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Ca^{2+} extrusion in aged smooth muscle cells

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ABSTRACT

We investigated the effects of aging in Ca^{2+} extrusion mechanisms in smooth muscle bladder cells from 4 and 20–24-month-old guinea pigs using fluorescence microscopy and fura-2. Cells were challenged with a pulse of KCl immediately before perfusion with a Ca^{2+} free solution containing no inhibitors (control, untreated cells) or inhibitors of plasma membrane Ca^{2+} pump (PMCA, 1 mM La^{3+}), Na^+/Ca^{2+} exchanger (NCX, 1 μM SEA0400) or the sarcoendoplasmic Ca^{2+} pump (SERCA, 1 μM thapsigargin). Treatment of young adult cells with the inhibitors allowed estimating a relative contribution of 55% for NCX, 27% for PMCA and 31% for SERCA. Combination of two inhibitors at the same time showed the presence of interaction between extrusion mechanisms. In aged cells the $[Ca^{2+}]_i$ extrusion was impaired due to decrease of PMCA activity, as revealed by the loss of effect of La^{3+}, and to inhibitory interactions between NCX and SERCA activities, indicated by acceleration of decay in response to their respective inhibitors. In conclusion, in smooth muscle cells aging decreases the overall Ca^{2+} extrusion activity and modifies the interactions between the activities of the main Ca^{2+} removing mechanisms.

1. Introduction

Numerous stimuli and conditions operate cellular responses through changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) termed Ca^{2+} signals. These signals in turn regulate multiple cellular functions, such as contraction, secretion or gene regulation. The presence of age-related changes leads to the proposal of a "Ca^{2+} theory of aging" [1]. $[Ca^{2+}]_i$ signals are shaped by cytosolic Ca^{2+} binding capacity and Ca^{2+} transport mechanisms. $[Ca^{2+}]_i$ increases are due to Ca^{2+} influx from extracellular medium and release from internal stores (mainly sarcoendoplasmic reticulum). Subsequent recovery of $[Ca^{2+}]_i$ to basal levels is achieved by active transport either to the external medium, via plasma membrane Ca^{2+} pumps (PMCA) and Na^+/Ca^{2+} exchange (NCX), or into subcellular stores through sarcoendoplasmic reticulum Ca^{2+} (SERCA) pumps. The relative importance of these systems depends on both the type and status of the cell [2].

The effects of aging in Ca^{2+} homeostatic parameters have been studied mainly in excitable cells (for reviews see Refs. [3,4]). Thus, aged neurones display impairment of mechanisms such as Ca^{2+} buffering and Ca^{2+} extrusion [5–8], expression [9] and operation [10] of sarcoplasmic Ca^{2+} release channels, and refilling of intracellular neural pools [11,12]. In cardiac cells, aging increases the frequency of spontaneous localized Ca^{2+} release events (sparks) [13], and reduces depolarization-evoked $[Ca^{2+}]_i$ responses [13,14], while skeletal muscle SERCA and brain PMCA activities are impaired by age-related redox modifications [15].

Reports on smooth muscle Ca^{2+} signals during aging are scant. Arterial myocites show age-induced alterations in Ca^{2+} release through ryanodine and IP_3 receptors [16,17] and

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impaired SERCA function [18], while intestinal smooth muscle cells from aged rats show increases in both Ca^{2+} release from intracellular stores [19] and Ca^{2+} entry [20]. It has also been reported that aged arterial muscle shows increased $[Ca^{2+}]_i$ [17] and proliferation rate [21]. To our knowledge, there is no additional information in other smooth muscle types with the exception of changes in sensitivity to Ca^{2+} blockers in bladder muscle strips [22] and our recent description of impairment of Ca^{2+} influx in aged gallbladder smooth muscle [23]. These changes are likely to be related to age-related functional changes of smooth muscle, such as loss of gallbladder contraction [23,24] or impaired arterial tone and shift to hypertension [18,25].

Bladder smooth muscle shows alterations in contractility during aging ([26]; Gomez-Pinilla, Camello and Pozo, unpublished observations), and some of them have been postulated to be related to changes in Ca^{2+} homeostasis [22]. Ca^{2+} extrusion systems are main determinants in bladder contraction [27]. Therefore, in the present study we have studied the possible modification of smooth muscle Ca^{2+} clearance mechanisms by aging.

2. Methods

2.1. Cell isolation

Single cells from detrusor smooth muscle of young adults (4-month-old) or aged guinea pigs (20–24-month-old) were obtained by enzymatic digestion. After anaesthesia and cervical dislocation, urinary bladder was removed, placed in a sylgard plate filled of Krebs-Henseleit solution (K-HS, see Section 2.3), trimmed free of fatty tissue and opened along its longitudinal axis. Later the bladder was pinned to the sylgard plate and the urothelium was removed carefully. Subsequently, about 20 mg of urinary bladder muscle was cut into small pieces and incubated during 35 min at 37 °C in enzyme solution (ES, for composition see Section 2.3) supplemented with 1 mg/ml BSA, 1 mg/ml papain, and 1 mg/ml dithioerythritol. The tissue was then transferred to fresh ES containing 1 mg/ml BSA, 1 mg/ml collagenase, and 100 μM $CaCl_2$ and incubated for 10 min at 37 °C. The tissue was then washed three times using ES, and the single smooth muscle cells were isolated by several passages of the tissue pieces through the tip of a fire-polished pipette. The resultant cell suspension was kept in ES at 4 °C until use, generally within 6 h. Both cell viability (assayed by fast trypan blue staining) and cell length was similar in young and adult cells (adult: 52.02 ± 1.48 μm; aged: 47.35 ± 2.52 μm). Experiments were performed at room temperature.

2.2. Cell loading and $[Ca^{2+}]_i$ determination

$[Ca^{2+}]_i$ was determined by epifluorescence microscopy using the fluorescent ratiometric Ca^{2+} indicator fura-2. Isolated cells were loaded with 4 μM fura-2-AM at room temperature for 25 min. An aliquot of cell suspension was placed in an experimental chamber made with a glass poly-D-lysine treated coverslip (0.17 mm thick) filled with Na^+-HEPES solution (for composition see Section 2.3) and mounted on the stage of an inverted microscope (Diaphot T2000; Nikon). After cell sedimentation, a gravity-fed system was used to perfuse the chamber with Na^+-HEPES solution in the absence or presence of experimental agents. For deesterification of the dye, >20 min were allowed to elapse before Ca^{2+} measurements were started. Cells were illuminated at 340 and 380 nm by a computer-controlled monochromator (Optoscan, Cairn Research) at 0.3 Hz, and the emitted fluorescence was selected by a 500-nm long-pass filter. The emitted images were captured with a cooled digital charge-coupled device camera (ORCAII-ER; Hamamatsu Photonics) and recorded using dedicated software (Metafluor, Universal Imaging). The ratio of fluorescence at 340 nm to fluorescence at 380 nm (F_{340}/F_{380}) was calculated pixel by pixel and used to indicate the changes in $[Ca^{2+}]_i$. For the purpose of the present study calibration of ratio values was not necessary due to the normalization procedure (see below).

2.3. Solutions and drugs

The K-HS contained (in mM): 113 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, and 11.5 D-glucose. This solution had a final pH of 7.35 after equilibration with 95% O_2–5% CO_2. The ES used to disperse cells was made up of (in mM): 10 HEPES, 55 NaCl, 5.6 KCl, 80 sodium glutamate, 2 $MgCl_2$, and 10 D-glucose, with pH adjusted to 7.3 with NaOH. The Na^+-HEPES solution contained (in mM): 10 HEPES, 140 NaCl, 4.7 KCl, 2 $CaCl_2$, 2 $MgCl_2$, and 10 D-glucose, with pH adjusted to 7.3 with NaOH. The Ca^{2+}-free Na^+-HEPES solution was prepared by substituting EGTA (1 mM) for $CaCl_2$. Drug concentrations are expressed as final bath concentrations of active species. Drugs and chemicals were obtained from the following sources: 1,4-dithio-D,L-threitol, La^{3+} and thapsigargin were from Sigma Chemical (St. Louis, MO); fura-2-AM was from Molecular Probes (Molecular Probes Europe, Leiden, Netherlands); collagenase was from Fluka (Madrid, Spain); and papain was from Worthington Biochemical (Lakewood, NJ). SEA0400 (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline) was synthesized in Taisho Pharmaceutical Co., Ltd. (Saitama, Japan). Other chemicals used were of analytical grade from Panreac (Barcelona, Spain). Stock solutions of fura-2-AM and thapsigargin were prepared in DMSO. The solutions were diluted such that the final concentrations of DMSO were ≤1% (v/v), which do not interfere with fura-2 fluorescence.

2.4. Data processing and statistics

To analyze $[Ca^{2+}]_i$ clearance the ratio traces were clipped from the start of the decay and averaged across different experiments for presentation purposes and to fit exponential decay equations (see below). When comparing between different experimental conditions, the raw data were previously normalized following the formula

$$\text{Normalized}_i = \frac{\text{raw}_i - \text{raw}_{\text{previous}}}{\text{raw}_{\text{start}} - \text{raw}_{\text{previous}}},$$

where $\text{raw}_{\text{start}}$ and $\text{raw}_{\text{previous}}$ are respectively the original ratio values at the beginning of the decay and before stimulation with KCl. This procedure allows direct comparison of the