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Gramicidin derivatives as membrane-based pH sensors

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Abstract

Ion channels provide a means for sensitive pH measurement at membrane interfaces. Detailed knowledge of the structure and function of gramicidin channels permits the engineering of pH-sensitive derivatives. Two derivatives, gramicidin-ethylenediamine and gramicidin-histamine, are shown to exhibit pH-dependent single-channel behaviour over the pH ranges 9–11 and 6.5–8.5, respectively. Thermal isomerization of a carbamate group at the entrance of the channels leads to a pattern of steps in single-channel recordings. The size of the steps depends on the time-averaged degree of protonation of the appended group (ethylenediamine or histamine). Measurement of the size of the steps thus permits single-molecule pH sensing under symmetrical pH conditions or in the presence of a pH gradient.

Keywords: Peptide; Engineering; Single molecule; pH gradient; Proton; Isomerization

1. Introduction

Proton movements play important roles in biology. For instance, pH gradients across mitochondrial membranes are used in the generation of ATP [1–3]. Bacteriorhodopsin pumps protons across the halobacterium cell membrane to generate a pH gradient that powers cellular functions [4,5]. As more detailed structural information has become available, efforts to understand the mechanisms of proton movement in a number of biological systems have intensified [6–11]. Of particular interest is the movement of protons at membrane–solution interfaces. A number of studies have suggested that lateral diffusion of protons at membrane surfaces can occur significantly faster than diffusion in the bulk [9,12–16]. Other studies have indicated that this is not the case [17–19].

A quantitative biophysical description of these proton transfer processes requires accurate methods for sensing localized pH changes near membrane surfaces. Lipid-bound pH-sensitive fluorescent compounds have been particularly useful in this regard [20–23]. Another strategy for measuring local pH in a membrane system is to employ a pH-sensitive ion channel. Ion channels are exquisitely sensitive sensor elements because individual ion channels can be readily detected electrically [24–28]. Ion flux through channels can be modulated by protonation/deprotonation at a single site [19,29–32] thus providing a

Abbreviations: BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid; CAPS, 3-cyclohexylamino-1-propanesulphonic acid; C:M:W:, chloroform:methanol:water; DMF, dimethylformamide; EDA, ethylenediamine; HPLC, high performance liquid chromatography; MeOH, methanol; TEA, triethylamine; TFA, trifluoroacetic acid; TLC, thin layer chromatography; Tris, tris(hydroxymethyl)aminomethane

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highly localized method of pH sensing. Single-channel electrical recording, together with noise analysis also offers the potential for kinetic measurements of protonation and deprotonation reactions to be made.

The ion channel formed by the peptide gramicidin (HCO-Val-Gly-Ala-D-Leu-Ala-D-Val-Val-D-Val-Trp-D-Leu-Trp-D-Leu-Trp-D-Leu-Trp-$NHCH_2CH_2OH$) provides an attractive scaffold for the design of a pH sensor that may be customized for a variety of applications. The structure and function of the gramicidin channel are well established [33–37]. Gramicidin readily incorporates into a wide variety of membrane types when added to the aqueous phase nearby. The channel is formed upon association of two gramicidin monomers in a N-terminal to N-terminal (head-to-head) fashion (Fig. 1) [38]. The $\beta^{6.3}$ helical structure of the peptide provides a pore $\sim$28 Å long and 4 Å in diameter that traverses the membrane when a dimer forms [35,39,40]. The pore is lined by backbone amide groups and permits the transmembrane flux of small monovalent cations at maximum rates of about 10^8 ions per second. Gramicidin is also permeable to protons (e.g. [7]).

Although the native gramicidin channel is not pH sensitive, enough is known about its structure and function that chemical modifications can be made that confer pH sensitivity on cation flux. Previous chemical modifications include C-terminal carboxylate groups, N-terminal carboxylate groups [41–43], amino groups [43] and sulphonate groups [44]. We have also reported gramicidin derivatives bearing amino groups at the C-terminal ends that were linked to the peptide backbone via carbamate linkages [45–47]. Thermal *cis-trans* isomerization of the carbamate linkages leads to steps in single-channel recordings as a result of differences in the average location of the (protonated) amino groups between the *cis* and *trans* isomers (Fig. 1). This effect is most pronounced when isomerization occurs at the channel entrance (where cations enter the pore) as opposed to the channel exit [47].

We show here that the size of the steps in the single-channel recordings (i.e. the relative current passed by the *trans* and *cis* isomers of the channel) depends on the degree of protonation of the terminal amino groups. The measurement of *cis* vs. *trans* current thus provides information about the local pH near the channel entrance. The pH at the channel exit (i.e. at the other side of the membrane) can be measured by reversing the applied voltage thereby reversing the direction of ion flux.

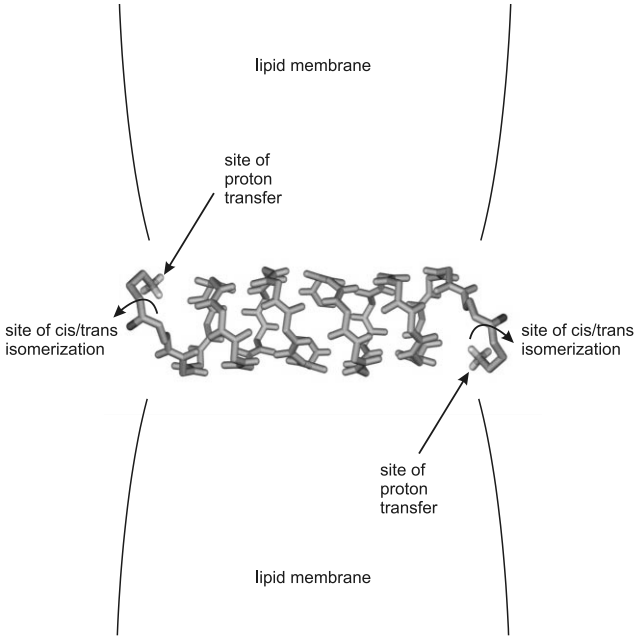

Fig. 1. Model of a gram-EDA channel in a lipid bilayer. The structure is based on coordinates obtained for native gramicidin [35]. Only the backbone of the channel is represented. Ion flux across the membrane occurs through the center of the channel. At each end of the channel an ionizable site (the primary amino group in gram-EDA; the imidazole group in gram-histamine) is connected to the gramicidin backbone via a carbamate linkage. Thermal *cis-trans* isomerization around the C–N atoms in the carbamate bond leads to different average positions of the ionizable sites relative to the channel entrance. This, in turn, leads to steps in single-channel recordings of ion flux.

2. Materials and methods

2.1. Synthesis of gramicidin-ethylenediamine (gram-EDA)

The C-terminal end of gramicidin was derivatized as described previously to install a primary amino group [45]. Gramicidin D was not initially separated into its components (gramicidins A, B, C) since the final HPLC purification of the product (vide infra) accomplished this [48]. Briefly, commercial gramicidin D (38 mg, 20 μmol; Sigma, Mississauga, ON, Canada) was combined with *p*-nitrophenyl chlorofor-

mate (200 μmol; Aldrich, Mississauga, ON, Canada) in dry tetrahydrofuran (2 ml, 4°C; Aldrich), then 100 μl triethylamine (TEA; Aldrich) were added and the mixture stirred for 1 h at 4°C. The resulting carbonate ester was filtered through celite into a 100-fold molar excess of ethylenediamine in 2 ml of dimethylformamide (DMF) (Aldrich). Solvent was removed under high vacuum and the residue redissolved in methanol (2 ml). The product (gram-EDA) was then separated by gel filtration using Sephadex LH-20 (2.5×20 cm gravity column) in methanol. The product was further purified using reverse-phase HPLC (Zorbax-RX-C8 column (4.6×250 mm), isocratic conditions, 80% MeOH/20% H_2O, 0.1% trifluoroacetic acid (TFA) adjusted to a pH of 3.0 with TEA, flow rate = 1 ml/min, retention time 6.2 min). Electrospray mass spectrometry: gram-(A)-EDA = $C_{102}H_{147}N_{22}O_{18}$ ($M+H^+$) calc'd = 1969.4, obs'd = 1969.5, TLC (C:M:W = 65:25:4): gramicidin: $R_f = 0.70$; gram-(A)-EDA: $R_f = 0.47$. Purity by HPLC was >98%.

2.2. Synthesis of gramicidin-histamine (gram-histamine)

The C-terminal end of gramicidin was derivatized exactly as described for gram-EDA except that the intermediate carbonate ester was filtered through celite into a 100-fold molar excess of histamine (Sigma) in 2 ml of DMF. Solvent was removed under high vacuum and the residue redissolved in methanol (2 ml). The product (gram-histamine) was then separated by gel filtration using Sephadex LH-20 (2.5×20 cm gravity column) in methanol. The product was further purified using reverse-phase HPLC (Zorbax-RX-C8 column (4.6×250 mm), isocratic conditions, 85% MeOH/15% H_2O, 0.1% TFA adjusted to a pH of 3.0 with TEA, flow rate = 1 ml/min, retention time 2.4 min). Electrospray mass spectrometry: gram-(A)-histamine = $C_{105}H_{147}N_{23}O_{18}$ ($M+H^+$) calc'd = 2110.4, obs'd = 2019.9, TLC (C:M:W = 65:25:4): gramicidin: $R_f = 0.70$; gram-(A)-histamine: $R_f = 0.55$. Purity by HPLC was >98%.

2.3. Single-channel recording

The general techniques for making single-channel conductance measurements of gramicidin derivatives have been described previously [49]. Gramicidin derivatives were HPLC-purified at least twice (using the conditions given above) before use in single-channel measurements to ensure highly uniform conductance events [50]. Peptides (~10 nM in methanol) were added to membranes formed from diphytanoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA) (50 mg/ml in decane; Aldrich). Membranes were formed by painting the lipid solution across a ~100 μm aperture in a polypropylene pipette tip mounted horizontally in a Teflon well. In the case of gradient experiments, a BCH-22A bilayer cell (150 μm aperture) from Warner Instruments (Hamden, CT, USA) was used. Silver/silver chloride electrodes were placed, one on either side of the membrane, and connected to the CV-4B headstage of an Axopatch 1D patch-clamp amplifier (Axon Instruments, Union City, CA, USA) controlled by Synapse (Synergy Research, Bromma, Sweden) software that permitted transmembrane voltage to be set and current recorded using the same pair of electrodes. Current records were filtered at 50 or 100 Hz, sampled at 1 kHz, stored directly on disk and analysed using Synapse and Igor (Wavemetrics, Lake Oswego, OR, Canada) software.

Solutions of CsCl (1 M) containing 5 mM buffer were used as the electrolyte. Buffers used were as follows: potassium phosphate (pH 3.95), BES (pH 6.1–7.7), Tris (pH 8.5), CAPS (pH 9.95–11.0), and sodium phosphate (pH ≥12.0). All buffers were obtained from Sigma Canada. The desired pH was obtained by addition of concentrated KOH or HCl to the buffered solution as required. A pH gradient across the membrane was established using 1 M CsCl, 5 mM CAPS, pH 8.2 on one side and 1 M CsCl, 5 mM sodium phosphate, pH 11.6 on the other side.

3. Results

Native gramicidin was modified by reacting the C-terminal alcohol moiety with *p*-nitrophenyl chloroformate to produce a *p*-nitrophenyl carbonate ester using methods described previously [47]. The *p*-nitrophenol group was then displaced with ethylenediamine to produce gram-EDA or with histamine to

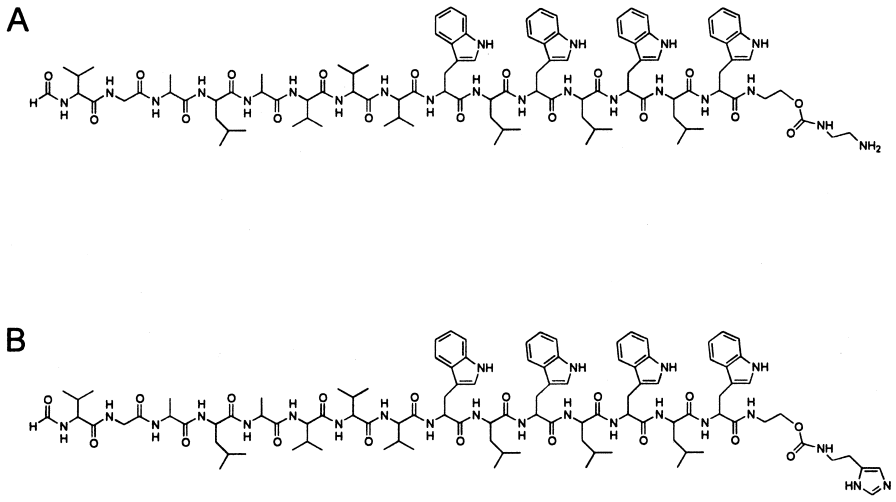

Fig. 2. Chemical structures of gram-EDA (A) and gram-histamine (B).

produce gram-histamine (Fig. 2). Although the imidazole end of histamine may also react with the intermediate *p*-nitrophenyl carbonate ester, this adduct is unstable and is expected to be converted to gram-histamine in the presence of excess histamine.

The effect of pH on cesium ion currents through single gram-EDA channels is shown in Fig. 3. Although gram-EDA is permeable to protons, the proton concentration is so low under the conditions of Fig. 3 as to make proton flux a negligible fraction of the total current. At pH 7 two main conducting levels are seen (Fig. 3). These have been previously identified as being due to *cis* and *trans* conformations of the carbamate group at the entrance to the channel [47]. The *cis* conformation results in a closer approach of the protonated amino group to the channel entrance and consequently smaller cation flux. Barely visible under the conditions of Fig. 3 are secondary steps in the recording just below each of the two main levels. These steps are due to *cis-trans* isomerization of the carbamate bond at the exit of the channel, which has a lesser effect on cation flux [47]. As the pH increases, the single-channel currents of all levels increase and the difference between the *cis* and *trans* levels becomes smaller so that, at very high pH values, no effects of *cis-trans* isomerization are seen (Fig. 3). Although, in principle, individual protonation/deprotonation events might be observable with such a system [30], these events presumably occur too quickly to be resolved by the recording apparatus under the present conditions.

A similar pattern of channel currents is observed for the gram-histamine derivative (Fig. 4) except that the maximum difference in conductance between *cis* and *trans* levels is smaller than in the gram-EDA case, and the range of pH over which changes occur

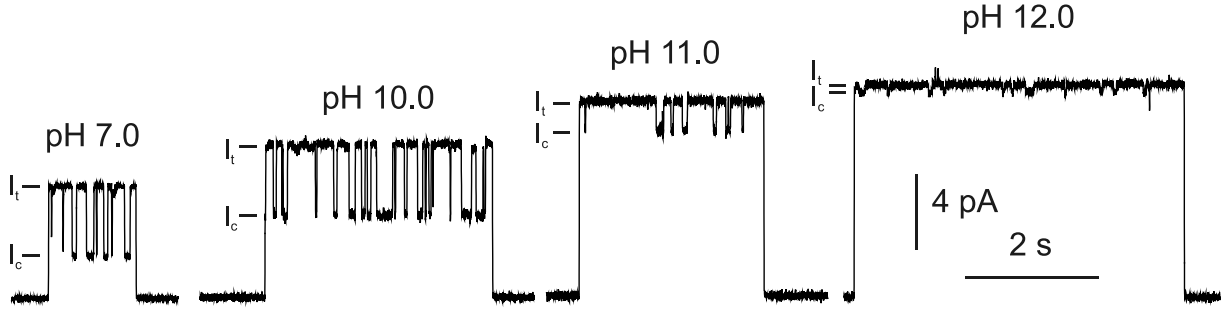

Fig. 3. Representative single-channel currents of gram-EDA at different pH values. Zero current (closed channel) is the lowest level in each case. The currents of the *trans* (I_t) and *cis* (I_c) states at the channel entrance are indicated. Applied voltage: 200 mV, 1 M CsCl, diphytanoylphosphatidylcholine/decane membranes.

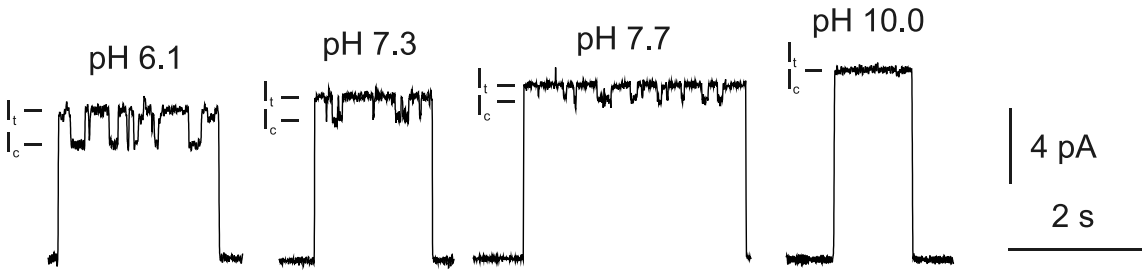

Fig. 4. Representative single-channel currents of gram-histamine at different pH values. Zero current (closed channel) is the lowest level in each case. The currents of the *trans* (I_t) and *cis* (I_c) states at the channel entrance are indicated. Applied voltage: 200 mV, 1 M CsCl, diphytanoylphosphatidylcholine/decane membranes.

is different (pH 6 to pH 9 instead of pH 9 to pH 12 for gram-EDA).

Fig. 5 shows the appearance of a single gram-EDA channel where a pH gradient exists across the membrane. For reference, we define the *trans* side of the membrane as electrical ground. The pH at the *trans* side of the membrane is 11.6 and the pH at the *cis* side of the membrane is 8.2. At the left hand side of the figure, a channel opens when the applied potential is +200 mV. The entrance of the channel (where cations are entering) is exposed to pH 8.2 buffer. Large steps in channel current are observed. While the channel remains open, the applied voltage is reversed (−200 mV) so that cations now enter from the high pH side of the membrane. After the capacitative transient decays, the channel current is larger than before with smaller steps.

4. Discussion

The data shown in Figs. 3 and 4 were analysed in terms of a model in which only the current changes associated with the large steps, which are due to *cis-trans* isomerization at the channel entrance, were considered. The observed current of the *trans* state (I_t) was assumed to be given by:

$$I_t = I_{tu} \cdot f_{tu} + I_{tp} \cdot f_{tp} \quad (1)$$

where I_{tu} is the single-channel current of the fully

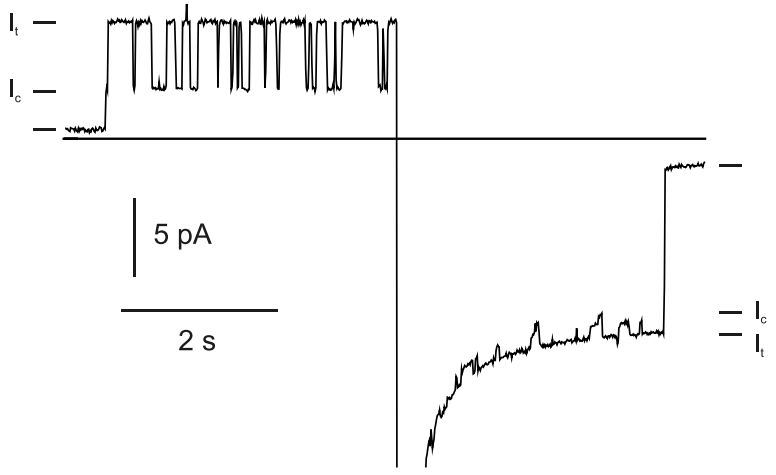

Fig. 5. Single-channel currents of gram-EDA in the presence of a pH gradient. The *trans* side of the membrane (electrical ground) was at pH 11.6. The *cis* side was at pH 8.2. At the beginning of the record, the applied voltage was +200 mV, so that the entrance of the channel was at pH 8.2. The ratio of the *trans* (I_t) and *cis* (I_c) currents (I_{trans}/I_{cis}) at the channel entrance is 2.6. Zero current (closed channel) is the lowest level indicated. After about 4 s, the voltage was reversed (vertical line). The entrance of the channel was then at pH 11.6. The ratio of the *trans* and *cis* currents (I_{trans}/I_{cis}) is then 1.1 (1 M CsCl, diphytanoylphosphatidylcholine/decane membranes).

unprotonated *trans* channel, I_{tp} is the single-channel current of the fully protonated *trans* channel, and f_{tu} and f_{tp} are the time-averaged fractions of unprotonated and protonated *trans* channel, respectively. These fractions depend on pH and the acid dissociation constant of the *trans* channel (K_{at}) as follows:

$$K_{at} = \frac{f_{tu} \cdot [H^+]}{f_{tp}} \tag{2}$$

Since

$$f_{tu} + f_{tp} = 1 \tag{3}$$

it follows that:

$$K_{at}(1 - f_{tu}) = f_{tu} \cdot [H^+] \tag{4}$$

so that:

$$f_{tu} = \frac{K_{at}}{K_{at} + [H^+]} \text{ and } f_{tp} = 1 - \frac{K_{at}}{K_{at} + [H^+]} \tag{5}$$

Similarly, the observed current of the *cis* state (I_c) was assumed to be given by:

$$I_c = I_{cu} \cdot f_{cu} + I_{cp} \cdot f_{cp} \tag{6}$$

where I_{cu} is the single-channel current of the fully unprotonated *cis* channel, I_{cp} is the single-channel current of the fully protonated *cis* channel, and f_{cu} and f_{cp} are the time-averaged fractions of unprotonated and protonated *cis* channel, respectively. Again:

$$K_{ac} = \frac{f_{cu} \cdot [H^+]}{f_{cp}}, \text{ and } f_{cu} + f_{cp} = 1 \tag{7}$$

so that:

$$f_{cu} = \frac{K_{ac}}{K_{ac} + [H^+]} \text{ and } f_{cp} = 1 - \frac{K_{ac}}{K_{ac} + [H^+]} \tag{8}$$

At sufficiently high pH values, the difference between *cis* and *trans* currents becomes negligible, indicating that $I_{cu} = I_{tu}$. These constants can therefore be represented by a single constant I_u.

The observed current of the *trans* state (I_t) can then be expressed as a function of pH:

$$I_t = I_u\left(\frac{K_{at}}{K_{at} + [H^+]}\right) + I_p\left(1 - \frac{K_{at}}{K_{at} + [H^+]}\right) \tag{9}$$

Similarly for the *cis* state:

$$I_c = I_u\left(\frac{K_{ac}}{K_{ac} + [H^+]}\right) + I_{cp}\left(1 - \frac{K_{ac}}{K_{ac} + [H^+]}\right) \tag{10}$$

Even with highly purified channel peptides, there is variability of a few percent in absolute single-channel currents. The ratio of the *trans* current to the *cis* current, however, is relatively insensitive to channel heterogeneity. That is, if a particular single channel has a *trans* current 5% greater than the mean, the *cis* current will also be about 5% greater than the mean. The ratio of the *trans* current to the *cis* current thus provides a robust measure of the pH that is not sensitive to small changes in absolute channel permeability. In addition, we find that the pK_a values of the *cis* and *trans* states do not appear to be different within experimental error ($K_{ac} = K_{at}$) and can be represented by a single constant K_a.

Eqs. 9 and 10 can therefore be combined to give an expression for the ratio of the *trans* current to the *cis* current (I_t/I_c):

$$\frac{I_t}{I_c} = \frac{I_u + I_{tp}(10^{pKa - pH})}{I_u + I_{cp}(10^{pKa - pH})} \tag{11}$$

Values for I_u, I_{tp} and I_{cp} were obtained from the limiting currents obtained at low and high pH values for gram-EDA and gram-histamine. These values are collected in Table 1. Fits of Eq. 11 to measured values of I_t/I_c for gram-EDA and gram-histamine are shown in Fig. 6. The pK_a values derived from the fitting are also given in Table 1. The calculated pK_a values correspond quite well to values expected for model compounds (10.7 for ethylamine; 7.0 for imidazole [51]).

Table 1
Collected constants

	I_u (pA)[a]	I_{cp} (pA)	I_{tp} (pA)	pK_a
Gram-EDA	12.2	2.2	5.8	10.8
Gram-histamine	10.2	5.7	7.5	7.6

[a] Measured with 200 mV applied. Electrolyte was 1 M CsCl.

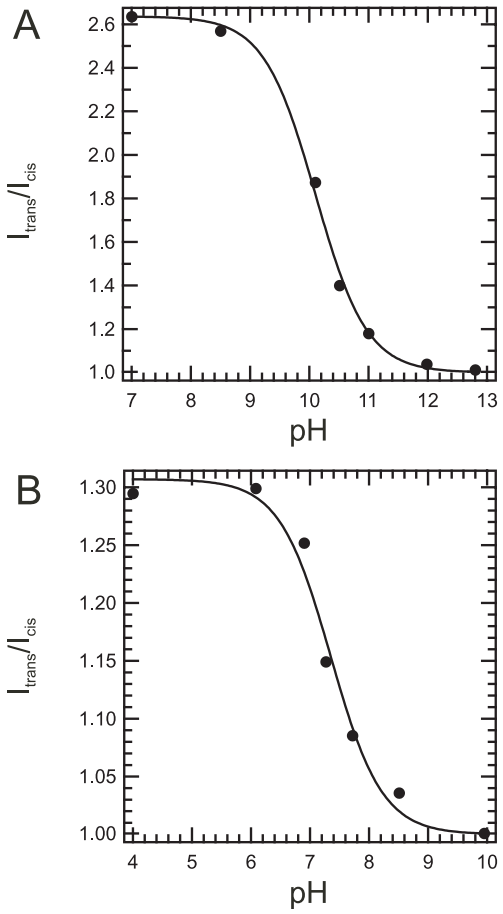

Fig. 6. Ratio of *trans* current to *cis* current (I_{trans}/I_{cis}) at the channel entrance as a function of pH (symmetrical conditions) for gram-EDA (A) and gram-histamine (B). The smooth lines are fitted curves (using Eq. 11) to experimental data (dots).

Curves such as those shown in Fig. 6 could be used to determine the local pH for an unknown case from a measurement of *cis* and *trans* currents. The size of local pH gradients could similarly be assessed by measuring relative *cis* and *trans* currents for each polarity of the applied voltage. A simple example of this is shown in Fig. 5 where I_t/I_c for gram-EDA is 2.6 on the *cis* side of the membrane and 1.1 on the *trans* side. These values can be used with Fig. 6A to estimate the pH on the *cis* side as between 8 and 9, and the pH on the *trans* side as between 11 and 12. The actual bulk pH values are 8.2 and 11.6 under the conditions of Fig. 5. pH values closer to the pK_a of the gramicidin analogue could be measured more precisely. Of course, gramicidin channels are permeable to protons so that their presence will perturb any pH gradients present. However, under single-channel recording conditions, and with buffer reservoirs of 2–3 ml, dissipation of the pH gradient is slow compared to the time required to make the measurements.

Although the present measurements were made with Cs^+ as the permeant ion, similar behaviour is observed with different permeant ions [46]. Also, steps in current recordings are still resolved at lower (e.g. physiological) salt concentrations [47]. In practice, relative *cis* and *trans* currents would have to be calibrated under the ionic conditions of interest. In situations where the signal to noise ratios are lower, larger numbers of single-channel I_t/I_c ratios could be averaged. Under the present conditions, the I_t/I_c ratios could be reliably determined from < 10 channel events.

Gram-EDA would be suitable for sensing pH values between 9 and 11; gram-histamine would be suitable for pH ranges between pH 6.5 and 8.5. If lower ranges were desired, a derivative could be constructed with an appropriate pK_a (e.g. gramicidin bearing a 4-fluorohistamine group (pK_a 3.5 [52]). Since the pK_a of the group attached at the C-terminal end of the channel can be altered as desired, this class of gramicidin derivatives may be useful for pH sensing under a variety of conditions. Since the structure of the channel and the location of the protonation site are known, these derivatives may also prove useful as tools for studying the kinetics of protonation/deprotonation reactions at membrane surfaces. By altering buffer concentrations, lipid type and/or substituting ^2H_2O for H_2O, measurements of rates of protonation/deprotonation might be possible in such a system (see e.g. [18]).

Acknowledgements

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