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RS-056812-198: partial agonist on native and antagonist on cloned 5-HT_3 receptors

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Abstract

Effects of (*R*)-*N*-(quinuclidin-3-yl)-2-(1-methyl-1*H*-indol-3-yl)-2-oxo-acetamide (RS-056812-198) on 5-HT_3 receptors have been investigated in whole-cell voltage-clamped N1E-115 mouse neuroblastoma cells and on 5-HT_3 receptors composed of either long (5-HT_3R-A_L) or short (5-HT_3R-A_S) subunits expressed in *Xenopus laevis* oocytes. In N1E-115 cells RS-056812-198 evokes small transient inward currents, which are completely and reversibly inhibited by the selective 5-HT_3 receptor antagonist MDL 72222 and cross-desensitizes with the 5-hydroxytryptamine (5-HT)-evoked current. The concentration-effect curve of RS-056812-198 yields an EC_{50} of 18 nM and a maximum amplitude of 15% of the maximum 5-HT-evoked current. In contrast to its effects on N1E-115 cells, RS-056812-198 does not evoke an ion current on cloned 5-HT_3 receptors expressed in *Xenopus* oocytes, but acts as an antagonist. For 5-HT_3R-A_L receptors, the IC_{50} of RS-056812-198 is 0.4 nM. The results show that (1) RS-056812-198 is a high-affinity partial agonist on 5-HT_3 receptors in N1E-115 cells, thus providing a valuable tool to study agonist-receptor interaction in more detail; (2) 5-HT_3 receptors in N1E-115 cells differ from the homo-oligomeric 5-HT_3 receptors expressed in *Xenopus* oocytes. Whether the difference is caused by differences in protein processing in the two preparations or by expression of additional, yet unidentified subunits in N1E-115 cells and consequent formation of hetero-oligomeric 5-HT_3 receptors remains to be determined.

Keywords: 5-HT_3 receptor; Ligand-gated ion channel; (Partial agonist); (Antagonist); N1E-115 mouse neuroblastoma cell; *Xenopus laevis* oocyte

1. Introduction

The 5-HT_3 receptor is a ligand-gated ion channel, presumably composed of five identical subunits (Jackson and Yakel, 1995). The 5-HT_3 receptor subunit (5-HT_3R-A_L) and a splice variant (5-HT_3R-A_S) have been cloned (Maricq et al., 1991; Hope et al., 1993), and each can form functional homo-oligomeric receptors with similar pharmacological properties when expressed in *Xenopus laevis* oocytes (Downie et al., 1994).

Mouse N1E-115 neuroblastoma cells endogenously express 5-HT_3 receptors and both the 5-HT_3R-A_L and 5-HT_3R-A_S subunit are present in these cells (Hope et al., 1993). The functional properties of 5-HT_3 receptor-mediated ion currents in these cells have been studied in detail (Neijt et al., 1986, 1989; Van Hooft and Vijverberg, 1995, 1996). In addition, the pharmacological profile of the receptor has been characterized extensively in binding assays on these cells with radiolabelled antagonists (Hoyer and Neijt, 1988; Lummis et al., 1990). Due to the lack of high-affinity 5-HT_3 receptor agonists, little is known about the agonist recognition site on the receptor. Only the full agonists 2,3,5-trichlorophenylbiguanide (Morain et al., 1994) and *meta*-chlorophenylbiguanide (*m*CPBG, Sepúlveda et al., 1991) have affinities for the 5-HT_3 receptor which are sufficiently high to perform binding experiments and to study agonist-receptor interaction in more detail (Lummis et al., 1993; Steward et al., 1993).

Recently, the compound (*R*)-*N*-(quinuclidin-3-yl)-2-(1-methyl-1*H*-indol-3-yl)-2-oxo-acetamide (RS-056812-198) has been shown to bind to 5-HT_3 receptors with high affinity, and to act as a partial agonist in 5-HT_3 receptor assays such as induction of the von Bezold-Jarisch reflex and depolarization of the rat vagus nerve (Clark et al., 1995). In this study, we examined the actions of RS-056812-198 on 5-HT_3 receptors in whole-cell voltage-

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clamped N1E-115 cells and on 5-HT_3 receptors expressed in *Xenopus* oocytes.

2. Materials and methods

2.1. Cell culture

Mouse neuroblastoma cells of the clone N1E-115 (Amano et al., 1972) were grown as described previously (Neijt et al., 1989). Subcultures of passages 31–42 were differentiated by addition of 1 mM dibutyryl-cyclic AMP and 1 mM 3-isobutyl-1-methylxanthine to the culture medium. This medium was refreshed every 2–3 days. Cells were used for experiments on day 7–14 after subculture.

2.2. Whole-cell voltage clamp

Membrane currents were recorded by a suction pipette technique for whole-cell voltage clamp (Neijt et al., 1989). Cells were continuously superfused with external solution during whole-cell voltage clamp (holding potential −70 mV) and ion currents were evoked by switching to agonist- and/or antagonist-containing external solution as described before (Van Hooft and Vijverberg, 1996). The ionic composition of the internal solution was (in mM): 100 K-glutamate, 20 Na-HEPES and 120 sucrose. The pH was adjusted to 7.25 with L-glutamic acid. The external solution contained (in mM): 125 NaCl, 5.5 KCl, 20 HEPES, 1.8 $CaCl_2$, 0.8 $MgCl_2$, 24 glucose and 37 sucrose. The pH was adjusted to 7.3 with approximately 7 mM NaOH.

2.3. Expression in Xenopus oocytes

Oocytes from mature specimens of *Xenopus laevis* were harvested, injected and incubated as described before (Zwart et al., 1995). cDNAs encoding either for the 5-HT_3R-A_L (Maricq et al., 1991) and 5-HT_3R-A_S (Hope et al., 1993) subunits were dissolved in distilled water. The volume injected in the nucleus of oocytes amounted to 18–32 nl (approximately 1 ng cDNA). Oocytes were incubated at 19°C in modified Barth's solution containing (in mM): 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.3 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 15 HEPES and 10 μg/ml gentamicin (pH 7.6 with NaOH).

2.4. Recordings from Xenopus oocytes

5-HT_3 receptor-mediated ion currents were recorded from oocytes 2–5 days after injection of cDNA by conventional two-microelectrode voltage clamp as described before (Zwart et al., 1995). Microelectrodes (≤ 1 MΩ) were filled with 3 M KCl. Oocytes were placed in a silicon tube (∅ 3 mm) which was continuously perfused with external solution containing (in mM): 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$ and 10 HEPES (pH 7.2 with NaOH) at a rate of approximately 20 ml/min. Ion currents were evoked by switching to agonist-containing external solution using a servo-motor operated valve. The membrane potential was held at −60 mV. All experiments were performed at room temperature (20–24°C).

2.5. Drugs

5-Hydroxytryptamine creatinine sulphate (5-HT) (Sigma, St. Louis, MO, USA), (*R*)-*N*-(quinuclidin-3-yl)-2-(1-methyl-1*H*-indol-3-yl)-2-oxo-acetamide (RS-056812-198; Syntex Research, Palo Alto, CA, USA), and 3-tropanyl-3,5-dichlorobenzoate (MDL 72222; Research Biochemicals International, Natick, MA, USA) were diluted in external solution from stock solutions in distilled water which were stored at −20°C.

2.6. Data analysis

Parameter estimates of concentration-effect curves were obtained by fitting the function:

$$i = i_{max} / \left\{1 + \left(EC_{50} / [\text{agonist}]\right)^{n_H}\right\}$$

Estimates of the apparent association and dissociation rate constants were obtained by fitting monoexponential functions to the activation and deactivation of the agonist-evoked current, respectively. All functions were fitted using a Levenberg-Marquardt non-linear least-squares algorithm (Marquardt, 1963). Results are expressed as mean ± S.D. of n independent experiments.

3. Results

In whole-cell voltage-clamped N1E-115 cells 100 nM RS-056812-198 evokes a transient inward current. This ion current is mediated by 5-HT_3 receptors because the current is completely and reversibly inhibited by 50 nM of the selective 5-HT_3 receptor antagonist MDL 72222 (Fig. 1A). In addition, after complete desensitization of the 5-HT_3 receptors by 5-HT, superfusion with RS-056812-198 does not evoke an ion current (Fig. 1B). The onset of RS-056812-198-induced desensitization is slow, because 4 min of superfusion with 100 nM RS-056812-198 is not sufficient to desensitize the 5-HT-induced current completely (Fig. 1B). Approximately 12 min of washing are required for complete recovery from RS-056812-198-induced desensitization (not shown).

Fig. 1C shows the concentration-effect curve of RS-056812-198. The EC_{50} and the Hill coefficient amount to 18.3 ± 6.0 nM and 1.6 ± 0.3, respectively ($n = 3$). The maximum inward current amplitude is 14.4 ± 3.5% ($n = 3$) of that of the 10 μM 5-HT-induced current. The kinetics

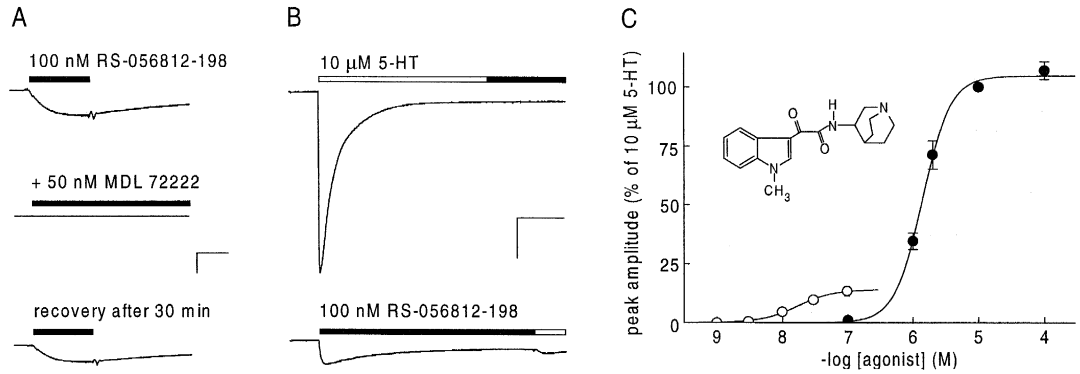

Fig. 1. Agonist effects of RS-056812-198 on 5-HT_3 receptors in N1E-115 cells. (A) Superfusion of 100 nM RS-056812-198 evokes a slowly activating inward current. After 4 min of pre-exposure to 50 nM MDL 72222, this current is completely inhibited. The inhibition by MDL 72222 is reversed after 30 min of washing. The solid bars on top of the records indicate the periods of RS-056812-198 applications. (B) After complete desensitization of the ion current evoked with 10 μM 5-HT (open bar), superfusion of 100 nM RS-056812-198 (solid bar) does not result in an inward current (upper trace). After 4 min of desensitization of the ion current evoked with 100 nM RS-056812-198 (solid bar), the amplitude of the ion current evoked by superfusion of 10 μM 5-HT (open bar) is greatly reduced (lower trace). The upper and lower traces have been recorded from the same cell. (C) Concentration-effect curve of the current induced by RS-056812-198 (open circles) and 5-HT (solid circles). All current amplitudes are expressed relative to the amplitude of the 10 μM 5-HT-induced current recorded from the same cell. Each point represents the mean (±S.D.) of the values obtained from 3 cells. Absence of error bars indicates that the standard deviation is smaller than the symbol size. The inset shows the chemical structure of RS-056812-198. Calibration bars are 5 s and 5 nA (A), 25 s and 10 nA (B, upper trace), and 50 s and 10 nA (B, lower trace).

of the RS-056812-198-induced ion currents are much slower than those of the 5-HT-induced ion currents (Fig. 1B). The kinetics of activation and deactivation of the 100 nM RS-056812-198-induced current are monoexponential with time constants of 2.5 ± 0.2 s ($n = 4$) and 25.2 ± 4.0 s ($n = 4$), respectively. Assuming that the activation and deactivation rates reflect agonist association (k_{obs}) and dissociation (k_{-1}), the apparent K_d of RS-056812-198, calculated from $K_d = k_{-1} \cdot [\text{agonist}]/(k_{obs} - k_{-1})$, is 11.5 ± 2.2 nM ($n = 4$), which is in good agreement with the EC_{50} obtained from the concentration-effect curve.

The effects of RS-056812-198 were also examined on the long (5-HT_3R-A_L) and short (5-HT_3R-A_S) subunits of the 5-HT_3 receptor expressed in *Xenopus* oocytes. Application of 5-HT to oocytes injected with either of the two subunits results in similar large, transient ion currents. These currents were shown to be mediated by 5-HT_3 receptors, because they were never observed in uninjected oocytes, they were blocked by nanomolar concentrations of *d*-tubocurarine and of the selective 5-HT_3 receptor antagonist MDL 72222 and they were mimicked by the selective 5-HT_3 receptor agonist *meta*-chlorophenylbiguanide (not shown), identical to previously published data (Maricq et al., 1991; Downie et al., 1994). Application of RS-056812-198 at concentrations up to 10 μM does not evoke any ion current in oocytes expressing 5-HT_3R-A_L ($n = 7$), in which the application of 10 μM 5-HT causes a large transient ion current. Instead, 100 nM RS-056812-198 inhibits the 5-HT-evoked current completely within 30 s and this effect is reversed after approximately 8 min of washing (inset Fig. 2). The 5-HT-evoked current in oocytes expressing 5-HT_3R-A_S is also completely inhibited by 100 nM RS-056812-198 (not shown). Fig. 2 shows the concentration dependence of the inhibition of the 5-HT-evoked current by RS-056812-198 in oocytes expressing 5-HT_3R-A_L. The IC_{50} and the Hill slope amount to 0.43 ± 0.08 nM and -0.70 ± 0.08, respectively ($n = 3$), indicating that RS-056812-198 is a high-affinity antagonist on homo-oligomeric 5-HT_3 receptors in *Xenopus* oocytes.

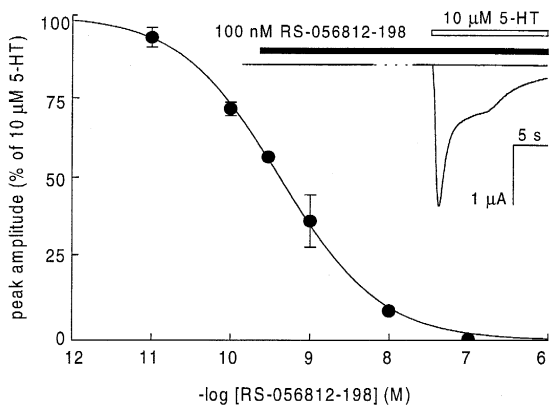

Fig. 2. Concentration-effect curve of the inhibition of the 10 μM 5-HT-induced current by RS-056812-198 in *Xenopus* oocytes expressing 5-HT_3R-A_L. Current amplitudes are expressed relative to the amplitude of the 10 μM 5-HT-induced current recorded from the same cell. Each point represents the mean (±S.D.) of the values obtained from 3 oocytes. Absence of error bars indicates that the standard deviation is smaller than the symbol size. The inset shows that application of 100 nM RS-056812-198 does not evoke a detectable ion current. After 30 s of superfusion with RS-056812-198, subsequent application of 10 μM 5-HT does not evoke an ion current. The inhibition by RS-056812-198 is reversed after 8 min of washing with external solution, as shown by the 5-HT-induced current trace superimposed. The solid and open bars indicate the periods of RS-056812-198 and 5-HT application, respectively.

4. Discussion

The results demonstrate that RS-056812-198 is a high-affinity partial agonist on 5-HT_3 receptors in N1E-115 neuroblastoma cells. RS-056812-198 and 5-HT act on the same population of 5-HT_3 receptors, as shown by the cross-desensitization experiments (Fig. 1B), but the efficacy of RS-056812-198 is only 15% of that of 5-HT. Despite the higher affinity of RS-056812-198, the ion currents induced by RS-056812-198 activate more slowly than those induced by 5-HT. Assuming that the activation and deactivation time constants of the RS-056812-198-induced current represent the upper limits of association and dissociation of RS-056812-198 to and from the receptor, respectively, the apparent affinity of RS-056812-198 was calculated and shown to be similar to the EC_{50} value obtained from the concentration-effect curve. This suggests that slow association is the rate-limiting step in the activation of the RS-056812-198-induced current. Therefore, the slow activation appears to reflect a property of the drug-receptor interaction. The EC_{50} value of RS-056812-198 to depolarize rat vagus nerve of 35 nM (Clark et al., 1995) is in the same low concentration range as the presently obtained values (12–18 nM), consistent with the similar pharmacological profiles of 5-HT_3 receptors in mouse N1E-115 neuroblastoma cells and rat vagus nerve (Hoyer and Neijt, 1988).

Partial 5-HT_3 receptor agonists studied thus far have lower affinities than full agonists (Neijt et al., 1986; Sepúlveda et al., 1991; Van Hooft and Vijverberg, 1996). However, the affinity of the partial agonist RS-056812-198 is comparable to that of the more potent full agonists, e.g., 2,3,5-trichlorophenylbiguanide with an EC_{50} of 27 nM (Morain et al., 1994). Therefore, the high-affinity partial agonist RS-056812-198 is a valuable tool to investigate agonist interaction with the 5-HT_3 receptor in both electrophysiological and radioligand binding studies.

Thus far, comparison of the biophysical and pharmacological properties of native and cloned 5-HT_3 receptors have revealed no gross differences (Hussy et al., 1994), apart from the observation that the cloned 5-HT_3 receptor is potentiated by low concentrations of zinc and the native receptor is not (Gill et al., 1995). The present results show that the novel high-affinity 5-HT_3 receptor ligand RS-056812-198 discriminates between cloned and native 5-HT_3 receptors by acting as a partial agonist on the 5-HT_3 receptors native to N1E-115 neuroblastoma cells and as an antagonist on homo-oligomeric 5-HT_3 receptors expressed in *Xenopus* oocytes. This qualitative difference, together with the marked difference in the apparent affinity of agonist (18 nM) and antagonist (0.4 nM) effects (Fig. 1C and Fig. 2), and the rapid block in oocytes as compared to the slow desensitization in N1E-115 cells strongly indicate receptor heterogeneity. It has been reported before that the pharmacological profile of 5-HT_3 receptors in various tissues is different (Richardson and Engel, 1986; Bonhaus et al., 1993), implicating the existence of distinct 5-HT_3 receptors. Here we have investigated 5-HT_3 receptors in N1E-115 cells and oocytes, expressing the same subunits (Hope et al., 1993). Whether the presently found difference is caused by differences in the processing of 5-HT_3 receptor proteins in N1E-115 cells and oocytes or by expression of additional, yet unidentified subunits in N1E-115 cells and consequent formation of hetero-oligomeric 5-HT_3 receptors remains to be determined.

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