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Cytosolic free [Ca^{2+}] in mononuclear blood cells from demented patients and healthy controls

Brigitta Bondy, Ute Klages, Franz Müller-Spahn, Christoph Hock

Psychiatric Clinic, Department of Neurochemistry, University of Munich, D-80336 Munich, Germany

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Summary. There is increasing evidence that the neurodegenerative processes in Alzheimer's disease (AD) may be related to alterations in calcium homeostasis and that these metabolic changes are not necessarily restricted to the central nervous system. However, previous studies investigating $[Ca^{2+}]_i$ in fibroblasts, lymphoblasts, platelets and lymphocytes of AD patients gave inconclusive results, since increase, decrease and no alteration in $[Ca^{2+}]_i$ were found in AD patients compared with controls. With respect to the importance of establishing altered Ca^{2+} homeostasis in peripheral cells, we have investigated $[Ca^{2+}]_i$ in circulating mononuclear cells of patients with AD, multi-infarct dementia, age-associated memory impairment and healthy controls. $[Ca^{2+}]_i$ was evaluated using the fluorescent dye fura-2 before and during stimulation with phythaemagglutinin (PHA). In our study we failed to find major differences in resting $[Ca^{2+}]_i$ and in response to stimulation with 25μg/ml and 100 μg/ml PHA in cells of AD patients as compared with all other groups investigated. There was only a tendency towards a decrease in $[Ca^{2+}]_i$ in AD after stimulation with PHA. Thus the present findings suggest that $[Ca^{2+}]_i$ evaluation in mononuclear cells does not have diagnostic value in discriminating AD patients from other demented patients. However, there might be some difference in $[Ca^{2+}]_i$ values between early- and late-onset AD, which could have pathophysiological importance.

Key words: Alzheimer's Disease – Lymphocytes – Calcium – Phytohaemagglutinin

Introduction

Recent effort in the biochemical characterisation of Alzheimer's disease (AD) has focused on calcium dependent cellular processes, suggesting that abnormalities in Ca^{2+} homeostasis might be involved in the pathophysiology of this disease (Gibson and Peterson 1987; Rizopoulos et al. 1988). Since free calcium ions act as an important messenger system and are crucially involved in many cellular functions (for review, see Verity 1992), alterations of calcium homeostasis and the resulting changes in free Ca^{2+} concentration could be an important link in the chain that leads to dysfunction and degeneration of the neurons during aging and in neurodegenerative disorders (Heizman and Braun 1992). This hypothesis was among others supported by the findings of a post-mortem study, where increased Ca^{2+} concentrations were found in neurons containing neurofibrillary tangles (Perl et al. 1982). In-vitro studies have further proposed the possible involvement of calcium and inositol phospholipid signalling in neurofibrillary degeneration (Mattson et al. 1991).

Based on recent findings that the cellular events in AD are not necessarily restricted to the central nervous system, peripheral tissues should have similar metabolic changes and would thus be an attractive model (Bosman et al. 1991). A number of studies have shown alterations in calcium homeostasis of aged donors and AD patients. Using cultured fibroblasts, a decreased calcium uptake and increased superficial binding was demonstrated in AD patients compared with young controls (Peterson et al. 1989). These authors have further shown that the resting concentration of cytosolic calcium was decreased in fibroblasts from aged donors and even more in AD patients. Furthermore, the ability of drugs, known to increase transiently intracellular calcium, was reduced in fibroblasts from AD patients (Peterson et al. 1988). These findings have tremendous implications not only on pathophysiology but also in the differential diagnosis of AD. However, other groups could not replicate the results. Neither the resting nor the stimulated values in fibroblasts were found to be markedly different from controls (Borden et al. 1992; Huang et al. 1991). Also, in cultured lymphoblasts no difference in $[Ca^{2+}]_i$ was observed between young, aged and AD donors (Gibson and Toral-Barza 1992). In contrast, an increase in $[Ca^{2+}]_i$ after stimulation was observed in platelets (Hajimohammadreza et al., 1990) and in circulating lymphocytes (Adunsky et al., 1991) of AD patients compared with controls, and in the latter study even compared with patients with multi-infarct dementia (MID).

Correspondence to: B. Bondy

With respect to the importance of establishing altered Ca^{2+} homeostasis in peripheral cells of AD patients, and since most of these studies have been carried out with a relatively small number of patients and have to be replicated, we have investigated the $[Ca^{2+}]_i$ concentration in resting and PHA stimulated mononuclear cells (MNC) of AD patients and a control group.

Subjects and methods

Subjects

24 demented patients were included in the study. 18 of them had been diagnosed as probable AD, based on NINCDS-ADRDA and DSM-III-R criteria (McKhann et al. 1984). 10 of these patients were females, 8 were males. The age of AD patients ranged from 49 to 83 years. 7 of them could be classified as "early onset" (from 49 up to 65 years), 11 as "late onset" Alzheimer (from 65 years onwards). These patients were hospitalised for diagnostic reasons. The duration of disease ranged between 3 and 6 years. The severity of the disorder was mild to moderate (16–25 points in Mini Mental State Score; MMS). The patients received different medications none of which are known to interfere with Ca^{2+}. None of the patients suffered from clinically significant organic disorders. Also included were 3 patients with age-associated memory impairment (AAMI; Crook et al. 1986) and 3 with multi infarct dementia (MID) (all females), MID, age: 81 ± 5, MMS: 20 ± 4. AAMI, age: 74 ± 9, MMS: 27. Four non-demented patients with major depression, mean age 67 ± 19 years, 3 females and 1 male, were further investigated as a psychiatric control group.

Thirty-five healthy persons (20 females, 15 males), receiving no medication, were investigated as a non-affected control group. Eight of them were aged from 21 to 30 years, 16 from 31 to 60 years and 10 from 62 to 87 years. In 15 of the healthy controls (aged from 21 to 70 years) the level of $[Ca^{2+}]_i$ was investigated twice with several weeks interval in order to assess the intraindividual variation over time.

Laboratory methods

Each donor gave 25 ml of blood by venipuncture using sodium citric acid (V/V 1/9) as anticoagulant. Mononuclear cells (MNC) were isolated using Ficoll-Paque (Biochrom) and standard procedures. In general we have strictly followed the laboratory procedure of Adunsky et al. (1991), except that we used Fura-2 as fluorescent dye instead of quin-2. After MNC preparation the cells were resuspended in 1 ml PBS buffer (137 m*M* NaCl, 2.7 m*M* KCl, 1 m*M* $MgCl_2$, 3.3 m*M* NaH_2PO_4, 20 m*M* HEPES, 5.6 m*M* glucose, 1 g/l bovine serum albumin, pH 7.4 with Tris) and loaded with Fura-2 AM (25 μ*M*) for 20 min at 37°C, then further diluted tenfold with fresh PSS buffer and incubated for another 30 min at 37°C. Autofluorescence was determined in parallel samples incubated with DMSO instead of Fura-2. To remove external dye, the samples were washed twice at 300 × *g* for 10 min and resuspended in fresh PSS buffer to yield a final cell concentration of 1×10^6 cells/ml. 1 m*M* $CaCl_2$ was added and the cells further incubated for 30 min at 37°C. $[Ca^{2+}]_i$ was determined using a luminiscence spectrometer (Perkin-Elmer LS-50B) before and during stimulation with phytohaemagglutinin (PHA-M; Sigma Biochemicals) in two different concentrations, 25 μg/ml and 100 μg/ml at 37°C for a period of 10 min. Digitonin (50 m*M*) and EGTA (10 m*M*) were used to obtain R_{max} and R_{min}.

Data analysis

Results of the $[Ca^{2+}]_i$ determination were compared for statistical significance ($P > 0.05$) using two-tailed Student's *t*-test.

Results

$[Ca^{2+}]_i$ in MNC of healthy controls

Resting and stimulated levels of $[Ca^{2+}]_i$ were determined in MNC of 35 healthy controls, aged from 21 to 87 years. All of them had been virtually drug free and without any history of hypertension. The mean value for resting $[Ca^{2+}]_i$ in the control group, unrelated to age, was 88 ± 17 nM. Stimulation with 25 μg/ml and 100 μg/ml PHA increased $[Ca^{2+}]_i$ to peak values of 211 ± 44 n*M* and 218 ± 48 respectively. In many controls, the stimulated values with 100 μg/ml PHA were equal or lower than those obtained with 25 μg/ml. This demonstrates, that saturation of the stimulatory influence was reached with a PHA concentration of 25 μg/ml.

Figure 1 shows the resting and stimulated $[Ca^{2+}]_i$ values (with 25 μg/ml PHA) in relation to age. Although there seems to be a tendency towards a decline in resting levels with increasing age (Fig. 1A), this does not reach statistical significance. After stimulation with 25 μg/ml PHA, both the total $[Ca^{2+}]_i$ values and the net increase above resting values do not show any relation to age (Fig. 1B).

We further have evaluated the intraindividual variation over time and reinvestigated 15 of our healthy controls (in the age from 21 to 70 years) after several weeks interval (Fig. 2). In our experiments the resting values appeared to be more stable than the increase in $[Ca^{2+}]_i$ after stimulation with PHA 25 μg/ml. The variation of these stimulated values was in the range from 5% to 40%, in one single case even more.

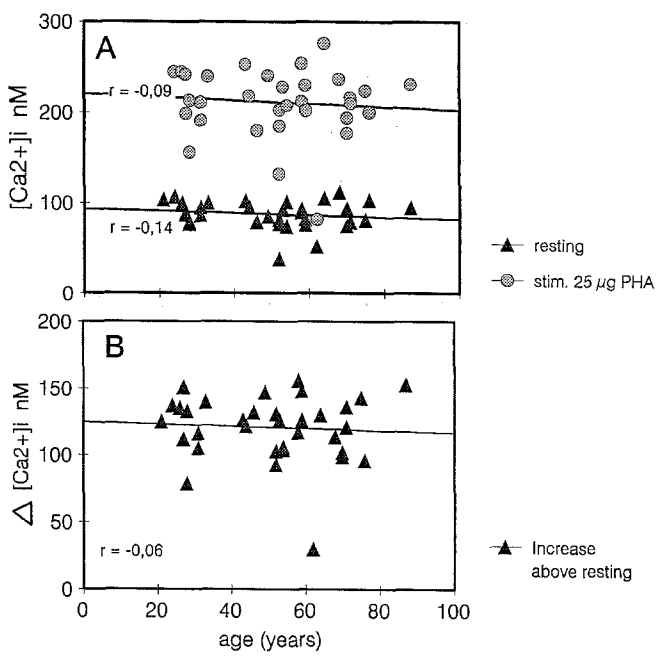

Fig. 1A and B. Correlation (individual data points and regression lines) between age and $[Ca^{2+}]_i$ (n*M*) in mononuclear cells of 35 healthy controls (from 21 to 87 years). Experiments were carried out as described in Methods. **A** represents values of resting cells and after stimulation with 25 μg/ml PHA; **B** shows the Delta $[Ca^{2+}]_i$ levels

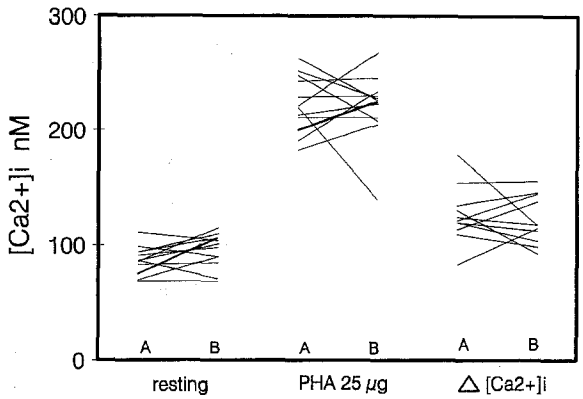

Fig. 2A and B. Intraindividual variation of $[Ca^{2+}]_i$ in mononuclear cells in 15 healthy volunteers. **A** = first determination; **B** = second determination after several weeks interval. The first and second value of each person are connected by line. $[Ca^{2+}]_i$ was determined before and after stimulation with 25 µg/ml PHA

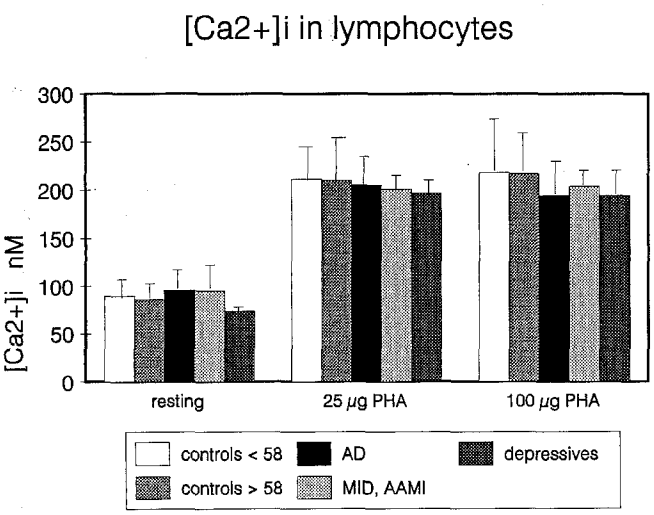

Fig. 3. Mean values (± SD) of $[Ca^{2+}]_i$ in mononuclear cells of healthy controls < 58 years (n = 20); healthy controls > 58 years (n = 15); AD patients (n = 18); MID and AAMI patients (n = 6), major depressives (n = 4). Resting and stimulated values after 25 µg/ml and 100 µg/ml PHA

$[Ca^{2+}]_i$ in MNC of patients

Investigated were 18 AD patients and the data compared with healthy controls, 6 non-DAT patients (AAMI and MID) with slight or moderate dementia and 4 patients with major depression. The results for all groups are demonstrated in Fig. 3. The healthy controls were split into two groups, one comprising those below 58 years (n = 20) and the other with those above 58 years (n = 15). The reason for that uncommon group distinction was the fact that the age of our AD patient group started with 58 years, with one exception of a male patient being 49 years old. We could not find any significant difference between the resting $[Ca^{2+}]_i$ values for young and old controls, AD patients, MID, AAMI patients and depressives. There was only a tendency towards higher resting levels in AD patients. The lower resting $[Ca^{2+}]_i$ levels of the depressive patients could become significant, but one has to keep in mind that this group comprises only 4 patients. After stimulation with 25 µg/ml PHA no significant difference was observed amongst all groups. Stimulation with 100 µg/ml PHA yielded a slight decrease in the $[Ca^{2+}]_i$ values of AD patients as compared to healthy, old and young controls. This decreased responsiveness reached statistical significance on a low level (P = 0.05). The $[Ca^{2+}]_i$ stimulated above resting values are shown in Table 1, again demonstrating a net, slightly reduced stimulation effect in AD patients using 100 µg/ml PHA.

Table 1. $[Ca^{2+}]_i$ (mean ± SD) stimulated over resting values in AD patients and controls

Subjects	Delta $[Ca^{2+}]_i$ (nM)	
	25 µg/ml PHA	100 µg PHA
Controls < 58 (n = 20)	121 ± 18	130 ± 40
Controls > 58 (n = 15)	119 ± 32	119 ± 20
AD (n = 18)	108 ± 21	101 ± 30*
MID, AAMI (n = 6)	105 ± 19	120 ± 15
Depressives (n = 4)	120 ± 16	120 ± 16

*P = 0.05

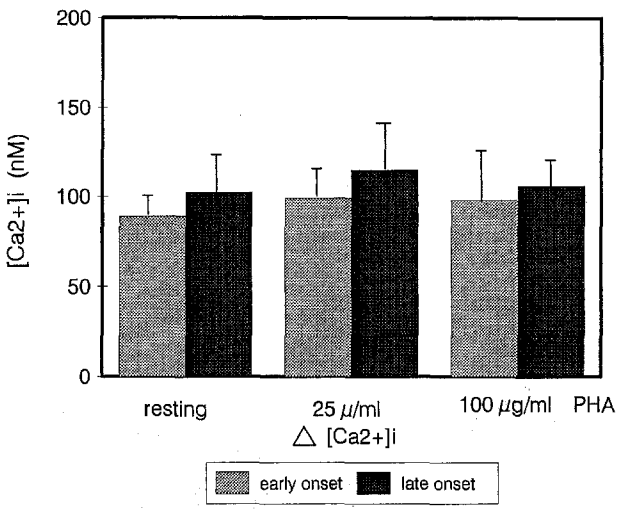

Fig. 4. Mean values (± SD) of resting $[Ca^{2+}]_i$ and Delta $[Ca^{2+}]_i$ in mononuclear cells after stimulation with 25 µg/ml and 100 µg/ml PHA in early-onset (n = 7) and late-onset AD patients (n = 11)

Our AD patient group comprised a wide range of age from 49 to 83 years. Seven of these patients have been early-onset patients (below the age of 65 years), 11 were late-onset patients (older than 65 years). Comparing the values of these two patient groups, there might be some difference with lower resting and stimulated $[Ca^{2+}]_i$ values in the early-onset patient group (Fig. 4), which was however not significant. The computer printout of the experiments of early-onset AD patients and age matched controls demonstrates the kinetics of PHA (25µg/ml) stimulation in MNC of both groups (Fig. 5). The increase in $[Ca^{2+}]_i$ began rapidly, and reached a plateau within about 100 s, and then declined only slightly or kept on a constant level over 10 min. There could be a possible difference not only in the increase of $[Ca^{2+}]_i$ with lower lev-

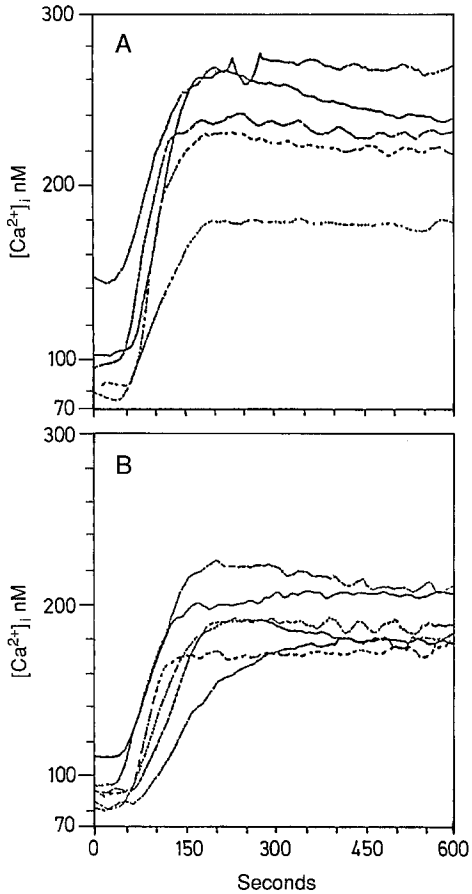

Fig. 5A and B. Computer printout of the kinetics of PHA stimulation (25 μg/ml) of mononuclear cells of early-onset AD patients (**B**) and age-matched controls (**A**). PHA was added after 30 s, the kinetics of stimulation was followed for 10 min

els for early onset AD patients, but maybe even in the shape, with a tendency towards a slight delay in the response to PHA.

Discussion

Investigating Ca^{2+} homeostasis in peripheral cells would offer an attractive model for functional biochemical investigations in AD. Since the development of fluorescent dyes that easily allow determination of cytosolic-free $[Ca^{2+}]_i$ (Tsien et al. 1982), several studies have been carried out on this subject with very different results. Decreased (Peterson et al. 1989) and increased (Adunsky et al. 1991) $[Ca^{2+}]_i$ was observed in different cells, and both findings were explained with respect to the pathophysiology of the disease. However, not all groups could replicate these findings (Borden et al. 1992; Huang et al. 1991), thus leaving many questions on possible differences in peripheral cells, different selection of patients, influence of drugs and methodology of the assay.

Our present study with circulating MNC contributes to these discrepancies, since we could not find overt differences in $[Ca^{2+}]_i$ levels between young and aged donors, AD patients, moderate or severely demented non AD patients and depressives. There was only a tendency towards a slight decrease in $[Ca^{2+}]_i$ increase after stimulating the cells with 25 μg/ml or 100 μg/ml PHA. Thus, these results sharply disagree with a previous study, also carried out with circulating MNC, where a tremendous increase in $[Ca^{2+}]_i$ levels was found after stimulation with 100 μg/ml PHA (Adunsky et al. 1991). Besides the possible pathophysiological significance of this finding, it additionally became important because of the clear separation of aged donors, AD and MID patients, thus having even marker qualities. It is presently not clear why we could not replicate the findings of Adunsky et al. (1991), since we have attempted to perform the experiments under similar conditions to those used by these authors with one single exception that we used fura-2 instead of quin-2. This, however, should not account for the discrepancies, since it was demonstrated that the results obtained with both fluorescent dyes are almost identical (Peterson et al. 1989). Discrepancies in age of the AD patients of both studies could also not be the reason, since the mean age of our AD patients was very similar in both studies (69 ± 9 versus 73 ± 1.5 years). There was, however, one serious difference, since our AD patients have been treated with several cardiovascular active or sedative drugs (neuroleptics, prothipendyl, melperone), but the patients of Adunsky had been drug-free for 4 weeks prior to the evaluation of $[Ca^{2+}]_i$. The possible influence of these drug effects has to be established.

Peterson and colleagues (1989), reported a marked decrease in resting $[Ca^{2+}]_i$ levels in cultured fibroblasts from AD patients. Although we have used MNC instead of fibroblasts, one could assume similar results for both cell types, since alterations in calcium homeostasis, if any, should then be found in the majority of peripheral cells. We did observe some decrease in the stimulated $[Ca^{2+}]_i$ values; however, this is far from being highly significant ($P = 0.05$) and far from the large difference observed earlier (Peterson et al. 1989), where not even an overlap occurred between aged donors and AD patients. In addition, since the intraindividual difference in the response to PHA stimulation is rather high (up to > 40%; Fig. 2), differences between AD patients and controls should be above that level for giving a meaningful statistical significance.

In our opinion the selection of patients could be a crucial point for the discrepant results. We have observed a tendency towards a decreased PHA stimulation of $[Ca^{2+}]_i$ in the early-onset AD patients as compared to age matched controls and late-onset AD patients. However, both our groups were not big enough for being able to distinguish between them. If early- and late-onset AD patients are mixed up to one single group as has been done in most studies (Peterson et al. 1988; Huang et al. 1991; Adunsky et. al. 1991), then differences might be hidden in the mean values. Thus, in our opinion several aspects such as methodology, selection of patients and drug effects should be considered very carefully, before final conclusions can be drawn concerning the evaluation of calcium homeostasis in peripheral cells. Furthermore, one should not assume that only such dramatic alterations, as observed by Peterson et al. (1989) and Adunsky et al. (1991) are relevant for the pathophysiology of AD. On the con-

trary, since calcium homeostasis is tightly regulated by many cellular events, even small alterations in this homeostasis could have tremendous effects on cellular function.

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