European Journal of Pharmaceutical Sciences 38 (2009) 533–547

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Lipid membrane interactions of indacaterol and salmeterol: Do they influence their pharmacological properties?

Dario Lombardi[a,*], Bernard Cuenoud[b,1], Stefanie D. Krämer[a]

[a] Institute of Pharmaceutical Sciences, ETH Zürich, Switzerland
[b] Novartis Institutes for BioMedical Research, Basel, Switzerland

ARTICLE INFO

Article history:
Received 2 September 2009
Received in revised form
28 September 2009
Accepted 1 October 2009
Available online 9 October 2009

Keywords:
Indacaterol
Salmeterol
Partitioning
Liposomes
Surface Plasmon Resonance (SPR)
Kinetics
Membrane fluidity
Beta-2-adrenoceptor agonist
Rafts

ABSTRACT

This study compares the lipid membrane interactions of indacaterol, an ultra long acting beta-2 agonist that is given once a day, to salmeterol, a twice a day beta-2 agonist, in order to elucidate the potential mechanisms leading to their different pharmacological properties. Salmeterol but not indacaterol perturbed dimyristoyl-phosphatidylcholine membranes. While the liposome partitioning of the two compounds was similar, independent of the lipid composition, the membrane affinity of indacaterol was two-fold greater than that of salmeterol when rafts, i.e. detergent-insoluble membrane domains, were used as the partition phase. The observed association kinetics with immobilized liposomes at physiological pH were two times faster for indacaterol than for salmeterol. A new model to explain the relationships between the drug/membrane interactions and drug's pharmacological properties considering multiple factors is proposed. The synergy between the higher partitioning of indacaterol into the raft micro domains and the faster membrane permeation of indacaterol could explain the faster onset and longer duration of therapeutic effect of indacaterol. The higher fluidizing effect of salmeterol on membrane fluidity may contribute to its lower intrinsic efficacy compared to indacaterol.

1. Introduction

Inhaled beta-2-adrenoceptor agonists are effective drugs in the management of pulmonary diseases such as asthma and chronic obstructive pulmonary disease (COPD). They induce bronchodilation via direct relaxation of airway smooth muscles, and give rapid relief of symptoms (Barnes, 1977; Waldeck, 2002). Indacaterol, also known as QAB149, is a novel, chirally pure inhaled ultra long beta-2-adrenoceptor agonist, in registration phase for the treatment of COPD. It provides a bronchodilating effect of 24 h after inhalation, combined with a fast onset of action (about 5 min) and an increased efficacy benefit compared with marketed inhaled beta-2-adrenoceptor agonists or the muscarinic receptor antagonist tiotropium (Beeh et al., 2007; Roig et al., 2009). This combination of fast onset, long duration and high efficacy benefit is unique when compared to marketed beta-2-adrenoceptor agonists.

In this study, we investigated whether the beneficial therapeutic profile of indacaterol, in particular the combination of fast onset and long duration, is related to its steady state and kinetic interactions with lipid membranes which provide the environment of the beta-2-adrenoceptor. We, therefore, compared lipid bilayer interactions of indacaterol with those of salmeterol, a beta-2 adrenoreceptor agonist that has been widely used in the past 30 years for the treatment of pulmonary diseases (Ullman and Svedmyr, 1988). Despite similar lipophilicity of the two agonists, salmeterol has a slower onset (around 15 min) and a shorter duration of action (about 12 h) than indacaterol (Lindberg et al., 2007; Palmqvist et al., 1997). Other fast acting agonists, such as salbutamol, have significantly shorter durations of therapeutic action, in agreement with their lower lipophilicity as compared to indacaterol and salmeterol. Beside the differences in time to onset and duration of action, salmeterol and indacaterol differ in their pharmacodynamic characteristics. While indacaterol is a highly efficacious partial agonist at beta-2 adrenoreceptor, salmeterol is a weak partial agonist, which might limit its clinical efficacy (Battram et al., 2006).

Solute-membrane interactions depend on the physicochemical characteristics of the solute and the membrane, respectively. The beta-2-adrenoceptor agonists are characterized by a basic amine

* Corresponding author. Current address: Laboratory of Organic Chemistry, ETH Zurich, Wolfgang-Pauli-Strasse 10, CH-8093 Zurich, Switzerland. Tel.: +41 44 632 4478; fax: +41 44 632 1292.
E-mail address: dario.lombardi@org.chem.ethz.ch (D. Lombardi).
[1] Current address: Merck Serono S.A., Geneva, Switzerland.

0928-0987/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejps.2009.10.001

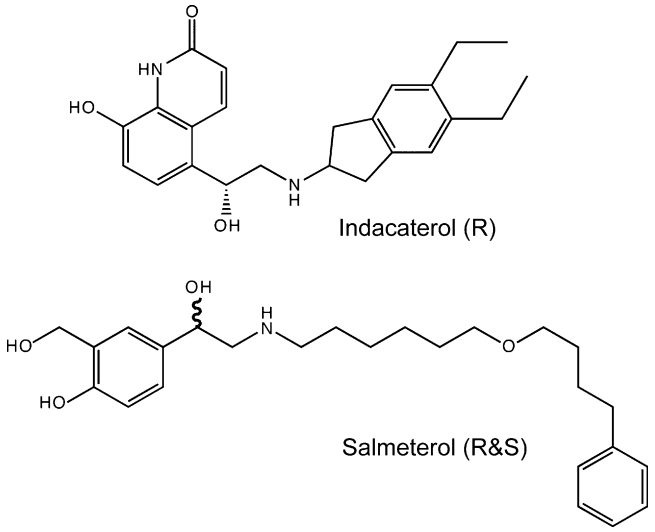


Fig. 1. Chemical structures of indacaterol and salmeterol.

group and an acidic phenol group, as shown in Fig. 1. In solution they can exist in four different ionization forms: the cation (C), two net neutral species (N) including the uncharged species as well as the zwitterionic species, and the anion (A). The acid–base equilibria are defined in terms of the macroscopic constants K_{a1} and K_{a2} that refer to the stoichiometric ionization (Bouchard et al., 2002) where the two net neutral species are treated collectively as being two tautomers of a single form (N). According to the titrated pK_a values of the two compounds, which are pK_a 6.7 and pK_a 8.3 for indacaterol and pK_a 8.8 and pK_a 9.8 for salmeterol, indacaterol is present as a combination of 4 ionization species, predominantly the zwitterion (54.1%) and the neutral one (21.5%) at physiological pH 7.4, while salmeterol is mainly in its cationic form (95.6%) (Cuenoud et al., manuscript in preparation). The fact that zwitterion/neutral species and cations may have different tissue interactions might explain, in part, why indacaterol has a significantly faster onset and duration of action than salmeterol. Indeed bulk hydrophobicity as well as the ionization state have been shown to influence the affinity for liposomes and hence the duration of action of a series of dual dopamine D_2 receptor/beta-2-adrenoceptor agonists (Austin et al., 2003).

We used liposomes of various lipid compositions to investigate a wide range of membrane properties that could influence the interactions with the beta-2-adrenoceptor agonists. Particular attention was given to negatively charged lipids since they are abundant in the lung (Rodgers et al., 2005). We also investigated the effect of cholesterol on the membrane affinities of the drugs, as well as their affinity to extracted membrane rafts since it has been suggested that highly ordered membrane micro-domains are encompassing the beta-2-adrenoceptors and could be of physiological relevance for their function (Ianoul et al., 2005; Pontier et al., 2008; Xiang et al., 2002). Liposomes made of bovine lipid extract surfactant (BLES) were used as a model to investigate the affinity of the agonists to the lung surfactant. Beside the lipid composition, experimental pH and temperature were varied in order to shed light on the influence of the ionization state of drug and lipids and of the physical membrane properties on drug/membrane interactions.

Despite the significant differences in the physicochemical properties of the two drugs, they displayed no major differences in their pH-distribution profiles and membrane interaction kinetics in the liposomal systems. However, the two agonists differed strikingly in their effect on membrane fluidity as determined with the anisotropy probe 1,6-diphenyl-1,3,5-hexatriene (DPH). While indacaterol had no effect, salmeterol significantly increased membrane fluidity at concentrations above 1 μM. Bilayer fluidization by salmeterol may indirectly influence the receptor activation, contributing to the partial agonism properties and the observed slow onset of action of salmeterol. Based on our results, we suggest an adapted model for the relationships between agonist-membrane interactions and their biological effects.

2. Materials and methods

2.1. Chemicals

Salmeterol was obtained from Tocris bioscientific (Ellisville, USA), indacaterol, ^{3}H-indacaterol and ^{3}H-salmeterol were from Novartis (Basel, CH). Propranolol hydrochloride and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma (Buchs, CH), Zwittergent® 3–14 R from Calbiochem (San Diego, USA). TritonX-100 was purchased from Fluka (Buchs, CH). Complete®, methanol, chloroform, trifluoro–acetic acid and acetonitrile (HPLC grade) for lipid extraction and HPLC investigations were from Merck (Darmstadt, DE). All other chemicals were of analytical grade.

2.2. Lipids

Egg phosphatidylcholine (PhC), spinal cord phospatidylserine (PhS), phosphatidylinositol (PhI) and sphingomyelin (SM), all grade 1, were purchased from Lipid Products (Nutfield, UK). Dipalmitoylphosphoglycerol (DPPG), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC), dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphoglycerol (DMPG) and dipalmitoylphosphoethanolammine (DPPE) were from Avanti polar lipids (Alabaster, USA). Cholesterol (Chol) was from Sigma (Buchs, CH). Bovine lipid extract surfactant (BLES) was purchased from BLES Biochemical (London, ON).

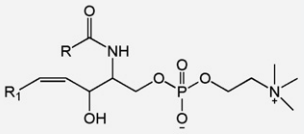
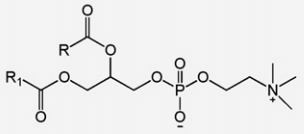
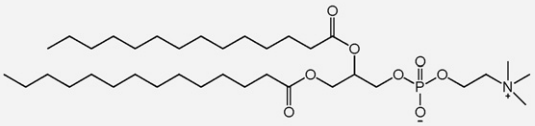
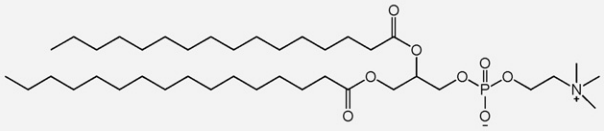
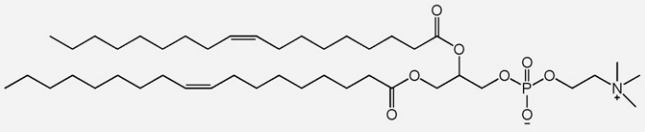
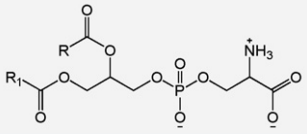
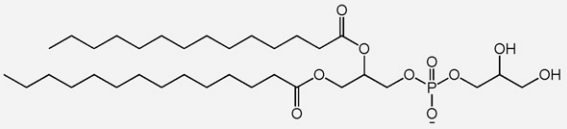
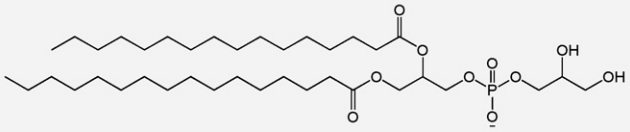
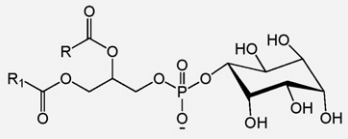
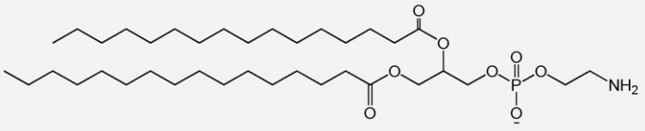
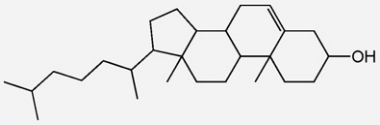
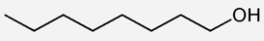
2.3. Octanol/buffer partitioning

The distribution profiles in the octanol/buffer system of indacaterol and salmeterol were determined between pH 4 and 13 by the shake flask method (Leo et al., 1971). Propranolol was used as control. Between 5 and 20 μl each of 2 mM methanolic stock solutions of the drugs were added to reaction tubes and the methanol was evaporated. The compounds were re-dissolved in 1 ml water-saturated 1-octanol and 5 ml of standardized universal buffer solutions (SUBS) containing citrate, borate, phosphate and NaCl, adjusted to 0.21 M ionic strength (Pauletti and Wunderli-Allenspach, 1994) at the desired pH. The tubes were shaken for 1 h at room temperature and centrifuged for 10 min at 9000 *g*. Shaking for 3 h revealed the same results, indicating that equilibrium was reached after 1 h. At the end of the experiments no significant pH shifts were observed in the buffer phases. Samples of 200 μl from both phases were diluted with 800 μl methanol and the concentrations were determined by LC/MS/MS with a Waters 2759 HPLC equipped with an Xterra column (C8, 3.5 m, 1 × 50 mm) and an MS Quattro Micro Mass detector. The drugs were eluted with a linear gradient of H_2O/acetonitrile 95%/5% to 100% acetonitrile. The partition coefficients were calculated as the ratios of the concentrations in the respective 1-octanol and aqueous phases.

2.4. Preparation and characterization of liposomes

Liposomes with various lipid compositions (PhC, DMPC, DPPC, DOPC, SM, DMPC/Chol 60/40 mol/mol, SM/Chol 60/40 mol/mol, DOPC/Chol 60/40 mol/mol, PhC/PhS 70/30 mol/mol, DPPC/DPPG 80/20 mol/mol, PhC/DPPG 70/30 mol/mol, DMPC/Chol/

Table 1
Molecular structure of phospholipids, cholesterol and octanol.

Sphingomyelin (SM)	
Phosphatidyl-choline (PhC)	
Dimyristoyl-phosphatidyl-choline (DMPC)	
Dipalmitoyl-phosphatidyl-choline (DPPC)	
Dioleoyl-phosphatidyl-choline (DOPC)	
Phospatidyl-serine (PhS)	
Dimyristoyl-phospho-glycerol (DMPG)	
Dipalmitoyl-phospho-glycerol (DPPG)	
Phosphatidyl-inositol (PhI)	
Dipalmitoyl-phospho-ethanolamine (DPPE)	
Cholesterol (Chol)	
Octanol	

R and R_1 denote naturally occurring fatty acid acyl chains; see Section 2 for details.

DMPG 30/40/30 mol/mol, SM/Chol/DPPE/PhI/PhS/DPPC 16/32.2/25.8/6.5/6.5/13 mol/mol (raftlike) and BLES), were prepared by extrusion (Kramer et al., 1998; Mayer et al., 1986). The molecular structures of the phospholipids are summarized in Table 1.

Since SM and PhS are extracted from bovine spinal cord while PhC and PhI are from eggs and wheat germ, respectively, they contain a mixture of fatty acid chains. The predominant molecular weight of SM, PhS and PhI species is 749, 788 and 856 respectively. The major fatty acids in PhC are palmitic and stearic acid.

Raft-like liposomes were prepared according to the composition of the raft-extract analyzed by (Kamau et al., 2005). BLES contains 3% cholesterol, 79% phospatidylcoline (53% is saturated), 11% phosphatidylglycerol (23% is saturated), phosphatidylethanolamine, phosphatidylinositol, lyso-bis-phosphatidic acid and sphingomyelin. Moreover it contains the two highly hydrophobic surfactant associated proteins "B" and "C" (Yu et al., 1983).

The lipids were dried from a chloroform/methanol solution to a thin layer in a round flask. The lipid film was subsequently resuspended with SUBS, pH 7.4, to form multilamellar vesicles (MLV) containing 20 mg lipid per ml. The MLV were submitted to 5 cycles of freeze and thaw. Large unilamellar vesicles (LUV) were prepared at 50 °C by extrusion of the MLV preparation through polycarbonate filters (0.1 or 0.2 μm pore size, Nucleopore R, Whatman) using the 10 ml Lipex extruder from Northern Lipids (Burnaby, Canada). Liposome preparations were equilibrated overnight before being used. The lipid content in liposome preparations was characterized with HPLC by injecting 50 μl of sample in a BDS Hypersil C8 column (150 mm × 4.6 mm I.D., 5 μm particle size, Thermo, Bellefonte, PA, USA) kept at 50 °C. The mobile phase was an isocratic mixture of acetonitrile:trifluoroacetic acid:water (86.9:0.1:13 v/v) for DPPC and DPPG containing liposomes or acetonitrile:water (87:13 v/v) for the other lipid compositions. The flow rate was 2 ml/min. The wavelength of the detector was 215 nm.

Liposome size distributions and zetapotentials were analyzed by dynamic light scattering and microelectrophoresis using a Zetasizer 3000 HSA (Malvern Instruments, UK). The hydrodynamic average mean diameters of all preparations were between 80 and 120 nm and 180 and 210 nm, respectively (intensity distribution). The polydispersity index was less than 0.1, indicating a narrow size distribution with a standard deviation of less than 32%, assuming a Gaussian distribution function. The size distribution was unchanged after equilibrium dialysis (5 h, see below) between pH 6.0 and 10.5. The zetapotential measurements of the liposomes were performed with liposome suspension containing 0.1 mg lipids per ml. Negatively charged liposomes revealed increased absolute values of negative zetapotential with increasing pH. This was assigned to an increase in the surface charge density according to the pH-dependence of the protonation/deprotonation equilibria of PhS, PhI, PhE or DPPG (Kramer et al., 1997, 1998; Sacre and Tocanne, 1977). Zetapotentials are summarized in Table 2.

Table 2
Zetapotentials of the investigated liposomes at 25 °C.

Lipid compositions	Zetapotential (mV) in the pH range 4–10
PhC	3 ± 4, pH-independent
DMPC	3 ± 4, pH-independent
DPPC	3 ± 4, pH-independent
DOPC	3 ± 4, pH-independent
SM	3 ± 4, pH-independent
DMPC/Chol 60/40	3 ± 4, pH-independent
SM/Chol 60/40	3 ± 4, pH-independent
DOPC/Chol 60/40	3 ± 4, pH-independent
PhC/PhS 70/30	−24 (pH 4) to −36 (pH 10)
DPPC/DPPG 80/20	−12 (pH 4) to −24 (pH 10)
PhC/DPPG 70/30	−14 (pH 4) to −26 (pH 10)
DMPC/Chol/DMPG 30/40/30	−14 (pH 4) to −26 (pH 10)
Raft-like	−10 (pH 4) to −32 (pH 10)
BLES	−16 (pH 4) to −26 (pH 10)

Lipid compositions according to Section 2.

2.5. Preparation of rafts

Rafts were prepared as previously described by Bucher et al. (2005). In brief, about 4×10^8 P388/ADR or P388 cells (Belli et al., 2009) were incubated on ice with 2 ml buffer 25 mM Tris, 150 mM NaCl, 5 mM EDTA (TNE), pH 7.4, containing Complete® and 1% (v/v) Triton X-100. The cell lysate was homogenized at 4 °C with 50 strokes at 1000 rpm using a Dounce homogenizer (Biotech International). The homogenate was mixed with an equal volume of 80% sucrose in TNE, transferred into a centrifuge tube and overlaid with 2 ml each of 30%, 20%, 10% sucrose and 1.5 ml of 5% sucrose in TNE. All sucrose solutions contained Complete®. After centrifugation at 200 000 × *g* for 19 h at 4 °C in an SW41Ti rotor (Beckman Instruments) the rafts were visible as a turbid band in the upper part of the centrifuge tube (density range between 1.04 and 1.09 g/cm^3) and were pooled in a 2.5 ml fraction. Protein concentrations of different raft preparations were typically between 75 and 120 μg/ml. Raft fractions showed an average mean diameter between 240 and 280 nm with polydispersity indices between 0.4 and 0.5, indicating a broad size distribution. The quantification of the total protein amount was performed with the DC protein assay kit from Bio-Rad (Hercules, CA, USA), the lipid content was estimated from the concentration of proteins assuming a ratio protein/lipid 1:3 (Brown and Rose, 1992). Aliquots of 400 μl were stored at −20 °C and used within 4 weeks.

2.6. Determination of membrane fluidity

To study the fluidity of the lipid bilayers, liposomes (0.25 mg lipids per ml) in SUBS pH 7.4 were incubated for 30 min with 10 μM DPH, which was added as a 10 mM solution in tetrahydrofuran, and subjected to anisotropy measurement with an LS50B Luminescence Spectrometer (Perkin Elmer) (Belli et al., 2009; Lentz, 1993; Seo et al., 2006). The wavelengths of excitation and emission were 355 and 430 nm, respectively, with a bandwidth of 5 nm each. Investigated lipid compositions and temperatures are summarized in Table 3. To investigate the influence of indacaterol and salmeterol

Table 3
Membrane fluidity.

Lipid compositions	T_{exp} (°C)	T_M (°C)	Anisotropy of DPH
DMPC	37	23[b]	0.074 ± 0.003
DMPC	4	23[b]	0.315 ± 0.001
DMPC/Chol 60/40	37		0.142 ± 0.002
PhC	37	−5[a]	0.038 ± 0.008
PhC	22	−5[a]	0.051 ± 0.002
PhC/Chol 60/40	37		0.089 ± 0.001
PhC/DPPG 70/30	37		0.047 ± 0.004
DPPC	37	41.5[b]	0.288 ± 0.004
DPPC/DPPG 80/20	37		0.284 ± 0.002
SM	37	41.4[b]	0.159 ± 0.002
SM	4	41.4[b]	0.362 ± 0.003
SM/Chol 60/40	37		0.272 ± 0.002
DOPC	37	−21[b]	0.089 ± 0.001
Raft-like	37		0.121 ± 0.001
Rafts[c]	37		0.082 ± 0.003
Octanol	22		0.019 ± 0.001

The anisotropy values are the mean of 3 independent experiments ± standard deviation. Octanol anisotropy was measured at 22 °C adding DPH to water-saturated octanol. Lipid compositions according to Section 2. T_{exp} is the experimental temperature, T_M is the main transition temperature of the membranes.

[a] Huang and Mason (1978).
[b] Marsh (1990).
[c] Rafts preparation according to Section 2.

on membrane fluidity as a function of temperature, the drugs were added in DMSO (100 mM drug) and the system was equilibrated for 10 min after each temperature change. The fluorescence intensity values of the vertical and the horizontal polarizers were converted to anisotropy according to Eq. (1):

$$\text{Anisotropy} = \frac{VV - (G \times VH)}{VV + (2G \times VH)} \quad (1)$$

where *VV* is the fluorescence intensity of the probe measured with vertical excitation and vertical emission polarizers, *VH* is the fluorescence intensity with vertical excitation and horizontal emission polarizers, and *G* is the instrument correction factor (*G* = *HV*/*HH*, with *HV* and *HH* fluorescence intensity with horizontal excitation and vertical (*HV*) or horizontal (*HH*) emission polarizers).

2.7. Determination of drug–membrane affinity by equilibrium dialysis

The membrane partitioning of indacaterol and salmeterol were characterized by equilibrium dialysis with liposomes of various lipid compositions. Control experiments with propranolol were performed with PhC liposomes. For each investigated pH, 1 μl of a methanolic 2 mM indacaterol, salmeterol or propranolol stock solution was dried in a reaction tube and 100 μl liposomes were added. The pH was adjusted to values between 6.0 and 10.5 by dilution with SUBS. The final lipid and drug concentrations were 1 mg/ml (1.3 mM) and 1 μM, respectively, if not stated otherwise. For experiments with rafts, 1 μl of drug stock solution was dried in a reaction tube and 1 ml of raft extract was added, TNE buffer pH 7.4 was used for the dialysis instead of SUBS.

Equilibrium dialysis experiments (Macro 1 cells, 1.0 ml; cellulose membrane, MW cut-off 10 000 (10.16) Dianorm, Germany) were performed for 5 h at 25 °C or 7 h at 4 °C and 37 °C against SUBS buffer of the respective pH. Control experiments confirmed that the partition equilibrium was reached at all pH values within this time (data not shown). At the end of the experiment, the pH was determined in the buffer phase. No significant pH shift was observed during the dialysis. The content of the chamber containing the liposomes was diluted 1:5 in methanol in order to solubilize the liposomes and the concentrations of drugs in both chambers were determined as described above for the octanol partitioning experiments. Experiments for the pH-distribution profiles were performed with up to three different compounds simultaneously and quantified by LC/MS/MS. Control experiments showed that this had no influence on the results under the applied experimental conditions (data not shown).

The lipid concentration was determined at the end of the dialysis by HPLC with a Hitachi L2130 pump equipped with a Thermo C8 column (5 m, 150 × 4.6 mm) and a Hitachi L2480 UV detector. Lipids were eluted with acetonitrile:water (87:13 v/v) and detected at 215 nm. A calibration curve was used for quantification. No degradation products such as free fatty acids or lysophosphatidylcholine were detected.

Control experiments performed with radio-labeled indacaterol and salmeterol and quantification by liquid scintillation counting revealed the same results as partition experiments with non labeled compounds and quantification by LC/MS/MS (data not shown). The pH-dependent partitioning of indacaterol and salmeterol was in addition analyzed after an overnight incubation of PhC liposomes with the drug. No significant differences were observed with or without overnight incubation (data not shown).

2.8. Concentration-dependence of indacaterol and salmeterol partitioning in the PhC liposome system

The influence of the drug concentration on the partitioning of indacaterol and salmeterol in PhC liposomes was investigated by means of equilibrium dialysis experiments at different indacaterol and salmeterol concentrations between 0.24 and 600 μM at pH 7.2. To solubilize the drugs, 0.2% DMSO was added at concentrations ≥20 μM and 2% at concentrations ≥300 μM.

2.9. Data analysis of the partitioning experiments by equilibrium dialysis

Partition coefficients (*D*) of indacaterol and salmeterol between lipid bilayers and buffer were calculated from the drug concentrations in the two dialysis chambers as previously described by Pauletti and Wunderli-Allenspach (1994) and shown in Eq. (2)

$$D = \frac{(C_{LB}C_B)V_{LB}}{(C_B V_L)} + 1 \quad (2)$$

where C_{LB} denotes the molar solute concentration in the liposomes-containing chamber and C_B the molar solute concentration in the buffer chamber. V_{LB} is the sample volume of the liposome suspension, V_L the volume of the lipophilic phase, i.e. the lipid bilayer (calculated with a density of 1 g/ml (Huang and Mason, 1978)), within V_{LB}.

The partition coefficients (*P*) of the single ionization species A, N and C were estimated by nonlinear curve fitting of the *D*/pH diagrams with the function described in Eq. (3) (Kramer, 2001):

$$D = \sum_{n=1}^{i} (\alpha_i \times P_i) \quad (3)$$

where α_i denotes the molar fraction of the ionization species *i* of the drug and P_i is the partition coefficient of the species *i*. P_C, P_N and P_A are defined for the cationic, net neutral and anionic species, respectively. For the analysis, the pK_a values of salmeterol and indacaterol were kept variable. Data were fitted with the Systat software TableCurve2D. For the curve fitting residuals were weighted by their reciprocal squares.

2.10. Surface Plasmon Resonance (SPR) measurements

The interactions of indacaterol and salmeterol with PhC bilayers were investigated at 25 °C in a pH range between 5 and 10 with a Biacore 3000 instrument equipped with an L1 chip according to (Lombardi et al., 2009). Sensor chips were pre-conditioned with two consecutive 30 s pulses of 20 mM Zwittergent® detergent followed by 50 mM HCl in 50% (v/v) isopropanol at a flow rate of 0.1 ml/min before their first use (Abdiche and Myszka, 2004). Liposomes were typically diluted in SUBS pH 7.4 to 5 mg lipids per ml and captured to saturation across isolated flow cells at 0.005 ml/min for 2 min. The flow rate was increased to 0.075 ml/min and the liposome surface was washed with running buffer at the respective pH (SUBS containing 0.2% DMSO) for 1000 s before drug injection.

To compare the pH-dependent kinetics of the interactions between the compounds and the lipid bilayers, 0.25 ml of indacaterol or salmeterol in running buffer containing 0.2% DMSO at pH values between 5 and 10 were injected at a flow rate of 0.05 ml/min. The injection type was “Kinject” and 400 s were chosen as dissociation time. After each binding cycle, the sensor surface was regenerated to the original matrix by injecting 50 mM HCl in 50% (v/v) isopropanol at 0.05 ml/min. The data were not corrected for the binding to the reference surface (unmodified lipid-free flow

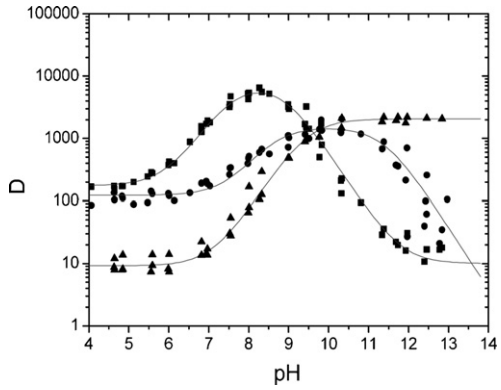


Fig. 2. Distribution profiles in the octanol/buffer system. pH-Distribution profiles of indacaterol (squares), salmeterol (circles) and propranolol (triangles). Experimental data were fitted with Eq. (3) (see Section 2). The fitted partition coefficients P and the inflection points are shown in Table 4.

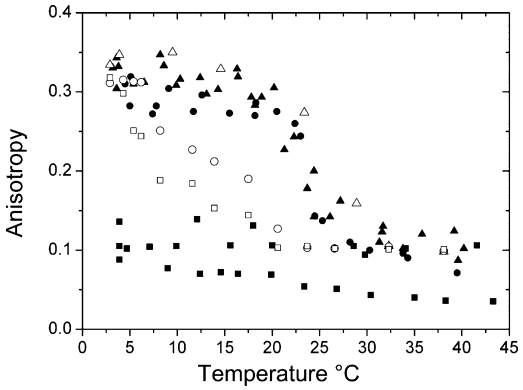


Fig. 3. Influence of indacaterol and salmeterol on DPH anisotropy in DMPC liposomes at pH 7.4. Closed circles, DMPC liposomes in the absence of drug (DMSO had no influence on the profile, not shown). Closed triangles, 100 μM indacaterol. Closed squares, 100 μM salmeterol; open squares, 10 μM salmeterol; open circles, 1 μM salmeterol; open triangles, 0.1 μM salmeterol.

cell) for avoiding artifacts due to non-specific binding to the chip as previously described by Lombardi et al. (2009).

At an excess of lipids over solute, i.e. at indacaterol or salmeterol concentrations <100 μM, the kinetics were independent of the drug concentrations (Lombardi et al., 2009). Kinetic experiments were therefore performed with 60 μM indacaterol or salmeterol. Absorption and desorption phases followed bi-exponential functions and data were analyzed with Eq. (4) (adsorption) and Eq. (5) (desorption),

$$\mathrm{RU}(t) = \mathrm{RU}(0) + \mathrm{RU}_{\max 1} \times e^{-k_1 \times t} + \mathrm{RU}_{\max 2} \times e^{-k_2 \times t} \quad (4)$$

$$\mathrm{RU}(t) = \mathrm{RU}(0) + \mathrm{RU}_{\max 1} + \mathrm{RU}_{\max 2} - (\mathrm{RU}_{\max 1} \times e^{-k_1 \times t} + \mathrm{RU}_{\max 2} \times e^{-k_2 \times t}) \quad (5)$$

where RU(t) and RU(0) are the $\mathrm{RU}_{\mathrm{Drug}}$ at time t and the RU at time zero, i.e. before injection of the drug, respectively. $\mathrm{RU}_{\max 1}$ and $\mathrm{RU}_{\max 2}$ are the fitted plateau values of the two exponentials and k_1 and k_2 are the faster and the slower apparent rate constants, respectively.

The faster apparent rate constants were not reproducible and in some cases beyond the time resolution of the instrument and were, therefore, not further analyzed. Kinetic data were analyzed as described earlier (Lombardi et al., 2009). Data points recorded for 150 s after the injection of drug or buffer were fitted with TableCurve2D neglecting the data of the first 5 s (Rebolj et al., 2006). The residuals were weighted by their reciprocal squares.

3. Results

3.1. Partitioning in the octanol/buffer system

The partitioning behavior of indacaterol, salmeterol and propranolol in the octanol/buffer system was examined between pH 4 and 13. The concentration was between 7.2 and 21.5 μM for indacaterol and salmeterol and between 80 and 330 μM for propranolol. The resulting D–pH diagrams were bell-shaped for indacaterol and salmeterol and sigmoidal for propranolol (Fig. 2). For indacaterol, D was highest at pH 8.2 (6500), decreasing to approximately 10 at pH 13 and 100 at pH 4. For salmeterol, D was highest at pH 10.1 (1250), decreasing to approximately 100 at pH 4 and 40 at pH 13. In order to quantitatively describe the D–pH diagrams we used a combination of Henderson–Hasselbalch equations (Eq. (3)). To fit the curves the inflection points ("pK_a" in Eq. (3), see Section 2) were kept variable. Fitted partition parameters and inflection points for indacaterol and salmeterol are shown in Table 4. Of all three drugs, the net neutral species had the highest affinity for octanol. Indacaterol was the most lipophilic compound (P_N 7720), followed by propranolol (P_N 2054) and salmeterol (P_N 1692). At pH 7.4, D was highest for indacaterol, medium for salmeterol and lowest for propranolol.

3.2. Membrane fluidity

The lipid order of liposome membranes was tested by fluorescence anisotropy measurements as described in Section 2. Liposomes of varying lipid composition were analyzed and anisotropy values calculated according to Eq. (1).

Table 3 summarizes the anisotropy values at different temperatures and shows the published transition temperatures (T_M) if available. Bilayers containing cholesterol in amount greater or equal to 30% (mol/mol) were considered in the *lo* (liquid ordered) phase (Laisc, 1993). In general, lipid bilayers with T_M below the experimental temperature, i.e. in their *ld* (liquid disordered) phase, had lower anisotropy values than those with higher T_M (*lo* phase), indicating that bilayers in *ld* phase were more fluid than bilayers in *lo* phase.

DOPC which contains two oleic acid residues with one unsaturation each, revealed a higher anisotropy value at 37 °C than the fully saturated DMPC and the partially unsaturated PhC at the same temperature, all in their *ld* phase (Nagle and Tristram-Nagle, 2000). The fluidity of DOPC bilayers appears to be lower than that of DMPC and PhC bilayers, despite its low T_M. Cholesterol generally enhanced the anisotropy, indicating a reduction in fluidity of the lipid bilayers.

3.3. Influence of the drug on the membrane fluidity

In order to investigate the influence of indacaterol and salmeterol on membrane fluidity, liposomes were incubated with the drugs at the indicated concentrations and anisotropy was determined in the temperature range between 4 and 40 °C. As shown in Fig. 3, salmeterol but not indacaterol had a prominent influence on DPH anisotropy in DMPC bilayers at temperatures lower than T_M. The effect of salmeterol was concentration-dependent starting at a concentration of 1 μM. Anisotropy was not affected at 4 °C and above T_M. No significant effect on DPH anisotropy was observed in SM and SM/Chol liposomes, all changes were less than 12% of the anisotropy value in the absence of drug (data not shown).

3.4. Concentration-dependence of salmeterol and indacaterol partitioning in the liposome system

To study concentration-dependent partitioning in the liposome systems and to investigate whether the concentration-dependent

Table 4
Membrane affinity parameters of indacaterol and salmeterol in different liposomal systems.

Lipid compositions	T_{exp} (°C)	Indacaterol				Salmeterol			
		Inflection points (pH)	P_C	P_N	P_A	Inflection points (pH)	P_C	P_N	P_A
DMPC	37	8.2/9.2	3647 ± 296	1183 ± 1079	281 ± 180	8.0/12.6	4710 ± 213	880 ± 64	[c]
DMPC	4	8.2/9.4	2061 ± 132	710 ± 942	415 ± 74	8.0/12.6	2628 ± 54	196 ± 64	[c]
DMPC/Chol	37	[a]	332 ± 106	332 ± 106	332 ± 106	[a]	310 ± 77	310 ± 77	310 ± 77
PhC	22	6.3/9.1	4424 ± 1114	5057 ± 235	865 ± 226	8.5/12.4	3545 ± 74	1480 ± 122	[c]
DPPC	22	[a]	1680 ± 233	1680 ± 233	1680 ± 233	[a]	560 ± 17	560 ± 17	560 ± 17
PhC/PhS	22	8.1/9.8	17001 ± 294	1386 ± 742	257 ± 167	8.2/11.2	10758 ± 148	2643 ± 511	[c]
DPPC/DPPG	22	7.1/9.0	1079 ± 206	3879 ± 446	631 ± 176	7.0/11.2	786 ± 180	2297 ± 186	[c]
DMPC/Chol/DMPG	37	8.6/9.6	427 ± 44	108 ± 147	171 ± 150	8.2/8.3	1294 ± 93	2567 ± 526	315 ± 2
PhC/DPPG	22	7.8/10.0	40456 ± 1649	3527 ± 1414	[c]	8.2/[b]	36537 ± 2602	2878 ± 738	[c]
SM	37	7.1/9.0	787 ± 376	3104 ± 576	719 ± 275	[a]	1389 ± 426	4768 ± 620	[c]
SM/Chol	37	[a]	1299 ± 261	1299 ± 261	1299 ± 261	[a]	1901 ± 250	1901 ± 250	1901 ± 250
DOPC	37	8.3/10.1	21253 ± 1453	5333 ± 9280	3255 ± 1026	8.2/[b]	19776 ± 2682	5162 ± 2281	[c]
DOPC/Chol	37	8.6/[b]	7006 ± 594	1839 ± 6642	[c]	8.2/[b]	9588 ± 1092	2325 ± 126	[c]
BLES	37	7.9/9.3	10006 ± 646	5319 ± 3152	2268 ± 749	8.1/8.5	11239 ± 466	2789 ± 4645	1608 ± 106
Raft-like	37	[a]	604 ± 97	604 ± 97	604 ± 97	[a]	809 ± 86	809 ± 86	809 ± 86
Octanol	22	7.6/8.9	164 ± 97	7720 ± 528	15 ± 101	8.9/11.3	152 ± 34	1692 ± 103	[c]

P_C, P_N and P_A are the partition coefficients for the cationic, net neutral and anionic species, respectively. Fitted values ± estimated standard errors are shown. Lipid compositions according to Section 2.

[a] No inflection points detectable.
[b] No second inflection point detectable.
[c] Outside experimental pH range.

effect of salmeterol but not indacaterol on DPH anisotropy is reflected in their membrane partitioning, we investigated the concentration-dependence of their partitioning in the PhC liposomal system at pH 7.2 and 22 °C. Drug concentrations varied from 0.24 to 600 μM.

As shown in Fig. 4, partitioning was concentration-independent up to 5 μM. At this concentration, the lipid/drug ratio within the lipid bilayer is greater than 230 (Lombardi et al., 2009). At higher concentrations, *D* values decreased in a concentration-dependent manner and *D* was about four times lower at 600 μM when compared to concentrations lower than 5 μM. No significant difference was observed in the concentration-dependence of the partitioning of the two drugs. To guarantee concentration-independent partition profiles, all other partition experiments with liposomes were started with 1 μM drug concentration in the liposome-containing dialysis cell half.

3.5. pH-Partition profiles in PhC liposomes

To investigate membrane partitioning of indacaterol and salmeterol, equilibrium dialysis experiments were carried out with liposomes as described under Section 2. *D* values were calculated according to Eq. (2) and *D*–pH diagrams were analyzed as described above for the octanol/buffer system. The fitted *P* values and the inflection points are reported in Table 4. Equilibrium dialysis experiments with PhC liposomes [$T_M - 5$ °C (Huang and Mason, 1978)] at 22 °C revealed a similar pH-partition profile for indacaterol and salmeterol (Fig. 5). The differences between the *P* values of the single ionization species were relatively small. For salmeterol, the cation showed the highest affinity to the membrane while P_N and P_C values were similar in the case of indacaterol. The pH-partition profile of propranolol is shown for comparison in Fig. 5. As reported before (Pauletti and Wunderli-Allenspach, 1994), membrane affinity was higher for the neutral species than for the cation.

3.6. Influence of the lipid order on partitioning

In order to better understand the differences between the pH-partitioning profiles in octanol and in PhC liposomes, experiments with more geometrically ordered membranes, i.e. DPPC, DMPC and DMPC/Chol 60/40 liposomes, were performed. Liposomes above and below the T_M were considered in the *ld* phase and in the *so* (solid ordered) phase, respectively (Laisc, 1993). The DMPC/Chol 60/40 bilayers were considered *lo* phase (Laisc, 1993). When compared to PhC liposomes, equilibrium dialysis experiments with DPPC liposomes [T_M 41.5 °C (Marsh, 1990)] at 22 °C (*so* phase)

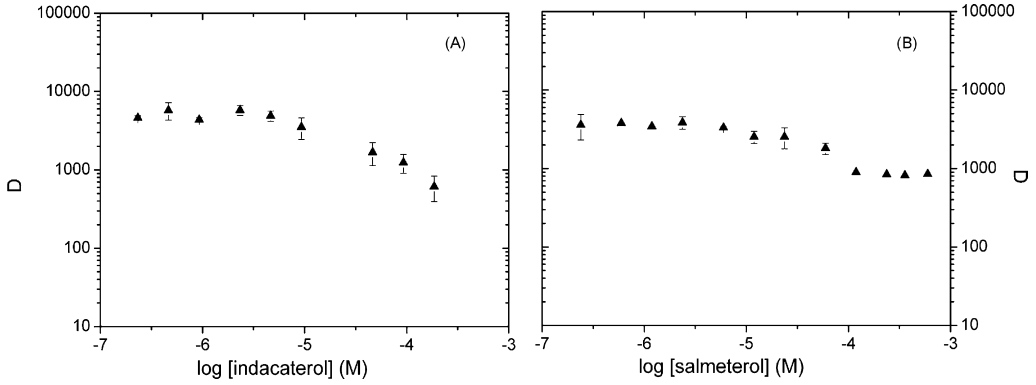


Fig. 4. Concentration dependence of liposome partitioning. Partitioning of (A) indacaterol and (B) salmeterol in PhC liposomes at pH 7.2 and 22 °C. At concentrations of agonists ≥20 μM and ≥300 μM, the samples contained 0.2% and 2% DMSO, respectively. As seen from the data, DMSO had no significant effect on partitioning. Salmeterol data are from Lombardi et al. (2009), published with permission from Elsevier Inc. and are shown here for comparison.

Table 5
D and log *D* values of indacaterol and salmeterol in various liposomal systems, rafts and octanol at pH 7.4.

Lipid compositions	T_{exp} (°C)	Indacaterol		Salmeterol	
		$D^{pH\ 7.4}$	$\log D^{pH\ 7.4}$	$D^{pH\ 7.4}$	$\log D^{pH\ 7.4}$
DMPC	37	3306 ± 605	3.52 ± 0.07	4009 ± 116	3.60 ± 0.01
DMPC	4	1861 ± 238	3.27 ± 0.05	2109 ± 89	3.32 ± 0.02
DMPC/Chol	37	332 ± 106	2.52 ± 0.12	360 ± 77	2.56 ± 0.08
PhC	22	4924 ± 166	3.69 ± 0.01	3406 ± 29	3.53 ± 0.00
DPPC	22	1680 ± 233	3.23 ± 0.06	560 ± 17	2.75 ± 0.01
PhC/PhS	22	14648 ± 1117	4.17 ± 0.03	9681 ± 212	3.99 ± 0.01
DPPC/DPPG	22	2845 ± 128	3.45 ± 0.02	1818 ± 228	3.26 ± 0.05
DMPC/Chol/DMPG	37	409 ± 63	2.61 ± 0.06	1409 ± 74	3.15 ± 0.02
PhC/DPPG	22	30971 ± 4116	4.49 ± 0.05	32122 ± 1748	4.51 ± 0.02
SM	37	2232 ± 424	3.35 ± 0.07	3324 ± 222	3.53 ± 0.03
SM/Chol	37	1299 ± 261	3.11 ± 0.08	1901 ± 250	3.28 ± 0.05
DOPC	37	16156 ± 2387	4.21 ± 0.06	17785 ± 3516	4.25 ± 0.08
DOPC/Chol	37	6686 ± 675	3.83 ± 0.04	8975 ± 117	3.95 ± 0.01
BLES	37	8756 ± 257	3.94 ± 0.01	9622 ± 551	3.98 ± 0.02
Raft-like	37	604 ± 76	2.78 ± 0.04	809 ± 76	2.91 ± 0.04
Rafts	37	10473 ± 731	4.02 ± 0.03	4527 ± 1426	3.66 ± 0.12
Octanol	22	3226 ± 101	3.51 ± 0.01	201 ± 47	2.30 ± 0.09

Fitted values ± estimated standard errors are shown. Lipid compositions according to Section 2.

revealed lower membrane affinities for the neutral and cationic species of both drugs. The partitioning was pH-independent for both agonists. Indacaterol had an approximately three times higher membrane affinity than salmeterol. Results are shown in Fig. 6, Tables 4 and 5.

Partitioning behavior in liposomes consisting of DMPC [T_M 23 °C (Marsh, 1990)] at 37 °C, DMPC/Chol 60/40 at 37 °C and DMPC at 4 °C, are shown in Fig. 7. For both compounds, the pH-partitioning profiles with DMPC at 37 °C [*ld* phase (Pokorny et al., 2000)] and 4 °C (*so* phase) were characterized by highest affinity of the cationic species. As seen with DPPC at 22 °C (*so* phase), the partitioning was pH-independent and similar for the two drugs in the presence of 40% cholesterol (*lo* phase).

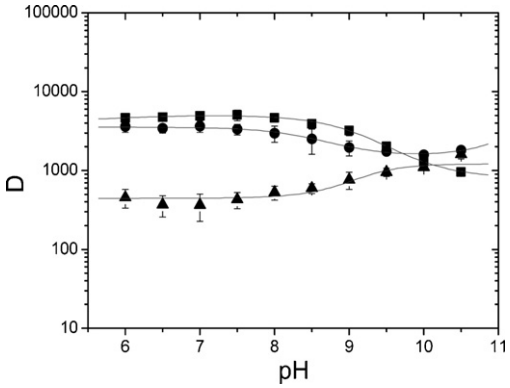


Fig. 5. pH-Dependent partitioning in PhC liposomes. pH-Distribution profiles of indacaterol (squares), salmeterol (circles) and propranolol (triangles) with PhC liposomes at 22 °C. The fitted values of *P* and the inflection points are reported in Table 4. Salmeterol and Propranolol data are from Lombardi et al. (2009), published with permission from Elsevier Inc. and are shown here for comparison.

3.7. *Influence of lipid head-group charge on membrane partitioning*

Next, we studied the influence of ionic and charge–charge interactions on the membrane partitioning of the two drugs. Lipid compositions were PhC/PhS 70/30 and PhC/DPPG 70/30 at 25 °C (*ld* phase), DPPC/DPPG 80/20 liposomes at 22 °C (*so* phase) and DMPC/Chol/DMPG 30/40/30 liposomes at 37 °C (*lo* phase). Results are shown in Fig. 8 and Tables 4 and 5. For both compounds the partition coefficients were highest with the negatively charged *ld* membranes at pH less than 7.5. P_C values were three- to four-fold higher in the PhC/PS than the PhC liposomes while P_N and P_A values decreased for indacaterol and P_N increased for salmeterol (Table 4).

Twenty percent DPPG in combination with DPPC at 22 °C did not show the increased attraction of cations observed with 30% DPPG in PhC membranes at a similar temperature. Indacaterol and salmeterol P_C values were lower than the respective P_N values in this system (Table 4).

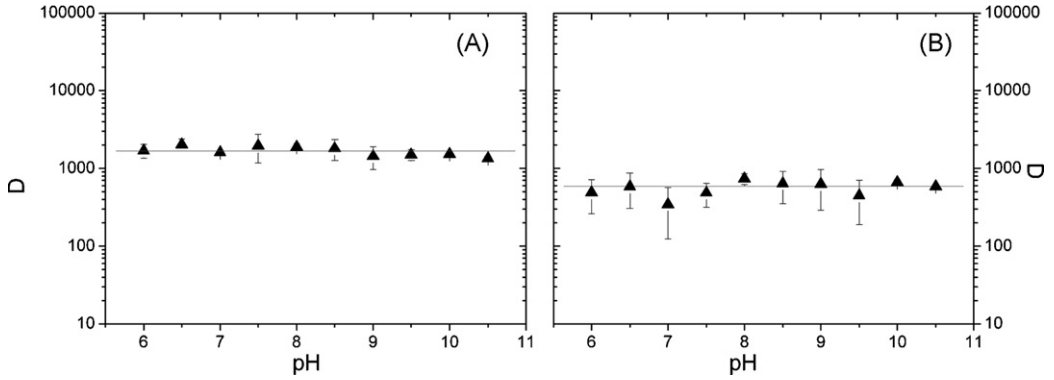


Fig. 6. Distribution profiles of indacaterol and salmeterol in DPPC liposomes. pH-Distribution profiles of indacaterol (A) and salmeterol (B) with DPPC liposomes at 37 °C. The fitted values of *P* are reported in Table 4.

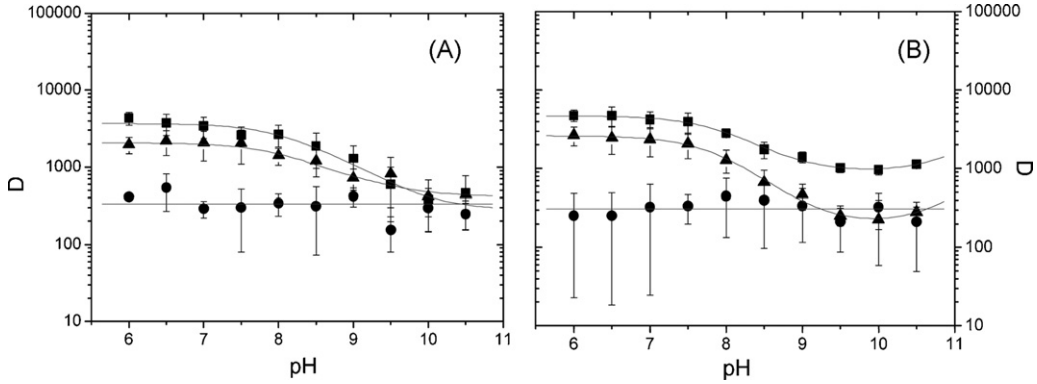


Fig. 7. Distribution profiles of indacaterol and salmeterol in DMPC and DMPC/Chol liposomes. pH-Distribution profiles of indacaterol (A) and salmeterol (B) with DMPC liposomes at 37 °C (squares) or 4 °C (triangles) and DMPC/Chol 60/40 liposomes at 37 °C (circles). The fitted values of *P* and the inflection points are reported in Table 4.

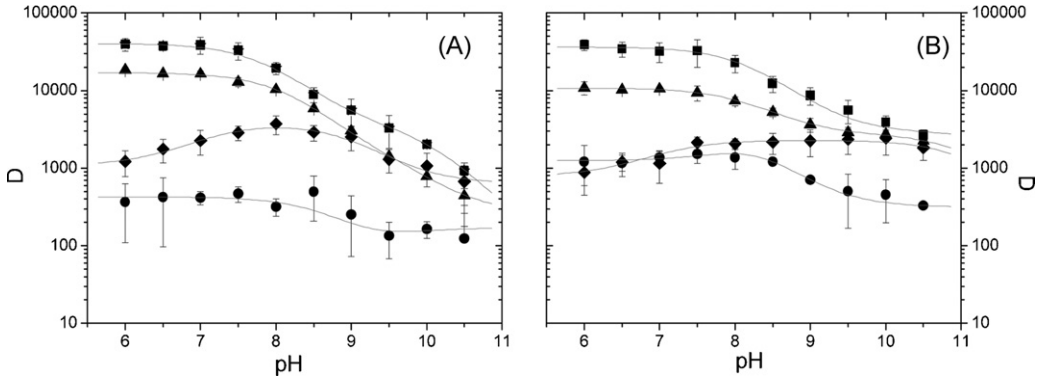


Fig. 8. Influence of negatively charged lipids on the partitioning of indacaterol and salmeterol. pH-Distribution profiles of indacaterol (A) and salmeterol (B) with PhC/DPPG 70/30 liposomes at 25 °C (squares) PhC/PhS 70/30 liposomes at 37 °C (triangles), DPPC/DPPG 80/20 liposomes at 25 °C (diamonds) and DMPC/Chol/DMPG 30/40/30 liposomes at 37 °C (circles). See Table 4 for fitted values of *P* and inflection points.

3.8. Influence of lipid acyl chain saturation on membrane affinity

To further investigate the influence of the membrane properties on partitioning, experiments with DOPC liposomes [$T_M - 21$ °C (Marsh, 1990)], at 37 °C, were performed (Fig. 9, Tables 4 and 5). The homogenous unsaturation pattern of DOPC significantly increased the membrane affinity of both drugs as compared to partly unsaturated PhC (Fig. 5) and saturated DPPC (Fig. 6) or DMPC (Fig. 7) systems. The two drugs had similar affinities and partition profiles in the DOPC system. The presence of 40% cholesterol reduced the affinity of both agonists by two- to three-fold. In contrast to the DMPC and DMPC/Chol systems, the pH-dependence was not affected when cholesterol was added to DOPC.

3.9. Influence of the lipid back bone hydrogen bonding properties on partitioning

Sphingomyelin has a ceramide backbone while phosphatidylcholines are built from the triglyceride backbone. The ceramide backbone can act as a hydrogen bond donor while the triglyceride backbone has only hydrogen bond acceptor capacities. Partitioning with SM liposomes [T_M 41.4 °C (Marsh, 1990)] at 37 °C revealed a bell-shaped partitioning profile for indacaterol and a sigmoidal profile with P_C value being lower than P_N value for salmeterol (Fig. 10, Tables 4 and 5). As in the other systems, cholesterol decreased the affinity of neutral indacaterol and salmeterol to SM membranes. As seen for DMPC/Chol (37 °C) but not DOPC/Chol

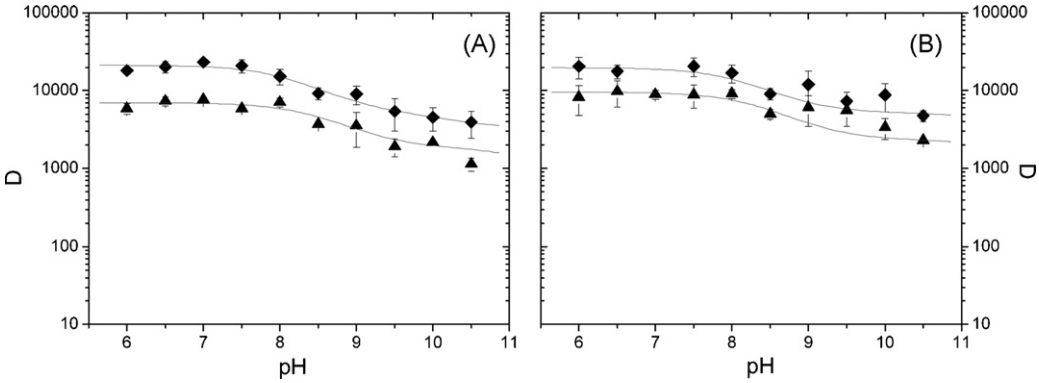


Fig. 9. Distribution profiles of indacaterol and salmeterol in DOPC and DOPC/Chol liposomes. pH-Distribution profiles of indacaterol (A) and salmeterol (B) with DOPC liposomes at 37 °C (diamonds) and DOPC/Chol 60/40 liposomes at 37 °C (triangles). The fitted values of *P* and the inflection points are reported in Table 4.

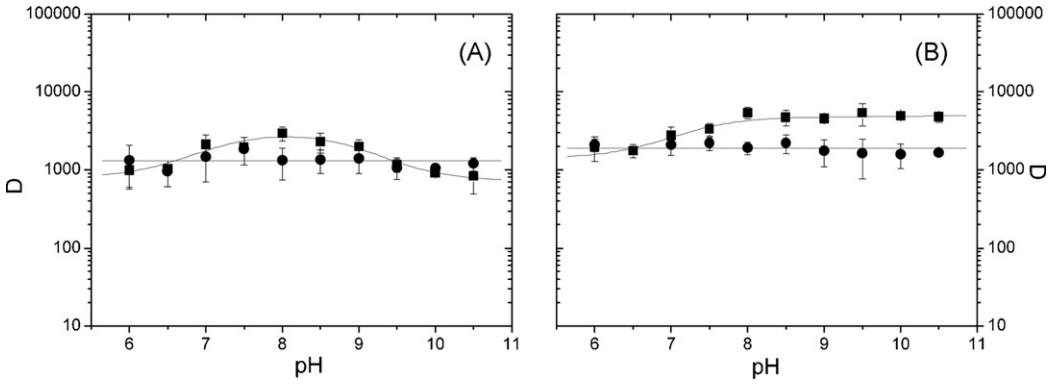


Fig. 10. Distribution profiles of indacaterol and salmeterol in SM and SM/Chol liposomes. pH-Distribution profiles of indacaterol (A) and salmeterol (B) with SM liposomes at 37 °C (squares) and SM/Chol 60/40 liposomes at 37 °C (circles). The fitted values of *P* and the inflection points are reported in Table 4.

(37 °C), pH-dependence was abolished in the SM/Chol (37 °C) system.

3.10. Influence of cholesterol on membrane partitioning

Cholesterol is known to bring *ld* membranes to an *lo* state above a certain cholesterol/lipid ratio (Laisc, 1993). Addition of cholesterol reduced the membrane affinities of both beta-2-adrenoceptor agonists in general. In DPMC and SM but not in DOPC membranes, 40% cholesterol equalized the partitioning of neutral and cationic species at 37 °C, flattening both the sigmoidal profile in DMPC with highest cation affinity as well as the bell-shaped profile in SM with highest affinity of the neutral species (Figs. 7, 9 and 10).

3.11. Partitioning in liposomes from BLES, liposomes mimicking rafts and extracted raft domains

Additional equilibrium dialysis experiments were performed with liposomes mimicking the lipid component of raft domains of cellular membranes and with rafts extracted from P388 or P388/ADR cells. As shown in Fig. 11, the experiments with the raft-like liposomes (see Section 2 for the lipid composition) revealed similar affinity for the two agonists and were independent of the pH, mimicking the situation observed in other cholesterol-containing liposomes (except DOPC/Chol and DMPC/Chol/DMPG). The affinity of the agonists to rafts extracted from cells revealed an approximately two-fold higher affinity of indacaterol at pH 7.4 and 37 °C (Table 5). It has to be noted that raft extract might contain proteins that also could affect affinity of the agonists. Experiments with the lipid extract from the bovine lung surfactant (BLES) are shown in Fig. 11. The affinity and pH-partition profiles were similar for indacaterol and salmeterol. Affinity was highest for the cations, as observed with the phosphatidylcholine and negatively charged liposomes.

3.12. Comparison of salmeterol and indacaterol membrane partitioning

In contrast to the octanol/buffer partition system where indacaterol *D* value, at pH 7.4, was 16 times higher than salmeterol, the two beta-2-adrenoceptor agonists showed similar partition characteristics in most of the liposomal systems studied. Significant differences in the pH-partition profiles were only observed in DPPC/DPPG (25 °C) and SM (37 °C) liposomes. Differences in *D* value, at pH 7.4, were highest in the DPPC (22 °C) system with a *D* ratio indacaterol/salmeterol of three and DMPC/Chol/DMPG (37 °C) with a ratio of 1/3. The respective ratio was two in extracted rafts (37 °C) (see Table 5).

3.13. Kinetics of membrane interactions of indacaterol and salmeterol

To measure the kinetics of the interactions of the different ionic species of indacaterol with PhC bilayers, liposomes were immobilized on a Biacore L1 sensor chip and binding kinetics were recorded between pH 5 and 10 as described under Section 2. The adsorption and desorption phases of indacaterol followed bi-exponential functions in the pH range under investigation (Fig. 12). In our earlier work (Lombardi et al., 2009), it was found that salmeterol has a bi-exponential kinetics up to pH 8.6 and mono or bi-exponential kinetics at pH above pK_{a1}.

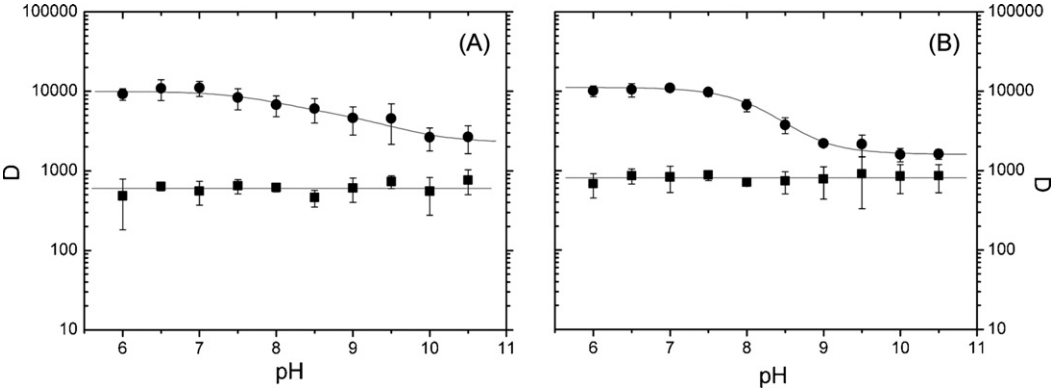


Fig. 11. Distribution profiles of indacaterol and salmeterol in BLES and raft-like liposomes. pH-Distribution profiles of indacaterol (A) and salmeterol (B) with raft-like liposomes (see Section 2) at 37 °C (squares) and BLES liposomes at 37 °C circles). See Table 4 for fitted parameters.

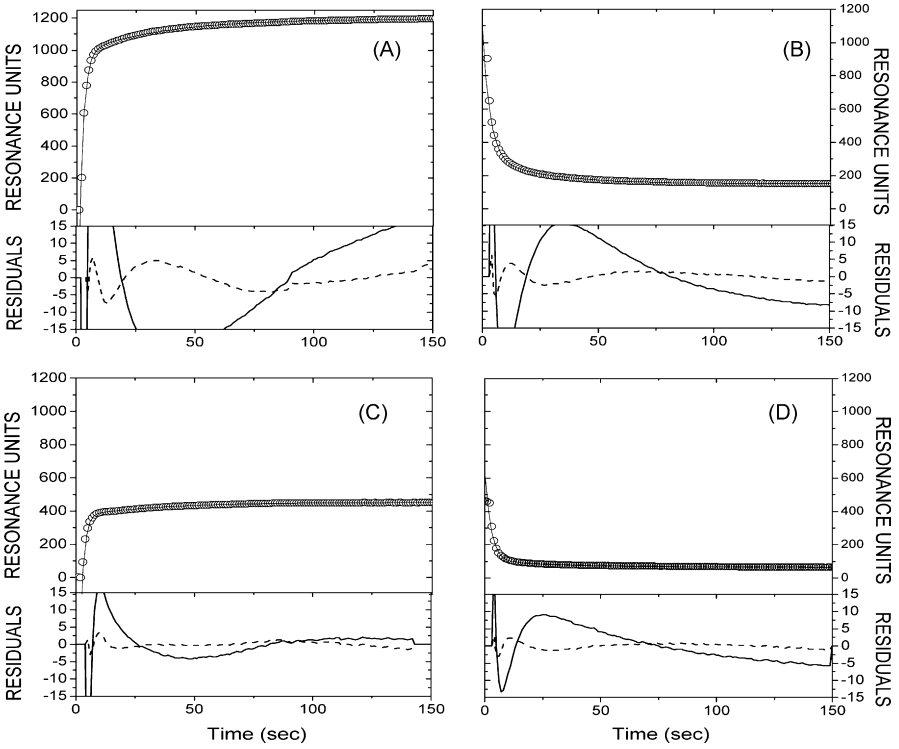


Fig. 12. Association and dissociation kinetics of indacaterol with PhC liposomes. Representative examples of the association phases (A, C) and the dissociation phases (B, D) at 60 μM indacaterol at pH 7.2 (A, B) and at pH 9.3 (C, D). Buffers contained 0.2% DMSO. The upper panels show the sensograms with the best-fit function; the lower panels the residuals for the mono- (solid line) and bi-exponential (dashed line) fits. Salmeterol kinetics are shown elsewhere (Lombardi et al., 2009).

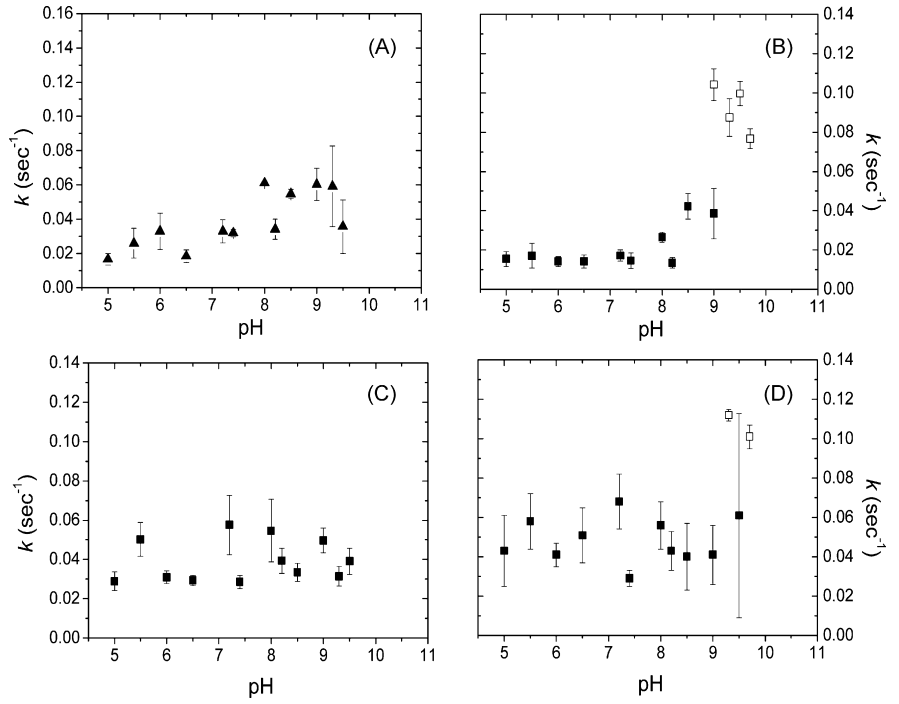


Fig. 13. pH-Dependence of the fitted apparent rate constants k_2 and k of the liposome association and dissociation phases of indacaterol and salmeterol. The association (A, B) and dissociation (C, D) phases of indacaterol (A, C) and salmeterol (B, D), respectively, to and from PhC liposomes were recorded at various pH values and the apparent rate constants of the slow phases of the bi-exponential functions (k_2, closed symbols) and of the mono-exponential functions (k, open symbols) were estimated from non-linear curve fitting as shown in Fig. 12. Mean values of 6 independent experiments with standard deviations. Salmeterol data are from Lombardi et al. (2009), published with permission from Elsevier Inc. and are shown here for comparison.

The k_2 values of the indacaterol association phase were between 0.02 and 0.06 s^{-1} in the investigated pH range; the respective apparent rate constants of the dissociation phase were between 0.03 and 0.06 s^{-1}. Our previous work with salmeterol revealed pH-independent k_2 values between pH 5 and pH 8. The respective apparent rate constants were between 0.01 and 0.03 s^{-1} for the association phase and between 0.03 and 0.07 s^{-1} for the dissociation phase. Salmeterol kinetics were mono-exponential above pK_{a1} with k values between 0.08 and 0.12 s^{-1} (Lombardi et al., 2009). Results are shown in Fig. 13.

4. Discussion

In this work, we investigated how the differences in time to onset and duration of action between indacaterol and salmeterol could be related to their steady state and kinetic interactions with lipid membranes. The two beta-2 adrenoreceptor agonists differed strikingly in their effect on membrane fluidity while their pH-distribution profiles were similar in most liposome systems, despite their different behavior in the octanol/buffer system.

The pH ranges with highest D values in the octanol/buffer partition system differed for indacaterol and salmeterol. A maximum log D value of 3.8 was found at pH 8.2 for indacaterol and of 3.2 at pH 10.1 for salmeterol. This is in agreement with the differences in pK_a values of the amine and phenolic groups of the two compounds. The pK_a values of indacaterol are lower than the respective values of salmeterol, indicating that at physiological pH indacaterol is mainly zwitterionic or uncharged while the cationic form prevails for salmeterol (Cuenoud et al., manuscript in preparation). Accordingly, the partition experiments in octanol/buffer at pH 7.4, revealed a 15-fold higher D value for indacaterol than for salmeterol. In contrast, a similar pH-distribution profile for indacaterol and salmeterol was observed in several liposome preparations regardless of differences in acyl chain length and saturation, in head group charge, or in cholesterol and/or sphingomyelin presence. Hence, neither the difference in the ionization pattern observed in aqueous solution nor the difference in length and flexibility of the compound tails seem to affect the affinity of salmeterol and indacaterol to lipid bilayers. Another striking difference to the octanol/buffer system was the extensive partitioning of the cationic forms of the two compounds.

Having a closer look at the lipid bilayer as a distribution system, the relatively high membrane affinity of charged species can be rationalized as follows: while charge neutralization within the zwitterion is favorable in octanol, leading to higher distribution coefficients for zwitterions than for net charged species (Avdeef et al., 1998; Betageri and Rogers, 1988; Hellwich and Schubert, 1995; Plember van Balen et al., 2001), in a lipid bilayer system, charge neutralization can be achieved by interactions with the phosphate and the amine moieties of the lipid head groups, independent of the presence of a counter-charge within the molecule. In support of this hypothesis, a similar behavior, as we found with the beta-2-adrenoceptor agonists in the present study, was observed with cetirizine, a histamine H_1 receptor antagonist with two basic and one acidic groups. The mono-cation had a higher affinity to lipid bilayers than the zwitterionic and the anionic species (Plember van Balen et al., 2001).

Depending on the sterical arrangement of the hydrophobic moiety/moieties and the charged groups, fewer charges may be favorable for a good fit within the bilayer. The zwitterions of salmeterol and indacaterol, which are the main ionization species between the two pK_a values and which have the highest partition coefficient in the octanol/buffer system, may need more energy to partition into the bilayer than the corresponding cations. It is reasonable to assume that the compounds' hydrophobic tails are buried in the acyl chain region, independently of the charges of the amine and phenol groups. In such a case, the positive charge of the cations, which is localized at the amine group of the compounds, may interact with the negatively charged phosphate groups of the lipids. At the same time the neutral phenolic moieties of the compounds could be located at the height of the carbonyl groups and the first C atoms of the acyl chains of the lipids, forming hydrogen bonds with the carbonyl oxygens. Upon deprotonation of the phenolic hydroxyl group, the preferred location of the phenolate may change and tend towards the head group/buffer region where it can neutralize its charge with the quaternary amine of the choline or with electrolytes in the water phase. Due to the loss of the hydrophobic interaction between the aromatic ring of the compound and the acyl chains of the lipids, the membrane affinity may decrease, although the charge distribution would fit with the charges of the head groups. This model is sufficient to explain why hardly any differences were detected between the pH-partition profiles into liposomes of the two drugs.

A striking difference between indacaterol and salmeterol was their effect on DPH anisotropy. Molecular dynamics modeling with DPPC bilayers at T value inferior to T_M value revealed a position of DPH in the middle region of the lipid hydrocarbon chains, in a parallel alignment to the chains (Repakova et al., 2005). The long tail of salmeterol, corresponding to an acyl chain of 15 C atoms in lengths, was able to perturb the membrane and therefore the environment of DPH. In contrast, the shorter and bulkier tail of indacaterol with less rotational freedom, corresponding in length to a C-chain of 7 atoms, did not cause significant changes in the lipid order in the vicinity of the probe.

Ochsner et al. (1999) demonstrated that the phase transition temperature of DPPC bilayers was depressed in the presence of salmeterol at concentrations greater or equal to 100 μM. This is in agreement with our findings from the anisotropy measurements. The perturbing effect of salmeterol is substantial, as it was observed at concentrations down to 1 μM, where the lipid/drug ratio was approximately 2000 within the bilayer. This may give rise to the beta-2-adrenoceptor independent effect observed with high concentrations of salmeterol (Chong et al., 1998; Nials et al., 1997).

In addition, the sustained membrane-fluidity perturbation induced by salmeterol may result in a reduced function of the receptor and hence only weak partial agonistic effect of salmeterol. Experimental observations made over the last 10 years led researchers to propose that highly ordered cholesterol-enriched membrane lipid microdomains, and more specifically caveolae, may influence beta-2-adrenoceptor signal transduction properties by controlling its association with its signaling partners (Ostrom et al., 2001; Razani et al., 1999; Rybin et al., 2000) and its dimerization. The latter was suggested to play a key role in its activation in airway smooth muscle cells (Cherezov et al., 2007; Ianoul et al., 2005; Rosenbaum et al., 2007; Xiang et al., 2002). Therefore, it is tempting to suggest that the membrane fluidity enhancement of salmeterol could indirectly reduce its coupling efficacy via perturbation of the cell membrane ordered micro-domains, resulting in a lower intrinsic efficacy.

In contrast to our results, Austin et al. (2003) found a bell-shaped distribution profile for salmeterol in DMPC liposomes. The net neutral species had a higher bilayer affinity than in our experiments while cation and anion affinities were in agreement with our data. In the present work, indacaterol and salmeterol revealed a concentration-dependent partitioning at concentrations greater than 5 μM. In addition, the influence of the concentration on salmeterol partitioning was pH-dependent (Lombardi et al., 2009). While at concentrations lower than 100 μM partitioning was higher at pH 7.2 than at pH 9.5, the opposite was true at concentrations greater than 100 μM, consistent with a bell-shaped distribution profile.

This may explain the discrepancies between the salmeterol pH-distribution profiles determined by other groups and the results shown in the present work.

It is noticeable that only with liposomes consisting of DPPC/DPPG 80/20 and SM we found a higher membrane affinity for the net neutral species than for the cations. In contrast to the other phospholipids, these liposomes are characterized by their hydrogen bond donor capacity, and their relatively high T_M that leads mainly to *so* membranes at 37 °C. Why these two liposomes revealed bell-shaped partition profiles while other liposomes with one of the two characteristics, such as DMPC at 4C° (*so* bilayer) and cholesterol-containing liposomes (hydrogen-bond donor characteristics), did not show this partition behavior, can only be speculated. First, the rigidity of the membrane could generate unfavorable conditions for the intercalation of the hydrophobic tail of the compounds. Second, the hydrogen-bond donors of PG and SM could shield the hydrogen-bond acceptor groups of the lipids and reduce their interaction with hydrogen-bond donors of the drugs and in particular of the fully protonated cationic drugs. Third, the hydrogen-bond donors of the lipids could interact in addition with the phenolate oxygen, favoring the membrane interaction of net neutral salmeterol and indacaterol.

The affinity of both indacaterol and salmeterol for DOPC bilayers was notably increased as compared to PhC bilayers. These two lipids have the same head groups but differ in their acyl chain pattern. While PhC contains a mix of unsaturated and saturated chains of different length, all acyl chains of DOPC membranes contain one unsaturation between C9 and C10 and are 18 C atoms in length. It appears that the higher rigidity of DOPC does not exclude a better intercalation of the solutes into DOPC than into PhC membranes. Cholesterol reduced the membrane affinity of the two drugs in general but did not abolish the pH-dependence as seen for partially (SM) or fully (DMPC) saturated phospholipids. This difference in effect may be related to the different interactions between cholesterol and the respective phospholipids (Tsamaloukas et al., 2006).

The micro-domain structure of biological membranes has attracted significant attention in the past decade [see Jacobson et al. (2007) for a review] and the function of the beta-2-adrenoceptor has been linked with the presence of membrane rafts and calveolae in airway smooth muscle (Halayko et al., 2008). We found that when raft vesicles, prepared by the cold TritonX-100 method, were used as the partition phase, indacaterol partition was two-fold greater than that of salmeterol at pH 7.4. This two-fold higher depot close to the receptors could contribute to the prolonged action of indacaterol as compared to salmeterol.

The kinetics of drug-membrane interactions investigated by SPR were pH-dependent according to the ionization state of the solutes. Under our experimental conditions, the apparent rate constant of salmeterol and indacaterol partitioning into the membrane at physiological pH was too fast for a quantitative analysis. The observed slower rate constants were assigned to the translocation process (flip–flop) at physiological pH (Lombardi et al., 2009). This process was two-fold faster for indacaterol than for salmeterol, indicative for faster membrane permeation and hence tissue diffusion of indacaterol over salmeterol. Faster tissue diffusion could contribute to the faster onset of therapeutic action of indacaterol.

Based on our results, we expand the existing 'micro-kinetic model' (Anderson et al., 1994) as shown in Fig. 14. After inhalation, the drug moves through the lung tissue to reach the beta-2-adrenoceptor on smooth muscles before being eliminated from the lung. In the case of compounds with moderate lipophilicity, such as salbutamol, a significant fraction of the drug is present in the aqueous phases. The compound diffuses rapidly to activate the beta-2-adrenoceptor before being rapidly eliminated from the lung

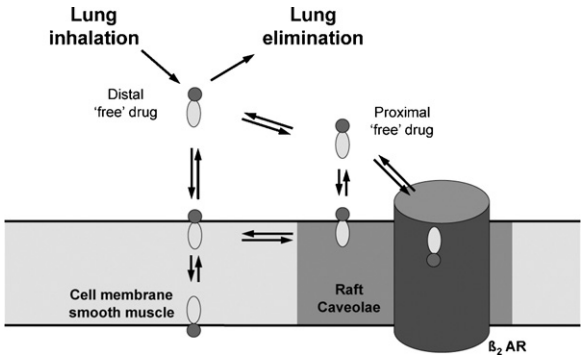


Fig. 14. Model to explain the pharmacological profile differences between salmeterol and indacaterol. See text for details.

resulting in a fast onset and short duration of action (Anderson et al., 1994). For the highly potent formoterol and the even more potent carmoterol, two compounds of intermediate lipophilicity, the high potency at the receptor is likely to play a determinant role in providing a sustained duration of action of 12 and 24 h, respectively (Voss et al., 1992).

The highly lipophilic indacaterol and salmeterol extensively partition into the membranes whilst diffusing into the lung tissues at a rate that can influence the onset of action. The proximal free drug concentration is sustained over-time by the local release from the lipid compartments long after the initial inhaled bulk free fraction has been eliminated (Anderson et al., 1994). For indacaterol this is likely to be one of the main mechanisms that provide extended duration of action, and its 2-fold higher affinity for raft micro-domains compared to salmeterol might contribute to the difference in duration of action. Regarding the fast onset of action for indacaterol, it has been postulated that zwitterionic lipophilic compounds might have a faster diffusion rate across tissues than the cations or anions (Jeppsson et al., 1989). At physiological pH indacaterol is mostly present as a zwitterionic species while salmeterol, that has a slow onset, is mostly present in its cationic form in aqueous solution. Therefore, the zwitterionic species of indacaterol could explain its fast onset of action when compared to salmeterol. This hypothesis is supported by our results showing a two fold faster translocation of indacaterol across lipid membranes when compared to salmeterol.

The shorter duration of action of salmeterol compared to indacaterol could be the result of the partial agonist properties of salmeterol, implying that occupancy of a higher number of receptors is needed for sustaining a pharmacologically relevant efficacy level, which might be more difficult to achieve for an extended time period. Supporting this view (Battram et al., 2006) have shown that higher than expected doses of salmeterol are required in *in vivo* pharmacological models to achieve a significant level of efficacy over time which might lead to a decrease in therapeutic index. The higher intrinsic efficacy of indacaterol implies that at equi-effective doses, a smaller number of beta-2-adrenoceptor need to be reached compared to a partial agonist with similar lipophilicity such as salmeterol.

Another aspect to be considered is the induced receptor desensitization upon prolonged agonist exposure. Duringer et al. (2009) correlated the lipophilicity of a panel of beta-2-adrenoceptor agonists with their capacity to induce loss in the receptor responsiveness after equi-effective stimulation. They showed that a high log *D* value in combination with a low efficacy was predictive of the loss of responsiveness. There were exceptions to this principle, and no single factor explained the rank order of reduced responsiveness to indacaterol and formoterol. Our suggestion is that the

tail length, responsible for the degree of membrane perturbation, influences the suppression of responsiveness.

Whether the membrane fluidizing effect of salmeterol contributes to the fact that it is only a partial agonist or to its high desensitization capacity remains unanswered here.

In conclusion, salmeterol and indacaterol show no major but several minor differences in their steady state and kinetic interactions with lipid membranes. The sum of these small differences including higher partitioning of indacaterol into the microenvironment of the receptor and faster membrane permeation of indacaterol is likely to contribute to the faster onset and longer duration of therapeutic action of indacaterol. A striking difference was observed in the effect of the two compounds on membrane fluidity. While indacaterol did not alter membrane fluidity, salmeterol drastically increased membrane fluidity. This may affect the function of the receptor reducing the intrinsic efficacy of the compound.

Acknowledgments

We are grateful to Jörg Scheuermann, Christoph Dumelin, Fabian Buller, and Dario Neri for making the SPR and HPLC–MS/MS instruments available to us. We thank Alexandre Trifilieff for fruitful discussions and for carefully reading the manuscript. This work was financially supported by Novartis Institutes for BioMedical Research, Basel, Switzerland.

References

Abdiche, Y.N., Myszka, D.G., 2004. Probing the mechanism of drug/lipid membrane interactions using Biacore. Anal. Biochem. 328, 233–243.

Anderson, G.P., Linden, A., Rabe, K.F., 1994. Why are long-acting beta-adrenoceptor agonists long-acting? Eur. Respir. J. 7, 569–578.

Austin, R.P., Barton, P., Bonnert, R.V., Brown, R.C., Cage, P.A., Cheshire, D.R., Davis, A.M., Dougall, I.G., Ince, F., Pairaudeau, G., Young, A., 2003. QSAR and the rational design of long-acting dual D2-receptor/beta-2-adrenoceptor agonists. J. Med. Chem. 46, 3210–3220.

Avdeef, A., Box, K.J., Comer, J.E., Hibbert, C., Tam, K.Y., 1998. pH-metric logP 10. Determination of liposomal membrane-water partition coefficients of ionizable drugs. Pharm. Res. 15, 209–215.

Barnes, P.B., 1977. Effect of beta-agonists in athsma treatment. In: Powels, R., O'Byrne, P.M. (Eds.), Beta2-Agonists in Athsma Treatment. Marcel Decker, New York, U.S., pp. 35–64.

Battram, C., Charlton, S.J., Cuenoud, B., Dowling, M.R., Fairhurst, R.A., Farr, D., Fozard, J.R., Leighton-Davies, J.R., Lewis, C.A., McEvoy, L., Turner, R.J., Trifilieff, A., 2006. *In vitro* and *in vivo* pharmacological characterization of 5-[(R)-2-(5,6-diethyl-indan-2-ylamino)-1-hydroxy-ethyl]-8-hydroxy-1H-quino lin-2-one (indacaterol), a novel inhaled beta(2) adrenoceptor agonist with a 24-h duration of action. J. Pharmacol. Exp. Ther. 317, 762–770.

Beeh, K.M., Derom, E., Kanniess, F., Cameron, R., Higgins, M., van As, A., 2007. Indacaterol, a novel inhaled beta2-agonist, provides sustained 24-h bronchodilation in asthma. Eur. Respir. J. 29, 871–878.

Belli, S., Elsener, P.M., Wunderli-Allenspach, H., Kramer, S.D., 2009. Cholesterol-mediated activation of P-glycoprote distinct effects on basal drug-induced ATPase activities. J. Pharm. Sci. 98, 1905–1918.

Betageri, G.V., Rogers, J.A., 1988. Thermodynamic of partitioning of beta-blokers in the n-octanol-buffer and liposome systems. Int. J. Pharm. 46, 95–102.

Bouchard, G., Pagliara, A., Carrupt, P.A., Testa, B., Gobry, V., Girault, H.H., 2002. Theoretical and experimental exploration of the lipophilicity of zwitterionic drugs in the 1,2-dichloroethane/water system. Pharm. Res. 19, 1150–1159.

Brown, D.A., Rose, J.K., 1992. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell 68, 533–544.

Bucher, K., Besse, C.A., Kamau, S.W., Wunderli-Allenspach, H., Kramer, S.D., 2005. Isolated rafts from adriamycin-resistant P388 cells contain functional ATPases and provide an easy test system for P-glycoprotein-related activities. Pharm. Res. 22, 449–457.

Cherezov, V., Rosenbaum, D.M., Hanson, M.A., Rasmussen, S.G., Thian, F.S., Kobilka, T.S., Choi, H.J., Kuhn, P., Weis, W.I., Kobilka, B.K., Stevens, R.C., 2007. High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. Science 318, 1258–1265.

Chong, L.K., Cooper, E., Vardey, C.J., Peachell, P.T., 1998. Salmeterol inhibition of mediator release from human lung mast cells by beta-adrenoceptor-dependent and independent mechanisms. Br. J. Pharmacol. 123, 1009–1015.

Duringer, C., Grundstrom, G., Gurcan, E., Dainty, I.A., Lawson, M., Korn, S.H., Jerre, A., Hakansson, H.F., Wieslander, E., Fredriksson, K., Skold, C.M., Lofdahl, M., Lofdahl, C.G., Nicholls, D.J., Silberstein, D.S., 2009. Agonist-specific patterns of beta(2)-adrenoceptor responses in human airway cells during prolonged exposure. Br. J. Pharmacol. 158, 169–179.

Halayko, A.J., Tran, T., Gosens, R., 2008. Phenotype and functional plasticity of airway smooth muscle: role of caveolae and caveolins. Proc. Am. Thorac. Soc. 5, 80–88.

Hellwich, U., Schubert, R., 1995. Concentration-dependent binding of the chiral beta-blocker oxprenolol to isoelectric or negatively charged unilamellar vesicles. Biochem. Pharmacol. 49, 511–517.

Huang, C., Mason, J.T., 1978. Geometric packing constraints in egg phosphatidylcholine vesicles. Proc. Natl. Acad. Sci. U.S.A. 75, 308–310.

Ianoul, A., Grant, D.D., Rouleau, Y., Bani-Yaghoub, M., Johnston, L.J., Pezacki, J.P., 2005. Imaging nanometer domains of beta-adrenergic receptor complexes on the surface of cardiac myocytes. Nat. Chem. Biol. 1, 196–202.

Jacobson, K., Mouritsen, O.G., Anderson, R.G., 2007. Lipid rafts: at a crossroad between cell biology and physics. Nat. Cell. Biol. 9, 7–14.

Jeppsson, A.B., Roos, C., Waldeck, B., Widmark, E., 1989. Pharmacodynamic and pharmacokinetic aspects on the transport of bronchodilator drugs through the tracheal epithelium of the guinea-pig. Pharmacol. Toxicol. 64, 58–63.

Kamau, S.W., Kramer, S.D., Gunthert, M., Wunderli-Allenspach, H., 2005. Effect of the modulation of the membrane lipid composition on the localization and function of P-glycoprotein in MDR1-MDCK cells. In Vitro Cell. Dev. Biol. Anim. 41, 207–216.

Kramer, S.D., 2001. Liposomes/wather partitioning: theory, techniques and applications. In: Testa, B., van de Waterbeemd, H., Folkers, G., Guy, R. (Eds.), Pharmacokinetic Optimization in Drug Research. VHCA Wiley-VHC Zurich, Zurich Weinheim, pp. 401–428.

Kramer, S.D., Braun, A., Jakits-Deiser, C., Wunderli-Allenspach, H., 1998. Towards the predictability of drug–lipid membrane interactions: the pH-dependent affinity of propanolol to phosphatidylinositol containing liposomes. Pharm. Res. 15, 739–744.

Kramer, S.D., Jakits-Deiser, C., Wunderli-Allenspach, H., 1997. Free fatty acids cause pH-dependent changes in drug–lipid membrane interactions around physiological pH. Pharm. Res. 14, 827–832.

Laisc, D.D., 1993. Liposomes: from Physics to Applications. Elsevier Science Publishers B.V., Amsterdam.

Lentz, B.R., 1993. Use of fluorescent probes to monitor molecular order and motions within liposome bilayers. Chem. Phys. Lipids 64, 99–116.

Leo, A., Hansch, C., Elkins, D., 1971. Partition coefficients and their uses. Chem. Rev. 71.

Lindberg, A., Szalai, Z., Pullerits, T., Radeczky, E., 2007. Fast onset of effect of budesonide/formoterol versus salmeterol/fluticasone and salbutamol in patients with chronic obstructive pulmonary disease and reversible airway obstruction. Respirology 12, 732–739.

Lombardi, D., Cuenoud, B., Wunderli-Allenspach, H., Kramer, S.D., 2009. Interaction kinetics of salmeterol with egg phosphatidylcholine liposomes by surface plasmon resonance. Anal. Biochem. 385, 215–223.

Marsh, D., 1990. Handbook of Lipid Bilayers. CRC Press, Boston.

Mayer, L.D., Hope, M.J., Cullis, P.R., 1986. Vesicles of variable sizes produced by a rapid extrusion procedure. Biochim. Biophys. Acta 858, 161–168.

Nagle, J.F., Tristram-Nagle, S., 2000. Structure of lipid bilayers. Biochim. Biophys. Acta 1469, 159–195.

Nials, A.T., Coleman, R.A., Johnson, M., Vardey, C.J., 1997. The duration of action of non-beta 2-adrenoceptor mediated responses to salmeterol. Br. J. Pharmacol. 120, 961–967.

Ochsner, M., Jaekel, K., Mutz, M., Anderson, M.G., John, P.E., 1999. Comparative biophysical analysis of the interaction of bronchodilating beta(2)-adrenoceptor agonists with lipid membranes. Eur. J. Med. Chem. 34, 451–462.

Ostrom, R.S., Gregorian, C., Drenan, R.M., Xiang, Y., Regan, J.W., Insel, P.A., 2001. Receptor number and caveolar co-localization determine receptor coupling efficiency to adenylyl cyclase. J. Biol. Chem. 276, 42063–42069.

Palmqvist, M., Persson, G., Lazer, L., Rosenborg, J., Larsson, P., Lotvall, J., 1997. Inhaled dry-powder formoterol and salmeterol in asthmatic patients: onset of action, duration of effect and potency. Eur. Respir. J. 10, 2484–2489.

Pauletti, G.M., Wunderli-Allenspach, H., 1994. Partition coefficients *in vitro*: artificial membranes as a standardized distribution model. Eur. J. Pharm. Sci. 1, 273–282.

Plember van Balen, G., Caron, G., Ermondi, G., Pagliara, A., Grandi, T., Bouchard, G., Fruttero, R., Carrupt, P.A., Testa, B., 2001. Lipophilicity behaviour of the Zwitterionic antihistamine cetirizine in phosphatidylcholine liposomes/water systems. Pharm. Res. 18, 694–701.

Pokorny, A., Almeida, P.F., Melo, E.C., Vaz, W.L., 2000. Kinetics of amphiphile association with two-phase lipid bilayer vesicles. Biophys. J. 78, 267–280.

Pontier, S.M., Percherancier, Y., Galandrin, S., Breit, A., Gales, C., Bouvier, M., 2008. Cholesterol-dependent separation of the beta2-adrenergic receptor from its partners determines signaling efficacy: insight into nanoscale organization of signal transduction. J. Biol. Chem. 283, 24659–24672.

Razani, B., Rubin, C.S., Lisanti, M.P., 1999. Regulation of cAMP-mediated signal transduction via interaction of caveolins with the catalytic subunit of protein kinase A. J. Biol. Chem. 274, 26353–26360.

Rebolj, K., Ulrih, N.P., Macek, P., Sepcic, K., 2006. Steroid structural requirements for interaction of ostreolysin, a lipid-raft binding cytolysin, with lipid monolayers and bilayers. Biochim. Biophys. Acta 1758, 1662–1670.

Repakova, J., Holopainen, J.M., Morrow, M.R., McDonald, M.C., Capkova, P., Vattulainen, I., 2005. Influence of DPH on the structure and dynamics of a DPPC bilayer. Biophys. J. 88, 3398–3410.

Rodgers, T., Leahy, D., Rowland, M., 2005. Tissue distribution of basic drugs: accounting for enantiomeric, compound and regional differences amongst beta-blocking drugs in rat. J. Pharm. Sci. 94, 1237–1248.

Roig, J., Hernando, R., Mora, R., 2009. Indacaterol, a novel once daily inhaled beta2-adrenoreceptor agonist. Open Respir. Med. J. 3, 27–30.

Rosenbaum, D.M., Cherezov, V., Hanson, M.A., Rasmussen, S.G., Thian, F.S., Kobilka, T.S., Choi, H.J., Yao, X.J., Weis, W.I., Stevens, R.C., Kobilka, B.K., 2007. GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function. Science 318, 1266–1273.

Rybin, V.O., Xu, X., Lisanti, M.P., Steinberg, S.F., 2000. Differential targeting of beta-adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signaling pathway. J. Biol. Chem. 275, 41447–41457.

Sacre, M.M., Tocanne, J.F., 1977. Importance of glycerol and fatty acid residues on the ionic properties of phosphatidylglycerols at the air-water interface. Chem. Phys. Lipids 18, 334–354.

Seo, P.R., Teksin, Z.S., Kao, J.P., Polli, J.E., 2006. Lipid composition effect on permeability across PAMPA. Eur. J. Pharm. Sci. 29, 259–268.

Tsamaloukas, A., Szadkowska, H., Heerklotz, H., 2006. Thermodynamic comparison of the interactions of cholesterol with unsaturated phospholipid and sphingomyelins. Biophys. J. 90, 4479–4487.

Ullman, A., Svedmyr, N., 1988. Salmeterol, a new long acting inhaled beta 2 adrenoceptor agonist: comparison with salbutamol in adult asthmatic patients. Thorax 43, 674–678.

Voss, H.P., Donnell, D., Bast, A., 1992. Atypical molecular pharmacology of a new long-acting beta 2-adrenoceptor agonist, TA 2005. Eur. J. Pharmacol. 227, 403–409.

Waldeck, B., 2002. Beta-adrenoceptor agonists and asthma—100 years of development. Eur. J. Pharmacol. 445, 1–12.

Xiang, Y., Rybin, V.O., Steinberg, S.F., Kobilka, B., 2002. Caveolar localization dictates physiologic signaling of beta 2-adrenoceptors in neonatal cardiac myocytes. J. Biol. Chem. 277, 34280–34286.

Yu, S., Harding, P.G., Smith, N., Possmayer, F., 1983. Bovine pulmonary surfactant: chemical composition and physical properties. Lipids 18, 522–529.