BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **245,** 50–52 (1998)
ARTICLE NO. RC988392

Upregulation of Functional Ryanodine Receptors during *in Vitro* Aging of Human Diploid Fibroblasts

Ming-Shang Huang, Olugbenga Adebanjo, Baljit S. Moonga, Samuel Goldstein, F. Anthony Lai, David A. Lipschitz, and Mone Zaidi

Department of Geriatrics, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205; Center for Osteoporosis and Skeletal Aging, Veterans Affairs Medical Center and Medical College of Pennsylvania–Hahnemann School of Medicine, Allegheny University of the Health Sciences; University of Pennsylvania, Philadelphia, Pennsylvania 19104; and Medical Research Council National Institute for Medical Research, Mill Hill, London NW7 1AA, United Kingdom

Received February 18, 1998

We demonstrate for the first time that cellular aging *in vitro* is accompanied by a dramatic elevation in the levels of ryanodine receptor-bearing Ca^{2+} channels. These channels normally reside within microsomal membranes and gate Ca^{2+} release from intracellular stores. We therefore measured cytosolic Ca^{2+} levels in 'young' (30 mean population doublings, MPDs) and 'senescent' (53 to 58 MPDs) human diploid fibroblasts (HDFs). Application of the known ryanodine receptor modulators, caffeine or cyclic adenosine diphosphate-ribose (cADPr), triggered cytosolic Ca^{2+} signals in both young and senescent cells. The signal magnitude however was significantly greater in senescent compared with young HDFs. In parallel, incubation with a highly specific anti-ryanodine receptor antiserum resulted in specific immunofluorescence only in senescent HDFs. We envisage that elevated levels of functional ryanodine receptors may underlie the defective Ca^{2+} handling and cellular degeneration that occurs with aging.

The release of Ca^{2+} from intracellular stores is critical to the survival, function and propagation of eukaryotic cells. One mechanism of Ca^{2+} release involves the activation of ryanodine receptor-bearing Ca^{2+} channels resident in cellular microsomal membranes (1). There are three known receptor isoforms. The type I receptor is restricted to skeletal muscle and gates voltage-activated Ca^{2+} release. Types II and III are more ubiquitous, although the former primarily mediates Ca^{2+}-induced Ca^{2+} release in cardiac muscle (2, 3). Physiologically, all three receptor isoforms can be activated by Ca^{2+} and cADPr, but not by inositol *tris*phosphate (IP_3) (4).

M-SH and OAA contributed equally to the work.

It is known that intracellular Ca^{2+} release becomes defective during cellular senescence. Agonist-induced Ca^{2+} release is attenuated in certain models of aging cells, including senescent fibroblasts (5), neutrophils (6), lymphocytes (7), and neurons (8). However, in degenerative disorders, such as Alzheimer disease, the harvested cells exhibit a marked enhancement in Ca^{2+} release (9, 10). The resulting elevation in cytosolic Ca^{2+} is thought to contribute to the characteristic degeneration of neurons and supporting cells. In the present study, we have examined changes in ryanodine receptor expression in a unique model of human cellular senescence, the HDF *in vitro* aging model (11 - 13).

MATERIALS AND METHODS

Materials. The Ca^{2+}-sensitive fluorochromes, indo 1, indo 1-AM, fura 2 and fura 2-AM, were purchased from Molecular Probes Inc. (Eugene, OR). Ethylene glycol-*bis*-N,N,N′,N′-tetraacetic acid (EGTA), N′-[2 hydroxyethyl] piperazine-N′-[2-ethanesulphonic acid] (HEPES), caffeine and FITC-labeled goat anti-rabbit antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Tissue culture media were obtained from GIBCO-BRL (St. Louis, MO). Cyclic ADPr was purchased from Calbiochem Corp. (San Diego, CA). The polyclonal antiserum, Ab^{34}, was raised in rabbits against a peptide corresponding to the *consensus* calmodulin-binding epitope on the ryanodine receptor (14). Thus, Ab^{34} does not differentiate between the three known ryanodine receptor isoforms (14).

Fibroblast culture. HDFs were derived from the anterior forearm skin of a normal 9 year-old female, A25 (11). The cells were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with fetal bovine serum (15%, v/v) and maintained in humidified 5% CO_2. A25 cells have a replicative life span of 58 MPDs. 'Young' HDFs were classified as those having a vigorous growth curve, being within the first half of their maximum MPD, i.e., having accrued ≤ 30 MPDs with a ^{3}[H]-thymidine labeling index (TLI) > 92%. 'Senescent' HDFs were late passage cells with a reduced growth potential being positioned within the last 10% of their replicative life span, i.e. having accrued between 53 and 58 MPDs (TLI ≤ 28%) (6, 11). Both young and senescent HDFs were sub-cultured, for between 1 and 3 days

0006-291X/98 $25.00

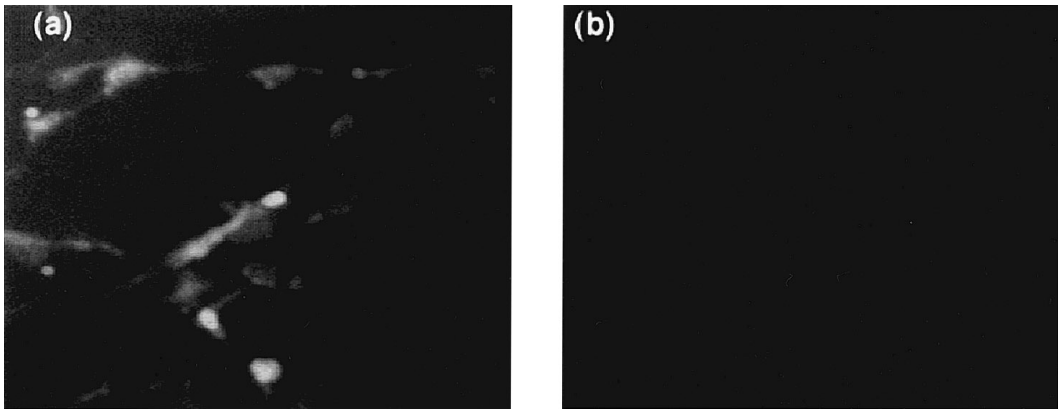

FIG. 1. Immunofluorescence micrographs of senescent (a) and young (b) human diploid fibroblasts (HDFs) staining with an anti-ryanodine receptor antiserum, Ab^{34}, raised to an eptiope corresponding to a *consensus* calmodulin-binding sequence.

before the experiment, on either four chamber slides (for imaging) or 22 mm-glass coverslips (for photometry).

Immunocytochemistry. Coverslips containing HDFs were first fixed with glutaraldehyde (10%, v/v) and permeabilized gently with Triton X-100 (0.1%, v/v, Sigma Chemical Co.). They were then incubated with normal goat serum (diluted in 10 mM-phosphate buffered saline, PBS, 1 in 10, pH - 7.4) in multiwell dishes for 15 minutes. The excess serum was removed and replaced with Hank's Balanced Salt Solution (HBSS) (GIBCO-BRL). The HDFs were then either incubated with non-immune rabbit serum (control) or with the anti-ryanodine receptor antibody, Ab^{34} (both diluted in HBSS, 1 in 100, v/v). After 1 hour of incubation, the coverslips were rinsed gently with HBSS, drained, and re-incubated for a further hour with goat anti-rabbit FITC (1 in 20, diluted in HBSS). Finally, the coverslips were washed gently and drained.

Imaging studies. For dye-loading, HDFs were incubated with 5 μM-indo 1-AM at 37°C for 60 minutes in HBSS containing HEPES (10 mM, HEPES-HBSS) and bovine serum albumin (BSA, recrystallized, 0.1% w/v). The cells were then washed three times with HEPES-HBSS, incubated for a further 30 minutes, and positioned on the stage of either an Interactive Laser Cytometer (ACAS 570, Meridian Instruments Inc., Okemos, MI). Briefly, clusters of HDFs were subjected to excitation by a 5-watt pulsed argon laser at a λ of 357 nm (range, 351 to 363 nm) and the emission was monitored every 30 seconds in the image scanning mode, at 405 and 485 nm. The intensities of the captured fluorescent images at the two wavelengths, I_{405} and I_{485}, were then transformed to yield the ratio, I_{405}/I_{485}. Indo 1 was calibrated using a protocol for intracellular calibration as described earlier (15).

Photometric studies. A fura 2-based, dual excitation method was used to complement the above studies and to make fast kinetic measurements in single cells. HDFs were exposed alternately to excitation λ of 340 and 380 nm, approximately every second, using a filter wheel. The emitted fluorescence was deflected to pass through a dichroic mirror (400 nm) and the transmitted light was filtered at 510 nm. Single photon currents from a distally attached photomultiplier tube (PM28B, Thorn EMI, London, UK) were converted to 5 volt, 25 nanosecond transistor-transistor-logic (TTL) pulses and counted by a photon counter (Newcastle Photometric Systems, Newcastle-upon-Tyne, UK). Photon counts per second were recorded by an IBM microcomputer. The ratio of emitted intensities due to excitation at λs of 340 and 380 nm, I_{340}/I_{380}, was then calculated and displayed as a line tracing.

RESULTS AND DISCUSSION

We have used the HDF *in vitro* aging model to obtain molecular insights into derangements in Ca^{2+} homeostasis that accompany cellular senescence. HDFs have a finite lifespan, and upon passage *in vitro*, undergo replicative senescence. The number of mean population doublings (MPDs) accrued before senescence is found to correlate inversely with donor age. Thus, cells from a given donor can be made to age *in vitro* and harvested at defined MPDs for studies into their biological characteristics (11 - 13).

Our immunocytochemical and pharmacological studies indicate that ryanodine receptor levels are markedly elevated in senescent HDFs. Figure 1 shows that, whilst senescent cells fluoresce intensely with a specific anti-ryanodine receptor antiserum, young HDFs barely show any staining. Likewise, compared with young cells, senescent HDFs display a marked enhancement in cytosolic Ca^{2+} release in response to the application of known ryanodine receptor modulators, caffeine (16) and cADPr (17). Figure 2 shows mean peak cytosolic

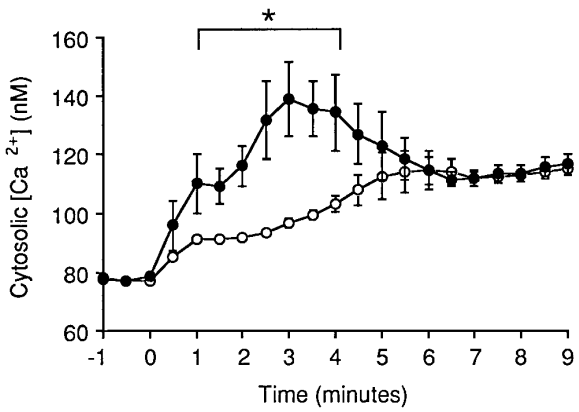

FIG. 2. Mean peak cytosolic Ca^{2+} levels (nM, ± standard error of the mean) of young (open circles) and senescent (closed circles) human diploid fibroblasts (HDFs), measured using an ACAS Cytometer and indo-1, following their exposure to 1 mM-caffeine (at time = 0). Differences were compared using Analysis of Variance with Bonferroni's Correction for Inequality (*$p < 0.01$).

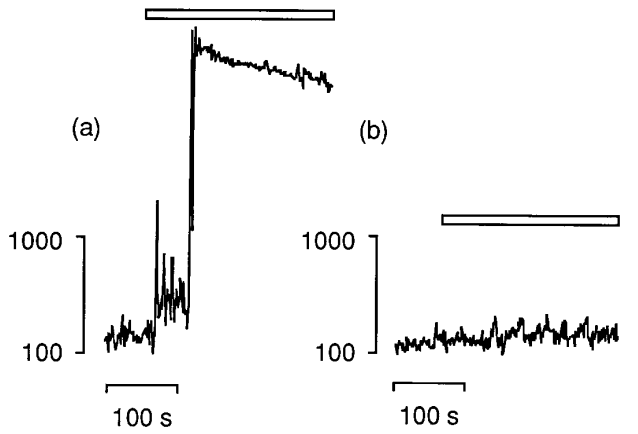

FIG. 3. Fast photometric measurements of cytosolic Ca^{2+} using fura 2 in gently permeabilized senescent (a) and young (b) human diploid fibroblasts before and after cyclic adenosine monophosphate-ribose (cADPr, 10 μM) application (open bars).

Ca^{2+} levels estimated for caffeine-exposed cells using the ACAS Cytometer. Senescent cells were noted to display a gradual rise in cytosolic Ca^{2+} to a peak followed by a plateau. In contrast, young HDFs showed a slower peak rise and significantly ($p < 0.01$) lower cytosolic Ca^{2+} levels, between 1 and 4 minutes following application (Figure 2). In parallel, when exposed to cADPr (10 μM), permeabilized HDFs exhibited fast Ca^{2+} transients. The signal magnitude was however far greater in senescent compared with young cells, the latter sometimes even failed to respond (Figure 3). Note that, non-permeabilized HDFs, whether young or senescent, were insensitive to the extracellular application of cADPr. Note also, that membrane permeabilization *per se* did not result in significant fluorochrome leakage monitored photometrically at F_{380}. Furthermore, HDFs continued to exclude the vital dye, trypan blue, even up to 15 minutes following permeabilization.

Ryanodine receptors are expressed widely in eukaryotic cells (1). By gating Ca^{2+} release, they mediate biological functions as critical as muscle contraction, neurotransmission, cell replication, macromolecule secretion, and hormone action. Thus, a senescence-associated elevation in ryanodine receptor expression could conceivably affect vital cellular processes. This, in turn, could contribute to the defective function and proliferative potential of aging cells (9, 10). Indeed, other molecules, such as calbindin, whose levels also change as a function of aging (18), may contribute similarly. In fact, there might occur compensatory, and perhaps paradoxical, changes in the levels of other Ca^{2+} release channels, such as the IP_3 receptor. This interesting possibility requires further investigation.

ACKNOWLEDGMENTS

MZ acknowledges the National Institutes of Health (NIA, RO1 AG14917-02), Department of Veterans Affairs (Merit Review Award), and Amgen Corp. (San Diego, CA). The authors are grateful to Ms. E. Moerman for culturing HDFs, and to Professors MacIntyre, FRS, Cristofalo, PhD, and Manolagas, MD, PhD, for helpful discussion.

REFERENCES

1. Berridge, M. J. (1993) *Nature* **361,** 315–325.
2. Lai, F. A., Erickson, H. P., Rousseau, E., Liu, Q. Y., and Meissner, G. (1988) *Nature* **331,** 315–319.
3. Gianini, G., Celmenti, E., Cici, R., Marziali, G., and Sorrentino, V. (1992) *Science* **257,** 91–94.
4. Lee, H.-C., Galione, A., Walseth, T. F. (1994) *Vitamins and Hormones* **48,** 199–258.
5. Huang, H. M., Toral-Barza, L., Thaler, T., Tofel-Grehl, B., Gibson, G. E. (1991). *Neurobiol. Aging* **12,** 469–473.
6. Lipschitz, D. A., Udupa, K. B., Indelicato, S. R., Das, M. (1991) *Blood* **78,** 1347–1354.
7. Grossman, A., Ledbetter, J. A., Rabinovitch, P. S. (1990) *J. Gerontol.* **45,** B81–B86.
8. Igwe, O. J., Ning, L. (1993) *Neurosci. Lett.* **164,** 167–170.
9. Eckert, A., Hartmann, H., Forstl, H., Muller, W. E. (1994) *Life Sci.* **55,** 2019–2029.
10. Peterson, C., Goldman, J. E. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2758–2762.
11. Goldstein, S., Moerman, E. J., Baxter, R. C. (1993) *J. Cell. Physiol.* **156,** 294–302.
12. Murano, S., Thweatt, R., Shmookler Riess, R. J., Jones, R. A., Moerman, E. J., Goldstein, S. (1991) *Mol. Cell. Biol.* **11,** 3905–3914.
13. Thweatt, R., Lumpkin, C. K., Goldstein, S. (1992) *Biochem. Biophys. Res. Comm.* **187,** 1–7.
14. Zaidi, M., Shankar, V. S., Tunwell, R., Adebanjo, O. A., Mackrill, J., Pazianas, M., O'Connel, D., Simon, B. J., Rifkin, B. R., Venkitaraman, A. R., Huang, C. L.-H., Lai, F. A. (1995) *J. Clin. Invest.* **96,** 1582–1590.
15. Zaidi, M., Alam, A. S. M. T., Bax, C., Shankar, V. S., Bevis, P. J. R., Huang, C. L.-H., Pazianas, M., and Moonga, B. S. (1994) *in* Methods in Molecular Biology, Vol. 27, Biomembrane Protocols II (Graham, J. M., Higgins, J. A., Eds.), pp. 279–293, Humana Press, Totowa, NJ.
16. Shankar, V. S., Pazianas, M., Huang, C. L.-H., Simon, B., Adebanjo, O., Zaidi, M. (1995) *Am. J. Physiol.* **268,** F447–F454.
17. Adebanjo, O. A., Shankar, V. S., Pazianas, M., Simon, B., Lai, F. A., Huang, C. L.-H., Zaidi, M. (1996) *Am. J. Physiol.* **270,** F469–F475.
18. Ishikawa, Y., Gee, M. V., Ambudkar, I. V., Bodner, L., Baum, B. J., Roth, G. S. (1988) *Bioch. Biophys. Acta.* **968,** 203–210.