AN ABSTRACT OF THE DISSERTATION OF

R. Ryan Preston for the degree of Doctor of Philosophy in Biochemistry and Biophysics presented on May 2, 2002. Title: Incorporation of Chromatophores into Multi-Cellular Biosensors.

Abstract approved:

Redacted for Privacy

Philip N. McFadden

Methodologies that detect biologically active substances have important potential applications for medical diagnostics, drug discovery, and chemical and biological anti-terrorism efforts. The wide spectrum of potential analytes that induce a physiological response dictates that novel techniques be developed to more rapidly screen and characterize agents that are more economical and have greater sensitivity than current practices. The research presented in this dissertation describes the development of a biosensor methodology that utilizes optical changes in naturally pigmented chromatophore cells from fish to detect and measure an array of biochemicals and protein toxins. The chromatophores used in this biosensor were harvested from teleost fish sources and the observed patterns of pigment aggregation and dispersion in response to added chemical modulators were

used as a reporter mechanism. Differential responses between chromatophore subtypes were utilized as simple cellular sensors for the detection of cholera toxin and in the study of the calcium signaling requirements in these cells.

A multi-cellular biosensor was developed that couples pigmentation changes in erythrophores from the teleost *Betta splendens* with mammalian nerve cell secretory activity. An apparatus was developed that placed PC12 cells, a neuroendocrine cell line, and erythrophores in adjacent chambers connected by a fluid network that allowed erythrophores to be exposed to effluent from PC12 cells; neurotransmitters secreted from PC12 cells induced pigment aggregation in erythrophores. By analyzing the extent of this erythrophore response, this method was capable of detecting the occurrence of substances that altered neurotransmitter secretion levels. A demonstration of this biosensor is presented that detected the inhibition of neurosecretory activity caused by the pathogenic bacterial toxin botulinum, the causative agent of human botulism.

Incorporation of Chromatophores into Multi-Cellular Biosensors

by

R. Ryan Preston

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented on May 2, 2002
Commencement June 2002

Doctor of Philosophy dissertation of R. Ryan Preston presented on May 2, 2002.

APPROVED:

Redacted for Privacy

Major Professor, Representing Biochemistry and Biophysics

Redacted for Privacy

Head of the Department of Biochemistry and Biophysics

Redacted for Privacy

Dean of Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Redacted for Privacy

R. Ryan Preston, Author

ACKNOWLEDGMENTS

I would first like to acknowledge the contributions made by my research advisor, Phil McFadden, who provided the resources necessary to perform this work and for ensuring that I became an independent scientist. Phil has an uncanny ability to discern the most salient details, a skill that was helpful during the editing process. Many thanks go to Drew Sellers, my lab mate for much of the time I've been at Oregon State University, for helpful scientific discussions and general advice on navigating graduate school. Shing Ho provided me with judicious advice and mentorship when it was needed most. Janine Trempy was exceedingly helpful in the approval of the BSL '2' laboratory that was necessary for me to complete an integral portion of this dissertation. Thanks also go out to Sam Bradford for cell culture advice, Kevin Ahern and Jeff Vargason for computing advice, Rosalyn Upson for initial guidance when I started on this project, and Barbara Hanson, Marilyn Walsh, and Anterra for assistance in all things bureaucratic. The most important acknowledgments go to my family, primarily my parents, Steve and Cynthea, and my wife, Åsa, whose support was absolutely imperative. My apologies to the many others who I haven't mentioned that contributed in numerous important ways.

CONTRIBUTION OF AUTHORS

Philip McFadden contributed to the design and analysis of each manuscript. Janine Trempy was integral in the development of the Biosafety Level (BSL) '2' laboratory that was necessary to perform the research for Chapter 5.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

DEDICATION

I dedicate this body of work to my wife, Åsa, who has given me boundless support and encouragement, the two things I now realize were most vital for the successful completion of this project.

Incorporation of Chromatophores Into Multi-Cellular Biosensors

Chapter 1

Introduction

Biosensors are devices that convert a biological activity induced by such analytes as environmental pollutants, pathogens, or metabolic byproducts into a practical measurement that represents the original agent. These devices have wide ranging applications including human disease diagnostics, environmental monitoring, and pathogen recognition. Conventional biosensor methodologies utilize antibody recognition, nucleic acid binding or amplification, enzymatic activity, or other chemical-based assays. These biological events are then translated into an output signal, typically using physical or chemical properties such as electrochemical, optical, or piezoelectric signals. For example, the widely used glucose biosensor employs an electrochemical reaction that accompanies the oxidation of glucose by the enzyme glucose oxidase to quantify blood sugar levels in diabetics (Heller 1999). However, circumstances exist where biosensors that utilize such conventional methods lack important requirements such as the ability to simultaneously detect large numbers of analytes, especially ones of unknown origin. Such is the case with environmental pollution, pathogenic screening, and

biological warfare where the specific properties of a threat are not known and sensor techniques based on specificity are insufficient. To remedy this shortcoming, recent efforts have been made to utilize cultured biological cells to characterize certain classes of analytes because the physiological responses of live cells can be perturbed by a multitude of externally applied agents. Several interesting examples exist including the excitability of neuronal networks of cells on microelectrode arrays (Gross *et al.* 1995), the analysis of gene expression patterns with cDNA microarrays following cellular exposure to an environmental threat (Schena *et al.* 1995), and the use of stem cells as a renewable source of mammalian cell types for incorporation into sensor methodologies (Parchment 1998)(Scholz *et al.* 1999). The studies that comprise this dissertation describe the use of chromatophores in the development of novel multi-cellular biosensors to detect important bioactive substances.

Chromatophores (reviewed in Fujii 2000; Fujii 1993) are terminally differentiated, pigmented cell types that originate from the neural crest of several classes of lower animals including teleosts (fish), amphibians, crustaceans, and mollusks. These cells are responsible for the unique color adaptation and camouflage capability of these animals. Several subtypes of chromatophores exist that can be generally divided into two groups: light-absorbing and light-reflecting. Light-absorbing chromatophores (melanophores, erythrophores, and xanthophores) have absorptive pigment-containing organelles dispersed throughout their cytoplasm that are motile in a process that is facilitated by motor protein movement

along the intracellular cytoskeletal network resulting in darkening (pigment dispersion) or blanching (pigment aggregation) of the cells and the animals (Figure 1.1). Light-reflecting chromatophores (iridophores and leucophores) have stacks of iridescent platelets encapsulated within organelles that reflect light of differing wavelength depending on their specific orientation. The optical properties (*e.g.* pigment motility) of chromatophores are sensitive to a range of pharmacological agents that affect intracellular second messenger concentrations, protein phosphorylation states, cytoskeletal integrity, and numerous receptor-mediated pathways which has made these cells a popular model system to study regulated intracellular transport mechanisms (Bagnara 1991). Chromatophores have also been incorporated into sensor applications for the detection of a variety of biologically active agents (Danosky and McFadden 1997); (Elwing 1990); (Lerner 1988); (Chaplen *et al.* 2002); (Preston and McFadden 2001) because the optical qualities of chromatophores can be analyzed and the response extrapolated back to the intensity of the stimulus.

There are unique requirements that need to be addressed in order to successfully incorporate live cells, such as chromatophores, into a practical application such as a biosensor. These include culture conditions that are conducive to cellular viability and durability, an observable physiological response, sensitivity to a variety of stimuli, and tissue that is readily available and economically feasible. In Chapter 2 of this dissertation, chromatophores were

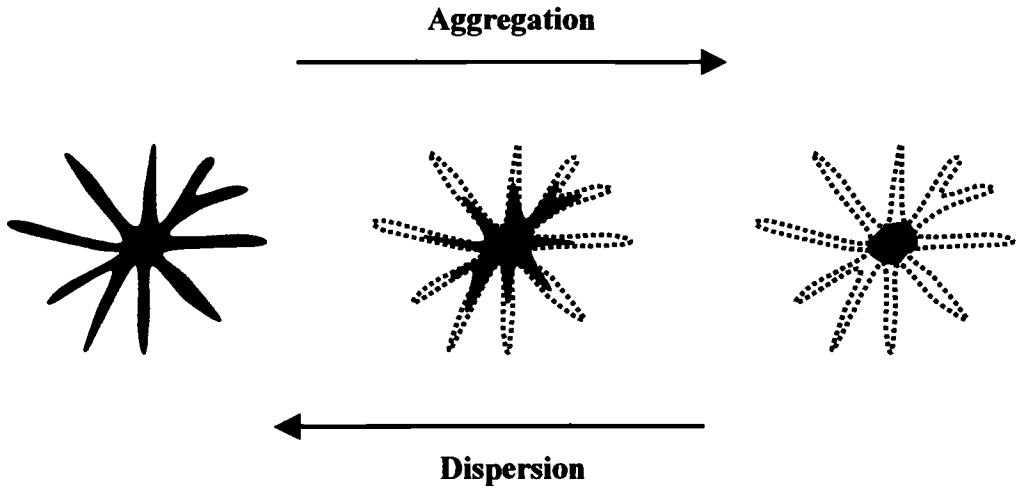


Figure 1.1: Schematic of pigment aggregation and dispersion in light-absorbing chromatophores. The pigments that impart the optical characteristics to light-absorbing chromatophores are melanin in melanophores and carotenoid/pteridine in erythrophores and xanthophores. These pigments are enclosed in distinct membrane-enclosed organelles within the cytoplasm of these cells and translocate along microtubules in a regulated manner in a process mediated by motor protein activity. The dispersion of chromatophore pigment results in a darkening appearance and the aggregation of pigment results in a lightening, or blanching, appearance.

screened from different fish species with these factors in mind to determine a suitable source of cells for use in biosensor methodologies. In this study, multiple subtypes of chromatophores were observed both in intact tissue and in primary cell culture on an artificial substrate. Further, cholera toxin was found to elicit a distinct, differential response between melanophores and erythrophores, suggesting that differences between chromatophore subtypes within a species can be used as a detector of this pathogenic toxin. From the screening studies in Chapter 2 it was determined that primary cell cultures from the teleost *Betta splendens* were the best choice for incorporation into a cellular biosensor. Differences in the signaling properties leading to pigment motility in melanophores and erythrophores from this species were further characterized in Chapter 3. *B. splendens* erythrophores were sensitive to intracellular Ca^{2+} levels, in contrast to melanophores from this species, which were largely unaffected by Ca^{2+} fluctuations. This variation in the response between chromatophore subtypes allows for further sensing capability, namely of Ca^{2+} modulating agents.

The application of chromatophores in the development of biosensor technologies can be expanded to include other cell types. This allows the sensitivity of other cells that do not have an easily observable output response to be monitored by coupling the activity of these alternative cells to regulated chromatophore pigment motility. The development of a specific application is described in Chapter 4 that used the secretion of neurotransmitters from mammalian neuroendocrine cells; the transformed cell line PC12 was chosen.

PC12 cells are derived from chromaffin cells and are characterized as synthesizing and secreting catecholamines in a regulated fashion (Greene and Tischler 1982). An apparatus was constructed that connected separate populations of *B. splendens* erythrophores and PC12 cells with a network of microfluidics that allowed secreted catecholamines from PC12 cells to bathe erythrophores. The extent of pigment aggregation in erythrophores was monitored microscopically and analyzed in a way that allowed for the amount of secreted catecholamines from PC12 cells to be quantified. Further, elevated catecholamine release from PC12 cells exposed to secretagogues (inducers of neurosecretion) was also observed with this method.

In Chapter 5, the PC12-erythrophore cellular biosensor was applied to the detection of botulinum neurotoxins. Botulinum toxins are produced by pathogenic strains of *Clostridium botulinum* and lead, in humans, to the inhibition of acetylcholine release at the neuromuscular junction (Hatheway 1988), resulting in flaccid paralysis that can persist for months and is fatal if untreated. These toxins are commonly present in spoiled food and have also been identified as potential biological warfare threats (Cohen and Marshall 2001). Therefore the development of novel and improved methods of detection are being sought. Using a modified version of the coupled-cell microfluidic device described in Chapter 4, the extent of erythrophore aggregation was used to detect botulinum toxins. These observations occurred after prior incubation of PC12 cells with purified toxin samples; prior incubation was necessary for the toxin to affect its intracellular target within PC12 cells. The response time, specificity, and sensitivity of this methodology were

characterized and compared with the merits of alternative detection methods. This technique demonstrates the use of cellular activity to both detect the pathology of these toxins and generate a measurable output.

The use of chromatophores in multi-cellular biosensor applications demonstrates the capability of these cells to detect a wide range of biologically active substances.

Chapter 2

Screening of Chromatophores from Teleost Sources for Incorporation Into Multi-Cellular Biosensor Methodologies

R. Ryan Preston and Philip N. McFadden

Formatted for Submission

2.1 Summary

Chromatophores are pigmented cells that are responsible for the coloration of numerous animal species and are a common model for the study of intracellular transport mechanisms. The pigments present within these cells rearrange in response to certain classes of pharmacological stimuli and pathogen or toxin exposure, a fact that has been previously exploited in developing cellular biosensors that utilize these cells. This study describes the screening of numerous teleost species as possible sources for chromatophores to be used as biosensors to detect biologically active agents. Characteristics such as chromatophore subtypes present, pigment responsiveness to pharmacological agents, cellular durability, and cost were considered. Scales removed from *Hemichromis bimaculatus* were found to be a reliable and self-contained source of numerous chromatophore subtypes that were used to characterize the presence of cholera toxin. Primary cell cultures from *Betta splendens* were considered to be the best choice for larger-scale biosensor development because of a combination of factors that include price, longevity in culture, responsiveness, and variety of available chromatophore subtypes including melanophores, erythrophores, and iridophores.

2.2 Introduction

Chromatophores are the richly colored cells in the dermis of vividly colored animals including fish, reptiles, and amphibians. These cells are responsible for an animal's ability to change its appearance in response to predator threats or breeding behaviors, for example. Toward this end, populations of these cells are capable of imparting a great variety of visible displays that are achieved due to the interplay of a number of factors, including the specific combination of chromatophore subtypes, their number, spatial arrangement, and the sex and stage of development of a particular species. On top of such fixed patterns, chromatophore behavior is then under the direct control of signaling from the endocrine and nervous systems. The rapid changing coloration of these cells has been utilized very effectively in cell biology studies of intracellular transport mechanisms because signaling principles leading to a cellular response are easily and unequivocally observed (Telzer and Haimo 2001). Chromatophore pigment motility has also been applied previously as a biosensor for the detection of catecholamines (Elwing *et al.* 1990), opioids (Karlsson *et al.* 2002), pertussis toxin (Karlsson *et al.* 1991), and odorants that affect cellular cAMP levels (Lerner *et al.* 1988); (Lundstrom and Svensson 1998). In a related technology, Xenopus chromatophores have been successfully induced to express transfected green fluorecesnt protein (GFP) and CD4 cell surface receptor suggesting these cells for the use of recombinant assays for the study of receptor-ligand interactions (Gatlin *et al.* 2001).

There are numerous subtypes of chromatophores with unique pigmentation characteristics (reviewed in Fujii 1993b). 'Light absorbing' chromatophores include melanophores, erythrophores, and xanthophores. These cells have a cytoskeletal structure that underlies their morphology, with numerous dendritic projections extending radially from a central cell body (Stearns 1984). These chromatophores contain up to thousands of pigment-containing organelles (melanin for melanophores and carotenoid/pteridine for erythrophores and xanthophores). Movement of these organelles by the motor proteins kinesin, dynein, and myosin are under the control of numerous signaling mechanisms (Cole and Lippincott-Schwartz 1995); (Thaler and Haimo 1996) that are initiated by external exposure to a variety of endogenous agents, including neurotransmitters and endocrine agents. The aggregation of pigmented organelles toward the cell body results in a blanching effect whereas dispersion results in a darker, more intense color. Information about intracellular transport mechanisms leading to aggregation and dispersion can be observed in at least two ways. A 'direct effect' indicates that exposure to an agent affects a pathway or mechanism resulting in observable pigment motility. An 'indirect effect' occurs when an agent impairs or otherwise modulates the effect of another agent. For example, blocking α_2-adrenergic receptors by yohimbine in melanophores prevents pigment aggregation induced by norepinephrine treatment, an agonist of the same receptor (Svensson *et al.* 1997). 'Light reflecting' chromatophores include iridophores and leucophores. Organelles within these cells contain crystal platelets, commonly composed of guanine and

other purines, which reflect (iridophores) or scatter (leucophores) light of a certain wavelength, resulting in an iridescent appearance.

In this study, we have examined the chromatophores from numerous freshwater fish sources for possible use as a cellular biosensor. It is envisioned that such a biosensor will utilize the physiological changes in appearance in chromatophores as an indicator for the presence of neurotransmitters, hormones, toxins, and other biologically active agents. The initial criteria for selecting fish were based on the visual characteristics of the skin and scales: fish were chosen that appeared to contain large quantities of chromatophores and a variety of chromatophore types. Chromatophore utility was studied both as primary tissue isolates and as primary dissociated cell cultures. For the purpose of initial screening, known modulators of chromatophore appearance were used. The chromatophore responses from various fish species were of major interest to us since we assumed that certain natural tissues and cells may be more suited for biosensor applications than others. The several chromatophore types within a fish species were compared to investigate the possibility that certain agents and toxins may act selectively on different chromatophore types. Other practical merits of melanophores, erythrophores, and iridophores (the most prevalent chromatophore types in the specimens chosen) were also compared including production and manufacturing issues and the costs associated with the incorporation of these cells into a biosensor.

2.3 Materials and Methods

The tropical fish discussed in this report are commonly available in the hobbyist trade and were obtained from local pet stores. The fish were maintained in freshwater aquariums at 27° C using hobbyist techniques, including under gravel and external water filtration and periodic feeding with commercially available prepared diets (Burgess and Bailey 2000).

Chromatophores were examined on intact fish scales or plated on polystyrene multi-well dishes after isolation from fin tissue. To obtain scales, fish were first anesthetized with 1 mM MS-222 (tricaine methanesulfonate) in 2 liters of water for approximately 5 minutes. Individual scales were plucked with fine-tipped forceps and washed for 2 hours with multiple solution changes in 'phosphate buffered saline' (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.6 mM KH_2PO_4, pH 7.4) containing 1 mM NaEDTA. Scales were then transferred to 'physiological saline solution' (PSS: 128 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.8 mM $MgCl_2$, 5.6 mM glucose, 10 mM Tris/HCl, pH 7.2) and stored at 20° C. Experiments were performed 2-10 days after plucking.

Isolation of chromatophores from individual fish was performed according to the method described previously (Preston and McFadden 2001; chapter 4 of this dissertation).

Chemical treatment of chromatophores was performed after placing a scale into a 35 mm dish containing PSS. Alternatively, solution in multi-well plates was

exchange with PSS. Agents to be screened (all were obtained from Sigma-Aldrich Co.) were introduced directly to the dish or plate to give the final concentration listed. Fish scales were observed on a Wild dissecting microscope (Wild-Heerbrugg, Gais, Switzerland). For cultures in multi-well plates, a Zeiss IM-35 inverted microscope (Carl Zeiss, Inc., Oberkochen, Germany) was used. All images were captured using a Panasonic GP-US502 color 3-CCD camera (Secaucus, NJ, USA) by a Flashpoint 3D capture card (Integral Technologies, Indianapolis, IN, USA) attached to a Pentium III computer.

2.4 Results

Teleost fish species were chosen as sources of chromatophores over other potential animal sources that contain chromatophores because of the variety of color characteristics present in fish and their ease of maintenance in freshwater aquariums. Specific fish species were generally selected for closer examination based on their visual impact: spectacular and colorful fish had similarly impressive chromatophore populations. For the purposes of developing a consistent and repeatable biosensor, we screened the specimens described to hopefully single out one optimum source for chromatophores. This optimum was based on several characteristics including specimen cost, chromatophore types present and the reactivity of each to numerous classes of chemicals, and longevity of the cells

either on scales or in culture (Table 2.1). Chromatophores from each of the fish chosen were harvested by plucking individual scales and/or by performing a primary isolation of cells from the fin tissue and growing the cells on an artificial substrate. In both cases, chromatophores were isolated at least two days before experiments could proceed because preparatory procedures used to isolate the cells had an aggregating effect on pigmentation of dendritic chromatophores that was overcome with time.

Preliminary examination of melanophores, erythrophores, and iridophores from each specimen in Table 2.1 involved exposure to 100nM norepinephrine and 1μM forskolin, signaling modulators that are known inducers of chromatophore pigment motility (Fujii 2000). Norepinephrine is an agonist of the α_1 and α_2 adrenergic signaling pathway (Exton 1985) and leads to the aggregation of pigment granules into the cell body of dendritic chromatophores and the reflection of longer wavelength light in iridophores (green/yellow). Forskolin is an agonist of the cAMP-producing enzyme adenelyl cyclase and has the converse effect on chromatophores: dispersion of pigment granules and the reflection of shorter wavelength light (blue/purple). Dendritic chromatophores in culture are generally in an intermediate state between aggregated and dispersed, making them sensitive to both aggregating and dispersing agents. Iridophore color change was judged in a similar manner. Chromatophores from each of the specimens listed in Table 2.1 responded in varying degrees to norepinephrine and forskolin treatment; specimens

Table 2.1: Teleost species and chromatophore characteristics of the specimens screened in this study

Species	Chromatophores Present[a] / Reactivity[b] *Melan*	*Eryth*	*Irid*	Market Price[c]	Primary Culture Longevity[d]	Observations
P. scalare (Angelfish)	✓ ++	✓ ++	✓ +	$$	< 2 weeks	Cells difficult to isolate; few cells per fish
B. splendens (Betta)	✓ +++	✓ +++	✓ +++	$	~ 1 month	Many cells per fish; good variety of chromatophores
S. discus (Discus)	✓ ++		✓ +	$$$	~ 1 week	Expensive; fish difficult to maintain
C.octofasciatum (Jack Dempsey)	✓ +++		✓ ++	$$	~ 2 weeks	many iridophores
H. bimaculatus (Jewel Cichlid)	✓ +++	✓ +++	✓ +++	$$	~ 3 weeks	Few cells per fish; excellent scales
C. characidae (Neon Tetra)	✓ +++	✓ ++	✓ ++	$	~ 2 weeks	Cells difficult to isolate
T. nilotica (Nile Tilapia)	✓ +++			$	~ 3 weeks	Few cells per fish; fish are easy to maintain
B. conchonius (Rosy Barb)		✓ ++	✓ +	$$	< 2 weeks	Cells difficult to isolate; few cells per fish
P. socolofi (Socolofi)	✓ +++		✓ ++	$$	< 2 weeks	Fish difficult to maintain

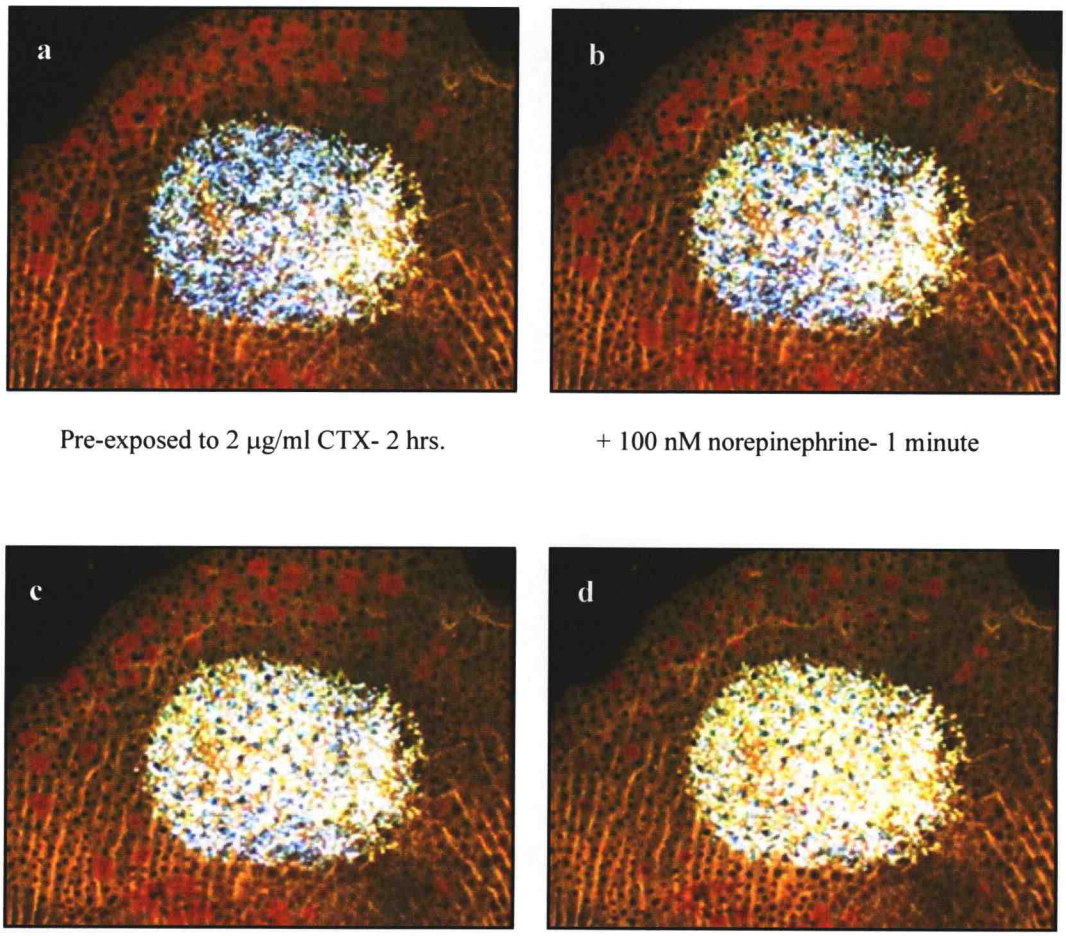
[a]: Chromatophore types present (denoted with ✓) on the skin and/or scales of each fish species (melan-- melanophores, eryth-- erythrophores; irid-- iridophores).
[b]: Overall responsiveness to an array of known stimulators of chromatophore pigment motility (+-- poor, ++-- average, +++-- good).
[c]: $-- less than $5; $$-- $5-$20; $$$-- greater than $20 per fish.
[d]: Length of time that chromatophores are viable (*e.g.*- active pigment motility) after isolation from fish.

that contained chromatophores of each subtype were ranked based on the quality of the pigment response. Among the species tested, *Betta splendens* and *Hemichromis bimaculatus* each contained highly responsive chromatophores of each subtype. Further, these cells were present and viable both in tissue isolates and in primary cultures. *Tilapia nilotica* contain very large, responsive melanophores that are commonly used to study pigment transport mechanisms within individual cells. However, melanophores from *T. nilotica* and melanophores and erythrophores from *H. bimaculatus* were present in low density in culture, which made it difficult to simultaneously observe larger numbers of these cells. Cost and chromatophore longevity after isolation from the fish were also important factors in judging the potential of chromatophores as biosensors (Table 2.1). Longevity was measured as the length of time after isolation that cells were still capable of transporting pigment in response to norepinephrine/forskolin and is important in determining the 'shelf life' of a biosensor application.

The simultaneous observation of melanophores and erythrophores was used to detect cholera toxin, a bacterial protein toxin produced by *Vibrio cholerae*, using an indirect assay method (Figure 2.1A). Prior incubation of a *H. bimaculatus* scale, which contained populations of melanophores, erythrophores, and iridophores, with 2μg/ml purified cholera toxin had no visible effect on these cells. Panel 'a' in Figure 2.1A shows chromatophores after a 2-hour exposure to the toxin. All three chromatophore types present in this panel looked identical to untreated cells (data

Figure 2.1: Differential response between melanophores and erythrophores after exposure to cholera toxin. **A).** Chromatophores on a *Hemichromis bimaculatus* scale were previously incubated with 2μg/ml purified cholera toxin (CTX) before exposure to 100nM norepinephrine. Panel 'a': after toxin exposure, before norepinephrine (at ~5x magnification). Panels 'b', 'c', and 'd': 1, 2, and 4 minutes after norepinephrine, respectively. Erythrophores (red), which normally aggregate at the same rate as melanophores (black) after norepinephrine exposure, display impaired ability to aggregate normally after CTX exposure. **B).** Similar experiment on a *H. bimaculatus* scale that was imaged at a greater magnification (16x). Upper panel: after 2μg/ml CTX. Lower panels: 1 and 4 minutes after 100nM norepinephrine.

Figure 2.1(A)



Pre-exposed to 2 μg/ml CTX- 2 hrs.

+ 100 nM norepinephrine- 1 minute

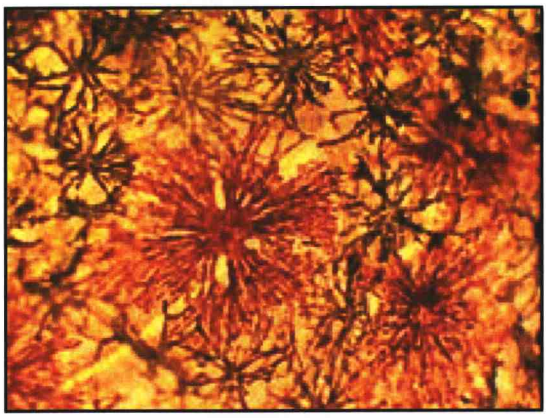
+ 100 nM norepinephrine- 2 minutes

+ 100 nM norepinephrine- 4 minutes

Bar = 200 μm

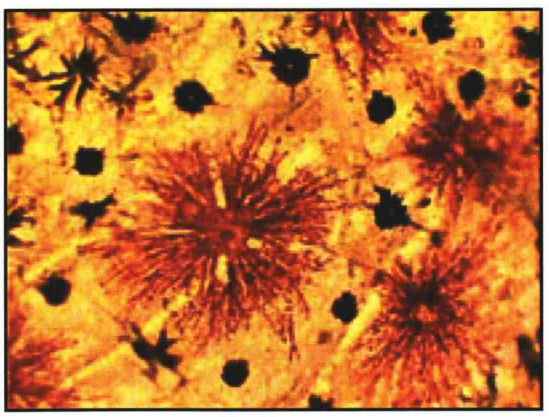
Figure 2.1(B)

Pre-exposure to
2μg/ml CTX- 2 hrs.



+ 100 nM norepinephrine-
1 minute

+ 100 nM norepinephrine-
4 minutes



Bar = 20 μm

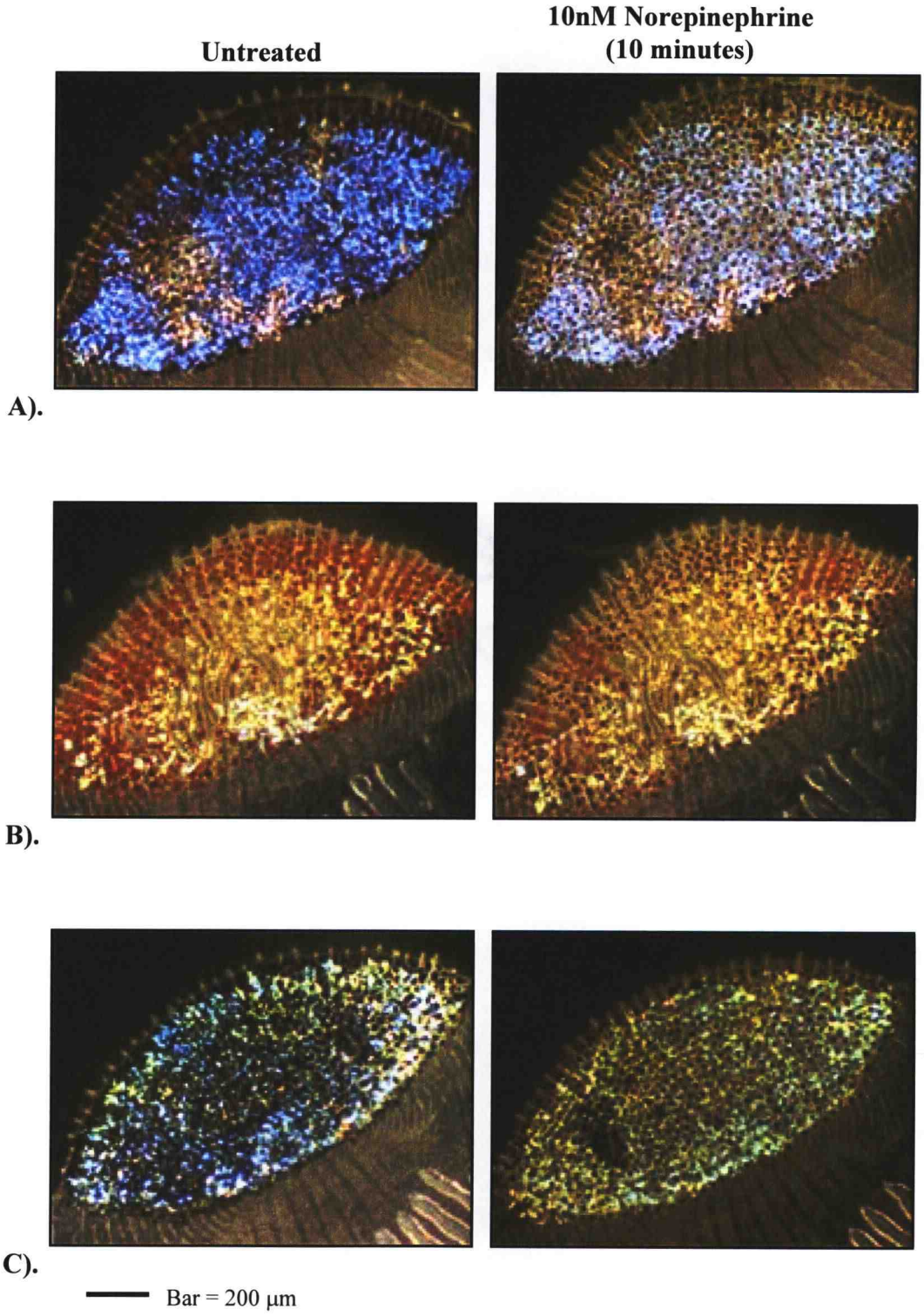
not shown), which indicate the lack of a direct effect by cholera toxin. Currently, the most commonly used technique to judge the morphology of dendritic chromatophore types is the Melanophore Index (M.I., Andersson *et al.* 1984; Hogben and Slome 1931). Using this method, a fully dispersed cell is rated '5' while a completely aggregated cell is rated '1'. Intermediate conditions are M.I.='2', '3', and '4'. Panels 'b' and 'c' show this scale at 1 minute and 2 minutes, respectively, after exposure to 100nM norepinephrine. When observed in real time, untreated melanophore and erythrophore pigment aggregated at approximately the same rate in response to norepinephrine treatment. On the cholera toxin-treated scale, however, the aggregation of erythrophore pigment was significantly slower than that of melanophores. By 4 minutes after exposure to norepinephrine (Panel 'd'), all melanophore pigment was completely aggregated (M.I.='1') while erythrophores were only partially aggregated (M.I.='3'). The complete aggregation of erythrophores after exposure with cholera toxin did not occur until greater than 20 minutes. The iridophores on this scale shifted from blue (Panel 'a') to yellow (Panel 'd'), as is typical following norepinephrine treatment; this response was unaltered by cholera toxin. The indirect effect of cholera toxin on erythrophores is seen even more clearly on a *H. bimaculatus* scale imaged at higher magnification in Figure 2.1B. The experimental treatment of this scale was identical to the scale in Figure 2.1A. After 4 minutes of norepinephrine exposure, melanophores on this scale aggregated almost completely (M.I.='1' or '2'). A clearly impaired response is seen in erythrophores, however, which only aggregated to M.I.= '4'.

B. splendens chromatophore responses to 10nM norepinephrine from primary tissue isolates on fish scales are seen if Figure 2.2. The scale in Figure 2.2A was removed from a blue fish and melanophores, erythrophores and iridophores are present. The pigment was dispersed within melanophores (black) and erythrophores (red) on the untreated scale; pigment aggregation in individual cells was evident on the norepinephrine treated scale, making the cells easier to distinguish from one another. The scale in Figure 2.2B was obtained from a red specimen and contains only erythrophores and iridophores. While erythrophore pigment aggregation occurred after norepinephrine treatment, there was no visually discernable change in the iridophores on this scale. The scale in Figure 2.2C came from a green fish and contained primarily melanophores and iridophores, with very few erythrophores. A color change from blue to green can be seen within many of the iridophores on this scale.

Chromatophore morphology and pigment responses to both norepinephrine and forskolin were also observed in cells that were isolated from the tissue of fish and reconstituted in primary cell culture. Isolated chromatophores from *B. splendens* plated on a polystyrene substrate are seen before and after exposure to 10nM norepinephrine in Figure 2.3. Within this field of view, melanophores, erythrophores, and iridophores are each indicated. The presence of norepinephrine for 3 minutes induced an obvious aggregation in both melanophores and erythrophores but little, if any, visual changes to iridophores. Although iridophores

Figure 2.2: Chromatophores on fish scales from *Betta splendens*. Scales were removed from specimens 3 days prior to experiment and stored in PSS. Each specimen was selected based on color: **A).** blue fish **B).** red fish **C).** green fish. Left panel shows untreated scale, right panel shows the same scale after exposure to 10nM norepinephrine for 10 minutes. Light-absorbing chromatophores (*e.g.* melanophores and erythrophores) are evident in the background and aggregate in response to norepinephrine exposure. Light-reflecting chromatophores (*e.g.* iridophores) are the bright, iridescent cells in the forefront and may reflect longer wavelength light in response to norepinephrine exposure. This is most clearly seen on the scale from the green fish, where individual iridophores changed from blue to green/yellow.

Figure 2.2



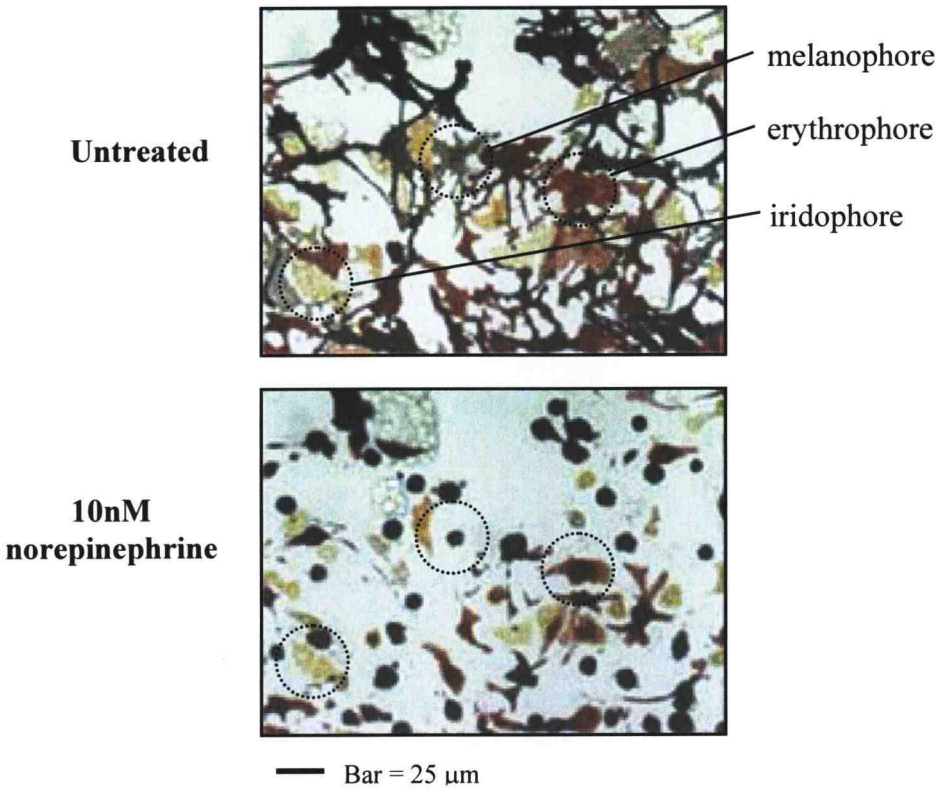


Figure 2.3: Primary chromatophore culture from *Betta Splendens.* Upper panel shows untreated cells in PSS 5 days after isolation from the specimen. Lower panel shows the same cells after exposure to 10nM norepinephrine for 3 minutes.

do persist in culture for many days, we have not reliably observed any optical changes in response to norepinephrine. This was in contrast to iridophores embedded in skin or scale tissue that changed color noticeably. The dendritic chromatophore types (*e.g.* melanophores and erythrophores), however, offer a measurable means of morphological change in response to norepinephrine or other pigment aggregating agents.

With cultured cells, it was possible to enrich for specific chromatophore types based on the physical characteristics of the specimen or isolation techniques performed during the harvest procedure (D. Sellers, personal communication). This enrichment is demonstrated in Figure 2.4, a culture comprised almost exclusively of erythrophores from a bright red *B. splendens* specimen. It is also evident from Figure 2.4 that very densely populated cell cultures can be achieved with *B. splendens* chromatophores because of their small size and high cell number per specimen.

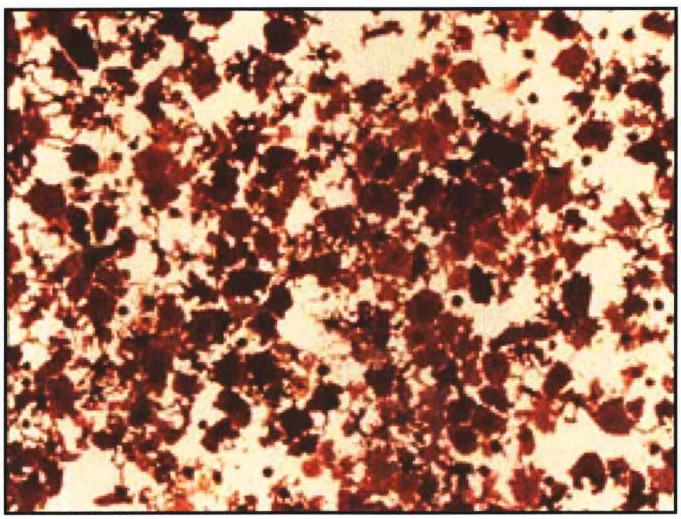


Figure 2.4: Primary culture of erythrophores from *Betta splendens.* These cells were isolated from a red fish, which allowed an almost pure population of erythrophores. Alternative chromatophore selection is possible using *B. splendens* of differing color properties.

2.5 Discussion

The process of screening for chromatophore sources for use as multi-cellular biosensors began by determining which species of animals to focus on. Chromatophores have been previously characterized in crustaceans, amphibians, reptiles, and teleosts (Nery and Castrucci 1997). For our purposes, teleosts (fish) were determined to be the best source for several reasons. Individual fish are relatively inexpensive and the maintenance of the necessary aquarium conditions is well understood. The low cost and local availability (all specimens discussed were obtained from local pet stores) made screening a wide variety of fish possible. Numerous subtypes of pigmented cells including melanophores, erythrophores, and iridophores were abundant in both the scales and skin of these fish. This allowed the use of single specimens to observe the different signaling and optical properties of each chromatophore type. In addition, initial observations determined that the optical quality of chromatophores available from particular fish was excellent: the pigmentation of these cells was motile in response to a variety of externally added chemicals.

It was determined that light absorbing chromatophores (*e.g.* melanophores and erythrophores) from the same fish responded similarly whether they were present on scales in tissue culture or in primary cell isolates attached to *in vitro* culture surfaces. Aggregation and dispersion of pigment occurred similarly upon application of stimuli in both cultures and scales. It was therefore evident that

melanophores and erythrophores in primary culture were able to reconstitute the molecular and cellular architectural requirements necessary for pigment motility. There are benefits of using either intact tissue (scales) or isolated cell cultures in biosensor applications. Scales offer the advantages of being easier to obtain than cultured cells and the chromatophores are allowed to remain in a less intrusive environment, which often allows them to respond better to stimuli. The cultured chromatophores in this study were plated on a monolayer substrate that made microscopic analysis easier than scales, which have a thicker depth of field. Cultured cells on glass or plastic also make it feasible to easily incorporate the cells into devices for automated sample delivery and data analysis. The iridophores observed in this study were typically responsive to agents when present on scales but were seldom responsive when grown in primary culture. Therefore, the iridescent color changes demonstrated on scales are likely dependent on the organization present in the tissue and the random orientation of iridophores in culture did not allow the concerted movements of reflective platelets that lead to color change. Xanthophores and other chromatophore types were not characterized in this study. Their presence on the specimens observed was too low to be useful as biosensors.

Only two fish exhibited near-optimal reactivity in each of the chromatophore subtypes (melanophores, erythrophores, and iridophores): *B. splendens* (Betta) and *Hemichromis bimaculatus* (Jewel Cichlid). *B. splendens* had two additional advantages: high cell density present per fish and the variety of

colors available, which allows a selection of chromatophore subtypes. The variety of coloration present in *B. splendens* is evident in Figure 2.2 where the choice of fish color allowed for the selection of erythrophores, melanophores, or iridophores that reflected light of different colors. Such variety allows for multiple chromatophore subtypes to be incorporated into multi-cellular biosensors that accentuate differing optical or signaling properties. Further, the high chromatophore density characteristic of *B. splendens* has several important advantages. First, similar numbers of cells can be harvested from fewer fish. Second, dense cell cultures allow for the analysis of more cells, which minimizes the slight variations in the pigment response that are inevitable between cells. In addition, the longevity of chromatophores in culture was greater in *B. splendens* than in any other specimen screened. The primary drawbacks of *B. splendens* were that cultures were at a higher risk for bacterial contamination than many other species, making sterility imperative, and melanophores and erythrophores were smaller than several other species. Although smaller cell size allows for more densely populated cultures, the optical detection of pigment changes is often more difficult. In contrast, *T. nilotica* melanophores are much larger and more dispersed, making individual cellular pigment changes easier to recognize. Some of the specimens analyzed contained non-motile chromatophores (cells that did not translocate pigment in response to a stimulus). For example, iridophores were numerous on scales from both *C. octofasciatum* and *B. conchonius*; however, no

visual changes were observed in either case after exposure to norepinephrine or forskolin.

Monitoring the optical changes of chromatophores as a biosensor methodology has some unique advantages. Being an intact biological system allows access to the full complement of signaling mechanisms present in these cells. Numerous signaling pathways in these cells are linked to the transport of pigment, which has made the use of chromatophores a useful model system for the study of intracellular transport (Bagnara 1991). Any chemical agent that affects the sequence of signals leading to regular pigment transport is capable of being detected by this system. The exact causative effect on the transport of pigment is not necessary for this system to be useful for detection. In fact, as long as the pattern of pigmentation is perturbed, it can reasonably be assumed that a causative agent is present. Further, the possible deviations away from normal pigmentation morphology can take many forms, including 'direct' effects and a variety of possible 'indirect' effects, any of which can be characterized and matched to certain classes of stimuli. The use of the Melanophore Index to quantify chromatophore changes is simple but introduces problematic human judgment. It is therefore more likely that chromatophores in future cellular biosensor applications will optically quantify pigment area or light absorbance in the dispersed and aggregated state as alternative means of measurement.

The differential activities of multiple chromatophore types were utilized on *H. bimaculatus* scales to characterize the presence of cholera toxin. After prior

incubation of chromatophores with the toxin, erythrophore pigment was impaired in its ability to aggregate in response to norepinephrine (Figure 2.1A and B). This observation was especially clear when the rate of erythrophore pigment aggregation was compared directly with that of melanophores that were present on the same fish scale. The characterized effect of cholera toxin is the selective ADP-ribosylation of the α-subunit of the stimulatory G protein (G_s), which ultimately results in elevated intracellular cAMP levels (Gill and Meren 1978). Therefore, erythrophore pigment motility seems to be under the mechanistic control of G_s. The delayed response of erythrophores to norepinephrine after cholera toxin exposure is a possible 'fingerprint' for this pathogen using this assay, as this result has not been induced by another agent added in a similar fashion. Pertussis toxin catalyzes the ADP-ribosylation of inhibitory G proteins (G_i) (Bokoch *et al.* 1983) and is another bacterial protein toxin that has a similar indirect effect on melanophores. Prior incubation of fish melanophores with pertussis toxin resulted in the blockage of norepinephrine-induced pigment aggregation (Karlsson *et al.* 1991). However, this result was not reported in erythrophores. The differential effects of these two protein toxins on melanophores and erythrophores demonstrates that there are distinct regulatory pathways that control pigment motility in these cells and that these differences can be utilized to detect and characterize agents that affect these pathways.

Chapter 3

Calcium Modulation of Pigment Transport in Erythrophores, But Not Melanophores, of *Betta splendens*

R. Ryan Preston and Philip N. McFadden

Formatted for Submission

3.1 Summary

The differential calcium (Ca^{2+}) signaling requirements leading to pigment migration observed between melanophores and erythrophores in *Betta splendens* were investigated as a potential two-cell cytosensor to detect Ca^{2+} modulating agents. Depletion of Ca^{2+} from erythrophore cultures resulted in the impaired ability to aggregate pigment in this chromatophore subtype. After mixed cell cultures of melanophores and erythrophores were exposed to the α-adrenergic agonist norepinephrine, dispersal of erythrophore pigment was observed following the pharmacological addition of Ca^{2+}-specific ion channel antagonists. The results presented suggest that pigment motility in erythrophores, but not melanophores, is sensitive to intracellular Ca^{2+} fluctuations.

3.2 Introduction

Chromatophores are the pigmented cells present in the dermal skin layer of numerous animals including fish, reptiles, amphibians, crustaceans, and mollusks. The movement of pigmented organelles along the cytoskeleton within these cells is under the regulation of the nervous and endocrine systems. The resulting color changes in the skin of these animals are responsible for camouflage, blanching, and other natural behaviors. Melanophores and erythrophores are two subtypes of

chromatophores that are differently colored due to their content of melanin (black) or carotenoid/pteridine (red) pigment (Fujii 1993a). Differences also exist in the signaling pathways that lead to pigment motility in these two cell types (Nery and Castrucci 1997).

The molecular signaling mechanisms that trigger pigment motility in chromatophores has been described in several fish species. Signaling pathways that modulate intracellular concentrations of cyclic adenosine triphosphate (cAMP) have been characterized in melanophores (Sammak *et al.* 1992)(Thaler and Haimo 1992) and erythrophores (Kotz and McNiven 1994)(Tuma and Gelfand 1999). cAMP levels are generally controlled in these chromatophore subtypes through the α_2-adrenergic receptor (Morishita 1987); (Svensson *et al.* 1997). Activation of this receptor leads to adenylyl cyclase inhibition, resulting in decreased cAMP levels and pigment aggregation (Andersson *et al.* 1984); (Negishi *et al.* 1982). Conversely, an intracellular increase in cAMP levels, as in the stimulation of adenylyl cyclase by forskolin, leads to pigment granule dispersion toward the cell periphery (Fujii 2000). The entry of Ca^{2+} across the plasma membrane has also been implicated in the control of pigment motility in chromatophores. In several species where erythrophores have been studied, Ca^{2+} was consistently involved in pigment migration (Luby-Phelps and Porter 1982); (McNiven and Ward 1988); (Kotz and McNiven 1994); (Oshima *et al.* 1988). However, the role of Ca^{2+} in pigment motility is species dependent in chromatophore model systems using

melanophores (Martensson and Andersson 2000); (Thaler and Haimo 1992); (Sammak *et al.* 1992).

In this study, the differential pigment responses in melanophores and erythrophores to pharmacological signaling agents have been explored as a possible multiple-cell biosensor to detect and characterize the cellular effects of Ca^{2+} modulating agents, including potential pharmaceutical targets. To accomplish this, the Ca^{2+} signaling requirements in chromatophores have been examined from a species of fish, *Betta splendens* (Siamese fighting fish), whose skin contains populations of both melanophores and erythrophores.

3.3 Materials and Methods

3.3.1 Cell Culture

Isolation of chromatophores from individual *Betta splendens* was performed according to the method described previously (Preston and McFadden 2001; chapter 4, page 55 of this dissertation).

Chromatophore pigment migration was induced pharmacologically by the direct addition of agents onto cells in culture on cover slips. All stimulating agents were obtained from Sigma-Aldrich. Prior to exposure with stimulating agents the cover slips were loaded into a continuous-flow imaging chamber (model RC-20H, Warner Instrument Corp., Hamden, CT, USA) and equilibrated for 5 minutes in

'physiological saline solution' (PSS, 128 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.8 mM $MgCl_2$, 5.6 mM glucose, 10 mM Tris/HCl, pH 7.2). Chemical agents were injected directly into the enclosed cell chamber at the given concentration in PSS. The volume added was sufficient to completely exchange the solution in the chamber.

3.3.2 Image Analysis

The assembled chromatophore chamber was mounted on the stage of a Zeiss IM-35 inverted microscope (Carl Zeiss, Inc., Oberkochen, Germany) fitted with a Plan 6.3x objective (numerical aperture = 0.16) and a Plan 16x objective (numerical aperture = 0.35). Digital images (uncompressed TIFF format, 640 X 480 resolution) were captured every 20 seconds from a Panasonic GP-US502 color 3-CCD camera (Secaucus, NJ, USA) by a Flashpoint 3D capture card (Integral Technologies, Indianapolis, IN, USA) attached to a Pentium III computer.

Image analysis of pigment aggregation and dispersion in melanophores and erythrophores after exposure to chemical agents was performed using the method described by Preston and McFadden (2001; chapter 4, page 57 of this dissertation).

3.4 Results

To compare how melanophores and erythrophores respond to stimulating agents, these cells were pharmacologically treated with active agents and the resulting pigment motility responses were monitored using light microscopy. In the first study, *B. splendens* melanophores and erythrophores were treated with a common pigment-aggregating stimulus, 10nM norepinephrine, in solution that contained either Ca^{2+} or solution where Ca^{2+} had been removed. In solution containing Ca^{2+} (1.8mM), treatment of erythrophores with norepinephrine caused most pigment to aggregate toward the cell body within minutes (Figure 3.1). A parallel experiment in Ca^{2+}-free PSS solution (*e.g.* 1mM EGTA and no added Ca^{2+}) resulted in the impaired ability of erythrophores to aggregate pigment. Melanophore pigment aggregation proceeded normally after exposure to norepinephrine (*e.g.* complete pigment aggregation within minutes) in solutions containing both Ca^{2+} and lacking Ca^{2+} (data not shown).

Cell cultures containing mixed populations of erythrophores and melanophores were used to test the different requirements for Ca^{2+} in pigment motility. Exposure to norepinephrine caused rapid pigment aggregation in both cell types. Subsequent exposure to verapamil resulted in the selective dispersion of erythrophores, but not melanophores (Figure 3.2). Ca^{2+} channel antagonists, such as verapamil, inhibit the inward flow of Ca^{2+} into the cytoplasm through Ca^{2+}-

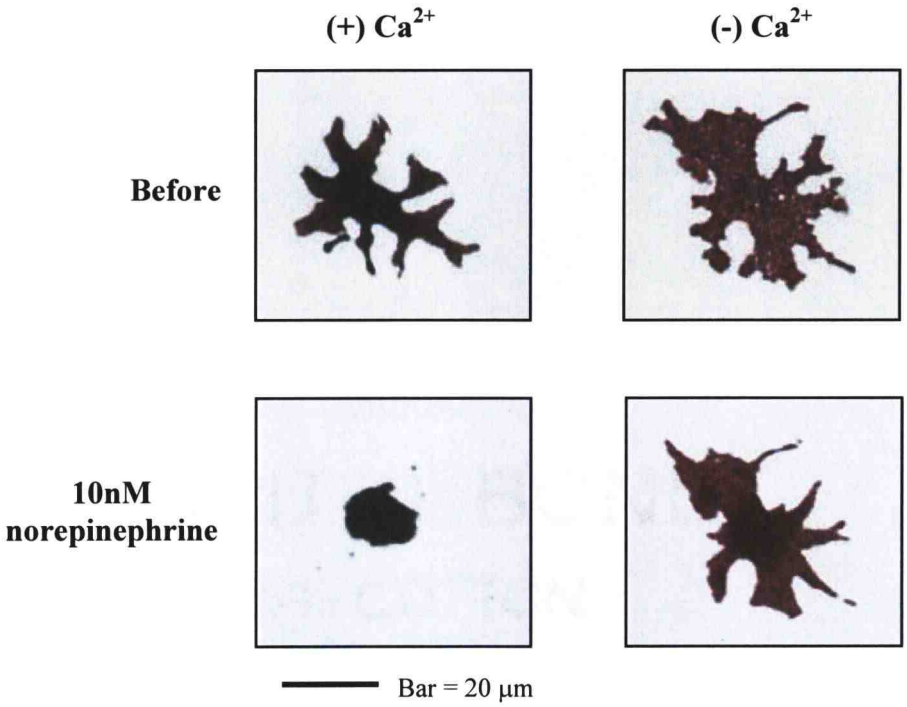


Figure 3.1: Demonstration of the dependence of Ca^{2+} on pigment aggregation in erythrophores. (+) Ca^{2+} (first column): Performed in solution containing 1.8mM added Ca^{2+}. (-) Ca^{2+} (second column): Performed in solution with no added Ca^{2+} plus 1mM EGTA. Before images were captured immediately prior to norepinephrine exposure and after images were captured 3 minutes after exposure.

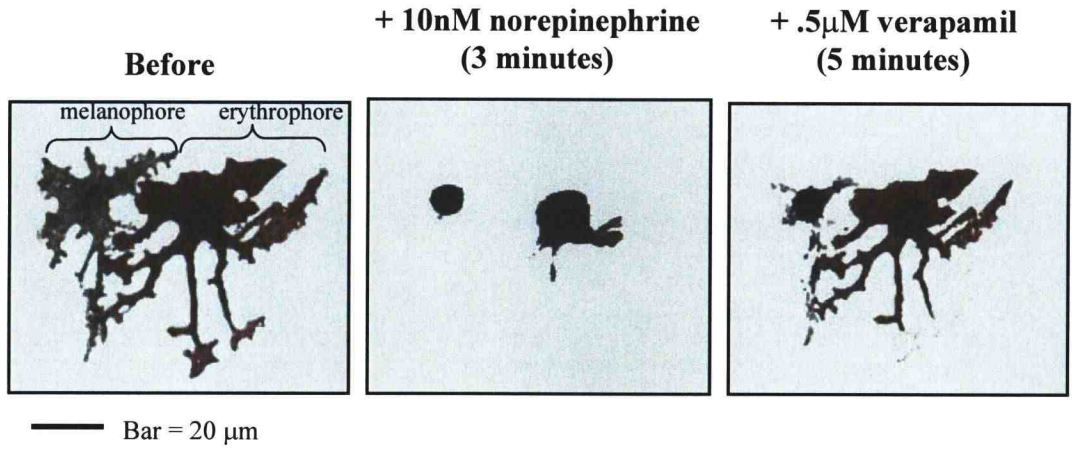


Figure 3.2: Differential responses between erythrophores and melanophores to verapamil, an antagonist of Ca^{2+} channels. Chromatophores were cultured from a specimen containing both cell types. Before any treatment, both cells persisted in a dispersed state. Addition of 10nM norepinephrine to the surrounding solution caused the pigment in both cell types to aggregate to the cell body. Subsequent addition of .5μM verapamil induced only erythrophores, not melanophores, to disperse.

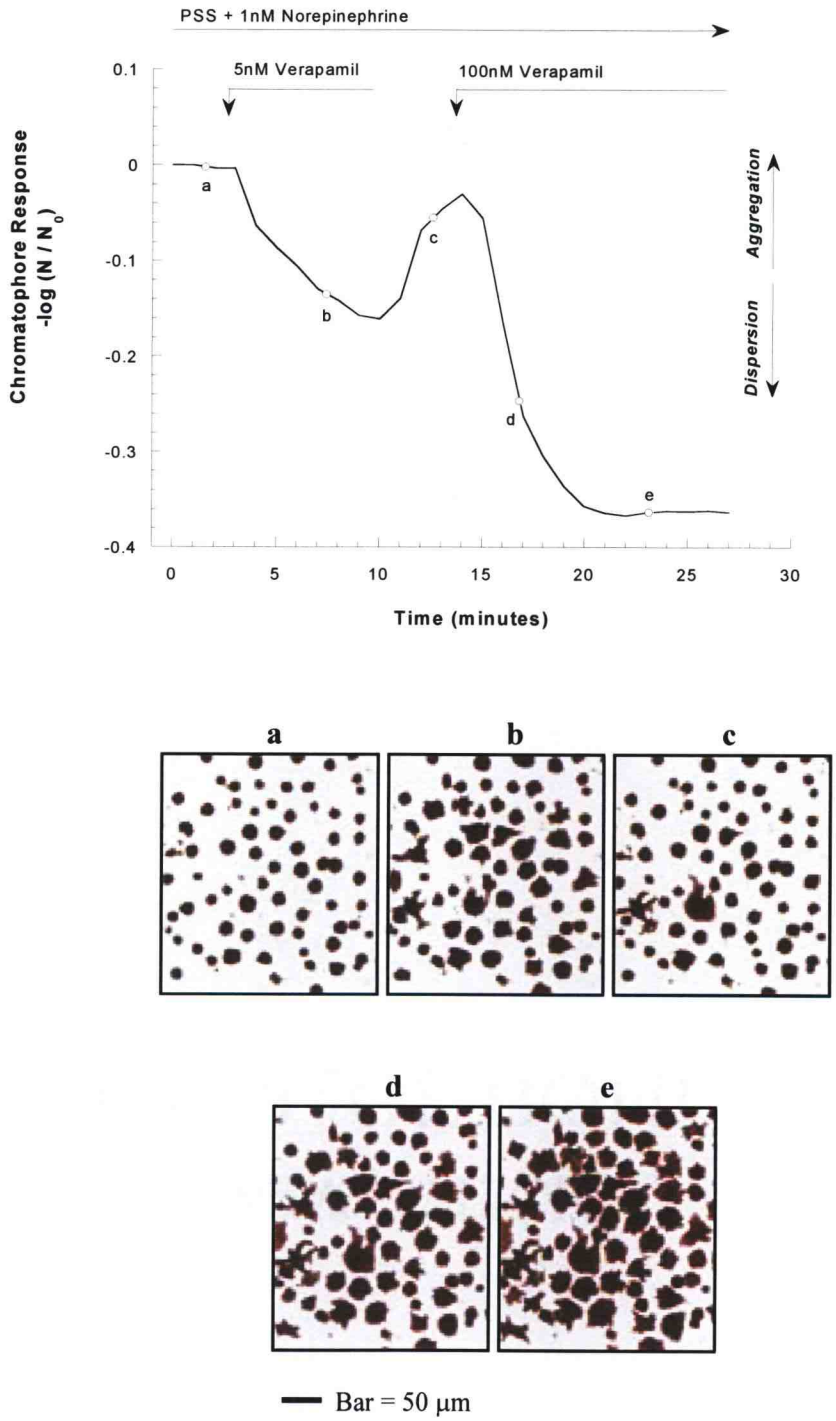
specific membrane ion channels (Lee and Tsien 1983), suggesting that a lowered intracellular Ca^{2+} level was sufficient to induce an observable pigment dispersion response in *B. splendens* erythrophores.

In order to further characterize the ability of erythrophore pigment to respond to Ca^{2+} channel antagonists, a homogeneous population of erythrophores was treated with two different verapamil concentrations in succession. Pre-treatment of erythrophores with PSS + 1nM norepinephrine caused pigment aggregation (Figure 3.3, image 'a'). Addition of a dose of 5nM verapamil resulted in pigment dispersion (Figure 3.3, image 'b') until the erythrophore cell chamber was flushed of verapamil by PSS + 1nM norepinephrine. This caused erythrophore pigment to re-aggregate (Figure 3.3, image 'c') until a second, stronger dose (100nM) of verapamil was added to the erythrophores. This stronger dose of verapamil induced erythrophore pigment to disperse to an extent that was consistent with the applied dose (Figure 3.3, images 'd' and 'e').

The efficacies of verapamil, diltiazem, and nifedipine were compared by performing a dose-dependent study of each agent's effect on the pigment response in erythrophores. A dose-dependency was observed for each agent after pre-treatment of erythrophores with norepinephrine, as recorded by the Chromatophore Response (Figure 3.4). Verapamil was found to be the most effective, with the maximum effect seen at 100nM whereas diltiazem and nifedipine had less potent, near-identical response curves. These response profiles between Ca^{2+} channel

Figure 3.3: Responsiveness of erythrophores to multiple stimulations with the Ca^{2+} channel antagonist verapamil. Erythrophores were pre-aggregated with 1 nM norepinephrine in PSS. This was at least partially due to Ca^{2+} entrance into the cytoplasm through plasma membrane Ca^{2+} channels. Subsequent blockage of these Ca^{2+} channels by exposure with verapamil in differing doses (5nM and 100nM) resulted in dose-dependent pigment dispersion. Flushing verapamil from the erythrophore chamber at t=10 minutes allowed re-aggregation of erythrophore pigment, allowing the response to recycle to the baseline level (fully aggregated state) before subsequent addition of a higher dose of verapamil at t=14 minutes. Images were captured and the area calculated every 20 seconds. The representative points labeled correspond with the images shown below.

Figure 3.3


PSS + 1nM Norepinephrine
5nM Verapamil
100nM Verapamil
Chromatophore Response
-log (N / N0)
Aggregation
Dispersion
0.1
0
-0.1
-0.2
-0.3
-0.4
0
5
10
15
20
25
30
Time (minutes)
a
b
c
d
e
Bar = 50 µm

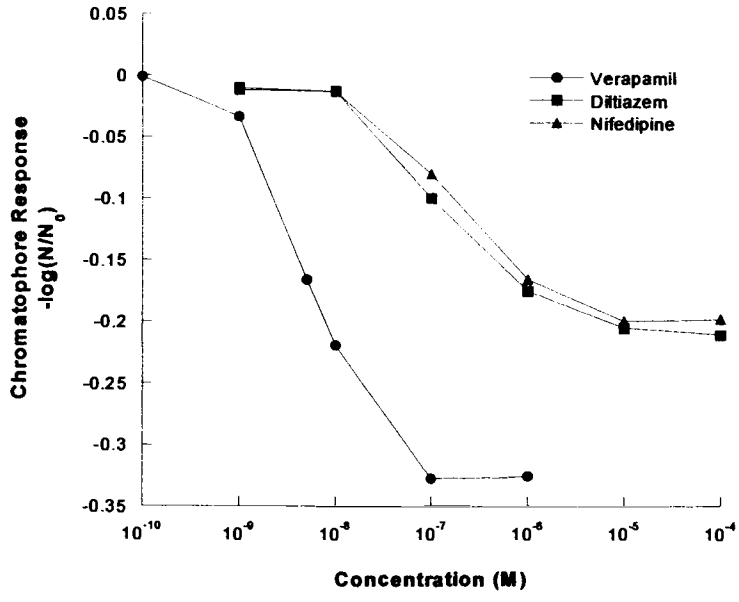


Figure 3.4: Erythrophore dose responses to the L-type Ca^{2+} channel antagonists verapamil, nifedipine, and diltiazem. Erythrophores were treated at each data point with 1nM norepinephrine for at least 5 minutes prior to exposure of the channel blockers. The Chromatophore Response [-log (N/N_0)] was calculated as area occupied by the cells after chemical exposure for 10 minutes divided by the area occupied before exposure.

antagonists are consistent with earlier reports in muscle cell Ca^{2+} channels (Triggle and Swamy 1983).

The results of further pharmacological studies pertaining to the role of Ca^{2+} on *B. splendens* erythrophore pigment motility are summarized in Table 3.1. The direct application of four L-type Ca^{2+} channel antagonists and four peptide toxins that were antagonists of N-, P-, and Q-type Ca^{2+} channels failed to directly induce either aggregation or dispersion in erythrophores. However, these Ca^{2+} channel antagonists induced erythrophore pigment dispersion when the cells were previously treated with norepinephrine to activate the opening of these ion channels. Ca^{2+} entry into erythrophores after the direct application of the L-type Ca^{2+} channel activator Bay K8644 did not induce pigment aggregation. However, treatment of erythrophores with the Ca^{2+} ionophores ionomycin and A23187 resulted in pigment aggregation, suggesting that elevated intracellular Ca^{2+} levels were sufficient to independently induce pigment aggregation. Direct treatment of erythrophores with the membrane permeable, Ca^{2+}-specific chelator BAPTA/AM had no observable effect on pigment distribution in cells. However, chelating intracellular Ca^{2+} pools with BAPTA/AM did impair the ability of erythrophores to aggregate pigment in response to norepinephrine treatment.

Table 3.1: Effects of Ca^{2+} modulating chemicals on *B. splendens* erythrophores.

	Pigment motility effect[a]		
	Dispersion	**Aggregation**	**Comments**
Ca^{2+} channel modulators			
Phenylalkylamines			
verapamil[b]	+	-	L-type channel antagonist
Benzothiazepines			
diltiazem[b]	+	-	L-type channel antagonist
1,4-Dihydropyridines			
nimodipine[b], nifedipine[b]	+	-	L-type channel antagonists
Bay K8644	-	-	L-type channel agonist
ω-Conotoxin GVIA[b]	+	-	N-type channel antagonist
ω-Conotoxin MVIIA[b]	+	-	N-type channel antagonist
ω-Conotoxin MVIIC[b]	+	-	N,P,Q-type channel antagonist
ω-Agatoxin IVA[b]	+	-	P-type channel antagonist
High [K+] (> 50mM)	-	-	depolarizes membrane; activates voltage-gated channels
Ca^{2+} chelating agents			
EGTA	-	-	chelates extracell. Ca^{2+}; impairs n.e.-induced aggregation
BAPTA/AM	-	-	chelates intracell. Ca^{2+}; impairs n.e.-induced aggregation
Ca^{2+} Ionophores			
A23187	-	+	
Ionomycin	-	+	

[a]: Pigment motility effects listed (+/-) are observed responses after each agent was added directly erythrophores.
[b]: The dispersion of erythrophore pigment observed after treatment with Ca^{2+} channel antagonists occurred after exposure to norepinephrine.

3.5 Discussion

These results suggest that there are differences in the signaling events that lead to bi-directional pigment motility in *B. splendens* melanophores and erythrophores. In erythrophores, but not melanophores, intracellular Ca^{2+} fluctuations mediated pigment aggregation. In solution where Ca^{2+} had been removed, erythrophore pigment aggregation was impaired following treatment with norepinephrine (Figure 3.1), suggesting that increased intracellular Ca^{2+} levels are required for pigment aggregation to occur in this chromatophore subtype. Norepinephrine is an agonist of both the α_1-adrenergic receptor, which is linked to the opening of agonist-operated Ca^{2+} channels on the plasma membrane (Exton 1985), and the α_2-adrenergic receptor, ultimately leading to a decrease in intracellular cAMP levels (Limbird 1988). Further, chelating intracellular Ca^{2+} pools with BAPTA/AM impaired norepinephrine-induced pigment aggregation (Table 3.1). In melanophores, however, pigment aggregation proceeded normally in the absence of Ca^{2+}, which suggests that melanophore pigment aggregation does not require Ca^{2+}. Instead, it is likely that cAMP mediates pigment aggregation in *B. splendens* melanophores. This is consistent with earlier studies in melanophores from other species that concluded that cAMP is exclusively responsible for aggregation in melanophores, regardless of fluctuations in Ca^{2+} levels (Andersson *et al.* 1984); (Sammak *et al.* 1992). A report describing erythrophores, however,

suggested that both cAMP and Ca^{2+} are involved in the control of pigment motility (Kotz and McNiven 1994).

The effect on pigment motility in erythrophores following the pharmacological inactivation of Ca^{2+}-specific ion channels has been described in this study. The entry of Ca^{2+} into the cytoplasm from the extracellular space is an event that is largely mediated by Ca^{2+} ion channels embedded in the plasma membrane (Mori *et al.* 1996). After Ca^{2+} channels were activated (opened) by exposure to norepinephrine, the treatment of erythrophores with Ca^{2+} channel antagonists resulted in pigment dispersion (Table 3.1). Further, the dispersion of pigment in erythrophores occurred in a dose dependent manner to three L-type Ca^{2+} channel inhibitors (Figure 3.4). Melanophores, however, did not disperse pigment following similar treatment with verapamil. This differential response between melanophores and erythrophores was observed in a co-culture of these two cell types (Figure 3.2), suggesting that these cells may be utilized to study the signaling effects induced by Ca^{2+} modulating chemicals.

The advantage of using multiple chromatophore subtypes for biosensors instead of single cell type is an increased breadth of sensitivity to active biological agents. Observing the differential responses between cell types can be utilized to screen the signaling effects induced by unknown drugs. For example, Ca^{2+} channel antagonists are widely used as therapeutic treatments for cardiac conditions such as arrhythmia and other peripheral conditions (Pine 1984). *B. splendens* provide a broad source of the various types of chromatophores for incorporation into

cytosensor applications. All chromatophore subtypes are represented in this species: melanophores, erythrophores, iridophores, xanthophores, and leucophores. Further study of the differential regulation leading to pigment motility in these subtypes can lead to a useful multi-cellular model to characterize a wide range of important compounds.

Chapter 4

A Two-Cell Biosensor that Couples Neuronal Cells to Optically Monitored Fish Chromatophores

R. Ryan Preston and Philip N. McFadden

Published in *Biosensors and Bioelectronics*

Elsevier Science, New York, NY, USA

2001, 16: 447-455

4.1 Summary

A two-cell biosensor was developed that uses optically detected changes in naturally colored fish chromatophores to measure the neurosecretory output of mammalian neuronal cells. The specific version of the biosensor described here is a continuous flow device that places red-pigmented, dendritic erythrophore cells directly downstream of an immobilized population of PC12 neuronal cells, a well-established model of neuroendocrine function. Agents known to stimulate catecholamine neurosecretion (secretagogues) were presented to the PC12 cells. It was found that the varying level of neurosecretion from the PC12 cells was measurable by judging the degree of pigment aggregation in the erythrophores. Increases in catecholamine secretion and consequent pigment aggregation were observed for several known secretagogues, including receptor agonists (ATP, acetylcholine), membrane depolarizing agents (high K^+ concentration), and specific neurotoxins (black widow spider venom, α-latrotoxin). This particular two-cell biosensor, which is applicable to the detection of any agents that affect the levels of catecholamine secretion from PC12 cells, demonstrates the general principle that the breadth of sensitivity of a biosensor is increased by employing coupled cell types.

4.2 Introduction

The use of living cells as an analytical tool is a promising approach in the field of biosensor development. Such biosensors utilize the physiological activities of living cells to analyze biologically active agents, such as drugs, metabolites, and toxins (Pancrazio *et al.* 1999). Live cells are particularly well suited to meet these needs because they express a wide variety of molecular targets for active agents, including enzymes, receptors, and antibodies. Moreover, the responses induced by the stimulation of these mechanisms are generally amplified by internal cellular mechanisms to yield a biologically relevant response. Desirable characteristics for any cell-based biosensor are high sensitivity, ease of material availability, and the ability to monitor an analyte continuously with a rapid response time and regeneration. The choice of cell type is an important issue toward these ends.

Chromatophores are pigmented cells responsible for the brilliant and changeable skin colors of many cold-blooded animals, including fish, amphibians, and reptiles (for reviews of these cells see Fujii 1993a; Fujii 1993b). These cells have been employed in biosensors because their appearance is changed by many types of biologically active agents (Danosky and McFadden 1997); (Elwing *et al.* 1990). Dendritic chromatophores, such as black-pigmented melanophores and red-pigmented erythrophores, contain thousands of pigmented organelles that are transported back and forth between the cell body and the cell periphery, depending on the exposure sequence of agents. Anterograde transport (aggregation) and

retrograde transport (dispersion) are facilitated by the motor proteins dynein and kinesin, respectively (Obika 1986). Typically, organelles spontaneously relocate to dispersed locations following the removal of an active agent (Kotz and McNiven 1994). The endogenous agents that trigger pigment movements in intact tissues are neurotransmitters and hormones under the control of the nervous and endocrine systems. For example, melanophores (Sugimoto *et al.* 1985) and erythrophores (Obika and Meyer-Rochow 1990; this study) are induced to aggregate their pigment by the neurotransmitter norepinephrine. The interaction is mediated by the α_2-adrenergic receptor (Fujii and Oshima 1986), the presence of which makes these cells suitable as sensors for catecholamines and the large class of pharmacologically related compounds. However, the receptors that respond to some other drug classes (*e.g.* cholinergic and purinergic drugs) are not found on chromatophores, and so drugs in those large pharmacological categories cannot be directly measured by their effects on the appearance of chromatophores.

In contrast, the neuronally related cell line PC12 expresses many of the receptors that are lacking in chromatophores. For example, PC12 cells have a sensitive response to cholinergic and purinergic drugs as well as neurotoxins that would presumably be ineffectual on chromatophores. PC12 cells were established in culture in 1976 by Greene and Tischler (1976) and are a widely used model system for neurophysiological studies, including neuronal differentiation, electrophysiology, and regulated secretion. Neurosecretion from PC12 cells occurs through a well-characterized pathway whose regulation is dependent on

intracellular Ca^{2+} concentration. Increased intracellular Ca^{2+} levels result from either agonist or voltage-activated Ca^{2+} channels, or through Ca^{2+} liberation from intracellular stores (Fasolato *et al.* 1990). Secretion by PC12 cells can be experimentally induced by secretagogues, agents like ATP, acetylcholine, and membrane depolarizing concentrations of K^+, that stimulate internal Ca^{2+} increases and the ensuing vesicle-mediated delivery of secreted material to the cell medium. The primary secreted products from PC12 cells are the catecholamines, dopamine and norepinephrine (Greene and Tischler 1982).

Given that secreted catecholamines can evoke optical changes in chromatophores, we investigated and developed a two-cell biosensor that uses changes in chromatophore appearance to monitor the regulated secretion from a population of PC12 cells.

4.3 Materials and Methods

4.3.1 Cell Culture

PC12 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown at 37°C / 5% CO_2 on plastic tissue culture flasks coated with 5 $\mu g/cm^2$ collagen IV (Sigma, St. Louis, MO, USA) in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% horse serum (Sigma, St. Louis, MO, USA) and 5% fetal bovine serum (HyClone

Laboratories, Logan, UT, USA). One hour before an experiment, cells were removed from the flask by passage through a syringe needle, counted on a Neubauer hemacytometer, and dispensed into 500 µl aliquots at a concentration of $4x10^4$ PC12 cells/ml in complete RPMI 1640 medium.

Erythrophore cultures were prepared 3-10 days prior to an experiment, as summarized below. The dorsal, caudle, and anal fins were clipped from a euthanized male *Betta splendens* (Siamese fighting fish) and diced into 2.0-4.0 mm squares. These were washed in six solution changes of phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.6 mM KH_2PO_4, pH 7.4) containing1 mM NaEDTA, then transferred into 7 ml of digestion solution (PBS containing 960 U/ml collagenase I and 230 U/ml hyalouronidase). After a 30-minute incubation, dissociated erythrophores were separated from the digestion solution by centrifugation for 3 minutes at 300x g and resuspended in L15 medium (Sigma, St. Louis, MO, USA) supplemented with 5% fetal bovine serum. Erythrophores from three successive digestion solution incubations were pooled and suspended in complete L15 medium at a concentration of 1 x 10^5 cells/ml. Cells were plated on 15 mm diameter glass cover slips that had previously been coated with 20 µg/cm^2 collagen IV and 15 µg/cm^2 fibronectin (both from Sigma, St. Louis, MO, USA) by placing one drop on each cover slip. After allowing the erythrophores to attach to the substrate for one hour, the cover slips were submerged in complete L15 medium and stored at room temperature.

4.3.2 Continuous Flow Device

The experimental apparatus was comprised of two cell-chambers, for PC12 cells and erythrophores, respectively. The chambers were connected by a network of 0.020-inch inner diameter polyetheretherketone (PEEK) tubes. Continuous flow of "Low K^+" solution (125 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM HK_2PO_4, 5.6 mM glucose, 25 mM HEPES, pH 7.4) was maintained through the tubing by a syringe pump (World Precision Instruments model sp200i, Sarasota, FL, USA) at a flow rate of 200 µl/min (unless otherwise stated). PC12 cells were retained in the first chamber, which was comprised of a 0.5-micron HPLC in-line filter (Cole-Palmer, Vernon Hills, IL, USA). The PC12 cells were introduced into their chamber as follows: 2×10^4 PC12 cells suspended in 500 µl were introduced upstream of the chamber by syringe injection through a three-way valve. A continuous flow of Low K^+ solution following injection facilitated the transport of the PC12 cells into the chamber. After the PC12 cells had been assembled into the device, cultured erythrophores on glass cover slips were inserted into their chamber, which was a closed-bath imaging chamber (Warner Instrument Corp. model RC-20H, Hamden, CT, USA) that was mounted in the line of flow downstream of the PC12 cell chamber. A flow rate of 200 µl/min was used during data collection in all experiments, unless otherwise noted. All secretagogues and α-latrotoxin were purchased from Sigma (St. Louis, MO, USA). Secretagogues were added upstream of the PC12 cell chamber through the

same injection port as the PC12 cells. A 20 second lag-time was required for effluent from PC12 cells to be pumped into the erythrophore chamber. All figures have been corrected to account for this lag time. Effluent was discarded after passing over the erythrophores.

4.3.3 Imaging and Image Processing

The erythrophore chamber was mounted on the stage of a Zeiss IM-35 inverted microscope (Oberkochen, Germany) fitted with a Plan 6.3x objective (numerical aperture = 0.16). Digital images (uncompressed TIFF format, 640 x 480 resolution) were captured every 20 seconds from a Panasonic GP-US502 color 3-CCD camera (Secaucus, NJ, USA) by an Integral Flashpoint 3.1.1 capture card (Integral Technologies, Indianapolis, IN, USA) attached to a Pentium II computer.

Image processing was performed using Image Pro 4.1 image analysis software (Media Cybernetics, Silver Springs, MD, USA). To determine the effect of PC12 cell effluent on pigment motility in chromatophores, software was used to define the *area occupied by chromatophore pigment* within the field of view of an image. (Individual images contained approximately 100 chromatophores). The *change in area* as the pigment aggregated (or dispersed) corresponded to a cellular response in chromatophores.

The trend of pigment aggregation and dispersion within a sequence of images was determined by calculating the area occupied by chromatophore pigment within each image in the sequence. The area occupied by pigment was converted

to a dimensionless value, the 'Chromatophore Response' (C.R.). This value was computed using the following formula:

$$\textit{Chromatophore Response (C.R.)} = -\log_{10}(N/N_0)$$

where 'N' was the area occupied by chromatophore pigment in each image of a sequence and 'N_0' was the area occupied by chromatophore pigment in the first image of a sequence.

4.4 Results

A schematic diagram of the apparatus used to optically monitor neurosecretion is shown in Figure 4.1. The flow rates, tubing lengths, and internal volume of this continuous-flow device were adjusted empirically to achieve a favorable configuration for the purpose of introducing analytes, detecting their presence in real time, and then flushing them out of the erythrophore chamber. A flow rate of 200 µl/minute and 2 x 10^4 PC12 cells per experiment was found to be optimal for sensitively coupling the neurosecretion of PC12 cells to the optically detected changes in pigment area of the erythrophores. The specific internal volumes for the apparatus components are given in Figure 4.1.

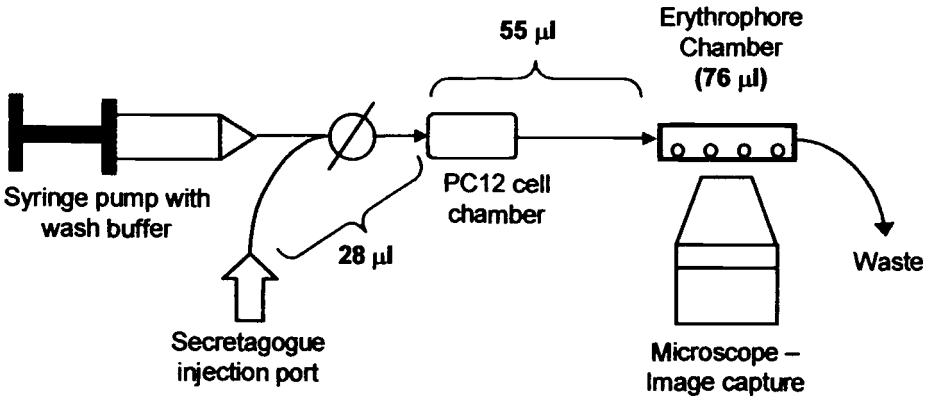
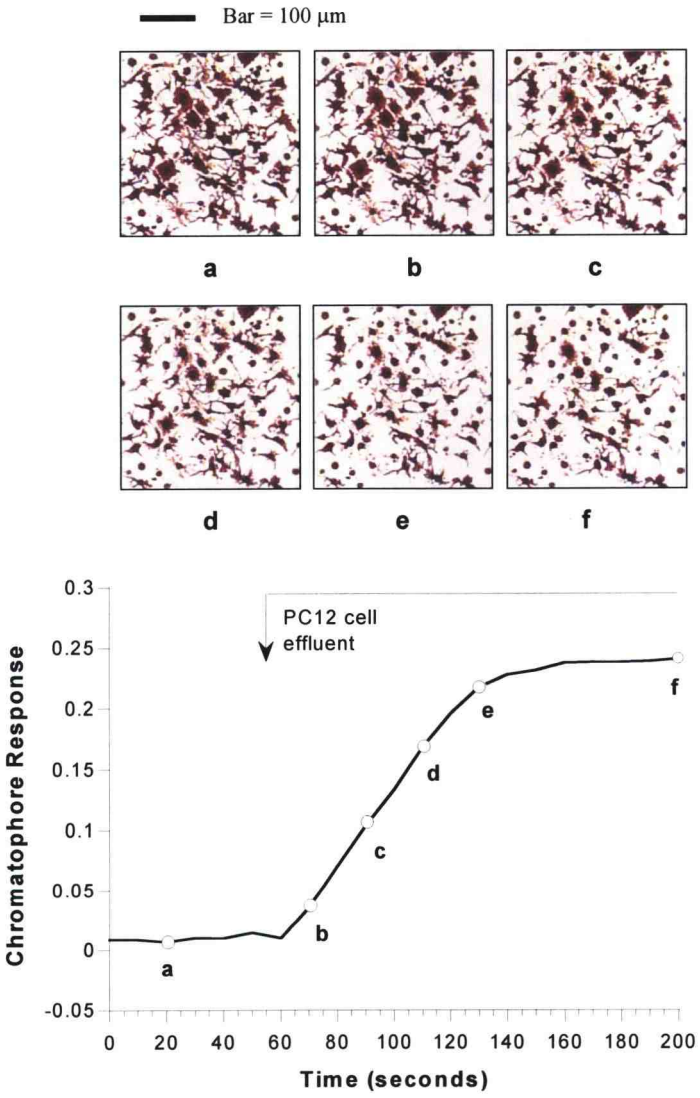


Figure 4.1: Erythrophore / PC12 cell continuous-flow apparatus. A syringe pump drove the flow of Low K+ buffer into the network of tubing. Internal volumes of the separate components are listed in the diagram. PC12 cells were suspended upon a 0.5 micron filter. Secreted norepinephrine from PC12 cells passed into the enclosed erythrophore cell chamber. Erythrophores were plated on a glass coverslip mounted over an inverted microscope fitted with a video camera. Secretagogues were injected as a 200 µl sample upstream of the PC12 cell chamber.

The two-cell nature of this biosensor is demonstrated in Figure 4.2. In the presence of a continuous flow of Low K^+ solution, erythrophores maintained a prolonged dispersed morphology (Figure 4.2, image 'a'). At the time point indicated, the flow to the erythrophore chamber was redirected to change from Low K^+ solution to Low K^+ that had just passed over the PC12 cells. The constitutive secretion of norepinephrine from the population of PC12 cells resulted in a rapid and sustained aggregation response of the erythrophores. The magnitude of the erythrophore response to PC12 cells was judged by comparing each image to the first image in that interval. The "Chromatophore Response" value was calculated as a measure of how the area of pigment changed relative to the initial highly dispersed state of the cells. When the erythrophores were exposed to the effluent from PC12 cells, aggregation proceeded within seconds (Figure 4.2, images 'b'-'e'). A population of 2×10^4 PC12 cells was determined to be optimum because the amount of continuous secretion from this number of cells maintained erythrophores in a partially aggregated state that could be further aggregated upon injection of secretion-inducing agent as described below (Figure 4.2, image 'f'). Maintaining erythrophores in this intermediate condition between an aggregated and dispersed morphology allowed the detection of increases in PC12 secretion above the constitutive level (described below). In Figure 4.2, the maximum Chromatophore Response attained was 0.24 (image 'f'), which corresponded to approximately half of the total aggregation possible by erythrophores.

Figure 4.2: Aggregation of erythrophores in response to norepinephrine secretion from $2x10^4$ PC12 cells. Erythrophores maintain a dispersed morphology under routine culture conditions, as seen in image 'a'. Approximately 100-120 erythrophores occupy a field of view and enter into the Chromatophore Response calculation. At the time point indicated, the effluent from $2x10^4$ PC12 cells was exposed to the erythrophores pictured. The aggregation of pigment from the cell periphery toward the cell body was initiated within seconds. Images 'b'-'f' demonstrate the visual appearance of this aggregation. The plot displays this trend by measuring the area occupied by erythrophores in this field of view. Labeled points on the plot ('a'-'f') correspond to the images shown above the plot. Chromatophore Response is a unitless value that increases as pigment aggregation occurs.

Figure 4.2


Bar = 100 µm
a
b
c
d
e
f
PC12 cell effluent
Chromatophore Response
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
0
20
40
60
80
100
120
140
160
180
200
Time (seconds)

Erythrophores that were stimulated with a saturating dose of norepinephrine leading to complete aggregation exhibited Chromatophore Response values of about 0.5 (data not shown).

The next question was whether it is possible to monitor changes in the level of neurosecretion induced by secretagogues. Figure 4.3 demonstrates how chromatophores responded to the expected increase in secretion from PC12 cells upon their exposure to a one-nanomole pulse of MgATP. ATP is a known agonist of the P_{2X}-purinergic receptor (Harden *et al.* 1995), which opens receptor-linked Ca^{2+} channels and promotes Ca^{2+} induced neurosecretion. The initial aggregation of chromatophores seen in Figure 4.3 at 2 minutes was in response to constitutive PC12 secretion resulting from the redirection of fluid flow to pass from the PC12 cells to the chromatophores. Further chromatophore aggregation then took place at 11 minutes, right after ATP was injected upstream of the PC12 cells. The increase in neurosecretion induced by ATP is known to be short-lived (Evans *et al.* 1992), with PC12 cell secretion returning to constitutive levels within minutes after ATP exposure. The aggregation response of chromatophores as shown in Figure 4.3 obeyed this pattern. The rapid aggregation in response to ATP was soon followed by redispersion of chromatophores.

Several other agents that are known to induce neurosecretion were tested in the two-cell system (Figure 4.4A). Each of the agents listed, with the exception of high K^+ solution, acts by binding as an extracellular ligand to receptors that directly

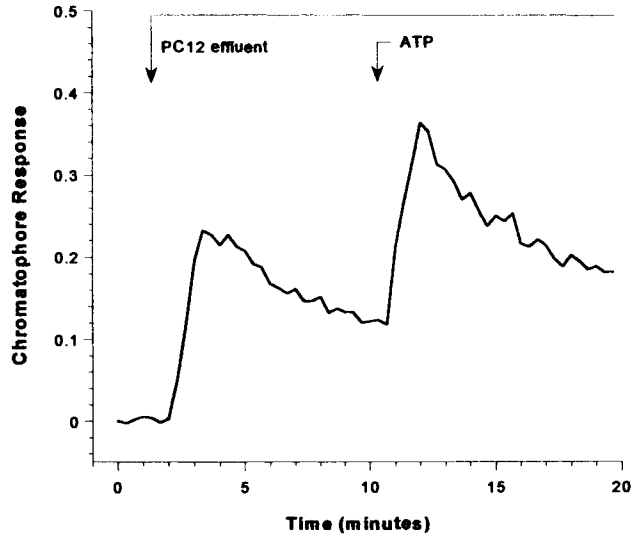
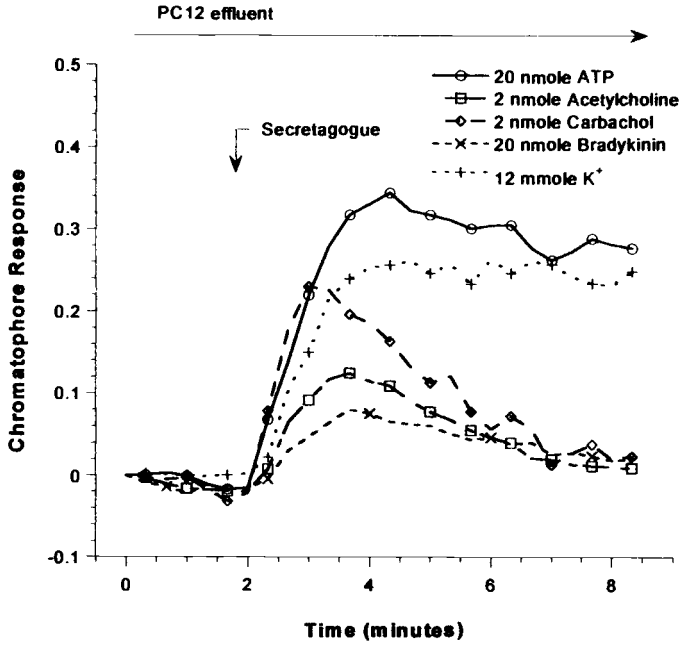


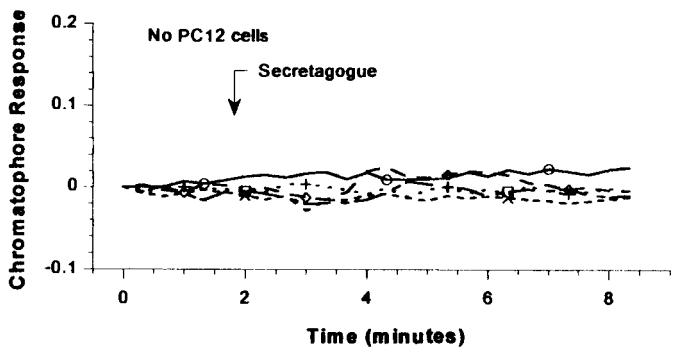
Figure 4.3: Erythrophore aggregation in response to norepinephrine secretion from PC12 cells stimulated with 5 μM ATP. At t=2 minutes, the effluent from $2x10^4$ PC12 was introduced to erythrophores. The constitutive release of norepinephrine from this population of PC12 cells resulted in partial aggregation of erythrophores. At t=11 minutes, a single pulse of 5 μM ATP (200 μl) was injected upstream of the PC12 cell chamber. The additional aggregation seen in erythrophores was rapidly followed by recovery of the cells toward the dispersed morphology. Flow rate=450 μl/min.

Figure 4.4: The induction of neurosecretion from PC12 cells following exposure to chemical secretagogues. A). The aggregation response observed occurred after erythrophores were pre-equilibrated in the effluent from $2x10^4$ PC12 cells. The concentrations used were determined to elicit a maximum secretion response from PC12 cells after a preliminary dose response for each secretagogue was performed. The data shown is from single experiments. **B).** Secretagogues were added to erythrophores without PC12 cells present. The secretagogue concentrations were the same as in part A (above). Each secretagogue was suspended in 200µl low K^+ buffer and injected at the time point indicated.

Figure 4.4


PC12 effluent
20 nmole ATP
2 nmole Acetylcholine
2 nmole Carbachol
20 nmole Bradykinin
12 mmole K+
Secretagogue
Chromatophore Response
Time (minutes)
0.5
0.4
0.3
0.2
0.1
0
-0.1
0
2
4
6
8

A).


No PC12 cells
Secretagogue
Chromatophore Response
Time (minutes)
0.2
0.1
0
-0.1
0
2
4
6
8

B).

modulate intracellular Ca^{2+} concentration. High K^+ concentration depolarizes the plasma membrane, which activates voltage-dependent Ca^{2+} channels. By recording the Chromatophore Response of chromatophores as an indicator of the level of secretion induced by each secretagogue, ATP elicited the strongest response, followed by carbachol and acetylcholine, both of which bind to the nicotinic (N_N-) subtype of cholinergic receptor ion channels receptors (Hardman and Limbird 1996). Bradykinin elicited the smallest response of the secretagogues tested. Bradykinin binds to G-protein linked bradykinin-B_2 receptors which leads to the activation of phospholipase C and thus an increase in IP_3 and the release of intracellular Ca^{2+} (Weiss and Atlas 1991). In control experiments, each of the secretagogues discussed was injected directly to erythrophore cultures and determined not to cause a direct morphological change on erythrophores (Figure 4.4B). At the conclusion of each of these control experiments, chromatophores were challenged with a 1μM dose of norepinephrine to ensure that each secretagogue did not have a detrimental effect on the cells' ability to aggregate. In each case aggregation proceeded normally (data not shown). These controls were important to establish that the effects on erythrophores were mediated exclusively through secretagogue effects upon PC12 cell neurosecretion, not upon erythrophores directly.

The protein neurotoxin α-latrotoxin, derived from black widow spider venom, is known to bind numerous nerve cell types, resulting in the rapid and massive release of stored neurotransmitter (Davletov *et al.* 1998); (Meldolesi *et al.*

1984). α-Latrotoxin was tested with the two-cell system to study how a specific neurotoxic compound affects the system (Figure 4.5). Upon injection of the toxin at the time point indicated, PC12 cell secretion occurred in a fashion similar to the other secretagogues. Maximum aggregation of erythrophores was followed rapidly by recovery to almost the original dispersed state. This showed that the erythrophores used in this trial were viable throughout the time of exposure to the toxin and were not adversely affected by α-latrotoxin exposure.

To quantify the levels of neurotransmitter secretion from PC12 cells using the two-cell system, a calibration study was performed in which known quantities of the catecholamine norepinephrine were added directly onto erythrophores and their responses recorded as shown in Figure 4.6A. These data showed that the initial rate of aggregation for each dose (spanning six orders of magnitude, 10^{-9} mole through 10^{-15} mole) was approximately equal. However, the magnitude of the Chromatophore Response value increased with higher norepinephrine doses. At each dose, washout of norepinephrine was followed by the eventual redispersion of erythrophores to their original morphology. At doses of norepinephrine greater than one nanomole, the extent of aggregation did not increase beyond the maximum Chromatophore Response level equal to 0.5. However, at those high norepinephrine doses, the norepinephrine washout and restoration of the basal state took over 30 minutes. This is demonstrated by the trace in Figure 4.5A showing the response of the system to a dose of 500 nanomole of norepinephrine. At very

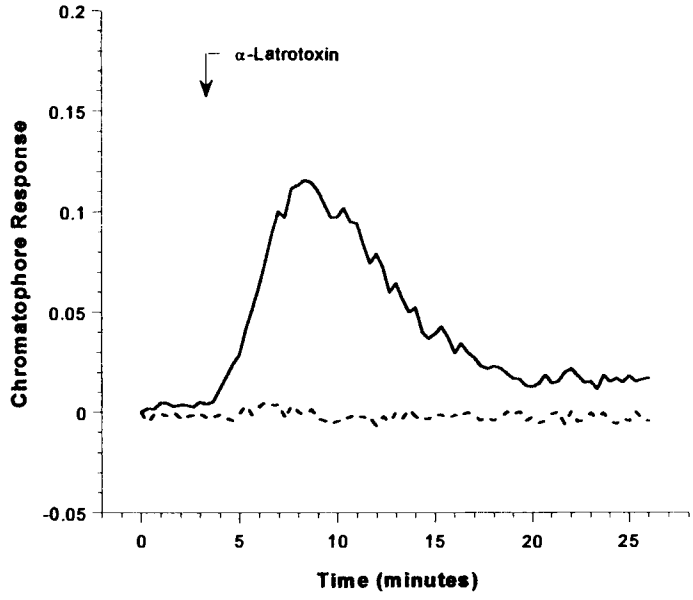
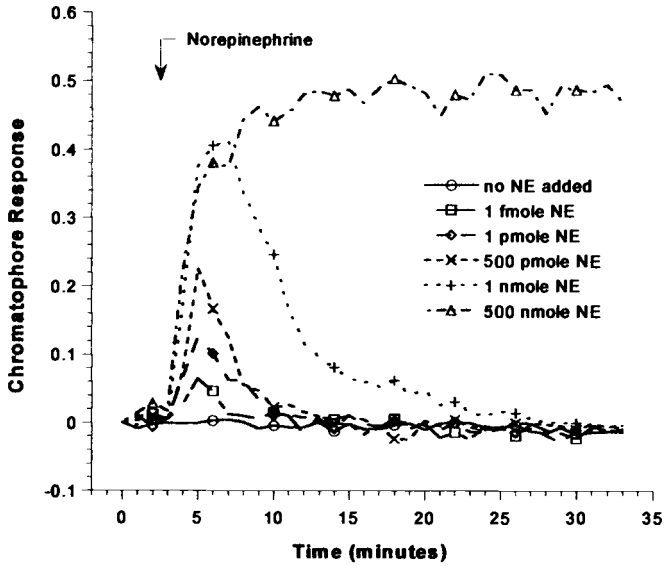


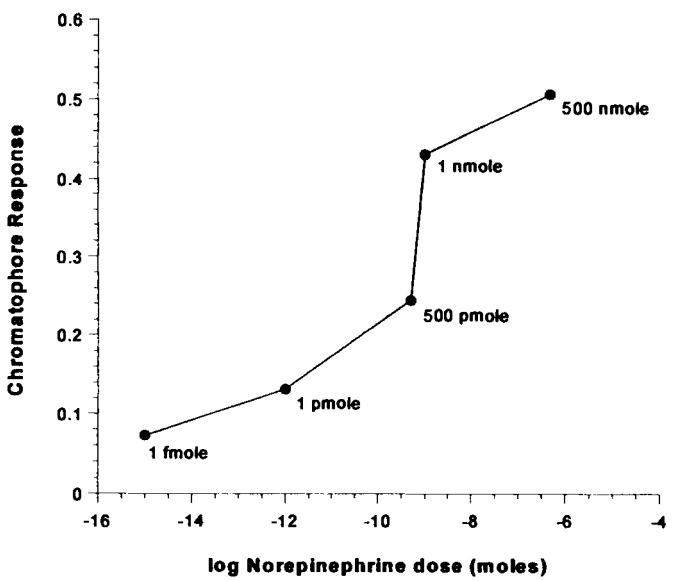
Figure 4.5: Neurotransmitter secretion from PC12 cells in response to α-latrotoxin. 5 μg/ml α-Latrotoxin induced the release of neurotransmitter from 2 x 10^4 PC12 cells (solid line). Rapid aggregation was followed by almost complete recovery of erythrophores to the original dispersed state. α-Latrotoxin exposure to erythrophores in the absence of PC12 cells did not cause aggregation (dashed line).

Figure 4.6: Erythrophore aggregation responses after exposure to varying doses of norepinephrine. **A).** Each indicated amount of norepinephrine was suspended in 200 µl of low K^+ solution and injected directly onto erythrophores. **B).** The maximum Chromatophore Response value achieved for each norepinephrine dose in part 'A' was replotted versus the log norepinephrine dose to produce a calibration curve of aggregation responses to increasing dose.

Figure 4.6


Norepinephrine
no NE added
1 fmole NE
1 pmole NE
500 pmole NE
1 nmole NE
500 nmole NE
Chromatophore Response
Time (minutes)
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
0
5
10
15
20
25
30
35

A).


500 nmole
1 nmole
500 pmole
1 pmole
1 fmole
Chromatophore Response
log Norepinephrine dose (moles)
0.6
0.5
0.4
0.3
0.2
0.1
0
-16
-14
-12
-10
-8
-6
-4

B).

low norepinephrine concentrations (*e.g.* 1 fmole), the aggregation response was only barely discernable above the background noise of the system. Therefore, the optimal range of norepinephrine doses that this biosensor is able to quantify reliably is between 1 pmole and 1 nmole.

The maximum responses (magnitude) of the erythrophores to norepinephrine doses up to 500 nanomole (Figure 4.6A) were replotted in Figure 4.6B as Chromatophore Response versus log norepinephrine dose. By measuring the magnitude of Chromatophore Response achieved after PC12 exposure to a pulse of each secretagogue, the curve in Figure 4.6B was used to judge the equivalent level of secreted norepinephrine from $2x10^4$ PC12 cells that caused the aggregation response. These levels are listed in Table 4.1 as moles of secreted norepinephrine per PC12 cell for each secretagogue. Again, the region of the dose response curve that fell between 1 pmole and 1 nmole was used to determine the induced secretory responses in PC12 cells from secretagogues.

Table 4.1: Calibrated levels of catecholamine secretion comparing the erythrophore/PC12 cell biosensor to alternative methods reported in the literature.

Secretagogue (dose units)	Equivalent NE released (moles/cell)
ATP (20 nmole)	$4x10^{-14}$
High K^+ (12 μmole)	$3x10^{-14}$
Carbachol (2 nmole)	$1x10^{-14}$
Acetylcholine (2 nmole)	$< 5x10^{-17}$ [b]
Bradykinin (20 nmole)	$< 5x10^{-17}$ [b]
α-Latrotoxin (1 nanogram)	$< 5x10^{-17}$ [b]
Literature Values	
Nicotinic stimulation of PC12 cells [c] (Greene 1976)	$1x10^{-20}$
Various stimulation of PC12 cells [d] (Greene and Rein 1977)	$1x10^{-17}$
Nicotinic stimulation of PC12 cells [e] (Chen *et al.* 1994)	$\geq 31x10^{-21}$
Various stimulation of bovine chromaffin cells [e] (Wightman *et al.* 1991)	$1\text{-}10x10^{-18}$

[a] NE released values for each secretagogue were estimated from the norepinephrine response calibration curve (Figure 4.6B).
[b] The measurements fell toward the lower extremity of the calibration curve and are therefore reported as the minimum value that can reliably be reported using this technique.
[c] Released catecholamines were measured using the trihydroxyindole-fluorometric technique (Nagatsu 1973).
[d] Released catecholamines were measured using ^{3}H-norepinephrine release assays (Greene and Rein 1977).
[e] Released catecholamines were measured amperometrically using carbon fiber electrodes (Cahill *et al.* 1996).

4.5 Discussion

These results demonstrate that an effective two-cell biosensor can be designed by using the optical appearance of erythrophores to measure neural secretion. This has been accomplished using the PC12 neurosecretory cell line that releases the catecholamine neurotransmitter norepinephrine, which in turn causes pigment aggregation in erythrophores. The optically measured levels of secretion induced by various secretagogues fit well with expectations based on literature work (Greene and Rein 1977); (Greene and Tischler 1976). For example, measurements based on radiolabeled pools of norepinephrine have shown similar orders of magnitude of norepinephrine release compared to our measurements with the two-cell biosensor (Table 4.1). While our calculations assume that all of the aggregation of erythrophores is caused by norepinephrine release from PC12 cells, this assumption is certainly not completely true since PC12 cells also release sizable quantities of other neurotransmitters, namely dopamine, that will also contribute to the aggregation response. The effects of these additional agents could explain the higher overall levels of secretion measured using the two-cell assay. Nonetheless, the sensitivity and the reversibility of secretion-induced erythrophore aggregation are consistent with what is known about both cell types.

While in theory a two-cell biosensor could be built from a single living cell of each type in close contact, in practice the system was most robust with small populations of each type held in separate chambers. The dual-chamber

configuration of the biosensor described allowed independent switching of fluid streams and a comparison, for example, of the effects of constitutive PC12 secretion versus stimulated secretion. The use of a continual exchange of solution to introduce solutes to chromatophores is advantageous because it has allowed this method to monitor samples in real time and over extended periods. Because erythrophores are capable of reversible transport of pigment within the cytoplasm, the presence of a dispersal-inducing chemical or the simple removal of aggregation-inducing chemical by continuous flow will cause erythrophores to disperse after an initial aggregation event, allowing regeneration of the sensitivity of the erythrophores. Thus, the system is poised as described to act as an in-line monitor of aqueous samples. With relatively simple modifications, we have set up multiple cell culture wells of PC12 cells, whose secretory output is evaluated by transferring their supernatant fluid to an optically monitored well containing erythrophores. This "two-pot" version of the technique is suited for scale-up to multi-sample screening technologies.

The technique reported here can screen potentially neurotoxic samples and a functional biological response is portrayed. The ability of this system to detect the presence of an active neurotoxic chemical was demonstrated with the use of α-latrotoxin. This protein, the active toxic constituent in black widow spider venom, acts on PC12 cells by binding to specific receptors on the surface membrane (Schiavo *et al.* 2000). This leads to an increase in intracellular Ca^{2+} resulting in a rapid, massive release of neurotransmitter. Denatured or inactive α-latrotoxin

would not trigger a response in such a function-based biosensor. The ability of the two-cell biosensor to detect neurotoxic responses suggests that this technique may be applied for discrimination between genuine toxic threats and benign samples.

The tissues of many fish species were screened as possible sources of chromatophores for incorporation into this assay. From this search, erythrophores from male *Betta splendens* were chosen. These fish have large populations of erythrophores in their fin tissues that are readily cultured in dense numbers allowing variations between individual cells to be minimized by analyzing a cross-section of at least 100 cells in a microscopic field of view. Both melanophores (melanin pigment) and erythrophores (pteridine pigment) are present in this fin tissue and each aggregates their pigment efficiently in response to norepinephrine. One cell type is enriched in relation to the other depending on the color of the fish; this simple means was used to select appropriate specimens. It was determined empirically that erythrophores from *Betta splendens* survived for longer periods of time in primary culture and were superior to other tested cells in withstanding a continuous fluid flow.

PC12 cells were chosen for incorporation into this two-cell cytosensor for two reasons. First, we were able to identify culture conditions for PC12 cells that were compatible with the culture conditions for erythrophores. Second, PC12 cells are known to release catecholamines, particularly norepinephrine and dopamine, in a regulated fashion. Of the two types of catecholamines, we have found norepinephrine to be substantially more effective at causing aggregation in

erythrophores. The neurosecretory properties of PC12 cells make them a good model of neuroendocrine behavior and an excellent choice for coupling to chromatophores in a two-cell biosensor. The device constructed to perform this analysis used the continuous flow of liquid to transport secreted norepinephrine from PC12 cells to erythrophores, allowing each cell type to function concomitantly in independent chambers. The population size of PC12 cells ($2x10^4$ cells) was empirically chosen and the effectiveness of that number of cells is likely related to the fluid volumes that the secreted norepinephrine enters (which is related to tubing and chamber dimensions) and to the concentration of norepinephrine needed to evoke a pigment change in erythrophores.

The addition of secretagogues to PC12 cells induced a transient increase in neural secretion, which was detected downstream by chromatophores. However, it was important in the development of this assay to choose secretagogues that did not directly lead to an aggregating response in erythrophores because the configuration of the flow apparatus dictated that any chemical introduced to PC12 cells would also encounter erythrophores downstream. As shown by control experiments, the secretagogues studied here did not cause a direct response on erythrophores. In future studies of unknown chemicals, the agents need to be added to erythrophores directly as well as through PC12 cells to fully understand the potential responses. Despite this procedural detail, the additive effect of coupling the sensitivity of two cell types expands the functionality of this assay. Using a two-cell configuration, the number of agents capable of being detected by erythrophore aggregation is

markedly expanded, from the set of agents that affect chromatophores directly, to the wider set of agents that affect erythrophores through neural mediation.

The two-cell biosensor has potential for qualitative and quantitative evaluation of unknown samples. The erythrophore aggregation response was used here as a quantitative indicator of the amount of norepinephrine secreted by PC12 cells upon stimulation with known secretagogues. These secretagogues were expected to induce secretion as a rapid pulse. Other secretagogues in unknown samples might behave differently, causing steady secretion, or nonreversible effects due to neurotoxicity and other mechanisms. Thus, the signature response (response rate, response amplitude, recovery, etc.) resulting from application of a sample offers a potential means of distinguishing different compounds.

As with alternative techniques for detecting catecholamine release from live cells, the results obtained in this study are an effective representation of nerve cell secretory behavior. A common neurosecretion assay employs the method of Greene and Rein (Table 4.1) in which PC12 cell populations are preloaded with [^{3}H]-norepinephrine followed by the measurement of regulated secretion as a percentage of the total label contained within the cells. The PC12 cells used in the method described here do not require a preloading step with a separate pool of catecholamines different from endogenous pools. This simplifies experimental preparation as well as avoiding possible artifacts that may result if [^{3}H] labeled norepinephrine is processed differently at the presynaptic terminal than biosynthesized norepinephrine. Recently, amperometric measurement of

catecholamines using carbon fiber electrodes (CFE) has been utilized to measure catecholamine release from individual chromaffin cells (Albillos *et al.* 1997); (Leszczyszyn *et al.* 1990). This electrochemical method is effective at recording the short (millisecond) time course of exocytotic events from single cells and has been shown to be exceedingly sensitive by Ewing and colleagues (Table 4.1) who detected catecholamine release from PC12 cells using amperometric detection with CFEs in the zeptomole range (10^{-21}). This technique requires the positioning of electronics into the experimental setup. In addition, a wide variety of responses are possible between similarly treated cells. Radiolabeled norepinephrine assays, amperometric detection, and erythrophore optical changes each have their merits as methods to detect catecholamine secretion; the two-cell biosensor described here was not developed to replace the other methods. Instead, we have developed a means of monitoring norepinephrine neurosecretion using unaltered populations of two distinct cell types which results in a technique that is comparatively simple to set up and capable of monitoring an array of potential analytes beyond catecholamines exclusively.

This technique offers an intriguing opportunity for developing an "artificial synapse" for incorporation into a biosensor. In nature, chromatophores are synaptically coupled to sympathetic neurons. Given that PC12 cells are closely similar to sympathetic neurons, the system described here mirrors the natural situation in which the skin colors of cold-blooded animals are adjusted according to the momentary balance of active agents (neurotransmitters and hormones). This

concept can be expanded beyond the application described here to include other cell types that form physiological junctions leading to a measurable effect. For example, an endocrine or immune system cell could be substituted for PC12 cells in this assay because erythrophores have been characterized as responding directly to numerous hormones and immunomodulatory substances. Bringing different cell types together will further extend the number of compounds and toxic substances that are capable of being detected using cell-based biosensors.

Chapter 5

A Two-Cell Biosensor for the Detection of Botulinum Neurotoxins

R. Ryan Preston, Janine E. Trempy, and Philip N. McFadden

Formatted for Submission

5.1 Summary

A biosensor that uses the joint physiological activity of two distinct cell types has been developed to detect the presence of neurotoxins that inhibit neurotransmitter secretion. The pathogenic bacterium *Clostridium botulinum* produces and secretes protein toxins that are among the most toxic substances known; each targets specific sites within the peripheral nervous system resulting in paralysis and possible death. These toxins have also been listed as possible biological warfare threats. In the biosensor device introduced here, PC12 neuronal cells were pre-incubated with toxin samples and their liquid effluent then bathes a population of red-pigmented *Betta splendens* erythrophores. Secreted catecholamines present in the effluent from PC12 cells induced a characteristic pigment change in these erythrophores. However, upon pre-exposure of PC12 cells to toxins, the level of catecholamine secretion was inhibited to a level that altered the response observed in erythrophores. Digital analysis of the 'Chromatophore Response' was therefore an accurate gauge of the presence or absence of botulinum neurotoxins in screened samples. Using this method, purified botulinum toxin subtype A, a form common in human botulism infections, elicited the strongest response; the presence of subtypes B-F also registered responses to differing degrees in this two-cell configuration. This technique is an effective potential

application for screening unknown samples containing neurologically toxic substances. Further, the principles discussed demonstrate the functionality of live cells in biosensor applications.

5.2 Introduction

The protein toxins produced by the pathogenic bacterium *Clostridium botulinum* are among the most potent toxic substances known, and so detection of these toxins in their active form is a practical problem in the area of analytical biochemistry. Botulinum neurotoxins are produced in seven serotypes, A-G, each of which inhibits acetylcholine release from peripheral cholinergic neurons resulting in flaccid paralysis. Serotypes A, B, and E have been described as having the greatest impact on human populations while subtypes C and D most commonly affect animal populations (Hatheway 1990). All are zinc proteases (Pellizzari *et al.* 1999); (Schiavo *et al.* 1992a) that are specific for components involved in exocytosis (Lomneth *et al.* 1991); (Shone and Melling 1992). Each serotype has a similar protein structure consisting of a 100 kD heavy chain and a 50 kD light chain connected by a single disulfide bond and non-covalent interactions (Lalli *et al.* 1999); (Umland *et al.* 1997). The heavy chain mediates receptor-specific membrane binding on target neurons (Halpern and Neale 1995); (Poulain *et al.* 1988), followed by internalization into acidic endosomes (Matteoli *et al.* 1996).

Reduction and dissociation of the two-chain form is followed by translocation of the light chain into the presynaptic cytosol (Pellizzari *et al.* 1999). Only three proteolytic targets exist, each of which is implicated in vesicular fusion with the presynaptic membrane: synaptobrevin (Schiavo *et al.* 1992), SNAP-25 (Blasi *et al.* 1993), and syntaxin (Schiavo *et al.* 1995).

Toxin-producing bacterial strains, primarily of *Bacillus anthracis* and *Clostridium botulinum*, have gained recent attention as potential biological warfare agents (Cohen and Marshall 2001). Widespread fears about such a possibility have generated the need for improved detection methods of these and other threats. The mouse bioassay (Hatheway 1988) is currently the most accepted means of detecting the toxicity of unknown samples. The primary benefits of this technique are that it measures a true physiological response and is very sensitive (*e.g.* toxin detection limit of 10-20 pg/ml, Notermans and Nagel 1989). However, it is also expensive, very difficult to analyze samples outside of a laboratory, and involves the use of live animals. Further, clinical symptoms of infection show up in mice only after several days. For these reasons, alternative methods of detection have been sought. One such method utilizes the polymerase chain reaction (PCR) to amplify template DNA from infectious bacterial strains using primers developed from known bacterial sequences (Franciosa *et al.* 1994); (Szabo *et al.* 1993); (Takeshi *et al.* 1996). Methods using PCR are very sensitive (*e.g.* capable of detecting as few as 5 bacteria/reaction, Fach *et al.* 1993) but are prone to false positives from dead or non-pathogenic strains and cannot detect toxin presence directly. Another method

uses toxin-specific antibodies in an adaptation of an enzyme-linked immunosorbent assay (ELISA) (Doellgast *et al.* 1993); (Lewis *et al.* 1981); (Shone *et al.* 1985). This technique is rapid and sensitive but depends on antibody quality and does not remain sensitive to mutated or denatured forms of the toxin. Another alternative method for detecting botulinum toxicity *in vitro* is based on the toxin's endopeptidase activity (Hallis *et al.* 1996); (Wictome *et al.* 1999). An immobilized synthetic substrate (*e.g.* representing synaptobrevin or SNAP-25) is cleaved in the assay and the truncated version is analyzed after the addition of an antibody-enzyme substrate. Each of these techniques aims to mirror a relevant physiological response using an *in vitro* assay. An improved technique would be one that measures a biologically relevant response, as in the mouse bioassay, but one that is not encumbered by the use of live specimens.

The use of cellular assays offers a unique potential for biosensor applications that utilizes the physiological response of live cells for the detection of a wide array of potential analytes. The application of live cells for this purpose allows for the simultaneous detection of large numbers of bioactive agents, including unknown ones. In addition, such cellular assays can characterize the functionality of known substances that have been previously identified by more conventional methods based on chemical and nucleic acid principles. The choice of cells for incorporation into such devices is usually based on their physiological output, such as electrical excitability, cytokine release, or functional genomic expression using cDNA microarrays (Stenger *et al.* 2001). Other important

determinants of successful cell-based assays are suitable cell culture conditions that allow for storage and portability, sensitivity, and rapidity of the response.

In this study, we report the demonstration and characterization of a cellular biosensor to detect toxicity induced by the protein toxin botulinum A. This technique pairs two unique cell types: the neurosecretory cell line PC12 and pigmented erythrophores from the teleost species *Betta splendens.* PC12 cells are a common model system for neurosecretion, especially of catecholamine neurotransmitters, which include dopamine and norepinephrine (Greene 1982). These neurotransmitters are released from PC12 cells both constitutively and in a regulated manner in response to a variety of secretagogues. Botulinum A exposure to PC12 cells results in inhibited catecholamine secretion after toxin binds to and disrupts the exocytotic machinery (Chen *et al.* 2001); (Lomneth *et al.* 1991); (McInnes and Dolly 1990); (Shone and Melling 1992). The chromatophore subtype chosen for use in this application, erythrophores, are dendritic cells that contain motile, pigmented organelles dispersed within their cytoplasm. Treatment of these chromatophores with catecholamines causes these organelles to be aggregated centripetally toward the cell body, an activity that can be observed optically. Conversely, removal of this aggregating signal allows pigment to redisperse out into the cell's periphery. The biosensor device described placed PC12 cells and erythrophores within close proximity in separate cell chambers so that catecholamine neurosecretion induced the aggregation/dispersion activity of erythrophores.

5.3 Materials and Methods

5.3.1 Cell Culture

Isolation of erythrophores from individual *Betta splendens* was performed according to the method described previously (Preston and McFadden 2001; chapter 4, page 55 of this dissertation).

PC12 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown at 37°C / 5% CO_2 on plastic tissue culture flasks coated with 5μg/cm^2 collagen IV in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% horse serum (Sigma) and 5% fetal bovine serum (HyClone).

5.3.2 Toxin Preparation and Exposure

All handling and experimentation with botulinum toxins was performed in a certified Biosafety level 2 (BSL 2) laboratory.

Purified botulinum toxin types A-F were obtained from Calbiochem (La Jolla, CA, USA) as stock solutions in acetate buffer (200 μM NaCl, 50 μM sodium acetate, pH 6.0). Toxin was added directly to PC12 cell culture flasks at the concentrations indicated. During toxin incubations, PC12 cells were cultured under normal conditions (*e.g.* serum supplemented RPMI 1640, 37°C, 5% CO_2). One hour before an experiment, PC12 cells were removed from the culture flask by repeated

passage through a pipet, counted on a Neubauer hemacytometer, and dispensed into aliquots at a concentration of 2 x 10^5 PC12 cells/ml in RPMI 1640 medium.

5.3.3 Biosensor Device Setup

The biosensor device used to collect data consists of two cell chambers (one each for PC12 cells and erythrophores) connected by a network of 0.020-inch inner diameter tubing that allowed liquid transfer from the PC12 chamber to the erythrophore chamber. The flow of liquid within the network was maintained by a syringe pump (World Precision Instruments model sp200i, Sarasota, FL, USA) at a flow rate of approximately 200 µl/min. Immediately prior to an experiment, 4 x 10^4 PC12 cells were injected into the chamber (a 0.22µm in-line HPLC filter) and equilibrated with continuous flow of 'low K^+' solution (125mM NaCl, 4.8mM KCl, 1.3mM $CaCl_2$, 1.2mM $MgSO_4$, 1.2mM HK_2PO_4, 5.6mM glucose, 25mM HEPES, pH 7.4). After the PC12 cells were assembled into the device, cultured erythrophores on glass cover slips were inserted into a closed-bath imaging chamber (Warner Instrument Corp. model RC-20H, Hamden, CT, USA) that was mounted in the line of flow downstream of the PC12 cell chamber. During data collection, flow through the network of tubing was changed from 'low K^+' to 'high K^+' (69mM NaCl, 56mM KCl, 1.3mM $CaCl_2$, 1.2mM $MgSO_4$, 1.2mM HK_2PO_4, 5.6mM glucose, 25mM HEPES, pH 7.4). The membrane depolarization that resulted from the elevated K^+ concentration acted as a secretagogue in order to

stimulate catecholamine release from PC12 cells. Effluent was discarded after passing over the erythrophores.

5.3.4 Data Analysis

The chromatophore chamber was mounted on the stage of a Leica DM IL inverted microscope (Leica, Inc., Wetzlar, Germany) fitted with a 10x objective. A Spot Insight Color CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) attached to a Pentium III computer captured digital images (1600 x 1200 resolution) every 20 seconds.

Image analysis of pigment aggregation and dispersion in erythrophores after exposure to PC12 cell effluent was performed using the method described by Preston and McFadden (2001; chapter 4, page 57 of this dissertation).

5.4 Results

To test whether the botulinum toxins had a direct effect on erythrophores, botulinum toxin subtypes were added to cultured *B. splendens* erythrophore cultures and these had no apparent effect on pigment motility (data not shown). None of the toxin samples containing subtypes A-F elicited aggregation or dispersion of pigment. Further, no impairment of pigment motility was observed, as toxin-treated erythrophores responded normally to a pigment-aggregating

stimulus (100nM norepinephrine). Therefore, to test whether erythrophores could indirectly report the presence of these agents, PC12 neuronal cells in cell culture were included as an intermediate cell-type that coupled botulinum toxicity to chromatophore pigmentation.

Figure 5.1 is a schematic illustration of the operation of the coupled-cell biosensor that was developed. PC12 cells were first exposed to toxin in their culture flasks at 37° C. The PC12 cells were then harvested from the flasks, counted, and an aliquot of cells was dispensed into the PC12 cell chamber. The apparatus consisted of two cell chambers connected by the pump-driven flow of liquid through a network of tubing. Effluent from PC12 cells that contained the secreted neurotransmitter product (catecholamines) was carried from the PC12 cell chamber to the second cell chamber that contained a cultured population of erythrophores mounted over an inverted microscope. Upon exposure to 'high K^+' solution, it was found that PC12 cells not exposed to botulinum toxin caused the aggregation of pigment in erythrophores. With prior exposure of PC12 cells to botulinum neurotoxins, the aggregation response of erythrophore pigment to PC12 cell effluent was decreased (Figure 5.1). Chromatophores that were not treated with toxin aggregated almost all of their pigment into the cell body, whereas the toxin treated cells had a much-diminished response.

A more complex demonstration of the erythrophore-PC12 arrangement to detect botulinum toxin A is shown in Figure 5.2. In this case, two separate

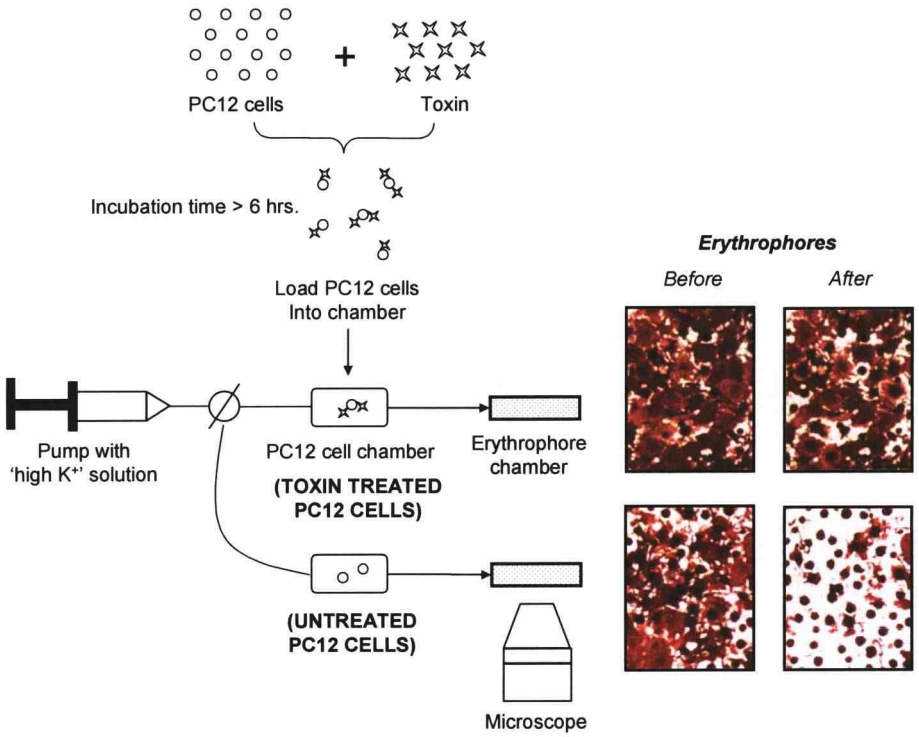


Figure 5.1: Schematic representation of the biosensor described in this study. PC12 cells and botulinum toxin samples were incubated at 37° C for greater than 6 hours prior to loading a defined population of cells into the PC12 chamber. A syringe pump moved the flow of 'high K^+' effluent (containing secreted catecholamine products) from the PC12 cell chamber into the erythrophore chamber. The resulting aggregation response of erythrophores ('Chromatophore Response') was tracked over time by collecting and analyzing digital images from an inverted microscope. Untreated PC12 cells elicited a much greater aggregation response than toxin-treated cells, as seen in the example images before and after effluent exposure to erythrophores.

populations of PC12 cells were prepared: routinely cultured, untreated cells and similarly cultured cells that had been pre-incubated with 10nM botulinum toxin A for 12 hours. The continuous flow of 'high K^+' solution served to induce secretion in PC12 cells and carry this effluent into a separate erythrophore chamber. Exposure of the effluent from $4x10^4$ PC12 cells (untreated) caused rapid pigment aggregation in erythrophores; this aggregation persisted as long as the flow of PC12 effluent was maintained. This number of PC12 cells secreted effluent that caused erythrophores to aggregate completely, which resulted in a 'Chromatophore Response' (C.R.) range of 0.3-0.4. When the flow of 'high K^+' was redirected to pass over $4x10^4$ toxin-treated PC12 cells, erythrophore pigment dispersed almost completely over several minutes. The dispersion of erythrophore pigment in this experiment demonstrated the difference in the concentration of catecholamines in the effluents of the two PC12 cell populations.

The ability of botulinum toxin A to inhibit catecholamine secretion in PC12 cells was counteracted if the protein toxin was first heat denatured (Figure 5.3). Prior to the addition of effluent at 2 minutes, erythrophores remained dispersed, as is normal for untreated cells in culture. Upon addition of effluent from untreated PC12 cells, aggregation was rapid and remained constant for the duration of exposure to effluent. Untreated PC12 cells elicited a much larger response than did the PC12 population that was treated with 10nM native botulinum A for 12 hours. A similar experiment was run where the toxin sample was denatured (boiled for 30

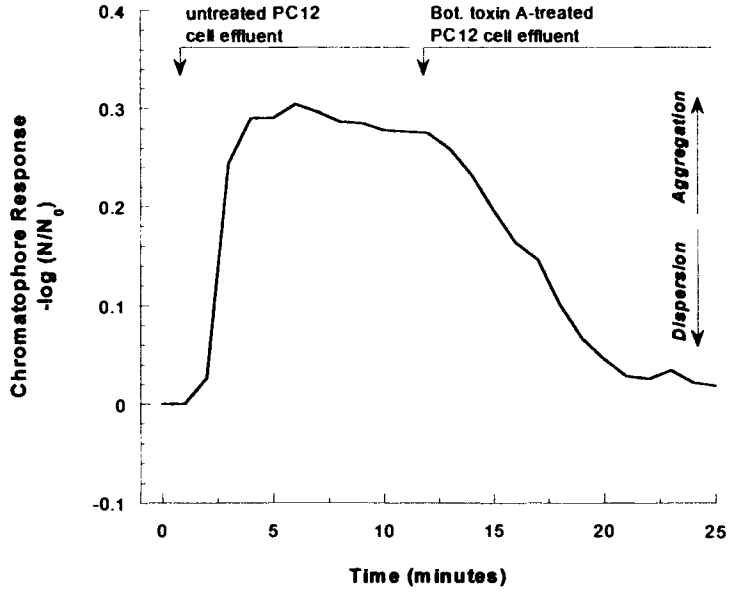


Figure 5.2: Response of erythrophores to treatment with untreated- and botulinum toxin A-treated PC12 cell effluent in sequence. Dispersed erythrophores in culture were first exposed (at 1 minute) with effluent from $4x10^4$ untreated PC12 cells. The resulting aggregation persisted as long as the erythrophores were exposed to the effluent. Flow was then redirected (at 12 minutes) over $4x10^4$ PC12 cells that were previously treated with 10nM botulinum A for 12 hours, which resulted in erythrophore pigment dispersion.

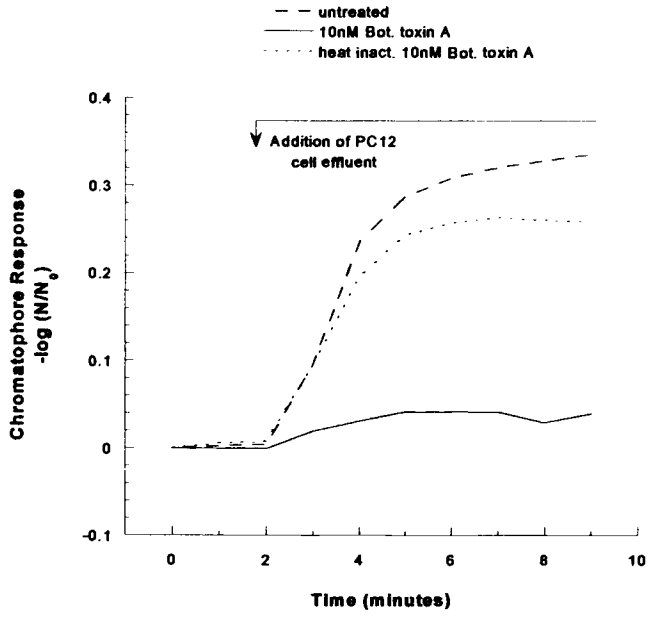


Figure 5.3: Biosensor response to native and inactivated botulinum A. Each experiment was performed with $4x10^4$ PC12 cells loaded into the apparatus. Botulinum toxin samples (10nM) were incubated with PC12 cells for 12 hours. Toxin inactivation (*e.g.* heat denaturation) was accomplished by boiling toxin samples for 30 minutes prior to incubation with PC12 cells.

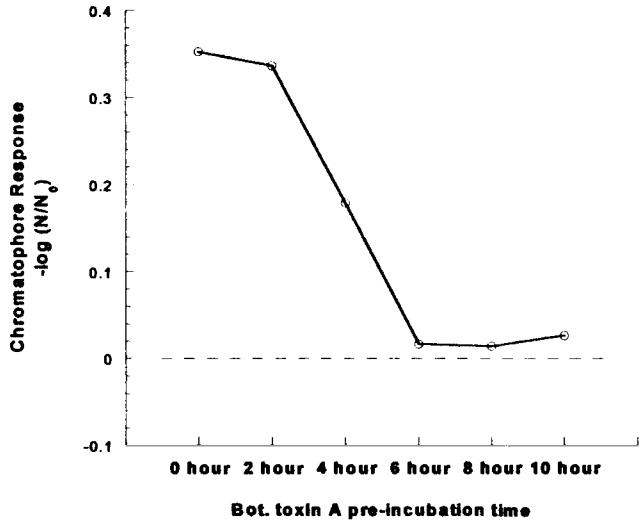
minutes) prior to the 12-hour incubation with PC12 cells. Heat treatment is generally thought to inactivate this protein toxin (*e.g.* suspect food samples are considered decontaminated after vigorous boiling). This denatured toxin resulted in erythrophore aggregation almost to the level of the untreated PC12 cells, which demonstrated the inhibitory action on secretion of the toxin on PC12 cells.

A series of experiments was performed in order to ascertain the minimum necessary pre-incubation time of exposure of PC12 cells with toxin (10nM botulinum A) before secretion attenuation was observed. The resulting time course (Figure 5.4A) revealed that secretion levels after incubation times of less than 2 hours were similar to untreated PC12 cells. Incubations of at least six hours maximally inhibited neurosecretion as measured using this two-cell method. Experiments to determine the sensitivity of this assay to botulinum A concentration (Figure 5.4B) showed that aggregation inhibition in erythrophores over untreated levels appeared at 10pM botulinum A in the pre-incubation medium (12 hours). However, this relatively small difference in aggregation (C.R. = 0.29 versus 0.31 for untreated PC12 cells) was not conclusive. A clearer inhibition of neurosecretion was observed at 100pM (C.R. = 0.19) and a strong response was observed at toxin concentrations larger than 1nM.

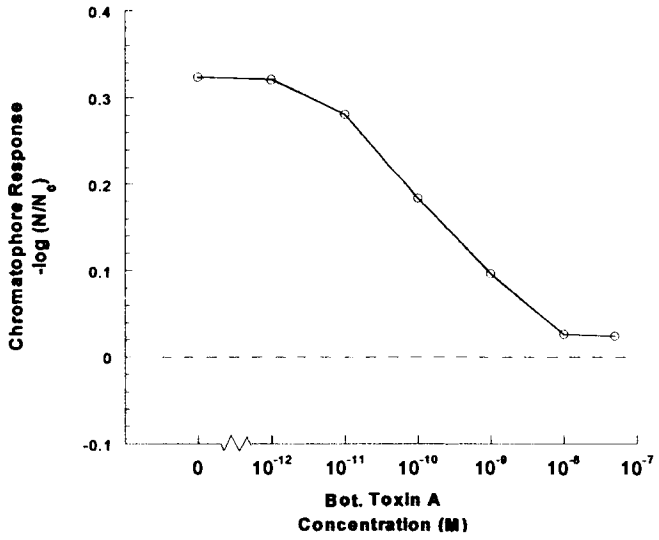
Initial screening studies of various botulinum subtypes using this assay revealed the strongest neurosecretory inhibition with botulinum A. Therefore, botulinum A toxin was used in the characterization of the biosensor presented in

Figure 5.4: A). Time course of biosensor response to botulinum A. PC12 cells were similarly treated with 10nM botulinum A for differing incubation times. $4x10^4$ PC12 cells were used in each experiment. The dashed line at 'Chromatophore Response = 0 represents no erythrophore pigment change (aggregation nor dispersion). **B). Dose response to botulinum toxin A.** PC12 cells were exposed to botulinum A at differing concentrations for 12 hours. (For all assays, $4x10^4$ PC12 cells were used.) See main text for discussion.

Figure 5.4


0.4
0.3
0.2
0.1
0
-0.1
Chromatophore Response
-log (N/N0)
0 hour 2 hour 4 hour 6 hour 8 hour 10 hour
Bot. toxin A pre-incubation time

A).


0.4
0.3
0.2
0.1
0
-0.1
Chromatophore Response
-log (N/N0)
0
10-12
10-11
10-10
10-9
10-8
10-7
Bot. Toxin A
Concentration (M)

B).

this study. When measured using erythrophore aggregation, subtype A resulted in a greater than 90% decrease in neurotransmitter secretion compared to untreated PC12 cells (Table 5.1). Among the B-F subtypes of botulinum toxins tested, each inhibited neurosecretion in PC12 cells to varying degrees. Further characterization and assay refinement may establish this coupled-cell biosensor as an effective method for the detection of multiple botulinum subtypes.

5.5 Discussion

In this report we describe a novel two-cell bioassay that effectively detects the neurosecretory inhibition in mammalian chromaffin-like secretory cells resulting from botulinum neurotoxin type A infection. In addition, we report results about preliminary studies for the use of this instrumentation to detect botulinum toxins B-F. The biosensor described couples the physiological activity of the PC12 cell line and the pigmented erythrophores from *Betta splendens*. The regulated secretion from PC12 cells, and the subsequent attenuation of secretion following toxin exposure, is monitored by the extent of pigment aggregation in erythrophores. This was accomplished using an apparatus consisting of two separate cell chambers, one each for PC12 cells and erythrophores, which were connected by a system of microfluidics. This configuration was optimized to meet

Table 5.1: Two-cell biosensor responses to botulinum neurotoxin subtypes A-F.

PC12 treatment [a]	***% Chromatophore Response*** [b] native toxin *(heat inact.)* [c]
untreated	100
A	8.0 ± 5 (*91 ± 7*)
B	35 (*85*)
C	56 (*96*)
D	67 (*80*)
E	45 (*83*)
F	48 (*87*)

[a]: Botulinum-treated PC12 cells were incubated for 12 hours with toxin samples prior to screening. $4x10^4$ PC12 cells were used in each experiment.
[b]: Chromatophore Response value (untreated) is the mean of 5 experiments. Percentages for toxin-treated samples were calculated relative to the aggregation of erythrophores after exposure to $4x10^4$ untreated PC12 cells.
[c]: Values listed for botulinum subtype A are the mean of 4 experiments. Values listed for botulinum subtypes B-F are each from single experiments.

several important criteria. First, continuous flow through separate cell chambers prevented the buildup of catecholamine concentration in a single chamber co-culture of both cell types that would over stimulate the erythrophore population, causing complete and irreversible aggregation. Second, the direct movement of effluent between chambers allowed catecholamine secretion to be monitored in real time. Third, this device allowed for differing treatments between each cell population before assembly into the biosensor. This is of primary importance because PC12 cells required the pre-incubation of neurotoxin; secretion of catecholamines from PC12 cells was inhibited greater than 90% after 6 hours of exposure with purified botulinum A. Further, *B. splendens* erythrophores also responded directly to numerous other signal transduction and cytotoxic agents under investigation (unpublished results), which expands the range of potential analytes for this cytosensor method to direct erythrophore pigment effects as well as effects mediated by PC12 cell secretion.

The decision to incorporate PC12 cells into this assay methodology was made because of their established use in the study of cellular secretion of catecholamines such as norepinephrine and dopamine. This cell line secretes these neurotransmitters at a constitutive, basal level and at elevated levels in a Ca^{2+} regulated fashion in response to certain secretagogues. We have chosen the use of high K^+ concentration (56mM) to depolarize the plasma membrane and subsequently open voltage-sensitive Ca^{2+} channels, ultimately leading to neurosecretion. The release of catecholamines is an important prerequisite for this

assay because *B. splendens* erythrophore pigment responded well to catecholamine treatment by aggregating their pigment from the periphery into the cell body. Further, PC12 cells have been previously used to study the mechanism of secretion inhibition caused by botulinum A neurotoxin in intact (Shone and Melling 1992) and permeabilized cell models (Lomneth *et al.* 1991); (McInnes and Dolly 1990), establishing this cell line as a viable model for botulinum toxicity.

As in the development of any alternative diagnostic and analytical technique, the comparison of sensitivity and specificity to current methods is an important consideration. The current benchmark for the detection of botulinum is the mouse bioassay. This method is an accurate predictor of infection and is extremely sensitive; however, it also expensive, requires a large number of laboratory animals, and lacks specificity unless antisera neutralization tests are performed in parallel. Other alternatives include PCR detection of toxin genes, ELISA, and enzymatic activity analysis of the toxins directly (Table 5.2). The sensitivity of the first generation method described here was able to detect toxin concentrations of approximately 100pM, or 15ng toxin/ml sample, a sensitivity that is similar to the endopeptidase activity test. While not as sensitive as the mouse assay or ELISA, it may be possible to improve sensitivity with further refinements. A possible drawback of the PC12-erythrophore assay described here is its broad specificity: the output of this assay, the aggregation of erythrophore pigment, cannot distinguish between toxin subtypes. Therefore, this technique is most suited

Table 5.2: Comparison of sensitivity and specificity of the PC12-erythrophore biosensor with other methods to detect botulinum A toxin

Technique	*Minimum toxin sensitivity*	*Toxin specificity*[a]	*Assay time*	*Reference*
PC12-erythrophore biosensor	15ng/ml	No	hours	This report
Mouse assay	10-20pg/ml	Yes[b]	days	(Hatheway 1988)
ELISA	< 10pg/ml	Yes	minutes	(Doellgast *et al.* 1993) (Shone *et al.* 1985) (Thomas 1991)
Endopeptidase activity	0.6-4.5ng/ml	Yes	minutes	(Hallis *et al.* 1996)
PCR	NA[c]	Yes	hours	(Fach *et al.* 1993) (Franciosa *et al.* 1994) (Szabo *et al.* 1993)

[a]: biosensor capability to distinguish between toxin subtypes
[b]: parallel live-animal tests with specific neutralizing antisera is necessary
[c]: sensitivity in the PCR method is based on template DNA present; direct toxin presence not tested

as an early screening method to produce possible targets for later corroboration with other, more specific techniques. The rapidity of response with this method would allow for results on the toxicity of a sample to be completed within one day (the time required for pre-incubation of toxin with PC12 cells: ≥ 6 hours).

Like the mouse assay, the output of this method is a physiological response. The inhibition of neurosecretion is ultimately the cause of the affliction botulism, and it is this activity in PC12 cells that was measured with this method. However, unlike the mouse assay, live animal specimens are not used. Unlike *in vitro* toxin detection methods, false positive and negative results may be minimized using the two-cell biosensor method described here because the actual physiological effect of the toxin is observed instead of an arbitrary marker. The output data generated by erythrophore pigment aggregation offers a novel form of analysis. Monitoring pigment translocation is beneficial in that it is a direct optical observation. The equipment necessary to observe changes are a low-power (*e.g.* 10x magnification) light microscope and a computer for analysis. By avoiding more complicated hardware, sample preparation, and data analysis requirements this technique may be modified for portability and cost effectiveness.

The two-cell biosensor method described here may also be applied to the detection of other neurotoxins. For example, tetanus toxin is closely related to botulinum in both structure and effect on neurosecretion. In addition, PC12 cells have also been applied to study the mechanism of tetanus (Sandberg *et al.* 1989); (Walton *et al.* 1988). Further advancement and characterization are needed before

this method can become a viable alternative to current detection techniques such as the mouse assay. However, the preliminary results for botulinum A (as well as subtypes B-F) suggest that this cytosensor technology may develop into a useful system for the diagnosis of toxicity in unknown samples.

Chapter 6

Concluding Remarks

The series of studies that comprise this dissertation describe the utilization of chromatophores as biosensors that were able to detect and characterize a range of biologically active substances. Chromatophores are uniquely suited for these applications because of the optical nature of pigment motility that occurs in these cells following exposure to a stimulating agent. The analysis of pigment motility in a biosensor application is a means of reporting the presence of a substance. A variety of signaling principles regulate the intracellular transport of pigmented organelles in chromatophores (Fujii 2000). An understanding of how these principles affect chromatophore pigmentation makes these cells useful in studying the specific cellular responses to active agents. However, the physiological nature of the chromatophore response also allows for the detection of an array of agents that impact live cells.

By incorporating chromatophores into biosensor methodologies that include other cell types, the breadth of agents capable of being detected is increased and the specificity of agent classification can be discerned. The work presented here demonstrates two general types of multi-cellular biosensors involving chromatophores. The first involves comparing the response induced by an agent of

interest in one chromatophore subtype to the response in another chromatophore subtype. This 'chromatophore comparison' biosensor is described in Chapters 2 and 3. The initial questions to be addressed in the development of this biosensor pertained to the choice of chromatophore subtypes to focus on and the source for these chromatophores. Numerous species of fish were examined and the responses of melanophores and erythrophores were chosen for comparison because each type was present in the same species and the pigment response mechanism is similar in each. Both melanophores and erythrophores have a dendritic morphology and aggregate and disperse pigment, which allowed for a similar comparison of cellular responses. The important differences between these two chromatophore subtypes were the color of their pigment (black: melanophores and red: erythrophores), which allowed for a convenient distinction between them, and the observation that the pigment within these two cell types responded differently to particular agents under investigation. It was observed in skin tissue from *Hemichromis bimaculatus* (Jewel chiclid) that exposure to cholera toxin impaired the norepinephrine-induced aggregation of pigment in erythrophores, but not melanophores (Chapter 2). In mixed primary cultures of melanophores and erythrophores from *Betta splendens* (Siamese fighting fish), Ca^{2+} channel antagonists selectively resulted in the pigment dispersion of erythrophores, but not melanophores, that had been exposed to norepinephrine (Chapter 3). These results suggest that bioactive agents can be detected and their effects characterized by observing the differential responses of two chromatophore subtypes.

The second multi-cellular biosensor described in this dissertation utilized pigment motility in chromatophores as a reporter for the activity of a second, non-chromatophore cell type. In this 'two cell' biosensor, catecholamine secretion from PC12 neuroendocrine cells was analyzed by observing the pigment aggregation response in a population of *B. splendens* erythrophores (Chapters 4 and 5). To accomplish this, instrumentation was built that connected separate populations of PC12 cells with erythrophores in a network of fluidics. Secretion of catecholamines from PC12 cells bathed erythrophores, inducing pigment aggregation. Elevated neurosecretion from PC12 cells in response to exposure with secretagogues was quantified by analyzing the extent of the erythrophore aggregation response (Chapter 4). Analysis of the inhibition of neurosecretion from PC12 cells after exposure to botulinum neurotoxins was also accomplished using a modified version of this coupled cell technique (Chapter 5). By using erythrophore pigment aggregation as a reporter of PC12 cell neurosecretory behavior, it was possible to detect the presence of agents that did not have a direct effect on erythrophore pigment motility.

BIBLIOGRAPHY

Albillos A, Dernick G, Horstmann H, Almers W, Alvarez dT, Lindau M (1997) The exocytotic event in chromaffin cells revealed by patch amperometry. *Nature* 389: 509-512.

Andersson RG, Karlsson JO, Grundstrom N (1984) Adrenergic nerves and the alpha 2-adrenoceptor system regulating melanosome aggregation within fish melanophores. *Acta Physiol Scand* 121: 173-179.

Bagnara JT (1991) An historical perspective on pigment cell biology from the editor. *Pigment Cell Res* 4: 2-6.

Blasi J, Chapman ER, Link E, Binz T, Yamasaki S, De Camilli P, Sudhof TC, Niemann H, Jahn R (1993) Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature* 365: 160-163.

Bokoch GM, Katada T, Northup JK, Hewlett EL, Gilman AG (1983) Identification of the predominant substrate for ADP-ribosylation by islet activating protein. *J Biol Chem* 258: 2072-2075.

Burgess P, Bailey M (2000) *Tropical Fishlopaedia : A Complete Guide to Fish Care*. Hungry Minds, Inc.: New York.

Cahill PS, Walker QD, Finnegan JM, Mickelson GE, Travis ER, Wightman RM (1996) Microelectrodes for the measurement of catecholamines in biological systems. *Anal Chem* 68: 3180-3186.

Chaplen FW, Upson RH, McFadden PN, Kolodziej W (2002) Fish chromatophores as cytosensors in a microscale device: detection of environmental toxins and bacterial pathogens. *Pigment Cell Res* 15: 19-26.

Chen TK, Luo G, Ewing AG (1994) Amperometric monitoring of stimulated catecholamine release from rat pheochromocytoma (PC12) cells at the zeptomole level. *Anal Chem* 66: 3031-3035.

Chen YA, Scales SJ, Duvvuri V, Murthy M, Patel SM, Schulman H, Scheller RH (2001) Calcium regulation of exocytosis in PC12 cells. *J Biol Chem* 276: 26680-26687.

Cohen J, Marshall E (2001) Bioterrorism. Vaccines for biodefense: a system in distress. *Science* 294: 498-501.

Cole NB, Lippincott-Schwartz J (1995) Organization of organelles and membrane traffic by microtubules. *Curr Opin Cell Biol* 7: 55-64.

Danosky TR and McFadden PN (1997) Biosensors based on the chromatic activities of living, naturally pigmented cells: digital image processing of the dynamics of fish melanophores. *Biosens* and *Bioelectr* 12: 925-936.

Davletov BA, Meunier FA, Ashton AC, Matsushita H, Hirst WD, Lelianova VG, Wilkin GP, Dolly JO, Ushkaryov YA (1998) Vesicle exocytosis stimulated by alpha-latrotoxin is mediated by latrophilin and requires both external and stored Ca2+. *EMBO J* 17: 3909-3920.

Doellgast GJ, Triscott MX, Beard GA, Bottoms JD, Cheng T, Roh BH, Roman MG, Hall PA, Brown JE (1993) Sensitive enzyme-linked immunosorbent assay for detection of Clostridium botulinum neurotoxins A, B, and E using signal amplification via enzyme-linked coagulation assay. *J Clin Microbiol* 31: 2402-2409.

Elwing J, Karlsson JOG, Grundstrom N, Gustafsson ALE, vonSchenck H, Sundgren, H, Odman S, Andersson RGG, and Lundstrom I (1990) Fish Scales as Biosensors for Catecholamines. *Biosens* and *Bioelectr* 5: 449-459.

Evans RJ, Derkach V, Surprenant A (1992) ATP mediates fast synaptic transmission in mammalian neurons. *Nature* 357: 503-505.

Exton JH (1985) Mechanisms involved in alpha-adrenergic phenomena. *Am J Physiol* 248: E633-E647.

Fach P, Hauser D, Guillou JP, Popoff MR (1993) Polymerase chain reaction for the rapid identification of Clostridium botulinum type A strains and detection in food samples. *J Appl Bacteriol* 75: 234-239.

Fasolato C, Pizzo P, Pozzan T (1990) Receptor-mediated calcium influx in PC12 cells. ATP and bradykinin activate two independent pathways. *J Biol Chem* 265: 20351-20355.

Franciosa G, Ferreira JL, Hatheway CL (1994) Detection of type A, B, and E botulism neurotoxin genes in Clostridium botulinum and other Clostridium species by PCR: evidence of unexpressed type B toxin genes in type A toxigenic organisms. *J Clin Microbiol* 32: 1911-1917.

Fujii R, Oshima N (1986) Control of chromatophore movements in teleost fishes. *Zoo Sci* 3: 13.

Fujii R (1993a) Cytophysiology of fish chromatophores. *Int Rev Cyt* 143: 191.

Fujii R (1993b) Coloration and chromatophores. In *The Physiology of Fishes*, pp 535-562. *CRC Press*.

Fujii R (2000) The regulation of motile activity in fish chromatophores. *Pigment Cell Res* 13: 300-319.

Gatlin J, Unett DJ, Lerner MR, Garcia JV (2001) Efficient, long-term transgene expression in Xenopus laevis dermal melanophores. *Pigment Cell Res* 14: 275-282.

Gill DM, Meren R (1978) ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase. *Proc Natl Acad Sci U S A* 75: 3050-3054.

Greene LA, Rein G (1977) Release of (3H)norepinephrine from a clonal line of pheochromocytoma cells (PC12) by nicotinic cholinergic stimulation. *Brain Res* 138: 521-528.

Greene LA, Tischler AS (1976) Establishment of a noradrenergic clonal line of rat pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci* 73: 2424-2428.

Greene LA, Tischler AS (1982) PC12 Pheochromocytoma Cultures in Neurobiological Research. *Adv Cell Neurobio* 3: 373-413.

Gross GW, Rhoades BK, Azzazy HM, Wu MC (1995) The use of neuronal networks on multielectrode arrays as biosensors. *Biosens Bioelectron* 10: 553-567.

Hallis B, James BA, Shone CC (1996) Development of novel assays for botulinum type A and B neurotoxins based on their endopeptidase activities. *J Clin Microbiol* 34: 1934-1938.

Halpern JL, Neale EA (1995) Neurospecific binding, internalization, and retrograde axonal transport. *Curr Top Microbiol Immunol* 195: 221-241.

Harden TK, Boyer JL, Nicholas RA (1995) P2-purinergic receptors: subtype-associated signaling responses and structure. *Annu Rev Pharmacol Toxicol* 35: 541-579.

Hardman JG, Limbird LE (1996) *The pharmacological basis of therapeutics.* McGraw-Hill: New York.

Hatheway CL (1988) Botulism. In *Laboratory diagnosis of infectious diseases: principles and practice, vol. 1*, Balows A, Hausler WJ, Ohashi M, Turano A (eds) pp 111-133. Springer-Verlag: New York.

Hatheway CL (1990) Toxigenic clostridia. *Clin Microbiol Rev* 3: 66-98.

Heller A (1999) Implanted electrochemical glucose sensors for the management of diabetes. *Annu Rev Biomed Eng* 1: 153-175.

Hogben LT, Slome D. (1931) The pigmentary effector system. IV. The dual character of endocrine coordination in amphibium colour change. *Proc Roy Soc B* 108: 10-53.

Karlsson AM, Bjuhr K, Testorf M, Oberg PA, Lerner E, Lundstrom I, Svensson SP (2002) Biosensing of opioids using frog melanophores. *Biosens Bioelectron* 17: 331-335.

Karlsson JO, Andersson RG, Askelof P, Elwing H, Granstrom M, Grundstrom N, Lundstrom I, Ohman L (1991) The melanophore aggregating response of isolated fish scales: a very rapid and sensitive diagnosis of whooping cough. *FEMS Microbiol Lett* 66: 169-175.

Kotz KJ, McNiven MA (1994) Intracellular calcium and cAMP regulate directional pigment movements in teleost erythrophores. *J Cell Biol* 124: 463-474.

Lalli G, Herreros J, Osborne SL, Montecucco C, Rossetto O, Schiavo G (1999) Functional characterisation of tetanus and botulinum neurotoxins binding domains. *J Cell Sci* 112 (Pt 16): 2715-2724.

Lee KS, Tsien RW (1983) Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialysed heart cells. *Nature* 302: 790-794.

Lerner MR, Reagan J, Gyorgyi RT, Roby A (1988) Olfaction by melanophores: What does it mean? *Proc Natl Acad Sci* 85: 261-264.

Leszczyszyn DJ, Jankowski JA, Viveros OH, Diliberto EJ, Jr., Near JA, Wightman RM (1990) Nicotinic receptor-mediated catecholamine secretion from individual chromaffin cells. Chemical evidence for exocytosis. *J Biol Chem* 265: 14736-14737.

Lewis GE, Jr., Kulinski SS, Reichard DW, Metzger JF (1981) Detection of Clostridium botulinum type G toxin by enzyme-linked immunosorbent assay. *Appl Environ Microbiol* 42: 1018-1022.

Limbird LE (1988) Receptors linked to inhibition of adenylate cyclase: additional signaling mechanisms. *FASEB J* 2: 2686-2695.

Lomneth R, Martin TF, DasGupta BR (1991) Botulinum neurotoxin light chain inhibits norepinephrine secretion in PC12 cells at an intracellular membranous or cytoskeletal site. *J Neurochem* 57: 1413-1421.

Luby-Phelps K, Porter KR (1982) The control of pigment migration in isolated erythrophores of Holocentrus ascensionis (Osbeck). II. The role of calcium. *Cell* 29: 441-450.

Lundstrom I, Svensson S (1998) Biosensing with G-protein coupled receptor systems. *Biosens Bioelectron* 13: 689-695.

Martensson LG, Andersson RG (2000) Is Ca2+ the second messenger in the response to melatonin in cuckoo wrasse melanophores? *Life Sci* 66: 1003-1010.

Matteoli M, Verderio C, Rossetto O, Iezzi N, Coco S, Schiavo G, Montecucco C (1996) Synaptic vesicle endocytosis mediates the entry of tetanus neurotoxin into hippocampal neurons. *Proc Natl Acad Sci U S A* 93: 13310-13315.

McInnes C, Dolly JO (1990) Ca2(+)-dependent noradrenaline release from permeabilised PC12 cells is blocked by botulinum neurotoxin A or its light chain. *FEBS Lett* 261: 323-326

McNiven MA, Ward JB (1988) Calcium regulation of pigment transport in vitro. *J Cell Biol* 106: 111-125.

Meldolesi J, Huttner WB, Tsien RY, Pozzan T (1984) Free cytoplasmic Ca2+ and neurotransmitter release: studies on PC12 cells and synaptosomes exposed to alpha-latrotoxin. *Proc Natl Acad Sci U S A* 81: 620-624.

Mori Y, Mikala G, Varadi G, Kobayashi T, Koch S, Wakamori M, Schwartz A (1996) Molecular pharmacology of voltage-dependent calcium channels. *Jpn J Pharmacol* 72: 83-109.

Morishita F (1987) Responses of the melanophores of the medaka, Oryzias latipes, to adrenergic drugs: evidence for involvement of alpha 2 adrenergic receptors mediating melanin aggregation. *Comp Biochem Physiol C* 88: 69-74.

Nagatsu T (1973) *Biochemistry of catecholamines*. University Park Press: Baltimore.

Negishi S, Masada M, Wakamatsu Y, Ohoka T, Obika M (1982) Epinephrine-induced changes in the cyclic nucleotide content of fish melanoma cells. *Gen Comp Endocrinol* 47: 88-93.

Nery LE, Castrucci AM (1997) Pigment cell signalling for physiological color change. *Comp Biochem Physiol A Physiol* 118: 1135-1144.

Notermans S, Nagel J (1989) Assays for botulinum and tetanus toxins. In *Botulinum neurotoxin and tetanus toxin*, Simpson LL (ed) pp 319-331. Academic Press: San Diego.

Obika M (1986) Intracellular transport of pigment granules in fish chromatophores. *Zoo Sci* 3: 1.

Obika M, Meyer-Rochow VB (1990) Dermal and epidermal chromatophores of the Antarctic teleost Trematomus bernacchii. *Pigment Cell Res* 3: 33-37.

Oshima N, Suzuki M, Yamaji N, Fujii R (1988) Pigment aggregation is triggered by an increase in free calcium ions within fish chromatophores. *Comp Biochem Physiol* 91A: 27-32.

Pancrazio JJ, Whelan JP, Borkholder DA, Ma W, Stenger DA (1999) Development and application of cell-based biosensors. *Ann Biomed Eng* 27: 697-711.

Parchment RE (1998) Alternative testing systems for evaluating noncarcinogenic, hematologic toxicity. *Environ Health Perspect* 106 Suppl 2: 541-557.

Pellizzari R, Rossetto O, Schiavo G, Montecucco C (1999) Tetanus and botulinum neurotoxins: mechanism of action and therapeutic uses. *Philos Trans R Soc Lond B Biol Sci* 354: 259-268.

Pine RM (1984) Calcium-channel blocking agents: current indications and potential uses. *Clin Ther* 6: 245-251.

Poulain B, Tauc L, Maisey EA, Wadsworth JD, Mohan PM, Dolly JO (1988) Neurotransmitter release is blocked intracellularly by botulinum neurotoxin, and this requires uptake of both toxin polypeptides by a process mediated by the larger chain. *Proc Natl Acad Sci U S A* 85: 4090-4094.

Preston RR, McFadden PN (2001) A two-cell biosensor that couples neuronal cells to optically monitored fish chromatophores. *Biosens Bioelectron* 16: 447-455.

Sammak PJ, Adams SR, Harootunian AT, Schliwa M, Tsien RY (1992) Intracellular cyclic AMP not calcium, determines the direction of vesicle movement in melanophores: direct measurement by fluorescence ratio imaging. *J Cell Biol* 117: 57-72.

Sandberg K, Berry CJ, Rogers TB (1989) Studies on the intoxication pathway of tetanus toxin in the rat pheochromocytoma (PC12) cell line. Binding, internalization, and inhibition of acetylcholine release. *J Biol Chem* 264: 5679-5686.

Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270: 467-470.

Schiavo G, Rossetto O, Santucci A, DasGupta BR, Montecucco C (1992a) Botulinum neurotoxins are zinc proteins. *J Biol Chem* 267: 23479-23483.

Schiavo G, Benfenati F, Poulain B, Rossetto O, Polverino dL, DasGupta BR, Montecucco C (1992b) Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature* 359: 832-835.

Schiavo G, Shone CC, Bennett MK, Scheller RH, Montecucco C (1995) Botulinum neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of syntaxins. *J Biol Chem* 270: 10566-10570.

Schiavo G, Matteoli M, Montecucco C (2000) Neurotoxins affecting neuroexocytosis. *Physiol Rev* 80: 717-766.

Scholz G, Pohl I, Genschow E, Klemm M, Spielmann H (1999) Embryotoxicity screening using embryonic stem cells in vitro: correlation to in vivo teratogenicity. *Cells Tissues Organs* 165: 203-211.

Shone C, Wilton-Smith P, Appleton N, Hambleton P, Modi N, Gatley S, Melling J (1985) Monoclonal antibody-based immunoassay for type A Clostridium botulinum toxin is comparable to the mouse bioassay. *Appl Environ Microbiol* 50: 63-67.

Shone CC, Melling J (1992) Inhibition of calcium-dependent release of noradrenaline from PC12 cells by botulinum type-A neurotoxin. Long-term effects of the neurotoxin on intact cells. *Eur J Biochem* 207: 1009-1016.

Stearns ME (1984) Cytomatrix in chromatophores. *J Cell Biol* 99: 144s-151s.

Stenger DA, Gross GW, Keefer EW, Shaffer KM, Andreadis JD, Ma W, Pancrazio JJ (2001) Detection of physiologically active compounds using cell-based biosensors. *Trends Biotechnol* 19: 304-309.

Sugimoto M, Oshima N, Fujii R (1985) Mechanisms controlling motile responses of amelanotic melanophores in the medaka, *Oryzias latipes*. *Zoo Sci* 2: 317.

Svensson SP, Adolfsson PI, Grundstrom N, Karlsson JO (1997) Multiple alpha 2-adrenoceptor signalling pathways mediate pigment aggregation within melanophores. *Pigment Cell Res* 10: 395-400.

Szabo EA, Pemberton JM, Desmarchelier PM (1993) Detection of the genes encoding botulinum neurotoxin types A to E by the polymerase chain reaction. *Appl Environ Microbiol* 59: 3011-3020.

Takeshi K, Fujinaga Y, Inoue K, Nakajima H, Oguma K, Ueno T, Sunagawa H, Ohyama T (1996) Simple method for detection of Clostridium botulinum type A to F neurotoxin genes by ploymerase chain reaction. *Microbiol Immunol* 40: 5-11.

Telzer BR, Haimo LT (2001) Chromatophores as tools for the study of organelle transport. *Methods Mol Biol* 161: 201-214.

Thaler CD, Haimo LT (1992) Control of organelle transport in melanophores: regulation of Ca2+ and cAMP levels. *Cell Motil Cytoskeleton* 22: 175-184.

Thaler CD, Haimo LT (1996) Microtubules and microtubule motors: mechanisms of regulation. *Int Rev Cytol* 164: 269-327.

Thomas RJ (1991) Detection of Clostridium botulinum types C and D toxin by ELISA. *Aust Vet J* 68: 111-113.

Triggle DJ, Swamy VC (1983) Calcium antagonists. Some chemical-pharmacologic aspects. *Circ Res* 52: I17-I28.

Tuma MC, Gelfand VI (1999) Molecular mechanisms of pigment transport in melanophores. *Pigment Cell Res* 12: 283-294.

Umland TC, Wingert LM, Swaminathan S, Furey WF, Schmidt JJ, Sax M (1997) Structure of the receptor binding fragment HC of tetanus neurotoxin. *Nat Struct Biol* 4: 788-792.

Walton KM, Sandberg K, Rogers TB, Schnaar RL (1988) Complex ganglioside expression and tetanus toxin binding by PC12 pheochromocytoma cells. *J Biol Chem* 263: 2055-2063.

Weiss C, Atlas D (1991) The bradykinin receptor--a putative receptor-operated channel in PC12 cells: studies of neurotransmitter release and inositol phosphate accumulation. *Brain Res* 543: 102-110.

Wictome M, Newton KA, Jameson K, Dunnigan P, Clarke S, Gaze J, Tauk A, Foster KA, Shone CC (1999) Development of in vitro assays for the detection of botulinum toxins in foods. *FEMS Immunol Med Microbiol* 24: 319-323.

Wightman RM, Jankowski JA, Kennedy RT, Kawagoe KT, Schroeder TJ, Leszczyszyn DJ, Near JA, Diliberto EJ, Jr., Viveros OH (1991) Temporally resolved catecholamine spikes correspond to single vesicle release from individual chromaffin cells. *Proc Natl Acad Sci U S A* 88: 10754-10758.