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Concentration of receptor and ligand revisited in a modified receptor binding protocol for high-affinity radioligands: [^{3}H]Spiperone binding to D_2 and D_3 dopamine receptors

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ABSTRACT

In receptor binding assays with ultra-high-affinity radioligands, it is difficult, in practice, to adhere the golden rule that the receptor concentration in the assay should be substantially (at least 10-fold) lower than the dissociation constant (K_d) of the radioligand and inhibition constant (K_i) of compound. Especially for low specific activity radioligands (usually tritiated ligands of a couple of TBq/mmol), routinely applied in concentrations at around or below the K_d, the use of extremely small amounts of receptor protein per assay will result in low levels of bound radioactivity; the alternative use of larger assay volumes will make it difficult to apply 96-well filtration devices. For assessing the inhibition constant (K_i) of competitive inhibitors under conditions violating the above golden rule, equations are available incorporating both [receptor] and [ligand] versus K_d; however, their application requires precise knowledge of [receptor] or initial bound/free [radioligand] ratio. In this study, we present the theoretical basis for determining the K_i for a competitive inhibitor in a new protocol at high [protein] and high [radioligand] with the simple Cheng–Prusoff correction without the need to correct for [receptor] or initial bound/free [radioligand] ratio. In addition, we present results on the binding of the ultra-high-affinity ligand [^{3}H]spiperone to dopamine D_2 and D_3 receptors validating the K_i values calculated with the new protocol for competitive inhibitors as compared with those calculated with the most comprehensive equation available to date, that of Munson and Rodbard (1988). Binding was measured at varying [radioligand] and [receptor], test compounds (including (−)5-OH-DPAT, (±)7-OH-DPAT, and ropinirole) were run with varying [receptor], and simulations were done at vastly varying [radioligand] for inhibitors with vastly different K_is. The modified high [radioligand] protocol presented here removes a major hindrance in the proper execution of binding assays with ultra-high-affinity tritiated ligands with K_d values in the sub-nanomolar range, allowing the use of 96-well plates with small volumes of 100–200 μl per binding assay.

1. Introduction

Dopamine (DA) receptors are G-protein-coupled receptors broadly categorized as D_1-like (stimulating adenylate cyclase) or D_2-like (inhibiting adenylate cyclase). Within the latter category, D_2 and D_3 subtype DA receptors have been studied abundantly as targets for antipsychotic and antiparkinsonian medications, respectively (Sokoloff et al., 1992; Girault and Greengard, 2004; McCall et al., 2005; Strange, 2008). Although D_2 and D_3 receptors are regulated differently, they share a similar molecular structure and pharmacological profile (Sokoloff et al., 1990; Werner et al., 1996; Levant, 1997). Site-directed mutagenesis and molecular modeling studies have provided insight into differences in the ligand-binding domain of D_2 and D_3 receptors (Mansour et al., 1992; Sokoloff et al., 1992; Lundstrom et al., 1998; Watts et al., 1998; Sartania and Strange, 1999; Strange, 2001; Dutta et al., 2002; Mach et al., 2003; McCall et al., 2005; Saur et al., 2007).

Because of the similarity between D_2 and D_3 receptors, development of compounds selective for one over the other receptor subtype is challenging. An essential component of the drug development process is the ability to test the receptor potency of compounds with a protocol that is devoid of artifactual distortions due to improper conditions for the receptor under study. In the case of D_2 and D_3 receptors, competitive inhibition of [^{3}H]spiperone binding has been used widely to measure compound affinities. Most antipsychotics, including spiperone, bind to both D_2 and D_3 receptors with high affinity, i.e. nanomolar or subnanomolar K_d (equilibrium dissociation constant) (Gardner et al.,

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1996; Strange, 2001). A ground rule for receptor binding studies with competitive inhibitors is that the concentration of radioactive ligand in relation to its K_d must be taken into account in converting the IC_{50} (concentration of compound to inhibit 50% of radioligand binding) to K_i (equilibrium dissociation constant of inhibitor) with the Cheng–Prusoff correction (Cheng and Prusoff, 1973). It is important to recall that in the Cheng–Prusoff equation, IC_{50} denotes the free (not totally added) concentration of inhibitor giving 50% inhibition, and the concentration of radioactive ligand is the free (not totally added) concentration. Differences between free and totally added concentrations can be minimized – creating valid conditions for applying the Cheng–Prusoff equation – by making sure that less than 10% of the total added radioligand is actually bound (zone A binding, see Bennett and Yamamura, 1985), and that the receptor concentration in the assay is substantially (at least 10-fold) lower than the K_d or K_i of radioligand or inhibiting compound, respectively (Chang et al., 1975; Bennett and Yamamura, 1985). In order to minimize the impact of the Cheng–Prusoff correction, most researchers also choose a radioligand concentration at or below its K_d. In the case of [^{3}H]spiperone or other tritiated ultra-high-affinity ligands that bind with a subnanomolar K_d, the above considerations necessitate the use of extremely low receptor concentrations that result in low levels of bound radioactivity with routinely used binding assay volumes of 0.2 ml or less in 96-well filtration devices combined with 96-format plate scintillation counting. With deep-well plates and some harvesters, one can increase the assay volume from 0.2 to 0.8 ml in order to get a higher amount of bound radioactivity without increasing the receptor concentration but this factor of 4 is not always enough for obtaining sufficient counts for proper counting; deep-well plates are also harder to work with in terms of making additions and ensuring proper mixing of assay components. One could, for ultra-high-affinity ligands, decide to increase the receptor concentration beyond the recommended 10% of K_d, and compute the K_i for a competitive inhibitor by applying equations that take into account the receptor concentration (Jacobs et al., 1975; Linden, 1982). This is problematic in experiments with brain tissue where the receptor density is not known unless specifically measured, and even in cell lines stably expressing receptors where receptor density can be affected by cell culture confluence, culture time and passage number, as well as culture medium conditions. The most comprehensive treatment of ligand and receptor concentration available to date is with an equation that requires the initial (without inhibitor) ratio of bound over free radioligand rather than the receptor concentration (Munson and Rodbard, 1988). The Munson–Rodbard equation shows that high receptor concentrations by themselves (even with radioligand concentrations substantially below K_d), can result in high corrections factors going from IC_{50} to K_i. For example, with an initial bound over free ratio of 5, radioligand over K_d ratio of 0.1, and $K_i \sim K_d$, the correction factor is 10. Day-to-day variation in initial bound over free will have substantial impact on the computed K_i values.

In this study, we present the theoretical basis for determining the K_i for a competitive inhibitor at high receptor protein and high radioligand concentration with the simple Cheng–Prusoff correction without the need to correct for receptor concentration or initial bound over free. In addition, we present results on [^{3}H]spiperone binding to DA D_2 and D_3 receptors validating the K_i values calculated in this manner as compared with those calculated with the Munson–Rodbard equation (1988). Simulations were run at vastly varying [radioligand] for inhibitors with vastly different K_is for assessing the accuracy of the new protocol. The method presented here allows small-volume assays in 96-well format and can be applied to other ultra-high-affinity radioligand binding studies.

2. Materials and methods

2.1. Materials

Human embryonic kidney (HEK) 293 cells, stably transfected with rat D_{2L} and rat D_3 receptor cDNAs, were gifts from Dr. Kim Neve (Portland VA Medical Center, Portland, Oregon). They were cultured in Dulbecco's modified Eagle's medium, supplemented with of 10% bovine calf serum, 2 mM glutamine and 2 μg/ml puromycin to keep selection pressure at 37 °C and 5% CO_2. [^{3}H]spiperone (15 Ci/mmol) was purchased from Perkin-Elmer (Waltham, MA). (−)5-OH-DPAT, (±)7-OH-DPAT, ropinirole, D341, D342, D343 and D344 were synthesized by Dr. Aloke Dutta (Wayne State University, Detroit, MI). Other chemicals were provided by commercial sources.

2.2. [^{3}H]Spiperone binding assays

"Hot saturation" assays were performed to determine the dissociation constant of [^{3}H]spiperone to DA D_2 and D_3 receptors. Crude membranes were prepared from HEK293-rD_2 and HEK293-rD_3 cells, as described before (Zhen et al., 2004). In a total reaction volume of 0.8 ml, [^{3}H]spiperone (0.3 pM to 3 nM, final concentration) and crude membranes were incubated together in assay buffer (50 mM Tris–HCl, pH 7.4, with 0.9% NaCl, 0.025% ascorbic acid and 0.001% bovine serum albumin) for 1 h at 30 °C in deep-well plates in a water-bath shaker. (+)-Butaclamol (2 μM) was used as non-specific binding definition for each concentration of [^{3}H]spiperone. Assays were terminated by the addition of ice-cold 0.9% saline and filtration through a glass fiber filtermat in the Brandel-96 cell harvester. The filtermat was being washed three times and dried under hot air. The radioactivity was counted in a Microbeta liquid scintillation counter (Perlin-Elmer) after adding 10 ml of Betaplate Scint (Perkin-Elmer) to each filtermat. In competitive inhibition assays, appropriate concentrations of non-radioactive spiperone were included. Kinetic parameters (K_d, B_{max} and IC_{50}) were calculated using the computer-fitting programs LIGAND (Biosoft, Cambridge, UK) and ORIGIN (Microcal Software, Inc., Northampton, MA, USA). Protein level was determined with the Lowry method (see Zhen et al., 2004). IC_{50} values were converted to inhibition constants (K_i) by the Cheng–Prusoff equation (Cheng and Prusoff, 1973) and Munson–Rodbard equation (Munson and Rodbard, 1988). To validate the modified protocol, the reaction volume was reduced from 0.8 ml (deep-well plates) to 0.2 ml (regular plates) with the increased concentration of [^{3}H]spiperone and protein level under conditions as described in the text.

2.3. Data simulation of competitive inhibition with random error added

Competitive inhibition was simulated to examine the accuracy of the modified binding protocol with the high concentration of radioligand. The K_d was set at 1 nM; K_i values were calculated as described by Cheng and Prusoff (1973) and Munson and Rodbard (1988) with varying concentrations of receptor and radioligand. The ratio of Cheng–Prusoff K_i to Munson–Rodbard K_i was calculated to judge the error introduced to K_i when [receptor] was higher than K_i. In a different simulation set, competition curves were generated based on a one-binding site model with relative random error added as follows. In the presence of inhibitor (K_i = 1 nM), we chose three conditions for generating binding curves, i.e. at concentrations of radioligand equal to 0.1 nM, 1 nM or 10 nM. Under all the conditions, the initial bound/free ratio was set to 0.1. The actual random values of bound equaled to the sum of the theoretic values and random errors calculated from a Gaussian distribution with a relative S.D. equal to 10% with the GraphPad Prism software (La Jolla,