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By

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Abstract

Osmotic Regulation of Axonal Elongation in Cultured Neurons

By

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Both osmotic dilution and mechanical tension have been reported to be potent stimulators of axonal growth. This dissertation represents an investigation of the mechanisms involved in mediating osmotic dilution-stimulated axonal growth, and we hope the study could help us get insight into mechanisms mediating tension-regulated growth.

In the first chapter, we used calibrated glass needles to apply constant force to elongate axons of cultured chick sensory neurons. We find that a neurite being pulled at a constant force will grow 50% to 300% faster following a 50% dilution of inorganic ions in the culture medium. That is, osmotic dilution appears to cause axons to increase their sensitivity to applied tensions. The osmolarity effect is reversible. Neuronal elongation rate decreased upon returning to original medium osmolarity.

In chapter two, activators/inhibitors of various signal mediators were used to see if they change neuronal growth sensitivity to tension or stimulate/inhibit the dilution-stimulated effect. Experiments suggest that the osmotic effect is not mediated by dilution of extracellular calcium,

or by the osmotic swelling-activated Cl^- channels, by osmotic-induced depolarization, to osmotic stimulation of adenylate cyclase, or by osmotic stimulation of mechano-sensitive ion channels, or PKC activation. Rather, experiments measuring the static tension normally borne by neurites suggest a direct mechanical effect on the cytoskeletal proteins of the neurite shaft. Our results are consistent with a formal thermodynamic model for axonal growth in which removing a compressive load on axonal microtubules promotes their assembly, thus promoting axonal elongation.

From the study, we also found out that although the Cl^- channel blocker and the PKC inhibitor inhibited tension-regulated growth, the activities of Cl- channel and PKC activation are not essential to tension-stimulated axonal growth.

To my parents and my sister.

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Introduction

I. The Significance of Osmotic Homeostasis and Cell Volume Regulation

Osmosis is the net diffusion of water from a region of high water potential to a region of lower water potential when the movement of solute is prevented. There are two components to osmosis: (1) the diffusion of water and (2) a barrier that will prevent solute movement but allow water movement. Since water constitutes about 70% of the body and the plasma membranes of most cells are relatively impermeable to many of the solutes of the interstitial fluid but are highly permeable to water, osmosis occurs between the intracellular and extracellular fluid compartments within an living organism. Surprisingly, up to one hundred times the volume of water in a cell crosses the plasma membrane every second (Sherwood, 1989). Changes in intracellular solute content or extracellular osmolarity and the consequent water flow can alter cell volume. A living cell whose volume is restricted by the cell membrane and cytoskeleton often behaves as an osmometer (Macleod et al.,1933; Guest, 1948) because it swells in a hypotonic environment and shrinks under hypertonic stress. Animal cells must control their

volume to prevent extreme swelling (and lysing) or shrinking under osmotic insults. The process in which a cell, when placed in an anisotonic conditions, controls its volume through the regulation of intracellular solute content is called "volume regulation" or "osmoregulation." Thus, although water is rapidly entering and leaving cells, most vertebrate cells normally do not experience any gain (swelling) or loss (shrinking) of volume over long times.

Since most vertebrate cells appear to be protected from osmotic volume changes by precise renal regulation of plasma solutes and water content, one would surmise that vertebrate cells would have little occasion to deal with osmotic homeostasis problems. This supposition is quite inaccurate, however. It is quite common for certain vertebrate cells to handle anisosmotic situations either during physiological or pathophysiological activities or medical treatment (Ballanyi et al., 1988; Strange et al., 1991). One example is the medullary epithelial cell of the kidneys. As mentioned above, the major renal function is to regulate the water content, mineral composition and acidity of the body by excreting each substance to achieve total body homeostasis and maintain normal concentrations in the extracellular fluid. During the excretion process, the medullary nephron epithelial cells face an local interstitial hyperosmolarity up to 1400 mOsmol/L (normally, it is 320 mOsmol/L in the interstitial fluid). Given such a environmental hypertonic stress, the epithelium must volume

regulate by uptake of inorganic ions or organic solutes (osmolytes) to keep itself from shrinking (Sun et al., 1994). Also, during passages through the renal medulla, the circulating mammalian red blood cells are challenged with large osmotic differences. The red blood cell is protected from shrinking by its ability to equilibrate urea very rapidly across its membrane through a specific transporter (Berkowitz et al., 1982) Another example of normal osmoregulation is the epithelium in the small intestine. Because the luminal concentrations of organic solutes (glucose and amino acids) fluctuate with the digestive process and those solutes are cotransported with Na^+ across the apical membrane, the epithelial cell volume in the small intestine tends to vary with time corresponding to the amount of net solute entry (Reuss et al., 1994). One more case concerns hepatocytes that are metabolically active (Haussinger et al., 1991). Insulin was reported to activate ion channels (mainly Na^+/H^+ exchangers and other electrolyte uptake paths) and to induce hepatocyte swelling. This swelling inhibited proteolysis in the liver, as effectively as experimental hyposmotic exposure. Conversely, cell shrinkage caused by glucagon or hyperosmotic exposure stimulates proteolysis and inhibits protein synthesis. It has been proposed (Haussinger et al., 1991) that the hormone-induced cell volume alterations act like a signal in hepatic metabolism: cell swelling triggers an anabolic reaction, while cell shrinkage generates an catabolic response.

All the physiological anisotonic volume changes are within cell volume control ranges, and the volume regulation can be regarded as part of normal cell function. However, many pathophysiological conditions involve long-term or dramatic changes in extracellular and plasma osmolarity. These have profound and vital effects on cell volume. In human beings, plasma hyposmolarity often results from congestive heart failure; the syndrome of inappropriate antidiuretic hormone secretion; Addision's disease, hepatic cirrhosis (where retained salt and water is effectively lost to edema formation); and malnutrition (Arieff and Guisado, 1976; Ho and Carroll, 1992; Strange et al., 1991). Increases in plasma osmolarity also are frequently observed with diarrheal syndromes, water deprivation, renal failure, central and nephrogenic diabetes insipidus, and diabetes mellitus (Arieff and Guisado, 1976; Ho and Carroll, 1992). Infants and children are the most vulnerable to changes in plasma osmolarity due to their small size and large surface area to volume ratio. For example, a disturbance of total body osmotic homeostasis can arise from such simple causes as inappropriate dilution of juice or formula preparation. The osmotic disturbance caused by malnutrition in children has been a specially serious concern in undeveloped countries (Keating et al., 1991).

Inducement of acute hypertonicity or hypotonicity by medical treatment may include mannitol or hypertonic saline administration for cerebral edema, intravenous infusion of

radiographic contrast material, and rapid infusion of certain medications such as sodium bicarbonate in the setting of cardiac arrest (Fisher et al., 1992; Mcmanus et al., 1994). However, those clinical treatments that cause acute changes in plasma tonicity are usually given with relative impunity, because cell volume regulatory mechanisms work in the background to maintain general homeostasis. In contrast, the rapid medical correction of chronic hypo- or hypertonic states can be met with serious complications (Sterns et.al., 1986; Sterns et al., 1989).

II. Cell Volume and Ion Transport, Gene Expression

Since cell volume is determined by the combination of extracellular fluid osmolarity and intracellular solute content, cell osmoregulation (volume regulation) involves the accumulation or loss of inorganic ions and organic solutes (osmolytes). In cells of higher vertebrates, inorganic ions such as K^+, Na^+, Cl^- and HCO_3^- comprise the majority of osmotically active ions. The general classes of organic osmolytes are sugars (e.g., glucose, mannose), polyols (e.g., inositol, sorbitol), amino acids (e.g., proline, taurine, alanine) and methylamines (e.g., betaine, glycerophosphoryl-choline).

Volume regulation under anisotonic conditions can occur within a few minutes or up to hours depending on the types of

cells (Lauf, 1985). When cells are exposed to a hypotonic environment, they respond initially by swelling followed by shrinkage to their initial volume. This regulatory volume decrease (RVD) occurs through water efflux accompanying the loss of intracellular solutes and ions , primarily by the loss of K^+ and Cl^-. On the other hand, a regulatory volume increase (RVI) following shrinking brings cells back to their previous volume upon an increase in external tonicity. This regulation is completed by taking up solutes and ions, primarily Na^+ and Cl^-, from the environment and the concomitant water influx (Melton et al., 1987; Schousboe et al., 1990; Pasantes-Morales et al., 1993b). For example, when resting salivary gland acinar cells are exposed to hypotonic solution, the cells initially swell, followed by RVD, and the whole process is essentially complete within 5 minutes (Reuss et al., 1994). In response to hyposmolarity stress, the release of osmolytes (for RVD) after initial cell volume swelling from cultured rat brain cortex astrocytes and neurons reached a maximum within 1-2 minutes (Pasantes-Morales et al. 1993a). For cultured rat cerebellar neurons, RVD was completed in 15 minutes, while RVI was not observed until an hour after the osmotic insult (Pasantes-Morales et al. 1993b). All these RVD and RVI processes are accomplished through the increased rate of ion fluxes that are activated by the osmotic imbalance between the intracellular and extracellular fluids.

There is good evidence that volume regulation also affects gene transcription and translation (Urban et al., 1992). The activation of osmoregulated genes during cell volume alterations has been reported to occur in molluscan and bacterial cells (Chamberlin et al., 1989) as well as in mammalian cells. For example, in cultured renal papillary cells, aldose reductase was induced during hyperosmotic cell shrinkage (Bedford et al., 1989). This enzyme is required for the synthesis of sorbitol which serves as an osmolyte in these cells. Also, hypertonic saline injection effectively induced *c-fos* protein expression in oxytocin neurons within 30 minutes, presumably through osmotically induced cell shrinkage (Giovannelli et al., 1992). In addition, some studies have reported that volume-regulatory transport activities change during cell maturation and cell proliferation (Meyer et al., 1991; Rao et al., 1991). These data implied a possible role of volume regulatory as part of signalling for cell growth. However, unlike the intensive studies in RVD and RVI that provided substantial information on ion and osmolyte transport pathways underlying volume regulation, the mechanisms linking osmoregulation to gene expression and cell proliferation in mammals is very poorly understood.

In spite of the intensive investigation on the mechanisms involving volume regulation, in general, the present state of knowledge on volume regulation is still limited. Much is known about the behavior of the inorganic

ion and organic solute transporters during volume regulation (Kimelberg et al., 1990; O'Conner et al., 1993; Hollows and Knauf, 1994). Conversely, little is known about the signalling systems that are involved in volume regulation and the mechanisms of how cell volume changes are perceived. In other words, the link between those second messenger pathways and the volume regulatory mechanism are unknown (Force et al., 1994).

III. Cell Volume Regulation in the Nervous System

Neurons, too, undergo osmoregulation during physiological and pathophysiological activities (Ballanyi and Grafe, 1988; Xu et al., 1994). During neuronal activities, the release of neurotransmitters alters ion permeabilities of the postsynaptic neuronal membrane, that then leads to transmembranal fluxes of ions. A shrinkage of extracellular space in the brain was observed *in vivo* during glutamate releases (Van Hareveld, 1972), which is an ubiquitous excitatory synaptic process in the mammalian central nervous system (Rogawski et al., 1985). Also, K^+-induced depolarization caused swelling of cerebral cortex both *in vivo* and *in vitro* (Schousboe et al., 1971; Bourke et al., 1972). It is possible that K^+ depolarization-induced release of neurotransmitter or other neuroactive substances served as mediators of cell swelling (Bourke et al, 1983; Kempski et

al., 1986; Walz, 1987). Antidiuresis regulation is postulated to be principally controlled by plasma osmotic changes. Neurons involved in body homeostasis detect and respond to osmotic changes in the circulation. In the rat, elevated plasma osmolarity increased the circulating concentration of both vasopressin and oxytocin, while a decrease in osmolarity reduced the plasma concentration of vasopressin and oxytocin (Stricker and Verbalis, 1986; Johnson et al., 1992). Oliet et al. (1994) showed that in the supraoptic and paraventricular nuclei of the hypothalamus, a non-selective cationic channel of vasopressin and oxytocin neurosecretory cells was effectively activated/inactivated by volume decrease/increase associated with osmotic changes in plasma. Quantitatively, a 10% cell volume decrease resulted in a 40% increase in channel conductance. This suggested neuronal volume changes as an important regulator for the control of vasopressin and oxytocin release, which in turn also regulated antidiuresis. In addition, isolated presynaptic nerve terminals exhibit RVD and RVI behavior. When induced by experimental hypotonic/hpertonic treatments, pinched-off presynaptic nerve terminals (synaptosomes) regulated their volume and respectively decreased/increased them back to within 5% of their original volume in 2-10 minutes (Bablia et al., 1990). Furthermore, osmolarity has been demonstrated to affect the release of neurotransmitter quanta. In motor nerve endings, a 20-30% elevation of extracellular osmolarity accelerated

the rate of neurotransmitter release about two times (Shimoni et al., 1977). Since nerve terminals constantly encounter local changes of solute concentration due to ion fluxes and neurotransmitter secretions, the physiological behavior of RVD and RVI in the nerve terminals might serve as a way of enabling the proper communication between the presynaptic and postsynaptic neurons.

Sometimes, the most serious consequence of plasma osmolarity changes and resultant fluid shifts is manifested by swelling or shrinking of the brain which can cause severe neurological impairment and high mortality (Pollock and Arieff, 1980). Clinical disorders caused by anisotonic volume disturbances include seizures, focal neurological injury, severe cerebral edema and diabetic coma (Kleeman, 1989; McManus et al., 1994). Despite the clinical importance of neuronal volume regulation in the brain, little is known because attempts to understand osmoregulation have been hampered by the structural complexity of the mammalian central nervous system.

Some peripheral nerve abnormalities also result from alternation of plasma osmolarity. One representative example is diabetic neuropathy, which is one of the most common chronic complications of diabetes (Greene et al., 1989). Slow motor and sensory nerve conduction and elevated sensory perception thresholds are some of the characteristics of diabetic neuropathy. Although insulin-induced hyperglycemia is considered to play a pivotal role in the development of

peripheral neuropathy (Greene et al., 1985), evidence also have suggested that the dysfunction might result from disruption of normal osmoregulation caused by accumulation of intracellular metabolites(McManus et al., 1994; Sango et al., 1994).

IV. Axonal Elongation and Osmotic Effect

Osmotic changes are involved in at least one other neuronal function, the control of axonal elongation. Normally, axonal elongation depends on forward advances of the highly motile growth cone at the distal end of neurons. One description of this growth cone-mediated axonal elongation phenomenon is that of a "leucocyte on a leash" (Pfenninger, 1986). In other words, the growth cone locomotes in its environment and the axon progressively elaborates from behind the advancing growth cone. The elongation of the axon is closely connected to growth cone advance. Oster and Perelson (1987) proposed that the osmotic effects could be the driving force for growth cone advance, based on the observations that all protrusive activities of filopodia and lamellopodia at the leading edge of cells are suppressed in hypertonic medium (Trinkaus, 1985). They proposed that the actin polymerization process, which is fundamental for protrusive activities of the neuronal filopodia and lamellopodia, involve the release of inositol

lipids or Ca^{++}-induced solation factors such as gesolin. These are osmotically active particles which upset the local osmotic pressure equilibrium. Then, water influx resulting from increased cytosolic osmolarity propels the protrusion of microspikes and lamellopodia, presumably leading to growth cone advance.

Bray et al. (1991) tested this hypothesis in cultured chick dorsal root ganglia (DRG), but observed an immediate and transient increase in filopodial length and number following an elevation in external osmolarity. The filopodial behavior argued against Oster and Perelsons' model if filopodial growth and growth cone extension were driven by turgor. However, Bray et al. did demonstrate that the elongation of DRG neurites showed a consistent osmotic response. In particular, reductions in osmotic strength through addition of water to the culture medium stimulated an immediate and prolonged increase in the rate of neurite outgrowth. The more dilute the medium, the faster the axonal growth. They observed up to a six fold increase in neurite elongation rate within 20 minutes with dilution to 50% of the basal medium osmolarity. The axon could lengthen at the stimulated rate for a couple of hours. Amazingly, even after 5 days of culture in 50% basal medium, many ganglia still had active growth cones at their periphery and possessed long dense axonal outgrowth. Thus, axonal elongation rate is intimately correlated with medium osmolarity. In view of the osmotic dilution vs. axonal lengthening, the idea that the

growth of the neuritic cylinder is purely expanded by water influx is inapplicable. For neurites to elongate, there are a lot of biochemical processes which are needed for the mass addition of neurites. Those processes include cytoskeleton assembly and reorganization, membrane addition and the synthesis of membraneous organelles such as mitochondria and synthetic vesicles. Especially, microtubule organization is closely related with axonal elongation and initiation (Zheng et al., 1993; Smith 1994; Tanaka et al., 1995). Apparently, osmotic dilution is one potent stimulator of axonal elongation in cultured neurons.

Osmotic dilution is an extrinsic input to axonal elongation, while tension is reported to be an intrinsic regulator and stimulator (Heidemann and Buxbaum, 1994). In neurons, "towed growth" (Weiss, 1944) of axons by the migration of their target cells after synaptogenesis indicates a cause-effect relationship between the pulling and axonal growth. Lamoureux et al. (1989) showed that growth cone advance and neurite tension are linearly related and accompanied by neurite elongation. There is now widespread agreement that neurites are under tension (Bray, 1979; Dennerll et al., 1988) which is exerted by the growth cones (Lamoureux et al., 1989) or their target cells. In neurons, the link between axonal development and tension seems unusually intimate in both the time scale and the simplicity of relationship (Heidemann and Buxbaum 1994). In response to experimentally applied tension by glass needles, axonal

elongation occurs over the course of seconds and minutes at physiological and far-above physiological rates. This elongation processes can continue for many hours without thinning of the neurites as long as the tension stimulation lasts (Bray 1984, Zheng et al. 1991) Under a variety of culture conditions, the rate of axonal lengthening of cultured chick neurons and PC-12 cells is a linear function of the applied force when the force is above the threshold (Dennerll et al., 1989; Zheng et al., 1991; Lamoureux et al., 1992). In addition, tension can initiate axons *de novo* from neuronal cell bodies (Bray 1984, Zheng et al. 1991).

The observations from neuronal responses to both hyposmotic treatment (Bray et al., 1991) and applied tension (Heidemann and Buxbaum 1994) indicate that neurons are quite mechanically robust. More support has been gained from *in vitro* studies which showed that neurons are quite resilient to survive in hyposmotic stress. For instance, Wan et al (1995) treated molluscan neurons with extreme osmotic insult, i.e., in distilled water for up to 60 minutes and which caused the cells to swell to several times their initial volume. They found that more than 50% of the neurons survived and reaborized within 24 hours after return to normal medium. For vertebrate neurons, the mouse dorsal root ganglion (DRG) neurons survive for more than 12 hours in a gradual reduction of osmolarity down to 1/4 osmolar of the normal culture medium (Sango et al., 1994). In molluscan neurons, rapidly elicited reversible membranous dilations can

be induced from a series of osmolarity decreases (downshocks) and increases (upshocks) (Reuzeau et al., 1995). In contrast, as is well known, erythrocytes rupture immediately in hypotonic solution and cultured heart cells lacking basement membrane lysed in one minute when exposed to 1/5 of normal saline (Morris et al., 1989). Neurons must extend out neuritic processes (axons and dendrites) to perform their fundamental task, which is to receive, conduct, and transmit signals. The mechanical robustness of neurons may contribute to the unique function and morphological plasticity characteristic of this cell type.

The close relationship of mechanical tension to elongation rate in cultured neurons and the increase of elongation rate stimulated by osmotic dilution suggests a possible link between osmotic dilution and axonal elongation. We postulated that osmotic dilution might be connected to the cytomechanics of elongation. A major goal of this dissertation was to test this hypothesis. Further, the results of Bray et al.(1991) suggested a robust coupling between the osmotic input and microtubule organization and membrane addition of neurite outgrowth. Although Bray et al.(1991) raised the possibility that osmotically derived internal pressure may contribute to axonal elongation, the possible mechanisms involved are still unknown. How is the osmotic effect mediated in stimulating axonal elongation over time periods of minutes? What physical/chemical signals are

involved? These questions were addressed in the current studied.

V. Plausible Mechanisms Involved in Osmotic Effects

One of the important questions we asked in these studies was "How is the osmotic dilution effect mediated in axonal elongation?" e.g., what mechanism(s) connects the osmotic effect to the consequent neuritic mass addition reactions? Is the osmotic effect simply a phenomenon of membrane expansion caused by the water influx, which in turn could directly alter the free energy equilibrium of microtubule assembly/ disassembly similar to the proposal of Buxbaum and Heidemann (1988, 1992)? Or are there protein mechanosensors, such as stretch-activated ion channels, that are directly activated by osmotic swelling, and the activation of which stimulates certain chemical signal transduction pathways (Force and Bonventre, 1994)? Do the chemical signals and the mechanical signals (if there are any) couple to each other?

So far, there are several proposals to explain osmotic effects. Some work suggests a "membrane-tension hypothesis" (Martin et al., 1990; Martin and Shain, 1993; Oliet and Bourque, 1994). According to this hypothesis, any swelling associated with hypotonic stimulation will increase the amount of tension experienced by the "tension sensors" (ion channels or receptors) on the membrane, thereby activating/inactivating channels or receptors. By contrast, elevated external osmolarity that causes cell shrinkage will attenuate membrane tension and therefore turn on/off the ion

channels or receptors. Other work implicates "classical" chemical signalling messengers such as protein kinase A (PKA) and protein kinase C (PKC) as possible mediators of the osmotic effect (Watson 1989). However, it is not known whether PKA and PKC signal pathways are directly activated by cell deformation caused by osmolarity changes or are indirectly turned on by other intracellular changes derived from osmolarity insults (Watson 1991).

As mentioned previously, unlike the ligand-activated signal transduction pathways that have been widely studied, how a cell perceives its volume changes, the signalling pathways coupling osmotic effect and the subsequent cellular biochemical reactions are still poorly understood. Studies about osmotic effects on axonal elongation are especially extremely scarce. In the following section are generally explored PKA and PKC signal transduction mechanisms as are other possible mediators that have been reported to be involved in osmoregulation. Similar signalling paradigms may characterize neuronal signal events activated by hypotonic challenge and stimulation of axonal growth.

a). Stretch-Activated and Stretched-Inactivated Ion Channels

Stretch-activated ion channels (SA channel) are among the few well-described direct cellular mechanotransducers. The open state probability (P_O) of these channels increases by membrane stretch brought about either by applying pipette hydrostatic pressure or hyposmotic-induced swelling (Sigurdson and Morris, 1989; Morris 1990). The effect of membrane stretch on P_O is reversible. SA channels are ubiquitous and have been identified in many cell types including yeast, neurons, heart cells, kidney cells, and muscle cells (Morris 1990). Generally, the selectivity of SA channels divides into two classifications: non-selective channels for monovalent and divalent cations, and channels that have prioritized selectivity for specific ions like K^+ or Cl^-, depending on the cell types (Christensen 1987; Morris and Sigurdson 1989; Ross et al., 1993). Interesting, no Na^+- or Ca^{++} -selective SA channels have been identified (Sachs, 1992).

Sigurdson and Morris (1989) reported the existence of stretch-activated K^+ channels (SAK channels) in the growth cones of snail neurons. In considering the tension-generating role of growth cone motility in axonal elongation, the authors raised the possibility that SA channels may serve as the primary mechanosensors in growth cones. The physical activities of growth cones open SAK channels and lead to membrane voltage changes that may activate some consequent voltage-dependent biochemical reactions. Thus, SAK channels

may provide a link between membrane tension and chemical reactions in growth cone activity.

Additionally, in neurons, stretch-inactivated K^+ ion channels (SIK channels) whose conductance is inactivated by stretch were found to coexist with stretch-activated ion channels (Morris and Sigurdson, 1989). SIK channels open in a coordinated way with SAK channels. When membrane stretching opens SAK channels, the conductance of SIK channels decreases at the same time. Thus, through differing stretch sensitivities, both SAK and SIK channels contribute to the net ion equilibrium and membrane voltage in the cells.

Although SA and SI channels have been described in many cell types, not much is known about the biological processes that may be regulated by or coupled to these channels (Watson 1991). The most commonly postulated biological role for SA and SI channels is in volume regulation. They may instantly detect cell swelling or shrinkage in anisotonic environments. This cellular deformation causes conductance changes of SA/SI channels and may alter membrane voltages. The activation of SA/SI channels may modulate the activity of other membrane transporters that are voltage-dependent or of other cellular events, leading to volume regulation.

In view of the role of SA and SI channels, it is very likely that SA or SI channels mediate osmotic dilution stimulated axonal growth. The gadolinium ion (Gd^{3+}) is a trivalent lanthanide with an ionic radius of 0.938 Å close to that of Na^+ and Ca^{++}. It has been reported to be a potent

but rather non-specific inhibitor of mechanosensitive (both SA and SI) ion channels (Yang and Sachs, 1989; Franco et al., 1991; Quasthoff, 1994). In T lymphocytes, 10 μM Gd^{3+} was shown to block cell volume regulation (Yang and Sachs, 1989). In the surrent studies, we used Gd^{3+} as the blocker to investigate the possible involvement of mechanosensitive ion channels.

b). Swelling-Induced Cl^- Channels

As described in the previous section, after initial swelling in a hypotonic condition, there are losses of intracellular ions accompanied by efflux of osmotically obligated water that enables a cell to restore its original volume (RVD). The lost ions are primarily proposed to be chloride ions and potassium ions through channels and/or transporters (Hallows and Knauf, 1994).

Many studies have shown a close relationship between efflux of chloride ions and osmotic swelling. For example, depletion of intracellular Cl^-, by long incubation with gluconate, markedly inhibited RVD in hypotonically swollen cerebellar granule neurons (Pasantes-Morales et al., 1993). Studies in *Xenopus laevis* oocytes, rat cardiac myocytes and human astrocytoma cells showed the development of a strong outwardly rectifying Cl^- current induced by hypotonic environments, and this anionic current is completely absent in isotonic solution (Coulombe and Coraboeuf 1992; Ackerman et al., 1994; Bakhramov et al., 1995). Several Cl^- channel

blockers including 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) were reported to inhibit this hypotonicity-induced Cl^- current as well as induced RVD in both fibroblasts and human astrocytoma cells (Bakhramov et al., 1995; Gschwentner et al., 1996). In cultured rat astrocytes, Cl^- channel blockers inhibited both hypotonicity-evoked Cl^- current and the fluxes of some osmolytes such as inositol and taurine which play important role in RVD (Pasantes-Morales et al., 1994; González et al., 1995). This inhibition raised the possibilities that either this anion pathway (swelling-activated Cl^- channel) might also serve as a common pathway for organic osmolytes during volume regulation or that the Cl^- and osmolytes fluxes are closely interconnected. For example, complete replacement of extracellular Cl^- with isethionate or gluconate either substantially reduced inositol effluxes and RVD by 80-90% in brain glial cells (Strange et al., 1993), or partially decreased the inositol effluxes (22%) in cultured rat astrocytes (González et al., 1995). Although the mechanisms are still poorly understood, the movement of Cl^- ions and RVD are apparently intimately related These data all indicated an increase in the membrane Cl^- conductances during osmotic swelling. However, the molecular basis of this Cl^- current (unidentified Cl^- channel) is still unknown (Sakkadi and Parker, 1991).

Several kinds of Cl^- channels have been postulated to play a role in volume regulation (swelling-activated Cl^-

channels), including Ca^{++}-activated Cl^- channels and cAMP-activated Cl^- channels. This is because those Cl^- channels have been reported to be involved in the secretion activities of some secretory cells, and cells must deal with volume regulation during the process of secretion (Reuss and Cotton, 1994, Reuss and Altenberg 1995). For example, Ca^{++}-activated Cl^- channels play a regulatory role in pituitary adrenocorticotropin (ACTH) secretion (Heisler and Jeandel, 1989; Heisler, 1991). In cystic fibrosis disease, the principal defect is in the cAMP-activated Cl^- channel critical for fluid secretion in exocrine gland and airway epithelia (Tabcharani et al., 1991; Schwiebert et al., 1995; Jentsch 1996). Although those Cl^- channels are well described in other systems, evidence for links between these channels and volume regulation is quite limited (Foskett 1994).

Expression cloning techniques and voltage clamp studies in lymphocytes and epithelial cells indicate that there exists one other type of Cl^- channel which is only activated by hypotonic solutions and is distinct from other stretch-activated ion channels, from the cAMP-activated, and from the Ca^{++}-calmodulin-activated chloride channels (Sarkadi and Parker, 1991; Jentsch 1996). One such putative Cl^- channel belongs to a specialized class of protein named I_{Cln} and was initially cloned from Madin Darby canine kidney (MDCK) epithelial cells (Paulmich et.al., 1992). Overexpression of I_{Cln} protein in *Xenopus laevis* oocytes produced a strong

outwardly rectifying chloride current which reversed at about 30mV, i.e., close to the equilibrium potential for chloride ions (Paulmich et.al., 1992). Hypotonicity-induced activity of I_{Cln} was inhibited by extracellular addition of NPPB and DIDS, substances known to block other chloride channels in a variety of cells (Gschwentner et al., 1996). More studies clearly are needed to identify the swelling-activated Cl^- channels. How these channels mentioned above are triggered by cell swelling still remains unknown.

c). Membrane depolarization

Depolarization has been reported to be involved in hyposmolarity challenges and volume regulation. In cultured astrocytes, membrane depolarizations could be elicited in proportion to the degree of hypotonicity-induced swelling, which ranged from 12 mV depolarization at 30% swelling to a peak about 60 mV at an 80-100% increase in cell volume (Kimelberg and O'Conner, 1988; Kimelberg and Kettenmann 1990; Olson 1995). On restoring the cells to an iso-osmotic medium, the membrane immediately repolarized back to the original potential. It has been suggested that swelling activation of certain ion channels, corresponding to the stretch-activated channels, caused the redistribution of ions that then leads to membrane depolarization (Kimelberg and O'Conner, 1988; Kimelberg and Kettenmann, 1990). Also, high $[K^+]_o$-induced depolarization, as well as hypotonicity, has been shown to cause a dose-dependent increase in cellular

release of the osmolyte taurine, the release of which has been proposed to play an active regulatory role during RVD in neurons (Schousboe and Pasantes-Morales, 1989). The efflux of other small organic molecules, such as aspartate and glutamate, has also been observed during RVD in cultured astrocytes swollen by exposure to elevated $[K^+]_o$ or hypotonic medium (Kimelberg et al., 1990).

In addition to a role in osmotic phenomena, membrane depolarization has been reported to play important roles in neuronal outgrowth (Franklin and Johnson, 1992). In injured nerve fibers, membrane depolarization, which probably functions to promote the survival and differentiation of cultured neurons, is one of the earliest detectable events after axotomy (Berdan et al., 1993; Yan et al., 1994). Depolarization was suggested to be a signal that could either activate or modulate events involved in nerve regeneration. In addition, Tolkovsky et al., (1990) found that the extent of cultured rat sympathetic neurite outgrowth was dependent on the degree of experimentally induced-depolarization.

Does the induced-depolarization of astrocytes elicited by a hypotonic environment also apply to neurons? Is the osmotic dilution effect due to swelling-induced depolarization, which leads to modulation of several neuronal activities, possibly including stimulation of axonal growth? In the current studies, we tested the possible role of K^+-depolarization in hypotonicity-stimulated axonal elongation.

d). Adenylyl Cyclase - cAMP

Since the discovery of adenylyl cyclase and cyclic AMP (cAMP) alternations in response to adrenergic hormones, the involvement of the cAMP cascade in a wide variety of biochemical pathways has been recognized. The classical mechanism of adenylyl cyclase activation involves a receptor coupled to one or more G proteins, and the catalytic unit of the cyclase. The Gs and Gi families of G proteins play stimulatory and inhibitory roles in this signalling pathway respectively. Both cAMP and protein kinase A activity change as a downstream result from the activation.

CAMP has been reported to be involved in osmoregulation in a variety of cells. Swelling of rat cardiac myocytes inhibited intracellular cAMP accumulation through activation of a pertussis toxin (PTX) sensitive Gi protein (Hilaldandan and Brunton 1995). In follicular cells and erythrocytes, cAMP also was suggested to play a role in volume regulation because it acted as a potent second messenger in regulating the osmo-dependent Cl^- current (London et al., 1989; Arellano and Miledi, 1994). Itzhak et al.(1994) reported an upregulation of peripheral benzodiazenpine (PBZD) receptors from rat astrocytes in the presence of hypo-osmotic stress and dibutyryl cAMP (dbcAMP). The PBZD receptors were suggested to participate in the control of astrocyte volume. Increases in volume of certain cells also caused intracellular cAMP accumulation. For example, hypo-osmolarity-induced swelling of turkey erythrocytes (Morgan et

al., 1989) and S49 mouse lymphoma cells (Watson, 1989) resulted in rapid increase in cAMP.

In addition to the experiments mentioned above, cAMP also has been reported to regulate the activities of many ion channels like Cl^- channels and transport pathways such as Na^+/H^+ and $Cl^-/HCO3^-$ exchange processes (London et al., 1989; Arellando and Miledi, 1994; Force and Bonventre, 1994). However, its role in volume regulation processes still remains poorly understood. No clear relationship between hypo-osmolarity and cAMP has been described. Some reports have demonstrated that mechanical deformation can activate adenylyl cyclase. For instance, stretch could induce cAMP increases in cardiac myocytes (Zimmer and Peffer, 1986; Watson, 1991; Komuro and Yazaki, 1993). Elevated cAMP has been linked to cardiac hypertrophy by increased cardiac protein synthesis due to pressure overload both *in vivo* and *in vitro*. This is important in adding evidence that adenylyl cyclase may be involved in volume regulation, since osmoregulation may be directly triggered by cell deformation induced by hyposmotic or hyperosmotic stimuli.

In addition to its possible involvement in osmoregulation, cAMP has been shown to be important in regulating neuronal growth cone activity (Lohof et al., 1992; Fawcett, 1993) and neuronal outgrowth and elongation (Mattson et al., 1988; Nakagawa-Yagi et al., 1992). Elevation of cAMP by forskolin and dibutyryl cAMP (dbcAMP) suppressed *Helisoma* neurite elongation in a dose-dependent fashion (Mattson et

al., 1988). In PC-12 cells, dbcAMP potentiated neurite outgrowth and stabilized microtubules (Heidemann et al., 1985; Ho and Raw, 1992). The activity of adenylate cyclase also increased concomitantly with neurite outgrowth and was proportional to neurite length in cultured rat sympathetic neurons (Tolkovsky, 1987). The effects of cAMP on neuronal activity vary, depending on the types of neurons. The proposed involvement of cAMP in volume regulation and in neuronal growth regulation thus makes the adenylyl cyclase signalling pathway a potential mediator in osmotic-stimulated axonal elongation.

e). Phospholipase C -PKC

Protein kinase C is critical to many of the signal transduction pathways activated by growth factors and neurotransmitters. At least ten isoforms have so far been identified in mammalian tissue (Tanaka and Nishizuka, 1994). The "conventional" isoforms (α, $\beta 1$, $\beta 2$, and γ) are Ca^{++}- and phospholipid-dependent and require diacylglycerol (DG) or phorbol ester (PMA) for activation *in vitro*.

Reports of the involvement of PKC activation in controlling volume regulation are very scarce and information is limited. The major postulated role of PKC in volume regulation is in the regulation of certain volume-sensitive ion transporters (Grinstein et al., 1992; Palfrey, 1994). It is probable that PKC may be one of the modulators of volume regulation because of its wide distribution and physiological

engagement. PKC is present in high concentrations in neuronal tissues and has been implicated in a variety of neuronal functions (Tanaka and Nishizuka, 1994). Activation of PKC has been related to enhancement of neurotransmitter release, regulation of ion channels, control of neuronal growth, and differentiation and modification of neuronal plasticity (Huang, 1989; Tanaka and Nishizuka, 1994) Phorbol ester (PMA) which activates PKC was shown to stimulate neurite outgrowth from both chick embryo sensory ganglia (Hsu et al., 1984) and rat sympathetic neurons (Camponot et al., 1991). PKC regulates neurites formation in PC-12 cells and may play an important role in morphological changes in neural cells (Glowacka and Wagner, 1990). Moreover, pharmacological data on hypertrophy research has suggested that PKC activation is responsible for *c-fos* and other immediate early gene induction by stretching in cardiomyocytes. The long term process of elevated protein synthesis in overloaded cardiomyocytes also appears to depend on PKC activation (Komuro et al., 1993). The activation of PKC by cell deformation plus the involvement of PKC in neuronal elongation stimulated our interest in investigating the possible role of PKC in osmotic dilution mediated axonal growth.

This dissertation is divided into two chapters that address questions about how the osmotic effect is mediated in stimulating axonal elongation. In Chapter One, our calibrated pulling glass needle methodology that resembles

growth cone-mediated axonal growth was used as the basic paradigm (Zheng et al., 1991) to investigate the effect of osmotic dilution on axonal growth.

Chapter Two focuses on pharmacological investigations. By towing the neurite at a constant force and keeping it at a constant growth rate, the roles of plausible mediators involving in the hypo-osmolarity induced axonal growth were assessed with the aid of some chemical inhibitors and activators.

Chapter 1. Osmotic Dilution Stimulates Axonal Elongation

1.1. Introduction

Previous work in our laboratory has indicated that axonal elongation and tension are closely related (Zheng et al., 1991; Lamoureux et al., 1992). When axonal elongation is experimentally stimulated by "towing" with a needle, the axon elongates immediately and continuously in response to tension. The rate of elongation of cultured chick neurons is a simple linear function of the applied force above the threshold tension.

Osmotic dilution is among the few extrinsic inputs that is documented to stimulate short term outgrowth rate of cultured neurons. Bray et al. (1991) measured a statistically significant increase in growth rate within 20 minutes with medium osmolarity reductions. The most dramatic growth rate elevation (as much as six fold) was seen following a shift to 50% of the basal medium. The axons had growth rates around 100-120 μm/hr at this stage, with apparent healthy growth cones.

The linear growth relationship and the short time scale of response between mechanical tension and axonal elongation

suggest that the application of mechanical tension directly stimulates subsequent cellular events for axonal elongation. In view of the effect of osmotic dilution on axonal elongation rate over times as equally short as the tension effect, we investigated whether there was any link between osmotic and tensile stimulation of growth. Are changes in growth rate following osmotic insults mediated by alternations in the tension/growth relationship? Will osmotic dilution cause an increase in the elongation rate of a neurite towed at the same constant tension before and after osmotic dilution? We measured the growth rate before and after osmotic dilution at the same force level to determine the linkage between the osmolarity effect and tension-mediated axonal growth.

1.2 Material and Methods

1.2.1. Materials

L-15 medium, L-glutamine, penicillin, streptomycin sulfate, laminin, poly-L-lysine and trypsin were purchased from Sigma Chemical Co. Fetal bovine serum (FCS) and phosphate-buffered saline (PBS) were from Gibco. 7S nerve growth factor (NGF) was from Harland (IN). Mouse monoclonal anti-β-tubulin was purchased from Amersham Inc. Fluorescein-labelled goat anti-mouse IgG was purchased from Kirkegaard and Perry Lab. Inc.

1.2.2. Preparation of Medium and Buffer:

1). L-15 Culture Medium

One bottle of L-15 medium was dissolved in 1 liter of reverse-osmosis glass-distilled water. The medium was fortified with 10% FCS, 100ng/ml of 7S NGF, 0.6% glucose, 2 mM L-glutamine and 100i.u./ml of penicillin and 136 mg/ml of streptomycin ($L\text{-}15^{+}$) and the pH was adjusted to 7.4.

2). Hyposmotic Medium

The medium was prepared containing all the amino acids, vitamins, sugars, and antibiotics found in fortified L-15 medium but lacking inorganic salts, which included Na^{+}, K^{+}, Ca^{++}, Cl^{-} and other inorganic ions. The medium pH was adjusted to 7.4. For osmotic dilutions, the initial culture medium was diluted 1:1 with this hyposmotic medium. The

final diluent medium contained approximately 50% of the inorganic salts of the basal medium, but normal concentrations of all other neutrients.

3). Hyperosmotic Medium

As in the preparation of hyposmotic medium, this medium was made containing all the amino acids, vitamins, sugars, and antibiotics found in L-15^{+} medium. The only difference was that this medium had 150% of normal ion concentrations, including Na^{+}, K^{+}, Ca^{++}, Cl^{-} and other inorganic ions, of the basal L-15 medium. The pH was 7.4.

4). Microtubule-Stabilized Buffer

The content of microtubule-stabilized buffer (MTSB) was as follows: 100 mM Pipes, 2 mM EGTA, 4% (W/V) polyethylene glycol (MW ca.8000), 0.025% sodium azide. The pH was 6.8.

1.2.3. Cell Culture

Chick sensory neurons were isolated and cultured as described by Sinclair et al. (1988) from lumbosacral dorsal root ganglia of 10-12 day old chicken embryos. Briefly, after dissection, gangla were treated with 0.1% trypsin for 30 minutes at 37^{0}C and were triturated with a pipette to disperse them into single cells. Cells were plated and cultured in 10 ml of supplemented L-15 medium as described above. Cells were grown on culture dishes pre-treated with

5mg/ml of laminin. Neurons were cultured at 37^0C for 16-24 hours prior to experimentation.

1.2.4. Neurite Elongation and Direct Axial Force and Length Measurement

The neurites of cultured cells were towed from their growth cone with calibrated glass needles as described by Zheng et al. (1991). Briefly, two needles were mounted in a micromanipulator (Narishige CO. LTD.) with one as a pulling needle and the other as a reference for bending of the towing needle and possible drift of the micromanipulator system. The bending constants of the pulling needles were between 4-11 μdyne/μm and needles were pre-treated with 0.1% polylysine and then 10 μg/ml of laminin to promote adhesion. Neurites were then towed with the calibrated pulling needles attached to their growth cones and a videotape record at 24X time lapse was made of the experiment. In the experiments reported here, the distance between pulling needle and reference needle was adjusted every 5 minutes, moving the needle approximately 10 mm over the course of a second. These adjustment conditions and the force for towing, usually between 100 and 300 μdynes, were chosen to allow the neurites to elongate at a visually observable pace. In our experience, the neurite does not respond sensitively to moderate differences in the period or amplitude of adjustments, instead producing a linear growth response to approximately constant applied force.

After towing untreated neurites for at least one hour to obtain a control value of the elongation rate, the culture was subjected to an experimental treatment of osmolarity shift (either increase or decrease depending on the need of the experiment) of the medium. We continued towing neurites after experimental treatments at the same tension, again for at least an hour for each experimental alteration. Following the completion of an experiment, the length of the neurite was measured every 5 minutes for the entire period of towing by analysis of the videotape record. From these measurements, neurite length was plotted as a function of time. The slope of this line was taken to be the elongation rate of the neurite, which was calculated for the data points before and after experimental manipulations.

1.2.5. Immunofluorescence

In some experiments, the microtubule cytoskeleton of towed neurites was observed by standard immunoflourescent methods, as described in detail by Zheng et al. (1993). Briefly, following neurite elongation, the distal ends of neurites were micromanipulated from the needle back onto the culture substrate. A diamond-tipped 'objective" was used to mark the experimental neuron by circling the dish beneath. Immunofluorescent staining of microtubules was carried out as follows: the medium was carefully removed and the culture was permeabilized in 0.5% Triton X-100 in MTSB, then fixed in 3.7% formaldehyde solution (in MTSB), all at 37^0C, followed

by extraction in methanol at -20^{0}C. The neurites were then incubated with mouse anti-β-tubulin mAb, rinsed and incubated with fluorescein labeled secondary Ab goat anti-mouse IgG. The prepared sample was observed through an Odyssey confocal microscope (Noran Instruments).

1.3. Results

1.3.1. Axonal elongation rate under constant applied tension

Previous work in our laboratory has demonstrated a robust linear relationship between axonal growth rates and applied forces in both PC-12 cells and cultured chick sensory neurons (Dennerll et al., 1989; Zheng et al., 1991; Lamoureux et al., 1992). That is, axonal elongation rate is proportional to neurite tension above a threshold. We here further confirmed that axons elongated at steady rates under constant towing forces. Figure 1 illustrates a uniform growth rate (as is manifested by the straight line) over the period of three hours from one of five neurites that were individually towed at different levels of constant force. Although the applied tension differed and growth rates varied from one neuron to another, all five neurons showed steady growth rate of axons. Table 1 compares growth rate from the first half of each experiment with that of the second half in these five constant force towing axons. Fig 1 represents neurite No.2 from Table 1. The stable growth rate of each neurite enabled us to use this constant force towing methodology as a fundamental approach for further investigations.

Figure 1.- Steady growth rate of axons under constant towing force. Throughout the experimental protocol, neurites were towed at a constant force (T value) suited to each neurite to produce easily visualized elongation rates. The lengthening of neurites in response to the applied tension was plotted as a function of time. As be observed from the straightness of the slope, the neurite elongated steadily under constant tension during a three hour experimental process. (n = 5)

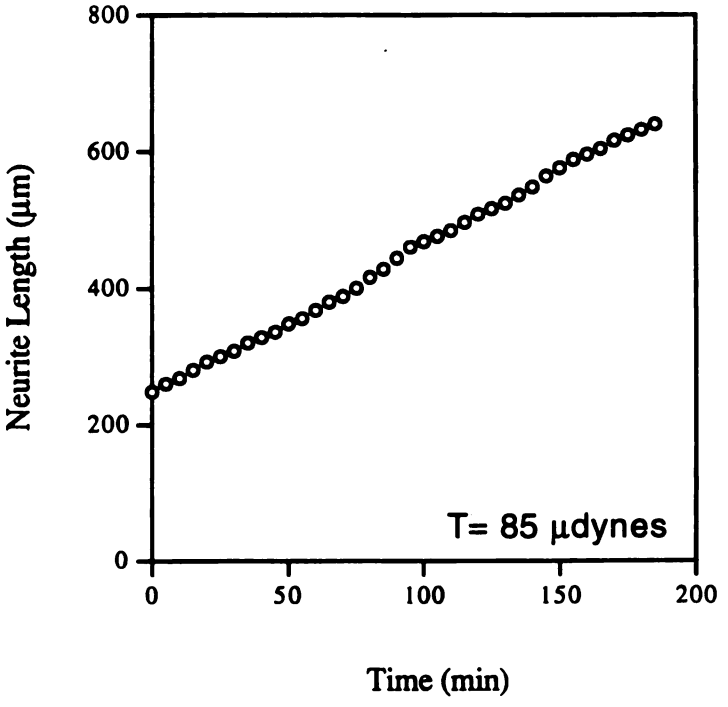


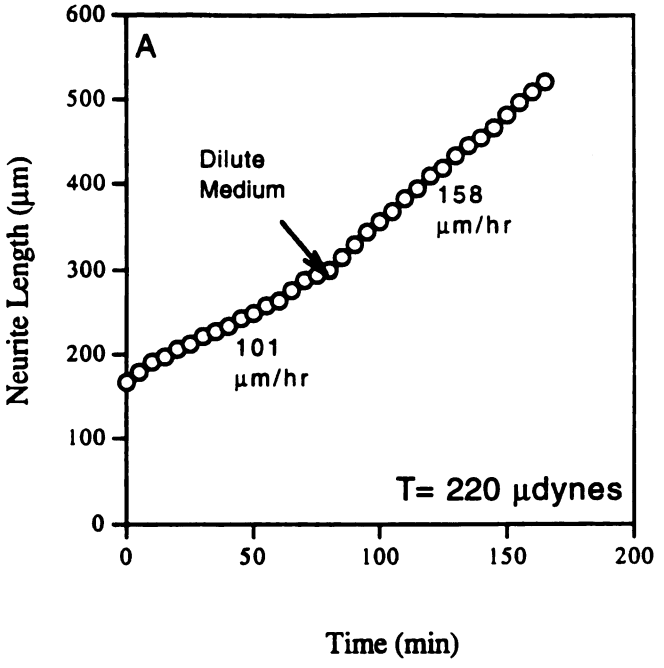
Table 1.-Axonal growth rates under constant towing tension. For each neurite, the experimental process was equally divided into two halves (about 60-90 minutes for each half) and growth rates were measured. Growth rates from the second halves were not statistically significant from those of the first halves (Student's T-test). In other words, each neurite lengthened steadily through the whole experiment at a given force.

No. of Trials	Tension Applied (udynes)	1st Half Elongation Rates (um/hr)	2nd Half Elongation Rates (um/hr)
1	232	140	143
2	85	120	122
3	157	52	54
4	243	55	52
5	133	80	70

1.3.2. Changes in axonal growth rate under osmotic dilution

To determine the relationship between osmotic dilution and tensile stimulation of axonal elongation, we elongated the neurite at a steady rate (constant force towing) for at least an hour. We then diluted the culture medium 1:1 with the hyposmotic medium lacking the inorganic salts, i.e. approx. a 50% reduction in medium osmolarity (see Materials and Methods for details of diluent medium). We found that osmotic dilution of culture medium causes an increase in elongation rate at a given towing force. The effect on towed elongation of diluted medium is illustrated in Fig 2A. Prior to dilution of the culture medium, the elongation rate of the neuron was 100 $\mu m\ h^{-1}$ and following medium dilution the elongation rate increased to 158 $\mu m\ h^{-1}$. That is, the neurite elongated more rapidly after osmotic dilution at the same applied tension, 220 μdynes in this case. All towed neurites (n = 8) showed an increase in the neurite elongation rate following dilution of the medium at a given tension (Table 2a). In most instances, the change in elongation rate was observed within 10 minutes, although in some cases the increase occurred more gradually, requiring 30 minutes to stabilize at a new value. The neuron shown in Fig 2A is experiment 5 in Table 2a. As shown, many neurons demonstrated substantially greater increases in their towed axonal elongation rate than the example shown in Fig. 2A.

Figure 2 - Effect of medium dilution on elongation rate at constant towing force. The experimental approach and analysis here was similar to figure 1, except that after at least one hour of towed growth, new medium was added to the dish. The lengthening response of the neurite in response to the applied tension was plotted as a function of time. T value is force applied in the experiment. Numbers shown adjacent to the line are growth rates (μm/hr). (panel A)- added medium contained no inorganic salt components thus reducing the osmolarity of the medium by approx. 50%. (Panel B) -Added medium was normal culture medium to serve as a "sham dilution" control.


A
Dilute
Medium
158
µm/hr
101
µm/hr
T= 220 µdynes
Neurite Length (µm)
Time (min)
600
500
400
300
200
100
0
0
50
100
150
200

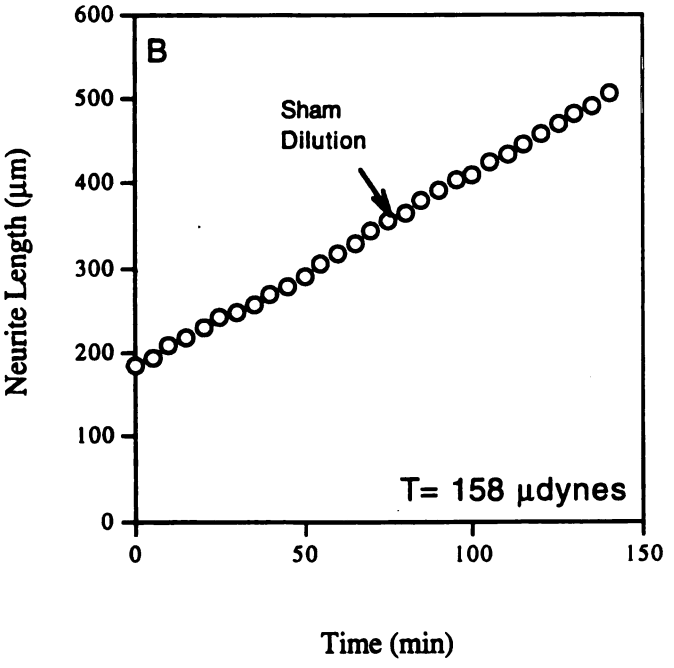

B
Sham
Dilution
T= 158 µdynes
Neurite Length (µm)
Time (min)
600
500
400
300
200
100
0
0
50
100
150

Table 2a. Changes in the axonal growth rate resulting from 50% osmotic reduction.

	Growth rate before osmotic shift (um/hr)	Growth rate after osmotic shift (um/hr)	Relative growth rate increase	Tension applied (udynes)
Exp.1	86	137	59%	251
Exp.2	145	313	116%	200
Exp.3	79	163	106%	267
Exp.4	80	116	45%	166
Exp.5	101	158	56%	220
Exp.6	37	168	354%	136
Exp.7	57	116	104%	228
Exp.8	119	228	92%	72

Table 2b. Axonal growth rates before and after sham osmotic dilution.

	Growth rate before sham osmotic shift (um/hr)	Growth rate after sham osmotic shift (um/hr)	Relative growth growth rate change	Tension aplied (udynes)
Control 1	124	131	6%	157
Control 2	133	137	3%	158
Control 3	19	19	0%	153
Control 4	115	125	9%	67
Control 5	129	150	16%	131
Control 6	149	181	21%	310

A sham dilution, to control for the intervention of the addition of an equal volume of medium, was without effect on elongation rate or the increased sensitivity to tension stimulated by osmotic dilution (Fig. 2B and Table 2b). An equal volume of complete medium was added to the culture (sham dilution) following at least one hour of towing and, as before, towing was continued at the same constant force. This change in culture medium volume and the accompanying disturbance to the cells and needles produced only small changes in the rate of towed elongation.

Osmotic dilution did not cause a disruption in the microtubules of the towed neurites. As revealed by immunofluorescence analysis, the microtubule array in gently lysed, towed neurites was intact and of normal appearance (Fig. 3A), indistinguishable from the microtubule array seen in neurites elongated by growth cone activity before and after osmotic dilution (Fig. 3B).

Changes of axonal elongation rates upon osmolarity stimulation were reversible. One hour after osmotic dilution, the medium was changed again and brought back up to 100% osmolarity of the basal medium. This was done by mixing diluted medium with concentrated medium (see Materials and Methods for details) at a volume ratio of 1:1. A typical experiment is shown in Fig. 4. In response to osmotic dilution, axonal growth rate increased from 55 $\mu m\ h^{-1}$ to 85 $\mu m\ h^{-1}$ and continued a steady elongation at this rate of 85 $\mu m\ h^{-1}$ as expected. However, when we increased the medium

Figure 3 - Microtubule cytoskeletons following osmotically-stimulated elongation in towed and untowed neurites. Fluorescence micrographs of neurons subjected to osmotic stimulation under conditions of A) elongation by towing and B) growth cone-mediated elongation and then lysed, fixed and "stained" for β-tubulin. A) The hollow arrow marks the point at which towing began in normal medium. The solid arrow marks the point at which the medium was diluted as in Fig. 2A and towing continued at the same constant force. The kink and bright spot in the neurite slightly distal to the hollow arrow, the curved segment, and the relatively bright staining at the distal tip are all artefacts of the process of coaxing the growth cone from the needle onto the dish surface, so the neurite can be fixed and processed for immunofluorescence. That is, following the towing process, the slow "thrashing" of the needle required to dislodge it from the neurite tip causes the axon to engage nearby cells and other detritus. The dim spots are fibroblasts, which stain less brightly than neuronal cell bodies. As shown here, the axial microtubule array was found to be essentially uniform all along the neurite shaft. The calibration bar is situated over the cell body and reflects a distance of 20 μm. B) Similar to panel A except that the neurite elongated via growth cone mediated elongation before and after osmotic challenge. The bright spots in the middle of the axon are neuronal cell bodies that the axon skirted during its growth. The arrow indicates the growth cone of the neuron extending the axon, same calibration as above.

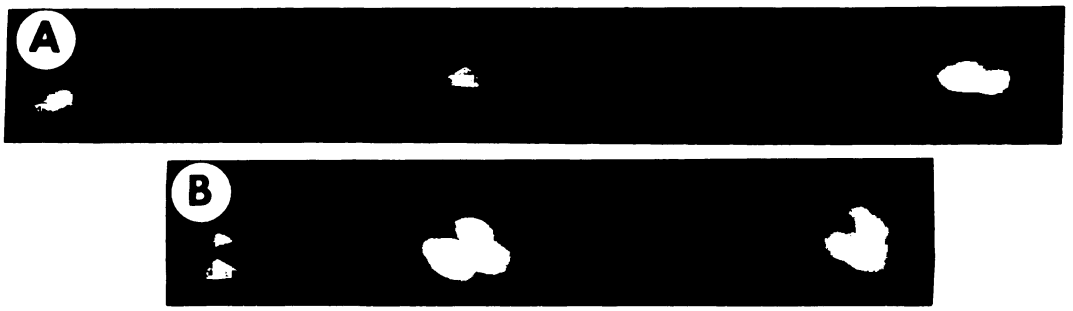
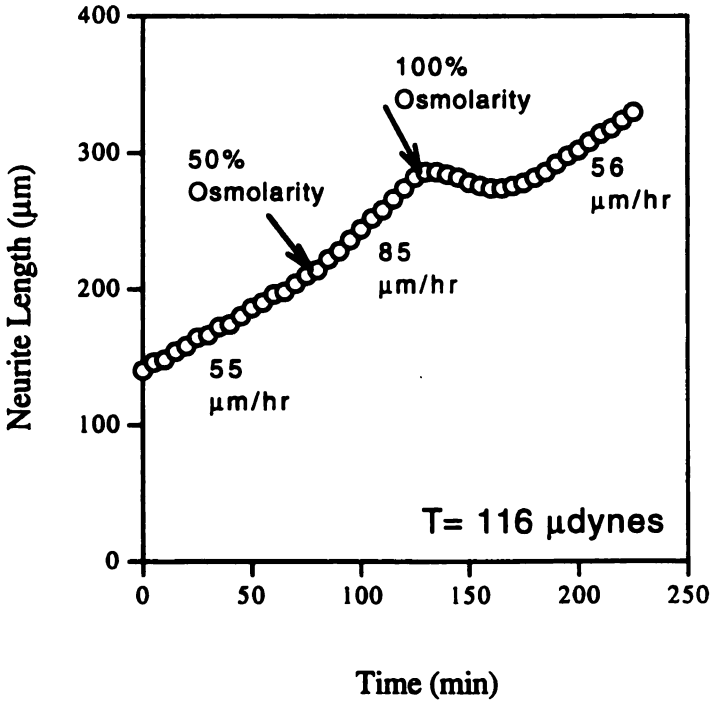

A
B

Fig.4 - Effect of osmolarity changes on elongation rate at constant towing force is reversible. The neurite was towed at a force of 116 µdynes. Medium dilution protocol and analysis is similar to Fig.2. In addition to osmotic dilution treatment, medium osmolarity was taken back to the original osmolarity at a later stage of the experiment. As expected, reduced osmolarity accelerated axonal growth. Although there was a growth rate decrease right after osmolarity increase from 50% to 100% of the basal medium, the lengthening rate eventually caught up with its initial speed.



osmolarity from 50% to normal medium osmolarity, the axonal elongation decreased and even came to a complete arrest at the same applied tension. In all instances (n = 3), neurites resumed their elongation in about thirty minutes after osmolarity increases and then restarted lengthening at near the original speed (56 $\mu m\ h^{-1}$ in Fig.4). In one experiment (data not shown here), an axon increased/decreased elongation rates twice corresponding to two cycles of osmolarity downshocks/upshocks. The reversibility of the osmolarity effect indicates that neurites had not become damaged in the process of osmotic insults, since the changes in growth rate are likely to be a sensitive measure of neurons morbidity.

1.4. Discussion

The axon elongates steadily at a given force

As expected, every axon that we pulled at a constant level of tension lengthened at a uniform speed throughout the whole experimental process (Fig.1 & Table 1). Our method was based on previous results that showed axonal elongation rate is a linear function of applied tension above some threshold (Zheng et al., 1991; Lamoureux et al., 1992). Although neurons have shown a very close fit to a linear function ($r \geq 0.9$ for 39 of 47 neurons to date), they vary in their values of the minimum threshold tension at which elongation begins (generally between 50 and 250 μdynes) and in their values of the proportionality between greater tensions and elongation rate (from 1-5 $^{\mu m/hr}/_{\mu dyne}$) (Zheng et al. 1991, Lamoureux et al. 1992). Similarly, as shown in Table 1, different neurons elongated at diferent rates at different tensions. As a result, only measurements of growth rate changes on the same neuron at a constant given tension before and after any treatment can be confidently interpreted. Our earlier work used a laborious method of observing elongation rates during multiple, one-hour steps of constant force, which were required to establish that the relationship is indeed a close fit to a straight line. Ideally, we would have liked to perform this study by the same stringent analysis used in those earlier studies. In preliminary osmotic dilution experiments (which we will

discuss next), however, we were unable to successfully coax a given neurite through the process of applying multiple steps of force in normal medium, changing or adding to the medium, and then subjecting the neurite to an additional round of multiple steps of tension. As a consequence, in this dissertation, I used a simplified method in which a neurite is towed at only one above-threshold force level for the entire experiment, and the elongation rate is measured before and after various experimental treatments for an hour or more. The limitation of this method is that what we call here "growth sensitivity" can reflect a change in threshold, proportionality, or both. The "growth sensitivity" is defined as growth rate per unit of applied force (μm./h /μdynes), which represents how fast the neurite grows in response to one μdyne force applied. A similar simplification is used in our formal mathematical model coupling the rate and thermodynamics of axonal MT assembly with the growth rate of axons in reponse to tension (Buxbaum and Heidemann 1988, 1992).

Osmotic dilution stimulated tension-regulated axonal growth

In response to applied tension greater than threshold values, axonal elongation occurs over short time scales of minutes (Bray et al., 1984; Lamoureux et al., 1992; Zheng et al., 1991). We chose to examine the effects of osmotic diltuion on chick sensory neurons because it is one of few interventions that is also documented to alter axonal

elongation rate over the equally short times (Bray et al., 1991). Is the osmotic stimulation of growth following medium dilution connected with the mechanical regulation of axonal growth? In support of our postulate of the coupling between osmotic stimulation of elongation and the tensile regulation of growth, we found that dilution of the osmotic environment of cultured chick sensory neurons causes these cells to grow faster at a given pulling force. That is, osmotic dilution appears to alter the intrinsic sensitivity of the neuron/neurite to a given tension stimulus for elongation.

The data of Fig.2 and Table 2 indicate that osmotic dilution of the medium surrounding chick sensory neurons shifts their growth sensitivity so that the neurite elongates faster at the same force. As expected from previous results, the neurite elongates at quite steady rates during the initial control period and also following each experimental alteration. As with other parameters of the tension/elongation relationship, the extent of osmotic-stimulation of elongation varies from cell to cell but the qualitative phenomena seem robust, similar to the variability observed by Bray et. al (1991) for osmotically stimulated changes in growth cone-mediated growth.

Results from Fig. 4 showed that first there were decreases or even complete arrests of axonal growth when neurons returned from hyposmolarity medium to 100% basal medium, then axons started to elongate again. Why did neurons axons behave this way when going from dilute medium

to original medium? One possibility is that axons from osmotic dilute environment react to 100% basal medium as going back to "hypertonic" medium. The notion that this "relative hypertonic" medium caused growth rate decrease in our work is quite consistant with the observation of Bray et al. (1991), which showed that increases in medium osmolarity caused a partial or total stop of axonal growth. As mentioned in the introduction previously, RVI occurs after cells are moved from an isotonic environment to a hypertonic one. However, when cells that have first been placed in a hypotonic medium and have adapted themselves to this hypotonic environment are removed back to "isotonic" medium, they volume regulate (2^0 RVI) again because the "isotonic" medium is now "hypertonic" relative to the cells (Halowa and Knauf, 1994). It took about 20-30 minutes before neurons adjusted themselves back to this isotonic medium and picked up the pace of lengthening again. Although we don't exactly know the mechanisms involved, the resumption of neurite extension to near their previous level further implied that the neuronal elongation mechinery was intact during the procedure. Different neurons seem to response to this "relative hypertonic" medium treatment slightly differently. Two growth cones out of five (one of the other three is shown at Fig. 4) detached from the needles minutes right after the osmolarity elevation and neurites retracted gradually (data not shown here). Thus, we were not able to conduct further experiments in these two cases.

Our data indicated that neurons could adapt themselves quite well to osmotic dilution treatment: the rate of elongation increased immediately and remained robust throughout the whole procedure (Fig.3). The available evidence on osmotic dilution stimulation of axonal elongation at a given force shows coupling between osmotic dilution stimulation and mechanical tension. It will be of interest to test whether the effects of various extrinsic regulators of axonal elongation involved in the osmotic dilution-stimulated elongation.

Chapter 2. Investigation Of Plausible Mediators Of Osmotic Dilution-Stimulated Axonal Growth

2.1. Introduction

Osmotic dilution of culture medium elicits immediate marked increases in the rate of neuronal elongation (Bray et el., 1991). This osmotic dilution stimulatory effect is linked to mechanical tension regulation of axonal growth (Ch.1). In view of the neuronal response to osmotic dilution over a time scale of minutes, this indicates a rather direct relationship between physical stimuli (osmotic swelling) and the subsequent biochemical alterations that leads to the stimulation of elongation of neurites. There must exist a machinery which connects the mechanical stimuli with the subsequent biological responses, i.e., a mechanotransduction mechanism.

How is the osmotic effect mediated? As discussesd in the Introduction, how a neuron perceives the osmotic insults and converts them into intracellular biochemical signals that lead to axonal growth rate changes is still poorly understood. It is thought to be unlikely that the dilution of intracellular solute as a result of cell swelling is the signal because this effect is too small (Kimelberg and

signal because this effect is too small (Kimelberg and O'Conner, 1988; Reuss and Cotton, 1994). First of all, volume regulation can take place upon very small changes in cell volume (Lau et al., 1984). Further, Lohr and Grantham (1986) showed that volume regulation could occur at the same slow rate at which the medium osmolarity was changed such that there was no measurable change in cell volume and therefore intracellular concentrations during the whole experimental process. However, judging from the significant neuronal response to osmolarity changes, one might guess that the signal is somehow amplified like an enzymatic cascade.

Based on previous osmoregulation studies, in the current studies, we generally divided mediators involving osmotic effects into several categories. The first group involves mechanical sensors such as mechanosensitive ion channels (both SA and SI channels) and swelling-activated Cl^- channels. The second group included "classic" G protein-linked second messenger elements such as cAMP and diacylglycerol. Prior evidence suggested osmotic dilution-stimulated growth might work through second messenger transduction pathways similar to those of agonist-receptor coupling (Watson 1991; Vandenburgh 1992; Davies and Tripathi 1993).

The third category by which cells may sense and respond to external osmotic perturbations is simply the mechanical effect. Since osmotic dilution causes cell swelling, it produces a mechanical stimulus. The mechanical load itself

(swelling-induced tension, in this case) has been proposed to be a direct signal that mediates axonal growth (Buxbaum and Heidemann, 1988, 1992). Is the osmotic effect mediated through chemical mediators or is it indeed a physical effect? Or are physical and chemical mediators both involved? In this portion of the current studies, we have tried to answer these questions by generally exploring the possible mediators mentioned above with pharmacological and cytomechanical investigations.

2.2. Material and Methods

2.2.1. Materials

Gadolinium chloride, potassium gluconate and sodium gluconate were purchased from Aldrich Chemical Co. Hepes (N-2-Hydroxyethyl piperazine-N'-2-ethane sulfonic acid) was from Reaearch Organics Inc. NPPB [5-Nitro-2-(3'-phenyl-propylamino) benzoic acid], chelerythrine chloride, synthetic diacetyl glycerol (1,2-dioctanoyl-*sn*-glycerol) were purchased from LC Laboratories. Forskolin, cholera toxin (CTX), dibutyryl cAMP (dbcAMP), phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12,13-didecanoate, and 1,3-dioctanoyl glycerol were purchased from Sigma Chemical Co.

2.2.2. Preparation of Medium and drugs:

1). L-15 culture medium (L-15^{+}) and hyposmotic medium

The media were prepared as previously described in Ch.1. The osmotic dilution procedures used here are also similar to those performed in former experiments.

2). Calcium Free Medium

The calcium-free medium consisted of all the components of L-15^{+} except that $CaCl_2$ was replaced with $MgCl_2$.

3). Hyposmotic Medium with Normal Concentration of Calcium

This medium was similar to the hyposmotic medium described in Ch.1, but contained the normal concentration of Ca^{++},

i.e., a 1:1 dilution of normal culture medium caused an approx. 50% dilution of all inorganic ions except Ca^{++}, which remained constant.

4). Hepes Buffered Medium

This medium had almost all the ingredients of fortified L-15 medium ($L\text{-}15^{+}$) except that we added 10 mM Hepes to substitute for Na_2HPO_4. This is to prevent the formation of precipitation between Gd^{3+} and PO_4^{3-} from the experiments of gadolinium ion treatment.

5). Low Cl^- $L\text{-}15^{+}$ Medium

Again, this medium contained all the ingredients of supplemented L-15 ($L\text{-}15^{+}$) except a few modifications: we substituted equimolar potassium gluconate for KCl, and sodium gluconate for NaCl. The trace amount of chloride ions (2.5mM) now left in the medium was from calcium chloride, for which we could not find a substitute and some amino acids which contained monohydrochloride. The original L-15 medium has chloride ion concentration at about 145 mM.

6). Preparation of Drugs

Gadolinium chloride, CTX and dbcAMP were prepared in H_2O. Forskolin, NPPB, PMA and other PKC activators or inhibitors were dissolved in DMSO. Those drugs were made in concentrated stock solutions and were added to the medium during experiments to obtain the desired final concentration

in the culture medium. In no case did the addition of drugs or ions change the volume of culture medium by more than 1%. Experiments to test the effects of DMSO on neuronal growth rate have been done as control (data not shown here). Generally, 0.1% of DMSO did not cause any change in the growth rate.

2.2.3. Cell Culture

Embryonic chick sensory neurons were cultured as previously described in Ch.1.

2.2.4. Neurite Elongation and Direct Axial Force and Length Measurement

The methods of measurement were the same as formerly illustrated in Ch.1. (Zheng et al., 1991). The growth rate was generally measured from the starting moment of one treatment till the end of that treatment unless indicated.

2.2.5. Rest Tension Measurement

The rest tension was measured as previously described (Dennerll et al., 1989; Lamoureux et al., 1992). Essentially, neurites were plucked to the side over the course of 3-5 seconds with calibrated glass needles while making video recordings. This highly dynamic loading condition does not allow sufficient time to engage the viscous elements of the passive, i.e. non-growth, response of axons to tension (see Fig. 5, Dennerll et al. 1989). That is, such plucking

produces nearly pure elastic behaviors on the part of neurites, although neurites are clearly viscoelastic over longer times of tens of minutes (Dennerll et al. 1989, Lamoureux et al., 1992). The relationship between neurite stretching and applied force was calculated by geometry and vector algebra from measurements of the lateral needle deflection, lateral neurite displacement, and neurite length at the time of plucking. The y-intercept of the elastic relationship (i.e. force at zero neurite deflection) was taken as the neurite rest tension and the slope is the spring constant. Comparisons were made of these parameters of the elastic response before and after experimental interventions over the course of about an hour. This method corrects for any changes in neurite length due to growth occurring between elastic measurements.

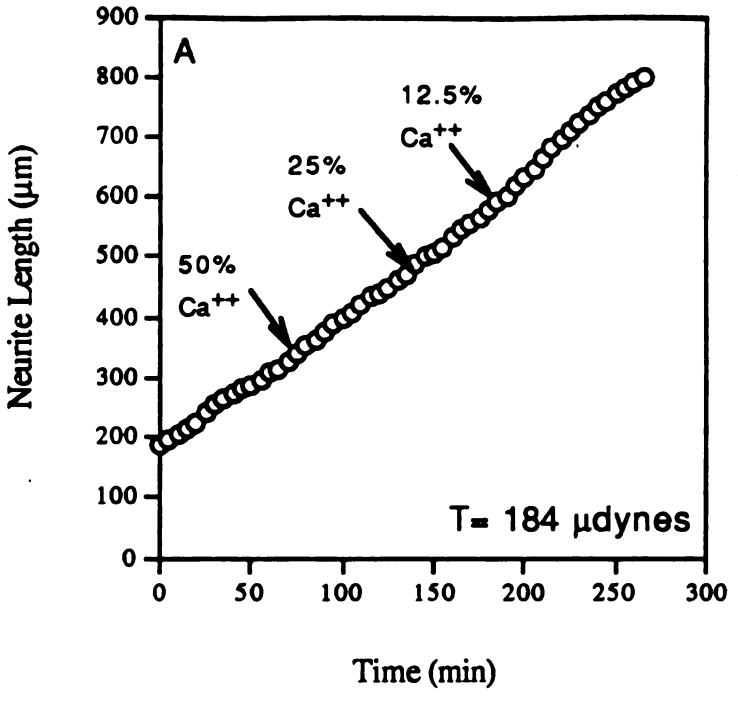
2.3. Results

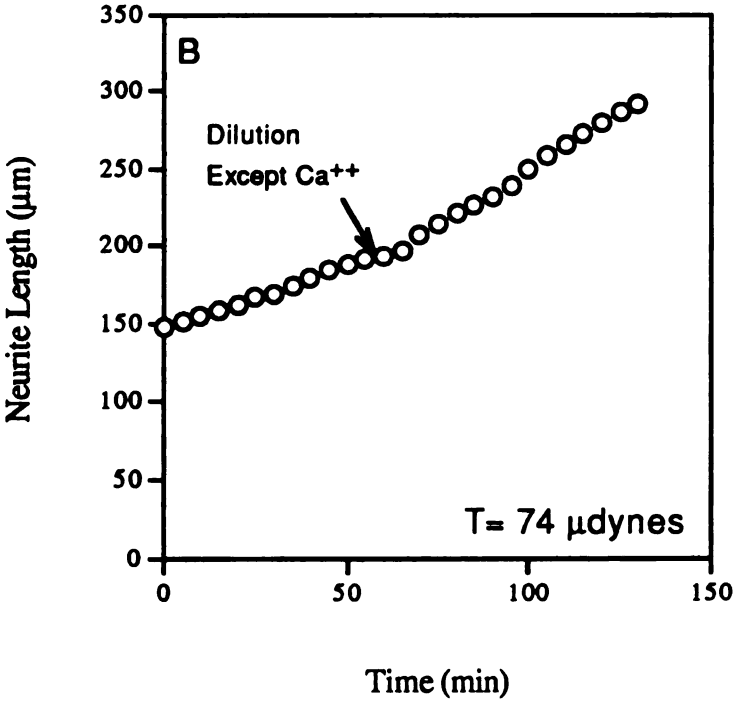
2.3.1. Effect of extracellular Ca^{++} reduction

Decreased extracellular Ca^{++} concentration have been shown to increase the rate of neurite elongation to 145% of the initial rate (Mattson et al.1987, Kater and Mills 1991). In the osmotic dilution experiments described in Chapter 1, the extracellular Ca^{++} concentration was reduced by half, along with all other ionic concentrations of the medium. We first would like to know whether the osmotic dilution effect was attributable to dilution of $[Ca^{++}]_o$ (Fig. 5). All the following experiments in this chapter were basically assessed with two experimental designs: 1). does the treatment influence sensitivity of tension-induced elongation? and 2). does the treatment affect osmotic stimulation of growth? The first question was used as a control for the second question.

To investigate the effect of extracellular Ca^{++} reduction, two types of experiments were performed. In the first experimental design, one hour after being towed at a given force, extracellular $[Ca^{++}]$ was serially diluted with Ca^{++}-free medium (please see Material and Methods) in three steps, each step reducing extracellular Ca^{++} by half. This intervention had no significant effect on the elongation rate of the neurite when $[Ca^{++}]$ was reduced down to 25% (Fig. 5A). Two other similar experiments in which extracellular Ca^{++} were reduced by half also had no effect on the elongation rate. However, there was a slight growth rate increase (27%

Figure 5 - Effect of extracellular Ca^{++} on elongation rate at constant force -- experimental protocol and analysis similar to Figure 2. Panel A - Three serial additions were made of Ca^{++}-free medium (other components in normal concentration) such that each addition reduced the extracellular [Ca^{++}] by half. As shown, reducing extracellular Ca^{++} to 25% had no effect on elongation rate at the constant force. The neuronal lengthening rate in 12.5% $[Ca^{++}]_o$ was about 27% higher than the initial rate. Panel B - Addition of medium in which all inorganic salts were excluded except $CaCl_2$, which was at normal concentration. As shown, an osmotic stimulation of axonal elongation rate (128%) continued to be observed in the presence of constant extracellular [Ca^{++}].


A
12.5%
Ca++
25%
Ca++
50%
Ca++
T= 184 µdynes
Neurite Length (µm)
Time (min)
0
100
200
300
400
500
600
700
800
900
0
50
100
150
200
250
300


B
Dilution
Except Ca++
T= 74 µdynes
Neurite Length (µm)
Time (min)
0
50
100
150
200
250
300
350
0
50
100
150

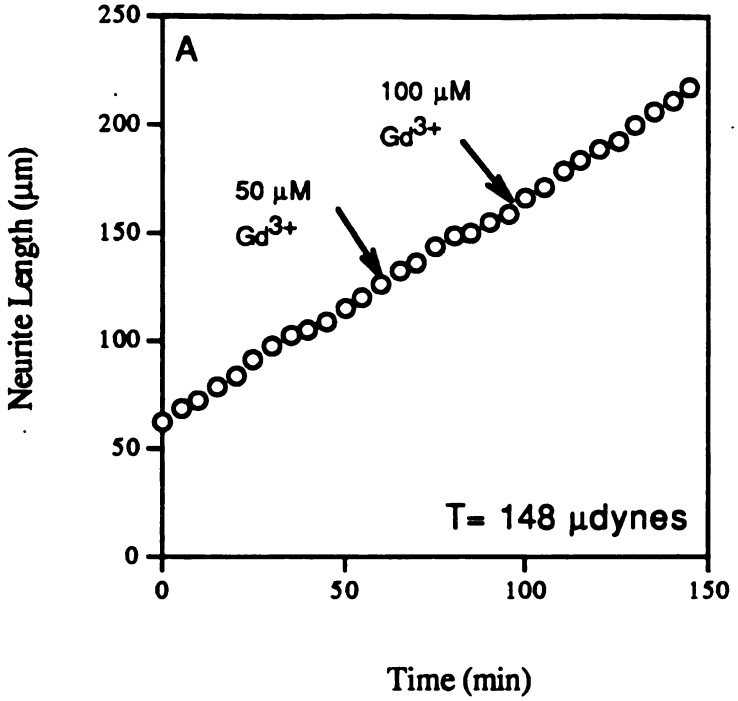
increase of the initial rate) with as low as 12.5% extracellular Ca^{++} in the medium.

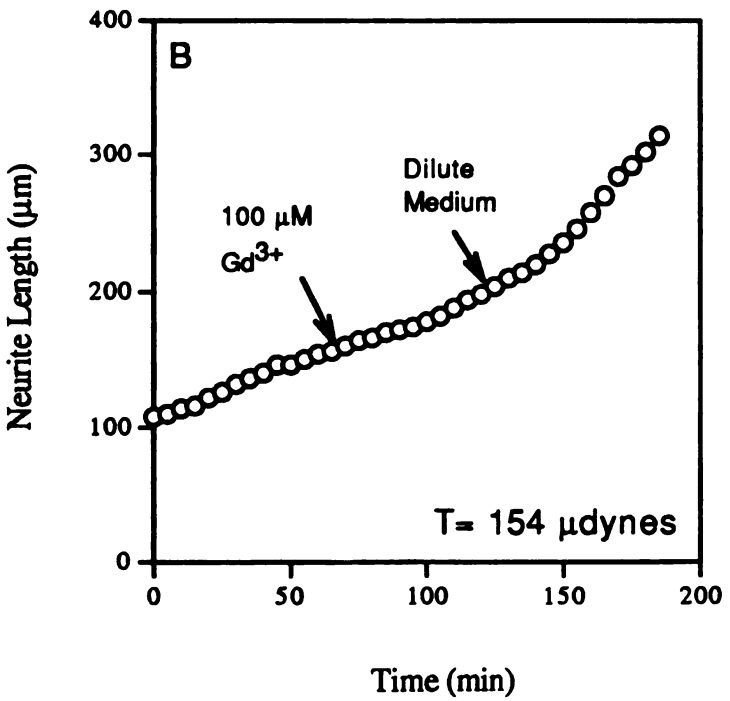
A second experimental design to control for possible Ca^{++} effects was to hold Ca^{++} concentrations constant but otherwise dilute inorganic ions by 50%, i.e. the normal culture medium was diluted 1:1 with medium lacking most inorganic salts but containing the normal concentration of Ca^{++} (1.25 mM). This osmotic dilution at constant extracellular [Ca^{++}] stimulated an increase in towed elongation rate, as shown in Fig.5B. In three such experiments, the increase in elongation rate at constant force increased from 51% to 128%, similar to the Ca^{++}-inclusive osmotic dilutions shown in Table 2a. The data from these two experimental designs indicated that the osmotic dilution stimulated elongation is not due to the reduction of extracellular Ca^{++}.

2.3.2. Effect of gadolinium ions

To examine the possible involvement of stretched-activated/-inactivated ion channels (SA or SI channels) in the mechano-transduction of axonal growth, a pharmacological approach was employed specifically. Gadolinium ions (Gd^{3+}) with concentrations of 1-50 μM have been shown to block the activities of SA and SI channels (Yang and Sach, 1989; Franco et al., 1991; Quasthoff, 1994). To prevent formation of Gd^{3+} and PO_4^{3-} precipitation in this experiment, DRG neurons were cultured in Hepes buffered L-15 medium, rather than PO_4^{3+}

Figure 6- Effect of Gd^{3+} on elongation rate and the osmotic dilution stimulation effect--experimental protocol and analysis similar to Fig. 2. Panel A -- Gadolinium chloride (10 mM in 10 mM HEPES buffer pH 7.4) was added to the culture medium to achieve final concentrations of 50 mM and 100 mM. As shown, these additions had no effect on the rate of elongation of the neurite at constant force. Panel B -- Following a period of towing in normal medium, $GdCl_3$ was added to a final concentration of 100 mM without effect on the elongation rate, as before. Following one additional hour of towed growth, the culture was diluted with an equal volume of medium lacking all inorganic salts, except 100 mM $GdCl_3$. As shown, the stimulation of axonal elongation by osmotic dilution persisted in the presence of Gd^{3+}.


A
100 µM
Gd3+
50 µM
Gd3+
T= 148 µdynes
Neurite Length (µm)
Time (min)
0
50
100
150
200
250

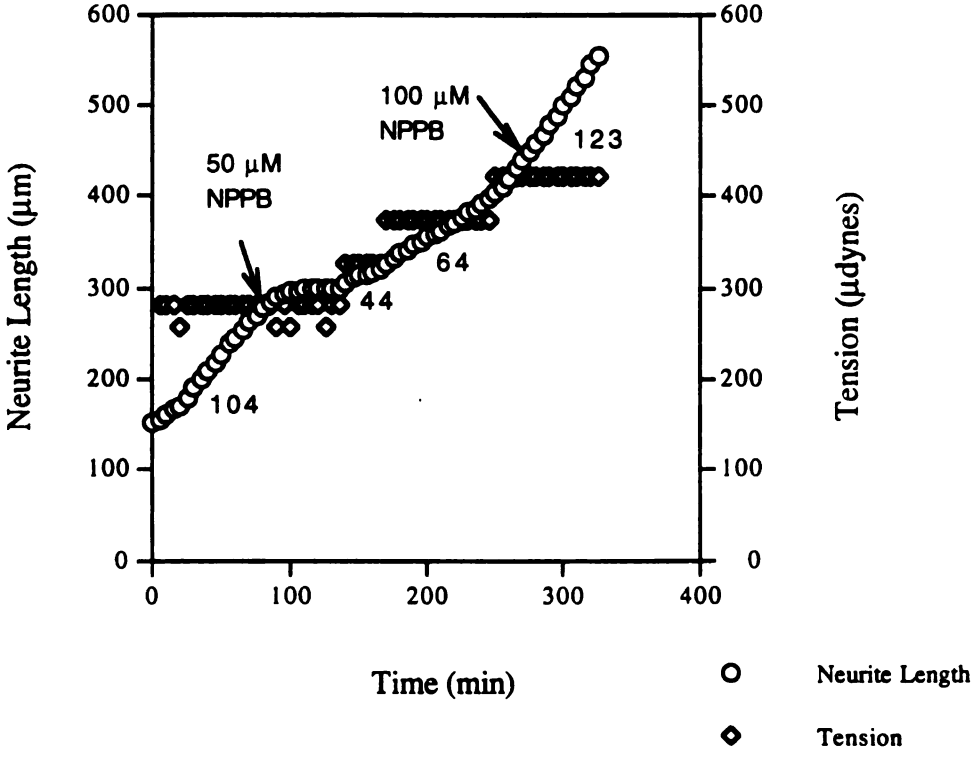

B
Dilute
Medium
100 µM
Gd3+
T= 154 µdynes
Neurite Length (µm)
Time (min)
0
100
200
300
400
50
150

containing medium, for 16-24 hours prior to one-hour towing and the following Gd^{3+} treatment. In three experiments in which Gd^{3+} was added to this culture medium to a final concentration of 100 µM, we observed no effect on the rate of experimentally induced neurite elongation (Fig. 6A). We also performed experiments in which 100 mM Gd^{3+} was added prior to 1:1 osmotic dilution (but Gd^{3+} kept constant at 100 µM). The presence of 100 mM Gd^{3+} failed to inhibit the increase in elongation rate observed after osmotic dilution (Fig. 6B). As illustrated in Fig. 6, the addition of gadolinium ions to block mechanosensitive ion channels was without effect on the tension-induced elongation rate or on the inhibition of growth stimulation by osmotic dilution.

2.3.3. Effect of NPPB and reduced extracellular [Cl^-]

50 µM-100 µM 5-nitro-2(3-phenylpropylamino)-benzoic acid (NPPB) has been demonstrated to markedly inhibit the activity of swelling-induced Cl^- channel and inhibited RVD (DreinhÖfer et al., 1988; Gschwentner et al., 1996). To examine the possible role of swelling-induced Cl^- channels, three experiment series were performed. We first tested the effect of NPPB on tension-regulated growth (n = 3). As shown in Fig. 7, 50 µM NPPB was added approximately one hour after the neurite had elongated steadily at a constant force; the addition of NPPB effectively inhibited the neuronal lengthening. However, when applied tension was increased, the neurite started elongating, and the rates of elongation

Figure 7- Inhibition effect of NPPB on tension-mediated elongation.-- experimental protocol and analysis similar to Fig. 1. Numbers adjacent to the line of neurite length were growth rates corresponding to that period of time. The neurite was towed for one hour at a constant force of about 280 µdynes and elongated at the rate of 104 µm/hr. Then 50 µM NPPB was added, which gradually slowed down and stopped neuronal elongation at the same towing force. When the applied tension was raised to 326 µdynes, the neurite began to elongate at the speed of about 44 µm/hr. The lengthening was faster in response to higher applied tension. As shown here, the second highest level of tension applied was 373 µdynes which resulted in a growth rate of 64 µm/hr. The highest level of force applied was 420 µdynes which induced a rate of elongation at 123 µm/hr. The neurite continued lengthening at the speed of 123 mm/hr even after 100 µM NPPB was added.

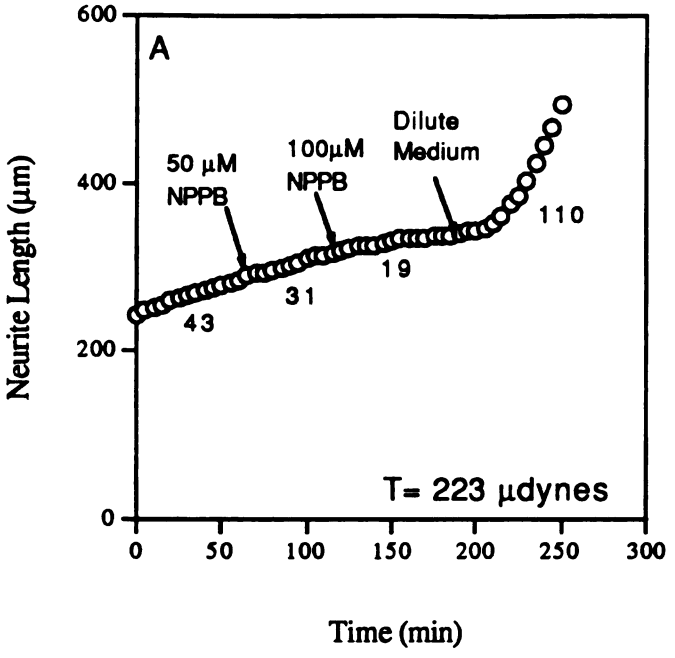


were proportional to tension applied. The highest tension we applied in Fig. 7 (420 μdynes in this case) caused even a bigger growth rate (123 μm/hr) than the initial rate (104 μm/hr). In addition, the neurite continued to elongate at the same rate of 123 μm/hr in spite of further addition of NPPB (100 μM). The results indicated that tension could overcome the inhibition of NPPB. The activity of this swelling-activated Cl- channel is not essential for tension-regulated growth.

We then tested the effect of NPPB on osmotic dilution. As illustrated in Fig. 8, there was a dose-dependent growth rate decrease (a lesser degree of rate decrease at 50 μM NPPB than at 100 μM NPPB) following the intervention of NPPB. However, when medium osmolarity was reduced by dilution, the neurite extension rate increased within 10-20 minutes and the relative rate increase varied from 81% to 156%, once again quite typical of osmotic dilution stimulation (Fig. 8A). We did another set of experiments with reverse order of treatments (n = 2). The medium osmolarity reduction accelerated rates of axonal lengthening as expected (Fig. 8B), while the addition of NPPB up to 100 μM did not cause significant changes on the elongation rate. In conclusion, although NPPB changed the sensitivity of tension-induced elongation, we observed that the stimulatory effect of osmotic dilution was not affected by NPPB treatment.

To further investigate the effect of chloride ions, we conducted experiments in the presence of low $[Cl^-]_o$, 2.5 mM

Figure 8- Effect of NPPB on elongation rate and the osmotic dilution stimulation effect--experimental protocol and analysis similar to Fig. 2. Numbers shown adjacent to the lines are neuronal growth rates (μm/hr). Panel A -- NPPB was added to the culture medium to achieve final concentration of 50 μM and 100 μM. Addition of NPPB dose-dependently reduced the axonal elongation at the same constant force but did not inhibit the osmotic stimulation of growth. The NPPB concentration was still kept at 100 μM after medium dilution. Fig. 8A is data from one of three experiments. Panel B -- Osmotic dilution was performed 45 minutes prior to the application of NPPB. As shown, the stimulation of axonal elongation by osmotic dilution persisted in the presence of up to 100 μM NPPB . Fig. 8B represents data from one of two trials.


A
600
400
200
0
Neurite Length (µm)
50 µM NPPB
100µM NPPB
Dilute Medium
43
31
19
110
T= 223 µdynes
0
50
100
150
200
250
300
Time (min)

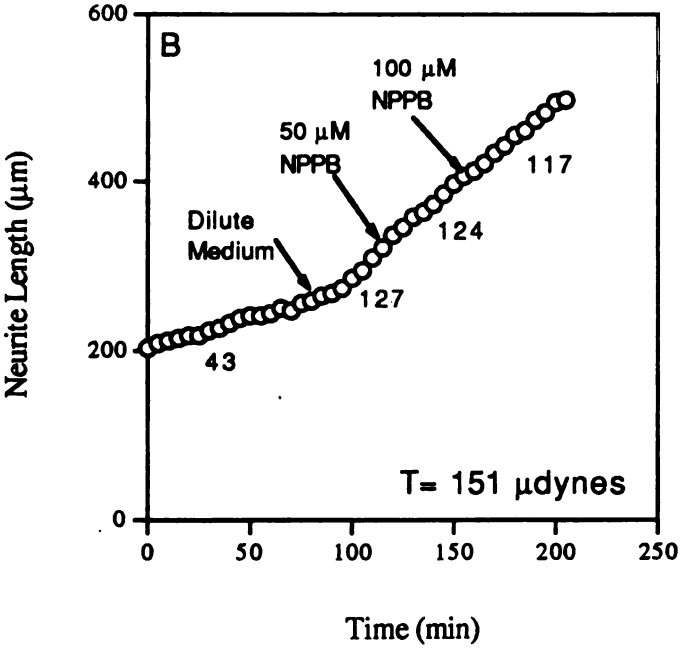
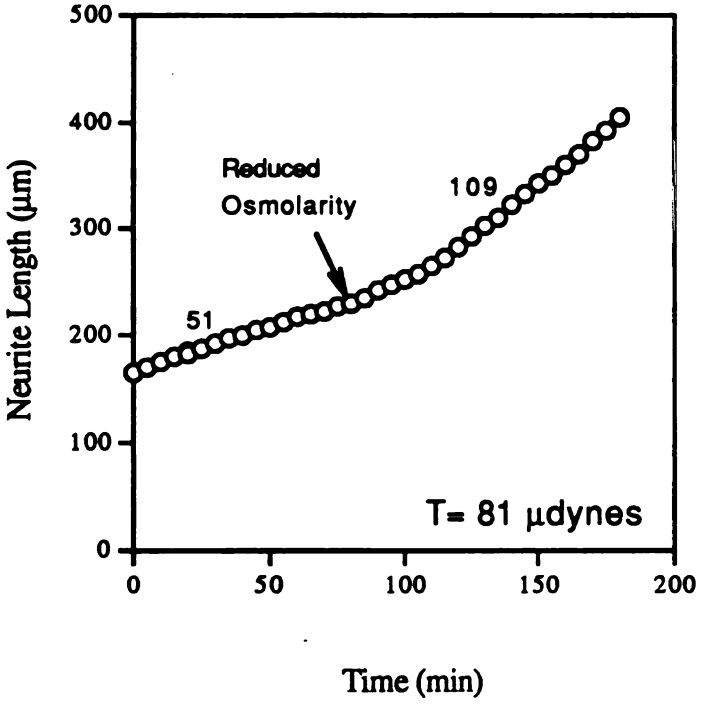

B
600
400
200
0
Neurite Length (µm)
100 µM NPPB
50 µM NPPB
Dilute Medium
43
127
124
117
T= 151 µdynes
0
50
100
150
200
250
Time (min)

Figure 9- Effect of reduced extracellular Cl^- on the osmotic dilution stimulation effect.-- experiment protocol and analysis were similar to Fig. 2, except that neurons were incubated for 90 minutes in an isosmotic low-Cl^- L-15^+ medium (see Material and Methods) in which chloride ions were replaced by gluconate salts before towing. After being towed for more than an hour at a given force, the neuron was subjected to osmotic dilution treatment. As shown, reduced extracellular Cl^- did not inhibit dilution stimulated growth. Figure 8 represents results from one of four experiments.



rather than the normal concentration of 145 mM (please see Material and Methods). In these experiments, neurons were first cultured in fortified L-15 medium for 16-24 hours, then were pre-incubated in low Cl^- $L\text{-}15^+$ medium, which was prepared basically by replacing most Cl^- salts with the corresponding gluconate salts, for 90 minutes prior to the towing experiments. All four neurites elongated steadily after being subjected to a given pulling force in the absence of extracellular Cl^-. A significant growth rate increase (68%-114%) was observed after the osmotic dilution. The reduction of extracellular chloride concentration did not hinder the growth rate increase stimulated by osmolarity dilution (Fig. 9).

2.3.4 Effect of K^+-depolarization

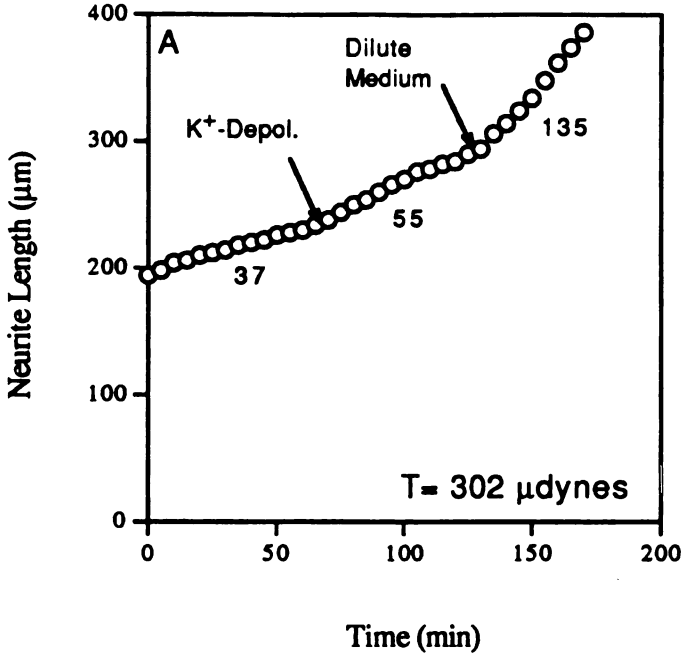
Hypotonicity-induced swelling has been shown to cause membrane potential depolarization in cultured rat astrocytes (Kimelberg and O'Conner, 1988; Kimelberg and Kettenmann, 1990). In many studies, depolarization conditions are known to elicit the release of some organic osmolytes such as taurine and aspartate (Schousboe and Pasantes-morales, 1989; Kimelberg et al., 1990). We investigated whether the osmotic effect was linked to membrane depolarization by elevating extracellular K^+ because high $[K^+]_o$ depolarizes membrane effectively and is widely used in *in vitro* studies. Previous reports indicated that addition of up to 25 mM extracellular KCl to the medium could elicit membrane depolarization in cerebellar granule neurons (Bessho et al., 1994) and *Helisoma*

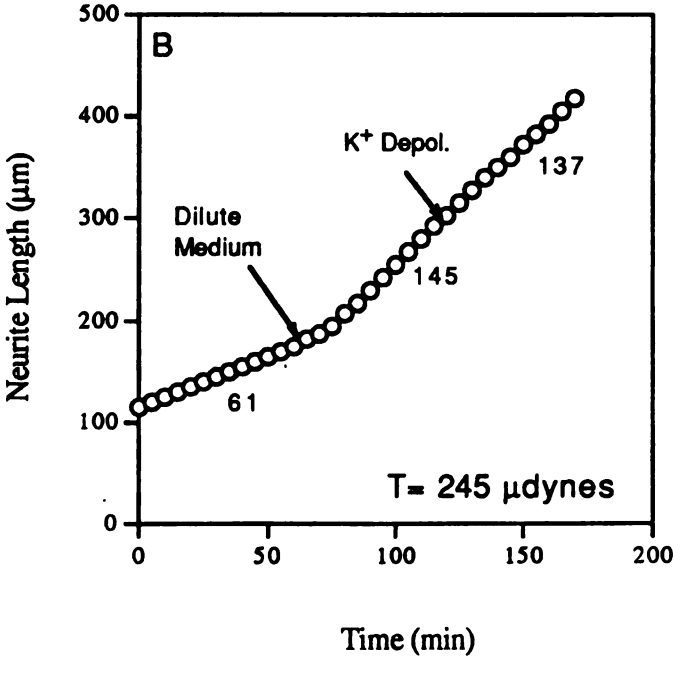
trivolvis B5 neurons (Berdan et al., 1993). Similarly, we used a final depolarizing $[KCl]_o$ concentration of 25 mM to ask whether there is any connection between osmotic dilution and membrane depolarization.

In a typical experiment illustrated in Fig. 10A, neuronal growth rate increased from 37 μm/hr to 55 μm/hr shortly after membrane depolarization induced by addition of KCl to the medium . The neurite extended quite steadily at the rate of 55 μm/hr for an hour until we diluted the ionic strength to 50%, which dramatically enhanced the outgrowth rate to 135 μm/hr (about 145% increase in this case). In three trials, the K^+-depolarization caused relative rate increases of 22%, 48% and 77% respectively, while osmotic dilution caused additional rate increases of 59%, 145% and 57%. We observed an additive effect on the rate of neuronal elongation when neurons were first depolarized by high K^+ and then treated with hyposmolarity shocks.

An alternative design to investigate the role of depolarization was to perform osmotic dilution before K^+-depolarization. Fig. 10B represented one of the experiments ($n = 3$) which K^+-depolarization was performed one hour following the osmotic dilution. Not surprisingly, the rate of neurite lengthening was enhanced from 61 μm/hr to 145 μm/hr shortly after hyposmolarity intervention. The growth rate was 137 μm/hr after high $[K^+]_o$ depolarization, which was slightly lower than 145 μm/hr. Interestingly, all three

Figure 10- Effect of K^+-depolarization on elongation rate and the osmotic dilution stimulation effect.-- experimental protocol and analysis similar to Fig. 2. K^+-induced depolarization was done by addition of extra KCl to the cultured medium to achieve a depolarizing final extracellular concentration of 25 mM KCl. Panel A -- K+-depolarization and osmotic reduction additively enhanced neuronal elongation. Fig. 9A is the result from one of three examples. Panel B -- Growth rate changes of the neurite that was first subjected to osmotic dilution treatment and then depolarized by 25 mM extracellular KCl. Panel B is the result from one of three examples.


A
Dilute
Medium
K+-Depol.
135
55
37
T= 302 µdynes
400
300
200
100
0
0
50
100
150
200
Neurite Length (µm)
Time (min)

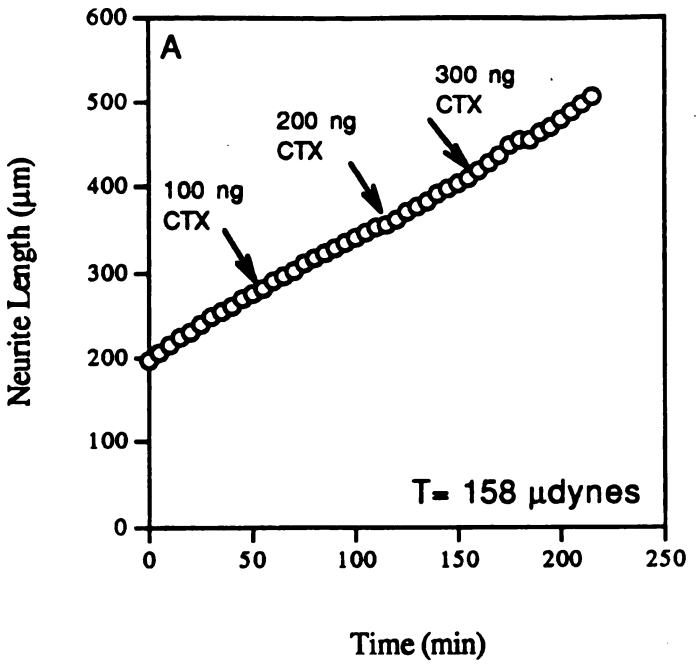

B
K+ Depol.
137
Dilute
Medium
145
61
T= 245 µdynes
500
400
300
200
100
0
0
50
100
150
200
Neurite Length (µm)
Time (min)

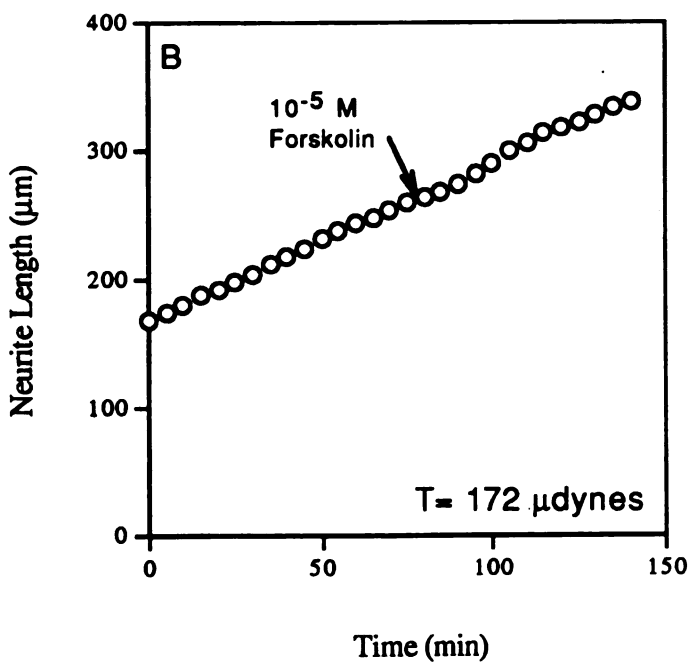
experiments showed rate reduction after K^+-depolarization, but all were very small (4%, 5.5% and 10% respectively).

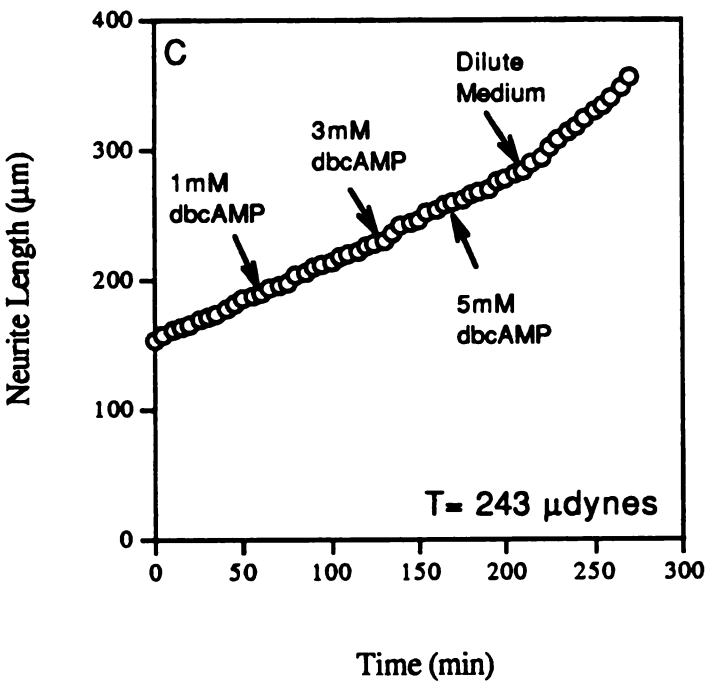
2.3.5. Effect of cyclic AMP

We wished to test the involvement of cAMP in osmotic dilution stimulation of elongation because: 1). osmotic swelling has been shown to induced cAMP accumulation in turkey erythrocytes (Morgan et al., 1989) and mouse lymphoma cells (Watson et al., 1989); and 2). cAMP has been shown to stimulate neuronal outgrowth in neuroblastoma (Nakagawa-Yagi et al., 1992) and sympathetic neurons (Tolkovsky 1987). Three different kinds of treatments were performed to assess a possible role of cAMP in neuronal towed elongation and/or mediation of the osmotic effect. As much as 300 ng cholera toxin, which stimulates Gs protein irreversibly, was without effect on the elongation rate of neurites at constant force (n = 2) (Fig. 11A). Application of forskolin (n = 3), which directly activates adenylate cyclase independent of Gs stimulation, did not cause any changes on the elongation rate of neurites, either (Fig. 11B). Finally, direct application of cAMP in the form of dibutyryl cAMP at increasing concentrations up to 5 mM was also without effect on the elongation rate of the towed neurons in five experiments, and, further, the presence of 5 mM dbcAMP did not inhibit the osmotic effect (Fig. 11C). Interventions intended to increase the cytoplasmic concentration of cyclic AMP were

Figure 11 -Effect of cyclic AMP on elongation rate and the osmotic dilution stimulation effect-- experimental protocol and analysis similar to Fig. 2. (panel A) -- Cholera toxin (CTX, 100 mg/ml) was added at various times during the course of towed axonal elongation to final concentrations of 100, 200, and 300 ng/ml of culture medium. As shown, the toxin had no effect on the elongation rate in response to constant tension. (panel B) Forskolin (2.5 mM in DMSO) was added during the course of towed axonal elongation to a final concentration of 10^{-5} M, without effect on towed elongation rate. (panel C) Dibutyryl cyclic AMP (dbcAMP, 100 mM in phosphate buffered saline) was added to the culture medium at various times during the course of towed axonal elongation to final concentrations of 1, 3, and 5 mM, with no effect on towed elongation rate. Following 1 hour of towing in medium containing 5 mM dbcAMP, the medium was diluted with medium lacking all inorganic salts. The axonal elongation rate at constant force increased 74% in the presence of the cyclic AMP.


A
300 ng
CTX
200 ng
CTX
100 ng
CTX
T= 158 µdynes
Neurite Length (µm)
600
500
400
300
200
100
0
0
50
100
150
200
250
Time (min)


B
10-5 M
Forskolin
T= 172 µdynes
Neurite Length (µm)
400
300
200
100
0
0
50
100
150
Time (min)

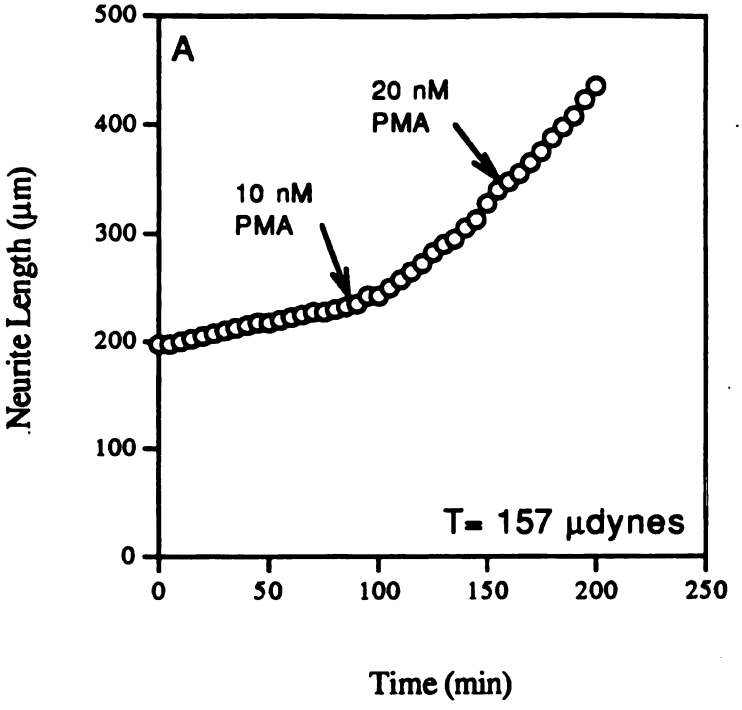

C
Dilute
Medium
3mM
dbcAMP
1mM
dbcAMP
5mM
dbcAMP
T= 243 µdynes
400
300
200
100
0
0
50
100
150
200
250
300
Neurite Length (µm)
Time (min)

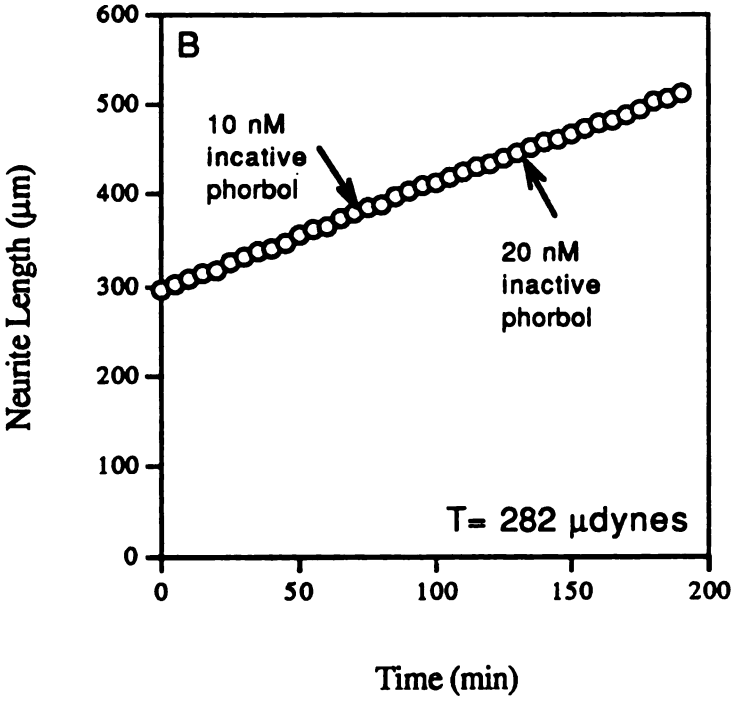
without effect on the tension-induced elongation rate, or its stimulation by osmotic dilution.

2.3.6 Effect of protein kinase C (PKC) activation on tension-induced elongation

Information on the role of PKC activation in osmotic swelling is quite scarce. PKC activation may play a regulatory role in some osmoregulation transporters (Grinstein et al., 1992). However, PKC activation has been shown to regulate neuronal outgrowth in a variety of neurons (Hsu et al., 1989; Camponot et al., 1991). We tested whether PKC is involved in osmotic dilution-stimulated elongation. Phorbol ester (PMA) is a potent activator of PKC. At namomolar concentrations, PMA significantly increases the affinity of PKC for Ca^{++} resulting in full activation of PKC (Nishizuka 1986). We first examined the effect of PKC activation on towed growth (n = 5). As shown in Fig. 12A, the neurite was towed at a given force and elongated steadily for more than an hour. Addition of 10 nM PMA caused a rapid (within 5-10 minutes) and significant increase (296% increase in this case) in the rate of neuronal lengthening. Additional PMA to 20 nM exerted a further stimulatory effect, while 5 nM PMA stimulated the neuronal growth only with slight increase (data not shown). Treatment of cells (n=3) with up to 20 nM 4α-12,13 didecanoate, an inactive phorbol, as a control for PKC involvement had no effect on the rate of elongation (Fig. 12B).

Figure 12 - Effect of PMA and 4α-phorbol 12,13-didecanoate on elongation rate at constant force -- Experimental protocol and analysis similar to Fig. 1. Panel A -- Phorbol 12-myristate 13 acetate (PMA) was added to the culture medium to achieve final concentrations of 10 nM and 20nM. As shown here, these additions caused a dramatically increase in the rate of elongation within 10 minutes. Fig. 12A is Exp. 4 in Table 4A. Panel B-- 10 nM and 20 nM 4α-12,13 didecanoate (inactive phorbol) were added to the culture medium as a negative control for PMA. Addition of 4α-12,13 didecanoate had no effect on the rate of elongation of the neurite at constant force. (n=3)


A
20 nM
PMA
10 nM
PMA
T= 157 µdynes
Neurite Length (µm)
Time (min)
0
100
200
300
400
500
0
50
100
150
200
250


B
10 nM
incative
phorbol
20 nM
inactive
phorbol
T= 282 µdynes
Neurite Length (µm)
Time (min)
0
100
200
300
400
500
600
0
50
100
150
200

The stimulatory effect of PKC activation elongation is intriguing to us, as it is the only pharmacological intervention we have tried that has caused an immediate and dramatic growth rate increase like the osmotic effect. Table 3a compares growth rate changes in all five neurons before and after PMA intervention (Fig 12A represents Exp.4 in Table 3a). Although the responses varied from cell to cell, the qualitative variability is similar to that we observed in osmotic effect. We wished to further investigate the effect of PKC activation on tension-induced elongation and to determine whether PKC-activation is a required mediator of axonal elongation and whether it also mediates dilution-stimulated axonal elongation.

Diacylglycerols (DAG) binds to and activates PKC (Mori et al., 1982) as the normal physiological stimulator of its activity. DAG with various fatty acids in the 1,2-*sn* configuration is active, while its isomer 1,3-DAG neither activates nor inhibits PKC (Boni et al., 1985). Like PMA, addition of 1,2-dioctanoyl-*sn*-glycerol (1,2-*sn*-DAG) induced rapid and significant extension rate increases for neurons at a given force (Fig. 13A). Again, different neurons responded differently as to the minimal doses of DAG needed for activation and relative rate changes (Table 3b), but, generally, application 20 nM 1,2-*sn*-DAG could elicit immediate and robust rate increase. Addition of 1,3-dioctanoyl glycerol (1,3-DAG) up to 60 nM showed no

Table 3a. Changes in the axonal growth rate (GR) before and after PMA treatment.

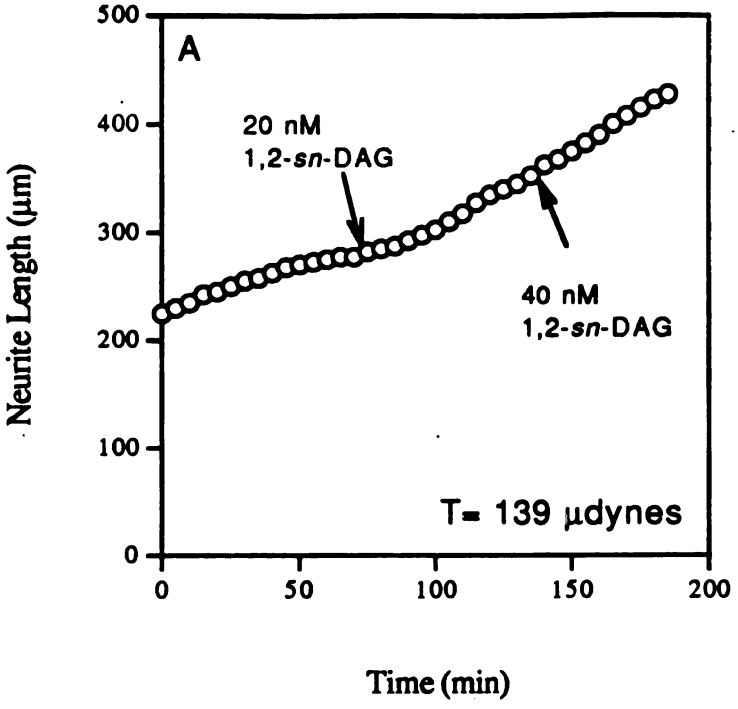
	Growth Rate before PMA treatment(um/hr)	Relative GR increase (10nM PMA treatment)	Relative GR increase (20nM PMA treatment)	Tension applied(udynes)
Exp.1	70	100 um/hr(43%)	137 um/hr (96%)	231
Exp.2	23	46 um/hr (100%)	47 um/hr (104%)	97
Exp.3	53	84 um/hr (59%)	100 um/hr (87%)	231
Exp.4	24	95 um/hr (296%)	129 um/hr (438%)	157
Exp.5	18	43 um/hr (139%)	78 um/hr (333%)	265

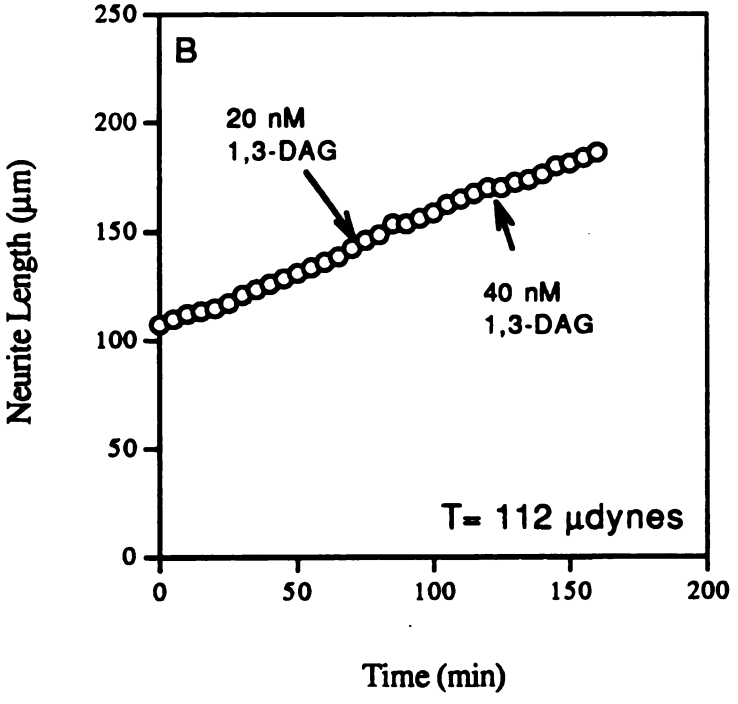
Table 3b. Changes in the axonal growth rate (GR) before and after 1,2 syn DG treatment.

	Growth Rate before treatment (um/hr)	Relative GR increase (20nM treatment)	Relative GR increase (40nM treatment)	Tension applied(udynes)
Exp.1	41	84 um/hr (105%)	92 um/hr (124%)	139
Exp.2	32	77 um/hr (141%)	101 um/hr (216%)	137
Exp.3	30	31 um/hr (3%)	56 um/hr (87%)	80
Exp.4	57	*82 um/hr (44%)	**200 um/hr (251%)	200
EXp.5	38	*79 um/hr (108%)		155
Exp.6	37	54 um/hr (46%)		251

* : 10 nM 1,2 syn DG treatment.
** : 20 nM 1,2 syn DG treatment.

Figure 13 - Effect of synthetic diacylglycerols on elongation rate at constant force.-- experimental protocol and analysis similar to Fig.1. Panel A -- An active from of synthetic diacylglycerol 1,2-dioctanoyl-*sn*-glycerol (1,2-*sn*-DAG) was added to the culture medium to achieve final concentrations of 20 nM and 40 nM. The addition of DAG significantly increased the rate of elongation at the same given force. Panel A is the Exp.1 from Table 3B. Panel B -- 1,3-dioctanoyl glycerol (1,3-DAG; inactive form) was used as a negative control for 1,2-*sn*-DAG. In two control neurites, application of 1,3-DAG had no influence on elongation rate.


A
20 nM
1,2-sn-DAG
40 nM
1,2-sn-DAG
T= 139 µdynes
Neurite Length (µm)
Time (min)
0
100
200
300
400
500
0
50
100
150
200


B
20 nM
1,3-DAG
40 nM
1,3-DAG
T= 112 µdynes
Neurite Length (µm)
Time (min)
0
50
100
150
200
250
0
50
100
150
200

stimulatory or inhibitory effects on the growth rate (n = 2) (Fig. 13B).

To further test for the role of PKC activation in tension-regulated axonal growth, we treated cells with PKC inhibitors to see if PKC activation is necessary for tension-induced elongation. Chelerythrine is a potent and rather specific PKC inhibitor. It inhibits only PKC activity at low micromolar concentrations. In all three trials, the tension-included elongation was retarded or even stopped in the presence of 5-10 μM chelerythrine (Fig. 14). However, neurites started to elongate at higher tension and the rates of lengthening were proportional to the amount of tension applied. Thus, inhibition of PKC inhibits towed elongation rate, but PKC does not appear to be required for tension to stimulate axon elongation.

2.3.7. PKC activation and the osmotic dilution effect

To determine whether osmotic dilution activated PKC, we combined osmotic dilution intervention with either PKC activator or inhibitor treatments. Treatment of 20 nM active DAG enhanced neurite lengthening, and the following osmotic dilution stimulated further significant increases of growth rate (Fig. 15A). However, when we reversed the order of treatments (Fig. 15B), application of 20 nM DAG caused only very subtle growth rate rise (or even no rate changes in the other two neurites) after the neurite elongation had been increased by osmolarity reduction. A similar pattern of

Figure 14- Inhibition effect of chelerythrine on tension-mediated elongation.-- experimental protocol and analysis similar to Fig. 1. T-values represented three steps of tension applied. 3 µM PKC inhibitor chelerythrine was first added to the culture medium after the neurite was towed for one hour at a constant force of about 166 µdynes. After chelerythrine concentration was increased to 10 µM, neurite elongation gradually slowed down and stopped at the same towing force. When the applied tension was raised, the neurite began to elongate. The lengthening was faster in response to higher applied tension. As shown here, the second highest level of tension applied was 211 µdynes which resulted in a growth rate of 76 µm/hr. The highest level of force applied was 255 µdynes which induced a rate of elongation at 100 µm/hr.

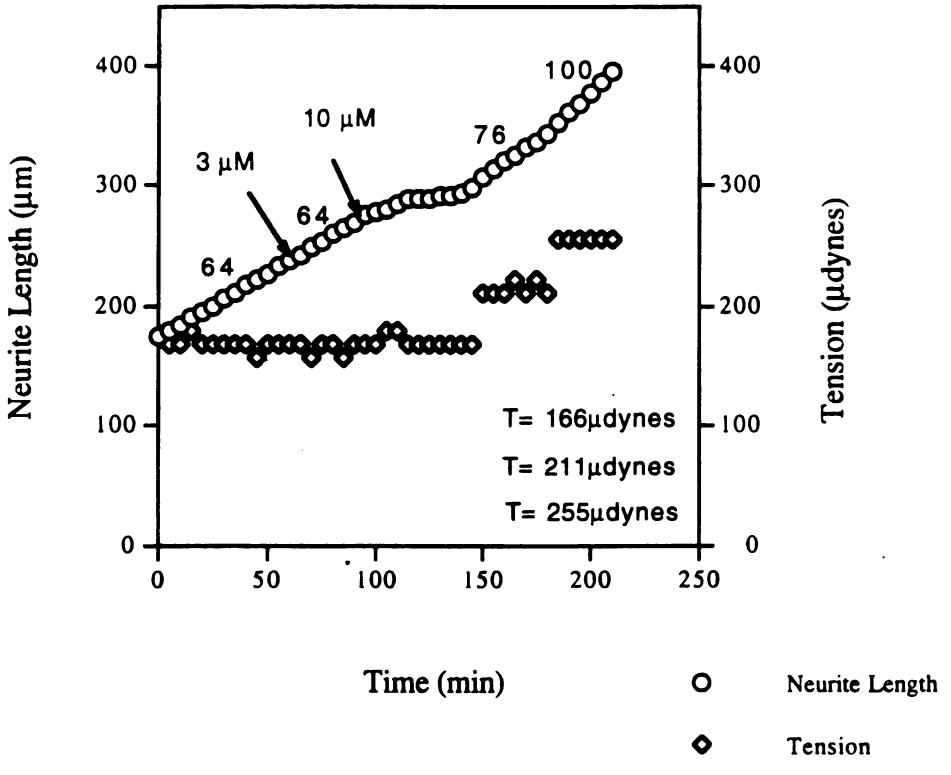
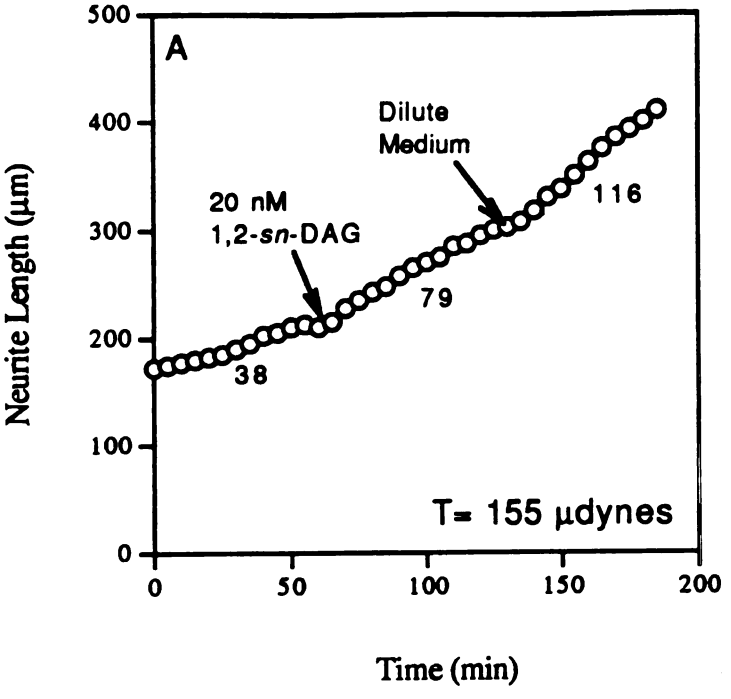
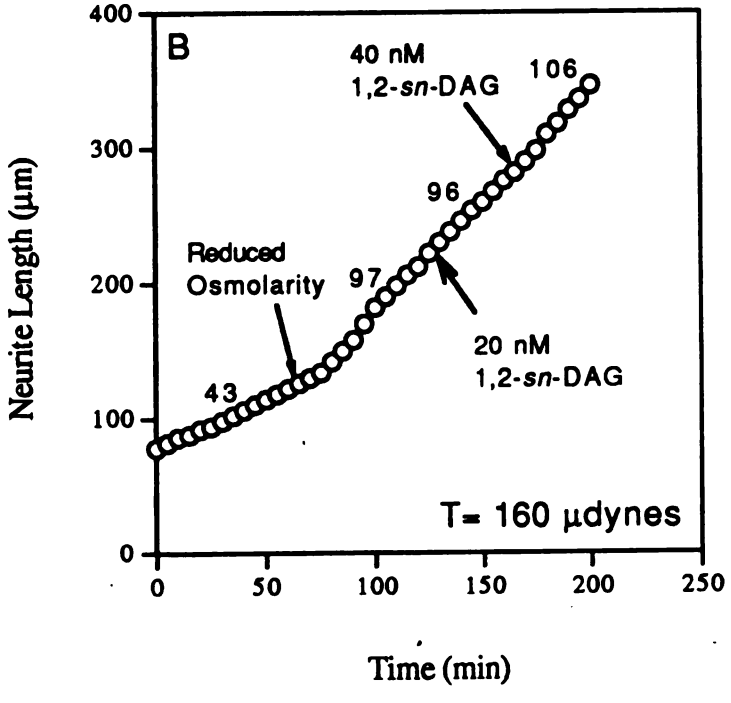


Figure 15- Effect of synthetic DAG on the osmotic dilution-stimulated elongation.-- experimental protocol and analysis similar to Fig. 2. Panel A -- Treatments of 1,2-*sn*-DAG and osmotic dilution in series resulted in additive effects on the rate of axonal elongation. As shown in the figure, the growth rate increased from 38 µm/hr to 79 µm/hr after the addition of 20 nM 1,2-sn-DAG. The following osmotic dilution intervention caused an even faster lengthening rate (116 µm/hr). Panel B -- Treatments of 1,2-*sn*-DAG following osmotic dilution did not yield further significant rate increases, as can be told from the graph. The growth rate 97 µm/hr was calculated from 10 minutes after the osmotic dilution till the time DAG was added.


A
Dilute
Medium
20 nM
1,2-sn-DAG
116
79
38
T= 155 µdynes
Neurite Length (µm)
Time (min)
0
100
200
300
400
500
0
50
100
150
200


B
40 nM
1,2-sn-DAG
106
96
Reduced
Osmolarity
97
20 nM
1,2-sn-DAG
43
T= 160 µdynes
Neurite Length (µm)
Time (min)
0
100
200
300
400
0
50
100
150
200
250

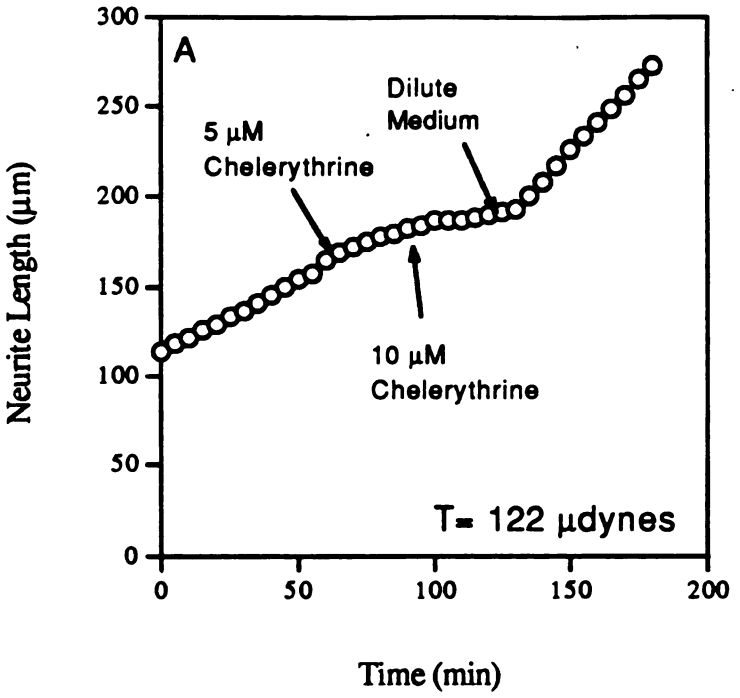
results was also obtained from similar experiments in which PMA was combined with osmotic dilution (data not shown).

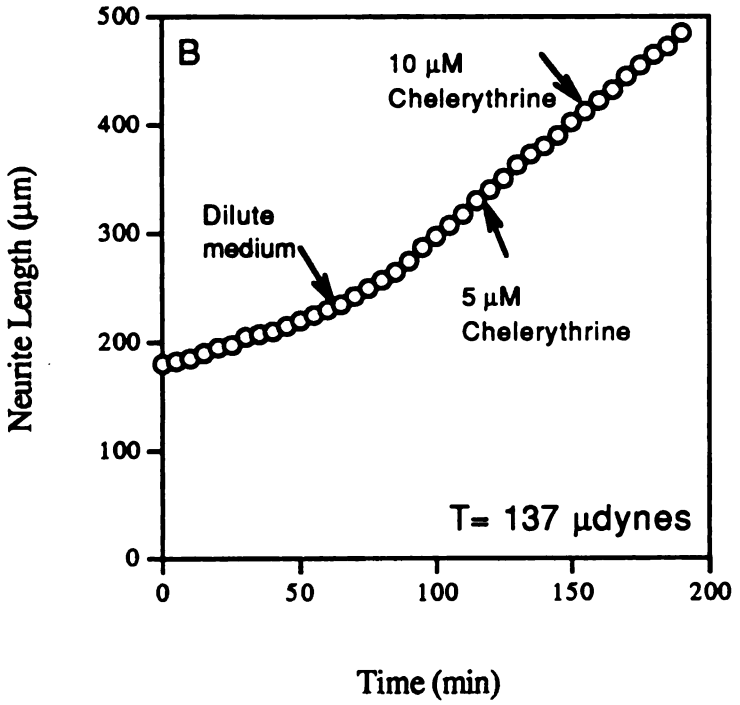
Although chelerythrine inhibits tension-regulated axonal growth, it did not have inhibitory effects on dilution -stimulated neuronal elongation. As illustrated in Fig. 16A, the presence of chelerythrine decreased rate of neuronal extension dramatically; however, reduction of medium osmolarity (the concentration of chelerythrine was maintained at 10 μM) soon increased the rate of elongation to 163% of the initial rate (n = 2). Three experiments in which treatments of chelerythrine followed osmotic dilution interventions did not alter the growth rate or caused only about 5% rate decrease (Fig. 16B).

2.3.8. Effect of osmotic dilution on the rest tension of neurites

To test the possibility that osmotic swelling itself might exert growth-related physical effect on the neurites, we measured the neurites rest tension. Interestingly, our results indicated that medium dilution exerted a direct mechanical effect on the neurite, viz. a decrease in the static tension normally borne by the neurite shaft as a result of growth cone pulling or other physiological phenomena. This "rest tension," as well as the stiffness of the neurite to distension (i.e. the spring constant of the neurite shaft) is measured by a neurite plucking technique outlined in Materials and Methods. In contrast to the

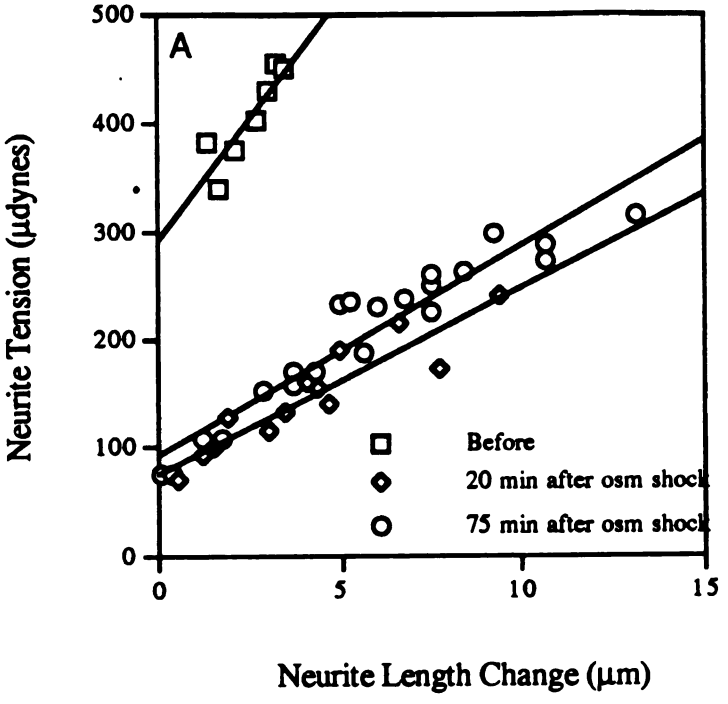
Figure 16- Effect of chelerythrine on the osmotic dilution-stimulated elongation.-- experimental protocol and analysis similar to Fig. 2. Panel A -- 5-10 μM chelerythrine reduced neuronal elongation at the constant towing force, but did not inhibit the osmotic stimulation effect. The concentration of chelerythrne was still kept at 10 μM after medium dilution. The initial growth rate in this graph was 51 μm/hr. The growth rate after medium dilution rose up to 83 μm/hr. Graph A is the result from one of two experiments. Panel B -- The order of treatments was reversed from Panel A. The osmotic dilution enhanced neuronal lengthening dramatically (about 140% increase), while further addition of PKC inhibitor did not have significant impact on the growth rate changes. Graph B represented the result from one of three experiments.

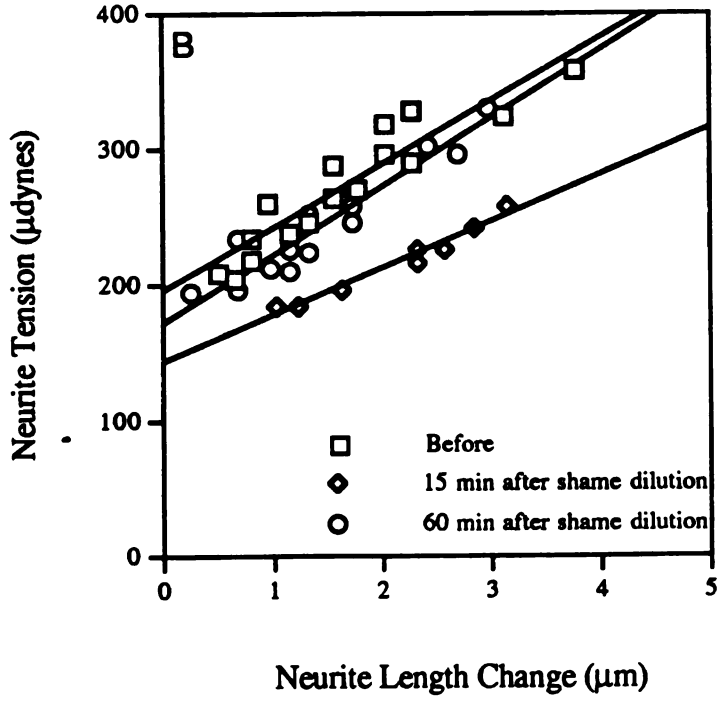

A
Dilute
Medium
5 µM
Chelerythrine
10 µM
Chelerythrine
T= 122 µdynes
Neurite Length (µm)
Time (min)
0
50
100
150
200
250
300


B
10 µM
Chelerythrine
Dilute
medium
5 µM
Chelerythrine
T= 137 µdynes
Neurite Length (µm)
Time (min)
0
100
200
300
400
500

technique of towing the neurite at constant force, neurites behave elastically during the rapid plucking intervention, and neurite tension increases linearly with neurite distension. The rest tension is taken to be the y-intercept (i.e. the extrapolated tension at zero experimental lengthening), and the slope of the line is the spring constant of the neurite. One of five experiments in which rest tension was measured before and after osmotic dilution is shown in Fig. 17A. In all five experiments the rest tension was found to decrease. In two cases, including that shown in Fig. 17A, the rest tension following dilution of the medium declined to less than half the original value; in the remaining three cases the rest tension declined to approx. 2/3 the original value. Control experiments in which rest tension was measured before and after a sham dilution (addition of normal medium) showed some scatter in the values of rest tension taken at various times following addition of medium, as shown in Fig. 17B, possibly reflecting physiological changes in rest tension as these neurites grew. In no case did the rest tension decline to a value less than 3/4 of the original. With respect to the stiffness of the neurite, the situation was somewhat more complex. When measured at times longer than 30 minutes, all experimental neurites showed a decline in spring constant. However, two neurites showed slightly increased spring constants when measured at 15 or 20 minutes. Furthermore, 2 of 3 control

Figure 17 --Rest tensions of chick sensory neurites following the osmotic dilution or sham dilution of the medium. Neurite tension was plotted as a function of the change in neurite length caused by plucking of the neurite with calibrated glass needles. The elastic response of neurites is extrapolated to the y-intercept to provide a value of the neurite's rest tension (i.e. without any applied distension). Panel A -- Elastic response of neurites prior to, and 20 and 75 minutes after, osmotic dilution as in Fig. 1a. The rest tension declined in all five experiments similar to that shown here. Panel B -- As in panel A except that the data points were taken after a sham dilution as in panel 1b.


A
500
400
300
200
100
0
0
5
10
15
Neurite Tension (µdynes)
Neurite Length Change (µm)
Before
20 min after osm shock
75 min after osm shock


B
400
300
200
100
0
0
1
2
3
4
5
Neurite Tension (µdynes)
Neurite Length Change (µm)
Before
15 min after shame dilution
60 min after shame dilution

neurites also showed declines of spring constant 30 or more minutes after sham dilution.

2.4. Discussion

Previous experiments have shown that in cultured chick sensory neurons, mechanical tension is a potent stimulator of axonal growth rate (Heidemann and Buxbaum, 1994), which is also stimulated by osmotic dilution (Bray et al., 1991). Our lab is the first group trying to seek a potential mechanism for the transduction between the mechanical tension and axonal growth (mechanotransduction). Attempts to understand the mechanotransduction mechanism(s) have been restricted by technical limitations which allow us to pull only one neurite at a time, and the lack of previous work. In this dissertation, we searched for the possible chemical signal mechanism and/or physical factor that mediate the osmotic dilution effect because of several reasons: 1). the axonal responses that characterized the immediate and prolonged increases in growth rate induced by both mechanical tension and osmotic dilution are quite similar; 2). osmoregulation plays an important role in neuronal physiological and pathophysiological activities (please refer to the general Introduction); 3). although still not well understood, mechanisms involved in neuronal responses to osmotic swelling are better studied than mechanotransduction per se, and this provided us with useful information. We hoped that searches of the chemical/physical candidates mediators of osmotic dilution effects could provide insight into signals involved in tension-regulated towed growth.

In chapter one, we demonstrated that axonal elongation is, indeed, coupled to the tensile regulation of growth, i.e., osmotic dilution of medium shifted the cultured chick sensory neurons' growth sensitivity so that the neurites elongate faster at the same force. In this chapter, the pharmacological strategy we used to explore a potential mechanism generally based on two aspects: 1). does treatment influence sensitivity of tension-induced elongation? 2). does treatment affect osmotic stimulation of growth? Approaches from these two aspects not only provided us with a general view of mediation of osmotic effect but also some insights into the possible regulators of tension-mediated growth, which we believe to be a proximate stimulus to axonal elongation in general.

The role of extracellular Ca^{++} in tension-regulated growth and osmotic dilution-induced elongation

Extracellular calcium has been a prime candidate as an regulator of neurite elongation and growth cone activity (Bandtlow et al., 1993; Kater and Lipton 1996). It has been shown that an specific level of calcium influx promoted neuronal elongation (Cohan et al., 1987), and decreased $[Ca^{++}]_o$ or moderate blockage of normal Ca^{++} influx could stimulate neuronal lengthening in snail B5 neurons (Mattson and Kater, 1987). Since in the osmotic dilution experiments, the extracellular Ca^{++} concentration was reduced by half,

along with other ionic concentrations, we investigated the effect of $[Ca^{++}]_o$ reduction on axonal growth.

Two sets of experimental designs were performed. We first serially reduced extracellular $[Ca^{++}]$ while keeping medium osmolarity constant and assessed towed growth sensitivity. The reduction of $[Ca^{++}]_o$ down to 25% of the basal medium failed to alter growth sensitivity (Fig. 5A). In other words, tension-regulated growth was not affected by reduction of $[Ca^{++}]_o$ to the extent of 25%. Although a further dilution of 12.5% did increase growth rate slightly (27%), it is premature for us to make any conclusion since this is the only experiment in which $[Ca^{++}]_o$ was reduced down to 12.5%. Judging from the variations of relative rate increases in Table 2B, this 27% increase probably is due to some sampling error. Our results indicated that extracellular Ca^{++} has little effect in promoting neuronal growth. This was consistent with observation from Tolkovsky et al.(1990) which showed Ca^{++} transients were not required as signals for long-term neurite outgrowth.

In another test of the role of extracellular calcium, we varied the osmolarity and kept extracellular $[Ca^{++}]$ constant, yet the osmotic sensitivity shift continued to be observed (Fig. 5B). From the data above, we concluded that the osmotic effect in chick sensory neurons did not result from the reduction of external calcium concentration.

The role of SA/SI channels in tension-regulated growth and dilution-stimulated elongation

The present work tested the possible involvement of one cellular mechanical sensor: stretch-activated ion channels (SA and SI channels) which are widely proposed to be involved in osmoregulation and mechanotransduction (Morris , 1990; Watson, 1991). High concentrations of Gd^{3+} to inhibit mechanosensitive channels (Yang and Sachs, 1989, Zhou et al. 1991) failed to alter growth sensitivity (Fig. 6A) or to alter the response to osmotic dilution (Fig. 6B). The Gd^{3+} concentration (50-100 μM) used for the experiments should have been sufficient to block all activities of Gd^{3+}-sensitive SA/SI channels, since it only takes 1 μM Gd^{3+} to suppress openings of the SA channels in patch-clamp recording (Sadoshima et al., 1992). Thus, the lack of Gd^{3+} effect suggests that the Gd^{3+}-sensitive SA and SI channels are probably not the mechanotransducer of tension-regulated growth and the osmotic dilution-induced elongation.

NPPB inhibits tension-regulated growth but does not inhibit the osmotic dilution-stimulated elongation

Another kind of mechanical sensor, swelling-induced Cl^- channels were assessed because of their well-described role in mediating RVD in response to osmotic dilution (Bakhramov et al., 1995; Jentsch, 1996). We tested the possible involvement of swelling-activated Cl^- channels by application of 50-100 μM of the phenol derivative NPPB which has been

shown to block the channels' activities and to abolish RVD (Gschwentner et al.,1996). Treatment of NPPB dose-dependently suppressed the tension-regulated axonal elongation (data not shown), i.e., NPPB shifted the neuronal sensitivity. The activity of NPPB-sensitive Cl^- channels apparently is not required for tension-regulated growth, since increased tension could overcome inhibitory effect of NPPB (Fig. 7). NPPB is known to block different chloride channels in a variety of cells (Dreinhöfer et al., 1988; Heisler, 1991; Gschwentner et al., 1996). Further studies on the roles of chloride channel(s) on tension-regulated axonal growth are needed for more information.

We found that the same doses of NPPB, which inhibited RVD in other kind of cells (Ackerman et al., 1994; Bakhramov et al., 1995) and markedly suppressed tension-regulated growth in chick sensory neurons, neither inhibited the osmotic stimulation of axonal growth (Fig. 8A) nor significantly retarded the osmotic stimulation effect (Fig. 8B). Judging from these results, the swelling-activated Cl^- channels are not involved in the osmotic dilution-stimulated elongation. Moreover, the stimulatory effect of osmotic dilution could easily overcome the inhibitory effect of NPPB. For example, in one experiment, application of 100 μM NPPB caused a small amount of axonal retraction, but the neurite started lengthening at almost twice as fast as the initial rate following subsequent osmotic dilution.

The removal of chloride ions had no significant effects on tension-regulated growth and dilution-stimulated-growth

The movement of chloride ions plays a major role in volume regulation. In hypotonicity-induced swelling cells, efflux of Cl^- causes subsequent water efflux and thus decreases cell volume (RVD) (Hallows and Knauf, 1994). Depletion of intracellular Cl^- inhibited RVD, even though the granule neurons were swollen to twice their normal volume (Pasantes-Morales et al., 1993). We further investigated the role of Cl^- movement on the osmotic effect. According to the Gibbs-Donnan equilibrium:

$$[K^+]_o \times [Cl^-]_o = [K^+]_i \times [Cl^-]_i$$

removal of extracellular Cl^- may result in depletion of intracellular Cl^- or even cell shrinkage if cation loss accompanies Cl^- efflux and thus affects RVD (Schousboe et al., 1990; Strange et al., 1993). In our experiments, most chloride ions were replaced with gluconate, which allowed reduction of extracellular $[Cl^-]$ from 145 mM to 2.5 mM. This concentration should effectively cause loss of intracellular K^+ and Cl^-. This intervention showed no inhibitory effects on the hyposmolarity-stimulated growth (Fig. 9). Ideally, we would like to totally replace the extracellular Cl^- with gluconate to completely eliminate chloride ions (Cl^- free medium), but due to the lack of availability of the gluconate salts of some cations, we could only decrease $[Cl^-]_o$ to 2.5 mM. Our data (Fig. 9) showed that even in the conditions of intracellular Cl^- loss: 1). neurite lengthened regularly upon

applied tension, and 2). osmotic dilution continued to stimulate neuronal lengthening effectively (68%-114%) (n=4). Our results imply that the tension-regulated growth and osmotic stimulated-growth is Cl^--independent, or Cl^- is not a major player in our system.

K^+-depolarization and osmotic dilution-stimulated elongation

Membrane depolarization in cultured astrocytes could be elicited by osmotic swelling (Kimelberg and O'Conner, 1988; Kimelberg and Kettenmann, 1990). We induced membrane depolarization by addition of KCl, which is widely used to elicit depolarization in *in vitro* studies, and investigated the relationship between osmotic dilution and depolarization. Based on the Nernst equation, elevation of extracellular K^+ to 25 mM could raise the resting membrane potential to about -43 mV (assuming that the cytoplasmic $[K^+]$ is 140 mM), a degree of depolarization that has been shown to enhance the neurite outgrowth of snail B5 neurons (Berdan et al., 1993). Additionally, depolarization can increase the calcium influx via voltage-gated calcium channels, which could affect neurite elongation (Hantaz-Ambroise and Trautmann, 1989) or enhance protein synthesis (Brostrom et al., 1983). Consistent with these results, we observed a rate increase in tension-regulated growth after K^+-depolarization (Fig. 10A) and the growth rate further increased significantly upon following osmotic dilution. In other words, we found that

the stimulatory effects of K^+-depolarization and osmotic dilution are additive.

When the order of treatments was reversed (Fig. 10B), the rate of neurite lengthening increased dramatically after the osmotic dilution, while following K^+-depolarization, the growth rate did not increase any further. According to observation of Kimelberg and Kettenmann (1990), exposure to hypotonic solution by decreasing 50 mM NaCl (100 mOsm) resulted in a 20 mV depolarization in cultured astrocytes. In our experiments, the osmotic dilution caused about 150 mOsm reduction in the medium osmolarity, which presumably would result in about 30 mV increase in membrane potential, while 25 mM external KCl increased membrane potential by about 40 mV. The much stronger osmotic dilution effect in stimulating elongation speaks against a possible involvement of K^+-depolarization in the dilution stimulated-growth, although K^+-depolarization significantly enhanced tension-regulated growth.

A possible ionic effect of high extracellular KCl

Results from Fig. 10B showed that treatment of K^+-depolarization succeeding osmotic dilution did not further enhance the growth rate but rather slightly decreased the growth (less than 10%), which was quite contrary to the additive effect from Fig. 10A. In view of this, we are puzzled about why the growth rate would not stay the same or slightly increase instead of slightly decreasing upon K^+-

depolarization in all three cases since K^+-depolarization has markedly enhanced growth in previous experiments. Because the percentages of rate decrease are so small (4%, 5.5% & 10% respectively), it might have been due to experimental variations or some other unknown factors. Alternatively, it might be the ionic effect brought about by addition of KCl.

In addition to depolarizing the membrane, high extracellular K^+ may have some ionic effect itself. For example, available evidence raised the possibility that the *in vitro* elevation of potassium ions itself may disturb the osmotic balances between the intracellular and extracellular fluids and thus change cell volume (Martin et al., 1990; Martin and Shain, 1993). In intact brain, $[K^+]_o$ rises significantly during normal neuronal activity and can reach values of 10-12 mM during periods of extreme activity such as seizures (Ballanyi and Grafe, 1988), and the increase in $[K^+]_o$ is accompanied by a decrease in extracellular space, indicating that the cell is swelling (Bourke et al, 1983; Kempski et al., 1986; Walz, 1987). Cells could be very sensitive to the osmolarity changes. A 6% decrease in osmolarity could elicit a large increase in ionic conductances of SA channel (Lau et al., 1984). A 5% increase in osmolarity (15 mM added sucrose) suppressed osmolyte releases (Martin et al., 1990). In our experiments, addition of 20 mM more KCl to the medium could increase the external osmolarity by about 13% (assuming that the medium osmolarity is approx. 300 mOsm), which might conteract the osmotic

dilution effect. This may explain the slightly decreased growth rate upon K^+-depolarization following medium dilution. Of course, the stimulated-growth effect from K^+-depolarization dominates the inhibitory effect of high potassium ions (if any); that is why K^+ depolarization could have enhanced the tension-regulated growth dramatically in Fig 10A.

PKA activation is not involved in tension-regulated axonal growth or dilution-stimulated growth

Osmotic swelling has been shown to induce accumulation of cAMP in some cells (Morgan et al., 1989; Watson et al., 1989) and cAMP has been reported to regulate the activities of some volume regulatory transport pathways (London et al., 1989; Force and Bonventre, 1994). In addition, cAMP is an important regulator of neuronal outgrowth (Tolkovsky, 1987; Mattson et al., 1988; Nakagawa-Yagi et al., 1992). We tested the possible involvement of cAMP in tension-regulated growth and osmotic dilution effect. Results of this study demonstrate that elevation of intracellular cAMP concentrations, which presumably activated PKA, by either application of cholera toxin or addition of forskolin (Daly et al. 1982) did not change the growth sensitivity of tension-regulated elongation (Fig. 11A & 11B). Further investigation by supplying cyclic AMP altered neither growth sensitivity nor the response to hyposmotic medium (Fig. 11C). These experiments argued that activation of PKA is not

involved in the tension-regulated growth nor in the osmotic dilution-stimulated growth.

PKC activation stimulates tension-regulated growth , but is not involved in the osmotic stimulation effect

Although some of the volume-sensitive transport systems have been postulated to be regulated by protein phosphorylation (Grinstein et al., 1992; Palfrey, 1994), there is lack of evidence showing any relationship between osmotic swelling and PKC activation (Force and Bonventre, 1994) . We tested the possible involvement of PKC in the osmotic effect because of the wide physiological engagement of PKC and its role in stimulating neuronal outgrowth (please refer to the General Introduction). Both PKC activators, PMA and synthetic DAG, significantly stimulated tension-regulated axonal growth (Fig.12-13 & Table 3). PKC inhibitor chelerythrine suppressed the tension-regulated growth (Fig. 14); nevertheless, the neurites started lengthening upon increased tension, and the rates were proportional to tension applied. Results from Fig. 12-14 indicated that tension-regulated axonal growth does not require the activation of PKC. However, PKC activation may play a role in modulating tension-mediated growth, probably through inducing reorganization of cytoskeletal proteins (Bershadsky et al., 1990; Tint et al., 1991)

As for investigations on PKC activation and osmotic effect, DAG and medium dilution treatments both separately

enhanced the neuronal elongation (Fig. 15). The dilution-stimulated growth was apparently much stronger than the effect of DAG: in all trials, the medium dilution following application of DAG still caused further significant increase in growth (Fig. 15A); the addition of DAG after the osmotic treatment, however, caused only slight increase of the growth rates (less than 10%) in all experiments (Fig. 15B). Further investigations by blockage of PKC activity with chelerythrine failed to inhibit the stimulatory effect of the osmotic dilution (Fig. 16). In summary, although PKC activation may modulate tension-regulated growth, our data argued against any involvement of PKC activation in the osmotic dilution stimulated-growth.

Osmotic dilution exerts direct mechanical effects on the neurites

In addition to these chemically mediated mechanisms for the shift in growth sensitivity, we also examined the possibility of a mechanical explanation for the osmotic effect. Most cell types behave as reliable osmometers over the time scale of hours, and neurons have been shown to swell in response to hyposmotic conditions (Wan et al. 1995). We postulated that osmotic swelling would have a direct mechanical effect on actin network of the cell, which previous results have shown bear the tension load in neurites (Dennerll et al. 1989). This speculation was based on a general theory of elasticity for all hydrophilic elastics

that has been extensively verified experimentally (Treyleor 1975). This theory suggests that the subplasmalemmal actin network would lose part of its stiffness due to swelling of the network, typically involving filament breakage, loss of crosslinks, but also in the case of dynamic actin cortex by additional actin assembly. Consistent with this notion, we found that dilution of the culture medium was accompanied by a modest decline in neurite rest tension. Osmotic dilution seemed also to cause a decline in spring constant, which would also be expected from osmotic swelling of a polymer network, although this effect was less clear than the decline in rest tension. The modest changes in mechanical properties of the neurite are consistent with the notion that the actin network is not significantly disrupted, as shown by the robust physiological accommodation of the neurite in increasing its subsequent rate of elongation. We attempted to assess the contribution of the microtubule cytoskeleton to these mechanical changes by using anti-microtubule drugs, as in previous investigations on PC12 cells (Dennerll et al. 1989). However, at concentrations of nocodazole sufficient to completely depolymerize the relatively resistant microtubules of chick sensory neurons (Baas and Heidemann 1986), attempting to pluck the neurite caused, in every case, its immediate detachment from the dish. As a result, we were unable to make these measurements.

Our principal conclusion is that a direct link exists between osmotic stimulation and the tensile "machinery" of

axonal development. We have eliminated several possible chemical signalling mechanisms that might plausibly mediate the connection between osmotic shock and growth rate. Given the mechanical impact of osmotic dilution on cells, it is perhaps not surprising that we find modest mechanical effects on the elastic behavior of neurites, in addition to the more robust growth effects. We propose that elastic loosening and growth stimulated by osmotic challenge may be linked. Although not conclusive, the data are consistent with our previous mechanical and thermodynamic model wherein the rate of microtubule assembly and axonal elongation is directly determined by an elastic tension/compression force balance within the axonal cytoskeleton. In this model, relief of compressive forces on axonal microtubules lowers microtubule free energy, promoting microtubule assembly and axonal elongation (Buxbaum and Heidemann 1988, 1992). In the simplest model, the osmotic influx of water "inflates" the actin cortex, exerting an outward force, consistent with observed osmotic swelling of neurons (Wan et al. 1995). (We did not observe an obvious inflation of the neurite. However, the magnifications we use for towing and observation of growth rate are too low to observe diameter changes less than approx. 2X.) The postulated inflation would partially compensate inward acting tension and relieve compressive load on internal microtubules. Compressive load on microtubules might be relieved further if the osmotic force also caused polymer network swelling (Treyloar 1975), in turn causing the

observed declines of rest tension and spring constant. Tubulin subunits, originally in a steady state with the compressed microtubules (i.e. the free energy of the polymer and monomer pool are equivalent), would now incorporate faster after the decline in polymer free energy caused by the decline in the compressive forces supported by the microtubules (Buxbaum and Heidemann 1992). Further work will be required to confirm this interpretation of the mechanism that links osmotic stimulation to tensile regulation of axonal elongation rate.

Conclusion:

The main purpose of this dissertation is to investigate the mechanisms mediating osmotic dilution-stimulated axonal growth and to begin investigation into signals involved in tension-regulated growth. In summary of our results, we came out with the following:

1).The osmotic dilution can cause a shift in the growth sensitivity of neurites to tension. This dilution-stimulated elongation is independent of external [Ca^{++}] reduction, Gd^{3+}-sensitive SA/SI channels, swelling-activated Cl^- channels, intracellular Cl^- depletion, depolarization, PKA and PKC activations.

2). Experiments on neurite rest tensions suggest that osmo-stimulation of growth rate can be accounted for by mechanical effect on the neurite shafts.

3). Tension-regulated axonal growth is not mediated by decreases in extracellular Ca^{++}, Gd^{3+}-sensitive SA/SI channels and PKA activation. Inhibition of NPPB-sensitive Cl^- ion channels suppresses the growth and activation of PKC stimulates growth, but activities of both Cl^- channels and PKC are not essential to tension-regulated growth. The involvement of K^+-depolarization needs further study. In view of our results and the thermodynamics model proposed by Bauxbaum and Heidemann (1988, 1992), it is possible that multiple intracellular messengers are involved.

4). From our data, we think there might be a possibility of medical application of the tension and osmotic effect on neuronal injury. Can application of tension or osmotic dilution prevent axonal retraction or even promote regeneration? This question clearly is worth of studying.

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