Assembly of an A kinase-anchoring protein–β_2-adrenergic receptor complex facilitates receptor phosphorylation and signaling

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Phosphorylation of G-protein-coupled receptors by second-messenger-stimulated kinases is central to the process of receptor desensitization [1–3]. Phosphorylation of the β_2-adrenergic receptor (β_2-AR) by protein kinase A (PKA), in addition to uncoupling adenylate cyclase activation, is obligatory for receptor-mediated activation of mitogen-activated protein kinase (MAP kinase) cascades [4,5]. Although mechanisms for linking G-protein-coupled receptor kinases to the activated receptor are well established, analogous mechanisms for targeting second messenger kinases to the β_2-AR at the plasma membrane have not been elucidated. Here we show that the A-kinase-anchoring protein, AKAP79/150, co-precipitates with the β_2-AR in cell and tissue extracts, nucleating a signaling complex that includes PKA, protein kinase C (PKC) and protein phosphatase PP2B. The anchoring protein directly and constitutively interacts with the β_2-AR and promotes receptor phosphorylation following agonist stimulation. Functional studies show that PKA anchoring is required to enhance β_2-AR phosphorylation and to facilitate downstream activation of the MAP kinase pathway. This defines a role for AKAP79/150 in the recruitment of second-messenger-regulated signaling enzymes to a G-protein-coupled receptor.

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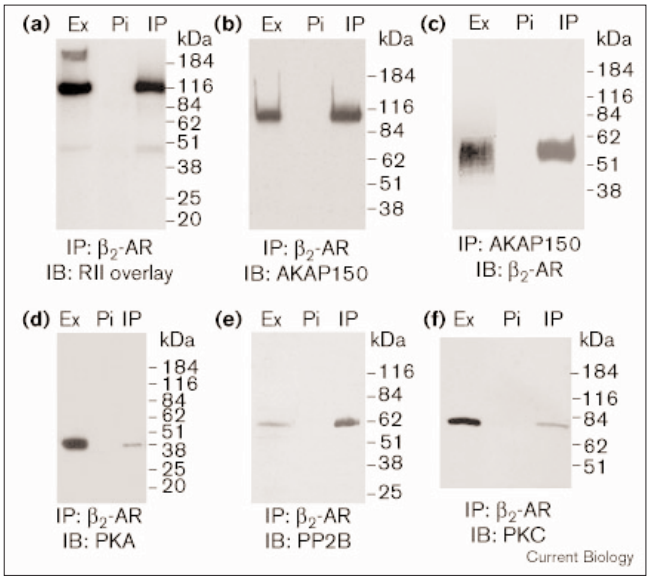
Results and discussion

To determine if kinase signaling complexes interact with G-protein-coupled receptors, a detergent (CHAPS)-solubilized rat brain lysate was immunoprecipitated with an antibody specific for β_2-AR. Immunoprecipitates were analyzed for co-purifying A-kinase anchoring proteins (AKAPs) by an *in vitro* overlay procedure using radiolabeled PKA regulatory subunit (RII) as a probe [6]. Several RII-binding proteins were detected in the brain extract upon longer exposure but only the predominant RII-binding protein of approximately 150 kDa co-precipitated with the β_2-AR (Figure 1a). The RII-binding protein was identified as AKAP150 (the rat homolog of human AKAP79) by immunoblotting (Figure 1b). The band at approximately 55 kDa that co-precipitated with AKAP150 is RII and was detected by dimerization with the labeled RII probe (Figure 1a). Reciprocal experiments detected β_2-AR upon immunoprecipitation of AKAP150 from brain lysates (Figure 1c).

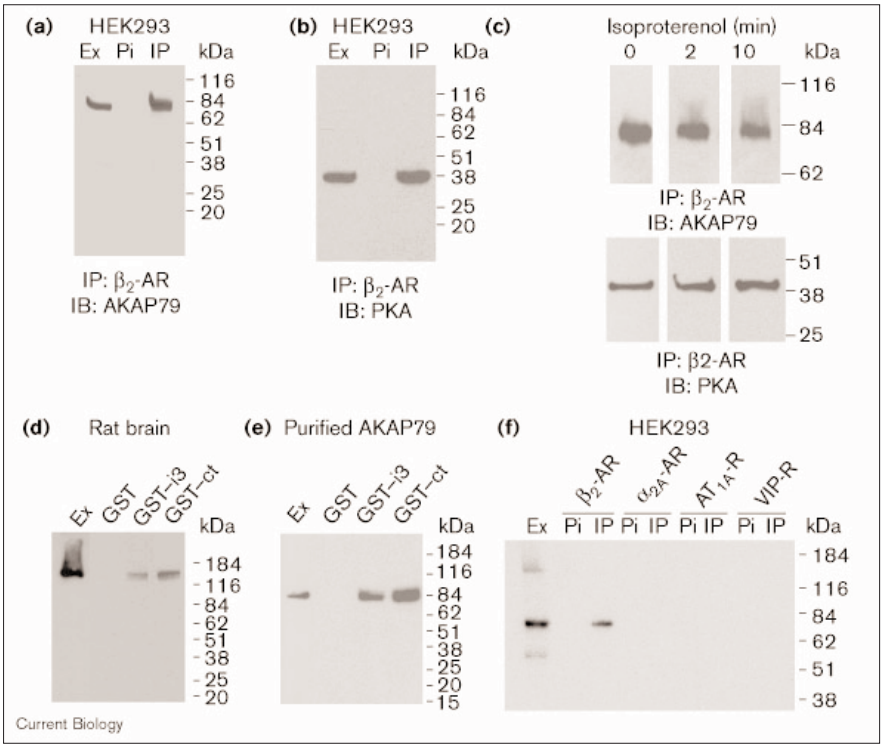
The AKAP79/150 family of anchoring proteins (comprising human AKAP79, rat/mouse AKAP150 and bovine AKAP75)

Figure 1



Identification of a β_2-AR–AKAP150 complex in rat brain by co-immunoprecipitation. **(a)** RII-binding proteins identified by overlay assay [6] in β_2-AR immunoprecipitate. **(b)** The major RII-binding band co-purifying with the β_2-AR is identified as AKAP150 by immunoblotting with specific antibody . **(c)** β_2-AR detected by immunoblot in AKAP150 immunoprecipitate. **(d–f)** Immunoblot detection of signaling enzymes. (d) PKA, (e) PP2B and (f) PKC in β_2-AR immunoprecipitates from rat brain extract. Blots are representative of at least three separate experiments. Ex, source extract ((a,b) 2 μg; (c–f) 20 μg); Pi, control pre-immune sera precipitation; IP, specific immunoprecipitation; IB, immunoblot. Complete experimental methods are given in the Supplementary material.

Figure 2



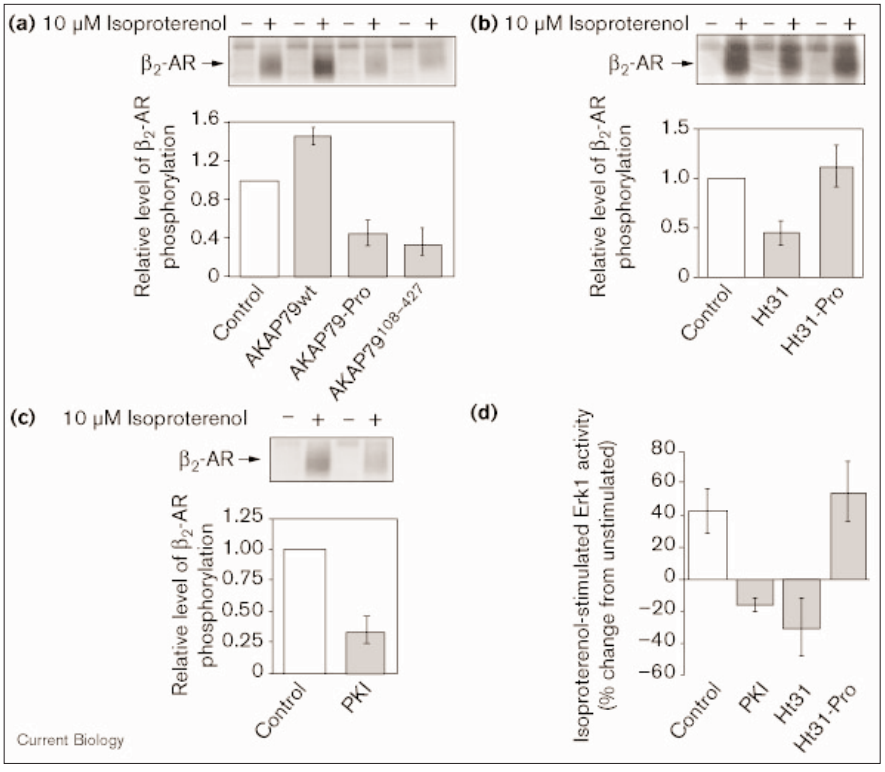
Characterization of the β_2-AR–AKAP79 interaction. **(a)** AKAP79 and **(b)** PKA co-precipitate with Flag–β_2-AR from transfected HEK293 cells. **(c)** The β_2-AR–AKAP79 interaction is not agonist dependent. AKAP79 and PKA co-immunoprecipitated with β_2-AR from HEK293 cells at specific time points (indicated above each lane) after application of the agonist isoproterenol (10 μM) are shown. **(d,e)** GST fusion proteins including the i3 (residues 222–270) and ct (residues 328–413) β_2-AR domains were used in pulldown assays. (d) AKAP150 immunoblot of eluates from glutathione–Sepharose resins incubated with rat brain extract. (e) AKAP79 immunoblot of eluates from glutathione–Sepharose resins incubated with purified AKAP79 protein. **(f)** RII-binding proteins identified by overlay assay in immunoprecipitates of specific receptors from HEK293 cells transfected with β_2-AR, α_{2A}-AR, angiotensin 1A receptor (AT_{1A}-R) or VIP receptor (VIP-R). AKAP79 specifically co-precipitates only with β_2-AR. Blots are representative of at least four separate experiments. Ex, source extract (a,b,f) 20 μg; (d) 2 μg, (e) 0.1 μg pure AKAP79 protein; Pi, control pre-immune sera precipitation; IP, specific immunoprecipitation; IB, immunoblot. Complete experimental methods are given in the Supplementary material.

has been shown to maintain a multi-enzyme signaling scaffold that includes the kinases PKA and PKC and the protein phosphatase PP2B [7,8]. Both kinases have a role in the control of β_2-AR function [2], and there is evidence for PP2B activity associated with the receptor [9]. All three signaling enzymes were detected by immunoblot when β_2-AR immunoprecipitates were analyzed with specific antibodies (Figure 1d–f). These data show that the AKAP150 protein maintains a signaling complex of three second-messenger-regulated enzymes with β_2-AR inside cells.

To investigate the functional consequences of the interaction between this AKAP and β_2-AR in a heterologous expression system, HEK293 cells were transfected with cDNAs for AKAP79 and for β_2-AR tagged with a Flag epitope. Despite the difference in size, AKAP79 and AKAP150 are highly conserved. An extended repeat sequence of unknown function accounts for the higher molecular weight of AKAP150. As expected, recombinant AKAP79 and endogenous PKA co-precipitated with the β_2-AR (Figure 2a,b). Most interactions of β_2-AR with other proteins occur transiently at particular stages of receptor activation and desensitization [3,10,11]. Several other signaling partners such as heterotrimeric G proteins, receptor kinases, arrestins and tyrosine kinases bind to β_2-AR in an agonist-dependent manner [3,10,11]. Thus, serum-starved HEK293 cells transfected with AKAP79 and β_2-AR were stimulated with the β_2-AR agonist isoproterenol to determine whether complex formation was dependent on the receptor being occupied. There was no difference in the levels of AKAP or PKA catalytic subunit that co-immunoprecipitated with β_2-AR in the absence or presence of isoproterenol (n = 9; Figure 2c). This indicates a constitutive association of β_2-AR with AKAP79/150 in the signaling complex. A stable β_2-AR–AKAP79/150 complex would be consistent with the maintenance of a signaling scaffold that integrates second-messenger-mediated signals.

Most regulatory phosphorylation sites and binding sites for β_2-AR-interacting proteins are contained within the third intracellular loop (i3) and the carboxy-terminal tail (ct) of the receptor [11,12]. Consequently, glutathione-*S*-transferase (GST) fusions with the i3 and ct domains were used as probes to investigate the β_2-AR–AKAP79/150 interaction. In GST pulldowns from rat brain extracts, endogenous AKAP150 interacted with both GST–i3 and GST–ct but not with a control GST construct (Figure 2d). Similar results were obtained using purified recombinant AKAP79 and the same GST–receptor fragments *in vitro*, indicating that association of AKAP79 with β_2-AR was a result of a direct interaction between the two proteins (Figure 2e). Thus, AKAP79/150 binds through two sites on the β_2-AR,

Figure 3



Effects of PKA anchoring on β_2-AR phosphorylation and activation of MAP kinase. **(a)** Agonist-stimulated β_2-AR phosphorylation is increased in cells overexpressing AKAP79 but is significantly inhibited by AKAP79-Pro and AKAP79$^{108–427}$ mutants. HEK293 cells were subsequently stimulated with 10 μM isoproterenol for 5 min. A representative gel shows basal (–) and isoproterenol-stimulated (+) phosphorylation of β_2-AR in cells transfected as indicated. Values are normalized with respect to receptor expression levels using FACS analysis and are expressed relative to isoprotenerol-stimulated β_2-AR phosphorylation in the absence of co-transfected AKAP79 (Control). **(b)** Disruption of PKA anchoring by Ht31 inhibits agonist-stimulated β_2-AR phosphorylation. **(c)** Agonist-stimulated β_2-AR phosphorylation is inhibited by the PKA inhibitor PKI. **(d)** Recruitment of PKA to the plasma membrane is required for β_2-AR-regulated Erk1 activation. β_2-AR and HA–Erk1 were expressed in HEK293 cells with pcDNA3 (Control), PKI, Ht31 or Ht31-Pro. Cells starved of serum overnight were stimulated with 10 μM isoproterenol for 5 min at 37°C before determination of Erk activity. Values represent means ± SEM of three to seven independent experiments. Complete experimental methods are given in the Supplementary material.

which may explain the constitutive nature of the interaction (Figure 2c). To investigate the specificity of the AKAP79/150 interaction with G-protein- coupled receptors, HEK293 cells were transfected with epitope-tagged expression plasmids for β_2-AR, α_{2A}-adrenergic receptor (α_{2A}-AR), angiotensin 1A receptor (AT_{1A}-R) or vasoactive intestinal polypeptide receptor (VIP-R). AKAP79 only co-precipitated with β_2-AR, suggesting selectivity for β_2-AR over other prototypic examples of transmembrane receptors coupled to G_s, G_i or G_q.

Many metabolic effects that follow agonist stimulation of the β_2-AR are propagated by G_s-mediated stimulation of adenylate cyclase, production of cAMP and subsequent activation of PKA [13]. PKA phosphorylation of the β_2-AR mediates a negative feedback through uncoupling of the receptor–G_s interaction [1]. We postulated that a β_2-AR–AKAP79/150 complex would function to enhance cAMP-dependent phosphorylation by bringing the kinase into close proximity to the receptor. Therefore, agonist-dependent phosphorylation of β_2-AR was measured in HEK293 cells transfected with Flag-tagged β_2-AR in the absence and presence of various AKAP79 proteins (Figure 3a). A 46 ± 10% (n = 4) increase in receptor phosphorylation was measured in cells expressing wild-type AKAP79 (Figure 3a). In contrast, cells transfected with the AKAP79-Pro mutant, which is unable to bind PKA [14,15], exhibited a 54 ± 14% (n = 4) decrease in β_2-AR phosphorylation (Figure 3a). Thus the AKAP79-Pro mutant may exert a dominant-negative effect by competing with the low levels of endogenous AKAP79 in HEK293 cells [16,17] for association with the β_2-AR. A more pronounced decrease in receptor phosphorylation (65 ± 15% (n = 4)) was observed upon co-expression of the truncated AKAP79$^{108–427}$ (Figure 3a). This AKAP79 fragment retains full PKA-binding activity but is unable to interact with the receptor (data not shown), thereby displacing the β_2-AR-associated pool of PKA holoenzyme. Additional controls confirmed that changes in the phosphorylation state of β_2-AR were mediated by anchored pools of PKA. The PKA-anchoring inhibitor Ht31, which contains the helical PKA-binding determinant conserved in AKAP proteins, has previously been used to uncouple anchored PKA from several substrates [18–20]. Co-expression of Ht31 caused a 52 ± 12% (n = 3) decrease in β_2-AR phosphorylation whereas expression of a control reagent that does not bind PKA (Ht31-Pro) had no effect (Figure 3b). Similarly, receptor phosphorylation was decreased by 65 ± 10% (n = 3) when the specific PKA inhibitor PKI was co-expressed with the β_2-AR (Figure 3c). Collectively, these results indicate that a functional consequence of AKAP79 targeting is facilitation of cAMP-dependent phosphorylation of β_2-AR by an anchored pool of PKA holoenzyme.

PKA phosphorylation of the β_2-AR induces a switch in G-protein coupling from G_s to G_i [4,5,21], which promotes a receptor-regulated mitogenic signaling cascade mediated by G_i, β-arrestin and the tyrosine kinase Src [22]. We therefore investigated the effect of PKA anchoring on agonist-dependent activation of the MAP kinase cascade in HEK293 cells transiently expressing a hemagglutinin (HA)-tagged MAP kinase, Erk1. MAP kinase activity was increased by 43 ± 13% (n = 7) in response to isoproterenol and, consistent with previous observations [4], activity was decreased 31 ± 17% (n = 4) by co-expression of the PKA-specific inhibitor PKI (Figure 3d). Overexpression of the PKA-anchoring inhibitor Ht31 decreased MAP kinase activity by 21 ± 3% (n = 7), whereas the Ht31-Pro control reagent had no inhibitory effect (Figure 3d). This implies that displacement of PKA from AKAP79 impairs the transduction of signals downstream from β_2-AR. Interestingly, a recent study suggested a role for the cytoskeletal anchoring protein gravin in β_2-AR resensitization [10]. There is, however, no evidence for plasma membrane localization of gravin, a predominantly soluble protein in most cell types [23,24], and gravin does not co-precipitate with β_2-AR in brain extracts (Figure 1a). Furthermore, we did not find evidence for *in vitro* interaction between gravin and the i3 and the ct receptor domains. These findings do not, however, preclude the possibility that gravin could be coupled to the internalized β_2-AR through an adaptor protein.

In conclusion, our data suggest a central role for AKAP79/150 in mediating the localization of signaling enzymes to the β_2-AR at the plasma membrane. They also emphasize that PKA anchoring contributes to effective β_2-AR-mediated mitogenic signaling. We also show that AKAP79/150 exists in a complex with the β_2-AR *in vivo* and regulates the phosphorylation state of the receptor. Given that the β_2-AR is the prototypic member of an extensive G-protein-coupled receptor family, these findings may have wider implications. Recruitment of second-messenger-regulated signaling enzymes by scaffolding proteins such as AKAP79/150 may prove to be a conserved mechanism in the regulation of G-protein-coupled receptors.

Supplementary material

Supplementary material including complete experimental methods is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

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