Mechanisms of Calcium Buffering and Aging in Neurons: Testing the Limits of Homeostasis

William James Pottorf II

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Mechanisms of Calcium Buffering and Aging in Neurons: Testing the Limits of Homeostasis

by

William James Pottorf, II

A Dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Pharmacology

September 2000
Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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DEDICATION

I would like to dedicate the writings of this dissertation to my family Bill, Peggy, Shawn, Grace, Jack and Trudy who have loved and supported me as a son, grandson, and brother. I would also like to dedicate this dissertation to those close to me whom I have developed an unending friendship with: Adam (a.k.a. The Bass), Allen, Alicia, Amanda (a.k.a. Weezer), Ben, Bobby, Christian, Crystal, David & Pat B., Debbie, Gina, Greg, Huong (a.k.a. Han Solo), Jane, Jay, Jeff D., Jeff K. (a.k.a. The Pres), Jenifer, Jesika, Jim (a.k.a. Jimbo), John B. (a.k.a. LB), John P., Kshama, Laura (a.k.a. Swanky Girl), Larry, Philip, Pierre (a.k.a. P-Funk), Marvin, Nicole (a.k.a. Mama Cole), Nicolus (a.k.a. Sasquatch), Rich, Rebecca, Ruby, Sherri, Tony, Tonya, Wanda and Wendy.
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<tr>
<td>[Ca(^{2+})](_i)</td>
<td>Intracellular Calcium Concentration</td>
</tr>
<tr>
<td>[K(^+)]</td>
<td>Potassium Concentration</td>
</tr>
<tr>
<td>[Na(^{+})](_e)</td>
<td>Extracellular Sodium Concentration</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis(2-Aminophenoxy)Ethane-N,N,N',N'-Tetraacetic Acid</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>BHQ</td>
<td>2,5-di(tert-butyl)-1,4-benzohydroquinone</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium Induced Calcium Release</td>
</tr>
<tr>
<td>CPA</td>
<td>Cyclopiazonic Acid</td>
</tr>
<tr>
<td>DHBA</td>
<td>Dihydroxybenzylamine</td>
</tr>
<tr>
<td>DNP</td>
<td>Dinitrophenol</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal Root Ganglia</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>F-344</td>
<td>Fisher-344</td>
</tr>
<tr>
<td>Fura-2/AM</td>
<td>5-Oxazolecarboxylic Acid, 2-(6-(Bis(2-((Acetyloxy)Methoxy)-2-Oxoethyl)Amino)-5-(2-(Bis(2-((Acetyloxy)Methoxy)-2-Oxoethyl)Amino)-5-Methylphenoxo)Ethoxy)-2-Benzofuranyl)-(Acetyloxy)Methyl Ester</td>
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<tr>
<td>HEPES</td>
<td>N-(2-Hydroxyethyl)Piperazine-N'-2-Ethanesulfonic Acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>IP(_3)R</td>
<td>Inositol 1,4,5-Triphosphate Receptor</td>
</tr>
<tr>
<td>K(_m)</td>
<td>Michaelis-Menton Constant</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NT</td>
<td>Neurotransmitter</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PMCA</td>
<td>Plasma Membrane Calcium ATPase</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl Fluoride</td>
</tr>
<tr>
<td>PMV</td>
<td>Plasma Membrane Vesicle</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Difluoride</td>
</tr>
<tr>
<td>RTA</td>
<td>Rat Tail Arteries</td>
</tr>
<tr>
<td>SCG</td>
<td>Superior Cervical Ganglia</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfide</td>
</tr>
<tr>
<td>SER</td>
<td>Smooth Endoplasmic Reticulum</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco/Endoplasmic Reticulum Calcium ATPase</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline Sodium Chloride</td>
</tr>
<tr>
<td>SSPE</td>
<td>Saline Sodium Phosphate EDTA</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetraethylammonium</td>
</tr>
<tr>
<td>TG</td>
<td>Thapsigargin</td>
</tr>
<tr>
<td>Va</td>
<td>Vanadate</td>
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ABSTRACT

MECHANISMS OF CALCIUM BUFFERING AND AGING IN NEURONS:
TESTING THE LIMITS OF HOMEOSTASIS

by

William James Pottorf, II
Doctor of Philosophy, Graduate Program in Pharmacology
Loma Linda University, September 2000
Dr. John N. Buchholz, Chairperson

Intracellular calcium ([Ca$^{2+}$]i) is an ubiquitous second messenger that integrates multiple and diverse neuronal pathways that include development and maturation, gene expression, synaptic plasticity, transmitter release, excitability, and even cell death. Upon neuronal excitation the [Ca$^{2+}$]i increases rapidly and the calcium buffering system reacts quickly to restore [Ca$^{2+}$]i to basal levels in order to reset the cell for the next stimulus and avoid prolonged exposure to cytotoxic levels of high [Ca$^{2+}$]i. The aging process appears to causes multiple changes in the ability of neurons to regulate [Ca$^{2+}$]i homeostasis and an age-related breakdown in the mechanisms controlling [Ca$^{2+}$]i homeostasis could contribute to decreased neuronal function or neurodegeneration. Thus, it is hypothesized that the aging process results in decreased function of sarco/endoplasmic reticulum calcium ATPase (SERCA) pumps, leading to greater or more sustained [Ca$^{2+}$]i levels, an increased reliance on remaining calcium buffering components to restore [Ca$^{2+}$]i homeostasis and regulate neurotransmitter release in adrenergic neurons.
CHAPTER I

INTRODUCTION AND REVIEW OF CALCIUM BUFFERING MECHANISMS AND AGING IN ADRENERGIC NEURONS

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1. INTRODUCTION

Intracellular calcium ([Ca\textsuperscript{2+}]\textsubscript{i}) is a universal second messenger integrating numerous cellular pathways. These include neuronal development and maturation, gene expression, synaptic plasticity, transmitter release, excitability, and cell death (Choi, 1992; Berridge, 1995, 1998; Clapham, 1995; Ginty, 1997; Malenka, Dauer, Perkel & Nicoll, 1989; Spitzer & Ribera, 1998). Mechanisms regulating [Ca\textsuperscript{2+}]\textsubscript{i} allow an enormous calcium gradient across neuronal membranes with resting [Ca\textsuperscript{2+}]\textsubscript{i} levels at 100-200 nM compared to extracellular calcium concentration of approximately 1 mM (Meldolesi, Huttner, Tsien & Pozzan, 1984; Nachshen, 1985; Ashley, 1986). Cellular excitation induces [Ca\textsuperscript{2+}]\textsubscript{i}-transients ranging from sub-micromolar to several micromolar, necessitating cellular mechanisms to rapidly return [Ca\textsuperscript{2+}]\textsubscript{i} to resting levels (Nachshen, 1985; Fontana & Blaustein, 1993; Ghosh & Greenberg, 1995). Therefore it is not surprising that neurons contain intricate systems for regulating [Ca\textsuperscript{2+}]\textsubscript{i}. Intracellular calcium buffering systems include calcium binding proteins, plasma membrane extrusion, and sequestration into intracellular organelles. In a concerted fashion these diverse calcium buffering systems attenuate [Ca\textsuperscript{2+}]\textsubscript{i}-transients and maintain resting [Ca\textsuperscript{2+}]\textsubscript{i} levels.

The aging process is thought to cause multiple changes in the ability of central and peripheral neurons to regulate [Ca\textsuperscript{2+}]\textsubscript{i} homeostasis (Duckles, Tsai & Buchholz, 1995; Michaelis et al., 1996; Murchison & Griffith, 1999). This is particularly important in the case of neurons, which have been shown to be highly vulnerable to calcium overload (Choi, 1994, 1995). The pervasive involvement of calcium in multiple neuronal pathways suggests that age-related alterations in the function of [Ca\textsuperscript{2+}]\textsubscript{i} homeostasis could contribute to age-related neuronal degeneration (Gibson & Peterson, 1987;
Numerous studies link dysregulation of 
\([\text{Ca}^{2+}]_i\) homeostasis to \([\text{Ca}^{2+}]_i\)-dependent neurotoxicity and cell death in the pathological condition of Alzheimer’s disease (Mattson et al., 1993a; Thibault et al., 1998; Chen & Fernandez, 1999). In addition to neurodegeneration, altered \([\text{Ca}^{2+}]_i\) homeostasis has also been linked to age-related changes in norepinephrine (NE) release from adrenergic nerve endings (Buchholz & Duckles, 1990; Tsai, Pottorf, Buchholz & Duckles, 1998; Mattson, Rydel, Lieberburg & Smith-Swintosky, 1993b), and this could contribute to development of hypertension and increased risk of stroke. Disruption of calcium homeostasis could account for numerous cellular pathologies and cell death associated with aging, concepts that deserve further exploration.

There is considerable knowledge of the mechanisms involved in regulating neuronal \([\text{Ca}^{2+}]_i\) and neurotransmitter release. Despite this knowledge the importance and participation of individual calcium buffering components and the impact of age on their function is not sufficiently understood. This dissertation examines the evidence for age-related alterations in neuronal calcium buffering and changes in the control of neurotransmitter release in the autonomic nervous system. Most of the knowledge concerning neuronal calcium regulatory systems is derived from studies of the central nervous system with only limited number addressing autonomic nerves. Therefore, the limited work in autonomic nerves will be emphasized, but supporting studies of the central nervous system will be discussed where gaps remain to be filled.
2. REGULATION OF INTRACELLULAR CALCIUM IN NEURONS

Calcium homeostasis in neurons involves several calcium buffering components working in concert to restore stimulation-evoked \([\text{Ca}^{2+}]_i\)-signals to basal levels (for review see Kostyuk & Verkhratsky, 1994). Calcium influx occurs via plasmalemmal voltage-gated and ligand-gated calcium channels and/or calcium release from endoplasmic reticulum (ER) stores via inositol-1,4,5-triphosphate receptors (IP$_3$R) and calcium-induced, calcium release (CICR), mediated by ryanodine receptors. In neurons, stimulation-evoked increase in \([\text{Ca}^{2+}]_i\) leads to activation of the neurotransmitter release mechanism, consequently inactivation of neurotransmitter release is in part regulated by calcium buffering mechanisms. These include calcium sequestration into ER by sarco/endoplasmic reticulum \(\text{Ca}^{2+}\)-ATPase (SERCA) pumps, mitochondrial calcium uptake, cytosolic calcium binding proteins, plasmalemmal calcium extrusion via plasma membrane \(\text{Ca}^{2+}\)-ATPase (PMCA) pumps, and \(\text{Na}^+ / \text{Ca}^{2+}\)-exchange. Each of these calcium regulatory systems is illustrated in Figure 1 and will be briefly discussed below.
3. CALCIUM INFLUX AND INTRACELLULAR RELEASE

3.1 Plasmalemmal Calcium Channels

Neuronal cells express multiple types of plasmalemmal calcium channels that can be activated by various excitatory stimuli. The two main families of plasmalemmal calcium channels include voltage-gated calcium channels (T-, L-, N-, P-, Q-, R) and ligand-gated calcium channels (including acetylcholine, glutamate, and ATP-activated channels) (Kostyuk, 1989; Miller, 1992; Lundy & Frew, 1996; Bean, Williams & Ceelen, 1990; Trouslard, Marsh & Brown, 1993; Burnashev, Zhou, Neher & Sakmann, 1995). The selectivity of the calcium channels results in a typical “calcium current” as these ions enter the cytosol and generate a calcium signal (Kostyuk, Mironiv & Shuba, 1983; Kim, Morii, Sun, Imoto & Mori, 1993).

3.2 Calcium Release from ER Stores

It is increasingly evident that $[\text{Ca}^{2+}]_i$ storage sites, like the ER, also play a crucial role in providing calcium for regulating and maintaining complex calcium signals and cellular functions such as excitability, neurotransmitter release, gene expression, and even apoptosis (Berridge, 1993; Hardingham, Cruzalegui, Chawla & Bading, 1998; Mattson, Guo, Furukawa & Pedersen, 1998; Verkhratsky & Petersen, 1998; Li, Miyata & Hatton, 1999). There are at least two distinct ER calcium stores, each of which is controlled by a functionally distinct second messenger. The IP$_3$R is activated by the intracellular second messenger IP$_3$ (for review see Berridge, 1993), while CICR is initiated by the rise in $[\text{Ca}^{2+}]_i$. Calcium ions themselves and ADP-ribose control the sensitivity of the CICR channel to calcium (Usachev & Thayer, 1997).
CALCIUM BUFFERING SYSTEMS

4.1 Intracellular Calcium Binding Proteins

Intracellular calcium binding proteins are mobile proteins belonging to the EF-hand family with the helix-loop-helix conformation. These calcium buffering proteins include parvalbumin, calmodulin, calretilin, calcineurin, and calbindin-D_{28k} (for review see Heizmann & Hunziker, 1991). These binding proteins, along with ATP-Ca^{2+} complexes, have an enormous capacity to buffer calcium ions. Collectively, these mobile buffers adsorb nearly 95-99% of the total cellular calcium load, leaving the rest available as free calcium ions (Belan, Kostyuk, Snitsarev & Tepikin, 1993). The mobile calcium buffer proteins are important for the facilitated transcellular diffusion of calcium ions and the propagation of calcium signals within the cell cytoplasm (Chard, Bleakman, Christakos, Fullmer & Miller, 1993).

4.2 ATP-Dependent Calcium Pumps

Two families of P-type Ca^{2+}-ATPase pumps are vital for the regulation of [Ca^{2+}]_i-transients: PMCAs that extrude calcium from the cytosol to the extracellular space (Werth, Usachev & Thayer, 1996; Carafoli, 1997) and SERCAs that sequester calcium from the cytosol into the ER lumen (Michaelis, Michaelis, Chang & Kitos, 1983; Inesi & Kirtley, 1992; Tsai et al., 1998). PMCAs utilize ATP hydrolysis to remove calcium from the cytosol with a 1:1 ratio (Niggli, Adunyah, Penniston & Carafoli, 1981). In contrast, SERCAs sequester two calcium ions for each ATP molecule used (MacLennan, 1970). PMCAs are expressed as multiple isoforms from four different genes (PMCA1,2,3,4), while SERCAs are expressed by three distinct genes (SERCA1,2,3), and each gene...
transcript is able to generate alternative splice variants (Wuytack et al., 1992). Expression of these Ca\(^{2+}\)-ATPase pumps is interdependent and is co-expressed in a tissue-specific manner (Liu, Xu, Fridman, Muallem & Kuo, 1996). In addition, Ca\(^{2+}\)-ATPase pump expression can be regulated by hormones, growth factors, and [Ca\(^{2+}\)]\(_i\) (Simonides et al., 1996; Kuo et al., 1997; Tajima et al., 1998). Although all these calcium pumps belong to the same family, they show considerable diversity in structure, function, and pharmacology (Lytton, Westlin, Burk, Shull & MacLennan, 1992; Kuo et al., 1997). For a more detailed description of the ATP reaction cycle and molecular structure of SERCAs, please refer to Appendix I (page 192).

4.3 Mitochondrial Calcium Uptake and Storage

Mitochondria display a robust capacity to sequester small and large calcium loads. Early studies suggested that mitochondria only buffer calcium under non-physiological conditions, such as when [Ca\(^{2+}\)]\(_i\) exceeds 2 \(\mu\)M (Nicholls, 1985), as in some pathological states (Wilson, Arnold, Burke & Schrier, 1984; Khandoudi, James & Feuvray, 1989). Recently mitochondria have been established as an important participant in the control of [Ca\(^{2+}\)]\(_i\)-transients and have been shown to buffer [Ca\(^{2+}\)]\(_i\) loads within normal physiological ranges and in the absence of pathology (Werth & Thayer, 1994; Buchholz, Tsai, Foucart & Duckles, 1996; David, Barrett & Barrett, 1998; Pinton et al., 1998; Rizzuto et al., 1998). Calcium uptake by the mitochondria occurs by a H\(^+\)/Ca\(^{2+}\)-uniporter, which is driven by the mitochondrial membrane potential established by the electron transport chain (Thayer & Miller, 1990). The mitochondrial uniporter is a high capacity system that can transport large as well as small calcium loads.
4.4  

Plasmalemmal Na\(^+\)/Ca\(^{2+}\)-Exchanger

The Na\(^+\)/Ca\(^{2+}\)-exchanger is found in the plasma membrane of a variety of excitable and non-excitable cells and uses the energy of the sodium electrochemical gradient to expel one calcium ion for every three sodium ions brought into the cell (Blaustein & Lederer, 1999). The K\(_m\) of the exchanger is approximately 2 \(\mu\)M; therefore, it is thought that the exchanger is primarily responsible for regulating large calcium loads and less important in adjusting small calcium loads and resting [Ca\(^{2+}\)]\(_i\) levels (Reeves & Sutko, 1979; Werth et al., 1996).

5.  

AGE-RELATED ALTERATIONS IN THE CONTROL OF NEUROTRANSMITTER RELEASE

There are numerous studies showing an age-related rise in systemic blood pressure is accompanied by increased levels of plasma catecholamines (Palmer, Zeigler & Lake, 1978; Esler et al., 1981; Barnes, Rashkind, Gumbrecht & Halter, 1982; Insel, 1993). In support of this idea, activity of tyrosine hydroxylase, the rate limiting step in NE and epinephrine synthesis, and levels of mRNA have been shown to increase with age in the adrenal medulla of F-344 rats (Tumer, Hale, Lawler & Strong, 1992). Elevated plasma catecholamines, especially NE, have been used as an index of increased sympathetic nerve activity occurring with age (Nishimura et al., 1979). Thus, these data point to the possibility that peripheral vascular adrenergic nerve activity may actually increase with age.

As an extension of this concept that adrenergic nerve activity actually increases with age, in arteries studied in vitro, including the rat tail artery and superior mesenteric artery, stimulation-evoked fractional NE release has been shown to increase with age.
(Buchholz & Duckles, 1990; Buchholz, Sexton & Hewitt, 1998). Furthermore, in the rat tail artery this age-related increase in NE release occurs over a wide range of stimulation frequencies from 0.25 to 8 Hz (Tsai, Duckles & Buchholz, 1995). These age-related increases in plasma catecholamines and peripheral adrenergic nerve activity, could contribute to increased incidence of cardiovascular disorders including coronary artery disease, heart failure, and hypertension.

There are a number of possible explanations for an age-related increase in NE release from adrenergic nerves. Age-related alterations in nerve density, neurotransmitter content and re-uptake, prejunctural $\alpha_2$-adrenergic receptors, and calcium regulation are possible sites of dysfunction that could lead to increased neurotransmitter overflow (Duckles, Carter & Williams, 1985; Handa & Duckles, 1987; Buchholz & Duckles, 1990; Buchholz, Tsai, Friedman & Duckles, 1992; Duckles et al., 1995; Buchholz et al., 1996). Elucidation of the etiology of age-related changes in cardiovascular function requires an understanding of the mechanisms controlling neurotransmitter release and how the regulation of adrenergic nerve function may change with age.

5.1 Neurotransmitter Content and Adrenergic Nerve Density

Norepinephrine content in the rat heart is reported to decrease with age (Martinez et al., 1981; Dawson & Meldrum, 1992). In rat blood vessels the consensus is less straightforward, with reports of increased neurotransmitter content (renal, femoral and saphenous arteries), no change (renal, femoral, saphenous and superior mesenteric veins), or a decrease (tail arteries) with advancing age (Handa & Duckles, 1987). Catecholamine histofluorescence, as a measure of adrenergic nerve density, has been shown to increase
with advancing age in rat superior mesenteric artery, renal artery, and portal vein (Mione, Erdo, Kiss, Ricci & Amenta, 1988). In contrast, density of adrenergic innervation of spinal cord blood vessels showed no age-related change (Amenta, Bronzetti, Ferrante & Ricci, 1990). Thus the impact of age on adrenergic nerve density appears to depend on the model and methodology utilized, so that no clear conclusion about advancing age and adrenergic nerve function can be drawn from studying adrenergic nerve density in isolation.

5.2 **Transmitter Uptake in Adrenergic Nerves**

The function of neuronal neurotransmitter reuptake mechanisms is to reduce levels of neurotransmitter in the neuroeffector junction and minimize spillover into the circulatory system (Esler *et al*., 1995). Using the selective blockers, cocaine and deoxycorticosterone, the impact of age on the contribution to NE uptake by adrenergic neuronal and extraneuronal systems, respectively, can be assessed. Effects of these blockers of uptake have been shown to be reduced with age in pithed rat and isolated rat atria (Borton & Docherty, 1989) and vas deferens (de Avellar, Kobashi & Markus, 1990), suggesting that NE uptake declines with advancing age. In contrast in rat heart (Limas, 1975) and rat tail artery (Buchholz & Duckles, 1990), the effect of cocaine and deoxycorticosterone on NE uptake was shown to increase with advancing age. However, in rat tail arteries when NE release due to blocked uptake was corrected for the total amount of NE release in absence of drugs, no change with age was observed (Buchholz & Duckles, 1990). Thus age-related alterations in the effect of uptake antagonists on NE release are not necessarily due to changes in the function of the NE transporters *per se*.
Rather age-related changes in the effect of these blockers may be due more to changes in the biophase concentration of NE in the junctional cleft. Thus, this approach suggests that with advancing age, the amount of NE taken up remains a constant fraction of the amount of NE released.

Direct measurement of the accumulation of $[^3H]$-NE as an index of uptake and storage showed no age-related change in femoral or renal arteries or renal veins of rats (Duckles et al., 1985). From both of these approaches, direct measurement of NE uptake or assessment of the effect of uptake blockers, there is no support for an age-related change in the function of NE uptake systems. Thus, the observed increase with age in stimulation-evoked fractional NE release cannot be explained by an age-related decline in the function of NE transporters.

5.3 Function of Prejunctional $\alpha_2$-Adrenergic Receptors

Prejunctional $\alpha_2$-adrenergic receptors mediate a negative feedback response whereby released NE modulates its own further release (for reviews see Illes, 1986; Langer & Arbilla, 1990). Direct measurements of NE overflow have shown an age-related decrease in effectiveness of prejunctional $\alpha_2$-adrenergic receptor blockade in pithed rats (Docherty & Hyland, 1986), isolated rat vas deferens (Hyland & Docherty, 1985), rat heart (Daly, Goldberg & Roberts, 1989), and rat tail artery (Buchholz & Duckles, 1990; Buchholz et al., 1992).

Superficially, these studies support the concept of a general age-related decline in the function of prejunctional $\alpha_2$-adrenergic receptors. However, although there was an age-related decline in sensitivity of prejunctional $\alpha_2$-adrenergic receptors to the
antagonist idazoxan, closer observation revealed no age-related difference in the maximal response to this drug (Buchholz et al., 1992). It is logical to assume that an age-related increase in NE release would lead to greater biophase concentrations of NE. This would result in more NE bound to the $\alpha_2$-adrenergic receptor and greater competition between higher levels of NE and a given antagonist concentration, resulting in apparent reduced sensitivity to the applied antagonist. Thus, the observed shift in potency of $\alpha_2$-adrenergic antagonists with age may merely reflect greater competition with increased levels of endogenous NE.

In light of the studies summarized above, age-related changes in NE release cannot be explained by changes in the function of NE uptake mechanisms or $\alpha_2$-adrenergic receptors. Other possibilities are that age-related changes in adrenergic transmitter release may be a consequence of alterations in calcium influx and/or efflux from intracellular calcium stores, intracellular buffering, or the sensitivity of the release mechanism. One approach to determining whether any of these factors might contribute to the age-related increase in NE release would be to determine whether there is any age-related change in sensitivity of stimulation-evoked transmitter release to variations in extracellular calcium.

5.4 Effects of Altering Extracellular Calcium Concentration

The effects of altering extracellular calcium on stimulation-evoked NE release were compared in tail arteries from young and old rats (Buchholz, Nikkah & Duckles, 1994). Stimulation-evoked NE release from adrenergic nerves of the tail artery from old rats was found to be more sensitive to an increase and less sensitive to a decrease in
extracellular calcium compared to nerves in tail arteries from young animals. Several possible explanations could account for this age-related difference in calcium sensitivity. First, it has been shown in rat hippocampal pyramidal neurons that calcium dependent potassium afterhyperpolarizations, calcium spike duration, and calcium current through calcium channels are all elevated with age (Landfield & Pitler, 1984; Pitler & Landfield, 1990). These data suggest that calcium channel function may be altered with age resulting in increased calcium influx into neurons. Second, the function of neuronal calcium buffering and extrusion mechanisms (e.g. calcium binding proteins, PMCA, Na⁺/Ca²⁺-exchanger, SERCA and mitochondria) has been suggested to decline with advancing age in both central and peripheral neurons (de Jong et al., 1996; Michaelis et al., 1996; Pottorf et al., 2000; Satrustegui, Villalba, Pereira, Bogonez & Martinez-Serrano, 1996). A decline in buffering and/or extrusion could possibly result in greater or more prolonged [Ca²⁺]i, leading to increased transmitter release. Third, the function of the release mechanism that is sensitive to calcium and responsible for vesicle mobilization during neurotransmitter release has been suggested to alter with age in rat adrenergic nerves (Parfitt, Hoffer & Browning, 1991). Thus there are multiple regulatory targets in adrenergic neurons whose function could be affected by the aging process.

5.5 Role of Calcium Influx

The contribution of calcium influx has been measured in several regions of the nervous system by both current-clamp and voltage-clamp methods. Calcium currents have been reported to increase (Landfield & Pitler, 1984), decrease (Kostyuk, Pronchuk, Savchenko & Verkhratsky, 1993), or remain unchanged (Murchison & Griffith, 1996)
with advancing age. To determine whether function of neuronal calcium channels is essential to age-related changes in NE release in the rat tail artery, calcium channels were bypassed using the calcium ionophore, ionomycin, to initiate calcium influx and NE release. However, ionomycin-induced NE release remained greater in adrenergic nerves of tail arteries from old as compared to young rats (Tsai, Hewitt, Buchholz & Duckles, 1997). Thus, despite the studies in central neurons suggesting that calcium influx may increase with age, this alone does not fully explain the age-related increase in NE release from peripheral adrenergic nerves.

5.6 Effects of Increasing Calcium Buffering Capacity

To test whether the capacity of calcium buffering declines with age an artificial buffer was used. The intracellular calcium chelator bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) was applied to rat tail arteries, and stimulation-evoked NE release was measured. Addition of BAPTA was more effective at reducing stimulation-evoked NE release in older rat tail arteries compared to arteries from young animals (Tsai et al., 1997). These data suggest that there is an age-related reduction in calcium buffering capacity.

5.7 Sensitivity of Norepinephrine Release Mechanism

Calcium influx and intracellular buffering only, in part, regulate the control of NE release from nerve endings. Mechanisms exist which can “sensitize” the release mechanism to a given stimulation-evoked [Ca^{2+}]i-transient (Trifaro & Vitale, 1993). Phosphorylation of synapsins by phorbol esters can potentiate NE release by increasing
the number of vesicles that can be mobilized and released in response to a given \([\text{Ca}^{2+}]_{i}\)-signal (Kotsonis & Majewski, 1996). Thus, observations discussed above showing an age-related increase in the sensitivity of adrenergic nerves to increased extracellular calcium could also be related to changes in the sensitivity of the release mechanism to calcium. There are no clear studies exploring the effect of advancing age on the sensitivity of the transmitter release mechanism itself. Thus, at this time it is difficult to come to any conclusion concerning the possible impact of age on function of the transmitter release mechanism.

6. SUMMARY: EFFECTS OF AGING ON CONTROL OF NEUROTRANSMITTER RELEASE

Clearly current knowledge of the consequence of aging on mechanisms controlling NE release from peripheral adrenergic nerves is limited. Nevertheless, several key points can be made concerning the impact of age on adrenergic nerve function. As discussed above, an age-related increase in stimulation-evoked NE release from adrenergic nerves has been observed in the rat model. It appears that neurotransmitter re-uptake remains constant with advancing age, or at least the amount of transmitter re-uptake remains a constant fraction of the amount of NE released from the nerve terminal. Secondly, the function of prejunctional \(\alpha_2\)-adrenergic receptors also appears to be maintained with age when competition between antagonist and the greater biophase concentration of NE is taken into consideration. Third, adrenergic nerves show a greater sensitivity to alterations in extracellular calcium with age. These data could be explained by any one or a combination of changes in calcium influx, buffering, or sensitivity of the release mechanism to calcium. In the particular studies discussed
above, bypassing calcium channels and the increased effect of an artificial buffer on NE release have led to a focus on calcium buffering mechanisms. Despite this focus on calcium buffering, the possibility that there are age-related changes in calcium influx or sensitivity of the NE release mechanism to calcium has not been ruled out and remains a subject for future investigation.

7. EFFECT OF AGE ON NEURONAL CALCIUM HOMEOSTASIS

Each component of the intracellular calcium buffering system functions in regulating stimulation-evoked calcium signals (Fig. 1). Therefore, impairment of any one component has the potential to affect \([\text{Ca}^{2+}]_i\) homeostasis. However, the existence of multiple calcium buffering components also allows for compensation by one for the decreased function of another. The focus of the discussion below will be on the impact of age on the function of SERCA calcium pumps, mitochondrial calcium uptake and plasmalemma calcium extrusion systems.

7.1 Function of SERCAs

One factor in calcium dysregulation with age appears to be a decline in the function of SERCAs. An age-related decline in transport of calcium by SERCA pumps has been demonstrated in the cerebral cortex. This was measured after intravenous infusion of \(^{45}\text{Ca}^{2+}\) into mice followed by fractionation of cortical SER. Uptake of \(^{45}\text{Ca}^{2+}\) into the SER was found to decline by 50% in cortex from old as compared to young mice (Gibson, Perrino & Dienel, 1986). Studies in autonomic nerves using the selective blockers thapsigargin and cyclopiazonic acid have revealed similar age-related declines
in the function of SERCAs (Tsai et al., 1998). In isolated rat superior cervical ganglion (SCG) cells, microfluorometric measurements of [K⁺]-evoked [Ca²⁺]i-transients in the presence of these SERCA blockers showed a significant decline in the rate of calcium recovery in young SCG cells but no significant change in calcium recovery in old cells. Further evidence of an age-related change in SERCA function was observed when NE release was measured in the presence of the same SERCA blockers, showing an increase in stimulation-evoked NE release in young rat tail arteries with no change in arteries from old animals (Tsai et al., 1998).

One explanation for such a decline in SERCA function with age may be changes in expression of SERCA isoforms. Indeed, SERCA mRNA expression declines with age in cardiac tissue and vascular smooth muscle (Maciel, Polikar, Rohrer, Popovich & Dillmann, 1990; Lompre, 1999). Conversely, changes in mRNA expression did not correlate with levels of SERCA proteins in rat skeletal muscle and myocardium, which were shown to remain stable with advancing age (Ferrington et al., 1997; Xu & Narayanan, 1998). However, despite unchanged SERCA content, calmodulin mediated activation of ⁴⁵Ca²⁺-uptake declined with age, corresponding to a decline in calmodulin mediated phosphorylation of SERCAs (Xu & Narayanan, 1998). Further studies are necessary to determine the mechanism by which function of SERCAs may be influenced by age. These might include a determination of the impact of age on fluidity of SER membranes, and how these changes, if any, may influence the function of these integral membrane proteins.
7.2 Mitochondrial Calcium Uptake

Studies of rat hippocampal neurons have suggested that age-related alterations in calcium homeostasis are due to a decline in mitochondrial calcium uptake (Vitorica & Satrustegui, 1986; Villalba, Pereira, Martinez-Serrano & Satrustegui, 1995; Satrustegui et al., 1996). The energy required for calcium uptake by the mitochondria is generated by maintenance of the mitochondrial proton gradient, providing the electrochemical potential to drive the H⁺/Ca²⁺-uniporter (Nicholls, 1985; Gunter & Pfeiffer, 1990). However, in rat liver microsomes and brain synaptosomes there is no significant age-related change in the mitochondrial proton gradient, respiratory rate, or membrane potential (Vitorica, Clark, Machado & Satrustegui, 1985; Paradies & Ruggiero, 1991). Therefore, if the mitochondrial proton gradient is maintained with advancing age one might also predict that the calcium buffering capacity of the mitochondria would be preserved. In recent studies, the presence of high concentrations of vanadate, which block both PMCsAs and SERCAs, together with replacement of sodium with tetraethylammonium to block the Na⁺/Ca²⁺-exchanger, essentially forced adrenergic neurons to rely on mitochondria to regulate [Ca²⁺]i-transients. Under these conditions the capacity for calcium uptake by mitochondria did not significantly change with age (Pottorff et al., 2000). Thus when multiple calcium buffering systems are blocked, mitochondria are still able to restore [Ca²⁺]i-transients to basal levels in adrenergic nerves, and this ability is maintained in nerves from older animals.

Increased reliance on mitochondrial calcium uptake in SCG cells from older animals has also been demonstrated. When SCG cells were exposed to the mitochondrial calcium uptake blocker, dinitrophenol (DNP), peak [K⁺]-induced [Ca²⁺]i-transients and
rate of rise of [Ca\(^{2+}\)]_{i} increased in cells from aged animals, but there was no significant effect of DNP in SCG cells from young animals (Buchholz et al., 1996). Similarly, exposure of tail arteries from aged animals to DNP resulted in increased stimulation-evoked NE release with no significant effect of DNP in young arteries (Tsai et al., 1995). Studies in rat pyramidal neurons have also shown that when SERCA calcium uptake is blocked mitochondria can compensate and effectively buffer [Ca\(^{2+}\)]_{i}-transients (Fiori & Mugnaini, 1981). Other studies in rat basal forebrain neurons from old animals showed that mitochondria are able to compensate for an age-related increase in calcium influx (Murchison & Griffith, 1998). Together these studies suggest that mitochondrial calcium uptake in peripheral and central neurons may become more important functionally with advancing age.

7.3 **Plasma Membrane Calcium Transport Systems**

It is plausible to assume that additional components of the calcium buffering system, other than mitochondria, may also compensate for a decline in calcium regulation with advancing age. Recent evidence has highlighted the role of the PMCAs as important participants in the dynamic regulation of [Ca\(^{2+}\)]_{i}-transients and as crucial players in calcium extrusion during normal (Carafoli & Stauffer, 1994; Werth et al., 1996) and pathological conditions (Garcia & Strehler, 1999). Additionally, Na\(^{+}/Ca^{2+}\)-exchange is a crucial component of [Ca\(^{2+}\)]_{i} regulation that interconnects both sodium and calcium homeostasis in neuronal cells (Blaustein & Lederer, 1999).

Alterations in calcium homeostasis due to blockade of SERCAs with thapsigargin have been shown to result in an induction of PMCA expression as measured by gene
transcription (mRNA expression) in rat aortic endothelial cells (Kuo et al., 1997). In failing myocardium, there is an increased dependence upon Na\(^+/\)Ca\(^{2+}\)-exchange when the function of SERCAs declines (Hasenfuss et al., 1999). In rat adrenergic neurons, when mitochondrial calcium uptake and SERCAs are blocked with DNP and thapsigargin, respectively, SCG cells are “forced” to rely on plasma membrane calcium extrusion systems (PMCAs and Na\(^+/\)Ca\(^{2+}\)-exchangers) to control high [K\(^+\)]-evoked [Ca\(^{2+}\)]i-transients. Under these conditions SCG cells from both old and young animals were able to fully recover from high [K\(^+\)]-evoked [Ca\(^{2+}\)]i-transients. These data suggest that plasmalemmal calcium extrusion can by itself control [Ca\(^{2+}\)]i-transients, and there is no age related change in the function of these systems in SCG cells (Tsai et al., 1998). In contrast to these results in rat SCG cells, however, the function of PMCAs in rat brain synaptosomes has been shown to progressively decrease with advancing age (Qin et al., 1998). Furthermore, activation of PMCA mediated calcium uptake by the cofactor calmodulin has been shown to decline with advancing age in the rat brain (Michaelis et al., 1996; Gao, Yin, Yao, Williams & Squier, 1998).

The ability of Ca\(^{2+}\)-ATPase pumps in plasma membranes and ER to control [K\(^+\)]-evoked [Ca\(^{2+}\)]i-transients was also assessed with advancing age in SCG cells. Blockade of PMCA function with a relatively low concentration of vanadate (0.25 μM), which does not significantly affect the function of SERCAs, induced a greater decline in the rate of recovery of [Ca\(^{2+}\)]i in SCG cells from old compared to young animals (Pottorf et al., 2000). Increasing the vanadate concentration to 1.0 μM, which can block both PMCAs and SERCAs, did not significantly alter the rate of recovery of [Ca\(^{2+}\)]i in old SCG cells; however, there was a further decline in calcium recovery in cells from young animals.
(Pottorf et al., 2000). These data suggest that PMCA function is maintained with age; furthermore, the plasma membrane extrusion system can apparently provide some compensatory buffering capacity for the age-related deficiency in SERCA function. However, even though PMCA function may decline with age in some neuronal models, this does not invalidate the possibility of compensation by additional calcium buffering components as we have observed in our SCG cells from aged animals. Studies in SCG cells suggest that the calcium buffering systems have several redundant mechanisms that allow for compensation when one or more components are compromised. Therefore, with advancing age the remaining calcium buffering components become a critical part of maintaining calcium homeostasis and preventing calcium overload in neurons.

8. HYPOTHESIS FOR AGE-RELATED CHANGES IN NEURONAL CALCIUM REGULATION

8.1 Hypothesis 1: Permanent Changes in Neuronal Calcium Regulation

Several current hypotheses have attempted to explain the relationship of aging to observed declines in neuronal function. One hypothesis postulates that age-related alterations in neuronal calcium homeostasis result in a permanent change in the regulation of [Ca$^{2+}$]i leading to neurodegeneration (Khachaturian, 1994). While studies have documented the neurotoxic effect of calcium overload in initiating neuronal death (Choi, 1994), this may not be the case in the normal physiological process of aging. For instance, Alzheimer’s disease is marked by a loss in hippocampal neurons (Jellinger, 1998); however, other studies examining normal aging have shown that the number of neurons is preserved in the dentate gyrus and CA1 pyramidal regions of the hippocampus (West, Coleman, Flood & Troncoso, 1994; Calhoun et al., 1998). Thus, the hypothesis of
[Ca\(^{2+}\)]_i dysfunction and neuronal loss may apply more to pathological conditions than to the process of normal neuronal aging.

8.2 Hypothesis 2: Subtle Changes in Neuronal Calcium Regulation

Another hypothesis is that the progression of aging induces a subtle change in [Ca\(^{2+}\)]_i homeostasis leading to impaired neuronal performance rather than neuronal loss. In theory, subtle changes in any component of the calcium buffering system could lead to dysregulation of calcium homeostasis and altered neurotransmitter release. In support of this hypothesis are data demonstrating the impact of age on NE release. As discussed, age-related increases in stimulation-evoked NE release from rat vascular adrenergic nerves cannot be accounted for by changes in NE content, the function of neuronal or extraneuronal uptake mechanisms, or the function of prejunctional \(\alpha_2\)-adrenergic receptors (Buchholz & Duckles, 1990; Buchholz et al., 1992). In addition, the SERCA antagonist cyclopiazonic acid elevated stimulation-evoked NE release only in adrenergic nerve endings from young animals (Tsai et al., 1998). Taken together these studies measuring NE release from adrenergic nerve endings suggest that the link between aging and increases in NE release may consist of subtle alterations in the function of SERCAs in nerve endings thus altering [Ca\(^{2+}\)]_i homeostasis. In support of this idea, measurement of [Ca\(^{2+}\)]_i-transients in SCG cells by microfluorometry reveal an age-related decline in ATP-dependent Ca\(^{2+}\)-uptake by SERCAs (Tsai et al., 1998; Pottorf et al., 2000). As a consequence of decreased SERCA function, neurons from older animals become more reliant on mitochondria to control [Ca\(^{2+}\)]_i compared to young (Tsai et al., 1995; Buchholz et al., 1996).
8.3  *Governing Hypothesis*

In adrenergic nerves from old animals, calcium uptake by ATP-dependent calcium buffering systems is reduced, leading to greater or more sustained neuronal 

$[\text{Ca}^{2+}]_i$ levels, a greater reliance on remaining calcium buffering systems to restore $[\text{Ca}^{2+}]_i$ homeostasis and increased neurotransmitter release (Fig. 2A,B).
Figure 2. Representation of the hypothesized age induced alteration in calcium buffering systems. In theory, alterations in one component of the calcium buffering system could lead to dysregulation of calcium homeostasis and increased neurotransmitter release. (A) In young neuronal cells, all components of the calcium buffering systems are working within normal limits (illustrated by thin, black arrows) and together regulate neurotransmitter (NT) release. (B) However, with advancing age the function of the sarco/endoplasmic reticulum calcium ATPase (SERCA) buffering component is reduced, leading to increased neurotransmitter release. Because of this deficit there is a shift towards dependence on the remaining calcium buffering components (illustrated by thick, black arrows) to prevent calcium overload, regulate NT release, and maintain calcium homeostasis. The red dotted arrow represents a decline in calcium uptake by SERCAs, while the large red arrow represents induction of NT release by calcium. The green dotted arrows represent calcium buffering pathways that attenuate increases in [Ca$^{2+}$]$_i$ and bring [Ca$^{2+}$]$_i$ back to basal levels.
A. Young Neuronal Cell: Normal Functioning Calcium Buffering Systems

Smooth Endoplasmic Reticulum

PMCA

Ca\(^{2+}\)

Ca\(^{2+}\)

Ca\(^{2+}\)

Ca\(^{2+}\)

Ca\(^{2+}\)

SERCA

Na\(^{+}\)

Calcium Binding Proteins

Ca\(^{2+}\) Ca\(^{2+}\) Ca\(^{2+}\) Ca\(^{2+}\)

Ca\(^{2+}\) Ca\(^{2+}\) Ca\(^{2+}\) Ca\(^{2+}\)

Calcium Binding Proteins

NT vesicle release

H\(^{+}\)

Mitochondria

Ca\(^{2+}\) H\(^{+}\) H\(^{+}\) H\(^{+}\)

Ca\(^{2+}\) H\(^{+}\) H\(^{+}\) H\(^{+}\)

H\(^{+}\) Ca\(^{2+}\)

B. Old Neuronal Cell: Decreased SERCA Uptake and Compensation

Smooth Endoplasmic Reticulum

PMCA

Ca\(^{2+}\)

Ca\(^{2+}\)

Ca\(^{2+}\)

Ca\(^{2+}\)

Ca\(^{2+}\)

SERCA

Na\(^{+}\)

Calcium Binding Proteins

Ca\(^{2+}\) Ca\(^{2+}\) Ca\(^{2+}\) Ca\(^{2+}\)

Ca\(^{2+}\) Ca\(^{2+}\) Ca\(^{2+}\) Ca\(^{2+}\)

Calcium Binding Proteins

NT vesicle release

H\(^{+}\)

Mitochondria

Ca\(^{2+}\) H\(^{+}\) H\(^{+}\) H\(^{+}\)

Ca\(^{2+}\) H\(^{+}\) H\(^{+}\) H\(^{+}\)

H\(^{+}\) Ca\(^{2+}\)
9. CONCLUSIONS

It is important to keep in mind that observed alterations in the activities or function of calcium regulatory systems may be an adaptive response to aging. It is possible that the aging process induces subtle changes in the components responsible for calcium regulation in adrenergic neurons, and this age-related effect may lead to dysfunction of calcium homeostasis and dysregulation of NE release. However, it is also possible that sustained changes in the regulation of [Ca\(^{2+}\)]\(_i\) may be one major cause of neuronal degeneration and altered peripheral adrenergic nerve function with advancing age. Despite our knowledge of the mechanisms involved in neuronal [Ca\(^{2+}\)]\(_i\) regulation, elucidation of the importance and participation of each calcium buffering component is still lacking, and additional studies are necessary to confirm their respective roles in normal aging of central and peripheral nerves.
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CHAPTER II

ADRENERGIC NERVE SMOOTH ENDOPLASMIC RETICULUM CALCIUM BUFFERING DECLINES WITH AGE

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1. ABSTRACT

Adrenergic nerve smooth endoplasmic reticulum calcium buffering declines with age. Calcium buffering capacity declines with age in sympathetic nerves of rat tail artery. To test whether smooth endoplasmic reticulum (SER) calcium buffering declines with age, effects of two SER calcium-ATPase inhibitors on norepinephrine release and intracellular were determined. Thapsigargin or cyclopiazonic acid caused a significant increase in stimulation-evoked norepinephrine release from 6 month tail arteries with much less effect in 20 months. In isolated superior cervical ganglion cells, the rate of rise of calcium with [K\(^+\)]-depolarization increased only in young cells with either cyclopiazonic acid or thapsigargin, with no effect in the old. In young cells, cyclopiazonic acid significantly influenced time to peak, rate of decline, and time to basal of [K\(^+\)]-evoked [Ca\(^{2+}\)]i-transients, but had no effect in old cells. Thapsigargin caused a significant increase in rate of decline in young, but not old, cells. These differential effects suggest an age-related decline in function of SER calcium buffering mechanisms in the sympathetic nervous system causing older nerves to become more reliant on mitochondria to buffer calcium.
α₂-adrenoceptors (Buchholz & Duckles, 1990; Buchholz, Tsai, Friedman & Duckles, 1992). Subsequent studies showed that in both the tail artery and isolated cells from the superior cervical ganglion (SCG), inhibition of mitochondrial calcium uptake had a greater impact in tissues from 20-month-old animals compared to those aged 6 months (Buchholz, Tsai, Foucart & Duckles, 1996). These findings lead to the hypothesis that an age-related decline in SER calcium handling mechanisms results in increased reliance on mitochondrial calcium buffering and increased stimulation-evoked fractional norepinephrine release.

To test this hypothesis, we used two structurally different SERCA inhibitors, cyclopiazonic acid and thapsigargin, to interrupt normal sequestration of calcium by the SER. If intracellular calcium homeostasis is less effective in older animals due to loss of SER function, then we might expect blockade of these mechanisms to have little impact on potassium ([K⁺])-evoked intracellular calcium concentration ([Ca²⁺]i)-transients in isolated SCG neurons from aged animals. In addition, we also measured stimulation-evoked norepinephrine release from tail arteries in the present of cyclopiazonic acid and thapsigargin. Using these SERCA inhibitors, we sought for evidence that a decline in SER calcium buffering systems is implicated in the increased norepinephrine release with advancing age in sympathetic nerves of the rat tail artery.
3. METHODS & MATERIALS

3.1 Experimental Animals

Male Fischer-344 (F-344) rats aged 6 and 20 months were obtained from the NIH-NIA colony maintained by Harland Sprague-Dawley, Inc. (Indianapolis, IN). Rats were anesthetized with CO\textsubscript{2} and sacrificed by decapitation. Animals took food and water \textit{ad libitum} and were maintained on a 12 hr light/dark cycle under controlled temperature (75 ± 2°F).

3.2 Measurement of Intracellular Calcium

SCG cells were isolated by acute dissociation as previously described (Schofield, 1990). Briefly, whole ganglia were removed from the carotid artery bifurcation and placed in cold Tyrode solution containing (in mM): NaCl, 150; KCl, 2; CaCl\textsubscript{2}, 2; MgCl\textsubscript{2}, 2; HEPES, 10; glucose, 10. Ganglia were minced, and the cells acutely dissociated in Earle's balanced salt solution to which were added trypsin (0.5 mg/mL), collagenase D (1 mg/mL), DNAse 1 type IV (0.1 mg/mL), 20 mM HEPES, 10 mM glucose, and 10 mM NaHCO\textsubscript{3}. The pH was adjusted to 7.4 with NaOH. Ganglia were incubated in a shaking water bath for 45 min at 34°C. The dissociated cells were resuspended in Hank’s balanced salt solution to which were added 10% fetal calf serum and 5 mM HEPES with final pH adjusted to 7.4. Cells were plated onto 22 mm cell-tak (3.5 µg/coverslip) coated coverslips (2 ganglia/coverslip). Cells were allowed to plate down for 2 h at room temperature.

Cells were loaded with fura-2 acetoxyxymethylester (fura-2 AM) (1 µM) for 20 min at room temperature and then washed and left for 20 min to allow cell esterases to
convert the dye to the free salt (Thayer, Sturek & Miller, 1988). Cover slips were mounted in a chamber and rinsed in a buffer composed of (in mM) NaCl, 138; CaCl₂, 2; MgCl₂, 1; KCl, 5; HEPES, 10; glucose, 10; pH adjusted to 7.4 with NaOH. The cell chamber was attached to a Nikon inverted microscope interfaced with a Video-Probe imaging system (ETM Systems, Irvine, CA). To change the solution in the chamber, a volume five times larger than the chamber volume of 200 μL was rapidly injected. Thus total volume exchange was achieved in less than 2 s. The fura-2 probe was illuminated alternately at 340 and 380 nm, and emission fluorescence at 510 nm was selected by a barrier filter. Images were collected at a rate of 20 images/min. The ratio of 340/380 nm fluorescence was converted to calcium concentration using the equation:

\[
[Ca^{2+}]_i = K_d \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \frac{S_f}{S_f}
\]

\(R_{\text{min}}\) and \(R_{\text{max}}\) are determined using calcium standards at 0 μM calcium (\(R_{\text{min}}\)) and 39 μM calcium (\(R_{\text{max}}\)). The constant \(K_d\) is the product of the dissociation constant of fura-2 (200 nM) and the ratio of the free and bound forms of the dye at 380 nm (Gryniewicz, 1985).

Rate of rise and rate of decline to 90% recovery of \([Ca^{2+}]_i\) were determined by linear fit (mean \(r\) values of 0.989 and 0.979, respectively).

In experiments using cyclopiazonic acid, cells were initially depolarized for 30 s with 68 mM [K⁺] followed by a 4-min equilibration period. The cells were then exposed to 5 μM cyclopiazonic acid to block SER calcium uptake followed 3 min later by another 30 s depolarization with 68 mM [K⁺]. In experiments using thapsigargin, cells were depolarized for 50 s with 68 mM [K⁺] followed by a 5-min equilibration period. The cells were then exposed for 10 min to 1 μM thapsigargin to block SER calcium uptake.
The drug was then washed out, followed by either a 5 min equilibration period or addition of 100 μM dinitrophenol for 5 min. Finally, cells were depolarized again with 68 mM [K⁺] for 50 s.

3.3  Tail Artery Norepinephrine Release

Proximal segments of tail artery, 6 cm in length, were dissected and cannulated at both ends with polyethylene tubing and placed into an in vitro low volume perfusion system (Budai, Buchholz & Duckles, 1990). The entire assembly was kept at 37°C in a circulating water bath. At the beginning of each experiment, tissues were perfused for 60 min with aerated (95% O₂,5% CO₂) Krebs' solution containing 10⁻⁵ M cocaine, 10⁻⁵ M deoxycorticosterone and 10⁻⁶ M idazoxan to inhibit neuronal uptake, extraneuronal uptake and prejunctional α₂-adrenergic receptors, respectively. The composition of the Krebs' solution was (in mM): NaCl, 118; KCl, 4.8; CaCl₂, 1.6; KH₂PO₄, 1.2; NaHCO₃, 25; MgSO₄, 1.2; ascorbic acid, 0.3; and glucose, 11.5.

Electrical field stimulation was delivered by a Grass S-48 stimulator through platinum electrodes at either end of the tissue chamber. In experiments with cyclopiazonic acid, parameters for excitation of perivascular nerves were 1.0 Hz, 60 volts, 1 ms duration, and 100 pulses. Tissues were stimulated (S1), then equilibrated in Krebs' with 5 μM cyclopiazonic acid for 30 min, and activated again, S2. In the thapsigargin study, parameters for excitation of perivascular nerves were 60 volts, 1 ms duration, and 100 pulses. Tissues were initially activated twice without thapsigargin at 0.25 Hz (S1) and 5.0 Hz (S2). Tissues were then equilibrated in Krebs' with 5 μM thapsigargin for 40 min and then washed for 30 min. Tissues were activated again, S3.
and S4, at 0.25 Hz and 5.0 Hz, respectively. A 20-min equilibration period was allowed between S1/S2 and S3/S4.

3.4 Norepinephrine Quantitation

The perfusate was collected continuously starting at the beginning of each stimulation period until 5 mL was collected. Basal norepinephrine release was monitored by collection of 5 mL of perfusate between each treatment. Perfusates were extracted and quantified with a dihydroxybenzylamine (DHBA) internal standard (300 pg) following a previously described protocol (Budai et al., 1990). One third of the total extracted sample of 300 µL (100 µL) was injected into an ESA coulochem 2 high performance liquid chromatograph (ESA Inc., Bedford Mass, USA) using an ESA reverse phase C-18 column and 1 L of ESA MD-TM aqueous mobile phase containing, in mM, Na₂HPO₄, 75; sodium dodecyl sulfate, 0.5; EDTA, 0.025, 20% acetonitrile, and 5% methanol. The amount of norepinephrine (NE) was calculated using the following formula:

\[
pg \text{ NE} = \frac{\text{NE Peak Ht of Sample}}{\text{Sample Peak Ht NE standard}} \times \frac{100 \text{ pg DHBA}}{\text{DHBA Peak Ht sample}}
\]

Recovery varied from 80-90%.

Quantitation of total tissue norepinephrine content has been previously described (Handa & Duckles, 1987). Briefly, arteries were taken at the end of the perfusion experiment and homogenized in 3 mL of 0.1 N perchloric acid and centrifuged. Tissue norepinephrine was then extracted similarly as the perfusate and used to calculate fractional norepinephrine release:

\[
\text{Fractional NE release} = \frac{pg \text{ NE release}}{(pg \text{ NE tissue content}) \times (\text{number of stimulation pulses})}
\]
3.5 Statistical Analysis

Effects of cyclopiazonic acid and thapsigargin on norepinephrine release and calcium transients were analyzed by paired Student’s t-test. All data are presented as mean ± SEM.

3.6 Materials & Solutions

DNAse-1 type IV, cyclopiazonic acid, thapsigargin, cocaine hydrochloride, deoxycorticosterone, idazoxan, norepinephrine bitartrate, dihydroxybenzylamine, and 2,4-dinitrophenol were obtained from Sigma Chemical (St. Louis, MO, USA). Earle’s and Hank’s balanced salt solutions were obtained from GIBCO (Grand Island, NY). Collagenase D and trypsin were obtained from Boehringer-Mannheim (Indianapolis, IN). Cell-Tak was obtained from Becton-Dickinson Labware (Bedford, MA). Fura-2 was purchased from Molecular Probe Inc. (Eugene, OR).

4. RESULTS

Stimulation-evoked norepinephrine release from the rat tail artery was measured with neuronal and extraneuronal uptake blocked by 10⁻⁵ M cocaine and 10⁻⁵ M deoxycorticosterone and α₂-adrenoceptors blocked by a maximal concentration of idazoxan (10⁻⁶ M; Buchholz et al., 1992). As in previous studies (Buchholz & Duckles, 1990; Tsai, Buchholz & Duckles, 1995), fractional norepinephrine release was significantly higher in the 20-month-old tail arteries when compared to the 6-month-old (132 ± 13, n = 9 compared to 98 ± 8, n = 120 pg/pg content/pulse X 10⁻⁶; p < 0.05).
When SER calcium uptake was blocked by 5 μM cyclopiazonic acid, fractional norepinephrine release evoked by 1 Hz stimulation increased in 6-month-old rat tail arteries, but cyclopiazonic acid had no significant effect in arteries from 20-month-old rats (Fig. 1). A structurally different inhibitor of SER calcium uptake, thapsigargin, had a similar differential effect on the two age groups. In the presence of 5 μM thapsigargin, stimulation-evoked norepinephrine release significantly increased in arteries from both 6- and 20-month old animals at frequencies of both 0.25 and 5 Hz. However, the effect of thapsigargin on 6-month-old arteries was greater than on the 20-month-old arteries.

Basal norepinephrine release from 20-month-old arteries was significantly lower than that of 6-month-old arteries in one set of experiments, but this was not noted in a second experimental set (Table 1). Decreased basal release with age has been found in a previous study (Tsai, Hewitt, Buchholz & Duckles, 1997). There were no differences in the basal norepinephrine release after treatment with cyclopiazonic acid or thapsigargin.

**EFFECT OF CYCLOPIAZONIC ACID OR THAPSIGARGIN ON BASAL NOREPINEPHRINE RELEASE FROM THE TAIL ARTERY**

<table>
<thead>
<tr>
<th>Age</th>
<th>Control</th>
<th>Cyclopiazonic acid (5 μM)</th>
<th>Control</th>
<th>Thapsigargin (1 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Months</td>
<td>0.16 ± 0.03 (12)</td>
<td>0.13 ± 0.02 (12)</td>
<td>0.15 ± 0.02 (16)</td>
<td>0.14 ± 0.01 (16)</td>
</tr>
<tr>
<td>20 Months</td>
<td>0.15 ± 0.03 (10)</td>
<td>0.17 ± 0.04 (10)</td>
<td>0.10 ± 0.01* (10)</td>
<td>0.11 ± 0.01* (10)</td>
</tr>
</tbody>
</table>

**Table 1.** Values represent the mean ± SEM with number of animals indicated in parentheses. All tissues were treated with 10^{-5} M deoxycorticosterone, 10^{-6} M cocaine, and 10^{-6} M idazoxan. * = significantly different as compared to 6 months (unpaired Student’s t-test, p < 0.05).
To extend our results, [Ca\textsuperscript{2+}]i was measured in acutely dissociated SCG cells prepared from the same rats. Dissociated SCG neurons loaded with fura-2 showed a stable basal calcium concentration that was not significantly different between cells from 6- and 20-month-old animals (58 ± 15, n = 11 and 73 ± 9 nM, n = 7, respectively; p = 0.50). When depolarized with 68 mM KCl, SCG neurons exhibited a rapid increase in [Ca\textsuperscript{2+}]i that was reversed when the solution was returned to 5 mM KCl (Fig. 2). The mean amplitude of the calcium transient (peak calcium concentration minus resting calcium concentration) due to 30 s depolarization was not significantly different between the two age groups (Table 2).

### TABLE 2

<table>
<thead>
<tr>
<th>Age</th>
<th>Control</th>
<th>Cyclopiazonic acid (5 µM)</th>
<th>Control</th>
<th>Thapsigargin (1 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Months</td>
<td>155 ± 13 (11)</td>
<td>187 ± 16 (11)</td>
<td>585 ± 81 (14)</td>
<td>683 ± 85 (14)</td>
</tr>
<tr>
<td>20 Months</td>
<td>173 ± 21 (7)</td>
<td>197 ± 32 (7)</td>
<td>659 ± 61 (10)</td>
<td>644 ± 78 (10)</td>
</tr>
</tbody>
</table>

Table 2. Values represent the mean ± SEM with number of animals indicated in parentheses.

Addition of 5 µM cyclopiazonic acid for 5 min resulted in a slight increase in [Ca\textsuperscript{2+}]i (Fig. 2), but there was no age-related difference in the calcium increase caused by cyclopiazonic acid (26 ± 7, n = 11 and 29 ± 6, n = 7 nM for cells from 6- and 20-month-old rats, respectively). However, treatment with cyclopiazonic acid revealed clear differences between nerve cells from 6- and 20-month-old rats when depolarization-induced calcium transients were analyzed. With respect to the increasing calcium phase,
rate of rise of [K\(^+\)]-evoked [Ca\(^{2+}\)]-transients was not different among the age groups (Fig. 3A). However, in the presence of cyclopiazonic acid, the rate of rise of [Ca\(^{2+}\)] in 6-month-old cells was significantly increased, whereas cyclopiazonic acid did not change rate of rise in 20-month-old cells. After exposure to cyclopiazonic acid, neurons from the young SCG showed decreased time to peak, but there was no significant effect in the aged tissues (Fig. 3B).

Analysis of the decreasing calcium phase also revealed clear and significant age-related differences in the effect of cyclopiazonic acid on the process of re-establishment of basal calcium following [K\(^+\)]-depolarization. In the presence of cyclopiazonic acid, the rate of decline of [Ca\(^{2+}\)] was significantly slowed in 6-month-old cells, but cyclopiazonic acid had no significant effect on rate of decline in 20-month-old cells (Fig. 3C). Time to reach basal [Ca\(^{2+}\)] was significantly lengthened in 6-month-old cells by cyclopiazonic acid treatment, but cyclopiazonic acid had no significant effect in cells from 20-month-old animals (Fig. 3D).

In studies with 1 \(\mu\)M thapsigargin, time of exposure to 68 mM [K\(^+\)] was increased from 30 to 50 s. This longer stimulation time produced a higher [K\(^+\)]-evoked [Ca\(^{2+}\)]i-transient (Fig. 4, Table 2). As in studies with cyclopiazonic acid, there were no age-related differences found in the ability of thapsigargin to increase [Ca\(^{2+}\)]i (110 ± 25, \(n = 11\) and 108 ± 44, \(n = 6\) nM for cells from 6- and 20-month-old rats, respectively).

Blockade of SER calcium uptake with thapsigargin also had no effect on the amplitude of [K\(^+\)]-evoked [Ca\(^{2+}\)]i-transients in 6- or 20-month-old neurons (Table 2).

As shown in Figure 5, when the rate of rise of the calcium transient was analyzed, in 6-month-old cells the rate of rise was increased by thapsigargin, but the drug had no
effect in 20-month-old cells (Fig. 5A). Thapsigargin had no significant effect on time to peak in either young or old neurons (Fig. 5B). Interestingly, the rate of decline was increased in the presence of thapsigargin, but only in 6-month-old cells (Fig. 5C). Thapsigargin had no significant effect on time to basal calcium (Fig. 5D).

Finally, to evaluate residual calcium buffering capacity when both mitochondrial and SER calcium uptake systems were blocked, cells were challenged with both 100 μM dinitrophenol to block mitochondrial calcium reuptake and 1 μM thapsigargin to block SER (Fig. 6). After treatment with both drugs, the peak amplitude of [K+]\textsuperscript{+}-evoked [Ca\textsuperscript{2+}]i-transients was significantly increased in both young and old neurons (Fig. 7A). Analysis of the increasing calcium phase showed that there was a significant increase in the rate of rise in both age groups with dinitrophenol and thapsigargin treatment (Fig. 7B). The rate of decline, however, only increased in neurons from 20-month-old animals (Fig. 7C).
Figure 1. Effects of cyclopiazonic acid (5 μM, CPA) and thapsigargin (1 μM, TG) on stimulation-evoked norepinephrine release in tail arteries from 6- and 20-month-old rats. Values are presented as the ratio of S2 (in the presence of treatment) to S1 (untreated). Nerves were activated with electrical stimulation at the frequency indicated (60 volts, 1 ms duration). $10^{-5}$ M deoxycorticosterone, $10^{-3}$ M cocaine, and $10^{-6}$ M idazoxan were present throughout. Values represent mean ± SEM ($N = 10$, 6-month and $N = 7$, 20-month-old animals). * = significantly different as compared to control, $p < 0.05$. 
Figure 2. Effects of cyclopiazonic acid (5 μM) on [Ca\(^{2+}\)]\(_i\) in single freshly dissociated SCG cells from (A) 6-month and (B) 20-month-old rats. Representative tracings are shown. Cells were initially depolarized for 30 s with 68 mM [K\(^+\)] followed by a 4 min equilibration period. The cells were then exposed to 5 μM cyclopiazonic acid followed by another 30 s depolarization with 68 mM [K\(^+\)].
Figure 3. Effect of cyclopiazonic acid (CPA) on indices of calcium increase (A, B) and decrease (C, D) phase in isolated SCG cells from 6-month and 20-month-old animals. Cells were depolarized for 30 s with 68 mM [K⁺]. Rates of rise (A) and decline (C) were determined by regression analysis of [Ca²⁺]ᵢ from base to peak and peak to 90% of recovery, respectively. Time to peak (B) and time to basal (D) calcium were determined, respectively, by the time periods from basal to peak and from peak to 90% recovery. * = significantly different from control, p < 0.05.
Figure 4. Effect of thapsigargin (1 μM) on [Ca^{2+}]i in single freshly dissociated SCG cells from (A) 6-month and (B) 20-month-old rats. Representative tracings are shown. Cells were depolarized for 50 s with 68 mM [K+] followed by 5 min equilibration period. The cells were then exposed for 10 min to 1 μM thapsigargin (TG). The drug was then washed out and followed by another 5 min equilibration period. Finally, cells were depolarized again with 68 mM [K+] for 50 s.
Figure 5. Effect of thapsigargin (TG) on indices of calcium increase (A, B) and decrease (C, D) phases in isolated SCG cells. Cells were depolarized for 50 s with 68 mM [K+] and equilibrated for 5 min. Cells were then exposed to 1 μM thapsigargin for 10 min and then washed out. The cells were allowed 5 min to equilibrate before they were depolarized again with 68 mM [K+]. Rates of rise (A) and decline (C) were determined by regression analysis of [Ca2+]i from base to peak and peak to 90% of recovery, respectively. Time to peak (B) and time to basal (D) calcium were determined, respectively, by the time periods from basal to peak and from peak to 90% of recovery. * = significantly different from control, p < 0.05.
Figure 6. Effect of thapsigargin (1 μM) and dinitrophenol (100 μM) on [Ca^{2+}]i in single freshly dissociated SCG cells from (A) 6-month and (B) 20-month-old rats. Representative tracings are shown. Cells were depolarized for 50 s with 68 mM [K+] followed by a 5 min equilibration period. The cells were then exposed for 10 min to 1 μM thapsigargin. The drug was then washed out and 100 μM dinitrophenol was added. Another 5 min equilibration period was allowed before cells were depolarized again with 68 mM [K+] for 50 s.
Figure 7. Effect of treatment with hypoglycemia (TG) and dimethylfumarol (DNP) on peak amplitude and rate of rise or decline.

A. Peak Amplitude (nM)

B. Rate of Rise (nM/sec)

C. Rate of Decline (nM/sec)

0 25 50 75 100

0 10 20 30 40 50

0 1600 3200 4800

20 months 6 months

Control

DNP + TG

* = significantly different from control,

< 0.05.

base to peak and peak to 90% of recovery, respectively. * = significantly different from control.

C. Rates of rise (b) and decline (c) were determined by regression analysis of Ca^2+ from

mM K_+ for 50 s. Peak amplitude (a) was determined by subtracting baseline Ca^2+ from peak

dimorphosed was added. After a 5 min calibration period, cells were depolarized again with 68 mM K_+

then exposed to 1 mM hypoglycemia for 10 min. Hypoglycemia was then washed out and 100 mM

cells. Cells were depolarized for 50 s with 68 mM K_+ and calibrations for 5 min. Cells were

(a') peak of rise (b) and rate of decline (c) of K_+-induced calcium transients in isolated SCG

effect of treatment with hypoglycemia (TG) and dimethylfumarol (DNP) on peak amplitude and rate of rise or decline.

0 20 40 60 80 100

0 1600 3200 4800
5. DISCUSSION

It has been suggested that neuronal calcium homeostasis declines with age, and much attention has focused on the mitochondrial calcium buffering system (Gibson & Peterson, 1987). Although extensive literature exists supporting the role of free radicals in causing mitochondrial senescence in a variety of tissues, the degree of cellular dysfunction due to mitochondrial damage or how much mitochondrial changes affect organ function is still unclear (Hagen et al., 1997). Other mechanisms may mediate aging in the nervous system. For instance, in hippocampal neurons, the increase in calcium influx with age is mediated by increased expression of L-type calcium channels (Campbell, Hao, Thibault, Blalock & Landfield, 1996). In the sympathetic nervous system, however, the mechanism for age-related changes in function is less clear. Here we show evidence that decline in SER calcium buffering occurs while mitochondrial calcium uptake is maintained in the peripheral sympathetic nervous system.

Previously, we have shown that when mitochondrial calcium buffering is blocked with dinitrophenol, stimulation-evoked norepinephrine release increases in tail arteries from 20-month-old animals whereas there is no change in 6-month-old arteries (Tsai et al., 1995). This is also true in acutely dissociated SCG neurons in which addition of a mitochondrial inhibitor increases peak calcium and rate of rise of $[K^+]$-evoked $[Ca^{2+}]_i$-transients only in cells from 20-month-old animals (Buchholz et al., 1996). Thus, it is of significance that, in the present study, the SERCA inhibitors, cyclopiazonic acid and thapsigargin, had a greater effect in increasing norepinephrine release from young tail arteries compared to old (Fig. 1). Likewise, in acutely dissociated SCG neurons prepared concurrently, cyclopiazonic acid and thapsigargin only had effects in cells from young
animals (Figs. 3 & 5). Together, these results clearly demonstrate age-related differences in calcium homeostatic mechanisms when tissues from 6- and 20-month-old animals are compared.

One interpretation of the results of the present study is that SER is the predominant buffer in the young tissues, while 20-month-old tissues rely more heavily on mitochondrial calcium buffering. In support of this interpretation, neither cyclopiazonic acid (Fig. 3) nor thapsigargin (Fig. 5) had any effect on calcium transients in SCG cells from 20-month-old animals, but the cells did have changes in peak amplitude, rate of rise, and rate of decline when a mitochondrial inhibitor, dinitrophenol, was added after thapsigargin treatment (Fig. 7). Interestingly, rate of decline in the 20-month-old SCG neurons increased in the presence of both dinitrophenol and thapsigargin, whereas there was no change in the young (Fig. 7C). Thus, it seems that all the calcium buffering systems, except for SER, are more activated in the aged neurons. This supports the hypothesis that decline in the SER calcium buffering system forces other calcium buffering systems such as the mitochondria to compensate.

Effects of cyclopiazonic acid and thapsigargin on young cells were not identical. This may be due to a longer [K⁺]-depolarization time of 50 s for studies with thapsigargin compared to 30 s in experiments with cyclopiazonic acid. Indeed, as shown in Table 2, control peak calcium transients were higher with 50 s exposures to high [K⁺] compared to 30 s exposure. Interestingly, with stimulation for 50 s the rate of decline was increased in the presence of thapsigargin (Fig. 5C), in striking contrast to the decreased rate of decline caused by cyclopiazonic acid after 30 s depolarization (Fig. 3C). One explanation is that, due to the kinetics of mitochondrial calcium uptake, at the higher
calcium concentrations seen with prolonged stimulation mitochondrial calcium uptake may be greater, especially when SER calcium buffering systems are blocked. To demonstrate that this increased rate of decline seen with thapsigargin in young cells could be due to mitochondrial activation, we added the mitochondrial inhibitor, dinitrophenol, together with thapsigargin, and in this case there was no change in the rate of decline in 6-month-old cells (Fig. 7C). Thus these results suggest that in cells from young SCG both the SER and mitochondrial calcium buffering systems are functional, and that the mitochondrial calcium buffer system is activated with longer [K+] depolarization and in the presence of thapsigargin.

It is unlikely that the differential effects seen with cyclopiazonic acid and thapsigargin on old versus young tissues were due to an age-related decrease in sensitivity to the drugs, because there were no age-related differences in the ability of cyclopiazonic acid or thapsigargin to release calcium from intracellular stores under basal conditions. Therefore, it seems that SERCA’s buffer calcium in the 20-month-old neurons under basal conditions, but do not function as well during [K+] induced depolarization. This may be due to changes with age in spatial relationships between intracellular organelles revealing that calcium signaling and buffering are highly compartmentalized (Golovina & Blausein, 1997; Thomas, Bird, Hajnóczky, Robb-Gaspers & Putney, 1996).

Another important issue that must be addressed concerns the possibility that there is an age-related difference in the amount of fura-2 taken up by the superior cervical ganglion cells or a difference in the activity of non-specific esterases that convert fura-2/AM to the free salt. Any age-related change in these parameters could result in
erroneous estimations of the [Ca\textsuperscript{2+}]i. One way to assess this is to examine the maximum intensity of the fluorescence signal at 510 nm when the dye is activated at 380 nm with zero Ca\textsuperscript{2+} (Negulescu & Machen, 1990). The intensity of the signal is then proportional to the amount of dye loaded into each individual cell. We have not found any age-related change in the fluorescence signal at 510 nm when fura-2 is activated at 380 nm, suggesting that dye loading is equivalent in young and old cells. Thus, age-related differences in dye loading do not seem to be an issue in our study.

The most significant finding in this study is that there is no effect in tissues from 20-month-old animals when SER calcium uptake mechanism is blocked, suggesting a decline with age in the function of SER. What are the mechanisms that underlie this age-related decline in SER? One possibility is that expression of SERCAs may be decreased with age. Sarcoplasmic reticulum buffering with age has been shown to decrease in cardiac myocytes (Hano et al., 1995), and this has been attributed to a reduction in expression of the gene coding for one SERCA subtype (Lompre, Lambert, Lakatta & Schwartz, 1991; Maciel, Poliker, Rohrer, Popovich & Dillmann, 1990). We are currently determining the subtypes and comparing the expression of SERCAs in the SCG with age to test this hypothesis.

In conclusion, we have clearly shown a significant age-related change in intracellular calcium buffering in adrenergic nerves. Loss of neuronal calcium homeostasis has been suggested to be the key final pathway in age-related decline in brain function (Landfield, 1992). Thus, our findings in adrenergic nerves innervating the rat tail artery and SCG neurons start to define an important aspect of cellular function that
is impacted by advancing age. Further studies would be needed to explore the possibility that this functional change defined in nerves of the autonomic nervous system may have more widespread applicability to age-related changes in other parts of the nervous system.
ACKNOWLEDGMENTS

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ADRENERGIC NERVES COMPENSATE FOR A DECLINE IN CALCIUM BUFFERING DURING AGING

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1. ABSTRACT

*Adrenergic nerves compensate for a decline in calcium buffering during aging.*

We explored the impact of age on the function of Ca$^{2+}$-ATPase systems of the plasma membrane and endoplasmic reticulum. We used the selective ranges of vanadate to block the effects of PMCAs (0.05 – 0.25 μM) over SERCAs (0.5 – 100 μM) and observed a biphasic effect on rate of recovery of [Ca$^{2+}$]i in young (6 month) SCG cells compared to only a single phase response on old (20 month) animals. These data suggests that PMCAs are functioning in both young and old SCG cells; however, the function of SERCAs declines in old SCG cells. We studied the contribution of mitochondrial calcium uptake with age by removing the contribution of Ca$^{2+}$-ATPase systems (PMCAs and SERCAs) with vanadate (100 μM) and Na$^+$/Ca$^{2+}$-exchangers with replacement of [Na$^+$]e with tetraethylammonium. In the presence of these blockers young and old SCG cells showed no change in recovery of [K$^+$]-evoked [Ca$^{2+}$]i-transients, suggesting that the capacity of mitochondrial calcium uptake does not change with age. These data together suggest that despite an age-related decline in SERCA function, PMCA and mitochondrial function appear to be maintained with age and provide compensation in calcium buffering capacity in neurons.
2. INTRODUCTION

Intracellular calcium ([Ca^{2+}]_i) is an ubiquitous second messenger that integrates multiple and diverse neuronal pathways including development and maturation, gene expression, cell death, synaptic plasticity, transmitter release, and excitability (Malenka et al., 1989; Choi, 1992; Clapham, 1995; Ginty, 1997; Spitzer & Ribera, 1998). Calcium homeostasis involves the cooperation of several calcium buffering components that work in concert to restore stimulation-evoked calcium signals to basal levels (Kostyuk & Verkhratsky, 1994). Mechanisms of calcium buffering include: calcium sequestration into endoplasmic reticulum (ER) by sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps (Wuytack et al., 1992; Wuytack et al., 1995); mitochondrial calcium uptake via an electrophoretic Ca^{2+}/H^{+}-uniporter (Werth & Thayer, 1994; David et al., 1998; Pinton et al., 1998); cytosolic calcium binding proteins (Miller, 1995); plasmalemmal calcium extrusion via plasma membrane Ca^{2+}-ATPase (PMCA) pumps (Carafoli, 1997) and plasma membrane Na^{+}/Ca^{2+}-exchange (Blaustein & Lederer, 1999).

Two families of P-type Ca^{2+}-ATPase pumps are vital for the regulation of [Ca^{2+}]_i transients: PMCAs that extrude calcium to the extracellular space (Werth et al., 1996; Carafoli, 1997) and SERCAs that sequester calcium into the ER (Inesi & Kirtley, 1992; Tsai et al., 1998). Functional characterization of SERCAs has been studied with several selective blockers, the most well known being thapsigargin, cyclopiazonic acid, and 2,5-di(tert-butyl)-1,4-benzoquinone (Seidler et al., 1989; Lytton et al., 1991; Khan et al., 1995).

To block the contribution of PMCAs has been more difficult. No rigidly selective, easily administered blockers can inhibit the activity of PMCAs alone.
However, a useful pharmacological tool for studying the function of Ca\textsuperscript{2+}-ATPase systems is the phosphate analog sodium orthovanadate (Na\textsubscript{3}VO\textsubscript{4}) (Simons, 1979; Bond & Hudgins, 1980; Caroni & Carafoli, 1981). The two Ca\textsuperscript{2+}-ATPase activities have been distinguished using vanadate in rat and pig purified brain synaptosomes (enriched PMCAs) and microsomes (enriched SERCAs) (Michaelis et al., 1987; Salvador & Mata, 1998). Salvador and Mata showed that PMCAs exhibit a greater sensitivity to vanadate (K\textsubscript{1/2} = 0.1 \textmu M) over SERCAs (K\textsubscript{1/2} = 0.4 \textmu M), and that this agent can be used to characterize the two Ca\textsuperscript{2+}-ATPase systems.

In recent years our understanding of mitochondria as another major component of the calcium buffering system has undergone substantial revision. Early studies suggested that mitochondria only buffer calcium under non-physiological conditions when [Ca\textsuperscript{2+}]	extsubscript{i} exceeds 2 \textmu M (Nicholls, 1985) or in pathological states (Wilson et al., 1984; Khandoudi et al., 1989). Recent studies have shown that mitochondria buffer [Ca\textsuperscript{2+}]	extsubscript{i} loads within normal physiological ranges and in the absence of pathology (Werth et al., 1994; Buchholz et al., 1996; David et al., 1998; Rizzuto et al., 1998).

The pervasive involvement of calcium in multiple neuronal pathways has led investigators to suggest that dysfunction of [Ca\textsuperscript{2+}]	extsubscript{i} homeostasis may be a primary mediator of age-related neuronal degeneration (Gibson & Peterson, 1987; Khachaturian, 1994; Landfield, 1996). While this is a generally accepted hypothesis, there is little information on age-related changes in specific calcium regulatory systems. Furthermore, as discussed above, multiple mechanisms can potentially compensate for an age-related decline in function of one or more components of the calcium buffering system. For example, our studies in superior cervical ganglion (SCG) cells have shown that there is
an age-related decline in SERCA function (Tsai et al., 1997; Tsai et al., 1998). In support, SERCA expression in cardiac tissue and pancreatic β-cells has also been shown to decline with age (Maciel et al., 1990; Varadi et al., 1996). However, despite the age-related decline in SERCA buffering, it has been suggested that mitochondria and plasmalemma calcium extrusion can compensate for either a decline in SERCA buffering capacity or an age-related increase in neuronal calcium influx (Buchholz et al., 1996; Tsai et al., 1998; Murchison & Griffith, 1998).

To explore the impact of age on the function of Ca\(^{2+}\)-ATPase in plasma membranes and ER, our first study investigated the contributions of PMCAAs and SERCAs to control [K\(^+\)]-evoked [Ca\(^{2+}\)]i-transients in SCG cells. Changes in [Ca\(^{2+}\)]i were measured by fura-2 microfluorometry. The governing hypothesis is that an age-related decline in SERCA function is partially compensated for by an increase in PMCA function. To test the function of Ca\(^{2+}\)-ATPases to control [Ca\(^{2+}\)]i transients in sympathetic neurons, we utilized the differential sensitivity of PMCAAs and SERCAs to vanadate. We anticipated that in young SCG cells the concentration vs. blockade of Ca\(^{2+}\)-ATPases would reveal a biphasic response over the concentration range used (0.01 – 100 μM). Furthermore, we expected that in aged SCG cells vanadate at high concentrations (＞0.25 μM) would have no further blocking effect after inhibition of PMCAAs, supporting a decline in SERCA function.

The second study examined the impact of age on the ability of mitochondrial calcium buffering to regulate [Ca\(^{2+}\)]i in the same young and old SCG cell model. To force the SCG to rely on mitochondrial calcium buffering, the contribution of PMCAAs and SERCAs was blocked with a high vanadate concentration (100 μM), and
extracellular sodium ([Na+]e) was replaced with tetraethylammonium (TEA) to block Na+/Ca2+-exchangers. Changes in [Ca2+]i were measured by fura-2 microfluorometry. This protocol allows for the investigation of the relative contribution and capacity of mitochondria to buffer Ca2+-transients in SCG cells. Our previous studies have demonstrated that there is a greater reliance on mitochondria to buffer Ca2+-transients with age. Therefore, our hypothesis in this study is that mitochondrial calcium uptake is maintained with age despite a decline in SERCA function.

3. MATERIALS & METHODS

3.1 Experimental Animals

Male Fischer-344 (F-344) rats aged 6- and 20-months were obtained from NIH-NIA breeding colony (Harlan Sprague-Dawley, Inc., Indianapolis, IN). Animals took food and water ad libitum and were maintained on a 12 hr light/dark cycle under controlled temperature (75 ± 2°F).

3.2 Superior Cervical Ganglion Preparation

SCG cells were isolated by acute dissociation as previously described (Tsai et al., 1998). Briefly, rats were anesthetized with CO2 followed by decapitation. SCG were dissected from the carotid artery bifurcation and placed in cold Tyrode’s solution containing (in mM): NaCl, 150; KCl, 4; CaCl2, 2; MgCl2, 2; N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), 10; and glucose 10. The ganglia were then acutely dissociated in 5 mL of Earle’s balanced salt solution (EBSS) containing: trypsin (0.5 mg/mL; ~6000 units/mL), collagenase D (1 mg/mL), DNAse-1 type IV (0.1
mg/mL), HEPES (20 mM), glucose (10 mM), and NaHCO₃ (10 mM), pH adjusted to 7.4 with NaOH. After incubation in a shaking water bath for 45 min at 34°C, the digestion reaction was terminated by the addition of 5 mL of modified Hank’s balanced salt solution (HBSS) with 10% fetal calf serum, CaCl₂ (1.3 mM), and HEPES (5 mM), pH adjusted to 7.4 with NaOH. The dissociated cells were then lightly pelleted at 600 rpm (60 x g) for 5 min, the supernatant removed, gently re-suspended in 5 mL of fresh HBSS, and pelleted again at 600 rpm (60 x g) for 5 min. Cells were dispersed in 0.5 mL of HBSS onto Cell-Tak protein coated glass coverslips modified by attaching a 2 cm Teflon ring to the surface with Sylgard. Coverslips were incubated for 2 hr at room temperature to allow cell attachment to the Cell-Tak protein coat before further treatment.

3.3 Measurement of Intracellular Calcium

A dual wavelength ratiometric fura-2 microfluorimetry system was used to measure [Ca²⁺]ᵢ in SCG cells. SCG cells were loaded with 10 µM fura-2 acetoxyethyl ester (fura-2/AM) for 20 min at room temperature, then washed with 5 mL of low [K⁺] (5 mM) buffer composed of (in mM): NaCl, 138; CaCl₂, 2; MgCl₂, 1; KCl, 5; HEPES, 10; glucose, 10; pH adjusted to 7.4 with NaOH. Incubation was continued for an additional 20 min to allow intracellular esterases to convert the fura-2/AM dye into the free acid form (Thayer et al., 1988). Coverslips were mounted into a superfusion chamber, which was attached to the stage of a Nikon inverted microscope (Nikon Instr., Tokyo, Japan). The microscope was attached to a Universal Imaging System running MetaFluor version 3.0 (Universal Imaging Corp., West Chester, PA). The perfusion system allowed the chamber volume (~250 µL) to be exchanged at the rate of 500 µL/sec
A suitable cell was distinguished as a rounded cell body isolated from other cells and has localized phase-contrast illumination of the fura-2 probe. A mercury vapor lamp illuminated the fura-2 probe, and fluorescence was excited alternately at wavelengths 340 and 380 nm by a Sutter-II rotating filter wheel. The fura-2 emission fluorescence at 510 nm was selected by a barrier filter and recorded by a CCD Hamamatsu imaging camera. Adjusting the microscope stage to a point with no cells in the field of view and capturing a background image before the start of the experiment corrected for background light levels. Prior to fura-2/AM loading, cellular autofluorescence was examined in SCG cells. Autofluorescence was found to be undetectable by our imaging system and did not significantly alter our $[\text{Ca}^{2+}]_i$ measurements. During the experiment, 340 and 380 images were collected at a rate of 4 boxcar-averaged images/sec, corrected for background fluorescence, calcium concentration calculated, and the data logged to a text file. During dye loading and data collection, ambient light levels were minimized and neurons were only illuminated during data acquisition to minimize bleaching and potential photo damage of the dye.

Intracellular calcium was estimated by both in vitro and in vivo calibration methods. The in vitro method was performed using a calcium calibration kit (Molecular Probes, Eugene, OR) with known $[\text{Ca}^{2+}]$ ranging from 0 – 40 μM. Each prepared calcium solution was loaded with 4 μM fura-2 pentapotassium salt. A droplet of each $[\text{Ca}^{2+}]$ was placed onto a glass slide and the fluorescent intensities from 340 and 380 excitation were measured and a curve of 340/380 ratio (R) vs. $[\text{Ca}^{2+}]$ was plotted. The in vivo method was performed on SCG cells by decreasing extracellular calcium concentration to 0 mM, and fluorescence from 380 ($F_{\text{min}}$) and 340/380 ratio ($R_{\text{min}}$) was
recorded for 1 min. The extracellular medium was then replaced with 10 mM \([\text{Ca}^{2+}]\) with ionomycin (1 \(\mu\)M) and depolarized with high potassium (68 mM). Values were then recorded for 1 min to obtain \(F_{\text{max}}\) and \(R_{\text{max}}\). The values for \(F_{\text{min}}\), \(R_{\text{min}}\), \(F_{\text{max}}\), and \(R_{\text{max}}\) were remarkably similar between the in vitro and in vivo method. Our in vitro calibration for \(K_d\) is comparable to in vivo values in neuronal cells (Neher, 1995; Murchison et al., 1998). Furthermore, we applied the same \(K_d\) for both young and old SCG neurons, since previous reports have shown no significant change in fura-2 \(K_d\) values for young and old neurons (Murchison et al., 1998). Since our in vitro method correlated well with in vivo measurements, the values obtained from the in vitro calibration were used to convert the experimental fluorescent intensity ratios (R) to \([\text{Ca}^{2+}]\) over the physiological range of \([\text{Ca}^{2+}]_i\) by iterative fit to the equation:

\[
[\text{Ca}^{2+}]_i = K_d \frac{R-R_{\text{min}}}{R_{\text{max}}-R} S_f
\]

\(R_{\text{min}}\) is the 340/380 ratio at zero \([\text{Ca}^{2+}]\) and \(R_{\text{max}}\) is the 340/380 ratio at 40 \(\mu\)M \([\text{Ca}^{2+}]\).

The value of \(K_d\) is the dissociation constant of fura-2, while \(S_f\) is a correction factor relating the ratio \(F_{\text{min}}/F_{\text{max}}\), which is the emission intensity at 380 nm when fura-2 is in the free (\(F_{\text{min}}; 0 \mu\text{M [Ca}^{2+}\)) or bound (\(F_{\text{max}}; 40 \mu\text{M [Ca}^{2+}\)) form (Gryniewicz et al., 1985). For this report, the \([\text{Ca}^{2+}]_i\) was estimated using averaged in vitro values for \(S_f\) (7.6), \(R_{\text{min}}\) (0.33), \(R_{\text{max}}\) (2.75), and \(K_d\) (251 nM).

### 3.4 Experimental Protocols

To characterize the effects of vanadate on PMCA and SERCA, SCG cells were first depolarized (S1, control) for 15 sec by superfusion with Tyrodes buffer containing...
68 mM KCl followed by a wash out with low [K⁺] (5 mM) buffer and a 2 min equilibration period. Cells were then exposed to a selected vanadate concentration ranging from 0.01 to 100 µM and allowed to equilibrate for 2 min. Again cells were depolarized for 15 sec with Tyrodes buffer containing 68 mM [K⁺] and vanadate (S2). Exposure to increasing concentrations of vanadate (0.01 - 100 µM) was repeated for S3 and S4. The SCG cells were divided into three groups for each age (6- and 20-month) and exposed to the following vanadate concentrations (in µM): Group A: 0.01, 0.05, 0.1; Group B: 0.25, 0.5, 1.0; and Group C: 1.0, 2.0, 100.

For studies using vanadate and TEA to study mitochondrial calcium uptake function, SCG cells were initially depolarized (S1, control) for 15 sec by superfusion with Tyrodes buffer containing 68 mM KCl followed by a wash out with low [K⁺] (5 mM) and a 2 min equilibration period. SCG cells were then exposed to 100 µM vanadate for 4 min to block the Ca²⁺-ATPase systems of PMCAs and SERCAs. Finally, zero sodium (Na⁺ replaced with TEA) was added to block the Na⁺/Ca²⁺-exchanger, and SCG cells depolarized (S2) for 15 sec in the presence of 68 mM KCl, 100 µM vanadate, and zero sodium.

3.5 Data Analysis

The resting [Ca²⁺]i for SCG cells was recorded as a long segment (50-60 sec) of fluorescence ratio signal before initial depolarization of the cell. The peak change in [Ca²⁺]i was measured by subtracting the baseline [Ca²⁺]i from the observed peak [Ca²⁺]i. The baseline [Ca²⁺]i was measured as the mean [Ca²⁺]i 1-2 sec immediately before each [Ca²⁺]i transient. The rate of recovery of [Ca²⁺]i (nM/s) was measured from the peak
change in \([Ca^{2+}]_i\) to within 90% recovery to baseline. Rate of rise of \([Ca^{2+}]_i\) (nM/s) was determined by measuring from baseline \([Ca^{2+}]_i\) to peak \([Ca^{2+}]_i\). The rate of decline and rate of rise of \([Ca^{2+}]_i\) were determined by linear fit regression (mean \(r\) values of 0.975 and 0.970, respectively).

3.6 Statistical Analysis

The effect of age on the response of calcium transients to treatment with vanadate and vanadate/TEA was analyzed by Student’s paired \(t\)-test and two-way ANOVA with Fischer-PLSD test. Data are presented as mean or mean % of control ± SEM.

3.7 Materials & Solutions

DNAse-1 type IV, trypsin, fetal calf serum, and vanadate were obtained from Sigma Chemical (St. Louis, MO). Fura-2/AM from Molecular Probes (Eugene, OR). Earle’s and Hank’s balanced salt solution from GibCO (Grand Island, NY). Collagenase D from Boehringer-Mannheim (Indianapolis, IN). Cel-Tak from Collaborative Biomedical Products (Bedford, MA). Sylgard from Dow Corning Corp. (Midland, MI).

4. RESULTS

4.1 Resting \([Ca^{2+}]_i\) Measurements

Acutely dissociated SCG cells prepared from 6- and 20-month-old rats were loaded with fura-2 and showed a stable basal \([Ca^{2+}]_i\) that was indistinguishable between the two age groups (6 month: 91 ± 6 nM, \(N = 35\) and 20 month: 98 ± 12 nM, \(N = 24\)).
4.2 *Effect of Ca\(^{2+}\)-ATPase Blockade*

When SCG cells were depolarized for 15 sec by superfusion with 68 mM KCl, [Ca\(^{2+}\)]\(_i\) increased rapidly, then was restored to basal levels when the solution was replaced with 5 mM KCl (Fig. 1). Exposure of SCG cells to low concentrations of vanadate (0.25 μM) to block the PMCA contribution resulted in a decline in rate of recovery of [Ca\(^{2+}\)]\(_i\) in 6-month-old, with a greater effect in 20-month-old cells (Fig. 1A, inset i; Fig. 1C, inset ii). However, when the vanadate concentration was increased (1.0 μM), to block both PMCs and SERCs, an additional decline in rate of recovery of [Ca\(^{2+}\)]\(_i\) occurred in 6-month, but no significant further effect was observed in 20-month-old cells (Fig. 1B, inset i; Fig. 1D, inset ii). Comparison of control values of time to recovery and rate of recovery for [K\(^+\)]-evoked [Ca\(^{2+}\)]\(_i\)-transients showed no significant difference between the two age groups, however, 20-month SCG cells showed a significant increase in peak height (Table 1). Exposure to high vanadate (100 μM) did not significantly alter peak height, time to peak, or rate of rise of [Ca\(^{2+}\)]\(_i\) when compared to control values in either age group (Table 2).

| CONTROL VALUES FOR [K\(^+\)]-EVOKED [Ca\(^{2+}\)]\(_i\)-TRANSIENTS FROM SCG CELLS |
|----------------------------------|----------------------------------|
|                                | Young (6-month) | Old (20-month) |
| Time to Recovery (sec)         | 10.4 ± 0.7      | 10.9 ± 1.0      |
| Rate of Recovery (nM/sec)      | 44.6 ± 4.0      | 45.3 ± 4.3      |
| Peak Height (nM)               | 436.5 ± 25.1    | 530.3 ± 34.5 *  |

*Table 1.* Values represent mean ± SEM. Values were compared by unpaired T-test. * = significantly different from 6 month control \((p < 0.05)\). \(N = 55\) cells from 24, 6-month-old animals and 47 cells from 24, 20-month-old animals.
EFFECT OF HIGH VANADATE (100 μM) ON [K⁺]-EVOKED [Ca²⁺]-TRANSIENTS IN SCG CELLS (PERCENT OF CONTROL)

<table>
<thead>
<tr>
<th></th>
<th>Young (6-month)</th>
<th>Old (20-month)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak Height (nM)</td>
<td>96.1 ± 3.1</td>
<td>96.8 ± 15.6</td>
</tr>
<tr>
<td>Time to Peak (sec)</td>
<td>104 ± 9.6</td>
<td>106 ± 6.6</td>
</tr>
<tr>
<td>Rate of Rise (nM/sec)</td>
<td>100 ± 7.9</td>
<td>105 ± 12.4</td>
</tr>
</tbody>
</table>

Table 2. Values represent mean % of control ± SEM. Effects of treatments were determined by two-way ANOVA with repeat measures and Fisher-PLSD-test, or paired Student’s t-test. N = 31 cells from 8, 6-month-old animals and 24 cells from 8, 20-month-old animals.

Exposure of SCG cells to the Ca²⁺-ATPase blocker vanadate at high concentrations (> 0.5 μM) that block both PMCAs and SERCAs consistently induced a cytosolic calcium spike in 14 of 18 SCG cells from 6-month-old compared to only 2 of 17 cells from 20-month-old animals (Fig. 1B,D). Low vanadate concentrations (< 0.5 μM), which selectively block PMCAs, did not induce cytosolic calcium spikes in either 6 or 20-month-old SCG cells (Fig. 1A,C).

In the presence of vanadate at low or high concentrations, the rate of recovery of [Ca²⁺]i was significantly reduced, and time to recovery of [Ca²⁺]i was significantly increased in both 6- and 20-month SCG cells (Fig. 2A,B). Analysis of rate of recovery and time to recovery of [Ca²⁺]i elucidated a number of points of interest. First, vanadate produced a biphasic response in SCG cells from 6-month-old animals, which contrasted to a single-phase response in 20-month-old (Fig. 2A,B). Secondly, the low vanadate range (0.01 – 0.25 μM), which is thought to selectively block PMCAs and not significantly affect SERCAs, produced a change in these parameters in both 6- and 20-
month-old SCG cells, with a greater effect in 20-month (Fig. 2A,B). Thirdly, the high vanadate range (0.5 – 100 μM), which is thought to block both PMCAs and SERCAs, produced an additional change in these parameters only in 6-month SCG cells, with no further significant change in 20-months (Fig. 2A,B).

4.3 Vanadate Selectivity for PMCAs

To confirm the effects of low vanadate concentrations on PMCAs we first blocked SERCAs with the selective SERCA blocker thapsigargin. Vanadate (0.1 μM) was then applied to observe if this concentration of vanadate had any additional affect on rate of recovery of high [K+]-evoked [Ca²⁺]i-transients. Young SCG cells exposed to thapsigargin (0.5 μM) showed a significant decline in rate of recovery of [Ca²⁺]i compared to control. Addition of a low vanadate concentration (0.1 μM), which is thought to primarily block PMCAs, showed a significant and further decline in rate of recovery of [Ca²⁺]i compared to treatment with thapsigargin alone. Subsequent exposure of thapsigargin treated cells to a high concentration of vanadate (1.0 μM) showed no significant further change in rate of recovery of [Ca²⁺]i (Table 3).
SELECTIVE EFFECTS OF VANADATE ON PMCA IN THE PRESENCE OF THAPSIGARGIN IN 6-MONTH-OLD SCG CELLS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Thapsigargin (0.5 μM)</th>
<th>Low Vanadate (0.1 μM)</th>
<th>High Vanadate (1.0 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of Recovery (nM/sec)</td>
<td>52.01 ± 9.9</td>
<td>33.28 ± 4.2 *</td>
<td>19.27 ± 2.9 +</td>
<td>18.13 ± 3.3</td>
</tr>
</tbody>
</table>

* Table 3. Values represent mean ± SEM. SCG cells were exposed to 68 mM KCl for 15 sec to evoke [Ca^{2+}]i-transients and then washed with 5 mM KCl to allow calcium buffering systems to restore [Ca^{2+}]i. Values were compared by paired Student’s t-test. * = significantly different from S1 control (p < 0.05). + = significantly different from S2 treatment with thapsigargin (0.5 μM) (p < 0.05). N = 6 cells from 4, 6-month-old animals.

4.4 Role of Mitochondria

In addition to studying the contributions of Ca^{2+}-ATPase systems to control [K^{+}]-evoked [Ca^{2+}]i-transients, we investigated the role of mitochondrial calcium uptake in SCG cells. Therefore, we blocked PMCA and SERCA with 100 μM vanadate and the Na^{+}/Ca^{2+} exchanger by replacing [Na^{+}]e with TEA. Under these conditions SCG cells will rely on mitochondria to control [K^{+}]-evoked [Ca^{2+}]i-transients. As shown in figure 3, following the initial high [K^{+}]-evoked [Ca^{2+}]i-transients (S1), cells were exposed to 100 μM vanadate. This induced a calcium spike in 20 of 28, 6-month-old cells, while only 5 of 23, 20-month cells responded in this way to the addition of vanadate. When [Na^{+}]e was replaced with TEA and 100 μM vanadate was present, there was a decline in rate of recovery of [K^{+}]-evoked [Ca^{2+}]i-transients (S2) in both 6- and 20-month cells (Fig. 3, inset i,ii). In the presence of 100 μM vanadate and TEA, the rate of recovery and time to recovery of [K^{+}]-evoked [Ca^{2+}]i-transients were both significantly altered with no significant differences in age (Fig. 4).
Figure 1. Representative tracings of the effects of vanadate on [K⁺]-evoked [Ca²⁺]-i-transients in single, freshly dissociated SCG cells from 6-month (A,B) and 20-month-old (C,D) rats. Effects of low vanadate concentration (0.25 μM) are shown in A and C, and effects of high vanadate concentration (1.0 μM) are shown in B and D. Figure insets (i-iv) illustrate rate of recovery of [Ca²⁺]-i after [K⁺]-evoked depolarization of control (S1) and treated (S2) cells with an expanded time scale to resolve changes in recovery. Rate of recovery was analyzed by best fit linear regression with an average R² of 0.982 for control and 0.972 for treated [Ca²⁺]-i-transients. Fura-2 loaded cells were depolarized (S1, Control) for 15 sec with Tyrodes buffer containing 68 mM [K⁺], followed by a 2 min equilibration in normal 5 mM [K⁺] Tyrodes. Cells were then exposed to Tyrodes with vanadate (0.25 or 1 μM) for 2 min and then depolarized for 15 sec with Tyrodes containing 68 mM [K⁺] and vanadate (S2) followed by a 2 min equilibration.
A. 6-month, Low Va Range

0.25 μM Va

S1

S2

B. 6-month, High Va Range

1.0 μM Va

S1

S2

Inset i.

6 Month

Control, S1
-69.7 nM/sec
Low Va, S2
-51.4 nM/sec
High Va, S2
-33.7 nM/sec

C. 20-month, Low Va Range

0.25 μM Va

S2

D. 20-month, High Va Range

1.0 μM Va

S2

Inset ii.

20 Month

Control, S1
-70.6 nM/sec
Low Va, S2
-35.2 nM/sec
High Va, S2
-32.6 nM/sec
Figure 2. (A) Effects of the Ca$^{2+}$-ATPase inhibitor, vanadate (0.01 - 100 μM), on rate of recovery of [Ca$^{2+}$]i in SCG cells from 6- and 20-month-old animals. (B) Effects of vanadate (0.01 - 100 μM), on time to recovery of [Ca$^{2+}$]i in SCG cells from the same age groups. The light gray area represents the low range of vanadate concentration, while the dark gray area represents the high range of vanadate concentration. Rate of recovery of [Ca$^{2+}$]i was determined by linear regression analysis from peak [Ca$^{2+}$]i levels to 90% of recovery to basal [Ca$^{2+}$]i, then adjusted to % of control. Time to recovery of [Ca$^{2+}$]i was determined by the difference in time from peak [Ca$^{2+}$]i levels to 90% of recovery to basal [Ca$^{2+}$]i, then adjusted to % of control. Values are expressed as % of control. Effects of treatments were determined by two-way ANOVA with repeat measures and Fisher-PLSD test, or paired Student’s t-test. * = Significantly different from control, $p < 0.05$. + = Significantly different from 0.25 μM (-6.6 on x-axis) vanadate, $p < 0.05$. N = 35 cells from 8, 6-month-old animals and 24 cells from 8, 20-month-old animals.
A. Rate of Recovery of [Ca\(^{2+}\)]\(_i\)

Low Vanadate Range (PMCA Blockade)

High Vanadate Range (PMCA/SERCA Blockade)

B. Time to Recovery of [Ca\(^{2+}\)]\(_i\)

Low Vanadate Range (PMCA Blockade)

High Vanadate Range (PMCA/SERCA Blockade)
Figure 3. Representative tracings of the effects of vanadate and zero Na⁺ on [K⁺]-evoked [Ca²⁺]-transients in SCG cells from (A) 6-month-old and (B) 20-month-old rats. Effects of addition of vanadate (100 μM Va) and zero Na⁺ (replacement of Na⁺ with TEA) are shown. Figure insets (i, ii) illustrate rate of recovery of [Ca²⁺]i after [K⁺]-evoked depolarization of control (S1) and treated (S2) cells with an expanded time scale to resolve changes in recovery. Rate of recovery was analyzed by best fit linear regression with an average R² of 0.98 for control and 0.96 for treated Ca²⁺-transients. Fura-2 loaded cells were depolarized (Control, S1) for 15 sec with Tyrodes buffer containing 68 mM [K⁺], followed by a 2 min equilibration. Cells were then exposed to 100 μM vanadate for 4 min. Again cells were depolarized (Va + TEA, S2) for 15 sec with Tyrodes buffer containing 68 mM [K⁺], vanadate, and zero Na⁺ (143 mM TEA) followed by a 2 min equilibration.
Significantly different from control, *p < 0.05, N = 24 cells from 16- and 20-month-old animals and 23

were determined by two-way ANOVA with repeated measures and Fisher-PLSD test. **

Figure 4. Effect of treatment with vanadate and zero Na⁺ (TEA) on high [K⁺]-evoked [Ca²⁺].}

**Figure 4. Effect of treatment with vanadate and zero Na⁺ (TEA) on high [K⁺]-evoked [Ca²⁺].**
5. DISCUSSION

5.1 Technical Considerations

The use of fluorescent calcium sensitive probes can introduce potential sources of error, which merit discussion (Moore et al., 1990; Kao, 1994; Neher, 1998). The \([\text{Ca}^{2+}]_i\) values we report are estimates and not absolute values. Therefore, the comparison of age-related changes in calcium buffering is the relative difference in \([\text{Ca}^{2+}]_i\) observed between the two age groups. For age comparisons, we pair depolarizations under treated conditions with control stimulations for each cell. Resting \([\text{Ca}^{2+}]_i\), peak \([\text{Ca}^{2+}]_i\), and the rates of rise and recovery (nM/sec) reported are well within the accepted physiological ranges reported in neurons and other cells (Neher, 1995; Murchison et al., 1998). In these experiments peak \([\text{Ca}^{2+}]_i\) transients are typically below 1.0 \(\mu\text{M}\) \([\text{Ca}^{2+}]_i\). Therefore, peak \([\text{Ca}^{2+}]_i\) transients fall within the linear range (0.02 – 2.0 \(\mu\text{M}\) \([\text{Ca}^{2+}]\)) of the in vitro calibration curve for fura-2. Additionally, there appeared to be little evidence of incomplete hydrolysis or compartmentalization of the fura-2/AM dye (Zhou & Neher, 1993). The short loading time and low concentration of fura-2/AM combined with low temperature and long de-esterification time minimizes these possible sources of error.

It has been suggested that the digestion process may differentially affect fura-2 loading for young and aged SCG cells and may be responsible for our observed age-related effect. When cells are at rest, the 380 nm signal is a comparative indicator of fura-2 loading (Negulescu & Machen, 1990). The 380 nm signals are remarkably similar between young and old SCG cells (6 month: 89.27 ± 2.57; 20 month: 90.72 ± 3.53 F<sub>380</sub>, emission intensity of fura-2 excited at 380 nm). This strongly suggests that the acute dissociation process does not introduce a sampling artifact, compromise data, or alter
fura-2 loading in SCG cells. Our basal [Ca\(^{2+}\)]_i levels are comparable with values reported in central and peripheral nerve models and this did not vary with age (Thayer et al., 1990; Garyantes & Regehr, 1992; Benham et al., 1992; Griffith & Murchison, 1995; Jasek & Griffith, 1998).

Vanadate (VO\(_4^{3-}\)) is analogous to the transition state of phosphate (PO\(_4^{3-}\)) involved in enzyme reactions, allowing it to inhibit P-type ATPase's (Smith et al., 1980; Stankiewicz et al., 1995). Given vanadate's similarity to the phosphate molecule, it is logical to assume vanadate may affect other ATPases, phosphatases, and kinases. Weak inhibition of phosphatase activity has been reported in cell cultures, red blood cells, and rat spleen (Brunati & Pinna, 1985; Boivin et al., 1986; Imes et al., 1987). In contrast, purified kinases exposed to vanadate showed no significant effect on pyruvate kinase, creatine kinase, or inorganic pyrophosphatase (Boyd et al., 1985). Vanadate partially blocks the Na\(^+/K^+\)-ATPase pump, thus possibly altering [Na\(^+\)] and [K\(^+\)] balance and in turn basal [Ca\(^{2+}\)]_i (Cantley et al., 1977; Bond & Hudgins, 1979). However, despite only a transient effect, vanadate had no sustained significant effect on basal [Ca\(^{2+}\)]_i in young or aged SCG cells, suggesting that blockade of Na\(^+/K^+\)-ATPase pumps does not appreciably affect basal [Ca\(^{2+}\)]_i (Fig. 1C,D; Fig 3A,B). Furthermore, if these non-specific actions of vanadate did have an effect on other parameters of the calcium transient, then these effects would be manifested in rate of rise and peak height. Our own studies (Table 2) and studies in dorsal root ganglion neurons have shown no significant effect of vanadate on peak [Ca\(^{2+}\)]_i (Benham et al., 1992). Therefore we may assume that the effects of vanadate appear to be localized to Ca\(^{2+}\)-ATPase systems, and the "nonspecific" effects of this treatment were minimal in SCG cells.
Complete \([\text{Na}^+]\) replacement will remove the inward sodium gradient that drives calcium efflux resulting in reversed exchange with calcium moving into the cell. The use of TEA to replace \([\text{Na}^+]\) had no significant effect on resting \([\text{Ca}^{2+}]\) (Fig. 3A,B), suggesting that the \(\text{Na}^+/{\text{Ca}^{2+}}\)-exchanger is not critical in determining resting \([\text{Ca}^{2+}]\) in these cells, and that any calcium entry via the reverse process of the exchanger is easily sequestered by other calcium buffering systems.

5.2 Impact of Age on SERCA Function

In this study a differential sensitivity of PMCA\s and SERCA\s to vanadate was established. Our study confirms the \(K_{1/2}\) values cited by Salvador and Mata. In the low vanadate concentration range (0.01 – 0.25 \(\mu\)M), our intent was to effectively block PMCA\s, while at higher vanadate concentrations (0.5 – 100 \(\mu\)M), blockade of both PMCA\s and SERCA\s was expected. Indeed, in SCG cells from young animals, vanadate produced a biphasic response in the rate of recovery and time to recovery of \([\text{Ca}^{2+}]\) over the entire concentration range. However, this was not the case in aged SCG cells, in which vanadate only produced a single response over the same concentration range. Vanadate had no further effect on aged SCG cells above a concentration of 0.25 \(\mu\)M, i.e. after PMCA blockade, whereas in young animals there was an additional and significant decline in rate of recovery and increase in time of recovery of \([\text{Ca}^{2+}]\). Vanadate above 0.25 \(\mu\)M frequently induced calcium spikes in 6-month-old cells only (Fig. 1C,D; Fig. 3A,B). The calcium spikes are produced because blockade of SERCA uptake prevents calcium sequestration, but not leakage, in calcium stores (Verkhratsky & Kettenman, 1996; Carlson et al., 1997). The greater effect of high vanadate on recovery dynamics of
[Ca$^{2+}$]i-transients and inducement of calcium spikes in young cells as compared to aged neurons is further evidence that SERCA function declines with age. These data strengthen our previous work suggesting that an age-related decline in SERCA function exists in SCG cells (Tsai et al., 1998).

One explanation for such a decline in SERCA function with age may be changes in expression of SERCA isoforms. Indeed, SERCA mRNA expression declines with age in cardiac tissue and pancreatic β-cells (Maciel et al., 1990; Varadi et al., 1996). In another study, SERCA proteins in rat myocardium were shown to remain stable with age. Despite unchanged SERCA content, $^{45}$Ca$^{2+}$-uptake declined, which corresponded to a decline in calmodulin mediated phosphorylation of SERCAs (Xu & Narayanan, 1998). Thus, an age-related decline in function does not necessarily imply a decline in expression of the protein. These data emphasize that other mechanisms underlying age-related changes in the function of calcium buffering systems need to be explored with a combination of functional and molecular tools.

5.3 Impact of Age on PMCA Function

These studies underscore a larger issue. That is, during normal healthy aging, alternative mechanisms can compensate for an age-related decline in a particular component of the calcium buffering systems. In the low vanadate concentration range (i.e. PMCA blockade), the effect of vanadate on rate of recovery and time to recovery of [Ca$^{2+}$]i was greater in SCG cells from 20-month-old animals compared to 6-months (Fig. 2). Furthermore, low vanadate concentrations ($< 0.5$ μM) in the presence of thapsigargin, which blocked the contribution of SERCAs, showed a significant effect on
rate of recovery of $[\text{Ca}^{2+}]_i$ in young SCG cells (Table 3). These data suggest that PMCA function is maintained with age, however, the plasma membrane extrusion system compensates for a deficiency in SERCA function. We hypothesized that when SERCA calcium uptake declines with age, other calcium buffering components, such as PMCAs may be able to compensate and restore $[\text{Ca}^{2+}]_i$ to basal levels. Together, our data using two different concentration ranges of the Ca$^{2+}$-ATPase antagonist vanadate support the hypothesis that in aged neuronal cells SERCA function declines and cells become more dependent on PMCAs to control $[\text{Ca}^{2+}]_i$.

5.4 Dependence on Mitochondrial Calcium Uptake

Recently mitochondria have been established as an important participant in the control of $[\text{Ca}^{2+}]_i$-transients (Werth et al., 1994; David et al., 1998; Pinton et al., 1998). Therefore, we wished to investigate the impact of age on the ability of mitochondria to buffer $[\text{Ca}^{2+}]_i$-transients when SCG cells are forced to rely on mitochondrial calcium uptake. In the present study we found that, when PMCAs and SERCAs were blocked with 100 μM vanadate and Na$^+$/Ca$^{2+}$-exchange was blocked by replacement of [Na$^+$]e with TEA, SCG cells could fully recover from high $[\text{K}^+]$-evoked $[\text{Ca}^{2+}]_i$-transients. In addition, there was a significant decline in the recovery dynamics of $[\text{Ca}^{2+}]_i$ in both 6- and 20-month SCG cells, with no age-related difference. These data suggest that there is no age-related change in the ability of the mitochondria to control $[\text{Ca}^{2+}]_i$-transients and that mitochondria may also compensate for a decline in SERCA function. Complementing these data, we have previously illustrated that the effect of the mitochondrial calcium uptake blocker, dinitrophenol, was greater in 20-month-old SCG
cells as compared to 6-month (Buchholz et al., 1996). Furthermore, mitochondria in aged basal forebrain neurons compensate for increased calcium influx (Murchison et al., 1998).

Our findings are in contrast to studies suggesting that mitochondrial calcium uptake declines with age in hippocampal neurons (Vitorica & Satrustegui, 1986; Villalba et al., 1995; Satrustegui et al., 1996). One explanation for this effect could be that these studies were done in synaptosomes, instead of single cell preparations, and mitochondrial calcium uptake was not directly measured. Instead, total $^{45}$Ca$^{2+}$ release from mitochondria was measured in the presence of the oxidative phosphorylation uncoupler, carbonyl cyanide $p$-trifluoromethoxyphenylhydrazone (FCCP). Release of mitochondrial calcium may not accurately measure the activity of mitochondrial calcium uptake by the Ca$^{2+}$/H$^+$-uniporter itself, but rather, may simply indicate a change in the capacity of mitochondria to store calcium. The primary factor in mitochondrial calcium uptake is maintenance of the mitochondrial proton gradient to provide electrochemical energy for calcium uptake. Indeed, in rat liver microsomes and brain synaptosomes there is no significant age-related change in the mitochondrial proton gradient, respiratory rate, or membrane potential (Vitorica et al., 1985; Paradies & Ruggiero, 1991). Thus, these studies support our data in SCG cells suggesting that the capacity of mitochondrial calcium uptake is maintained with age.

In conclusion, we have provided data that support the hypothesis that advancing age in SCG is associated with a significant decline in SERCA function, reinforcing the importance of Ca$^{2+}$-ATPase uptake and extrusion as a component of the neuronal calcium
buffering system. Additionally, to compensate for the age-related decline in SERCA activity, PMCA and mitochondrial function appear to be maintained with age and provide ancillary calcium buffering capacity. Despite knowledge of the mechanisms involved in neuronal [Ca$^{2+}$]i regulation, elucidation of the importance and participation of each calcium buffering component is still lacking and additional studies are necessary to confirm their roles in neuronal degeneration.
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CHAPTER IV

SERCA FUNCTION DECLINES WITH AGE IN ADRENERGIC NERVES FROM THE SUPERIOR CERVICAL GANGLION

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1. **ABSTRACT**

*SERCA function declines with age in adrenergic nerves from the superior cervical ganglion.* In excitatory cells intracellular calcium ([Ca$^{2+}$]i)-signals are regulated by multiple calcium buffering mechanisms to restore [Ca$^{2+}$]i to basal levels and maintain [Ca$^{2+}$]i homeostasis. The involvement of [Ca$^{2+}$]i in multiple neuronal pathways suggests that dysfunction of [Ca$^{2+}$]i homeostasis may be a primary mediator of age-related neuronal degeneration. Previous work in sympathetic neurons supports the hypothesis that a decline in sarco/ endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) function occurs with advancing age. To test for age-related functional changes in SERCA activity, [K$^+$]-evoked [Ca$^{2+}$]i-transients were measured by fura-2 microfluorometry in acutely dissociated superior cervical ganglion (SCG) cells from F-344 rats. SCG cells were forced to rely on SERCAs by exposure to low vanadate (0.1 μM) to selectively block plasma membrane Ca$^{2+}$-ATPase pumps, dinitrophenol (100 μM) to block mitochondrial Ca$^{2+}$-uptake, and replacement of [Na$^+$]e with tetraethylammonium to block Na$^+$/Ca$^{2+}$-exchange. Analysis of rate of recovery of [Ca$^{2+}$]i showed a decline in both young and old SCG cells with a greater effect in old animals. These findings suggest that, when the calcium buffering system is forced to rely on SERCA Ca$^{2+}$-uptake to maintain [Ca$^{2+}$]i homeostasis, calcium transients are affected to a greater extent in old compared to young SCG cells.
2. INTRODUCTION

Calcium homeostasis in neurons involves multiple calcium buffering components working in concert to restore stimulation-evoked intracellular calcium concentration ([Ca$^{2+}$]i)-signals to basal levels (Kostyuk & Verkhratsky, 1994). After neuronal excitation, the cell must quickly restore [Ca$^{2+}$]i to basal levels in order to reset the cell for the next stimulus and avoid prolonged exposure to cytotoxic levels of high [Ca$^{2+}$]i (Miller, 1991). Major components involved in neuronal calcium regulation include calcium sequestration into the endoplasmic reticulum by sarco/endoplasmic reticulum calcium ATPase (SERCA) pumps and into mitochondria by an electrophoretic Ca$^{2+}$/H$^+$-uniporter. Additional buffering mechanisms include a mobile group of cytosolic calcium binding proteins, plasmalemmal calcium extrusion via plasma membrane Ca$^{2+}$-ATPase (PMCA) and plasma membrane Na$^+/Ca^{2+}$-exchange (Heizmann & Hunziker, 1991; Rizzuto, Brini, Murgia & Pozzan, 1993; Carafoli, 1997; MacLennan, Rice & Green, 1997; Blaustein & Lederer, 1999). The SERCA pumps have a high affinity for calcium ions and fast turnover rates (Lytton, Westlin, Burk, Shull & MacLenna, 1992), making them essential for regulating stimulation-evoked [Ca$^{2+}$]i-signals to maintain [Ca$^{2+}$]i homeostasis (Pozzan, Rizzuto, Volpe & Meldolesi, 1994; Tsai, Pottorf, Buchholz & Duckles, 1998; Hussain & Inesi, 1999).

In central and peripheral neurons it is hypothesized that the aging process causes multiple changes in the ability of neurons to regulate [Ca$^{2+}$]i homeostasis (Duckles, Tsai & Buchholz, 1995; Buchholz, Tsai, Foucart & Duckles, 1996; Michaelis et al., 1996; Murchison & Griffith, 1999). Since intracellular calcium is a universal second messenger integrating numerous cellular pathways, an age-related breakdown in mechanisms...
controlling [Ca\textsuperscript{2+}]i homeostasis could contribute to neuronal degeneration (Gibson & Peterson, 1987; Khachaturian, 1994; Thibault et al., 1998). One component of neuronal calcium regulation believed to decline with age is the function of SERCA pumps. Previous studies using microfluorometric measurements of [K\textsuperscript{+}]-evoked [Ca\textsuperscript{2+}]i-transients from rat superior cervical ganglion (SCG) cells have suggested an age-related decline in SERCA function (Tsai, Hewitt, Buchholz & Duckles, 1997; Tsai et al., 1998; Pottorf, Duckles & Buchholz, 2000). Other reports in dorsal root ganglion cells and skeletal muscle also have shown declines in SERCA function with advancing age (Gafni & Yuh, 1989; Kirischuk & Verkhratsky, 1996). In support of this decline in function, SERCA mRNA expression in cardiac tissue and vascular smooth muscle and pancreatic β-cells has also been shown to decline with age (Maciel, Polikar, Rohrer, Popovich & Dillmann, 1990; Lompre, 1999).

However, despite the age-related decline in SERCA expression and function, the neuronal cell contains robust alternative calcium buffering mechanisms that can compensate for a deficit in SERCA calcium uptake. The calcium buffering components of mitochondrial sequestration and plasma membrane extrusion have been shown to compensate for either an age-related decline in SERCA buffering capacity or an increase in neuronal calcium influx (Buchholz et al., 1996; Tsai et al., 1998; Murchison & Griffith, 1998). Nevertheless, even though neurons contain multiple backup systems to control stimulation-evoked [Ca\textsuperscript{2+}]i-transients and to maintain [Ca\textsuperscript{2+}]i homeostasis, aged neurons may still be highly vulnerable to calcium overload (Choi, 1992). Thus, alternative buffering mechanisms may be able to compensate for decline in function of a
single buffering component during normal function. However, under more stressful conditions, it may become impossible to maintain $[Ca^{2+}]_i$ homeostasis.

To date, evidence for a decline in function of SERCA pumps has relied on somewhat indirect approaches, since many different buffering mechanisms simultaneously contribute to calcium homeostasis. Therefore we sought to isolate the function of SERCA pumps from other calcium buffering components by blocking plasma membrane $Ca^{2+}$-ATPase (PMCA) pumps with vanadate, mitochondrial calcium uptake with dinitrophenol (DNP) and $Na^+/Ca^{2+}$-exchange by replacement of extracellular sodium ($[Na^+]_e$) with tetraethylammonium (TEA). In the presence of these blockers SCG cells are forced to rely primarily on SERCA pumps to regulate $[K^+]$-evoked $[Ca^{2+}]_i$-transients. Using this paradigm, we investigated the impact of age on the capacity of SERCA pumps to control $[K^+]$-evoked $[Ca^{2+}]_i$-transients in SCG cells from 6- and 20-month-old Fisher-344 rats. If an age-related decline in SERCA function exists in aged animals, then we would expect the ability of SERCAs to sequester calcium to be lower in SCG cells from aged compared to young animals. Forcing SCG cells to rely on SERCA uptake to control $[Ca^{2+}]_i$ homeostasis, we sought for evidence that an age-related decline in function of SERCA pumps occurs in sympathetic nerves.

3. METHODS & MATERIALS

3.1 Experimental Animals

Male Fischer-344 (F-344) rats aged 6- and 20-months were obtained from the NIH-NIA breeding colony (Harlan Sprague-Dawley, Inc., Indianapolis, IN). Animals
took food and water *ad libitum* and were maintained on a 12 hr light/dark cycle under controlled temperature (72 ± 2 °F).

3.2 *Superior Cervical Ganglion Preparation*

SCG cells were isolated by acute dissociation as previously described (Schofield, 1990). Briefly, rats were anesthetized with CO₂ followed by decapitation. SCG were dissected from the carotid artery bifurcation and placed in cold Tyrode’s solution containing (in mM): NaCl, 150; KCl, 4; CaCl₂, 2; MgCl₂, 2; N-(2-hydroxyethyl) piperazine-N’-2-ethanesulfonic acid (HEPES), 10; and glucose 10. The ganglia were then acutely dissociated in 5 mL of Earle’s balanced salt solution (EBSS) containing: trypsin (0.5 mg/mL; ~6000 units/mL), collagenase D (1 mg/mL), DNAse-1 type IV (0.1 mg/mL), HEPES (20 mM), glucose (10 mM), and NaHCO₃ (10 mM), pH adjusted to 7.4 with NaOH. After incubation in a shaking water bath for 45 min at 34°C, the digestion reaction was terminated by the addition of 5 mL of modified Hank’s balanced salt solution (HBSS) with 10% fetal calf serum, CaCl₂ (1.3 mM), and HEPES (5 mM), pH adjusted to 7.4 with NaOH. The dissociated cells were then lightly pelleted at 600 rpm (60 x g) for 5 min, the supernatant removed, pellet gently re-suspended in 5 mL of fresh HBSS, and cells pelleted again at 600 rpm (60 x g) for 5 min. Cells were dispersed in 0.5 mL of HBSS onto Cell-Tak protein coated 22 mm glass coverslips (8 µg/slide) modified by attaching a 2 cm Teflon ring to the surface with Sylgard. Coverslips were incubated for 2 hr at room temperature to allow cell attachment to the Cell-Tak protein coat before further treatment.
Measurement of Intracellular Calcium

A dual wavelength ratiometric fura-2 microfluorimetry system was used to measure \([\text{Ca}^{2+}]_i\) in SCG cells. SCG cells were loaded with 10 μM fura-2 acetoxyethyl ester (fura-2/AM) for 20 min at room temperature, then washed with 5 mL of low \([\text{K}^+]\) (5 mM) buffer composed of (in mM): NaCl, 138; CaCl₂, 2; MgCl₂, 1; KCl, 5; HEPES, 10; glucose, 10; pH adjusted to 7.4 with NaOH. Incubation was continued for an additional 30 min to allow intracellular esterases to convert the fura-2/AM dye into the free acid form (Thayer, Sturek & Miller, 1988). Coverslips were mounted into a superfusion chamber, which was attached to the stage of a Nikon inverted microscope (Nikon Instruments, Tokyo, Japan). The microscope was attached to a Universal Imaging System running MetaFluor version 3.0 (Universal Imaging Corporation, West Chester, PA). The perfusion system allowed the chamber volume (~250 μL) to be exchanged at the rate of 500 μL/sec (i.e. 2 times/sec). A suitable cell was distinguished as a rounded cell body isolated from other cells with localized phase-contrast illumination of the fura-2 probe. A mercury vapor lamp illuminated the fura-2 probe, and fluorescence was excited alternately at wavelengths 340 and 380 nm by a Sutter-II rotating filter wheel (Sutter Instrument Company, Novato, CA). The fura-2 emission fluorescence at 510 nm was selected by a barrier filter and recorded by a CCD Hamamatsu imaging camera (Hamamatsu Corporation, Bridgewater, NJ). Adjusting the microscope stage to a point with no cells in the field of view and capturing a background image before the start of the experiment corrected for background light levels. Prior to fura-2/AM loading, cellular autofluorescence was examined in SCG cells. Autofluorescence was found to be undetectable by our imaging system and did not
significantly alter our [Ca\(^{2+}\)]\(_i\) measurements. During the experiment, 340 and 380 images were collected at a rate of 4 boxcar-averaged images/sec. These were corrected for background fluorescence, calcium concentration calculated, and the data logged to a text file. During dye loading and data collection, ambient light levels were minimized, and neurons were only illuminated during data acquisition to minimize bleaching and potential photo damage of the dye.

Intracellular calcium was estimated by both \textit{in vitro} and \textit{in vivo} calibration methods. The \textit{in vitro} method was performed using a calcium calibration kit (Molecular Probes, Eugene, OR) with known [Ca\(^{2+}\)] ranging from 0 – 40 \(\mu\)M. Each prepared calcium solution was loaded with 4 \(\mu\)M fura-2 pentapotassium salt. A droplet of each [Ca\(^{2+}\)] was placed onto a glass slide, the fluorescent intensities from 340 and 380 excitation were measured, and a curve of 340/380 ratio (R) versus [Ca\(^{2+}\)] was plotted. Excitation of calcium solution at zero [Ca\(^{2+}\)] and recording of 340/380 ratio and 380 nm fluorescence intensities for 1 min allowed for the determination of \(R_{\text{min}}\) and \(F_{\text{min}}\), respectively. Replacing the calcium solution with 40 \(\mu\)M [Ca\(^{2+}\)] and recording fluorescence intensities revealed \(R_{\text{max}}\) and \(F_{\text{max}}\) values.

The \textit{in vivo} method was performed on SCG cells by decreasing extracellular calcium concentration to 0 mM to determine \(R_{\text{min}}\) and \(F_{\text{min}}\). Replacing the extracellular medium with 10 mM [Ca\(^{2+}\)] with ionomycin (1 \(\mu\)M) and depolarizing with high potassium (68 mM) gave \(R_{\text{max}}\) and \(F_{\text{max}}\). The values for \(R_{\text{min}}, F_{\text{min}}, R_{\text{max}},\) and \(F_{\text{max}}\) were remarkably similar between the \textit{in vitro} and \textit{in vivo} methods. Our \textit{in vitro} calibration for \(K_d\) is comparable to \textit{in vivo} values previously reported in neuronal cells (Neher, 1995; Murchison & Griffith, 1998). We applied the same \(K_d\) for both young and old SCG
neurons, since previous reports have shown no significant difference in fura-2 \( K_d \) values for young and old neurons (Murchison & Griffith, 1998).

Since our *in vitro* method correlated well with *in vivo* measurements, the values obtained from the *in vitro* calibration were used to convert the experimental fluorescence intensity ratios (R) to \([Ca^{2+}]\) over the physiological range of \([Ca^{2+}]\) by iterative fit to the equation:

\[
[Ca^{2+}] = K_d \left( \frac{R - R_{min}}{R_{max} - R} \right) S_f
\]

\( R_{min} \) is the 340/380 ratio at zero \([Ca^{2+}]\) and \( R_{max} \) is the 340/380 ratio at 40 \( \mu \)M \([Ca^{2+}]\). The value of \( K_d \) is the dissociation constant of fura-2, while \( S_f \) is a correction factor relating the ratio \( F_{min}/F_{max} \), which represents emission intensities at 380 nm when fura-2 is in the free (\( F_{min} \); 0 \( \mu \)M \([Ca^{2+}]\)) or bound (\( F_{max} \); 40 \( \mu \)M \([Ca^{2+}]\)) form (Grynkiewicz, Peonie & Tsien, 1985). For this report, \([Ca^{2+}]\) was estimated using averaged *in vitro* values for \( S_f \) (7.6), \( R_{min} \) (0.33), \( R_{max} \) (2.75), and \( K_d \) (251 nM).

### 3.4 Experimental Protocol

To assess the function of SERCAs in young and old sympathetic neurons, SCG cells were first depolarized (S1, control) for 15 sec by superfusion with Tyrodes buffer containing 68 mM KCl followed by a wash out with low \([K^+]\) (5 mM) buffer and a 2 min equilibration period. Cells were then exposed to a low concentration of vanadate (0.1 \( \mu \)M) to preferentially block PMCA (Salvador & Mata, 1998; Pottorf *et al.*, 2000) and allowed to equilibrate for 1 min. Next, dinitrophenol (DNP, 100 \( \mu \)M) was added to remove the contribution of mitochondrial calcium uptake, and cells were allowed to
equilibrate for 3 min in the presence of both vanadate and DNP. Finally, zero sodium ([Na\(^+\)]e replaced with TEA) was added to block the function of Na\(^+\)/Ca\(^{2+}\)-exchangers, and SCG cells were depolarized (S2) for 15 sec in the presence of 68 mM KCl, 0.1 \(\mu\)M vanadate, 100 \(\mu\)M DNP and zero sodium.

3.5 Data Analysis

The resting [Ca\(^{2+}\)]i for SCG cells was recorded as a long segment (50 - 60 sec) of fluorescence ratio signal before initial depolarization of the cell. The peak change in [Ca\(^{2+}\)]i was measured by subtracting the baseline (or resting) [Ca\(^{2+}\)]i from the peak [Ca\(^{2+}\)]i. The baseline [Ca\(^{2+}\)]i was measured as the mean [Ca\(^{2+}\)]i 2-5 sec immediately before each [Ca\(^{2+}\)]i transient. Rate of rise of [Ca\(^{2+}\)]i (nM/s) was determined by measuring from baseline [Ca\(^{2+}\)]i to peak [Ca\(^{2+}\)]i. The rate of recovery of [Ca\(^{2+}\)]i (nM/s) was measured from the peak change in [Ca\(^{2+}\)]i to within 90% recovery to baseline. The rates of rise and recovery of [Ca\(^{2+}\)]i were determined by linear fit regression (mean \(r\) values of 0.973 and 0.972, respectively).

3.6 Statistical Analysis

The effect of vanadate/DNP/TEA treatment on calcium transients within age groups was analyzed by paired Student’s \(t\)-test. The effect of age on the response of calcium transients to treatment with vanadate/DNP/TEA was analyzed by unpaired Student’s \(t\)-test or two-way ANOVA with Fischer-PLSD test. Data are presented as mean \(\pm\) SEM.
blockers significantly increased the time to recovery of $[\text{Ca}^{2+}]_i$ by 100% in young SCG cells and by 170% in aged SCG cells as compared to control values (Fig. 2B). As shown in figure 2, both of these effects of treatment with vanadate, DNP, and zero Na$^+$ were significantly greater in cells from old compared to young animals.

4.3 Basal $[\text{Ca}^{2+}]_i$ measurements

Acutely dissociated SCG cells prepared from 6- and 20-month-old rats showed a stable basal $[\text{Ca}^{2+}]_i$ that was indistinguishable between the age groups (6 month: $99 \pm 4$ nM, $N = 30$ and 20 month: $102 \pm 3$ nM, $N = 21$). Basal $[\text{Ca}^{2+}]_i$ levels 1 min after washout of high $[\text{K}^+]$ remained elevated in both 6- and 20-month-old SCG cells compared to before exposure to high $[\text{K}^+]$; however, there was no significant difference between the age groups (Fig. 3). Application of low concentrations of vanadate (0.1 μM) to block the function of PMCAs produced no significant change in basal $[\text{Ca}^{2+}]_i$ in either young or old SCG cells. However, exposure of SCG cells to DNP (100 μM), a mitochondrial calcium uptake blocker, produced a significant and sustained increase in basal $[\text{Ca}^{2+}]_i$ in cells from aged animals (Fig. 3). In cells from young animals, addition of DNP produced a transient rise in basal $[\text{Ca}^{2+}]_i$ which was not sustained. After recovery from high $[\text{K}^+]$-depolarization in the presence of vanadate, DNP, and zero Na$^+$, basal calcium levels remained elevated. The increase in basal $[\text{Ca}^{2+}]_i$ was significant in both 6- and 20-month-old SCG cells compared to control basal levels; however, there was no significant difference with age (Fig. 3).
Figure 1. Representative tracings of the effects of vanadate, dinitrophenol, and zero Na⁺ on [K⁺]-evoked [Ca²⁺]i-transients in SCG cells from (A) 6 and (B) 20-month-old rats. Effects of addition of vanadate (0.1 μM Va), dinitrophenol (100 μM DNP) and zero Na⁺ (replaced with TEA) are shown. Figure insets (i, ii) illustrate rate of recovery of [Ca²⁺]i after [K⁺]-evoked depolarization of control (S1) and treated (S2) cells with an expanded time scale to resolve changes in recovery. Rate of recovery was analyzed by best fit linear regression with mean r values of 0.970 for control and 0.969 for treated [Ca²⁺]i-transients. Fura-2 loaded cells were depolarized (Control, S1) for 15 sec with Tyrodes buffer containing 68 mM [K⁺], followed by a 2 min equilibration. Cells were then exposed to 0.1 μM Va for 1 min. Next, DNP (100 μM) was added and cells allowed to equilibrate for 3 min in the presence of Va and DNP. Finally, [Na⁺]e was removed (Na⁺ replaced with TEA) and SCG cells depolarized (Va/DNP/Zero Na⁺) for 15 sec in the presence of Tyrodes buffer containing 68 mM KCl, Va, DNP and zero Na⁺ followed by a 2 min equilibration.
Figure 2. Effect of treatment with vanadate (Va), dinitrophenol (DNP), and zero Na⁺ (Na⁺ replaced with TEA) on rate of recovery and time to recovery of [Ca²⁺]i in 6- and 20-month-old SCG cells. (A) Rate of recovery of [Ca²⁺]i was determined by linear regression analysis from peak [Ca²⁺]i level to 90% of basal [Ca²⁺]i. (B) Time to recovery was determined by the difference in time from peak [Ca²⁺]i to 90% of basal [Ca²⁺]i. Freshly isolated SCG cells loaded with fura-2 were depolarized (Control) for 15 sec with Tyrodes buffer containing 68 mM [K⁺], followed by a 2 min equilibration. Cells were then exposed to low concentrations of Va (0.1 μM) for 1 min. Next, DNP (100 μM) was added and cells allowed to equilibrate for 3 min in the presence of Va and DNP. Finally, [Na⁺]e was removed and SCG cells depolarized (Va/DNP/Zero Na⁺) for 15 sec in the presence of Tyrodes buffer containing 68 mM KCl, Va, DNP and zero Na⁺ followed by a 2 min equilibration. Differences between control and Va/DNP/Zero Na⁺ treatment were determined by two-way ANOVA with repeat measures and Fisher-PLSD test or unpaired Student’s t-test. * = significantly different from control, p < 0.05. + = significantly different from 6-month-old SCG cells, p < 0.05. N = 30 cells from twelve, 6-month-old animals and 21 cells from twelve, 20-month-old animals.
Figure 3. Effect of age on basal [Ca$^{2+}$]i in the presence and absence of calcium uptake blockers in SCG cells. Basal [Ca$^{2+}$]i was recorded in fura-2 loaded cells for 1 min prior (Control) and 1 min following each treatment. Effects of treatments were determined by two-way ANOVA with repeat measures and Fisher-PLSD test or unpaired Student’s t-test. * = significantly different from control, $p < 0.05$. + = significantly different from 6-month-old SCG cells, $p < 0.05$. $N = 30$ cells from twelve, 6-month-old animals and 21 cells from twelve, 20-month-old animals.
5. DISCUSSION

5.1 Technical Considerations

The use of fluorescent calcium sensitive probes can introduce potential sources of error, which merit discussion (Moore, Becker, Fogarty, Williams & Fay, 1990; Kao, 1994; Neher, 1998). The [Ca^{2+}]_{i} values we report are estimates and not absolute values. Therefore, comparison of age-related changes in calcium buffering relies on relative differences in [Ca^{2+}]_{i} between the two age groups. Within each age group, depolarizations of each cell under treated conditions are paired with control stimulations for the same cell. Resting [Ca^{2+}]_{i}, peak [Ca^{2+}]_{i}, and the rates of rise and recovery (nM/sec) reported are well within the accepted physiological ranges reported in neurons and other cells (Neher, 1995; Murchison & Griffith, 1998). In the present experiments, peak [Ca^{2+}]_{i} transients are typically below 1.0 μM [Ca^{2+}]_{i}. Therefore, peak [Ca^{2+}]_{i} transients fall within the linear range (0.02 – 2.0 μM [Ca^{2+}]) of the in vitro calibration curve for fura-2. Additionally, there appears to be little evidence of incomplete hydrolysis or compartmentalization of the fura-2/AM dye (Zhou & Neher, 1993). The short loading time and low concentration of fura-2/AM combined with low temperature and long de-esterification time minimizes these possible sources of error.

It has been suggested that the cell dissociation process may differentially affect fura-2 loading for young and aged SCG cells and may be responsible for our observed age-related effect. When cells are at rest, the 380 nm signal is a comparative indicator of fura-2 loading (Negulescu & Machen, 1990). The 380 nm signals are remarkably similar between young and old SCG cells (6 month: 89.27 ± 2.57; 20 month: 90.72 ± 3.53 F_{380}, emission intensity of fura-2 excited at 380 nm). This strongly suggests that the cell
dissociation process does not introduce a sampling artifact, compromise data, or alter fura-2 loading in SCG cells. Our basal $[\text{Ca}^{2+}]_i$ levels are comparable with values reported in central and peripheral nerve models, and these do not vary with age (Thayer & Miller, 1990; Garyantes & Regehr, 1992; Benham, Evans & McBain, 1992; Griffith & Murchison, 1995; Jasek & Griffith, 1998).

Orthovanadate ($\text{VO}_4^{-3}$) inhibits P-type ATPase’s and is similar to the transition state of phosphate ($\text{PO}_4^{-3}$) involved in enzyme reactions (Smith, Zinn & Cantley, 1980; Stankiewicz, Tracey & Crans, 1995). The similarity of vanadate to phosphate may affect other ATPases, phosphatases, and kinases. In cell cultures, red blood cells, and rat spleen vanadate produces weak inhibition of phosphatase activity (Brunati & Pinna, 1985; Boivin, Galand & Bertrand, 1986; Imes, Kaplan & Knowles, 1987). In contrast, after exposure to vanadate there was no significant effect on the activity of purified forms of pyruvate kinase, creatine kinase, or inorganic pyrophosphatase (Boyd, Kustin & Niwa, 1985). Vanadate can also partially block the $\text{Na}^+$/K$^+$-ATPase pump, thus possibly affecting $[\text{Na}^+]$ and $[\text{K}^+]$ balance and in turn basal $[\text{Ca}^{2+}]_i$ (Cantley et al., 1977; Bond & Hudgins, 1979). However, exposure of young and aged SCG cells to vanadate had no significant effect on basal $[\text{Ca}^{2+}]_i$, suggesting that blockade of Na$^+$/K$^+$-ATPase pumps does not appreciably affect basal $[\text{Ca}^{2+}]_i$ in our cell model (Fig. 2; Pottorf et al., 2000). Other studies in dorsal root ganglion and SCG neurons have also shown no significant effect of vanadate on peak $[\text{Ca}^{2+}]_i$ (Benham et al., 1992; Pottorf et al., 2000). Therefore, these data suggest that the effects of vanadate are localized to Ca$^{2+}$-ATPase systems, and that “nonspecific” effects of this treatment were minimal in SCG cells.
Mitochondrial calcium uptake blockers like DNP, carbonyl cyanide chlorophenyl hydrazone (CCCP), ruthenium red, and antimycin used to disrupt Ca\(^{2+}\)-uptake can potentially affect other cellular processes. However, the application of DNP to resting 6-month-old SCG cells produced only a transient rise in [Ca\(^{2+}\)]\(_i\) that returned to basal levels even with continued presence of the mitochondrial blocker (Fig. 1A; Fig. 2). Other studies also showed only a transient rise in [Ca\(^{2+}\)]\(_i\) when CCCP was applied to dorsal root ganglion cells (Werth & Thayer, 1994). These data suggest that the mitochondria contain very little sequestered calcium when neuronal cells are at rest. Only when the neuronal cell is depolarized do we observe the full effect of mitochondrial calcium blockade, which produces large increases in peak [Ca\(^{2+}\)]\(_i\) in dorsal root ganglion and SCG cells (Buchholz et al., 1996; Werth, Usachev & Thayer, 1996).

Complete [Na\(^+\)]\(_e\) replacement will remove the inward sodium gradient that drives calcium efflux resulting in reversed exchange with calcium moving into the cell. The K\(_m\) of the Na\(^+\)/Ca\(^{2+}\)-exchanger is approximately 2 \(\mu\)M (Reeves & Sutko, 1979). Therefore, it is thought that the exchanger is primarily responsible for regulating large calcium loads and less important in adjusting small loads and resting [Ca\(^{2+}\)]\(_i\) levels in neurons of dorsal root ganglion and SCG cells (Benham et al., 1992; Werth et al., 1996; Pottorf et al., 2000). Additionally, in our SCG cell model the use of TEA to replace [Na\(^+\)]\(_e\) had no significant effect on resting [Ca\(^{2+}\)]\(_i\) (Fig. 2), suggesting that the Na\(^+\)/Ca\(^{2+}\)-exchanger is not critical in determining resting [Ca\(^{2+}\)]\(_i\) in these cells.
5.2 Function of SERCA Calcium Uptake with Age

The focus of this study was to investigate the impact of age on \([\text{Ca}^{2+}]_i\) homeostasis when SCG cells were forced to rely on SERCA pumps to restore \([\text{K}^+]\)-evoked \([\text{Ca}^{2+}]_i\)-transients back to basal levels. SCG cells from young and old animals exposed to vanadate (0.1 μM), DNP (100 μM) and \([\text{Na}^+]_e\) replaced with TEA could restore \([\text{K}^+]\)-evoked \([\text{Ca}^{2+}]_i\)-transients to levels close to basal. Exposure to these blockers resulted in a significant decline in the recovery phase of \([\text{Ca}^{2+}]_i\) in both age groups, but there was clearly a greater effect in cells from old compared to young animals. The age-related decline in calcium recovery could not be attributed to age-related differences in sensitivity to the calcium blocking agents, since the increase in basal \([\text{Ca}^{2+}]_i\) in the presence of these blockers was the same for both age groups, except in the presence of vanadate and DNP (Fig. 3). Therefore, these findings suggest that the function of SERCAs declines with advancing age.

Previous studies investigating age-related changes in SERCA function used the approach of applying selective SERCA blockers, such as thapsigargin or cyclopiazonic acid. For example, in isolated rat SCG cells, microfluorometric measurements of \([\text{K}^+]\)-evoked \([\text{Ca}^{2+}]_i\)-transients showed a significant decline in the rate of calcium recovery in the presence of SERCA blockade in young SCG cells, but no significant change in old cells (Tsai et al., 1998). Further evidence of an age-related change in SERCA function was observed when noradrenaline release was measured in rat tail arteries in the presence of these same SERCA blockers, showing an increase in stimulation-evoked noradrenaline release in young arteries with no change in arteries from old animals (Tsai et al., 1998). Therefore, current data underscore conclusions from our previous work with selective
SERCA blockers, indicating a decline in SERCA function with advancing age in autonomic nerves (Tsai et al., 1998; Pottorf et al., 2000).

5.3 Compensation for Declines in SERCA Function with Age

Selective SERCA blockers have been used to provide evidence for a decline in SERCA function with advancing age. However, blockers of other buffering components have shown a greater contribution to intracellular calcium regulation in older cells, presumably due to a deficit in calcium regulation by SERCAs. Since each component of the \([\text{Ca}^{2+}]_i\) buffering system contributes to the regulation of stimulation-evoked \([\text{Ca}^{2+}]_i\)-transients and maintenance of \([\text{Ca}^{2+}]_i\) homeostasis in neurons, dysregulation of \([\text{Ca}^{2+}]_i\) homeostasis by the impairment of one of these components has the potential to increase the functional contribution of other calcium buffering mechanisms. Thus one or more calcium buffering mechanisms may prevent adverse effects by compensating for a decline in function by another component.

It has been demonstrated in several neuronal cell types that mitochondrial calcium uptake takes on a crucial role in compensating for alterations in calcium regulation with advancing age. In acutely dissociated neurons of rat SCG, addition of mitochondrial calcium uptake blockers resulted in a greater effect on high \([\text{K}^+]\)-evoked \([\text{Ca}^{2+}]_i\)-transients in old (20 month) compared to young (6 month) animals (Buchholz et al., 1996). In basal forebrain neurons, activation of voltage-gated calcium channels by voltage clamp techniques resulted in an age-related increase in calcium influx; however, simultaneous microfluorometric analysis of \([\text{Ca}^{2+}]_i\) levels also revealed a decline in peak \([\text{Ca}^{2+}]_i\) (Murchison & Griffith, 1998). When rat basal forebrain neurons were exposed to
CCCP and [Ca\textsuperscript{2+}]i-transient elicited by caffeine, there was a significantly greater increase in peak [Ca\textsuperscript{2+}]i in neurons from old compared to young animals (Murchison & Griffith, 1999). These data suggest a greater dependence upon mitochondrial calcium uptake when a component of the calcium regulatory system fails to restore stimulation-evoked [Ca\textsuperscript{2+}]i-transients back to basal levels. The greater dependence upon mitochondria to compensate for changes in calcium regulation correlates with the increased sensitivity of basal [Ca\textsuperscript{2+}]i to the presence of DNP in SCG cells from 20-month-old animals (Fig. 3).

The role of plasma membrane extrusion systems (PMCA and Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchange) have been highlighted as important participants in the dynamic regulation of [Ca\textsuperscript{2+}]i-transients and as crucial players in calcium extrusion (Carafoli & Stauffer, 1994; Werth et al., 1996; Blaustein & Lederer, 1999). In rat aortic endothelial cells, there is believed to be interdependence between PMCA and SERCA gene expression. This was tested by blocking of SERCAs with thapsigargin, which resulted in an increased level of mRNA expression of PMCAs with concurrent increases in PMCA protein levels (Kuo et al., 1997). In another study, levels of SERCA and Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchange proteins were correlated with force-frequency relations in ventricular muscle strips from failing human myocardium (Hasenfuss et al., 1999). A decreased diastolic function was correlated with decreased SERCA protein levels; however, ventricular strips showing compensated diastolic function demonstrated increased expression of Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchange protein levels apparently to compensate for declines in SERCA function. These studies reveal that when [Ca\textsuperscript{2+}]i homeostasis is compromised, redundant calcium buffering mechanisms exist that can compensate for a decline in function of another component.
5.4 Possible Mechanisms of Age-Related Decline in SERCA function

To date the mechanisms responsible for age-related declines in SERCA function have not been identified; however, there are several possibilities to account for a decline in SERCA function with advancing age. One obvious possibility would be that there is a decline in levels of mRNA expression for one or more SERCA isoforms, translating into decreased levels of SERCA protein. Indeed, SERCA mRNA expression in several cell types has been shown to decline with age (Maciel et al., 1990; Varadi et al., 1996; Lompre, 1999). However, age-related declines in SERCA mRNA expression could not be correlated with levels of SERCA protein in rat skeletal muscle or myocardium (Ferrington et al., 1997; Xu & Narayanan, 1998). These studies have been repeated in rat cerebral cortex, which also show an age-related decline in SERCA mRNA expression, but no corresponding change in protein levels (Pottorf, De Leon, Hessinger, Buchholz; unpublished data).

Despite unchanged SERCA protein levels, an age-related decrease in $^{45}$Ca$^{2+}$-uptake into microsomes has been observed in rat myocardium. This reduction in calcium uptake was suggested to be the result of age-related declines in calcium/calmodulin kinase (CAMK) protein expression and the ability of CAMK to phosphorylate SERCAs (Xu & Narayanan, 1998). It has also been suggested that the effectiveness of the calmodulin dependent activation of CAMK in rat brain synaptic membranes declines with advancing age (Zaidi, Gao, Squier & Michaelis, 1998). Such effects would manifest as an age-related decline in phosphorylation of SERCA and could result in depressed SERCA function. Still another group has proposed that in rat skeletal muscle SERCA molecules are more prone to undergo self-association to form inactive oligomers with
advancing age (Ferrington et al., 1997). These studies suggest possible mechanisms of
calcium regulation, independent of changes in SERCA mRNA and protein levels, which
may be sensitive to the aging process and could account for observed age-related declines
in SERCA function. Further studies are necessary to elucidate which mechanisms are
responsible for age-related declines in SERCA function.

In conclusion, these studies document a significant reduction in the function of
SERCA pumps in SCG cells with advancing age. Neuronal dysregulation of $[Ca^{2+}]_{i}$
homeostasis is suggested to be a critical factor in age-related neuronal degeneration
(Gibson & Peterson, 1987; Khachaturian, 1994; Thibault et al., 1998). Therefore, a
decline in SERCA function with age could lead to altered $[Ca^{2+}]_{i}$ regulation and may
ultimately contribute to calcium overload in neurons. Although an age-related decline in
SERCA function appears to occur in SCG cells, other calcium buffering mechanisms are
able to compensate for a deficit in $[Ca^{2+}]_{i}$ homeostasis in order to restore basal $[Ca^{2+}]_{i}$
and prevent calcium overload (Tsai et al., 1997; Tsai et al., 1998; Pottorf et al., 2000).
The exact mechanism responsible for an age-related decline in SERCA function has not,
as yet, been identified; however, there are several possibilities. Despite current
knowledge of the mechanisms involved in neuronal $[Ca^{2+}]_{i}$ regulation, understanding of
the importance and participation of each calcium buffering component is still lacking,
and additional studies are needed to confirm the role of each separate component in
$[Ca^{2+}]_{i}$ dysregulation with advancing age.
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CHAPTER V

EFFECTS OF AGING ON FUNCTION AND EXPRESSION OF SERCA IN RAT CEREBRAL CORTEX

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1. ABSTRACT

*Effect of aging on function and expression of SERCA in rat cerebral cortex.*

Calcium acts as a second messenger in biological systems to integrate numerous cellular pathways. Previous work in sympathetic neurons suggests that a decline in sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) function occurs with advancing age. Therefore, an age-related breakdown in the mechanisms controlling intracellular calcium homeostasis could contribute to altered neuronal function and/or neuronal degeneration. In this study we sought to determine age-related changes in SERCA function and expression in rat cerebral cortex from young (6-month) and old (20-month) animals. Functional studies compared ATP-dependent, oxalate-facilitated \(^{45}\)Ca\(^{2+}\)-uptake into microsomes and plasma membrane vesicles (PMV) with advancing age. For both microsomal (thapsigargin-sensitive) and PMV (thapsigargin-insensitive) fractions, there was no significant difference in \(^{45}\)Ca\(^{2+}\)-uptake with age. Expression studies measured SERCA3 mRNA and protein content in rat cerebral cortex in young and old animals. Old animals showed a significant decline in SERCA3 mRNA levels compared to young. However, comparison of SERCA3 protein content did not reveal a corresponding decline with advancing age; therefore, implying that SERCA turnover rates are greater in young animals. Although the present work with rat cerebral cortex does not indicate an age-related decline in SERCA function or content, previous work from our laboratory with sympathetic nerves suggests that such a decline does exist. In comparison to studies in peripheral neurons our present study on central neurons suggest fundamental differences in the ways aging affects critical calcium homeostatic mechanisms in central and peripheral autonomic neurons.
2. INTRODUCTION

The free intracellular calcium content of neuronal cells at rest consists of only a small fraction of the total cellular content of calcium (1/10,000\textsuperscript{th}), including that which is buffered and sequestered (Blaustein, 1988). In neuronal cells the level of free intracellular concentration ([Ca\textsuperscript{2+}]i) is fastidiously regulated by multiple calcium buffering mechanisms to maintain [Ca\textsuperscript{2+}]i between 100 – 200 nM (Nachshen, 1985; Ashley, 1986; Hartmann, Eckert & Muller, 1994). Upon neuronal excitation the [Ca\textsuperscript{2+}]i rapidly increases and the calcium buffering system quickly reacts to restore [Ca\textsuperscript{2+}]i to basal levels in preparation for the next stimulus (Nachshen, 1985; Fontana & Blaustein, 1993; Ghosh & Greenberg, 1995). However, prolonged exposure of neurons to elevated [Ca\textsuperscript{2+}]i causes calcium cytotoxicity and possible neurodegeneration (Choi, 1992, 1994).

Because high [Ca\textsuperscript{2+}]i can be damaging or fatal to mature neurons, powerful regulatory mechanisms exist to attenuate [Ca\textsuperscript{2+}]i-transients and restore [Ca\textsuperscript{2+}]i to resting levels (Kostyuk & Verkhratsky, 1994). The primary components involved in intracellular calcium regulation in neurons include calcium sequestration into the endoplasmic reticulum by sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPases (SERCA) pumps and into the mitochondria by electrophoretic Ca\textsuperscript{2+}/H\textsuperscript{+}-uniporters. Secondarily, calcium buffering mechanisms include cytoplasmic calcium binding proteins, plasmalemmal calcium extrusion via plasma membrane Ca\textsuperscript{2+}-ATPases (PMCA), and Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchange (Carafoli, 1997; Heizmann & Hunziker, 1991; Rizzuto, Brini, Murgia & Pozzan, 1993; MacLennan, Rice & Green, 1997; Blaustein & Lederer, 1999). In a concerted fashion these diverse calcium buffering systems attenuate [Ca\textsuperscript{2+}]i-transients and maintain resting [Ca\textsuperscript{2+}]i.
The SERCAs actively accumulate two calcium ions into the endoplasmic reticulum for each hydrolyzed ATP molecule (MacLennan, 1970). Three different genes encode the SERCAs and alternative splicing produces five different isoforms (Wuytack et al., 1992). The SERCA1a (adult) and 1b (fetal) isoforms are exclusively found in skeletal muscle. The SERCA2a is found in both skeletal and heart muscle and the SERCA2b and 3 are found in the endoplasmic reticulum of brain and several other cell types (Wu, Lee, Wey, Bungard & Lytton, 1995). Although the SERCA isoforms belong to the same gene family, they are diverse in structure, function, and sensitivity to pharmacological agents (Lytton, Westlin, Burk, Shull & MacLennan, 1992).

Increasing evidence suggests that the control of $[Ca^{2+}]_i$ in some central and peripheral neurons is altered with advancing age (Duckles, Tsai & Buchholz, 1995; Buchholz, Tsai, Foucart & Duckles, 1996; Michaelis et al., 1996; Tsai, Hewitt, Buchholz & Duckles, 1997; Tsai, Pottorf, Buchholz & Duckles, 1998; Murchison & Griffith, 1999). An age-related change in calcium homeostasis could contribute to changes or loss of function, and possibly neuronal degeneration (Gibson & Peterson, 1987; Khachaturian, 1994; Thibault et al., 1998).

In peripheral sympathetic neurons, microfluorometric measurements of $[K^+]_i$-evoked $[Ca^{2+}]_i$-transients from rat superior cervical ganglion (SCG) cells strongly implicate an age-related decline in SERCA function (Tsai et al., 1998; Pottorf, Duckles & Buchholz, 2000). Uptake measurements of $^{45}Ca^{2+}$ by microsomes isolated from dorsal root ganglion cells and skeletal muscle also reveal age-related declines in SERCA function (Gafni & Yuh, 1989; Kirischuk & Verkhratsky, 1996). However, the mechanism of the age-related decline in SERCA function is not yet known.
3.2 Isolation of Microsomes and Plasma Membrane Vesicles

Microsomes and plasma membrane vesicles (PMV) were prepared by the method of Salvador and Mata (1998) with minor modifications. Briefly, rats were anesthetized with CO₂ followed by decapitation. Whole brains were removed from the cranial cavity and the cerebral cortex collected, minced, and placed in 5 ml of fresh homogenizing buffer (Buffer A) contained (in mM): HEPES (10), sucrose (320), MgSO₄ (0.5), phenylmethylsulfonyl fluoride (0.25), and adjusted to pH 7.4 with KOH. Tissues were homogenized using 10-15 passes with a motorized glass-Teflon homogenizer at 4°C. Homogenates were centrifuged for 15 min at 2000 x g to pellet cellular debris. The supernatant fraction (SN-I) was transferred to a new sterilized tube and centrifuged for 30 min at 20,000 x g to obtain a new pellet (P-II) and supernatant fraction (SN-II). PMV (enriched with PMCAs) were prepared from the P-II fraction by re-suspending in 1 ml of Buffer A, aliquoting, and storing at -80°C. The SN-II fraction was transferred to a new tube and further centrifuged for 75 min at 100,000 x g to obtain a final pellet (P-III). Microsomes (enriched with SERCAs) were prepared by re-suspending the P-III fraction in 0.5 ml Buffer A, aliquoting, and storing at -80°C. Protein concentration was determined by the colorimetric method (Smith et al., 1985) using a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical) with bovine serum albumin (BSA) as a standard. The assay was measured spectrophotometrically at 570 nm using a Bio-Rad microplate reader, model 3550.
3.3 **Calcium Uptake Assay**

Calcium uptake was determined by ATP-dependent, oxalate-facilitated uptake of $^{45}\text{Ca}^{2+}$ by microsomes and PMV isolated from 6- and 20-month rat cerebral cortex using the Millipore filtration technique as described by Narayanan (1981). The assay medium (pH 7.2) contained (in mM): HEPES (50), KCl (100), MgCl$_2$ (3.5), NaCl (20), Na$_2$ATP (5), potassium oxalate (1), and ruthenium red (0.025). Ruthenium red, a mitochondrial calcium uniporter blocker, was added to block the effect of possible mitochondrial contamination in microsomal and PMV fractions. The medium was thermo-equilibrated with 5 µM $^{45}\text{CaCl}_2$ (2000 mCi/mmol) for 5 min at 37°C. Adding 25 µg of microsomes or PMV initiated the reaction, which was then incubated at 37°C for incubation times of 0.5 – 10 min. For assays selectively blocking SERCAs, the pre-incubation medium contained thapsigargin (0, 0.1 or 5.0 µM) and microsomes or PMV incubated for 10 min at 37°C. Calcium uptake was terminated by filtering 50 µl aliquots of each sample through HAWP Millipore filters (0.45 µm pore size, 25 mm diameter) followed by an immediate 10-ml wash with ice-cold buffer (50 mM HEPES, 100 mM KCl, 3.5 mM MgCl$_2$, 20 mM NaCl, pH 7.2). The filters were washed an additional 4x with 10-ml of ice-cold buffer. Filters were then transferred to scintillation vials, dried for 30 min at 40°C, and 10 ml of scintillation cocktail added to each vial. Radioactivity was determined by liquid-scintillation spectrometry. Appropriate controls for assays without membranes (Blank) and without ATP (Control) were included for each experiment.
3.4 *Northern Blot Analysis*

Isolation of RNA and Northern blot analysis were performed on young and old rat cerebral cortex using the modified protocol of De León *et al.* (1991). Briefly, tissues from rat cerebral cortex were minced, homogenized in a glass-Teflon homogenizer, and total RNA collected by a guanidium thiocyanate/phenol/chloroform precipitation method (Chomczynski & Sacchi, 1987). The collected RNA was dried using a Speed Vac (Labconco, Model 78120-00) and then dissolved in 0.5% sodium dodecyl sulfide (SDS). Total RNA samples (20 μg/well) were separated by electrophoreses in a 1% agarose-formaldehyde gel at constant voltage (100 mV) for 150 min. The gel was then soaked in 10X sodium/saline phosphate/EDTA (SSPE) for 45 min and then RNA transferred to nylon membrane (Hybond-N, Amersham Pharmacia Biotech) with 10X SSPE for 3 hr (10X SSPE = 1.5 M NaCl, 0.1 M NaH2PO4, 0.01 M EDTA). Heating at 80°C for 30 min dried the membrane and then RNA was affixed to membrane by UV cross-linking (Fisher Scientific SB-UVXL 1000). Membranes were pre-hybridized at 37°C for 1 hr with 10 ml of Express-Hyb solution (Clontech Laboratories). A 50-mer oligonucleotide probe corresponding to nucleotides 707 – 756 (5’ – tgcattccagccccgagccgtgaaccaggacaagaag aacatgctgt – 3’) of the SERCA3 rat cDNA (Burk, Lytton, MacLennan & Shull, 1989) was synthesized and used to determine the relative SERCA3 mRNA expression in RNA samples of rat cerebral cortex from young and old animals. The selective SERCA3 oligonucleotide probe (10 pmol) was labeled with [α-32P]dATP (3000 Ci/mmol) using a 3’-end DNA labeling kit with terminal transferase (Boehringer-Mannheim). The labeled probe was purified from unincorporated nucleotides by separating on a Chroma-Spin-30 size exclusion column (Clontech Laboratories). The probe was diluted in 10 ml of fresh
Express-Hyb solution (final activity $10^7$ cpm/ml) and allowed to hybridize with the membrane for 1 hr at 37°C. After hybridization, membranes were washed 3X for 15 min each with 2X sodium/saline citrate solution (SSC) + 0.05% SDS at 25°C followed by washing 3X for 15 min each with 0.5X SSC + 0.1% SDS at 35°C (2X SSC = 0.15 M NaCl, 0.015 M sodium citrate). The membrane was then exposed to Kodak X-OMAT AR x-ray film (Eastman Kodak) at -70°C for 3 to 6 hr. Densitometric analysis of autoradiographic films was analyzed to determine relative SERCA3 mRNA expression with age. The SERCA3 mRNA level in each lane was standardized to cyclophilin mRNA expression using a 0.7-kb rat cyclophilin cDNA probe.

3.5 Western Blot Analysis

Western immunoblotting techniques were used to detect and measure the relative amounts of SERCA3 isoform protein in young and aged rat cerebral cortex using a modified protocol of Lytton et al. (1992). Microsomal samples were prepared in Laemmli sample buffer containing 5% 2-mercaptoethanol (Laemmli, 1970). Protein samples were heated for 5 min at 95°C, loaded onto a 7.5% Tris-HCl precast SDS polyacrylamide gel (Bio-Rad), and then separated under constant voltage (200 mV) for 60 min in a Mini-Protean electrophoresis module (BioRad). Proteins were transferred onto polyvinylidiene difluoride (PVDF) membranes by electroblotting with a semi-dry transfer cell (BioRad Laboratories) at room temperature for 30 min at constant voltage (15 V). Completion of electroblotting was verified by staining the gel with Coomasie blue to show absence of residual protein. Detection of SERCA3 isoform protein was performed by initially blocking membranes overnight at 4°C in phosphate buffered saline.
(PBS, pH 6.9) containing 1.0% nonfat dried milk (w/v) and 0.1% Tween-20. The next day, the blocking buffer was poured off and replaced with the polyclonal (rabbit) primary anti-SERCA3 IgG antibody diluted 1:1000 in PBS/Tween-20. Incubation of membranes with primary antibody continued for 3 hr at room temperature. The primary antibody was removed and membranes washed 3X for 5 min each with PBS/Tween-20. The membranes were then incubated for 1 hr at room temperature with horseradish peroxidase (HRP) conjugated to secondary anti-rabbit IgG antibodies diluted to 1:2000 in PBS/Tween-20. After incubation the secondary antibody was poured off and the membranes washed 3X for 5 min each with PBS/Tween-20. Protein bands were detected by exposing membranes to enhanced chemiluminescence detection solution (ECL+; Amersham Pharmacia Biotech) for 2 min followed by autoradiography (Hyperfilm ECL; Amersham Pharmacia Biotech) for 10 min. Several protein concentrations (5 – 75 µg) were used from both age groups for comparing SERCA3 protein content with age. Densitometric analysis of autoradiographic films was used to determine relative protein content with age.

3.6 Data Analysis

$^{45}$Ca$^{2+}$-uptake values were generated by converting counts per min (cpm) to nmol calcium and then expressed as uptake activity (nmol/µg protein). Control assays were performed with microsomal and PMV fractions in the absence of ATP, while blank assays contained ATP but did not contain membrane fractions. Experiments were done in duplicate and corrected values (control subtracted) were averaged.
Relative band density of Western and Northern blots was determined using a ChemiNova CCD camera connected to an imaging system (ChemilImager, Alpha Innotech Corporation) running ChemilImager 4000 software (version 3.3). Densitometric values were given as integrated density values (IDV) with the area of measurement within each band remaining constant. Each Western blot exhibited multiple protein concentrations from both age groups. For instance, protein amounts (in μg) of 5, 10, 25, 50, and 75 for both age groups were run in parallel on the same gel as a relative comparison of band density with age. Western blots for each age group were performed in triplicate and values averaged together. The molecular weight of the target protein (SERCA3, ~97.4 KDa) was determined by running molecular weight standards with the following markers: myosin (220 KDa) from rabbit muscle, phosphorylase b (97 kDa) from rabbit muscle, and albumin (66 KDa) from bovine serum. For each Northern blot, samples were run in duplicate and values averaged together. Differences in sample loading were corrected for by determining the level of cyclophilin mRNA expression for each age group as an internal standard, which has been shown to be maintained with age in neurons (Iacopino & Christakos, 1990; Kuchel, Rowe, Meaney, & Richard, 1997). Determination of SERCA3 and cyclophilin mRNA bands were compared with a RNA molecular weight ladder with RNA range of 0.24 – 9.5 kb.

3.7 Statistical Analysis

Data presented in results are expressed as mean ± SEM. Statistical significance was determined by unpaired Student’s t-test or two-way ANOVA with Fischer-PLSD test. A $p < 0.05$ was accepted as the level of significance.
4.2 Effects of Age on Calcium Uptake into Microsomal and PMV Fractions

The time-courses of ATP-dependent, oxalate-facilitated Ca\(^{2+}\)-uptake into microsomes and PMV isolated from 6- and 20-month-old rat cerebral cortex were compared (Fig. 1A, B). For both microsomal and PMV fractions, the extent of Ca\(^{2+}\)-uptake increased linearly with time. There was, however, no significant difference between 6- and 20-month rats. Rate of calcium uptake detected by least-squares comparison of the slope over the entire range of incubation times showed no significant difference between 6- and 20-month animals (Table 2). The amount of Ca\(^{2+}\)-uptake at 10 min was greater in microsomes (~2800 nmol/µg) compared to PMV (~550 nmol/µg) (Fig. 1). Calcium uptake plateaued at incubation times greater than 10 min for both microsomes and PMV with no significant difference with age (data not shown).

<table>
<thead>
<tr>
<th>Age</th>
<th>Microsomes (enriched with SERCA) (nmol/µg/min)</th>
<th>Plasma Membrane Vesicles (enriched with PMCA) (nmol/µg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Month</td>
<td>247 ± 34</td>
<td>51.4 ± 5.8</td>
</tr>
<tr>
<td>20 Month</td>
<td>244 ± 48</td>
<td>49.0 ± 1.9</td>
</tr>
</tbody>
</table>

**Table 2.** Values represent mean ± SEM. Values were generated by least-squares comparison of calcium uptake (nmol/µg) to incubation time (min) and converted to the slope value (nmol/µg/min) and averaged together. Values were compared by unpaired Student’s t-test. N = 6 experiments with microsomes and N = 4 experiments with PMV, repeated in duplicate. Microsomal and PMV fractions isolated from rat cerebral cortex were prepared from six, 6-month and six, 20-month-old animals.
4.3 Sensitivity of Microsomal Fractions to Thapsigargin with Advancing Age

The sensitivity of microsomal fractions to thapsigargin was assessed with advancing age. In microsomes, 0.1 μM thapsigargin decreased Ca^{2+}-uptake by ~40% in both age groups (Fig. 2). Little further decline in Ca^{2+}-uptake occurred when thapsigargin was increased to 5 μM. Maximal blockade of ^{45}Ca^{2+}-uptake by thapsigargin only approached ~50%, suggesting the presence of a thapsigargin-insensitive component of calcium uptake. Since the microsomes were insensitive to low concentrations of vanadate (Table 1), the likelihood of the thapsigargin-insensitive calcium uptake being due to contamination by PMCAs is minimal.

4.4 Aging Effects on SERCA3 mRNA Expression in Rat Cerebral Cortex

The expression of the SERCA3 mRNA isoform was identified in cerebral cortex from 6- and 20-month-old rats using an oligonucleotide probe derived from the SERCA3 rat cDNA (Burk et al., 1989). The probe identified a single 4.5 kb RNA band in both 6- and 20-month animals (Fig. 3A). The SERCA3 mRNA content of rat cerebral cortex from old animals was significantly lower than from young animals (Fig. 3B). Age-related changes in SERCA3 expression was normalized with cyclophilin mRNA as an internal control (Iacopino & Christakos, 1990; Kuchel et al., 1997). Cyclophilin expression appeared to be the same in both age groups (Fig. 3A,B). Comparison of SERCA3 to cyclophilin mRNA expression in rat cerebral cortex revealed a significant age-related decline of ~25% in cyclophilin-normalized SERCA3 mRNA content from aged compared to young animals (Fig. 3C).
4.5 *SERCA3 Protein Expression with Advancing Age*

Age-related changes in SERCA3 protein levels were examined by Western immunoblotting of microsomes isolated from rat cerebral cortex for 6- and 20-month animals. A polyclonal anti-SERCA3 IgG antibody was used to probe for the SERCA3 isoform (Lytton et al., 1992; Wuytack et al., 1994). A single band at 97 kDa occurred in both age groups corresponding to the SERCA3 protein isoform (Fig. 4A). The relative amounts of SERCA3 protein in rat cerebral cortex was linear over the range 5 – 75 μg of microsomal protein (Fig. 4B). Analysis by least squares fit revealed no age-related difference in slope values of SERCA3 protein level versus total protein in microsomes isolated from rat cerebral cortex (6-month, 1.71 ± 0.09; 20-month, 1.74 ± 0.13 integrated density value/μg total microsomal protein). Thus, there was no significant age-related difference in SERCA3 protein levels.
A. Microsomes

B. Plasma Membrane Vesicles

**Figure 1.** Calcium uptake in microsomes and plasma membrane vesicles (PMV) isolated from 6- and 20-month-old rat cerebral cortex. $^{45}$Ca$^{2+}$-uptake was initiated by the addition of 25 µg of microsomes (A) or PMV (B) in the presence of ATP (5 mM), oxalate (1 mM) and ruthenium red (25 µM). The assays were incubated for the times indicated at 37°C. $^{45}$Ca$^{2+}$-uptake values represent nmol Ca$^{2+}$/µg protein – control, but are expressed as nmol/µg for simplification. Control assays are determined by the amount of $^{45}$Ca$^{2+}$-uptake by microsomes or PMV in the absence of ATP. Data shown represent mean ± SEM. Comparison of Ca$^{2+}$-uptake with age was determined by unpaired Student’s t-test. No statistical significant difference was observed in Ca$^{2+}$-uptake in microsomes or PMV with age, $p > 0.05$. Experiments with microsomes ($N = 6$) and with PMV ($N = 4$) were repeated in duplicate. Microsomal and PMV samples were prepared from individual rat cerebral cortex from six, 6-month and six, 20-month-old animals.
Figure 2. Sensitivity of calcium uptake to thapsigargin in microsomes. Microsomes isolated from rat cerebral cortex were incubated for 10 min at 37°C in the presence and absence of thapsigargin (0, 0.1, 5 μM). Measured $^{45}$Ca$^{2+}$-uptake values are corrected for control values and then expressed in normalized form as "percent of control." Maximum Ca$^{2+}$-uptake (Control) was determined from the amount of uptake by microsomes or PMV in the absence of thapsigargin. All assays were corrected for by the amount of Ca$^{2+}$-uptake by microsomes or PMV in the absence of ATP. Data shown represents mean ± SEM. * = significantly different from control, $p < 0.05$. Comparison of Ca$^{2+}$-uptake with age was determined by unpaired Student's $t$-test. $N = 3$ experiments with microsomes and $N = 3$ experiments with PMV, repeated in duplicate. Microsomal and PMV samples isolated from rat cerebral cortex were prepared from six, 6-month and six, 20-month-old animals.
Figure 3. (A) Representative Northern blot of SERCA3 mRNA expression in rat cerebral cortex isolated from 6- and 20-month-old animals. (B) Estimation of the relative amounts of SERCA3 and cyclophilin mRNA expression in rat cerebral cortex from 6- and 20-month-old animals. (C) Comparison of SERCA3/cyclophilin mRNA ratios with advancing age. The SERCA3 mRNA was probed using a selective 50-mer oligonucleotide probe correlating to positions 707 – 756 of the SERCA3 rat cDNA. SERCA3 and cyclophilin mRNA sizes were determined by the position of the RNA markers. Variance in RNA loading was corrected for by the level of cyclophilin expression as an internal standard. Autoradiograms were analyzed by densitometry and band density was reported as integrated density values (IDV) with the area around each band remaining constant. Data presented are given as mean ± SEM. Significance was determined by unpaired Student’s t-test. * = significantly different from 6-month, p < 0.05. Data presented in B & C represent N = 12 Northern blots with RNA samples done in duplicate for each age group. RNA samples isolated from rat cerebral cortex were prepared from six, 6-month and six, 20-month animals.
A. SERCA3 mRNA Expression

B. Band Density

C. SERCA3/Cyclophilin Ratio

Integrated Density Value (IDV x 1000)

SERCA3
Cyclophilin

SERCA3/Cyclophilin Ratio

20 month
6 month

*
Figure 4. (A) Representative Western blot of SERCA3 protein expression in rat cerebral cortex isolated from 6- and 20-month-old animals. (B) Estimation of the relative amounts of SERCA3 protein expression in rat cerebral cortex from 6- and 20-month-old animals. A protein concentration range (5 – 75 µg total protein) was separated by SDS-PAGE and transferred to PVDF membrane. A polyclonal anti-SERCA3 IgG antibody was used to selectively probe for SERCA3 isoform protein. SERCA3 molecular weight was determined by the position of the standardized protein markers. Autoradiograms were analyzed by densitometry and band density was reported as integrated density values (IDV) with the area around each band remaining constant. Data presented are given as mean ± SEM. Significance was determined by unpaired Student’s t-test. No statistical significant difference was observed in SERCA3 protein expression with age, p > 0.05. Data presented represents N = 4 Western blots with each protein concentration paired between age groups and blots performed in triplicate. Microsomal samples isolated from rat cerebral cortex were prepared from four, 6-month and four, 20-month-old animals.
A. SERCA3 Protein Expression

<table>
<thead>
<tr>
<th>MW ladder (kDa)</th>
<th>6 month</th>
<th>20 month</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>75</td>
<td>50</td>
</tr>
<tr>
<td>Protein amount (μg)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![SERCA3 protein expression diagram]

B. Band Density

![Band density graph]

- 6 Month
- 6 Month Linear Fit
- 20 Month
- 20 Month Linear Fit
5. DISCUSSION

5.1 Effects of SERCA Blockade on Calcium Uptake into Microsomal and PMV Fractions with Advancing Age

Our previous studies, and those from other groups, have shown an age-related decline in SERCA function in peripheral sympathetic neurons and in myocardial and skeletal muscle. In this study we sought to determine if an age-related decline in SERCA function is also manifested in central nervous tissue. Therefore, we focused on possible age-related changes in SERCA-mediated calcium uptake into microsomal membranes from neurons of the cerebral cortex.

The ATP-dependent \(^{45}\text{Ca}^{2+}\)-uptakes by microsomal and PMV fractions isolated from young (6-month) and old (20-month) cerebral cortex were not significantly different. The selective SERCA blocker, thapsigargin, significantly inhibited calcium uptake in microsomal fractions (enriched with SERCAs) at 0.1 \(\mu\text{M}\) in both age groups (Fig. 2). However, in PMV factions (enriched with PMCAs), thapsigargin did not significantly inhibit calcium uptake, even at the maximal concentration of 5 \(\mu\text{M}\) (Table 1). The insensitivity of PMV fractions to thapsigargin suggests that isolated PMVs from rat cerebral cortex are primarily enriched with PMCAs and that SERCA contamination is minimal. Conversely, the higher sensitivity of the microsomal fraction to thapsigargin indicates that this subcellular fraction contains a higher content of SERCAs compared to PMV fractions.

Although an age-related change in calcium uptake was not observed in microsomes isolated from rat cerebral cortex, our previous microfluorometric studies in superior cervical ganglion (SCG) cells indicate an age-related decline in SERCA activity (Tsai et al., 1998; Pottorf et al., 2000). The selective SERCA blocker, cyclopiazonic acid
(5 μM), caused a significant decline in the recovery phase of [K⁺]-evoked [Ca²⁺]i-transients in SCG cells from young animals with no effect in cells from old animals (Tsai et al., 1998). Vanadate concentrations greater than 1 μM, which will also antagonize SERCAs, also caused a significant decline in rate of recovery of [K⁺]-evoked [Ca²⁺]i-transients in young with no effect in SCG cells from old animals (Pottorf et al., 2000). These studies suggest that in peripheral autonomic neurons there is an age-related decline in the function of SERCAs. Similarly, in other peripheral neurons and excitable tissues calcium uptake by SERCAs has also been shown to decline. For example, in microsomes isolated from rat skeletal muscle and myocardium calcium uptake via SERCAs declined with age (Narayanan, 1981; Gafni & Yuk, 1989; Xu & Narayanan, 1998). In addition, a decline in SERCA function has been suggested to be responsible for more prolonged calcium transients in dorsal root ganglion cells (Kirischuk & Verkhratsky, 1996). Thus, in peripheral neurons and muscle tissues there seems to be mounting evidence that the functional capacity of SERCAs declines with age.

Since SERCA function appears to decline in peripheral excitable cells, it may be tempting to suggest that an age-related decline in SERCA function occurs in all neurons. However, caution is warranted in making such a generalization, as the present study and those of other investigators suggest. Interestingly, two studies reported an age-related increase in thapsigargin-sensitive calcium uptake in whole rat brain microsome (Hanahisa & Yamaguchi, 1998; Yamaguchi et al., 1999). In the first study, an increase in SERCA activity with age was, in part, related to enhanced protein kinase C activity (Hanahisa & Yamaguchi, 1998). Protein kinase C is involved in the regulation of SERCA activity and the data showed that staurosporine, a protein kinase C antagonist, decreased Ca²⁺-ATPase
activity in brain microsomes from aged rats, but did not affect activity in microsomes from young animals (Hanahisa & Yamaguchi, 1998). In the second study, the expression of regucalcin with advancing age was monitored since regucalcin appears to negatively modulate SERCA activity. With advancing age the expression of regucalcin declined, thus, attenuating inhibitory modulation of SERCAs (Yamaguchi et al., 1999). Data from both of these studies suggest that there may be subtle age-related alterations in the regulation of SERCAs that may, in effect, preserve or maintain SERCA activity in some central neurons. Contrasting studies, with respect to calcium buffering in central and peripheral neurons, underscore the need for caution in suggesting universal mechanisms causing age-related changes in SERCA function.

Thapsigargin, even at high concentrations (5 μM), did not completely inhibit $^{45}$Ca$^{2+}$-uptake by microsomal fractions (Fig. 2). Failure of thapsigargin to completely inhibit calcium uptake in microsomes isolated from rat or pig brain has also been noted by other investigators (Kijima, Ogunbunmi & Fleischer, 1991; Salvador & Mata, 1998; Wells & Abercrombie, 1998). Several possibilities may explain this. Firstly, microsomal fractions may be contaminated with other calcium translocating systems. However, vanadate, which selectively blocks PMCAs below 0.25 μM (Salvador & Mata, 1998), does not alter calcium uptake in our microsomal fraction (Table 1). These data are consistent with the conclusion that our microsomal fraction was not significantly contaminated with PMCAs. Secondly, thapsigargin-resistant, ATP-dependent mechanisms capable of sequestering calcium may exist in some cell and microsomal preparations (Waldron, Short & Gill, 1995; Pizzo, Fasolato & Pozzan, 1997). Indeed in the presence of cyclopiazonic acid (500 μM), 2,5-di(tert-butyl)-1,4-benzohydroquinone
(1.5 mM), or orthovanadate (2 μM), pig brain microsomes retain ~25% of $^{45}$Ca$^{2+}$-uptake (Salvador & Mata, 1998). Thirdly, the incomplete blockade of microsomal calcium uptake could be due to the differential sensitivity of the SERCA isoforms to thapsigargin. In one study, COS-1 cells, in which the four major SERCA isoforms (1, 2a, 2b, 3) were overexpressed, were used to provide microsomes for measuring oxalate-facilitated, ATP-dependent calcium uptake (Lytton, Westlin & Hanley, 1991). Overexpressed SERCA3 isoform was less sensitive to thapsigargin (80% blockade) than SERCA1, 2a, and 2b isoforms (90-95% blockade). Since neuronal tissue primarily contains SERCA3 and 2b isoforms, the rat cerebral cortex used in our present study may include a higher ratio of SERCA3 to 2b isoform; thereby, resulting in thapsigargin-insensitive calcium uptake in isolated microsomes.

5.2 SERCA3 mRNA Expression in Rat Cerebral Cortex with Advancing Age

To obtain additional information regarding molecular regulation of SERCAs, we examined mRNA expression of the SERCA3 transcript in rat cerebral cortex from young and old animals. Northern blot analysis revealed a 4.5 kb band in both young and old animals corresponding to the SERCA3 mRNA isoform from previous reports with brain tissues (Burk et al., 1989; Wu et al., 1995). Comparing SERCA3 mRNA levels with cyclophilin levels, as an internal control, we demonstrated a significant decline in the SERCA3 mRNA with advancing age (Fig. 3C). To the best of our knowledge, this is the first reported age-related change in SERCA mRNA expression in central neurons. Other studies have investigated age-related changes in SERCA expression in rat cardiac tissue and vascular smooth muscle (Maciel et al., 1990; Lompre, 1999). These studies have
also revealed a significant age-related decline in the mRNA expression of SERCAs. The SERCA3 isoform also decreases in pancreatic β-cells in diabetes (Varadi, Molnar, Ostenson & Ashcroft, 1996), and in the myocardium of failing heart (Hasenfuss et al., 1994; Peters et al., 1997). Thus, it appears that a decline in SERCA mRNA expression may commonly accompany aging and disease in various tissues.

Information regarding the regulation of SERCA mRNA expression is limited, but it can be affected by hormones, growth factors, and [Ca$^{2+}$]i levels (Simonides et al., 1996; Kuo et al., 1997; Tajima et al., 1998). Additionally, SERCA mRNA is downregulated when the PMCA gene is overexpressed in rat aortic endothelial cells, suggesting an interdependency between PMCA and SERCA gene expression (Liu, Xu, Fridman, Muallem & Kuo, 1996). Although the link between PMCA and SERCA regulation may be calcium itself, the means by which calcium affects PMCA or SERCA gene expression has yet to be elucidated (Ghosh & Greenberg, 1995).

5.3 Absence of Age-Related Changes in SERCA3 Protein Levels

To determine whether age-related declines in SERCA3 mRNA expression (Fig. 3) reflect a decrease in SERCA protein or a decline in SERCA turnover, we measured protein levels of the same isoform in rat cerebral cortex. SERCA3 protein levels remained the same in both age groups (Fig. 4). Therefore, in rat cerebral cortex the observed age-related decline in SERCA3 mRNA expression did not coincide with a decrease in SERCA protein levels nor a decline in SERCA activity. This has also been observed in rat skeletal muscle and myocardium (Ferrington et al., 1997; Xu & Narayanan, 1998). In these studies, SERCA protein levels did not show a corresponding
age-related change as predicted from measurements of mRNA expression. Additionally, myocardial SERCA protein levels were compared between patients with terminal heart failure and individuals with no signs of heart disease (Munch et al., 1998). In heart failure, SERCA protein levels did not show a parallel decline compared to mRNA expression even though a decline in SERCA function existed. Together, these studies suggest that declines in SERCA mRNA expression in peripheral tissues and central neurons do not necessarily correlate to declines in SERCA protein levels. Therefore, age-related declines in \([\text{Ca}^{2+}]_i\) regulation by SERCAs, as observed by us in peripheral neurons, may be due to changes in the function or regulation of SERCAs rather than due to changes in gene expression.

Although declines in SERCA mRNA expression do not seem to be reflected by corresponding changes in SERCA protein levels, there is often observed an age-related decline in SERCA function. An age-related decline in rat myocardial microsomal calcium uptake correlated with declines in calcium/calmodulin kinase (CAMK) protein expression and in the ability of CAMK to phosphorylate SERCAs (Xu & Narayanan, 1998). Also, calmodulin-dependent activation of CAMK in rat brain synaptic membranes declines with advancing age and may depress SERCA function via declines in the ability of CAMK to phosphorylate SERCA pumps (Zaidi, Gao, Squier & Michaelis, 1998). Others have proposed that SERCAs in rat skeletal muscle form inactive oligomers with advancing age (Ferrington et al., 1997). Thus, in peripheral neurons and tissues there may exist age-sensitive mechanisms of calcium regulation, independent of changes in SERCA mRNA and protein levels, which account for declines in SERCA function.
In conclusion, the mechanism of possible age-dependent changes in brain [Ca^{2+}]i regulation is still unresolved. Although our measures of SERCA mRNA and protein levels are in agreement with studies in peripheral tissues, we did not observe an age-related decline in brain SERCA function as we have observed in isolated SCG neurons (Tsai et al., 1998; Pottorf et al., 2000). Indeed, age-related declines in SERCA function appear to be restricted to peripheral excitable tissues. Thus, it seems possible that SERCA function in peripheral neurons and other excitable tissues is more sensitive to aging than that of central neurons.
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REFERENCES


CHAPTER VI

INTEGRATIVE DISCUSSION AND CONCLUSIONS

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1. INTRODUCTION

Descriptions of aging often assume an undesirable process:

'Nothing is more dishonorable than an old man, heavy with years, who has no other evidence of his having lived long except his age.'

Seneca (B.C. 3-65 A.D.).

However, the process of aging does not have to be met with disdain:

'If wrinkles must be written upon your brows, let them not be written upon the heart. The spirit should not grow old.'

James Garfield (1831-1881).

The statements above reflect the conundrum of the aging process. The first statement suggests that as one ages, deterioration of a once energetic and youthful image is all that remains after the inhospitable process of aging. Thus, many students of the aging process have assumed a general deterioration of all cellular processes resulting in increased susceptibility of the organism to pathology. The second statement is one of hope. Many elderly individuals have weathered the aging process quite well, maintaining remarkable peripheral physiological function and mental acuity. Despite much work on this problem, however, the impact of the aging process on the function of central and peripheral neurons remains a mystery in many respects (Verkhratsky & Toescu, 1998).

One facet of cellular function that has come to the forefront, in studies of the impact of advancing age on neuronal function, is the involvement of the ubiquitous ion, calcium. One prominent theory is that, in neurons, the aging process leads to
dysregulation of calcium homeostasis (Duckles, Tsai & Buchholz, 1995; Michaelis et al., 1996; Murchison & Griffith, 1998, 1999). Given that calcium appears to be a universal second messenger in neurons, altered calcium homeostasis with age has been suggested to be a fundamental mediator of age-related changes in neuronal function (Gibson & Peterson, 1987; Khachaturian, 1994; Thibault et al., 1998).

The idea that altered calcium homeostasis with advancing age is responsible for an age-related change in neuronal function is currently quite popular. However, despite the attraction of this idea, evidence of altered calcium regulation in central and peripheral neurons is difficult to sort out. For example as detailed below, studies have shown declines in calcium buffering with advancing age in peripheral and some central neurons. However, despite possible declines in any one component or multiple components of the calcium buffering system, compensatory mechanisms seem to be able to maintain overall calcium homeostasis. It remains to be seen whether similar changes also occur in other types of nerves.

2. SUMMARY OF DISSERTATION FINDINGS

This investigation of alterations in neuronal calcium buffering components with advancing age led to four major findings. First, calcium imaging and norepinephrine release studies revealed a decline in SERCA function to regulate calcium loads with advancing age (Tsai, Pottorf, Buchholz & Duckles, 1998; Pottorf, Duckles & Buchholz, 2000b, 2000c). Each component of [Ca^{2+}]_i regulation functions to modulate stimulation-evoked [Ca^{2+}]_i-signals in neuronal cells; therefore, an age-related impairment of any one component, such as SERCA, has the potential to affect [Ca^{2+}]_i homeostasis. Second,
although there appears to be a decline in function of SERCA with advancing age, compensatory mechanisms such as PMCA, mitochondria and Na\(^+\)/Ca\(^{2+}\)-exchange are mobilized to attenuate [Ca\(^{2+}\)]\(_i\)-transients in spite of the loss of a major component of the calcium buffering system (Buchholz, Tsai, Foucart & Duckles, 1996; Pottorf \textit{et al.}, 2000b). The existence of multiple calcium buffering components allow for compensation when one or more components are compromised. Therefore, with advancing age the remaining calcium buffering components become a critical part of maintaining calcium homeostasis and preventing calcium overload and excess neurotransmitter release in neurons. Third, in rat cerebral cortex there is an age-related decline in the mRNA expression of SERCA3 isoform; however, this decline did not translate to corresponding age-related changes in SERCA3 protein content (Pottorf, De Leon, Hessinger & Buchholz, 2000a). Although this seems rhetorical to have a decline in mRNA expression without a parallel shift in protein content, this phenomenon is reproduced in several other tissues with advancing age (Maciel, Polikar, Rohrer, Popovich & Dillmann, 1990; Ferrington \textit{et al.}, 1997; Xu & Narayanan, 1998; Lompre, 1999). Fourth, \(^{45}\)Ca\(^{2+}\)-uptake into microsomes isolated from rat cerebral cortex revealed no age-related change in calcium uptake or sensitivity to thapsigargin (Pottorf \textit{et al.}, 2000a). The consensus on calcium uptake into microsomes is not clear, with measurements of decreased, increased, or no change in calcium uptake with advancing age in neuronal tissue (Kirischuk & Verkhratsky, 1996; Yamaguchi, Hanahisa & Murata, 1999; Pottorf \textit{et al.}, 2000a). Although an age-related change in calcium uptake was not observed in microsomes isolated from central nerves of rat cerebral cortex, our microfluorometric studies of peripheral nerves from rat superior cervical ganglion (SCG) cells suggest such a decline
in SERCA activity does exists. Contrasting studies, with respect to calcium buffering in central and peripheral neurons, underscore the need for caution in suggesting universal mechanisms causing age-related changes in SERCA function. The exact mechanism responsible for an age-related decline in SERCA function has not, as yet, been identified; however, there are several possibilities that remain to be investigated. Determination of the mechanism responsible for regulation of SERCA and the other calcium buffering components may provide the evidence necessary in understanding declines in [Ca$^{2+}$]$_i$ regulation with advancing age.

3. DISCUSSION

3.1 Mechanisms of Intracellular Calcium Regulation

Neurons and other excitable cells use calcium as a second messenger to integrate numerous cellular pathways; these include neuronal development and maturation, gene expression, synaptic plasticity, transmitter release, excitability, and cell death (Malenka, Dauer, Perkel & Nicoll, 1989; Choi, 1992; Berridge, 1995, 1998; Clapham, 1995; Ginty, 1997; Spitzer & Ribera, 1998). In neurons intracellular calcium ([Ca$^{2+}$]$_i$) homeostasis relies on multiple calcium buffering components working in concert to restore stimulation-evoked [Ca$^{2+}$]$_i$-signals to basal levels (Kostyuk & Verkhratsky, 1994). After neuronal excitation, the cell must quickly restore [Ca$^{2+}$]$_i$ to basal levels in order to reset the cell for the next stimulus and avoid prolonged exposure to cytotoxic levels of high [Ca$^{2+}$]$_i$ (Miller, 1991). Neurons utilize several calcium buffering components to regulate [Ca$^{2+}$]$_i$. These include calcium sequestration into the endoplasmic reticulum by sarco/endoplasmic reticulum calcium ATPase (SERCA) pumps and into mitochondria by
an electrophoretic Ca\(^{2+}\)/H\(^{+}\)-uniporter. Additional buffering components include a mobile group of cytosolic calcium binding proteins, plasmalemmal calcium extrusion via plasma membrane Ca\(^{2+}\)-ATPase (PMCA), and plasma membrane Na\(^{+}\)/Ca\(^{2+}\)-exchange (Heizmann & Hunziker, 1991; Rizzuto, Brini, Murgia & Pozzan, 1993; Carafoli, 1997; MacLennan, Rice & Green, 1997; Blaustein & Lederer, 1999).

### 3.2 Loss of Buffering Capacity with Advancing Age

The overall consensus is that there is a prevailing age-related decline in the ability of neuronal cells to control stimulation-evoked [Ca\(^{2+}\)]\(i\)-transients and to maintain [Ca\(^{2+}\)]\(i\) homeostasis (Gibson & Peterson, 1987; Khachaturian, 1994; Thibault et al., 1998). Such a loss of calcium regulation could lead to alterations in neurotransmitter release with advancing age (Mattson, Rydel, Lieberburg & Smith-Swintosky, 1993). One neuronal system that has consistently been shown be functionally altered with advancing age is the adrenergic innervation of the cardiovascular system. An age-related rise in systemic blood pressure and increased levels of plasma catecholamines has repeatedly been demonstrated in humans and laboratory animals (Palmer, Ziegler & Lake, 1978; Esler et al., 1981; Barnes, Rashkind, Gumbrecht & Halter, 1982). Therefore, investigations were begun to explore the impact of age on function of vascular adrenergic nerves. Interestingly, an age-related increase in stimulation-evoked norepinephrine release from rat vascular adrenergic nerves was actually found (Buchholz & Duckles, 1990). Additional investigations demonstrated that this could not be accounted for by changes in norepinephrine content, the function of neuronal or extraneuronal uptake mechanisms, or the function of prejunctional \(\alpha_2\)-adrenergic receptors (Buchholz & Duckles, 1990;
Buchholz, Tsai, Friedman & Duckles, 1992). Therefore, investigations ensued to explore the possibility that there is a fundamental age-related change in the process of regulation of stimulation-evoked transmitter release in vascular adrenergic nerves.

3.2.1 Enhanced calcium buffering. To test whether alterations in norepinephrine release result from age-related changes in [Ca^{2+}]i homeostasis, the calcium chelator, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetatic acid (BAPTA), was applied to determine if norepinephrine release would be attenuated when calcium buffering capacity was augmented. Addition of BAPTA was more effective at reducing stimulation-evoked norepinephrine release in older rat tail arteries compared to arteries from young animals (Table 1; Tsai, Hewitt, Buchholz & Duckles, 1997). These data suggest that there is an age-related reduction in calcium buffering capacity from adrenergic nerves. However, as discussed above, there are multiple components involved in the regulation of [Ca^{2+}]i and any one component alone or several components together could be affected with advancing age. Therefore, multiple mechanisms involved in [Ca^{2+}]i regulation were investigated to determine which components of the [Ca^{2+}]i buffering system are affected by the aging process.

3.3 Effects of Age on the Function of SERCAs

3.3.1 Blockade of SERCAs. One factor in calcium dysregulation with age in vascular adrenergic nerves does appear to be a decline in the function of SERCAs. Studies in autonomic nerves using the selective blockers thapsigargin and cyclopiazonic acid suggest declines in SERCA function with advancing age. In isolated rat superior cervical ganglion (SCG) cells, microfluorometric measurements of [K^+] evoked [Ca^{2+}]i-
transients in the presence of these SERCA blockers showed a significant decline in the rate of calcium recovery in young cells but no significant change in calcium recovery in old cells (Table 1; Tsai et al., 1998). Further evidence of an age-related change in SERCA function was observed when norepinephrine release was measured in the presence of the same SERCA blockers. Blockade of SERCAs caused an increase in stimulation-evoked norepinephrine release in young rat tail arteries but there was no effect of SERCA blockade in arteries from old animals (Table 1; Tsai et al., 1998). These data suggest that SERCA uptake plays an important primary role in modulating [Ca\textsuperscript{2+}]i-transients in adrenergic nerves from young animals, while the function or contribution of calcium uptake by SERCAs appears to be lost in nerves from old animals.

3.3.2 Reliance on SERCAs. The contribution of SERCA function with age was further assessed by blocking all calcium buffering components except SERCAs in order to force the system to rely primarily on endoplasmic reticulum calcium uptake to control increased [Ca\textsuperscript{2+}]i (Pottorf et al., 2000c). Acutely dissociated SCG cells from young and old rats were exposed to vanadate (0.1 μM) to selectively block PMCA and dinitrophenol (100 μM) to block mitochondrial calcium uptake, and [Na\textsuperscript{+}]e was replaced with tetraethylammonium (TEA) to block Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchange. Acutely dissociated SCG cells were then depolarized by high [K\textsuperscript{+}], and recovery of [Ca\textsuperscript{2+}]i back toward basal levels was monitored (Table 1; Pottorf et al., 2000c). Exposure of SCG cells to these blockers resulted in a significant decline in the recovery phase of [Ca\textsuperscript{2+}]i in both age groups compared to control. However, there was clearly a greater effect of this treatment in cells from old compared to young animals (Table 1). Thus when forced to rely primarily on SERCAs, cells from old animals were not able to recover from increases in [Ca\textsuperscript{2+}]i as
well as cells from young animals. Therefore, these data, together with studies using selective blockers of SERCAs, support the conclusion that there is a decline in SERCA function with advancing age in adrenergic nerves (Tsai et al., 1998).

During blockade of SERCA function the recovery phase of 
$[K^+]$-evoked $[Ca^{2+}]_i$-transients is extended. However, intracellular calcium is still restored to basal levels in neuronal cells from both young and old animals. This finding suggests that compensatory mechanisms are mobilized to attenuate the $[Ca^{2+}]_i$-transients in spite of the loss of a major component of the calcium buffering system in nerves from aged animals. Each component of the neuronal $[Ca^{2+}]_i$ buffering system contributes to the regulation of stimulation-evoked $[Ca^{2+}]_i$-transients and maintenance of $[Ca^{2+}]_i$ homeostasis. Therefore dysregulation of $[Ca^{2+}]_i$ homeostasis by impairment of any one of these components can lead to an increase in the functional contribution of other calcium buffering components. Thus one or more calcium buffering components may prevent adverse effects by compensating for a decline in function by another component. This leads to the question: Which component(s) of the calcium buffering system might compensate when a deficit in $[Ca^{2+}]_i$ homeostasis occurs?

3.4 Contribution and Capacity of Mitochondrial Calcium Uptake with Advancing Age

3.4.1 Blockade of mitochondria. Mitochondria are important organelles in the control of $[Ca^{2+}]_i$-transients as they have been shown to buffer $[Ca^{2+}]_i$ loads within the normal physiological range and in the absence of pathology (Werth & Thayer, 1994; Buchholz et al., 1996; David, Barrett & Barrett, 1998; Pinton et al., 1998; Rizzuto et al.,
1998). Previous studies have demonstrated an increased reliance on mitochondrial calcium uptake in neurons from aged compared to young animals. Addition of the mitochondrial calcium uptake blocker dinitrophenol (DNP) depressed the recovery phase after high [K⁺]-evoked [Ca²⁺]i-transients to a greater extent in acutely dissociated SCG neurons from old compared to young rats (Table 1; Buchholz et al., 1996). Similarly, exposure to DNP of tail arteries from aged animals caused an increase in stimulation-evoked norepinephrine release. In contrast, DNP had no significant effect on norepinephrine release in young arteries (Table 1; Tsai, Duckles & Buchholz, 1995). Together these studies suggest that mitochondrial calcium uptake in peripheral neurons may become more important functionally with advancing age.

3.4.2 Reliance on mitochondria. To complement this study we wished to determine if calcium buffering capacity changed with age when SCG cells were forced to rely solely on mitochondria to regulate [Ca²⁺]i-transients. Young and aged SCG cells were forced to rely on mitochondria by blocking the contributions of both PMCA and SERCA with vanadate (100 μM), as well as by blocking Na⁺/Ca²⁺-exchange by replacement of [Na⁺]e with TEA (Pottorf et al., 2000b). Interestingly, in the presence of these blockers both young and aged SCG cells were still able to recover from [K⁺]-evoked [Ca²⁺]i-transients. Furthermore, there was no difference in the extent of this recovery with age (Table 1; Pottorf et al., 2000b). These data and the previous study suggest that when multiple calcium buffering systems are blocked, mitochondria are still able to restore [Ca²⁺]i-transients to basal levels in adrenergic nerves, and this ability is maintained in nerves from older animals.
3.5 Effects of Age on Plasma Membrane Calcium Transport Systems

Along with mitochondria, additional components of the calcium buffering system could also compensate for a decline in calcium regulation with advancing age. Recent evidence has highlighted the role of the PMCAs as important participants in the dynamic regulation of [Ca^{2+}]i-transients and as crucial players in calcium extrusion during normal (Carafoli & Stauffer, 1994; Werth, Usachev & Thayer, 1996) and pathological conditions (Garcia & Strehler, 1999). Additionally, Na^+/Ca^{2+}-exchange is an important component of [Ca^{2+}]i regulation that interconnects both sodium and calcium homeostasis in neuronal cells (Blaustein & Lederer, 1999).

3.5.1 Blockade of mitochondria and SERCAs. In rat adrenergic neurons, when mitochondrial calcium uptake and SERCAs are blocked with DNP and thapsigargin, respectively, SCG cells are "forced" to rely on plasma membrane calcium extrusion systems (PMCAs and Na^+/Ca^{2+}-exchange) to control high [K^+] evoked [Ca^{2+}]i-transients (Tsai et al., 1998). Under these conditions SCG cells from both old and young animals were able to fully recover from high [K^+] evoked [Ca^{2+}]i-transients. While the rate of recovery in neurons from young rats was unchanged by this treatment, there was an increase in the rate of recovery in aged neurons (Table 1; Tsai et al., 1998). These data suggest that plasmalemmal calcium extrusion alone can effectively control [Ca^{2+}]i-transients and that there is an induction of remaining calcium buffering components (i.e. PMCA and Na^+/Ca^{2+}-exchanger) during advancing age.

3.5.2 Blockade of PMCAs and SERCAs. The ability of Ca^{2+}-ATPase pumps in plasma membranes and ER (PMCA and SERCA, respectively) to control [K^+] evoked [Ca^{2+}]i-transients was also assessed with advancing age in SCG cells (Pottorf et al.,
Blockade of PMCA function with a relatively low concentration of vanadate (0.1 μM), which does not significantly affect the function of SERCAs, induced a greater decline in the rate of recovery of [Ca\(^{2+}\)]\(_i\) in SCG cells from old compared to young animals (Table 1; Pottorf et al., 2000b). Increasing the vanadate concentration to 1.0 μM, which can block both PMCAs and SERCAs, did not produce a further effect on the rate of recovery of [Ca\(^{2+}\)]\(_i\) in old SCG cells; however, there was a further decline in calcium recovery in cells from young animals (Table 1; Pottorf et al., 2000b). Therefore, the differential effect of vanadate on Ca\(^{2+}\)-ATPase pumps revealed a biphasic effect on rate of recovery of [Ca\(^{2+}\)]\(_i\) in young SCG cells compared to only a single phase response in cells from old animals. These data suggest that PMCA function is maintained with age; furthermore, the plasma membrane extrusion system can apparently provide some compensatory buffering capacity for this age-related deficit in SERCA function. Studies in SCG cells suggest that the calcium buffering system has several redundant mechanisms that allow for compensation when one or more components are compromised. Therefore, with advancing age, when SERCA function is attenuated, the remaining calcium buffering components become a critical part of maintaining calcium homeostasis and preventing calcium overload in adrenergic neurons.
DIFFERENTIAL EFFECT OF AGE ON RESPONSE TO PHARMACOLOGICAL MANIPULATION AFFECTING INTRACELLULAR CALCIUM BUFFERING

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[Ca²⁺]-Transient Rate of Recovery (SCG)</th>
<th>Stimulation-Evoked Norepinephrine Release (RTA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young</td>
<td>Aged</td>
</tr>
<tr>
<td>Enhanced Calcium Buffering</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAPTA (100 µM) (5)</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Blockade of Buffering Components</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERCA (cyclopiazonic acid) (6)</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>Mitochondria (dinitrophenol) (1,4)</td>
<td>↓↓</td>
<td>↓↓↓</td>
</tr>
<tr>
<td>SERCA &amp; Mitochondria (thapsigargin + dinitrophenol) (6)</td>
<td>↓</td>
<td>↓↓</td>
</tr>
<tr>
<td>PMCA (low vanadate) (2)</td>
<td>↓↓</td>
<td>↓↓</td>
</tr>
<tr>
<td>PMCA &amp; SERCA (high vanadate) (2)</td>
<td>—</td>
<td>↑</td>
</tr>
<tr>
<td>Reliance on Single Buffering Component</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reliance on SERCA (vanadate + dinitrophenol + zero Na⁺) (3)</td>
<td>↓</td>
<td>↓↓</td>
</tr>
<tr>
<td>Reliance on Mitochondria (vanadate + zero Na⁺) (2)</td>
<td>↓↓</td>
<td>↓↓</td>
</tr>
</tbody>
</table>

Table 1. The following symbols represent changes from control values: (↑) increase from control; (↓) decrease from control; (—) no change from control; and (n/d) no data available. BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetatic acid. Fisher-344 rats were used for all experiments and divided into two age groups of young (6 month) and aged (20 month). [Ca²⁺]-transients were recorded by microfluorometry using fura-2 in acutely dissociated rat superior cervical ganglion (SCG) cells, and rate of recovery of [Ca²⁺] (nM/sec) compared to control within each age group. Electrical stimulation-evoked norepinephrine release was measured in rat tail arteries (RTA) and compared to control values within each age group. Norepinephrine was collected from isolated arteries during and after electric field stimulation, and samples analyzed by HPLC with electrochemical detection.

3.6 Comparison of the Effects of Age on Function and Expression of SERCA in Rat Cerebral Cortex

Thus far studies in SCG cells and tail arteries have provided evidence for functional declines in SERCA activity and an induction of compensatory mechanisms by alternate calcium buffering components with advancing age. The molecular regulation of this calcium pumps has been identified and quantified in neuronal and several other tissues; however, no comparison of SERCA expression with advancing age has been studied. Studies measuring $^{45}$Ca$^{2+}$-uptake by microsomes isolated from dorsal root ganglion cells and skeletal muscle also have shown declines in SERCA function with advancing age (Gafni & Yuh, 1989; Kirischuk & Verkhratsky, 1996). However, the mechanism of an age-related reduction in SERCA function has, as yet, to be resolved. Investigators have struggled to explain age-related changes in SERCA function and mRNA expression without corresponding changes in protein levels. Age-related declines in SERCA function have been supported with similar reductions in mRNA expression in cardiac tissue and vascular smooth muscle (Maciel et al., 1990; Lompre, 1999). However, the expression of SERCA proteins in the same tissues does not show corresponding changes in protein levels with advancing age (Ferrington et al., 1997; Xu & Narayanan, 1998). Therefore, further studies are necessary to answer the question of age-related changes in SERCA expression and the connection with declines in SERCA function.

We sought to answer functional changes in SERCA activity with changes in molecular regulation of SERCA expression and protein levels in young (6 month) and old (20 month) Fisher-344 (F-344) rat cerebral cortex. Functional studies were conducted by using ATP-dependent, oxalate-facilitated $^{45}$Ca$^{2+}$-uptake into microsomes isolated from rat
cerebral cortex and comparing the two age groups. From this same tissue model, age-related functional changes in SERCA activity were compared with changes in mRNA expression and protein levels of the SERCA3 isoform.

3.6.1 $^{45}$Ca$^{2+}$-uptake into microsomal and plasma membrane vesicle fractions.

The focus of this study was to investigate the impact of age on calcium uptake into two subcellular fractions of microsomes and plasma membrane vesicles (PMV) in the presence and absence of the selective SERCA blocker thapsigargin (Pottorf et al., 2000a). The two subcellular fractions isolated from young and old rat cerebral cortex revealed increasing $^{45}$Ca$^{2+}$-uptake with incubation time; however, there was no difference with advancing age (Table 2; Pottorf et al., 2000a). In the presence of thapsigargin (0.1 μM) ATP-dependent, oxalate-facilitated $^{45}$Ca$^{2+}$-uptake into microsomes was significantly declined in both age groups compared to control values. Whereas, PMV fractions exposed to even higher concentrations of thapsigargin (5 μM) had no significant effect on $^{45}$Ca$^{2+}$-uptake compared to control values for either age group (Table 2; Pottorf et al., 2000a). The lack of effect of thapsigargin in PMV fractions suggests that isolated PMVs from rat cerebral cortex are primarily enriched with PMCA and that SERCA contamination is not a major factor. Conversely, the higher sensitivity to thapsigargin blockade observed in microsome fractions suggests that this subcellular fraction contains a higher content of SERCAs compared to PMV fractions. Although an age-related change in calcium uptake was not observed in microsomes isolated from rat cerebral cortex, our functional studies in superior cervical ganglion (SCG) cells suggest such a decline in SERCA activity does exist (Table 1). Calcium uptake by microsomes isolated from skeletal muscle and dorsal root ganglion cells has shown declines in SERCA
function with advancing age (Gafni & Yuh, 1989; Kirischuk & Verkhratsky, 1996). However, the opposite effect of increased calcium uptake has been shown in rat brain microsomes (Hanahisa & Yamaguchi, 1998; Yamaguchi et al., 1999). This discrepancy between studies measuring calcium uptake by microsomes in brain, dorsal root ganglion, or skeletal muscle suggests that a clear mechanism for age-related changes still need further clarification. Additionally, our microfluorometric studies with SCG cells suggest a significant decline in the ability of SERCAs to control [Ca\textsuperscript{2+}]i-transients and maintain calcium homeostasis with advancing age (Table 1; Tsai et al., 1998; Pottorf et al., 2000b; 2000c). Contrasting studies, with respect to calcium buffering in central and peripheral neurons, underscore the need for caution in suggesting a universal mechanism causing age-related changes in SERCA function. It may indeed be that age-related declines in SERCA function are restricted to peripheral excitable tissues and that SERCA function in these tissues is more sensitive to aging than that of central neurons.

### 3.6.2 SERCA3 mRNA expression and protein content

Molecular regulation of the SERCA3 isoform was examined in rat cerebral cortex from young and aged animals. Comparison of SERCA3 mRNA expression demonstrated a significant decline in the transcript with advancing age (Table 2; Pottorf et al., 2000a). Our investigation of the literature revealed no other studies that have attempted to determine levels of SERCA mRNA expression with advancing age in neuronal tissue. However, there are a few studies that have investigated age-related changes in SERCA expression in rat cardiac tissue and vascular smooth muscle (Maciel et al., 1990; Lompre, 1999). Our study correlates well with these studies that revealed a significant decline in the mRNA expression of SERCAs with advancing age.
To verify age-related declines in SERCA3 mRNA expression, we measured protein levels of the same isoform in rat cerebral cortex. SERCA3 protein levels were shown to remain stable with advancing age (Table 2; Pottorf et al., 2000a). Therefore, an age-related decline in SERCA3 mRNA expression did not coincide with a parallel decrease in protein levels. However, this same phenomenon has also been observed in rat skeletal muscle and myocardium (Ferrington et al., 1997; Xu & Narayanan, 1998). In these studies, measurement of SERCA protein levels also did not show a corresponding age-related change as predicted from measurements of mRNA expression in the same tissues. These studies investigating molecular changes of SERCAs suggest that a down-regulation in SERCA mRNA expression does not necessarily result in a parallel shift in SERCA protein levels with advancing age.

**SUMMARY OF FUNCTIONAL AND MOLECULAR STUDIES IN RAT CEREBRAL CORTEX**

<table>
<thead>
<tr>
<th>Study</th>
<th>Young</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect of TG on $^{45}$Ca$^{2+}$-Uptake in Microsomes</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Effect of TG on $^{45}$Ca$^{2+}$-Uptake in PMV</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SERCA3 mRNA Expression (compared to young)</td>
<td>—</td>
<td>↓</td>
</tr>
<tr>
<td>SERCA3 Protein Levels (compared to young)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 2. The following symbols represent changes from control values: (↑) increase from control and (—) no change from control. Cerebral cortex from Fisher-344 rats were used for all experiments and divided into two age groups of young (6 month) and aged (20 month). $^{45}$Ca$^{2+}$-uptake studies in microsomes (enriched with SERCA) and plasma membrane vesicles (PMV, enriched with PMCA) were exposed to the selective SERCA blocker thapsigargin (TG) and compared to control (absence of TG) within each age group. SERCA3 mRNA expression was determined by Northern blot analysis and compared with cyclophilin as an internal control. SERCA3 protein levels were determined by Western blot analysis. Functional and molecular data reported in this table represents a previous study submitted for publication (Pottorf et al., 2000a).
4. CONCLUSIONS & FINAL THOUGHTS

There were two hypotheses proposed at the beginning of this dissertation concerning possible mechanisms of dysregulation of \([Ca^{2+}]_i\) homeostasis with advancing age (page 22-24). First, a permanent change in the regulation of neuronal calcium may occur with advancing age leading to neurodegeneration. Second, instead of permanent neuronal loss, subtle changes in \([Ca^{2+}]_i\) homeostasis may occur leading to altered neuronal performance. However, in this case functional compensation by remaining calcium buffering components may be effective in controlling \([Ca^{2+}]_i\)-transients and preventing calcium overload. In this model, functional consequences of age-related changes may be subtle because of effective cellular compensation.

The evidence presented in this dissertation supports the second hypothesis in the case of peripheral adrenergic neurons. These studies document a significant reduction in the function of SERCA pumps to control \([Ca^{2+}]_i\)-transients with advancing age in SCG cells and tail artery adrenergic nerves. However, multiple calcium buffering components such as PMCA, mitochondria, and \(Na^+/Ca^{2+}\)-exchange are also present, and these can functionally compensate for a deficit by another buffering component. Thus, our governing hypothesis is conserved stating that in peripheral adrenergic nerves calcium uptake by ATP-dependent calcium buffering components of the endoplasmic reticulum is reduced with advancing age, leading to greater or more sustained \([Ca^{2+}]_i\) levels, a greater reliance on remaining calcium buffering components to restore \([Ca^{2+}]_i\) homeostasis and increased neurotransmitter release (page 24; representative graph of governing hypothesis refer to Fig 2, page 26).
It is important to understand that the aging process is complex and multifaceted. The hypothesis proposed is just one of many possible theories that attempt to define and understand the role of $[Ca^{2+}]_i$ regulation and aging in neurons. Despite our knowledge of the mechanisms involved in neuronal $[Ca^{2+}]_i$ regulation, elucidation of the importance, participation, and regulation of each calcium buffering component is still lacking, and additional studies are necessary to confirm their respective roles in normal aging of neurons, both in the peripheral and central nervous systems.

‘No wise man ever wished to be younger.’

Swift (1667-1745).
REFERENCES


APPENDIX I:
SERCA CALCIUM TRANSPORT AND MOLECULAR STRUCTURE

Reaction Cycle for Calcium Transport by SERCA

The sarco/endoplasmic reticulum calcium ATPase (SERCA) pump is an integral membrane protein and a member of the P-type ATPases. The SERCA pump transports two calcium ions against a concentration gradient into the organelle from the hydrolysis of ATP (Fig 1). The $E_2$ state exposes the high-affinity calcium-binding site, which binds two calcium ions forming the $E_1$ conformation state. After calcium binding, the SERCA enzyme is phosphorylated by ATP at specific residue within the catalytic domain forming the high-energy phosphoenzyme intermediate ($E_1P$). The phosphoenzyme intermediate undergoes a conformational change in the SERCA structure, producing the low-affinity calcium binding state $E_2P$, which expels the calcium ions into the lumen. Hydrolysis of the phosphate group regenerates the high-affinity calcium binding state ($E_2$) and resets the enzyme for the next cycle.

3-Dimensional Molecular Modeling of SERCA1

The crystal structure of the calcium ATPase of skeletal muscle (SERCA1) has been resolved at 2.6 Å obtained by X-ray crystallography (Fig. 2). The SERCA crystals were prepared in lipid membranes forming planar stacks and grown in the presence of 10 mM CaCl$_2$. The SERCA enzyme is primarily composed of four major domains: 1. Actuator domain (pink and green); 2. Catalytic domain (dark blue); 3. ATP-binding and hinge domain (red); and 4. Membrane and stalk domain (light blue). The two calcium ions (purple) are located side-by-side and are surrounded by ten transmembrane $\alpha$-helices
forming the stalk and membrane domain. The four transmembrane domains of M4, M5, M6, and M8 are clustered within the membrane domain and form the two calcium-binding sites. The cytoplasmic region consists of three major domains, with the ATP phosphorylation site at Asp^{351} residue (yellow) located within the catalytic domain. The adenosine-binding and hinge domain stabilizes the ATP molecule and forms the main head region. The actuator domain is formed between the M2 and M3 helices of the membrane domain.
Figure 1. Conformational states of SERCAs. There are at least four interconvertible conformational states that have been identified during the cycle of ATP-dependent calcium transport. 
(a) Increases in $[\text{Ca}^{2+}]_{i}$ saturates the two high-affinity calcium binding sites to form the $E_1$ state. 
(b) The enzyme then binds ATP forming a high-energy phosphoenzyme intermediate ($E_1\text{P}$). 
(c) The rate-limiting step results in a conformational change leading to a low-affinity calcium state, $E_2\text{P}$, and release of the calcium ions into the lumen. 
(d) The enzyme then undergoes hydrolysis to remove the phosphate group regenerating the $E_2$ state.
Figure 2. Molecular structure of the SERCA1 enzyme at a resolution of 2.6 Å obtained by X-ray crystallography. The molecular structure of SERCA1 can be divided into 4 main domains. The SERCA enzyme is primarily composed of four major domains: (1) Actuator domain (pink and green); (2) Catalytic domain (dark blue); (3) ATP-binding and hinge domain (red); and (4) Membrane and stalk domain (light blue). The four α-helices of the membrane domain M4, M5, M6, and M8 form the two calcium-binding sites. The ATP phosphorylation site at Asp$^{351}$ residue (yellow) if located within the catalytic domain. The actuator domain is formed between M2 and M3 α-helices of the membrane domain. Inset, shows the bottom view of the membrane domain from the lumenal face. The four membrane regions (M4, M5, M6, M8) are clustered around the calcium ions to form the two calcium-binding sites.