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β-Amyloid protein enhances the mitogen-induced calcium response in circulating human lymphocytes

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The role of β-amyloid in Alzheimer's disease and its cellular mechanism of action on neurons are still unclear. There is growing evidence that β-amyloid or its fragment, 25–35, influence neuronal calcium regulation. To investigate the effects of β-amyloid on calcium homeostasis in man we used peripheral human lymphocytes as a model system for central neurons. β-Amyloid fragment 25–35 exposed to lymphocytes for 60 s elevates the phytohemagglutinin (PHA)-induced Ca^{2+} rise in a dose-dependent manner. Small effects were already seen at concentrations as low as 50 nmol/l. Similar effects were also observed with fragment 1–40, whereas fragments 1–28 or 12–28 did not affect the Ca^{2+} response after PHA stimulation. Our findings support the hypothesis of an enhanced calcium response as a general feature of β-amyloid's neurotoxicity The lymphocyte seems to be a valuable model to study this effect in man.

β-Amyloid; Human lymphocyte; $[Ca^{2+}]_i$

1. INTRODUCTION

The formation of senile plaques, mainly consisting of β-amyloid protein, represents one of the characteristic features of the pathology of Alzheimer's disease (AD). β-Amyloid is derived from the larger β-amyloid precursor protein (βAPP) and seems to be one of the causative factors in AD [1]. β-Amyloid, and its biologically active fragment, 25–35, have been shown to possess neurotrophic or neurotoxic activity depending on the concentration applied [2]. At neurotoxic concentrations, β-amyloid renders neurons more vulnerable to stimuli which elevate intracellular calcium [3]. Accumulating evidence supports the hypothesis that altered regulation of intracellular free calcium concentration ($[Ca^{2+}]_i$) may play a role in neurodegeneration and in neuronal damage [3,4]. Thus, β-amyloid-mediated neurotoxicity might be due to a direct or indirect disturbance of intracellular Ca^{2+} homeostasis. So far, effects of β-amyloid on neuronal Ca^{2+} regulation have only be demonstrated in neuronal cells in tissue culture including embryonic neurons from human brain [3,5].

It is widely accepted that $[Ca^{2+}]_i$ also plays a key role in the signal transduction system of human peripheral lymphocytes. Accordingly, human lymphocytes have already been used to investigate effects of aging or AD on calcium homeostasis. While similar age-related changes of intracellular Ca^{2+} regulation were observed in lymphocytes [6,7] as in central neurons [4,8,9], studies with lymphocytes from AD patients have only demonstrated minor additional changes [7,10]. Similar to the situation in central neurons, activation of lymphocytes (e.g. by mitogens) leads to the secretion of βAPP [11]. In the present study, we report the use of freshly prepared human lymphocytes to study the destabilizing effect of β-amyloid on intracellular Ca^{2+} regulation in individual humans.

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Abbreviations. AD, Alzheimer's disease; βAPP, β-amyloid precursor protein; βA25–35, β-amyloid fragment 25–35; $[Ca^{2+}]_i$, intracellular free calcium concentration; EGTA, ethylene glycol-bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; PHA, phytohemagglutinin.

2. MATERIALS AND METHODS

2.1 *Subjects*

Fresh blood was obtained from healthy young donors (male or female) aged between 20–30 years (mean age = 27). None took any medication except one taking an oral contraceptive.

2.2. *Lymphocyte preparation*

Mononuclear cells (>95% lymphocytes) were isolated from heparinized blood by centrifugation on Ficoll-Hypaque (Lymphoprep; Immuno Heidelberg, Germany) according to the method of Boyum [12]. The lymphocyte suspension (10^7 cells/ml) was loaded with Fura 2-AM (3 μmol/l) (Molecular Probes, USA) for 40 min at 37°C. After washing out external dye with Hank's balanced salt solution (6 mmol/l glucose, 1 mmol/l $MgSO_4$, 5 mmol/l KCl, 137 mmol/l NaCl, 0.3 mmol/l Na_2HPO_4, 1 mmol/l $CaCl_2$, 10 mmol/l HEPES), lymphocyte suspension was adjusted to 2.5×10^6 cells/ml.

2.3. *$[Ca^{2+}]_i$ measurement*

Before fluorescence measuring in a SLM Aminco 4800 spectrofluorometer, samples of 1 ml were equilibrated in a cuvette at 37°C for 3 min under magnetic stirring. Lymphocytes were stimulated with phytohemagglutinin (PHA) at the appropriate concentration. To test the effects of β-amyloid fragments (Sigma, München, Germany) on basal and PHA-induced $[Ca^{2+}]_i$, aqueous stock solutions of the peptides were prepared and stored in aliquots at −20°C. The aliquots were

freshly diluted in water to yield the corresponding concentrations (0.05–5 μmol/l). The solutions were added into the cuvette in a volume of 10 μl. After preincubating (60 s) the cell suspension with the β-amyloid fragments, cells were stimulated with PHA (5–20 μg/ml). $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. [13].

2.4. *Statistics*

Differences were assessed by paired *t*-test or by analysis of variance (ANOVA) (SAS-Institute, Cary, NC).

3. RESULTS

After PHA stimulation (15 μg/ml) the maximal increase of $[Ca^{2+}]_i$ in human lymphocytes was reached within 45–60 s (Fig. 1). Preincubation of the cells (60 s) with β-amyloid fragment 25–35 (βA25–35, 1 μmol/l), which seems to represent the neurotoxic sequence of β-amyloid [2], resulted in an amplification of the PHA-induced Ca^{2+} response (Fig. 1). Comparison of the time-courses of $[Ca^{2+}]_i$ in control lymphocytes and lymphocytes pretreated with βA25–35 suggests that the $[Ca^{2+}]_i$-enhancing effect of βA25–35 is more pronounced in the plateau phase than during the initial increase (Fig. 1). Since the plateau phase of the mitogen-induced elevation of $[Ca^{2+}]_i$ is mainly dependent on Ca^{2+} influx, we investigated the effect of extracellular Ca^{2+} on the β-amyloid effect. In the absence of free Ca^{2+} in the extracellular space, the mitogen response was reduced to about 30%. Moreover, the amyloid effect disappeared after chelating extracellular Ca^{2+} with EGTA (1.5 mmol/l) (Fig. 2).

The Ca^{2+} increasing effect is dependent on the β-amyloid concentration (Fig. 3). Interestingly, significant amplification of the Ca^{2+} response is already seen at concentrations as low as 50 nmol/l) $n = 4$, $P < 0.01$). At a final concentration of 1 μmol/l, βA25–35 enhanced the

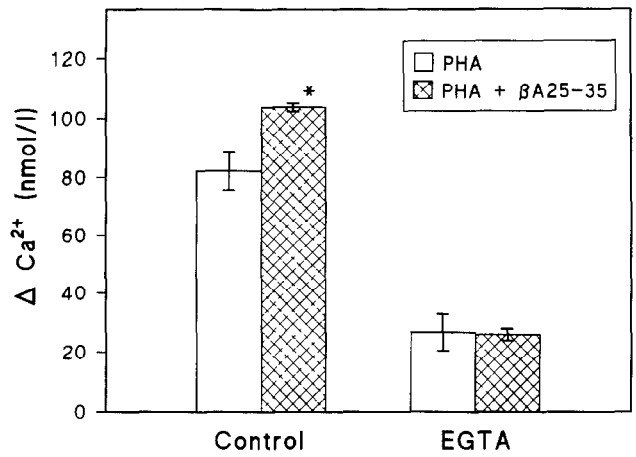

Fig. 2. Effect of βA25–35 on the PHA-induced Ca^{2+} response in lymphocytes in control buffer containing Ca^{2+} (1 mmol/l) and Ca^{2+}-free buffer (EGTA, 1.5 mmol/l). Values are means ± S.D. ($n = 3$). $^*P < 0.05$

PHA-induced Ca^{2+} increase (Δ Ca^{2+}) in six volunteers (3 male, 3 female) with an average increase over control of about 15 nmol/l (Fig. 3, inset). Incubation of lymphocytes with βA25–35 in concentrations up to 1 μmol/l did not destabilize basal $[Ca^{2+}]_i$ (Fig. 1). Only in very high concentrations (>10 μmol/l) did βA25–35 itself cause a direct elevation in $[Ca^{2+}]_i$ (data not shown). This is in accordance with findings of direct neurotoxic effects [2] or alterations of membrane permeability [14,15] induced by high concentrations of β-amyloid or its fragments. The amplifying effect of βA25–35 on the Ca^{2+} response after PHA stimulation was more pronounced at submaximal than at low PHA concentrations (Fig. 4).

The effect of βA25–35 on the Ca^{2+} homeostasis was

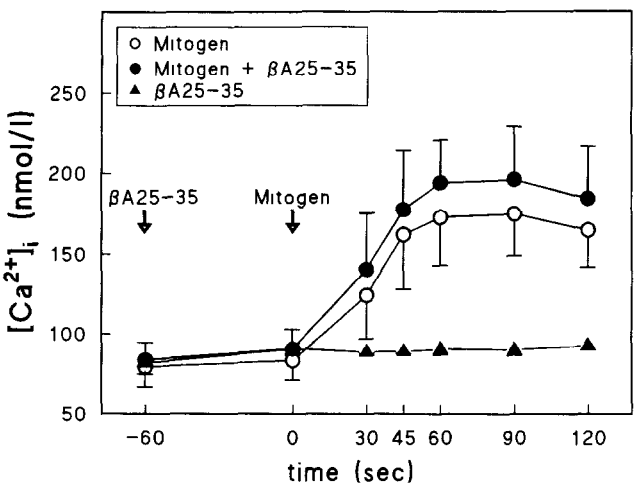

Fig. 1. Time-course of $[Ca^{2+}]_i$ in lymphocytes in the presence of mitogen (PHA, 15 μg/ml), mitogen + βA25–35 (1 μmol/l), and βA25–35 separately. Arrows indicate time points of mitogen and βA25–35 (60 s preincubation) addition Values are means ± S.D. ($n = 9$). Ca^{2+} responses to mitogen in lymphocytes pretreated with βA25–35 were significantly higher than in controls ($P = 0.001$, ANOVA).

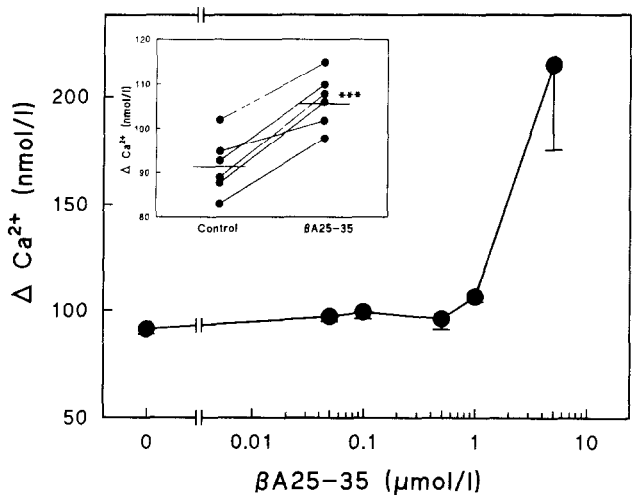

Fig. 3. Effects of preincubation (60 s) with increasing concentrations of βA25–35 on PHA-induced (15 μg/ml) Ca^{2+} rise (Δ Ca^{2+}) in lymphocytes. Data are means ± S.E M. ($n = 4$–6). (Inset) Effect of βA25–35 (1 μmol/l) on PHA-induced (15 μg/ml) Ca^{2+} in lymphocytes of 6 individuals. $^{***}P < 0.001$.

specific for the biologically active sequence of β-amyloid. The PHA-induced increase in $[Ca^{2+}]_i$ was not significantly influenced by fragment 1–28 or 12–28 (1 μmol/l). However, β-amyloid itself (βA1–40) had similar effects on the Ca^{2+} response as fragment 25–35 (Fig. 5). These findings agree with other studies investigating the neurotoxic effects of β-amyloid fragments and their interaction with excitatory stimuli [2,3,5].

4. DISCUSSION

The present study clearly indicates that β-amyloid and its neurotoxic fragment βA25-35 enhance the mitogen-induced rise of $[Ca^{2+}]_i$ in circulating human lymphocytes, probably by increasing Ca^{2+} influx. This effect is already present at nanomolar concentrations, which per se do not affect $[Ca^{2+}]_i$. Only at very high concentrations did βA25–35 alone destabilize intracellular Ca^{2+} regulation. This parallels findings in PC12 cells [5], whereas in human cortical neurons exposed to high β-amyloid concentrations no significant elevation in $[Ca^{2+}]_i$ has been observed [3].

Contrary to its amplifying effect on stimulation-induced elevations of $[Ca^{2+}]_i$ on various neuronal cell cultures established from embryonic or fetal cells, which has only been observed after days of exposure, the effect of βA25–35 was already observed within seconds. A similar rapid effect of β-amyloid on $[Ca^{2+}]_i$ regulation was previously found using dissociated neurons from the adult mouse brain [16]. In contrast to embryonic or fetal neurons in tissue culture, the two latter cell types represent adult, fully differentiated cells. It seems reasonable to assume that the balance between neurotrophic and neurotoxic effects of β-amyloid might be different in embryonic or fully differentiated cells.

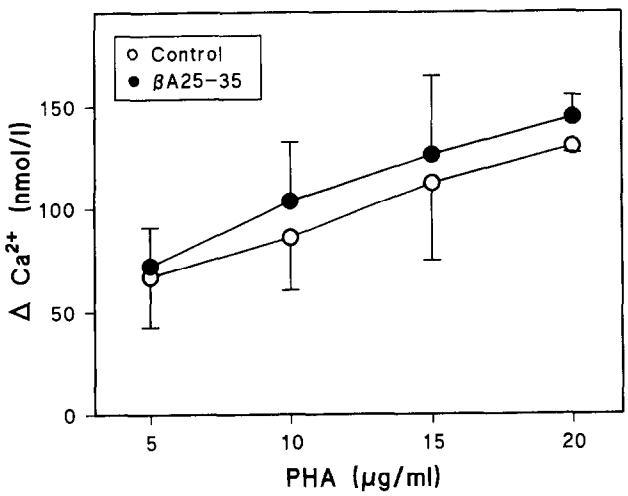

Fig. 4. Effect of preincubation (60 s) with βA25–35 (1 μmol/l) on Ca^{2+} increase (Δ Ca^{2+}) after stimulation with increasing concentrations of PHA (5–20 μg/ml). Values are means ± S.D. (n = 3). Δ Ca^{2+} in lymphocytes treated with βA25–35 was significantly higher than in controls ($P < 0.01$, ANOVA).

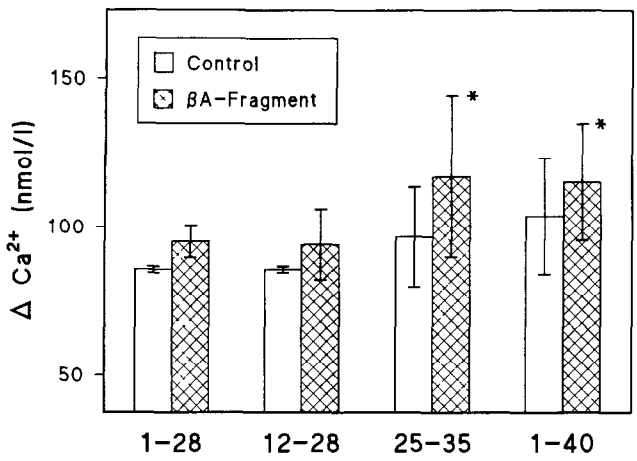

Fig. 5. Comparison of the effects of β-amyloid fragments 1–28, 12–28, 25–35 and 1–40 (1 μmol/l) on PHA (15 μg/ml)-induced Ca^{2+} response in lymphocytes. Values are means ± S.D. (n = 3–5).*$P < 0.05$.

The biological mechanism of action of β-amyloid is still unknown. β-Amyloid amplifies the mitogen-induced Ca^{2+} signal only in the presence of extracellular calcium, suggesting an enhancing effect on the Ca^{2+} influx, which presents the main component of the mitogen-induced Ca^{2+} signal [17], rather than on the mitogen-induced Ca^{2+} release from intracellular storage organelles. These findings are in accordance with observations on single neurons and artificial membranes where β-amyloid incorporated into the membrane bilayer forms structures acting like Ca^{2+} channels [14,15]. Studies on cell cultures have suggested that this Ca^{2+} enhancing effect of β-amyloid might be counterbalanced by secreted forms of βAPP, which promote a rapid decrease of $[Ca^{2+}]_i$ within minutes [18]. Very interestingly, these βAPP fragments can be released in central neurons, as well as in human lymphocytes, by activation of cell surface receptors coupled to phospholipase C [11,19]. Accordingly, it might be possible to use circulating lymphocytes to further investigate the hypothesis of a bidirectional regulation of neuronal $[Ca^{2+}]_i$ by the two alternative products of the cellular βAPP processing in man.

In conclusion, Ca^{2+} regulation in human lymphocytes is affected by β-amyloid in a fashion similar to its effects on central neurons. Accordingly, the lymphocyte represents an excellent model to study β-amyloid's neurotoxicity in man.

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