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Special Issue on Galanin

Galanin (2–11) binds to GalR3 in transfected cell lines: limitations for pharmacological definition of receptor subtypes

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Abstract

The neuropeptide galanin regulates a variety of physiological and pathophysiological processes through three G protein coupled receptors, GalR1, GalR2, and GalR3. The studies on galanin receptor subtype specific effects have been hampered by the lack of high affinity subtype selective antagonist and/or agonist to any of these three galanin receptor subtypes. Since its recent introduction in 2003, galanin (2–11) has been widely used as a GalR2 selective agonist in several in vitro and in vivo studies. In the present paper, we demonstrate that galanin (2–11) binds to rat GalR3 receptors in transfected cell lines with a similar affinity as it binds to GalR2. As none of the available antagonists are galanin receptor subtype selective, as shown here for M35 and M40, more work is needed to confirm whether a galanin (2–11) effect is GalR2 mediated and there is an urgent need for high affinity galanin receptor subtype selective ligands. For now one needs to interpret the data obtained at lower galanin (2–11) concentrations as effects mediated by non-GalR1 type galanin receptors, i.e., GalR2 and/or GalR3.

Galanin, the 29/30 aminoacid long neuropeptide (Tatemoto et al., 1983), exerts its actions through three known GPCR type galanin receptors: GalR1–R3 (Branchek et al., 1998). The advancement of galanin receptor pharmacology is critically dependent on receptor subtype selective ligands or other means that can selectively alter the expression of the different galanin receptor subtypes. The generation of antisense oligonucleotides (peptide nucleic acid) to selectively suppress translation of GalR1 (Pooga et al., 1998; Rezaei et al., 2001) and GalR2 (Mazarati et al., 2004a) proteins has been used in studies on GalR1 and GalR2 mediated effects in pain (Pooga et al., 1998; Rezaei et al., 2001) and seizure (Mazarati et al., 2004a), respectively. The PNA-antisense oligonucleotide mediated knock-down of GalR1 and GalR2 but not yet of GalR3 has given useful insights regarding galanin receptor subtype mediated effects, but the extensive application of this technique is prohibited by the difficulty in quantifying the extent of knock down, owing to the absence of antibodies and ligands specific to GalR1 and GalR2. Transgenic mouse strains with null mutations of GalR1 (Blakeman et al., 2003; Grass et al., 2003; Holmes et al., 2003; Holmes et al., 2004; Jacoby et al., 2002; Mazarati et al., 2004b; Wrenn et al., 2004) and GalR2 (Kinney et al., personal communications) have provided an additional tool to the study of galanin effects mediated through GalR1 and GalR2 receptor subtypes, with no data regarding a GalR3 null mutation available. While a large amount of effort has been devoted to the generation and phenotyping of GalR1 and GalR2 knockout mice, very limited abnormalities have been identified in these stains to date. As with all other studies using conventional knockout or transgenic animals, the developmental

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compensations could potentially mask the effects or complicate the interpretation of these data. Developmental compensatory changes will be of extraordinary relevance in studies on galanin receptors since accumulating data suggest that galanin acting at GalR2 has trophic effects on the PNS (Mahoney et al., 2003) and CNS (Mazarati et al., 2004a) neurons.

Against this background the introduction of galanin (2–11) (AR-M1896) (Liu et al., 2001), a highly conserved (100%) sequence for all galanin species, as a "selective" GalR2 ligand was an important advance in the field. This short galanin fragment was shown to act as an agonist for GalR2 receptors in the locus coerules (Ma et al., 2001) and spinal cord (Liu et al., 2001). The equilibrium displacement studies on cells lines expressing GalR1 and GalR2 receptors have shown that galanin (2–11) displaces ^{125}I-galanin from GalR2 receptors with a higher affinity than from GalR1 receptors (Liu et al., 2001). However, the affinity of galanin (2–11) for GalR3 has not yet been determined. Neither has radiolabeled galanin (2–11) been introduced to enable direct equilibrium binding studies rather than displacement binding studies.

In the present study, we determined the affinity of galanin (2–11) for the GalR3 receptors using membranes from cells transiently transfected with rat GalR3 plasmid in the displacement binding assay (Fig. 1). Rat GalR3 cDNA (Wang et al., 1997) was introduced into COS-7 cells by the use of LipofectAMINE 2000 method (Life Technologies, Inc.) according to the manufacturer's instructions. Two days following the transfection of the COS-7 cells, membranes were prepared and competition binding experiment was performed as previously described (Wang et al., 1997). The radioligand ^{125}I-procine galanin (2200 Ci/mmol, Perkin–Elmer Life Science, Boston, MA) was used at a concentration of 0.25 nM and the amount of COS-7 membrane proteins per assay was 40 μg. The data were analyzed by nonlinear regression (Prism, GraphPad, San Diego, CA) and the K_i calculated according to the method of Cheng and Prusoff (Cheng and Prusoff, 1973). Radioligand competition binding experiment was also performed on membranes prepared from Bowes melanoma cell lines (expressing human GalR1) and CHO cells that were stably transfected with rat GalR2 plasmid (Wang et al., 1998) to determine the affinity of galanin (2–11) for GalR1 and GalR2 receptors, respectively, under similar conditions. Our data show that galanin (2–11) binds to GalR3 with a K_i value of 271 ± 14 nM (n = 3), which is comparable with its affinity for GalR2 receptors (K_i = 88 ± 22 nM) (n = 3) (Fig. 1). Galanin (2–11) at concentrations up to 5 μM was unable to displace ^{125}I-porcine galanin from GalR1 expressing Bowes cell membranes. Galanin (2–11) was initially reported to have a much higher affinity for GalR2 receptor than reported here, with a K_i value of 1 nM (Liu et al., 2001). The reason for this discrepancy is not clear, however, one possible explanation could be that ^{125}I-human galanin, instead of ^{125}I-porcine galanin, was employed in the earlier binding study and these two ^{125}I-labeled galanin species have been shown to have different binding properties (Kolakowski et al., 1998).

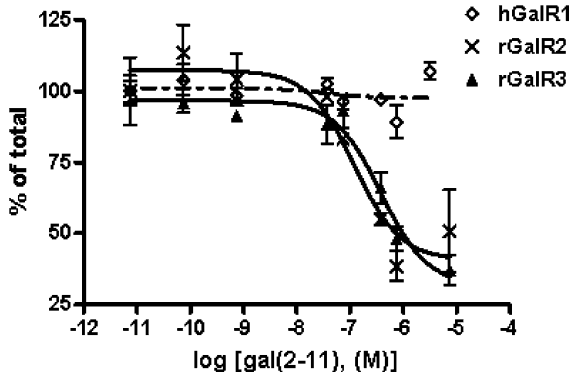

Fig. 1. Competition of ^{125}I-porcine galanin for GalR1, GalR2 and GalR3 by galanin (2–11). Data were plotted with Prism Software as percent of total binding. Forty micrograms membrane proteins prepared from Bowes' melanoma cells (hGalR1), CHO cells expressing rat GalR2, or COS-7 cells expressing rat GalR3 were used in the assay and the ^{125}I-procine galanin concentration was 0.2 nM.

These results indicate that interpretation of the data using galanin (2–11) needs to take into account the binding of this ligand to GalR3 receptors in addition to the binding to GalR2 receptors. As there is a substantial controversy in the literature regarding the distribution of GalR3 mRNA in the CNS (Mennicken et al., 2002; Smith et al., 1998; Wang et al., 1997), and as we continue to suffer from the lack of GalR3 selective antiserum, we can not tell with certainty which of the galanin (2–11) evoked effects are GalR3 receptor mediated. For now, we have to say that these effects are mainly mediated by non-GalR1 receptors. In the absence of high affinity, subtype selective antagonist to the GalR1–3, novel ligands will thus be needed to determine whether an action of galanin (2–11) is mediated by GalR2 or GalR3 receptors. The commonly used high affinity chimeric galanin receptor antagonists M40 and M35 are not receptor subtype selective (Table 1) and furthermore, they can behave as partial agonists at higher concentrations (Kask et al., 1995). The newly

Table 1
Affinities of two chimeric peptide type galanin receptor antagonists for GalR1–3

	M35	M40
hGalR1	4.8 ± 1.1	1.8 ± 0.8
rGalR2	8.2 ± 3.1	5.1 ± 1.0
rGalR3	4.7 ± 1.8	63 ± 19

IC_{50} values from competitive displacement of porcine ^{125}I-galanin binding to galanin receptors were converted to K_i values according to the Chang-Prusoff equation ($K_i = IC_{50}/(1 + [L]/K_d)$) and reported as K_i ± SE (n = 3).

synthesized GalR3 antagonist, 3-[(3,4-dichlorophenyl)-imino]-1-(6-methoxy-3-pyridinyl)-1,3-dihydro-2H-indol-2-one (Konkel, Wetzel, Talisman, 2004, US patent 514/323), does not bind to GalR1 and GalR2 (data not shown) at up to 10 μM. Thus, the effects of galanin (2–11) that can be blocked with the GalR3 antagonist are probably GalR3 mediated while those which cannot be blocked by this antagonist will be considered a GalR2 mediated. The use of galanin (2–11) in GalR2 (−/−) mice will provide a measure of GalR3 mediated effects by galanin (2–11).

Our conclusion is that galanin (2–11), an agonist peptide, is a useful agonist for the study of non-GalR1 receptors. In the near future systemically active, receptor subtype selective non peptide type ligands are expected to supplement the pharmacological use of such difficult and blunt tools as galanin (1–29) or galanin (2–11) peptides in determining receptor subtype selectivity.

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