, 1993; Allen, Pringle, Smith, Conlon & Burgmann, 1993; Debuf, 1994; Gardner, Davis, Weina & Theune, 1995). It has been shown that sodium pentobarbital changes the waveform of the corneal electroretinogram (ERG) in rabbits and monkeys (Noell, 1958; Bornschein, Hanitzsch & Lutzow, 1966; Winkler, 1972; Satoh, Fukuda, Kuriki, Maki, Nomura & Saji et al., 1980). It has also been shown that sodium pentobarbital affects the b-wave increment thresholds in rats (Brown & Green, 1984). The mechanisms that underlie the pentobarbital effects on mammalian retina remain unknown. The present study is an attempt to define the mechanisms by determining which types of cells in the rat retina are affected by sodium pentobarbital.

The ERG is a combination of several components with different polarities, originating from distinct types of retinal cells (Granit, 1933; Penn & Hagins, 1969, 1972; Hood & Birch, 1990, 1992; Breton, Schueller, Lamb & Pugh, 1994; Robson & Frishman, 1995; Hood & Birch, 1996; Robson & Frishman, 1996). In the present study we have used a new approach by combining a new method for the preparation of isolated rat retina with a microelectrode technique (Green & Kapousta-Bruneau, 1999a). We isolated the components of the ERG which belong to photoreceptors, depolarizing bipolar cells, Müller cells and inner retina neurons (Green & Kapousta-Bruneau, 1999b).

The reference handbooks for veterinarians recommended pentobarbital doses for the anesthesia of rat (via IP or IV injections) in the range of 30–60 mg/kg of body mass (Allen et al., 1993; Debuf, 1994). Several researchers measured the concentration of pentobarbital in rat serum and plasma 1 h after such injections by

* Tel.: +1-734-763-2280; fax: +1-734-936-2690.
E-mail address: nvb@umich.edu (N.V. Kapousta-Bruneau)

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using gas chromatography and spectrophotometry; all concentrations fall in the range 15–30 μg/ml in serum (Guthrie, Cooper, Thurman & Linnoila, 1987; Widerlöv, Bissette & Nemeroff, 1987) and 15–100 μg/ml in plasma (Chu, Stella, Bruckner & Jiang, 1977; Lang, Bagby, Hargrove, Hyde and Spitzer, 1987; Hatanaka, Sato, Endon, Katayama, Kakemi & Koizumi, 1988; Jung & Prasad, 1989; Sato, Koshiro, Kakemi, Fukasawa, Katayama & Koizumi, 1995; Stetinova, Grossmann, Kvetina & Sramek, 1995). Using the molecular mass of pentobarbital $M = 226$ (or $M = 250$ for sodium pentobarbital) these concentrations correspond to 67–126 μM in serum and 66–420 μM in plasma, respectively. We used 100 μM of sodium pentobarbital to study its effects on retina neurons because we believe that this concentration belongs to the range of relevant concentrations for general anesthesia in mammals. All effects of the anesthetic on the ERG in the isolated rat retina reported here were reversible. We supplied evidence for reversibility by repeated ERG measurements while the perfusate solution was changed from normal Ringer, to Ringer containing pentobarbital, and back to normal Ringer. These experiments also demonstrated that our isolated retina preparation is a suitable object for studies of the reversible effects of anesthetics under controlled experimental conditions. We have applied computerized data analysis to isolate the ERG components and to compare their magnitudes and shapes in the presence and absence of pentobarbital.

2. Methods

2.1. Preparation

The retinas were excised from albino rats (Sprague–Dawley) which were reared in 12/12-h cycle of dim light and darkness to avoid retinal light damage. Before each experiment rats were kept in the dark for 10–12 h. The animals were anesthetized with intraperitoneal injections of sodium pentobarbital (30 mg/kg) prior to decapitation. Only infrared light was used during the preparation and recording procedures. All manipulations were done using night vision devices (NAV-3, Intevac, Palo Alto, CA). The preparation technique, superfusion solutions, construction of the perfusion chamber, and recording procedures have been described in detail (Green & Kapousta-Bruneau, 1999a,b). Briefly, both eyes were enucleated, the retinas were removed and then placed in a bath of oxygenated Ringer solution at the room temperature. A portion of the retina ($\approx 3 \times 3.5$ mm) was placed photoreceptor side up on a piece of Millipore filter (type SC; Millipore, Bedford, MA). In the experiments presented in this paper we used only a single piece of retina from one eye in each experiment. The filter and the retina were then positioned between two pieces of nylon mesh in a perfusion chamber. The retina was perfused at flow rates of 3 ml/min with oxygenated preheated Ringer solution. The temperature of the perfusing solution was monitored inside the chamber and maintained at 36–37°C.

2.2. Perfusion solutions

The basic Ringer solution used to superfuse the retina had the composition (in mM): NaCl, 110; KCl, 5; Na_2HPO_4, 0.8; NaH_2PO_4, 0.1; $NaHCO_3$, 30; $MgSO_4$, 1; $CaCl_2$, 1.8; glucose, 22; glutamine, 0.25. The solution was bubbled in storage bottles with a mixture of 95% O_2 and 5% CO_2 using fine-pored air stones. The solution flowing over the retina could be rapidly switched to other solutions containing the components of the basic Ringer solution plus known concentrations of pentobarbital (Sodium Pentobarbital Injection 65 mg/ml (Butler, Columbus, OH, USA)), APB (DL2-amino-4-phosphonobutyric acid), NMDA (*N*-methyl-D-aspartic acid), DNQX (6,7-dinitroquinoxaline-2,3-dione) (Sigma, St. Louis, MO), either singly or in various combinations. The pH of all solutions was 7.45–7.55. The time required for the new solution to reach the retina was approximately 2 min, and the fluid exchange was 90% complete within another min.

2.3. Recordings

Transretinal ERG recordings were made using a glass micropipette filled with Ringer solution positioned at the tips of the outer segments and referenced to an Ag–AgCl electrode (model E202, IVM, Healdsburg, CA) located under the retina. The borosilicate (Corning no. 7740; Sutter Instruments) micropipettes had tip diameters of 3–5 μm and resistances of 5–25 MΩ. Rod photovoltages were recorded between a micropipette, which was advanced 45–50 μm into the retina and a second micropipette at the tips of the rod outer segments (Green & Kapousta-Bruneau, 1999b). Light evoked responses from isolated retina were recorded with a high gain amplifier (CyberAmp, Axon Instruments, CA), filtered at 400 Hz, digitized (A/D converter model NB-MIO-16X; National Instruments, Austin, TX) and then stored and subsequently analyzed with a minicomputer (Apple Computer, Cupertino, CA) using LabVIEW software (version 4.0, National Instruments). Each record contained 2400 points with 1 ms sampling period, including 50 pre-stimulus points, which were used for estimates of the noise level. In data analysis, we used the record beginning from the 51st point in order to

avoid the artefacts induced by the brief electronic flash (see below), which were occasionally observed in the records at the 50th point.

2.4. Stimulation

The light stimulus was brief (<1 ms duration) and monochromatic (500 nm), derived from a commercial photoflash unit. The intensity and color of the light were controlled by placing calibrated neutral-density and interference filters in the stimulus beam. The timing of the flash presentation was under computer control. Data were collected using a fixed set of stimuli in which the light intensity increased progressively in ≈ 0.5 log steps. Low intensity flashes were interposed between brighter flashes to assure that there was sufficient time between flashes for the sensitivity of the retina to be restored. A 12 s interstimuli interval (ISI) with low intensity responses, and a 60 s ISI with the highest intensity responses were sufficient for the measured response amplitudes to be unaffected by earlier flashes.

The quantum flux on the retina was estimated in the following manner. The photon flux ($\lambda = 500$ nm) falling on the isolated retina from a 150 W xenon arc lamp was directly measured using a calibrated radiometric detector (United Detector Technology, S370, Hawthorne, CA). The flux per rod was calculated by assuming half the light is absorbed (Hagins, Penn & Yoshikami, 1970; Penn & Hagins, 1972) and each rod has a cross-sectional area of 2.3 μm^2 (Cone, 1963). Stimulus duration from the xenon arc lamp (40 ms) was controlled by an electronic shutter (Uniblitz, Model VS25, Vincent Associates, Rochester, NY) under computer control. For the purposes of calibrating at low light intensities transretinal ERGs were measured using both brief electronic flashes and 40 ms shuttered stimuli. When equal amplitudes of b-wave responses were obtained for both types of stimuli it was assumed that the retina had absorbed equal numbers of quanta/flash. In all experiments presented in this paper only brief electronic flash was used.

2.5. Data analysis

The digitized responses were analyzed and plotted using MATLAB (version 4.2, Math Works, Natick, MA). The peak amplitudes of the recorded ERGs and their components were measured by fitting second order polynomials to the segments of the response containing the peak (see (Green, Herreros de Tejada & Glover, 1991) for additional details). The amplitude of the first negative peak of the ERG was defined as the a-wave amplitude. The voltage measured from the negative peak (a-wave) to the first positive peak defines the b-wave amplitude.

To measure the response latencies a least squares straight line was fitted to the 50 prestimulus baseline points and another straight line was fitted to points from the early part of the light response. The start of the response was determined by measuring the time at which the response was three standard deviations above the baseline noise, and then defining the early response to be ten points on either side of the start time point. Latency was defined by the intersection of these two lines. The adequacy of the fitted lines could be assessed by displaying the fits to the data on the computer monitor. In some records at lower light intensities latency was sufficiently long that the possible baseline drift adversely affected our estimates. To minimize these errors an automated interactive procedure was used to add points to the baseline. These points were determined using the latency estimate from the previous iteration and the procedure was repeated until a stable latency value was measured. This procedure provided a good operationally defined value for the response latency.

We isolated the OPs from the total ERG using a bandpass Butterworth filter of the 5th order (a program in MATLAB version 4.2, Math Works). This filter has a magnitude response that is maximally flat in the passband between -3 dB points at 75 and 300 Hz. In the transretinal ERG recorded in response to bright light stimuli (2065 quanta/rod/flash) we can identify several OPs, which are observed at specific delay times after the flash of light. We measured the overall power of the oscillations, which we calculated as the mean of squared values of filtered ERG in the time frame between 1 ms after the flash (point $\#51$) and 91 ms after the flash (point $\#141$).

Statistical significance was set at $P < 0.05$ using Student's paired t-test with 2-tails distributions (Microsoft Excel 5, Microsoft Corporation, Seattle, WA).

2.6. Dissection of the ERG into components

2.6.1. Direct measurements of the activity of photoreceptors

In order to isolate components of the ERG, which belong to distinct types of cells in the rat retina, we used three electrodes and simultaneously recorded two signals: the transretinal ERG across the entire retina and photovoltages across the rod outer segments (see Green & Kapousta-Bruneau, 1999b, for more details). For the transretinal ERG recordings we used a glass micropipette filled with Ringer solution positioned at the tips of the outer segments and referenced to an Ag–AgCl electrode located under the retina. Rod photovoltages were recorded by measuring voltages across the rod outer segments between two micropipettes, one of which was inserted 45–50 μm into the retina, while the other was positioned at the tips of the outer seg-

ments. Recorded potentials were proportional to the radial extracellular currents flowing along the rod outer segments. After subtracting the field potentials coming from the proximal retina, we found that these measurements were equivalent to those obtained using suction electrodes, except that these photovoltages came from a group of about 10 000 rods, rather than from a single rod outer segment (Green & Kapousta-Bruneau, 1999b). The electric response, which we measured from the photoreceptor layer, we designate as the $P_3(t)$ component, where (t) is time (Green & Kapousta-Bruneau, 1999b). Simultaneous measurements of the transretinal ERG and the $P_3(t)$ component under a variety of conditions (light intensity, drug application, etc.) allow us to separate out the contributions of photoreceptors and other types of cells to the total ERG. At the same time, we use the waveforms of $P_3(t)$ as a test for normality of photoreceptor function under various experimental conditions.

2.6.2. Use of APB, a functional blocker of ON-depolarizing bipolar cells

We isolated the retina and performed the measurements under infrared light. Under these scotopic conditions, it is likely that the light-evoked responses in the rod-dominated rat retina are generated mostly by neurons from the rod pathway, which involves signal transduction through a single type of ON-depolarizing bipolar cell (Dacheux & Raviola, 1986; Yamashita & Wässle, 1991; reviewed by Kolb, 1994; de la Villa, Kurahashi & Kaneko, 1995). Metabotropic glutamate receptors (mGlu6) sensitive to DL2-amino-4-phosphonobutyric acid (APB) are localized on the postsynaptic part of ON-type bipolar cells in the rat retina (Nomura, Shigemoto, Nakamura, Okamoto, Mizuno & Nakanishi, 1994; Masu, Iwakabe, Tagawa, Miyoshi, Yamashita & Fukuda et al., 1995; Euler, Schneider and Wässle, 1996). Thus, APB can be used to selectively block the depolarizing bipolar cell activity in rat and other mammals (Massey, Redburn & Crawford, 1983; Knapp & Schiller, 1984; Robson & Frishman, 1995; Hanitzsch, Lichtenberger & Mattig, 1996; Robson & Frishman, 1996).

We have found that all measurable effects of 50 μM APB, are reversible (Green & Kapousta-Bruneau, 1999b). The effects are reversible in the sense that there is complete restoration of the waveform of the transretinal ERG after the drug is washed out of the retina. Typically, all signals are restored within 15–20 min. In order to isolate the ERG component suppressed by APB, transretinal recordings obtained with APB in the perfusate were subtracted from those obtained without APB in the medium, as shown in Fig. 1A.

We have found that 50 μM APB completely blocks the b-wave of the ERG recorded transretinally in the isolated rat retina (Kapousta-Bruneau, Green & Lui, 1996). We also found that low concentrations of barium ions (50–200 μM), which block the potassium conductance of the Müller cells (Newman, 1985, 1989; Reichelt & Pannicke, 1993; Puro & Stuenkel, 1995), do not block the $P_2(t)$ component (Green & Kapousta-Bruneau, 1999b). Therefore, we believe that the extracellular radial currents from ON-depolarizing bipolar cells is the major source for the b-wave origin in rat retina. Although the APB-isolated $P_2(t)$ component contains some influence from the third order neurons we use this component as a reflection of ON-depolarizing bipolar cells activity.

2.6.3. Isolation of the electrical response of the Müller cells

In our experiments we take advantage of the simplified ERG recorded from APB-treated retina. This entirely negative signal basically consists of the fast- and slow-PIII components which reflect, correspondingly, the activity of the photoreceptors (Weinstein, Hobson & Dowling, 1967; Penn & Hagins, 1969; Arden, 1976; Breton et al., 1994; Robson & Frishman, 1996) and the activity of Müller cells (Ripps & Witkovsky, 1985; Frishman & Steinberg, 1989; Steinberg, Frishman & Sieving, 1991; Xu & Karwoski, 1997). In our studies the $P_3(t)$ component (photovoltages) described above corresponds to the fast-PIII. We have found that the kinetics of the $P_3(t)$ component do not depend on the presence of APB in the perfusion medium (Kapousta-Bruneau et al., 1996; Green & Kapousta-Bruneau, 1999b). Thus, by subtracting the $P_3(t)$ component from the transretinal ERG recorded simultaneously in the presence of APB, we can obtain the slow component, which we named the $P_3^*(t)$, as shown in Fig. 1B. The $P_3^*(t)$ component corresponds to what others call slow-PIII and have identified as the Müller cell response to the decrease in potassium around photoreceptors (Oakley & Green, 1976; Newman & Odette, 1984; Frishman & Steinberg, 1989). Our conclusion is based on our results which have shown that a low concentration of barium ions (50–200 μM), known to block the potassium conductance of the Müller cell membranes (Newman, 1985, 1989; Reichelt & Pannicke, 1993; Puro & Stuenkel, 1995), completely suppresses the $P_3^*(t)$ component (Kapousta-Bruneau et al., 1996; Green & Kapousta-Bruneau, 1999b).

In Fig. 1C we show the transretinal ERG obtained at high light intensity (2065 quanta/rod/flash) as an algebraic sum of these three major components: $P_3(t)$, $P_2(t)$ and $P_3^*(t)$.

2.6.4. Detection of signals from inner retina neurons

The approaches described above allow us to separate contributions to the ERG from three types of retinal neurons: photoreceptors, bipolar cells, and Müller cells.

In addition, we can separate the contributions of the inner retina neurons to the total ERG by analyzing minor components of the ERG which we can record in our isolated rat retina preparation (Green & Kapousta-Bruneau, 1999a).

Under scotopic conditions we measured the scotopic threshold response (STR), which is known to come from amacrine or ganglion cells, possibly mediated by K^+ currents in Müller cells (Sieving, Frishman & Steinberg, 1986; Frishman & Steinberg, 1989). In response to bright flashes, we detect the wavelets or oscillatory potentials (OPs)—high frequency components usually superimposed on the rising part of the b-wave (Ogden, 1973; Heynen, Wachtmeister & Norren, 1985; Vaegan, Graham, Goldberg & Millar, 1991). It has been recently shown that the OPs and the STR originate from different types of cells in cat inner retina: the OPs are generated close to the ganglion cell layer, proximal to the STR origination (Vaegan & Millar, 1994). We used the STR and the

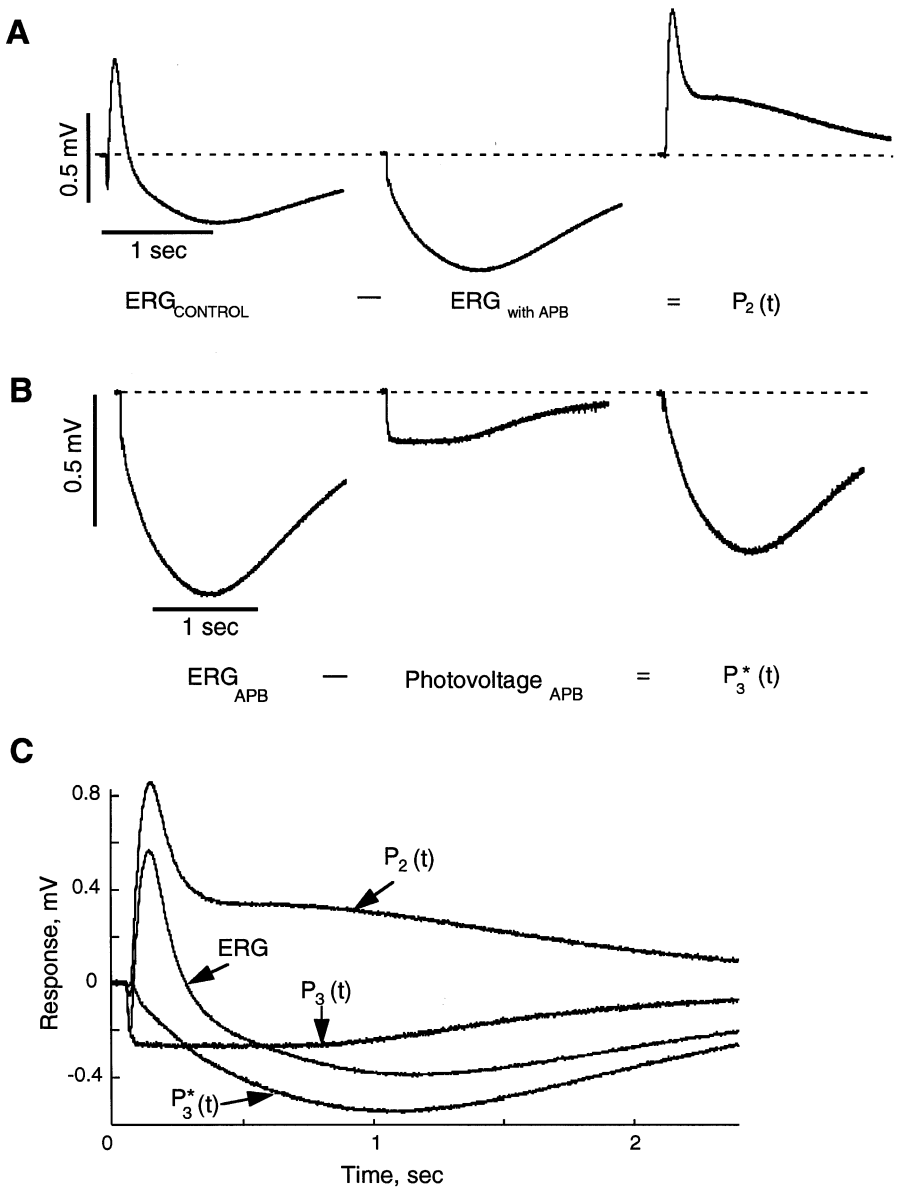

Fig. 1. Dissecting of the transretinal electroretinogram (ERG) into components. (A) The $P_2(t)$ component is obtained as the algebraic difference between the control ERG and the ERG measured in the presence of 50 μM APB. (B) The $P_3^*(t)$ component is obtained by subtracting the $P_3(t)$ component (photovoltage) from the transretinal ERG recorded simultaneously in the presence of 50 μM APB. (C) Transretinal ERG as a combination (algebraic sum) of three components: $P_3(t)$, $P_2(t)$ and $P_3^*(t)$. The traces in A, B and C represent average responses from nine retinas. Signals from each retina were measured according to the same protocol R-APB-R, with complete recovery of the b-wave after washing out APB. Although at each stage of our experiments, the ERGs were measured in entire intensity range, only the records obtained at 2065.4 quanta/rod/flash are chosen for the presentation of the method.

OPs as indicators of the activity of the inner retina neurons.

2.7. Protocols

We used several protocols in our study. Our controls were measurements of ERGs and photovoltages in the isolated rat retina perfused with normal Ringer solution and for which we obtained the entire set of responses to light stimuli of varied intensities (see Section 2 for details). Then we switched to the perfusion solution containing 100 μM of sodium pentobarbital and repeated all measurements. To ensure that the effects of pentobarbital were reversible, we then switched back to the normal Ringer solution and monitored the recovery of signals, which was indicated by restoration of the b-wave kinetics in the transretinal ERG recorded at low light levels (1–4 quanta/rod/flash). After full recovery, we changed the perfusion solution again, switching to Ringer containing 50 μM APB, and measured the entire set of responses in order to isolate the $P_2(t)$ and $P_3^*(t)$ components. Then we washed out the APB-contained Ringer for 10–15 min to monitor the recovery of the b-wave as we described above for pentobarbital. After the recovery was completed we switched to the Ringer, which contained both 50 μM APB and 100 μM pentobarbital in order to obtain the $P_2(t)$ and $P_3^*(t)$ components from the pentobarbital treated retina. This protocol can be summarized as $R \rightarrow PB \rightarrow R \rightarrow APB \rightarrow R \rightarrow PB$ and $APB \rightarrow R$, where R denotes normal Ringer; PB indicates Ringer solution with pentobarbital; PB and APB indicates the Ringer solution containing both pentobarbital and APB. We used this sequential protocol for half of the experiments. In other experiments, we used two reversed protocols to monitor possible effects of drug interactions as well as possible influence of deterioration of the retina samples. The reversed protocols were: $R \rightarrow APB \rightarrow R \rightarrow PB \rightarrow PB$ and $APB \rightarrow R$, and $R \rightarrow PB \rightarrow PB$ and $APB \rightarrow R \rightarrow APB \rightarrow R$. Each protocol took 1.5–2 h to complete. We did not find any differences in the effects of the drugs or in the recovery rates of the responses due to protocol sequences, thus ruling out drug interactions or insufficient recoveries.

With our isolated rat retina preparation, stable responses can be recorded for over 2 h. As a criterion for survival of a retina sample, we used the amplitude of the b-wave in response to flashes of low intensity (1–4 quanta/rod/flash), which corresponds to the steepest part of the response-intensity function (see e.g. Fig. 2C below). Using this criterion deterioration of our samples was less than 10% over 2 h. We applied the same criterion to the recovery of a retina sample monitoring the removal of APB or PB, singly or in combination, from the perfusate. If we could not obtain a complete recovery (within 10%), the experiment was stopped at that stage. All ERG components compared in this paper were obtained from retinas with full recovery.

3. Results

3.1. Effects of sodium pentobarbital on the transretinal ERG

It has been reported that sodium pentobarbital increased the amplitudes and latencies of a- and b-waves of the corneal ERGs from rabbit and monkey (Bornschein et al., 1966; Winkler, 1972; Satoh et al., 1980) and in the transretinal ERGs from the isolated retina of rabbit (Bornschein et al., 1966; Honda & Nagata, 1971). We expected that 100 μM pentobarbital could affect the isolated rat retina ERG in the same manner that has been reported for the ERG in rabbit and monkey. To determine which types of cells are affected by pentobarbital in rat retina we analyzed its effects on the major components of ERG, $P_3(t)$, $P_2(t)$, $P_3^*(t)$, and on its minor components, the STR and the OPs, which we believe reflect the activity of photoreceptors, Müller cells, ON-depolarizing bipolar cells, and the third order neurons, respectively.

Figure 2 represents the effects of pentobarbital on the transretinal ERGs recorded from the isolated rat retina.

Figure 2A shows sample responses to a bright flash (2065 quanta/rod/flash) obtained from the same retina with and without pentobarbital. Both a- and b-wave are increased in the presence of pentobarbital. Figure 2B shows the comparison of the b-waves obtained at five low and medium light intensities (0.09, 0.32, 1.1, 4.1, and 14.3 quanta/rod/flash) in isolated rat retina perfused with normal Ringer solution (solid lines) and in the presence of 100 μM pentobarbital (dotted lines). It is evident that b-wave kinetics are slower in the presence of pentobarbital, and the latencies of each response are increased with pentobarbital in the medium. The amplitude of the a-wave increases in the entire range of intensities (Fig. 2C). However the b-wave amplitude increases only at high intensity, while at low light level these amplitudes are almost unchanged (at 0.04 and 0.09 quanta/rod/flash) and even significantly reduced ($P < 0.005$) at the next two intensities (at 0.32 and 1.1 quanta/rod/flash). The latencies for the b-wave are significantly increased in the presence of pentobarbital at low and medium light intensity levels ($P < 0.01$). The latencies of responses to flashes at high intensities are difficult to determine because of the development of the a-wave. The latencies of the a-wave in the presence and absence of pentobarbital were significantly different ($P < 0.05$) only at the first two intensities when the a-wave begins to develop (at 4.1 and 14.3 quanta/rod/flash). The earlier development of the a-wave in the presence of pentobarbital could be partly due to the increased latency of the b-wave.

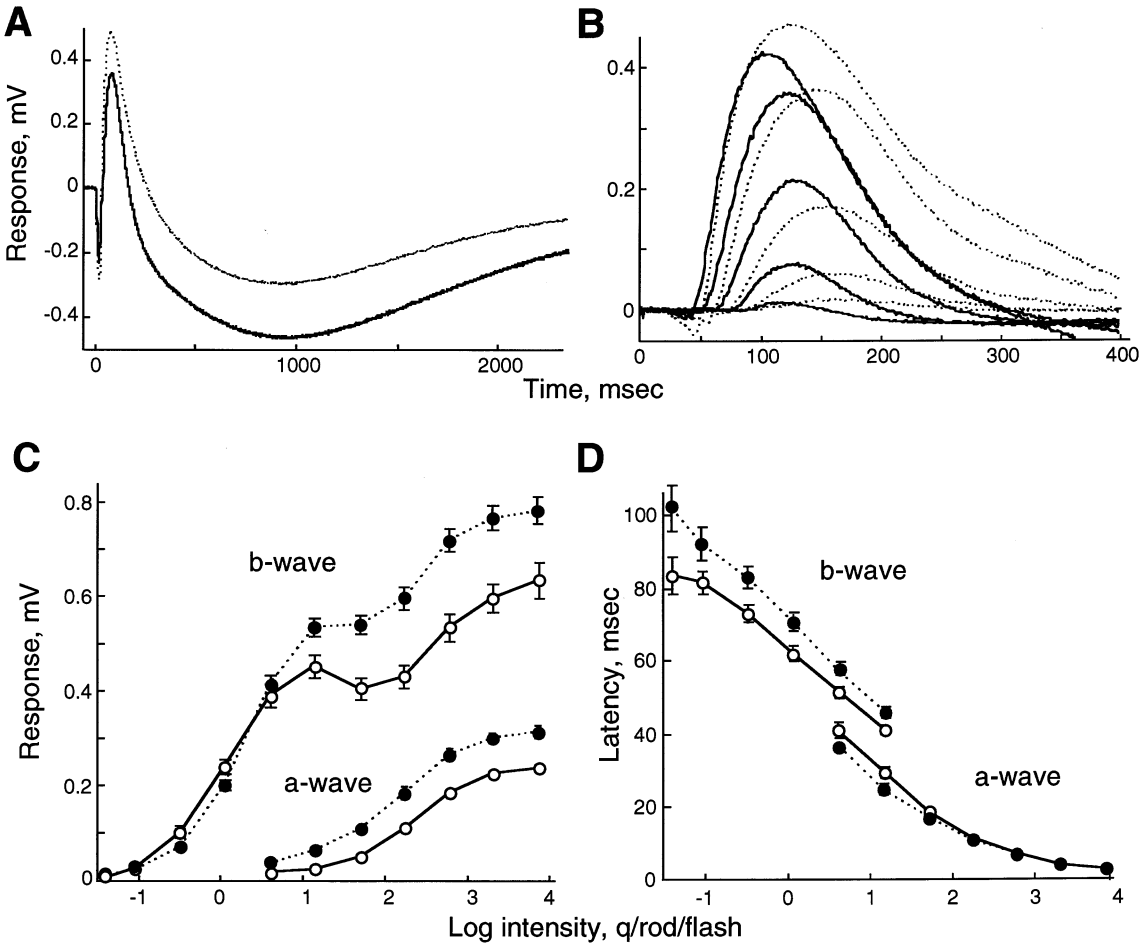

Fig. 2. Effects of 100 μM sodium pentobarbital on isolated rat retina ERG. (A) An example from the single experiment superimposing ERG responses recorded from the same retina with pentobarbital (dotted line) and without (solid line). The responses are evoked by intensity of 2065.4 quanta/rod/flash. (B) Kinetics of the b-wave comparison: with pentobarbital (dotted line) and without (solid line). The b-waves were obtained at intensities varied in approximately 0.5 log units steps, with actual intensities being 0.09, 0.32, 1.14, 4.1, and 14.3 quanta/rod/flash. Responses are averaged from 16 retinas. (C) Amplitude of the a-wave and the b-wave as a function of the flash intensity: with pentobarbital (●, dotted line) and without (○, solid line). Each point is the average of 16 experiments. Vertical lines represent standard error. (D) Latency of the a-wave and the b-wave as a function of the flash intensity with (●, dotted line) and without pentobarbital (○, solid line). Plots are averaged from 16 retinas with standard error bars.

Some of the effects of pentobarbital on isolated rat retina ERGs are similar to those reported by others in studies of rabbits and monkeys. Namely, we confirm that pentobarbital increases the amplitudes of the b-wave and a-wave (Fig. 2C), and increases the latency of the b-wave (Fig. 2D) as observed earlier by several groups (Bornschein et al., 1966; Honda & Nagata, 1971; Winkler, 1972; Satoh et al., 1980). In addition, we confirm that at low light levels pentobarbital decreases the amplitudes of the b-wave as observed by (Noell, 1958). At the same time, we found that pentobarbital did not affect the latency of the a-wave, except at the lowest two intensities (Fig. 2D).

The increase of a-wave amplitude could be caused by a direct effect of pentobarbital on photoreceptors or it could be a consequence of the increased latency of the b-wave. To test whether pentobarbital has a direct effect on photoreceptors we have compared the $P_3(t)$ components obtained in the presence and in the absence of pentobarbital.

3.2. Effect of sodium pentobarbital on the $P_3(t)$ component

According to Granit's analysis of ERG, the leading edge of the a-wave is produced by a negative component which he named PIII (Granit, 1933). Later it was shown that the same kinetic model can be used for the description of the rising part of the photocurrent response of the isolated rod and for the leading edge of the a-wave of the corneal recorded ERG (Hood & Birch, 1990; Kraft, Schneeweis & Schnapf, 1993; Breton et al., 1994). In our recent experiments we have shown that our $P_3(t)$, the Granit's PIII (Granit, 1933), as well as the $P_3(t)$ (Hood

& Birch, 1990) and $R_r(t)$ (Breton et al., 1994) represent the same component of the ERG (Green & Kapousta-Bruneau, 1999b). In our experiments we directly measure the rod photovoltages in the outer segments layer of isolated rat retina. The $P_3(t)$ components measured at various light intensities, with and without 100 μM sodium pentobarbital, are compared in Fig. 3.

There is no significant difference in the kinetics (Fig. 3A) and response-intensity (Fig. 3B) functions ($0.1 < P < 0.8$) of the $P_3(t)$ components measured in the presence and in the absence of pentobarbital. Thus, we can conclude that 100 μM sodium pentobarbital does not affect photoreceptor function in the rat retina. Therefore, the increase of the a-wave amplitudes induced by pentobarbital (Fig. 2C) is probably related to the increase in latency of the b-wave (Fig. 2D).

Because depolarizing bipolar cells are the major source for the b-wave generation in rod dominated rat retina ERG (Green & Kapousta-Bruneau, 1999b), we have tested whether pentobarbital affects the $P_2(t)$ component in the same manner as the b-wave.

3.3. Effects of sodium pentobarbital on the $P_2(t)$ component

We found that pentobarbital has multiple effects on the $P_2(t)$ component (Fig. 4).

Pentobarbital slows the kinetics of the $P_2(t)$ component (Fig. 4A) in a way similar to its effect on the kinetics of the b-wave of the transretinal ERG (Fig. 2B). Pentobarbital also increases the latency of $P_2(t)$ component in the entire range of light intensities (Fig. 4C) as it does for the latency of the b-wave (Fig. 2D). Response-intensity functions for the $P_2(t)$ component (Fig. 4B) reveal that at the two low intensities, 0.32 and 1.1 quanta/rod/flash, pentobarbital decreases the $P_2(t)$ component, which is similar to its effect on the b-wave. This decrease is statistically significant ($0.01 < P < 0.05$). At medium light intensities the effect of pentobarbital on the $P_2(t)$ component is not pronounced. At high-intensity stimuli we observe a tendency that the $P_2(t)$ component increases in the presence of pentobarbital, although the increase was not statistically significant ($0.1 < P < 0.5$).

As we mentioned above, the $P_2(t)$ component may contain some contribution from the inner retina neurons which is not eliminated by APB. To clarify that pentobarbital directly affects ON-depolarizing bipolar cells we repeated these experiments in the presence of NMDA and DNQX, which are known to suppress the inner retina neurons responses (Gurevich & Slaughter, 1993; Vaegan & Millar, 1994; Robson & Frishman, 1995, 1996). In our experiments, 500 μM NMDA and 25 μM DNQX completely eliminated the STR and the OPs from the transretinal recorded ERG in rat retina. Thus the $P_2(t)$ component obtained in the presence of inner retina neurons blockers supposedly reflects only bipolar cell activity. The results of these experiments are presented on Fig. 4D. Pentobarbital noticeably increases the latencies and affects the kinetics of the $P_2(t)$ component. These findings support our assumption that the growth of the b-wave amplitudes at high intensities of stimuli observed in the ERG in the presence of pentobarbital could be due, at least in part, to

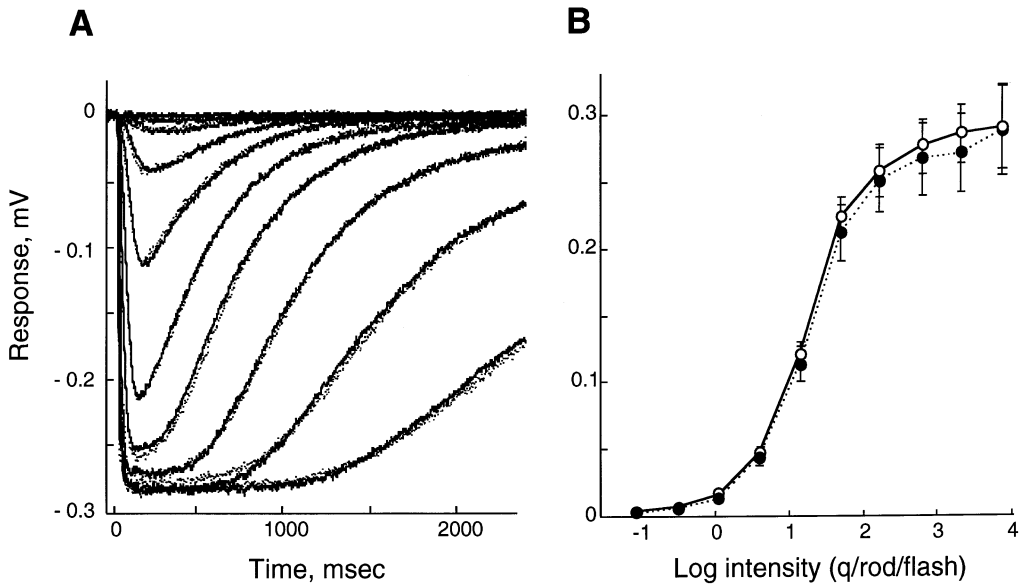

Fig. 3. Effects of 100 μM sodium pentobarbital on the photovoltages, $P_3(t)$ component. (A) Comparison of the kinetics of the $P_3(t)$ components obtained with pentobarbital (dotted line) and without pentobarbital (solid line). Responses are averaged from 12 retinas. The responses have been lowpass filtered (0–125 Hz). (B) Amplitude of the $P_3(t)$ component as a function of the flash intensity: with pentobarbital (●, dotted line) and without (○, solid line). Plots are averaged from 12 retinas with bars indicating the standard error.

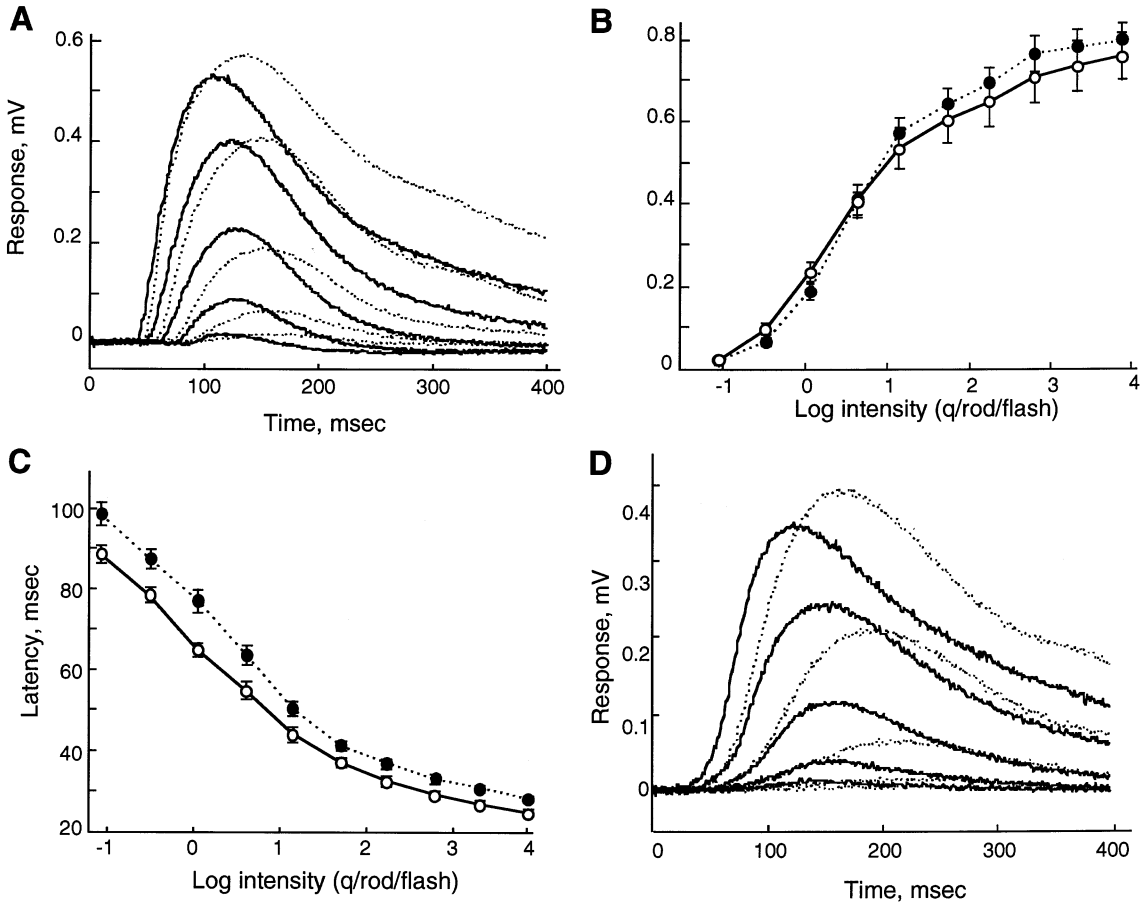

Fig. 4. Effects of 100 μM sodium pentobarbital on the $P_2(t)$ component of the transretinal ERG. (A) Kinetics of the $P_2(t)$ components with pentobarbital (dotted line) and without (solid line). The $P_2(t)$ components were obtained at intensities varied in approximately 0.5 log units steps, with actual intensities being 0.09, 0.32, 1.14, 4.1, 14.3 quanta/rod/flash. Responses are averaged from eight retinas. (B) Amplitude of the $P_2(t)$ component as a function of flash intensity: with pentobarbital (●, dotted line) and without (○, solid line). Plots are the averages from eight retinas with bars indicating the standard error. (C) Latency of the $P_2(t)$ component as a function of flash intensity with (●, dotted line) and without (○, solid line) pentobarbital. Plots are averaged from eight retinas with bars indicating the standard error. (D) Kinetics of the $P_2(t)$ components obtained in the presence in the perfusion media 500 μM *N*-methyl-D-aspartic acid (NMDA), 25 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) (solid line) and in the presence 500 μM NMDA, 25 μM DNQX, and 100 μM pentobarbital (dotted line). The $P_2(t)$ components were obtained at intensities varied in approximately 0.5 log units steps, with actual intensities being 0.09, 0.32, 1.14, 4.1, 14.3 quanta/rod/flash. Responses are averaged from six retinas.

a direct effect of pentobarbital on the extracellular current produced by the ON-depolarizing bipolar cells.

3.4. Effects of sodium pentobarbital on third order neurons

At least two ERG components are known to represent the activity of third order neurons in retina: the OPs and the STR (Cobb & Morton, 1954; Ogden, 1973; Heynen et al., 1985; Sieving et al., 1986; Wachtmeister, 1987; Frishman & Steinberg, 1989; Vaegan et al., 1991; Vaegan & Millar, 1994). We tested whether 100 μM pentobarbital affected these minor ERG components in the isolated rat retina.

We found that pentobarbital strongly suppresses the STR (Fig. 5 A,B).

In Fig. 5A transretinal ERGs recorded at three stimuli of lowest light intensities in the presence and absence of pentobarbital are superimposed. The recordings are averaged from nine retinas. The transretinal ERG responses evoked from retinas in the presence of pentobarbital become almost completely positive and represent the b-wave. Comparison of the amplitudes of the STRs measured at the same time frame: between 200 and 350 ms (Fig. 5B) shows that pentobarbital strongly suppresses the STR, but does not eliminate it.

Fig. 6 shows the effects of pentobarbital on the OPs.

The OPs, which are usually seen on the leading edge of the b-wave in ERGs recorded at high light intensities, are not seen in the presence of pentobarbital (Fig.

6A). This finding agrees with the reports of others who showed that barbiturates inhibited the OPs in the ERGs of rabbit and monkey (Bornschtein et al., 1966; Honda & Nagata, 1971; Winkler, 1972; Satoh et al., 1980). However, computerized data analysis (see Section 2 for details) revealed that the OPs obtained by the filtering procedure were only reduced in amplitudes in the presence of pentobarbital ($P < 0.002$), but were not completely blocked (Fig. 6C,D). Pentobarbital not only reduces the amplitudes of the OPs, but it also changes their latencies. The overall power values (Fig. 6D), which we calculated as the mean of squared values of filtered ERG in the time frame between 1 ms after the flash and 91 ms after the flash, reveal the suppressing effect of pentobarbital on the OPs. From these results we conclude that the third order neurons were strongly suppressed by pentobarbital in the rat retina.

3.5. Effects of sodium pentobarbital on the $P_3^*(t)$ component

We have shown that pentobarbital strongly suppressed the STR (Fig. 5). Since it was shown that Müller cells rather than photoreceptors are involved in the origin of the STR (Frishman & Steinberg, 1989; Naarendorp & Sieving, 1991; Robson & Frishman, 1996), we tested whether pentobarbital has a direct effect on another ERG component. The $P_3^*(t)$ components obtained at various light intensities with and without 100 μM sodium pentobarbital are compared in Fig. 7.

There is a difference between the kinetics of the $P_3^*(t)$ components: the peaks in responses to stimuli of medium and high intensities occurred later in time in the presence of pentobarbital than in the absence of pentobarbital (Fig. 7A). On the other hand, the difference between the response-intensity functions (Fig. 7B) was not statistically significant ($0.05 < P < 0.8$). Therefore, as no effects of pentobarbital on the $P_3(t)$ component were found (Fig. 2), this kinetic change of the $P_3^*(t)$ component may be due to a direct effect of pentobarbital on Müller cells or due to a secondary effect from the changes in potassium redistribution around Müller cells caused by the effect of pentobarbital on rod synaptic terminals.

4. Discussion

We have found that 100 μM pentobarbital has multiple effects on the ERG of the isolated rat retina. It increases the amplitudes of the b-wave and the a-wave at high levels of light stimuli and decreases the b-wave amplitudes at low light stimuli. It also increases the latency of the b-wave. By dissecting the ERG into components, which belong to distinct types of retinal

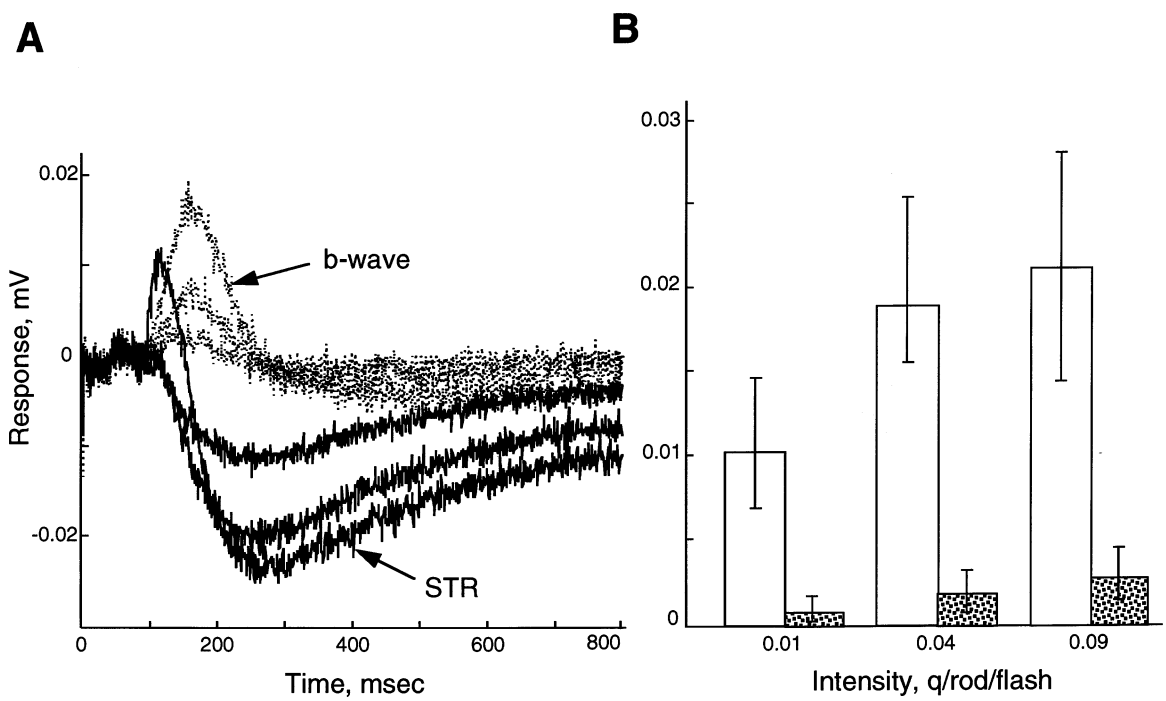

Fig. 5. Effect of 100 μM sodium pentobarbital on the scotopic threshold response (STR). (A) Transretinal ERGs in response to dim light (0.01, 0.04, 0.09 quanta/rod/flash), measured with pentobarbital (dotted line) and without pentobarbital (solid line). Responses are averaged from nine retinas. (B) Effect of 100 μM sodium pentobarbital on the amplitudes of the STRs. Open bars, amplitudes of the STRs recorded in basic Ringer at the lowest light intensities (0.01, 0.04, 0.09 quanta/rod/flash). Filled bars, amplitude of STR recorded at the same lowest light intensities in the presence of pentobarbital. Error bars are standard deviations ($n = 9$).

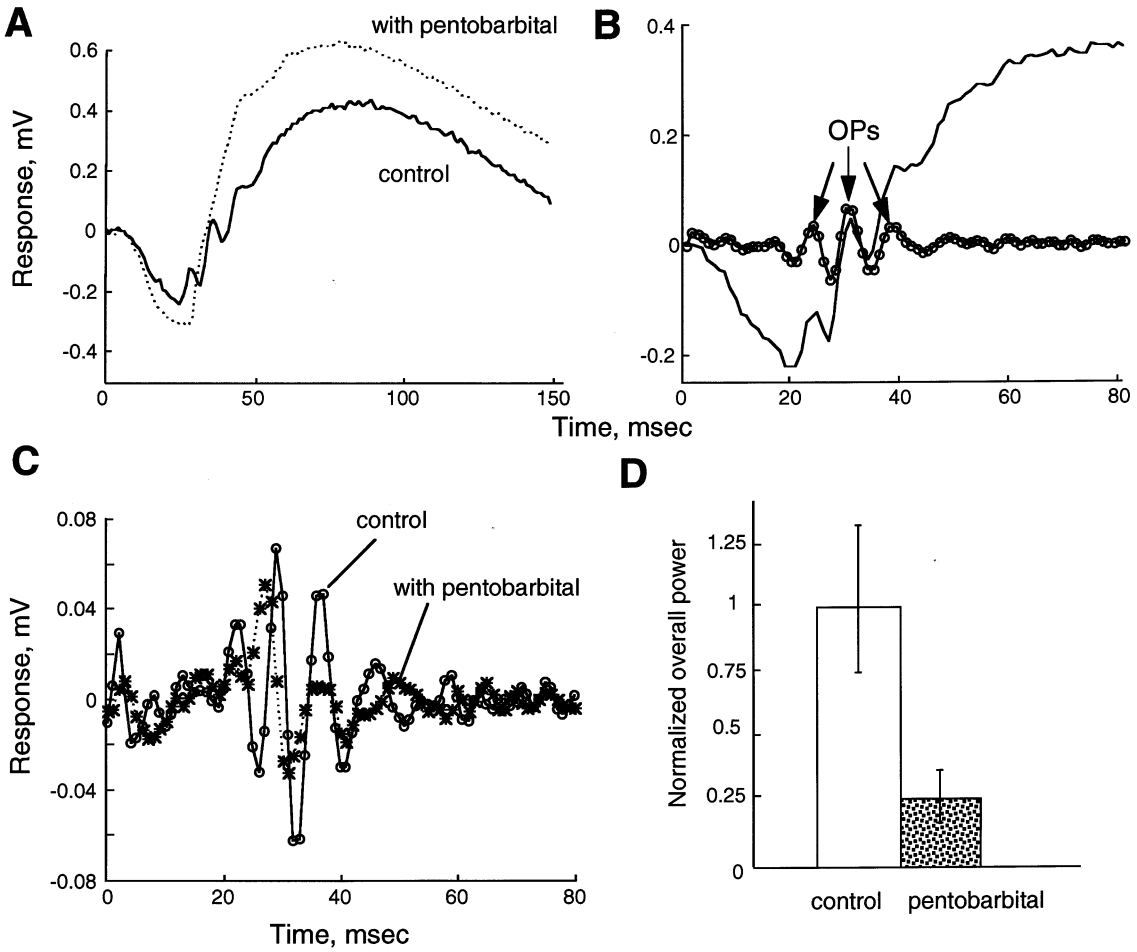

Fig. 6. Effect of 100 μM sodium pentobarbital on the oscillatory potentials (OPs). (A) Typical transretinal ERG recorded in response to a bright flash (2065.4 quanta/rod/flash), measured without pentobarbital (solid line) and with pentobarbital (dotted line). (B) Typical transretinal ERG response to a bright flash (2065.4 quanta/rod/flash) and the OPs obtained from this ERG after filtering it at 75–300 Hz. (C) The OPs in the presence of 100 μM sodium pentobarbital (*, dotted line) and in the absence of pentobarbital (●, solid line) obtained by filtering the transretinal ERG, as shown in A, at 75–300 Hz. (D) Effect of 100 μM sodium pentobarbital on the OPs isolated from ERGs recorded at the high intensity flash (2065.4 quanta/rod/flash). Open bar, overall power of the OPs, which we calculated as the mean of squared values of filtered ERG in the time frame between 1 ms after the flash and 91 ms after the flash, in basic Ringer solution. Filled bar, overall power of the OPs, obtained in the presence of pentobarbital. Error bars are standard deviations ($n = 16$).

cells, we have found that pentobarbital has specific effects on these individual components. It slows the kinetics of the $P_2(t)$ component and increases its latency. Pentobarbital slightly reduces the slow $P_3^*(t)$ component and affects its kinetics. The fast $P_3(t)$ component was not affected by pentobarbital. The minor components of ERG, the STR and the OPs, were strongly suppressed by pentobarbital. These results suggest that in rat retina sodium pentobarbital affects depolarizing bipolar cells, may affect Müller cells, does not affect the photoreceptor function, and suppresses the third order neurons.

The component analysis has shown that the $P_2(t)$ component, which we believe to originate from depolarizing bipolar cells, is one of the targets for pentobarbital action. Pentobarbital slowed the kinetics of the $P_2(t)$ component and increased its latency in the entire range of light intensities (Fig. 4). We did not find a significant increase in the amplitude of the $P_2(t)$ component in the presence of pentobarbital, but there was a tendency for the post pentobarbital responses to be larger (Fig. 4B). We did not find any effect of pentobarbital on the $P_3(t)$ component (Fig. 3). But we cannot completely exclude the photoreceptors from the list of possible targets for pentobarbital. In our experiments we were able to measure only the outer segment photocurrents and could not track possible effects of the anesthetic on the rods' synaptic terminal. Therefore the effects of pentobarbital on the $P_2(t)$ component could be either presynaptic affecting the rods' synaptic terminals, or postsynaptic directly affecting depolarizing bipolar cells.

The next possible target for pentobarbital action in the rat retina involves the third order neurons, whose activity is reflected by the minor components of the ERG—the STR and the OPs. Our experiments show that pentobar-

bital suppresses the STR and the OPs in isolated rat retina, and these effects are distinct. The STR was suppressed by a factor of six (Fig. 5), while the OPs were suppressed much less substantially (Fig. 6). This distinction is consistent with different origins of the STR and the OPs, and thus agree with earlier studies suggesting different sources for the STR and the OPs in the mammalian retina (Vaegan & Millar, 1994). As we mentioned above, Müller cells may participate in the STR origin (Sieving et al., 1986; Frishman & Steinberg, 1989). Since we have found only a slight effect of pentobarbital on the kinetics of the $P_3^*(t)$ component (Fig. 7A), pentobarbital's effects on Müller cells probably cannot explain the strong effect of pentobarbital on the STR. It seems more likely that pentobarbital has a direct effect on the inner retinal neurons. Suppression of the OPs observed in our experiments (Fig. 6) is in agreement with early reports that pentobarbital eliminated the wavelets in the mammalian ERG (Noell, 1958; Bornschein et al., 1966; Winkler, 1972; Satoh et al., 1980), although our results have shown that the OPs were only suppressed not eliminated (Fig. 6C,D). The sources of the OPs in mammalian retina are not known. Heynen et al. made a conclusion that the OPs directly originate from bipolar cells by analysis of the current profiles in the primate retina (Heynen et al., 1985). At the same time, the majority of authors agree that the OPs are not directly generated by a single group of the inner retina neurons, but rather reflect feedback from the inner retina neurons to bipolar cells (Ogden, 1973; King-Smith, Loffing & Jones, 1986; reviewed by Wachtmeister, 1987). Moreover, the individual OPs may have different origins (Wachtmeister & Dowling, 1978; Peachey, Alexander & Fishman, 1987; Tremblay & Lam, 1993; Janaky, Goupland & Benedek, 1996). Hence our results, which show that pentobarbital affects individual OPs with different strengths, may support the likelihood of a synergistic nature of OP origin.

Thus we did not find a single target for the pentobarbital action in the rat retina at the cellular level. This is not surprising, because the mechanism of action of barbiturates occurs at the molecular level and involves specific receptor (Franks & Lieb, 1994; Dilger, Liu & Vidal, 1995; Kress, 1995; Richards, 1995; Ito, Suzuki, Wellman & Ho, 1996). Pentobarbital is known as an endogenic modulator of $GABA_A$ receptors (Olsen, 1982; Ticku & Rastogi, 1986); it can also affect ionotropic glutamate (iGlu) receptors (Teichberg, Tal, Goldberg & Luini, 1984; Milkovic & MacDonald, 1986; Collins & Anson, 1987; Marszalec & Narahashi, 1993), and interact with all types of acetylcholine (ACh) receptors (Gage & McKinnon, 1985; Firestone, Sauter, Braswell & Miller, 1986; Jacobson, Pocock & Richards, 1991; Charlesworth, Jacobson, Pocock & Richards, 1992). Therefore possible targets for the action of sodium pentobarbital in the rat retina may include the cells that contain either iGlu receptors, ACh receptors, $GABA_A$ receptor, or combinations of the receptors. Immunocytochemical and electrophysiological studies on vertebrate retina have shown that bipolar cells have $GABA_A$ receptor (Grigorenko & Yeh, 1994; Pan & Lipton, 1995; Enz, Brandstätter, Wässle & Bormann,

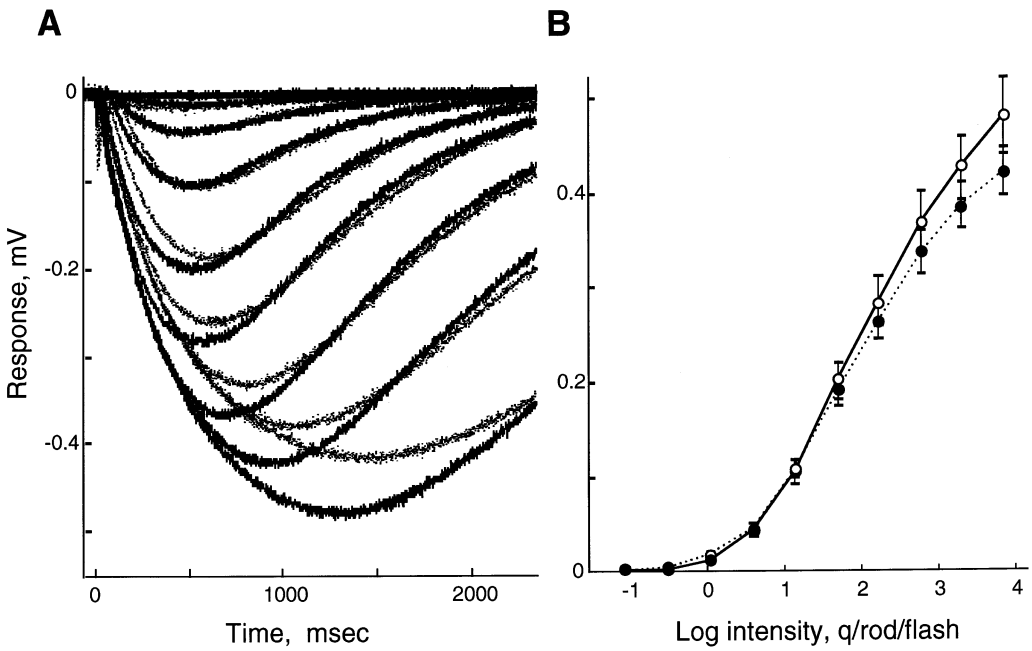

Fig. 7. Effects of 100 μM sodium pentobarbital on the $P_3^*(t)$ component. (A) Comparison of the $P_3^*(t)$ components: with pentobarbital (dotted line) and without pentobarbital (solid line). Responses are averaged from 11 retinas. (B) Amplitudes of $P_3^*(t)$ component as a function of the flash intensity: with pentobarbital (●, dotted line) and without pentobarbital (○, solid line). Error bars indicating the standard error ($n = 11$).

1996; Yeh, Grigorenko & Veruki, 1996). Third order neurons also contain all these receptor types (Lipton, 1988; Voigt, 1988; Aizenman, Loring & Lipton, 1990; Naarendorp & Sieving, 1991; Greferath, Muller, Wässle, Shivers & Seeburg, 1993; Zhang, Sucher & Lipton, 1995; Enz et al., 1996; Kittila & Massey, 1997; Brandstätter, Koulen & Wässle, 1998; Wässle, Koulen, Brandstätter, Fletcher & Becker, 1998). Therefore, one might expect sodium pentobarbital to affect all third order neurons and depolarizing bipolar cells through its action on their membranes receptors.

Our findings may provide a basis for the next step of investigation: to identify the pharmacologically distinct receptor types on membranes of depolarizing bipolar cells, Müller cells, and third order neurons, which are affected by sodium pentobarbital. To achieve this goal we plan to use specific antagonists known for each type of receptors and have already started to study the effects of $GABA_A$ and $GABA_C$ receptor antagonists on the components of ERG (Kapousta-Bruneau, 1998).

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