AN INVESTIGATION OF THE PHOTOTRANSDUCTION CASCADE AND TEMPORAL CHARACTERISTICS OF THE RETINA OF THE CUTTLEFISH, *SEPIA OFFICINALIS*

by

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A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

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In collaboration with
The Marine Biological Association

October 2003

ABSTRACT

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Title: An Investigation of the Phototransduction Cascade and Temporal Characteristics of the Retina of the Cuttlefish, *Sepia Officinalis*

Cephalopods have extremely well developed visual systems which are of particular interest due to the well known morphological similarity of the cephalopod eye to the vertebrate eye. This similarity ends at the level of the photoreceptors where vertebrates and invertebrates have been found to use different intracellular second messengers. Although the effect of extracellular ion manipulation on the light response has been examined and some very useful biochemical studies carried out, the pathway has not been investigated by the use of pharmacological intervention; a method which has proved to be useful in other preparations. This study examines various properties of the photoreceptors of the cuttlefish, *Sepia officinalis*, with particular interest in the second messenger signalling pathway. Both extracellular and whole cell patch clamp recording has been utilised.

The second messenger signalling pathway, which mediates phototransduction in the retina of *S. officinalis*, was investigated by recording the electroretinogram and examining how this changed with the application of various extracellularly applied, membrane permeable pharmacological agents. Invertebrate phototransduction utilises the phosphoinositide (PI) signalling pathway therefore specific activators and inhibitors targeted at precise sites of this pathway were applied to the extracellular bathing solution. These studies indicated that cleavage of phosphatidylinositol-4,5-bisphosphate is essential for the production of a light response and that the inositol trisphophate (IP$_3$) branch of this pathway is of greatest importance in this preparation, as opposed to the diacylglycerol branch.

How this second messenger cascade transfers the incoming information into a temporally coded signal was studied by measuring maximum critical flicker fusion frequency. The effect of cell size on this property was investigated and also how cell sensitivity was affected and whether these properties appeared to fit the animal's environmental conditions or whether they were restricted by cellular properties. The animals were found to have relatively "slow" eyes. However the younger age group studied, with shorter photoreceptors, was found to be both faster and more sensitive. This was an unexpected finding considering temporal resolving power is often sacrificed for sensitivity. It is suggested that the observed differences between age groups was attributable to the effects of increased cell size on the cell membrane time constant and that deterioration of signalling molecules with aging may also be a contributing factor.

An investigation of the cell signalling pathway at the level of individual cells was also carried out using the whole cell patch clamp technique. Using this technique, two voltage activated currents were found; an inward sodium current characterised by its voltage and tetrodotoxin sensitivity, and an outward potassium current characterised by its tetraethylammonium sensitivity. As well as finding further evidence for the involvement of the IP$_3$ branch of the PI pathway there is also evidence of a role for cyclic guanosine monophosphate. A suitable mode of measuring light-induced fluctuations in the intracellular calcium levels was also investigated with a view to observing the impact of the pharmacological agents on intracellular calcium concentration.

This investigation has enhanced the understanding of the *S. officinalis* visual system by greatly adding to the present knowledge of the second messenger signalling cascade and by giving an insight into how this transfers into the animal's temporal resolving power. Some preliminary information regarding the membrane currents activated by light has also been presented. This has all been possible by the development of a versatile retinal slice preparation that has been proven to be accessible to extracellular recording and whole cell patch clamp recording combined with pharmacological manipulation.

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$[Ca^{2+}]_e$	Concentration of extracellular calcium
$[Ca^{2+}]_i$	Concentration of intracellular calcium
2-APB	Diphenylboric acid 2-aminoethyl ester
4-BPB	4-bromophenacylbromide
AA	Arachodonic acid
ASW	Artificial seawater
Ca^{2+}	Calcium ion
cDNA	Complimentary deoxyribonucleic acid
CFASW	Calcium free artificial seawater
CFF	Critical flicker fusion frequency
CG-1	Calcium green 1
CG-5N	Calcium green 5N
cGMP	Cyclic guanosine monophosphate
Cm	Membrane capacitance
CPA	Cyclopiazonic acid
DAG	Diacylglycerol
DMSO	dimethylsulphoxide
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ERG	electroretinogram
GC	Guanylate cyclase
GMP	Guanosine monophosphate
G-protein	Guanine nucleotide binding protein
Gq	Gq type G-protein
Gt	transducin
GTP	Guanosine triphosphate
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2[ethanesulfonic acid])
IP_3	Inositol trisphosphate
IP_3R	Inositol trisphosphate receptor
K^+	Potassium ion
La^{3+}	Lanthanum ion
LY-83583	6-Anilinoquinoline-5,8-quinone
MBA	Marine Biological Association
mCFF	Maximum critical flicker fusion frequency
ML	Mantle length
Na^+	Sodium ion
NDGA	Nordihydroguaiaretic acid
OS	Outer segment
PDE	phosphodiesterase
PI	phosphoinositide
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMT	Photomultiplier
PUFA	Polyunsaturated fatty acid
Rm	Membrane resistance
SE	Standard error
SOC	Store-operated calcium channel
SOCE	Store-operated calcium entry

t_c	Critical duration
TEA	Tetraethylammonium chloride
t_{lat}	Latency
Trp	Transient receptor potential
Trpl	Transient receptor potential-like
TTX	Tetrodotoxin
U-73122	1-(6-[(17b]-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl)-1H-pyrrole-2,5-dione
Vmax	Maximum response amplitude
XeC	Xestospongin C
μW	Micro watts
τm	Cell membrane time constant

ACKNOWLEDGEMENTS

AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

A programme of advanced study was undertaken, which included training in various techniques such as *in vitro* extracellular electrophysiological recordings, light microscopy, fluorescence microscopy, cell dissociation and whole cell patch clamp recording.

Relevant scientific seminars and conferences were regularly attended at which work was presented.

Publications:

Nelson, L. & Williamson, R. (2003). The temporal resolution of the cuttlefish photoreceptors changes with growth. *Comparative Biochemistry and Physiology Part A.* 134: S74.

Meetings and Conferences Attended (in chronological order):

1. The Marine Biological Association. Student forum. 2000. Oral presentation

2. The Marine Biological Association. MBA Council Meeting. April 2000. Poster presentation: "Second messengers systems involved in phototransduction in cephalopods".

3. The Marine Biological Association. MBA Council Meeting. April 2001. Poster presentation: "Phototransduction in the cuttlefish retina: second messenger pathways".

4. International Conference on Invertebrate Vision, Lund, Sweden. 7-12 August 2001. Poster presentation: "Phototranduction in the cuttlefish retina: second messenger pathways".

5. University of Cambridge. 24-25 September 2001. 4th Young Physiologists Meeting, Cambridge. Oral presentation: "The cuttlefish photoresponse".

6. MBA seminar series, Plymouth marine fund. 2002. Oral presentation: "Phototransduction in the cuttlefish retina – the role of the phosphoinositide cascade".

7. University of Plymouth. Inaugural meeting of the Plymouth Institute of Neuroscience (PION). 2002. Poster presentation: "Inhibition of the cuttlefish photoresponse by 4-BPB and 2-APB".

8. The Marine Biological Association. MBA Council Meeting. April 2002. Poster presentation: as for PION meeting (see 7).

9. University of Southampton. SEB Meeting. 31 March – 4 April 2003. Poster presentation: "The temporal resolution of the cuttlefish photoreceptors changes with growth".

10. The Marine Biological Association. MBA Council Meeting. April 2003 Poster presentation: as for SEB meeting (see 9).

Courses Attended:

Cell Physiology Workshop

CRAC course / Graduate Schools Residential Workshop

Signed... Lisa Nelson

Dated... 10 October 2003

CHAPTER 1: General Introduction

The invertebrates possess a wide variety of eye types reflecting the varied environments in which these animals live, ranging from the simple pin-hole eye of the *Nautilus* to the compound eye of insects to the single chamber, camera type eye used by coleoid cephalopods and indeed by the vertebrates (Land & Nilsson, 2002). The cephalopods are one of the most developed classes of invertebrate and in particular they have an extremely well developed visual system. The cephalopod eye is morphologically very similar to the vertebrate eye consisting of the same major components i.e. lens, iris and cornea. This is often used as an excellent example of convergent evolution (Packard, 1972). In *Sepia officinalis*, ~70% of the brain is devoted to visual processing (Young, 1988), reflecting the importance of vision to these animals which live by hunting and require sight to locate their prey.

As well as its morphological similarity to the vertebrate eye, the cephalopod eye's performance is also comparable with that of the vertebrate eye. The eye has the capability to form a sharp image at both long and short distances and to adjust to large changes in light intensity (Budelmann et al., 1997). In the aquatic environment, animals cannot use a curved cornea to form an image as is commonly used in the terrestrial environment. This is due to the fact that the refractive indexes of the media on both surfaces of the interface are approximately the same; therefore a cornea would provide no advantage. Therefore all of the focusing power is in the lens (Land, 1990). The lens of a cephalopod contains a gradient of refractive index, ranging from $n = 1.33$ in the periphery, to $n = 1.52$ in the centre (Sivak, 1982 squid), this allows the lens to have a focal length to lens radius ratio of 2.5 which conforms to Matthiessen's ratio (Pumphrey, 1961). The spatial resolution of an eye, i.e. its ability to resolve fine detail, is dependent on the packing density and diameter of its photoreceptor cells. This has been measured as 1.7 minutes of arc in *S. officinalis* (Farley, unpublished) which is comparable to the human retina whose visual acuity is 0.5

minutes of arc under optimal conditions (McIlwain, 1996). So it can be seen that these eyes are equally capable of forming a sharp image, over a range of light intensities, with a comparable resolving power to the vertebrate eye.

The most likely underlying signalling cascade utilized by the cephalopod phototransduction cascade, or at least parts thereof, is commonly used throughout the animal kingdom to mediate various physiological processes. The signalling cascade in question is the phosphoinositide (PI) cascade, which is shown diagrammatically in Figure 1.1. This cascade has been found to be employed in cell growth, fertilization, digestion and also in other sensory systems (Berridge, 1993). Evidence relating to the use of this cascade in cephalopods is presented in Section 1.4 and Section 2.1. The *S. officinalis* retinal preparation could potentially provide a relatively simple model for its investigation due to the presence of only one type of photoreceptor and the ease of control over the characteristics of the stimulus as has been the case for the use of vertebrate photoreceptors in the investigation of the cyclic guanosine monophosphate (cGMP) signalling system (Stryer, 1991). Additionally, this would be particularly attractive due to the biochemical accessibility of this preparation which has already produced the majority of the evidence for the use of the PI pathway, some of which will be described later.
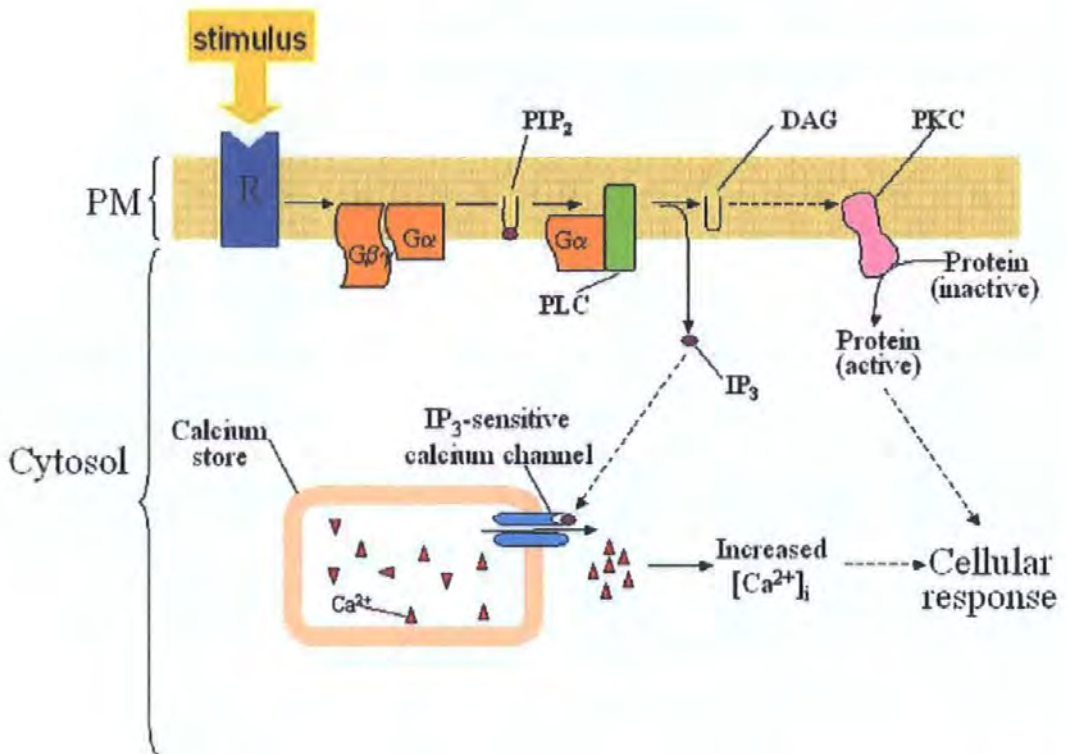
Figure 1.1 **A diagrammatic representation of the phosphoinositide (PI) cascade. The action of an external stimulus on its specific receptor leads to activation of a G-protein, which consists of α, β and γ subunits. When a G-protein is activated, it undergoes a conformational change allowing the activated α-subunit to dissociate from the other subunits. The α-subunit then activates the enzyme PLC which then cleaves the membrane phospholipid PIP_2, releasing DAG which activates PKC, and IP_3 which causes the release of Ca^{2+} from internal stores. Both active PKC and Ca^{2+} then exert various cellular effects. The end cellular response is dependent on which stimulus has acted on which cell type. G – G-protein type q, α, β, γ – subunits of the G-protein, PLC – phospholipase C, PIP_2 – phosphoinositide bisphosphate, DAG – diacylglycerol, PKC – protein kinase C, IP_3 – inositol trisphosphate (Figure compiled and adapted from information sourced from (Berridge (1993), Lodish et al. (1995) and Voet & Voet (1995)).**

1.1 Overview of phototransduction

Phototransduction is the process whereby incident light acts on photoreceptor cells, which are cells specialised to convert light to an electrical signal, ultimately resulting in cellular hyperpolarisation or depolarisation. It is the absorption of a photon by the photopigment rhodopsin which ultimately leads to a change in cell membrane potential and production of the visual signal. The phototransduction process consists of excitation, adaptation and

deactivation. Excitation involves the photochemical cycle of rhodopsin, the subsequent enzyme cascade and the activation of ion channels by intracellular messengers. Adaptation occurs when prolonged or bright illumination adapts the photoreceptor by reversibly reducing the light-activated membrane permeability, in other words the regulation of sensitivity to light. This allows these cells to have a broad dynamic range. The deactivation phase is the process of response shut down, also known as inactivation and various mechanisms are employed in this stage. The efficiency and rate of inactivation of the cascade will determine whether a ready supply of transduction intermediates is available and therefore influence the temporal resolving power of the photoreceptor. The more quickly the cell is returned to its resting state, the sooner it can be activated again and hence the greater the temporal resolving capacity. The cascade of events involved in these component processes is well understood in vertebrate cells however in invertebrates it has proved more difficult to elucidate and there remain many gaps in the knowledge as will be explained in the subsequent two sections.

1.2 Phototransduction in other animals

1.2.1 Vertebrates

The process of phototransduction in vertebrates is best understood in vertebrate rod cells as they are large, and easy to isolate (Saibil, 1986). The vertebrates use a cGMP mediated transduction cascade which results in cellular hyperpolarisation (for review see Stryer (1991)). The activation and inactivation of this process is shown diagrammatically in Figure 1.2.

Rhodopsin

↓ light

*Rhodopsin ⟹ Gt ⟹ *Gt ⟹ PDE ⟹ ↓cGMP

↓

Channel opening ⟸ ↑cGMP ⟸ GC ⟸ ↓[Ca²⁺]ᵢ ⟸ Channel closure

↓

Hyperpolarisation
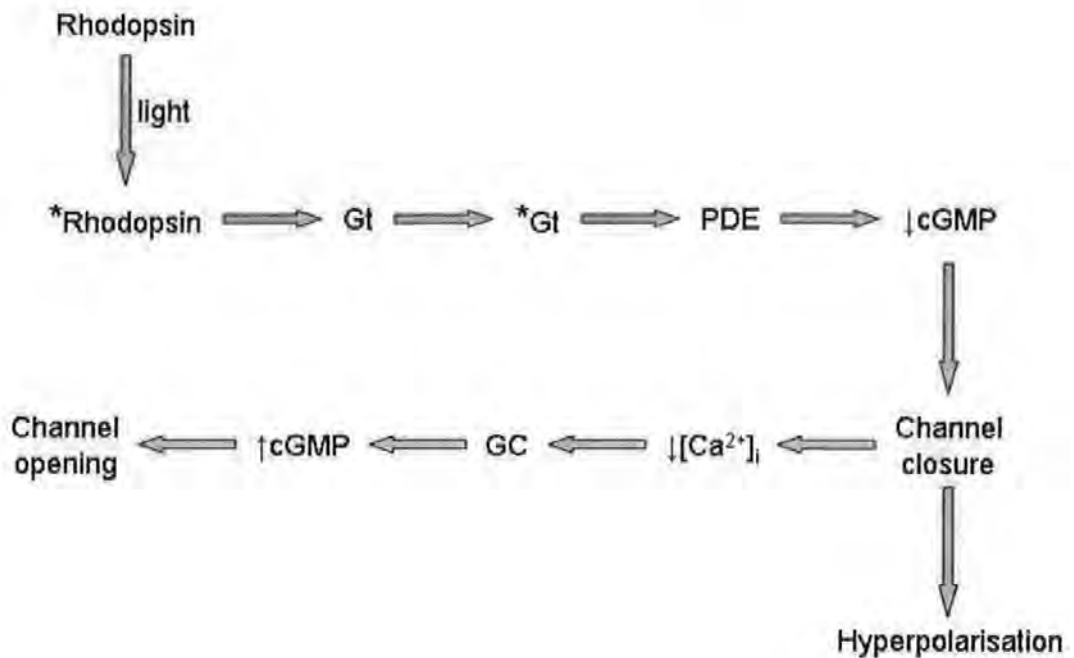
Figure 1.2 Activation and inactivation of vertebrate phototransduction (adapted from Stryer (1991)). This process is described in the text. * - activated form, Gt – transducin, PDE – phosphodiesterase, GC – guanylate cyclase.

In the dark, high intracellular levels of cGMP keep sodium channels open and the cell in a depolarized state. These sodium channels are distinct from those in normal nerve cells in that they are voltage insensitive, very small and less cation selective (Saibil, 1986). When a light stimulus is applied, rhodopsin undergoes a conformational change to its active form metarhodopsin, which leads to activation of cGMP phophodiesterase (PDE) via the guanine nucleotide binding protein (G-protein), transducin (Gt). cGMP hydrolysis lowers the intracellular level of cGMP resulting in cellular hyperpolarisation due to closure of the cation channels. Inactivating Gt and the rhodopsin molecules shuts down the cascade. The intrinsic guanosine triphosphatase activity of the G-protein α-subunit hydrolyses bound guanosine triphosphate to guanosine diphosphate, returning Gt to its resting state. Rhodopsin is inactivated by a number of mechanisms including phosphorylation by rhodopsin kinase, followed by binding of arrestin, which hinders activation of G-protein. cGMP levels are also influenced by the intracellular calcium concentration whereby cGMP

levels increase in low calcium and decrease in high calcium. This is because PDE activity increases at elevated calcium levels (Kawamura & Bownds, 1981) and guanylate cyclase (enzyme which synthesises cGMP) activity is reduced (Fleischman & Denisevich, 1979).

1.2.2 Other invertebrates

The two invertebrates, in which visual transduction has been most extensively studied are the fruit fly, *Drosophila melanogaster* and the horseshoe crab, *Limulus polyphemus*. The information that has already been obtained in these animals will act as a useful guide in the investigation of the transduction cascade in *S. officinalis*. Evidence has been emerging that these animals utilize distinct branches of the PI signalling cascade (see Figure 1.1). In overview, it has been accepted that both branches follow the same initial pathway which involves activation of a G-protein by rhodopsin resulting in activation of the enzyme phospholipase C (PLC) causing cleavage of the membrane phospholipid, phosphatidyl inositol bisphosphate (PIP$_2$), producing diacylglycerol (DAG) and inositol trisphosphate (IP$_3$). The PI cascade is triggered by a G-protein type receptor in this system and in many others, but it is worth noting that it can also be activated by tyrosine-kinase type receptors (Rang et al., 1996). As mentioned above, this is a widely used signalling cascade, however it is evident from the following discussion that there is more than one way in which it can be utilized by cells.

Recent evidence suggests that *D. melanogaster* uses the DAG branch of the signalling pathway to open light activated ion channels. DAG is a potential precursor for polyunsaturated fatty acids (PUFAs) and it has been shown that PUFAs are capable of opening the ion channels that are activated by light in *D. melanogaster* photoreceptors (Chyb et al., 1999). Further, it has been shown that the IP$_3$ branch is not necessary for vision, as a mutant lacking the IP$_3$ receptor is not blind (Acharya et al., 1997; Raghu et al.,

2000). Light stimulation results in the opening of two types of cation channel, one of which has a high permeability for calcium (Ca^{2+}) ions (Hardie, 1991). Studies in *D. melanogaster* are probably closest to finding the answer to the complete phototransduction pathway, however there are still some unknowns. Exactly how DAG is converted to / produces the correct PUFA required to open the membrane ion channels has still to be demonstrated directly in the preparation.

In *L. polyphemus* ventral photoreceptors the other product of PIP_2 cleavage appears to be utilized, namely IP_3. This second messenger causes an increase in intracellular calcium levels by binding to its receptor on the intracellular calcium stores causing calcium channels to open. Although the timing of the increase in intracellular calcium is closely correlated with response activation (Payne & Demas, 2000), calcium does not appear to directly open membrane ion channels (Bacigalupo et al., 1991). Hence, there is a missing link between the light stimulus and cellular depolarization. The light-activated ion channels have however been shown to be opened by the cyclic nucleotide, cGMP (Bacigalupo et al., 1991), thus more recent research has focused on finding a link between calcium and cGMP. The present evidence suggests that the changing levels of intracellular calcium may act by controlling cGMP metabolism (Shin et al., 1993; Johnson & O'Day, 1995). So far, IP_3 induced calcium release, although important for response amplification, does not appear to be capable of opening the membrane ion channels. However, cGMP can and to date it is not known if cGMP levels are controlled intracellular calcium levels, or by another means. Also, there is still the possibility of a parallel pathway, as the effects of IP_3 / Ca^{2+} / cGMP have still not been shown to be exclusively responsible for the light response.

1.3 The cephalopod eye

As can be seen in Figure 1.3A, the gross physical organization of the coleoid cephalopod eye closely resembles that of vertebrates, and consists of the same major components, i.e. lens, iris and cornea. The retina is orientated so that the photoreceptors are directly exposed to the incident light (Young, 1962; Yamamoto et al., 1965) and consists of only one type of neural cell, the photoreceptor. The simplicity of this arrangement is similar to that of a single insect ommatidium. Although containing only photoreceptors, and supporting cells, the cephalopod retina (Figure 1.3B) can be divided into three layers: the outer or distal segment, which is found proximal to the 5 μm limiting membrane (Zonana, 1961) and containing the retinal cell outer segments and supporting cells; the inner or proximal segment containing the retinal cell bodies and blood capillaries; and below this is the plexiform layer. Just below the plexiform layer, the inner segments taper to form axons of 0.1–1.5 μm diameter (Cohen, 1973; Karita & Tasaki, 1973). These axonal fibres come together to form the optic nerves, which make up the optic chiasma before entering the optic lobe.

A   **B**

Ciliary muscle   Iris   Lens   Cornea

rhabdomere   limiting membrane

retinal cell

supporting cell   basement membrane

retinal cell body

Retina

Optic nerves   glial cell
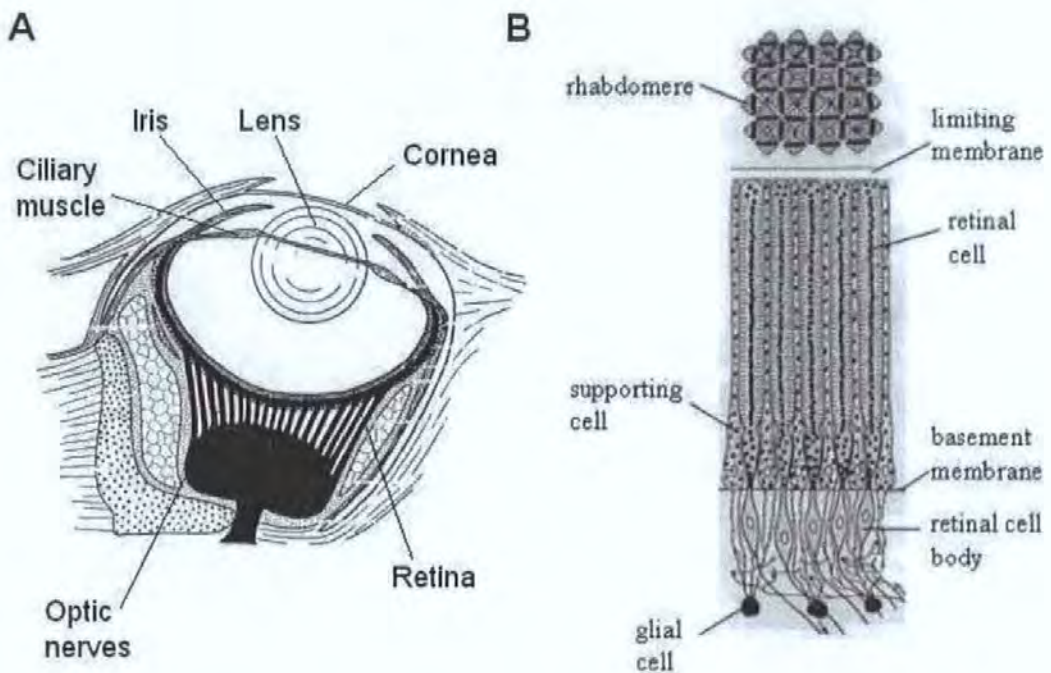
Figure 1.3 Diagrams of the cuttlefish eye (A) and the cephalopod retina (B) taken from Tompsett (1939) and Young (1971) respectively. The eye closely resembles the vertebrate eye, however the retina is functionally different. The retina consists of only one type of photoreceptor with its distinctive microvillar membrane in the outer segment. A layer of pigment granules is found at the basement membrane, which marks the boundary between outer and inner segments. Note that these diagrams are not to scale.

The photoreceptor cells are between 4 and 10 μm in diameter (Budelmann et al., 1997), and from 200 to 800 μm long (Young, 1963; Messenger, 1981; Michinomae et al., 1994). The photoreceptor outer segments are composed of a central core with two rows of finger-like projections known as microvilli at right angles to this core and on opposite sides (Yamamoto et al., 1965; Budelmann et al., 1997). The microvilli are grouped into structures known as rhabdomeres that are the photosensitive structures containing the photopigment rhodopsin. It has been shown that the rhabdomeres of four adjacent cells come together radially to form structures called rhabdomes in such a way that each cell contributes to two rhabdomes (Moody & Parriss, 1961). Pushing up between the photoreceptor outer segments are the supporting cells that are wholly found in the outer

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layer. These cells are fewer in number than the receptor cells and are thought to either secrete the limiting membrane or actually form it (Zonana, 1961). The supporting cells contain a screening pigment that is involved in retinal cell adaptation. Screening pigment is also found within the photoreceptors and this is found densely packed in an area called the spindle region by Young (1962). The spindle region is the part of the outer segment, next to the basement membrane, which lacks microvilli. This screening pigment migrates distally when exposed to light and retreats proximally towards the basement membrane in the dark (Hagins & Liebman, 1962; Daw & Pearlman, 1974).

1.4 Phototransduction in cephalopods

The discovery that the PI pathway has been utilized differently by different types of photoreceptor makes it interesting to learn which is used by the cephalopods or to see if they employ a different method altogether. So far, the existing information on cephalopod transduction has resulted almost exclusively from biochemical and molecular investigations (discussed in detail in Section 2.1). For these types of investigation the cephalopod retina has proved to be an excellent preparation due to the relative simplicity of the retina (see Figure 1.3 above) and the ease with which the outer transducing layer could be isolated for biochemical analysis. Additionally, early investigations into the effects of changing the composition of the extracellular bathing solution have been carried out (Hagins & Liebman, 1962; Duncan & Weeks, 1973; Clark & Duncan, 1978). In this study the assumption has been made that all cephalopods use the same second messenger signalling cascade to mediate their response to light. Therefore all evidence relating to phototransduction, in any cephalopod species, will be examined and used as the basis for investigation into *S. officinalis* phototransduction.

To date investigations have identified Gq as the principal G-protein type present in the retinal photoreceptors, including *S. officinalis* (Schraermayer et al., 1995). A PLC has been identified with PIP_2-hydrolytic activity and can be stimulated by active Gq (Suzuki et al., 1999). A role for cGMP has also been identified (Huppertz, 1995; Brown & Kelman, 1996) suggesting that the cephalopod photoreceptor uses a transduction method either the same as, or closely the related to, that used by *L. polyphemus,* as opposed to the *D. melanogaster* pathway. Additionally, calcium appears to play an important and complex role as it has been shown to be necessary for response inactivation and to play a role in controlling the photoreceptor sensitivity (Duncan & Weeks, 1973; Clark & Duncan, 1978). Many investigations have also indicated possible feedback mechanisms involving Ca^{2+}, IP_3 and PLC, which are thought to have a role in response inactivation (Fyles et al., 1991; Kishigami et al., 2001; Mayeenuddin et al., 2001). However, the complete pathway has yet to be elucidated. The evidence to date will be discussed in more detail in the introduction to Chapter 2 (Section 2.1). To add both pharmacological and electrophysiological data to the existing information would help to build up a more complete picture of cephalopod phototransduction and also demonstrate that other experimental techniques can be applied to this potentially very useful preparation.

1.5 Temporal properties

One of the ways in which the efficiency of the retina and the reaction kinetics of the underlying cellular mechanisms can be investigated, is by examining its temporal response properties. The ability of the individual cells to recycle the messengers used in phototransduction will have a strong bearing on the retina's temporal characteristics, as will the speed with which the incoming visual information is transferred into an electrical signal for processing. The temporal resolution can be determined by measuring the

maximum critical flicker fusion frequency (mCFF). This measurement has only been made once in a cephalopod species, and this was in the intact, anaesthetized octopus. This measurement of a cephalopod's mCFF produced a value of 72 Hz (Hamasaki, 1968); this is a relatively low value in comparison to the fast eyes of flies which can easily resolve images moving at frequencies in excess of 100 Hz (Laughlin & Weckstrom, 1993) and it could be that their different methods of transduction may influence this characteristic. A recent study carried out in this lab (Farley, unpublished) discovered that there is a large increase in photoreceptor length as *S. officinalis* grows. The size of the photoreceptor cells is thought to influence the temporal abilities of the retina, hence a study looking at two different age groups would be pertinent, to take account of differences in cell size. Why this is thought to be so is discussed extensively in Chapter 3.

1.6 Aims and objectives

This study will look at how *S. officinalis* uses this eye to transfer incoming light signals into information by examining some of the properties of the eye's light sensitive cells. The intracellular second messenger cascade of the photoreceptors will be investigated as well as the retina's temporal properties. In doing so it will be possible to determine how the light signal is converted to an electrical signal and to obtain an estimate of how long this process takes to complete a full cycle. This can be compared to the mechanisms used by other invertebrate species as well as the vertebrates, which use such a similar eye design. It is becoming apparent that there is no one common phototransduction mechanism utilized by invertebrates therefore it will be interesting to see if these mechanisms are as varied as the types of eyes, or if cephalopods use an intracellular cascade common to other species. This study will therefore address the following four aims, which will then be examined by more specific objectives.

Aim 1: To describe the second messenger signalling cascade by the use of extracellular application of pharmacological agents to the intact cells of the retinal preparation.

Objectives:

- To determine whether the action of PLC is essential for the *S. officinalis* light response by the use of pharmacological inhibitors.

- To determine whether *S. officinalis* utilise the IP_3 or DAG branch of the PI pathway.

- To identify the role played by calcium in the *S. officinalis* phototransduction pathway.

Aim 2: To investigate the temporal resolving power of the retina and the sensitivity and to determine how cell size influences these parameters.

Objectives:

- To determine the maximum CFF for this animal to obtain an indication of temporal resolution.

- To determine whether the temporal resolution changes as the animal grows from juvenile to adult.

- To measure threshold sensitivity and response latency in both juvenile and adult animals.

- To determine how the levels of background illumination affect the temporal resolving power of the *S. officinalis* retina.

Aim 3: To investigate the fluctuations in intracellular calcium concentration induced by light and to ascertain the role of intracellular calcium in phototransduction.

Objectives:

- To load the retinal slice preparation with fluorescent calcium indicator dye.

- To measure a light-induced change in intracellular calcium concentration by using membrane permeable fluorescent calcium indicators.

- To determine the effect of some of the pharmacological agents used in Chapter 2, on the intracellular free calcium concentration.

Aim 4: To investigate the electrophysiological properties of individual photoreceptors and to further study the signalling cascade by direct intracellular application of pharmacological agents.

Objectives:

- To develop a successful preparation for the reliable use of the whole cell patch clamp technique in order to study the properties of the cuttlefish photoreceptors.

- To use the whole cell patch clamp technique to record membrane currents and to characterize them using various voltage pulse protocols and pharmacological intervention.

- To record cell capacitance in order to estimate cell surface area.

- To record light induced currents in these isolated cells and to submit these currents to pharmacological manipulation in order to further our understanding of the phototransduction cascade.

- To identify the terminal intracellular signalling molecule in the phototransduction cascade that is responsible for opening the light-activated ion channels.

CHAPTER 2: An Investigation of the Extracellular Electrophysiology and Pharmacology of the *Sepia officinalis* Retina

2.1 Introduction

The primary event in visual processing is the transduction of the external signal by photoreceptors into an electrical signal which can be interpreted by processing centres in the brain. Phototransduction occurs in the retina and is the process whereby photons of light are converted into a change in the ionic permeability of the cell membranes. This is mediated via a second messenger signalling pathway, the first step of which is the absorption of a photon of light by the visual pigment rhodospin. Rhodopsin is found entirely in the outer segments of the photoreceptor cells localised to the microvillous membrane of the rhabdomes (Hara & Hara, 1976). Rhodopsin is a conjugated protein composed of the chromophore retinaldehyde bound to a protein moiety opsin, which is species specific (Hubbard & St. George, 1958; Messenger, 1991). In the resting state, i.e. in the dark, the retinaldehyde exists in the 11-*cis* form which, when exposed to light, undergoes a conformational change to the all-*trans* configuration (Messenger, 1981, 1991). This is the active form and this triggers the intracellular signalling cascade that leads to cellular depolarisation in response to light.

2.1.1 The electroretinogram

The simplest way to study the electrophysiological properties of the cephalopod retina is to employ the electroretinogram (ERG). The ERG is an extracellular recording of the electric field potential change, not a recording from an individual cell. When the photoreceptor is activated it is nonuniformly polarised, i.e. different parts of the cell are at different potentials. These spatial nonuniformities cause the flow of current through the extracellular solution. The photoreceptors effectively act as an electric dipole, where the outer segment is an active current sink and the inner segment is a passive current source. This is what is measured when we look at potential change / difference. These potential differences can be complex in that they vary with space and time.

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By recording the potential change in response to a light stimulus it is possible to look at how the ERG recording changes under different physiological conditions. Figure 2.1 illustrates some of the different parameters that we can measure from the ERG. We can then compare the values of these parameters when exposing the tissue to different conditions to give an indication of how, for instance, a chemical exerts its effect on the light response.
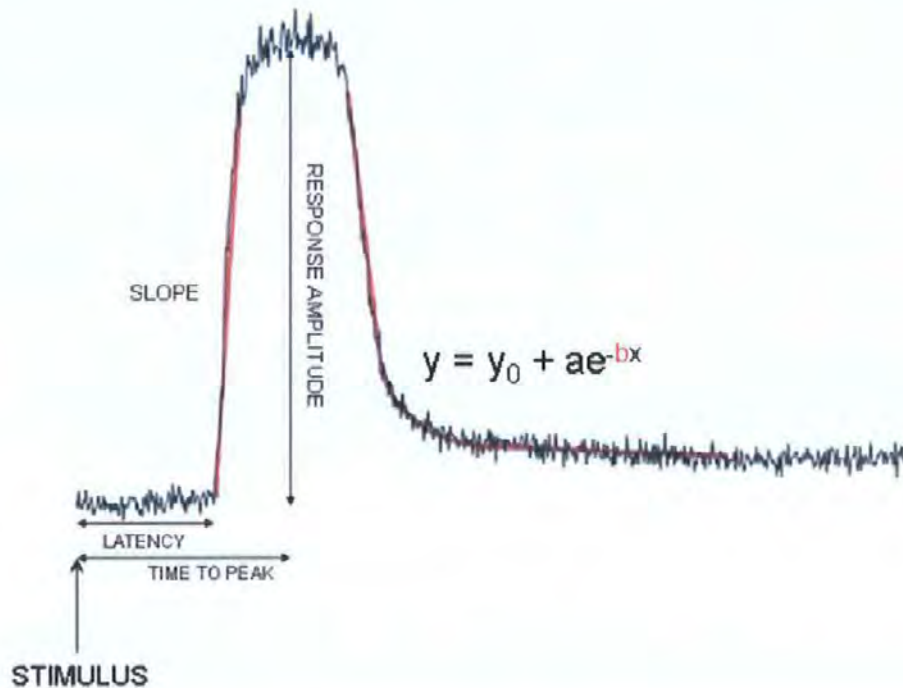
Figure 2.1 Schematic of an ERG based on Rayer et al. (1990). Some of the parameters used to analyse the ERG are shown. Latency and time to peak are measured from the start of the stimulus to the start and the peak of the response respectively. The slope is taken from the linear part of the rapid rising phase and the exponential decay is fitted with the above equation where 1/b is the decay constant.

Latency is the time from the starts of the light stimulus to the start of the light response. Measuring latency gives an indication of the time it takes for the threshold number of photons to be absorbed to initiate the response. Slope of rise phase indicates the level of amplification / the speed at which the cells can convert the light signal into an electrical signal via the internal second messenger cascade. Response amplitude is indicative of the number of ion channels opened or closed by the terminal second messenger. Finally, decay constant measures the rate of response inactivation.

2.1.2 Transduction

The existing information concerning the transduction cascade in the cephalopods is somewhat limited compared to what is known about *L. polyphemus* and *D. melanogaster*. The present knowledge is mainly the result of biochemical and molecular studies as a preparation lending itself to electrophysiological examination of phototransduction is not in use. The evidence to date will be discussed in the following sections.

2.1.2.a G-proteins

The major plasma membrane G-protein types utilised in physiological systems are Gs, Gi, Gq and Gt. Gs and Gi both influence the same membrane enzyme, adenylyl cyclase which catalyses the conversion of ATP to cAMP; Gs is stimulatory and Gi is inhibitory (Rang et al., 1996). Gt, also known as transducin, is the G-protein utilised by vertebrate photoreceptors which leads to the activation of cGMP phosphodiesterase (Saibil, 1986). The mechanism of action of Gq is as follows. The enzyme phospholipase C (PLC) is triggered causing it to cleave phosphatidylinositol bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and diacylglycerol (DAG), two cellular signalling molecules. G-protein inactivation involves the activity of GTPase, an enzyme which hydrolyses GTP, returning the G-protein to its resting state. GTPase activity has been observed in octopus (Calhoon et al., 1980) and squid (Saibil & Michel-Villaz, 1984; Vandenberg & Montal, 1984). Later, cloning experiments identified cDNA clones corresponding to eye-specific G-protein subunits from squid (Pottinger et al., 1991; Ryba et al., 1991) and the existence of up to three distinct, light sensitive G-proteins has been reported (Robinson et al., 1990; Tsuda & Tsuda, 1990; Fyles et al., 1991).

Suzuki et al. (1993) ruled out the presence of Gt in the cephalopod phototransduction cascade. Their study using both *Octopus vulgaris* and *Todarodes pacificus* suggested Gq

as the major G-protein in photoreceptor membranes. Their method used polyclonal antibodies and did not exclude the possible involvement of a Gs-type G-protein. It has been suggested that the multiple G-protein types that have been detected are very minor components of the photoreceptor response and that Gq is the major G-protein involved (Nobes et al., 1992). Many other studies have identified Gq as the predominant candidate for the most abundant G-protein in the rhabdomeres. This has been found by Pottinger et al. (1991) who partially purified G-protein from squid photoreceptor membrane and whose sequence suggested Gq-type. Ryba et al. (1993) found similar results when investigating the squid *Loligo forbesi*. Light stimulation has been shown to promote guanine nucleotide binding to squid photoreceptor Gαq (Nobes et al., 1992). Additionally, using antibodies, Gq has been identified in both *S. officinalis* (Schraermayer et al., 1995) and *Octopus vulgaris* and the Japanese flying squid *Todarodes pacificus* (Suzuki et al., 1993). Finally, the production of a cDNA library from *Octopus vulgaris* eye (Iwasa et al., 2000) found four classes of G-protein α subunits however Gq expression was by far the most abundant.

2.1.2.b The phosphoinositide (PI) pathway

As already stated, Gq activates the enzyme PLC which cleaves membrane bound PIP_2 into IP_3 and DAG. This proposed rhodopsin / Gq / PLC cascade is supported by the results of Baer & Saibil (1988) who observed light stimulated PIP_2 hydrolysis and IP_3 release in the squid (*Alloteuthis subulate*). A PLC has now been identified in the Japanese squid, *Watasenia scintillans*, which has PIP_2-hydrolytic activity stimulated by an active form of Gqα (Suzuki et al., 1999). There have also been various other studies in squid photoreceptors where a light has been shown to result in IP_3 production (Szuts et al., 1986; Brown et al., 1987; Wood et al., 1989). Finally, in common with *L. polyphemus*, there is evidence of a role for cGMP in cephalopod phototransduction. Saibil (1984) found that exposure of squid retina to light led to an increase in intracellular cGMP levels. This issue,

however, remains controversial and will be examined more closely in the relevant section of this chapter's discussion.

Experiments on *Sepiola atlantica*, where the ionic composition of the extracellular bathing solution has been altered also give us some information about the response to light. Duncan & Weeks (1973) concluded that calcium ions are essential for the recovery process. Calcium has also been shown to affect response sensitivity. Low calcium increased sensitivity initially, but following 10-15 minutes exposure, light sensitivity was reduced irreversibly (Clark & Duncan, 1978). Calcium's effect on sensitivity was also found to be dependent on the intensity of the stimulus. Replacing the sodium in the extracellular solution dramatically reduced response amplitude, however a small response could still be elicited (Clark & Duncan, 1978). This shows that the majority of the current was carried by sodium ions, but that there was also a small proportion carried by another ion.

The evidence presented has shown that, in cephalopods, there is light induced hydrolysis of PIP_2, by PLC, resulting in release of IP_3. Due to the known path of this reaction, one can state that DAG would also be released as a result of the action of PLC. There has however been no measure of a light induced increase in intracellular calcium levels. It is however expected that IP_3 will release calcium from internal stores, as this is its known action, as previously stated (see Figure 1.1) and it is also possible that calcium will enter from the extracellular solution. Evidence so far does not give any indication as to whether the action of IP_3 or DAG, or both, is responsible for the light response in the cell membrane. Even although it is known that there is an increase in IP_3 and a decrease in levels of PIP_2 following light stimulation, this does not prove that IP_3 is involved in producing the light response or indeed, that the action of PLC is necessary, just because it does occur. For instance, $[IP_3]$ increases in the *D. melanogaster* retina following light stimulation, however

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it is not involved in production of a light response (Acharya et al., 1997; Raghu et al., 2000a).

By taking electrophysiological recordings and observing the effect of various pharmacological agents (as outlined in Table 2.1) it will be possible to determine which intracellular signalling molecules actually have a direct influence on the cellular response to light. The existing knowledge of phototransduction in *L. polyphemus* and *D. melanogaster*, as outlined in the general introduction, will be used as a guide to direct this investigation. This chapter addresses the hypothesis that cephalopod phototransduction utilises the IP_3 branch of the PI pathway and that calcium plays an important regulatory role.

Chemical	Action
TTX	Voltage sensitive Na^+ channel blocker
4-BPB	PLC inhibitor
2-APB	IP_3R inhibitor
XeC	IP_3R mediated calcium release
CFASW	Eliminates Ca^{2+}_e
Co^{2+}	Ca^{2+} channel blocker
La^{3+}	Ca^{2+} channel blocker
CPA	Ca^{2+}ATPase inhibitor
Thapsigargin	Ca^{2+}ATPase inhibitor
CFASW + CPA	Removes calcium sources
(-)-Indolactam V	PKC activator
Chelerythrine chloride	PKC inhibitor
NDGA	Lipoxygenase inhibitor
8-Br-cGMP	Membrane permeable cGMP analogue
DB-cGMP	Membrane permeable cGMP analogue
LY-83583	Soluble guanylate cyclase inhibitor

Table 2.1 Table listing the pharmacological agents used in this study along with their action as stated in the Sigma chemicals catalogue.

The objectives for this chapter, as outlined in the general introduction, are as follows:

- To determine whether the action of PLC is essential for the *S. officinalis* light response by the use of pharmacological inhibitors.

- To determine whether *S. officinalis* utilise the IP_3 or DAG branch of the PI pathway.

- To identify the role played by calcium in the *S. officinalis* phototransduction pathway.

2.2 Materials and Methods

2.2.1 Animals

The common cuttlefish, *S. officinalis*, was used in this study. Adult animals were caught offshore near the coast of Plymouth, UK and transported to laboratory holding tanks at the Marine Biological Association (MBA). Tanks contained circulating aerated seawater maintained at $18°C$. *S. officinalis* were given live crabs (*Carcinus maenas*), one per day. Some of the animals caught in the wild were not initially used for experimentation and instead were used for breeding. Animals have been successfully bred over 2 generations. There was therefore a large range of animal sizes available and both wild and captive bred animals were used in these experiments. All the results presented in this chapter are from experiments performed in juvenile animals.

2.2.2 Slice preparation

Prior to an experiment an animal was dark adapted by being left in a dark room with only dim red light for approximately 30 minutes. It was then anaesthetised with 2 % alcohol in seawater and decapitated under dim (6 μWcm^{-2}) red light. All subsequent manipulations were also carried out under dim red light. The eyes were removed and then hemisected in cold calcium free artificial seawater (CFASW). Once the sclera was removed from the back of the eye, the retina was cut into ~1 cm^2 pieces.

One of these was then cut into 300 μm wide strips through the following procedure and the remainder kept in oxygenated CFASW on ice for later use. The retina piece was floated onto a glass slide with the inner segment upward and excess solution was blotted off the slide. A Millipore membrane filter (HABG02500) was gently placed on the retina and then turned over to expose the attached retina with the outer segments now upwards. The

filter with the attached retina was then sliced using a McIlwain mechanical tissue chopper. The use of the Millipore filter was thanks to the recommendation of Dr David Becker (University College London, UK). A slice was then positioned in a recording dish of 2-3 ml volume, using the Millipore, on its side so that the length of the photoreceptors was visible. This was held down by a slice anchor and perfused with artificial seawater (ASW) at a flow rate of ~2 ml min^{-1}. The set up of the recording dish is shown diagrammatically in Figure 2.2.
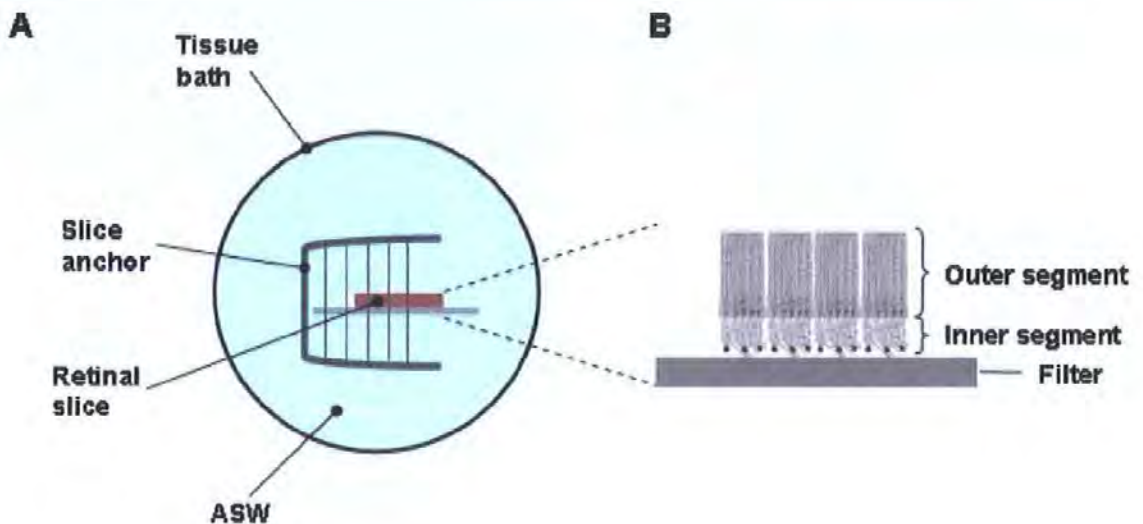
Figure 2.2 Diagrammatic representation of the recording dish and the retinal slice preparation. Part A shows an overhead view of the recording dish containing a retinal slice on filter held down by a slice anchor. Part B shows an expanded view of the slice preparation showing clearly how the slice is orientated on the filter and indeed in the dish. It can be seen that the inner segment containing the cell bodies is nearest to the filter and the outer segment furthest away, with the whole length of the cells visible to the experimenter.

2.2.3 Fixing

For photography, retinal slices were taken at the appropriate stage, fixed in 4% paraformaldehyde and kept, wrapped in foil, in the fridge for processing later. Slices were kept no longer than 2 days before processing. Dehydration was performed using, in succession, 30%, 50%, 70%, 90% and 100% (twice) alcohol for ~10 minutes each. The slices were then cleared using methyl salicylate and then photographed using a digital camera.

2.2.4 LIVE/DEAD® Viability/Cytotoxicity Assay Kit

The viability of the slice preparation was determined before commencing work on this preparation by using a commercial assay purchased from Molecular Probes. 5-10 slices were tested and this was considered adequate confirmations that the preparation was suitable for continued study.

Principle – This assay provides a two-colour fluorescence to distinguish between live and dead cells. This is based on two recognised parameters of cell viability, namely intracellular esterase activity and cell membrane integrity. Cell permeant calcein AM is converted to intensely fluorescent calcein by cell esterase activity and hence labels live cells green (exitation/emission ~495 nm/~515 nm). Ethidium homodimer (EthD-1) enters damaged cells and undergoes an enhancement of fluorescence upon binding to nucleic acids. Dead cells therefore fluoresce a bright red (excitation/emission ~495 nm/~635 nm). EthD-1 cannot enter cells with an intact plasma membrane.

Dilution – A 5 ml assay solution was made at any one time and contained 2 μM calcein AM and 4 μM EthD-1. Stock solutions were removed from the freezer and allowed to warm to room temperature. 10 μl of 2 mM stock EthD-1 was added to 5 ml ASW and vortexed. When these were thoroughly mixed, 2.5 μl of 4 mM stock calcein AM was added and the solution was again vortexed. Working solutions were used within one day.

Assay – This working solution, containing 2 μM calcein AM and 4 μM EthD-1, could be added directly to the tissue. Slices were incubated in the viability/cytotoxicity assay for 40 minutes whilst oxygenated, on ice. Slices were then removed and viewed whilst in ASW.

Fluorescence Microscopy – Calcein and EthD-1 can be viewed simultaneously using a standard fluorescein bandpass filter of 495 nm and a mercury light source. Typically, cell viability was >90%.

2.2.5 Electroretinogram (ERG) recording

Extracellular electrical recordings were made with ASW filled borosilicate glass microelectrodes with 1-2 MΩ resistance when measured in ASW. The indifferent electrode was a chlorided silver pellet placed directly in the bathing solution. In retinal slice recordings, the electrode was generally placed in the inner segment region of the retina where the larger response was found. The electrode was coupled via a silver/silver chloride wire to a high impedance microelectrode amplifier and a low pass bessel filter. This signal was visualised on an oscilloscope screen and then sent to a computer via a Cambridge Electronic Design 1401 (digital to analogue converter and vice versa).

Light stimulation came from a Schott KL1500 LCD halogen light source via a fibre optic illuminator whose output went straight to a mechanical shutter. This Uniblitz shutter took ~5-7 ms to fully open, determined by using a photodiode, and another fibre optic was positioned at the other side of the shutter to direct the light onto the preparation so as to illumination all of the tissue. The computer-sampling program, Signal 2, was used to control the Uniblitz shutter driver (Model VMM-D3) and therefore to determine the timing and duration of the light flash. The maximum light intensity available at the level of the preparation was 200-300 μWcm^{-2} and neutral density filters were used to attenuate this maximum intensity. The distance of the output of the light guide from the preparation was kept constant throughout all experiments. The intensity at the level of the preparation was measured with a light meter (Advantest TQ8210). Unless otherwise stated the stimulus protocol consisted of a 50 ms flash delivered every minute with a moderate intensity of 20-30 μWcm^{-2}. This protocol was chosen to take account of the refractory period of the response and also to use a stimulus intensity bright enough to produce a good sized response for study whilst still being close to the light levels experienced by the animal in the natural environment (Clarke & Denton, 1962).

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2.2.6 Solutions

The composition of artificial seawater (ASW) was (in mM) 470 NaCl, 10 KCl, 10 $CaCl_2$, 55 $MgCl_2$ and 10 HEPES pH 7.8, osmolality 1100 $mOsmkg^{-1}$ measured using a Wescor vapour pressure osmometer (Model 5500). The perfusion solution was kept on ice, which resulted in a bath temperature of 18-22 °C. Calcium free ASW (CFASW) had the same composition as ASW minus the calcium and zero calcium ASW (0 Ca^{2+}ASW) was as CFASW plus 6 mM EGTA. All chemicals were obtained from Sigma. 5- 50 mM stock solutions were made in the appropriate solvent and stored according to suppliers' instructions. Stock solutions were diluted using ASW to the appropriate working concentrations when necessary. Test substances were applied extracellularly either by perfusion (2-3 ml min^{-1}) or by pipetting directly into the edge of the bath. The final concentration of DMSO or EtOH never exceeded 0.5%, a concentration which has been tested and found to have no effect on the ERG. The calcium channel blockers were added directly to ASW as 5 or 10 mM $CoCl_2$ and $LaCl_3$. Although bath perfusion was the preferred method of drug application, it was sometimes necessary to pipette the test chemical directly into the tissue bath. This was due to the expense of these chemicals and the fact that the larger amounts required for bath perfusion would have been too costly.

2.2.7 Viability of preparation

When maintained in ASW in the conditions described, above the preparation continued to be viable for at least 2 hours. This was determined by monitoring the ERG amplitude whilst the preparation was flashed with moderate intensity light for 50 ms every minute. Over a two hour period the amplitude stayed within a 10 % range after an initial adjustment period.

2.3 Results

2.3.1 Retinal slice preparation

Initial experiments to investigate the signalling pathway were carried out using pieces of retinal tissue. This preparation was successful in that it provided a preliminary indication of the nature of the underlying transduction cascade and which of the two branches already described was of more importance in this animal. However, some unexpected results were also obtained where chemicals known to alter the transduction process had no apparent effect. This may have been due to the fact that the tight packing arrangement of the tissue was preventing chemicals gaining access to individual photoreceptor cells. For this reason the viability of slices was investigated, as a more sensitive means of examining this signalling cascade.

A retinal slice preparation has been used previously for intracellular recordings (Hagins et al., 1962) but generally not to investigate the signalling cascade. In other invertebrates successful single cell recording is utilized, however as yet recording from dissociated cells in cephalopods has not been long-lived enough to investigate transduction. If a slice preparation can be developed, where the cells remain healthy, this would provide a relatively simple preparation for further investigations.

Figure 2.3 shows that, morphologically, the sliced tissue appears to be intact and healthy with the limiting membrane clearly visible distal to the photoreceptors and both inner and outer segments easily distinguishable. One of the advantages of this preparation over the blocks of tissue is that the electrode can be accurately positioned in a known area of the retina. It can be seen that this slice is from a dark adapted preparation as the screening pigment is localized at the bottom of the outer segment, close to the limiting membrane.
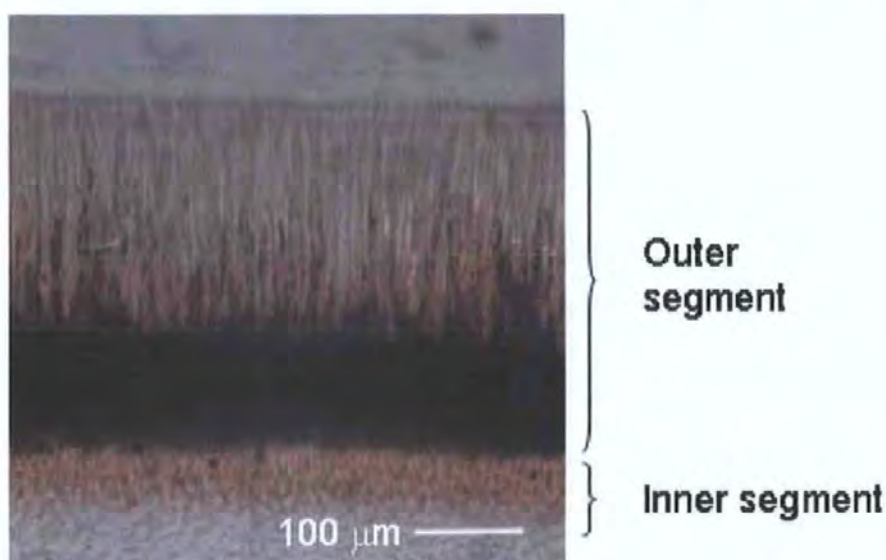
Figure 2.3 Photograph of a 300 μm thick slice of retina taken from an animal of mantle length 16 cm. Both the rhabdome containing outer segment and the cell body containing inner segment can be clearly distinguished.

The health of the cells within the tissue was confirmed by the LIVE/DEAD® viability/cytotoxicity assay (Figure 2.4). The dye clearly shows a large amount of green fluorescence that represents live cells interspersed with a lesser amount of red fluorescence (dead cells).
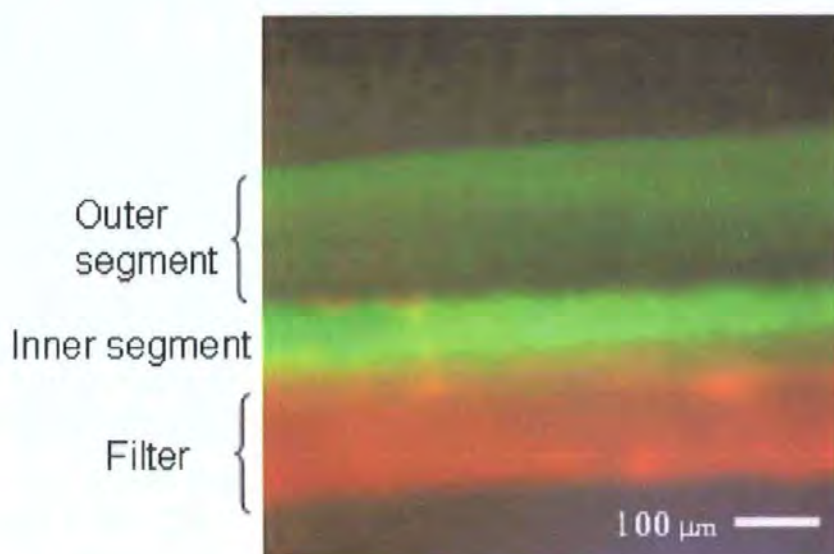
Figure 2.4 A 300 μm thick slice loaded with LIVE/DEAD dye. Photograph shows that the majority of the tissue is alive indicated by the areas which fluoresce green. In addition to this there is a much smaller proportion of the sample which has fluoresced red indicating the presence of dead cells.

2.3.2 Features of the ERG

As shown in the introduction (Figure 2.1) the characteristics of the ERG can be defined by

a number of features, namely latency, amplitude, slope of rise phase and time constant of

decay. It has been possible to measure how these parameters are affected by a change in

stimulus intensity by applying increasing intensity flashes, of constant duration, to slice

preparations from juvenile animals. Figure 2.5 to Figure 2.9 illustrate the changes that

occur in these parameters as the preparation is exposed to increasing light intensity over a

range of ~ 3 log units. Example traces obtained over the full stimulus intensity range, in

response to a 50 ms flash, are shown in Figure 2.5. The differences in the previously

mentioned ERG parameters can be seen in this figure and will be examined later.
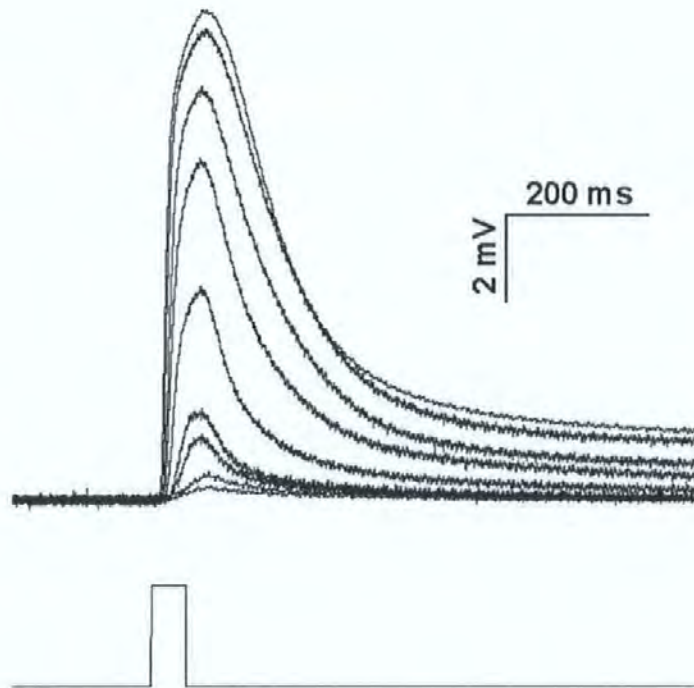
Figure 2.5 Example ERG recordings in response to increasing intensity stimulation from 0.6 - 211 μWcm^{-2}. Traces show responses to a 50 ms flash as indicated by the stimulus monitor shown below. The stimulus was applied at 5 minute intervals.

It can clearly be seen that amplitude (Figure 2.6) and slope (Figure 2.8) increase with

increasing intensity and that latency decreases (Figure 2.7). The decay constant also

increases (Figure 2.9) although the effect is less dramatic. Clark & Duncan (1978)

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observed similar relationships in *Sepiola atlantica*. When the voltage-activated sodium channel blocker, tetrodotoxin (TTX) was applied to the preparation (10^{-6} M, n = 4) no change was seen in the ERG suggesting that the response was purely a photoreceptor transduction response in origin, i.e. no component originated from the photoreceptor axon action potential. Similarly, TTX (~300 μM) has been shown to have no effect on the light induced ERG in *Sepiola atlantica* (Duncan & Weeks, 1973).

The stimulus intensity was increased from 0.6 μWcm^{-2} to 211 μWcm^{-2}, and it can be seen that this covered nearly the full dynamic range of the ERG Figure 2.6. A threshold response was produced at the lowest intensity being just discernible from the noise levels and as the stimulus intensity increased, near saturation was eventually reached. Saturation would be when further increases in stimulus intensity do not result in an increase in response amplitude. By looking at Figure 2.6 it can be predicted that a further increase in light intensity of 0.5-1 log unit would result in saturation due to the near levelling off of the graph at the highest intensity.
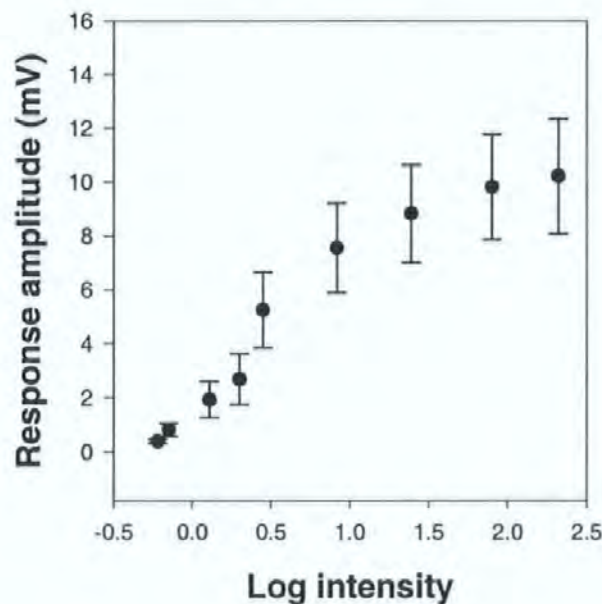
Figure 2.6 Graph of the change in response amplitude with increasing light intensity. Data taken from 3 animals with a mean mantle length of 36 mm. Each data point is the mean ± SE where n=3 or 4.

Response latency has been shown to be inversely related to stimulus intensity, i.e. as the stimulus intensity increased, response latency decreased. Response latency (Figure 2.7) fell from 24 ms at 0.6 μWcm^{-2} to 10 ms at the maximum intensity of 211 μWcm^{-2}. The relationship appears to be almost linear however this plot might be expected to level out with further increases in stimulus intensity as the rate of activation reaches its maximum for the dark adapted state.
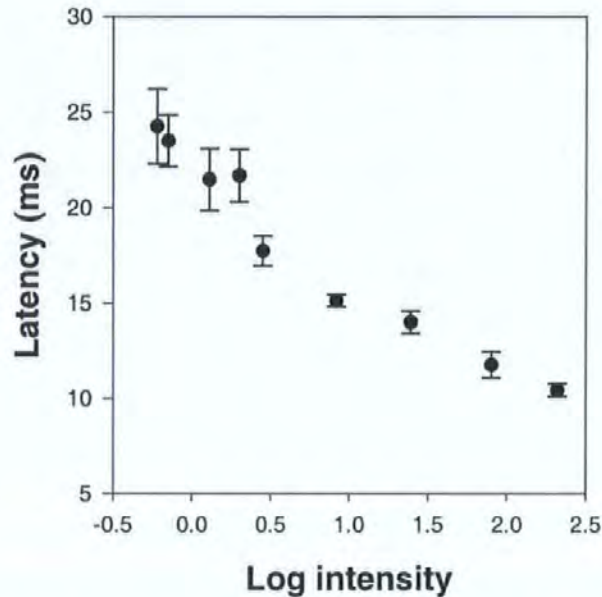
Figure 2.7 Graph of changes in response latency with increasing light intensity. Values taken from the same ERG recordings as used for Figure 2.4. Each data point is the mean ± SE where n=3 or 4.

The slope of the rising phase of the ERG response (Figure 2.8) increases with increasing stimulus intensity in much the same way as amplitude. Slope increases from 6 mVs^{-1} with the lowest stimulus light intensity to 434 mVs^{-1} with the highest stimulus intensity. This value would also be expected to level out with further increases in intensity as the transduction cascade reaches its limit.
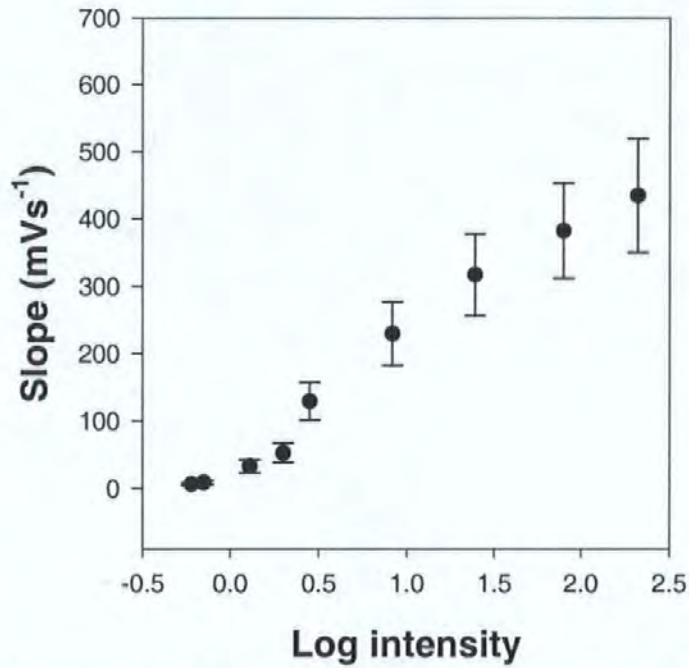
Figure 2.8 Slope of the ERG rising phase with increasing stimulus light intensity. Values taken from the same ERG recordings as used for Figure 2.4. Each data point is the mean ± SE where n=3 or 4.

Finally, the decay phase of the photoresponse does not appear to be directly proportional to stimulus light intensity (Figure 2.9) but has a more complex association. It was relatively high at 150 ms with the lowest stimulus intensity (0.6 μWcm^{-2}) and then decreased rapidly to ~50 ms at 1.3 μWcm^{-2}. There was then a gradual increase and levelling off at 90 ms by ~80 μWcm^{-2}. The decay constant stayed at this value for any remaining increases in stimulus amplitude within the range investigated. It can be seen that the decay constant is dependent on another factor as well as intensity. Clark & Duncan (1978) demonstrated that the decay constant was also dependent on the external calcium concentration.
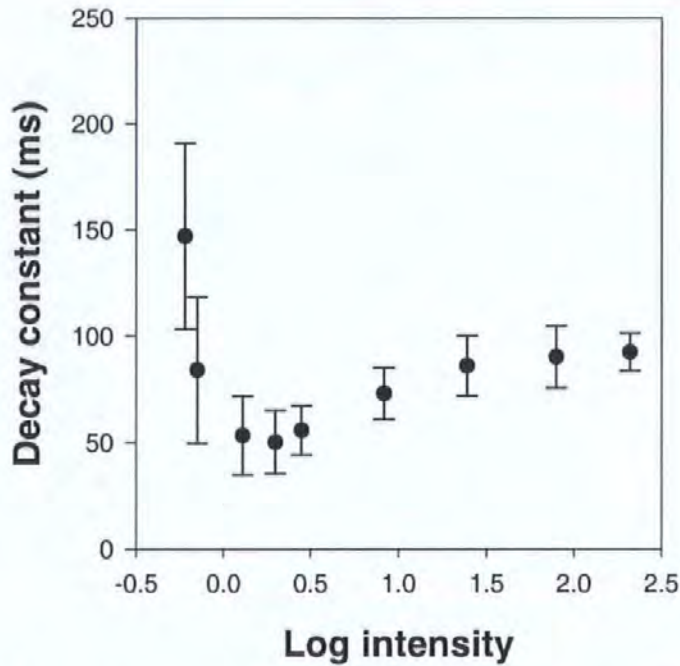
Figure 2.9 Changes in decay constant with increasing stimulus intensity. These values were taken from the same ERG recordings used for Figure 2.4. Each data point is the mean ± SE where n=3 or 4. These single exponential fits (y = y$_0$ + ae^{-bx}) had R^2 values ranging from 0.77 to 0.99, P<0.001. The goodness of fit tended to increase with increasing intensity.

2.3.3 Phospholipase C inhibition

Previous work has shown PLC to be essential for vision in the insect *D. melanogaster* as

the *norp*A mutant, which lacks eye specific PLC, has been shown to be blind. In *L.*

polyphemus, PLC appears to be needed for at least a component of the light response

(Nagy & Contzen, 1997) although as yet has not been shown to be essential. It was

therefore a logical step to determine whether PLC was also essential for phototransduction

in the cephalopod eye. This was investigated by using the phospholipase inhibitor, 4-

bromophenacyl bromide (4-BPB), which is known to inhibit both PLC and PLA$_2$.

Extracellular application of 100 μM 4-BPB led to complete abolition of the light response

(Figure 2.10) in 3 experiments and caused a dramatic reduction in a fourth. This

experiment was also performed previously in pieces of retinal tissue in which 4-BPB was

unable to block completely the ERG even following prolonged exposure, although it did

substantially reduce the response. This therefore indicated that a retinal tissue slice was an

improved preparation for the investigation of the effects of membrane permeable

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pharmacological agents. This is presumably due to the improved penetration for these chemicals when using a slice as opposed to a piece of tissue, which did not appear to be fully exposed to the test chemicals.
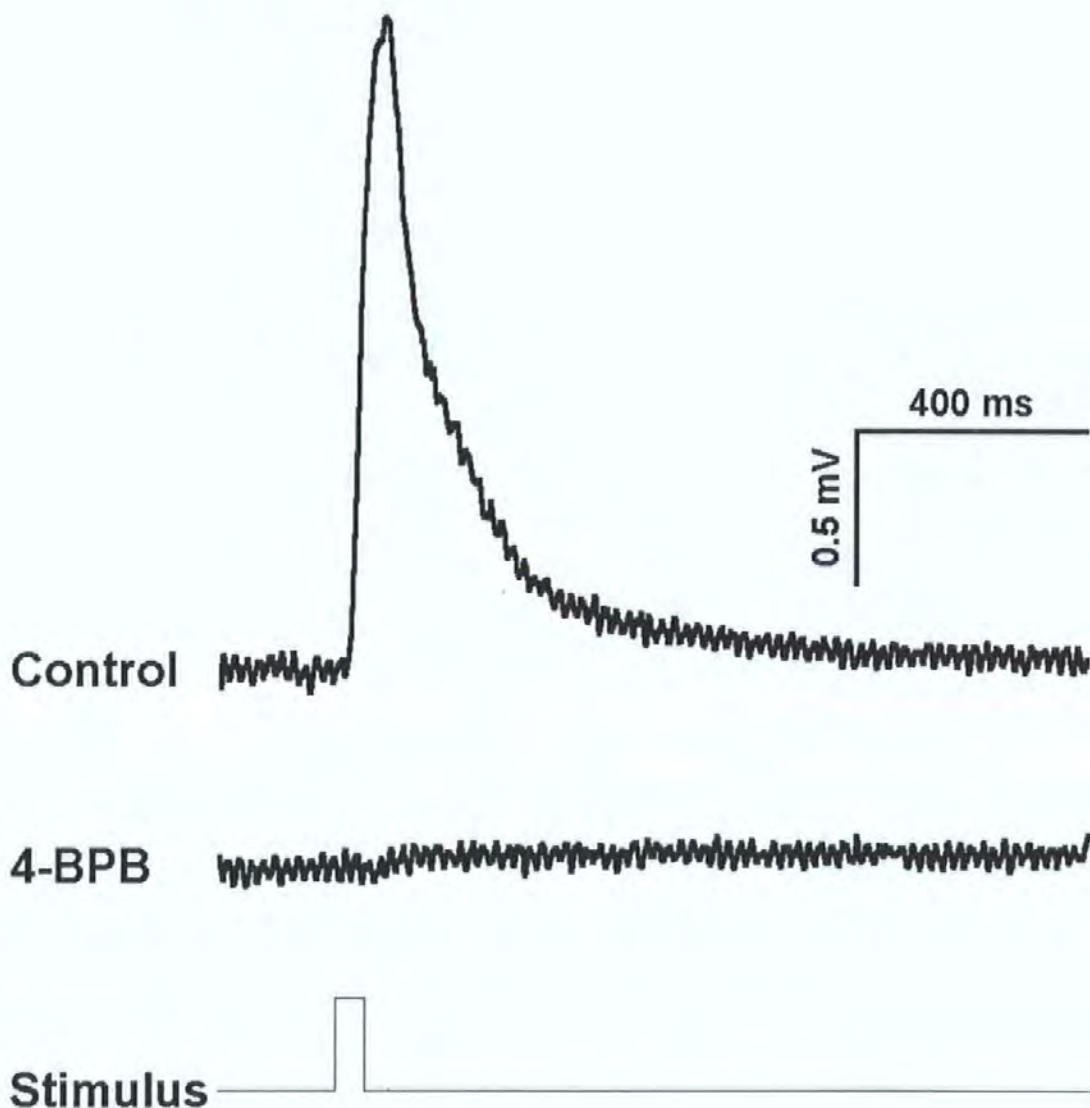
Figure 2.10 Traces to show the effect of 100 μM 4-BPB on a 300 μm thick slice of retina. These were responses elicited by a 50 ms light flash of light (~30 μWcm^{-2}) applied at 1 minute intervals. The control response was taken just before perfusion of the test chemical and the trace following drug application was taken at 27 minutes post application. This experiment was performed in a juvenile animal.

The effect of 4-BPB was also studied at 50 μM and 25 μM and when it was pipetted directly into the tissue bath rather than perfused. The decision to pipette the chemical directly into the bath rather than to perfuse was taken because the effect of one chemical at a certain concentration could be tested much more quickly. Therefore, more experiments

could be performed in a shorter time and, with the lower concentrations, it was possible to be much surer that a chemical effect was observed rather than a natural run down of the response over time. The findings when pipetting directly into the bath are presented in Table 2.2. A similar relationship was found when perfusing the solutions differing only in the fact that the overall time for the chemical to take effect was longer at each concentration. For instance, it took 17.3 ± 2.7 minutes (mean $\pm$ SE, n = 4) for 100 μM perfused 4-BPB to block the light response by 50 %. The ERG was consistently abolished by 100 μM 4-BPB under all conditions and was also blocked the majority of the time by 50 μM. Figure 2.11 demonstrates that by pipetting the chemical directly into the bath the end effect was the same as when perfused (Figure 2.10) except that the effect occurred within a shorter time scale. Full block was never achieved with 25 μM.
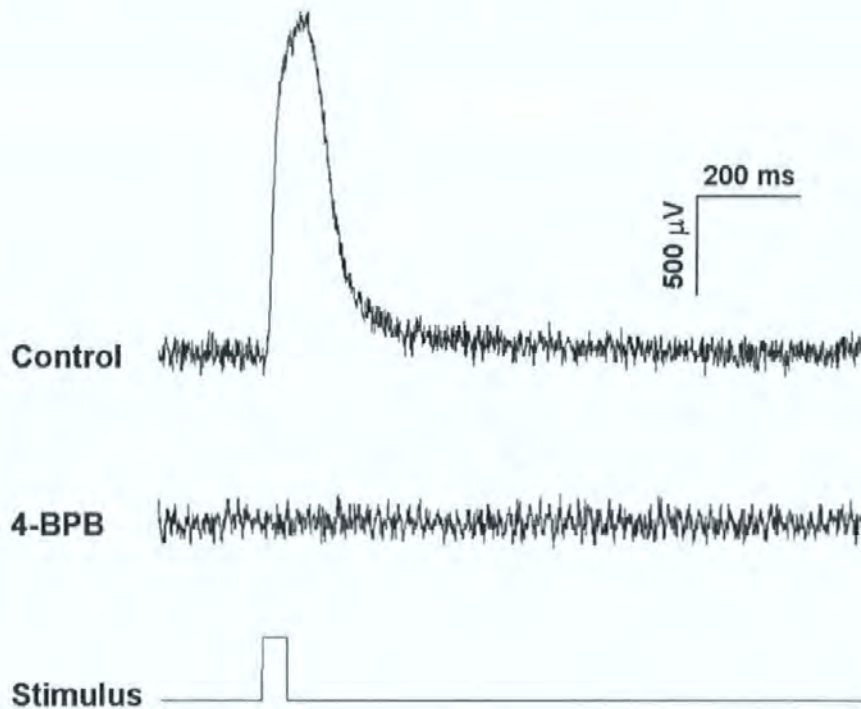
Figure 2.11 Traces to show the effect of pipetting 100 μM 4-BPB directly into the tissue bath. The stimulus light intensity was 41 μWcm^{-2} and 50 ms in duration. The middle trace was recorded following 5 minutes exposure to 4-BPB.

To enable a comparison of the effect of the different concentrations, the time it took to reduce the ERG to 50% of its maximum was determined, as total block was not reached

with all concentrations. It can be seen that there is a concentration dependent effect of this chemical, with block taking longer as the concentration decreased and the final level of block is also being reduced.

4-BPB concentration (μM)	Mean time to 50% control amplitude (minutes)	Mean time to block (minutes)
25 (n=2)	Doesn't reach 50%	Levels at ~60% at ~20 min
50 (n=3)	4	17.5 (n=2)
100 (n=2)	1.25	5

Table 2.2 **The effect of 4-BPB on the ERG is concentration dependent. These are results from experiments where the chemical was added direct to the bath.**

As well as showing that the use of a slice of retinal tissue is more effective than that of an unsliced piece, it appears that the mode of application of the test chemical is also significant. When pipetting, the chemical took effect much more rapidly. This was probably because the effective concentration was reached in the bath much more quickly. It was possible with the perfusion that it took some time for test concentration to be reach due to the volume of the bath; it is possible that the test solution was perfusing through but not completely replacing the ASW already in the bath over the initial test period.

Examination of the other ERG parameters revealed that they are also affected by the application of 4-BPB. There is a significant change in both latency and slope determined by performing a Student's t-test ($P<0.05$) however the decay constant was not shown to be significantly different (Table 2.3). The changes in slope and latency follow the pattern that would be expected with a reduction in response amplitude suggesting that blocking PLC has the same effect as reducing response amplitude by reducing stimulus light intensity. This is further illustrated in Figure 2.12 where example recordings are shown under control conditions and following 23 minutes exposure to 100 μM 4-BPB which resulted in a 50% reduction in response amplitude.

	ERG parameter (Mean +/- SE)		
	Slope (mV/s)	Latency (ms)	Decay (ms)
Control	75.3+/-12.7	14.4+/-1.2	92.3+/-27.9
50% max	27.9+/-7.2	21.7+/-2.4	137.1+/-15.6

Table 2.3 Values for the ERG parameters slope, latency and decay measured in a slice preparation (n = 4). Each parameter was measured under control conditions and following a 50 % reduction in response amplitude due to perfusion with 100 μM 4-BPB.
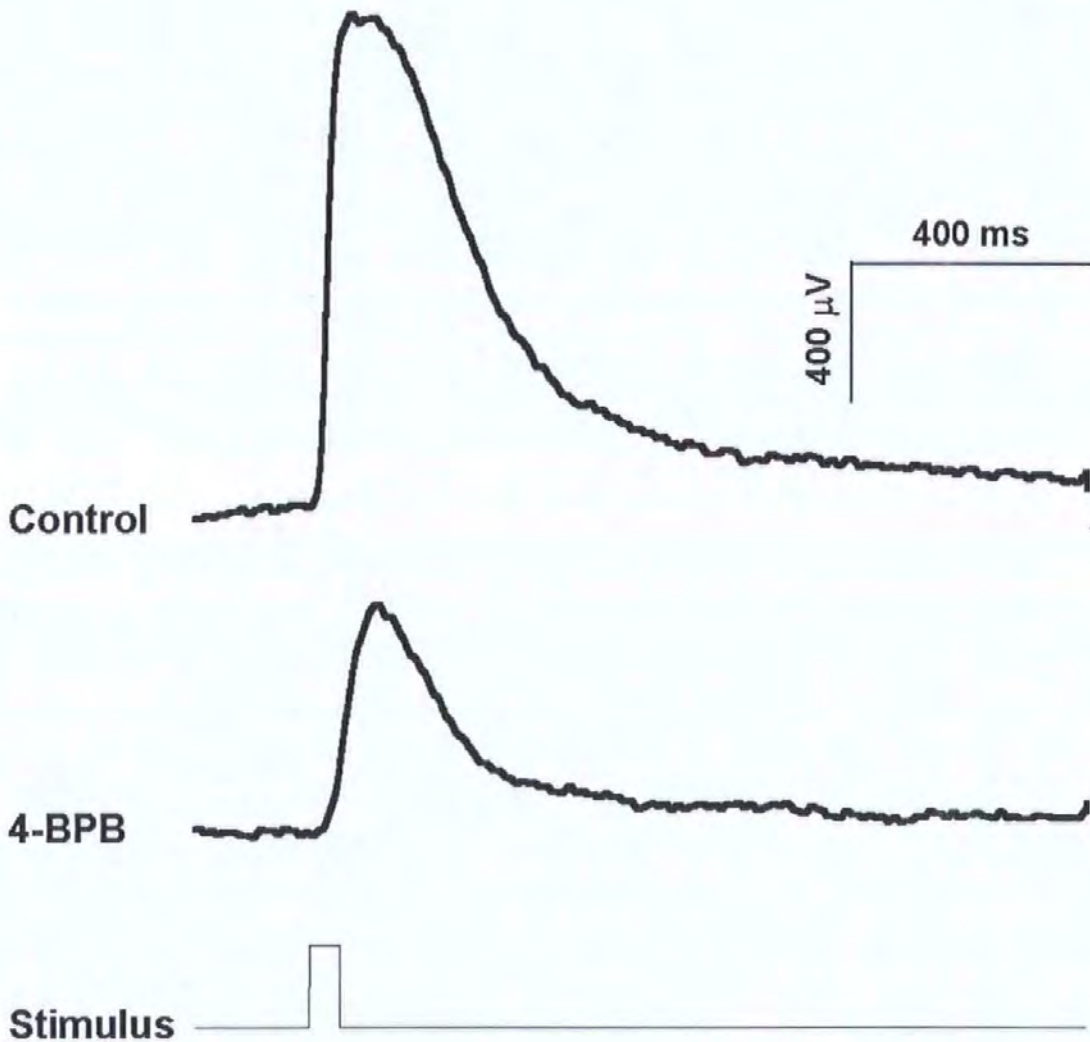
Figure 2.12 Control ERG and following 50 % block with 100 μM 4-BPB. The flash stimulus is shown in the lowest trace, LI – 26 μWcm^{-2}.

2.3.4 Inhibition of IP$_3$ receptors

2.3.4.a 2-aminoethoxydiphenyl borate (2-APB)

The role of IP$_3$ in the transduction cascade was investigated by applying the IP$_3$ receptor inhibitor, 2-APB. 2-APB inhibits IP$_3$ receptors and channels activated by the depletion of calcium stores (Wang et al., 2002). Perfusion with this agent resulted in complete abolition of the light response in slices of retina, whereas a significant response remained after the same duration exposure when a piece of tissue was used (Figure 2.13). The effect of 2-APB was concentration dependent as shown in Table 2.4 and also took effect quicker when pipetted directly into the tissue bath (Table 2.5) although still having the same final effect.

[2-APB] μM	Mean time to 50% control amplitude (minutes)	Mean time to total block (minutes)
25 (n=2)	66.0 +/- 6.0	Not blocked
50 (n=2)	41.0 +/- 16.0	72 +/- 18
100 (n=3)	25.3 +/- 6.0	40.5 +/- 4.5

Table 2.4 The effect of 2-APB is concentration dependent. For these experiments the chemical was perfused at a rate of 2-3 ml/min.

2-APB concentration (μM)	Time to 50% control amplitude (minutes)	Time to total block (minutes)
25 (n=1)	60% at 20 min	Not blocked
50 (n=1)	3.5	11
100 (n=1)	3	7

Table 2.5 Effect of 2-APB is quicker when added directly to the tissue bath.
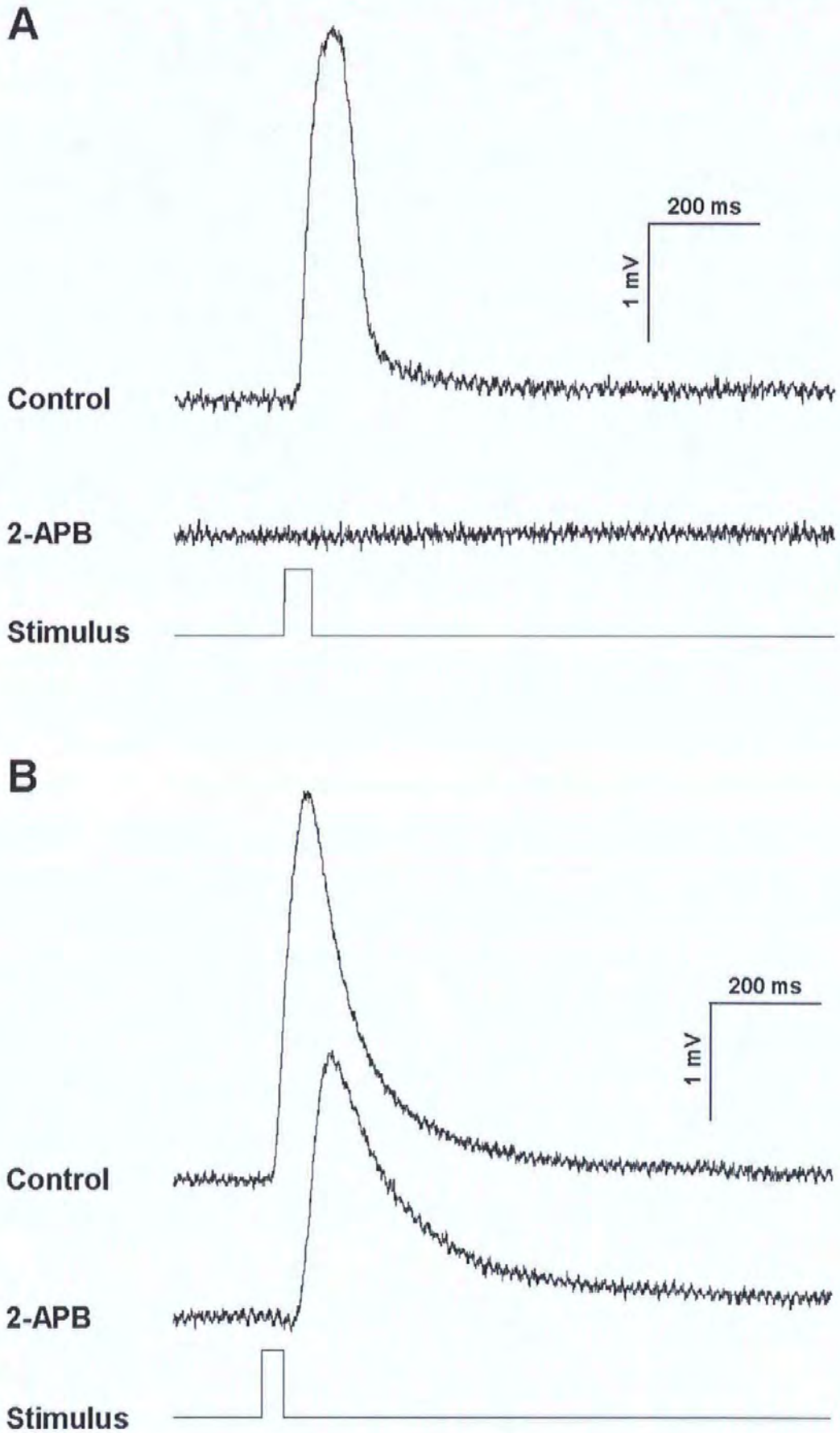
Figure 2.13 The effect of 100 μM 2-APB on the ERG. Figure shows the control ERG and that following exposure to 2-APB in both a slice preparation (A) and in a piece of tissue (B), with the stimulus pulse shown below. The slice was perfused with the test chemical for 36 minutes and the piece of tissue for 40 minutes.

It is apparent from Figure 2.14 that when the piece of tissue was exposed, as well as reducing response amplitude, 2-APB caused an increase in response latency which would suggest that the mechanism for excitation had been retarded. The same effect was also seen in the slices with latency increasing to approximately 40 ms when response amplitude had dropped to 50 % of the control value. This is longer than seen with even the lowest intensity light and is significantly longer than expected from a response of this size. It can therefore be suggested that 2-APB does not bring about its effect in the same way as reducing light intensity or blocking PLC. It is likely, due to its known properties, to be working at a number of sites within the cells. As the ERG is a combination of the potential change of many cells, its latency will also be a combination. Therefore if a messenger involved in excitation is inhibited it may take longer for individual cells to reach threshold levels of messengers necessary to open ion channels, leading to an overall increase in ERG latency.

Thus the action of 2-APB was found to be not only concentration dependent, but intensity dependent as well. 2-APB is not as effective at all light intensities and we have shown the effect to be partially reversible (Figure 2.14). Perfusion with 100 μM 2-APB appears to shift the graph to the right indicating reversible competitive antagonism or that another parallel pathway takes over at higher light intensities. Up to ~50 μWcm^{-2}, 2-APB can completely abolish the response to light within 60 minutes. However at higher intensities the ERG still persists, although at much reduced amplitude, therefore 2-APB reduces the sensitivity of the light-induced activation process.

There are a number of explanations for these findings. IP$_3$ or subsequent messengers may be necessary to obtain maximum amplification but not directly involved in channel opening as the response to high intensity stimulus is reduced but not abolished by the action of 2-APB. Alternatively, another parallel pathway may dominate at higher

intensities, or 2-APB may be acting as a competitive inhibitor and the increase in light

intensity produced a great enough amount of second messenger to overcome the action of

2-APB.
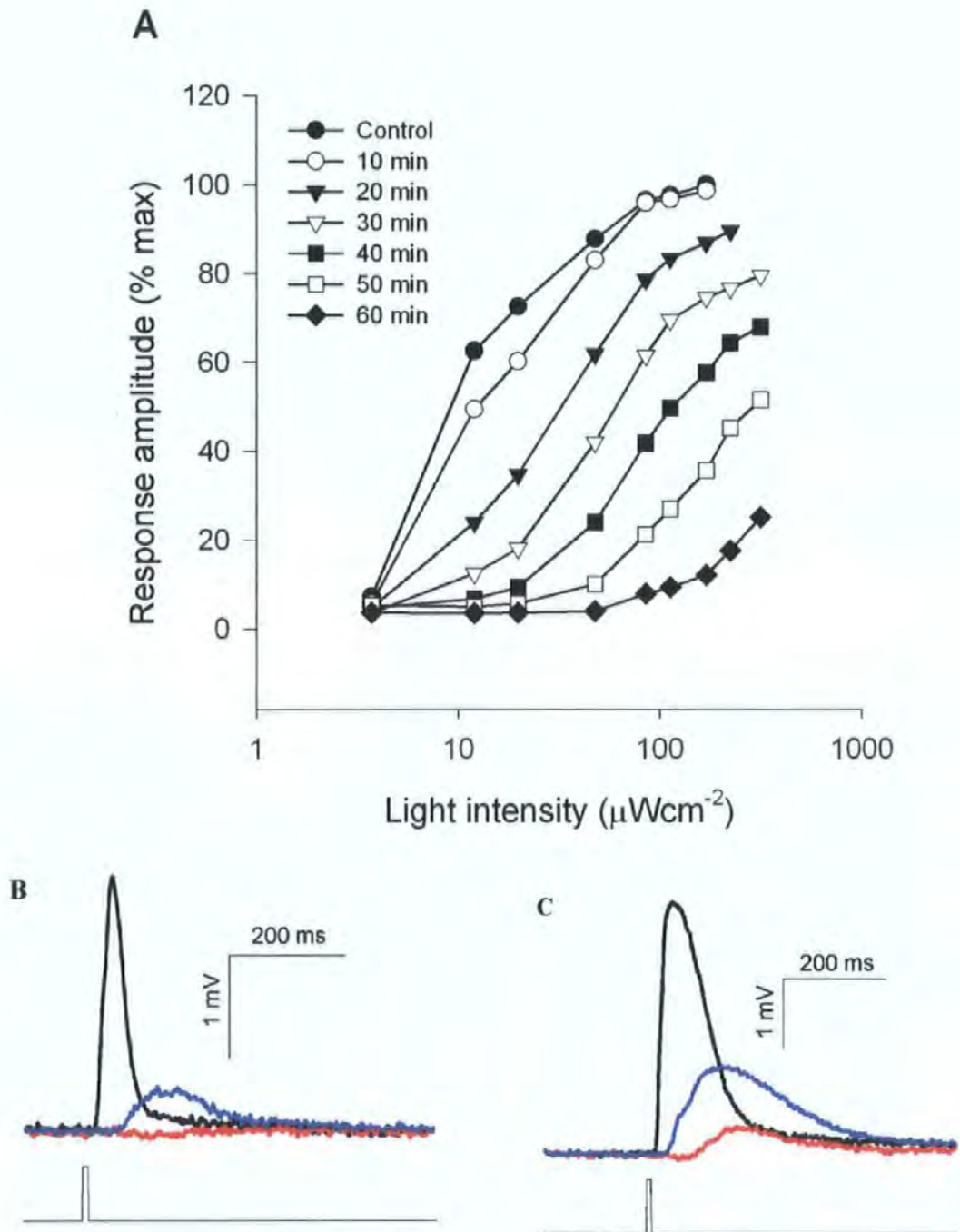
Figure 2.14 The effect of 2-APB is intensity dependent. The voltage response to increasing light intensity under control conditions and following exposure to 100 μM 2-APB at 10 minute intervals, from 10-60 minutes (A). The response-intensity plot is effectively shifted to the right along the x-axis rather than being vertically depressed. Example traces in response to low (12 μWcm^{-2}) and high (169 μWcm^{-2}) intensity stimuli are shown in figures B and C with the stimulus pulse represented below. Each figure shows a control response (black), following 60 minutes exposure to 2-APB (red) and partial recovery following 120 minutes wash in ASW.

2.3.4.b Xestospongin C

2-APB has potentially more than one site of action namely, IP_3R and channels activated by calcium store depletion. In order to gain a more specific idea of the role of IP_3 the non-competitive blocker of the IP_3R, Xestospongin C (XeC), was tested on the evoked ERG. 10 μM XeC was applied to slice preparations by pipetting directly into the bath. An effect was produced in 2 of the 3 preparations tested. The response amplitude was reduced to 50 % by ~ 30 minutes (Figure 2.15); however the response levelled out at this size and was not abolished within the time scale of the experiment. Due to the small quantities available higher concentrations were not tested. However, XeC is almost 100 times more effective than 2-APB as its IC_{50} value (concentration required to produce 50 % inhibition) is 358 nM compared to 42 μM for 2-APB.
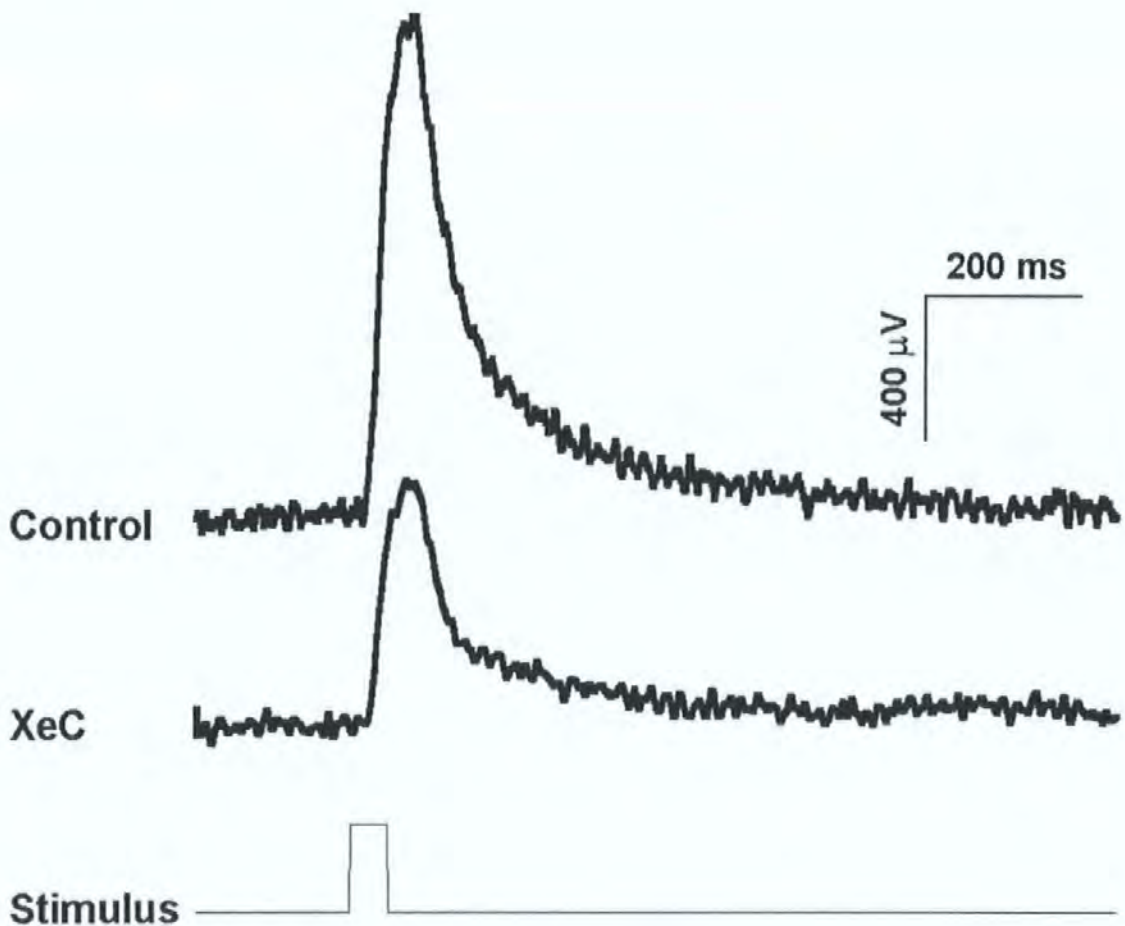
Figure 2.15 Xestospongin C reduces the size of the ERG. Example showing a control ERG in ASW and following 30 minutes exposure to 10 μM XeC which was pipetted directly into the tissue bath.

A chemical effect might have been expected to occur more quickly however, looking at the previous results this is actually more effective than 25 μM of both 4-BPB and 2-APB under similar conditions. Additionally, the light response has been tested in these conditions, in ASW with no added chemicals, and was found to be stable for well over 2 hours. Although the response amplitude is depressed, the other ERG parameters appear to be unaffected. This is in contrast to the very obvious increase in latency, which was observed when applying 2-APB. This would suggest that blocking IP$_3$ induced calcium release results in the observed reduction in response amplitude produced by 2-APB and XeC, and that 2-APB increases response latency by a different means.

2.3.5 The role of calcium

Calcium has been implicated in cephalopod photoreceptor inactivation and sensitivity (Duncan & Corghan, 1973; Clark & Duncan, 1978) as well as excitation, response amplification and adaptation to different light levels in *L. polyphemus* (Levy & Fein, 1985; Payne et al., 1986; Nagy, 1991; Ukhanov & Payne, 1997). Because it is involved in such a wide range of the features of transduction it might be expected to exert its effect in a number of ways, have more than one site of action and probably have a concentration dependent effect. Another factor also has to be taken into account, namely that calcium is present both in the extracellular bathing solution and intracellular stores, as well as the concentrations found in the cytosol. This part of the study was approached by trying to separate the effects of extracellular calcium found in the external bathing solution and the intracellular calcium released from internal stores.

2.3.5.a Extracellular calcium

The involvement of extracellular calcium (Ca^{2+}$_e$) was investigated by changing the concentration in the extracellular bathing solution and by applying calcium channel

blockers externally. When pieces of retinal were perfused with 0 Ca^{2+}ASW (n = 6) or CFASW (n = 12), there was an initial increase in response amplitude and duration, followed by a gradual decrease in amplitude accompanied by an increase in time to peak, partly due to a reduction in the slope of the rise phase, and slowing of the decay phase (Figure 2.16 & Figure 2.17). This effect was usually reversible following a long wash ($\geq$ 1 hour) in ASW.
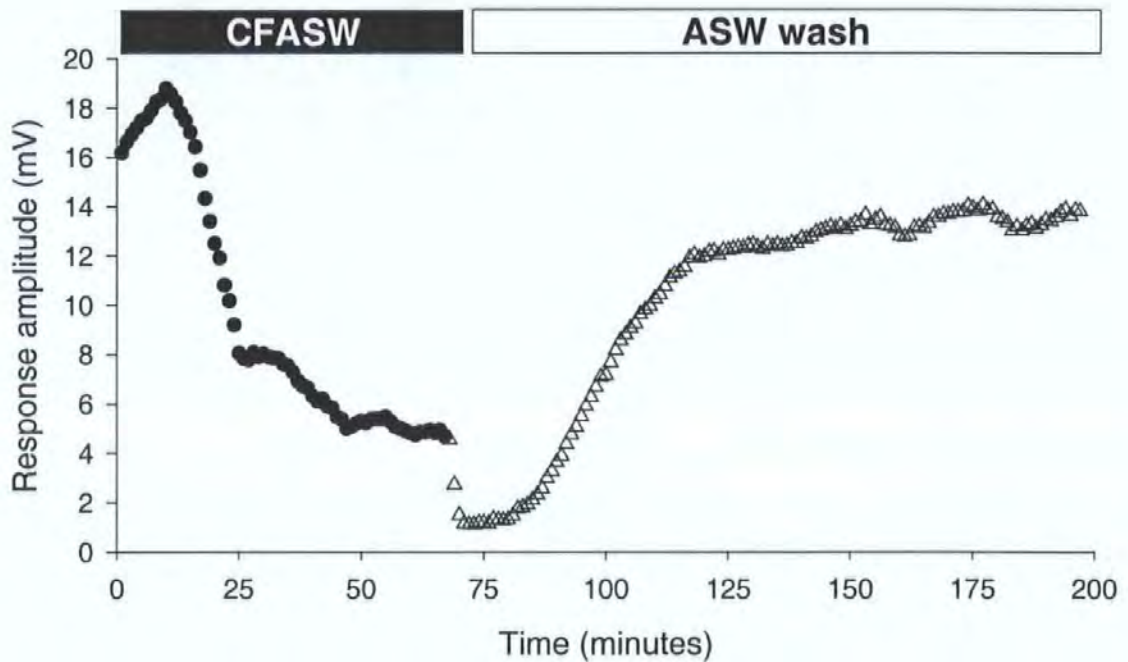
Figure 2.16 Change in response amplitude over time when perfused with CFASW and following wash with ASW.

ASW
10 min CFASW
60 min CFASW
130 min wash
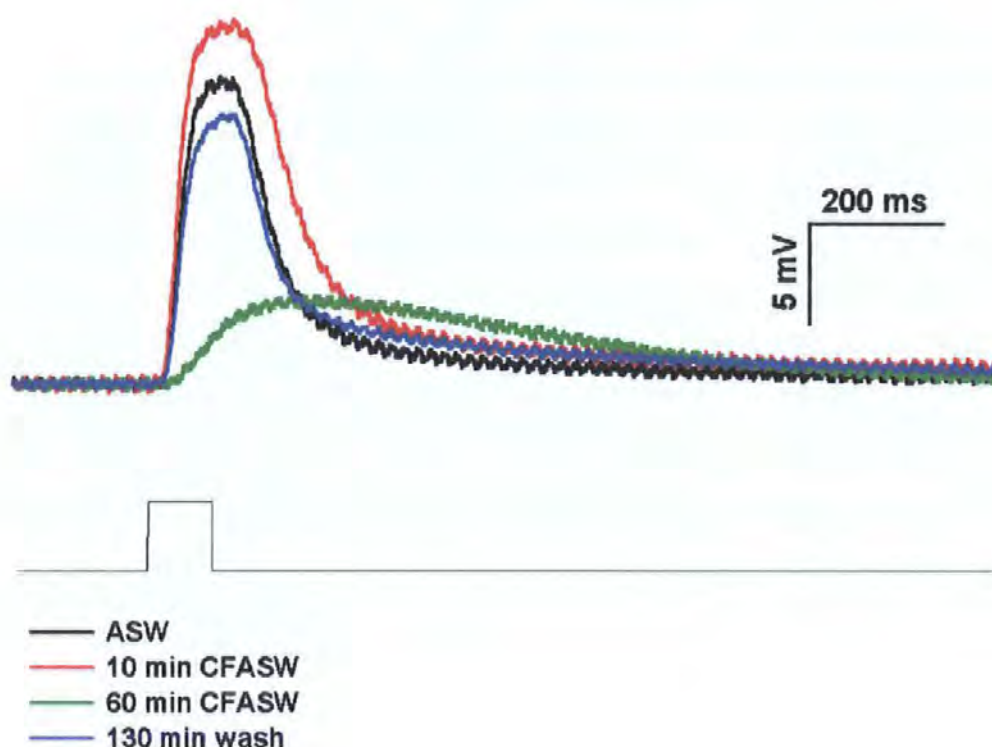
Figure 2.17 Some example traces taken from the same data as used to plot Figure 2.16 above. Conditions were as stated in the legend.

The calcium channel blocker cobalt (Co^{2+}) increased photoresponse duration in both pieces (Figure 2.18A) and slices of retinal tissue (Figure 2.18B) and also reduced the response amplitude in the slices. The response was dramatically reduced but not completely abolished. In one preparation the effect of Co^{2+} was tested over an intensity range from 3-160 μWcm^{-2} (Figure 2.19). There seems to be a cut off point at ~50 μWcm^{-2} where at intensities below this value, Co^{2+} caused an increase in response amplitude during early exposure and above this intensity the response amplitude was reduced. At all intensities examined, response duration was increased and latency was unaffected. The effects of Co^{2+} could not be reversed by washing and the photoresponse was eventually lost. Co^{2+} has previously been used in blowfly photoreceptors and the effect was also found not to be reversible (Mojet, 1993). It is interesting to note that the effect in the piece of tissue was reversible, and it is suggested that this may depend on whether Co^{2+} actually enters the cells or not. This will be discussed later.
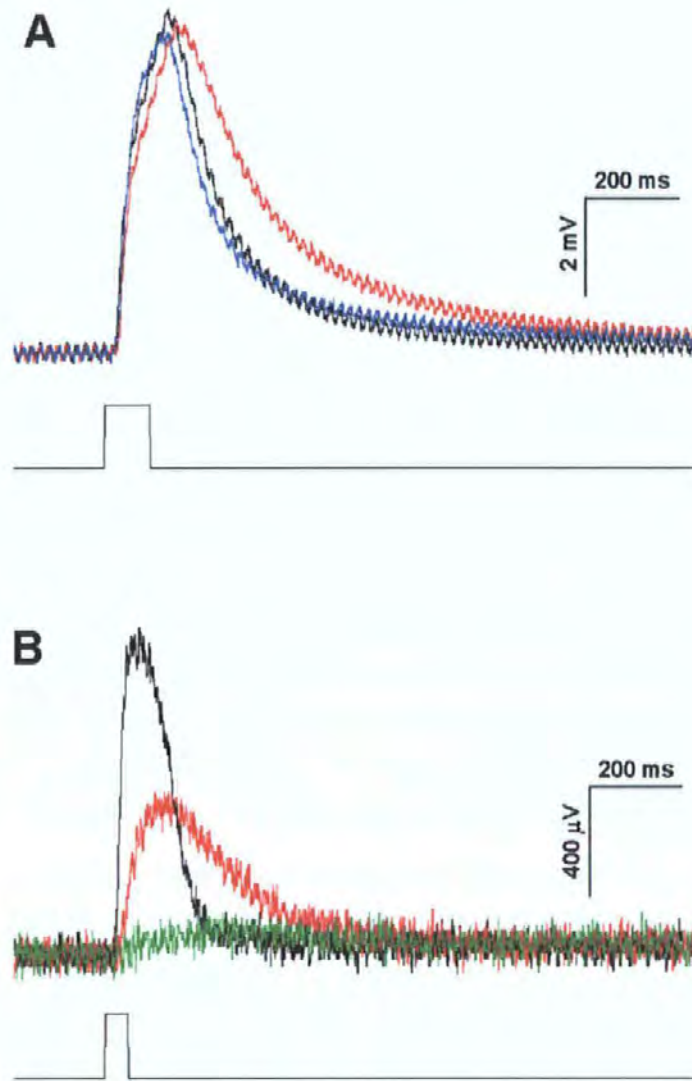
Figure 2.18 The effects of 10 mM $CoCl_2$ on a piece of tissue (A) and a slice (B). Traces in A are control (black), 20 minutes $CoCl_2$ (red) and 60 minutes wash (blue). Control (black), 25 minutes $CoCl_2$ (red) and 55 minutes $CoCl_2$ (green) are shown in B. The stimulus pulse is shown below each figure.
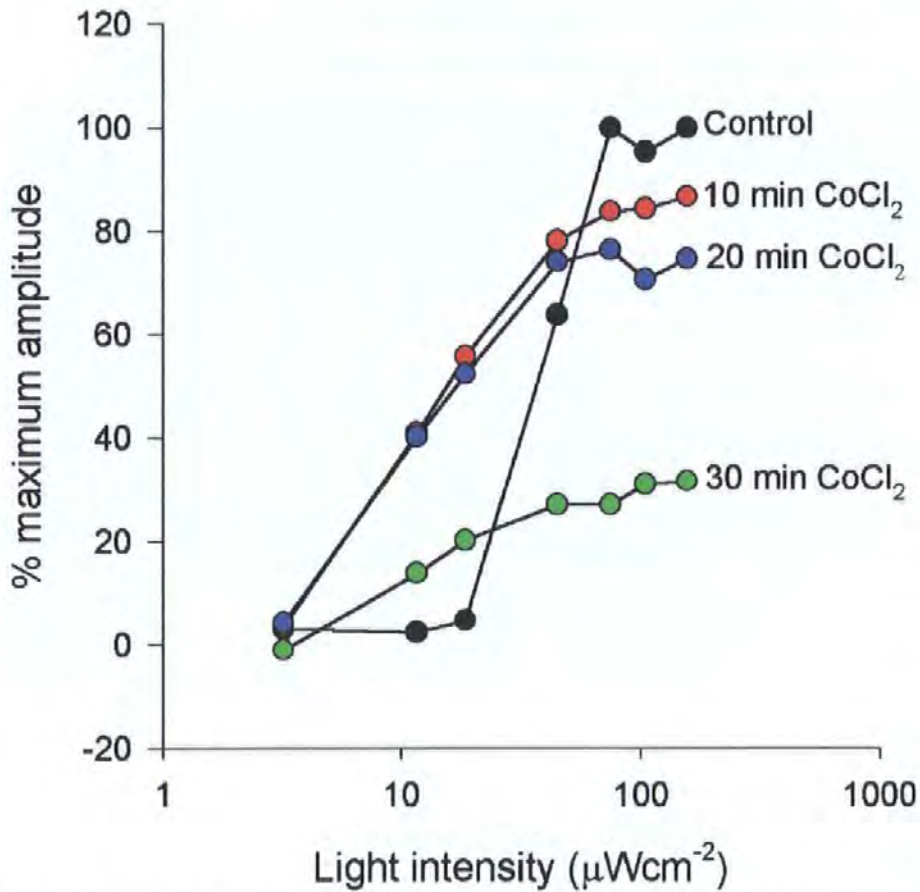
Figure 2.19 The effect of CoCl₂ is intensity dependent. A range of intensities were applied to a slice preparation at 10 minute intervals, under control conditions and with CoCl₂ added.

Lanthanum (La^{3+}) is another calcium channel blocker which was tested. This has a much greater efficacy than cobalt (Hoth & Penner, 1993) and is specific for the *D. melanogaster* Trp channel (Suss-Toby et al., 1991). La^{3+} was tested at 5 mM (n = 4) and 10 mM (n = 2) in retinal slices and at both concentrations, latency and duration were increased. An example following perfusion with 5 mM La^{3+} is shown in Figure 2.20 and the corresponding traces under control conditions and following 8 minutes exposure to ASW containing 5 mM $LaCl_3$ (Figure 2.21). When the response amplitude had reduced to half its original size, latency had increased by more than 35 ms and 50 ms for 5 mM and 10 mM respectively. After this time the response started to become unstable and could not be recovered by washing with ASW, however a response could still be detected up to 50 minutes later.
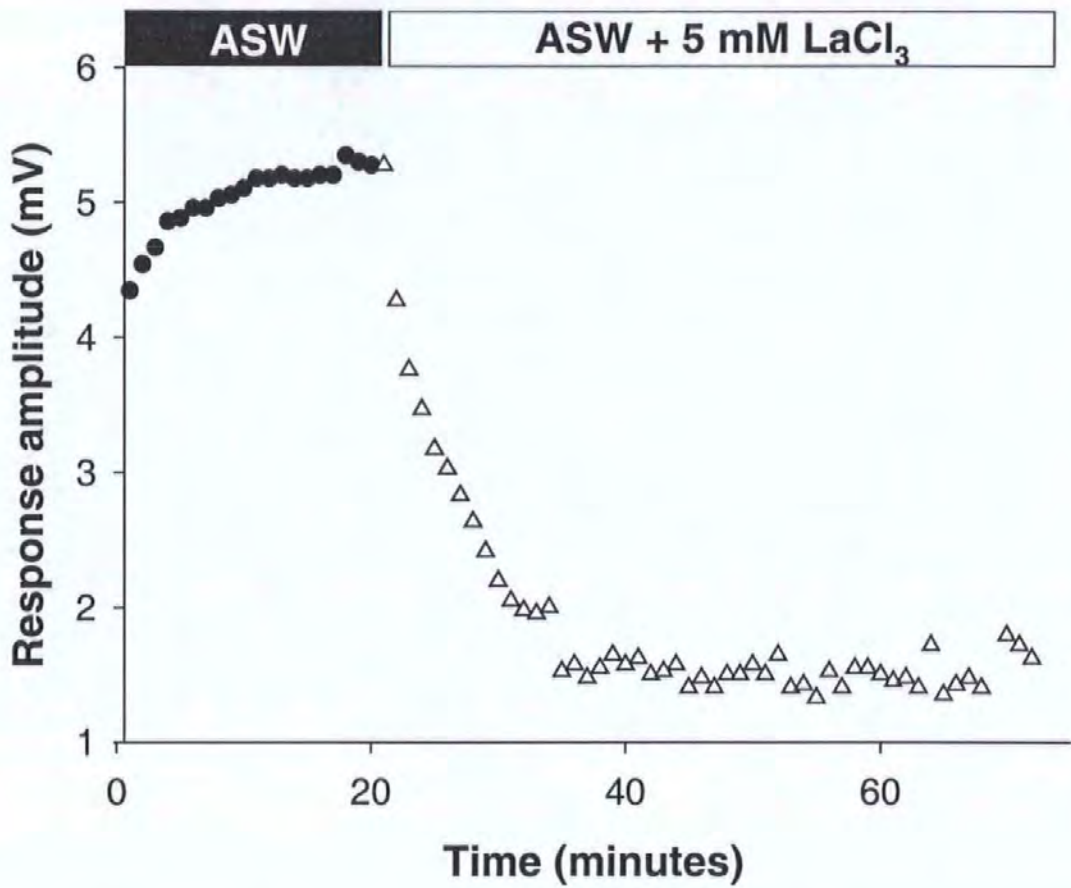
Figure 2.20 Changes in response amplitude with time measured at one minute intervals in ASW and then following the addition of 5 mM LaCl₃ to the perfusate.
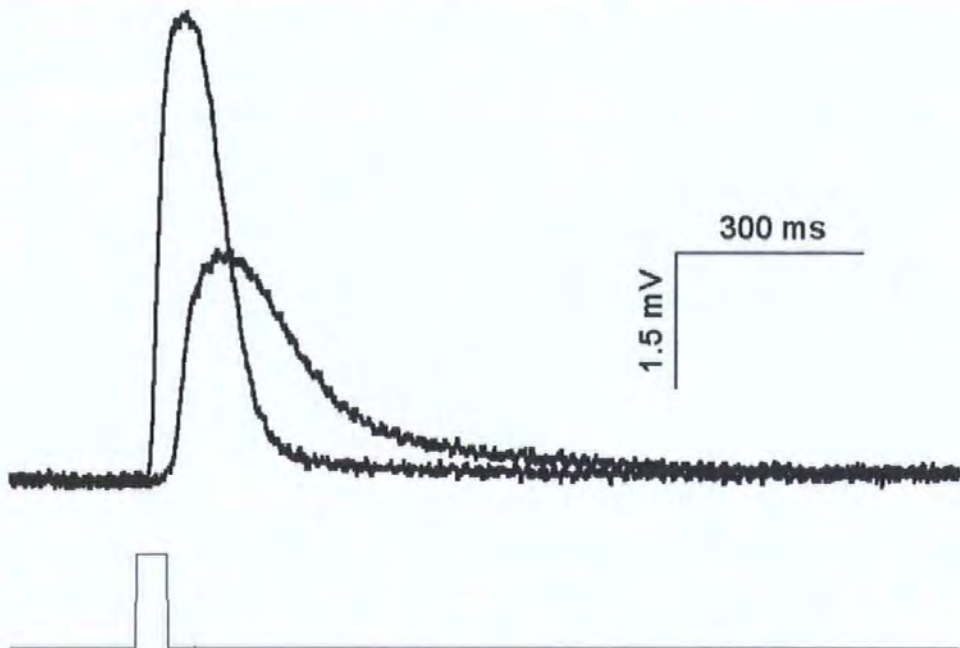
Figure 2.21 Effect of 5mM La³⁺ on the ERG. Control ERG and after 8 minutes perfusion with La³⁺. The stimulus pulse is shown below. We can see that amplitude has decrease and that both latency and duration have increased.

2.3.5.b Intracellular calcium

It is likely that there are internal calcium stores within the photoreceptor cells because such

stores have been identified in other molluscan species (Walz, 1979, 1982) and organelles

resembling calcium stores have been identified in squid (Walrond & Szuts, 1992). Release

from these stores can produce large, rapid elevations in the intracellular calcium

concentration. Following release, calcium is then taken back up into the stores by

Ca^{2+}ATPase. This transporter actively pumps Ca^{2+} out of the cytosol at the expense of

ATP hydrolysis. This source of calcium was therefore manipulated by the use of

Ca^{2+}ATPase inhibitors with the intention of depleting the internal calcium stores and

therefore removing the effect of calcium release from these stores. Cyclopiazonic acid

(CPA) is one such chemical and its effect at 100 μM is shown in Figure 2.22. Although

reduced in amplitude there was no effect on latency, slope or decay. The same effect was

observed in 3 preparations. Another Ca^{2+}ATPase inhibitor, thapsigargin, was also tested

however, no change in the ERG was observed at 80 μM or 100 μM in tissue slices. It is

possible that the thapsigargin could not gain access to the cell interior as readily as CPA.
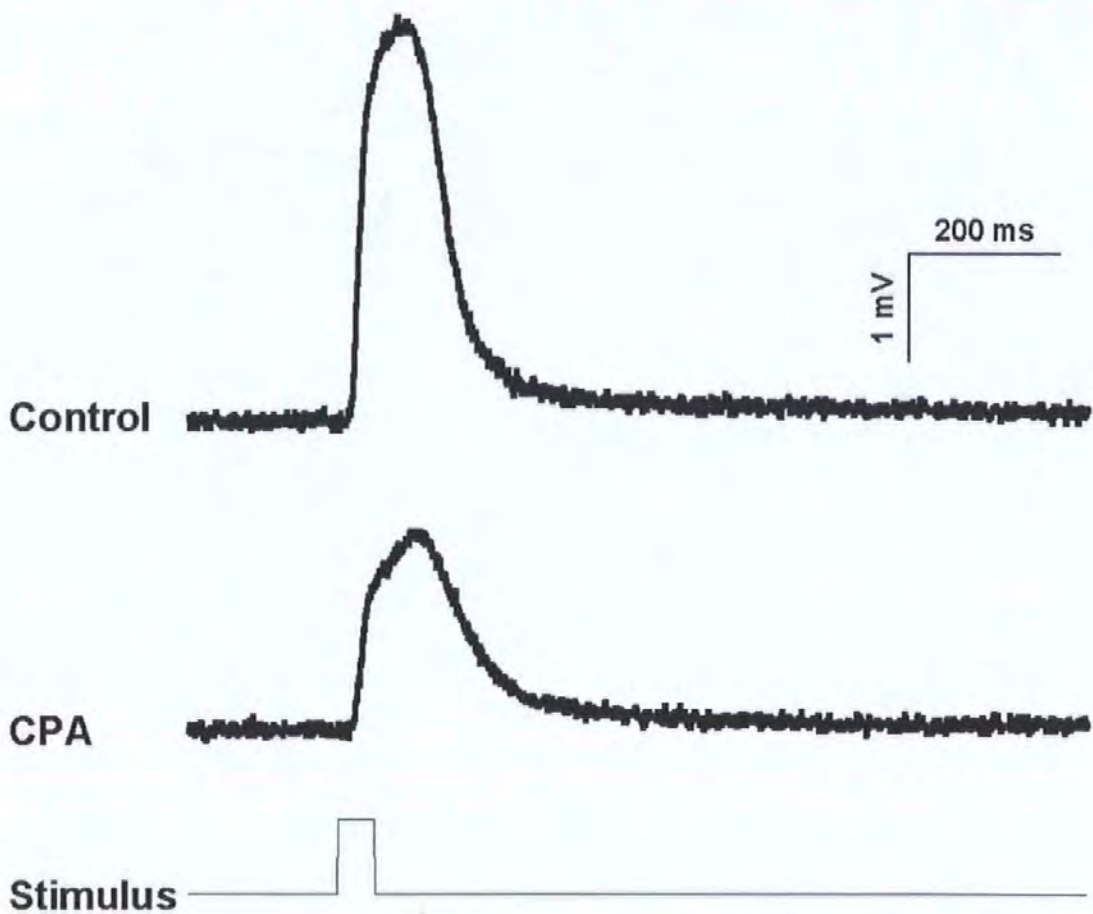
Figure 2.22 Cyclopiazonic acid reduces the ERG response amplitude in a slice of tissue when added directly to the tissue bath. The control ERG was reduced in amplitude by 50 % after 26 minutes exposure. The amplitude continued to decrease to ~40% where it levelled out.

When CFASW and CPA were combined, therefore completely eliminating the effects of calcium, whether it be from an internal or external source, the ERG was completely abolished (n = 2) as shown in Figure 2.23. Total block was achieved within 40 minutes. It is possible to totally abolish the ERG by exposing the tissue to CFASW for a prolonged period of time (i.e > 60 minutes). It can therefore be seen that this has been quicker by eliminating the effects of both intra- and extracellular calcium suggesting that both sources have a role to play in the photoresponse.
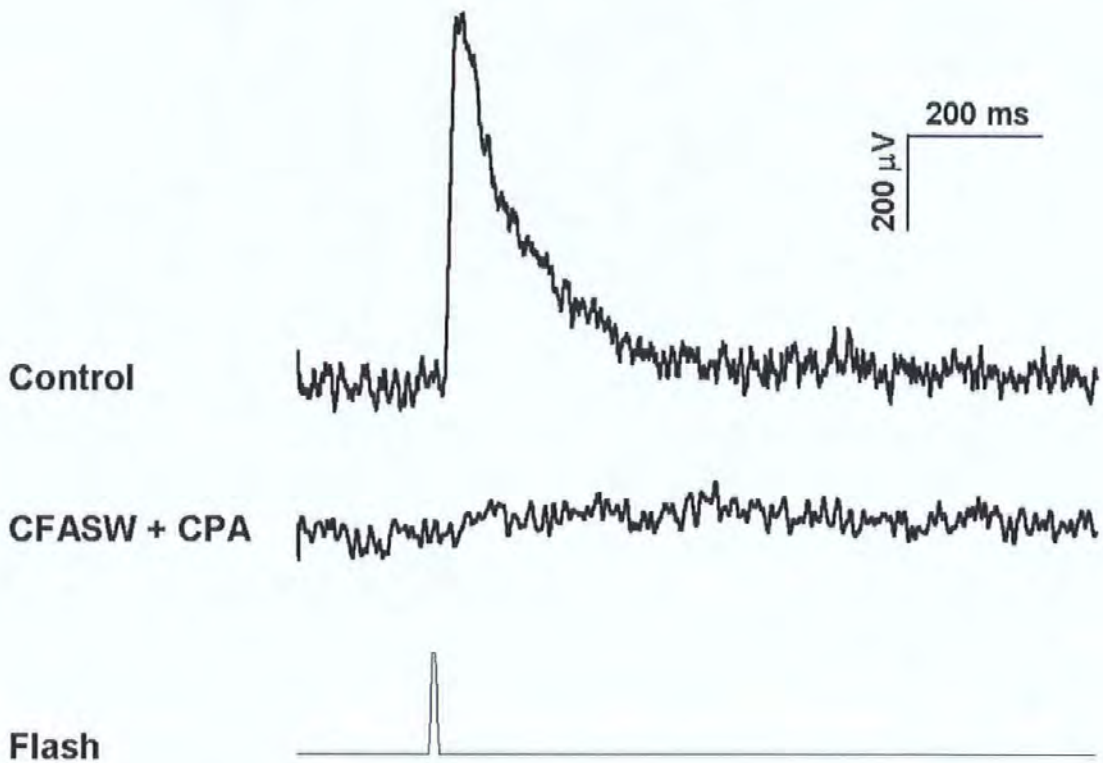
Figure 2.23 Removal of all calcium sources completely abolished the response to light. The response to a 10 ms flash of light (top) as indicated below traces, with an intensity of ~180 μWcm^{-2} was completely abolished at 40 min (middle).

2.3.6 Investigation into other potential pathways (DAG / cGMP)

2.3.6.a Diacylglycerol (DAG)

The results presented so far have looked specifically at the IP$_3$ branch of the PI pathway.

However, DAG is also produced following cleavage of PIP$_2$ by PLC, hence the potential

involvement of this additional branch was also investigated. Figure 2.24 shows

schematically the potential cellular products subsequent to DAG production. Protein

kinase C (PKC) exerts its effect by causing phosphorylation of intracellular proteins and

has numerous sites of action and hence many physiological effects (Rang et al., 1996).
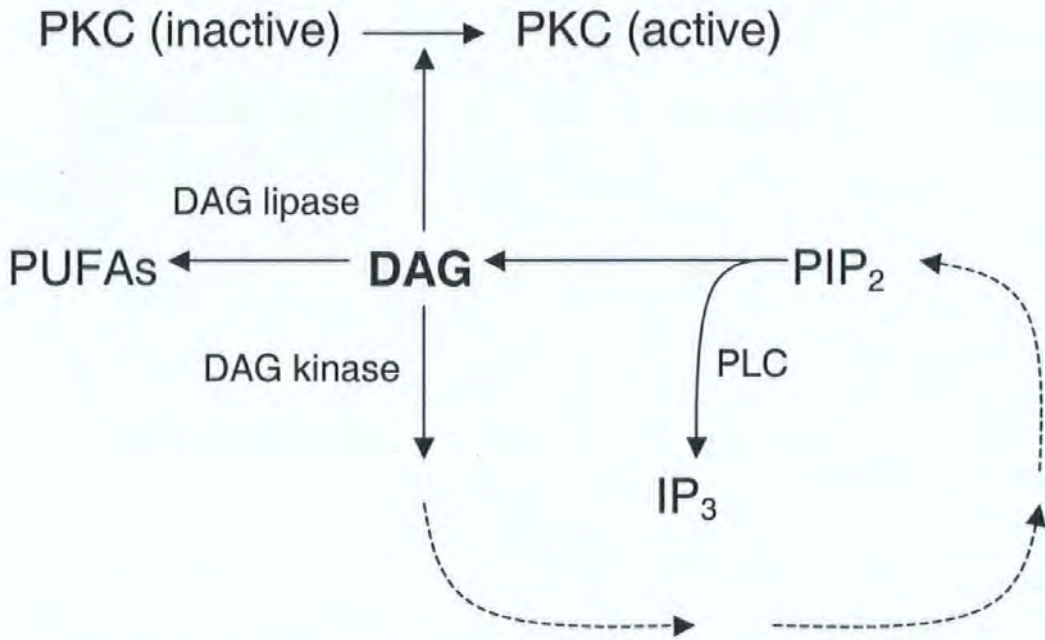
Figure 2.24 The DAG signalling pathway. PIP$_2$ hydrolysis by PLC produces DAG as well as IP$_3$. DAG kinase is the first enzyme in the recycling process catalysing the breakdown of DAG ultimately resulting in the regeneration of PIP$_2$. DAG lipase catalyses one of the steps involved in producing polyunsaturated fatty acids (PUFAs) from DAG and DAG also activates PKC. This diagram was adapted from Raghu et al. (2000b) with additional information from (Rang et al. (1996).

As PKC activators and inhibitors have been tested and shown to have an effect in other invertebrate photoreceptors (*D. melanogaster* (Hardie et al., 1993); *L. polyphemus* (Dabdoub & Payne, 1999)) they were also tested in this preparation. The PKC activator (-)-indolactam V (15 µM) had no effect on the ERG induced in a tissue slice by light flashes with an intensity of 15 µWcm^{-2} (Figure 2.25). The same was true when pieces of tissue were tested (n = 3). Due to the cost of this chemical it was not considered cost effective to test higher concentrations however this chemical has been shown to be effective over a concentration range of 0.03-30 µM in *L. polyphemus* ventral photoreceptors (Dabdoub & Payne, 1999). Additionally the PKC inhibitor, 50 µM chelerythrine chloride was tested in pieces of tissue (n = 3) and again had no clear effect on the ERG. This chemical has however been found to be effective in inhibiting the effect of PKC activators in *Lima scabra* photoreceptors at this concentration (Gomez & Nasi, 1998).
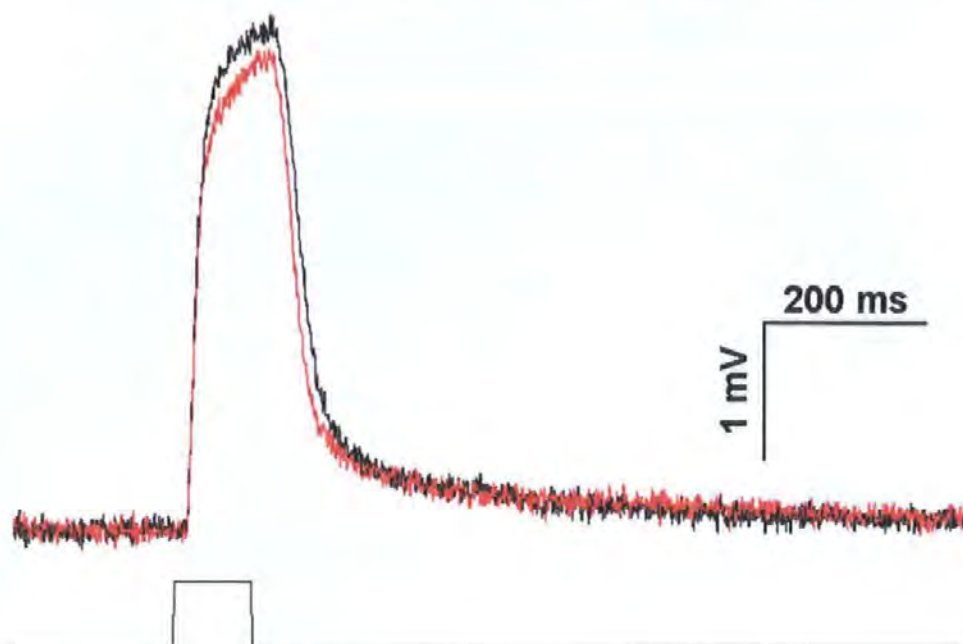
Figure 2.25 (-)-Indolactam V had no effect on the ERG. A control trace evoked in ASW is shown in black and a response following 15 minutes exposure to 15 µM (-)-indolactam V in red. It can be seen that there was no change in any of the ERG components.

The potential role of polyunsaturated fatty acids was also investigated as they have been shown to play a key role in opening ion channels in *D. melanogaster* photoreceptors (Chyb et al., 1999). Nordihydroguaiaretic acid (NDGA) is a lipoxygenase inhibitor and therefore would inhibit fatty acid breakdown. Hence it may have enhanced the ERG response if PUFAs were involved. This chemical was tested at 100 µM (n = 3) in pieces of tissue and no clear effect was observed. This chemical was however capable of inducing a response at concentrations as low as 10 µM in *D. melanogaster* photoreceptors (Chyb et al., 1999).

2.3.6.b   Cyclic guanosine monophosphate (cGMP)

Due to the evidence presented by Takagi (1994a) and Huppertz (1995), supporting a possible role for cGMP in opening of membrane ion channels, the involvement of this messenger was also investigated. The membrane permeable analogues of cGMP, 8-Br-cGMP and dibutryl-cGMP have both been bath applied to the retinal preparation at a

concentration of 100 μM and 1 mM respectively, however neither caused a change in the ERG. These agents can be effective at 100 μM (Bacigalupo et al., 1991) however often, mM concentrations are used (Takagi, 1994a; Chyb et al., 1999). Both retinal pieces (n = 4) and retinal slices of tissue (n = 1) were tested with 8-Br-cGMP and a slice with dibutryl-cGMP. Example traces from one of these experiments is shown in Figure 2.26.
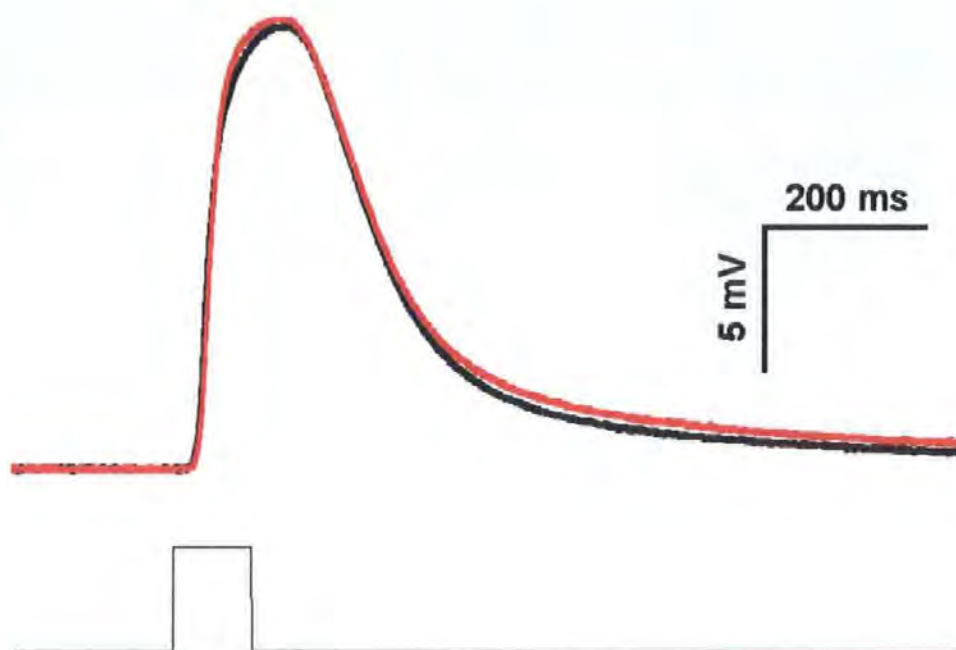
Figure 2.26 8-Br-cGMP had no effect on the ERG. Recordings were taken from a piece of tissue from a juvenile animal in response to light with an intensity of ~ 30 μWcm^{-2}. The control response is shown in black and the response following 35 minutes exposure to 100 μM 8-Br-cGMP is shown in red. The 100 ms stimulus pulse is shown below the traces.

Finally LY-83583 was tested on pieces of tissue (n = 2, up to 50 μM). This chemical inhibits soluble guanylate cyclase, the enzyme which catalyses the production of cGMP and therefore was predicted to have the ability to reduce the ERG amplitude if cGMP is responsible for channel opening. Again no change was observed.

The results presented in this chapter are summarised in Table 2.6.

Chemical tested	Action	ERG Effect in *S. officinalis*
TTX	Voltage sensitive Na^+ channel blocker	No change (Section 2.3.2)
4-BPB	PLC inhibitor	Complete block (Fig. 2.10 & 2.11, Section 2.3.3)
2-APB	IP_3R inhibitor	Increased latency, reduced sensitivity (Fig. 2.14, Section 2.3.4a)
XeC	IP_3R mediated calcium release	Reduced amplitude to ~50% (Fig. 2.15, Section 2.3.4b)
CFASW	Eliminates Ca^{2+}_e	Increased duration, reduced amplitude with repetitive stimulation (Fig. 2.16 & 2.17, Section 2.3.5a)
Co^{2+}	Ca^{2+} channel blocker	Increased duration, greatly reduced amplitude (Fig. 2.18 & 2.19, Section 2.3.5a)
La^{3+}	Ca^{2+} channel blocker	Increased latency and duration, reduced amplitude (Fig. 2.20 & 2.21, Section 2.3.5a)
CPA	Ca^{2+}ATPase inhibitor	Reduced amplitude by ~50% (Fig. 2.22, Section 2.3.5b)
Thapsigargin	Ca^{2+}ATPase inhibitor	No effect (Section 2.3.5b)
CFASW + CPA	Removes calcium sources	Complete block (Fig. 2.23, Section 2.3.5b)
(-)-Indolactam V	PKC activator	No effect (Fig. 2.25, Section 2.3.6a)
Chelerythrine chloride	PKC inhibitor	No effect (Section 2.3.6a)
NDGA	Lipoxygenase inhibitor	No effect (Section 2.3.6a)
8-Br-cGMP	Membrane permeable cGMP analogue	No effect (Fig. 2.26, Section 2.3.6b)
DB-cGMP	Membrane permeable cGMP analogue	No effect (Section 2.3.6b)
LY-83583	Soluble guanylate cyclase inhibitor	No effect (Section 2.3.6b)

Table 2.6 Table summarising chemical effects.

2.4 Discussion

2.4.1 The preparation

This study has demonstrated the advantage of using the thin retinal slice preparation as opposed to whole or large pieces of retinal tissue, for the investigation of the effects of various pharmacological agents. It has shown by comparison, that chemicals applied to the extracellular bathing solution exerted a lesser effect on a piece of tissue than on a thin slice. It can be suggested that this is due to the dense packing arrangement of the retina preventing access of the test chemical to the target site. It is thought that the chemical was failing to penetrate to the central cells, at the site from which recording was made, which tended to be well within the preparation rather than at the periphery. However recordings from tissue slices do not appear to differ in shape from those from pieces of tissue, suggesting that the physiology remains the same.

2.4.2 Characteristics of the electroretinogram

Increasing stimulus light intensity increases both response amplitude and slope of the rising phase, decreases response latency and has a varying effect on the time constant of decay. These are fairly classical effects of increasing stimulus light intensity on the evoked ERG as has been shown by Weeks & Duncan (1974) and Takagi (1994b). Weeks & Duncan (1974) examined some of these characteristics in the retina of *Sepiola atlantica*. They also found that response amplitude increased with stimulus intensity and found the dynamic range to cover ~4 log units. It is interesting to note that they observed large seasonal variations in sensitivity. Although they did not take direct measurements of slope, it can be seen from the traces that slope also increased with intensity. They also note that response latency decreased as stimulus intensity increased. Takagi (1994b) also found an increase in response amplitude and slope, with increasing stimulus intensity,

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when examining the ERG of *Octopus vulgaris*. Additionally the latent period decreased and rate of rise increased while response amplitude increased. The data for inactivation differs from that presented here, in that inactivation time increased as light intensity increased. Takagi (1994b) suggests this is due to the opening of an excess of the ion channels involved in excitation, producing a long lasting depolarisation against which the repolarising current was less effective. The results presented in this study can be explained as follows. The increased number of photons arriving at the photoreceptors would results in activation of a greater number of second messenger molecules and hence opening of more ion channels, resulting in the increase in response amplitude. Similarly, the threshold number of messenger molecules required to open the light-activated ion channels would be reached within a shorter time resulting in the reduction in response latency. The increase in slope, i.e. rate of activation, would also be due to opening of more ion channels within a shorter time, hence resulting in a greater rate of change in potential. Finally the increase in rate of inactivation may be due to activation of more voltage-activate ion channels involved in repolarisation (i.e. K^+ channels) resulting in more rapid inactivation. At higher intensities it may be that the repolarising current was less effective against the greater depolarisation, resulting in levelling of the intensity-decay plot.

2.4.3 Role of the phosphoinositide pathway

By blocking the action of the enzyme PLC with 4-BPB it was possible to completely abolish the response to a short flash of light over a range of light intensities. It should be noted that, as stated in the results section, 4-BPB is a general PL blocker and therefore would also block PLA_2, the enzyme which catalyses the conversion of phospholipids to arachidonic acid (AA). AA is the precursor to the eicosinoids which are best known for their role in the inflammatory reaction (Rang et al., 1996). There has however been no evidence to suggest that PLA_2 is involved in phototransduction in any invertebrate or

vertebrate preparation. The action of PLC therefore appears to be essential for the production of the photoresponse under these conditions. PLC is also essential for activation of the light sensitive channels in the insect *D. melanogaster*, demonstrated by the *norpA* mutant which lacks eye-specific PLC (Bloomquist et al., 1988) and is blind. 4-BPB has been used by Hardie & Raghu (1998) to examine the effect of PLC inhibition on the activity of expressed TRPL channels. Both spontaneous and agonist induced activity was depressed by 4-BPB. PLC inhibition has also abolished one of the three components of the photoresponse in *L. polyphemus*, although the other two components remained at a reduced amplitude (Nagy & Contzen, 1997). This latter study suggests that there are three parallel pathways operating within the *L. polyphemus* photoreceptor, one of which results in IP$_3$-induced calcium release. This calcium is suggested to regulate the other two pathways. There has been no direct evidence that PLC is essential in *L. polyphemus*. In the above mentioned experiments Nagy & Contzen (1997) injected the PLC inhibitor U-73122. This chemical was tried here but found not to cause any inhibition of the photoresponse at concentrations up to 10 µM. Hardie & Raghu (1998) applied U-73122 by puffer pipette to expressed *D. melanogaster* Trpl channels and found concentrations above 10 µM caused "large non-specific leak". Both of these groups observed an inhibitory effect of this chemical at concentrations <10 µM. However, Cobb & Williamson (1999) also observed no effect with U-73122 (10 and 100 µM) when applied extracellularly to the photoreceptor cells from the epistellar body of *Eledone cirrhosa*.

Although 2-APB is described as a modulator of intracellular IP$_3$-induced calcium release, it has also been found to have multiple sites of action independent of the IP$_3$ receptor being a modulator of both store operated calcium channels (SOCs) and Trp channels (Prakriya & Lewis, 2001) as well as being capable of blocking voltage activated K$^+$ channels (Wang et al., 2002). The present study has clearly shown that 2-APB has the ability to block the

light induced response in the *S. officinalis* retina at a range of concentrations and that, as well as reducing peak response amplitude, it also increases latency and time to peak. 2-APB completely abolished responses to dim flashes of light however a proportion of the response to bright flashes still remained even after 60 minutes exposure. Concentrations from 25–100 μM were tested which fall within the effective range found in other preparations (Chorna-Ornan et al., 2001; Wang et al., 2002). The results would suggest that activation of the IP_3R is necessary for production of a light response to dim stimuli but that it is only required for maximum gain in response to bright flashes. This is most likely due to the subsequent release of calcium which has been shown to be necessary for maximum amplification in *L. polyphemus* (Payne & Demas, 2000) and 2-APB had been directly shown to reduce IP_3-induced calcium release (Wang et al., 2002). There are a number of possible explanations as to why this differential intensity dependent effect occurs and at this stage, without further investigations, this is a matter for speculation. Firstly, it is possible that 2-APB is acting as a competitive inhibitor and that at higher light intensities is overcome by transmitter molecules. Alternatively, it is possible that IP_3-induced calcium release is necessary for the light response a low light intensities but at higher intensities a different mechanism takes over and the calcium release is only required for maximum amplification. As well as blocking calcium release in *L. polyphemus*, 2-APB also inhibits the opening of cation channels in the plasma membrane by calcium. Wang et al. (2002) do not speculate as to what type of channels these may be, however based on the known actions of this chemical it is likely that they are some type of calcium channel, similar to SOC or Trp.

It is clear from this discussion that it is not possible to say exactly what has caused the observed changes in the ERG following exposure to 2-APB, other than that by inhibiting a rise in intracellular calcium from store release and/or influx from the extracellular solution, ERG excitation is slowed, and amplitude is reduced in an intensity dependent manner.

Further to this, XeC was tested, another chemical antagonist of IP_3-induced calcium release. This chemical also reduced response amplitude however there was no change in latency or time to peak suggesting that these effects, which were observed with 2-APB, were due to 2-APB's activity on membrane ion channels rather than at the IP_3R. This result would further support the idea that IP_3-induced calcium release is necessary for response amplification. However, other studies have shown that XeC may not exclusively inhibit IP_3-induced Ca^{2+} release. For example, it has been shown to inhibit the $Ca^{2+}ATPase$ pump on internal calcium stores in some vertebrate preparations (e.g. Castonguay & Robitaille (2002) and Solovyova et al. (2002)). However this action would ultimately reduce the amount of calcium available for release from internal stores, so the end effect is the same, i.e. a reduced amount of calcium released from internal stores.

2.4.4 Evidence for a Ca^{2+} channel in *S. officinalis* photoreceptor membrane

To investigate the possibility of an additional site of action for 2-APB besides the IP_3R, evidence was sought for this site in the cephalopod retina. The site in question would be a plasma membrane calcium channel based on the finding that 2-APB can block both SOC and Trp channels (Prakriya & Lewis, 2001), both of which are cell membrane calcium channels. A trp-like channel has been isolated and characterised in squid photoreceptor membranes (Monk et al., 1996) which has been named strp. This makes it a distinct possibility that this type of channel exists in *S. officinalis* photoreceptors and that it could be a target for 2-APB. The carboxyl terminal domain of the squid strp protein is considerably truncated compared to Trp. The carboxyl domain is thought to be the region involved in channel activation and in the case of Trp, where interaction with intracellular calcium stores may take place. It is therefore suggested that in this case sTrp is actually more like Trpl (for more on Trpl, see Hu & Schilling (1995)). sTrp also shows some similarity to the individual domains of the dihydropyridine-sensitive voltage-gated Ca^{2+}

channel. The sTrp protein carboxyl terminal domain bound calmodulin in a calcium dependent manner which indicates a possible mechanism for regulation of the channel. Overall Monk et al. (1996) suggest that it is a cation channel, primarily mediating Ca^{2+} influx following light stimulation with potential regulation by calmodulin and cytoskeletal interactions.

2.4.5 The role of calcium

Reducing the effect of extracellular calcium by either removing it from the extracellular bathing solution or by using non-specific calcium channel blockers, was found here to generally increase response duration and decay constant, and decrease response amplitude with prolonged stimulation. With CFASW there was usually an increase in response amplitude before it started to decline, and this was also seen at all intensities except the highest when 10 mM $CoCl_2$ was added to the bathing solution, hence demonstrating the intensity dependence of this effect. These findings appear consistent with what one would expect from previous studies in *Sepiola atlantica* (Duncan & Weeks, 1973; Clark & Duncan, 1978). Clark & Duncan (1978) obtained similar results; their main findings being that there was always an increase in duration, the rising phase was insensitive, and the effect was intensity dependent with there being an initial increase in amplitude at low intensities which was not seen at highest intensities. The retina is effectively sensitised by removing external calcium in the same way as it would be by increasing stimulus intensity. Duncan & Weeks (1973) also found removal of calcium to slow the recovery of the ERG, again indicating that calcium is necessary for inactivation / shut down. Similarly, in *L. polyphemus*, Levy & Fein (1985) measured a simultaneous decrease in intracellular calcium and an increase in sensitivity when bathing the photoreceptor in zero calcium ASW, and found exposure to low calcium for longer than ~10 minutes resulted in a loss of sensitivity. This was not correlated with an increase in $[Ca^{2+}]_i$, therefore sensitivity is not

always strictly coupled to intracellular calcium levels. In fact, it has been suggested that time to peak is affected by a change in the capacity to bind Ca^{2+}_i and not by resting Ca^{2+} concentrations (Levy & Fein, 1985). These differing effects on sensitivity might reflect both inhibitory and facilitatory roles for calcium, dependent on its concentration. They also concluded that the increase in calcium is due mainly to release from internal stores as they could still produce a light–induced calcium rise in zero calcium ASW. Although it was not possible to measure intracellular calcium here, a light-induced response was observed following prolonged exposure to CFASW; the response was only reduced when repeated stimuli were applied.

So the main effects of removing extracellular calcium are an initial increase in response amplitude, increased duration and slowed inactivation. Each of these changes can be explained by calcium having a role in response termination exerted through negative feedback. The possible sites of calcium negative feedback are discussed later. Whether this is directly dependent on calcium influx from the extracellular solution or its effect on intracellular calcium concentration and store content cannot be determined at this stage. In *L. polyphemus*, the calcium increase is thought to be due to both influx and release from internal stores as the increase is affected by the amount of calcium in the extracellular solution (Brown & Blinks, 1974). However, the majority of the increase is due to release from internal stores (Payne & Demas, 2000). This is in contrast to *D. melanogaster* where the opposite is true, i.e. the majority of the Ca^{2+} increase is due to influx (Ranganathan et al., 1995). Although there have been no measurements of calcium release from internal stores in the cephalopod retina evidence of potential internal calcium stores have been found (Walrond & Szuts, 1992) and they have been shown to be present in many other invertebrates, closely associated with the photoreceptive microvillar membrane (e.g. Walz (1979)). The later reduction in response amplitude may be due to depletion of internal

Ca^{2+} stores resulting in slowed excitation or the slowed inactivation and hence prolonged response duration may have resulted in a lack of time for recovery.

Mojet (1993) performed a similar experiment in blowfly retina, but in response to longer light flashes. Lowering $[Ca^{2+}]_e$ was found to slow the excitation kinetics, abolish the plateau phase and slow repolarisation. The slowed excitation eventually leads to complete abolition of the peak. As well as removing extracellular calcium, the effects of Co^{2+} at 1, 3 and 10 mM were investigated. 1 mM was found to have no effect and the effect of 3 mM was virtually indistinguishable from that of 10 mM. Interestingly, Co^{2+} did not produce all the effects of removing extracellular Ca^{2+}; namely, the plateau phase was only slightly reduced. Prolonged exposure (i.e. >10 minutes) was found to result in secondary effects probably due to Co^{2+} entry into the cell body. Based on this, it is suggested that the Co^{2+} effect observed was reversible in the piece of tissue (Figure 2.18) because it had not gained access to the cell interior. It is also suggested that the accelerated decline in response amplitude seen in Figure 2.19 after 20 minutes exposure to Co^{2+} was also due to entry into the cell, and not solely due to its action as a calcium channel blocker. Similarly it is suggested that the response became unstable following prolonged exposure to La^{3+} for the same reason.

So to summarise, the effects of Co^{2+} as a Ca^{2+} channel blocker were i) slowed excitation resulting in disappearance of the peak and ii) slowed repolarisation; the plateau was unaffected. This study suggested that there were two distinct binding sites for La^{3+} and Co^{2+}, as other studies have found La^{3+} to abolish the plateau phase in flies (Suss-Toby et al., 1991). La^{3+} is a known blocker of the light-sensitive trp channels which are thought to be involved in Ca^{2+} re-uptake possibly due to store depletion. Mojet (1993) suggests that Co^{2+} may affect a calcium channel involved in accelerating transduction. Obviously it is not possible to differentiate between these two effects as this study has only looked at

responses to relatively short flashes of light and the plateau phase is yet to be investigated. From the results presented there does not appear to be much difference between the effect of La^{3+} and Co^{2+} other than that La^{3+} increases response latency, but this could be explained by its greater efficacy for calcium channels therefore producing a more pronounced slowing of excitation. However it is still possible that this is due to a specific effect on a trp-like channel or a store-operated calcium channel, as an increase in latency was also seen with 2-APB; an effect also thought to be due to an action on membrane calcium channels.

CPA was used in an attempt to differentiate between the roles of calcium influx and calcium release from internal stores. CPA is an inhibitor of Ca^{2+}ATPase, therefore preventing uptake of calcium into the internal stores. This is therefore expected to deplete the internal stores and subsequently eliminate any effects due to release of calcium from these stores. In addition, CPA might also cause an increase in $[Ca^{2+}]_i$ by reducing uptake. CPA reduced the ERG response amplitude, but did not abolish the response and did not affect activation or inactivation. This would suggest that release of calcium from internal stores is necessary for maximum amplification. An alternative possibility would be that the depleted stores trigger opening of SOCs in the plasma membrane raising $[Ca^{2+}]_i$ and triggering response inactivation. These channels would be open more than under normal conditions and therefore the response is inactivated before it can reach its maximum attainable amplitude. A similar effect could also occur if CPA elevates $[Ca^{2+}]_i$ by preventing uptake. Dorlochter et al. (1999) found that CPA greatly reduced single photon responses in *L. polyphemus* ventral photoreceptors without altering the response kinetics. If however, $[Ca^{2+}]_e$ was reduced the waveform was also changed. CPA did not affect the maximal response to bright flashes in *L. polyphemus* (Ukhanov & Payne, 1995); the effect was similar to that of 2-APB in that respect. When *L. polyphemus* photoreceptors were bathed in $0Ca^{2+}$ASW containing CPA the light response was greatly diminished but was

never completely abolished showing that there was a small element of the response which was independent of calcium (Payne & Demas, 2000). However, when the *S. officinalis* retina was exposed to CFASW containing CPA, the response was completely abolished. This would suggest that the *S. officinalis* photoresponse is solely dependent on calcium. However, this was not tested at higher light intensities and therefore the observed effect my just have been a reduction in sensitivity. It is quite possible that a light response could have been elicited with brighter light stimuli and that it also required sodium ions.

2.4.6 Regulation by calcium

It can clearly be seen that calcium plays a complex role in the transduction process, having roles in adaptation, inactivation and excitation. Calcium has been shown to have an effect at nearly every stage of the PI cascade, usually with a concentration dependency. In *D. melanogaster*, trp channels (primarily permeable to Ca^{2+} ions) appear to be important for the plateau phase and La^{3+} mimics the trp phenotype. It has been suggested that trp encodes a calcium transporter which refills IP_3–sensitive calcium stores during light, activated by capacitative Ca^{2+} entry (Vaca et al, 1994). However, recently Hardie et al. (2001) have shown that the calcium entering through trp channels is required to maintain PIP_2 levels suggesting that the trp channels may not be required for refilling stores, however they do require store depletion unlike trpl (Hu & Schilling, 1995). Purified squid PLC activity was examined (Mitchell et al., 1995) by looking at the calcium dependence of its PIP_2 hydrolytic activity. Activity increased steeply up to a maximum at 3-10 μM and then decreased when calcium levels were increased further. The IP_3R has also been shown to have a calcium dependency, being facilitated up to 300 nM and inhibited at higher concentrations in *D. melanogaster* (Hardie, 1993). Payne et al. (1986) have suggested that desensitization of *L. polyphemus* photoreceptors is associated with micromolar calcium concentrations and that excitation is associated with higher concentrations.

2.4.7 Alternative pathways & transmitters

2.4.7.a cGMP

Bacigalupo et al. (1991) applied cGMP to excised patches from *L. polyphemus* and the channels that opened appeared to be like those activated by light. These channels could also be opened by 8-Br-cGMP but they could not be opened by calcium. Also in *L. polyphemus*, injection of cGMP produced a depolarisation with a reversal potential similar to that of the light induced current (Johnson et al., 1986). This effect was shown to be independent of a rise in intracellular calcium levels by the use of the calcium buffer, EGTA. The effect was, however, isolated to specific cGMP sensitive regions. This may be an explanation as to why others have failed to observe a cGMP induced depolarisation in the past (Stern & Lisman, 1982) and indeed why we observed no effect when exposing *S. officinalis* retina to the membrane permeable cGMP analogues 8-Br-cGMP and DB-cGMP. These membrane permeable analogues may not have reached the localised site of action for cGMP. However there is evidence for cGMP gated channels in cephalopods. Huppertz (1995) used Neutral red to measure cyclic nucleotide induced sodium fluxes into reconstituted *S. officinalis* photoreceptor membranes and found evidence for gating by cGMP. Takagi (1994a) found evidence of cGMP channel gating in octopus reconstituted membranes with cGMP blocking, activating and having no effect at all on the different channels present. He also observed a change in the ERG waveform when DBcGMP was bath applied, however the concentration was very high at 25 mM. In addition to this there have been several attempts to detect a light-induced increase in cellular levels of cGMP. A light-stimulated increase in cGMP has been detected in isolated photoreceptor outer segments from squid retina using a radioimmunoassay (Saibil, 1984). However, Brown et al. (1992) found no significant changes in the cGMP content of either *Loligo pealei* or *L. polyphemus* photoreceptors following a light flash and Seidou et al. (1993) saw no change in the nucleotide content of octopus photoreceptors following an intense light flash.

Dorlochter & de Vente (2000) were also unable to detect a light dependent change in cGMP levels however they found strong immumoreactivity for cGMP in the light sensitive lobe of *L. polyphemus* photoreceptors. Lisman et al. (1991) suggest that these negative results can be explained by a large but localised change in cGMP levels which overall would not appear to be a significant increase.

The enzymes controlling cGMP metabolism have also come under investigation, specifically guanylate cyclase (GC) the enzyme which catalyses cGMP production and phosphodiesterase (PDE) which mediates cGMP breakdown. There was no light-induced change in GC activity in squid (Robinson & Cote, 1989) but more recently Garger et al. (2001) have shown that inhibitors of GC can dramatically reduce the response to light in *L. polyphemus* ventral photoreceptors and present evidence supporting the involvement of particulate GC as opposed to soluble GC. Further studies in *L. polyphemus* have detected a light-induced decrease in PDE activity (Inoue et al., 1991). In support of this, injection of cGMP PDE blocked the maintained component of the *L. polyphemus* photoresponse (Nagy, 1993). This study also blocked the inositol cascade with neomycin and the results indicated that both cGMP and Ca^{2+} may participate, but in different components of the response. Finally Johnson & O'Day (1995) found that PDE inhibitors (zaprinast and IBMX) enhanced the response to light in an intensity dependent manner, having a greater effect on responses to bright light, and that lowering calcium greatly increased the effect suggesting that calcium modulates cGMP metabolism. The concentration of LY-83583 used in the present study was within the range shown to be effective in other preparations (Nishi & Gotow, 1998). Neither membrane permeable cGMP analogues nor the cGMP PDE inhibitor zaprinast had any effect on the light response in *D. melanogaster* photoreceptors.

The lack of an effect of LY-83583 in this study may indicate that modulation of GC activity does not play a role in production of the photoresponse. However, LY-83583 is an inhibitor of the soluble form of GC (Mulsch et al., 1989) so it is possible that the particulate form of GC is involved in *S. officinalis* phototransduction as Garger et al. (2001) found to be the case in *L. polyphemus*.

2.4.7.b The DAG branch

As mentioned in the introduction, PUFAs (see Figure 2.24) have been shown to open the light sensitive channels in *D. melanogaster* photoreceptors (Chyb et al., 1999) however, neither arachodonic acid (AA) nor linoleic acid (LNA) activated light sensitive channels in *L. polyphemus*, whether applied externally or injected (Fein & Cavar, 2000). This adds to the growing body of evidence indicating that different transduction mechanisms are used by different species of invertebrates. Lipoxygenase enzymes are involved in metabolising PUFAs (Rang et al., 1996) hence the use of a lipoxygenase inhibitor (NDGA) would be expected to result in a build up of PUFAs and hence enhance or prolong their effect, if indeed they have one in *S. officinalis* photoreceptors. NDGA has been shown to produce events similar to the light-induced quantum bumps in *D. melanogaster* photoreceptors at concentrations as low at 10 μM (Chyb et al., 1999) however no alteration of the ERG in pieces of *S. officinalis* retina was seen suggesting that PUFAs do not play a role in *S. officinalis* phototransduction. Alternatively, it is possible that the chemical did not gain sufficient access to the cells in the slice.

The involvement of PKC (see Figure 2.24) was tested by applying the PKC activator (-)-indolactam V and the PKC inhibitor chelerythrine chloride. Neither elicited any change in the light induced ERG. Smith et al. (1991) have shown that the *D. melanogaster* inaC mutant (inactivation-no-afterpotential) lacks eye specific PKC. They have also shown that this PKC is required for rapid deactivation and desensitization by recording light activated

currents in response to short and long light flashes. Their study suggests that PKC exerts its effect by negative feedback, probably by phosphorylating one of the messengers involved in the transduction cascade. PKC has also been show to be required for adaptation due to a role in termination of individual quantum bumps (Hardie et al., 1993). In the clam, *Lima scabra*, both PKC activators produced large inward currents and an increase in conductance when applied by both puffer pipette and intracellularly suggesting a possible involvement in excitation (Gomez & Nasi, 1998). The effect was reversible with the PKC antagonist, chelerythrine chloride however chelerythrine had no effect alone. The effect of (-)-indolactam V was facilitated by an increased $[Ca^{2+}]_i$. PKC activators have been shown to greatly reduce light sensitivity in an intensity dependent manner in *L. polyphemus* ventral photoreceptors (Dabdoub & Payne, 1999). It was also established that PKC modulates sensitivity at a site in the cascade after rhodopsin and before IP_3, i.e. at the G-protein or PLC stage. PKC inhibitors alone had only a small effect on sensitivity. As PKC inhibitors appear to be effective in opposing PKC activators, but not in exerting an effect alone, it is not surprising that chelerythrine chloride elicited no change in the ERG. However, various PKC activators have been effective in numerous preparations as described above therefore it is very unlikely that they would have no role in the *S. officinalis* retina. It is therefore suggested that a more likely explanation was that they did not reach their site of action. Therefore before a role for PKC can be discounted, activators and inhibitors would have to be put directly into photoreceptor cells by means of injection or whole cell patch clamp recording in order to ensure they had access to the cell interior. The effect of a PKC activator, applied intracellularly, will be investigated in Chapter 5.

2.4.8 Proposed transduction pathway

To sum up one can postulate the possible mechanism of transduction utilised by the *S. officinalis* retina, based on the findings presented. All experiments were performed on

responses to short light flashes, therefore these findings hold true for the transient part of the ERG; the plateau phase was not investigated in this study. The findings and proposed mechanism of transduction in the *S. officinalis* retina are as follows:

i. The action of PLC, which regulates the breakdown of PIP_2 to produce IP_3 and DAG, is essential for production of the light response.

ii. IP_3-induced Ca^{2+} release is involved in response sensitivity.

iii. Increase in $[Ca^{2+}]_i$ is due to both influx from the extracellular space and release from internal stores.

iv. A channel that can be blocked by 2-APB but not XeC is involved in response activation. The channel is consistent with a trp-like calcium channel.

v. Ca^{2+} both excites and inactivates the photoresponse; its role being highly concentration dependent.

vi. There is still the possibility of a role for cGMP as a terminal transmitter responsible for opening the light dependent channels.

CHAPTER 3: A Study of Temporal Resolution and Sensitivity in the Retina of *Sepia Officinalis*

3.1 Introduction

3.1.1 Resolution of the visual system

The eye's ability to extract visual information from its environment is defined by its resolving power. This falls into two main categories; spatial resolution and temporal resolution. Most studies of invertebrate species tend to focus on spatial resolution (Muntz & Gwyther, 1988; Watanuki et al., 2000), leaving temporal properties less well investigated. The spatial resolving power of a visual system is a measure of how close two objects can be in the visual field and still be seen by the eye as two separately distinguishable objects. There will be a critical point where these two objects will appear as one. Similarly, temporal resolution looks at how close in time two stimuli can be presented to the eye before they merge into one. The former is a function of structure and optics of the photoreceptor (McIlwain, 1996) and the latter is a function of the cell membrane properties and kinetics of the underlying cellular processes (Cuttle et al., 1995; Weckstrom & Laughlin, 1995). Spatial resolution can be measured by performing behavioural experiments where the animals follow alternating bands of black and white stripes of equal width. The stripe width is decreased until the animal no longer follows them in a rotating drum and this gives a measure of the minimum separable angle (MSA), a commonly used measure of spatial resolution or "acuity" as it is often referred to. Temporal resolution can be determined by measuring critical flicker fusion frequency (CFF) and this test can be performed in both the intact animal and the isolated retina. CFF is the transition point where an intermittent light ceases to flicker and appears continuous. As CFF is dependent on light intensity (Hart, 1987) it is preferable to measure the maximum CFF (mCFF). This is the frequency at which increases in light intensity no longer lead to an increase in CFF (Frank, 1999).

In the case of both spatial and temporal resolution, rather than intensity, per se, being of importance, it is actually the level of contrast that it produces. Therefore a flash of light in a well-illuminated room may not be detected, but if the same intensity flash is applied in a darkened room it will then become visible because the contrast has increased. Contrast can be defined as the maximum luminance difference between dark and light areas divided by the sum of these luminances (McIlwain, 1996). In the following experiments the background light levels remain constant, except when being studied specifically. This therefore means that contrast increases as the intensity of stimulus light increases.

Temporal resolution, and hence CFF can be influenced by many factors: stimulus intensity, as shown by the Ferry-Porter law (which states that CFF is proportional to the logarithm of the luminance of the flickering light stimulus is based on the work of Ferry (1892) and Porter (1902)), spectral composition, retinal recording position, size of test field, age/health of the animal, duration of exposure, level of light or dark adaptation (Landis, 1954; D'Eath, 1998; Kalloniatis & Luu, 2002). In cephalopods, the evidence so far suggests that all photoreceptors are of the same type (Yamamoto et al., 1965; Cohen, 1973a), except in the case of the one known exception, the firefly squid *Watasenia scintillans*, which has three types of visual pigment (Seidou et al., 1990). Cuttlefish photoreceptors all contain the photopigment rhodopsin therefore resulting in the same spectral sensitivity (Brown & Brown, 1958) although they do differ in diameter and length across the retina (Young, 1971). This would therefore suggest that spectral composition would affect all cells uniformly. This is in contrast to the situation in the vertebrate retina where there are two populations of photoreceptors, i.e. rods and cones, which respond differently to different wavelengths of light and are present in changing proportions across the retina. It is only diameter and length of photoreceptor that changes across the retina of the cuttlefish and only length changes as the animal grows, with more photoreceptors being added rather than the existing ones increasing in diameter (Farley, unpublished). However, it is

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important to remember that the cephalopod retina also has two populations of photoreceptors differing in their polarized light sensitivity which is attributable to cell orientation (Moody & Parriss, 1960, 1961; Tomita, 1968).

Temporal resolution determined by ERG measurements has only been performed once for cephalopod species. Hamasaki (1968) measured flicker fusion frequency in *Octopus vulgaris* and *Octopus briareus*. These experiments were performed in intact anaesthetised animals and produced an mCFF value of 72 cycles per second at the maximum luminance of 1.3×10^6 ftL (foot Lamberts). This value was determined by taking an average of increasing and decreasing thresholds.

3.1.2 Temporal resolution relates to habitat and lifestyle

Temporal resolution is an important aspect of vision as most animals live in a changing visual environment. Excellent spatial resolution would be of no use if this were not combined with good temporal resolution in order to follow the moving image. One of the most obvious situations, encountered by most animals, would be prey capture. Although some animals are efficient stalkers and can surprise their prey, usually there is some sort of chase involved, which requires the ability to keep an accurate fix on the target.

Terrestrial animals have been shown to display temporal resolution characteristics that can be closely associated with their environment (Autrum, 1950, 1984). This relationship should also apply to the marine environment, where fast moving animals hunting fast moving prey would require more rapid temporal resolution than more sedentary animals, for example. Additionally, sensitivity might also be expected to correlate with habitat. For instance, an animal living deep in the oceans or an animal active at night would not be expected to experience rapid or large changes in background illumination. It would

therefore be important for them to respond strongly to every small change in intensity. Such high gain eyes tend to have a low CFF but high sensitivity and this has been shown to be the case in some species of crustaceans (Frank, 1999). However, the more brightly lit environment is correlated with higher temporal resolution and low gain eyes that would be better equipped to deal with the changing light levels (e.g. light versus shade). Similarly it is logical that fast moving insects have particularly fast temporal resolution (Laughlin & Weckstrom, 1993).

3.1.3 Relating cell properties to temporal resolution

Temporal properties are dependent on the dynamics of the photoresponse and the cell membrane properties. As described in the previous chapter, the result of the invertebrate phototransduction cascade is a depolarization, due to the opening of ion channels and, as stated by Frank (2000), "temporal resolution is a function of the membrane properties of the receptor cells" which encompasses the activity of these ion channels. Temporal resolution may give an indication of the different classes of ion channels active in the rhabdomeres. Also important would be the area of the membrane and the length of the photoreceptor available for photon capture as this will influence how many photons can be captured within a certain time period, i.e. a longer cell should be able to capture more photons than a shorter cell within the same time period.

Whether the retina detects two flashes of light as one or two signals, depends on the temporal integration time of the individual cells as illustrated in Figure 3.1. The temporal integration time is the time over which the cell will sum the effects of quantal absorptions. This summing of quantal effects is known as temporal summation and the maximum time over which this can occur is the critical duration (t_c); no summation occurs beyond the critical duration. Beyond the critical duration the response elicited by a stimulus light is

dependent on its intensity alone; rather than the product of intensity and duration (Hart, 1987). Different species have been shown to have different t_c, however how the cell properties and the transduction cascade impose these differing values is unknown (de Souza & Ventura, 1989). A short integration time will improve temporal resolution (Figure 3.1), however longer integration times would improve sensitivity by allowing a longer time to collect photons.
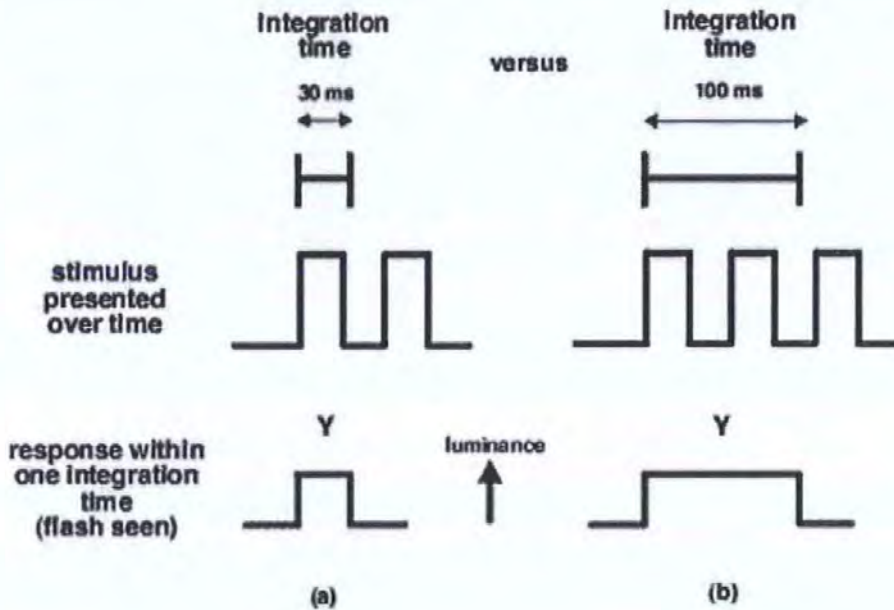
Figure 3.1 Figure taken from Kalloniatis & Luu (2002) illustrating the effect of having short versus long integration time. It can be seen that in situation (a) where the integration time is short the stimulus flash is seen as presented. However in (b) where the integration time is longer, the flash observed is longer than that presented as the individual flashes were summed over time.

So essentially, integration time is the time it takes the cell to produce a photoresponse and to return to a state where it is ready to produce another light response. So speed of integration will determine whether intermittent stimuli will be detected or not, assuming that the transduction kinetics are sufficiently fast so as not to be a limiting factor in this process.

The cell integration time is determined by a number of factors:

- intensity of stimulus

- speed / rate of the phototransduction cascade and available messengers

- number / population of ion channels

- size of receptor / receptor surface area, etc.

- recovery time, for instance rate of second messenger recycling.

3.1.4 Experimental design

In order to examine how temporal resolution is affected by stimulus intensity and animal size, it was first necessary to take account of the other factors which influence temporal resolution. These were listed in Section 3.1.1. The effects of the duration of exposure, size of test field and adaptation can be eliminated in the experimental design. Keeping it constant in all experiments can eliminate the effect of duration of exposure. Any effects of the size of the test field can be eliminated by illuminating the whole preparation and by keeping the size of the preparation (in terms of area of retina) constant in all experiments. An attempt was also made to take account of retinal recording position by replicating all measurements of CFF in both central and peripheral locations. However no difference was found and therefore the results recorded at both locations were collated. One would have expected there to be a difference due to the differences in cell size, however the recording setup used did not allow particularly accurate electrode placement therefore the extremes of cell length and diameter may not have been found. Subsequently more average sized cells may have been recorded from in this instance.

As *S. officinalis* grow, there is a very distinct change in the size of the photoreceptors. As well as the total length of the photoreceptor changing, the length of the outer segment in proportion to the inner segment changes with age. This chapter addresses the question that

cell size is a limiting factor in determining the temporal characteristics and sensitivity of the *S. officinalis* retinal cells. Temporal resolution can give an indication of the efficiency of the transduction cascade and whether this has a limiting effect on the cell's temporal characteristics or if, indeed differences in cell size limit transduction efficiency.

The objectives for this chapter, as outlined in the general introduction, are as follows:

- To determine the maximum CFF for this animal to obtain an indication of temporal resolution.

- To determine whether the temporal resolution changes as the animal grows from juvenile to adult.

- To measure threshold sensitivity and response latency in both juvenile and adult animals.

- To determine how the levels of background illumination affect the temporal resolving power of the *S. officinalis* retina.

3.2 Materials and Methods

3.2.1 Animals

The species used in the following experiments was the common cuttlefish, *Sepia officinalis*. A range of animal sizes was used as defined below. All juvenile animals used had been hatched and reared at the Marine Biological Association (MBA). Adult animals were a mixture of those reared at the MBA and those caught locally off the coast of Plymouth by the RV Squilla. Captured animals were brought back to the laboratory and kept in aerated tanks for up to a number of months, some being used for experimentation and others being integrated into the breeding program.

3.2.2 Animal categorisation

Animals have been classified according to their dorsal mantle length (ML), which is illustrated in Figure 3.2. This categorization has been based on that used by Hanlon & Messenger (1988). Four age groups have been specified and are defined in Table 3.1.
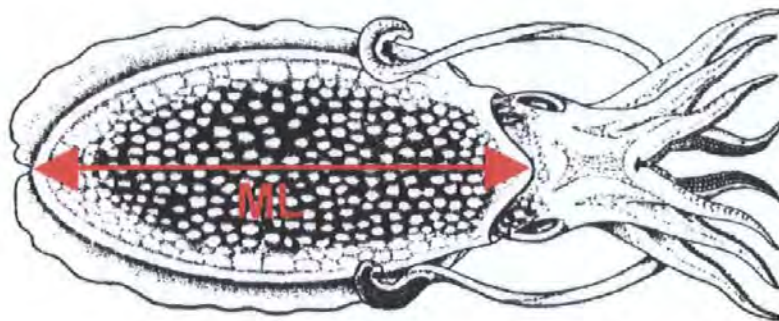
Figure 3.2 Diagram showing the cuttlefish mantle length (ML) which is used as the standard size measurement.

Definition	Size (mm)	age (weeks)
Hatchling	7-10	<1
Early Juvenile	16-20	<7
Late Juvenile	35-45	<17
Post juvenile / Adult	>45	>17

Table 3.1 Animal categorization. *S. officinalis* **can be classified according to size and age. Data taken from Hanlon & Messenger (1988).**

For these experiments animals from the early juvenile and adult categories were used with mean ML of 19.7 mm and 141 mm respectively.

3.2.3 Electrophysiological recordings

Prior to experimentation, the animal was dark adapted for 30 minutes. For electrophysiological recordings, animals were decapitated following anaesthesia in 2 % alcohol, and then the eyes dissected out under dim red light whilst bathed in artificial seawater (ASW). The lens was removed leaving the eye cup, which was either used whole in the case of the small animals or a piece was cut out in larger animals. With the larger animals, an equal number of samples were taken from both central and peripheral regions. The final piece of tissue used was always 5-10 mm^2. The tissue was then pinned down in the recording dish and perfused with oxygenated, ASW. The perfusion solution was kept on ice, which resulted in a bath temperature of 18-22 °C. Standard ERG recording was used; therefore see ERG methods for more details. In this configuration, experiments on a single preparation could be carried out for up to 3 hours before the response amplitude started to decline, which was thought to be indicative of reduced cell viability.

In all of the data presented in the results sections, the n number is the number of individual pieces of tissue and is, in some cases, also a different animal.

3.2.4 Pulse protocols / light stimuli

Light stimuli were applied from a Schott 1500 LCD cold light source via a light guide positioned so that the light at the output, when at maximum intensity, was 200 μWcm^{-2}. A tungsten lamp was used to apply an adapting background light when appropriate. In the case of both light sources, any infrared component was filtered out, and both were tested with a polarizing filter and found to be non-polarized. The circle of light at the output of both light sources illuminated an area larger than the eyecup or the piece of tissue in the case of adult animals. Flash duration was controlled by a Uniblitz shutter (model T132) under computer control so that a 50:50 light : dark cycle was maintained. Values used to apply flickering light with increasing frequency are shown in Table 3.2.

Frequency (Hz)	Duration/Interval (ms)	M
10	50.0	5
12	41.7	6
14	35.7	7
16	31.2	8
18	27.5	9
20	25.0	10
22	22.5	11
24	20.8	12
26	19.2	13
28	17.8	14
30	16.7	15
32	15.6	16
34	14.7	17
36	13.8	18
38	13.1	19
40	12.5	20
42	11.9	21
44	11.4	22
46	10.9	23
48	10.4	24
50	10.0	25

Table 3.2 Flicker stimulus protocol. M is the number of flashes applied per stimulus sequence.

Light intensity was controlled by neutral density filters which were manually changed, and by changing the aperture setting on the Schott light source. The intensity of the light was measured at the level of the preparation with a light meter (Advantest TQ8210). CFF was

only correct to within 2 Hz due to the 0.5 s test stimulus duration. To apply intermediate frequencies would require half pulses.

3.2.5 Procedure

After electrode placement on the retina, the specimen was dark-adapted until the response to a low intensity (3 μWcm^{-2}) test flash, given every 5 minutes, had not changed in amplitude or waveform for 30 minutes thus, indicating the eye was fully dark adapted. A flickering light stimulus of 0.5 second duration was then presented to the dark adapted eye and the ERG response examined to determine whether the flickering stimulus was followed. To ensure every flicker stimulus was presented to a fully dark-adapted eye, the 50 ms test flash was used to test for recovery between presentations of the flicker stimuli. The test flash was applied at 5 minute intervals until the test flash amplitude had recovered to its original state. Flicker rate for subsequent stimuli was increased until flicker fusion was achieved; this is defined as the point where the eye can no longer produce a modulated signal in time with the flickering stimulus. The stimulus light was then increased and the flicker rate once again increased until flicker fusion was achieved. This continued until the response to the test flash started to decline and the preparation was no longer considered viable for experimentation, or the maximum critical flicker fusion frequency was reached.

In order to determine the effect of background light on the critical flicker fusion frequency, the same procedure as described above was repeated whilst introducing constant levels of background light ranging from 300 $nWcm^{-2}$ to 1100 $nWcm^{-2}$. The lower of these light levels had no effect on CFF; therefore the light intensity was increased in increments of 200 $nWcm^{-2}$ until an effect was observed. The results presented were in the presence of ~ 1 μWcm^{-2}. In the case of these experiments the response to the test flash was allowed to

settle in the presence of the background light, as opposed to the dark conditions of the previous experiment.

3.2.6 Sensitivity

Sensitivity was calculated for a sample of juvenile and adult animals. In order to determine the sensitivity of the eye, responses to 50 ms flashes of light were recorded with increasing irradiance intensity. Intensity was increased up to the maximum available intensity or until the response amplitude no longer increased. Response amplitude was then plotted as a function of log intensity and from this, the relative sensitivity of the eyes can be determined. Sensitivity is often obtained by determining the log I_{10} value which is the intensity at 10 % maximum response amplitude (V_{max}) (after de Souza & Ventura (1989)). However with the intensity range available, V_{max} was rarely reached, therefore it was decided to use different criteria to determine sensitivity. Following Ziedins & Meyer-Rochow (1990), sensitivity was determined by taking the minimum stimulus intensity required to produce a threshold response, defined as the threshold intensity.

3.2.7 Latency

Latency (T_{lat}) was used as an indicator of the speed of activation of the phototransduction process (Frank, 1999) and was indicative of how quickly the threshold number of photons is reached in order to trigger the cellular signalling pathway. This parameter was taken as the time from start of stimulus to start of response and was also measured and compared for both juvenile and adult animals.

3.2.8 Tissue fixation and scanning electron microscopy

The opportunity arose to perform the same experiments in the octopus, *Eledone cirrhosa*. It was considered an interesting experiment to carry out as the previous measure of cephalopod CFF was carried out in *O. vulgaris* and *O. briareus* (Hamasaki, 1968). As the size of the photoreceptors forms part of the argument it was necessary to prepare the tissue for examination using the scanning electron microscope in order to measure the outer segment length. *E. cirrhosa* retinal tissue was treated with 0.05 % trypsin (Sigma) in ASW for 10 minutes on a shaker and washed twice in ASW. It was then possible to mechanically remove the limiting membrane with forceps. The tissue was then fixed overnight in 2.5 % gluteraldehyde in ASW. It was then dehydrated as described in Chapter 2, but for 20-30 minutes at each concentration due to the large size of the tissue and was cut into appropriately sized sections when in 70 % alcohol. The tissue was then transferred into absolute acetone and transferred to the critical point drier and sputter coated with gold. Measurements were then taken whilst viewing the preparations using a scanning electron microscope (SEM).

3.3 Results

Although both positive and negative ERG responses are obtainable, depending on where the recording electrode is placed within the retina, it was decided to use only the positive response in this case as it was more easily obtained and was of greater amplitude. It is considered to be acceptable to use either, as they are both the result of the same cellular process (Hagins et al., 1962; Pynsent & Duncan, 1977; Tsacopoulos et al., 1989).

The response to the test flash was used to monitor the adaptational state of the retina to ensure that it was fully dark-adapted before application of each flicker stimulus. A flicker stimulus was therefore not applied until the response to the test stimulus had returned to its original amplitude indicating the retina had re-dark-adapted. The term "dark-adapted" was used here, however there were very low light levels in the experimental room, of the order of 200-300 $nWcm^{-2}$ at the level of the preparation. This was necessary as the minimum amount of light required to carry out the experiment. However, the preparation was essentially considered dark-adapted and the steady state response amplitude used as a reference as to the adaptational state of the retina.

Three parameters have been measured using the ERG recording in order to characterise *S. officinalis*'s temporal resolution and light sensitivity, namely mCFF, threshold sensitivity and response latency. By examining these parameters it can be deduced how this animal has adapted to best cope with its environmental conditions and how this differs between adult and juvenile animals.

3.3.1 Critical flicker fusion frequency

Some example traces of the response to flickering light stimuli are shown in Figure 3.3.
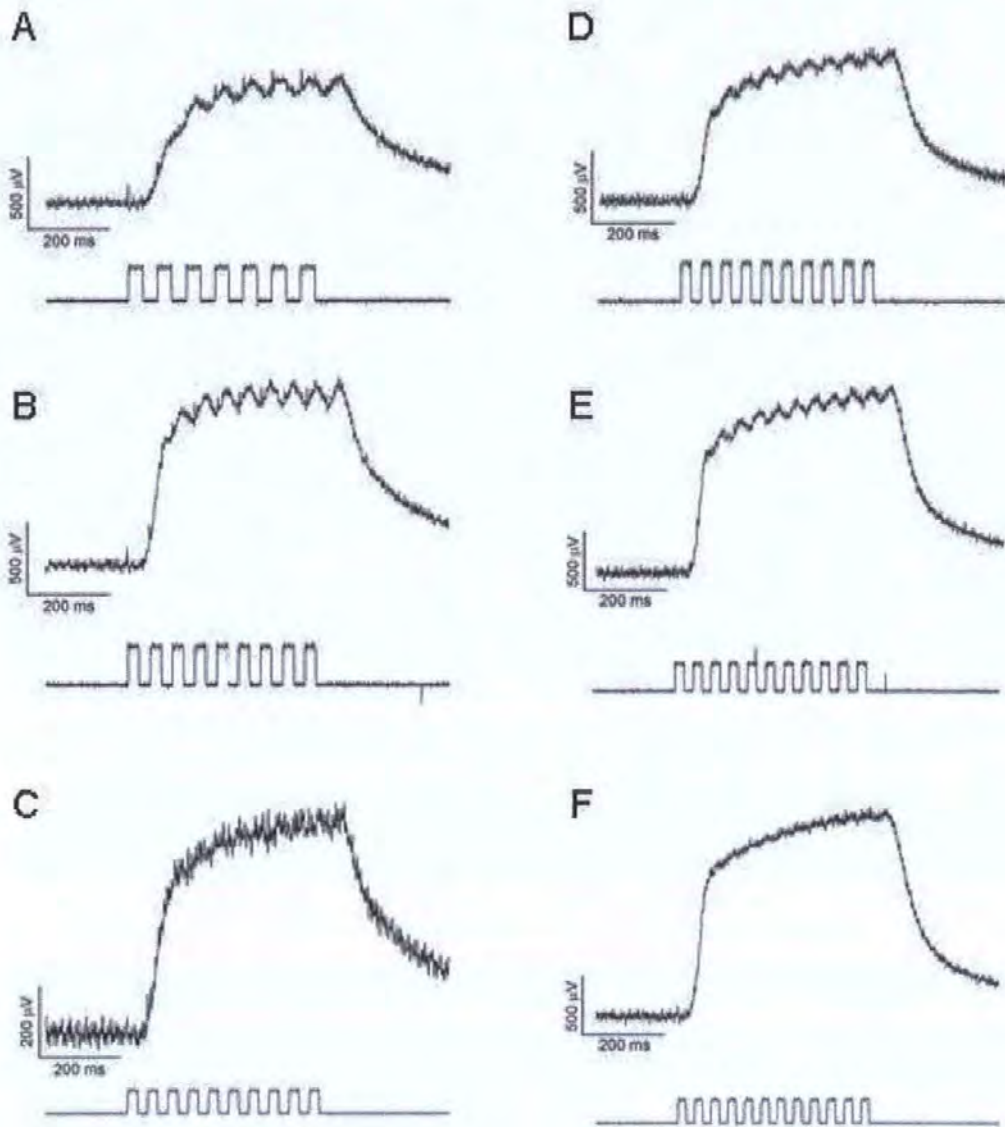
Figure 3.3 Representative traces showing response to flickering light stimuli. Upper traces are the ERG responses and lower traces are the light stimulus. These traces are all taken from results obtained from juvenile animals. Figures A and B are from the same animal and show the response to 14 Hz and 18 Hz stimuli respectively. These frequencies were the maximum followed at 0.3 μWcm^{-2} and 0.5 μWcm^{-2} for this particular preparation. C is the same response as B, but at 20 Hz where the response has fused. Figures D and E were taken from a different individual and show the ERG following light stimuli at 20 Hz and 22 Hz respectively at 1.3 and 2.0 μWcm^{-2}. F is the same response as E but at 24 Hz where the response no longer follows the flickering stimulus.

3.3.1.a Juvenile animals

Within 30-45 minutes of applying the test flash a steady test response was obtained, determined by its constant amplitude and shape, and presentation of flickering light stimuli could commence. The amplitude of the response to a test flash varied from preparation to preparation. This was attributed to the known differences in ERG amplitude obtainable from various depths within the retina and differences in electrode configuration. Tasaki et

al. (1963) have demonstrated this in octopus and it has been replicated in the *S. officinalis* retina where differences were observed in response amplitude when the ERG was recorded from various depths within a slice of retina (Figure 3.4). These variations were unavoidable and have been observed in numerous other preparations (Tsacopoulos et al., 1989; Frank, 1999), however every effort has been made to minimize them by always recording from the narrower inner segment (relative to the outer segment) therefore minimizing the scope for variation.

When taking these recordings an equal number from central and peripheral areas were used. No difference was observed between the results obtained from peripheral and central areas. Therefore the results can be taken as being representative of a sample of the whole eye.
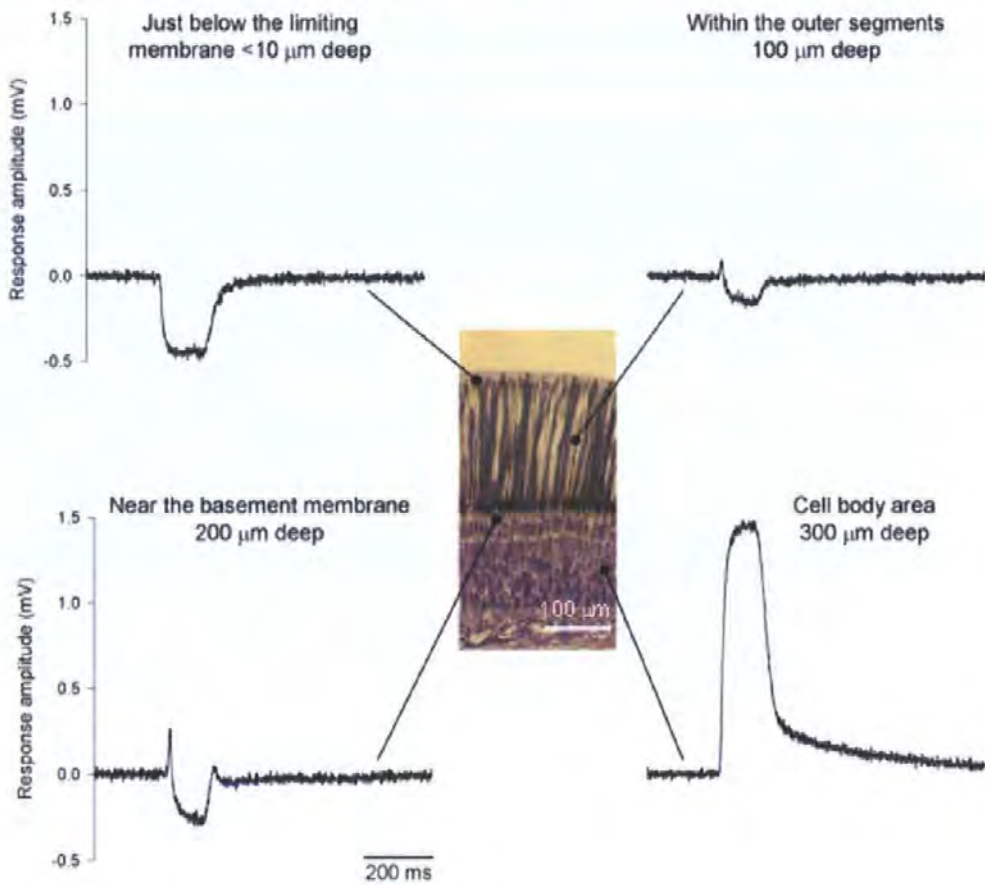
Figure 3.4 ERG recordings from different depths within the retina. Electrode was placed in the specified positions by visualising a slice of retina under infrared illumination. A photo of a slice of tissue is shown in the middle showing approximately, where the recordings were taken from.

As stated in the introduction, CFF is known to be dependent on stimulus intensity. This is clearly illustrated for early juvenile animals in Figure 3.5. CFF is lower at low light intensities and higher at high light intensities. From this graph it can be seen that the mCFF obtained was 42 Hz at 23.3 μWcm^{-2}. Further increases in irradiance above this level did not produce any further increase in CFF; in fact CFF starts to drop off after this point. The first five points of this graph can be fitted with a straight line with the following equation:

$$y = 21x + 22, R^2 = 0.97 \ (P<0.01, DF = 3)$$

This shows that there is a linear relationship between luminance and CFF up to mCFF which conforms to the Ferry-Porter law (Landis, 1954). This law states that CFF (Hz) is

directly proportional to the log of luminance (Note that the light intensity was measured in μWcm^{-2} in this study, which is a measure of irradiance rather than luminance however the law still applies). This is illustrated in Figure 3.6.
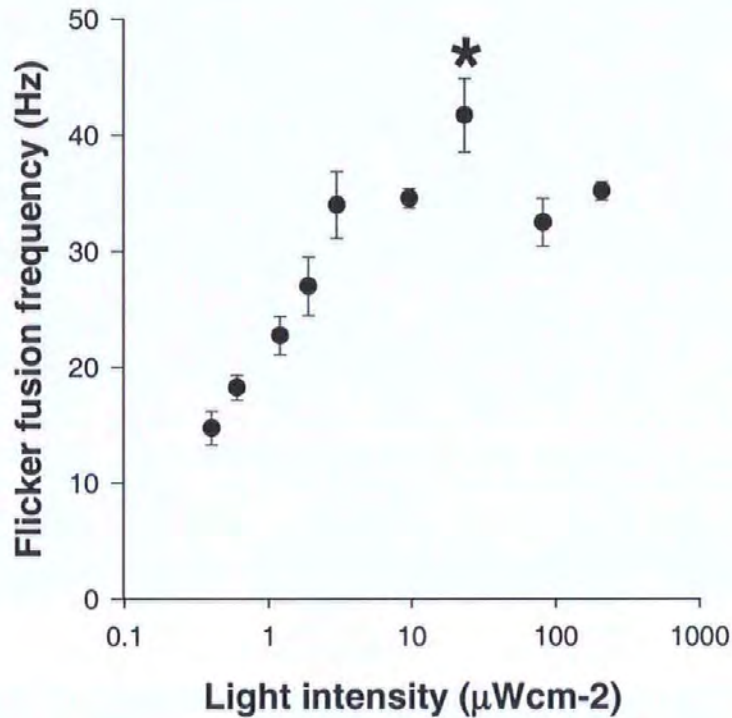
Figure 3.5 **Flicker fusion frequency in early juvenile cuttlefish.** **Flicker fusion frequency increases with increasing stimulus intensity up to the maximum CFF (indicated by *).** **Beyond this intensity, further increases do not produce higher CFF values.** **The early part of the graph can be fitted with a straight line with the equation y = 21 x + 22, R^2 = 0.97 (P<0.01, DF = 3) to demonstrate the adherence to the Ferry-Porter law.** **Each data point the mean +/- SE, n > 5.**
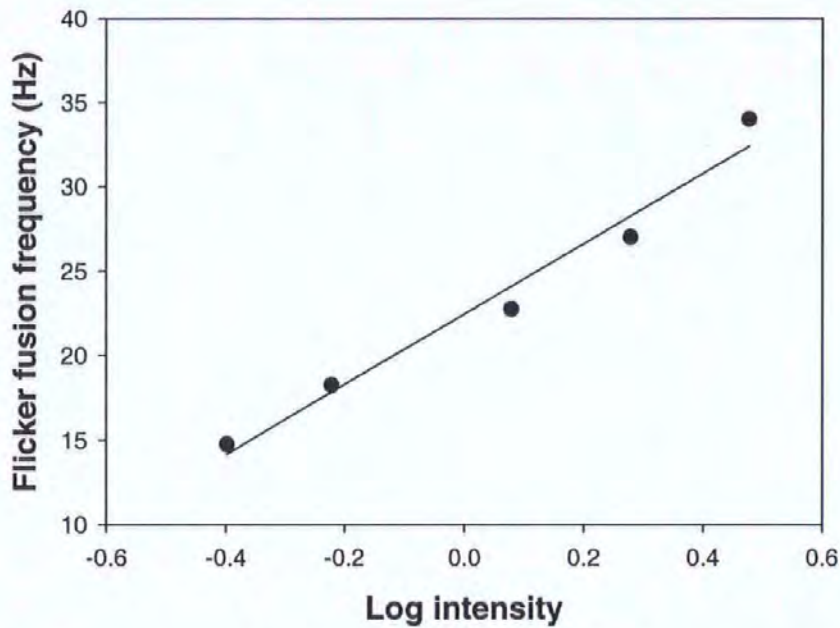
Figure 3.6 First five data points from the above juvenile CFF graph have been used to demonstrate the adherence to the Ferry-Porter law. The linear part of Figure 3.5 has been fitted with a straight line with the equation $y = 21x + 22$, $R^2 = 0.97$ ($P<0.01$, $DF = 3$). This shows that, during the linear part of the graph up to mCFF, CFF is directly proportional to log I.

3.3.1.b Adult animals

During adaptation the response to the test flash usually declined rapidly in amplitude during the first 5-10 minutes and then proceeded to increase gradually during the duration of the experiment. The test flash amplitude rarely levelled out in the adult animals as it did in juveniles, however once the gradual rise had started, flicker stimuli were applied and tests were continued as long as gradual rise persisted. Experimentation was halted when the test response amplitude started to decline, which usually occurred after 2-3 hours.

As was observed in the juvenile animals, CFF was also lower at low light levels and higher at high light levels in the adult animals as shown in Figure 3.7. However the mCFF for adults was much lower at 24 Hz than that for the juveniles which is thought to be, at least in part, due to the difference in size of the photoreceptor cells, and was attained within a much shorter stimulus intensity range, being reached at a low light intensity of 1.3 µWcm
2
.
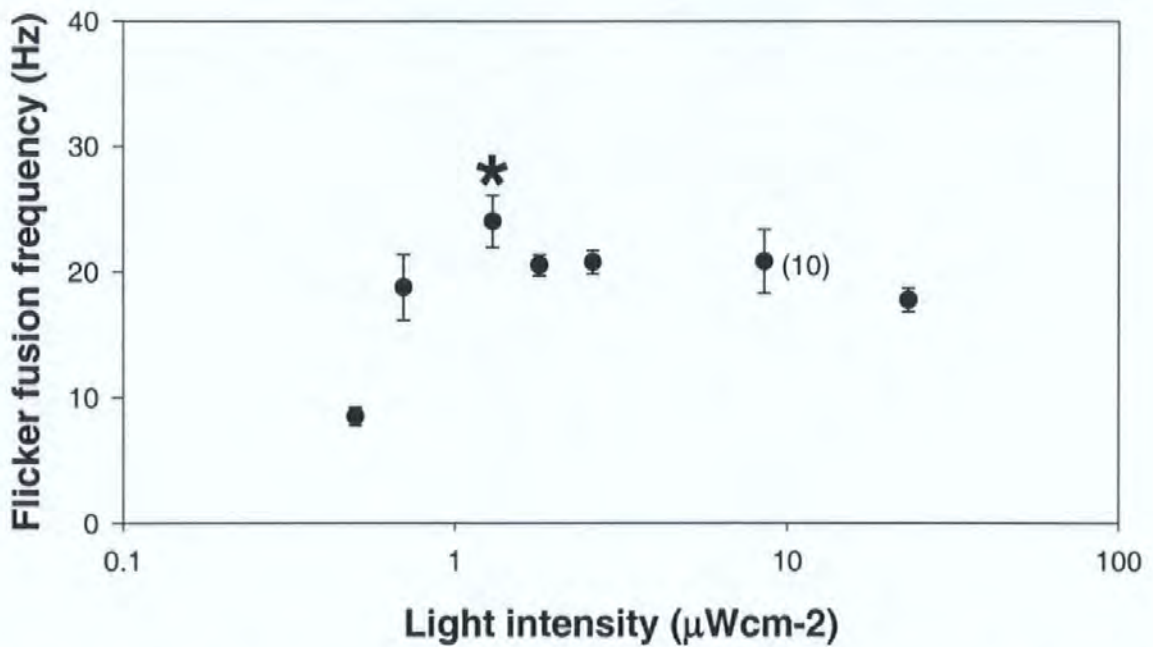
Figure 3.7 Flicker fusion frequency in adult animals. mCFF is 24 Hz reached at 1.3 μWcm^{-2}, indicated by *. Graph has been compiled from data from 5 individuals and each data point is the mean +/- SE, n = 8, except where indicated in brackets.

3.3.2 Effect of background light

Table 3.3 shows the effect on mCFF of applying an adapting background light to the juvenile preparation. This was only tested in juvenile animals but a similar effect would be expected in the adult animals. This degree of adaptation did not have a significant effect on the mCFF value attainable by the photoreceptors. Although mCFF was unaffected, the graph was shifted to the right indicating a reduction in sensitivity which is the typical effect of light adaptation in the photoreceptor response (e.g. insects (Matic & Laughlin, 1981); crustaceans (Frank, 2000); cephalopods (Groeger, personal communication)).

Maximum CFF (Hz)	
Dark-adapted (n = 4)	Light-adapted (n = 4)
37 +/- 1.7	36 +/- 2.2

Table 3.3 Effect of an adapting background light on mCFF in early juvenile animals. Data are the mean +/- SE mCFFs for dark- and light-adapted preparations. The intensity of the background light was ~1 μWcm^{-2}.

3.3.3 <u>Sensitivity</u>

When comparing the threshold intensity of juvenile and adult animals, the juvenile animals were found to be more sensitive. Threshold intensity was measured as 250 μV, as this was the size of response that could be consistently distinguished from noise. The results are shown in Table 3.4. A Student's t-test was performed and the mean threshold intensity found to be significantly different between adults and juveniles at a 95 % confidence level.

To examine sensitivity, an intensity-response (V/logI) plot was constructed by recording the amplitude of the photoresponse elicited by flashes of increasing intensity stimuli (Figure 3.8). Sensitivity has been measured as threshold sensitivity due to the range of light intensity available. This data could also have been used to give a measure of the animals' dynamic range and contrast sensitivity, however to do this both plots would really need to have levelled out in order to obtain a value for the maximum response amplitude (V_{max}). To measure the full dynamic range, it is necessary to record the full range of response amplitudes from threshold to the plateau, when V_{max} is reached; and to obtain a measure of contrast sensitivity it is necessary to fit the data with a function containing a value for V_{max} (Eguchi & Horikoshi, 1984).

Animal group	n	Mean threshold intensity (μWcm^{-2})	SEM
Juvenile	8	1.2	0.2
Adult	10	2.1	0.3

Table 3.4 Intensities required to produce a threshold response in juvenile and adult animals. n - number of samples, SEM - standard error of the mean.
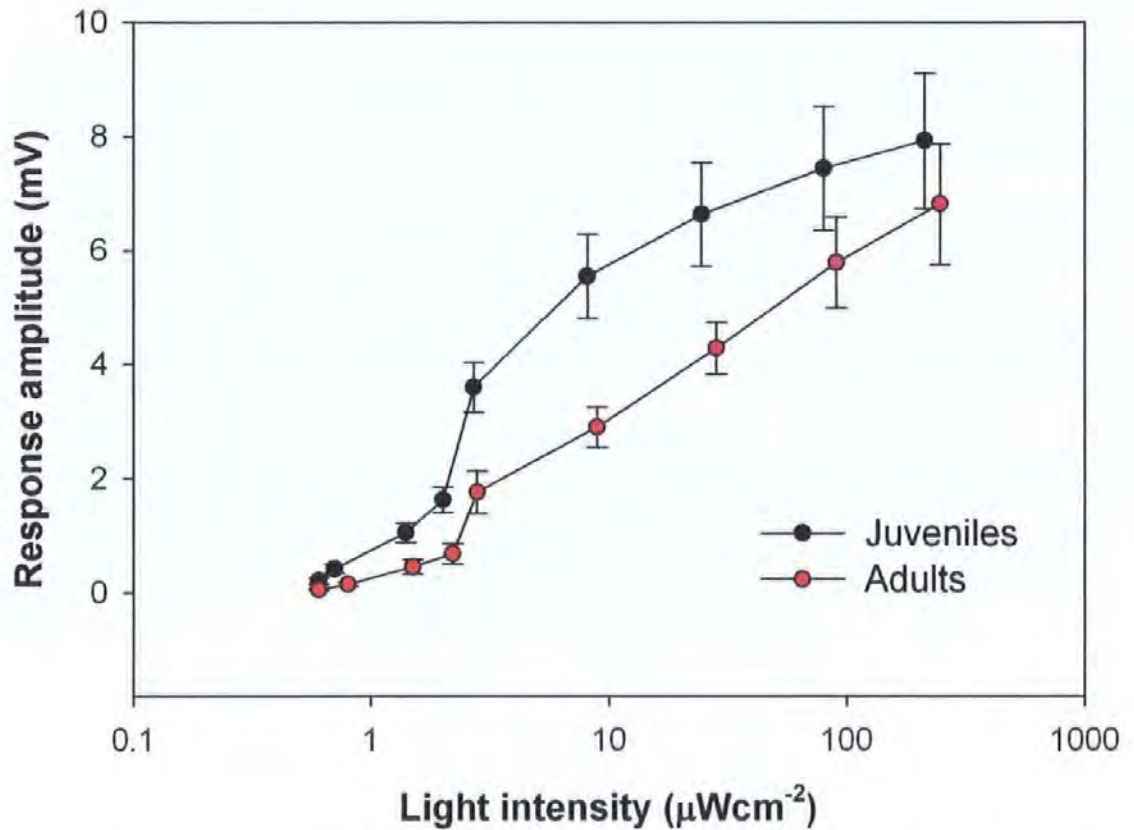
Figure 3.8 Intensity response plots from a sample of juvenile animals (black) and adults animals (red). Each data point is the mean ± SE where n = 6 and n = 9 for the juvenile and adult age groups respectively.

3.3.4 Latency

Figure 3.9 illustrates the relationship between response latency and stimulus intensity. This was taken from the same experimental results used to plot the intensity response plots (Figure 3.8). This graph clearly shows that in both juveniles and adults, response latency decreased as stimulus light intensity increased, indicating that the rate of response activation was increasing. This is easily explained by the greater number of photons being applied, resulting in the threshold number of photon being reached within a shorter period of time. Overall, responses from the adult animals were slower with longer latency values showing that the animals with a lower CFF are correspondingly slower to respond and that the animals with a higher CFF are faster.
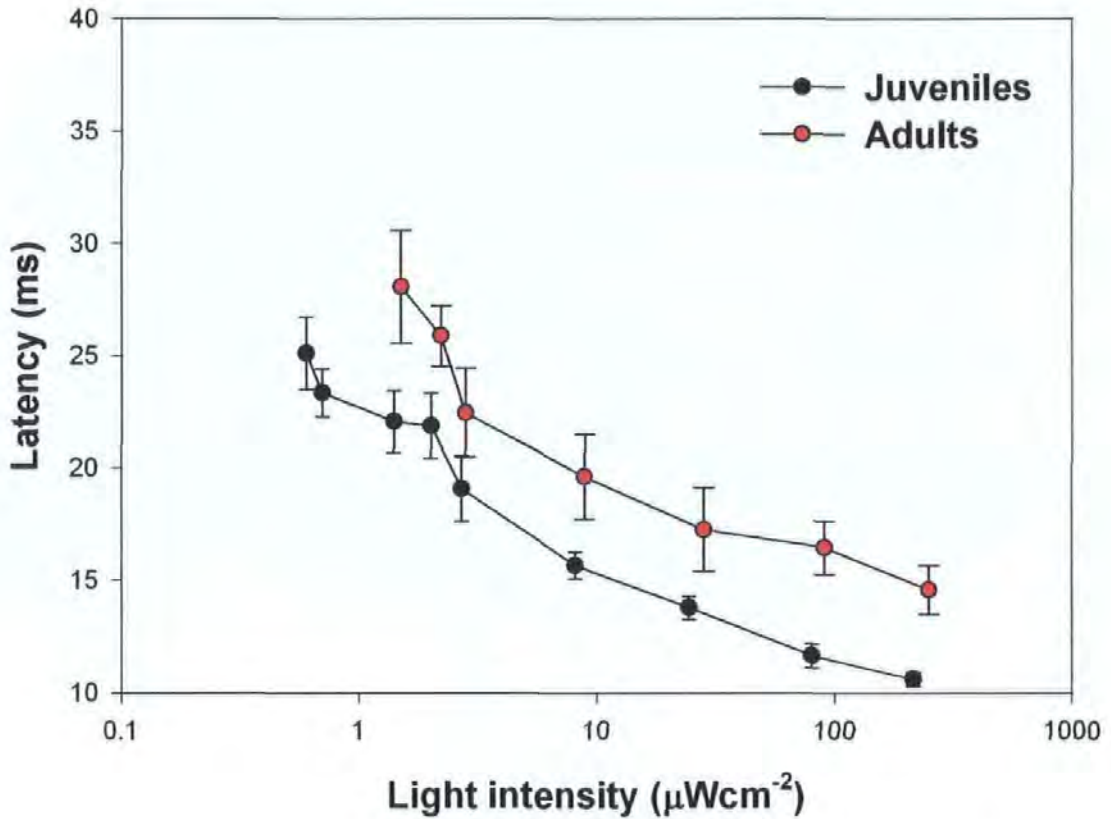
Figure 3.9 Graph to show how ERG response latency changes with increasing stimulus intensity. In both adult and juvenile animals, response latency decreases with increasing light intensity.

Some example traces of intensity response runs from juvenile and adult animals are shown in Figure 3.10. From these traces it is possible to see that the slope of the rising phase of the ERG increased with intensity, indicating that the rate of the transduction cascade had also increased. In order to determine if the slope of the rising phase of the ERG was different in the two age groups these values were measured and the results shown in Figure 3.11. When plotted on a double log scale, these data can be fitted with a best fit straight line. The slope of the adult regression was not significantly different from the juvenile regression (ANCOVA $F(1,86) = 1.21$, $P>0.01$) but there was a significant difference between intercepts ($P(t)<0.01$). This shows that light intensity has the same effect on both adults and juveniles, i.e. as light intensity increased slope also increased however the slope, overall, was great in juveniles suggesting that the rate of transduction excitation was greater.
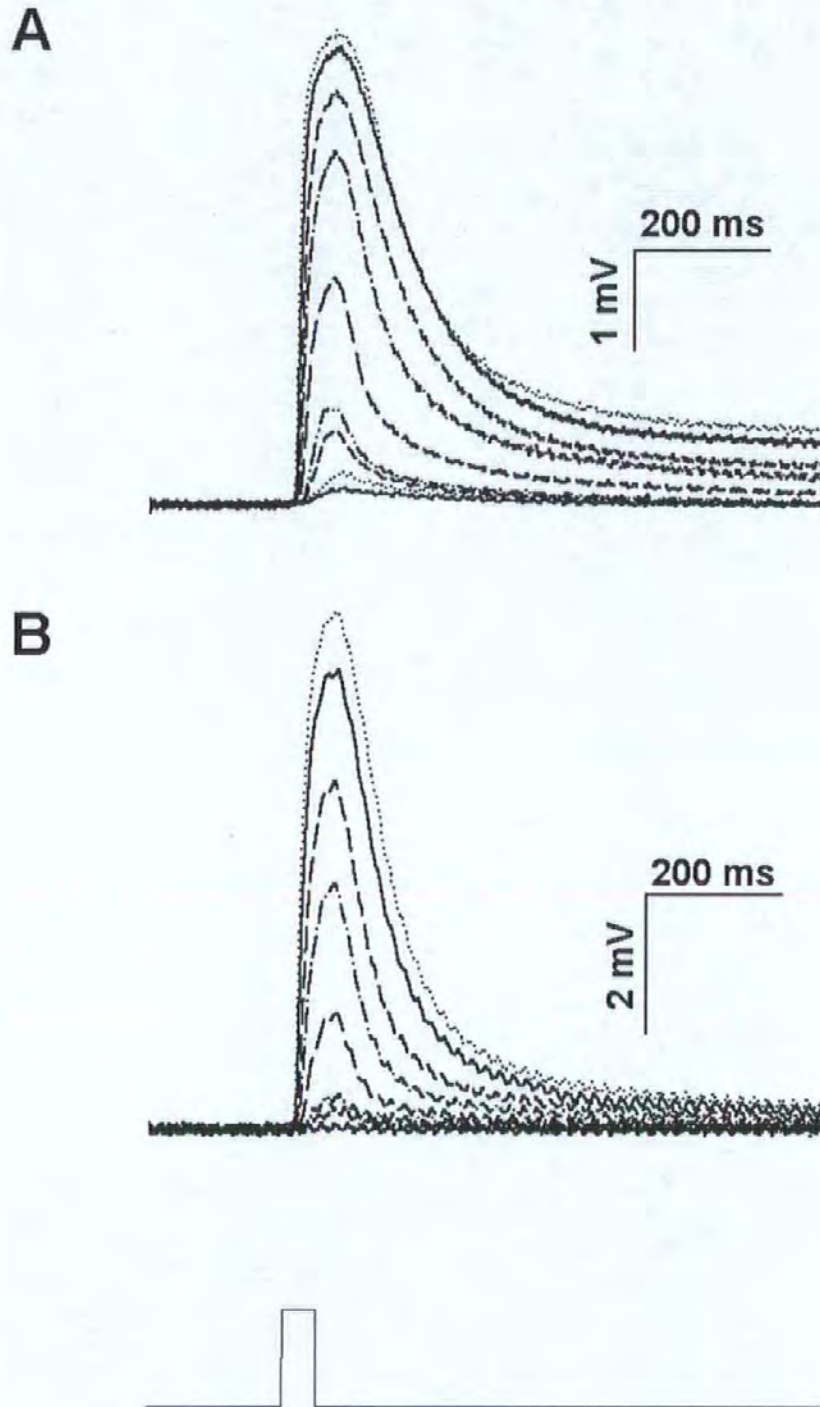
Figure 3.10   Superimposed photoresponses elicited by increasing intensity light stimuli.   The photoresponse was recorded every 5 minutes in response to a 50 ms light flash as indicated below the traces.   The light intensity increased from 0.6 μWcm^{-2} to ~200 μWcm^{-2}.   Parts A and B show the responses recorded in a juvenile and an adult animal respectively.
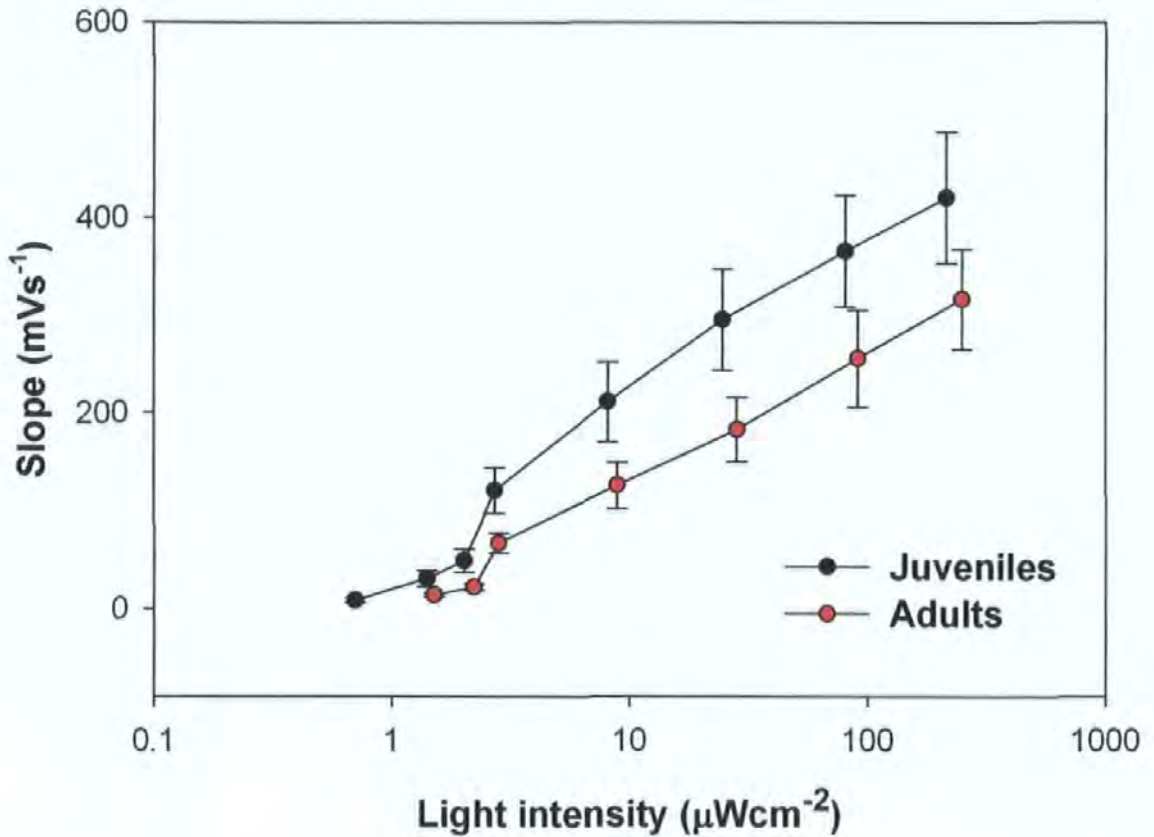
Figure 3.11 Plot of the slope of the rising phase of the ERG against light intensity for both juvenile (black) and adult (red) animals.

3.3.5 Eledone cirrhosa

3.3.5.a Electrophysiological measurements

The opportunity to test the temporal characteristics of the octopus, *E. cirrhosa* arose when the MBA boat fortuitously caught one of these animals. This was a useful experiment to perform as the only other measurement of CFF in cephalopods was done in octopus (*Octopus vulgaris* and *Octopus briareus* (Hamasaki, 1968)). The results presented are from one individual and therefore only give a preliminary indication of this animal's temporal properties. The mCFF was found to be 24 Hz (Figure 3.12), the same as that of the adult *S. officinalis*. Also in common with adult *S. officinalis*, this value was reached at the relatively low light intensity of 2.6 μWcm^{-2}.
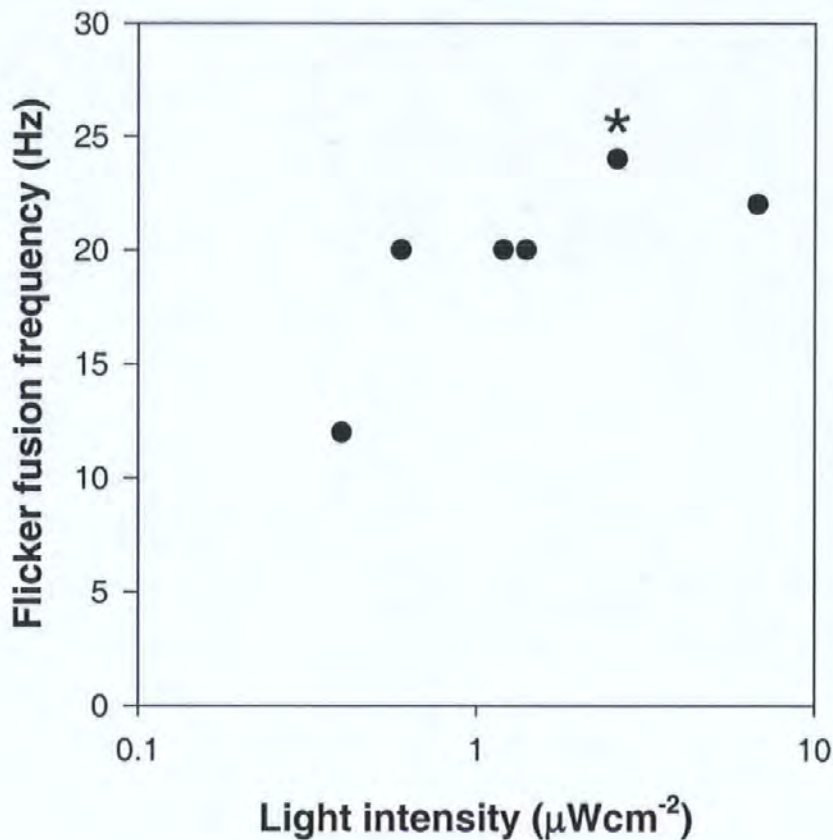
Figure 3.12 CFF in an adult *Eledone cirrhosa*. CFF was measured at increasing light intensities and mCFF (*) was found to be 24 Hz and this was at 2.6 μWcm^{-2}. This graph illustrated the data obtained from one individual.

Based on the criteria used to determine threshold sensitivity in *S. officinalis*, the sensitivity threshold of *E. cirrhosa* was 0.4 μWcm^{-2}. This would suggest a greater sensitivity than both juvenile and adult S. officinalis, however it must be reiterated that this was only one measurement and some juvenile *S. officinalis* also responded to this intensity. It should be noted that the threshold intensity was the lowest intensity available with the present set up and therefore a threshold response may have occurred at an even lower intensity. It can be seen that the response amplitude increased from 0.3 mV to 8.7 mV as the stimulus intensity increased from 0.4 to 194 μWcm^{-2} showing a dynamic range of ~3 log units (Figure 3.13).
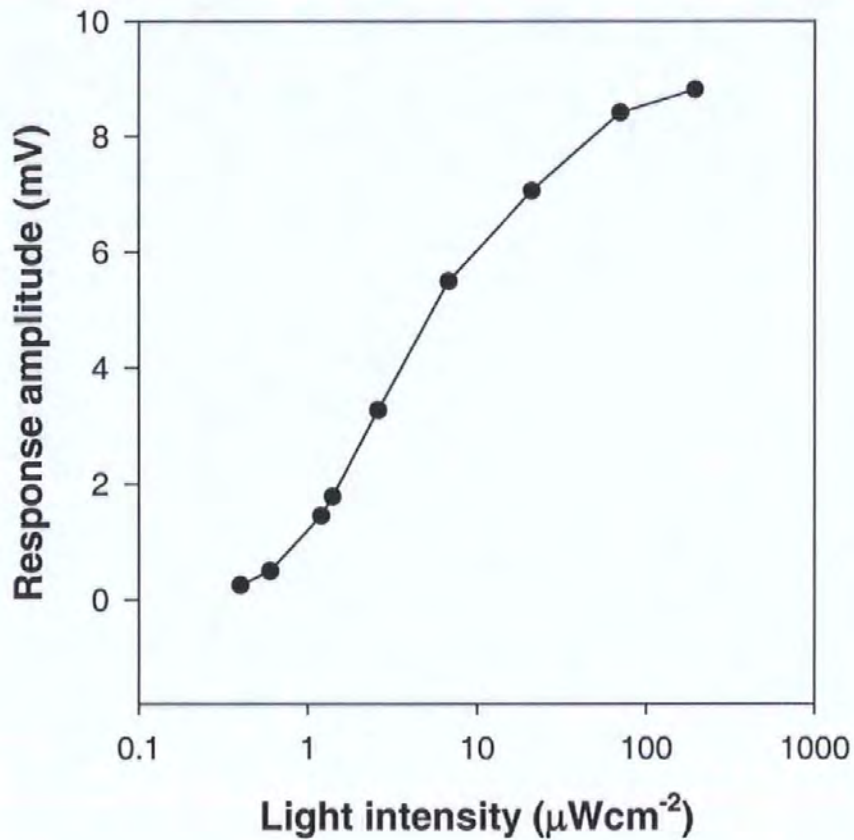
Figure 3.13 Response amplitude measured in retinal slices of *E. cirrhosa* with increasing stimulus intensity. Each data point is one recording from one individual measured at each light intensity.

Response latency decreased from 35 ms to 15 ms as the stimulus intensity increased from 0.4-194 μWcm^{-2}. This again indicates an increased rate of activation with increased stimulus intensity. The range of latency values measured in *E. cirrhosa* follow more closely those of the adult *S. officinalis* than the juveniles.
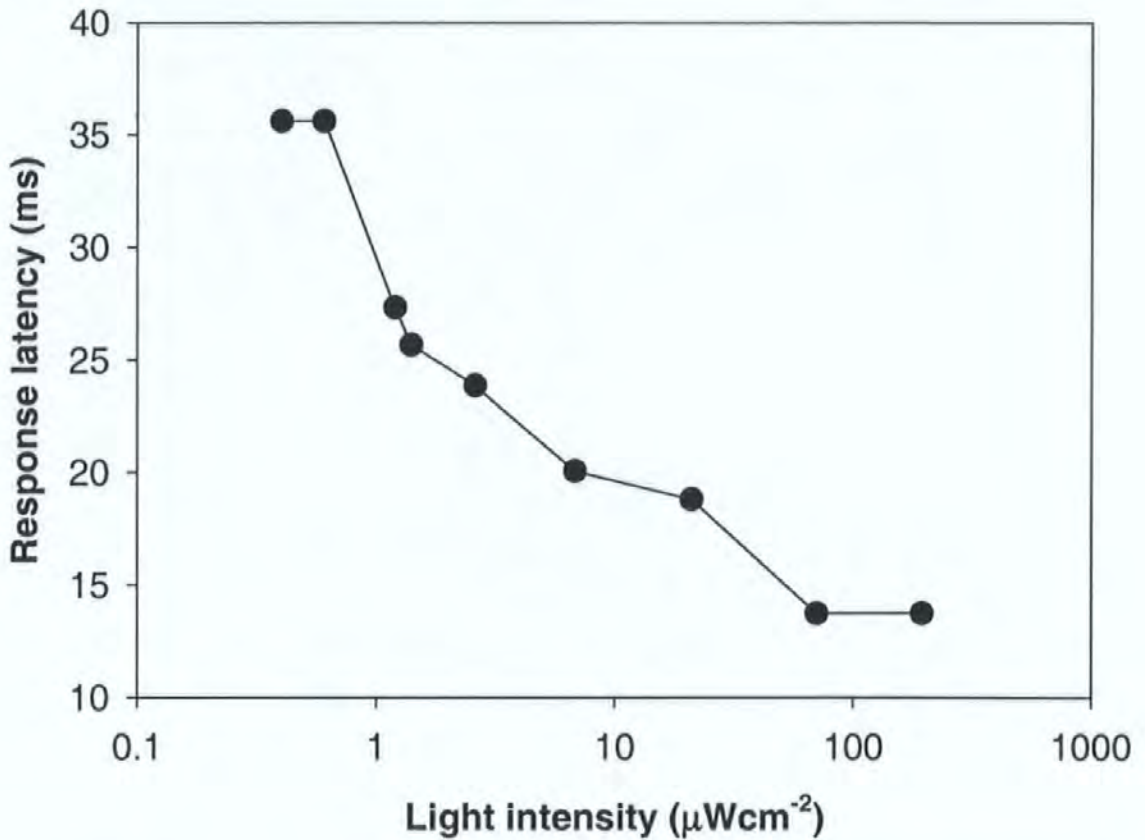
Figure 3.14 Response latency recorded in *E. cirrhosa* taken from one individual with increasing light intensities from 0.4 to 194 μWcm⁻².

3.3.5.b Measurements of outer segment length

Retinal photoreceptor outer segment (OS) length was also measured as it is thought that the size of the photoreceptor cells may be an important factor in determining the cells' temporal characteristics. This was done by examining sections of tissue under the scanning electron microscope (SEM). Ten measurements were taken at equally spaced intervals from specified areas of the retina to give an average outer segment length. Measurements were taken from central and peripheral areas to give a range representative of the outer segment lengths throughout the retina. Previous examination of outer segment length both in this group and by others (Young, 1963) would suggest that measurements from these areas are where the photoreceptors are shortest and longest. It was assumed that all other areas would have OS lengths falling within this range. The sections used to

measure OS length are shown diagrammatically in Figure 3.15A and Figure 3.15B, and indicate where on these sections the measurements were made. These measurements were taken from an eye with a diameter of 2.2 cm (previous work in this lab has shown that this was a similar sized eye to that found in a *S. officinalis* with a mantle length of 20-23 cm).
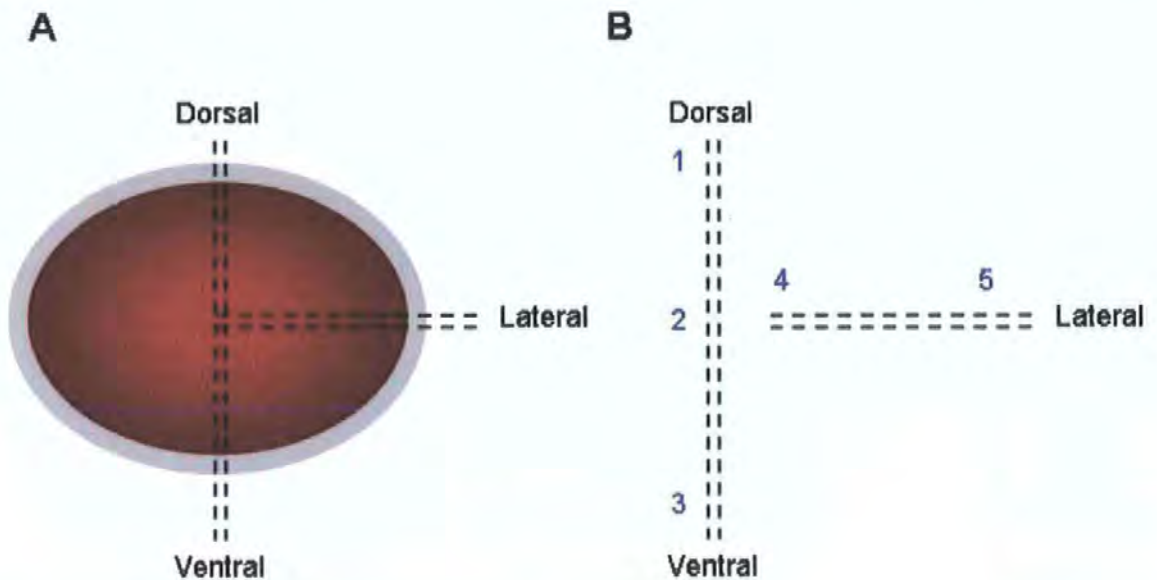
Figure 3.15 Diagrammatic representation of a cephalopod eyecup and sections. This diagram shows a view of the eyecup when looking directly down onto it from above (A). This has been used to show where the sections were taken from for the measurement of outer segment length. A dorsal ventral section was taken as indicated by the vertical dashed lines, and a lateral section was taken as indicated by the horizontal dashed lines). The mean outer segment length was then calculated for each of the positions 1-5 indicated in B.

Location	Mean OS length (µm) n = 10	Standard Error
1	92.4	1.1
2	149.8	1.6
3	83.1	2.3
4	178.2	5.1
5	73.9	2.1

Table 3.5 The outer segment length measured at 5 locations in the *E. cirrhosa* retina as indicated in Figure 3.15B. The OS length was measured at 10 equally spaced points at each location within a 100 µm wide section of the tissue.

These measurements show that the mean outer segment length ranged from 74 μm in the periphery to 178 μm in the central region of the eye. These values fall within the range measured by Young (1963) however, Young's more detailed study found values both slightly lower and higher than these, ranging from near 0 to 250 μm.

3.3.6 Summary

Thus the results can be summarized as follows:

- Smaller animals, with shorter photoreceptors, have been found to have higher temporal resolution than the larger, older animals with longer photoreceptors.

- The smaller animals have also been shown to have faster activation kinetics and to have greater sensitivity than the adults.

- The single *E. cirrhosa* tested was found to have temporal resolving powers and activation kinetics similar to the adult *S. officinalis* however its sensitivity was greater than that of both the juveniles and the adults.

3.4 Discussion

In this chapter it has been possible to determine the temporal resolution of the photoresponse from juvenile and adult *S. officinalis* visual systems by measuring the mCFF from isolated pieces of retina. It was determined that the mCFF for juveniles was around 42 Hz, almost twice that of the young adult animals which was found to be around 24 Hz. These values are similar to those obtained from the human retina which has a mCFF of ~15-60 Hz depending on the proportion of rods and cones being stimulated (Hart, 1987). It was observed that the mCFF for adult animals was reached at a much lower light intensity than for juveniles. These mCFF values are much lower than those obtained in the only other study in which CFF was measured in a cephalopod species. Hamasaki (1968) measured a mCFF of 72 Hz with a full intensity stimulus of 1.3×10^6 ftL, in octopus. He also measured the slope of the straight portion of the plot and obtained a value of 16 Hz/log unit which is a similar rate of increase as found in the present studies (e.g. Figure 3.6), of 21 Hz/log unit. Hamasaki's experiments were performed in intact anaesthesised animals, which may be a contributory factor to the discrepancy between the mCFF values. However it is also possible that this is a species difference especially considering the difference found here between two age groups of the same species. Hamasaki (1968) combined results obtained from *Octopus vulgaris* and *Octopus briareus* ranging from 120 to 390 grams in weight. These are shallow living species found on coral reefs or rocky ground (Norman, 2000). This is a slightly different environment to that encountered by *S. officinalis* therefore light levels experienced may be higher, and the speed of available prey may also differ. Another possible explanation for the large difference between the measured mCFF values may be the duration of stimulation. Hamasaki (1968) does not state how long the flickering light stimulus was applied for, but it can be determined from the figure that it was at least 1 second. However in this study the stimulus was applied for 0.5 second. Landis (1954) discusses the fact that CFF in humans is dependent on duration

of exposure and that CFF increases with duration from 0.1-1 seconds. But their figure (Figure 10 in Landis (1954)) shows that by 0.5 seconds further increases in stimulus duration have very little effect on CFF, however the effect of stimulus duration is possibly quite different in cephalopods.

The possibility that the maximum spiking frequency of the cells could influence the mCFF value has been considered. If the firing rate of retinal action potentials is low then there would be no advantage in having a higher mCFF. Higher mCFF would be wasteful, as the high temporal resolution would be lost when being passed centrally via action potentials. However, the evidence suggests that this is very unlikely. Lange & Hartline (1974) have used suction electrodes to record nerve action potentials from single retinal units in squid. Over a range of stimulus intensities, they observed a broad range of firing rates up to 400 Hz. It would be highly unlikely that the spiking rate would be so much lower in *S. officinalis* so as to have any impact on a useful mCFF. In fact, any spiking activity observed in this laboratory has been at a rate of around 100 Hz (Groeger, personal communication), which is also in excess of the mCFF values measured.

3.4.1 Why does CFF increase with increasing stimulus intensity?

Previous experiments have shown CFF to be dependent on the stimulus light intensity (Crozier & Wolf, 1939; Landis, 1954) and this is indeed what was found to be the case for this preparation. At low light levels it takes longer for the threshold number of photons to be reached and therefore the integration time is longer resulting in a low CFF. As light intensity increases, the threshold number of photons is reached in a shorter time, resulting in a shorter integration time and higher CFF. This relationship (defined by the Ferry-Porter law, based on the work of Ferry (1892) and Porter (1902)) continues up to a critical point where an increase in intensity no longer results in a higher CFF. Here the response to

intermittent light fuses and an individual response to each applied flash cannot be distinguished. The response fuses because the temporal integration time is too slow to detect every response at this rate of stimulation. It would be sensible to assume that at this frequency and at frequencies above, the amount of light being supplied is no longer a limiting factor in the speed of the cell's response. The speed of integration could now be limited by a number of other factors such as amount of available second messenger, rate of second messenger recycling, ion channel properties, number of ion channels, membrane properties, and diffusion of messengers within the cell. Further increases in light intensity beyond the mCFF resulted in a decrease in CFF. Landis (1954) states that other investigators have also found further increases in stimulus intensity to result in a decrease in CFF, but unfortunately no references are available to support this suggestion. However, Conner (1982) also found CFF to be depressed at very bright light levels, but this was in humans and was attributed to saturation of rod vision. One could speculate that CFF starts to decline in *S. officinalis* with bright light due to saturation of the transduction cascade therefore reducing availability of intracellular second messengers to produce a modulating response in time with higher frequency flicker.

3.4.2 Relating differences in mCFF to outer segment length

The mCFF is reached at a lower light intensity in adults than in juveniles, and is correspondingly lower. One possible explanation would be that this phenomenon is attributable to structural changes in the photoreceptors that occur with age. Farley (unpublished) has shown that there are some very distinct changes in the length and proportions of *S. officinalis* photoreceptors between juveniles and adults. As well as the receptors growing in length with age, the proportion of outer segment to inner segment also increases. This is obviously significant as the outer segment is the phototransducing

part of the cell. Possible ways in which this could affect the temporal resolution will now be discussed.

3.4.3 Membrane time constant

The cell membrane time constant is a measure of how long it takes for a cell to change the voltage across its membrane. This value is measured in milliseconds (ms) and is denoted by the symbol τ_m. τ_m is dependent on two factors, membrane resistance (R_m) and membrane capacitance (C_m). These variables are related by the following equation:

$$\tau_m = R_m C_m$$

The lipid membrane acts as an insulator between the two conductors of the intra- and extracellular solutions that together form a capacitor. As the surface area of the parallel plates of a capacitor increases, the capacitance also increases. Similarly, as the area of the lipid membrane increases capacitance will increase and result in a greater membrane time constant. As capacitance is dependent on the area of membrane, membrane resistance is dependent on ionic conductance.

3.4.3.a Rhabdome area & outer segment length

Based on the above reasoning it can be assumed that variations in photoreceptor outer segment length could result in variations in membrane time constant and hence, flicker fusion frequency. Therefore it would be predicted that an increase in outer segment length and hence membrane area would increase the time constant resulting in lower temporal resolution which would manifest itself in a lower mCFF. The results presented here state that the mCFF is lower in adult animals than in juveniles therefore, following this argument the rhabdomes would be expected to be longer in the adult animals. This is indeed what has been observed in our laboratory (Farley, unpublished). The photoreceptor outer segment length was significantly greater in adult animals than in juveniles.

Furthermore, outer segment length is known to change depending on the adaptational state of the preparation, namely, illumination causes rhabdomes to contract (Young, 1963). However, any possible contribution of this change has been eliminated by the experimental procedure. By applying the short, low intensity test flash it was ensured that the cells were always in the same adaptational state, i.e. dark-adapted, before presenting each flickering stimulus.

As well as increasing membrane time constant, greater outer segment area could also affect the diffusion rate of the internal messenger molecules. This again could slow down the speed of the response resulting in a lower temporal resolution. A correlation between rhabdom cross-sectional area (and hence cell volume) and critical duration (as defined below) was found by de Souza & Ventura (1989) when examining the photoreceptor properties of seven species of hymenopteran. Critical duration (t_c) is the maximum time over which temporal summation occurs, i.e. the summing of quantal events. The shorter the t_c the greater the temporal resolving capacity of a cell. If however t_c is long, temporal resolving capacity will be reduced as individual events may be summed and interpreted as one (refer back to Figure 3.1). The critical duration of a cell gives a good measure of the speed of the photoreceptor process and de Souza & Ventura (1989) found critical durations of 10-46 ms, which correlated with both rhabdomal cross-sectional area and the animals' lifestyle. They suggest that differences in messenger diffusion rate may account for differences in critical duration between species. Matin (1968) found that CFF is equal to the reciprocal of t_c, therefore for this study t_c was equal to 24 ms for juveniles and 42 ms for adults. This corresponds to the intermediate and slow groups from the study of de Souza & Ventura (1989). They found that the animals which were slowest temporally were nocturnal and relatively slow moving.

As stated, adult animals reached their mCFF at a lower light intensity than juveniles. This would suggest that a factor other than light intensity becomes limiting. Again, this can be explained by the structural differences between the two groups, i.e. outer segment length. It could be suggested that the physical constraints of the adult's photoreceptors prevent them from attaining a higher temporal resolution, no matter how much light they can capture.

It is interesting to note that photoreceptors have been found to be longer in *S. officinalis* retina than in *Octopus vulgaris* (Young, 1963) which, based on the above argument, may be a contributing factor to the difference between the mCFF value obtained here, and that measured by Hamasaki (1968). The *O. vulgaris* used by Young (1963) to measure photoreceptor length weighed 350g which is within the range used by Hamasaki (1968) to measure CFF, unfortunately Young (1963) does not mention the size of the *S. officinalis* used in his experiments. However, more recently a much more detailed study of photoreceptor size in *S. officinalis* has been undertaken in this lab (Farley, unpublished). Using these data it has been possible to expand on the information given in Table 3.5 to include values for outer segment diameter and for *S. officinalis* as well as *E. cirrhosa* allowing the determination of an approximate value for cell volume (Table 3.6). Cell volume was estimated using the measured values for OS length and diameter using the following equation:

$$V = l\pi r^2,$$

where V = OS volume, l = OS length and r = OS radius. Cell surface area was also estimated due to its impact on cell capacitance. Cell surface area (SA) was estimated using a calculation based on the assumptions that there is no spacing between the microvilli, the microvilli have a diameter of 700 Å and are 1 μm long, and that 60 % of half the surface of the outer segment (OS) is covered in microvilli (i.e. 30% of the total OS surface area). These assumptions were based on measurements made by Cohen (1973a) in the squid,

Loligo pealei. The surface area estimate is for the OS only, as this is the part of the photoreceptor cells for which measurements of length and diameter exist (Farley, unpublished; Young, 1963). Thus, the photoreceptor cell SA was estimated as follows:

Total estimated OS SA =
total OS SA assuming cylindrical shape – 30% + SA of microvilli

This equation was solved using the following equations:

SA of a cylinder (or outer segment) = πdl

SA of one microvillus = $\pi \times 700\text{Å} \times 1\mu m = 2.2 \times 10^{-13} \text{ m}^2$

Number of microvilli = (0.6 x OS length / 700Å) x (0.5 x circumference / 700Å)

SA of all microvilli = number of microvilli x $2.2 \times 10^{-13} \text{ m}^2$

Adult animals used to compile the data in Table 3.6 had an eye diameter of ~ 2.5 cm and a mantle length of 20 cm and the juvenile animals had an eye diameter of ~ 1.6 mm and a mantle length of 1 cm. Values presented are the mean taken from a number of animals except those marked with [b] which are as described in the result section 3.3.5.

	OS length (μm)		OS diameter (μm)		Estimated volume (μm³)		Estimated surface area (μm²)	
	C	DP	C	DP	C	DP	C	DP
Adult *S. officinalis*	320[a]	250[a]	3[a]	9[a]	2262	15904	42720	100125
Juvenile *S. officinalis*	160[a]	190[a]	3[a]	9[a]	1131	12087	21360	76095
Eledone cirrhosa	178[b]	92[b]	3[a]	4[a]	1258	1156	23763	16376

Table 3.6 Comparison of photoreceptor OS length and diameter and hence volume from central (C) and dorsal peripheral (DP) locations in the retina of *Sepia officinalis* and *Eledone cirrhosa*. All values marked with [a] have been taken from figures in Farley, unpublished and those marked with [b] were taken from Table 3.5. Values for the estimated volume were calculated using these values and clearly indicate that the photoreceptor cell volume is greater in *Sepia officinalis* than in *Eledone cirrhosa* at both central and dorsal peripheral locations.

Overall, from the data presented in Table 3.6, it can be seen that the volume and surface area of individual photoreceptors is greater in this cuttlefish species than in the octopus. Such a large difference in values between these species of cephalopod highlights the possibility that the discrepancy between the measured mCFF presented here for adult *S. officinalis* and the mCFF value measured by Hamasaki (1968) in octopus may also be due to a species difference. A factor which has not been considered so far, and which should also be taken into account, given that field potentials were measured rather than individual cell recordings, is receptor density. The design of this experiment does not allow for comparison of receptor density between preparations. However one can see from Table 3.6 that the photoreceptor density would be much greater in central regions than dorsal peripheral regions. Therefore volume and total surface area of membrane in this area may be much greater. A more detailed study, taking receptor density into account, would be necessary before a clear picture of the influence of cell volume and surface area on temporal resolving power could be formed.

3.4.3.b Ionic conductance

Membrane resistance is the second factor that contributes to the cell membrane time constant, which is dependent on ion channel populations and their conductance. As with cell capacitance, an increase in membrane resistance results in a greater time constant that could contribute to a lower temporal resolution. A correlation between channel distribution and biological function has been demonstrated in insect photoreceptors (Laughlin & Weckstrom, 1993; Laughlin, 1996). They have found that fast and slow cells maintain their cell membrane potassium channels in different states in order to manipulate the membrane resistance and hence cell time constant. Fast cells open their potassium channels following depolarization in order to reduce the membrane time constant whereas slow cells inactivate their potassium channels hence ensuring a longer time constant. The

state of the potassium channels is effectively filtering the signal produced by the transduction cascade. It is possible that the *S. officinalis* uses a similar strategy to modulate its cell time constant. To date there is no direct evidence to support this as a preparation for the study of *S. officinalis* ion channels has yet to be developed (see Chapter 5). There is therefore very little information as to the types of channels present and whether there is any difference in their activity with age. Nasi & Gomez (1992) have managed to successfully isolate individual photoreceptors from the squid, *Loligo pealei*. However, although they were able to obtain high resistance seals relatively easily, there was a high incidence of patches showing no channel activity hence they were unable to characterize the light- and voltage-activated channels present.

As latency is an indicator of the speed of the transduction process, the arguments for greater temporal resolution also apply to shorter latency values with increases in stimulus intensity. Namely, as a greater number of photons are delivered to the cell the integration time is reduced and hence the light response is speeded up. It is common to find animals with greater flicker fusion frequency to have shorter latencies and those with low flicker fusion frequency to have longer latencies and hence slower dynamics. Frank (1999) has found the same correlation when examining the visual systems of a range of mesopelagic crustaceans.

One would expect light adaptation state to enhance temporal characteristics, as has been found in humans (Lythgoe & Tansley, 1929; Conner, 1982), flies (Juusola & Hardie, 2001), and crustaceans (Johnson et al., 2000). An adapting background light would be expected to depolarize the cells resulting in the opening of ion channels and this may significantly change the membrane resistance (R_m). Remembering that CFF is dependent on τ_m, a reduction in R_m would reduce τ_m (any change in C_m due to background illumination would be expected to be negligible) therefore increasing mCFF. However

mCFF was found to be unchanged by the presence of background illumination, with the predominant effect being a loss of sensitivity where greater stimulus intensity was required to reach the CFF values obtained in the dark-adapted preparations. As there was no increase in the mCFF attainable, it can be said that either any change in R_m or C_m was not great enough to influence τ_m, or that the background light was not bright enough or of long enough duration to bring about changes in the tissue's temporal characteristics. Although light adaptation generally enhances temporal properties, there are exceptions. Frank (2000) observed no difference in mCFF between dark- and light-adapted *Funchalia villosa* and Moeller & Case (1995) observed no difference in *Gnathophausia ingens* and *Oplophorus spinosus* (note that these are all crustaceans).

3.4.4 Gap junctions

The cephalopod retina has been found to possess gap-junctions (GJs) in the inner segment between individual retinal cells and retinal cell collaterals (Cohen, 1973b; Yamamoto & Takasu, 1984). Intercellular communication via GJs appears to be a common component of retinal circuitry having been reported to be present in groups such as mammals (Vaney, 1991), fish (Marc et al., 1988) and insects (Stebbings et al., 2002). It is also becoming apparent that GJs play an important role in retinal development (Becker et al., 1998; Becker et al., 2002; Stebbings et al., 2002). It is possible that the extent of cell coupling could have an influence on the mCFF attainable by the retina. If the photoreceptors within the retina are well coupled, this could effectively result in a large cell capacitance which in turn would increase the cell time constant producing a lower mCFF. Conversely, increased coupling could be viewed as reducing the membrane resistance due to the increased number of channels in the membrane. This would have the opposite effect on the cell time constant and result in a higher mCFF. As mentioned, the cephalopod retina does contain

gap junctions however there has yet to be an investigation into the frequency of occurrence in different age groups.

3.4.5 Sensitivity

Sensitivity is often measured along with temporal resolution as it can give a further indication of the possible light environment of the animal of interest. The first step in determining sensitivity is usually to plot response amplitude against log intensity. This plot tends to follow the shape of a hyperbolic function with the following equation $V = (V_{max}I^n)/(I^n + K^n)$ where n is the slope of the near linear part of the graph, I is the stimulus intensity, K is the intensity required to produce a half maximal response and V_{max} is the maximum response amplitude. From this plot various values can be taken in order to compare sensitivity between, for instance, species or different adaptational states. Commonly used comparative measurements are $logI_{10}$ and $logI_{50}$ which are the intensities required to produce a response which is 10 % and 50 %, respectively, of the maximum response amplitude (V_{max}) generated by the eye. In the present study, the plot did not achieve plateau values for both age groups as the adult intensity response plot was still increasing when the maximum light intensity available had been reached and needed brighter light to reach a plateau. Therefore an alternative measurement was used to compare sensitivity, namely, threshold sensitivity. The threshold is the minimum intensity required to produce a response that is just discernable from the noise.

Using this method, the juvenile animals were found to be more sensitive than the adults. This was unexpected, as it would have been predicted that longer photoreceptors would be more sensitive than shorter cells, as they would be capable of capturing a greater fraction of the available photons. Also the adults are thought to inhabit deeper waters, which would also require greater sensitivity due to the reduced light levels. So why in practice,

have the juvenile animals with shorter photoreceptors been shown to be more sensitive? It may be that *S. officinalis* photoreceptors reach their optimal length at a young age and that as they grow they become less sensitive. Nilsson & Warrant (2001) have been able to accurately predict a range of photoreceptor lengths by assuming that receptor length is limited by dark-noise. Beyond the optimum length the signal-to-noise ratio is reduced and the cost to the cell out-weighs the sensitivity benefits.

Alternatively, it may be possible that the adult photoreceptors would have responded better at a lower temperature. In this lab, photoreceptors have been observed contracting when exposed to lower temperatures (by ~10-15 μm). It is possible that under the experimental conditions, the adult photoreceptors were actually longer than they would be in the natural environment. This is something that could be tested by examining sensitivity over a range of temperatures. This would assume that the adults spend more time in deeper waters than the juveniles. Nilsson & Warrant (2001) also predicted that photoreceptors used at low temperatures should be longer than those used at higher temperatures. However, one usually associates an increase in temperature with an increase in sensitivity and temporal characteristics (e.g. Tatler et al. (2000)) and therefore this would be one of the less likely explanations. Additionally, as the adult animals used were < 20 cm ML, it is most likely that these animals had yet to migrate out to deeper waters and had therefore not lived at depths much greater than 50 metres (the depth to which the MBA boats fish for cephalopods).

Another possible explanation for the surprisingly low comparative sensitivity of the adult animals could be that perhaps fewer cells were being stimulated in the adult preparations. However, every effort was made to avoid this by using the same sized piece of tissue in each case and taking samples from both peripheral and central regions where there are variations in packing density and cell diameter. And in fact, it has been shown in this lab

that photoreceptor diameter does not increase with age, rather it is the overall number of cells that increases. So there are actually the same numbers of individual photoreceptors per unit area in adults and in juveniles.

However, it is also possible that the adult animal's low sensitivity is due to a circadian effect that is present in adults and not in juveniles whereby the adults are less sensitive during the day than at night, when they are more active. It is possible that the photoreceptors have reduced sensitivity during the day, which is when all of the experiments presented here were performed. Ventura et al. (1976) found a reduction in sensitivity of ~1log unit in the ant *Atta sexdens* during the day, determined by measuring the ERG. These experiments were performed under constant darkness therefore showing that the change in sensitivity is not triggered by light but by an endogenous circadian clock. de Souza & Ventura (1989) report a similar finding were they found a nocturnal ant species to have both lower temporal resolution and lower sensitivity then a group of bee species tested. One of their suggested explanations for this surprisingly low sensitivity was that the recordings were taken during the day when the species was known to be significantly less sensitive. However, it would seem unlikely that there would be a circadian effect in the adult animals and yet not in the juveniles.

It is interesting to note that the adult retina responded to the lowest intensity of light when it was a 0.5 s flickering stimulus, but not the 50 ms test flash. This is a simple illustration of temporal summation where with the 50 ms low intensity flashes, either intensity or duration had to be increased to produce a response. This was clearly demonstrated when the flickering light of longer duration was applied and a clearly discernable response was produced. This would suggest that threshold sensitivity may in fact have been greater in adult animals had the duration of the flash been longer allowing temporal summation to occur.

3.4.6 Adapting background light

Applying a constant background light to the preparation effectively caused a shift to the right of the CFF-intensity plot indicating that the background light caused the photoreceptors to become less sensitive. Exposing the retina to a constant level of illumination has caused an adaptational change to occur within the cells which required a greater intensity stimulus to produce the same size response than was required in the dark adapted state. This was most likely due to one of two things or a combination of both; namely, an increase in integration time or migration of screening pigment. As screening pigment migrates distally it effectively reduces the amount of light gaining access to the cells and is capable of causing a sensitivity change in the order of 0.6 log units (Daw & Pearlman, 1974). The pigment granules would have had an adequate length of time to migrate before the flickering stimuli were applied, as it has been shown to take 5-20 minutes to fully migrate outwards in squid (Hagins & Liebman, 1962; Daw & Pearlman, 1974) and the preparation was allowed to settle for at least 30 minutes under these conditions whilst only being stimulated by the low intensity test flash. Young (1963) showed that cephalopod photoreceptors contract when light adapted. Further to this Torres et al. (1997) showed that rhabdome cross-sectional area also reduces following light adaptation. This cyclic formation and breakdown of microvilli is linked with movement of the photopigments rhodopsin and retinochrome.

If the cell's integration time is increased, this could also be responsible for the reduced sensitivity. The integration time could be increased by changes in the state of the membrane ion channels or the availability of second messengers. The adapting light will utilize a proportion of the molecules involved in the second messenger cascade, therefore a greater stimulus than previously necessary would be required to produce the same sized response due to the reduced electromotive driving force. It is widely accepted that

calcium ions play a role in adaptation. A relationship has been found between the level of light adaptation and intracellular calcium levels in *L. polyphemus*. Ukhanov et al. (1995) used fluorescent calcium indicators to show that small increases in calcium due to background illumination can produce relatively large changes in sensitivity. Increases in intracellular calcium levels have also been reported to mediate light adaptation in squid (Pinto & Brown, 1977). Both intracellular injections of the calcium buffer EGTA and reduction of extracellular calcium cause a reduction in the peak to plateau decline characteristic of a response to a relatively long light flash. The mCFF however, is ultimately unaltered because it is sensitivity and not temporal resolution that has been reduced so all that is needed is a brighter stimulus.

3.4.7 How does CFF relate to habitat and behaviour?

A relationship between temporal characteristics and habitat and lifestyle was first proposed by Autrum (1950, 1984) following the study of insect photoreceptors. This initial hypothesis has been extensively investigated and it has been confirmed that indeed, for terrestrial insects, temporal characteristics are indeed related to habitat and lifestyle. Subsequent to this, there have been some studies into the response dynamics of marine crustaceans (Moeller & Case, 1995; Frank, 1999, 2000; Johnson et al., 2000) to try to discover if Autrum's hypothesis could be extended into the marine environment. Frank (1999) discovered that the hypothesis was not supported when using down welling light levels as the determining environmental factor. However, when the bioluminescence of prey was taken into account the data fitted with the hypothesis. Moeller & Case (1995) found a difference in the temporal characteristics in the eyes of the deep-sea crustacean *Gnathophausia ingens*, between adults and juveniles. This was attributed to the fact that the juveniles are found in shallower waters than the adults, and that the two age groups had adapted to the different light levels. They confirmed that the juvenile's eyes were not

faster due to the warmer temperature of shallower waters by performing the experiments at the same temperature for both age groups.

The current experiments found that the juvenile animals had higher temporal resolution and shorter response latency. This correlated well with the fact that juveniles would be expected to be found living in shallower waters than adult animals. They would therefore be able to sacrifice a level of sensitivity in order to improve temporal resolution, as the light levels would not be limiting in the shallower waters. It was therefore surprising that the juveniles actually proved to be more sensitive than the adults. Similarly, the adults' lower temporal resolution would be expected to be coupled with a greater level of sensitivity due to the reduced light levels found in deeper waters. It may be that these results do not fully conform to the expected pattern or it may be that another environmental factor has to be taken into account to reconcile this unexpected result.

The animals used in these experiments would not be expected to be found living in particularly deep waters; probably no deeper than 50 metres. From the surface to this depth the animals would experience light levels ranging from 10^5 μWm^{-2} down to ~10^1 μWm^{-2} (Clarke & Denton, 1962), a range of 4 log units. For ease of comparison, this is 10 μWcm^{-2} to 0.01 μWcm^{-2} in the units used in this study. These animals come inshore to spawn and the young hatchlings remain in these shallow coastal waters for the best part of a year by which time they could have grown to anything up to 20 cm mantle length. This is when they migrate out to deeper waters where they stay, and continue to grow until they return to the shallow waters to spawn (Norman, 2000). It is therefore possible that animals larger than 20 cm mantle length would need to have been studied in order to see an adaptational change in sensitivity, as it would not be until then that they would move into deeper waters and have the need for enhanced sensitivity. It would therefore be plausible to suggest that the differences observed in this study were purely due to growth and that

any specific adaptational changes to their environmental conditions had not been necessary within the age range examined. It would therefore appear that the *S. officinalis* photoreceptors were in their optimal state for sensitivity and temporal resolving power at the juvenile stage and that these senses diminished with age. However, it would appear that the animals have not adapted to compensate for this, suggesting that greater sensitivity and/or temporal resolution was not a necessary or economical adaptation.

With reference to the original questions posed, the following conclusions can be drawn:

- CFF was limited by light intensity up to mCFF, beyond which cell length appeared to be a limiting factor.

- Sensitivity was inversely related to outer segment length. This may be explained by a reduced signal to noise ratio as the cell length increased.

- In *S. officinalis*, mCFF was inversely related to outer segment length. This finding was explained by the increase in cell membrane time constant produced by increased cell surface area.

- Rate of transduction did not appear to be a limiting factor in adult *S. officinalis* photoreceptor temporal resolution, however conversely, the efficiency of the transduction cascade appeared to be impeded by longer cells.

CHAPTER 4: Calcium Imaging

4.1 Introduction

From the work presented in Chapter 2, it can be seen that it would be sensible to expect

there to be a change in the photoreceptor intracellular calcium levels following excitation

by light. This is based on the fact that both removing calcium from the extracellular

bathing solution (see Figures 2.16 & 2.17) and application of the Ca^{2+}ATPase inhibitor

cyclopiazonic acid (see Figure 2.22) both cause a change in the size and shape of the ERG,

as well as the addition of general calcium channel blockers such as cobalt and lanthanum

(see Figures 2.18 to 2.21). Additionally, modulation of intracellular calcium is a widely

used regulatory technique not only in both vertebrate and invertebrate photoreceptors, but

also in many other physiological systems such as in skeletal muscle contraction and

exocytosis of neurotransmitters during synaptic transmission (Vander et al., 1994).

Although calcium signals are used extensively, the mechanisms by which they are

controlled and implemented vary widely. Measurement of the intracellular calcium

concentration in a cephalopod retina has only been carried out once. Takagi et al. (1994)

measured the dark adapted photoreceptor cell calcium to be 0.01 mmoll^{-1} in *Octopus*

vulgaris. This was calculated from electron probe X-ray microanalysis of the cell

cytoplasm, combined with measurements of the density and dry mass fraction of the retina.

This method is extremely complex and is only useful for measuring $[Ca^{2+}]_i$ in either the

light or dark adapted states. It does not allow Ca^{2+} to be monitored with time to follow all

fluctuations and is also not compatible with studies of the intracellular signalling cascade.

Although it is fairly certain from the results presented in Chapter 2 that calcium does play a

role in the cuttlefish photoresponse, additional data are required to determine the details of

this role. Fluorescence calcium imaging can be used to monitor the timing and magnitude

of any calcium changes occurring within the photoreceptors and should therefore be able to

indicate whether there is a calcium increase and whether this is rapid enough to play a role

in excitation. An understanding of intracellular calcium fluctuations is vital to the understanding of how the phototransduction cascade controls the conversion of an incoming light signal into an electrical membrane response. In order to understand this it is necessary to know exactly where these calcium fluxes occur and whether they are extremely localized as has been shown to be the case in fly photoreceptors (Oberwinkler, 2000) or whether the changes are more uniform.

Calcium fluctuations have been successfully recorded using fluorescence techniques in a number of invertebrate photoreceptors, most notably in flies and *Limulus polyphemus* using both conventional epi-illumination systems and confocal microscopy with a range of calcium indicators (Hardie, 1991b; Peretz et al., 1994; Ukhanov & Payne, 1995, 1997; Oberwinkler & Stavenga, 1998; Payne & Demas, 2000). In *L. polyphemus*, intracellular calcium (Ca^{2+}_i) levels have been shown to increase by >100 fold following light-induced excitation. Ukhanov et al. (1995) used fura-2 to measure the resting intracellular calcium concentration ($[Ca^{2+}]_i$) in dark-adapted ventral photoreceptors which gave a resting value of 0.4 μM. They found that fura-2 could not be used to measure $[Ca^{2+}]_i$ during the response to a bright flash because it saturated and therefore underestimated the change in calcium. Instead they used mag-fura 2 and calcium green–5N (CG-5N) and found peak values of Ca^{2+}_i in the light of 60-70 μM. Although they found a correlation between $[Ca^{2+}]_i$ and both sensitivity and adapting light levels, the calcium increase did not commence prior to the electrical response and therefore the results did not support the proposed role of calcium as the messenger of excitation. However, subsequent experiments using confocal microscopy detected sufficiently rapid calcium elevations to support a role in producing the photoresponse (Ukhanov & Payne, 1997). Fluorescence calcium imaging has also been used to determine which source of calcium is responsible for the light-induced elevation. Payne & Demas (2000) have demonstrated that the rise in

$[Ca^{2+}]_i$ is due to release from internal stores rather than influx from the extracellular solution.

The development of a dissociated ommatidial preparation (Hardie, 1991b) allowed for the first time the simultaneous measurement of changes in $[Ca^{2+}]_i$ and the light response in flies. Peretz et al. (1994) showed that, in contrast to *L. polyphemus*, the light-induced increase in Ca^{2+}_i in *D. melanogaster*, was mainly due to influx from the extracellular solution. Subsequently, other preparations have been developed for the measurement of Ca^{2+}_i in flies. Oberwinkler & Stavenga (1998) used an intact preparation of the blowfly, *Calliphora vicina* to show that there is a linear relationship between Ca^{2+}_i levels and the intensity of a background adapting light, as has been found in *L. polyphemus*. So although *L. polyphemus* and flies use a different source of calcium, they both use it to regulate their state of light adaptation. By including confocal imaging and modelling, it has been shown that the $[Ca^{2+}]_i$ changes are highly localized; both influx and extrusion. This translates into rapid dark adaptation which may be particularly important for the fast eyes of flies.

The objectives for this chapter, as outlined in the general introduction, are as follows:

• To load the retinal slice preparation with fluorescent calcium indicator dye.

• To measure a light-induced change in intracellular calcium concentration by using membrane permeable fluorescent calcium indicators.

• To determine the effect of some of the pharmacological agents used in Chapter 2, on the intracellular free calcium concentration.

4.2 Materials and Methods

4.2.1 Animals and preparation

The animals used and the slice preparation are as described in Chapter 2. Once the 300 μm thick retinal slices were prepared, they were kept in ASW on ice prior to dye loading. All manipulations were performed under dim red light.

4.2.2 Dye loading

Slices were loaded with one of the following acetoxymethyl-ester (AM-ester) forms of Ca^{2+}-sensitive fluorescent indicator dyes Calcium Green-1 (CG-1), Calcium Crimson (CC), Fluo-3 or Calcium Green-5N (CG-5N), all from Molecular Probes. AM-ester type dyes passively load into cells where they are cleaved by intracellular esterases to a cell impermeant form, which is sensitive to the relevant ion (Haugland, 1996). The dissociation constant (K_d) for each indicator is shown in Table 4.1. This value gives us an indication of the concentration range over which they can detect calcium. Indicators were made as stock solutions of either 1 or 2 mM made up in DMSO containing 10% w/v pluronic acid F-127 (Sigma), which helps to disperse indicator in the loading medium. Stock dye solution was pipetted into ASW to make a 5 μM working solution to which 6 – 8 slices were added. The Petri dish was gently shaken and then left to incubate for 30 minutes at room temperature. Following incubation the slices were washed at least three times in ASW before being visualized. This was the standard procedure used with all dyes however different incubation times had been tried and CG-5N was also tested at 10 μM.

Indicator	K_d
Calcium Green-1	190 nM
Calcium Crimson	185 nM
Fluo-3	390 nM
Calcium Green-5N	14 μM

Table 4.1 Properties of the indicators which have been used in these experiments giving an indication of their affinity for calcium. The working range is usually taken as from 0.1 to 10 times the K_d of the indicator (data taken from Haugland (1996)).

4.2.3 Sampling and microscopy

In order to view the loaded slices, they were held down by a slice anchor in a small Petri dish half filled with ASW. The epi-illumination setup consisted of an upright microscope with X20, NA: 0.50 and X40, NA: 0.75 water immersion objectives. The light source used was either a mercury (Nikon, 100W) or a Xenon lamp (AMKO, 75W), controlled by a Uniblitz electromechanical shutter with a time to fully open of 5-7 ms and 1-2 ms (tested with a photodiode and the photomultiplier) respectively, for the two lamps, as different shutter types were used due to the slightly different configurations required. The shutter was controlled by computer via a Uniblitz shutter driver (Model VMM-D3) to deliver the fluorescence excitation light which was also the light used to excite the cells. To excite the dyes and collect fluorescence emission the appropriate dichroic filter block (see Table 4.2) was inserted into the light path and focused light onto the slice via the objective lens.

Dye	Block type	Dichroic mirror	Excitation filter	Barrier Filter
CG-1, CG-5N, Fluo-3	Nikon B-2A	510 nm	450-490 nm	520 nm
CC	Omega Optical XF 43	600 nm	580 nm	630 nm

Table 4.2 Table showing the dichroic filter blocks used to excite the fluorescent indicator dyes and to collect the emitted light.

Emission was collected from an area selected by a variable rectangular diaphragm and fed to a photomultiplier tube (Thorn EMI, type QL3OF/RFI). It was possible to select the recording area by visualising the preparation on a monitor, using an infra-red video camera. Sampling from the PMT was triggered by the pulse used to open the shutter and Signal 2 or Spike 2 was used for data acquisition and storage. The 1 kHz PMT filter was used and data was sampled at a rate of 5000 Hz at the computer.

Any ERG recordings were performed as described in Chapter 2, methods.

4.2.4 Confocal

Images were obtained with a Nikon Diaphot 300 inverted microscope coupled to a MRC 1024 scanning laser confocal system (running Bio-Rad LaserSharp 2.1T). Dye excitation was at 488nm; emitted light was collected at 522 ± 32 nm and at >585 nm. Each image consisted of 512 x 512 pixels, representing ten scans of 1 second each, added to each other using Kalman filtering.

4.2.5 Solutions

The composition of ASW was as described previously. The composition of high potassium solution (high K^+) was (in mM) 350 NaCl, 130 KCl, 10 Hepes, 55 $MgCl_2$ and 10 $CaCl_2$ pH 7.8, osmolality 1100 $mOsmkg^{-1}$ measured using a Wescor vapour pressure osmometer (Model 5500).

4.2.6 Brain cell dissociation

Brain cells were dissociated from the cuttlefish with $3mgml^{-1}$ trypsin in CFASW for 60 minutes. The suspension was then pelleted in a miniature centrifuge (Tomy Kogyo Co.

Ltd. PMC-060) at 6200 rmin^{-1} for 30 – 40 seconds and the enzyme solution removed. The cells were then wash 2-3 times in fresh CFASW and the resulting cell suspension plated on poly-L-lysine coated coverslips. This was left on ice for at least one hour to allow the cells to adhere to the coverslip. Cells adhered successfully and were then incubated for 30 minutes in 5 µM CG-1 in ASW. The indicator was then washed off and the fluorescence observed under the microscope.

4.3 Results

4.3.1 Loading

With all dyes, even fluorescence throughout the tissue could be observed with the fluorescence microscope at X10 and X20 magnification. Fluo-3 loading was also checked using the confocal microscope, which showed it to be uniform with no visible compartmentalization. However although the slices appeared to have loaded successfully, it was not possible to detect a change in the calcium fluorescence signal with the PMT. As the K_d for these dyes are all in the nM range, CG-5N was tested which has a higher K_d, in case the calcium concentration was at such a level that the other dyes had saturated. Unfortunately this was not the problem and this dye also produced no relevant calcium signal even although it was still possible to elicit a response to light showing that the tissue was still responsive.

It was evident when viewing the preparation under the fluorescence microscope that the dyes had loaded in spite of the fact that it was not possible to pick up any change in fluorescence when the preparation was illuminated by the excitation light. It was thought that a possible problem could be that the shutter was taking too long to open. If the calcium concentration change was occurring within the opening time, the "on" step of the calcium fluorescence signal would be masked. However, when the lighting configuration was changed to incorporate a faster shutter (see section 4.2.3 for shutter speeds) there was still no relevant signal.

4.3.2 Viability of the tissue following loading

To demonstrate that the tissue was still viable and light responsive following incubation, ERG recordings were made (as describe previously in Chapter 2) in response to both

normal white light (Figure 4.1) and fluorescence excitation light. Due to the intensity of the excitation light necessary to excite the dyes, generally only one response was obtained from the slice, which completely saturated the transduction process. By using neutral density filters it was possible to attenuate the light to a level at which repeated responses could be recorded, however the signal to noise ratio of the PMT then tended to be too low. In an attempt to reconcile these problems calcium crimson (CC) was used which is excited at ~590 nm. This is outside the peak absorption range of cephalopod rhodopsin (Brown & Brown, 1958) and it was therefore thought that it would be possible to use a brighter light and hence improve the PMT signal to noise ratio without saturating the light response. Nonetheless, it was still not possible to detect a calcium concentration change.
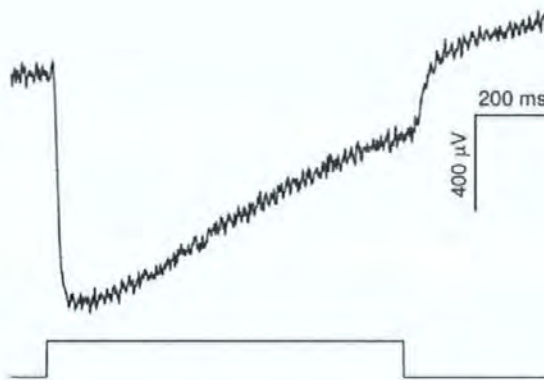
Figure 4.1   Retinal slice is still light responsive following incubation with fluorescent indicator. Example trace from a retinal slice loaded with 5 µM CG-1 in response to a 1s flash of white light.

4.3.3 Autofluorescence

The confocal microscope was used to look at fluorescence in slices loaded with calcium green (CG-1) and unloaded slices. When looking for autofluorescence in the unloaded cells, there was virtually none when collecting with the 522 ± 32 nm filter, however when images were collected using the 585 nm band pass filter there was a large amount of fluorescence which was exclusive to the outer segment. This is most likely to be photopigment. It was very convenient that the autofluorescence occurred at wavelengths

greater than 585 nm as this meant that it could not be confused with indicator fluorescence which occurred at ~530 nm. Nothing was picked up at this wavelength in unloaded slices but there was clearly fluorescence throughout the whole slice, apart from at the dark layer of screening pigment, when loaded with the calcium indicator dye.

4.3.4 The rise phase observed

It was noticed that with the first flash of the excitation light, the signal from the PMT was not completely square; there was a slightly rounded off rise phase. Initially it was thought that this may have been a small effect due to the Ca^{2+} signal, however this was unconvincing for two main reasons: 1) the size of the rise phase was no different with CG-5N in comparison to CG-1 or Fluo-3 and 2) there was no distinct delay phase indicating the start of the calcium signal. A slice which had not been loaded with calcium indicator dye, and would therefore not have the capability to respond to changes in intracellular calcium with a change in fluorescence, was tested in the same way with the fluorescent excitation light. The rise phase observed with CG-1, Fluo-3 and CG-5N loaded slices was also present when an unloaded slice was stimulated with the blue excitation light (Figure 4.2). This phenomenon was only observed in the outer segment area, not in the inner segment (Figure 4.2). Also, this rise was not observed when exciting with the yellow light used to excite CC. These findings suggest that the rise phase observed is due to a light induced change in one or more of the photopigments in the outer segment, possibly causing an increase in autofluorescence and hence leading to the rise.
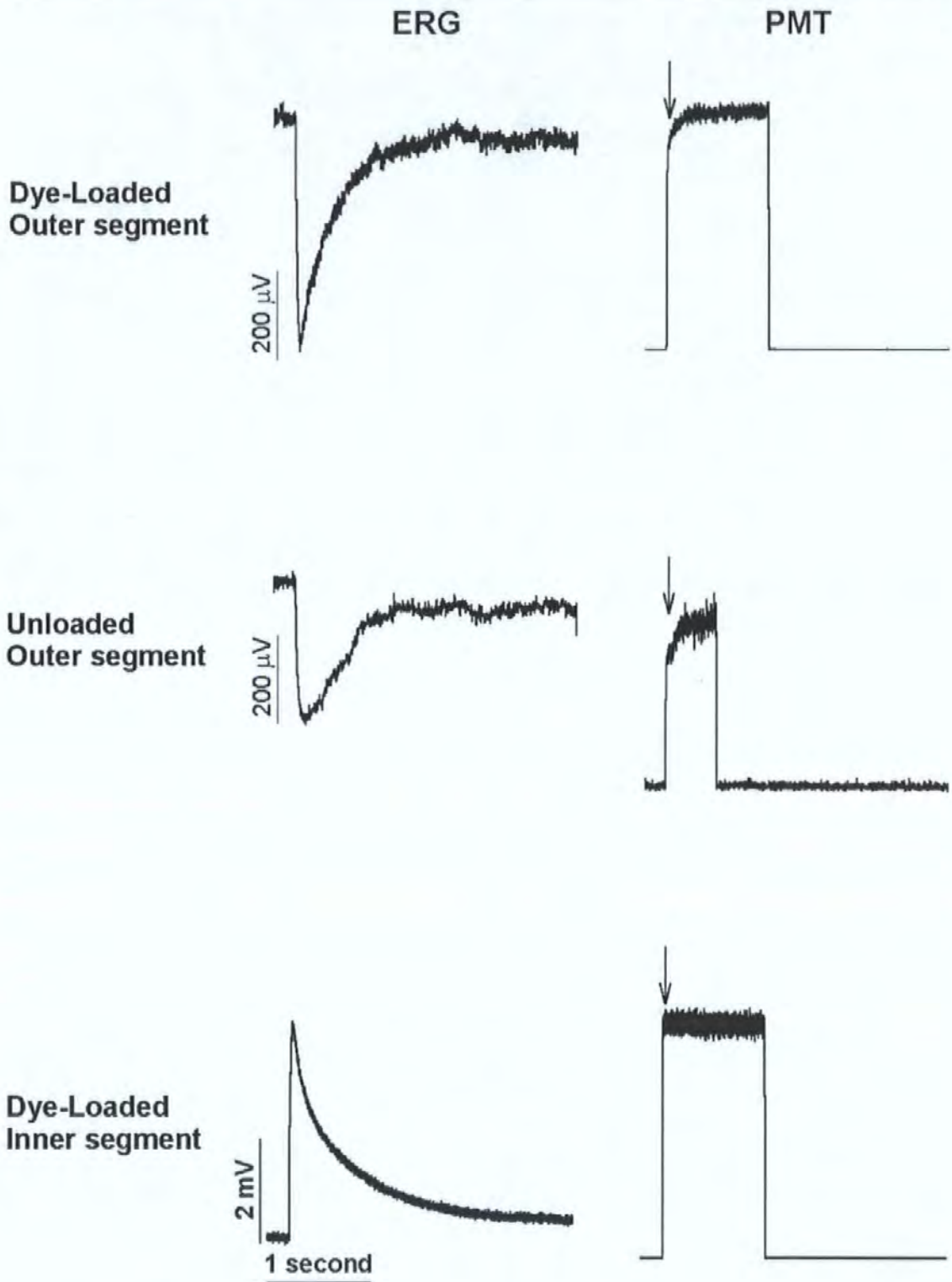
Figure 4.2 ERG recordings (left) and simultaneous recording from the photomultiplier (right) from three different slice preparations. It is evident that the rise phase is present in the photomultiplier signal when recording from the outer segment, independent of whether the slice is loaded or not. However, when collecting from the inner segment, no rise phase was observed suggesting that this is a result of a photopigment reaction and not a change in fluorescence of the calcium indicator dye.

4.3.5 Test with high K⁺ and ionomycin

In an attempt to determine if the dye was actually responsive to changes in calcium levels the tissue was exposed individually, to high K^+ solution and ionomycin (a calcium ionophore) in an attempt to flood the cells with calcium. This also was unsuccessful in producing the expected change in fluorescence from the indicator dye.

4.3.6 Dissociated brain cells

To test the experimental set up, dissociated brain cells were loaded with 5 μM CG-1. When observed under the fluorescence microscope it could be seen that the cells had successfully taken up the dye and when 5 μM ionomycin was added to the bath solution the fluorescence started to increase after ~3-4 minutes and then continued to gradually increase over the next 10 minutes before starting a slow decline. This gave a good indication that there were no problems with the experimental setup and that the problem was the retinal preparation. When exactly the same test was carried out with a retinal slice the fluorescence started to decrease within ~ 10 s and had reduced to baseline within 120 s. This would suggest that the dye loaded slice did not have the capability to produce a fluorescence response to an increase in intracellular free calcium induced by the use of a calcium ionophore. The decrease in fluorescence may be due to bleaching of the dye.

4.4 Discussion

4.4.1 Why was no calcium change detected?

Why have all attempts been unsuccessful in detecting a light induced change in fluorescence from any of the calcium indicator dyes in the cuttlefish retina? There are a number of possible explanations as to why this is so, and these will be discussed in turn.

4.4.1.a Dye distribution

In order for a change in cytosolic calcium concentration to be detected the indicator obviously has to be in the cell cytosol. When using the AM ester form of the dye, which is designed to pass through membranes, there is always the possibility that it passes through internal membranes and enters into cell organelles such as mitochondria, endoplasmic reticulum, etc. When using the slice preparation as opposed to isolated cells there is also the possibility that a proportion of the dye is taken up into the supporting cells. If the cell organelles or supporting cells take up some of the dye there may not be enough dye in the photoreceptor cytosol to produce a detectable change in fluorescence. However, this is believed that this is unlikely to be the main problem in this case, for two reasons. Firstly, dye distribution appeared to be even when observed using the confocal microscope with no obvious compartmentalization. Secondly, the supporting cells send only a fine process up through the photoreceptor outer segments (Saibil, 1990). As the dye fluoresced strongly and evenly throughout the outer segment, based on the previous statement, there must have been dye loading into the photoreceptors.

4.4.1.b Dye is no longer calcium responsive

It is also possible, that for some reason, the dye is no longer responsive to calcium. It may be that some stage of the procedure has caused a transient elevation in calcium levels. If

the calcium levels were already elevated this could have resulted in the dye becoming

saturated. This could be feasible with regards to the higher affinity dyes however it would

be very unlikely that the calcium could be elevated to such an extent so as to saturate CG-

5N.

4.4.1.c No light-induced increase in $[Ca^{2+}_i]$

There is of course the possibility that there is in fact no light-induced increase in

intracellular calcium levels. There are examples of photoreceptors from molluscs that do

not regulate their state of light-adaptation with the intracellular calcium concentration

(Gomez & Nasi, 1997); however those are rather the exception than the rule. Additionally,

very strong pharmacological evidence was presented in Chapter 2 pointing towards a role

for intracellular calcium in the photoresponse. Also, the fact that it was not possible to

induce a rise in fluorescence by applying ionomycin or high K^+ solution would suggest that

the problem is at the stage of calcium fluorescence detection.

4.4.1.d Slice preparation

A review of the literature suggests that it may be the combination of a slice preparation and

ester loading that is the problem. The majority of successful measurements of a light

induced increase in $[Ca^{2+}]_i$ in invertebrate photoreceptors has been performed in dye

injected isolated cells (Ukhanov et al., 1995; Ukhanov & Payne, 1997; Oberwinkler &

Stavenga, 2000; Payne & Demas, 2000) or intact tissue (Walz et al., 1994; Oberwinkler &

Stavenga, 1998). There are some examples were ester loaded slices have been used

(Hochstrate & Juse, 1991; Ukhanov et al., 2001), however is has been pointed out that in

the case of Hochstrate and Juse's (1991) study it was difficult to reconcile these results

with newer data using different techniques. Additionally, there are other reports of

difficulties in ester loading retinal slices (Walz et al., 1994). As yet there has been no measurement of intracellular calcium levels in any cephalopod species.

4.4.1.e Esterase activity

Another possible explanation could be that there was low esterase activity within the cells (Enrico Nasi, personal communication), not high enough to hydrolyse all of the indicator molecules, resulting in a lack of calcium responsiveness. The AM ester form of the indicator is membrane permeant and insensitive to ions however, once internalized the cellular esterases hydrolyse the ester releasing the ion sensitive form of the indicator (Haugland, 1996). However, the cells did fluoresce when excited with the appropriate wavelength light suggesting at least some level of calcium sensitivity. It is possible that there was enough cellular esterase activity to activate some of the indicator; enough to show up dark adapted resting cellular calcium levels. However further increases in calcium concentration induced by light stimulation may have produced no further change in fluorescence because there was no more active indicator.

4.4.2 Explanation for observed rise phase

As stated in the results section, the findings indicate that the rise phase observed is due to a change in the photopigments found in the photoreceptor outer segment. Rhodopsin is found entirely in the outer segments of the photoreceptor cells localised to the microvillous membrane of the rhabdomes and exists with metarhodopsin at a molar ratio of 100:3 (Hara & Hara, 1976). This ratio does not markedly change even during light adaptation however the bright blue light required for fluorescence imaging would be expected to shift the ratio of rhodopsin concentration to metarhodopsin concentration.

Light converts rhodopsin to metarhodopsin resulting in a mixture of the two; this reaction is also photoreversible (Goldsmith, 1972; Hara & Hara, 1972). *S. officinalis* rhodopsin has a peak absorbance (λ_{max}) of 492 nm and is red in colour and metarhodopsin has a λ_{max} of 497 nm (Brown & Brown, 1958). Metarhodopsin is highly coloured, being red in acidic or neutral solutions and yellow in alkaline (Hara & Hara, 1972). When *S. officinalis* rhodopsin is exposed to light it deepens in colour rather than bleaching (Brown & Brown, 1958). Rhodopsin only bleaches on exposure to light in alkaline solution (Hara & Hara, 1972). Metarhodopsin has greater absorbance than rhodopsin and its λ_{max} is always slightly longer than that of rhodopsin.

This red autofluorescence was observed when using the confocal microscope with the 582 nm filter and it is this which is suggested to increase on exposure to the blue excitation light, due to a shift in the concentration ratio of rhodopsin to the darker metarhodopsin. This effect was not observed with CC because the yellow excitation light was not near the λ_{max} for rhodopsin and hence did not cause such a shift in the concentration ratio.

4.4.3 Future experiments

The use of membrane permeable calcium indicators has proved unamenable to experimentation in the present experimental model. It would be necessary to find an alternative preparation for the investigation of fluctuations in $[Ca^{2+}]_i$ in the retina of *S. officinalis*. The development of the whole cell patch clamp preparation described in Chapter 5 would surely lend itself well to calcium imaging using non-AM ester-type indicators.

CHAPTER 5: Whole Cell Recording

5.1 Introduction

The electrical response to light which has been recorded in the retina so far in this work, has been the electroretinogram (ERG). This is the sum of the photoresponse from many individual cells. In turn the photoresponse from each individual cell is the product of the electrical events taking place in the membrane as a result of the second messenger cascade which is triggered by light. Light leads to cellular depolarization, which probably involves an influx of sodium ions as reducing the concentration of sodium in the external solution reduced the size of the photoresponse (Clark & Duncan, 1978); however this effect was less pronounced at high stimulus intensities, showing that a large component of the response may be independent of sodium. There are likely to be numerous ionic currents involved in depolarization and the subsequent repolarization to the membrane resting potential. By using the whole cell patch clamp technique, combined with some pharmacological intervention, it was possible to characterize some of the membrane currents and to therefore gain an insight into the ionic movements taking place across the photoreceptor cell membrane. In addition, the whole cell preparation ensured entry of test chemicals into the cell and also allowed the use of non-membrane permeable agents. It was therefore possible to produce supportive evidence of the findings from Chapter 2 and to expand the understanding of the phototransduction cascade by providing evidence of a potential role for cGMP, which has previously not been demonstrated electrophysiologically.

5.1.1 Patch clamp recording

The whole cell patch clamp technique is widely used in the field of electrophysiology. It was first used by Neher & Sakmann (1976) to record from frog muscle cells and was subsequently refined by Hamill et al. (1981). Briefly the patch clamp technique involves forming a high resistance seal between a glass pipette and the surface of the cell of interest,

therefore isolating a patch of membrane from the external solution and allowing measurement of current flow. There are then further adaptations of this technique; one of which is the whole cell recording method, which involves rupturing the patch of membrane isolated by the pipette by applying strong suction. The method of making a whole cell recording is shown diagrammatically in Figure 5.1. This technique can be applied to both dissociated cells and cells in slices of tissue.

Whole cell patch clamp recording allows voltage clamping of the cells in order to investigate ionic currents across the membrane. In the case of photoreceptors, these currents can be both voltage- and light-activated. Additionally, as the micropipette internal solution is continuous with the cell internal solution when using this technique, the method also allows the addition of chemicals to the cell interior via the pipette internal solution. The patch clamp technique can also be used to investigate single channel conductance and kinetics (before going to the whole cell mode), however this is outside the remit of this project.
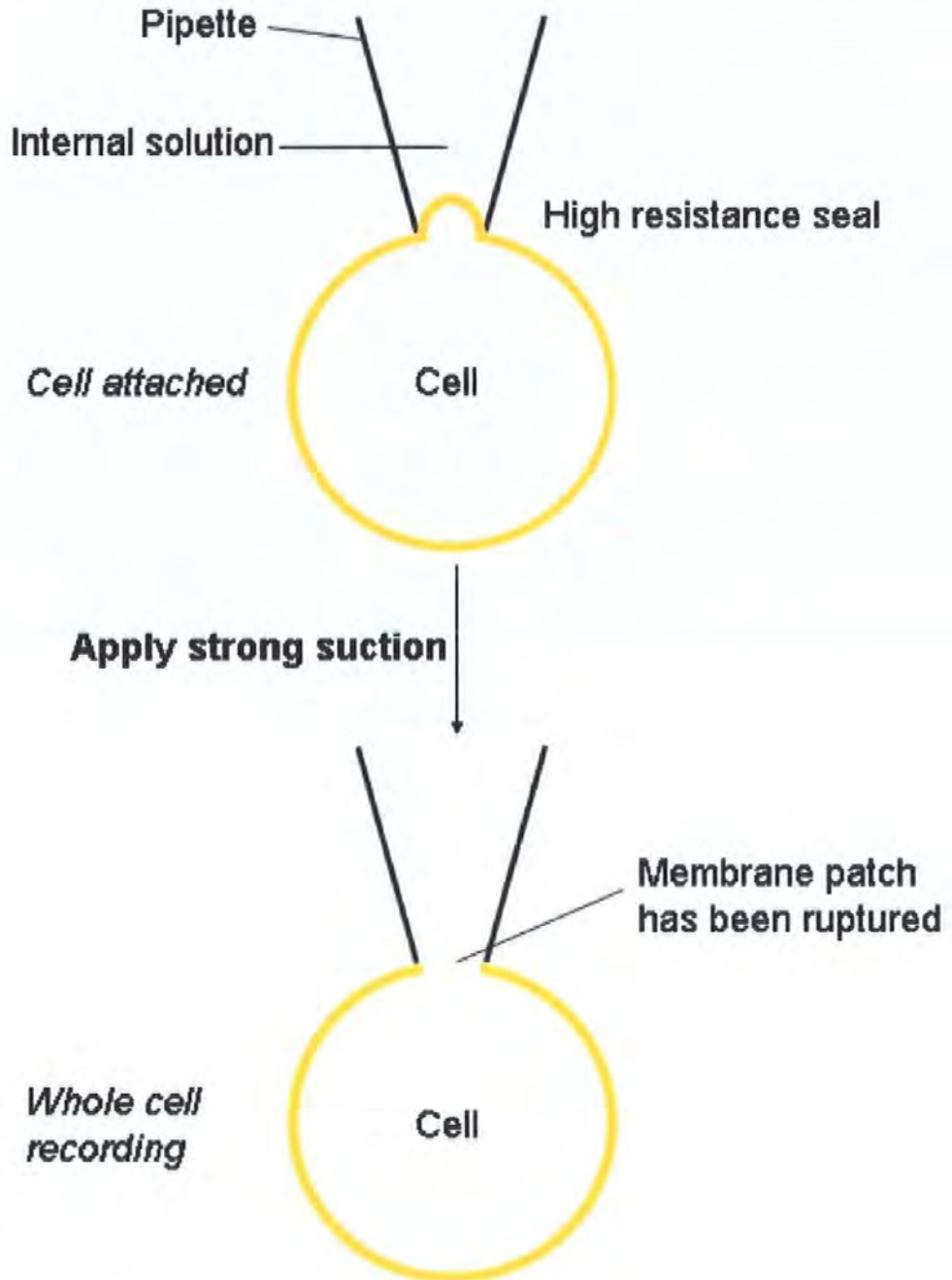
Figure 5.1 Diagram showing the method of making a whole cell recording. This has been adapted from Ogden & Stanfield (1994).

5.1.2 Cephalopod photoreceptor cell dissociation

The only work which has been carried out using whole cell patch clamp in a cephalopod species was by Nasi & Gomez (1992). They used dissociated squid photoreceptors from the species, *Loligo pealei* and attempted a number of recording techniques. Therefore, initial attempts to find a suitable preparation for the study of currents from individual

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cuttlefish photoreceptor cells started with the dissociation procedure used by Nasi & Gomez (1992). Maria Gomez was consulted to ensure that the techniques and procedures used in their study were followed exactly in this study. The procedure proved to be ineffective in producing viable isolated cells from the cuttlefish retina, however when the opportunity arose to test this dissociation procedure on *L. pealei*, it proved successful showing that the technique was species specific and was obviously not compatible with the *S. officinalis* retina. Alternative dissociation methods were therefore pursued based on the procedure used by Nasi & Gomez (1992) but using different enzymes and combinations of enzymes, and under different conditions of temperature and incubation time in order to elucidate a suitable isolation procedure. Collagenase P (7-8 mgml^{-1}) at room temperature for 45-60 minutes in a gentle shaker proved to be most successful in producing cells however whole cell recordings were rarely achieved and light induced currents were never recorded. This proved to be the case in both adult and juvenile animals. The possibility of recording from tissue slices, similar to those used for ERG recordings was then explored. Unfortunately, this proved difficult due to fragments of cells, etc, remaining attached to the surface of the slice preventing access of the microelectrode, or causing blockage of the pipette tip. This was still the case when various enzyme treatments were used in an attempt to clean the surface of the slice. However, this did not prove to be the case with hatchling and pre-hatchling animals where the slices were very clean in comparison and the cell surfaces could be accessed much more easily allowing pipette tight seals on the cells to be formed. Whole cell recording from thin slices (200 µm) of hatchling and pre-hatchling retinal tissue was therefore developed as a new preparation for cephalopod phototransduction studies.

5.1.3 The cephalopod photocurrent

The only cell membrane current recordings obtained from cephalopod photoreceptors to date were from the squid, *L. pealei* (Nasi & Gomez, 1992). Suction electrode, whole cell patch clamp and perforated patch methods were used and photocurrents were recorded with all three methods. Additionally, bursts of channel activity were induced by light when using on cell recordings and a voltage dependent current was recorded using whole cell patch clamp. This was a slow activating outward current which was induced by depolarization; no inward current was observed during the application of the voltage steps.

Takagi (1994a) recorded some single channel currents using reconstituted bilayer lipid membranes containing fractions of octopus photoreceptor membrane. Conductances ranging from 5-400 pS were recorded, with the majority occurring at the conductance range less than ~50 pS. Data was also provided which indicated that channels with a conductance of < 50 pS were likely to be the light activated channels. Additionally, 10 pS channels were shown to be activated by cyclic guanosine monophosphate (cGMP). In octopus photoreceptors the light activated channels have been shown to be non-selective cation channels (Takagi, 1994b; Takagi et al., 1994). Poor selectivity of membrane ion channels is a feature which has been found to be common in sensory cells (Torre et al., 1995). It is thought that the repolarisation phase of the photoresponse is dependent, at least in part, on the opening of potassium (K^+) channels, as the K^+ channel blocker tetraethylammonium (TEA) has been found to prolong this phase (Takagi, 1994b). A role in regulating the K^+ conductance was proposed for calcium, as removing calcium from the extracellular solution also prolonged the repolarisation phase.

There is also direct evidence of the presence of calcium (Ca^{2+}) channels in the cephalopod photoreceptor with the identification of the *trp* homologue *strp*, in squid photoreceptors (Monk et al., 1996). It was proposed that this channel may mediate Ca^{2+} influx and may be

regulated by a Ca^{2+}-calmodulin interaction. The discovery of the sTrp protein added to the growing number of Trp-like channels which appear to be a wide-spread group (Montell, 1997). sTrp is a protein which has been isolated from *Loligo forbesi* photoreceptor membranes. The *strp* gene shares greatest sequence similarity with the products of *D. melanogaster trp* and *trpl* genes. These genes encode the light-activated cation channels in *D. melanogaster* photoreceptors.

5.1.4 Unanswered questions from Chapter 2

From the results presented in Chapter 2 it has been possible to further the understanding of the cuttlefish phototransduction pathway by determining which components of the PI cascade proved to be essential for the production of a light response. There still remain a number of unanswered questions and points which require clarification as the use of ERG recording limited investigations to those where membrane permeable pharmacological agents were available. Now, with the development of the whole cell patch clamp preparation an attempt was made to resolve some of these unanswered questions and to step closer to elucidating the full transduction pathway. It was of particular interest to know the precise role of the internal calcium stores and whether or not cGMP plays an important role in the transduction cascade. By using the whole cell patch clamp technique it was anticipated that it would be possible to obtain much more specific answers as to the role of these two internal messengers. This chapter addressed the question of whether it is possible to investigate phototransduction in the retina of *S. officinalis* by the use of the whole cell patch clamp technique.

The objectives for this chapter, as outlined in the general introduction, are as follows:

- To develop a successful preparation for the reliable use of the whole cell patch clamp technique in order to study the properties of the cuttlefish photoreceptors.

- To use the whole cell patch clamp technique to record membrane currents and to characterize them using various voltage pulse protocols and pharmacological intervention.

- To record cell capacitance in order to estimate cell surface area.

- To record light induced currents in these isolated cells and to submit these currents to pharmacological manipulation in order to further our understanding of the phototransduction cascade.

- To identify the terminal intracellular signalling molecule in the phototransduction cascade that is responsible for opening the light-activated ion channels.

5.2 Methods

5.2.1 Animals

As explained in the introduction, this technique proved most successful in very young animals. The data presented in this chapter was recorded from the retina of pre-hatchling animals and newly hatched animals. Pre-hatchlings were animals which were taken from the egg and therefore were still feeding from their yolk sac, but were developed to the stage where the lens had formed in the eye and were due to hatch within a matter of a few weeks. This stage corresponds to approximately stage XIX of development according to the classification used by Naef (1928). It is expected that there will be some developmental differences between these animals and the juvenile and adult animals used in the previous chapters. However, whole cell patch clamp recording from older animals has so far not been possible therefore this was the only preparation available at this time. It is hoped that these initial studies will lead the way to whole cell recording from animals of all ages.

5.2.2 Whole cell recording

Currents were recorded using the whole cell patch clamp technique. Borosilicate glass tubes were pulled using a Flaming/Brown micropipette puller (Model P-97) to produce the microelectrodes for recording. Patch electrodes were filled with an internal solution as described below. Electrode resistance measured in ASW ranged from 5 MΩ to 8 MΩ. The microscope stage was cooled by a Peltier device and all experiments were conducted at 14-15 °C. The preparation was visualized on a television monitor by means of a Hamamatsu CCD camera (Model C7500-11/-21) attached to the port of an Olympus upright microscope (Model BX50WI) while illuminated by infrared light. The microelectrode was positioned using a Sutter Instruments micromanipulator (Model MP-

285) under X10 (NA 0.30) and then X40 (NA 0.08) water immersion objectives. Following formation of a tight seal the electrode potential was set to a holding potential of -60 mV and then the membrane patch was ruptured.

Whole cell recordings were made using an Axopatch 200A amplifier with a current –to-voltage converter headstage. Signals were low pass filter at 2 kHz and sampled at a rate of 3.3 kHz for the photoresponse and 10 kHz for the voltage protocols, and then stored on computer for off-line analysis. Membrane capacitance and series resistance were compensated using the Axopatch internal electronic circuitry. Stimulation pulse protocols were generated by the Digidata digitizer computer controlled by Clampex 8.1 software.

5.2.3 Data acquisition and analysis

All data was transferred to computer via a Digidata 1322A digitizer and sampled using the Clampex 8.1. Linear subtraction was used on all voltage activated currents in order to study them without interference from the passive electrical characteristics. This technique uses a small stimulus that does not activate any ionic current, to measure the "leak" current which can then be scaled up and subtracted from the recorded traces. The linear component was cancelled on-line using P/N leak subtraction. Any off-line analysis was performed in Clampfit 8.1.

5.2.4 Optical stimulation

Light stimulation was from a Schott KL1500 LCD halogen light source via a fibre optic light guide whose output was directed to a mechanical Uniblitz shutter. Another fibre optic light guide was positioned at the other side of the shutter so that the output illuminated the entire tissue bath. The computer-sampling program, Clampex, was used to

control the Uniblitz shutter driver (Model VMM-D3) and therefore to determine the timing and duration of the light flash. The maximum light intensity available at the level of the preparation was 200-300 μWcm^{-2} and neutral density filters were used to attenuate this maximum intensity. The intensity at the level of the preparation was measured with a light meter (Advantest TQ8210). Unless otherwise stated, when examining the photocurrent and chemical effects on it, a low intensity flash (~4-8 μWcm^{-2}), 50 ms in duration, was applied every minute.

5.2.5 Photographic Images

Photographic images were taken using a Brian Reece Scientific Limited framestore, via a Hamamatsu CCD camera (Model C7500-11/-21). Images were imported directly into Photoshop 7.

5.2.6 Solutions

Unless otherwise stated, tissue was perfused with aerated ASW, composition as described in Chapter 2. The composition of the pipette internal solution was (in mM) 200 K-gluconate, 100 KCl, 9 $MgCl_2$, 6 ATP-Na_2, 10 HEPES, 500 sucrose and 1 EGTA, pH 7.3 adjusted using KOH. This recipe was adapted from Nasi & Gomez (1992). The sucrose was added to maintain osmotic balance. A 1 mM stock solution of tetraethyl ammonium (TEA) was made up in ASW and diluted down to the appropriate concentrations. Stock solutions of dibutryl guanosine cyclic monophosphate (DB-cGMP) (10 mM), 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP) (5 mM) and guanosine 3',5'-cyclic monophosphate (cGMP) (20 mM) were made up in internal solution and then diluted down appropriately. Heparin sodium was added directly to the internal solution at the appropriate concentration. Stock solutions of U-73122 (2 mM), zaprinast (20 mM), and phorbol-12,13-dibutyrate (10 mM) were made up in DMSO and diluted to the desired

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concentration in internal solution. The DMSO concentration did not exceed 0.5 %, a concentration shown to have no effect on the cell responses.

When adding test chemicals to the pipette internal solution, the first photoresponse was elicited soon after patch rupture and was called the control response. There is therefore no true control response but it was considered that there would be very little perfusion of test chemical into the cell cytosol at this stage.

5.3 Results

5.3.1 The preparation

The dissociation procedure did not provide a reliable yield of well dissociated cells for whole cell patch clamp. There was extensive variability in yield even when the same protocols and enzymes were used and successful recordings were rare. Attempts to patch cells from slices taken from adult animal retina had previously been unsuccessful probably due to the cell surfaces not being clean enough to form a high resistance seal. However slices of retinal tissue obtained from hatchlings and pre-hatchlings proved to be far cleaner and much more successful and reliable for whole cell patch clamp experiments. Stable recordings could be made for a maximum of 20-30 minutes. This appeared to be dependent on the continued integrity of the cell. Figure 5.2 shows photos of an example slice preparation from a pre-hatchling animal, the type of which has provided successful whole cell recordings. It can be seen in Figure 5.2B that the cell bodies are clearly visible, without anything obstructing their surface therefore providing clear access for the recording microelectrode. In slice preparations from adult animals it was extremely difficult to clearly pick out individual cell bodies and the route to the cells was almost always obstructed by what looked like dead cells and other debris.
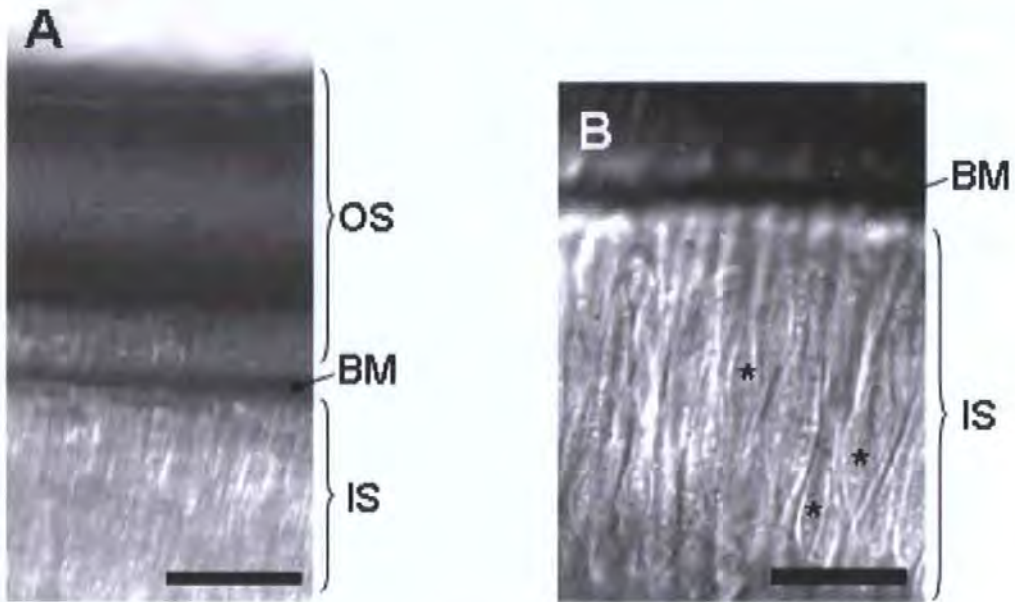
Figure 5.2 The slice preparation from a pre-hatchling animal as seen on the TV monitor. Part A is at X20 magnification where the whole slice can be seen to help with orientation. Part B was at X40 magnification which was used to position the microelectrode. The microelectrodes used had a tip diameter of <1 μm. Some of the cell bodies have been pointed out (*). Part A, scale bar 50 μm; Part B, scale bar 25 μm. OS – outer segment, BM – basement membrane, IS – inner segment.

5.3.2 Cell membrane passive properties

Whole cell capacitances in the pre-hatchling animals ranged from 4.1 pF to 18.9 pF with an average value of 10.1 ± 0.8 pF (mean ± SE, n = 30). For pre-hatchlings the estimated cell membrane area (using the calculation explained in Chapter 3) would be ~23000 - 40000 μm^2, calculated from measurements of outer segment length and cell diameter. Assuming a specific membrane capacitance of 1 μFcm^{-2} which is typical for most neurons (Johnston & Wu, 1995), the recorded capacitance values would appear very low.

5.3.3 Light-induced currents

The dynamic range of the photoreceptors was determined by recording the photoresponse to increasing intensity stimuli. Some sample traces from one cell are shown in Figure 5.3.
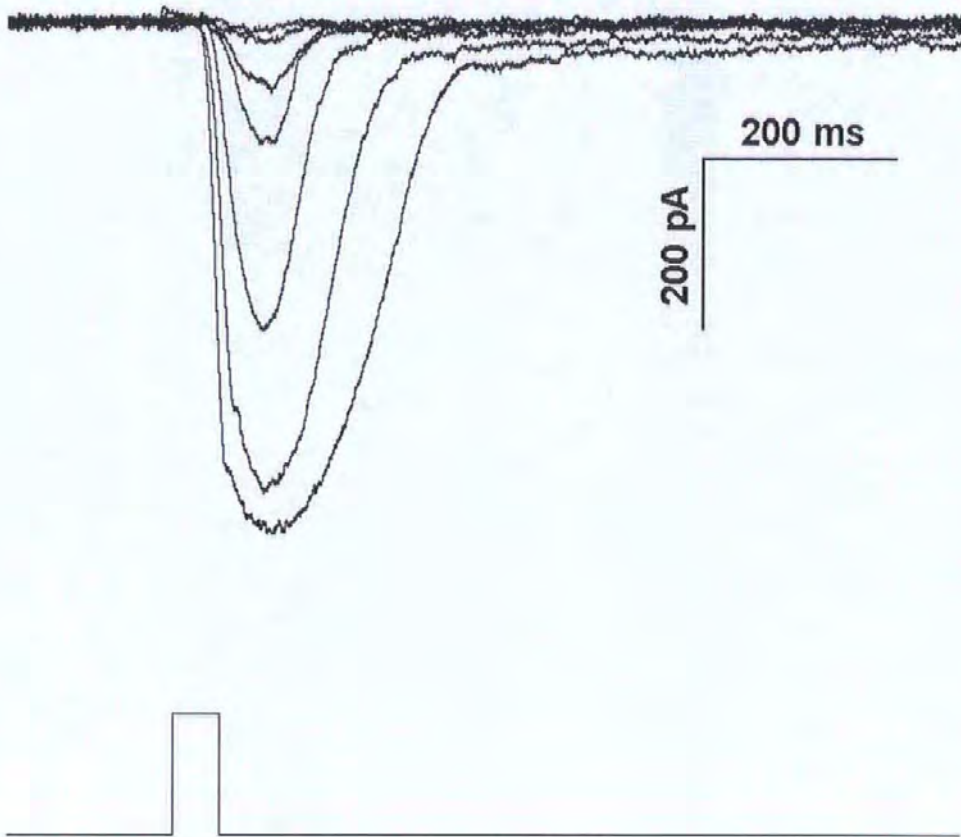
Figure 5.3 **Superimposed photocurrents elicited by increasing intensity light stimuli.** **The photocurrent was recorded every minute in response to a 50 ms light flash as indicated below the traces. The light intensity increased in steps of ~0.5 log unit from 0.04 μWcm^{-2} to 200 μWcm^{-2}. In this particular cell, further increases in stimulus intensity produced no further increase in response amplitude.**

The intensity response relationship was tested in 4 cells and the results are shown in Figure 5.4, each data point is the mean ± SE. The light intensity was increased from a sub-threshold value of 0.04 μWcm^{-2} up to a maximum available value of 200 μWcm^{-2}, in order to see close to the full range of these photoreceptors. It can be seen that the photoreceptors have a range of ~4 log units, with a threshold response being elicited at ~0.3 μWcm^{-2} and a response of 700 pA being elicited with the maximum intensity available of ~200 μWcm^{-2}.
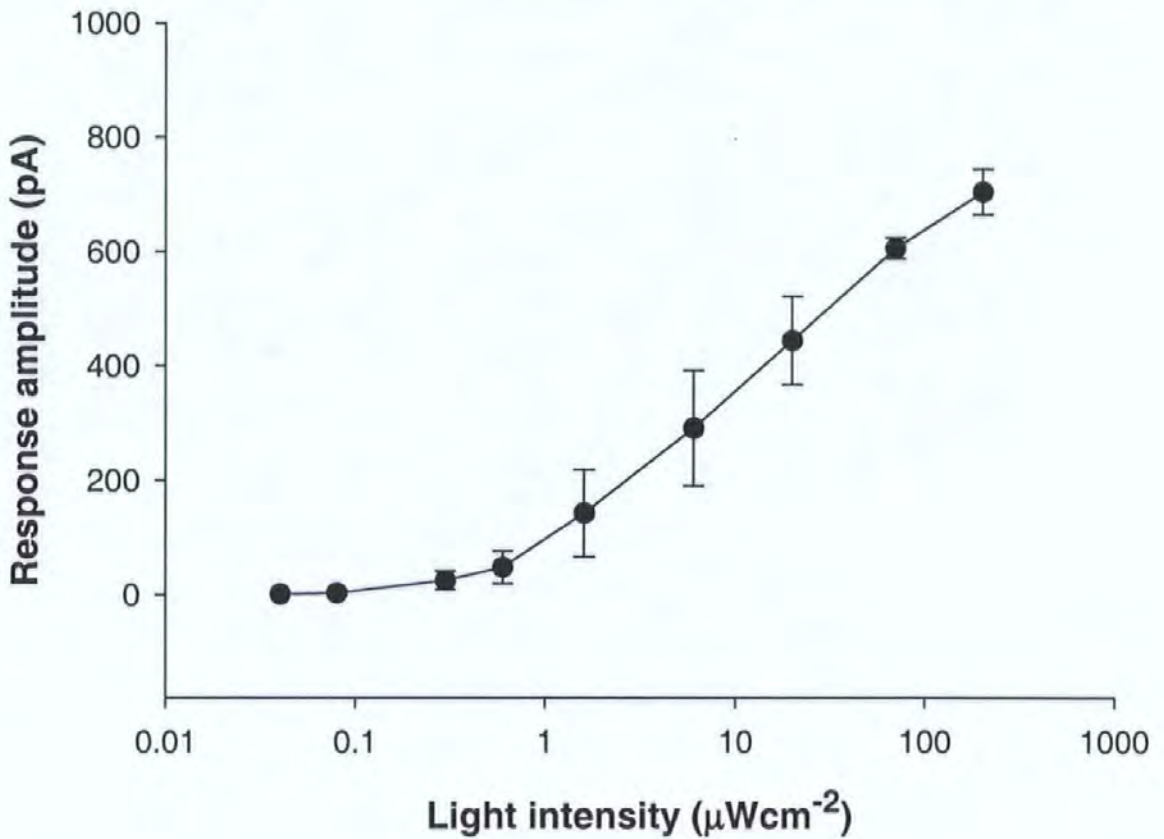
Figure 5.4 Plot of the peak current amplitude vs. light intensity. Each data point is the mean ± SE (n = 4)

It was evident from Figure 5.3 that as the light intensity increased response latency decreased and the slope of the rising phase increased; this is indicative of an increase in response kinetics with increasing stimulus intensity, as was observed when recording the ERG. The response latency decreased from ~60 ms at 0.6 μWcm^{-2} to 25 ms at the maximum intensity of 200 μWcm^{-2} and this is shown in Figure 5.5. There are no measurements for the lower light intensities as there was either no response, or the response was so small that is was difficult to measure response latency accurately.
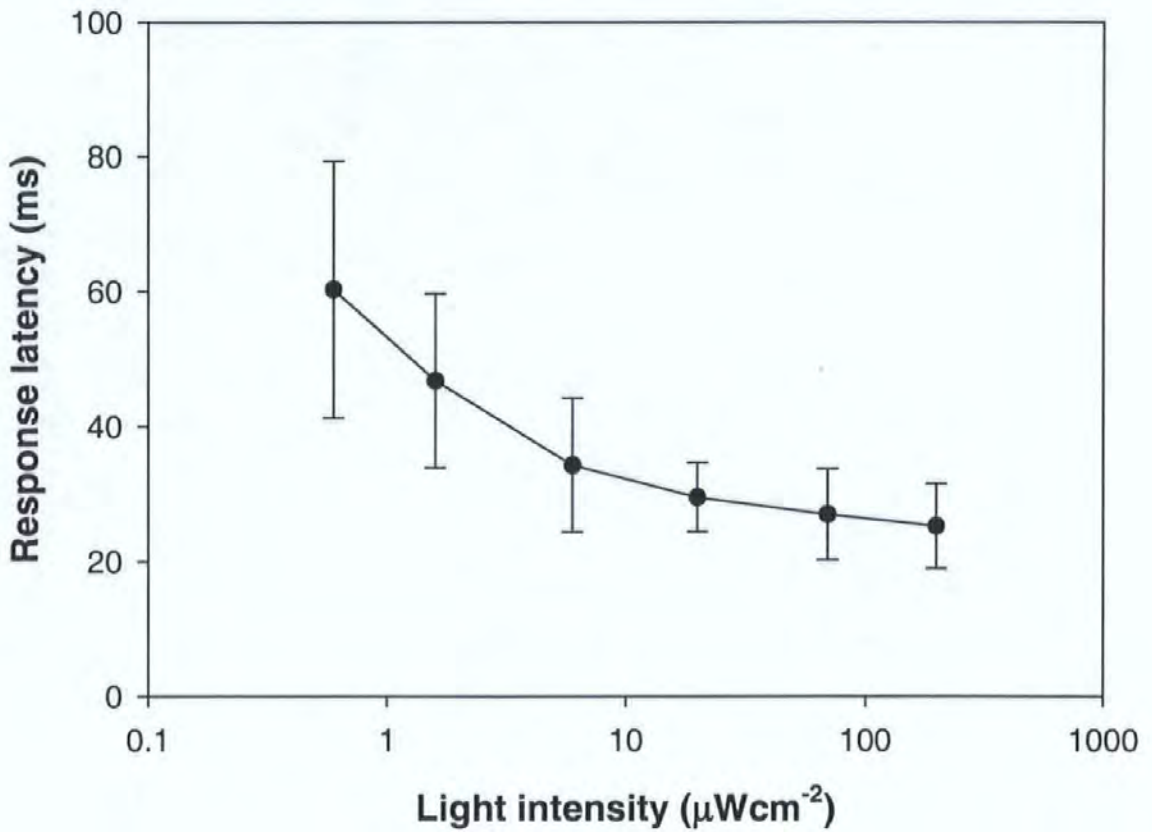
Figure 5.5 Response latency decreases as the stimulus intensity increases. Each data point if the mean ± SE, n = 4.

Reversal of the photocurrent through manipulation of the cell membrane potential was usually not observed as the preparation became increasingly unstable at more depolarized holding potentials (n = 10). However reversal was observed once between +40 mV and +60 mV and once between 0 mV and +20 mV as shown in Figure 5.6. It is likely that the difficulty in measuring the cell reversal potential was due to poor space clamp of these very elongated cells.
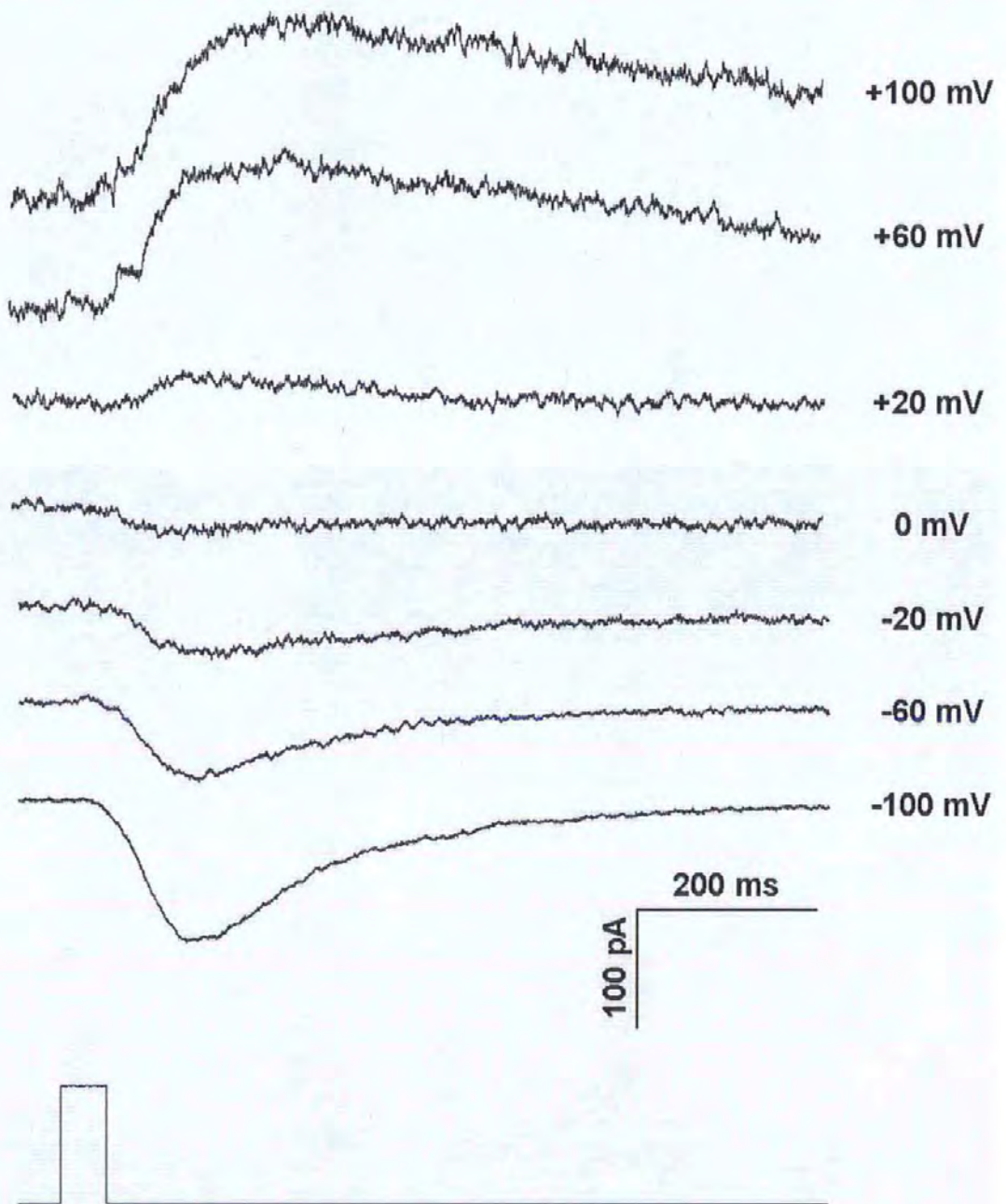
Figure 5.6 Reversal of the photoresponse elicited by relatively dim (7 μWcm^{-2}) light flash of 50 ms duration as indicated below the traces. The cell was held at a holding potential of -60 mV and stepped from -100 mV to +100 mV in 20 mV steps. The potential reversed somewhere between 0 mV and +20 mV.

5.3.4 Pharmacological intervention

From the extracellular recording of the ERG, it was shown that the enzyme PLC was essential for the production of a light response (Chapter 2). The PLC inhibitor U-73122 was added to the internal pipette solution at a concentration of 10 μM. This inhibitor has

been shown to cause inhibition when injected into *L. polyphemus* (Nagy & Contzen, 1997) photoreceptors and to inhibit agonist induced activation of the light activated channel Trpl in *D. melanogaster* (Hardie & Raghu, 1998). In the cuttlefish photoreceptor, this chemical greatly reduced the light induced response in all cells tested and completely abolished the response in 3 out of 5 cells. Representative traces are shown in Figure 5.7. A photoresponse was elicited soon after rupture of the membrane patch, by a 50 ms low intensity light flash when the slice was perfused with ASW. This response was completely abolished after 17 ± 4 minutes (mean $\pm$ SE, n = 3) exposure to U-73122.
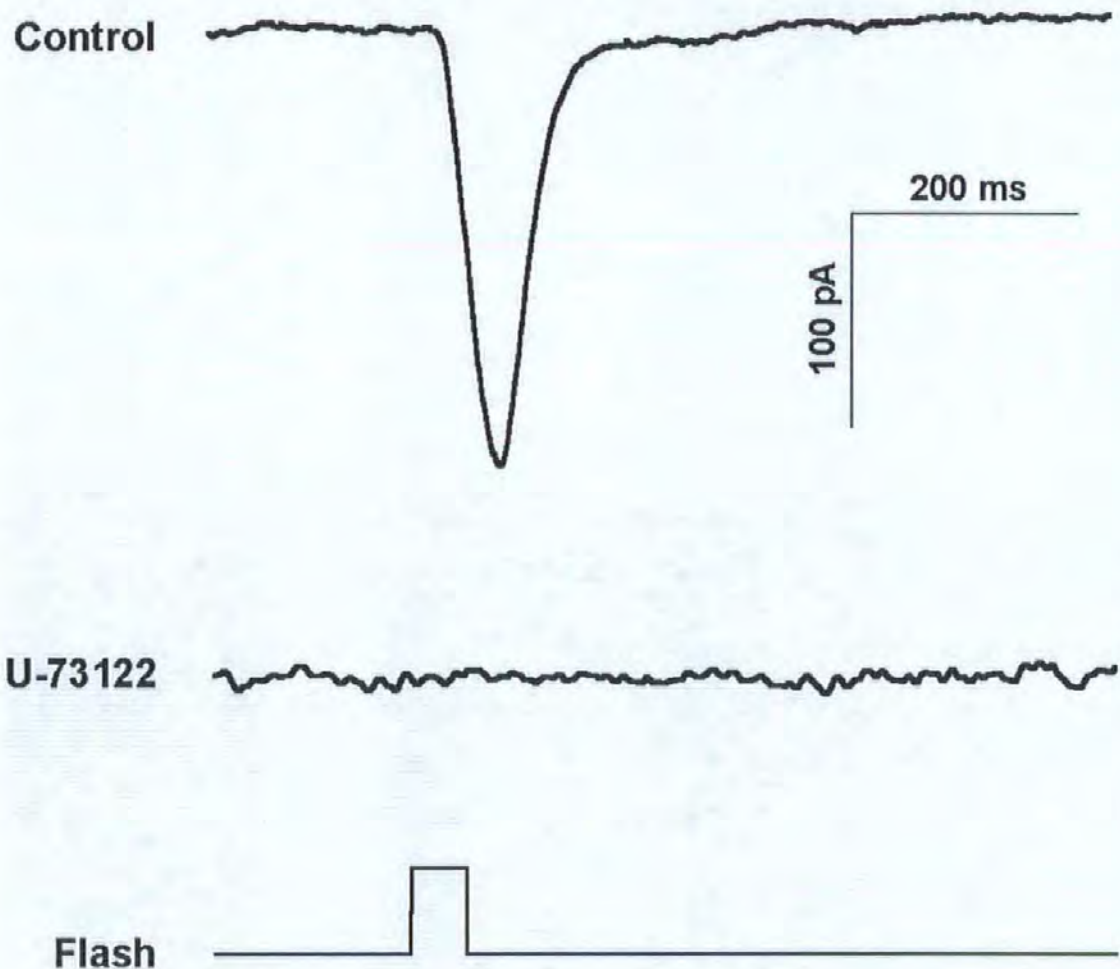
Figure 5.7 Inhibition of PLC can cause complete abolition of the photoresponse. The top trace shows an example control response elicited by a 50 ms flash of light (7.5 μWcm^{-2}) as indicated by the lowest trace. The same flash was applied following 25 minutes exposure to 10 μM U-73122 and it can be seen (middle trace) that the response was abolished.

When the non-membrane-permeable IP$_3$ receptor inhibitor heparin was applied internally to the photoreceptor cells, the photoresponse was abolished in 5 out of 6 preparations. The results from a representative experiment are shown in Figure 5.8. It can be seen that the trace of the control light response was completely abolished following exposure to 25 mg/ml heparin. A time series of this experiment is shown in Figure 5.9 to demonstrate the kinetics of this effect.

Control

200 ms

100 pA

Heparin
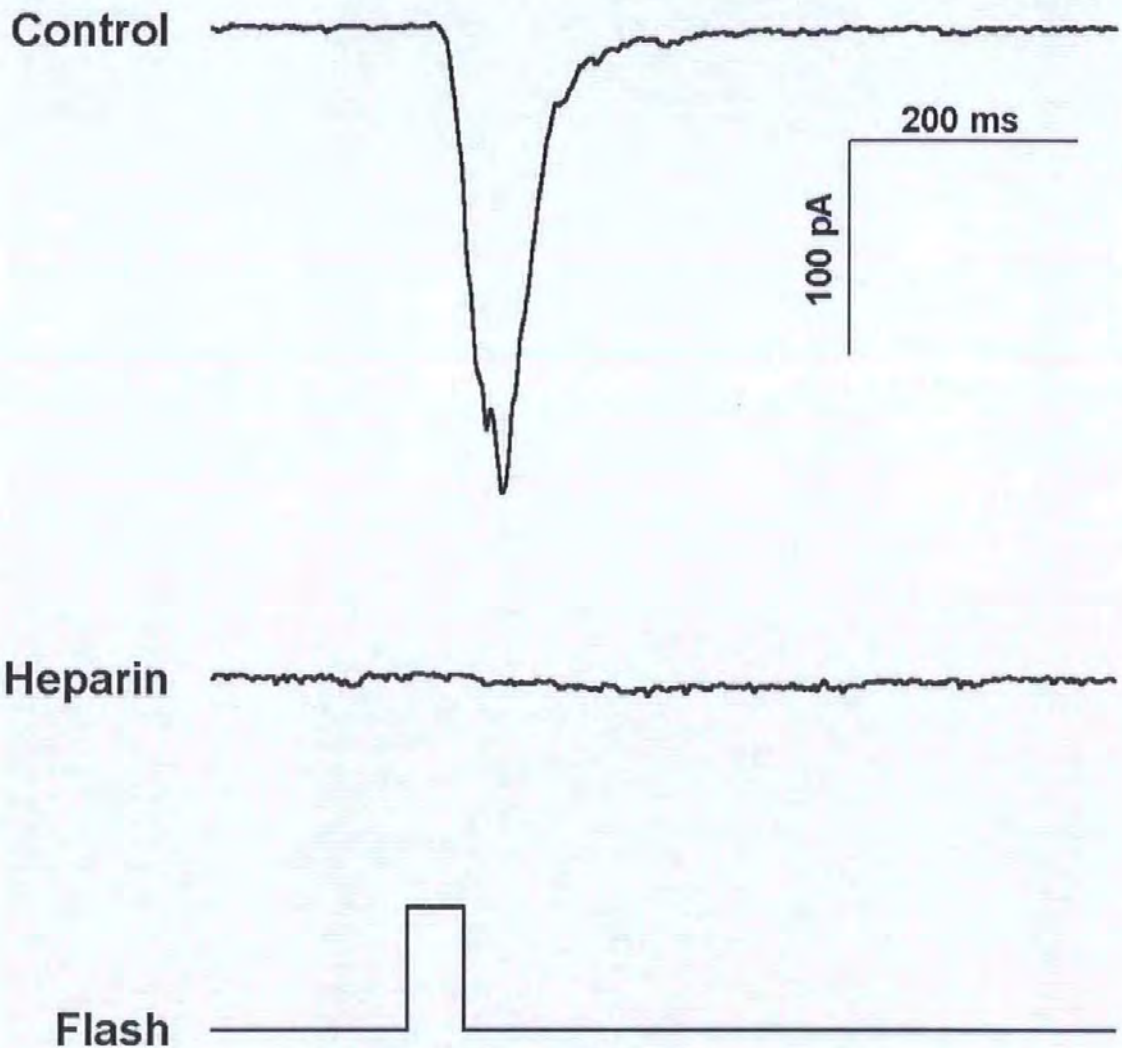
Flash

Figure 5.8 The light-induced response can be abolished by blocking the IP$_3$ receptor. The top trace shows the control response to a 50 ms low intensity (7 μWcm^{-2}) light flash as represented by the lower trace. The cell produced no response to the same light stimulus following 24 minutes exposure to 25 mg/ml heparin.
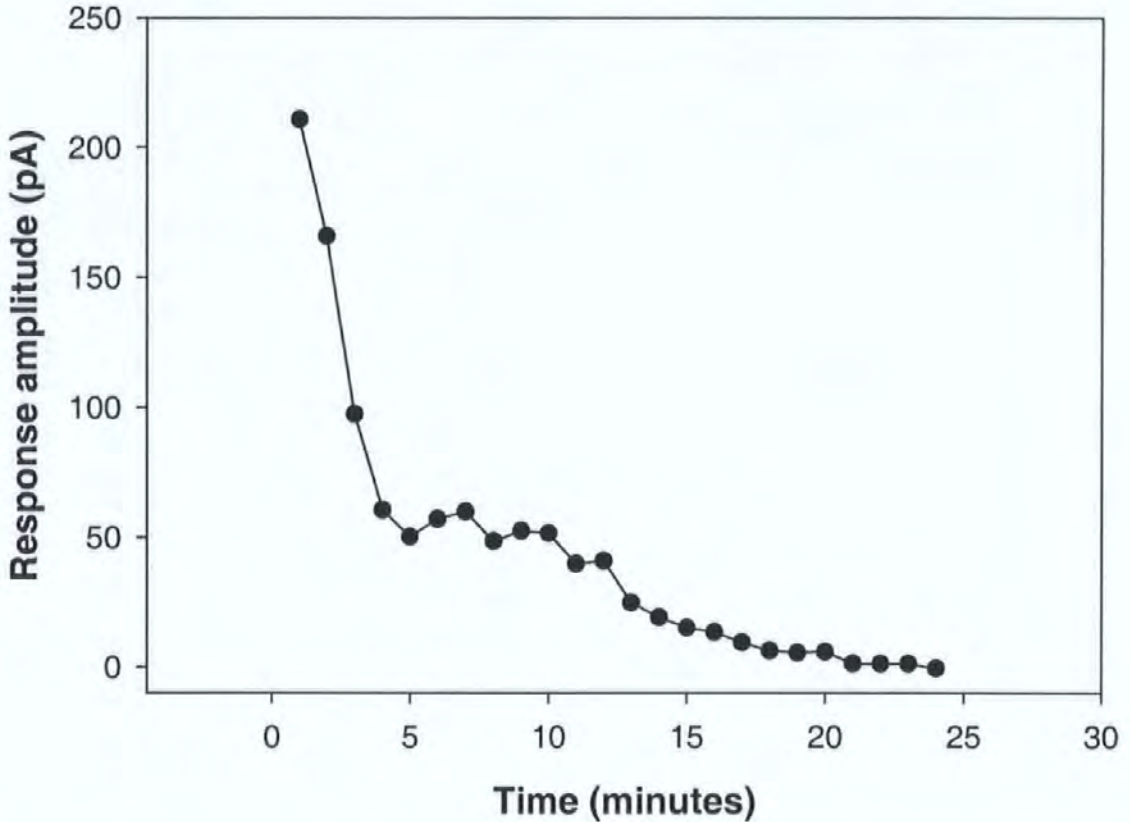
Figure 5.9 Plot showing how the response latency decreased as length of exposure to 25 mg/ml heparin increased.

Heparin has been shown to be an effective inhibitor of the IP_3R in other invertebrate photoreceptor preparations (Contzen et al., 1995; Ukhanov & Walz, 2000). When 25 $mgml^{-1}$ heparin sodium was added to the pipette internal solution, the photoresponse was abolished in 9.4 ± 3.8 minutes (mean ± SE, n = 5). This effect was shown to be concentration dependent; increasing the concentration from 25 $mgml^{-1}$ to 40 $mgml^{-1}$ reduced the time to abolish the response to 4 ± 0.6 minutes (n = 3). This time was reduced to ≤1 minute by increasing the concentration to 50 $mgml^{-1}$. At the lower concentration, in some cases, it was still possible to elicit a response at higher light intensities when the response was abolished at the low test intensities. However, with the higher concentrations, the response to brighter light was also blocked.

The effect of Zaprinast on the photoresponse was also tested, as this agent is known to inhibit cGMP phosphodiesterase (PDE). cGMP PDE catalyses the breakdown of cGMP therefore the application of Zaprinast would be expected to slow down cGMP breakdown and hence potentially increase the cellular cGMP level or prolong the time for which it was present. Johnson & O'Day (1995) tested the effects of Zaprinast on the light response induced in *L. polyphemus* ventral photoreceptors at low and high stimulus intensities. They found the effect to be much more prominent at higher light intensities. Based on the findings of Johnson & O'Day (1995) and assuming the *S. officinalis* uses a similar second messenger cascade to *L. polyphemus*, Zaprinast application would be expected to increase overall conductance in some way. However, no clear effect on the light induced current was observed (n = 7).

Further investigation of a potential role for cGMP was carried out by adding DB-cGMP and 8-Br-cGMP to the pipette solution. Again no clear change in the response was observed. This may be attributed to these agents being membrane permeable analogues of cGMP therefore may have moved out of the cell being recorded from and hence any effect was too small to produce a visible effect. Therefore cGMP was used in its sodium salt form, a form which is not membrane permeable. Example traces showing a control response and a response following exposure to 100 μM cGMP are shown in Figure 5.10. It can be seen that the response amplitude was increased and that the trace was also displaced to the right. The same effect was observed in 3 cells, with a mean maximum increase in amplitude of 37 % ± 5.5 (mean ± SE, n = 3) occurring within 9 minutes of exposure. It can also be seen that the kinetics of response activation were retarded by cGMP.
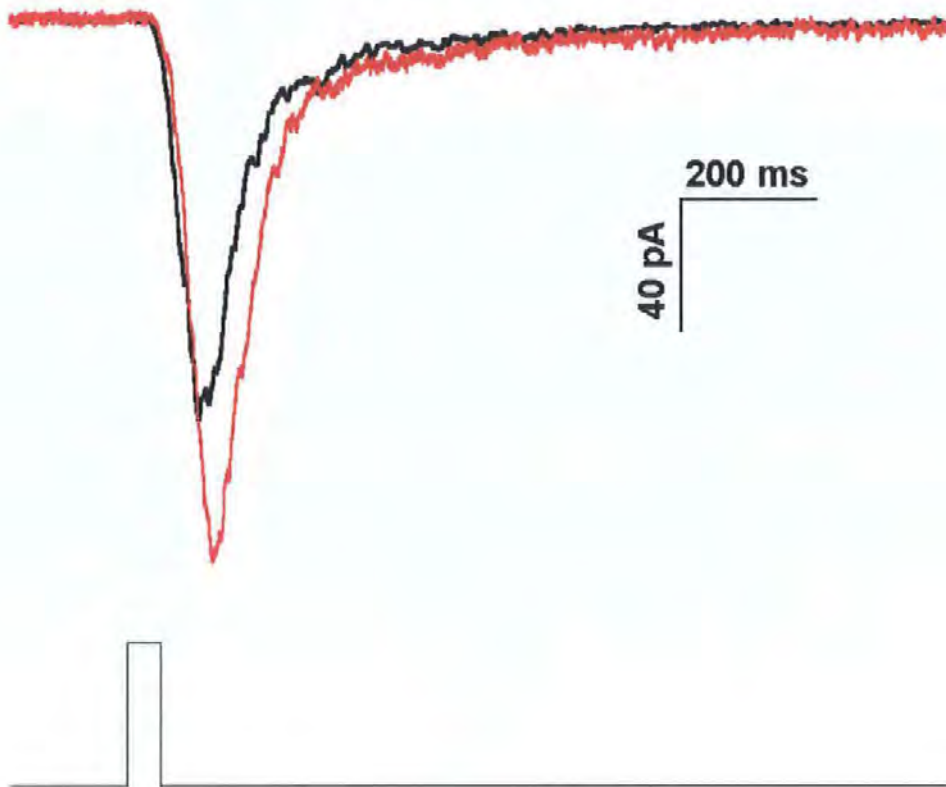
Figure 5.10 Traces showing the effect of 100 μM cGMP on the light response induced by a 50 ms flash (10 μWcm^{-2}) as indicated below. The control response is shown in black and the response elicited following exposure to cGMP in red. Each trace is the average of 3 individual traces.

The protein kinase C (PKC) activator, phorbol-12,13-dibutyrate (50 μM) was also tested by adding to the pipette internal solution. So far there is still the possibility of an alternative means of opening the light-activated ion channels other than via IP$_3$-mediated calcium release, the most likely of which would be via diacylglycerol (DAG). As DAG is commonly known to activate PKC it was thought that a PKC activator may give an indication of how this may affect the light response. However, as was found when this chemical was tested using ERG recording, this chemical produced no clear change in the light response (n = 3).

5.3.5 Voltage-activated currents

Voltage-activated currents were found to be present in all cells tested with outward currents being most common and inward currents occurring less often. These currents would also be activated by the membrane depolarization induced by light and would hence contribute to the photoresponse. An example of the current traces induced by 100 ms depolarizing voltage steps applied every 2 seconds is shown in Figure 5.11.
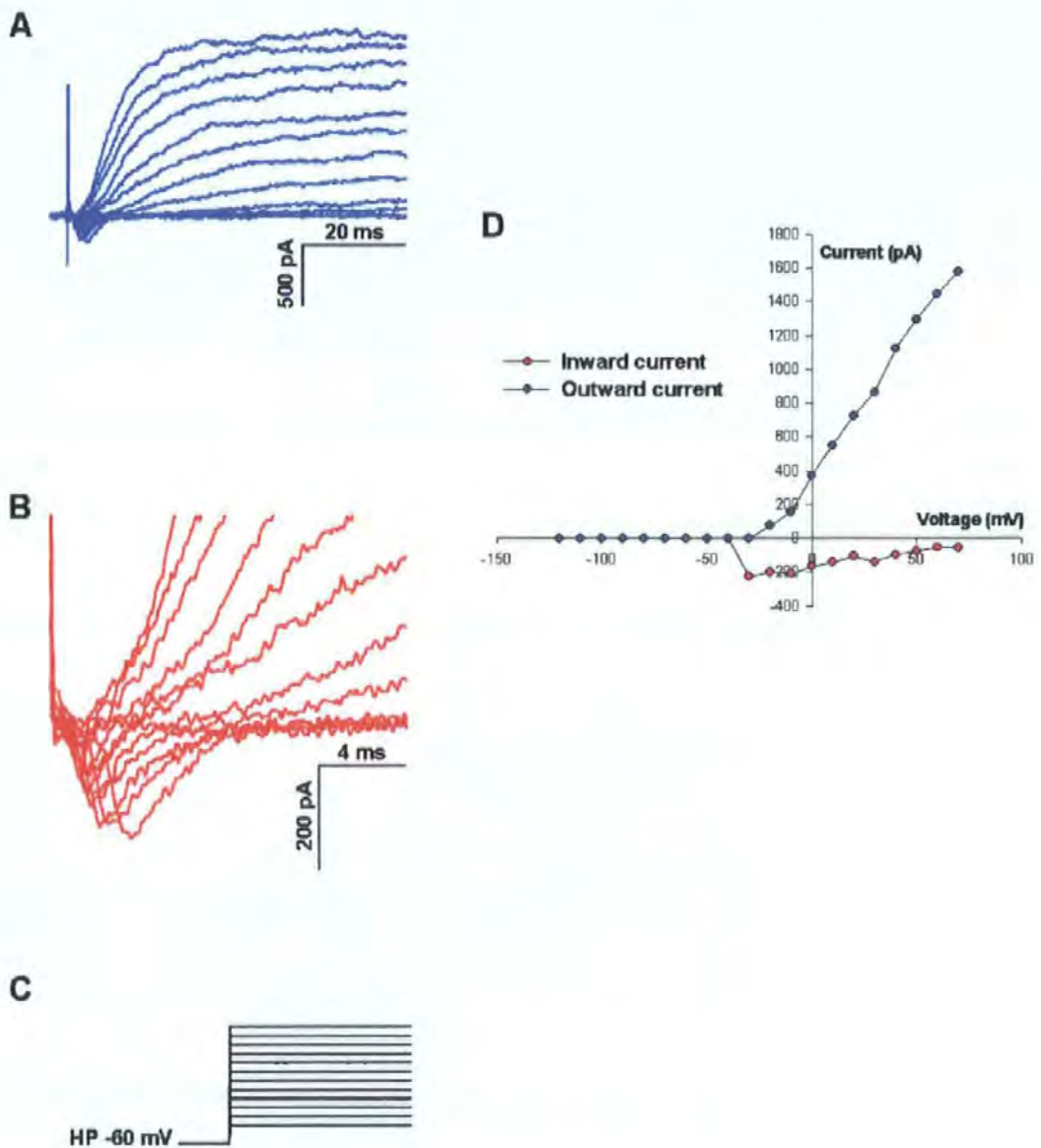
Figure 5.11 Voltage-dependent current responses recorded in the whole cell configuration. Part A shows the current traces recorded in the dark in response to depolarising voltage steps from a holding potential of -60 mV as indicated in C. Voltage steps were increased by increments of 10 mV from an initial step to -40 mV. Part B show the same traces as in A but on an expanded time and current scale to show more clearly, the inward current. The I-V curves for both the inward and outward currents are shown in Part D.

These current traces were in response to voltage steps from a holding potential of -60 mV at 10 mV increments whilst the slice was perfused with ASW. A small, early transient inward current was observed followed by a sustained outward current. Both currents were activated at similar potentials; the outward current was activated at a potential slightly more positive than -30 mV and the inward current at a potential slightly more positive than -40 mV (Figure 5.11, D) in this particular cell.

5.3.5.a Outward Currents

An outward current was recorded in both dissociated cells and in cells in the slice preparation. The current was elicited by depolarizing voltage steps and was activated at potentials more positive than -30 mV (n = 15) and increased in amplitude with further increases in depolarization. In slice preparations from juveniles and pre-hatchlings the maximum amplitude of the outward current was 2384 pA $\pm$ 213 (mean $\pm$ SE, n = 15). This outward current was most likely carried by K^+ ions and therefore the K^+ channel blocker TEA was bath applied to the preparation. This chemical has been shown to be effective in other invertebrate retinal preparations (e.g. *L. polyphemus* (Lisman et al., 1982) and *Lima scabra* (Nasi, 1991b)). Figure 5.12 shows the representative effect of 10 mM TEA on the voltage activated outward current induced by stepping from a holding potential of -60 mV to +70 mV. Due to its relatively slow activation, lack of inactivation for the duration of the test pulse and TEA sensitivity, it was deduced that this current was a delayed rectifier type K^+ current.
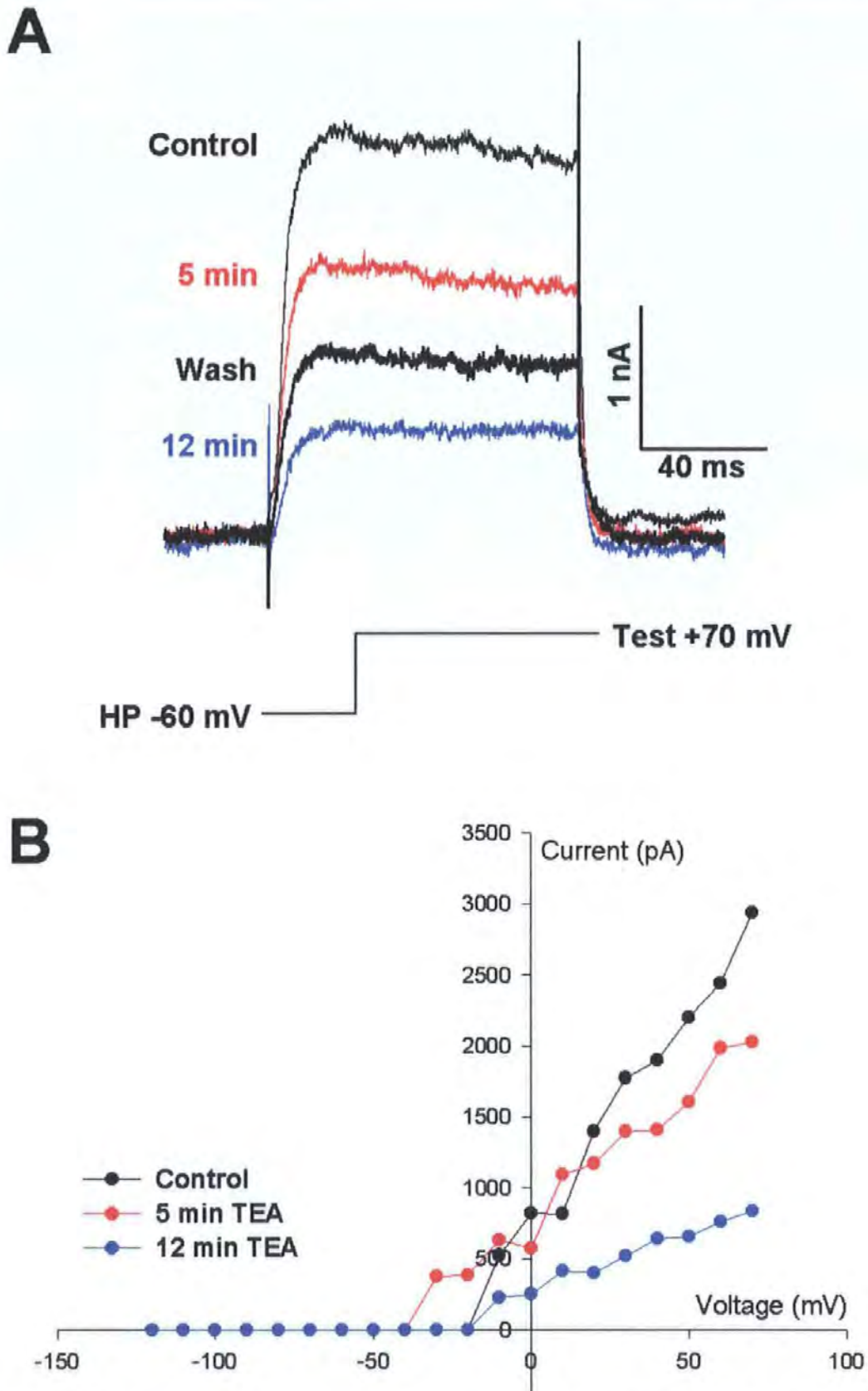
Figure 5.12 Reduction of the voltage activated outward current by TEA. Part A shows the current activated by a voltage step from the holding potential of -60 mV to a test value of +70 mV, as indicated below, under control conditions, following 5 and 12 minutes exposure to 10 mM TEA and following 25 minute wash in ASW. The current amplitude was reduced by ~70 % following exposure to TEA. Part B shows the peak current voltage relationship following 5 and 12 minutes exposure to TEA. n=3

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5.3.5.b Inward Currents

The inward current, which was observed in some of the preparations, was investigated more fully in order to determine its properties. This current was activated at potentials more positive than -40 mV (n = 5) and it had a maximum amplitude of 178 pA ± 27 (mean ± SE, n = 5). It activated quickly, with the peak occurring as early as 2 ms, and also inactivated rapidly. By changing the holding potential from -60 mV to -40 mV (n = 2) it was possible to completely inactivate the inward current (Figure 5.13). This suggested that the ion channels carrying this inward current were inactivated at -40 mV and therefore could not be opened by depolarization. This inactivation was removed when the cell was held at the more hyperpolarized holding potential of -60 mV.
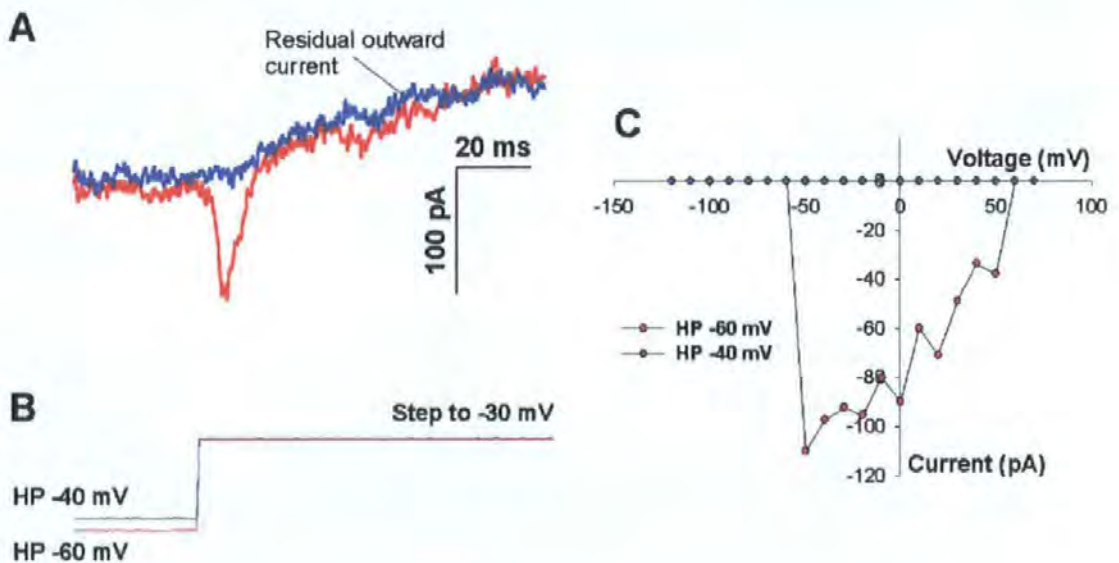
Figure 5.13 Depolarising the cell to a holding potential of -40 mV inactivated the inward current. Part A shows two currents traces elicited by voltage steps to -30 mV from holding potentials of -60 mV (red) and -40 mV (black) as indicated in part B. Part C shows the current voltage relationships at both holding potentials.

It was also possible to block the inward current by adding the voltage-sensitive Na^+ channel blocker, tetrodotoxin (TTX) to the perfusate. Current traces induced by a step to +30 mV from a holding potential of -60 mV under control conditions (ASW) and in the presence of 10^{-6} M TTX are shown in Figure 5.14A. It can be seen that TTX completely abolished the inward current. TTX exposure abolished the inward current at all potentials

(Figure 5.14B). A similar effect was observed by substituting normal ASW for choline ASW (n = 1) firmly establishing this as a Na^+ current. No other inward currents were observed in any of the preparations.
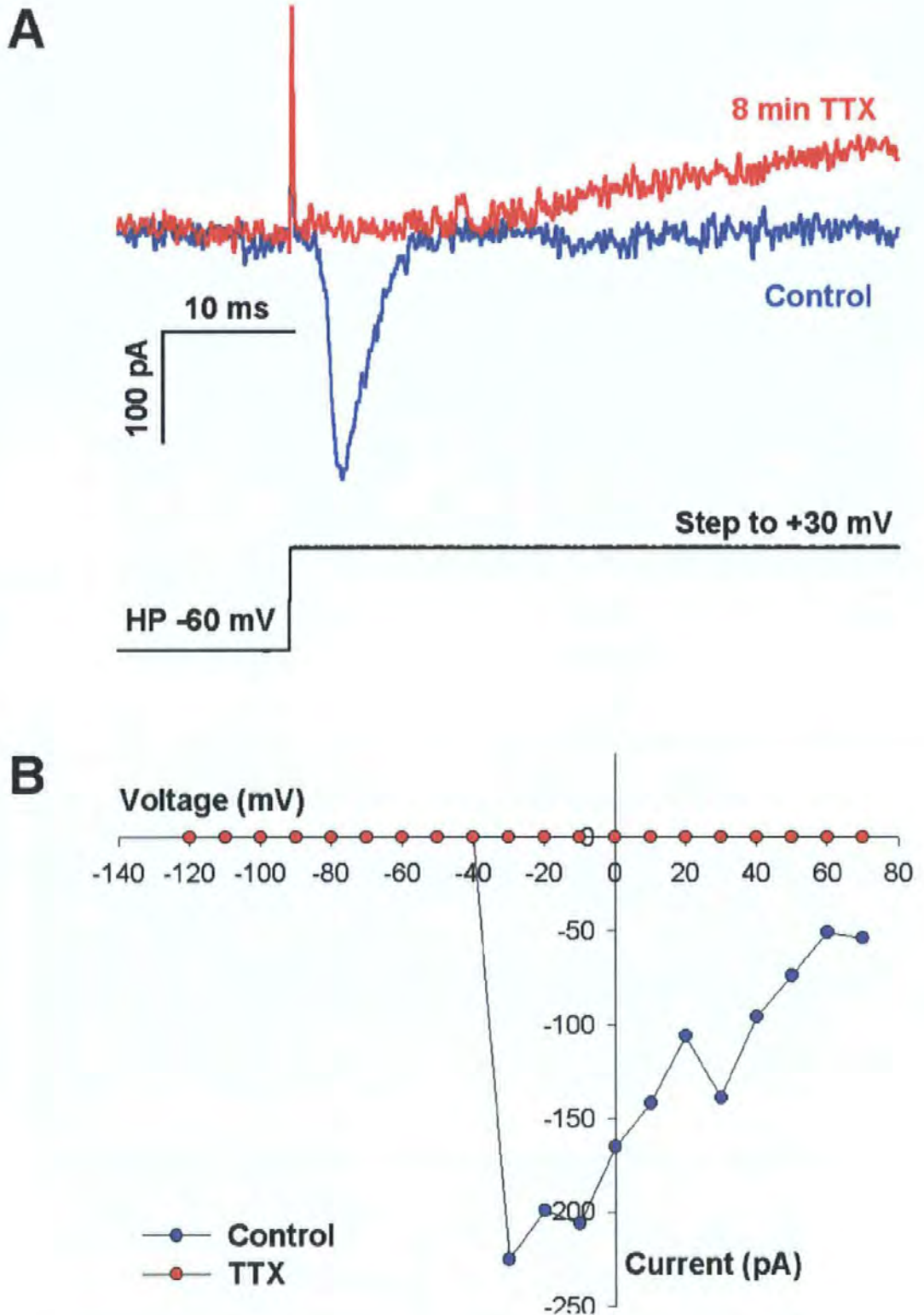
Figure 5.14 The voltage dependent inward current was blocked by TTX. Part A shows the current traces induced by voltage steps from a holding potential of -60 mV to a test potential of +30 mV when the tissue was perfused with ASW (blue) and with the addition of 10^{-6} M TTX (red). The current voltage plot (Part B) shows that the current was blocked at all test potentials.

The results presented in this chapter can be summarized as follows:

- Retinal slices from pre-hatchling animals provide a more suitable preparation for whole cell patch clamp recordings than dissociated cells or retinal slices from older animals.

- *S. officinalis* photoreceptors have a dynamic range of ~4 log units and a threshold sensitivity of approximately 0.3 μWcm^{-2}. The maximum response amplitude attained with the intensity range available was ~700 pA.

- Photocurrent reversal was found to occur somewhere between 0 mV and +60 mV, although in most preparations reversal was not observed.

- Inhibition of PLC abolished the photoresponse.

- Inhibition of the IP_3R abolished the photoresponse; being more effective at lower stimulus intensities.

- Addition of cGMP to the pipette internal solution resulted in an attenuation of the photocurrent amplitude and a retardation of the activation kinetics.

- Two voltage-activated currents were observed; an inward sodium current and an outward potassium current.

5.4 Discussion

5.4.1 The preparation

The slice preparation developed in this work has again proved to be extremely useful in investigating the light response and transduction cascade in the retina of *S. officinalis*. Prolonged investigation of the viability of using dissociated cells proved fruitless, not to say that under the correct conditions it would not be successful. Although a method for dissociation of the squid *L. pealei* photoreceptors has been developed (Nasi & Gomez, 1992) it produced no viable cells when applied to this preparation. Why this should be so is a matter of speculation. General observations show that there are visible differences in the photoreceptors from squid retina compared to cuttlefish retina. Squid photoreceptors tend to be much thinner and smoother looking than cuttlefish photoreceptors. Cellular differences such as these may influence their reaction to different enzyme types and hence result in the need for very different dissociation protocol.

By taking slices of retina from very young animals it was possible to have a preparation which had healthy looking cells with no cell debris on the surface blocking the path of the micropipette. This allowed good seal formation on the cell surface and a light response was recorded in most preparations although it was not always long-lived, a feature determined to be due to the fragility of the cells which were sometimes seen to shrink or swell up. This relative ease of recording was not available in older animals, where obtaining recordings became progressively more difficult with age. This was due to access to a clean cell surface becoming increasingly more difficult. Healthy cells were visible but they were nearly always occluded by dead cells and debris on the cut surface; even when access was gained, good membrane seals were rarely formed in older animals.

5.4.2 Responses to light

This preparation gave the opportunity to study the light response in a single photoreceptor from the retina of *S. officinalis*. Single cell recordings have been carried out previously using intracellular electrodes (Pinto & Brown, 1977; Duncan & Pynsent, 1979) however they did not allow the study of voltage activated currents or the pharmacological manipulation of the light response; both of which are possible with this preparation. Additionally, the Nasi & Gomez (1992) exploration using the whole cell patch clamp technique did not involve any investigation of the phototransduction cascade. Intracellular recordings from the retina of *Sepiola atlantica* have shown that generally the ERG was a good representative of the single cell light response (Duncan & Pynsent, 1979). In this study, the light responses elicited in the whole cell configuration were the same in form, as the ERG responses presented in the previous chapters. It was therefore possible to confirm the validity of the use of the ERG in the previous chapters, as an accurate representation of the single cell response.

In order to determine which ions carried the current in the *S. officinalis* photoreceptor, an attempt was made to measure the photocurrent reversal potential. The reversal potential is the equilibrium potential of the ion/s carrying the current across the photoreceptor membrane. It was surprising to find that in most cases there was no reversal. When reversal did occur, it was once between 0 and +20 mV and once between +40 mV and +60 mV. In other invertebrate photoreceptor preparations the reversal potential has been found to occur between 0 mV and +10 mV in *Lima scabra* (Nasi, 1991a), at +10 mV in *L. polyphemus* (Bacigalupo et al., 1986) and between 0 mV and +40 mV in a calcium dependent fashion in *D. melanogaster* (Hardie, 1991b). Why then was a reproducible reversal not observed in this preparation? The current would be predicted to be mainly

carried by sodium and calcium however the measured reversal potentials were below the

Nernst equilibrium potential for either ion as indicated in Table 5.1.

Ion	Concentration in internal solution (mM)	Concentration in external solution (mM)	Calculated equilibrium potential (mV)
Na^+	6	470	108
K^+	300	10	-84
Ca^{2+}	see legend	10	see legend
Mg^{2+}	9	55	45
Cl^-	124	610	-40

Table 5.1 Equilibrium potentials calculated for the ions present in the internal and external solutions using the Nernst equation. The $[Ca^{2+}]$ in the internal solution is not known exactly but is thought to be in the nanomolar (nM) range. However the resting dark level of cellular Ca^{2+} is thought to be in the μM range; measured as 10 μM in *Octopus vulgaris* (Takagi et al., 1994). Therefore assuming a maximum $[Ca^{2+}]_i$ of 10 μM the calculated equilibrium potential would be 86 mV. However, the actual value would be lower than this as the internal pipette solution equilibrated with the cell cytosol; the equilibrium potential would increase with lower $[Ca^{2+}]_i$.

These values were calculated using the ionic concentration in the internal pipette solution

and that in ASW. It is possible that the ion channels were also permeable to potassium

ions, hence the lower reversal potential measured. However, as most cells continued to

respond with a depolarization, even when being held at very positive holding potentials, it

is possible that the light-activated channels are selective for Ca^{2+} and/or Na^+ and hence,

from the table, it can be seen that reversal would not occur until a much more depolarized

holding potential. As the evidence points towards the light activated channels being fairly

non-specific (Takagi, 1994b; Takagi et al., 1994), it is likely that the recordings were just

not stable enough to withstand the large depolarizing voltage steps needed. The light

response was always greatly diminished and rapidly lost following a test for reversal

potential. As mentioned, it is possible, due to the morphology of the photoreceptor cells,

that there was poor voltage control of the outer segment. If the voltage of the outer

segment was poorly controlled then it would be unlikely that the membrane channels were

being influenced as expected and this could account for the unpredictability in measuring

reversal potential. It is likely that the successful measurement was made in particularly short cells.

5.4.3 Pharmacology

The results of pharmacological intervention presented, were in good agreement with the findings from ERG recording and extracellular perfusion of test chemicals in Chapter 2. The use of an alternative phospholipase C (PLC) inhibitor, in this case U-73122, further supports the suggestion of an essential role for PLC in the production of a light response in the retina of *S. officinalis*. Again, the effect of an IP$_3$R inhibitor was tested but this time a membrane impermeant agent was used, namely heparin. This chemical has been widely used as an IP$_3$R inhibitor, e.g. Frank & Fein (1991); Contzen et al. (1995), and in the retina of *S. officinalis*, abolished the photocurrent induced by dim light at all concentrations tested. In contrast to the effects of the other IP$_3$R inhibitors previously tested, the response to brighter light was also abolished. The extracellular studies presented in Chapter 2 found that IP$_3$R inhibitors essentially shifted the intensity response plot to the right; therefore light responses were still present but appeared only at higher stimulus intensities. However, there are a number of possible explanations for the difference between the findings from the two sets of experiments. In the whole cell patch clamp experiments with heparin added to the pipette internal solution, the response to a high intensity flash was evoked after the response to dim light had been blocked by heparin. As stated in Section 5.3.1, the maximum lifetime of the whole cell recordings was ~30 minutes. It is suggested that by the time the response to dim light was blocked by heparin, the whole cell recording was possibly no longer viable. This is a possible reason why no response was elicited by the bright light stimuli. To test this hypothesis is would be necessary to test the effect of heparin on responses to high intensity flash stimuli without prior stimulation with dim light. However, it may be that brighter light was required, or alternatively that activation

of the IP$_3$R was essential for production of a light response and that the inhibitors used in Chapter 2 did not permeate all cells in the slice preparation. From this discussion, it can be seen that the action of IP$_3$ is certainly required for responses to dim light, but that it is not possible to determine whether it is essential for the production of bright light evoked photocurrents. Further discussion of the roles of PLC and IP$_3$R were presented in Chapter 2.

It was important to try to identify the terminal intracellular signalling molecule in the phototransduction cascade that is responsible for opening the light-activated ion channels, in an attempt to form a complete picture of the transduction cascade. There were various pieces of evidence in support of cGMP as the terminal transmitter (previously discussed), and indeed, when cGMP was added to the pipette solution, this suggestion was supported. The light response was increased in response amplitude by the addition of cGMP, suggesting that cGMP has the ability to increase membrane conductance and might play a role in excitation. These findings support a role for cGMP as a terminal transmitter. The only other conditions which had had the ability to increase response amplitude in this preparation were exposure to low extracellular calcium solutions. As both produced a similar effect it is possible that Ca^{2+} some how has the ability to alter cGMP levels, potentially by regulation of cGMP metabolism. A simplified diagram of cGMP metabolism is shown in Figure 5.15.

<div align="center">

guanylate   cGMP
cyclase   PDE

GTP $\longrightarrow$ cGMP $\longrightarrow$ GMP

</div>

Figure 5.15 Diagram showing the enzymes involved in cGMP metabolism. GTP – guanosine trisphosphate, cGMP – cyclic guanosine monophosphate, GMP – guanosine monophosphate, PDE – phosphodiesterase (Stryer, 1991).

There is a growing body of evidence in support of the hypothesis that phototransduction in the *L. polyphemus* photoreceptor terminates with cGMP gated channels regulated by Ca^{2+}. Johnson & O'Day (1995) have shown that cGMP modulates the light response in *L. polyphemus* ventral photoreceptors in a calcium dependent manner. They used cGMP PDE inhibitors and found their application to result in an increase in the amplitude of the light response to bright light stimuli and also found the effect to be enhanced in a low calcium environment. There is also evidence of Ca^{2+} regulation of cGMP metabolism in squid retinal photoreceptors. Brown & Kelman (1996) found that the activity of cGMP PDE was increased by physiological levels of Ca^{2+}. So in both *L. polyphemus* and squid the evidence points towards a light induced increase in $[Ca^{2+}]_i$ resulting in activation of cGMP PDE hence speeding up cGMP metabolism. As cGMP has been shown to open cation channels in *L. polyphemus* (Bacigalupo et al., 1991) and in *S. officinalis* (Huppertz, 1995) this would appear to be a method of inactivation, and still does not explain how Ca^{2+} could result in the opening of ion channels. However, it is possible that Ca^{2+} also has an effect on guanylate cyclase (GC) which catalyses the production of cGMP (see Figure 5.15) and that it affects the two enzymes in a concentration dependent manner. Evidence of a role for particulate GC in *L. polyphemus* photoreceptors has been demonstrated by the use of GC inhibitors. Garger et al. (2001) have shown that inhibition of GC can reduce the light response; however their study does not explore any possible regulation by calcium. In contrast to cGMP, the cGMP PDE inhibitor Zaprinast and the membrane permeable analogues of cGMP resulted in no clear effect on the photocurrent. It is suggested that once again, the fact that these chemicals were membrane permeable caused them to leak out of the cell of interest resulting in a greatly reduced effect; reduced in fact, to the point where it was not evident in the photoresponse.

It was surprising that the PKC activator phorbol-12,13-dibutyrate (P-12,13-diB) still produced no change in the light response, even when introduced to the cell interior via the patch pipette. PKC has been shown to play a role in the light response a number of invertebrate photoreceptor preparations. This has already been discussed in Section 2.4.6b of Chapter 2 therefore will just be mentioned briefly here. In *D. melanogaster* photoreceptors, PKC has been shown to be involved in deactivation, desensitization (Smith et al., 1991) and adaptation (Hardie et al., 1993). PKC also reduced light sensitivity in *L. polyphemus* (Dabdoub & Payne, 1999). However in the clam, *Lima scabra*, PKC activators produced large inward currents, suggestive of a role in excitation (Gomez & Nasi, 1998). There are a number of possible reasons why P-12,13-diB did not have an effect on the photocurrent in *S. officinalis*. This is a membrane permeable agent, so once again, it is suggested that it may have leaked out of the cell of interest before any effect was observed. Alternatively if PKC plays no role in deactivation, desensitization or excitation but is involved in adaptation, this may not have been detected with the flash protocol used. It is also possible that DAG may exert an effect via another means as it appears to do in *D. melanogaster*, where it is effective as a precursor of PUFAs (Chyb et al., 1999).

5.4.4 Voltage-dependent properties

This is only the second time that ionic currents have been studied in cephalopod retinal photoreceptors and the first time that an inward current has been measured. The outward current recorded in *Loligo pealei*, by Nasi & Gomez (1992) appeared around -30 mV as did the outward current recorded in this preparation. As there are no further data regarding the light- or voltage-activated currents in cephalopod photoreceptors, these results will be discussed with respect to other invertebrate photoreceptors where both the light- and voltage-activated currents have been extensively studied.

In *L. polyphemus* ventral photoreceptors, both inward and outward voltage-activated currents have been found to be present and their properties were reviewed in Nagy (1991). Briefly, the transient inward current was reduced in amplitude by reducing either the $[Na^+]$ or $[Ca^{2+}]$ in the extracellular bathing solution and removal of both ions completely abolished the current. This indicates that the inward current could be carried by these two ions. This current was found to be insensitive to the voltage-activated sodium channel blocker, TTX. Two outward currents were observed, namely transient (A) type and delayed rectifier type K^+ currents which could be reduced by the K^+ channel blockers, 4-aminopyridine and TEA respectively. No Ca^{2+}-activated K^+ current was observed (Lisman et al., 1982). These currents were all activated by depolarizing voltage steps hence would be expected to play a role in the light response which produces depolarization. Similarly, in the mollusc, *Lima scabra*, both inward and outward voltage-activated currents were observed (Nasi, 1991b). The inward current was found to be TTX insensitive and also insensitive to Na^+ substitution. However it was reduced by cadmium and almost completely abolished by Ca^{2+} removal showing that this inward current was carried primarily by Ca^{2+} ions. Three outward currents were identified; transient A-type, delayed rectifier and Ca^{2+}-activated K^+ currents.

The cephalopod photocurrent would be expected to be composed of both light-activated currents and voltage-activated currents induced by the depolarization this produces. The outward current observed in this preparation was similar in characteristics to the delayed rectifier type K^+ current seen in other cell types and this was the only outward current observed. It was thought that there may have been more than one type of K+ current based on the findings from other animals. However it is possible that an A-type K^+ current had not developed yet in the pre-hatchling animals used in this study. Hardie (1991a) found that the transient A-current developed at a later date than the delayed rectifier in *D. melanogaster* photoreceptors. In fact in our preliminary investigations, an outward current

with a transient component as well as a sustained component was observed in an older animal however its properties were not investigated. Further investigations at difference stages of development would be required to determine if any other outward currents were present and their developmental progress.

The inward current recorded in this study was found to be TTX-sensitive, as were the spikes which were sometimes observed when the cell was depolarised. However, the photoresponse was only slightly reduced by exposure to TTX. This would suggest that the inward current was not a significant component of the photoresponse as such, but that it was part of the propagated response which fires the action potential. It would be interesting to know at which point along the cell that the firing starts, possibly at the basement membrane. It can therefore be said that the inward current recorded in the cell body region was only a very small component of the light activated inward current. It was thus surprising that no other inward currents were evoked which could carry the light induced current. It is possible that there are no other voltage activated currents, and that the current is carried exclusively by channels opened via the second messenger pathway. Alternatively it may be that a component of the light response is carried by Ca^{2+} and/or Na^{+} ions but that these currents are only present in the outer segment and are not observed when recording from the cell body. Unfortunately all attempts to record from the outer segments were unsuccessful; quite possibly due to the microvillar nature of the outer segment. More detailed study of the inward current and the photocurrent using ionic substitution would be required to determine which ions carry these currents.

5.4.5 Summary

In this study the hypothesis that the whole cell patch clamp technique could be used to investigate the phototransduction cascade in the retina of *S. officinalis* was addressed. It

has been shown that it was possible to use this technique on the *S. officinalis* retinal preparation and that it was indeed useful and viable. It has been shown that there are at least two voltage-activated currents present in this *S. officinalis* photoreceptor; on inward sodium current and one outward potassium current. Additionally it has been possible to measure the light-activated currents and investigate the effect of pharmacological agents on this current.

CHAPTER 6: General Discussion

6.1 Discussion of original aims

The overall findings of this thesis will be discussed in the context of the original objectives outlined in the introduction and then an overall conclusion will be drawn based on this. However, at this stage is it worth mentioning how useful the slice preparation has proven to be. The development of this slice preparation, with the help of Dr David Becker (University College London, UK) has given the opportunity to greatly increase our knowledge of the phototransduction cascade in the cephalopod retina using a variety of techniques. A slice preparation has previously been used to make electrophysiological recordings from the retina of *Loligo pealii*. Hagins et al. (1962) recorded extracellular potentials, from 100 μm thick retinal slices, induced by stimulating the retina at various positions along the length of the photoreceptors. Later, Pinto & Brown (1977) used 500 μm thick slices of *L. pealii* retina to make intracellular recordings. A slice preparation has, however, never been used in *S. officinalis* and has also never been exploited for the investigation of the phototransduction cascade by pharmacological intervention.

6.1.1 To describe the second messenger signalling cascade by the use of extracellular application of pharmacological agents to the intact cells of the retinal preparation

This program of research has provided new information on the identity of the second messengers utilized by the *S. officinalis* retina for the process of phototransduction. By systematic testing of pharmacological inhibitors, activators and analogues of the various stages of the PI pathway, it has been possible to establish which branch of the pathway was of greatest importance for the excitation of these photoreceptors. The action of PLC has been shown to be essential for the production of a light response and the results support the hypothesis that the *S. officinalis* retina uses the IP_3 branch of the PI pathway to mediate phototransduction. The light induced response has been shown to be greatly diminished by the application of chemicals capable of either inhibiting release of calcium from internal

stores or reducing the amount of calcium in the stores. It was evident that calcium played a complex role in the process, appearing to have the ability to both enhance and inactivate the photoresponse. This came as no surprise as studies of other phototransduction cascades, in both vertebrates and invertebrates, have discovered that calcium plays a complex regulatory role in the cascade *D. melanogaster* (Ranganathan et al., 1995; Zuker, 1996), *L. polyphemus* (Nagy, 1991) and vertebrates (Jindrova, 1998).

It appears that release of calcium from internal stores is necessary for maximum response amplification but that it is not a requirement for production of a light response. It can therefore be said that calcium appears to regulate sensitivity. Also, by examining the effect of the removal of calcium from the external bathing solution, it was shown to play a role in response inactivation. It is suggested that there is a greater increase in $[Ca^{2+}]_i$ due to calcium influx than due to release from internal stores, and that at these higher concentrations calcium becomes inhibitory and inactivates the response through various negative feedback mechanisms. So removal of external calcium had the ability to increase response amplitude and duration, essentially enhancing the overall response to light. The same effect was also seen when blocking cell membrane calcium channels; hence the cells appear to be sensitized by reducing calcium influx. This may be due to a reduced inactivation effect due to the lower concentration of calcium entering from the extracellular solution or a secondary effect due to a reduction in internal calcium concentration. However, a lack of external calcium may also reduce the available calcium for replenishment of the internal stores. This is thought to be why there was eventually a reduction in response amplitude as was also seen with any other manipulations involving the internal stores.

There is one point which arose from the investigation which was more difficult to reconcile. Both lanthanum and 2-APB increased response latency as well as reducing

response amplitude. The one thing they both have in common it that they have the ability to block store-operated calcium channels (SOC). This has been shown by the specificity of lanthanum for Trp channels in *D. melanogaster* photoreceptors (Suss-Toby et al., 1991), the first SOC identified (Montell, 1997) and by 2-APB's ability to block SOCs in various preparations, e.g. *L. polyphemus* photoreceptors (Wang et al., 2002). The phenomenon of store operated calcium entry (SOCE), or capacitative calcium entry as it has also been called, is one which is far from being fully described however it has become evident that it is extremely widespread. SOCE is a calcium conductance which is activated by the depletion of intracellular calcium stores. The mechanism of activation is as yet unknown, however it is not thought to be due directly to calcium ions as the conductance can still be triggered in the presence of calcium chelators. The phenomenon of SOCE has been well reviewed in Berridge (1995) and Montell (1997).

There is evidence for a Trp-like channel in *Loligo forbesi* which has been named sTrp (Monk et al., 1996) suggesting the possibility of a SOC. However, the carboxyl terminal domain was truncated. This is where the interaction with depleted calcium stores is thought to occur therefore suggesting this may not be a SOC and may be more like the *D. melanogaster* non-specific cation channel, Trpl (Montell, 1997). There is a possible mechanism of regulation as this channel binds calmodulin in a calcium dependent manner. This information does not rule out the possibility of a SOC in the photoreceptor membrane of cephalopods, it just suggests that it is not sTrp. Why the block of store operated calcium channels would increase response latency is not clear at this stage. The simplest explanation would be that activation of SOCE initiates the response to light and then activates subsequent conductances via the calcium elevation; possibly sTrp. This could then be followed by opening of voltage-activated channels. However this is contradicted by the fact that removing extracellular calcium did not increase response latency and also that inhibiting the release of calcium from the internal stores had no effect. It was thought

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that this would stop SOCE and hence increase latency. Alternatively it could be that inhibiting these calcium channels would result in a reduced amount of stored calcium and therefore less calcium would be released by the action of IP_3. If the calcium was involved in excitation, it may then take more time for the threshold level to be reached necessary for activation of the terminal transmitter. However one would therefore also expect a similar effect when inhibiting IP_3R or when perfusing the preparation in CFASW, neither of which increased latency.

It is clear that this result cannot be explained without further investigation, but overall the transduction mechanism has been shown to be most closely related to that used by *L. polyphemus*. Nevertheless they do appear to differ in one key respect; *L. polyphemus* photoresponse was not abolished by PLC inhibition suggesting the possibility of another parallel pathway. It is still likely they use a very similar system, however further investigation would be necessary to clarify this suggestion.

It seems that release of calcium from internal stores was not essential for the production of a light response. This would suggest that there must be another means by which light can mediate the opening of membrane ion channels. As PLC was shown to be essential, it is possible that this alternative pathway is via DAG. There was no evidence of a role for the DAG branch of the PI pathway in this study; however it cannot be discounted completely at this stage. Nonetheless it is also possible that the calcium release *is* essential and that the chemicals used were not of a high enough concentration to block completely the release of Ca^{2+}, or that competitive inhibition occurred with light producing enough second messenger to overcome the inhibitors.

This study also found no evidence of a role for cGMP which was somewhat unexpected due to evidence of its involvement in both *L. polyphemus* (Johnson et al., 1986; Bacigalupo

et al., 1991) and squid phototransduction (Saibil, 1984). However, the potential role of cGMP remains controversial with numerous studies having found no evidence of a light induced change in cGMP concentration (e.g. Brown et al. (1992) and Seidou et al. (1993)).

6.1.2 To investigate the temporal resolving power and sensitivity of the retina and to determine how cell size influences these parameters

It has been possible to measure, for only the second time, the maximum critical flicker fusion frequency (mCFF) in a cephalopod species. mCFF was measured in two age groups, juveniles and young adults, based on the assumption that the length of their outer segments would differ enough to confer properties to the cells which would result in different mCFF values. The values *were* found to differ, and were also lower than the measurement made by Hamasaki (1968) in octopus. Juvenile animals had a mCFF value of 42 Hz and adult animals had a value of 24 Hz; both of which were far below that of 72 Hz measured by Hamasaki (1968). It is suggested that this difference may have arisen due to the differences in experimental methods. Hamasaki used an intact, anaesthetised animal whereas the results presented here were obtained from the isolated retina. It is suggested that by isolating the retina, and in doing so severing the optic nerve, there was a loss of some input from the brain which aided the temporal resolution of the retina. By severing the optic nerve the efferent input to the retina is lost. As well as any direct input, this could also affect lateral interactions between the cells, which may have a mechanism of improving temporal resolution, for instance via gap junctions. Yamamoto & Takasu (1984) have shown gap junctions to be present between visual cell inner segments as well as between visual cell axons and efferent fibres. Also, it has been shown that screening pigment loses its ability to migrate proximally when the optic nerve has been cut (Gleadall et al., 1993). The result of this would be that the incoming light may be filtered more than normal resulting in less light gaining entry to the cell for each particular light level. As CFF is dependent on stimulus light intensity (discussed in Chapter 3) this filtering effect

would reduce the light received and hence reduce CFF. One would however expect that this could be overcome by further increases in light intensity, which did not prove to be the case. It is unlikely that the light source was not bright enough to elicit any further increase in mCFF, as there was a degree of levelling out of the intensity-CFF plots. However in support of the findings presented in this study, Bullock & Budelmann (1991) measured mCFF of 20-30 Hz from the brain of an unanaesthetised, unrestrained cuttlefish, in response to visual stimuli.

The differences observed between the two age groups of animals were predicted to be related to the difference in the size of the photoreceptor cells. In fact, the temporal resolution and sensitivity were found to be inversely proportional to photoreceptor outer segment length. The increase in OS length could have a number of effects, any one of which could be responsible for the observed differences, or any combination. Additionally there may also be some other factors that change with age or cell growth which were not identified or examined in this study. Various potentially influential factors were discussed in the chapter discussion and are summarized below.

Changes which could occur due to an increase in outer segment length:

- Increased cell surface area resulting in increased capacitance and hence increased cell time constant

- Increased cell surface area may result in an increased number of ion channels which would affect cell membrane time constant

- Increased cell volume which could cause diffusion of the second messenger molecules to take longer

Other changes which may occur with age which are not related to OS length:

- Changes in types of ion channel populations or density of ion channels

- Changes in number of gap junctions

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Potential reasons for the differences between the two animal size groups have been put forward, however further investigations would be required to determine which hypothesis or combination of hypotheses is correct. The most plausible of these hypotheses will be discussed and an attempt will also be made to suggest why the animal group with greater temporal resolving power was also found to be more sensitive. This appeared to go against convention as sensitivity is often sacrificed for temporal resolution (Laughlin, 1990; Frank, 1999). The mCFF values measured in this study were similar to those found in humans (Hart, 1987), some deep sea crustacean (Frank, 1999, 2000) and fish (Mora-Ferrer & Gangluff, 2002), and are not particularly fast. As these animals are hunters and live off live prey it may be thought that they would require faster eyes, however they tend to stalk their prey, animals which themselves are not particularly fast moving such as crabs, shrimps and fish. As there is a minimal chase involved, if any, there is no need for their temporal resolution to be particularly fast. So it would appear that it has been matched, at least to some degree, to the lifestyle conditions of the animal. However this does not explain why juvenile animal's temporal resolution was almost twice as good as the adult's and that they were also more sensitive.

The reduced temporal resolution observed in adult animals can be explained by the effects that increased cell size could have on the cell membrane time constant. Adult sensitivity is thought to be less than juvenile's based on the hypothesis that they reached their optimum length in the younger animals and that the signal to noise ratio decreased with age due lengthening of the photoreceptors with no cellular mechanism to compensate. An alternative hypothesis may be that, with age, there is deterioration of a component of the transduction cascade which may affect not only sensitivity but the cell's temporal properties as well.

6.1.3 To investigate the fluctuations in intracellular calcium concentration induced by light and to ascertain the role of intracellular calcium in phototransduction

This study, unfortunately, was only able to fulfil one of its aims, namely to "load the retina slice preparation with fluorescent calcium indicator dye". It was concluded that although it was possible to load the dye into the photoreceptor cells, it did not retina its calcium sensitivity. It is suggested that the reason for this was too low an esterase activity within the cells, hence the indicator was not converted to its active state. It has not been possible to examine light induced intracellular calcium fluctuations or how these change following the application of pharmacological agents. However it has been established that the AM-ester type fluorescent calcium indicators are not compatible with this preparation. It is likely that cell impermeant type indicators, which are introduced to the cell interior directly, would be more successful. The whole cell preparation (Chapter 5), which will be discussed in the next section, is expected to be ideal for this approach. Unfortunately, time limitations prevented this being tested during the course of these studies.

6.1.4 To investigate the electrophysiological properties of individual photoreceptors and to further study the signalling cascade by direct intracellular application of pharmacological agents

This study has shown that successful whole cell patch clamp recording can be carried out in the retinal preparation of hatchling and pre-hatchling *S. officinalis*. This is the first study of its kind in a cuttlefish species and only the second in a cephalopod; the first of which was performed in the isolated photoreceptors of the squid *Loligo pealei* (Nasi & Gomez, 1992). Nasi & Gomez (1992) however did not use any pharmacological agents and were only able to measure an outward voltage-activated current. Therefore this was the first study in which the whole cell patch clamp technique was used to investigate the phototransduction cascade in a cephalopod species and the first recording of a voltage-activated inward current.

The results of the pharmacological investigations, which looked at the effects of introducing various agents directly into the photoreceptor cell via the patch pipette, have both reinforced the results of the tests with extracellularly applied pharmacological agents, and added to these. At the level of the individual cell, PLC has been shown to be essential. Block of the IP$_3$R has been shown to abolish the response evoked by dim flashes of light and to reduce the response to bright light. In addition, evidence has been presented showing that cGMP has the ability to enhance the response to light. This suggests that either, cGMP is capable of opening the light-activated membrane ion channels or, that cGMP has the ability to stimulate the cascade responsible for opening these ion channels. As previously discussed, there is evidence for the presence of a cGMP gated channel in the *S. officinalis* photoreceptor membrane (Huppertz, 1995) however more work is needed before the role of cGMP can be decided.

As well as recording light responses, two voltage-activated currents were also identified. An outward K$^+$ current and an inward Na$^+$ current was recorded. The inward current could be blocked without appreciably blocking the photocurrent showing that the current induced by light stimulation is not a TTX-sensitive sodium current; this however does not discount the possibility that the light-induced current is carried by sodium. It was suspected that this current may not have been detected due to that fact that recordings were taken from the photoreceptor cell bodies rather than from the phototransducing outer segment. It is possible, due to the cell morphology, that there was poor control of the currents in the outer segment and hence it had not been possible to evoke the currents responsible for the light response. Alternatively, the light-activated current does not necessarily have to have a voltage-activated component, however this would seem unlikely. The sodium current induced by depolarising voltage steps is most probably that responsible for the propagated action potential.

6.2 Conclusions

The conclusions drawn from the results presented in Chapters 2 and 5 were based on the effects of various pharmacological agents targeted at the postulated transduction pathway. PLC is thought to be essential, however the action of IP_3 is thought to be necessary for maximum amplification and further investigation would be needed to determine if its action is also essential for production of the light response. However, at present there is still the possibility of another pathway, working alongside IP_3, capable of opening membrane ion channels. As PLC is essential, it would be most likely that this alternative pathway is via DAG, although this study has not been able to produce any evidence of this. Finally evidence has been presented in support of the hypothesis that cGMP may gate a light-activated membrane ion channel in the photoreceptors of *S. officinalis*, as has been suggested in *L. polyphemus* (Johnson & O'Day, 1995). These findings show that phototransduction in *S. officinalis* retina uses the PI pathway and that IP_3 is particularly important in producing a fully amplified response. The importance of IP_3 and hence calcium release, make calcium regulation of cGMP metabolism a very attractive possibility.

By measuring the temporal resolution of the retina it has been shown that the photoreceptor cells are not particularly fast in producing a response to light stimulation. It is interesting to note that the very fast eyes of the flies use an alternative intracellular signaling cascade and it would be interesting to determine whether this is a contributing factor in the speed of their response. Is it that the IP_3 route of excitation utilized by *L. polyphemus* and *S. officinalis* is a slower process, hence reducing the cell's temporal resolution. It has also been shown that the size of the photoreceptor cells was inversely related to the temporal resolution and sensitivity; with cells becoming slower and less sensitive as they became longer. This increase in cell size would slow the transduction cascade, as the greater cell

volume would result in diffusion taking longer. However it is also possible that the age of the animals is more relevant than the size of its photoreceptors. It may be that there is deterioration in a vital component of the signalling cascade with age therefore reducing temporal resolution and sensitivity.

6.3 Future Work

Preliminary investigations into imaging the $[Ca^{2+}]_i$ changes in the *S. officinalis* photoreceptors have been carried out, the results of which have been presented previously. Membrane permeable AM ester forms of fluorescent calcium indicator dyes were loaded into the cells of slice preparations. There did not appear to be a problem with dye loading, however no calcium signal was detected when the preparation was stimulated by light. It was deduced that a likely explanation was that the esterase activity of the cells was not great enough to cleave a sufficient amount of dye to detect light induced calcium changes. This was a particularly likely finding due to the fact that Nasi & Gomez (personal communication) found exactly the same problem with photoreceptors from other marine mollusks; a problem which was eliminated by switching to intracellular perfusion of photoreceptors with the salt form of the indicator dye. Therefore it is extremely likely that successful measurement of intracellular calcium changes could be carried out by introducing non-membrane permeable calcium indicator dyes to the cell interior via the patch pipette. This would allow the measurement of the timing of calcium elevations giving an indication of whether it was possible for calcium to be responsible for photoreceptor excitation, for example. It would also be possible to determine how changes in extracellular calcium concentrations and manipulation of stored calcium, affects intracellular calcium levels and the photoresponse. This would hence result in a much clearer understanding of the role of calcium.

In addition to this, it would be interesting to carry out a detailed investigation of the membrane currents in order to clearly establish which ions carry the light-induced current and indeed which types of voltage-activated current occur across the photoreceptor membrane. It would be particularly useful to record from the phototransducing outer segment where all of the light-activated currents would be expected to be found. This proved to be particularly challenging when attempted in the slice preparation.

With the development of the slice preparation for whole cell patch clamp recording it would be useful to use this to carry out further pharmacological investigation combined with varying stimulus protocols. It would be important to look at responses to longer flash stimuli to determine if the same second messengers are responsible or if there is evidence for another pathway. It would also be interesting to design and carry out experiments to attempt to test the hypothesis that the metabolism of cGMP is regulated by calcium as has been found in *L. polyphemus* (Johnson & O'Day, 1995).

Finally it would be of great interest to carry out a more detailed examination of mCFF and how it relates to age and cell outer segment length. This could include examination of more light intensities and different species of cephalopod. It would then be possible to determine if there is a strong correlation between outer segment length and mCFF over the whole range of animal sizes from pre-hatchling to adult. It would also be of interest to try to perform experiments in an intact animal to attempt to reconcile the difference between mine and Hamasaki's results.

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