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Eliminating membrane depolarization caused by the Alzheimer peptide Aβ(1–42, aggr.)☆

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Abstract

A high-throughput screen found compounds that eliminate the dramatic membrane depolarization caused by the aggregated Alzheimer Aβ1–42 peptide, which activates mGluR1 receptors. The library was composed of known biologically active compounds; the cell-based assay measured the changes of membrane potential with a slow-acting voltage-sensitive dye. We found 10 potentially useful compounds, some of which reduce the Aβ-induced membrane depolarization up to 96%. Interestingly, the active compounds include specific tyrosine kinase inhibitors and inhibitors of certain chloride channels. We deduce that mGluR1 receptors, activated by Aβ1–42 or otherwise, can control the membrane potential via downstream activation of certain tyrosine kinases and certain ion channels. Dopaminergic and serotonergic agonists that emerged from the screen presumably compensate for the Aβ-induced membrane depolarization by themselves causing a hyperpolarization. The hit compounds, whose pharmacokinetics are known, show promise for the restoration of cognitive function in the treatment of early and mid-stage Alzheimer's disease.

Keywords: Alzheimer's disease; Aβ1–42 peptide; Membrane depolarization; Voltage-sensitive dye; Tyrosine kinase inhibitors; mGluR1 Receptors

It is generally thought [1] that the proportion of the longer Alzheimer Aβ peptide, Aβ1–42, over Aβ1–40 is greatly increased in Alzheimer's disease (AD). This feature has been linked to the pathology of AD. The peptide Aβ1–42 is also a form of peptide that spontaneously aggregates more readily to β-sheet-containing fibrils [2]. Since the visible Aβ aggregates in AD are mostly, perhaps entirely, extracellular, we might look for interaction with receptors in the neuronal membrane. In the event we found strong interaction of aggregated Aβ1–42 peptide with Ca-permeant AMPA/NMDA ionotropic receptors [3,4] and with metabotropic glutamate receptors of type I [14].

In AD it is very important to find a way to eliminate the lasting Aβ-induced membrane depolarization, since this can lead to hyperexcitability of affected neurons in the brain. The hyperexcitability is expected to occur before cell death induced by calcium influx, but it can lead to cognitive deficits of early or mid-stage AD. We have developed a high-throughput screen in which PC12 cells in 384-well plates are exposed to a suspension of preincubated Aβ1–42 aggregates in the presence of low micromolar concentrations of ~1540 compounds with known biological activities. We found 10 leading compounds that we have characterized.

Materials and methods

Aggregation of Aβ1–42. The Aβ1–42 peptide was prepared as described [4]. It was aggregated in 30 μM solution in Tyrode's/2Ca buffer (150 mM NaCl, 3 mM KCl, 10 mM HEPES, pH 7.4, 2 mM $CaCl_2$, 10 mM D-glucose), pH 7.4.

High-throughput screen. The library of 1540 compounds was selected from biologically active compounds available from Sigma, Aldrich, RBI, and Fluka (Stockwell, manuscript in preparation). These compounds were dissolved in DMSO and distributed in six 384-well plates, each of which was screened in triplicate, as follows: 384-well

☆ *Abbreviations*: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DiB $AC_4(3)$, bis-(1,3-dibutylbarbituric acid)trimethine oxonol; NMDA, *N*-methyl-D-aspartate; Tyrode's/2Ca, Tyrode's solution with 2 mM Ca^{2+}.

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plates (Costar #3712) were first coated with poly-D-lysine + collagen in water (final concentrations of 10 μg/ml and 1 mg/ml, respectively) at room temperature for 2 h, and then washed three times with sterile water using a Multidrop automatic dispenser. Undifferentiated PC12 neuronal cells were then seeded at 60,000 cells/well/40 μl in medium (RPMI, 10% horse serum, 5% fetal bovine serum, 1% L-glutamine, 1% pen–strep) and incubated at 37 °C and 6% CO_2 for 48 h. The cells were washed three times with Tyrode's/2Ca, pH 7.4, using a Tecan automatic power washer; the final wash left 40 μl of buffer/well.

Preincubated 30 μM Aβ1–42 (in Tyrode's/2Ca, pH 7.4, 37 °C, 48 h) was dispensed with the Multidrop automatic dispenser at 20 μl/well to a final concentration of 10 μM peptide/well. Each plate was set up to include Aβ1–42 and Tyrode's/2Ca controls. The voltage-sensitive fluorescent dye $DiBAC_4(3)$ [5–8] was added to the Aβ1–42 and Tyrode's/2Ca solutions to a final concentration of 97 nM. The compounds from the 384-well library plate (thawed at RT and quickly centrifuged) were pin transferred (Genetix #X5050) into the plates with PC12 cells in columns #3–22. The pin transferred 50 nl of stock compound that was at 4 mg/ml in DMSO giving a final concentration of ~35 μg/ml/well. The approximate concentration of tested compounds was ~8.3 μM, if we assume an average molecular size for the compounds to be 400.

Fluorescence was recorded within 5–15 min at Ex = 485 nm, Em = 530 nm using a fluorescein filter set in an LJL Biosystems Analyst AD 96-384. Data analysis normalized each well by dividing the gross fluorescence observed by the mean of the gross fluorescence in Aβ(1–42, aggr.) containing wells without test compounds giving RFU values (relative fluorescence units). Thus the depolarization of untreated cells is equal to 1.00; buffer alone (Tyrode's/2 mM Ca) is ~0.6. For further characterization we selected compounds that showed a reduction in the membrane depolarization (caused by Aβ1–42) that gave values of <0.80.

The compounds to be examined (Sigma Chemical) were assayed as described above using $DiBAC_4(3)$ but in 96-well biocoated poly-D-lysine black/clear plates (VWR #35-6640) in a volume of 100 μl/well. Fluorescence was read at Ex = 485 nm, Em = 538 nm on a Fluoroskan II. The initial search of the library uses compounds dissolved in the "universal" solvent DMSO. Since DMSO itself changes the membrane potential a little, the later characterizing concentration curves were done in aqueous solution, whenever possible. Otherwise, experiments included the appropriate solvent controls.

Results and discussion

A total of 1540 biologically active compounds have been screened from a library in six 384-well plates. Fig. 1 shows the results of the screen after sorting the compounds by their gross fluorescence values relative to the gross "Aβ1–42" controls in the figure. The identifying numbers given to individual compounds in Fig. 1 are arbitrary, but reference to the corresponding wells in the library plates gave us the chemical identity of the compounds. Many of the compounds that showed reductions in the Aβ-induced membrane depolarization to <80% were examined in a "second characterization" in 96-well plates (e.g., Fig. 2A and B).Of the surprisingly high number of potential "hits" (37 out of 1540 compounds) six proved on re-examination to be viable hits (Table 1); another four compounds were picked up because of their close chemical relationship to one or other of the six hits. Several of the apparently most effective hits were considered to be false positives, because they were deeply colored compounds that would quench the fluorescence (Fig. 1, #20,29,36).

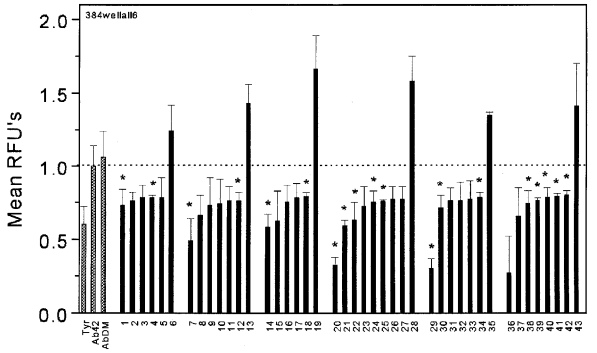

Fig. 1. Library compounds that show the greatest reduction in membrane depolarization caused by Aβ(1–42, aggr.); they gave values of <0.80 RFUs (see Materials and methods). Each column represents the mean RFUs of triplicate assays (five repeats for #1–6) for individual compounds. Each group of columns represents one of the six plates that comprised the library. The last column of each group is the maximum mean value for that plate. The first three columns are the controls: Tyrode's/2Ca, Aβ(1–42, aggr.), and Aβ(1–42, aggr.) + DMSO, respectively. $^{*}p \leqslant 0.05$.

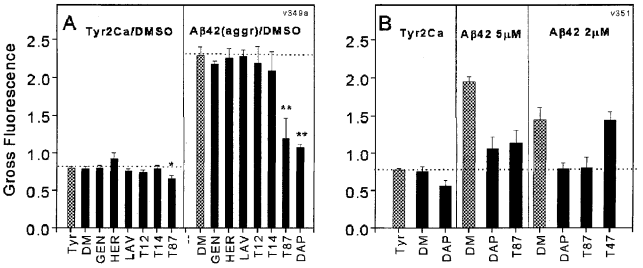

Fig. 2. Characterization of tyrosine kinase inhibitors ("hit compounds") and other functionally related compounds in 96-well plates, as described in Materials and methods. (A) Seven tyrosine kinase inhibitors were examined at 10 μM; aggregated Aβ1–42 = 10 μM; final concentration of DMSO = 0.1%; $n = 4$. Each column represents the mean of four wells. Tyr = Tyrode's/2Ca; DM = DMSO only control; GEN = genistein; HER = herbimycin A; LAV = lavendustin A; T12 = tyrphostin AG1295; T14 = tyrphostin AG1478; T87 = tyrphostin AG879; T47 = tyrphostin 47; DAP = 4,5-dianilinophthalimide (DAPH1). $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$. (B) Further characterization of tyrosine kinase inhibitors ("hit compounds") at higher molar ratios of {compound @ 10 μM}/{Aβ(1–42, aggr.)} in 96-well plates; $n = 4$.

For further study we selected the six compounds most active in eliminating membrane depolarization (Fig. 1, #1, 3, 5, 7, 21, 22; Table 1). We also tested four structurally related compounds (Table 1). The list includes dopamine itself, although this did not show up in the screen, but two dopamine agonists, SKF81297 ((±)-6-chloro-PB hydrobromide) and rauwolscine, had appeared (Fig. 1, #3, #5). The neuropeptide substance P, an NK1 agonist, was also included, because in an early publication Yankner et al. had described the ability of the peptide to eliminate the neurotoxicity of Aβ25–35 [9]. Since the tyrosine kinase inhibitor tyrphostin 47 had

Table 1
"Hit compounds" that decrease Aβ-induced membrane depolarization or cause hyperpolarization

Compound	% Decrease of Aβ-induced depolarization: compound at 10 μM			Hyperpolarization[a]
	10 μM Aβ	5 μM Aβ	2 μM Aβ	
Tyr-kinase inhibitor (EGF)				
4,5-Dianilinophthalimide (DAPH1)	−79, −74	−75	−96	−26
Tyrphostin 47	−13	ND	−43	ND
Tyr-kinase inhibitor (TrkA)				
Tyrphostin AG879	−73	−68	−95	−16
Cl^- Channel antagonists				
Nafoxidine	−49	−100, −74	−124	−41
Clomiphene	−19	−16	−38	−11
Dopamine agonists				
SKF81297 (6-Cl-PB)	−30, −29	−14	ND	−2
Vanillylmandelic Acid	−26, −17, −17	−21	ND	−7
Dopamine	−14, −48	ND	−55	−5
α2-Adrenergic Antagonist; 5-HT1A Serotonergic agonist				
Rauwolscine (α-yohimbine)	−79, −14	−12	−37	−27
TK1 Antagonist				
Substance P	−25	ND	ND	−25

For details, see Materials and methods; values represent the means of 4–8 wells.
[a] Percentage change of membrane potential by compound alone at 10 μM.

appeared in the screen, several other tyrphostins and several other tyrosine kinase inhibitors were also tested in the secondary screen (Fig. 2A and B). One of these, tyrphostin AG879, was found to be very active; the others tested were not. The characterization of these hit compounds was by multiple assays at different concentrations, using 96-well plates and quadruplicate fluorescence measurements. Grouping the hit compounds by function gives the list shown in Table 1.

The most effective elimination of depolarization was achieved with two tyrosine kinase inhibitors, DAPH1 (4,5-dianilinophthalimide, EGF-receptor tyrosine kinase-specific) (Fig. 1, #7; Fig. 2A and B) and tyrphostin AG879 (TrkA-receptor tyrosine kinase-specific) (Fig. 2A and B). In addition, nafoxidine (an anti-estrogen-receptor compound and chloride channel antagonist) (Fig. 1, #21; Table 1) was also very effective. These were active in low micromolar concentrations (Fig. 2A and B). Tyrphostin 47 (EGF-receptor tyrosine kinase inhibitor) (Fig. 1, #22; Fig. 2B) is less effective.

Five other tyrosine kinase inhibitors were inactive (Fig. 2): genistein, herbimycin A, lavendustin A, tyrphostin AG1295, and tyrphostin AG1478. They had not been detected in the initial screen, but were tested because they are functionally related to those tyrosine kinase inhibitors that did appear in the original screen.

The dopamine agonists vanillylmandelic acid, SKF81297 (Fig. 1, #1,#3; Table 1), and dopamine (Table 1) itself were also effective in reducing depolarization, but it was less than some of the above compounds. The serotonergic receptor agonist rauwolscine (α-yohimbine) (Fig. 1, #5; Table 1) was also effective.

Some of these compounds, when added to PC12 cells without Aβ1–42, produce a hyperpolarization (Table 1, Fig. 2A and B).

Finding so many "hit compounds" in our high-throughput screen gives exciting insights into the cellular mechanisms for maintaining and modulating the all-important membrane potential of neuronal cells. Our evidence shows that the Aβ-induced membrane depolarization is due to the activation of mGlu receptors of Group I (especially mGluR1) by the aggregated form of Aβ1–42 [14] (Fig. 3). This activation includes the activation of linked G-proteins, which might include the long-term inhibition of particular K-channels [10–13], leading to membrane depolarization. The modulation of other ion channels might also be involved. Our findings suggest steps in the mechanism whereby glutamate can control the excitability of its target neurons.

It will be remembered that Aβ(1–42, aggr.) also causes an influx of Ca^{2+} from the external medium by activating AMPA/kainate and NMDA ionotropic receptor channels [4]. It is likely that this activation is also a consequence of the initial mGluR1/G-protein activation (Fig. 3). However, the depolarization pathway that we are now describing does not involve changes in cytosolic calcium levels [14], since the membrane phenomenon is observed when external calcium is zero.

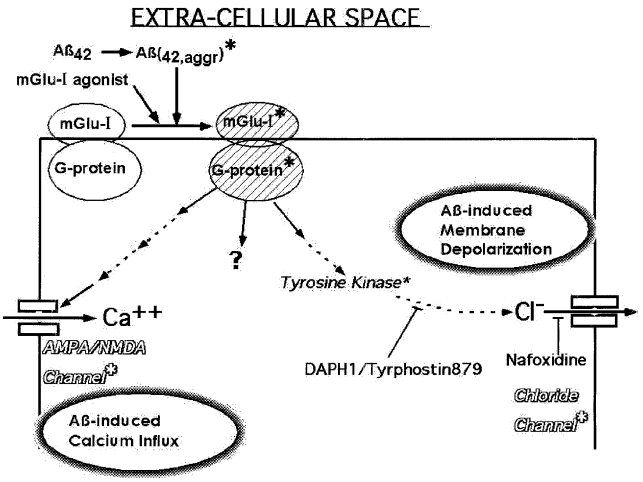

Fig. 3. A possible scheme showing how Aβ(1–42, aggr.) might induce membrane depolarization and calcium influx and how depolarization is reduced by certain "hit compounds" from the high-throughput screen.

There is no release of calcium from internal stores under these circumstances, which would have been detected by the calcium-sensitive dye fura-2 which was present.

The screen identified (A) compounds that reduce or eliminate the depolarization effect and (B) at least some of the intermediaries by which the activated mGluR1 receptors control the membrane potential.

(A) The compounds found fall into two categories: those that interfere with steps in the cascade postulated to produce depolarization, for instance, certain tyrosine kinase inhibitors and others that compensate for the depolarization by causing a hyperpolarization of the membrane (Table 1). In the first category are the most effective compounds (Table 1), such as tyrosine kinase inhibitors (DAPH1, tyrphostin 47, and tyrphostin AG879). The chloride channel inhibitors (nafoxidine, clomiphene), though effective, belong to a different category.

(B) We assume that these kinases and channels are normally activated to some extent by glutamate via mGluR1 receptors. We deduce that they contribute to the control of normal membrane potential of the cell. Presumably their activation is greatly increased by the aggregated Aβ1–42 peptide.

In the second group of compounds are dopaminergic and serotonergic agonists (dopamine itself, vanillylmandelic acid, rauwolscine (α-yohimbine) (Table 1), which are normally involved in signal transmission rather than maintenance. They compensate by causing hyperpolarization. The compound SKF81297, although described as a dopamine agonist, has no detectable hyperpolarizing effect, unlike other agonists.

The pharmacokinetics and toxicology of these compounds are known. Some were developed as anti-cancer agents. Some are presently in clinical use, but not for AD. The Aβ-induced membrane depolarization is concentration-dependent and reversible. It is very large and lasting. We assume that cognitive processes in vivo are sensitive to changes in the resting membrane potential. Apparently, compensation can greatly decrease the "deleterious" membrane depolarization, but we do not yet know how much compensation would be needed to restore normal cognitive function. The compounds described here will lead to improved therapy for early and mid-stage AD. They should be particularly useful when neurons are dysfunctional, not dead, i.e., in early and mid-stage AD.

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