

THE EFFECTS OF NERVE-CONTACT, MEMBRANE  
EXCITABILITY, AND SYNAPTIC ACTIVITY ON  
THE REGULATION OF SODIUM AND POTASSIUM  
VOLTAGE GATED ION CHANNELS IN EMBRYONIC  
*Xenopus* MUSCLE CELLS AFTER ONE DAY IN CULTURE

CENTRE FOR NEWFOUNDLAND STUDIES

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SCOTT STEPHEN HANCOCK









The Effects of Nerve-Contact, Membrane Excitability,  
and Synaptic Activity on The Regulation of Sodium and Potassium  
Voltage Gated Ion Channels in Embryonic *Xenopus* Muscle Cells After  
One Day in Culture

By

Scott Stephen Hancock

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### Abstract

The kinetics, density and distribution of ion channels on myocytes are altered significantly during development, by genetic cues and extrinsic factors such as excitability, synaptic activity, and nerve-contact. The effects of such extrinsic factors on the developmental expression of ion channels in embryonic *Xenopus* myocytes is the focus of this work. Whole cell currents were recorded from embryonic *Xenopus* muscle cells after one day in culture using a List patch clamp and standard recording conditions. (Moody-Corbett and Gilbert, *Dev. Br. Res.*, 1990, 55: 139-142).  $\text{Na}^+$  currents ( $I_{\text{Na}}$ ) and three types of  $\text{K}^+$  currents inward rectifier (IR), inactivating ( $I_{\text{K}}$ ), and non-inactivating ( $I_{\text{K}}$ ) outward rectifiers were recorded from both nerve-contacted and non-innervated muscle cells. The influence of membrane excitability and synaptic activity were examined by adding tetrodotoxin (TTX) and/or  $\alpha$ -bungarotoxin ( $\alpha$ -BTX) to the culture medium at the time of plating. Nerve-contact significantly increased the proportion of cells which displayed an  $I_{\text{Na}}$  and this increased expression was blocked when membrane excitability or synaptic activity was blocked. These results suggest that  $I_{\text{Na}}$  was maintained by a positive feedback mechanism while the upregulation of  $I_{\text{Na}}$  following nerve-contact was dependent on synaptic function. Nerve-contacted muscle cells had a significantly increased probability of expressing  $I_{\text{K}}$  than non-contacted muscle cells and this increase was not blocked

when membrane excitability or synaptic activity was blocked. These results suggest that  $I_{IK}$  was maintained by a separate mechanism than  $I_{Na}$ . It was concluded that nerve-contact, membrane excitability, and synaptic activity all play distinct and different roles in altering  $Na^+$  and  $K^+$  current expression.

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## CHAPTER 1

### Introduction

A variety of muscular disorders exist which have been linked to abnormalities of ion channels, including  $K^+$  channels in hypertension (Brooksby et al, 1993), acetylcholine receptors (AChR) in myasthenia gravis,  $Na^+$  channels in periodic paralysis,  $Cl^-$  channels in some myotonic disorders, and others (see review by Barchi, 1993). Disorders also arise from reinnervation of skeletal muscles following nerve damage (see Buxton, 1980). This work is the first comprehensive study to date which reports the effects of nerve contact on the expression of ion channels in embryonic skeletal muscle. Such a study will be invaluable to further our understanding of neuromuscular disorders.

Adult skeletal muscle from a variety of species including rat, frog, and chick have been shown to contain a plethora of ion channels including sodium ( $Na^+$ ) channels (Weiss and Horn, 1986; Campbell and Hille, 1976), potassium delayed rectifiers ( $I_K$ ), inactivating outward  $K^+$  channels ( $I_{KX}$ ), inward rectifiers (IR), ATP-

regulated  $K^+$  channels ( $K_{ATP}$ ),  $Ca^{2+}$ -dependent  $K^+$  channels ( $I_{K(Ca)}$ ), (Stefani and Chiarandini, 1983; Rudy, 1988),  $Ca^{2+}$  channels, (Hagiwara and Byerly, 1981) and  $Cl^-$  channels (Bretag, 1987).

The  $Cl^-$  channels and IRs are thought to be important for maintaining a high resting conductance to prevent hyperexcitability in adult skeletal muscles (Almers, 1972; Bretag, 1987). In embryonic muscle, however, the  $Cl^-$  current is small and the IR channel is thought to maintain the cell's resting membrane potential, (Kubo, 1991; Moody-Corbett and Gilbert, 1990; Hancock et al, in press). The delayed rectifier  $I_K$  is responsible for repolarisation of the muscle membrane following an action potential (Adrian et al, 1970; Duval and Leoty, 1980; Rudy, 1988). Another  $K^+$  channel of interest is the  $I_{K1}$  or rapidly inactivating  $K^+$  channel, which passes  $K^+$  out of the cell rapidly, to return the cell membrane potential to rest following an action potential. This is similar to the A-type  $K^+$  channel described in molluscan ganglion neurons, which is thought to lengthen the refractory period between action potentials, resulting in a reduced frequency of firing, (Neher, 1971). The activation of the  $I_{K(Ca)}$  current is thought to be responsible for the slow afterhyperpolarization following an action potential, which lasts as long as it takes to remove the excess  $Ca^{2+}$  from the cell (Stefani and Chiarandini, 1983). This current may act to slow down the rate of action potential generation, (Hille, 1992). The  $K_{ATP}$  channels

are known to open in the absence of ATP. They serve to hyperpolarize the cell during times of energy depletion, possibly to protect the cell from prolonged depolarizations (Spruce et al, 1987). The  $\text{Ca}^{2+}$  channels of skeletal muscles are involved in excitation-contraction coupling, spike generation, and may be involved in AChR clustering (Moody-Corbett et al, 1989; Peng, 1984; Hille, 1992). It is known that  $\text{Na}^{+}$  channels are highly concentrated at the neuromuscular junction since their high densities may be necessary to allow the fibre to respond to rapid action potential generation in the motor neuron (Beam et al, 1984; Caldwell and Milton, 1988).

The literature is less complete in describing the complement of ion channels in embryonic muscle preparations. Studies of satellite cells from human muscle (Trautmann et al, 1986), embryonic and neonatal rat muscle (Kidokoro, 1981), rat muscle cell line L6 (Kidokoro, 1973), mouse mesodermal stem cell line (Kubo, 1991), fetal mouse muscle (Gonoi and Hasegawa, 1991), ascidian larval muscle (Davis et al, 1995), and embryonic chick muscle (Fischbach et al, 1971; Engelhardt et al, 1978; Kano, 1975), have given pieces of information about the developmental regulation of ion channels. However, the most complete description of embryonic ion channel development to date comes from the *Xenopus* system.

Several researchers have studied *Xenopus* myocytes in culture in the

absence of nerve, and have determined that acetylcholine receptors (AChRs), then IR,  $I_K$ ,  $I_{KX}$ ,  $Na^+$  and finally  $Ca^{2+}$  voltage-gated channels appear in these cells starting about stage 15 or just when the neural tube is formed (Spruce and Moody, 1992; Kidokoro, 1992; Moody-Corbett and Gilbert, 1990, 1992a,b). These studies, although informative, did not investigate the role of nerve contact in altering ion channel expression. *Xenopus* myocytes are nerve contacted as early as stage 19 *in vivo*, and this event has been shown to be important in development (Chow, and Cohen, 1983; Kullberg, Lentz and Cohen, 1977; Blackshaw and Warner, 1977).

### 1.1 The Role Of Nerve Contact In Altering Ion Channel Expression

In all systems that have been studied, the motor neuron alters the expression and localization of transmitter receptors at the synaptic site (for review see Hall and Sanes, 1993). Spontaneous release of ACh at synaptic terminals which causes muscle twitching in mixed cultures from *Xenopus* embryos is also thought to enhance development of cross striations of myofibrils (Kidokoro, and Saito, 1988). However, the role of nerve contact in altering the developmental expression of voltage regulated ion channels is less well described in the literature.

Following nerve contact in adult muscle,  $\text{Na}^+$  channels have been shown to become highly concentrated at the synaptic site (Boudier et al, 1992; Barchi, 1988; Beam et al, 1984; Angelides, 1986). There is some evidence for an increased IR current density at the NMJ as well (Katz and Miledi, 1982). Conversely,  $\text{Cl}^-$  currents have been shown to increase in the extrajunctional muscle membrane following nerve contact (Heathcote, 1989) while they are found to be reduced at the site of nerve contact (Betz et al, 1984). These studies indicate that nerve-contacted muscle cells have an altered regulation and distribution of ion channels when compared to the embryonic muscle. However since these studies were carried out on adult muscle, the effects of nerve-contact cannot be truly separated from the role of muscle maturity in altering ion channel expression. Following denervation, it has been shown that  $\text{Cl}^-$  channels (Heathcote, 1989),  $I_K$ , and  $I_{K\text{A}}$  channels (Escobar et al, 1993), IR channels (Gonoi and Hasegawa, 1991) and  $\text{Na}^+$  channel densities (Caldwell and Milton, 1988) decrease. The types of  $\text{Na}^+$  channel present also changes from a TTX sensitive type to a TTX insensitive one similar to the  $\text{Na}^+$  channel found in early development (Redfern and Thesleff, 1971). Taken together these findings suggest the importance of nerve-contact in regulating ion channel expression.



## 1.2 Role Of Chemical Factors In Altering Ion Channel Expression

Nerves release chemicals from growth cones even before they contact muscle cells (Hall and Sanes, 1993). It was postulated that a chemical factor contained in vesicles and released by impulse activity in the nerve, such as agrin, calcitonin gene related peptide (CGRP), or acetylcholine receptor-inducing activity (ARIA) may be involved in maintaining AChRs in postsynaptic muscle membranes (Andreose, Fumagalli, and Lomo, 1995; McMahan et al, 1993). Basic fibroblast growth factor (bFGF) is another chemical which is present in the myotomal region of *Xenopus* embryos during synaptogenesis. Local application of bFGF can mimic the effects of innervation by inducing AChR clustering (Peng et al, 1991). These soluble chemicals may alter the muscle cell's complement of ion channels as well.

DeCino and Kidokoro in 1985 found that muscle cells grown in the presence of neural tube appeared to have an increased  $I_{Na}$  compared with muscle cells grown without nerve. These results were based on single channel recordings which were summed to generate a macroscopic current; the whole cell  $I_{Na}$  was not measured. Corfas and Fischbach, in 1993 demonstrated that ARIA a substance able to aggregate AChRs could also cause an increase in  $Na^+$  current density in embryonic chick muscle cells in culture.  $Na^+$  channels in chick

embryonic skeletal muscle were also found to increase in current density when cultured in the presence of nerve free brain extracts (Sato et al, 1995). Studies of *Xenopus* myocytes found that the presence of neurons in the culture dish could accelerate the development of the  $I_{K}$  current in myocytes (Ribera and Spitzer, 1991), suggesting that a soluble neural factor could influence ion channel development. These experiments suggested that a soluble chemical factor, secreted by a nerve, could cause the upregulation of ion channels.

### 1.3 Role Of Depolarization In Altering Ion Channel Expression

Electrical activity has been shown to increase the level of junctional and decrease the level of extrajunctional AChRs on skeletal muscle preparations (Fambrough, 1979; Lomo and Rosenthal, 1972). Reducing depolarizations by blocking  $Na^{+}$  channels with tetrodotoxin (TTX) treatment has been shown to increase AChR expression in denervated cultured cells (Cohen, and Fischbach, 1973; Shainberg et al, 1976). AChRs are present in embryonic muscle membrane at very early times, and as a consequence the earliest nerve-contacts will result in depolarization of the muscle membrane (Kullberg et al, 1977; Xie and Poo, 1986; Evers et al, 1989). Other experiments showed that excitability or depolarizations were important for neural maturation (Bergy et al, 1981),

neuronal segregation (Shatz, and Stryker, 1988), receptor localization (Peng et al, 1988), and Na<sup>+</sup> channel expression (Sherman, and Caterall, 1984).

Recently, Linsdell and Moody, 1994 demonstrated that rat brain Na<sup>+</sup> channels expressed in embryonic *Xenopus* myocytes, increased the density of IR and I<sub>K</sub> channels and the time course of expression. Further, block of the Na<sup>+</sup> channels by TTX blocked the increased expression of the K<sup>+</sup> channels, suggesting a role for membrane excitability in the developmental expression of K<sup>+</sup> channels. However, work by Spruce and Moody, 1992 has demonstrated that Na<sup>+</sup> channels are normally expressed after K<sup>+</sup> channels, not before. One problem with this and other studies looking at developmental expression of the ion channels, is that they have been done on isolated muscle cells and may not be representative of the developmental expression at the time of innervation. Preliminary studies (Moody-Corbett, and Hancock, 1995) from this lab indicated that muscle cells contacted by nerve have a higher probability of expressing I<sub>Na</sub> than muscle cells grown in the absence of nerve. Normally, *Xenopus* myocytes develop Na<sup>+</sup> currents about the same time that they are innervated. Both events (AChR and Na<sup>+</sup> channel expression) contribute to the electrical excitability of the cell, which may alter the ion channel expression in these cells (Spruce and Moody, 1992).

#### 1.4 Objectives Of The Study

The main objective of this study was to determine the effects of nerve-contact in altering the expression of  $I_{Na}$ ,  $I_K$ ,  $I_{K1}$ , and  $I_R$  currents in *Xenopus* myocytes after one day in culture. The role of synaptic activity and membrane depolarization in altering these channels was also examined. The alterations in  $I_{K(ATP)}$ ,  $I_{K(Ca)}$ , and  $I_{Ca}$  were not investigated. Whole cell currents were recorded from embryonic *Xenopus* muscle cells after one day in culture. Cells were cultured in the presence of nerve so that some muscle cells were nerve-contacted for periods up to 24 hours (some of these data were previously reported Moody-Corbett and Hancock, 1995).

*Xenopus* myocytes cultured with TTX can up-regulate AChRs at nerve contact sites (Davey and Cohen, 1986). Thus, the objective of the second experiment was to determine whether membrane excitability altered ion channel expression in nerve-contacted and non-contacted myocytes grown in culture.

Nerves can release ACh spontaneously when they approach muscle cells (Evers et al, 1989; Young and Poo, 1983). Thus, the objective of the third experiment was to examine the role of synaptic activity in nerve-contacted myocytes and the role of spontaneous AChR openings in non-contacted myocytes in altering ion channel expression in muscles grown in culture.

A fourth experiment was performed using a combination of TTX and the AChR channel blocker  $\alpha$ -bungarotoxin ( $\alpha$ -BTX) to eliminate synaptic activity and membrane excitability for the 24 hour culture period. The objective of this experiment was to eliminate all membrane excitability in myocytes growing in culture in order to determine the role of excitability in altering ion channel expression in developing myocytes before and after nerve-contact.

These data confirm that the expression of  $\text{Na}^+$  currents and the onset of innervation both play important but distinctly different roles in the regulation of ion channel expression in embryonic myocytes.

## CHAPTER 2

### Materials and Methods

#### 2.1 Preparation of Cultures

Female *Xenopus laevis* were injected with 500 IU of human chorionic gonadotrophin (Ayerst Laboratories, Montreal, Canada) while males received 300 IU injections into the dorsal lymphatic sac. Embryos were extruded within 24 hours and were grown for one day in Petri dishes in batches of 20-100 at room temperature (21-23° C). Some embryos were kept in the fridge at 4° C to slow their development. In this way embryos from the same batch could be developed to the proper stage, over a period of two or three days, similar to the procedure used by Spruce and Moody, 1992.

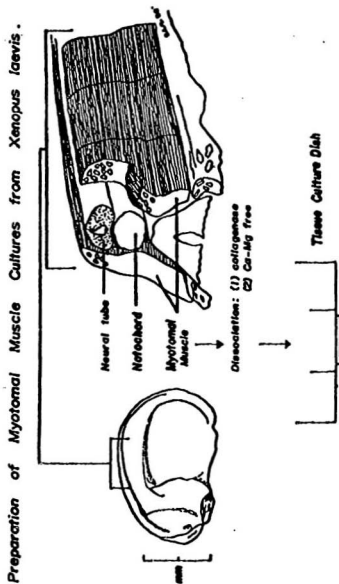
Embryos were staged according to Nieuwkoop and Faber, 1967. Dissections were performed on embryos of stages 19-23 using the procedure of Moody-Corbett and Gilbert, 1990. Embryos were removed from their vitelline membrane and jelly coat. The dorsal 1/3 of the embryo containing myocytes, notochord, neural tube, and ectoderm was isolated (see Figure 1) and placed

Figure 1: Preparation of Cultures

**Figure 1: Preparation of Myotomal Muscle Cultures From *Xenopus laevis*.** Shown is a stage 19 embryo, from which the back portion (shown between the lines) was removed. As depicted, the myotomal muscle and neural tube are then easily visualized and separated from the rest of the tissue, following enzymatic treatment (20 minutes in 1mg/ml collagenase). One hour of further treatment in  $\text{Ca}^{2+}/\text{Mg}^{2+}$  - free Ringers solution allowed individual muscles and neurons to be plated in a culture dish chamber as shown.  
(courtesy of Cynthia Mercer)



Figure 1: Preparation of Cultures



into a chamber containing dissection medium (see below). The dorsal portions were placed into a collagenase solution (1mg/ml in 2/3 L15) for 15 minutes which separated the myotomes and neural tube from the other tissue. Following this enzyme treatment, the myotomes and neural tubes were placed in separate dishes and washed in dissecting medium. The myotomes and neural tubes were placed in  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Ringers solution for one hour. This treatment separated the tissue into single myocytes and neurons which were resuspended in plating medium (see below). Two types of cultures were made; muscle only cultures in which only dissociated single myocytes were plated, and mixed cultures which contained both nerve and muscle cells in co-culture.

The culture dishes were made from Falcon 35mm Petri dishes (VWR, Canlab, ON, Canada). A lathe was used to cut a hole in the bottom of the Petri dish and a glass coverslip was adhered to the bottom of the culture dish while a glass ring was placed around the hole on the inside of the Petri dish in order to make a chamber with a capacity of approximately 1.5 ml. The glass ring and coverslip were attached to the Petri dish using silicon grease. The coverslips were coated with collagen type VII prior to constructing the culture chamber. One hour prior to the addition of cells the culture chamber was treated with ECL (entactin, collagen 4, and laminin) Cell Attachment Matrix. After the cells were added, the chambers were filled with culture medium and sealed with a sterile

glass coverslip using silicon grease. Following 24 hours of growth at room temperature in the dark, patch clamp recordings were performed on the myocytes.

Membrane excitability was eliminated by culturing cells in the presence of 3  $\mu$ M TTX, a concentration which has been shown to block  $\text{Na}^+$  channels (Cohen, 1972; Kullberg et al, 1977). Prior to recordings, the culture medium, containing TTX was washed out of the recording chamber using sterile, extracellular recording solution. The solution was replaced four times prior to the start of a recording session in order to remove all traces of TTX from the chamber.

In another experiment synaptic activity and spontaneous AChR openings were eliminated by culturing cells in the presence of 1  $\mu$ M  $\alpha$ -BTX for 24 hours, a concentration known to block the AChRs (Anderson and Cohen, 1974). Co-cultures of nerve and muscle were also plated in the presence of  $\alpha$ -BTX for 24 hours.  $\alpha$ -BTX is a specific and irreversible blocker of the nicotinic AChRs on this preparation (Anderson et al, 1977). Similar to the TTX experiment, recording chambers were washed with external recording solution four times prior to initiating a recording session.

In a fourth experiment both 3  $\mu$ M TTX and 1  $\mu$ M  $\alpha$ -BTX were added to culture chambers at the time of plating, in both muscle only cultures and co-

cultures of nerve and muscle cells to eliminate all means of excitability. Culture medium was washed out four times prior to commencement of recordings.

## **2.2 Patch Clamp Recordings**

Whole cell patch clamp was performed with a List patch clamp (EPC 7, Medical Systems, Greenvale, NY, USA) using the tight seal recording technique of (Hamill et al, 1981). Cells were visualized under 400x magnification on an IM 35 Zeiss inverted microscope (Zeiss, Canada) and the electrode was positioned using a motorized micromanipulator (Zeiss, Canada).

Patch electrodes were made from fiber filled WPI borosilicate capillary tubes (World Precision Instruments, Sarasota, Florida, USA) fabricated on a Narishige Scientific PP-83 two stage vertical puller (Narishige, Japan). When filled with 140 mM KCl solution, the electrodes had resistances of 5-10 MΩ. Electrodes were coated with Sigmacote (Sigma Chemical labs, Mississauga, On, Canada) to reduce electrostatic noise. By gentle suction seals between 10-100 GΩ were made with the muscle cell surfaces (Milton and Caldwell, 1990). The membrane potential was held at -60 mV (close to RMP) and further suction allowed rupturing of the cell membrane to voltage clamp the entire cell. Capacitances and series resistances were measured from the patch clamp

amplifier. Eighty percent series resistance compensation was used for most cell recordings.

Pclamp software (Axon Instruments, CA, USA) was used for experimental protocols, data collection, and analysis. Depolarizing pulses of 100 msec duration were applied from a holding potential of -80 mV using Clampex software. Steps in 10 mV increments were applied from -70 mV to a final potential of +50 mV. There was a 10 second interval between each test pulse, during which the cell was maintained at its holding potential. This protocol was used to activate the outward potassium and inward sodium channels. To activate and record from the IR channels, 100 msec long pulses of 10 mV increments in 10 mV steps in the hyperpolarized direction were applied (one pulse every 10 seconds, as for the depolarizing pulses). In these protocols the muscle cells were held at -60 mV and stepped from -70 mV to -160 mV.

The current data were filtered at 3 KHz at the patch clamp amplifier and digitized at 200  $\mu$ sec. The currents were analysed using Clampfit software (Pclamp). Cell capacitances were read from the Cs dial of the EPC7 patch clamp amplifier. Current densities were then determined by dividing the peak current by the cell capacitance. Current density versus voltage plots were generated using Origin software (Microcal Software, USA). For the  $I_{Na}$ , the peak current, time to peak current and potential of maximum current were recorded (see

Figure 3). For the outward  $K^+$  currents, the peak current and the average current during the last 10 msec of each pulse were recorded using Clampfit (see Figure 8). As with the  $Na^+$  current, current densities were determined and current density vs voltage plots were generated using Origin software. The outward potassium currents were separated into non-inactivating ( $I_{K1}$ ) and inactivating ( $I_{K2}$ ) types using the following criterion. The current was said to be inactivating ( $I_{K2}$ ) if the average current of the last 10 msec of the current trace (measured at the +50 mV potential) inactivated to a level less than 80% of the peak current. If the average current of the last 10 msec was not less than 80% of the peak current then we determined an  $I_{K1}$  current to be present. For the  $IR$  current, peak current and current density were measured. The kinetics of this current were not reported since this channel class has been well characterized in this preparation (Moody-Corbett and Gilbert, 1992; Hancock and Moody-Corbett, 1995).

### 2.3 Analysis

Currents were recorded from myocytes in muscle only cultures and compared to currents recorded from myocytes which were nerve-contacted in mixed nerve-muscle cocultures. Nerve-contact was determined from visual

inspection under 400x magnification (see Figure 2). The proportion of cells in each experimental group which expressed  $I_{Na}$ ,  $I_{IK}$ ,  $I_K$ , and  $I_R$  was measured, and compared between groups using binomial or chi-squared tests as appropriate. Data were reported as means  $\pm$  standard errors. As well, the current densities of the four currents examined were compared between groups using a Fischer ANOVA.

## 2.4 Solutions

Dissection medium contained Leibovitz's L15 diluted to 2/3 with sterile water, gentamycin, (0.01mg/ml), nystatin (5U/ml), pH 7.4 (with NaOH or HCl). Plating medium contained 2/3 L15, and 5% dialysed horse serum (DHS), pH 7.4. Culture medium contained 2/3 L15, 0.5% DHS, pH 7.4. Collagenase, Leibovitz's L15, dialysed horse serum, nystatin and gentamycin were obtained from (Gibco BRL., Burlington, ON, Canada). The glass coverslips were coated with rat tail collagen (Sigma Chemical Co., Mississauga, Canada) prior to plating the cells. The coverslips were coated with 10  $\mu$ g/ml ECL (Upstate Biotechnology Inc, Lake Placid, NY, USA) dissolved in 2/3 L15, for one hour, immediately prior to plating the cells.

External recording solution contained in mM: 140 NaCl, 5 KCl, 1

CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>, 10 HEPES, pH 7.4. Internal (patch electrode) solution contained in mM: 140 KCl, 1 BAPTA, 5 MgCl<sub>2</sub>, 10 HEPES, pH 7.4. Ground electrodes were made from 50  $\mu$ l Drummond micropipettes (Fisher Scientific, Ottawa, ON, Canada) and contained external recording solution in 2% agar (w/w). TTX,  $\alpha$ -BTX, HEPES, and collagen(Type VII) were obtained from Sigma Chemicals, Mississauga, ON, Canada.

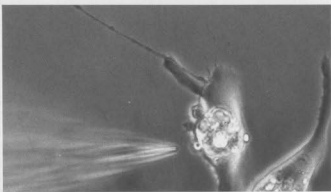


**Figure 2:** Phase Contrast Micrographs of a Nerve-Contacted and a Muscle Only  
Muscle Cell at 400x Magnification

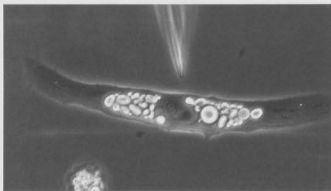


**Figure 2: (A). Phase Contrast Micrograph of a Nerve-Contacted Muscle Cell. (B) Phase Contrast Micrograph of a Muscle Cell Grown in The Absence of Nerves. The cells were taken from stage 19 embryos, were plated for 24 hours and are typical of cells used for all treatment groups. A glass electrode can be seen which was used for whole cell patch clamping. (calibration) 1cm = 20  $\mu$ m**

**A**



**B**



## CHAPTER 3

### Results

Muscle cells in all of the groups were matched in size and age as shown in Table 1. Nerve-contact, and cell size were determined from visual observation (400x magnification). Only cells which were  $150\text{ }\mu\text{m}$  in length, as determined by an eyepiece graticule, were used in these experiments. Examples of a nerve-contacted and a non-contacted muscle cell are shown in Figure 2. Capacitance is a measure of cell size, since  $1\text{fF} = 0.1\text{ }\mu\text{m}^2$  (Fernandez et al, 1984). Cell capacitances were not significantly different between nerve-contacted ( $44.5 \pm 2.1\text{ pF}$ ) and non-contacted muscle cells ( $44.2 \pm 2.4\text{ pF}$ ), or among the different treatment groups. Resting membrane potentials were similar between nerve-contacted ( $-62.6 \pm 0.9\text{ mV}$ ) and non-contacted muscle cells ( $-64.8 \pm 0.6\text{ mV}$ ), and similar among the different treatment groups, as shown in Table 1.

### 3.1 Sodium Channel Expression

#### 3.1.1 Effects of Nerve Contact

**Table 1: Average Resting Membrane Potential (RMP), Membrane Capacitance, and Age in Culture for Muscle Cells in the Various Treatment Groups**

Treatments	RMP (mV)	Capacitance (pF)	# of Cells	Age (hr)
Muscle Control	-64.8 $\pm$ 0.6	44.2 $\pm$ 2.4	n = 45	23.1 $\pm$ 0.5
Muscle TTX	-61.2 $\pm$ 0.7	41.2 $\pm$ 4.3	n = 16	21.0 $\pm$ 0.6
Muscle BTX	-64.5 $\pm$ 1.4	43.2 $\pm$ 3.4	n = 17	22.5 $\pm$ 0.3
Muscle TTX/BTX	-63.6 $\pm$ 0.6	46.3 $\pm$ 2.4	n = 16	23.8 $\pm$ 0.2
Nerve Control	-62.6 $\pm$ 0.9	44.5 $\pm$ 2.1	n = 49	23.6 $\pm$ 0.3
Nerve TTX	-58.3 $\pm$ 1.0	38.1 $\pm$ 2.4	n = 21	23.5 $\pm$ 0.4
Nerve BTX	-59.2 $\pm$ 1.4	36.4 $\pm$ 2.3	n = 23	22.3 $\pm$ 0.5
Nerve TTX/BTX	-59.2 $\pm$ 2.0	49.4 $\pm$ 2.9	n = 15	24.1 $\pm$ 0.5

*Xenopus* muscle cells express sodium currents after one to two days grown in culture (Spruce and Moody, 1992). The sodium current expression in muscle cells grown in the absence of nerves and in nerve-contacted muscle cells one day after plating are shown in Figures 3 and 4. The  $\text{Na}^+$  currents were similar to those reported by Spruce and Moody, 1992. The peak  $\text{Na}^+$  current in nerve-contacted myocytes was found to be at a potential of  $-24 \pm 1.5 \text{ mV}$ ,  $n=33$ , with a time to maximum activation of  $1.81 \pm .14 \text{ msec}$ . Myocytes in muscle only cultures were found to contain the same sodium current with a peak current at  $-21 \pm 2.3 \text{ mV}$ ,  $n=11$  and a time to maximum activation of  $1.5 \pm .13 \text{ msec}$ . These results agree with previously reported values; a time to peak activation of  $1.5 \text{ msec}$  and a peak current at a potential of  $-15 \text{ mV}$ , in adult frog muscle (Campbell and Hille, 1976) and a time to peak activation of  $1.8 \text{ msec}$  and a peak current at a potential of  $-26 \text{ mV}$ , in slow twitch rat muscle (Duval and Leoty, 1980). However, because of our slow digitization rate ( $200 \mu\text{sec}$ ), statistical comparisons of  $I_{\text{Na}}$  activation rates between these results and those of past researchers were not possible. Sodium currents in both groups were found to be sensitive to TTX block (see Figure 6).

In the cells which expressed a sodium current, there was no difference in current density between the muscle cells grown with no nerve and those contacted by nerve (see Table 2). However, as shown in Figure 5, muscle cells

contacted by nerve had significantly higher probability ( $p < 0.01$ ) of containing a  $\text{Na}^+$  current (73%,  $n=49$ ) than muscle cells grown without nerve (38%,  $n=45$ ). These results indicated that nerve-contact altered the developmental expression of  $I_{\text{Na}}$  without changing the  $I_{\text{Na}}$  density. In order to examine the influence of membrane depolarization on  $\text{Na}^+$  channel expression, cells were grown in the presence of the  $\text{Na}^+$  channel blocker TTX.

### 3.1.2 Membrane Excitability

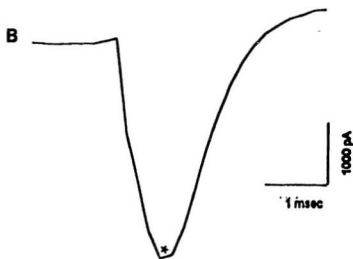
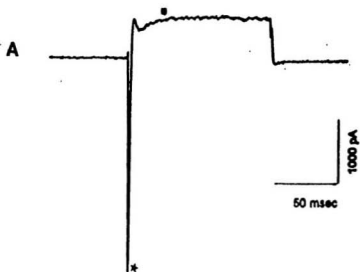
In order to test which component of nerve-contact caused the increased expression of  $\text{Na}^+$  channels, we first tested the effects of  $\text{Na}^+$  channel autoregulation by blocking  $\text{Na}^+$  channels with TTX during the 24 hour growth period prior to recording. As shown in Figure 6 and described below,  $3 \mu\text{M}$  TTX was sufficient to block  $\text{Na}^+$  currents in these muscle cells in culture. The culturing of muscle cells in the presence of TTX was found to decrease the developmental expression of  $\text{Na}^+$  channels in both nerve-contacted muscle cells and in muscle cells cultured without nerves. It was found that only one (5%) of the TTX cultured nerve-contacted muscle cells contained an  $I_{\text{Na}}$ ,  $n=21$ . This was significantly lower than  $I_{\text{Na}}$  expression in nerve-contacted cells grown without TTX (73%;  $P < 0.001$   $n=49$ ) and significantly lower than  $I_{\text{Na}}$  expression

**Figure 3:** Sodium Current Trace Recorded From a Muscle Cell in a Culture Which Contained no Nerve Cells.



**Figure 3: Sodium Current Trace Recorded From a Muscle Cell After 24 Hours in a Culture Which Contained no Nerve Cells.**(A) The fast inward spike (\*) is an  $I_{Na}$  recorded from a 100 msec long pulse to a membrane potential of -30 mV, from a holding potential of -80 mV. Following the  $I_{Na}$  spike an outward potassium current can be seen (■). (B) The same  $I_{Na}$  is shown at a faster time scale to indicate the rise time ( 1.3 msec ) which is similar to previous work (Campbell and Hille, 1976).

**Figure 3:** Sodium Current Trace Recorded From a Muscle Cell in a Culture Which Contained no Nerve Cells.



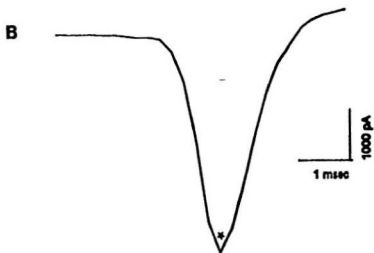
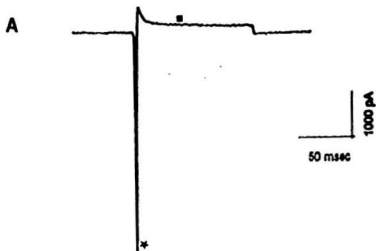
**Figure 4: Sodium Trace From a Nerve-Contacted Muscle Cell**

1

2

**Figure 4:** Sodium Current Trace Recorded From a Nerve-Contacted Muscle Cell After 24 Hours in Culture. (A) The fast inward spike (\*) is an  $I_{Na}$  recorded from a 100 msec long pulse to a membrane potential of -30 mV, from a holding potential of -80 mV. Following the  $I_{Na}$  spike an outward potassium current can be seen (■). (B) The same  $I_{Na}$  is shown at a faster time scale to indicate the rise time ( 1.0 msec ) which is similar to previous work (Campbell and Hille, 1976).

Figure 4: Sodium Trace From a Nerve-Contacted Muscle Cell



**Table 2:** The Effects of Blocking Membrane Excitability and Synaptic Activity on  $I_{Na}$  Expression in Muscle Only and Nerve-Contacted Muscle Cells

treatments	Muscle only			Nerve contact		
	# of Cells	% of cells with $I_{Na}$	$I_{Na}$ (pA/pF)	# of Cells	% of cells with $I_{Na}$	$I_{Na}$ (pA/pF)
no Treatment	45	38%	$23.8 \pm 4.8$	49	73%	$23.8 \pm 4.1$
TTX	16	0% <sup>1</sup>	-	21	5% <sup>2</sup>	2 *
BTX	17	24%	$32 \pm 5.3$	23	22% <sup>2</sup>	$16.1 \pm 2.9$
TTX/BTX	16	6% <sup>1</sup>	13.5 *	15	0% <sup>2</sup>	-

\* - This was only one cell

<sup>1</sup> - Significantly different from muscle only no treatment ( $p < 0.01$ , binomial test)

<sup>2</sup> - Significantly different from nerve contacted no treatment ( $p < 0.01$ , binomial test)

**Figure 5: Effect of Nerve-Contact on  $I_{Na}$  Expression**

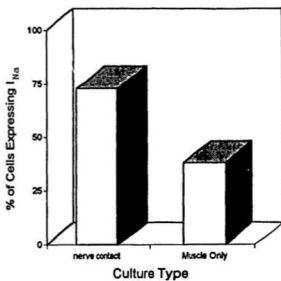
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**Figure 5: Effect of Nerve-Contact on  $I_{Na}$  Expression.** Shown are the percentages of nerve-contacted (nerve contact) and muscle cells grown without nerves (muscle only) which exhibited an  $I_{Na}$ . Nerve-contacted muscle cells expressed  $I_{Na}$  a significantly ( $p < 0.01$ , binomial test) greater proportion of the time (73%,  $n=49$ ) than muscle cells grown without nerves (38%,  $n=45$ ).



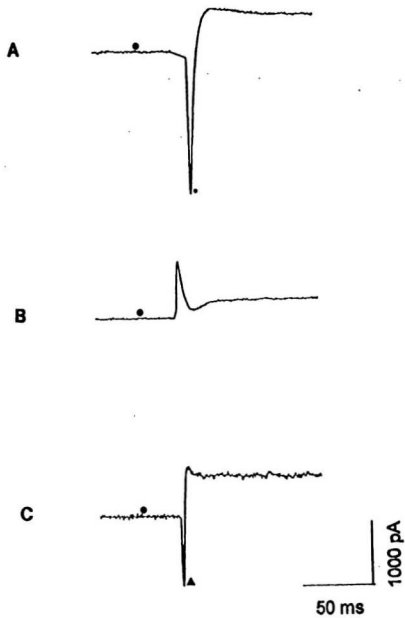
**Figure 5: Effect of Nerve Contact on  $I_{Na}$  Expression**



**Figure 6:** Acute Effect of TTX Treatment on  $I_{Na}$  Expression

**Figure 6: Acute Effect of TTX Treatment on  $I_{Na}$  Expression.** (A) Typical  $I_{Na}$  recorded from a nerve-contacted muscle cell after 24 hours in culture. (Peak current = \*). 3  $\mu$ m TTX was added to the bath solution following this recording. (B) While TTX was in the bath solution recordings from the nerve-contacted muscle cells did not exhibit an  $I_{Na}$ . Shown is a current trace which is representative of the 5 cells recorded from with TTX in the bath solution. (C) Following washout of the TTX solution with extracellular solution which contained no TTX  $I_{Na}$  could be recorded again (Peak current =  $\blacktriangle$ ). Shown is a current trace which is representative of the 4 cells recorded from following TTX washout. TTX treatment did not alter the kinetics or size of the current. Current traces from A, B, and C were taken at a test potential of -30 mV from a holding potential of -80 mV ( $\bullet$ ).

Figure 6: Acute Effect of TTX Treatment on  $I_{Na}$  Expression



in muscle cells of muscle only cultures (38%;  $p < 0.01$ ,  $n = 45$ ). These results are summarized in Figure 7 and Table 2. Similarly, muscle cells plated with TTX in the absence of nerve had 0% probability of expressing a  $I_{Na}$  ( $n = 16$ ), which was a significantly lower probability than nerve-contacted (73%;  $p < 0.01$ ,  $n = 49$ ) and nerve free cultures (38%;  $p < 0.01$ ,  $n = 45$ ) grown without TTX, see Figure 7 and Table 2.

These results were not due to TTX remaining in the culture dish during the recordings. An acute experiment was performed in which  $Na^+$  currents were recorded from nerve-contacted muscle cells prior to, during, and after one hour exposure to TTX. In the presence of TTX no  $I_{Na}$  could be recorded ( $n = 5$ ). TTX was removed by washing four times with an extracellular solution without TTX. Following TTX wash out  $I_{Na}$  could once again be recorded from the cultured cells ( $n = 4$ ). These results demonstrate that  $3 \mu M$  TTX blocked  $I_{Na}$  in culture and the washout procedure was sufficient to remove the drug following one hour exposure (Figure 6). However it is not known whether 24 hours of TTX treatment caused irreversible changes in the  $Na^+$  channel or cell membrane conformation, resulting in permanent block of  $Na^+$  channels that were present in the membrane. TTX ( $3 \mu M$ ) blocked not only muscle  $Na^+$  channels but was found to block the nerve  $Na^+$  channels at this concentration (Cohen, 1972;

**Figure 7: Drug Treatment Effects on  $I_{Na}$  Expression**

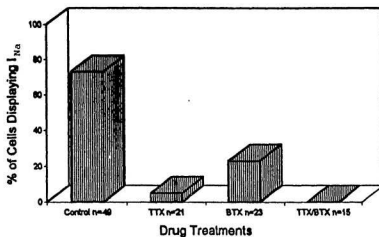
Figure 7: Drug Treatment Effects on  $I_{Na}$  Expression

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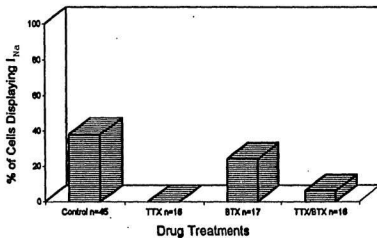
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**Figure 7: Drug Treatment Effects on  $I_{Na}$  Expression** ..(A) Shown is the percentage of nerve-contacted muscle cells in each drug treatment group which exhibited an  $I_{Na}$ . All drug treatments resulted in a significant ( $p < 0.01$ , binomial test) decline in probability of expressing an  $I_{Na}$  as compared to untreated nerve-contacted muscle cells (73%,  $n=49$ ). TTX treatment resulted in only 5% of cells ( $n=21$ ) displaying an  $I_{Na}$ .  $\alpha$ -BTX treatment resulted in only 23% of cells ( $n=23$ ) displaying an  $I_{Na}$ , while both drug treatments when combined resulted in 0% of cells ( $n=15$ ) displaying an  $I_{Na}$ . (B) Shown is the percentage of muscle only cells in each drug treatment group which displayed an  $I_{Na}$ . TTX treatment resulted in 0% of cells ( $n=16$ ) displaying an  $I_{Na}$ .  $\alpha$ -BTX treatment resulted in 24% of cells ( $n=17$ ) displaying an  $I_{Na}$ , while both drug treatments when combined resulted in 6% of cells ( $n=16$ ) displaying an  $I_{Na}$ . The probability of expressing an  $I_{Na}$  in the TTX treatment and the combined treatment of TTX and  $\alpha$ -BTX were significantly lower than the muscle only control group (38%,  $n=45$ ,  $p < 0.01$ , binomial test).

**Figure 7a: Drug Effects on  $I_{Na}$  Expression in Nerve-Contacted Muscle Cells**



**Figure 7b: Drug Effects on  $I_{Na}$  Expression in Muscle Only Cells**





Kullberg et al, 1977; Willard, 1980). By blocking neuronal  $\text{Na}^+$  channels, ACh release was reduced, resulting in a reduction of post-synaptic activity. To test the role of synaptic activity on  $\text{Na}^+$  channel expression,  $\alpha$ -BTX (an irreversible blocker of AChR channels) was added to the culture media for the 24 hour growing period.

### 3.1.3 Synaptic Activity:

Nerve-contacted muscles and muscle cells grown without nerves were cultured in the presence of  $1 \mu\text{M}$   $\alpha$ -BTX to block the effects of AChR openings. It was found that only 22% of nerve contacted muscle cells grown in the presence of  $\alpha$ -BTX had  $\text{Na}^+$  currents ( $n=23$ ). see Figure 7 and Table 2. This was significantly lower than nerve-contacted muscle cells grown in the absence of  $\alpha$ -BTX (73%,  $p<0.001$ ,  $n=49$ ), but similar to the number of cells grown without nerve which expressed  $I_{\text{Na}}$  (38%,  $n=45$ ). Furthermore, muscle cells in muscle only cultures with  $\alpha$ -BTX showed no significant difference in the probability of expressing a  $I_{\text{Na}}$  (24%,  $n=17$ ) compared to muscle cells grown in the absence of  $\alpha$ -BTX (38%,  $n=45$ ).

It appears that  $\alpha$ -BTX, like TTX prevented the nerve-contacted cells from upregulating their  $\text{Na}^+$  channel expression. Assuming that  $\alpha$ -BTX treated

cultures had functional neurons, the effects of  $\alpha$ -BTX can be explained by a decreased muscle membrane depolarization following  $\alpha$ -BTX treatment. Skeletal muscles may need depolarization or functional pulsatile ACh release to maintain  $\text{Na}^+$  channels in nerve-contacted cells, while it would seem that muscle only cells require less depolarization to maintain their lower level of  $\text{Na}^+$  channels, because some cells with  $\alpha$ -BTX treatment still displayed  $\text{Na}^+$  currents. These cells may have had functional  $\text{Na}^+$  channels prior to nerve-contact, which could maintain themselves by spontaneous openings causing small depolarizations.

No nerve-contacted cell contained  $\text{Na}^+$  currents when cultured with a combination of TTX and  $\alpha$ -BTX, ( $n = 15$ ), a result significantly different from nerve-contacted controls ( $p < 0.001$   $n = 49$ ), see Figure 7 and Table 2. The probability of displaying a  $\text{Na}^+$  current was reduced to 6% in muscle only cells cultured with TTX and  $\alpha$ -BTX ( $n = 16$ ), significantly lower than muscle only controls (38%,  $p < 0.01$ ,  $n = 45$ ), see Figure 7 and Table 2.

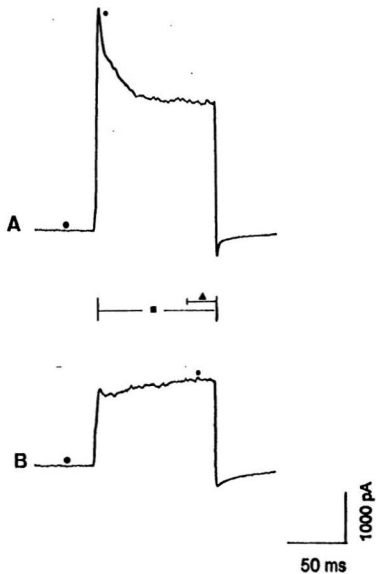
### 3.2 Outward $\text{K}^+$ Channel Expression

Whole-cell current recordings from *Xenopus* myocytes show that the myocytes may develop both an inactivating ( $I_{K1}$ ) and a non-inactivating ( $I_{Kr}$ ) outward potassium current during the first day in culture (Spruce and Moody,

**Figure 8: Outward  $K^+$  Currents From Muscle Cells Cultured in the Absence of Nerve Cells**

**Figure 8: Outward  $K^+$  Currents From Muscle Cells Cultured in the Absence of Nerve Cells.** (A) Shown is a typical  $I_{K1}$  recorded from a muscle cell grown for 24 hours in the absence of nerves. The voltage step was 100 msec long and was recorded at a membrane potential of +50 mV (●) from a holding potential of -80 mV (■). The rapid activation rate and the inactivation to a level lower than 80% of the peak current classifies this current as an  $I_{K1}$ . (B) Shown is a typical  $I_K$  current recorded from a muscle cell grown for 24 hours in the absence of nerves. The voltage step was 100 msec long and was recorded at a membrane potential of +50 mV (●) from a holding potential of -80 mV (■). The  $I_K$  current depicted slowly activates, however some  $I_K$ s were found to inactivate slightly during the 100 msec long pulse. Peak current was measured at a point depicted by (\*). Inactivation was measured by comparing the late current (the last 10 msec of the voltage step) depicted by (▲) to the peak current.

**Figure 8:** Outward  $K^+$  Currents From Muscle Cells Cultured in the Absence of Nerve Cells



1992; Moody-Corbett and Gilbert, 1992a,b; Ribera and Spitzer, 1991). These two currents are shown in Figure 8. In muscle only cultures the  $I_{K1}$  declined during the 100 msec voltage step to +50 mV to a value  $62.5\% \pm 2.7\%$  ( $n=22$ ) of the peak current. The  $I_{K1}$  could be distinguished from the  $I_{K2}$  since it inactivated to a lesser degree of ( $94\% \pm 2.9\%$   $n=20$ ) after the same 100 msec pulse to +50 mV. As well  $I_{K1}$  was found to be substantially larger in peak value and current density than the  $I_{K2}$  in all treatment groups. Both current types have similar voltage sensitivities and reversal potentials (Moody-Corbett and Gilbert, 1992a), while TEA and 4-AP do not discriminate between the two currents (Moody-Corbett and Gilbert, 1992b). The roles of nerve-contact, membrane excitability, and synaptic activity were examined on the regulation of both types of outward  $K^+$  current.

### 3.2.1 Nerve Contact

The proportion of nerve-contacted muscle cells containing  $I_{K1}$  was significantly higher (73%,  $n=49$ ) than in muscle cells grown without nerves (49%  $p < 0.01$ ,  $n=45$ ), see Figure 9a, while the opposite effect was found for the  $I_{K2}$  (see Figure 9b). The densities of the two outward potassium currents were unchanged by nerve-contact (see Figure 10 and Table 3). The inactivation

**Figure 9: Effects of Nerve-Contact on Outward  $K^+$  Current Expression**

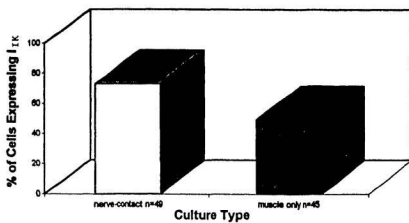
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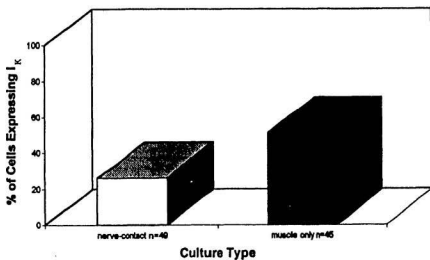
**Figure 9: Effect of Nerve-Contact on Outward Potassium Current Expression.** (A) Shown is the effect of nerve-contact on  $I_{K}$  expression. Nerve-contacted cells (open bars) were found to have a significantly ( $p < 0.01$ ,  $\chi^2$  test) higher probability (73%,  $n=49$ ) of expressing an  $I_{K}$  than muscle cells grown in the absence of nerves (solid bars) (49%,  $n=45$ ). (B) Shown is the effect of nerve-contact on  $I_{K}$  expression. Nerve-contacted cells (open bars) were found to have a significantly ( $p < 0.01$ ,  $\chi^2$  test) lower probability (26%,  $n=49$ ) of expressing an  $I_{K}$  than muscle cells grown in the absence of nerves (solid bars) (51%,  $n=45$ ).



**Figure 9a: Effect of Nerve-Contact on  $I_{IK}$  Expression**



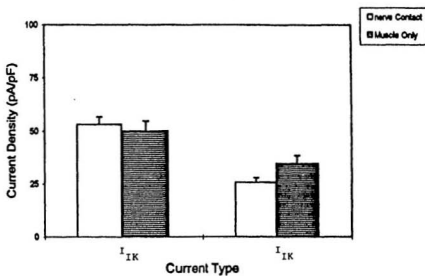
**Figure 9b: Effect of Nerve-Contact on  $I_K$  Expression**



**Figure 10:** Effects of Nerve-Contact on Outward  $K^+$  Current Density

**Figure 10: Effects of Nerve-Contact on Outward  $K^+$  Current Density.** The current density for  $I_K$  and  $I_{K1}$  in muscle cells contacted by nerve (open bars) and muscle cells grown in the absence of nerve (cross hatched bars) is shown. Nerve-contact was found to have no significant effect on the current density of either  $I_K$  or  $I_{K1}$ . However,  $I_{K1}$  was significantly larger ( $p < 0.01$ , binomial test) than  $I_K$  in both nerve-contacted and muscle only cultures.

Figure 10: Effect of Nerve-Contact on Outward K<sup>+</sup> Current Density



**Table 3:** Effects of Nerve-Contact and Drug Treatments on  $I_{Kx}$ 

treatments	Muscle Only			Nerve Contact		
	# of Cells with $I_{Kx}$	$I_{Kx}$ (pA/pF)	$I_{Kx}$ (pA/pF)	# of Cells with $I_{Kx}$	$I_{Kx}$ (pA/pF)	$I_{Kx}$ (pA/pF)
no Treatment	22/45 (49%)	50 ± 4.6	34.7 ± 3.7	36/49 (73%)	53 ± 3.6	25.7 ± 2.1
TTX	3/16 (19%)	39.4 ± 0.5	33.5 ± 4.3	16/21 (76%)	51.9 ± 5.3	29.3 ± 3.2
$\alpha$ -BTX	10/17 (59%)	63.8 ± 12	47.2 ± 4.5	17/23 (74%)	45 ± 4.5	27.2 ± 5.6
TTX/BTX	8/16 (50%)	53 ± 5.8	46.2 ± 5.5	6/15 (40%)	52.9 ± 8.9	37.6 ± 4.2

of the  $I_{IK}$  and  $I_K$  were unchanged as well by nerve-contact. The  $I_{IK}$  inactivated to  $66.4\% \pm 1.7\%$  following a 100 msec pulse, while the  $I_K$  inactivated to  $92.9\% \pm 2.3\%$  following a 100 msec pulse, indicating that the channel kinetics were unchanged by nerve-contact, see Figure 11 and Table 4.

Since nerve-contacted muscle cells also had a higher probability of containing  $I_{Na}$ , the occurrence of cells with both  $I_{Na}$  and  $I_{IK}$  was examined. Regardless of nerve-contact, cells with  $Na^+$  currents had a significantly higher probability of containing an  $I_{IK}$  than cells without  $Na^+$  currents. (see table 5 and Figure 12). These results suggest that nerve-contact causes an increase in  $I_{IK}$  channel expression in these cells, and that  $I_{IK}$  was correlated with  $I_{Na}$  expression.

### 3.2.2 Membrane Excitability

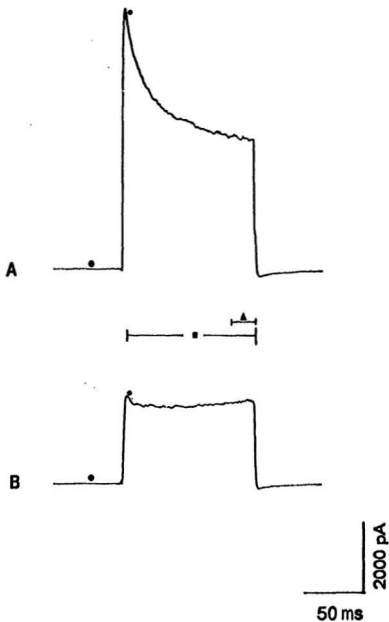
Following nerve-contact  $I_{IK}$  expression was found to increase significantly, while the current density was unchanged. To test the role of membrane excitability on  $I_{IK}$  expression, cells were cultured with or without nerves in the presence of  $3 \mu M$  TTX for 24 hours. Nerve-contacted cells cultured with  $3 \mu M$  TTX had the same probability of expressing  $I_{IK}$  (76%,  $n = 21$ ) as did nerve-contacted muscle cells grown without TTX (73%,  $n = 49$ , see

**Figure 11: Outward  $K^+$  Currents From Nerve-Contacted Muscle Cells**

**Figure 11: Outward  $K^+$  Currents From Nerve-Contacted Muscle Cells.** (A) Shown is a typical  $I_K$  current recorded from a nerve-contacted muscle cell grown for 24 hours in culture. The voltage step was 100 msec long and was recorded at a membrane potential of +50 mV (●) from a holding potential of -80 mV (■). The rapid activation rate and the inactivation to a level lower than 80% of the peak current classifies this current as an  $I_K$ . (B) Shown is a typical  $I_K$  current recorded from a nerve-contacted muscle cell grown for 24 hours in culture. The voltage step was 100 msec long and was recorded at a membrane potential of +50 mV (●) from a holding potential of -80 mV (■). The  $I_K$  current depicted inactivates slightly, however some  $I_K$ s were found to activate slowly during the 100 msec long pulse. Peak current was measured at a point depicted by (\*). Inactivation was measured by comparing the late current (the last 10 msec of the voltage step) depicted by (▲) to the peak current.



**Figure 11:** Outward  $K^+$  Currents From Nerve-Contacted Muscle Cells



**Table 4:** Effects of Nerve-Contact on  $I_{hK}$  and  $I_K$  Inactivation

**Figure 4: Effects of Nerve-Contact on  $I_{K1}$  and  $I_K$  Inactivation**

	Nerve Contacted Muscle Cells				Muscle Cells Without Nerve Contact			
	Control	TTX	$\alpha$ -BTX	TTX/ $\alpha$ -BTX	Control	TTX	$\alpha$ -BTX	TTX/ $\alpha$ -BTX
$\%I_{K1}$ Inactivation	$66.4 \pm 1.7$	$65.6 \pm 2.4$	$68 \pm 1.8$	$65.6 \pm 2.6$	$62.5 \pm 2.7$	$75 \pm 2.3$	$71.6 \pm 3.1$	$72.9 \pm 3.2$
$\%I_K$ Inactivation	$92.9 \pm 2.3$	$90.7 \pm 2.9$	$90 \pm 2.3$	$85.3 \pm 1.6$	$94 \pm 2.9$	$89 \pm 1.3$	$89.2 \pm 2$	$89.8 \pm 2.6$

**Table 5:** Effects of Nerve Contact and Occurrence of  $I_{Na}$  on  $I_{IK}$  Expression

	Nerve Contact		Muscle Only	
	With $I_{Na}$	Without $I_{Na}$	With $I_{Na}$	Without $I_{Na}$
% of Cells	29/34 (85%)	8/15 (53%)	12/17 (71%)	10/28 (36%)
With $I_{IK}$	*		**	
% of Cells	5/34 (15%)	7/15 (47%)	5/17 (29%)	18/28 (64%)
With $I_K$				

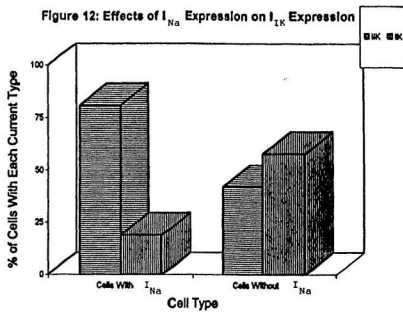
\* - Significantly higher probability than Nerve-Contacted cells without  $I_{Na}$  ( $p < 0.01$ , binomial test)

\*\* - Significantly higher probability than Muscle Only cells without  $I_{Na}$  ( $p < 0.01$ , binomial test)

**Figure 12: Effects of  $I_{Na}$  Expression on  $I_{IK}$  and  $I_K$  Expression**

**Figure 12:** Effects of  $I_{Na}$  Expression on  $I_{Kx}$  and  $I_K$  Expression. The percentage of cells which displayed either  $I_{Kx}$  or  $I_K$  is shown. Regardless of nerve-contact, muscle cells which displayed an  $I_{Na}$  had a significantly ( $p < 0.01$ , binomial test) higher probability of expressing an  $I_{Kx}$  (81%,  $n=51$ ) than muscle cells which did not display an  $I_{Na}$  (42%,  $n=43$ ). (cells with  $I_{Kx}$  are shown as horizontal hatched bars, cells with  $I_K$  are shown as vertical hatched bars).

Figure 12: Effects of  $I_{Na}$  Expression on  $I_{IK}$  Expression



**Figure 13: Drug Effects on  $I_{h3}$  Expression**

21

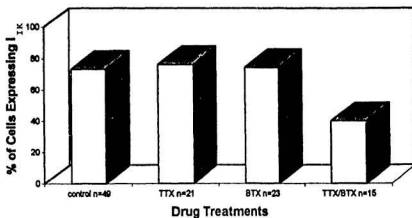
22

23



**Figure 13: Drug Effects on  $I_{K}$  Expression.** The percentage of cells which displayed an  $I_{K}$  in each drug treatment group is shown. (A) In nerve-contacted muscle cells, treatment of cultures with either TTX or  $\alpha$ -BTX alone did not alter the muscle cell's probability of expressing  $I_{K}$ . However the combined treatment of cultures with both TTX and  $\alpha$ -BTX significantly ( $p < 0.01$ , binomial test) reduced the probability of  $I_{K}$  expression (40%,  $n=15$ ) from control cultures (73%,  $n=49$ ). (B) In muscle cells grown in the absence of nerves, TTX treatment significantly ( $p < 0.05$ , binomial test) reduced the probability of cells expressing  $I_{K}$  (19%,  $n=16$ ) from muscle only control levels (49%,  $n=45$ ). However  $\alpha$ -BTX treatment or the combined treatments of TTX and  $\alpha$ -BTX did not affect the expression of  $I_{K}$  in muscle only cells.

**Figure 13a: Drug Effects on  $I_{IK}$  Expression in Nerve-Contacted Muscle Cells**



**Figure 13b: Drug Effects on  $I_{IK}$  Expression in Muscle Only Cells**

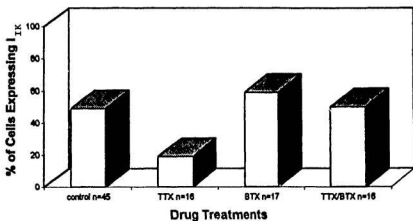


Figure 13a). Given that nerve-contacted muscle cells that were grown in the presence of TTX, have a greatly reduced probability of expressing an  $I_{Na}$ ; these results indicate that the expression of  $I_{IK}$  is not dependent on the coexpression of  $I_{Na}$ . Similarly, the other treatments which caused a reduction in  $I_{Na}$  expression on nerve-contacted muscle cells did not affect the expression or current density of  $I_{IK}$ . In contrast to the effect of TTX on nerve-contacted muscle cells, muscle only cells cultured with TTX had significantly lower probability of containing an  $I_{IK}$  current (19%,  $n=16$ ) than muscle controls (49%,  $n=45$ ,  $p < 0.05$ , see Figure 13b).

### 3.2.3 Synaptic Activity

Nerve-contacted muscle cells grown with  $\alpha$ -BTX had a similar probability of expressing  $I_{IK}$  (74%,  $n=23$ ) as nerve-contacted muscle cells grown without blocking agents, see Figure 13a. There was no difference in the outward potassium current densities in nerve-contacted muscle cells grown in the presence or absence of  $\alpha$ -BTX, see Table 3.

Alpha-bungarotoxin in the muscle only dishes did not affect the expression of  $I_{IK}$  in muscle cells in these dishes. Fifty-nine percent of the muscle only cells grown in the presence of  $\alpha$ -BTX had  $I_{IK}$  ( $n=17$ ), a result similar to

muscle only controls, see Figure 13b. However, the cells grown in the presence of  $\alpha$ -BTX had increased  $I_{IK}$  ( $63.8 \pm 12$ ) and  $I_K$  ( $47.2 \pm 4.5$ ) densities over control cells, see Table 3.

When both TTX and  $\alpha$ -BTX were added to the culture media of nerve-contacted muscle cells,  $I_{IK}$  expression was significantly ( $p < 0.01$ ) reduced (40%,  $n = 15$ ) from nerve-contacted controls grown without blocking agents (73%,  $n = 49$ ). The  $I_{IK}$  expression in the nerve-contacted muscle cells cultured with both TTX and  $\alpha$ -BTX was similar to muscle only controls, while the current density was unaffected. The presence of both TTX and  $\alpha$ -BTX in the growth medium resulted in muscle only cells expressing  $I_{IK}$  and  $I_K$  with a similar probability as untreated controls. The  $I_K$  current density was increased over control levels as well, however, the values were not significant.

In nerve-contacted muscle cells it appears that  $I_{IK}$  and  $I_{Na}$  are regulated by different mechanisms, with  $I_{Na}$  dependent upon synaptic activity while the  $I_{IK}$  expression is dependent upon some other mechanism. However the regulation of  $I_{IK}$  expression in muscle only cultures follows a different mechanism still. The muscle only TTX results suggest that before nerve-contact  $Na^+$  and  $I_{IK}$  channels are regulated together, while the  $\alpha$ -BTX results indicate that outward potassium channels are regulated by AChR levels prior to nerve-contact.

### 3.3 Inward Potassium Current

The inward rectifier is the earliest voltage dependent ion channel expressed in embryonic *Xenopus* myocytes, being found in almost all cells by stage 15 of development (Spruce and Moody, 1992). This current has been found colocalized with AChRs at the earliest times in culture, (Hancock et al, in press). IR currents in nerve-contacted muscle cells were found to be similar to the IR currents recorded in the muscle only cultures, which were described previously (Moody-Corbett and Gilbert, 1992) in this preparation. An example of an IR recorded in both a nerve-contacted muscle cell and a muscle cell in a nerve free dish are shown in Figure 14. All cells displayed an IR current after one day in culture regardless of nerve-contact. The IR current density remained unchanged following nerve-contact and subsequent increases in  $\text{Na}^+$  current, see Figure 15. The presence of IRs was not affected by growing the cells in the presence of TTX,  $\alpha$ -BTX or the combination of the two (TTX/ $\alpha$ -BTX). Muscle cells always expressed an IR. The density of the IR current did not vary in nerve-contacted muscle cells under these different treatment conditions and was not significantly different from IR current density in muscle cells grown in

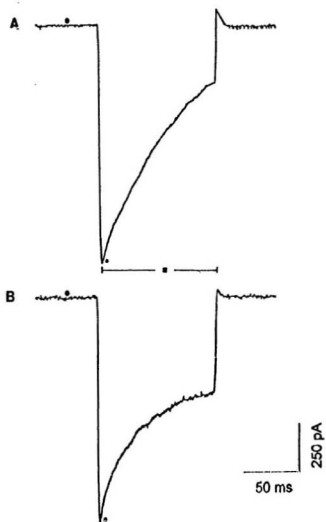
the absence of nerve. IR current density in muscle cells grown in the absence of nerves however, were affected by TTX but not  $\alpha$ -BTX. In the presence of TTX, there was a significant reduction in IR current density compared to muscle controls ( $p < 0.05$ , see Figure 15).

**Figure 14:** Inward Rectifier Current Traces in Muscle Only and Nerve-Contacted Muscle Cells

**Figure 14: Inward Rectifier Current Traces in Muscle Only and Nerve-Contacted Muscle Cells.** (A) Shown is an IR current recorded from a muscle only cell grown for 24 hours in the absence of nerves. The voltage step was 100 msec long and was recorded at a membrane potential of -160 mV ( ● ) from a holding potential of -60 mV ( ■ ). The size and inactivation rate is similar to IR currents recorded with the same protocol in nerve-contacted muscle cells as shown in (B). Peak current was measured at a point depicted by ( \* ).



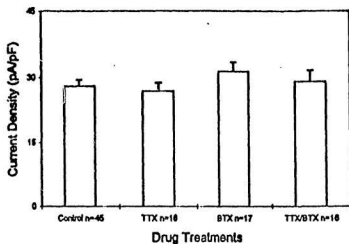
**Figure 14:** Inward Rectifier Current Traces in Muscle Only and Nerve-Contacted Muscle Cells



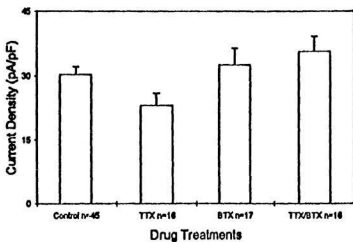
**Figure 15: Drug Treatment Effects on IR Current Density**

**Figure 15: Drug Treatment Effects on IR Current Density.** (A) Shown are the effects of drug treatments on IR current density in nerve-contacted muscle cells. It was found that drug treatments did not affect IR current density in nerve-contacted muscle cells. (B) Shown are the effects of drug treatments on IR current density in muscle cells grown in the absence of nerve cells. TTX treatment significantly ( $p < 0.05$ , Fisher) reduced IR current density ( $23.1 \pm 2.9$  pA/pF,  $n=16$ ) from muscle control values ( $30.4 \pm 1.8$  pA/pF,  $n=45$ ).  $\alpha$ -BTX treatment and the combined treatment of both TTX and  $\alpha$ -BTX did not alter the IR current density from control values. Standard error bars are shown for each treatment group.

**Figure 15a: Drug Effects on IR Current Density in Nerve-Contacted Cells**



**Figure 15b: Drug Effects on IR Current Density in Muscle Only Cells**



## CHAPTER 4

### 4. Discussion

The purpose of this work was to test whether nerve-contact, membrane excitability and synaptic activity altered the developmental expression of  $\text{Na}^+$  and  $\text{K}^+$  currents in *Xenopus* myocytes in culture. The results show that nerve-contact increased the probability of  $I_{\text{Na}}$  and  $I_{\text{K}}$  expression while it did not alter the current densities of the four currents examined ( $I_{\text{Na}}$ ,  $I_{\text{K}}$ ,  $I_{\text{K}}$ , and  $I_{\text{R}}$ ). It would appear from these results that  $\text{Na}^+$  channels require depolarizations most likely from synaptic activity, to become upregulated following nerve-contact. Blocking either synaptic activity or membrane excitability did not prevent  $I_{\text{K}}$  upregulation following nerve-contact, suggesting that  $I_{\text{K}}$  currents are more dependent on physical contact or the effect of undetermined synaptic chemical factors. In muscle only cultures  $I_{\text{Na}}$  and  $I_{\text{K}}$  expression were differentially altered by TTX and  $\alpha$ -BTX treatment.

## 4.1 Sodium Channel Expression

### 4.1.1 Nerve Contact

The  $I_{Na}$  was found more often in nerve-contacted muscle cells than in muscle cells grown in the absence of nerves. Studies by DeCino and Kidokoro, 1985; and Corfas and Fischbach, 1993 have suggested that nerve-contact may alter the density and localization of  $Na^+$  channels in embryonic muscles. This present study is in agreement with these past studies and provides evidence that nerve-contact increases or accelerates the expression of  $I_{Na}$  in embryonic myocytes in culture. Cohen et al, 1979, found that 73% of nerve-contacts, in nerve-muscle cocultures from *Xenopus* embryos (the same as those used in this work) formed functional synapses which were able to cause accumulation of AChR receptors. Results of the present study show that 73% of nerve-contacted cells express  $I_{Na}$  and  $I_{K}$ . It is tempting to speculate that the cells which expressed  $I_{Na}$  and  $I_{K}$  in the present study represent the population of cells with functional synapses. That is, only functional cholinergic synapses can induce the acceleration of  $I_{Na}$  and  $I_{K}$  expression in these muscle cells. Consistent with these results it was found that  $\alpha$ -BTX, which blocked synaptic activity, also blocked the up-regulation of  $I_{Na}$ . As discussed below,  $\alpha$ -BTX did not effect

the up-regulation of  $I_{K\alpha}$ , suggesting that the expression of these two currents are affected by different aspects of synaptic contact.

In addition to the effects of nerve-contact, it was found that  $I_{Na}$  expression was influenced by synaptic activity in nerve-contacted muscle cells, similar to the TTX sensitive  $Na^+$  channel described in other muscles (Sherman and Caterall, 1984; Caldwell and Milton, 1988).

In muscle only cultures, AChRs, not  $Na^+$  channels are the main depolarizing channel. Harpur et al, 1993 suggested that the fast depolarization provided by  $Na^+$  channels are present in small quantities in early myocytes because their fast depolarizations may be harmful to the cell prior to nerve-contact. This idea is supported by the findings in *Xenopus* muscle in which  $I_{Na}$  is developed after the  $I_R$ ,  $I_{K\alpha}$ , and  $I_{K\beta}$  currents (Spruce and Moody, 1992) and it has likewise been suggested that this sequence may act to prevent depolarizations prior to nerve-contact (Linsdell and Moody, 1994). The fact that some myocytes in muscle only cultures contained an  $I_{Na}$  should be noted. It could be argued that these myocytes were nerve-contacted *in vivo* prior to dissection, which accelerated their development, similar to the nerve-contacted myocytes in culture. However, Chow, 1980 reported that at the earliest stage of dissection (stage 19) only 6% of embryos display nerve contact staining in the anterior three myotomes (the percentage of nerve-contact at the latest stage

of dissection used (stage 23) may be some what higher). Thus the probability that myocytes were nerve-contacted *in vivo* prior to culturing was low. As well, Spruce and Moody, 1992 reported finding  $I_{Na}$  in muscle cells from muscle only cultures after one day in culture. Since their dissections were performed at stage 15 none of the cells were nerve-contacted prior to culturing. Thus,  $I_{Na}$  development may occur in muscle only cells which are not nerve-contacted.

#### 4.1.2 Membrane Excitability and Synaptic Activity

In this *Xenopus* system one of the first nerve-muscle interactions is a spontaneous pulsatile release of ACh within seconds of nerve-contact, which results in pulsatile synaptic currents within the muscle, (Xie and Poo, 1986). In the present study it was found that blocking synaptic activity prevented the upregulation of  $I_{Na}$ . This result suggests that the increased  $Na^+$  channel expression following nerve-contact is probably dependent on pulsatile depolarizations, generated by AChR openings in response to nerve signalling. Synaptic activity in embryonic muscle is known to regulate muscle development. For example, Kidokoro and Saito, 1988, found that nerve-contacted muscle cells develop cross-striations earlier than muscle cells grown in the absence of nerve. Blocking synaptic activity with  $\alpha$ -BTX delayed cross



striations and spontaneous twitching compared to nerve-muscle cocultures grown without  $\alpha$ -BTX. This delayed maturation was not found when the nerve-muscle cocultures were grown in the presence of 3  $\mu$ M TTX further supporting the findings that synaptic activity is the regulatory mechanism responsible for *Xenopus* myocyte development following nerve-contact.

Block of the AChRs with  $\alpha$ -BTX treatment prevented  $I_{Na}$  development, yet the release of neural factors was not inhibited. Therefore, it can be deduced that chemicals which have been suggested as possible modulators of ion channel expression following nerve-contact, such as agrin (McMahan, 1990) CGRP (Sala et al, 1995), ARIA (Corfas and Fischbach, 1993), or bFGF (Peng et al, 1991) do not appear to play a role in the developmental expression of  $I_{Na}$  in nerve contacted *Xenopus* myocytes after one day in culture.

Calcium is an important ion in the regulation of a number of cellular processes (Hille, 1992). Most relevant to this work, researchers have reported downregulations in  $Na^+$  channels following increases in intracellular  $Ca^{2+}$  concentration in rat skeletal muscle (Sherman and Caterall, 1982; Sherman et al, 1985; Brodie et al, 1989) and rat cardiac myocytes (Chiamuimonvat et al, 1995). These results suggest that sites of local increases in intracellular calcium would be expected to have lower sodium current density. It is known that there is an influx of  $Ca^{2+}$  through AChR (Vervico et al, 1994), but despite this

increase in  $\text{Ca}^{2+}$  during synaptic activity  $\text{Na}^+$  channels are found to be highly concentrated at the site of nerve-contact in mature neuromuscular junctions (Boudier et al, 1992; Barchi, 1988; Beam et al, 1985). The results of the present experiment show that  $I_{\text{Na}}$  expression is increased in nerve-contacted muscle cells, which would be expected to have an increase in intracellular  $\text{Ca}^{2+}$  concentration through AChRs. Further, it was found that the proportion of cells with  $I_{\text{Na}}$  was reduced by  $\alpha$ -BTX block, which would be expected to result in a decrease in  $\text{Ca}^{2+}$  influx. Thus, in contrast to the expression of  $\text{Na}^+$  channels in rat muscle,  $I_{\text{Na}}$  in nerve-contacted *Xenopus* myocytes was increased in cells which would be expected to have increases in intracellular  $\text{Ca}^{2+}$ . An increase in  $\text{Ca}^{2+}$  influx has also been shown to facilitate the aggregation of AChRs in embryonic *Xenopus* muscle cells (Peng, 1984; Henderson et al, 1984). Taken together, these results suggest that increasing the influx of  $\text{Ca}^{2+}$  may also facilitate the expression of  $I_{\text{Na}}$  in embryonic *Xenopus* muscle.

Abu-Shakra et al, in 1993 found that nerve-contacted cells express a different set of immediate early genes (IEGs) than denervated muscle cells and these IEGs which are upregulated with AChR increases may also function to regulate ion channels differently following nerve-contact. The IEG's (*c-myc*, *c-jun*, and *c-fos*), are expressed transiently in skeletal muscles during development (Leibovitch et al, 1987; Wilkinson et al, 1989). In adult muscle

nerve stimulation caused the IEG (zif-268) to rise after 30 minutes and (c-jun) to rise within 45 minutes, suggesting that synaptic activity can initiate gene and subsequent AChR expression (Abu-Shakra et al, 1993). These recent experiments when combined with the present study indicate that following nerve-contact another set of regulatory mechanisms may be required to maintain and upregulate both  $I_{K}$  and  $I_{Na}$ . Future studies should attempt to correlate IEG levels with the developmental expression of ion channels.

#### 4.1.3 Autoregulation of the $I_{Na}$

In both muscle only and nerve-contacted muscle cells TTX in the culture medium blocked the expression of  $Na^+$  channels. It would seem that  $Na^+$  channels are autoregulated, since blocking  $Na^+$  channels with TTX prevents the development of  $I_{Na}$  prior to nerve-contact. Following nerve-contact synaptic activity upregulates the  $I_{Na}$ , however, the basal level is still maintained by a positive feedback mechanism. This result is in opposition to data from rat muscle, where TTX-sensitive  $Na^+$  channels were reported to be upregulated by blockade of electrical activity with TTX (Sherman and Catterall, 1984).

Future studies should investigate the time in culture required for TTX to prevent the expression of  $I_{Na}$ , since one hour of TTX treatment did not block  $I_{Na}$  expression while 24 hours of TTX treatment did. The results suggest that

functional  $\text{Na}^+$  channels are needed for the upregulation of  $\text{Na}^+$  channels. To further test this idea, cells from which  $I_{\text{Na}}$  have been recorded should be cultured for periods of time up to 24 hours in the presence of TTX or other  $\text{Na}^+$  channel blocking agents (saxitoxin) to determine if  $I_{\text{Na}}$  are maintained by a positive feedback mechanism.

The combined application of TTX and  $\alpha$ -BTX, also caused a decline in the number of cells expressing  $I_{\text{Na}}$  as would be expected from the TTX group results. However, one cell did display an  $I_{\text{Na}}$ . It may be that the cell which expressed an  $I_{\text{Na}}$  in the TTX/ $\alpha$ -BTX treatment group was nerve-contacted prior to dissection and thus may have contained a more developed  $I_{\text{Na}}$ . Another explanation for this anomalous result would be that there exists a competitive effect between these two drugs. To further test this possibility,  $\mu$ -conotoxin may be used to selectively block voltage-dependent  $\text{Na}^+$  channels in skeletal muscle (Cruz et al, 1985).  $\alpha$ -conotoxin may be used to selectively block neuromuscular AChR instead of  $\alpha$ -BTX (Hashimoto et al, 1985). By using these alternative blocking agents, any unknown interactions between TTX and  $\alpha$ -BTX may be circumvented. As well single channel recordings should be performed to determine if current density differences were the result of a change in channel number, channel type or channel kinetics.

## 4.2 Outward $K^+$ Currents

### 4.2.1 Nerve Contact

Recently, Ernsberger and Spitzer, 1995, demonstrated that *Xenopus* myocytes could express both  $I_{IK}$  and  $I_K$  type potassium channels after one day in culture. Previous whole cell data suggested that some myocytes express different proportions of these two types of channels (Moody-Corbett and Gilbert, 1992a). Thus, the amount of inactivation of the outward potassium current, will depend on the proportion of  $I_{IK}$  to  $I_K$  type channels in a muscle cell. The present data suggest that the contact of a nerve not only upregulates  $I_{Na}$  expression but it also upregulates the expression of the fast inactivating outward potassium channel resulting in  $I_{IK}$ . Thus, even if a cell contains both channel classes, nerve-contact upregulates the  $I_{IK}$  resulting in these cells displaying a faster inactivating outward potassium current than muscle only myocytes.

Similar to the  $I_{Na}$ , the density of  $I_{IK}$  was unchanged by nerve-contact. The  $I_K$  density likewise was unchanged by nerve-contact, and was similar in size to that reported by Spruce and Moody, 1992 for  $I_K$  after 24 hours in culture. Since neither Spruce and Moody, 1992 or Linsdell and Moody, 1994 reported current

densities for  $I_{IK}$  the present results cannot be compared to theirs.

The expression of  $I_{IK}$  and  $I_{Na}$  were highly correlated in nerve-contacted muscle cells. In fact, cells which expressed  $I_{Na}$  had a higher probability of expressing  $I_{IK}$  than cells without  $I_{Na}$  regardless of nerve-contact. These results are in agreement with those of Linsdell and Moody, 1994 who reported that increasing  $I_{Na}$  by expression of exogenous  $Na^+$  channels in *Xenopus* muscle resulted in an increased expression of  $I_K$ . However Linsdell and Moody reported an increased potassium current density in cells which contained an  $I_{Na}$ , which was not found in this work. This discrepancy may be accounted for by considering that Linsdell and Moody did not separate the outward potassium current into  $I_{IK}$  and  $I_K$ . Since  $I_{Na}$  containing cells have a higher proportion of  $I_{IK}$  than  $I_K$ , and  $I_{IK}$  is larger than  $I_K$ , data from the study by Linsdell and Moody may be accounted for by a change in the proportion of  $I_{IK}$  channels rather than an increase in current density.

Hamann et al, 1994, reported that outward potassium channels seem to be regulated by different mechanisms than  $Na^+$  channels in human satellite cells. Similarly, in the present study,  $I_{IK}$  and  $I_{Na}$  were found to be correlated but not coregulated. Following block of excitability by TTX or block of synaptic activity with  $\alpha$ -BTX,  $I_{Na}$  expression was reduced dramatically while  $I_{IK}$  expression remained elevated similar to nerve-contacted control levels. Similarly,  $I_K$  and  $I_{Na}$

were found to be regulated by different mechanisms in *Xenopus* spinal neurons where one day of TTX treatment did not affect  $I_K$  development (Desarmenien and Spitzer, 1991). Taken together these results suggest that  $I_{K}$  current is regulated by a different mechanism than the  $I_{Na}$  following nerve-contact.

Following 24 hours of culture with both blocking agents TTX and  $\alpha$ -BTX, the probability of  $I_{K}$  expression was similar to muscle only controls. This suggests that  $I_{K}$  expression is not dependent solely on membrane excitability or synaptic activity following nerve-contact, but may require some basal level of excitability to be upregulated. Alternatively, the set of IEGs which should be implemented following nerve-contact or neural activity, may not be initiated when both membrane excitability and synaptic activity are interrupted during the NMJ development.

Future studies should, as suggested previously, examine the role of IEGs in altering ion channel development, prior to and following nerve-contact. Alternatively, these data may have resulted from a drug interaction between TTX and  $\alpha$ -BTX. As discussed above this could be tested by substituting  $\mu$ -conotoxin and  $\alpha$ -conotoxin for these drugs respectively.

#### 4.2.2 Muscle Only

In the absence of nerve-contact, muscle cells had an equal probability of expressing either  $I_{IK}$  or  $I_K$ . Unlike nerve-contacted muscle cells, the outward potassium currents in muscle only cells were sensitive to block by  $\alpha$ -BTX and TTX. These results suggest that the  $Na^+$  channels and AChRs play different roles in regulating  $I_{IK}$  currents in muscle only cultures.

Muscle only cells grown in the presence of TTX showed a decreased expression of  $I_{IK}$  and a slight decrease in the density of  $I_{IK}$ . These data are consistent with the results reported by Linsdell and Moody, 1994 who also found decreased  $I_K$  density following TTX treatment. However, these results are inconsistent with the nerve-contacted data and suggest that the mechanisms regulating  $I_{IK}$  maybe different in muscle only and nerve-contacted muscle cells. Specifically, it may be that  $I_{IK}$  and  $I_{Na}$  are coregulated in muscle cells prior to nerve-contact but following nerve-contact regulation of these two channel classes are dependent on different neuronal signals.

In contrast to the TTX results, muscle cells grown in the presence of  $\alpha$ -BTX showed a slight increase in the expression of  $I_{IK}$ . Ribera and Spitzer in 1990 reported increases in  $I_{IK}$  in *Xenopus* spinal neurons during times when there is a decrease in excitability. The same conditions may exist on muscle only cells



following  $\alpha$ -BTX blockage. Several lines of evidence lend support to this idea. In adult muscle denervation or blockade of synaptic activity by blocking neuronal release of ACh or the AChRs caused increases in extrajunctional AChRs. Similar increases in AChR numbers were found to occur following denervation, or blockage of  $\text{Na}^+$  channels with TTX (see review by Fambrough, 1979; Cohen, and Fischbach, 1973; Shainberg and Burstein, 1976). To extend this idea further, it is known that in rat muscle AChRs and  $\text{Cl}^-$  channels are expressed in an "anticoordinate" manner, such that when excitability is blocked, or a muscle is denervated, AChR levels increase extrajunctionally while  $\text{Cl}^-$  channel levels decrease (Heathcote, 1989). Thus during times of low excitability AChR levels increase. As in *Xenopus* spinal neurons (Ribera and Spitzer, 1990), a decrease in membrane excitability may increase expression of the outward  $\text{K}^+$  channels in skeletal muscle. From this, it may be deduced that outward potassium channel expression may be coregulated with AChR levels. Thus, the cells may upregulate  $\text{K}^+$  channels to provide a means of repolarisation and to retain homeostatic balance prior to innervation. Alternatively, lack of depolarization in muscle only cells may allow  $\text{K}^+$  channel upregulation, if depolarizing channels were acting as regulators of  $\text{K}^+$  channels.

The combined treatments of TTX and  $\alpha$ -BTX were found to be additive. TTX treatment decreased  $I_{Kx}$  expression while  $\alpha$ -BTX alone enhanced outward

K<sup>+</sup> current, it would be expected that treatment of muscle only cells with both TTX and  $\alpha$ -BTX would also decrease  $I_{K}$  expression. Since  $I_{K}$  expression was not found to decrease following TTX/ $\alpha$ -BTX treatment it may be suggested that TTX and  $\alpha$ -BTX may be interacting competitively in this system. Further experiments, as suggested above in the  $I_{Na}$  section, may be required to isolate the effects of AChR and membrane excitability on  $I_{Na}$  and  $I_{K}$  expression in muscle only cells.

Further studies should include single channel recordings to determine if differences in current density were the result of changes in channel number, channel type or channel kinetics.

### 4.3 Inward Rectifier Regulation

The inward rectifier is the earliest ion channel expressed in embryonic *Xenopus* myocytes, being found in almost all cells by stage 15 of development (Spruce and Moody, 1992). The IR channel also seems to be unique in this system, in that it was the only channel which was not altered by nerve-contact. All cells displayed an IR current, and the current was unchanged by nerve-contact. Since Cl<sup>-</sup> currents are negligible in these cells (unpublished results) the IR current is the main conductance at rest. This may be the reason for the IR

current to be expressed early in development, and for its being less susceptible to factors which alter other ion channels.

A recent study by Linsdell and Moody, 1995, and work by Gono and Hasegawa, 1991, suggested that  $\text{Na}^+$  channel expression could increase and accelerate the developmental appearance of the IR current. They suggested that increases in excitability caused a subsequent rise in IR production, while decreased excitability would slow IR development. In the present study TTX block in muscle only cultures was found to cause a significant decrease in IR current density, compared to muscle only controls, in agreement with Linsdell and Moody. However, in the nerve-contacted cells no drug treatment had any effect on the IR current density. The current may develop regardless of the level of excitability by some mechanism initiated before culturing, or the factor regulating IR expression following nerve-contact may be a chemical signal which was unaccounted for in this study. To study this effect botulinum toxin may be used to prevent vesicular release of chemicals from the nerve terminals. Further investigation may require manipulation of the extracellular matrix at the synapse, to determine the role of extracellular matrix proteins, such as agrin.

In the muscle only cells, AChR block caused an increase in IR current density, in opposition to the effect of TTX. The combined treatment of TTX and  $\alpha$ -BTX increased the IR current density greater than the treatment of  $\alpha$ -BTX

alone. Similar to the outward  $K^+$  channels described above, it would appear from our data that the IR is tightly regulated with the first depolarizing channel found on these cells (AChRs). This is supported by the finding that the AChR and IR channel were found colocalized in embryonic *Xenopus* muscle membrane (Moody-Corbett and Hancock, 1994; Hancock et al, 1996). The increase in AChR levels following  $\alpha$ -BTX blockade may be correlated with an increase in IR current.

#### 4.4 Conclusions

It would appear from these data that nerve-contact does cause an increased rate of  $I_{Na}$  and  $I_K$  expression. These two currents are correlated at a high rate, however, similar to human satellite cells I must conclude that the expression of  $Na^+$  and  $K^+$  currents are not controlled in the same manner, (Hamann et al, 1994). Basal levels of  $I_{Na}$  are maintained through a positive feedback mechanism where functional  $Na^+$  channels are required to maintain an  $I_{Na}$ . Following nerve-contact, synaptic activity is needed to cause  $Na^+$  channel upregulation, while  $I_{K}$  current expression would not appear to be dependent on synaptic activity but dependent instead on some aspect of the physical nerve-contact. In muscle only cultures, it appears that AChR expression is related to IR and  $I_K$  current

expression, as these currents are found to be most dense when AChRs are blocked.  $I_{K1}$  currents are not found often on muscle only cells without  $Na^+$  currents, suggesting that these currents are coregulated before nerve-contact.

## CHAPTER 5.

### References

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