RESEARCH PAPER

Acta Neurobiol Exp 2020, 80: 192–204
DOI: 10.21307/ane-2020-018

since 1928 ACTA NEUROBIOLOGIÆ EXPERIMENTALIS

Effects of HCN channel blockade on the intensity-response function of electroretinographic ON and OFF responses in dark adapted frogs

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Hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels are well expressed in the vertebrate retina. Their role in formation of electroretinographic (ERG) responses to stimulus onset (b-wave) and stimulus offset (d-wave) are largely unknown. In this study we investigated the effects of pharmacological blockade of HCN channels (with ZD7288 or ivabradine) on the ERG b- and d-waves in dark adapted frog eyecup preparations. Initially, the dose-response relationship of ZD7288 effects on the b- and d-waves was investigated. Afterwards, the effects of 75 μM ZD7288 on the stimulus - response function of the ERG b- and d-waves were explored over a wide intensity range (10 log units). Finally, the effects of 30 μM ivabradine on the same function were studied. Perfusion with 75 μM ZD7288 did not change the absolute and relative sensitivity of the ERG ON and OFF responses. It caused an enhancement of the d-wave amplitude at all suprathreshold stimulus intensities, while the b-wave amplitude was slightly enhanced only in the range of higher intensities. As a result of the greater blocker effect on the OFF response amplitude, the b/d amplitude ratio was significantly decreased over the whole intensity range. ZD7288 caused a prolongation of the b-wave half-width duration, but a shortening of the d-wave half-width duration at higher intensities. Similar results were obtained when 30 μM ivabradine was used for HCN channel blockade. Our results clearly demonstrate that the blockade of retinal HCN channels changes the balance between the ON and OFF responses in the distal frog retina. This ON/OFF imbalance may be one of the causes for visual disturbances reported in ivabradine treated patients.

Key words: electroretinogram, HCN channel, ZD7288, ivabradine, retina

INTRODUCTION

Hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels are cation channels that open when the membrane potential is hyperpolarized. The HCN family consists of four isoforms (HCN1–4), which possess different kinetics, voltage-dependence and modulation by cyclic nucleotides (for review: Sartiani et al., 2017). Expression of all isoforms has been demonstrated in the vertebrate retina: tiger salamander (Barrow and Wu, 2009), rat (Müller et al., 2003; Ivanova and Müller, 2006; Fyk-Kolodziej and Pourcho, 2007; Stradleigh et al., 2011), mouse (Koizumi et al., 2004; Cangiano et al., 2007; Knop et al., 2008; Della Santina et al., 2012; Pan et al., 2014), rabbit (Müller et al., 2003; Kim et al., 2003). An unanswered question in retinal physiology is how HCN channels participate in generation of responses to stimulus onset (ON) and stimulus offset (OFF) in the distal retina.

One of the easiest ways to investigate electrophysiologically the global distal retinal function is by recording electroretinogram (ERG). The most prominent ERG components in response to long-lasting stimuli are the b-wave (ON response) and the d-wave (OFF response). In the ERGs obtained with brief (flash) stimuli, the OFF response is small and merges with the preced-

Received 26 December 2019, accepted 26 March 2020

ing ON-response. The role of the HCN channels in generation of ERG responses to stepped light stimuli has been mainly assessed by studying the effects of pharmacological blockade or genetic ablation of HCN channels on the flash ERGs. Contradictory results, however, have been reported in these studies. Some authors reported that the HCN channel blockade (with zetabradine or Cs^+) slightly reduced the amplitude and delayed the latency and implicit time of the b-wave to mesopic light stimuli in dark adapted cats (Gargini et al., 1999). Other authors (Della Santina, 2009; Della Santina et al., 2010) observed that the acute blocker application (ivabradine) in dark adapted rodents (mice and rats) caused a slight enhancement of the b-wave amplitude, accompanied with unaltered sensitivity and implicit time, but delayed decay phase of b-wave to dim and intermediate stimuli. The effects of genetic HCN channel manipulation on the flash ERG depends on the isoform manipulated. In $HCN2^{-/-}$ mice, a prolongation of the b-wave implicit time and decay phase was observed in dark-adapted responses to dim light stimuli. The amplitude of the responses was not significantly changed, although a tendency for its enhancement could be seen (Della Santina et al., 2012; Fig. 5C). In $HCN1^{-/-}$ animals, the dark adapted b-wave implicit time remained unaltered, while a prolonged response recovery was demonstrated at all intensities with the effect being most pronounced at higher intensities (Della Santina, 2009; Della Santina et al., 2012). The amplitude of the responses was not significantly altered, although a tendency for its diminution could be seen (Della Santina et al., 2012, Fig. 5B).

It has been shown that the effects of HCN channel ablation on the flash stepped ERG may depend on the state of light adaptation. Lei et al. (2007) reported that the flash b-wave amplitude was increased in dark adapted $HCN1^{-/-}$ mice, but it was greatly diminished in conditions of light adaptation. Reduced photopic b-wave amplitude in $HCN1^{-/-}$ mice was demonstrated also by Knop et al. (2008), while the scotopic b-wave amplitude remained unaltered in this study. The duration of both scotopic and photopic ERG responses was strikingly prolonged. Considerable prolongation of the b-wave to trains of mesopic stimuli with low temporal frequency was reported in $HCN1^{-/-}$ mice by Seeliger et al. (2011). The response amplitude to the lowest frequency stimuli (0.5 Hz) was unchanged, but it decreased dramatically with increasing frequency and essentially no responses were observable above 5 Hz. Because the cone system dominates the responses to stimuli higher than 3 Hz, the authors concluded that HCN1 channels have an important role in mesopic vision by preventing saturation of the pathways receiving inputs from the rod system. In none of the papers cited one could find any information concerning the role of HCN channels in the OFF response generation in distal retina, which is reflected in the ERG d-wave.

In this study we investigated the effects of pharmacological blockade of HCN channels (with ZD7288 or ivabradine) on the ERG ON and OFF responses, obtained with a wide range of stimulus intensities in dark adapted frog eyecups. Both blockers inhibit all isoforms of HCN channels, although in a different manner. ZD7288 is an open-state blocker of HCN channels (Shin et al., 2001; Wu et al., 2012), while ivabradine is a closed-channel blocker of HCN1 channels and an open-channel blocker of HCN4 channels (Bucci et al., 2006).

METHODS

Subjects and drug application

The experiments were carried out on frog eyecup preparations (n=67). The frogs (*Rana ridibunda*) of either sex were supplied by a licensed supplier and were bred locally in the vivarium of the Medical University of Sofia. They were anesthetized with Tricaine (Sigma) dissolved in the bathing water to a concentration of 500 mg/l and then decapitated and pithed. All procedures performed in the study were approved by the Committee for ethics in scientific research of Medical University of Sofia and the experiments were allowed by the Bulgarian Food Safety Agency (license No 242).

The eyecup preparations were placed in a chamber, where they were continuously superfused with Ringer solution (NaCl 110 mM, KCl 2.6 mM, $NaHCO_3$ 10 mM, $CaCl_2$ 1.6 mM, $MgCl_2$ 0.8 mM, Glucose 2 mM; HCl 0.5 mM to adjust pH to 7.8) at a rate of 1.6-1.8 ml/min, temperature 18-20°C and supplied with moistened O_2 (for details see Kupenova et al., 2017; Popova and Kupenova, 2011; 2013). The HCN channels were blocked by using ZD7288 (Tocris Bioscience) or Ivabradine hydrochloride (Tocris Bioscience). Concentrations of 5, 50, 75 and 100 μM ZD7288 were tested in this study similar to other authors working on retinal preparations: 1-5 μM (Cangiano et al., 2007; Della Santina, 2009), 30 μM (Chen and Yang, 2007), 50 μM (Cui et al., 2017; Barrow and Wu, 2009; Ma et al., 2003; Vinberg et al., 2009), 100 μM (Hellmer et al., 2016, Lee and Ishida, 2007; Yang et al., 2013, Van Hook and Berson, 2010; Satoh and Yamada, 2000). We chose concentration of 75 μM ZD7288, because it had saturating effect for both the b- and d-wave amplitude changes. Ivabradine was used in a concentration of 30 μM on the basis of our preliminary experiments, where concentrations of 3, 30 and 60 μM were tested in accordance with other retinal studies: 3 μM (Della Santina et al., 2012; Bemme et al., 2017), 30 μM (Demontis et al., 2009).

Experimental procedure and groups

The animals were dark adapted for 24 h prior to the beginning of the experiments and then the eyecups were prepared under dim red light. The eyecups were rhythmically stimulated with diffuse white light stimuli (150 W tungsten halogen lamp) with 5 s ON and 25 s OFF periods presented in the dark. The maximal stimulus intensity (log I=0) was 6×10^8 quanta s^{-1} μm^{-2} at the plane of the retina.

Two main groups of experiments were performed. In the first group light stimuli with constant intensity (log I=-6) were used throughout the experiments. The rhythmic light stimulation began after an additional dark adaptation period of the eyecups for 10 minutes. In the control experiments of this group (n=8), the eyecups were perfused with Ringer solution throughout the entire course of the experiment (22 min). In the test experiments (n=30), the eyecups were perfused with Ringer solution in the first 6 min (control period) and afterwards with solution containing ZD7288. In a subgroup of experiments, the dose-response relationship of ZD7288 effects on the b- and d-wave amplitude was evaluated. One or two concentrations of ZD7288 were tested in each eyecup. In a few cases, all four concentrations were applied in an ascending manner to the eyecup. In another subgroup of experiments, the dynamics of 75 μM ZD7288 effects on the b- and d-wave amplitude was investigated.

In the second main group of experiments, light stimuli with increasing intensity over a range of 10 log units were applied in the dark in order to investigate the stimulus-response function of the ERG b- and d-waves. The rhythmic light stimulation began after an additional adaptation of the eyecups to the dark for 30 minutes. The very wide intensity range of the test stimuli allowed us to obtain rod-dominated responses by using lower intensity stimuli (log I≤-8), mixed rod-cone responses by using middle intensity stimuli (-8<log I<-5) and cone-dominated responses by using higher intensity stimuli (log I ≥-5). In each eyecup, two V – log I functions were obtained, which were separated by a 30 min adaptation period (without rhythmic stimulation). In the control experiments (n=11), both functions were investigated during Ringer solution perfusion. In the test experiments, the first V - log I function (control) was obtained during Ringer solution perfusion and the second one (test) - during perfusion with 75 μM ZD7288 (n=11) or 30 μM ivabradine (n=7).

ERG recording and data analysis

The electroretinograms were recorded by means of non-polarized Ag/AgCl electrodes at a bandpass of 0.1 - 1000 Hz and digitized at 2 kHz (Biopac system MP 150, Biopac Systems, Inc., 42 Aero Camino, Goleta, California 93117, USA, AcqKnowledge 4.3.1 software). The amplitude of the b-wave was measured from the peak of the a-wave to the peak of the b-wave, while that of the d-wave was measured from the baseline to the peak of the wave. The latency of the ERG waves was measured from stimulus onset (for b-wave) or offset (for d-wave) to the beginning of the wave, while their implicit time was measured from stimulus onset (for b-wave) or offset (for d-wave) to the peak of the wave. The half-width duration of the responses was determined from the time when the amplitude rise to half of its peak to the time when the amplitude decayed to half of the peak. In the first main group of experiments, the amplitudes of the ERG waves were normalized to the values obtained in the last minute of the control period. This was done for both the control and test experiments in order to follow the time course of the amplitude changes. In order to more precisely evaluate the changes in the waveform of the ERG responses, 5 consecutive responses were averaged during the initial control period and during the period of stable ZD7288 effect (or the corresponding time period in the control group). The latency, implicit time and half-width duration of the ERG b- and d-waves were measured from the averaged responses. The amplitude of the averaged responses was used for construction of the dose-response relationship for the b- and d-waves. Because of the small a-wave amplitude, it was measured from the averaged responses only.

In the second main group of experiments, the amplitude of the responses to stimuli of different intensities was used for V – log I function evaluation. For assessment of the relative amplitude change at each stimulus intensity, the values obtained in the second intensity series were normalized to the values obtained in the first series. This was done for both the control and test experiments. Similar normalization was performed for the values of the b/d amplitude ratio in the control and test experiments. The b-wave V - log I function was approximated by a generalized form of the Naka-Rushton equation: $V=V_{max} \times I^n / (I_\sigma^n + I^n)$, where V, amplitude of the ERG b-wave; V_{max}, b-wave maximum amplitude; I, stimulus intensity above the background; I_σ, stimulus intensity at half-maximum amplitude; n, an exponent, related to the steepness of the V - log I function (Naka and Rushton, 1966). The value of I_σ was used as an index of the response relative sensitivity, while the threshold intensity eliciting 5 μV criterion amplitude was used as an index of the response absolute sensitivity. The V - log I function of the d-wave was estimated by smoothing the experimental data using inductive algorithm for smooth approximation of data (Kupenova, 2011). The threshold intensity (5 μV criterion response amplitude), V_{max} and I, producing 0.5 V_{max} (I_σ), were derived

from the approximating curves. The a-wave amplitudes were measured only in the higher intensity range (log I≥-5), where an accurate measurement of single responses was possible (without their averaging).

For statistical evaluation of the data, the Student's t-test, one-way ANOVA and two-way ANOVA with Bonferroni *post hoc* test were used (OriginPro 2019 (v.9.6), OriginLab Corporation, Northhampton, MA). A p value <0.05 was considered significant.

RESULTS

Dose-response relationship of ZD7288 effects

The perfusion with 5 μM ZD7288 caused no significant changes of the b-wave amplitude in comparison with the corresponding values obtained in the control experiments (Fig. 1A). On the other hand, a small, but statistically significant increase of the d-wave was evident (t-test, P<0.0222) (Fig. 1B). A progressive enhancement of the d-wave amplitude and a small enhancement of the b-wave amplitude were seen with increasing ZD7288 concentration to 50 μM and 75 μM. Concentration of 100 μM ZD7288 caused no additional enhancement of the b- and d-wave amplitudes (Fig. 1A-C). From the dose-response relationship was evident that concentration of 75 μM ZD7288 was a saturating one for the effects of the blocker on the b- and d-wave amplitude. That is why we chose this concentration to investigate the dynamics of the blocker effects on the ERG waves and afterwards its effects on the intensity-response function of the ERG ON and OFF responses.

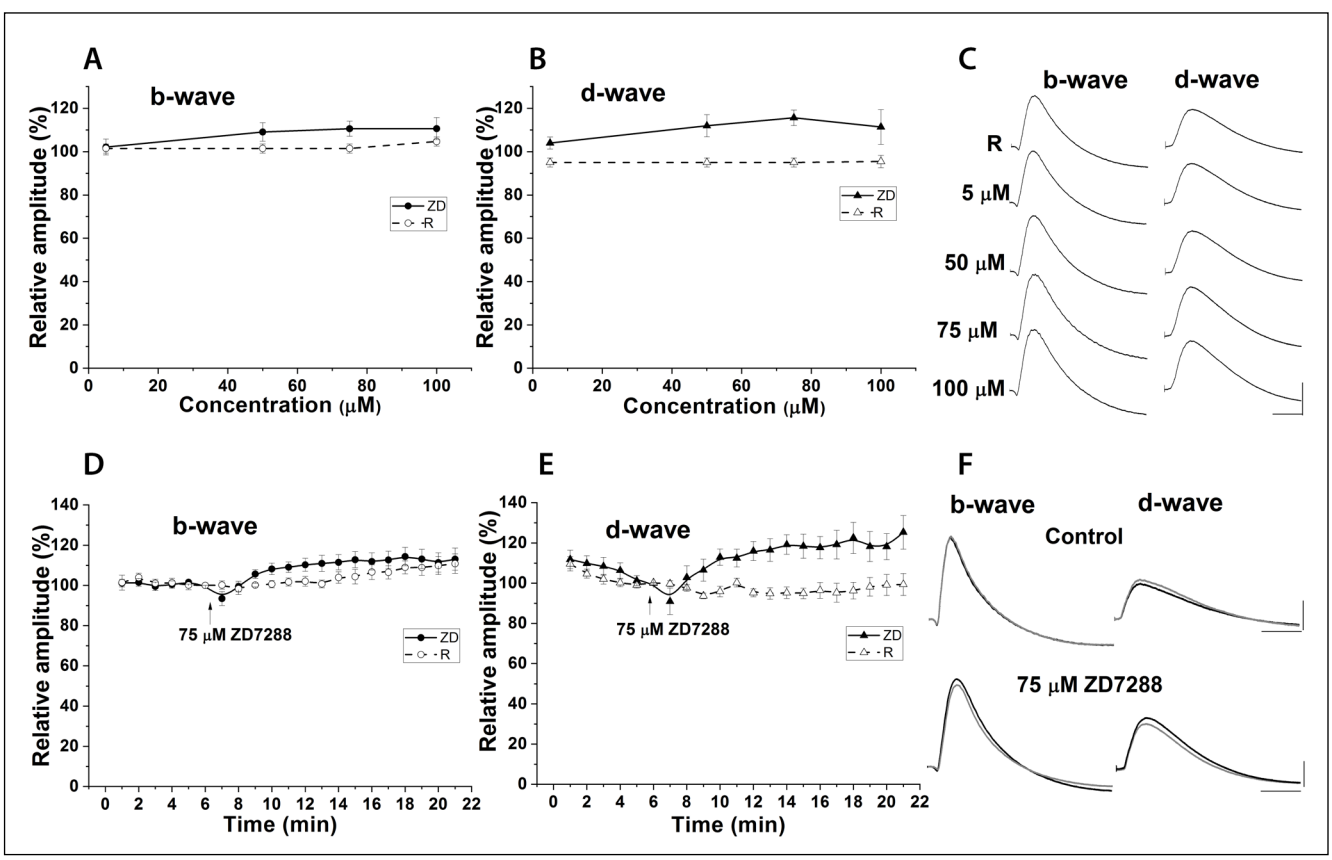

Fig. 1. (A), (B) Dose-response relationship of ZD7288 effects on the b-and d-wave amplitude. The amplitudes of the ERG waves during treatment with 4 different concentrations of ZD7288 (5 μM, 50 μM, 75 μM and 100 μM) are normalized to the values in the control period (6th minute from beginning of the experiment). Each point represents mean ± SEM of the averaged amplitude values (n=5) obtained during maximal expression of ZD7288 effect (filled symbols) or corresponding time periods in control experiments (open symbols). (C) Original ERG records obtained during the control period (R) and during application of four different concentrations of ZD7288 in one and the same eyecup. Calibration: time – 0.4 s; amplitude – 100 μV. (D), (E) Time course of the effects of 75 μM ZD7288 on the amplitudes of the ERG b- and d-waves. The amplitudes of the ERG waves were normalized to the values obtained in the 6th minute from experiment beginning. Results of both test experiments (field symbols; n=12) and control experiments (open symbols; n=8) are represented. Mean values ± SEM are shown. (F) Original ERG records of a control and a test (75 μM ZD7288) experiment. The curves recorded during the control period (grey lines) and the period of maximal ZD7288 effect or corresponding time interval in control experiment (black lines) are superimposed. The beginning of the records coincides with the stimulus onset (b-wave) or offset (d-wave). Calibration: time – 0.4 s; amplitude – 100 μV.

Dynamics of the ZD7288 effects

These experiments were carried out in order to evaluate the time course of the blocker effects on the ERG waves. In the control experiments, the d-wave amplitude showed a slight tendency for diminution through the course of the experiment, while the b-wave amplitude was relatively stable with exception for a slight increase at the end of the experiment (Fig. 1D-F). These amplitude changes were probably due to sensitivity changes, developing during the course of intermittent light stimulation. Perfusion with 75 μM ZD7288 caused a small increase of the b-wave amplitude and a greater increase of the d-wave amplitude (Fig. 1D, E). The effect developed rapidly and reached a plateau at the 5th minute from the beginning of the blocker application. During the plateau period (from the 12th to 21st minute, timing from the beginning of the experiment) the amplitudes of the b- and d-waves were significantly higher than those obtained in the control experiments (two-way ANOVA, $F_{1,199}$=9.46, P<0.00244 for b-wave; $F_{1,199}$=34.88, P<0.00001 for d-wave). From Fig. 1D, E is seen that the difference in amplitude values between test and control experiments was greater for the d- than b-wave. The stability of the blocker effect on the b- and d-wave amplitude was confirmed by comparison of the mean normalized amplitude values obtained at different time points during the plateau period. No significant differences between these values were found with the Bonferroni *post hoc test* and One-way repeated measures ANOVA with Greenhouse-Geisser correction ($F_{2.46,27.06}$=1.13, P>0.05 for b-wave; $F_{2.64,29.03}$=0.94, P>0.05 for d-wave). This allowed us to obtain the V – log I function of the ERG waves in the second main group of experiments during the period of constant ZD7288 effect. Perfusion with 75 μM ZD7288 did not cause any significant changes in the b-and d-wave time characteristics (Fig. 1C, F, lower row). The values of the b-wave latency (88±3.67 ms), implicit time (238±11.35 ms) and half-with duration (357±13.33 ms) during the blocker application were practically the same as those obtained during the control period (latency: 86±3.62 ms; implicit time: 241±11.18 ms; half-width duration: 359±12.34 ms). Similar results were obtained for the OFF response. The d-wave latency (71±5.94 ms), implicit time (321±17.90 ms) and half-width duration (687±57.56 ms) during ZD7288 treatment did not differ significantly from the control values of latency (70±6.27 ms), implicit time (299±12.54 ms) and half-width duration (660±57.56 ms). The a-wave amplitude during the blocker application (9.52±2.04 μV) did not differ significantly from the value obtained in the control period (7.69±1.13 μV).

Intensity-response function of control experiments

In the control experiments, there were some differences between the V – log I function obtained with the first and second intensity series. The changes in the amplitudes of the responses to lower (log I≤-8), middle (8<log I<-5) and higher (log I≥-5) intensities were separately evaluated. The amplitudes of the b- and d-waves to lower intensity stimuli were not significantly changed (Fig. 2A, B, D). The absolute sensitivity of the ERG ON and OFF responses (determined by their thresholds) was practically the same in both intensity series (Table I). The same was true for their relative sensitivity (determined by I_{σ} value) (Table I). The b-wave amplitude was unaltered also in the range of middle intensities, while that of the d-wave showed some small diminution (non-significant but with p value close to 0.05; two-way ANOVA, $F_{1,109}$=3.62, P=0.05978). As a result of this tendency, the b/d amplitude ratio was significantly increased in the middle intensity range (two-way ANOVA, $F_{1,109}$=11.89, P=0.00043) (Fig. 2C). In the higher intensity range, an amplitude decrease of the a-, b- and d-waves waves was observed during the second intensity series (two-way ANOVA, $F_{1,131}$=50.53, P<0.00001 for a-wave; $F_{1,131}$=28.02, P<0.00001 for b-wave; $F_{1,131}$=27.96, P<0.00001 for d-wave) (Fig. 2A, B). This amplitude decline was probably produced by the adaptational effect of the test stimulation itself and it was also seen in other ERG recordings in dark adapted frog eyecups (Popova and Kupenova, 2016; Kupenova et al., 2017). Because the d-wave amplitude diminished to a greater extent than that of the b-wave (Fig. 2A, B), the b/d amplitude ratio was significantly increased in the higher intensity range (two-way ANOVA, $F_{1,131}$=8.21, P<0.00491) (Fig. 2C). The waveform of the responses during the second intensity series did not differ from that during the first series (Fig. 2D). The amplitude decline in the higher intensity range was not accompanied with changes of latency and half-width duration of the ERG b- and d-wave. This was true for the eyecups, where the amplitude changes were minimal (Fig. 2D) as well as for the eyecups, where the amplitude decrease was well seen (Fig. 2E). The d-wave implicit time, however, was delayed in this intensity range (at log I=-3.5, first series: 163±14.72 ms; second series: 193±16.41 ms; P<0.0151).

Effects of ZD7288 on the intensity-response function

Perfusion with 75 μM ZD7288 caused no significant changes of the b-wave V – log I function in comparison

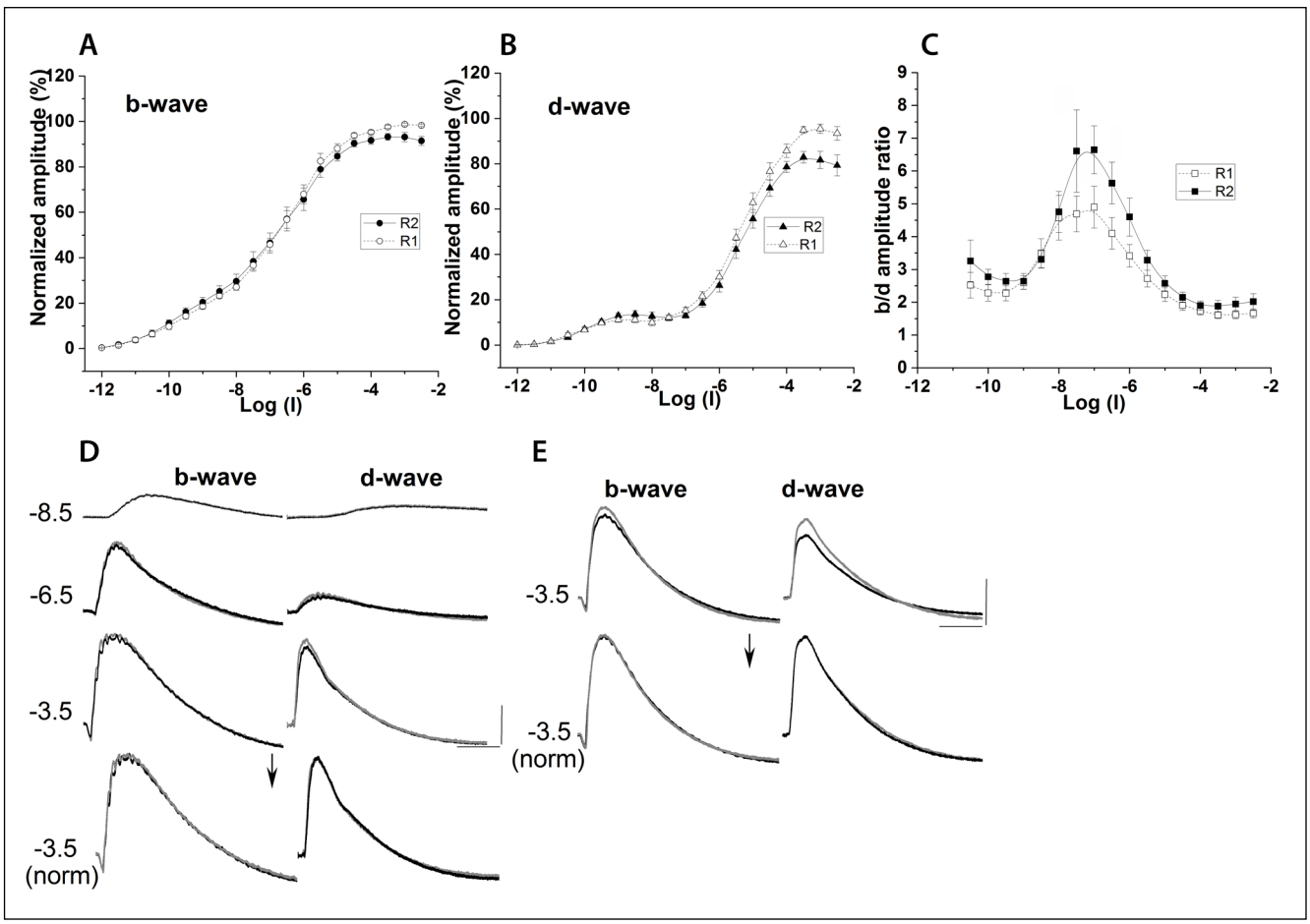

Fig. 2. (A), (B) V – log I function of the b- and d-waves obtained in the control experiments. The symbols, representing the responses obtained during the first (R1) and second (R2) intensity series, are denoted in the legends. The amplitudes of the ERG waves were normalized to V_{max} of the responses obtained in the control (first) V – log I function in each eyecup. Mean values ± SEM are shown (n=11). (C) Values of the b/d amplitude ratio during the first (R1) and second (R2) intensity series in the control experiments. Mean values ± SEM are shown. (D), (E) Original ERG records obtained in eyecups perfused with Ringer solution only. The curves recorded during the first (grey lines) and second (black lines) intensity series are superimposed in the same way as in Fig. 1F. Numbers on the left of the curves represent log I. Calibration: time – 0.4 s; amplitude – 100 μV in (D), 200 μV in (E). The arrows point to curves obtained with log I=-3.5, which were normalized to their maximal values. This way of representation allows for measuring the half-width duration of the ERG waves.

Table I. Absolute and relative sensitivity of the b- and d-wave V – log I function.

Influence	Threshold (logI)		I_σ (logI)	
ERG wave	I series	II series	I series	II series
Controls				
b-wave	-11.34±0.20	-11.28±0.20	-7.02±0.14	-7.09±0.27
d-wave	-10.67±0.20	-10.73±0.18	-5.37±0.16	-5.43±0.17
75 μM ZD7288				
b-wave	-11.27±0.22	-11.44±0.20	-6.96±0.13	-7.16±0.15
d-wave	-10.51±0.15	-10.67±0.26	-5.43±0.12	-5.40±0.09
30 μM ivabradine				
b-wave	-11.36±0.19	-11.60±0.15	6.90±0.18	-7.05±0.20
d-wave	-10.67±0.17	-10.99±0.18	5.29±0.16	-5 36±0.13

The threshold and I_σ values (mean ± SEM) during the first and second intensity series obtained in the control experiments (n=11) and during HCN channel blockade with 75 μM ZD7288 (n=11) and 30 μM ivabradine (n=7) are represented.

with the control one (Fig. 3A). The absolute and relative sensitivity of the ON response were similar in both intensity series (Table I). Because nonspecific changes of the response amplitude were observed in the control experiments, we compared the relative b-wave amplitude change in the test and control experiments in the three intensity ranges (Fig. 3C). A statistically significant difference was obtained only in the range of higher intensities (two-way ANOVA, $F_{1,131}$=4.2893, P<0.0406), where the amplitudes of the b-wave during ZD7288 treatment were relatively higher compared to those in the control experiments. The same was true for the relative change of the a-wave amplitude in the higher intensity range, where the amplitudes obtained during the blocker application were relatively higher compared to those in the control experiments (two-way ANOVA, $F_{1,131}$=22.49, P<0.00001) (Fig. 3C, inset). The small changes of the cone-dominated b-wave amplitude were accompanied with increased half-width duration of the response. This was evident in the eyecups, where the b-wave amplitude was not enhanced during the second series (Fig. 4B) as well as in the eyecups, where it was enhanced (Fig. 4C). The half-width duration (at log I=-3.5) during ZD7288 treatment (668±39.11 ms) was significantly longer (paired t-test, P<0.0231) than that in the first series (608±47.78 ms). Because the implicit time during the blocker application (239±12.50 ms) was not increased (control: 249±13.73 ms), it was evident that the prolongation of the half-width duration was due to a delayed decay phase of the response. No similar changes in the b-wave waveform were seen in the lower and middle intensity range (Fig. 4B, C).

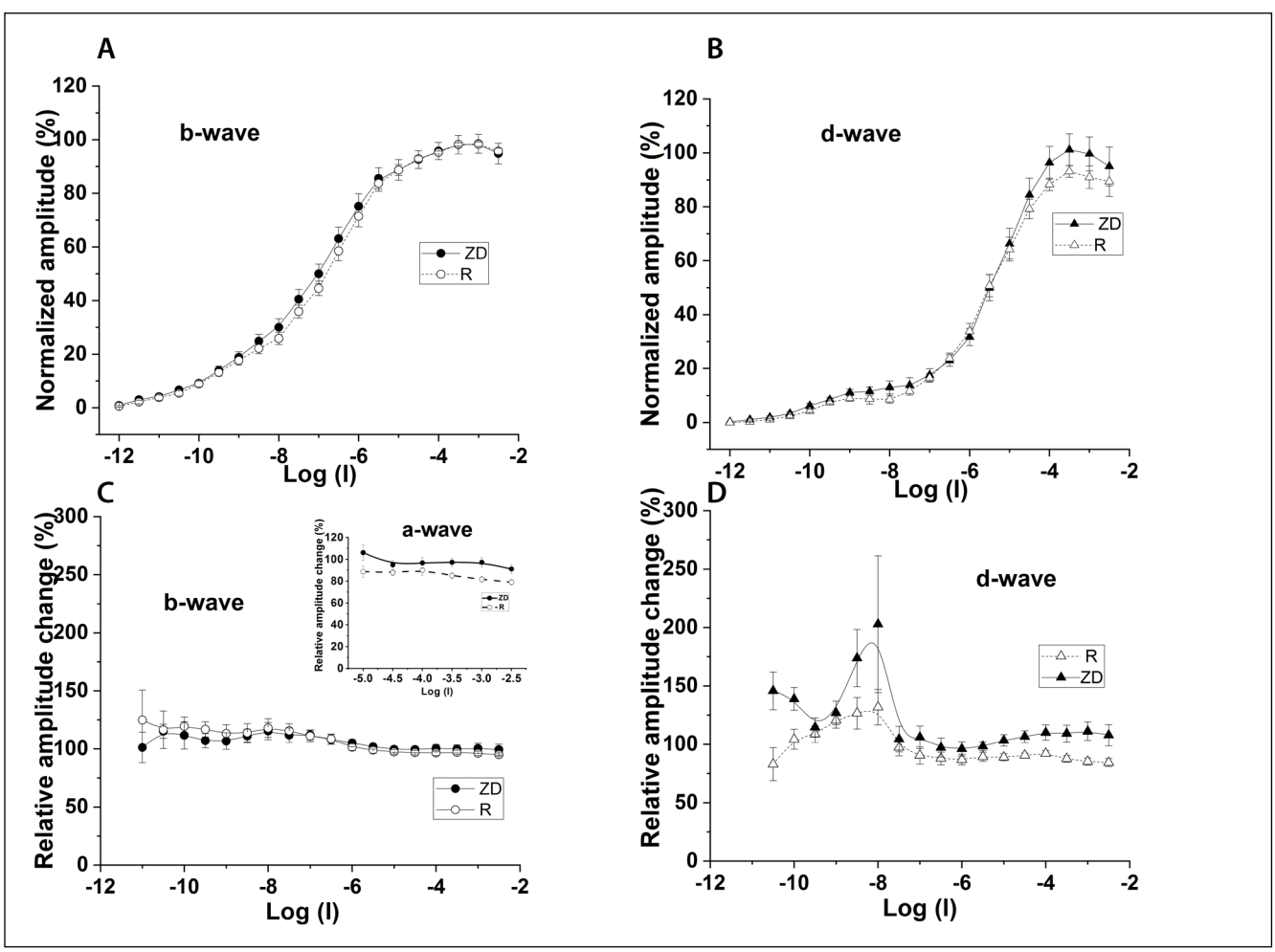

Fig. 3. (A), (B) Effects of 75 μM ZD7288 on the V – log I function of the b- and d-waves. The amplitudes of the ERG waves were normalized to V_{max} of the responses obtained in the control (first) V – log I function in each eyecup. The symbols, representing the responses obtained during the first (R) and second (ZD) intensity series, are denoted in the legends. Mean values ± SEM are shown (n=11). (C), (D) Relative change of the b- and d-wave amplitude in the control experiments (open symbols) and 75 μM ZD7288 experiments (filled symbols). The amplitudes of the ERG waves, obtained at each stimulus intensity during the second series were normalized to those, obtained during the first series. Mean values ± SEM are shown. (C) inset Relative change of the a-wave amplitude in the control experiments (open symbols) and 75 μM ZD7288 experiments (filled symbols). The amplitude normalization is made as in (C).

In contrast to the small changes in the ON response, perfusion with 75 μM ZD7288 caused well manifested changes in the OFF response. The amplitudes of the d-waves during ZD7288 treatment were significantly higher than those obtained in the first series in the range of lower (two-way ANOVA, $F_{1,131}$=9.15, P<0.00304) and higher intensities (two-way ANOVA, $F_{1,131}$=4.33, P<0.03947), while in the middle intensity range no significant difference was obtained (Fig. 3B). The latter fact probably accounted for the unaltered relative sensitivity of the OFF response (determined by I_d value, which was in the middle intensity range) (Table I). The absolute sensitivity of the OFF response was not significantly changed (Table I), indicating that the enhancing effect of the blocker on the scotopic d-wave amplitude was developed at stimulus intensities eliciting d-wave amplitude greater that 5 μM. Similar to the b-wave, we compared the d-wave relative amplitude change in the test and control experiments separately in the three intensity ranges. A statistically significant difference was obtained in the lower (two-way ANOVA, $F_{1,128}$=9.46, P<0.00262), middle (two-way ANOVA, $F_{1,109}$=4.95, P<0.02835) and higher intensity range (two-way ANOVA, $F_{1,130}$=43.59, P<0.00001). Thus, the application of ZD7288 had an enhancing effect on the d-wave amplitude over the entire intensity range in the dark adapted frog eyes (Fig. 3D). To compare the effect of the blocker on the amplitude of the OFF *vs.* ON response, we had to account for the nonspecific change of the b/d amplitude ratio in the control experiments (Fig. 2C). That is why we compared the relative changes of the b/d amplitude ratio during the second intensity series between the test and control experiments in the three intensity ranges (Fig. 4A). A statistically significant difference was obtained in the lower (two-way ANOVA, $F_{1,126}$=11.43, P<0.00099), middle (two-way ANOVA, $F_{1,109}$=7.51, P<0.00726) and higher intensity range (two-way ANOVA, $F_{1,131}$=46.98, P<0.00001). This result demonstrated that the blockade of HCN channels with ZD7288 caused an ON/OFF imbalance in the distal retina favoring the amplitude of the ERG OFF response. This imbalance was independent of the photoreceptor input and it was evident in conditions, when the ERG responses are mediated predominantly by rods, cones or both photoreceptor types.

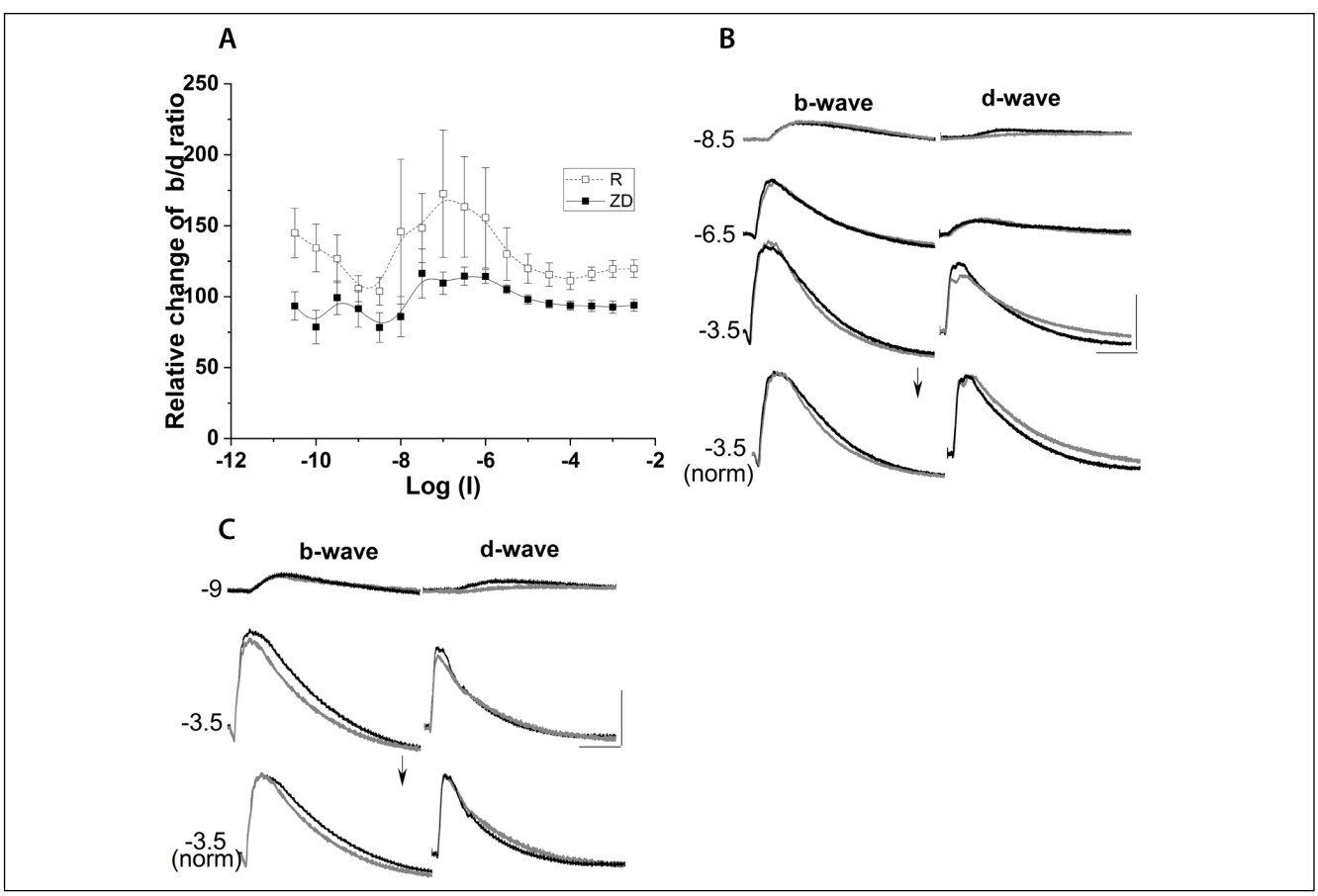

Fig. 4. (A) Effects of 75 μM ZD7288 on the b/d amplitude ratio. The values of the b/d amplitude ratio at each stimulus intensity during the second intensity series were normalized to the values during the first series. Results of both control experiments (open symbols) and test experiments (filled symbols) are represented. Mean values ± SEM are shown. (B), (C) Original ERG records obtained in eyecups perfused with 75 μM ZD7288. Designations are the same as Fig. 2D, E. Calibration: time – 0.4 s; amplitude – 100 μV in b, 150 μV in (C).

The blocker did not cause marked changes in the time characteristics of the ERG OFF response with exception of the shortened half-width duration obtained at higher intensities (Fig. 4B, C). The d-wave half-width duration during ZD7288 application (339±28.06 ms; log I=-3.5) was significantly shorter than the corresponding value obtained in the first series (383±32.54 ms; paired t-test, P<0.01311). The implicit time of the OFF response at higher intensities remained unaltered (control: 175±12.50 ms; test: 174±13.72 ms, log I=-3.5), although it was delayed in the control experiments. Thus, the blockade of HCN channels with ZD7288 enhanced and speeded up the cone-dominated ERG OFF response, but slowed the cone dominated ERG ON response.

Effects of ivabradine on the intensity-response function

Perfusion with 30 μM ivabradine caused no significant differences between control and test b-wave V – log I function (Fig. 5A). The absolute and relative sensitivity of the ON response remained unchanged (Table I). Comparison of the b-wave relative amplitude change between the test and control experiments revealed a statistically significant difference only in the higher intensity range (two-way ANOVA, $F_{1,107}$=4.31, P<0.04061). In this range, the relative b-wave amplitude values during ivabradine treatment were slightly greater than the corresponding values in the control experiments (Fig. 5D). Similar results were obtained for the a-wave, which amplitudes

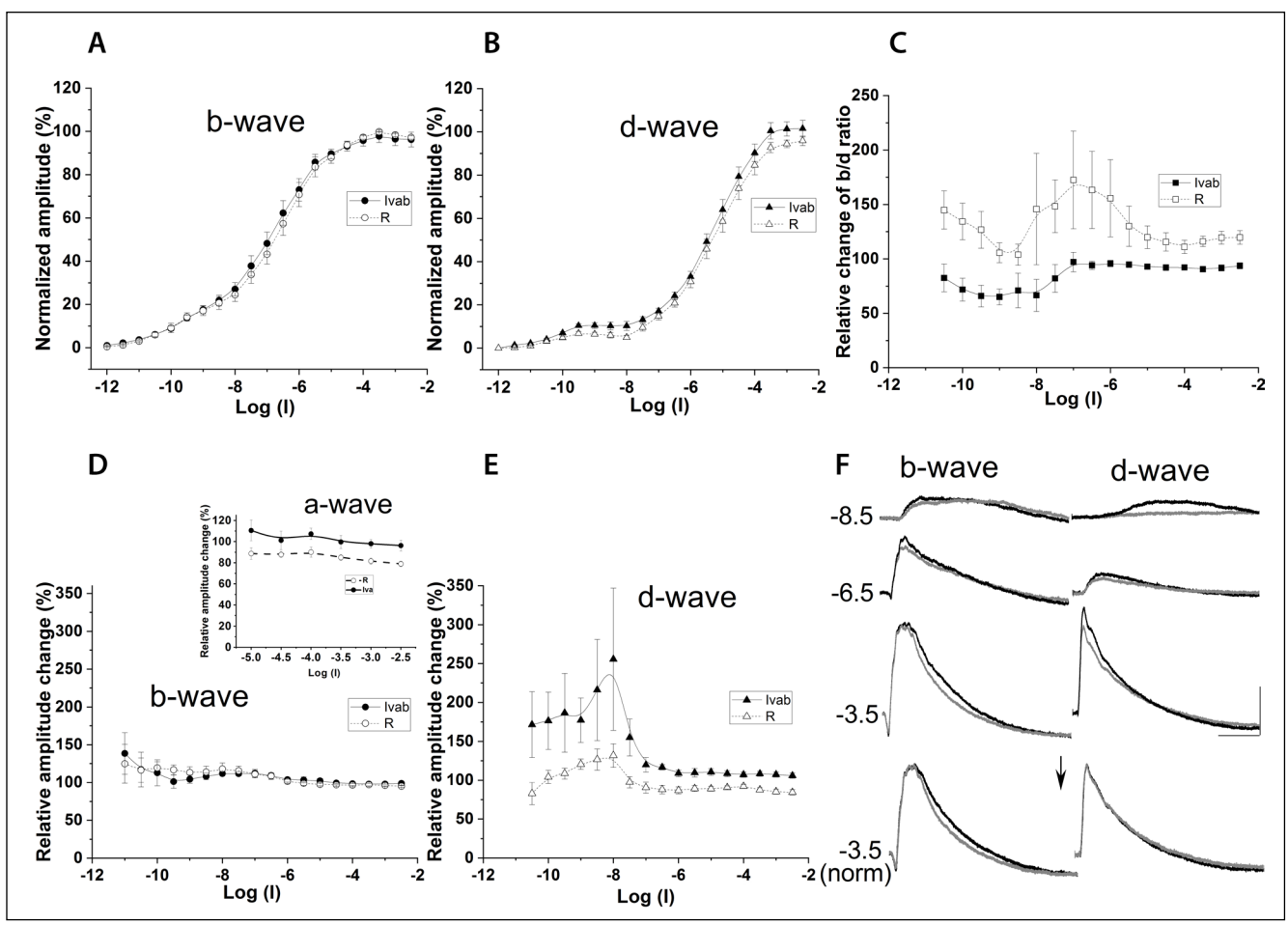

Fig. 5. (A), (B) Effects of 30 μM ivabradine on the V – log I function of the b- and d-waves. The amplitudes of the ERG waves were normalized to V_{max} of the responses obtained in the control (first) V – log I function in each eyecup. The symbols, representing the responses obtained during the first (R) and second (Ivab) intensity series, are denoted in the legends. Mean values ± SEM are shown (n=7). (C) Effects of 30 μM ivabradine on the b/d amplitude ratio. The values of the b/d amplitude ratio at each stimulus intensity during the second intensity series were normalized to the values during the first series. Results of both control experiments (open symbols) and test experiments (filled symbols) are represented. (D), (E) Relative change of the b- and d-wave amplitude in the control experiments (open symbols) and 30 μM ivabradine experiments (filled symbols). The amplitudes of the ERG waves, obtained at each stimulus intensity during the second series were normalized to those, obtained during the first series. Mean values ± SEM are shown. (D) inset Relative change of the a-wave amplitude in the control experiments (open symbols) and 30 μM ivabradine experiments (filled symbols). The amplitude normalization is made as in (D). (F) Original ERG record obtained in an eyecup treated with 30 μM ivabradine. Designations are the same as Fig. 2D, E. Calibration: time – 0.4 s; amplitude – 100 μV.

during the blocker application were relatively higher compared to those in the control experiments (two-way ANOVA, $F_{1,107}$=33.52, P<0.00001) (Fig. 5D, inset). These results were identical to those obtained for the effect of ZD7288 on the b-wave V – log I function and a-wave amplitude in the higher intensity range. Similar to ZD7288, the slight enhancement of the cone-dominated b-wave amplitude was accompanied with a slowed time course of the response (Fig. 5F). The half-width duration of the b-wave during ivabradine application (614±21.78 ms; log I=-3.5) was significantly longer compared to its control value (561±36.61 ms; paired t-test, P<0.0133). Because the b-wave implicit time remained unaltered (control: 238±13.04 ms; test: 237±7.21 ms), the prolonged half-width duration was due to the prolonged decay phase of the ON response. No slowing of the b-wave time course was seen in the lower and middle intensity ranges under the influence of ivabradine (Fig. 5F).

The d-wave V – log I function during ivabradine perfusion showed changes similar to those reported for ZD7288 action (Fig. 5B). The absolute and relative sensitivity of the OFF response remained unaltered (Table I), while the d-wave amplitude values were significantly higher than those during the first intensity series in the lower (two-way ANOVA, $F_{1,83}$=26.05, P<0.00001) and the higher intensity range (two-way ANOVA, $F_{1,83}$=7.45, p<0.00795). However, when the d-wave relative amplitude changes in the test experiments were compared to those in the control experiments, a statistically significant difference was obtained in the lower (two-way ANOVA, $F_{1,107}$=19.91, P<0.00002), middle (two-way ANOVA, $F_{1,89}$=35.15, P<0.00001) and higher intensity range (two-way ANOVA, $F_{1,107}$=113.56, P<0.00001) (Fig. 5E). Thus, the blockade of HCN channel with ivabradine had an enhancing effect on the d-wave amplitude over the entire intensity range similar to that reported for ZD7288. Similarly to ZD7288, the b/d amplitude ratio was decreased over the entire intensity range under the influence of ivabradine (Fig. 5C). The relative changes of this ratio in the text experiments differed significantly from those in the control experiments at lower (two-way ANOVA, $F_{1,106}$=17.76, P<0.00006), middle (two-way ANOVA, $F_{1,89}$=10.49, P<0.00174) and higher stimulus intensities (two-way ANOVA, $F_{1,107}$=42.49, P<0.00001). It is evident that both blockers of HCN channels caused a marked ON/OFF amplitude imbalance favoring the OFF response irrespectively of the predominant photoreceptor input. Ivabradine did not cause any significant changes in the OFF response time characteristics (Fig. 5F). However, the lengthening of the d-wave implicit time at higher intensities, reported for control experiments, was not observed in ivabradine treated eyecups (first series: 167±18.30 ms; second series: 154±18.23 ms; log I=-3.5). It seems that ivabradine similarly to ZD7288 speeded up the cone-dominated OFF response, although to a lesser extent.

DISCUSSION

Our results clearly show that a pharmacological blockade of retinal HCN channels leads to a marked imbalance in ON/OFF response amplitude in distal frog retina. The blockers (ZD7288 and ivabradine) caused an enhancement of the suprathreshold d-wave amplitude over the entire intensity range, while the b-wave amplitude was slightly enhanced only in the range of higher intensities. As a consequence, the b/d amplitude ratio was diminished at all stimulus intensities. It is evident that the absence of HCN channel function favors the OFF over ON response amplitude in the distal frog retina irrespectively of the type of the photoreceptor input. The effect is seen in rod-dominated (at lower intensities), mixed rod-cone (at middle intensities) and cone-dominated (at higher intensities) responses. The enhancing effect of the blockers on the OFF response amplitude was developed at stimulus intensities elicited scotopic d-wave amplitude greater than 5 μV and was especially expressed at those intensities, where transition from rod-dominated to mixed rod-cone dominated responses occurred (-8.5<I<-7.5). The diminution of the d-wave amplitude, which was evident at those intensities in control conditions, was not seen during HCN channel blockade, indicating a possible involvement of HCN channels in this phenomenon. The d-wave amplitude changes were not accompanied with manifest changes of the response time characteristics with an exception of speeded responses in higher intensity range. Thus, it appears that one of the functions of HCN retinal channels is to diminish the amplitude of the ERG OFF responses irrespectively of the photoreceptor input and to slow the time course of the cone-dominated ones. HCN retinal channels are probably not essential for the ERG ON response formation in dark adapted frog eyes, except for responses to stimuli of the higher intensity range, where they slightly diminish the amplitude and speed up the time course of the responses. To the best of our knowledge this is the first study where the effects of HCN channel blockade on the amplitude and waveform of the ERG ON and OFF responses were investigated over a broad stimulus intensity range.

We cannot directly compare our results with the results of other authors investigating the effects of pharmacological blockade or genetic ablation of HCN channels on the flash ERG, because in none of these studies the effects on the OFF response have been

commented. Although different changes of the b-wave amplitude have been reported in the absence of HCN channel function (see Introduction), most of the authors insist that the main change in flash ERG is a prolongation of the b-wave waveform. This prolongation is responsible for a marked reduction in the ability of the responses to follow high-frequency stimulation (Gargini et al., 1999; Knop et al., 2008; Della Santina et al., 2010; 2012, Seeliger et al., 2011; Pan et al., 2014). We could not see any prolongation of the latency and implicit time of the b-wave, while its half-width duration was prolonged only in the higher intensity range. Our results are similar, but not identical with the results obtained during acute ivabradine application in dark adapted rodents (Della Santina, 2009; Della Santina et al., 2010). The b-wave sensitivity and implicit time remained unaltered, while the b-wave amplitude was slightly enhanced in both rodents and frogs. In rodents, however, the delayed decay phase of the b-wave was to dim and intermediate stimuli, while in frogs it was to high intensity stimuli. It remains to be determined if retinal HCN channel blockade in frogs will reduce the ability of ERG responses to follow high frequency stimulation.

Our study indicates that the main consequence of retinal HCN channel blockade is a diminished b/d amplitude ratio over the entire intensity range in dark adapted frogs. The amplitude imbalance is accompanied with opposite changes in time course of the cone-dominated ON and OFF responses. The ON responses are prolonged, while the OFF responses are speeded up. The observed ON/OFF imbalance may account for the visual symptoms commonly associated with abrupt changes in light intensity seen in ivabradine treated patients (Borer et al., 2003; Cervetto et al., 2007). Similar suggestion was made by Bemme et al. (2017), who demonstrated that ivabradine differentially affects the activity of the ON and OFF ganglion cells in mouse retina. The ON cells showed reduced response gain, whereas OFF cells experienced an increase in response threshold under temporal white noise stimulation. The OFF cells, in contrast to ON cells, also showed reduced baseline activity during mesopic and photopic visual stimulation, reduced spontaneous activity and increased burst-like spiking activity in the presence of ivabradine. The authors proposed that "differential effects of the HCN channel block on the ON and OFF ganglion cells result from interactions between the drug-induced changes in photoreceptor activation and the signal transmission at the synapse between photoreceptors and bipolar cells". Another possibility is a direct action of ivabradine on the ganglion cells, because some of them were shown to express HCN channels (Müller et al., 2003; Stradleigh et al., 2011). An ON-OFF imbalance on ganglion cell level was seen also by Yang et al. (2013), who reported that the light-evoked OFF, but not ON responses were significantly reduced in dark adapted mouse retina during HCN blockade with ZD7288. We demonstrated an opposite change in the ON/OFF response balance in frog ERG, which may be due to species specific differences, but also to a different retinal level where this balance was investigated. It is well known that the main generator of the ERG b- and d-wave is the activity of the ON and OFF bipolar cells respectively (reviews: Frishman, 2006; 2013). We may suggest that the ERG ON/OFF imbalance during HCN channel blockade, obtained in the present study, occurred at the level of bipolar cells.

Many data indicate that HCN channel isoforms are well expressed in both types of bipolar cells: rat (Müller et al., 2003; Ivanova and Müller, 2006; Fyk-Kolodziej and Pourcho, 2007), mouse (Koizumi et al., 2004; Cangiano et al., 2007; Knop et al., 2008; Della Santina et al., 2012; Pan et al., 2014), rabbit (Kim et al., 2003). There is no consensus among the authors concerning the isoforms expression in the ON and OFF bipolar cells. Some authors reported that ON bipolar cells expressed HCN1 and HCN2 isoforms, while OFF bipolar cells expressed HCN4 isoform (rat: Müller et al., 2003, Ivanova and Müller, 2006; Stradleigh et al., 2011; mouse: Mataruga et al., 2007; Della Santina, 2009). Other authors, however, found expression of the HCN1 isoform in both ON and OFF bipolar cells (Fyk-Kolodziej and Pourcho, 2007). Still other authors observed expression of HCN1 isoform only in OFF bipolar cells (Kim et al., 2003). We could not find any information concerning the expression of different HCN channel isoforms in frog bipolar cells. We could not find also any data concerning the effects of HCN channel blockade (or absence) on the light-evoked responses of the ON and OFF bipolar cells. Their light responses could be changed as a result of blockade of their own HCN channels and/or blockade of HCN channels expressed on the photoreceptors. Della Santina et al. (2012) suggested that HCN2 channels on the ON bipolar cells control the b-wave temporal profile at dim luminance, while HCN1 channels on photoreceptors control the b-wave temporal profile at bright luminance. Knop et al. (2008) also suggested that the prolongation of the b-wave observed at high, but not low light intensities in HCN1 knock-out mice, was due to loss of HCN1 channels in photoreceptors and not in bipolar cells themselves. Because the prolongation of the b-wave half-width duration in our study was also seen only at higher intensities, we may suggest that this phenomenon was due to a blockade of HCN1 channels in frog photoreceptors. It was shown that HCN1 is the predominant HCN isoform in tiger salamander pho-

toreceptors and its blockade led to an enhancement of the amplitude and time course of photoreceptor light responses (Barrow and Wu, 2009). Our present finding that the a-wave, which reflects the activity of photoreceptors, had slightly increased amplitude in the higher intensity range during HCN channel blockade is in line with these results. Therefore, we may suggest that the blockade of HCN1 channels on the frog photoreceptors may account for the changes of the waveform of the cone-dominated b-wave as well as the amplitude of the cone-dominated b- and d-waves. However, such blockade could not account for the acceleration of the d-wave time course observed at higher stimulus intensities. Other mechanisms are probably involved in shaping the responses of frog OFF bipolar cells, whose activity is reflected in the ERG d-wave.

CONCLUSIONS

Our results indicate that the HCN channels contribute to the balance between the ON and OFF responses in the distal frog retina. Their blockade causes a marked change in the ERG ON/OFF response amplitude ratio, favoring the OFF response over a wide range of stimulus intensities. In the higher intensity range, the HCN channels affect also in a specific manner the time course of the ERG ON and OFF responses.

ACKNOWLEDGEMENTS

This work was supported by grant 67/23.04.2019 from the Council for Medical Science, Medical University of Sofia.

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