

Calcium stores in cultured fibroblasts and their changes with Alzheimer's disease

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Abstract

The experiments in this paper identify multiple calcium compartments in cultured human fibroblasts and reveal abnormalities in one of these pools in cells from Alzheimer patients. In the presence of external calcium, bradykinin (BK) increased cytosolic free calcium ($[Ca^{2+}]_i$) about 3-fold and then $[Ca^{2+}]_i$ rapidly declined. Omission of calcium from the media did not affect the BK-induced peak, which indicates that the peak reflects internal stores. Other compounds that also released calcium from internal stores included A23187 (a calcium ionophore), thapsigargin (Tg; an inhibitor of endoplasmic reticulum ATPase), and FCCP (an uncoupler of oxidative phosphorylation). The $[Ca^{2+}]_i$ response to sequential addition of compounds in calcium-free media identified discrete internal calcium stores. BK depleted internal calcium pools such that subsequent stimulation with BK, FCCP or bombesin did not increase $[Ca^{2+}]_i$. However, A23187 or thapsigargin still elicited responses. A23187 depleted essentially all internal calcium pools. Either Tg or FCCP reduced the calcium stores that could be released by BK or A23187. Thus, cellular calcium compartments that respond to BK and A23187 partially overlap. The common pool includes Tg- and FCCP-sensitive compartments. Calcium stores were examined in cells from Alzheimer disease patients, because previous studies suggest that their calcium homeostasis is altered. A23187 addition to BK-treated cells produced a 95% greater response in cell lines from Alzheimer patients ($n = 7$) than in those from controls ($n = 5$). Thus, various calcium stores can be pharmacologically distinguished in fibroblasts and at least one of these compartments is abnormal in Alzheimer's disease.

Keywords: Calcium; Alzheimer's disease; Fibroblast; Bradykinin; Mitochondrion

1. Introduction

Although assessment of cytosolic free calcium ($[Ca^{2+}]_i$) is now routine in many laboratories throughout the world, evaluation of calcium in cellular compartments is still difficult and indirect. Cellular calcium compartments can be an important source of calcium following stimulation (e.g., release of calcium from the endoplasmic reticulum) and, in addition, the calcium concentrations within these organelles can regulate calcium-dependent processes. Two well-documented examples of the latter are processing of proteins in the endoplasmic reticulum [1] and mitochondrial oxidation [2,3]. Cross talk exists between these vari-

ous calcium compartments. For example, in some cell types depletion of non-nuclear pools can arrest cell division [4]. In some preparations, such as synaptosomes, external calcium can be the major source of the elevated calcium that accompanies stimulation [5]. However, in many cellular systems, the primary calcium response to stimulation is from internal stores and external calcium is only required for refilling depleted internal calcium pools [6]. Thus, in the absence of external calcium, changes in $[Ca^{2+}]_i$ can be used to monitor internal calcium stores. In the current studies, the internal calcium compartments were evaluated in calcium-free media by sequential addition of compounds that interact with various calcium stores. Bradykinin (BK) activates phospholipase C and subsequently releases IP_3 to release internal calcium stores. BK-sensitive pools were depleted and then the following drugs were added to test whether additional calcium stores could be released: FCCP (an uncoupler of oxidative phosphorylation that collapses the mitochondrial membrane potential and releases mitochondrial calcium), thapsigargin

Abbreviations: Fura-2AM, acetoxymethylester of fura-2; AD, Alzheimer's disease; BK, bradykinin; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; $[Ca^{2+}]_i$, cytosolic free calcium; DMEM, Dulbecco's modified Eagles's medium; SNK, Student-Newman-Keuhls'; Tg, thapsigargin.

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(an inhibitor of the endoplasmic reticulum ATPase), A23187 (a calcium ionophore) or bombesin (an activator of IP_3 production by a different mechanism than BK). This approach allowed delineation of discrete calcium pools within fibroblasts.

Several studies suggest that calcium homeostasis is altered in fibroblasts from Alzheimer's Disease (AD) patients. Recent reports indicate that the abnormal gene in the majority of Alzheimer families (i.e., early-onset AD) is related to 'calcium cycling' within cellular organelles [7]. Previous studies report a reduced ^{45}Ca uptake [8]. Whether a consistently altered response to stimulation occurs in AD fibroblasts has proven difficult to document. Although enhanced sensitivity of $[Ca^{2+}]_i$ to bombesin or BK [9] has been found in AD fibroblasts, the response of $[Ca^{2+}]_i$ to stimulation is controversial. Published reports show decreases [10–12], increases [13] or no change [14,15]. Whether or not resting $[Ca^{2+}]_i$ changes in AD cells is also controversial [10,14,15]. A possible explanation for the lack of reproducible modifications with AD is that internal calcium stores have been variably altered by unique manipulations in the various laboratories. Thus, in the current studies the internal stores of calcium in fibroblasts from a young control are characterized and then one of these internal pools is compared in fibroblasts from Alzheimer patients and age-matched controls.

2. Materials and methods

2.1. Materials

Cultured human fibroblasts were from the Coriell Institute for Medical Research (Camden, NJ). Fura-2AM and 4-Br-A23187 were from Molecular Probes (Eugene, OR). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Gibco Laboratories (Grand Island, NY). Bradykinin (BK), thapsigargin (Tg), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), Hepes and EGTA were from Sigma Chemical Co. (St. Louis, MO). Bovine albumin fraction V was from United States Biochemical Corp. (Cleveland, OH).

2.2. Methods

2.2.1. Cell culture

Fibroblasts were routinely maintained in DMEM supplemented with 10% FBS and incubated at 37°C in a humidified atmosphere with 5% CO_2 in air. For the experiments, confluent cells were subcultured onto 25 mm round glass cover slips at a density of $1 \cdot 10^4$ cells/cm². The comparison of multiple cell lines required that all lines be maintained under highly controlled conditions [16].

A cell line from an apparently normal, young male donor (GM03652, 24 yr) was grown for 3–4 days after subculturing. One day before the experiment the growth

medium was removed and the cells were rinsed and incubated in serum-free DMEM for 24 h. Since some of the cell lines from age-matched control and AD donors did not reach confluence in 3–4 days, the cell lines for these comparisons were allowed to grow for seven days and were not serum-deprived until the day of the experiment. The age-matched control cell lines consisted of four apparently normal donors (AG03524, AG09878, GM04260, AG06010) and one escapee from the Canadian pedigree (AG07657) with an average age of 66 ± 6 yr. An escapee is an individual in that family who has passed the age of onset for AD without symptoms of the disease. The Alzheimer cell lines included three donors from the Canadian pedigree (AG06848, AG06840, AG04159) and four other confirmed Alzheimer donors (AG05809, AG04400, AG07377, AG04401) with an average age of 55 ± 2 yr.

All cell lines were used at similar passage numbers. The studies were done with cells that had cumulative population doubling level of less than 30. In general, these cell lines stop dividing (i.e., phase out) at a cumulative population doubling level of 43. Whether the donor has AD does not affect the cumulative population doubling level to phase out [17–19].

2.2.2. Incubation media

Two different incubation media were used for the experiments on calcium regulation in the cell line from the young donor. The control incubation medium was DMEM with 20 mM-Hepes and 1% BSA similar to the loading medium (see below) and had a calcium concentration of 1.8 mM. Calcium-free medium was made by adding 2.5 mM-EGTA to the control medium, and adjusting the pH to 7.4 at 37°C. This provides a calculated calcium concentration of 116 nM (MaxChelator v 6.63; Chris Patton, Stanford University, Hopkins Marine Station, Pacific Grove, CA 93950-3094).

2.2.3. $[Ca^{2+}]_i$ measurements

Fibroblasts were loaded with 2 μM Fura-2AM in DMEM with 20 mM-Hepes and 1% bovine serum albumin (loading medium) for one hour at room temperature to prevent the sequestration of the dye in organelles [14,20,21]. After the loading incubation, the coverslip was rinsed three times with 37°C medium that was the same as above except that the Fura-2AM was omitted. Even under high magnification, no punctate fluorescence was apparent. Furthermore, excitation scans of these cells showed that no unhydrolyzed fura-2AM was present in the cells. All experiments were performed at 37°C. The cover slips were secured in a Leiden coverslip dish and placed on a temperature controlled (37°C) micro-incubator with 2 ml incubation medium. The micro-incubator was mounted onto the stage of an Olympus inverted microscope equipped with a Nikon 40X UV Fluor objective lens. To minimize the temperature gradient between different parts of the coverslip, the micro-incubator was continuously gassed with 5%

CO₂/95% air at a flow rate of 3 standard cubic feet/h [22].

These studies utilized a photometric method in which the signal from a microscope field was detected with a photomultiplier tube as opposed to imaging. This allowed us to average the response of the field that included up to six cells. Since the approach is much faster than imaging, a continuous monitoring of the response to stimulation can be made. Calcium measurement was initiated after a 3-min equilibration period by alternately exciting the cells with 350 and 380 nm wavelengths five times per second using a PTI Delta scan (Photon Technology International; S. Brunswick, NJ) and emission was measured at 510 nm. Treatments were done by removing about 90% of the incubation medium and then adding it to the drug. The solution was then reapplied to the cells. These manipulations required less than 10 s. Three to four samples were run for each experimental group or cell line on a given day and the experiments were repeated on at least two separate days. $[Ca^{2+}]_i$ was calculated by the method of Grynkiewicz et al. [23] after correction for background fluorescence.

2.2.4. Statistical analysis

Statistical analysis was by analysis of variance followed by Student-Newman-Keuls' test with software from SPSS Inc (Chicago, IL).

3. Results

The effects of external calcium on the temporal response of $[Ca^{2+}]_i$ to BK were determined (Fig. 1). Our previous results [14,20] demonstrate that 10 nM-BK is the optimal concentration to resolve the effects of various treatments on the magnitude and time of peak response. In medium with normal calcium (1.8 mM), 10 nM-BK immediately increased $[Ca^{2+}]_i$ 2.6 fold and then $[Ca^{2+}]_i$ declined to a new equilibrium value that was 36% higher than the baseline after three minutes. Removal of calcium at the time of BK stimulation (acute) did not alter the basal $[Ca^{2+}]_i$ (91 ± 1 nM (control) vs. 88 ± 1 nM (acute)), time to peak (13 ± 1 s) nor the peak $[Ca^{2+}]_i$ (325 ± 22 nM (control) vs. 334 ± 21 nM (acute)), but eradicated the sustained increase above baseline (143 ± 2 nM (control) vs. 83 ± 4 nM (acute) at 3 min after stimulation; $P < 0.05$). Similarly, reduction of the calcium immediately after fura loading (short term): (1) slightly, but significantly, diminished resting $[Ca^{2+}]_i$ (from 91 ± 1 nM to 82 ± 1 nM; $P < 0.05$), (2) did not significantly reduce the peak $[Ca^{2+}]_i$ and (3) abolished the sustained increase after the peak. Thus, the $[Ca^{2+}]_i$ peak after BK was largely independent of external calcium, while the sustained elevation after BK required external calcium. For subsequent experiments, 'short term' was used rather than acute because it is technically easier to control temperature and calcium.

The observation in calcium-free media that the BK-in-

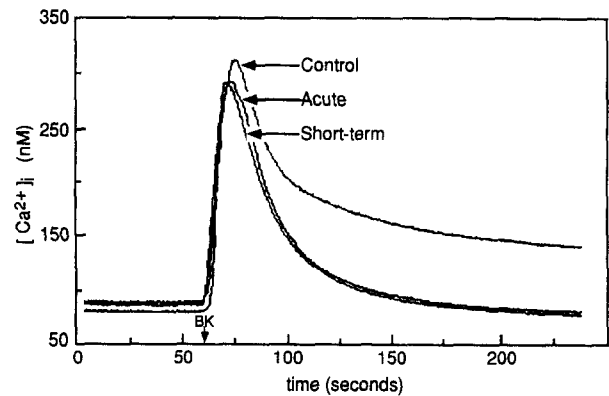


Fig. 1. The temporal response of cellular $[Ca^{2+}]_i$ to bradykinin (10 nM) without external calcium at the time of stimulation (acute) or for 5 min (short term). The calcium in the control medium (1.8 mM) was decreased to calcium-free (i.e., control medium + 2.5 mM-EGTA) either at the time of BK-stimulation (acute) or immediately after loading with fura-2 (short term). Cells from the young donor were loaded as described in 'Methods'. The tracing for each treatment is the average of 8 samples that were run on two different days. On each experimental day, all of the samples from each treatment were measured alternately to prevent any bias. For statistical comparisons, values (means \pm S.E.) were calculated at multiple time points. Control values (nM) were basal (91 ± 1), peak after BK (325 ± 22), 60 s after BK (182 ± 4), 120 s after BK (152 ± 2) and 180 s after BK (143 ± 2). The time to peak in controls was 13 ± 1 s and did not vary with treatment.

duced peak of $[Ca^{2+}]_i$ was primarily related to internal calcium compartments provided a method to evaluate internal stores. The calcium-free media was used in the rinses and all subsequent steps after the fura-2 loading as described for the 'short term' incubations in Fig. 1. The basic design of the experiments was that an agent was added to release the internal calcium stores that were sensitive to that drug. The compound was left in the media to block the refilling of that particular calcium pool. The effects of subsequent addition of other drugs were then determined to delineate various calcium stores.

To characterize BK-insensitive calcium pools, cells were stimulated with BK in calcium-free medium to deplete BK-sensitive stores and then subsequently treated with either BK, thapsigargin, FCCP, A23187 or bombesin (Fig. 2). When used as the initial drug, 10 nM-BK increased $[Ca^{2+}]_i$ by $304 \pm 20\%$ over basal with a peak time = 16 ± 3 s ($n = 6$). By two min $[Ca^{2+}]_i$ returned to baseline. This concentration of BK completely blocked subsequent stimulation with 10 nM-BK as long as BK was still present in the media. If BK was removed and the cells were restimulated with BK, a small response was elicited (results not shown). If BK was followed by A23187, a second peak occurred that was $54 \pm 13\%$ ($n = 6$) above the 3-min value (Fig. 2). Thapsigargin caused a $46 \pm 12\%$ ($n = 7$) elevation, whereas no increase was observed with FCCP ($n = 4$) or bombesin ($n = 6$). Clearly, releasable internal stores of calcium persist after BK stimulation.

To test whether the BK-insensitive, but Tg- or A23187-sensitive pools were a function of the initial con-

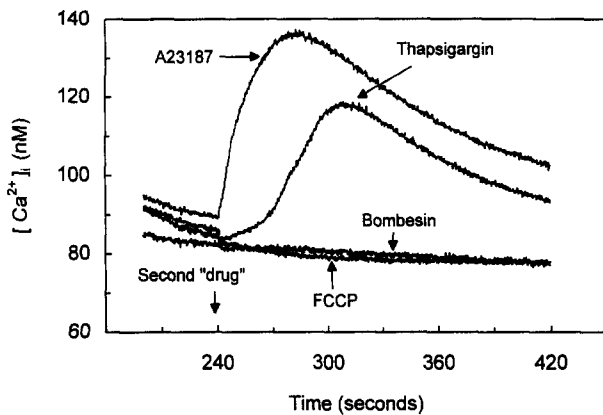


Fig. 2. Characterization of BK-insensitive internal calcium stores. Cells in calcium-free media were first stimulated with BK. The response paralleled the 'short term' tracing in Fig. 1. This tracing only shows the last 40 of the response after bradykinin. 3 min after BK either A23187 (20 μ M; $n = 6$), FCCP (10 μ M; $n = 4$), bombesin (1 μ M; $n = 6$) or thapsigargin (100 nM; $n = 7$) was added. Each tracing is the average of the number of samples indicated in parentheses. No response was observed after a second addition of BK.

centration of BK, multiple BK concentrations were added before Tg or A23187 (Fig. 3). The $[Ca^{2+}]_i$ response to the indicated concentration of BK was determined (\bullet). Increasing the BK concentrations released progressively more $[Ca^{2+}]_i$ from the BK sensitive pool. The maximal peak was at 1 μ M-BK and the response declined at higher concentrations. The effect of 100 nM Tg after each concentration of BK was determined (Δ). The BK-insensitive, Tg-sensitive pool did not decline significantly once the BK concentrations were higher than 10 nM. The response to 20 μ M-A23187 after each concentration of BK was also determined (\square). The BK-insensitive, A23187-sensitive com-

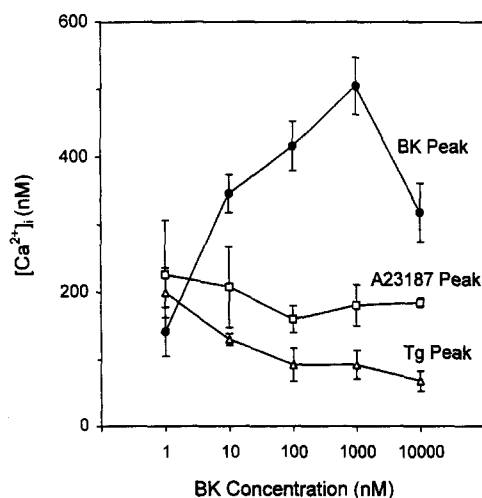


Fig. 3. Characterization of BK-insensitive but Tg- or A23187-sensitive internal calcium stores. BK was added at the indicated concentration. The \bullet represents the initial BK peak. 3 min later, Tg (100 nM) or A23187 (20 μ M) were added. The \square represents the A23187 peak after the addition of BK. The Δ represents the Tg peak after the addition of BK.

partment persisted at all concentrations of BK that were tested.

The response of internal calcium pools to stimulation with the calcium ionophore A23187 was also characterized. Experiments were designed to determine if A23187 released all calcium stores from the fibroblasts, because A23187 released a significant amount of calcium after BK. The responses to 2, 10 and 20 μ M-A23187 were evaluated to determine which concentration should be used. Two μ M-A23187 increased $[Ca^{2+}]_i$ about 40%. Ten and 20 μ M-A23187 increased $[Ca^{2+}]_i$ more than 2 μ M and the increase was similar at both concentrations (330–350%; Data not shown). Because the response at 20 μ M was no greater than 10 μ M, 20 μ M-A23187 was found to be saturating and was used to characterize A23187 sensitive stores. The 20 μ M-A23187 peak was about 10% larger than the peak after BK addition and returned to baseline more slowly (3 min) than following BK (2 min) and continued to slowly decrease. The subsequent addition of BK or FCCP did not increase $[Ca^{2+}]_i$ above the 3 min value. Tg caused a small increase in $[Ca^{2+}]_i$. Tg after A23187 increased $[Ca^{2+}]_i$ by a maximum of $4.4 \pm 0.7\%$. Thus, A23187 depleted essentially all releasable calcium stores.

To determine if any of the Tg-releasable calcium pool overlapped the A23187 and BK compartments, the effects of Tg on the BK- or A23187-stimulated increase in $[Ca^{2+}]_i$ was determined. Thapsigargin gradually increased $[Ca^{2+}]_i$ (Fig. 4). The maximal change was observed after 120 ± 4 s compared to peaks at about 15 s with BK or A23187. The increase over the baseline was $62.2 \pm 2.5\%$ ($n = 14$). $[Ca^{2+}]_i$ did not return to baseline for 10 minutes. Although $[Ca^{2+}]_i$ was still $51 \pm 2\%$ above basal at 3 min, the second stimulation was done three minutes after Tg to allow comparison with the experiments on A23187 and BK. Preliminary studies showed that the BK-response was similar at either 3 or 10 minutes after the initial Tg addition.

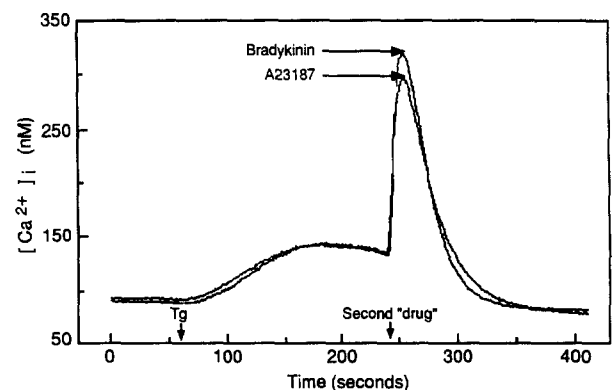


Fig. 4. Characterization of thapsigargin (Tg)-sensitive internal calcium compartment and its relation to BK and A23187-sensitive stores. Cells were stimulated with Tg (100 nM) and 3 min later either BK (10 nM; $n = 7$) or A23187 (20 μ M; $n = 7$) was added. Each tracing is the average of the number of samples indicated in parentheses.

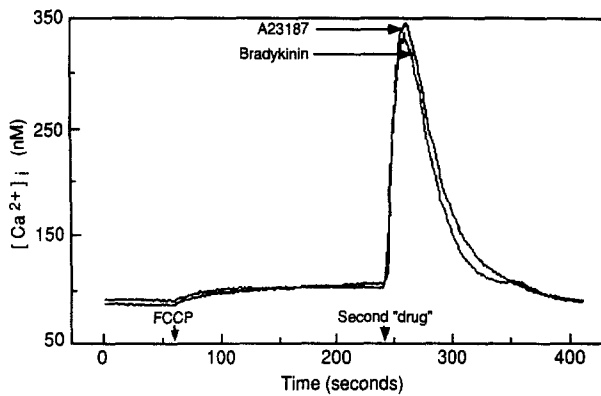


Fig. 5. Characterization of the FCCP-sensitive calcium compartment and its relation to BK and A23187-sensitive stores. Cells were stimulated with FCCP (10 μ M) and 3 min later either BK (10 nM; $n = 4$) or A23187 (20 μ M; $n = 5$) was added. Each tracing is the average of the number of samples indicated in parentheses. Table 1. Altered internal calcium stores in fibroblasts from Alzheimer donors and age-matched controls.

Prior addition of Tg reduced the BK-induced change in $[Ca^{2+}]_i$ from 272 ± 25 nM (above basal) to 201 ± 12 nM (i.e., about a 26% reduction; $n = 7$; $P = 0.008$). In the same manner, Tg reduced the A23187-induced increase in $[Ca^{2+}]_i$ from 306 ± 16 nM to 179 ± 20 nM (i.e., a 41% reduction; $n = 7$; $P = 0.0001$). Thus, Tg diminished the BK- and A23187-releasable stores of calcium.

Although FCCP did not release calcium following either BK (Fig. 2) or A23187 (Fig. 4; data not shown), FCCP releases calcium from internal stores (Fig. 5). Thus, to test if the FCCP-releasable calcium pool overlapped the A23187 or BK compartments, the effects of FCCP on the BK- or A23187-stimulated increase in $[Ca^{2+}]_i$ were determined. FCCP had a less profound effect on $[Ca^{2+}]_i$ than the other compounds that were tested (Fig. 5). FCCP gradually increased $[Ca^{2+}]_i$ by $20.5 \pm 3\%$ ($n = 9$) after 3 min. To allow comparison with the results on the BK- and

A23187-sensitive compartments previously discussed, BK or A23187 was added three minutes after FCCP. The prior addition of FCCP reduced the maximal BK-induced increase from 321 ± 17 nM to 239 ± 16 nM (i.e., a 26% reduction; $P = 0.004$). Prior stimulation with FCCP diminished the response to A23187 from 321 ± 42 nM to 261 ± 16 nM (i.e., a 19% reduction). Thus, although FCCP did not alter $[Ca^{2+}]_i$ by very much, it diminished both BK- and A23187-releasable calcium pools.

Calcium stores in fibroblasts from Alzheimer patients differed from controls (Table 1). Basal values and the changes in $[Ca^{2+}]_i$ following BK stimulation were identical in control and Alzheimer fibroblasts, in agreement with our previous studies [14,20]. However, the response to stimulation with A23187 following BK was exaggerated in the Alzheimer cells. If control cells were first stimulated with BK, the second stimulation with A23187 increased $[Ca^{2+}]_i$ from 90 ± 4 nM to 126 ± 7 nM (i.e., a 40% increase), whereas the $[Ca^{2+}]_i$ in the Alzheimer cells increased from 87 ± 4 nM to 153 ± 17 nM (i.e., a 77% increase). The percent increase in the Alzheimer cells was 92% greater than in the controls.

4. Discussion

The results demonstrate that manipulation of $[Ca^{2+}]_i$ after an initial stimulation with BK, Tg, FCCP or A23187 can clearly distinguish internal calcium stores. Multiple studies have examined calcium compartments in cultured fibroblasts [6,24]. Generally, these experiments concentrated on how these pools – particularly BK sensitive compartments – are refilled after depletion of internal stores [6]. Those results demonstrate that BK and ionomycin deplete BK-sensitive calcium pools and that refilling of these stores requires extracellular calcium. The

Table 1
Altered internal calcium stores in fibroblasts from Alzheimer donors and age-matched controls

	Line	Age	Basal $[Ca^{2+}]_i$	$\Delta [Ca^{2+}]_i$ after BK	Basal $[Ca^{2+}]_i$ 3 min after BK	$\Delta [Ca^{2+}]_i$ after A23187
Aged control	7657 ♀ ^a	88	96 ± 2	497 ± 51	102 ± 3	40 ± 26
	6010 ♀	53	90 ± 1	458 ± 47	92 ± 7	34 ± 1
	3524 ♀	67	92 ± 3	360 ± 53	92 ± 7	42 ± 4
	9878 ♀	61	83 ± 2	286 ± 51	80 ± 8	38 ± 8
	4260 ♂	60	77 ± 1	215 ± 21	85 ± 13	23 ± 1
	mean \pm S.E.	66 ± 5	88 ± 4	363 ± 58	90 ± 4	36 ± 4
Alzheimer	5809 ♀	63	91 ± 3	380 ± 120	88 ± 3	88 ± 8
	4400 ♀	61	86 ± 6	302 ± 75	93 ± 24	94 ± 62
	6848 ♀ ^a	55	81 ± 2	476 ± 65	75 ± 1	43 ± 16
	7377 ♂	47	79 ± 12	272 ± 50	81 ± 14	46 ± 18
	6840 ♂ ^a	56	80 ± 3	330 ± 88	77 ± 5	40 ± 5
	4159 ♀ ^a	52	105 ± 2	365 ± 138	101 ± 3	122 ± 1
	4401 ♀	53	91 ± 6	446 ± 89	89 ± 7	39 ± 3
	mean \pm S.E.	55 ± 2	88 ± 4	367 ± 30	87 ± 4	67 ± 14^b

^a Cell lines from the Canadian family with the chromosome 14 defect [7].

^b Denotes that AD cells are significantly ($P = 0.023$) higher than controls. Cells were first stimulated in calcium-free media with BK. The response paralleled the 'short term' tracing in Fig. 1. 3 min after BK, A23187 (20 μ M) was added. Each cell line was evaluated on multiple days and a median value for each measurement was obtained for that day. The values in the table are means \pm S.E. of the daily medians.

current studies revealed multiple calcium pools and their interactions by examining the effects on $[Ca^{2+}]_i$ after releasing one pool and then testing the effects of subsequent stimulation with BK, A23187, thapsigargin, bombesin or FCCP. Although some of the changes in $[Ca^{2+}]_i$ with the addition of the second drug were small, they likely reflect large changes in particular subcellular compartments. Even though the compartments can be clearly defined, the size of the various calcium pools is more difficult to determine. This is shown by the observation that Tg and FCCP do not release much calcium but diminish the release of calcium after BK or A23187 to a considerable extent. Morphological correlates of these pools cannot be assigned, because none of the compounds that we examined are absolutely specific.

The manipulation of $[Ca^{2+}]_i$ following BK revealed multiple compartments. The peak after BK clearly reflects internal stores under the conditions of these experiments (short time intervals and calcium-free media). This agrees with previous results of our own [14,20] and others [6]. Bombesin and bradykinin appeared to release the same calcium pools, since bombesin was inactive after bradykinin addition and produced a large peak if no BK was present. Although BK released most of the calcium from the A23187 compartment, A23187 still released calcium after BK addition. This is clear evidence of a BK-insensitive, A23187-sensitive pool of calcium in fibroblasts. Following BK, Tg released nearly as much calcium as A23187. Although Tg alone did not release much calcium, Tg addition before BK diminished the action of BK. If the Tg and BK calcium pools were separate, an additive action would have occurred. Since BK is not generally considered to act on mitochondria, the observation that BK released all of the calcium in the FCCP-sensitive pool was surprising. Although FCCP alone did not release much calcium, it diminished the effects of BK. If the FCCP and BK calcium pools were separate, the prior addition of FCCP would not diminish the BK response. These results demonstrate considerable interaction of these various pools.

Sequential addition of the various compounds after A23187 also revealed multiple calcium pools. The entire BK-sensitive compartment was released by A23187. Although Tg after A23187 released minimal calcium, Tg addition before A23187 diminished the action of A23187 considerably. This suggests that there is a large Tg-sensitive compartment that overlaps A23187-sensitive stores. If the Tg and A23187 sensitive store were separate, prior addition of Tg would not be expected to diminish the A23187 response. A23187 released all of the calcium in the FCCP-sensitive pool. Although FCCP alone did not release much calcium, it diminished the effects of A23187. If the FCCP and A23187 pools were separate, an additive effect of the drugs on $[Ca^{2+}]_i$ would have been apparent.

Mitochondrial stores of calcium in cultured fibroblasts have not been previously examined. FCCP collapses mitochondrial proton gradients and diminishes the mitochon-

drial membrane potential. Our previous studies in synaptosomes show that FCCP reduces the mitochondrial membrane potential and diminishes their ability to buffer calcium from outside the cell [25]. The current studies only examined the interaction of the mitochondrial calcium with other internal pools. The lack of an increase in $[Ca^{2+}]_i$ with FCCP after BK or A23187 suggests that both BK and A23187 release some mitochondrial pools of calcium. The observation that FCCP reduces both the A23187- and BK-induced elevation of $[Ca^{2+}]_i$ further supports this possibility. Methods are now evolving to directly study mitochondrial calcium [26] so that this hypothesis can be tested. Although considerable evidence suggests that an oxidative deficit accompanies AD [27], the current experiments did not test whether mitochondrial calcium stores were abnormal in AD fibroblasts.

The current studies demonstrate that internal calcium pools are altered in fibroblasts from Alzheimer patients. The stores that are not released by BK, but are released by A23187, are substantially elevated in Alzheimer fibroblasts. The compartment that is altered by AD probably does not reflect the mitochondrial compartment, since BK releases the mitochondrial store (i.e., FCCP sensitive) and the calcium compartment that is altered in AD is BK insensitive. Previous reports of altered calcium regulation in Alzheimer fibroblasts are controversial [see summary in [28]]. They disagree on whether agonist stimulated changes in $[Ca^{2+}]_i$ are elevated [13], decreased [10–12] or not changed [14,15]. A reasonable explanation of these difficulties in replication is that the internal stores of calcium were variably loaded and released in various laboratories. Since the calcium content of internal stores can regulate the rate of calcium uptake [29], the current results that show increased calcium in internal stores are consistent with our original observation that showed diminished uptake of ^{45}Ca [8] and the studies that showed increased calcium content in these cells [11]. Further support for altered internal calcium stores in AD fibroblasts is shown by findings that demonstrate bombesin-releasable calcium pools are larger in AD fibroblasts and that AD fibroblasts are more sensitive than controls to pM concentrations of BK [9]. Complete elucidation of the AD-related changes in calcium regulation requires comparison of the other calcium compartments. Recent studies suggest that the abnormal gene in AD families with chromosome 14 linkage (the lines listed as Canadian in this study) codes for a protein that is likely to be involved in calcium cycling in internal stores [7]. Thus, the current approach will be useful to examine the pathophysiological importance of this gene defect.

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