

CALCIUM DYSREGULATION AND MEMBRANE DISRUPTION AS A UBIQUITOUS NEUROTOXIC MECHANISM OF SOLUBLE AMYLOID OLIGOMERS

Angelo Demuro¹⁺, Erene Mina²⁺, Rakez Kaye², Saskia C. Milton², Ian Parker^{1*}, Charles G. Glabe^{2*}

From the ¹Departments of Neurobiology and Behavior and ² Department of Molecular Biology and Biochemistry, University of California Irvine, CA 92697, USA.

Running title: Membrane permeabilization by soluble amyloid oligomers.

⁺* These authors contributed equally to this work.

Address correspondence to: Dr. Charles Glabe, 3238 McGaugh Hall, Dept. of Molecular Biology and Biochemistry, UC Irvine, Irvine, CA 92697-3900, Tel. (949) 824-6081; Fax (949) 824-8551: cglabe@uci.edu

Increasing evidence suggests that amyloid peptides associated with a variety of degenerative diseases induce neurotoxicity in their intermediate oligomeric state, rather than as monomers or fibrils. To test this hypothesis and investigate the possible involvement of Ca²⁺ signaling disruptions in amyloid-induced cytotoxicity, we made homogeneous preparations of disease-related amyloids (A β , Prion, IAPP, polyglutamine, and lysozyme) in various aggregation states and tested their actions on fluo-3-loaded SH-SY5Y cells. Application of oligomeric forms of all amyloids tested (0.6 - 6 μ g ml⁻¹) rapidly (~ 5s) elevated intracellular Ca²⁺, whereas equivalent amounts of monomers and fibrils did not. Ca²⁺ signals evoked by A β 42 oligomers persisted after depletion of intracellular Ca²⁺ stores, and small signals remained in Ca²⁺-free medium; indicating contributions from both extracellular and intracellular Ca²⁺ sources. The increased membrane permeability to Ca²⁺ cannot be attributed to activation of endogenous Ca²⁺ channels, because responses were unaffected by the potent Ca²⁺-channel blocker cobalt (20 μ M). Instead, observations that A β 42 and other oligomers caused rapid cellular leakage of anionic fluorescent dyes point to a generalized increase in membrane permeability. The

resulting unregulated flux of ions and molecules may provide a common mechanism for oligomer-mediated toxicity in many amyloidogenic diseases, with dysregulation of Ca²⁺ ions playing a crucial role owing to their strong trans-membrane concentration gradient and involvement in cell dysfunction and death.

Introduction

Alzheimer's disease (AD) is characterized by the appearance in the brain of plaques - containing extracellular deposits of amyloid β -peptide (A β) that result from altered proteolytic processing of amyloid precursor protein - together with intracellular neurofibrillary tangles containing misfolded tau (1). Brain regions with plaques and tangles exhibit reduced numbers of synapses, and neurites associated with plaques and tangles are often damaged, suggesting a pivotal role for A β in the neuropathology of AD (2-5). Moreover, numerous other neurodegenerative disorders (including Huntington's, Parkinson's and prion diseases) are also associated with the formation and accumulation of amyloid fibrils in specific brain areas (6,7). These commonalities suggest a general mechanism of action for the more than 100 human amyloid-related diseases, whereby normally soluble peptides and proteins undergo aberrant folding (8).

Aggregation of A β proceeds through several conformational states, including dimers, spherical oligomers composed of 10-24 monomers and strings of oligomers (protofibrils), before finally assuming an insoluble fibrillar conformation (9). The initial formulation of the "amyloid hypothesis" of AD specifically implicated fibrillar amyloid deposits (10). However, more recent evidence suggests that soluble oligomers may be the principal neurotoxic agent (11-15). Soluble A β oligomers are found in the cerebrospinal fluid of AD patients (12): the soluble A β content of human brain is better correlated with the severity of the disease than are the classical amyloid plaques containing insoluble A β deposits (12,16): and fibril-free oligomers are toxic to cultured cells and neurons (11,15,17). Moreover, soluble oligomeric forms of several amyloids (including islet amyloid polypeptide (IAPP), α -synuclein, prion, and polyglutamine) are respectively implicated as the primary toxic species in type 2 diabetes mellitus, Parkinson's disease, spongiform encephalopathies, and Huntington's disease (6,15,18). This toxicity of amyloid oligomers appears to be intrinsically related to their aggregation state, because oligomeric forms of proteins and peptides that are not disease-related are equally toxic (14,19). Taken together, these findings support the notion of 'conformational disease' (20), whereby a common structural motif formed by otherwise unrelated amyloid oligomers leads to a common mechanism of pathogenesis (14,15). However, it remains to be established precisely which aggregation state is primarily responsible for the neurotoxicity; and difficulties in preparing highly homogeneous and stable populations of monomers, oligomeric intermediates and fibrils may account for observations of highly variable actions among different cells (21).

Questions also remain regarding the mechanism of amyloid toxicity. Growing evidence points to a disruption of intracellular Ca²⁺ homeostasis in AD and other amyloidogenic diseases (1,21,22), and elevated intracellular Ca²⁺ levels are known to trigger apoptosis and/or excessive phosphorylation of key proteins that ultimately

lead to cell death (23-28). Pre-fibrillar amyloid aggregates have been shown to elevate cytosolic Ca²⁺ in neurons (19,21), but such actions have been proposed to be secondary to the generation of reactive oxygen species (1,29); to arise from activation of cell surface receptors coupled to Ca²⁺ influx (22,30-32); or to result from Ca²⁺ influx across the plasma membrane as a result of either cation-selective channels formed by A β itself (21,33) or through a general disruption of lipid integrity (34).

To address these issues we prepared pure samples of monomeric, oligomeric and fibrillar forms of several disease-related amyloids and examined their respective actions on cytosolic Ca²⁺ levels in SH-SY5Y cells. We show that amyloid oligomers consistently produce rapid and dramatic elevations in [Ca²⁺], whereas equivalent concentrations of monomers or fibrils do not. The action of amyloid oligomers appears to involve a channel-independent disruption of the integrity of both plasma and intracellular membranes, and we propose that this may be a neurotoxic mechanism common to many amyloidogenic diseases.

Materials and Methods

Materials - Fluorescent dyes (fluo-3AM, calcein) and thapsigargin were from Molecular Probes (Eugene, OR). Ionomycin, cell culture media and other reagents were obtained from Sigma-Aldrich. Peptides were synthesized as described previously (35). Lyophilized peptides and proteins were resuspended in 50% acetonitrile in water and re-lyophilized.

Preparation of homogeneous populations of amyloid peptide monomers, oligomers and fibrils. - Soluble monomer and oligomers were prepared by dissolving 1.0 mg of peptide in 400 μ l hexafluoroisopropanol (HFIP) for 10-20 min at room temperature. 100 μ l of the resulting seedless solution was added to 900 μ l double-distilled water in a siliconized Eppendorf tube. After 10-20 min incubation at room temperature, the samples were centrifuged for 15 min at 14,000 x G and the

supernatant fraction (pH 2.8-3.5) was transferred to a new siliconized tube and subjected to a gentle stream of N₂ for 5-10 min to evaporate the HFIP. The monomer solutions were used immediately after evaporation of HFIP. For soluble oligomers, the samples were then stirred at 500 rpm using a Teflon-coated micro stir bar for 24-48 h at 22°C. Aliquots (10 µl) were taken at 6-12h intervals for observation by electron microscopy (EM) and size exclusion chromatography (SEC). For prion 106-126, the samples were heated at 65°C for 30min before stirring. Poly Q was heated for 2h at 37°C.

Fibrils were prepared as described above for oligomers, except they were stirred at room temperature for 6-9 days and sodium azide was added (0.02%). The final peptide concentration was 0.3 mg/ml. Fibril formation was monitored by thioflavin T fluorescence and UV light scattering. Once fibril formation was complete, the solutions were centrifuged at 14,000 x G for 20min, the fibril pellet was washed three times with double-distilled water, and then resuspended in the desired buffer. The morphology was verified by negative stain EM as previously reported (34).

Cell culture and dye loading - SH-SY5Y human neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum, glutamine (4 mM), penicillin (200unit/ml), streptomycin (200 µg/ml), and sodium pyruvate (1 µM). Cells were maintained at 37°C in 5% CO₂ and the medium was replaced every 2 days. Cells (~10,000) were plated in 35mm glass-bottom culture dishes (MatTek Corporation) and grown overnight. Loading with the Ca²⁺ indicator fluo-3 was accomplished by incubating with fluo-3-AM (7 µM in Hank's Balanced Salt Solution) for 30 min at room temperature, washing 3 times with HBSS and maintaining at 37°C for 20 min to ensure complete hydrolysis. A similar loading protocol was utilized to load cells with calcein, by incubating with 7 µM calcein-AM.

Fluorescence Imaging - The imaging system consisted of an inverted microscope (Olympus IX 71) equipped with a Leitz 16X

objective. Fluorescence excitation was by a 488 nm argon ion laser, and emitted fluorescence ($\lambda > 510$ nm) was imaged by a cooled c.c.d. camera (Cascade 650, Roper Scientific). Time-lapse images (1 frame s⁻¹) were captured using the MetaMorph software package (Universal Imaging, Westchester, PA), and fluorescence intensities were measured from regions of interest centered on individual cells. Signals are expressed as a pseudo ratio ($\Delta F/F$) of the change in fluorescence (ΔF) divided by the resting fluorescence before treatment (F). A small proportion (9 %) of cells failed to load with fluo-3, having low initial fluorescence and failing to respond to ionomycin. These were excluded from analysis.

Amyloids were applied by pipetting a fixed aliquot (70 µl) of a diluted stock solution into the recording chamber (1 ml volume) directly above the microscope objective. To estimate the resulting concentration experienced by the cells under observation we pipetted the same volume of a fluorescent dye (calcein) into the chamber, and measured the resulting fluorescence in the vicinity of the cells relative to that of the initial, undiluted solution of dye. This calibration yielded a dilution factor of about 5, which was assumed in calculating the effective concentrations of amyloids.

To avoid complications from differences in molecular weights between monomers, oligomers and fibrils, we express these concentrations in units of µg ml⁻¹. As a rough guide, a concentration of 0.6 µg ml⁻¹ Aβ42 corresponds to 200 nM monomer, and approximately 7 nM oligomer and 70 pM fibrils.

RESULTS

Aβ42 oligomers, but not monomers or fibrils, increase intracellular free Ca²⁺ - Homogeneous populations of monomeric, oligomeric and fibrillar Aβ were prepared as described above and characterized by size exclusion chromatography and electron

microscopy. The oligomeric preparation had an approximate molecular mass of 90 kDa, contained very little material of lower molecular mass, and was comprised of spherical vesicles with diameters of 2-5 nm (Figure 1). The monomeric preparation contained no detectable oligomeric aggregates as analyzed by size exclusion chromatography (Figure 1). The morphology of the fibrillar preparations was as previously published (34).

The actions of homogeneous monomeric, oligomeric and fibrillar preparations of soluble A β 42 amyloid were examined by adding aliquots of the samples to fluo-3 loaded SH-SY5Y cells (Figure. 2). Figure 2A illustrates images and corresponding Ca²⁺-dependent fluorescence measurements in a representative cell. Applications of monomers or fibrils at final concentrations of 6 $\mu\text{g ml}^{-1}$ evoked no detectable change in fluorescence, whereas subsequent application of the same amount of oligomer evoked large and rapid (~ 5 s) increases in Ca²⁺-dependent fluorescence. Similar results were obtained in > 200 cells examined. For example, Figure 2B shows mean fluorescence signals from 22 cells, where A β 42 oligomers evoked a fluo-3 signal with a peak increase of $\Delta F/F = 3.3 \pm 0.5$ (SEM).

The fact that monomeric and fibrillar samples were inactive rules out the possibility that the increase in Ca²⁺ was due to trace contaminants in the solvent, because the solvent remained the same over the time of incubation for the conversion of monomer to oligomer and then ultimately to fibrils. Solvent controls in the absence of peptide had no effect on Ca²⁺ levels (data not shown).

Actions of other disease-related amyloids -

To explore the possibility of a common neurotoxic mechanism among amyloidogenic diseases, we selected five disease-related amyloid peptides (A β 42, prion, IAPP, polyglutamine, and lysozyme) and tested the effects of purified monomers, oligomers and fibrils of each of these amyloids on cytosolic Ca²⁺. Figure 3A illustrates representative fluorescence records obtained with IAPP at a concentration of 6 $\mu\text{g ml}^{-1}$. Applications of IAPP monomers and fibrils evoked no

detectable Ca²⁺ signals, even though the same cells showed large increases in fluorescence when subsequently challenged with the Ca²⁺ ionophore ionomycin (Figure 3A, traces a, b). In marked contrast, equivalent amounts of purified IAPP oligomers produced a large fluorescence signal (Figure 3A, trace c), closely resembling the response evoked by A β 42 oligomers.

Mean results obtained with all five amyloids are summarized in Figure 3B. In each case the oligomeric forms evoked large increases in intracellular [Ca²⁺] (respective peak $\Delta F/F$ values A β 42 = 3.3 ± 0.08 ; prion = 2.18 ± 0.1 ; IAPP = 2.45 ± 0.19 ; polyglutamine = 3.58 ± 0.07 ; lysozyme = 2.77 ± 0.1), whereas monomers and fibrils failed to produce any appreciable signal. Among a total of 1320 cells analyzed, 1280 showed Ca²⁺ responses to oligomers, whereas we never observed appreciable ($\Delta F/F > 0.4$) fluorescence increases with monomers or fibrils.

Concentration-dependence of oligomer action -

We next examined the concentration-dependence of the Ca²⁺ signals on oligomer concentration, using IAPP and A β 42 as representative amyloids. The protocol is illustrated in Figure 4A, and involved sequential additions of oligomer to the imaging chamber, resulting in stepwise increases in concentration. Concentrations of IAPP oligomer as low as 0.6 $\mu\text{g/ml}$ (corresponding to 7.5 nM assuming a molecular mass of 80 kDa) already evoked a detectable fluorescence increase, and 12 $\mu\text{g/ml}$ (about 150 nM) produced a maximal response, as assessed by the failure of ionomycin to cause any further increase in fluorescence (not shown). Figure 4B shows mean measurements of relative fluorescence signals evoked by increasing concentrations of A β 42 and IAPP oligomers. Both amyloids showed similar concentration-response relationships, with half-maximal responses at respective concentrations of 2.7 and 3.5 $\mu\text{g/ml}$. These values may, however,

underestimate the true EC₅₀ of the oligomers if the indicator dye were saturated at higher concentrations.

Elevation of cytosolic free Ca²⁺ from Aβ42 oligomers involves both intra- and extracellular sources - To discriminate whether the increase in cytosolic Ca²⁺ concentration evoked by amyloid oligomers arises from influx of extracellular Ca²⁺ across the plasma membrane or from Ca²⁺ liberated from intracellular stores, we reduced the free extracellular Ca²⁺ concentration to very low levels by bathing cells for 20 min in a Ca²⁺-free medium that included 5 mM Mg²⁺ and 10 mM EGTA. Application of Aβ42 oligomers (6 μg/ml) then evoked only a small, slowly rising fluorescence signal, which reached a peak amplitude of about 30% of that in Ca²⁺-containing medium (Figure 5A). Thus, the large, immediate Ca²⁺ increase induced by oligomers appears to result from influx of extracellular Ca²⁺, whereas the remaining small, slow component may arise from intracellular Ca²⁺ liberation.

To confirm the latter interpretation we then depleted the endoplasmic reticulum Ca²⁺ stores of cells bathed in normal [Ca²⁺] medium by applying thapsigargin, a specific SERCA pump inhibitor. Thapsigargin evoked a small rise in the Ca²⁺ signal, consistent with leakage of Ca²⁺ from intracellular stores into the cytosol, but subsequent application of Aβ42 oligomers produced a large, rapid increase, similar to that observed in control cells without thapsigargin treatment (Figure 5B).

We examined the possibility that amyloidogenic peptides may induce Ca²⁺ influx via endogenous Ca²⁺-permeable plasma membrane ion channels (30,31) by applying Aβ42 oligomers (6 μg/ml) in the presence of 20 μM cobalt, a non-specific Ca²⁺ channel blocker (Figure 5A). Fluorescence signals were of almost identical amplitude in the presence (black trace) and absence (gray trace) of cobalt, suggesting that Ca²⁺ entry through cobalt-sensitive plasma membrane channels does not contribute significantly to the oligomer-induced rise in cytosolic [Ca²⁺]. Similarly, the AMPA receptor antagonist, CNQX (40 μM) failed to block Ca²⁺ entry

(22): the mean fluorescence signal evoked by Aβ42 oligomers (6 μg/ml) in the presence of CNQX ($\Delta F/F = 1.80 \pm 0.24$, SEM, n = 32) was similar to that in parallel control experiments ($\Delta F/F = 1.70 \pm 0.27$, n = 47).

Decay of the oligomer-induced fluorescence signal is due to dye leakage - As evident in Figures 2-5, high concentrations of Aβ42 consistently evoked a transient fluorescence signal that decayed over tens of seconds or a few minutes even in the continued presence of amyloid oligomers. The experiments illustrated in Figure 6 indicate that this decay does not reflect a fall in cytosolic free [Ca²⁺], but rather that Aβ42 induces a general increase in membrane permeability, thereby allowing leakage of dye into the extracellular medium.

Firstly, ionomycin failed to induce any rise in fluorescence when applied late in the decay phase of a response to Aβ42 oligomers (Fig. 6A). Secondly, Aβ42 oligomers evoked an almost complete loss of fluorescence from cells loaded with the Ca²⁺- and pH-insensitive dye calcein (Figure. 6B). Finally, measurements of fluorescence in the extracellular fluid adjacent to calcein-loaded cells showed a transient rise synchronous with the abrupt drop in intracellular fluorescence evoked by oligomers (Figure. 6C). Fluo-3 fluorescence also appears in the medium coincident with its loss from the cells (data not shown). Interestingly, ionomycin caused no change in fluorescence of intracellular calcein (Figure. 6B), indicating that the increase in dye permeability induced by Aβ42 oligomers results from a direct action on the cell membrane and is not secondary to a rise in intracellular [Ca²⁺].

The effect of Aβ42 to induce a leakage of anionic dyes was shared by other soluble oligomers. Loss of fluo-3 fluorescence like that illustrated in Fig. 6A was consistently observed with equivalent concentrations of prion peptide (n = 51 cells), IAPP (n = 69), polyglutamine (n = 44) and lysozyme (n = 102).

DISCUSSION

We show that extracellular applications of spherical oligomeric forms of

A β 42 and several other disease-related amyloidogenic proteins and peptides cause an immediate and large increase in cytosolic free [Ca²⁺], whereas equivalent amounts of monomeric and fibrillar forms are without any detectable effect. These results, obtained with highly homogeneous preparations, support a growing body of evidence that the aggregation state of these amyloids, rather than their specific amino acid sequences, is the key factor determining their toxicity (14,15).

Several mechanisms have been proposed to account for the Ca²⁺ mobilizing actions of amyloids in their oligomeric and fibrillar aggregation states. These include a direct interaction with membrane components to destabilize the membrane structure (36-39); insertion into the membrane to form a cation-conducting pore (21,34,40-44); activation of cell surface receptors coupled to Ca²⁺ influx (30-32); and oxidative stress leading to dysregulation of mitochondrial Ca²⁺ homeostasis (1). Among these possibilities, our data favor the hypothesis of a generalized increase in membrane permeability induced specifically by spherical amyloid oligomers. The rise in cytosolic Ca²⁺ following addition of oligomers is so rapid (one or a few seconds) as to preclude it being a secondary effect resulting from metabolic impairment and reduced Ca²⁺ pumping or sequestration. Moreover, several observations argue against Ca²⁺ influx through either endogenous Ca²⁺ membrane channels, or through cation-selective channels formed by the amyloid proteins themselves. Firstly, Ca²⁺ signals were not reduced in the presence of 20 μ M cobalt, a non-specific blocker of many Ca²⁺-permeable channels (45), or after applying CNQX to block Ca²⁺-permeable AMPA receptor/channels (22). Secondly, all the amyloid oligomers we tested induced a rapid leakage of fluorescent dyes from cells loaded with fluo-3 and calcein, in agreement with reports showing release of dye from phospholipid vesicles (46,47). Both fluo-3 and calcein are polyanionic molecules that are not expected to permeate through cation-selective channels, and have molecular masses (respectively, 854 and 622 Da) greater than the size cut-off for most ion channels. Finally,

we note that although the major part of the oligomer-induced Ca²⁺ elevation arose through entry of extracellular Ca²⁺ ions, a remaining component in Ca²⁺-free medium points to liberation of Ca²⁺ from intracellular stores. We thus propose that amyloid oligomers exert an immediate action by increasing the permeability of the plasma membrane, and subsequently penetrate into cells, as previously proposed (19), where they similarly disrupt intracellular membranes to cause leakage of sequestered Ca²⁺.

Given the non-specific increase in membrane permeability, it is probable that amyloid oligomers will disrupt the intracellular homeostasis of many ions and other cellular constituents in addition to Ca²⁺. Their actions on Ca²⁺ signaling are, however, likely to mediate the major cytotoxic effects. Firstly, resting cytosolic free [Ca²⁺] is normally maintained at very low levels despite enormous trans-membrane concentration gradients, so that even a small increase in Ca²⁺ permeability will result in a proportionately large elevation of cytosolic [Ca²⁺]. In agreement, Ca²⁺ signals were evident at concentrations of oligomers that failed to induce any detectable dye leakage. Secondly, Ca²⁺ dyshomeostasis is definitively implicated in both necrotic and apoptotic cell death (23), and amyloid-induced Ca²⁺ changes are directly correlated with cell viability (19). Nevertheless, it remains possible that perturbations of other ions and molecules may contribute to the long term pathogenesis of amyloid diseases. An intriguing possibility in neurological diseases is that an enhanced plasma membrane ion conductance may cause a sustained depolarization and increased electrical excitability, leading to excitotoxic injury.

We observed half-maximal Ca²⁺ elevations with A β and IAPP oligomers at concentrations of about 3 μ g ml⁻¹, and consistent signals remained at 0.6 μ g ml⁻¹ (equivalent to about 7 nM). These concentrations are considerably lower than used in most previous studies, and the Ca²⁺ signals were immediate, rather than rising over several minutes or after long latencies (19,21), possibly reflecting a higher purity of the active

amyloid oligomers in our preparations. In comparison to the levels of soluble A β measured in the brain of AD patients, however, the oligomer concentrations required to evoke immediate Ca²⁺ signals are 100 - 1000 times greater (12). The obvious difference is that whereas high concentrations of amyloid oligomers evoke an acute and dramatic increase in cytosolic free [Ca²⁺], the pathophysiology of AD likely involves the cumulative effects of a much smaller but chronic disruption of Ca²⁺ homeostasis over

several years. Moreover, the Ca²⁺ mobilizing action of A β oligomers may be only one mechanism contributing toward Ca²⁺ signaling disruptions as a common pathway for neurotoxicity in AD. Early-onset AD is associated with mutations in the *presenilin* (*PS*) genes, and neurons from knock-in mice expressing an AD-linked mutation in *PS1* display greatly exaggerated release of Ca²⁺ from intracellular stores (48).

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FIGURE LEGENDS

Fig. 1. Size exclusion chromatography and morphology of pure A β 42 oligomers. The homogeneity and purity of A β 42 monomer and oligomer preparations were analyzed using size exclusion chromatography. Oligomers eluted between 12 and 15 min, which corresponds to an approximate molecular weight of 90,000 Da. Monomers eluted between 22 and 25 min. Inset image shows typical spherical oligomers (~3-5nm in diameter) formed after 48h of stirring.

Fig. 2. A β 42 oligomers, but not monomers or fibrils, elevate intracellular free [Ca²⁺]. (A) Time course of Ca²⁺-dependent fluorescence recorded from a single fluo-3-loaded SH-SY5Y cell in response to sequential application of monomers, fibrils, and oligomers of A β 42 (each at a final concentration of 6 μ g/ml). Inset images of the cell were captured at the times indicated during the trace, and are depicted on a pseudocolor scale with 'warmer' colors on a rainbow scale corresponding to higher fluorescence. (B) Trace shows the average responses from 22 cells, evoked using the same protocol. Error bars indicate \pm 1 S.E.M.

Fig. 3. Ca²⁺ elevations induced by other amyloids depend upon their aggregation state. (A) Fluorescence records illustrating typical responses to applications of 6 μ g/ml of A β 42 monomer (a), fibrils (b), and oligomers (c) Each trace was obtained from a different cell. No responses were observed to monomers or fibrils, even though the same cells gave large signals when subsequently challenged with ionomycin (6 μ M). (B) Pooled data showing mean fluorescence signals (\pm S.E.M.) from 40 - 200 cells evoked by A β 42, prion, IAPP, polyQ, and lysozyme; each at a final concentration of 6 μ g ml⁻¹. Measurements were obtained from records like those in (A). For each amyloid, the histogram bars show responses evoked by monomers, fibrils and oligomers.

Fig. 4. Dose-dependence of A β 42 and IAPP oligomers. (A) Trace show Ca²⁺-dependent fluorescence signals averaged from 26 cells in response to successive additions of IAPP oligomers, resulting in stepwise concentration increases to final levels of 0.6, 2.4, 6 and 12 μ g/ml. Inset images show pseudocolor representations of a single cell, captured at the times indicated by the trace. (B) Mean peak fluorescence signals evoked by different concentrations of A β (red bars) and IAPP (blue bars) oligomers. Data are from 78 cells for IAPP and 85 for A β 42, and are expressed as a percentage of the maximal response evoked by 12 μ g ml⁻¹ oligomer.

Fig. 5. A β 42 oligomer-induced rise in cytosolic [Ca²⁺] involves Ca²⁺ ions from both extracellular and intracellular sources. (A) Ca²⁺ signals were reduced, but not abolished, when A β 42 oligomers (6 μ g ml⁻¹) were applied to cells bathed in Ca²⁺-free medium. The trace shows the mean fluorescence (\pm 1 S.E.M.) from 31 cells. (B) Depletion of ER Ca²⁺ stores does not abolish oligomer-induced Ca²⁺ signals in cells bathed in normal (1.8 mM Ca²⁺) medium. Following pretreatment with 5 μ M thapsigargin, a specific blocker of SERCA Ca²⁺ pumps, applications of 6 μ g ml⁻¹ A β 42 oligomers induced a large, rapid rise in fluorescence with an amplitude ($\Delta F/F = 0.7$) similar to that obtained in the absence of thapsigargin ($\Delta F/F = 0.5$). Trace shows mean \pm 1 S.E.M. of results in 38 cells. (C) Ca²⁺ signals evoked by A β 42 oligomers are not reduced by cobalt, a nonspecific Ca²⁺ channel blocker. Cells were pretreated with 20 μ M cobalt for 10 min. Subsequent application of 6 μ g/ml of A β 42 oligomers evoked a mean Ca²⁺-dependent fluorescence signal (black trace) of similar amplitude and kinetics to that in the absence of Cobalt (gray trace). Data with cobalt were obtained from 27 cells. Control data (gray trace) are reproduced from Figure. 3A, trace c.

Fig. 6. A β 42 oligomers cause a general increase of membrane permeability. (A) The decline of fluo-3 fluorescence following high doses of A β 42 oligomers results from dye leakage, not a decline of cytosolic [Ca²⁺]. Trace shows fluo-3 fluorescence from a single cell challenged with the Ca²⁺ ionophore ionomycin following decay of the fluorescence signal evoked by A β 42 oligomers. (B) A β 42 oligomers induce cellular leakage of the Ca²⁺-insensitive dye calcein. Trace shows mean fluorescence monitored from the soma of cells loaded with calcein by incubation with AM ester. Application of ionomycin failed to cause any change in fluorescence, whereas subsequent addition of 6 μ g ml⁻¹ of A β 42 oligomers resulted in almost complete loss of cellular fluorescence. The record is a mean (\pm 1 S.E.M.) from 57 cells, and the inset images show representative fluorescence of a group of cells captured at the times marked on the trace. (C) Loss of intracellular calcein is accompanied by a rise in extracellular fluorescence. Traces show representative examples from three cells, illustrated (before addition of oligomer) in the panels at the right. In each case fluorescence was monitored from the cell soma (black traces) and from regions of interest positioned just outside the cells (red traces: regions marked by white outlines on the images).

Figure 1.

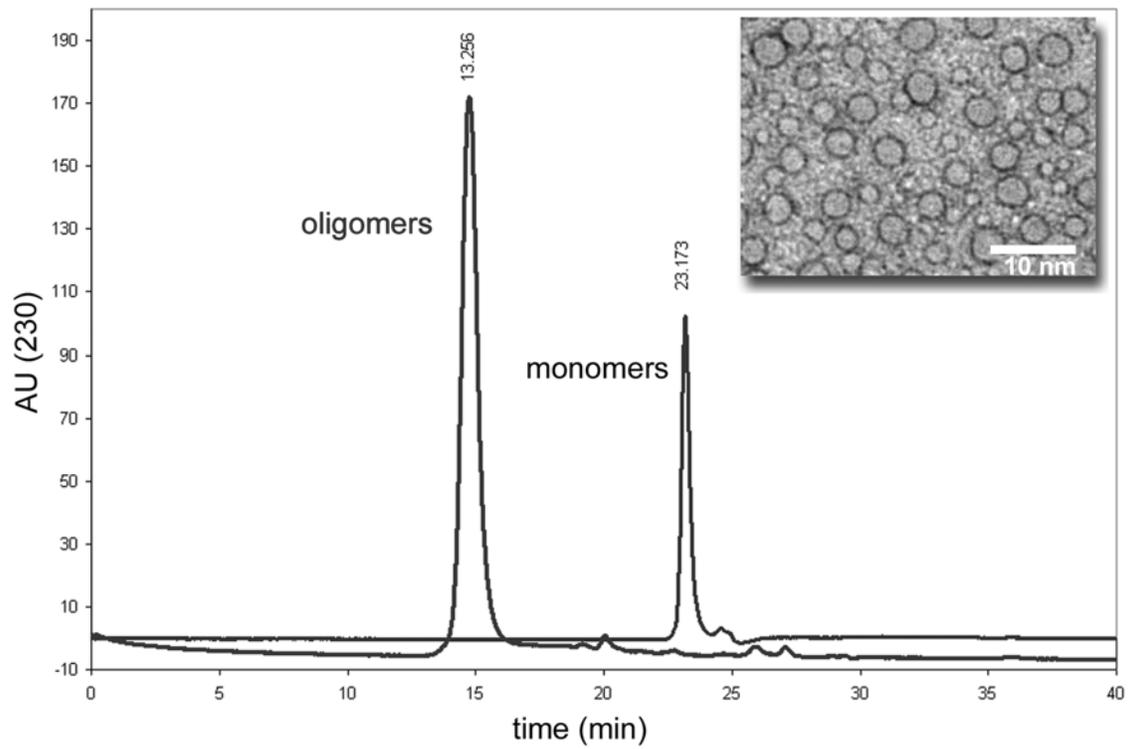


Figure 2.

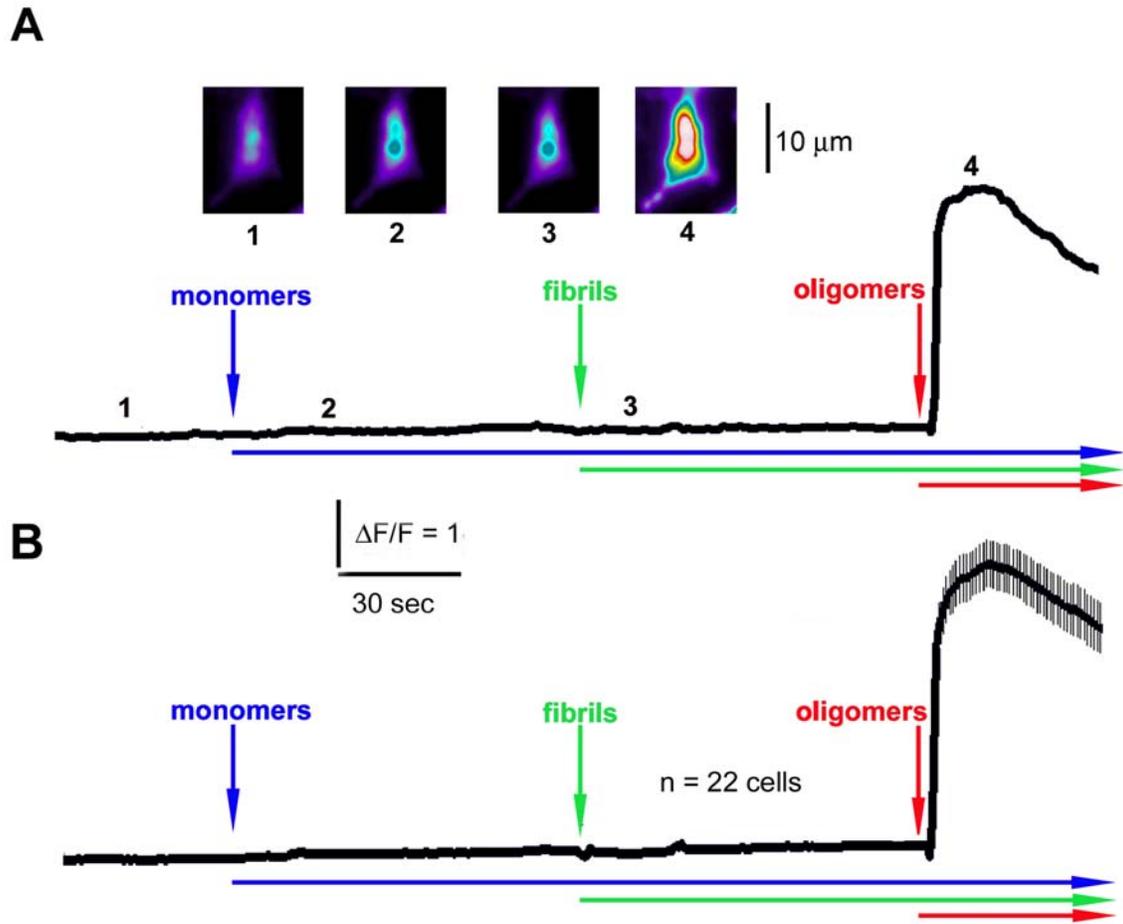


Figure 3.

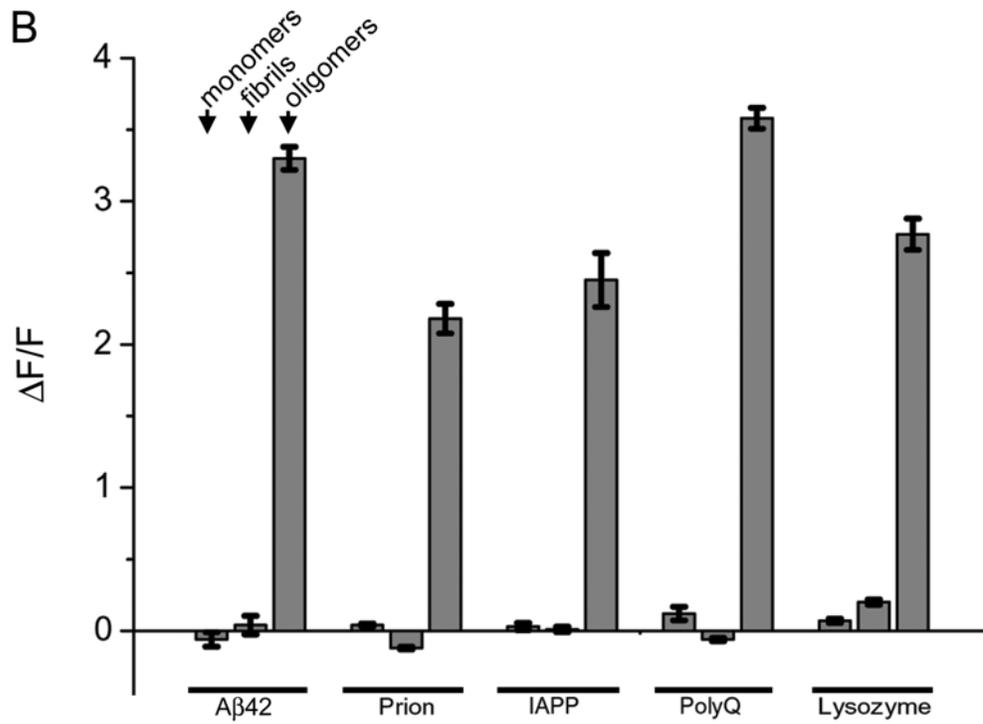
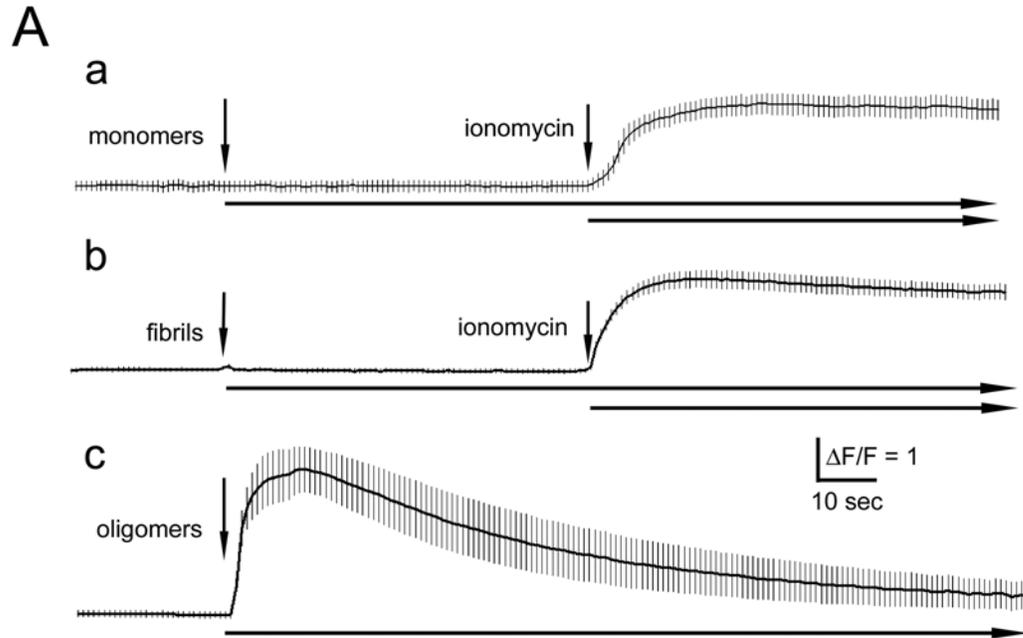


Figure 4.

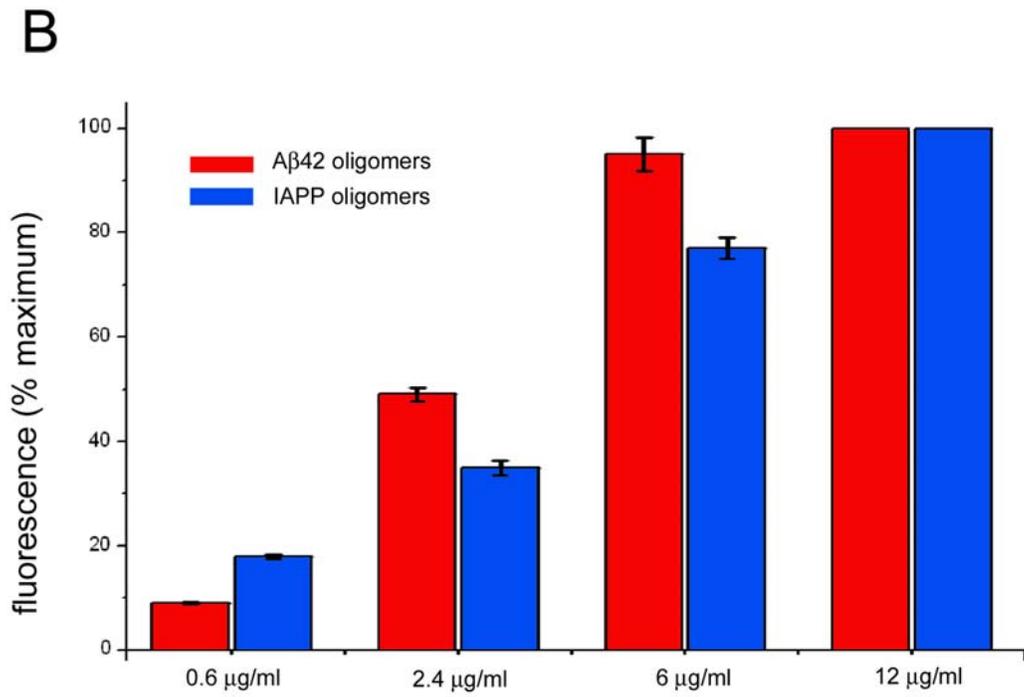
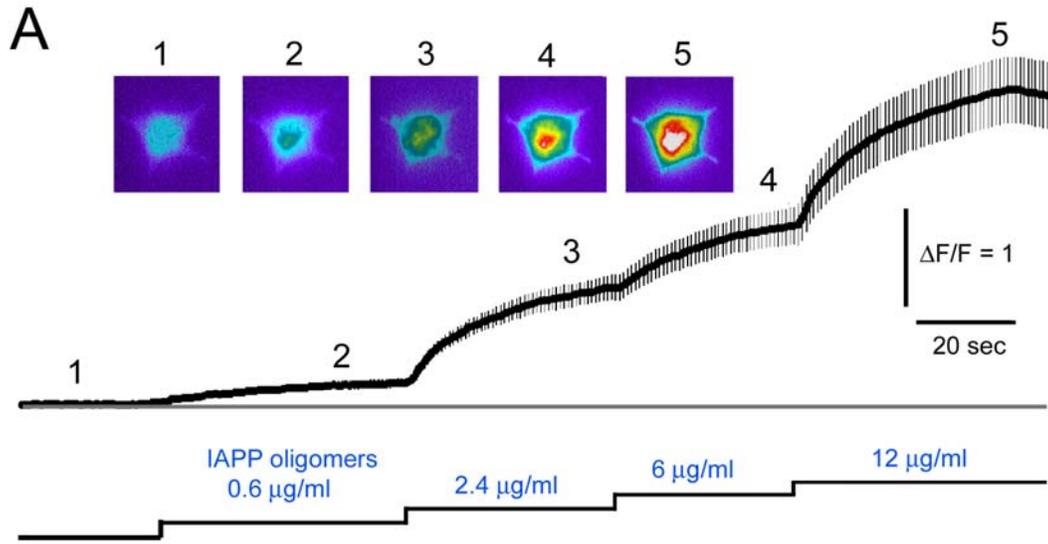


Figure 5.

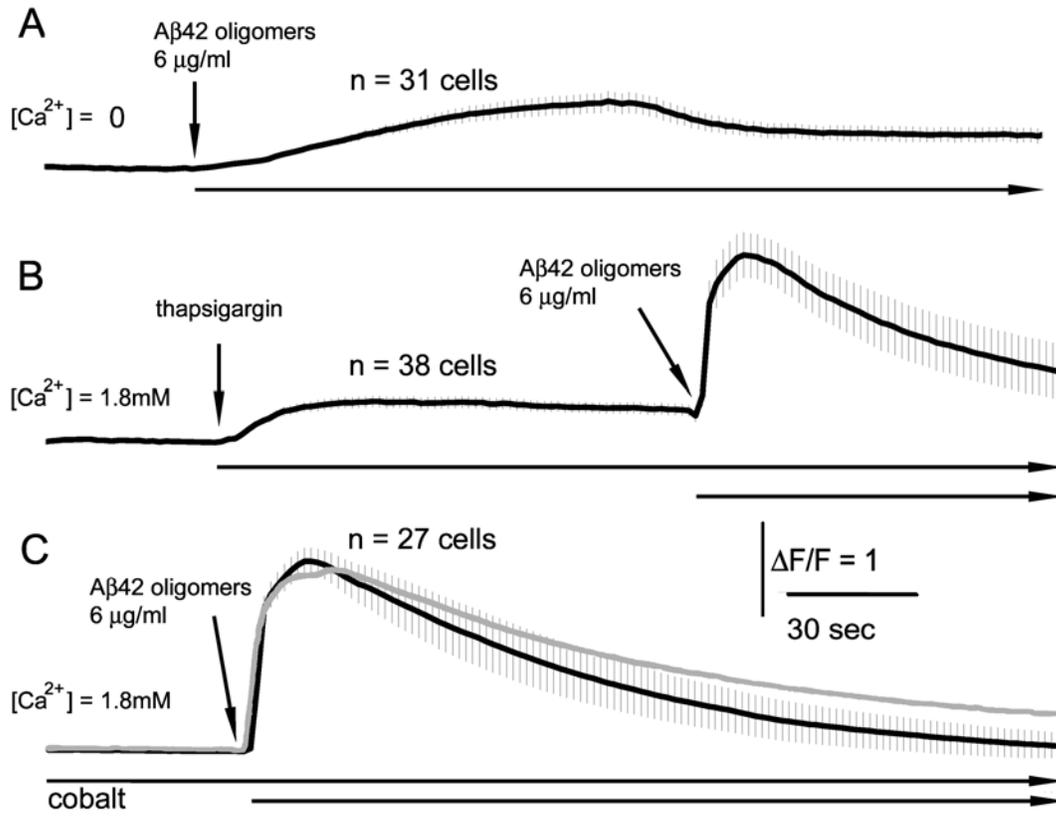


Figure 6.

