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ENDCAPPED ANTISENSE OLIGONUCLEOTIDES DECREASE CHROMAFFIN CELL SCINDERIN EXPRESSION AND STIMULATION-EVOKED F-ACTIN DISASSEMBLY AND EXOCYTOSIS

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ABSTRACT

Scinderin is a Ca^{2+}-dependent F-actin severing protein discovered in our laboratory. It has been suggested that scinderin controls F-actin dynamics and chromaffin vesicle availability for exocytosis. We have recently designed a 20-mer scinderin phosphorothioate endcapped substituted antisense oligodeoxynucleotide (ODN) sequence, which encompasses the scinderin initiation (ATG) site, and evaluated its impact on scinderin expression and amine output from cultured chromaffin cells. Chromaffin cells cultured for 48 hours in serum-free medium were treated with 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN or a 20-mer mismatch phosphorothioate endcapped ODN sequence for 2-4 days. Cellular viability was not compromised when chromaffin cells were plated in serum-free medium or in serum-free medium containing antisense. Uptake of the antisense was evident when 5'-fluorescein labelled 20-mer scinderin phosphorothioate endcapped antisense ODN entered chromaffin cells within 1 hour of treatment and remained highly concentrated in the nucleus for 24 hours post-treatment suggesting that the antisense was targeting gene expression. Moreover, a 60% decrease in scinderin mRNA levels was accompanied by a decrease of 50% in scinderin levels when chromaffin cells were treated with the 20-mer scinderin phosphorothioate endcapped antisense ODN compared to those chromaffin cells treated with the 20-mer mismatch phosphorothioate endcapped ODN sequence. Furthermore, stimulation of chromaffin cells with a depolarizing concentration of K^+ showed that

both F-actin disassembly and evoked release of amines in 20-mer phosphorothioate endcapped scinderin antisense treated chromaffin cells was 50% lower than those chromaffin cells untreated or treated with the 20-mer phosphorothioate endcapped mismatch sequence. Previous work from out laboratory with permeabilized chromaffin cells treated with recombinant scinderin showed potentiation of amine release in response to 10μM Ca^{2+}. Conversely, in the present experiments a reduction of scinderin expression by 20-mer scinderin phosphorothioate endcapped antisense ODN treatment decreases significantly stimulation-induced release of amines. The present results suggest that scinderin is an important component of the exocytotic machinery.

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ABBREVIATIONS

DEPC = diethylpyrocarbonate

DTT = DL-dithiothreiton (Cleland's Reagent)

EDTA = ethylenediamine tetraacetic acid

EGTA = glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid

HEPES = N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]

MOPS = 3-[N-morpholino]propane-sulfonic acid

PIPES = piperazine-N,N'-bis[2-ethanesulfonic acid] dipotassium salt

PMSF = phenylmethylsulfonyl fluoride

PVP = polyvinylpyrrolidine

SDS = sodium dodecyl sulfate

SSC = sodium chloride/sodium citrate

TCA = trichloroacetic acid

TEMED = tetramethylethylene diamine

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CHAPTER 1 - INTRODUCTION

CHAPTER 1 - INTRODUCTION

PART I: Chromaffin Cell Exocytosis

1. (1). General overview of the adrenal medulla

Adrenal glands are essential for life with their general function being to protect the organism against acute and chronic stress. In humans, the glands are paired organs located on top of the kidneys with each gland weighing approximately 4 grams. Adrenal glands consist of an outer yellow cortex which surrounds a pale grey medulla. These two anatomically distinct regions were first noted in 1805 by George Cuvier who described the glands as consisting of a cortex and a solid medulla; however, the term cortex and medulla were first used by Huschke in 1845 (Carmichael, 1987). Although the two regions of the adrenal gland were first described in the nineteenth century, the adrenal medulla was first mentioned by Caspar Bartholinus in 1611 as a cavity full of black bile and not until 1856 did Alfred Vulpian describe the unusual staining of the medulla following treatment with ferric chloride. This portion of the adrenal gland turned green and furthermore, the medullary secretions reduced the ferric chloride to ferrous chloride (Carmichael, 1987). Secretions from the adrenal medulla are controlled via the cholinergic fibers of the splanchnic nerves, which synapse directly onto individual cells within the gland (Kaplan, 1996). Furthermore, due to this "preganglionic" innervation, adrenal cells are considered to be part of the peripheral autonomic nervous system

(Anderson, 1989).

In 1865, Jacob Henle reported that chromium salts produced a characteristic colour reaction when applied to the adrenal meduila. Also, around this time, von Kölliker concluded that the cortex and the medulla were structurally and functionally distinct (Carmichael, 1987). As a result in 1902, Kohn was the first to use the name "chromaffin cell" when referring to cells which react to chromium salts in the afore described manner (Carmichael, 1987; Kohn, 1902).

1. (2). Adrenal Medullary Chromaffin Cells

Histological studies have shown that the adrenal medulla is comprised mainly of chromaffin cells, or pheochromocytes, which are spherical or ovoid in shape and have a diameter of approximately 10-20μm. Within the adrenal medulla, chromaffin cells are arranged in nests or alveoli which are separated by nerves, connective tissue and blood vessels. Chromaffin cells contain nuclei which are oval to irregular in shape and usually have one or two nucleoli. Other organelles within the cytoplasm include lysosomes, multivesicular bodies, centrioles, microtubules, mitochondria and a moderately developed Golgi complex (Carmichael, 1987). These organelles are not randomly distributed within the cytoplasm, but rather, are polarized with respect to nerve ending on one end of the cell and blood vessels on the other side of the cell (Carmichael, 1987) (Figure 1). The blood vessels are on

the venous sinusoids side, while the nucleus is on the opposite side. Further the golgi complex is located near the nucleus on the venous side. Chromaffin cell polarity does not appear to affect the distribution of chromaffin vesicles, although it does favour an organization which is appropriate for cellular secretion whereby nervous stimulation occurs at one end of the cell while catecholamines are released from the vesicles at the other end, the basolateral surface (Carmichael, 1987).

The granular appearance of the chromaffin cell cytoplasm is due to the presence of chromaffin vesicles. These vesicles have a diameter of 150 to 250 nm and are electron-dense membrane-bound vesicles which when treated with potassium dichromate turn dark brown, thus the name chromaffin cell and chromaffin vesicles or granules (Vitale *et al.*,1995; Sjöstrand and Wetzstein, 1956; Lever, 1955). Each cell contains between 17,000 to 20,000 vesicles (Vitale *et al.*, 1995; Nordmann, 1984; Phillips, 1982) which resemble dense-core vesicles of sympathetic nerve endings and are responsible for the storage and secretion of catecholamines (Aunis and Bader, 1988; Sjöstrand and Wetzstein, 1956). Biochemical studies and concurrent morphological observations have shown the presence of three types of secretory cells which are adrenaline containing cells, noradrenaline-containing cells and small granule chromaffin cells. Adrenaline and noradrenaline containing cells are large dense-core vesicles (LDCV) (150-250nm in diameter), which are related to the LDCV in neurons of the sympathetic nervous system. LDCV are mainly involved with the storage and release of peptides, although they also store the

classical neurotransmitters acetylcholine, noradrenaline, glutamate, serotonin, GABA, *etc.* (Trifaró *et al.*, 1992). Small granule chromaffin cells are comprised of small dense-core vesicles, 400-500Å, which are also found in sympathetic nerves (Trifaró *et al.*, 1992). These vesicles store and release the classical neurotransmitters and they seem to undergo exocytosis and/or endocytosis at nerve terminals (Cecarelli *et al.*, 1973; Heuser and Reese, 1973). In addition, small electrotranslucent vesicles, 400-500Å, have been identified in chromaffin cells; however, their function has not yet been elucidated (Hökfelt, 1973; Bisby and Fillenz, 1971; Tranzer *et al.*, 1969; Grillo, 1966). These secretory vesicles serve several purposes in secretory tissue: they synthesize and store large amounts of secretory material in a small volume, they protect the secretory material from intracellular degradation and they allow for efficient transport and subsequent release of fixed amounts of secretory material (Trifaró 1990; Trifaró and Poisner, 1982).

The distinction between adrenaline and noradrenaline containing chromaffin cells was first reported by Eränkö in 1955, whereby the noradrenaline containing vesicles were more dense than adrenaline containing vesicles after fixation with gluteraldehyde-osmium tetroxide. Further, the distribution of the two types of cells within the adrenal medulla is such that adrenaline containing cells comprise 85-95% of the chromaffin cell population in most mammals, although extensive species

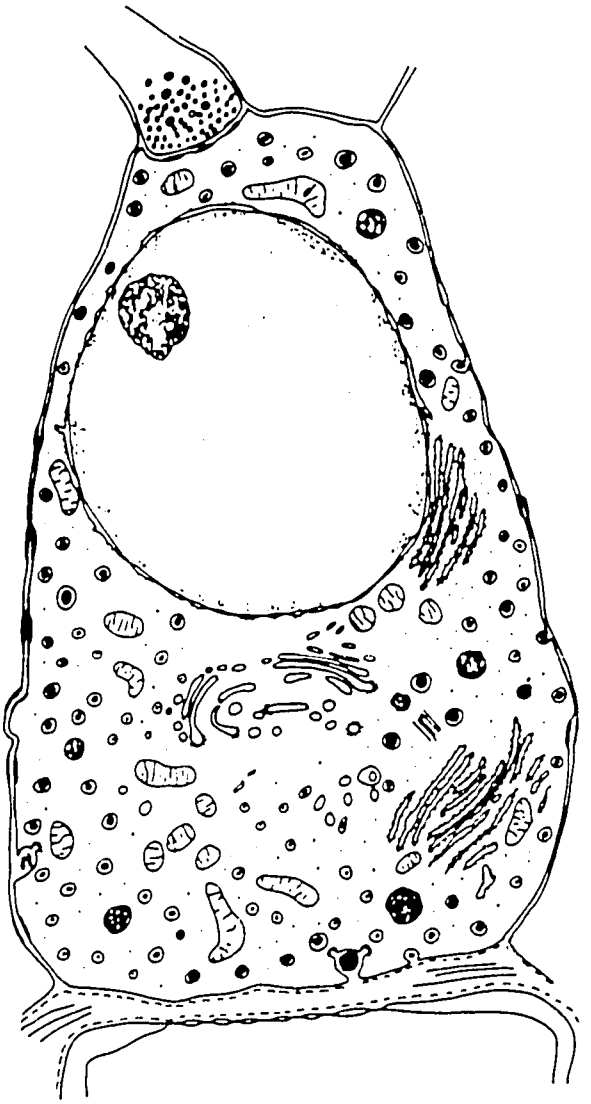


Figure: 1
Schematic of adrenal chromaffin cell illustrating the typical ultrastructural features. The membrane-bound, dense-cored vesicles are chromaffin vesicles containing noradrenaline. A nerve terminal is illustrated at the top, and a fenestrated endothelial cell is at the bottom (Taken from Carmichael, 1987).

variation does exist (Benchimol and Cantin, 1977). Therefore, the general process of secretion demands that chromaffin vesicles containing catecholamines move from their point of origin within a cell toward the cell membrane where they fuse with the membrane and release their contents into the cell exterior. In order for this elaborate process to occur there must be a balance between the cytoskeleton and its regulatory proteins.

1. (3). Adrenal Chromaffin Cells in Culture

Chromaffin cells of the adrenal medulla are amongst the best studied endocrine cells in terms of neuronal function, endocrine mechanisms, hormone storage and response to a variety of stimulating agents (Schäfer *et al.*, 1987; Carmichael, 1986; Baker and Knight, 1984; Burgoyne, 1984; Trifaró, 1982; Winkler and Westhead, 1980). Homogenous populations of isolated chromaffin cells (adrenaline and noradrenaline containing cells) are easily obtained and they are an excellent model for studying exocytosis using pharmacological, electrophysiological, biochemical and molecular biological approaches (Strittmatter, 1988; Trifaró and Lee, 1980). At the ultrastructural and biochemical levels, chromaffin cells exhibit many features that are characteristic of other endocrine cells while sharing a primordial origin as well as a variety of features such as electrical stability, storage and release of catecholamines and almost identical carrier and enzyme proteins with sympathetic adrenergic neurons (Unsicker *et al.*, 1980). This type of cell is commonly referred

to as a paraneuron (Fujita and Kobayashi, 1975) (Figure 2). Other paraneurons include gastroenteric endocrine cells, mast cells, melanocytes, pancreatic islet cells, pinealocytes, adenohypopohysial cells, parafollicular cells and Merkel cells (Fujita, 1980). Freshly isolated attach to collagen-coated plastic culture dishes within 8 to 16 hours and are spherical in shape with no discernable polarity; however, cells that have been maintained in culture for several days begin to develop one or more processes displaying varicosity-like structures which increase in length in proportion to the time in culture (Trifaró and Lee, 1980).

1. (4). Chromaffin Cell Exocytosis

The intricate process of cellular secretion, or exocytosis, is a fundamental mechanism which is required for chemical intracellular communication and it is a process which involves a significant degree of interaction between cellular components and secretory vesicles (Strittmatter, 1988; Trifaró *et al.*, 1993; Trifaró and García, 1995; Walker *et al.*, 1996). More precisely, this process allows for the release of stored, presynthesized compounds into the extracellular space in response to specific stimuli (Strittmatter, 1988; Douglas and Rubin, 1961). The exocytosis of secretory vesicles can be either constitutive or regulated (Trifaró, 1990; Kelly, 1985; Gumbiner and Kelly, 1982; Tartakoff *et al.*, 1978). Constitutive secretion is unregulated and follows the rate of synthesis of secretory products and it occurs in almost all cell types including lymphocytes, hepatocytes, yeast, *etc.*,

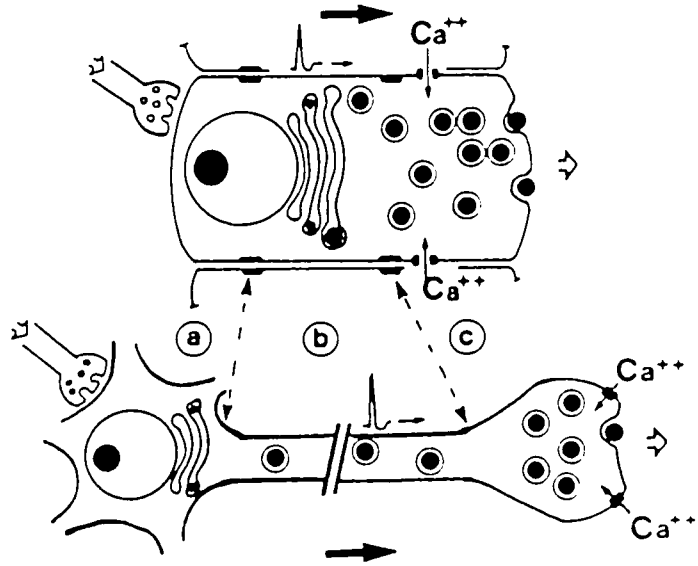


Figure: 2

Schematic representation of a neuron and paraneuron. The functional characteristics in three different regions *a*, *b*, and *c* are indicated by broken lines. The input region (*a*) is the reception site (receptors) for the stimulus. The stimulus-induced membrane changes in (*a*) are accompanied by the inward movement of Na^+ and Ca^{2+}, with the generation of "all or none" action potentials that travel down the intermediate conductive region (*b*). The arrival of the action potential to the output region (*c*) produces an opening of slow Ca^{2+} channels with a concomitant rinse in intracellular Ca^{2+}. The increase in intracellular Ca^{2+} triggers the secretory machinery and the secretory vesicles discharge their content by exocytosis. Paraneurons are then functionally polarized cells receiving information in one end, and releasing material for export at the opposite side (Taken from Trifaró, 1990).

whereas regulated secretion is localized to cells which store their secretory products in membrane bound vesicles or granules (Buckley and Kelly, 1985; Kelly, 1985; Trifaró, 1990; Trifaró and García, 1995). This latter form of cellular secretion is characteristic of neurons, endocrine and exocrine cells and is triggered by an increase in intracellular Ca^{2+} (Trifaró and García, 1995; Trifaró *et al.*, 1993).

Depolarization of chromaffin cells by stimulation of the nicotinic receptor or elevation of extracellular K^+ or alternatively, stimulation of the muscarinic receptor results in an increase in intracellular Ca^{2+} (Trifaró and García, 1995; Aunis and Bader, 1988; Strittmatter, 1988). Despite the fact that the role of Ca^{2+} in secretion was observed many years ago (Douglas 1968; Douglas and Rubin, 1961; Harvey and McIntosh, 1940; Houssay and Molinelli, 1928), the exact mechanism by which Ca^{2+} triggers secretion is still poorly understood (Trifaró and García, 1995; Trifaró *et al.*, 1993). Therefore, an increase in intracellular Ca^{2+} is followed by numerous steps which result in the release of chromaffin vesicle contents which include catecholamines, ATP, dopamine β-hydroxylase, chromogranins A and other neuropeptides into the cell exterior (Strittmatter, 1988; Trifaró, 1990). In order for the chromaffin vesicle contents to be released the vesicle membrane must fuse with the plasma membrane. Zucker (1996) hypothesizes that the vesicle and the plasma membrane may fuse completely resulting in subsequent endocytosis or, alternatively, a transient "kiss-and-run" fusion may occur. This latter possibility would allow the vesicle contents to escape through a briefly opened fusion pore

(Zucker, 1996).

Another attractive hypothesis is that the action of Ca^{2+} in secretion is mediated through its control of the cytoskeleton network (Figure 3)(Trifaró and Vitale, 1993). This theory suggests that the chromaffin vesicles are transported from the Golgi complex to the plasma membrane where they are docked and then subsequently fused with the plasma membrane resulting in the expulsion of the vesicle contents into the cell exterior. In resting or unstimulated chromaffin cells most of the vesicles are located approximately 250nm from the plasma membrane thus indicating that they are trapped within an actin-rich subplasmalemmal cytoskeleton of the cell cortex thus making their interaction with the plasma membrane impossible (Trifaró and Vitale, 1993; Burgoyne and Cheek, 1987). While in stimulated chromaffin cells the movement of chromaffin vesicles towards the cell membrane occurs when there is a reorganization of the cortical F-actin network (Vitale *et al.*, 1995; Marcu *et al.*, 1994; Trifaró *et al.*, 1993; Rodríguez Del Castillo *et al.*, 1990).

1. (5). The Cytoskeleton and Exocytosis in the Chromaffin Cell

The cytoskeleton is a dynamic, rather than static, structure which is continuously changing in response to cellular function. The discovery of a prominent cytoskeleton in the adrenal medulla was subsequently followed by the observation that an abundant supply of microtubules exists in the Golgi complex, the neurite-like

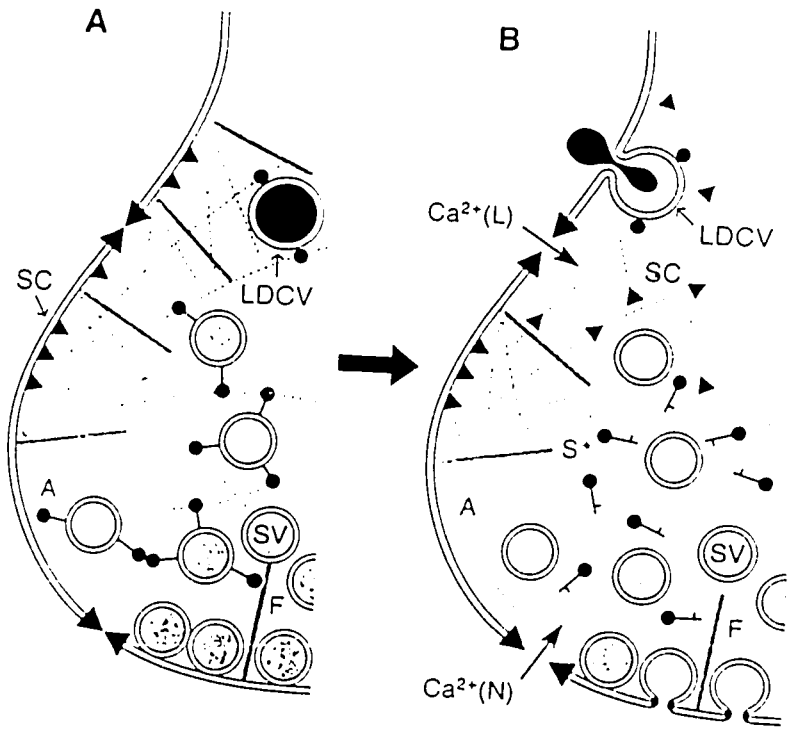


Figure: 3

Diagram of a nerve terminal, illustrating the possible participation of the cytoskeletal elements in the release of fast-acting neurotransmitters. **(A)** In resting conditions, a cluster of synaptic vesicles (SV), positioned close to the presynaptic membrane, is immediately available for secretion. At a distance from the plasma membrane, another set of synaptic vesicles that are linker to each other, to the actin network (A) and to the presynaptic membrane through synapsin I (S) and fodrin (F), constitute a reserve pool. **(B)** Nerve depolarization causes the opening or N-type Ca^{2+} channels and the release of synaptic vesicles via exocytosis. Phosphorylation of synapsin I (S*) and activation of proteins that sever actin filaments results in a partial disassembly of the actin filament networks. Synaptic vesicles are then free to move towards the active zone of the nerve endings (Taken from Trifaró and Vitale, 1993).

expansions and in the subplasmalemmal space of cultured chromaffin cells (Aunis *et al.*, 1987; Lee and Trifaró, 1981; Bader *et al.*, 1981; Poisner and Cooke, 1975). Immunocytochemical studies have shown at least three types of filamentous systems: microtubules, microfilaments and intermediate filaments. Also, a large variety of cytoskeleton associated proteins have been characterized (Trifaró, 1990; Trifaró *et al.*, 1985*a*, *b*). They include proteins that favour (actinogelin) or disrupt (gelsolin, scinderin) microfilament formation, proteins that serve as anchorage for cytoskeletal elements to membranes (vinculin, spectin or fodrin, α-actinin, ankyrin) and proteins that regulate the phosphorylation of cytoskeleton elements (calmodulin) (Trifaró, 1990). Morphological studies have shown that these cytoskeletal structures of the subplasmalemmal zone form a three-dimensional network which interconnects membrane-limited organelles, microtubules and the cell membrane (Aunis and Bader, 1988; Aunis *et al.*, 1987). In addition to the afore mentioned components of the cytoskeleton, another major component is actin, which comprises up to 10% of the cells total protein content (Forsher, 1989).

1. (5). (i). ***Cortical Actin Network***

The actin microfilament network is localized underneath the plasma membrane of chromaffin cells and it opposes the movement of secretory vesicles to releasing sites (Trifaró *et al.*, 1982; Lee *et al.*, 1981). In resting chromaffin cells a 200-400 nm deep region of the cell cortex is largely devoid of secretory vesicles (Burgoyne

et al., 1982) suggesting that the vesicles are excluded from this region due to the presence of a cortical actin network (Burgoyne and Cheek, 1987). The presence of actin in chromaffin cells was first conceived following work in which glycerol-treated chromaffin cells were exposed to fluorescein-labelled heavy meromysin (Trifaró and Lee, 1978). Lee *et al.* (1978) further reinforced the presence of actin by purifying actin from chromaffin cell cytosol by DNase I affinity chromatography and separating the eluted fractions on SDS-PAGE. Two major bands were identified: (i) a major band (92%) which had a molecular weight of 42kD and comigrated with muscle-actin and; (ii) a minor band (10%) having a molecular mass of 90-91kD. The latter was most likely an actin binding proteins such as gelsolin (Lee *et al.*, 1979). Further, two isomeric forms of actin (β and α) which have the same molecular weight as muscle actin, but different isoelectic points, were identified by two-dimensional electrophoresis (Lee *et al.*, 1979). Indirect immunofluorescence studies on freshly isolated one day old chromaffin cells showed that actin was widely distributed throughout the cell and that it associated with numerous cellular structures (Trifaró and Lee, 1981; Aunis *et al.*, 1980; Trifaró and Lee, 1978). Subsequently, immunohistochemical studies on stimulated chromaffin cells using rhodamine-labelled phalloidin [a substance which binds specifically to filamentous actin (F-actin) and not globular actin (G-actin)] have demonstrated the presence of a continuous ring of actin filaments in the subplasmalemmal area of chromaffin cells, confirming the presence of an actin network in this region (Trifaró and Vitale, 1993; Trifaró *et al.*, 1989; Burgoyne and

Cheek, 1987).

The association of actin with chromaffin vesicles was demonstrated at the electron microscope level with the protein A-gold technique (Bendayan *et al.*, 1982). These studies revealed that electron opaque gold particles where localized near dense-core vesicles, suggesting that actin-binding sites are closely associated with secretory vesicles (Trifaró *et al.*, 1985*a*, *b*). Also, in addition to the labelling around the granules, a small number of gold particles was observed in the subplasmalemmal area. Finally, stereo-electron microscopy of polyethylene glycol-embedded rat adrenal medulla has revealed a three-dimensional lattice of microtrabeculae that is continuous with the surface of chromaffin vesicles as well as the inner surface of the plasma membrane (Kondo *et al.*, 1982). Nicotinic stimulation of chromaffin cells induces a Ca^{2+} influx which results in the activation of Ca^{2+}-dependent F-actin severing proteins such as gelsolin and scinderin and in turn the transient disassembly of cortical F-actin network (Trifaró and García, 1995; (Trifaró and Vitale, 1993; Aunis and Bader, 1988). This disassembly allows the secretory vesicles to move freely toward the plasma membrane and in turn interact with it (Trifaró *et al.*, 1994; Vitale *et al.*, 1991; Aunis and Bader, 1988)(Figure 4). Cytochemical experiments with rhodamine-labelled phalloidin, a probe for filamentous actin, revealed that under resting conditions a continuous cortical fluorescent ring is present; however, stimulation with nicotine or depolarizing concentrations of K^+ produced a disruption of the rhodamine-phalloidin cortical

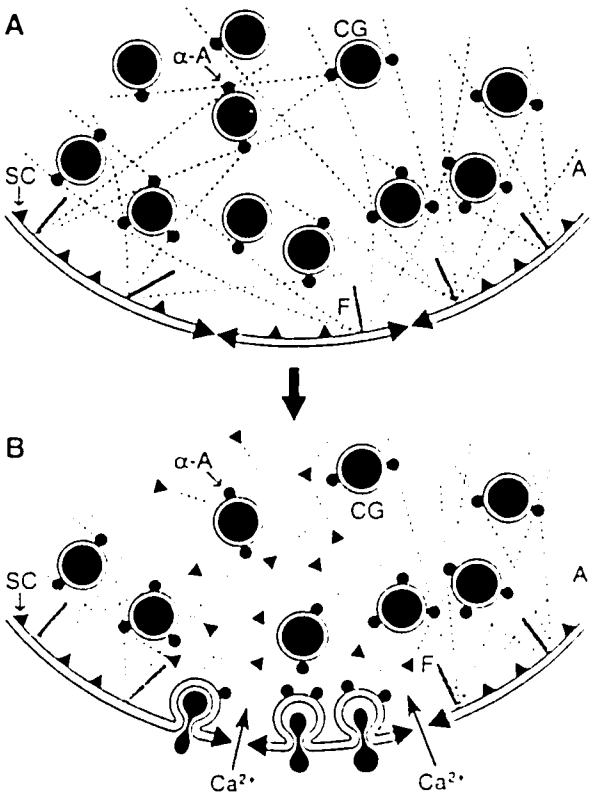


Figure: 4

The possible involvement of the cortical actin filament network in secretory granule exocytosis from chromaffin cells. **(A)** In resting cells, cortical actin filaments (A) are anchored to the plasma membrane and chromaffin granules (CG) through fodrin (F) and α-actinin (α-A). The cortical actin filament network restrains chromaffin granules from interacting with exocytotic sites on the plasma membrane. **(B)** Cell stimulation induced the influx of Ca^{2+}, which in turn detaches chromaffin granules bound to actin filaments and simultaneously activate proteins that sever actin filaments [such as scinderin (SC)]. This creates subplasmalemmal areas of low viscosity in which chromaffin granules are highly mobile; these areas are the exocytotic sites (Taken from Trifaró and Vitale, 1993).

fluorescent pattern and removal of the barrier (Vitale *et al.*, 1991). The absence of the cytoskeletal barrier in these zones of low cytoplasmic viscosity and high secretory vesicle mobility, would allow the secretory vesicles to interact with the plasma membrane and subsequently release the vesicle contents to the cell exterior by exocytosis (Vitale *et al.*, 1991). Vesicles that are relatively close to the membrane constitute the vesicle release-ready pool, and they release their contents milliseconds after stimulation (fast release), while a second pool (or depot pool) of secretory vesicles is responsible for the slow-rate secretion, which occurs after the depletion of the first pool (Vitale *et al.*, 1995). It should be noted that the actin-filaments reassemble within one minute of stimulation (Trifaró and Vitale, 1993; Burgoyne and Cheek, 1987).

PART II: Actin Severing Proteins

The dynamics of actin cytoskeleton (depolymerization and polymerization) is regulated by specific actin filament-severing proteins. This family of proteins consists of six proteins: severin, fragmin and fragmin 60, villin, gelsolin and scinderin (Hartwig and Kwiatkowski, 1991). All members of this family of proteins have the ability to sever actin filaments in a stoichiometric manner in the presence of micromolar concentrations of Ca^{2+} (Hartwig and Kwiatkowski, 1991).

1. (6). Gelsolin

Gelsolin is one of the actin filament-severing proteins which in addition to severing actin filaments (Hartwig and Kwiatkowski, 1991) also promotes the transition of cytoplasmic extracts from a gel phase to a sol phase (solation) upon the addition of Ca^{2+}, thus the name gelsolin (Forscher, 1989). The gel-sol transition is due primarily to actin filament severing and the resultant dramatic reduction of the length distribution of actin filaments (Forscher, 1989). In addition to severing actin filaments in the presence of micromolar concentrations of Ca^{2+}, gelsolin can tightly "cap" the barbed end of actin filaments (K_d= $<10^{-9}$M) (Pollard and Cooper, 1986) thus blocking monomer exchange and preventing filament reannealing after severing (Forscher, 1989). Also, gelsolin can bind actin monomers and oligomers to form nucleation sites, causing rapid actin assembly under optimal conditions (Forscher, 1989).

Gelsolin is a 85 kDa protein encoded by a single gene which is alternatively spliced and its proteins are expressed as a cytoplasmic and a secreted forms (Xian *et al.*, 1995; Vanderkerckhove, 1990). The protein is widely distributed and immunofluorescence studies have shown that it is mainly concentrated in the cortical cytoplasm under the plasma membrane in bovine brain, anterior and posterior pituitary, kidney, salivary gland, testis, liver, skeletal muscle, heart muscle, platelets and plasma (Tachakarov *et al.*, 1990; Rodríguez Del Castillo *et al.*, 1990).

Further, two-dimensional electrophoresis of adrenal medullary gelsolin showed the presence of three isoforms of pI = 5.8, 5.9, and 6.0 (Rodríguez Del Castillo *et al.*, 1990). The protein consists of a sixfold repeat sequence of 125-130 residues with amino terminal possessing the ability of bind monomeric actin in a calcium-dependent manner (Xian *et al.*, 1995; Rozycki *et al.*, 1994; Forscher, 1989). Limited proteolysis in which the protein is cut between these domains results in a 14 kDa N-terminal fragment which forms a complex with G-actin (this interaction is Ca^{2+}-independent); a 28 kDa fragment, which constituted the remaining portion of the N-terminal, binds mainly to F-actin and this interaction is reversed by phosphatidylinositol 4,5-bisphosphate (PIP_2) (Vandekerckhove, 1990). The C-terminal half (38 kDa) of gelsolin contains the third actin-binding domain and only binds in the presence of Ca^{2+} (Vandekerckhove, 1990).

1. (7). Scinderin

Scinderin, a Ca^{2+}-dependent F-actin severing protein, which controls F-actin dynamics has been suggested to be involved in regulating chromaffin vesicle availability for exocytosis (Vitale *et al.*, 1995) by binding to actin and severing actin filaments (Rodríguez Del Castillo *et al.*, 1990). The name scinderin (a name derived from the Latin "*scindere*") was chosen because of the proteins' actin filament-severing property (Rodríguez Del Castillo *et al.*, 1990). Conversely, scinderin is also known as adseverin (Nakamura *et al.*, 1994; Sakurai *et al.*, 1991a, b;

Maekawa and Sakai, 1990). This protein, first described by Bader *et al.* (1986) and Rodríguez Del Castillo *et al.* (1990) in chromaffin cells, has subsequently been isolated via actin-DNase I-Sepharose and immunoprecipitation experiments performed on the adrenal medulla, brain, salivary glands, anterior and posterior pituitaries and platelets (Rodríguez Del Castillo *et al.*, 1992; Tchakarov *et al.*, 1990). Also, scinderin is found in renal tissue and testis, although at much lower levels; however, the protein is not found in skeletal and heart muscles, liver and plasma, tissues known to contain gelsolin (Tchakarov *et al.*, 1990). Therefore, scinderin seems to be expressed in neuronal and endocrine tissues; systems in which secretion is a main function (Rodríguez Del Castillo *et al*, 1992, 1990; Tchakarov *et al.*, 1990; Sakurai *et al.*, 1990).

The molecular weight of scinderin as determined by SDS-PAGE and confirmed by immunoblotting is 79,600 ± 450 daltons (Rodríguez Del Castillo *et al.*, 1990). In 1994, Marcu *et al.* reported that when the first possible start codon of the open reading frame is taken as a translational start site, that polypeptide consists of 7.15 amino acids with a calculated molecular weight of 80,800 daltons. This molecular weight derived from the sequence agrees with the value obtained by Rodríguez Del Castillo *et al.* (1990). The protein contains two Ca^{2+} binding sites (K_d 5.85 x 10^{-7}M, B_{max} 0.81 mol Ca^{2+}/mol protein; K_d 2.85 x 10^{-6}M, B_{max} 1.87 mol Ca^{2+}/mol protein) within the N-terminal portion (Rodríguez Del Castillo *et al.*, 1990). Two-dimensional electrophoresis showed three distinct isoforms (pI = 6.0, 6.1, and 6.2), while

chymotryptic digestion of scinderin (using a protein:enzyme ratio of 400:1) produced two main proteolytic fragments of molecular weights 40 kd (two isoforms of pI 6.0 and 6.1) and 34 kd (two isoforms of pI 5.7 and 5.9) respectively, and a small 32 kd (pI = 5.8) fragment (Rodríguez Del Castillo *et al.*, 1990). Additionally, in the presence of Ca^{2+}, the viscosity of actin solutions was decreased with molar ratios of scinderin to actin of more than 1:3,200, while at a molar ratio of 1:800 there was a decrease in viscosity from 480 to 10 cp (Rodríguez Del Castillo *et al.*, 1990). Further, two molecules of actin form a complex with one molecule of scinderin in a Ca^{2+}-dependent manner (Trifaró *et al.*, 1993; Rodríguez Del Castillo *et al.*, 1990). More recent data from the lab suggest that a third actin binding site exists on the C-terminal portion of the molecule (Marcu *et al.*, 1997; submitted for publication).

Sequence analysis of scinderin revealed that bovine chromaffin cell scinderin is a protein of 715 amino acids which share 63 and 53% homology with gelsolin and villin, respectively (Marcu *et al.*, 1994). Furthermore, scinderin also shares six internal repeats of short sequence motifs (A, B, C) with gelsolin and villin and alignment of these motifs reveals six domains (1-6) each having 120 to 130 amino acid residues (Marcu *et al.*, 1994). There are strong similarities between domains 1 and 4, 2 and 5, and 3 and 6 in gelsolin, villin and scinderin (Marcu *et al.*, 1994). Two other filament severing proteins, severin and fragmin, contain homologies to scinderin although these proteins have about half of the molecular mass of scinderin (Marcu *et al.*, 1994). Also, severin and fragmin have better homologies

with the N-terminal half of villin, gelsolin and scinderin suggesting that the latter three proteins have been derived by gene duplication from an ancestral actin filament-severing protein which was similar to the N-terminal half of these molecules (Marcu *et al.*, 1994). Moreover, Marcu *et al.* (1994) reported that the glutathione S-transferase (GST) fusion protein, F-Sc1, which was obtained from a full length scinderin cDNA clone 1 (Sc-1) was immunologically identical to native scinderin, was bound to actin, phosphatidylserine (PS) and phosphatidlylinositol 4,5-bisphosphate (PIP_2) in a Ca^{2+}-dependent manner. Also, antibodies raised against the fusion protein produced the same cellular staining patterns for scinderin as with antibodies against native scinderin (Marcu *et al.*, 1994).

Immunofluorescence studies on cultured chromaffin cells have shown that under resting conditions scinderin displays a diffuse cytoplasmic staining and a continuous cortical fluorescent ring, suggesting that the protein may be bound to plasma membrane phospholipids such as phosphatidylinositol, phosphotidylserine (Rodríguez Del Castillo *et al.*, 1992; Vitale *et al.*, 1991). Stimulation of the cells with either nicotine, depolarizing concentrations of K^+ or micromolar concentrations of Ca^{2+} results in the fragmentation of the scinderin cortical fluorescent ring, suggesting that the protein is released from subplasmalemmal regions (Vitale *et al.*, 1991). Therefore, the scinderin redistribution which occurs along with F-actin disassembly, is a Ca^{2+}-dependent process which preceeds exocytosis (Vitale *et al.*, 1991). Further, the close association between scinderin redistribution and F-actin

SCINDERIN PROPERTIES

- Ca^{2+}-dependent F-actin severing protein
- molecular weight = 80,800
- three isoforms (pI: 6.0, 6.1, 6.2)
- six domains (Sc_1 to Sc_6) (see above)
- two Ca^{2+} binding sites
- three actin binding sites
- two PIP_2 binding sites
- present in tissues with high secretory activity

disassembly resulting from stimulation evoked-Ca^{2+} influx, suggests that during stimulation scinderin associated with actin filaments and promotes its severing activity (Rodríguez Del Castillo *et al.*, 1992; Vitale *et al.*, 1991). Moreover, removal of the stimulus results in the recovery of the cortical scinderin fluorescent ring as well as the cortical F-actin ring although the scinderin recovery is faster than F-actin indicating that when Ca^{2+} levels return to basal, scinderin reassociates with plasma membrane phospholipids (Rodríguez Del Castillo *et al.*, 1992). Finally, PS and PIP_2 inhibit scinderins severing and polymerization-promoting activities (Rodríguez Del Castillo *et al.*, 1992; Maekawa and Sakai, 1990).

Recent studies conducted by Zhang *et al* (1996) have shown that recombinant scinderin potentiates catecholamine release in permeabilized chromaffin cells. These effects are inhibited by peptides with sequences corresponding to two actin binding sites or a PIP_2-binding site of scinderin. Also, a truncated $scinderin_{254-715}$, which lacks the actin-severing domains (domain 1 and 2), did not potentiate exocytosis. These results suggest that scinderin-evoked cortical F-actin disassembly is required for secretion and that scinderin is an important component of the exocytotic machinery (Zhang *et al.*, 1996). To further expolore scinderins role in exocytosis and validate the afore mentioned findings scinderin levels within chromaffin cells could be reduced though the use of antisense technology. In turn, this treatment is expected to produce opposite effects to those obtained with recombinant scinderin treatment.

PART III: Antisense Oligodeoxynucleotides

In recent years science has witnessed the advent of innovative technologies for disrupting the expression of virtually any gene desired (Gewirtz *et al.*, 1996). One of these new technologies focuses on a class of chemicals, oligonucleotides, that have not been studied as potential drugs before and uses them to intervene in biological procedures that likewise have not been studied as sites at which drugs might act (Crooke, 1993). Although this new antisense technology is still in its infancy it has the potential to become one the most powerful tools available in modern medicine to treat illnesses such as cancer, AIDS and other currently non-treatable diseases simply because antisense molecules bind with high specificity to messenger RNA (mRNA) to selectively turn off genes.

1. (8). Oligodeoxynucleotides

In 1978, Zamecnik and Stephenson reported that antisense oligonucleotides can be used to inhibit the Rous sarcoma virus. Since then antisense oligonucleotides, although still in the early stages of development, have been emerging as a potentially new class of therapeutics. This new class of therapeutic agents allow researchers to control gene expression through the most basic of elements required for cell survival: RNA and DNA. These novel agents modulate the information transfer from the gene to protein; in essence they alter the intermediary metabolism

of RNA (Crooke, 1995; Mirabelli and Crooke, 1993; Crooke, 1992). Simply, in order for a protein to be made, the unique gene that specifies its composition must be expressed (transcribed or copied) from double-stranded DNA into individual molecules of single-stranded messenger RNA (mRNA) which is subsequently translated into the specified protein (Cohen and Hogan, 1994). Using the basic concepts of Watson-Crick base pairing researchers have developed synthetic strings of antisense nucleotides or deoxynucleotides, thus their name oligonuclotide (ON) or oligodeoxynuclotide (ODN), which are 15 to 30 bases long (Crooke *et al.*, 1996) which are complementary for the desired target mRNA with the consequent inhibition of protein synthesis. For simplicity both ONs and ODNs will be referred to as antisense ODNs.

1. (9). Molecular Mechanism of Action of Antisense ODNs

Although researchers, to date, have not yet been able to elucidate the exact mechanism of action of antisense ODNs several possibilities have been put forth. Based on the concept that antisense ODNs inhibit gene expression in a sequence specific manner through Watson-Crick base pairing it is commonly accepted that once the synthetic antisense ODN binds to or hybridizes with its target mRNA (either precursor mRNA in the nucleus or cytoplasmic mRNA) it wili prevent translation of a specific mRNA transcript, alternatively this interaction may cause translational arrest by steric hinderance of ribosome progression (Akhtar and

Agrawal, 1997; Coulson *et al.*, 1996; Miller, 1996; Brysch and Schlingensiepen, 1994; Kim and Wold, 1985)(Figure 5a). Also, hybridization of the antisense ODN with the target mRNA may interfere with mRNA splicing or the antisense ODN may disrupt secondary or tertiary structures in the mRNA which form recognition and binding sites for nonribosomal proteins involved in RNA processing, RNA transport from the nucleus, regulation of translation, and stabilization of the mRNA (Vickers *et al.*,1991; Cazenave and Hélène, 1990; Kim and Wold, 1985). Another mode of action that has been suggested is the activation of RNase H, a ubiquitous enzyme required for DNA replication which specifically cleaves the RNA strand of RNA:DNA hybrids (Akhtar and Agrawal, 1997; Coulson *et al.*, 1996; Miller, 1996; Brysch and Schlingensiepen, 1994; Cazenave and Hélène, 1991; Walder and Walder, 1988). Activation of this enzyme results in the cleavage of the mRNA at the RNA:DNA heteroduplex and as a result the mRNA cannot be translated and is in turn degraded within the cells (Cohen and Hogan, 1994; Cazenave and Hélène, 1991; Walder and Walder, 1988). Yet another mechanism of action for antisense ODNs that has been proposed is that deoxynucleotide sequences may bind to double-stranded DNA in the nucleus forming triple-stranded DNA complexes resulting in transcriptional inhibition and in turn down-regulation of the target gene (Miller, 1996; Scanlon *et al.*, 1995; Wahlestedt, 1994; Brysch and Schlingensiepen, 1994; Crooke, 1993; Mirabelli and Crooke, 1993; Ramanathan *et al.*, 1993; Cazenave and Hélène, 1991; Weintrub, 1990; van der Krol *et al.*, 1988)(Figure 5b). The advantage of this mechanism of action over other strategies is that the triplex-

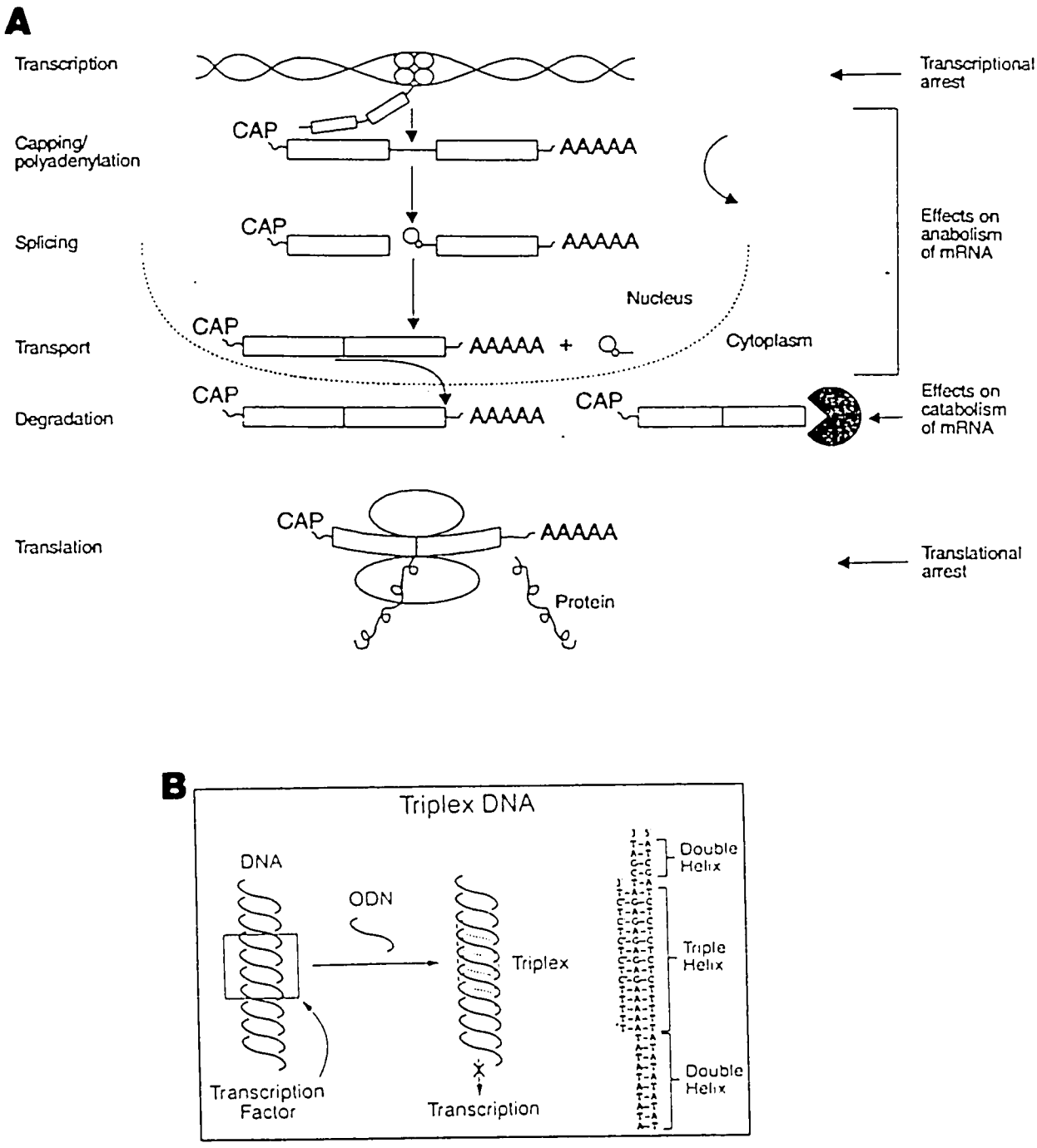


Figure:5

(A) Schematic of RNA processing and potential mechanisms of action of oligonucleotides (Taken from Crooke, 1995). **(B)** Structure and mechanism for triplex DNA (Taken from Scanlon *et al.*, 1995)

forming antisense ODNs target the gene directly rather than its mRNA (Scanlon *et al.*, 1995). Antisense ODNs which target DNA rather than mRNA are commonly referred to as antigenes.

1. (10). Cellular Uptake of ODNs

Antisense technology has been successfully applied in cell culture systems where by the expression of a desired gene has been inhibited (Sachinidis *et al.*, 1996; Valerio *et al.*, 1994; Mattson *et al.*, 1993; Wahlestedt *et al.*, 1993). Regardless of the antisense ODN application, *in vivo* or *in vitro*, it is essential that a significant amount of antisense ODN is taken up by the cell and that it accumulates in sufficient quantity at the target site (cytoplasm or nucleus) so that it may exert its biological effect. Cellular uptake of antisense ODNs is temperature and time dependent and it is influenced by cell type, cell-culture conditions, media, antisense ODN length and sequence and modification (Crooke *et al.*, 1995; Gao *et al.*, 1993; Crooke, 1992; Ghosh and Cohen, 1992). Unmodified antisense ODNs have a negative charge and are highly water soluble while phosphorothioate antisense ODNs, are negatively charged, but because of the sulphur atom, they are slightly more lipophilic than the unmodified antisense ODNs and tend to bind nonspecifically to serum proteins (Crooke, 1992).

The negative charge of the antisense ODNs rules out the possibility of simple

diffusion across the cell membrane as a mechanism of cellular uptake. Instead cellular uptake is thought to be an energy-dependent mechanism such as absorptive endocytosis or fluid-phase endocytosis, which may be triggered in part by the binding of the antisense ODN to receptor-like proteins present on the surface of a wide variety of cells (Gewirtz *et al.*, 1996; Beltinger *et al.*, 1995; Gao *et al.*, 1993). Yakubov *et al.* (1989) suggests that absorptive endocytosis is the predominant mechanism at lower antisense ODN concentrations, when a substantial amount of the antisense ODN present is absorbed by the cell surface while at higher antisense ODN concentrations the dominant mechanism of uptake is fluid-phase endocytosis. Confocal and electron microscopy have shown that once the antisense ODN is internalized it enters the endosome/lysosome compartment from which it subsequently escapes intact, enters the cytoplasm and then diffuses into the nucleus where it presumably binds to its target mRNA (Gewirtz *et al.*, 1996).

1. (11). Antisense ODN Affinity

The affinity between the antisense ODN and its receptor, mRNA or DNA, results from hybridization interactions (Crooke and Bennett, 1996; Mirabelli and Crooke, 1993). The free energy of binding is affected by hydrogen bonding (usually Watson-Crick base pairing) and base stacking in the newly formed double helix (Crooke and Bennett, 1996; Crooke, 1995; Crooke, 1993; Mirabelli and Crooke,

1993). Affinity is affected by ionic strength which results from the hydrogen bonding between complementary base pairs (A-T bases form two hydrogen bonds while C-G bases form three hydrogen bonds) while stacking of the coplanar bases decreases entropy (Crooke and Bennett, 1996; Crooke, 1995; Crooke, 1993; Mirabelli and Crooke, 1993). Therefore, affinity increases as the length of the antisense ODN receptor complex increases while overall affinity is determined by the affinity per nucleotide unit and the number of hybridizing nucleotide pairs (Crooke and Bennett, 1996; Mirabelli and Crooke, 1993). Further, affinity varies as a function of the sequence in the duplex and the stability of these duplexes varies as a function of the sequence (Crooke and Bennett, 1996; Mirabelli and Crooke, 1993). The free energy of binding for DNA-DNA, RNA-RNA, DNA-RNA and RNA-antisense ODN hybrids can be determined with high precision using the nearest neighbour rules, with the RNA-RNA duplex being most stable (Freier, 1993; Breslaur *et al.*, 1986; Freier *et al.*, 1986).

A minimal amount of affinity must exist between the antisense ODN and its receptor in order that the desired interaction and in turn biological effect may occur. For many targets and types of antisense ODNs, this can be achieved with an antisense ODN that is a least 11-15 (Wahlestedt, 1994) or 12-14 (Brysch and Schlingensiepen, 1994; Mirabelli and Crooke, 1993; Crooke, 1992) or 15-18 nucleotides long (Crooke, 1993). Based on the differences in affinity of antisense ODNs for their complementary target sequence, calculations suggest that

unmodified antisense ODNs between 11-15 in length should be able to bind selectively to a single RNA species in the cell (Crooke, 1992; Cazenave and Hélène, 1991).

1. (12). Antisense ODN Specificity

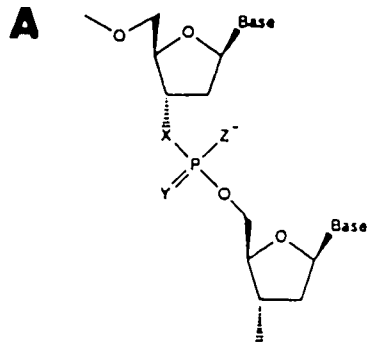
The attraction of antisense ODNs as potential therapeutic agents comes from their ability to target a single gene product without influencing other cellular functions, therefore specificity is highly important. Specificity implies that under constant physiological conditions, *in vivo*, perfect hybrids will form at the complementary site in the target mRNA, and no imperfect complexes will be produced through interactions between the antisense ODN and regions of partial complementarity in other mRNA species (Tidd, 1996). In order for an antisense ODN to be highly specific, the affinity for the correct target must be substantially larger than the affinity for any other sites (Freier, 1993). Therefore, the introduction of a single base mismatch results in a change in affinity of approximately 500-fold (Crooke and Bennett, 1996; Crooke, 1993; Crooke, 1992; Freier *et al.*, 1992). In addition, specificity is effected by chain length, structure, accessibility of the targeted RNA to antisense ODN binding, stability of the RNA-antisense ODN duplex in cells, susceptibility of the RNA-antisense ODN duplex to RNase H cleavage, presence or absence of secondary structures at the binding site and sequence of the antisense ODN (Wagner *et al.*, 1996; Brysch and Schlingensiepen, 1994; Mirabelli

and Crooke, 1993; Crooke, 1992; Weintraub, 1990; van der Krol *et al.*, 1988). Further, specificity is influenced by the antisense ODNs nucleotide composition. A high G-C content will cause unspecific effects since the hybridization of even a short portion of the antisense ODN to an unrelated target might be stable enough to cause inhibition, while antisense ODNs consisting only of A's and T's will not bind well enough to their designated target to inhibit mRNA metabolism (Brysch and Schlingensiepen, 1994). Moreover, unmodified antisense ODNs, less than 12-14 nucleotides in length, should not be used as they are not unique in the mRNA population or complementary to the target RNA and would therefore inhibit nontargeted genes (Brysch and Schlingensiepen, 1994; Woolf *et al.*, 1992; Weintraub, 1990). Conversely, longer antisense ODNs may form more stable duplexes, however, they may also interact with multiple mRNAs through partial base-pairing involving 5 to 10 contiguous bases within their sequence (Wagner, *et al.*, 1996; Brysch and Schlingensiepen, 1994; Ghosh and Cohen, 1992). As a result, optimal antisense ODN activity is obtained with chain length of 14-20 bases (Crooke and Bennett, 1996; Tidd, 1996; Ghosh and Cohen, 1992). It should also be noted that antisense ODN length and concentration are highly interlinked: lower concentrations (usually between 1-10μM) are required for antisense ODNs of 17-20 bases, compared to antisense ODNs of 10 bases or less for which much higher concentrations are needed for biological activity (Ghosh and Cohen, 1992).

1. (13). Phosphorothioate Antisense ODNs

Unmodified phosphodiester antisense ODNs have several problems of which the most important is nuclease digestion. These antisense ODNs are digested by ubiquitous endo- and exo-nucleases in the serum and intracellular space within 15 to 30 minutes (Peyman and Uhlmann, 1996; Crooke, 1992; Wickstrom, 1986). As a result the use of these antisense ODNs is limited to serum-free culture systems or organs which have low nuclease content such as the central nervous system (CNS) and in cerebrospinal fluid, even then intracellular stability is low (Brysch and Schlingensiepen, 1994). In order to overcome this problem researchers have enhanced the pharmacodynamic and pharmacokinetic properties of antisense ODNs by introducing chemical modifications in the sugar, base or phosphate backbone with the latter modification being most common since the phosphorous center is the site of nucleolytic attack (Crooke, 1993; Hoke *et al.*, 1991). Although numerous modifications have been characterized (Figure 6) this report will focus on the phosphorothioate modification.

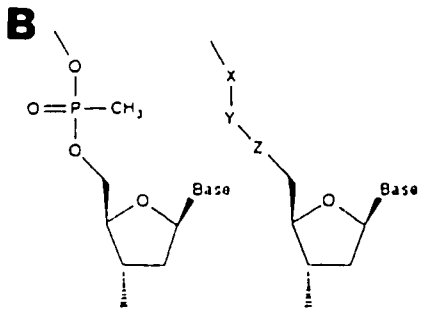
In phosphorothioate antisense ODNs (antisense SODN) the non-bridging oxygen of the phosphate backbone is replaced with a negatively charged sulphur atom, making the antisense SODN polyanionic (Crooke and Bennett, 1996; Peymann and Uhlmann, 1997; Brysch and Schlingensiepen, 1994; Cazenave and Hélène, 1991; Weintraub, 1990). This substitution dramatically increases the antisense SODNs



Analog	X	Y	Z
1a	O	O	O
1b	O	O	S
1c	O	S	S
1d	O	BH_3	O
1e	NH	O	O

1

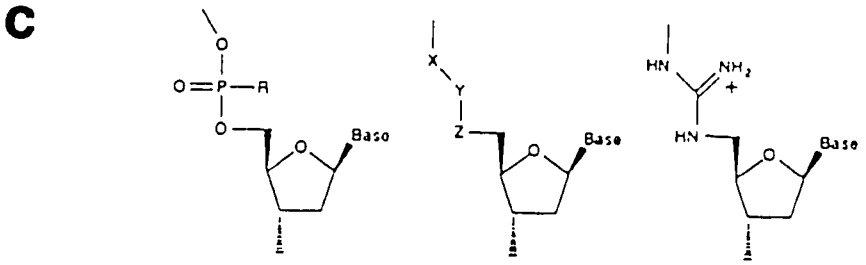
. Anionic phosphodiester analogs



Analog	X	Y	Z
3a	S	CH_2	O
3b	O	CH_2	O
3c	CH_2	NMe	O
3d	CH_2	C=O	NH
3e	CH_2	SO_2	CH_2
3f	NH	SO_2	CH_2
3g	O	SO_2	NH

2 3

Neutral backbones including methylphosphonate 2



4 5 6

Analog	R	Analog	X	Y	Z
4a	$-NHCH_2CH_2N^+Me_2H$	5a	CH_2	CH_2	NH
4b	$-CH_2CH_2N^+H_3$	5b	CH_2	NH	CH_2
		5c	NH	CH_2	CH_2

Cationic linkages

Figure: 6

Various ODN modifications. **(A)** Phosphorus modifications. **(B)** Sugar modifications. **(C)** Backbone modifications (Taken from Cook, 1993).

resistance to both exo- and endo-nucleases (Akhtar *et al.*, 1991; Hoke *et al.*, 1991; Eckstein and Gish, 1989) by approximately 2 to 45 fold in tissue culture media, sera or other biological fluids (Crooke, 1992; Campbell *et al.*, 1990; Stein *et al.*, 1988). Phosphorothioate antisense ODNs, unlike unmodified antisense ODNs or methylphosphonates, are substrates for RNase H (Cook, 1993; Chiang *et al.*, 1991; Mirabelli *et al.*, 1991; Baker *et al.*, 1990; Hélène and Toulmé, 1990) suggesting that this may be one of the possible mechanisms of action. Chiang *et al.* (1991) reported that the antisense SODNs potently inhibited ICAM production via extensive degradation of ICAM mRNA by RNase H activation in addition to other mechanisms.

1. (14). Toxicity of Phosphorothioate Antisense ODNs

Although there are numerous advantages of using antisense SODNs as compared to unmodified antisense ODNs there are some drawbacks. Most apparent is the problem of nonspecific side effects and toxicity. The nonspecific effects of antisense SODNs is due to their ability to bind to a variety of proteins, including serum albumin which may produce pharmacological activity easily confused with antisense activity (Crooke and Bennett, 1996; Crooke, 1992). Ghosh *et al.* (1992) reported that direct comparison of binding to human serum albumin using a filter assay with ^{32}P end labelled 17-mer phosphodiester or phosphorothioate anti-β-globin antisense ODNs, 25 ± 6% of the phosphodiester antisense ODN was bound

compared to 66 ± 3% of the phosphorothioate antisense ODN. The nonspecific effects of the antisense SODNs are length-dependent with the nonspecific toxicity increasing with antisense SODN length (Cazenave *et al.*, 1989; Matsukura *et al.*, 1987).

Another problem with antisense SODNs is that the replacement of the nonbridging oxygen with a sulphur atom results in the formation of many chiral centers within the antisense ODN and in turn a duplex of the modified antisense ODN with a complementary RNA target is less stable (Chaix *et al.*, 1996). The number of chiral centers increases with the number of bases that are modified and the length of the sequence. Therefore, antisense ODNs that are minimally modified, *i.e.*, several nucleotide bases on either the 3', 5' or both the 3' and 5' end are modified have been developed. When compared to fully modified antisense ODNs, the phosphorothioate endcapped antisense ODNs have less nonspecific side effects and Hoke *et al.* (1991) and Stein *et al.* (1988) reported that endcapping the 3' end is sufficient to confer stability from exonuclease digestion in serum containing cell culture medium. Alternatively, endcapping on the 5' did not confer any nuclease resistance to the antisense ODN (Hoke *et al.*, 1991).

1. (15). Statement of the Problem

The process of neurotransmission requires the involvement of several components

and elaborate cellular processes, one of which, is exocytosis. Exocytosis or cellular secretion can be either constitutive or regulated (Kelly, 1985; Green and Shields, 1984; Gumbiner and Kelly, 1992; Tartakoff *et al.*, 1978). The latter form of secretion has been prominently studied in adrenal chromaffin cells as these cells share a common embryological origin with the sympathetic neurons in that they are both derived form the neural crest and in their differentiated states share some morphological and functional properties (Euler, 1972; Stjärne, 1972). In addition, cellular secretion in chromaffin cells is triggered by an increase in intracellular Ca^{2+} and the secretory products, which are released from the cells during stimulation, are stored in membrane-bound secretory vesicles or granules (Trifaró *et al.*, 1993; Trifaró and Poisner, 1982; Trifaró, 1977).

Although the exact mechanism in which Ca^{2+} is involved in triggering exocytosis is still poorly understood Trifaró (1990) has suggested that the action of Ca^{2+} is mediated through the control of the cellular cortical cytoskeleton network. Several researchers have shown that the cortical actin network acts as a barrier to the secretory vesicles thus blocking their movement toward release sites on the plasma membrane (Burgoyne *et al.*, 1989; Sontag *et al.*, 1988; Burgoyne and Cheek, 1987; Cheek and Burgoyne, 1986; Trifaró *et al.*, 1982, 1984, 1989). Vitale *et al.* (1995) showed that in resting chromaffin cells 1-3% of the total chromaffin vesicles are either docked to the plasma membrane or within 50nm from it. This group of vesicles constituted the so called "release-ready vesicle pool". The remaining

chromaffin vesicles (97-99%) form a reserve pool and remain within a barrier of cortical filamentous actin (F-actin) (Vitale *et al.*, 1995). Immunofluorescence studies have shown that stimulation of the cells with either micromolar Ca^{2+} concentrations, nicotine or depolarizing concentrations of K^+ results in the disassembly of the cortical F-actin network and in turn the movement of the secretory vesicles from the reserve pool to release sites as the plasma membrane (Vitale *et al.*, 1991, 1995; Cheek and Burgoyne, 1986). Thus, the cortical network dynamics control the size of the release-ready vesicle pool (Vitale *et al.*, 1991). Rodríguez Del Castillo *et al.* (1992; 1990) and Vitale *et al.* (1991) have suggested that chromaffin cell cortical actin network dynamics is controlled by scinderin, a Ca^{2+}-dependent F-actin severing protein discovered in our laboratory (Rodríguez Del Castillo *et al.*, 1990).

Immunocytochemical studies have also shown that during cellular stimulation subplasmalemmal scinderin is redistributed in chromaffin cells and that this redistribution precedes exocytosis (Vitale *et al.*, 1991). In addition work, also from Vitale *et al.* (1991) has demonstrated that exocytosis sites are preferentially localized to areas of F-actin disassembly. Furthermore, redistribution of scinderin and F-actin disassembly are Ca^{2+}-dependent events which have similar patterns of distribution following stimulation (Vitale *et al.*, 1991). Moreover, scinderin redistribution and actin filament disassembly, induced by either nicotine or high K^+, precedes catecholamine release (Vitale *et al.*, 1991). In 1996, Zhang *et al.* showed

that catecholamine release was potentiated in permeabilized chromaffin cells treated with 0.1μM recombinant scinderin and subsequently stimulated with 10μM Ca^{2+} compared to cells stimulated with only 10μM Ca^{2+}. These data suggest that scinderin may play a significant role in the exocytotic process. To further demonstrate and validate these findings scinderin levels within chromaffin cells could be reduced and the opposite effect to those obtained with recombinant scinderin treatment should be observed.

Therefore, one method of reducing scinderin levels without drastically effecting the physiological state of the cultured chromaffin cells was to utilize antisense technology. The advantage of this technology, when used properly, is that it allows researchers to target a specific gene without introducing other potentially misleading variables. Therefore, the primary objective of this project was to reduced cellular expression of scinderin and study the effects of scinderin reduced levels on events directly related to the secretion process. Consequently the aim of these studies were:

(1) To design a scinderin antisense ODN with the following characteristics:
   (a) antisense ODN sequence which encompasses the scinderin starting codon (ATG)
   (b) optimal length, between 15 to 20 nucleotides, to produce biological effects

(c) modified backbone to confer nuclease resistance

(d) 100% sequence specificity for scinderin

(2) To develop reliable assay for scinderin cellular levels

(3) To test the scinderin antisense ODN designed according to the parameters described in (1) for their effect on scinderin expression

(4) If scinderin expression and its message (mRNA) are successfully reduced, the impact of the reduced scinderin levels on secretion will be evaluated. Specific parameters to be studies are:

(a) Effects on stimulation induced F-actin disassembly

(b) Effects on tissue distribution of scinderin as evaluated by immunocytochemistry

(c) Effects on stimulation (high K^+) induced catecholamine secretion

CHAPTER 2 - MATERIALS AND METHODS

CHAPTER 2 - METHODS

2. (1). Bovine Chromaffin Cell Culture

2. (1). (i). Dissection and Preparation of the Bovine Adrenal Gland

A primary culture consisting of chromaffin cells (90%-95%), fibroblasts (4%-9%) and cortical cells (1%) was obtained utilizing the method previously described by Trifaró and Lee (1980). Six to eight bovine adrenal glands were obtained from a local slaughterhouse and immediately upon excision were rinsed in buffer I, a Ca^{2+} and Mg^{2+}-free Locke's solution (in mM: NaCl, 154; KCl, 2.6; K_2HPO_4, 1.25; KH_2PO_4, 0.5; glucose, 10; pH 7.0) which also contained phenol red, penicillin (200 mg/l), streptomycin (50 mg/l), gentamycin (50 mg/l) and ceftazidime (20μg/ml).

In the laboratory the glands were rinsed in 70% ethanol and subsequently perfused with buffer I in a retrograde fashion with a polyethylene tube inserted in the adrenal vein to ensure that buffer I was perfused through all of the cells within the gland. Once the glands were cannulated and perfusion initiated the cortex was removed and the adrenal medulla placed in a closed circuit perfusing chamber and perfused with buffer I for 10 minutes at a flow rate of 10ml/min.

2. (1). (ii). Chromaffin Cell Isolation from the Adrenal Gland

Perfusion of the adrenal medulla was then carried out, in a closed circuit perfusing

chamber, for 60 minutes at 37°C with 25-30 ml of buffer II which was circulated throughout the chamber at a rate of 10ml/minute. Buffer II was an enzyme mixture which consisted of 5.3 mg DNase I and 548 units of collagense/gland per 400 ml of Ca^{2+} and Mg^{2+}-free Locke's solution containing phenol red, penicillin (100 mg/l), streptomycin (100 mg/l), mycostatin (25,000 U/l), gentamycin (50 mg/l) and ceftazidime (20 μg/ml).

Following enzymatic digestion the remaining adrenal cortex was removed from the flaccid adrenal medulla which was then minced with scissors. The minced tissue was transferred into a trypsinizing flask containing 200 ml of buffer II prewarmed at 37°C and stirred for 30 minutes in a 37°C water bath. The single cell mixture was then filtered through a sterile 44μ/cloth mesh into a graduated cylinder containing 50 ml of Locke's solution (in mM: NaCl, 154; KCl, 2.6; K_2HPO_4, 1.25; KH_2PO_4, 0.5; $MgCl_2$, 1.2; $CaCl_2$, 2.2; glucose, 10; pH 7.2) which also contained phenol red, penicillin, streptomycin, mycostatin, ceftazidime and gentamycin in the afore mentioned concentrations. The undigested tissue was rinsed with up to 400 ml of the solution described above. An aliquot of this total dispersion was taken and the cell number determined using a hemocytometer (Neubauer, Levy Chamber cat. no. 500).

2. (1). (iii). ***Chromaffin Cell Purification***

The crude cell mixture was centrifuged at 50 x *g* for 10 minutes at room temperature. The pellet was washed with 100 ml of the above mentioned solution and re-centrifuged at 50 x *g* for 15 minutes at room temperature. Following re-centrifugation the pellet was then suspended in Eagle's Balanced Salt Solution (EBSS 1x) (6 ml loading volume of cells/tube, 27 million cells/gradient) and was added to a mixture of Percoll (colloidal silica coated with polyvinylpyrrolidone) (pH 7.2) (6.72 ml/tube), EBSS 10x and antibiotics (0.75 ml/tube). The total volume of each gradient was 13.5 ml. A ratio of 8:9:1 for cell suspension:Percoll:EBSS 10x was obtained for optimal chromaffin cell isolation. The Percoll gradient was then centrifuged in a Sorvall centrifuge at 45,000 x *g* for 20 minutes at 20°C. To obtain the desired chromaffin cell population the top 4.5 ml of the gradient was discarded and the next gradient band, which contains the chromaffin cells, was collected. The collected volume of chromaffin cells was washed by diluting with 5 volumes of EBSS 1x and centrifuging at 50 *x g* for 15 minutes at room temperature. The supernatant was discarded and the pellet washed once more with 50 to 100 ml EBSS containing antibiotics (penicillin, 100 U/l; streptomycin, 100 mg/l; nystatin, 25,000 U/l; gentamycin, 50 mg/l; ceftazidime, 20 μg/ml). An aliquot of the cell suspension was taken and the number of cells counted using the Levy & Levy Hausser counting chamber.

2. (1). (iv). Plating of Chromaffin Cells

Once the chromaffin cells were obtained they were resuspended in Dulbecco's modified Eagle's medium (DMEM) fortified with 10% fetal calf serum, 0.1 mM ascorbic acid, 0.1% glucose and 15 mM Hepes, pH 7.2. The culture media also contained penicillin (100 μg/ml), streptomycin (100 μg/ml), gentamycin (50 μg/ml), ceftazidime (20 μg/ml), nystatin (25,000 U/l) and 5-fluorodeoxyuridine (10^{-5}M) as well as, fresh cytosine arabinose (cytostar, 10^{-5}M) to arrest cell division and in turn, the proliferation of fibroblasts.

Cells utilized for all experiments contained within this thesis were plated on collagen-coated 35mm petri dishes at a density of 0.5 x 10^6 cells/35mm petri dish. It should be noted that cells utilized for immunohistochemistry purposes where plated at a density of 0.5 x 10^6 cells/dish with the following modification. The cells were plated on collagen-coated glass cover slips which were placed into 35mm petri dishes. Once the cells were plated they were cultured in a NAPCO 6300 humidified incubator under a 5% CO_2 and 95% air atmosphere at 37°C for an initial rest period of at least 24 hours. This rest period allowed for cell adhesion to the collagen-coated surface. After this period the cells were treated according to the protocols described below.

2. (2). Evaluation of Cell Survival using Trypan Blue Exclusion

2. (2). (i). Chromaffin Cells Cultured in a Serum-free Medium

Following a 24 hour period, the above specified culture medium was changed to a culture medium which was not fortified with 10% fetal calf serum (DMEM, 0.1 mM ascorbic acid, 0.1% glucose and 15 mM Hepes, pH 7.2); hence, termed serum-free medium. The serum-free culture medium also contained penicillin (100 μg/ml), streptomycin (100 μg/ml), gentamycin (50 μg/ml), ceftazidime (20 μg/ml), nystatin (25,000 U/l) and 5-fluorodeoxyuridine (10^{-5}M) as well as, fresh cytosine arabinose (cytostar, 10^{-5}M).

2. (2). (ii). Determination of Cell Survival in Serum-free Medium

Phase-contrast microscopy was used to inspect dishes for cell death or changes in cellular morphology. Chromaffin cells were cultured in serum-free medium for 4 or 7 days at which time cell survival was determined by adding 50 μl of 0.4% trypan blue in saline solution to each 35mm petri dish. One hundred cells were examined per 35mm petri dish and the percentage of cell survival calculated (see below). Cells were deemed dead if they turned blue.

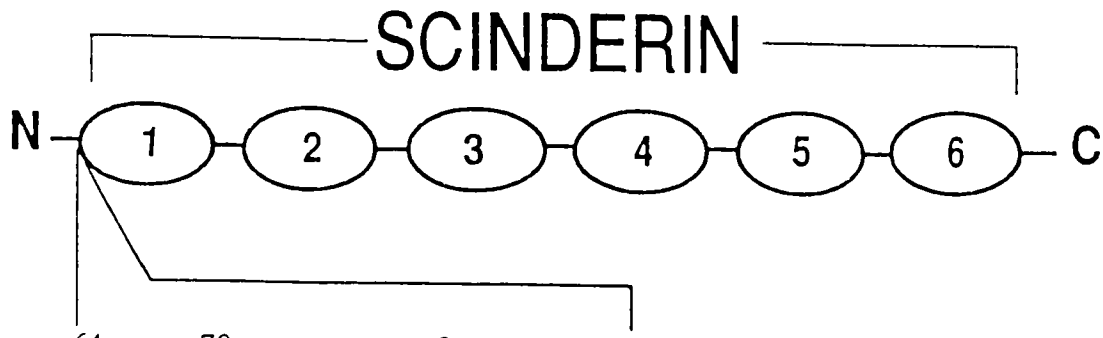
Calculation of cell survival:

$$\frac{\text{number of dead (or blue) cells}}{\text{number of living (or clear) cells}} \times 100\% = \%\text{ of intact cells}$$

2. (3). Scinderin Oligodeoxynucleotide Antisense Sequence

2. (3). (i). Scinderin Oligodeoxynucleotide Antisense Sequence

The scinderin oligodeoxynucleotide (ODN) antisense sequence used was analysed for stem loop structure, loop melting temperature, homodimer formation, homodimer melting temperature and target melting temperature (by %GC). These parameters were determined using *OligoTech* software (*Oligos Etc.* Inc., Wilsonville, OR., U.S.A.). Two ODN antisense sequences, an 18-mer and 20-mer sequence, which encompassed the methionine (ATG) start codon were chosen (Figure 7). Several modifications were made: (i) endcapped phosphorothioate antisense ODN (only three bases on the 5' and 3' end where phosphorothioate substituted); (ii) complete phosphorothioate backbone modification. In addition, a 20-mer endcapped phosphorothioate mismatch ODN sequence and a 20-mer phosphorothioate scramble ODN sequence were designed. All four ODN sequences selected were checked against known sequences of the GeneBank by a computer search and were found not to resemble any other known sequences. The ODNs were custom synthesized by *Oligos Etc.*, Inc. (Wilsonville, OR, U.S.A.). ODN stocks were prepared in DEPC treated water at a concentration of 10^{-4}M and stored at -70°C.



64 70 81 89

5'...CGCGTGCCCGAGTC***ATG***GCCCAGGGG...3' (scinderin)

3'.....GGGCTCAGTACCGGGTCCCC...5'(20mer scinderin antisense ODN)

(i) * * * * * *

(ii) **********

3'.....CGCGTCAGTAGGCCCTGGGC...5' (20mer mismatch)

* * * * * *

3'.....CCCCTGGGCCATGACTCGGG...5' (20mer scramble)

****

3'...GCGCACGGGCTCAGTACC.....5' (18mer scinderin antisense ODN)

****

* = phosphorothioate substitution

__ = start codon

Figure: 7

The schematic illustration of scinderin. In addition, the various oligodeoxynucleotide sequences used for this project are depicted below the scinderin schematic.

2. (4). Uptake of Scinderin Antisense ODN by Chromaffin Cells

2. (4). (i). Source of 5'-Fluorescein Labelled Scinderin Phosphorothioate Endcapped Antisense ODN

The fluorophore, fluorescein was linked by a covalent bond to the 5'-terminus of the 20-mer scinderin phosphorothioate endcapped ODN antisense by *Oligos Etc.*, Inc (Wilsonville, OR., U.S.A). The labelling was performed during synthesis and as a result the finished ODN was not contaminated with unreacted label. The labelling compound was designed such that the fluorescein structure of the labelled oligonucleotide is the same as that obtained using fluorescein isothiocyanate (FITC). The stock was dissolved in DEPC treated water at a concentration of 10^{-4}M and stored at -70°C until the experiment was carried out.

2. (4). (ii). Treatment of Cultured Chromaffin Cells with 5'-Fluorescein Labelled Scinderin Phosphorothioate Endcapped Antisense ODN

Chromaffin cells utilized for the uptake of 20-mer scinderin antisense studies were cultured at a density of 0.5 x 10^6 cells/35mm petri dish with glass cover slip bottoms in a serum- containing medium for 24 hours. The serum-containing medium was removed and replaced with serum-free medium. Twenty four hours later the cell preparations were incubated at 37°C under 5% CO_2 and 95% O_2 with 2μM 5'-fluorescein labelled 20-mer scinderin phosphorothioate endcapped antisense ODN

in serum-free medium for 1, 2, 4, 6, 24, 48, 72 and 96 hours. After the afore mentioned times had lapsed the chromaffin cells were washed three times with 1 ml of Locke's solution (see page 4 for composition) and mounted with PBS:glycerol (1:1). The uptake of the fluorescein labelled 20-mer scinderin phosphorothioate endcapped antisense ODN was observed using a Olympus BX-FLA fluorescent microscope equipped with a 100-W high pressure lamp and a Universal reflected light fluorescent vertical illuminator possessing an U-MNB filter cube (DM500 dichroic mirror, BP470-490 exciting filter, BA515 barrier) for fluorescein. All photographs were taken with Kodak Tri X-pan film (400 ASA).

2. (5). Treatment of Cultured Bovine Chromaffin Cells with Antisense Oligodeoxynucleotides (ODN)

2. (5). (i). Treatment of Cultured Bovine Chromaffin Cells with Scinderin Antisense ODN

Bovine chromaffin cells were cultured as previously described at a density of 0.5 x 10^6 cells/35mm petri dish in serum containing medium which was replaced with serum-free medium 24 hours after culturing. Following the latter incubation all of the medium was replaced with serum-containing medium (controls) or serum-free medium containing 2μM 18-mer or 20-mer scinderin phosphorothioate antisense ODN, 20-mer scinderin phosphorothioate endcapped antisense ODN, 20-mer mismatch phosphorothioate endcapped ODN sequence or 20-mer scramble phosphorothioate ODN sequence. This was termed day 1 of treatment. On days

2, 3 and 4 of the treatment protocol 20% of the medium was replaced with serum (control) or serum-free medium containing the previously specified treatments. After the fourth day of treatment cell survival, scinderin levels, catecholamine release, actin disassembly and mRNA levels were examined. It should be noted that several concentrations, ranging from 0.5 to 10μM, of antisense were first used in order to determine the concentration which would reduce scinderin levels most profoundly without adversely effecting chromaffin cell survival.

2. (6). Scinderin Assay

A method utilising SDS-PAGE - Western blot analysis was developed to measured chromaffin cell scinderin levels.

2. (6). (i). Source of Scinderin Antiserum

Scinderin antiserum was produced by raising polyclonal antibodies to purified bovine scinderin in rabbits. The antiserum used for all of the immunohistochemical experiments described in this thesis was derived from blood obtained by bleeding rabbit number 6, hence termed scinderin antiserum #6. Scinderin antiserum #6 has been determined to possess a high degree of specificity toward scinderin and does not cross-react with the other cytoskeleton proteins (Vitale *et al.*, 1991) and actin-binding proteins such as gelsolin (Rodríguez Del Castillo *et al.*, 1990; Tchakarov

et al., 1990). Due to inadequate amounts of scinderin antiserum #6 it was necessary to utilize scinderin antiserum #7 for all the Western blot analysis described in this thesis. Scinderin antiserum #7 was derived from blood obtained by bleeding rabbit number 7, hence termed scinderin antiserum #7. Using Western blot analysis the results obtained with scinderin antiserum #7 were comparable to those obtained with scinderin antiserum #6 indicating that this antiserum possed a high degree of specificity toward scinderin and it did not cross-react with gelsolin or other cytoskeleton proteins.

2. (6). (ii). ***Sample Preparation***

Chromaffin cells were washed two times with one ml phosphate buffered saline (PBS) (in mM: NaCl, 140; KCl, 2.6; Na_2HPO_4, 10; KH_2PO_4, 1.8; pH 7.4) and then harvested with 200 μl lysis buffer of the following composition: 100mM Tris-HCl, 0.1M EDTA and 0.4% SDS. Using a rubber policeman the cells were then scraped from the petri dish, transferred to an ependorf tube and stored on ice until all of the samples were prepared. Next, 20 μl of 100mM PMSF in isopropynol was added and the cell emulsion sonicated with 2 pulses (Sonifier Cell Disruptor 350, Brunson Sonic Power Company) and then centrifuged at 12,000 x *g* for 10 minutes at 4°C. The supernatant was transferred to clean ependorf tubes and the pellet discarded. Ninety microliters of the supernatant were mixed with 30 μl of 2X solubilizing buffer (140 mM Tris-HCl, 10M urea; 200mM DTT; 3% SDS and bromophenol blue) and

the samples were denatured by heating at 100°C for 2 minutes prior to loading.

2. (6). (iii). ***Scinderin Standard Curve***

A scinderin standard curve was run for every experiment concurrently with the samples in each gel. Samples (containing increasing amounts of scinderin) for the standard curve were prepared from cells cultured only in serum-free medium in the above specified manner with the following exceptions. After centrifugation, supernatants from three petri dishes were pooled and four different aliquots taken. The volumes for each standard was adjusted to 90 μl with PBS. Thirty microliters of 2X solubilizing buffer were then added and the standards were denatured by heating at 100°C for 2 minutes prior to loading.

2. (6). (iv). ***Monodimensional SDS-PAGE Electrophoresis***

Monodimensional SDS-PAGE was performed according to Doucet and Trifaró (1988). Gels were prepared from stock solutions of 25% acrylamide and 0.25% *N,N'*methylenebisacrylamide. The final composition of the running gel was 10% acrylamide, 0.1% *N,N'*methylenebisacrylamide, 0.4% SDS, 5% glycerol, 100mM glycine and 200mM Tris, pH 9.0. Polymerization was produced by the addition of 0.1% ammonium persulphate and 0.05% TEMED prior to pouring. Once the running gel mixture was loaded, the surface was slowly covered with ethanol and

the mixture was allowed to polymerize for approximately 30 minutes. Once polymerized the stacking gel was layered on top of the running gel. The stacking gel contained 4% acrylamide, 0.04% *N,N'*methylenebisacrylamide, 0.4% SDS, 5% glycerol, 4 mM EDTA and 70mM Tris-HCl and was degassed for 15 minutes prior to adding 0.1% ammonium persulphate and 0.05% TEMED. Once poured the stacking gel was allowed to polymerize for 60 minutes prior to sample loading.

Once the gel was loaded with samples 600 ml of upper tank buffer (0.1% SDS, 150 mM glycine and 100 mM Tris) was added to the upper tank and 1200 ml of lower tank buffer (600 ml of upper tank buffer diluted with 600 ml of distilled water) was added to the lower tank. The gels were run at a constant voltage of 60V overnight in a Bio-Rad Protean I apparatus (Bio-Rad Laboratories Inc., Richmond, CA, U.S.A.).

2. (6). (v). ***Western Blot of SDS-PAGE Gel***

After, SDS-PAGE, the porous slab gel was first soaked for 15 minutes in cold transfer buffer [in mM: Tris-HCl, 25; glycine, 150; and 20% methanol (v/v) (pH 8.3)]. Proteins that were resolved on the gel were electrotransferred onto a nitrocellulose membrane (Hoefer Scientific Instruments, San Francisco, CA., U.S.A) for 1.5hr at 90V (setting 100%, with water cooling; current increase from 0.8 to 1.4A) in a LKB 2005 Transphor electroblotting unit. The nitrocellulose membranes were first

blocked with 5% Carnation non-fat dry milk in PBS for 1 hour and then incubated for 1.5 hours with scinderin antiserum #7 (1:1000 dilution) or anti-α-tubulin (Sigma Immuno Chemicals, St. Louis, MO., U.S.A) (1:3000 dilution) or anti-gelsolin (Chemicon, Mississauga, ON., Canada) (1:1000). Next, the membranes were washed three times for 30 minutes (first wash - 0.1% BSA in PBS; second wash - 0.1% BSA and 0.05% Tween-20 in PBS; third wash - 0.1% BSA in PBS) and then incubated for 1 hour with donkey anti-rabbit IgG-horseradish peroxidase-linked antibody (Amersham, Oakville, ON., Canada) (1:3000 dilution) or anti-mouse IgG horseradish peroxidase-linked antibody (Amersham, Oakville, ON., Canada) (1:3000 dilution). The membranes were washed again three times, as specified above, for 30 minutes.

*2. (6). (vi). **Detection of Scinderin***

The desired protein was visualized using enhanced chemiluminescence (ECL) which is a highly sensitive light emitting non-radioactive method for detecting immobilized specific antigens, conjugated directly or indirectly with horseradish peroxidase-labelled antibodies (Figure 8). An equal volume of ECL detection solution 1 was mixed with detection solution 2 (Amersham, Oakville, ON., Canada). Nitrocellulose membranes were then incubated in the detection cocktail for 60 seconds. Excess detection cocktail was drained from the membrane which was then placed into a clear zip-lock bag, sealed and exposed to autoradiographic

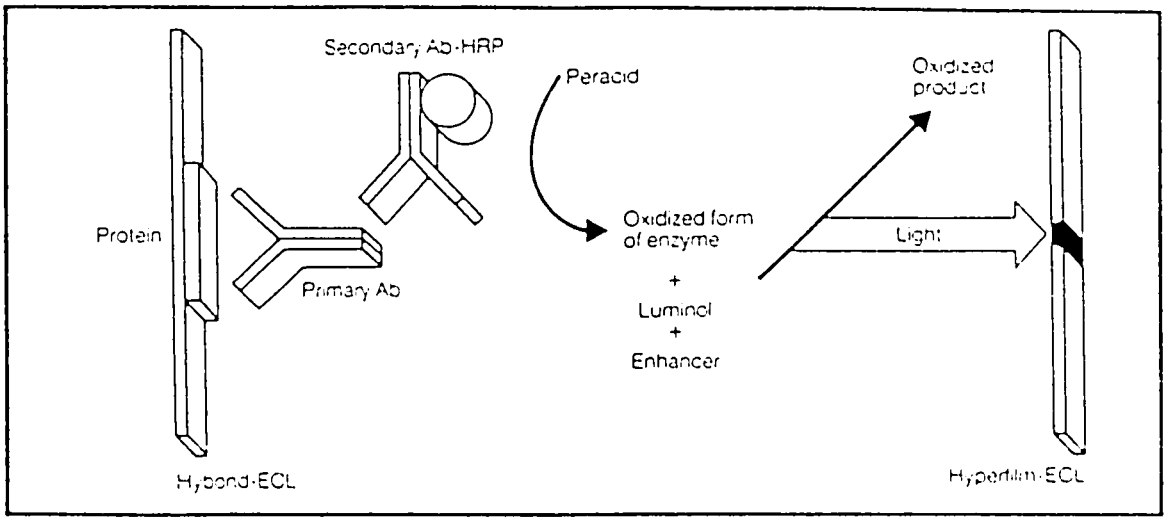


Figure: 8
Schematic of the principle behind ECL, the light emitting non-radioactive method for detecting immobilized antigens which have been conjugated with horseradish peroxidase-labelled antibodies (Taken from ECL Western Blotting Protocols, Amersham, Oakville, ON., Canada).

Hyperfilm-ECL (Amersham, Oakville, ON., Canada) for 45 seconds. The Hyperfilm-ECL was developed using a Kodak film developer. The protein bands were quantified using an Ultroscan XL Laser Densitometer (Beckman, CA., U.S.A).

2. (7). Coomassie Blue

2. (7). (i). Coomassie Blue Staining of SDS-PAGE Gel

An alternative method of ensuring that each lane of the monodimensional SDS-PAGE gels contained equivalent amounts of protein was to stain the gels, following electrophoresis, with coomassie blue (a dye which binds to protein). Each gel was loaded with protein as previously described [see section 2. (6). (iii)] and the proteins were subsequently electrophoresed as described in section 2. (6). (iv). Following electrophoration the proteins were fixed within the matrix of the SDS gel for 30 minutes in 40% methanol and 7% acetic acid. The proteins were then stained with 40% methanol, 7% acetic acid and 0.1% coomassie blue for 60 minutes. The gel was then destained overnight in 25% methanol and 10% acetic acid and then subsequently dried on 3M filter paper in a Gel Slab Dryer Model 224 (Bio-Rad Laboratories, Mississauga, ON., Canada) for 2 hours.

2. (8). Northern Blot

2. (8). (i). Scinderin DNA Probe

The ds DNA restriction fragment bovine scinderin cDNA (500bp) produced by treatment of scinderin clone 6 (2.7 Kb) with Sal I and Hind III was chosen as probe. Twenty nanograms of DNA were labelled with (γ^{32}P)dCTP (Amersham, Oakville, ON., Canada) using the Rediprime DNA Labelling System (Amersham, Oakville, ON., Canada). Briefly, 20 ng of the DNA probe was diluted to a final volume of 45 μl with sterile water and heated to 95-100°C for 5 minutes in a boiling water bath. The denatured DNA was then added to the Rediprime labelling mix (Amersham, Oakville, ON., Canada) and the contents mixed by gentle flicking of the tube until the contents where evenly distributed. Five microliters of Redivue [γ^{32}P]dCTP (specific activity 1.9 x 10^9CPM/μg) (Amersham, Oakville, ON., Canada) were added to the denatured DNA which was then incubated at 37°C for 10 minutes. The reaction was stopped by adding 5 μl of 0.2M EDTA and the labelled DNA mixture was then heated to 95-100°C for 5 minutes in a boiling water bath and then chilled on ice.

2. (8). (ii). GAPDH DNA Probe

The 600bp dsDNA restriction fragment of human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained as a gift from Dr. C. Pratt's laboratory. The

GAPDH restriction fragment was produced by inserting the desired fragment between PST I and XBAI in pBluescript SK. Twenty nanograms of DNA were labelled with (γ^{32}P)dCTP (Amersham, Oakville, ON., Canada) using the Rediprime DNA Labelling System (Amersham, Oakville, ON., Canada) as described above.

2. (8). (iii). Percentage of (γ^{32}P)dCTP Incorporation into the DNA Probe

Two microliters of the denatured labelled DNA probe were added to 498 μl of distilled water. A 5 μl aliquot of the diluted DNA probe was spotted on a glass filter disk (Whatman GF/C) which was then washed three times with 50 ml of ice cold 10% (w/v) trichloroacetic acid (TCA) containing 1% (w/v) sodium pyrophosphate, and once with 50 ml of 95% ethanol at room temperature. The glass filter was dried and then added to a scintillation vial containing 10 ml of Cytoscinct scintillation cocktail (ICN Biochemicals Inc., Irvine, CA., U.S.A.). The precipitable radioactivity obtained from this glass filter was a representation of the incorporated [γ^{32}P]dCTP. Total radioactivity was determined by spotting a second 5 μl aliquot of the diluted DNA probe onto a glass filter disk. The filter was dried and then subsequently added to a scintillation vial containing 10 ml of Cytoscinct scintillation cocktail.

$$\% \text{ of } [\gamma^{32}\text{P}]\text{dCTP incorporation} = \frac{\text{precipitable radioactivity}}{\text{total radioactivity}} \times 100\%$$

2. (8). (iv). ***RNA Isolation***

Bovine chromaffin cell total RNA was harvested from samples treated for 4 days with 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN or 20-mer mismatch phosphorothioate ODN sequence as described previously. Twenty samples per treatment group were collected 96 hours post-treatment by aspirating the medium from each 35-mm petri dish and replacing it with one ml of Trizol reagent (GibcoBRL, Burlington, ON., Canada). The cell lysate was passed through a 20 gauge needle and syringe three times and then transferred to a clean tube. The lysate was incubated for 5 minutes at room temperature to ensure complete dissociation of the nucleoprotein complexes. Two hundred and fifty millilitres of chloroform were added per 1 ml Trizol reagent and the samples were shaken vigorously for 15 seconds, incubated at room temperature for 2 to 3 minutes and then centrifuged at 12,000 x *g* for 15 minutes at 4°C. Following centrifugation, the clear aqueous phase, containing the RNA was transferred to a clean ependorf tube and the RNA precipitated from the aqueous phase by adding one to two volumes of 100% ice cold ethanol to each sample. The samples were then incubated for 15 minutes and then centrifuged at 12,000 x *g* for 30 minutes at 4°C. The RNA pellet was washed two times with ice cold 80% ethanol and then air dried for 10 to 15 minutes. The RNA pellet was redissolved in 25 μl of sample gel-loading buffer which contained deionized formamide, formaldehyde, 5X MOPS (pH 7.0)[MOPS, 3M sodium acetate, 0.5M EDTA (pH 8.0)] and DEPC water. Following

quantification, 2μl of ethidium bromide (10μg/μl) and 4 μl of formaldehyde gel-loading buffer (50% glycerol, 1mM EDTA [pH 8.0], 0.25% bromophenol blue, 0.25% xylene cyanol FF) was added to each 20 μg RNA sample. Each sample was then heated for 10 minutes at 70°C and then placed on ice.

2. (8). (v). ***Northern Blot***

Prior to loading the RNA samples onto a 1% agarose-formaldehyde gel the gel was pre-run at 100V for 30 minutes in a 1 x formaldehyde gel-running buffer consisting of 100 ml 5X MOPS, 18 ml 37% formaldehyde and 978 ml DEPC water. Once the gel was loaded, it was electrophoresed for 4 hours at 80V in the afore mentioned buffer. Prior to transferring the separated RNA from the 1% agarose-formaldehyde gel to a Magna Nylon Transfer Membrane (MSI, Westborough, MA., U.S.A.) the gel was rinsed several times with DEPC water, soaked for 20 minutes in 0.05N sodium hydroxide, rinsed several times with DEPC water and then finally soaked for 45 minutes in 20x SSC (3M sodium chloride, 0.3M trisodium citrate; pH 7.0). The RNA was then transferred overnight by capillary action from the gel to a nylon membrane in 10x SSC. Once the RNA was transferred to the nylon membrane, it was then cross-linked to the membrane with 1,500 J in a CL-1000 Ultraviolet Crosslinker UVP (Diamed Laboratory Supplies, Mississauga, ON, Canada). Prehybridization was carried out for 6 hours in prehybridization buffer which consisted of 5X SSC, 5X Denhardt's reagent (0.1% Ficoll 400, 0.1% PVP, 0.1% BSA), 50% deionized

formamide, 0.5% SDS and 100μg/ml salmon sperm DNA (GibroBRL., Burlington, ON., Canada) at 42°C. Next, the membrane was hybridized overnight at 42°C in the afore described buffer which also contained 1.9 x 10^9CPM of [γ^{32}P]dCTP random prime scinderin and GAPDH DNA probes per ml. This was followed by stringency washes in: (I) 5x SSC, 0.5% SDS for 5 minutes at 65°C; (ii) 5x SSC, 0.5% SDS at 65°C for 10 minutes; (iii) 0.1x SSC, 1% SDS for 1 hour at 50°C; (iv) 2x SSC at room temperature for 10 minutes and; (v) 2x SSC at room temperature for 15 minutes. The membrane was exposed to Kodak Biomax MR Scientific Imaging Film (InterScience, Oakville, ON, Canada) for 24 to 48 hours at -70°C using a cassette with intensifying screens. The film was developed using a Kodak film developer.

2. (9). Catecholamine Uptake

2. (9). (i). <u>Catecholamine Release Studies</u>

Catecholamine output was determined by utilizing the method previously described by Trifaró and Lee (1980). Chromaffin cells which are loaded with ^{3}[H]noradrenaline (^{3}[H]NA) under carefully controlled conditions (Kenisberg and Trifaró, 1980) exhibit concomitant and parallel release of endogenous catecholamines and ^{3}[H] labelled amines upon stimulation by secretagogues (Trifaró and Lee, 1980; Trifaró and Bourne, 1981).

In brief, the experiments were started by washing the cells three times with 1 ml of special medium (in mM: NaCl, 110; $NaHCO_3$, 40; KCl, 5; $MgSO_4$, 1; NaH_2PO_4, 1; Na-pyruvate, 1; $CaCl_2$, 2; $Fe(NO_3)_3$, 2×10^{-4}, ascorbic acid, 0.1; pH 7.2 adjusted with 0.6 g/ml Hepes) and then incubated in 750 µl of this same medium with 10^{-7} M [^{3}H]NA (sp act 43.7 Ci/mmol) (Amersham, Oakville, ON., Canada) at room temperature for 5 minutes. Following this labelling step, the cells were then rinsed five times with regular Locke's solution [see section 2. (1). (ii) for composition] over a 50 minute period. Next, cells were rinsed once with Ca^{2+}-free Locke's solution (in mM: NaCl, 154; KCl, 2.6; K_2HPO_4, 1.25; KH_2PO_4, 0.5; $MgCl_2$, 1.2; EGTA, 2; glucose, 10; pH 7.2). Cells were then incubated with Locke's solution [see section 2. (1). (ii) for composition] (control) or high potassium Locke's (in mM: NaCl, 100; KCl, 53; K_2HPO_4, 1.25; KH_2PO_4, 0.5; $MgCl_2$, 1.2; $CaCl_2$, 2; glucose, 10; pH 7.2) (stimulation) for 40 seconds. After incubation, the entire 500 μl incubation media was collected and the radioactivity measured. The total [^{3}H]NA cell content of each dish was determined by treating each dish with 500 µl of 10% TCA for 10 minutes followed by two washes with 250 µl of 6% TCA; the three aliquots were combined in a scintillation vial. Ten millilitres of Cytoscinct scintillation cocktail was added to each vial and radioactivity measured using a Beckman Model LS-7800 liquid scintillation spectrometer (Beckman Instruments, Fullerton, CA., U.S.A).

2. (9). (ii). Data Analysis

Total [^{3}H]NA cell content [decay per minute/0.5 x 10^6 cells (dpm/0.5 x 10^6 cells)] was obtained by adding the amount of [^{3}H]NA secreted during the 40 second stimulation period to the [^{3}H]NA extracted with TCA. The percentage of catecholamine output was obtained by dividing the amount of [^{3}H]NA released into the medium by the total [^{3}H]NA content. The sensitivity of this protocol was that it allowed for the determination of an amount of catecholamine released corresponding to 0.35% of total cell catecholamine content (Vitale *et al.*, 1991). A minimum of 8 dishes were used per condition and the mean ± S.E.M. of each value obtained was plotted.

2. (10). Immunohistochemistry

2. (10). (i). Indirect Fluorescence of F-Actin in Chromaffin Cells

Chromaffin cells utilized for immunohistochemistry studies were processed for immunoflourescence using the technique described by Lee and Trifaró (1981), Vitale *et al.* (1991) and Zhang *et al.* (1996). As previously described, chromaffin cells were cultured on glass cover slips at a density of 0.5 x 10^6 cells/35mm petri dish in serum containing medium for 12 hours after which time the medium was replaced with serum-free medium. Cells were then treated with 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN or the 20-mer mismatch

phosphorothioate endcapped ODN sequence for 48 hours. After the antisense treatment, cells were washed three times with one ml of Locke's solution [see section 2. (1). (ii) for composition] and then incubated in Locke's (control) or high potassium Locke's [see section 2. (9). (i). for composition] (stimulation) for 40 seconds. The cells were then fixed on the glass coverslips with 3.7% formaldehyde for 20 minutes at room temperature. Coverslips were then rinsed six times with PBS, permeabilized by three successive exposures of 5 minutes each to 50%, 100% and 50% acetone, respectively, and then rinsed again six times with PBS. Chromaffin cells were stained for F-actin with rhodamine-phalloidin (0.25U/ml; Molecular Probes, Eugene, OR., U.S.A.) for 40 minutes in the dark at room temperature. The coverslips were then rinsed six times with PBS and mounted in glycerol-PBS (1:1).

*2. (10). (ii). **Data Analysis***

Slides were observed using a Leitz-Ortholux II fluorescent microscope equipped with a 100-W high pressure lamp and Ploemopack II incident light illuminator possessing an I-filter block (KP 490 plus 1mm GG 455 exciting filter, TK dichronic beam splitting mirror, K 515 suppression filter) for rhodamine as previously described in Vitale *et al.* (1991) and Zhang *et al.* (1996). All photographs were taken with Kodak-Tri-X Pan films (400 ASA). To study the effect of treatment on the percentage of cells showing cortical F-actin disassembly, 100 single-rounded

chromaffin cells per coverslip (generally 6 coverslips per experimental condition) were examined. Each cell was classified as having either a "continuous" or "discontinuous" cortical rhodamine (F-actin) fluorescent ring. The percentage of chromaffin cells showing cortical F-actin disassembly (discontinuous rhodamine fluorescent ring) was calculated for each experimental condition. Personal bias was avoided by coding the individual coverslips. The samples were examined and classified without knowing whether they were from control or treated preparations. Once all of the preparations were examined and the results recorded, the codes were revealed (single-blind design).

2. (10). (iii). Scinderin Immunohistochemistry

Chromaffin cells utilized for scinderin immunohistochemistry studies were processed for immunoflourescence using the technique described by Lee and Trifaró (1981), Vitale *et al.* (1991) and (1992). As previously described, chromaffin cells were cultured on glass cover slips at a density of 0.5×10^6 cells/35mm petri dish in serum containing medium for 12 hours after which time the medium was replaced with serum-free medium. Cells were then treated with 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN or the 20-mer mismatch phosphorothioate endcapped ODN sequence for 48 hours. After the antisense treatment, cells were washed three times with 1 ml of Locke's solution [see section 2. (1). (ii) for composition] and then fixed on the glass coverslips with 3.7%

formaldehyde in Locke's solution for 20 minutes at room temperature. Coverslips were then rinsed six times with PBS, permeabilized by three successive exposures of 5 minutes each to 50%, 100% and 50% acetone, respectively, and then rinsed again six times with PBS. Chromaffin cells were then blocked with 1% BSA in PBS for 60 minutes and then incubated with scinderin antiserum #6 (1:200 dilution) for 60 minutes. Next, the cells were rinsed six times with PBS and then incubated for 60 minutes with rhodamine conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA., U.S.A) (1:25 dilution). The coverslips were then rinsed six times with PBS and mounted in glycerol-PBS (1:1).

2. (10). (iv). Data Analysis

Slides were observed using a Leitz-Ortholux II fluorescent microscope equipped with a 100-W high pressure lamp and Ploemopack II incident light illuminator possessing an I-filter block (KP 490 plus 1mm GG 455 exciting filter, TK dichronic beam splitting mirror, K 515 suppression filter) for rhodamine as previously described in Vitale *et al.* (1991) and Zhang *et al.* (1996). All photographs were taken with Kodak-Tri-X Pan films (400 ASA). Personal bias was avoided by coding the individual coverslips. The samples were examined and classified without knowing whether they were from control or treated preparations. Once all of the preparations were examined and the results recorded, the codes were revealed (single-blind design).

2. (11). Video-Enhanced Microscopy of Antisense Treated Chromaffin Cells

2. (11). (i). Description of Equipment for F-actin Disassembly

After the cells had been viewed under the fluorescence microscope they were once again classified as exhibiting either "continuous" or "discontinuous" cortical F-actin fluorescence. The fluorescent intensity was quantitatively analysed using a Hammamatsu Photonics K.K. Argus-50/CL image processor. The image observed under the fluorescent microscope was fed into a Zeiss Venus III camera head which was in turn connected to a Hamamatsu Argus-50/CL Image processor. The image processor was linked to a Compaq Desk Pro 386s/20 personal computer equipped with Argus-50 version 3.0 software for Windows.

2. (11). (ii). Computer Analysis of F-Actin Disassembly and Graphic Representation of the Data

Fluorescent intensity of the afore mentioned cellular preparations [section 2. (10). (i)] was conducted by employing the three-dimensional display option within the Argus computer program. This function transforms a two-dimensional distribution of the intensities of a chromaffin cell displayed on the monitor and designated by a window into a three-dimensional graphic representation. The three-dimensional representation may be viewed in four distinct directions and at four levels of resolution with respect to intensity of the sample. The coordinates of the surface

of a cell of interest are represented on the X and Y axes and the intensity of fluorescence of the chromaffin cell on the Z axis. The output from the image processor was displayed on a Sony 300 colour monitor and subsequently printed with a Sony Marvigraph UP-3000 Colour Video Printer. Images of cells printed in pseudocolour were then analysed according to fluorescence intensity.

2. (11). (iii). Description of Equipment for Scinderin Fluorescent Intensity

After the cells had been viewed under the fluorescence microscope the fluorescent intensity was quantitatively analysed using a Hammamatsu Photonics K.K. Argus-50/CL image processor. The image observed under the fluorescent microscope was fed into a Zeiss Venus III camera head which was in turn connected to a Hamamatsu Argus-50/CL Image processor. The image processor was linked to a Compaq Desk Pro 386s/20 personal computer equipped with Argus-50 version 3.0 software for Windows.

2. (11). (iv). Computer Analysis of Scinderin Intensity and Graphic Representation of the Data

Fluorescent intensity of the afore mentioned cellular preparations [section 2. (10). (iii)] was conducted by employing the area analysis option within the Argus computer program. This function calculates the total fluorescent intensity, average

fluorescent intensity, maximum and minimum fluorescent intensities as well as the standard deviation between the maximum and minimum fluorescent intensities for the chromaffin cells within a select area. In addition, the function calculates the number of pixels within the selected area. The output from the image processor was displayed on a Sony 300 colour monitor and subsequently printed with a Sony Marvigraph UP-3000 Colour Video Printer. Images of cells printed in pseudocolour were then analysed according to total fluorescence intensity for a specified area.

CHAPTER 2A - MATERIALS

Materials used for the culture of bovine adrenal chromaffin cells were obtained from the following sources: Earl's Balanced Salt Solution, Dulbecco's modified Eagle's medium, streptomycin stock and penicillin stock were purchased from GibroBRL (Burlington, ON., Canada). Nystatin stock, cytostar, gentamycin, collagenase, 5-fluorodeoxyuridine, FITC-IgG was obtained from Sigma Chemical company (St. Louis, MO., U.S.A) while rhodamine-IgG was obtained Jackson Immunoresearch Laboratories, Inc. (West Grove, PA., U.S.A). DNase type I was obtained from Boehringer Mannheim (Laval, PQ., Canada). Ceftazidime was obtained from Eli Lilly Canada (Toronto, ON., Canada). Culture dishes were obtained from Corning (Kirkland, PQ., Canada), glass coverslips were obtained from Fisher Scientific (Nepean, ON., Canada) and Percoll was obtained from Pharmacia Biotech (Baie d'Urfé, PQ., Canada). All oligodeoxynucleotide sequences utilized for this project were obtained from *Oligos Etc.* (Wilsonville, OR., U.S.A). The antibodies against α-tubulin and gelsolin were obtained from Sigma Chemical company (St. Louis, MO., U.S.A.) and Chemicon (Mississauga, ON., Canada), respectively, while the anti-rabbit IgG-horseradish peroxidase and anti-mouse IgG horseradish peroxidase antibodies were obtained from Amersham (Oakville, ON., Canada). In addition, the ^{3}H]NA, (γ^{32}P)dCTP and the Rediprime DNA Labelling System were also obtained from Amersham (Oakville, ON., Canada). The rhodamine-phalloidin was obtained from Molecular Probes (Eugene, OR., U.S.A.).

CHAPTER 3 - RESULTS

CHAPTER 3 - RESULTS

3. (1). Chromaffin Cell Viability in Serum-Free Medium: Effect of Antisense ODN Treatment

Unmodified antisense ODN analogues are unstable in cell culture systems (Wickstrom, 1986; Akhtar *et al.*, 1991) and they should be employed in serum-free cultures or in systems which have very low nuclease content, such as the CNS or the cerebrospinal fluid (Brysch and Schlingensiepen, 1994). In agreement with Coulson *et al.* (1996), Mattson *et al.* (1993) and Wahlestedt *et al.* (1993), who specified that A431 cells and cortical neurons, respectively, were cultured in serum-free medium, prior to beginning elaborate work with antisense, it was necessary to determine whether chromaffin cells would survive in a serum-free environment for an extended period of time (maximum of 7 days). Figure 9 shows that up to 80% of the cultured chromaffin cells remained viable in a serum-free environment for up to seven days compared to 95% for chromaffin cells maintained in a serum environment for an equivalent period of time. Statistical analysis using one-way ANOVA revealed that there was a significant decrease in cellular viability ($p<0.05$) when chromaffin cells were maintained in a serum containing medium. More specifically, post analysis using Tukey's pair wise comparison revealed that the decrease was significant when cells were maintained in a serum containing medium for seven days. Also, in serum-free medium cellular viability was significantly decreased ($p<0.05$; Tukey's pair wise comparison) after five and seven days in culture when compared to control cells (day one) and between days two and five

(Figure 9). Although, cellular viability decreased significantly the longer the cells were maintained in culture, more than 80% of the cells were still viable on day 4, the day of the experiment. In addition, phase-contrast microscopy revealed that there was no visible morphological difference between cells maintained in a serum-free environment for four days and those cells grown in a serum containing medium for an equivalent period of time (Figure 10).

Unmodified antisense analogues have very little toxicity at micromolar concentrations and, due to their short half life, higher concentrations must be used to impart biochemical or physiological effects (Brysch and Schlingensiepen, 1994). In order to overcome this problem of degradation by nucleases, many researchers have switched to modified antisense analogues (Coulson *et al.*, 1996; Crooke and Bennett, 1996; Brysch and Schlingensiepen, 1994; Valerio *et al.*, 1994; Wahlestedt *et al.*, 1993). Although these modified ODNs are more stable within the cell and micromolar concentrations can be utilized to obtain the desired effect they have been reported to be somewhat more toxic to cells. Coulson *et al.* (1996) reported that phosphorothioate modified antisense produced a concentration-dependent effect on A431 cell morphology in cells cultured in either serum-free or serum containing medium after 24 hours of treatment. Also, these effects were reversible once the antisense was removed. Since phosphorothioate modified antisense was to be added to chromaffin cell cultures the impact of this treatment on chromaffin

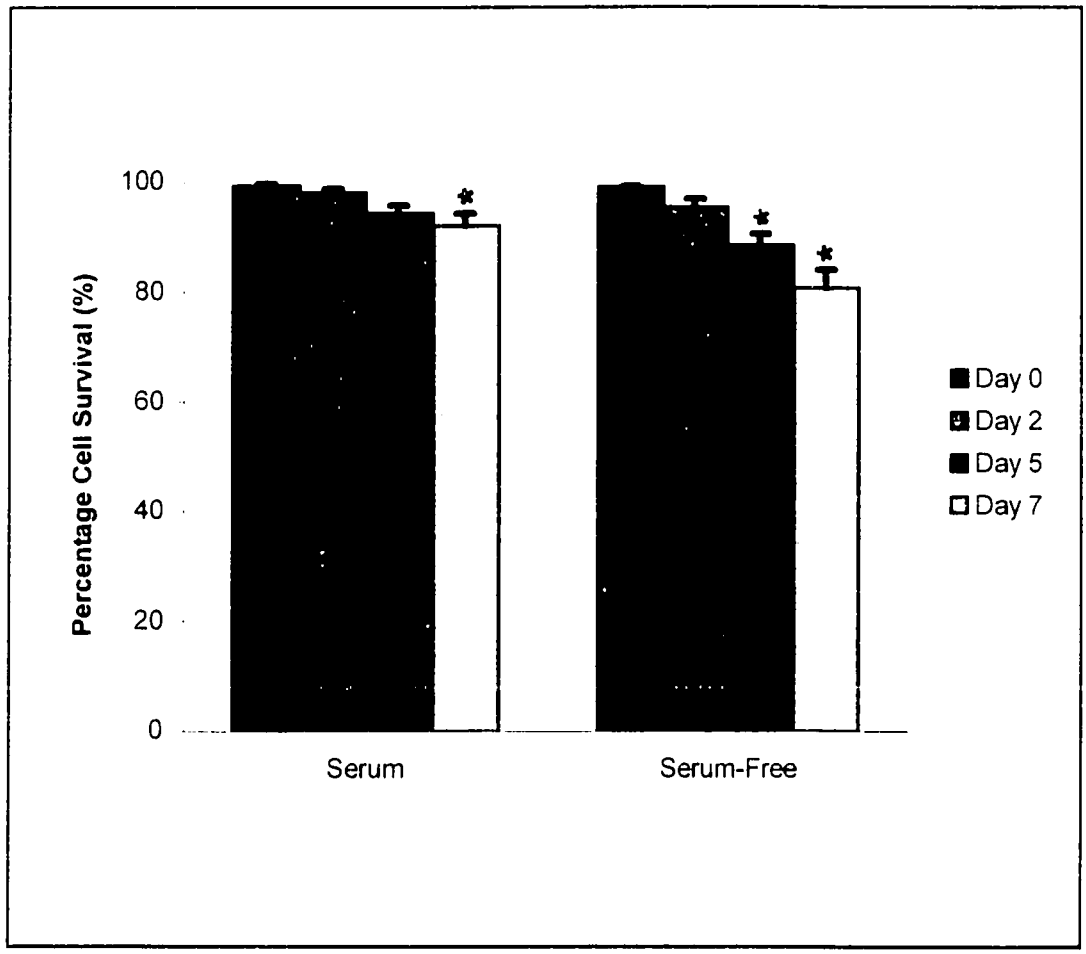
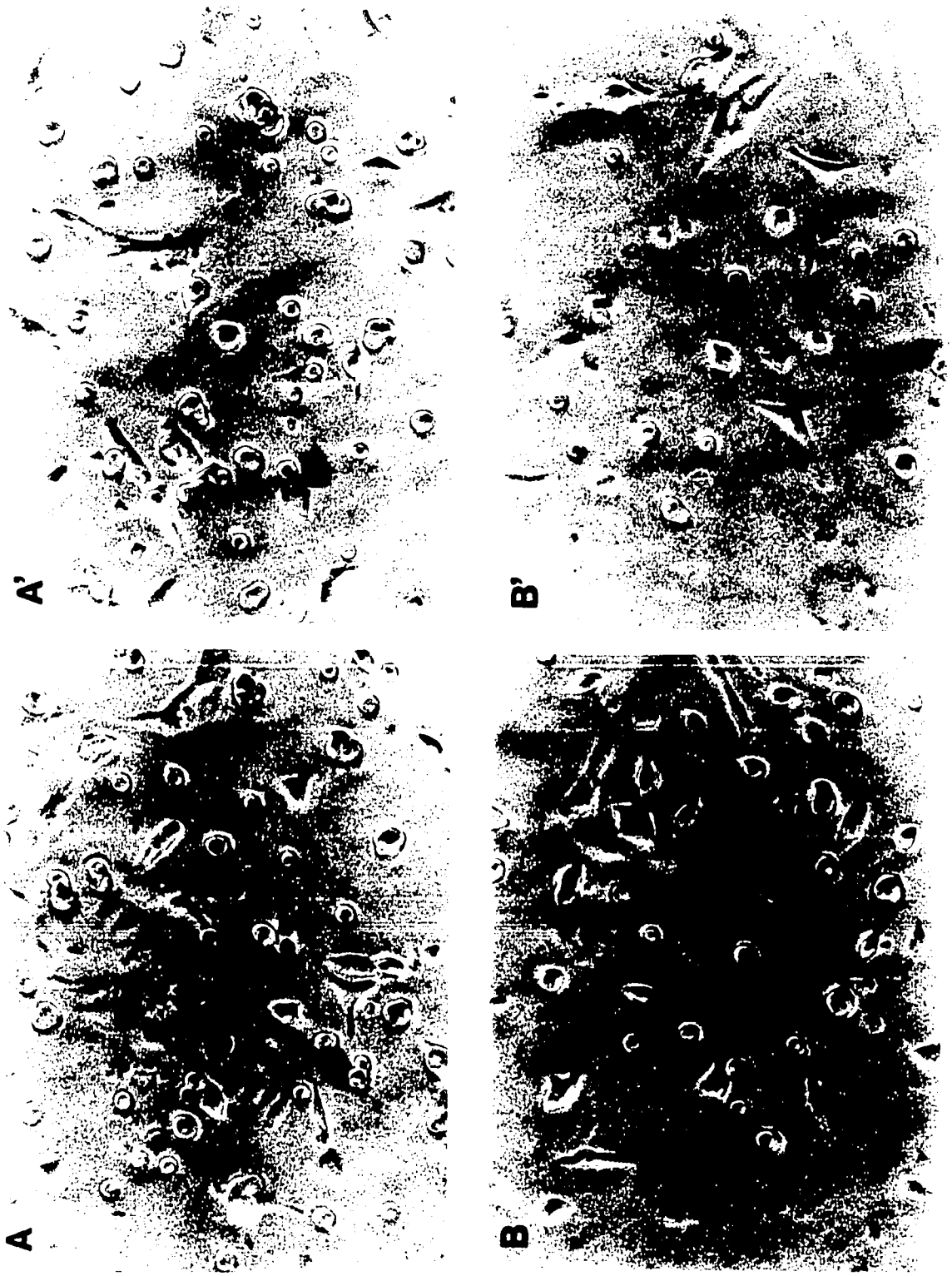


Figure: 9

Chromaffin cell viability in serum-free medium. Chromaffin cells were plated at a density of 0.5 x 10^6 cells/35 mm petri dish in culture medium containing 10% fetal calf serum (serum medium) for 24 hours. After this resting period, the medium was replaced with either fresh serum-containing medium or serum-free medium. Cellular viability was assessed 2, 5 and 7 days later using 0.4% trypan blue in saline. One hundred cells were counted per 35mm petri dish and each treatment was carried out in triplicate. Each bar represents data from 300 chromaffin cells. (*) statistically significant at $p<0.05$ using one-way ANOVA followed by Tukey's pair wise comparison.

Figure: 10

Chromaffin cell morphology in serum-free medium. Chromaffin cells were plated at a density of 0.5 x 10^6 cells/35 mm petri dish in culture medium containing 10% fetal calf serum (serum medium) for 24 hours. After this resting period, the medium was replaced with either fresh serum-containing medium or serum-free medium. Phase-contrast microscopy (40x) was used to assess variations in cellular morphology in serum (A, B) or serum-free (A', B') environments after 24 (A, A') and 96 (B, B') hours.


A'
B'
A
B

cell viability was examined. Figure 11 shows that no significant difference in cellular viability was evident between cells cultured in either serum or serum-free medium after 4 or 7 days. In addition, trypan blue exclusion revealed that cellular viability remained at 80% for controls (cells cultured in serum-free medium only) as well as treatment groups (cells cultured in serum-free medium and treated with either 1.5μM 20-mer scinderin phosphorothioate antisense ODN, 20-mer phosphorothioate scramble ODN sequence or 20-mer sense phosphorothioate ODN sequence or 20-mer mismatch phosphorothioate endcapped ODN). Further, contrary to what Coulson *et al.* (1996) have described, no significant morphological differences were observed with phase-contrast microscopy in chromaffin cells cultured in serum-free medium, serum-free medium containing 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN or 20-mer mismatch phosphorothioate endcapped ODN sequence at 24, or 96 hours (Figure 12).

3. (2). Transport of Antisense ODNs into Chromaffin Cells

Phosphorothioate modified antisense ODN is taken up by cells in culture in a saturable, size-dependent manner (Loke *et al.*, 1989). Brysch *et al.* (1996) reported that 2mM FITC-labelled phosphorothioate antisense ODN was internalized in human SK-Br-3 mammary carcinoma cells within 10 hours of treatment, while similar results were reported by Valerio *et al.* (1994) for pituitary cells. In addition, the persistence of intracellular fluorescence is an informative intracellular stability

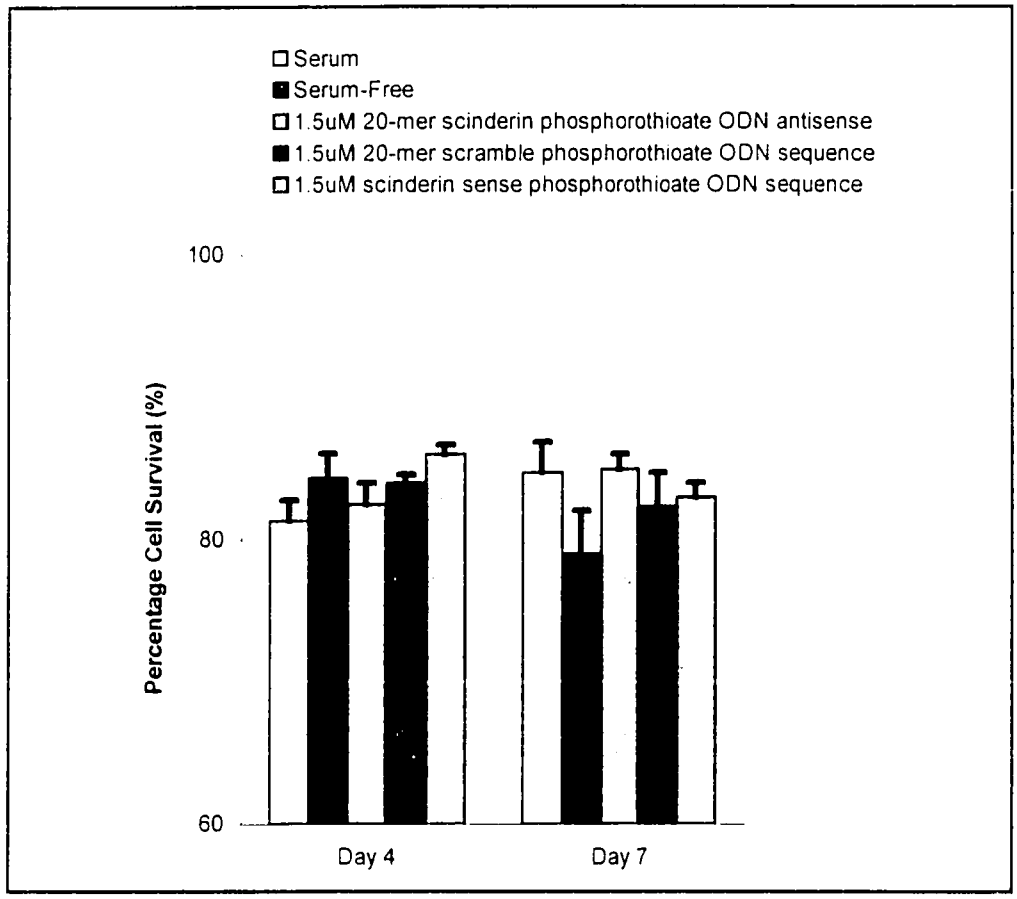
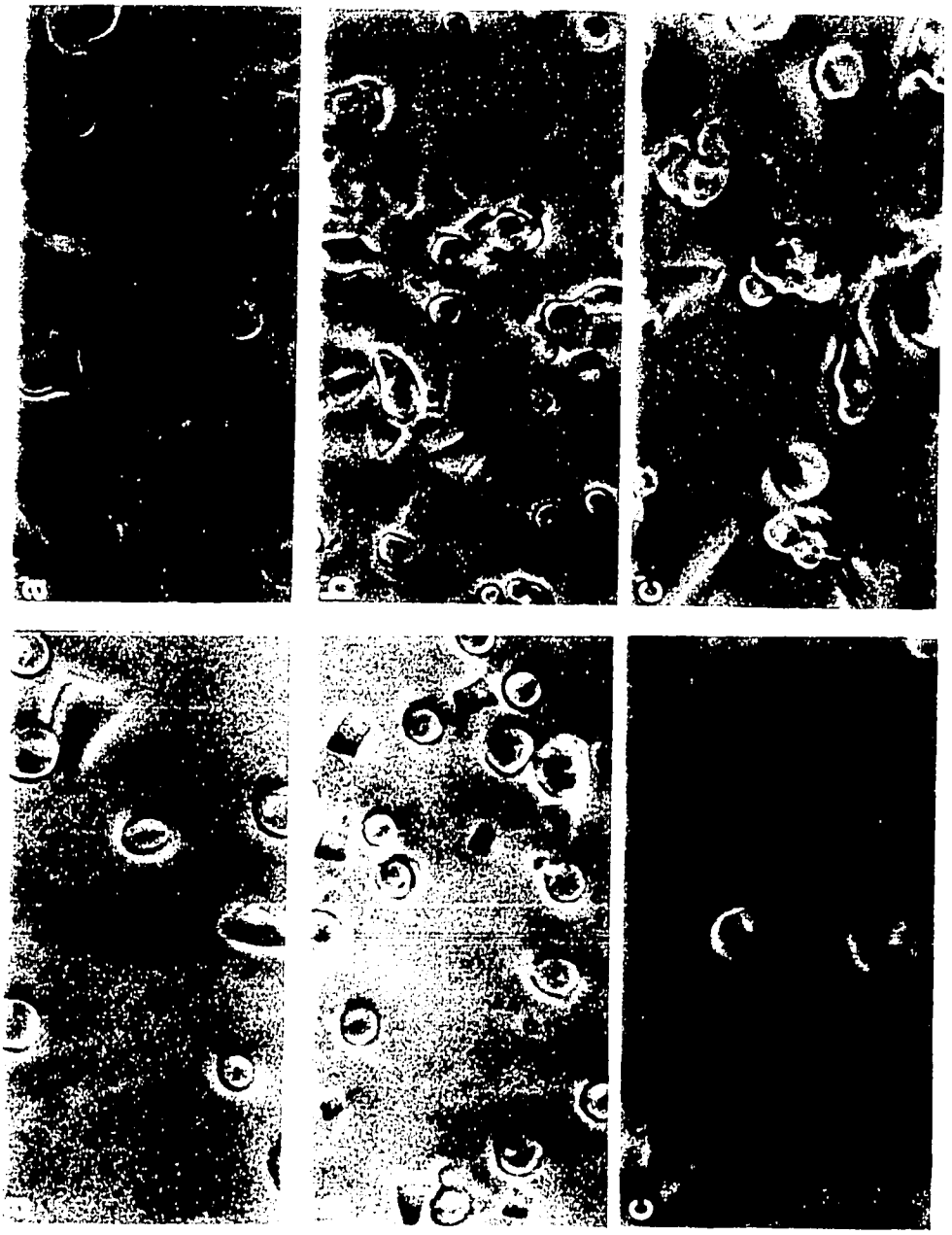


Figure: 11

Cellular viability following treatment with 1.5μM 20-mer scinderin phosphorothioate antisense ODN. Chromaffin cells were plated at a density of 0.5 x 10^6 cells/35 mm petri dish in culture medium containing 10% fetal calf serum (serum medium) for 24 hours. After this resting period, the medium was replaced with either fresh serum-containing medium (negative control), serum-free medium (positive control) or serum-free medium containing either 1.5μM 20-mer scinderin phosphorothioate antisense ODN, 20-mer scramble phosphorothioate ODN sequence or 20-mer sense phosphorothioate ODN sequence. Cellular viability was assessed four days and seven days later using 0.4% trypan blue in saline. One hundred cells were counted per 35mm petri dish and each treatment was carried out in triplicate. Each bar represents data from 300 chromaffin cells.

Figure: 12

Cellular morphology of chromaffin cells following 96 hours of treatment with 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN. Chromaffin cells were plated at a density of 0.5 x 10^6 on collagen coated 35mm petri dishes in serum containing medium. After an initial rest period of 24 hours the medium was replaced with serum-free medium. The cells were again allows to rest for another 24 hour after which time the medium was replaced with either serum-free medium (a, a') or serum-free medium containing 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN (b, b') or 20-mer mismatch phosphorothioate endcapped ODN sequence (c, c'). Cellular morphology was assessed at 24 (a, b, c), or 96 (a', b', c') hours post-treatment using phase-contrast microscopy (40x).



assay for antisense as the fluorescently labelled antisense ODNs are too large to undergo efflux from cells (Matteucci, 1996; Fisher *et al.*, 1993). Fluorescent efflux will only occur once the antisense ODN has been degraded. Cellular uptake of 2μM 5'-fluorescein labelled 20-mer scinderin phosphorothioate endcapped antisense ODN into chromaffin cells was followed over a 96 hour period. After one hour a major portion of the signal was nuclear (Figure 13a). However, after 2 and 6 (Figure 13b and c) hours significant amounts of signal was still concentrated within the nucleus and remained this way for up to 24 hours (Figure 13d). Only after 96 hours (data not shown) did the signal become very diffuse with punctate labelling visible throughout the cell. Also, marked differences in cellular labelling was apparent between cells on the same slide preparation. The diffuse and punctate labelling which was apparent after 96 hours (data not shown) suggested that the antisense ODN had been degraded.

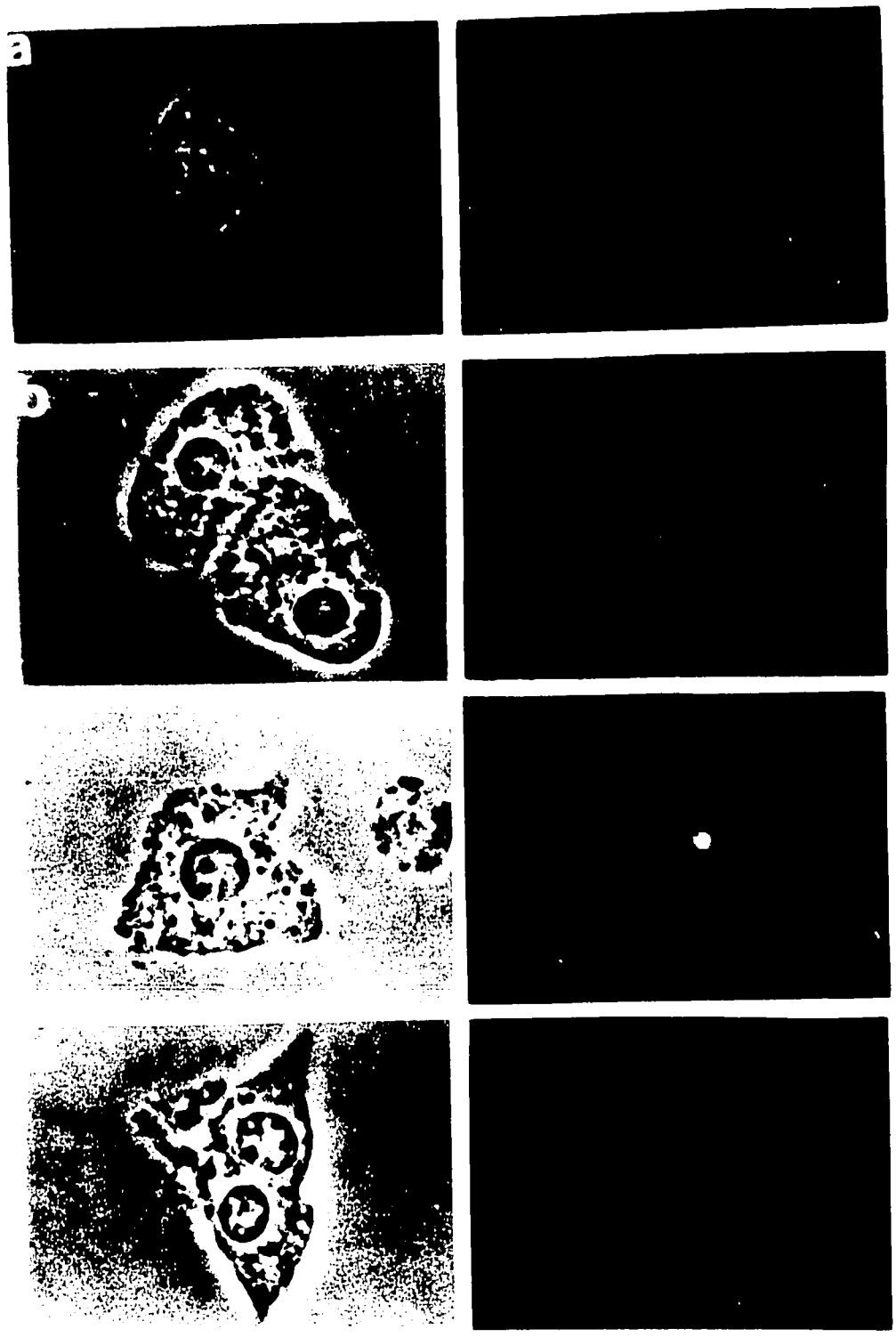
3. (3). Effect of Treatment of Chromaffin Cells with 18-mer or 20-mer Scinderin Phosphorothioate (full SDNA) or 20-mer Scinderin Phosphorothioate Endcapped Antisense ODN on Scinderin Levels

3. (3). (i). Development of a Scinderin Assay

Before any conclusions or results could be obtained, with respect to an increase or decrease in scinderin levels in cultured chromaffin cells, a sensitive and reproducible assay was required. Figure 14a shows that when 10×10^{10}

Figure: 13

Time course of 5'-fluorescein labelled scinderin phosphorothioate endcapped antisense ODN entry into chromaffin cells. Chromaffin cells were plated at a density of 0.5 x 10^6 on 35 mm petri dishes with glass cover slip bottoms in serum containing medium for 24 hours. The medium was replaced with serum-free medium containing 2μM 20-mer 5'-fluorescein labelled scinderin phosphorothioate endcapped antisense. The degree of ODN uptake was observed after 1 (a), 2 (b), 6 (c), and 24 (d) hours under oil immersion (100x, 1.30).


a
b

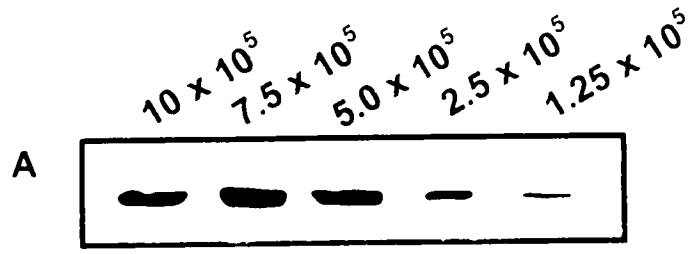
chromaffin cells are loaded onto an SDS-PAGE gel and developed by ECL, the scinderin signal is very intense, almost saturated; however, when the number of chromaffin cells is decreased by 8-fold, the scinderin signal is decreased proportionally after an equivalent exposure period. Therefore, a standard curve in which scinderin levels are correlated to chromaffin cell number was obtained through monodimensional SDS-PAGE and Western Blot followed by ECL development (see Figure 8). This technique proved to be very accurate in that scinderin content is linearly correlated to chromaffin cell number with the limit of sensitivity of scinderin level being in 60,000 chromaffin cells (Figure 14b).

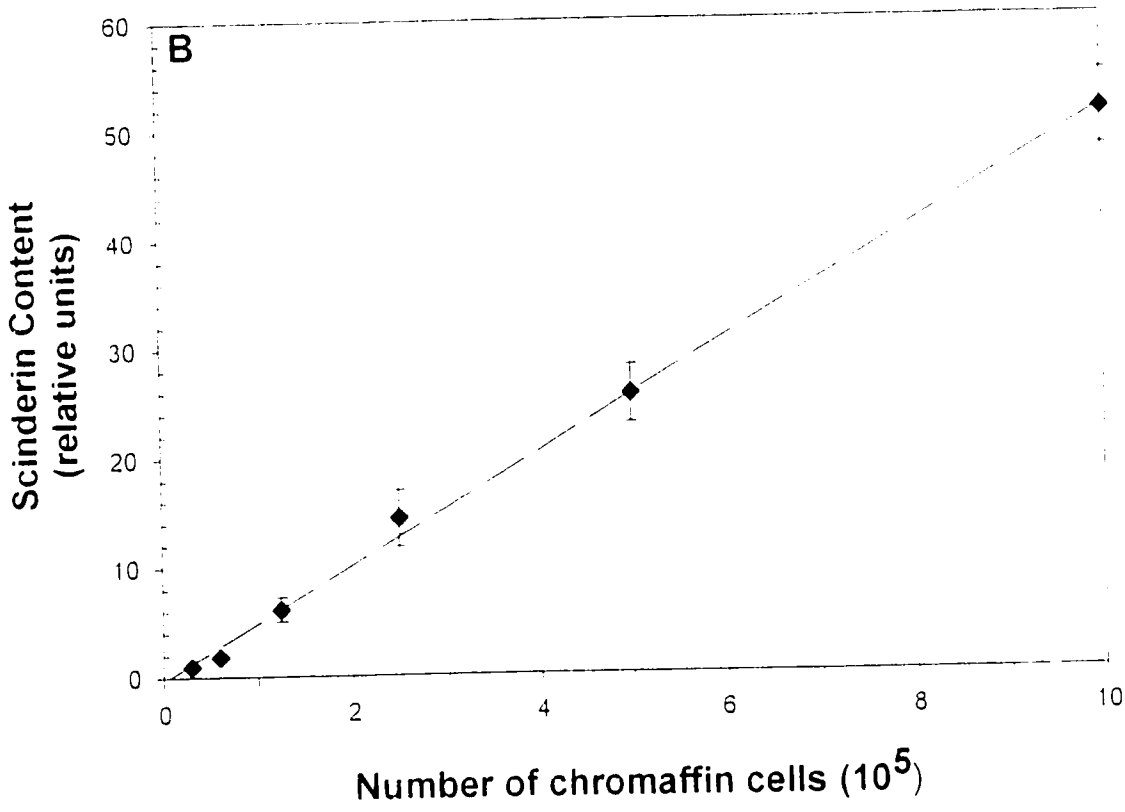
3. (3). (ii). ***Effect of Antisense ODNs on Scinderin Levels***

The percentage of decrease in a specific protein due to antisense ODN treatment varies between proteins and cell types and, as a result, it would be difficult to determine to what degree scinderin antisense ODN will be effective in a primary cell culture such as chromaffin cells. Mattson *et al.* (1993) reported that treatment of cultured hippocampal neurons with antisense ODN for four days resulted in a 48% decrease in the target protein. Similarly, treatment of cultured chromaffin cells for four days with 2μM 20-mer scinderin phosphorothioate antisense ODN resulted in a 60% decrease in scinderin levels (highly significant decrease, $p<0.001$ using one-way ANOVA followed by Tukey's pair wise comparison), while treatment of chromaffin cells with 2μM 18-mer scinderin phosphorothioate antisense ODN for

Figure: 14

Scinderin Assay. Chromaffin cells were plated at a density of 0.5 x 10^6 cells/35mm petri dish in serum containing medium for 48 hours. Cells were then lysed with 100mM Tris-HCl, 0.1M EDTA and 0.4% SDS and 10mM PMSF. Dilutions were based on cell number, with the final dilutions being 0.6, 1.25, 2.5, 5.0, 7.5 and 10 x 10^5 cells/70 μl final volume. The samples were mixed with 2X solubilizing buffer in a ratio of 3:1 and then loaded onto a 10% SDS-PAGE gel. The proteins in the gel were subsequently transferred to a nitrocellulose membrane and immunoblotted with scinderin antiserum (1:1000). The second antibody, was anti-rabbit IgG labelled peroxidase antibody raised in donkey (1:3000). The membrane was developed using the Amersham ECL kit (A) and the Amersham ECL Hyperfilm quantified by laser densitometer (B) as described in the Materials and Methods section.





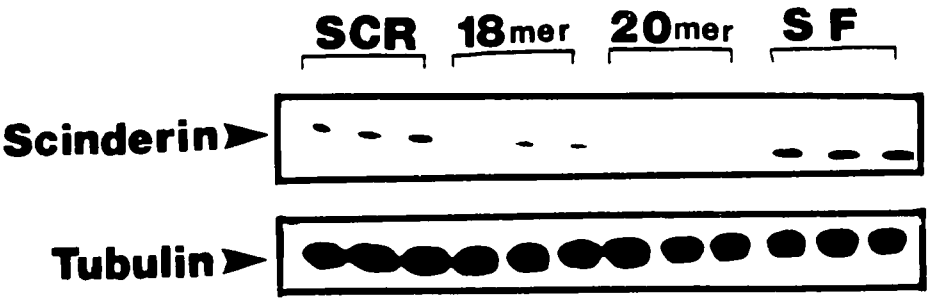
1 unit = scinderin content in 30.000 chromaffin cells

four days significantly reduced scinderin levels by 40% when compared to chromaffin cells treated with 2μM 20-mer phosphorothioate scramble ODN sequence (Figure 15a). Following quantification of the ECL Hyperfilm it was evident that scinderin levels were decreased with both scinderin antisense sequences, although a more significant decrease in protein levels was achieved with the 20-mer scinderin phosphorothioate antisense ODN (Figure 15b). As a result the 18-mer scinderin phosphorothioate antisense ODN was not utilized for further experiments (*e.g.*, catecholamine output, Northern Blot analysis and F-actin disassembly) since it was apparent that scinderin levels could be decreased more effectively with the 20-mer scinderin phosphorothioate antisense ODN. Although the standard error for 20-mer scramble ODN sequence treatment was fairly large, possibly due to a small variation in sample loading, the difference between this treatment and the 20-mer scinderin phosphorothioate antisense ODN treatment was still statistically significant with $p<0.05$ (one-way ANOVA followed by Tukey's pair wise comparison). Moreover, when chromaffin cells were treated for four days with 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN the scinderin levels where significantly ($p<0.05$; one-way ANOVA followed by Tukey's pair wise comparison) reduced by 50% compared to cells treated with 20-mer mismatch phosphorothioate endcapped antisense ODN (Figure 16a). In addition, the mismatch sequence, in which 11 bases were changed from C to G or vice versa, did not effect scinderin levels due to the fact that a single base mismatch results in a change in affinity of approximately 500-fold (Freier *et al.*, 1992). Therefore, a

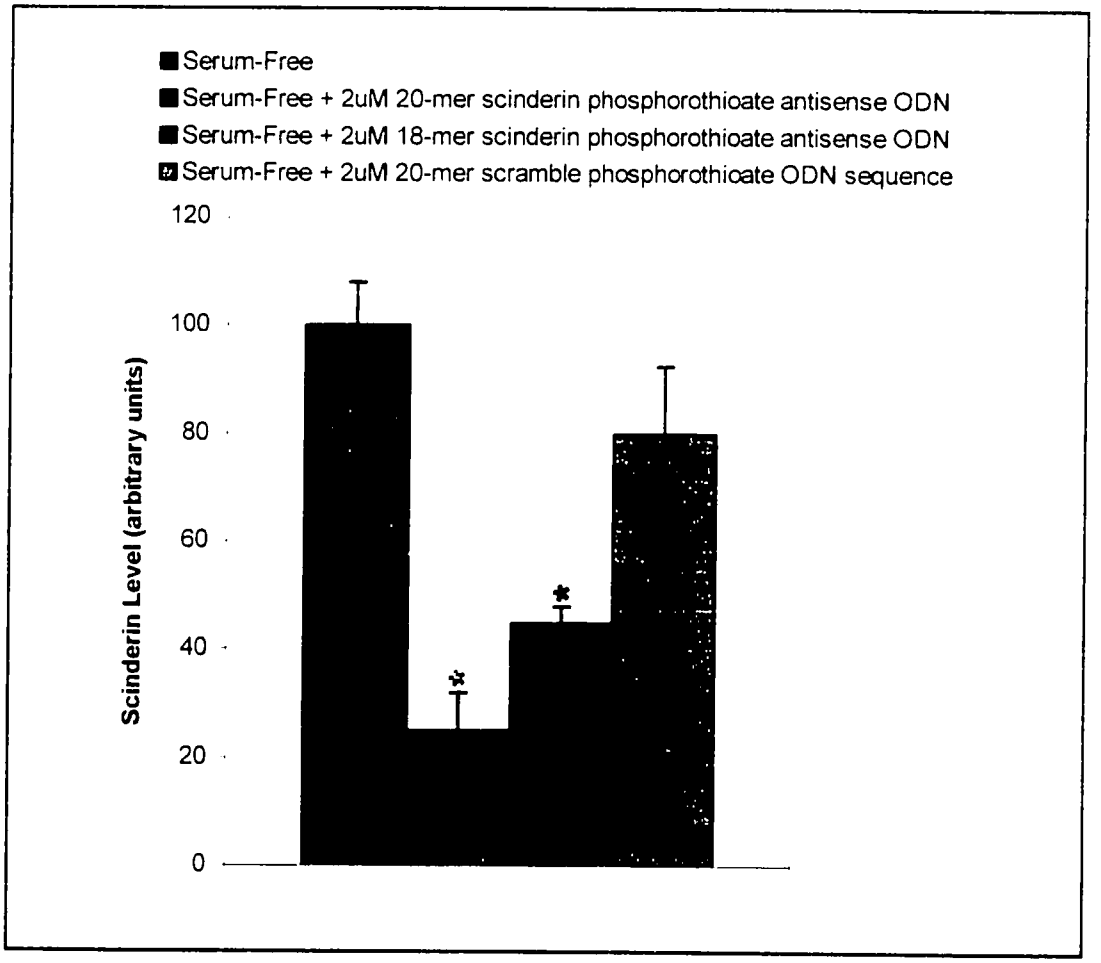
Figure: 15

Effect of 2μM 18-mer and 20-mer scinderin phosphorothioate antisense on scinderin levels following 96 hours of treatment. Chromaffin cells were plated at a density of 0.5 x 10^6 cells/35 mm petri dish in serum-containing medium. The medium was replaced 24 hours later with serum-free medium and the cells were allowed to rest for another 24 hours after which time the medium was replaced with serum-free medium (control) or serum-free medium containing 2μM 18-mer and 20-mer scinderin phosphorothioate antisense or 2μM 20-mer scramble phosphorothioate sequence. Twenty percent of the medium was replaced by medium containing 2μM fresh 18-mer or 20-mer scinderin antisense or scramble sequence daily for four days after which time scinderin levels (A) were assayed as described in legend to Figure 14. In addition to immunoblotting with scinderin antiserum (1:1000) the nitrocellulose membrane was immunoblotted with α-tubulin (1:3000) antiserum. The second antibodies were anti-rabbit IgG labelled peroxidase antibody (1:3000) and anti-mouse IgG labelled peroxidase antibody (1:3000). The membrane was developed using the Amersham ECL kit (A) and the Amersham ECL Hyperfilm quantified by laser densitometer (B). (*) statistically significant from serum-free treatment at $p<0.001$ using one-way ANOVA followed by Tukey's pair wise comparison.

A


SCR
18mer
20mer
S F
Scinderin
Tubulin

B


Serum-Free
Serum-Free + 2uM 20-mer scinderin phosphorothioate antisense ODN
Serum-Free + 2uM 18-mer scinderin phosphorothioate antisense ODN
Serum-Free + 2uM 20-mer scramble phosphorothioate ODN sequence
Scinderin Level (arbitrary units)
120
100
80
60
40
20
0
*
*

more optimal control would have been an ODN seqeunce idential to the 20-mer scinderin phosphorothioate endcapped antisense ODN with the exception of one base, *i.e.*, a single base mismatch. Furthermore, SDS-PAGE gels stained with coomassie blue (Figure 17) show that each lane was loaded with an equivalent amount of protein. Following quantification of the ECL Hyperfilm it was evident that scinderin levels were significantly lower in cells treated with 20-mer scinderin phosphorothioate antisense ODN although the level of protein decrease was greatest in cells treated with the 20-mer scinderin phosphorothioate endcapped antisense ODN (Figure 16b). In order to ensure that the afore mentioned results were accurate the nitrocellulose membranes were also blotted for α-tubulin and gelsolin, two internal controls. Figure 16a shows that the levels of both internal controls was consistent between various treatments indicating, specifically, that the decreases in scinderin levels were due to antisense ODN treatment.

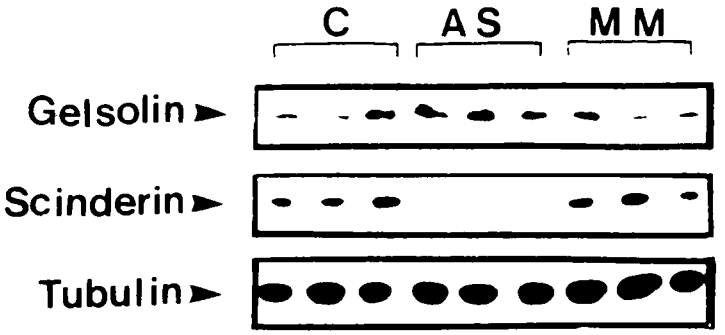
3. (3). (iii). ***Immunohistochemical Detection of Scinderin Levels Following Treatment with Antisense ODNs***

In accordance with the results obtained from scinderin immunoblotting [section 3. (3). (ii)] the immunohistochemical data also show that scinderin levels are significantly decreased when chromaffin cells are treated with 20-mer scinderin phosphorothioate endcapped antisense ODN. Chromaffin cells utilized for

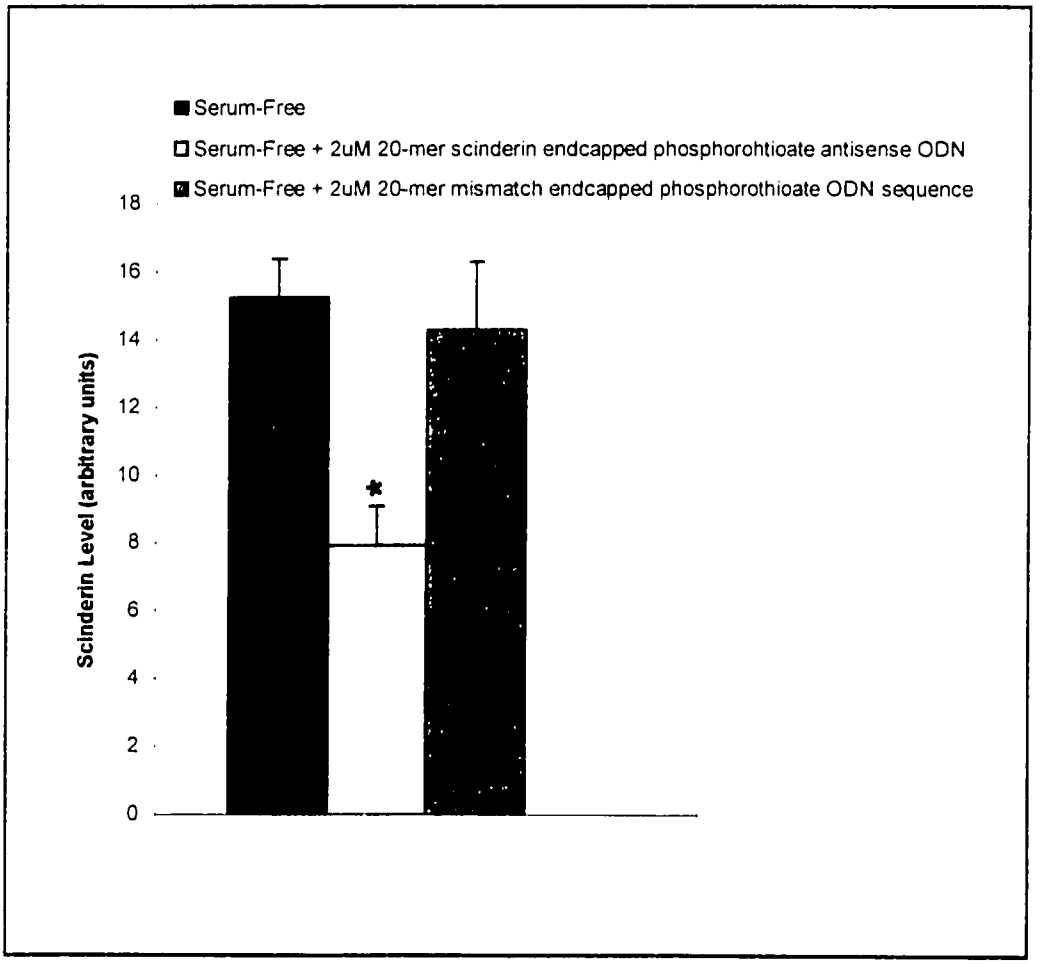
Figure: 16

Effect of 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN on scinderin levels following 96 hours of treatment. Chromaffin cells were plated and treated as described in Figure 15 with the following exception. Cells were treated with either serum-free medium (control) or serum-free medium containing 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN or 2μM 20-mer mismatch phosphorothioate endcapped ODN sequence. After 96 hours of treatment scinderin levels (A) were assayed as described in legend to Figure 14. In addition to immunoblotting with scinderin antiserum (1:1000) the nitrocellulose membrane was immunoblotted with α-tubulin (1:3000) and gelsolin (1:1000) antiserum. The second antibodies were anti-rabbit IgG labelled peroxidase antibody (1:3000) and anti-mouse IgG labelled peroxidase antibody (1:3000). The membrane was developed using the Amersham ECL kit (A) and the Amersham ECL Hyperfilm quantified by laser densitometer (B). (*) statistically significant ($p<0.05$) from the scramble and mismatch treatments using one-way ANOVA followed by Tukey's pair wise comparison.

A



B



1 unit = scinderin content in 30,000 chromaffin cells

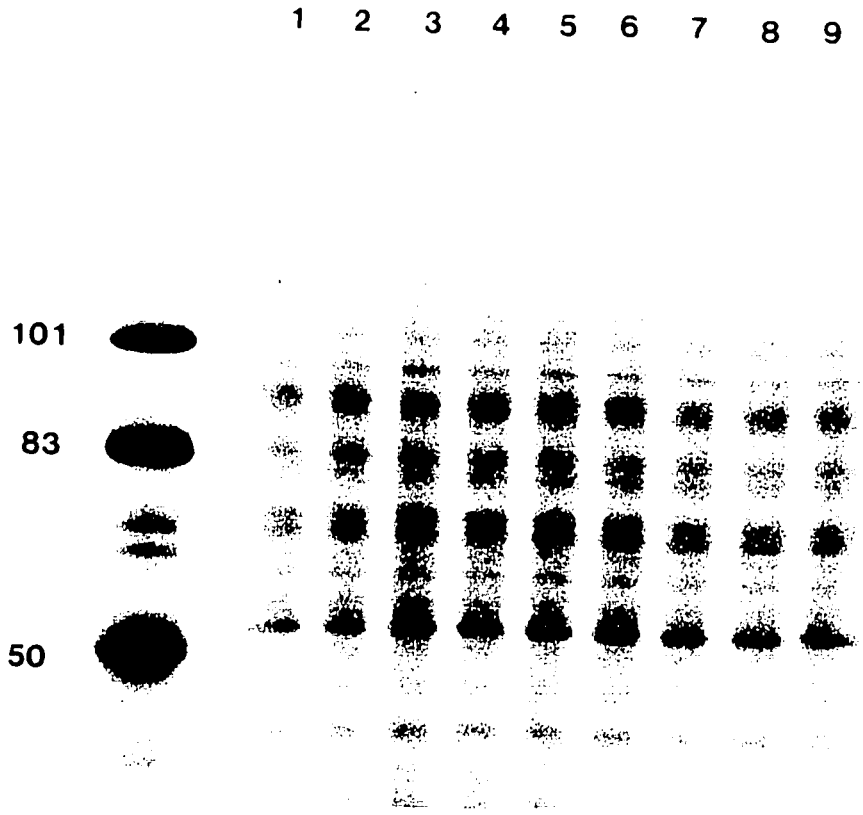


Figure: 17

Sample loading of protein for determination of scinderin levels. Chromaffin cells were plated at a density of 0.5 x 10^6 cells/35 mm petri dish in serum-containing medium and were treated for 96 hours with either serum-free medium (control) or serum-free medium containing 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN or 2μM 20-mer mismatch phosphorothioate endcapped ODN sequence as previously described. Cells were then lysed with 100mM Tris-HCl, 0.1M EDTA and 0.4% SDS and 10mM PMSF. Seventy microliters of sample was mixed with 2X solubilizing buffer in a ratio of 3:1 and then loaded onto a 10% SDS-PAGE gel. The proteins in the gel were subsequently fixed and stained with 0.1% coomassie blue, and the dried on filter paper. Lanes 1-3 represent chromaffin cells cultured in serum-free medium; lanes 4-6 represents cells cultured in serum-free medium containing 20-mer scinderin phosphorothioate endcapped antisense ODN; lanes 7-9 represent cells cultured in serum-free medium containing 20-mer mismatch phosphorothioate endcapped ODN sequence.

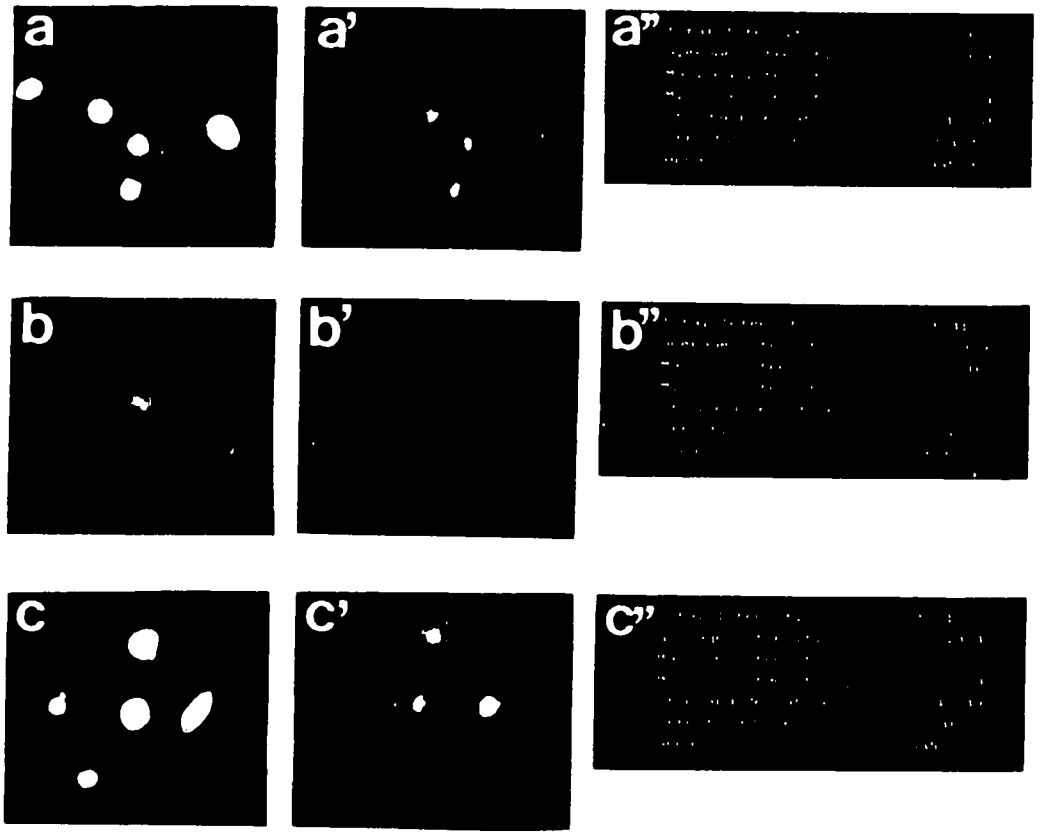
immunohistochemical experiments were treated for 48 hours with either serum-free medium (control) or serum-free medium containing 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN or 2μM 20-mer mismatch phosphorcthioate endcapped ODN sequence, as previously described, then fixed and subsequently stained with scinderin antiserum #6 followed by donkey anti-rabbit IgG conjugated to rhodamine. Figure 18 shows that the fluorescent intensity of chromaffin cells treated with 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN (b, b') is significantly reduced when compared to control (a, a') or mismatch phosphorothioate endcapped ODN sequence (c, c') treated cells. Furthermore, image analysis using the area analysis function of the Argus 50 imaging software shows that when an area of 676000μm^2 (containing 170352 pixels) was analysed for total fluorescent intensity, the total intensity was 1250074 ± 164358 for serum-free treated cells and 1264120 ± 143717 for mismatch phosphorothioate endcapped ODN sequence treated cells. Also, the total intensity decreased significantly, 177546 ± 29868, when cells were treated with the 20-mer scinderin phosphorothioate endcapped antisense ODN (Table 1).

3. (4). Scinderin mRNA Levels

Antisense ODNs are designed to modulate protein expression by altering the intermediary metabolism of mRNA (Crooke, 1995). However, the precise

Figure: 18

Immunohistochemical analysis of scinderin levels in chromaffin cells treated for 48 hours with 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN. Chromaffin cells were plated on collagen coated glass cover slips at a density of 0.5 x 10^6 cells/35 mm petri dish in serum-containing medium. Twelve hours later the medium was replaced with serum-free medium. The medium was subsequently replaced 12 hours later with serum-free medium containing either 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN or 2μM 20-mer mismatch phosphorothioate endcapped ODN sequence. Twenty percent of the medium was replaced 24 hours later and the cells were again allowed to rest for another 24 hours after which time the cells fixed with 3.7% formaldehyde and subsequently stained for scinderin with scinderin antiserum #6 (1:200) and then donkey anti-rabbit IgG conjugated rhodamine (1:25). Control cells (a) or cells treated with 2μM 20-mer mismatch phosphorothioate endcapped ODN sequence (c) shows "normal" scinderin levels while (b) displays the decreased scinderin levels in treated cells. Quantitative fluorescent intensity of chromaffin cell scinderin was performed by using a Hamamatsu Photonic KK Argus-50/CL Image Processor. The results from the area analysis of pseudoimages a', b' and c' from cells depicted in a, b and c, respectively, are shown in a", b", and c". Five areas were analysed per treatment.


a
a'
a"
b
b'
b"
c
c'
c"

Treatment	Area (μm^2)	Effective Pixels	Total Intensity (arbitrary units) (Mean ± S.E.M)
Serum-Free (n = 5)	676000	170352	1250074 ± 164358
Scinderin Endcapped ODN Antisense (n = 5)	676000	170352	177546 ± 29868**
Mismatch Endcapped ODN Sequence (n = 5)	676000	179352	1264120 ± 143717

Table: 1

Area analysis of scinderin immunohistochemical pseudoimages. Quantitative fluorescent intensity of chromaffin cell scinderin was performed by using a Hamamatsu Photonic KK Argus-50/CL Image Processor. The mean ± S. E. M from five different areas per treatment are shown above. (**) Statistically significantly different from serum-free ($p<0.001$) and mismatch endcapped ODN sequence treatment ($p<0.001$) using one-way ANOVA followed by Tukey's pair wise comparison.

mechanism by which these molecules inhibit gene expression is unknown, although it is clear that antisense ODNs must hybridize to their complementary RNA sequence, otherwise the biological effects would not be due to antisense inhibition (dependence on sequence) (Crook, 1993). As a result, it follows that treatment of cells in culture with antisense ODNs should produce a decrease in mRNA levels. Several studies have demonstrated that mRNA levels can be decreased by up to 80% in chicken myoblast cells, A549 lung carcinoma cells, mouse C127 mammary epithelial cells and rat hippocampal cells treated with antisense ODNs from 12 hours to four days (Monia *et al.*, 1996; Dean and McKay, 1994; Mattson *et al.*, 1993; Thinkaran and Bag, 1991). In conjunction with this data, scinderin mRNA levels were reduced by 60% following treatment of chromaffin cells for 96 hours with 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN compared to cells treated with 20-mer mismatch phosphorothioate endcapped ODN sequence (Figure 19). The Kodak BioMax MR film was quantified by laser densitometer and following results for scinderin mRNA levels were obtained (expressed in arbitrary units): serum-free, 3.939; 20-mer scinderin phosphorothioate endcapped antisense ODN, 1.8 and; 20-mer mismatch phosphorothioate endcapped ODN sequence, 4.301. The decreases in scinderin mRNA were normalized with GAPDH. The GAPDH values were: serum-free, 12.802; 20-mer scinderin phosphorothioate endcapped antisense ODN, 10.160 and; 20-mer mismatch phosphorothioate endcapped ODN sequence, 11.091. The results were expressed as a ratio of

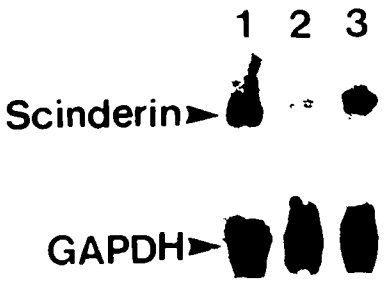


Figure: 19

Scinderin mRNA levels following 96 hours of treatment with 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN and 2μM 20-mer mismatch phosphorothioate endcapped ODN sequence. Chromaffin cells were plated at a density of 0.5 x 10^6 cells/35 mm petri dish and were treated with either serum-free medium (control) or serum-free medium containing 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN or 2μM 20-mer mismatch phosphorothioate endcapped ODN sequence as previously described. After 96 hours cells were lysed with Trizol (Gibco) and the mRNA harvested with 100% ethanol. Twenty micrograms of RNA was loaded per lane onto a 1% agarose-formaldehyde gel. The RNA was transferred onto a nylon membrane as previously described in the materials and methods section [section 2. (8). (v)] and then probed with 1.9 x 10^9 CPM of [γ^{32}P]dCTP random primed 500bp scinderin DNA probe or 1.9 x 10^9 CPM of [γ^{32}P]dCTP random primed GAPDH DNA probe. Following the stringency washes [see section 2. (8). (v)] and then exposed to Kodak BioMax MR Scientific Imaging Film for 24 hours at -70°C. The film was developed using a Kodak film developer (A). Lane 1 represents scinderin mRNA from 20 dishes of serum-free treated chromaffin cells; lane 2 represents scinderin mRNA from 20 dishes of chromaffin cells treated with 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN and; lane 3 represents scinderin mRNA from 20 dishes of chromaffin cells treated with 2μM 20-mer mismatch phosphorothioate endcapped ODN sequence.

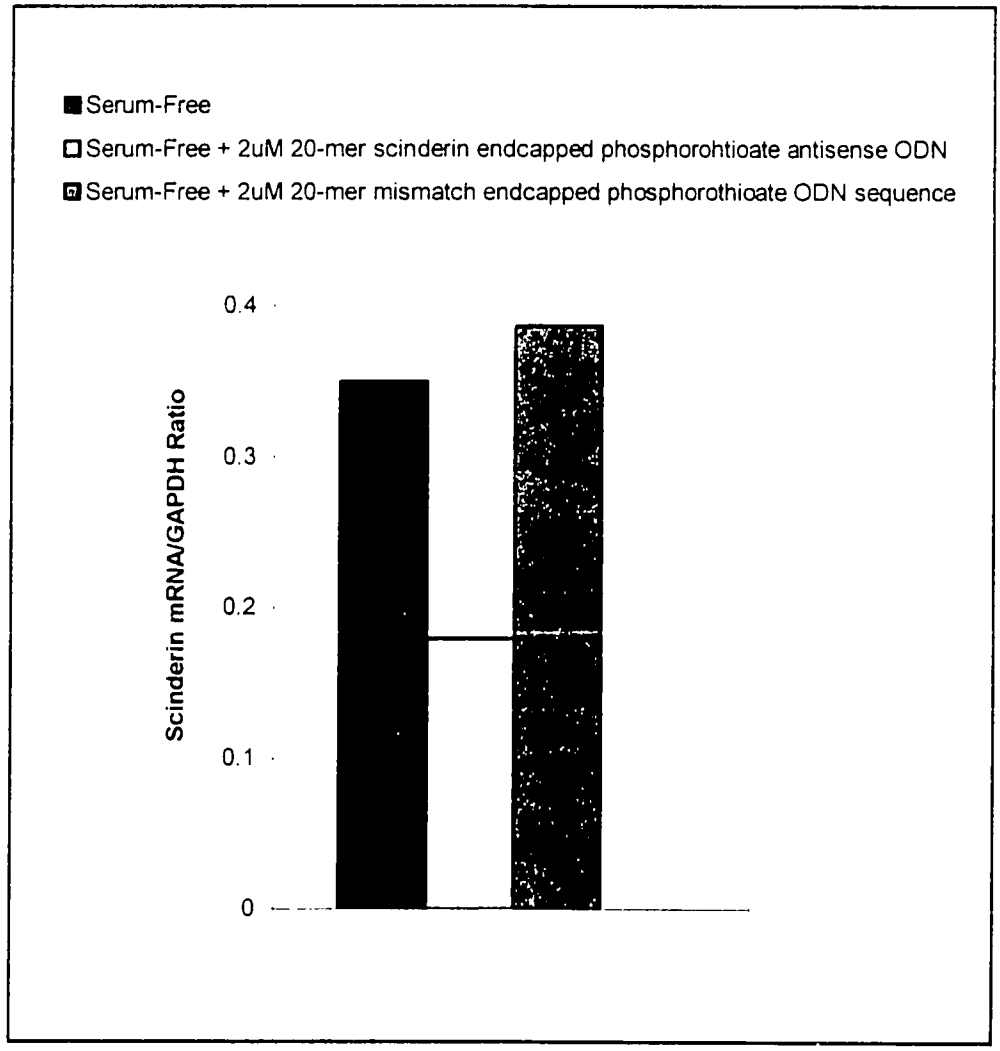


Figure: 20

Scinderin mRNA quantification. The Kodak BioMax MR film from Figure 19 was quantified by laser densitometer. Each bar represents data from 20 dishes (each dish contained 0.5 x 10^6 cells/35 mm petri dish) of chromaffin cells. Both the scinderin and GAPDH bands were scanned and the results expressed as a ratio of scinderin mRNA/GAPDH.

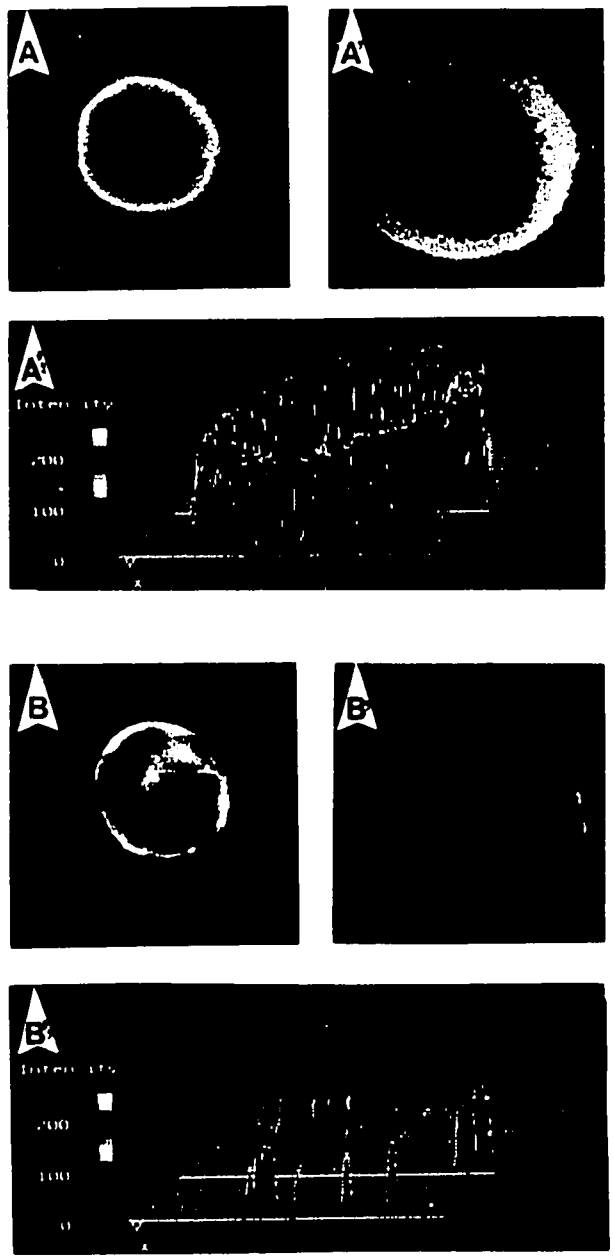
scinderin mRNA/GAPDH levels (Figure 20) to account for variations in sample loading.

3. (5). F-actin Disassembly

The chromaffin cell subplasmalemmal F-actin network opposes the movement of chromaffin vesicles to exocytotic sites on the plasma membrane (Vitale *et al.*, 1995; Trifaró *et al.*, 1982). K^+-evoked chromaffin cell depolarization or nicotine receptor stimulation induces cortical F-actin disassembly thus allowing the movement of secretory vesicles to release sites at the plasma membrane (Zhang *et al.*, 1996; Vitale *et al.*, 1995; Trifaró *et al.*, 1993). Rodríguez del Castillo *et al.* (1990) and Vitale *et al.* (1991) suggested that cortical F-actin disassembly is controlled by scinderin. Therefore, histochemical experiments were performed to determine the effect of antisense treatment on cytoskeleton dynamics. Histochemical studies where carried out in chromaffin cells cultured in serum-free medium and treated with either 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN or 20-mer mismatch phosphorothioate endcapped ODN sequence for 48 hours. Cells were incubated for 40 seconds with regular Locke's (control) or stimulated with high-K^+ Locke's and then stained for F-actin with rhodamine-labelled phalloidin (phalloidin is a heptapeptide which binds preferentially to F-actin and not G-actin) (Vitale, *et al.*, 1995), to visualize the actin network. Cells cultured in serum-free medium and incubated with Locke's solution (resting cells) (Figure 21) displayed

Figure: 21

F-actin disassembly in high K^+ stimulated chromaffin cells treated. Chromaffin cells were plated on collagen coated glass cover slips at a density of 0.5×10^6 cells/35 mm petri dish. Cells were stimulated with 40 seconds with high-K^+ (56 mM) and then fixed with 3.7% formaldehyde and subsequently stained for F-actin with rhodamine-phalloidin (1:250). A control cell (A) shows a continuous and intense ring of fluorescence for F-actin. Stimulated cells (B) display a disrupted cortical fluorescent pattern. Quantitative three dimensional analysis of cortical rhodamine fluorescence was performed by using a Hamamatsu Photonic KK Argus-50/CL Image Processor. The results from the 3-D analysis of pseudoimages A' and B' from cells depicted in A and B, respectively, are shown in A" and B". One hundred cells were examined per cover slip. Using this method, the percentage of cells showing intact fluorescent rings or fragmented fluorescent rings (F-actin disassembly) were recorded for each experimental condition (*i.e.*, antisense, mismatch and serum-free control).


A
A'
A''
B
B'
B''

a bright cortical fluorescent ring. These results are in accordance with previous histochemical findings obtained in our laboratory (Vitale *et al.*, 1991). Figure 21b shows that upon K^+-evoked depolarization, the F-actin fluorescent ring became fragmented and patchy in most cells previously incubated in serum-free medium or treated with 20-mer mismatch phosphorothioate endcapped ODN sequence. On the other hand, the percentage of F-actin fluorescence ring fragmentation was much lower in K^+-depolarized cells previously treated with 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN. The percentage of F-actin disassembly in control treated chromaffin cells was 48.8 ± 3.4 and 48.0 ± 3.6 in chromaffin cells treated with 20-mer mismatch phosphorothioate endcapped ODN sequence. However, when chromaffin cells were treated with 20-mer scinderin phosphorothioate endcapped antisense ODN F-actin disassembly was significantly reduced to 27.2 ± 4.8. It should be noted that although the maximum level of F-actin disassembly is 48% this does not imply that only half of the cells were responding to the stimuli, but rather that the degree of F-actin disassembly differs between cells as the cells are not synchronized with repect to Ca^{2+} entry, scinderin activation and in turn F-actin disassembly. Figure 22 shows that F-actin disassembly in chromaffin cells treated with 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN is inhibited by 50% when compared to cells treated with 2μM 20-mer mismatch phosphorothioate endcapped ODN sequence.

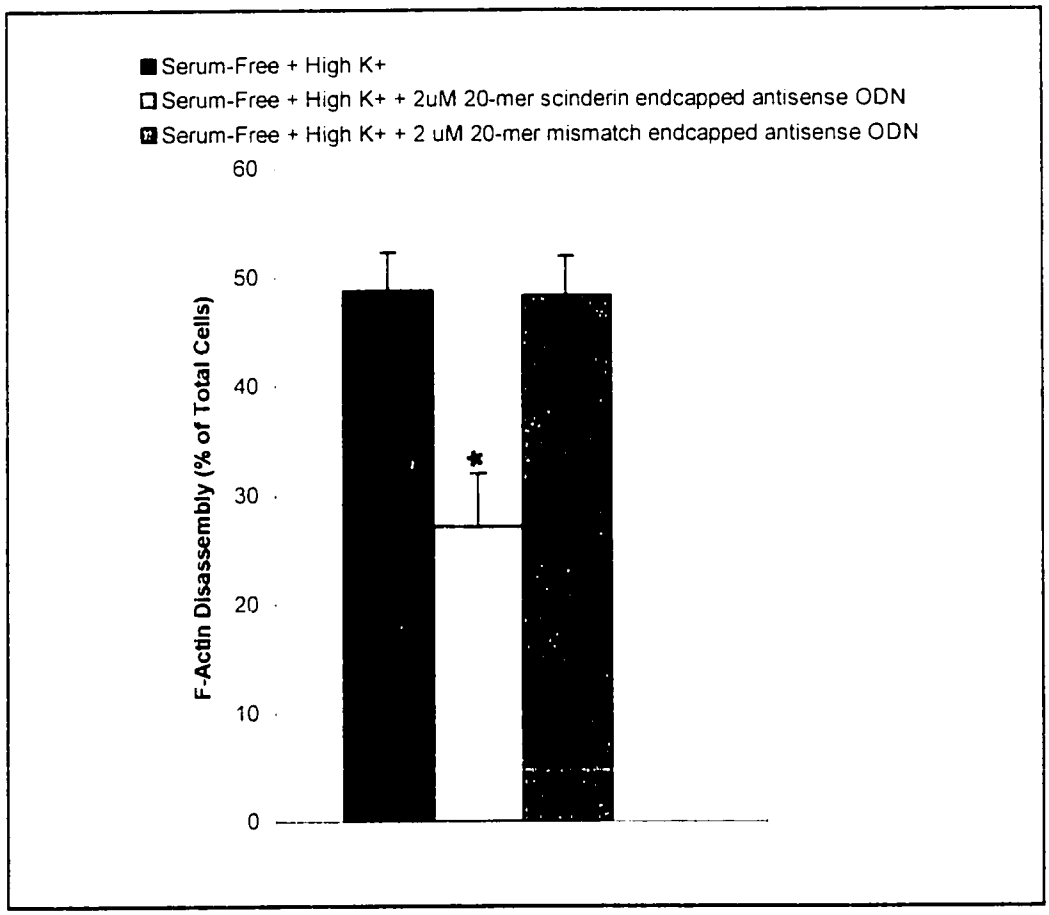


Figure: 22

Quantification of cortical F-actin disassembly. Chromaffin cells were plated on collagen coated glass cover slips at a density of 0.5 x 10^6 cells/35 mm petri dish in serum-containing medium. Twelve hours later the medium was replaced with serum-free medium. The medium was replaced 12 hours later with serum-free medium containing either 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN or 2μM 20-mer mismatch phosphorothioate endcapped ODN sequence. Twenty percent of the medium was replaced 24 hours later and the cells were again allowed to rest for another 24 hours after which time they were stimulated for 40 seconds with high-K^+. The cells were fixed with 3.7% formaldehyde and then stained for F-actin with rhodamine-phalloidin (1:250). One hundred cells were counted per cover slip. Each bar represents 600 cells per treatment. (*) statistically significant ($p<0.05$) from either the serum-free or 20-mer mismatch phosphorothioate endcapped ODN sequence treatment as determined by a one-way ANOVA followed by Tukey's pair wise comparison.

3. (6). Catecholamine Uptake

As previously mentioned chromaffin cells share a common embryological origin with sympathetic neurons, and like sympathetic neurons, chromaffin cells are capable of the synthesis, storage, and release of transmitter substances (Trifaró and Cubeddu, 1979). Another similarity between sympathetic neurons and chromaffin cells is the presence of two distinct catecholamine uptake mechanisms, which correspond to neuronal and extra-neuronal uptake, respectively (Rang *et al.*, 1995; Kenisberg and Trifaró, 1980). When chromaffin cells are cultured in a serum containing environment, noradrenaline accumulation is linear from 1 to 6.25 minutes (Kenisberg and Trifaró, 1980) and one of the amine uptake components (high affinity) operates at low incubating concentrations of [^{3}H]NA (saturates before 1μM, follows Michaelis-Menton kinetics and is absolutely dependent on the presence of external Na^+), while the second uptake component also functions at low incubating concentrations of [^{3}H]NA, becomes more apparent at and beyond [^{3}H]NA concentrations of 1μM, with no signs of saturability at [^{3}H]NA concentrations of 4μM (Kenisberg and Trifaró, 1980). When chromaffin cells were cultured in serum-free medium in the presence or absence of 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN or 20-mer mismatch phosphorothioate endcapped ODN sequence the total cellular uptake of [^{3}H]NA did not differ significantly between antisense ODN treatment or controls (Table 2). Moreover, the rate of [^{3}H]NA uptake for 0.5 x 10^6 cells maintained in a serum-free medium was

Treatment	Total Cellular [^{3}H]NA Uptake (dpm/0.5 x 10^6 cells)	Rate of [^{3}H]NA Uptake (dpm/second)
Serum-Free (n=8)	57802 ± 2583	193 ± 9
Scinderin Endcapped Antisense ODN (n=8)	61008 ± 6156	203 ± 21
Mismatch Endcapped ODN Sequence (n=7)	60324 ± 4503	201 ± 15

Table: 2

Cellular uptake of [^{3}H]NA in chromaffin cells cultured in a serum-free medium containing either 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN or 20-mer mismatch phosphorothioate endcapped ODN sequence. Chromaffin cells were treated with serum-free medium or serum-free medium containing either 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN or 20-mer mismatch phosphorothioate endcapped ODN sequence for 96 hours. Following treatment, cells were washed two times with special medium, loaded with 10^{-7}M [^{3}H]NA in special medium for 5 minutes and then washed 6 times with regular Locke's at 10 minute intervals. Total cellular [^{3}H]NA uptake (dpm/0.5 x 10^6 cells) was obtained by adding the radioactivity of the medium to that in the 10% TCA extracts. The rate of [^{3}H]NA uptake (dpm/second) was obtained by dividing total cellular [^{3}H]NA uptake (dpm/0.5 x 10^6 cells) by 300 seconds.

193 ± 9 dpm/0.5 x 10^6 cells; for serum-free medium containing 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN was 203 ± 21 dpm/0.5 x 10^6 cells and; for serum-free medium containing 2μM 20-mer mismatch phosphorothioate endcapped ODN sequence was 201 ± 15 dpm/5 x 10^{-5} cells (Table 2). No statistically significant differences were evident between control and antisense ODN treatments suggesting that the antisense ODN treatment did not impair the uptake mechanism.

3. (7). Catecholamine Release

In chromaffin cells catecholamine release is measured by labelling endogenous stores of catecholamines with [^{3}H]NA, a method which can detect catecholamine concentrations equal to 0.35% of the total content (Vitale *et al.*, 1991). Previous experiments in our laboratory have shown that when intact chromaffin cells are loaded with [^{3}H]NA under controlled conditions (Kenisberg and Trifaró, 1980), there is a concomitant and parallel release of endogenous catecholamines and [^{3}H]NA following stimulation (Trifaró and Bourne, 1981; Trifaró and Lee, 1980). Therefore, the use of [^{3}H]NA-labelled cells increases the sensitivity of the catecholamine assay and allows the used of fewer cells per experiment (Vitale *et al.*, 1992). Also, only [^{3}H]NA output was monitored in these experiments. Therefore, chromaffin cells with catecholamine stores labelled with [^{3}H]NA were used in the experiments treated with 2μM of the 20-mer scinderin phosphorothioate antisense ODN, 20-mer

scinderin phosphorothioate endcapped antisense ODN, 20-mer scramble phosphorothioate ODN sequence or 20-mer mismatch phosphorothioate endcapped ODN sequence, as described in the methods section, where loaded with [^{3}H]NA and then stimulated for 40 seconds with 56mM K^+ (Figure 23). High K^+-evoked depolarization induced catecholamine release was reduced by 30% in chromaffin cells treated with 2μM 20-mer phosphorothioate scinderin antisense ODN when compared to cells treated with either serum-free medium, 20-mer scramble phosphorothioate ODN sequence (Figure 23). The decrease in catecholamine release was very significant ($p<0.001$; one-way ANOVA followed by Tukey's pair wise comparison) between the serum-free and 20-mer scinderin phosphorothioate endcapped antisense ODN as well as the 20-mer scinderin phosphorothioate endcapped antisense ODN and the 20-mer mismatch phoshorothioate endcapped ODN sequence. Further, when cells were treated with 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN for four days, depolarization-evoked catecholamine release was significantly reduced by 50% from 1.5 ± 0.1% (40 seconds) and 1.4 ± 0.2% (40 seconds) in serum-free and 20-mer mismatch phosphorothioate endcapped ODN sequence, respectively, to 0.7 ± 0.1% (40 seconds) in 20-mer scinderin phosphorothioate endcapped antisense ODN (Figure 24).

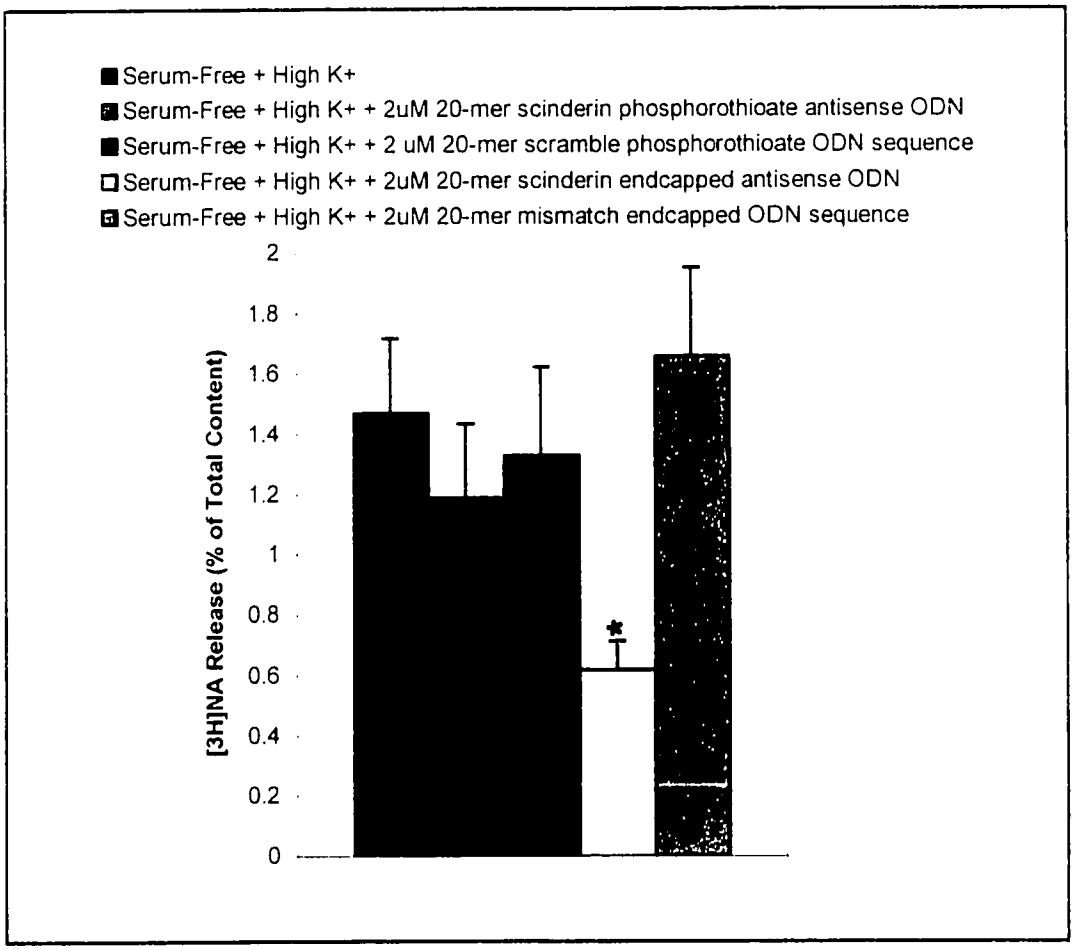


Figure: 23

Depolarization-evoked catecholamine from chromaffin cells treated with either serum-free (control) or serum-free medium containing 2μM 20-mer scinderin phosphorothioate antisense or 2μM 20-mer scramble phosphorothioate sequence for 96 hours. Following treatment, cells were washed two times with special medium, loaded with 10^{-7}M [^{3}H]NA in special medium for 5 minutes and then washed 6 times with regular Locke's at 10 minute intervals. The cells were then incubated with either regular Locke's (control) or high-K^+ (56 mM) Locke's (stimulation) for 40 seconds. Total radioactivity was obtained by adding the radioactivity of the medium to that in the 10% TCA extracts. (*) statistically significant ($p<0.001$)(one-way ANOVA followed by Tukey's pair wise comparison) compared to serum-free treatment and treatment with the 20-mer mismatch phosphorothioate endcapped ODN sequence.

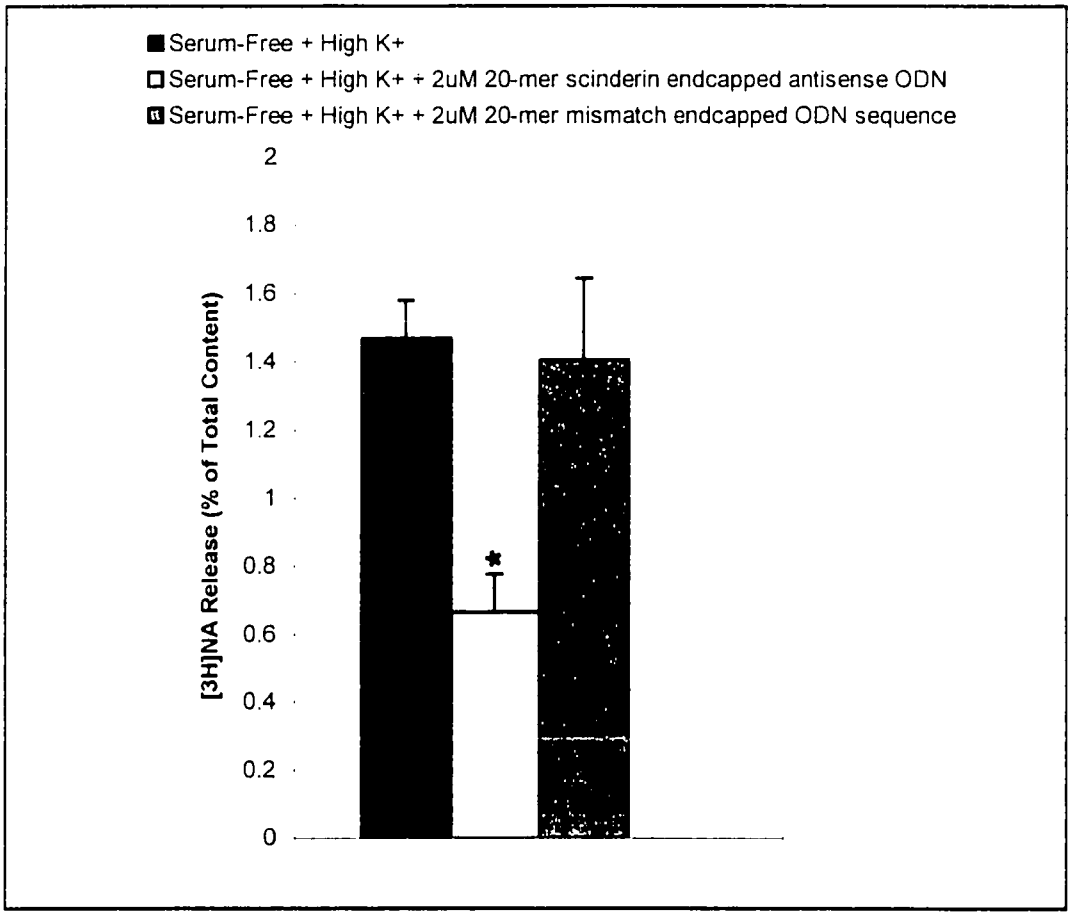


Figure: 24

Depolarization-evoked catecholamine output was examined in chromaffin cells that had been treated with serum-free medium or serum-free medium containing either 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN or 2μM 20-mer mismatch phosphorothioate endcapped ODN sequence for 96 hours. Following treatment, cells were washed two times with special medium, loaded with 10^{-7}M [^{3}H]NA in special medium for 5 minutes and then washed 6 times with regular Locke's at 10 minute intervals. The cells were then incubated with either regular Locke's (control) or high-K^+ (56 mM) Locke's (stimulation) for 40 seconds. Total radioactivity was obtained by adding the radioactivity of the medium to that in the 10% TCA extracts. (*) statistically significant ($p<0.001$) from serum-free and 20-mer mismatch phosphorothioate endcapped ODN sequence treatment. Statistical method used was a one-way ANOVA followed by Tukey's pair wise comparison.

3. (8). Correlation Between Scinderin Levels, F-actin Disassembly and Catecholamine Release Following Treatment of Chromaffin Cells with 2μM Scinderin Endcapped Antisense ODN

Table 3 shows that the significant decrease in scinderin levels was accompanied by a parallel decrease in catecholamine release and F-actin disassembly following treatment with the 20-mer scinderin phosphorothioate endcapped antisense ODN sequence. Ratios of F-actin disassembly to scinderin levels are not significantly different between serum-free, 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN or 2μM 20-mer mismatch phosphorothioate endcapped ODN sequence treated cells. Furthermore, the ratios for [^{3}H]NA release to scinderin levels do not differ significantly different between control or 20-mer scinderin phosphorothioate endcapped antisense ODN treated cells.

Treatment Conditions	Scinderin Levels (arbitrary units)*	F-Actin Disassembly (% of Total Cells)	[^{3}H]NA Release (% of Total Content)	Ratio	
				Scinderin Levels / F-Actin Disassembly	Scinderin Levels / [^{3}H]NA Release
Serum-Free	15.2 ± 0.5	48.8 ± 3.4	1.5 ± 0.1	0.30 ± 0.01	10.1 ± 0.3
Serum-Free + 2μM Scinderin endcapped antisense	7.8 ± 0.5	27.2 ± 4.8	0.7 ± 0.1	0.29 ± 0.16	10.1 ± 0.7
Serum-Free + 2μM Mismatch endcapped antisense	14.3 ± 1.0	48.0 ± 3.6	1.4 ± 0.2	0.29± 0.19	10.2 ± 0.7

*1 unit = scinderin content in 30,000 chromaffin cells

Table: 3

Correlation between scinderin levels and cortical F-actin disassembly and catecholamine release. The results show that the degree of cortical F-actin disassembly or catecholamine release is proportional to scinderin levels. When ratios of scinderin levels to F-actin disassembly or catecholamine release are calculated, the ratios remain the same regardless of treatment indicating that the level of scinderin within a cell directly influences cortical F-actin disassembly and catecholamine release.

CHAPTER 4 - DISCUSSION

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4. (1). Chromaffin Cell Viability Following Antisense ODN Treatment and Antisense ODN Uptake

Antisense ODNs targeting the scinderin gene were used in an attempt to decrease scinderin expression in primary culture chromaffin cells. Although antisense ODNs are highly specific for their gene target they must first enter the cells and accumulate in sufficiently high concentrations within the same cellular compartment as the mRNA or pre-mRNA before any biological effects are produced (Lewis *et al.*, 1996; Bennett *et al.*, 1992). Cellular uptake of antisense ODNs is influenced by cell type and is an energy dependent process which is influenced by antisense ODN length and sequence, antisense ODN concentration, temperature and culture conditions (Crooke and Bennett, 1996; Wahlestedt, 1994; Bergan *et al.*, 1993; Loke *et al.*, 1989). Pilowsky *et al.* (1994) reported that *in vitro* studies have shown that phosphorothioated antisense ODNs are taken up by cells and retained at high concentrations, with the intracellular concentrations reaching a maximum after 2 to 16 hours in most cases. Similar results were obtained in this project. The cellular uptake of the 5'-fluorescein labelled scinderin endcapped antisense ODN by chromaffin cells was highly efficient. Using fluorescent microscopy and a 5'-fluorescein labelled endcapped antisense ODN, the antisense ODN was internalized and concentrated within the nucleus one hour post-treatment indicating that the antisense ODN was targeting gene expression. In addition, the distinct

nuclear staining in chromaffin cells was maintained for 24 hours post-treatment and only after 96 hours was the punctate staining of the cytoplasm apparent suggesting that the antisense ODN was stable for up to 24 hours. It should also be noted that the 5'-fluorescein modification does not prevent nuclear uptake nor does it alter antisense ODN efficacy (Krieg *et al.*, 1989). In contrast to our results, Zhao *et al* (1993) reported that fluorescently labelled phosphorothioate endcapped antisense ODN was located predominantly in the cytoplasm of primary cultured mouse spleen cells with relatively little nuclear staining. The difference in cellular localization of the antisense ODN could possibly be due to the different methods for assessing cellular distribution, cell density, cell type, differing antisense ODN concentrations or the possibility that modified antisense ODNs may be in different intracellular compartments or different structural modifications may induce the exit from endosomes at different rates (Mirabelli and Crooke, 1993; Zhao *et al.*, 1993). Also, the choice of tissue culture medium can dramatically influence uptake of antisense ODN into cultured cells, generally much less antisense ODN is taken up into cells in the presence of defined, serum-free media such as fibroblast growth medium (FGM) or OptiMem I (Crooke *et al.*, 1995). Although, the reason for these differences in uptake due to different serum-free medium are not known, but may be the result of higher affinity of the antisense ODN for specific growth factors or proteins in defined medium than those found in media containing fetal calf serum (Crooke *et al.*, 1995). Other methods which have been successfully used in other cell types are complexing phosphorothioate modified antisense with Sendai virus

coat protein containing liposomes (Morishita *et al.*, 1993), electroporation (Bergan *et al.*, 1993) and cationic lipids (Lewis *et al.*, 1996; Bennett *et al.*, 1992). The latter two methods were utilized with chromaffin cells in an attempt in increase ODN uptake; however, neither method was adequate (data not shown). That is, cellular viability was decreased to 40% following electroporation and only 2% of chromaffin cells were effectively transfected using commercial lipid preparations such as Lipofectamine™, Lipofectin™, DMRIE™ or Cellfectin™ (data not shown).

Although the exact mechanism of cellular uptake is still unknown several mechanisms have been proposed including passive diffusion, receptor-mediated active transport, fluid-phase endocytosis (pinocytosis) or adsorptive endocytosis (Stein and Narayanan, 1996; Gibson, 1994; Wahlestedt, 1994; Stein and Cheng, 1993; Akhtar and Juliano, 1992; Budker *et al.*, 1991). The latter two processes are believed to be the predominant mechanisms (Stein and Narayanan, 1996). Adsorptive endocytosis is thought to be a possible mechanism because antisense ODNs that adsorb well to the cell surface, such as phosphodiesters and phosphorothioates, tend to be internalized to a higher degree than antisense ODNs that do not (Stein *et al.*, 1993). Transport across the plasma membrane may also involve binding of the antisense ODN to an 80 kDa plasma membrane protein (Gibson, 1994; Loke *et al.*, 1989). Gibson (1994) suggests that the greater the amount of this protein, the greater the uptake of the antisense ODN into the cells. As mentioned above antisense ODN uptake is influenced by antisense ODN

concentration. The efficiency of uptake at higher antisense ODN concentrations is similar to that of polyvinylpyrrolidone, a substance known to be taken up by pinocytosis (Roberts *et al.*, 1977). Low antisense ODN concentrations (<0.5μM), have considerably higher uptake efficiencies with the average concentration of the antisense ODN derivative in cells exceeding the derivative concentration in the medium (Yakubov *et al.*, 1989). Yakubov *et al.* (1989) suggest that is can be understood by assuming that the cells can absorb a limited amount of antisense ODN on their surface. Although, the efficiency of uptake was not evaluated in the case of the scinderin antisense ODN, it was evident that a limited window of opportunity for biological effects was present since antisense ODN concentrations less than 1.5μM did not produce any effects nor did they effect cellular viability. However, when antisense ODN concentrations between 2 and 10μM were utilized cellular viability was decreased significantly and in turn no increase in biological effects were observed. Another factor which influenced antisense ODN uptake was cellular density. As with antisense ODN concentration, when chromaffin cells were treated with 2μM antisense ODN and plated at a density of less than 0.5 x 10^6 cells or more than 0.5 x 10^6 cells/35mm petri dish no decrease in scinderin levels was observed, suggesting that the optimum density was 0.5 x 10^6 cells/35mm petri dish. Similarly, Yakubov *et al.* (1989) reported than when the cell monolayer density of mouse fibroblasts was increased from 8 x 10^4 cells per cm^2 to 5 x 10^5 cells per cm^2 there was a concomitant decrease in the maximal binding of the antisense ODN (per cell) by a factor of 3. Further, Wu-Pong (1996a, b) has suggested that the

mechanism of uptake may be influenced by various cations, specifically Ca^{2+}. In Rauscher Red 5-1.5 mouse erythroleukemia cells, antisense ODN uptake was Ca^{2+}-dependent, whereby antisense ODN uptake was increased 20-fold by the addition of excess Ca^{2+} and virtually eliminated in a Ca^{2+}-free medium (Wu-Pong, 1996a, b). Also, cadmium and lanthanum significantly increased uptake while magnesium and sodium had no effect (Wu-Pong, 1996a, b). These cation-mediated effects may occur at the cell surface by improving the access of the antisense ODN to the negatively-charged cell surface by charge neutralization or since some metal ions destabilize DNA conformations by binding simultaneously to the phosphate backbone and base, the cations may alter the conformation or properties of the antisense ODN and in turn influence the mechanism and/or extent of cellular uptake (Wu-Pong, 1996a, b). The efficiency of antisense ODN uptake into chromaffin cells may have been influenced by the fact that the cells were cultured in a Ca^{2+}-containing medium. Uptake may also be influenced by the cellular environment.

Optimal results, using antisense ODNs, can be obtained by switching from a serum containing medium to a serum-free medium which ideally contains very low concentrations of nucleases, especially 3'-exonucleases. The major problem with serum-free medium is its effect on cellular viability since in addition to containing nucleases, the serum contains growth factors, hormones and nutrients which are essential for cell survival. In the case of chromaffin cells, cellular viability was not drastically affected by a serum-free environment because following isolation from

the adrenal gland the chromaffin cells were initially cultured in a serum containing medium for at least 12 hours. This allowed the cells to rest, attach to the collagen matrix and adapt to the new environment. As a result, when the serum medium was replaced with serum-free medium approximately 92% of the cells survived for up to 4 days and their morphology did not differ from chromaffin cells maintained in a serum environment for the same time period. However, cellular viability was greatly compromised (less than 50% of the cells remain viable) when after isolation from the adrenal gland the chromaffin cells were placed in serum-free medium and not serum containing medium.

Despite the mechanism(s) of cellular uptake, the fate of antisense ODNs once in the cell depends on the antisense ODN analog used. Unmodified phosphodiester antisense ODN analogues are rapidly degraded by nuceleases having a relatively short half-life of approximately 30 minutes in serum medium (Brysch and Schlingensiepen, 1994). The nuclease activity of fetal calf serum is more active than mouse serum and human serum has the least nuclease activity; however, all sera possess substantial nuclease activity and there is significant lot-to-lot variation (Mirabelli and Crooke, 1993). The primary nuclease in all sera are 3'-exonucleases and heat inactivation, heating to 65°C for 30 minutes, does not inactivate all nucleases (Uhlmann and Peyman, 1990). To overcome this problem of rapid degradation by serum nucleases, modified antisense ODN analogues which are nuclease resistant while still retaining Watson-Crick base pairing specificity are

commonly utilized (Shoji *et al.*, 1991). From the first generation analogues the most promising is the phosphorothioate antisense ODN which is stable in media, cells and cells extracts, serum, various tissues, urine, cerebrospinal fluid and most nucleases (Agrawal and Goodchild, 1987; Miller and Ts'o, 1987; Ts'o *et al.*, 1987; Miller *et al.*, 1985, 1983, 1981; Agrawal and Rifitina, 1979). Also, *in vitro* studies have shown that phosphorothioate antisense ODNs are taken up by cells and retained at high concentrations with intracellular concentrations reaching a maximum after 2 to 16 hours (Pilowsky *et al.*, 1994). Since phosphorothioate antisense ODNs are more resistant to 3' and 5' exo- and endonucleases they have a half-life of 12-24 hours in tissue culture (Crooke *et al.*, 1995; Cook, 1993; Crooke, 1993, 1992; Hoke *et al.*, 1991). Although phosphorothioate analogues are distributed broadly within the cell and are fairly resistant to nuclease digestion, each of the phosphorothioate modifications contributes negatively to other required antisense ODN properties, such as binding affinity (Cook, 1993). Also, the presence of the sulphur at one of the non-bridging oxygen atoms in the backbone of the DNA makes this antisense ODN analog toxic to cells even at micromolar concentrations (Brysch and Schlingensiepen, 1994; Bennett *et al.*, 1992). This was evident when chromaffin cell viability was decreased by approximately 20% following treatment with 2μM of the 18-mer (data not shown) or 20-mer scinderin phosphorothioate ODN for 96 hours; however, the antisense ODN toxicity and non-specific side effects such as binding to proteins (*e.g.*, serum albumin, cell surface proteins) or inhibiting polymerases (Cohen, 1993) can be reduced by using

endcapped antisense ODNs.

The gap technique in which the antisense ODN backbone is partially modified reduces non-specific effects while as the same time conferring nuclease resistance to the antisense ODN (Peyman and Uhlmann, 1996). As mentioned earlier the primary nuclease in sera is the 3'-exonuclease, therefore, capping the 3'-end of the antisense ODN enhances the antisense ODN resistance to this type of nuclease degradation (Peyman and Uhlmann, 1996; Cook, 1993; Gamper *et al.*, 1993; Hoke *et al.*, 1991; Shaw *et al.*, 1991). A single 3'-endcap modification using any analog except the 2'-fluoro modification, increases antisense ODN stability more than 10-fold (compared to unmodified antisense ODNs) in sera (Cook, 1993; Gamper *et al.*, 1993). Also, when compared to fully modified antisense ODNs, endcapped antisense ODNs form more stable DNA-RNA hybrids which are substrates for RNase H (Gamper *et al.*, 1993; Hoke *et al.*, 1991). This may explain the greater decrease in scinderin levels obtained when chromaffin cells were treated with the scinderin endcapped antisense ODN compared to treatment with the fully modified scinderin phosphorothioate antisense ODN. Gamper *et al.* (1993) and Shaw *et al.* (1991) reported that the presence of 3' or 3' plus 5' end modification of an antisense ODN may impart nuclease resistance while making the antisense ODN stable for up to 24 hours in serum and cells; however, modification of only the 5'-end did not enhance the antisense ODNs resistance to nucleases. When chromaffin cells were treated with the scinderin endcapped antisense ODN cellular viability increased

from approximately 80% to 92% suggesting that the endcapped antisense sequence was less toxic at the equivalent concentration. Also, the non-specific binding observed when cells were treated with the 5'-fluorescein labelled scinderin phosphorothioate antisense ODN (data not shown) was significantly reduced when cells were treated with the 5'-fluorescein labelled scinderin endcapped antisense ODN.

4. (2). Scinderin Antisense Decreases Scinderin mRNA Levels in Chromaffin Cells

Regardless of the mechanism of cellular uptake, once the antisense ODN has been internalized the bulk of the antisense ODNs enter the endosome/lysosome compartments (Gewirtz *et al.*, 1996; Stein and Narayanan, 1996; Beltinger *et al.*, 1995). The endocytosed ODNs will localize first within the endosomal compartment and from there they may transfer into secondary lysosomes (Hudson *et al.*, 1996). Subcellular fractionation of K562 cells treated with ^{35}S-labelled phosphorothioate antisense ODN showed that the phosphorothioate antisense ODN accumulated in vesicular structures and in the nucleus but significantly less in the cytoplasm (Beltinger *et al.*, 1995). Tonkinson and Stein (1994) using fluorescein labelled unmodified antisense ODNs, phosphorothiotate ODNs and 3'-endcapped phosphorothioate antisense ODNs reported that upon internalization, phosphorothioate antisense ODNs accumulate in acidic compartments in HL60

cells, while unmodified antisense ODNs do not and endcapped antisense ODNs do not reside in acidic compartments but rather are located in the cytoplasm, nucleus or both. In turn they suggested that oligos with different sequences are trafficked differently in HL60 cells. These results can be extrapolated to chromaffin cells since cells treated with the fully modified scinderin phosphorothioate antisense ODN were labelled in both the cytoplasm and nucleus (data not shown) while cells treated with the scinderin endcapped antisense ODN were labelled only in the nucleus. Alternatively the differences in fluorescent labelling may be due to non-specific binding of the phosphorothioate antisense ODN as previously discussed. In the case of phosphorothioate antisense ODNs, they must escape from the acidic endosome compartments if they are to become bioavailable and reach their intended target, and it is unlikely that they can leave by passive diffusion (Akhtar *et al.*, 1991b). Although the exact mechanism is unknown, efflux from endosomal compartments may be mediated by one or more proteins present in the lipid bilayer or since endosomes regularly fuse with other vesicles simple leakage of the phosphorothioate antisense ODN during rupture or membrane fusion cannot be eliminated (Akhtar *et al.*, 1991b). None the less, the fate of phosphorothioate antisense ODNs once they escape from endosomal compartments remains unclear (Akhtar and Juliano, 1992) although some researchers suggest that they bind rapidly to the nuclear proteins ranging from 36 to 50 kDa (Chin *et al.*, 1990) or are taken up into the nucleus through nuclear pores by passive diffusion (Beltinger *et al.*, 1995). Conversely, others have shown that phosphorothioate antisense ODNs

become localized predominantly in the cytoplasm (Ceruzzi *et al.*, 1990). Bergan *et al.* (1993) suggest that entry of phosphorothioate endcapped ODNs into the nucleus involves a specific transport system or systems since the nuclear efflux of this form of ODN is energy dependent.

The precise mechanism of action at the nuclear level is unknown and depends on several factors including choice of target site (Akhtar and Juliano, 1992). Specifically, if the target site is DNA then the antisense ODN may inhibit transcription, the formation of a triple helix and inhibition of RNA polymerase; however, if the target site is mRNA then the antisense ODN may inhibit RNA processing (through RNase H mediated degradation of pre-mRNA or by interfering with activities of splisosome complexes which inhibit splicing) or mRNA transport (by interfering with the transport of mRNA from the nucleus to cytoplasm (Akhtar and Juliano, 1992). Finally, if the antisense ODN targets mRNA it may cause translational arrest through RNase H-mediated degradation of mRNA or by interfering with ribosomal activity (Akhtar and Juliano, 1992). In the case of the scinderin antisense ODN the target site was unknown and therefore the mode of action at the nuclear level was not apparent. Another important factor to determine the mechanism of action at the nuclear levels is target sequence; however, identifying a good target sequence is difficult due to lack of knowledge about the three-dimensional structure of most RNA targets within their natural environment, the difficulty in predicting the accessible nucleic acid sequences within

ribonucleoprotein complexes or chromatin structures (Leonetti *et al.*, 1993). Therefore, the target sequence of choice should be selected empirically to avoid biasing the outcome and the target should be accessible, complementary to either a part of the coding region or the untranslated but transcribed part of the gene, a splice site on the pre-mRNA or a sequence in the 5'-untranslated region of mRNA (Brysch and Schlingensiepen, 1994; Wagner, 1994; Leonetti *et al.*, 1993). Since the afore mentioned information, with respect to scinderin, is unknown the general rule for designing ODNs was used: that is, utilising the most straightforward target region - the start codon and its surrounding bases (Probst and Skutella, 1996; Wagner, 1994; Kitajima *et al.*, 1993). The 20-mer scinderin antisense ODN sequence used encompassed the ATG start codon and it was assumed that this antisense ODN would interfere with the formation of a translation initiation complex and thus block protein synthesis (Probst and Skutella, 1996). This proposed mechanism of action may have occurred in chromaffin cells; however, this may not have been the only method of impeding the flow of information from gene to protein. Another probable method of antisense ODN action may have been hybrid-arrested translation via RNase H-mediated RNA cleavage (Probst and Skutella, 1996; Scanlon *et al.*, 1995; Brysch and Schlingensiepen, 1994; Gao *et al.*, 1992; Walder and Walder, 1988; Cazenave *et al.*, 1987; Minshull and Hunt, 1986). Since the scinderin antisense ODN sequence used in these experiments decreased scinderin mRNA levels by 50% it can be suggested that the mechanism of action was at the mRNA level rather that through the formation of DNA triple helices.

4. (3). Scinderin Antisense ODNs Decreases Scinderin Expression

The severing action of scinderin is important for F-actin disassembly and ultimately the movement of chromaffin vesicles to the plasma membrane. Therefore, a decrease in scinderin levels should directly affect catecholamine release. Scinderin levels were decreased by 50% when chromaffin cells were treated with 2μM of 20-mer scinderin endcapped antisense ODN for up to four days. Similarly, Brysch *et al.* (1996) reported that treatment of SK-Br-3 mammary carcinoma cells with 2μM of their sequence-specific phosphorothioate antisense ODN for Erb-2, p53, c-JUN and JUNB reduced each respective protein by 75%. This decrease in protein levels was also evident when chromaffin cells were treated with the 18-mer scinderin phosphorothioate antisense ODN sequence which also targeted the start codon, rather than the 20-mer scinderin endcapped antisense ODN sequence. The difference between the 18-mer and the 20-mer sequences was not only two nucleic acid bases but also the 18-mer antisense ODN targeted bases 64 to 81 of the scinderin sequence with the start codon being at 5' end of the sequence, while the 20-mer antisense sequence targeted bases 70 to 89 with the start codon in the middle of the sequence. The 18-mer antisense ODN had a %G-C ratio of 64.5°C and Gibbs free energy value of 2.2 kcal/mol while the 20-mer antisense ODN sequence had a %G-C ratio of 78.5°C and a Gibbs free energy value of -0.6

kcal/mol. These differing parameters suggest that the 18-mer antisense ODN sequence may have had lower affinity and/or specificity for the mRNA target, or it was unable to hybridize adequately due to phosphorothioate modifications along the entire backbone. Also, changing the number of phosphorothioate substitutions in the 20-mer antisense ODN from 20 to 6 (three bases on the 3' and 5' ends) allowed for improved antisense ODN-target hybridization, less sequence-specific and non-sequence specific side effects and in turn a greater decrease in scinderin protein levels.

Furthermore, the decrease in scinderin levels followed by treatment of chromaffin cells with the 20-mer scinderin antisense ODN was not due sequence-specific side effects such as reduced levels of the highly related actin severing protein gelsolin. Although scinderin and gelsolin belong to the same protein family (Hartwig and Kwiatkowski, 1991), are composed of six internal domains and are 63% homologous (Marcu *et al.*, 1994) scinderin antibodies do not cross react with gelsolin and gelsolin antibodies do not cross react with scinderin (Rodríguez Del Castillo *et al.*, 1990). Using this latter point, we were able to show that a decrease in scinderin levels is not accompanied by a parallel decrease in gelsolin ruling out the possibility of sequence-specific side.

4. (4). Decreases in Scinderin Levels are Followed by Parallel Decrease in F-actin Disassembly and Catecholamine Release

Two laboratories have shown that filamentous actin is mainly localized in the cortical surface of the chromaffin cell (Vitale *et al.*, 1991; Trifaró *et al.*, 1989, 1984; Cheek and Burgoyne, 1986; Lee and Trifaró, 1981). In addition, it has also been suggested that cortical F-actin acts as a barrier to the secretory vesicles, impeding their contact with the plasma membrane (Trifaró *et al.*, 1992, 1982; Vitale *et al.*, 1991; Trifaró, 1990; Trifaró and Fournier, 1987). Stimulation of chromaffin cells produces disassembly of the actin network and removal of the barrier (Burgoyne *et al.*, 1989; Trifaró *et al.*, 1989, 1984, 1982; Cheek and Burgoyne, 1987, 1986). Furthermore, cytochemical studies with rhodamine labelled phalloidin and actin antibodies have shown that a strong fluorescent ring is present in resting cells as an indication of the distribution of F-actin in the cortical region of the chromaffin cell. Simulation of intact cells with either nicotine or high K^+ produces fragmentation of the fluorescent ring leaving cell cortical areas devoid of fluorescence (Zhang *et al.*, 1996; Trifaró *et al.*, 1989; Cheek and Burgoyne, 1987, 1986; Lee and Trifaró, 1981). Moreover, Vitale *et al.* (1991) showed that during cell stimulation, subplasmalemmal scinderin, but not gelsolin, is redistributed in chromaffin cells, and that this redistribution precedes catecholamine release and that exocytosis sites are preferentially localized to areas of F-actin disassembly. The effects of stimulation on F-actin disassembly and scinderin redistribution are Ca^{2+}-dependent

and precede exocytosis (Vitale *et al.*, 1991). These observations imply that cellular stimulation and Ca^{2+} entry bring about activation of scinderin with consequent disassembly of cortical actin filament networks (Zhang *et al.*, 1996).

Nucleotide and amino acid sequence analysis has revealed that scinderin contains two actin and two PIP_2 binding sites within the first two domains of its structure (Marcu *et al.*, 1994). Therefore, in order to further determine the role of scinderin in exocytosis several different scinderin preparations were tested. Zhang *et al.* (1996) reported that catecholamine release in response to 10μM Ca^{2+} was potentiated when permeabilized chromaffin cells were treated with 0.1μM recombinant full-length scinderin. However, recombinant truncated scinderin (scinderin protein with only domains 3 to 6) did not alter catecholamine release in response to 10μM Ca^{2+}. Furthermore, the scinderin potentiating effects on catecholamine release were inhibited by scinderin derived peptides (Sc-ABP_1 and Sc-APB_2) with sequences corresponding to two active actin binding sites of scinderin (Zhang *et al.*, 1996). In addition, when the two peptides were used simultaneously the inhibitory effect achieved was much greater then when the peptides were used individually (Zhang *et al.*, 1996). This suggests that these two actin binding domains are the F-actin severing domains of scinderin and are most likely the domains responsible for the scinderin potentiation of Ca^{2+}-induced catecholamine release (Zhang *et al.*, 1996). Finally, the inhibitory effects of PIP_2 on Ca^{2+}-induced catecholamine release and F-actin disassembly are eliminated if

the PIP_2 binding site is blocked with scinderin-derived PIP_2-binding peptide (Zhang *et al.*, 1996).

Conversely, when scinderin levels are decreased through the use of antisense ODNs targeting the scinderin gene, the decrease in this protein is accompanied by a parallel decrease in F-actin disassembly. Similarly, the decrease in scinderin levels mirrored the decrease in depolarization evoked catecholamine release. Moreover, it should be noticed that the ratio between scinderin levels and F-actin disassembly (Table 3) remain constant regardless of the levels of scinderin (*i.e.*, decreased levels due to antisense treatment or control levels in serum-free or mismatch treated cells). Similarly, the ratio between scinderin and depolarization evoked catecholamine relase where also shown to be constant regardless of sample treatment. These observations strongly suggest that scinderin levels regulate the degree of F-actin disassembly and exocytosis in response to chromaffin cell stimulation.

4. (5). Scinderin a Component of the Exocytotic Machinery

Since it's discovery in 1989 and the subsequent functional studies which followed, scinderin's involvement in secretory vesicle exocytosis has become much more evident. The exocytotic process in chromaffin cells has been studied by various laboratories (Trifaró *et al.*, 1997, 1993, 1992a, 1992b; Andrea and Thorn 1996;

Burgoyne *et al.*, 1996; Roth and Burgoyne, 1995; Trifaró and García, 1995; Vitale *et al.*, 1995, 1991; Trifaró and Vitale, 1993; Strittmatter, 1988; Burgoyne and Cheek, 1987; Schäfer *et al.*, 1987; Baker and Knight, 1978) and it has become apparent from the research that the exocytotic process in which stored, presynthesized compounds are released into the extracellular space in response to specific stimuli (Strittmatter, 1988) is complex. This process requires that proteins associated with secretory vesicles such as synapsin I (Zucker, 1996), and α-actinin (Trifaró *et al.*, 1992a, 1992b; Strittmatter, 1988) as well as secretory vesical integral membrane proteins [synaptogamin, synaptobrevin, synaptophysin (p38), glycoprotein SV_2, synaptotagmin (65 kDa calmodulin-binding protein, 65-CMBP, p65] (Trifaró *et al.*, 1992a, 1992b; Fournier *et al.*, 1989) interact with cellular components such as cytosolic proteins [*N*-ethylmaleimide sensitive factor (NSF), soluble NSF attachment proteins (SNAP)], plasma membrane proteins [syntaxin, SNAP-25 and N-type Ca^{2+} channels], GTP-binding proteins, cytoskeleton proteins [fodrin and caldesmon], Ca^{2+}-dependent binding proteins [calmodulin, calpactin] and actin filament severing proteins [scinderin] (Trifaró *et al.*, 1997, 1992a, 1992b; Zucker, 1996; Gratzl, 1995).

In chromaffin cells, exocytosis is a highly regulated (Kelly, 1985; Gumbiner and Kelly, 1982) Ca^{2+}-dependent process (Gratzl, 1995; Holz *et al.*, 1982). During resting conditions secretory vesicles are "trapped" within a subplasmalemmal actin network which acts as a physical barrier to the movement of vesicles toward exocytotic sites on the plasma membrane (Cheek and Burgoyne, 1986; Perrin and

Aunis, 1985; Trifaró *et al.*, 1985a; Vitale *et al.*, 1991; Trifaró *et al.*, 1992b). Therefore, as previously mentioned the reorganization of the cortical cytoskeleton network is necessary but not a sufficient part of exocytosis (Aunis and Bader, 1988; Muallem *et al.*, 1995) and is accomplished by the activation of Ca^{2+}-dependent actin severing proteins such as scinderin (Rodríguez Del Castillo *et al.*, 1990). Prior to the discovery of scinderin is was believed that gelsolin was the sole actin severing protein responsible for the disassembly of the cortical cytoskeleton (Bader *et al.*, 1986). However, Rodríguez Del Castillo *et al.* (1990) reported that a novel actin filament severing protein, scinderin, which has a different molecular weight, isoelectric points and amino acid composition may be a valuable component of the exocytotic machinery.

Subsequent studies, after it's discovery have shown that scinderin is restricted to tissues with high secretory activity (Tchakarov *et al.*, 1990) and that scinderin is colocalized with F-actin both during the resting state and following cellular stimulation with either nicotine or depolarizing concentrations of K^+ (Trifaró *et al.*, 1993; Vitale *et al.*, 1991). That is, immunofluorescent studies have shown that during the resting state a continuous fluorescent ring is present for both F-actin and scinderin; however, when chromaffin cells are stimulated areas in which F-actin fluorescence is absent are also devoid of scinderin fluorescence (Trifaró *et al.*, 1993; Vitale *et al.*, 1991) suggesting that during the resting state scinderin is bound to plasma membrane phospholipids that position the protein in close proximity of

this substrate, the cortical F-actin network (Rodríguez Del Castillo *et al.*, 1992b). Further, cellular stimulation produces a rise in intracellular Ca^{2+} resulting in the translocation of scinderin from the plasma membrane to the F-actin network and the activation of its severing activity (Rodríguez Del Castillo *et al.*, 1990). Moreover, this type of cellular redistribution does not occur with gelsolin, suggesting that gelsolin may not participate in the disassembly of the cortical cytoskeleton that precedes exocytosis (Vitale *et al.*, 1991).

More recently, as discussed above, recombinant full length scinderin has been shown to potentiate Ca^{2+}-evoked F-actin disassembly and exocytosis in permeabilized chromaffin cells, an effect blocked by Sc-ABP_1 and Sc-APB_2 (Zhang *et al.*, 1996). In addition, the current experiments show that a reduction in scinderin levels by 50%, as evidenced by immunoblotting and immunocytochemical analysis is accompanied by an equivalent and parallel decrease in F-actin disassembly and catecholamine release, two processes that are explicit indicators of exocytosis. Therefore, scinderin seems to control the dynamics of the cortical F-actin network in a Ca^{2+}-dependent manner and this disassembly will in turn determine the availability of secretory vesicles for exocytosis suggesting that scinderin is an important component of the exocytotic machinery (Zhang *et al.*, 1996).

4. (6). Conclusions

From the data presented within this thesis it is apparent that antisense technology provides a more precise method of targeting specific genes to evaluate the importance of their protein products. Although this technology is still in its infancy careful precautions must be taken to ensure that the biological effects are due to the antisense treatment and not to nonspecific biological effects. Therefore, in an ideal antisense experiment: (I) target RNA or protein levels should be measured and compared to internal RNA or protein controls; (ii) the target sequence should be chosen empirically to avoid biasing the outcome; (iii) mismatch controls should differ from the antisense sequence no more than is necessary to prevent specific hybridization; (iv) ODNs should be nuclease-resistant; (v) ODNs should be able to cross cellular membranes efficiently (Wagner, 1994). Taking this into consideration all of the necessary precautions were used to obtain results which will assist in our understanding of scinderin's involvement in the exocytotic process. Using the above mentioned criteria the results obtained for this project reflect the biological effects due to treatment of chromaffin cells with scinderin phosphorothioate endcapped antisense ODN, not nonspecific side effects.

Cellular viability was not compromised when the chromaffin cells were cultured in serum-free medium or serum-free medium containing 20-mer scinderin phosphorothioate antisense ODN or 20-mer scramble phosphorothioate ODN

sequence. The 20-mer scinderin phosphorothioate endcapped antisense ODN sequence not only enters the cells but it was also concentrated within the nucleus where it was able to exert its biological effect as was evident when scinderin mRNA levels and consequently scinderin levels were decreased by 50% following treatment with 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN for four days compared to cells treated with only serum-free medium or 20-mer mismatch phosphorothioate endcapped ODN sequence for an equivalent period of time. The decrease in scinderin mRNA levels was accompanied by a parallel decrease in scinderin protein levels and subsequently depolarization-evoked catecholamine release. Further, depolarization-evoked catecholamine release was decreased by 50% when chromaffin were cells treated with 2μM 20-mer scinderin phosphorothioate endcapped antisense ODN for four days compared to cells treated with only serum-free medium or 20-mer mismatch phosphorothioate endcapped ODN sequence for an equivalent period of time. These results suggest that the decrease in scinderin levels caused a decrease in F-actin disassembly and subsequently a decrease in catecholamine release, the final endpoint in the exocytotic process. The present data taken together with previous observations with recombinant scinderin from our laboratory (Zhang *et al.*, 1996) strongly indicate that scinderin plays an important role in exocytosis.

In conclusion, in can be said that scinderin is a component of the molecular machinery which controls this highly intricate process of exocytosis. Although the

importance of scinderin for F-actin disassembly and catecholamine release from chromaffin cells in culture has been clearly demonstrated through the use of antisense technology, the relationship between scinderin and other physiological processes related to secretion have yet to be elucidated.

CHAPTER 5 - REFERENCES

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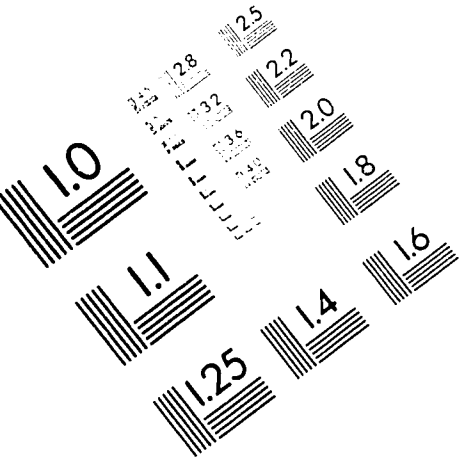

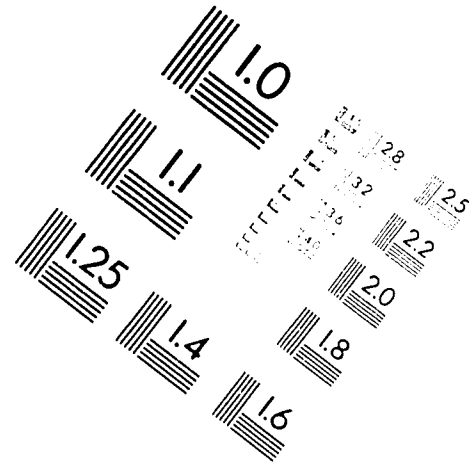

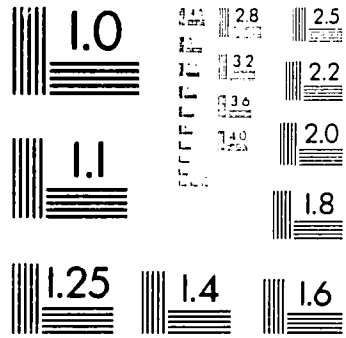

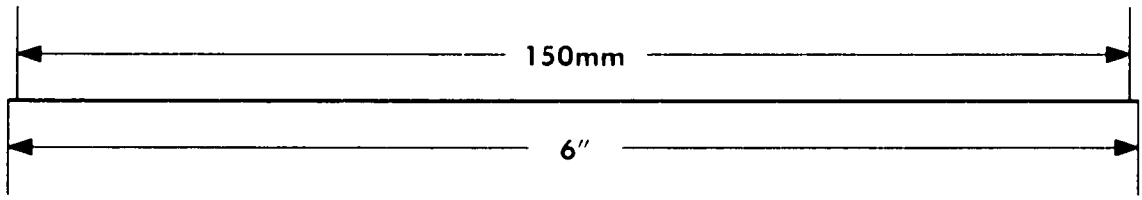

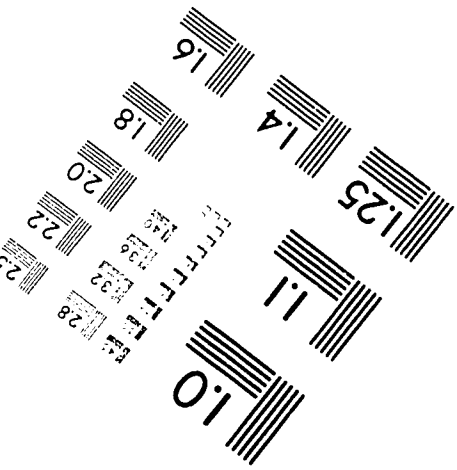

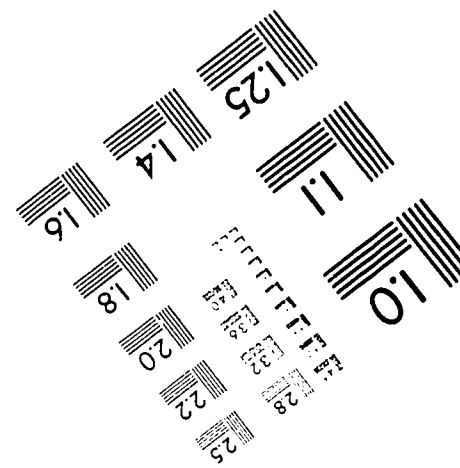

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