Protein Aggregation

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Selectively N-Methylated Soluble IAPP Mimics as Potent IAPP Receptor Agonists and Nanomolar Inhibitors of Cytotoxic Self-Assembly of Both IAPP and Aβ40**

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The aggregation of islet amyloid polypeptide (IAPP) is linked to β-cell degeneration and the pathogenesis of type 2 diabetes (T2D).[1] IAPP is a 37-residue polypeptide hormone secreted by the pancreatic β-cells.[1] IAPP amyloid deposits are found in the pancreata of many T2D patients.[1,2] In its soluble form, however, IAPP acts as a brain–gut neuropeptide regulator of glucose homeostasis.[3,4] Its main physiological functions comprise delay of gastric emptying by inhibiting gastric contractions and suppression of food intake.[3–6] IAPP receptors are G protein coupled receptors (GPCRs) that form through heterodimerization of the calcitonin receptor with receptor activity modifying proteins (RAMPs).[7–10] Due to its extreme insolubility IAPP is not suited for medicinal application.[4,11] However, a soluble IAPP receptor agonist, the IAPP analogue [P25,P28,P29]-IAPP or pramlintide, was approved for diabetes therapy a few years ago and its use results in improved control of blood sugar levels.[4,10,11] Therefore, the application of soluble IAPP receptor agonists has lately become a very promising new approach in diabetes treatment.[4,10,11]

We reported the design of the soluble IAPP analogue IAPP-GI some time ago.[12] IAPP-GI is a conformationally constrained IAPP mimic that was generated by N-methylation of the amide bonds of G24 and I26, which are located within the minimum IAPP self-recognition region IAPP(22–27) (Figure 1).[12,13] So far IAPP-GI has been a unique IAPP analogue as it is a nanomolar-affinity inhibitor of IAPP cytotoxic self-assembly and at the same time a soluble full

Figure 1. Primary structures of IAPP, IAPP-GI, and the novel IAPP mimics. IAPP(22–27) is shown in red. N-methyl groups (N-Me) are in blue and N-methylated residues are blue and highlighted in yellow.

IAPP receptor agonist.[12] However, the presence of the two N-methyl residues, though highly beneficial with regard to solubility and amyloid inhibitory properties, also results in a strong decrease of IAPP receptor binding and agonistic potency.[12]

More recently, we have found that IAPP-GI binds with high affinity to the β-amyloid peptide (Aβ) of Alzheimer's disease (AD) and inhibits Aβ fibrillogenesis and cytotoxicity.[14,15] In addition, increasing evidence suggests that AD and T2D are linked, which increases the interest in developing strategies targeting both diseases.[1,4,16,17] Thus, soluble IAPP analogues that inhibit the aggregation and toxicity of both IAPP and Aβ40 and also display potent IAPP receptor agonism could become leads for therapeutics for both diseases. However, the molecular basis of IAPP receptor activation is not known yet.

Here we show that by N-methylating specific amide bonds within IAPP(22–27) we can generate soluble IAPP mimics that exhibit IAPP receptor agonism that is strongly improved over that of IAPP-GI and also maintain nanomolar-affinity inhibitory effects on the aggregation and cytotoxicity of both

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IAPP and Aβ40. In addition, all mimics exhibit drastically enhanced stability in human plasma relative to that of IAPP. Most importantly, the most potent IAPP receptor agonist in vitro was found to inhibit gastric muscle contractions ex vivo with the same potency as rIAPP, the most potent naturally occurring soluble IAPP analogue, and to ameliorate Aβ40-induced impairment of hippocampal synaptic plasticity.

For analogue design, the N-methyl residues present in IAPP-GI were systematically moved to other amide bonds within IAPP(22–27).[12] Two analogues with alternate N-methylations ([(N-Me)A25, (N-Me)L27]-IAPP (IAPP-AL) and [(N-Me)F23, (N-Me)A25]-IAPP (IAPP-FA)) and one with consecutive N-methylations ([(N-Me)I26, (N-Me)L27]-IAPP (IAPP-IL)) were synthesized and studied (Figure 1).

We first studied the solubility, self-assembly, and amyloidogenic and cytotoxic properties of the mimics by using various biophysical assays. Similarly to IAPP-GI, the novel mimics were at least 100-fold more soluble (pH 7.4), less amyloidogenic, and less cytotoxic than IAPP (Figure 2).[12] However, they self-aggregated into soluble, nonfibrillar, and nontoxic oligomers at concentrations in the low nanomolar range as was also found for IAPP-GI (Figure 2c; see also Figure S1 and text in Supporting Information).[12] Our results suggested that the self-association of IAPP into nonfibrillar and nontoxic assemblies is polymorphic in nature and does not depend on the H-bonding/β-sheet-forming ability of IAPP(22–27). By contrast, misfolding to form cytotoxic oligomers and amyloid fibrils appears to be directly linked to the β-sheet-forming ability of IAPP(22–27), consistent with models of IAPP fibrils (see the Supporting Information).[13,15,18,19]

We next investigated whether IAPP-AL and IAPP-FA are able to inhibit IAPP self-assembly. In the presence of either IAPP-AL or IAPP-FA (1:1), a strong suppression of IAPP amyloidogenesis was observed according to the ThT binding assay and transmission electron microscopy (TEM; Figure 3a and Figure S2 in the Supporting Information).[12,20] Moreover, a strong reduction of IAPP-mediated cell damage in RIN5fm cells was found by the MTT reduction assay (Figure 3b).[21] Titrations of cytotoxic IAPP with IAPP-AL or IAPP-FA showed that the maximum effect is reached at a molar ratio of 1:1 and yielded IC_{50} values of 48(±6) and 33(±1) nM, which were very similar to the IC_{50} value of IAPP-GI (Figure 3c).[12]

We then used far-UV CD, TEM, and fluorescence spectroscopy to study the interaction of IAPP-AL and IAPP-AF with IAPP. The mimics were found to bind IAPP monomers and low-molecular-weight oligomers with low nanomolar affinities and to form soluble, nonfibrillar, and ordered hetero-oligomers (see text and Figure S3 in the Supporting Information). The determined affinities were in good agreement with the nanomolar IC_{50} values and nearly identical to the affinities of IAPP-GI (Table S1).[12] Thus, the N-methylations within IAPP(22–27) did not affect either the binding affinities or IC_{50} values.

Next, the addition of IAPP-AL and IAPP-FA (1:1) to nonfibrillar and nontoxic Aβ40 was found to block both Aβ40 fibrillogenesis and formation of cytotoxic assemblies (Figure 3d,e and Figure S4). Titrations of cytotoxic Aβ40 with the mimics suggested that a 1:1 interaction results in maximal inhibition, and IC_{50} values of 164(±27) and 96(±3) nM for IAPP-AL and IAPP-FA, respectively, were obtained (Figure 3f). Far-UV CD, TEM, and fluorescence spectroscopy suggested that the mimics bind Aβ40 monomers and low-molecular-weight oligomers with nanomolar affinities and form soluble, nonfibrillar, and ordered hetero-oligomers (Figures S4 and S5, Table S1). Notably, the properties of the consecutively N-methylated mimic IAPP-IL were nearly identical to those of the other mimics (Figures S2, S4, S6, and S7, Table S1). Finally, the mimics were found to block already nucleated fibrillogenesis of both

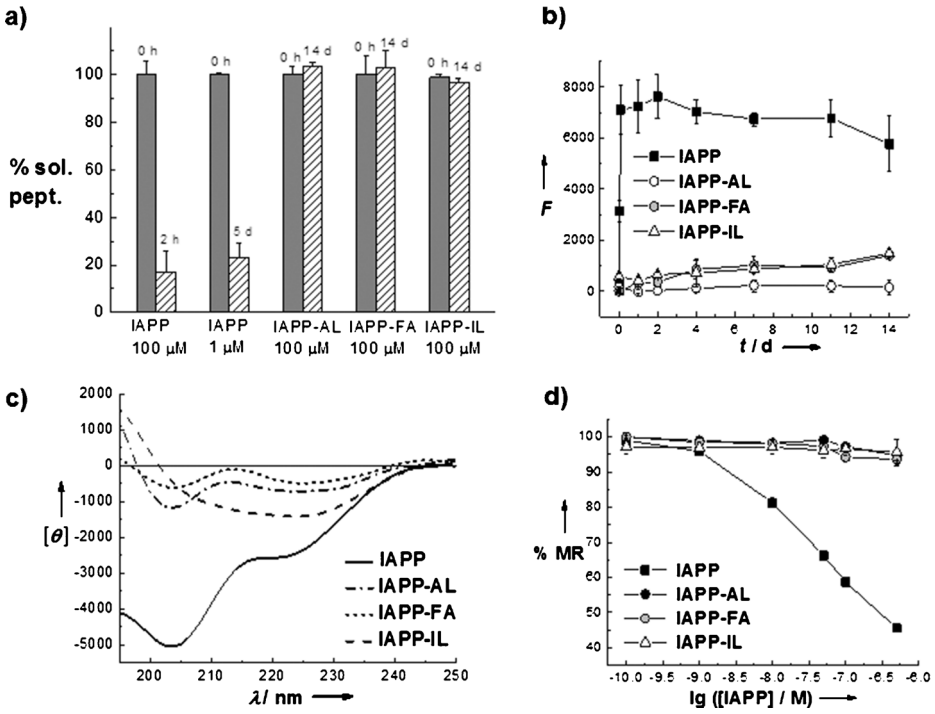

Figure 2. Solubility (a), amyloidogenicity (b), conformation (c), and effects on cell viability (d) of mimics and IAPP.[12] a) Solubilities (100 μM) determined by a sedimentation assay. The previously determined solubility of IAPP (1 and 100 μM) is shown.[12] Results are means (±SEM) from three to five assays (% sol. pept.: % soluble peptide). b) Amyloidogenicities as determined by the thioflavin T (ThT) binding assay (62.5 μM). Results are means (±SEM) from three assays (F: ThT fluorescence). c) Far-UV CD spectra of IAPP and mimics (5 μM, aqueous buffer, pH 7.4). d) Effects of aged (7 days) solutions of IAPP or mimics on rat insulinoma RIN5fm cell viability as determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay in rat insulinoma cells (%MR, MTT reduction, % of control). Results are means (±SEM) from three assays ($n=3$).

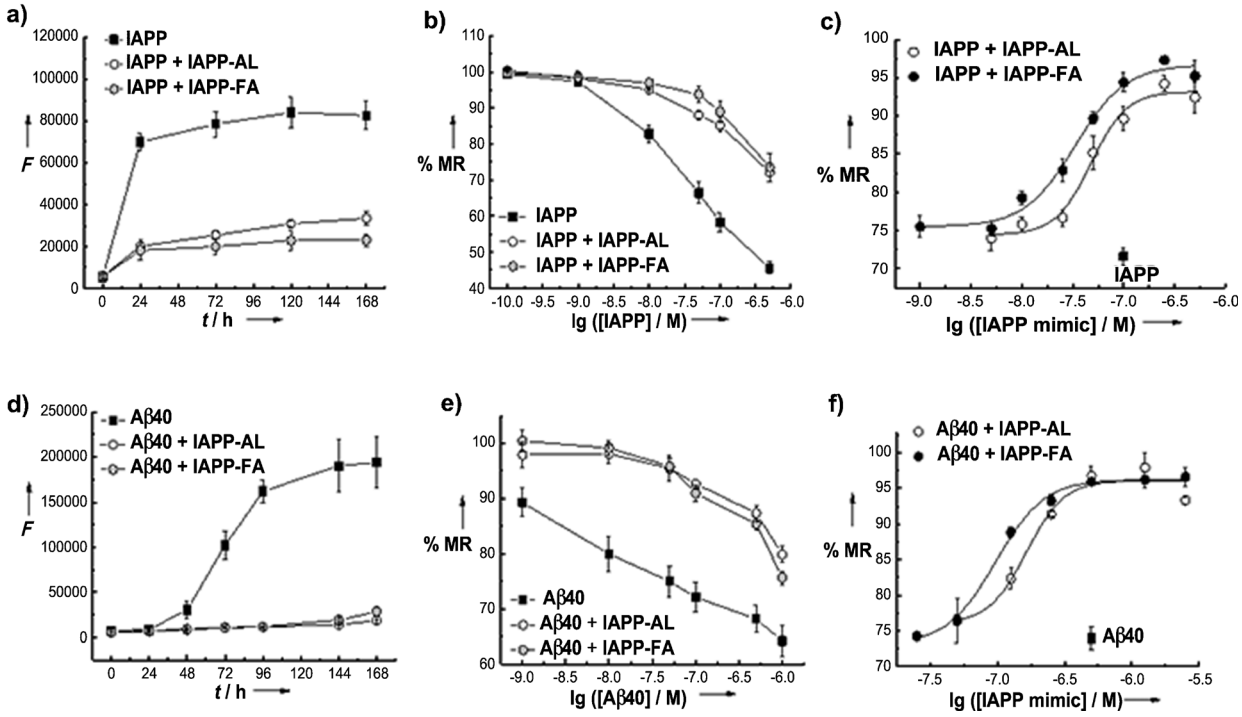

Figure 3. Inhibitory effects of IAPP-AL and IAPP-FA on IAPP (a–c) or Aβ40 (d,e) aggregation and cytotoxicity. a) Effects on IAPP fibrillogenesis. IAPP (16.5 μM) was aged alone or with a mimic (1:1) and fibrillogenesis was assessed by the ThT assay. Data are means (±SEM) from six assays. b) Effects of mimics on the formation of cytotoxic IAPP assemblies. Aged (7 days) IAPP or mixtures of IAPP with the mimics (from (a)) were added to RIN5fm cells and cell damage was assessed by the MTT reduction assay. Results are means (±SEM) from three assays ($n=3$). c) Determination of the IC_{50} of the inhibitory effect of IAPP-AL and IAPP-FA on IAPP cytotoxicity by titration of IAPP (100 nM) with mimics and the MTT reduction assay. Data are means (±SEM) from three assays ($n=3$). d) Fibrillogenesis of Aβ40 (16.5 μM) alone or with mimics (1:1) was followed by the ThT assay. Data are means (±SEM) from three assays. e) Effects of mimics on Aβ40 cytotoxicity. Aged Aβ40 or its mixtures with mimics (7 days aged; from (d)) were added to PC12 rat pheochromocytoma cells and cell damage was assessed by MTT reduction. Data are means (±SEM) from three assays ($n=3$). f) IC_{50} of the inhibitory effect of IAPP-AL and IAPP-FA on Aβ40 cytotoxicity by titration of Aβ40 (500 nM) with mimics and MTT reduction assay. Data are means (±SEM) from three assays ($n=3$).

IAPP and Aβ40 and to convert large parts of preformed fibrils and cytotoxic assemblies into nonfibrillar and less toxic ones likely through aggregate disassembly and remodeling processes (Figures S8–S20).[12,14]

Taken together, our previous and current results suggest that the mimics bind IAPP and Aβ40 with high affinity through polymorphic interactions.[12,14] These interactions likely result in the sequestering of nonfibrillar and nontoxic Aβ40 and IAPP species in the form of nonfibrillar and nontoxic hetero-oligomers and in the disassembly and remodeling of fibrils and cytotoxic assemblies into less fibrillar and toxic ones (see also Figure S20 and Table S2).[12,14,15] Our findings are consistent with models of IAPP assembly and with a crucial role of IAPP(22–27) in both IAPP self-association and its hetero-association with Aβ40.[13–15,18,19,22] Our results also suggest that the conformational features underlying nonamyloidogenic IAPP self- or hetero-association with Aβ40 are different from those underlying its amyloidogenic self-association, though interactions between the same regions mediate these processes.[15]

Next, the bioactivity of the mimics was studied in vitro using the human breast carcinoma cell line MCF-7, which expresses high-affinity IAPP receptors.[8] First, receptor binding affinities were determined by studying the competitive inhibition of the specific receptor binding of radioactively labeled rat IAPP (rIAPP), a high-affinity IAPP receptor ligand, by the mimics.[8,9,12] All three mimics exhibited markedly improved IAPP receptor binding relative to that of IAPP-GI (Figure 4a).[12] The binding affinities of IAPP-IL were tenfold, of IAPP-AL sevenfold, and of IAPP-FA threefold stronger than that of IAPP-GI (Figure 4a and Table S3).

IAPP receptor agonistic potencies were determined then by studying adenylate cyclase activation in MCF-7 cells (Figure 4b).[8] All mimics were full receptor agonists. Most importantly, IAPP-AL was found to be a three times stronger receptor agonist than IAPP and its potency was nearly identical to that of rIAPP. IAPP-IL and IAPP-FA were as potent as IAPP.[8,12] In comparison to IAPP-GI, IAPP-AL was 19 times while IAPP-FA and IAPP-IL were 4 times more potent (Figure 4b and Table S3).[12] This data suggested that IAPP-AL is the most potent designed IAPP receptor agonist and revealed a crucial role for IAPP(22–27) in IAPP bioactivity.[8,9]

IAPP-mediated inhibition of stomach contractions underlies its delaying effect on gastric emptying.[4,10,11,23] Therefore,

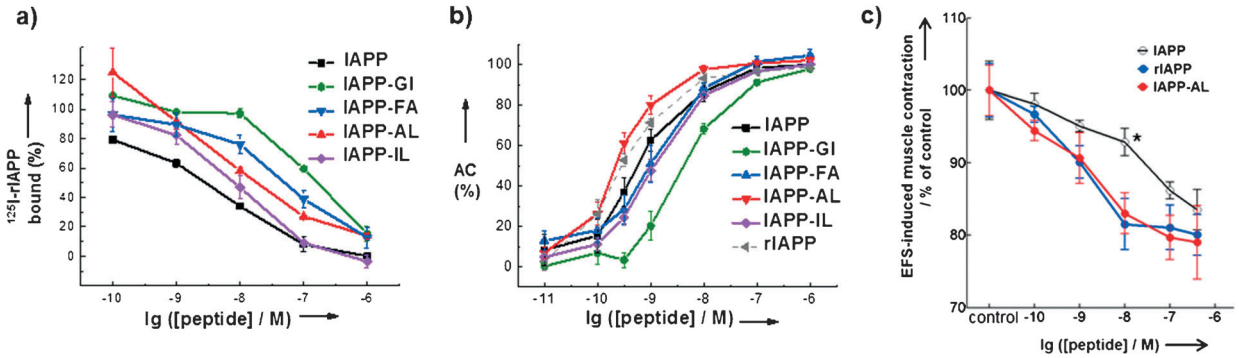

Figure 4. In vitro and ex vivo bioactivities of IAPP, rIAPP, and the IAPP mimics. a,b) In vitro human IAPP receptor binding and activation potencies in MCF-7 cells. The previously determined effects of IAPP and IAPP-GI are also shown.[12] a) Receptor binding affinities were assessed by competitive inhibition of receptor binding of ^{125}I-rIAPP by IAPP or mimics. A plot of specifically bound ^{125}I-rIAPP versus the concentration of IAPP or mimics is presented. Data are means (±SEM) of two assays ($n=4$). b) Adenylate cyclase activation was determined by quantification of intracellular cAMP. Adenylate cyclase activity (AC) above basal levels versus peptide concentration is shown. Results are means (±SEM) of three assays ($n=2$ each). c) Dose-response curve of the inhibitory effect of IAPP, rIAPP, and IAPP-AL on contractions of isolated murine smooth stomach muscle. Significant inhibitory effects ($p<0.05$) were found for all peptide concentrations versus control ($n=6$) and a significant difference was found for 10 nM IAPP(*) versus IAPP-AL ($p=0.044$, $n=6$) or versus rIAPP ($p=0.025$, $n=6$).

we next studied the effects of IAPP-AL, rIAPP, and IAPP on contractions of isolated murine stomach smooth muscle.[5] All three peptides significantly reduced electrical field stimulation (EFS)-evoked stomach contractions in a concentration-dependent manner (Figure 4c). Most importantly, identical dose–response curves were obtained for IAPP-AL and rIAPP while human IAPP exhibited markedly weaker effects (Figure 4c).

Synaptic plasticity is required for learning and memory and its damage in the hippocampus by synaptotoxic Aβ assemblies is believed to be primarily responsible for AD pathology.[24,25] Long-term potentiation (LTP) reports on the long-lasting increase in synaptic strength upon tetanic stimulation and is a major cellular mechanism for learning and memory.[24,25] We therefore tested IAPP-AL for its ability to ameliorate Aβ40-induced LTP deficits in brain slices ex vivo.[26,27] Hippocampal slices were first treated with Aβ40 aggregates and LTP in CA1 neurons was induced by tetanic electrical stimuli (high-frequency stimulation, HFS).[27] Under control conditions, field excitatory postsynaptic potentials (fEPSPs) were potentiated to 138% of baseline 60 min after HFS (Figure 5a,b). However, after treatment with Aβ40, the same stimulus produced only short-term potentiation and fEPSP slopes returned to 108% after 60 min (Figure 5a,b), consistent with a marked inhibition of LTP. By contrast, in the presence of mixtures of Aβ40 with IAPP-AL the potentiation of fEPSP was maintained (Figure 5a,b). These results demonstrated that IAPP-AL prevents Aβ40-induced hippocampal LTP impairment.

Peptides are highly susceptible to proteolytic degradation in body fluids. However, the mimics were expected to exhibit improved proteolytic stabilities due to the N-methylations.[28] To address this issue, incubations in human blood plasma at 37°C were performed and the amounts of intact peptides were determined by SDS-PAGE and western blot analysis (Figure 5c,d). IAPP was degraded fast with a half-life ($t_{1/2}$) of less than 1 h (Figure 5c, d). By contrast, the $t_{1/2}$ values of IAPP-AL, IAPP-IL, and IAPP-GI were 7–8 h while that of IAPP-FA was approximately 6 h (Figure 5c, d). Thus, the $t_{1/2}$ values of all mimics were six to eight times higher than that of IAPP. Of note, the half-life of [P25,P28,P29]-IAPP was determined to be roughly 5 h (Figure 5c,d). These results were confirmed by HPLC analysis and MALDI-MS measurements. Thus, all mimics exhibited strongly enhanced proteolytic stabilities relative to that of IAPP while three of them have even a longer half-life than [P25,P28,P29]-IAPP.

The presented multifunctional IAPP mimics constitute a unique class of IAPP analogues in that they are a) soluble, nonamyloidogenic and nontoxic, b) markedly stable toward proteolytic degradation in human plasma, c) potent IAPP receptor agonists, and d) nanomolar-affinity inhibitors of cytotoxic self-assembly and fibrillogenesis of both IAPP and Aβ40. For comparison, of the other known soluble IAPP analogues, IAPP-GI is only a weak agonist while rIAPP only weakly interferes with cytotoxic self-assembly of IAPP or Aβ40 if at all.[12,14,29] In addition, no reports on the effects of [P25,P28,P29]-IAPP or other IAPP analogues on IAPP or Aβ40 cytotoxicity have been yet available.[30] Notably, the rIAPP-derived [P25,P28,P29]-IAPP is so far the only therapeutically applied soluble IAPP receptor agonist.[4,10,11] Furthermore, short N-methylated IAPP- or Aβ40-derived peptide sequences that have been reported to inhibit IAPP or Aβ aggregation or other amyloid inhibitors would be expected to be unable to potently inhibit aggregation and cytotoxicity of both IAPP and Aβ and to function as IAPP receptor agonists.[31–36] Most importantly, the identified strong inhibitory effects of IAPP-AL on stomach contractions and on the Aβ40-mediated impairment of synaptic plasticity ex vivo suggest that this mimic may also delay gastric emptying and ameliorate AD-related synaptic dysfunction in vivo. IAPP-AL and other IAPP mimics with similar properties could thus become promising candidates for T2D and AD treatment and in vivo studies in animal models are now of high priority.

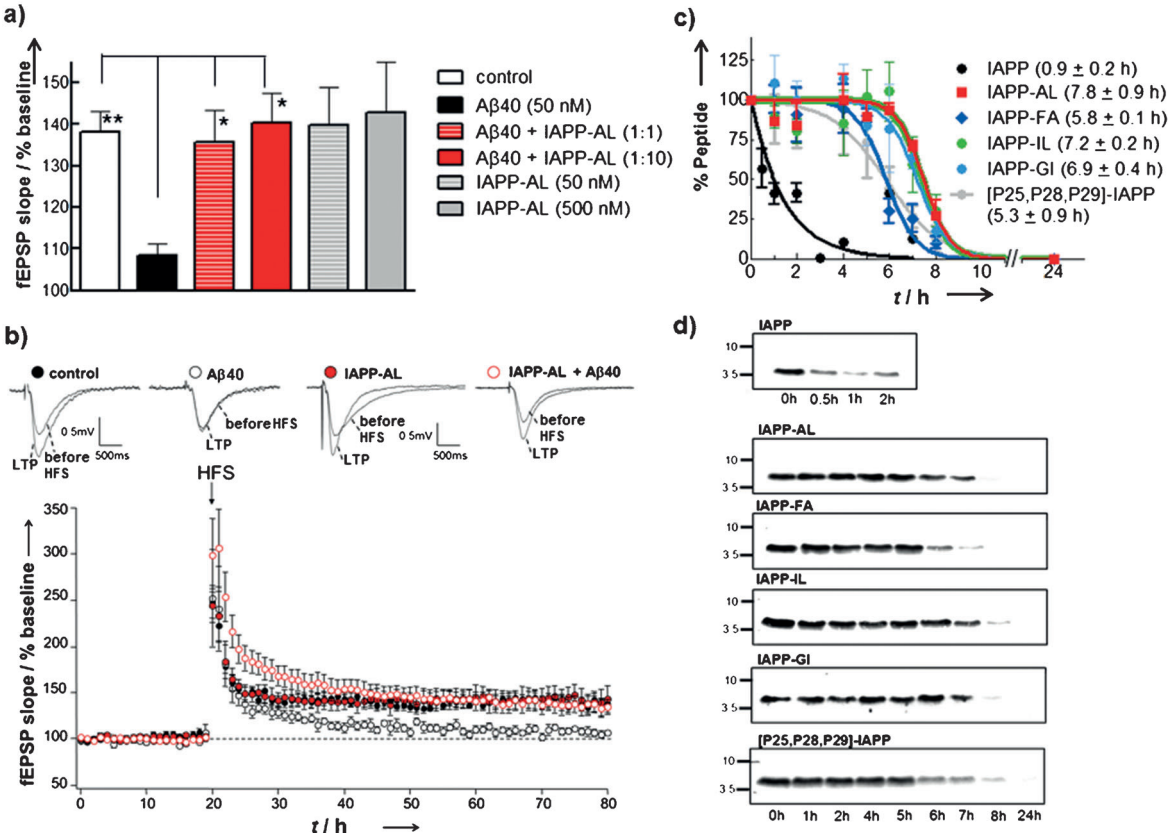

Figure 5. Effects of IAPP-AL on Aβ40-induced impairment of hippocampal LTP (a,b) and in vitro proteolytic stabilities of mimics, IAPP, and [P25,P28,P29]-IAPP in human blood plasma (c,d). a) Effects of controls, Aβ40 aggregates (50 nM), Aβ40 + IAPP-AL (1:1 or 1:10), and IAPP-AL on LTP in murine acute hippocampal slices. LTP magnitudes averaged from the last 10 min of recordings are shown as % of baseline fEPSP slopes (means (±SEM)). Significant effects were found for Aβ40 versus control ($p<0.01$, $n=7$) and versus the mixtures Aβ40 + IAPP-AL (1:1 and 1:10) ($p<0.05$; $n=7$ and $n=6$). Controls, $n=9$; IAPP-AL (500 nM), $n=5$, and IAPP-AL (50 nM), $n=6$. b) LTP measurements (peptides as indicated): Aβ40 (50 nM) was applied for 90 min before LTP induction by HFS (arrow). Responses (% of baseline fEPSP slopes) were measured for the next 60 min. The presence of IAPP-AL (500 nM) prevented Aβ40-induced LTP impairment. IAPP-AL (500 nM) alone did not affect LTP. The insets show representative fEPSP traces before and after HFS. Horizontal calibration bars, 500 ms; vertical bars, 0.5 mV. Data are means (±SEM) ($n=5$–9). c) Determination of proteolytic stabilities of peptides in human plasma by incubation (37°C) and quantification at various time points of remaining intact peptide via NuPAGE and western blot (WB) with anti-IAPP antibody. Remaining peptide (% of total) is plotted versus incubation time; $t_{1/2}$ values are shown in parentheses. Data are means (±SEM) from three to four assays (except for IAPP time points ≥3 h). d) Representative WBs following NuPAGE of incubations of peptides in human plasma (as in (c)) following removal of plasma proteins are shown (100% input (0 h), 3 μg).

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