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**ISOLATION AND CHARACTERIZATION OF CAVEOLAE FROM CANINE AIRWAY
AND INTESTINAL SMOOTH MUSCLE**

By

PETER JAMES DARBY, B.Sc (Hons), M.Sc.

A Thesis

Submitted to the School of Graduate Studies

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INVOLVEMENT OF CAVEOLAE IN SMOOTH MUSCLE CALCIUM HANDLING

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AUTHOR: Peter James Darby, B.Sc. (Hons), M.Sc. (McMaster University)

SUPERVISOR: Professor Edwin E. Daniel

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ABSTRACT

Calcium (Ca^{2+}) for contraction of smooth muscle comes from two sources: release of Ca^{2+} from an internal site, the sarcoplasmic reticulum (SR), and influx of Ca^{2+} from the extracellular space across the plasma membrane (PM). Previous contractility experiments in canine tracheal and bronchial smooth muscle preparations have suggested a close association between the PM and SR. In canine trachea, refilling of the SR from the extracellular space can occur via a "preferred pathway", which involves L-type Ca^{2+} channels on the PM. In canine bronchial rings, contractile agents produce a sustained contraction in nominally Ca^{2+} -free medium. These contractions are dependent upon a functioning SR, and influx of Ca^{2+} across the PM via L-type Ca^{2+} channels, since inhibitors of the SR Ca^{2+} pump or of L-type Ca^{2+} channels prevent the sustained phase of the contraction. This extracellular source of Ca^{2+} is protected since low levels (μM) of EGTA, a Ca^{2+} chelating agent, are without affect, while higher levels (mM) inhibit the sustained phase of the contraction.

We hypothesize that caveolae, flask-shaped invaginations of the PM, are the protected source of Ca^{2+} . Ultrastructurally, there are frequent close associations between the PM and SR. More specifically, this peripheral SR is often associated with caveolae, forming a network between and surrounding the caveolae. This thesis provides biochemical evidence that supports the hypothesis of caveolae

involvement in Ca^{2+} handling.

Caveolae were isolated from canine tracheal smooth muscle by detergent treatment of PM-derived microsomes. The detergent-resistant membranes were enriched in caveolin, which forms the protein coat on caveolae, and is a specific marker for caveolae. These detergent-resistant membranes were also enriched in: L-type Ca^{2+} channels, as determined by Western blotting and radioligand binding experiments, the Ca^{2+} binding proteins calsequestrin and calreticulin, and the neuronal form of nitric oxide synthase (nNOS), as determined by Western blotting. Western blots also demonstrated that the PM Ca^{2+} pump was present, but not connexin-43, a non-caveolae PM protein, the SR Ca^{2+} pump, or the Type 1 IP_3 receptor, indicating that SR-derived membranes were not a contaminant.

Immunoprecipitation experiments confirmed the presence of calsequestrin and calreticulin in caveolae. Antibodies to caveolin coimmunoprecipitated caveolin with calsequestrin and calreticulin. These experiments also indicated that at least some of the associated calsequestrin and calreticulin are located on the cytoplasmic face of each caveola, since no part of the caveolin protein crosses into the luminal side of each caveola. Immunohistochemistry of fixed tracheal smooth muscle cryosections confirmed that the PM Ca^{2+} pump, nNOS, and caveolin were all located on the cell periphery, while the SR Ca^{2+} pump is located deeper in the cell.

Recently, many investigators have questioned the validity of detergent

treatment as a method for isolating caveolae, suggesting that this approach may cause artifactual clustering of proteins into detergent-resistant membrane domains. To address this possibility, a second caveolae isolation method was employed using treatment with Na_2CO_3 , followed by sonication, and separation by discontinuous sucrose gradient centrifugation. Using canine intestinal smooth muscle, results of this method were compared with the results of detergent treatment. A number of fractions were isolated from the Na_2CO_3 -treated membranes. Western blotting indicated that in the caveolin-enriched fraction, the α_1 chain of the L-type Ca^{2+} channel, calsequestrin, calreticulin, and the PM Ca^{2+} pump were present, but connexin-43, the SR Ca^{2+} pump, and type 1 IP_3 receptor were excluded. These results confirmed the results obtained by the detergent treatment method.

Based on the results presented here and our previous results of contractility experiments, a model of Ca^{2+} handling for airway smooth muscle is proposed. This is an extension of the superficial buffer barrier hypothesis first proposed by van Breemen (1986). In our model, caveolae provide the physical basis for the junctional space between the PM and SR. Ca^{2+} can move into the lumen of the caveolae via the PM Ca^{2+} pump, and be released from caveolae into the junctional space via L-type Ca^{2+} channels. Calsequestrin and calreticulin, located on the cytoplasmic face of caveolae, may act as a physical barrier facilitating the direct refilling of the closely associated SR with Ca^{2+} . Similarly, nitric oxide, produced by

the nNOS located on the caveolae, may inhibit release of Ca^{2+} by the SR, thereby enhancing refilling. The Ca^{2+} in the lumen of the caveolae may be retained by calsequestrin, calreticulin, or another unidentified Ca^{2+} binding protein located in the caveolae lumen. Alternatively, the extracellular matrix, which crosses over the neck but does enter the lumen of each caveola, may prevent or impede the loss of Ca^{2+} to the surrounding extracellular space.

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List of Abbreviations

AT ₁ :	Angiotensin II receptor (Type 1)
Ca ²⁺ :	Calcium
cGMP:	guanosine 3',5'-cyclic monophosphate
8Br-cGMP:	Membrane permeant analog of cGMP
CHAPS:	3-[(3-Cholamidopropyl)dimethyl-ammonio]1-propanesulfonate
cy3:	Indocarbocyanide
ECL:	Enhanced Chemiluminescence
EGTA:	ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid
ER:	Endoplasmic Reticulum
FITC:	Fluorescein Isothiocyanate
F1,1.5,2,2.5,3:	Sucrose Gradient Fractions (4/14% interface, 14% layer, 14/25% interface, 25% layer, 25/35% interface, respectively)
GPI:	Glycosyl Phosphatidyl Inositol
I _{CRAC} :	Calcium Release-activated Calcium Current
K ⁺ :	Potassium
LES:	Lower Esophageal Sphincter
L-NAME:	N ^ω -nitro-L-arginine methyl ester
L-NNA:	N ^G -Nitro-L-arginine
Mg ²⁺ :	Magnesium

MIC I: Microsomal Pellet I

MIC II: Microsomal Pellet II

MIT I: Mitochondrial Pellet I

MIT II: Mitochondrial Pellet II

MOPS: 3-[N-Morpholino] propanesulfonic acid

M3C: Microsomal Pellet 3

M3T: Detergent-insoluble Microsomal Pellet (3)

Na⁺: Sodium

Na₂CO₃: Sodium Carbonate

NOS, eNOS, iNOS, nNOS: Nitric Oxide Synthase (endothelial, inducible, neuronal)

PM: Plasma Membrane

PMSF: Phenylmethylsulfonyl Fluoride

PNS: Post-nuclear Supernatant

SDS: Sodium Dodecyl Sulfate

SDS-PAGE: SDS-Polyacrylamide Gel Electrophoresis

SEM: Standard Error of the Mean

SMB: Sucrose MOPS buffer

SNAP: S-nitroso-N-acetylpenicillamine

SOL: Soluble fraction

SR: Sarcoplasmic Reticulum

TBS: Tris-buffered Saline
TTBS: Tris-buffered Saline + 0.05% Tween-20
Tris: Tris(hydroxymethyl)aminomethane

Chapter 1: INTRODUCTION

This thesis describes evidence that caveolae may act as a novel site for Ca^{2+} storage and handling in airway smooth muscle. Before this evidence is presented, it is necessary to look first at the history of caveolae in smooth muscle and other cell types. This first chapter will examine five main areas. First, morphological data will be discussed, demonstrating the existence of caveolae on the plasma membrane (PM) of airway smooth muscle, their association with the internal Ca^{2+} store, the sarcoplasmic reticulum (SR), and initial evidence that caveolae may be a source of Ca^{2+} for contraction. Next, models of Ca^{2+} handling will be presented, as well as limitations of these models with regard to results in bronchial smooth muscle. The third section will present proposed models of caveolar function in various cell types, including smooth muscle cells. Proteins localized to caveolae, with emphasis on the caveolar structural protein caveolin and its potential role as a scaffolding protein will comprise the fourth section. The final section will describe methods used to biochemically isolate caveolae and their associated proteins, as well as their drawbacks.

1.1 Structure of Tracheal Smooth Muscle

In the dog trachea, the muscle forms a narrow band running transverse to the longitudinal axis of the trachea, connecting the horseshoe-shaped cartilage. The muscle is arranged in bundles, separated by wide interstitial spaces (Kannan and Daniel, 1980), running approximately parallel. However, the bundles are not independent, but there are clefts and splits in the bundles, leading Cameron et al. (1982) and others to conclude that the muscle cells exist not as discrete bundles, but as groups which interconnect in a syncytial pattern.

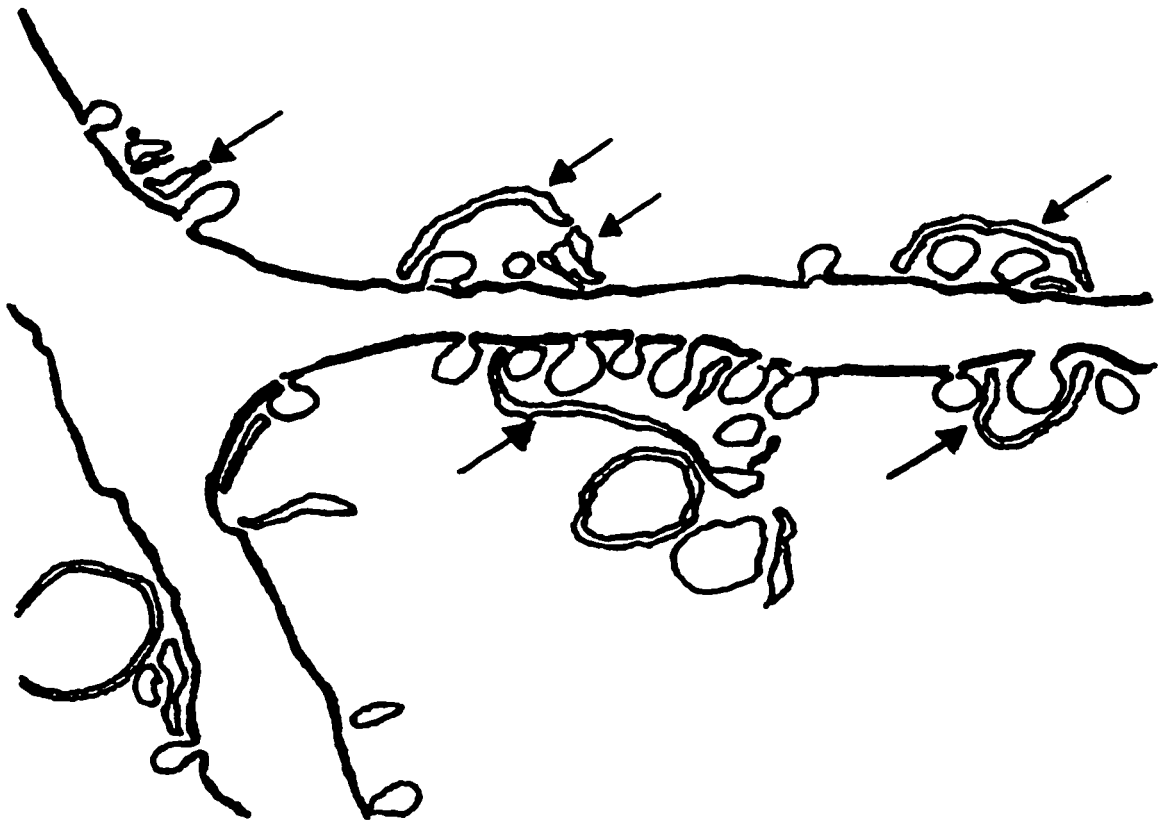
Ultrastructurally, tracheal smooth muscle cells have a basic spindle shape along the axis of the bundles, while in transverse sections, they are irregular in shape with several processes and invaginations (Suzuki et al., 1976; Gabella, 1989). Gap junctions can be seen connecting such processes on occasion (Kannan and Daniel, 1980). Although no detailed morphological data for dog trachea exists, it does for bovine tracheal muscle. Smooth muscle cells from bovine trachea have an average length of $806\mu\text{m}$, volume of $2950\mu\text{m}^3$, surface area of $7375\mu\text{m}^2$, and volume to surface area ratio of 0.4, considered to be low when compared with other smooth muscles, but expected due to the number of processes and invaginations (Cameron et al., 1982). Intracellular organelles (nucleus, Golgi apparatus, mitochondria, SR, and caveolar intracellulares) occupy 10.5% of cell volume with SR occupying 1.94% (Cameron et al., 1982) This value is low compared with other smooth muscle, which range from 1.8 to 5.1% (Devine et al., 1972).

1.1.1 Caveolae

Caveolae, first described in smooth muscle tissues fixed for electron microscopy by Caesar et al. (1957), are flask-shaped invaginations of the PM with a narrow neck partially closing off the contents of each caveola from the surrounding extracellular space (Figure 1). Additionally, the basal lamina surrounding the smooth muscle cells does not penetrate into the caveolae but passes over their necks (Gabella, 1981b), which may provide a further barrier between the interior of the caveolae and extracellular space. However, in fixed tissues, it has not been shown that caveolae are completely closed off from the extracellular space, and further, the interior of each caveola is accessible to extracellular tracers such as ferritin, colloidal lanthanum, or peroxidase, even when there is no visible connection in a thin section (Devine et al., 1972; Gabella, 1981b).

Smooth muscle caveolae are generally uniform in size, with an internal diameter of 75 x 100nm (Cameron et al., 1982). The neck region is approximately 35nm wide at its narrowest point (Gabella, 1981b). In bovine trachea, they are arranged in long rows in longitudinal sections, and as groups of 3-5 in transverse sections (Cameron et al., 1982). This ordered arrangement is similar to that found in other visceral smooth muscles (Gabella, 1989). Studies in other smooth muscles have reported that caveolae occupy 29-40% of the cell surface area (Gabella, 1976; Suzuki and Sugi, 1989). However, Gabella (1976) calculated the surface area of

Figure 1: Artist's depiction of electron micrograph of circular muscle layer of guinea pig ileum. Three smooth muscle cells transversely sectioned show several flask shaped caveolae closely associated with sarcoplasmic reticulum (arrows). Magnification: 67,000 x. Figure adapted from: Gabella, G. "Structure of muscles and nerves in the gastrointestinal tract" in Physiology of the Gastrointestinal Tract. L.R. Johnson, ed. Raven Press, New York 1981a. pp197-242.



a single caveola, and reported that although they occupy 29% of the cell surface, they increase the cell surface area by 73%. For bovine tracheal smooth muscle, Cameron et al. (1982) reported that caveolae increase the cell surface area by 90%, and occupy 1.64% of the cell volume. This value is comparable to the volume occupied by the SR in this tissue (1.94%) and led Cameron et al. (1982) to suggest that caveolae, in addition to SR, may be acting as a site for Ca^{2+} storage. Studies in guinea pig taenia cecum have reported that caveolae occupy 0.8% (Gabella, 1976) or 3.5% (Popescu et al., 1974) of the cell volume.

1.1.2 Association Between Plasma Membrane and Sarcoplasmic Reticulum

The SR is found distributed throughout smooth muscle cells and is generally divided into two basic regions: peripheral and deep, with the peripheral SR in close association with the PM. Numerous investigators have reported that SR in this region is often associated with the PM in various smooth muscles, including trachea (e.g. Gabella, 1971; Devine et al., 1972; Suzuki et al., 1976; Cameron et al., 1982). In many instances, the SR forms a network spread among caveolae (Gabella, 1971, 1978; Devine et al., 1972; Popescu et al., 1974). An example of the association between caveolae and SR is illustrated in Figure 1. In bovine tracheal smooth muscle, Cameron et al. (1982) described the SR as either forming caps over or a fenestrated network around caveolae. The distance between caveolae and SR (or

PM and SR) is variable, with the distance between them as close as 10nm (Gabella, 1971; Devine et al., 1972). However there is no evidence of direct contact between SR and caveolae or fusion of membranes (Gabella, 1971) although electron-opaque material spanning between SR and PM have been reported in some smooth muscles (Devine et al., 1972).

Further evidence for the association between caveolae and SR was provided by Moore et al. (1993) using toad stomach smooth muscle. Using histochemical techniques viewed with a digital imaging microscope and processed by a complex algorithm, a resolution of 150nm, or perhaps as high as 50nm was achieved. The authors demonstrated that both the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and the Na^+/K^+ pump were localized in discrete sites on the PM at regular intervals which were suggested to be caveolae. Further, the SR Ca^{2+} binding protein, calsequestrin, was also found to colocalize, suggesting that caveolae and peripheral SR were closely associated.

In a study investigating SR Ca^{2+} binding proteins using histochemistry and immunoelectron microscopy, calsequestrin was demonstrated to be the chief Ca^{2+} binding protein in peripheral SR of rat vas deferens (Villa et al., 1993). They reported that calreticulin was uniformly distributed in both peripheral and deep SR, and calsequestrin, while also present in both peripheral and deep SR, was approximately five times more concentrated in peripheral SR as determined from immunoelectron microscopy. The peripheral SR structures were seen to be often running parallel to the PM at a distance of 25nm from the PM. Although the authors

state that there was no gold labelling of caveolae when stained for calsequestrin, it can be seen in the figures that there were some structures close to the cell surface with a round shape resembling caveolae, although no contact to the PM or neck region was present, which were positively stained for calsequestrin. The significance of this will be discussed further in later chapters.

The identity of SR Ca^{2+} binding proteins has also been sought in biochemical studies. While some smooth muscles, such as rat uterus (Milner et al., 1991; Volpe et al., 1994), bladder (Volpe et al., 1994), pig aorta, and pulmonary artery (Raeymaekers et al., 1993) contain calreticulin and no detectable calsequestrin, pig stomach, ileum, trachea (Raeymaekers et al., 1993), rat vas deferens, aorta, and stomach (Volpe et al., 1994) do contain calsequestrin. Those tissues which contain calsequestrin also have calreticulin present, although relative amounts have not been determined. In all smooth muscles which express calsequestrin, the cardiac isoform of calsequestrin is present to a variable extent. In the only study looking at relative amounts of the calsequestrin isoforms, rat vas deferens expressed approximately equal amounts of the cardiac and skeletal isoforms, while in rat stomach and aorta, approximately 80% was the skeletal muscle isoform.

1.2 Models for Calcium Handling

Although many possible physiological roles have been proposed for smooth

muscle caveolae, none have been proven. Some have speculated on a role for caveolae in stretch, as is seen in skeletal muscle (Dulhunty and Franzini-Armstrong, 1975), where under conditions of extreme stretch, the caveolae opened up and provided the membrane needed for the increased surface area. However, Gabella and Blundell (1978) demonstrated that, at least in guinea pig taenia cecum, stretch or contraction affected neither the packing density, nor the shape of the caveolae. Others have speculated on a role for caveolae in sodium handling (Garfield and Daniel, 1977a,b), which has remained unproven. The frequent close association between caveolae and SR, and the cell volume occupied by caveolae in bovine tracheal smooth muscle led Cameron et al. (1982) to speculate on a possible role for caveolae in Ca^{2+} movement and storage. A role in Ca^{2+} handling will be discussed further in later sections. In order to better define a role for caveolae in Ca^{2+} handling, a general description of models for Ca^{2+} handling must first be addressed.

It is generally agreed that in smooth muscle, there are two sources of Ca^{2+} for contraction. The intracellular Ca^{2+} pool, located within the SR and released via Ca^{2+} - or inositol 1,4,5-trisphosphate- (IP_3 -) induced Ca^{2+} release channels, is involved in the initial quick rise in intracellular Ca^{2+} leading to contraction. However, this source is limited. Thus, the influx of extracellular Ca^{2+} , essentially a limitless supply of Ca^{2+} , across the PM via L-type (or other) Ca^{2+} channels allows for the contraction to continue indefinitely. However this is an oversimplification of

the processes involved. There are two main theories of Ca^{2+} handling in smooth muscle, the superficial buffer barrier postulated by van Breemen (van Breemen et al., 1986, 1995), and Putney's capacitative Ca^{2+} entry (Putney, 1986), a theory which was derived from studies in non-excitabile cells.

1.2.1 Capacitative Calcium Entry

This model, first put forward by Putney (1986), and later revised by him and others (Putney, 1990, 1997; Holda et al., 1998), states that a decrease or depletion of Ca^{2+} in the peripheral SR acts to stimulate influx of Ca^{2+} across the PM. This influx is postulated to occur through a unique set of channels (capacitative Ca^{2+} entry channels, also referred to as I_{CRAC}), which have been identified on the basis of electrophysiological data in various non-excitabile cell types.

Since SR and PM in smooth muscle and non-excitabile cells are not physically connected (Gabella, 1971; Devine et al., 1972), for the loss of Ca^{2+} from SR to stimulate influx of Ca^{2+} , it has been suggested that a signalling mechanism must exist. Many hypotheses have been proposed (reviewed in Holda et al., 1998). Potential signalling factors include a low molecular weight (<500Da) phosphorylated factor, cGMP, IP_4 , and metabolites of the cytochrome P450 system. Another possibility is that the SR and PM are in close contact, with a structure analogous to the foot structure between t-tubules and the SR in skeletal muscle. However, as

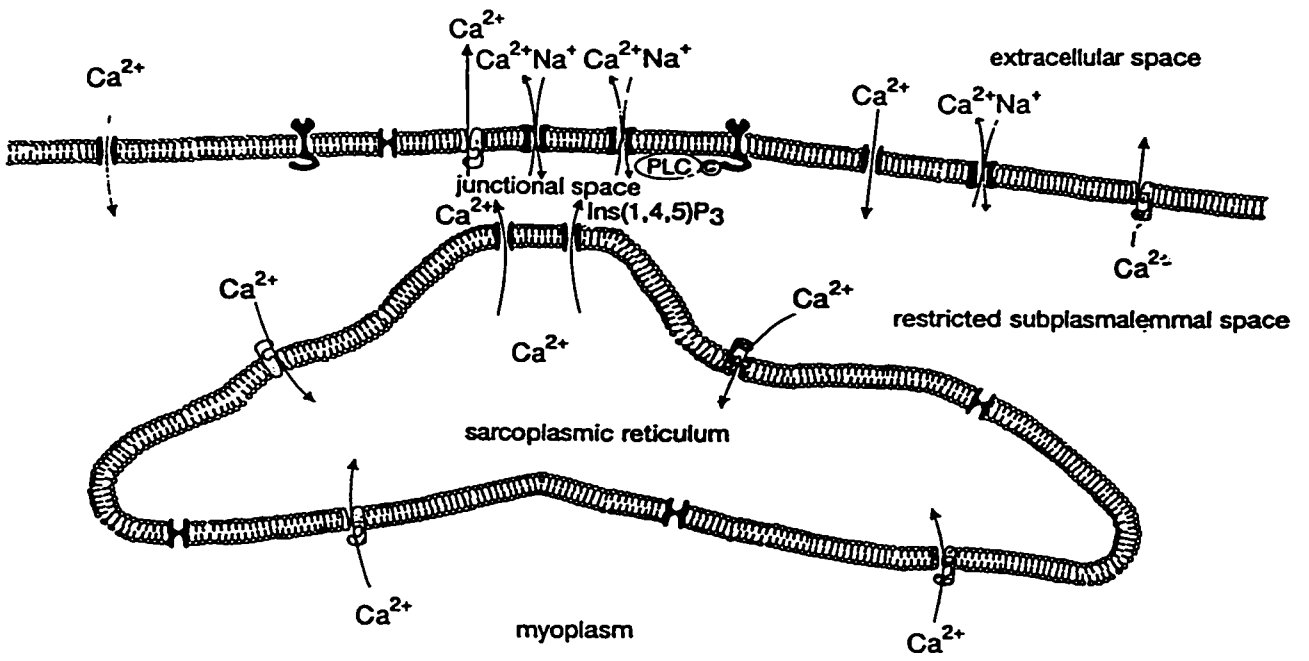
stated earlier, at least in airway smooth muscle, there is no evidence, at the ultrastructural level, that such a structure exists (Gabella, 1971; Devine et al., 1972). The most recent theory by Putney (1997) postulates that the type 3 IP_3 receptor may act as the capacitative Ca^{2+} entry channel in some cell types. However in other cell types, such as cultured vascular endothelial cells (Holda et al., 1998) there is no evidence of expression of such a channel, despite evidence of capacitative Ca^{2+} entry in these cells.

1.2.2 Superficial Buffer Barrier

This model, put forward by van Breemen (1986) and depicted in Figure 2, also involves the peripheral SR acting to modulate Ca^{2+} diffusion to the myoplasm. In order for the peripheral SR to continuously regulate the intracellular Ca^{2+} levels, it must be able to take up Ca^{2+} entering from the extracellular space if unfilled and release accumulated Ca^{2+} vectorially towards the PM when filled, allowing Ca^{2+} to be removed from the cell by the Na^+/Ca^{2+} exchanger and PM Ca^{2+} pump. Evidence for this model has been derived from studies in vascular smooth muscle and endothelial cells. Therefore this model, rather than the capacitative Ca^{2+} entry model, may be more relevant towards an understanding of Ca^{2+} handling in airway smooth muscle. It has been demonstrated that after depletion of Ca^{2+} from the SR, stimulation of the smooth muscle cells with high K^+ did not result in immediate

contraction (van Breemen et al., 1995). Instead there was a delay, while the rate of Ca^{2+} influx was unchanged, during which the SR was refilled by the entering Ca^{2+} via the SR Ca^{2+} pump. Agonist stimulation of smooth muscle cells releases Ca^{2+} from the SR and stimulates Ca^{2+} influx via voltage-gated or receptor-operated Ca^{2+} channels. In addition, agonist stimulation inhibits the functioning of the superficial buffer barrier by preventing refilling of the SR which results in a greater driving force of Ca^{2+} for contraction. The superficial SR also is postulated to play a role in Ca^{2+} extrusion. Nazar and van Breemen (1998) demonstrated that in vena cava smooth muscle, inhibition of the SR Ca^{2+} pump by the selective Ca^{2+} pump inhibitor cyclopiazonic acid (Darby et al., 1993) resulted in a reduced rate of decline of intracellular Ca^{2+} . They postulated that the Ca^{2+} taken up by the SR was not simply sequestered but instead was subsequently released vectorially toward the PM, where it was removed via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. However, there is no evidence of a functional $\text{Na}^+/\text{Ca}^{2+}$ exchanger in canine airway smooth muscle (Janssen et al., 1997), so this mechanism requires some modifications in order to apply to the airway.

Figure 2: Model of superficial buffer barrier hypothesis. Ca^{2+} crossing the PM is sequestered into the SR from the restricted subplasmalemmal space by the SR Ca^{2+} pump. Ca^{2+} mobilizing agonists, which produce IP_3 (labelled $\text{Ins}(1,4,5)\text{P}_3$) inhibit the functioning of the superficial buffer barrier and enhance flow of Ca^{2+} into the myoplasm for contraction. The superficial SR also plays a role in Ca^{2+} extrusion. Ca^{2+} taken up by the SR can be released vectorially into the junctional space, where it is removed from the cell via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Reprinted from: Trends in Pharmacological Sciences, Vol 16, No 3, C. van Breemen, Q. Chen, and I. Laher, Superficial buffer barrier function of smooth muscle sarcoplasmic reticulum, pp.98-105, Copyright 1995, with permission from Elsevier Science.



1.2.3 Evidence for Non-classical Calcium Handling in Airway Smooth Muscle

In tracheal smooth muscle, there is evidence of refilling of the SR with Ca^{2+} entering from the extracellular space without initiating contraction, similar to that demonstrated by van Breemen. This refilling could be enhanced by raising extracellular Ca^{2+} or opening L-type Ca^{2+} channels with Bay K 8644, or blocked by addition of an L-type Ca^{2+} channel blocker (Bourreau et al., 1991, 1993). A study by Janssen and Sims (1995) confirmed that the Ca^{2+} entry was by a voltage-dependent Ca^{2+} channel. However, this 'preferred pathway' did not require a functioning SR Ca^{2+} pump since cyclopiazonic acid did not block the refilling. This was in contrast to van Breemen's model which stated that the refilling of the peripheral SR from the extracellular space is mediated via Ca^{2+} influx and the SR Ca^{2+} pump.

Contractility experiments using canine bronchial smooth muscle demonstrated that in a Ca^{2+} -free medium, agonist stimulation resulted in a sustained contraction which could be reproduced several times, with only the magnitude reduced on subsequent contractions (Montano et al., 1993, 1996). The sustained phase of the contraction could be prevented by addition of an L-type Ca^{2+} channel blocker or by raising the EGTA concentration of the medium to mM levels (Montano et al., 1993). This suggested that the source of Ca^{2+} was "extracellular" but was somehow protected from the extracellular medium since concentrations of

EGTA (100 μ M) which removed all extracellular Ca^{2+} (<9nM) did not prevent these bronchial contractions. This concentration of EGTA results in only a transient contraction in vascular smooth muscle (Guan et al., 1988; Low et al., 1991). Although other researchers have suggested that the source of this Ca^{2+} may be the cartilage (Raeburn et al., 1986, 1987; Gupta et al., 1991), this does not appear likely since pretreatment with cyclopiazonic acid resulted in only a transient contraction, suggesting that the sustained contraction also involved a functional SR (Montano et al., 1993). Moreover, studies of $^{45}\text{Ca}^{2+}$ uptake into, and efflux from cartilage revealed very rapid exchange, too fast to supply Ca^{2+} for long periods (Montano et al., 1996).

The identity of this protected, extracellular source of Ca^{2+} remains unknown, but we postulate that caveolae may be this source. The notion of caveolae involvement in Ca^{2+} handling/signalling in smooth muscle is not novel (Popescu et al., 1974; Cameron et al., 1982; Suzuki and Sugi, 1989), and will be discussed further in section 1.3.1. Additionally, there is precedent in studies using non-muscle cell types which demonstrate caveolae involvement in a number of cellular processes.

1.3 Involvement of Caveolae in Cellular Signalling

A growing body of evidence implicates caveolae in various cellular pathways.

Caveolae have been implicated in protein sorting to the apical PM involving the trans-Golgi network in epithelial cells (Dupree et al., 1993) and in protein shuttling across endothelial cells (trancytosis, Schnitzer et al., 1994, 1995a,b). In relation to trancytosis, caveolae have been shown to contain the proteins necessary for vesicle budding, docking and fusion (Schnitzer et al., 1995a,b). As well, the cholesterol binding drug, filipin, which flattens caveolae and disassembles its protein coat (Rothberg et al., 1992), inhibits the movement of insulin and albumin across cultured endothelial cells and the capillary permeability of albumin in rat lungs *in situ* (Schnitzer et al., 1994).

Others have looked at caveolae as sites for internalization of macromolecules (potocytosis, Montesano et al., 1982; Rothberg et al., 1990; Anderson et al., 1992). Specifically, the internalization of cholera and tetanus toxins (Montesano et al., 1982), and of folate (Rothberg et al., 1990; Anderson et al., 1992). With regard to the movement of folate, it is hypothesized that folate binds to its glycosyl phosphatidylinositol- (GPI-)anchored receptor, located either in caveolae or which moves to caveolae upon binding of folate, followed by the closing or detachment of the caveolae from the PM in order for the folate to be released from its receptor and internalized (Rothberg et al., 1990; Anderson et al., 1992).

The concept of caveolae as a site for cell signalling is being rapidly elaborated. Found in caveolae are numerous receptors, including the β -adrenergic,

muscarinic acetylcholine, bradykinin, angiotensin, and endothelin receptors (Chun et al., 1994; Lisanti et al., 1994a; de Weerd and Leeb-Lundberg, 1997; Ishizaka et al., 1998), numerous G proteins (Sargiacomo et al., 1993; Chang et al., 1994; Lisanti et al., 1994a,b; Li et al., 1995; de Weerd and Lundberg, 1997), and non-receptor tyrosine kinases (Sargiacoma et al., 1993; Lisanti et al., 1994a,b; Song et al., 1996a).

1.3.1 A Role for Caveolae in Calcium Handling

The final cellular function described for caveolae is Ca^{2+} handling. Using immunoelectron microscopy and immunofluorescence microscopy, Fujimoto and co-workers demonstrated the presence of the PM Ca^{2+} pump (Fujimoto, 1993) and an IP_3 receptor-like protein (Fujimoto et al., 1992, 1995) in caveolae from endothelial and epithelial cells, cultured fibroblasts, and smooth muscle cells (mouse ileum). Caveolae isolated from rat lung endothelium have also been shown to contain the PM Ca^{2+} pump and an IP_3 receptor (Schnitzer et al., 1995c).

As mentioned in an earlier section, the concept of caveolar involvement in smooth muscle Ca^{2+} handling has been postulated for some time. Popescu et al. (1974) demonstrated that in guinea pig taenia cecum, Ca^{2+} -oxalate precipitates could be found in caveolae, as well as in intracellular organelles (SR, nucleus, mitochondria), suggesting that caveolae may be one of the sources of Ca^{2+} for

contraction. The estimated amount of Ca^{2+} in caveolae appeared to be comparable to that required to initiate contraction, although similar estimations of Ca^{2+} content in SR indicated that only 20% would be necessary to initiate contraction (Popescu et al., 1974).

Further studies by Popescu (1977) suggested that the PM Ca^{2+} pump is also present in caveolae of guinea pig taenia cecum. The Ca^{2+} pumps were localized as electron dense granules of phosphate, Ca^{2+} , and citrate in electron micrographs. Enzymatic cleavage of ATP by the Ca^{2+} pump produced inorganic phosphate which was precipitated when Ca^{2+} and citrate were present on or near the Ca^{2+} pump (Popescu, 1977). These electron dense granules were found mainly associated with caveolae, although they were also found in non-caveolar regions of the PM. Popescu hypothesized that caveolae act as a source of Ca^{2+} for contraction, and in association with the SR, as a route for Ca^{2+} extrusion. He hypothesized that the frequent close association of caveolae and SR allows for Ca^{2+} taken up by the SR to be removed from the cell via an undetermined interaction between SR and caveolar membranes.

Further evidence that caveolae could be involved in Ca^{2+} handling in smooth muscle comes from an ultrastructural study in dog coronary artery (Suzuki and Sugi, 1989). Using the pyroantimonate method of localizing Ca^{2+} , they found that in relaxed tissues, the pyroantimonate precipitate containing Ca^{2+} was most prominent in the caveolar lumen. In contracted fibres, the precipitate was diffusely distributed

in the myoplasm, while the precipitate in the caveolar lumen was much less prominent. This suggests that at least in coronary artery, caveolae are acting as a source of activator Ca^{2+} .

In contrast with these findings, a study by Wheeler-Clark and Buja (1995) in canine coronary artery using $^{45}\text{Ca}^{2+}$ autoradiography and electron probe microanalysis, looked at the changes in Ca^{2+} in tissues fixed in control and high potassium medium in the presence and absence of nitrendipine. They showed that the Ca^{2+} required for contraction came from the PM region and localized the changes to the glycocalyx surrounding the smooth muscle cells. However, the data presented demonstrated some changes in Ca^{2+} associated with the region of the PM (including caveolae), although these were not significant. As well, few caveolae could be seen in contrast with the results of Suzuki and Sugi (1989) using the same tissue source. It is unclear why there is a discrepancy between these two studies.

Some have questioned the validity of the precipitation methods mentioned previously. Osmium, potassium, and antimony are present in the pyroantimonate precipitates and can potentially be the main component of some cell surface precipitates with Ca^{2+} present in trace amounts (Garfield and Somlyo, 1985). As well, there is the potential for translocation of Ca^{2+} or of the precipitates during the fixation process (Garfield and Somlyo, 1985). However, the results of Fujimoto (1993) demonstrating the PM Ca^{2+} pump in caveolae using immunoelectron microscopy would suggest that the localization of the PM Ca^{2+} pump to caveolae

using precipitating agents (Popescu, 1977) was not an artifact. Collectively, these results suggest a potential role for caveolae in Ca^{2+} handling. However, due to the limitations of the experimental techniques, other methods are required to elucidate the role of caveolae in Ca^{2+} handling.

1.4 Proteins Associated with Caveolae

As indicated from the list of pathways caveolae are involved in, numerous proteins are associated with caveolae. These proteins have been assigned to caveolae either by immunohistochemistry, immunoelectron microscopy, or by biochemical isolation of caveolae. This isolation of caveolae relies on detergent treatment of membranes, although more recently, detergent-free methods have been developed. In order to confirm that the membranes isolated are in fact caveolae, a marker of caveolae is needed. Caveolin, a 22kDa protein originally identified as a substrate for v-src tyrosine kinase (Glennay and Soppet, 1992), is located exclusively in caveolar membranes on the PM surface.

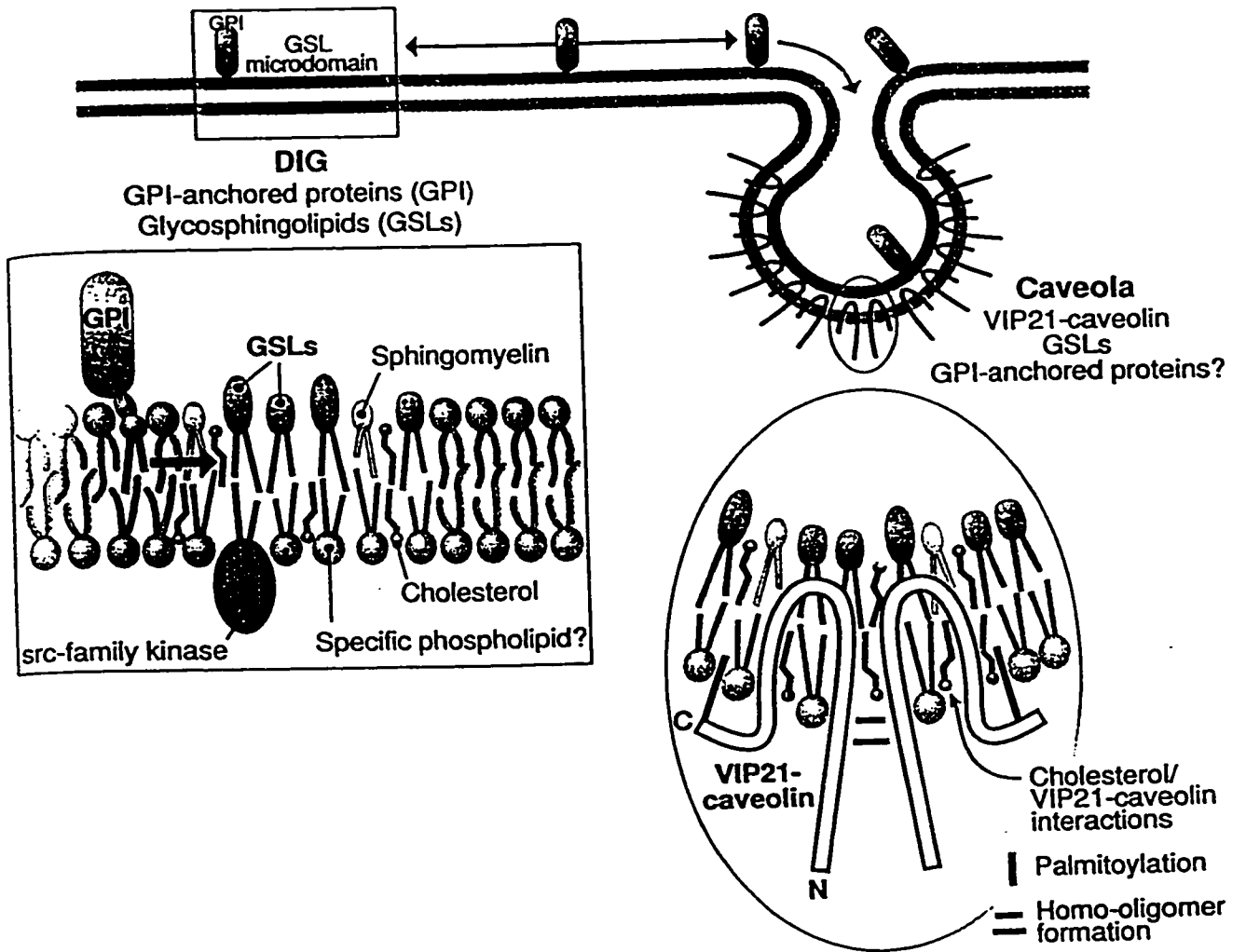
1.4.1 Caveolin

Scanning electron micrographs of endothelial cells show a striped bipolar surface structure (Peters et al., 1985), which has also been described by others as

a striated coat or electron dense threads. This coating has been identified, at least in part, as caveolin. Caveolae also contain a high density of cholesterol, sphingomyelin, and glycosphingolipids but are depleted of phospholipids (Brown and Rose, 1992; Sargiacoma et al., 1993; Feng and Kraus-Friedmann, 1993, Parton and Simons, 1995). It is the sphingolipids which render these membranes insoluble in detergent (Brown and London, 1997). Caveolin has a 30-40 amino acid hydrophobic region, proposed to form a β -sheet which forms a hairpin loop and acts as a membrane spanning region, resulting in both the N- and C-terminals being located on the cytoplasmic side of the caveolae (Glennay, 1992; Glennay and Soppet, 1992; Kurzchalia et al., 1992; Dupree et al., 1993; depicted in Figure 3). Isolation of caveolin (and other potential caveolar proteins) using detergent solubilization and separation by SDS-PAGE resulted in the detection of high molecular weight complexes (~200, 400, 600kDa) which were found to be caveolin oligomers (Monier et al., 1995). Further, Monier et al. (1995) demonstrated that *in vitro*, caveolin molecules formed these high molecular mass oligomers. Expression of caveolin in cells lacking endogenous caveolin results in the formation of caveolar structures, supporting a role of caveolin in the formation of caveolae (Fra et al., 1995).

It has been suggested that cholesterol plays a role in the association of caveolin with the plasma membrane. Cholesterol is apparently required to maintain the integrity of the striated coat on caveolae (Rothberg et al., 1992) since removal

Figure 3: Artist's depiction of caveolin insertion in caveolae membrane. Caveolin is inserted in the membrane with both the N- and C-terminals protruding into the cytosolic side of the caveolae. Note that caveolin does not cross into the luminal side of the caveolae. Figure also depicts the hypothesis of Parton and Simons (1995) that detergent-insoluble glycosphingolipid-enriched complexes (DIG's) enriched in GPI-linked proteins may move freely from non-caveolar membrane domains to caveolae. These movements may be triggered by agonist signalling *in vivo*, or under experimental conditions by antibody crosslinking or detergent treatment. This hypothesis is discussed further in Section 1.5.2. Reprinted with permission from: Science, Vol 269, R.G. Parton and K. Simons, Digging into caveolae, pp.1398-1399, Copyright 1995, American Association for the Advancement of Science.



of cholesterol with sterol binding drugs, such as filipin and nystatin, resulted in the flattening of the invagination and caused the coat to unravel. Furthermore, treatment of cells with cholesterol oxidase causes the redistribution of caveolin to the Golgi apparatus and ER (Smart et al., 1994). Caveolin binds to cholesterol in an artificial membrane system, and this interaction promotes the formation of protein oligomers (Murata et al., 1995).

1.4.2 Caveolin Isoforms

Caveolin-1, also referred to as VIP21 (Vesicular Integral-membrane Protein of 21kDa) in earlier reports, was the first caveolin isoform to be sequenced (Kurzchalia et al., 1992; Glenney and Soppet, 1992; Glenney, 1992). This 21kDa protein is palmitoylated (Dietzin et al., 1995) and appears on Western blots as two bands at 22 and 24 kDa. These two bands have subsequently been identified as two forms of caveolin-1, α and β , a result of two start sites on the mRNA, where the β isoform lacks the first 31 amino acids (Scherer et al., 1995). Caveolin-1 is expressed in many cell and tissue types, especially adipose tissue, smooth muscle, endothelial, and epithelial cells, and cultured fibroblasts (Glenney, 1989; Kurzchalia et al., 1992; Glenney and Soppet, 1992; Rothberg et al., 1992; Chang et al., 1994).

Caveolin-2 is a 20kDa protein which is 38% identical, and 58% homologous (conservative amino acid substitutions) to caveolin-1 (Scherer et al., 1996). It is

found in most cell types and tissues at low levels, with higher levels in smooth muscle, endothelial cells, fibroblasts and adipocytes (Scherer et al., 1997). Whereas caveolin-1 can exist as a high molecular mass oligomer when expressed in cells, caveolin-2, when overexpressed in transfected COS cells, exists mainly as a monomer or dimer (Scherer et al., 1996). Cells which express only caveolin-2 have no caveolae-like structures visible by electron microscopy (Scherer et al., 1997). When both caveolin-1 and caveolin-2 are coexpressed in a cell, caveolin-2 is associated with caveolin-1, as demonstrated by immunoprecipitation, and they are colocalized in caveolae (Scherer et al., 1997). As well, caveolin-1 and caveolin-2 are associated with high molecular mass hetero-oligomers.

Caveolin-3, also referred to as M-caveolin, is a muscle specific isoform of 18-20kDa which is 65% identical, and 85% homologous to caveolin-1 (Way and Parton, 1995; Tang et al., 1996). It is expressed only in skeletal, cardiac, and smooth muscle cells (Song et al., 1996b). Similar to caveolin-1, caveolin-3 forms high molecular mass homo-oligomers (Tang et al., 1996). When caveolin-1 and -3 are coexpressed in cell lines, they are colocalized to discrete points at the cell surface thought to be caveolae (Way and Parton, 1995; Tang et al., 1996).

The expression of the various caveolin isoforms can be altered in cultured cells. Differentiation of 3T3-L1 fibroblasts to adipocytes increases synthesis of both caveolin-1 and caveolin-2 (Scherer et al., 1996). Differentiation of C2C12 myoblasts to myotubes up-regulates caveolin-3 synthesis (Tang et al. 1996).

Interestingly, transformation of NIH 3T3 cells resulted in the down-regulation of caveolin-1, while the expression of caveolin-2 remained unchanged (Scherer et al., 1997). The functional significance of these changes and their relevance to cell function *in vivo* have not been determined.

1.4.3 Caveolin and its Role as a Scaffolding Protein

More recently, the role of caveolin in formation of caveolae, and specifically its role in sequestering into and maintaining other proteins in caveolae has been further investigated. The self-association of caveolin has been further studied, as well as evidence that caveolin can specifically interact with other proteins localized to caveolae. Evidence of an interaction between caveolin and another protein came first from a study by Chun et al. (1994) which demonstrated that caveolin could be coimmunoprecipitated with the endothelin receptor (ET_A). The list of proteins which can specifically interact with caveolin has grown to include G protein α subunits (Li et al., 1995), c-Src tyrosine kinase, Ras (Li et al., 1996), eNOS (Feron et al., 1996) and nNOS (Venema et al., 1997). Amino acids 82-101 of caveolin-1 form a region termed a 'scaffolding domain' (Li et al., 1996) which binds to a motif on other proteins (as well as caveolin itself) rich in aromatic amino acid residues. The consensus sequence for this caveolin binding domain is: ArXXArXXXXArXAr, where "Ar" is any aromatic amino acid (phenylalanine, tyrosine,

tryptophan) and "X" is any amino acid. This interaction is responsible for the self-association of caveolin into high molecular mass oligomers, and the association of caveolin with other proteins localized to caveolae. A similar scaffolding domain is also present in caveolin-3, and is highly conserved with six amino acid changes, all of which are conservative substitutions (Tang et al., 1996). In caveolin-2, the region corresponding to the scaffolding domain is less well conserved, with twelve amino acid changes, with many non-conservative substitutions (Scherer et al., 1996). Couet et al. (1997) investigated the sequences of various proteins localized to caveolae and provided a partial list demonstrating that all contained at least part of the caveolin binding motif. This list includes the IP₃-sensitive Ca²⁺ channel, numerous G protein-coupled receptors which includes the endothelin A receptor, G protein α subunits, protein kinase C α , Src-like kinases, and the endothelial NOS.

Caveolin may not only act simply as a scaffolding protein, but may also have another physiological role. Specifically, interactions with caveolin-1 can inhibit the activity of various proteins. For example, the GTPase activity of G protein α subunits is inhibited by caveolin-1 (Li et al., 1995) and caveolin-3 (Tang et al., 1996), while it is stimulated by caveolin-2 (Scherer et al., 1996). The interaction of caveolin-1 with eNOS (Ju et al., 1997; Michel et al., 1997a,b) inhibits synthase activity. This inhibition has been localized to the scaffolding peptide region of caveolin-1 as well as another C-terminal region (Garcia-Cardena et al., 1997). The corresponding sequences in caveolin-3 have also been shown to inhibit eNOS

activity (Garcia-Cardena et al., 1997) in addition to iNOS and nNOS (Garcia-Cardena et al., 1997; Venema et al., 1997), while the sequence in caveolin-2 which corresponds to the scaffolding region had no effect on synthase activity of any NOS isoforms (Garcia-Cardena et al., 1997). The functional significance of this difference between caveolin isoforms, especially in cell types such as smooth muscle cells which express all three isoforms, still needs to be investigated.

Immunoprecipitation of caveolin with its associated proteins has proved to be a useful method for circumventing the problems either of isolating a particular subset of the PM or of proteins resorting in the membranes due to the isolation procedure. However, while some proteins directly interact with caveolin and others contain the motif necessary for interacting with the scaffolding domain, it has not been directly demonstrated that these other proteins directly interact with caveolin. The binding motif must be located on the protein in a region accessible to caveolin in order for the interaction to occur. Additionally, some proteins localized to caveolae but lacking the necessary binding motif may instead be interacting with another caveolar protein. Thus while immunoprecipitation can confirm the localization of a protein in caveolae, an inability to coimmunoprecipitate with caveolin does not necessarily prove that the protein is not localized to caveolae.

1.5 Biochemical Isolation of Caveolae

1.5.1 Detergent Extraction Using Triton X-100

A study investigating the proteins associated with the membrane cytoskeleton in murine tumour and lymphoid cells demonstrated that treatment with detergent did not completely dissolve the PM. Instead, there was a detergent-insoluble membrane fraction which could be pelleted by ultracentrifugation at 100,000g (Mescher et al., 1981). This fraction contained actin as well as other unidentified proteins, the molecular weights of which did not correspond with any known cytoskeleton components. In addition, these investigators demonstrated that 5'-nucleotidase, a marker for PM membranes, was also enriched in this fraction, The enzyme 5'-nucleotidase is a member of a family of proteins located on the cell surface and associated with the PM via a C-terminal glycosyl phosphatidylinositol (GPI). They do not have any membrane spanning region anchoring them to the membrane, and thus do not have any direct connection with the inner membrane or cytosol.

Investigators studying protein sorting in epithelial cells discovered that GPI-anchored proteins were resistant to detergent solubilization during transport to the apical surface (Brown and Rose, 1992). Previous knowledge that various GPI-linked proteins could be found clustered in caveolae as determined by immunohistochemistry and immunoelectron microscopy (e.g. Rothberg et al., 1990), led Sargiacoma et al. (1993) and many other groups to investigate whether other

proteins were also clustered in these detergent-insoluble membrane complexes. The discovery that caveolin was enriched in these complexes (Sargiacoma et al., 1993) led to the conclusion that caveolar membranes comprised a major component of these detergent-insoluble complexes. In addition to caveolin and GPI-linked proteins, annexin II, c-Yes (a non-receptor tyrosine kinase), GTP binding proteins, and various α subunits of heterotrimeric G proteins (s, q/11, i2, i3) were also localized to these complexes derived from an epithelial cell line (Sargiacoma et al., 1993). Others have confirmed these findings and added to this list: annexin V and VI (Parkin et al., 1996), the β subunit of heterotrimeric G proteins (Chang et al., 1994), the PM Ca^{2+} pump (Schnitzer et al., 1995c), and an IP_3 -like receptor (Feng and Kraus-Friedmann, 1993; Schnitzer et al., 1995c; Parkin et al., 1996).

To this point, three biochemical studies have been conducted in smooth muscle; one using whole tissue (Chang et al., 1994), and two using cultured smooth muscle cells (de Weerd and Leeb-Lundberg, 1997; Ishizaka et al., 1998). Chang et al. (1994), using chicken gizzard, reported the presence of GPI-linked proteins, α (s, i1, i2, i3) and β G protein subunits, and a GTP binding protein (Rap-1) in caveolin-enriched, detergent-resistant membranes. de Weerd and Leeb-Lundberg (1997) demonstrated the α_i and α_q G protein subunits in detergent-resistant, caveolin-enriched membranes isolated from cultured DDT₁ MF-2 smooth muscle cells. They confirmed these results using immunoprecipitation experiments, where α_i and α_q coimmunoprecipitated with caveolin, and also using an alternate

detergent-free caveolae isolation method (OptiPrep, discussed further in Section 1.5.3). The amounts of α_i and α_q in caveolae increased when the B2 bradykinin receptors were occupied by an agonist (bradykinin) but not an antagonist. The alternate caveolae isolation method demonstrated the sequestration of B2 bradykinin receptors in caveolae following agonist stimulation. Further, these agonist-receptor complexes were resistant to acid washing, suggesting that the receptors located in caveolae were closed off from the surrounding extracellular space. Using cultured rat aortic smooth muscle cells, Ishizaka et al. (1998) demonstrated that the angiotensin II Type 1 receptor (AT_1) was sequestered in caveolin-enriched membrane fractions following agonist stimulation. Caveolin-enriched membranes were prepared by the Na_2CO_3 treatment method described further in Section 1.5.3. Although all three caveolin isoforms could be detected in the cells, as determined by Northern blot analysis, only caveolin-1 was affected by agonist stimulation of the AT_1 receptor. Agonist stimulation resulted in a transient decrease in caveolin-1 protein levels, an increase in caveolin-1 mRNA levels, and an increase in caveolin-1 biosynthesis. Caveolin-1 coimmunoprecipitated with the AT_1 receptor following agonist stimulation, indicating a direct interaction between caveolin-1 and the AT_1 receptor. No data was presented on the relative amounts of the caveolin isoforms. Although receptors and G protein subunits have been localized to caveolin-enriched membranes, the localization of the PM Ca^{2+} pump, and IP_3 -like receptor to smooth muscle caveolae has been demonstrated only by

immunolectron microscopy (Fujimoto et al., 1992; Fujimoto, 1993).

1.5.2 Is Detergent Insolubility Equal to Caveolar Localization?

Some have questioned whether the detergent-insoluble complexes isolated from various cells and tissues are caveolae. Arguments against this are based on both histochemical and biochemical methods. In a study by Mayor et al. (1994) they discovered that if various cell lines were fixed with paraformaldehyde and glutaraldehyde prior to addition of the secondary antibody, various GPI-linked proteins were diffusely distributed on the cell membrane. If the secondary antibody was added prior to fixation, a punctate pattern of fluorescence was observed. These clusters of GPI-linked proteins were shown to colocalize with caveolin. These results of clustering of GPI-linked proteins were confirmed by Fujimoto (1996) who demonstrated by immunolectron microscopy that these clusters were located in caveolae. Further, in a study by Nomura et al. (1997), the G protein α_2 subunit, previously localized to caveolar membranes by detergent treatment (Sargiacoma et al., 1993; Chang et al., 1994), was also found to have a diffuse distribution if the cells were fixed prior to addition of the secondary antibody. However, quantification of the distribution by immunolectron microscopy showed that the labelling density in caveolae was slightly higher (2 times) in caveolae than in the rest of the PM. Another study looking at distribution of GPI-linked proteins

(Mayor and Maxfield, 1995) found that whole cells treated with detergent prior to localization redistributed the fluorescence into clusters. This detergent treatment did not affect the overall intensity of the signal, suggesting that the GPI-linked proteins did exist in a detergent-insoluble membrane domain, although it was not possible to determine by their methodology if these domains were caveolae.

Fujimoto (1996) demonstrated that sphingomyelin and glycosphingolipids were diffusely distributed on the PM if fixed prior to detection with the secondary antibody, but these were found in clusters if fixed after the addition of the secondary antibody. Immunoelectron microscopy localized these clusters to caveolae. In a cell line (Caco-2) lacking any detectable caveolin, as determined by immunoprecipitation, Western blotting and Northern blotting, and by the fact that no caveolae-like structures were detectable, a detergent-insoluble complex could be isolated which contained GPI-linked proteins, as well as the non-receptor tyrosine kinase, Src, and a G protein α , subunit (Mirre et al., 1996). The isolated membranes were heterogeneous in size, 100-400nm, and too large to be caveolae.

Collectively, these results suggest that while proteins such as GPI-linked proteins may exist in detergent-insoluble domains, these domains are not necessarily caveolae. Some groups have hypothesized that these detergent-insoluble complexes, enriched in glycosphingolipids, and containing GPI-linked proteins exist as "rafts" in the PM, able to move freely within the membrane (Parton and Simons, 1995; Kurzchalia et al., 1995; depicted in Figure 3). Caveolae are one

part of the PM which contain these "rafts". Thus GPI-linked proteins would be diffusely distributed on the PM and could move to clusters due to experimental conditions or to specific protein-protein interactions under physiological conditions. An example would be the clustering of the folate receptor in caveolae when bound to folate (Rothberg et al., 1990; Anderson et al., 1992). The caution from these results is that detergent insolubility does not equate with caveolar localization. Therefore other experimental methods or methods of isolation are necessary to confirm the proteins associated with caveolae.

1.5.3 Alternative Methods for Isolating Caveolae

A study of endothelial cells (Schnitzer et al., 1995b) used cationic colloidal silica to coat the luminal endothelial cell PM from rat lung. These membranes could be isolated by centrifugation to give a membrane enriched in endothelial cell surface markers. The silica coating did not enter caveolae, probably due to the large size of the particles (>50nm, Chaney and Jacobson, 1983), and so these membranes could be separated from the rest of the PM by homogenization in the presence of Triton X-100, followed by sucrose density gradient centrifugation. Using this methodology, the caveolar membranes were enriched in caveolin, the PM Ca²⁺ pump, and an IP₃-like receptor, but did not contain GPI-linked proteins or β-actin. Comparison of these caveolar membranes with membranes isolated by

detergent solubilization without prior coating of silica revealed differences in their size and shape by electron microscopy. The caveolar membranes were a homogeneous population of vesicles <100nm, while the detergent-extracted membranes were a heterogeneous population which included membranes resembling caveolae in shape and size, as well as larger spherical vesicles and linear membrane sheets. In some instances, the caveolar membranes were attached to the larger vesicles. Immunoelectron microscopy revealed that GPI-linked proteins were localized to the membrane domains adjacent to the caveolae, suggesting that they may cluster in the neck region of the caveolae. The results suggest that there are two populations of detergent-insoluble membranes, consistent with the model by Parton and Simons (1995), with one being caveolae, enriched in caveolin and other proteins, and the other not containing caveolin, but enriched in GPI-linked proteins. While this method is useful for isolation of caveolae from endothelial cells or cell culture monolayers, it would not be practical for studies with whole tissues where access to the PM by the colloidal silica particles is limited.

A method which can be used for whole tissues is that employed by Chang et al. (1994). Using differential centrifugation a membrane fraction was isolated from chicken gizzard smooth muscle, which the authors claimed to be a caveolar membrane fraction. This fraction was then treated with Triton X-100 to produce a detergent-insoluble membrane fraction, characterized to be enriched in caveolin,

GPI-linked proteins, and other cell-signalling proteins, as discussed above. The fraction also contained the G protein β subunit, although it was not enriched, compared to the total PM fraction. Immunoelectron microscopy of the membrane fraction prior to detergent treatment demonstrated that the β subunit was present in cup-shaped membranes similar in size to caveolae. Based on the electron micrographs which showed that the membrane fraction contained similar sized membranes as seen in the detergent-treated membrane fraction, the authors concluded that their procedure of differential centrifugation produced a fraction enriched in caveolar membranes. However, the electron micrographs show that the membrane fraction is heterogeneous in size, with some small dense-cored fragments and others which appear larger than caveolae ($>100\text{nm}$). As well, the micrographs of the detergent-insoluble membrane fraction also contain membranes of various sizes, with some larger than caveolae ($>100\text{nm}$), although they stain positively for caveolin. It is unclear whether the membrane fraction obtained by differential centrifugation is caveolae, or if it contains other membrane domains of comparable buoyant density, similar to those reported by Schnitzer et al. (1995b).

A detergent-free method of isolation of caveolae from cultured cells (Smart et al., 1995) may also be applicable to whole tissues. Similar to the method of Chang et al. (1994), this method also relies on the buoyant density of caveolae, but employs the use of sonication to disrupt the membrane and floatation on OptiPrep (GIBCO) density gradients. Immunoblots of the resultant caveolin-enriched

membrane fraction indicated that GPI-linked proteins, α and β G protein subunits, protein kinase C_{α} , and actin were all present, consistent with previous results from detergent-treated membranes. However, the recovery of total caveolin using this method was only 1-5%, which may make this method impractical when there are limits on tissue availability.

A final detergent-free method employed in cultured cells but which can be adapted to whole tissues is that proposed by Song et al. (1996a) which substituted a sodium carbonate buffer (pH 11.0) and a sonication step to disrupt the membranes, prior to floatation on a discontinuous sucrose gradient. Using this method, the caveolin-enriched membrane fraction was shown to be enriched in Ras, c-Src, and the G protein α_{12} and β subunits, but to exclude GPI-linked proteins. Further they confirmed these results by co-eluting these proteins with a polyhistidine tagged form of caveolin which was expressed in the MDCK cells used. The recovery of caveolin was reported to be 90-95% of total caveolin. While this method appears to purify caveolar membranes and excludes most other cellular proteins, the authors did not provide evidence that other membrane domains such as ER were also excluded. The sucrose gradient used would allow for ER membranes to be located at the same interface (5/35%) as the caveolar membranes. At least in smooth muscle, the SR membranes are usually isolated at the 25/35% interface while PM is located at the 14/25% interface (*e.g.* Ahmad et al., 1987). Thus while this method may be useful in non-muscle cells, it may need

modifications to be applicable to smooth muscle tissues or cells.

1.6 Summary of Proteins to be Investigated in Smooth Muscle Caveolae

Caveolin-1, as discussed in previous sections, is expressed in most cell types, including smooth muscle (Chang et al., 1994; de Weerd and Leeb-Lundberg, 1997; Ishizaka et al., 1998) with two protein bands of 22 and 24 kDa, corresponding to alternate start sites for translation (Scherer et al., 1995). More recently, it has been shown that other caveolin isoforms (caveolin-2 and -3) are also present in smooth muscle (Ishizaka et al., 1998), although these isoforms were not investigated here.

The L-type Ca^{2+} channel is composed of four subunits in smooth muscle, termed α_1 , α_2 , β , and δ (reviewed in Hofmann et al., 1994). The α_1 chain forms the actual Ca^{2+} channel, and is the site where the various inhibitors bind, such as dihydropyridines (Hofmann et al., 1994). In smooth muscle, the α_1 chain is an alternatively spliced product of the cardiac isoform, termed CaCh2b (Biel et al., 1990; Hofmann et al., 1994). It encodes a protein of 2166 amino acids with a predicted molecular mass of 240kDa (Biel et al., 1990).

Calreticulin is an SR/ER resident Ca^{2+} binding protein, which is expressed in all cell types (excluding erythrocytes) including smooth muscle (reviewed in Krause and Michalak, 1997). It is a protein of 417 amino acids which has an N-

terminal signal sequence which is cleaved during translation to form a protein of 400 amino acids, with a predicted molecular mass of 46kDa (Rokeach et al., 1991; Krause and Michalak, 1997). However, separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) typically reveals a protein of approximately 60kDa (McCauliffe et al., 1990; Milner et al., 1991).

Calsequestrin in smooth muscle is predominantly the cardiac isoform. It is a protein of 410 amino acids, with the first 19 amino acids cleaved during translation (Scott et al., 1988). The predicted size is 45kDa, but it has two glycosylation sites, which can alter the molecular mass by SDS-PAGE (Scott et al., 1988). The molecular mass has been estimated to be 55kDa in cardiac muscle (Scott et al., 1988).

Connexins are a family of proteins which form gap junctions on the PM of cells, allowing communication between cells via diffusion of ions or small molecules between them. Connexin-43 is a member of the connexin family, and is expressed in various smooth muscle tissues including canine intestinal circular muscle (Li et al., 1993) and rat uterine smooth muscle (Lang et al., 1991). This isoform is a protein of 382 amino acids with a molecular mass of 43kDa (Lang et al., 1991).

The IP₃-induced Ca²⁺ release channel is a family of 3 genes, with the Type 1 IP₃ receptor the predominant isoform in smooth muscle (De Smedt et al., 1994). It encodes a protein with a molecular mass of approximately 220-230kDa (Chadwick et al., 1990)

The neuronal form of nitric oxide synthase (nNOS) is a protein of 1429 amino acids with a molecular mass of 160kDa in rat cerebellum (Bredt et al., 1991). This membrane-associated nNOS isoform has also been demonstrated in skeletal muscle, although due to alternate splicing, it has a 34 amino acid insert, resulting in a protein of approximately 164kDa (Silvagno et al., 1996).

The PM Ca²⁺ pump in smooth muscle is the product of the PMCA1 gene, predominantly the PMCA1b isoform (De Jaegere et al., 1990; Khan and Grover, 1991). Rabbit stomach and aortic smooth muscle expresses predominantly the PMCA1b isoform (Khan and Grover, 1991) while pig stomach smooth muscle expresses only the PMCA1b isoform (De Jaegere et al., 1990). This isoform is a protein of 1220 amino acids, which gives rise to a band of approximately 135kDa when separated by SDS-PAGE (De Jaegere et al., 1990; Khan and Grover, 1991).

The smooth muscle SR Ca²⁺ pump is an alternatively spliced form of the cardiac SR Ca²⁺ pump, termed SERCA2b (reviewed in Missiaen et al., 1991). The protein has a molecular mass of 115kDa, which is slightly larger than the cardiac isoform (110kDa, Spencer et al., 1991).

Chapter 2: EXPERIMENTAL DESIGN AND RATIONALE

As stated earlier (Section 1.2.3), results obtained in contractility experiments in canine bronchial smooth muscle suggested that there was a source of activator Ca^{2+} in a nominally Ca^{2+} -free medium which was located somewhere in the extracellular space. This source of Ca^{2+} was protected from the surrounding extracellular space since $100\mu\text{M}$ EGTA was unable to sequester it. The Ca^{2+} from this source could enter the cell via L-type Ca^{2+} channels since its uptake could be blocked with the use of nifedipine. A functional SR was also required. However, the exact location of this Ca^{2+} store is unknown. Based on the growing body of evidence summarized previously, we hypothesize that the PM caveolae may act as this Ca^{2+} store.

In order for caveolae to act as a source of Ca^{2+} , a number of proteins have to be located within this region. L-type Ca^{2+} channels must be localized here to allow Ca^{2+} to enter the cell. Similarly, a PM Ca^{2+} pump is also necessary to remove Ca^{2+} from the cytosol to the lumen of the caveolae. Also, some type of Ca^{2+} binding protein is required in order to sequester the stored Ca^{2+} and limit its loss through the opening of the caveolae at its neck region to the surrounding extracellular space.

In order to test whether caveolae contain the necessary proteins for Ca^{2+} handling, an isolation procedure is needed. Detergent solubilization of non-caveolar membranes was chosen based on the numerous results from other cell types studying a role for caveolae in other cell processes. Due to the difficulties in obtaining large quantities of bronchial smooth muscle devoid of any contaminating epithelial cells (which are an even greater source of caveolar membranes), it was decided to devise a method of isolation using canine tracheal smooth muscle. Trachea provides a larger mass of smooth muscle, and also can be isolated free from any contaminating epithelial cells.

More recent studies have demonstrated that detergent isolation may provide erroneous results (Mayor et al., 1994; Mayor and Maxfield, 1995; Schnitzer et al., 1995b; Fujimoto, 1996; Song et al., 1996a). This is because some proteins isolated in association with caveolae by the detergent method may be artifacts of the treatment as described above. These may not be located within caveolae but instead in other membrane domains, which upon detergent solubilization, are relocated into the caveolar membranes. Based on available literature, the sodium carbonate treatment of membranes (Song et al., 1996a) was chosen as an alternative method of isolating caveolae. Additionally, it was decided to take advantage of the specific interactions of caveolin with other caveolar proteins and employ immunoprecipitation experiments under non-denaturing conditions (Feron et al., 1996) to test for the specific interaction of caveolin with the various Ca^{2+}

handling proteins postulated to be in caveolae.

2.1 Specific Goals:

1. To develop a suitable biochemical method for the isolation of caveolae-enriched preparations from airway smooth muscle (canine trachea) using detergent solubilization.

2. To characterize the detergent solubilized fraction biochemically, in order to identify the proteins present, specifically those required for smooth muscle Ca^{2+} handling. The techniques used include: enzyme assays (5'-nucleotidase and Mg^{2+} -ATPase); Western blot analysis with selected antibodies, to demonstrate the presence of L-type Ca^{2+} channels, the PM Ca^{2+} pump, calsequestrin, calreticulin, and nNOS; and radioligand binding using [^3H]PN200-110, a selective L-type Ca^{2+} channel antagonist, to confirm the presence of L-type Ca^{2+} channels.

3. To confirm the localization of proteins in caveolae:
 - i.- Using the technique of immunoprecipitation of caveolin under non-denaturing conditions to demonstrate which proteins interact directly with caveolae

ii.- Using the technique of immunocytochemistry of tracheal smooth muscle cryosections to localize these proteins to caveolae

4. i.- Develop an alternative isolation procedure for caveolae from canine intestinal smooth muscle: Na_2CO_3 treatment of microsomes, followed by sonication, and separation on a discontinuous sucrose gradient.

ii.- Compare the results from the two isolation procedures (detergent treatment and Na_2CO_3 treatment) in intestinal smooth muscle to confirm the co-localization of proteins with caveolae.

Chapter 3: METHODS

3.1 Animal Handling

Mongrel dogs (20-40 kg) of either sex were euthanized by sodium pentobarbital overdose (100mg/kg body weight). All procedures were approved by the McMaster University Animal Care Committee. The trachea was removed and immediately placed in ice cold Ringers solution except for tissues used for histochemistry experiments, which were placed in 1% paraformaldehyde in 0.1mM phosphate buffer (pH7.4). The small intestine was removed and immediately placed in ice cold sucrose magnesium MOPS buffer (SMB, 250mM sucrose, 10mM MgCl₂, 25mM MOPS (3-[N-Morpholino] propanesulfonic acid), pH7.4).

3.2 Preparation of Tissues for Membrane Fractionation

The tracheal smooth muscle was dissected from the cartilage and epithelium, and then cleaned of any connective tissue and blood vessels using fine scissors and forceps under a dissecting microscope. Once cleaned, the muscle was placed into ice cold SMB for 5-10 min, blotted dry, weighed, and frozen at -20°C until used

for membrane preparation.

For the isolation of small intestine circular smooth muscle, approximately 15cm pieces were cut and cleaned of fat, connective tissue and mesenteric arcade. The pieces were opened along the mesenteric line and placed mucosal side down on a dissecting dish on ice. The longitudinal muscle was carefully peeled off with a pair of forceps. The circular muscle layer was then peeled off and placed in ice cold SMB. The muscle was then blotted dry, weighed, and frozen at -20°C until used for membrane preparation.

3.3 Preparation of Caveolar Membranes from Tracheal Smooth Muscle Using Detergent Treatment

Microsomal membranes, enriched in PM- and SR-derived microsomes were prepared using differential centrifugation according to the method of Grover et al. (1980). All steps were carried out at 4°C . Briefly, canine tracheal smooth muscle from approximately 6-8 mongrel dogs was pooled and minced finely with scissors in a small volume of SMB containing 1mM phenylmethylsulfonyl fluoride (PMSF), homogenized for 10 sec (Polytron PT20 homogenizer, approximately 15,000 rpm) at a tissue:volume ratio of 1g:10ml, and centrifuged at 1,000g (10min). The resulting post-nuclear supernatant (PNS) was centrifuged at 10,000g (10min) to remove mitochondria (MIT I) and the resulting supernatant centrifuged at 100,000g

(45min). The pellet (MIC I) was resuspended in SMB and centrifuged at 10,000g (10min) to pellet further contaminating mitochondria (MIT II). The supernatant (MIC II) is enriched in PM and SR membranes. MIC II was divided in half with one half serving as control, the other treated with 1% Triton X-100 in SMB (v/v) for 1hr at 4°C. The membranes were recentrifuged at 100,000g (45min) with the resultant pellets designated M3T for the Triton X-100 resistant membranes and M3C for the control membranes.

3.4 Preparation of Caveolar Membranes from Intestinal Smooth Muscle Using Detergent or Sodium Carbonate Treatment

Microsomal membranes were prepared according to the method of Ahmad et al. (1987). All steps were carried out at 4°C. Briefly, circular muscle from one dog was finely minced with scissors in a small volume of SMB. The tissue was homogenized for 3 x 7sec (Polytron PT20 homogenizer, approximately 15,000 rpm) at a tissue:volume ratio of 1g:10ml, and centrifuged at 1,000g. The resultant PNS was centrifuged at 10,000g to remove mitochondria and synaptosomes (MIT I). The supernatant was then centrifuged at 170,000g for 60min. The resultant pellet was either resuspended in SMB for preparation of detergent-treated membranes (according to the method used for trachea, above) or resuspended in 500mM Na₂CO₃, pH 11.0 with a final protein concentration of 1.5-2.5mg/ml.

The preparation of sodium carbonate-treated membranes used a modification of the methods of Song et al. (1996a) and Chang et al. (1994). The MIC I membranes were resuspended in sodium carbonate, sonicated 3 x 20sec, then adjusted to 250mM Na₂CO₃ by addition of an equal volume of SMB (final pH 11.0). The membranes were then layered on top of a discontinuous sucrose gradient (14, 25, 35, 48% w/w). Each sucrose concentration was prepared in a solution containing 250mM Na₂CO₃, 10mM MgCl₂, 25mM MOPS (3-[N-Morpholino] propanesulfonic acid), pH 11.0. The gradient tubes were centrifuged in a swinging bucket (SW40) rotor at 30,000 rpm for 100 min. Various fractions were collected at the sucrose interfaces as well as the regions between according to the scheme shown in Figure 4.

3.5 Enzyme Assays (5'-nucleotidase and Mg²⁺-ATPase)

The specific activities of 5'-nucleotidase and Mg²⁺-ATPase (markers for smooth muscle PM) were determined spectrophotometrically based on the amount of inorganic phosphate liberated from 5'-AMP over 60 min (for 5'-nucleotidase) or 5'-ATP over 15 min (for Mg²⁺-ATPase) on all membrane fractions (Matlib et al., 1979). Duplicate samples of membranes (1-20µg/tube) were incubated at 37°C in a medium containing 50mM imidazole (pH 7.2) and 10mM MgCl₂. The reaction was started with the addition of 5mM Na-AMP (for 5'-nucleotidase) or 5mM Na₂-ATP (for

Mg²⁺-ATPase) to a final reaction volume of 200µl, and terminated by the addition of 50µl of ice-cold 25% TCA (trichloroacetic acid). The samples were immediately centrifuged at 3000g for 15min on a desktop centrifuge at 4°C to remove precipitated proteins and 100µl of the resultant supernatant was removed to a 96 well plate for determination of inorganic phosphate according to the method of Taussky and Shorr (1953). To each sample 100µl of 0.3M FeSO₄/1% NH₃·molybdate·H₂SO₄ was added and incubated for 10min prior to measuring the absorbance at 595nm using a Titertek Multiskan PLUS MK II photometer. Absorbance was converted to µmol inorganic phosphate by measuring the absorbance at 595nm of a series of phosphate standards (10-100µmol phosphate in 100µl water, measured in 10µl increments) treated the same as the experimental samples.

3.6 Protein Determination

Protein was determined by using colorimetric assays based on the methods of Lowry et al. (1951) or Bradford (1976) using bovine serum albumin as standard.

Figure 4: Scheme for preparation of membrane fractions from canine intestinal smooth muscle. Each step was carried out at 4°C. Abbreviations are the same as used in the methods section.

3.7 [³H]PN200-110 Binding

[³H]PN200-110 binding was carried out on the various membrane fractions in 1.5ml Eppendorf tubes at 25°C in 50mM Tris-HCl, pH 7.4 with 100nM [³H]PN200-110 for 60 min. The reaction was started by addition of 5-50µg of protein and terminated by placing the tubes on ice. The tubes were then immediately centrifuged at 10,000g for 60 min at 4°C. The supernatant was removed by aspiration and the bottom of the Eppendorf tube containing the pelleted proteins cut off and placed in a scintillation vial, 4.5ml of Beckman ReadySafe scintillation fluid added, and counted for ³H in a Beckman (Model LS6800) scintillation counter. Binding was done in reduced lighting by covering the water bath with a cardboard box. Nonspecific binding was defined as the [³H]PN200-110 binding in the presence of 1µM nitrendipine. Specific binding was defined as total [³H]PN200-110 binding minus nonspecific binding.

3.8 Electrophoresis and Immunoblotting

Isolated membrane fractions were first concentrated either by drying under vacuum or by precipitating with 5% TCA, centrifuging for 20min at 10,000g on a desktop centrifuge at 4°C, and then aspirating the supernatant. The concentrated proteins were then resuspended in Laemmli sample buffer containing 0.0625M Tris-

HCl (pH 6.8), 3% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue, and boiled for 5min prior to separation by SDS-PAGE according to the method of Laemmli (1970). The 1.5mm or 0.75mm thick mini-gels (BioRad) consisted of a 5% stacking gel (0.125mM Tris-HCl (pH6.8), 1% SDS) and 7.5% or 12% separating gel (0.375mM Tris-HCl (pH8.8), 1% SDS). After electrophoresis, the proteins were transferred overnight onto 0.45 μ m nitrocellulose at 20V according to the method of Towbin et al. (1979).

For immunoblotting, all incubations and washes were done at room temperature with constant shaking. The nitrocellulose was first incubated with 5% milk powder in TTBS (20mM Tris-HCl (pH7.5), 500mM NaCl, 0.05% Tween-20) for 1 hour to block nonspecific protein binding prior to incubation with the primary antibodies for 1 hour diluted in the same solution (TTBS + 5% milk powder). The blots were washed for 30 min with 3 changes of TTBS prior to 1 hour incubation with the horse radish peroxidase (HRPO)-linked secondary antibodies (diluted in TTBS + 5% milk powder). The blots were washed for 30 min with 3 changes of TTBS and for 10 min with 2 changes of TBS (20mM Tris-HCl (pH7.5), 500mM NaCl; 2 changes) prior to immunodetection using enhanced chemiluminescence (ECL, Amersham) according to the manufacturer's directions. Proteins were detected by exposing the immunoblots to ECL Hyperfilm (Amersham) for various time periods (10 sec to 30 min).

3.9 Immunoprecipitation

Immunoprecipitation experiments were conducted using MIC II membranes under non-denaturing conditions, according to the method of Feron et al. (1996). All steps were carried out at 4°C. Briefly, approximately 200-400µg protein was incubated in a 4-fold dilution of CHAPS buffer (50mM Tris-HCl, pH7.4, 20mM CHAPS, 125mM NaCl, 2mM DTT, 0.1mM EGTA (ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid), 1mM PMSF) for 30min with constant agitation. Anti-caveolin antibody (4µg) was added and incubated for 60min with constant agitation. The solution was centrifuged at 10,000g for 30 min on a desktop centrifuge to pellet any insoluble membranes. The supernatant was removed to a new 1.5ml Eppendorf tube, and 50µl of a 50% slurry of Protein A Sepharose beads (in CHAPS buffer) was added. The solution was vortexed and incubated with constant agitation for 60min. A sample was removed (20µl) to serve as positive control for the presence of caveolin or associated proteins. The beads were then pelleted (10,000g, 30sec), the supernatant aspirated and the pellet then washed 6 times with a small volume (150µl) of CHAPS buffer. The beads were pelleted and the supernatant aspirated after each wash. After the final wash the beads were pelleted (10,000g, 4min) and all of the supernatant was removed. The samples were prepared for immunoblotting by adding 50µl Laemmli buffer to the pelleted beads, boiling for 5min, briefly centrifuging, and the supernatant loaded onto a gel.

3.10 Immunocytochemistry

Preparation of cryosections and immunohistochemistry were performed according to the methods of Salapatek et al. (1998b).

3.10.1 Preparation of Cryosections

The tracheae from euthanized dogs were removed and placed in 0.1M phosphate buffer (pH7.4) containing 4% paraformaldehyde for 3 hours at room temperature. The fixed tissues were washed with 0.1M phosphate buffer and then placed in 30% sucrose in 0.1M phosphate buffer for cryoprotection overnight at 4°C. Small pieces of tissue were sectioned at 10µm thickness in a cryostat (Leitz 1720 digital) and collected on glass slides coated with gelatin.

3.10.2 Histochemical Staining

The slides with attached cryostat sections were washed (3 x 15min) with 0.1M phosphate buffer containing 0.4% Triton X-100, incubated for 1 hour in 0.1M phosphate buffer containing 0.4% Triton X-100 and 3% BSA (to block nonspecific binding, both steps at room temperature), and incubated overnight in the same buffer containing the various antibodies at 4°C in a humid chamber. The slides

were then washed (3 x 15min) with 0.1M phosphate buffer containing 0.4% Triton X-100, and incubated with the various secondary antibodies (FITC-labelled goat anti-rabbit IgG, indocarbocyanide (cy3)-labelled anti-guinea pig or anti-mouse IgG (all from Jackson ImmunoResearch)) for 1-2 hours in the dark (in a humid chamber) at room temperature. The slides were washed in reduced lighting first with 0.1M phosphate buffer (3 x 15min) and then with 4mM Na₂CO₃ (10min). The tissues were mounted in 80% glycerol in 0.1M phosphate buffer (pH 10) and viewed on a Leitz microscope equipped with fluorescence epiluminator, and an I₂ filter for cy3 and an N₂ filter for FITC. Kodak ISO400/27° 35mm film was used for colour photography.

3.11 Antibodies

Antibodies used for immunoblotting, immunoprecipitation, or immunohistochemistry experiments are listed, along with their source, specificity, and immunogen used for production. Anti-caveolin (polyclonal, Transduction Laboratories, Lexington, KY) recognizes all caveolin isoforms and was generated from amino acids 1-97 of human caveolin-1. Anti-caveolin-3 (polyclonal, Transduction Laboratories, Lexington, KY) recognizes the muscle specific caveolin isoform and was generated from amino acids 3-24 of rat caveolin-3. Anti-Ca²⁺ channel α 1 subunit (polyclonal, gift from Dr. Schwartz, University of Cincinnati)

recognizes both the cardiac and smooth muscle Ca^{2+} channel $\alpha 1$ subunit. Anti-PMCA ATPase (monoclonal, clone 5F10, Affinity Bioreagents, Golden, CO) recognizes all four isoforms of the PM Ca^{2+} pump and specifically recognizes an epitope between amino acids 724-783 of the human erythrocyte Ca^{2+} pump. Anti-SERCA2 ATPase (monoclonal, clone IID8, Affinity Bioreagents, Golden, CO) recognizes both isoforms of the SERCA2 Ca^{2+} pump and was generated using purified canine cardiac sarcoplasmic reticulum. Anti-calreticulin (polyclonal, Affinity Bioreagents, Golden, CO) recognizes the protein from various animal and tissue sources and was generated from recombinant human calreticulin. Anti-canine cardiac calsequestrin (polyclonal, Upstate Biotechnology, Lake Placid, NY) recognizes the cardiac but not skeletal muscle isoform of calsequestrin, and was generated from amino acids 39-48 of canine cardiac calsequestrin. Anti-connexin-43 (monoclonal, Transduction Laboratories, Lexington, KY) recognizes connexin-43 from various species, and was generated from amino acids 252-270 of rat connexin-43. Anti- IP_3 receptor (polyclonal, Calbiochem, San Diego, CA) recognizes the IP_3 receptor from various cell types and animal species and was generated from a short synthetic peptide corresponding to the C-terminal cytoplasmic domain. Anti- IP_3 receptor type-1 (polyclonal, Affinity Bioreagents, Golden, CO) recognizes the type-1 IP_3 receptor ($\text{IP}_3\text{R-1}$) found in various animal and tissue sources, including canine vascular smooth muscle, and was generated from amino acids 1829-1848 of human $\text{IP}_3\text{R-1}$.

3.12 Statistics

Data for enzyme assays and radioligand binding were expressed as mean \pm SEM. Statistical comparisons were made using ANOVA with post-comparisons using the Bonferroni technique. Significant differences were reported if a $p < 0.05$ was found.

Chapter 4: RESULTS

4.1 Studies in Tracheal Smooth Muscle

4.1.1 Isolation and Characterization of Detergent-resistant Membranes

A protocol was developed, based on methods used by others studying caveolae in non-muscle cell types (Sargiacoma et al., 1993) and studying chicken gizzard smooth muscle (Chang et al., 1994), to isolate caveolar membranes from canine tracheal smooth muscle microsomes. PM-enriched microsomes, prepared according to the method of Grover et al. (1980), were treated with 1% Triton X-100 for 1 hour at 4°C and then subjected to centrifugation to pellet any detergent-resistant membranes. These membranes were then studied (along with appropriate control membranes) using various biochemical methods (enzyme assays, immunoblotting, radioligand binding) to evaluate whether these detergent-resistant membranes contained caveolar membranes, and also to determine which proteins were associated with caveolar membranes.

4.1.1.1 PM Marker Enzyme Activities

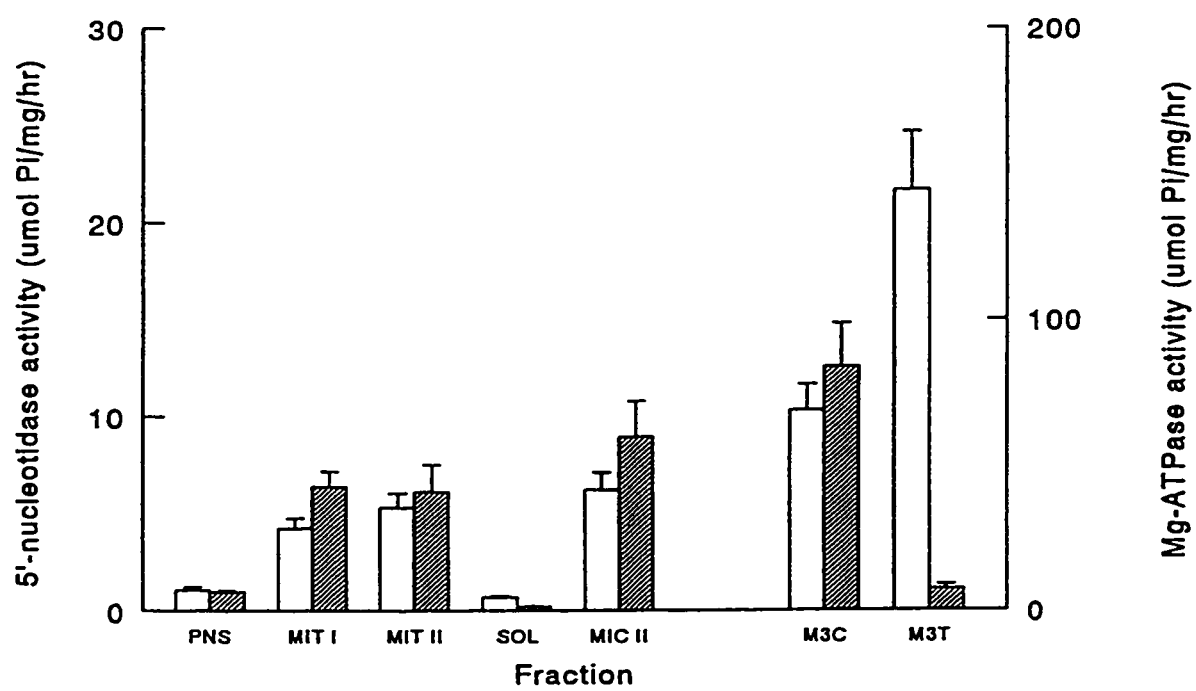
Analysis of PM marker enzyme activities on the various membrane fractions indicated that the specific activities of both 5'-nucleotidase and Mg^{2+} -ATPase were highest in the PM-enriched membrane fraction, MIC II, and also high in the mitochondrial fractions, MIT I and MIT II (Figure 5). The 5'-nucleotidase and Mg^{2+} -ATPase specific activities in MIT I, MIT II, and MIC II were significantly different from those in PNS and SOL ($p < 0.05$, 0.05 , and 0.01 for MIT I, MIT II, and MIC II respectively). There were no significant differences between specific activities in MIT I, MIT II, and MIC II. The values for 5'-nucleotidase specific activity were comparable with previously reported values (Grover et al., 1980).

Detergent-resistant membranes were prepared from the PM-enriched, MIC II fraction. The membranes were divided in two, with one half serving as control and the other half treated with 1% Triton X-100 (in SMB) for 1hr at 4°C. Both control and detergent-treated membranes were then centrifuged at 100000g for 45min, and the resultant pellets resuspended in SMB. These fractions were labelled M3C for control membranes, and M3T for the detergent-resistant membranes. PM marker enzyme activities for the control membranes, M3C, were increased slightly compared with MIC II, with 5'-nucleotidase specific activity significantly different from that in MIC II ($p < 0.05$). The detergent-treated membranes, M3T, had 5'-nucleotidase specific activities which were approximately

3-fold higher than MIC II ($p < 0.001$), and significantly higher than M3C ($p < 0.001$), while Mg^{2+} -ATPase specific activities were almost eliminated ($p < 0.01$ compared with MIC II and M3C, Figure 5). This would suggest that detergent treatment resulted in the isolation of a subset of the PM-enriched fraction which is enriched in 5'-nucleotidase activity, and lacking in Mg^{2+} -ATPase activity.

An alternative explanation for the increase (5'-nucleotidase) and decrease (Mg^{2+} -ATPase) of enzyme activities in the detergent-treated membrane fraction is that the detergent had a direct effect on the enzymes, without affecting enrichment or loss of the proteins. To address this possibility, enzyme reactions were carried out on MIC II or M3C microsomes treated with 0.1% and 1% Triton X-100 for 105 minutes (the same time as used in the preparation of detergent-resistant microsomes - 60min incubation and 45min centrifugation). The microsomes (+ detergent) were then added to the enzyme reaction mixture for determination of enzymatic activity. The results of 4 separate experiments indicate a slight increase in 5'-nucleotidase specific activity (no significant difference) and a marked decrease in Mg^{2+} -ATPase activity (specific and total activity, $p < 0.05$ for both, Table 1). While the decrease in Mg^{2+} -ATPase specific activity approaches that of M3T, total activity of the fraction, expressed as a percentage of MIC II in the absence of detergent, is still significantly higher than that in M3T ($p < 0.05$). Collectively, these results suggest that the increase in 5'-nucleotidase activities and decrease in Mg^{2+} -ATPase activities are not solely an artifact of detergent affecting the enzyme reaction. A

Figure 5: Distribution of specific activities of 5'-nucleotidase (open bars) and Mg²⁺-ATPase (shaded bars) in fractions isolated from canine tracheal smooth muscle. Data are expressed in the units: $\mu\text{mol P}_i \cdot \text{mg protein}^{-1} \cdot \text{hr}^{-1}$. Fractions PNS to MIC II were isolated by differential centrifugation. MIC II microsomes were treated with 1% Triton X-100 for 60 min prior to ultracentrifugation. The detergent-resistant membranes which were pelleted were termed M3T. Parallel experiments omitting detergent treatment produced control membranes, termed M3C. Each bar represents the mean \pm SEM from 9 separate experiments.



	MIC II	MIC II 0.1% Tx-100	MIC II 1% Tx-100	M3C	M3C 1% Tx-100	M3T
5'-nucleotidase (Specific Activity)	53.6±6.3	64.4±8.5	74.9±10.9	nd	nd	143.1±20.6
Mg ²⁺ -ATPase (Specific Activity)	40.7±8.1	9.0±2.1	4.1±0.7	63.3±7.2	10.8±7.2	2.3±0.8
Mg ²⁺ -ATPase (Total Activity of fraction) (% MIC II)	(100)	42.2±7.1	16.6±3.0	77.6±7.0	10.1±5.5	2.5±0.6

Table 1: Effect of detergent (0.1 and 1% Triton X-100) on 5'-nucleotidase and Mg²⁺-ATPase activities. Specific activity is expressed in the units: $\mu\text{mol P}_i \cdot \text{mg protein}^{-1} \cdot \text{hr}^{-1}$. Membranes were treated with detergent for 105 minutes prior to addition to the enzyme reaction. Although 5'-nucleotidase activity is increased, although not significantly ($p > 0.05$), it does not increase to that seen in the detergent-resistant fraction, M3T ($p < 0.05$ compared with MIC II 1% Tx-100). In contrast, Mg²⁺-ATPase activity is significantly decreased by detergent treatment, to levels near those seen in M3T, although it is still significantly higher ($p < 0.05$; comparison of M3T with MIC II 1% Tx-100 or M3C 1% Tx-100). Similarly, comparisons of total activity of the fractions (expressed as a percentage of total activity in untreated MIC II) reveals that detergent treatment does not reduce activity down to the level in M3T ($p < 0.05$, comparison of M3T with MIC II 1% Tx-100 or M3C 1% Tx-100). Boxes marked "nd" indicate data not determined. Each value is the mean \pm SEM from 3-6 separate experiments.

certain component of the change is due to the elimination of membranes which contain Mg^{2+} -ATPase, and an enrichment of membranes which contain 5'-nucleotidase.

4.1.1.2 Immunodetection of Caveolin in Membrane Fractions

Experiments in endothelial cells have suggested the enrichment of GPI-linked proteins to detergent-resistant membrane domains is an artifact of the treatment (Schnitzer et al., 1995b). That is, the detergent treatment results in the movement of GPI-linked proteins from membrane domains nearby caveolae into the detergent-resistant caveolar membranes. Since 5'-nucleotidase is a GPI-linked protein, then 5'-nucleotidase activity may not be useful as a marker of caveolar membranes. In order to confirm that the detergent-resistant membrane fraction in fact contain caveolae, a caveolae-specific marker protein must be identified in the fraction. To this point, only one protein has been identified as a true marker of caveolar membranes: caveolin, which forms the protein coat of caveolae (Rothberg et al., 1992).

Various membrane fractions from canine tracheal smooth muscle were tested for the presence of caveolin. Using 5 μ g of total protein per fraction, the proteins were separated on a 12% polyacrylamide gel, transferred to nitrocellulose, and probed using a polyclonal antibody to caveolin (Transduction Laboratories).

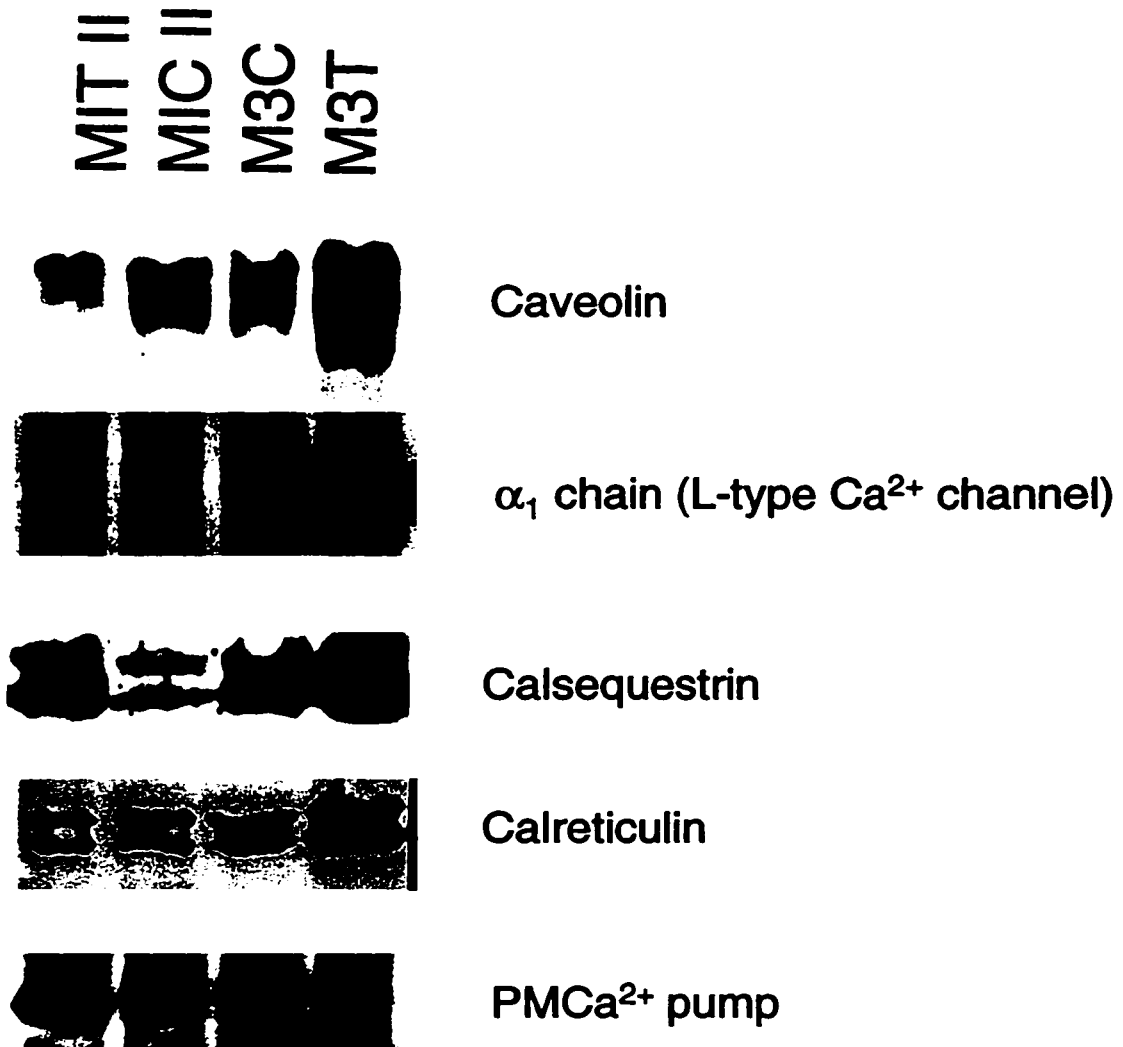
Immunoblots consistently demonstrated that all of the fractions tested (MIT II, MIC II, M3C, M3T) contained caveolin in varying amounts (Figure 6). In all cases, caveolin was greatly enriched in the detergent-resistant membrane fraction, M3T, compared with all other fractions. This would suggest that the detergent-resistant membrane fraction is enriched in caveolar membranes. With the confirmation that the detergent-resistant membrane fraction was enriched in caveolar membranes, further immunoblots were done to test for the presence of other proteins, specifically those involved in Ca^{2+} handling.

4.1.1.3 Immunodetection of Proteins Involved in Ca^{2+} Handling

We have hypothesized that caveolae are involved in Ca^{2+} handling in airway smooth muscle. For caveolae to function in this role, then a number of Ca^{2+} handling proteins must be present, such as the L-type Ca^{2+} channels, PM Ca^{2+} pump, and Ca^{2+} binding proteins. The presence of these proteins was investigated using immunoblotting. Using the same membrane fractions as used for the caveolin immunoblots (MIT II, MIC II, M3C, M3T), 60-80 μg of total protein per fraction was separated on a 7.5% polyacrylamide gel, transferred to nitrocellulose, and probed with the various antibodies.

As seen in Figure 6, immunoblots indicated the presence of the α_1 chain of the L-type Ca^{2+} channel, the PM Ca^{2+} pump, calsequestrin, and calreticulin in M3T,

Figure 6: Immunoblots demonstrating the distribution of caveolin and various Ca^{2+} handling proteins in the canine tracheal smooth muscle membrane fractions: MIT II (mitochondria), MIC II, M3C, and M3T. There is an enrichment of caveolin, the α_1 chain of the L-type Ca^{2+} channel, calsequestrin, and calreticulin in the detergent-resistant fraction, M3T. M3T also appears to contain the PM Ca^{2+} pump, although it is not enriched. Immunoblots are representative of 4-9 separate experiments.



as well as the other membrane fractions. L-type Ca^{2+} channels, calsequestrin, and calreticulin are enriched in M3T, suggesting an enrichment in caveolar membranes. The presence of calsequestrin and calreticulin, both of which are considered SR/ER resident proteins was surprising. Results confirming their presence in caveolae will be presented in later sections (4.1.2, 4.2.2.3) and possible explanations for their localization to caveolae will be presented in the discussion. Collectively, these results suggest that caveolae contain the proteins necessary for involvement in Ca^{2+} handling.

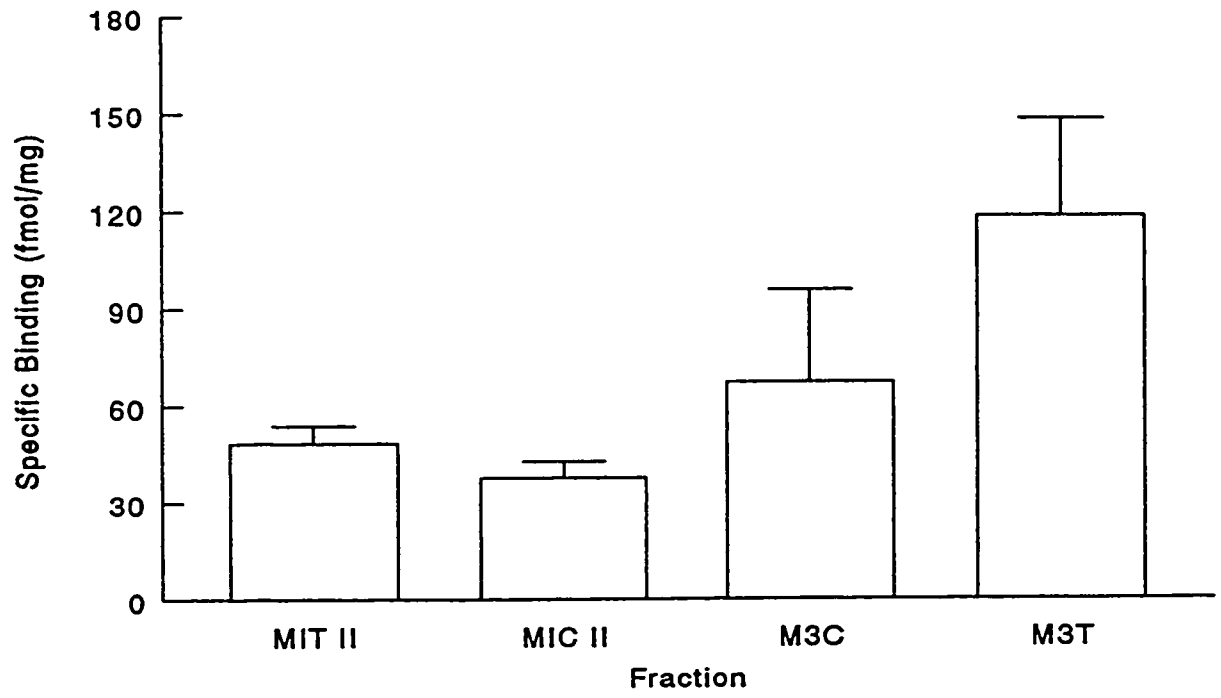
4.1.1.4 [3H]PN200-110 Binding

To confirm the presence of L-type Ca^{2+} channels in the detergent-resistant membrane fraction, [^3H]PN200-110 radioligand binding experiments were conducted. Preliminary experiments measuring distribution of binding on all of the membrane fractions indicated that [^3H]PN200-110 specific binding was highest in the MIT II and MIC II fractions, similar to results in other smooth muscle types using another dihydropyridine, nitrendipine (Triggle et al., 1982; Grover et al., 1984; Ahmad et al., 1989). Binding experiments were conducted on the detergent-treated membrane fraction, M3T, and compared with binding in M3C, MIC II, and MIT II. Specific binding (mean \pm SEM) from 4 separate experiments are shown in Figure 7. As can be seen in both the figure and the summary Table 2A, specific binding

in the control membranes, M3C, is almost double that in MIC II ($p=0.09$) while in the detergent-treated membranes, M3T, the specific binding is three times that in the MIC II fraction ($p<0.05$). Further, as seen in Table 2B, total recovery of [^3H]PN200-110 binding in M3T is comparable to that in M3C (expressed as a percentage of MIC II, no significant difference). Collectively, these results confirm the results of the immunoblot experiments, demonstrating the presence and enrichment of dihydropyridine-sensitive, L-type Ca^{2+} channels in the detergent-resistant, caveolar-enriched membrane fraction.

The results summarized in Table 2B indicate that although detergent treatment resulted in the loss of a significant amount of protein (80% compared with MIC II, 50% compared with M3C, $p<0.01$ and 0.05 , respectively), there is not a comparable loss in either the 5'-nucleotidase total activity or the number of dihydropyridine binding sites (no significant difference). There is significant ($p<0.01$) loss of Mg^{2+} -ATPase total activity, and most, but not all is a direct result of detergent affecting enzyme activity, as indicated previously in Table 1. Collectively, the results presented to this point suggest that detergent treatment resulted in the purification of a subset of the PM, enriched in 5'-nucleotidase, L-type Ca^{2+} channels, calsequestrin, and calreticulin, and containing the PM Ca^{2+} pump (though not enriched). The similarities in total recovery of dihydropyridine binding in M3C and M3T suggest that almost all of the L-type Ca^{2+} channels are present in caveolar membranes.

Figure 7: Distribution of [³H]PN200-110 binding in canine tracheal smooth muscle membrane fractions. Data are expressed in the units: fmol [³H]PN200-110•mg protein⁻¹. Specific binding in M3C is almost double that in MIC II, while for the detergent-resistant fraction, M3T, the specific binding is three times that in the MIC II fraction. Calculation of total recovery of specific binding in M3C and M3T indicates an approximate equal recovery between the two fractions (shown in Table 2). Each data point represents the mean ± SEM from 4 separate experiments.



	MIC II	M3C	M3T
5'-nucleotidase	6.2±0.9	10.3±1.4	21.7±3.0
Mg ²⁺ -ATPase	59.7±12.1	83.8±14.9	7.4±1.4
[³ H]PN200-110	37.6±5.1	67.3±28.3	118.6±29.7

Table 2A: Summary of PM enzyme activities and [³H]PN200-110 specific binding in the different PM (MIC II and M3C) and caveolae-enriched (M3T) fractions isolated from canine tracheal smooth muscle. Values are taken from Figures 5 and 7. Enzyme activities are expressed in the units: $\mu\text{mol P}_i \cdot \text{mg protein}^{-1} \cdot \text{hr}^{-1}$, and [³H]PN200-110 specific activity is expressed in the units: $\text{fmol } [^3\text{H}]\text{PN200-110} \cdot \text{mg protein}^{-1}$.

	M3C	M3T
5'-nucleotidase	66.7±6.0	62.4±13.6
Mg ²⁺ -ATPase	73.4±12.6	2.3±0.5
[³ H]PN200-110	36.3±3.0	38.4±0.6
Protein Recovery	43.1±6.9	19.2±3.0

Table 2B: Total Recovery of PM enzyme activities, [³H]PN200-110 binding, and total protein in the M3C and M3T fractions isolated from canine tracheal smooth muscle. Data are expressed as a percentage of that in MIC II. There is no significant difference between total recoveries of 5'-nucleotidase activity or [³H]PN200-110 specific binding while there is a significant difference between protein recovery ($p < 0.05$).

4.1.1.5 Immunodetection of Non-caveolar PM Proteins

To confirm that the detergent-resistant membranes are a subset of the PM, and do not represent a purification of the total PM, immunoblots were conducted using an antibody specific for a protein which should not be localized in caveolae. Connexin-43, which is a protein localized to and involved in the formation of gap junctions, should not be found in caveolae. Using 80µg of total protein per fraction, the MIT II, MIC II, M3C, and M3T fractions were separated on a 12% polyacrylamide gel, transferred to nitrocellulose, probed using the antibody to connexin-43, and detected by ECL. As indicated in Figure 8, although connexin-43 was found in MIC II and M3C, it was not detected in M3T, even at longer time exposures of the nitrocellulose to film. This supports the hypothesis that detergent treatment results in the purification of a subset of PM which is enriched in caveolar membranes, and not the purification of the PM from membrane sources which contain gap junctions.

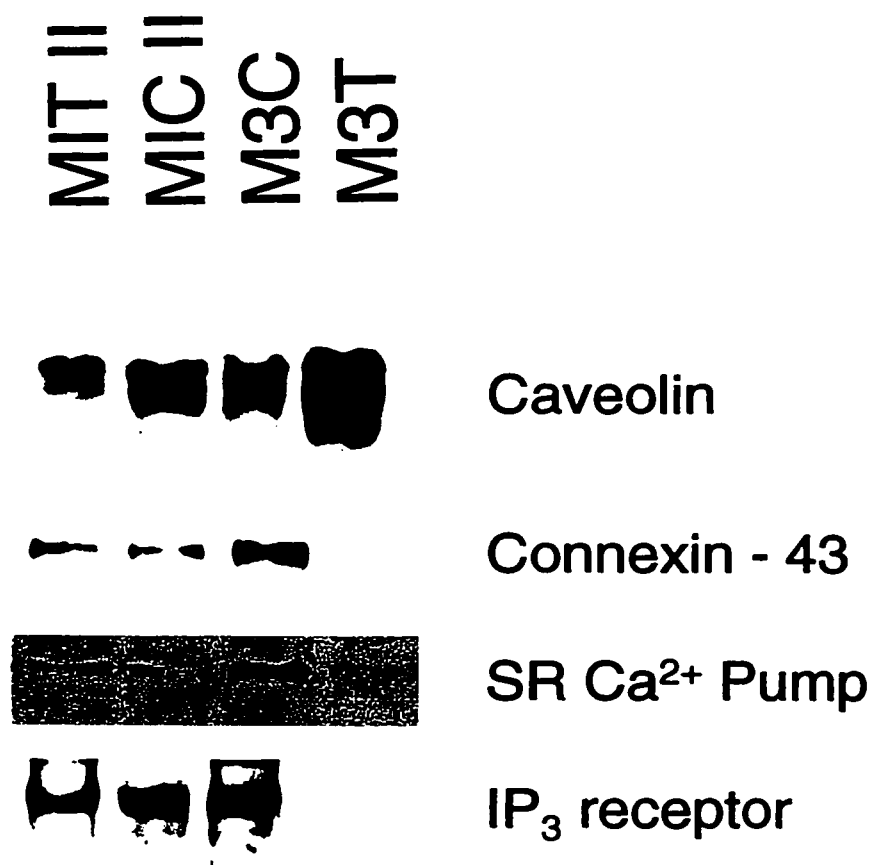
4.1.1.6 Immunodetection of SR Proteins

An alternative interpretation of the results demonstrating the presence of calsequestrin and calreticulin in M3T is that either some SR membranes are also detergent resistant or that the detergent treatment results in the movement of

proteins from SR membranes into the detergent-resistant membranes. Additionally, as discussed in the introductory chapter, it has been demonstrated that an IP₃-like protein may be localized to caveolae in smooth muscle and other cell types (Fujimoto et al., 1992, 1995).

To address these issues, immunoblots were conducted using antibodies specific for the SR Ca²⁺ pump (SERCA2b), and IP₃ receptor (Type 1). As indicated in Figure 8, although detected in both the originating MIC II fraction and the M3C fraction, neither the SR Ca²⁺ pump, nor the IP₃ receptor were detected in the M3T fraction. These results suggest that the SR membranes are not detergent resistant, and that membrane-inserted SR proteins are not being translocated from SR to detergent-resistant membranes. However, while the SR membrane proteins are not being translocated, this does not prove that SR resident proteins which are not inserted in the membrane but localized in the lumen (such as calsequestrin and calreticulin), are not translocated to the detergent-resistant membranes. As will be discussed in the next section, this possibility however, is unlikely.

Figure 8: Immunoblots demonstrating the distribution of caveolin and "non-caveolar" proteins in the canine tracheal smooth muscle membrane fractions: MIT II (mitochondria), MIC II, M3C, and M3T. The immunoblot for caveolin is the same as in Figure 6 and is included for comparison. There is no detectable signal for the IP₃ receptor in M3T, although it is present in MIT II and MIC II. Similarly, the detergent-resistant fraction M3T does not show detectable levels of the SR Ca²⁺ pump or connexin-43, even at longer exposure times (data not shown). Immunoblots are representative of 4-9 separate experiments.



4.1.2 Immunoprecipitation of caveolin and associated proteins

In addition to forming the protein coat on caveolae, more recently caveolin has been shown to bind to numerous proteins localized to caveolae via an interaction between the caveolin scaffolding domain (amino acids 82-101) and an aromatic-rich domain on the various proteins (Couet et al., 1997). As discussed in the introduction, investigators have taken advantage of this interaction, conducting immunoprecipitation experiments under non-denaturing conditions to determine which proteins are associated with caveolin.

The method used was that of Feron et al. (1996) who demonstrated in isolated cardiac myocytes and cultured aortic endothelial cells the immunoprecipitation of eNOS with caveolin. Immunoprecipitation experiments were conducted using the PM-enriched MIC II membranes from tracheal smooth muscle. Using these non-denaturing conditions, antibodies to caveolin were able to immunoprecipitate caveolin from MIC II membranes isolated from canine tracheal smooth muscle, confirming the immunoprecipitation was successful (Figure 9A, B). In addition to caveolin, calsequestrin (Figure 9A, B) and calreticulin (Figure 9C) were also immunoprecipitated, but not connexin-43, the α_1 chain of the L-type Ca^{2+} channel, or the PM Ca^{2+} pump (data not shown).

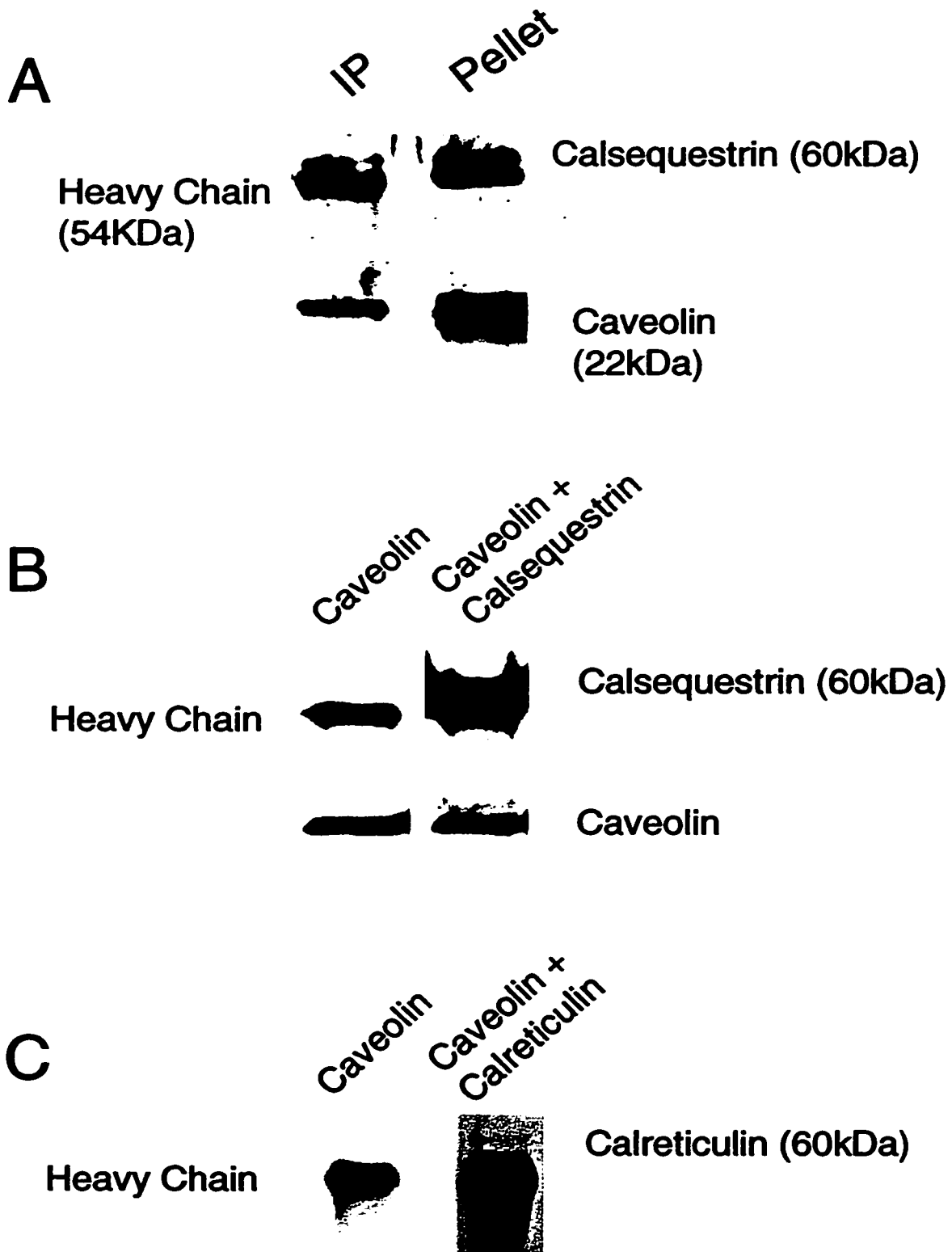
As seen in Figure 9A, when probed with antibodies to caveolin (the same rabbit polyclonal antibody as used for immunoprecipitation) and calsequestrin, the

immunoprecipitated protein lane (IP) shows a band at ~22kDa corresponding to caveolin, as well as a single band at ~54kDa which corresponds to the caveolin antibody heavy chain used for the immunoprecipitation, and a band at ~60kDa which corresponds to calsequestrin. The lane labelled 'pellet' (Figure 9A) corresponds to any membranes (and associated proteins) which were insoluble in the CHAPS detergent. These membranes and proteins were pelleted by centrifugation at 10000g (30min) prior to addition of the Protein A-Sepharose beads. Two bands are visible at ~57kDa and ~60kDa when antibodies to calsequestrin (Figure 9A, pellet) or calreticulin (data not shown) were used for immunodetection consistent with other results reported here (Figures 6, 17, and 19). This lower band can not be seen in the immunoprecipitation lanes (Figure 9A, IP), due to the strong signal from the rabbit heavy chain at ~54kDa. However, the upper band at ~60kDa is present in both the immunoprecipitation and pellet lanes only when antibodies to calsequestrin or calreticulin are used for immunoblots (Figure 9). When caveolin antibodies alone are used for immunodetection of immunoprecipitated proteins, a single band at ~54kDa is present (Figure 9B, C) and the bands at ~60kDa are not visible, even at longer exposures of the nitrocellulose to film (data not shown).

These results demonstrate an interaction between caveolin and calsequestrin and calreticulin, and suggest that their presence in the detergent-resistant membranes is not an artifact of protein sorting during detergent treatment.

In addition, since caveolin is located only on the cytosolic face of caveolae and does not traverse the membrane, and since calsequestrin and calreticulin are membrane-associated proteins which are not inserted in the membrane, then both calsequestrin and calreticulin must be located on the cytoplasmic side of the caveolar membrane *in situ*. The possibility exists that calsequestrin and/or calreticulin are also located in the caveolar lumen (extracellular) though no experiments were done to either prove or disprove this possibility.

Figure 9: Co-immunoprecipitation of caveolin, calsequestrin, and calreticulin. Using an antibody to caveolin, proteins in tracheal smooth muscle microsomes (MIC II) were immunoprecipitated under non-denaturing conditions according to the method of Feron et al. (1996). **A.** Following incubation with anti-caveolin antibodies and prior to incubation with Protein A-sepharose beads, a 10,000 g centrifugation step was added to pellet any undissolved membranes or proteins, and were labelled "pellet". The lane labelled "IP" refers to those proteins immunoprecipitated by anti-caveolin antibodies. Western blots using antibodies directed against caveolin and calsequestrin indicate that caveolin and calsequestrin were both immunoprecipitated. However, the molecular weight of the heavy chain of the anti-caveolin antibodies is very close to the molecular weight of calsequestrin (54kDa and 60kDa, respectively), although in our hands, the antibody to calsequestrin produced two bands (at 57 and 60kDa, refer to Figures 6, 17 and 19), the lower band being obscured by the anti-caveolin antibody heavy chain. **B.** Western blot of immunoprecipitated proteins using anti-caveolin antibodies, and then re-blotted using both anti-caveolin and anti-calsequestrin antibodies. Note that the heavy chain for the anti-caveolin antibody appears in the blot marked "caveolin" at ~54kDa, while in the blot marked "caveolin + calsequestrin", two overlapping bands are visible, one at ~54kDa, and a second at ~60kDa, indicating positive staining for calsequestrin. **C.** Western blot of proteins immunoprecipitated by anti-caveolin antibodies (IP) and stained with antibodies to calreticulin. Similar to the results with calsequestrin, two overlapping bands are visible at ~54kDa and ~60kDa, which correspond to the anti-caveolin heavy chain and calreticulin, respectively. Each blot is representative of 4 separate experiments.



4.1.3 Immunocytochemistry

Immunoprecipitation experiments confirmed the association between calsequestrin and calreticulin with caveolin and thus, caveolae. However, immunoprecipitation experiments failed to detect an association between caveolin and either the PM Ca^{2+} pump or the α_1 chain of the L-type Ca^{2+} channel, although both contain the consensus sequence for binding to the caveolin scaffolding domain.

Immunocytochemistry was performed using cryosections of fixed tracheal smooth muscle (10 μm thick, cut transverse to the smooth muscle bundles). Antibodies to caveolin stained only the periphery of each smooth muscle cell, consistent with its localization to caveolae on the PM (Figures 10A, 11A, and 14A). No staining was seen deeper in the cells. The staining was particulate, although all regions of the cell surface were stained. As mentioned in the introduction, caveolae of smooth muscle are arranged in rows running parallel to the long axis of the cells. However, if the rows are not straight, or if the transverse sections are not perfectly perpendicular to the long axis of the cells, then caveolae will appear to be located throughout the cell periphery of a 10 μm section, as opposed to discrete sites.

Antibodies directed against the PM Ca^{2+} pump also stained the periphery of the smooth muscle cells (Figure 10B, 12B, and 13B). The staining was not as

strong as the caveolin staining. Numerous 'hot spots' were seen on the cell periphery, randomly distributed. These 'hot spots' were visible only on the cell periphery. Double staining of cryosections with antibodies to caveolin and the PM Ca^{2+} pump indicated colocalization of staining on the periphery of the same smooth muscle cells (Figure 10), although the limits of the microscope resolution made it impossible to colocalize the particulate staining to the same sites on the cell surface. As a control for nonspecific staining, either the caveolin or PM Ca^{2+} pump antibody were omitted. This resulted in a lack of staining with their respective secondary antibodies. Similar control experiments were done for all double staining experiments.

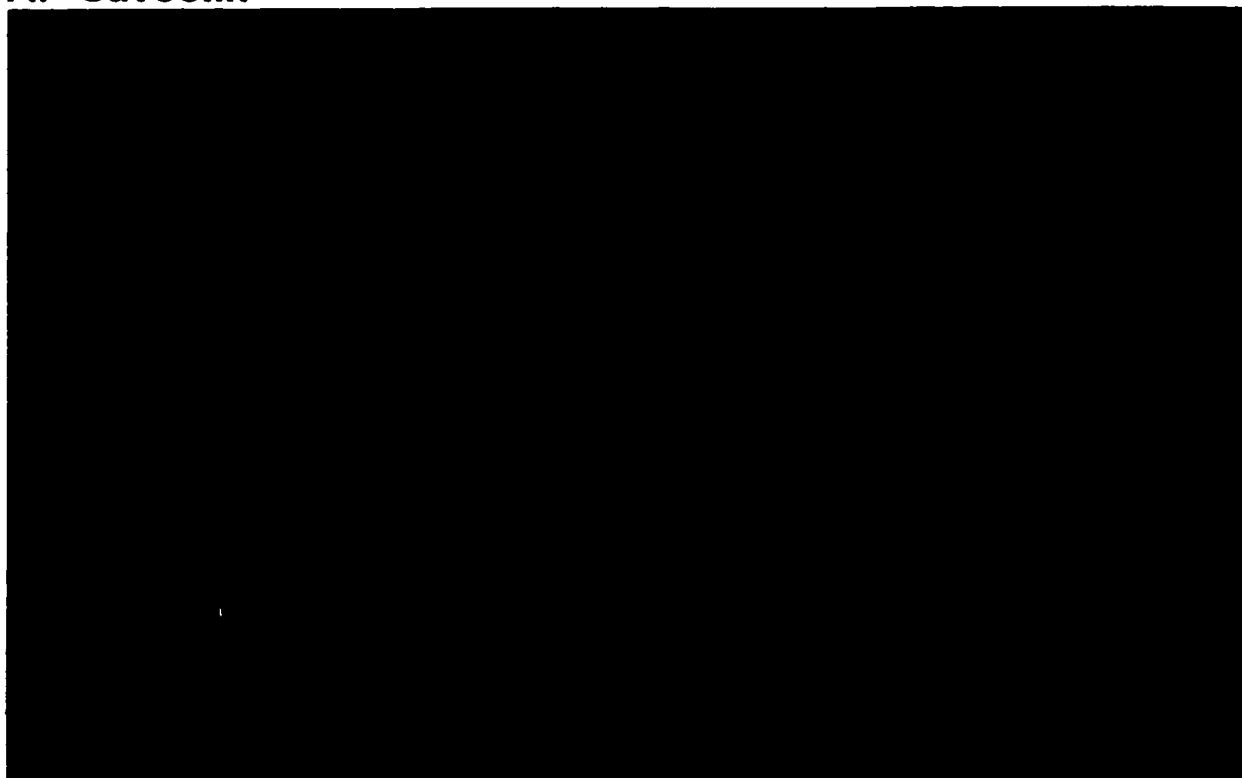
In contrast to the results with the caveolin or PM Ca^{2+} pump antibodies, antibodies directed against the SR Ca^{2+} pump did not stain the cell surface. Instead, staining was found throughout the cell interior (Figure 11B). In particular, there was intense staining in regions just below the cell surface, which may correspond to peripheral SR. Double staining with antibodies to caveolin and the SR Ca^{2+} pump indicate no colocalization of staining. Instead, as seen in Figure 11, in regions where caveolin staining is in focus, the staining for the SR Ca^{2+} pump is not, and conversely, in regions where staining for the SR Ca^{2+} pump is in focus, the staining for caveolin is not in focus. This would suggest that staining for caveolin is on the cell surface, while staining for the SR Ca^{2+} pump is located at regions just below the cell surface, on the peripheral SR.

Immunocytochemistry using antibodies directed against calsequestrin or calreticulin produced ubiquitous staining of the smooth muscle cells, with no structures visible when viewed in the microscope. The photo images appear to show stronger staining at or just below the cell surface (Figures 12A and 13A). Double staining with antibodies to the PM Ca^{2+} pump and calsequestrin or calreticulin indicated staining for the PM Ca^{2+} pump on the periphery of the cell surface while staining for the Ca^{2+} binding proteins was throughout the smooth muscle cells (Figures 12 and 13). This staining may include areas at or just below the cell surface.

Figure 10: Immunohistochemical staining of canine tracheal smooth muscle cryosections using antibodies to caveolin (A) and PM Ca²⁺ pump (B). Sections (10µm thick) were cut transverse to the smooth muscle bundles. Bar =25µm.

A: Caveolin

88



B: PM Ca²⁺ Pump

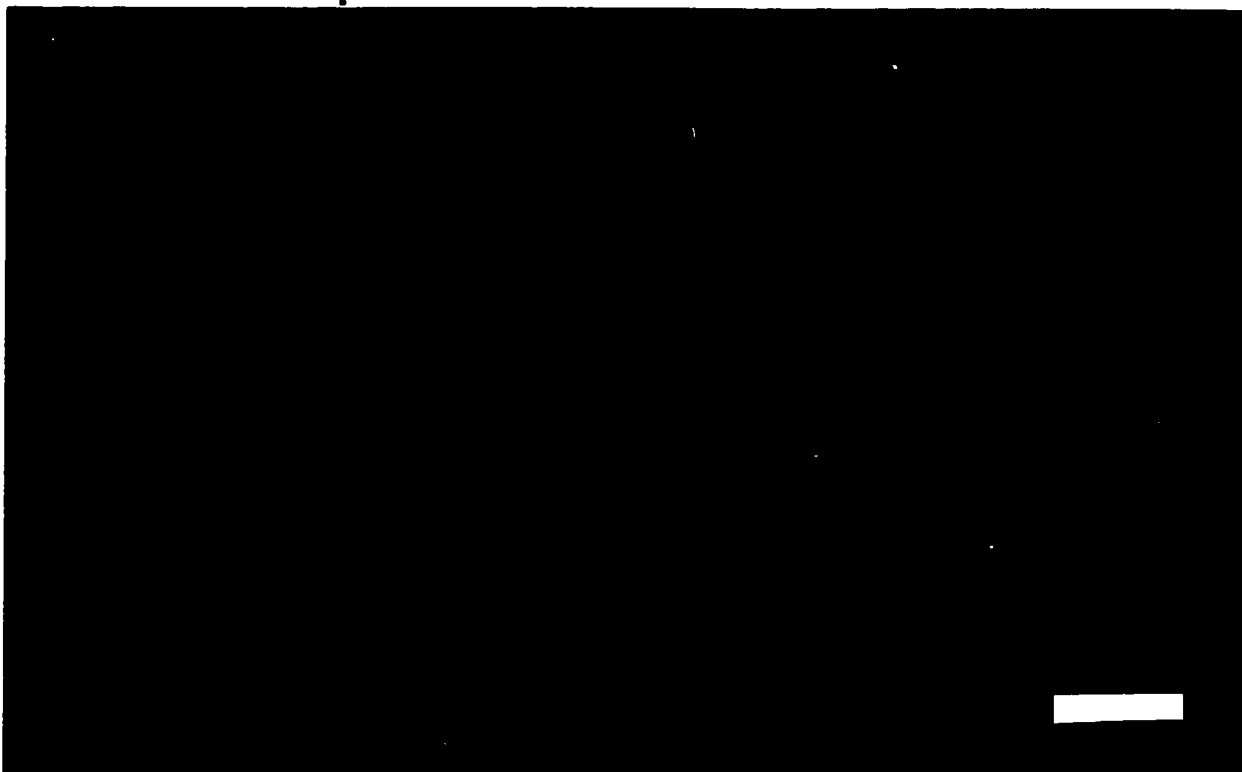


Figure 11: Immunohistochemical staining of canine tracheal smooth muscle cryosections using antibodies to caveolin (A) and SR Ca²⁺ pump (B). Sections (10µm thick) were cut transverse to the smooth muscle bundles. Bar =25µm.

A: Caveolin



B: SR Ca²⁺ Pump

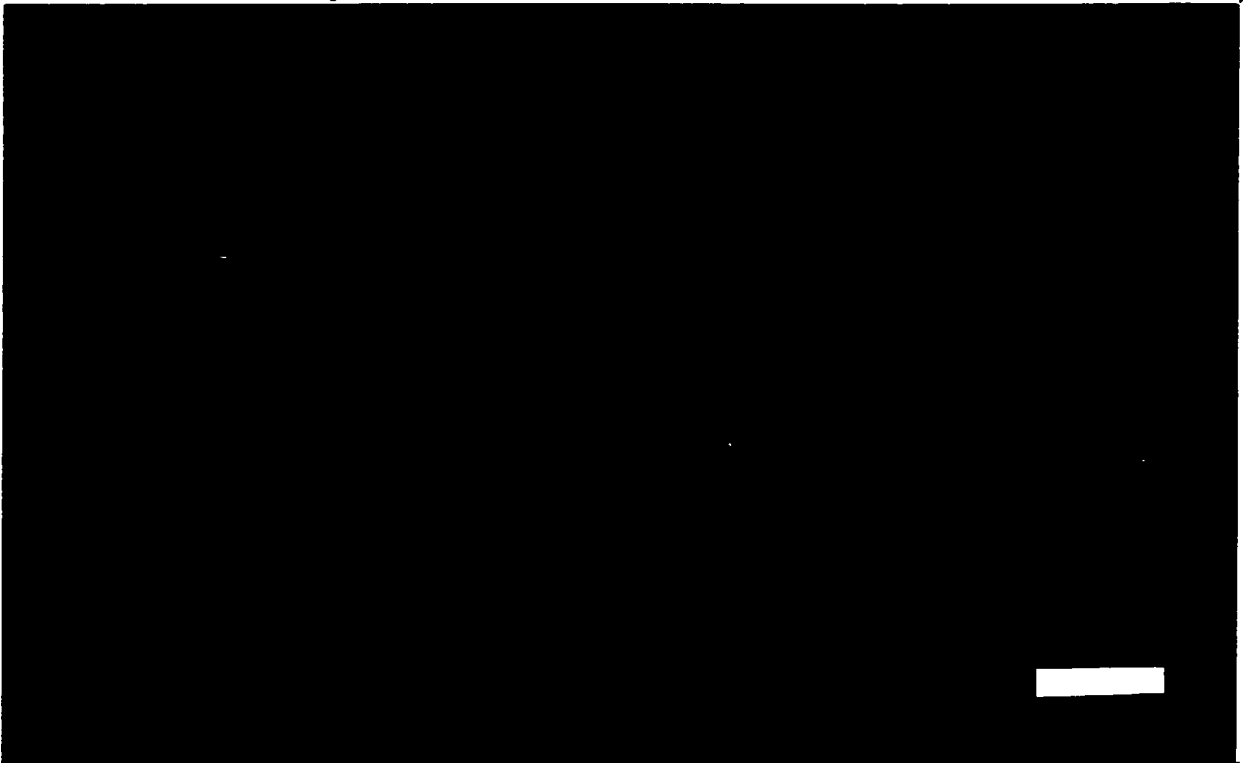


Figure 12: Immunohistochemical staining of canine tracheal smooth muscle cryosections using antibodies to calsequestrin (A) and PM Ca²⁺ pump (B). Sections (10µm thick) were cut transverse to the smooth muscle bundles. Bar =25µm.

A: Calsequestrin



B: PM Ca²⁺ Pump

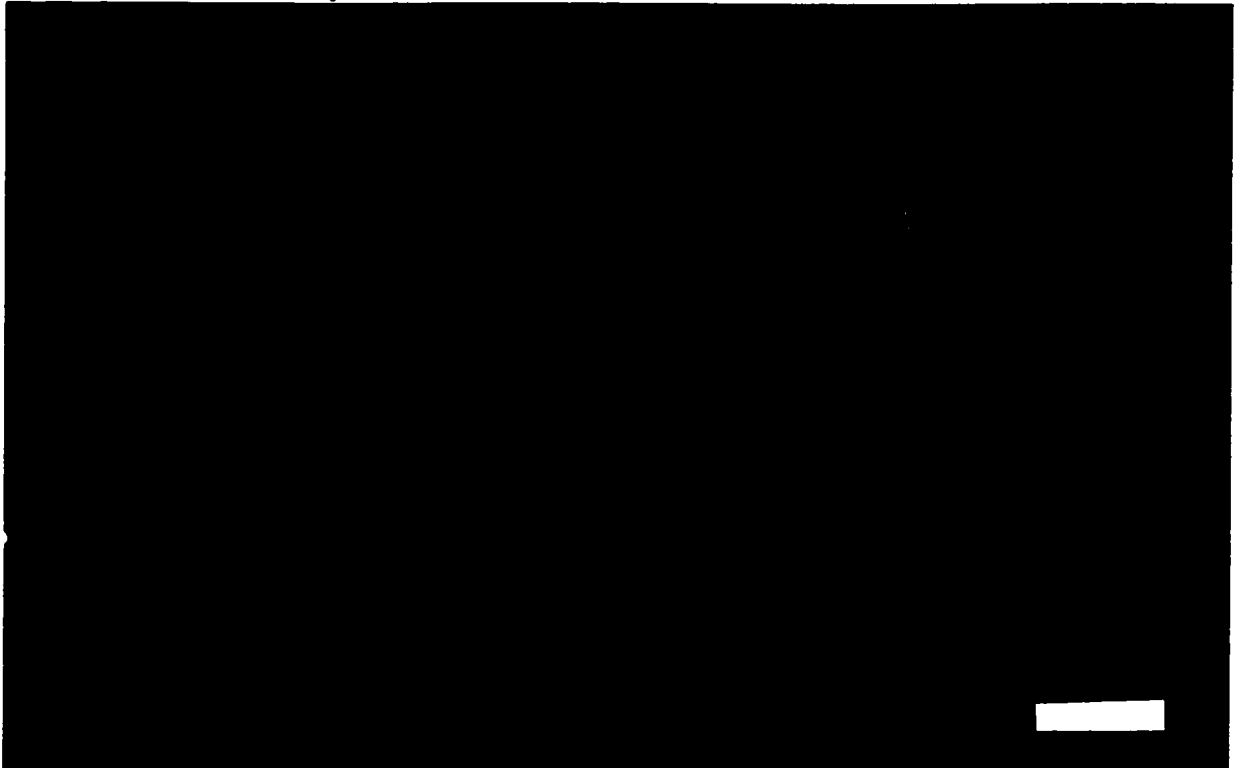
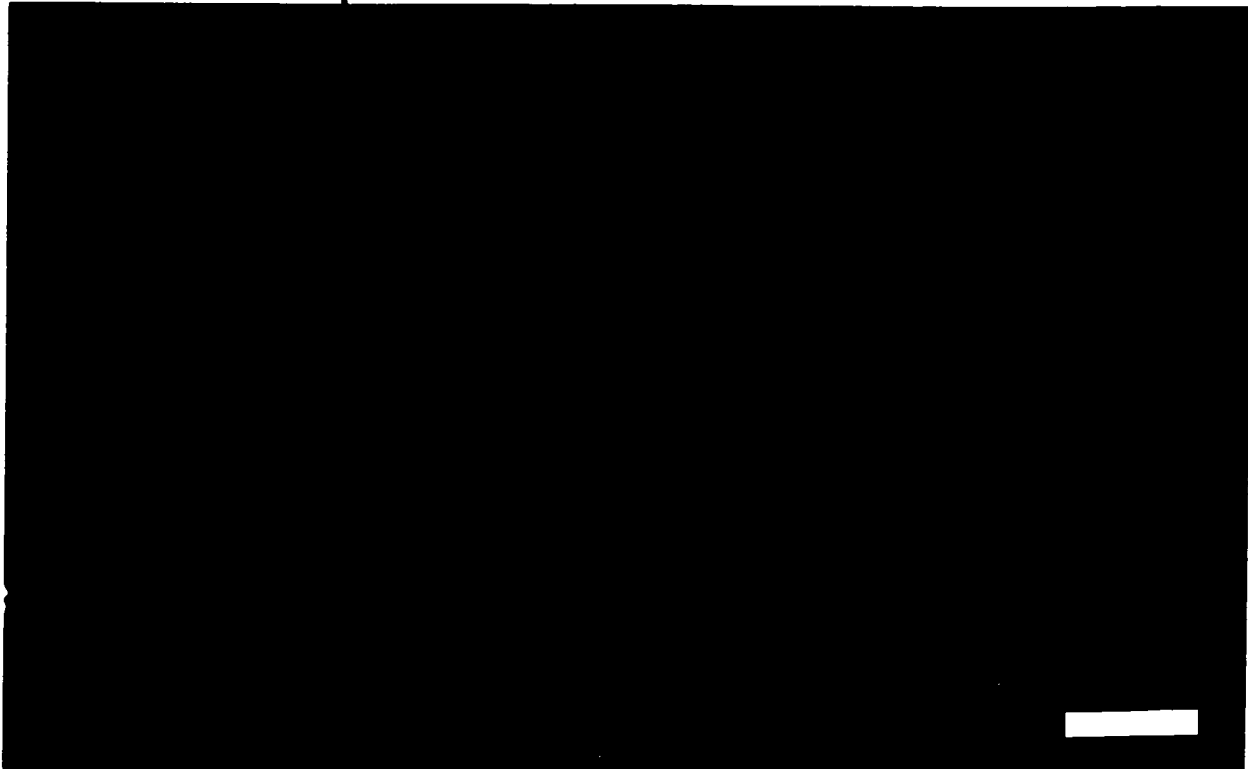


Figure 13: Immunohistochemical staining of canine tracheal smooth muscle cryosections using antibodies to calreticulin (A) and PM Ca²⁺ pump (B). Sections (10µm thick) were cut transverse to the smooth muscle bundles. Bar =25µm.

A: Calreticulin



B: PM Ca²⁺ Pump



4.1.3.1 Immunocytochemical Detection of nNOS in Tracheal Smooth Muscle Cryosections

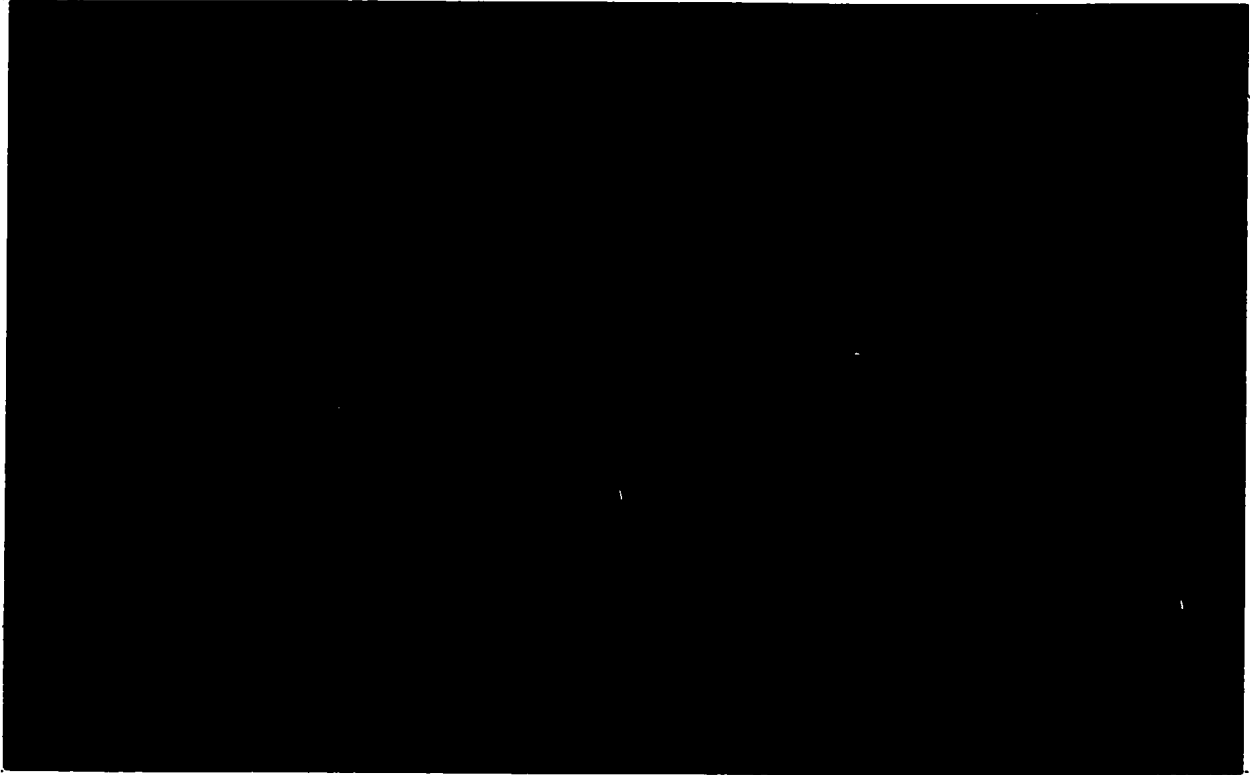
Recently, it has been demonstrated that an antibody raised in guinea pigs and directed against the C-terminal of rat neuronal NOS stains the cell periphery of canine lower esophageal sphincter smooth muscle (Salapatek et al., 1998b). Further, this staining has been shown to colocalize with caveolin staining. In cryosections from tracheal smooth muscle, intense staining was seen on the cell surface using this nNOS antibody (Figure 14B). Further, double staining with antibodies directed against caveolin and nNOS indicated colocalization of both caveolin and nNOS on the cell surface of each tracheal smooth muscle cell (Figure 14).

The question was raised as to whether the antibody specifically recognized nNOS or if there was a possibility that another protein was being stained. Although eNOS has been found to co-localize with caveolin in various cultured cells (endothelial and epithelial) and to co-immunoprecipitate with caveolin in endothelial cells and cardiac myocytes (Feron et al., 1996), and nNOS has been shown to co-immunoprecipitate with caveolin-3 in rat skeletal muscle homogenates (Venema et al., 1997), no isoform of NOS has ever been demonstrated in tracheal smooth muscle. Immunoblots using the nNOS antibody indicate that it recognizes a protein of approximately 160kDa in membranes from tracheal smooth muscle, which

corresponds to the molecular mass of nNOS (Figure 15). Staining is weak in all of the fractions tested (MIC II, MIT II, M3C, M3T) with high background staining, although this high background is due to nonspecific effects of the secondary antibody used. Interestingly, it appears that staining in M3T is the strongest, suggesting that nNOS is located in the caveolar region in tracheal smooth muscle cells.

Figure 14: Immunohistochemical staining of canine tracheal smooth muscle cryosections using antibodies to caveolin (A) and nNOS (B). Sections (10 μ m thick) were cut transverse to the smooth muscle bundles. Bar =25 μ m.

A: Caveolin



B: Nitric Oxide Synthase (Neuronal)



Figure 15: Immunoblot demonstrating the presence of nNOS in the canine tracheal smooth muscle membrane fractions: MIT II (mitochondria), MIC II, M3C, and M3T. A band is visible in all fractions at ~160kDa, which corresponds to the molecular weight of nNOS. There is an apparent enrichment in the fraction M3T, compared with the other fractions, suggesting an enrichment in caveolae-derived membranes. Immunoblots are representative of 3 separate experiments.

MIT II
MIC II
M3C
M3T



Nitric Oxide Synthase
(neuronal)

4.2 Studies in Intestinal Smooth Muscle

Many investigators have questioned the validity of detergent treatment as a method of caveolae isolation. The immunoprecipitation experiments using tracheal smooth muscle microsomes confirmed that the presence of calsequestrin and calreticulin in the detergent-resistant membrane fraction was not an artifact. Immunocytochemistry confirmed the localization of caveolin, nNOS, and the PM Ca^{2+} pump to the cell surface, while the SR Ca^{2+} pump was located in regions beneath the cell surface. However, to determine if the proteins localized to the cell surface were located in caveolae was not possible due to limits of microscope resolution. To confirm these findings, an alternative biochemical method of isolating caveolae was needed. However, there was insufficient tracheal tissue for our purposes, so an alternative smooth muscle was used. Intestinal smooth muscle was chosen, because large quantities were available, and more importantly, because it is a tissue which has been shown to have numerous caveolae (Gabella, 1971, 1976, 1981a). The isolation procedure chosen was the Na_2CO_3 method described by Song et al. (1996a), based on its high yield of caveolar membranes compared with other methods.

The Ca^{2+} handling properties of intestinal smooth muscle, specifically the close association between PM and SR, have not been investigated to date. To this point, the proteins localized to caveolae in intestinal smooth muscle were not

known. Comparisons of results obtained using Na_2CO_3 isolation methods in intestinal smooth muscle to results obtained using detergent treatment in trachea would be difficult. Thus it was necessary to determine which proteins were localized to detergent-resistant membranes obtained from intestinal smooth muscle.

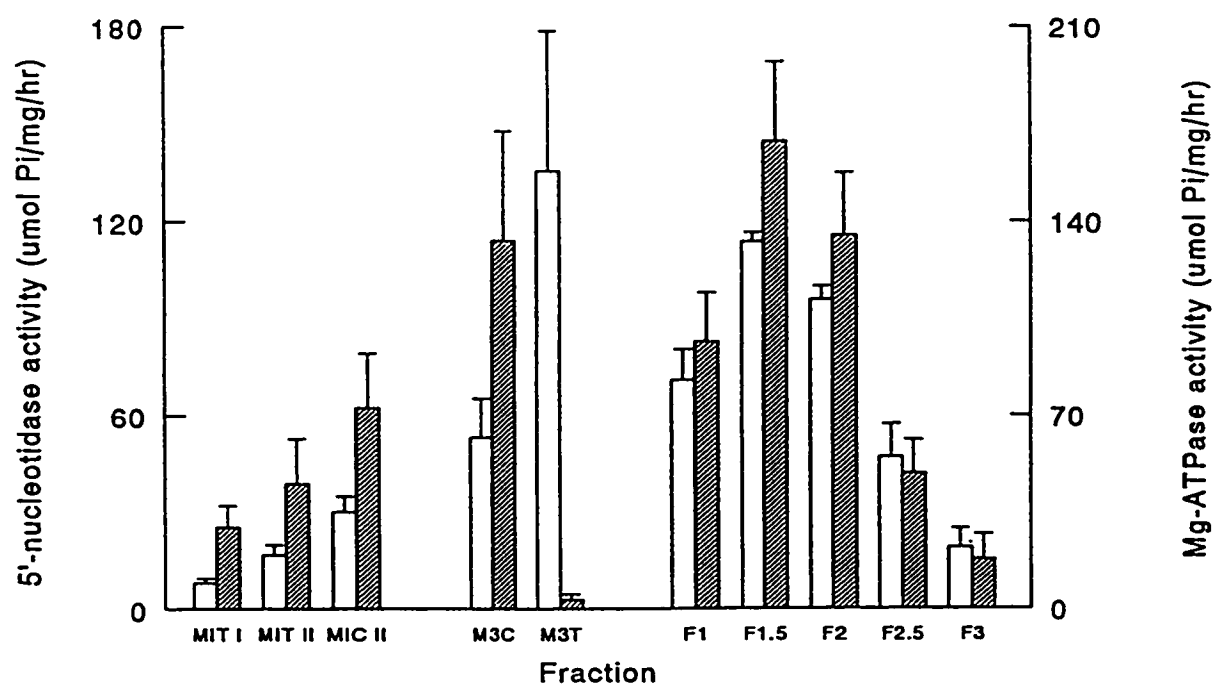
4.2.1 Detergent Treatment of Intestinal Smooth Muscle Microsomes

Detergent-resistant membranes were prepared from canine intestinal smooth muscle using the same methods as for tracheal smooth muscle. All enzyme assays and immunoblots performed on tracheal smooth muscle membranes were repeated using the membranes from intestinal smooth muscle.

4.2.1.1 PM Marker Enzyme Activities

The specific activities of 5'-nucleotidase and Mg^{2+} -ATPase in the fractions obtained by differential centrifugation were similar to previously reported values in intestine (Ahmad et al., 1987). The 5'-nucleotidase and Mg^{2+} -ATPase specific activities in the mitochondrial fractions, MIT I and MIT II, and PM-enriched fraction, MIC II (Figure 16), were significantly higher than those in PNS and SOL ($p < 0.05$ for all, data not shown). The 5'-nucleotidase specific activities were increased in M3T, compared with M3C and MIC II ($p < 0.05$ and 0.01 , respectively), while Mg^{2+} -ATPase

Figure 16: Distribution of specific activities of 5'-nucleotidase (open bars) and Mg^{2+} -ATPase (shaded bars) in various fractions isolated from canine intestinal smooth muscle. Data are expressed as $\mu\text{mol } P_i \cdot \text{mg protein}^{-1} \cdot \text{hr}^{-1}$. Fractions PNS to MIC II were isolated by differential centrifugation. Microsomes (MIC II) were treated with 1% Triton X-100 prior to ultracentrifugation. The resultant detergent-resistant membranes were termed M3T. Parallel experiments omitting detergent treatment produced the membrane fraction, M3C. In another set of experiments, microsomes (MIC I) were resuspended in 500mM Na_2CO_3 buffer (pH 11.0), sonicated and then layered on a discontinuous sucrose gradient (4/14/25/35/48%). The resulting fractions correspond to the 4/14% interface (F1), the 14% layer (F1.5), the 14/25% interface (F2), the 25% layer (F2.5), and the 25/35% interface (F3). Each bar represents the mean \pm SEM from 4 separate experiments.



specific activities were nearly eliminated in M3T ($p < 0.01$ compared with both MIC II and M3C). These results were qualitatively similar to those in trachea and are consistent with the hypothesis that detergent treatment produces a membrane fraction enriched in caveolae.

4.2.1.2 Immunodetection of Proteins Contained in Detergent-resistant Membrane Fraction

Immunoblots were conducted to determine which proteins were localized in the detergent-resistant membrane fraction, M3T. Other fractions (MIT II, MIC II, and M3C) were also analyzed. The results obtained were qualitatively similar to those obtained in trachea. The detergent-resistant membrane fraction was enriched in caveolin, suggesting that the fraction was enriched in caveolar membranes (Figure 17). Additionally, M3T was enriched (compared with MIC II and M3C) in the α_1 chain of the L-type Ca^{2+} channel, calsequestrin, calreticulin, and contained the PM Ca^{2+} pump (Figure 17). In contrast, connexin-43, the IP_3 receptor, and SR Ca^{2+} pump could not be detected in the M3T fraction, although they were detected in the other fractions (Figure 18). Thus, the fraction containing detergent-resistant caveolar membranes from intestinal smooth muscle contained the same Ca^{2+} handling proteins as we found colocalized to tracheal caveolar membranes, and appeared to not be contaminated with SR membranes.

Figure 17: Immunoblots demonstrating the distribution of caveolin and various Ca^{2+} handling proteins in the canine intestinal smooth muscle membrane fractions: MIT II (mitochondria), MIC II, M3C, and M3T. There is an enrichment of caveolin, the α_1 chain of the L-type Ca^{2+} channel, calsequestrin, and calreticulin in the detergent-resistant fraction, M3T. M3T also appears to contain the PM Ca^{2+} pump, although it is not enriched. Immunoblots are representative of 4 separate experiments.

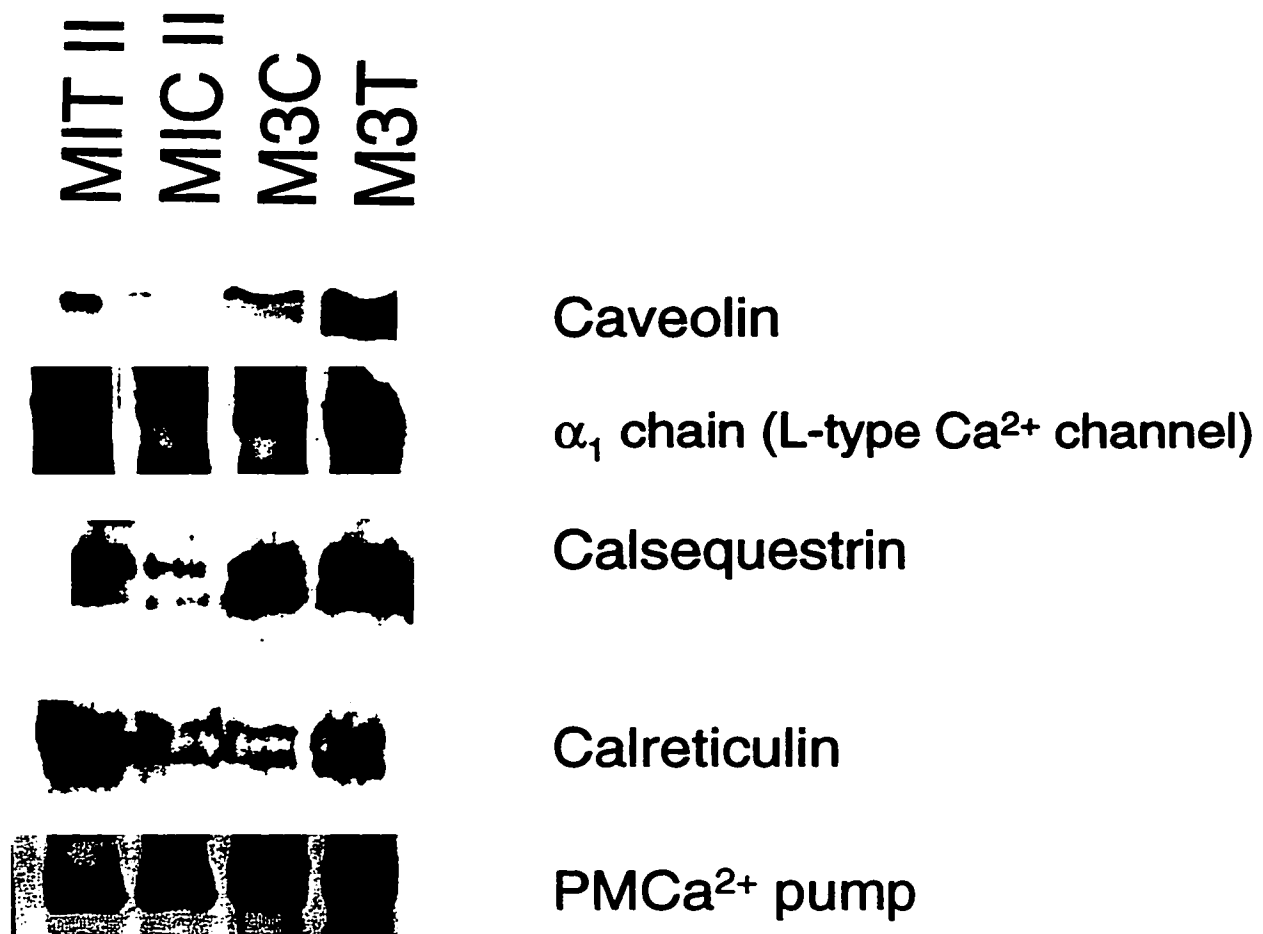
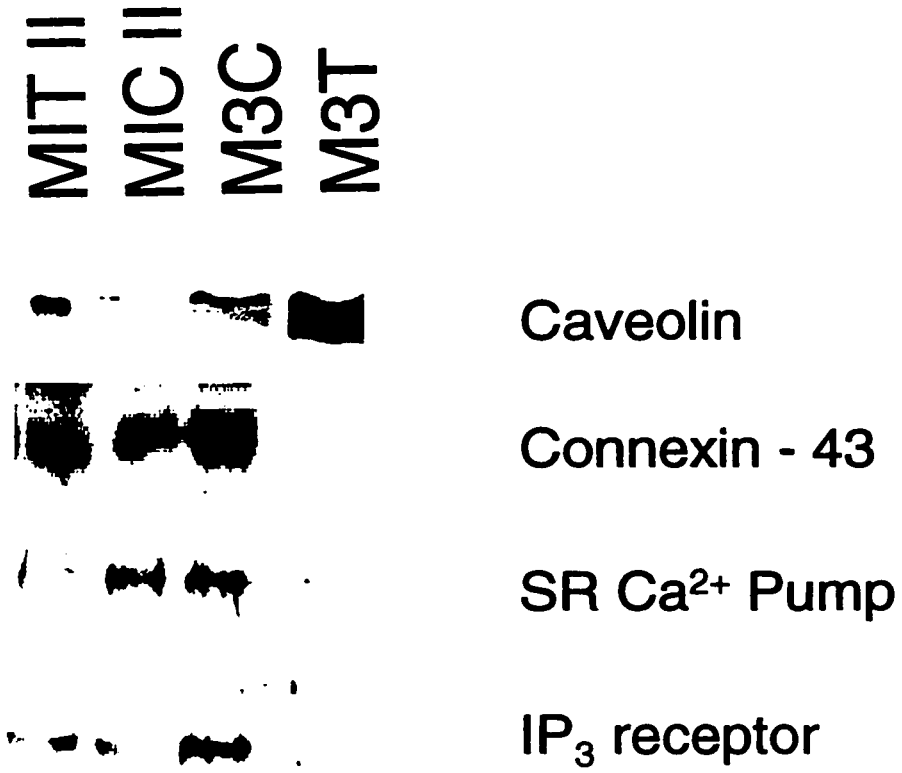


Figure 18: Immunoblots demonstrating the distribution of caveolin and "non-caveolar" proteins in the canine intestinal smooth muscle membrane fractions: MIT II (mitochondria), MIC II, M3C, and M3T. The immunoblot for caveolin is the same as in Figure 17 and is included for comparison. There is no detectable signal for the IP₃ receptor in M3T, although it is present in MIT II and MIC II. Similarly, the detergent-resistant fraction M3T does not show detectable levels of the SR Ca²⁺ pump or connexin-43, even at longer exposure times (data not shown). Immunoblots are representative of 4 separate experiments.



4.2.2 Alternative preparation of caveolar membranes: Na₂CO₃ treatment of intestinal smooth muscle microsomes

Recently, a detergent-free method for preparing caveolae has been described. Song et al. (1996a), using cultured epithelial cells, treated the cells with 500mM Na₂CO₃ (pH 11.0), and disrupted the cells by homogenization, polytroning, and sonication. The membranes were then layered on the bottom of a discontinuous sucrose gradient (5/35/45%). The 5/35% interface was collected and shown to contain caveolin and various proteins involved in cell signalling: Ras, G₂α, Gβ, and c-Src. The GPI-linked protein, carbonic anhydrase IV was not found in this fraction.

Modifying this method, the microsomal pellet, MIC I, from canine intestinal circular smooth muscle was resuspended in 500mM Na₂CO₃ (pH 11.0) and the microsomes disrupted by sonication (3 x 20s). The microsomes were then adjusted to 250 mM Na₂CO₃ by addition of an equal volume of SMB, before being layered on the top of a discontinuous sucrose gradient (4/14/25/35/48% w/w). This gradient is similar to gradients used previously in this laboratory for purifying PM-derived microsomes, and similar to the gradient used by Chang et al. (1994) in purifying caveolae from chicken gizzard using detergent treatment, except that the various sucrose concentrations were made in buffer containing 25mM MOPS, 10mM MgCl₂ and 250mM Na₂CO₃ (pH11.0). Based on the findings of Chang et al. (1994),

caveolae and their associated proteins were expected to be found at the 14/25% interface (F2) and within the 25% sucrose layer (F2.5).

4.2.2.1 PM Marker Enzyme Activities

PM marker enzyme activities of the various fractions indicated that the distribution of both 5'-nucleotidase and Mg^{2+} -ATPase specific activities were similar to one another (Figure 16). The 5'-nucleotidase and Mg^{2+} -ATPase specific activities were highest in the 14% layer (F1.5) and the 14/25% interface (F2). They were significantly different than those in F2.5 and F3 ($p < 0.05$ for all) and 5'-nucleotidase specific activity in F1.5 was also significantly different from that in F1. Specific activities in F1, F1.5, and F2 were all significantly different than MIC II ($p < 0.05$). The specific activity of 5'-nucleotidase in F1.5 and F2 was similar to that in the detergent-resistant membrane fraction, M3T ($p < 0.05$). In contrast to the results of Song et al. (1996a), 5'-nucleotidase was not excluded from any of the fractions tested.

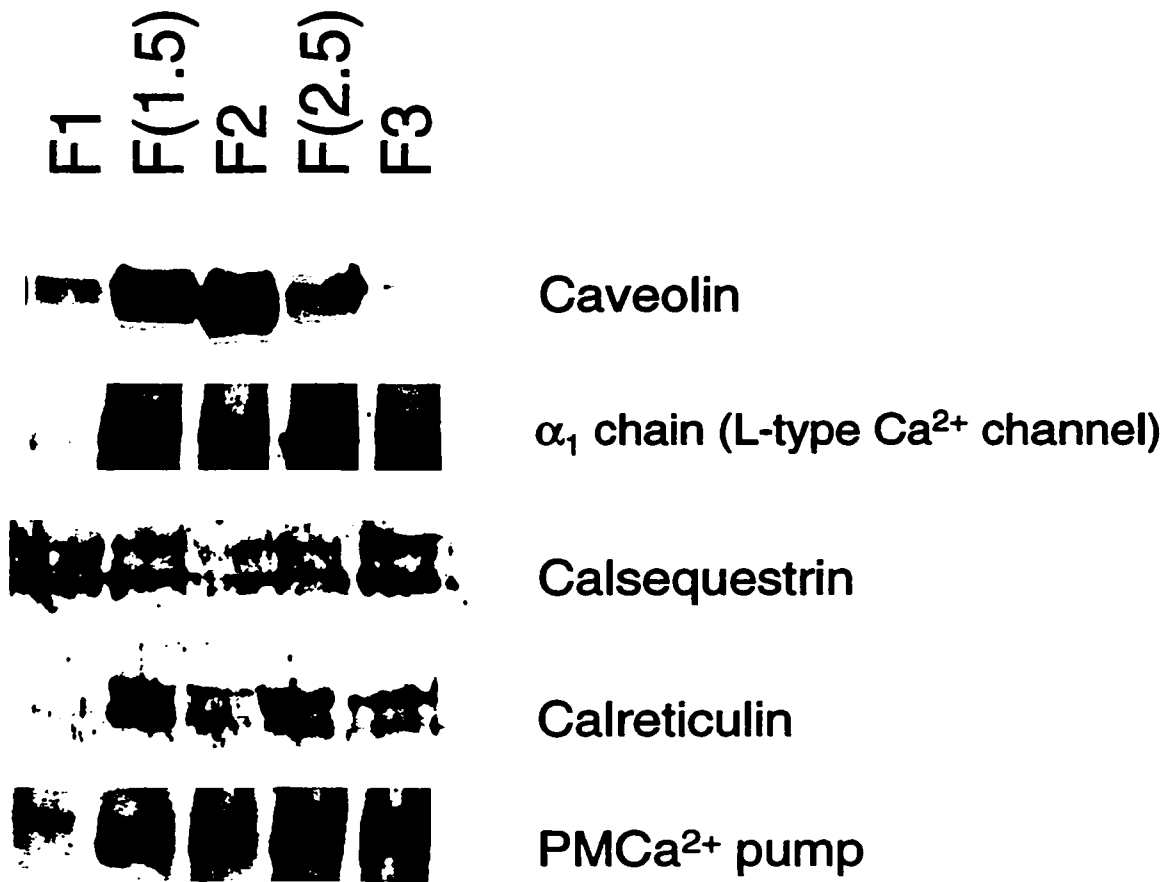
4.2.2.2 Immunodetection of Caveolin

Immunoblots using antibodies to caveolin indicated that it was present in all of the fractions tested, although the signal was weak in F1 and F3 (Figure 19). The

signal was strongest in the F1.5 and F2 fractions. Thus, caveolar membranes were enriched within the 14% layer and 14/25% interface and are also located in the 25% layer to a lesser extent. The enrichment of caveolar membranes at these sucrose concentrations represents a slight shift from their location in the results of Chang et al. (1994) who reported detergent-resistant caveolar membranes in the 14/25% interface and 25% layer.

The distribution of caveolin (highest in F1.5 and F2) corresponds with the distribution of 5'-nucleotidase and Mg^{2+} -ATPase enzyme activities. These results contrast with the results of Song et al. (1996a), in which GPI-linked proteins were excluded from the caveolin-enriched membrane fraction, and agree with the results of Chang et al. (1994) and the results presented here for detergent-treated membranes from trachea and intestine, where GPI-linked proteins were enriched in caveolin-enriched membranes. However, the enrichment of 5'-nucleotidase (and Mg^{2+} -ATPase) in F1.5 and F2 is not as clear as the results of detergent-treated membranes. The specific activities in F1 and F2.5, although 50-60% lower than F1.5 and F2, are still approximately double the specific activity in MIC II. Thus, while 5'-nucleotidase and Mg^{2+} -ATPase may be associated with caveolar membranes, they are not enriched.

Figure 19: Immunoblots demonstrating the distribution of caveolin and various Ca^{2+} handling proteins in the fractions isolated by discontinuous sucrose gradient centrifugation of Na_2CO_3 -treated, sonicated MIC I canine intestinal smooth muscle membranes. Caveolin is present in all of the fractions tested, and enriched in the fractions, F1.5 and F2. The α_1 chain of the L-type Ca^{2+} channel, calsequestrin and calreticulin are also enriched in the fraction F1.5, although they are present in the other fractions. The PM Ca^{2+} pump is also present in F1.5, though not enriched. Immunoblots are representative of 4 separate experiments.



4.2.2.3 Immunodetection of Ca²⁺ Handling Proteins

Results of immunoblots performed on the various fractions (F1-F3) are consistent with the results of detergent-treated membranes, in that the various Ca²⁺ handling proteins located in the detergent-treated membranes are also located in the fractions enriched in caveolin (F1.5, F2). More specifically, the α_1 chain of the L-type Ca²⁺ channel, the PM Ca²⁺ pump, calsequestrin, and calreticulin are all present in F1.5 and F2 (Figure 19). There is a strong signal for the PM Ca²⁺ pump in F1.5 and F2, but an even stronger signal in F2.5, suggesting that the PM Ca²⁺ pump is located not only in caveolae, but in other membrane regions as well, consistent with the results in detergent-treated membranes. In the cases of calsequestrin, calreticulin, and L-type Ca²⁺ channels, there appear to be two separate populations. That is, there appears to be an enrichment in F1.5, as well as an enrichment in F2.5, suggesting two separable populations of these proteins. While the population in F1.5 is likely caveolin-associated membranes, the identity of the denser fraction, F2.5 is unknown. It may be non-caveolar PM, SR, or synaptosomal membranes.

4.2.2.4 Immunodetection of Non-Caveolar PM Proteins

To confirm that the fractions F1.5 and F2 were caveolae-derived

membranes, and not simply PM-derived membranes, further immunoblots were done to test for the presence of connexin-43. As stated earlier, this protein is involved in gap junction formation, and should not be located in caveolae. A signal corresponding to connexin-43 could be detected in the fractions F2, F2.5, and F3, while in F1 and F1.5, no signal was detected (Figure 20). These results demonstrate that there is some "crossover" between caveolar membranes and other PM membranes at the 14/25% interface (F2). However, the lack of signal at F1.5 for connexin-43, compared with the strong signal for caveolin, would indicate that it is possible to separate caveolar membranes from other PM-derived membranes, at least that region of the PM which contains gap junctions. The fraction F1.5, corresponding to the 14% sucrose layer, is enriched in caveolae-derived membranes, and is not contaminated by other PM-derived membranes.

4.2.2.5 Immunodetection of SR Proteins

In addition to investigating the contamination of non-caveolar PM membranes, the presence of SR-derived membranes in the various fractions was also investigated. Specifically, immunoblots were done to test for the presence of the SR Ca^{2+} pump and IP_3 receptor (Type 1). As seen in Figure 20, both the IP_3 receptor and SR Ca^{2+} pump have strong signals in the more dense fractions, F2.5 and F3, while there is no detectable signal in the other fractions. That is, they are

not found in the caveolae-enriched fractions. The presence of the SR Ca^{2+} pump and IP_3 receptor in F2.5 and F3 indicates that SR-derived membranes are located in the more dense fractions, consistent with previous results (Ahmad et al., 1987). The lack of "crossover" between caveolae-derived membranes and SR-derived membranes suggests that the presence of calsequestrin and calreticulin in the caveolin-enriched fractions is not an artifact of SR contamination. Instead, these results demonstrate that these Ca^{2+} binding proteins are located in caveolae, in addition to their presence in SR. These results are consistent with those obtained with detergent-treated membranes from both tracheal and intestinal smooth muscle, and with the immunoprecipitation experiments on tracheal smooth muscle microsomes, demonstrating the co-immunoprecipitation of both calsequestrin and calreticulin with caveolin.

Collectively, the results from an alternate preparation of caveolae using Na_2CO_3 confirm the results obtained on the detergent-treated membranes. That is, the detergent-resistant membranes are caveolar-derived membranes, which contain the proteins necessary for a role in Ca^{2+} handling, specifically L-type Ca^{2+} channels, the PM Ca^{2+} pump, and the Ca^{2+} binding proteins calsequestrin and calreticulin. However, it is also possible that these proteins are not associated with caveolae or caveolin-enriched membranes but are located in similar buoyant density membranes or in non-caveolar, detergent-insoluble membranes.

Figure 20: Immunoblots demonstrating the distribution of caveolin and "non-caveolar" proteins in the fractions isolated by discontinuous sucrose gradient centrifugation of Na_2CO_3 -treated, sonicated MIC I canine intestinal smooth muscle membranes. The immunoblot for caveolin is the same as in Figure 19 and is included for comparison. While caveolin is enriched in the fractions F1.5 and F2, the SR Ca^{2+} pump and IP_3 receptor are found only in the fractions F2.5 and F3. Connexin-43 is found in the Fractions F2, F2.5, and F3, indicating some crossover between caveolae-derived and other non-caveolae PM, although the absence of a signal for connexin-43 in F1.5 indicates that it is possible to separate caveolae from other membranes. Immunoblots are representative of 4 separate experiments.

F1
F(1.5)
F2
F(2.5)
F3



Caveolin



Connexin - 43



SR Ca²⁺ Pump



IP₃ receptor

Chapter 5: DISCUSSION

This thesis describes for the first time, the isolation of a caveolae-enriched, detergent-resistant membrane fraction from canine tracheal smooth muscle. Further, it demonstrates the presence of L-type Ca^{2+} channels, the PM Ca^{2+} pump, calsequestrin, and calreticulin in this caveolin-enriched fraction. The presence of these proteins is consistent with our model of caveolae involvement in Ca^{2+} handling in airway smooth muscle.

These results were confirmed using other biochemical techniques. The association of calsequestrin and calreticulin with caveolae was confirmed by immunoprecipitation experiments where both calsequestrin and calreticulin were coimmunoprecipitated with caveolin, the marker protein of caveolae. The association of calsequestrin, calreticulin, PM Ca^{2+} pump, and L-type Ca^{2+} channels with caveolae was confirmed by isolation of caveolae from canine intestinal smooth muscle using a detergent-free procedure (Na_2CO_3 treatment of membranes followed by sonication and separation by discontinuous sucrose gradient centrifugation). In addition to Ca^{2+} handling proteins, nNOS was investigated in tracheal smooth muscle, and was present in the detergent-resistant membrane fraction. This result was confirmed by immunocytochemistry experiments demonstrating

	Trachea Detergent treated	Intestine Detergent treated	Intestine Na ₂ CO ₃ treated
Caveolin	enriched	enriched	enriched
5'-nucleotidase	high activity	high activity	moderate activity
Mg ²⁺ -ATPase	very low activity	very low activity	moderate activity
α ₁ chain of L-type Ca ²⁺ channel	enriched	enriched	present
[³ H]PN200-110 binding	2-3 fold enrichment	nd	nd
Calsequestrin	enriched	enriched	present
Calreticulin	enriched	enriched	present
PM Ca ²⁺ pump	present	present	present
SR Ca ²⁺ pump	absent	absent	absent
IP ₃ receptor	absent	absent	absent
Connexin-43	absent	absent	absent

Table 3: Comparison of results obtained of caveolae isolated using the different biochemical isolation methods. Results are expressed as the relative amount in the detergent-resistant membrane fractions, M3T, or in the caveolin-enriched Na₂CO₃-treated membranes, F1.5, compared with their respective control membranes (M3C for detergent-treated membrane fractions or MIC II for Na₂CO₃-treated membranes). Boxes marked "nd" indicate data not determined.

the colocalization of nNOS and caveolin at the cell periphery of fixed tracheal smooth muscle cryosections. A summary comparing the different caveolae isolation techniques employed is presented in Table 3, listing the various proteins which were tested for, using either enzyme assays or Western blot analysis.

5.1 Validity of Detergent Treatment Method of Caveolae Isolation

The isolation procedure used for tracheal smooth muscle was based upon methods used in other cell types (Sargiacoma et al., 1993; Schnitzer et al., 1995c; Parkin et al., 1996) and chicken gizzard smooth muscle (Chang et al., 1994) with some modification. The separation of detergent-resistant membranes from the rest of the membranes by discontinuous sucrose gradient centrifugation (with the total membrane fraction loaded on the top (Chang et al., 1994) or bottom (Sargiacoma et al., 1993; Schnitzer et al., 1995c) of the gradient was replaced with a faster and simpler centrifugation at 100,000g for 45 min to pellet the detergent-resistant fraction. The advantage of this method is convenience: 45min centrifugation versus 2hr or overnight for sucrose gradient centrifugation, as well as the time saved in not having to make the sucrose gradients. There are two potential disadvantages to this method: the presence of residual amounts of detergent in the isolated fraction, and the possibility that contaminating non-caveolar membranes are pelleted, leading to erroneous conclusions regarding the proteins located in

caveolae. These disadvantages will be addressed in later sections.

As discussed in the Introduction, the only true marker of caveolae-derived membranes is caveolin. It forms the protein coat on the cytoplasmic face of caveolae and is a structural protein, interacting with other proteins localized to caveolae via a protein-protein interaction between a scaffolding domain in caveolin and a domain rich in aromatic amino acids on the other proteins (Li et al., 1996). The results presented in Figures 6 and 17 (Sections 4.1.1.2 and 4.2.1.2) show an enrichment of caveolin in the detergent-resistant membrane fractions when compared with the starting materials (untreated microsomal membranes). These results are consistent with the results of other investigators using detergent treatment in other cell types (Sargiacoma et al., 1993; Schnitzer et al., 1995c) or chicken gizzard smooth muscle (Chang et al., 1994). This would suggest that our detergent treatment produced a fraction enriched in caveolae-derived membranes.

The antibody used for identifying caveolin in the various membrane fractions is a polyclonal antibody, generated by using amino acids 1-97 of the human caveolin-1 protein as immunogen (Transduction Laboratories, Product Information Sheet C13630). In our hands it consistently identified two proteins of approximately 22 and 24kDa, which corresponds to the two caveolin-1 isoforms (Scherer et al., 1995). No band was visible at 18kDa, which would correspond to the muscle-specific isoform, caveolin-3, although it is unclear whether the antibody recognizes this isoform. Although antibodies to the muscle specific isoform are now

commercially available, the presence of the muscle specific caveolin isoform was not investigated.

As mentioned, there are potential disadvantages of the methodology used. The first is the presence of detergent in the detergent-insensitive membrane fraction which can interfere with some of the biochemical methods used for analysis of the M3T fraction. The detergent-insensitive membrane pellet is resuspended in the centrifuge tube which contained 1% Triton X-100. Even with careful rinsing of the sides of the tube to attempt to remove any detergent, it is virtually impossible to remove all traces of detergent without risking disrupting and losing part or all of the detergent-resistant membrane pellet. The presence of residual Triton X-100 does not affect Western blot analysis, since the samples are placed in a buffer containing 3% SDS and β -mercaptoethanol, which are intended to disrupt the membranes and denature all proteins in order to separate them by size. Levels of Triton X-100 exceeding 0.1% can affect some protein assays such as the Bradford protein microassay (Bradford, 1976), while the Lowry assay is unaffected by Triton X-100 concentrations of less than 1%. Other enzyme assays (5'-nucleotidase and Mg^{2+} -ATPase) were affected at levels of detergent one-tenth that used for isolation of the detergent-resistant fraction, as indicated in Table 2. The detergent increased 5'-nucleotidase activity and significantly decreased Mg^{2+} -ATPase activity in the MIC II or M3C fractions ($p < 0.05$ for both MIC II and M3C) down to a level approaching that seen in the detergent-resistant fraction, M3T. However, the activities in the

detergent-treated MIC II and M3C membranes were still significantly higher than those in M3T ($p < 0.05$), indicating that the loss of Mg^{2+} -ATPase activity in M3T could not be explained solely as an effect of detergent on the assay itself, but was also due to the loss of some membranes which contained Mg^{2+} -ATPase activity. Presumably these were derived from non-caveolar regions of the PM.

The second potential disadvantage of this methodology, and of detergent treatment in general, is the possibility of non-caveolar membranes or proteins being contained in the detergent-insensitive membrane fraction. The presence of these membranes could be due to incomplete disruption by the detergent treatment, or due to protein sorting during the treatment, or lastly, to the presence of non-caveolar, detergent-insensitive membranes. The absence of connexin-43, the SR Ca^{2+} pump, and IP_3 receptor in the detergent-resistant fraction isolated from either tracheal or intestinal smooth muscle argues against the possibility that non-caveolar PM or SR were contaminating the detergent-resistant membrane fraction, M3T. However the possibility exists that regions of the PM which do not contain gap junctions could be present in the detergent-resistant fraction. To rule out the presence of these other regions of the PM in the detergent-resistant fraction, further immunoblotting could be done using, for example, anti-vinculin antibodies to test for the presence of those PM-derived membranes which contain dense bodies.

The presence of calsequestrin and calreticulin, two SR resident proteins, in the detergent-insensitive fraction from both tracheal and intestinal smooth muscle

suggests that protein resorting during the detergent treatment is possible. However, the results of the immunoprecipitation experiments using tracheal smooth muscle microsomes (Figure 9, Section 4.1.2) would indicate that both Ca^{2+} binding proteins are associated with caveolin prior to detergent treatment. Further, results in intestinal smooth muscle using an established detergent-free method of isolation of caveolae (Section 4.2.2.3) demonstrated that calsequestrin and calreticulin are present in the caveolin-enriched membrane fractions. Alternatively, the association of calsequestrin and calreticulin with caveolin or caveolin-enriched membrane fractions may be an artifact of the different methods. The solubilization of all membranes during the immunoprecipitation experiments may allow calsequestrin or calreticulin to be released from SR-derived vesicles and then associate with caveolin. The Na_2CO_3 treatment should cause the disruption of all membrane vesicles resulting in the loss of all luminal contents. The release of calsequestrin and calreticulin, which are luminal SR proteins, may result in their association with caveolin-enriched membranes. However, the alkaline pH should prevent any protein-protein interactions, arguing against this possibility. The most likely explanation of these results is that the presence of calsequestrin and calreticulin in the detergent-insensitive membrane fractions is not an artifact of protein sorting from detergent-sensitive membranes to detergent-insensitive caveolar membranes, but represents the localization of calsequestrin and calreticulin to caveolae, in addition to their previously established location in SR.

5.2 Validity of Na₂CO₃ Treatment Method of Caveolae Isolation

The presence of non-caveolar membranes in the detergent-insensitive membrane fraction has been an issue of intense debate over the last few years. As discussed in the introduction (Section 1.5.2), many investigators have argued that detergent treatment leads to erroneous conclusions, with some proteins redistributed to detergent-resistant membranes which are not located in caveolae *in vivo*. Specifically, the presence of GPI-linked proteins in detergent-resistant membranes is considered an artifact by some (e.g. Mayor and Maxfield, 1995; Fujimoto, 1996), while others have argued that it is not an artifact (Anderson, 1998). For this reason, a second method of caveolae isolation was employed. This detergent-free method involved exposure of the membranes to a Na₂CO₃ buffer (pH 11.0), sonication to disrupt the membranes, and discontinuous sucrose gradient centrifugation to separate the caveolae from other membranes. Previously, this method has been employed using cultured epithelial cells (Song et al., 1996a). Song et al. (1996a) demonstrated that the caveolin-enriched fraction contained G protein α_{12} and β subunits, c-Src, and Ras, and excluded most other cellular proteins, including the GPI-linked protein, carbonic anhydrase IV. More recently, Ishizaka et al. (1998), using cultured vascular smooth muscle cells localized the angiotensin type II receptor to caveolin-enriched membranes following stimulation of the cells with angiotensin II. Further, they stated that G protein $\alpha_{q/11}$ and β

subunits, phospholipase C- γ 1, and phospholipase D were also present in these membranes, although no data were presented.

Canine intestinal smooth muscle was chosen as a model for comparing the proteins associated with detergent-treated membranes and caveolin-enriched Na_2CO_3 -treated membranes. Intestinal smooth muscle has not been demonstrated to share similar Ca^{2+} handling properties as tracheal or bronchial smooth muscle. That is, there is no evidence in the literature demonstrating that agonist stimulation of intestinal smooth muscle in a Ca^{2+} -free medium results in a sustained contraction (as in bronchial smooth muscle) or that refilling of the SR occurs via a mechanism involving L-type Ca^{2+} channels (as in tracheal smooth muscle). However, intestinal smooth muscle has numerous caveolae (Gabella, 1971, 1976, 1981a), increasing the PM surface area by 73% (Gabella, 1976). There is a close association between caveolae and peripheral SR, similar to the structures seen in tracheal smooth muscle (Gabella, 1971). Large quantities of the circular smooth muscle layer can be separated from other cell types (e.g. epithelium) quickly and easily, and a protocol for the preparation of PM-enriched microsomes has already been established (Ahmad et al., 1987). While caveolae from intestinal smooth muscle may not have the same Ca^{2+} handling proteins as tracheal smooth muscle, their presence is not essential to our purposes. Rather, the goal of this study was to demonstrate that proteins localized to detergent-treated membranes isolated from intestinal smooth muscle could also be localized to the caveolin-enriched, Na_2CO_3 -

treated membranes isolated from intestinal smooth muscle.

The method of Song et al. (1996a) was modified in that the sonicated, Na_2CO_3 -treated membranes were loaded on the top, instead of the bottom, of a discontinuous sucrose gradient, which was also altered. Instead of a 5/35/45% gradient, the gradient used was 4/14/25/35/48%, similar to that used by Chang et al. (1994) and that used previously in our laboratory (Ahmad et al., 1987). Caveolae were expected to be localized in the 14/25% interface and 25% layer according to the results of Chang et al. (1994). The additional sucrose concentrations were included to allow for a separation of caveolae from any contaminating SR-derived, or synaptosomal membranes, both of which should migrate at the 25/35% interface (F3) according to previous results (Ahmad et al., 1987). Caveolin was detected in all of the fractions, with the strongest signal in the F1.5 and F2 fractions (14% layer and 14/25% interface) while detection of the SR Ca^{2+} pump and IP_3 receptor (Type 1) was limited to the F2.5 and F3 fractions (25% and 25/35% interface). These results confirmed a separation between caveolae-derived and SR-derived membranes. The presence of caveolin in the denser fractions (F2.5 and F3) may be due to caveolin associated with Golgi-derived membranes or may be due to the association of caveolae-derived membranes with other denser membrane fractions. That is, caveolar membranes that are still attached to other membranes or trapped within denser membranes. Connexin-43 was detected in the F2, F2.5, and F3 fractions (14/25% interface, 25% layer,

25/35% interface), indicating some "crossover" between caveolar membranes and other PM-derived membranes at the 14/25% interface. However, the absence of a connexin-43 signal in F1.5 indicated that caveolin-enriched membranes could be separated from other PM-derived membranes, at least those membranes containing gap junctions. The presence of proteins from other regions of the PM, such as dense bodies, was not investigated, and so the possibility exists that other regions of the PM are also located in the caveolin-enriched membrane fractions. However, the results of other investigators would argue against this possibility. Previous studies (Song et al., 1996a; Ishizaka et al., 1998) demonstrated that the caveolin-enriched fraction isolated using the Na_2CO_3 method excluded other non-caveolar PM proteins (carbonic anhydrase IV, clathrin, CD36, and dynamin). Further, Ishizaka et al. (1998) demonstrated that the AT_1 receptor was localized in the caveolin-enriched fraction when cells were stimulated with agonist, and located at the bottom of the centrifuge tube in the absence of agonist. This would suggest that the non-caveolar regions of the PM, including those regions very close to the caveolae, can be separated from the caveolin-enriched fractions.

There were some differences in our results compared with previous results. The presence of SR proteins and non-caveolar proteins in the fractions isolated from intestinal smooth muscle was not expected based on the claims of other investigators (Song et al., 1996a; Ishizaka et al., 1998). Neither study indicated if SR membrane proteins were found in their isolated fractions, and no data were

presented indicating that SR proteins were investigated. The study by Song et al. (1996a) stated that SR membranes (and other internal cell membranes) were excluded based on the use of similar methods (discontinuous sucrose gradient centrifugation) to those in previous studies, although some of these used detergent treatment of membranes prior to separation on the sucrose gradients (e.g. Li et al., 1995). Both studies did present data indicating the exclusion of non-caveolar PM proteins in the caveolin-enriched fraction (carbonic anhydrase IV, clathrin, CD36, and dynamin). These proteins were found at the bottom of the gradients. It may be that loading of the proteins on the bottom of the gradient and/or the addition of high sucrose concentrations (45%, final concentration) to the sonicated, Na_2CO_3 -treated membranes results in the disruption of all but caveolar membranes. Regardless, the results presented here indicate that while the method employed did not give a definitive separation of caveolar and non-caveolar membranes, it did separate some of the caveolin-enriched membranes from non-caveolar membranes.

The caveolin-enriched membrane fractions from intestinal smooth muscle were not found at the expected sucrose concentrations. They were located at the 14% layer and 14/25% interface. It had been previously shown that caveolae isolated from smooth muscle (chicken gizzard) using detergent treatment were found at the 14/25% interface and 25% layer (Chang et al., 1994). The sucrose concentrations reported by Chang et al. (1994) were calculated as weight/volume while the concentrations reported here were calculated as weight/weight. Further,

the sucrose concentrations used for this study were made in a buffer solution containing 250mM Na₂CO₃. Measurement of the densities of the various sucrose concentrations used here indicate that the densities correspond to sucrose concentrations of 5/18/28/38/52% (w/w). Measurement of the densities of the sucrose concentrations used by Chang et al. (1994) made in a buffer solution containing 300mM NaCl, indicate that the 14 and 25% concentrations correspond to 13 and 24% (w/w), respectively. The higher densities employed here would explain the apparent shift in localization of the caveolin-enriched membranes to higher fractions (F1.5 and F2). Further, it is possible that the change in densities affected the separation of caveolin-enriched membranes and non-caveolar membranes.

Total protein recovery from the fractions collected from canine intestinal smooth muscle was only calculated from two membrane preparations. Total recovery of the 5 fractions (F1-F3) was 26-30% of the starting material. The caveolin-enriched fractions F1.5 and F2 had protein recoveries of 6.0-6.5% and 6.3-6.9% of total starting material, respectively. Both of the previous studies using cultured cells (Song et al., 1996a; Ishizaka et al., 1998) indicated that the majority of the cellular protein was contained at the bottom of the centrifuge tubes, with only a small proportion of protein in the caveolin-enriched fractions. Approximately 2.7% of total cellular protein was in the caveolin-enriched fraction isolated from vascular smooth muscle cells (Ishizaka et al., 1998). Although it appears that the recovery

from intestinal smooth muscle was greater than the previous studies using cultured cells, these results should be interpreted cautiously. Recoveries from intestinal smooth muscle were only calculated from two membrane preparations and further, the starting material from the previous studies was a total cell lysate, whereas a partially purified membrane fraction (MIC I) was used as the starting material for the membrane fractions isolated from intestinal smooth muscle.

The most unanticipated result from the Na_2CO_3 -treated membrane fractions isolated from intestinal smooth muscle was the distribution of PM marker enzyme activities. Specifically, the activity of 5'-nucleotidase, a GPI-linked protein, in the fractions isolated indicated that all but F3 had higher activity than MIC II, with F1, F1.5, and F2 all significantly higher ($p < 0.05$ for all versus MIC II). Fractions F1.5 and F2 had the highest activity, paralleling the distribution of caveolin. These results contrast those of Song et al. (1996a) which indicated exclusion of carbonic anhydrase IV, another GPI-linked protein, in the caveolin-enriched fraction using the Na_2CO_3 method of isolating caveolae, and support the results of Chang et al. (1994) who demonstrated the presence of GPI-linked proteins in their detergent-insensitive membrane fraction isolated from chicken gizzard smooth muscle. It is unclear whether this discrepancy results from differences in tissues (smooth muscle versus epithelium) or from differences in the methods used.

One possibility is that 5'-nucleotidase behaves differently than other GPI-linked proteins, and is located in caveolae *in vivo*. Using cytochemical techniques,

5'-nucleotidase has been localized to guinea pig ileum smooth muscle caveolae (Forsman and Gustafsson, 1985). However, the methods used could allow artifactual clustering in caveolae. The tissues were fixed with 4% paraformaldehyde prior to the enzyme assay procedure, and then fixed with glutaraldehyde. Some have argued that fixation with paraformaldehyde, which provides minimal protein cross-linking, could allow movement of the protein or the reaction product during the enzyme assay (Mayor and Maxfield, 1995; Fujimoto, 1996)

Alternatively, it may be that the composition of smooth muscle caveolae differs from caveolae in other cell types. Gabella (1978, 1981b) reported that smooth muscle caveolae are unique compared to other cell types ultrastructurally. While caveolae in smooth muscle are uniform in size, caveolae from cardiac muscle cells are smaller, non-uniform, and can be irregular in shape (Gabella, 1978). Further, while smooth muscle caveolae are usually arranged in rows along the long axis of the cell, caveolae from cardiac muscle cells and endothelial cells are randomly distributed (Gabella, 1978, 1981b). Sarcoplasmic reticulum is often associated with smooth and cardiac muscle caveolae, while endoplasmic reticulum is not or rarely associated with endothelial caveolae (Gabella, 1978, 1981b). Many investigators consider endothelial caveolae to be unique compared to other cell types since they are involved in transport of macromolecules across the endothelium (i.e. transcytosis; Schnitzer et al., 1994, 1996; Anderson, 1998). The

results presented here indicate that smooth muscle caveolae may differ in their protein components as well as in their structure compared with other cell types. While the PM Ca^{2+} pump has been localized to caveolae of endothelial cells as well as other smooth muscle cells by immunoelectron microscopy (Fujimoto, 1993), L-type Ca^{2+} channels and the Ca^{2+} binding proteins calsequestrin and calreticulin have not been previously localized to caveolae in any other cell types. However, no investigators have specifically investigated the association of these proteins with caveolae. The results indicating that 5'-nucleotidase is associated with caveolae-derived membranes may reflect a unique property of smooth muscle caveolae.

5.3 Proteins Localized to Smooth Muscle Caveolae

5.3.1 Plasma Membrane Ca^{2+} Pump

The presence of the PM Ca^{2+} pump in caveolar membranes is consistent with previous immunoelectron microscopy studies (Fujimoto, 1993) demonstrating the PM Ca^{2+} pump in caveolae of mouse intestinal smooth muscle. However, our results do not indicate an enrichment of the PM Ca^{2+} pump in caveolae, using either detergent- or Na_2CO_3 -treatment methods of caveolae isolation, in contrast with the results of Fujimoto (1993). It is unclear why there is this discrepancy. It could be due to species differences between mouse and dog, or due to differences between

the experimental methods used.

The failure of immunoprecipitation experiments supports the Western blot results indicating that the PM Ca^{2+} pump is not enriched in caveolae. The available protein sequence for the smooth muscle PM Ca^{2+} pump (De Jaegere et al., 1990) has no sequences corresponding to the caveolin binding domain. Thus it is not surprising that the PM Ca^{2+} pump was not enriched in detergent-resistant membranes, and failed to coimmunoprecipitate with caveolin. Instead, it is more likely that the PM Ca^{2+} pump is evenly distributed across the entire PM (including caveolae). Our immunocytochemical results indicated that caveolin and the PM Ca^{2+} pump were not located at discrete sites on the cell surface but diffusely distributed. However, due to the limits of the microscope resolution, it is impossible to determine with certainty whether they are colocalized. Confocal microscopy methods or immunoelectron microscopy are required to address whether or not these two proteins were colocalized at a subcellular level.

5.3.2 L-type Ca^{2+} Channels

The localization and enrichment of L-type Ca^{2+} channels to caveolar membranes isolated from either intestinal or tracheal smooth muscle is a novel finding. This was demonstrated by immunoblotting using an antibody directed against the α_1 chain of the smooth muscle L-type Ca^{2+} channel in both the

detergent-treated membranes from tracheal and intestinal smooth muscle, and in the Na_2CO_3 -treated membranes from intestinal smooth muscle. The results were also confirmed in detergent-treated tracheal smooth muscle membranes using radioligand binding (Section 4.1.1.4). Although these results are novel, they are consistent with our model of Ca^{2+} entry (and recycling) in airway smooth muscle through L-type Ca^{2+} channels located in caveolae.

Calculations of total recovery of [^3H]PN200-110 binding in the detergent-treated and control membranes (M3T and M3C, respectively) isolated from tracheal smooth muscle indicated that both fractions contained comparable amounts of dihydropyridine binding sites (Table 2). This suggested that most of the L-type Ca^{2+} channels in tracheal smooth muscle were located in caveolae. The enrichment of the α_1 chain of the L-type Ca^{2+} channel in M3T isolated from both tracheal and intestinal smooth muscle as indicated by Western blotting (Figures 6 and 17) was consistent with this. Total recovery of [^3H]PN200-110 binding in M3T and M3C was only 36.3% and 38.4%, respectively, of the original MIC II fraction. This suggests that while there is an enrichment of L-type Ca^{2+} channels in the detergent-insensitive caveolar membranes, some L-type Ca^{2+} channels may be located on other areas of the PM.

The association of L-type Ca^{2+} channels with caveolin-enriched membranes was confirmed using an alternate caveolae isolation procedure (Na_2CO_3 treatment). There was a strong signal corresponding to the α_1 chain of the L-type Ca^{2+} channel

in the caveolin-enriched fractions, F1.5 and F2, in Western blots. However, a second population of α_1 chains was also found in the F2.5 and F3 fractions. This second population may be synaptosomes present in the originating MIC I fraction. The MIC I fraction can be separated by a 10,000g centrifugation into the MIT II and MIC II membrane fractions. As was indicated by Western blotting of intestinal and tracheal membrane fractions (Figures 6 and 17), and by radioligand binding using tracheal membrane fractions (Figure 7), the MIT II fraction contains a considerable amount of L-type Ca^{2+} channels, which at least in the intestine, have been attributed to synaptosomal membranes (Ahmad et al., 1989). These membranes have been isolated by sucrose gradient centrifugation at the 25/35% interface, which corresponds to the F3 fraction by the methods used here. Thus, the relative abundance in Western blots of the α_1 chain in the caveolin-enriched fractions compared with the F2.5 and F3 fractions may be due to contamination of the latter fractions by synaptosomes containing large amounts of the α_1 chain.

Caveolin and the α_1 chain of the L-type Ca^{2+} channel did not coimmunoprecipitate, in spite of the presence of a caveolin binding domain on a region of the α_1 chain located on the cytoplasmic face of the protein (Mikami et al., 1989). Several possibilities explain why the immunoprecipitation experiments failed. The L-type Ca^{2+} channel is as a complex of 4 subunits (α_1 , α_2 , β , and δ). It is possible that the interaction with the other 3 subunits makes the caveolin binding domain on the α_1 chain inaccessible to caveolin. Alternatively, it is possible

that caveolin does not bind to the α_1 chain, but to one of the other subunits. The immunoprecipitation experiments were done under non-denaturing conditions, according to the method of Feron et al. (1996), in order to maintain native protein-protein interactions. More specifically, the conditions were designed to maintain the interaction between caveolin and eNOS. However, these conditions may disrupt the interactions between caveolin and the α_1 chain or the other subunits of the L-type Ca^{2+} channel. Additionally, these conditions may disrupt the interactions between the different L-type Ca^{2+} channel subunits. If this latter were the case, then even if caveolin binds to another of the L-type Ca^{2+} channel subunits, this would not be detected since we used an antibody directed against the α_1 subunit. Irrespective of the immunoprecipitation experiments, the enrichment of the L-type Ca^{2+} channel to detergent-insoluble caveolar membranes isolated from tracheal or intestinal smooth muscle was confirmed using a detergent-free method of isolating caveolae.

5.3.3 Type 1 IP_3 Receptor

The absence of the type 1 IP_3 receptor in both the detergent-resistant membrane fractions and the caveolin-enriched Na_2CO_3 -treated fractions contrasts with the previous results in smooth muscle (Fujimoto et al., 1993) and other cell types (Fujimoto et al., 1993; Schnitzer et al., 1995c). Immunoelectron microscopy

demonstrated that in mouse endothelial and epithelial cells, and intestinal smooth muscle cells, the type 1 IP₃ receptor was localized to caveolae (Fujimoto et al., 1993). These results were not due to antibody-induced clustering. The protein could not be recognized by all IP₃ receptor antibodies used, which suggested that the protein may not be the IP₃ receptor, but a related protein. Further work by Fujimoto et al. (1995) has shown that this protein has a molecular mass of 240kDa, which corresponds to the IP₃ receptor, although Fujimoto hypothesized that the protein may be a structural protein, linked to the actin cytoskeleton. More recently, studies by other investigators have localized the type 3 IP₃ receptor subtype to the PM by immunoelectron microscopy in *Xenopus* oocytes overexpressing the type 3 receptor (DeLisle et al., 1996) which may be localized to PM caveolae. The antibody used in our studies was specific for the type 1 IP₃ receptor, which was absent from caveolar membranes using both isolation methods. Our results do not exclude the possibility that the type 3 IP₃ receptor is located in caveolae from tracheal and intestinal smooth muscle.

Some have suggested that the capacitative Ca²⁺ entry model be modified in smooth muscle such that store-operated Ca²⁺ channels (analogous to I_{CRAC}, mentioned in Section 1.2.1) have a variable role in Ca²⁺ entry (Gibson et al., 1998). That is, while the majority of Ca²⁺ entry is attributed to voltage-gated Ca²⁺ channels, a smaller or variable component is voltage-independent, and attributed to these store-operated Ca²⁺ channels. However, previous results indicate that the agonist-

induced, sustained contraction by bronchial smooth muscle in Ca^{2+} -free medium could be blocked by the addition of L-type Ca^{2+} channel blockers (Montano et al., 1993, 1996). If there were functional IP_3 receptors in caveolae, then agonist stimulation, which produces IP_3 , should have resulted in sustained contractions. The refilling of the SR in trachea was dependent upon functional L-type Ca^{2+} channels (Bourreau et al., 1991, 1993). Refilling of the SR, in the presence of an SR Ca^{2+} pump inhibitor, was augmented by Bay K 8644, an L-type Ca^{2+} channel opener, and was almost completely blocked by the addition of nifedipine, although a small component was not blocked. These contractility results in airway smooth muscle would suggest that the route of Ca^{2+} entry is via L-type Ca^{2+} channels, and not via an IP_3 receptor. It is possible that the type 3 IP_3 receptor, if located in caveolae, may have a role other than as a functional Ca^{2+} channel. One such role is the postulated structural role suggested by Fujimoto et al. (1995).

5.3.4 Calsequestrin and Calreticulin

Immunoblots of detergent-resistant membranes and caveolin-enriched Na_2CO_3 -treated membranes, and immunoprecipitation experiments all consistently demonstrated the association of calsequestrin and calreticulin with caveolin or caveolin-enriched membranes. The presence of calsequestrin and calreticulin in the denser Na_2CO_3 -treated membrane fractions (F2.5, F3) indicates an additional

association of these proteins with a second population of membranes, likely SR-derived membranes. Collectively, these results indicate that in addition to the established cellular location of calsequestrin and calreticulin in SR, these Ca²⁺ handling proteins are also associated with caveolae.

The presence of calreticulin and calsequestrin in smooth muscle caveolae is a novel finding. Calreticulin is a resident SR protein which has the C-terminal KDEL amino acid sequence which is the ER/SR retention signal (Munro and Pelham, 1987). Despite this, there is a growing body of evidence that calreticulin is not located exclusively in SR. Calreticulin has been detected in plasma at low levels (Sueyoshi et al., 1991). It has been identified on the surface of cultured mouse melanoma cell line, where it plays a role in cell spreading (White et al., 1995), and on the surface of cultured fibroblasts, where it binds to fibrinogen (the B β chain) and mediates their mitogenic activity (Gray et al., 1995). Calreticulin binds purified coagulation factors (Factor IX/IXa) in a concentration-dependent and saturable manner (Kuwabara et al., 1995). Further, Kuwabara et al. (1995) demonstrated that calreticulin binds to the extracellular surface of endothelial cells *in vitro* and *in vivo*. Calreticulin bound cultured endothelial cells and stimulated nitric oxide production in a concentration-dependent manner. Infused calreticulin bound to endothelial cells and prevented thrombosis in a canine thrombosis model. In addition to its extracellular localization, calreticulin has been localized to other intracellular locations, such as the nuclear envelope and cytoplasm (reviewed in

Nash et al., 1994; Krause and Michalak, 1997).

Studies in smooth muscle have investigated the presence of calreticulin by Western blot in total membrane fractions (Villa et al., 1993) and in purified SR membrane fractions (Milner et al., 1991; Raeymaekers et al., 1993) or by immunoelectron microscopy in fixed cryosections (Villa et al., 1993). In those studies which used purified SR membranes, purified PM fractions were not tested, leaving the possibility that calreticulin could be located in regions other than SR. In the lone study using immunoelectron microscopy (Villa et al., 1993), calreticulin was distributed evenly between peripheral and deep SR. However, some micrographs showed gold particles attributed to peripheral SR located on circular structures just below the PM. These structures could be caveolae, in sections where the plane of the section misses the necks of the caveolae. Similar structures have been seen in other electron microscopy studies of smooth muscle (e.g. Devine et al., 1972) which have been shown to be connected to the extracellular space using extracellular tracers, and identified as caveolae.

Prior to this study, calsequestrin had been localized only to SR in all muscle cells investigated. Studies in smooth muscle using Western blot techniques have employed purified SR membrane fractions (Raeymaekers et al., 1993; Volpe et al., 1994) or total membrane fractions (Villa et al., 1993). Similar to the studies of calreticulin, those studies where purified SR membranes were investigated, purified PM fractions were not tested (Raeymaekers et al., 1993; Volpe et al., 1994). Thus,

the possibility exists that calsequestrin may also have additional cellular locations in addition to the SR. In the immunoelectron microscopy study by Villa et al. (1993), calsequestrin was localized predominantly in peripheral SR. However, some micrographs appeared to show gold particles in circular structures just below the PM which may be caveolae. As mentioned in the introduction, a study by Moore et al. (1993) investigating the distribution of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, Na^+/K^+ ATPase, and calsequestrin in toad stomach smooth muscle cells by immunocytochemical methods, colocalized all three proteins to discrete sites on or near the PM. The authors concluded that these sites were consistent with localization to caveolae (for the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and Na^+/K^+ ATPase) and closely associated peripheral SR (for calsequestrin). Based on our findings presented here, we suggest that these conclusions be reinterpreted. That is, calsequestrin localized to these discrete sites is located in caveolae. However, based on our results of Na_2CO_3 -treated membranes indicating a second population of calsequestrin in non-caveolar membranes, it is likely that calsequestrin is also associated with the peripheral SR.

The coimmunoprecipitation of calsequestrin and calreticulin with caveolin indicates that these caveolae-associated Ca^{2+} binding proteins are located on the cytoplasmic surface of caveolae. Analysis of the available protein sequences for calsequestrin (Scott et al., 1988) and calreticulin (Rokeach et al., 1991) indicates that both have a sequence corresponding to the caveolin binding domain. The experimental methods used allow only a qualitative analysis, so we are unable to

compare the relative amounts of calsequestrin or calreticulin associated with the detergent-treated membranes, Na_2CO_3 -treated membranes, or immunoprecipitated caveolin. Thus, it is unknown if all of the calsequestrin and calreticulin associated with caveolae are located on the cytoplasmic surface or if some of the calsequestrin and calreticulin is also located on the extracellular/luminal surface. Further no experiments were done to test if calsequestrin or calreticulin were located on the extracellular surface of the cell.

An alternative explanation for the immunoprecipitation experiments is that the calreticulin and calsequestrin were released from SR-derived vesicles during the solubilization step and associated with caveolin. Further, the detergent solubilization step used for isolation of detergent-resistant membranes could also release calsequestrin and calreticulin from SR vesicles and allow them to associate with caveolin or caveolin-enriched vesicles. That is, both the detergent solubilization method and immunoprecipitation method may allow artifactual association of calsequestrin and calreticulin with caveolin or caveolin-enriched vesicles. The results of the Na_2CO_3 -isolation method indicated that both calsequestrin and calreticulin were associated with the caveolin-enriched fractions, as well as the other fractions isolated. Treatment of membrane vesicles with Na_2CO_3 at alkali pH has been shown to cause the release of luminal contents and remove non-membrane bound proteins. Further, electron micrographs of Na_2CO_3 -treated membranes indicate that they exist as membrane sheets, and not intact

vesicles (Fujiki et al., 1982a,b), although these studies only investigated mitochondrial, ER, and peroxisomal membranes, and not PM-derived membranes. Based on the results of Fujiki et al. (1982a,b), calsequestrin and calreticulin, which are non-membrane bound proteins contained in the lumen of SR vesicles, should not remain associated with any membranes using this isolation method. This allows the possibility that our results using the Na_2CO_3 treatment method, indicating the presence of calsequestrin and calreticulin, were also an artifact of the experimental method. However, more recent studies (Vaugh et al., 1998, 1999) have demonstrated that using the method of Song et al. (1996a), the caveolin-enriched membrane fractions consist mainly of intact vesicles ranging in size from 40-250nm in diameter. Further, these vesicles could be immunoprecipitated with caveolin antibodies (Vaugh et al., 1998). The immunoprecipitated vesicles ranged in size from 50-75nm, similar in size to caveolae *in vivo*. The range of vesicle sizes would suggest that the Na_2CO_3 treatment method results in the isolation of both caveolin-enriched membranes, as well as other membranes at similar buoyant densities. This is consistent to the results presented here indicating some "cross-over" between caveolin-enriched membrane fractions and membranes containing connexin-43. More importantly, the finding that the Na_2CO_3 method can result in intact membrane vesicles suggests that the localization of calsequestrin and calreticulin to the caveolin-enriched fraction may not be an artifact of the treatment method. It is possible that the Na_2CO_3 treatment method may allow membrane

vesicle contents to be released and then taken up by other vesicles prior to those vesicles resealing. This possibility could be tested by looking for the presence of another SR luminal protein in the caveolin-enriched membrane fractions which lacks the caveolin binding motif.

5.3.5 Nitric Oxide Synthase (Neuronal)

The colocalization of nNOS with caveolin at the cell periphery in tracheal smooth muscle cryosections is a novel finding. Western blots confirmed the results of immunohistochemistry. A band was visible in tracheal smooth muscle membrane fractions at ~160kDa which corresponds to nNOS. Further, there was an apparent enrichment in the M3T fraction, indicating enrichment in caveolar membrane domains. There is no previous evidence for the presence of a functional NOS in airway smooth muscle. Studies in guinea pig indicated that the addition of N^ω-nitro-L-arginine methyl ester (L-NAME), an NOS inhibitor, had no effect on histamine-induced contractions of isolated perfused tracheal tubes following removal of the epithelium (Nijkamp et al., 1993). However, similar functional studies have not been conducted on airway smooth muscle tissues from the dog. Recent studies in canine lower esophageal sphincter (LES) indicate that the smooth muscle cells contain a membrane-bound form of nNOS (Salapatek et al., 1998a,b). This nNOS is unique in that it is recognized only by an antibody made in guinea pig against the

C-terminal of rat cerebellum nNOS (the same antibody used in our study) and not by other antibodies specific for nNOS. This enzyme is functional, since the addition of the NOS inhibitor, N^G-Nitro-L-arginine (L-NNA) increased tone of the isolated muscle strips, and L-NAME blocked NOS activity in a biochemical assay using microsomes isolated from LES smooth muscle tissues or isolated LES smooth muscle cells (Salapatek et al., 1998a). More recent work indicates that this antibody also recognizes a membrane bound form of NOS in other visceral smooth muscle cells of the dog, including the circular muscle layer of the small intestine (Wang, Y.F. and E.E. Daniel, unpublished observations), although evidence that the nNOS in these tissues is functional has yet to be obtained. Additionally, nNOS has been detected in the smooth muscle cells of the opossum internal anal sphincter and other gastrointestinal tissues, and in cultured human intestinal smooth muscle cells as determined by Northern blot hybridization techniques (Chakder et al., 1997)

The localization of the neuronal form of NOS to non-neuronal tissue is not novel. It has been well characterized in skeletal muscle of mouse and rat. This PM-associated protein is linked to the dystrophin glycoprotein complex (Brenman et al., 1995, 1996) and is involved in modulating contractile force (Kobzik et al., 1994). The nNOS in skeletal muscle is a novel isoform termed nNOS_μ. Alternative splicing results in a protein which is slightly larger than brain nNOS, owing to a 34 amino acid insert near the calmodulin binding site and has similar catalytic activity

to cerebellar nNOS (Silvagno et al., 1996). Antibodies specific for nNOS μ have localized the protein to skeletal and cardiac muscle in the rat (Silvagno et al., 1996). No smooth muscle tissues were investigated.

In addition to its interaction with the dystrophin complex in skeletal muscle, nNOS also interacts with caveolin-3 (Venema et al., 1997), as discussed in the introduction (Section 1.4.3). Caveolin-3 and nNOS could be coimmunoprecipitated from rat quadriceps muscle homogenates, and further, peptides corresponding to the caveolin binding domain from both caveolin-1 and -3 inhibited the enzymatic activity of purified recombinant nNOS (Venema et al., 1997). Similarly, the endothelial form of NOS also interacts with caveolin-1 and -3 (Feron et al., 1996; Garcia-Cardena et al., 1997; Ju et al., 1997; Michel et al., 1997a,b). Interestingly, eNOS has been localized to cardiac muscle cells, where it interacts with both caveolin-1 and -3 (Feron et al., 1996), further confusing the present nomenclature of NOS isoforms.

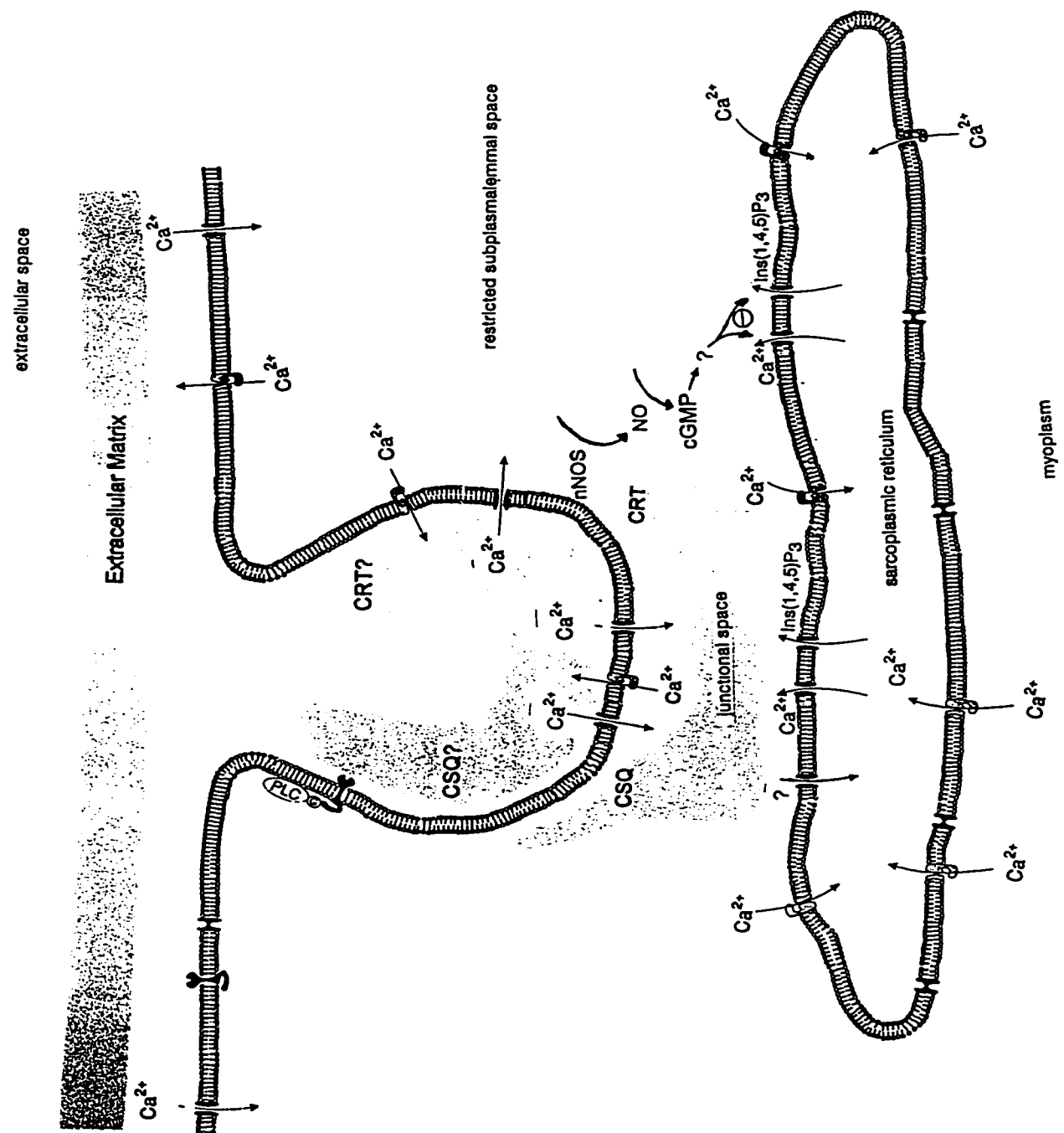
5.4 Model of Ca²⁺ Handling

As stated in the introduction and design/rationale chapters, our initial model for Ca²⁺ handling in airway smooth muscle involved a close association between the SR and a specialized region of the PM which we hypothesized to be caveolae. We proposed that these caveolae contain L-type Ca²⁺ channels, to allow Ca²⁺ entry, a

PM Ca^{2+} pump for Ca^{2+} removal/extrusion, and Ca^{2+} binding proteins to sequester Ca^{2+} within the lumen of the caveolae to prevent its loss to the surrounding extracellular space. The results presented here demonstrated the presence of L-type Ca^{2+} channels and PM Ca^{2+} pump in caveolae, and the association of two Ca^{2+} binding proteins, calsequestrin and calreticulin, and nNOS with caveolae. The results of immunoprecipitation experiments indicate that calsequestrin and calreticulin are located on the cytoplasmic surface of caveolae, although it is possible that one or both are also located on the extracellular/luminal side. We propose a model, modified from the superficial buffer barrier model of van Breemen (described in Section 1.2.2 and Figure 2) which incorporates the results presented here, shown in Figure 21. The key difference between this model and that of van Breemen is that caveolae provide the physical basis for the junctional space between the SR and PM, as opposed to a projection from the SR towards the PM. Further, the caveolae provide a physical structure which localizes the various proteins involved in Ca^{2+} handling. The remainder of this section will discuss the specific aspects of this model.

If calsequestrin and calreticulin are not located in the lumen of caveolae, then, as required in our model, how is Ca^{2+} retained inside the caveolae, and not lost into the surrounding extracellular space? One possibility is that calsequestrin and/or calreticulin are located in caveolae. While the immunoprecipitation experiments indicate that both proteins are associated with caveolin on the

Figure 21: Proposed model of Ca^{2+} handling in canine airway smooth muscle. This is an updated schematic of the superficial buffer barrier model depicted in Figure 2, with caveolae providing the physical basis for the junctional space between the PM and SR. It takes into consideration the results of contractility experiments in airway smooth muscle and the results presented in this thesis on the biochemical isolation of caveolae from tracheal smooth muscle. Ca^{2+} entry, through L-type Ca^{2+} channels located in caveolae, can be sequestered into SR via the SR Ca^{2+} pump or an unidentified Ca^{2+} channel (labelled ?). Calsequestrin (CSQ) and calreticulin (CRT), located on the luminal side of caveolae may be one of the components of the electron dense material which can occur between PM and junctional SR (shaded area), and therefore may act as a physical barrier which enhances refilling of the SR. Also associated with caveolae, nNOS may prevent release of Ca^{2+} by the SR via a cGMP-dependent mechanism, thereby enhancing refilling. Since there is no functional evidence of a $\text{Na}^+/\text{Ca}^{2+}$ exchanger in canine tracheal smooth muscle, the PM Ca^{2+} pump, located on caveolae, is responsible for Ca^{2+} extrusion into the caveolae lumen. Ca^{2+} may be sequestered into the caveolae lumen by binding to calsequestrin, calreticulin, or another Ca^{2+} binding protein (light shading). Alternatively, the extracellular matrix may provide a physical barrier preventing or slowing the loss of Ca^{2+} to the surrounding extracellular space.



cytoplasmic side of the caveolae, they do not rule out the possibility that some of the calsequestrin and calreticulin are located in the lumen of the caveolae. The results in other cell types demonstrating the presence of calreticulin on the extracellular cell surface (Gray et al., 1995; White et al., 1995) supports this possibility. Alternatively, another unidentified Ca^{2+} binding protein may be located within the caveolae lumen.

A final possibility is that the extracellular matrix acts as a physical barrier preventing or slowing the loss of Ca^{2+} to the surrounding extracellular space. As stated in the introduction, the extracellular matrix does not enter the caveolar lumen, but passes over the neck, which would act as a barrier. Electron micrographs have shown that the extracellular matrix surrounding bronchial smooth muscle cells is much thicker than that of other smooth muscle, including tracheal smooth muscle, at least in human (Gabella, 1989). This thicker extracellular matrix may act as a physical barrier capable of stopping or slowing the loss of Ca^{2+} , and may provide a partial explanation for the sustained contraction observed by Montano et al. (1993, 1996) in bronchial smooth muscle tissues *in vitro*. Alternatively, the net negative charge of the surface membrane and extracellular matrix may stop or impede the loss of Ca^{2+} to the surrounding extracellular space.

In support of the potential role of the extracellular matrix, Wheeler-Clark and Buja (1995) have demonstrated in coronary artery smooth muscle that the Ca^{2+}

required for smooth muscle contraction is mainly associated with the PM region, specifically the extracellular area within 100nm of the membrane using electron probe x-ray microanalysis. Depolarization-induced contractions mobilized Ca^{2+} from this extracellular area, which the authors hypothesized was trapped by the extracellular matrix acting as a passive diffusion barrier. While the authors concluded that the area near the PM (which included the caveolae lumens) was not the source of Ca^{2+} , they did not specifically investigate caveolae. Further, they stated that caveolae were rarely seen, which contrasts with a previous study in coronary artery smooth muscle (Suzuki and Sugi, 1989). In the latter study, the authors demonstrated that the extracellular Ca^{2+} for contraction derived mainly from caveolae. We suggest that the model of Wheeler-Clark and Buja (1995) be expanded to incorporate that the Ca^{2+} used for contraction, at least in airway smooth muscle, is located within the space between the extracellular matrix and the PM which includes the lumens of caveolae.

The localization of calsequestrin and calreticulin to the cytoplasmic side of caveolae is a novel finding, and at this point, their exact roles are unknown. The identity of the proteins which form the electron dense material between the PM and junctional SR is unknown in smooth muscle. We hypothesize that calsequestrin or calreticulin play some role in forming this structure. This junctional space may be the route of Ca^{2+} entry described by Bourreau et al. (1991, 1993) which involves refilling of the SR through an SR Ca^{2+} pump-independent mechanism and Ca^{2+}

entry through L-type Ca^{2+} channels. The electron dense material, and more specifically calsequestrin and calreticulin, could facilitate the movement of Ca^{2+} between caveolae and SR, and prevent loss of Ca^{2+} to the adjacent restricted subplasmalemmal space or surrounding cytosolic space.

The mode of entry of Ca^{2+} into the SR remains unknown, but is assumed to be a channel of some form. It may be the IP_3 -gated channel or ryanodine channel, working in reverse, or an as yet unidentified channel. For this mode of entry to function, then the concentration of Ca^{2+} in the junctional space must exceed the concentration of Ca^{2+} in the SR lumen. Since the concentration of Ca^{2+} in the SR lumen is in the mM range, then the concentration in the junctional space must also be in this range. However, while the total concentration of Ca^{2+} in the SR lumen is in the mM range, much of this is bound to the matrix of Ca^{2+} binding proteins in the SR lumen (calsequestrin and calreticulin). The free concentration of Ca^{2+} in the lumen is much lower, likely in the μM range. Thus, the concentration of Ca^{2+} in the junctional space need only be in μM range to allow for the movement of Ca^{2+} into the SR lumen.

As stated previously, the localization of nNOS to airway smooth muscle caveolae specifically, and airway smooth muscle in general, is also a novel observation. There is no evidence in the literature of a functional constitutive form of NOS localized in normal airway smooth muscle. The finding that nitric oxide can inhibit the release of Ca^{2+} from SR (Kannan et al., 1997) provides a possible role

for nNOS associated with caveolae. Using isolated porcine tracheal smooth muscle cells, Kannan et al. (1997) measured the intracellular Ca^{2+} concentration by Fura-2 fluorescence imaging. The nitric oxide donor, S-nitroso-N-acetylpenicillamine (SNAP), inhibited the release of Ca^{2+} from the SR induced by either acetylcholine or caffeine, indicating that release by either the IP_3 -induced or Ca^{2+} -induced Ca^{2+} release channels was inhibited. Further, the membrane permeant analogue of guanosine 3',5'-cyclic monophosphate (cGMP), 8-bromo-cGMP, also inhibited SR Ca^{2+} release, indicating that the effect of NO was via a cGMP-dependent mechanism. SNAP affected only the release of Ca^{2+} by the SR since it did not affect either Ca^{2+} influx across the PM, or reuptake of Ca^{2+} by the SR. Inhibition of SR Ca^{2+} release may be related to the direct refilling of SR proposed by Bourreau et al. (1991, 1993). A functional nNOS, via a cGMP-dependent mechanism, may facilitate the refilling of the SR by preventing its subsequent release into the cytosol. However, as yet, this remains highly speculative, given the lack of evidence of a functional NOS in airway smooth muscle.

5.5 Conclusions

This study demonstrated that smooth muscle caveolae, isolated by detergent- or Na_2CO_3 -treatment, were enriched in the α_1 chain of the L-type Ca^{2+} channel, calsequestrin, calreticulin, and nNOS. They also contained the PM Ca^{2+}

pump, but excluded connexin-43, the SR Ca^{2+} pump, and type 1 IP_3 receptor. The localization of calsequestrin and calreticulin to caveolae was confirmed by immunoprecipitation experiments. Antibodies to caveolin coimmunoprecipitated caveolin with calsequestrin and calreticulin. Further, these experiments indicated that at least some of the associated calsequestrin and calreticulin were located on the cytoplasmic side of the caveolae. Immunohistochemistry of fixed tracheal smooth muscle cryosections confirmed the localization of caveolin, the PM Ca^{2+} pump, and nNOS to the cell periphery, while the SR Ca^{2+} pump was located deeper in the cell. These results support the hypothesis that caveolae are involved in Ca^{2+} handling in smooth muscle. However, as will be discussed in the final section, some aspects require further study.

5.6 Future Studies

1. Demonstrate the localization of the Ca^{2+} handling proteins to airway smooth muscle caveolae at the ultrastructural level.

Although the various Ca^{2+} handling proteins were localized to caveolae-derived membranes using two separate biochemical isolation methods or by direct interaction with caveolin, contamination by other non-caveolar membranes is still a possible alternate explanation. Further, the isolation of bronchial smooth muscle

in sufficient amount for biochemical caveolae isolation methods is extremely difficult. Using immunoelectron microscopy in airway smooth muscle (trachea and bronchi) would confirm or directly prove localization of the Ca^{2+} handling proteins to caveolae. An alternative method would be confocal microscopy. Energy transfer experiments would determine the distance between different proteins.

2. Provide experimental evidence to support the role of caveolae in airway smooth muscle Ca^{2+} handling using contractility experiments.

The results presented in this thesis as well as potential results of the methods in the previous paragraph, while providing evidence that Ca^{2+} handling proteins are localized or enriched in caveolae, do not prove that caveolae are involved in Ca^{2+} handling in airway smooth muscle. Contractility experiments in isolated tissues could provide further functional evidence that caveolae are involved in Ca^{2+} handling. Filipin has been shown at the ultrastructural level to disrupt caveolae, causing the protein coat to unravel, and the invaginations to flatten (Rothberg et al., 1992). The use of filipin or other cholesterol binding agents in contractility experiments could result in the disruption of caveolae, and therefore the loss of caveolae function in Ca^{2+} handling. However, filipin, in addition to disrupting caveolae, also results in the formation of holes in the PM at slightly higher concentrations (50 $\mu\text{g}/\text{ml}$ versus 5 $\mu\text{g}/\text{ml}$; Flaadt et al., 1993) which may cause

difficulties in conducting and interpreting contractility experiments. Further work would be needed to find a compound which could disrupt caveolae or caveolae function with minimal nonselective effects.

3. Investigate the role of nNOS in airway smooth muscle.

Finally, it is necessary to determine the functional significance of nNOS in airway smooth muscle cells. Contractility experiments on isolated tracheal smooth muscle strips (without epithelium) in the presence and absence of NOS inhibitors, such as L-NAME or L-NNA, would provide the evidence of a functional NOS in the smooth muscle cells. Since there are no selective inhibitors of the various NOS isoforms, further methods would be necessary to confirm that it is the neuronal form of NOS which is present in the muscle cells. Measurement of NOS activity by biochemical methods could determine if the enzyme is Ca^{2+} dependent, and if so, would rule out the inducible form of NOS. Western blotting using selective antibodies for the endothelial form of NOS would be required to determine if this isoform is also present in the smooth muscle cells.

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