Amyloid β (Aβ) Peptide Directly Activates Amylin-3 Receptor Subtype by Triggering Multiple Intracellular Signaling Pathways*§

Received for publication, December 6, 2011, and in revised form, April 10, 2012. Published, JBC Papers in Press, April 12, 2012, DOI 10.1074/jbc.M111.331181

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Background: Aβ and human amylin peptides share similar biophysical and neurotoxic properties.

Results: Aβ directly activates amylin-3 receptor (AMY3) isoform and triggers multiple signaling pathways.

Conclusion: Aβ actions are expressed via AMY3 receptors.

Significance: AMY3 could serve as a therapeutic target for attenuating Aβ toxicity.

The two age-prevalent diseases Alzheimer disease and type 2 diabetes mellitus share many common features including the deposition of amyloidogenic proteins, amyloid β protein (Aβ) and amylin (islet amyloid polypeptide), respectively. Recent evidence suggests that both Aβ and amylin may express their effects through the amylin receptor, although the precise mechanisms for this interaction at a cellular level are unknown. Here, we studied this by generating HEK293 cells with stable expression of an isoform of the amylin receptor family, amylin receptor-3 (AMY3). Aβ1–42 and human amylin (hAmylin) increase cytosolic cAMP and Ca2+, trigger multiple pathways involving the signal transduction mediators protein kinase A, MAPK, Akt, and cFos. Aβ1–42 and hAmylin also induce cell death during exposure for 24–48 h at low micromolar concentrations. In the presence of hAmylin, Aβ1–42 effects on HEK293-AMY3-expressing cells are occluded, suggesting a shared mechanism of action between the two peptides. Amylin receptor antagonist AC253 blocks increases in intracellular Ca2+, activation of protein kinase A, MAPK, Akt, cFos, and cell death, which occur upon AMY3 activation with hAmylin, Aβ1–42, or their co-application. Our data suggest that AMY3 plays an important role by serving as a receptor target for actions Aβ and thus may represent a novel therapeutic target for development of compounds to treat neurodegenerative conditions such as Alzheimer disease.

Alzheimer disease and type 2 diabetes mellitus are age-related diseases that are associated with the deposition of proteinaceous amyloid proteins within the brain (amyloid β protein, Aβ) and pancreatic islet cells (islet amyloid polypeptide, amylin), respectively (1–4). Aβ and amylin also share biophysical and biochemical characteristics including a propensity to aggregate and form fibrillar structures composed of a core of β-pleated sheets (5). Furthermore, amylin inhibits self-association of Aβ into cytotoxic aggregates through direct amylin and Aβ interactions (6, 7). A recent proteomics study also indicates that Aβ1–42 and human amylin (hAmylin)² share a common pathway for induction of toxicity via mitochondrial dysfunction (8). Also, evidence from pathophysiological, clinical, and epidemiological studies suggests that these two amyloidoses are linked to each other (9–11). In line with these findings, our studies on cultured human or rat fetal neurons, have shown that electrophysiological and neurotoxic actions of Aβ are strikingly similar to those for hAmylin and that such effects can be blocked by the amylin receptor antagonists AC253 and AC187 (12, 13). Additionally, we identified that down-regulation of the AMY3 isoform in neurons using shRNA can blunt the neurotoxic effects of Aβ (12). Collectively, these observations support the notion that Aβ and amylin share a common pathophysiological mechanism possibly via AMY3.

G protein-coupled receptors (GPCRs) constitute a superfamily of cell surface signaling proteins that mediates transduction of a large variety of extracellular stimuli across cell membranes and is involved in numerous key brain neurotransmitter systems, which may be disrupted in pathophysiological conditions such as Alzheimer disease (14). Amylin receptors belong to class B GPCRs and are heterodimeric complexes, formed by calcitonin receptor (CTR) association with receptor activity-modifying proteins (RAMPs) (15). The CTR component of this receptor shares the same general GPCR architecture: seven membrane-spanning interconnected α-helices that transmit extracellular signals to the intracellular cytoplasmic signaling cascade. Activation of amylin receptors in mammalian cells has been shown previously to raise cAMP and presumed to involve Gs protein (Gs α-guanine nucleotide-binding signal transduction protein) and stimulation of adenylate cyclase (16, 17). Recent binding data indicate that amylin has a high affinity for AMY3 receptors.

The abbreviations used are: hAmylin, human amylin; AMY3, amylin receptor-3; rAmylin, rat amylin; CGRP, calcitonin gene-related peptide; CTR, calcitonin receptor; GPCR, G protein-coupled receptor; MTT, Thiazolyl blue tetrazolium bromide; PKA R2, type II subunit of cyclic AMP-dependent protein kinase A; PKA, protein kinase A; RAMP, receptor activity modifying protein.

* This work was supported by Canadian Institutes of Health Research Grant CIHR MOP 93601 (to J. H. J.), the Canada Foundation of Innovation, and a scientist award from the Alberta- Heritage Foundation for Medical Research (to K. B.).

This article contains supplemental Figs. 1 and 2 and Videos 1–7.

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for the AMY3 isoform of amylin receptors (18). However, the precise intracellular signal transduction pathways following AMY3 activation are not fully understood, and it is not known whether Aβ directly activates AMY3. In the present study, we have for the first time expressed the AMY3 using human embryonic kidney 293 (HEK293) cells to study intracellular signal transduction pathways that are activated by Aβ or hAmylin and to further investigate whether their cytotoxic effects are mediated via this particular isoform of the amylin receptor.

**EXPERIMENTAL PROCEDURES**

**AMY3 Gene Construction and Expression**—AMY3 component gene cDNAs were purchased from Open Biosystems (Huntsville, AL): CTR (catalog no. MHS1010-98052186, gene access no. BC075028.2), RAMP3 (catalog no. MHS1010-9205630, gene access no. BC053852.1), RAMP1 (catalog no. MHS1011-59255, gene access no. BC000548.2), and RAMP2 (catalog no. MHS1010-7430169, gene access no. BC027975.1). CTR and RAMP3 genes were further subcloned in pcDNA3.1(+) (Invitrogen) and pBud-gfp vector (19), respectively. (The pBud-gfp vector was provided by Dr. David Westaway from University of Alberta.) The pBud-gfp-RAMP3 is a bigenic vector, which originally generated from the pBud CE4 vector and contained two distinct promoters upstream for GFP and RAMP3 genes. All of the CTR and RAMP3 gene sequences were confirmed by further DNA sequencing. HEK293 cells were cultured with DMEM (Invitrogen) with 10% FBS (Invitrogen) and grown at 37 °C, 5% CO₂. An AMY3-expressing HEK293 (AMY3-HEK293) cell line was generated by co-transfecting pcDNA3-CTR with pBud-gfp-RAMP3 with a 1:1 molecule ratio using Lipofectamine 2000 transfection reagent (Invitrogen). For controls, AMY1 (CTR + RAMP1) and AMY2 (CTR + RAMP2) expressing cells were generated using similar procedures as above. The blank plasmid pcDNA3 and pBud-gfp also were transfected and generated GFP-positive control wild type HEK293 cells. Zeocin™ was used for stable cell line selection and maintenance (200 ng/ml of final concentration). Passages 5–15 of AMY3-HEK293 cells were used for all experiments.

**Immunohistochemistry**—For CTR expression, AMY3-HEK293 cells were plated on glass coverslips precoated with poly-L-ornithine (Sigma) in DMEM, 10% FBS, Zeocin medium for 12–24 h. Cells on coverslips were then fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100, blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE), and stained with the following target antibodies. The phospho-p44/42 MAPK (ERK1/2, Thr-202/Tyr-204), phospho-Akt (Ser-473), and phospho-PKC (Thr-410/Tyr-416) were used as primary antibodies. The cFos rabbit polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibody was AMY3-HEK293 cell line was generated by co-transflecting pcDNA3-CTR with pBud-gfp-RAMP3 with a 1:1 molecule ratio using Lipofectamine 2000 transfection reagent (Invitrogen). For controls, AMY1 (CTR + RAMP1) and AMY2 (CTR + RAMP2) expressing cells were generated using similar procedures as above. The blank plasmid pcDNA3 and pBud-gfp also were transfected and generated GFP-positive control wild type HEK293 cells. Zeocin™ was used for stable cell line selection and maintenance (200 ng/ml of final concentration). Passages 5–15 of AMY3-HEK293 cells were used for all experiments.

**Captitative CAMP Measurements**—AMY3-HEK293 cells were plated on 24-well plates overnight. Cells were stimulated for 30 min with hAmylin, rat amylin (rAmylin), human calcitonin gene-related peptide, human adrenomedullin, Aβ1–42, and salmon calcitonin 8–32 over a concentration range (1 pm–1 μM). Cellular CAMP levels were measured using a parameter cyclic AMP assay kit (catalog no. KGE002B, R&D Systems) according to the manufacturer’s instructions. Data were plotted, and nonlinear regression was fitted with four parameters using Prism software (version 5, GraphPad Software, La Jolla, CA).

**Signal Profiling**—Intracellular signaling profiles were determined using in-cell Western blot techniques. AMY3-HEK293 cells were seeded at 10,000 cells/well in a 96-well plate (Nalg Nunc Intl., Rochester, NY) in DMEM, 10% FBS, and Zeocin medium. After culturing for 12–16 h, cells were pretreated for 24 h either or not with AC253 (10 μM) and then treated with either hAmylin or Aβ1–42 in culture medium for time periods between 10 min and 30 h. Subsequently, cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100, blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE), and stained with the following target antibodies. The phospho-p44/42 MAPK (ERK1/2, Thr-202/Tyr-204), phospho-Akt (Ser-473), and phospho-PKC (Thr-410/Tyr-416) were used as primary antibodies. The cFos rabbit polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibody was IRDye 800CW goat anti-rabbit antibody, whereas Sapphire700 and DRAQ5 were used for cell number normalization (LI-COR). For in-cell Western blot cyclic adenosine monophosphate (cAMP) quantification, mouse mononclonal anti-cAMP (R&D Systems) was used as a primary antibody, and IRDye 700 goat anti-mouse antibody (LI-COR) was used as a secondary antibody. Plates were imaged using an Odyssey Infrared Imaging System (LI-COR), and the integrated intensity was normalized to the total cell number on the same well.

**Ca²⁺ Imaging**—Dynamic changes of the free cytosolic Ca²⁺ concentration were monitored with confocal microscopy as described previously (21). For this, AMY3-HEK293 cells were plated on glass coverslips precoated with poly-L-ornithine and incubated at 37 °C for 12–36 h with DMEM, 10% FBS, and
Zeocin medium. For Ca\(^{2+}\) imaging, superfusate of ion content similar to extracellular brain fluid thus containing the following: 130 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, and 10 mM glucose (pH 7.35) was applied at a rate of 3 ml/min using a roller pump (Watson-Marlow Alitea, Sin-Can, Calgary, AB, Canada). For zero calcium experiment, superfusate of ion content similar to extracellular brain fluid thus containing the following: 130 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, and 10 mM glucose (pH 7.35) was applied at a rate of 3 ml/min using a roller pump (Watson-Marlow Alitea, Sin-Can, Calgary, AB, Canada). For zero calcium experiment, superfusate of ion content similar to extracellular brain fluid thus containing the following: 130 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, and 10 mM glucose (pH 7.35) was applied at a rate of 3 ml/min using a roller pump (Watson-Marlow Alitea, Sin-Can, Calgary, AB, Canada). For zero calcium experiment, superfusate of ion content similar to extracellular brain fluid thus containing the following: 130 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, and 10 mM glucose (pH 7.35) was applied at a rate of 3 ml/min using a roller pump (Watson-Marlow Alitea, Sin-Can, Calgary, AB, Canada). For zero calcium experiment, superfusate of ion content similar to extracellular brain fluid thus containing the following: 130 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, and 10 mM glucose (pH 7.35) was applied at a rate of 3 ml/min using a roller pump (Watson-Marlow Alitea, Sin-Can, Calgary, AB, Canada). For zero calcium experiment, superfusate of ion content similar to extracellular brain fluid thus containing the following: 130 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, and 10 mM glucose (pH 7.35) was applied at a rate of 3 ml/min using a roller pump (Watson-Marlow Alitea, Sin-Can, Calgary, AB, Canada). For zero calcium experiment, superfusate of ion content similar to extracellular brain fluid thus containing the following: 130 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, and 10 mM glucose (pH 7.35) was applied at a rate of 3 ml/min using a roller pump (Watson-Marlow Alitea, Sin-Can, Calgary, AB, Canada). For zero calcium experiment, superfusate of ion content similar to extracellular brain fluid thus containing the following: 130 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, and 10 mM glucose (pH 7.35) was applied at a rate of 3 ml/min using a roller pump (Watson-Marlow Alitea, Sin-Can, Calgary, AB, Canada). For zero calcium experiment, superfusate of ion content similar to extracellular brain fluid thus containing the following: 130 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, and 10 mM glucose (pH 7.35) was applied at a rate of 3 ml/min using a roller pump (Watson-Marlow Alitea, Sin-Can, Calgary, AB, Canada). For zero calcium experiment, superfusate of ion content similar to extracellular brain fluid thus containing the following: 130 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, and 10 mM glucose (pH 7.35) was applied at a rate of 3 ml/min using a roller pump (Watson-Marlow Alitea, Sin-Can, Calgary, AB, Canada). For zero calcium experiment, superfusate of ion content similar to extracellular brain fluid thus containing the following: 130 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, and 10 mM glucose (pH 7.35) was applied at a rate of 3 ml/min using a roller pump (Watson-Marlow Alitea, Sin-Can, Calgary, AB, Canada). For zero calcium experiment, superfusate of ion content similar to extracellular brain fluid thus containing the following: 130 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, and 10 mM glucose (pH 7.35) was applied at a rate of 3 ml/min using a roller pump (Watson-Marlow Alitea, Sin-Can, Calgary, AB, Canada). For zero calcium experiment, superfusate of ion content similar to extracellular brain fluid thus containing the following: 130 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, and 10 mM glucose (pH 7.35) was applied at a rate of 3 ml/min using a roller pump (Watson-Marlow Alitea, Sin-Can, Calgary, AB, Canada).
ance followed by Tukey’s test when appropriate using Prism software. \( p \leq 0.05 \) was taken as significant.

**RESULTS**

**Stable Expression of AMY3 in HEK293 Cells**—We first generated the novel, AMY3-HEK293 stable cell line using co-transfection of the genes for the AMY3 constituents, CTR, and RAMP3 (Fig. 1a), in a 1:1 molecule ratio with the assumption of equal CTR and RAMP3 expression in individual cells. As an indication of RAMP3 expression, 60–70% of AMY3-HEK293 cells were GFP-positive 24 h following co-transfection, whereas >95% of cells were GFP-positive already after 3 passages with...
zeocin selection using 200 ng/ml. RT-PCR further confirmed RAMP3 gene expression in the AMY3-HEK293 cells. There is weak endogenous RAMP1 and RAMP2 gene expression in HEK293 cells that does not change after AMY3 expression. There is also little endogenous RAMP3 gene expression in HEK293 cells (Fig. 1b). Fig. 1c shows GFP-positive cells are all stained with CTR, which indicated successful AMY3 expression.

AMY3 Activation with Aβ1–42 and hAmylin Increases Cellular cAMP—As indication that the AMY3 is functional in the new cell line, we first identified that cAMP increases observed after 30 min of exposure to the established agonist for this receptor, hAmylin (1 μM), could also be evoked following application of Aβ1–42 (1 μM) (Fig. 2a). As expected, hAmylin and Aβ1–42 did not evoke cAMP increases in control wild type cells (Fig. 2b). There also was no significant increase in cAMP following exposure to hAmylin or Aβ1–42 in HEK293 cells that expressed single components of AMY3, i.e. either a functional CTR (supplemental Fig. 1) or RAMP3. Next, we quantified the effects of hAmylin (0.1–10 μM) on cAMP levels with in-cell Western blot. In fact, both agents increased cAMP in a very similar, concentration-dependent manner (Fig. 2c). In contrast, cAMP was not affected by Aβ42–1, human calcitonin gene-
related peptide or human adrenomedullin (Fig. 2d). To further validate functional expression of AMY3 in the HEK cells, a cAMP assay over a full concentration range of the different peptides was performed. Aβ1–42, hAmylin, and rAmylin concentration-response curves were non-linearly fitted (Fig. 2e). The EC$_{50}$ values for Aβ1–42, hAmylin, and rAmylin are 7.7, 2.4, and 6.9 nM, respectively.

**AMY3 Activation with Aβ1–42 and hAmylin Increases Cytosolic Ca$^{2+}$**—Confocal microscopy was used to investigate whether signaling pathways in AMY3 activation include the important intracellular second messenger Ca$^{2+}$ (26). Under control conditions, fluorescence intensity in cells loaded with the fluorescent Ca$^{2+}$ dye Fluo-8L-AM did not show notable spontaneous fluctuations of cytosolic Ca$^{2+}$ (Fig. 3a). Bath application of hAmylin (0.1–2 μM) for 30 s produced a major Ca$^{2+}$ increase within <1 min after entry of the peptide within the imaging chamber. These Ca$^{2+}$ increases displayed a sharp peak, indicating that return to base line already started at the end of the application period of the peptide or within <30 s after start of recovery to superfusate. Recovery to base line was achieved typically within <2 min after return to control perfusing solution (Fig. 3b). Also Aβ1–42 (0.1–2 μM) increased cytosolic Ca$^{2+}$ in a fashion very similar to that observed for hAmylin (Fig. 3c). In contrast, Ca$^{2+}$ did not rise in response to hAmylin (0.5 μM, 30 s) following superfusion of cells with AC253 (2 μM) for 30 s prior to application of the agent, whereas AC253 alone (1–10 μM) also did not change Ca$^{2+}$ base line (Fig. 3e and f). Similarly, the Ca$^{2+}$ increase due to Aβ1–42 (0.5 μM, 30 s) was abolished by preincubation of AC253 (2 μM) (Fig. 3g). Co-application of Aβ1–42 (0.25 μM) with hAmylin (0.25 μM) elevated Ca$^{2+}$ levels to a similar extent as when each drug was applied alone, and this response to a combined application of the two peptides also was abolished by 2 μM AC253 (Fig. 3, d and h). Bar graphs in Fig. 3i show quantification of these data. Fig. 3j and the accompanying videos (supplemental data) show time-lapsed recordings of Ca$^{2+}$ signals from the same AMY3-HEK293 cell in response to hAmylin, Aβ1–42, hAmylin + Aβ1–42, AC253, AC253 + hAmylin + Aβ1–42, and recovery. In wild type (non-transfected) HEK293 cells, Aβ1–42 or hAmylin did not induce increases in cytosolic Ca$^{2+}$ (data not shown).

Increases in cytosolic Ca$^{2+}$ levels after AMY3 activation with hAmylin and Aβ1–42 mainly depend on extracellular calcium. In the presence of calcium-free superfusate, hAmylin and Aβ1–42 only produced small and delayed cytosolic Ca$^{2+}$ increases (~20% of peak increases of cytosolic Ca$^{2+}$ under normal calcium concentration, Fig. 4).

**Signaling Pathways Involved in Aβ1–42 Activation of AMY3**—In addition to their effects on cAMP and Ca$^{2+}$, we found here that Aβ1–42 and hAmylin also increase phosphorylation of the type II subunit of cAMP-dependent protein kinase A (PKA R2) (Fig. 5a), activate mitogen-activated protein kinase (ERK1/2) (Fig. 5b), increase cellular levels of the transcription factor cFos (Fig. 5c), and increase phosphorylation of Akt (protein kinase B (pAkt)) (Fig. 5d). All of these effects were blocked by AC253 (Fig. 5, a–d) and
not mimicked by Aβ42–1 (data not shown). In another series of experiments, phosphorylation of protein kinase C was not observed to change with exposure to either Aβ1–42 or hAmylin (data not shown).

In a further set of experiments, we elucidated that phosphorylation of ERK1/2 or PKA depends on the duration of exposure to hAmylin or Aβ1–42. The phosphoERK1/2 peak is reached ~10 min after start of application and returns to base-
AMY3 activation using KH7 (adenylate cyclase inhibitor) or FR180204 (an ERK1/2 inhibitor) also protected AMY3 cells from hAmylin or Aβ1–42 cytotoxicity (Fig. 7d).

DISCUSSION

Our data demonstrate that both Aβ1–42 and hAmylin act as agonists at the AMY3 subtype that has been expressed here for the first time using HEK293 cells. Amylin receptors are heterodimerized by CTR and one of three RAMPs, thus generating multiple amylin receptor subtypes, AMY1–3 (Fig. 1a) (15). There are several CTR isoforms in the human (27). In this study, hCTRA was used for AMY3 receptor construction, which is insert-negative and modulates cell cycle progression (28). The importance of receptor splice variation in AMY physiology remains to be elucidated. These amylin receptor subtypes are pharmacologically distinct and demonstrate different binding affinities to members of the calcitonin peptide family, which includes CGRP, adrenomedullin, and amylin (15). However, due to the lack of selective pharmacological tools, significant complexity of this system and lack of specific RAMP antibodies, it has been difficult to confidently assign specific amylin functions to one of these receptor subtypes. Data from functional bioassays, including binding studies (16, 29, 30), demonstrate that AMY3, which is a heterodimeric complex of CTR and RAMP3, has a high affinity for amylin. Amylin receptor components (CTR and RAMPs) are reported to be distributed widely in the central nervous system with pronounced expression within spinal cord, brain stem, cortex, hypothalamus, and hippocampus (31, 32). However, data on distribution of specific subtypes of RAMPs within the CNS is lacking. Moreover, at present, there is no information on the functional effects of co-localization of CTR with RAMP3 to generate AMY3. Herein, we provide evidence that the AMY3 subtype is indeed the specific target receptor for direct actions of Aβ (and hAmylin) at the level of the cellular membrane.

For the cAMP production assay, the mean EC_{50} for hAmylin (2.4 nM) in our AMY3 cell line is close to a previous reported value for rAmylin (18). We have identified that AMY3 activation results in Gα_{s}-mediated adenylate cyclase activation, with a subsequent increase in cAMP and activation of PKA. This occurred in a manner similar to that reported for the CGRP receptor, which is also a member of the same family of CTR (33). PKA is a multiunit protein kinase that mediates signal transduction of GPCRs following their activation by adenylyl cyclase-mediated cAMP formation and is involved in a wide range of cellular processes. PKA R2 is one of the regulatory isoforms of the enzyme, which is predominantly expressed in adipose tissue and brain (34). Nearly all PKA activity in adipose tissue and 50% of PKA activity in the striatum, hypothalamus, and cortex is attributed to the subunit. Disruption of PKA R2 affects physiological mechanisms known to be associated with healthy aging in mammals, which include increased lifespan and decreased incidence and severity of a number of age-related diseases in PKA R2 null mice. In that context, our data further indicate that PKA R2 is secondarily activated after Aβ or hAmylin stimulation of AMY3 and that such an effect may contribute to the long term deleterious neuronal actions of Aβ.

**FIGURE 6.** AMY3-related signaling depends on hAmylin and Aβ1–42 application time. a, after exposure to either hAmylin (hA; 0.2 μM) or Aβ1–42 (0.5 μM), phosphoERK1/2 levels peak within 10 min and return to base-line level after 0.5–1 h. b, maximum phosphoPKA R2 increase is also reached within ~10 min, but the phosphorylation lasts longer and returns to base line only after 4–24 h. *p < 0.05, compared to 0 time point.

*line level within 0.5 h (or slightly more or less) after exposure to either hAmylin (0.2 μM) or Aβ1–42 (0.5 μM) (Fig. 6a). The phospho-PKA R2 also reaches a maximum level at ~10 min, but the phosphorylated form of this protein lasts longer and returns to base line level at 2 h (or slightly more or less) for Aβ and hAmylin (Fig. 6b). Aβ1–42 Activation of AMY3 Triggers Cell Death at Higher Concentrations and Longer Exposure Times—Our previous observations that Aβ1–42 and hAmylin induce apoptotic cell death in cultured neurons suggested that these effects may require AMY3 activation (12, 13). This hypothesis is supported by our present findings using an MTT assay that 48 h incubation with either Aβ1–42 (2–20 μM) or hAmylin (0.2–2 μM) causes cell death of AMY3-HEK293 cells in a concentration-dependent manner (Fig. 7a). Next, we confirmed that hAmylin and Aβ1–42 induced cell death is dependent on AMY3 expression. In cells expressing other amylin receptor subtypes (AMY1 or AMY2, supplemental Fig. 2) or single components of AMY3 (CTR or RAMP3), hAmylin, and Aβ1–42 did not induce significant cell death (Fig. 7b). The cell death observed when AMY3-HEK cells were exposed to hAmylin or Aβ1–42 occurred after 24 h (Fig. 7c). Furthermore, cell death induced from hAmylin and Aβ1–42 is attenuated significantly by pretreatment with AC253 (Fig. 7d). Interference with downstream mediators of...
or amylin as indicated by the present findings of increased cell death of AMY3-HEK293 cells exposed to these peptides.

The increases in cytosolic Ca\(^{2+}\) observed here following hAmylin or A\(\beta\)-42 application could also occur via Goq activating. Goq proteins activate phospholipase C, which cleaves phosphatidylinositol-4,5-bisphosphate into diacyl-glycerol and inositol trisphosphate, leading to mobilization of Ca\(^{2+}\) from cellular stores (35). Ca\(^{2+}\) represents a ubiquitous intracellular second messenger with enormous versatility (26). The versatility of Ca\(^{2+}\) as a signaling molecule is based on its binding kinetics, varying amplitude, spatiotemporal distribution, and ability to cross-talk with multiple other signaling cascades within the GPCR-activated pathways, including ERK and Akt. The Akt serine/threonine kinase (also called protein kinase B) has emerged as a critical signaling molecule within eukaryotic cells and regulates diverse aspects of neuronal cell function protein translation and cell size, axonal outgrowth, suppression of apoptosis, and synaptic plasticity. Akt activation could result from activation of phosphatidylinositol 3-kinase (PI3K) or Goq activation.

Inhibition of adenylyl cyclase at a lower concentrations of KH7 (1–2 μM) can protect AMY3 cells from hAmylin and A\(\beta\)-42 damage. However, at higher concentrations (4 μM), KH7 did not demonstrate a protective effect, which could be related to interruption of normal cellular function at such concentrations. Altering activity of adenyl cyclase results in changes in cAMP second messenger levels, which in turn affects PKA and protein phosphatase A activity. Protein phosphatase A is inhibited by increased PKA activity, thus maintaining Akt in an activated state (36). Both hAmylin and A\(\beta\)-42 activate Akt, which indicates that AMY3 may play also an important role in controlling cell fate. Most likely, changes in Akt activity that we observed are secondary to alterations in cAMP and PKA that result from AMY3 activation. The rapid Ca\(^{2+}\) increase associated with AMY3 activation could also contribute to Akt activation.

ERK1/2 is a member of the mitogen-activated protein kinase family that is centrally involved in many processes during the lifetime of a cell. This kinase has been not only associated with proliferation, differentiation, and protection against apoptosis but also has been linked to cell death (37). A selective ERK1/2 inhibitor, FR180204, can protect AMY3 against hAmylin or A\(\beta\)-42 toxicity, but this protective effect does not appear to be concentration dependent. The magnitude and the duration of activities can be significant in a cell.

FIGURE 7. A\(\beta\)-42 and hAmylin activation of AMY3 triggers cell death in a concentration- and time dependent manner. a, using an MTT assay, hAmylin and A\(\beta\)-42 induced cell death in a concentration (conc.)-dependent manner (n = 4 for each treatment group). b, unlike AMY3-HEK cells, hAmylin and A\(\beta\)-42 (1 μM 48 h) did not induce significant cell death in control HEK-gfp cells, cells expressing single AMY3 receptor components (CTR or RAMP3), or other amylin receptor subtypes (AMY1 or AMY2) (n = 8 for each). c shows cell death occurred after 24 h when AMY3-HEK cells were exposed to either hAmylin or A\(\beta\)-42 (0.2 and 2 μM). d shows cell survival after 48 h exposure to hAmylin or A\(\beta\)-42 (1 μM) and following preincubation with AC253 (2.5 μM), KH7 (1, 2, and 4 μM), or FR180204 (FR; 1 and 2 μM) for 12 h, (n = 8 for each treatment group). *, p < 0.05; **, p < 0.01, one-way analysis of variance followed by Tukey’s test.
Aβ and may play an important role in pathogenesis of conditions, where these amyloidogenic proteins have been implicated, namely type 2 diabetes mellitus and Alzheimer disease. Thus, it may be possible, for example, to develop novel therapies for Alzheimer disease by altering AMY3 function or its downstream signaling pathways through the design of highly selective antagonists for this isoform of amyl receptor family.

Acknowledgments—We thank The Pulmonary Research Group and Chris Laurent (University of Alberta) for access to ICW instrumentation and technical assistance with experiments utilizing this technique. We thank Dr. Khem Jhamandas for useful comments and suggestions on the manuscript.

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FIGURE 8. Aβ1–42-activated cellular signaling pathways and AMY3 role in AD. AMY3 is a multimeric complex formed by CTR and RAMP3 (see also Fig. 1a). Direct activation of AMY3 with hAmylin and Aβ1–42 results in Gqα-mediated adenylate cyclase (AC) stimulation, with subsequent increase in cellular cAMP, stimulation of PKA R2, downstream activation of ERK1/2, and an increase transcription factor cFos expression. The observed cytosolic Ca2+ rise could be due to Gqα activation. Akt is also activated followed cAMP and PKA activation. The cytosolic Ca2+ changes could also contribute to Akt activation. Long term AMY3 activation could trigger an ERK1/2 pathway that may cause Ca2+ imbalance and perturb homeostasis of the endoplasmic reticulum (ER), leading to cell death. MI, mitochondria.

of ERK1/2 activity may determine its role in regulating different aspects of cellular function. Following AMY3 activation, temporal changes in ERK1/2 phosphorylation followed alterations in cAMP and PKA activity. At early stages or with short term activation, ERK1/2 is associated with cell proliferation, regulation of cellular function, and increases in the transcription factor, cFos. However, after longer periods of activation, ERK1/2 appears to trigger cell death pathways, an observation that seems consistent with our finding of phosphorylation of this kinase and the resultant cell death as shown by our MTT assay data.

In conclusion, we provide for the first time evidence that Aβ1–42 directly activates AMY3 and triggers several intracellular signaling pathways, including cytosolic cAMP and Ca2+ rises. AMY3 likely regulates cellular functions by changing activity of PKA, ERK1/2, and Akt. The sustained activation of AMY3 triggers phosphorylation of ERK1/2 resulting in cell death. Putative uncontrolled elevations of cytosolic Ca2+ as a result of prolonged AMY3 activation may also perturb homeostasis of the endoplasmic reticulum, produce mitochondrial dysregulation and engagement of caspases that contribute to apoptosis. The possible pathophysiological mechanisms whereby Aβ1–42 activates AMY3 and triggers multiple signaling pathways are illustrated in Fig. 8. Our data suggest the AMY3 is receptor target for the actions of human amylin and...
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VOLUME 287 • NUMBER 22 • MAY 25, 2012