CHAPTER III

EXPERIMENTAL METHODS

ANIMALS

Twelve (six, 12 months of age and six, 27 months of age) virus antibody-free male Brown Norway-Fischer 344 (F344BNF1/Nia) hybrid rats were obtained from NIA and housed in Virginia Tech's central Laboratory Animal facility. Where they were housed in pairs in a room maintained at 21°C on a 12h light-dark photoperiod. All animals were fed a standard lab diet. The F344BNF1/Nia rat is the rat of choice for aging research because it develops the fewest detrimental pathologies. Also, the onset of clinical disorders for this rat occurs at a later age than other crosses (source: Information Notice to Recipients of NIA Rats & Mice, 1995) (Weindruch, 1995). These rats are an alternate model to the traditionally used Fischer 344. The life span for F344BNF1/Nia crosses has a mean and maximum span of 28 and 35 months, respectively (National Center for Toxicological Research, NCTR).

At the time of sacrifice, animals were weighed and anesthetized with 50mg/kg of a pentobarbital sodium solution administered intraperitoneal. Supplemental dosages were given as needed. After tissue (gastrocnemius) removal, animals were sacrificed by pentobarbital overdose (>100mg/kg ip). All methods of anesthesia and sacrifice were approved through the Virginia Tech Animal Use Committee.

SARCOPLASMIC RETICULUM VESICLE ISOLATION

The homogenization buffer (HB) consisted of 20mM HEPES, 250mM sucrose, 0.2% sodium azide and 0.2mM PMSF (added daily) at pH 7.4 (Luckin, Favero, & Klug, 1991). Gastrocnemius muscles were minced with scissors and the homogenized using a VirTis Virtshear. Homogenization was initially performed at a low speed to disperse large pieces. Increasing speeds were used until a visually homogenous liquid was established. The homogenate was placed in a 50ml polycarbonate centrifuge tubes and then was

centrifuged at 1600g for 10 minutes 5° C. The supernatant was filtered through 4 layers of gauze and the pellet, consisting of fascia, cell debris and fat, was discarded. The supernatant was weight-balanced visually and centrifuged at 10,000g for 20 minutes. The white, fluffy mitochondrial pellet was then discarded. The supernatant was centrifuged at 49,000rpm for 90 minutes. Next, the supernatant was discarded and the remaining pellet (SR and myofibrillar proteins) was resuspended in a centrifuge tube with approximately 500µl HB supplemented with 0.6M KCl to solublize the contractile proteins. Samples were added to clean tubes and weight-balanced using 0.6M KCl-HB. The final centrifugation was at 49,000g for 60 minutes. The supernatant was discarded and the remaining pellet was rinsed once with HB. The SR pellet was then resuspended with a final volume of 500-700µl with HB, depending on size of pellet. Protein content was determined by the Bradford protocol according to Bio-Rad. SR samples were immediately stored at -80°C.

CALCIUM UPTAKE AND RELEASE

Ca^{2+} uptake and release by the SR vesicles were measured fluorimetrically in SR vesicles using fura2 as the extravesicular Ca^{2+} indicator. The incubation buffer solution consisted of 100mM KCl, 20 mM HEPES, 7.5 mM pyrophosphate, 10µM $CaCl_2$, and 1mM $MgCl_2$. Ca^{2+} uptake and release were initiated by the addition of 0.5 mM ATP. The rates of Ca^{2+} uptake and release were determined as the steepest negative slope (uptake) and positive slope (release) of the extravesicular free- Ca^{2+} vs. time curve and normalized by the SR protein concentration.

A Jasco CAF-110 intracellular calcium analyzer interfaced with a microcomputer was used for data collection. Excitation light was obtained from a xenon high pressure lamp (75W) equipped with two monochromators containing 340 and 380 nm interference filters. Emission fluorescence was measured by a photomultiplier tube (detect electrical

signal and divides it into two signals synchronized with a chopping mirror) using a 500nm filter. The ratio of the fluorescence due to excitation at 340 and 380 nm was sampled via microcomputer (2Hz) and converted into free Ca^{2+} concentrations using the equation: $[Ca^{2+}]_f = K_d \bullet \beta \bullet (R - R_{min}) \bullet (R - R_{max})^{-1}$, where K_d is the dissociation constant for Ca^{2+} and Fura2 (200nM), β is the ratio of fluorescence measured at 380nm in the presence of zero and 100μM free Ca^{2+}. R_{min} and R_{max} represent fluorescence ratios in the presence of zero and 100μM free Ca^{2+}.

Prior to calibration and assay procedures, HOCl, H_2O_2, and fura2 stock solutions were placed in ice with fura2 wrapped in foil. SR vesicle samples were thawed on ice for approximately ten minutes and remained on ice during procedure. All other solutions were at room temperature. Cuvettes filled with 1ml buffer solution pre-warmed to 37° C via a warming block. A calibration procedure was performed by loading a cuvette with buffer, fura2, and 1mM EGTA. The cuvette was then placed in the Jasco CAF-110 analyzer and F_{380} min (fluorescence intensity when all fura2 are free) & Rmin (fluorescence intensity ratio) values were recorded. Next, another cuvette was loaded with buffer, fura2, and ~100mM $CaCl_2$ ($CaCl_2$ was added until the R value plateaus) and F_{340}max (fluorescence intensity when all fura2 are bounded) & Rmax values were recorded.

Ca^{2+} uptake activity was measured in the presence of 1 mM HOCl. Ca^{2+} release was determined in the presence of 10 μM cyclopiazonic acid using 3mM H_2O_2 or 5 μM $AgNO_3$ (control condition).

ANALYTICAL PROCEDURES

Statistical analyses were performed using SigmaStat, (Jandel Scientific). Two-way analysis of variance was used to determine differences in each varible between age, group and treatment. A Student Newman-Keuls method was performed to discern the difference of means. The level of significance will be set at $p < 0.05$.