A Temporal Analysis of Testosterone-Induced Changes in Electric Organs and Electric Organ Discharges of Mormyrid Fishes

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SUMMARY

The electric organ discharge (EOD) of several species of mormyrid fishes within the genus *Brienomyrus* is sexually dimorphic during the breeding season: the duration of the male's EOD is much longer than the duration of the female's (for a review see Hopkins, 1986). The mormyrid used here, *Brienomyrus* sp., exhibits similar alterations in the duration of the triphasic EOD after treatment with testosterone, as do other members of this genus (for reviews see Bass, 1986a,b). In this experiment, animals were intraperitoneally implanted with pellets of either 11-ketotestosterone or 17 a-methyltestosterone, and the time course of the changes in the duration of each of the three phases of the EOD were quantified. Additionally, the time course of changes in the morphology of the electric organ, after testosterone treatment, was also quantified using electron microscopic techniques. The results suggest that the change in the duration of the first phase of the EOD is due exclusively to the change in the thickness of the electrocyte body: this is consistent with a model proposed by Bennett and Grundfest (1961) for the electrogenesis of a triphasic EOD. Changes in the duration of the second and third phases of the EOD are highly correlated with changes in the surface area of the posterior and anterior faces of the electrocyte, respectively. The results support the hypothesis that gonadal steroid hormone-induced changes in the EOD are due to structural changes in the electrocyte's membranes, and that all of the observed changes in the discharge of this system can be explained by the action of steroid hormones on the peripheral target cells (electrocytes).

INTRODUCTION

The effect of steroid hormones on sexually dimorphic behaviors has been studied in a number of vertebrate systems (for a review see Kelley, 1988). In many of these cases, the effects of steroids on both the central and peripheral structures mediating the behavior are inseparable due to the complex interaction between these two sites of hormone action. It is often difficult to determine whether the primary site of hormonal action is in the peripheral target tissue, and the observed central changes are a response to these peripheral changes, or whether the peripheral targets are changed in response to central modifications. One dimorphic system that is not confounded by these complex

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interactions between central and peripheral effects of steroid hormones is that of the electromotor system of mormyrid fishes.

Several lines of evidence indicate that the electric organ is the primary site of steroid hormone action in the electromotor system of mormyrids. In mormyrids, the sexually dimorphic, hormone-sensitive output, an *e*lectric *o*rgan *d*ischarge (EOD), is generated by the electric organ (for reviews see Bass, 1986a,b; in press). The structure of the discharge is closely associated with the gross and ultrastructural morphology of the specialized cells (electrocytes) whose activity generates the electrical output of the system (Bennett and Grundfest, 1961; Schwartz, Pappas, and Bennett, 1975; Bass, 1986c; Bass, Denizot, and Marchaterre, 1986a). Steroid hormones can induce changes in EODs that resemble naturally occurring sex differences (for reviews see Bass, 1986a,b). Neurophysiological recordings from individual electrocytes show that changes in the EOD are paralleled by changes in the properties of the action potentials generated by the electric organ (Bass and Volman, 1987). Finally, biochemical studies have shown that the steroid sensitivity of the electric organ and the EOD correlates with high levels of androgen-binding activity in electrocytes (Bass, Segil, and Kelley 1986b). In contrast, central electromotoneurons do not appear to concentrate steroids as shown with autoradiography (Bass et al., 1986b). For these reasons, mormyrids represent an ideal system in which to study the effects of steroid hormones on a sexually dimorphic behavior at both the level of the behavior itself and at the structural, mechanistic level.

Bennett and Grundfest (1961) proposed a model describing the electrogenesis of the electric organ discharge. They demonstrated that the wafer-shaped electrocytes of mormyrids are composed of three distinct, electrically excitable membranes: anterior face, posterior face, and stalk. The geometry of these three components is species-typical and is an integral part of the production of species-typical EOD waveforms (Bass, 1986c; Bass et al., 1986a). The EOD of many species of mormyrids is a sexually dimorphic characteristic (for reviews see Bass, 1986b; Hopkins, 1986). In the nonbreeding season, the EODs of males and females are often indistinguishable. During the breeding season, however, the waveform of the male EOD undergoes various changes. Changes in the morphology and physiology of the electric organ and the EOD, of both males and females, after exposure to gonadal steroid hormones have been documented in *Brienomyrus* sp., a commercially available mormyrid species, which has a triphasic EOD waveform (Bass and Hopkins, 1984; Bass, 1986a; Bass et al., 1986a,b; Bass and Volman, 1987). These studies documented changes in both the morphology and the electrical properties of these cells after animals were treated with testosterone. The goal of the present study was to quantify changes in the amplitude and duration of each phase of the EOD and to compare the time course of these changes with possible changes in electrocyte morphology using quantitative electron microscopy.

Portions of these results have appeared elsewhere (Freedman, Olyarchuk, Marchaterre, and Bass, 1988).

METHODS

Experimental Paradigm

Twelve fish, ranging in standard length from 7.5 to 11 cm (mean, 10 cm) were divided into three groups without regard for the sex of the animal. As noted above, both males and females respond

similarly to testosterone treatment; determination of sex was performed at the end of the observation period. No differences between males and females were found, and the data from males and females has been pooled. Six fish were each implanted with testosterone pellets (one group of three with 11 ketotestosterone, the other group with 17 a-methyltestosterone). 11 Ketotestosterone was used because it is the principle form of circulating testosterone so far documented in teleost fishes (e.g., see Wingfield and Grimm, 1977). 17 a-Methyltestosterone was used for comparative purposes since it is the form of testosterone used in a number of previous anatomical and physiological studies addressing a similar set of questions (Bass et al., 1986a; Bass and Volman, 1987). This form of experimental treatment results in elevated plasma levels of testosterone similar to circulating levels found in several teleosts (Bass and Volman, 1987). Measurements of the EOD amplitude and duration in these two groups of fish were carried out every day beginning 1 week prior to implantation.

The remaining six fish comprised the control group. Three were treated in a fashion identical to that for the experimental group (including surgery) but were not implanted. The final group of three was isolated and measurements were taken daily, but these animals underwent no additional treatment. The additional control of cholesterol implants was not done in this series of experiments, but previous experiments using cholesterol pellet implants have been done (Bass et al., 1986a; Bass and Volman, 1987). The change in amplitude and duration of the EOD was tracked in all groups beginning 1 week prior to treatment for up to 35 days after implantation. Fourteen additional fish were implanted with 17 a-methyltestosterone, allowed to survive for varying lengths of time after treatment, sacrificed, and perfused transcardially with 0.1 *M* phosphate buffer followed by 2.5% glutaraldehyde. The electric organ was dissected out, cut into 1 mm blocks, and then prepared for electron microscopy as described elsewhere (Bass et al., 1986a). At the conclusion of each experiment, the hormone pellet was retrieved to confirm that treatment extended to the time of sacrifice. The EODs of these animals were also tracked over time and showed changes of a magnitude similar to those documented for the long-term study group shown in Fig. 4.

Hormone Treatments

Brienomyrus sp. were anesthetized with tricaine methanosulfate (MS222). A small incision was made anterior to the anal pore. Small pellets (2–3 mg) of 11-ketotestosterone (Sigma Chemical) or 17 a-methyltestosterone (Sigma Chemical) were placed in the intraperitoneal cavity. The incision was then sutured closed and the animal revived by respiration with fresh water. Immediately after surgery, animals were placed in 0.4% NaCl to reduce infection and were allowed to remain in this solution for at least 12 hours. Following exposure to this high salt solution, animals were removed to and maintained in individual aquaria, separately aerated and filled with fresh aquarium water. Special care was taken to keep the conductivity of the water constant at 500 mho/cm^2; changes in the conductivity of the water in the holding tanks can affect the EOD amplitude and waveform (Bratton and Kramer, 1988).

Measurements of EOD Amplitude and Duration

Measurements of the EOD amplitude and duration were taken as follows. Fish were placed in a PVC tube, similar to those kept in their home aquaria as hiding places. Once in the tube, fish remained still for the duration of the measurement period (sometimes up to 30 mins.). A small silver-wire ring (the active electrode) was placed around the tail of the animal until the posterior limit of the tail touched a stop placed approximately 1.5 cm behind the ring. In this way the electrode was always in the same place along the longitudinal axis of any given fish. The electrode output was digitized (100 kHz, Neurodata 6100, Analogic) and recorded on a video cassette recorder (GE 9-7520). Waveforms were analyzed using the Data 6100 wave analyzer. All measurements were based on averages of 128–256 successive EODs. Peak amplitudes of the three phases of the EODs were read directly from the Data 6100. The duration of each of the three phases was similarly measured. The duration of each phase was defined as the time from the first deviation from the baseline to the next zero-crossing (see Fig. 1).

Surface Area Measurements

One-μm-thick plastic embedded sections, stained with toluidine blue, were used for the quantification of electrocyte thickness (after Bass et al., 1986a). Thin sections (0.5 μm) of the stalk, anterior face, and posterior face were then photographed at a magnification of 5000×. All measurements were then based on photographic prints with a final magnification of 25,000×. In order to estimate the surface area of both faces and the stalk, a 10 × 30 cm grid marked off in 1 cm squares was placed over each photograph with the 10 cm dimension along the membrane surface. The maximum length of an invagination within each of the 10 1-cm columns was measured. The 10 lengths were then summed, and this was taken as an approximation of the amount of membrane surface area in the sample. This method takes both the invagination density and length into account. At least 10 pictures of each electric organ were taken by an observer unaware of the treatment group.

Statistics

All statistical measures are based on the results of one-way ANOVA tests using Scheffe's method for multiple comparisons (Sokal and Rohlf, 1981) unless stated otherwise.

RESULTS

Changes in EOD Duration and Amplitude

As shown in Fig. 1, the EOD of *Brienomyrus* sp. is triphasic and includes an initial, small amplitude, head-negative phase (Phase 1) followed by a major positive (Phase 2) and then a major negative phase (Phase 3). Figure 1 is a qualitative overview of the changes in the EOD documented in a more quantitative fashion in subsequent figures. To the left is shown a series of EOD waveforms from an untreated animal monitored at the same time intervals as shown for the EOD waveforms to the right from a specimen treated for 27 days with 11-ketotestosterone.

Phase 1

In order to account for the natural animal-to-animal variation in duration and amplitude of the EOD, the data were normalized by expressing the results in terms of the *change* in amplitude or duration: average pretreatment values of amplitude and duration of each phase of the EOD were subtracted from the values recorded subsequently for each individual animal at the posttreatment time intervals. Four days after implantation with either 11-ketotestosterone or 17 a-methyltestosterone, the duration of the initial negative phase of the EOD (Phase 1, see Fig. 1) begins to show its first significant increase (all levels of significance are $p < 0.05$ unless otherwise indicated; Fig. 2a,b). The duration of this phase continues to increase for about 9 days after initial implantation.

After 9 days, the duration no longer changed but remained at its increased length for the rest of the measurement period (35 days after treatment; see Fig. 2). The *amplitude* of this phase of the EOD does not show any significant changes in testosterone-treated individuals ($p > 0.05$).

Phases 2 and 3

Figures 3 and 4 show the *changes* in duration of phases 2 and 3 for individuals with an implant of either 11-ketotestosterone (Fig. 3) or 17 a-methyltes-

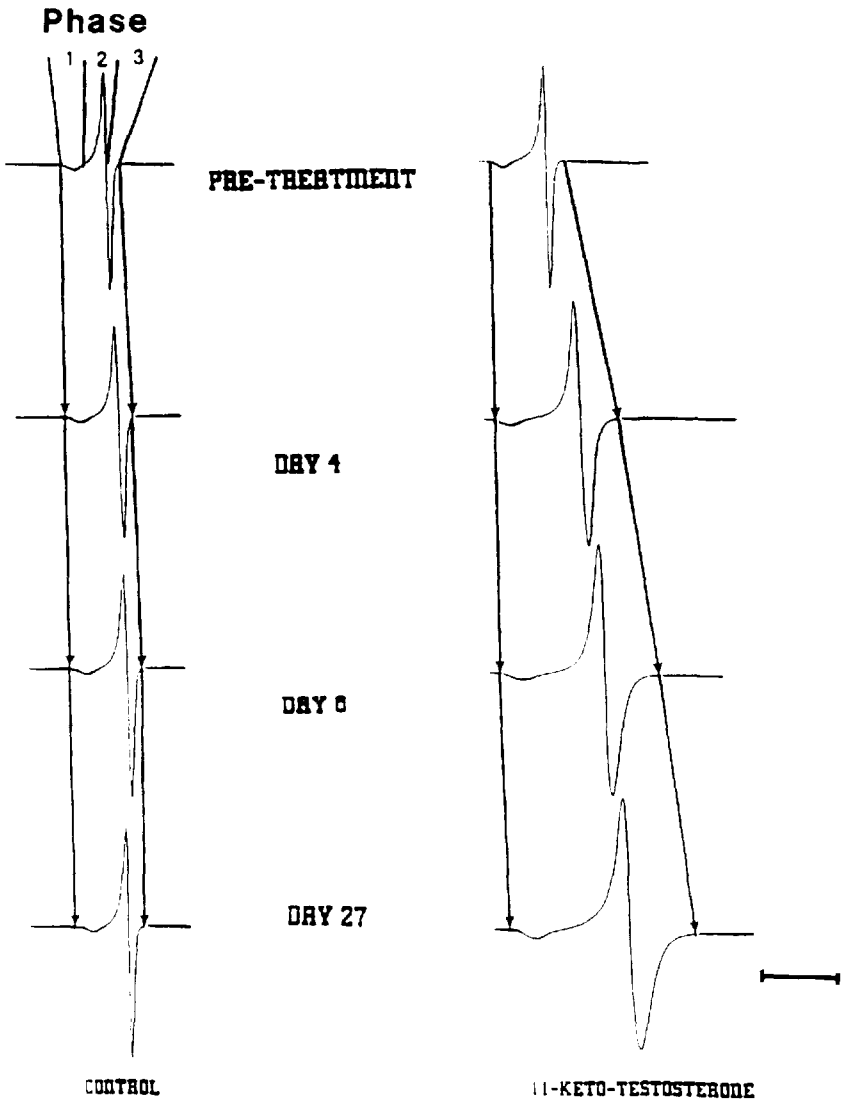

Fig. 1. Observed changes in EOD waveform after testosterone treatment. The left column shows four EODs from a control animal; sample times correspond with those of the treated animal shown in the right column. Amplitudes are normalized to the same peak-to-peak amplitude to facilitate accurate duration comparisons. Arrows indicate the beginning and end of the discharge. The three phases of the EOD are defined in the upper left corner. Scale bar = 1 ms.

tosterone (Fig. 4). There are no significant changes in EOD duration in untreated controls as shown in Fig. 5. As with Phase 1, the first significant changes in EOD duration are seen after 4 days of treatment. The change in duration of both phases in animals with implants is significantly different both from controls as well as from their own pretreatment baseline. The duration of both phases continues to increase for the duration of the experiment. Unlike the duration of phase 1, which stops increasing after about 9 days (Fig. 2), the duration of phases 2 and 3 do not stop increasing after only 9 days, although

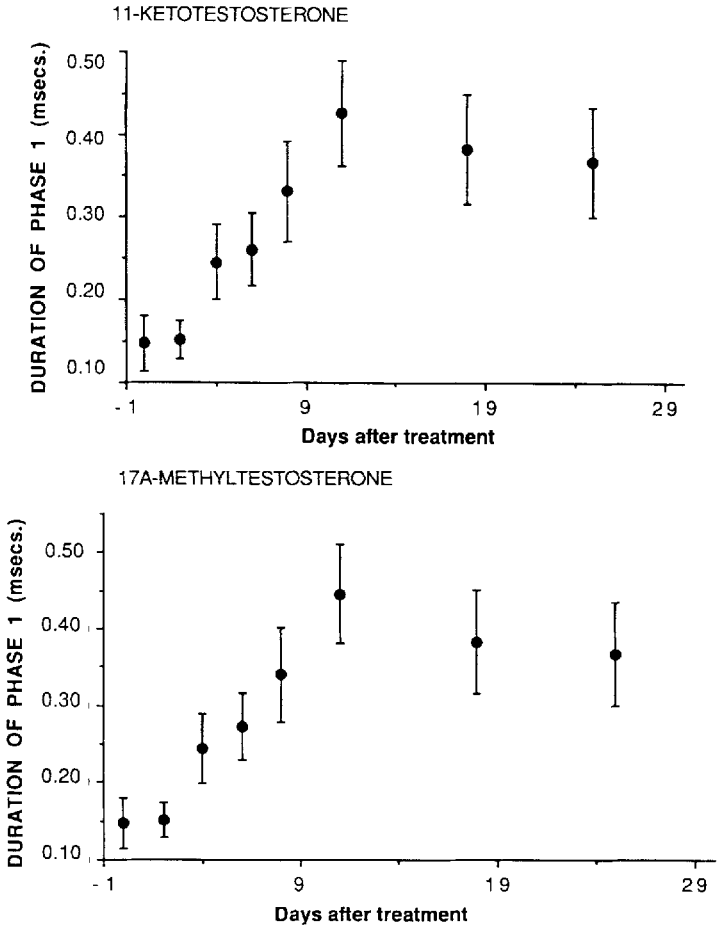

Fig. 2. The duration of phase 1 is plotted against the number of days after initial implantation. There were no significant differences between the 11-ketotestosterone (A) and 17 a-methyltestosterone (B) treatments. The first significant ($p < 0.05$) increase in duration of this phase occurs after 4 days for both treatment groups.

the rate of increase seems to fall off after extremely long periods (upwards of 20 days), and measurements taken at the end of the experiment (30–35 days) were not different from previous measurements (days 23–30). As with Phase 1, there are no significant differences between the animals treated with 11-ketotestosterone and those treated with 17 a-methyltestosterone. This suggests that the action of 17 a-methyltestosterone is similar to that of naturally occurring forms of testosterone (11-ketotestosterone).

The changes in EOD *amplitude* are best expressed as the ratio of Phase 2 to Phase 3, which reflects changes that are obvious at a qualitative level (Fig. 1, right). The amplitudes of Phases 2 and 3 also change as a function of the duration of the EOD. As shown in Fig. 6 the ratio of the amplitude of Phase 2 to Phase 3 is constant (at 0.7) in untreated animals, while in hormone-treated animals the ratio increases from control levels up to 1 as the duration of

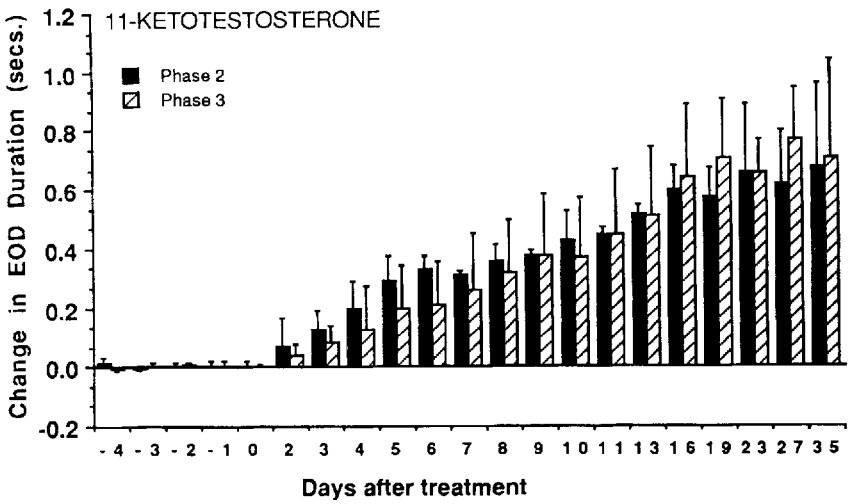

Fig. 3. This histogram shows the *change* in duration of the second (dark) and third (light) phases of the EOD as a function of the number of days after the initial 11-keto-testosterone treatment. Pretreatment values were averaged and then subtracted from subsequent values to normalize animal-to-animal variation in EOD duration. Each bar is the average change in duration of the three treated animals for the given day. Error bars are one standard deviation from the mean. The first significant ($p < 0.001$) change occurs at 4 days of treatment.

treatment increases. Thus, the amplitudes of the second and third phases of the EOD become more similar as treatment progresses. As noted earlier, the amplitude of Phase 1 (not shown) showed no significant changes over the treatment period from pretreatment or control values.

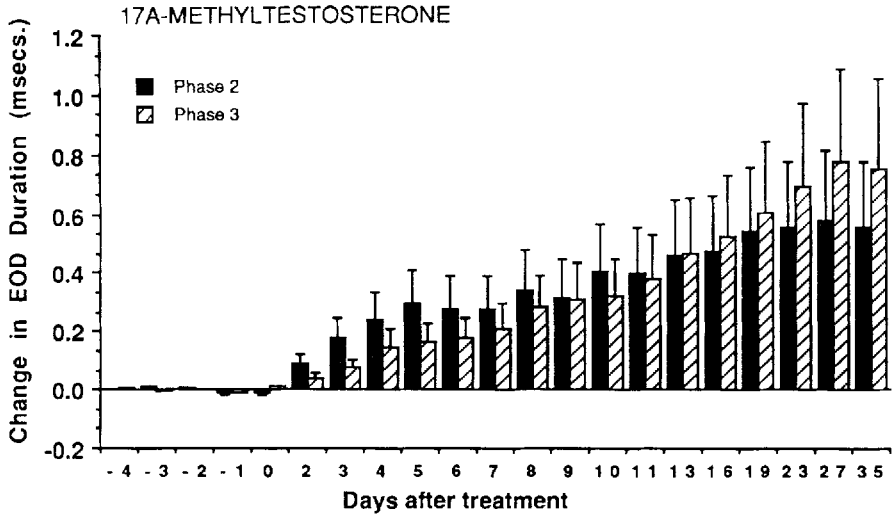

Fig. 4. Changes in duration of phases 2 and 3 after treatment with 17 a-methyltestosterone. As with 11-ketotestosterone, the first significant changes occur at 4 days. See Fig. 3 for further details.

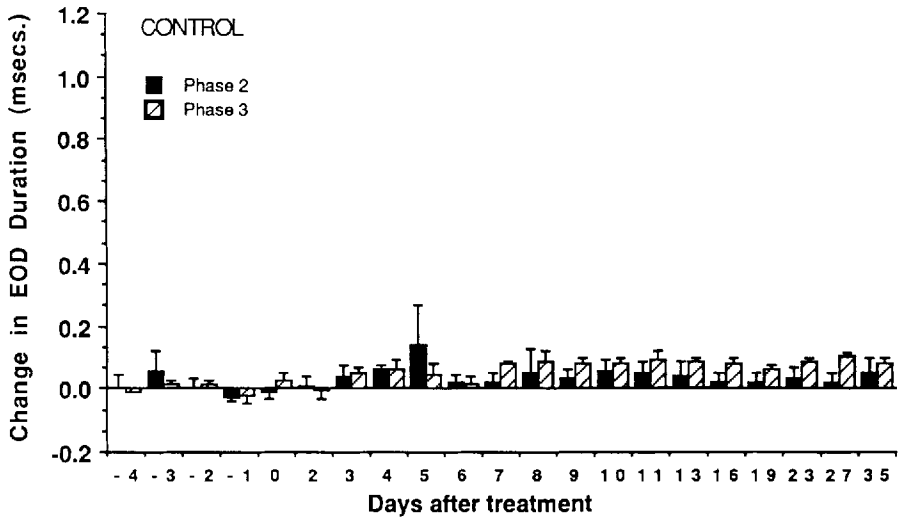

Fig. 5. Changes in duration of phases 2 and 3 in control animals. Details are similar to Fig. 3. There are no significant changes in duration ($p > 0.05$). These data include untreated ($n = 3$) and sham-operated ($n = 3$) controls.

Changes in Electrocyte Surface Area

The surface area of the stalk region of electrocytes showed no significant changes in response to hormone treatment ($p > 0.1$). However, there were significant changes in surface area in both the anterior and posterior faces of the electrocytes of steroid-treated animals. Figures 7 and 8 show representative examples of light (Fig. 7) and electron (Fig. 8) micrographs of electrocytes

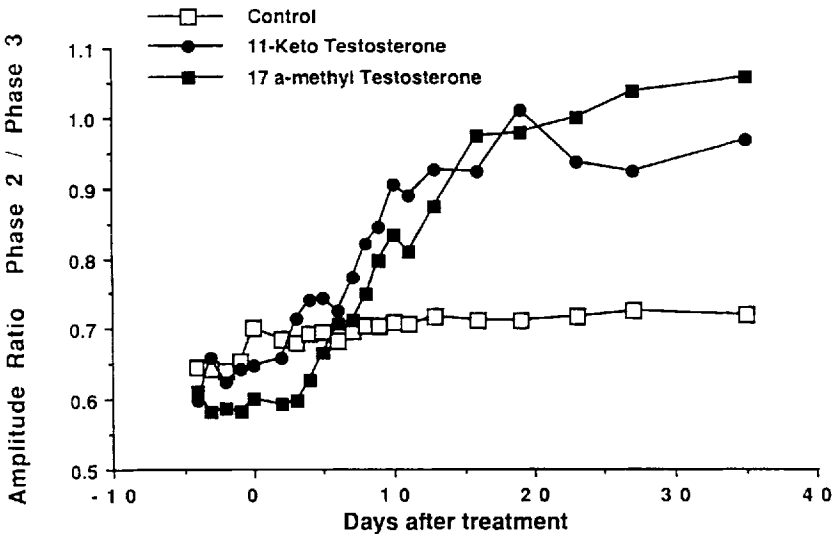

Fig. 6. The amplitude of phase 2 divided by the amplitude of phase 3 for the three treatments are shown plotted against the number of days after the hormone implantation. See text for description and discussion of these data.

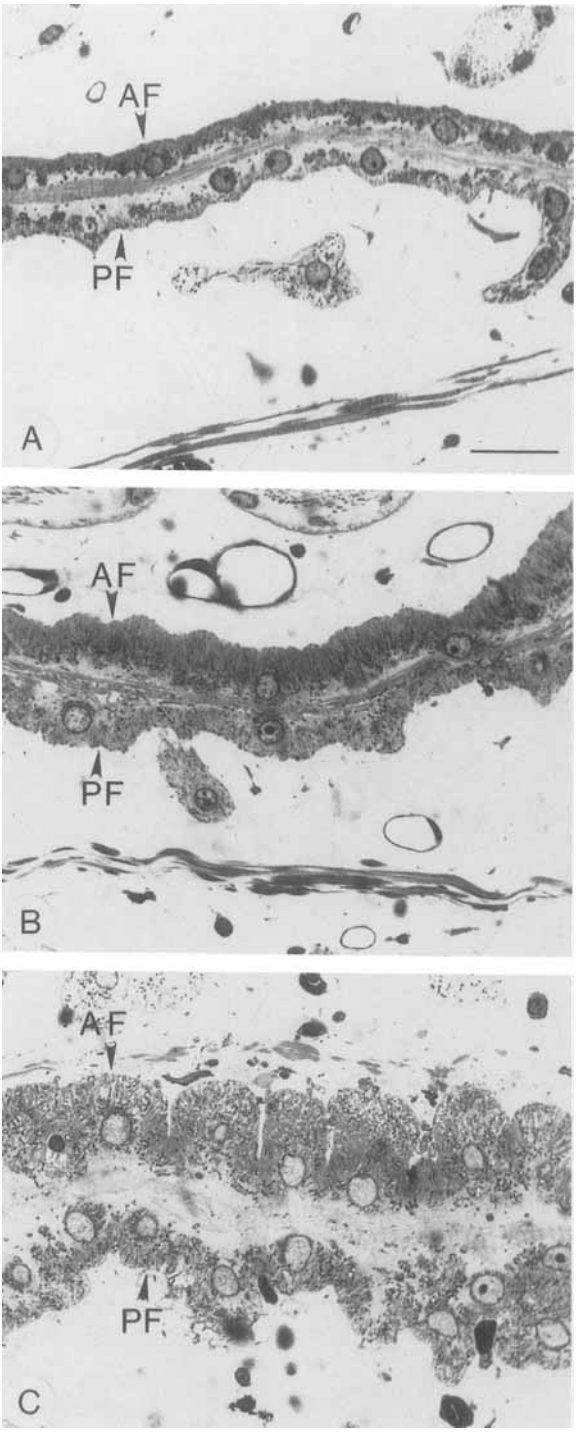

Fig. 7. Representative light micrographs of sagittal sections through electrocytes taken at three treatment times: (A) pretreatment, (B) 4 days after treatment, (C) 25 days after treatment. After 4 days of treatment there is the first significant increase in electrocyte width ($p < 0.05$; see text and Fig. 10). Scale bar = 15 μm. Abbreviations: AF, anterior face; PF, posterior face.

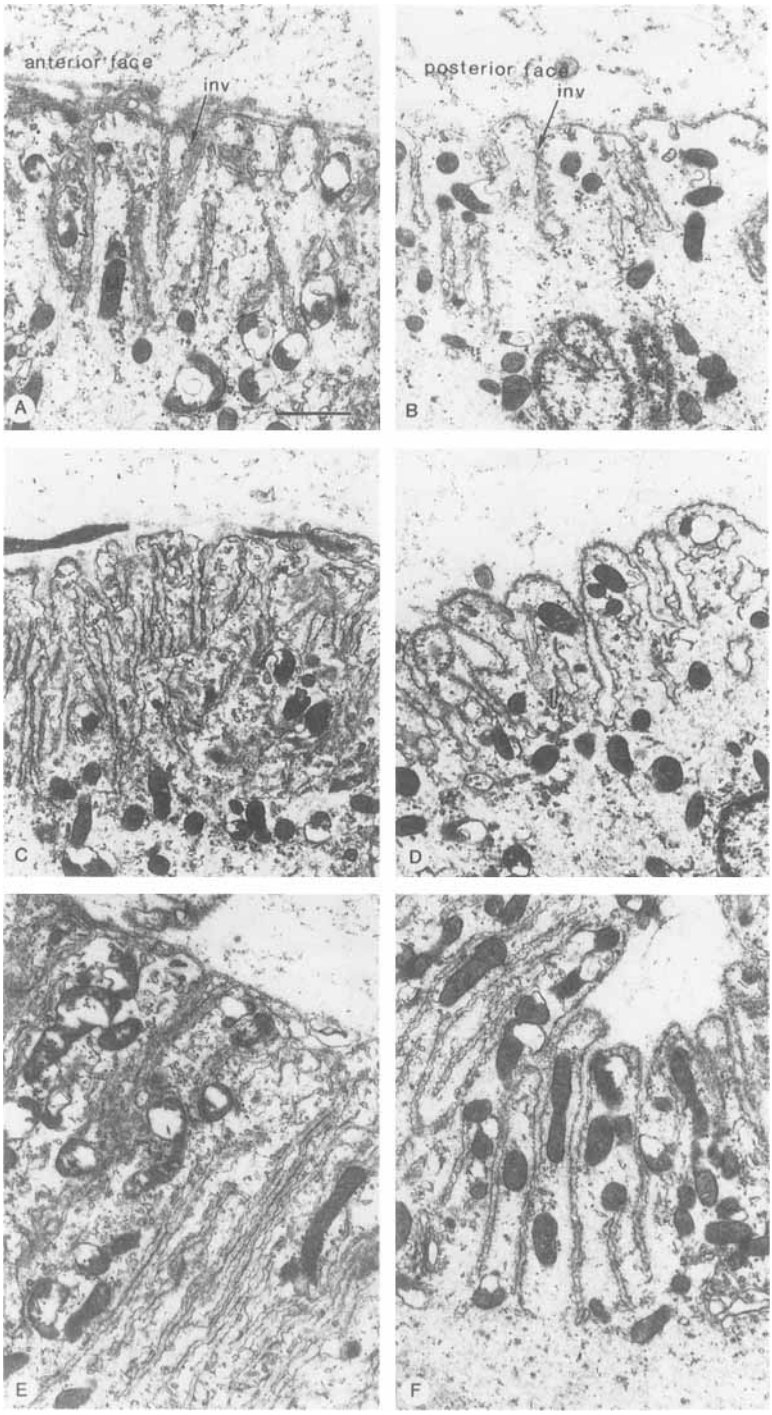

Fig. 8. Representative electron micrographs showing anterior (A,C,E) and posterior (B,D,F) faces of electrocytes at three treatment times: (A,B) pretreatment, (C,D) 4 days after treatment, (E,F) 25 days after treatment. Four days after implantation there is a significant increase in the surface area of both anterior and posterior faces ($p < 0.05$; see text and Fig. 9). Scale bar = 0.8 μm. Abbreviation: inv, surface invaginations along each face.

from untreated animals [Figs. 7(A) and 8(A)] and ones treated for 4 [Figs. 7(B) and 8(B)] and 25 [Figs. 7(C) and 8(C)] days. There are two principle changes in electrocyte morphology. First, there is a general increase in overall electrocyte width or thickness (Fig. 7). Second, the dramatic change observed in electrocyte width is due primarily to increases in the depth of surface invaginations (inv, Fig. 8) that characterize the anterior and posterior faces of *Brienomyrus*' electrocytes (Bass et al., 1986a).

Figure 9 shows the quantitative changes in electrocyte surface area for the two faces. The first significant changes in surface area are seen after 4 days following implantation of testosterone. Both anterior and posterior faces of the electrocyte show significant increases in membrane surface area. Most importantly, the surface area of these two faces increased with a time course that parallels the changes in duration of phases 3 and 2, respectively.

The width of electrocytes, measured from the outside edge of the posterior face to the outside edge of the anterior face (see Fig. 8), increases for about 8 days after steroid implantation and then plateaus and maintains its increased width for the duration of the experiment (Fig. 10).

DISCUSSION

The EOD Waveform of Mormyrids

Figure 11 presents a schematic representation of transverse and sagittal views of a representative electrocyte from the genus *Brienomyrus* (see Bass, 1986c, for details). As shown in transverse view, the stalk of the electrocyte has a branched, arbor-like structure and is raised above the surface of the disk-shaped electrocyte. The stalk is innervated in one focal region near its base by electromotor axons. As shown in sagittal view, the stalk actually arises as a series of evaginations from the posterior face of the electrocyte. These evaginations fuse with one another into larger branches that eventually pierce the body of the electrocyte at penetration sites. The stalk's many branches are

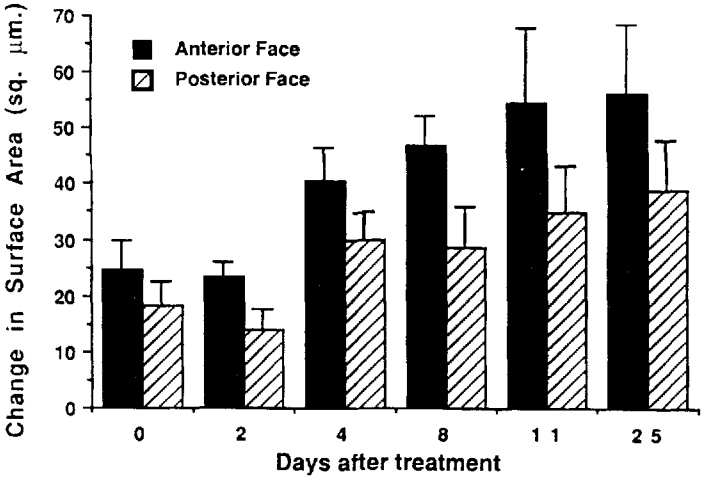

Fig. 9. Histogram showing the *changes* in the surface area of the anterior (dark) and posterior (light) faces of the electrocyte. The first significant changes in surface area appear after 4 days of treatment (see Fig. 8).

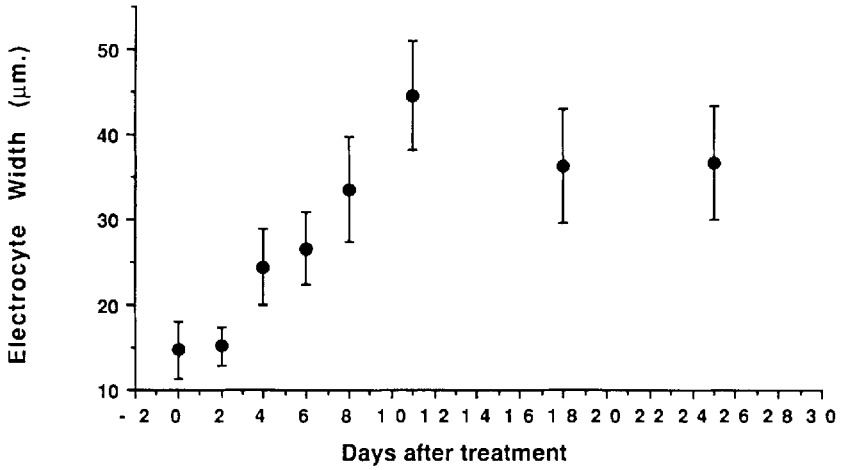

Fig. 10. Change in the width of electrocytes as a function of the number of days after treatment. Measurements were made from the outside of the anterior face to the outside of the posterior face using 1-μm-thick plastic tissue sections stained with toluidine blue (see Fig. 7).

continuous with one another after they penetrate through the electrocyte body. It is important to emphasize that the stalk is only a portion of this multinucleated cell (the electrocyte is essentially a modified striated muscle fiber; see review in Bass, 1986a). Bennett and Grundfest's (1961) model states that the first phase of the EOD—a small head negative deflection (as measured differentially with a positive electrode at the head of the animal)—arises from an action potential initiated in the stalk region of the electrocyte by electromotoneuron innervation. As current passes through the body of the electrocyte at a penetration site, it becomes directed along the longitudinal body axis of the fish, producing the first phase of the EOD. Phase 2 is a head positive phase that is initiated by the activation of the posterior face of the electrocyte. Activation of this face allows current to flow in the posterior-anterior direction and generates the head positive deflection of the EOD. The anterior face of the electrocyte is then activated as current flow across the posterior face depolarizes the electrocyte. Activation of the anterior face produces anterior-posterior current flow and creates the third, negative deflection phase of the EOD.

Correlative Changes in EODs and Electrocyte Morphology

EOD Duration. The above model suggests that the first phase of the EOD arises as the action potential generated in the stalk region of the electrocyte becomes directed along the body axis of the fish as it enters the electrocyte body. The duration of this phase should then depend solely on the time during which the current remains directed, i.e., the time it takes for the action potential to pass through the electrocyte and out the other side where current is no longer constrained to flow along the body axis. The width of the electrocyte should thus have a direct relationship with the duration of the first phase of the EOD. As shown in Figure 10, electrocyte width changes as a function of the number of days after hormone treatment. Figure 2 shows the relationship

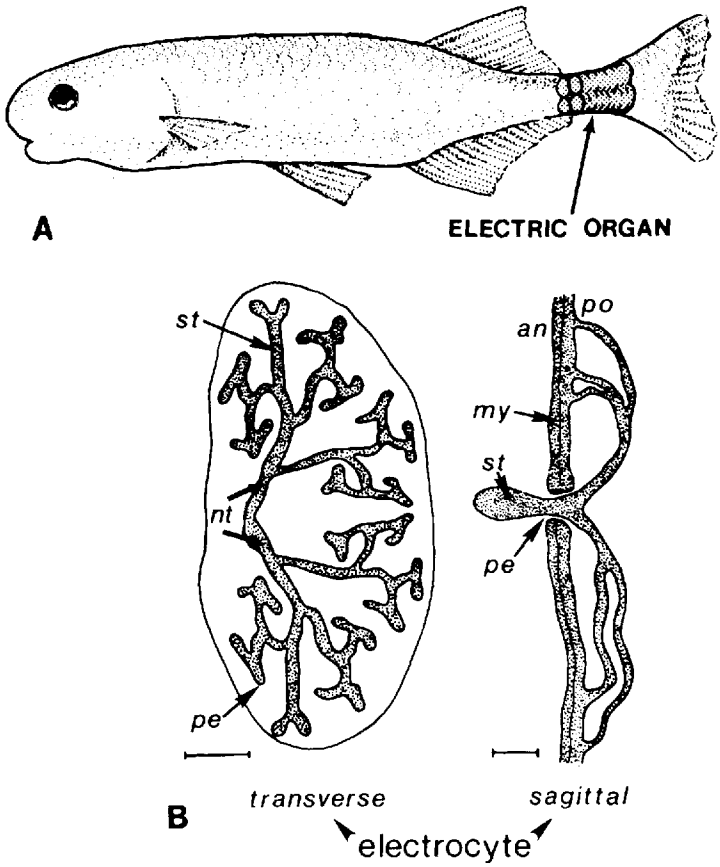

Fig. 11. (A) A depiction of a mormyrid showing the position of the electric organ which includes two dorsal and two ventral columns of electrocytes that surround the spinal cord. (B) Transverse and sagittal views of an electrocyte showing the penetration site (pe) of the stalk (st) as it passes the anterior face (an) and then fuses with the posterior face (po). After Bass and Volman (1987). Other abbreviations: nt, site of electromotor axon terminals; my, position of myofilaments.

between the duration of the first phase and the number of days after testosterone treatment. The similarity of these curves is self-evident, and the correlation of electrocyte width and duration of the first phase of the EOD is demonstrated in Fig. 12. The data clearly support the extrapolation of Bennett and Grundfest's model, which suggests that the first phase duration is a function of the width of the electrocyte.

The effects of hormone treatment on the second and third phases of the EOD are quite different from the effects of treatment on the first phase. It has been suggested that the surface area of the anterior and posterior faces of the electrocytes play a major role in determining the duration of the third and second phases of the EOD, respectively (Bass et al., 1986a; Bass and Volman, 1987). Quantitative evaluation shows there are significant changes in the surface area of both faces. The time course of these changes is similar to the time course of changes in duration of Phases 2 and 3. The relationship between anterior and posterior face surface area and the duration of the EOD is shown

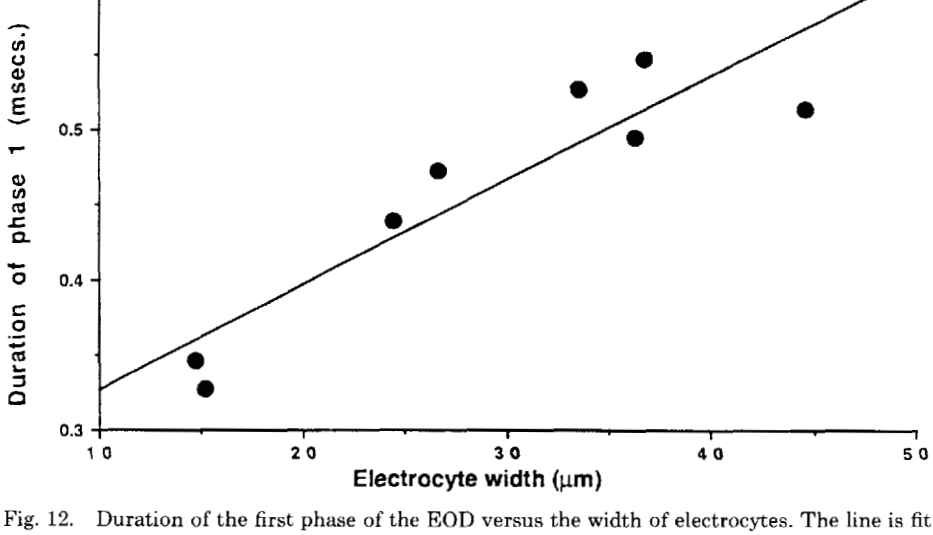

Fig. 12. Duration of the first phase of the EOD versus the width of electrocytes. The line is fit using a least squares method. Correlation coefficient (R) is as shown.

in Fig. 13. There is a strong positive correlation between the hormone-induced change in surface area of the two faces and the hormone-induced change in duration of the respective EOD phases. Thus, these data strongly suggest that the increase in membrane surface area is the proximate cause underlying changes in the EOD.

By significantly increasing the surface area of the membranes responsible for second and third phase production, testosterone must alter the electrical properties of the membranes which ultimately result in an increase in duration of these two phases. Such changes may involve both active (ion channels) and

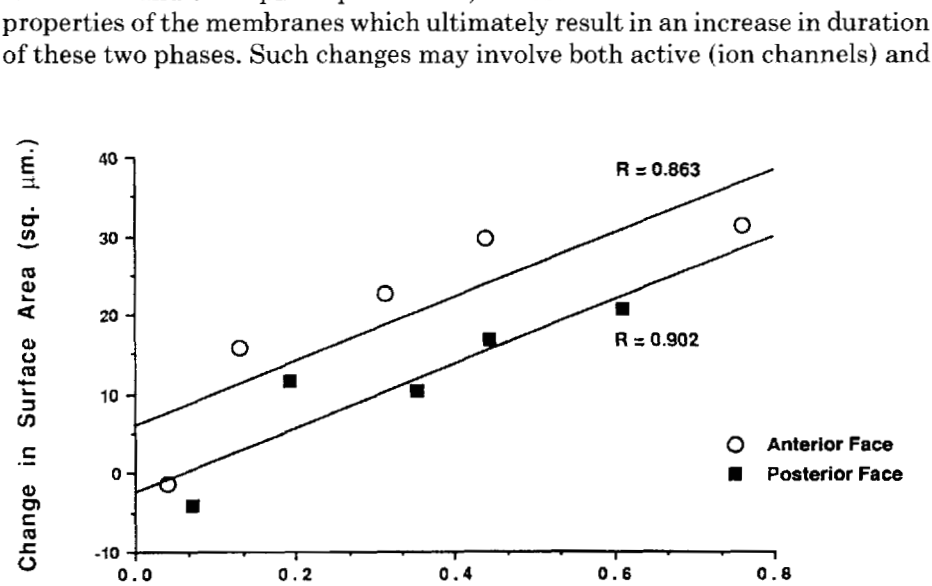

Fig. 13. The duration of phases 2 and 3 versus changes in the surface area of the posterior and anterior faces, respectively. Lines are fit using the least squares method, and correlation coefficients (R) for each line are shown.

passive (capacitative) elements. As regards passive properties, increases in the capacitance of the membrane (associated with increased surface area; e.g., see Neher and Marty, 1982) may increase the initial onset of a spike in either the anterior or posterior face. This may prevent temporal overlap of the action potentials generated across the posterior and anterior faces during long duration EODs. It is of interest that changes in surface area do not extend to the stalk, which is the first site of spike generation in the electrocyte. Increases in capacitance could also prolong the rise-fall time of the action potential across each face (see Bass and Volman, 1987, for an extended discussion).

Surface area changes have also been shown to be associated with changes in the active electrical properties of other excitable cells (Neher and Marty, 1982; Moody and Lansman, 1983). Changes in membrane area may thus determine the selective addition of certain species of ion channels, which would then have a direct effect on the active properties of those membranes that may ultimately determine changes in action potential duration. In this way, surface area changes could be affecting changes in both active and passive membrane properties.

EOD Amplitude. Increases in the duration of Phases 2 and 3 are correlated with relative increases and decreases, respectively, in their amplitude. Intracellular recordings indicated significant increases in the average amplitude of action potentials associated with changes in their duration in testosterone-treated specimens, but these data showed wide overlap with controls (cholesterol-treated and untreated animals; Bass and Volman, 1987). Thus, it seems unlikely that the changes in amplitude of Phases 2 and 3 of the EOD are linked to the direct effect of steroids on action potential amplitude (in sharp contrast to their robust effect on action potential duration; see Bass and Volman, 1987). It is more likely that changes in amplitude of the EOD phases are due to the interaction between current flow across the two faces, which in turn will be somewhat dependent upon the interval between excitation of the two faces (see Fig. 12 in Bennett and Grundfest, 1961; see also Bell, Bradbury, and Russel, 1976). Thus, in this more indirect manner, changes in membrane capacitance could also affect the amplitude of each phase of the EOD.

Qualitatively, steroid-induced changes in the relative amplitudes of the various phases of the EOD are seen in other species of mormyrids (Bass and Hopkins, 1983, 1985; Bass, 1986c; Landsmann and Moller, 1988; also see Hagedorn and Carr, 1985; Westby, 1988; and Mills, Zakon, and Bass, 1988 for gymnotifom fish). Indeed, a major species-typical feature of mormyrid EODs is the relative amplitude of each phase. The functional significance of such differences is not clear, although it may be related, at least in part, to the capacity of the electrosensory system to detect temporal features of the EOD waveform itself (Hopkins and Bass, 1981).

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