Arcemisbéhère L *et al*.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Construction of G protein-coupled receptor (GPCR) fusions. The coding sequence for the α_5 integrin (α_5I) fragment (amino acid residues 231 to 514) was amplified by polymerase chain reaction (PCR) using the pET15b-α_5I prokaryotic vector as a template (1). The two primers used in the PCR were designed to introduce a NdeI restriction site at the 5' end and a BamHI restriction site at the 3' end. The PCR fragment was purified using the Qiaquick gel extraction kit (Qiagen), digested with NdeI and BamHI, and inserted into the corresponding sites of the pET21a(+) prokaryotic vector (Novagen). An equivalent protocol was used to subclone the coding sequence of the *Bacillus cereus* phospholipase C (amino acid residues 1 to 258) into the NdeI/BamHI restriction sites of pET21a(+) using the plasmid pMR1 as a template (2). The *Escherichia coli* gene PurF, coding for the glutamine phosphoribosylpyrophosphatase amidotransferase (amino acid residues 1 to 504), was amplified using the same protocol with the pET24a-PurF as the template (3), although the two primers were designed to incorporate a NheI restriction site and a BamHI restriction site at the 5' and 3'ends, respectively. The PurF PCR fragment was inserted at the NheI/BamHI sites of the pET21a(+) expression vector. The resulting pET21a-α_5I, pET21a-PLC, and pET21a-PurF plasmids were verified by direct DNA sequencing (Cogenics, Meylan, France). Apart from the human chemokine-like ChemR23 and the arginine-vasopressin (AVP) V1b receptors, synthetic GPCR genes optimized for bacterial expression were constructed by a three-step PCR using a set of 24 overlapping oligonucleotides, as described previously (4). The final PCR reaction contained short flanking primers incorporating restriction sites for subsequent cloning of the amplified products into the expression vector. Each PCR product was digested and inserted into the corresponding pET21a(+) restriction sites. For the AVP V2 receptor expressed as an isolated protein, the optimized coding sequence was inserted into the NdeI/EcoRI restriction sites. For GPCRs expressed as fusions, the AVP V2 and V1b, catecholamine β3-adrenergic, leukotriene BLT2, CysLT1, CysLT2, and cannabinoid CB1 receptor coding sequences, BamHI/EcoRI restriction sites were chosen. For insertion of the transglutaminase recognition sequence (TGase tag; (5)) at the C-terminus of the BLT2 receptor, two oligonucleotides corresponding to the sense and antisense sequence encoding the TGase tag (Pro-Lys-Pro-Gln-Gln-Phe-Met) flanked by a SalI and XhoI sites were synthesized and cloned into the corresponding sites of the pET21a-α_5I-BLT2 vector. The ChemR23 receptor coding sequence was inserted into the BamHI/XhoI sites of the pET21a-α_5I vector. The resulting pET21a-GPCR vectors were verified by direct DNA sequencing, and errors were corrected by site-directed mutagenesis with the QuickChange multisite-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The optimized version of the oxytocin (OT) receptor (OTR) was amplified by PCR and inserted into the SacI/XhoI restriction sites of the pET21a-α_5I-V2. The optimized versions of the CXCR4 and CCR5 chemokine receptor coding sequences were amplified by PCR using two primers designed to introduce SacI/HindIII restriction sites at the 5' and 3' ends, respectively. After purification of the PCR products and digestion with restriction enzymes, the different GPCR coding sequences were subcloned into the SacI/XhoI or SacI/HindIII sites of the pET21a-α_5I-V2 prokaryotic vector. The resulting pET21a-α_5I-V2-GPCR vectors were verified by direct DNA sequencing and errors were corrected by site-directed mutagenesis. Each cDNA construct was checked to ensure that it was in phase with a 6xHIS tag coding sequence that was present in the vector downstream of the multicloning site region.

In the first set of fusions (α_5I-GPCR), a thrombin cleavage site (LVPR/GS) was added between the α_5I module and the GPCR coding sequence. A 5'-phosphorylated oligonucleotide designed to introduce LVPR residues (GS being already present due to the BamHI restriction site) was used in the mutagenesis PCR reaction. In addition, to increase the accessibility of the thrombin to its cleavage site, a sequence encoding a spacer arm was added upstream of the LVPR/GS cleavage site. The sequence, $DP(G)_8$, was also inserted by mutagenesis. In both cases, the same protocol was followed with the Quickchange multisite-directed mutagenesis kit from Stratagene. The addition of the sequences coding for LVPR and $DP(G)_8$ was verified by direct DNA sequencing. In the second set of fusions (α_5I-V2-GPCR), a thrombin cleavage site and two spacer arms, $DP(G)_8$ and $(G)_6$, located 5' and 3' to the LVPR/GS sequence, respectively, were added using the same mutagenesis protocol. In all cases,

insertion of the different sequences was confirmed by direct DNA sequencing. The final α_5I-GPCR and α_5I-V2-GPCR constructs were all in phase with a C-terminal 6xHIS tag sequence.

Expression of the GPCR fusions. Rosetta^{TM}(DE3) bacteria were transformed with the different pET21a-α_5I-GPCR or pET21a-α_5I-V2-GPCR expression vectors following the manufacturer's (Novagen) instructions and grown overnight at 37°C on Luria-Bertani (LB)-agar plates containing 100 μg/ml ampicillin. For each construct, an isolated colony was picked up from the plate and the bacteria grown in 10 ml LB broth containing 100 μg/ml carbenicillin at 37°C to an absorbance of 0.6 at 600 nm. This preculture was used to inoculate 100 ml of LB medium in a 500-ml flask for the extraction/purification of the recombinant fusions. Otherwise, for a rapid screening, 0.1 mM of isopropylthio-β-D-galactoside (IPTG) was added to the 10 ml culture to induce expression, and bacteria were grown for another 4 hours. 1 ml of suspension was harvested and the absorbance was measured. The bacteria were centrifuged and the supernatant discarded. If for instance absorbance was 1.8, the pellet was solubilized first in 180 μl of electrophoresis running buffer (Tris 25 mM, Glycine 182 mM and SDS 0.1%). Then, 180 μl of Laemmli buffer (Bio-Rad) was added. The solubilized sample was sonicated on ice (10 seconds) and total bacterial proteins separated onto 12% SDS-polyacrylamide gels. This relation between the value of absorbance and the volume of running and Laemmli buffers was always followed in order to put equivalent quantities of proteins in each electrophoresis well. For purification of the α_5I-GPCR or α_5I-V2-GPCR fusions, 2 ml of the preculture were used to inoculate 100 ml of LB medium supplemented with 100 μg/ml carbenicillin and 0.2% glucose, and bacteria were grown at 37°C until the OD was 0.6 at 600 nm. Then, 0.1 mM IPTG was added to the culture to induce expression of the recombinant proteins. Bacteria were harvested 4 hours later, pelleted by centrifugation (4,000 x *g* for 15 min at 4°C) and kept frozen at –80°C until use.

Extraction/purification of the GPCR fusions. All steps were carried out at 0-4°C. The cell pellet was thawed, resuspended in 9 ml of ice-cold lysis buffer (20 mM Tris-HCl pH 8.00 supplemented with the protease inhibitors benzamidine (10 μg/ml), leupeptine (5 μg/ml), and phenylmethylsulfonylfluorid (PMSF; 10 μg/ml)), and lysed by sonication (6 times 30 sec, duty cycle 50%, output control 5, on ice, using a Branson sonifier 250). The lysates were centrifuged at 27,000 x *g* for 30 min. The lysis, sonication, and centrifugation steps were repeated. The resulting pellet was suspended in 10 ml wash buffer (20 mM Tris-HCl pH 8.00, 1 M urea and protease inhibitors), incubated for 90 min on ice, and centrifuged again at 27,000 x *g* for 30 min. Solubilization of inclusion bodies (IB) was carried out in 10 ml of solubilization buffer (20 mM Tris-HCl pH 8.00, 6 M urea, 0.2 % SDS, 150 mM NaCl, protease inhibitors) for 3.5 hours on ice with slow stirring. The solubilized sample was centrifuged at 27,000 x *g* for 30 min and the supernatant collected. 5 mM imidazole was added to the supernatant, which was incubated overnight at 4°C with 6 ml of 50% Ni-NTA (Nickel-nitrilotriacetic acid) superflow slurry (Qiagen) equilibrated with solubilization buffer. The sample-resin suspension was allowed to settle down into a 1 cm diameter Econo-column (Bio-Rad). The resin was washed with 50 ml of solubilization buffer supplemented with 10 mM imidazole. The recombinant GPCR fusions were eluted with 12 ml of elution buffer (20 mM Tris-HCl pH 8.00, 6 M urea, 0.2 % SDS, 150 mM NaCl, protease inhibitors, and 100 mM imidazole). The eluted fractions were analyzed using 12% SDS-polyacrylamide gels and the GPCR fusions were visualized by Coomassie blue staining. Each eluted fraction (12 ml) was concentrated to 3 ml using Centriplus YM-50 (Amicon). In the case of α_5I-V2-GPCR fusions such as α_5I-V2-OTR, the lysis and washing steps were equivalent, but the solubilisation was carried out in a different buffer (5 ml Tris 20 mM pH 8.00, SDS 1%, NaCl 150 mM, and protease inhibitors) for 3.5 hours at 20°C. After centrifugation (27,000 x *g* for 30 min), the supernatant was collected and diluted 5-fold to obtain a final 0.2% SDS concentration (Tris 20 mM pH 8.00, SDS 0.2%, NaCl 150 mM, protease inhibitors supplemented with 5 mM imidazole). Incubation with 3 ml pre-equilibrated Ni-NTA resin was performed at 20°C for 16 hours. The IMAC procedure was equivalent to that described above for other fusions, but without urea. With the CB1 receptor, all the buffers contained 0.8% SDS and 10% glycerol to limit non-specific protein:protein interactions.

Thrombin cleavage of the GPCR fusions and purification of isolated GPCRs. 3 ml of each purified GPCR fusion were dialysed overnight at 4°C, except for the α_5I-V2-OTR fusion which was kept at 20°C, in 1 litre 20 mM Tris-HCl pH 8.00 using Slide-A-Lyser dialysis cassettes (Pierce) with a 10 KDa molecular weight cut-off (MWCO). After dialysis, the SDS concentration was decreased enough to be compatible with thrombin cleavage. Despite this low SDS concentration, the fusions were still soluble. The quantity of protein was determined by UV spectrophotometry using the Beer-Lambert law and extinction coefficients calculated using the method of Gill and Von Hippel (5). According to Nominé et al. (6), the solubility of the fusions and its correlated "aggregation rate" were measured by recording fluorescence emission spectra between 240 and 400 nm on a PTI spectrofluorimeter with an excitation wavelength at 280 nm (bandwidth 2 nm). The buffer contribution was subtracted under the same experimental conditions. Comparing the maximum intensity value of the scattered light (I_{280}) to that of the fluorescence emission (I_{330}) allowed us to calculate a I_{280}/I_{330} ratio defining the aggregation rate. This ratio, < 0.2, indicated that fusions were not aggregated and their cleavage was done at 20°C using restriction grade Thrombin and commercial cleavage buffer (20 mM Tris-HCl pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$) from Novagen. For each fusion, both an optimized enzyme:fusion ratio and an incubation time were determined. For instance, complete cleavage of the α_5-ChemR23 fusion was achieved using 13 U of thrombin per mg of fusion and the reaction was stopped 15 min later by adding 1 mM PMSF. At this point, two different procedures were developed. With the α_5I-GPCR fusions, the cleaved sample was equilibrated in denaturing buffer (20 mM Tris-HCl pH 8.00, 8 M urea, 0.2% SDS, 150 mM NaCl, protease inhibitors, 5 mM imidazole) and incubated at 20°C with 3 ml of 50% Ni-NTA superflow slurry for 16 hours. The sample-resin suspension was packed into an Econo-column, washed with 30 ml of the same buffer supplemented with 10 mM imidazole, and the purified GPCR eluted with 6 ml of elution buffer (20 mM Tris-HCl pH 8.00, 8 M urea, 0.2 % SDS, 150 mM NaCl, protease inhibitors, 100 mM imidazole). The purity of the different GPCRs was examined after migration onto 12% SDS-polyacrylamide gels and detection by Coomassie blue staining. In the case of α_5I-V2-OTR, the reaction was stopped by adding 1 mM PMSF and 0.2% SDS. The mixture was directly loaded onto a HighPrep Sephacryl S300 high-resolution column (16 X 60 mm, GE Healthcare Amersham Biosciences), equilibrated in thrombin cleavage buffer supplemented with SDS 0.2%. The flow rate was kept constant at 0.3 ml/min, and 2-ml fractions collected. Fractions known to contain proteins on the basis of UV detection at 280 nm were analysed onto 12% SDS-polyacrylamide gels and stained with Coomassie blue. The eluted fractions containing a mix of OTR, thrombin and the α_5I-V2 module were pooled and the OTR was purified by IMAC in the equilibration buffer containing 20 mM Tris pH 8.00, NaCl 150 mM, SDS 0.2%, 5 mM imidazole, and protease inhibitors. The protocol for incubating the sample with the Ni-NTA resin and eluting the pure OTR was equivalent to that described above for other GPCRs such as V2 or ChemR23.

1. Banères, J.-L., Roquet, F., Green, M., LeCalvez, H., and Parello, J. (1998) *J. Biol. Chem.* **273**, 24744-24753
2. Tan, C.A., Hehir, M.J., and Roberts, M.F. (1997) *Protein Expr. Purif.* **10**, 365-372
3. Tso, J.Y., Zalkin, H., Van Cleemput, M., Yanofsky, C., and Smith, J.M. (1982) *J. Biol. Chem.* **257**, 3525-3531
4. Mouillac, B., Sen, T., Durroux, T., Gaibelet, G., and Barberis, C. (2002) *Prog. Brain Res.* **139**, 163-177
5. Jäger, M., Nir, E., and Weiss, S. (2006) *Prot. Sci.* **15**, 640-646
6. Gill, S.C., and Von Hippel, P.H. (1989) *Anal. Biochem.* **182**, 319-326
7. Nominé, Y., Ristriani, T., Laurent, C., Lefèvre, J.-F., Weiss, E., and Travé, G. (2001) *Protein Eng.* **14**, 297-305