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Research report

Effects of xanthine derivatives on electroretinographic responsiveness

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Abstract

In view of the use of synthetic propentofylline (PPF) as a protective agent in brain ischemia, its possible side effects on vision capacities have been explored by electroretinography in comparative experiments with theophylline. We used eyecup preparations of small-spotted dogfish sharks and of European eels, particularly suitable for long-lasting experiments. The drug exerted profound but reversible modifications of ERG records: (1) a dose-dependent increase of the amplitude and duration of the chemically isolated late receptor potential (LRP), (2) a partial unmasking of LRP, (3) a strong potentiation of the LRP-unmasking effect of low temperature, (4) a potentiation of light adaptation effects, and (5) a strong potentiation of the post-illumination hyperexcitability. The effects were explicable as due to a strong phosphodiesterase (PDE) inhibiting, cyclic guanosine monophosphate (cGMP) promoting, action of the drug. The effects were considerably stronger, or even of opposite sign, in comparison to those of the chemically related theophylline. PPF did not seriously affect the ERG c-wave originating in the pigment epithelium. The results suggested that the effects of PPF on vision may not seriously hamper the therapeutic use of the drug. They indicated, on the other hand, that PPF was a retinoactive drug of potential usefulness in the exploration of the complex biochemical events underlying visual transduction.

Theme: Sensory systems

Topic: Retina and photoreceptors

Keywords: Electroretinography (ERG); Propentofylline; Theophylline; Vision; Xanthines

1. Introduction

It has been shown by Grome and Stefanovich [16] that propentofylline (PPF) can concomitantly induce an overall increase in cerebral blood flow and a generalized decrease of glucose utilization by brain tissue, thus resetting to a higher level the coupling of blood flow to energy metabolism in the brain. On the basis of this observation, a brain energy-sparing action of PPF has been envisaged, of particular value under conditions of anoxia and brain ischemia. The existence of such an action of PPF was strongly supported by our own experiments with brain synaptosomes [5]. PPF proved to be most effective in markedly reducing DNP-uncoupled oxidations of synaptosomes, particularly in association with hypothermia. It has been subsequently found that the post-ischemic administration of PPF, described as an adenosine agonist, reduces neuronal damage in gerbils, inhibits the transport of adenosine into cultured cells, inhibits free radical production by microglia cells, stimulates growth factor production, and improves glucose metabolism in all brain regions in acute stroke patients (see review article [30]). PPF acted as an adenosine uptake blocker [15], and improved in a dose-dependent manner the post-ischemic recovery in the rabbit spinal cord [12].

Xanthine derivatives, including PPF, are known for their strong phosphodiesterase (PDE)-inhibiting activity [21,29]. PPF was shown to be the best inhibitor of different PDE isoforms among a number of xanthine derivatives tested [22]. On the other hand, it is known that PDE plays a

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crucial role in molecular mechanisms of visual transduction, and that PDE-inhibitors (such as isobutyl methylxanthine, IBMX) affect profoundly the excitability of rod photoreceptors [21]. In view, therefore, of the use of PPF as a protective agent in anoxia and brain ischemia, in stroke patients, patients with dementia or organic brain disorders [23,30] its possible side effects on vision capacities due to its PDE-inhibiting properties must be seriously considered. Side effects of PPF in other organic systems have already been the subject of several reports [23,30].

The electroretinographic approach was presently chosen to check the effects of PPF on vision. Two model systems, developed in our laboratory, have been exploited: (1) the isolated eyecup preparation of the small-spotted dogfish shark (*Scylliorhinus canicula*), and (2) the in situ eyecup preparation of the immobilized European eel (*Anguilla anguilla*). Both species possess mainly rod retinas with rhodopsine as the rod pigment, characteristic also of the human retina [28]. Our preparations exhibit stable and reproducible responses to photic stimuli and are particularly suitable for a long-lasting exploration by quantitative electroretinography [3–6]. Two preparations were used in parallel, because the responsiveness to drugs may differ in different preparations. For example, the phenomenon of post-illumination excitability, first described in detail using skate retinal preparations [14], was prominent in our elasmobranch (dogfish) preparation as well, but not in our eel preparation. Besides, the two-preparation approach was considered safer when attempting to compare the results of our PPF studies to those obtained with other PDE inhibitors, in preparations of different animal origin. We used the easily prepared isolated eyecups of the dogfish, but in situ eyecups of the eel (the eel being not suitable for simple eyecup isolation). The use of the two preparations might, therefore, help in avoiding shortcomings or defective information due eventually to the isolation procedure itself. Literature data on PDE inhibitors, to which our results had to be compared, were obtained using a variety of preparation techniques (from eyecup isolation to rod impaling).

Theophylline, the ‘classical’ and naturally occurring methyl xanthine, has been shown by Grome and Stefanovich [16] to elicit effects opposite to those of PPF on cerebral blood flow and glucose utilization. In our experiments with brain synaptosomes, demonstrating a strong inhibition of uncoupled oxidations by PPF, theophylline also elicited opposite effects or remained ineffective [5]. While propentofylline, as an adenosine agonist, was found to protect against ischemia, theophylline, on the contrary, exacerbated post-ischemic nerve cell damage, acting as an adenosine receptor antagonist [32]. A number of subsequently published data pointed also to opposite effects of the two xanthines (see Ref. [15]). In the present study, the effects of PPF were again compared to those of theophylline, concerning this time the electroretinographic responsiveness.

Some of the data were the subject of previous preliminary communications and conference papers [5,6].

2. Materials and methods

2.1. Animals

Small-spotted dogfish sharks (*Scyliorhinus canicula*; 150–250 g body mass) were caught by trawler nets in the South Adriatic. They were maintained for at least 1 month prior to the experiments in a sea-water recirculation system for experimental aquaculture, located in a dark and temperature controlled room, at 15°C. At no time were the dogfishes long exposed to light as this is known to be damaging to the elasmobranch photoreceptors [17]. European eels (*Anguilla anguilla*) were electrofished during summer months in coastal waters running along Kotor Bay (Montenegro) and kept subsequently for at least 20 days prior to the experiments in fresh-water aquaria located in the same dark and temperature-controlled room (15°C) as the sea-water aquaculture system with dogfishes. Only eels of the silver developmental stage have been used. The developmental stage was identified by eye index measurements according to Pankhurst [27].

2.2. Preparations

Isolated eyecups were prepared under dim red light from dogfish eyeballs (about 10 mm in diameter) excised after rapid decapitation of the fish. The preparations were surgically deprived of cornea, lens and most of the vitreous. The eyecup was filled with elasmobranch Ringer [33] and placed on a cotton-wool bed soaked with the same solution, in a plastic temperature-controlled chamber inside a lightproof Faraday cage. After mounting, the preparations were dark adapted for additional 30 min before ERG recording. The temperature within the eyecup was measured with thermistors.

Eels were anesthetized (phenobarbital sodium) and curarized (tubocurarine) following procedures recommended by Hamasaki et al. [17] and by adjusting the dosage to arrest respiratory movements. Artificial respiration was provided continuously by forcing aerated and temperature-controlled water through the gills. The immobilized eel was positioned laterally on a plastic platform inside a lightproof Faraday cage. The in situ eyecup was prepared in the same way as in the case of isolated preparations of the dogfish (removal of cornea, lens and most of the vitreous), and it was filled with teleosts Ringer. At the conclusion of experiments, eels were killed by rapid decapitation.

In experiments with iodate, the eyecup was filled with physiological solutions in which a given amount of $NaIO_3$ was substituted for an equivalent amount of NaCl. A built-in dose-dispensing and sucking device (polyethylene

tubing) allowed for the replacement of solutions and intermittent washing without causing mechanical disturbances or changes in illumination. After 20 min, the iodate solution was washed out and replaced with the physiological medium.

Propentofylline (3-methyl-(5′oxohexyl)-7-propyl xanthine, HWA 285, Hoechst AG, Wiesbaden, Germany) was introduced into the eyecup preparation, using different concentrations of the drug (1–32 mM), a given amount of PPF being substituted for an osmotically equivalent amount of NaCl. The same substitution procedure was applied in comparative experiments with theophylline (1,3-dimethylxanthine) and aspartate (sodium-L-aspartate, 75 mM).

2.3. Electroretinography

ERG potentials were detected with nonpolarizable chlorided silver ($Ag/AgCl_2$) electrodes, the active one being introduced in the interior of the saline filled eyecup. The reference electrode was in contact with the cotton-wool bed underneath the isolated preparations of the dogfish, or in the retro-orbital space behind the in situ eyecup of the eel. It was connected to the input stage of a directly coupled differential preamplifier, and responses were recorded by means of a Polaroid camera from a storage oscilloscope display.

Photic stimuli were delivered by a dual-beam optical system using an 8-V, 50-W tungsten-halogen lamp as the light source, and providing independent control of intensity (neutral density filters), duration (electromagnetic shutter), and spectral composition (interference filters) of the test flashes. A heat filter virtually eliminated wavelengths longer than 700 nm. The stimuli consisted of single flashes guided through a fiber optic positioned normal to the surface of the eyecup that cast a circular patch of light covering the external surface of the preparation. Unless otherwise specified, the duration of the light stimulus was 200 ms, the same as in the experiments of Dowling and Ripps [13] with the eyecup preparation of the skate. Preliminary experiments showed that in the dogfish as well this duration of the light stimulus was well beyond the duration-sensitive range of the b-wave amplitude. Intervals between test flashes were kept sufficiently long so as not to influence subsequent responses. Background light was delivered through the second fiber optic, its intensity being checked by ‘physiological calibration’ (recording the amplitude of the response to the onset of the background light, and comparing it to responses of the dark-adapted preparation to light flashes of incremental intensity).

Light intensities were calibrated and checked by placing the active surface of the radiometer probe in the position usually occupied by the eyecup preparation. Non-attenuated, the energy flux delivered by the test field was of the order of 2×10^{-2} mW/cm^2. When comparing intensity/amplitude relations in different preparations, relative intensity (I_R) scales were used, plotting ERG amplitudes (voltage) against attenuation in log units.

The regularity of the intensity–amplitude relation has been checked by fitting experimental data with the basic model [13]:

$$V_o = I^a/(I_o^a + I^a)$$

where V_o is the normalized voltage (V/V_{max}) of the ERG signal (b-wave or LRP), I_o is the stimulating light intensity corresponding to $V_o = 1/2$, and exponent a is a constant. I_o is a determinant of the position of the curve on the horizontal intensity axis, while V_{max} is a measure of response compression or amplification on the vertical axis. Simple linear regression was applied to estimate log dose/response relationships (Figs. 3 and 4).

3. Results

3.1. Responses of untreated and chemically treated preparations

Fig. 1 (left panels) illustrates typical ERG records obtained from dark-adapted eyecups of the dogfish shark, untreated (uppermost record), and after treatment with sodium iodate (unmasked late receptor potential, LRP). The lower two panels depict series of records obtained by incremental stimulation before and after iodate-induced LRP unmasking (left and right panels, respectively). Typical ERG records provided by the in situ eyecup of the immobilized eel are shown on the right, again before and after iodate-induced LRP unmasking. The time course of unmasking is illustrated by the sequence of records labeled by numbers (intervals in minutes after filling the eyecup with 50 mM iodate). Log sigmoids, defining intensity–amplitude relations at the bottom of Fig. 1, show that in the dogfish (left) as well as in the eel preparation (right), iodate-unmasked LRP behaves in the same way as the b-wave amplitude of the chemically untreated preparations. The b-wave and LRP data-points are both adequately fitted by template sigmoids of practically identical slopes (the a constant), with a net lateral shift, however, of the LRP sigmoids to the right (towards higher flash intensities).

While the b- and c-wave are of extrareceptor origin (ascribed, respectively, to indirect responses of Müller glial cells, and the pigment epithelium [26,31]), the a-wave and LRP unmasked by the elimination of both positive extrareceptor components represent a direct reflection of the rod photocurrent [10]. This is why in our experiments particular attention has been devoted to the effects of PPF on the isolated LRP.

3.2. Dose-dependent effects of PPF on the chemically isolated LRP

Fig. 2 illustrates the effects of PPF on the chemically

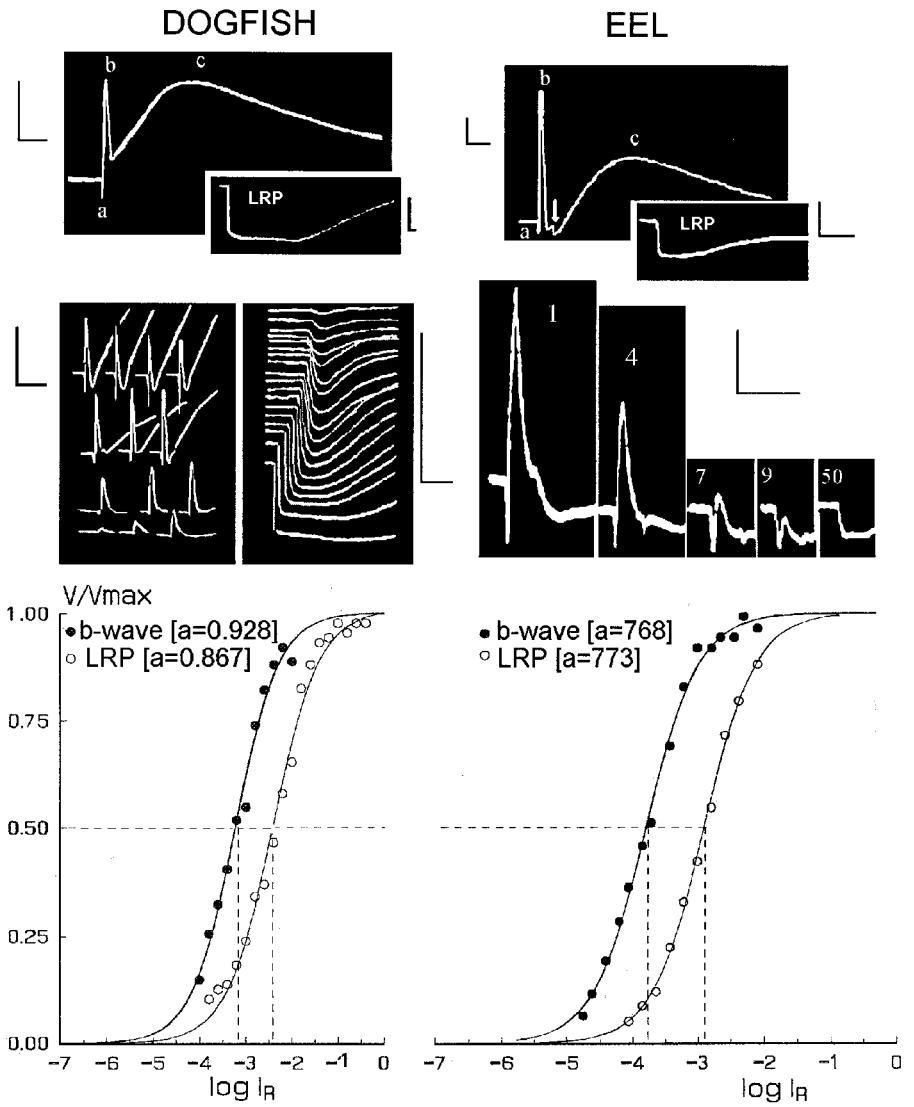

Fig. 1. Examples of ERG waveforms obtained with dogfish (left) and eel preparations (right). All calibrations: 0.1 mV, 2s. *Dogfish data*. Two upper records: normal ERG and iodate-isolated LRP, respectively. Lower two series of records: incremental stimulation (from below upwards) before and after iodate-induced LRP unmasking (left and right panels, respectively). *Eel data*. First upper record: normal ERG (1.35-s test flash; off-response indicated by arrow). Next record: iodate-unmasked LRP (0.2-s test flash). Sequence of records below: five responses during LRP unmasking by 50 mM $NaIO_3$ (1.0-s test flashes; numbers indicate minutes after intraocular administration of iodate). Fitted log sigmoids at the bottom (V/V_{max} plotted against I_R): results of incremental stimulation concerning b-wave and LRP amplitudes (closed and open circles, respectively) in dogfish (left) and eel preparations (right). Other indications in the figure.

isolated LRP of the eel. The in situ eyecup was first treated with sodium iodate and PPF was administered only after LRP was completely unmasked. The amplitude of the iodate-isolated LRP exhibited a rapid 4-fold increase. The effect was equally well expressed when using the usual short (0.2 s) and the prolonged (1.0 s) light flashes for stimulation. In both cases, the effects were spontaneously reversible: after some 10 min the LRP amplitude returned towards its initial value, and regained it after eyecup washout with regular saline.

Fig. 3 illustrates the dose dependence of the effects of PPF and theophylline on the dark-adapted and iodate-isolated LRP of the eel. The effects of the two xanthines were qualitatively similar. Obviously, however, the effects of PPF were markedly stronger. When expressed as percent increases above control values (left inset), the effects increased linearly with the logarithm of the dose, the slope concerning PPF being much steeper than the one concerning theophylline (slope values of 177 and 44, respectively).

The dose-dependence of the effect of PPF on the iodate-isolated LRP (in situ eyecup of the eel) is shown in greater detail in Fig. 4. The amplitude recorded 3 min after the administration of PPF increased linearly with the logarithm of the concentration of PPF (Fig. 4B). The effects of different PPF concentrations on the duration (width) of

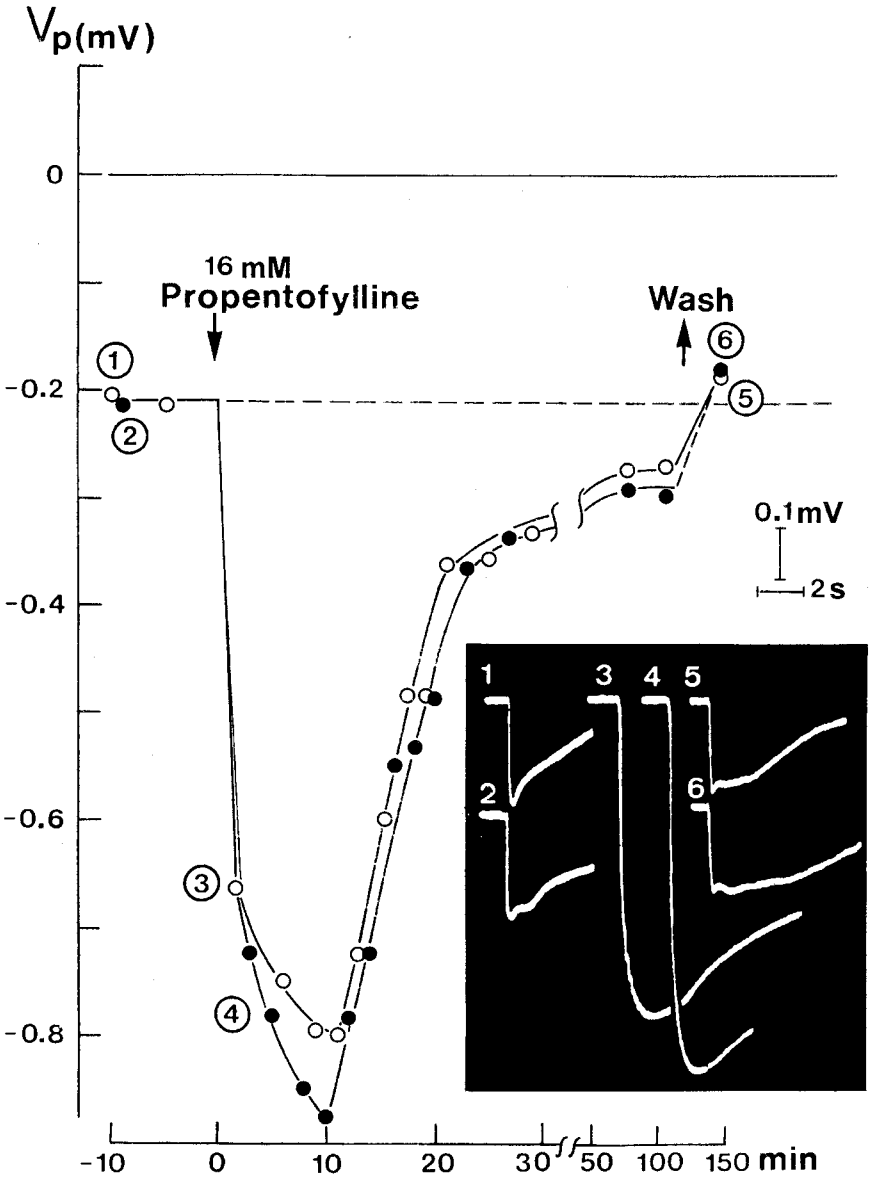

Fig. 2. Augmentative but spontaneously reversible effect of PPF on the iodate-isolated LRP of the eel. Maximal response amplitudes to light flashes of short (0.2 s) and long (1.0 s) duration (open and closed circles, respectively) at different intervals (min) after PPF (16 mM) administration at time zero. Numbered LRP records refer to equally numbered data-points in the two superimposed curves.

LRP, was evaluated using 'the time of 50% recovery', t_R, as an appropriate indicator. After rapidly reaching its maximum, t_R decreased abruptly, but reached its initial value only after washout. Peak values of t_R, the latency-to-peak and the time of recovery increased with the dose of PPF (from 0.5 to 8 mM in the figure). The reversibility of the PPF effect was remarkable.

The dependence on dose and temperature is illustrated by data in Fig. 5. All of the data shown were obtained with the same preparation, but at two temperatures: first at 15°C, and then after increasing the temperature of the preparation to 25°C. Obviously, the duration of the PPF effect shortens with increased temperature: the period of increased response amplitudes is shorter, and the recovery of initial values is speedier, particularly in case of the larger doses. Values recorded 3 min after application onset are thus substantially smaller at 25 than at 15°C, although retaining their logarithmic relationship to PPF concentrations (Fig. 5 framed inset). Responses narrow with increased temperature and the recovery of t_R becomes much speedier.

3.3. Effect of PPF in association with low temperature

PPF itself has been shown to induce hypothermia [18], and some of our previous experiments, as already mentioned in the Introduction, indicated that the protective

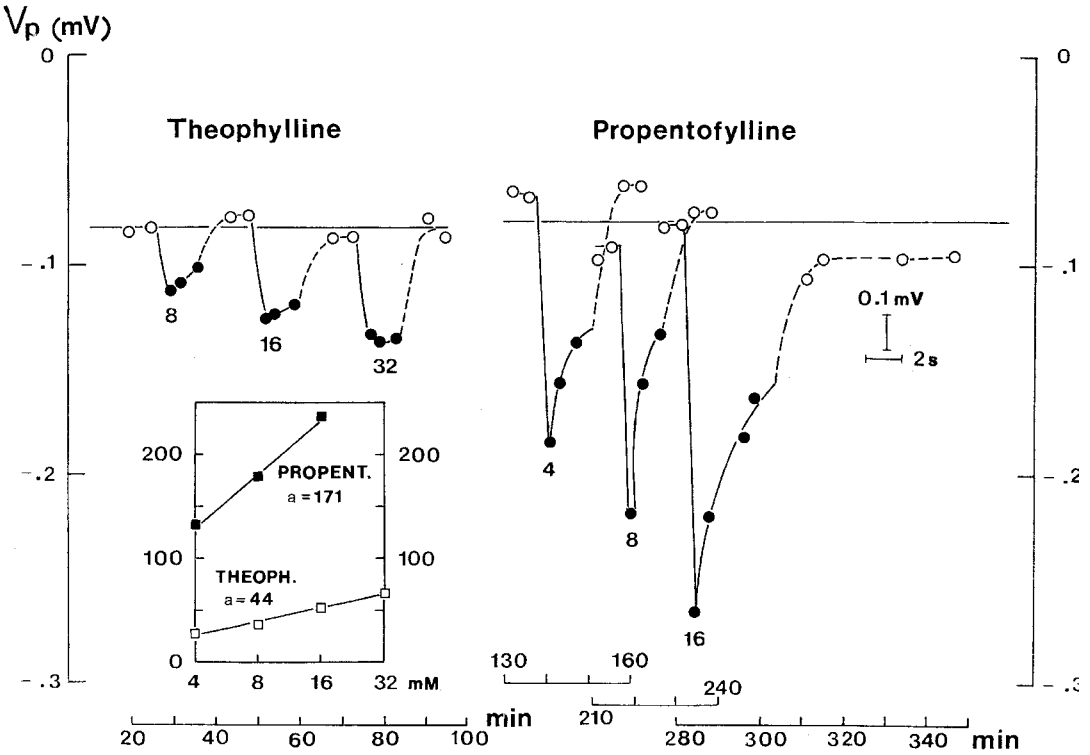

Fig. 3. Dose dependence of the effects of propentofylline and theophylline on the iodate-isolated LRP of the eel. Peak LRP amplitudes (V_p) recorded after drug administration (closed circles); drug concentrations indicated by numbers (4–32 mM). Left inset: dose dependence of the effects expressed as per cent increases above control values (a: slope value). Other indicators in the figure.

energy-sparing role of PPF in brain synaptosomes can be strongly potentiated by hypothermia [2]. In view of such a coupled application (PPF and low body temperature), our present experiments were designed to check the effects of the association of PPF and low temperature on ERG potentials.

As shown by the series of original ERG records in Fig. 6A, during cooling of the dogfish eyecup from 15°C (temperature to which the fishes were acclimated) down to 6°C, the ERG becomes gradually deprived of its most prominent positive component, the b-wave. A simplified U-shaped negative wave is left behind (third row of ERG records), testifying to a partial LRP unmasking similar to the one obtainable by the action of aspartate (b-wave eliminated, but the c-wave still present; aspartate-unmasking illustrated for comparison by the two records in Fig. 6B). Upon rewarming, the b-wave of extrareceptor origin reappears, but only at temperatures substantially higher than the temperature of their disappearance (a phenomenon of 'temperature hysteresis'). In the example illustrated in Fig. 6A, the temperature was raised from 6 to 11°C without affecting the simplified shape of the ERG profile obtained by cooling, although increasing appreciably its amplitude. The rewarmed eyecup was then filled with a 4-mM solution of PPF in elasmobranch Ringer, and a dramatic increase of the responses to light flashes soon appeared. The peak amplitudes of the responses were doubled, their duration prolonged manifold, and the latency-to-peak substantially increased.

A similar experiment, illustrated in Fig. 6C, shows that, at constant low temperature (6°C), the effect of PPF slowly increases with time, maximal amplitudes of the negative responses being obtained only some 50 min after the introduction of the drug into the eyecup. Incremental stimulation of the precooled eyecup leads to a much higher saturation level of the responses after PPF. The drug did not, however, produce a substantial lateral shift of the log V/log I profile along the log I axis: sensitivity was little affected (not shown graphically).

Fig. 6D displays ERG records obtained during a 4-h experiment with cooling and rewarming of the dogfish eyecup. Control ERG records (upper row) were obtained during the cooling phase, and PPF was introduced at the beginning of rewarming. Records in the lower row (marked HWA) were obtained after PPF and during rewarming, at exactly the same temperatures as the first two records in the upper row, obtained in the absence of PPF and during cooling. Again, the records demonstrate strong augmentative effects of the drug on LRP partially isolated by low temperature.

According to ERG criteria, therefore, the association of PPF and low temperature appears as markedly effective, in agreement with previous observations in other experimental systems (brain synaptosomes) [2].

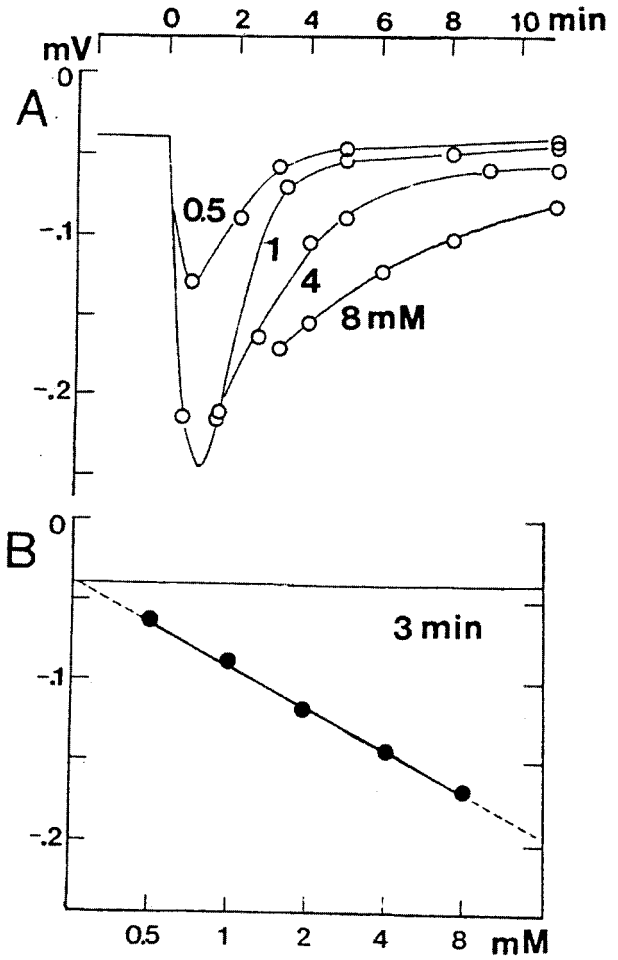

Fig. 4. Details of the dose dependence of PPF effects on the iodate-isolated LRP (eel in situ eyecup). (A) Series of superimposed LRP–amplitude profiles obtained with different PPF concentrations (0.5–8 mM, as indicated by numbers along the curves) applied at zero time. (B) Logarithmic dependence of LRP amplitude (mV), measured 3 min after the intraocular application of PPF, on the concentration of the drug applied (mM).

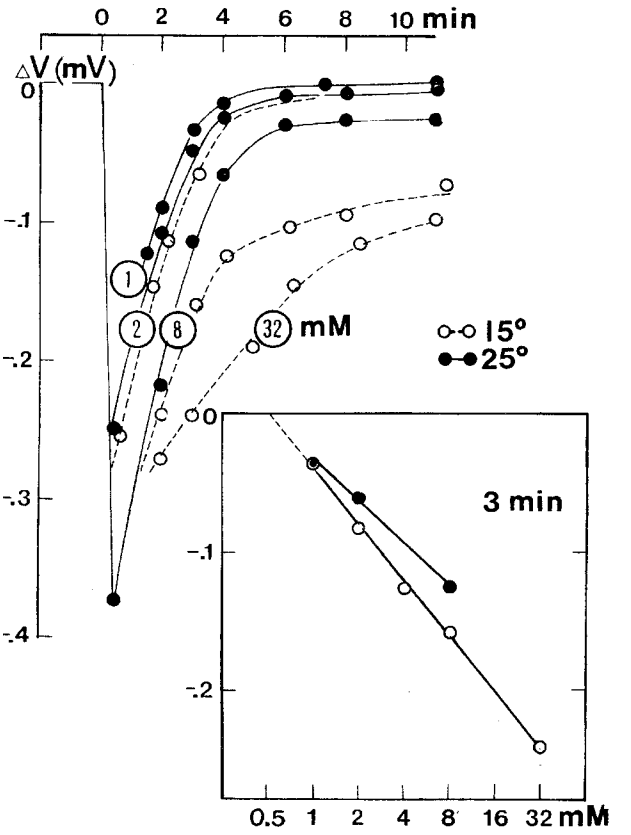

Fig. 5. Dose and temperature dependence of the PPF-induced modifications of the iodate-isolated LRP (in situ eyecup of the eel). Time course of LRP–amplitude changes after different doses of PPF applied at time zero, at 15°C (open circles, dashed lines) and after warming the preparation to 25°C (closed circles, continuous lines). Concentrations of PPF applied indicated by circled-number labels (mM) attached to the corresponding curves (2 and 8 mM labels belong to two curves each, one dashed and one continuous). Framed inset: logarithmic dependence of LRP amplitude (mV), measured 3 min after the intraocular application of PPF, on the concentration of the drug (mM) applied at 15°C (open circles) and at 25°C (closed circles).

3.4. Propentofylline versus theophylline in precooled preparations

In experiments with precooled preparations of the dogfish, the effects of PPF were compared to those of theophylline. Clearly opposite effects were observed of the sequentially added theophylline and PPF, at the level of both the amplitude and the duration of the responses: 16 mM theophylline decreased, while 16 mM PPF increased both characteristics of the responses. The differential effects of the two xanthines were also clearly expressed at the level of the $V/\log I_R$ profiles obtained by incremental stimulation of the precooled eyecup (treatment similar to the one described in the preceding paragraphs). In an experiment of the same type, the left eye preparation did not respond at all to 8 mM theophylline, while the subsequently added 8 mM PPF induced remarkable augmentative effects. When, however, the right eye preparation of the same fish was treated with increased doses of both xanthines (16 mM), a decrease of the response amplitude was recorded after theophylline, and a substantial increase above the control level in response to the subsequently added PPF.

Therefore, as far as the electroretinographic responsiveness of our dogfish eyecup preparations is concerned, theophylline remained ineffective or elicited modifications opposite to those of PPF, just as in the case of the effects of these two xanthines in other experimental systems mentioned in the Introduction.

3.5. Partial LRP unmasking by PPF itself

Fig. 7 shows the time course of ERG changes induced by injecting 16 mM PPF (at time zero) into an isolated eyecup of the dogfish. The ‘a+b’ accident (sum of the a- and b-wave amplitudes, open symbols) rapidly decreased and vanished in less than an hour. Concomitantly, the

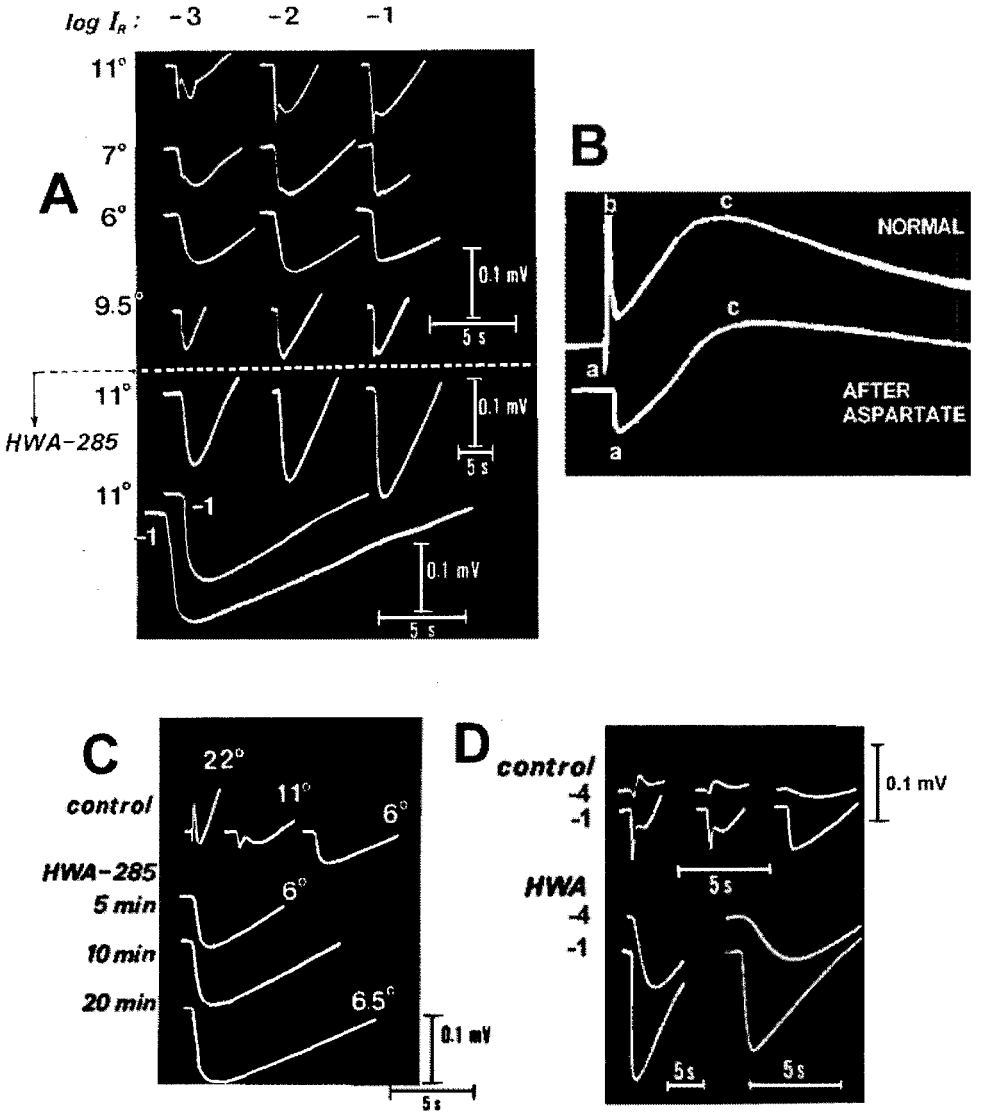

Fig. 6. Effects of the association of propentofylline and low temperature. (A) Three records, obtained in response to three flash intensities (I_R indicated by log values of the neutral density filters), are shown for each temperature level. PPF (16 mM) added after rewarming to 11°C. Detailed description in the text. (B) Effect of 50 mM sodium aspartate (partial LRP unmasking). (C) Records obtained before, and at different intervals after the application of PPF, in a dogfish eyecup pre-cooled to, and maintained at, 6°C. (D) Pairs of ERG records obtained before (control) and after PPF (HWA) at same temperatures during cooling (upper row) and rewarming (lower row) of the preparation. Each pair of records represents responses to two different flash intensities indicated by the logarithmic value of the neutral density filters. See text for detailed explanations.

amplitude of late negativity (closed symbols) increased, and after the b-notch (visible in records 2 and 3) was completely eliminated, appeared as a simplified U-shaped tracing reminiscent of LRP partially unmasked by the action of aspartate. Its amplitude continually increased to reach a maximum (record 4) after some 1.5 h after the beginning of the action of PPF. Later on, responses to light flashes became reduced in amplitude and the tracings (numbered 5 and 6) rapidly deteriorated. At 5 h (record 6) their amplitude was reduced to one third of the maximum recorded at 1.5 h, and the negative light-induced deflection was extremely slow to regain the baseline.

Fig. 8 illustrates ERG modifications elicited by four different concentrations of PPF (1.5, 4, 8, and 16 mM). The effect of each of the concentrations is represented by a series of ERG records obtained by incremental stimulation. The effect of 1.5 mM PPF was barley discernible and consisted in a slight potentiation of the late negativity at sufficiently high stimulus intensities. With increasing dose, the b-wave component was increasingly affected, becoming reduced (at 4 mM) to a notch on the descending branch of the negative deflection, and finally vanished (at 8 mM). The prominent c-wave was the last to become affected, and remained visible even in the presence of the highest PPF concentration (16 mM), in the form of a steep rising branch of the negative deflection, making thus the tracing U-shaped and comparable to the partially unmasked LRP obtainable by the action of aspartate.

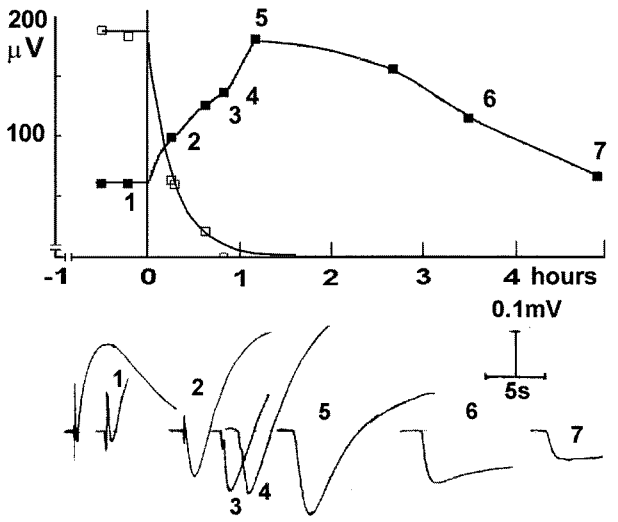

Fig. 7. Time course of the effect of 16 mM PPF (numbered ERG records obtained at instances labeled by same numbers along the LN/LRP profile).

Fig. 9 illustrates the effects of propentofylline on the electroretinogram of the chemically untreated in situ eyecup of the eel. Soon after the intraocular application of propentofylline (16 mM) the b-wave became gradually suppressed and followed by an increasing late negativity (LN). After the complete elimination of the b-wave deflection, LN became transformed into a partially unmasked LRP (record 2). It reached its maximal value of 350 mV some 9 min after the introduction of PPF into the eyecup. Thereafter, its amplitude spontaneously decreased towards its initial value, and the b-wave reappeared. In the beginning, it had the appearance of a barely visible notch on the descending branch of the tracing (record 3); its amplitude then increased and its peak started protruding from the electronegative into the electropositive area (record 4), before the tracing regained its initial complex appearance (record 5; complete recovery of amplitudes not shown). The same dose of PPF, applied some 50 min after the first application, evoked identical effects: an abrupt unmasking of the late receptor component and a marked increase of its amplitude. The PPF-induced modifications were again spontaneously reversible.

3.6. Light adaptation and hyperexcitability

The basic post-illumination hyperexcitability phenomenon is illustrated by the two control experiments (A and B) in Fig. 10a. In comparison to saturated photoresponses of the dark-adapted dogfish eyecup (solid circle at the start), after exposing the preparation to a steady background illumination the response to superimposed flashes of the same saturating intensity became substantially reduced. When, however, background illumination was extinguished, responses recorded during dark readaptation (closed circles) exceeded by far the dark-adapted maximum (lower baseline). Fig. 10b shows that propentofylline (8 mM), introduced into the eyecup during the action of background illumination, increased considerably the attenuated responses to superimposed flashes, thus mimicking, if only transiently, the effect of light adaptation. After extinguishing the background illumination, however, the phenomenon of post-illumination hyperexcitability appeared as greatly enhanced in comparison to both control experiments in Fig. 10a: responses reached much higher levels, and their decay during dark readaptation was considerably retarded.

The two consecutive experiments with one and the same preparation, illustrated in Fig. 10c, show that the same type of hyperexcitability appeared during short (1 min) interruptions of background illumination, and that it increased with the duration of light adaptation. As shown by the first (control) experiment, flashes delivered during each of the consecutive 1-min intervals of darkness elicited responses of steadily increasing voltage (solid circles, dashed line), greatly exceeding the control, dark-adapted level. On the other hand, flashes delivered during intervals of continuous background illumination provoked greatly depressed responses (open circles), but tending to recover and testifying therefore to progressing light adaptation. After definitely discontinuing the intermittent light regime, dark readaptation was followed by an exponential return of the grossly augmented responses to their initial pre-exposure level (the lower baseline). The procedure was then repeated, but this time propentofylline was introduced into the eyecup (arrow) during the phase of adaptation to intermittent background illumination. The effect consisted in a transient potentiation of light adaptation (open circles, continuous line), accompanied by a further abrupt increase of hyperexcitability, recorded during the intermittent 1-min intervals of darkness (solid circles, dashed curve). Again, the end of exposure to intermittent background illumination was followed by a rapid decrease of the responses, although after propentofylline the return to the initial level was retarded, response amplitudes tending to level out at a considerably higher level than in the preceding control test.

Obviously, therefore, the dogfish eyecup preparations exhibited pronounced light adaptation and post-illumination hyperexcitability, both phenomena being strongly potentiated by propentofylline.

4. Discussion

In our experiments propentofylline was capable of inducing profound but spontaneously reversible modifications of the electroretinogram, a complex light-evoked global potential generated by the retina. A reversible and dose-dependent increase of the amplitude of the chemically isolated late receptor potential (LRP), a greatly increased latency to peak and the prolonged overall duration of the responses to light flashes were among the most prominent

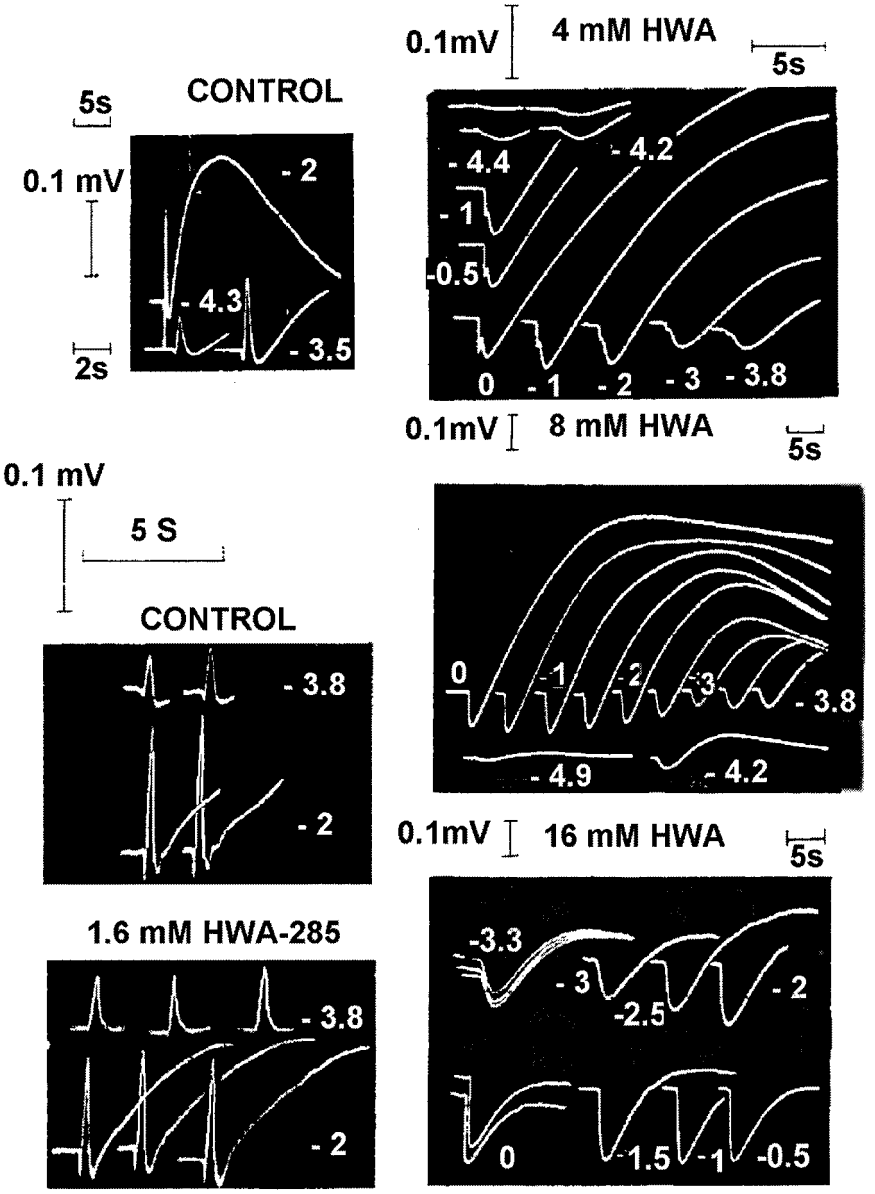

Fig. 8. Effects of four PPF concentrations, illustrated by series of records obtained by incremental stimulation (log values of neutral density filters as indicated). Other indications in the figure. Detailed description in the text.

PPF effects. Besides, PPF exerted a strong potentiation of the LRP-unmasking effect of low temperature, and was itself capable of producing a reversible partial unmasking of LRP, similar to the one obtainable by the action of aspartate. Propentofylline was shown to antagonize the attenuating effect of a steady background illumination and to enable greater responses to superimposed flashes, thus mimicking the effects of light adaptation. PPF was also shown to greatly enhance the phenomenon of hyperexcitability following the interruption of a background illumination. In comparison to the action of theophylline explored in parallel, the PPF effects were substantially stronger or even of opposite sign.

According to still prevailing theories of the molecular mechanism of visual transduction (for review, see Ref. [35]), the light induced closing of cationic channels in the plasma membrane of rod outer segments, and the resulting late receptor potential (LRP), are due to the hydrolysis of cGMP which is catalyzed by a photoactivated phosphodiesterase (PDE). PDE inhibitors should therefore modify the reactivity of rods to photostimulation. In 1972, Zaret [38] found indeed that the aspartate-isolated PIII component of the frog ERG (comparable to a partially unmasked LRP) increases in amplitude and broadens in shape after caffeine and aminophylline. In subsequent experiments with rods and rod membranes of the toad, PDE-inhibitors strongly influenced the light evoked responses, determining in particular a considerable increase of the late receptor potential [11,20,39].

All our results are explicable as due to induced modi-

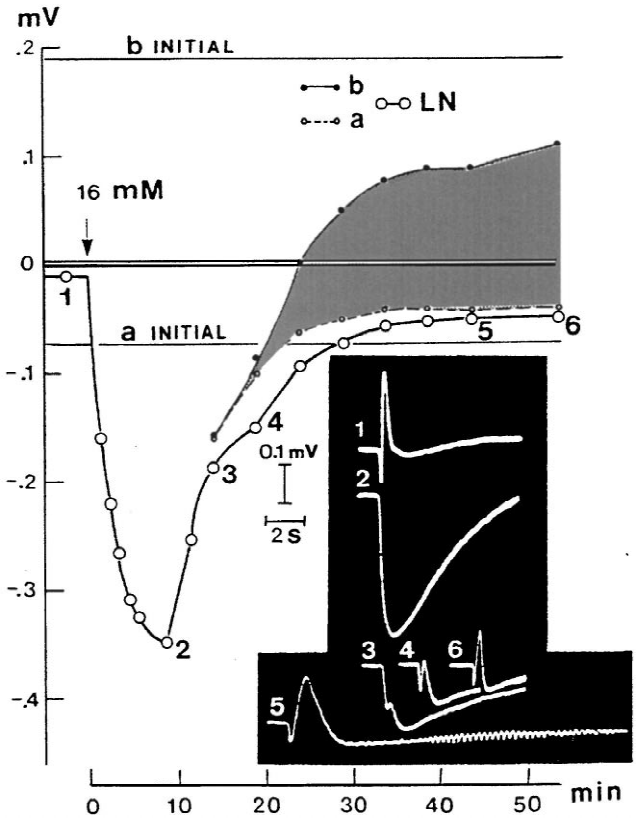

Fig. 9. Spontaneously reversible LRP unmasking by 16 mM propentofylline (in situ eyecup of the eel; 15°C). Initial (pre-treatment) levels of b- and a-wave amplitudes (peak distances from zero baseline) indicated by correspondingly labeled horizontals. Large open circles, continuous line: late negativity (LN, amplitude of the negative deflection following the b-wave) and the partially isolated late receptor potential (LRP, amplitude of the negative deflection in the absence of the b-wave); small open circles, dashed line: a-wave amplitude; dots, plain line: b-wave amplitude. Shaded area: distance between a- and b-wave peaks during recovery. The numbered ERG records were obtained at instances labeled by same numbers along the LN/LRP profile. Record 5: time base increased 4-fold to make discernible the oscillatory potentials (ripples) appearing regularly on the rising branch of the LN tracings.

fications of the kinetics of cyclic GMP formation or destruction. By favoring, for instance, cGMP formation (by inhibiting PDE), propentofylline would increase the concentration of cGMP in the dark-adapted state, thus opening additional cationic channels and further depolarizing the outer segment membrane. This would actually be the cause of the increased amplitudes of the subsequent hyperpolarizing LRP responses to light flashes recorded in our experiments with PPF: starting from a substantially higher depolarization level, the light-induced hyperpolarization would result in a correspondingly larger negative deflection of the ERG tracing. This would also explain the greatly increased latency-to-peak and the prolonged overall duration of the responses.

By acting as a cGMP promoter, PPF may also act as a blocker of events at stages later than photoreceptors. In the continuous presence of cGMP, the light-sensitive channels would be kept permanently open, maintaining a continuous flooding of bipolar cells with glutamate, thus preventing them to respond to light (as if permanently in the dark), so that no voltage change is induced in them and consequently in Müller cells responsible for b-wave generation by the light flash. Only the photoreceptors would react, thus producing unmasked LRP (which itself slowly deteriorates). Experiments with a direct administration of PPF through microdialysis probes into brain tissue (hippocampus) showed that the drug does not alter the release of excitatory amino acids (glutamate) during ischemia and reperfusion [18]. It should be mentioned, in connection with the PPF-induced elimination of the b-wave, that a direct effect of PPF on glial cells other than Müller glial cells has been described (suppression of the microglial production of reactive oxygen intermediates [7]; inhibition of the proliferation of cultured microglial cells [25]). On the other hand, it is known that excessive activation of glutamate receptors in the retina may lead to excitotoxic effects through an increase in intracellular calcium concentration [1]. Whether this kind of effects is operative during the supposed PPF-induced glutamate flooding of bipolar cells, remains to be elucidated. In any case, the effects of PPF proved in our experiments to be spontaneously reversible. On-bipolar cells possess glutamate APB receptors and a cGMP cascade generates on-bipolar cell responses. It is now known that much of the b-wave originates ultimately from on-bipolar cells and a contribution of on-bipolar cells to the mammalian electroretinogram has been described [19]. Inhibition of PDE in the bipolar cells might, therefore, account for part of the b-wave effects observed in our experiments with fish preparations as well. The action of PPF, mimicking the effect of light at the level of on-bipolar cells, would result in a decrease of the positive response of on-bipolar cells to light, and consequently a decrease in b-wave amplitude. In the presence of sufficient PPF concentrations, the b-wave would disappear altogether, as it was the case in some of our experiments (Fig. 8).

Experiments designed in the past to investigate the effect of PDE inhibitors on intracellularly explored rods are highly relevant to the interpretation of our results and deserve particular attention. For example, the PPF-induced huge but reversible increase of the amplitude of the chemically isolated LRP reminds of similar increases elicited in impaled toad rods by the action of IBMX, renown for its PDE-inhibiting properties, alone or in combination with cGMP [21]. Responses of 42 mV in amplitude, 3.6 times greater than the control amplitude, were described as larger than any receptor responses ever recorded in the vertebrate retina. The increase in amplitude of the rod responses to IBMX was accompanied by a prolongation in response time course, and this was also the case in our whole-eyecup ERG experiments with PPF. The amplitude of the ‘rod mass receptor potential’, recorded in the aspartate treated isolated retina of the frog (equivalent

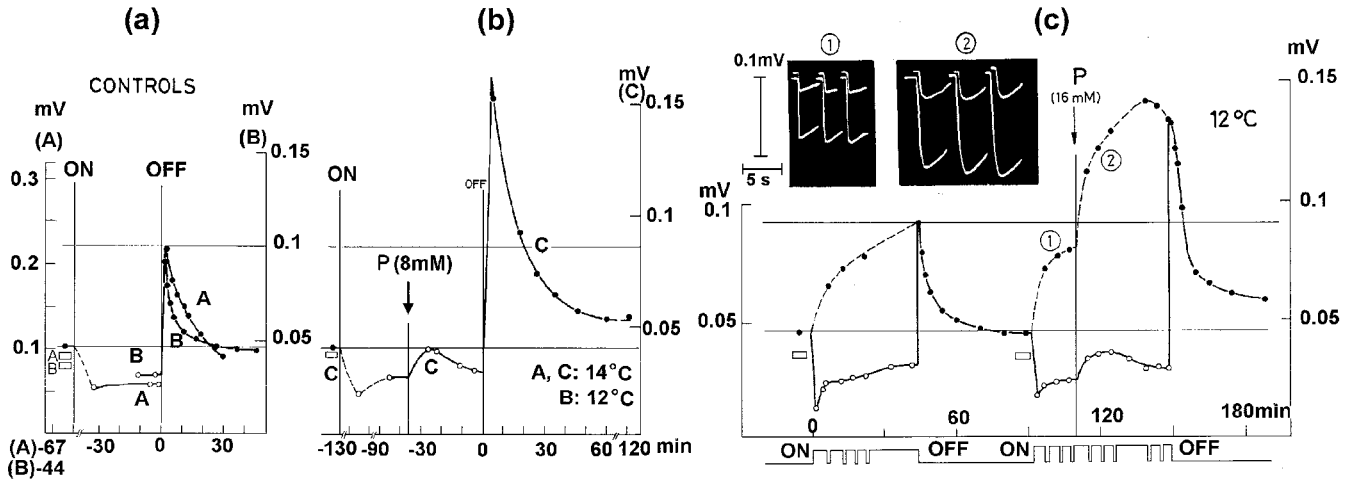

Fig. 10. Light adaptation and post-illumination hyperexcitability (isolated dogfish eyecup). (a) Two control experiments (A and B; clear bars: potential elicited at the onset of the background light; note calibration differences between the mV ordinates referring to A and B). (b) A similar experiment (C) with the addition of propentofylline (P 8 mM, framed). Amplitudes (mV) of the negative deflections during background illumination (turned off at $t=0$), and in its absence indicated by open and closed circles, respectively. (c) Hyperexcitability within intermittent 1-min intervals of darkness, its increase during adaptation to background light, and its decay during dark readaptation (light marker at the bottom). Propentofylline added during the repeated experiment with the same preparation. The two sets of record pairs: tracings obtained before and during intermittent dark intervals (upper and lower members of each pair, respectively); sets 1 and 2 refer to data-points indicated by same (circled) numbers along the curve in the main panel. Other symbols same as in (b).

to the partially aspartate-isolated LRP in our experiments), exhibited also a huge, 5-fold increase after IBMX, accompanied by an increase of the latency-to-peak and a shift of sensitivity towards lower flash intensities [9].

Similarly to our findings in experiments with PPF, in the experiments with impaled rods [21] the effect of IBMX was time and dose-dependent. The beginning of the increase of response amplitudes after IBMX coincided with the beginning of rod depolarization. Their amplitudes reached, however, a maximum only at about 4 min, and the increase in response amplitude (ΔV) was, within experimental error, equal in absolute magnitude to the depolarization of the plasma membrane. At later times, however, the extent of depolarization was greater than the increase in response amplitude. With prolonged exposure to IBMX the rods lost responsiveness: response amplitudes diminished and sensitivity decreased. In detached frog retinas, the aspartate-isolated LRP also rapidly decreased after a huge initial increase induced by high-dose IBMX [9]. Such a time-dependent behavior resembles the time course of events observed in our own experiments (Fig. 7): in the dogfish eyecup, after a sufficiently long time (although after considerably longer delays than in the previously mentioned experiments) the effect of 16 mM propentofylline ended in rapidly deteriorating late receptor potentials of markedly reduced amplitudes. The drug then effectively antagonized the effect of light stimuli (see also Fig. 4 in Ref. [5]). With prolonged action, therefore, and particularly after large doses, the depolarizing effect of PPF would approach limits incompatible with excitability, just as it was the case with the prolonged action of IBMX in the experiments of the above mentioned authors.

As to the dose-dependent effects of PPF described in our present study, it should be recalled that in other systems effects of PPF were not dose-dependent, while in still others the dose-dependence was well expressed. This was taken as pointing to regional and/or species differences of propentofylline action [12]. Doses of PDE inhibitors (IBMX) shown to substantially depolarize the membrane of isolated rods and induce markedly augmented LRP responses to subsequent light flashes were often appreciably lower than those used in our whole-eyecup experiments (1–32 mM). For example, concentrations of IBMX equal to, or smaller than 0.5 mM, were applied to isolated *Bufo bufo* rods [11]. It should be reminded, however, that in contrast to the isolated rod preparations used by the quoted authors, we dealt with whole-eye preparations, supplied moreover by a continuous circulation of oxygenated blood in the case of the in situ eyecup of the eel. Even in some of the experiments with impaled cells (rods of *Bufo marinus* [21]), a concentration of 5 mM IBMX was applied, comparable to PPF concentrations used in our experiments.

After short-term application, the effect of IBMX in toad rods was of transient nature: the $V-\log I$ curve recorded after 12 min and washout perfectly matched the control curve. In some experiments, however, recovery of the IBMX treated rods was observed only after a 15-min washout [21]. A complete recovery after PPF was also observed in our whole-eyecup preparations. In the eel in situ eyecup preparation the recovery proceeded to a great extent even before washout. Even the small-size oscillatory potentials (ripples) were discernible on the recovery tracings (Fig. 9). This pre-washing recovery might have been due to drug removal and/or metabolic degradation, since in these preparations circulation of oxygenated blood was not interrupted. In the completely isolated dogfish prepara-

tions, however, recovery was only partial even after extensive washouts.

It is known that rods are capable of pooling their responses, this capacity being particularly effective at dim intensities [14]. It contributed apparently to the responses recorded from toad rods in the experiments of Lipton et al. [20]. To what extent this contributed to the large, 4-fold increase of LRP amplitudes by PPF in our experiments remains unknown.

In our experiments with background illumination, saturation plateaus of the partially isolated LRP were appreciably higher after PPF, just as it was the case in the dark-adapted state. As estimated by I_o values, however, the sensitivity was somewhat reduced after PPF. It was shown in experiments with *Bufo bufo* rods, that background illumination hyperpolarized the membrane potential by 17 mV, while IBMX depolarized the dark-adapted resting potential by 15–17 mV [11]. In the experiments with impaled *Bufo marinus* rods, IBMX was shown to increase, under the light-adapted conditions tested, the response amplitudes relative to those recorded from rods in control Ringer's, similarly to what was observed in the dark-adapted state. In the latter experiments, however, IBMX virtually did not shift the curve on the intensity axis: sensitivity was nearly identical in the partially light-adapted rod whether the rod was in control or in the IBMX containing Ringer's [21]. The authors underlined, however, that sensitivity, according to the I_o criterion, was not affected by IBMX addition that lasted up to 6 min. Differences, therefore, between our and Lipton's experiments as far as drug-induced sensitivity changes are concerned may be due to different degrees of bleaching achieved in our and Lipton's experiments and/or to different effects of the duration of the PDE-inhibiting drug action.

The capacity of PPF to further increase the light adapted level of response amplitudes to superimposed flashes during background illumination (Fig. 10), may be regarded as PPF's capacity to antagonize the effects of bright light and to mimic light adaptation. In the experiments with isolated rods, 'mimicking light adaptation ' was also mentioned as an effect of PDE inhibition by IBMX, since it was found that the PDE inhibitor opposed the effect of background illumination. A competition between light and IBMX on the activity of PDE was taken as responsible, since light activates the PDE and IBMX inhibits the enzyme [20]. From experiments with detached frog retinas and the aspartate-isolated LRP, it was also concluded that the effects of natural light adaptation compete with those of PDE inhibitors [9]. Obviously, our results point to a similar competition between light and PPF.

According to Baylor [8], light adaptation prevents moderate background light from closing all the cGMP-gated channels, which would defeat the cell's ability to register changes in light intensity. Light adaptation is mediated by a light-induced fall in $[Ca^{2+}]_i$ that speeds the recovery of the response to a flash presented in darkness. $[Ca^{2+}]_i$ falls because closure of the channel blocks Ca^{2+} influx while extrusion by the Na/Ca-K exchanger in the rod outer segment continues. The fall in $[Ca^{2+}]_i$ antagonizes the light-induced closure of channels by action at several sites, through various effects that oppose channel closure and tend to reopen channels. These effects include cyclase activation, a lowering of the PDE activation gain, and an increase of channel affinity for cGMP. Recent evidence suggests, however, that the Ca^{2+} effect on light-evoked PDE activation is most important in producing adaptation [8]. According to this data, PPF would facilitate light adaptation by enhancing, through its PDE-inhibiting action, the effect of the lowering of $[Ca^{2+}]_i$ induced by background illumination. Whether, in addition, PPF produces its effect on light adaptation by enhancing the activity of guanylate cyclase in rods through mechanisms described in other systems [37], remains to be elucidated.

The phenomenon of post-illumination hyperexcitability is likewise attributable to an enhancement of the kinetics of cGMP formation due to light adaptation. It was particularly well described in an ERG study of the isolated retina of the skate [13]. The beginning of dark readaptation of a light-adapted retina, or a retina previously exposed to a step of (unbleaching) light, is characterized by a transient and rapidly decaying state of hyperexcitability: responses to saturating flashes are greatly increased in amplitude and greatly exceed the dark-adapted maximum. The level of cGMP recovers rapidly and overshoots its dark-adapted (resting) level, thus producing a depolarization (greater number of open, deblocked Na^+-channels, in the outer segment plasma membrane). In our present experiments with another elasmobranch, the dogfish shark, the phenomenon of post-illumination hyperexcitability was even better expressed and PPF was shown to strongly enhance this phenomenon. It is reasonable to ascribe the underlying cGMP promoting action of PPF to its known PDE-inhibiting properties.

In comparison to PPF, theophylline was found in our experiments to remain without any effect, to elicit weaker effects, or to provoke even effects of opposite sign. Opposite effects were particularly well expressed in experiments with dogfish eyecups, in which theophylline was shown to decrease, and PPF to increase the amplitude of LRP. A similar effect of theophylline (decrease of the amplitude of the aspartate-isolated LRP) was previously recorded in experiments with detached frog retinas [34]. Opposite effects observed in some of our experiments with theophylline and PPF are reminiscent of a number of findings obtained by comparing the effects of the two xanthines in other test systems. In the pioneering findings by Grome and Stefanovich [16] opposite effects of theophylline and PPF were demonstrated on cerebral blood flow and glucose utilization. In our earlier experiments with brain synaptosomes, revealing a strong inhibition of uncoupled oxidations by PPF, theophylline elicited also

opposite effects or remained ineffective [2]. While propentofylline, as an adenosine agonist, was found to protect against ischemia, theophylline exacerbated post-ischemic nerve cell damage, acting as an adenosine receptor antagonist [32]. PPF altered the pattern of purine release in ischemia (increasing specifically adenosine), while theophylline increased indiscriminately the release of all purines [15].

In the presently described experiments, the difference between the two xanthines was remarkable and similar to differences established in other experimental paradigms where they were due to the differential influence of the two xanthines on the adenosine system. Indications have been obtained in the past, in other systems, that xanthines might enhance the activity of guanylate cyclase by competing with adenylate cyclase for adenosine receptors [37]. Increased intracellular levels of cGMP have indeed been found after treatment with PPF [36]. Since guanylate cyclase is the enzyme responsible for cyclic GMP generation in rods, the possibility should be left open that propentofylline, besides inhibiting PDE, may also produce its effect in the retina, particularly at high concentrations, by enhancing the activity of guanylate cyclase in rod outer segments. One should have in mind, however, that PPF was shown to have minimal effects on adenosine mechanisms under basal physiological conditions [30].

Rod Na^+–K^+–ATPase may represent another possible site of PPF action, since the drug was shown to modulate the activity of this widely spread membrane-bound enzyme (to counteract, namely, the reduction of its activity in the postischemic brain [24]). In the dark adapted rods, Na^+–K^+–ATPase is responsible for the constant removal of Na^+ ions entering through cationic channels, and thus for the maintenance of the 'dark current'. Weather the opening of additional channels through PPF-induced PDE inhibition, and the resulting increase of rod membrane depolarization, are accompanied by a PPF-induced modification of Na^+–K^+–ATPase activity, remains to be elucidated.

It has been known for a long time now that elevated eye temperature greatly shortens the time of exposure required to produce a damaging effect of light and that exposure to light is particularly damaging to the elasmobranch photoreceptors [17]. Warming of the dogfish eyecup preparation to 25°C (a temperature close to the upper tolerance limit of the animal according to our unpublished observations) resulted in a shortening of the PPF effect, a narrowing of responses, and a speedier recovery, particularly in case of the larger doses (Fig. 5). Cooling, on the other hand, greatly prolonged responses to flashes delivered in the presence of PPF and potentiated its effects. The association of PPF and low temperature was particularly effective in modifying the electroretinographic responsiveness, reminding of the effectiveness of this association in depressing uncoupled oxidations in the brain synaptosome system [2].

The effects described here for PPF were fully reversible: even before washout, ERG records from in situ preparations of the eel regained almost completely their pretreatment appearance. The LRP-unmaskig effect of PPF was only partial, without eliminating the c-wave component. PPF did not, therefore, appear to seriously affect pigment epithelium cells responsible for c-wave generation. The results suggest, therefore, that PPF's effects on vision may not seriously hamper the therapeutic use of the drug. On the other hand, the capacity of PPF to partially unmask LRP, to antagonize the effects of bright light stimuli and to mimic light adaptation makes this compound an interesting retinoactive drug, potentially useful as a tool for the exploration of the complex biochemical events underlying visual transduction.

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