Neurobiology of Disease **5,** 37–45 (1998)
Article No. NB980176

Calcium Responses in Fibroblasts from Asymptomatic Members of Alzheimer's Disease Families

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Received October 22, 1997; revised February 4, 1998; accepted for publication February 20, 1998

We have previously identified alterations of K^+ channel function, IP_3-mediated calcium release, and Cp20 (a memory-associated GTP binding protein) in fibroblasts from Alzheimer's disease (AD) patients vs controls. Some of these alterations can be integrated into an index that distinguishes AD patients from controls with both high specificity and high sensitivity. We report here that alterations in IP_3-mediated calcium responses are present in a large proportion of AD family members (i.e., individuals at high risk) before clinical symptoms of Alzheimer's disease are present. This was not the case if such members later "escaped" AD symptoms. This preclinical calcium signal correlate of later AD does not reflect, however, the presence of the PS1 familial AD gene.

Key Words: **Alzheimer's disease; calcium; fibroblast.**

INTRODUCTION

Fibroblasts of patients suffering from Alzheimer's disease (AD) exhibit alterations at the cellular and molecular level (Baker *et al.,* 1988; Peterson *et al.,* 1988; Govoni *et al.,* 1993; McCoy *et al.,* 1993; Scott, 1993; Etcheberrigaray *et al.,* 1993, 1994; Huang *et al.,* 1994, 1995; Ito *et al.,* 1994; Kim *et al.,* 1995; Gibson *et al.,* 1996a,b; Hirashima *et al.,* 1996; Scheuner *et al.,* 1996). Despite the lack of complete agreement—particularly in relation to the regulation of intracellular calcium (Borden *et al.,* 1991; Huang *et al.,* 1991; McCoy *et al.,* 1993; Matsuyama *et al.,* 1995; Tatebayashi *et al.,* 1995; Gibson *et al.,* 1996a)—and the lack of methodological standardization, most studies do identify significant cellular and molecular alterations in fibroblasts from AD patients. More recently, we have identified potassium channel dysfunction and alterations of IP_3-mediated calcium release in AD fibroblasts. Patch-clamp techniques revealed the functional absence of an ≈113-pS tetraethylammonium (TEA)-sensitive K^+ channel (Etcheberrigaray *et al.,* 1993). It was also established that intracellular calcium elevations in response to TEA resulted from blockade of functional TEA-sensitive K^+ channels and subsequent depolarization. In consequence, TEA-induced Ca^{2+} elevations were primarily observed in control cell lines and were

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0969-9961/98 $25.00

virtually absent in AD cell lines. Thus, the presence/absence of the TEA-induced calcium response is a reflection of the functional status of K^+ channels in fibroblasts (Etcheberrigaray *et al.*, 1993, 1994; Etcheberrigaray & Alkon, 1997). In addition, IP_3-mediated Ca^{2+} release was found enhanced in AD fibroblasts (Ito *et al.*, 1994; Hirashima *et al.*, 1996). Cp20, a memory-associated GTP binding protein, was also found significantly reduced in fibroblasts from AD patients (Kim *et al.*, 1995). Two of these AD-associated changes, K^+ channel alteration and Cp20 reduction, can be induced by treatment of control fibroblasts with low doses of soluble β-amyloid (the main component of AD plaques) (Etcheberrigaray *et al.*, 1994; Kim *et al.*, 1995). We have also devised a combined scoring system or index that integrates both alterations in K^+ channel function and in IP_3-mediated intracellular calcium release (Hirashima *et al.*, 1996). The index also takes into account the degree of "responsiveness" of each particular cell line. The index is the result of the algebraic sum of values assigned to each alteration (i.e., responses to TEA, bradykinin, and bombesin) where negative values indicate "abnormal" responses while positive values indicate "normalcy." Briefly, the values of each component are determined as follows (in terms of % of responding cells): TEA, $<5 = 0$, $\geq 5 = 0.5$, $\geq 16 = 1$ (latency to onset $\leq$60 s); BK, $<1.5 = 0$, $\geq 1.5 = -0.5$, $\geq 4 = -1$ (latency to onset $\leq$135 s); and bombesin, <50 or integrated area $<23{,}000 = 0$; ≥ 50 and area $\geq 23{,}000 = -0.5$, ≥ 50 and area $\geq 30{,}000 = -1$ (latency to onset $\leq$60 s). Additional details on construction of the index can be found elsewhere (Hirashima *et al.*, 1996). We now focus our attention on asymptomatic members of AD families to determine whether some of these molecular alterations can be detected before clinical symptoms are evident. We also assessed whether the sole presence of a PS1 mutation correlated with early molecular alterations. We report here that such molecular alterations are indeed present in a significant number of these individuals, particularly reflected in IP_3-mediated calcium responses, even before clinical manifestations occur. In our sample, however, the presence of the mutation alone did not determine the appearance of the calcium alterations.

METHODS

Cell lines and cell culture procedures. Cultured fibroblasts from asymptomatic members of AD families were obtained from the Coriell Cell Repositories (Camden, NJ). The asymptomatic group includes cells from nine individuals "at 50% risk" (@R) having at least one direct relative affected (AG07629, AG07867, AG08172, AG08185, AG08709, AG07653, AG07583, AG07671, and AG08129; age, 49.33 ± 5.36, mean ± SD) and six escapees (Es) (AG06838, AG06842, AG07603, AG08265, AG06846, and AG07657; age, 60.17 ± 10.63). Es are asymptomatic individuals beyond the expected age of the onset of disease for a given family (National Institute of Aging, 1994; Gibson *et al.*, 1996b). Preliminary data for lines AG06838, AG06842, and AG07603 were reported previously (Hirashima *et al.*, 1996). The cell lines are from individual members of the Canadian pedigree 964, except for AG07867 of Italian pedigree 1079 and AG08265 of pedigree 2090. Es and @R status information was obtained from this reference (National Institute of Aging, 1994). For six cell lines the data collection and analyses were performed blindly. Three of those cell lines classified as @R at the time of the biopsy developed symptoms in recent years (L. Nee, pers. comm.). This information was also only revealed after the analysis was completed. The responses to different pharmacological challenges (see below) were compared to previously obtained results (Etcheberrigaray *et al.*, 1993; Ito *et al.*, 1994; Hirashima *et al.*, 1996) from AD and control cell lines. The sources for control and AD cell lines were the Coriell Cell Repositories and the Laboratory of Molecular and Cellular Neurobiology, Sacred Heart Hospital of Brescia, Italy. Additional information about these cell lines can be obtained elsewhere (Etcheberrigaray *et al.*, 1993; Govoni *et al.*, 1993; Ito *et al.*, 1994; National Institute of Aging, 1994; Hirashima *et al.*, 1996). Cells were seeded (Hirashima *et al.*, 1996) in 35-mm Nunc petri dishes (≈10 to 15 cells/mm^2) and used 3 to 4 days after seeding, at comparable levels of confluence (≈60 to 80 cells/mm^2). Culture medium was Dulbecco's modified Eagle's supplemented with 10% fetal calf serum (Gibco). The passage numbers of the experimental groups ranged from 2 to 11. A number of previous studies have demonstrated that passage number does not affect calcium responses (Borden *et al.*, 1991; McCoy *et al.*, 1993; Ito *et al.*, 1994). A significant proportion of members of the Canadian family have been included in those studies; therefore, no effort was made to have the cell lines at the same passage for measurements in the present study. Cells were maintained in complete culture medium until just before the experiments.

Calcium imaging. Culture medium was removed and cells were washed at least three times with BSS (in mM: NaCl 140, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1.5, Hepes 10, glucose 5, pH 7.4). The fluorescent probe was loaded

by incubating the cells in 2 μM (in BSS) Fura 2-AM (Molecular Probes) for 60 min at room temperature. After loading, cells were washed thoroughly with BSS or BSS–0 Ca^{2+} (in mM: NaCl 140, KCl 5, $CaCl_2$ 0.1, $MgCl_2$ 1.5, EGTA 1, Hepes 10, glucose 5, pH 7.4; free Ca^{2+} ~5 nM, calculated with program developed and kindly provided by Dr. T. J. Nelson). After washes, 1 ml of fresh solution was added for intracellular Ca^{2+} baseline measurements. TEA (Sigma) challenge was done by adding to the dish 3 ml of TEA-modified BSS (TEA–MBSS) solution (in mM: TEA 133.3, NaCl 6.7, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1.5, Hepes 10, glucose 5, pH 7.4). This solution was adjusted to introduce no osmolality changes. One milliliter of bradykinin (BK) (0.2 nM) (Calbiochem) in BSS was added to the experimental dish to achieve a final BK concentration of 0.1 nM. Bombesin stimulation was accomplished by adding 1 ml of BSS–0 Ca^{2+} plus bombesin to achieve final bombesin (Calbiochem) concentrations of 1 μM. Fluorescent images at 340 and 380 nm were acquired at a rate of 1 ratio / 2.6s for 200 s, with a Hamamatsu Argus 50 system (Hamamatsu Photonics). The objective lens was a 10× Nikon fluor. Only a ¼ evenly illuminated area of the whole image was acquired. Individual ratio values were obtained off line for all cells within the area of interest. For each cell line the data were obtained from experiments conducted on at least two separate occasions. In each experimental session at least five dishes were used per condition / treatment. The total number of cells per condition was ≥44. For faster processing, we analyzed the experiments using the 340 / 380 ratio values directly (see also Hirashima *et al.*, 1996). The established criteria for "response" (Etcheberrigaray *et al.*, 1993) of an elevation of at least 100% from base line were maintained here to allow direct comparisons to previous studies.

Statistical analyses. Conventional statistical methods included Mann–Whitney and contingency table analyses (Remington & Schork, 1985; Motulsky, 1995). Nonparametric statistics were used for comparisons between groups that did not follow a normal distribution (Motulsky, 1995). All calculations were made with the help of the commercially available software package Graphpad Prism.

Genotype. Information on the presence / absence of the presenilin 1 (PS1A246E) mutation for individuals of the Canadian family 964 was kindly provided by Dr. R. E. Tanzi. Briefly, the transcript for PS1 was recovered by RT-PCR and later sequenced. The analysis of the nucleotide sequence revealed an Ala → Glu substitution at codon 246 (see also Sherrington *et al.*, 1995; Scheuner *et al.*, 1996; Tanzi *et al.*, 1996).

RESULTS

TEA-induced responses. The responses to the TEA challenge of @R and Es were evaluated and compared with those previously obtained from our pool of AD (22 cell lines) and controls (23 cell lines) (AD and control cell lines listed in Etcheberrigaray *et al.*, 1993; Ito *et al.*, 1994; Hirashima *et al.*, 1996). As a group, the responses (measured as % of responding cells) of @R did not differ from AD ($P = 0.26$, Mann–Whitney) and were significantly lower than the controls ($P < 0.02$, Mann–Whitney) (see Fig. 1A). Es group responses were significantly different from those of the AD group ($P < 0.003$, Mann–Whitney), while they did not differ from those of the control group ($P = 0.16$, Mann–Whitney) (Fig. 1A). There was, however, significant data dispersion among both groups. In the @R group, four cell lines had clear control-like responses while five had virtually no responses (in an AD-like manner) (Fig. 1A). Representative traces of TEA-induced calcium responses in Es are depicted in Fig. 1B. A contingency table analysis [≥5% responding / <5% responding; % values according to previously established criteria (see Hirashima *et al.*, 1996)] confirmed the group results showing a profile closer to AD in @R cell lines. There were no significant differences between @R vs AD ($P = 0.22$, Fisher's exact test), while there was a clear difference between @R vs controls ($P < 0.004$, Fisher's exact test). Individual Es cell lines' responses distributed like those of the controls; five of six Es cell lines had ≥5% of cells responding, which is in the range previously identified as that of control-like responses (Hirashima *et al.*, 1996). The contingency table analysis confirmed no significant differences between Es and controls ($P = 0.37$, Fisher's exact test). The same statistic revealed significant differences between Es vs AD ($P < 0.02$).

Intracellular calcium release responses. The BK responses of the @R group showed a pattern comparable to that observed in AD (14 cell lines) ($P = 0.4$, Mann–Whitney) and it was significantly different from that of the controls (11 cell lines) ($P < 0.009$, Mann–Whitney) (Fig. 2A) (list of AD and control cell lines in Hirashima *et al.*, 1996). The Es group responses to BK were significantly different from those in AD ($P < 0.006$, Mann–Whitney). In contrast, the Es group was remarkably similar to the control group ($P = 0.22$, Mann–Whitney) (Fig. 2A). When cell lines were analyzed individually, the responses to BK appeared more uniform than those elicited by TEA. Clearly Es responses, with one exception, distributed like those of the controls, and @R responses distributed like those of the

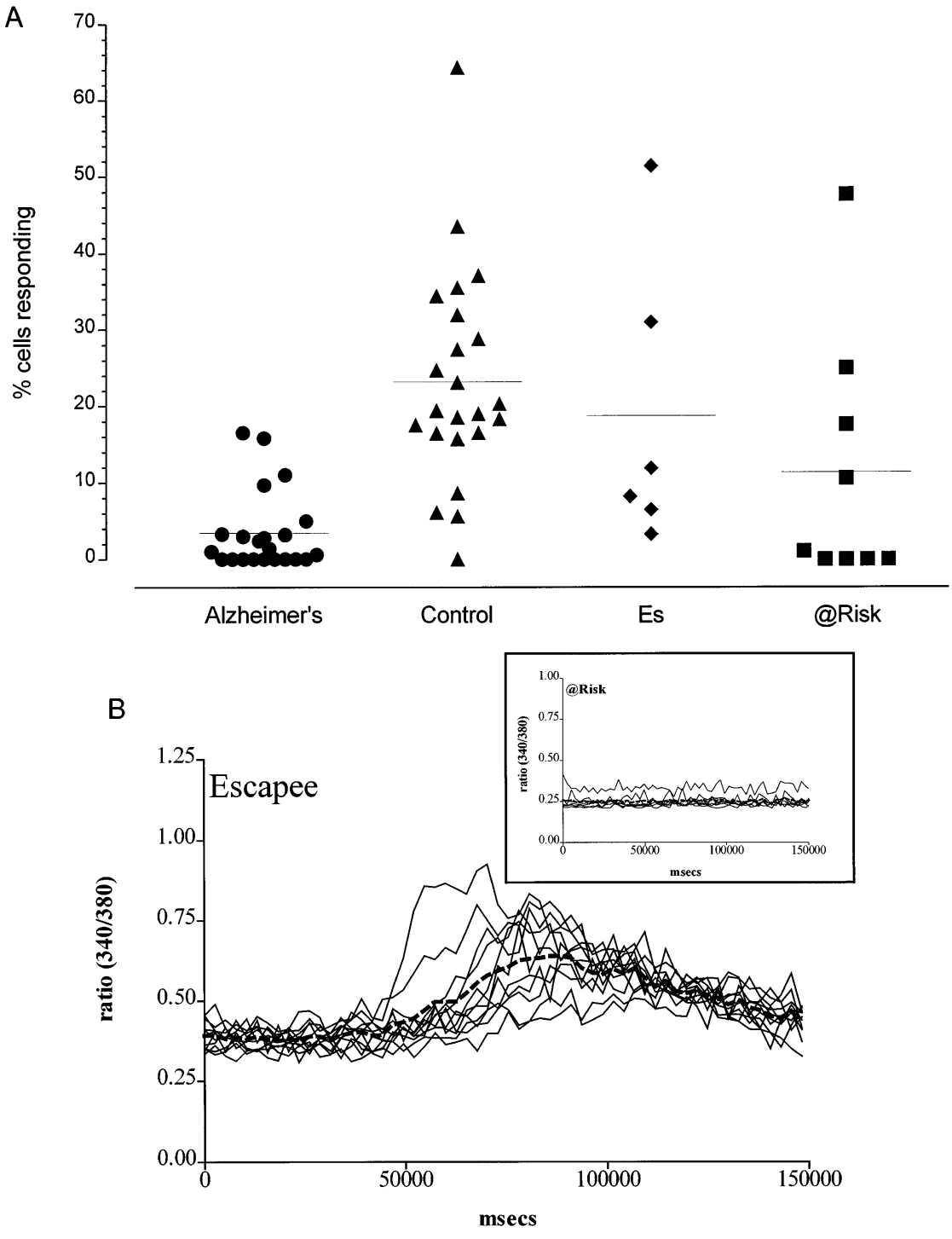

FIG. 1. TEA-induced responses. Each point in the graph (scatter plot, A) represents the % of responding cells in each cell line. The solid horizontal line indicates the group's average. The group results show a similar % of responding cells for controls and Es. The % of responding cells was significantly reduced in @R compared with controls. As a group, @R was not significantly different from the AD group (see text for statistics). The distribution of the responses of individual cell lines also shows a similarity between Es and controls. Although the distribution of @R cell lines was statistically no different from that of AD (and significantly different from controls, see text), they clearly were almost equally distributed between the AD- and the control-like ranges. Representative traces of Es with normal-like responses are depicted in B. The inset illustrates an @R cell line with lack of responses to TEA in an AD-like manner. Broken lines represent average responses.

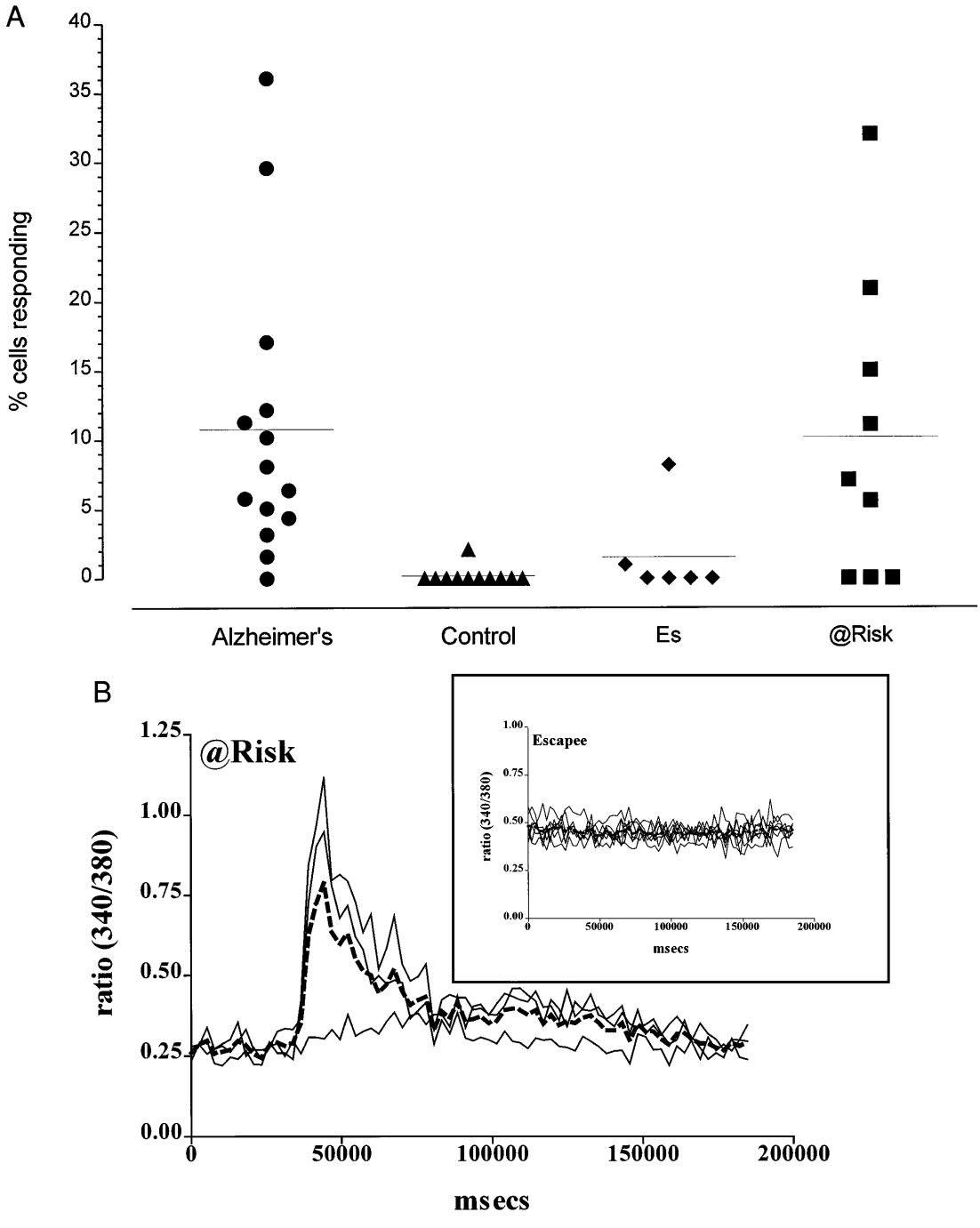

FIG. 2. Bradykinin-induced responses. Calcium elevations in response to 100 pM bradykinin were almost exclusively observed in @R and AD cell lines. The highly significant group differences are shown by the horizontal lines representing the average % of responding cells across cell lines in each group (A, see text for statistics). Even more marked than the TEA responses, BK responses are similar and very low for controls and Es. In contrast, they are enhanced and similar for @R and AD. The majority of @R cell lines have responses distributing in a manner remarkably similar to that of AD (scatter plot, A). Cell lines from Es, except one cell line, had a lack of responses comparable to the control group. Traces are examples of @R responding to BK in an AD-like manner and lack of responses in Es (inset). Broken lines indicate average responses.

AD group (Fig. 2A). Examples of BK-induced calcium responses are depicted in Fig. 2B. Contingency table analyses [<1.5% responding / ≥1.5% responding (Hirashima *et al.*, 1996)] confirmed this observation, showing no significant statistical differences between @R and AD and between Es and controls. Accordingly, significant differences were observed between @R and controls and between Es and AD ($P < 0.015$ and $P < 0.003$, respectively, Fisher's exact test). As seen in our previous studies, bombesin elicited responses in a large percentage of cells in all cell lines (AD, 60.54 ± 4.86; control, 49.16 ± 5.73; Es, 59.73 ± 9.11; @R, 63.7 ± 5.79, mean ± SE). The magnitudes of the responses (integrated area) were AD-like for @R (AD vs @R, $P = 0.13$, Mann–Whitney) and similar to controls for Es (control vs Es, $P = 0.31$). Conversely, @R had larger responses than controls ($P < 0.0001$) and Es had smaller responses than AD ($P < 0.05$). Using the established criteria for "enhanced" bombesin responses (area at least ≥23,000 and ≥50% responding, see Hirashima *et al.*, 1996), 5 of 8 @R cell lines had enhanced bombesin responses, statistically no different than AD with 11 of 14 cell lines exhibiting enhanced responses ($P = 0.37$, Fisher's exact test). Accordingly, @R was different from the control group that had only 2 of 10 cell lines with enhanced responses ($P = 0.0521$). Es had 2 of 6 cell lines with enhanced responses, which was statistically no different from control or AD cell lines.

Integrated index for Alzheimer's disease. The @R group had the majority of cell lines with AD-like scores. Only three of nine cell lines had normal-like index values. Contingency table analyses (−values / or +values, see Hirashima *et al.*, 1996) indicated significant differences from controls ($P < 0.01$, Fisher's exact test) and to a lesser degree from AD ($P < 0.05$). The index values showed that Es cell lines, as expected from their control-like profile for TEA and BK, distribute similarly to controls. Five of six cell lines had "normal" index values. The statistical analysis indicated significant differences from AD ($P < 0.0005$) and no statistical differences from controls ($P = 0.35$) (Fig. 3).

DISCUSSION

The main finding of this study is that some of the molecular changes previously reported in AD fibroblasts (K^+ channels and IP_3-mediated calcium release defects) can be detected even before clinical symptoms appear. @R individuals (with one exception, AG07867 member of Italian family 1079) are asymptomatic

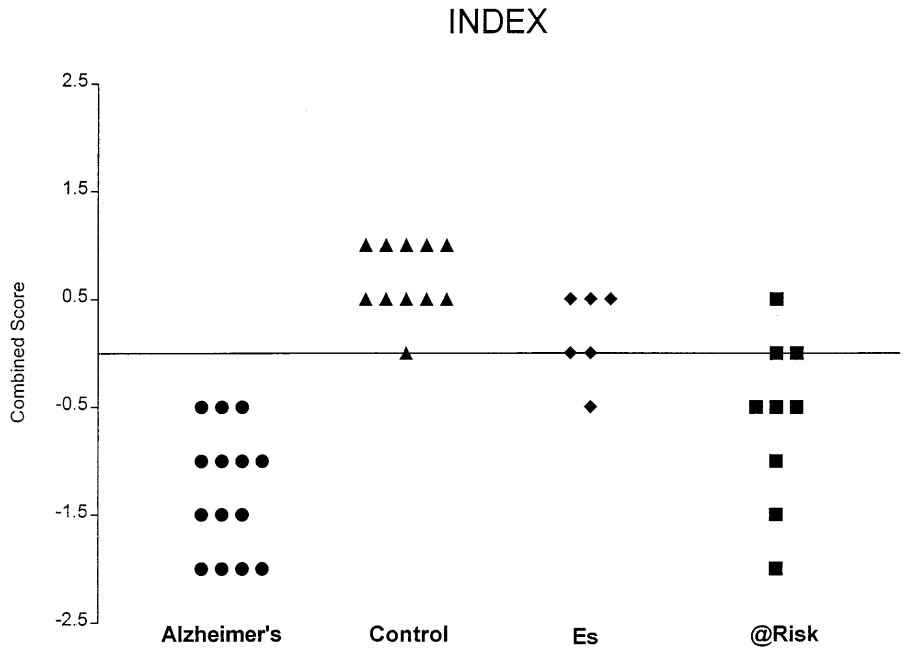

FIG. 3. Index for AD. Symbols represent the combined score value for each particular cell line. As can be clearly observed, Es have distribution and index values closer (and statistically no different, see text) to those of the controls. In contrast, six of nine @R cell lines distributed within the AD range.

members of the Canadian family 964 originally studied and reported by Nee *et al.* (1983). This family follows a transmission pattern compatible with an autosomal dominant transmission mode. Thus, a percentage risk can be assigned to individuals given the pedigree. For instance, a 50% risk can be assigned to the offspring of affected individuals. This family has also been linked to chromosome 14 and more recently, mutations in presenilin 1 have been identified in affected members of this family (Sherrington *et al.*, 1995; Scheuner *et al.*, 1996; Tanzi *et al.*, 1996; Hardy, 1996) (Table 1). We only used cells from individuals with a 50% risk. Es are also asymptomatic members of the AD Canadian family (except AG08265, member of family 2090). Unlike @R individuals, they have ''escaped'' from the disease process and remain asymptomatic beyond the average age of the onset of disease for that family (National Institute of Aging, 1994; Gibson *et al.*, 1996b). In some instances, it has been shown that they also do not carry the presenilin 1 mutation(s) [AG06842 and AG06846 have been demonstrated to be negative for PS1A246E mutation (R. E. Tanzi, pers. comm.); see also Sherrington *et al.*, 1995]. Thus, @R and Es share many common characteristics but also have some important differences in genetic terms. Differences are also evident in terms of their calcium responses (or lack of them) as Es and @R significantly differ compared with AD and controls. The TEA-induced responses of Es cell lines were very similar to control and significantly different from AD cell lines. This finding is consistent with a group of individuals who are essentially free of disease, with no apparent clinical symptoms, presumably free of pathology, and eventually carrying no mutation. Only one of six cell lines had a responsiveness level characteristic of AD. Cells from three of these individuals (AG06846, AG07657, and AG07603) were tested and analyzed blindly. Es responses to BK were also remarkably similar to those from controls and significantly different from those of AD cell lines. Only one cell line had a level of responsiveness comparable to that observed in AD. It is important to note than in a previous communication (Hirashima *et al.*, 1996), in which only three Es cell lines were studied, we had observed somewhat altered TEA patterns. Two cell lines had responses higher than 5% but below 10% and one had responses below 5%, thus they appeared closer to AD. In the expanded sample (which includes the previous results) a normal-like pattern becomes clear. The only cell line with a clear AD pattern was tested a second time as part of the blind subsample with virtually identical results (percentages of responding cells were 3.2 and 3.4, respectively). For the same three cell lines we originally reported a normal pattern for BK-induced responses that held true for the present expanded sample.

An opposite and more complex pattern can be observed in the @R group. These individuals have a significant risk for carrying the identified mutation and thus developing the disease. However, all the samples were obtained prior to clinically evident symptoms (National Institute of Aging, 1994). An important consideration is that for three (tested blindly) of these nine individuals, new information was revealed after the analysis was completed. These three individuals have recently developed symptoms of AD (L. Nee, pers. comm.) and proven to be PS1A246E mutation carriers (R. E. Tanzi, pers. comm.; Scheuner *et al.*, 1996) approx 10 years after the biopsies were obtained during the asymptomatic period. The PS1A246E mutation was proven positive in one additional individual and negative in the other two (R. E. Tanzi, pers. comm.). There is no information available for the remaining three individuals (Table 1). Of the six individuals from whom genotypes were obtained, the TEA response alteration coincided with the presence of

TABLE 1
Cell Lines from At-Risk and Escapee Individuals

@Risk	TEA	BK	Bom.	Index	PS1A246E	Clin. status
A[a]	1	−1	0	0	+	+ now
B[a]	1	−1	−0.5	−0.5	−	−
C[a]	0	−1	−1	−2	−	−
D[a]	0	0	0	0	+	−?
E[a]	0	−1	0	−1	+	+ now
F[a]	1	0	−0.5	0.5	+	+ now
G[a]	0.5	−1	NT	−0.5		−
H[a]	0	0	−0.5	−0.5		−
I[b]	0	−1	−0.5	−1.5		−
Escapee						
AG06842[a]	0.5	0	−1	−0.5	−	−
AG06846[a]	1	0	−0.5	0.5	−	−
AG07603[a]	0	0	0	0		−
AG07657[a]	1	−1	0	0		−
AG06838[a]	0.5	0	0	0.5		−
AG08265[c]	0.5	0	0	0.5		−

Note. Columns indicate the score assigned to each cell line's responses to the various pharmacological challenges as well as the integrated ''index'' value. The presence or absence of the PS1 mutation is indicated for eight cell lines. Clinical status as indicated reflects the latest available information at the time of completing this article @R individuals are coded due to bioethical considerations.

[a]Canadian kindred 964.

[b]Italian kindred 1079.

[c]Kindred 2090.

mutation in only two cases, suggesting that the sole presence of the mutation was not enough to alter the TEA response. Apparently, a pathological process with additional cellular/molecular alterations must take place before observable TEA changes occur. Testing cell lines of these same patients from biopsies in the symptomatic phase might provide a better understanding of these phenomena. Based on this and our previous studies, these changes (with a few exceptions) appear to be concomitant with the appearance of clinical symptoms. The BK responses were also similar to AD, with six of nine cell lines exhibiting an AD pattern. The BK response was altered in two of the three cell lines from patients that later developed symptoms and were positive for PS1 mutation. However, the BK response did not correlate with the presence of mutations in the other two individuals.

The use of combined responses in the index also indicated that Es scores were distributed as in the controls. Only one cell line had values within the "AD range" (Hirashima *et al.,* 1996). The majority of @R cell lines (six of nine) had values in the AD range, which can be expected from the largely AD-like responses to BK (and bombesin), and at least half had altered TEA responses. The index was accurate in identifying Es as "normal," including three cell lines tested blindly. The abnormal index values for "at-risk" patients are consistent with an increased likelihood for Alzheimer's disease. However, there was no clear correlation between the presence of PS1 mutation and the AD index abnormality. This suggests that other predisposing factors are necessary to interact with the PS1 gene in order to produce an AD phenotype. These results should not be considered an unusual finding giving the multifactorial nature of AD. The limited number of individuals from whom genetic characterization was available may be another factor contributing to the lack of complete correlation between the index values and the presence of the PS1 mutation. It is interesting to note that an individual identified as "normal" by the index, who tested positive for the mutation, seems to remain free of symptoms (Table 1, "D"). It also is important to emphasize that the index was developed originally in a sample of symptomatic individuals and identified as a good test for distinguishing AD vs normals and individuals affected by other non-AD neuropsychiatric conditions with no assumptions of predictability of future symptoms.

We confirm here that the index remains a good tool for identifying as such non-AD individuals, exemplified by the normal values of Es cell lines. Additional studies will be required to understand how the genetic factors interact with other processes to result in altered K^+ channels and Ca^{2+} responses. The summary index will require further studies that include the future clinical outcomes and pathologies. From this and previous studies, it becomes apparent that the above-mentioned alterations are the reflection of pathophysiological processes to which the genetic mutations may be contributors but not the only determinant factors. It is also important to note that all but two cell lines from the Es and @R groups belong to one pedigree (the Canadian family) with specific mutations. It remains to be established whether these results on asymptomatic individuals can be generalized to pedigrees carrying different mutations. This is particularly important in light of recent evidence that carriers of the Swedish mutations do not show clear alterations in PKC (Vestling *et al.,* 1995) and BK calcium responses (Gibson *et al.,* 1997), common to most sporadic and other familial forms of AD.

From these results we can conclude that a significant proportion of asymptomatic AD family members do have significant alterations in calcium responses to various pharmacological challenges, particularly to IP_3-mediated agonists. These are particularly salient in individuals that have at least one direct relative affected (i.e., 50% risk). Individuals from the same families that have lived free of symptoms long after the expected onset (i.e., Escapees) have "normal"-like calcium responses and index values within the normal range. These results also strongly suggest that in addition to genetic factors, other pathophysiological processes must occur prior to alterations in the calcium responses in AD fibroblasts.

ACKNOWLEDGMENTS

We thank Hamamatsu Photonics for providing the calcium imaging system.

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