Mass Spectrometric Analysis of Agonist Effects on Posttranslational Modifications of the β-2 Adrenoceptor in Mammalian Cells†

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ABSTRACT: Posttranslational modifications (PTMs) of the β-2 adrenoceptor (B2AR) play a fundamental role in receptor regulation by agonists. We have examined the effects of several agonists on net levels of B2AR palmitoylation and phosphorylation using epitope tagging in stably transfected human embryonal kidney (HEK) 293 cells, immunoaffinity purification, and mass spectrometry combined with the method of stable isotope labeling by amino acids in cell culture (SILAC). Palmitoylation of Cys341 was confirmed and did not change detectably after 30 min exposure of cells to saturating concentrations of dopamine, epinephrine, or isoproterenol. However, all of these agonists produced a marked increase in net phosphorylation. Phosphorylation of the third cytoplasmic loop was increased to a similar degree by all three agonists, whereas differences between agonists were observed in net phosphorylation of the carboxyl-terminal cytoplasmic domain (isoproterenol $\sim$ epinephrine $\gg$ dopamine). Interestingly, agonist-induced phosphorylation of the carboxyl-terminal cytoplasmic domain was observed exclusively in a proximal portion (between residues 339−369). None of the agonists produced detectable phosphorylation in a distal portion of the cytoplasmic tail, which contains all sites of agonist-induced phosphorylation identified previously by in vitro reconstitution. These results provide insight to agonist-dependent regulation of the B2AR in intact cells, suggest the existence of significant differences in regulatory phosphorylation events occurring between in vitro and in vivo conditions, and outline a general analytical approach to investigate regulated PTM of receptors in mammalian cells.

Ligand-induced posttranslational modifications (PTMs)[1] of G protein-coupled receptors (GPCRs) play a critical role in controlling the functional activity of receptors. Important regulatory roles of PTMs have been amply demonstrated in studies of the light-activated GPCR rhodopsin as well as for a number of ligand-activated GPCRs (*1*−*6*). PTMs of ligand-activated GPCRs are of particular interest because there is evidence, from studies of a number of GPCRs, that distinct agonists can mediate different effects on receptor signaling or regulation (*7*−*14*).

PTMs of GPCRs such as the β-2 adrenoceptor (B2AR) have been investigated using both cell-free preparations and intact cells. In vitro reconstitution experiments are advantageous for mechanistic studies and typically yield sufficient quantities to facilitate direct biochemical analysis or "readout" of PTMs (*15*−*17*). Studies carried out in intact cells more closely reflect physiologically relevant conditions of receptor regulation, but biochemical analyses of receptors isolated from cultured mammalian cells have been limited by the small amount of purified receptor protein that is typically obtained, which compounds other difficulties inherent to analyzing highly hydrophobic proteins such as GPCRs (*18*). Therefore, many studies carried out in intact cells have relied primarily upon site-directed mutagenesis combined with metabolic labeling to infer potential sites of PTMs and to examine agonist effects on the formation and/or turnover of PTMs (*4*, *19*−*22*). Indeed, with the exception of rhodopsin, relatively few studies have utilized direct readout methods to define GPCR PTMs occurring in cells or tissues (*6*, *17*, *23*, *24*). Furthermore, to our knowledge no previous studies have applied such methods to compare the effects of distinct agonists on net PTM of GPCRs in intact cells.

Several methodological advances could facilitate such an analysis of GPCR PTMs. Epitope tagging and heterologous expression methods have improved the ability to purify GPCRs from cultured cells in amount and purity sufficient for chemical analysis (*25*). Significant improvements have been made in mass spectrometry (MS) and related procedures of protein analysis, to the degree that MS is now regarded as a major tool for analyzing PTMs (*26*, *27*). MS analysis of hydrophobic membrane proteins has been enhanced by the identification and utilization of "MALDI-MS friendly" detergents (*18*), and enrichment procedures such as im-

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[1] Abbreviations: B2AR, β-2 adrenergic receptor; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; FLAG, DYKDDDDK; IMAC, immobilized metal ion chromatography; PTM, posttranslational modification; SILAC, stable isotope labeling by amino acids in cell culture; TCEP, tris(carboxyethyl)phosphine; ISO, isoproterenol; Epi, epinephrine; Dopa, dopamine.

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mobilized metal affinity chromatography (IMAC) have been developed to increase MS detection of phosphorylated protein species when present in low amounts relative to unmodified species (*26*, *28*). Furthermore, isotope labeling methods have facilitated the detection and quantification of differences in specific PTMs between samples or under different conditions (*29*, *30*). In particular, deuterated leucine has been used successfully in the method called "stable isotope labeling by amino acids in cell culture" (SILAC) (*31*−*33*).

In the present study, we have applied these methods to investigate agonist effects on PTM of the β-2 adrenoceptor (B2AR) by palmitoylation and phosphorylation in stably transfected human embryonal kidney cells (HEK293), a model cell system in which these PTMs are known to be functionally significant. We have compared the effects of three agonists (dopamine, epinephrine, and isoproterenol) on net levels of these PTMs under conditions (saturating concentrations for 30 min) that approximate steady-state conditions of receptor activation (*34*). Our results indicate that, while distinct agonists have similar overall effects on B2AR PTMs, agonists differ in their ability to promote multiple phosphorylation of a proximal portion of the carboxyl-terminal cytoplasmic domain. Our results also suggest the existence of significant differences between the B2AR phosphorylation occurring in intact cells relative to in vitro and articulate an experimental approach that could have broad utility for the analysis of GPCR PTMs occurring under physiologically relevant conditions.

MATERIALS AND METHODS

Materials. Deuterated leucine (L-leucine-*5,5,5-d*$_3$) was from Isotec (a member of the Sigma-Aldrich family) (Miamisburg, OH). All other cell culture materials including amino acids, dialyzed serum, and the cell culture media [1:1 Dulbecco's modified Eagle's medium (DME) and Ham's F-12 nutrient mixture, deficient in L-leucine] were from Sigma Chemicals (St. Louis, MO). (−)-Isoproterenol and (−)-epinephrine were from Sigma Chemicals (St. Louis, MO). Dopamine hydrochloride was from Research Biochemicals International (Natick, MA). Sequencing grade endoproteases Glu-C (*Staphylococcus aureus* V8) and modified bovine trypsin were purchased from Roche Applied Science (Indianapolis, IN). [^{3}H]Dihydroalprenolol (85−90 Ci/mmol) was from Amersham (Arlington Heights, IL). *n*-Dodecyl β-D-maltoside (DDM) was from Calbiochem (San Diego, CA). The 4−20% polyacrylamide gels (in Tris−glycine buffer) were obtained from Invitrogen. Pro-Q Diamond phosphoprotein stain and SYPRO Ruby protein stain were from Molecular Probes (Eugene, OR). Handee minispin columns were from Pierce, and the microcon centrifugal filter devices and C18 zip tips were from Millipore (Bedford, MA). Reversed-phase packing, Oligo R3, and POROS 50 R2 were purchased from Applied Biosystems.

Cell Culture and Isotope Labeling. A previously described clone of stably transfected HEK293 cells expressing an amino-terminally FLAG-tagged human B2AR at moderate levels (~1 pmol/mg) (*35*) was propagated in a 1:1 mixture of Dulbecco's modified Eagle's medium (DME) and Ham's F-12 nutrient mixture, made specifically deficient in L-leucine, containing 10% dialyzed fetal bovine serum (*31*), penicillin (100 μg/mL), and streptomycin (100 units/mL). Media were made either "light" by adding regular L-leucine or "heavy" by adding deuterated L-leucine to a final concentration of 59 mg/L. Cells were maintained in the indicated culture medium for at least five cell doublings, and with frequent changes, and uniform isotopic labeling was confirmed by MALDI-TOF MS (not shown).

Agonist Stimulation and Cell Harvest. Cells were grown to ~70% confluence in 15 cm round cell culture dishes (Falcon) containing 20 mL of the appropriate (heavy or light) culture medium. Five dishes were used for each of the incubation conditions. Control dishes were incubated in the absence of any added agonist, while treated dishes were supplemented with the appropriate agonist (isoproterenol, 10 μM, epinephrine, 10 μM, or dopamine, 100 μM). Dishes were incubated for 10 min at 37 °C, media were removed and replaced by 10 mL of calcium-free PBS supplemented with 1 mM EDTA and the appropriate agonist (or no ligand for control dishes), and dishes were incubated for an additional 20 min at room temperature to dissociate cells from the dish. Cells were harvested by gentle aspiration, cell suspensions from the five replicate dishes were combined in a tared 50 mL conical tube (Falcon) and pelleted (500*g* for 10 min), and aspirated pellets were quickly frozen at −80 °C.

Receptor Purification. Cell pellets were solubilized in buffer A (10 mL/g wet mass) containing protease inhibitors (10 μg/mL leupeptin, 10 μg/mL benzamidine) and phosphatase inhibitors (50 mM β-glycerophosphate, 50 mM NaF, and 0.1 mM sodium orthovanadate) and 1 μM alprenolol to stabilize the solubilized B2AR as described previously for receptor purification from insect cells (*25*). Cell extracts were supplemented to 1 mM $CaCl_2$ and centrifuged at 18000 rpm (in a Sorvall centrifuge using an SS-34 rotor) for 20 min to clarify the extract. The supernatant was applied to an M1-FLAG affinity resin (Sigma) equilibrated in buffer B and cycled five times. The volume of resin used was 0.04 mL/mL of extract. The column was washed with 1 column volume of buffer C plus 1 mM $CaCl_2$ followed by 1 column volume of buffer B plus 1 mM $CaCl_2$, and this alternate washing was repeated five times. The column was then washed with an additional two column volumes of buffer B plus 1 mM $CaCl_2$ prior to elution with buffer B supplemented to 200 μM FLAG peptide and 1 mM EDTA. The buffers used are as follows: buffer A, 100 mM NaCl, 20 mM Tris, pH 7.5, 1% DDM; buffer B, 100 mM NaCl, 20 mM Tris, pH 7.5, 0.1% DDM; buffer C, 500 mM NaCl, 20 mM Tris, pH 7.5, 0.1% DDM. The eluted receptor fraction was immediately frozen on dry ice and kept frozen prior to subsequent biochemical analysis.

Radioligand Binding Assay. Radioligand binding assays of the purified, solubilized B2AR were carried out using 5 nM [^{3}H]dihydroalprenolol and rapid gel filtration on G50 minicolumns to separate bound from free radioligand, as described previously (*25*). Nonspecific binding was defined in the presence of 1 μM alprenolol and was typically <5% of total binding.

Sample Preparation and Receptor Electrophoresis. Purified B2AR was combined with SDS−PAGE sample buffer containing 2% 2-mercaptoethanol (room temperature, 20 min) and electrophoresed through 4−20% polyacrylamide gels. ProQ and SYPRO staining reagents were applied in

sequence, per the manufacturer's instructions, and imaged using a Typhoon 9400 (Amersham Biosciences) with excitation at 555 and 610 nm, respectively, to detect the ProQ and SYPRO signals. Images were processed and staining intensities quantitifed using ImageQuant software (Amersham Biosciences).

Sample Preparation for Mass Spectrometric Analyses of Proteolytic Digests. (a) To separate FLAG peptide (DYKDDDDK) from the B2AR, gel filtration was performed using Handee minispin columns filled with Sephadex G-50 fine resin. Purified receptor (~10 pmol) was loaded and eluted from the resin using a Tris buffer (20 mM, pH 7.5) containing NaCl (100 mM) and 0.1% DDM. (b) A modified procedure of Trester-Zedlitz et al. (*36*) was used for the reduction, alkylation (typically with iodoacetamide), and in solution proteolytic digests (with Glu-C or trypsin) of the B2AR. Some samples were depalmitoylated on the microconcentrator membrane prior to enzymatic digestion by adding 1 M hydroxylamine (pH to ~8 with NaOH) and leaving samples in the dark for 60 min at room temperature. Reduction of disulfides with tris(carboxyethyl)phosphine (TCEP) was carried out as described (*36*) except that reactions were carried out for 1 h at room temperature (instead of 35 °C) to minimize receptor aggregation. IMAC procedures for phosphopeptide enrichment were carried out using the phosphopeptide kit (Pierce Biotechnology). Phosphopeptides were subsequently purified using a step-elution reversed-phase procedure [Oligo R3:POROS R2 (1:2) loaded on top of zip tips] (*37*).

Modified Sample Preparation for Mass Spectrometric Analysis of Receptor Palmitoylation. The above procedure (with TCEP reduction and iodoacetamide alkylation) was used to identify the palmitoylated tryptic peptides. For SILAC analysis, the procedure was modified as follows: After reduction with TCEP (1 μmol, pH 7, room temperature, 60 min), exposed cysteines were alkylated with *N*-methylmaleimide (2 μmol, pH 7, room temperature, 60 min). After several washes, the palmitoyl group was released by cleavage with hydroxylamine (38 μmol, pH 8, room temperature, dark, 60 min), and then exposed thiols were alkylated with *N*-ethylmaleimide (2 μmol, pH 7, room temperature, 60 min). Mass signals of peptides modified with *N*-ethylmaleimide (representing the depalmitoylated thiols) were used in the SILAC analysis.

Mass Spectrometry. MALDI-MS was performed on a MALDI time-of-flight mass spectrometer Voyager-DE STR (PE Biosystems, Foster City, CA) equipped with a nitrogen laser delivering pulses of ultraviolet light (wavelength 337 nm) at 3 Hz to the matrix spot. Acquisitions by the MALDI-TOF MS were made in both linear and reflectron, delayed extraction modes. MALDI-TOF data were smoothed, calibrated, and analyzed using Data Explorer (PE Biosystems). Peptides were identified with assistance from the web-based tool, MS-Digest (prospector.ucsf.edu). Relative intensity values, for use in quantitative analysis, were derived only from spectra collected using linear, delayed extraction modes. All spectra for preparation of figures used the program M-over-Z (Genomic Solutions, Ann Arbor, MI). The MALDI matrix used was 4-hydroxy-α-cyanocinnamic acid (4HCCA) mixed with a mixture of trifluoroacetic acid (0.1%), acetonitrile (33%) and water (67%).

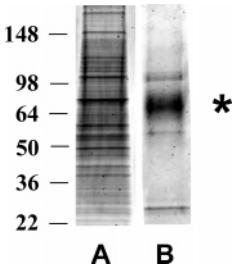
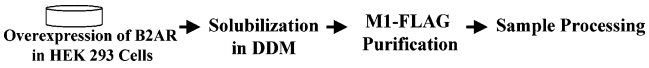


FIGURE 1: Immunoaffinity purification of FLAG-B2AR: (A) SYPRO Ruby total protein stain of the cell extract and (B) SYPRO Ruby total protein stain of the purified B2AR fraction.

Scheme 1: Overview of Immunopurification of B2AR from Mammalian Cells



Calculation of Agonist Effects on PTMs. To examine agonist effects on B2AR PTMs, light and heavy B2AR preparations (representing different treatment conditions) were mixed in equal amount prior to enzymatic digests to ensure identical handling. MS signal intensities were measured according to peak height, and small variations in mixing were adjusted for by normalizing all MS signal intensities with an internal B2AR proteolytic peptide, which did not incur any modifications upon agonist treatment and consistently ionized well in the mass spectrometer. The MS signal intensity of the tryptic B2AR peptide, [240−253], or the Glu-C peptide, [238−249], fulfilled this role as the internal standard for quantitation experiments. For determining quantitative ratios when there was overlap between heavy and light isotope distributions, an isotopic correction factor was applied as described previously by Ong et al. (*31*). Mixing experiments conducted using heavy and light B2AR preparations from untreated cells (i.e., an identical condition) confirmed the accuracy of this method for quantitating relative amounts to a standard deviation of ~3% (e.g., see Figure 5B).

RESULTS

Expression and Isotope Labeling of the β-2 Adrenoceptor. As a first step toward analyzing PTMs, we developed a method to rapidly purify receptors from intact cells. The human B2AR retains its functional properties with an N-terminal FLAG epitope tag (*38*, *39*). This epitope greatly facilitates rapid immunopurification of receptors (*25*), suggesting a single step method for isolating receptors from cell extracts (Scheme 1). A previously characterized (*35*) clone of stably transfected human embryonal kidney (HEK293) cells, expressing the FLAG-B2AR at moderate levels (~1 pmol/mg), was used throughout the studies. Detergent extracts prepared from five 15 cm dishes yielded ~10 μg of purified FLAG-B2AR. SDS−PAGE analysis revealed a complex protein mixture in crude detergent extracts and a single major species (indicated by an asterisk) migrating ~70 kDa in the immunopurified fraction (lanes A and B of Figure 1, respectively). This species is consistent with the electrophoretic mobility with the mature receptor species identified previously in these cells by immunoblotting (*35*), and this identification was confirmed by peptide mapping using MALDI-TOF MS (data not shown). The specific activity of

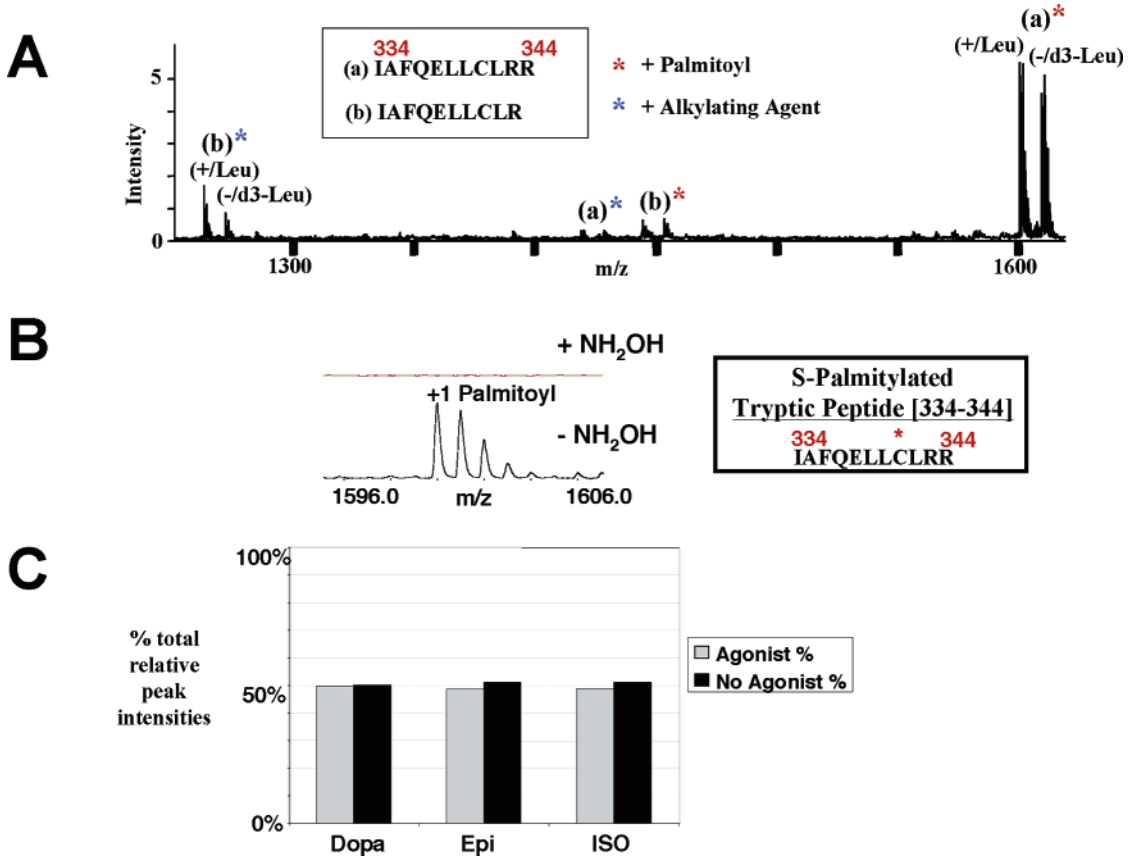


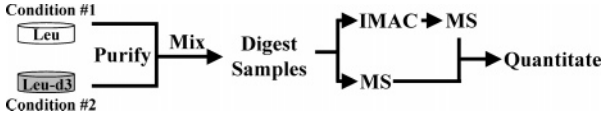
FIGURE 2: Characterization of B2AR palmitoylation. (A) MALDI-TOF MS spectrum of B2AR regions, [334−343] and [334−344], with addition of either one palmitoyl ([334−344] + 1 palmitoyl, calc *M* (mono) = 1598.995; obs *M* = 1598.981; and [334−343] + 1 palmitoyl, calc *M* (mono) = 1442.893; obs *M* = 1442.898) or one carboxyacetamide (CAM) moiety ([334−344] + 1 CAM, calc *M* (mono) = 1417.786; obs *M* = 1417.826; and [334−343] + 1 CAM, calc *M* (mono) = 1261.685; obs *M* = 1261.721). The sample includes an ~1:1 mix of the reduced and alkylated, ISO-treated B2AR (+/Leu) (labeled with the light amino acid, Leu) and ISO-untreated B2AR (−/d3-Leu) (labeled with the heavy amino acid, d3-Leu). (B) MALDI-TOF MS of region [334−344] + 1 palmitoyl moiety with and without NH_2OH treatment. (C) Quantitation of B2AR palmitoylation using alkylation strategy described in Materials and Methods. No change between the palmitoylation levels between 30 min of agonist treatments (gray bars) and no agonist treatments (black bars) was observed.

this fraction, estimated by the proportion of purified receptor protein specifically binding [^{3}H]DHA was calculated to be ~60%.

To investigate agonist-dependent regulation, it was necessary to quantitatively compare MS analyses of B2ARs isolated from cells incubated in the absence or presence of various agonists. Quantitative comparisons using MS typically require the use of internal peptide standards or isotope labeling. We found the SILAC approach (*31*) to be useful for isotope labeling of our HEK293 cell line. We selected to label with light and heavy (deuterated) Leu residues, because there were more Leu residues evenly distributed in the intracellular regions of interest than other candidate residues (e.g., Lys or Arg) (*31*, *40*). As described by Ong et al. (*31*), the cells were allowed to double at least five times prior to agonist stimulation and harvesting to allow for complete deuterated Leu incorporation (data not shown).

As shown in Scheme 2, two cell populations were grown in medium containing either the light or heavy Leu, each representing a different condition. This allowed different agonist conditions to be quantitatively compared within a single MS analysis of a single sample spot. These advantages and details of the SILAC approach have been extensively reviewed (*32*, *33*). We closely followed the methods of Ong et al. (*31*) with the minor difference that isotope-labeled receptor preparations were mixed after purification, so that

Scheme 2: Overview of Sample Processing for Comparing Agonist-Induced Posttranslational Modifications of the B2AR with Mass Spectrometry



we could analyze multiple treatment conditions in a pairwise manner at a later time.

Detection of B2AR Palmitoylation. Palmitoylated receptor fragments were inefficiently extracted following routine in-gel trypsin digest procedures. Therefore, we utilized an in-solution digestion method as described previously by Trester-Zedlitz et al. (*36*). Similarly, we used microconcentrators as a porous surface to facilitate easy exchange of experimental conditions (chemistries for reduction, alkylation, and, when necessary, depalmitoylation) prior to enzymatic digestion. Unfortunately, the microconcentrators were ineffective for size exclusion of the FLAG peptide and required that we use size exclusion chromatography prior to sample processing. Without this step, the FLAG peptide, DYKDDDDK, became a competitive substrate for enzymatic digests (data not shown). MALDI-TOF MS (Figure 2A) identified the mass signals of the tryptic peptides, IAFQELLCLR and IAFQELLCLRR, representing respectively regions [334−343] and [334−344], with either one addition of palmitate

or one addition of carboxyacetamide. The major mass signal was of [334−344] plus one addition of palmitate. Small signals of the palmitoylated region, [334−343], in addition to regions [334−343] and [334−344] modified by one carboxyacetamide were also observed. The relative signal intensities of the peaks representing alkylated and palmitoylated fragment [334−343] differed from the relative intensities for [334−344]. This difference suggests that trypsin preferred to cleave after Arg344 (instead of Arg343) due to the steric bulk of the large palmitoyl group. The palmitoylated peptide signal disappeared upon hydroxylamine treatment (Figure 2B), consistent with hydrolysis of a palmitoyl−Cys thioester bond. As there is only one Cys in this peptide region, this observation confirmed that Cys341 is modified by the palmitate moiety.

By using SILAC, it was possible to compare steady-state levels of B2AR palmitoylation under different conditions. For this purpose we applied a different alkylating strategy, using both *N*-methylmaleimide and *N*-ethylmaleimide, as described in Materials and Methods. To correct for any error in preparing 1:1 mixes of receptor preparations, MS signal intensities were normalized with an internal B2AR proteolytic peptide that did not incur any modifications upon agonist treatment and consistently ionized well in the mass spectrometer. The mass signal of the tryptic B2AR peptide, [240−253], fulfilled this role as the internal standard for palmitoylation quantitation. When normalized peak intensities were compared, none of the agonists produced a detectable difference in palmitoyl occupancy of the B2AR (Figure 2C). Because we could not exclude the possibility that the reducing conditions used might produce some depalmitoylation of receptors after purification, we did not attempt to estimate "absolute" ratios of palmitoylated: unpalmitoylated receptors in the samples.

Detection of B2AR Phosphorylation. We began detection of B2AR phosphorylation by using standard SDS−PAGE procedures followed by two commercial fluorescent staining methods. Pro-Q Diamond phosphoprotein dye technology (ProQ) was used for the detection of phospho-containing proteins (*41*, *42*), followed by the SYPRO Ruby total protein dye (panels A and B of Figure 3, respectively). Fluorescent images of each stain were individually analyzed, and normalized intensity values derived from a representative experiment are displayed in Figure 3C. This analysis suggested quantitative differences in the relative degree to which distinct agonists promoted net phosphorylation of the B2AR (ISO ∼ Epi > Dopa). Using ovalbumin as a reference for comparing ProQ/SYPRO intensity ratios (ovalbumin contains ∼2 phosphate residues per protein) (*41*), we estimated that B2AR phosphorylation stoichiometries ranged from ∼1 mol/mol of phosphate (no agonist) to ∼4 mol/mol of phosphate (ISO) (Figure 3C, numbers over bars).

MS analysis was then employed to define regions of the B2AR containing phosphorylated residues. While the detection of phosphorylated peptides using MS can be challenging (*26*), our access to substantial amounts of purified receptor enabled us to overcome some of the hurdles through application of multiple enzymatic strategies, in addition to phosphopeptide enrichment using IMAC (*28*).

As mentioned with the palmitoylation study, in-solution digests of the B2AR were performed on microconcentrators for easy exchange of experimental conditions. The B2AR

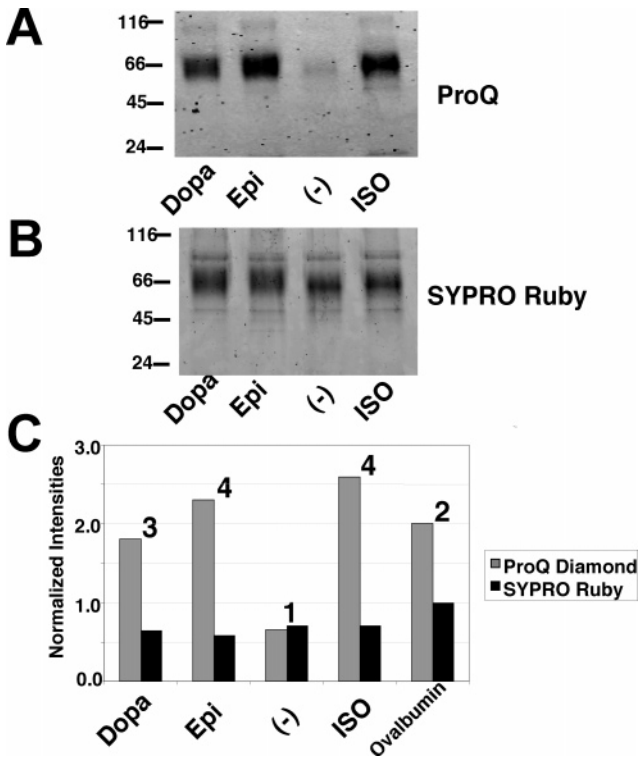


FIGURE 3: Detection of B2AR phosphorylation by in-gel fluorescence assay. SDS−PAGE (Tris−glycine, 4−20%) of the M1-FLAG purified B2AR samples treated with dopamine (Dopa), epinephrine (Epi), no agonist (−), and isoproterenol (ISO). The gel was stained with both (A) Pro-Q phosphoprotein stain (ProQ) and (B) SYPRO Ruby total protein stain. (C) No agonist (−) < Dopa < Epi ∼ ISO = the relative phosphorylation stoichiometries of B2AR under different agonist treatments. The ratios of ProQ/SYPRO fluorescent stain intensities were normalized to ovalbumin intensities (∼2 phosphorylated residues per protein).

(∼500 ng or 10 pmol) was digested using two different endoproteinases (Glu-C or trypsin) to maximize coverage of the regions of the B2AR which are thought to contain major sites of regulatory phosphorylation (Figure 4). In-solution digests were critical for proteolysis by Glu-C because the enzyme behaves poorly in gel experiments. It is noteworthy that only one putative phospho residue (Thr274) in the intracellular B2AR regions of interest was not accounted for in these digests by MS. All relevant peptide assignments are reported in Table 1.

Similar to normalization used in quantifying receptor palmitoylation, B2AR MS signals were referenced to either the tryptic peptide, [240−253], or the Glu-C peptide, [238−249]. Shown in Figure 5A, this B2AR region did not incur any modifications upon agonist treatment and consistently ionized well in the mass spectrometer. Normalized peak intensity ratios correlated as expected (over a 9-fold range) upon mixing different ratios of the light and heavy amino acid-labeled B2AR, and this analysis was reproducible within a standard deviation of ∼3% (Figure 5B).

Results from a representative analysis of the B2AR region [250−268], containing the consensus PKA sequence in the third cytoplasmic loop, are displayed in Figure 5C. B2AR isolated from isoproterenol-treated cells [(+i), labeled in light medium] exhibited a pronounced increase in a singly phosphorylated form of this peptide (1 Pi), relative to B2AR isolated from untreated cells [(−), labeled in heavy medium], with a corresponding decrease in the unphosphorylated form

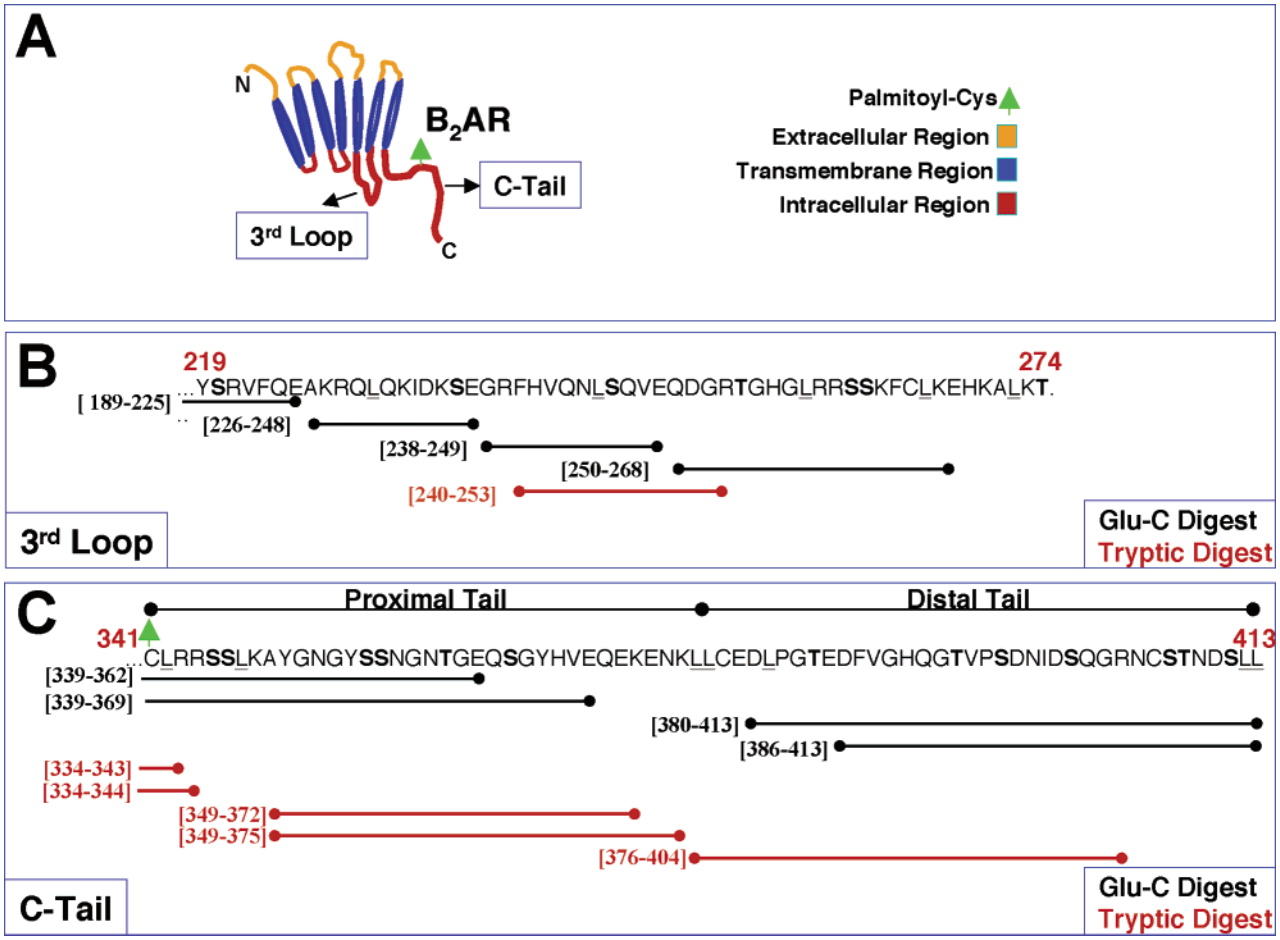


FIGURE 4: MALDI-TOF MS coverage of B2AR enzymatic digests. (A) Schematic of B2AR. The B2AR extracellular regions are shown in orange, transmembrane regions are shown in blue, and the intracellular regions are shown in red. Overview of the MS coverage of the B2AR intracellular (B) third loop region and the (C) C-terminal tail region, for both the Glu-C and tryptic digests.

Table 1: B2AR Peptide Mass Identification[a]

B2AR peptide	enzyme	*M* (calc)	*M* (obs)	no. of Leu	Δ (calc)	Δ (obs)
[250−268]	Glu-C	2231.13	2231.09	2	6	6
[339−362]	Glu-C	2616.27	2616.22	4	12	12
[339−369]	Glu-C	3416.62	3416.53	4	12	12
[386-413]	Glu-C	3032.35	3432.33	2	6	6
[386-413]	Glu-C	3644.63	3644.64	3	9	9
[348−372]	trypsin	2575.09	2574.99	0	0	0
[348−375]	trypsin	2946.27	2946.15	0	0	0
[376−404]	trypsin	3209.46	3209.46	3	9	9

[a] Unphosphorylated forms of relevant B2AR peptides were identified by MALDI-MS in the reflectron mode. Peptide assignments correspond to those listed in the figures and text. *M* (calc) is the calculated mass of the relevant peptides, and *M* (obs) is the observed mass. The number of Leu residues in each peptide is listed, and the corresponding calculated and observed isotope shifts are listed. Glu-C peptides were alkylated with iodoacetamide, and tryptic peptides were alkylated with *N*-methylmaleimide.

(0 Pi). Quantification of these effects, calculated from normalized peak intensities (as described in Materials and Methods), is shown in Figure 5D. These results were highly reproducible, and control experiments indicated that cell labeling with heavy or light medium had no effect on receptor phosphorylation detected by MS (not shown). We then carried out a similar analysis of the [250−268] peptide derived from receptors isolated following exposure of cells to Epi or Dopa. In each case, the SILAC method was used to provide internal standards for agonist effect references to receptors isolated from untreated cells. All of the agonists tested produced similar depletions of the 0 Pi species (Figure 6A), together with a corresponding increase in the 1 Pi form (Figure 6B).

We then used the same approach to examine agonist effects on phosphorylation of peptides representing the carboxyl-terminal cytoplasmic domain of the B2AR, including proximal ([339−362] and [339−369]) and distal ([386-413], [380−413], and [376−404]) C-tail peptides (Figure 4). Regardless of agonist type, minimal changes to any of the 0 Pi species representing unphosphorylated distal C-tail regions were observed, while all of the agonists produced a pronounced depletion of both peptides representing the unphosphorylated proximal C-tail (Figure 6C). This suggests that regulated phosphorylation of the cytoplasmic tail, in response to all three agonists, occurred primarily in the proximal portion. Consistent with this, 1 Pi forms of both proximal tail peptides were detected, while no signals were observed from 1 Pi forms of any of the distal tail peptides (Figure 6D). It was interesting that 1 Pi forms of the proximal tail peptides were observed even in untreated cells. Furthermore, we noted that none of the agonists tested increased the 1 Pi species, despite pronounced depletion of the 0 Pi species. Together, these observations suggest that the proximal cytoplasmic tail is singly phosphorylated in the absence of agonist and agonist ligands promote multiple phosphorylation of this region.

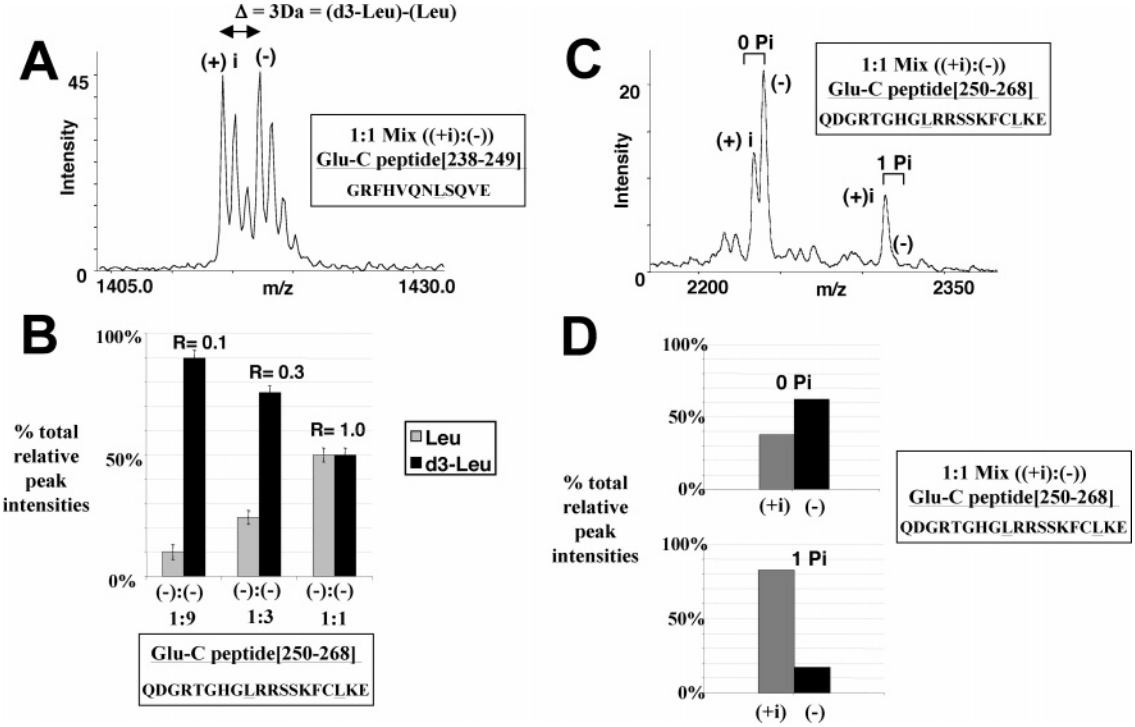


FIGURE 5: Analysis of B2AR phosphorylation in region [250−268] using SILAC and MALDI-TOF MS. (A) MALDI-TOF MS of the isotope pair of the B2AR reference peptide, [238−249], after a 1:1 mix of the B2AR isolated from cells treated with ISO (+i) containing the light amino acid (Leu) and the B2AR isolated from untreated cells (−) containing the heavy amino acid (d3-Leu). (B) B2AR isolated from untreated cells (−) containing the light amino acid (Leu) was mixed 1:9, 1:3, and 1:1 with B2AR isolated from untreated cells (−) containing the heavy amino acid (d3-Leu). R indicates the normalized mass signal intensities of the ratio Leu/d3-Leu. Error bars indicate the standard deviation of the measurements (∼3%). (C) MALDI-TOF MS of the Glu-C digest B2AR peptide, [250−268], after a 1:1 mix of the (+i),Leu)/(−),d3-Leu. (D) Percent total relative peak intensities and corresponding relative (+i),Leu/(−),d3-Leu ratios for the peptide, [250−268], were calculated and plotted from the MALDI-TOF MS displayed in (C).

Agonist Effects on Multiple Phosphorylation of the Proximal Carboxyl-Terminal Cytoplasmic Domain. We were unable to detect higher order phosphorylated forms of the proximal C-tail peptides directly but were able to do so readily following IMAC enrichment (Figure 7A). While these "non-SILAC" analyses of tryptic peptides [349−372] and [349−375] were not sufficient for quantitative assessment, they clearly revealed the following effects: (1) the 1 Pi form of the proximal C-tail region was already present without agonist, (2) agonist stimulation changed the 1 Pi minimally relative to the unstimulated receptor, (3) the 2 Pi form increased upon agonist stimulation, and (4) ISO and Epi were more effective than Dopa in producing higher order (2 Pi and 3 Pi) forms of the proximal carboxyl-terminal cytoplasmic domain.

Glu-C digests bracketed similar B2AR regions of the proximal tail to the trypsin digests and included four Leu residues that enabled SILAC analysis (Figure 7B). This analysis was repeated with the three agonist treatments and confirmed that 1 Pi species were present in similar amounts regardless of agonist type. Higher order forms (2 Pi and 3 Pi) were only apparent upon agonist treatment and differed depending upon agonist type (Figure 7). The IMAC enrichment procedure prevented us from carrying out the previous normalization to an unmodified "reference" peptide because unphosphorylated species were removed to a large (and somewhat variable) extent by this procedure. However, as both Figure 7B and the "non-IMAC" analysis (Figure 6D) indicated that agonists did not greatly change total amounts of 1 Pi peptides, we used the 1 Pi form of the proximal tail peptides as an internal standard in the IMAC analysis. Thus, to make comparisons of agonist-treated with untreated B2AR phosphorylations, we calculated peak intensity ratios of the higher phosphopeptide signals derived from agonist-treated cells with reference to the 1 Pi species from the untreated condition (indicated by 1 Pi* in Figure 7B). Analysis of [339−362] in this manner confirmed quantitatively that ISO and Epi produced 2 Pi and 3 Pi forms of the proximal B2AR tail to a much greater degree than Dopa (Figure 7C, left set of bars). No detectable 3 Pi form and relatively little of the 2 Pi form were observed in B2AR purified from Dopa-treated cells, whereas both higher phosphorylated forms were readily detected in B2AR purified from ISO and Epi-treated cells. Analysis of [339−369], resulting from partial enzymatic digest, further confirmed this agonist difference (Figure 7C, right set of bars). Additionally, while dopamine effects were equivalent on [339−362] and [339−369], enhanced 2 Pi and 3 Pi levels produced by Epi and ISO were even more pronounced in the [339−369] peptide. Together, these results demonstrated the following relative order of agonists for producing higher order phosphorylated forms of the proximal carboxyl-terminal cytoplasmic domain: ISO ∼ Epi ≫ Dopa. They also suggest that residues [363−369] may contain additional site(s) whose phosphorylation is variable with respect to agonist type.

DISCUSSION

In the present study, we have applied biochemical purification and MS analytical methods to investigate B2AR palmitoylation and phosphorylation in intact mammalian

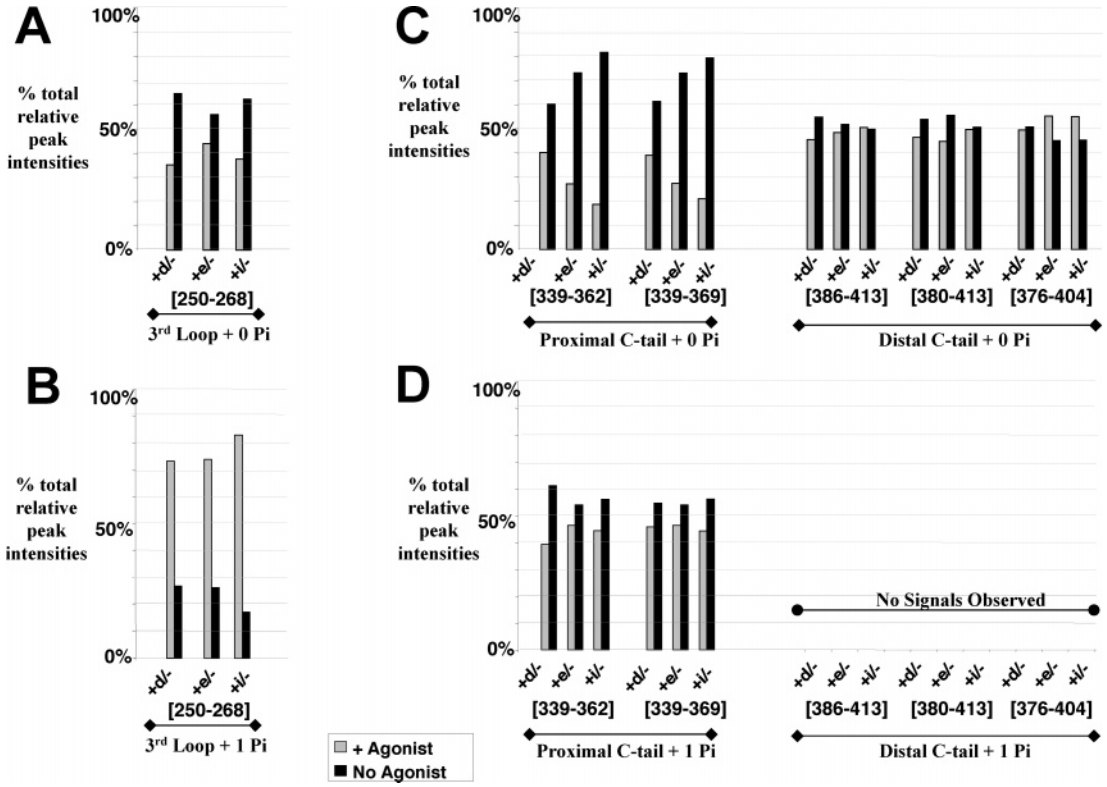


FIGURE 6: Summary of B2AR phosphorylation analysis conducted using SILAC and MALDI-TOF MS. After mixing a 1:1 ratio of the agonist [Dopa (+d), Epi (+e), or ISO (+i)] treated B2AR samples with untreated B2AR (−), the percent total relative peak intensities were calculated and plotted from the MALDI-TOF spectra for the intracellular B2AR peptides. (A) 0 Pi states of B2AR region, [250−268]; (B) 1 Pi states of B2AR region, [250−268]; (C) 0 Pi states of B2AR C-terminal tail regions; and (D) 1 Pi states of B2AR C-terminal tail regions.

cells. Our approach took advantage of recent advances in GPCR purification and MS analysis to analyze these PTMs in the absence or presence of several agonist ligands. We focused on effects of saturating concentrations of agonists when applied for a sufficient period of time (~30 min) to approximate steady-state conditions of receptor activation. Our analysis covered essentially the entire third cytoplasmic loop and carboxyl-terminal cytoplasmic domain of the receptor, providing a relatively comprehensive view of agonist effects on net incorporation of specific PTMs throughout these domains.

Palmitoylation of the B2AR at Cys341 was confirmed unambiguously by MS analysis and sensitivity to hydroxylamine. Protein palmitoylation is a reversible PTM, and metabolic labeling experiments indicate that B2AR palmitoylation is regulated by agonists in several cell types (*43*, *44*). The present observation that several agonists produced no detectable change in net palmitoylation was initially surprising. However, based on previous evidence that agonists regulate both palmitoylation and depalmitoylation of the B2AR (*45*, *46*), our data support the hypothesis that these opposing processes can be closely balanced under steady-state conditions. While this idea has been suggested previously for the B2AR (*45*), and shown for the α subunit of G_s heterotrimeric G protein by direct determination of net palmitate incorporation (*47*), to our knowledge a direct analysis of net B2AR palmitoylation has not been accomplished previously in intact cells. We note that palmitoylation of another ligand-activated GPCR, the B2 bradykinin receptor, was shown previously by MS analysis from intact cells (*6*). In this study it was not possible to examine ligand effects on receptor PTMs because receptors were purified by affinity chromatography using an immobilized agonist peptide. The immunopurification method used in the present study is independent of ligand binding to the receptor, thereby facilitating the study of ligand effects on receptor PTMs.

In contrast to the lack of agonist effects on B2AR palmitoylation, all agonists tested produced a marked increase in net phosphorylation of B2AR under the same conditions. This was evident both by fluorescence (ProQ) detection of phosphate in the intact B2AR and by MS analysis of multiple B2AR proteolytic fragments. Our experiments estimated a B2AR phosphorylation stoichiometry up to ~4 mol/mol of phosphate in the presence of isoproterenol or epinephrine. This stoichiometry is comparable to incorporations (~6 mol/mol) estimated previously in intact cells by metabolic labeling at a similar time point (*2*). Furthermore, our estimates of net phosphate incorporation and location were highly reproducible from experiment to experiment. Thus, we believe that the present results provide reliable information on the major sites/regions of B2AR phosphorylation occurring in an intact cell model.

Our analysis also detected some phosphorylation of the B2AR in the absence of agonist. This was identified both by fluorescence assay of the intact B2AR and by MS analysis revealing single phosphorylation in peptides representing the proximal cytoplasmic tail. When examining B2AR phosphorylation in the presence of agonist, a first observation from MS analysis was the overall similarity in the effects of distinct agonists. All of the agonists tested produced comparable increases in phosphorylation of the third cytoplasmic

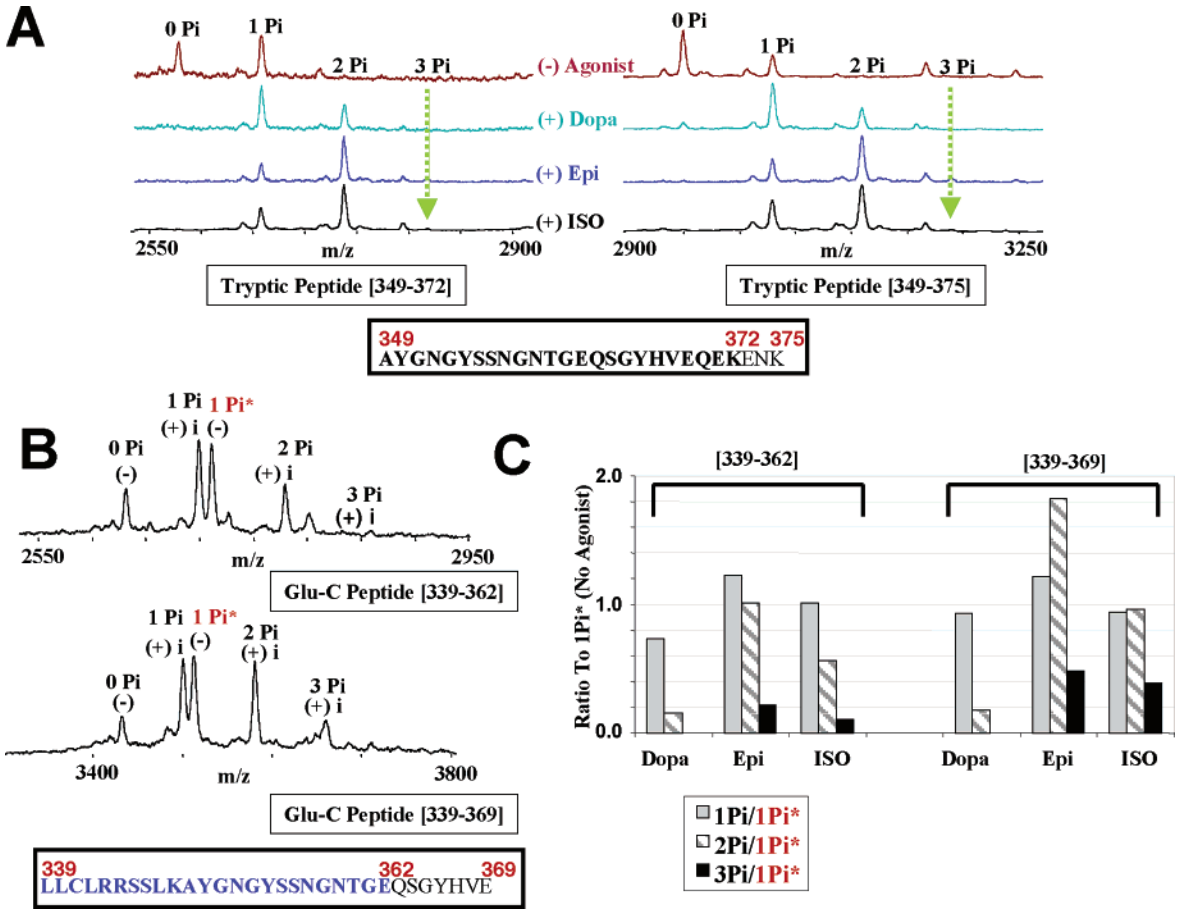


FIGURE 7: Detection and analysis of higher order B2AR phosphopeptides using IMAC enrichment. (A) MALDI-TOF spectra of IMAC-purified peptides from the tryptic digests of untreated B2AR (top), Dopa-treated B2AR, Epi-treated B2AR, and ISO-treated B2AR (bottom). (B) MALDI-TOF spectrum of IMAC-purified peptides from the Glu-C digest after mixing the ISO-treated B2AR containing the light amino acid (Leu) and the untreated B2AR containing the heavy amino acid (d3-Leu) with a ratio of 1:1. (C) Relative quantitation of phosphopeptides of B2AR regions, [339−362] and [339−369], under different agonist conditions versus the 1 Pi form detected in untreated cells.

loop, consistent with PKA-mediated phosphorylation inferred from assays using a phospho-selective antibody (*48*). All of the agonists also produced phosphorylation in the carboxyl-terminal cytoplasmic domain. Nevertheless, some differences between agonists were noted. First, direct MS analysis revealed that distinct agonists differ quantitatively in the degree to which they promote phosphorylation of the B2AR tail. Second, IMAC enrichment identified multiple phosphorylated forms of these peptides in agonist-treated cells and revealed significant differences between agonists in the mixture of phosphorylated forms present. The first observation is consistent with the idea that agonists differ only in the degree to which they promote a single phosphorylated state of the B2AR (*15*, *48*). However, the second observation is not easily reconciled with this hypothesis and suggests that agonists may differ in their ability to promote the formation of distinct phosphorylated state(s). It is conceivable that this phenomenon is a direct consequence of agonist differences in B2AR conformation, consistent with recent biophysical studies (*9*). However, we cannot exclude other possibilities because net B2AR phosphorylation in intact cells is determined by multiple factors, including distinct phosphorylation and dephosphorylation reactions occurring during the process of agonist-induced desensitization and resensitization (*49*). We also note that a previous study, conducted using purified components in vitro, did not reveal agonist-specific differences in the location of B2AR phosphorylation sites (*15*). Thus further analysis, probably using both in vitro and intact cell approaches, will be required to investigate the mechanism of the agonist differences in B2AR phosphorylation identified in the present study.

Another interesting observation from the MS analysis is the location of agonist-induced phosphorylations in the carboxyl-terminal cytoplasmic domain of the B2AR. Previous studies using purified B2AR and in vitro reconstitution defined major sites of phosphorylation, both by GRK2 and by GRK5, in a distal portion of the carboxyl-terminal cytoplasmic domain (*16*). We analyzed three overlapping peptide fragments covering this region and were unable to detect significant agonist-induced phosphorylation in any of them (neither a quantitative reduction in the observed unphosphorylated peptides nor a formation of their phosphorylated forms). In contrast, all of the agonists produced clear phosphorylation of peptides covering a proximal portion of the B2AR tail, which contains none of the phosphorylation sites identified as GRK2 sites in vitro (*16*). Nevertheless, GRK2 is thought to be the major kinase mediating non-PKA phosphorylation of the B2AR in HEK293 cells (*50*). Previous mutational studies indicate that agonist-induced desensitization of the B2AR requires Ser/Thr residues located in a proximal (*51*) but not distal (*52*) portion of the carboxyl-terminal cytoplasmic domain, and a recent immunochemical

study detected agonist-induced phosphorylation of the human B2AR on Ser355 and/or Ser356 (located in the proximal tail) in intact cells (*48*). Our results are fully consistent with these previous findings, and they extend these results by demonstrating that agonist-induced phosphorylation in the proximal tail occurs without any detectable phosphorylation in the distal tail domain. A limitation to this conclusion is that we examined B2AR phosphorylation at a single time point (30 min) after agonist addition. Thus we cannot presently exclude the possibility that additional, transient phosphorylation(s) may occur at earlier times after receptor activation. While further studies will be required to address this question and reconcile in vitro and in vivo phosphorylation data mechanistically, the present observations emphasize the importance of investigating agonist effects on phosphorylation of the wild-type B2AR in intact cells.

Beyond the specific relevance of the present study to B2AR cell biology, the experimental approach described herein could have broad utility for the study of other GPCRs. The present immunochemical purification combined with MS analysis could, in principle, be applied to any GPCR. The inherent versatility of the SILAC method, including the ability to use other amino acids (in addition to leucine) for isotopic labeling (*30*, *31*, *40*, *53*), should facilitate quantitative studies of GPCRs containing a wide range of amino acid compositions. MALDI-TOF MS offers a robust and relatively affordable methodology, which can provide an initial screen for locating specific PTMs in GPCRs and for examining ligand effects on net PTM incorporation. The ability of this approach to provide a comprehensive view of net PTM has inherent value for examining overall regulatory effects of ligands, as in the present study, and can serve as a first step toward later definition of individual sites of modification by other methods (such as tandem MS or chemical microsequencing). Thus, we suggest these methods as a general analytical approach to the study of regulated PTMs of GPCRs in intact cells.

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