Formamidinium-induced Dimer Stabilization and Flicker Block Behavior in Homo- and Heterodimer Channels Formed by Gramicidin A and *N*-Acetyl Gramicidin A

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ABSTRACT Compared to the *N*-formyl gramicidin A (GA), the *N*-acetyl gramicidin A (NAG) channel has unchanged conductance in 1 M NH_4^+ (γ_{NN}/γ_{GG} = 1, conductance ratio) but reduced conductance in 1 M K^+ (γ_{NN}/γ_{GG} = 0.6) methylammonium (γ_{NN}/γ_{GG} = 0.3), and formamidinium (γ_{NN}/γ_{GG} = 0.1) solutions. Except with formamidinium, "flicker blocks" are evident even at low cutoff frequencies. For all cations studied, channel lifetimes of *N*-acetyl homodimers (NN) are ~50-fold shorter than those of the GA homodimer (GG). The novel properties of GA channels in formamidinium solution (supralinear current-voltage relations and dimer stabilization (Seoh and Busath, 1993)) also appear in NN channels. The average single channel lifetime in 1 M formamidinium solution at 100 mV is 6–7-fold longer than in K^+ and methylammonium solutions and, like in the GA channel, significantly decreases with increasing membrane potential. Experiments with mixtures of the two peptides, GA and NAG, showed three main conductance peaks. Oriented hybrids were formed utilizing the principle that monomers remain in one leaflet of the bilayer (O'Connell et al., 1990). With GA at the polarized side and NAG at the grounded side, at positive potentials (in which case hybrids were designated GN) and at negative potentials (in which case hybrids were designated NG), channels had the same conductances and channel properties at all potentials studied.

Flicker blocks were not evident in the hybrid channels, which suggests that both *N*-acetyl methyl groups at the junction of the dimer are required to cause flickers. Channel lifetimes in hybrids are only ~threefold shorter than those of the GG channels, and channel conductances are similar to those of GG rather than NN channels.

We suggest that acetyl-acetyl crowding at the dimeric junction in NN channels causes dimer destabilization, flickers, and increased selectivity in *N*-acetyl gramicidin channels.

INTRODUCTION

Gramicidin A is a hydrophobic linear pentadecapeptide which dimerizes to form monovalent-cation-selective channels of ~3.7-Å diameter (Busath et al., 1988) in lipid bilayer membranes. The ions and water move through the channel length in single-file (Urry, 1971; Eisenman et al., 1980; Finkelstein and Andersen, 1981). The dimer consists of two single-stranded right-handed (Arseniev et al., 1990) β-helices associated head-to-head at the amino termini by six inter-chain N—H—O=C hydrogen bonds (Urry, 1971; Bamberg et al., 1977; Weinstein et al., 1979; Arseniev et al., 1990). The dimer model has been challenged (Stark et al., 1986; Strassle et al., 1989) but Cifu et al. (1992) verified that the elementary conducting unit is a dimer with hybrid experiments using gramicidin A and *O*-pyromellityl gramicidin A where only four main conductance levels were observed.

Any modification of the amino end of the gramicidin A channel either completely eliminates the capacity to form the channels (e.g., *N*-pyromellityldesformyl or *N,O*-bissuccinyldesformyl dimers) or dramatically changes the channel lifetimes (e.g., the *N*-acetyl dimer, the malonyl, succinyl, and oxalyl covalent dimers, and the succinyldesformyl dimer) (Urry, 1971; Bamberg and Benz, 1976; Bamberg and Janko, 1977; Szabo and Urry, 1979; Urry, 1979), whereas modifications of the carboxyl end has limited effects on channel activity. For example, *O*-acetylation of the ethanolamine at the carboxyl terminus does not alter the steady state conductance properties nor the CD spectrum of the molecule (Wallace et al., 1982).

A single channel study (Szabo and Urry, 1979) of the conductances and single channel lifetimes for alkali metal cations in the NAG homodimer channels (Goodall, 1971) showed them to have 50-fold reduced lifetimes, slightly reduced conductances, and frequent flicker blocks. Structurally, NAG has a methyl group instead of hydrogen at the amino terminus as follows.

N-Formyl gramicidin A (GA):

$$\mathbf{H}CO\text{-L-}Val^{1}\text{-}Gly^{2}\text{-L-}Ala^{3}\text{-}\ldots$$

$$\text{-L-}Trp^{13}\text{-D-}Leu^{14}\text{-L-}Trp^{15}\text{-}NHCH_2CH_2OH$$

N-Acetyl gramicidin A (NAG):

$$\mathbf{CH_3}CO\text{-L-}Val^{1}\text{-}Gly^{2}\text{-L-}Ala^{3}\text{-}\ldots$$

$$\text{-L-}Trp^{13}\text{-D-}Leu^{14}\text{-L-}Trp^{15}\text{-}NHCH_2CH_2OH$$

Szabo and Urry (1979) concluded that NAG channel lifetimes were short because the methyl residues interfere with the hydrogen bonding at the head-to-head junction of single stranded β-helices. The flicker blocks have been ascribed to a nonconductive state which occurs when an acetyl methyl rotates into and blocks the channel (Szabo, 1981; Sigworth and Shenkel, 1988). Prior to the present report of hybrid experiments with GA and NAG peptides, it was not known

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whether only one methyl group or two methyl groups are required for flickers.

We report here that GA and NAG readily form hybrid channels and compare the single channel conductances and single channel lifetimes for heterodimers and homodimers. We report that our results support the previous suggestion for the mechanism for flickers by Szabo and Urry (1979) and further clarify the mechanism of destabilization. In a prior study (Seoh and Busath, 1993), we found that organic cations provide another sensitive assay of GA channel structure. In particular, formamidinium ($HC(NH_2)_2^+$), a rigid planar molecule of dimensions similar to the channel interior, has supralinear current-voltage relations at low concentrations, causes substantial open channel noise at low voltages (<100 mV) (but not flicker blocks like guanidinium (Hemsley and Busath, 1991)), and prolongs channel lifetimes dramatically. Channel lifetimes are reduced to levels observed with the alkali metal ions at high voltage or low concentration. The study reported here was motivated partly by the expectation that, if channel prolongation is due to interactions at the dimeric junction, the pattern of lifetime prolongation should be different in the altered-junction analog like the NN channels. Surprisingly, both this expectation and our additional expectation that the acetyl-flicker blocks would be exaggerated in formamidinium solutions proved wrong, although the overall formamidinium permeability was much lower in NN than in GG, as we expected.

METHODS

Single channel currents were recorded in planar lipid bilayers formed from 50 mg of glyceryl-1-monoolein (GMO, NuCheck, Blysian, MN) in 1 ml of *n*-decane (Aldrich Chemical Company, Inc., Milwaukee, WI) or hexadecane (Aldrich). The experimental procedures were as described previously (Busath and Szabo, 1988; Seoh and Busath, 1993). All the solutions (K^+, NH_4^+, methylammonium, and formamidinium solutions) were augmented with 0.009 M $MgCl_2$. All were prepared freshly using distilled water purified to >18.2 MΩ-cm with a Barnstead NANO pure II system (VWR Scientific, San Francisco, CA) and were filtered just before use with a 0.2-μm Nalge Filter (Fisher Scientific, Pittsburgh, PA). Formamidine HCl and methylamine HCl were used without purification. The unbuffered solution had pH ~6 so both organic molecules were fully ionized, and H^+ conductance by the channels was negligible. In aqueous solution formamidinium undergoes slow hydrolysis producing the very permeant ammonium ion (Seoh and Busath, 1993). Methylammonium is more stable than formamidinium under normal conditions, but also undergoes slow hydrolysis judging from the conductances of GG channel which increase 15–20% after 5–6 h. To avoid the effect of ammonium contamination, all the data for formamidinium and methylammonium solutions were collected within 1.5 h of the solution preparation. The transmembrane current was measured in symmetrical solutions, low-pass filtered with an eight-pole Bessel filter using a cut-off frequency of 30–200 Hz, and digitized with 12-bit resolution at 100–600 samples/s for subsequent analysis with a Masscomp computer (Concurrent Computer Corp., Oceanport, NJ).

All the single channel currents were collected within 10–15 min after formation of the lipid membrane. The electrode potential was checked before and after every experiment. All the data collected during experiments where the electrode potential was more than 1 mV were discarded. All the experiments were performed at room temperature (19–24°C) and conductances were corrected to 25°C using Q_{20} = 1.9 (Hladky and Haydon, 1972).

For oriented hybrid experiments with NAG and GA peptides, either NAG or GA was applied to the baths on both sides of the membrane first, and single channel data for homodimers were recorded at 100 mV in several different membranes. Then the applied voltage lowered nominally to 0 mV, and the other peptide was added to the grounded side. The bath containing the second peptide was stirred for ~60 s with the capillary micropipette, the voltage was reapplied, and currents were measured starting immediately. Throughout this procedure the integrity of the lipid bilayer was monitored by measuring the membrane current.

For channels without frequent flickers, conductances and lifetimes were evaluated as described previously (Seoh and Busath, 1993). Single channel lifetimes were measured by matching channel appearance steps with disappearance steps. When ambiguities existed due to simultaneous appearance of multiple channels with equal conductances, the match was randomized. For those with flickers, the amplitude of the open state was measured as the maximal conductance. This was estimated as the difference between the average of the prior baseline current and the average initial segment of the channel preceding a flicker event. Channels with flickers appearing before 5 data points, or opening to a state not representative of the maximal conductance, were not utilized. Because the open state dominates, this simple expedient gave consistently reliable estimates as judged by eye.

For the analysis of mean single channel lifetimes in all the hybrid experiments, the channels were grouped according to their conductances and the mean of the lifetimes in each conductance group was computed.

Unless otherwise noted, statistics reported for conductances and lifetimes are the mean of the standard channels ± the standard deviation (S.D.) between several (>3) experiments with the total number of channels from all experiments (n) given in parentheses. When the S.D. is less than 0.05, it is designated as 0.0.

For estimation of open channel noise, channels were selected which lasted more than 100 sample durations and which were preceded or followed by a baseline segment of similar length. Care was taken to exclude periods of "membrane breakdown" noise. All current samples during the channel opening were used (excluding the five points that occurred during the channel opening or closing). No glitch removal was employed so that the reported noise reflects gaps and flickers as well as formamidinium-induced noise. The baseline variance was subtracted from the open channel current variance to eliminate instrumental and bilayer sources of noise. Because the filter cuts the noise at a frequency well below the characteristic frequency of the noise, the open channel current-power spectral density can be estimated as the difference between the variance of the single open channel current and that of the baseline divided by the cutoff frequency. Rare flicker induced noise (Heinemann and Sigworth, 1993) in the open channel current has spectral density:

$$S(f) = \frac{4i^2\gamma\tau}{1 + (2\pi f\tau)^2} \quad (1)$$

where i is the channel current for the open state, γ is the flicker frequency, and τ is the average flicker duration. We therefore normalize the estimate dividing by the measured current i^2 using the mean channel currents as an approximate estimate of the open state current to allow comparison of channels of different conductance:

$$S' = \frac{\text{Var(open)} - \text{Var(closed)}}{f_c * i^2} \quad (2)$$

RESULTS

Fig. 1, *a–d*, shows single channel currents from NN channels in 1 M K^+ (*a*), NH_4^+ (*b*), and methylammonium (*c*) at 100 mV and formamidinium (*d*) at 200 mV. There are significant current fluctuations (so-called "flicker blocks," though partially filtered here) for K^+, NH_4^+ and methylammonium salt solutions (Fig. 1, *a–c*) but not for the formamidinium solution. The current amplitudes in formamidinium solutions were too small to distinguish flickers from the baseline at 100 mV. Shown instead is the current trace (Fig. 1 *d*) at 200 mV which does not show any significant current fluctuations (see

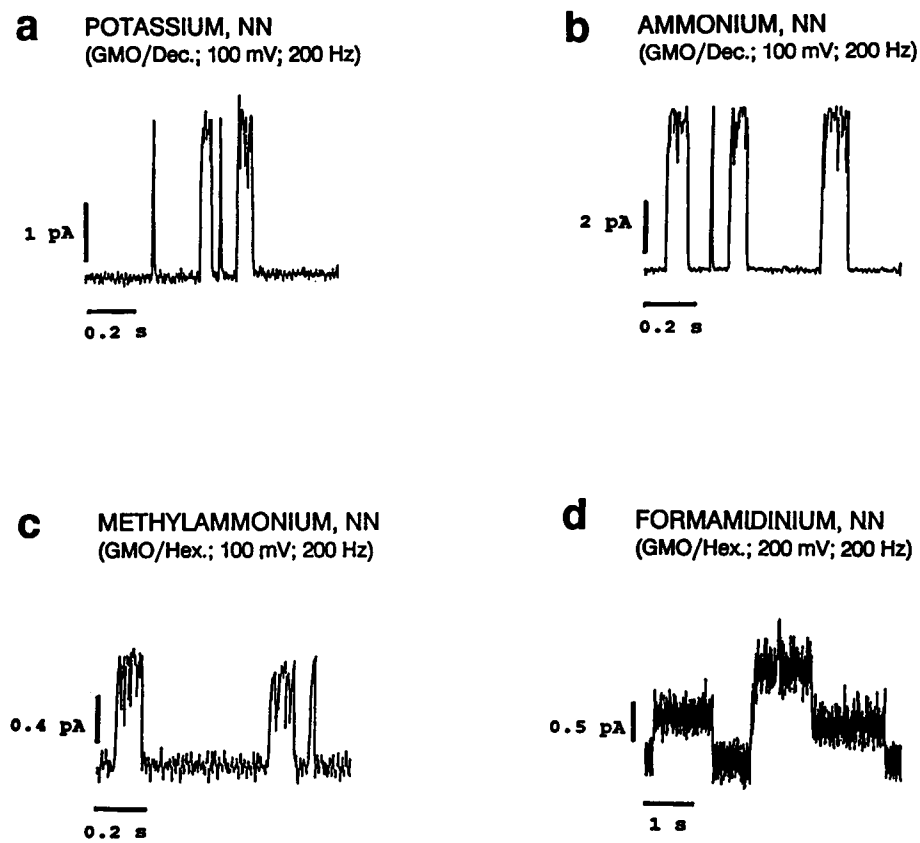

FIGURE 1 Single channel currents with 1 M salt solutions in NN at 100 mV (*a–c*) and at 200 mV (*d*) and at room temperature, 19.5–21°C. In parentheses the lipid, applied membrane potential, and the cut-off frequency are noted.

also below, Table 3, for quantification of the noise). The open state current magnitudes in 1 M K^+, NH_4^+, methylammonium, and formamidinium solutions at 100 mV in the NN channel were 2.9 ± 0.2 pA (n = 125), 6.6 ± 0.1 pA (n = 497), 0.8 ± 0.1 pA (n = 175), and 0.2 ± 0.0 pA (n = 784), respectively; (the data for each solution was collected from an average of five different membranes). K^+, methylammonium, and formamidinium yielded reduced current sizes compared to those in the GG channels whereas NH_4^+ currents were the same (see also the Table 1).

Single channel lifetimes for NN channels were much shorter than those of GG channels for all salt solutions. It was previously found that 1 M formamidinium solution produces approximately four to five times longer GG single channel lifetime than 1 M methylammonium or 1 M hydrazinium solutions (Seoh and Busath, 1993) in GMO/decane membranes at 100 mV. In NN channels, 1 M formamidinium solution produces approximately 7–15 times longer single channel lifetime (0.3 ± 0.1 s, n = 208) than does 1 M K^+ (0.02 ± 0.00 s, n = 324) or 1 M NH_4^+ (0.04 ± 0.01 s, n = 917) solutions in GMO/decane membranes at 100 mV (Fig. 3). 1 M formamidinium solution produces 7 times longer channel lifetimes than does 1 M methylammonium and 1 M K^+ in GMO/hexadecane membranes.

Fig. 2 shows the current-voltage relationship (*I-V*) for 1 M K^+, NH_4^+, methylammonium, and formamidinium solutions in NN channels. There is no significant supralinearity of the *I-V* in K^+, NH_4^+, and methylammonium solutions (this can be quantified using the ratio, G_{200}/G_{100} which was 1.02, 1.01, and 0.97 for the three solutions, respectively). The *I-V* of the channel in formamidinium solution, however, was supralinear (*inset* in Fig. 2) like in GG channels (Seoh and

TABLE 1 Open state current amplitudes (mean ± S.D., pA)

	K^+	Methylammonium	Formamidinium		NH_4^+
V(mV)	100	100	100	200	100
GG	4.5 ± 0.2	2.8 ± 0.0	1.6 ± 0.0	5.5 ± 0.1	(6.7)
NG and GN	4.2 ± 0.1	1.9 ± 0.0	0.8 ± 0.0	2.9 ± 0.2	
NN	2.9 ± 0.1*	0.8 ± 0.1*	0.2 ± 0.0	0.6 ± 0.1	6.6 ± 0.1*
I_{NN}/I_{GG}	0.6	0.3	0.1	0.1	1.0
I_{NG}/I_{GG}	0.9	0.7	0.5	0.5	

The comparison of current amplitudes and the presence of flickers (designated with *) of two heterodimers, GA-NAG (GN) and NAG-GA (NG), and two homodimers, NAG-NAG (NN) and GA-GA (GG). 1 M K^+, methylammonium, and formamidinium solution were used with GMO/hexadecane membranes at 100 mV. GMO/decane membranes were used with 1 M formamidinium solution where 200 mV was used and with 1 M NH_4^+ solution. For the cases with flickers the maximum current (open state) was measured. Each current is the average of 288–1131 standard channels. S.D. is the standard deviation from more than four experiments. The data in parentheses is from Urban et al. (1980).

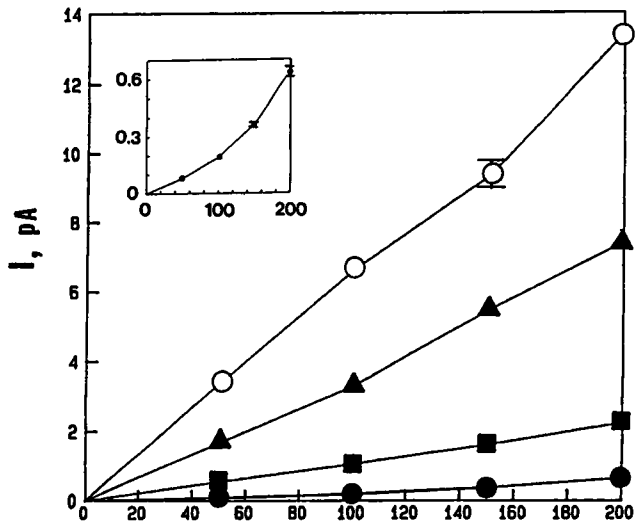

FIGURE 2 The current-voltage (*I-V*) relationship for NH_4^+ (*open circle*), 1 M K^+ (*filled triangle*), methylammonium (*filled square*), and formamidinium (*filled circle*) salt solutions in the NN channel in GMO/decane (for K^+, NH_4^+, and formamidinium solutions) and in GMO/hexadecane membranes (for methylammonium solution) at 25°C. The applied membrane potential was stepped from 50 to 200 mV. Each data point reflects the averaged standard single channel current averaged from 136 to 763 channels. The inset shows *I-V* relation in formamidinium solution on an expanded scale. Data points for K^+, NH_4^+, and methylammonium reflect mean maximum (open state) single channel currents. Standard error bars represent ±1 S.E. of the mean of at least six experiments for each point and are not shown when less than the symbol size.

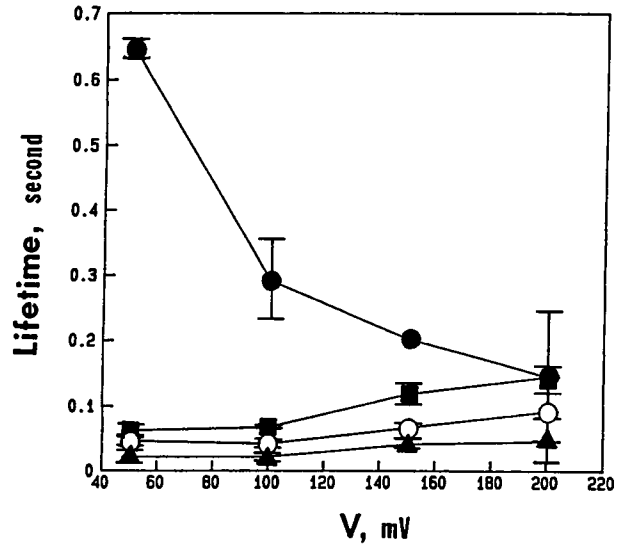

FIGURE 3 Average NN channel lifetimes in 1 M K^+, NH_4^+, methylammonium, and formamidinium solutions for GMO/decane (K^+, NH_4^+, and formamidinium solutions) or GMO/hexadecane membranes (methylammonium solution) as a function of membrane potential. Filled triangle: K^+ solution at 20.5°C ($203 < n < 635$); open circle: NH_4^+ solution at 21°C ($167 < n < 917$); filled square: methylammonium solution at 20°C ($179 < n < 874$); filled circle: formamidinium solution at 21°C ($147 < n < 208$). The vertical bars are %1 S.E. of the mean of at least three experiments except in two cases where the result of one experiment is shown without error bars.

Busath, 1993). G_{200}/G_{100} which was 1.68 for NN channels in 1 M formamidinium solution was not different, $G_{200}/G_{100} = 1.72$ in GG channels (Seoh and Busath, 1993). These observations indicate that the relative significance of the highly voltage-dependent translocation step is not altered in the open state of the NN channel.

Fig. 3 shows the average single channel lifetime in 1 M K^+, NH_4^+, methylammonium, and formamidinium solutions in NN channels with increasing applied membrane potential. Single channel lifetimes in 1 M K^+ and 1 M NH_4^+ solutions in GMO/decane membranes were 0.022 and 0.046 s at 50 mV, respectively, and increased approximately twofold to 0.046 and 0.09 s, respectively, at 200 mV. Similarly, the average lifetime in 1 M methylammonium solution in GMO/hexadecane membranes increased from 0.06 to 0.14 s. In contrast, the single channel lifetime in formamidinium solution decreased approximately four times from 0.65 to 0.14 s with the same voltage change (from 50 to 200 mV). The relative increase of single channel lifetimes in K^+, NH_4^+, and methylammonium solutions and the decrease in single channel lifetime in formamidinium solution with increasing applied membrane potential are very similar to those in GG channels (Seoh and Busath, 1993) although single channel lifetimes of NN channels are much shorter than those of GG channels. In addition, the NN channels in formamidinium solution show much longer lifetimes compared with other solutions, like GG channels. This suggests that the NN channel lifetimes are affected by two independent mechanisms, one of which is specific to formamidinium solutions, the other which is not. They are shortened due to dimer destabilization resulting from steric crowding between acetyl methyl groups at the center of the channel, whereas they are stabilized by binding of formamidinium.

The observations in NN channels of pronounced flickers in K^+, NH_4^+, and methylammonium solutions but not in formamidinium solution motivated hybrid experiments using two peptides. We hoped to find some clue for the mechanism of flickers in K^+, NH_4^+, and methylammonium solutions in the NN channel. The results are shown in Figs. 4-6. Fig. 4 shows single channel currents and the current amplitude histogram in 1 M potassium solution. In the symmetrical solution with only NAG on the two sides of the membrane (Fig. 4, *a* and *e*), there is only one conductance peak (NN) with mean current size of 2.9 ± 0.1 pA. After obtaining a very stable membrane in the symmetrical solution, the GA peptide was added to the grounded solution without breaking the membrane (see Methods). Because of the protocol only NG (or NAG-GA, denoting NAG peptide to be at the entry) hybrid channels were expected at the applied membrane potential at +100 mV and only GN (or GA-NAG, denoting NAG peptide to be at the exit) hybrid channels at −100 mV. The new peaks with mean current size of 4.2 ± 0.1 pA at +100 mV (Fig. 4, *b* and *f*) and 4.3 ± 0.1 pA at −100 mV (Fig. 4, *c* and *g*) were identified and assigned as NG and GN channels, respectively. These two hybrid channels had similar conductances and single channel lifetimes. Their conductances were similar to those of GG channels (4.5 ± 0.2 pA, Fig. 4, *d* and *h*), while their lifetimes were 4-fold shorter

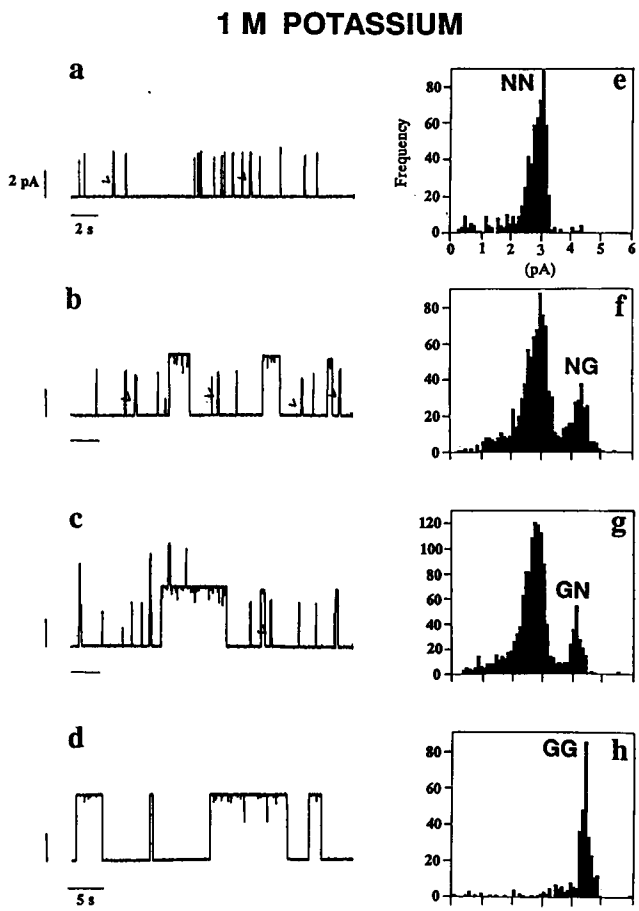

FIGURE 4 Current fluctuations and current amplitude histograms of two heterodimers (NG, GN) and two homodimers (NN, GG) in GMO/hexadecane membrane in 1 M potassium solution at 100 mV, 23°C. (*a* and *e*) Only NAG peptides were symmetrically applied to the both sides of the membrane. (*b* and *f*) GA peptides was next added to the grounded solution without breaking the membrane and a voltage of +100 mV was applied. (*c* and *g*) The same asymmetrical condition as *b* and *f* but a membrane potential of −100 mV was applied. (*d* and *h*) Only GA peptides were applied to both sides of a different membrane. All the data were collected at a sampling frequency of 600/s and the cut-off frequency of 200 Hz. NN channels in all the current traces showed frequent flickers on expanded scales as shown in Fig. 1 *a*. Peak statistics: (*e*) NN peak: 2.85 ± 0.08 pA (N = 496); (*f*) NN peak: 2.82 ± 0.14 pA (N = 695); NG peak: 4.25 ± 0.16 pA (N = 244); (*g*) NN peak: 2.68 ± 0.13 pA (N = 1000); GN peak: 4.16 ± 0.12 pA (N = 192); GG peak: 4.45 ± 0.11 pA (N = 264); (*h*) GG peak: 4.45 ± 0.11 pA (N = 264).

than those of GG channels (Table 2). In mixed solution with GA and NAG on both sides of the bilayer, these hybrid peaks could not be separated from the GG peak on the current histogram, even at 200 mV (data not shown).

Interestingly these two hybrid channels did not show the flickers which were obvious in NN channels. Arrows in Fig. 4, *a–c*, point to the (partially resolved) base of the flicker blocks in the NN channels. The blocks are so frequent that they produce a filled-in appearance which covers more than one half of the channel conductance height at this time scale (see also Fig. 1 *a* where the time scale is expanded 10-fold). Compared with the current traces of NG and GN channels, which have only occasional partial flickers like the GG channels in Fig. 4 *d*, the flickers in NN channels are considerably more prominent.

With the same protocol, the oriented hybrid experiment was performed in 1 M formamidinium solution. NN channels did not show flickers. The results are shown in Fig. 5. Fig. 5, *a* and *e*, show the initial results obtained with NAG only on both sides of the bilayer. When the GA was added to the grounded solution, a new peak with mean current size of 0.82 ± 0.02 pA appeared at +100 mV and was assigned as NG channels. Upon reversing the polarity to −100 mV the mean current size of the new peak was 0.80 ± 0.03 pA which was assigned as GN channels. Conductances (Table 1) and single channel lifetimes (Table 2) of the two hybrid channels were intermediate between those of the two homodimers. Like the NN homodimers the hybrid channels did not show flickers. In a completely mixed solution of the two peptides, three main peaks were identified. Two had the conductances of the NN and hybrid channels. The third peak with the highest conductance (1.6 ± 0.0 pA) had the same conductance as previously observed for GG channels (Fig. 2 and Seoh and Busath (1993)).

Fig. 6 shows the oriented hybrid experiment in 1 M methylammonium solution. After identifying the GG peak in the symmetrical solution with GA (Fig. 6, *a* and *d*), NAG was added to the grounded solution (Fig. 6, *b* and *e*). The GN peak was identified as before. The hybrid channel has an intermediate conductance (Table 1) and single channel lifetimes (Table 2) and did not show flickers. NG channels behaved similar to the GN channels (data not shown). In the symmetric mixed solution, three main peaks were found, the new peak having the conductance illustrated for NN channels in Fig. 1 *c*.

Table 1 summarizes the results for current amplitudes and the presence of flickers in 1 M K^+, methylammonium, formamidinium, and ammonium solution. The appearance of flickers was easily discerned in the raw data and was also analyzed quantitatively using the channel current noise. Interestingly flickers for K^+ and methylammonium appeared only in the NN channel, not in hybrid channels. In formamidinium solution, flickers were negligible in all types of channels. The current sizes for NN channels are the same as those of GG channels in ammonium solution, while I_{NN}/I_{GG} in 1 M K^+, methylammonium, and formamidinium solutions are 0.6, 0.3, and 0.1, respectively (Table 1). The ratios for K^+, methylammonium, and formamidinium are approximately proportional to the inverse of the ionic radius. Ammonium represents an exception: it's ionic crystal radius is ~12% greater than that of potassium (Hille, 1992), yet it's conductance is not decreased in the NN channel.

The current sizes for hybrid channels in potassium solution are similar to those of GG channels, while those in methylammonium and formamidinium solutions are approximately one half those of GG channels. The current sizes of hybrid channels for ammonium were not measured, but can be expected to have the same conductances as those of GG channels and NN Channels, which have the same as each other.

TABLE 2 Mean channel lifetimes (mean ± S.D., s)

	K^+	Methylammonium	Formamidinium	Ammonium
GG	6.4 ± 1.6	1.5 ± 0.4	11.5 ± 0.9	3.2 ± 0.4
NG and GN	1.5 ± 0.3	0.6 ± 0.2	5.0 ± 2.0	
NN	0.1 ± 0.0	0.1 ± 0.0	0.8 ± 0.2	0.1 ± 0.0
τ_{NN}/τ_{GG}	0.02	0.07	0.07	0.02
τ_{NG}/τ_{GG}	0.23	0.43	0.43	

A comparison of the mean single channel lifetimes (s) in 1 M K^+, methylammonium, or formamidinium solution and 0.1 M ammonium solution in GMO/hexadecane membranes at 100 mV. Each value is the average of 160–621 channels. Each standard deviation was obtained from at least three (up to 12) different experiments. The temperature ranged from 21 to 23°C.

The single channel lifetimes of heterodimers and homodimers in 1 M K^+, methylammonium, and formamidinium solutions and 0.1 M ammonium solutions are summarized in Table 2. The single channel lifetime of NN channels in 1 M formamidinium solution is approximately 7-fold longer than those in potassium and methylammonium solution. The magnitude of the NN channel-stabilization effect in the formamidinium solution (Seoh and Busath, 1993) is similar to that in GG channels. For hybrid channels single channel lifetimes are only two to four times shorter than those in GG (τ_{NG}/τ_{GG} = 0.23–0.43). In summary, conductances and single channel lifetimes of hybrid channels are similar to those of GG rather than NN, and furthermore they are similar in that they do not have obvious flickers (Table 1). It is, however, obvious that NN channels have very different conductance levels, single channel lifetimes, and flickers from those of GG channels.

One method of analyzing the flicker blocks is to utilize high bandwidth measurements to measure flicker frequency and duration. Because the bandwidth in our measurements was not sufficient for such measurements, we use an alternative measure of flicker occurrences, the open channel noise (described in Methods). Table 3 contrasts the noise in GG channels and NN channels. In GG channels, the noise is low with K^+ and methylammonium solutions. In formamidinium solutions, it is high at 100 mV, but reduces to a level similar to the other two solutions at 200 mV. The noise in NN channels is measurably higher than in GG channels for K^+ and methylammonium. In formamidinium, the noise, though significantly higher than in GG channels, is much lower than for the other two ions in NN channels.

DISCUSSION

The results presented here indicate that the NN conductance is reduced by 90% compared to GG in formamidinium solution but not reduced in ammonium solution, that the *I-V* shape is the same for organic cations and K^+ in the open state of NN channels as in GG channels, and that the lifetimes of NN channels are reduced when compared to GG but have the same voltage-dependent stabilization by formamidinium. The presence of two acetyl methyl groups at the dimeric junction is required for flickers and the very low stability in NN channels. All of these effects can be explained simply in terms of the acetyl methyl group which, in NAG, replaces the formyl hydrogen of GA.

Conductance and I-V of NAG

The variations in single-channel conductances, which are related to changes in the ion and water mobility in the channel, reflect changes in the energy barriers for ion and water movement through the channel (Läuger, 1973; Russell et al., 1986; Roux and Karplus, 1991). NAG channels are more selective than GA channels for the monovalent cations reported here (I_{NN}/I_{GG} are 1.0, 0.6, 0.3, and 0.1, respectively, for 1 M NH_4^+, K^+, methylammonium, and formamidinium solutions (Table 1)) indicating that they are slightly more constricted in the interior.

The single channel current-voltage characteristics for 1 M K^+ and methylammonium solutions were linear like those in *N*-formyl GA and the *I-V* in 1 M formamidinium solution was supralinear like that in *N*-formyl GA channels. Either the acetylation caused a uniform decrease of kinetics for the various steps of ion passage, or it caused a localized decrease in only one of the steps which, alone, isn't very voltage-dependent. We can safely say that the *N*-methylation did not cause a graded decrease in kinetics (increasing barriers from the exterior to the center of the bilayer), because a graded decrease should increase the supralinearity of the *I-V*. It seems most logical from a structural point of view to suppose that the *N*-methylation causes a very localized constriction at the center of the channel.

NN and GG channels have the same conductance in 1 M NH_4^+ solution even though NN channels have obvious flickers. On the other hand, the NN channel conductance is 10-fold lower than the GG channel conductance in 1 M formamidinium solution, but the NN current doesn't show flickers like in other solutions. Thus, the constriction in the open NN channel which confers increased selectivity does not inevitably predispose to flicker blocks. However, at this stage, we can only suppose that both the conductance changes and the flickers are mediated by the acetyl methyls.

Single channel lifetimes of NN channels

Apell et al. (1979) suggested that one additional pair of hydrogen bonds between two carboxyl groups in *N*-glutaryl gramicidin channels could explain the ~30-fold increase in single channel lifetime over that of GA. In contrast, the average lifetime of the *N*-acetyl homodimer, NN, is 15–50-fold shorter than that of GG homodimer (Table 2 and Szabo and Urry (1979)). The lower stability of NN suggests that fewer

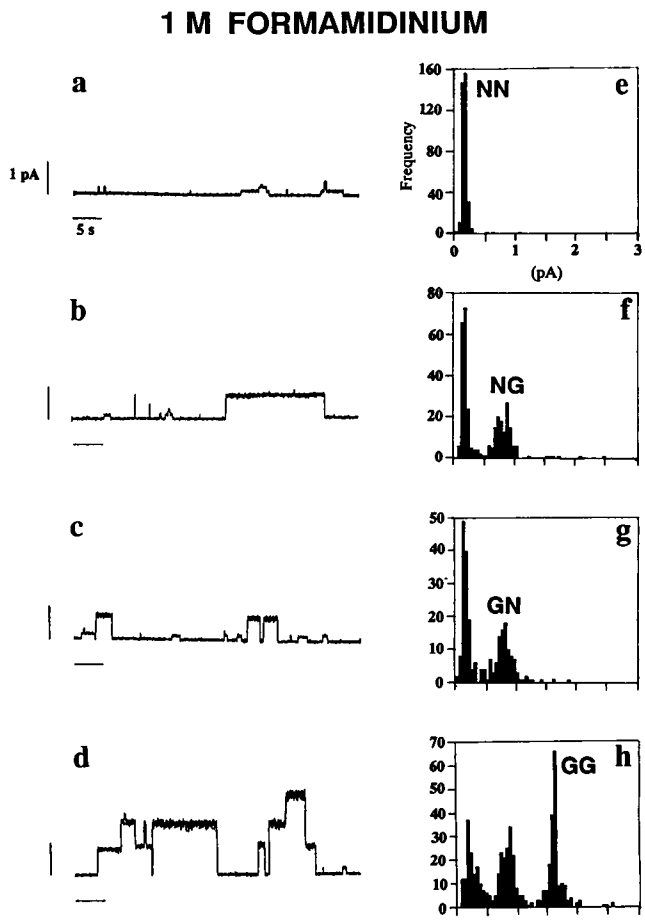

FIGURE 5 Single channel currents and the current amplitude histogram using NAG and GA with GMO/hexadecane membranes in 1 M formamidinium solution at 100 mV, 21.5–22°C. (*a* and *e*) Only NAG was symmetrically applied to both sides of the membrane; (*b* and *f*) GA was next added to the grounded solution without breaking the membrane and the voltage of +100 mV was applied; (*c* and *g*) A membrane potential of −100 mV was next applied; (*d* and *h*) two different peptides, GA and NAG, were added symmetrically to both sides of a different membrane. All the data were collected with sampling frequency of 100/s and the cut-off frequency of 30 Hz. The reason for the low cut-off frequency was to improve the ratio of signal to noise with the low conductance levels of NN channels in the formamidinium solution. Peak statistics: (*e*) NN peak: 0.202 ± 0.002 pA (N = 375); (*f*) NN peak: 0.181 ± 0.005 pA (N = 198); NG peak: 0.803 ± 0.019 pA (N = 138); g) NN peak: 0.167 ± 0.004 pA (N = 126); GN peak: 0.797 ± 0.020 pA (N = 98); (*h*) NN peak: 0.241 ± 0.012 pA (N = 135); NG peak: 0.780 ± 0.016 (N = 159); GG peak: 1.606 ± 0.024 pA (N = 184).

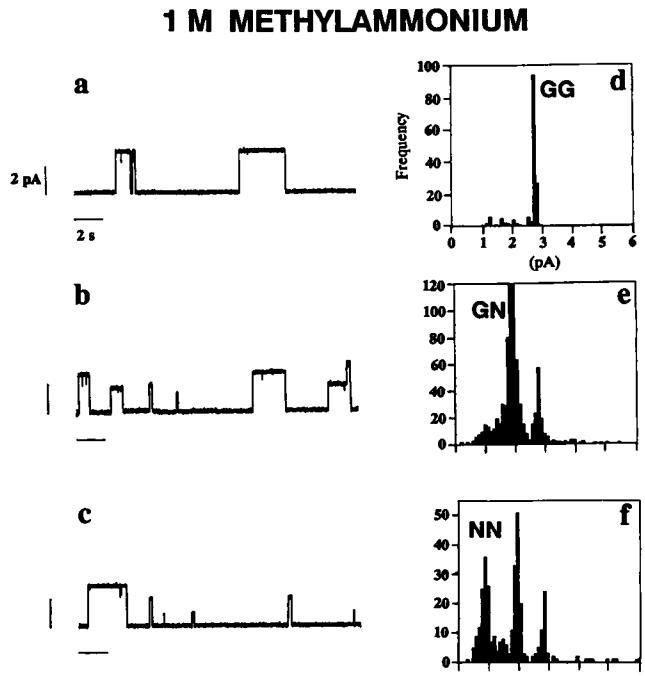

FIGURE 6 Current fluctuations and current amplitude histograms of heterodimers and homodimers in GMO/hexadecane membranes, 1 M methylammonium solution at 100 mV, 21°C. (*a* and *d*) GA was symmetrically applied to the both sides of the membrane; (*b* and *e*) NAG was next added to the grounded solution without breaking the membrane and a membrane potential +100 mV was applied; (*c* and *f*) GA and NAG were added symmetrically to both sides of the membrane. All the data were collected with a sampling frequency of 600/s and a cut-off frequency of 200 Hz. NN channels which have the lowest conductance in current trace of *c* showed well distinguished flickers on expanded scales. Peak statistics: (*d*) GG peak: 2.76 ± 0.06 pA (N = 132); (*e*) GG peak: 2.77 ± 0.08 pA (N = 141); GN peak: 1.93 ± 0.04 pA (N = 397); (*f*) GG peak: 2.80 ± 0.17 pA (N = 52); GN peak: 1.92 ± 0.04 pA (N = 123); NN peak: 0.84 ± 0.04 pA (N = 133).

TABLE 3 Open channel noise, S' (10^{-4} s^{-1})

	Potassium		Methylammonium		Formamidinium	
V	GG	NN	GG	NN	GG	NN
mV						
100	0.05	2.22	0.01	1.36	0.40	0.53
200	0.03	1.69	0.01	1.47	0.02	0.14

A comparison of the open channel noise in 1 M K^+, methylammonium, or formamidinium solutions in GMO/hexadecane membranes at 100 mV. Each value is the average of 15–38 channels obtained from at least two different experiments. The standard deviation in S' was of the same magnitude as the mean in each case and the mean value of S' was similar from day to day when experiments on different days were performed. The temperature ranged from 21 to 23°C.

(perhaps ~5) intermolecular hydrogen bonds sustain the dimer structure.

In formamidinium solution, NN channels are stabilized compared to in other solutions and lifetimes have the same dependence on membrane potential (Fig. 3 and Table 2). We speculate that the stabilization effect of formamidinium must occur away from the center of the channel, because the pattern of behavior would otherwise be expected to vary dramatically upon methylation of the amino terminus. Furthermore, formamidinium would probably be more likely, if anything, to disrupt hydrogen bonds at the center of the channel, rather than stabilize them, particularly in NN channels which appear from their selectivities to be slightly constricted at the center. In a prior analysis (Seoh and Busath, 1993), we suggested that formamidinium stabilization may be mediated by interactions with the channel backbone in an interior binding site in such a way as to affect the Trp side chain positions, enhancing Trp hydrogen bonding with the lipid bilayer. We also observed that Ag^+ and Tl^+ produced

strong lifetime stabilization in GG channels (data not shown) as has been reported by McBride (McBride, 1981; Andersen and Procopio, 1979). By our measurements the magnitude of relative Ag^+ stabilization of NN channels was similar to that of GG channels (by comparison to K^+ solution).

Conductances and single channel lifetimes in hybrid channels

In formamidinium and methylammonium solution conductances and single channel lifetimes of heterodimers are intermediate between those of homodimers. In K^+ solution, the conductances of hybrid channels are the same as those of GG channels, while those due to larger cations, like methylammonium and formamidinium, are intermediate between those of the two homodimers. The hybrid behaves as though it has a slight narrowing which does not affect K^+ flux but does affect the flux of the larger organics.

Single channel lifetimes in hybrid channels are similar to those of GG channels rather than NN channels. Single channel lifetimes with hybrid channels in 1 M potassium, methylammonium, and formamidinium solution imply that one methyl group at the dimeric junction is not enough to disrupt the hydrogen bonds at the dimeric junction.

The mechanism of flicker in NN dimers

The duration and the frequency of flickers in NN channels are insensitive to membrane potential and membrane lipid composition (Sigworth and Shenkel, 1988; Szabo, 1981; Seoh and Busath, unpublished data). The underlying mechanism of flickers in NN channels is thus different from that of gaps observed with GG channels (GG) which have ~1-ms durations in GMO/decane membrane, decrease with voltage, and are considered to be an intermediary conformation before reassociation of the dimer (Ring, 1986). The flicker frequency in NN channels is 100-fold higher than that in GG (Sigworth and Shenkel, 1988) but much lower than the ion hopping rate. The flickers in NN channels may come from the steric block caused by the methyl group as it rotates deeply into the center of the channel. Flickers appear only with homodimeric NAG-NAG channels. The fact that flickers occur in only NN channels suggests that one methyl pushes the other into the channel to form the blocked state. This conclusion was further supported by molecular mechanics described next.

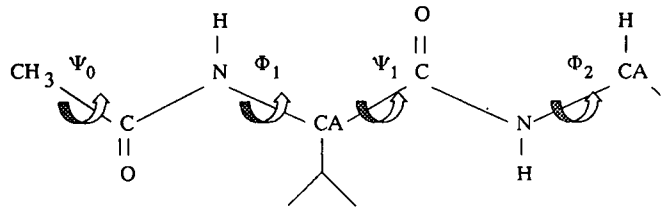

FIGURE 7 The amino-terminal backbone structure of NAG. Rotations about bonds labeled (Φ) and (Ψ) are expected, whereas rotations about peptide bonds (unlabeled) are not, due to resonance stabilization. (Ψ_0) rotations are of minor consequence because of the tetrahedral symmetry and small atomic radius of the hydrogens affected.

To evaluate the mechanics of the dimer junction, we used the CHARMM force field (Brooks et al., 1983) and computed the potential energies of optimized structures for the GG, GN/NG, and NN dimers. When the GG channel is mutated to NN by replacing the formyl hydrogens with methyl carbons and no additional conformational changes, the potential energy is 6.2×10^5 kcal/mol due to van der Waals contact between the two methyls. A conformational search using the first three relevant backbone dihedrals, Φ_1, Ψ_1, and Φ_2 (Fig. 7), indicated that there are two major stable conformations in the NN channel where this steric conflict is relieved, one with both acetyl methyls outside the average C_α diameter and one with one of the acetyl methyls inside the channel and the other out.

Table 4 gives the properties of GG, GN (=NG) and these NN structures, designated NN-o and NN-b1, respectively, including the radial positions of the acetyl methyl carbons, the number of hydrogen bonds in the dimer junction, the

TABLE 4 Molecular mechanics of GG, GN, and NN channels

Structure	Radial Positions*		Hydrogen-bonds‡	Energy§	Dihedral angles‖		
					Φ_1	Ψ_1	Φ_2
	Å			kcal/mol			
GG	3.4 H	3.1 H	6	0.0	−90	100	170
GN	4.4 C	2.9 H	6	0.1	−90	90/110¶	170
NN-o	4.6 C	4.3 C	6	6.2	−100	110	170
NN-b1	1.1 C	5.0 C	6	−2.8	−80	90	150
NN-b2	0.5 C	3.7 C	5	4.7	30	50	160
NN-b3	1.2 C	4.9 C	4	−1.1	0	90	140

*Distance from the channel axis of the formyl aliphatic hydrogen (H) or of the carbon of the acetyl CH_3 (C).
‡H—O length < 2 Å.
§Total dimer potential energy (in vacuo) after 300 steps adopted basis Newton-Raphson energy minimization relative to that of the GG channel. Final RMS force, 0.1–0.5 kcal/Å.
‖In degrees, rounded to the nearest decade. For NN-b structures, only the angles of the inward projecting NAG are given. For GG and NN-o, the angles for the two monomers were within 5° except for the case of Ψ_1 which varied by as much as 13°. Here the mean is represented.
¶The GA angle is listed first, NAG second. For the other two dihedrals, GA and NAG are approximately equal.

local-minimum potential energy (relative to that of the GG channel), and the backbone dihedral angles of interest. The average C_α radial position for residues 3–14 is 3.8 Å. Thus NN-o, with both acetyl carbons out, by virtue of a −10° rotation in Φ_1, is an open channel. NN-b1 is a blocked state with one methyl 1.1 Å from the channel axis by virtue of 10–20° rotations of the first three relevant backbone dihedrals. There are two additional variants of the one-acetyl blocked state (NN-b2 and NN-b3) having increasing degrees of the acetyl peptide plane rotation. NN can also be constructed with both acetyls projecting symmetrically into the channel (similar to the blocking acetyl of NN-b2), but the structure has a high potential energy, +40 kcal/mol relative to GG, so we did not consider it further.

GN is stable without outward or inward rotations of the NAG acetyl. When GN is constructed from NN-b1 by mutating the inward pointing methyl to hydrogen, minimization causes the inward pointing group to rotate back to the flush position, indicating no metastable blocked state for the GN channel, consistent with the observed lack of flicker blocks. The energy of GN is similar to that of GG, reflecting the lack of steric conflict between the acetyl methyl and the formyl hydrogen, although the acetyl methyl and formyl hydrogens do overlap slightly requiring that one be slightly out (4.4 Å) and the other slightly in (2.9 Å).

The open state of NN (NN-o) has a higher potential energy than GG, suggesting that the NN dimer would be destabilized compared to GG and thus yield shorter channel lifetimes. Energy decomposition revealed that bonds and angles were stretched in this structure due to the close contacts of the acetyl methyls. NN-b1 has a lower potential energy than NN-o, the open state, an inimical result, because the open time has been measured in NN channels to be 3.3-fold (Sigworth and Shenkel, 1988) or 6.0-fold (Szabo, 1981) greater than the block time, indicating that the free energy of the blocked state is higher by 0.7–1.1 kcal/mol than the open state. But it must be noted that our computations contained the channel alone. The separation of waters in the channel by the acetyl methyl would increase the water-water interaction energy by 3–6 kcal/mol to yield a net relative potential energy of 0.2–3.2 kcal/mol for the first blocked state. In addition, there may be a dynamical effect due to water and ion flow and/or a thermodynamic effect due to pressure from an ion in the channel under the force produced by the membrane field which destabilize the blocked state.

The structures of NN-o and NN-b2 (in which the peptide plane of the blocking acetyl has rotated about 120° from parallel to the axis) are shown in Fig. 8. NN-b2 is reasonably representative of all NN-b3-blocked states in that it illustrates the occlusion of the channel. The second and third blocked states have fewer intermolecular hydrogen bonds, five and four, respectively, and consequently have monomer-monomer interaction energies (relative to that of GG) of +10.7 and +15.0 kcal/mol, respectively. From either of these states, the channel would be likely to dedimerize. Thus NN-b1 is most likely to be the principal intermittent block state. In summary, the molecular mechanics computations suggested that GN channels would not be likely to undergo blocks, as we observed, and they provide reasonable coordinates for open and blocked states in NN channels, as well as suggesting a logical reaction coordinate for future molecular dynamics computations.

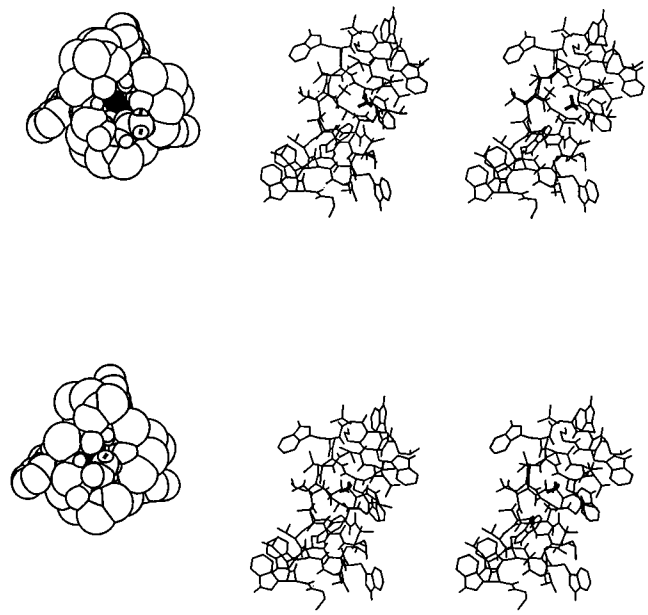

FIGURE 8 On the left are Van der Waals plots of the right-handed $\beta^{6.3}$ NAG helix viewed from the amino-terminal (*upper*) in the "flush" conformation (acetyl methyl carbon at ~3.7 Å from the channel axis) and (*lower*) in the "in" conformation (NN-b2 in Table 4). The acetyl methyl hydrogens are labeled and the pore space is black. The pore is occluded in the "in" conformation (*lower*) and would correspond to the flicker block state. On the right are stereo wireframe-tilted views (left-eye image on the left) of the NN channel in the (*upper*) open conformation and (*lower*) the second blocked (NN-b2) conformation. The acetyl methyl group in the upper monomer is highlighted.

The relation of flickers and single channel lifetimes in heterodimers and homodimers to the number of hydrogen bonds at the dimer junction

Gramicidin A channels are dimers with six intermolecular hydrogen-bonds at the junction. Two of them come from the two formyl groups in each monomer according to Urry's model (Urry, 1971) as confirmed by Arseniev's model (Arseniev et al., 1990). Szabo and Urry (1979) suggested that in the NN channels there is an oscillation between head-to-head dimeric structures of six intermolecular hydrogen bonds (an open state that is destabilized by crowding between the acetyl methyl groups) and four intermolecular hydrogen bonds (a closed state where the crowding between methyl groups is relieved by a conformational change that briefly terminates conductance).

We observed that, in formamidinium solution, NAG-containing channels do not undergo transitions to the closed state. Yet, the average single channel lifetime of the NN channel in the solution was ~15-fold shorter than that of the GG channel (Table 2). This is consistent with the hypothesis

that the open state is destabilized by methyl-methyl crowding. Evidently the methyl-methyl crowding is very important in destabilizing the dimer. This is especially well-demonstrated with formamidinium where no flicker blocks are observed: destabilization is primarily a property of the open state and not a consequence of the conformational change that produces the flicker block.

From our data then, a more complete picture of the behavior of the *N*-acetyl gramicidin A channel emerges. 1) In heterodimers when the one acetyl methyl groups is flush with the backbone, the channel is in the open state. In this position, an intermolecular hydrogen bond is probably stretched producing the 2- to 4-fold decrease in channel lifetime. 2) In NN channels the methyl-methyl crowding reduces the lifetime by a factor of 15–50, consistent with an energy change of about 3–4 *RT*, suggesting that the average number of intermolecular hydrogen bonds is reduced to approximately five whether there are flickers or not. 3) The flicker block state must not be the source of dimer destabilization.

Implications of single channel noise measurements: formamidinium-induced noise is independent of flicker block noise

There is evidence in the single channel noise reported in Table 3 that there are two independent conformational fluctuations in NN channels when formamidinium is the permeant ion: one which is also observed with formamidinium in GG channels (Seoh and Busath, 1993) and is eliminated at 200 mV, and an attenuated flicker block noise associated with the acetyl rotations in NN channels. The noise term, S', drops in GG channels with formamidinium from 0.40×10^{-4}/s at 100 mV to 0.02×10^{-4}/s at 200 mV, which is the same order of magnitude as we observed in GG channels with potassium and methylammonium at either potential ($0.01–0.05 \times 10^{-4}$/s).

On the other hand, NN channels have flicker blocks reflected in the noise with potassium and methylammonium and shown there to be essentially voltage-independent ($1.36–2.22 \times 10^{-4}$/s). The S' then for NN channels in formamidinium at 200 mV should reflect only this flicker noise if the two noise processes are independent. Although the noise in this case, 0.14×10^{-4}/s is 10-fold lower than the flicker noise in potassium or methylammonium, it is nevertheless 7-fold higher than the noise induced by formamidinium in GG channels at this potential, and we ascribe the excess to acetyl fluctuation in NN. If the flicker noise and the formamidinium-induced noise result from independent conformational fluctuations, the noise in NN channels at 100 mV (0.53×10^{-4}/s) should be the sum of the formamidinium-induced noise (0.14×10^{-4}/s) and the voltage-independent NN-acetyl flicker block noise (0.40×10^{-4}/s), which it is. The conclusion that the two types of noise are independent is also supported by the observation that flicker noise in the NN channels (Fig. 1, *a–c*) has a visibly slower time course than the formamidinium-induced noise in the GG channels (Fig. 5 *d*), although our limited frequency resolution precludes any definitive statement in this regard.

The finding that the flicker block noise in NN channels is 10-fold lower with formamidinium than with potassium or methylammonium suggests a specific interaction between the permeating cations and the NAG acetyl groups. Both formamidinium and methylammonium have eight atoms, but in formamidinium, three are large atoms (two nitrogens and one carbon), and the eight atoms are coplanar arranged around a trigonal carbon. The maximum dimension of the minimal profile is 4.5 Å (Hemsley and Busath, 1991). Methylammonium consists of two tetrahedral atoms and has a minimal-profile maximum dimension of only 3.3 Å, similar to that of potassium. It may be that passage of the large formamidinium ions sterically inhibit formation of the blocked state in NN dimers.

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