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Research paper

A new procedure for amyloid β oligomers preparation enables the unambiguous testing of their effects on cytosolic and mitochondrial Ca^{2+} entry and cell death in primary neurons

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HIGHLIGHTS

- How oligomers of amyloid β peptide ($Aβ_o$) kill neurons in AD remain controversial.
- $Aβ_o$ may promote Ca^{2+} influx but solvents used for oligomerization may do it as well.
- Different procedures, solvents and supplements were used for oligomer preparation.
- New small oligomers promote Ca^{2+} influx, mitochondrial Ca^{2+} overload and apoptosis.
- Results foster role of mitochondrial Ca^{2+} overload in Alzheimer's disease.

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ABSTRACT

Oligomers of the amyloid β peptide ($Aβ_o$) are becoming the most likely neurotoxin in Alzheimer's disease. Controversy remains on the mechanisms involved in neurotoxicity induced by $Aβ_o$ and the targets involved. We have reported that $Aβ_o$ promote Ca^{2+} entry, mitochondrial Ca^{2+} overload and apoptosis in cultured cerebellar neurons. However, recent evidence suggests that some of these effects could be induced by glutamate receptor agonists solved in F12, the media in which $Aβ_o$ are prepared. Here we have tested the effects of different media on $Aβ_o$ formation and on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in rat cerebellar and hippocampal cell cultures. We found that $Aβ_o$ prepared according to previous protocols but solved in alternative media including saline, MEM and DMEM do not allow oligomer formation and fail to increase $[Ca^{2+}]_{cyt}$. Changes in the oligomerization protocol and supplementation of media with selected salts reported to favor oligomer formation enable $Aβ_o$ formation. $Aβ_o$ prepared by the new procedure and containing small molecular weight oligomers increased $[Ca^{2+}]_{cyt}$, promoted mitochondrial Ca^{2+} overload and cell death in cerebellar granule cells and hippocampal neurons. These results foster a role for Ca^{2+} entry in neurotoxicity induced by $Aβ_o$ and provide a reliable procedure for investigating the Ca^{2+} entry pathway promoted by $Aβ_o$.

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder associated to excess of amyloid β ($Aβ_{1-42}$) peptide and deposit of amyloid plaques [1]. In the last few years, small soluble assemblies (oligomers) of $Aβ_{1-42}$ ($Aβ_o$) are becoming the proximate neurotoxin in AD [1–4]. However, mechanisms by which $Aβ_o$ promote cell death in AD remain controversial. Early reports proposed that $Aβ_o$ neurotoxicity was associated to enhanced Ca^{2+}influx [5,6] by a yet unknown pathway. Consistently, we showed that $Aβ_o$, but not fibrils, promote Ca^{2+} influx into rat cerebellar granules leading to mitochondrial Ca^{2+} overload and neuron cell death [7,8]. Furthermore, it was proposed that $Aβ_o$ activates NMDA receptors to induce Ca^{2+} entry into neurons [9]. However, Zempel et al. reported that effects of $Aβ_o$ [10] could be contaminated by the presence of glutamate receptor agonists in the F12 medium used for oligomer preparation. In fact, a survey of publica-

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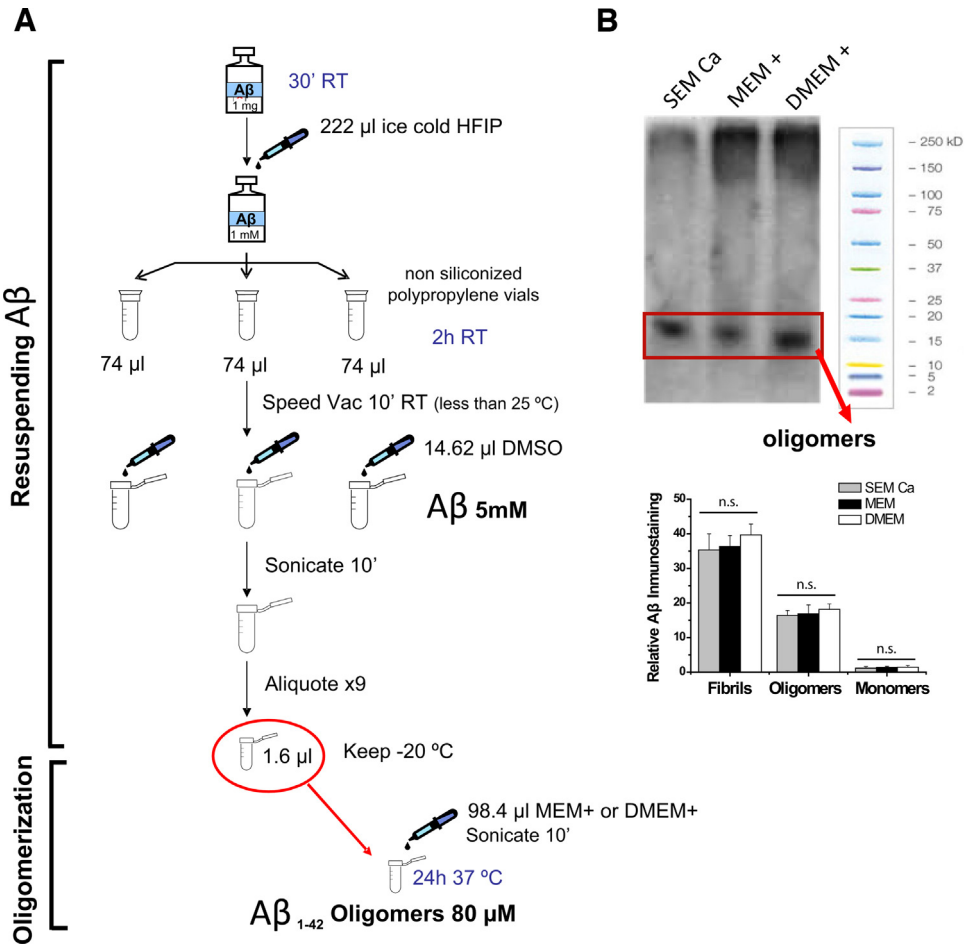

Fig. 1. New procedure for $A\beta_{1-42}$ oligomer formation. A. $A\beta_{1-42}$ (1 mg) is allowed to equilibrate at RT for 30 min and then solved in 222 µl of ice cold 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) for a final concentration of 1 mM. The solution is quickly spared in aliquots of 74 µl in 3 eppendorf tubes (cap closed) and incubated for 2 h at RT. Next, vials are opened and $A\beta_{1-42}$/HFIP solution is concentrated using a speedVac centrifuge (800 g × 10 min at RT but less than 25 °C). Peptide film is solved in 14.6 µl of DMSO to reach a concentration of 5 mM. Solutions are sonicated for 10 min, distributed in 1.6 µl aliquots and frozen at −20 °C. For oligomerization, one aliquot is unfrozen and 98.4 µl of supplemented oligomerization medium (DMEM, MEM or saline) are added to bring the peptide to a concentration of 80 µM. Then, solutions are sonicated for 10 min and incubated at 37 °C for 24 h before use. B. Western blot analysis of samples prepared as stated above. Molecular weight ladder is shown at right. Bars are relative integrated optical density values of Aβ immunostaining corresponding to fibrils (>100 kDa), oligomers (10–20 kDa) and monomers (<5 kDa) present in samples prepared in different media (SEM Ca, MEM+ and DMEM+). Data are mean ± se of 3 experiments (n.s. $p > 0.05$).

tions on amyloid β oligomers shows that nearly 40% of publications used F12 medium as solvent for oligomer formation containing significant concentrations of glutamate (0.06–6.50 µM) and glutamine (3–312 µM) in the experiments. Other publications (14%) use culture media without reporting glutamine concentrations. The remaining publications employed PBS or water as solvent. The above results may challenge many reports including our own on the effects of $A\beta_o$ on $[Ca^{2+}]_{cyt}$ and the role of NMDA receptors [5–9]. Here we have investigated the influence of different solvents on oligomer formation and on $[Ca^{2+}]_{cyt}$ in rat cerebellar and hippocampal neurons. In addition, we have modified Kleinís protocol to use alternative media for oligomer preparation devoid of glutamate and supplemented with selected salts that favor oligomer formation. Finally, the effects of the new $A\beta_o$ preparations were tested on cytosolic and mitochondrial $[Ca^{2+}]$ and on cell death in cultured cerebellar and hippocampal neurons.

2. Materials and methods

2.1. Reagents

Fura2/AM, Lipofectamine 2000 and coelenterazine n were purchased from Invitrogen (Barcelona, Spain). DMEM-F12 (ref: 21041-025), MEM (ref: 51200-046), fetal bovine serum, horse serum, neurobasal medium, B27, penicillin and streptomycin are from Gibco (Madrid, Spain). DMEM (ref. BE12-917F) is from Lonza (Barcelona, Spain). Papain solution is from Worthington (Lakewood, NJ). $A\beta_{1-42}$ and $A\beta_{42-1}$ peptides were purchased from Bachem AG (Bubendorf, Switzerland). The mGA plasmid was kindly donated by P. Brulet (CNRS, Gif-sur-Yvette, France). Poly-D-lysine and Annexin V are from BD (Madrid, Spain). Antibodies against $A\beta_{1-42}$ are from Chemicon International (Millerica, MA). Dispasa II is from Roche (Basel, Switzerland). Other reagents are from Sigma or Merck.

2.2. Primary rat cerebellar and hippocampal neuron culture

Wistar rats were obtained from the Valladolid University animal facility and treated according to the Valladolid University Ethical Committee. Cerebellar granule cells were obtained from 5-day old Wistar rat pups killed by decapitation [7]. Granule cells were plated on poly-L-lysine coated, 12 mm diameter glass coverslips and cultured in high-glucose, low K^+, DMEM plus 10% FBS, 5% horse serum, 100 u/ml penicillin and 100 µg/ml streptomycin for 1 day. Then the culture medium was replaced by Sato's medium plus 5% horse serum [7]. Cells were cultured for 2–4 days before experi-

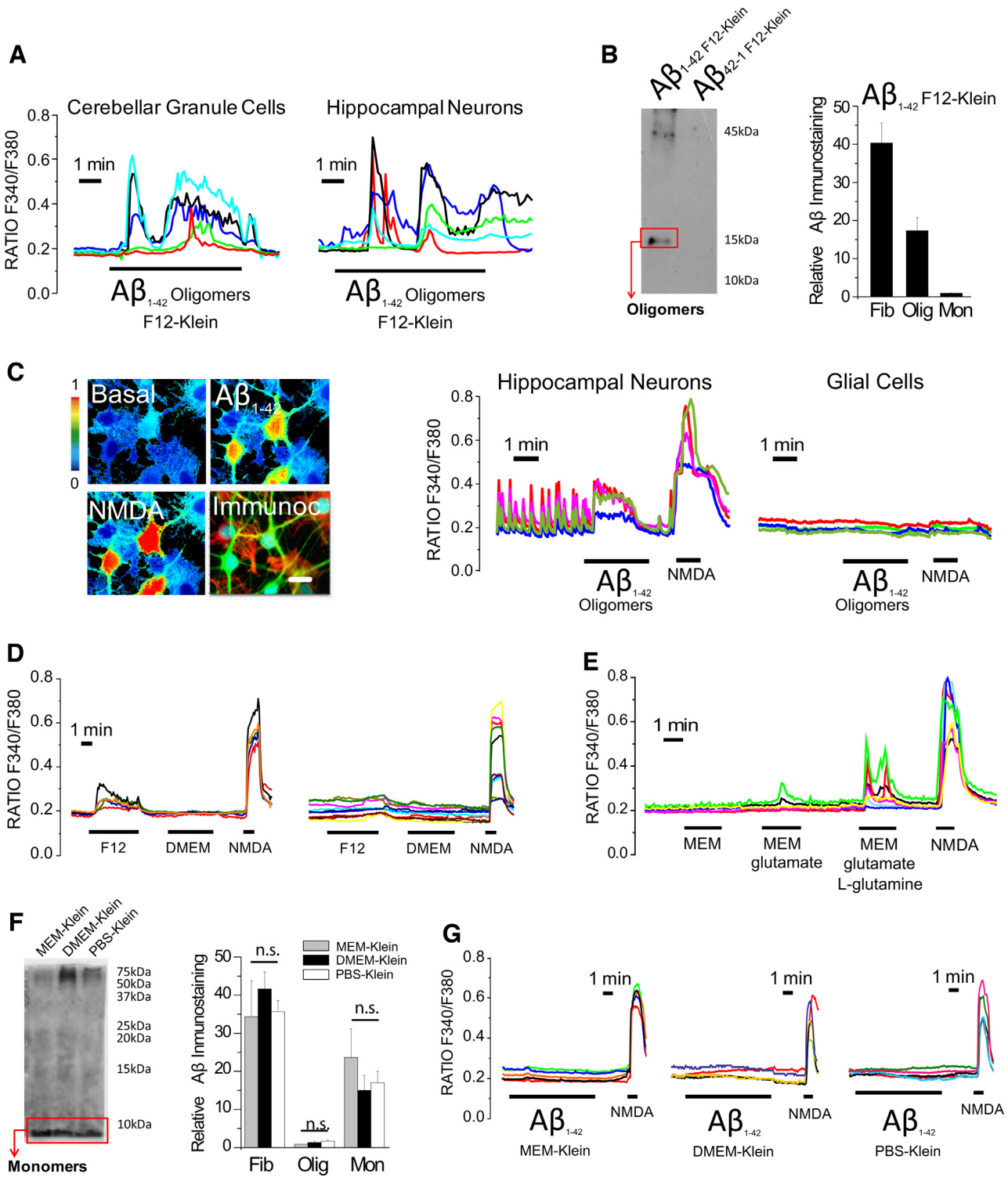

Fig. 2. Effects of $A\beta_{1-42}$ oligomers prepared in DMEM-F12 on $[Ca^{2+}]_{cyt}$ in cultured neurons. A. $A\beta_{1-42}$ oligomers (500 nM) were prepared by the Klein's protocol and effects on $[Ca^{2+}]_{cyt}$ were tested on cerebellar and hippocampal neurons, respectively ($n = 181–231$ cells, 6 experiments). B. Western blotting analysis of $A\beta_{1-42}$ and $A\beta_{42-1}$ preparations. Bars are relative Aβ immunostaining as in Fig. 1 (mean ± se, $n = 3$) C. Calcium images of fura2 loaded neurons before (Basal) and after treatment with Aβ oligomers (500 nM $A\beta_{1-42}$) and NMDA 100 μM. Double immufluoroescence (Inmunoc) for β-tubulin III (green, neurons) of GFAP (red, glia). Traces are representative $[Ca^{2+}]_{cyt}$ recordings of hippocampal neurons and glial cells ($n = 117$ cells, 3 experiments). D. Effects of solvents F12, DMEM and NMDA 100 μM on $[Ca^{2+}]_{cyt}$ in hippocampal neurons ($n = 112$ cells, 12 independent experiments). E. Effects of solvents MEM, MEM supplemented with glutamate (1.25 μM), MEM supplemented with glutamate (1.25 μM) and glutamine (62.5 μM) and NMDA 100 μM on $[Ca^{2+}]_{cyt}$ in hippocampal neurons ($n = 45$ cells, 3 experiments). F. Western blotting analysis of $A\beta_{1-42}$ samples made according to Klein's protocol but prepared in solvents MEM, DMEM and PBS. Bars are relative Aβ immunostaining as in Fig. 1 (mean ± se, $n = 3$), n.s. $p > 0.05$. G. Efects of oligomer preparations shown in F on $[Ca^{2+}]_{cyt}$ in hippocampal neurons (representative of 82–103 cells, 16 experiments).

ments. Hippocampal neurons were prepared from P0 Wistar rat pups as reported [11,12]. Briefly, after brain removal, meninges were discarded and the hippocampus was separated from cortex. Hippocampal tissue was cut in small pieces, transferred to papain solution (20 u./ml) and incubated at 37 °C for 30 min with gentle shaking. Tissue pieces were washed with Neurobasal medium and dissociated into single cells. Hippocampal cells were plated onto poly-D-lysine coated, 12 mm diameter glass coverslips at 40×10^3 cells/dish, and grown in Neurobasal medium supplemented with 2% B27 and 10% FBS without medium exchange as reported [11,12]. Cells were cultured for 7–10 days before experiments.

2.3. Preparation of amyloid β oligomers

$A\beta_{1-42}$ oligomers ($A\beta_o$) were prepared by two different protocols. The first one was reported previously by Dahlgren et al. [4,13]. The second one is a modified version as indicated below (Fig. 1). First, 1 mg of $A\beta_{1-42}$ is allowed to equilibrate at room temperature (RT) for 30 min and then solved in 222 μl of ice cold 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to reach a concentration of 1 mM. The solution is quickly distributed in aliquots of 74 μl in three vials (cap closed) and incubated for 2 h at RT to allow monomerization. Next vials are opened and $A\beta_{1-42}$/HFIP solution is concentrated using a SpeedVac centrifuge (800 g × 10 min at RT). The peptide film is solved in 14.6 μl of DMSO. After sonication (10 min), the solution is distributed in 1.6 μl aliquots and frozen at −20 °C. For oligomerization, aliquots are unfrozen and 98,4 μl of oligomerization medium (DMEM, MEM or saline) supplemented with cupric, ferric and zinc sulfates ($FeSO_4$–$7H_2O$, 0.15 μM, $CuSO_4$–$5H_2O$, 5.2 nM and $ZnSO_4$–$7H_2O$, 0.15 μM) is added to bring the peptide to a final concentration of 80 μM. Saline external solution (SEM) is made of (in mM) NaCl 145, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, glucose 10 and HEPES 10 (pH 7.4). Finally, samples are sonicated for 10 min and incubated at 37 °C for 24 h before use or characterization by western blotting. As in the original protocol no further step is intended to separate fibrils from oligomers.

2.4. Western blotting

In order to assess the aggregation states of the peptide under our bioassay conditions, a standard 15% PAGE-SDS was prepared [14]. Briefly, 5 μl samples of either $A\beta_{1-42}$ oligomers or $A\beta_{42-1}$ preparations (80 μM) were incubated with 1 × loading buffer. Samples were loaded in the gel without boiling and then it was run at constant amperage of 40 mA and transferred to nitrocellulose. Membranes were then blocked for 1 h in a solution of 5% nonfat-dry milk in TBS-T. Clone wo-2 was used as primary antibody against amyloid β (mouse monoclonal 1:1000) and IgG antimouse (goat) conjugated to biotin as secondary antibody (1:500). Finally, anti-biotin-peroxidase chromogen was used. Pierce ECL western blotting substrate kit was used (Thermo Fisher Scientific). Molecular mass was estimated by Rainbow molecular weight markers (Bio-Rad).

2.5. Fluorescence imaging of cytosolic [Ca^{2+}]

Cells were incubated in SEM and loaded with fura2/AM (4 μM) for 60 min at RT. Coverslips were placed on the perfusion chamber of a Zeiss Axiovert 100 TV, perfused continuously with SEM warmed at 37 °C and epi-illuminated alternately at 340 and 380 nm excitation lights. Light emitted at 520 nm was recorded every 5 s with a Hamamatsu ER camera (Hamamatsu Photonics France). Pixel by pixel ratios of consecutive frames were captured and $[Ca^{2+}]_{cyt}$ of regions of interest corresponding to individual neurons were expressed as the ratio of fluorescent emission following excitation at 340 and 380 nm [7,12]. For differential identification of responsive cells, β tubulin III (neurons) and glial fibrillary acidic protein (GFAP, glia) were assessed in the same cells by indirect immunofluorescence [7]. For this end, cells were fixed after imaging with p-formaldehyde and incubated with anti β tubulin III (1:300) and anti GFAP (1:300) for 1 h at 37 °C. Then, cells were washed and incubated with 1:100 labeled anti IgG antibodies. Nuclei were stained by incubation with DAPI 0.2 mg/ml for 5 min.

2.6. Bioluminescence imaging of mitochondrial [Ca^{2+}]

Cultured neurons were transfected with the mitGAmut plasmid using lipofectamine® 2000. The mitGAmut probe contains a low affinity aequorin targeted to mitochondria and a GFP sequence to select transfected neurons. 24 h after transfection, cells were incubated for 2 h with 4 μM coelenterazine n at RT to enabling Ca^{2+}-dependent light (bioluminescence) emission [15,16]. Cells were then washed and placed into a perfusion chamber under a Zeiss Axiovert S100 TV microscope. During bioluminescence imaging, cells were perfused at 5–10 ml/min with test solutions made in SEM and warmed at 37 °C. After the experiment, cells were permeabilized with 0.1 mM digitonin in 10 mM $CaCl_2$ to release all the residual aequorin counts, a parameter required for calibration. Bioluminescence images were taken with a Hamamatsu VIM photon counting camera handled with an Argus-20 image processor. Photonic emissions were integrated for 10 s periods using the Hamamatsu Aquacosmos software and converted into mitochondria free Ca^{2+} concentration ($[Ca^{2+}]_{mit}$) values [15,16].

2.7. Cell death and apoptosis

For cell death, cerebellar granule cells were treated with or without $A\beta_o$ 500 nM overnight. Then, dead cells were estimated by staining with fluorescein diacetate (50 μg/ml, 3 min in PBS) and propidium iodide (20 μg/ml, 30 s in PBS) and assessed by fluorescence microscopy using a Nikon Eclipse 80i microscope (objective 20×). For apoptosis, hippocampal neurons were treated for 1 h with or without $A\beta_o$ 500 nM and then neurons were washed once and then returned to the original Neurobasal medium for 24 h. Cells were then washed with phosphate buffered saline (PBS) and apoptosis was evaluated using Annexin V (1:20, 10 min) in annexin binding buffer 1× (in mM) NaCl, 140; $CaCl_2$, 2.5; Hepes, 10 (pH 7.4) and assessed by fluorescence microscopy using a Nikon Eclipse TS100 microscope (objective 40×).

2.7. Statistical analysis

When only 2 means were compared, student's t test was used. For more than 2 groups, statistical significance of the data was assessed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests using Origin Lab 7.0. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Effects of Aβ oligomers prepared by previous protocol in DMEM-F12 medium and of DMEM-F12 medium alone on [Ca^{2+}]$_{cyt}$

Initially, $A\beta_o$ were prepared according to the original protocol reported by Klein and co-workers where oligomerization takes place in F12 medium [4]. Oligomers prepared from $A\beta_{1-42}$ promote large rises in $[Ca^{2+}]_{cyt}$ in rat cerebellar granules (Fig. 2A) and hippocampal neurons. This protocol produces both oligomers and fibrils of $A\beta_{1-42}$ as shown by western blotting (Fig. 2B). These results are similar to those previously reported by us and other authors [4–7]. However, Zempel et al. [10] showed recently that DMEM-F12 medium alone induces small $[Ca^{2+}]_{cyt}$ increases as well,

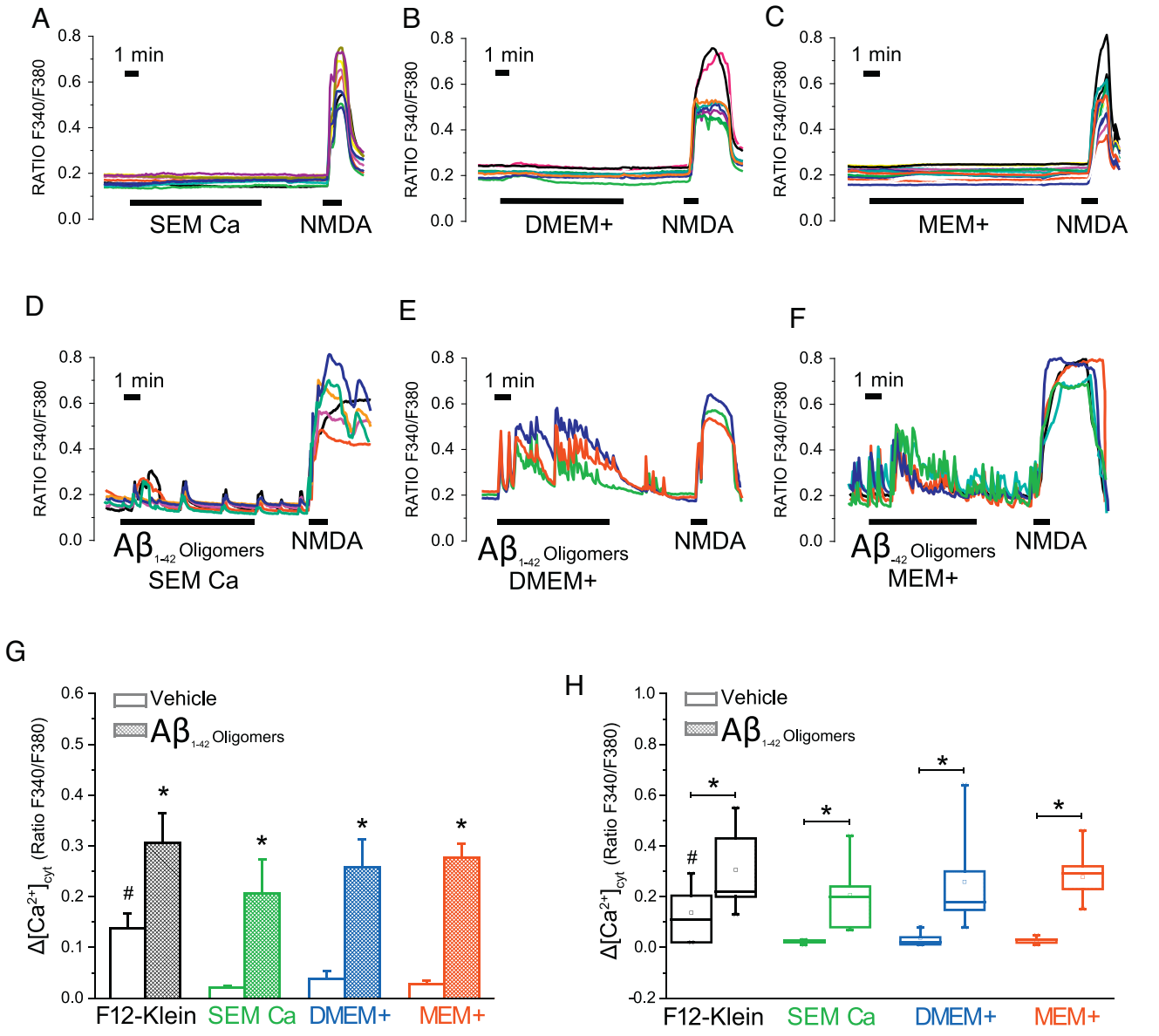

Fig. 3. $A\beta_{1-42}$ oligomers prepared by the new protocol in supplemented media, but not supplemented media alone, increase $[Ca^{2+}]_{cyt}$ in hippocampal neurons. $A\beta_{1-42}$ oligomers were prepared by the new protocol with different supplemented media SEM 2 mM Ca^{2+}, DMEM+ and MEM+. Effects of the supplemented media alone (A–C), oligomers (500 nM) prepared in them (D–F) and NMDA 100 μM on $[Ca^{2+}]_{cyt}$ in hippocampal neurons representative of 96–108 cells studied in 26–31 independent experiments. G. Bars $\Delta[Ca^{2+}]_{cyt}$ induced by different solvents alone (vehicle) and oligomers prepared in them. Data are mean ± se of 112 cells, 12 experiments (F12-Klein vehicle), 231 cells, 6 experiments (F12-Klein oligomers); 104 cells, 10 experiments (SEM Ca vehicle), 53 cells 4 experiments (SEM Ca oligomers); 96 cells, 9 experiments (DMEM+ vehicle), 263 cells, 17 experiments (DMEM+ oligomers); and 108 cells, 12 experiments (MEM+ vehicle), 121 cells, 11 experiments (MEM+ oligomers). H. Box plots of data from G are shown to compare $\Delta[Ca^{2+}]_{cyt}$ induced by the above treatments $^*p < 0.05$ *vs.* vehicle. #$p < 0.05$ *vs.* solvents.

thus rising doubts about the actual Ca^{2+} mobilizing effects of $A\beta_o$. In our hands, DMEM-F12 medium alone, but not DMEM, also increases $[Ca^{2+}]_{cyt}$ in some cases (Fig. 2D). Analysis of DMEM-F12 composition reveals it contains glutamate (50 μM) and glutamine (2.5 mM). This medium is diluted about 50 fold when presented to cells as a solvent of oligomers during recordings but it still may potentially activate glutamate receptors. In contrast, DMEM lacks glutamate receptor agonists and does not increase $[Ca^{2+}]_{cyt}$. Media devoid of glutamate but supplemented with the same concentrations of glutamate and glutamine that can be reached during recordings also increase $[Ca^{2+}]_{cyt}$ (Fig. 2E).

Next, we prepared oligomers according to Klein's protocol but using media devoid of glutamate receptor agonists (MEM, DMEM and PBS). Western blotting analysis of these preparations revealed the presence of fibrils and monomers but not oligomers (Fig. 2F). Consistently, these preparations did not increase $[Ca^{2+}]_{cyt}$ in neurons in spite that the same cells responded normally to NMDA (Fig. 2G). These results pose the question on whether effects of preparations made in F12 medium are due to oligomers or to the presence of glutamate receptor agonists in the medium.

3.2. *New protocol for preparation of $A\beta_{1-42}$ oligomers*

We aimed next at modifying the oligomer preparation protocol using the above media devoid of glutamate receptor agonists but changing conditions to favor oligomerization. Two new protocols have been reported recently by Fa et al. [17] and Itkin et al. [18]. The protocol by Fa et al. is similar to Klein's except that it reduces the suspension time from 72 h to 24 h [17]. The protocol by Itkin et al. is also similar to Klein's except that oligomerization takes place

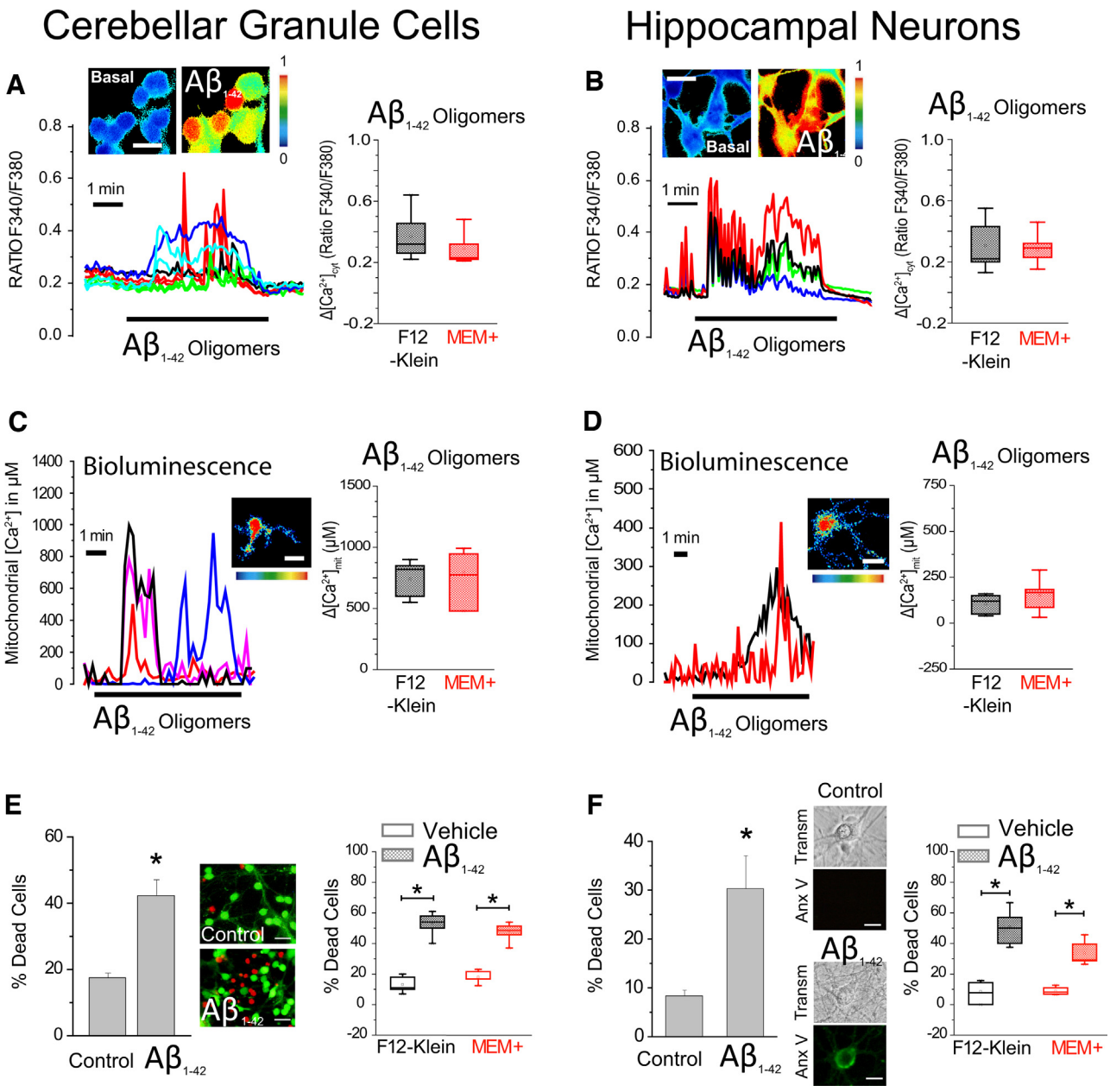

Fig. 4. $A\beta_{1-42}$ oligomers prepared by the new protocol increase cytosolic and mitochondrial $[Ca^{2+}]$ and promote apoptosis in neurons. $A\beta_{1-42}$ oligomers were prepared by the new protocol, solved in MEM+ and their effects on $[Ca^{2+}]_{cyt}$ (A,B), mitochondrial $[Ca^{2+}]$ (C,D) and cell death (E,F) were tested in cerebellar (Left) and hippocampal neurons (Right) using fluorescence imaging and bioluminescence imaging, respectively. Traces are recordings of 421–563 individual neurons from 84 $[Ca^{2+}]_{cyt}$ experiments or 17–32 cells from 6 $[Ca^{2+}]_{mit}$ experiments. Pictures on top of recordings represent pseudocolor $[Ca^{2+}]_{cyt}$ or $[Ca^{2+}]_{mit}$ images according to scales shown at right and below, respectively. Effects of oligomers on cerebellar granule cell death as shown by fluorescein diacetate (green) and propidium iodide (red) staining (E). Effects of oligomers on apoptosis in hippocampal neurons as shown by Annexin V staining (F). Bars are mean ± SE of the percent of dead cells. Cerebellum, control, $n = 2276$ cells; $A\beta_{1-42}$ $n = 3129$ cells studied in 8 independent experiments. Hippocampi, control, $n = 325$ cells; $A\beta_{1-42}$ $n = 382$ cells studied in 5 independent experiments ($^*p < 0.05$). Box plots compare $\Delta[Ca^{2+}]_{cyt}$ (A,B), $\Delta[Ca^{2+}]_{mit}$ (C,D) and% dead cells (E,F) induced by oligomers prepared according to Klein's protocol (F12 Klein) and the new protocol (MEM+). $^*p < 0.05$. Data from Klein's protocol are from 181 cells, 6 experiments (A), 231 cells, 6 experiments (B), 36 cells, 3 experiments (C) and 28 cells, 5 experiments (D). Cell death data are from at least 3 independent experiments.

at 37 °C instead of 4 °C. We have included both modifications in a novel protocol shown in Fig. 1A.

We have also analyzed in detail the chemical composition of DMEM-F12 in search for key components contributing to $A\beta_o$ formation. We noticed that some inorganic salts including cupric, ferric and zinc sulfates were present at low concentrations in DMEM-F12 medium but not in other media. Interestingly, some of these salts have been reported to contribute to Aβ oligomerization [19,20]. Accordingly, we decided also to change further the oligomerization protocol by supplementing media with inorganic salts including ferric sulfate ($FeSO_4$–$7H_2O$, 0.15 μM), cupric sulfate ($CuSO_4$–$5H_2O$, 5.2 nM) and zinc sulfate ($ZnSO_4$–$7H_2O$, 0.15 μM). It has been reported also that oligomerization is favored by the presence of calcium [18]. Since culture media (DMEM and MEM) contain a high calcium concentration, we also added 2 mM calcium to SEM to obtain a supplemented SEM (SEM Ca). Western blotting analysis of the preparations obtained by the novel procedure using different supplemented media revealed the presence of small molecular weight oligomers (Fig. 1B) in a similar fraction than in the preparations obtained using the Klein's protocol (Fig. 2B).

3.3. $A\beta_o$ preparations made in supplemented media, but not supplemented media alone, increase $[Ca^{2+}]_{cyt}$ in cultured neurons

Next we tested the effects of supplemented media alone and oligomer preparations made in these media on $[Ca^{2+}]_{cyt}$ in cultured neurons. Fig. 3A–C shows that none of the supplemented media alone increases $[Ca^{2+}]_{cyt}$ in hippocampal neurons despite cells responded normally to the glutamate receptor agonist NMDA. In contrast, $A\beta_o$ preparations made in these media induced large $[Ca^{2+}]_{cyt}$ increases in hippocampal neurons (Fig. 3D–F). Average data in Fig. 3G indicates that all preparations yielded similar results as shown by box plots of the same data (Fig. 3H). Thus, the supplemented media tested do not increase $[Ca^{2+}]$ but allow oligomer formation that are able to increase $[Ca^{2+}]_{cyt}$ in neurons.

3.4. $A\beta_o$ prepared with the new protocol promote not only Ca^{2+} entry but also mitochondrial Ca^{2+} overload and cell death in cerebellar and hippocampal neurons

$A\beta_o$ were prepared using the new procedure described in Fig. 1 in supplemented media (MEM+) devoid of glutamate receptor agonists and their effects on $[Ca^{2+}]_{cyt}$, $[Ca^{2+}]_{mit}$ and cell death in cultured neurons were tested. We found that the newly prepared $A\beta_o$ induce large increases in $[Ca^{2+}]_{cyt}$ in both cerebellar granules (Fig. 4A) and hippocampal neurons (Fig. 4B). Oligomers prepared in MEM+ also induced mitochondrial Ca^{2+} overload as shown by bioluminescence imaging of mitochondria-targeted aequorin in cerebellar granules (Fig. 4C) and hippocampal neurons (Fig. 4D). The same preparations promoted cell death in cerebellar granules (Fig. 4E) and apoptosis in hippocampal neurons (Fig. 4F). The effects of oligomers prepared by the new procedure in MEM+ devoid of glutamate receptor agonists on all three parameters were compared with the effects on oligomers prepared in F12 medium using the Klein's protocol. We found that the rises in $[Ca^{2+}]_{cyt}$, the mitochondrial Ca^{2+} overload and cell dead induced by both preparations were similar as displayed in the box plots shown next to each panel of Fig. 4A–F.

4. Discussion

It is becoming increasingly clear that amyloid β_{1-42} oligomers rather than monomers, fibrils or amyloid plaques are the most likely neurotoxin in Alzheimer's disease [1–4]. However, considerable controversy remains as to how these small assemblies induce neurotoxicity, particularly regarding the role of intracellular Ca^{2+} in this process. First reports suggested an important role of Ca^{2+} entry in neuron cell death induced by ADDLs and oligomers [5,6]. We reported that $A\beta_o$, but not fibrils, promote Ca^{2+} entry and mitochondrial Ca^{2+} overload leading to neuronal apoptosis [7]. This effect could be mediated by activation of endogenous Ca^{2+} channels, particularly NMDA receptors [9,21] and/or the formation of the so-called amyloid channels [22,23]. However, Zempel et al. challenged these previous proposals by showing that the solvent in which oligomers are prepared increases $[Ca^{2+}]_{cyt}$ [10]. Here we have confirmed that DMEM-F12 medium alone increases $[Ca^{2+}]_{cyt}$, a response that can occlude actual oligomer effects. The effects of media alone are always lower than those induced by oligomers prepared in DMEM-F12 media suggesting genuine effects of oligomers on top of those induced by DMEM-F12 medium.

We used here different media to prepare oligomers and found that all three tested media failed to allow oligomer formation indicating that media composition and other characteristics may be critical for oligomer formation. Interestingly, these preparations that contained monomers and fibrils, but lacked oligomers, did not increase $[Ca^{2+}]_{cyt}$ consistently with a critical role for oligomer formation in Ca^{2+} entry. Consistently, it has been reported that $A\beta_{1-42}$ monomers and fibrils do not promote Ca^{2+} entry into neurons and are far less toxic than oligomers [3,13]. Therefore, our results support the view that $A\beta_o$ toxicity depends largely on Ca^{2+} entry. Unfortunately, the target of $A\beta_o$ oligomers responsible for Ca^{2+} entry cannot be easily tested with preparations contaminated with glutamate receptor agonists.

Dahlgren et al. [13] reported the first study using a single chemically and structurally homogeneous unaggregated starting material and demonstrated that the formation of oligomers, fibrils, and fibrillar aggregates is determined by different parameters including time, concentration, temperature, pH, ionic strength, and Aβ species [3]. Recently, two new protocols for oligomer formation were reported [17,18] that included critical changes in oligomerization temperature and incubation time. We have applied both changes to a novel oligomerization protocol. However, these changes cannot explain why DMEM-F12 medium is permissive for oligomer formation but the other media are not. A simple comparison of solvent composition reveales that some uncommon salts including cupric, zinc and ferric sulfates reported to promote Aβ oligomer formation [17,18] are present in DMEM-F12 medium but not in the other media. Consistently, we found that preparations made with the improved protocol using supplemented media contained small molecular weight oligomers. As a caution note, we acknowledge that western blot is not the optimal method for the identification of Aβ oligomer sub-types and further analysis is warranted. In any case, these preparations containing small molecular weight Aβ oligomers but not glutamate receptor agonists promoted Ca^{2+} entry, mitochondrial Ca^{2+} overload and neuron cell death to an extent similar to that induced by oligomers prepared according to Klein's. These results not only provide a novel, improved procedure for obtaining Aβ oligomers in solvents devoid of glutamate receptor agonists, but also yield unambiguous evidence that Ca^{2+} entry and mitochondrial Ca^{2+} overload are critical to the neurotoxicity induced by Aβ oligomers, the most likely toxin in Alzheimer's disease. The new procedure reported here may provide the basis for searching new targets of Aβ oligomers involved in Ca^{2+} dishomeostasis in Alzheimer's disease.

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