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Original article

Comparative biophysical analysis of the interaction of bronchodilating β_2-adrenoceptor agonists with lipid membranes

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Abstract – Two selective β_2-adrenoceptor agonists, formoterol and salmeterol, have recently become commercially available. After inhalation of appropriate doses, both bronchodilators are capable of inducing a sustained relaxation of airway smooth muscle for ≈12 h. Remarkably, formoterol has a higher potency and shows a faster onset of relaxation than salmeterol. In this paper, evidence is presented that the long-acting relaxant effect of these drugs primarily results from an accumulation within plasmalemma lipid bilayers of airway smooth muscle and that the delayed response of salmeterol is due to its higher lipophilicity. Once having partitioned into the membrane lipid bilayer, the drugs remain trapped therein and available to interact with the β_2-adrenoceptor. In the case of salmeterol, the majority of drug molecules approach the active site(s) of β_2-adrenoceptor glycoproteins by lateral diffusion via the plasma membrane and poorly bind to β_2-adrenergic receptors from the extracellular space. This model clearly expands the traditional picture of a ligand approaching the β_2-receptor exclusively via the aqueous biophase. © Elsevier, Paris

β_2-adrenoceptor agonists / n-octanol/water partition coefficients / lipid membrane/water partition constants / thermodynamics of drug-membrane interactions / membrane microviscosity

1. Introduction

Contraction of airway smooth muscle is largely responsible for the bronchospasm which is one of the characteristic features of an asthmatic attack [1]. The pathological processes underlying asthmatic disease are inflammatory in nature, with a concurrent involvement of various mediators, such as histamine, prostaglandins, hydroxyeicosatetraenoic acid and the sulfidopeptide leukotrienes (LTC_4, LTD_4 and LTE_4) [2–4]. International guidelines therefore recommend inhaled corticosteroids or non-steroidal anti-inflammatory drugs, such as cromolyn sodium and nedocromil sodium, as first-line therapy for the management of asthma [5–7]. Nevertheless, there are still sufficient unanswered questions concerning long-term therapy with inhaled steroids, that such treatment should be reserved for adults or for children with severe asthma [8]. In clinical practice the prescription of bronchodilatory β_2-adrenoceptor agonists remains the mainstay especially for handling acute asthmatic attacks [9].

Two long-acting selective β_2-adrenoceptor agonists, formoterol and salmeterol, have recently become available for the treatment of airflow obstruction in asthma [10, 11]. The important differences between these drugs are that formoterol has a higher potency and shows a faster onset of relaxation (1–3 min) than salmeterol, which reaches its maximum therapeutic response up to 11 h after administration [10–12]. Following inhalation of appropriate doses of salmeterol (50 μg) [12] or formoterol (12 μg) [10–13], bronchodilatation is maintained for at least 12 h, and protection afforded by both drugs against histamine or methacholine challenges is provided for up to 24 h.

The mechanisms behind the long duration of action and the origin of the delayed bronchodilating response of

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salmeterol remain to be elucidated. Two models have been proposed to account for these effects. The exo-site hypothesis is based on the concept that the large lipophilic N-substituent of long-acting β_2-adrenoceptor agonists, interacts with a distinct non-polar region in the cell membrane in the vicinity of the β_2-adrenoceptor, the exo-site region [14, 15]. This model consistently explains the long-lasting bronchodilating effect of salmeterol by suggesting that the long aliphatic tail remains anchored at the hydrophobic exo-site domain, while the saligenin head activates the β_2-adrenoceptor in a continuous manner. However, the idea of a specific exo-site binding region fails to rationalize the long duration of action of formoterol, since its lipophilic side chain is much too short to allow anchorage outside the β_2-adrenoceptor glycoprotein [16]. It has therefore been proposed that accumulation and retention of lipophilic β_2-stimulating bronchodilators in the plasma membrane accounts for the pharmacodynamic effects observed and results in a long-lasting stimulation of β_2-adrenoceptors being embedded in plasmalemma lipid bilayers of airway smooth muscle [17–20].

The current study concentrates on an extensive analysis of the physicochemical properties of salmeterol and formoterol in comparison with the short-acting (< 5 h) β_2-adrenoceptor agonist salbutamol. In addition, a thorough characterization of the interaction of these drugs with lipid membranes is presented. In our opinion, the acquired results not only contribute to the understanding of the pharmacological behavior of β_2-adrenoceptor agonists, but they reveal general principles relevant for the development of drugs targeting membrane receptors.

For the characterization of the lipophilic properties of the studied drugs, n-octanol/water partition coefficients and pKa values were determined, and the lipid membrane/water partition constants, K_p^0, measured using 1-palmitoyl-2-oleoyl-phosphatidylcholine and 1,2-dioleoly-phosphatidyl-L-serine as artificial membrane lipid components reconstituted as liposomes. Since changes in the enthalpy, entropy and free energy (ΔH, ΔS and ΔG) occur upon binding of β_2-adrenoceptor agonists to lipid membranes and act as driving forces for drug-membrane interactions, the thermodynamics of the partitioning was studied. The enthalpy, ΔH, released per mole of drug bound to lipid membranes was measured by isothermal titration calorimetry, and the Gibbs free energy, ΔG, calculated from the partition constant K_p^0, thus yielding the entropic part of the binding reaction.

Furthermore, the influence of increasing drug concentrations on the phase transition temperature of synthetic 1,2-dipalmitoyl-phosphatidylcholine lipid bilayers was analysed with a differential scanning calorimeter to resolve whether the drugs simply attach to the membrane surface or actually incorporate into the core of lipid bilayers.

Since all experiments described so far exclusively characterize physicochemical interactions of β_2-adrenoceptor agonists with synthetic lipid membrane vesicles, the effect of salmeterol, formoterol and salbutamol on the microviscosity of plasmalemma lipid bilayers of glomerular mesangial cells, a specialized type of vascular smooth muscle cells [21], was examined. Mesangial cells are known to adequately respond to a number of smooth muscle agonists and were thus used as analogues of human pulmonary smooth muscle cells [21].

2. Materials and methods

2.1. Chemicals

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), formoterol and salmeterol were synthesized at Novartis Pharma AG; 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), salbutamol and HEPES (4-(2-hydroxy-ethyl)-piperazine-1-ethanesulfonate) were obtained from Sigma (St. Louis, MO, USA); RPMI 1640, penicillin and streptomycin sulfate from Boehringer Mannheim (FRG); trypsin buffer solution (0.25%) and tissue culture flasks from Gibco BRL (Basel, Switzerland) and 1,6-diphenyl-1,3,5-hexatriene (DPH) from Molecular Probes (Eugene, OR, USA). All other chemicals and solvents were from Fluka (Buchs, Switzerland).

2.2. Preparation of lipid vesicles

POPC and DOPS were dissolved in chloroform in a weight ratio of 9:1, shell dried under a stream of nitrogen, and desiccated for 2 h in a high vacuum chamber. Buffer (buffer A: 10 mM phosphate buffer, pH 7.0, 150 mM NaCl; buffer B: 5 mM phosphate buffer, pH 7.0) was added thereafter to the phospholipid film and the lipid concentration adjusted to 30 mg/mL. The suspension was equilibrated at room temperature for 2 h, periodically vortexed, and sonicated for 3 min at 0 °C under a nitrogen atmosphere. Metal debris from the sonication tip was removed by centrifugation (2 500 *g*, 5 min, 25 °C). Aliquots of the lipid vesicle suspension were prepared and stored at –20 °C. The particle size of POPC /DOPS phospholipid vesicles was analysed by quasi elastic light scattering (Nano-Sizer, Coulter Electronics Ltd., Luton, UK). As the results indicated, the described procedure yielded stable lipid vesicles with a

characteristic diameter of 140 ± 20 nm, which didn't change during experiments.

2.3. pKa values and n-octanol/water partition coefficients

The partition coefficients, D_{ow}, of β_2-adrenoceptor agonists between buffer-saturated n-octanol and buffer solution A were determined at pH 7.0 by the shake flask method of Leo, Hansch and Elkins and expressed in terms of their logarithms to base 10 [22]. In both phases, drug concentrations were measured spectrophotometrically (Lambda 16, Perkin Elmer, Norwalk, CT, USA). To determine whether bronchodilators were dissolved at pH 7.0 as neutrals or ions, their pKa values were measured by photometric titration at 22 °C in aqueous solution [23].

To complete the data set obtained, theoretical log D_{ow} values of uncharged drug molecules were calculated with the CLOGP 3.42 program (Med Chem Software, Medical Chemistry Project, Pomona College, Claremont, CA, USA).

2.4. Lipid membrane/water partition constants

The lipid membrane/water partition constants of β_2-adrenoceptor agonists were determined by equilibrium dialysis at room temperature using POPC and DOPS as artificial membrane lipid components reconstituted as uni- and/or multi-lamellar liposomes. The teflon dialysis vessel (Dianorm, Prochimie, Zürich, Switzerland) consisted of two 1 mL sample cells separated by a regenerated cellulose dialysis membrane (Visking, Union Carbide, Chicago, USA) with pores of 2.4 nm diameter. Typically, the first sample chamber was filled with lipid vesicle suspensions in the concentration range 4–8 mM, while the second chamber contained aqueous drug solutions in the range 40–70 μM. After an equilibration period of 18 h, both sample solutions were diluted with isopropanol (1:1) and the molar concentrations of free (c_f) and of membrane-bound (c_b) β_2-adrenoceptor agonists determined spectrophotometrically (Lambda 16, Perkin Elmer). Mass balances of drugs were checked to verify the concentrations measured and exclude disintegration of drug molecules. In addition, the concentrations and integrity of the lipids prior to, and after, the experiments were analysed by semi-quantitative thin-layer chromatography. In general, no degradation of phospholipids was detected, and the total amount of lipids lost by diffusion across the dialysis membrane into the lipid free dialysis chamber was below 0.3%.

Based on these experimental data, standard lipid membrane/water partition constants of β_2-adrenoceptor agonists were calculated from the formula: $K_p^0 = c_b/(c_f \times c_L)$, where cL denotes the concentration of phospholipids [24, 25][1]. Averaged from at least 4 independent measurements, the K_p^0s thus obtained were finally used to calculate the free energy of binding, $\Delta G = -RT \times \ln (K_p^0 \times 55.6\ M)$, where the cratic factor, 55.6 M, corresponds to the molar concentration of water in dilute aqueous solutions [26].

To ensure reproducible results, a modified dialysis vessel was constructed to measure the partition constants of drugs with K_p^0 values beyond 10 000 M^{-1}. For this purpose, two dialysis membranes with an area of 7 cm^2 were mounted in a teflon block to enclose a dialysis volume of 4.5 mL. Thereafter, the dialysis chamber was filled with lipid vesicle suspensions, and the teflon device submerged in 300.0 mL of magnetically stirred, aqueous drug solutions. After an equilibration period of 18 h, molar drug concentrations were measured spectrophotometrically as described above. However, to overcome the limitation of the spectrophotometer, residual drug in the aqueous phase was concentrated by extraction into n-octanol (volume ratio: 250.0:1.00) and the measured drug concentration corrected by taking account of the corresponding log D_{ow} value.

2.5. Isothermal titration calorimetry

The enthalpies of the binding of β_2-adrenoceptor agonists to POPC/DOPS lipid vesicles were determined at 25 °C using a MCS-ITC isothermal titration calorimeter (MicroCal, Northampton, MA, USA). The sample cell (1.3 mL) was filled with lipid vesicle suspension in the range 20–40 mM. As soon as baseline stability was better than 0.4 μJ/s, heat of binding curves were monitored after consecutive injections of 5–15 μL of ≈1 mM drug solution at characteristic time intervals of 250 s. Thereafter, the isothermal titration curves were analysed using the ORIGIN software package provided by the MCS-ITC instrument supplier. The reaction enthalpy Δh, expressed in kJ per mole of drug added (c_D), was calculated from the peak areas after single injections and corrected for the heat of dilution Δh_{dil}, measured in a separate control experiment. The binding enthalpy ΔH refers to the actual amount of drug bound to lipid bilayers (c_b) and was computed from: $\Delta H = c_D \times \Delta h/c_b$ with $c_b = c_D \times (K_p^0 \times c_L)/(K_p^0 \times c_L + 1)$.

[1] K_p^0 is connected to the dimensionless lipid membrane/water partition constant, K_{pmem}, through the expression: $K_{pmem} = K_p^0 \times 55.6\ M \times MW_W/MW_L$, where MW_L and MW_W refer to the molecular weights of the phospholipids and water, respectively [25].

2.6. *Differential scanning calorimetry*

The influence of increasing concentration of β_2-adrenoceptor agonists on the solid-to-liquid phase transition temperature profile of DPPC membranes was studied by high-sensitivity differential scanning calorimetry (MicroDSC II, Setaram, Caluire, France). Lipid vesicle suspensions were prepared on demand by hydrating dry, crystalline phospholipid powder with phosphate buffer A containing increasing amounts of drug dissolved. These suspensions were inserted into the calorimeter without any further preparation step. To ensure a complete hydration of the phospholipids, each sample was in a first run heated up and cooled down with a temperature gradient of 0.5 °C/min in the range 25–50 °C. Thereafter DSC scans were monitored at a lower temperature gradient of 0.1 °C/min.

2.7. *Fluorescence anisotropy of the plasmalemma membrane of mesangial cells*

2.7.1. *Cultivation of mesangial cells*

Primary cultures of rat mesangial cells were kindly provided by Prof. J. Pfeilschifter (Biocentre, University of Basel, Switzerland). Grown in an RPMI 1640 medium supplemented with 10% foetal calf serum, penicillin (100 U/mL), streptomycin sulfate (100 µg/mL) and bovine insulin (0.66 U/mL), mesangial cells were cultivated in 75 cm^2 flasks at 37 °C in air/CO_2 (19:1) essentially as described previously [27, 28]. Prior to starting the measurements, the cells ($3 \times 10^5/cm^2$) were harvested using trypsin (0.25%) buffer solutions, centrifuged (70 *g*, 5 min, 25 °C) and suspended in a saline Hepes buffer solution at a density of (10^7 cells/mL [29]. Viability was assessed thereafter by the Trypan blue exclusion method, which indicated a survival rate of 96 ± 1%. To investigate changes in the microviscosity, the cell suspension was subdivided into four fractions and incubated with DPH (5 µM, 37 °C, 30 min), a fluorescent anisotropy indicator known to rapidly partition into the acyl side chain region of cellular membranes [30]. Whereas the first fraction served as a control cell group and exclusively contained DPH in the buffer medium, the incubation medium of the remaining cell batches additionally contained 50 nM formoterol, 100 nM salmeterol and 650 nM salbutamol, respectively, i.e. drugs at concentrations in proportion to their inhaled β_2-adrenergic bronchodilator doses [31, 32]. After the incubation period, the cells were washed twice, resuspended in saline buffer medium at a density of 10^6 cells/mL and transferred to a magnetically stirred and thermostated (30 °C) sample cuvette.

2.7.2. *Measurement of the fluorescence anisotropy of cultivated mesangial cells*

The apparatus for fluorescence anisotropy measurements has been described in much more detail elsewhere [33]. Briefly, the DPH molecules, anchored in hydrophobic regions of plasmalemma lipid membranes of mesangial cells, were alternatively excited by a vertically and horizontally polarized, power-locked argon-ion laser beam (Spectra Physics, SP 2045-15S; mid-UV range: 351.1–363.8 nm, Mountain View, CA, USA). The correlated fluorescence photons were measured at a right angle to the excitation laser using an interference filter (Corion, IF-filter, λ = 442 nm, FWHM = 10 nm, Franklin, MA, USA) to select the wavelength and a polarizing beam splitter cube (Newport, 10FC16.PB3, Irvine, CA, USA) to separate the vertically and horizontally polarized fluorescence components. Detected by two separate photomultipliers operating in the single-photon-counting mode (Hamamatsu, R 928, Hamamatsu City, Japan), the photoelectrons released from the individual detection tubes were registered by a two channel single-photon-counting unit (Stanford Research System, SR 400, Palo Alto, CA, USA). Typically, incoming pulses were accumulated over a time period of 5 s. At the end of each interval, count rates (photons/s) were digitized and transferred to an IBM-AT computer.

Some limitations of the described experimental approach should be clearly recognized. At first, fluorescence anisotropy of DPH does not uniquely reflect plasmalemma properties, but refers to the ensemble of cellular membranes [34]. Moreover, diffusive rotational motions of DPH are generally hindered in membranes, since natural lipid bilayers possess a highly organized, anisotropic structure [35]. That is, the fluorophore probably does not rotate beyond a particular angle and its limiting anisotropy r_∞ may be different from zero. Care was thus advised when converting measured anisotropy data into standard microviscosity units (cP). As a result, the microviscosity of the cell membrane was expressed in terms of its steady-state anisotropy, $\langle r \rangle = (I_{VV} - G \times I_{VH})/(I_{VV} + 2 \times G \times I_{VH})$ throughout the study. The parameters I_{VV} and I_{VH} refer to the vertically (I_{VV}) and horizontally (I_{VH}) polarized emission intensities after excitation with a vertically polarized excitation laser. The gain factor, G [= I_{HV}/I_{HH}], represents the ratio of the orthogonally polarized fluorescence intensities (I_{HV} and I_{HH}) measured by the detection system at an angle of 90° with respect to a horizontally aligned excitation laser [35]. To get reasonable anisotropy values, $\langle r \rangle$, the autofluorescences (I_{VV}, I_{VH}, I_{HV} and I_{HH}) from unlabeled cells (stemming

Table I. pKa and log D_{ow} values of β_2-adrenoceptor agonists and thermodynamic data of drug-membrane interactions measured in buffer A (10 mM phosphate buffer, 150 mM NaCl, pH 7.0).

	Salmeterol	Formoterol	Salbutamol
pKa	–	7.88, 9.33	9.01, 10.10
log D_{ow}	1.45 ± 0.03	0.06 ± 0.03	–2.30 ± 0.03
log D_{ow} (calc.)	3.26	1.26	0.11
K_p^0 (M^{-1})	12 500 ± 300	420 ± 40	55 ± 10
K_{pmem}	16 400	550	72
ΔG (kJ/mol)	–33.4	–24.9	–19.9
Δh^a (kJ/mol)	–14.7 ± 0.4	–21.3 ± 0.7	–0.6 ± 0.1
ΔH^b (kJ/mol)	–14.7	–22.6	–0.9
$T\Delta S$ (kJ/mol)	18.7	2.3	19.0

[a]Experimental reaction enthalpy per mole of drug added. [b]Binding enthalpy per mole of drug bound to lipid vesicles as calculated from the corresponding K_p^0 value.

mainly from $NADP^+$ and NADPH) were determined in a preliminary experiment and subtracted from the data set obtained.

3. Results

3.1. Partition behaviour and pKa values

Measured at pH 7.0, the logarithms of the n-octanol/water partition coefficients, log D_{ow}, of salmeterol, formoterol and salbutamol were equal to 1.45, 0.06 and –2.30, respectively (*table I*). Determined by photometric titration, the pKa values of the secondary amino groups of formoterol and salbutamol were 7.88 and 9.01. Unfortunately, the solubility of salmeterol was too low at basic pH to allow an experimental determination of the corresponding pKa value. As a result, the pKa value of the aliphatic nitrogen atom of salmeterol was theoretically predicted using quantum chemical procedures [36, 37]. Interestingly, the calculated pKa value (pKa = 9.04 ± 0.12) closely matched the pKa value of salbutamol. This result seems quite reasonable, since both of these β_2-adrenoceptor agonists contain a saligenin-ethanolamine head as proton accepting/donating substituent. Altogether, the pKa values obtained indicate that all studied bronchodilators were dissolved as cations under the experimental conditions used. Consequently, the measured log D_{ow} values specify the rank order of lipophilicity of positively charged β_2-adrenoceptor agonists. In harmony with these results, the theoretically predicted log D_{ow} values, which refer to the uncharged molecules, were 1–2 log units higher.

With reference to POPC/DOPS lipid membrane vesicles, the standard lipid membrane/water partition constants K_p^0 of salmeterol, formoterol and salbutamol were 12 500 M^{-1}, 420 M^{-1} and 55 M^{-1}, respectively, and followed the same rank order of lipophilicity as their associated log D_{ow} values (*table I*).

To ensure reproducible results, the lipid membrane/water partition constant, K_p^0, of formoterol was determined at molar lipid-to-drug concentration ratios (L/D) in the range 5.5–100. As shown in *figure 1*, measured K_p^0 values of formoterol increased in parallel with the L/D ratio and reached a plateau phase at an L/D ratio of ≈30. Observation indicates that saturation effects dominate the scenario at L/D ratios below 30, e.g. electrostatic repulsion between positively charged drug molecules supposedly precludes a further partitioning of formoterol into membrane lipid bilayers [38]. In support of this hypothesis, K_p^0 values essentially remained constant at L/D ratios beyond 30 owing to the large excess of phospholipids. As a result, all the K_p^0 values listed in *table I* were determined at an L/D ratio ≥ 50, i.e. under conditions which reasonably reflect the in vivo situation.

Since surfaces of POPC/DOPS lipid vesicles were negatively charged due to high content (10%) of DOPS phospholipids, it was expected that electrostatic attraction between cationic drug molecules and the membrane surface plays an important role in the binding reaction. Consequently, the partitioning of β_2-adrenoceptor agonists into POPC/DOPS lipid bilayers was studied under hypotonic conditions, where electrostatic attractions should be enhanced due to reduced charge shielding effects. The partition constants measured in 5 mM phosphate buffer solution, which contained no NaCl supplement (buffer B), are summarized in *table II*. Consistent with our expectation, the K_p^0 value of formoterol, K_p^0 = 860 M^{-1}, was about twice as large as the partition constant measured in a buffer solution of higher ionic strength (*tables I* and *II*). However, contrasting with our assumption, the K_p^0 values of salmeterol and salbutamol

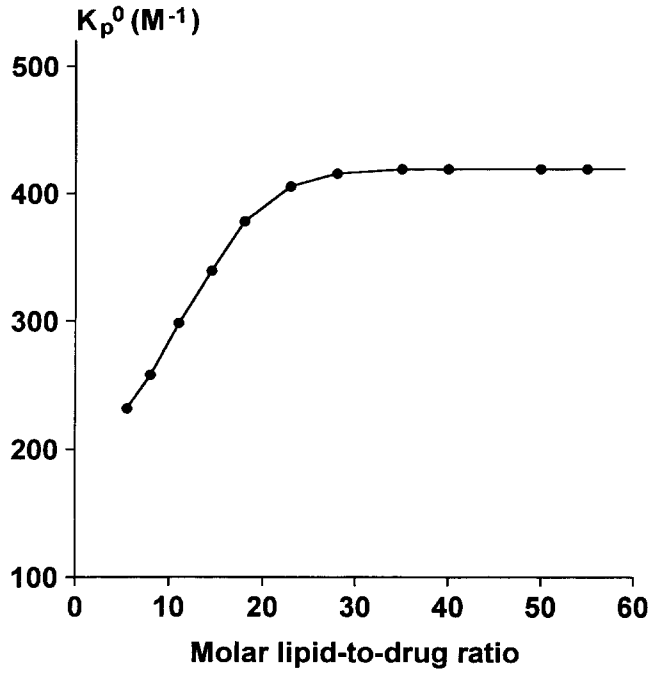

Figure 1. Standard lipid membrane/water partition constants K_p^0 characterizing the partitioning of formoterol into POPC/DOPS lipid vesicles, expressed as a function of increasing lipid-to-drug concentration ratios.

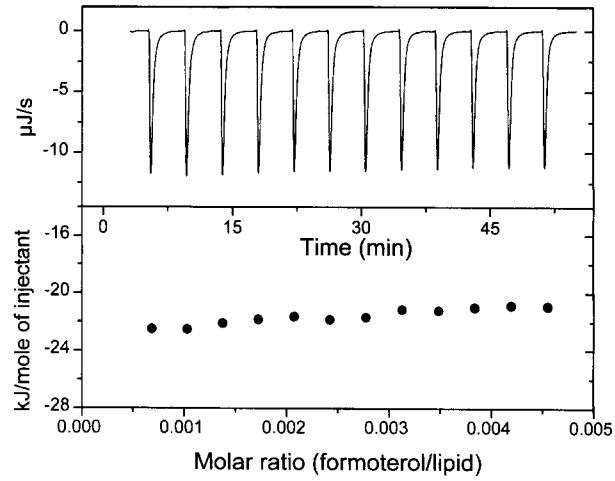

Figure 2. A. (upper panel) Isothermal titration curve monitored after consecutive injections of equivalent amounts (c_D = 15.5 µmol) of formoterol to a POPC/DOPS lipid vesicles suspension. **B.** (lower panel) Calculated heat of reaction, expressed in kJ per mol of drug added (c_D), at molar drug-to-lipid ratios in the range 0.000–0.005 and at a total lipid concentration c_L ~ 10 mg/mL.

slightly decreased to 10 600 M^{-1} and 39 M^{-1} under hypotonic conditions.

3.2. Isothermal titration calorimetry

Figure 2A displays the isothermal titration curve monitored after 11 consecutive injections of equivalent amounts (15.5 µmol) of formoterol to a POPC/DOPS lipid vesicle suspension. As indicted in *table I*, the reaction of cationic drug molecules with synthetic POPC/DOPS lipid bilayers was highly exothermic. Since all experiments were carried out at a high L/D concentration ratio (L/D ≥ 100), the heat of reaction remained almost constant for consecutive injections due to the large excess of phospholipids. The reaction enthalpy of formoterol (Δh = –21.3 ± 0.7 kJ/mol) was calculated by integration over individual peaks and corrected by the heat of dilution (*table I, figure 2B*). Based on a partition constant K_p^0 of 420 M^{-1}, an estimated 94% of formoterol molecules were bound to lipid membranes of POPC/DOPS vesicles and the binding enthalpy thus determined was equal to: ΔH = –22.6 kJ/mol. As shown in *table I*, the binding enthalpies ΔH of salmeterol and salbutamol were –14.7 kJ/mol and –0.9 kJ/mol, respectively, i.e. significantly lower than the value found for formoterol.

Table II. Thermodynamic data of drug-membrane interactions in buffer B (5 mM phosphate buffer, pH 7.0).

	Salmeterol	Formoterol	Salbutamol
K_p^0 (M^{-1})	10 600 ± 300	860 ± 60	39 ± 10
K_{pmem}	13 900	1 130	51
ΔG (kJ/mol)	–33.0	–26.7	–19.0
Δh^a (kJ/mol)	–12.4 ± 0.3	–23.7 ± 0.5	–0.4 ± 0.1
ΔH^b (kJ/mol)	–12.4	–24.4	–0.7
$T\Delta S$ (kJ/mol)	20.6	2.3	18.3

[a]Experimental reaction enthalpy per mole of drug added. [b]Binding enthalpy per mole of drug bound to lipid vesicles as calculated from the corresponding K_p^0 value.

Remarkably, the binding enthalpy of formoterol decreased by 1.8 kJ/mol to $\Delta H = -24.4$ kJ/mol in hypotonic buffer solution, whereas the binding enthalpies of salmeterol and salbutamol increased to –12.4 kJ/mol and –0.7 kJ/mol, respectively.

3.3. Differential scanning calorimetry

Pure DPPC membranes exhibit a sharp transition from the gel phase Lβ to the liquid crystalline phase Lα at a temperature T_m = 41.70 °C. This phase transition is known to be very sensitive to the presence of impurities in the lipid membrane bilayer and can therefore be used to monitor the incorporation of drugs.

Figure 3 shows DSC scans in the range 39–44 °C of pure DPPC membranes and of membranes containing formoterol at decreasing lipid-to-drug (L/D) molar ratios. Strikingly, the line profile broadened and the phase transition temperature T_m decreased with increasing content of formoterol in the lipid bilayer. Measured DSC scans indicated that salmeterol and salbutamol also shifted the phase transition temperatures of DPPC membranes to lower values and broadened the line profiles (data not shown). Remarkably, at identical L/D ratios, salmeterol was much more potent than formoterol, and the later more powerful than salbutamol in depressing T_m. The plots in *figure 4* compare the efficiencies of the drugs to induce changes in the phase transition temperature (ΔT_m = 41.70 °C – T_m) and demonstrate that the temperature shift ΔT_m increased in linear proportion to the drug concentration for all β_2-adrenoceptor agonists studied.

Much to our satisfaction, measured line profiles and temperature shifts did not depend on the procedure used to incorporate the drug molecules into the lipid vesicles. Recorded DSC scans were identical independent of whether the drugs were incorporated under standard conditions or whether the DPPC vesicles were incubated with equivalent drug concentrations for time periods of up to 4 d at 45 °C, i.e. above the phase transition temperature of pure DPPC membranes. Results indicating a very good reproducibility and stability of the samples.

3.4. Fluorescence anisotropy of cellular membranes

Fluorescence anisotropy measurements have been widely used to estimate changes in the microviscosity of cellular membranes and to discuss consequences thereof, including the molecular order and the dynamics within plasmalemma lipid membranes [30, 35, 39]. Since all experiments reported so far referred to interactions with synthetic lipid bilayers, it was of principal interest to

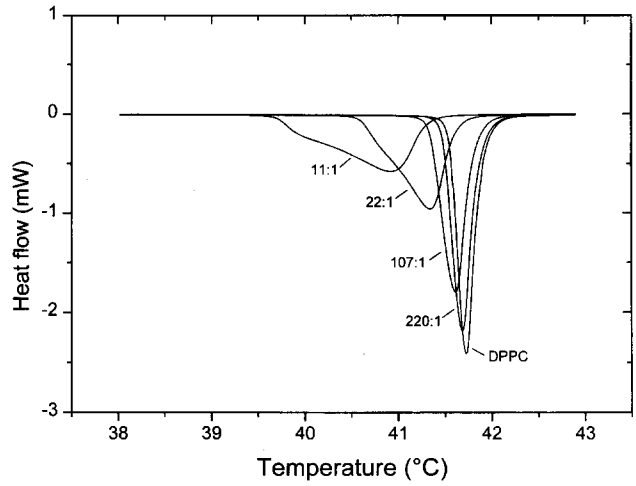

Figure 3. DSC scans of the phase transition temperature profile of pure DPPC membranes and of membranes containing formoterol at decreasing lipid-to-drug molar ratios. Characteristically, pure DPPC lipid bilayers exhibit a sharp phase transition at a temperature T_m = 41.70 °C.

study whether the binding of β_2-adrenoceptor agonists to suspended mesangial cells could be detected in terms of changes in the microviscosity of the cell membranes. To compare the effects of the considered anti-asthmatic drugs under conditions being relevant for the therapy, the drug concentrations used for incubation of mesangial cells varied in proportion to their inhaled β_2-adrenergic bronchodilator doses [31, 32]. The steady-state anisotropy of control cells was $\langle r \rangle$ = 0.265 (*table III*). Salbutamol, with concentrations up to 650 nM, showed no significant effect on the fluorescence anisotropy, i.e. on the microviscosity of the plasma membrane. Pretreatment of the cells with 100 nM salmeterol slightly increased the anisotropy to $\langle r \rangle$ = 0.289. In contrast, formoterol led, at a concentration of 50 nM, to a distinct decrease in steady-state anisotropy, $\langle r \rangle$ = 0.222. These results are in excellent harmony with the observation that both salmeterol and formoterol strongly accumulate in lipid membranes. However, contrasting with salmeterol, the partitioning of formoterol was associated with a significant decrease in the microviscosity of plasmalemma lipid bilayers of suspended mesangial cells.

4. Discussion

The logarithm of the n-octanol/water partition coefficient, log D_{ow}, has been widely used to characterize the lipophilic properties of drugs [22, 40]. Since the studied

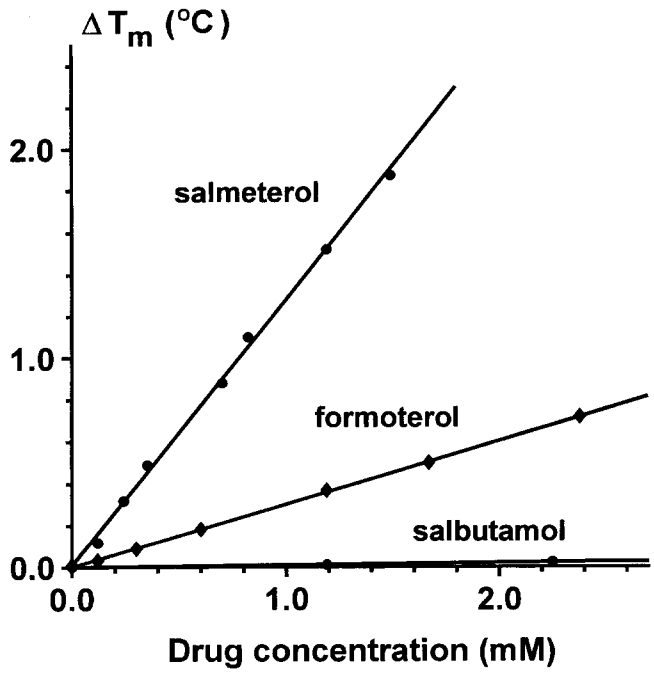

Figure 4. Comparative plot of the efficiencies of β_2-adrenoceptor agonists to depress the phase transition temperature ($\Delta T_m = 41.70\ °C - T_m$) of DPPC membranes, expressed in terms of increasing drug concentrations. In the case of salbutamol, the slope and linearity of the fitting function was derived from measurements performed at drug concentrations in the range 0–20 mM.

β_2-adrenoceptor agonists were dissolved as cations under the experimental conditions used, measured log D_{ow} values indicate that the lipophilicity of the positively charged drug molecules increases in the sequence: salbutamol < formoterol < salmeterol. Interestingly, the log D_{ow} values of β_2-stimulating agents grew in parallel with the standard lipid membrane/water partition constants K_p^0, results to suggest correlations between these two parameters. This seems quite reasonable, since n-octanol, with its polar head group and lipophilic part, adequately represents the lipoid body phase of natural membranes. Nevertheless, we consider the available data set (consisting of three pairs) to be too small to procure sound mathematical correlations. In support of our hypothesis, Gobas and co-workers analysed the partitioning behaviour of a wide variety of halogenated aromatic hydrocarbons and demonstrated that the logarithm of the dimensionless partition constant K_{pmem} increased in linear proportion to the log D_{ow} value and the molar volume of the chemical [40]. Interestingly, compounds with log $D_{ow} \geq 5.5$, or molar volumes $\geq 230\ cm^3/mol$, displayed marked differences in partitioning properties, and their data didn't match linear correlation functions [40]. In general, care is advised to postulate correlations between thermodynamic quantities, since such conclusions are prone to contradictory discussions [25, 41]. For a reliable mathematical analysis of functional dependences, it is indispensable to carefully analyse the experimental conditions used to procure the data, i.e. to take into account the charges of the solutes (pH values), the sizes of the hydrophobic and hydrophilic parts of drug molecules, and the composition, charge and state of the lipid membranes.

Table III. Effect of salmeterol (100 nM), formoterol (50 nM) and salbutamol (650 nM) on the steady-state anisotropy of DPH anchored in plasmalemma lipid membranes of mesangial cells. The errors represent one standard deviation.

Cells	Samples	Anisotropy ⟨r⟩
Control	12	0.265 ± 0.007
Salmeterol	8	0.289 ± 0.010
Formoterol	12	0.222 ± 0.007
Salbutamol	8	0.269 ± 0.006

The binding of β_2-adrenoceptor agonists to POPC /DOPS lipid bilayers was highly exergonic for all drugs studied and the concomitant changes in the Gibbs free energy decreased with increasing lipophilicity. In the case of salmeterol, changes in the entropy and enthalpy both contributed by considerable amounts to cause a remarkable decrease in the Gibbs free energy (ΔG = –33.4 kJ/mol; *table I*). In contrast, the binding enthalpy of formoterol was the major determinant responsible for the large change in free energy, and the binding of salbutamol to lipid bilayers was almost completely driven by an increase in entropy. Consistent with these data, Seelig and co-workers studied the interaction of a wide variety of drugs with lipid membranes and demonstrated that the decrease in the free energy resulted both from changes in the enthalpy and entropy [42, 43].

Based on the idea that charge shielding effects grow in parallel with the ionic strength of the medium, the partitioning of β_2-adrenergic drugs was studied in hypotonic and isotonic environments to understand to what extent electrostatic interactions contribute to the binding reaction. Strikingly, the partition constant K_p^0 of formoterol increased in hypotonic medium by a factor of ≈2, thus decreasing the free energy of binding by 1.8 kJ/mol to ΔG = –26.7 kJ/mol. Interestingly, the binding reaction was not associated with a change in the entropic part, but the enhanced decrease in the Gibbs free energy resulted purely from changes in the enthalpy. Altogether, these

data support the concept that electrostatic attraction facilitates the partitioning of cationic formoterol molecules into POPC/DOPS lipid bilayers.

The situation was completely different for salbutamol and salmeterol, i.e. for those drugs which exhibit an extremely low or high affinity for membrane lipid bilayers. Contrasting with our expectation, the K_p^0 values of these drugs decreased slightly when measured in hypotonic solution, causing changes in the free energy to marginally increase. It is quite remarkable that in hypotonic medium the binding enthalpy of salmeterol increased by a substantial amount (2.4 kJ/mol), but this increase was almost perfectly compensated by an increase in the reaction entropy, thus restraining the rise in the free energy of binding to 0.4 kJ/mol. In the case of salbutamol, changes in the binding enthalpy and entropy did not cancel each other, but both contributed to make ΔG more positive, although by a small amount.

Measurements with a differential scanning calorimeter demonstrated that the phase transition temperature of synthetic DPPC bilayers was depressed in linear proportion to the content of β_2-adrenoceptor agonists in the lipid membrane. Remarkably, the potency of the drugs to induce changes in the phase transition temperature grew in parallel with the lipophilicity and strongly increased from salbutamol to salmeterol. These data support the hypothesis that lipophilic β_2-adrenoceptor agonists incorporate into hydrophobic domains of membrane lipid bilayers. β_2-adrenergic drugs are presumably anchored with their positively charged nitrogen atoms at polar head groups of phosphocholine constituents, and are dipping their hydrophobic moieties into the core of the lipid membranes. In harmony with these suggestions, recent low-angle neutron diffraction studies demonstrated that in natural bilayers, salmeterol molecules orient along the phospholipids with their saligenin heads in the plane of the phosphate groups and their aliphatic tails extending into the acyl chain region [44]. Remarkably, the available diffraction data indicate a preferential accumulation of salmeterol in the outer monolayer of lipid membranes, and there is no evidence that drug molecules "flip-flop" from the outer to inner monolayers of phospholipid bilayers, at least within the time frame of the experiment (< 30 min) [44]. Consequently, the initial conformation of salmeterol in the surface layer of the plasmalemma membrane is likely to be the one which interacts with the β_2-adrenoceptors.

It is well accepted today that stimulation of β_2-adrenergic receptors increases the formation of cAMP, a small nucleotide derived from ATP, and considered to be the primary second messenger responsible for relaxation of airway smooth muscle. cAMP activates specific protein kinases which in turn phosphorylate a number of key enzymes involved in the delicate regulation of smooth muscle contraction [33, 45, 46]. As a major result, cAMP-dependent protein kinases reduce cytosolic Ca^{2+} concentration and decrease the Ca^{2+} -sensitivity of the contractile system [45, 46]. Altogether, these processes lead to a decreased phosphorylation of 20 kDa myosin light chain and to a weaker coupling between the actin and myosin components, thus causing relaxation [33, 46].

From a biophysical point of view, agonist-activated β_2-adrenoceptors are linked to the ultimate cellular response, i.e. smooth muscle relaxation, by a transduction mechanism that consists of a stimulatory G protein (G_s) and the catalytic subunit of adenylate cyclase [47, 48]. This type of transmembrane signal transmission has been termed "collision coupling" and was found to be enhanced when the microviscosity of the cell membrane was lowered [47, 48]. In harmony with these conclusions, it has been demonstrated that a decrease in membrane microviscosity often leads to substantial modifications in the local organization within the plasmalemma (e.g. lateral phase separations) and potentiates the generation of relaxant cAMP due to a higher mobility and activity of adenylate cyclase [49].

For highly lipophilic drugs, such as salmeterol, the partition equilibrium is very much in favor of membrane lipid bilayers. Evidence supports the hypothesis that in vivo, the plasma membrane of airway smooth muscle acts as a depot for lipophilic β_2-adrenoceptor agonists [10, 16–20]. Once having partitioned into membrane lipid bilayers of airway smooth muscle, the drugs remain trapped therein and are available to interact with the β_2-adrenoceptor, which is composed of seven transmembrane spanning protein sequences arranged in α-helices [50, 51]. Due to its high lipophilicity, the majority of salmeterol molecules approach the active site(s) of β_2-adrenoceptor glycoproteins by lateral diffusion via the plasma membrane and bind poorly to β_2-adrenergic receptors from the extracellular space. This model clearly expands the traditional picture of a ligand approaching the β_2-receptor exclusively via the aqueous biophase and rationalizes the prolonged anti-asthmatic effect of salmeterol as well as the slow onset of bronchodilatation observed clinically. Lateral diffusion may also account for the difficulties to get, with salmeterol, a true dose-response relationship for relaxation of airway smooth muscle, i.e. even submaximally effective drug concentrations may result in a long-acting stimulation of the β_2-adrenoceptor-coupled adenylate cyclase activity which is sufficient to cause a sustained relaxant effect [11, 16, 47–49].

In contrast to salmeterol, the partition equilibrium of hydrophilic drugs, such as salbutamol, is strongly in favour of the extracellular aqueous compartment (*table I*). Consistent with our model, the onset of bronchodilating action is rapid after inhalation of salbutamol, but no persistent relaxation of airway smooth muscle results, since the drug does not partition into lipid bilayers to a substantial amount and the therapeutic agent is readily removed from the aqueous biophase by diffusion into the microcirculation.

In the case of formoterol, the partition equilibrium forces the molecules to accumulate within the plasmalemma lipid membrane and to be retained therein for a long time period. As compared to the more lipophilic salmeterol, there are, however, sufficient drug molecules available in the aqueous biophase to permit an immediate interaction with the active site(s) of β_2-adrenergic receptors. Remarkably, the formoterol concentration (EC_{50}) required for relaxation of isolated human bronchus was below 0.3 nM, i.e. by about two orders of magnitude lower than that of salmeterol ($EC_{50} \approx 25$ nM) or salbutamol ($EC_{50} \approx 75$ nM) [11]. The high potency of formoterol and the moderate lipophilicity of the drug rationalize the rapid bronchodilatation observed clinically. Altogether, the pharmacodynamic profile of formoterol is characterized both by an immediate activation of β_2-adrenoceptors via the aqueous biophase, and a delayed, but persistent activation due to drug molecules approaching the active site(s) of β_2-receptors via the plasmalemma lipid bilayer. It is quite remarkable that formoterol was capable of decreasing the microviscosity of the plasma membrane of mesangial cells. In airway smooth muscle, this effect on membrane microviscosity supposedly enhances the lateral diffusion of drug molecules to the active site(s) of β_2-adrenergic receptors and amplifies the signal transduction to adenylate cylase. The relaxing effect of the drug is thus potentiated due to a higher activity and mobility of adenylate cyclase, i.e. the catalyzed generation of cAMP strongly depresses phosphorylation of the myosin light chain and reduces the coupling between the actin and myosin components.

It has been estimated that a single inhalation of salbutamol, salmeterol and formoterol instantaneously leads to topical concentrations of $\approx$ 20, 2 and 1 μM in the main bronchi of patients [10]. These drug concentrations represent a substantial bulk, which appears sufficient to promote an efficient permeation of β_2-stimulating agents across the epithelium into the lamina propria towards airway smooth muscle. However, it should be clearly noticed that the flux of drug molecules across epithelial tissue layers not only depends on the drug gradient, but also on its permeability coefficient, which in turn depends on the lipid membrane/water partition constant [52]. Towards the goal of understanding the pharmacokinetic profiles of bronchodilators, Jeppsson and co-workers studied the permeation of a variety of β_2-stimulating agents across the tracheal epithelium of guinea-pigs under in vitro conditions [53]. The hydrophilic β_2-adrenoceptor agonist terbutaline permeated, from the tracheal lumen to the external medium, with a time constant ($\tau \approx 400$ min) that closely matched the duration of the bronchodilating action of this drug after inhalation in vivo. Characteristically, the time constants of lipophilic compounds were approximately 10 times longer. A result suggesting a very slow permeation of salmeterol molecules across the bronchial epithelium towards airway smooth muscle, which may contribute to the delay of the onset of therapeutic action of this drug.

5. Concluding remarks and therapeutic implications

In this study, the physicochemical properties of the β_2-adrenoceptor agonists salmeterol, formoterol and salbutamol and their interaction with lipid membranes were extensively characterized. The data support the concept that transitory retention of salmeterol and formoterol in plasma membranes of smooth muscle determines the long duration of the bronchodilating action after inhalation in vivo. The presented model expands the traditional picture of a ligand approaching the receptor exclusively via the aqueous biophase and attributes the long-acting relaxant effect of these drugs to their high lipophilicity. Nevertheless, the limitations of this simple model should be clearly recognized. It still remains to be elucidated, to what extent the prolonged relaxation observed in vivo results from an enhanced accumulation and/or retention of lipophilic β_2-adrenoceptor agonists in plasmalemma lipid bilayers of smooth muscle, or from a hindered permeation of lipophilic drugs across the bronchial epithelium towards airway smooth muscle. The lipophilicity of salmeterol and formoterol may prove to be a required, but not a sufficient precondition for induction of a sustained relaxation. As far as the delayed therapeutic response of salmeterol is concerned, it is also very likely that diverse mechanisms operate in parallel. Since this drug extremely rapidly partitions into lipid bilayers ($\tau_{1/2} \leq 1$ min), and since the lateral diffusion of amphiphilic phospholipids in membranes is quite rapid (up to 2 μm s^{-1} [54]), the retarded onset of bronchodilatation observed clinically at least partially results from the low permeability of bronchial epithelium towards highly lipophilic salmeterol molecules.

Currently, inhaled β_2-stimulating agents remain the drugs of choice for handling acute asthmatic attacks,

since these therapeutic agents rapidly reverse airway obstruction within the bronchial tree, and are safe to use [9, 55]. However, while short-acting bronchodilators were completely ineffective in suppressing late phase reactions, long-acting β_2-adrenoceptor agonists exerted potent inhibitory effects on late asthmatic bronchoconstriction which reaches maximum 6–8 h after challenge and lasts for up to 24 h [56]. This result explains why the clinical importance of long-acting β_2-adrenergic drugs in the treatment of asthma receives growing acceptance from the medical community [7–9, 12, 13, 55].

Available clinical data suggest that adverse reactions are significantly lower for long-acting β_2-adrenoceptor agonists [32, 57]. In support of this hypothesis, inhaled therapeutic doses of salmeterol and formoterol failed to provoke the characteristic side-effects of hydrophilic β_2-stimulating bronchodilators in patients [57]. To understand the influence of lipophilicity on the affinity of bronchodilators for β_1- and β_2-adrenoceptors, El Tayar et al. analysed a wide variety of in vitro studies and demonstrated that β_2/β_1-selectivity of bronchodilating agents increased in linear proportion to the log D_{ow} value measured at pH 7.4 [58]. Thus the higher the lipophilicity, the lower the cardiovascular side-effects (tachycardias, arrhythmias, extrasystoles, etc.) expected due to stimulation of cardiac β_1-adrenergic receptors. In harmony with these conclusions, salmeterol and formoterol showed a higher selectivity for β_2-adrenoceptors in animal airways than salbutamol [16]. Moreover, salmeterol exerted a better β_2/β_1-selectivity than formoterol in a variety of experiments using guinea-pig trachea and rat atria [59].

Hopefully, the current study has shed some light on the underlying mechanisms of the pharmacological basis of long-acting β_2-adrenoceptor agonists. In our opinion, a deeper understanding not only improves asthma therapy, but reveals general principles applicable to a wide diversity of drugs exerting effects on cell membrane receptors.

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