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Cytosolic free calcium and cell spreading decrease in fibroblasts from aged and Alzheimer donors

(aging/quin-2/fura-2/actin/immunofluorescence)

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ABSTRACT **Aging and Alzheimer disease lead to alterations in calcium homeostasis. The concentration of cytosolic free calcium in cultured skin fibroblasts during aging and Alzheimer disease was determined with the calcium-sensitive fluorescent dyes quin-2 and fura-2. The Alzheimer donors showed a decline of 70% when compared to age-matched controls ($P < 0.001$) and 81% when compared to cells from young adult donors ($P < 0.001$). This reduction in quin-2–calcium fluorescence does not appear to be due to quenching by heavy metals or alterations in intracellular pH. Similar decreases in free cytosolic calcium were observed with fura-2. In addition, cells from aged and Alzheimer donors spread more slowly than those from young donors, and this deficit can be partially reversed by treatment with the calcium ionophore A23187. These studies agree with accumulating evidence that, at the cellular level, Alzheimer disease is a systemic, as well as cerebral, disease. The precise molecular basis of the decreased cytosolic calcium in fibroblasts is unknown, but there is evidence that it may be pathophysiologically important.**

Alzheimer disease presents as a disorder of the central nervous system, characterized by specific neuropathological abnormalities (1). Consequently, it is common to think of Alzheimer disease as a uniquely neurological disorder and, this being the case, research has concentrated primarily on the brain. The lack of an animal model, the limitations of current *in vivo* neurochemical techniques, as well as ethical considerations, confine much of the research and diagnosis of this disease to the use of postmortem brain. Alterations have been reported in nonneuronal tissues (2–9) from patients with Alzheimer disease, which suggest that the disease is a systemic disorder with the most prominent pathology in the central nervous system. In addition, the appearance of Alzheimer disease-like neuropathology in older (>35 years) Down syndrome patients (10, 11) and the increased risk of first-degree relatives of Alzheimer patients developing this disorder (12) support the possibility of a genetic component of Alzheimer disease (13).

Calcium homeostasis is altered in cultured skin fibroblasts from aged and Alzheimer donors. Peterson *et al.* (8) have found that calcium-45 uptake by fibroblasts declines (−14%) with aging and decreases even further (−43%) in Alzheimer disease, but that total cell calcium increases in cells from aged (+52%) and Alzheimer (+197%) donors when compared to young adult donors (14). These data suggest that levels of free calcium could also be abnormal, so the concentration of cytosolic free calcium ($[Ca^{2+}]_i$) was investigated with the calcium-sensitive fluorescent binding dyes quin-2 (15) and fura-2 (16).

Deficits in calcium metabolism may be important in fibroblast cytoskeletal organization and spreading. Changes in surface morphology and spreading of epithelial cells occur in the presence of decreased extracellular calcium (17) and resemble the effects induced by EGTA or other chemicals that alter cytoskeletal integrity (18, 19). These changes in cell spreading are reversed by the addition of calcium, but they do not appear to be modulated by a calcium/calmodulin interaction since they are not prevented by chlorpromazine treatment (20). Since there are alterations in the calcium metabolism of fibroblasts due to aging or Alzheimer disease, the present study correlated this to changes in cell spreading.

METHODS

Cultured skin fibroblasts from eight young persons (26.2 ± 0.6 years) and eight aged persons (61.1 ± 3.1 years) who were apparently normal were obtained from the National Institute on Aging Cell Repository (Camden, NJ). Eight cell lines from age- (61.0 ± 3.3 years) and sex-matched donors with neuropathologically confirmed Alzheimer disease or with several first-degree relatives who had confirmed Alzheimer disease were used in these studies. All Alzheimer donors had classical symptoms of global impairment. In two patients (cell lines AG4401 and AG5770) Alzheimer disease was confirmed neuropathologically. Donors of other cell lines (AG0364a, AG5809, AG5810, AG4402, AG4400, AG6264) had at least two parents or siblings with confirmed Alzheimer disease. The eight lines represent patients from six different families.

Fibroblasts that were matched for early passage number (9.1 ± 0.6) were cultured in antibiotic-free Dulbecco's modified Eagle's medium with 15.6% (vol/vol) heat-inactivated fetal bovine serum as described previously (8, 14). All cell lines were coded before plating and the studies were carried out in a double-blind manner. Three days after the cells had been seeded in coverslip-bottomed tissue culture dishes (1000 cells per 35-mm dish), a serum-free replacement medium was substituted. On day 4, cytosolic calcium in fibroblasts from the donors was estimated with the fluorescent dyes. Day 4 represents a preconfluent stage, with all cell lines normally reaching confluence by day 7 (14).

Cells were incubated with 20 μM acetoxymethyl ester of quin-2 (2-{2-[bis(carboxymethyl)amino-5-methylphenoxy]-methyl}-6-methoxy-8-bis(carboxymethyl)aminoquinoline; Lancaster Synthesis, Eastgate, England) in 20 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.4/150 mM NaCl/1 mM $CaCl_2$/10 mM glucose/5 mM KCl for 45 min at 37°C (21). After loading with quin-2, cells were washed twice with fresh phenol red-free medium before quin-2–calcium fluorescence was quantitated. In experiments with fura-2, cells were incubated for 5 min in the presence of 100 μM fura-2 pentapotassium salt (Molecular Probes, Eugene, OR) in a permeabilization buffer [137 mM NaCl/2.7 mM KCl/20 mM Hepes, pH 7.6/5.6 mM glucose/3

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μM ATP (22)]. To halt dye uptake 1 mM $MgCl_2$ was added; this closes the ATP-induced channels (22). The cells were then washed twice and allowed to recover for 1 hr at 37°C in antibiotic- and serum-free Dulbecco's medium before analysis.

Emission of quin-2 or fura-2 in response to excitation at 340 nm is highly calcium dependent. Excitation of quin-2 at 360 nm is calcium independent (15), while that of fura-2 at 380 nm (16) is inversely dependent on the calcium concentration. Thus the ratio of these two intensities (I_{340}/I_{360} or I_{340}/I_{380}) depends upon ionized calcium and not distribution of the probes resulting from changes in cell thickness or loading efficiencies. Ratios of I_{340}/I_{360} or I_{340}/I_{380} were converted to calcium concentrations by use of quin-2 and fura-2 calibration curves. The dyes were calibrated in buffers designed to approximate the cells' intracellular environment. Microviscosity and protein binding could alter the response of the dyes to calcium (16), but calcium concentrations calculated from I_{340}/I_{380} ratios in intact smooth muscle cells or solutions are similar (23). The fluorescence intensities were recorded with a Leitz microscope photometer with a quartz epi-illuminator and a Nikon UV-Fluor objective as previously described (21, 24, 25). Cells were maintained at 37°C during all determinations. Probe-calcium fluorescence was measured in a minimum of two separate cultures per cell line, three cells per dish, and the value for each cell was determined in duplicate. Experiments with fura-2 were done with only four cell lines from each group. Results are presented as mean ± SEM. Fluorescent probe–calcium binding was 10 times autofluorescence.

To study cell spreading, cells from young, aged, and Alzheimer donors were seeded onto 13-mm coverslips (1000 cells per coverslip) in 24-well plates. Two hours after plating, duplicate coverslips from each group were removed, rinsed twice with phosphate-buffered saline, and fixed with 10% buffered Formalin for 30 min. The cells were rinsed and permeabilized with 1% Nonidet P-40 (30 min), and filamentous actin was stained by rhodamine-labeled phalloidin (30 min at 37°C). The fluorescent cells were examined with an Olympus BH2 microscope equipped with epifluorescent optics and a rhodamine filter. Four hundred cells per coverslip were counted, and those cells that were rectangular were considered spread and are reported as a percent of the total number of cells.

RESULTS

Cytosolic free calcium, as measured with quin-2, declined due to aging and even further due to Alzheimer disease (Fig. 1). The apparent concentration decreased from 54.8 ± 2.1 nM (young) to 36.3 ± 2.8 nM (aged) to 12.6 ± 2.0 nM (Alzheimer). When the cells were loaded with fura-2 by ATP permeabilization, the values for cytosolic calcium were quantitatively similar to those with quin-2 (young, 63.3 ± 2.8 nM; aged, 37.0 ± 1.3 nM; Alzheimer, 15.3 ± 2.8 nM). Values differed significantly ($P < 0.001$) by analysis of variance with the least significant difference test (26). In spite of the fact that the fibroblasts from Alzheimer donors came from eight individuals in six unrelated families, the cytosolic free calcium values were tightly clustered and did not overlap with age-matched controls.

The values observed with quin-2 were not due to endogenous heavy metal binding. There were no changes in quin-2 fluorescence after an additional incubation for 15 min at 37°C with 20 μM TPEN [*N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (27, 28)], a lipid-soluble chelating agent.

Decreased cytosolic calcium was not due to changes in intracellular pH. Fibroblasts were incubated at 37°C with 20 μM (5 and 6)-carboxy-4,5-dimethylfluorescein diacetate or 20 μM (5 and 6)-carboxyfluorescein diacetate (Molecular Probes) in 20 mM Hepes buffer, pH 7.4, for 10 min, washed twice, and then allowed to recover for 15 min. There were no differences in intracellular pH among the groups.

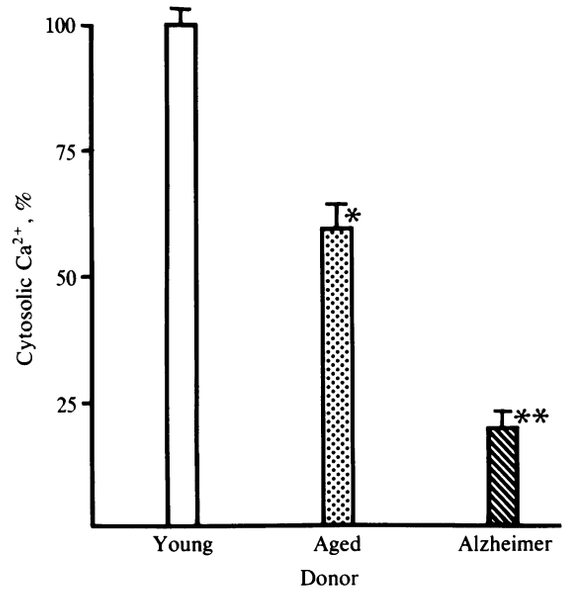

FIG. 1. Decreased cytosolic calcium during aging and Alzheimer disease. Cytosolic free calcium was measured in cell lines from young, aged, and Alzheimer donors with the calcium-sensitive fluorescent dye quin-2. Results are presented as percent of values for young donors.
*Values differ from young ($P < 0.001$).
**Values differ from young and aged ($P < 0.001$).

Cells from aged and Alzheimer donors spread more slowly than those from young individuals (Fig. 2). After 2 hr, 79 ± 1% of the fibroblasts from young donors were spread, while cells from aged and Alzheimer donors were spread only 54 ± 1% or 39 ± 1%, respectively. If after cell attachment, the medium was replaced by one that also contained 0.5 μM calcium ionophore A23187 (Calbiochem–Behring), the percentage of spread cells from aged (75 ± 4%) and Alzheimer (72 ± 2%) donors increased. Treatment with 0.5 mM EGTA, pH 7.4, detached the cells in all groups similarly. Plating cells from aged and Alzheimer donors at a higher density did not increase the rate of cell spreading.

DISCUSSION

The concentration of cytosolic free calcium in cells from young donors is quite similar to that reported for interphase PtK2 rat kangaroo kidney epithelial cells (21). The values for the fibroblasts from Alzheimer donors, however, are less than half the lowest free calcium estimated during any phase of the PtK2 cell cycle. The free calcium level of 12 nM is probably insufficient to activate calmodulin, protein kinase C, or other calcium-dependent systems optimally (for review, see ref. 29). This could lead to hyperstability of the cytoskeleton (30), receptor hyposensitivity (31), and defects in other aspects of cell metabolism (14). The decline in intracellular calcium may underlie the decreases in cell spreading (present study), since treatment with A23187, which causes a transient increase in cytosolic free calcium in neutrophils (32), reverses the deficit.

The effects of chronically low cytosolic calcium levels may be especially detrimental in neurons, which are rich in calcium-dependent structural and secretory processes. Formation of paired helical filaments (33, 34) and neurofibrillary tangles (35) may be related to central nervous system alterations in calcium homeostasis. The neuropathological findings of Alzheimer dis-

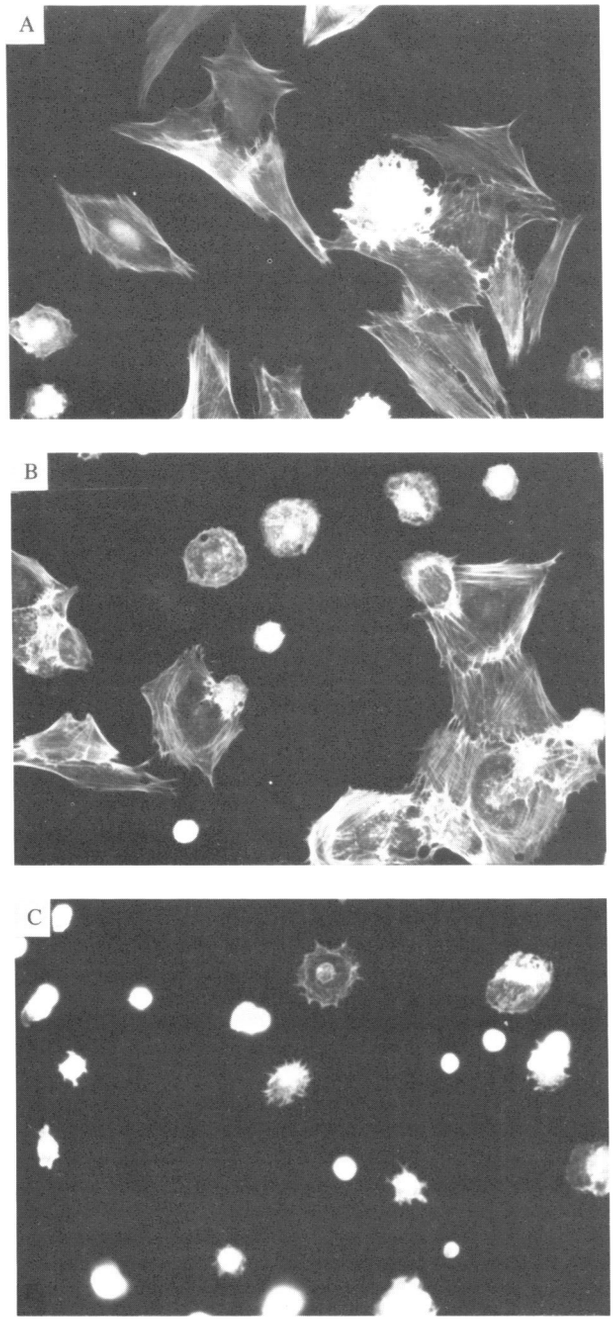

FIG. 2. Spreading of fibroblasts from young (*A*), aged (*B*), and Alzheimer (*C*) donors. Two hours after plating, cells were stained with rhodamine-phalloidin to visualize filamentous actin. Cells that were rectangular were considered fully spread, although the cells from Alzheimer donors were slower to spread radially as well. (×80.)

ease would suggest that neurons are more susceptible to altered calcium metabolism than are cultured skin fibroblasts. Whether the low calcium levels are primary or secondary to other metabolic problems is unresolved.

Fibroblasts from aged and Alzheimer donors demonstrate alterations in calcium homeostasis, but the molecular basis for these abnormalities is unknown. Increased intracellular binding (14), coupled with the decreased calcium uptake (8), could produce low free calcium levels, for example. These studies now require extension to other cell types and other neurological disorders. The observation of decreased cytosolic calcium could prove diagnostically valuable if it holds true in larger populations. Investigation of the pathophysiological consequences of low cytosolic calcium is also a high priority.

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