Observed Drug-Receptor Association Rates Are Governed by Membrane Affinity: The Importance of Establishing “Micro-Pharmacokinetic/Pharmacodynamic Relationships” at the $\beta_2$-Adrenergceptor\(^{\text{1521}}\)

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ABSTRACT

Current pharmacological models for determining affinity and kinetics of drugs for membrane receptors assume the interacting molecules are homogeneously distributed in the bulk aqueous phase. The phospholipid membrane can, however, provide a second compartment into which drugs can partition, particularly lipophilic/basic compounds. In this study we measured the phospholipid affinity and receptor binding kinetics of several clinically relevant $\beta_2$-adrenergceptor agonists and antagonists and demonstrated that the degree of phospholipid interaction directly affects the observed kinetic association rate ($k_{\text{on}}$) and dissociation constant ($K_d$), but not the dissociation rate ($k_{\text{off}}$) from the target, by concentrating drug in the local environment around the receptor. When the local drug concentration was accounted for, the $k_{\text{on}}$ was comparable across the cohort and the corrected $K_d$ was directly related to the $k_{\text{off}}$. In conclusion, we propose a new approach to determining the pharmacology of drugs for membrane targets that accounts for differences in local drug concentration brought about by direct affinity for phospholipids, establishing “micro-pharmacokinetic/pharmacodynamic relationships” for drugs.

Introduction

The affinity and kinetics of a drug binding to its target receptor are quantities fundamental to our understanding of structure activity relationships, allowing more rational drug design. These values are almost exclusively calculated using equations that assume the interacting molecules are homogeneously distributed in a solvent, with the concentration of drug available to bind target being equal to that in the bulk aqueous phase. While this assumption applies well to soluble enzymes, it is less satisfactory for membrane-associated targets (e.g., G protein–coupled receptors), where the protein is embedded in a phospholipid bilayer. This is because the inclusion of phospholipid adds an additional amphiphilic compartment into which drugs may partition, depending on their physicochemical properties. Partitioning of a drug into the membrane compartment not only potentially depletes drug from the aqueous compartment but also concentrates drug in the local environment around the receptor, which may alter the observed pharmacology of a compound (Sargent and Schwyzser, 1986), particularly if the drug is able to access the target receptor from this compartment.

For many G protein–coupled receptors the binding site is deeply embedded within the central pocket formed by their seven transmembrane–spanning $\alpha$-helical domains. Traditionally this pocket is thought to be accessible to hydrophilic ligands that reside in the aqueous phase, but it has also been proposed that membrane-associated amphiphilic and lipophilic ligands could still gain access to these pockets via lateral diffusion between the $\alpha$-helical transmembrane domains of the receptor, e.g., for the $\beta_2$-adrenergceptor, cannabinoid, and opsin receptors, and also ion channels (Rhodes et al., 1985; Mason et al., 1991; Hildebrand et al., 2009, Hurst et al., 2010). More recently, crystal structures of the sphingosine-1-phosphate (S1P) receptor suggest an initial interaction of S1P with the outer leaflet of the cell membrane, which is followed by lateral diffusion of S1P into the binding pocket, thereby effectively circumventing an aqueous entry pathway (Hanson et al., 2012). It is not necessary, however, to evoke a direct lipid pathway for accessing the receptor, because hydrophilic basic ligands may merely concentrate at the membrane-water interface through electrostatic interactions with phosphate head groups without...
necessarily fully embedding within the membrane (Avdeef et al., 1998). In this way they would still be able to readily access an aqueous entry pathway.

In addition to the concentrating effects of membrane-associated drug, lateral diffusion of drug across the two-dimensional surface (rather than three dimensions in aqueous bulk) could also increase reaction rates with receptors in a mechanism referred to as “reduction of dimensionality” rate enhancement (McCloskey and Poo, 1986). The result would be a further enhancement of association rates and corresponding affinity values for lipophilic/basic drugs.

The aim of this work was to investigate the kinetic properties of several clinically relevant β2-adrenoceptor agonists and antagonists with widely varying physicochemical properties to determine if a relationship exists between observed affinity and degree of membrane interaction. The association and dissociation rates of β2-adrenoceptor ligands were measured using a kinetic competition radioligand binding method (Motulsky and Mahan, 1984; Sykes and Charlton, 2012). The interaction of drugs with membrane phospholipids is multifaceted, driven not only by lipophilicity (logD) but also electrostatic interactions with both acidic and basic moieties on the phosphate head groups. We therefore used an artificial immobilized phospholipid column (Baur et al., 2010) to derive a macroscopic membrane partition coefficient (logKIAM) that encapsulates all the different ligand–phospholipid interactions into a single coefficient for each drug. This partition coefficient was then used to estimate the local concentration of drug in the membrane.

**Materials and Methods**

[3H]DHA (1-[4,6-propyl-3H]dihydroalprenolol, specific activity 91 Ci·mmol⁻¹) was obtained from PerkinElmer Life and Analytical Sciences (Beaconsfield, UK). Ninety-six-deep well plates and 500 cm² culture plates were purchased from Fisher Scientific (Loughborough, UK). Millipore 96-well GF/B filter plates were purchased from Receptor Technologies (Warwick, UK). Sodium bicarbonate, ascorbic acid, EDTA, sodium chloride, GTP, bisoprolol hemifumarate, (S)-atenolol, labetalol hydrochloride, (±) metoprolol, carvedilol, (S)-propranolol hydrochloride, salmeterol xinafoate, CI1118,551 hydrochloride, (±) sotalol hydrochloride, nadolol, (S)-cyanopindolol hemifumarate and formoterol fumarate, were obtained from Sigma Chemical Co Ltd. (Poole, UK). Bucindolol, (S)-timolol maleate and CGP12177 hydrochloride were obtained from Tocris Cookson, Inc. (Bristol, UK). All cell culture reagents including Hanks' balanced salt solution and HEPES were purchased from Gibco (Invitrogen, Paisley, (Bristol, UK). All cell culture reagents including Hanks' balanced salt solution, pH 7.4, 0.01% ascorbic acid, and 100 μM GTP. GTP was included to remove the G protein–coupled population of receptors that can result in two binding sites in membrane preparations, because the Motulsky–Mahan model is only appropriate for ligands competing at a single site. In all cases, non-specific binding was determined in the presence of 1 μM propranolol. After the indicated incubation period, bound and free radiolabels were separated by rapid vacuum filtration using a FilterMate Cell Harvester (PerkinElmer Life and Analytical Sciences) onto 96-well GF/B filter plates (Millipore, Watford, UK) previously coated with 0.5% (w/v) polyethyleneimine and rapidly washed three times with ice-cold 75 mM HEPES, pH 7.4. After drying (>4 hours), 40 μl of Microscint 20 (PerkinElmer Life and Analytical Sciences) was added to each well and radioactivity was quantified using single-photon counting on a TopCount microplate scintillation counter (PerkinElmer Life and Analytical Sciences). Aliquots of radiolabel were also quantified accurately to determine how much radioactivity was added to each well using liquid scintillation spectrometry on an 300SLL scintillation counter (LabLogic, Sheffield, UK). In all experiments, total binding never exceeded more than 10% of that added, limiting complications associated with depletion of the free radioligand concentration (Carter et al., 2007).

**Saturation Binding Studies**

CHO cell membranes containing the β2-adrenoceptor were incubated in 96–deep well plates at 37°C in assay binding buffer with a range of concentrations of [3H]DHA (3 – 0.001 nM) at 15 μg per well, for 180 minutes with gentle agitation to ensure equilibrium was reached. Saturation binding was performed in a final assay volume of 1.5 ml to avoid significant ligand depletion.

**Determination of the Association Rate and Dissociation Rate of [3H]DHA**

To accurately determine association rate (k.on) and dissociation rate (k.off) values, observed rate of association (k(obs)) was calculated using at least three different concentrations of [3H]DHA. The appropriate concentration of radioligand was incubated with β2-adrenoceptor CHO cell membranes (15 μg per well) in assay binding buffer with gentle agitation (final assay volume, 1000 μl). Exact concentrations were calculated in each experiment by liquid scintillation counting. Free radioligand was separated by rapid filtration at multiple time points to construct association kinetic curves as described previously by Sykes and Charlton (2012). The resulting data were globally fitted to the association kinetic model (eq. 2) to derive a single best-fit estimate for k.on and k.off as described under “Data Analysis.”

**Determination of Dissociation Constants**

To obtain affinity estimates of unlabeled ligand, [3H]DHA competition experiments were performed at equilibrium. [3H]DHA was used at a concentration of approximately 0.6 nM (~25 000 cpm final assay volume of 0.5 ml), such that the total binding never exceeded more than 10% of that added. Radioligand was incubated in the presence of the indicated concentration of unlabeled ligand and CHO cell membranes (15 μg per well) at 37°C, with gentle agitation for 180 minutes.

**Competition Binding Kinetics**

The kinetic parameters of unlabeled ligand were measured using a competition kinetic binding assay originally described by Motulsky and Mahan (1984) and developed for the β2-adrenoceptor by Sykes and Charlton (2012). This approach involves the simultaneous addition of both radioligand and competitor to receptor preparation, so that at t = 0 all receptors are unoccupied.

Approximately 0.6 nM [3H]DHA (a concentration which avoids ligand depletion in this assay volume) was added simultaneously with the unlabeled compound (at t = 0) to CHO cell membranes containing the human β2-adrenoceptor (15 μg per well) in 500 μl of assay buffer.

**Cell Culture and Membrane Preparation**

Chinese hamster ovary (CHO) cells stably transfected with the human β2-adrenoceptor were grown adherently in Ham’s F-12 Nutrient Mix Glutamax-1, containing 10% fetal calf serum, and 0.5 mg·ml⁻¹ Geneticin (G-418). Cells were maintained at 37°C in 5% CO₂ humidified air and routinely subcultured at a ratio between 1:10 and 1:20 twice weekly using trypsin-EDTA to lift cells. Cell membranes were prepared and stored as described previously (Sykes and Charlton, 2012).

**Common Procedures Applicable to All Radioligand Binding Experiments**

All radioligand binding experiments using [3H]DHA were conducted in 96-deep well plates, in assay binding buffer, Hanks’ balanced salt solution, pH 7.4, 0.01% ascorbic acid, and 100 μM GTP. GTP was included to remove the G protein–coupled population of receptors that can result in two binding sites in membrane preparations, because the Motulsky–Mahan model is only appropriate for ligands competing at a single site. In all cases, non-specific binding was determined in the presence of 1 μM propranolol. After the indicated incubation period, bound and free radiolabels were separated by rapid vacuum filtration using a FilterMate Cell Harvester (PerkinElmer Life and Analytical Sciences) onto 96-well GF/B filter plates (Millipore, Watford, UK) previously coated with 0.5% (w/v) polyethyleneimine and rapidly washed three times with ice-cold 75 mM HEPES, pH 7.4. After drying (>4 hours), 40 μl of Microscint 20 (PerkinElmer Life and Analytical Sciences) was added to each well and radioactivity was quantified using single-photon counting on a TopCount microplate scintillation counter (PerkinElmer Life and Analytical Sciences). Aliquots of radiolabel were also quantified accurately to determine how much radioactivity was added to each well using liquid scintillation spectrometry on an 300SLL scintillation counter (LabLogic, Sheffield, UK). In all experiments, total binding never exceeded more than 10% of that added, limiting complications associated with depletion of the free radioligand concentration (Carter et al., 2007).

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Approximately 0.6 nM [3H]DHA (a concentration which avoids ligand depletion in this assay volume) was added simultaneously with the unlabeled compound (at t = 0) to CHO cell membranes containing the human β2-adrenoceptor (15 μg per well) in 500 μl of assay buffer.
The degree of [3H]DHA bound to the receptor was assessed at several time points by filtration harvesting and liquid scintillation counting, as described previously. Nonspecific binding was determined as the amount of radioactivity bound to the filters and membrane in the presence of propanolol (1 μM) and was subtracted from each time point, meaning that t = 0 was always equal to zero. Each time point was conducted on the same 96-deep well plate incubated at 37°C with constant agitation. Reactions were considered stopped once the membranes reached the filter, and the first wash was applied within 1 second. A single concentration of unlabeled competitor was tested, as rate parameters were shown to be independent of unlabeled ligand concentration (data not shown). All compounds were tested at either 1-, 3-, 10-, or 100-fold of their respective Kᵢ, and data were globally fitted using eq. 3 to simultaneously calculate kᵦᵣ and kᵦᵣ. Different ligand concentrations were chosen as compounds with a long residence time and equilibrate more slowly, so a higher relative concentration is required to ensure the experiments reach equilibrium within a reasonable time frame (90 minutes), while still maintaining a good signal-to-noise. The actual concentrations used were selected from a preliminary experiment using three different concentrations of each ligand (data not shown).

LogD<sub>7.4</sub> and Immobilized Artificial Membrane Chromatography

All high-performance liquid chromatography experiments were carried out in gradient mode using 100% 50 mM ammonium acetate (pH 7.4) buffer as mobile phase A and 100% acetonitrile as mobile phase B. High-performance liquid chromatography logD<sub>7.4</sub> was measured on a 5-cm × 3-mm, 3-μm Gemini C<sub>18</sub> column following a methodology originally described by K erns et al. (2003). The gradient was: 0 minutes/0% B, 2.5 minutes/95% B, 4.0 minutes/95% B, 4.1 minutes/0% B, 5.5 minutes/0% B. The mobile-phase flow rate was 1.0 ml/min. A set of standards (atenolol, metoprolol, labetolol, diltiazem, triprohenylene) were selected, and a calibration graph of retention time versus LogD<sub>7.4</sub> (as described in K erns et al., 2003) was constructed.

Immobilized artificial membranes (IAMs) consist of monolayers of phospholipid covalently immobilized on a silica surface, mimicking the lipid environment of a fluid cell membrane on a solid matrix. The lipophilicity index obtained from IAM chromatography is the capacity factor logK<sub>IAM</sub> in 100% aqueous phase. For hydrophilic compounds, logK<sub>IAM</sub> can be determined directly by using the aqueous mobile phase. For lipophilic drugs it is necessary to add an organic modifier, in this case acetonitrile, to the mobile phase to facilitate the elution by the use of gradient elution. A method described by V alko et al. (2000) was used to measure the chromatographic hydrophobicity index for immobilized artificial membrane chromatography at pH 7.4 (CHI IAM<sub>7.4</sub>) values of all test drugs on a 10-cm × 4.6-mm, 10-μm Regis IAM PC DD2 column (where the stationary phase support is immobilized phosphatidylcholine). The gradient was: 0 minutes/0% B, 6.0 minutes/100% B, 6.5 minutes/100% B, 7.0 minutes/0% B, 9.0 minutes/0% B. The mobile phase flow rate was 1.0 ml/min. With a set of alkyphenone standards, (acetophenone, propiophenone, valerophenone, octanophenone) the gradient retention times can be converted to CHI IAM<sub>7.4</sub>, which approximates to an acetonitrile concentration at which an equal distribution of compound can be achieved between the mobile phase and IAM. The gradient method is an ideal way to assess phosphatidylcholine affinity as some samples retain extremely well on the phosphatidylcholine stationary phase and are extremely difficult to accurately analyze on a 100% aqueous mobile phase. Additionally, the gradient CHI IAM<sub>7.4</sub> values were used to calculate a logK<sub>IAM</sub> extrapolated value (value of retention factor with 100% buffer-only aqueous phase) as described by V alko et al. (2000).

Data Analysis and Statistical Procedures

As the amount of radioactivity varied slightly for each experiment (<5%), data are shown graphically as the mean ± range for individual representative experiments, whereas all values reported in the text and tables are mean ± S.E.M. for the indicated number of experiments unless otherwise stated. All experiments were analyzed by either Deming regression or nonlinear regression using Prism 5.0 (GraphPad Software, San Diego, CA).

**Competition Binding.** Competition displacement binding data were fitted to sigmoidal (variable slope) curves using a four-parameter logistic equation:

\[
Y = \text{Bottom} + \left(\text{Top} - \text{Bottom}\right) \left(1 + 10^\left(\log IC_{50} - X\right)\right) \cdot \text{HillSlope}
\]  

(1)

IC<sub>50</sub> values obtained from the inhibition curves were converted to Kᵢ values using the method of Cheng and Prusoff (1973).

**Association Binding.** [3H]DHA association data were globally fitted to eq. 2, where L is the concentration of radioligand in nM using GraphPad Prism 5.0 to determine a best-fit estimate for kᵦᵣ and kᵦᵣ:

\[
k_{ob} = \left|L\right| \cdot k_{ob} + k_{af}
\]  

(2)

**Competition Kinetic Binding.** Association and dissociation rates for unlabeled agonists were calculated using the equations described by Motulsky and Mahan (1984) using a global fitting model:

\[
K_A = k_1 \left[L \cdot k_2 \right]
\]

\[
K_B = k_3 \left[I \cdot k_4\right]
\]

\[
S = \sqrt{\left(\left(K_A - K_B\right) \cdot 2 + 4 k_1 k_2 L \cdot 10^{-10}\right) + K_B + K_A + S - K_A - K_B + S} - \sqrt{K_A + K_B - S}
\]

\[
\text{DIFF} = K_P - K_S
\]

\[
Q = \frac{P_{max} \cdot K_L \cdot 10^{-9}}{\text{DIFF} + k_3 \cdot k_2 \cdot \text{exp}\left(-k_2 X\right) - k_2 \cdot k_3 \cdot k_4 \cdot \text{exp}\left(-k_3 X\right)}
\]

where X is time (min), Y is specific binding (cpm), k₁ is kᵦᵣ [3H]DHA, k₂ is kᵦᵣ [3H]DHA, I is the concentration of [3H]DHA used (nM) and L is the concentration of unlabeled agonist (nM). Fixing the above parameters allowed the following to be simultaneously calculated: B<sub>max</sub> is total binding (cpm), k₃ is association rate of unlabeled ligand (M⁻¹ min⁻¹) or kᵦᵣ, and k₄ is the dissociation rate of unlabeled ligand (min⁻¹) or kᵦᵣ.

**Using K<sub>IAM</sub> to Estimate Concentration of Ligand in the Membrane Compartment and Generate “Corrected” kᵦᵣ Values.** To correct for the increased concentration of drug in the membrane we first estimated how much each ligand concentrates in the membrane and then used this new concentration to calculate a “corrected” kᵦᵣ. K<sub>IAM</sub> is a partition coefficient that essentially describes the degree to which a ligand concentrates from aqueous into the phospholipid compartment, so we estimated membrane concentration by simply multiplying the ligand concentration in the aqueous compartment by the K<sub>IAM</sub>. For example, if a ligand is at an aqueous concentration of 1 nM, then a K<sub>IAM</sub> of 100 (log K<sub>IAM</sub> of 2) would result in 100 nM in the membrane. We then used this new concentration to recalculate kᵦᵣ by substituting it into the Motulsky–Mahan equation during the fitting process instead of using the aqueous concentration. kᵦᵣ is not affected by changing the competitor concentration in the fitting process.

**Linear Correlations.** The correlation between datasets was determined by calculating a Pearson correlation coefficient (r<sup>2</sup>) in GraphPad Prism 5.0.

**Results.** Equilibrium and Kinetic Binding Parameters for β₂-Adrenoceptor Ligands. Initially the binding affinity of the β₂-adrenoceptor ligands (shown in Fig. 1) was measured at
equilibrium in Hanks’ buffered saline solution containing GTP (0.1 mM) at 37°C to ensure that agonist binding only occurred to the uncoupled form of the receptor. Binding affinities (Ki values) are summarized in Table 1 and associated curves are presented in Fig. 2. To determine the association and dissociation rates of the β2-adrenoceptor ligands, we used a competition kinetic radioligand binding assay as previously described by Sykes and Charlton (2012). First we characterized the binding kinetics of the radiolabeled ligand [3H]DHA by monitoring the observed association rates at four different ligand concentrations (Fig. 3A). The observed rate of association was related to [3H]DHA concentration in a linear fashion (Fig. 3B). Kinetic rate parameters for [3H]DHA were calculated by globally fitting the association time courses, resulting in a kon of 1.83 ± 0.13 × 109 M⁻¹ min⁻¹ and a koff of 0.23 ± 0.02 min⁻¹. The resulting Kd (koff/kon) of 125 ± 7 pM was comparable to that obtained from equilibrium saturation experiments (83 ± 11 pM).

Representative kinetic competition curves for the β2-adrenoceptor ligands are shown in Fig. 4, A–D. Progression curves for [3H]DHA alone and in the presence of competitor were globally fitted to eq. 3 enabling the calculation of both kon (k3) and koff (k4) for each of the ligands, as reported in Table 1. There was a very wide range in dissociation rates for the different ligands, with t1/2 values between 0.1 minutes for bisoprolol to 5.8 hours for cyanopindolol. To validate the rate constants, the kinetically derived dissociation constant (Kd) values (koff/kon) were compared with the dissociation constant (Ki) obtained from equilibrium competition binding experiments (Fig. 5). There was a very good correlation (r² = 0.99, P < 0.0001) between these two values, indicating the kinetic parameters were accurate.

**Measurements of Lipophilicity and Membrane Interactions.** The calculated logP (clogP) and the measured partition coefficient logD₇.₄ are detailed in Table 2. There was a good correlation between the calculated and measured values (r² = 0.91, P < 0.0001), although in most cases logD₇.₄ was lower than clogP. To assess the degree of membrane interaction a chromatographic method was applied (Jiang et al., 2008). This method utilizes IAMs consisting of monolayers of phospholipid covalently immobilized on a silica surface, mimicking the lipid environment of a fluid cell membrane on a solid matrix. Compounds with longer retention times on this column have higher affinity for phospholipids and will have a higher calculated membrane partition coefficient, denoted KIAM (see Materials and Methods for full description).
The log\(K_{IAM}\) values for the \(\beta_2\)-adrenoceptor ligands were measured at pH 7.4 and are summarized in Table 2. There was a good correlation between these values and both the clogP (\(r^2 = 0.85, P < 0.0001\)) and log\(D_{7.4}\) (\(r^2 = 0.89, P < 0.0001\); Supplemental Fig. 1), although the values for log\(K_{IAM}\) were higher in almost all cases.

### Relationship between Kinetics and Membrane Interactions

The dissociation rate of a drug is not dependent on drug concentration so should be independent of the affinity of interaction with the membrane. Reassuringly, when the \(k_{off}\) for each compound was compared with either its log\(D_{7.4}\) or log\(K_{IAM}\) no correlation was observed (\(P > 0.05\), Fig. 6A and B, respectively). In contrast, the \(k_{on}\) is calculated from the observed on rate (\(k_{obs}\), which is highly dependent on concentration of drug. As such, it might be expected that an increase in the local concentration of compound might influence the calculated \(k_{on}\) with drugs possessing a high membrane affinity appearing to have a more rapid association rate. As predicted, the test-compound log\(D_{7.4}\) values were significantly correlated (\(r^2 = 0.67, P < 0.0001\)), with \(k_{on}\) determined in the competition kinetic assay (Fig. 6C) and lipophilic compounds having a faster association rate. Furthermore, an improved correlation was observed when log\(K_{IAM}\) were correlated with \(k_{on}\) (Fig. 6D, \(r^2 = 0.81, P < 0.0001\)), confirming that drug interactions with phospholipids are complex and cannot be completely mimicked by simple isotropic models. This was supported by comparisons between clogP and \(k_{on}\), which also show a poorer correlation than observed with \(K_{IAM}\) (\(r^2 = 0.71, P < 0.0001\); data not shown).

### Correcting for Local Drug Concentration to Calculate “Corrected” \(k_{on}\) and Affinity

The observation that membrane affinity correlates with our calculated association rate suggests that the concentration of drug in the environment close to the receptor is different from what we assume is in the bulk aqueous phase. To correct for this, we have used the log\(K_{IAM}\) data to estimate relative distribution of drug between the aqueous and membrane compartments and used these new concentrations of drugs in the membrane to recalculate \(k_{on}\) values for each of the compounds, denoted “corrected” \(k_{on}\.” In this calculation we have assumed that the rate of equilibration of drug with membrane is very rapid. This is supported by the work of Herbette and colleagues (1989) which suggests that partitioning would be complete within 0.5 seconds.

The corrected \(k_{on}\) values are compared with the original observed association rates in Fig. 7A. In contrast to the measured \(k_{on}\) values that vary by up to three orders of magnitude, there was little difference in the corrected \(k_{on}\) values across the whole

**Fig. 2.** Competition binding between \(^{3}H\)DHA and \(\beta_2\)-adrenergic ligands for human \(\beta_2\)-adrenoceptors expressed in the CHO cells in the presence of GTP. Displacement of \(^{3}H\)DHA (0.6 nM) by increasing concentrations of (A) bisoprolol, bucindolol, atenolol, labetalol, metoprolol, carvedilol, propranolol, salmeterol, and (B) timolol, ICI 118,551, sotalol, (S)-cyanopindolol, nadolol, CGP12177. Data are presented as the mean ± range from a representative of three experiments performed in duplicate.
range of \(\log K_{IAM}\) (also see Table 2). This suggests that the very rapid association rate observed for some of the ligands is likely to be an artifact of the higher local concentrations of compounds, driven by high phospholipid affinity.

This also suggests that the observed \(K_d\) of these compounds may be artificially high. We therefore used these new corrected \(k_{on}\) values to calculate a corrected \(K_d\), using \(k_{off}\)/corrected \(k_{on}\) (shown in Table 2). Finally, we compared the corrected \(K_d\) values with our measured dissociation rates (\(k_{off}\)). As shown in Fig. 7B, there was an excellent correlation between the two parameters, suggesting that true affinity at the \(\beta_2\)-adrenoceptor is largely dependent on the dissociation rate of a ligand, while differences in association rate are mainly driven by interactions with the local environment around the receptor.

A More Practical Approach for Correcting Affinity Values. These data suggest that it could be very informative to calculate the “true” affinity of a ligand at a receptor, but the approach we have taken using measured kinetic parameters can be technically challenging and time consuming. Instead it is more common to measure the affinity at equilibrium using competition binding experiments to calculate a \(K_d\) from an IC\(_{50}\) curve. A more practical approach to correcting for local drug concentration using such data are to use the relationship \(K_d = K_d' \times K_{mem}^{-1}\), where \(K_d'\) is the local equilibrium dissociation constant (Mason et al., 1991; Castanho and Fernandes, 2006; Vauquelin and Packeu, 2009). We have used this relationship to calculate “true” \(K_d\) values from our data using eq. 4, where \(p\) is the negative logarithm (Table 2).

\[
pK_d = pK_i + pK_{IAM}
\]

(4)

These values compare very well to the corrected \(K_d\) values derived from our kinetic data (Fig. 7C; \(P < 0.0001, r^2 = 0.98\)) and represent a simpler and more practical method for determining true \(K_d\). It should be noted that this term is similar to lipophilic efficiency, which is used as a practical way of assessing the drug-like qualities of a compound (Leeson and Springthorpe, 2007). But, whereas lipophilic efficiency is an abstract descriptor comparing affinity with logP, our “true” \(K_d\) has a direct molecular origin, based on including the membrane as a compartment from which the drug may bind receptor. Importantly, it utilizes direct measurements of phospholipid interactions rather than octanol/water partition ratios.

Discussion

Our initial hypothesis was that the different physicochemical properties of drugs affect their local concentration in the vicinity of receptors embedded in biologic membranes and therefore directly influence observed pharmacology. We have measured kinetic rate parameters for a series of \(\beta_2\)-adrenoceptor ligands and compared them to the degree of interaction with phospholipids.

Direct kinetic binding studies with radiolabeled \(\beta_2\)-adrenoceptor antagonists have been performed in the past but seldom in one study and often using a variety of different buffers and temperatures, making direct comparisons difficult. To our knowledge, our current study is the first time that kinetic rate constants have been derived for a large number of \(\beta_2\) adrenoceptor antagonists and agonists in a binding assay performed at physiologic temperature and sodium concentrations in the presence of guanine nucleotide. The fastest and the slowest dissociating ligands tested in this study differed in \(k_{off}\) by up to three orders of magnitude. Interestingly we also observed a similar correlation between the equilibrium dissociation constant and \(k_{on}\) \((r^2 = 0.78, P < 0.0001)\), a phenomenon we have observed previously when studying the kinetics of \(\beta_2\) agonists (Sykes and Charlton, 2012) and muscarinic agonists (Sykes et al., 2009). This can now be rationalized on the basis of differences in the physicochemical properties of these ligands.

We have used several approaches to assess the physicochemical properties of the ligands, ranging from calculated logP to measured \(\log D_{7.4}\) and \(K_{IAM}\) for an immobilized artificial membrane. In general there was a good correlation between these values, although \(K_{IAM}\) was higher in almost all cases. This is to be expected, because although logP and \(\log D_{7.4}\) values provide information about ligand hydrophobicity, they do not account for the ability of the ligand to interact with the polar head groups of lipid membranes. For ionized drug molecules partitioning into lipid membranes is significantly enhanced over octanol, with the ionized species being strongly associated with the membrane through electrostatic interactions with the zwitterionic phospholipid. Furthermore, ionized bases tend to interact more favorably with phosphatidylcholine membranes than acids due to the anisotropic makeup of the membrane itself. Additional membrane stabilization can be
achieved if the drug species has a suitably positioned H-bond donor, due to interactions with the carbonyl groups of membranes that act as H-bond acceptors (Avdeef et al., 1998). As a consequence, the log $K_{IAM}$ values are likely to provide a more accurate estimate of drug membrane interactions than clogP and logD$_{7.4}$, although clogP may be preferred on a practical level for the simplicity of obtaining this value.

At the initiation of this study we predicted that the association rate, but not the dissociation rate, of a compound would be influenced by the magnitude of its interaction with the membrane surrounding the receptor. We have found this to be the case, with $k_{on}$ being highly correlated to $K_{IAM}$. We cannot exclude the possibility that drug binding to the $\beta_2$-adrenoceptor involves a direct lipid membrane pathway that predominates for highly lipophilic ligands, but what seems certain is that the amphipathic lipid bilayer helps to concentrate lipophilic and suitably charged ligands in the immediate vicinity of the receptor, enhancing the observed association rate, without necessarily suggesting differences in interaction at the receptor.

This is supported by literature investigating the kinetics of isomers that share identical logD$_{7.4}$ and phospholipid affinity (CHI IAM$_{7.4}$ values) (Jiang et al., 2008). Both the $S$ and $R$-isomers of CGP12177 and pindolol share the same kinetic on-rates but differ only in their measured off-rate (Affolter et al., 1985). Similar findings were also made by Hoyer et al. (1982) using the two radiolabeled enantiomers of cyanopindolol. Therefore, observed differences in $K_d$ or $K_i$ values for these enantiomeric molecules with identical physicochemical properties appear to be purely the result of differences in $k_{off}$.

By accounting for local ligand concentrations we have generated “corrected $k_{on}$” values that more likely reflect the
true rate of association at the receptor protein. We have then used these new values to calculate what we believe to be the “true” affinity of these compounds at $\beta_2$-receptors, better describing the interaction of the compound with the receptor itself. These calculations suggest that the true affinity of compounds at the receptor may be much closer than previously believed. This is well illustrated by the structurally related partial agonists salmeterol and salbutamol, which possess an identical adrenaline-mimicking saligenin-pharmacophore (see Fig. 1). Surprisingly, despite sharing the same pharmacophore, the measured affinity of salmeterol is three orders of magnitude higher than salbutamol (Sykes and

### Table 2

| $\beta_2$ Ligand | clogP | logD$_{7.4}$ | log$K_{IAM}$ | Corrected $k_{on}$ | Corrected p$K_d$ |  \( \text{"True" p}K_d \)  
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Bisoprolol</td>
<td>1.831</td>
<td>0.77 ± 0.01</td>
<td>2.35 ± 0.09</td>
<td>2.06 × 10$^5$</td>
<td>4.48</td>
<td>4.44</td>
</tr>
<tr>
<td>Atenolol</td>
<td>−0.109</td>
<td>−1.36 ± 0.01</td>
<td>0.65 ± 0.02</td>
<td>1.02 × 10$^6$</td>
<td>5.93</td>
<td>5.64</td>
</tr>
<tr>
<td>Labetalol</td>
<td>2.50</td>
<td>0.87 ± 0.01</td>
<td>2.89 ± 0.08</td>
<td>2.61 × 10$^6$</td>
<td>5.31</td>
<td>5.56</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>1.486</td>
<td>0.16 ± 0.01</td>
<td>2.09 ± 0.08</td>
<td>3.57 × 10$^6$</td>
<td>4.81</td>
<td>4.66</td>
</tr>
<tr>
<td>Bucindolol</td>
<td>3.294</td>
<td>1.66 ± 0.01</td>
<td>3.09 ± 0.08</td>
<td>1.13 × 10$^6$</td>
<td>7.47</td>
<td>7.43</td>
</tr>
<tr>
<td>Carvedilol</td>
<td>4.041</td>
<td>2.14 ± 0.01</td>
<td>3.00 ± 0.05</td>
<td>1.85 × 10$^6$</td>
<td>7.77</td>
<td>7.88</td>
</tr>
<tr>
<td>Propranolol</td>
<td>2.75</td>
<td>1.10 ± 0.01</td>
<td>3.07 ± 0.14</td>
<td>3.11 × 10$^6$</td>
<td>8.63</td>
<td>6.69</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>3.063</td>
<td>1.99 ± 0.00</td>
<td>3.52 ± 0.18</td>
<td>0.16 × 10$^6$</td>
<td>5.95</td>
<td>5.97</td>
</tr>
<tr>
<td>ICI 118,551</td>
<td>3.40</td>
<td>1.61 ± 0.01</td>
<td>3.36 ± 0.15</td>
<td>3.33 × 10$^5$</td>
<td>6.20</td>
<td>6.00</td>
</tr>
<tr>
<td>Sotalol</td>
<td>0.226</td>
<td>−1.27 ± 0.02</td>
<td>0.91 ± 0.05</td>
<td>2.82 × 10$^6$</td>
<td>5.96</td>
<td>5.75</td>
</tr>
<tr>
<td>Nadolol</td>
<td>0.379</td>
<td>−0.61 ± 0.01</td>
<td>1.71 ± 0.07</td>
<td>1.96 × 10$^6$</td>
<td>7.16</td>
<td>7.11</td>
</tr>
<tr>
<td>Formoterol</td>
<td>1.26</td>
<td>0.35 ± 0.01</td>
<td>2.58 ± 0.09</td>
<td>4.68 × 10$^5$</td>
<td>5.19</td>
<td>4.70</td>
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<tr>
<td>Timolol</td>
<td>1.214</td>
<td>0.06 ± 0.00</td>
<td>2.20 ± 0.12</td>
<td>2.38 × 10$^6$</td>
<td>7.86</td>
<td>7.70</td>
</tr>
<tr>
<td>Cypipindolol</td>
<td>2.03</td>
<td>0.41 ± 0.00</td>
<td>2.87 ± 0.10</td>
<td>2.47 × 10$^6$</td>
<td>9.17</td>
<td>8.87</td>
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<tr>
<td>CGP12177</td>
<td>1.176</td>
<td>−0.77 ± 0.01</td>
<td>2.06 ± 0.10</td>
<td>2.51 × 10$^6$</td>
<td>7.72</td>
<td>7.61</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>0.153</td>
<td>−2.22 ± 0.01</td>
<td>0.76 ± 0.04</td>
<td>4.27 × 10$^6$</td>
<td>6.13</td>
<td>5.96</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>0.061</td>
<td>−1.49 ± 0.01</td>
<td>0.79 ± 0.04</td>
<td>3.32 × 10$^6$</td>
<td>5.86</td>
<td>5.75</td>
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</table>

**Fig. 6.** Correlating $\beta_2$-adrenocceptor ligand physiochemical parameters with kinetically derived parameters. Correlation plot showing the relationship between (A) log$K_{on}$ and logD$_{7.4}$ and (B) log$K_{on}$ and log$K_{IAM}$. Correlation plot showing the relationship between (C) log$k_{on}$ and logD$_{7.4}$ and (D) log$k_{on}$ and log$K_{IAM}$. All data used in these plots are detailed in Tables 1 and 2. Data are presented as mean ± S.E.M. from three or more experiments.
Charlton, 2012), driven by large differences in association rates (Fig. 6D). This difference in affinity has been attributed to an interaction of the lipophilic tail of salmeterol with an “exosite” on the β2 receptor (Green et al., 1996). However, previous investigations using artificial membranes have established an approximate 5000-fold difference in the membrane partition coefficient of salmeterol compared with salbutamol (Rhodes et al., 1985), agreeing well with the data presented in our current study. If we take these large differences in local drug concentration into account by calculating a “corrected” $K_d$ as described above, we find that the ligands now have a similar affinity, likely represents their “true” affinity at the receptor (Fig. 7B). This suggests that the “exosite” described by Green and colleagues likely reflects the phospholipid membrane rather than specific residues on the receptor (also explored by Szczuka et al., 2009).

These findings are of particular importance to those who seek to understand how affinity and kinetic parameters are governed by specific ligand-receptor interactions using structure-based modeling. If such models do not consider the influence of local drug concentrations on observed affinity, they are likely to be poorly predictive. This is supported by the finding that the efficiency index $\log(-\Delta G/P)$, where $P$ is the partition coefficient, can provide better correlations between experimental and calculated values in molecular docking studies (Garcia-Sosa et al., 2010). We have observed that the corrected affinity is highly correlated to the dissociation rate, suggesting that an alternative approach would be to use the off-rate to understand ligand-receptor interactions. For example bucindolol and carvedilol share a similar affinity for the β2-adrenoceptor and both are expected to exhibit slow receptor dissociation based on their measured off-rates (estimated $t_{1/2}$ of dissociation = 17.8 minutes and 21 minutes, respectively). Interestingly, docking studies have suggested that these two molecules possess more close-contact residues with the transmembrane domains and extracellular loops than the more rapidly dissociating propranolol and ICI 118,551 (δ(S*,S*)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]2-butanol hydrochloride) ($t_{1/2}$ of dissociation = 1.5 minutes and 3.3 minutes, respectively) (Audet and Bouvier, 2008). This increase in the rate of dissociation for propranolol and ICI 118,551 is responsible for their reduced affinity for the β2-adrenoceptor when compared with bucindolol and carvedilol, since all four compounds share very similar $k_{on}$ values.

Having established that a membrane bilayer serves as a medium by which drug molecules interact or locate low concentrations of receptor, the significance of this finding for
Establishing “Micro PK/PD” at the β2-Adrenoceptor

Ceng Y and Prussoff WH (1973) Relationship between the inhibition constant (KI) and the concentration of inhibitor which causes 50 per cent inhibition (50%) of an enzymatic reaction. Biochem Pharmacol 22:3999–4104.


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