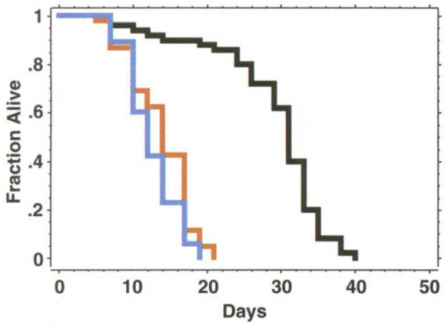

Fig. 4. Loss of *daf-2* function during development does not increase life-span. Wild-type animals were grown on bacteria expressing *daf-2* dsRNA from hatching until the first day of adulthood and then transferred to bacteria expressing dsRNA of *dcr-1*. Red line, life-spans of wild-type animals grown on *daf-2* RNAi bacteria during development and then shifted during day 1 of adulthood to bacteria expressing *dcr-1* dsRNA. Blue line, life-span of wild-type animals grown on the control RNAi bacteria during development and then shifted during day 1 of adulthood to *dcr-1* RNAi bacteria. Black line, life-span of wild-type animals grown on *daf-2* RNAi bacteria during development and adulthood. Life-span studies were conducted at 25°C. For statistics, see table S1.

through this pathway would invariably be associated with impaired growth or reproduction. Instead, our findings suggest that, in other organisms as well, it may be possible to manipulate insulin/IGF-1 signaling during adulthood so as to extend youthfulness and life-span without affecting growth or reproduction.

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Table S1

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Targeting of Cyclic AMP Degradation to β_2-Adrenergic Receptors by β-Arrestins

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Catecholamines signal through the β_2-adrenergic receptor by promoting production of the second messenger adenosine 3′,5′-monophosphate (cAMP). The magnitude of this signal is restricted by desensitization of the receptors through their binding to β-arrestins and by cAMP degradation by phosphodiesterase (PDE) enzymes. We show that β-arrestins coordinate both processes by recruiting PDEs to activated β_2-adrenergic receptors in the plasma membrane of mammalian cells. In doing so, the β-arrestins limit activation of membrane-associated cAMP-activated protein kinase by simultaneously slowing the rate of cAMP production through receptor desensitization and increasing the rate of its degradation at the membrane.

Many hormones elicit their effects on cells by binding to and activating cell-surface guanine nucleotide binding protein (G protein)–coupled receptors (GPCRs) (*1*). Once activated, GPCRs couple to and activate specific G protein isoforms that promote the production of intracellular second messengers such as cAMP, thus initiating signaling cascades that result in diverse cellular responses. To limit the magnitude of GPCR signals, and to return the cell to its unstimulated state, further receptor–G protein coupling must be prevented and the already synthesized second messenger molecules must be degraded. Receptor uncoupling occurs through desensitization (*2*, *3*), whereby activated receptors become phosphorylated and bind to β-arrestin proteins, inhibiting further interaction with G proteins. Cyclic AMP is degraded by the phosphodiesterase (PDE) family of enzymes (*4*, *5*). Because many PDE isoforms are targeted to subcellular structures through association with signaling and scaffolding proteins (*4*–*11*), the rate of cAMP degradation likely depends on the type and amount of PDEs present at a specific subcellular location (*12*, *13*). In this manner, free diffusion of cAMP within the cell is impeded and microdomains of cAMP signaling are created where generation of cAMP by adenylyl cyclase is highest and PDE activity is lowest (*13*, *14*).

In addition to receptor desensitization, the β-arrestins also function as multivalent adaptor proteins that recruit a variety of cytosolic proteins to their sites of action at the plasma membrane (*15*). In doing so, β-arrestins allow diverse plasma membrane-associated signals to be targeted to and regulated by GPCRs. We investigated whether PDE targeting to the plasma membrane is also regulated by GPCR stimulation. In transfected human embryonic kidney (HEK293) cells overexpressing recombinant stimulatory G protein (G_s) and adenylyl cyclase–coupled β_2 adrenergic receptors, stimulation with the β agonist isoproterenol resulted in time-dependent targeting of endogenous PDE4D3 and PDE4D5 to cell membranes (Fig. 1, A and B), the major PDE4 isoforms detected in these cells (*16*). The increase was detected 2 min after stimulation, continued to increase until 5 min after stimulation, and then declined. This transient nature of the recruitment was similar to that observed for β-arrestins (Fig. 1, A and B)

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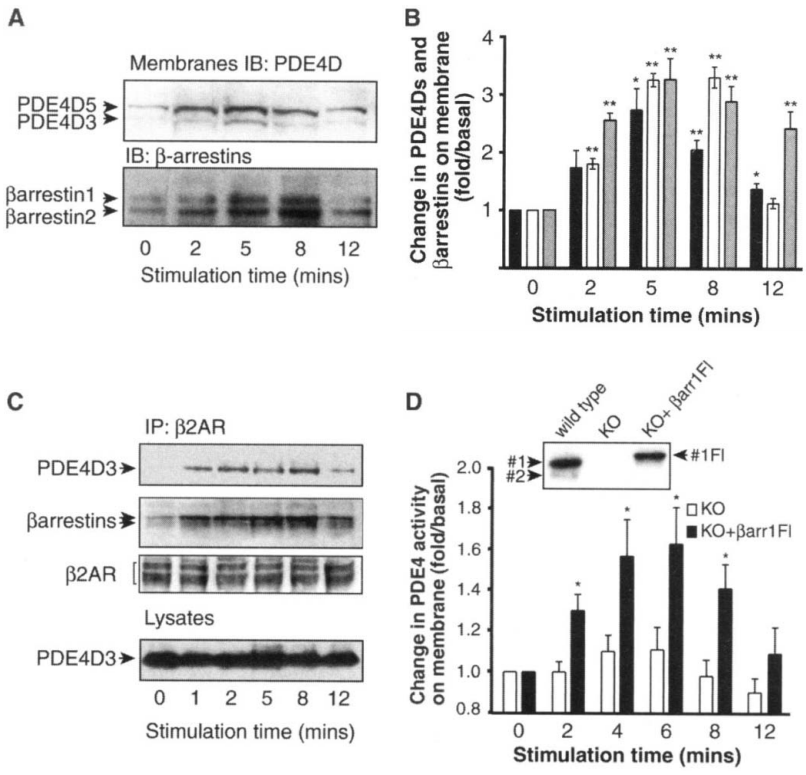

Fig. 1. β_2-adrenergic receptor-mediated recruitment of PDE4D and β-arrestins. (**A**) Recruitment to membranes. We stimulated HEK293 cells expressing a FLAG–β_2-adrenergic receptor–green fluorescent protein (GFP) construct with 10 μM (−)-isoproterenol for 0 to 12 min and examined 50 μg of membrane proteins (determined by a Bradford protein assay) for the presence of PDE4D3 and PDE4D5 (**upper**) and β-arrestin1 and β-arrestin2 (**lower**) by immunoblotting with antibodies to PDE4D and β-arrestin (A1CT). Representative immunoblots from four identical experiments are shown. (**B**) Quantification of PDE4D (black bars) and β-arrestin (white bars) protein levels and PDE4 activity (gray bars) associating with membranes after (−)-isoproterenol stimulation. $^*P < 0.05$; $^{**}P < 0.01$ (compared with 0 min of stimulation; mean ± SEM from four or five experiments). Basal PDE4 activity on membranes was 17.9 ± 1.1 pmol min^{-1} mg^{-1}, or 56% of the total cAMP-hydrolyzing PDE activity measured. This increased to 79% after 5 min of stimulation. (**C**) Recruitment of PDE4D3 to β_2-adrenergic receptors. HEK293 cells expressing the FLAG–β_2-adrenergic receptor-GFP construct and PDE4D3 were stimulated with 10 μM (−)-isoproterenol for 0 to 12 min, cell lysates were prepared, protein levels were normalized, and the receptors were immunoprecipitated with an antibody to FLAG (M2) conjugated to agarose (IP: β2AR). Representative immunoblots of three experiments showing recruitment of PDE4D3 (**top**, first row) and β-arrestins (**top**, second row) to the β_2-adrenergic receptor (**top**, third row); expression levels of recombinant PDE4D3 in cell lysates (**bottom**). Immunoblotting used antibodies to PDE4D, β-arrestin (A1CT), and FLAG (M2). (**D**) β-arrestin dependence of PDE4 recruitment. Mouse embryonic fibroblasts (MEFs) lacking expression of both β-arrestins (derived from β-arrestin1$^{-/-}$ β-arrestin2$^{-/-}$ knockout mice), stably transfected with pcDNA3.1-Zeo (white bars) or β-arrestin1–FLAG (black bars), were stimulated with 10 μM (−)-isoproterenol, and membrane-associated PDE4 activity was determined. $^*P < 0.05$, comparing cells expressing β-arrestin1–FLAG with control cells at the same time points (mean ± SEM from five to nine experiments). We compared expression levels of β-arrestin1 (#1), β-arrestin1–FLAG (#1Fl), and β-arrestin2 (#2) by immunoblotting 70 μg of total cellular protein from wild-type MEFs, knockout MEFs transfected with pcDNA3.1-Zeo (KO), and knockout MEFs transfected with pcDNA3.1-Zeo–β-arrestin1–FLAG (KO+βarr1Fl) with antibody to β-arrestin (A1CT).

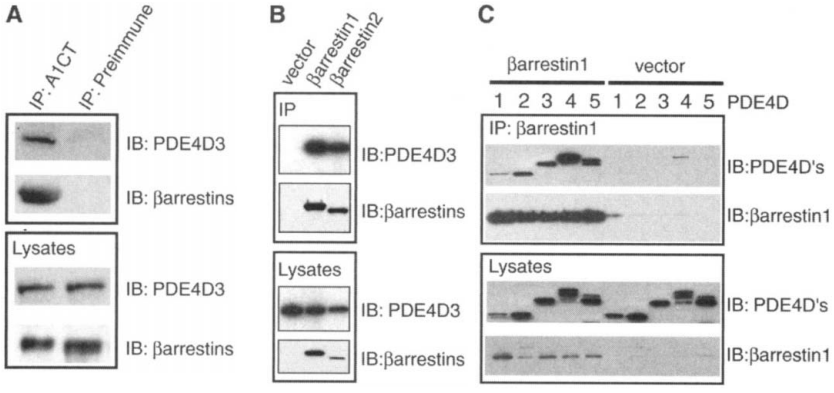

Fig. 2. Interaction between β-arrestins and PDE4D isoforms in cells. (**A**) Endogenous β-arrestin1 was immunoprecipitated from Rat-1 cell cytosol by using the antiserum to β-arrestin A1CT (IP: A1CT) and the paired preimmune serum (IP: Preimmune); the presence of endogenous PDE4D3 and β-arrestin1 in the immune complexes was detected by immunoblotting with the indicated antibodies (IB: first and second rows; representative of three experiments). Endogenous protein levels were detected by immunoblotting cell lysates (IB: third and fourth rows). (**B**) Interaction of PDE4D3 with both β-arrestins. Lysates from COS7 cells overexpressing PDE4D3 with empty vector, β-arrestin1–FLAG, or β-arrestin2–FLAG were subjected to immunoprecipitation (IP) with an antibody to FLAG (M2) conjugated to agarose. We detected FLAG-tagged β-arrestins and associated PDE4D3 in immune complexes by immunoblotting with the indicated antibodies (IB: first and second rows); expression was confirmed by immunoblotting the cell lysates (IB: third and fourth rows). Representative blots of three similar experiments are shown. (**C**) Interaction of five PDE4D isoforms with β-arrestin1. Lysates from COS7 cells expressing one each of all five PDE4D isoforms with β-arrestin1–FLAG or empty vector were subjected to immunoprecipitation with an antibody to FLAG (M2) conjugated to agarose. β-arrestin1–FLAG and associated PDE4D isoforms were detected in the immune complexes by immunoblotting with the indicated antibodies (IB: first and second rows); expression was confirmed by immunoblotting the cell lysates (IB: third and fourth rows). Representative blots of three similar experiments are shown.

and may represent a regulated dissociation of the complex, possibly as the receptors are internalized. We also observed a parallel increase in membrane-associated PDE4 activity (Fig. 1B).

When we stimulated transfected cells overexpressing PDE4D3 and the β_2-adrenergic receptor with isoproterenol, both PDE4D3 and β-arrestins were recruited to receptors (Fig. 1C) with time courses similar to those observed for recruitment to membranes (Fig. 1A). This suggests that β-arrestins could serve as adaptors for the translocation of PDE4Ds to activated receptors. No recruitment of PDE4 to membranes was observed in a mouse embryonic fibroblast cell line lacking both β-arrestin1 and β-arrestin2 but expressing endogenous β-adrenergic receptors (*17*) when it was treated with isoproterenol. However, an amount of membrane recruitment of PDE4 equivalent to that observed in wild-type fibroblasts (*18*) was reestablished in these cells when βarrestin1 was exogenously expressed to wild-type levels (Fig. 1D), demonstrating that β-arrestin is required for the recruitment of PDE4 to the membrane.

If β-arrestins recruit PDE4Ds to the plasma membrane, the two proteins might be expected to associate with each other in the cytosol of cells at their normal, endogenous levels of expression. We detected complexes containing both PDE4D3 and β-arrestin1 in the cytosol of untransfected Rat-1 cells by immunoprecipita-

tion (Fig. 2A). When overexpressed in cells, both β-arrestin1 and β-arrestin2 immunoprecipitated with PDE4D3 (Fig. 2B). Through alternative splicing and the use of multiple promoters, the PDE4D gene encodes five isoforms (PDE4D1 to -5) with identical catalytic domains and carboxyl termini but different amino termini (*19*, *20*) that are responsible for specific interactions with scaffolding and signaling proteins (*4*–*6*, *9*–*11*). However, when expressed in cells with β-arrestin1 (Fig. 2C) or β-arrestin2 (*18*) all five isoforms immunoprecipitated with both β-arrestins, indicating that the common catalytic and carboxyl domains may be involved in β-arrestin association. β-arrestin1 also immunoprecipitated with other PDE4 family members (PDE4A4, -4B1, -4B2, and -4C2) (fig. S1) but not guanosine 3′,5′-monophosphate–hydrolyzing PDE5A1, which shows only weak sequence homology to the PDE4s (*18*). We observed a direct interaction between β-arrestin1 (Fig. 3) and β-arrestin2 (*18*) and purified fusion proteins composed of maltose binding protein (MBP) and either PDE4D3 or PDE4D5 (Fig. 3). Yeast two-hybrid analysis further confirmed this, as both β-arrestins bound the region common to all PDE4Ds but not, for example, the unique amino terminus of PDE4D3 (table S1).

An agonist-induced recruitment of cAMP-hydrolyzing enzymes to GPCRs would be expected to enhance the rate of cAMP degradation at the plasma membrane. This could both reduce G_s-coupled receptor signaling and enhance inhibitory G protein (G_i)–coupled receptor inhibition of cAMP levels. To determine the functional significance of this recruitment, we tested activation of membrane-associated protein kinase A (PKA) by cAMP in cells in which recruitment of PDE4s to the membrane was blocked by overexpression of a mutant PDE4D that lacked catalytic activity but that could still interact with β-arrestins. We generated a PDE4D5 mutant that retains <0.1% of the activity of the wild-type enzyme (*18*) by making a discrete single mutation, Asp^{556} to Ala (D556A), in its catalytic site. When overexpressed in cells, PDE4D5(D556A) retained its ability to bind β-arrestin1 (Fig. 4A) but inhibited recruitment of the endogenous wild-type PDE4 to membranes in response to β_2-adrenergic receptor stimulation (Fig. 4B). Overexpression of this mutant enhanced isoproterenol-stimulated PKA activity on membranes compared with mock-transfected cells (Fig. 4C). Moreover, this effect was specific to agonist-stimulated PKA activity associated with the membrane as PDE4D5(D556A) did not alter cytosolic or membrane-bound PKA activity in unstimulated cells (fig. S2).

Our results demonstrate a mechanism by which the β-arrestins attend to the degradation of cAMP. By recruiting cAMP phosphodiesterases to ligand-activated receptors, the β-arrestins target cAMP degradation to sites of localized PKA activity at the plasma membrane. In this manner, β-arrestins coordinate both receptor desensitization and the quenching of PKA activity.

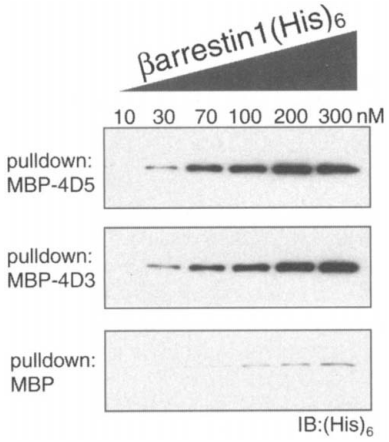

Fig. 3. Analysis of the interaction between β-arrestins and PDE4D in vitro. MBP fusion proteins of PDE4D3, PDE4D5, and MBP alone, expressed in and purified from bacteria, were incubated with 10 to 300 nM purified β-arrestin1$(His)_6$. The quantity of β-arrestin that remained bound after washing was measured by immunoblotting [IB: $(His)_6$; blots are representative of three similar experiments].

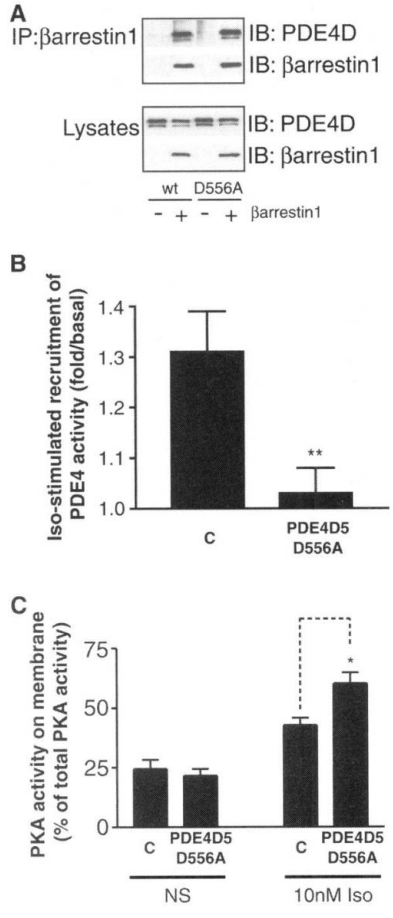

Fig. 4. Effects of catalytically inactive PDE4D5(D556A) on β_2-adrenergic receptor signaling. (**A**) Comparison of β-arrestin1 binding to wild-type and mutant PDE4D5. Lysates from COS7 cells overexpressing wild-type (wt) PDE4D5 or PDE4D5(D556A) with or without β-arrestin1–FLAG were subjected to immunoprecipitation (IP) with an antibody to FLAG (M2) conjugated to agarose. β-arrestin1–FLAG and associated PDE4D5 were detected in the immune complexes by immunoblotting with the indicated antibodies (IB:) (**upper**); equivalent levels of expression were confirmed by immunoblotting the cell lysates (IB:) (**lower**). Representative blots of three similar experiments are shown. (**B**) Increases in membrane-associated PDE4 activity after 5 min of treatment with 10 nM (−)-isoproterenol (Iso) in HEK293 cells overexpressing both FLAG–β_2-adrenergic receptor and empty vector (c) or PDE4D5(D556A). $^{**}P < 0.02$ (mean ± SEM of five experiments). (**C**) Membrane-associated PKA activity in cells treated in an identical manner to those in (B) before stimulation (NS) and after stimulation with 10 nM (−)-isoproterenol (Iso) for 5 min. PKA activity is expressed as percent of total PKA activity stimulated with 10 μM cAMP. $^{*}P < 0.02$ (mean ± SEM of four experiments).

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Supporting Online Material

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Materials and Methods
Figs. S1 and S2
Table S1

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