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Permeation characteristics of a hydrophilic basic compound across a bio-mimetic artificial membrane

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Abstract

In the present study, the permeation characteristics of a hydrophilic basic compound (HBC) in a bio-mimetic parallel artificial membrane permeability assay (bio-mimetic PAMPA) were investigated in detail. The bio-mimetic PAMPA membrane was constructed on a hydrophobic filter by impregnating a lipid solution consisting of phosphatidylcholine (0.8%, w/w), phosphatidylethanolamine (0.8%, w/w), phosphatidylserine (0.2%, w/w), phosphatidylinositol (0.2%, w/w), cholesterol (1.0%, w/w), and 1,7-octadiene (97.0%, w/w). The pH–permeability curve (pH 3–10), the effect of lipid composition, concentration dependency (0.02–2.00 mM), and inhibition by other cationic compounds, were investigated for several HBCs. Ketoprofen and methylchlorpromazine were also employed as an acidic and a quaternary ammonium compound, respectively. At pH 3–6, the permeability of timolol, a HBC, was higher than expected from the pH-partition hypothesis, especially in the PI-containing membrane, whereas the pH–permeability curve of ketoprofen followed the pH-partition hypothesis. Permeation of HBC was saturable and inhibited by basic and quaternary ammonium compounds. Similar results were also found for methylchlorpromazine. The permeation characteristics of HBC observed in the present study are not usually expected in a passive permeation process across an artificial membrane. The participation of facilitated permeation of cationic species was suggested, in addition to a simple passive diffusion of un-dissociated species. Ion pair transport was suggested as a possible permeation mechanism of cationic species. However, further investigation is necessary to clarify the reason for the permeation characteristics of HBC.

Keywords: Permeability; Phospholipid; Physicochemical properties; Hydrophilic basic compound

1. Introduction

The parallel artificial membrane permeation assay (PAMPA) is currently used in many pharmaceutical companies as a rapid in vitro assay of passive bio-membrane permeation in the drug discovery stage (Kansy et al., 1998; Kerns, 2001; Sugano et al., 2001a; Wohnsland and Faller, 2001; Barton et al., 2002; Veber et al., 2002; Zhu et al., 2002; Di et al., 2003). PAMPA is an application of a filter-supported lipid membrane, and is completely artificial, without pores or active transport systems (Thompson et al., 1982). Previously, we constructed a bio-mimetic version of PAMPA (bio-mimetic PAMPA) (Sugano et al., 2001a). The lipid composition of the bio-mimetic PAMPA membrane is similar to the intestinal brush border membrane, e.g. phosphatidylcholine (PC)/phosphatidylethanolamine (PE)/phosphatidylserine (PS)/phosphatidylinositol (PI)/cholesterol (CHO) (Proulx, 1991; Lipka et al., 1991). The bio-mimetic PAMPA membrane was negatively charged by anionic phospholipids, i.e. PS and

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PI. The bio-mimetic PAMPA was found to quantitatively predict the oral absorption of structurally diverse compounds including hydrophilic basic compound (HBC) (Sugano et al., 2001a, 2002, 2003). Several other PAMPA membrane variations also contain anionic lipids (Barton et al., 2002; Avdeef, 2003; Di et al., 2003). Addition of anionic lipids to the membrane was found to increase the permeability of HBC, resulting in the satisfactory prediction of in vivo absorption (Sugano et al., 2001a; Barton et al., 2002; Avdeef, 2003). However, reason(s) behind the HBC permeation enhancement by anionic lipids remains unclear. Previously, in the bio-mimetic PAMPA, permeation of acidic compounds was found to follow the pH-partition hypothesis (Hogben et al., 1959; Sugano et al., 2001b). However, permeation of some HBCs, i.e. timolol, pindolol, etc. did not follow the pH-partition hypothesis (Sugano et al., 2001b). In the present study, the permeation characteristics of HBC across a bio-mimetic PAMPA membrane were investigated in detail.

2. Materials and methods

2.1. Materials

Pindolol, timolol maleate, metoprolol tartrate, ketoprofen, tetrahexyl ammonium chloride, propantheline bromide, procainamide hydrochloride, PS, PI, and CHO were purchased from Sigma Chemical (St. Louis, MO, USA). PC and PE were purchased from Nippon Oil & Fats Corporation (Tokyo, Japan). Propranolol hydrochloride, diphenhydramine hydrochloride, chlorpheniramine hydrochloride, tetra-alkyl ammonium chlorides except tetrahexyl ammonium chloride, trialkylamines, choline, methyliodide and 1,7-octadiene were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Chlorpromazine hydrochloride and guanidine hydrochloride were purchased from Wako Pure Chemicals (Tokyo, Japan). Other reagents were of analytical grade. The hydrophobic filter plate (PVDF, pore size 0.45 μm) was purchased from Millipore Corporation (Bedford, MA, USA). Methylchlorpromazine iodide was synthesized from chlorpromazine and methyliodide in our laboratory (Huang et al., 1970). The structure of methylchlorpromazine was ascertained by ^{1}H NMR (270 MHz).

2.2. Permeability studies

Permeability measurement was performed in the same manner as described previously (Sugano et al., 2001a). A 96-well microplate (acceptor compartment) was filled with a buffer solution. The buffer solutions consisted of 50 mM sodium citrate (pH 3.0–5.5), 50 mM sodium phosphate (pH 6.0–7.5) and 50 mM sodium borate (pH 8.0–10.0). pH 6.0 was used except in the pH dependency study. The ionic strength of the buffer was adjusted to 0.23 M by NaCl. A hydrophobic filter plate (donor compartment) was fixed on the buffer-filled plate. The filter surface was impregnated with 5 μl lipid solution in 1,7-octadiene. (1,7-Octadiene is an irritant and inhalation should be avoided.) PE, PS, and PI were added at 0.8% (w/w), 0.2% (w/w), and 0.2% (w/w), respectively, and the total phospholipid concentration was adjusted to 2.0% (w/w) by PC. CHO was added at 1.0% (w/w). The lipid composition used in each assay is indicated in the text as an enumeration of lipid species. For example, PC (1.2%), PE (0.8%), CHO (1.0%) in 1,7-octadiene is indicated as PC/PE/CHO. A sample solution (0.5 mM, 100 μl or otherwise noted in the text) of the same buffer was added to the filter plate and incubated at 30 °C for 2 h (or 15 h in the methylchlorpromazine study). The filter plate was then carefully removed. The concentration of the solution in the acceptor compartment was determined by UV spectroscopy, using the microtiter plate reader Spectramax 190 (Molecular Devices) at 240–440 nm at intervals of 10 nm (Kansy et al., 1998). A blank assay without a sample was also performed and the UV spectrum of the blank assay was subtracted from that of the sample assay. The apparent permeability through the artificial membrane (P_{am}) was calculated using Eq. (1). In the concentration dependency study and the inhibition study, the concentration in the acceptor compartment was quantified by HPLC (Alliance 2790 separation module, Waters) using a C18 column (Develosil ODS-UG-3, 4.6 mm × 75 mm, Nomura Chemical Co., Ltd.) with 1 ml/min flow rate. The mobile phase was consisted of 20% acetonitril and 0.1% trifluoroacetic acid. Detection wavelengths of timolol, pindolol, and metoprolol were, 290, 265, and 270 nm, respectively. The flux rate (J, mmol/cm^2/s) was calculated as a product of initial concentration (mM) and P_{am}.

$$P_{am} = -2.303 \times \frac{V_{dn}V_{ac}}{V_{dn}+V_{ac}} \times \frac{1}{St} \times \log\left(1 - \frac{\text{flux}\,\%}{100}\right) \quad (1)$$

$$\text{Flux}\,\% = \frac{C_{ac}}{C_{do}} \times \frac{V_{dn}+V_{ac}}{V_{dn}} \times 100 \quad (2)$$

where V_{dn} (ml) = volume of the donor compartment (0.1 ml), V_{ac} (ml) = volume of the acceptor compartment (0.30 or 0.38 ml), C_{ac} = concentration in the acceptor compartment after incubation (mM), C_{do} = concentration of the initial donor compartment (mM), S (cm^2) = membrane area (0.266 cm^2), t (s) = incubation time.

2.3. pH–permeability equation

The pH–permeability equation is based on the pH-partition hypothesis and corrected for the effect of the unstirred water layer (UWL) (Walter and Gutknecht, 1984; Avdeef, 2001). In a buffered medium, dissociable compounds exist as an equilibrium of un-dissociated and dissociated (charged) species. Membrane permeation of dissociable compound is sum of permeation of both un-dissociated and dissociated species. In the pH–permeability equation, the following assumptions are made: (a) membrane permeation of charged species is negligible, (b) membrane permeation of un-dissociated species is a simple passive diffusion across a membrane and constant over the employed pH range, (c) UWL permeation is a simple passive diffusion and constant over the employed pH range, and (d) effects of other factors on apparent permeability is negligible. From the assumptions (a) and (b), we obtain,

$$P_m = \frac{P_0}{1+10^{pK_a\text{-pH}}} \quad \text{(base)} \quad (3)$$

$$P_m = \frac{P_0}{1+10^{\text{pH-}pK_a}} \quad \text{(acid)} \quad (4)$$

where P_m is the membrane permeability, P_0 is the intrinsic membrane permeability of un-dissociated species, and pK_a is the dissociation constant. It may be assumed that total resistance to permeation is the sum of the resistances of the membrane and UWL on each side of the membrane. Resistance is the inverse of permeability. Therefore, the apparent permeability (P_{am}) was expressed as,

$$\frac{1}{P_{am}} = \frac{1}{P_m} + \frac{1}{P_{UWL}} \quad (5)$$

By combining Eqs. (3)–(5), P_{am} was obtained as,

$$\frac{1}{P_{am}} = \frac{1+10^{pK_a\text{-pH}}}{P_0} + \frac{1}{P_{UWL}} \quad \text{(base)} \quad (6)$$

$$\frac{1}{P_{am}} = \frac{1+10^{\text{pH-}pK_a}}{P_0} + \frac{1}{P_{UWL}} \quad \text{(acid)} \quad (7)$$

Eqs. (6) and (7) were fitted to the observed pH–P_{am} curves of timolol and ketoprofen, respectively. Curve fitting of Eqs. (6) and (7) was calculated by the least square method (EXCEL 2000, Microsoft, Redmont, WA, USA). Sum of squares of the difference between calculated and observed P_{am} was minimized using the Quasi-Newton method.

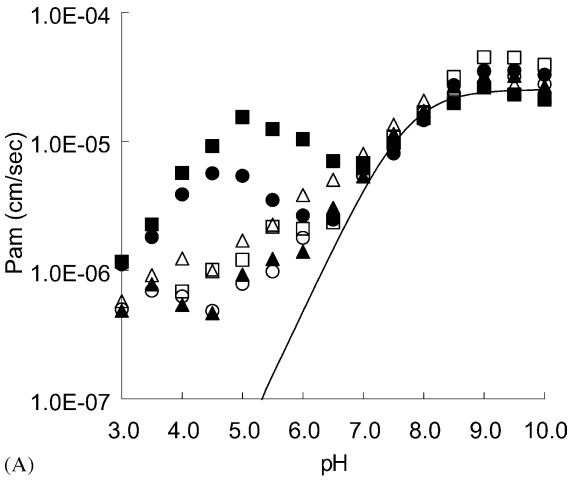
2.4. Statistical analysis

The paired t-test, Dunnett's test or Tukey's test was used to evaluate the significance of difference. A minimum P-value of 0.05 was used as the significance level for all tests.

3. Results

3.1. pH–P_{am} curve and its membrane composition dependency

In the case of timolol (Fig. 1A), in the range of pH 7.5–10.0, the pH–P_{am} curves were similar for each membrane composition. The pH–P_{am} curve in this pH range was analyzed by Eq. (6). In this pH range, the pH–P_{am} curve adhered to Eq. (6). The P_0 and P_{UWL} of timolol in the PC/PE/PS/PI/CHO membrane were obtained as 1.6×10^{-3} and 2.6×10^{-5} cm/s, respectively. However, at pH < 7.5, the pH–P_{am} curves deviated from Eq. (6) and differed for each membrane composition. The deviation from Eq. (6) was more markedly observed in the PC/PI and PC/PE/PS/PI/CHO membranes than in the PC, PC/PE, PC/PS, and PC/CHO membranes. At pH < 5 in the PC/PE/PS/PI/CHO membrane, timolol was more than 100-fold more permeable than predicted from Eq. (6). At the physiolog-



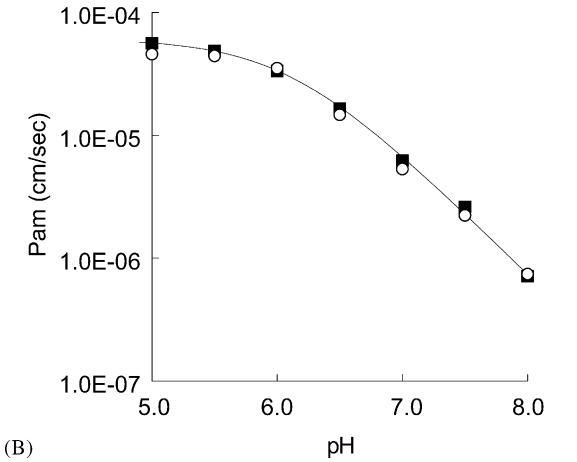


Fig. 1. pH–permeability (P_{am}) profile of (A) timolol and (B) ketoprofen. Lipid composition: PC (○), PC/PE (△), PC/PS (□), PC/PI (●), PC/CHO (▲), and PC/PE/PS/PI/CHO (■). P_{am} of timolol at pH < 4 in the PC/PS membrane and P_{am} of ketoprofen at pH > 8 were less than the detection limit. Each point represents the mean of $n = 3$–6. In typical cases, CV was less than 20%. The solid line in (A) and (B) indicates the fitting line of Eqs. (6) and (7), respectively.

ical microclimate pH of the intestinal membrane surface (pH 6.0) (Maxwell et al., 1968), the P_{am} in the PC/PE/PS/PI/CHO membrane was 6-fold larger than that in the PC membrane, and 20-fold larger than expected from Eq. (6). However, the P_{am} in the PC/PI membrane was the same as that in the PC membrane at pH 6.0. A similar pH–permeability profile was also found in the case of pindolol (data not shown).

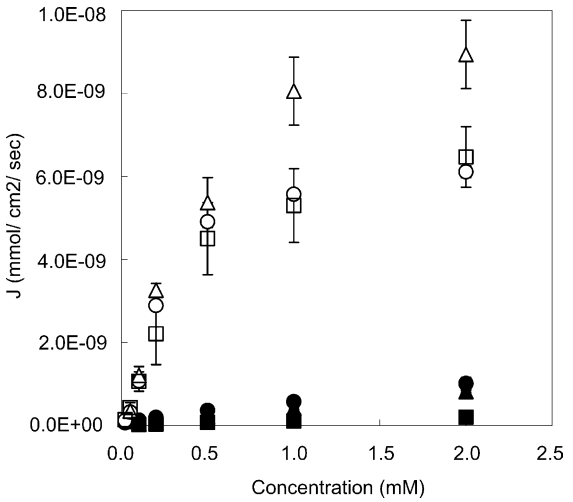


Fig. 2. Concentration dependency of flux. Keys: timolol (circle), metoprolol (triangle), pindolol (square), PC/PE/PS/PI/CHO membrane (open), and PC membrane (closed). Each point represents the mean ± S.D., $n = 6$.

In the case of ketoprofen (Fig. 1B), the pH–P_{am} curves of the PC and PC/PE/PS/PI/CHO were similar for all pH ranges. The pH–P_{am} curves in the range of 5.0–8.0 were analyzed by Eq. (7). No deviation from Eq. (7) was observed. The P_0 and P_{UWL} of ketoprofen in the PC/PE/PS/PI/CHO membrane were obtained as 9.4×10^{-3} and 6.1×10^{-5} cm/s, respectively.

3.2. Concentration dependency of the HBC flux rate

The concentration dependency of the timolol, metoprolol, and pindolol flux rates is shown in Fig. 2. In the PC/PE/PS/PI/CHO membrane, the flux rate of these compounds showed saturable concentration dependency. In the PC membrane, the flux rate was also found to show saturable concentration dependency, although to a lesser extent than in the PC/PE/PS/PI/CHO membrane. The flux rate in the PC/PE/PS/PI/CHO membrane was larger than that in the PC membrane in the 0.02–2.0 mM range.

3.3. Inhibitory effect of basic compounds and quaternary ammonium compounds on HBC permeation

To investigate the inhibitory effect of basic compounds and quaternary ammonium compounds (QAC)

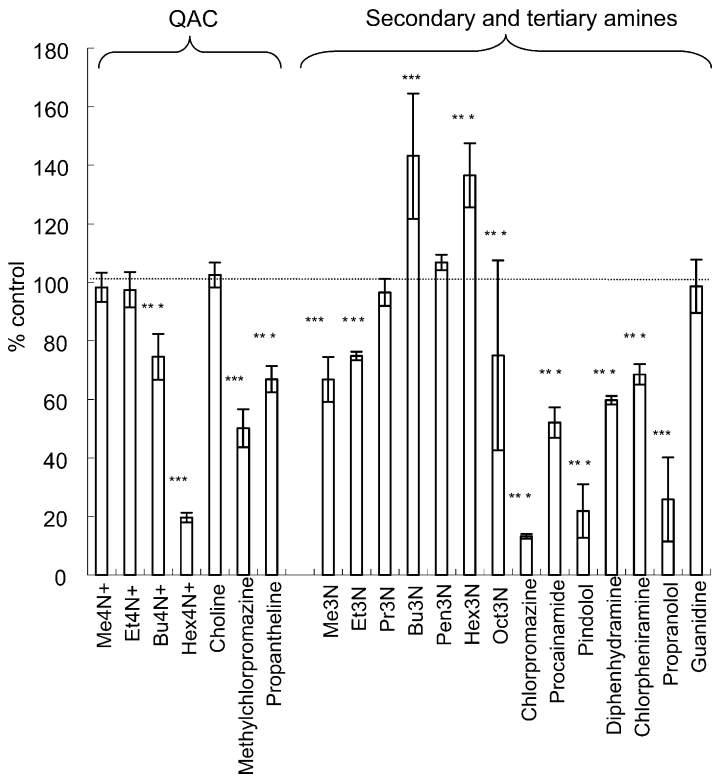


Fig. 3. Inhibitory effect of secondary amine, tertiary amine, and quaternary ammonium compounds on the permeation of timolol across the PC/PE/PS/PI/CHO membrane. Permeability ratio against the control is indicated (control = 100%, $1.62 \pm 0.06 \times 10^{-5}$ cm/s (mean ± S.D., $n = 12$)). The concentration of timolol and inhibitors was 0.25 and 2.00 mM, respectively. Each point represents the mean ± S.D., $n = 6$. *** $P < 0.001$ (Dunett's test against control).

on the permeation of timolol across the PC/PE/PS/PI/CHO membrane, 2.0 mM of basic compound and QAC was added to the donor compartment at the same time as the addition of timolol (Fig. 3). Several structurally diverse basic compounds and QACs were found to inhibit the transport of timolol. QACs with longer alkyl chain length inhibited the transport of timolol more strongly. However, obvious alkyl chain length dependency was not observed for basic compounds. In addition, tributylamine and trihexylamine increased the transport of timolol. Transport of pindolol, metoprolol, and procainamide were also inhibited by THA (Table 1).

3.4. Permeation characteristics of methylchlorpromazine

The permeation characteristics of methylchlorpromazine (a QAC) at pH 6.0 are shown in Table 2. Methylchlorpromazine permeated the PC/PE/PS/PI/CHO membrane, but did not permeate the PC membrane (less than the detection limit). In the PC/PE/PS/PI/CHO membrane, tetrahexylammonium (THA) inhibited the transport of methylchlorpromazine ($P < 0.001$, Tukey's test).

Table 1
Effect of THA on permeability[a]

Compound	P_{am} ($\times 10^{-6}$ cm/s)	
	Control	+THA[b]
Timolol	16.2 ± 0.65	3.18 ± 0.26***
Pindolol	9.11 ± 1.84	1.60 ± 0.42***
Metoprolol	12.8 ± 1.03	2.31 ± 0.19***
Procainamide	6.70 ± 0.28	0.40 ± 0.00***

[a] Substrate concentration was 0.25 mM. Values represent means± S.D., $n = 6$.
[b] Tetrahexylammonium (2.0 mM).
*** $P < 0.001$ (paired t-test against control).

Table 2
Permeability and membrane binding of methylchlorpromazine

Lipid composition	Concentration (mM)	THA[a]	P_{am} ($\times 10^{-6}$ cm/s)[b]
PC/PE/PS/PI/CHO	0.50	−	0.76 ± 0.10
PC	0.50	−	<0.09[c]
PC/PE/PS/PI/CHO	0.25	−	1.20 ± 0.10
PC/PE/PS/PI/CHO	0.25	+	0.49 ± 0.17

[a] Tetrahexylammonium (2.0 mM).
[b] Values represent means ± S.D., $n = 6$.
[c] Less than the detection limit. Detection limit was set at $OD_{ac} = 0.005$.

4. Discussion

4.1. pH–P_{am} curve of HBC and its membrane composition dependency

Previously, in the PC/PE/PS/PI/CHO membrane (bio-mimetic PAMPA membrane), the pH dependency of the P_{am} for some HBCs was found to deviate from pH-partition hypothesis in the pH 5.5–7.4 range (Sugano et al., 2001b). Therefore, we investigated the pH–P_{am} curve of timolol and pindolol for a wider pH range and various membrane compositions (Fig. 1A). In addition, ketoprofen was employed as an acidic compound.

We firstly examined the possibility of the decomposition and precipitation of the permeants (timolol, pindolol, and ketoprofen), and membrane breakage in the employed pH range. Stability and solubility of the permeants had been ensured (data not shown). In addition, leakage of trypan blue (non-permeable marker) was not observed, indicating that no aqueous pores existed in the membrane. Therefore, artifacts from the decomposition and precipitation of the permeants, and membrane breakage, can be negligible.

Deviation of the observed pH–P_{am} curve from Eq. (6) suggested that permeation of HBC was not only a simple passive permeation of un-dissociated species.

The appearance of a peak in the low-pH range (pH 3–6) was markedly observed in the PI-containing membranes, suggesting that PI caused the low-pH range peak. However, at pH 6, the PC and the PC/PI membranes showed similar permeability, whereas the PC/PE/PS/PI/CHO membrane showed greater permeability. These results suggested that, at pH 6, some combination of phospholipids is required to increase the permeability more than PC membrane permeability.

The pH–permeability curve of ketoprofen was found to adhere to Eq. (7) (Fig. 1B). The P_{am} became larger as the pH lowered, with a slope of −1, eventually leveling off at about 6×10^{-5} because of the resistance of the unstirred water layer (Ruell et al., 2003). This result suggested that permeation mechanism of ketoprofen was a simple passive diffusion of un-dissociated species across the membrane.

4.2. Saturation and inhibition of HBC permeation

Concentration dependency and inhibition by other basic compounds and QACs were then investigated (Fig. 2). Saturation and inhibition were observed in the PC/PE/PS/PI/CHO membrane, suggesting the participation of facilitated permeation.

In the PC membrane, saturation was only slightly observed. Therefore, the saturable portion of permeation in the PC/PE/PS/PI/CHO membrane was caused by the addition of lipids other than PC. Even though bio-mimetic PAMPA is an assay of passive transport, this result indicates that concentration could be an important determinant for the permeation of HBC. Usually in the drug discovery stage, a 0.2–0.5 mM sample concentration is employed for routine PAMPA because UV spectroscopy is utilized for concentration measurement. In this concentration range, permeation was not saturated.

Several structurally diverse basic compounds and QACs inhibited timolol permeation across the PC/PE/PS/PI/CHO membrane at pH 6.0 (Fig. 3). In addition, permeation of HBCs was also inhibited by THA. These results suggested that these compounds might (at least partly) share a common permeation process. In the case of QACs, the size of the hydrophobic part correlated with the inhibitory effect. Therefore, hydrophobic interaction was suggested to play a role in the inhibition mechanism, as well as the electrostatic interaction (Saitoh et al., 1990). In contrast to QAC, the inhibitory effect of basic compounds does not appear to be simply explained by the size of the hydrophobic part. In basic compounds, steric hindrance around the cationic center might influence the inhibition mechanism (Saitoh et al., 1990). The reason for the increase in the P_{am} of timolol by tributylamine and trihexylamine remains unclear.

4.3. Permeation characteristics of methylchlorpromazine

Previously, methylchlorpromazine was found to permeate across the intestinal membrane (Kitagawa et al., 1996), and therefore, it was used in the present study. The results of methylchlorpromazine permeation are surprising, given the assumption of the pH-partition hypothesis that the passive permeation of cationic species across a lipid membrane is negligible. The permeation characteristics of methylchlorpromazine were similar to those of HBC permeation, suggesting that there is a common permeation process between QAC and HBC.

4.4. Permeation mechanism of HBC

As described above, the permeation characteristics of HBC in the PC/PE/PS/PI/CHO membrane (i.e. deviation of the pH–P_{am} curve from Eq. (6), saturation, and inhibition) suggested the participation of facilitated permeation, in addition to a simple passive diffusion of un-dissociated species. The membrane permeability of dissociable compound is sum of both the un-dissociated and dissociated species permeability. In the case of ketoprofen, the permeability of un-dissociated species (P_0) was found to be unaffected by pH and membrane composition. In addition, previously, the permeability of un-dissociable compounds was found to be unaffected by pH and membrane composition (Sugano et al., 2001a,b). These findings suggested that permeation of un-dissociated species of HBC was also unaffected by pH and membrane composition. Therefore, permeation of cationic species was suggested as a reason for the permeation characteristics of HBC. The similarity of the permeation characteristics between HBC and methylchlorpromazine (a permanent cation) also supported this suggestion. Previously, as a permeation mechanism of cationic species across a membrane, the active transport system (Tsuji and Tamai, 1996), electric potential difference across the charged membrane (Saitoh et al., 1988), and ion pair transport with counter anions (Hallen et al., 1985; Neubert, 1989; Takács-Novák and Szasz, 1999) have been reported. The bio-mimetic PAMPA membrane has neither an active transport system nor the an electric potential difference across the membrane. Therefore, in the bio-mimetic PAMPA, of the previously reported mechanisms, ion pair transport remains a possible transport mechanism of cationic species. Ion pair transport can explain the saturation and inhibition, because counter anion may act as a carrier (Ozaki et al., 2000). The results of membrane composition dependency suggested that PI could be a counter anion for cationic species. Another anionic phospholipid, PS, did not cause the low pH range peak in the pH–P_{am} curve, probably because the PS–cationic species complex contains two ion pairs and requires more energy to partition into the hydrophobic part of the membrane (anion = phosphate and carboxylate of PS; cation = ammonium of PS and substrate cation). In addition, PS became less negatively charged at pH < 5.5 (pK_a of carboxylate of PS is 5.5 in the membrane; Avdeef, 2003).

However, further investigation is necessary to clarify the reason for the permeation characteristics of HBC. At present, the structure of the PAMPA membrane is not known with certainty. It is noteworthy that some deviation of the pH–P_{am} curve from the pH-partition hypothesis (i.e. slope of pH–log P_{am} curve <1) was also observed in the PC membrane. A similar pH–P_{am} curve was also previously reported in the DOPC/dodecane membrane (Avdeef, 2003).

5. Conclusion

The pH–permeability curve of HBC was not explained by the pH-partition hypothesis and unstirred water layer resistance. Permeation of HBC was saturable and inhibited by other basic compounds and QACs. These results suggested the participation of facilitated permeation of cationic species, in addition to a simple passive diffusion of un-dissociated species. Ion pair transport was suggested as a possible permeation mechanism. However, further investigation is necessary to clarify the reason for the permeation characteristics. The findings of the present study would be beneficial for improving PAMPA, understanding PAMPA data, and constructing an in silico prediction method.

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