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Stable bellows cup electrode demonstrates low-frequency properties of long-term electroretinographic recordings in the *Limulus* lateral eye

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Abstract

Conventional methods have long been used to record electroretinograms (ERGs) from the surface of the lateral eye of *Limulus*, the horseshoe crab. But, using these methods, the convexity of this eye has sometimes led to electrode problems that deterred acceptance of the validity of unexpected and unfamiliar phenomena. To deal with the electrode problem, a new gel/bellows cup electrode has been devised which was created from a small bellows suction cup. Coated with a recording gel and positioned by a massive apparatus arrangement, it maintains a secure connection to the convexity of the lateral eye for many days without requiring any attention after its placement. This new electrode has now been used for thousands of hours of ERG research during which crabs have often been left undisturbed in the apparatus for many days. This new method has revealed the existence of a novel low-frequency phenomenon demonstrated by the occurrence of noise-like fluctuations in successive ERGs. The frequency of these fluctuations is low relative to the properties of the ERG itself. Several converging tests of this new electrode system indicated that this new phenomenon is not an artifact but rather a genuine expression of endogenous bioelectric events.

Keywords: *Limulus*; Horseshoe crab; Vision; Cornea; Potential; Electroretinogram; Electrode; Circadian rhythm; Secular variation; Neuromodulator

1. Introduction

The lateral eye of the horseshoe crab, *Limulus polyphemus*, has been recognized as a valuable preparation for investigating visual neurophysiology since Hartline and Graham (1932) recorded extracellularly from a single optic nerve fiber in this eye almost 75 years ago. The lateral eye is the main image-forming eye of this animal. It has a compound structure with about a thousand facets (or ommatidia) that collectively form a virtual image. Further important early discoveries were derived from subsequent intracellular recordings (Hartline, 1948) because this eye has large photoreceptors that are easily penetrated by microelectrodes. Early investigations in this preparation using both cellular techniques powerfully contributed to the understanding of many of the fundamental mechanisms of vision.

For this reason, a number of attempts were made to condition visually guided behaviors in *Limulus* so that modern psychophysical techniques could be brought to bear. However, what was thought to represent evidence of associative conditioning turned out to reflect the sensitization of an unconditioned response and subsequent behavioral work has accordingly been limited by the consequent absence of reinforcement control (cf. Wasserman and Patton, 1970).

By contrast, non-invasive corneal electroretinographic (ERG) techniques have been particularly useful in long-term investigations of the lateral eye's powerful circadian rhythm. The lateral eye ERG is a negative-going wave that is monophasic (Chapman and Lall, 1967). Owing to its relatively simple neural architecture, the lateral eye ERG does not have the complex waveform interactions that are found in ERGs produced by animals with more complex retinal circuitry. Instead, it is mainly a mass receptor response. And it has a simple dependence on stimulus intensity: *Limulus* ERGs show characteristic increases in amplitude and decreases in response latency as the total light-flash energy increases (cf. Wasserman and Cheng, 1996; Fig. 3 and Lucas et al., 2003, Fig. 2) as well as characteristic accelerations under light adaptation (cf. Lucas et al., 2003, Fig. 4) which are robust enough to be reliably manifested under different stimulating and recording conditions.

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It should be noted, however, that other things being equal, the recorded amplitudes of lateral eye ERGs actually express the operation of two factors: the size of the photoreceptor potentials that engender the ERGs and the relative impedances of the electrical pathways through which these signals pass en route to being recorded.

Similar effects can sometimes be observed in cellular studies in lateral eye slices when a microelectrode tip is placed outside a photoreceptor cell; such responses are about an order of magnitude larger than corneal ERGs but otherwise similar in waveform. When the microelectrode tip penetrates the photoreceptor, the shape of the even larger intracellular responses is the same but inverted. These properties make it very likely that mass photoreceptor potentials are the main contributors to ERG recordings, as has been previously concluded (Kass and Barlow, 1984).

The visual system of *Limulus* also exhibits a robust circadian rhythm, first demonstrated by Barlow et al. (1977a). It is expressed in a dramatic increase in the amplitude of visual responses at night. Severing the lateral optic nerve abolished this nighttime increase, which led to the conclusion that efferent fibers mediated the lateral eye circadian rhythm. Moreover, shocking the optic nerve stump produced the same effects as had been observed after endogenous activity, namely an increase in ERG amplitude that diminished when the optic nerve stimulation terminated (Barlow, 1983; Barlow et al., 1977b). This led to the early proposal that a pacemaker that controlled the circadian rhythm governing lateral eye sensitivity must reside in the brain.

Our further interest in the ERG developed when we began a program of research into the role of neuromodulators released by efference on the responses of the lateral eye to light. This program began with intracellular recordings taken from excised eye slices under perfusion and, because of our continuing interest in neural timing matters (reviewed in Bolbecker et al., 2002), we used brief light flashes separated by long intervals. This procedure demonstrated dramatic effects of octopamine on the temporal properties of receptor potentials in vitro. We particularly found (Lim and Wasserman, 2001) that octopamine radically prolonged receptor responses, an effect that had not been observed by others who had used very long steps of light which produced stimulus-bound responses which were prolonged ipso facto. This raised the question of whether comparable timing changes occurred in intact animals.

Because of the powerful circadian rhythm, such investigations required the use of a recording technique that could produce stable results for days. For similar reasons, other investigators had felt the need for such an improvement. As a result, a variation on the classical salt bridge electrode was devised by others in which a transparent seawater-filled chamber covered the entire eye as well as its surrounding carapace and served as a salt bridge electrode (Khadilkar et al., 2002; Pieprzyk et al., 2003, Fig. 1). Preferring to record from smaller regions of the cornea, we developed the more compact salt bridge electrode that is described below.

The present report is therefore partly concerned with the resolution of the methodological issues that arose in the course of refining the new ERG technique to meet this need. In addition, however, the present report also communicates that these improvements convincingly revealed that *Limulus* ERGs have certain endogenous low frequency properties that were not previously reported. These have been designated as DELFN, for reasons that will be explained below.

Preliminary presentations of various aspects of this work have been made at professional meetings. These include papers by Carlson et al. (2003), Bolbecker et al. (2005a) as well as by Bolbecker et al. (2005b).

2. Materials and methods

2.1. Animals

Mature *Limulus* with clear lateral eyes were obtained from the Supply Department of the Marine Biological Laboratory in Woods Hole, MA and maintained in a 570 l Marineland Commercial Aquarium filled with Instant Ocean (Aquarium Systems) artificial seawater. Salinity was kept at 1.025 and pH was adjusted to be near 8.0. Aquarium temperature was kept at 8 °C. Animals were fed clams once per week. Room lights came on at 0700 and went off at 1900, giving a 12 h light/dark cycle. A 75 l aquarium, which was maintained at room temperature, was used to bring animals into equilibrium with the ambient temperature prior to their use in an experiment. Crabs were taken from the cold tank and placed in the room-temperature tank at least 4 h prior to experimentation.

2.2. Test chambers

Three similar ERG recording stations were used. Animals were clamped into Plexiglas or metal cages that were placed inside lightproof chambers, where they were allowed to dark-adapt for 1 h before data were collected. The cages differed slightly in their materials and construction but each held its occupant motionless and moist material was always placed under the animals' ventral surfaces, where their gills are located.

These multiple recording chambers played an important role in the recognition of the endogenous nature of the DELFN findings reported below. Had only one chamber been employed, it would have been easier to speculate that exogenous factors were at work. But the fact that the new phenomena occurred in only one chamber at a time clearly ruled out such speculations.

Macintosh MacLab/400 (ADInstruments) interfaces were used to record ERGs. This system digitized bioelectric signals that were recorded by a differential bioamplifier. Its input leads were connected to a reference electrode that was near the eye and to a gel/bellows cup electrode (see below) that was placed directly on the eye. MacLab BioAmps (ADInstruments ML132) and Stimulus Isolators (ADInstruments ML180) allowed ERG recording and delivery of light stimuli to be controlled via Macintosh host computers. The BioAmp input filters were adjusted to provide a bandpass that extended from 0.1 to 100 Hz.

The MacLab stimulators delivered light flashes from super bright LEDs (Radio Shack 276-316) with peak emission at 478 nm. Dual 2.5 ms flashes separated by 75 ms were delivered