Temporal evolution of intracellular signaling and gene expression following patterns of membrane depolarization in the pheochromocytoma cell line, PC12

by

Amir H. Nashat

B.S. Mechanical Engineering
B.S. Materials Science and Mineral Engineering
University of California, Berkeley (1994)

M.S. Materials Science and Mineral Engineering
University of California, Berkeley (1996)

SUBMITTED TO THE DEPARTMENT OF CHEMICAL ENGINEERING IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF SCIENCE IN CHEMICAL ENGINEERING
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

OCTOBER 2002

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Signature of Author: [Signature]
Department of Chemical Engineering

[Signature]  September XX, 2002
OCTOBER 28

Certified by: [Signature]
Robert S. Langer
Germeshausen Professor of Chemical and Biomedical Engineering
Thesis Supervisor

Accepted by: [Signature]
Daniel Blankschtein
Professor of Chemical Engineering
Chairman, Committee for Graduate Students
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Submitted to the Department of Chemical Engineering
On October 28, 2002 in Partial Fulfillment of the Requirements for the Degree of Doctor of Science in Chemical Engineering

ABSTRACT

An experimental study was performed to characterize the effects of different patterns of membrane depolarization on undifferentiated pheochromocytoma (PC12) cells. In response to chronic depolarization, cytoplasmic free Ca\(^{2+}\) ([Ca\(^{2+}\)\(_i\)]\(_c\)) levels increased transiently, then decreased to intermediate levels that were maintained for more than 90 minutes. Short pulses of depolarization, from 1 to 5 minutes in length, also resulted in transient increases in [Ca\(^{2+}\)\(_i\)]. Following a recovery period in a polarized state, a second increase in [Ca\(^{2+}\)\(_i\)] could be induced by repeated depolarization.

The activity of signal transduction pathways was also characterized following patterns of depolarization. Chronic depolarization elicited transient (less than 10 minutes in duration) activation of the mitogen-activated protein (MAP) kinases Erk1 and Erk2, while the cAMP-response element binding protein (CREB) remained active at intermediate levels for over 60 minutes. Pulsatile depolarization also stimulated Erk1/2 and CREB activation, and the rate of deactivation of the MAP kinases was not found to depend on pulse duration. Lastly, both the MAP kinases and CREB were successfully reactivated by pulsatile stimulation, following recovery periods of greater than 10 minutes in duration. Thus, pulsatile stimulation may be a means of maintaining signaling activity over long periods of time.

The effect of depolarization on gene expression was determined. Gene expression profiling of PC12 cells over the course of 8 hours following a single or double pulse of stimulation confirmed that a diverse set of genes were regulated by electrical activity. These genes included neural differentiation-specific genes, as well as genes involved in cell cycle control and intracellular signaling. Finally, the gene expression profiles of PC12 cells that were depolarized for one week were also measured. Prolonged depolarization induced a new set of diverse genes, and some of which also have roles in cell cycle control and differentiation. Thus, we conclude from our studies that different patterns of electrical stimulation can have numerous instructive influences on undifferentiated neuron-like cell lines.

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ACKNOWLEDGEMENTS

Now that I am nearing the end, I begin to think that one of the conditions of completing a thesis is to have exhausted your supply of favors from most everyone you know. I have been fortunate to get support from and forge relationships with a truly wonderful group of people. I will try and list the most important here.

I begin with my advisor, Professor Robert S. Langer. Bob (there, I finally said it!) gave me the one thing I value most, his trust. Amazingly, he trusted me from the very beginning, and even more surprising, he maintained his faith in me during the long, bleak “middle” years. For me, the most sustaining force throughout this process has been Bob’s belief in me, and his readiness to support me at any turn in the road. Not many advisors will let you create your own project, or allow you to head off to Europe to trade bonds. Thank you, Bob, for your unrelenting trust in me.

I would also like to thank the many other professors that took the time to help and teach me. These include my committee members, Professors Stephanopoulos, Sasisekharan and Snyder, who kindly sat through many a presentation on kinase phosphorylation, and most importantly, told me what I was doing wrong. Also, Professors Liu, Sur and Quinn taught me enough about neurons to convince me that I’d never be a real neuroscientist. I would also like to thank the following people in the various labs I crashed: Drs. Dube, Egles, Lyckman, and Huang. Each was kind enough to give freely of their time, resources and understanding, and never made me feel like an intruder. I have been fortunate enough to find the most generous collaborators and teachers. Thank you, all.

For support of a different kind, I would like to thank the Hertz Foundation, the National Science Foundation, and the Biotechnology Training Grant, for contributing to my education.

And to the Langer Lab, I’d like to say thank you for all of your understanding. Since this is section to do it in, I acknowledge that my music was not the most soothing. And special shout-outs to the folks that really brought out the personality of the Lab: Dan Anderson, Dave Lynn, Betty Yu, Karen Fu, Prasad Shastri, Ivan Martin, Bojana O., Rubin Sinistera, and Maria Papadaki. I thank everyone for helping to create a really fun (and funny) lab.

I come now to the last group of relationships, the ones built on uncommonly large amounts of friendship and love. In the past six years, I have met an amazing group of individuals that would not otherwise have entered my life. These friendships have made my thesis well worth the time and struggle. To Jacob, Sachiko, Tommy, Akin, Henry, Suzi, Predrag and Nenad, the Cookies, Camillo, Ioana, Serafim, Julie and Sami (and so many people I am forgetting), I thank you all for being my friends throughout this process; for understanding when I needed to work instead of play, and play instead of work. I’ll be a lucky person if I have another chance to meet such outstanding people again.
Last, none of this would be possible without my family. I am thankful to have a mother, father and brother that love me unequivocally and unconditionally. They have patiently cheered me on, and have always understood my moods and needs. My mother and father, in particular, have taught me the importance of determination and character. And, I would like to especially thank my little brother for not rubbing it in that he finished school four years before me.

So saving the very best for very last, I would like to thank my wife for being an absolute inspiration. She has supported me, loved me, and believed in me with an unyielding spirit. And she was kind enough to threaten me with violence everytime I wanted to quit. She has made every corner of my life and my soul warmer and brighter, and without her love, this thesis, and so much else in my life, would not have been possible. Thank you, Carmen Barnés, for being amazing.
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CHAPTER 1. INTRODUCTION

The brain, that pound and a half of chicken-colored goo so highly regarded (by the brain itself), that slimy organ to which is attributed such intricate and mysterious powers ...

— Tom Robbins
Even Cowgirls Get the Blues

Since the turn of the century, starting with the work of Santiago Ramon y Cajal, biologists, physicians, and now increasingly computer scientists and engineers, have tried to understand how the brain develops from a mass of unconnected cells into a highly ordered structure that can simultaneously perform numerous calculations in specialized regions (reviewed in Albright et al., 2001). What is now accepted as a central tenet of neural development is that electrical activity helps pattern the connections within the brain. Thus, to a large extent, the input signals help design the cortical regions that ultimately will be receiving and processing these same signals (reviewed in Sur and Leamey, 2001). In this manner, the brain is able to adapt itself to new patterns and functions. While engineers have increasingly used feedback control to improve a wide variety of devices, from antilock breaking systems in automobiles to active vibration-damping systems in skis, none of these designs incorporate flexibility and feedback on the scale of the brain. None have the ability to re-engineer themselves, as the brain does. From a broad perspective, our aims in the current studies have been to contribute to the increasing understanding of the brain's adaptability to different inputs.

At the start of the current studies in 1996, neuroscientists had established that electrical activity could significantly influence neuronal survival and the strength of connections between neurons (reviewed in Kennedy, 1989) and (Ghosh and Greenberg, 1995)). The mechanisms for these phenomena were being uncovered, and in most cases involved the influx of Ca$^{2+}$ through voltage-stimulated Ca$^{2+}$ channels (VSCC's) during electrical activity. The influx of calcium ions was found to trigger the activation of various enzymes that mediated neuronal survival and adaptation. For example, it was observed that blocking the activation of Ca$^{2+}$/calmodulin dependent kinase II (CaMKII) following electrical stimulation abolished long-term potentiation (LTP, a process
currently believed to be central to memory formation in the brain) in the hippocampus (Malinow et al., 1989). Furthermore, it was found that electrical activity led to endogenous release of neurotrophins, such as brain derived neurotrophic factor (BDNF), that helped support cell survival (reviewed in (Marty et al., 1997)). Thus, in terminally differentiated neurons, electrical activity had been established as a major influence in maintaining the health and function of the cells.

The role of electrical stimulation in neuronal differentiation was much less established, however ((Gu and Spitzer, 1995), (Kocsis et al., 1994)). It was known that during the course of neural development, cells slowly acquired the electrophysiological properties that mark them as fully differentiated neurons. We were interested to know at what point during this progression could electrical stimulation be used as an instructive influence, or in other terms, as a "non-chemical" growth factor. In support of this hypothesis, Ca$^{2+}$ influx was found to activate most of the major intracellular signaling cascades following electrical stimulation (Ghosh and Greenberg, 1995). These same transduction cascades were activated following stimulation with a variety of growth factors and cytokines. Furthermore, it was increasingly being established that the temporal characteristics of signal transduction activity carried instructive influences as well (Marshall, 1995). It was feasible that the appropriate patterns of electrical stimulation would activate transduction cascades and transcription factors with the same temporal patterns as particular growth factors, and thus give the same instructions.

We initiated the current studies to address two issues. As a first step, we wanted to characterize the transient activation of signal transduction cascades by electrical depolarization as a function of time. We further wanted to determine whether the cascades could be activated in a pulsatile fashion, such that unique profiles of activity over time could be obtained with appropriate patterns of stimulation. These studies are presented in Chapter 3. Second, we wanted to characterize the instructive influence of electrical stimulation on cells that could decide whether or not to commit to neural differentiation. We studied expression of a wide variety of genes following depolarization of PC12 cells, a cell line that can reversibly differentiate in response to neurotrophins. In Chapter 4, we present the gene expression changes that occur in the
hours following short pulses of depolarization, and in Chapter 5 we present gene
expression that occurs following chronic depolarization of the cells for one week. In
Chapter 6, we conclude the present work with a summary of the results, and a few
thoughts on future directions. Finally, supplementary observations and results are
presented in the Appendix.

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in the century after Cajal (and the mysteries that remain). *Annals of the New York
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CHAPTER 2. BACKGROUND

2.1 PATHWAYS OF CALCIUM STIMULATION

2.1.1 Transduction Pathways Stimulated by Calcium

Calcium can enter neurons in a voltage-dependent manner through several types of voltage-stimulated Ca\(^{2+}\) channels (VSCC's): T-type, P/Q-type, N-type, L-type, and the NMDA-subtype of the glutamate receptor. Influx through the last two channel types has been shown to induce transcription of immediate-early genes (Bading et al., 1993). The L-type channel is activated solely by membrane depolarization, while the NMDA channel requires simultaneous application of both the neurotransmitter (glutamate) and depolarization. Since we focused on membrane depolarization only during the current study, this review will discuss mainly Ca\(^{2+}\) influx through L-type channels.

Even after focusing attention on only one mode of entry, the effects of Ca\(^{2+}\) influx are far from simplified. Influx through L-type channels has been shown to activate at least three pathways, depicted in Figure 1 (Ghosh and Greenberg, 1995). One of these pathways is the well-studied Ras/MAP kinase (MAP K) cascade. This pathway is central to cell signaling, since it is activated by stimuli that bind to the tyrosine kinase (Trk) family of receptors. These stimuli include neurotrophins and growth factors (Segal and Greenberg, 1996). It appears that Ca\(^{2+}\) can stimulate Ras through several mechanisms, depicted in Figure 2 (Finkbeiner and Greenberg, 1996). The implicated starting points (the tyrosine kinases PYK2, Src and the EGF receptor; and the adapter protein Shc) all appear to sequester the Grb2/Sos complex to the cell membrane. Sos then activates the membrane bound G protein Ras (p21) via GTP exchange. Activated Ras sets into action the generic “MAP K” pathway (Marshall, 1995). This pathway consists of three steps: phosphorylation of a serine/threonine kinase (Raf), which in turn phosphorylates a dual specificity protein kinase (MEK), which then activates another serine/threonine kinase (MAP kinase). Once activated, this last enzyme translocates into the nucleus, where it alters transcription factors (TF's) and ribosomal S6 kinases (pp90rsks). Examples of MAP kinases are extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases
(JNKs), or p38 kinases. Several transcription factor targets of MAP kinases have been identified: c-Jun, ATF-2 and Elk-1 (Enslen et al., 1996). The latter regulates transcription through the serum response element (SRE) promoter, in conjunction with the serum response factor (SRF) protein.

Figure 1. Calcium influx through VSCC's can activate at least three known pathways. These three pathways cross-talk at various points. Calcium influx may also activate PI 3-kinase and/or Akt, based on studies of neuronal apoptosis. In
addition, calcium influx through NMDA receptors may also activate the same pathways. Solid arrows indicate known pathways; broken arrows indicate proposed pathways; arrowheads indicate activation; T-heads indicate inhibition. Reproduced, in part, from Ghosh & Greenberg (1995).

Figure 2. Several pathways have been proposed linking calcium influx to the Ras/MAP K pathway. Solid arrows indicate known relationships or pathways; dashed lines indicate possible mechanisms of activation. The pathways are not mutually exclusive and may overlap. Although not depicted above, the interaction between Grb2/Sos and Ras occurs at the cell membrane. Reproduced from Finkbeiner & Greenberg (1996).
Calcium influx can stimulate at least two other pathways, both of which depend on the activity of the Ca\(^{2+}\)-calmodulin (CaM) complex. CaM can activate adenylate cyclases, such as type I Ca\(^{2+}\)-sensitive adenylate cyclase (I-AC; (Ghosh and Greenberg, 1995)). The cyclases regulate concentrations of cyclic AMP (cAMP), which in turn activates protein kinase A (PKA). Activated PKA translocates into the nucleus, where it alters the cAMP-response element binding protein (CREB). This last is a transcription factor for the cAMP-response element (CRE) promoter site. Closely related family members of CREB (members of the bZIP family) are ATF-1, CREM, and c-Jun; these TF's have also been shown to be Ca\(^{2+}\) sensitive.

A second CaM-dependent pathway involves the family of CaM-dependent protein kinases (CaM kinases). An upstream member of this cascade has been identified as well: CaM kinase kinase (CaMkk; (Enslen et al., 1996)). Two members of the CaM kinase family, CaM kinase I and CaM kinase IV, have been shown to activate the transcription factors ATF-1 and CREB (Sun et al., 1996). The CaM kinases can also regulate transcription via the SRE by activating SRF (Miranti et al., 1995). It should be noted that this latter regulation is independent of the Elk-1 site. Thus, the MAP K pathway and the CaM kinase pathway can both regulate the same promoter (namely, SRE) independently (see below for cross-talk, however).

### 2.1.2 Cross-talk Between Pathways

There is evidence within the last few years that the Ca\(^{2+}\)-sensitive pathways can regulate each other as well. First, it was observed that constitutively active forms of CaM kinase IV can activate c-Jun-dependent transcription (Enslen et al., 1996). Interestingly, activation by CaM kinase IV requires a functional JNK, and is not due to direct modification of c-Jun by CaM kinase. Thus, activated CaM kinase IV can activate JNK (also ERK and p38), in the absence of Ras-stimulation. Since MAP kinase and CaM kinase activate transcription factors for different portions of the same promoter region (Elk and SRF, respectively; both regulating the SRE), this positive cross-talk may exist to ensure that the pathways act cooperatively.
An example of inhibitory cross-talk is observed between the cAMP kinase (PKA) and CaM kinase cascades. PKA can phosphorylate CaM kinase kinase, leading to inhibition of the latter. In four different cell lines, activation of PKA by forskolin caused 30%-90% inhibition of CaMkk (Wayman et al., 1997). This inhibitory cross-talk is mutual, since CaM kinase IV can inhibit I-AC (Wayman et al., 1996). Interestingly, the PKA and CaM kinase pathways can both regulate the CRE promoter site.

2.1.3 Specificity of Pathways based on Temporal Characteristics

It is becoming clear that cellular responses to different stimuli can be determined by the kinetics of the signaling cascades, as well as their identity. An example of this phenomenon is the differences in pheochromocytoma cell (PC12) response to epidermal growth factor (EGF) and nerve growth factor (NGF). NGF-stimulation of PC12 induces these cells to stop dividing and extend neurites, indicative of a differentiation-like phenotype. Conversely, EGF-stimulation elicits a proliferative response in the PC12 cells. Both growth factors act through Trk receptors, and both activate the MAP K pathway. However, stimulation with EGF only transiently activates the MAP K pathway and ERK’s, whereas NGF results in prolonged activation. It has been shown that this temporal difference between the two stimuli is sufficient to induce the different effects. Manipulations have shown that in all cases where ERK activation is transient, a differentiation stimulus is not delivered (even by NGF). Conversely, over expression of the EGF receptor results in long-term expression of ERK and PC12 differentiation after treatment with EGF (Marshall, 1995).

Two hypotheses have been put forward for this phenomenon. First, it has been observed that in all cases where ERK stimulation is transient and PC12 cells proliferate, the ERK’s do not translocate into the nucleus. In long-term stimulation of ERK’s, and subsequent differentiation, activated ERK’s enter the nucleus. Thus, the two stimuli might be distinguished by the cellular distribution of ERK’s (Marshall, 1995). It is not clear precisely how the cell can differentiate between a cytoplasmic versus a nuclear ERK. A second hypothesis is that the delayed response (or effector) genes stimulated by NGF contain both CRE and IEG transcription factors in their promoter regions. Therefore, if CREB is activated transiently, by the time the TF’s expressed by the IEG’s
are synthesized and translocate into the nucleus to initiate effector gene expression, CREB is no longer active (Segal and Greenberg, 1996).

Along these lines, it has been shown in AtT20 cells (a mouse pituitary cell line) that free Ca\(^{2+}\) in the cytoplasm induces c-fos transcription via the SRE promoter, whereas Ca\(^{2+}\) in the nucleus acts through the CRE promoter (Hardingham et al., 1997). Unfortunately, the kinases that are activated by a cytoplasmic or nuclear distribution of Ca\(^{2+}\) were not determined. But, if genes for a particular behavioral response are regulated by CREB, a prolonged stimulus that delivers Ca\(^{2+}\) into the nucleus would be required. In support of this hypothesis, the relationship between neurite outgrowth and the nuclear concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_n\)) was studied (Kocsis et al., 1994). Based on their observations, these authors hypothesize that high [Ca\(^{2+}\)]\(_n\) induces expression of genes that promote neurite outgrowth. They also propose that a small influx of calcium at the cell membrane can reach the nucleus by stimulating calcium-induced calcium release (CICR) from the endoplasmic reticulum.

As a final point, it should not be concluded that prolonged activation correlates uniquely with differentiation in all cell types. In fibroblasts, long-term activation of ERK's results in proliferation, not differentiation. Given these caveats, any study of the patterns of signaling pathways must include analysis of the temporal characteristics of activation and deactivation.

2.2 IMMEDIATE EARLY GENES

2.2.1 Nature and Role of Immediate Early Genes

The immediate early genes (IEG's) are a broad class of genes that are involved in eukaryotic cell growth, mitosis, differentiation, and death. IEG's are activated in eukaryotic cells in response to many stimuli, including serum, growth factors, and ion channel activators; they encode for secretory proteins, enzymes, membrane-bound receptors and, most importantly for this project, transcription factors (Herdegen and Zimmermann, 1994). TF's important in the nervous system include the Fos (c-Fos and FosB), Jun (c-Jun, JunB and JunD) and Krox (Krox-20 or Egr-2, Krox-24 or NGFI-A, Egr-1, Zif/268 and Tis 8) families.
Unlike other gene regulators, such as SRF and CREB, IEG transcription factors are not continuously expressed. They must first be synthesized by the cell, and then may undergo further post-translational modification (by JNK, for example). We will thus classify IEG transcription factors as secondary TF’s. SRF and CREB, on the other hand, are always expressed by the cell, and hence will be classified as primary TF’s. Primary TF’s are activated by post-translational events resulting from the transduction cascades, and are frequently regulators of the secondary TF’s. For example, the CRE promoter regulates c-fos, c-jun, junB, and krox-24 (Herdegen and Zimmermann, 1994). By having two layers of TF’s that ultimately control expression of effector genes, cells can diversify the response to transduction cascades. As was discussed in 2.1.3, the temporal characteristics of a given signaling pathway can determine the type of response as much as the specific kinases activated by that cascade. This extra degree of control over response may result from the interplay between the primary and secondary TF’s.

To activate gene transcription, members of the Fos and Jun families dimerize after translation. The Fos:Jun dimers comprise the set of activator protein-1 (AP-1) complexes which regulate gene expression through the AP-1 binding site. The composition of a specific AP-1 complex has an effect on the affinity of that complex for the AP-1 binding site; for example, JunB attenuates the transcriptional potency of c-Jun (Herdegen and Zimmermann, 1994). Phosphorylation can further modulate the affinity and action of the AP-1 complex by altering the transcriptional potency of each constituent in the dimer.

In the nervous system, a variety of stimuli can induce IEG expression: noxious afferent stimulation; changes in blood pressure; epileptic seizures; ischemia; cortical spreading depression; and long-term potentiation (Herdegen and Zimmermann, 1994). The kinetics of IEG expression differ for each TF. In general, within 2 hours after stimulation, c-Fos, c-Jun, JunB and Krox-24 have reached maximal expression; FosB and JunD are slower and reach maximal levels after approximately 5 hours. The patterns of persistence and decay, on the other hand, are a function of stimulus intensity and duration. Exceptions to the above observations exist, however. For example, KCl depolarization of neurons in vitro and cortical spreading depression in vivo both lead to expression of c-Fos and JunB, without expression of c-Jun.
The Jun family appears to have an interesting functional role, since it is implicated in learning and memory during development, and is up-regulated for weeks or months after axotomy or until regeneration is complete ((Herdegen and Zimmermann, 1994); (Dragunow, 1992), (Leah et al., 1991)). This evidence leads one to conclude that Jun may be associated with the regenerative program in neurons. Apoptotic neurons also express high levels of c-Jun, however (Estus et al., 1994). Thus, it is unclear whether the Jun family induces a neuronal program of survival (or attempted survival) after axotomy in the animal or after apoptotic stimulus in culture, or is a preparatory step prior to cell death or degeneration. Due to their involvement in a variety of cellular responses and their combinatorial behaviour via dimerization and post-translational modification, it is not possible at this point in time to designate a clear functional role for the IEG transcription factors.

2.2.2 Response of IEG's to Electrical Stimulus

Several members of the IEG transcription factors have been studied in the context of patterned electrical stimulus. From these studies, it has become clear that IEG expression is differentially affected by the frequency, pattern (i.e. burst versus continuous), and duration of electrical stimulus (Sheng et al., 1993). For example, c-fos expression increased from basal levels with stimulation at 0.01 Hz to 4 times the basal level with stimulation at 1 Hz or greater. Furthermore, expression levels were 4 to 8 times higher when stimulus was applied for 2-4 hours than when it was applied for one-half, one or six hours. Lastly, c-fos expression was maximal when bursts of 6 impulses (at 10 Hz) were delivered once a minute, versus continuous stimulus or 12 impulses (again, at 10 Hz) once every 2 minutes. Expression of nur/77, on the other hand, was the same for 6 impulses per minute and 12 impulses every two minutes. Thus, PES can affect expression of each IEG in a unique manner, and the pattern, frequency and duration of stimulation are all important parameters.

In a later study of the AP-1 complex after electrical stimulus, the expression levels of combinations of c-fos, c-jun and jun-B were determined after KCl depolarization (Sheng et al., 1995). It was found that in unstimulated cultures, 3 times more cells expressed both c-jun and jun-B than expressed c-jun, junB, and c-fos; no cells expressed the c-
The fos/junB combination. After stimulation with KCl, 70% of the cells expressed c-fos/junB, 5% expressed c-jun/junB, and 10% expressed all three genes. Thus, the result of depolarization was a shift in the composition of AP-1 complexes, from a mostly c-jun containing distribution of AP-1’s to a mostly c-fos containing distribution of AP-1’s. Unfortunately, the effect of patterned electrical stimulation was not studied. It seems likely, given the previously shown frequency and pattern dependency of IEG expression, that even more complex changes in AP-1 distribution will result from patterned electrical stimulation.

2.3 PC12 CELLS AS A MODEL SYSTEM

2.3.1 Background

Pheochromocytoma (PC12) cells are a clonal cell line, derived from a rat adrenal medullary tumor in 1975 (Greene and Tischler, 1976). This cell line was initially found to differentiate reversibly in response to nerve growth factor (NGF). In addition, it was observed that NGF could prevent PC12 cell death following serum withdrawal (Greene, 1978). Many studies in PC12 cells subsequently focused on the mechanisms by which neurotrophins and survival factors communicated their messages to the nucleus. Consequently, many early discoveries of the events that follow growth factor stimulation, such as activation of signal transduction pathways and transcription of immediate-early genes, modulations in cellular adhesion, and induction of synapse formation, were made in PC12 cells ((Halegoua and Patrick, 1980), (Kruijer et al., 1985), (Bartel et al., 1989), (Schubert and Whitlock, 1977), (Schubert et al., 1977), (Stallcup, 1979), reviewed in (Levi et al., 1988)).

Naïve, unstimulated PC12 cells are normally dividing with a doubling time of between 2.5 to 4 days (Greene et al., 1987). Morphologically, the cells are round and phase-bright, aggregate in culture, and attach to tissue culture surfaces only moderately well. PC12 cells can be grown either in monolayers on coated surfaces, or in suspension cultures. Furthermore, while PC12 cells are relatively stable as a cell line, spontaneous variations and generation of sub-clones is an issue after prolonged passaging ((Greene et
al., 1987), (Yamada et al., 1996)). As a result, the cells are typically used in experiments over a well-defined range of passage number.

2.3.2 Response of PC12 Cells to Various Stimuli

The utility of the PC12 cell as a model system arises from its ability to make several phenotypic decisions in response to various stimuli. In response to mitogenic stimuli, such as epidermal growth factor (EGF), PC12 cells were shown to increase their proliferation rate, but remained rounded and phase bright (Boonstra et al., 1985). In contrast, in response to other growth factors, such as NGF or basic fibroblast growth factor (bFGF), PC12 cells exited the cell cycle and proceeded down a path of neurogenesis ((Greene and Tischler, 1976), (Schubert et al., 1987)). The cells extended neurites that contacted neighboring cells, they increased expression of various synaptic vesicle proteins, and voltage-activated Na⁺ and Ca²⁺ currents increased as the cells differentiated electrophysiologically ((Hilborn et al., 1997), (Schubert et al., 1977), (Possenti et al., 1989)). Studies in PC12 cells were critical for developing the paradigm that differentiating signals resulted from sustained activation of signaling cascades, while mitogenic stimuli only activated intracellular signaling for short periods of time (reviewed in (Marshall, 1995) and discussed in depth above). PC12 cells are also able to make a third phenotypic decision, to undergo programmed cell death or not. In cultures that had differentiated in response to NGF treatment, it was observed that withdrawal of NGF induced apoptosis in a subset of these cells (Mesner et al., 1992). As a result, PC12 cells were extensively used to elucidate the mechanisms behind growth factor withdrawal-induced neuronal degeneration (reviewed in (Mills et al., 1995)).

The effects of membrane depolarization on intracellular signaling and gene transcription have also been investigated on PC12 cells. Two types of voltage-stimulated calcium channels (VSCC's) are expressed in undifferentiated PC12 cells, L (long-lasting) and N(neuronal)-type, both of which have been shown to contribute to the Ca²⁺ influx upon depolarization (Janigro et al., 1989). As described above in detail, the depolarization-induced Ca²⁺ influx in PC12 cells activated various signal transduction cascades and led to transcription of IEG's (reviewed in (Ghosh and Greenberg, 1995)). Several studies observed that sustained depolarization of PC12 cells also led to
phenotypic changes. For example, it was previously shown that chronic depolarization with KCl induced neurite outgrowth in up to 27% of the cells in the absence of other chemical agents (Hilborn et al., 1997). Other methods of depolarization have also been used to induce phenotypic differentiation. For example, PC12 cells that were grown on electrode surfaces, such as polypyrrole (PPy) and indium-tin oxide (ITO), and were subjected to various patterns of electrical stimulation also extended neurites ((Schmidt et al., 1997), (Kimura et al., 1998)). Thus, a great variety of different factors, both chemical and physical, have been used to stimulate PC12 cells to make phenotypic choices.

2.3.4 Gene Expression Studies in PC12 Cells

The phenotypic changes in PC12 cells described above are also accompanied by changes in gene expression. As described above, both growth factor and electrical stimulation of PC12 cells have been shown to induce expression of a variety of IEG's. Expectedly, many delayed-response genes (DRG's) have also been identified as being expressed in response to one or more of these stimuli. The first differentiation-specific DRG found to be expressed in PC12 cells was the vgf gene (Levi et al., 1985). VGF expression was induced within 6 hours of NGF stimulation of PC12 cells, to a lesser extent by electrical stimulation, and minimally by EGF stimulation (Salton et al., 1991). Other delayed response genes, such as genes for tyrosine hydroxylase and cyclooxygenase-1, were also identified as up-regulated in NGF-treated PC12 cells ((Leonard et al., 1987), (Kaplan et al., 1997)). In another study, representational difference analysis (RDA) was used to identify a set of four genes that were preferentially induced by NGF within 4 hours of treatment of PC12 cells compared to EGF-treated cells (Vician et al., 1997). These genes were collagenase 1, plasminogen activator inhibitor-1, activity-regulated cytoskeletal protein and VH6/MKP-3. A similar study employing the serial analysis of gene expression (SAGE) method identified a group of approximately 150 genes that were induced after 7 days of NGF-mediated differentiation (Angelastro et al., 2000).

PC12 cells have also been used to identify genes induced by depolarization. In addition to the vgf gene, the gene for tyrosine hydroxylase gene was also shown to be up-regulated after 1 to 12 hours of depolarization (Mishra et al., 1998)). Several studies
have also identified synapse-specific genes, such as the neuron-specific IEG synaptotagmin IV, secretogranin II, and chromogranin B, that are induced by depolarization (Vician et al., 1995). Thus, similar to chemical factors, electrical activity can stimulate the expression of a variety of both immediate-early and delayed-response genes.

REFERENCES


CHAPTER 3. ACTIVATION OF SIGNAL TRANSDUCTION BY SHORT PERIODS OF DEPOLARIZATION: RESTIMULATION AND DEACTIVATION KINETICS

3.1 SUMMARY

Electrical stimulation of neurons, as a result of Ca^{2+} entry into the cytoplasm, induces many cellular changes that occur from minutes to hours or even days after stimulation. Many of the downstream effects of Ca^{2+} influx have been uncovered in previous research. In the present study, we focus specifically on the transient nature of rises in cytoplasmic free Ca^{2+} ([Ca^{2+}]). We have characterized changes in [Ca^{2+}], versus time during both chronic and pulsatile depolarization of the neuron-like PC12 cell line. During chronic depolarization, [Ca^{2+}], peaks and subsequently falls off to intermediate values within 10 minutes. Both 1- and 5-minute pulses of depolarizing KCl can induce sharp rises in [Ca^{2+}], which fall back to baseline within 30 seconds of KCl washout and repolarization. If cells are allowed sufficient time to recover, a second pulse of KCl can induce another rise in [Ca^{2+}]. Mirroring the trends in [Ca^{2+}], phosphorylation of the MAP kinases Erk1 and Erk2 is also transient, peaking and falling to low levels within ten minutes of depolarization. Short pulses of depolarization can phosphorylate Erk1 and Erk2, and the rate of deactivation of the Erk's is not affected by the duration of depolarization (over the range 1 to 5 minutes) and appears to be minimally affected by [Ca^{2+}]. Phosphorylation of the transcription factor CREB also peaks as a result of chronic depolarization, and drops to intermediate levels that are maintained for over 1 hour. As predicted from observations of [Ca^{2+}], both Erk1/2 and CREB phosphorylation can be re-induced by a second round of depolarization that follows a recovery period. To our knowledge, this is the first report of pulsatile activation of the Erk's and CREB. The effects of the durations of depolarization and inter-pulse recovery on reactivation of Erk’s and CREB were characterized. Recovery periods as short as ten minutes and pulse durations from 1 to 5 minutes in length can be combined to effectively re-stimulate signal transduction kinases and transcription factors. Thus, pulsatile activity may be a means of maintaining signaling activity over long periods of time.
3.2 INTRODUCTION:

Electrical simulation of neurons in culture activates a series of cellular processes that unfold over time, starting within seconds of stimulation and continuing several hours to days later. Membrane depolarization immediately activates, by way of voltage-gated calcium channels, various intracellular signaling pathways. These pathways include the MAP kinase cascade ((Rosen et al., 1994), (Egea et al., 1999)), the PKA cascade ((Impey et al., 1998), (Brosenitsch and Katz, 2001), (Nakao, 1998)), the CaMK cascade (Aletta et al., 1996), and the PKC cascade (reviewed in (Huang, 1989)). The combined action of these intracellular signaling pathways leads to activation of various transcription factors within 30 seconds to 1 minute of depolarization, the best characterized of which is the transcription factor CREB ((Thompson et al., 1995), reviewed in (Finkbeiner and Greenberg, 1998)).

The Ca$^{2+}$-activated transcription factors subsequently bind to regulatory regions and initiate transcription of various immediate-early genes (IEG’s). Since no new protein synthesis is required, transcription of IEG’s begins within minutes of electrical stimulation ((Thompson et al., 1995), (Bartel et al., 1989)). Some of these IEG’s, such as c-fos, jun, NGFI-A (zif 268) and NGFI-B (nur 77), are transcription factors that proceed to activate other genes ((Enslen and Soderling, 1994), (Bartel et al., 1989)). Others, such as arg3.1/arc and BDNF, are involved in synaptic plasticity and neuronal survival, respectively ((Walterete et al., 2001), (Ghosh et al., 1994)). At least one IEG, MKP-1, is a phosphatase that exerts feedback on the signaling cascades by inactivating MAP kinase (Sun et al., 1993).

As a result of the expression of the IEG’s, delayed-response genes (DRG’s) begin to be expressed. Examples include the vgf gene, which starts to be expressed 2 hours after the start of depolarization and peaks after 6 hours; and the gene for Tyrosine Hydroxylase, which is expressed after 6 to 24 hours of stimulation ((Salton et al., 1991), (Brosenitsch and Katz, 2001)). Thus, a wide array of events begin immediately after stimulation and progress over the course of many hours.
Interestingly, different patterns of electrical stimulation lead to diverse results. For example, studies have shown that long-term potentiation (LTP) is induced most effectively by specific patterns of high-frequency stimulation ((Yun et al., 2002), (Hoffman et al., 2002)). To better understand these phenomena, subsequent studies focused on the stimulation-dependent activation of specific proteins. The intracellular kinase CaMKII becomes autonomously activated when exposed to Ca\(^{2+}\)/CaM at a frequency of 4 Hz, but not at 1 Hz (De Koninck and Schulman, 1998). In a study of hippocampal neurons, electrical stimulation at 5 Hz for 180 sec lead to phosphorylated CREB levels greater than stimulation for 18 sec (Bito et al., 1996). Consequently, 18 sec stimulation did not induce c-Fos or somatostatin expression, while 180 sec stimulation did lead to expression of these proteins. In a study of frequency dependence, c-fos transcription was specifically induced by a burst of 6 stimuli, delivered once a minute at 10 Hz, but not by 6 stimuli delivered uniformly 0.1 Hz (Sheng et al., 1993).

One hypothesis for the frequency dependence of kinases and transcription factors is that levels of free Ca\(^{2+}\) do not reach an equilibrium after electrical activity, but rather rise and fall transiently. As a result, cellular reactions that depend on Ca\(^{2+}\)/CaM as a cofactor are also activated transiently. Timing then becomes an important factor when one considers the effects of transient activation of a given reaction on downstream reactions, positive and negative feedback within the intracellular signaling networks, and the requirement for coordinated action of many of the signaling reactions. Thus, the complex and non-equilibrium nature of intracellular signaling cascades gives rise to a variety of timing-dependent phenomena.

We were interested in characterizing how a transient rise free cytoplasmic Ca\(^{2+}\) would activate downstream kinases and transcription factors. We focused on MAP kinase and CREB activation in the neuron-like cell line PC12, since this model system has been extensively studied with a diverse array of stimuli. In particular, it has been shown that the activation of CREB and the MAP kinases can last from minutes to hours as a result of different stimuli ((Qiu and Green, 1992), reviewed by (Marshall, 1995)). In the present study, we investigate the variation of [Ca\(^{2+}\)], with time during chronic and pulsed depolarization. We also characterize the resulting time-courses of MAP kinase and
CREB activation during chronic and short-term depolarization. Lastly, we determine whether the MAP kinases and CREB can be reactivated following a recovery period. The significance of pulsatile stimulation is discussed in the context of stimulus-specific responses of cells.

3.3 MATERIALS AND METHODS

3.3.1 Cell Culture

Rat pheochromocytoma PC12 cells (ATCC) were cultured in complete Dulbecco’s Modified Eagle’s Medium (cDMEM), which consists of DME supplemented with 10% heat-inactivated horse serum (HS), 5% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin (all components from Gibco BRL). Cells were maintained in a humidified atmosphere of 5% CO₂ and 37 °C. Cells were plated at a density of 2 x 10⁴ cells/cm² on tissue culture plates (Corning) coated with 0.1 mg/ml of poly-L-ornithine (PLO; Sigma: P3655) in water. Cells were fed cDMEM every 5 days and passaged every 10 days. Briefly, cells were detached after incubation in a 0.05% trypsin-EDTA solution (Gibco BRL) at 37 °C for 5 mins. Cell clumps were broken up with mild tituration or passage through a 20G needle and syringe, and reseeded at the density noted above. Cells were not used in experiments past passage number 20.

3.3.2 Stimulation for assessing protein phosphorylation

For stimulation experiments where phosphorylation level was to be determined with Western blots, cells were seeded 24 to 48 hours before experiments on 35mm Corning dishes pre-coated with PLO (as above) at a density of 1 x 10⁵ cells/cm². Prior to stimulation, cells were maintained in serum-free DME for 3 hours to remove any artifacts of protein phosphorylation due to serum factors. Cells were depolarized by adding an equal volume of HiK solution (135 mM KCl, 30 mM D-glucose, 1 mM MgCl₂, 2 mM CaCl₂, 40 mM NaHCO₃, 1 mM NaH₂PO₄; all components cell-culture grade from Sigma) to the cells in DME. Since DME contains 5 mM KCl, the final concentration of KCl during chronic depolarization was 70 mM.

In experiments where cells were returned to a polarized state to measure the dephosphorylation rate in the absence of elevated [Ca²⁺]ᵢ, the HiK solution was washed
out with two successive dilutions with serum-free DME. The final KCl concentration after washout was between 5 and 10 mM, sufficiently low to prevent significant depolarization of the cells. In experiments where cells were re-stimulated with KCl, cells remained in the resting solution of DME with trace amounts of HiK for the indicated period of time, and subsequently an equal volume of HiK was added. Final concentrations of KCl were between 70 and 72.5 mM for the second pulse.

In all cases, negative controls were cells stimulated at the indicated times and for the indicated durations with a solution of HiNa (125 mM NaCl, 30 mM D-glucose, 1 mM MgCl₂, 2 mM CaCl₂, 40 mM NaHCO₃; 1 mM NaH₂PO₄), which is iso-osmolar with HiK. An equal volume of NiNa was added to cells resting in DME. Washout was performed exactly as described for stimulation with HiK. Two positive controls were used. For Westerns of Erk1/2 phosphorylation, PC12 cells were stimulated for 10 minutes with epidermal growth factor (EGF; Boehringer Mannheim) at a concentration of 30 ng/ml in DME. For Westerns of CREB phosphorylation, PC12 cells were stimulated for 10 minutes with 10 μM forskolin (Sigma) in DME.

In a control experiment to determine the effect of inhibitors of Ca²⁺ influx and action, cells were seeded as described above. To block Ca²⁺ influx through L-type channels, cells were pre-treated with 5 μM nifedipine (Sigma) for 15 minutes prior to depolarization with 70 mM KCl for 3 minutes. To inhibit Ca²⁺-calmodulin activity, cells were pretreated with the CaM inhibitor W-13 (Sigma) at a concentration of 70 μM for 1 hour prior to depolarization with 70 mM KCl for 3 minutes.

3.3.3 Western Blotting

In all cases of stimulation, cells were washed quickly with two rinses of ice-cold PBS at the time points of interest. After removal of the second PBS rinse, 50 μl of lysis solution was added to each dish. The lysis solution consisted of 1% SDS, 50 mM Tris (pH 7.4), 4.46 mg/ml sodium pyrophosphate, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 2 mM sodium orthovanadate, 10 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride (PMSF, added within 2 minutes of use; Na₂VO₄ from Fisher, all other components from Sigma). The culture dishes were kept on ice and cells
were homogenized using a disposable plastic cell scraper. All of the cell lysate (approximately 100 μl, due to residual film of PBS that remained after the last aspiration) was carefully transferred to a 1.5 ml Eppendorf tube, kept on ice, and sonicated to shear the DNA. The lysates were then centrifuged at 10,000 rpm for 15 minutes at 4 °C to pellet the DNA. The supernatant from each tube, containing the proteins of interest, was removed and placed in a fresh Eppendorf tube. From this fraction, 20 μl was set aside for protein concentration measurements using the Bio-Rad DC Protein Assay, a version of the Lowry method. The remaining solution was mixed with an equal volume of Laemmli sample buffer (BioRad) containing β-mercaptoethanol, boiled for 10 minutes and frozen. The samples were boiled again before use.

Cellular proteins were separated using gel electrophoresis. Proteins were loaded on 7.5% pre-cast Tris-glycine acrylamide gels (Bio-Rad) and run at 100 V. For the analysis of CREB phosphorylation, 5 μg of total protein was used in each lane. For analysis of phospho-Erk1/2, 7 μg total protein was used per lane. Proteins were transferred from the acrylamide gels to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore) at 300 mA for 70 minutes in transfer buffer (20% methanol, 25 mM Tris, 192 mM glycine; Mallinckrodt), and then air-dried at room temperature. To prevent non-specific adsorption, membranes were bathed for 4 hours at room temperature in a block solution (BS) of phosphate buffered saline (PBS) containing 5% non-fat milk, 0.05% Tween-20. Membranes were then exposed overnight to primary antibody in fresh BS at 4 °C. Free primary antibody was removed with two successive 10 minute washes in BS followed by two successive 15 minute washes in 0.05% Tween-20/PBS (PBS-T). Horse radish peroxidase-conjugated (HRP-) secondary antibody was then added in fresh BS. Excess secondary antibody was removed with the same wash protocol used for removal of primary antibody. Membranes were then rinsed several times in PBS, and the presence of immobilized HRP was detected using enhanced chemiluminescence (ECL; Amersham Pharmacia). Luminescence from the membranes was detected using Hyperfilm ECL film (Amersham Pharmacia), developed on a photoprocessor (M35A X-OMAT, Kodak). The luminescence from each membrane was digitized by scanning the film on a transparency scanner at 600 dpi. The optical density (OD) of each lane was calculated using the Scion Image 4.02 software, with the GelPlot macro installed. In
most cases, the OD counts of lanes of interest were divided by the OD count from the positive control. In this manner, OD counts for the same experimental conditions could be pooled from different Westerns blots, and semi-quantitative comparisons between different experimental conditions could be made. All reported values are averages of 4 separate measurements. In the case of Figure 8, panel C, the OD counts from double-stimulated cells were reported relative to the signal from single-stimulated cells.

The following antibodies were used. For detection of phospho-CREB, polyclonal rabbit IgG against Ser-133-phosphorylated rat CREB (06-519, Upstate Biotechnology) was used at a concentration of 1:3000. For detection of whole CREB molecule, polyclonal rabbit IgG (9192, New England Biolabs) was used at a concentration of 1:2000. Phosphorylated MAP kinases Erk1/2 were detected with mouse monoclonal IgG raised against phospho-Tyrosine/phospho-Threonine peptide analog of Erk1 and Erk2 (05-481, Upstate Biotechnology) at a concentration of 1:2000. HRP-conjugated secondary antibodies against mouse and rabbit IgG were purchased from Santa Cruz Biotechnology and used at a dilution of 1:2000.

3.3.4 Calcium Imaging

Relative changes in calcium concentration during depolarization and repolarization of PC12’s were followed with a single-wavelength fluorescent dye, Fluo-4 (Molecular Probes). The quantum yield of Fluo-4 increases approximately 10-fold when $[\text{Ca}^{2+}]_{\text{free}}$ is increased from 50 nM to 1 μM, which is the range over which $[\text{Ca}^{2+}]_{\text{free-cyto}}$ changes during depolarization ((Haugland, 2001), (Park et al., 1998)).

Several days prior to imaging, cells were seeded on number zero glass coverslips (Carolina Sciences) that had been pre-treated with poly-l-ornithine (0.1 mg/ml, Sigma), followed by mouse laminin (25 μg/ml in PBS, Gibco BRL). The cells were maintained at 37 °C, 5% CO$_2$ in cDMEM until the day of experiments, when the coverslips were transferred to a new dish containing a mixture of 4 μM Fluo-4 AM and Pluronic (Molecular Probes) in Tyrode’s solution (125 mM NaCl, 5 mM KCl, 30 mM D-glucose, 25mM HEPES, 2 mM CaCl$_2$, 1 mM MgCl$_2$, pH 7.4, 300 mOsm). Cells were left in the dye-containing solution for 40 minutes at 37 °C, after which they were washed three
times with Tyrode's solution, and then left for 10 minutes in Tyrode's at room temperature before imaging. The coverslip containing Fluo-4-loaded PC12's was transferred to a microscope stage (Warner Instruments) containing an imaging chamber that allowed continuous flow of solutions. In all cases the chamber initially contained room temperature Tyrode's solution.

Imaging was performed using an epifluorescence microscope (Zeiss) equipped with a CCD camera for image collection. Prior to imaging the cells, the gain of the CCD camera was set using a solution of 1 µM Fluo-4 pentapotassium salt (F4-K5) (Molecular Probes) in Tyrode's containing only 0.5 mM CaCl₂. This calibration procedure ensured a large response of the camera over the range of [Ca²⁺] variations during the experiments with a low incidence of camera saturation. Excitation of the Fluo-4 dye was achieved with a Mercury arc lamp and a 488 nm filter. The exposure of the cells to the excitation beam was limited to 100 msec per time point using a computer-controlled shutter assembly. The total number of images collected during a single time series did not exceed 100 images in any of the experiments. Thus, the cells were exposed to the excitation beam for less than 10 seconds total in any of the experiments, minimizing photo-bleaching. Cells resting in Tyrode's solution were imaged at the beginning and end of each experiment to determine baseline fluorescence levels, and the effects of bleaching.

Extensive efforts were made to relate changes in fluorescence to changes in [Ca²⁺]. A series of calibration experiments were performed to characterize the response of Fluo-4 over the range of [Ca²⁺], expected in the cytoplasm during depolarization. At these low concentrations, impurities in the various solutions necessitate use of a calcium buffer if precise concentrations are desired. The calcium buffer EGTA was used to control the amount of [Ca²⁺]free over the range of interest (50 to 500 nM; obtained from Molecular Probes). These experiments confirmed that over this range of free Ca²⁺, the change in fluorescence, F - F₀, was linearly proportional to the logarithm of the ratio of free calcium ions, log([Ca²⁺]free/[Ca²⁺]free,₀). However, the ratio of proportionality depended strongly on the concentration of Fluo4 used. Since uptake of the Fluo4 was heterogeneous and each individual cell contained a different concentration of the dye, we
could not accurately calculate ratio $[\text{Ca}^{2+}] / [\text{Ca}^{2+}]_{i,0}$ from $F - F_0$. Thus, we reported $F - F_0$ versus time in the various stimulation experiments.

### 3.4 RESULTS

#### 3.4.1 MAP Kinase and CREB Phosphorylation During Chronic Depolarization

Previous studies have shown that chronic membrane depolarization of PC12 cells elicits both immediate and delayed responses, from activation of intracellular signaling molecules and transcription of immediate early genes to expression of the neuronal differentiation-specific delayed-response gene vgf ((Rosen et al., 1994), (Finkbeiner and Greenberg, 1998), (Salton et al., 1991)). We thus wanted to first determine the length of time MAP kinases and CREB remained phosphorylated during chronic depolarization.

PC12 cells were depolarized with 70 mM KCl and the phosphorylation of Erk1, Erk2 and CREB were followed over the course of 2 hours using Western blots. As shown in Figure 3, the level of phosphorylation of the MAP kinases Erk1/2 peaked within the first 5 minutes of depolarization, and then decreased to a low and stable level that was maintained for up to 2 hours of depolarization. While over an order of magnitude lower than their peaks, the steady state levels of Erk1 and Erk2 phosphorylation were still greater than in negative control cells exposed to Tyrode’s solution. In addition, while chronic depolarization clearly induced phosphorylation of Erk1 and Erk2, the degree of phosphorylation was approximately a factor of 3 to 10 lower than the phosphorylation of Erk1/2 induced by 30 ng/ml EGF. This latter observation is in agreement with previously reported results ((Zwick et al., 1999),(Rosen and Greenberg, 1996)).

The phosphorylation of CREB at serine 133 showed a similar temporal profile as Erk1/2, with a peak occurring within the first 2 to 5 minutes of depolarization (Figure 3). However, in contrast to the approximately 10-fold dephosphorylation of Erk1/2, CREB phosphorylation decreased by approximately a factor of 2 and was maintained at this intermediate level for up to 60 minutes of depolarization. The amplitude of CREB phosphorylation was a factor of 2 lower as that induced by 10 μM forskolin, and approximately an order of magnitude greater than that induced by EGF or NGF, in agreement with previous results (Ginty et al., 1993).
Figure 3. MAP kinase and CREB activation during chronic depolarization. A: Western of phosphorylated Erk1 and Erk2 during chronic depolarization for various durations, from 2 to 60 minutes (lanes 3 to 8), with 70 mM KCl. Lane 1 is a positive control, stimulation with 30 ng/ml EGF for 10 minutes. Lane 2 is a negative control, stimulation with Tyrode's solution for 5 minutes. B: Western of phosphorylated CREB protein during chronic depolarization for various durations, from 2 to 120 minutes (lanes 3 to 9), with 70 mM KCl. Lane 1 is a positive control, stimulation with 10 \* M forskolin for 10 minutes. Lane 2 is a negative control, stimulation with Tyrode's solution
for 5 minutes. C: Optical density (OD) counts from Western blots versus duration of depolarization. Values are relative to OD of positive control in each Western, n = 4 for each measurement. D: Western of phosphorylated CREB protein after treatment with inhibitors. Lane 1 is a positive control, stimulation with 10 μM forskolin for 10 minutes. Lane 2 is a negative control, stimulation with Tyrode’s solution for 3 minutes. Lane 3 is pCREB from cells depolarized for 3 minutes with 70 mM KCl, while cells in lanes 4 and 5 were pretreated with 5 μM nifedipine and 70 μM W-13 prior to depolarization, respectively.

To confirm that intracellular signaling cascades were being activated as a result of Ca^{2+} influx during our experiments, we pretreated cells with nifedipine, an inhibitor of L-type Ca^{2+} channels. As shown in Figure 3, panel D, CREB phosphorylation was reduced to baseline levels when cells were first treated with nifedipine. This result is in agreement with previously reports that nifedipine reduced signal transduction following membrane depolarization of PC12 cells (Rosen et al., 1994). In addition, CREB phosphorylation was reduced, though not completely eliminated, when cells were pretreated with the CaM antagonist W-13. This latter observation indicated that membrane depolarization activates CREB partially through a CaM-independent mechanism, since pCREB was not reduced to baseline levels. It was previously shown in PC12 cells that W-13 completely eliminated the activation of Erk1 and Erk2 following membrane depolarization (Egea et al., 1999). Our results support these previous observations, since CREB lies downstream of Erk1 and Erk2. Taken together, pretreatment with nifedipine and W-13 both confirmed that the activation of signal transduction following membrane depolarization was due to Ca^{2+} influx L-type channels, and was partly mediated by the Ca^{2+}/CaM complex.

Since PC12 cells are depolarized throughout their exposure to high KCl for long periods of time, we concluded that the dephosphorylation of Erk1/2 and CREB after the first 5 minutes of depolarization must have been due to one of two general mechanisms, or the combination of both. First, previous research has shown that neurons counter-act rises in [Ca^{2+}], by inactivating voltage-stimulated calcium channels, increasing the Ca^{2+}-buffering capacity of the cytoplasm, and increasing the rates Ca^{2+} efflux ((Vyas et al.,
1994), (Di Virgilio et al., 1987)), all of which would act to reduce $[Ca^{2+}]_i$. Second, increases in $[Ca^{2+}]_i$ could lead to activation of phosphatases that could deactivate Erk1/2 and CREB. Calcineurin is an example of this latter type of CREB regulation (Bito et al., 1996).

3.4.2 Cytoplasmic Free $Ca^{2+}$ Concentration During Chronic Depolarization

To better understand why chronic depolarization induced only transient activation of Erk1/2, $[Ca^{2+}]_i$ in single cells was followed over time using fluorescence microscopy. A calcium-sensitive dye was introduced into the cytoplasm of the PC12s, and subsequent changes in $[Ca^{2+}]_i$ resulted in proportional changes in cellular fluorescence. Since we were interested in relative changes in $[Ca^{2+}]_i$, rather than absolute $Ca^{2+}$ concentrations, we used the single-wavelength dye Fluo-4.

As shown in Figure 4, $[Ca^{2+}]_i$ increased dramatically upon depolarization of PC12s with KCl, peaked within the first 5 minutes of depolarization, and subsequently decreased to intermediate levels after 10 minutes of depolarization. The amplitude of response differed from cell to cell, partly due to heterogenous uptake of Fluo-4, and partly due to variations in the electrical properties of PC12 cells ((Pun and Behbehani, 1990), (Janigro et al., 1989)). However, the increase, peak and decrease of fluorescence occurred simultaneously in all cells imaged, indicating strong temporal uniformity of the calcium response. The decrease in cell fluorescence after the rapid rise and peak were not due to either bleaching or dye saturation/quenching, as these effects were corrected for and ruled out with pre-experiment calibrations, respectively (see 3.3.4). Thus, the decrease in fluorescence after 5 minutes of depolarization was due solely to decreases in $[Ca^{2+}]_i$.

Undifferentiated PC12 cells contain mainly two types of voltage gated calcium channels (VSCC’s): L (long-lasting) and N(neuronal)-type (Janigro et al., 1989). In response to sustained depolarization and rises $[Ca^{2+}]_i$, most calcium channels in PC12 cells close, and only a few remain open (Di Virgilio et al., 1987). As a result of this incomplete inactivation, $[Ca^{2+}]_i$ falls from peak levels to an intermediate steady state; and over the longer term the steady state $[Ca^{2+}]_i$ slowly rises ((Di Virgilio et al., 1987). (Park
et al., 1998), (Rossi et al., 1993)). Thus, the rise and fall of [Ca$^{2+}$], observed in the present study are in agreement with reports in the literature.

Figure 4. Imaging of free cytoplasmic Ca$^{2+}$ during chronic depolarization. A: Representative time series images of PC12 cells during chronic depolarization. Cells were initially resting in Tyrode's solution in a polarized state (top row, left image). Upon addition of 70 mM KCl, fluorescence emission from the cells increased dramatically (top row, center and right images), due to free Ca$^{2+}$-binding by the dye Fluo-4. Images in the bottom row depict the slow decrease in fluorescence after 10 min or greater in depolarizing solution. B: The change in fluorescence versus time in depolarizing solution was plotted. Each value is the average of 6 cells in each time series.
3.4.3 Free Ca^{2+} Concentrations During Short Periods of Depolarization

To better characterize the ability of the cell to remove excess free calcium, the PC12s were subjected to short pulses of depolarizing 70 mM KCl and then re-polarized by washout of the HiK solution and replacement with Tyrode’s solution. Time series images of Ca^{2+}-induced fluorescence from 1- and 5-minute pulses are shown in the top rows of panels A and D of Figure 5, respectively. As plotted in Figure 5, panels B and E, [Ca^{2+}], increased rapidly in response to either a 1 or 5 minute pulse of HiK. However, [Ca^{2+}], returned to baseline, pre-depolarization levels within 30 seconds after re-polarization in both cases. The temporal uniformity observed during chronic depolarization was also present in response to the short pulses of depolarization: every cell analyzed in Figure 5 responded rapidly, simultaneously and with nearly identical rates of Ca^{2+} uptake and removal.

Comparing the cytoplasmic free Ca^{2+} clearance times measured in the three cases studied (1 min, 5 min and chronic depolarization), several conclusions about Ca^{2+} influx and efflux rates could be drawn. First, PC12s removed excess Ca^{2+} approximately 10 times faster when the cell was at resting voltage than when the cell was depolarized. Also, [Ca^{2+}], never dropped to initial values during chronic depolarization, whereas [Ca^{2+}], did return quickly (within 30 sec) to starting values after KCl washout and replacement with Tyrode’s (after both 1 and 5 min pulses). Thus, even though VSCC’s inactivate in response to sustained depolarization (Di Virgilio et al., 1987), our results confirm that there is still considerable Ca^{2+} influx. Second, Ca^{2+}-clearance times after KCl-washout were the same after both the 1 and 5 min exposures to KCl. We thus concluded that either efflux mechanisms were not affected by length of depolarization (to within the sensitivity of our methods); or the effects occurred within the first minute and thus were the same for the two time periods we used.
Figure 5. Imaging of free cytoplasmic Ca\(^{2+}\) during chronic depolarization. A: Representative time series images of Ca\(^{2+}\)-induced fluorescence resulting from two 5-min pulses of depolarization. Cells were initially resting in Tyrode's solution in a polarized state (top row, left image). Fluorescence emission from the cells at the beginning and end of the first 5-min pulse are shown in the top row, center-left and center-right images. Cells were returned to a polarized resting state in Tyrode's solution in the top row, right and bottom row, left images. Fluorescence emission increased again during the second 5-min pulse, shown in the center-left and center-right images in the bottom row. B: The change in fluorescence versus time during the first 5-min KCl pulse and washout was plotted. Each data point is the average of 5 cells. C: Fluorescence change versus time is plotted over the entire course of series
A, showing the response to the second 5-min KCl pulse relative to the first. Each data point is the average of 5 cells. D: Representative time series images of \( \text{Ca}^{2+} \)-induced fluorescence resulting from two 1-min pulses of depolarization. Cells were initially resting in Tyrode’s solution in a polarized state (top row, left image). Fluorescence emission from the cells at the middle and end of the first 1-min pulse are shown in the top row, center and right images. Cells were returned to a polarized resting state in Tyrode’s solution in the bottom row, left and center images. Fluorescence emission increased again during the second 1-min pulse, shown in the bottom row, right image. E: The change in fluorescence versus time during the first 1-min KCl pulse and washout was plotted. Each data point is the average of 6 cells. F: Fluorescence change versus time is plotted over the course of series D, showing the response to the second 1-min KCl pulse relative to the first. Each data point is the average of 6 cells.

3.4.4 Free \( \text{Ca}^{2+} \) Concentrations After a Second Period of Depolarization

Previous studies have shown that cells limit the influx of calcium during prolonged depolarization by inactivating/inhibiting VSCC’s (Di Virgilio et al., 1987). To determine whether the inhibition of VSCC’s disappears if cells are allowed to recover for a period of time in a polarized state, PC12s were subjected to a second pulse of HiK after resting for 30 minutes in Tyrode’s solution. As shown in the bottom rows of panels A and D in Figure 5, a second pulse of either 1- or 5-minute duration caused a rise from resting levels of fluorescence. Comparing the amplitudes of the fluorescence response plotted in panels C and F of Figure 5, in both cases the second exposure to KCl produced a significantly smaller rise in fluorescence (and thus \( \text{Ca}^{2+} \)) than the first depolarization event (\( P < 0.05 \) for both cases using single-factor ANOVA). The amplitude of the second 1 minute pulse was 61% lower than the first, while the second 5 minute pulse was 50% lower than the first. Since the effects of Fluo-4 bleaching were corrected for during image processing (discussed in 3.3.4), these results suggest that VSCC’s were still under partial inhibition during the second pulse.

However, recovery is more complete after a 1-minute depolarization pulse versus a 5-minute pulse. This can be seen by comparing the average amplitudes of the second pulse relative to the first pulse in the two cases. Furthermore, in a sub-population of the cells depolarized for 1 minute, the second pulse elicited an equivalent effect as the first. When 5-minute pulses were used, the second pulse always resulted in a decreased response
amplitude. Thus, the duration of depolarization does affect the degree of VSCC inhibition, and longer rest periods are needed for full recovery after longer periods of depolarization. In our studies, a 30-minute recovery period is sufficient for full recovery of at least a subset of PC12 cells.

3.4.5 Effect of Duration of Depolarization on Deactivation of MAP Kinases

Measurements of \([\text{Ca}^{2+}]_i\) confirmed that upon re-polarization, PC12’s can quickly clear unbound \(\text{Ca}^{2+}\), and that the time required for clearance did not depend on the duration of depolarization. We thus wanted to determine if the rate of MAP kinase deactivation was dependent on \([\text{Ca}^{2+}]_i\) or the duration of depolarization. Cells were depolarized for either 1 or 5 minutes, and then immediately placed in physiological (re-polarizing) solution. The phosphorylation levels of Erk1 and Erk2 were measured at various times after the end of the depolarizing pulse using western blots. As shown in Figure 6, the rate of de-phosphorylation was the same order of magnitude after either 1 or 5 minutes of depolarization. We note that since optical density (OD) measurements of Western blots are semi-quantitative at best, we use the term “rate of de-phosphorylation” here to represent the inverse of the time required for complete Erk1/2 de-phosphorylation. Similar to the measurements of calcium efflux, if the duration of depolarization did have an effect on deactivation of Erk1/2, either the effect occurred within the first minute of depolarization, or our methods were not sensitive enough to measure the difference. In addition, the rates of de-phosphorylation observed in Figure 6 are equivalent to the de-phosphorylation rate of the MAP kinases from peak levels during chronic depolarization. Since \([\text{Ca}^{2+}]_i\) is significantly lower after washout of KCl and during depolarization (see 3.4.3), it appears that the rate of MAP kinase deactivation is not strongly affected by \([\text{Ca}^{2+}]_i\).
Figure 6. MAP kinase deactivation after 1 and 5 minute periods of depolarization. A: Western blot showing phosphorylation of Erk1 and Erk2 at various times following a 1-minute pulse (lanes 3 to 5) and a 5-minute pulse (lanes 6 to 8) of 70 mM KCl. KCl was washed out and replaced with Tyrode's solution at time point 0 minute. Lane 1 is a positive control, stimulation with 30 ng/ml of EGF for 10 minutes. Lane 2 is a negative control, stimulation with Tyrode's solution for 5 minutes. B: Optical density counts following 1- and 5-minute pulses of depolarization versus time after KCl washout. Values are relative to OD counts of the positive control for each Western, n = 4 for each measurement.

3.4.6 Reactivation of MAP Kinases with Two Pulses of Depolarization: Effect of Recovery Period

The fluo-4 imaging measurements showed that a second depolarizing stimulus could elicit a second increase in [Ca^{2+}], if sufficient time had elapsed after the first stimulus. We were thus interested to determine if this second depolarization event also led to re-phosphorylation of the MAP kinases Erk1/2 and CREB, or if other deactivating mechanisms were in effect as a result of the first stimulus. Thus, PC12s were stimulated with two 5 minute pulses of HiK separated by a 30 minute, 70 minute or 100 minute recovery period in Tyrode's solution. The phosphorylation states of Erk1/2 and CREB
were determined at the end of the second stimulus period using western blots. As shown in Figure 7, panel A, Erk1 and Erk2 were both phosphorylated in response to the second stimulus at all inter-pulse intervals. Furthermore, the amount of phosphorylation was the same order of magnitude in the second pulse as in the first pulse in all three cases, indicating that any inhibitory effects from the first pulse, such as deactivation of VSCC’s and up-regulation of phosphatases, had disappeared after a 30-minute recovery period. Similarly, CREB was re-phosphorylated in response to a second depolarizing event at all three intervals (Figure 7, panel B). Given that 100 minutes is sufficient time for protein translation, we confirmed that the levels of total CREB had not changed as a result of the first stimulation event (Figure 7, panel D). Thus, the second stimulation event induced the same degree of phosphorylation (both total and as a % of total available substrate) as the first at all three intervals.
Figure 7. Re-phosphorylation of Erk1, Erk2 and CREB with a second 5-minute pulse of depolarization. A. Western blot of re-phosphorylation of Erk1 and Erk2 with a second 5-minute pulse of depolarizing KCl delivered from 30 to 100 minutes after the initial pulse (lanes 4 to 6). Lane 3 is the phosphorylation resulting from a single, 5 min pulse of KCl. Lane 1 corresponds to a positive control, treatment with 30 ng/ml EGF for 10 minutes. Lane 2 is a negative control, treatment with Tyrode’s solution for 5 minutes. B: Western of re-phosphorylation of CREB protein with a second 5-min pulse of depolarization, from 30 to 100 minutes after the first pulse (lanes 4 to 6). Lane 3 is phosphorylation after a single, 5-min pulse of KCl. Lane 1, positive control, treatment with 10 •M forskolin for 10 min. Lane 2, negative control, treatment with Tyrode’s solution for 5 min. C: Optical density (OD) counts in four experimental conditions for both phospho-Erk1/2 and for phospho-CREB. Values are relative to positive control in each Western, n = 4 for all
measurements. D: Western of whole CREB protein. Lanes are exactly same samples as in B.

To further characterize the ability of MAP kinases Erk1/2 and CREB to be reactivated, we applied two 2-minute pulses of depolarization with inter-pulse rest periods of 10, 30 and 60 minutes. As can be seen in Figure 8, panel A, reactivation of Erk1/2 was possible after only a 10-minute recovery period, although reactivation appears qualitatively lower than a single stimulation or a second stimulation following after 30 or 60 minutes of recovery. Similarly, CREB phosphorylation was equally strong after recovery periods as short as 10 minutes (Figure 8, panel C). Thus, CREB can be reactivated in a pulsatile fashion at inter-pulse intervals of 10 minutes with little loss of activity. It is possible that reducing the duration of depolarization in each pulse further might allow for even smaller inter-pulse rest periods.
Figure 8. Re-phosphorylation of Erk1/2 and CREB at short inter-pulse intervals with a second 2-minute pulse of depolarization. A. Western blot of re-phosphorylation of Erk1 and Erk2 with a second 2-minute pulse of depolarizing KCl delivered from 10 to 60 minutes after the initial pulse (lanes 4 to 6). Lane 3 is the phosphorylation resulting from a single, 5 min pulse of KCl. Lane 1 corresponds to a positive control, treatment with 30 ng/ml EGF for 10 minutes. Lane 2 is a negative control, treatment with Tyrode's solution for 5 minutes. B: Western of re-phosphorylation of CREB protein with a second 2-min pulse of depolarization, from 10 to 60 minutes after the first pulse (lanes 4 to 6). Lane 3 is phosphorylation after a single, 2-min pulse of KCl. Lane 1, positive control, treatment with 30 ng/ml EGF for 10 min. Lane 2, negative control, treatment with Tyrode's solution for 5 min. C: Optical density (OD) counts of re-phosphorylated Erk1/2 and CREB at the three inter-pulse intervals tested. Values are relative to the OD from a single pulse of depolarization, n = 4 for all measurements.

3.4.7 Reactivation of MAP Kinases with Two Pulses of Depolarization: Effect of Duration of Depolarization

To further characterize the relationship between depolarization and recovery, we varied the duration of depolarization in two successive pulses while keeping the recovery period constant. Cells were depolarized by two pulses of KCl that lasted 1, 2 or 5 minutes each, with a fixed recovery period of 30 minutes in all cases. The phosphorylation of Erk1/2 and CREB were measured at the end of the second depolarization period. As shown in Figure 9, panel A, the reactivation of the MAP kinases was most successful following a 2-minute pulse duration; 1 and 5 minute depolarizations gave qualitatively lower Erk1/2 activity. In contrast, CREB phosphorylation was equivalent with 1 or 2 minute pulses, and slightly lower with a 5 minute pulse (see Figure 9, panel B). Thus, pulses from 1 to 5 minutes in duration are effective at reactivating MAP kinases and CREB following 30 minute recovery periods.
Figure 9. Re-phosphorylation of Erk1/2 and CREB by successive 1-, 2- or 5-minute pulses of depolarization with a 30 minute interpulse interval. A: Western blot of phosphorylated Erk1 and Erk2 induced by a second pulse of 1-, 2- or 5-minute duration (lanes 3, 4 and 5, respectively), 30 min after a first pulse of equivalent duration. Lane 1 is positive control, treatment with 30 ng/ml EGF for 10 min. Lane 2 is negative control, treatment with Tyrode’s solution for 5 min. B: Western blot of phosphorylated CREB protein induced by a second pulse of 1-, 2- or 5-minute duration (lanes 3, 4 and 5, respectively), 30 min after a pulse of equivalent duration. Lane 1 is positive control, treatment with 30 ng/ml EGF for 10 min. Lane 2 is negative control, treatment with Tyrode’s solution for 5 min.

3.5 DISCUSSION

3.5.1 Transient Availability of Free Ca^{2+} in the Cytoplasm

The present measurements of Ca^{2+}-induced fluorescence clearly indicate that [Ca^{2+}]_{i} rises only transiently in response to chronic depolarization, but can be raised in a pulsatile fashion if depolarization occurs for short periods of time. These results are in agreement with recent reports in the literature. In a study of the effect of the A_{2a} adenosine receptor, Park and colleagues showed that [Ca^{2+}]_{i} could be raised by two, successive 30-second
periods of chronic depolarization if the cells were allowed to rest for 10 minutes between pulses (Park et al., 1998). Unfortunately, in the cited study, cells were treated with exogenous inhibitors of Ca$^{2+}$ influx during the second stimulation event, and thus it is not possible to determine if full recovery had occurred during the 10 minute recuperation period. Taken in conjunction with the results of Park and coworkers, our studies indicate [Ca$^{2+}$], can be reliably increased by multiple, short periods of depolarization (1 minute or less in duration) if sufficient (10 minutes or greater) time is allowed between stimuli for recuperation.

While we have considered the role of inhibition of VSCC's and Ca$^{2+}$ influx during chronic depolarization, the decrease in [Ca$^{2+}$], over time can also be viewed in terms of up-regulation of cytoplasmic Ca$^{2+}$-buffering mechanisms as a result of sustained depolarization. Studies have shown that calbindin-D 28K, a Ca$^{2+}$-binding protein widely distributed in the brain, is expressed in response to membrane depolarization, cytoplasmic Ca$^{2+}$ increases, or stimulation with excitatory amino acids ((Vyas et al., 1994), (Batini et al., 1993)). For example, in differentiated PC12's, calbindin-D 28K mRNA increased 10-fold after 2 hours in depolarizing KCl concentrations; protein levels also increased to a similar magnitude (Vyas et al., 1994). However, undifferentiated PC12 cells express low levels of calbindin-D 28K. Also, depolarization-induced up-regulation of calbindin-D 28K requires sufficient time for both transcription and translation to occur. Since we used undifferentiated PC12's and in most cases looked at events less than one hour after the first depolarization event, we conclude that the role of buffering up-regulation is small in interpreting our results.

3.5.2 Transient Activity of MAP Kinases and CREB

The present findings show that in PC12 cells, the activation of the MAP Kinases Erk1/2 is transient in response to chronic membrane depolarization. These results are consistent with observations in vivo (Sgambato et al., 1998), and in PC12 cells (Rosen and Greenberg, 1996). Studies of another calcium-activated messenger, CaM Kinase I in PC12 cells, also showed a peak in activity within the first 10 minutes of depolarization and a subsequent drop to intermediate levels that were maintained for up to 60 minutes (Aletta et al., 1996). Previous studies of CREB phosphorylation in PC12 cells showed
the presence of phospho-CREB after 5, 20 and 45 min in depolarizing concentrations of KCl, but not after 1, 2 or 4 hours (Thompson et al., 1995), in agreement with our results. Thus, in PC12's, chronic depolarization seems to transiently activate several intracellular signaling factors. We note that the transient activation of CREB might be cell-type specific, as a recent study in primary sensory neurons found that CREB phosphorylation did not decrease at all after more than 2 hours of depolarization in cultures of primary sensory neurons (Brosenitsch and Katz, 2001). Our results, supported by the literature ((Rosen and Greenberg, 1996), (Bitto et al., 1996), (Wu et al., 2001), (Thompson et al., 1995)), indicate that while both Erk1/2 and CREB are activated transiently during chronic depolarization, CREB phosphorylation is maintained at intermediate levels for up to 60 minutes.

Since several messenger pathways can activate CREB (Johnson et al., 1997), it is also conceivable that while the MAP Kinase pathway remains active for only a short time during depolarization, other pathways remain maximally or partially active and thus maintain CREB activity throughout depolarization. In the present study, depolarization induced CREB phosphorylation an order of magnitude greater than stimulation with 30 ng/ml EGF. However, the same depolarizing stimulus induced Erk1/2 phosphorylation approximately an order of magnitude less efficiently than 30 ng/ml EGF. Thus, a large portion of the CREB activation must have resulted from one of the other Ca²⁺-sensitive pathways. The signaling pathways PKA and CaM kinase have both been shown to induce CREB phosphorylation and to be activated by calcium entry into the cell (reviewed in (Ghosh and Greenberg, 1995)). In a recent study in hippocampal neurons, the MAP kinase pathway was shown to contribute to the later stages of CREB phosphorylation (approximately 60 minutes after a 3-minute pulse of KCl), while the CaM kinase pathway was the dominant contributor to phosphorylation of CREB in the first 10 minutes following depolarization (Wu et al., 2001). Along these lines, we have found that CREB activity mirrors MAP kinase activity in some cases (during the early minutes of depolarization and during pulsatile re-stimulation), but not in others (during the later stages of chronic depolarization).
Interestingly, the activity of CREB might be under the influence of several dephosphorylating factors as well. In a recent study, it was found that electrical stimulation with 0.2 msec pulses of depolarization delivered continuously at 5 Hz, rather than chronic depolarization, maintained CREB phosphorylation for only 1 hour (Brosenitsch and Katz, 2001). The authors suggest that several phosphatases respond differentially to the patterns of stimulation, and thus give the diverse response. Similarly, it was observed in hippocampal neurons that calcineurin, a negative regulator of CREB activity, was modulated by different patterns of stimulation (Bito et al., 1996). When the duration of stimulation was increased from 18 seconds to 180 seconds, CREB activity was prolonged as a result of the inactivation of calcineurin at longer stimulation times.

Comparing the temporal profiles of \([\text{Ca}^{2+}]_{i}\) and Erk1/2 phosphorylation during chronic depolarization, we can draw conclusions about the deactivation of the MAP kinases Erk1/2. First, \([\text{Ca}^{2+}]_{i}\) decreased to initial base-line concentrations within 30 seconds of KCl washout. However, complete dephosphorylation of Erk1/2 required 10 minutes after washout. Second, during chronic depolarization, while \([\text{Ca}^{2+}]_{i}\) was maintained at intermediate levels, Erk1/2 was dephosphorylated completely. The deactivation of Erk1/2 also occurred after washout in the short-pulse experiments, when \([\text{Ca}^{2+}]_{i}\) levels were at baseline. These observations suggest that the deactivation of the MAP kinases from peak levels requires approximately 10 minutes and occurs independent of \([\text{Ca}^{2+}]_{i}\). We would predict from the present study that given a particular pattern of \([\text{Ca}^{2+}]_{i}\), versus time, Erk1/2 activity would follow this pattern if the peaks in \([\text{Ca}^{2+}]_{i}\) were at least 10 minutes apart.

In a similar study in PC12's, CaMKI activity was followed over time as a result of Ca$^{2+}$ entry through different routes (Aletta et al., 1996). The study observed that the dephosphorylation of CaMKI was not greatly affected whether the cells were continuously depolarized with KCl or subjected to calcium-clamp conditions using Ionomycin. Unfortunately, a subsequent study has shown that \([\text{Ca}^{2+}]_{i}\) rises to a peak and subsequently drops to steady-state level after 1 minute following both KCl depolarization and Ionomycin treatment (Park et al., 1998); albeit the peak is 30% higher and the steady-state 40% lower in the case of chronic depolarization. Thus, \([\text{Ca}^{2+}]_{i}\) varies similarly over
time in the two different stimulation methods, and it is not possible from the study of Aletta and colleagues to uncover the dependencies of CaMKI on $[\text{Ca}^{2+}]_i$.

3.5.3 Pulsatile Activation of the MAP Kinases and CREB

While $[\text{Ca}^{2+}]$ and Erk1/2 phosphorylation decrease during chronic depolarization, the present study shows that if duration of simulation is short and is followed by a sufficiently long recovery period in a polarized state, the kinases can be successfully reactivated. Re-stimulation of Erk’s was possible as soon as 10 minutes after the first depolarization event, and led to full restimulation of CREB. Although the $[\text{Ca}^{2+}]_i$ measurements showed some effects of buffering with 5 minute pulses separated by a 30 minute interval, there was no statistically significant effect on Erk1/2 or CREB phosphorylation. Since Erk1/2 phosphorylation closely mirrored $[\text{Ca}^{2+}]_i$ in the pulsed studies as well, patterns of stimulation where electrical activity occurs repetitively for short periods of time might be a mechanism to maintain CREB activity over long periods of time.

Considering the physiological responses to depolarization, the transient activation of Erk1/2 has several implications with respect to delayed effects, such as gene expression and morphological changes. For example, the expression of vgf begins after 2 hours of depolarization, peaks after 6 hours and is still maintained 12 hours after the onset of depolarization (Salton et al., 1991). Expression of vgf is Ras- and MAP kinase-dependent (D’Arcangelo et al., 1996). Interestingly, the vgf promoter requires both activated CREB as a transcription factor, as well as the transcription factor NGFI-A. This latter factor is an immediate early gene that is expressed as a result of CREB activation. Thus, it appears that expression of the vgf gene will require CREB activity for a length of time that is sufficient for transcription and translation of NGFI-A. Stimulation of PC12 cells with NGF induces CREB activity for up to 4 hours, while EGF-treatment of PC12’s activates CREB for only minutes. Consistent with the above model for vgf transcription, NGF induces robust expression of vgf, while EGF does not. Since chronic depolarization also induces vgf expression, there must be sufficient CREB activity, even at the intermediate levels we observed during sustained depolarization, to drive transcription of this gene.
We have observed in our study that pulsatile depolarization can induce well-defined increases in CREB phosphorylation. It would be interesting to determine if short pulses of depolarization can be properly timed such that the first induces expression of NGFI-A, and the second coincides with the presence of NGFI-A protein in the nucleus, and thus can drive vgf expression. If the model proposed by Salton and co-workers (D'Arcangelo et al., 1996) for the regulation of vgf represents a general paradigm for gene regulation, as depicted schematically in Figure 10, then it is possible that a number of genes will respond to specific patterns of electrical stimulation.

![Diagram showing gene regulation](image)

**Figure 10. Schematic of gene regulation requiring sustained or pulsatile activation of a Ca²⁺-activated transcription factor.** A first Ca²⁺ transient activates a transcription factor (TF), such as CREB, that regulates expression of a second transcription factor, denoted P1. P1 acts at the promoter of a second protein, denoted P2, whose expression induces delayed, phenotypic cellular changes. However, expression of P2 requires both functional P1 protein as well as the Ca²⁺-activated TF. Thus, delayed cellular changes occur only as a result of a second Ca²⁺ transient.
The present findings also have implications for modeling cellular networks and phenomena. While our studies yield relative measurements of activity levels, clear correlations between $[\text{Ca}^{2+}]_i$ transients and activation of the MAP kinases and CREB can be drawn from the above results, which may lead to improved prediction of signaling cascade activity as a result of various stimuli. Already several attempts have been made to model complex behavior of the intracellular signaling networks and calcium transients ((Bhalla and Iyengar, 1999), (Shvartsman et al., 2002), (Hofer et al., 2002), reviewed in (Weng et al., 1999) and (Normile, 1999)). One such attempt, the Virtual Cell project developed by the National Resource for Cell Analysis and Modeling, has two modules for $\text{Ca}^{2+}$ transients: Calcium Dynamics in Neuroblastoma Cell; and Calcium waves in Fertilized Egg. Thus, the present study reflects a growing body of work that recognizes the dynamic, non-equilibrium nature of cellular signaling and reactions.

3.6 CONCLUSIONS

Electrical stimulation of neurons, as a result of $\text{Ca}^{2+}$ entry into the cytoplasm, induces many cellular changes that occur from minutes to hours or even days after stimulation. Many of the downstream effects of $\text{Ca}^{2+}$ influx have been uncovered in previous research. In the present study, we focus specifically on the transient nature of rises in cytoplasmic free $\text{Ca}^{2+}$ ($[\text{Ca}^{2+}]_i$). We have characterized changes in $[\text{Ca}^{2+}]_i$ versus time during both chronic and pulsatile depolarization of the neuron-like PC12 cell line. During chronic depolarization, $[\text{Ca}^{2+}]_i$ peaks and subsequently falls off to intermediate values within 10 minutes. Both 1- and 5-minute pulses of depolarizing KCl can induce sharp rises in $[\text{Ca}^{2+}]_i$ which fall back to baseline within 30 seconds of KCl washout and repolarization. If cells are allowed sufficient time to recover, a second pulse of KCl can induce another rise in $[\text{Ca}^{2+}]_i$. Mirroring the trends in $[\text{Ca}^{2+}]_i$, phosphorylation of the MAP kinases Erk1 and Erk2 is also transient, peaking and falling to low levels within ten minutes of depolarization. Short pulses of depolarization can phosphorylate Erk1 and Erk2, and the rate of deactivation of the Erk’s is not affected by the duration of depolarization (over the range 1 to 5 minutes) and appears to be minimally affected by $[\text{Ca}^{2+}]_i$. Phosphorylation of the transcription factor CREB also peaks as a result of chronic depolarization, and drops to intermediate levels that are maintained for over 1 hour. As predicted from
observations of \([\text{Ca}^{2+}]_i\), both Erk1/2 and CREB phosphorylation can be re-induced by a second round of depolarization that follows a recovery period. To our knowledge, this is the first report of pulsatile activation of the Erk's and CREB. The effects of the durations of depolarization and inter-pulse recovery on reactivation of Erk's and CREB were characterized. Recovery periods as short as ten minutes and pulse durations from 1 to 5 minutes in length can be combined to effectively re-stimulate signal transduction kinases and transcription factors. Thus, pulsatile activity may be a means of maintaining signaling activity over long periods of time.

REFERENCES


CHAPTER 4: GENE EXPRESSION CHANGES RESULTING FROM SHORT PERIODS OF DEPOLARIZATION

4.1 SUMMARY

Electrical stimulation of neuron sets into motion a series of events leading to long-term changes, such as modifications in the electrophysiological properties of the cells that are associated with long-term potentiation. These changes are mediated by up- and down-regulation of various genes. Expression of a number of immediate-early genes has been shown to occur in the hours following depolarization. Furthermore, several delayed-response genes begin to be expressed starting several hours after depolarization, and some remain up-regulated for days following the stimulus. We wanted to study the expression of a large set of diverse genes in the eight hours following depolarization, over the time period that captures the transition from immediate-early to delayed-response genes. We stimulated PC12 cells with either one or two pulses of depolarizing KCl, and then analyzed gene expression in the cells at 1, 3, 4, 6 and 8 hours following the stimuli using gene expression arrays. We identified five genes that were up-regulated following depolarization: namely, the genes for VGF, MEK5, MAD1, prostaglandin E2 receptor, and brain gastrin receptor. Previous to the current study, only the first of these, VGF protein, was shown to be regulated by depolarization. Furthermore, we identified five genes whose expression level decreased following depolarization. Two of these genes, the tyrosine phosphatase SHP2 and the transcription factor NF-κB, are expressed in neurons and neuronal tissue. The three other genes, organic cation transporter 1, aquaporin 3 and the chemokine receptor LCR-1, do not appear to have any specific relation to neuronal tissue. Lastly, we observed that the δ subunit of the γ-aminobutyric acid type A (GABA_A) receptor was regulated differentially by one pulse of stimulation versus two. Thus, we identified a wide variety of genes which responded to short periods of depolarization.
4.2 INTRODUCTION

Electrical stimulation leads to long-term changes in neurons (reviewed in (Kandel, 2001) and (Ghosh and Greenberg, 1995)). These changes require expression of new sets of genes following the depolarizing stimuli (Brosenitsch and Katz, 2001). Previous research has identified both immediate-early genes (IEG’s) and delayed-response genes (DRG’s) that are regulated at the level of transcription by electrical stimulation. The former are genes that do not require protein synthesis before their expression, while the latter group require synthesis of new proteins (in many cases, transcription factors) before they can be expressed.

Electrical stimulation of neurons leads to Ca\(^{2+}\) influx through voltage-gated calcium channels (Clapham, 1995). A number of Ca\(^{2+}\)-activated transcription factors that are normally present in the cytoplasm subsequently bind to regulatory regions and initiate transcription of various IEG’s. The best-studied of these transcription factors is the cAMP response element binding protein, CREB ((Bito et al., 1996), (Johnson et al., 1997)). Since new protein synthesis is not required, transcription of IEG’s begins within minutes of electrical stimulation ((Thompson et al., 1995), (Bartel et al., 1989)). Some of these IEG’s, such as c-fos, jun, NGFI-A (zif 268) and NGFI-B (nur 77), are transcription factors that proceed to activate other genes ((Enslen and Soderling, 1994), (Bartel et al., 1989)). Others, such as arg3.1/arc and BDNF, are involved in synaptic plasticity and neuronal survival, respectively ((Waltereit et al., 2001), (Ghosh et al., 1994)). And at least one IEG, MKP-1, is a phosphatase that exerts feedback on the signaling cascades by inactivating MAP kinase (Sun et al., 1993).

The expression of IEG’s begins a cascade of events that lead to activation of other stimulation-induced genes, namely delayed-response genes. Examples include the vgf gene, which starts to be expressed 2 hours after the start of depolarization and peaks after 6 hours (Salton et al., 1991). VGF expression is one of the first signs that a neurogenic program has been initiated in NGF-treated PC12 cells (Possenti et al., 1989). Tyrosine hydroxylase is another delayed-response gene that is up-regulated after depolarization, as it is expressed after 6 to 24 hours of stimulation ((Brosenitsch and Katz, 2001), (Mishra et al., 1998)). Cyclooxygenase 2 (COX-2), an enzyme responsible for biosynthesis, is
induced after electrical activity in the cortex of rats (Meittinen et al., 1997). Thus, electrical activity can stimulate the expression of a variety of genes hours after the stimulation has ceased.

Gene expression arrays have become a powerful tool for analyzing the changes in gene expression following various stimuli ((Roberts et al., 2000), (Tepperman et al., 2001), (Chaudhry et al., 2002)). Most studies of genes that are induced immediately following depolarization have focused on one or two genes as part of a particular pathway ((Johnson et al., 1997), (Snyder et al., 1998), (Sun et al., 1993)). Expression of the genes was tracked over a series of time points following stimulation, in many cases showing the transient expression of these genes ((Bartel et al., 1989), (Thompson et al., 1995)). Several studies have used subtractive or differential library methods to isolate new mRNA molecules that appeared after stimulation ((Nedivi et al., 1993), (Herschman et al., 2000), (Vician et al., 1995)). In these studies, the library wide comparison of the mRNA was performed at a single time point, 3 hours or 6 hours after stimulation. We were interested to combine the advantages of these two approaches and analyze the expression of a large number of diverse genes at a series of time points following electrical stimulation.

We subjected PC12's to brief periods of depolarization and tracked gene expression changes during the following eight hours. We elected to study the PC12 cell line for two reasons. First, naive PC12 cells (i.e. untreated with growth factors other than serum) are cycling and have the ability to make several cell-fate choices, namely continue to proliferate, differentiate, or undergo programmed cell death ((Greene and Tischler, 1976), (Greene, 1978)). Thus, stimulation of naive PC12 cells can induce a wide variety of gene expression programs. Second, PC12 cells have been studied extensively to understand the effect of depolarization on immediate-early and delayed-response genes ((Bartel et al., 1989), (Salton et al., 1991), (Herschman et al., 2000)). In the present studies we have identified genes that were up- or down-regulated as a result of short depolarizations. Furthermore, we were able to compare the cellular response to a two depolarizing pulses versus the response to one pulse. Our results confirm that a diverse set of genes is regulated by electrical stimulation of PC12 cells.
4.3 MATERIALS AND METHODS

4.3.1 Cell Culture

Rat pheochromocytoma PC12 cells (ATCC) were cultured in complete Dulbecco’s Modified Eagle’s Medium (cDMEM), which consists of DME supplemented with 10% heat-inactivated horse serum (FHS), 5% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin (all components from Gibco BRL). Cells were maintained in a humidified atmosphere of 5% CO₂ and 37 °C. Cells were plated at a density of 2 x 10⁴ cells/cm² on tissue culture plates (Corning) coated with 0.1 mg/ml of poly-L-ornithine (PLO; Sigma: P3655) in water. Cells were fed cDMEM every 5 days and passaged every 10 days. Briefly, cells were detached after incubation in a 0.05% trypsin-EDTA solution (Gibco BRL) at 37 °C for 5 mins. Cell clumps were broken up with mild tituration or passage through a 20G needle and syringe, and reseeded at the density noted above. Cells were not used in experiments past passage number 20.

4.3.2 Stimulation for Assessing Short-term Gene Expression

For stimulation experiments where gene expression was to be determined by expression array analysis, cells were seeded 24 hours before experiments on 100mm Corning dishes pre-coated with PLO (as above) at a density of 9 x 10⁴ cells/cm². Cells were maintained in a humidified atmosphere of 5% CO₂, at 37 °C, in cDMEM until stimulation.

Cells were depolarized by adding an equal volume of 2xHiK solution (130 mM KCl, 30 mM D-glucose, 1 mM MgCl₂, 2 mM CaCl₂, 37 mM NaHCO₃; 1 mM NaH₂PO₄; all components cell-culture grade from Sigma) to the cells in culture. Since cDMEM contains 5 mM KCl, the final concentration of KCl during chronic depolarization was 70 mM. Depolarization was maintained for 5 minutes in 5% CO₂, 37 °C, after which elevated KCl was washed out and replaced with cDMEM. For cells that received a single stimulus, cells were maintained in cDMEM at 37 °C, 5% CO₂ for the appropriate time and then were lysed as described below. For cultures that received two depolarizing stimuli, the cells were maintained in cDMEM for 2 hours, after which an equal volume of 2xHiK was added. After 5 minutes of depolarization, this second pulse of excess KCl
was also washed out and replaced with cDMEM. Cells were maintained in cDMEM for the appropriate time and then lysed as well.

In each experiment, one 100mm dish was of PC12 cells was randomly chosen as the reference culture (discussed below with regards to data analysis). This dish was not stimulated with any factors, and the gene expression levels in these cells were determined with the same protocols as for stimulated cultures. Furthermore, to account for gene expression that resulted from addition and wash-out of solutions, a culture of PC12 cells was treated with a 5 minute pulse of NaCl. An equal volume of 2xHiN (130 mM NaCl, 30 mM D-glucose, 1 mM MgCl₂, 2 mM CaCl₂, 37 mM NaHCO₃; 1 mM NaH₂PO₄; all components cell-culture grade from Sigma) was added to the culture, and after 5 minutes the solution was replaced with cDMEM. The culture was maintained in cDMEM for 2 hours prior to lysis and RNA extraction.

4.3.3 RNA Extraction and Purification

At the appropriate times after one or two depolarizing stimuli, PC12 cells were lysed and RNA was isolated according to the following protocol. Cells were washed twice with ice-cold PBS to remove serum factor, and cell lysis buffer was added to each dish (RLT buffer containing β-mercaptoethanol). Cells were lysed by scraping and the lysate was collected and diluted with an equal volume of 70% ethanol. This solution was passed repeatedly through a 20G needle and syringe, and then each sample was added to an individual RNeasy MINI column (Qiagen). Total RNA was bound, purified and eluted in water following the protocol of the Rneasy column.

After purification, total RNA from each sample was analyzed on an UV/Visible spectrophotometer (50Bio model from Cary Instruments) to determine the concentration of RNA, and to confirm that the sample did not contain contaminating DNA. RNA concentration was determined in water by measuring the absorbance at 260 nm wavelength (A₂₆₀) and using the standard formula:

\[ C_{RNA} = A_{260} \times DF \times EC \]
where \( C_{\text{RNA}} \) was mg of total RNA per ml of solution, \( DF \) was the dilution factor for the measurement, and \( EC \) was the extinction coefficient, which had a value of 40 for the experimental apparatus we used. The level of DNA contamination was evaluated by measuring the absorbance at 280 nm and at 260 nm in phosphate-buffered saline (PBS, pH 7.4). The ratio \( A_{260}/A_{280} \) will be greater than 1.9 for solutions that are free of contaminating DNA and proteins. All RNA samples used in our experiments had \( A_{260}/A_{280} \) greater than 2.0.

To confirm that RNA degradation had not occurred during extraction and purification, samples were analyzed by electrophoresis and ethidium bromide (EthBr, Gibco BRL) staining. Samples were separated on 1.2% agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The gels were stained in TAE containing 1 \( \mu \)g/ml EthBr for 60 minutes. Gels were then washed for 60 minutes in TEA buffer without EthBr, and then imaged on a UV light table. Images were captured on a digital camera. A representative image is shown in Figure 11. The 18S and 28S bands are clearly visible, with little sign of degradation.

![Electrophoresis of total RNA](image)

**Figure 11.** Electrophoresis of total RNA to determine degradation. Each lane was loaded with 2 •g of total RNA isolated from KCl-stimulated PC12 cells. RNA molecules were separated on 1.2% agarose gel/TAE with electrophoresis. RNA was stained with 1 •g/ml EthBr for 1 hour, de-stained for 1 hour, and imaged on UV light table.
4.3.4 Fabrication of Radio-labeled cDNA Probes from Isolated RNA

Isolated total RNA from each experimental condition was used as a template to fabricate cDNA probes for hybridization with expression arrays. The protocol described in the Array Advantage-AA product (Ambion) was used, with minor changes. In a separate tube for each sample, 4 µg of total RNA was mixed with 2 µl of CDS-Primer mix, provided with the expression array (Atlas Nylon Rat Expression Array, Clontech) and incubated at 70 °C for 10 minutes, followed by 10 minutes at 48 °C. In a second tube, also separate for each sample, the following components were mixed: 2 µl of 10x Reaction Buffer (Ambion kit), 2 µl of 10x StripEZ dNTP cocktail (Ambion kit), 3 µl of \(^{33}\)P-α-dATP (10 mCi/ml, ICN Biochemicals) and a volume of nuclease-free water needed to raise the total volume of the corresponding first and second tubes to 18 µl. The contents of the second tube were incubated for 5 minutes at 48 °C. The two tubes were mixed, and 2 µl of Reverse Transcriptase (Ambion kit) was added to the resulting mixture for each sample, raising the total volume to 20 µl. This final solution was incubated for 2.5 hours at 48 °C, to ensure complete fabrication of cDNA probes. We were able to use total RNA in this procedure because the CDS-Primer mix contains primers specific for each spot on the Atlas Array, and is not a mixture of random primers.

To purify the cDNA probes from the other components in the mixture, we used the Nucleospin columns provided with the Atlas arrays (Clontech). Since we did not have good results using the columns provided with the Array Advantage product from Ambion. Upon completion of the probe-fabrication reaction, the contents of each sample tube were diluted 1:10 with NT2 buffer (Clontech) and loaded on each column. The columns were centrifuged at 14,000 rpm for 1 minute, and the eluted fraction was collected and reloaded on the column to ensure that any un-bound cDNA had a second chance to bind to the column. The columns were again centrifuged (1 min at 14,000 rpm), the eluted fraction was discarded, and 400 µl of buffer NT3 (Clontech) was added to each column. The NT3 buffer was eluted by centrifugation (1 min, 14,000 rpm) and the flow-through discarded. The columns were washed twice more with NT3 in the same manner. Finally, 100 µl of nuclease-free water was added to each column and allowed to
soak for 2 minutes. The column was then spun at 14,000 rpm for 1 min, and the flow-through, containing purified cDNA probes in water, was collected in a sample tube. The elution of cDNA probes with 100 µl of water was repeated a second and third time, and all the collected fractions were pooled. To prevent degradation of probes in subsequent steps, the Ca²⁺-chelator, EDTA, was added to each sample to a final concentration of 10 mM. A small volume from each sample was collected and mixed with 5 ml of scintillation cocktail (ScintiSafe Plus 50%, Fisher) for measurement of radioactivity. Samples were counted on a Packard 2200CA Liquid Scintillation Analyzer, with the energy range for ³²P set to 0 to 250 kev. Probes with total radioactivity less than 1.5 x 10⁶ cmp were discarded and re-fabricated, since ample hybridization signal to the arrays could not be obtained if probes did not contain this level of radioactivity. After counting the radioactivity, the remainder of each sample (between 250 and 275 µl) was frozen and stored at −20 °C until hybridization. Due to the short half-life of ³²P (25.4 days), probes were hybridized to arrays within three days of fabrication.

4.3.5 Hybridization of cDNA Probes to Expression Arrays

Gene expression levels were determined by hybridizing cDNA probes to commercially available hybridization arrays. Atlas Rat cDNA Expression Nylon Arrays, purchased from Clontech, were used in our study. Each array contained 588 cloned genes, spotted in duplicate. The arrays also contained alignment spots, and nine spotted genes were nominally designated as house-keeping genes.

Hybridization of radio-labeled probes to arrays was performed with the following protocol. Prior to hybridization, the arrays were placed in 35x100 mm roller bottles and pre-hybridized in 10 ml of pre-warmed UltraHyb buffer (Ambion) at 50 °C for greater than 1 hour. Herring-sperm DNA was denatured in boiling water for 5 minutes, and 50 µl was added to each array 10 minutes before addition of probes. Radio-labeled probes were also denatured for 5 minutes in boiling water, and then added to each array. Care was taken to pipet the probe solution into the hybridization solution and not directly onto the array. Probes were allowed to hybridize to the arrays over-night for 16 to 18 hours at 50 °C with gentle agitation (roller bottles were rotated at 6 rpm for hybridization).
Following over-night hybridization, un-hybridized probes were washed from the nylon membranes. The hybridization solution was discarded and membranes were washed twice in 50 ml of pre-warmed Low Stringency Wash Buffer (LSWB, 0.3 M NaCl, 0.03 M Na₃Citrate·2H₂O, 1% SDS) for 40 minutes per wash, at 50 °C, with medium agitation (roller bottles were rotated at 12 rpm during washing). Next, membranes were washed twice in 50 ml of pre-warmed High Stringency Wash Buffer (HSWB, 0.075 M NaCl, 7.5 mM Na₃Citrate·2H₂O, 1% SDS) for 40 minutes per wash, at 50 °C.

Once all four washes were completed, the arrays were wrapped and exposed to a phosphor screen. Each array was removed from its roller-bottle and placed face-down on a single sheet of plastic wrap. A square of blotting paper, slightly bigger than the size of the membrane, was soaked in water and placed on top of the membrane. The damp blotting paper ensured that the membrane would not dry out during the long periods of exposure to the screen, as drying of the array would make stripping and re-use impossible. The membrane and blotting paper were carefully wrapped and sealed, and each membrane was arranged face-up in the cassette. Any air bubbles between the plastic wrap and the membrane surface were carefully eliminated, and finally the phosphor screen (LE Phosphor Screen, Molecular Dynamics) was placed in contact with the membranes. The screen was exposed to the membranes for between 1 and 6 days at room temperature, depending on the radioactive strength of the probes (measured after probe fabrication). After the phosphor screen had been exposed to the arrays for a sufficient length of time, the screen was removed and placed on a phosphor imager scanner (STORM reader, Molecular Dynamics). The screen was scanned at a resolution of 50 µm per step. The digital image of the radioactive emission from each membrane was stored and processed as described below for quantification of gene expression levels. A representative image of a membrane is shown in Figure 12. After imaging was complete, the phosphor screens were blanked by exposure to white light for 30 minutes and stored in the dark until the next use.
Figure 12. Image of Atlas Rat Nylon Expression Membrane. A total of 588 genes are included on each array. The genes are divided into 6 subsets, and 9 of the genes are designated as house-keeping genes. Each cloned gene is represented by two spots. The last row at the bottom and the last two columns on the right are alignment spots.

4.3.6 Stripping Membranes for Re-hybridization

The use of nylon membranes and the Array Advantage kit for radio-labeled probe fabrication allowed us to strip and re-use expression arrays. The Array Advantage method was developed so that stripping conditions were more gentle than the standard protocols, minimizing damage to the spotted genes on the array during the strip steps. After exposure to the phosphor screen was completed, the membranes were washed in a solution of 0.2% SDS at 75 °C for 30 to 45 minutes with mild agitation. The wash solution was then discarded and the membranes were rinsed in room temperature distilled water (DIW) for 1 to 2 minutes. The membranes were then washed in Degradation Wash (Ambion) for 10 minutes at room temperature. The Degradation Wash acted to degrade the probes at specific locations, due to the use of the StripEZ dNTP Mix during probe fabrication. As a result, the probes that remained hybridized to the array after the previous SDS wash were degraded to 4-mers during the Degradation Wash step. Finally, the remaining 4-mers were stripped from the arrays by washing in Reconstitution Wash (Ambion) at 68 °C for 15 minutes. The arrays were then wrapped and stored at -20 °C.
until further use. Using this method, we were able to re-use membranes up to five times without any noticeable decrease in hybridization signal due to stripping.

### 4.3.7 Data Analysis

The digital image of each membrane obtained from the scan of the phosphor screen was saved as a .GEL file and then analyzed with the Atlas Image 2.01 software to obtain expression levels for each of the spotted genes. A digital template of the Rat cDNA Expression Array was placed on top of the image from each membrane, and each spot on the image was manually aligned with the corresponding spot on the template. The intensities of the two spots corresponding to each gene were averaged, and the background intensity from the array was subtracted to give a single, adjusted intensity measurement for each gene. This adjusted intensity measurement thus corresponded to the level of radioactivity hybridized to each spot on the array, which corresponded to the amount of mRNA from that gene isolated from the cells (since one radiolabeled cDNA probe molecule was fabricated from each mRNA molecule). A culture of PC12 cells that had not been stimulated with KCl or any other factors was taken as the reference state for the analysis of all stimulated samples. RNA isolation, hybridization and imaging of the reference cells were performed in parallel and in exactly the same manner as for samples from stimulated cells.

The expression levels of all genes in stimulated conditions were measured as the ratio of the stimulated expression level to the reference expression level. Before normalizing the expression level in each gene to the level in the reference state, an array-wide normalization was performed to reduce the effects of variations in probe fabrication and hybridization efficiency (see Kroll and Wolfl, 2002) and (Goryachev et al., 2001) for reviews of normalization methods). Two general options for normalization were available with the Image 2.01 software. Array-wide normalization could be performed with the set (or a subset) of the genes denoted as house-keeping genes by the array manufacturers, or normalization could be performed by measuring the sum of the expression level of all of the spots on the array (termed global sum normalization). In our experience, both normalization procedures were equivalent for samples that were
collected over the 8 hours after stimulation. We thus elected to use global sum normalization.

Once normalization was completed, the data for each gene was represented as a series of expression ratios versus time after one or two depolarizing stimuli. Twelve data points constituted the time series for each gene. Two types of comparative analysis were performed. In the first, we were interested to know which genes were up- or down-regulated, at the transcriptional level, following either a single or a double pulse of stimulation. We used a z-test of the means to determine whether the average of the twelve expression ratios was statistically different from an expression ratio of 1 at a confidence level of P<0.05. We also used analysis of variance (ANOVA) to determine if a subset of the time series was statistically different from the rest of the data points. In the second type of analysis, we were interested to know which genes were up- or down-regulated as a result of two pulses of depolarization compared to a single pulse of depolarization. The Student’s t-test for unpaired data was used to determine if the two time series were statistically different from each other at a confidence level of P<0.05.

4.4 RESULTS AND DISCUSSION

4.4.1 Estimation of Variability in Gene Expression Measurements

Gene expression arrays are powerful tools for studying changes in the expression level of a large number of genes simultaneously. However, since expression arrays are expensive and hybridization steps are time-consuming, expression array data usually involve smaller sample sizes than experiments using Northern blots, or quantitative or semi-quantitative RT-PCR. Thus, a significant step in analyzing gene expression data is to understand the statistical variations that arise from the experimental manipulations so that statistically relevant conclusions can be drawn from the data. Our preliminary experiments with gene expression arrays were focused on quantifying the reproducibility of expression data with the Atlas Rat Nylon Expression membrane.

Variations in measurements of a particular gene’s expression level can arise from two general sources. First, errors and variations arise in experimental manipulations. These include variations in the efficiencies of RNA isolation and purification, probe synthesis,
hybridization and washing of membranes, and imaging of arrays. Also, small levels of nuclease contamination can greatly affect the results. A second source of variation is due to the regulation of the expression of the particular gene of interest. Recent research has uncovered the role of noise in gene expression, and it is now understood that certain genes are “noisier” than others (Ozbudak et al., 2002), (Thattai and van Oudenaarden, 2001)). While the exact biological role of expression noise is under investigation, the effects of this phenomenon on analysis of gene expression data must be taken into account. We will term the first type of measurement variability “extrinsic,” and the second “intrinsic.” To establish confidence in our gene expression data, we performed two reproducibility experiments to estimate the contributions of each type of variability to our data.

To estimate the extrinsic variability in expression data, we synthesized cDNA probes from a single sample, HiN-stimulated cells, in two separate reactions, and hybridized the probes to two different membranes. Thus, error was introduced in the probe synthesis, hybridization, and washing procedures. We estimated that exposure of the membranes to the phosphor screen introduced little error that was not corrected for during normalization. Figure 13 shows the comparison of gene expression in the two hybridizations. Each spot shown in the figure corresponds to a single gene, and the x and y positions represent gene expression level in the two hybridizations (in arbitrary units obtained from scanning the images). If extrinsic variability was zero, all points would fall on the line $x = y$. As shown in Figure 13, the real data were spread about the line $x = y$ with a Pearson Correlation Coefficient of 0.982. A linear regression to the data had a slope of 0.92. Thus, pooling the 588 measurements of gene expression, we found a bias error of 8% and a standard deviation of 19%, implying that gene expression ratios greater than 2 or less than 0.5 were the result of extrinsic error at a probability of less that 0.001 (statistical analysis methods described in (Howell, 1989)).
Figure 13. Estimate of gene expression error introduced by experimental manipulations. Gene expression from the same sample measured in two separate instances. A best-fit line through the data had slope of 0.92, with a Pearson Correlation Coefficient of 0.98.

To estimate the intrinsic variability in gene expression data, we measured gene expression in two separate cultures of PC12 cells which had been stimulated with a single pulse of depolarizing KCl. To ensure maximum independence of gene expression levels, the two cultures were grown to different passage numbers (passaged 3 and 7 times, respectively). Stimulation of cultures with KCl, RNA isolation and purification, probe synthesis, hybridization and imaging of membranes were all performed on separate occasions in the two cases. Thus, both intrinsic and extrinsic sources of gene variation were included in the measurements. Gene expression data from the two experiments were compared, as shown in Figure 14. A best-fit linear regression to the 588 measurements had a slope of 0.94 and a Pearson’s Correlation Coefficient of 0.83. Interestingly, the bias error of 6% was very close to the bias error observed from extrinsic sources. However, the standard deviation of the measurements was 41% when both intrinsic and extrinsic sources of variance were included. Assuming zero correlation between gene expression noise and variance introduced in experimental manipulations,
we calculate the noise from gene expression to be approximately 36% of the signal strength, or approximately 2 times greater than noise due to experimental error. Taking the measure of combined, total variance as 41%, an expression ratio of 2 would be attributable to statistical error (and not a biological phenomenon) at a probability of less than 0.01. Based on these studies of experimental error and variance, we thus denoted expression ratios greater than a factor of 2 or less than a factor of 0.5 to be statistically relevant, meaning not attributable to error or expression noise. Notably, of the 588 gene expression measurements plotted in Figure 14, only 3 were outside the range 0.5 to 2.

![Figure 14. Variability in measurements of gene expression taking into account both gene noise and experimental manipulations. Gene expression from the same sample measured in two separate instances. A best-fit line through the data had slope of 0.94, with a Pearson Correlation Coefficient of 0.83.](image)

While the standard deviation of gene expression measurements calculated above was a useful guide in experimental design, it was also an over-simplification. Studies have shown that genes are noisy to differing degrees (Ozbudak et al., 2002). However, the
above analysis assumes that the 41% standard deviation is applicable to all genes. Thus, three genes that fell outside the range 0.5- to 2-fold expression may have been noisier than the other genes analyzed. Since it was impractical to perform large numbers of preliminary hybridizations in parallel to estimate the variance of each gene individually, we elected to demand that genes be regulated significantly over the course of the entire (or a statistically significant subset of the) time series. Thus, genes were designated as up- or down-regulated due to a particular stimulus only if gene expression changes were maintained over a period of time.

4.4.2 Short-term Regulation of Gene Expression after Short Pulses of Depolarization

Using the confidence intervals established from the reproducibility studies above, we examined changes in gene expression after either a single or double pulse of depolarization. The time period of interest, designated “short-term”, covered the first eight hours after either a single pulse, or after the second of two pulses. A total of 12 measurements of gene expression, covering the eight-hour time period and obtained in three separate experiments, comprised the time series. In four cases, gene expression measurements were duplicated for the same experimental conditions: one hour after single-stimulation; one hour after double-stimulation; four hours after double-stimulation; and six hours after single-stimulation. In the cases of these duplications, the cells were from cultures at different passage numbers and all experimental manipulations were performed completely independently. These cases thus provided true replicate measurements.

We analyzed the set of expression data from 12 measurements of 588 genes after depolarizing stimulation in two steps. First, we flagged all genes where at least one measurement in the time series was greater than 2-fold or less than 0.5-fold the level in the un-stimulated reference culture. This subset encompassed 158 genes. As discussed above, we were interested in identifying genes that had consistent, statistically relevant expression. To identify such genes, we took the product of the expression ratio in all 12 measurements for these 158 genes. For noisy genes that were not consistently up- or down-regulated, this product was close to 1. For genes with consistent changes in
expression, the product quickly deviated from 1. We next looked in detail at these approximately 30 genes. We determined whether the average expression ratio of the 12 measurements was statistically different from 1, or whether the ratios in subset of the time points were significantly different from the ratios of the points in the series. This latter screen allowed us to identify five genes that were up-regulated, and six genes that were down-regulated, as a result of depolarization. As discussed in the following sections, many of these genes have been previously linked to neurons or neuronal differentiation. The genes for VGF, Mad1, brain gastrin receptor, MEK5, the prostaglandin E2 receptor and NF-κB fall into this category. However, several of the genes we identified, such as for aquaporin 3, organic cation transporter 1 and the chemokine receptor LCR-1, do not appear to have any link to neuronal tissues. Thus, short depolarizing stimulations regulate a diverse group of genes.

4.4.3 Depolarization Induces Expression of the Neuronal Differentiation Marker, VGF

The vgf gene is an important marker of neuronal differentiation, and is expressed almost exclusively in central and peripheral neurons ((D'Arcangelo et al., 1996)). As shown in Figure 15, transcription of vgf was induced approximately 4 hours after either one or two depolarizing stimuli (p<0.001). Over the period four to eight hours after stimulation, expression of vgf was between 2.5-to 3.5-fold higher than in un-stimulated cells. Conversely, in the first three hours following stimulation, expression was unchanged relative to un-stimulated cells.
Figure 15. Induction of vgf transcription following one or two pulses of KCl. PC12 cultures were stimulated with either one (filled circles) or two pulses (open circles) of KCl. Transcription of the vgf gene significantly increased four hours following depolarization, and was maintained for the next four hours (p<0.001). The increase in expression of vgf was 2.5- to 3.5-fold.

The vgf gene is neuron-specific and is induced by a variety of stimuli in PC12 cells. VGF was one of the first mRNA molecules found to be induced by nerve growth factor (NGF) treatment of PC12 cells (Levi et al., 1985). Since NGF-treatment of PC12 cells induced neurite outgrowth and cell-cycle exit, the VGF protein was hypothesized to be involved with neuronal differentiation. Further studies showed that expression of VGF was specific to neuronal tissue (van den Pol et al., 1989), and that the VGF protein was stored and secreted from vesicles in PC12 cells (Possenti et al., 1989). Thus, the connection of VGF to neuronal differentiation, in particular in PC12 cells, became well established. Subsequently it was found that epidermal growth factor (EGF) treatment of
PC12 cells induced expression of *vgf* only 2-fold compared to unstimulated cells, while NGF treatment induced 15-fold up-regulation of the gene (Salton et al., 1991). In both cases, expression of the VGF mRNA began 1 to 3 hours after stimulation, peaked 3 to 6 hours after stimulation, and finally dropped back to control levels 48 hours after stimulation. These authors also found that chronic depolarization of PC12 cells with 40 mM KCl induced *vgf* expression, 4.1-fold over the period of 2 to 6 hours following depolarization. Thus, while the *vgf* gene is strongly induced by NGF and is linked to neuronal differentiation, its expression can be induced by other stimuli as well, including depolarization.

The *vgf* gene encodes a polypeptide precursor, and the physiological role of the VGF polypeptide is still being understood. In the rat, VGF mRNA is expressed throughout the CNS and PNS, and most strongly during the first two weeks of postnatal development (Salton et al., 1991). Given that the polypeptide is localized to synaptic vesicles and *in vitro* is up-regulated during neuronal differentiation, it is likely that VGF is involved in synaptogenesis or synapse formation. The VGF polypeptide precursor is regulated *in vivo* by electrical activity. Inhibition of retinal activity blocked expression of VGF mRNA in the dorsal lateral geniculate nucleus (where electrical activity activity from the retina is first processed in the cortex) (Snyder et al., 1998). Furthermore, electrical activity resulting from kainite-induced seizures led to transient expression of VGF mRNA in several brain regions. Lesions in the cortex also induced *vgf* gene expression. These observations provide a strong link between the VGF polypeptide and neuronal development. Surprisingly, the polypeptide appears to have a strong role in maintaining energy homeostasis. In a recent study, knock-out mice lacking the *vgf* gene were found to be hyperactive, hypermetabolic, infertile and small (Hahm et al., 1999). These mice had lower leptin levels and fat stores compared to wild-type mice. The authors also found that fasting of normal mice induced expression of *vgf* in the hypothalamus (the key brain structure regulating food intake and energy output). Thus, the physiological role of the VGF polypeptide precursor is multi-faceted and central to nervous system development and function.
The regulation of the vgf gene at a molecular level is also complex and requires the coordinated action of several factors. The vgf promoter contains a cAMP response element (CRE) motif, and thus both NGF and cAMP were found to strongly induce promoter activity (Hawley et al., 1992). However, several growth factors, which can activate CRE-binding (CREB) protein, did not induce strong vgf promoter activity ((Hawley et al., 1992), (Salton et al., 1991)). These factors included EGF, basic fibroblast growth factor (bFGF) and acidic fibroblast growth factor (aFGF). Subsequent studies found that the VGF promoter is regulated by different factors in the neuronal versus non-neuronal cells (Mandolesi et al., 2002). In neuronal tissues, a multifactor complex induces promoter activity upon CREB activation (either by NGF or by another stimulus, possibly depolarization). In contrast, in non-neuronal tissues, an alternate multifactor complex binds to the promoter and represses transcription. This mechanism thus provides the neural tissue-specific expression of the vgf gene. Furthermore, a region was identified in the vgf promoter that greatly amplifies transcriptional activity (D'Arcangelo et al., 1996). This region was found to bind the transcription factor NGFI-A (zif268), which is induced by NGF and other stimulators of CREB activity. The interesting implication of this finding is that if CREB activity is maintained for long periods of time (on the order of hours), then sufficient time will pass for translation of NGFI-A, and subsequent amplified transcription of vgf. Short activation of CREB will lead to transcription of vgf in the absence of amplification. This mechanism would thus allow vgf to respond differentially to persistent activators of CREB, such as NGF, rather than to transient activators of CREB, such as EGF, thus explaining the factor-specific induction of this gene. While much remains to be understood about the molecular events regulating the vgf promoter, the complex and multifactor regulation of the gene have been well-established.

The results we have obtained with gene expression analysis of KCl-treated cells are consistent with previously reported results. The level of vgf induction after short exposure to KCl we observed, 3.5-fold peak expression relative to untreated cells (Figure 15), is close to the 4.1-fold increase in expression reported after chronic exposure to KCl (Salton et al., 1991). It is interesting that a 5-minute period of depolarization was sufficient to induce the same level of expression as hours of chronic depolarization.
Furthermore, vgf expression was observed to peak after 2 to 6 hours of chronic depolarization, while we observed induction of vgf starting 4 hours after stimulation (Figure 15). Thus, in both amplitude and timing, our observations with short pulses of depolarization agree with previous reports using chronic depolarization. The gene expression arrays we used in this study did not include the NGFI-A gene. Thus, we were not able to determine if short pulses of depolarization triggered an amplified response of the vgf promoter. Comparing the last time point measured after either a single or a double pulse in Figure 15, it does appear that double stimulation leads to stronger activation of the vgf promoter. While we did not make enough measurements to make statistically significant conclusions, the model of sustained activity of CREB would suggest that multiple depolarizing events would trigger the amplified expression of the vgf gene, while a single stimulus would not.

4.4.4 Depolarization Induces Expression of Another Pro-differentiation Factor, MAD1

MAD1 is a protein involved in the switch between proliferation and differentiation in several cell types. In our gene expression studies, we observed that MAD1 mRNA levels increased starting 3 hours after a single or the second of two depolarizations, and continued to increase for up to 8 hours after stimulation (shown in Figure 16; p<0.05). The peak increase in mad1 expression was 3.5-fold versus unstimulated PC12 cells. To our knowledge, this is the first report that depolarization induces expression of the mad1 gene.
Figure 16. Induction of MAD1 transcription following one or two pulses of KCl. PC12 cultures were stimulated with either one (filled circles) or two pulses (open circles) of KCl. MAD1 mRNA increased significantly (p<0.05) starting three hours after depolarization, and was maintained at an elevated level for the next five hours. The peak increase in MAD1 mRNA was 3.5-fold, occurring 8 hours after double stimulation.

The physiological role and mechanism of action of MAD1 is currently being uncovered. MAD1 belongs to the MAD family of bHLH-Zip transcription factors. These proteins were identified by their ability to bind the transcription factor MAX ((Ayer et al., 1993), (Hurlin et al., 1995)). In this function, the Mad proteins disrupt the ability of c-Myc and other Myc family members to bind MAX and induce transcription (reviewed in (Foley and Eisenman, 1999)). Thus, Mad1 is an antagonist of c-Myc and promotes the switch between a proliferative phenotype and differentiation ((Queva et al., 1998), (McArthur et al., 2002), (Foley et al., 1998)).

The role of Mad1 in neuronal differentiation was described in vitro using the P19 teratocarcinoma cell line (Queva et al., 1998). P19 cells underwent differentiation in
vitro after treatment with retinoic acid (RA), during the course of which each Mad family member was expressed sequentially. Mad1 was the last of the Mad family to be expressed, approximately 7 to 9 days after addition of RA. Expression of Mad1 coincided with terminal differentiation of the cells into a post-mitotic neuronal phenotype. Similarly, Mad1 was upregulated in post-mitotic cells in both embryonic and adult rats in vivo ((Queva et al., 1998), (Hurlin et al., 1995)). The fact that Mad1 expression increased after we stimulated PC12 cells with KCl indicates that a program for cell cycle exit may be triggered by depolarization. Taken together, the increased expression of Mad1 and VGF both suggest that PC12 cells shifted towards a differentiating phenotype in the 8 hours following short periods of depolarization.

4.4.5 Depolarization Induces Expression of Neurotrophin-Signaling Specific Kinase, MEK5

The Mitogen-Activated Protein (MAP) kinases play a central role in transducing signals originating from neurotrophins and neuronal activity in neurons. A recently identified member of this family, ERK5, is the only member so far observed to mediate signals only from neurotrophins, and not from neuronal activity (Cavanaugh et al., 2001). Our studies with gene expression arrays showed that expression of an up-stream activator of ERK5, MEK5, is up-regulated following short depolarizing stimuli. As shown in Figure 17, MEK5 mRNA levels began to increase 3 hours after a single or the second of two pulses of depolarizing KCl, and reach significant levels of up-regulation 4 hours after depolarization (p < 0.01). MEK5 mRNA levels are between 2- and 5-fold higher than in unstimulated cultures over the time period of 4 to 8 hours after stimulation. To our knowledge, this is the first report that expression of the mek5 gene is regulated by electrical activity or any other stimulus.
Figure 17. Induction of MEK5 transcription following one or two pulses of KCl. PC12 cultures were stimulated with either one (filled circles) or two pulses (open circles) of KCl. MEK5 mRNA increased significantly (p<0.01) starting three hours after depolarization, and stayed an average of 3-fold higher than in unstimulated cells for the next four hours.

The MEK5 protein and its downstream kinase ERK5, were discovered only recently (Zhou et al., 1995); (English et al., 1995). They were found to be present in many tissues, including strongly expressed in brain and skeletal muscle. Subsequent studies of ERK5 found that in neurons and PC12 cells, this kinase was specifically involved in signaling by neurotrophins (Cavanaugh et al., 2001). Furthermore, ERK5 was shown to be involved in the retrograde trafficking of NGF/TrkA receptor complexes from synapses to the cell soma, and appeared to remain active for up to 24 hours after addition of NGF to PC12 cells (Shao et al., 2002). The neuro-protective role of ERK5 was also described recently (Suzaki et al., 2002). It was observed that ERK5 is activated in response to hydrogen peroxide treatment of PC12 cells, and inhibition of ERK5 activity led to
increased apoptotic cell death. In muscle cells, it has been shown that activation of ERK5 is involved in differentiation of myoblasts into myocytes (Dinev et al., 2001).

Our observation of increased MEK5 mRNA after depolarization is interesting in light of the current understanding of MEK5’s and ERK5’s roles in signal transduction. MEK5 transcription may have been induced in an attempt to increase resistance to apoptosis and excitotoxicity, given ERK5’s role in protecting against oxygen radicals (Suzaki et al., 2002). Alternatively, there might exist a feedback between depolarization and neurotrophin signaling during differentiation of PC12 cells. If short depolarizing pulses stimulate PC12 cells into a differentiation pathway, as suggested by the observations of VGF and MAD1 expression, then an increase in MEK5 levels might presage an increase in neurotrophin (in particular, NGF) signaling. During neural development, target-derived neurotrophins induce axons to form proper connections, and electrical activity helps to stabilize these connections (reviewed in (Marty et al., 1997), (Ghosh and Greenberg, 1995)). Thus, the induction of MEK5 might represent one regulatory element of this process.

4.4.6 Prostaglandin E2 Receptor mRNA Increases Following Depolarization

The prostaglandins are a family of local hormones that have a variety of physiological effects, including control of ion transport across plasma membranes and modulation of synaptic transmission. We observed that a receptor for a member of the prostaglandin family, the prostaglandin E2 receptor (PGE2R), was up-regulated following depolarization of PC12 cells. As shown in Figure 18, starting 4 hours after stimulation, PGE2R mRNA increased to on average 2-fold higher levels than in unstimulated cells (p<0.01). The increase was equivalent in double- or single-stimulated cultures. To our knowledge, this was the first observation of regulation of PGE2R by electrical activity in PC12 cells.
Figure 18. Prostaglandin E2 receptor mRNA increases following one or two pulses of KCl. PC12 cultures were stimulated with either one (filled circles) or two pulses (open circles) of KCl. Transcription of prostaglandin E2 receptor increased significantly (p<0.01) starting four hours after depolarization, and stayed an average of 2.5-fold higher than in unstimulated cells for the next four hours.

Previous studies have shown that prostaglandin E2 signaling in PC12 cells increased cAMP levels (Gusovsky et al., 1989), and that the prostaglandins modulated ion-channels, neurotransmitter production and synapse function in PC12 cells (Li et al., 1994), (Nakamura et al., 1998)). The involvement of prostaglandins in differentiation of PC12 cells has also been described (DeGeorge et al., 1988). Stimulation of PC12 cells with NGF rapidly induced increased the rate of metabolism of arachidonic acid (AA, the metabolic precursor of prostaglandin) and the production of prostaglandin E. This increase in AA metabolism occurred prior to neurite outgrowth. However, inhibition of AA metabolism blocked NGF-induced neurite outgrowth. Also, stimulation of PC12 cells with NGF rapidly increased cyclooxygenase-1 (COX-1) levels (Kaplan et al., 1997). This latter enzyme synthesizes prostaglandin H2 from AA. Thus, our observation that
PGE2R was up-regulated in response to electrical stimulation may be related to the increase in prostaglandin synthesis when PC12 cells begin a program of neuronal differentiation.

4.4.7 Brain Gastrin B Receptor mRNA Increases After Depolarization of PC12 Cells

Gastrin, also known as cholecystokinin (CCK), is a peptide that was initially identified in the gastrointestinal tract, but has been shown to be widely expressed in the mammalian brain (reviewed in (Noble and Roques, 1999)). We observed significant increases in brain gastrin B receptor (CCKB-R) mRNA, starting 4 hours after stimulation of PC12 cells (p<0.001). As seen in Figure 19, CCKB-R mRNA increased between 2- and 3-fold, with no statistically significant differences between a single- or double-pulse of KCl. To our knowledge, this was the first observation of CCKB-R regulation by electrical activity.
Figure 19. Brain gastrin receptor mRNA increases following one or two pulses of KCl. PC12 cultures were stimulated with either one (filled circles) or two pulses (open circles) of KCl. Levels of messenger RNA for brain gastrin receptor increased significantly (p<0.001) starting four hours after depolarization, and stayed an average of 2.5-fold higher than in unstimulated cells for the next four hours.

Studies of CCB-R have shown that it is widely expressed in the rat brain (Honda et al., 1993). The receptor is a G-coupled receptor that can putatively activate phospholipase C (PLC) and protein kinase C (PKC) mediated signaling, can induce the formation of inositol trisphosphate (IP₃) and diacylglycerol (DAG) signaling molecules, and can stimulate Ca²⁺ release from intracellular stores (reviewed in (Noble and Roques, 1999)). The molecular role of CCKB-R in neurons is poorly understood. However, studies with agonists and antagonists have shown that disrupting gastrin signaling through the CCKB-R can block anxiety attacks in humans, and agonizing the receptor can induce anxiety (reviewed in (Bourin et al., 1996)), increase sensitivity to pain (Verge et al., 1993), and can perturb memory function (Dauge et al., 1992). Based on current understandings of gastrin signaling through CCKB-R receptors, we could not hypothesize a mechanism for up-regulation of gastrin receptor mRNA following depolarization of PC12 cells.

4.4.8 SHP2, the SH-2 Containing Tyrosine Phosphatases, Is Down-Regulated Following Depolarization

The tyrosine phosphatase, SHP2, is involved in neurotrophin signaling in neurons. As shown in Figure 20, SHP2 mRNA briefly increased following depolarization (1.5-fold increase relative to unstimulated cultures, p<0.1), and after a 3 hour period, dropped rapidly to 4-fold lower levels than unstimulated controls (p<0.01). The response of the SHP2 gene was similar after one or two pulses of KCl. To our knowledge, this was the first observation of the regulation of the SHP2 gene by depolarization.
Figure 20. Messenger RNA for SHP2 increases briefly, then drops following one or two pulses of KCl. PC12 cultures were stimulated with either one (filled circles) or two pulses (open circles) of KCl. Levels of messenger RNA for SHP2 increased 1.5-fold (p<0.1) in the hour following stimulation, then dropped significantly (p<0.01) starting four hours after depolarization. From four to eight hours after stimulation, mRNA for SHP2 was on average 4-fold lower than in unstimulated cells.

Expression of the tyrosine phosphatase SHP2 has been observed in all tissues and cell lines investigated so far, and it is strongly expressed in the brain, heart and skeletal muscle (reviewed in (Stein-Gerlach et al., 1998)). The protein is a phosphatase containing two src homology 2 (SH2) domains that allow it to dimerize with other proteins that contain phosphorylated tyrosine residues. SHP2 has been shown to be activated after stimulation by EGF, platelet-derived growth factor (PDGF, (Feng and Pawson, 1993)), and brain-derived neurotrophic factor (BDNF, (Yamada et al., 1999)), and to be involved in neurotrophin receptor signaling (Okado et al., 1996). Phosphatase activity of SH2 is required for activation of signal transduction pathways downstream of the receptor tyrosine kinases to which it binds (Hadari et al., 1998). We have not found
any reports that indicate that depolarization or Ca\(^{2+}\) influx stimulate the activity of SHP2 in neurons. Thus, although SHP2 plays an essential role in signal transduction in neurons, we could not hypothesize a mechanistic link between depolarization and the down-regulation of the syp/shp2 gene that was observed in our studies.

4.4.9 Nuclear Factor κB (NF-κB) mRNA Decreases After Depolarization

The transcription factor NF-κB is an important mediator of neuronal survival and plasticity. We observed a decrease in NF-κB mRNA levels following a single- or double-pulse of KCl (p<0.001). As shown in Figure 21, NF-κB mRNA levels dropped by more than a factor of 2, starting 4 hours after stimulation, and the response was the same after one or two stimuli. To our knowledge, this was the first observation of the regulation of NF-κB transcription by depolarization.

![Diagram showing mRNA levels over time after stimulation](image)

**Figure 21.** Messenger RNA for NF-κB increases briefly, then drops following one or two pulses of KCl. PC12 cultures were stimulated with either one (filled circles) or two pulses (open circles) of KCl. Levels of messenger RNA for NF-κB decreased sharply (p<0.001) starting four hours...
after depolarization. From four to eight hours after stimulation, mRNA for NF-κB was on average 3-fold lower than in unstimulated cells.

NF-κB was originally discovered as promoting immunoglobulin expression in B lymphocytes (Sen and Baltimore, 1986), but since has been shown to play an important role in neuronal survival and plasticity (Foehr et al., 2000), reviewed in (Mattson et al., 2000)). In PC12 cells, apoptosis was induced when the activity of NF-κB was inhibited (Taglialatela et al., 1997), and in sympathetic neurons, NGF-dependent survival was mediated by NF-κB activation (Maggirwar et al., 1998). The anti-apoptotic stimulus provided by NGF is transduced specifically through the low-affinity p75NTR receptor, as activation of NF-κB as a result of p75 stimulation prevented apoptosis in PC12 cells (Foehr et al., 2000). Surprisingly, activation of NF-κB by the tyrosine kinase TrkA receptor after NGF stimulation was required for neurite outgrowth in these cells. Thus, in PC12 cells, NF-κB promoted both neuronal survival and differentiation through independent signaling pathways. The role of NF-κB in protecting against neuronal death has been confirmed in vivo (Yu et al., 1999). Also, the involvement of NF-κB during long-term potentiation (LTP, a process hypothesized to be central in learning and memory) was observed in hippocampal slice preparations and in vivo ((Albensi and Mattson, 2000), (Meberg et al., 1996)). Thus, considerable evidence suggests that NF-κB activation has a role in both neuronal survival and plasticity.

NF-κB normally resides in the cytoplasm, and activation of the transcription factor involves its translocation into the nucleus (Mattson et al., 2000). Translocation of NF-κB is normally inhibited by a regulatory subunit, IκBα. Inhibition is relieved when IκBα is phosphorylated in response to an activating stimulus. Activators include TNF, which binds to the TNF receptor (p55) and induces phosphorylation of IκBα. Elevated intracellular Ca²⁺, either released from intracellular stores or entering the cell through glutamate or voltage-gated channels, have been shown to phosphorylate IκBα ((Mattson et al., 2000), (Aizman et al., 2001), (Wellmann et al., 2001)). Interestingly, it was shown in both T-lymphocytes and renal epithelial cells, that oscillations in cytoplasmic Ca²⁺ can enhance activation of NF-κB ((Aizman et al., 2001), (Dolmetsch et al., 1998)). Although we did not measure the level of NF-κB activation in the present study, based on
previous research, it is likely that the transcription factor had been activated after the depolarizing pulses, due to the increases in cytoplasmic Ca\textsuperscript{2+}.

Regulation of the gene for NF-κB has not been extensively studied. Thus, we cannot hypothesize why mRNA levels for NF-κB decreased in the wake of depolarization. Whether NF-κB is under negative feedback from its own activation, or decreases in PC12 cells after depolarization, remains to be determined. Given the role of NF-κB in cell survival, however, it would be predicted from our results that PC12 cells stimulated with short pulses of KCl would be less resistant to pro-apoptotic stimuli.

4.4.10 Depolarization Induces Down-Regulation of Various Unrelated Genes

Analysis of the gene expression data uncovered several other genes that were down-regulated at the level of transcription following depolarization. As shown in Figure 22, mRNA for aquaporin 3, organic cation transporter 1 (OCT1) and the chemokine receptor LCR-1, had decreased by over a factor of 2 by eight hours after stimulation (p<0.01 in all three cases). Transcription of aquaporin 3 and OCT1 responded equivalently (to within experimental variation), as mRNA for both proteins decreased starting 4 hours after either a single or a double pulse. Interestingly, the LCR-1 gene showed a differential response to the pattern of stimulation. LCR-1 mRNA decreased sometime between 1 and 3 hours following a single pulse of depolarization. When pulsed twice, the mRNA levels for LCR-1 had already decreased 2-fold by the time of the first measurement, 1 hour after the second pulse. Thus, the decrease in LCR-1 transcription resulting from the first exposure to KCl had already occurred in this latter case.
A. Aquaporin 3 expression

B. Organic cation transporter OCT1A expression
Figure 22. Down regulation of three un-related genes occurs following depolarization. A. Expression of Aquaporin 3 water channel decreases starting 4 hours after depolarization with KCl (p<0.001). From 4 to 8 hours after stimulation, Aquaporin 3 mRNA is on average 3-fold less abundant than in reference cells. B. Expression of organic cation transporter 1 decreases starting 4 hours after depolarization with KCl (p<0.001). C. LCR-1 mRNA decreases starting 3 hours after a single depolarization and 1 hour after a double pulse of KCl (p<0.05).

Previous research does not indicate any connection between these three genes and depolarization of PC12 cells. OCT1 is a non-neuronal monoamine transporter with strong expression in the liver, kidney and intestine (Grundemann et al., 1999). Its association with neuronal tissue has not been established. Aquaporin-3 is a water transporter. The related protein, aquaporin-4, has been localized to the brain and the optic nerve (Wen et al., 1999). However, aquaporin-3 has only been localized to the basolateral membrane of renal collecting duct cells (Connolly et al., 1998). Lastly, LCR-1 has not been characterized in neuronal cells yet. Based on previous studies of these proteins, we could not propose a mechanism for regulation of these genes by depolarizing stimuli in PC12 cells.
4.4.11 GABA Receptor δ-subunit is Preferentially Induced by Two Pulses of Depolarization

In addition to identifying genes that were regulated by electrical stimulation, we analyzed the expression data for genes that responded differentially to one pulse versus two. The time series of gene expression measurements following one pulse was compared to the time series following two pulses of KCl. Due to the noise in gene expression measurements, in only one case were the two time series significantly different, namely, the delta subunit of the gamma amino butyric acid receptor (GABA(A)r-δ, p<0.01). As shown in Figure 23, mRNA levels for GABA(A)r-δ dropped relative to the level in unstimulated cells following a single pulse of depolarization, by approximately a factor of two 4 hours after stimulation. Conversely, by one hour after a second pulse of KCl, the levels of mRNA for GABA(A) receptor had rose to greater than 1.5-times the level in unstimulated cells, and mRNA levels were maintained at this elevated level for the next eight hours. Thus, the GABA(A)r-δ gene responds differentially to repeated electrical stimulation.

![Figure 23. Expression of GABA(A)-r receptor is responds differentially to one pulse of stimulation versus two. Single pulse leads to a slight decrease in](image)

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GABA(A)- receptor mRNA over the eight hours following stimulation. Conversely, mRNA for GABA(A)- receptor increases following a second pulse of depolarization. Response to two stimuli is different (p<0.01).

The GABA(A) receptor is a ligand-gated Cl⁻ channel that is found in a variety of cell types in both neuronal and non-neuronal cells in the central and peripheral nervous systems ((Bormann and Clapham, 1985), (Bormann and Kettenmann, 1988), (Olsen and Tobin, 1990)). Numerous subunits of GABA(A)-r have been indentified, and these subunits combine in heteromers to form functional GABA(A)-r channels. In a study of 13 cell lines, the delta subunit of GABA(A)-r was found to be expressed in PC12 cells, as well as in the neuroblastoma cell line NB41A3, and the endocrine pancreatic cell lines RINm5F and βTC3 (Tyndale et al., 1994). Naïve PC12 cells were also found to express the β3 and γL&S subunits of GABA(A)-r. Interestingly, it has been show that while PC12 cells express mRNA for several subunits of GABA(A)-r, they do not contain functional GABA receptor channels (Hales and Tyndale, 1994). Thus, while our present results indicate that at least one subunit of GABA(A)-r can be regulated by repetitive electrical stimulation, this observation does not have electrophysiological significance. It will be interesting to determine, however, if the expression of other neurotransmitter channels can also be regulated by different protocols of depolarization.

4.4.12 Minimal Response of Immediate-Early Genes to Depolarization

Interestingly, we did not identify any immediate early genes (IEG's) that were strongly induced by depolarization. Of the five genes that were up-regulated, expression levels increased between 2 and 4 hours after stimulation, and none have been identified as IEG's (as discussed above). It has been previously shown that depolarization induces expression of several IEG's, including the transcription factors c-fos, jun-B, NGFI-A (zif 268) and NGFI-B (nur 77); and neuronal proteins arg3.1/arc and BDNF ((Ensen and Soderling, 1994), (Bartel et al., 1989), (Waltereit et al., 2001)). Of these genes, c-fos, nur77 and jun-B were all spotted on the Atlas Rat Expression membrane we used.

Both jun-B and nur77 had undetectable mRNA levels in unstimulated cultures (shown in Figure 24), consistent with previous reports (Bartel et al., 1989). Expression of nur77 did rise above background 1 hour after both a single and a double pulse of
depolarization (see Figure 24, panel B). However, the increase in mRNA level was not very large in the two cases, and mRNA levels had fallen back to undetectable levels by 3 hours after stimulation. The transient and relatively weak response of nur77 to KCl depolarization we observed was consistent with the previous results (Bartel et al., 1989). In contrast, we did not observe any increase in jun-B mRNA levels, which contradicts previous observations (Bartel et al., 1989). Interestingly, mRNA for the transcription factor Ear-3 were undetectable in unstimulated cultures, and similar to nur77, expression of Ear-3 increased briefly after stimulation (compare panels A and B, Figure 24). However, elevated levels of Ear-3 mRNA were only observed one hour after a single or double pulse of depolarization (n=4), and had dropped back to baseline levels 3 hours after stimulation.

Figure 24. Expression of immediate-early genes in reference and stimulated cultures of PC12 cells. A. Radioactive signal from spots of five IEG’s in unstimulated cultures of PC12 cells. Messenger RNA for c-fos and c-jun were present in high levels (note strength of spots relative to other spots on array), while mRNA for junB, nur77 and Ear-3 were present in low amounts. B. One hour following depolarization with KCl, hybridization signal from Ear-3 and nur77 increased slightly, indicating slight increases in levels of mRNA for the two genes.
In contrast to nur77 and jun-B, we observed that mRNA for the transcription factors c-fos and c-jun were abundant in unstimulated cultures (shown in Figure 24, panel A), contradicting previously published results that unstimulated PC12 cells had low levels of these mRNA (Bartel et al., 1989). Furthermore, we did not observe up-regulation of c-fos transcription following depolarization, also in contradiction of previous results ((Sheng et al., 1988), (Bartel et al., 1989)). Since transcriptional activity of the c-fos gene was aberrantly strong in our unstimulated cultures, it is possible that depolarization was not able to increase upon this activity, and thus we observed a false negative. The strong level of activity of the c-fos and c-jun genes may be due to one of several reasons. It is possible that these genes had been incorrectly spotted on the Atlas Array, or that the spotted sequences bound some other mRNA species non-specifically. Also, it may be that over the 10 passages of our cultures, we selected sub-clones of PC12 cells that had constitutively active c-fos and c-jun. Lastly, cells used in our experiments were passaged 24 hours prior to stimulation and RNA isolation. At the time of passage, the cells were fed with fresh media containing 15% serum. It is possible that these IEG’s were still active as a result of the re-plating and addition of fresh serum 24 hours earlier.

The induction of fos and jun by serum addition to cultured cells has been well-established ((Lamph et al., 1988), (Cohen and Curran, 1988)). These studies have shown that the induction of IEG’s by serum is transient (on the timescale of hours). However, it is possible that in our system, transcriptional activity of IEG’s was still maintained 24 hours later. In the studies cited above IEG expression was induced by depolarization, cells were maintained in serum-free media for a day or longer prior to stimulation ((Enslen and Soderling, 1994), (Suzaki et al., 2002)), which acts to both synchronize the population and to quiet expression of IEG’s. Alternatively, cells had been in serum-containing media for two days or longer prior to stimulation ((Bartel et al., 1989), (Sheng et al., 1988), (Waltereit et al., 2001)), which again leads to shut-off of IEG expression. Thus, it is likely that we did not observe up-regulation of IEG’s in response to depolarization because our cells were unsynchronized and had been fed serum soon before the start of the experiments.
4.5 CONCLUSIONS

Building upon previous studies of immediate-early and delayed-response genes, we wanted to study the expression of a diverse set of genes in the eight hours following depolarization. This time period captures the transition from immediate-early to delayed-response genes. We stimulated PC12 cells with either one or two pulses of depolarizing KCl, and then analyzed gene expression in the cells at 1, 3, 4, 6 and 8 hours following the stimuli using gene expression arrays. We identified five genes that were up-regulated following depolarization: namely, the genes for VGF, MEK5, MAD1, prostaglandin E2 receptor, and brain gastrin receptor. Previous to the current study, only the first of these, VGF protein, was shown to be regulated by depolarization. Furthermore, we identified five genes whose expression level decreased following depolarization. Two of these genes, the tyrosine phosphatase SHP2 and the transcription factor NF-κB, are expressed in neurons and neuronal tissue. The three other genes, organic cation transporter 1, aquaporin 3 and the chemokine receptor LCR-1, do not appear to have any specific relation to neuronal tissue. Lastly, we observed that the δ subunit of the γ-aminobutyric acid type A (GABA_A) receptor was regulated differentially by one pulse of stimulation versus two. Thus, we have identified a wide variety of genes that respond to short periods of depolarization.

REFERENCES


CHAPTER 5.  GENE EXPRESSION CHANGES FOLLOWING LONG-TERM DEPOLARIZATION OF PC12 CELLS

5.1 SUMMARY

Cells respond to numerous stimuli with a sequential pattern of physiological and gene expression changes. Days after the stimulus, cells will undergo phenotypic changes that are accompanied by changes in gene expression. In the previous chapter, we had identified a group of genes that were regulated by depolarization during the 8 hours following stimulation. In this chapter, we were interested to know whether long-term depolarization of PC12 cells will induce a different set of genes to be expressed. We cultured PC12 cells in media that contained depolarizing concentrations of KCl, and measured the expression level of 588 known genes. The gene expression resulting from this depolarizing media was compared to gene expression changes induced by hypertonic, non-depolarizing media, and to gene expression in unstimulated cells. We identified seven genes that were specifically induced by long-term depolarization: the nuclear tyrosine phosphatase, PRL-1; clusterin; synapsin II; cyclin D1; 40S ribosomal protein S12; 60S ribosomal protein L21; and the adipocyte fatty acid-binding protein. Expression of the first four of these genes has been found in previous studies to coincide with neuronal or glial differentiation or survival, and to our knowledge, none of these genes had been shown previously to be regulated by depolarization. We also identified ten diverse genes that were expressed after seven days of culture in hypertonic conditions. These included proteins involved in signal transduction, transmembrane ion transport, lipid metabolism proteins and cell adhesion. Finally, we identified a group of genes that were down-regulated following hypertonic culture conditions. Notably, the proliferating cell nuclear antigen (PCNA) was included in this group, and thus the decrease in PC12 growth rate that resulted from culturing in hypertonic conditions. Taken together, our results identified for the first time several groups of genes that were differentially regulated at the transcriptional level by long-term exposure to combinations of depolarization and hypertonic extracellular environment.
5.2 INTRODUCTION

Cells respond to a variety of chemical and non-chemical signals by initiating programs of gene expression that ultimately lead to changes in cell fate. While signal transduction cascades respond immediately to these stimuli, the progression of events may take days to weeks before noticeable phenotypic changes occur. For example, stimulation of unsynchronized, naïve PC12 cells with nerve growth factor (NGF) induces a program of neuronal differentiation in these cells (Greene et al., 1987). However, neurite outgrowth, one of the early phenotypic markers of the differentiation program, begins after a lag of 18 hours following addition of NGF to unsynchronized, unprimed PC12 cells (Greene et al., 1982). Only after 6 to 8 days of exposure to NGF do a majority of the cells bear neurites. Similarly, treatment of PC12 cells with epidermal growth factor stimulates a proliferative response in these cells (Huff et al., 1981). As is the case with NGF, the response to EGF is not observed for almost one week after addition of the compound.

While phenotypic changes occur on a slow time-scale, gene expressional changes begin almost immediately following stimulation. In the short-term, immediate-early and the first round of delay-response genes are expressed ((Bonni et al., 1995), reviewed in (Segal and Greenberg, 1996)). However, a whole new set of genes becomes induced in the long-term following a stimulus, as the cell changes to a new phenotype. In a recent study of gene expression in NGF-treated PC12 cells, the authors identified a large number of genes that were up- or down-regulated in the differentiated cells after 9 days of neurotrophin stimulation (Angelastro et al., 2000). None of the genes identified in this study were found to be regulated in the 4 hours following NGF stimulation ((Vician et al., 1997), (Segal and Greenberg, 1996)). Our results in the previous chapter, along with previous studies ((Nedivi et al., 1993), (Herschman et al., 2000)), identified numerous genes that were induced in the hours following depolarizing stimuli in neurons and neuron-like cells. However, in analogy to NGF-treated PC12 cells, it remains to be shown whether a new set of genes are induced in the long-term following depolarization.

Long-term stimulation of PC12 cells in culture does induce phenotypic changes in these cells. It was previously shown that chronic depolarization with KCl induced neurite
outgrowth, in the absence of other chemical agents (Hilborn et al., 1997). The program of differentiation induced by KCl was termed "morphological" to distinguish it from the "physiological" differentiation stimulated by NGF. PC12 cells cultured in depolarizing media adopted a flattened morphology, distinguishing them from the round, phase bright shape they have in the naive phenotype. In addition, the cells began to extend neurites, which in some cases formed junctions with neighboring cells. By 7 days of chronic depolarization, nearly 30% of cells expressed neurites. However, the length of these neurites was less than after stimulation with NGF. Furthermore, it was observed that depolarization had no effect on membrane conductance, while cells stimulated with NGF showed increases of up to 10-fold in Na\(^+\) and Ba\(^{2+}\) currents after 7 days (the latter is a measure of Ca\(^{2+}\) conductance). Thus, long-term depolarization of PC12 cells induced the morphological, early steps of the neurogenic differentiation program.

Other methods of electrically stimulating PC12 cells also induce phenotypic changes in these cells. Neurite outgrowth was promoted in PC12 cells grown on polypyrrole films and subjected to a constant voltage relative to the culture medium (Schmidt et al., 1997). In another study, PC12 cells grown between two platinum wires were exposed to 1 msec pulses of depolarization, delivered at 100 Hz in short bursts (Nakae, 1991). After 11 days, significant neurite outgrowth was observed in these cells. In two further studies, PC12 cells were grown on indium-tin oxide (ITO) electrodes and stimulated with 1 msec pulses of depolarization ((Kimura et al., 1998a), (Kimura et al., 1998b)). After 4 days, 25% of the cells had extended neurites, and a small increase in c-fos mRNA was detected in these cells. Thus, electrical stimulation of PC12 cells, by KCl-induced depolarization or via electric fields, can stimulate phenotypic changes in these cells.

In addition to acting alone, KCl-induced depolarization has been shown to enhance the action of some growth factors, and completely alter the action of others. In combination with NGF, long term chronic depolarization induced significantly greater neurite outgrowth than stimulation with NGF alone (Mark et al., 1995). Also, in combination with EGF, a significant number of PC12 cells exited the cell cycle and began a neurogenic program ((Mark et al., 1995), (Mark and Storm, 1997), (Hilborn et al., 1997)). This was in contrast to the normal action of EGF, which stimulated increased
proliferation in the absence of depolarization. The effect of chronic depolarization was found to be as a result of elevation of cAMP levels in the cytoplasm, and could be mediated in the absence of extracellular Ca$^{2+}$ (Mark et al., 1995). Thus, several lines of evidence support the conclusion that long-term depolarization of naïve PC12 cells can induce phenotypic changes in these cells.

We were interested to study changes in gene expression after long-term electrical stimulation that induced the phenotypic changes described above. Thus, we used naïve, unsynchronized PC12 cells, and depolarized them for 7 days with 70 mM KCl in the presence of serum factors. To measure the mRNA levels of a large number of genes simultaneously, we used commercially available gene expression arrays. Gene expression changes were measured relative to controls: unstimulated cells, and cells grown in hypertonic, non-depolarizing conditions. Previous research had shown that hypertonic conditions have pronounced effects on depolarization-induced gene expression (Kilbourne et al., 1991). Thus, we isolated genes whose expression was regulated solely by long-term membrane depolarization. A subset of these genes had significant connections to cell-fate decisions, such as differentiation and apoptosis.

5.3 MATERIALS AND METHODS

5.3.1 Cell Culture

Rat pheochromocytoma PC12 cells (ATCC) were cultured in complete Dulbecco's Modified Eagle's Medium (cDMEM), which consists of DME supplemented with 10% heat-inactivated horse serum (HS), 5% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin (all components from Gibco BRL). Cells were maintained in a humidified atmosphere of 5% CO$_2$ and 37 °C. Cells were plated at a density of 2 x 10$^4$ cells/cm$^2$ on tissue culture plates (Corning) coated with 0.1 mg/ml of poly-L-ornithine (PLO; Sigma: P3655) in water. Cells were fed cDMEM every 5 days and passaged every 10 days. Briefly, cells were detached after incubation in a 0.05% trypsin-EDTA solution (Gibco BRL) at 37 °C for 5 mins. Cell clumps were broken up with mild tituration or passage through a 20G needle and syringe, and reseeded at the density noted above. Cells were not used in experiments past passage number 20.
5.3.2 Long-Term Depolarization of PC12 Cells

For long-term stimulation experiments, cells were seeded 72 hours before experiments on 100mm Corning dishes pre-coated with PLO (as above) at a density of 1.5 x 10^4 cells/cm². Cells were maintained in a humidified atmosphere of 5% CO₂, at 37°C, in cDMEM until stimulation. Cells were depolarized by replacement of normal cDMEM with cDMEM that contained 70 mM KCl (final concentration, 420 mOsm, referred to as depolarizing cDMEM in the following). Cells were fed every 48 hours with a half volume of fresh depolarizing cDMEM. After seven days of chronic depolarization, total RNA was isolated from cultured cells as described below.

Two negative controls were used in the studies of gene expression changes. To account for the effect of the high osmolarity of the depolarizing cDMEM, parallel cultures of PC12 cells were treated with cDMEM containing an extra 65 mmols of NaCl per liter of solution (174 mM final concentration of NaCl, 420 mOsm, referred to as hypertonic cDMEM in the following). These cultures were fed every 48 hours with a half volume of hypertonic cDMEM. A second group of cells was fed with normal cDMEM (330 mOsm) every 48 hours, and thus were a control of unstimulated, unsynchronized, naïve PC12 cells. Total RNA was isolated after 7 days from both of the control-stimulated cultures as described below.

5.3.3 RNA Extraction and Purification

After seven days in either a depolarized, hyperosmotic or unsynchronized growth state, the cultures of PC12 cells were were lysed and RNA was isolated and purified. Total RNA from each sample was analyzed on an UV/Visible spectrophotometer to determine the concentration of RNA, and to confirm that the sample did not contain contaminating DNA. Electrophoresis of the RNA on agarose gels and ethidium bromide staining confirmed that RNA degradation had not occurred during extraction and purification. Protocols for all of these steps were described in Chapter 4.
5.3.4 Fabrication of Radio-Labeled cDNA Probes From Isolated RNA and Hybridization to Expression Arrays

Isolated total RNA from the three experimental conditions were used as templates to fabricate cDNA probes for hybridization with expression arrays using the Array Advantage product from Ambion. The protocol for fabrication of $^{33}$P-labeled probes was described in the Materials and Methods section of Chapter 4. Briefly, radio-labeled cDNA probes were purified on Nucleospin columns provided with the Atlas arrays (Clontech). Purified cDNA probes were eluted in nuclease-free water containing 10 mM EDTA. The radioactivity of each sample was measured with a scintillation counter to assess the success of the probe fabrication steps. Probes with total radioactivity less than $1.5 \times 10^6$ cmp were discarded and re-fabricated, and probes were hybridized to arrays within three days of fabrication due to the short half-life of $^{33}$P (25.4 days).

Gene expression levels were determined by hybridizing cDNA probes to commercially available hybridization arrays. Atlas Rat cDNA Expression Nylon Arrays, purchased from Clontech, were used in our study. Each array contained 588 cloned genes, spotted in duplicate. The arrays also contained alignment spots, and nine spotted genes were nominally designated as house-keeping genes.

Hybridization of radio-labeled probes to arrays was performed following the protocol described in Chapter 4. Briefly, arrays were prehybridized for greater than one hour at 50 °C in UltraHyb buffer (Ambion). Heat-denatured herring-sperm DNA was added to the arrays 10 minutes before heat-denatured, radio-labeled probes were added. Hybridization occurred overnight (16 to 18 hours) at 50 °C with gentle agitation. The next day, unhybridized probes were washed from the nylon membranes with two washes in Low Stringency Wash Buffer (LSWB, 0.3 M NaCl, 0.03 M Na$_2$Citrate·2H$_2$O, 1% SDS), followed by two washes with High Stringency Wash Buffer (HSWB, 0.075 M NaCl, 7.5 mM Na$_2$Citrate·2H$_2$O, 1% SDS). All washes were at 50 °C and lasted 40 minutes each.

After washing was completed, the arrays were wrapped and exposed to a phosphor screen. As described in Chapter 4, each array was carefully removed and wrapped with a single sheet of plastic wrap. A square of damp blotting paper was also wrapped with the
array to prevent drying out of the membrane. Air bubbles between the plastic wrap and the membrane surface were carefully eliminated, and the membranes were exposed to the phosphor screen for between 1 and 6 days at room temperature, depending on the radioactive strength of the probes (measured after probe fabrication). The phosphor screen was scanned at a resolution of 50 μm per step on a phosphor imager, and a digital image of the radioactive emission from each membrane was stored and processed as described below.

After imaging was complete, the nylon membranes were stripped and re-used for subsequent hybridizations, as described in Chapter 4. Briefly, the membranes were first stripped in a solution of 0.2% SDS at 75 °C for 30 to 45 minutes. Probes that were still hybridized to the array were degraded by exposure to Degradation Wash (Ambion) for 10 minutes at room temperature, and arrays were fully stripped by washing in a Reconstitution Solution (Ambion) at 68 °C for 15 minutes. The arrays were then wrapped and stored at −20 °C until further use.

5.3.5 Data Analysis

The scan of the phosphor screen following hybridization produced a digital image of the radioactive emission from each membrane. This digital image was analyzed with the Atlas Image 2.01 software to obtain expression levels for each of the spotted genes. The initial alignment of the image to the array template was performed as described in Chapter 4. The expression level of each gene spotted on the array was thus measured as the intensity of the corresponding spot on the digital image.

The gene expression profiles of PC12 cells in three different conditions were compared to each other to determine the effects of depolarization and hypertonic culture. For each culture condition, the gene expression profiles were measured in multiple cultures of cells: n=5 for chronic depolarization; n=6 for hypertonic; and n=4 for unstimulated. As a first step to perform the comparison, we determined the average expression ratio in each culture condition. Based on our studies of short-term gene expression, we assumed that global levels of transcription in different cultures at the same conditions should be the same, and that measurement variation would be due to intrinsic
and extrinsic noise (described and discussed in Chapter 4). As a result, we normalized
the gene expression level of a given gene on each membrane to the sum of the expression
level of all genes on that membrane (see (Kroll and Wolfl, 2002) and (Goryachev et al.,
2001) for reviews of normalization methods). After global sum normalization, we took
the average of the measurements of expression level for each gene in each of the three
culture conditions.

The second step of the data analysis was to compare the average expression profiles
in the three conditions to each other. Normalization was now complicated by the fact that
global gene expression might be different in the three culture conditions. For example, in
a proliferative state, overall gene expression might be higher than in growth-arrested
cells. Furthermore, some of the genes denominated as house-keeping genes by the array
manufacturers (Clontech) have been shown to be differentially expressed depending on
the phenotype of the cells. For example, polyubiquitin gene expression has been shown
to be regulated during development-induced cell death (Schwartz et al., 1990). In
addition, we observed increases in mRNA levels for another nominal house-keeping
gene, ornithine decarboxylase (Table 1, group II in 5.4). Ornithine decarboxylase gene
expression also increases dramatically following NGF stimulation (Volonte and Greene,
1990). We thus searched the literature for a house-keeping gene that had been previously
used to normalize expression measurements in PC12 cells. We identified GAPDH as a
commonly employed internal standard for expression levels ((Walteriet et al., 2001), (Tao
et al., 1998)). Thus, the average expression profiles in the three culture conditions were
compared to each other after being normalized to the GAPDH expression level in each
condition.

Comparison of the average profiles identified genes that were up- or down-regulated
in depolarized or hypertonic conditions relative to unstimulated cells. As a first screen,
we screened for all genes that were up or down regulated by 1.5-fold relative to
unstimulated cells. However, since the variance in expression measurements was not the
same for each gene, we performed a statistical analysis of the identified genes to
determine confidence intervals. For each selected gene, all 15 measurements of
normalized expression level were first compared using analysis of variance (ANOVA).
If a statistically significant difference between the three groups existed, at a cut-off of p<0.05, we next used a two-tailed Student’s t-test with unequal variance to compare the stimulated groups to the unstimulated group and determine the probability that the expression changes were due to a biological phenomenon, and not due to statistical variation. We reported all genes when the probability of statistical error was less than 5% (p<0.05).

5.4 RESULTS AND DISCUSSION

5.4.1 Comparison of Gene Expression Profiles After Long-Term Depolarization in Hypertonic Growth Conditions to Unstimulated PC12 Cells

Following seven days in either depolarizing conditions or hypertonic conditions, the expression level of a wide array of genes in PC12 cells was determined with gene expression arrays. The expression level of each of 588 genes in the two stimulation conditions was compared to the expression level of unstimulated cells. Figure 25 shows the comparison of depolarized cells to unstimulated cells. The majority of genes on the array had either undetectable expression levels (217 genes), or expression ratios within the interval of 1.5-fold up- or down-regulated (248). Of the remaining genes, 40 had expression ratios that were outside the interval of 0.67 to 1.5, and had expression levels above 10. While the actual value of the expression level is an arbitrary measurement, for the experiment of Figure 25, the region bounded by the x- and y- axes and the lines x=10 and y=10 contains genes where measurement noise is larger than expression level, as can be seen by the wide scatter of the points.

Of the 40 genes that remained for analysis, we next determined which were significantly up- or down-regulated after 7 days of chronic depolarization. Following the statistical analyses described in 5.3.5, we found 11 genes that were significantly up-regulated and 6 genes that were significantly down-regulated (shown as large, filled circles in Figure 25). Genes that were up-regulated are listed in Table 1, while those that were down-regulated are listed in Table 2.
Figure 25. Comparison of gene expression profiles of PC12 cells depolarized for 7 days and unstimulated PC12 cells. The average expression level of each gene (arbitrary units) is shown on a log-log scale for the two conditions: chronic depolarization for 7 days in 70 mM KCl; and unstimulated, unsynchronized, naïve cells. The expression level in KCl stimulated cells is the average of 5 measurements, while the unstimulated expression profile is the average of 4 measurements. The long-dashed line is the line $x=y$, while the two short-dashed lines demarcate the region 1.5-fold up- to 1.5-fold down-regulated. Large filled circles denote genes that fell outside this region and were regulated at a statistically significant difference in the two conditions ($p<0.05$).

To correct for the fact that the depolarizing conditions were hypertonic, we also analyzed the gene expression in cells that were maintained in hypertonic but non-depolarizing conditions. Figure 26 shows the comparison of the average expression profile of cells maintained in hypertonic cDMEM for seven days to the profile of cells in normal cDMEM. As in the comparison of depolarized cultures to unstimulated cells, the gene expression ratios and levels were put through a
series of statistical tests. We finally identified 11 genes that were significantly up-regulated, and 9 genes that were significantly down-regulated, depicted in Figure 26 as large, filled circles. The former group are listed in Table 1, and the latter group are in Table 2.

![Figure 26](image)

**Figure 26.** Comparison of gene expression profiles of PC12 cells maintained for 7 days in hypertonic conditions and unstimulated PC12 cells. The average expression level of each gene (arbitrary units) is shown on a log-log scale for the two conditions: hypertonic media for 7 days in 174 mM (final) NaCl; and unstimulated, unsynchronized, naïve cells. The expression level in NaCl-stimulated cells is the average of 6 measurements, while the unstimulated expression profile is the average of 4 measurements. The long-dashed line is the line x=y, while the two short-dashed lines demarcate the region 1.5-fold up- to 1.5-fold down-regulated. Large filled circles denote genes that fell outside this region and were regulated at a statistically significant difference in the two conditions (p<0.05).

The genes that were observed to be up-regulated in the two stimulated cases fell into three categories, as shown in Table 1. In group I, we listed genes which were up-regulated in response to chronic depolarization relative to both unstimulated and
hypertonic conditions. Of the 7 genes in group I, only one, the tyrosine phosphatase PRL-1, was up-regulated by hypertonic cDMEM relative to unstimulated cells (by a factor of 3, p<0.05). The mRNA levels for the other six genes showed no significant increase due to the hypertonic media. Thus, the increased expression of these genes was due only to the depolarizing action of the elevated KCl in the culture media, and not due to the high osmolarity of the solution. While at least one of the genes in group I showed a connection to depolarization in neurons, namely synapsin II, many of the others did not appear linked to neurons or depolarization. Thus, we reviewed the literature for each of these genes to determine the connection to our observations. The results of the literature review are discussed below.
Table 1. Genes which showed increases in mRNA levels following either chronic depolarization or hypertonic culture. In the column marked KCl:Unstim, mRNA levels of cells that were maintained in depolarizing (70 mM KCl) cDMEM for 7 days were compared to mRNA levels in unstimulated cells. In the column marked NaCl:Unstim, mRNA levels of cells that were maintained in hypertonic (174 mM NaCl) cDMEM for 7 days were compared to levels in unstimulated cells. All genes listed had significant up-regulation in one of the two stimulated conditions relative to unstimulated cells (p<0.05 cut-off). For genes in group I, mRNA levels in depolarizing conditions were significantly higher than hypertonic conditions (p<0.05). For genes in group II, mRNA levels in depolarizing and hypertonic conditions were not significantly different from each other. For genes in group III, mRNA levels in hypertonic conditions were significantly higher than in depolarizing conditions (p<0.05). The up-regulation of PKB by depolarizing media (bracketed) was at a statistical confidence of p<0.15, but was listed in group II rather than in group III due to the high expression ratio.

<table>
<thead>
<tr>
<th>Gene</th>
<th>KCl : Unstim</th>
<th>NaCl : Unstim</th>
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</thead>
<tbody>
<tr>
<td><strong>I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nuclear tyrosine phosphatase, PRL-1</td>
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<tr>
<td>clusterin (CLU or ApoJ)</td>
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<td>G1/S-specific cyclin D1 (CCND1)</td>
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</tr>
<tr>
<td><strong>II</strong></td>
<td>[3.7]</td>
<td>5.9</td>
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<tr>
<td>Protein kinase B (PKB or AKT1)</td>
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<td>ornithine decarboxylase (ODC)</td>
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<td>serine phospholipid-specific phospholipase A precursor (PS-PLA1)</td>
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<td>2.4</td>
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<td><strong>III</strong></td>
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<td>voltage-dependent P/Q-type calcium channel alpha-1A subunit (CACNA1A)</td>
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<td>cadherin 6 precursor (K-cadherin)</td>
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<td></td>
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</tbody>
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Genes that were up-regulated at the level of transcription in both stimulating conditions are listed in group II of Table 1. Since mRNA for these genes increased to approximately the same level in both depolarizing and hypertonic cDMEM, we could not conclude if these effects would occur in isotonic, depolarizing conditions. Furthermore, we observed that the growth rate of PC12 cells had decreased drastically after 2 to 3 days in depolarizing cDMEM or hypertonic cDMEM (data presented in Appendix). Thus, it is possible that the increased expression of the genes in group II was triggered by the cell’s exit from the cell cycle, regardless of whether this exit resulted from chronic depolarization or hypertonic conditions. Exit from the cell cycle exit is a required step in neuronal differentiation, and thus may predispose cells to a neuronal fate (reviewed in (Lu et al., 2000)).

The genes listed in group III of Table 1 were found to be up-regulated only after prolonged culture in hypertonic cDMEM. For example, the voltage-gated P/Q-type Ca$^{2+}$ channel was up-regulated more than 3-fold in solely hypertonic conditions, but was unaffected when cells were depolarized in hypertonic media. Thus, membrane depolarization must have counter-acted up-regulation of the genes in group III, perhaps through increased Ca$^{2+}$ influx through L-type voltage-gated channels. It would be interesting to determine if the repressive action of depolarization is by a common pathway for all of the genes in group III.

Comparison of the group II and group III genes highlights the complex effect of osmotic strength on the cellular response to membrane depolarization. Trans-membrane voltage and osmotic strength in some cases did not affect or agonized each other (group II genes), and in other cases antagonized each other (group III genes). Evidence of the complex interplay between depolarization and osmotic strength had been obtained in a previous study ((Kilbourne et al., 1991)). It was shown in PC12 cells that the tyrosine hydroxylase gene was differentially up-regulated during isotonic depolarization, but not during hypertonic depolarization. Meanwhile, mRNA for dopamine β hydroxylase decreased and actin mRNA levels increased in response to hypertonic depolarization. While we were able to identify genes that responded differentially to the action of the two
stimuli, we were not able to elucidate the mechanism of this differential regulation in the current studies.

In Table 2, we listed genes whose mRNA levels dropped following one week in depolarizing or hypertonic growth conditions. In all cases but one, the effects of depolarizing cDMEM and hypertonic cDMEM were similar and not significantly different from each other. Interestingly, there were no genes that were down-regulated by the sole action of membrane depolarization. However, mRNA levels for the transcription factor, COUPg, decreased in hypertonic conditions, but not in depolarizing, hypertonic media. Thus, while membrane depolarization did not induce down-regulation of any genes by itself, it did act to repress down-regulation of COUPg in response to elevated osmotic strength. Furthermore, down-regulation of proliferating cell nuclear antigen (PCNA) confirmed our observation that the growth rate of PC12 cells in both stimulation conditions had decreased greatly. The possible role of PCNA will be discussed below.

Table 2. Genes which showed decreases in mRNA levels following either chronic depolarization or hypertonic conditions. In the column marked KCl: Unstim, mRNA levels of cells that were maintained in depolarizing (70 mM KCl) cDMEM for 7 days were compared to mRNA levels in unstimulated cells. In the column marked NaCl: Unstim, mRNA levels of cells that were maintained in hypertonic (174 mM NaCl) cDMEM for 7 days were compared to levels in unstimulated cells. All genes listed had significant down-regulation in one of the two stimulated conditions relative to unstimulated cells (p<0.05). Only one gene, COUPg, was down-regulated by only one of the two stimuli, namely hypertonic conditions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>KCl : Unstim</th>
<th>NaCl : Unstim</th>
</tr>
</thead>
<tbody>
<tr>
<td>High mobility group protein 2 (HMG2)</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>signal transducer CD24 precursor; heat stable antigen (HSA); nectadrin</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Glut/Glut-R glutamate transporter</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>RAB-related GTP-binding protein</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>mast cell protease 7 precursor (RMCP-7)</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>cAMP-dependent 3',5'-cyclic phosphodiesterase 4 (DDPDE3)</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>endothelin 1 precursor (ET1)</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Ras-related protein m-Ras</td>
<td>0.6</td>
<td>0.6</td>
</tr>
</tbody>
</table>
5.4.2 Long-Term Depolarization Induces Expression of the Nuclear Protein-Tyrosine Phosphatase PRL-1

The nuclear tyrosine phosphatase, PRL-1, is an immediate-early gene that was first identified in mitogen stimulated cells and regenerating liver (Diamond et al., 1994). We observed that PRL-1 gene expression increased almost 10-fold following 7 days of chronic depolarization, and that expression increased 3-fold in cells that were maintained in a hypertonic, non-depolarizing medium. Thus, PRL-1 expression occurred to a greater part solely as the result of membrane depolarization.

The PRL-1 gene has been shown to be induced in the liver immediately following heptectomy, and in several cell lines in culture following mitogenic stimulation (Diamond et al., 1994), (Peng et al., 1999)). Thus, PRL-1 protein is postulated to control cell growth. PRL-1 mRNA has also been found widely distributed in the adult rat brain, in regions including the cerebral cortex, hippocampus and cerebellum (Takano et al., 1996). PRL-1 was also found expressed during neural development, as mRNA levels increased starting at embryonic day 15, and continued to be expressed at post-natal day 1 in the rat (Takano et al., 1996). Similarly, in mice, PRL-1 was expressed in the brain, neural tube and dorsal root ganglia between embryonic day 10 and embryonic day 18 (Rundle and Kappen, 1999). In a recent study of oligodendroglial differentiation, it was found that PRL-1 expression increased more than 3-fold as progenitor cells differentiated into primary immature oligodendroglial cells in culture (Scarlatto et al., 2000). Interestingly, PRL-1 expression was also induced following forebrain ischemia in the rat, peaking between 6 and 9 hours after injury. Thus, while the role of PRL-1 in neuronal tissue and cells still remains to be determined, increasing evidence points towards involvement of the tyrosine phosphatase with development, differentiation and regeneration of cells in these tissues. We hypothesize that induction of the gene in our
studies may have been linked to the neurite outgrowth and neurogenic phenotype of the PC12 cells following chronic depolarization.

5.4.3 Long-Term Depolarization Induces Expression of the Extracellular Glycoprotein Clusterin

The extracellular glycoprotein has been found expressed in a variety of tissues and in association with several cellular processes, including differentiation and cell death ((Jones and Jomary, 2002), (Klock et al., 1998), (French et al., 1994)). We observed in the current study that mRNA for Clusterin protein increased 3-fold after 7 days in depolarizing, hypertonic media, but did not increase in non-depolarizing hypertonic media (Table 1). Thus, sustained membrane depolarization was able to up-regulate expression of the clusterin gene.

Previous to our observations, up-regulation of clusterin had been observed in various situations of neural degeneration. For example, studies in albino rats showed that clusterin expression increased in the retina after retinal degeneration was induced by exposure to intense light (Wong et al., 2001). Clusterin was also found to be associated with neuronal degeneration in Alzheimer’s disease (Choi-Miura and Oda, 1996). However, research in several systems suggests that clusterin protein may not be a mediator of cell death, but rather may be expressed in neighboring cells as a means of protecting against apoptosis ((French et al., 1994), (Klock et al., 1998), (Schwochau et al., 1998)). In addition, clusterin was induced as a result of non-apoptotic stimuli in neuron-like cells. In PC12 cells, both epidermal growth factor (EGF) and nerve growth factor (NGF) were shown to stimulate expression of the clusterin gene (Gutacker et al., 1999). In these studies, it was observed that clusterin was expressed before neurite outgrowth occurred in NGF-treated PC12 cells. Also, the Ras/MAP kinase/AP-1 signaling pathway mediated the expression of clusterin after treatment with either EGF or NGF.

The expression of the clusterin gene that we observed in the current studies may have resulted from one of two processes. First, it has been well established that depolarization of PC12 cells activates the Ras/MAP kinase signaling cascade ((Rosen et al., 1994),
(Egea et al., 1999)), and that long-term depolarization leads to neurite outgrowth ((Hilborn et al., 1997), and results presented in Appendix). Thus, clusterin expression may have occurred after long-term depolarization in a similar fashion as after EGF or NGF treatment of PC12 cells. Second, expression of clusterin in our studies may have been due to the initiation of a program of cellular protection. Long-term culture of primary neurons, such as cerebellar granule cells, requires elevated KCl (25 mM) for survival of these cells (Miller and Johnson Jr., 1996). Thus, chronic depolarization may mediate enhanced cell survival, and clusterin expression may be a marker of this program.

5.4.4 Long-Term Depolarization Induces Expression of the Gene for Synapsin II

The synapsin II gene encodes two members of the synapsin family of phosphoproteins, Synapsin IIa and IIb. The synapsins are essential elements of synaptic structures in neurons, and play a role in neurite elongation, synaptogenesis and neurotransmitter release (reviewed in (Scarlato et al., 2000)). In the current study, we observed that expression of the synapsin II gene increased in response to sustained membrane depolarization (Table 1). Since synapsin II expression did not occur following a week of culture in hypertonic conditions, we concluded that the up-regulation of the gene was a result of chronic membrane depolarization.

Synapsin II has been shown to be essential for neurite elongation and axon formation ((Ferreira et al., 1994), (Ferreira et al., 1998)). In addition, disruption of synapsin II translation using antisense molecules blocked synapse formation in hippocampal neuron cultures, and led to decreases in several other synaptic vesicle proteins (Ferreira et al., 1995). Also, expression of synapsin IIb in neuroblastoma cells gave rise to presynaptic terminal structures (Han et al., 1991). Interestingly, expression of the synapsin II gene in fibroblasts led to the formation of synaptic architectures within these non-neuronal cells (Han and Greengard, 1994). Thus, expression of synapsin II is critical in the early stages of axonal formation and synaptogenesis. We hypothesized that the expression of this gene after chronic depolarization was linked to the neurite outgrowth we observed by phase-contrast microscopy. In a study of the human synapsin II gene promoter, it was observed that the promoter was regulated in part by the transcription factor zif268
(Petersohn et al., 1995). It has been previously shown that zif268 expression is induced by depolarization in PC12 cells (Bartel et al., 1989). Mechanistically, it is possible that expression of synapsin II by depolarization is mediated by zif268, and thus leads to a neuritogenic program in the cells.

5.4.5 Long-Term Depolarization Induces Expression of the Cyclin D1 Gene

Cyclin D1 protein helps regulate the transition from the G1 to S phase of the cell cycle. In particular, cyclin D1 forms a complex with the cyclin dependent kinases (cdk's) 4 and 6 and activates the transition from G1 to S phase by inhibiting the action of the retinoblastoma protein (Rb, reviewed in (Zhang, 1999)). We observed that expression of cyclin D1 increased 1.7-fold following 7 days in depolarizing media, but was unchanged in hypertonic media (Table 1). Thus, cyclin D1 expression increased as a result of a depolarization-sensitive pathway.

The increased expression of cyclin D1 that we observed may have occurred for one of two reasons. First, studies with PC12 cells have shown that following treatment with NGF, PC12 cells accumulated in the G1 phase and did not progress into S phase ((van Grunsven et al., 1996b), (van Grunsven et al., 1996a), (Yan and Ziff, 1995)). This block in cell cycle progression was accompanied by an increase in cyclin D1 expression, which was an unexpected finding, since it had been established that cyclin D1, in association with cdk4, inactivated Rb and induced the transition from G1 to S phase. The increase in cyclin D1 levels coincided with neurite outgrowth in NGF-treated PC12 cells (Tamaru et al., 1994). Also, over-expression of cyclin D1 from a vector induced G1-phase growth arrest of PC12 cells (Yan and Ziff, 1995). While cyclin D1 expression increased, it was observed that p21Cip1 levels increased as well (van Grunsven et al., 1996a). This latter protein is an inhibitor of cyclin D1/cdk4 complexes, and thus prevents progression of cells into S phase. Correspondingly, despite the rise in cyclin D1 levels, cyclin kinase activity actually decreased, and cells remained in G1 ((van Grunsven et al., 1996a), (Yan and Ziff, 1995)). Thus, it is possible that the expression of cyclin D1 that we observed in the current study resulted from the activation of the early steps of a neurogenic program that coincide with the growth arrest of the cells in G1. This hypothesis is consistent with
our observation that cell growth rate dropped dramatically following depolarization (presented and discussed in Appendix).

A second possible reason for the increase in cyclin D1 expression may be due to an increase in cell death. There is evidence linking cyclin D1 expression to programmed cell death in neurons (see (Bergeron and Yuan, 1998) for a review). In particular, it was shown in vivo that excitotoxic cell death, induced by kainic acid, resulted in expression of cyclin D1 and cdk4 in regions of neuronal apoptosis (Ino and Chiba, 2001). While we did not observe apoptosis in PC12 cells within the 8 hours after short-term depolarization, we did observe significant cell death and cell cycle arrest in long-term cultures in depolarizing conditions (see Appendix). Thus, it is possible that cyclin D1 expression is linked to excitotoxic cell death as a result of sustained membrane depolarization. Evidence against this latter explanation comes from the fact that cyclin D1 expression did not increase in hypertonic culture conditions, while we did observe similar levels of cell loss. Thus, we hypothesize that PC12 cells accumulate in the G1 phase following sustained depolarization, and concomitantly cyclin D1 expression is increased.

As shown in Table 1, three other genes not yet discussed were induced solely by long-term membrane depolarization (group I of Table 1). These genes encode the 40S ribosomal protein S12, the 60S ribosomal protein L21, and the adipocyte fatty acid binding protein (AFABP). We reviewed the literature and found that little information is available on these three genes and their connection to either membrane depolarization, neuronal tissue or development, or PC12 cells. Thus, we cannot hypothesize a mechanistic link between expression of these genes and long-term membrane depolarization.

5.4.6 Diverse Genes are Up-Regulated Following Long-Term Culture in Hypertonic Conditions

We observed that the expression of ten diverse genes increased when cells were cultured in hypertonic media (groups II and III in Table 1). Two of these genes, protein kinase B and protein kinase C-δ, encode signal transduction proteins and are expressed in all tissues and cells. Many studies have established the role of protein kinase B (PKB) in
transducing survival signals from a variety of chemical growth factors to the nucleus (reviewed in (Lawlor and Alessi, 2001)). Increased expression of PKB might have acted to protect the cells from increased cell death. However, there are no previous reports of the regulation of PKB by hypertonic or depolarizing conditions. Thus, we could not hypothesize on the functional roles of the increased expression of either of these proteins in hypertonic conditions.

Two genes encoding ion-channel proteins, namely the P/Q-type voltage-gated Ca^{2+} channel and the K⁺ channel protein, RK5, were found to be up-regulated following long-term hypertonic culture. Increased expression of the P/Q Ca^{2+} channel was only observed in hypertonic, non-depolarizing media. Thus, membrane depolarization, perhaps via Ca^{2+} influx, acted to repress expression of the Ca^{2+} channel. Previous reports of the regulation of P/Q type Ca^{2+} channels by depolarization in PC12 cells have not been reported. The second ion channel, RK5, was expressed in both depolarizing and non-depolarizing hypertonic media. RK5 has shown to be widely expressed in neurons and myocytes, but has not been previously reported in PC12 cells ((Roberds and Tamkun, 1991), (Klumpp et al., 1995)).

The prostaglandin E2 receptor gene was also up-regulated following hypertonic culture, but only in non-depolarizing conditions. Interestingly, we had observed increased expression of the gene for PGE2 receptor following short pulses of depolarization in isotonic conditions, as discussed in Chapter 4. Thus, our studies showed that PGE2 receptor expression increased in the short-term following isotonic depolarization, but was not expressed in the long-term following hypertonic depolarization. Prostaglandin biosynthesis has been shown to increase following NGF stimulation of PC12 cells, and prostaglandin E2 has been shown to affect neurotransmitter and ion channels in these cells ((DeGeorge et al., 1988), (Li et al., 1994), (Nakamura et al., 1998)). Based on the limited understanding of the PGE2 receptor, we cannot hypothesize on the mechanisms for regulation of this gene by hypertonic culture conditions.
Hypertonic culture in non-depolarizing conditions also led to the increase in expression of a cell adhesion molecule. The adhesion molecule cadherin 6 is a type II cadherin that has been associated with carcinomas of the liver (Shimoyama et al., 1995). However, expression of the cadherin 6 gene has also been observed in murine motoneurons and Schwann cells, presenting a possible link to its expression in PC12 cells in the current study (Padilla et al., 1998).

We observed that cathepsin E, a cytoplasmic aspartic proteinase that is involved in antigen processing and programmed cell death in neurons (reviewed in (Tsukuba et al., 2000)), was expressed following hypertonic culture. Of relevance to our present study is the observation that excitotoxicity in neurons has been shown to involve cathepsin E activity (Tominga et al., 1998). Thus, it is possible that the expression of cathepsin E increased as a result of hypertonic media-induced programmed cell death.

Three other genes were up-regulated following hypertonic culture in both depolarizing and non-depolarizing conditions. Ornithine decarboxylase (ODC) expression was shown to increase transiently following stimulation of PC12 cells with NGF ((Volonte and Greene, 1990), (Marschall and Feinstein, 1995)). In addition, Fos protein has been shown to induce expression of ODC (Wrighton and Busslinger, 1993). As discussed in Chapter 4, we measured high levels of c-Fos mRNA in all of our cultures, whether the cells were stimulated or not. It is possible that ODC expression was induced as a result of high levels of c-Fos expression. However, some other cofactor must be needed, since ODC was preferentially expressed in hypertonic media, while c-Fos expression remained unchanged. Interestingly, cells with sustained ODC activity were found to respond poorly to NGF and displayed other aberrant behavior (Marschall and Feinstein, 1995). Thus, our observation of increased ODC mRNA following hypertonic culture may be an indicator of the abnormal effects of prolonged exposure to hypertonic media. The two other genes, for serine-phospholipid phospholipase (PS-PLA1) and for lecithin:cholesterol acyl transferase (LCAT), are both involved in lipid metabolism. PS-PLA1 was first isolated in rat platelet cells, and has since been identified in several non-neural human tissues ((Sato et al., 1997), (Sonoda et al., 2002)). We could not find any reports in the literature showing a functional role for PS-PLA1 protein in
PC12 cells. Similarly, LCAT protein was only observed in one instance in cultures of cells from neuronal tissue ((Collet et al., 1999)). In this study, two human glioma cell lines, U343 and U251, were found to express and secrete functional LCAT enzyme. However, the role of the plasma lipid enzyme LCAT in these cells was not established. In summary, hypertonic culture conditions induced the expression of a diverse group of genes, some of which exhibited functional roles in neuronal survival and differentiation, and others that had not been previously described in PC12 or neuronal cells.

5.4.7 Proliferating Cell Nuclear Antigen (PCNA) Gene is Down-Regulated Following Depolarization and Hypertonic Culture

The proliferating cell nuclear antigen (PCNA) protein is essential for cell replication, as it plays a central role in DNA replication and repair, and interacts with cell-cycle regulatory proteins (reviewed in (Kelman, 1997)). We observed that following 7 days of culture in either hypertonic or depolarizing cDMEM, levels of PCNA mRNA dropped relative to unstimulated cells (Table 2). The decrease in PCNA expression was greater in purely hypertonic media (more than 2-fold) than in depolarizing, hypertonic media (1.3-fold decrease). Decreases in PCNA expression were consistent with our separate observations that cell growth rates were decreased in depolarizing and hypertonic media (see Appendix).

The PCNA protein was first discovered as specifically expressed in proliferating cells (Miyachi et al., 1978). Subsequently, it was observed that PCNA was elevated during the S phase of dividing cells (Bravo and Celis, 1980). Since the initial discoveries, the role of PCNA in DNA replication has been elucidated. PCNA forms a trimeric ring structure that surrounds double-stranded DNA and forms a sliding scaffold (Krishna et al., 1994). PCNA thus mediates the interactions of various proteins, including DNA polymerase complexes and DNA repair molecules, with the DNA (Kelman, 1997). Interestingly, PCNA also interacts with various cell-cycle regulatory proteins, including p21CIP1 (Gulbis et al., 1996). As a result of this interaction, p21CIP1 can competitively inhibit association of DNA polymerase complexes with PCNA, and thus block DNA replication (Podust et al., 1995). Given its essential role in DNA replication, the decreased expression of PCNA observed in both hypertonic and depolarizing media is consistent with our
simultaneous observations that cyclin D1 expression increased and cell growth rate decreased.

5.4.8 Diverse Genes are Down-Regulated Following Long-Term Culture in Hypertonic Conditions

In addition to PCNA, mRNA for numerous other genes also decreased following hypertonic culture, with or without depolarization. Two of these genes, for β-nerve growth factor and the glutamate transporter, GluT/GluTR, have roles in neuronal cells. Others, such as m-Ras and the RAB-related GTP binding protein, are elements of the signal transduction cascades. Only one of the genes identified, for the transcription factor ovalbumin, was differentially regulated in by the two stimuli. COUPg expression did not decrease after 7 days of hypertonic depolarization, but did decrease by a factor of 2 following one week in hypertonic, non-depolarizing media. Since all of the other genes were down-regulated to approximately the same degree in both conditions, we could not specifically identify any genes that responded to long-term chronic depolarization.

5.5 CONCLUSIONS

Cells respond to numerous stimuli with a sequential pattern of physiological and gene expression changes. Days after the stimulus, cells will undergo phenotypic changes that are accompanied by changes in gene expression. In the previous chapter, we had identified a group of genes that were regulated by depolarization during the 8 hours following stimulation. In this chapter, we were interested to know whether long-term depolarization of PC12 cells will induce a different set of genes to be expressed. We cultured PC12 cells in media that contained depolarizing concentrations of KCl, and measured the expression level of 588 known genes. The gene expression resulting from this depolarizing media was compared to gene expression changes induced by hypertonic, non-depolarizing media, and to gene expression in unstimulated cells. We identified seven genes that were specifically induced by long-term depolarization: the nuclear tyrosine phosphatase, PRL-1; clusterin; synapsin II; cyclin D1; 40S ribosomal protein S12; 60S ribosomal protein L21; and the adipocyte fatty acid-binding protein. Expression of the first four of these genes has been found in previous studies to coincide
with neuronal or glial differentiation or survival. To our knowledge, none of these genes had been shown previously to be regulated by depolarization. We also identified ten diverse genes that were expressed after seven days of culture in hypertonic conditions. These included the signal transduction proteins protein kinase B and protein kinase C-d, voltage-gated potassium and calcium ion channel proteins, lipid metabolism proteins and a cell adhesion molecule, amongst others. Finally, we identified a group of genes that were down-regulated following hypertonic culture conditions. With one exception, all of these genes were similarly regulated in depolarizing and non-depolarizing conditions. Notably, the proliferating cell nuclear antigen (PCNA) was included in this group, and thus underscored the decrease in PC12 growth rate following hypertonic culture conditions. Taken together, our results identified for the first time several groups of genes that were differentially regulated at the transcriptional level by long-term exposure to combinations of depolarization and hypertonic extracellular environment.

REFERENCES


CHAPTER 6. CONCLUSIONS

6.1 SUMMARY OF RESULTS

Electrical stimulation of neurons, as a result of Ca$^{2+}$ entry into the cytoplasm, induces many cellular changes that occur from minutes to hours or even days after stimulation. Previous research has uncovered many of the downstream effects of Ca$^{2+}$ influx, including the modulation of electrophysiological properties of the cells, changes in synaptic connections between neurons, enhanced survival of the cells, and in a few cases morphological differentiation of neuronal precursors. These results were reviewed in Chapter 2.

In Chapter 3 of the present study, we focused specifically on the transient nature of rises in cytoplasmic free Ca$^{2+}$ ($[\text{Ca}^{2+}]_{i}$). We characterized changes in $[\text{Ca}^{2+}]_{i}$ versus time during both chronic and pulsatile depolarization of the neuron-like PC12 cell line. During chronic depolarization, $[\text{Ca}^{2+}]_{i}$ peaked and subsequently fell off to intermediate values within 10 minutes, while short pulses of depolarizing KCl induced sharp rises in $[\text{Ca}^{2+}]_{i}$ which fell back to baseline within 30 seconds of repolarization. If cells were allowed sufficient time to recover, a second pulse of KCl induced another rise in $[\text{Ca}^{2+}]_{i}$. Mirroring the trends in $[\text{Ca}^{2+}]_{i}$, phosphorylation of the MAP kinases Erk1 and Erk2 was also transient, peaking and falling to low levels within ten minutes of depolarization. Short pulses of depolarization also successfully activated Erk1 and Erk2, and the rate of deactivation of the Erk's was not affected by the duration of depolarization. Phosphorylation of the transcription factor CREB also peaked as a result of chronic depolarization, and dropped to intermediate levels that were maintained for over 1 hour. We showed for the first time that both Erk1/2 and CREB phosphorylation could be re-induced by a second round of depolarization that followed a recovery period. The effects of the durations of depolarization and inter-pulse recovery on reactivation of Erk's and CREB were characterized. Recovery periods as short as ten minutes and pulse durations from 1 to 5 minutes in length could be combined to effectively re-stimulate signal transduction kinases and transcription factors. Thus, we concluded from our observations...
in Chapter 3 that pulsatile stimulation may be a means of maintaining signaling activity over long periods of time.

In Chapter 4, we focused our attention on the instructive effects of short pulses of depolarization. We measured the expression of a large set of diverse genes in the eight hours following depolarization. This time period captures the transition from immediately-early to delayed-response genes. We identified five genes that were up-regulated following depolarization: namely, the genes for VGF, MEK5, MAD1, prostaglandin E2 receptor, and brain gastrin receptor. Previous to the current study, only the first of these, VGF protein, was shown to be regulated by depolarization. Furthermore, we identified five genes whose expression level decreased following depolarization. Two of these genes, the tyrosine phosphatase SHP2 and the transcription factor NF-κB, are expressed in neurons and neuronal tissue. The three other genes, organic cation transporter 1, aquaporin 3 and the chemokine receptor LCR-1, do not appear to have any specific relation to neuronal tissue. Lastly, we observed that the δ subunit of the γ-aminobutyric acid type A (GABA<sub>A</sub>) receptor was regulated differentially by one pulse of stimulation versus two. Thus, we concluded from our studies in Chapter 4 that pulses of depolarization that last on the order of minutes can have instructive influences up to eight hours later.

In the final phase of our study, we were interested to know whether long-term depolarization of PC12 cells would induce a different set of genes to be expressed than over the short-term. We cultured PC12 cells in depolarizing, hypertonic media for seven days and compared gene expression in these cells to expression resulting from prolonged exposure to hypertonic, non-depolarizing media, and to gene expression in unstimulated cells. We identified seven genes that were specifically induced by long-term depolarization: the nuclear tyrosine phosphatase, PRL-1; clusterin; synapsin II; cyclin D1; 40S ribosomal protein S12; 60S ribosomal protein L21; and the adipocyte fatty acid-binding protein. Expression of the first four of these genes has been found in previous studies to coincide with neuronal or glial differentiation or survival, and to our knowledge, none of these genes had been shown previously to be regulated by depolarization. Furthermore, none of these genes were identified as being regulated by
short pulses of depolarization in Chapter 4. We also identified ten diverse genes that were expressed after seven days of culture in hypertonic conditions. These included proteins involved in signal transduction, transmembrane ion transport, lipid metabolism proteins and cell adhesion. Finally, we identified a group of genes that were down-regulated following hypertonic culture conditions. Notably, the proliferating cell nuclear antigen (PCNA) was included in this group, and thus the decrease in PC12 growth rate that resulted from culturing in hypertonic conditions. We concluded form our studies in Chapter 5 that prolonged depolarization or hypertonic growth induce a new set of diverse genes to be expressed, and that some of these genes play a role in cell cycle control and differentiation.

Taken together, our present studies have characterized the activity of intracellular signaling molecules following various protocols of depolarization in a neural precursor model system, and have shown that these depolarization patterns can confer instructions on the cells in both the short- and the long-term.

6.2 FUTURE DIRECTIONS

While our studies have characterized the effects of depolarization in a model of neuronal differentiation, much remains to be understood. First, while PC12 cells can differentiate in response to growth factors and, thus, are a powerful model system for neural differentiation, they are not primary neuronal precursors. We showed in Chapter 3 that short periods of intense depolarization that were separated by recovery periods could reactivate CREB. It remains to be determined what the kinetics of activation and deactivation of short bursts of depolarization are in vivo, and if pulsatile activation of signaling molecules and transcription factors can be reproduced in functional neuronal tissues.

The presence and importance of several types of Ca\(^{2+}\) transients have already been described in the developing nervous system ((Gu and Spitzer, 1995), (Spitzer et al., 1994)). For example, in spinal cord neurons, spontaneous activity precedes the formation of synapses, and Ca\(^{2+}\) transients serve an important role in the continued differentiation of these cells in vivo (Spitzer, 2002). Notably, these spontaneous Ca\(^{2+}\)
transients occur with a period of between 5 and 30 minutes (Gu and Spitzer, 1995). Thus, the recovery periods between pulses observed in these cells are on the same scale as what we observed to be necessary for faithful reactivation of the kinases in our studies. It will be interesting to determine if these endogenous Ca^{2+} transients occur in a pulsatile fashion to maintain activity of kinases and transcription factors over long periods of time, as we observed in our studies.

It will also be important to extend our studies to neural stem cells, and to determine if these cells can be induced to make particular phenotypic choices following various protocols of depolarization. In addition to the observations we and others have made with PC12 cells (see Appendix and (Hilborn et al., 1997)), it has also been shown that depolarization can induce differentiation of the neuronal cell line, RN46A, into serotonergic cells (White et al., 1994). Thus, membrane depolarization may be a general differentiating influence for a wide array of neuronal cell lines and neural stem cells. Also, RN46A cells can differentiate into either cholinergic or serotonergic cells, depending on the differentiating factors used (Rudge et al., 1996). As we showed in Chapter 4, GABA receptor expression was differentially induced following two pulses of depolarization, whereas expression was slightly depressed following one pulse. Perhaps different patterns of depolarization can also induce differential effects on RN46A cells, in particular with regards to the choice of a serotonergic versus cholinergic cell fate.

In addition to the GABA receptor, we identified numerous other genes that were regulated in the short and long-term following depolarization. As discussed in chapters 4 and 5, many of these genes had previously been identified during various instances of neuronal differentiation or survival. As the role of pulsatile depolarization and Ca^{2+} transients is studied in neural stem cells and in vivo, it will be important to understand which of the genes we identified behave similarly in these new systems, and whether their roles are causative or secondary.

Finally, we identified only one gene that was differentially regulated by one pulse versus two pulses in Chapter 4. However, the ability of neuronal cells to respond differentially to patterns of stimulation has been confirmed in other studies ((Bito et al.,
1996), (Sheng et al., 1993), (Yun et al., 2002)). We hypothesize that the presence of serum factors and the fact that we did not synchronize PC12 cells prior to stimulation in our studies served to decrease the coordinated expression of immediate-early genes in our studies and increased the noise of gene expression. Thus, to properly study the response to different patterns of stimulation, it will be important to synchronized PC12 cells, possibly by serum-starvation, prior to stimulation. Given that we have already characterized the temporal profiles of signaling activity following depolarization in these cells, unique patterns of stimulation can be designed, and their effects on gene expression can be monitored in these cells.

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APPENDIX

A.1 LONG-TERM DEPOLARIZATION OF PC12 CELLS: MORPHOLOGICAL EFFECTS

During long-term depolarization of PC12 cells, we observed that the cells underwent morphological phenotypic changes in depolarizing conditions, and to a lesser extent in hypertonic culture conditions. These changes were similar to the morphological differentiation observed by others (Hilborn et al., 1997). To characterize these observations in a quantitative manner, we used phase contrast light microscopy to observe the morphology of the cells grown for seven days in each of three conditions: hypertonic, depolarizing media; hypertonic, non-depolarizing media; and normal media. We analyzed the images quantitatively for two features: the number of cells extending neurites in each field of view, and the average length of the neurites in each field of view.

A.1.1 Methods

All culture methods were exactly as described in Chapter 5. Cells were seeded at a density of $1.5 \times 10^4$ cells per cm$^2$, and were grown for three days in culture dishes prior to the addition of stimulating media. The depolarizing cDMEM, hypertonic cDMEM, and normal cDMEM were exactly the same as described in Chapter 5. Cells were fed every other day with a half volume of the stimulating media. After 7 days, cells were observed on a phase-contrast light microscope (Zeiss Axiovert 200). To minimize experimental artifacts, the experiment was performed twice, with cells that were passaged four times more in the second experiment than in the first. In the first experiment, 5 culture wells were used for each stimulating condition, and in the second experiment, 9 culture wells were used. Three separate fields were imaged and quantified in each culture well. Since variation in the morphology of the unstimulated cultures was minimal, we used two separate culture wells in each of the two experiments.

We were interested in measuring two parameters: the number of cells extending neurites, and the average length of these neurites. To obtain percentages for comparison of images and cultures, we also counted the total number of cells in each field. The number of neurite-bearing cells was reported as an average. Figure 27 below shows
representative images from each experimental group. For the first experiment, a total of 6 images were analyzed for unstimulated cell cultures, and a total of 15 images were counted for each of the stimulated conditions (depolarizing and hypertonic cDMEM). In the second experiment, 6 images were counted for unstimulated cells, 27 images were counted for each of the two stimulated cultures.

To compare the measurements obtained from the above image analysis, we used analysis-of-variance (ANOVA) to compare the five groups: hypertonic depolarization in each of the two experiments; hypertonic, non-depolarized growth in each of the two experiments; and unstimulated growth in normal media (the data from the two experiments were combined into one group, due to minimal statistical variation). The five groups were deemed significantly different from each other at a cut-off of p<0.05.
Figure 27. Phase contrast imaging of PC12 cells in three culture media.
A. PC12 cells grown in normal cDMEM. Cells were phase-bright and proliferating. B. PC12 cells cultured for 4 days in depolarizing, hypertonic media. Significant number of cells adopted a flattened morphology and extended neurites that connected to each other. C. PC12 cells in depolarizing, hypertonic media for 7 days. Similar to previous panel, some cells show morphological differentiation. D. PC12 cells in hypertonic media for 7 days. A few cells in the field had extended neurites and adopted a flattened morphology. Bar, 30 μm.
A.1.2 Results

Quantitative comparison of the three different conditions showed that growth in depolarizing, hypertonic conditions induced significantly more neurite-bearing cells than growth in the other two conditions (p<0.01 for both comparisons). Depolarization induced 2.5-fold more cells to extend neurites (6% of the population) than hypertonic culture (2.5% of the population), and 10-fold more cells than in unstimulated cultures. Interestingly, hypertonic growth conditions had a statistically significant effect on neurite outgrowth compared to unstimulated cells (p<0.01). The results are summarized graphically in Figure 28.

![Graph showing neurite outgrowth comparison]

Figure 28. Chronic depolarization induces neurite outgrowth in PC12 cells. Cells were cultured in depolarizing, hypertonic or normal media for seven days. Phase contrast optical imaging was used to quantify the percentage of cells bearing neurites, denoted as “% cells differentiated”. Cells grown in depolarizing conditions were significantly more differentiated than in hypertonic or unstimulated growth conditions (**, p<0.01), and cells grown in hypertonic media were more differentiated than in unstimulated cultures (*, p<0.01).
Similarly, the average neurite length was influenced by the culture conditions, as summarized in Figure 29. In depolarizing cDMEM, PC12 cells extended neurites that were 40 μm long, while hypertonic media induced neurites with an average length of 28 μm. Unstimulated cells extended neurites of less than 20 μm in length. Taken together, morphological analysis of the PC12 cells showed that chronic membrane depolarization significantly induced more cells to extend longer neurites.

![Graph showing average neurite length](image)

**Figure 29. Chronic depolarization induces growth of longer neurites.** Cells were cultured in depolarizing, hypertonic or normal media for seven days. Phase contrast optical imaging was used to quantify the average length of neurites. Cells grown in depolarizing conditions extended significantly longer neurites than in hypertonic or unstimulated growth conditions (**, p<0.01), and cells grown in hypertonic media extended longer neurites than in unstimulated cultures (*, p<0.01).

Previous research has shown that membrane depolarization induces morphological differentiation of PC12 cells (Hilborn et al., 1997). Differentiation of PC12 cells, as measured by the percentage of cells bearing neurites, was shown to depend on the concentration of KCl in the depolarizing media. A concentration of 30 mM KCl induced 6% of the cells to extend neurites, while 27% of cells extended neurites in 60 mM KCl. The authors of this study did not mention whether cells were depolarized in hypertonic or
isotonic solution. However, in another study, the effect of isotonic versus hypertonic depolarization was investigated, and it was found that at the level of gene expression, significant differences between the two protocols could be observed. Our gene expression results in Chapter 5 further supported the observation that membrane depolarization and osmotic strength have interacting yet differential effects on PC12 cells. While our current results showed that membrane depolarization significantly increased morphological differentiation of PC12 cells compared to hypertonic, non-depolarizing culture, neither our results nor any others reported in the literature have definitively shown that chronic depolarization in isotonic conditions could induce phenotypic changes in these cells.

A.2 LONG-TERM DEPOLARIZATION OF PC12 CELLS: CELL DEATH

Depolarization in some cases promotes the survival of cultured primary neurons, such as primary cerebellar granule neurons (Franklin and Johnson, 1994). However, the role of depolarization and Ca$^{2+}$ influx in promoting neuronal death, such as in excitotoxicity, is equally well established ((Randall and Thayer, 1992), (Hartley et al., 1993)). Thus, we were interested in determining whether depolarization of PC12 cells induced cell death. First, we quantified cell viability using light microscopy in hypertonic and depolarizing culture conditions over the course of 7 days. Second, to gain insight into the mechanism of cell death, we used fluorescence microscopy to monitor one marker of apoptosis in the hours following depolarization.

A.2.1 Methods

For studies of cell viability over the course of days following depolarization, 3 x 10$^6$ cells were seeded per culture dish at day one with the protocol described in Chapter 5. Cells were grown in one of three culture media: depolarizing cDMEM, hypertonic cDMEM, or normal cDMEM (all prepared as described in Chapter 5). Cells were fed every other day with a half volume of fresh media. At days 4 and 7, the culture media containing any detached cells was collected, and cells attached to the tissue culture surface were collected following a brief exposure to trypsin (0.05%, containing EDTA). The two fractions of cells from each dish were pooled together and suspended in a known
volume of liquid. A small volume of the cell suspension was removed and mixed with Trypan Blue (5x dilution of Trypan Blue from Sigma). The concentration of viable cells (identified as cells that were able to exclude the dye from their cytoplasms) in each sample was measured using a hemacytometer, and thus the total number of viable cells in each culture dish was calculated. Five dishes were counted for each of the three culture conditions on day 4 and day 7.

To determine whether programmed cell death was initiated in the cultures during chronic depolarization or hypertonic growth, we used the commercially available dye Merocyanine 540. Merocyanine 540 has been used as an indicator of one of the initial steps in apoptosis, namely the loss in plasma membrane asymmetry ((Pradhan et al., 1997), (Schlegel et al., 1993)). Cells that stain positive with Merocyanine 540 will progress down an apoptotic path in the following hours. We cultured PC12 cells at a density of 2 x 10^4 per cm^2 and allowed them to grow in normal cDMEM for 3 days. Following this lag time, we replaced the culture media with either depolarizing cDMEM, hypertonic cDMEM or normal cDMEM. At 3 or 7 hours following the switch to stimulating media, we added Merocyanine 540 (100x dilution, Upstate Biotechnology) and incubated the cells with the dye for 10 minutes at room temperature. Cells were then washed twice with phosphate-buffered saline and immediately imaged under a microscope equipped with a mercury arc lamp and appropriate optical filter set (maximum excitation of Merocyanine 540 occurs at 555nm; maximum emission at 578nm). Three fields were imaged for each culture dish, and three culture dishes were analyzed for each condition. Each field was imaged once with phase contrast white light to assess total number of cells in the field, then imaged with fluorescence to determine the number of Merocyanine-positive cells. The total exposure to fluorescence was limited to less than 2 seconds for each image to reduce the effects of photo-bleaching.

**A.2.2 Results**

Cell viability studies, as assessed by Trypan Blue exclusion, confirmed that both hypertonic and depolarizing conditions induce a large decrease in viable cells compared to unstimulated cultures (p<0.001). The results are summarized graphically in Figure 30. Approximately 75% of the cells that were seeded on day one were no longer viable after
four days in depolarizing or hypertonic cDMEM. Also, an additional three days in culture did not significantly decrease the number of viable cells in the two groups. Finally, the measurements of viability showed that PC12 cells grew normally in isotonic, non-depolarizing cDMEM, confirming that the decrease in viability was due to hypertonic culture (with or without depolarization). We were not able to conclude from the present study whether chronic depolarization in isotonic media would have induced a similar loss in PC12 cell viability. However, studies with cerebellar granule neurons and other primary neurons in culture have shown that no loss in cell viability occurs after chronic, isotonic depolarization for over 3 weeks in these cells ([Lasher and Zagon, 1972], [Franklin et al., 1995], reviewed in [Berridge et al., 1998]). Taken together with previous research, our studies suggest that the loss in cell viability we observed was due largely to the hypertonic conditions in the depolarizing media.

**Figure 30.** Depolarizing and hypertonic culture conditions induce decreases in cell viability. PC12 cells were cultured for prolonged periods of time in depolarizing, hypertonic and normal cDMEM. Cell viability was assessed with the Trypan Blue exclusion assay. Unstimulated cells continued to grow normally, while both depolarized and hypertonically cultured cells experienced a large loss in cell viability in the first four days (p<0.001).
To determine if the loss in cells occurred via programmed cell death in the hours following depolarization, we stained cells in the three conditions with Merocyanine 540. As summarized in Figure 31, PC12 cells were not more likely to be Merocyanine-positive in either depolarized or hypertonic media than in normal media. Rather, depolarizing cDMEM significantly decreased the number of Merocyanine-positive cells at the 3 hour time point (p<0.01). This anti-apoptotic effect was abolished after 7 hours of depolarization. Thus, the loss in cell viability that we observed to occur after 4 days in culture either did not occur via an apoptotic pathway within the first 7 hours of chronic depolarization.

![Graph showing the percentage of cells stained with Merocyanine 540 under different conditions](image)

**Figure 31.** Depolarizing and hypertonic conditions did not induce significant apoptosis within the first 7 hours of depolarization compared to unstimulated cultures. PC12 cells were transferred to depolarizing or hypertonic cDMEM, and cells were analyzed for loss of membrane asymmetry 3 and 7 hours after the switch. Unstimulated cells had media replaced with normal cDMEM. There was no significant increase in Merocyanine 540 staining in either stimulating media. Conversely, cells depolarized for 3 hours showed a significant decrease in positively-stained cells (p<0.01).

**REFERENCES**


