Characterization of G Protein and Phospholipase C-Coupled Agonist Binding to the Y₁ Neuropeptide Y Receptor in Rat Brain: Sensitivity to G Protein Activators and Inhibitors and to Inhibitors of Phospholipase C¹,²

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Accepted for publication March 26, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

Binding of a Y₁-subtype-selective agonist of neuropeptide Y (NPY) receptor, (Leu³¹,Pro³⁴)human peptide YY (LP-PYY), to particulates from four rat brain areas (parietal cortex area 1, piriform cortex, anterior hypothalamus and hippocampus) showed a distinct response to LP-PYY and PY, a uniformly low sensitivity to ligands selective for the Y₂, Y₄ and Y₅ NPY receptor subtypes and high sensitivity to a Y₁ site-selective antagonist, BIBP-3226. The Y₁ binding was sensitive to guanine nucleotide-binding protein (G protein) agonist and antagonist nucleotides, with the rank order of guanosine 5'-O-(thiotriphosphate) (GTPγS) > GTP > GDP > guanosine 5'-O-(thiodiphosphate). However, guanine nucleotides did not affect about one third of the specific Y₁ binding. Most of Y₁ binding could be inhibited by a G protein nucleotide site/docking site receptor mimic, mastoparan analog MAS-7. In all areas examined, the Y₁ binding of LP-PYY was little affected by up to 100 μM of the antagonists of K⁺, Na⁺ and Ca²⁺ channels, protein kinase C, phospholipase A₂, phospholipase D and phosphati- dylinositol 3-kinase, phospholipase substrate phospholipids, steroids or detergents. However, the binding was potent- ly inhibited by phospholipase C inhibitors (especially the aminosteroid U-73122), which also dissociated the bound Y₁ ligand in steady-state conditions. U-73122 also displaced the Y₁ binding insensitive to GTPγS. Ligand association with the brain Y₁ NPY receptor thus strongly depends on activity of both G proteins and phospholipase C, implying specific interactions of these transducers/effectors with the receptor molecule in ligand binding. A portion of brain Y₁ sites could be directly coupled to phospholipase(s) C.

NPY, a 36-residue peptide abundant especially in the forebrain, is known to participate especially in regulation of vascular tone (Malmstrom, 1997), of feeding (e.g., Stanley et al., 1992) and of neuropeptide and anterior pituitary hormone secretion (Kalra and Crowley, 1992). At least six subtypes of NPY receptor have been described to date, showing a large degree of sequence variation (Larhammar, 1997). All of these peptides possess the seven hydrophobic folds characteristic of the rhodopsin family of G protein-linked receptors.

Among the known mammalian NPY receptors, the Y₁ species (including possible splicing variants—see Larhammar, 1997) apparently represents a large fraction of physiologically expressed NPY binding molecules, especially in the neural matrix. Activation of this receptor is known to stimulate the in vivo release of oxytocin (Parker and Crowley, 1993), and the release of LHRH from hypothalamic tissue (Kalra et al., 1992). Stimulation of the peripheral vascular Y₁ complement is vasotonic (Malmstrom, 1997) and diuretic (Bischoff et al., 1997).

Most of these activities could be coordinated through a multiple regulation of the Y₁ agonist signal by ions, transducers and effectors, which appears to have evolved both at the level of the Y₁ receptor structure and in the features of the agonistic peptides. Thus, agonist binding requires interaction with several extracellular and transmembrane domains of the Y₁ receptor molecule (for a review, see Du et al., 1997), resulting in a high sensitivity of the attachment to chaotropic influences, including those related to the ionic environment (Parker et al., 1996a). This is distinct from the

ABBREVIATIONS: NPY, neuropeptide Y; PYY, peptide YY; LP-PYY, (Leu³¹,Pro³⁴)human peptide YY; PYY(3-36), human peptide YY(3-36); rPP, rat pancreatic polypeptide; hPP, human pancreatic polypeptide; PAR1, parietal cortex area 1; PIR, piriform cortex; AHA, anterior hypothalamus; HIPP, hippocampus; EIPA, ethylisopropylamiloride; GTPγS, guanosine 5'-O-(3-thiotriphosphate); GDPβS, guanosine 5'-O-(2-thiodiphosphate); ATPγS, adenosine 5'-O-(3-thiotriphosphate); ADPβS, adenosine 5'-O-(3-thiodiphosphate); PIP₂, phosphatidylinositol-4,5-bisphosphate.
binding to, for example, the Y2 site, which shows little ion or chaotrope sensitivity (possibly due to accommodation of a long carboxyl-terminal primary binding epitope; Parker et al., 1996a, 1996b). The Y1 receptor also appears to be easily down-regulated by agonistic peptides (Parker et al., 1996b), possibly in connection to a large susceptibility to secondary interactions with neighboring proteins. Regulatory interactions could also be facilitated by the noncontinuous localization of the binding epitopes on both termini of Y1-active NPY analogue peptides (see Daniels et al., 1995).

Binding of agonists to many receptors coupled to G proteins is attenuated by high levels of guanosine polyphosphates, which force a change in the conformation of the nucleotide-binding site of the transducer molecule that in turn modifies the conformation of the attached receptor to decrease the affinity of ligand association (Mixon et al., 1996). Based on the existing reports using subtype-nonspecific unmodified NPY analogs (Unden and Bartfai, 1984; Walker and Miller, 1988), association of agonist peptides with the brain Y1 receptor should also be sensitive to G protein activators and inhibitors. The Y1 receptor is also linked to activity of phosphoinositide-specific phospholipase C (Selbie et al., 1995) and could be involved in the activity of ion channel systems (Hastings et al., 1997). Although these classes of physiological regulators are generally presumed to act as effectors located apart from a receptor molecule properly, there is evidence of direct association of rhodopsin family receptors with phospholipase C (e.g., Aiyar et al., 1989; Biddlecome et al., 1996). Such interactions could result in perturbations sufficient to affect the highly sensitive agonist binding to the Y1 receptor. Physical engagement of metabolic effectors could facilitate the regulation of activity, especially for metabotropic peptide receptors triggering cascade events, including the Y1 receptor. Indeed, it is known that at least Gαi-associating receptors would extensively interact with the phospholipase C effectors that serve as physiological amplifiers of the GTPase activity of G-protein (Biddlecome et al., 1996). However, the influence of such association on ligand-binding parameters would be expected to vary considerably among G protein-linked receptors, depending on organization of binding sites and properties of the agonists involved.

Phospholipase inhibitors, especially those of phospholipase A2, are known to influence ligand-binding activity of both G protein-associated receptors and ion channel receptors. Thus, quinacrine is known to alter the affinity of cholinergic receptor (O’Donnell and Howlett, 1991). Ion channel-active drugs are also known to cross-react in the binding of ligands to several classes of receptors, as shown for nicotinic receptors with Ca2+ channel blockers (Siegel and Lukas, 1986). It is therefore of interest to also examine the possible interactions of these antagonists with NPY-binding sites.

In this report, we show that ligand association with the Y1 receptor in four areas of the rat brain is strongly and uniformly sensitive to guanosine polyphosphates or to inhibitors of phospholipase C, much less sensitive to inhibitors of other phospholipase groups and insensitive to antagonists of several ion channels.

**Methods**

**Chemicals.** LP-PYY, hPYY(3-36) and hPP were purchased from Bachem California (Los Angeles, CA). pPYY and rPP were purchased from Peninsula Laboratories (Los Angeles, CA). Other chemicals were purchased from either Sigma Chemical (St. Louis, MO) or Calbiochem (La Jolla, CA). BIBP-3226, a selective Y1 receptor antagonist, was a gift from Dr. Karl Thomae GmbH (Biberach, Germany).

The guanine nucleotides tested included GTPγS, GDPβS, GMP, GDP and GTP. Other nucleotides used were ADP, ATP, ADPβS, ATPγS, UTP and CTP. MAS-7 (1NLKALAAALKAK-NH2) was used as a very potent mastoparan analogue.

Phospholipase C inhibitors used were ET-18-OCH3 (1-O-octadecyl-2-O-methyl-ν-cyano-3-phosphorylcholine), U-73122 (1-[6-[(17β-3-methoxyestra-1,3,5(10)-tri-en-17-yl)-aminol(2-hexyl-1H-pyrrrole-2,5-dione) and D609 (tricyclodecanyl-9-yl-xanthogenate). U-73343 (1-[6-[(17β-3-methoxyestra-1,3,5(10)-tri-en-17-yl)aminol(2-hexyl-1H-pyrrolidinedione] was used as a control compound for U-73122. L-α-Phosphatidylinositol-4,5-bisphosphate and L-α-phosphatidylethanolamine were used as the control phospholipase substrate phospholipids. Wortmannin was tested as an inhibitor of phosphatidylinerose-3-kinase and of phospholipase D. Quinacrine and cytidine diphosphocholine were tested as inhibitors of phospholipase A2. H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazone) was tested as an inhibitor of protein kinase C.

The steroids tested were cholesterol, estradiol, progesterone and pregnenolone 4-sulfate (Steraloid, Woburn, MA). The surfactants tested included Tween 80 (polyoxyethylene sorbitan monooleate) and CHAPS (3-(3-cholamidopropyl)diamethylylammonio)-1-propane sulfonate).

**Phospholipase assays.** These assays were performed similar to the procedure of Claro et al. (1989). Briefly, the particles were resuspended in Tris-maleate buffer (20 mM; pH 7.0) containing 10 mM LiCl, 2 mM sodium cholate, 6 mM MgCl2, 3 mM EDTA (neutralized to pH 7.0 with NaOH) and 1 mM CaCl2 (corresponding to ~100 mM free Ca2+ as estimated by the procedure of Raeflaub, 1960). At 0.5 mg of particle protein/ml and 10 µM [3H]phosphatidylinositol-4,5-bisphosphate [inositol-2-3H(HN); DuPont-NEN, Cambridge, MA] and after the addition of 100 µM of inhibitors incubated for 20 min at 24°C, in a total volume of 0.2 ml. The reaction was stopped by adding 0.8 ml of 2:1:0.4 (v/v) mixture of chloroform, isopropanol and 1 N HCl and 0.25 ml of 1 N HCl/4 mM EDTA and shaking. After centrifugation for 5 min at 6000 g for 3% of the input [3H]inositol. The upper phase was mixed with 20 volumes of a liquid scintillation medium and counted in a Beckman (Palo Alto, CA) model LS 3801 liquid scintillation counter.

**Iodinated peptides.** [125I]Leu31,Pro34]hPYY, hPYY(3-36) and hPP were either purchased from NEN (Cambridge, MA), or iodinated as described (Parker et al., 1996a). The commercial peptides, moniodinated by the chloramine-T procedure, had specific activities close to the theoretical (2170 Ci/mmol) and were labeled mainly in the carboxyl-terminal tyrosine residue (75–90%, as ascertained by exhaustive tryptic digestion followed by Bio-Gel P-4 chromatography; Parker et al., 1996a) or by high-performance liquid chromatography using the procedures of Walker and Miller (1988). Full-length PYY analogs iodinated in our laboratory had specific activities in excess of 1000 Ci/mmol and contained both carboxyl-terminally and amino-terminally labeled peptides. No important affinity differences were observed in the binding of these ligands relative to carboxyl-terminally moniodinated peptides. All of the assay paradigms were tested at least once with commercial iodinated peptides. No significant differences were noted for either Y1 or Y2 site-selective tracers. The iodinated peptides were stored at −60°C. Preservation of NPY/PYY analogs over assay incubations was evaluated by Bio-Gel P-4 chromatography (Parker et al., 1996a). Routinely, <3% of the input of [125I]labeled NPY or PYY derivatives was fragmented over the assay incubation, and no degraded peptides could be detected in particle-bound tracers by either gel filtration or high-performance liquid chromatography.

**Tissue preparation.** Rat brains were rapidly excised and frozen in dry ice before slicing with a cryomicrotome to obtain 0.5-mm-thick
coronal sections. All stereotaxic coordinates listed below refer to the atlas of Paxinos and Watson (1986). Tissue was excised from the following areas: PARI, the parietal cortex area 1, ~1 mm deep and 4 mm long at 0.6 to 3 mm behind bregma; PIR, the piriform cortex area, ~1 mm deep and 3 mm long at 0.6 to 3 mm behind bregma; AHA, the anterior hypothalamic area, trapezoidal cuts 1 to 1.5 mm deep on either side of the third ventricle, 0.8 to 2.0 mm behind bregma; and HIPP, the anterior hippocampal area (mainly the CA1–CA3 zones), triangular cuts (side length ~1.5 mm) taken 1.8 to 3 mm behind bregma.

**Particle isolation.** This was accomplished as described (Parker et al., 1996a). The particles were stored at ~60°C.

**Ligand-binding assays.** The assay buffer and most of the conditions were similar to those described previously (Parker et al., 1996a). The assay buffer contained 10% sucrose, 20 mM HEPES-NaOH (pH 7.4), 0.25 mg/ml bacitracin, 10 μg/ml each of leupeptin, pepstatin, aprotinin, chymostatin and antipain, 0.5 mM each of phenylmethylsulfonyl fluoride, benzamidine and diisopropylfluorophosphate and 2 mg/ml of proteinase-free bovine serum albumin. Before all assays, the particulates were resuspended once (for 10 min at 6000 × gmax; Eppendorf 5413 centrifuge; Brinkmann, Westbury, NJ, operated at 5°C) from the assay buffer. The assay volume was 0.4 ml. The assays were incubated for 100 min at 24°C, using 25 μg/ml of particulate protein (as measured by the Coomassie Brilliant Blue procedure). The [125I]-labeled peptides were input at 50 pM. Competition assays utilized the nonlabeled NPY or PYY analogs at 0.01 to 300 nM, using up to 16 different concentrations. Unlabeled (Leu31,Pro34)hPYY and hPYY(3-36) were used as displacement agents at 12 to 14 different concentrations in the range of 5 to 100,000 pM.

**Results**

**Displacement of the Y1 ligand by NPY receptor agonists and antagonists.** As expected from previous studies, proportions of the binding of subtype-selective ligands varied greatly among the brain areas examined, whereas the apparent affinity was generally much higher with the Y2 ligand (table 1). At 50 pM of [125I]-labeled ligands, the Y1 binding represented >85% of the specifically bound radioactivity in the parietal cortex but <35% in circumventricular hypothalamic particulates (table 1). The affinity ranges found for PYY/NPY analogs at the Y1 receptor were generally similar for the respective compound across the brain areas tested (fig. 1).

**Data analysis.** Receptor binding parameters were calculated in the LIGAND program (Munson and Rodbard, 1980). Constants for modulation of NPY analog binding by nonpeptide ligands were obtained from biexponential or logistic curve fitting. Mean values of the binding data recorded at discrete molar inputs (usually 100 μM) of various drugs were compared by Tukey’s tests after an analysis of variance and in some cases by Student’s t tests.

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**Competition of [125I]LP-PYY with nonlabeled LP-PYY, pPYY(1-36) (used as a specific NPY receptor ligand that is relatively subtype nonselective) and the Y2-receptor selective ligand hPYY(3-36) are shown in figure 1A. For each ligand, a similar characteristic affinity was found across the areas studied. With particles from any of the areas, a significantly lower affinity in displacement of [125I]LP-PYY was observed for pPYY(1-36) compared with LP-PYY. Above 10 nM, both peptides displaced ≥90% of the total [125I]LP-PYY binding. Inhibition of [125I]LP-hPYY binding by hPYY(3-36), on the other hand, was not complete even at 300 nM. The Y2/Y1 molarity ratio at half-displacement of [125I]LP-PYY (the ligand selectivity ratio) was at least 100.

The Y1-selective antagonist BIBP-3226 showed a similar activity vs. [125I]LP-PYY at particulates from all areas tested, with an average Kd of ~8 nM (fig. 1B). Above 30 nM of BIBP-3226, the displacement was essentially the same as found with 100 nM LP-PYY (i.e., ≥90% of the labeled Y1 ligand binding at an input of 50 pM). As expected, the binding of the Y2-selective tracer [125I]hPYY(3-36) was very weakly competed by the Y1 antagonist, with a half-inhibition close to 100 μM, and a selectivity ratio in excess of 1000 (fig. 1B). After exposure of PIR, AHA or HIPP particulates to 100 μM BIBP-3226 for 20 min at 24°C followed by a single washing, the Y1 binding was <10% of control values. Since at high inputs of LP-PYY there was no significant increase in the residual binding, the inhibition by BIBP-3226 was largely irreversible. On the other hand, the same particulates showed no significant change in either the affinity or the capacity of the Y2-selective binding in any of the areas (Parker SL, Parker MS and Crowley WR, manuscript in preparation).

**The activity vs. the Y1 binding of LP-PYY of the Y4 recep-
For human peptide YY(3-36) (hPYY(3-36)) at 100 nM, the displacement of total [125I]LP-PYY binding by 1 μM of any of the guanosine polyphosphates were in no case significantly different in the inhibition of the Y₁ binding by the various guanosine polyphosphates were noted among the tissues studied at a saturating concentration of 100 μM. The four-tissue means for Y₁ binding at 100 μM of any of the guanosine polyphosphates were not active at up to 100 μM (also see Results). For abbreviations and other details, see the text.

As anticipated from previous work (e.g., Salomon and Rodbell, 1975), profiles of inhibition of the Y₁ binding to brain area particulates by the physiologically occurring guanosine polyphosphates could not be meaningfully assessed without protection against strong phosphate activities present in the membranes. Thus, as shown in figure 3, GTP in the absence of other phosphatase substrates inhibited the Y₁ binding to parietal cortex particulates with an apparent IC₅₀ of ~15 μM. In the presence of 1 mM ATP, however, GTP was nearly as potent as GTP·S in modulation of the Y₁ binding (IC₅₀ <100 nM). As expected, the potency of GTP·S, and especially that of GDP·S, was much less affected by particulate phosphatases than the activity of GDP or GTP (fig. 3). The apparent activity of GTP increased >3 orders of magnitude in the presence of 1 mM ATP, surpassing that of GDP, which, however, was also greatly augmented. GDP·S was 15- to 20-fold less active than GTP·S in modulating the Y₁ binding. At 1 mM ATP, GDP·S was also significantly less active than GDP as an inhibitor of the Y₁ binding.

Fig. 1. A, Competition of [125I]Leu³¹,Pro³⁴hPYY binding to particulates from four areas of rat brain by peptide YY-derived ligands. All binding data were corrected for the binding at 100 nM nonlabeled LP-PYY. The Kᵦₐ values for nonlabeled (Leu³¹,Pro³⁴hPYY (LP-PYY) ranged from 74 to 106 μM (n = 6–12; see table 1). For porcine/rat peptide YY (pPYY(3-36)), the corresponding values, in pM, were 234 ± 33 with PAR1, 290 ± 79 with PIR, 300 ± 45 with AHA and 293 ± 50 with HIP (n = 3 for all). For human peptide YY(3-36) (hPYY(3-36)) at 100 nM, the displacement was between 50% and 60% of that obtained at 100 nM unlabeled LP-PYY, and half of this displacement was reached between 8 and 18 nM of hPYY(3-36) (n = 2 for all). For abbreviations and other details, see the text.

B, Competition binding of [125I](Leu³¹,Pro³⁴)hPYY to particulates from four areas of rat brain by the Y₁-site selective antagonist BIBP-3226 and by NPY/PYY ancestral peptide, rPP. The displacement of the Y₁-selective ligand [125I]hPYY(3-36) by BIBP-3226 from anterior hypothalamic particulates (AHA) is included for comparison. The Kᵦₐ values of the Y₁ ligand with BIBP-3226, in nM, were 6.5 ± 0.8 with PAR1, 11 ± 2 with PIR, 6.8 ± 1.3 with AHA and 8.5 ± 2.1 with HIP (n = 3 for all). For rPP, the corresponding values for the Y₁ ligand with the above tissues (in nM) were 12.6 ± 0.9, 12.8 ± 7.4, 23.3 ± 9.9 and 13.4 ± 6.1 (n = 2 for all). The displacement of total [125I]LP-PYY binding by 1 μM rPP was <5%, and that of the specific binding (as defined at 100 nM LP-PYY) was 55% to 60%; there was 45%, and that of the specific binding (as defined at 100 nM LP-PYY) was 55% to 60%; there was <5% displacement at inputs up to 1 nM. As detailed in the text, similar profiles were obtained with hPP. For abbreviations and other details, see the text.

Fig. 2. Nucleotide sensitivity of steady-state Y₁ receptor binding in four rat brain areas. The data are expressed as percentage of the binding for a nucleotide relative to the binding in the absence of nucleotides. The results represent averages (plus S.E.M.) of at least two separate assays in the presence of 100 μM of the respective nucleotide, with the nonspecific binding defined at 100 nM of unlabeled LP-PYY. Any adenine nucleotides (including the purinergic ligands ATP·S and ADP·S), UTP and CTP were not active at up to 100 μM (also see Results). For abbreviations and other details, see the text.
Lack of sensitivity of Y₁ binding to ion-channel blockers, a protein kinase C inhibitor and adrenergic blockers. Steady-state Y₁ binding was not sensitive to any of the ion channel blockers tested; the maximum decrease at 100 μM was <20% of the control binding (data not shown). The channel blockers inactive toward the steady-state brain particulate Y₁ receptor binding included dihydropyridine antagonists of the L-type Ca²⁺ channel, nimodipine and nitrendipine, the phenylalkylamine L-channel antagonist verapamil, the inhibitors of ATP-sensitive K⁺ channels glyburide and tolazamide and the Na⁺ channel/transport inhibitors, amiloride and ethylisopropylamilorida (EIPA). Pretreatment with any of these agents at 100 μM did not induce a significant reduction in the subsequent Y₁ binding. The protein kinase C inhibitor H-7 also was not active at 100 μM either in inhibition of the steady-state Y₁ binding or by way of pretreatment.

Prazosin, an alpha-1 adrenergic receptor blocker, yohimbine, an alpha-2 adrenergic receptor antagonist, propranolol, a lipophilic beta adrenergic blocker and a phospholipase D/phosphatidyldiacylphosphohydrolase inhibitor did not inhibit the Y₁ binding at up to 100 μM (data not shown). Pretreatment with any of these antagonists at 100 μM also did not produce a significant inhibition or affinity change of the subsequent Y₁ binding to particles from any of the rat brain areas studied.

Sensitivity of Y₁ binding to phospholipase inhibitors. The phospholipase C inhibitors U-73122, ET-18-O-CH₃ and D609 at 100 μM produced different degrees of reduction of the steady-state Y₁ binding to particulates from the brain areas studied (fig. 5). For the above agents, the degree of dissociation of the steady-state binding was quite similar to the extent of irreversible Y₁ binding inhibition produced by pretreatment at 100 μM. Data for PAR1 Y₁ binding after change over a large range of particle protein input (legend of fig. 4).
preincubation with U-73122 over the dose range of 0.3 to 100 μM are shown in figure 6. U-73122 was consistently the most active inhibitor, with IC₅₀ values ranging from 2 to 6 μM in direct competition (fig. 6). Pretreatment of PAR1 particulates with U-73122 at 0.3 to 100 μM reduced the subsequent [¹²⁵I]LP-PYY binding with an inflexion at 10 μM (fig. 6), parallel and quantitatively similar to the profile of direct inhibition by U-73122 at PAR1 shown in the same graph. The control aminosteroid U-73343 over the same molarity range reduced the Y₁ binding insignificantly by <15% (figs. 5 and 6). The phospholipid ether ET-18-O-CH₃ induced a strong inhibition of the Y₁ binding, but its potency was much lower than that of U-73122 (fig. 5). The xanthate drug D609 was also active in all assay conditions, although its activity at 100 μM was significantly lower than that of U-73122 with particulates from all areas and also somewhat lower than that of ET-18-O-CH₃ (fig. 5).

Quinacrine, a phospholipase A₂ inhibitor, was only weakly active under steady-state conditions (<25% reduction of the binding at 100 μM; fig. 5). A nucleotidic phospholipase A₂ inhibitor, CDP-choline, was not active at 100 μM (data not shown). The phosphatidylinositol 3-kinase/phospholipase D inhibitor wortmannin was weakly active only at hipocampal particulates (fig. 5).

**Lack of sensitivity of Y₁ binding to phospholipase substrates, detergents and steroids.** In view of the pronounced sensitivity of the brain area particulate Y₁ binding to phospholipase C inhibitors, it was of interest to check for possible sensitivity of the binding to phospholipase substrates or activators. Phospholipase C substrates and activators carboxylic phosphatidylethanolamine at 100 μM did not significantly decrease the specific binding of [¹²⁵I]LP-PYY. (It should be noted that both compounds at 100 μM, but not at 10 μM, strongly increased both the apparent total and nonspecific binding of the Y₁ agonist.) The nonionic detergent Tween 80 and the zwitterionic detergent CHAPS, tested as controls of the possible role of surfactant cocinellation in the observed inhibition of Y₁ binding by phospholipase C blockers (see James et al., 1995), did not inhibit the steady-state Y₁ binding at 100 μM (i.e., at a molar excess over total particulate phospholipid of ≥2) over 20 min at 24°C. It should be noted, however, that treatment at 0° to 4°C with 1 to 10 mM of CHAPS (but not of Tween 80) resulted in a progressive loss of the Y₁ binding, linked to solubilization of the particulate lipid and to an inactivating extraction of the Y₁ receptor. Cholesterol, estradiol, progesterone and pregnenolone 4-sulfate (used as steroid controls against PLC-active aminosteroid U-73122, and also to test for possible sex steroid cross-reactivity in the Y₁ binding) were not inhibitory at 100 μM (data not shown).

**Inhibition of phospholipase C activity by the antagonists tested.** U-73122, ET-18-OCH₃, and, to a lesser extent, D609 significantly inhibited the activity of phosphatidylinositol-hydrolyzing phospholipase C as assayed in AHA or PAR1 particulates (fig. 7). U-73343 was not significantly active. Under the assay conditions used (free Ca²⁺ of ~100 nM, at pH 7.0), ET-18-O-CH₃ appeared to be the most active phospholipase C blocker.

**Sensitivity of the Y₁ binding to cotreatment with G protein and phospholipase-active drugs.** As seen in figure 8, U-73122 inhibited, with particulates from three brain areas, most of the Y₁ binding not sensitive to guanine nucleotides. At 20 to 100 μM GTPγS, this binding represented about one third of the specific Y₁ binding displaceable by 100 nM of LP-PYY (see also figs. 2 and 3). Scatchard estimates from saturation assays (fig. 8, insets and legend) showed, with particulates from all areas, a somewhat higher affinity for this component relative to the total specific Y₁ binding. Similar affinity trends for the guanine nucleotide-insensitive Y₁ binding were noted in competition of [¹²⁵I]LP-PYY by unlabeled LP-PYY (data not shown).

The interaction of G protein and phospholipase C-associated processes in the Y₁ binding was further examined by parallel competitions pairing the appropriate inhibitors over the same set of molar inputs, using particulates from piriform cortex (fig. 9). As seen in figure 9A, the inhibitory potency of either GTPγS or U-73122 was apparently maintained, rather than augmented by pairing (because the gua-

**Fig. 6.** Profiles of inhibition of the binding of [¹²⁵I]Leu³¹,Pro³⁴]hPYY to particulates from rat brain areas by phospholipase inhibitor U-73122. A profile of LP-PYY binding after pre-treatment of PAR1 particulates by 0.3 to 100 μM of U-73122 (see the text) is also shown. The IC₅₀ values, in μM, were 2.1 ± 0.36 for PAR1 (n = 5), 2.2 ± 0.23 for PIR (n = 8), 4.4 ± 0.85 for AHA (n = 3) and 5.3 ± 1.1 for HIPP particulates (n = 5). After pretreatment of PAR1 particulates with U-73122, the half-maximal decrease of [¹²⁵I]LP-PYY binding was found at 10.7 ± 1.1 μM (n = 2).
nine nucleotide-associated IC_{50} remained in the range of 30–50 nM and the half-inhibition by the phospholipase C blocker stayed in the range of 1.5–2 μM; see legend for fig. 9A). On the other hand, cotreatment with the G protein receptor-docking site agonist MAS-7 and U-73122 (fig. 9B) resulted in a synergic response at up to 75% of the total displacement, and these differences were significant in point-to-point t testing.

**Discussion**

Sensitivity to guanine nucleotides of the brain binding of [125I]NPY and [125I]PYY, agonists that have no clear preference for NPY receptor subtypes, was shown previously by Unden and Bartfai (1984) and by Walker and Miller (1988), before discovery of the subtypes. The present study provides, to our knowledge, the first direct characterization of the sensitivity to guanosine polyphosphates for brain sites labeled by a ligand selective for the Y_1 subgroup of the NPY receptor. The Y_1-selective ligand used can also label, with high affinity, the structurally related Y_4 and Y_5 sites (Gehlert et al., 1996, 1997). However, the low affinity and displacement activity against [125I]LP-PYY observed with pancreatic polypeptides for rat forebrain sites in this and previous work (Parker et al., 1996a) indicate that most of the binding is to the Y_1 subtype. We also present the first evidence for sensitivity of Y_1 ligand binding to inhibitors of phosphatidylinositol-specific PLC. With rat forebrain Y_1...
sites, there could exist a direct link between regulation of a peptide-ergic signal through an interaction of the classic G protein nucleotide exchange stimulated by the receptor (Mixon et al., 1995) and of GTP hydrolysis stimulated by a phospholipase effector known to potently act on G alpha subunits (Biddlecome et al., 1996).

The affinity of the Y₁ receptor in homologous competition and saturation assays found in this work is essentially in agreement with previous reports on selective Y₁ binding in areas of rodent brain (Dumont et al., 1995; Parker et al., 1996a). Unlabeled LP-PYY was significantly more potent than pPYY(1-36) in displacement of [³²P]LP-PYY, probably due to a closely similar carboxyl-terminal epitope. The affinity profiles for LP-PYY were quite similar with perticulates from the four brain areas studied, indicating an absence of major differences in structure or environment of the binding sites. The binding parameters observed in this work for the Y₁-selective antagonist BIBP-3226 are close to values reported for displacement of [¹²⁵I]NPY from rat brain areas by this chemical (Wieland et al., 1995), as is the low activity of BIBP-3226 at the Y₂ site. The low affinity that we observed with perticulates from four brain areas in competition of the Y₁ binding by rPP confirms a generally low number of Y₁/rPP sites in rat forebrain (Parker et al., 1996a). This affinity is in a good agreement with displacement of the binding of PYY or LP-PYY by pancreatic polypeptides in cell lines expressing only the Y₁ subtype (Mannon et al., 1994; Gehlert et al., 1997).

The uniform sensitivity of a considerable portion of the Y₁ binding to guanosine polyphosphates across the areas studied indicates the importance of a direct coupling of the Y₁ receptor with G proteins in its attachment of agonist peptides. Optimization of the apparent activity of GTP and GDP by ATP should be due to saturation of nonselective phosphatase activities (e.g., Salomon and Rodbell, 1975) and highlights participation of the guanine nucleotide-site “switch” in ligand association with the Y₁ receptor.

Our findings do not support an important participation of common neuronal ion channels in the process of Y₁ ligand binding. Among the compounds tested, the phenylalkylamine L-channel blocker verapamil is known to influence binding of radioligands to many classes of both peptide and non-peptide receptors (e.g., Siegel and Lukas, 1986). While NPY and Y₁ receptors arelx radioligands to many classes of both peptide and non-peptide binding. Among the compounds tested, the phenylalkylamine L-channel blocker verapamil is known to influence binding of radioligands to many classes of both peptide and non-peptide receptors (e.g., Siegel and Lukas, 1986). While NPY and Y₁ receptors are

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receptor to a PLC molecule. Elucidation of a more precise mechanism for this interaction would require reconstitution of the corresponding complex in a form capable of high-affinity ligand binding, as already done for the m1 muscarinic receptor (Biddlecome et al., 1996). The multiple interaction of PLC, G protein and receptor triggered by the binding of agonistic peptides could be shared by other peptide receptors known to interact with phospholipase systems, including the vasopressin Y1 and oxytocin receptors (see also Conklin et al., 1996). Thus, evidence was presented that the liver vasopressin V1 receptor could directly associate with a PLC isoenzyme (Aiyar et al., 1989). Precoupling of the epidermal growth factor receptor with PLC-β in the absence of ligand attachment was also documented (Langgut and Ogilvie, 1995). A physical association of PLC and the Y1 receptor is strongly supported by our finding of sensitivity to U-73122 for a sizable component of the Y1 binding that is insensitive to guanine nucleotides. Sensitivity of all Y1 binding to MAS-7, on the other hand, could indicate that the guanine nucleotide-refractory component interacts with aspects of PLC attached to the docking region of the G protein involved. The PLC/mastoparan sensitive region of Gα subunits can have important epitopes located close to its ultimate carboxyl terminus (Conklin et al., 1996).

The high sensitivity to guanosine polyphosphates and phospholipase inhibitors shown in this study augments the mechanism of activation of Y1 receptor activity. The Y1 receptor is to some degree unique among neuropeptide receptors in possessing a highly segmented agonist-binding domain (see Du et al., 1997) and also in requiring noncontinuous and widely separated binding epitopes in agonist peptides (Daniels et al., 1995). This delicate binding assembly can be easily perturbed by an array of chaotropic influences, including temperature (Parker MS, Crowley WR and Parker SL, in preparation), common ions (Parker et al., 1996a) and alkylating agents and nonionic chaotropes (Parker et al., 1996b), thus weakening or even terminating the association of the Y1 receptor with G protein transducers, and perhaps also with PLC effectors. However, as already shown for another neuropeptide receptor (Aiyar et al., 1989), association of the Y1 receptor with PLC enzymes may not be easy to disrupt and may require proteolysis (observed with PLC-β in a number of systems; see, e.g., Blank et al., 1993). This could point to a novel signal transduction mechanism connected to phosphoinositide signaling. On the other hand, attachment to a large PLC molecule could also serve to vectorially promote receptor sequestration and internalization, known to be readily induced by agonist peptides in the case of the brain Y1 receptor (Parker et al., 1996b).

References


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