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Internal Ca^{2+} mobilization is altered in fibroblasts from patients with Alzheimer disease

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ABSTRACT **The recent demonstration of K^+ channel dysfunction in fibroblasts from Alzheimer disease (AD) patients and past observations of Ca^{2+}-mediated K^+ channel modulation during memory storage suggested that AD, which is characterized by memory loss and other cognitive deficits, might also involve dysfunction of intracellular Ca^{2+} mobilization. Bombesin-induced Ca^{2+} release, which is inositol trisphosphate-mediated, is shown here to be greatly enhanced in AD fibroblasts compared with fibroblasts from control groups. Bradykinin, another activator of phospholipase C, elicits similar enhancement of Ca^{2+} signaling in AD fibroblasts. By contrast, thapsigargin, an agent that releases Ca^{2+} by direct action on the endoplasmic reticulum, produced no differences in Ca^{2+} increase between AD and control fibroblasts. Depolarization-induced Ca^{2+} influx data previously demonstrated the absence of between-group differences of Ca^{2+} pumping and/or buffering. There was no correlation between the number of passages in tissue culture and the observed Ca^{2+} responses. Furthermore, cells of all groups were seeded and analyzed at the same densities. Radioligand binding experiments indicated that the number and affinity of bombesin receptors cannot explain the observed differences. These and previous observations suggest that the differences in bombesin and bradykinin responses in fibroblasts and perhaps other cell types are likely to be due to alteration of inositol trisphosphate-mediated release of intracellular Ca^{2+}.**

A number of cellular changes have been observed in fibroblasts from patients with Alzheimer disease (AD). These include abnormality of glucose and energy-related metabolism (1), defective release of a cholinergic factor (2), abnormal β-amyloid expression and processing (3), changes in Ca^{2+} metabolism (30–34), and altered β-adrenergic-induced cAMP formation (4). The recent demonstration of K^+ channel dysfunction in AD fibroblasts (5, 6) and past observations of Ca^{2+}-mediated K^+ channel modulation during memory storage (7) suggested that AD, which is characterized by memory loss and other cognitive deficits (8, 9), might also involve dysfunction of intracellular Ca^{2+} mobilization. Bombesin (10–12), an agent that activates phospholipase C (PLC) to generate inositol 1,4,5-trisphosphate (IP_3) (13–15), is shown here to cause a markedly enhanced but greatly abbreviated increase of intracellular Ca^{2+} in fibroblasts from AD patients compared with control and other individuals suffering from a variety of neurodegenerative or psychiatric disorders. Bradykinin, another activator of PLC (16–18), caused a similar difference between AD and control fibroblasts. Neither increased external K^+, which triggers voltage-dependent Ca^{2+} influx, nor thapsigargin, an agent that acts directly on the endoplasmic reticulum (ER) (19–22) rather than through IP_3, caused Ca^{2+} increases that were different for AD and control fibroblasts. β-Amyloid protein (23–25) itself, while causing the previously observed inactivation of K^+ channels in AD fibroblasts, had no effect on the bombesin-elicited Ca^{2+} signals. These and other findings, together with measurements of bombesin receptor number, suggest that PLC/G-protein coupling and/or IP_3 receptors are responsible for differences in Ca^{2+} responses between AD and non-AD fibroblasts.

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METHODS

Cell Lines. Human skin fibroblasts (Table 1) were purchased from the Coriell Cell Repositories (Camden, NJ). Cells were seeded and maintained as described (5). The number of passages was not significantly different between groups [AD, 10.9 ± 1.3 (mean ± SEM), $n = 10$; AC, 11.5 ± 0.8, $n = 8$; YC, 8.4 ± 1.0, $n = 6$] and always <20 (see also Fig. 1*D*). As previously determined (5), to reach a standard density (50 cells per mm^2 after seeding at 5 cells per mm^2) for analysis, fibroblasts from AD, AC, ND, and YC donors required virtually the same time in culture [AD, 3.5 ± 0.1 days (mean ± SEM), $n = 10$; AC, 3.4 ± 0.1 days, $n = 8$; YC, 3.6 ± 0.2 days, $n = 6$; all n values refer to the number of cell lines unless otherwise specified]. Furthermore, Ca^{2+} signal characteristics were not at all correlated with the number of passages in tissue culture (Fig. 1*D*). Additional information regarding these cell lines can be found elsewhere (26, 27).

Ca^{2+} Imaging. The cells were incubated with 2 μM fura-2 acetoxymethyl ester in basal salt solution (BSS: 140 mM NaCl/5 mM KCl/2.5 mM $CaCl_2$/1.5 mM $MgCl_2$/5 mM glucose/10 mM Hepes·NaOH, pH 7.4) at room temperature for 60 min. After three washes with BSS, the cells were used for determinations of intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$. Cell fluorescence was measured with a Ca^{2+}-imaging system (Hamamatsu Photonics Argus 50, Hamamatsu, Japan). For excitation, 340-nm and 380-nm bandpath filters with a neutral-density filter were used. Images of fluorescence were obtained with a 400-nm dichroic mirror and a 510-nm long-pass barrier filter. The objective lens was a 10× Nikon UV fluor and only one-quarter of a whole image was recorded to avoid uneven illumination. The averaged Ca^{2+} responses within 15 × 15 pixels in cytosolic cellular compartments obtained were quantified with ratios of fluorescence emitted at 510 nm with activation at 340 nm to fluorescence emitted at 510 nm with activation at 380 nm. The ratio values were transformed to absolute $[Ca^{2+}]_i$ values after

Abbreviations: AD, Alzheimer disease; AC, age-matched control; ND, non-AD neurodegenerative disease and schizophrenia; YC, young control; ER, endoplasmic reticulum; IP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration.

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Table 1. Human fibroblast lines and Ca^{2+} responses in AD and control cells stimulated by 1 μM bombesin

Cell line	Age, years	Gender	Race	Diagnostic criteria	Bombesin response	
					Enhanced	Abbreviated
AD						
AG06840*	56	Male	White	Clinical; family history	+	−
AG06848*	55	Female	White	Clinical; family history†	+	−
AG07637*	55	Female	White	Clinical; family history	+	−
AG08170*	56	Male	White	Clinical; family history	+	−
AG04401	53	Female	White	Clinical; family history†	+	−
AG05809	63	Female	White	Clinical; family history	+	−
AG06263	67	Female	White	Clinical; no family history	+	−
AG07375	71	Male	White	Clinical; no family history	+	−
AG07376	59	Male	White	Clinical; no family history	+	−
AG08243	72	Male	White	Clinical; no family history	+	−
Age-matched control (AC)						
AG07603*	61	Female	White	Normal; family history	−	+
GM03524	67	Female	Black	Normal; no family history	−	+
GM04260	60	Male	White	Normal; no family history	−	+
AG04560	59	Male	White	Normal; no family history	−	+
AG06010	62	Female	White	Normal; no family history	−	+
AG06241	61	Male	White	Normal; no family history	−	+
AG08044	58	Female	Black	Normal; no family history	−	+
AG09878	61	Female	White	Normal; no family history	−	+
Parkinson disease						
AG08395	85	Female	White	Clinical; no family history	−	+
Schizophrenia						
GM01835	27	Female	White	Clinical; family history	−	+
GM02038	22	Male	White	Clinical; ?	−	+
Huntington chorea						
GM00305	56	Female	White	Clinical; uncertain family history	−	+
GM01061	51	Male	White	Clinical; family history	−	+
GM01085	44	Male	White	Clinical; family history	−	+
GM02165	55	Male	White	Clinical; family history	−	+
GM04777	53	Male	White	Clinical; family history	−	+
GM05030	56	Male	White	Clinical; family history	−	+
GM06274	56	Female	White	Clinical; family history	−	+
Wernicke–Korsakoff						
7504	50	Male	White	Clinical; ?	−	+
7505	52	Female	White	Clinical; ?	−	+
7507	63	Male	White	Clinical; ?	−	+
7508	64	Male	White	Clinical; ?	−	+
Young control (YC)						
GM02987	19	Male	White	Normal; no family history	−	−
GM03377	19	Male	White	Normal; no family history	−	+
GM03651	25	Female	White	Normal; no family history	−	+
GM03652	24	Male	White	Normal; no family history	−	+
GM04390	23	Female	White	Normal; no family history	−	+
GM08399	19	Female	?	Normal; no family history	−	+

AD fibroblasts were from familial (n = 6) and nonfamilial (n = 4) cases. Five lines (*) are from members of the Canadian family (no. 964), only AG06840 and AG06848 are immediate relatives (sibs). AD was confirmed by autopsy in two cases (†). One of the AC cell lines is from an unaffected member of the Canadian family (no. 964). All YC cell lines (n = 6) were from normal individuals without known AD family history. Enhanced $[Ca^{2+}]_i$ responses (>300 nM), measured at 42 sec after 1 μM bombesin stimulation in the absence of extracellular Ca^{2+}, indicated as +, were observed in all of the AD cell lines used. None of the AC, the ND [non-AD neurodegenerative (Parkinson, Huntington, Wernicke–Korsakoff) and schizophrenia], or the YC cell lines exhibited enhanced responses. Furthermore, abbreviated $[Ca^{2+}]_i$ responses (<110 nM) measured at 120 sec after 1 μM bombesin stimulation in the presence of extracellular 2.5 mM Ca^{2+}, indicated as −, were observed in all of the AD cell lines used. None of the AC or ND, and only one of six YC cell lines exhibited abbreviated responses.

calibration based on the equation $R = R_{max} + (R_{min} - R_{max})/[1 + ([Ca^{2+}]_i/K_d)^b]$. Here R denotes the ratio of fluorescence with activation at 340 nm to that with activation at 380 nm, and R_{max} and R_{min} are the values of R when $[Ca^{2+}]_i$ is at a maximum and a minimum, respectively. K_d is a dissociation constant of fura-2 for Ca^{2+} and was determined as 240 nM. The value of b, which determines the degree of asymmetry, was 1.2.

Radioligand Binding Assay. *Preparation of ^{125}I-labeled bombesin.* [^{125}I-Tyr^4]Bombesin was prepared by reaction of 10 μg of synthetic [Tyr^4]bombesin (Sigma) with Iodo-Gen (Pierce) and Na^{125}I (Amersham) (28). The reaction was terminated with 1 mM NaI and the labeled product was purified by reversed-phase HPLC (0–60% acetonitrile in 0.1% trifluoroacetic acid in 0–60 min). Both [^{125}I-Tyr^4]bombesin and bombesin were eluted at 33 min. The fractions containing the product were combined, and trifluoroacetic acid and acetonitrile were removed by lyophilization. The purity of the final product was determined by HPLC.

Bombesin receptor assay. Cells ($\approx 10^6$) were scraped from culture flasks, collected by centrifugation, and washed with phosphate-buffered saline. The cells were lysed by sonication and crude membranes were prepared by centrifugation at 50,000 × g for 30 min at 4°C. Aliquots containing $\approx$154 μg of

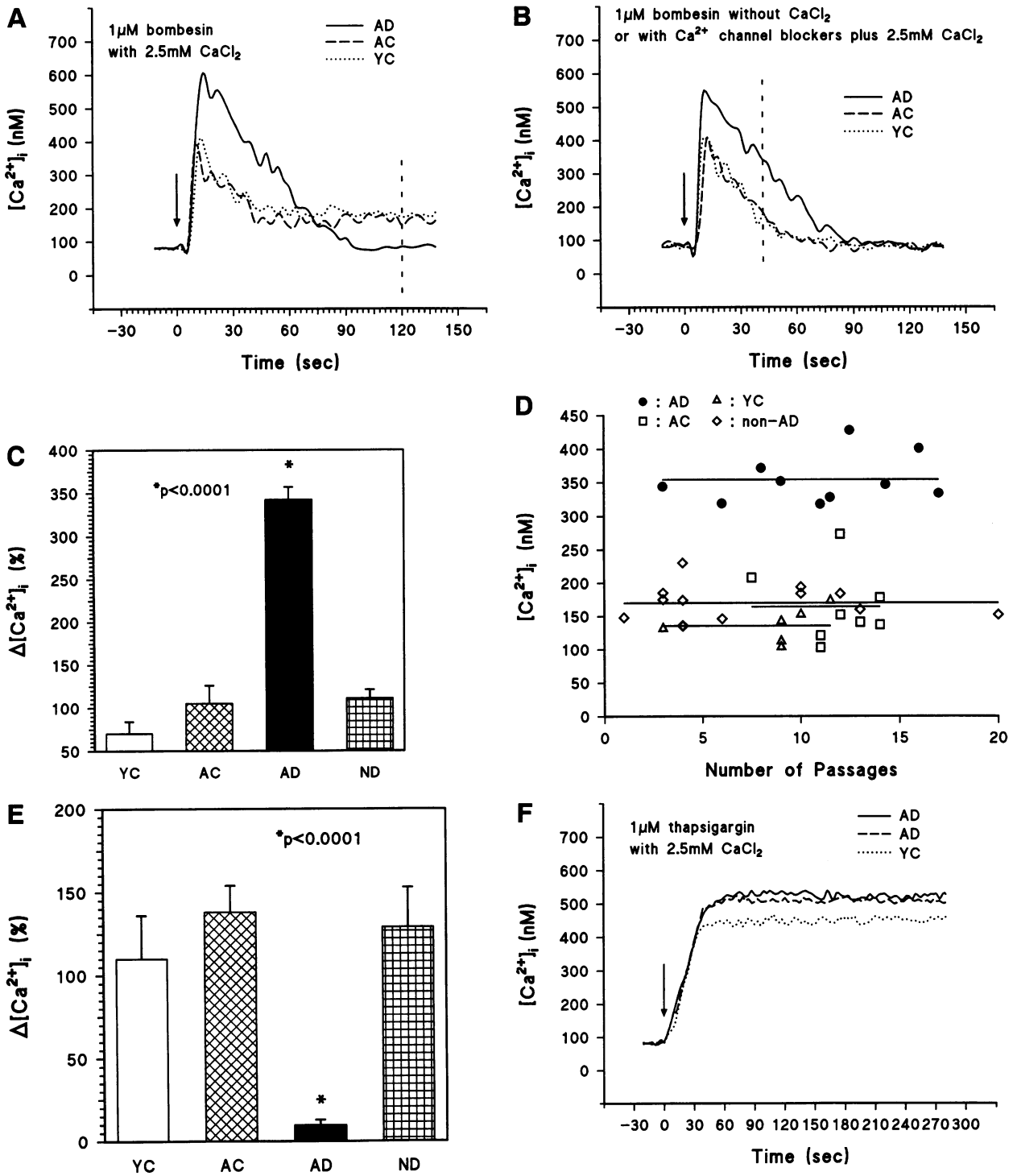

FIG. 1. Ca^{2+} responses induced by 1 μM bombesin. (*A*) Bombesin elicited a large initial transient peak of $[Ca^{2+}]_i$ followed by a sustained phase for YC and AC cells, but not for AD cells, in the presence of extracellular 2.5 mM Ca^{2+}. Arrow indicates the time of drug application. (*B*) The sustained phase in YC and AC cells disappeared in the absence of extracellular Ca^{2+} (nominally Ca^{2+} free) or in the presence of a mixture of divalent cation Ca^{2+} channel blockers (1 mM Co^{2+}, Ni^{2+}, and Cd^{2+}). The Ca^{2+} signals of AD fibroblasts were unaffected by removal of extracellular Ca^{2+} or by addition of Ca^{2+} channel blockers. The experiments without extracellular Ca^{2+} or with blockers plus 2.5 mM Ca^{2+} were performed independently of those experiments when 2.5 mM Ca^{2+} alone was present in the bathing medium. (*C*) Ca^{2+} responses in all of the AD fibroblasts measured at 42 sec after 1 μM bombesin stimulation in the absence of extracellular Ca^{2+} were still >300% above the basal level. In contrast, all of the AC, ND, and YC cells had <270%, <230%, and <120% Ca^{2+} responses, respectively. (*D*) As previously reported for resting and transient $[Ca^{2+}]_i$ in fibroblasts (30), the bombesin-induced Ca^{2+} responses had no correlation with the number of passages. Ca^{2+} responses at 42 sec after applications of 1 μM bombesin without extracellular Ca^{2+} were plotted. (*E*) Ca^{2+} responses of all the AD fibroblasts measured at 120 sec after 1 μM bombesin application in the presence of 2.5 mM extracellular Ca^{2+} were usually negligible but were never >30% above the basal level. In contrast, Ca^{2+} levels of all of the AC, all of the ND, and five out of six YC cell lines were still 50%, 60%, and 60% greater than their basal levels, respectively. (*F*) Thapsigargin (1 μM) induced the same increase of $[Ca^{2+}]_i$ in the AD and the AC fibroblasts, both of which were greater than those of the YC cells.

protein were incubated in 100 μl for 2 hr at 37°C with various concentrations of [^{125}I-Tyr^4]bombesin in bombesin binding buffer (130 mM NaCl/25 mM Pipes, pH 6.8/4 mM $MgCl_2$/1 mM dithiothreitol/1 mM 1,10-phenanthroline/1 mM capto-

pril/1 mM EGTA/0.025% bacitracin/0.1% bovine serum albumin with chymostatin, at 2 μg/ml). The incubation mixture was then filtered through a Whatman CG/C glass fiber filter which had been soaked in 0.3% polyethyleneimide for 3 hr (29). Blank values were obtained for each bombesin concentration by substituting an equal amount of bovine serum albumin for cell membrane suspension. The blank values were analyzed by least squares and the refined blank values were subtracted from the binding data.

RESULTS

Ca^{2+} signals were measured as fluorescence in human skin fibroblasts that were loaded with the calcium indicator fura-2. Ca^{2+} signals elicited by bombesin from normal fibroblasts (AC and YC) showed a large initial transient peak followed by a much smaller sustained phase. Ca^{2+} signals elicited by bombesin from AD fibroblasts, however, showed only the large initial transient phase (Fig. 1*A*). In response to 1 μM (but not 0.1 or 10 μM) bombesin, the AD peak transient [558 ± 17 nM (mean ± SEM), $n = 10$] was larger (AD vs. controls, $P = 0.0002$, two-tailed Student t test) than those of AC (472 ± 23 nM, $n = 8$), ND (483 ± 11 nM, $n = 14$), and YC (388 ± 31 nM, $n = 6$).

The sustained phase of the Ca^{2+} signals elicited by bombesin from AC ($n = 8$), ND ($n = 14$), and YC ($n = 6$) fibroblasts was eliminated by removal of extracellular Ca^{2+} (Fig. 1*B*). A mixture of divalent cation Ca^{2+} channel blockers (1 mM Co^{2+}, Ni^{2+}, and Cd^{2+}) also eliminated the sustained phase of the Ca^{2+} signals in the control cells (Fig. 1*B*). The Ca^{2+} signals of AD fibroblasts which showed no sustained phase were unaffected by removal of external Ca^{2+} or Ca^{2+} channel blockade (Fig. 1*B*).

Features of the bombesin-elicited Ca^{2+} signals described above were so consistently and distinctively different for AD fibroblasts compared with AC, ND, and YC fibroblasts as to suggest their diagnostic potential. We therefore obtained isochronal values for Ca^{2+} levels as satisfying a minimal criterion for enhancement. Enhancement measured 10–40 sec the peak transient signal in the absence of extracellular Ca^{2+} was observed for all of the AD fibroblasts ($n = 10$) and none of the control ($n = 14$) or the ND ($n = 14$) fibroblasts (Tables 1 and 2 and Fig. 1 *C* and *D*). Thus, when we considered Ca^{2+} signal enhancement measured in this way as a diagnostic criterion for AD, there were no false positives and no false negatives. As an AD test, then, bombesin-induced Ca^{2+} signal enhancement was both highly sensitive and highly specific. Another feature of potential diagnostic value is the absence of a sustained bombesin-induced Ca^{2+} signal from AD fibroblasts in the presence of extracellular 2.5 mM Ca^{2+}. The absence of a sustained response measured as an isochronal value 120 sec after the onset of drug application was observed for all of the AD fibroblasts ($n = 10$) and 1 out of 14 control cell lines and none of the ND fibroblasts ($n = 14$) (Tables 1 and 2 and Fig. 1*E*).

Table 2. A laboratory diagnosis for AD

	No. of cell lines		
		Bombesin response	
Condition	Total	Enhanced	Abbreviated
AD	10	10	10
AC	8	0	0
ND	14	0	0
YC	6	0	1

Bombesin-induced Ca^{2+} responses were enhanced (>300 nM) in the absence of extracellular Ca^{2+} at 42 sec after drug application for all of the AD cell lines ($n = 10$) and none ($\chi^2 = 38.00$, $P < 0.0001$) of the AC ($n = 8$), ND ($n = 14$), and YC ($n = 6$) cell lines. In the presence of extracellular 2.5 mM Ca^{2+}, abbreviated Ca^{2+} responses (<110 nM) were observed at 120 sec after onset of drug application in all of the AD cell lines ($n = 10$) and 1 out of 28 control and ND cell lines ($\chi^2 = 33.95$, $P < 0.0001$).

Various agents and experimental procedures were used to constrain the mechanisms by which bombesin might elicit different Ca^{2+} responses in AD cells. Ca^{2+} signals elicited by perfusion with elevated external K^+ have been shown not to differ between AD and AC cells (5). Ca^{2+} channel blockers completely eliminated all Ca^{2+} signals elicited (presumably by depolarization-induced Ca^{2+} influx) with elevated external K^+ ($n = 11$) (5). Thapsigargin, an inhibitor of the Ca^{2+}-ATPase in the endoplasmic reticulum, mobilizes Ca^{2+} without an increase in inositol phosphates or significant activation of protein kinase C (19–22). Application of 1 μM (as well as 0.1 μM) thapsigargin elicited the same Ca^{2+} signals for AD (521 ± 12 nM, $n = 10$) and AC (516 ± 9 nM, $n = 8$) fibroblasts. These signals were larger than those of YC fibroblasts (441 ± 11 nM, $n = 6$) (Fig. 1*F*). Thapsigargin-elicited Ca^{2+} signals did not depend on extracellular Ca^{2+} (AD, 524 ± 14 nM, $n = 10$; AC, 512 ± 6 nM, $n = 8$; YC, 449 ± 11 nM, $n = 6$). These results indicate that stored Ca^{2+} pools and Ca^{2+} buffering systems do not exhibit AD-specific differences; thus they cannot explain the observed differences of the bombesin-induced Ca^{2+} responses.

Radioligand binding revealed a lower number of bombesin binding sites in AD fibroblasts [173 ± 14 fmol/mg of protein (mean ± SEM), $n = 8$ cell lines] than in AC (368 ± 133 fmol/mg, $n = 3$) and YC (450 ± 110 fmol/mg, $n = 3$) fibroblasts (AD vs. controls, $P = 0.006$, Mann–Whitney test) (Fig. 2). The K_d was slightly but not significantly (Mann–Whitney test) lower in AD fibroblasts (AD, 8.4 ± 1 nM, $n = 8$; AC, 16.3 ± 4.6 nM, $n = 3$; YC, 12.2 ± 1.8 nM, $n = 3$). Since the number of bombesin receptors is lower in AD, the larger Ca^{2+} response observed in this group cannot be attributed to differences in number of receptors between groups. Since all Ca^{2+} responses were obtained at saturation levels, the slightly higher affinity in AD cells cannot account for the enhanced responses in AD fibroblasts.

Further, a low concentration of bradykinin (0.1 pM), another activator of PLC, elicited large Ca^{2+} signals in AD cells (29 out of 145 cells in 5 cell lines), but in no control cells (0 out of 191 cells in 5 cell lines) ($P < 0.0001$, Fisher's exact test).

Finally, preliminary results (35) indicated that incubation with 10 nM β-amyloid did not affect the bombesin–Ca^{2+} response in control cells but did impair the function of K^+ channels that were previously shown to be absent in AD

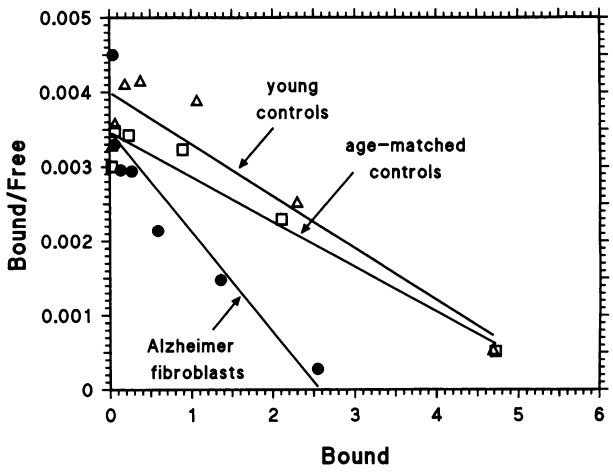

FIG. 2. Scatchard plots of bombesin binding to bombesin receptors. B_{max} was lower in cells from AD patients. B_{max} was virtually identical in AC and YC fibroblasts. K_d values were also similar in AC and YC cells and slightly lower in AD cells.

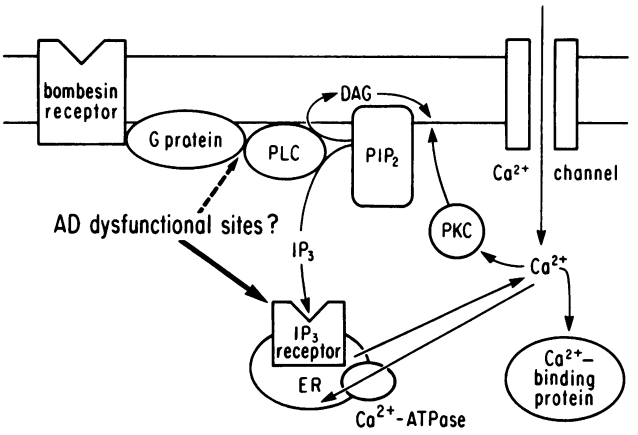

FIG. 3. Model for the bombesin-induced Ca^{2+} signaling pathway. A schematic representation, incorporating the results of Berridge and coworkers (13–15) as well as our own results, indicating the steps involved in the bombesin-induced Ca^{2+} signal and its termination (22) in human fibroblasts. Also indicated are the site(s) likely to be responsible for the observed AD-specific differences in the Ca^{2+} signal. DAG, diacylglycerol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C.

fibroblasts (5). These results suggest that the AD-specific differences in Ca^{2+} signaling elicited by bombesin are not secondary to β-amyloid toxicity or β-amyloid-induced metabolic derangements (23).

DISCUSSION

Based on current models of receptor-linked Ca^{2+} signaling (13–15) and/or Ca^{2+} buffering (22), the bombesin-induced Ca^{2+} responses are likely to involve bombesin receptors, G-protein transduction, PLC cleavage, IP_3 generation, activation of IP_3 receptors in the ER, and Ca^{2+} release. Several Ca^{2+} buffering systems can contribute to termination of the Ca^{2+} signal: organelles associated with the ER, Ca^{2+}-binding proteins, Na^+/Ca^{2+} exchangers, Ca^{2+} pumps, and, under certain conditions, mitochondria (22). Our results allowed us to exclude Ca^{2+} buffering and Ca^{2+} pumping systems as responsible for the observed Ca^{2+} signal differences in AD, since voltage-dependent Ca^{2+} influx, as well as thapsigargin block of Ca^{2+}-ATPase, caused similar Ca^{2+} signals in AD and control cells. The thapsigargin results also indicate that there were no AD-specific differences in intracellular Ca^{2+} pools. Differences in bombesin receptor number or affinity as causes of enhanced Ca^{2+} signaling were ruled out as discussed above. These results suggest, therefore, that the AD-specific differences in the bombesin-induced and bradykinin-induced Ca^{2+} signals are most likely due to derangement of the PLC system. Possible sites of dysfunction in this system include the ER IP_3 receptors or, less likely, G protein–PLC coupling (Fig. 3).

Our finding of AD-specific alteration of Ca^{2+} signaling in peripheral tissue (skin fibroblasts) suggests that defective IP_3-mediated Ca^{2+} metabolism may have systemic expression in AD. This peripheral defect, observable in both familial and non-familial AD fibroblasts, although possibly present in a variety of cell types, may only have clinical expression when neurons of the aging brain are involved. Indeed, a recent examination of AD and control olfactory neuroblasts (unpublished work) suggests an alteration of Ca^{2+} signaling with AD similar to that found here for fibroblasts with AD.

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