Distinction between Dipolar and Inductive Effects in Modulating the Conductance of Gramicidin Channels†

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ABSTRACT: The ion permeability of transmembrane channels formed by the linear gramicidins is altered by amino acid sequence substitutions. We have previously shown that the polarity of the side chain at position one is important in modulating a channel's conductance and ion selectivity [Russell et al. (1986) *Biophys. J. 49*, 673–686]. Changes in polarity could alter ion permeability by (through-space) ion–dipole interactions or by (through-bond) inductive electron shifts. We have addressed this question by investigating the permeability characteristics of channels formed by gramicidins where the NH_2-terminal amino acid is either phenylalanine or one of a series of substituted phenylalanines: *p*-hydroxy-, *p*-methoxy-, *o*-fluoro-, *m*-fluoro-, or *p*-fluorophenylalanine. The electron-donating or -withdrawing nature, as quantified by the Hammett constant, ranges from −0.37 to +0.34 for these side chains. Channels formed by these gramicidins show a more than 2.5-fold variation in their Na^+ conductance, but the conductance variations do not rank in the order of the Hammett constants of the side chains. Inductive effects cannot therefore be of primary importance in the modulation of the gramicidin single-channel conductance by these side chains. The results support previous suggestions that electrostatic interactions between side chain dipoles and permeating ions can modify the energy profile for ion movement through the gramicidin channel and thus alter the conductance.

The linear gramicidins are a family of polypeptides that form well-defined ion channels that are ideally selective for monovalent cations [for reviews, see Andersen (1984) and Hinton and Koeppe (1985)]. The parent [Val^1]gramicidin A molecule, isolated from *Bacillus brevis*, has the following amino acid sequence (Sarges & Witkop, 1965a): formyl-L-Val^1-Gly^2-L-Ala^3-D-Leu^4-L-Ala^5-D-Val^6-L-Val^7-D-Val^8-L-Trp^9-D-Leu^{10}-L-Trp^{11}-D-Leu^{12}-L-Trp^{13}-D-Leu^{14}-L-Trp^{15}-ethanolamine. In addition, there exist two naturally occurring mutants: gramicidin B, with a phenylalanine at position 11, and gramicidin C, with a tyrosine at 11 (Sarges & Witkop, 1965b). This functional and structural simplicity makes the gramidicin channel well-suited for examining general aspects of structure–function relations in membrane proteins.

The peptides are very hydrophobic (Segrest & Feldman, 1974). When inserted into lipid bilayers, the ion-conducting channels are formed as (formyl-NH-terminal)-to-(formyl-NH-terminal) dimers of single-stranded $\beta^{6.3}$-helical monomers (Urry, 1971; Urry et al., 1971; Finkelstein, 1974; Bamberg & Janko, 1977; Bamberg et al., 1977; Veatch & Stryer, 1977; Weinstein et al., 1980, 1985; Urry et al., 1983; Boni et al., 1986). The hydrophobic side chains form the exterior surface of the channel, while the polar polypeptide backbone lines a lumen with a diameter of 0.4 nm that readily accommodates partially dehydrated alkali metal cations (Venkatachalam & Urry, 1983; Koeppe & Kimura, 1984; MacKay et al., 1984; Jordan, 1987; Pullman, 1987).

In β-helical dimers, the side chains are not in direct contact with the permeating ions. Molecular models of the gramicidin channel show that the side chains are positioned at distances ranging from 0.5 to 1 nm from the ion permeation path (Venkatachalam & Urry, 1983). Despite this separation, one finds that chemically produced side chain substitutions can significantly affect the rate of ion permeation through the mutant channels [for reviews, see Andersen et al. (1987) and Koeppe and Andersen (1987)]. Importantly, these conductance variations do not result from gross alterations of channel structure (Mazet et al., 1984; Durkin et al., 1986; Russell et al., 1986); they instead reflect side chain dependent modulation of the energy profile for ion permeation. The modulation could result from two mechanisms: "through-space" electrostatic interactions between the side chain dipoles and permeating ions and "through-bond" inductive electron redistributions, where electron-donating or -withdrawing side chains alter the charge density on the carbonyl oxygens that solvate the permeating ions. Either mechanism, or a combination, will alter the energy profile for ion movement through the channel. In this paper, we address the relative importance of these two mechanisms through investigations on channels formed by a series of gramicidins having phenylalanine derivatives of different electron-donating or -withdrawing power as the formyl-NH-terminal amino acids.

To avoid single-channel conductance changes due to steric modifications, a set of side chains with similar geometry was

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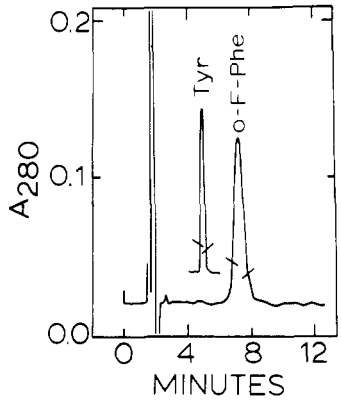

FIGURE 1: Examples of the final purification of semisynthetic [X-Phe[1]]gramicidins by reversed-phase HPLC. The tracings represent the second passage of the *p*-OH and *o*-F analogues through the octylsilica column. The peaks were pooled as indicated, diluted with ethanol, and used directly for single-channel studies.

required. Isosteric pairs of nongenetic amino acids had proven useful for this purpose previously [e.g., see Russell et al. (1986)]. For the present study, [Phe[1]]gramicidin A (rather than the naturally occurring [Val[1]]gramicidin A) was chosen as the reference compound because channels formed by [Phe[1]]gramicidin A are structurally equivalent to [Val[1]]-gramicidin A channels (Mazet et al., 1984; Durkin et al., 1989). Additionally, [Phe[1]]gramicidin A and [Val[1]]gramicidin A channels have comparable conductances: In 1.0 M NaCl, the single-channel conductances are 10.1 and 12.5 pS, respectively (Mazet et al., 1984). Finally, the inductive and dipolar characteristics of the first side chain of [Phe[1]]gramicidin can be easily altered by substitutions on the ring.

Some of this material has been presented in preliminary form (Koeppe et al., 1984).

Materials and Methods

Chemical Modification. Commercial mixtures of linear gramicidins were deformylated, and the N-terminal amino acid was removed as described previously (Morrow et al., 1979; Weiss & Koeppe, 1985). The amino acids were formylated with formic–acetic anhydride (Weiss & Koeppe, 1985), recrystallized from ethyl acetate, and coupled to 2-mg samples of a des(formylvalyl)gramicidin (A + C) mixture using stoichiometric amounts of diphenylphosphoryl azide and triethylamine in dimethylformamide (Weiss & Koeppe, 1985). Aliquots of the crude coupling reaction mixtures were diluted with 10–15 volumes of methanol and injected directly onto an octylsilica reversed-phase HPLC column. The gramicidin A and C components for each of the semisynthetic analogues were collected by hand and repurified by a second passage on the same HPLC column, as previously described (Weiss & Koeppe, 1985). Examples of the collection of the final samples, and of their chemical purity, are given by the HPLC tracings shown in Figure 1. The resulting solutions of gramicidin analogues in ~80% methanol + 20% water (2–5 μg/mL) were diluted into 10–100 volumes of ethanol and used for the single-channel experiments.

The hydroxy- and methoxyphenylalanines were formylated and coupled as L-amino acids, with minimal racemization (Weiss & Koeppe, 1985). The *o*-, *m*-, and *p*-fluorophenylalanines were formylated and coupled as the racemic DL mixtures. Mixtures of gramicidins having either D or L amino acids at position 1 were thus produced. This should not be a problem for the single-channel work. Indeed, on the one hand, pure [D-Val[1]]- or [D-Phe[1]]gramicidin A does not appear to form channels (Morrow et al., 1979; Weiss & Koeppe, 1985).[1] On the other hand, mixtures of gramicidins that have either D or L isomers of the fluorophenylalanine at position 1 exhibit histograms of single-channel events with single narrow major peaks (Figure 3), which argues that the D isomer at position 1 in each case does not contribute to the single-channel events.

Single-Channel Measurements. Planar lipid bilayer membranes were formed at 25 ± 1 °C using the pipet technique of Szabo et al. (1969) across a hole (area ~1.6 mm^2) in a Teflon partition separating two Teflon chambers containing 5 mL of unbuffered 0.1 or 1.0 M NaCl or CsCl. The membrane-forming solution was diphytanoylphosphatidylcholine dissolved in *n*-decane, 2–3% w/v. A 5–20-μL aliquot of an ethanolic dilution of one of the gramicidin analogues was added to each aqueous phase after membrane formation. The final peptide concentration was in the range of 10–100 pM.

The single-channel measurements were done by using the "bilayer punch" (Andersen, 1983). The reported small-signal conductances were in most cases based on measurements at 25 and 50 mV, at least four measurements (two at each polarity) at each potential. The average conductances were calculated as weighted averages of the conductances in the individual experiments, using the number of channels in the experiment as the weight function. Average single-channel durations were estimated from lifetime histograms assembled from stripchart recordings as previously described (Russell et al., 1986).

Materials. Linear gramicidin (gramicidin D), diphenylphosphoryl azide, L-phenylalanine, L-tyrosine (*p*-HO-Phe), and *o*-, *m*-, and *p*-fluoro-DL-phenylalanine were from Sigma Chemical Co. (St. Louis, MO). L-Tyrosine *O*-methyl ether (*p*-CH_3O-Phe) was from Vega Biochemicals (Tuscon, AZ). Methanol (HPLC grade) and acetic anhydride were from Fisher Scientific Co. (Memphis, TN). Ethanol was from U.S. Industrial Chemicals (Tuscola, IL). Formic acid, 95–97%, was from Aldrich Chemical Co. (Milwaukee, WI). Phenyl isothiocyanate, pyridine, dimethylformamide, triethylamine, and 4 N HCl in dioxane were "Sequanal" grade from Pierce Chemical Co. (Rockford, IL). A prepacked HPLC column of "Zorbax-C8", a 5 μM octyl reversed-phase spherical packing, was obtained from Dupont Corp. (Wilmington, DE).

Diphytanoylphosphatidylcholine from Avanti Polar Lipids (Birmingham, AL) was purified by ion-exchange chromatography (Andersen, 1983). *n*-Decane from Wiley Organics (Columbus, OH) was used without further purification. NaCl and CsCl (Suprapur grade) from E. Merck Darmstadt (through MCB, Cincinnati, OH) were roasted for at least 24 h at 550–600 °C before use and stored in an evacuated desiccator over NaOH. The water was deionized Millipore Corp. Milli-Q water (Bedford, MA). All other chemicals were reagent grade.

Results

Introduction of different polar substituents on the phenyl ring of the formyl-NH-terminal phenylalanine produces more than 2-fold changes in Na^+ permeation through channels formed by the different peptides, as illustrated by the single-channel current traces and amplitude histograms shown in Figures 2 and 3. Furthermore, the conductance amplitude histograms show single (sharp) peaks, indicating that we de-

[1] When the formyl-NH-terminal L-amino acid is replaced by its D isomer, one does not observe recognizable gramicidin channel activity unless one adds 100–1000-fold larger amounts than would be necessary to observe single-channel activity with the "standard" peptide.

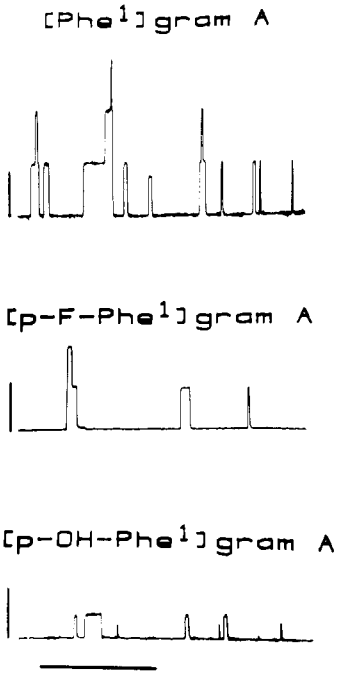

FIGURE 2: Single-channel current traces obtained with ring-substituted [Phe^1]gramicidin A analogues. Top: [Phe^1]gramicidin A channels. Middle: [*p*-F-Phe^1]gramicidin A channels. Bottom: [*p*-HO-Phe^1]gramicidin A channels. Conditions: 1.0 M NaCl, 200 mV. The calibration bars denote: 2 pA (vertically) and 2.5 s (horizontally).

Table I: Mean Single-Channel Durations for [Phe^1]Gramicidin (gram) Analogues[a]

substituent on Phe-1	mean channel duration (ms) for gram A	gram C
none	250	100
p-OCH_3	150	250
p-OH	20	40
o-F	130	420
m-F	130	330
p-F	110	280

[a] 1.0 M NaCl, 200 mV.

termine the characteristics of a well-defined channel conformation (Andersen et al., 1987). Ring-substituted phenylalanines are thus useful for studying structure–function relations in gramicidin channels. [In experiments with the fluoro-substituted phenylalanines, a substantial fraction of the transitions fall outside the major peak in each histogram. This occurs, in part, because the peptides are difficult to separate by HPLC. The relative mobilities of the [*o*-, *m*-, and *p*-F-Phe^1]gramicidins are essentially the same, which means that the sample purity depends upon the isomeric purity of the starting amino acids, the commercially obtained fluorinated phenylalanines themselves, which appears not to have been 100%.]

The possibility that the current variations could result from alterations in the basic peptide structure was addressed by examination of channel lifetimes and of the properties of hybrid channels. Table I summarizes information about the mean durations of the channels under consideration here. The durations are fairly similar, indicating that the different channels have a similar structure of the peptide backbone at the formyl-NH-terminal regions. [*p*-HO-Phe^1]gramicidin A and C channels have shorter durations than channels formed by the other peptides.

A more stringent test involved experiments that were designed to prove that the mutant peptides could form hybrid channels with [Phe^1]- and with [Val^1]gramicidin A. When

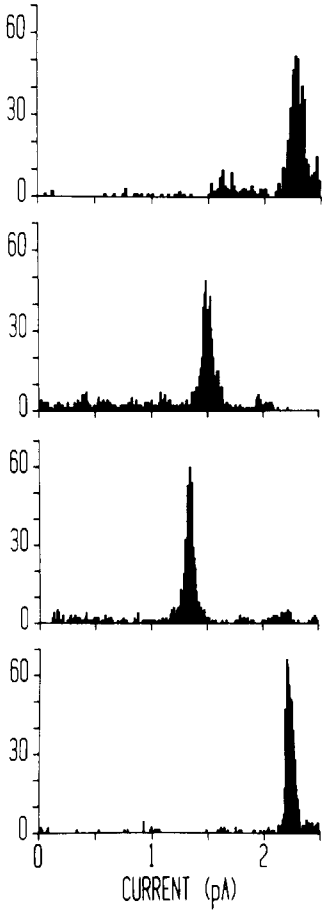

FIGURE 3: Current transition histograms obtained with channels formed by ring-substituted [Phe^1]gramicidin analogues. The four histograms denote, from top to bottom, results obtained with [Phe^1]gramicidin A, 519 events in the histogram of which 370 (or 71%) are in the main peak, the average current for these channels being 2.27 ± 0.07 pA (mean ± SD); [*p*-F-Phe^1]gramicidin A, 912 events in the histogram of which 561 (or 58%) are in the main peak, the average current for these channels being 1.54 ± 0.06 pA; [*p*-HO-Phe^1]gramicidin A, 813 events in the histogram of which 609 (or 75%) are in the main peak, the average current for these channels being 1.34 ± 0.05 pA; and [*p*-CH_3O-Phe^1]gramicidin A, 609 events in the histogram of which 535 (or 88%) are in the main peak, the average current for these channels being 2.23 ± 0.04 pA. Conditions: 1.0 M CsCl, 50 mV.

[X-Phe^1]gramicidin A or C is added together with either [Phe^1]- or [Val^1]gramicidin A, one observes not only the channels formed by each of the peptides but also a new channel type that is formed by two chemically dissimilar monomers [e.g., see Mazet et al. (1984)]. In Figure 4, we illustrate the results of such an experiment using [Val^1]gramicidin A and [*o*-F-Phe^1]gramicidin C.

That two different peptides can form hybrid channels indicates that the peptide backbone conformations of the chemically dissimilar monomers must be very similar (Mazet et al., 1984; Durkin et al., 1986; Russell et al., 1986). Furthermore, if two chemically dissimilar monomers have such similar a backbone folding that they can join with no energetic disadvantage, the frequency of appearance of hybrid channels should be predicted from the appearance frequencies of the pure channels (Mazet et al., 1984):

$$n_H \geq 2(n_a n_b)^{0.5} \quad (1)$$

where the equality holds if the two orientations of the hybrid channels (*a*–*b* and *b*–*a*) occur with equal probability [see Mazet et al. (1984) for details]. For each mutant peptide tested, we find that the number of hybrid channels with

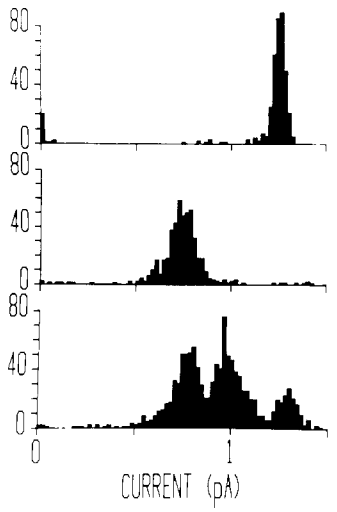

FIGURE 4: Current transition histograms from a [o-F-Phe[1]]gramicidin C/[Val[1]]gramicidin A hybrid channel experiment. Top: histogram of [Val[1]]gramicidin A channels. There were 374 events in the histogram, of which 352 (or 94%) were in the main peak; the average current for these channels was 1.24 ± 0.03 pA (mean ± SD). Middle: histogram of [o-F-Phe[1]]gramicidin C channels. There were 497 events in the histogram, of which 449 (or 90%) were in the main peak; the average current for these channels was 0.73 ± 0.07 pA. Bottom: histogram obtained with a mixture of [Val[1]]gramicidin A and [o-F-Phe[1]]gramicidin C. The peaks due to single-component channels can be identified by comparison with the upper histograms. The new peak in the center corresponds to the hybrid channels. The average currents for the three channels were, from left to right, 0.75 ± 0.07 (431), 0.99 ± 0.07 (482), and 1.29 ± 0.05 (162). The numbers in parentheses denote the number of events underlying each peak. There were 1128 events in the histogram, and we can account for 95% of these. Conditions: 1.0 M NaCl, 100 mV.

Table II: Hybrid Channel Relative Appearance Rates

peptide pair	$n_H/2(n_a n_b)^{0.5}$
[Phe[1]]gram A/[Val[1]]gram A	1.1 ± 0.2[a]
[p-CH_3O-Phe[1]]gram A/[Val[1]]gram B	1.0 ± 0.2
[p-F-Phe[1]]gram C/[Val[1]]gram A	1.2 ± 0.3
[m-F-Phe[1]]gram C/[Val[1]]gram A	1.0 ± 0.1
[o-F-Phe[1]]gram C/[Val[1]]gram A	1.3 ± 0.1
[p-HO-Phe[1]]gram C/[Val[1]]gram B	1.3[b]

[a] From Durkin et al. (1989). [b] From Mazet et al. (1984). The hybrid channel duration was so short (3.8 ms) that it was comparable to the minimal duration that was used to assign an event (4.4 ms). Consequently, a large number of hybrid events were not detected, and the observed ratio $[\eta_H/2(\eta_a\eta_b)^{0.5}]$ was 0.4 ± 0.1. After correction for lost events, the ratio is 1.3.

[Val[1]]gramicidin A or B conforms to eq 1 (see Table II). This shows that the channels formed by each of these peptides are $\beta^{6.3}$-helical dimers. (Hybrid channels involving [p-HO-Phe[1]]gramicidin A or C have very short durations, indicating that the heterodimer is destabilized relative to the respective pure channel types. We do not understand the molecular basis for this destabilization.)

Since hybrid channel formation is transitive (Durkin et al., 1989), the results in Table II imply that channels formed by the different peptides are structurally equivalent; that is, the substitutions (other than possibly the -OH) in the phenyl ring have minimal effects on the folding of the peptide backbone. It is therefore appropriate to use these compounds to evaluate the relative importance of electron redistributions and ion–dipole interactions in modulating ion permeation.

The small-signal (25–50 mV) conductances of channels formed by the substituted [Phe[1]]gramicidin A and C analogues are summarized in Table III. The p-OCH_3 substitution leads to a slight conductance increase in both 0.1 and 1.0 M NaCl, but not in 1.0 M CsCl. The other substitutions all decrease

Table III: Small-Signal Conductances of Substituted [Phe[1]]Gramicidin Analogues

substituent on Phe-1	g(0.1 M NaCl)	g(1.0 M NaCl)	g(1.0 M CsCl)
gramicidin A (Trp-11)			
none	4.65 ± 0.27	10.09 ± 0.45	44.8 ± 1.9
p-OCH_3	5.06 ± 0.42	10.44 ± 0.89	42.3 ± 1.7
p-OH	2.32 ± 0.18	3.94 ± 0.08	26.0 ± 0.7
o-F	3.52 ± 0.02[a]	9.14 ± 0.18	40.0 ± 2.7
m-F	3.19 ± 0.06[a]	6.08 ± 0.12	32.6 ± 1.7
p-F	3.17 ± 0.03[a]	5.86 ± 0.17	31.2 ± 1.1
gramicidin C (Tyr-11)			
none		7.39 ± 0.69	
p-OCH_3		7.72 ± 0.35	
p-OH		3.41 ± 0.39	
o-F		7.47 ± 0.15	
m-F		4.39 ± 0.12	
p-F		4.23 ± 0.07	

[a] The indicated conductances were measured at 50 mV only. Each other conductance is the mean value from an aggregate amplitude histogram based on measurements at 25 and 50 mV. Conductances are expressed as picosiemens ± the standard deviation.

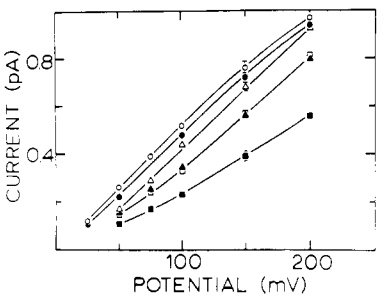

FIGURE 5: Current–voltage behavior for a series of substituted [Phe[1]]gramicidins with 0.1 M NaCl as the permeant ion. The curves change from sublinear for the p-OCH_3 substituent (○) to superlinear for the p-OH substituent (■) as the conductance decreases. The other curves are for unsubstituted Phe-1 (●), o-F-Phe-1 (△), m-F-Phe-1 (▲), and p-F-Phe-1 (□) gramicidins. The standard deviations are shown for the currents observed at 150 mV. The standard deviations for the m- and p-F-Phe compounds are less than the widths of the corresponding symbols.

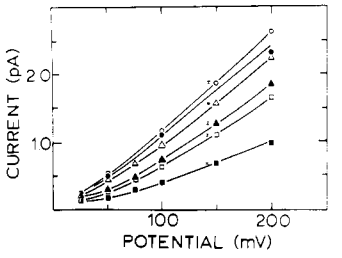

FIGURE 6: Current–voltage behavior for a series of substituted [Phe[1]]gramicidins with 1.0 M NaCl as the permeant ion. The substituents on the phenylalanine ring are (○) p-OCH_3, (●) none, (△) o-F, (▲) m-F, (□) p-F, and (■) p-OH. The standard deviations for the currents observed at 150 mV are shown to the left of the corresponding symbols.

the conductance. The relative decreases are in all cases larger in 1.0 M NaCl than in either 0.1 M NaCl or 1.0 M CsCl. The relative conductance changes are comparable for the gramicidin A and C series.

The substitutions affect not only the small-signal conductance but also the shape of the single-channel current-voltage relation. In 0.1 M NaCl, the shape varies with the substituent on the phenyl ring. The current–voltage characteristics are sublinear for unsubstituted and [p-CH_3O-Phe[1]]gramicidin A channels (Figure 5), similar to that observed with [Val[1]]-gramicidin A channels (Andersen & Procopio, 1980). The current–voltage relations are linear for channels formed by

Table IV: Values for σ, μ, $\mu_{\beta-\gamma}$, and $\mu_{\alpha-\beta}$

substituent on Phe-1	σ^a	μ^b (D)	$\mu_{\beta-\gamma}{}^c$	$\mu_{\alpha-\beta}{}^d$
none	0.00	0.4	0.4	0.16
p-OCH_3	−0.27	1.2		
p-OH	−0.37	1.6		
o-F	0.06	1.4	−0.4	−0.16
m-F	0.34	1.8	1.2	0.47
p-F	0.06	2.0	2.0	0.78

[a] See p 29 of Exner (1972). [b] The values of μ (in debyes) are those of substituted toluenes in benzene solution or in the gas phase [see pp 321–332 of Smyth (1955)]. [c] The component of μ along the C_β–C_γ bond is calculated, assuming free rotation of the phenyl ring about the C_β–C_γ bond, by adding the contribution from the unsubstituted ring (0.4 D) to the product of the fluorine group moment [1.6 D; see p 253 of Smyth (1955)] times the cosine of the angle between the vectors defined by ring–fluorine bond and the C_β–C_γ bond [see pp 231–235 of Smyth (1955)]. The assumption of free rotation represents an approximation. Because the orientation and dynamics of the phenyl side chain are unknown, the actual values may vary from those shown. [d] The component of μ along the C_α–C_β bond is calculated, once again assuming free rotation, by multiplying $\mu_{\beta-\gamma}$ by the cosine of 113°, the angle between the the C_α–C_β bond and the C_β–C_γ bond.

[Phe[1]]- and [*o*-F-Phe[1]]gramicidin A, and superlinear for channels formed by [*m*-F-Phe[1]]-, [*p*-F-Phe[1]]- and [*p*-HO-Phe[1]]gramicidin A.

In 1.0 M NaCl, the current–voltage relations are superlinear for channels formed by all the analogues (Figure 6); similar results were obtained with the gramicidin C analogues (not shown). The currents are larger in magnitude, and the differences among the channels are more apparent than in 0.1 M NaCl. Channels formed by [*m*-F-Phe[1]]- or [*p*-F-Phe[1]]-gramicidin A are, for example, virturally indistinguishable in 0.1 M NaCl. In 1.0 M NaCl, the [*m*-F-Phe[1]]gramicidin A channels clearly have the higher conductance.

Changes in the shape of single-channel current-voltage relations as a function of permeant ion concentration are due to changes in the relative importance of the different kinetic steps that underlie the ion translocation (Hladky & Haydon, 1972; Hladky et al., 1979; Andersen, 1983). At low permeant ion concentration (when the channel is mostly empty), the rate of ion movement is determined primarily by ion entry and movement through the channel interior; at high permeant ion concentration (when the channel is mostly occupied), the rate of ion movement is determined primarily by ion movement through the channel interior and ion exit. Changes from sublinear or linear at low [Na^+] to superlinear at high [Na^+], as for the [Phe[1]]-, [*o*-F-Phe[1]]-, and [*p*-CH_3O-Phe[1]]gramicidin A, can result if the concentration-dependent ion association step is fairly voltage independent and may become rate limiting at high voltage. Comparatively, the invariance of the superlinear current–voltage relation, with respect to changes in [NaCl], for [*m*-F-Phe[1]]-, [*p*-F-Phe[1]]-, and [*p*-HO-Phe[1]]-gramicidin A, indicates that the limiting rate constant is voltage-dependent; i.e., the central barrier is high for these latter three analogue channels.

Data Analysis. The results on the hybrid channel frequency (Table II) show that the conductance changes induced by the phenyl ring substituents cannot result from gross changes in the peptide structure. We will in this section evaluate the relative importance of side chain induced inductive electron shifts and of dipolar interactions between the phenyl ring and permeating ions in modulating the ion permeability.

Inductive electron shifts induced by phenyl ring substitutions should be reflected by Hammett's σ parameter which is an expression of the influence of the substituted phenyl ring at another point in the molecule (Hammett, 1937). Dipolar

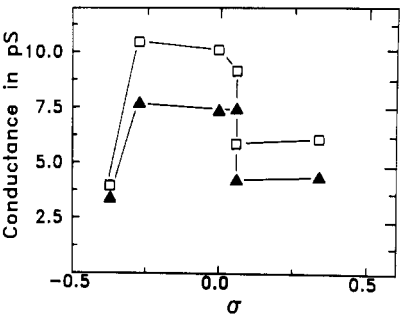

FIGURE 7: Small-signal conductance versus the Hammett constant, σ, for two series of substituted [Phe[1]]gramicidins: gramicidin A (□) and gramicidin C (▲). The Hammett constants on the abscissa are listed in Table IV.

effects should be related to the side chain dipole moment, μ. Values for σ and μ, as well as estimates for the components of μ along the β–γ and α–β bonds, $\mu_{\beta-\gamma}$ and $\mu_{\alpha-\beta}$, are listed in Table IV.

On the Hammett scale, negative σ values are assigned to electron-donating groups, and positive σ values are assigned to electron-withdrawing groups. For the dipole moments, positive μ values are assigned to dipoles that point toward the channel lumen. The substituents had σ values ranging from −0.37 for *p*-OH to +0.34 for *m*-F (Exner, 1972). The dipole moments of the phenyl rings were estimated as the dipole moments of the corresponding toluenes and ranged from 0.4 D for unsubstituted phenylalanine to 2.0 D for *p*-F-phenylalanine (Smyth, 1955).

A plot of single-channel conductance versus σ in 1.0 M NaCl or CsCl, where the conductance is close to its maximal value, shows no correlation between the conductance changes and the inductive properties of the substituents (Figure 7). (A similarly shaped plot would result if the data obtained in 1.0 M CsCl were plotted. Even if the [*p*-HO-Phe[1]]gramicidin points are exluded, there is no correlation with σ.) Factors other than electron-inductive effects are responsible for the conductance variations among the channels formed by these gramicidin analogues.

The [Phe[1]]-, [*o*-F-Phe[1]]-, [*m*-F-Phe[1]]-, and [*p*-F-Phe[1]]-gramicidin analogues can be used to evaluate the importance of dipolar (and orientation) effects, as the dipoles associated with the fluorine substitutions have their moments in the plane of the phenyl ring [see pp 320–333 of Smyth (1955)], and the rather similar sizes of fluorine and hydrogen suggest a priori that there should be little steric crowding associated with the H → F substitution [e.g., see p 331 of Smyth (1955), and Goldman (1969)]. This assertion is supported by the results on hybrid channel frequencies (Table II). This does *not* imply that the (time-averaged) side chain positions may not vary for the different ring-substituted phenylalanines, only that the consequences of such variations for the backbone structure are small. A plot of the conductance in 1.0 M NaCl or CsCl against estimated $\mu_{\alpha-\beta}$ or $\mu_{\beta-\gamma}$ shows that the conductance is correlated with the dipole component along the α–β or β–γ bond (Figure 8). The deviations from linearity can be accounted for by differences in the time-averaged orientation of the phenyl ring about the β–γ bond, which should arise because fluorine is slightly larger than hydrogen. In the [*o*-F-Phe[1]]- and [*m*-F-Phe[1]]gramicidin analogues, steric interactions with the channel wall should thus move the fluorine atom away from the channel axis. The effective dipole vector of the two analogues seen by the ion in the channel would thus be more positive than estimated in Table III. Given this correction, ion–dipole interactions appear to be important in modulating the single-channel conductance.

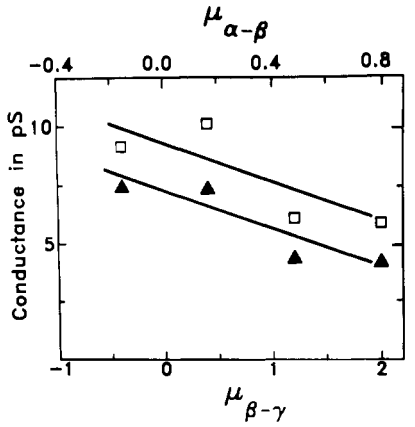

FIGURE 8: Small-signal conductance versus projections of μ for Phe[1]-substituted gramicidin A (□) and gramicidin C (▲) analogues. The dipole moments are listed in Table IV. The two scales for the abscissa denote the estimated component of μ along C_α–C_β (top) and along C_β–C_γ (bottom).

Discussion

Amino acid substitutions in the linear gramicidin sequence lead to alterations in the rate of ion movement through the channel (Bamberg et al., 1976; Morrow et al., 1979; Heitz et al., 1982; Mazet et al., 1984; Prasad et al., 1986; Durkin et al., 1986; Russell et al., 1986). The physical mechanisms that underlie the permeability changes have remained somewhat obscure however.

We have previously shown that alterations in the polarity of amino acids substituted at position 1 can lead to more than 10-fold changes in single-channel conductance and that these conductances should be ascribed to the side chain substitution per se rather than to alterations in the backbone structure (Mazet et al., 1984; Russell et al., 1986). The present results show that the predominant functional consequences of altering the side chain polarity are mediated via ion–dipole interactions between the permeating ion and the side chain dipole. Inductive through-bond effects are less important—at least for side chains where the polar substitutent is two carbons away from the peptide group.

Structural Equivalence. Mechanistic interpretations of our results depend on the *assumption* that the side chain substitutions leave the basic channel structure intact. A priori, all of the analogue channels should have the same backbone structure because the substitutions are in the phenyl ring, somewhat removed from the peptide backbone. We also addressed this problem experimentally by using a functional criterion for structural equivalence: hybrid channel formation (Figure 4 and Table II). That hybrid channels form between chemically dissimilar peptides shows that the peptide backbones in the two monomers can adapt to each other. Further, when the hybrid channel appearance rate conforms to the predictions of eq 1, there cannot be a significant energetic cost associated with hybrid channel formation (Mazet et al., 1984; Durkin et al., 1989). The amino acid substitutions are at position 1, i.e., at the join between the two monomers, and even subtle alterations of the peptide backbone should result in deviations from predictions based on eq 1. However, one should note that this criterion is not sensitive to small alterations in side chain orientation and dynamics. With this proviso, we conclude that the backbone folding patterns are equivalent and that the functional changes result from the side chain substitutions per se.

Hybrid channels involving [*p*-HO-Phe[1]]gramicidins are destabilized relative to the pure channel types, and these hybrid channels also have abnormally low conductances (Mazet et al., 1984). Nevertheless, the hybrid channel appearance rates match the predictions of eq 1; we therefore believe any structural perturbations of the [*p*-HO-Phe[1]]gramicidin channels are quite small. The nature of the possible subtle conformational variations that may be reflected here has yet to be determined, and we do not know whether they by themselves alter the rate of ion permeation through the channel.

Electrostatic vs Inductive Interactions. On the assumption that the dispersion and repulsion forces (steric crowding) have negligible effects in our set of analogues, two other forces may be invoked to explain the changes of conductances: inductive interactions and electrostatic interactions. The lack of correlation between σ and the single-channel conductance (Figure 7) suggests that the inductive character of the phenyl ring is not a major determinant of the rate of ion permeation through the channels. On the contrary, in view of the correlation between the single conductance and the component of the side chain dipole moment along the the $C_{\alpha-\beta}$ or $C_{\beta-\gamma}$ bond (Figure 8), it appears that the conductance changes may result from electrostatic interactions between the first side chain dipole and the permeating ion.

We note, in particular, that among the fluoro-substituted phenylalanines, both the *o*-F and *p*-F groups are slightly electron-withdrawing, with a σ value of +0.06, while the *m*-F group is quite electron-withdrawing with a σ of +0.34. The dipole moments of the p- and m-substituted phenyl rings are comparable, pointing toward the channel lumen, and should thus give rise to repulsive ion–dipole interactions of comparable magnitude. The similar conductances observed with the [*p*-F-Phe[1]]- and [*m*-F-Phe[1]]gramicidin channels show that inductive electron shifts induced by a phenyl ring attached to the β-carbon at position 1 cannot be an important determinant of the conductance, while the results are consistent with the notion that ion–dipole interactions are important in modulating the conductance of gramicidin channels. Additionally, the *o*-F and *p*-F substituents have similar σ values while the dipole moments associated with the *o*-F and *p*-F substituents point in generally opposite directions. The two analogues form channels that have different conductances; the higher conductance of the [*o*-F-Phe[1]]gramicidin channels probably results from favorable ion–dipole interactions. These results support the hypothesis that inductive effects are not of major importance.

The *p*-OH group is the most electron-donating of the substituents, while the *p*-OCH_3 group is weakly electron-donating. If inductive effects were dominant in modulating ion permeation through the channels, the [*p*-HO-Phe[1]]gramicidin channels should have higher conductances than [*p*-CH_3O-Phe[1]]gramicidin channels. In constrast, we found that the conductance of the [*p*-CH_3O-Phe[1]]gramicidin channels is more than 2-fold higher than that of the [*p*-HO-Phe[1]]gramicidin channels (for both series of analogues). Admitting the possibility of small conformational effects here, the results nevertheless once again argue against inductive effects being of major importance. The conductance changes, at least in part, are the result of alterations in the central barrier for ion translocation through the channel (cf. Figures 5 and 6).

The dipoles associated with the *p*-OH and *p*-OCH_3 substituents have similar moments, but their moment vectors are not in the plane of the phenyl ring, and we have no simple way to evaluate the component pointing in the general direction of the channel lumen. Inspection of CPK[2] models of $\beta^{6,3}$-

[2] Abbreviation: CPK models, Corey–Pauling–Koltun models.

helical dimers indicates furthermore that the moment vectors should point toward the C-termini of the channel. At this time, we cannot use these substituents to evaluate the importance of ion–dipole interactions.

Conceptually, the distinction between inductive and electrostatic effects is fairly straightforward. The former, a through-bond effect, should be much less sensitive to the side chain orientation than the latter, due to a through-space interaction. In practice, difficulties exist because electrostatic field changes will alter the electron distribution in polarizable groups and inductive electron shifts will conversely lead to the appearance (or alteration) of permanent dipoles. Hammett's parameter (σ) therefore provides a measure of the total electronic influence (polar, inductive, field effects) of a substituted phenyl group on a distant active site of the same molecule. The discrepancy between the σ values and the single-channel conductance of modified gramicidin can be easily explained if the balance between the various effects is not the same in our experiments as when the values of σ were measured. The most obvious difference in the experimental conditions is that the reported values of σ for substituted phenyl rings were measured in water, where through-space electrostatic effects are shielded, whereas the side chain of gramicidin lies in a medium of very low dielectric constant, where the electrostatic field is much greater. This gives theoretical support to our conclusion that the conductance changes are due primarily to electrostatic interactions and not to inductive effects.

The conductance changes result from changes in the energy profile for ion movement throughout the channel, where the major change should be close to the formyl-NH-termini, at least for the unsubstituted and fluoro-substituted phenylalanines. Attempts to quantitate the conductance changes based on ion–dipole interactions are complicated, however, by the dielectric anisotropy of the medium which lies between the dipole and the ion, and by possible restrictions on the side chain movement. Nevertheless, it is possible to obtain estimates for the effective dielectric constant of the channel wall by noting that the conductance varies ~2-fold for a 1–2-D change in $\mu_{\beta-\gamma}$ (Figure 8). Neglecting complications due to the inhomogeneous media, ion–dipole interactions can formally be described by

$$\Delta E = \mu e / 4\pi\epsilon_0\epsilon_r r^2 \qquad (2)$$

where ΔE is the interaction energy, e is the elementary charge, ϵ_0 is the permittivity of free space, ϵ_r is an effective dielectric constant, and r is the distance between ion and dipole. From CPK models, r is estimated to be ~0.7 nm, and ϵ_r can then be estimated to be 3–6, which is comparable to estimates for the dielectric constant of relatively nonpolar peptides (Tredgold et al., 1977; Gilson & Honig, 1986). Our estimate for ϵ_r may, however, be an overestimate. The central barrier is not a rate-determining factor for ion movement through [Phe^1]- and [*o*-F-Phe^1]gramicidin A channels. It is thus possible that the changes in the central energy barrier for ion movement through the channel are larger than would be inferred from the relative conductance changes per se.

One caveat exists with regard to our conclusion that inductive electron interactions are relatively unimportant. While this conclusion is valid for the series of [X-Phe^1]gramicidins, it is possible that inductive interactions could be important for other polar side chains (e.g., trifluorovaline or hexafluorovaline) where the polar moiety is closer to the backbone carbonyl groups. Indeed, binding the phenyl ring to the α-carbon instead of the β-carbon should multiply σ by a factor of 2–3 (Ritter & Miller, 1964). This possibility could be tested by moving the phenyl ring one carbon closer to the peptide backbone, i.e., by eliminating the β-carbon of phenylalanine and using instead a series of substituted phenylglycine side chains at position 1.

Orientation Effects. The conductance of gramicidin channels is modulated by the polar side chains at position 1 (Morrow et al., 1979; Mazet et al., 1984; Russell et al., 1986). This modulation is not due to the mere presence of polar side chains, however, but is a result of their particular average orientation with respect to the channel's lumen. This is most clearly seen by comparing the results for [*p*-F-Phe^1]- and [*m*-F-Phe^1]gramicidin A, on the one hand, with the results for [*o*-F-Phe^1]gramicidin A on the other. The side chains have similar dipole moments, but the single-channel conductances in 1.0 M NaCl vary 2-fold (Table III). The conductance changes can be attributed to the different orientations of the dipole moment vectors.

The conductances of [*p*-HO-Phe^1]- and [*p*-CH_3O-Phe^1]-gramicidin A channels are 2.5-fold different in 1.0 M NaCl (Table III). The side chain dipole moments are again very similar. Restrictions on the side chain orientation coupled with the different bulk of the polar substituents, and the possibly different orientation of the dipole moment vectors, should probably be invoked to explain these observations. As argued above, small conformational differences for the [*p*-HO-Phe^1]analogues cannot be ruled out.

We finally note that the indole ring of tryptophan has a dipole moment of 2 D (Smyth, 1955), while [Trp^1]- and [Val^1]gramicidin A channels have similar conductances (Mazet et al., 1984). This shows again that not only the side chain polarity but also its orientation is important for modulating the conductance [see also Pullman (1987)].

Implications for Structure–Function Studies. In gramicidin channels, the side chains form the exterior surface of the channel, such that each β-carbon is about 0.55 nm from the channel center. Side chain substitutions *cannot* therefore affect ion permeation by means of direct contact between the permeating ion and the side chains (Morrow et al., 1979; Mazet et al., 1984). Rather, the effects are mediated over some distance; i.e., side chain substitutions can alter the function of ion channels even when the region of chemical alteration is *spatially separate* from the region where the functional modulation is induced. Our conclusion that through-space electrostatic interactions dominate the side chain dependent conductance modulation is not surprising, since dipolar field effects generally seem to be more important than inductive effects (Stock, 1972; Reynolds, 1983).

The quantitative importance of the electrostatic field effects is also in agreement with the view that the dielectric constant of the interior of membranes or proteins appears to be quite low (Gilson et al., 1985). Given the long-range character of electrostatic interactions, the replacement of nonpolar side chains by dipolar or charged residues (or vice versa) will produce significant ($>kT/e$) electrostatic potential alterations quite far from the substituted side chain. For $\epsilon_r = 3$, a dipole moment change of 3.6 D (e.g., produced by replacing a valine by an asparagine) will produce this potential change up to 1.1 nm from the side chain, in a line along the direction of the moment vector. The introduction of a monopole (e.g., replacing a valine by an aspartate) could produce this same potential change up to 18 nm from the side chain. In either case, the actual potential profile will be modified by polarization changes at the membrane or protein/solution interface (Parsegian, 1969; Honig et al., 1986; Jordan, 1986). Lateral interactions (parallel to the plane of the membrane) will

therefore be less than predicted from estimates based on isotropic media calculations [e.g., see Andersen (1978)]. This does not, however, affect the general conclusion that electrostatic interactions can be significant over large distances, as the effect of a single charge is by any means larger than the one that we have observed for a dipole. This suggests the possibility that the function of membrane channels could be controlled by mechanisms other than a simple occlusion/deocclusion of the channel pore [see also Huang et al. (1978, 1979)]. Alterations in the intra- and intermolecular arrangement(s) of polar groups in the vicinity of the pore can profoundly alter the energy profile—and thereby the rate—for ion movement through the channel, while leaving the pore intact.

Finally, we wish to emphasize the advantage brought by chemical over genetic modifications to understand a mechanism of transport in precise detail. By a chemical substitution, the graded change of a single parameter could be introduced into the side chain of a peptide. This allowed demonstration that the electrostatic interaction, a long-range force, was very important in determining the conductance of a transmembrane channel. Although the energy change due to an electrical dipole for an ion crossing a membrane may seem weak with respect to other energetic contributions (Lennard–Jones, image potential, etc.), its magnitude is of the right order for changing significantly the height of the main energy barrier to ion transfer. This kind of result should indicate the direction for refinement of molecular dynamics simulations of channel properties. Thus, at the present state, gramicidin may be used to bridge the gap between theory and experiment on ionic channel activity.

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Registry No. [Phe^1]gram A, 89314-65-8; [Phe^1]gram C, 89314-71-6; [*p*-OCH_3-Phe^1]gram A, 124042-34-8; [*p*-OCH_3-Phe^1]gram C, 124042-35-9; [*p*-OH-Phe^1]gram A, 89314-66-9; [*p*-OH-Phe^1]gram C, 89314-72-7; [*o*-F-Phe^1]gram A, 124042-36-0; [*o*-F-Phe^1]gram C, 124042-37-1; [*m*-F-Phe^1]gram A, 124042-38-2; [*m*-F-Phe^1]gram C, 124042-39-3; [*p*-F-Phe^1]gram A, 124042-40-6; [*p*-F-Phe^1]gram A, 124042-41-7; [Val^1]gram B, 124042-42-8; [Val^1]gram A, 4419-81-2; Na, 7440-23-5.

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Direct Stoichiometric Evidence That the Untransformed M_r 300 000, 9S, Glucocorticoid Receptor Is a Core Unit Derived from a Larger Heteromeric Complex†

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ABSTRACT: We have used three methods to measure the stoichiometry of the glucocorticoid receptor and the 90-kDa heat shock protein (hsp90) in L-cell glucocorticoid receptor complexes that were purified by immunoadsorption to protein A–Sepharose with an anti-receptor monoclonal antibody, followed by a minimal washing procedure that permits retention of receptor-associated protein. In two of the methods, receptor was quantitated by radioligand binding, and receptor-specific hsp90 was quantitated against a standard curve of purified hsp90, either on Coomassie blue stained SDS gels by laser densitometry or on Western blots by quantitative immunoblotting with ^{125}I-labeled counterantibody. The stoichiometry values obtained by densitometry and immunoblotting are 7 and 6 mol of hsp90/mol of receptor, respectively. In a third method, which detects total receptor protein rather than just steroid-bound receptor, the ratio of hsp90 to receptor was determined by immunopurifying receptor complexes from [^{35}S]methionine-labeled L cells, and the amount of ^{35}S incorporated into receptor and hsp90 was corrected for the established methionine content of the respective proteins. In complexes from L cells which are labeled to steady state (48 h), the ratio of hsp90 to GR is 4:1. When immunoadsorbed receptor complexes are washed extensively with 0.5 M NaCl and 0.4% Triton X-100 in the presence of molybdate, the ratio of hsp90 to GR is 2:1. In addition to hsp90, preparations of [^{35}S]methionine-labeled untransformed receptor complex also contain a 55-kDa protein that resolves into several isoelectric forms on two-dimensional gel electrophoresis. These observations lead to the conclusion that the untransformed L-cell glucocorticoid receptor exists in cytosol in a much larger heteromeric complex than considered to date. We propose that the 9S receptor form that is commonly observed by density gradient centrifugation, and by gel filtration chromatography, must be a "core unit" containing two hsp90 and one GR which is derived from this larger structure.

The untransformed[1] glucocorticoid receptor (GR)[2] can exist in cytosolic preparations as an 8–9S (M_r 300 000) heteromeric complex consisting of a single molecule of the steroid-binding protein (Gehring & Arndt, 1985; Okret et al., 1985) and two molecules (Mendel & Orti, 1988) of a non-steroid-binding phosphoprotein (Joab et al., 1985; Sullivan et al., 1985; Housley et al., 1985; Mendel et al., 1986) which has been identified as the 90-kDa heat shock protein (hsp90) (Sanchez et al., 1985; Schuh et al., 1985; Catelli et al., 1985; Denis et al., 1987). There is strong evidence that this heteromeric untransformed receptor complex is derived from the physiologically inactive form of the receptor that is turned on by the hormone in intact cells: (1) Mendel et al. (1986) have shown that hsp90 is associated with GR obtained from hormone-free cells but not from cells that were exposed to hormone at 37 °C, a condition that ensures transformation; (2) Rexin et al.

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[1] The term transformation will be used throughout this paper to describe the process whereby the receptor is converted from a non-DNA-binding to a DNA-binding form.

[2] Abbreviations: GR, glucocorticoid receptor; TA, triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; hsp90, 90-kDa heat shock protein.

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