LETTERS

Insulin disrupts β-adrenergic signalling to protein kinase A in adipocytes

Jin Zhang[1]*†, Christopher J. Hupfeld[2]*, Susan S. Taylor[3,4], Jerrold M. Olefsky[2] & Roger Y. Tsien[1,3,4]

Hormones mobilize intracellular second messengers and initiate signalling cascades involving protein kinases and phosphatases, which are often spatially compartmentalized by anchoring proteins to increase signalling specificity[1]. These scaffold proteins may themselves be modulated by hormones[2–4]. In adipocytes, stimulation of β-adrenergic receptors increases cyclic AMP levels and activates protein kinase A (PKA)[5], which stimulates lipolysis by phosphorylating hormone-sensitive lipase and perilipin[6–8]. Acute insulin treatment activates phosphodiesterase 3B, reduces cAMP levels and quenches β-adrenergic receptor signalling[9]. In contrast, chronic hyperinsulinaemic conditions (typical of type 2 diabetes) enhance β-adrenergic receptor-mediated cAMP production[10]. This amplification of cAMP signalling is paradoxical because it should enhance lipolysis, the opposite of the known short-term effect of hyperinsulinaemia. Here we show that in adipocytes, chronically high insulin levels inhibit β-adrenergic receptors (but not other cAMP-elevating stimuli) from activating PKA. We measured this using an improved fluorescent reporter and by phosphorylation of endogenous cAMP-response-element binding protein (CREB). Disruption of PKA scaffolding mimics the interference of insulin with β-adrenergic receptor signalling. Chronically high insulin levels may disrupt the close apposition of β-adrenergic receptors and PKA, identifying a new mechanism for crosstalk between heterologous signal transduction pathways.

In order to assess the effect of chronically high insulin levels on PKA activation (a signalling step immediately following cAMP production), we took advantage of genetically encoded A-kinase activity reporters (AKARs) for monitoring PKA activity in living cells. Such reporters serve as surrogate substrates for PKA and, when phosphorylated, generate a change in fluorescence resonance energy transfer (FRET) between two green fluorescent protein (GFP) mutants as the result of phosphorylation-induced intramolecular complex formation between a phosphoamino acid binding domain and the phosphorylated peptide[11]. The previously described AKAR1 reporter has successfully monitored compartmentalized PKA activity. However, its poor sensitivity to cellular phosphatases impedes reversal of the FRET response, limiting the utility of AKAR1 for physiological studies.

To generate an improved AKAR reporter, we hypothesized that within the AKAR construct, tight binding of the phosphorylated peptide by the 14-3-3 module efficiently protects it from dephosphorylation[12]. Thus, a phosphoamino acid binding domain that has reduced binding affinity for the phosphorylated peptide would be more suitable for constructing a reversible reporter. To this end, we chose the forkhead associated domain 1 (FHA1), a modular phosphothreonine binding domain with submicromolar binding affinity[13], one order of magnitude weaker than that of 14-3-3 and substrate peptides. Furthermore, peptide library screening has identified FHA1-binding peptides that favour specific amino acids around the phosphorylation site, particularly in the positions immediately C-terminal to the phospho-threonine (pT)[13]. We therefore modified the PKA substrate sequence to LRRATLVD by incorporating the near-optimal sequence for FHA1 binding at positions +1, + 2 and +3 with respect to pT, while maintaining the PKA phosphorylation consensus motif at the −2, −3 positions (Fig. 1a).

We incorporated three linkers of different lengths between the substrate sequence and FHA1, and found that one construct provided a greater response and faster kinetics compared to the other two constructs (data not shown). This reporter is termed AKAR2, and when tested in HEK293 cells, it showed a reversible response. After the response induced by the β-adrenergic agonist isoproterenol reached a maximum, removal of the stimulant and addition of H89 (a relatively specific PKA inhibitor) led to a decrease in emission ratio, which returned to the basal level over 30–50 min (Fig. 1b). A second increase in emission ratio was generated by removal of H89 and addition of the adenylyl cyclase activator forskolin, showing that activation of AKAR2 was fully reversible. Pulsed photolytic release ('uncaging') of cAMP from a membrane-permeant precursor[14] led to repeated cycles of increased and decreased emission ratios, presumably involving intracellular release then rapid degradation of cAMP followed by rapid changes in the activity balance between PKA and phosphatases (Fig. 1c).

To test the specificity of the reporter, we incubated AKAR2-expressing HEK293 cells with H89. As seen in Fig. 1d, this pretreatment prevented the response of AKAR2 to isoproterenol. The reporter itself was not compromised, as removal of H89 restored the ability of AKAR2 to respond to forskolin. Furthermore, co-expression of the specific PKA inhibitor PKI also abolished the response (Fig. 1e), confirming that the FRET response is PKA-specific. In addition, treatment of AKAR2-expressing HEK293 cells with phorbol dibutyrate (PDBu, a protein kinase C (PKC) activator) or thapsigargin (to stimulate calcium/calmodulin-dependent protein kinase II, CaMKII) did not alter the emission ratio (Fig. 1f, g). Thus, AKAR2 senses PKA but not PKC or CaMKII activation in living cells.

To confirm that changes in emission ratio result from phosphorylation of AKAR2 at the designated threonine residue, we changed the threonine to alanine in the PKA substrate sequence (LRRATLVD). Figure 1h shows that this single mutation, T391A, completely abolished the response to increases in cAMP. To compare the FRET change with the phosphorylation state of the reporter, immunoblotting analysis was performed using an antiphospho-PKA-substrate antibody before and after forskolin stimulation. AKAR2

[1]Department of Pharmacology, [2]Division of Endocrinology and Metabolism, [3]Department of Chemistry and Biochemistry, and [4]Howard Hughes Medical Institute, University of California at San Diego, La Jolla, California 92093, USA. †Present address: Department of Pharmacology and Molecular Sciences and Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA.
*These authors contributed equally to this work.

migrated at the expected molecular weight, showing no proteolysis. Forskolin increased phosphorylation, correlating with the increase in FRET (Fig. 1i, lanes 1, 2). In contrast, no phosphorylation was detected for the variant T391A with or without forskolin stimulation (Fig. 1i, lanes 3, 4), indicating that the single threonine in the substrate sequence is the crucial phosphorylation site responsible for the emission ratio change.

As recently shown[10], chronic insulin treatment leads to down-regulation of β-arrestin1 protein, thus impairing desensitization of β-adrenergic receptor (β-AR):G_s signalling. This results in a 25–63% increase in β-AR-mediated cAMP production compared with non-insulin-treated cells (Supplementary Fig. 1; see also ref. 10). As PKA is the major downstream target of cAMP, we measured the effect of insulin treatment on β-AR-mediated, cAMP-induced activation of PKA using AKAR2. In 3T3-L1 adipocytes microinjected with AKAR2 plasmid DNA, AKAR2 was expressed throughout the cell, and was excluded only from the lipid droplets (inset of Fig. 2b). Cells were pretreated with insulin for 8 h, and then acutely stimulated with varying concentrations of the β-adrenergic agonist isoproterenol. Insulin treatment (100 ng ml^{-1} for 8 h) did not alter basal AKAR2 phosphorylation (data not shown), but did delay PKA responses to isoproterenol stimulation, despite super-sensitized cAMP production. As the isoproterenol dose decreased, these delays increased from 20 s (10 μM) to 150 s (1 μM) to 300 s (0.1 μM) (Fig. 2a, b).

β-AR signalling alters the expression of adipocyte-specific gene products such as leptin[15], adiponectin[16] and resistin[17]. Many transcriptional responses to PKA activation are mediated by PKA phosphorylation at Ser 133 of the cAMP response element binding protein (CREB)[18], which is almost exclusively a nuclear protein. As shown in Fig. 2c, CREB was rapidly phosphorylated at Ser 133 upon isoproterenol stimulation, and chronic insulin treatment inhibited this phosphorylation. The prolonged delay in CREB Ser 133 phosphorylation (relative to cytoplasmic AKAR2) is consistent with a 5–10 min delay in the response of the nuclear-localized AKAR2 (Supplementary Fig. 2). Thus, the antagonistic effect of chronic insulin treatment on β-AR activation of PKA is detectable by an endogenous effector protein (CREB), not just the exogenous fluorescent reporter.

Treatment of cells with the adenylyl cyclase activator forskolin or ultraviolet photolysis of caged cAMP raises intracellular cAMP and activates PKA independently of β-AR and G_s signalling. As shown in Fig. 3a, b, chronic hyperinsulinaemia did not delay AKAR2 phosphorylation induced by forskolin or by cAMP uncaging, suggesting that insulin treatment does not impair the total available pool of intracellular PKA. We conclude from these experiments that the insulin-induced delay in PKA activity is specific for a β-adrenergic-related pool of PKA.

Distinct pools of PKA can result from spatial compartmentalization of PKA activity by A-kinase anchoring proteins (AKAPs)[1], which bind to the regulatory subunit of PKA and direct the holoenzyme to discrete locations within the cell. To test whether AKAPs are involved in β-adrenergic-coupled PKA activation in adipocytes, we measured AKAR2 phosphorylation in the presence of Ht31, a synthetic peptide that blocks the interaction between AKAP and the regulatory subunit of PKA, thereby disrupting AKAP-mediated PKA targeting[19]. Ht31 and its inactive analogue Ht31p were microinjected as intact peptides. As shown in Fig. 4a, we found that microinjected Ht31 blocked the isoproterenol-stimulated FRET response, and slightly delayed but did not block forskolin-induced phosphorylation. On the other hand, the functionally inactive analogue Ht31p had no effect on the isoproterenol-stimulated FRET response (Supplementary Fig. 3).

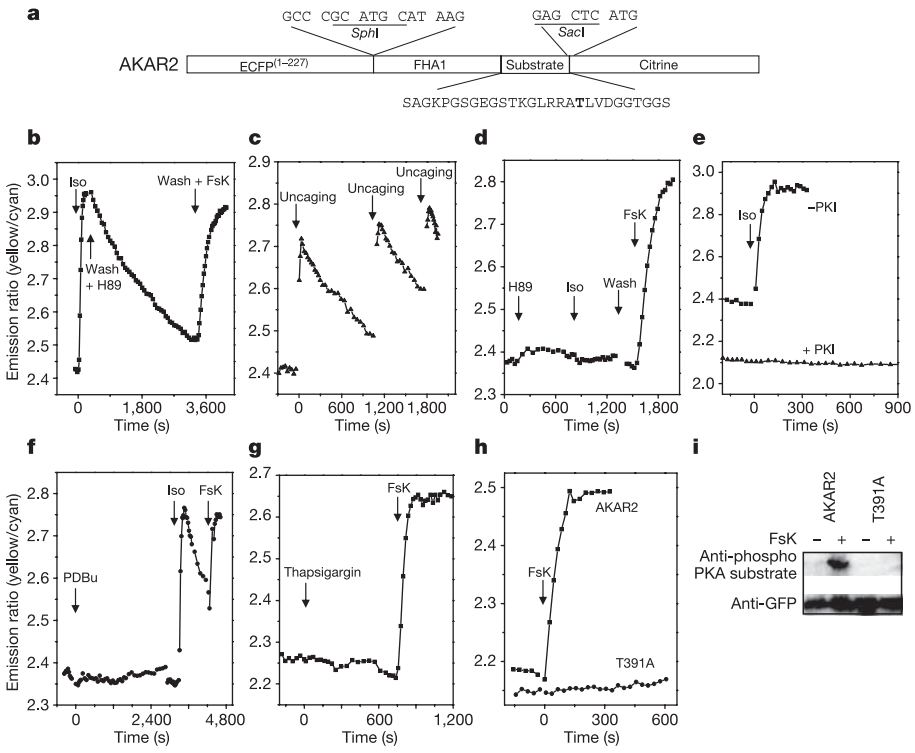

Figure 1 | Re-engineering of AKAR2. **a**, Domain structure of AKAR2. **b**, **c**, Representative FRET data in HEK293 cells in response to agonist stimulation (**b**) or cAMP uncaging (**c**). FsK, forskolin; Iso, isoproterenol. All time courses are representative of at least three experiments, total number of cells $n \geq 8$. **d**, **e**, Pretreatment with H89 (**d**) or co-expression of PKI (**e**, triangles, PKI; squares, control) blocks the response of AKAR2 to isoproterenol. **f**, **g**, AKAR2 does not sense PKC (**f**) or CaMKII (**g**). **h**, FRET responses of the T391A mutant (circles) and wild-type AKAR2 (squares). **i**, Antiphospho-PKA-substrate (top) and anti-GFP (bottom) western blot analyses of wild-type and T391A AKAR2 from untreated and forskolin-treated cells.

To further examine the effect of chronic insulin treatment on PKA targeting, we measured the association between β_2-AR and the RIIβ subunit of PKA, in the presence or absence of chronic insulin. We found that RIIβ and β_2-AR were constitutively associated, and that this association was maintained or slightly increased after isoproterenol stimulation. Insulin pretreatment led to a pronounced decrease in β_2-AR:RIIβ association after isoproterenol stimulation (Fig. 4b, c). These data suggest that AKAPs are involved in coupling a specific pool of PKA to β-AR, an association weakened by insulin pretreatment.

Chronic hyperinsulinaemia thus not only amplifies total cAMP production[10], but more importantly, disrupts the linkage of β-ARs to a specific pool of PKA. The targeting of PKA to β-ARs by AKAPs[20,21], compartmentalized cAMP generation and PKA activation[22,23] are well documented. Here we show that in adipocytes, compartmentalization of PKA with β-ARs is dynamically disrupted by insulin, providing a new mechanism for heterologous crosstalk. Previous examples of AKAP modulation[2–4] were triggered by agents that directly increase cAMP levels and therefore represent homologous feedback. We propose that chronically high insulin levels weaken the close apposition of β-ARs and PKA (Supplementary Fig. 4) through modulation of AKAP scaffolding proteins, similar to the action of Ht31. Isoproterenol then seems to complete the dissociation between β-AR and PKA in insulin-treated cells. In contrast, the association between β-AR and PKA in other systems is independent of or strengthened by agonist stimulation, as exemplified by AKAP79/150 (ref. 20) or gravin[24], respectively. We speculate that once chronic insulin treatment weakens the complex containing β-AR and PKA, PKA activation might phosphorylate one or more components and destroy the complex, thus terminating the β-AR/PKA association by very rapid negative feedback. The molecular components that constitute such signalling microdomains in adipocytes, and the mechanisms by which chronic hyperinsulinaemia affect their integrity, will be questions for future study. The improved AKARs presented here are reversible and targetable reporters allowing real-time imaging of PKA activity, and are valuable for analysing compartmentalized kinase activities.

METHODS

Gene construction. cDNA for enhanced cyan fluorescent protein (ECFP) and citrine[25] (an optimized version of YFP) were fused to forkhead associated domain 1 (FHA1) (Rad53p 22–162) created by polymerase chain reaction (PCR) using the Rad53-FHA1 template and primers containing the gene sequence for the linkers and phosphorylation substrate regions. The T391A mutation was incorporated by the QuickChange method (Stratagene). For expression in mammalian cells, the chimaeric proteins were subcloned into a modified pcDNA3 vector (Invitrogen) behind a Kozak sequence.

HEK293 cell culture and immunoblot analysis. HEK293 cells were plated onto sterilized glass coverslips in 3-cm dishes or 10-cm plates and grown to 50–90% confluency in DMEM medium supplemented with 10% FBS at 37 °C, 5% CO_2. Cells were then transfected using FuGENE-6 transfection reagent (Roche). For immunoblot analysis, cells expressing the reporters were stimulated with 50 μM forskolin for 30 min at 25 °C. The cells were lysed with an ice-cold lysis buffer and the crude protein samples were concentrated, separated by SDS–polyacrylamide

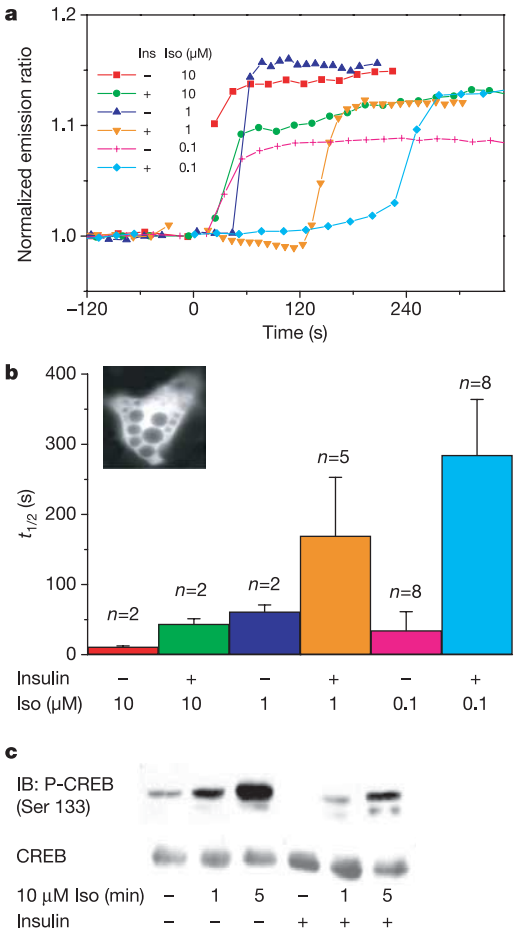

Figure 2 | Effect of insulin pretreatment on PKA activity. **a**, Representative data from cells stimulated with 10 μM, 1 μM or 0.1 μM isoproterenol (Iso), with or without insulin (Ins) pretreatment. **b**, The mean time ($t_{1/2}$) for n cells to generate 50% of the maximal response with different doses of isoproterenol. Error bars indicate s.d. The inset shows a representative adipocyte expressing AKAR2. **c**, Effect of insulin treatment on CREB phosphorylation (P-CREB). Immunoblots (IB) are representative of two independent experiments.

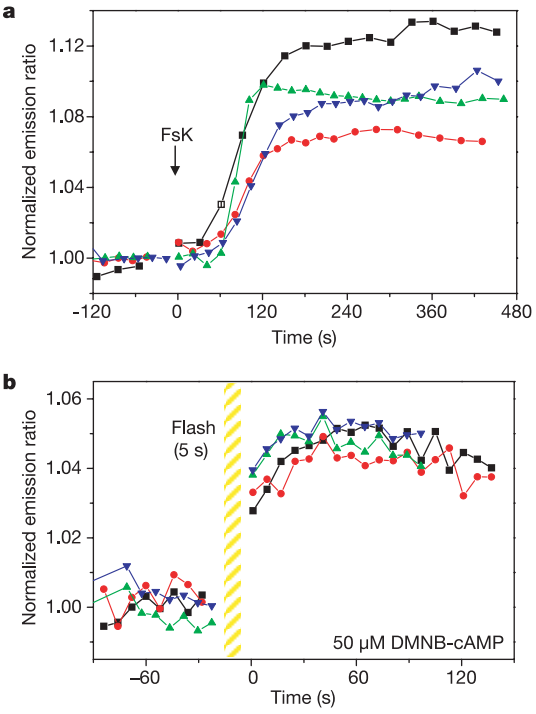

Figure 3 | The insulin-induced delay in PKA activity is specific for a β-adrenergic-coupled pool of PKA. **a**, Cells were stimulated acutely by the addition of forskolin. Insulin-treated cells in blue and green triangles ($t_{1/2} = 93 \pm 12$ s, mean ± s.d., $n = 8$); control cells in black squares and red circles ($t_{1/2} = 90 \pm 11$ s, $n = 7$). **b**, Cells were stimulated acutely by ultraviolet uncaging of DMNB-cAMP. Insulin-treated cells in blue and green triangles, representative data from three uncaging experiments ($n = 7$); control cells in black squares and red circles, representative data from three uncaging experiments ($n = 5$).

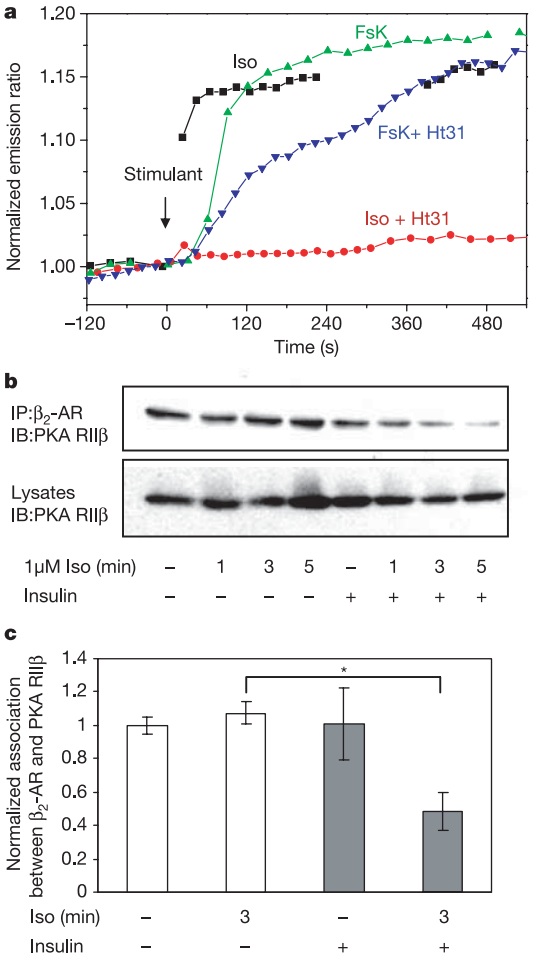

Figure 4 | β-adrenergic-coupled pool of PKA. a, Representative data from cells microinjected with Ht31 and stimulated with 10 μM isoproterenol ($n = 7$) or 50 μM forskolin ($n = 6$). **b**, Effect of insulin pretreatment on the amount of PKA RIIβ co-immunoprecipitated with β_2-AR. **c**, Quantification of PKA RIIβ co-immunoprecipitated with β_2-AR, before (white) and after (grey) a 3-min treatment with isoproterenol. Data shown are mean ± s.e.m. from three experiments. Asterisk, $P = 0.045$, two-tailed *t*-test. Data are corrected for total amount of PKA RIIβ in lysates. Normalization is relative to the mean for cells without insulin or isoproterenol treatment.

gel electrophoresis and probed with an antiphospho-PKA-substrate antibody (New England Biolabs).

T3-L1 adipocyte culture, microinjection and immunoblot analysis. 3T3-L1 fibroblasts were grown to confluence at 37 °C, 10% CO_2, and differentiated into mature adipocytes as previously described[26]. On day 7 post-differentiation, adipocytes were re-seeded to 3-cm dishes containing embedded glass coverslips, and microinjections were performed between days 10 and 14. AKAR2 expression plasmid was dissolved in microinjection buffer (5 mM NaH_2PO_4, 100 mM KCl, pH 7.2) to a final concentration of 0.1 mg ml^{-1}. Four hours before stimulation, 10 μM Ht31 (DLIEEAASRIVDAVIEQVKAAGAY) was microinjected into nuclei along with the AKAR2 plasmid DNA. For analysing CREB phosphorylation, serum-starved 3T3-L1 adipocytes were treated, where indicated, with insulin (17 nM) for 8 h. After washes with PBS, cells were stimulated with isoproterenol (10 μM) for 1 or 5 min. Proteins from cell lysates were probed on a western blot using a phospho-specific (Ser 133) anti-CREB antibody or an anti-CREB antibody.

Imaging. After transfection (24–72 h) or microinjection (4 h), the cells were washed twice with HBSS buffer, and maintained in buffer in the dark at 20–25 °C. Forskolin (Calbiochem) and isoproterenol (Aldrich) were then added as indicated. For inhibition studies of PKA activity, 10 μM H89 was added to the cells before forskolin stimulation.

For uncaging of cAMP, cells were incubated with 50 μM of a membrane-permeant photolysable derivative, 4,5-dimethoxy-2-nitrobenzyl adenosine-3′,5′-cyclic monophosphate (DMNB-cAMP), then exposed for 5 s to approximately 0.5 W cm^{-2} of 340–370 nm illumination from the microscope's xenon lamp filtered through a 330WB80 filter. This ultraviolet light exposure was calculated to uncage >90% of the DMNB-cAMP within the cell.

Cells were imaged on a Zeiss Axiovert microscope with a ×40/1.3 NA oil-immersion objective lens and a cooled CCD camera (Photometrics), controlled by Metafluor 2.75 software (Universal Imaging). Dual-emission ratio imaging used a 440DF30 excitation filter, a 455DRLP dichroic mirror and two emission filters (480DF30 for ECFP, 535DF25 for citrine) alternated by a filter changer (Lambda 10-2, Sutter Instruments). Fluorescence images were background-corrected. Exposure time was 100–1000 ms, and images were taken every 5–15 s (see Supplementary Methods).

Immunoprecipitation. 3T3-L1 adipocytes were treated with the indicated ligands, and whole-cell lysates were prepared. Lysate protein (500 μg) was immunoprecipitated with 2 μg of rabbit polyclonal anti-β_2-AR antibody (Santa Cruz) overnight at 4 °C, and the resulting immune complexes were captured by incubating with Protein A beads for 2 h. After three washes with PBS, beads were dissolved in Laemmli buffer, boiled for 5 min, and proteins were separated by SDS gel electrophoresis and probed with an anti-RIIβ antibody (Biomol). Densitometry was performed using a Kodak ImageStation scanner and software.

Received 26 May; accepted 15 August 2005.

1. Wong, W. & Scott, J. D. AKAP signalling complexes: focal points in space and time. *Nature Rev. Mol. Cell Biol.* **5,** 959–970 (2004).
2. Salvador, L. M. *et al.* Neuronal microtubule-associated protein 2D is a dual A-kinase anchoring protein expressed in rat ovarian granulosa cells. *J. Biol. Chem.* **279,** 27621–27632 (2004).
3. Carr, D. W., DeManno, D. A., Atwood, A., Hunzicker-Dunn, M. & Scott, J. D. Follicle-stimulating hormone regulation of A-kinase anchoring proteins in granulosa cells. *J. Biol. Chem.* **268,** 20729–20732 (1993).
4. Feliciello, A., Rubin, C. S., Avvedimento, E. V. & Gottesman, M. E. Expression of a kinase anchor protein 121 is regulated by hormones in thyroid and testicular germ cells. *J. Biol. Chem.* **273,** 23361–23366 (1998).
5. Taylor, S. S. *et al.* PKA: a portrait of protein kinase dynamics. *Biochim. Biophys. Acta* **1697,** 259–269 (2004).
6. Londos, C., Honnor, R. C. & Dhillon, G. S. cAMP-dependent protein kinase and lipolysis in rat adipocytes. III. Multiple modes of insulin regulation of lipolysis and regulation of insulin responses by adenylate cyclase regulators. *J. Biol. Chem.* **260,** 15139–15145 (1985).
7. Egan, J. J., Greenberg, A. S., Chang, M. K. & Londos, C. Control of endogenous phosphorylation of the major cAMP-dependent protein kinase substrate in adipocytes by insulin and beta-adrenergic stimulation. *J. Biol. Chem.* **265,** 18769–18775 (1990).
8. Greenberg, A. S. *et al.* Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. *J. Biol. Chem.* **266,** 11341–11346 (1991).
9. Elks, M. L. & Manganiello, V. C. Antilipolytic action of insulin: role of cAMP phosphodiesterase activation. *Endocrinology* **116,** 2119–2121 (1985).
10. Hupfeld, C. J., Dalle, S. & Olefsky, J. M. β-Arrestin 1 down-regulation after insulin treatment is associated with supersensitization of β2 adrenergic receptor Gαs signalling in 3T3–L1 adipocytes. *Proc. Natl Acad. Sci. USA* **100,** 161–166 (2003).
11. Zhang, J., Ma, Y., Taylor, S. S. & Tsien, R. Y. Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering. *Proc. Natl Acad. Sci. USA* **98,** 14997–15002 (2001).
12. Muslin, A. J., Tanner, J. W., Allen, P. M. & Shaw, A. S. Interaction of 14-3-3 with signalling proteins is mediated by the recognition of phosphoserine. *Cell* **84,** 889–897 (1996).
13. Durocher, D. *et al.* The molecular basis of FHA domain:phosphopeptide binding specificity and implications for phospho-dependent signalling mechanisms. *Mol. Cell* **6,** 1169–1182 (2000).
14. Nerbonne, J. M., Richard, S., Nargeot, J. & Lester, H. A. New photoactivatable cyclic nucleotides produce intracellular jumps in cyclic AMP and cyclic GMP concentrations. *Nature* **310,** 74–76 (1984).
15. Kosaki, A., Yamada, K. & Kuzuya, H. Reduced expression of the leptin gene (ob) by catecholamine through a Gs protein-coupled pathway in 3T3–L1 adipocytes. *Diabetes* **45,** 1744–1749 (1996).
16. Fasshauer, M., Klein, J., Neumann, S., Eszlinger, M. & Paschke, R. Adiponectin gene expression is inhibited by β-adrenergic stimulation via protein kinase A in 3T3–L1 adipocytes. *FEBS Lett.* **507,** 142–146 (2001).
17. Fasshauer, M., Klein, J., Neumann, S., Eszlinger, M. & Paschke, R. Isoproterenol inhibits resistin gene expression through a Gs-protein-coupled pathway in 3T3–L1 adipocytes. *FEBS Lett.* **500,** 60–63 (2001).
18. Mayr, B. & Montminy, M. Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nature Rev. Mol. Cell Biol.* **2,** 599–609 (2001).
19. Scott, J. D. & Faux, M. C. Use of synthetic peptides in the dissection of protein-targeting interactions. *Methods Mol. Biol.* **88,** 161–185 (1998).

20. Fraser, I. D. *et al.* Assembly of an A kinase-anchoring protein–β_2-adrenergic receptor complex facilitates receptor phosphorylation and signalling. *Curr. Biol.* **10,** 409–412 (2000).
21. Lin, F., Wang, H. & Malbon, C. C. Gravin-mediated formation of signalling complexes in β_2-adrenergic receptor desensitization and resensitization. *J. Biol. Chem.* **275,** 19025–19034 (2000).
22. Jurevicius, J. & Fischmeister, R. cAMP compartmentation is responsible for a local activation of cardiac Ca^{2+} channels by β-adrenergic agonists. *Proc. Natl Acad. Sci. USA* **93,** 295–299 (1996).
23. Zaccolo, M. & Pozzan, T. Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. *Science* **295,** 1711–1715 (2002).
24. Tao, J., Wang, H. Y. & Malbon, C. C. Protein kinase A regulates AKAP250 (gravin) scaffold binding to the β_2-adrenergic receptor. *EMBO J.* **22,** 6419–6429 (2003).
25. Griesbeck, O., Baird, G. S., Campbell, R. E., Zacharias, D. A. & Tsien, R. Y. Reducing the environmental sensitivity of yellow fluorescent protein. Mechanism and applications. *J. Biol. Chem.* **276,** 29188–29194 (2001).
26. Dalle, S. *et al.* Insulin induces heterologous desensitization of G-protein-coupled receptor and insulin-like growth factor I signalling by downregulating β-arrestin-1. *Mol. Cell. Biol.* **22,** 6272–6285 (2002).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature. A summary figure is also included.

Acknowledgements We thank Y. Ma for technical assistance and discussions and M. B. Yaffe for providing FHA cDNA. This work was supported by the Howard Hughes Medical Institute, the Alliance for Cell Signaling (R.Y.T.), the NIH (R.Y.T, C.J.H. and S.S.T) and the Johns Hopkins University School of Medicine and W. M. Keck Foundation (J.Z.).

Author Contributions J.Z. and C.J.H. performed all the experiments; all authors contributed to planning the experiments, interpreting the data and writing the paper.

Author Information Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to R.Y.T. (rtsien@ucsd.edu).