Cryptdin 3 forms anion selective channels in cytoplasmic membranes of human embryonic kidney cells

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Received 10 April 2001; accepted in final form 10 January 2002

Yue, Gang, Didier Merlin, Michael E. Selsted, Wayne I. Lencer, James L. Madara, and Douglas C. Eaton. Cryptdin 3 forms anion selective channels in cytoplasmic membranes of human embryonic kidney cells. Am J Physiol Gastrointest Liver Physiol 282: G757–G765, 2002. First published February 20, 2002; 10.1152/ajpgi.00152.2001.—Cryptdins are antimicrobial peptides secreted by Paneth cells located at the base of intestinal crypts. In addition to their antimicrobial function, cryptdins may also regulate salt and water secretion by intestinal epithelial cells. Recent work with short-circuit current measurements indicated that at least one cryptdin peptide, cryptdin 3, induces apical conductance(s) in Cl− secretory, including cystic fibrosis, epithelia. In the present study, we characterized the cryptdin 3-induced anion channel activity in human embryonic kidney (HEK) cells with single-channel patch-clamp techniques. The patch pipette was filled with solution containing different concentrations of cryptdin 3, and, after gigaseal formation, the channel activity was recorded with either cell-attached or inside-out patch modes. We found an anion selective channel with a conductance of 15 pS and open probability of 0.19, regardless of cryptdin 3 concentration. The mean open and closed times varied with the cryptdin 3 concentration. For cryptdin 3 concentrations of 10, 4, 1, and 0.5 μg/ml in the pipette, the corresponding mean open times were 1.2, 7.0, 9.0, and 17.4 ms and the corresponding mean closed times were 1.1, 1.6, 4.2, and 12.5 ms. These results suggest that cryptdin 3 forms anion-selective channels on the cytoplasmic membrane of HEK cells and that the kinetics of one such channel are affected by its interaction with other such channels.

INTESTINAL SALT AND WATER secretion depends on vectorial Cl− transport across the epithelial cells lining the crypt and secretory glands of the intestine (49). Cl− ions cross the basolateral membrane through cotransporters, then they are secreted into the intestinal lumen through Cl− channels in the apical membrane. This transepithelial Cl− movement drives Na+ and water across interepithelial tight junctions to produce a secretory response. Movement of Cl− through apical Cl− channels is the rate-limiting step for Cl− secretion. Therefore, regulation of Cl−-channel activity from crypt cells plays an important role in intestinal fluid secretion.

The production of antimicrobial peptides such as defensins is an important means of host defense against microbial invasion in multicellular organisms (33). Cryptdins are defensins produced by Paneth cells located in the small intestine at the crypt base adjacent to the Cl−-secreting cells (40). Mouse and human cryptdins are potent antimicrobial agents especially to Escherichia coli, Listeria, and Salmonella (12). Six mouse defensins, cryptdins 1–6, have been purified to homogeneity from the small intestine (32, 40). In addition to their antimicrobial function, cryptdins may also regulate the salt and water secretion in intestinal epithelial cells. Recent work based on short-circuit current measurements indicated that cryptdin 2 and 3 reversibly stimulate chloride ion secretion when administered apically to T84 cells, a human intestinal epithelial cell line (22). This cryptdin 3-stimulated Cl− transport is not inhibited by pretreatment with 8-phenylthioethanol or dependent on a concomitant rise in intracellular cAMP or cGMP, indicating that cellular signaling pathways that often stimulate Cl− transport are not involved (22). Interestingly, it has been shown recently that cryptdin 3 promotes a current in permeabilized cystic fibrosis (CF) epithelial monolayers, demonstrating that cryptdin 3 induces a restoration of Cl− secretion in CF cells that is probably not cAMP or CF transmembrane conductance regulator (CFTR) dependent (27). In addition, neutrophil defensins induce the formation of an anion channel when incorporated into artificial phospholipid bilayers (19). This activity may explain the bactericidal effect of neutrophil defensins. Because cryptdins are homologous in structure and function to defensins isolated from mammalian phagocytic cells (31, 32, 40) and Cl−-secreting crypt cells are exposed to cryptdin released from Paneth cells in vivo,
the cryptdins may induce Cl\(^{-}\) secretion by formation of anion channels in the cytoplasmic membrane of secretory gland cells. In the present study, we characterize the cryptdin 3-induced single-channel activity in HEK cells with cell-attached and inside-out patch-clamp techniques. We find that extracellular application of mouse cryptdin 3 induces anion-selective channels with a conductance of 15 pS and an open probability of 0.19 regardless of cryptdin concentration.

**MATERIALS AND METHODS**

**Cryptdin synthesis.** Studies were carried out using synthetic, folded, and oxidized cryptdin 3 prepared using a protocol identical to that previously described for the synthesis of cryptdin 4 (30). Synthetic and natural cryptdin 3 peptides have been shown to have identical physicochemical and antimicrobial characteristics (Ref. 39 and D. Tran and M. E. Selsted, unpublished data).

**Human embryonic kidney cells preparation, patch recording, and data analysis.** Human embryonic kidney (HEK) cells were purchased from American Type Culture Collection (Bethesda, MD). The HEK cells were grown in 35-mm culture dishes in a humidified incubator gassed with 5% CO\(_2\)-95% air. The culture media contain 95% DMEM and 5% fetal bovine serum supplemented with 2 mM glutamine.

After 2–4 days of culture, a dish containing HEK cells was placed on an inverted microscope for patch-clamp experiments. We used the cell-attached and inside-out patch-clamp methods following standard procedures. Patch pipettes were fabricated from TW 150 glass (World Precision Instruments, New Haven, CT) and fire-polished to produce tip resistances of 5–10 M\(\Omega\) when filled with pipette solution. Experiments were performed at room temperature (22–23°C). Patch pipette solution contained (in mM) 150 NaCl, 2.8 KCl, 2 MgCl\(_2\), 1 CaCl\(_2\), and 10 HEPES (pH 7.4). For cell-attached patch experiments, the bath solution had the same ionic composition as that of the pipette. For inside-out patch experiments, the bath was changed to a solution containing (in mM) 3 NaCl, 150 KCl, 1 MgCl\(_2\), 5 EGTA, and 10 HEPES. Cryptdin 3 appeared to interfere with seal formation. Therefore, to investigate the effects of cryptdin 3 on single-channel activity, the patch pipettes were filled with regular pipette solution in the tip portion, then they were backfilled with solutions containing various concentrations of cryptdin 3. In other experiments using similar electrodes backfilled with blocking agents for channels, which we observed in other cells, we have shown that it takes ~5 min for the tip to reach equilibrium. We always waited 10 min before beginning recording. Single-channel events were measured with an Axopatch 200 amplifier, low-pass filtered at 5 kHz, recorded on a digital video recorder with a bandwidth of 44 kHz (Sony, Tokyo, Japan), and then filtered at 500 Hz and digitized at 2 kHz using a Scientific Solutions analog-to-digital converter and a Pentium computer equipped with Axotape software (Axon, Redwood City, CA). The convention for applied voltage to the membrane patch represents the voltage deflection from the patch potential (i.e., the resting membrane potential for cell-attached patches). Outward anionic current (cell to pipette) is represented as downward transitions in the single-channel records. Small offset potentials associated with reduction of chloride in the bath are subtracted from current-voltage records.

**Data analysis.** Ten-minute data records were low-pass filtered at 500 Hz using a software Gaussian filter and analyzed using pCLAMP 6.03 (Axon). Amplitude histograms were generated to obtain unit current values. Although patches containing only a single channel are ideal for performing kinetic analysis, many patches contain multiple opening channels but can be analyzed using previously reported methods (26, 34). In this case, activity of a single channel (\(N_p\)) and open probability (\(P_o\)) of a single channel is given by

\[
N_p = \frac{\sum_{i=0}^{N} N \cdot t_i}{T} \tag{1}
\]

where \(T\) is the total recording time, \(N\) is the apparent number of channels within the patch, determined as the highest observable current level, \(i\) is the number of channels open, and \(t_i\) is the time during which \(i\) channels are open. We used this method for calculating \(N_p\) because the calculations were facilitated by having the event tables generated by pCLAMP. If channels are uniformly distributed in the membrane and the exact number of channels in a patch is known, then the mean \(P_o\) of one single channel can be calculated by dividing \(N_p\) by the number of channels in a patch. The total number of functional channels (\(N\)) in the patch was determined by observing the number of peaks detected in all-points amplitude histograms. The histograms were constructed from event records of long enough duration to provide 95% confidence of determining the correct \(N\) according to methods we have previously described (20, 26). The mean open time (\(t_o\)) of one of the \(N\) channels can be calculated as

\[
t_o = \frac{TNP_o}{n^2} \sum_{i=0}^{N} \frac{N \cdot t_i}{n^2} \tag{2}
\]

where \(n\) is the total number of transitions between states during \(T\) and the other parameters are the same as in Eq. 1. This value represents the average time the channel spends open (in any open state) and should not be confused with the mean residency time of the channel in a specific state [sometimes called the mean open time (\(t_o\)) for the state]. Alternatively, in patches with multiple channels, we determined the most likely value for \(N\) and then calculated values for \(t_o\) by measuring the duration of the events in which all \(N\) channels were open (\(t_o\)) and remembering that \(t_o = t_N \times N\). The difficulty with this method is the assumption that \(N\) can be determined accurately and that there are enough events with all channels open to obtain a meaningful distribution of intervals. To ensure the accuracy of this approach, we only used patches for which we had a >95% probability of having estimated the number of channels correctly [following the methods of Marunaka and Eaton (26) or in which \(t_o\) can be determined unambiguously (patches with no overlapping open events)]. Either measure we used to calculate \(t_o\) provided an easy way to distinguish whether experimental manipulations (e.g., cryptdin 3) modify \(P_o\) by affecting the channel’s open or closed states.

Besides the method described above to calculate \(P_o\), in theory, one could also calculate \(P_o\) according to

\[
P_o = \frac{t_o}{t_o + t_{closed}} \tag{3}
\]

However, in general, the variability in the two parameters, mean open and closed times, produces an estimate for \(P_o\) that tends to have a large error (for an example of the problem, see Ref. 46). For the cryptdin channels described in this work,
the problem is exacerbated by two properties of the channels. First, at high cryptdin concentrations, the mean open and closed times are short, making determinations of the mean values from interval histograms somewhat inaccurate, although we used methods described elsewhere to improve the accuracy (4, 41). Second, there is a large cell-to-cell variability in mean open and closed times. However, the variability in mean open and closed times appears to be on a cell by cell basis (see Fig. 8); that is, if mean \( t_o \) is short, mean closed time is also short. This means that estimating \( P_o \) from \( N P_o \), is more accurate than calculating \( P_o \) from the mean open and closed times.

Statistics. The data were presented as means ± SD. Student’s \( t \)-tests were used as appropriate to compare experimental groups. For more than one group, one-way ANOVA was used. For comparison of fits, a \( \chi^2 \)-test was used. Results were considered significant when \( P \) was <0.05.

RESULTS

A recent report suggests that cryptdin 3 may induce anion permeability in T84 cell monolayers (22). However, T84 cells express large numbers of several different types of Cl\(^-\) channels. Therefore, it might be difficult to determine whether cryptdin 3 induced new channels or activated preexisting ones. On the other hand, HEK cells express few endogenous channels of any type, and no anion channels have been reported (see DISCUSSION). Therefore, we used HEK cells to determine whether cryptdin 3 could form anion channels. As shown in Fig. 1, there was no single-channel activity when cryptdin 3 was absent from the pipette solution (and none in 142 additional untreated patches). Application of 10 \( \mu \)g/ml cryptdin 3 in the pipette elicited multiple channel openings with very rapid transitions between open and closed states. As cryptdin 3 concentration increased, the number of channel openings increased and open and closed times decreased (as summarized in Table 1). The relationship between the cryptdin 3 dose and the number of channels is shown in Fig. 2. At cryptdin 3 concentrations higher than 10 \( \mu \)g/ml, it was not possible to count the number of channels and patches were not stable so that it was not possible to determine whether there was a saturating dose of cryptdin 3. Figure 3 illustrates the current-voltage relationship (\( I-V \)) from inside-out patch experiments. This result shows that formation of cryptdin 3 channels does not require any cytoplasmic components and does not involve changes in cell volume. The \( P_o \) of the channels was not voltage dependent, and the channels did not rectify. A conductance of 15 pS was obtained from the slope of the \( I-V \) curves. In five experiments, when part of the Cl\(^-\) in the pipette was replaced with 120 mM gluconate, a nonpermeable anion, the reversal potential shifted from 0.2 ± 1.6 to 39.6 ± 2.9 mV, close to the calculated equilibrium potential for Cl\(^-\) (+42 mV). This indicated that cryptdin 3 induced channels that were strongly selective for anions over cations in HEK cells.
The mean open and closed times of these channels increased with decreasing concentration of cryptdin 3 in the pipette (Fig. 4). Examination of the open and closed interval histograms revealed only a single exponential component of each histogram. However, despite the changes in open and closed times, there is no significant variation of the $P_o$ at different concentrations of cryptdin 3 in pipettes (Fig. 5). These results suggest that the kinetics of one channel in the membrane is modified by its interaction with other cryptdin 3 molecules.

**DISCUSSION**

In this study, we report that cryptdin 3 induces an anion-selective channel in the cytoplasmic membrane of HEK cells. HEK293 cells were originally described in 1990 (42, 43). We used HEK293 cells because they have few endogenous ion channels and are often used for heterologous expression of cloned ion channels. By using these cells, we hoped to eliminate the possibility that cryptdin 3 was activating an endogenous chloride channel. In 823 PubMed references to HEK293 cells, only four describe any endogenous channels, and these are all cation channels. However, more information about endogenous channels is available from expression studies in which investigators often establish that there are no endogenous channels of a specific type before transfecing and expressing cloned channels. In this context, several chloride channels (CLC; CLC-0, CLC-1, CLC-2, CLC-4, CLC-5, CLC-6, and CLC-7) cannot be detected in HEK293 as protein (by Western blotting), as mRNA (by RT-PCR), or functionally (by patch clamp) (3, 8, 9, 14–17, 24, 35, 37, 44, 45). In addition, unit conductance, single-channel kinetics, and voltage dependence of these channels as well as CLC-3 are all different from the cryptdin channels we describe in this work (3, 16, 17, 24, 36, 37, 45). Thus all known members of the CLC family are unlikely candidates for activation by cryptdin 3 unless the peptide is capable of changing virtually every biophysical characteristic of the channel.

Rabbit neutrophil defensin NP3A stimulates a calcium-dependent volume reduction in villus enterocytes (25). This cell shrinkage is prevented by 9-anthracene-carboxylic acid, a Cl$^-$ channel blocker. The results might suggest that NP3A activates Ca$^{2+}$-dependent Cl$^-$ channels in enterocytes, but this activation could easily be due to an NP3A-mediated increase in the membrane permeability to calcium that indirectly activates calcium-dependent chloride channels. However, it is unlikely that cryptdin 3 activates Ca$^{2+}$-dependent Cl$^-$ channels in HEK cells, because such channels have never been reported in HEK cells (in the 893 references mentioned above). Moreover, cryptdin 3-induced channels have very different biophysical properties (e.g., no voltage dependence and a very different conductance) from those of Ca$^{2+}$-dependent Cl$^-$ channels (2, 11). Also, cryptdin can induce channels in excised patches when the calcium concentration...
on the cytosolic surface is well below the level necessary for calcium-dependent chloride channel activation. CFTR, a cAMP-activated anion channel with a conductance of 7–10 pS, also does not appear to be a candidate for a cryptdin 3-induced channel, because the biophysical properties are different (5, 15). Moreover, cryptdin 3 still induces a current in basolaterally permeabilized epithelial monolayers derived from airway cells with a nonfunctional F508 mutation of CFTR (27), and cryptdin 3 does not increase intracellular cAMP (22), which is necessary for CFTR activation. Volume-sensitive anion channels, which are stimulated by extracellular hyposmolality, are broadly expressed in animal cells (47). They are also poor candidates for a cryptdin 3-induced channel, because they are all voltage dependent, outwardly rectifying channels (6, 10, 50) with unit conductances in excess of 60 pS (29). Moreover, there was no alteration of osmolality during our patch-clamp recording in HEK cells, making activation of such channels unlikely. Also, cryptdin channels appear in excised patches, a condition under which volume-sensitive channels are not active.

Therefore, cryptdin 3 channels in HEK cells do not appear to be associated with any of the major categories of anion channels often present in animal cell membranes.

The remaining possibility is that cryptdin 3 forms new anion channels in the cytoplasmic membrane. Previous studies show that a rabbit neutrophil defensin (NP-1) forms ion channels in a planar lipid bilayer membrane (19). This channel is voltage dependent and weakly anion selective with heterogeneous single-channel conductance ranging from 10 to 1,000 pS. Because the structure of NP-1 is similar to cryptdin 3, it is possible that cryptdin 3 might also form new membrane channels. However, the biophysical properties of NP-1 channels are very different from those we observed for cryptdin 3-induced anion channels: cryptdin 3 channels are voltage-independent, highly anion-selective channels with a constant conductance of 15 pS. The differences could be due to differences between incorporation in native cell membranes rather than artificial bilayer membranes or from intrinsic differences in amino acid structure between the two defensins. The second explanation is supported by the recent study showing that cryptdin 3 induces an apical

Fig. 3. Current-voltage relation of cryptdin 3-induced anion channels from inside-out patch recordings. For recording conditions with almost equal chloride in the bath and pipette, the reversal potential in 5 experiments was 0.2 ± 1.6 mV (bath contained (in mM) 3 NaCl, 150 KCl, 1 MgCl₂, 5 EGTA, and 10 HEPES; and pipette contained (in mM) 150 NaCl, 2.8 KCl, 1 CaCl₂, and 10 HEPES, with 0.4 µg cryptdin). When the chloride concentration in the pipette was reduced, the reversal potential shifted to 39.6 ± 2.9 mV in a manner expected for a chloride permeable channel (bath contained (in mM) 3 NaCl, 150 KCl, 1 MgCl₂, 5 EGTA, and 10 HEPES; and pipette contained (in mM) 30 NaCl, 120 Na⁺ gluconate, 2.8 KCl, 2 MgCl₂, 1 CaCl₂, and 10 HEPES, with 0.4 µg cryptdin 3). Lines are the best-fit linear least squares regression line through the data points.

Fig. 4. Mean open and closed times of cryptdin 3-induced single anion channel activity vary with cryptdin 3 concentration. Both open and closed times were dependent on the cryptdin 3 concentrations in the pipette solution. Both open time and closed times decreased with increasing cryptdin 3 concentrations from 0.5 to 10 µg/ml.

Fig. 5. Single channel open probability (Pₒ) was not affected by the cryptdin 3 concentrations in the pipette solution. Despite changes in mean open and closed times, Pₒ remained near 0.2 when cryptdin 3 concentration was varied from 10 to 0.5 µg/ml.
conductance that is voltage independent in basolaterally permeabilized epithelial cell monolayers (T84) (27). Defensins are cationic molecules with spatially separated hydrophobic and charged residues (51). This arrangement allows their hydrophobic regions to be buried within interior apolar regions of membrane lipid and their cationic regions to interact with the anionic polar head group of phospholipid and water so that the defensin molecules can incorporate into cytoplasmic membranes. Beyond the primary structure, defensins are folded to form antiparallel β-sheets, which are stabilized by three disulfide bonds (13, 40). Despite these general structural similarities among the defensin peptide family, mouse cryptdin 3 and rabbit NP-1 differ in amino acid sequence. Figure 6 shows the sequences of these two peptides. There are a number of structural differences between NP-1 and cryptdin 3. By analogy with the neutrophil defensins HNP-1, HNP-3, NP-2, and NP-5, amino acids at positions 10 and 15 are predicted to be located at conserved turns on the surface of the molecule (31). Positively charged Arg-11, Lys-14, and Arg-15 in cryptdin 3 are replaced by neutral residues Ala, Leu, and Pro in the corresponding positions in NP-1. Positive charges in the turn regions are critical for defensin molecules to interact with negatively charged polar head groups of membrane lipid. Thus such positive charges might logically favor the anion selectivity of cryptdin 3 channels compared with NP-1 channels. The extra positive charges on cryptdin 3 molecules might also produce a different peptide stoichiometry for the channel than NP-1, thus leading to the conductances which differ. Alternatively, the longer NH₂ terminus of cryptdin 3 compared with NP-1 could contribute to differences in stoichiometry and gating.

In our work, we observe cryptdin-induced channels that are remarkably selective for chloride over sodium or potassium, but we have not examined the permeability sequence for different anions. Nonetheless, the anion-to-cation selectivity is maintained at all of the cryptdin concentrations we have examined. Although the concentrations we examined tend to be lower than those used by other investigators, our results are still in marked contrast to reports that, after exposure to high cryptdin concentrations, cells become much more permeable and the cryptdin-induced permeability becomes much less selective. For example, at high concentrations, cryptdin 3 increases the permeability of T84 cells to even allow entry of the usually membrane-impermeant fluorophore 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acid (22). This observation appears to imply that larger, less selective pores are formed at high cryptdin 3 concentrations. However, an alternative explanation, consistent with the actions of other pore-forming peptides, is that as the cryptdin concentration and the number of cryptdin channels increase, the selectivity of the channel remains unchanged. However, when the concentration is high enough, there is a generalized disruption of the cell membrane and cell function that leads to a increased nonspecific permeability of the cell membrane to large molecules even though the cryptdin pore is still only permeable to anions. This idea is consistent with the actions of a variety of pore-forming agents such as filipin, nystatin, and amphotericin (7, 21, 23, 28, 38), which form pore complexes with fixed properties but at high concentrations disrupt cellular membranes.

In brief, the present studies show that cryptdin 3 is, at the least, associated with and is likely capable of forming anion channels in the cytoplasmic membranes of HEK cells. Thus cryptdin 3 in vivo, after release from Paneth cells at the base of the crypt, likely forms channels in the apical membrane of adjacent glandular cells. Such channel formation would lead to salt and water secretion, causing the crypt lumen to be flushed after Paneth cell discharge, removing bacteria as well as diluting the cryptdin 3 concentration to levels below the effective cytotoxic concentration for epithelial cells in the crypt lumen. Meanwhile, cryptdin 3 channels could also be removed from the apical membrane by partitioning into the aqueous phase or by endocytosis. This balance between luminal cryptdin 3 concentrations sufficient to induce Cl⁻ secretion but below the cytotoxic range coupled with membrane recycling of cryptdin 3 channels may serve to protect crypt epithelial cells from possible cytotoxic effects while delivering these antimicrobial peptides to pathogen cell surfaces at high enough concentrations in the crypt or within the gut lumen itself to promote lysis of the bacterial cells.

APPENDIX

A Potential Mechanism for Cryptdin 3 Channel Formation

Presently, there is not enough information available to propose a complete structural model of cryptdin channels; however, some conclusion can be drawn about the mechanism of the interaction of cryptdin 3 with the membrane. Of course, other small molecules produced in eukaryotic cells can form pores. For example, a 27-amino-acid fragment of the so-called minK potassium channel forms a cation pore by forming a homotrimer (1, 18). Nonetheless, it seemed unlikely to us that one cryptdin 3 peptide could form a channel, because the

Fig. 6. Amino acid sequences of mouse cryptdin 3 and rabbit defensin (NP-1). Primary structures are shown in single-letter amino acid code. Charged residues are marked + or − as positive or negative charge. Three disulfide bonds are indicated as line connections.

Cryptdin 3
NP-1

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size of the molecule (∼4 kDa) would appear to preclude a single peptide from having the multiple membrane spanning domains usually required for channel formation. In an attempt to gain more information about this issue, we reexamined the dose-response data of Fig. 2. If n peptide molecules in the aqueous phase are necessary to form one channel in the membrane according to the following reaction

\[
n\text{Cryptdin}_{\text{aqueous}} \leftrightarrow n\text{Cryptdin}_{\text{channel}} \quad (A1)
\]

then at equilibrium

\[
K_{eq} = \frac{[\text{Cryptdin}_{\text{channel}}]}{[\text{Cryptdin}_{\text{aqueous}}]} \quad (A2)
\]

and

\[
\log (\text{Cryptdin}_{\text{channel}}) = n \log (\text{Cryptdin}_{\text{aqueous}}) + \log K_{eq} \quad (A3)
\]

Therefore, in a plot of the log of the number of channels vs. the log of the cryptdin 3 concentration, the slope will be equal to the number of cryptdin 3 molecules forming a channel and the value at a cryptdin 3 concentration of 1 [i.e., log [cryptdin concentration] = 0] will be the log of the equilibrium distribution (log \(K_{eq}\)) of cryptdin 3 molecules between the aqueous and membrane phases. An examination of such a plot (Fig. 7) shows that, surprisingly, the slope is 1.07 ± 0.094 and the value at a cryptdin concentration of 1 (the log of equilibrium constant) is 0.734 ± 0.133, implying that only one cryptdin 3 molecule is required to form a channel and that cryptdin 3 molecules are about fivefold more likely to reside in the membrane as a channel as in the aqueous phase.

An alternative method for approaching the issue of the distribution of cryptdin molecules depends on the observation that the open- and closed-interval histograms for cryptdin channels indicate that there is only one open and one closed state of the channel. Also, once we observed activity, we never subsequently observed a loss of activity before seal breakdown (usually ∼30–40 min). If the channel in the membrane was changing from a closed to an open conformation and, in addition, was partitioning and departioning between membrane and aqueous phases, we would expect two components to the closed-interval histogram or we would expect occasional loss of activity. Therefore, the observed pattern is only consistent with two possibilities. The first is that the open partitions into the membrane and never partitions out. This seems a thermodynamically unlikely possibility. The second is that the transitions actually represent partitioning and departioning of channels, in which case we would expect only one component of the closed- and open-interval histograms (as we see) and the reciprocal of the closed time is the partitioning rate and the reciprocal of the open time is the departioning rate. A calculation of the equilibrium partition constant from the mean open and closed time gives a value of 2.78 ± 1.56, which is not significantly different from the value of 5.42 ± 2.06 obtained from Fig. 7.

However, these results do not necessarily imply that only one peptide forms a channel. The alternative explanation is that the peptides can only insert into the membrane as a multimer of fixed stoichiometry. On the basis of a variety of

\[
\text{Fig. 7. A plot of the log of the number of channels vs. the log of the cryptdin 3 concentration. In this plot, the slope will be equal to the number of cryptdin molecules forming a channel, and the value at a cryptdin 3 concentration of 1 (at which log concentration is 0) will be the log of the equilibrium distribution of cryptdin 3 molecules between the aqueous and membrane phases. An examination of the plot shows that the slope is 1.07 ± 0.094 and the intercept is 0.734 ± 0.133, implying that only one cryptdin 3 molecule or only one cryptdin 3 molecular complex in solution is required to form a channel and that cryptdin 3 molecules are ∼5-fold more likely to reside in the membrane as a channel as in the aqueous phase. The means include patches with 0 channels for lower cryptdin concentrations, because the patches with no channels also reflect the probability that cryptdin at low concentration will enter the membrane.}
\]

\[
\text{Fig. 8. Mean open and closed times are correlated. Plotting all mean open times as a function of closed times yields a linear relationship with an r value of 0.804. Despite a large variability in the mean open and closed times from cell to cell, in any given cell, the mean open and closed times are correlated; i.e., if the mean open time is short, the mean closed time is also. Thus the ratio of the mean open to closed time remains close to the same so that P0 does not appreciably change as cryptdin concentration increases.}
\]

\[
\text{Fig. 9. Plots of the log of the number of channels vs. the log of the cryptdin 3 channel mean open and closed times. In these plots, the slope will be equal to the average number of cryptdin complexes in the membrane that interact to alter mean open and closed times. An examination of the plot shows that the slopes are −1.10 ± 0.082 for open time and −0.958 ± 0.193 for closed time, values not significantly different from 1. This result is consistent with the idea that a single dimer can form a pore but that 2 dimers in the membrane can associate, and when they are associated, the rate of transitions between the open and closed states is increased.}
\]
approaches including the crystal structure and nuclear magnetic resonance measurements, investigators have reported that some defensins appear to form a dimer in aqueous phase (13, 51). If cryptdin forms a dimer, the dimeric channel would appear to have a structure that might be consistent with the formation of an aqueous pore (13). If cryptdin 3 were either present in aqueous solution or at least inserted into the membrane as a dimer, then it would be possible to explain the first-order power dependence of the number of channels on cryptdin 3 concentration and still invoke a multimeric channel.

It is not surprising that increasing cryptdin 3 concentrations increase the channel density (number of channels per patch). Some investigators have suggested that as the concentrations of some defensins increase, the number of peptides that form a channel also increases (51). In these cases, the conductance increases with increasing cryptdin concentration, and the selectivity decreases (implying a larger pore). However, for cryptdin 3, the fact that the unit conductance and even the \( P_o \) do not change with increasing cryptdin 3 concentration implies that the stoichiometry of a cryptdin 3 channel, once formed, is constant. Nonetheless, when there are a large number of channels in the membrane, the properties of channels do change: both mean open and closed times decrease with increasing cryptdin 3 concentration.

Despite a large variability in the mean open and closed times from cell to cell, in any given cell, the mean open and closed times are correlated; i.e., if the mean open time is short, the mean closed time is also (Fig. 8). Thus the ratio of the mean open to closed time remains close to the same so that \( P_o \) does not appreciably change. This result also implies that the ratio of partitioning to departitioning of cryptdin into and out of the membrane does not change. The nature of this interaction can best be understood by examining the properties of a simple kinetic model that describes the properties of cryptdin 3 channels once a cryptdin molecule in aqueous solution has entered the membrane to form a channel.

\[
\text{Closed} \xrightarrow{k_1} \text{Open} \xrightarrow{k_1} \text{Closed}
\]  

The transition from a closed to an open state represents a conformational change of the cryptdin 3 molecule. Such a change requires a certain amount of energy, and the mean duration in the open or closed state is proportional to the amount of energy required to cause a transition. Likewise, the rate of transition from one state to another is inversely proportional to the mean duration and inversely proportional to the transition energy. A decrease in mean duration can only mean that the energy barrier between the two states is uniformly decreased. Therefore, addition of extra cryptdin 3 molecules does not alter the fact that functional conducting molecules do not change with increasing cryptdin 3 concentration and still invoke a multimeric channel.

\[
\text{Open} \leftrightarrow \text{Closed} \quad \text{with} \quad k_1 \quad \text{and} \quad k_2
\]

for closed times). Adding additional power components does not improve the goodness of fit (based on \( x^2 \) analysis). Cryptdin 3 (10 \( \mu \)g/ml) produces a flickering channel with shorter open and closed durations. Five micrograms per milliliter or less are required to observe clear single-channel records. This result is consistent with the idea that a single cryptdin molecule (regardless of its stoichiometry) can form a pore but that two molecules in the membrane can associate and, when they do associate, the rate of transitions between the open and closed states is increased. This result is also consistent with the finding that low (5 \( \mu \)g/ml) but not high concentrations (50 \( \mu \)g/ml) of NP-1 are necessary to observe a stable unitary conductance in artificial lipid bilayers (19). The formation of a multimeric channel depends on the interaction among cryptdin 3 peptides, which is related to their concentration. Thus the kinetics of one channel are affected by interaction with other cryptdin 3 molecules.

We thank B. J. Duke for maintaining the cells in culture. This work was supported by R01 AI-22931 and the Large Scale Biology (to M. E. Selsted), P01 DK-33506 (to J. L. Madara), K01 DK-02833 (to D. Merlin), R01 DK-56305, P01 DK-50268, and R01 DK-37963 (to D. C. Eaton), and the Center for Cell & Molecular Signaling.

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