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Differences in cation sensitivity of ligand binding to Y_1 and Y_2 subtype of neuropeptide Y receptor of rat brain

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Abstract

The binding of selective ligands to the Y_1 subtype of neuropeptide Y receptor in rat brain particulates was promoted by Ca^{2+} and also stimulated by Sr^{2+}, but reversibly reduced by Ba^{2+}, Mg^{2+}, Mn^{2+}, by the organic polycations neomycin and spermidine, and by chelating agents. The alkali monovalent cations inhibited the Ca^{2+}-enabled Y_1 subtype binding with some selectivity ($Cs^+ \geq NH_4^+ > Li^+ > Na^+, K^+$), with half-inhibition between 70–120 mM. The specific Y_2 subtype binding was enhanced by all alkaline-earth divalent cations, Mn^{2+}, neomycin and spermidine in the range of 0.1–10 mM, and by alkali cations at up to 100 mM, and also by Na^+ salts of the chelators EGTA and EDTA. The large disparity in cation sensitivity indicates substantial differences in the structure of the binding sites of the Y_1 and Y_2 receptors, predictable from known distinct features of ligand epitopes and of primary structure of the receptors.

Keywords: Neuropeptide Y receptor, Y_1 subtype; Neuropeptide Y receptor, Y_2 subtype; Cation regulation

1. Introduction

Neuropeptide Y, an abundant neuropeptide released in multiple brain areas (O'Donohue et al., 1985), is known to participate in regulation of the release of neuropeptides (Kalra and Crowley, 1992), and in the control of blood pressure (Wahlestedt and Reis, 1993) and feeding (Stanley et al., 1992). While four subtypes of neuropeptide Y receptor are now recognized in the mammal, the Y_1 subtype (which prefers as ligands intact neuropeptide Y or neuropeptide Y analogs bearing C-terminal hydrophobic substitutions (Fuhlendorff et al., 1990)), and the Y_2 subtype (which binds intact neuropeptide Y as well as N-terminally truncated neuropeptide Y fragments (Dumont et al., 1993)), are well represented in major visceral and neural tissues, and especially in the forebrain. The Y_1 and Y_2 receptors are activated by both neuropeptide Y and peptide YY (a neuropeptide Y-related peptide found in the gut and hindbrain) as subtype-non-selective neuropeptide Y receptor agonists. Peptide YY and peptide YY analogues show a lower non-specific binding in receptor assays than neuropeptide Y and its derivatives; recently introduced peptide YY derivatives highly selective for Y_1 and Y_2 subtypes (Dumont et al., 1993, 1995) were employed in this study. The Y_3 subtype, apparently insensitive to peptide YY, is not strongly expressed in the forebrain (Dumont et al., 1994), and this work finds few Y_4-like sites (Bard et al., 1995) in this part of rat brain.

The activity of many receptors for neurotransmitters and neuropeptides is known to be strongly influenced by cationic environment (e.g. Horstman et al., 1990; Parker et al., 1991; Quintana et al., 1993), in relation to specific aspects of receptor structure (Horstman et al., 1990; Quintana et al., 1993). The two principal subtypes of rat neuropeptide Y receptor differ greatly in overall sequence, in structure of extracellular segments (Eva et al., 1992; Rose et al., 1995), and in ligand epitopes (Grundemar et al., 1992), which could result in distinct profiles of pharmacological reactivity, but their ion sensitivity was not compared thus far. We therefore examined the influence of major cation species encountered in vivo, and of some analogous cations, upon the ligand binding parameters of brain Y_1 and Y_2 receptors.

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2. Materials and methods

2.1. Animals

Holtzman male rats (280–300 g) from Harlan Sprague-Dawley (Indianapolis, IN, USA) were maintained on a 12 : 12 h light/dark cycle, with ad libitum food and water.

2.2. Chemicals

Chlorides of the inorganic cations were obtained from Aldrich (Milwaukee, WI, USA). Percoll medium for particle fractionation was purchased from Pharmacia Biotech (Piscataway, NJ, USA). All other non-peptide reagents and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). The following neuropeptide Y-related peptides (Bachem-California or Peninsula Laboratories, Los Angeles, CA, USA) were used: human/rat neuropeptide Y; porcine/rat peptide YY; Y_1 subtype-selective neuropeptide Y receptor agonist [Leu^{31},Pro^{34}]human peptide Y (Dumont et al., 1995); N-terminally truncated human peptide YY-(3–36), selective for the Y_2 subtype of the neuropeptide Y receptor (Dumont et al., 1995); and rat pancreatic polypeptide, selective for the pancreatic polypeptide/neuropeptide Y-Y_4 subtype of neuropeptide Y receptor (Bard et al., 1995).

2.3. Collection of tissue from rat brain areas

For most preparations used in this study, brains were excised quickly and frozen in dry ice. Sections of 0.3 mm were sliced with a cryomicrotome. Tissue was excised from the anterior hypothalamic area (0.6–3 mm behind bregma, 1 mm bilateral to the third ventricle), and the parietal cortex area (about 1 mm deep and 4 mm long at 0.6–3 mm behind bregma). In control experiments employing fresh tissue, anterior and medial hypothalamic tissue (from the optic chiasm to the area immediately behind the median eminence) and frontoparietal cortex shavings were excised by iris scissors and immediately processed as detailed below.

2.4. Particle isolation and fractionation

The tissue was homogenized in 0.32 M sucrose-10 mM Hepes-NaOH buffer (pH 7.4) containing 1 mM EDTA, and sedimented for 5 min at $600 \times g$ to remove the nuclei and cell debris, and then for 20 min at $12{,}000 \times g_{max}$. The $12\,000 \times g_{max}$ pellets were resuspended in the assay buffer (see below) containing 1.67 mM $CaCl_2$, resedimented for 15 min at $6000 \times g_{max}$ in an Eppendorf Model 5413 centrifuge (Brinkman, Westbury, NY, USA) and stored at −60°C. In control experiments employing fresh tissue, the $12\,000 \times g_{max}$ pellet was processed on Percoll/sucrose gradients by the procedure of Verhage et al. (1989) to obtain a synaptosome-enriched particle fraction, and the synaptosomal pellets were stored at −60°C.

2.5. Ligand iodination and purification

The ligands were iodinated and purified as described (Parker et al., 1991). The iodinated peptides were stored at −60°C. Binding parameters for human peptide YY-(3–36) made by us corresponded well with those obtained with commercially available human peptide YY-(3–36) (DuPont/New England Nuclear, Boston, MA, USA).

Preservation of neuropeptide Y/peptide YY analogues was evaluated by Bio-Gel P-4 (Bio-Rad, Richmond, CA, USA) chromatography in 1.5 M CH_3COOH-0.1% bovine serum albumin (similar to the procedure of Walker et al., 1988). Routinely, less than 3% of the input of ^{125}I-labeled neuropeptide Y or peptide YY derivatives was fragmented over the assay incubation.

2.6. Ligand-binding assays

The assay buffer contained 10% sucrose, 10 mM Hepes-NaOH (pH 7.4), 0.25 mg/ml of bacitracin, 10 μg/ml each of leupeptin, pepstatin, aprotinin, chymostatin and antipain, 0.5 mM each of phenylmethylsulfonyl fluoride and benzamidine, and 2 mg/ml of proteinase-free bovine serum albumin. The pH of EGTA and EDTA solutions was adjusted to 7.4 with NaOH. Prior to all assays, the particulates were resedimented once (for 10 min at $6000 \times g_{max}$; Eppendorf #5413 centrifuge at 5°C) from the assay buffer in the presence of 1.67 mM Ca^{2+}. In some cases, a preincubation of 10 min at 0°C was done in the assay buffer containing cations tested for possible effects on subsequent neuropeptide Y receptor agonist binding, followed by resedimentation as above to remove the ion used. The assay volume was 0.4 ml. The assays were incubated for 100 min at 23–24°C, using 25 μg/ml of particulate protein (Bradford, 1976). The ^{125}I-labeled peptides were input at 15 pM except where noted otherwise. The non-labeled neuropeptide Y or peptide Y analogs were input at 0.005–100 nM, using up to 16 different concentrations. Rat pancreatic polypeptide was used at 0.1–300 nM. The incubations were terminated by centrifugation for 10 min at $6000 \times g_{max}$ in an Eppendorf Model 5413 centrifuge, at 5°C. The pellets were surface-washed with cold assay buffer, and counted in a gamma-scintillation counter.

2.7. Data analysis

Receptor binding parameters were calculated in the LIGAND program (Munson and Rodbard, 1980). Hill coefficients were derived by linear least-square fitting (Segel, 1976). Constants for ion modulation of neuropeptide Y analogue binding were obtained from exponential or

logistic curve fitting. Amino acid sequences and motifs were compared by FASTA programs (Pearson, 1990). Statistical comparisons of regression parameter means were done by *F*-testing (Sokal and Rohlf, 1990).

3. Results

3.1. Characterization of the Y_1-subtype-selective neuropeptide Y receptor binding to particulates from rat parietal cortex

As expected (Dumont et al., 1995), the binding of the Y_1-selective neuropeptide Y analog [^{125}I][Leu^{31},Pro^{34}]human peptide YY to particulates from rat parietal cortex was little affected by the non-labeled Y_2-selective neuropeptide Y receptor agonist, human peptide YY-(3–36), which at 20 nM displaced less than 20% of the labeled Y_1 neuropeptide Y receptor agonist (while, as seen in Table 1, up to 95% of the binding could be displaced by the non-labeled [Leu^{31},Pro^{34}]human peptide YY). As seen in Table 1, the binding of [^{125}I][Leu^{31},Pro^{34}]human peptide YY to particulates from parietal cortex was homogenous, with Hill slopes close to unity. At 1.67 mM Ca^{2+}, no changes in either the affinity or the cooperativity of the binding were detected in competition assays employing [^{125}I][Leu^{31},Pro^{34}]human peptide YY at an input of 150 pM (Table 1), or in saturation assays using inputs of this ligand as high as 600 pM. A similar binding affinity for the Y_1-selective ligand at 1.67 mM Ca^{2+} was found with synaptosomal particulates isolated from fresh frontoparietal cortex, with a considerably higher maximum binding due to removal of intracellular particulates that show little or no specific neuropeptide Y binding (S.L. Parker, M.S. Parker, T. Sweatman and W.R. Crowley, manuscript in preparation). For any of the regimens shown in Table 1, the binding of [^{125}I][Leu^{31},Pro^{34}]human peptide YY could not be satisfactorily defined in terms of more than one component (Munson and Rodbard, 1980). Up to 95% of the binding could be displaced by 20–100 nM of unlabeled [Leu^{31},Pro^{34}]human peptide YY (Table 1), human neuropeptide YY or porcine peptide YY. However, only 31% of [^{125}I][Leu^{31},Pro^{34}]human peptide YY binding (at an input of 15 pM) could be displaced by rat pancreatic polypeptide even at 300 nM, with a K_i of 69 nM (Table 1), and the displacement of [^{125}I]human neuropeptide Y (also input at 15 pM) by 300 nM of rat pancreatic polypeptide was less than 20% of the total binding. A minor component of higher affinity in rat pancreatic polypeptide competition of the [^{125}I][Leu^{31},Pro^{34}]human peptide YY binding (K_i 642 pM), possibly similar to the rat pancreatic polypeptide/Y_4 receptor binding (Bard et al., 1995; Lundell et al., 1995), represented only about 4% of the total binding of this ligand (Table 1). At 1 nM, rat pancreatic polypeptide increased the K_d value in the homologous competition of [^{125}I][Leu$_{31}$,Pro$_{34}$]human peptide YY by unlabeled [Leu^{31},Pro^{34}]human peptide YY, without affecting the B_{max} (Table 1).

Table 1
Parameters of the binding of [^{125}I][Leu^{31},Pro^{34}]human peptide YY to Y_1 sites of rat parietal cortex

mM pretreatment cation/cation(s) in the assay	K_d (pM)	B_{max} (fmol/mg) [a]	% of total	Hill coefficient (n_H) [c]
Competitions with (Leu^{31},Pro^{34}) human peptide YY				
1.67 Ca^{2+}/0.033 $CaCl_2$	125 ± 24	34.6 ± 9	17	0.98 ± 0.12
1.67 Ca^{2+}/1.67 $CaCl_2$	98.7 ± 9.4	167 ± 6.6	85	0.96 ± 0.2
1.67 Ca^{2+}/1.67 $CaCl_2$ at 150 pM of the Y_1 agonist [d]	119 ± 16	177 ± 21	53	0.98 ± 0.04
1.67 Ca^{2+}/1.67 $CaCl_2$ at 1 nM rat pancreatic polypeptide	124 ± 7.6	169 ± 12	86	1.05 ± 0.12
1.67 Ca^{2+}/1.67 $CaCl_2$ with fresh-tissue synaptosomes [e]	88 ± 22	279 ± 42	90	0.94 ± 0.1
1.67 Ca^{2+}/10 $CaCl_2$	89.3 ± 11.2	149 ± 13	94	0.93 ± 0.1
1.67 Ca^{2+}/10 $BaCl_2$ + 1.67 $CaCl_2$	254 ± 65	51.7 ± 12	92	1.14 ± 0.11
10 Ba^{2+}/1.67 $CaCl_2$	107 ± 24	167 ± 18	95	1.0 ±0.03
1.67 Ca^{2+}/10 $MnCl_2$ + 1.67 $CaCl_2$	303 ± 65	11 ± 2.6	93	0.97 ± 0.05
10 Mn^{2+}/1.67 $CaCl_2$	103 ± 3	147 ± 8	94	1.05 ± 0.12
Competition with rat pancreatic polypeptide				
1.67 Ca^{2+}/1.67 $CaCl_2$	69 300 ± 29 400		31	1.45 ± 0.16
Component 1	642 ± 380		4	
Component 2	89 200 ± 12 200		27	

[a] fmol of [^{125}I][Leu^{31},Pro^{34}]human peptide YY bound per mg particulate protein.
[b] Percentage of the total binding displaceable by 20 or 100 nM of unlabeled [Leu^{31},Pro^{34}]human peptide YY, or by 300 nM of rat pancreatic polypeptide.
[c] The Hill coefficients were derived by linear least-square fitting of $\log_{10}$ of (Bound/(B_{max} − Bound)) vs. $\log_{10}$[Free ligand] (Segel, 1976).
[d] All other assays employed 15 pM of the ^{125}I-labeled Y_1 ligand (see Materials and methods).
[e] Synaptosomes from fresh frontoparietal cortex cuts were obtained by the procedure of Verhage et al. (1989) (also see Materials and methods). All other assays employed particulates isolated from cryotome slices of parietal cortex, as detailed in Materials and methods. The particulates were resedimented once from the assay buffer (see Materials and methods) containing the indicated pretreatment cation, and then resuspended in assay buffer containing the divalent cation(s) shown. The parameter estimates are averages of at least three assays for each of the above treatments.

Table 2
Parameters of the binding of [^{125}I]human peptide YY-(3–36) to anterior hypothalamic Y_2 sites

Compound and mM	n_H [a]	K_{d1}	% K_{d1} [b]	B_{max1}	K_{d2}	B_{max2}
Buffer only [c]	1.86 ± 0.2 [a]	8.6 ± 2.8	61	5.6 ± 3		72 ±32
Na_3EDTA 5	1.2 ±0.16 [a]	7.4 ± 0.65	79	6.6 ± 0.52	409 ± 85	15.2 ± 9.4
Na_3EDTA 20 [d]	1.38 ± 0.2	8.6 ± 0.32	64	16.8 ± 2.6	129 ± 32	36.8 ± 15.4
NaCl 40	1.40 ± 0.2	7.3 ± 0.89	62	17 ±3	359 ± 195	89.6 ± 28
KCl 140	1.45 ± 0.27	8.6 ± 4.1	54	9.6 ± 0.52	196 ± 59	80.8 ± 19
$CaCl_2$ 3.3	1.45 ± 0.06	8.4 ± 1.5	64	18 ±4.4	366 ± 63	46.2 ± 9.4
$CaCl_2$ 3.3 at 150 pM Y_2 ligand [e]	1.57 ± 0.21	10.4 ± 3.5	46	19.7 ± 5.8	589 ± 329	90 ±66
$CaCl_2$ 3.3 with fresh-tissue synaptosomes [f]	1.30 ± 0.08	11.4 ± 0.28	64	31 ±0.8	249 ± 49	146 ±12
$SrCl_2$ 3.3	1.47 ± 0.26	9.1 ± 1.3	65	13.8 ± 4	337 ± 67	91.2 ± 33.8
$MgCl_2$ 3.3	1.29 ± 0.2	8.5 ± 1.55	61	11.5 ± 0.9	412 ± 12	43 ±25
$MnCl_2$ 3.3	1.34 ± 0.13	5.3 ± 0.13	57	10.6 ± 3.4	431 ± 89	76.4 ± 34
Spermidine 10	1.65 ± 0.19	10.9 ± 6.7	68	13.7 ± 2.68	174 ± 89	21.4 ± 20
Neomycin 10	1.44 ± 0.17	11.8 ± 3.5	63	13.3 ± 4	320 ± 34	39.6 ± 14

[a] Hill coefficients were estimated as explained in Table 1. The apparent disparity found with regimens detecting only a fraction of the available sites (assay buffer only, and assay buffer with 5 mM Na_3EDTA) reflects the large variation due to a high level of non-displaceable binding.
[b] Percentage counts bound for the high-affinity component, derived by numerical integration of predicted counts bound (Munson and Rodbard, 1980).
[c] All assays included 10 mM Hepes-NaOH buffer (pH 7.4).
[d] This concentration of EDTA anion would correspond to at least 60 mM Na^+ in the assay, assuming $EDTA^{3-}$ as the principal conjugate base.
[e] All other assays employed 15 pM of the ^{125}I-labeled Y_2 ligand.
[f] Synaptosomes from fresh anterior hypothalamic cuts were obtained by the procedure of Verhage et al. (1989) (also see Materials and methods). All other assays employed particulates isolated from cryotome slices of anterior hypothalamus, as detailed in Materials and methods. Data are averages of at least two assays for each of the compounds tested. At 15 pM of [^{125}I]human peptide YY-(3–36) input, the binding displaceable by ≥ 20 nM of unlabeled human peptide YY-(3–36) was more than 80% of the total binding for all regimens resulting in sufficient activation of the Y_2 binding (also see Fig. 1C,D). At 3.3 mM $CaCl_2$ and 150 pM of the labeled Y_2 ligand, the displaceable binding was only 42% of the total binding.

3.2. Effects of di- and polyvalent cations on Y_1 binding

In 10 mM Hepes-NaOH buffer, a large fraction of ^{125}I-labeled ligand binding to Y_1 sites was not competed by 100 nM of unlabeled neuropeptide Y analogs. Ca^{2+} in the incubation buffer greatly reduced such binding and increased the overall specific Y_1 binding up to fourfold employing either the Y_1-selective neuropeptide Y receptor agonist [^{125}I][Leu^{31},Pro^{34}]human peptide YY, or [^{125}I]human neuropeptide Y 'masked' by 1 nM human peptide YY-(3–36) (a concentration at least twice in excess of the low-affinity K_d for the Y_2 binding of this ligand; Table 2)

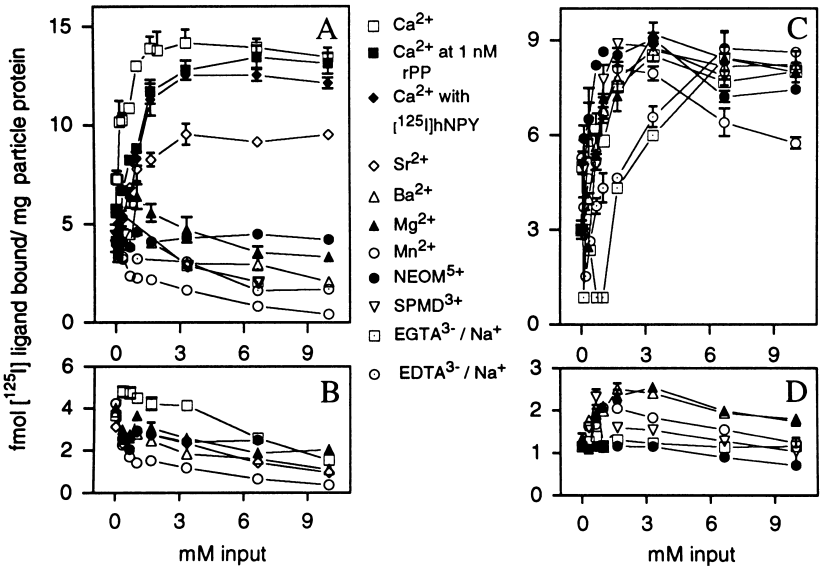

Fig. 1. NPY receptor ligand binding in the presence of di- and polyvalent cations. (A,B) The specific and non-specific binding of Y_1-selective ligand [^{125}I][Leu^{31},Pro^{34}]human peptide YY to particles from parietal cortex. [^{125}I]human neuropeptide Y was also used, with 1 nM of unlabeled human peptide YY-(3–36) as a 'masker' of Y_2 sites, to label Ca^{2+} sensitive Y_1 sites. (C,D) The specific and non-specific binding of the Y_2-selective ligand [^{125}I]human peptide YY-(3–36) to particles from anterior hypothalamus. The data are means of two assays (five in the case of Ca^{2+} without rat pancreatic polypeptide) ± one standard error of measurement (1 S.E.M.). Non-standard abbreviations used in graph inscriptions: rPP, rat pancreatic polypeptide; hNPY, human neuropeptide Y; NEOM, neomycin; SPMD, spermidine.

to predominantly label the Y_1 sites (Fig. 1A,B). This activation occurred without large affinity changes (Table 1) and saturated below 2 mM Ca^{2+}, with half-maximal change close to 0.3 mM Ca^{2+}. In the presence of 1 nM rat pancreatic polypeptide (used to exclude the potential high-affinity Y_4 sites), the half-activation of Y_1 binding by Ca^{2+} was shifted significantly rightward to about 1 mM, while the maximum response was not significantly inhibited (Fig. 1 and Fig. 3). Among other divalent cations tested, only Sr^{2+} supported a significant specific Y_1 binding, but to a lower capacity than Ca^{2+} (Fig. 1). Mn^{2+} and Ba^{2+} strongly reduced both the specific and the non-specific Y_1 binding (Fig. 1A,B), including the binding enabled by Ca^{2+} (Table 1), while Mg^{2+} was a weak inhibitor of both (Fig. 1A,B). Neomycin and spermidine also inhibited the Y_1 binding. Pretreatment for 10 min at 0°C with 10 mM of the cations shown in Tables 1 and 2 did not induce significant changes in Y_1 binding as measured subsequently at 1.67 mM Ca^{2+} (as seen in Table 1 for Ba^{2+} and Mn^{2+} pretreatments), indicating no irreversible receptor alteration. Chelating agents EGTA and EDTA at 1–10 mM strongly inhibited the Y_1 binding (Fig. 1A). EDTA at 5 mM entirely eliminated the stimulation of the Y_1 binding by Ca^{2+} at up to 3.33 mM. Di- and polyvalent cations shown in Tables 1 and 2 could not be tested above 10 mM, due to increased particle aggregation. Zn^{2+}, Cd^{2+} and the lanthanide cations Tb^{3+}, Gd^{3+} and Hf^{4+} at ≥ 100 μM induced aggregation of tracer peptides, particulates and bovine serum albumin. However, no important stimulation of the binding was observed at up to 100 μM of any of these ions. The reversible lowering of the Y_1 binding affinity induced by inhibitory ions occurred without changes in cooperativity of the binding (Table 1).

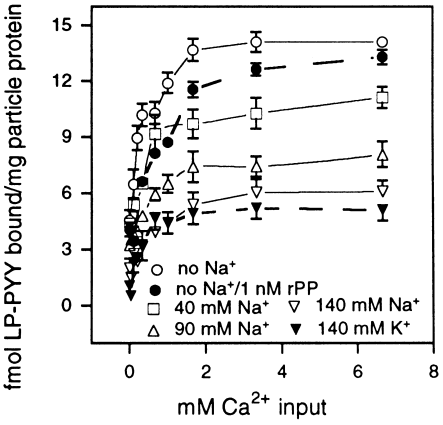

Fig. 3. Effect of Ca^{2+} ion on Y_1 binding of [^{125}I][Leu^{31},Pro^{34}]human peptide YY to rat parietal cortex particulates in the presence of various concentrations of alkali chlorides. The point means are shown ±1 S.E.M. The half-maximal stimulation by Ca^{2+} (mM): 0.28±0.08 in the assay buffer (10 mM Hepes-NaOH) ($n=7$; see also Fig. 1A), 0.43±0.07 at 40 mM NaCl ($n=2$), 0.56±0.06 at 90 mM NaCl ($n=2$) 0.58±0.14 at 140 mM NaCl ($n=2$), and 0.32±0.06 at 140 mM KCl ($n=2$). The half-maximal stimulation by Ca^{2+} at 1 nM rat pancreatic polypeptide without added Na^+ was 1.0±0.15 mM ($n=4$; see also Fig. 1A). At 1.67 mM Ca^{2+}, and 15 pM of the labeled ligand input, the K_d values in competition of [^{125}I][Leu^{31},Pro^{34}]human peptide YY by unlabeled [Leu^{31},Pro^{34}]human peptide YY were 99±9 ($n=3$; Table 1), 131±15 ($n=3$), 159±18 ($n=2$) and 180±19 ($n=3$) pM with 0, 40, 90 and 140 mM NaCl in the assay. The K_d for [^{125}I][Leu^{31},Pro^{34}]human peptide YY binding at 1.67 mM Ca^{2+} in the presence of 1 nM rat pancreatic polypeptide was 124±8 pM ($n=3$; Table 1). Non-standard abbreviations used in graph inscriptions: LP-PYY, [Leu^{31},Pro^{34}]human peptide YY; rPP, rat pancreatic polypeptide.

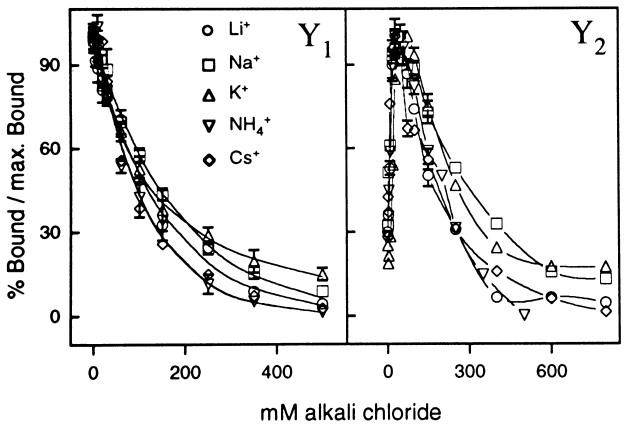

Fig. 2. Influence of alkali cations on NPY receptor binding. $\mathbf{Y_1}$: the binding of [^{125}I][Leu^{31},Pro^{34}]human peptide YY to parietal cortex particulates. All assays contained 1.67 mM Ca^{2+}. The half-inhibition (K_i), mM: CsCl, 76±7 ($n=2$), NH_4Cl, 80±8 ($n=3$), LiCl, 91±9 ($n=2$), NaCl 116±12 ($n=4$), and KCl, 129±14 ($n=4$). Slope differences between KCl or NaCl and CsCl or NH_4Cl profiles were significant in regression variance tests (Sokal and Rohlf, 1990). $\mathbf{Y_2}$: The binding of [^{125}I]human peptide YY-(3–36) to particles from anterior hypothalamus. K_i, mM: Li^+, 174±21; Na^+, 254±25; K^+, 202±16; NH_4^+, 213±20; Cs^+, 237±14 ($n=2$ for all). The point and parameter means are shown ±1 S.E.M.

3.3. Effects of the alkali monovalent cations on Y_1 binding

Alkali metal chlorides and ammonium chloride potently reduced the Ca^{2+}-enabled Y_1 binding, with some selectivity (Fig. 2). Cs^+ and NH_4^+ were clearly more potent inhibitors than Na^+ or K^+ (see the legend to Fig. 2). With up to 140 mM of Na^+, this inhibition occurred with less than a twofold decrease in the apparent affinity of [^{125}I][Leu^{31},Pro^{34}]human peptide YY binding (see the caption to Fig. 3). The inhibition by Na^+ or K^+ could be significantly counteracted by increasing [Ca^{2+}] in the assay (Fig. 3). The half-activation of the Y_1 binding by Ca^{2+} was observed at 0.3–0.5 mM of this cation with [Na^+] in the range of 40–140 mM (and also at 140 mM [K^+]) (Fig. 3), indicating lack of a direct competition of these ions in the binding response, as different from a significant competitive shift induced by 1 nM rat pancreatic polypeptide in half-stimulation of the Y_1 binding by Ca^{2+} (Fig. 3; see also Fig. 1A) and also in the K_d for [^{125}I][Leu^{31},Pro^{34}]human peptide YY binding (legend of Fig. 3).

3.4. Characteristics of the Y_2-selective neuropeptide Y receptor agonist binding to rat hypothalamic particulates

As expected (Dumont et al., 1995), the binding of [^{125}I]human peptide YY-(3–36) to particulates from rat anterior hypothalamus was essentially unaffected by the non-labeled Y_1-selective neuropeptide Y receptor agonist, [Leu^{31},Pro^{34}]human peptide YY (less than 5% displacement at 20 nM of the Y_1 ligand), while (at an input of 15 pM) up to 90% of the binding could be displaced by non-labeled human peptide YY-(3–36) (Fig. 1C,D and the legend to Table 2). This Y_2-selective binding in most assay conditions showed Hill coefficients significantly above unity, and two statistically acceptable components (Munson and Rodbard, 1980). The high-affinity component (K_d range 5.3–11.8 pM) accounted for more than 50% of the binding in all environments. There were no significant affinity shifts for Y_2 binding related to saturation of the lower-affinity site with the ligand at 150 pM relative to 15 pM of [^{125}I] human peptide YY-(3–36) input (Table 2), or in saturation assays using up to 500 pM of the labeled ligand. The binding of [^{125}I]human peptide YY-(3–36) to rat hypothalamic particulates was less than 10% displaced by 300 nM of rat pancreatic polypeptide. Similar affinities and relative abundance for the higher-affinity and the lower-affinity component of the Y_2 binding were found with particulates from rat hippocampus and piriform cortex.

3.5. Effects of di- and polyvalent cations on Y_2-selective neuropeptide Y receptor agonist binding to rat hypothalamic particulates

The high-affinity Y_2 binding was enhanced by di- or polyvalent cations at up to 10 mM, without a clear selectivity. Thus, Ca^{2+}, Sr^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+}, neomycin^{5+} and spermidine^{3+} all increased the specific Y_2 binding about threefold relative to that at 10 mM Hepes-NaOH, without major changes in the affinity of this binding, or the amount of the apparent non-specific binding (Table 2 and Fig. 1D). Neomycin and spermidine also activated the Y_2 binding, yielding somewhat bell-shaped response profiles, with maxima between 2–5 mM of these cations (Fig. 1C). Mn^{2+} produced a pronounced bell-shaped change in the specific Y_2 binding over the range of 1–10 mM (Fig. 1C). Concentrations above 10 mM were not tested, since both the divalent and the polyvalent cations can induce particle aggregation and artifacts in neuropeptide Y receptor ligand binding in this molarity range.

3.6. Effects of the alkali monovalent cations on Y_2 binding

The alkali monovalent cations stimulated the Y_2 binding at up to 100 mM, and inhibited the binding at higher inputs, with K_i values in the range of 170–250 mM. Li^+ was the most potent, and Na^+ the weakest inhibitor (see the legend of Fig. 2). Very similar responses to most of the cations tested were found for Y_2 binding to particulates from rat hippocampus and piriform cortex (data not shown). Sodium salts of the chelating agents EGTA and EDTA (both mainly present as trivalent anions at pH 7.4) enabled the Y_2 binding with half-activation close to 3 mM (Fig. 1C; see also Table 2) to maxima at 10–20 mM anion (i.e., 30–60 mM Na^+) similar to those obtained with divalent cations at 3–10 mM (Fig. 1C), or with monovalent cations, including Na^+, at 30–70 mM (Fig. 2, Y_2 graph).

4. Discussion

Our results show major differences in cation sensitivity and selectivity of ligand binding to rat brain Y_1 and Y_2 receptors. The Y_1 subtype binding is selectively enabled by Ca^{2+} and strongly attenuated by Na^+ (and other alkali cations), while the Y_2 binding shows little cation selectivity or sensitivity. To our knowledge, this is the first demonstration of such differences in the ligand-binding properties between the Y_1 and the Y_2 subtype of the neuropeptide Y receptor.

Promotion of the high-affinity Y_1 binding by Ca^{2+} and also by Sr^{2+} ion (the only alkaline-earth cation possessing an ionic radius close to that of Ca^{2+} (Weast, 1982)) might involve chelation of aspartate residues, especially those in the third extracellular (ec3) loop of the receptor, which were shown to participate in fashioning the Y_1 binding site (Walker et al., 1994). Two of the three essential (Walker et al., 1994) aspartate residues in this loop are flanked by basic and aromatic-cation (Dougherty, 1996) residues (YKD^{193}KYY and FD^{199}KYY; see Eva et al., 1990), similar to some of the known low-affinity calcium binding sites. This is not found in the ec3 loop of the Y_2 receptor (Rose et al., 1995). Failure of the alkali cations to competitively increase the molarity for half-maximum stimulation of Y_1 binding by Ca^{2+} points to an allosteric contribution of these ions to the regulation of Ca^{2+}-sensitive region(s) of the Y_1 binding site. The binding of the Y_1 site-selective neuropeptide Y analogue [Leu^{31},Pro^{34}]human peptide Y to parietal cortex particulates did not entail a significant high-affinity pancreatic polypeptide-sensitive component, and appeared to almost entirely reflect attachment to the Y_1 neuropeptide Y receptor.

The only Asp residue found in the second transmembrane (tm2) segment of neuropeptide Y receptors could be important for an allosteric monovalent cation regulation of the Y_1 binding, by analogy with the binding of the agonists of aminergic receptors (Horstman et al., 1990) and with that of some protein hormones liganding the family 1 G-protein-enabled receptors (Quintana et al., 1993). This residue is found at the C-terminus of an LXXXD (X = any amino acid residue) motif of the tm2 segment in more than eighty family 1 G-protein-linked receptors. Several receptors from this group that are highly sensitive to monovalent cations in terms of agonist binding affinity possess at least one serine residue close to the proposed (Horstman et

al., 1990; Ceresa and Limbird, 1994) aspartate allosteric cation 'switch'. Two of the most cation-sensitive family 1 members, the neuropeptide Y-Y_1 and the luteinizing hormone receptor (Quintana et al., 1993), also have Phe close to Asp in the tm2 segment (Eva et al., 1990; Minegishi et al., 1990). Both Ser and Phe can assist ion access to hydrophobic pockets (e.g. Oblatt-Montal et al., 1993), which may sensitize the Asp 'switch' to ion fluxes. The corresponding sequence in the tm2 segment of the Y_2 receptor (LAVAD; Rose et al., 1995) lacks both serine and aromatic amino acid residues. Examination of the possible role of these residues in the activity of neuropeptide Y receptor subtypes would thus be of interest.

The low cation selectivity and sensitivity of ligand binding to the Y_2 sites could be due to receptor preference for long C-terminal epitopes in neuropeptide Y and related peptides. The Y_2 receptor is known to accommodate larger C-terminal neuropeptide Y epitopes than the Y_1 site (Grundemar et al., 1992). The invariant C-terminal stretch of all known neuropeptide Y molecules (residues 24–36; LRHYINLITRQRY.amide (Wahlestedt and Reis, 1993)), which is also highly similar in peptide YY molecules (Wahlestedt and Reis, 1993), could be utilized in the Y_2 binding as a super-epitope entity. The primary binding could be accomplished via the C-terminal Tyr amide and multiple polycationic Arg residues (Grundemar et al., 1992), while the Tyr aromatic 'benzene cations' (Dougherty, 1996) and other hydrophobic residues in the above sequence could strengthen the attachment via entropy-driven secondary interactions (Clackson and Wells, 1995).

The selective activation of the Y_1 binding by Ca^{2+} ion and the attenuation of this effect by Na^+ occur close to the concentration ranges of these cations encountered in vivo, and this antagonism might have physiological relevance. The Y_2 binding apparently requires an ionic environment only in terms of charge neutralization that enables ionic elements of the binding epitope(s) to adequately attach to the receptor site. The Y_2 binding could also be much less sensitive to non-ionic chaotropes than the Y_1 binding. A lower efficacy of disruption of the Y_2 receptor binding by common ions (coupled to a higher affinity of the attachment) could result in longer signaling activity per binding event than in the case of the Y_1 site.

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