β₂-Adrenergic receptor activation mobilizes intracellular calcium via a non-canonical cAMP-independent signaling pathway

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Beta adrenergic receptors (βARs) are G-protein-coupled receptors essential for physiological responses to the hormones/neurotransmitters epinephrine and norepinephrine which are found in the nervous system and throughout the body. They are targets of numerous widely used drugs, especially in the case of the most extensively studied β₁AR, β₂AR, which are used for asthma and cardiovascular disease. βARs signal through Gαᵪ G-proteins and via activation of adenyl cyclase and cAMP-dependent protein kinase, but some alternative downstream pathways have also been proposed that could be important for understanding normal physiological functioning of βAR signaling and its disruption in disease. Using fluorescence-based Ca²⁺ flux assays combined with pharmacology and gene knock-out methods, we discovered a previously unrecognized endogenous pathway in HEK-293 cells whereby β₂AR activation leads to robust Ca²⁺ mobilization from intracellular stores via activation of phospholipase C and opening of inositol trisphosphate (InsP₃) receptors. This pathway did not involve cAMP, Gαᵪ, or Gαᵢ or the participation of the other members of the canonical β₂AR signaling cascade and, therefore, constitutes a novel signaling mechanism for this receptor. This newly uncovered mechanism for Ca²⁺ mobilization by β₂AR has broad implications for adrenergic signaling, cross-talk with other signaling pathways, and the effects of β₂AR-directed drugs.

Among G-protein-coupled receptors (GPCRs), encoded by the largest gene family in the human genome, receptors of the β-adrereceptor family (βARs), are perhaps the most thoroughly studied and some of the most commonly targeted by therapeutic drugs. βARs are divided into three subtypes: β₁AR, β₂AR, and β₃AR, differing in their localization (1) and responsiveness to drugs (2–4). These receptors are ubiquitously expressed throughout the body and respond to the hormones/neurotransmitters epinephrine and norepinephrine (5). The β₂AR in particular has been widely studied and has been often used as a model for studying GPCR structure and function (6).

Canonically, all βARs signal through coupling to Gαᵪ G-proteins, adenyl cyclase (AC), and cAMP-dependent protein kinase A (PKA) (1), but in recent years there has been increasing interest in alternative downstream pathways (7). In some cell types, β₂AR activation has been linked to mobilization of Ca²⁺ from intracellular stores, but these have been attributed to the actions of cAMP acting on PKA (8, 9) or exchange protein activated by cAMP (EPAC) (10–12). Understanding all of the pathways downstream from β₂AR is of great importance for comprehending normal physiological functioning of adrenergic signaling and its disruption in disease, cross-talk between cyclic nucleotide-mediated and Ca²⁺-mediated signaling, and the effects of β₂AR-directed drugs.

Results

Activation of endogenous β₂ARs in HEK cells leads to an increase in cytoplasmic Ca²⁺

In the course of testing responsiveness of mutant dopamine receptors to various agonists (15), we observed that treatment of HEK-293 cells with norepinephrine (NE) led to a dramatic increase in intracellular Ca²⁺ (Fig. 1a). To determine which receptors are responsible for this response, we tested the cells with adrenergic agonists and antagonists of known specificity. Epinephrine (Epi), norepinephrine (NE), and isoproterenol (ISO) all activated the response with a rank order of potency (ISO > Epi > NE), consistent with that of β₂AR (2), and the β₂AR-selective terbutaline (Ter) activated with high potency (Fig. 1b). Whereas propranolol, an inhibitor of β₁AR and β₂AR, but not β₂AR, and the β₂AR-specific inhibitor ICI 118,551 did not block responses of purinergic-P₂Y receptors to adenosine triphosphate (ATP; Fig. 1c), they blocked the response to isoproterenol (Fig. 1, d and e). In contrast, α₁/2AR inhibitor yohimbine and α₂AR-selective inhibitor prazosin did not block signaling (Fig. 1, f and g). These results point strongly to β₂AR, known to be expressed in this cell line (16), as the receptor
**cAMP-independent Ca\(^{2+}\) mobilization by \(\beta_2\)AR**

![Diagram](https://via.placeholder.com/150)

**Figure 1. Endogenous \(\beta_2\)AR activation increased cytoplasmic (Ca\(^{2+}\)) in HEK cells.** Continuous changes in Fluo-4 fluorescence intensity with time (a and c) or peak increases in intensity (b) as a function of drug concentration (b and d–h) are plotted. a, NE treatment increased cytoplasmic calcium release (Fig. 1d). b, NE response is mimicked by AR agonists epinephrine (Epi) and isoproterenol (ISO) and the \(\beta_2\)AR-selective terbutaline (Ter), the \(\beta_2\)AR inhibitor propranolol and the \(\beta_2\)AR-selective ICI 118,551 do not suppress P2Y receptor signaling. d and e, \(\beta_2\)AR-selective ICI 118,551 do not suppress P2Y receptor signaling. f and g, \(\alpha_1\)-adrenergic inhibitors do not suppress ISO responses. h and i, loss of Ca\(^{2+}\) response in cells lacking \(\beta_2\)AR and restoration by \(\beta_2\)AR expression. h, \(\beta_2\)AR deletion mutant cells (KO) were transfected with pcDNA3.1 or HA-tagged \(\beta_2\)AR and tested for Ca\(^{2+}\) responses over a range of ISO concentrations. i, \(\beta_2\)AR deletion mutant cells (KO) or wildtype (WT) cells were treated with 10 \(\mu\)M ISO at the indicated times, and Ca\(^{2+}\) responses were monitored over time. For all panels Ca\(^{2+}\) traces represent three or more independent experiments; error bars indicate internal replicate S.E. AU absorbance units.

**Potential isoproterenol stimulation of Ca\(^{2+}\) release requires a functional \(\beta_2\)AR gene**

To test for the role of \(\beta_2\)AR in the observed Ca\(^{2+}\) release by a genetic approach, we supplement the strong pharmacological evidence, we created an ADRB2 gene deletion using the CRISPR/Cas9 system. This knock-out line was transfected with control or HA-\(\beta_2\)AR-expressing constructs and tested for Ca\(^{2+}\) response to ISO. In the absence of \(\beta_2\)AR (Fig. 1, h and i) there was no detectable Ca\(^{2+}\) response to ISO up to its EC\(_{50}\) in WT cells (Fig. 1d), and at much higher concentrations only a very attenuated response was observed, likely due to nonspecific effects on other targets. The response was completely rescued by transfection with a plasmid directing expression of \(\beta_2\)AR (Fig. 1h).

**The cytoplasmic [Ca\(^{2+}\)] increase is due to release from thapsigargin-sensitive intracellular stores through the actions of phospholipase C (PLC) and the inositol trisphosphate receptor (InsP\(_3\)R)**

Removal of extracellular Ca\(^{2+}\) with EGTA did not block \(\beta_2\)AR-mediated Ca\(^{2+}\) release (Fig. 2, a and b), although it did eliminate the characteristic long-term plateau of the Ca\(^{2+}\) signal, suggesting the latter may be due to store-operated Ca\(^{2+}\) entry. In contrast, treatment of cells with thapsigargin to inhibit the endoplasmic-reticulum-resident SERCA Ca\(^{2+}\)/ATPase pump and deplete intracellular Ca\(^{2+}\) stores resulted in a transient increase in intracellular Ca\(^{2+}\) concentration and almost completely blocked Ca\(^{2+}\) release in response to \(\beta_2\)AR activation (Fig. 2c). Inhibition of PLC with U73122 or of the InsP\(_3\)R with 2-APB blocked \(\beta_2\)AR-mediated Ca\(^{2+}\) release (Fig. 2d). These results point strongly to a mechanism in which \(\beta_2\)AR activation leads to PLC activation, release of InsP\(_3\), and Ca\(^{2+}\) release from thapsigargin-sensitive intracellular stores via the InsP\(_3\) receptor.

**Activity of cAMP-dependent protein kinase is not necessary or sufficient for \(\beta_2\)AR-mediated Ca\(^{2+}\) signaling**

To test for canonical signaling through cAMP-dependent PKA we treated with the cell-permeant PKA activator, 8-bromo-cAMP (8-Br-cAMP). Challenge of HEK cells with 2 mM 8-Br-cAMP after pretreatment for 1 h with 200 \(\mu\)M 3-isobutyl-
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HEK-μOR), which couples to the AC-inhibiting G-protein, G\(_{\alpha_i}\). Activation of μ-opioid receptor (μOR) with 3 μM DAMGO did not suppress β\(_2\)AR-mediated Ca\(^{2+}\) mobilization (Fig. 4a) but did activate the G\(_{\alpha_i}\)-activated TRPC4\(_{\beta}\) channel, depolarizing the membrane (Fig. 4b), a response that was abolished by pertussis toxin (PTX; Fig. 4c).

To investigate potential G\(_{\alpha_i}\)-insensitive roles of AC in our Ca\(^{2+}\) pathway, we used varying concentrations of the AC inhibitors 9-(tetrahydrofuryl)-adenine (SQ 22536) and 2′,5′-dideoxyadenosine (ddAd) and found that both inhibitors failed to suppress Ca\(^{2+}\) signaling (Fig. 4, d and e) even at 1 mM (Fig. 4, d, e, and g). Control experiments confirmed the ability of these compounds to block cAMP accumulation (Fig. 4f), leading to the conclusion that AC activity is not necessary for the Ca\(^{2+}\) response.

To determine whether increases in cAMP concentration mimic or enhance β\(_2\)AR-induced responses, we treated HEK cells with the phosphodiesterase inhibitor IBMX and found that although this reagent increased isoproterenol-induced CREB phosphorylation (supplemental Fig. S1) and promoted robust cAMP production in response to ISO (Fig. 4f), it failed to potentiate isoproterenol-induced increases in intracellular Ca\(^{2+}\) (Fig. 4, h and i). These results demonstrate that activation of AC and increasing cAMP levels inside the cell are neither necessary nor sufficient for the mobilization of Ca\(^{2+}\) by β\(_2\)AR.

β\(_2\)AR-induced calcium release from intracellular stores is independent of G\(_{\alpha_i}\) and G\(_{\alpha/o}\) G-proteins

To test for the participation of β\(_2\)AR’s canonical signaling partner G\(_{\alpha_o}\), which may act through effectors other than AC, we treated cells with cholera toxin (CTX), which leads to persistent activation of G\(_{\alpha_i}\). CTX treatment neither induced Ca\(^{2+}\) release (not shown) nor potentiated β\(_2\)AR-induced Ca\(^{2+}\) responses (Fig. 5a), whereas CTX treatment generated a very large increase in cAMP (Fig. 5b). These results not only indicate that G\(_{\alpha_i}\) does not play an important role but also further confirm that cAMP, the canonical second messenger regulated by β\(_2\)AR, does not play any role in the Ca\(^{2+}\) response.

It has been reported that upon PKA phosphorylation β\(_2\)AR can couple to the PTX-sensitive G-protein G\(_{\alpha_i}\) (16). We treated cells with PTX and found that it failed to suppress signaling with ISO (Fig. 5c), whereas in control experiments in cells expressing G\(_{\alpha_i}\)-activated TRPC4\(_{\beta}\), it strongly suppressed activation of G\(_{\alpha_i}\) by the dopamine D\(_2\) receptor (Fig. 5d) or the μ-opioid receptor (Fig. 4c). Moreover, G\(_{\alpha_i}\) activation with DAMGO did not lead to increases in cytoplasmic Ca\(^{2+}\) concentration (Fig. 4e), and isoproterenol treatment alone did not lead to G\(_{\alpha_i}\) activation of TRPC4\(_{\beta}\) (Fig. 4b). Thus, neither G\(_{\alpha_i}\) nor G\(_{\alpha_o}\) was necessary or sufficient for β\(_2\)AR-induced Ca\(^{2+}\) release.

Discussion

Our results reveal that in HEK-293 cells, β\(_2\)AR activation led to rapid and robust Ca\(^{2+}\) signaling that relied on phospholipase C, the InsP\(_3\) receptor, and intracellular Ca\(^{2+}\) stores but not on the canonical downstream signaling partners, G\(_{\alpha_i}\), G\(_{\alpha_o}\), AC, or PKA. For a summary of the drugs used to test for involvement of these molecules, please refer to supplemental Table S1.
There have been previous reports of intracellular Ca\textsuperscript{2+} mobilization or modulation by adrenoreceptor activation, but compelling evidence for a pathway leading to InsP\textsubscript{3} release without involvement of G\textsubscript{s}, G\textsubscript{i}, or cAMP has been lacking. For example, Ca\textsuperscript{2+} release upon adrenergic stimulation of rat submandibular and parotid cells, although not specifically attributed to \(\beta_2\)AR (17–19) and subject to dispute (20), was reported to be downstream of cAMP production (19). In ostensibly the same cell line as that studied here, it was reported that activation of overexpressed recombinant \(\beta_2\)AR led to Ca\textsuperscript{2+} mobilization but that the pathway proceeded through cAMP and EPAC (12). More recently, in an HEK-293-derived cell line overexpressing a tagged recombinant \(\beta_2\)AR, Ca\textsuperscript{2+} release from intracellular stores was reported to contribute to impedance changes resulting from \(\beta_2\)AR activation, but the pathways leading from receptor to release were not explored (21).

The involvement of \(\beta_2\)AR in the modulation of Ca\textsuperscript{2+} release from intracellular stores has been previously observed during cross-talk between \(\beta_2\)AR-mediated pathways and those of Ga\textsubscript{s}-coupled receptors such as the M\textsubscript{3} muscarinic receptor (22, 23) and \(\alpha_1\)AR (24). In these studies AR agonist effects were shown to depend on co-activation of both receptors, in contrast to our findings that clearly show that treatment with \(\beta_2\)AR agonists alone leads to increasing cytoplasmic Ca\textsuperscript{2+}. Additionally,
cAMP was found to be a necessary mediator for cross-talk, whereas our data demonstrate that cAMP is not necessary for β2AR agonist-induced Ca2+ mobilization. Thus the previously observed cross-talk between β2AR and Gq-coupled receptors likely represents a signaling cascade distinct from the one studied here. Previous failure to observe the robust Ca2+ release seen here may be attributable to differences in conditions, e.g., loading cells with indicator dye at 37 °C (25), known to increase dye accumulation in intracellular compartments (26), culturing cells in suspension (25), or clonal differences in HEK-293 cells. In this regard it is important to note that we have observed this phenomenon in five different clonal HEK-derived cell lines from two different laboratories as well as in cells freshly obtained from ATCC. In addition, we have shown that a lack of β2AR leads to a huge reduction in Ca2+ release, which can be rescued by transient transfection with an HA-tagged β2AR.

The lack of involvement of Gq, G11, and cAMP in this pathway is quite surprising; even currently recognized “alternative” signaling mechanisms for β2AR primarily go through at least one of these effectors. Gq, G11, couple a variety of GPCRs to phospholipase C; however, β2AR failed to couple to Gq, G11, and Gα14 to activate PLC in co-expression studies (27, 28) and has only been shown to couple to Gq in overexpression systems where the G-protein and receptor were fused together (29). Moreover, treatment of HEK-TRPC4β cells with carbachol, an agonist for the Gq-coupled M3 muscarinic receptor, leads to a robust change in membrane potential. In a previous study we confirmed that these changes are dependent on activation of Gq, G-proteins (30). In contrast, we found that treatment with ISO does not lead to a change in membrane potential (Fig. 6), further arguing against a role for Gq.

Two promiscuous G-proteins, human Gα16 and the mouse version, Gα15, are known to couple a wide range of GPCRs to PLC. We determined previously that D2 dopamine receptors and Group II and Group III metabotropic glutamate receptors do not couple to PLC and Ca2+ release significantly through endogenous G-proteins in HEK-293 cells but produce robust Ca2+ responses upon activation in cells co-transfected with plasmids directing expression of Ga15 or Ga16, or in cell lines stably expressing Ga16 (15, 30), arguing strongly against a role for Ga16 in the responses observed here. Another candidate for future study is Ga15, potentially acting through a previously unknown pathway. Presumably, any number of Gβγ subunits could be involved, as these have been shown to activate PLC (32). Another possible mechanism for this response could involve the participation of β-arrestins, which initiate an array of signaling cascades. Activation of β-arrestin is not typically associated with PLC activation or the opening of InsP3Rs (33–35), so a β-arrestin-linked pathway would represent a novel branch of signaling mediated by these molecules. Moreover, the rapid nature of the responses we observed would also represent a novel feature of β-arrestin-mediated responses, which are generally much slower than those mediated by G-proteins (31).

Exploration of the mechanisms linking receptor activation to phospholipase C and the implications of this novel pathway for responses to endogenous β2AR agonists and β2AR-directed drugs will be important impacts of these observations.

Experimental procedures

Reagents

Fluo-4-AM, thapsigargin, pluronic F-127, Lipofectamine 2000 were purchased from Life Technologies (Grand Island, NY). ATP, terbutaline, propranolol, yohimbine, prazosin, ddAd, U73122, epinephrine, and norepinephrine were purchased from Sigma-Aldrich. ICI 118,551, H-89, isoproterenol, KT 5720, IBMX carbachol, and 2-APB were purchased from EMD Millipore (Darmstadt, Germany). DAMGO was purchased from TOCRIS (Bristol, UK). CTX and PTX were purchased from List Biological Laboratories (Campbell, CA). 8-Bromo-cAMP and 9-(tetrahydrofuryl)-adenine (SQ 22536) were purchased from Enzo Life Sciences (Farmingdale, NY). Phospho-CREB (pCREB) antibody (Ser-133) (1B6) antibody and CREB chase antibody from Enzo Life Sciences (Farmingdale, NY). IR dye-conjugated secondary antibodies were purchased from LI-COR (Lincoln, NE).

Cell culture and transfection

Human embryonic kidney 293 wild type (HEK) cells were obtained from the American Type Culture Collection (Manassas, VA). HEK-293 cells, HEK-293 cells stably expressing transient receptor potential channel-4-β (TRPC4β) (HEK-TRPC4β), and HEK-293 cells stably expressing both β2AR and TRPC4β (HEK-β2AR) were kindly donated by Dr. Michael X. Zhu from the University of Texas Health Science Center (Houston, TX). All cell cultures were maintained at 37 °C, 5% CO2 in a humidified incubator in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. Wild-type cells were maintained in antibiotic free DMEM, whereas stably transfected cells were maintained in media containing 0.5 g/liter G418, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transfections were performed in 96-well plates with Lipofectamine 2000 following the manufacturer’s recommendations.

Electroportation of the ADRB2 knock-out cell line to introduce control or rescue constructs was performed using a BTX ECM830 square wave machine (Harvard Apparatus, Holliston, MA).
**CAMP-independent Ca\(^{2+}\) mobilization by \(\beta_2\)AR**

MS). For each electroporation, 400 \(\mu\)l of \(5 \times 10^5\) cells/ml in PBS were combined with 20 \(\mu\)g of plasmid DNA in H\(_2\)O. The ADRB2 rescue construct contains an N-terminal HA\(_3\)-tagged human ADRB2 cloned into pcDNA3.1 (cDNA Resource Center). The electroporation was done with 3–2 ms pulses of 260 V in 4 ms intervals. Cells were allowed to recover for 1–3 days and were then plated as described for 96-well Ca\(^{2+}\) release assays.

**Generation of ADRB2 knock-out HEK-293 cell line**

The ADRB2 gene deletion was generated in HEK-293 cells using the CRISPR/Cas9 method (13). Several 20-bp DNA targeting sequences upstream of protoscaler adjacent motif sites were identified in both the 5’ and 3’ ends of ADRB2 using Benchling. These sequences were cloned into px458 (Addgene #48138), which contains the Cas9 gene from Strepococcus pyogenes and a single-guide RNA (sgRNA) sequence. The constructs were transfected into HEK-293 cells and tested for efficiency using the surveyor assay (13, 14). The mismatch-specific endonuclease CEL1 used for this assay was extracted from celery as described (36). Two constructs targeting the 5’ (-GCCGGACCAACGAGTACGC-3’) and 3’ (-AGCGAT-AACATTGATTCACAC-3’) ends of the gene were co-transfected into HEK-293 cells and tested for gene deletion using PCR. Clonal cell lines were generated at the Baylor College of Medicine Cell Based Assay Screening Service core facility by limited dilution and expansion and screening of clones by PCR.

**Calcium mobilization assay**

Cells were plated in clear, flat-bottom, poly-D-lysine-coated 96-well plates (Corning Life Sciences, Corning, NY) at 80,000 or 150,000 cells per well and allowed to grow for ~48 or 24 h, respectively. The day of the experiment plating medium was removed, and cells were washed with Krebs/Ringer/HEPES (KRH) buffer (120 mm NaCl, 4.7 mm KCl, 2.2 mm CaCl\(_2\), 10 mm HEPES, 1.2 mm KH\(_2\)PO\(_4\), 1.2 mm MgSO\(_4\)) supplemented with 1.8 g/liter glucose, 2.5 mm probenecid (to prevent dye efflux), and ascorbic acid (to prevent oxidation of assayed drugs). KRH buffer that was supplemented with glucose, probenecid, and ascorbic acid is abbreviated as KRH-A. After washing, cells were loaded with Fluo-4-AM in DMSO (final concentration 2 \(\mu\)M) and cells were allowed to equilibrate to 37 °C for 10 min before drug injection. Data were analyzed using GraphPad Prism software. Blot raw images were adjusted in GraphPad Prism software (GraphPad Software for Science, Inc., La Jolla, CA).

**Gene activation assay**

Activation of Go\(_i\) was assessed by measuring changes in membrane potential caused by the opening of TRPC4\(\beta\) ion channels. These assays were done in HEK-TRPC4\(\beta\) and HEK-\(\mu\)OR cells, which were plated as described for the Ca\(^{2+}\) mobilization assay. The day of the experiment the plating media was removed, and cells were washed with KRH buffer. The cells were loaded with a membrane potential dye and quencher combination (Molecular Devices) diluted in KRH buffer and incubated at 32 °C for 30 min. Dye was not removed during the assay. Differences in membrane potential were measured by reading fluorescence (excitation/emission 530/565 nm) from the bottom with a Flexstation 3 plate reader. A baseline of fluorescence was collected for 30 s before drug injection. Data were analyzed using GraphPad Prism software.

**Total intracellular cyclic AMP measurements**

Total intracellular cAMP was measured with the Amersham Biosciences cAMP Biotrak Enzyme Immunoassay system RPN2251 (GE Healthcare). Measurements were acquired by following the manufacturer’s recommendations. In brief, HEK-293 cells were plated at 50,000 cells per well in 96-well plates 24 h before the assay. Drug treatments were performed as described under “Results.” Total cAMP ELISA was done following manufacturer’s instructions, followed by optical density measurements at 450 nm using a Flexstation 3 plate reader.

**Immunoblotting**

Cells were plated at a density of 400,000 cells per well in 24-well plates and assayed the next day. After treatment with pertinent drugs, cells were lysed by the addition of sample application buffer (50 mM Tris HCl, pH 6.8, 6% v/v glycerol, 2% w/v sodium dodecyl sulfate, 10 \(\mu\)M dithiothreitol, and 1% v/v \(\beta\)-mercaptoethanol) with cOmplete protease inhibitor mixture (Roche Diagnostics) and PhosSTOP (Roche Diagnostics) phosphatase inhibitor mixture. Cell lysates were collected into prechilled microcentrifuge tubes and sonicated in an ethanol/ice bath for 30 s. Equal volumes of all samples were separated on 10 or 12% polyacrylamide gels in Tris-glycine-SDS running buffer. Protein bands were transferred onto nitrocellulose membranes in Tris-glycine-SDS buffer + 20% methanol at 350 mA for 90 min at 4 °C. Membranes were blocked with 5% milk for 1 h and incubated overnight at 4 °C in 1:1000 primary antibody solutions (anti-CREB antibody anti-phospho-CREB antibody (Cell Signaling Technologies)) made in 5% milk. Membranes were washed three times in Tris-buffered saline with Triton X-100 before secondary antibody incubation. IR dye-conjugated antibodies (donkey-anti-mouse 800CW and goat-anti-rabbit 680RD (LICOR)) were diluted 1:5000 in 5% milk. Membranes were incubated in this solution for 1 h at room temperature. Incubation was followed by 3 washes in Triton X-100. After washing blots were scanned using an Odyssey scanner (LICOR), light intensity in both the 700 and 800 channels was quantified using Odyssey software, and data were analyzed with GraphPad Prism. Blot raw images were adjusted in...
Adobe Photoshop by inverting colors and adjusting brightness on all blots at the same time to make bands clear.

Data analysis

Ca²⁺ and membrane potential traces

Data shown are representative of at least three independent experiments. Both Ca²⁺ and membrane potential experimental data were corrected for by subtracting the average fluorescence from baseline measurements. For Ca²⁺ and membrane potential traces three replicates of each condition were averaged, and standard error between these replicates was used to determine error bars, which indicate the internal replicate error.

Dose-response curves

For dose-response curves the maximum Ca²⁺ response from individual Ca²⁺ traces was plotted against drug concentration. These data were fitted to a sigmoidal dose-response curve with a Hill coefficient of 1.0 using GraphPad Prism. The error bars shown in these curves correspond to the S.E. obtained from three independent experiments.

cAMP accumulation assays

All cAMP experiments were carried out alongside a standard cAMP curve, which was used to convert optical density measurements into cAMP concentration. The data from three independent experiments were averaged to construct cAMP bar graphs, and the statistical differences between samples treated with test drugs and their respective controls were determined by use of an unpaired two-tailed t test. Specific p values for each figure are indicated in their respective figure legends. The error bars in these plots represent the S.E. of the three independent experiments. The statistical significance in the difference of cAMP accumulation from various samples was determined by applying two-tailed non-parametric t tests to the data from three or more independent experiments, and the corresponding p values are reported. The use of a t test for statistical analysis assumes that samples that are compared with each other have similar variances (which logically applies in our case as in our experiments the only difference between drug-treated samples and controls is the actual drug treatment). However, in experiments testing the effect of AC inhibitors, the variance for measurements after dDA treatment is substantially lower than for the other samples, likely as an artifact of a low n. We, therefore, analyzed using both the observed sample variance (p = 0.0059) and the larger variance for the samples treated with SQ 22,536 (p = 0.0086), with the latter considered the more reliable analysis. Likewise, the variances of control and CTX-treated samples are different because the variance increases as the size of the signal (i.e. relative sample S.D. are similar but absolute variances are not) so that the non-CTX-treated samples with virtually no signal yield an artificially low variance. Assuming a larger variance for the control samples (i.e. the variance of the CTX treated samples), the p value continues to be significant (p = 0.0014 versus p = 0.0004 with the observed sample variance).

Phospho-CREB accumulation semiquantitative immunobLOTS

After blotting, membranes were scanned on an Odyssey scanner. The blots shown are representative of two internal replicates and three independent experiments. Data from CREB and pCREB bands were collected with Odyssey software. After background subtraction, pCREB/CREB values were calculated, and the data were normalized by dividing by the respective control value so that control samples have a pCREB/CREB value of 1.0. The data from three independent experiments were averaged and used to plot pCREB/CRES bar graphs. One-sample t tests were used to determine whether the differences between the means of drug-treated samples were significantly different to a theoretical mean of 1.0 representing the control sample. Specific p values for each plot are indicated in figure legends. The error bars in these plots also represent the S.E. from the three independent experiments.

Author contributions—M. G.-M. designed, performed, and analyzed most experiments and contributed to the writing of the manuscript. S. J. W. designed, performed, and analyzed some of the experiments and contributed to the writing of the manuscript. G. J. R. made the initial discovery that led to the project. O. L. provided input on experiments and project directions. T. G. W. provided guidance with experimental design and project directions and contributed to the writing of this manuscript. All authors reviewed the results and approved the final version of the manuscript.

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After blotting, membranes were scanned on an Odyssey scanner. The blots shown are representative of two internal replicates and three independent experiments. Data from CREB and pCREB bands were collected with Odyssey software. After background subtraction, pCREB/CREB values were calculated, and the data were normalized by dividing by the respective control value so that control samples have a pCREB/CREB value of 1.0. The data from three independent experiments were averaged and used to plot pCREB/CRES bar graphs. One-sample t tests were used to determine whether the differences between the means of drug-treated samples were significantly different to a theoretical mean of 1.0 representing the control sample. Specific p values for each plot are indicated in figure legends. The error bars in these plots also represent the S.E. from the three independent experiments.

Author contributions—M. G.-M. designed, performed, and analyzed most experiments and contributed to the writing of the manuscript. S. J. W. designed, performed, and analyzed some of the experiments and contributed to the writing of the manuscript. G. J. R. made the initial discovery that led to the project. O. L. provided input on experiments and project directions. T. G. W. provided guidance with experimental design and project directions and contributed to the writing of this manuscript. All authors reviewed the results and approved the final version of the manuscript.

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