A β_2 Adrenergic Receptor Signaling Complex Assembled with the Ca^{2+} Channel $Ca_v1.2$

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The existence of a large number of receptors coupled to heterotrimeric guanine nucleotide binding proteins (G proteins) raises the question of how a particular receptor selectively regulates specific targets. We provide insight into this question by identifying a prototypical macromolecular signaling complex. The β_2 adrenergic receptor was found to be directly associated with one of its ultimate effectors, the class C L-type calcium channel $Ca_v1.2$. This complex also contained a G protein, an adenylyl cyclase, cyclic adenosine monophosphate–dependent protein kinase, and the counterbalancing phosphatase PP2A. Our electrophysiological recordings from hippocampal neurons demonstrate highly localized signal transduction from the receptor to the channel. The assembly of this signaling complex provides a mechanism that ensures specific and rapid signaling by a G protein–coupled receptor.

Neurons contain more than a thousand G protein–coupled receptors (GPCRs). The mechanism that preserves specificity in signaling by such an abundance of GPCRs remains obscure. G proteins and their cognate receptors and immediate downstream effectors, such as adenylyl cyclases, may be colocalized in subcellular microdomains (*1, 2*). Spatial proximity of these components may afford specific signal transduction, but evidence for the existence of preassembled macromolecular signaling complexes that would target defined GPCRs to their ultimate effectors is lacking. We show that the β_2 adrenergic receptor (β_2AR) is directly linked to one of its final effectors, the class C L-type Ca^{2+} channel $Ca_v1.2$. This receptor-channel complex also contains a G protein, an adenylyl cyclase, cyclic adenosine monophosphate–dependent protein kinase (PKA), and a counteracting phosphatase, PP2A.

β adrenergic signaling via adenylyl cyclase and PKA acutely increases L-type channel activity in the heart and brain (*3, 4*). The predominant L-type channel in the heart and brain is the class C channel $Ca_v1.2$ (*5, 6*). It consists of the central pore-forming α_{1C} subunit and several auxiliary subunits including $\alpha_2\delta$ and β (*6*). Phosphorylation of α_{1C} on Ser^{1928} near the COOH-terminus and β (Ser^{478} and Ser^{479} in the β_{2a} isoform) contributes to the upregulation of channel activity by PKA (*7–10*). PKA is kept in close proximity to a

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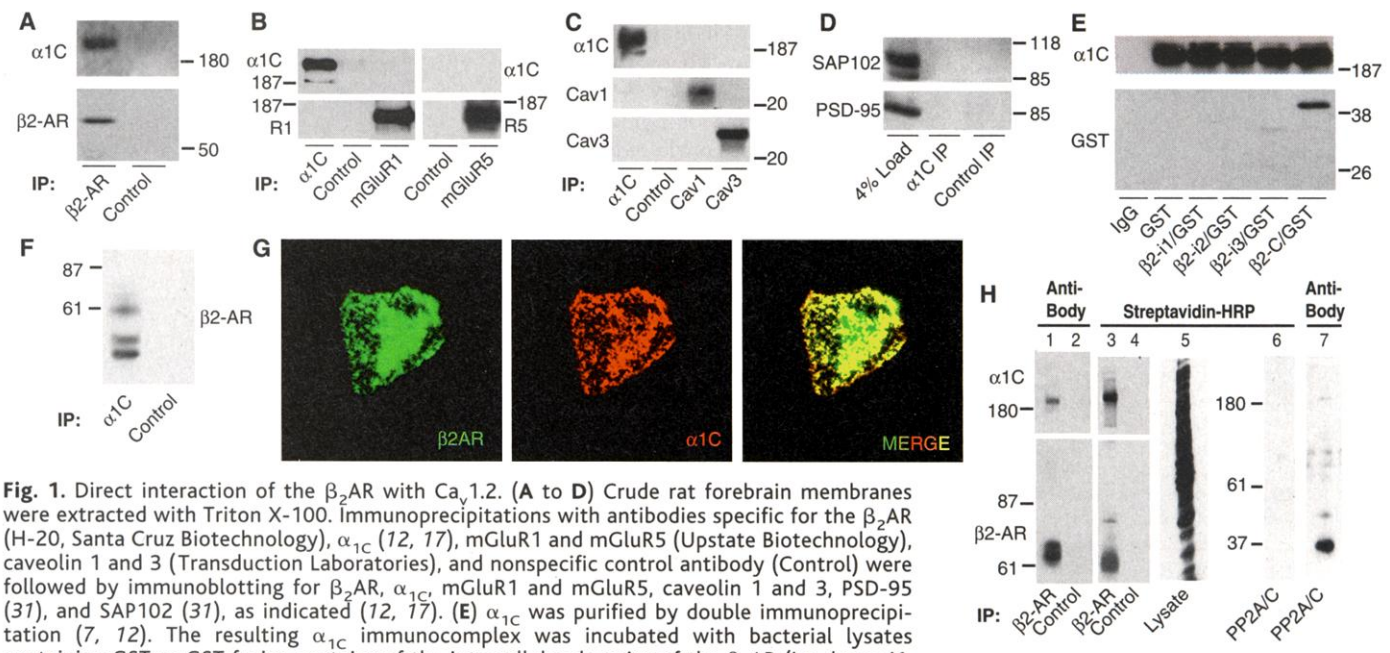

Fig. 1. Direct interaction of the β_2AR with $Ca_v1.2$. (**A** to **D**) Crude rat forebrain membranes were extracted with Triton X-100. Immunoprecipitations with antibodies specific for the β_2AR (H-20, Santa Cruz Biotechnology), α_{1C} (*12, 17*), mGluR1 and mGluR5 (Upstate Biotechnology), caveolin 1 and 3 (Transduction Laboratories), and nonspecific control antibody (Control) were followed by immunoblotting for β_2AR, α_{1C}, mGluR1 and mGluR5, caveolin 1 and 3, PSD-95 (*31*), and SAP102 (*31*), as indicated (*12, 17*). (**E**) α_{1C} was purified by double immunoprecipitation (*7, 12*). The resulting α_{1C} immunocomplex was incubated with bacterial lysates containing GST or GST fusion proteins of the intracellular domains of the β_2AR (i.e., loops i1, i2, and i3 and the COOH terminus) (*15*). Association of α_{1C} with the receptor fragment was detected by immunoblotting with anti-GST (bottom). Upper blot was probed for α_{1C}. (**F** to **H**) The β_2AR and $Ca_v1.2$ consisting of α_{1C}, $\alpha_2\delta$, and β_{2a} were coexpressed in HEK293 cells (*16*). (F) Immunoprecipitations were done with anti-α_{1C} or control antibody before immunoblotting for the receptor, which exists in differentially glycosylated isoforms in HEK293 cells. (G) Immunofluorescent confocal microscopy of α_{1C} and β_2ARs. (H) Intact HEK293 cells were surface-biotinylated 24 hours after transfection (*32*). Triton X-100 extract was either directly loaded (20 μl, lane 5) or used for immunoprecipitation (500 μl) with antibodies to the β_2AR (lanes 1 and 3), control antibodies (lanes 2 and 4), or PP2A/C (lanes 6 and 7). Blots were probed with antibodies to α_{1C} (lanes 1 and 2, upper part), β_2AR (lanes 1 and 2, lower part), or PP2A/C (lane 7) (*17*), stripped with SDS, and reprobed with horseradish peroxidase–streptavidin (lanes 3 to 6).

variety of its substrates by A kinase (PKA) anchoring proteins (AKAPs) (*11*). The microtubule-associated protein MAP2B is an AKAP that recruits PKA to $Ca_v1.2$ in neurons (*12*). We hypothesized that the channel complex may assemble signaling components upstream of PKA, including GPCRs.

Immunoprecipitation of β_2ARs from Triton X-100 extracts of rat forebrain and subsequent immunoblotting for α_{1C} revealed that the receptor was associated with $Ca_v1.2$ (Fig. 1A). The α_{1C} immunoreactive band was a mixture of full-length long form and COOH-terminally truncated short form; these components are not completely resolved on 10% acrylamide gels (*5, 7, 13*). The COOH-terminal fragment, which results from post-translational proteolytic processing and con-

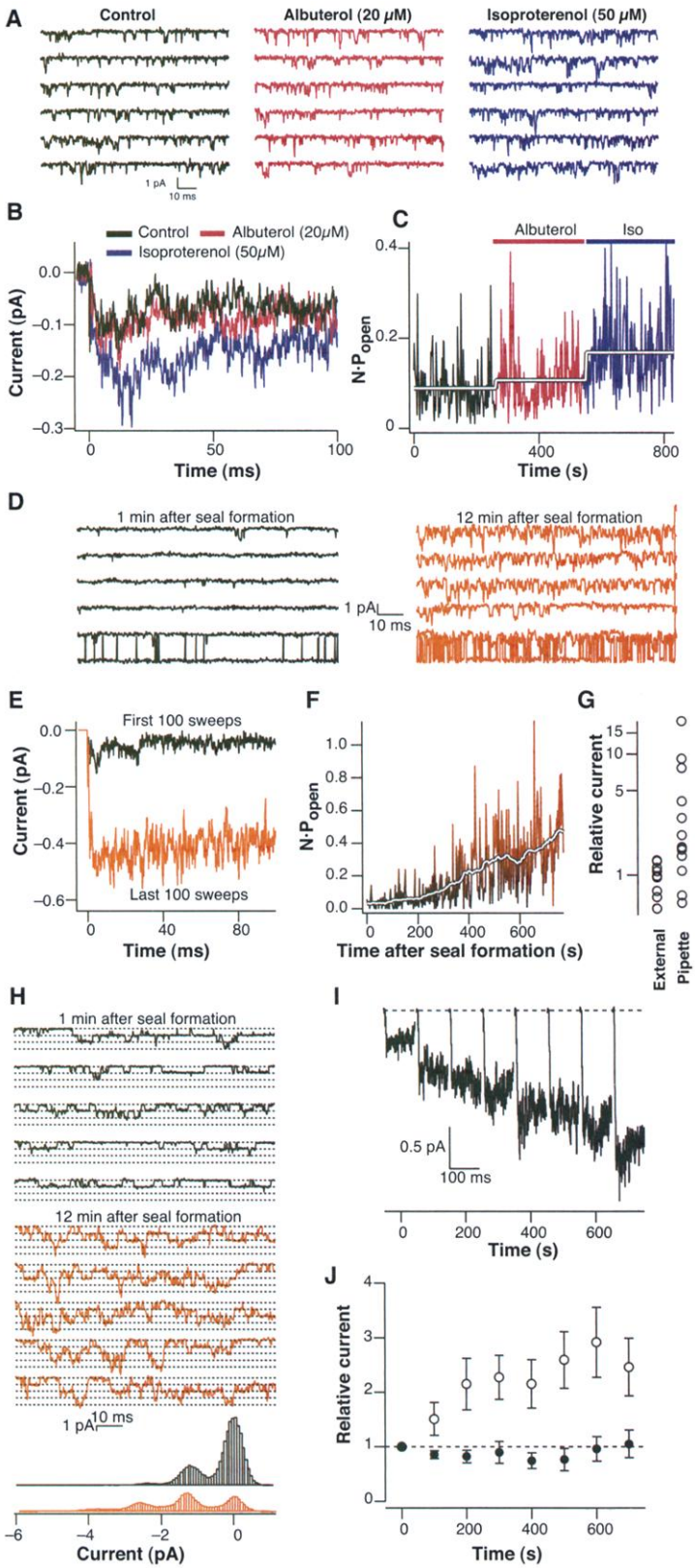

Fig. 2. Highly localized up-regulation of L-type channel activity by β_2ARs in neurons. Cell-attached patch clamp recording of L-type channel activity from primary hippocampal cultures during 100-ms test pulses from −80 mV to 0 mV (*18*). (**A**) Sweeps show channel activity (downward deflection) before (left) and after addition of 20 μM albuterol (middle) and then 50 μM isoproterenol (right) to the bath. (**D**) Sweeps 1 and 12 min after seal formation with a pipette backfilled with 20 μM albuterol. For each condition, ensemble averages of 100 sweeps (**B** and **E**) and open probability NP_{open} versus time (**C** and **F**) are shown [center line in (C), mean NP_{open} averaged over each experimental condition; center line in (F), running average calculated at each time point from 50 consecutive sweeps]. (**G**) Current sizes from an average of 100 sweeps after bath (left, $n = 9$) or pipette (right, $n = 14$) application of albuterol normalized to those before the application. Mean values are significantly different ($p < 0.05$, t test). (**H**) Sweeps 1 min (black) and 12 min (red) after seal formation with a pipette backfilled with albuterol in the presence of 100 nM ωCTx GVIA, 100 nM ωCTx MVIIC, and 1 μM BayK8644. Amplitude histograms from 50 sweeps at the beginning (black) and end (red) of the experiment are shown at bottom. (**I**) Ensemble averages of 50 consecutive sweeps over the time course of the experiment shown in (H). (**J**) Current amplitudes measured from ensemble averages with albuterol either applied to the bath ($n = 10$; open circles) or by pipette backfilling ($n = 10$; closed circles) versus time with 100 nM ωCTx GVIA, 100 nM ωCTx MVIIC, and 1 μM BayK8644 present. Current amplitudes were normalized to the first value (1 at $t = 0$) obtained after bath application of albuterol or after seal formation in backfilling experiments (bars, SEM). Mean values after 700 s were significantly different ($p < 0.05$, t test).

tains the PKA phosphorylation site Ser[1928] (*7, 13*), stays physically and functionally associated with the channel in intact cells (*14*). The channel was not associated with the metabotropic glutamate receptors mGluR1 and mGluR5 (two GPCRs concentrated at postsynaptic sites), nor with caveolin 1 and 3 (two caveolae markers), nor with PSD-95 and SAP102 (two postsynaptic density markers) (Fig. 1, B to D). Next, $Ca_v1.2$ was immunoprecipitated from brain extract, auxiliary subunits and other associated proteins were removed by dissociation with SDS at 60°C, and α_{1C} was reprecipitated (*7, 12*). The resulting immunocomplexes were incubated with glutathione S-transferase (GST) fusion proteins of the cytosolic domains of the β_2AR (*15*). Immunoblotting with antibodies to GST showed that the COOH terminus of the β_2AR specifically bound to α_{1C} (Fig. 1E).

The β_2AR coprecipitated and colocalized with $Ca_v1.2$ after ectopic coexpression in human embryonic kidney (HEK) 293 cells (Fig. 1, F and G) (*16*). We sought to determine whether the channel interacts with the receptor in the plasma membrane. Proteins on the surface of transfected HEK293 cells were biotinylated with a membrane-impermeable reagent. Membrane proteins were then solubilized and subjected to immunoprecipitation with antibodies to the β_2AR, and α_{1C} and the β_2AR were identified by immunoblotting (Fig. 1H). Stripping and reprobing the blot with streptavidin showed that not only the β_2AR but also the coprecipitating α_{1C} was thoroughly biotinylated (Fig. 1H). PP2A is an abundant protein present throughout the cell and also specifically interacts with α_{1C} in brain and HEK293 cells ectopically expressing $Ca_v1.2$ (*17*). After immunoprecipitation of PP2A, the catalytic C subunit of PP2A showed a strong signal by immunoblotting, but biotinylation was not detectable (Fig. 1H). Accordingly, the biotinylation reagent had no access to intracellular proteins.

Assembly of a β_2AR-$Ca_v1.2$ complex may locally restrict signaling from the receptor to the channel. We recorded channel activity in the cell-attached patch configuration from neurons in 2- to 3-week-old primary hippocampal cultures (*18*), which show largely L-type channel activity (*19*). When the β_2 adrenergic agonist albuterol was applied outside of the recording pipette to the bath (after recording under control conditions with no agonist), no statistically significant increase in channel activity was observed (Fig. 2, A to C and G). Subsequent bath application of the general β adrenergic agonist isoproterenol raised the L-type channel open probability NP_{open} in the patch, presumably via β_1 adrenergic receptors (β_1ARs), which, as in the heart (*20*), may result in a more widespread activation of PKA.

To evaluate whether localized stimulation of the β_2AR within the patch would increase channel activity, we partially filled the tip of the recording pipette without agonist and then backfilled it with agonist. During 10 of 14 recordings, channel activity significantly increased within the first few minutes of seal formation as the drug diffused to the pipette tip (Fig. 2, D to G). If no agonist was present, channel activity was either stable or declined slightly (Fig. 2C, control condition) (*21*). Openings of the Ca^{2+} channels observed in our experiments were prolonged by the L-type channel agonist BayK8644 in the presence of non–L-type channel blockers ωCTx GVIA and ωCTx MVIIC (Fig. 2H), confirming the identity of these Ca^{2+} channels as L-type. The channel activity observed with BayK8644 was significantly up-regulated over time by albuterol when applied inside the recording pipette, but bath application of albuterol had no effect (Fig. 2, H to J). Single-channel amplitudes were not affected by albuterol (Fig. 2H, bottom). These results show that β_2 adrenergic stimulation can strongly increase L-type channel activity only if it occurs in the vicinity of the channel.

We next sought to determine where β_2ARs and $Ca_v1.2$ are colocalized in vivo. Rat hippocampal sections were double-la-

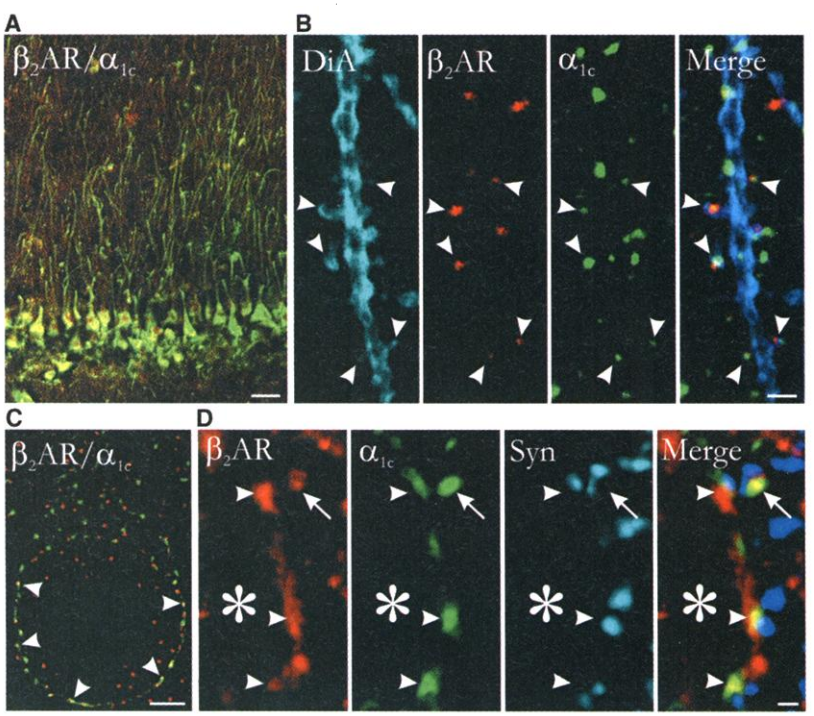

Fig. 3. Synaptic colocalization of the β_2AR with $Ca_v1.2$. Rat hippocampal sections were stained with antibodies to the β_2AR (red) and α_{1C} (green) (*22*). (**A**) Colocalization of the β_2AR and $Ca_v1.2$ in pyramidal cell bodies and apical dendrites in the CA1 region, as indicated by the yellow color from overlay of the red and green signal. (**B** to **D**) Apical dendrite (B) and cell body [(C) and (D); asterisk marks cell interior] in CA1 stained for the receptor and α_{1C} and with the membrane tracer DiA to outline the dendritic shaft studded with spines [blue in (B)] or for synaptophysin [blue in (D)] to identify synapses. Arrowheads indicate spines (B) or axosomatic synapses [(D); arrow on right indicates an adjacent axodendritic synapse] on which both β_2AR and α_{1C} are detectable. Arrowheads in (C) point to examples of puncta surrounding a cell body in CA1 that are immunoreactive for both β_2AR and α_{1C}. Scale bars, 50 μm (A), 2 μm (B), 5 μm (C), 1 μm (D).

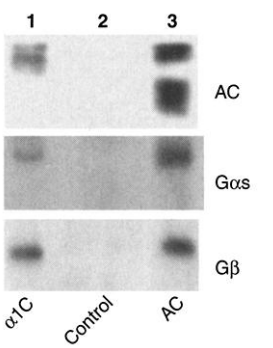

Fig. 4. Association of G proteins and adenylyl cyclase with the β_2AR-$Ca_v1.2$ complex. Triton X-100 extracts of rat forebrain membranes were used for immunoprecipitations (*12, 17*) with anti-α_{1C}, nonspecific control IgG from rabbit, and an antibody that recognizes multiple adenylyl cyclases (*25*) as indicated, followed by immunoblotting (*12, 17*) with the adenylyl cyclase antibody (top) or antibodies (*28*) to $G\alpha_s$ (middle) and Gβ (bottom).

beled for immunofluorescence microscopy (*22*); in the CA1 region, most cells and dendrites in which $Ca_v1.2$ was detectable also had β_2ARs (Fig. 3A). In dendrites stained with the membrane tracer DiA (blue) in addition to the antibody labeling, β_2AR (red) and α_{1C} (green) colocalized at tips of dendritic spines (postsynaptic sites of excitatory synapses; Fig. 3B). Extensive colocalization was also observed on the surface of the soma (Fig. 3C). Earlier immunoelectron microscopy had suggested a postsynaptic localization for both proteins (*13, 23*). Triple labeling with antibodies to the presynaptic marker synaptophysin confirmed that the observed colocalization of the receptor and $Ca_v1.2$ occurs at axodendritic and axosomatic synapses (Fig. 3D) (*21*); in dendrites, 80 ± 3% of synaptic β_2 AR clusters contained $Ca_v1.2$ and 77 ± 3% of synaptic $Ca_v1.2$ clusters had β_2ARs (*22*). This observation suggests that $Ca_v1.2$ may often, but not always, be associated with β_2ARs.

Like $Ca_v1.2$ and β_2ARs (*13, 23*), adenylyl cyclase is concentrated at postsynaptic sites in the hippocampus (*24*). We tested whether adenylyl cyclase and G proteins are also components of the β_2AR-$Ca_v1.2$ complex. Immunoprecipitation of α_{1C} and adenylyl cyclase was followed by immunoblotting with an antibody to adenylyl cyclase (*25*). The antibody recognized several isoforms indicated by multiple bands around the 200-kD region of the blot, including a doublet at the top of the blot (Fig. 4). This doublet was also present in the channel complex. We have not identified the adenylyl cyclase isoforms in the channel complex.

Earlier findings indicated that $G\alpha_s$ and $G\beta$ copurify with adenylyl cyclase (*26*) and that the $G\beta\gamma$ dimer can directly bind to various GPCRs (*27, 28*). Beyond short catalytic interactions of G proteins with GPCRs and adenylyl cyclases, G proteins may be tied up in stable interactions that keep them in the immediate vicinity of signaling complexes for rapid and specific signal transduction (*1, 2*). Indeed, $G\alpha_s$ and $G\beta$ specifically coprecipitated not only with the adenylyl cyclase but also with $Ca_v1.2$ (Fig. 4). In addition, PKA and the phosphatase PP2A are associated with $Ca_v1.2$ and phosphorylate and dephosphorylate α_{1C}, respectively (*7, 8, 12, 17*). Accordingly, $Ca_v1.2$ assembles a signaling complex that consists of the β_2AR, trimeric G proteins, adenylyl cyclase, PKA, and the counterbalancing PP2A. A similar complex might exist in the heart, where activation of β_1ARs results in PKA-mediated phosphorylation of substrates distributed throughout the cell, whereas stimulation of β_2ARs acts more selectively on $Ca_v1.2$ (*29*). Our electrophysiological studies indicate that signaling from the β_2AR to the channel is spatially highly restricted in neurons. Colocalization of GPCRs with their ultimate targets in macromolecular complexes could be a general mechanism to ensure that signaling is both specific and fast.

References and Notes

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15. Complementary DNA templates encoding hemagglutinin (HA)-tagged human β_2AR (*30*) were amplified by the polymerase chain reaction (PCR), subcloned into pGEX4T1 in-frame with GST, and confirmed by DNA sequencing (*17*). GST fusion proteins of the intracellular loops i1, i2, and i3 and of the COOH-terminus carry residues 59 to 74, 127 to 153, 217 to 277, and 326 to 413, respectively. Expression of all fusion proteins, absorption of equal amounts of fusion proteins on glutathione-Sepharose, and interaction assays including immunoblotting were performed as described (*12, 17*).
16. Class C channels (α_{1C}, $\alpha_2\delta$, and β_{2a}) and β_2ARs (*30*) were coexpressed in HEK293 cells and analyzed by immunoprecipitation and immunoblotting (*17*). For colocalization studies by immunofluorescence confocal microscopy, cells were cultured on cover slips coated with collagen (10 μg/ml) and poly-D-lysine (1 μg/ml) and transfected with Lipofectamine (Gibco-BRL) following manufacturer's protocols. After 24 hours, cells were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.3% Triton X-100 in phosphate-buffered saline (PBS) for 20 min, incubated with solution 1 (PBS containing 2% glycerol, 25 mM NH_4Cl, 2.5% fetal bovine serum, and 0.5% donkey serum), then with primary antibodies [anti-α_{1C} (1:100) and mouse anti-HA tag (Jackson ImmunoResearch; 1:1000) to detect the HA-tagged β_2AR (*30*)] in solution 1, washed several times with PBS, incubated with lissamine rhodamine-conjugated donkey antibody to rabbit immunoglobulin G (IgG) and fluorescein-conjugated donkey antibody to mouse IgG (both from Jackson ImmunoResearch; 1:400) in solution 1, washed five times with PBS and twice with H_2O, and mounted. Control experiments were routinely performed to exclude the possibility of nonspecific labeling or cross-reactivity of secondary antibodies with primary antibodies as well as bleed-through from one channel into another.
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18. Primary hippocampal cultures were grown for 2 to 3 weeks (*19*). L-type channel activity, characterized by relatively long openings and high single-channel conductance, was recorded (AxoPatch 200A, 5-kHz filter) in the cell-attached configuration during test pulses from a holding potential of −80 mV (in some cases −50 mV) to 0 mV [bath solution: 140 mM KCl, 3 mM $MgCl_2$, 10 mM EGTA, 10 mM glucose, 10 mM Hepes (pH 7.3); pipette solution: 90 mM $BaCl_2$, 10 mM tetraethylammonium chloride, 10 mM Hepes (pH 7.3) containing in some experiments (Fig. 2, H to J) 0.1 μM ωCTx GVIA and 0.1 μM ωCTx MVIIC to further exclude non–L-type channels]. BayK8644 (1 μM) was added in some cases to bath and pipette solutions to identify and better resolve L-type channel openings (Fig. 2, H to J).
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22. Vibratome sections of hippocampus were prepared from Sprague-Dawley rats perfused with 0.5% paraformaldehyde in phosphate buffer, pH 7.4. Because antibodies to the β_2AR (H-20, Santa Cruz Biotechnology) and α_{1C} were produced in rabbits, we used tyramide signal amplification for double labeling (*31*). After β_2AR staining with H-20 (1:25,000 dilution) and a Cy3 kit (Du Pont NEN), α_{1C} was detected by conventional fluorescent staining with the antibody to α_{1C} (*5, 12, 17*) (1:1000) and Cy5-conjugated donkey antibody to rabbit IgG (Jackson ImmunoResearch). Some sections were also labeled for synaptophysin with SVP-38 (Sigma, 1:1000) and fluorescein isothiocyanate–conjugated donkey antibody to mouse IgG. For membrane staining, DiA crystals (Molecular Probes) were applied to the immunostained sections at 4°C for 24 to 72 hours. Fluorescent images were acquired with a Leica TCS confocal microscope. For quantification of colocalization, α_{1C} labeling of all puncta immunoreactive for the receptor and synaptophysin, and receptor staining of all puncta immunoreactive for α_{1C} and synaptophysin, was determined in eight randomly selected fields from CA1 stratum radiatum.
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33. We thank J. D. Hildebrandt for antibodies to $G\alpha_s$ and $G\beta$, R. D. Green for antibody to adenylyl cyclase, R. B. Clark for the β_2AR mammalian expression vector, and P. J. Bertics, S. S. Blair, B. S. Ganetzky, and E. R. Weiss for critically reading the manuscript. Supported by NIH grants NS35563 (J.W.H.), AG17502 (J.W.H.), NS39444 (R.J.W.), GM56900 (M.C.H.), and HL61645 (T.H.); American Heart Association Established Investigator Grant 0040151N (J.W.H.); a Shaw Scientist Award (J.W.H.); a postdoctoral fellowship from the American Heart Association (V.A.); and NIH training grants GM08688 and AG00213 (M.A.D. and D.D.H.).

12 October 2000; accepted 3 May 2001