Solid-Phase Synthesis of Melittin: Purification and Functional Characterization†

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ABSTRACT: The main component of the honey bee venom, melittin, is a cationic polypeptide containing 26 amino acids. Exposure of lipid bilayers to this peptide results in the formation of anion-selective channels with a variety of unit conductances. One of the possible causes for this heterogeneity in the conductance could be heterogeneity of the melittin preparation, and indeed, the existence of two prominent forms of naturally occurring melittin, differing only at the N-terminal amino group, has been documented. This paper describes the synthesis of the major form of melittin, using stepwise solid-phase methodology and the demonstration that the synthetic melittin, devoid of the minor component (*N*-formylmelittin) and other contaminants, interacts with lipid bilayers to form channels which are qualitatively indistinguishable from the ones formed by the naturally occurring toxin. This result indicates that the heterogeneity in the channels produced in bilayers by bee venom is not due to differences in the channel-forming properties of the formyl and non-formyl melittin but rather to differences in the number and orientation of melittin monomers of identical primary structure as they aggregate to form channels in the lipid bilayer.

Melittin, the principal component of *Apis mellifera* venom, is an amphipathic peptide consisting of 26 amino acids (Habermann & Jentsch, 1967): H-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH_2. The bee venom toxin has been shown to bind to phospholipids and to cell membranes and to produce increased ionic permeability and subsequent lysis of a variety of cells (Dufourcq & Fauçon, 1977; Habermann, 1972; Tosteson et al., 1985a,b). It has also been shown that melittin forms anion-permeable channels in lipid vesicles and induces a time- and voltage-dependent, anion-selective conductance in planar bilayers. This conductance arises from channels which have a variety of single-channel conductances, ranging from 20 to 2000 pS (Sessa et al., 1969; Tosteson & Tosteson, 1981; Hanke et al., 1983; Tosteson et al., 1985a,b).

Further work on the determination of the molecular characteristics of the voltage- and time-dependent conductance induced by melittin has been hampered by the existence of two predominant forms of melittin in the toxin when isolated from natural sources (Habermann & Jentsch, 1967; Kreil & Kreil-Kiss, 1967). These components, which differ in the structure and, consequently, charge at the N-terminus, have proven to be difficult to separate (Maulet et al., 1982). The major component (90–98%) contains 26 amino acids and a free primary α-amino group on glycine at the N-terminus. In the minor form of melittin (2–10%), the glycine at the N-terminus is formylated.

In order to continue our work on the molecular mechanism of melittin's action, it became necessary to obtain each derivative in pure form, in order to be able to compare the characteristics of the conductance which each of them induces in lipid bilayers to those induced by the mixture of the two forms. This would permit us to establish the form of the toxin responsible for the previously observed voltage- and time-dependent conductance and would make possible the further characterization of the melittin-induced conductance.

We therefore undertook the stepwise, solid-phase synthesis of melittin described in this paper, and will show that we have obtained melittin with a free N-terminus, devoid of *N*-formylmelittin. We will further show that this synthetic, free N-terminus, melittin induces a time- and voltage-dependent conductance in asolectin bilayers which is qualitatively the same as the one induced by the unfractionated, natural product. Moreover, the single channels which give rise to the macroscopic conductance were found to be multisized, as observed previously when using the naturally occurring compound. These results suggest that the conductance induced in asolectin bilayers does not depend on the presence of *N*-formylmelittin in the aqueous solutions bathing the bilayers. The role of this form of melittin in the voltage-dependent conductance remains to be determined.

Experimental Procedures

Peptide Synthesis. Melittin was assembled by stepwise solid-phase synthesis (Merrifield, 1963; Barany & Merrifield, 1980) on an Applied Biosystems Model 430A peptide synthesizer (version 1.2 software) with 4-methylbenzhydrylamine resin as the solid support (0.82 mmol of N/g, 0.61 g). Side-chain protecting groups for amino acids used were as follows: N^{ω}-(2-chlorobenzyl)oxycarbonyl for lysine; benzyl for threonine and serine; N^{in}-formyl for tryptophan; and N^{G}-*p*-toluenesulfonyl for arginine. N^{α} protection for all amino acids was provided by the *tert*-butyloxycarbonyl (Boc) group. All amino acids were purchased from Applied Biosystems Inc.

A typical coupling cycle was as follows: the N-protected peptide resin was treated with 65% trifluoroacetic acid (TFA) in CH_2Cl_2 for 5 min followed by a second treatment for 16 min to remove the N-protecting group. The peptide resin was then washed in CH_2Cl_2 and neutralized twice with 10% diisopropylethylamine in *N*,*N*-dimethylformamide (DMF) for 1 min each, followed by washing with DMF.

Coupling was performed by using a preformed symmetric anhydride of the next amino acid in DMF. The preformed symmetric anhydride was prepared on the synthesizer by dissolving 2 mmol of the Boc-amino acid in 6 mL of CH_2Cl_2

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and adding 1 mmol of *N,N'*-dicyclohexylcarbodiimide (DCC) in 2 mL of CH_2Cl_2. After approximately 5 min, the activated amino acid solution was transferred to another vessel where CH_2Cl_2 was evaporated with a stream of N_2 and replaced with DMF. The preformed symmetric anhydride solution, now in DMF, was transferred to the neutralized peptide resin (0.5 mmol) and allowed to react for 16 min. The peptide–resin was then washed with CH_2Cl_2 in preparation for the next cycle.

All amino acids were coupled in similar fashion, with the exception of glutamine and arginine. These amino acids were coupled twice as preformed 1-hydroxybenzotriazole (HOBt) esters using 2 mmol of Boc-amino acid, 2 mmol of HOBt, and 2 mmol of DCC in the activation stage for each coupling.

After the chain was assembled, the N^{α}-Boc protecting group was removed with 65% TFA in CH_2Cl_2, and the resin was washed and then dried in a desiccator.

The side-chain protecting groups and the peptide resin anchoring bond were cleaved by treatment with anhydrous hydrogen fluoride. The peptidyl resin was first treated as described in a procedure of Tam et al. (1983), with 25% HF, 7.5% *p*-cresol, and 2.5% thiocresol in dimethyl sulfide (DMS) for 2 h at 0 °C, followed by treatment with 90% HF, 7.5% *p*-cresol, and 2.5% *p*-thiocresol for 1 h at 0 °C.

The crude peptide was precipitated from the HF reaction mixture with ether and extracted into 10% acetic acid and lyophilized.

Peptide Purification. The crude synthetic melittin was purified by high-performance liquid chromatography (HPLC) on a Waters Delta Prep 3000. A Waters PrepPak radial compression cartridge (57 mm × 30 cm) was used, with Vydac C_{18} bonded silica, 15–20-μm particle size, and 300-Å pore size. Purification of 0.510 g of melittin was done in a single load, using a 60-min linear gradient of 20–50% CH_3CN in 0.1% TFA at a flow rate of 101.5 mL/min. The eluted material (30-mL fractions) was analyzed on a Waters RadPak radial compression cartridge (8 mm × 10 cm) with the same packing material as described above. The fractions containing the major peak were combined and analyzed for purity.

Determination of Peptide Purity. The purified peptide was characterized by amino acid analysis, analytical HPLC, and sequence analysis. Amino acid analysis was performed on a Beckman amino acid analyzer (Spackman et al., 1958). Analytical HPLC was performed on the SpectraPhysics SP8000 using a Vydac steel column (4.6 × 15 cm; C_4 bonded silica, 5-μm particle size, and 300-Å pore size). The material was eluted by using a 40-min linear gradient of 24–43% CH_3CN in 0.1% TFA at a flow rate of 1.5 mL/min. Sequence analysis was performed on an Applied Biosystem Sequences Model 470A by stepwise Edman degradation (Hewick et al., 1981; Spiess et al., 1979).

Bilayers. Lipid bilayers were formed from asolectin (20 mg/mL pentane) by apposition of two monolayers spread at the air/solution interfaces (Montal & Mueller, 1972). The hole in the Teflon partition (approximately 100 μm in diameter) was pretreated with a 3% solution of squalene in pentane (v/v). The aqueous solutions contained unbuffered 0.5 M $K(Na)NO_3$ + 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA). All experiments were conducted at room temperature (20 °C).

Current flowing across the bilayer in response to an applied potential difference was measured by using a fast-settling, high-input impedance amplifier (LF 157A, National Semiconductors). The sign of the potential refers to the compartment to which melittin is added (cis). Positive charge flowing from the cis to the trans compartment is plotted as upward current. The silver–silver chloride electrodes were connected to the solutions via salt bridges. The current signals were recorded on FM tape (Racal Store 4). Signals from the tape were filtered at 0.5–3 kHz and the records digitized with a Tracor Northern 1710.

Natural melittin, purified from bee venom, was purchased from Sigma Chemical Co. (St. Louis, MO) (catalog no. M 2272 and M 7129).

Results

Purification and Chemical Characterization of Synthetic Melittin. The yield of protected peptide resin from the synthesis of free N-terminus was 3.34 g, and the yield of crude peptide from the HF reactions was 1.05 g of which 23.9% was the desired product. Fractions representing the top portion of the principal peak were selected in order to obtain highly purified material, intentionally sacrificing yield. Hence, the generation of 5.4 mg of highly purified material from 510 mg of crude peptide does not reflect accurately the potential yield of melittin from this synthesis.

One of the criteria used to determine the purity of the synthetic N-free melittin was analytical HPLC. Figure 1 shows the HPLC analysis of the purified peptide (C) as well as the analysis of two different batches of commercially available melittin, shown for comparison (A and B). Integration of the peaks indicates that the major peak contains greater than 99.7% of the product. In contrast, using the same criterion, the major peaks in Figure 1A,B are, at best, 62.0% and 73.1% pure, respectively. The arrow in Figure 1 indicates the elution position of a formylmelittin marker. A shallower gradient, designed to resolve additional, close-running impurities of the synthetic peptide, is shown in Figure 2. The major peak integrates to 98.9% of the material.

Amino acid analysis of the synthetic melittin showed a close correlation with the known composition of melittin, as indicated [observed (theory)]: Lys, 2.91 (3); Arg, 2.00 (2); Thr, 2.09 (2); Ser, 1.03 (1); Glu, 1.97 (2); Pro, 1.04 (1); Gly, 3.00 (3); Ala, 2.06 (2); Val, 2.00 (2); Ile, 2.83 (3); Leu, 3.94 (4).

Confirmation that the correct sequence had been generated by chemical synthesis was obtained by automated sequence analysis utilizing stepwise Edman degradation of 26 cycles, utilizing HPLC analysis of aminoacyl-phenylthiohydantoin (AA-PTH) derivatives. Furthermore, using the method described by Tregear, which tends to overemphasize the presence of contaminants, we detected less than 3% cumulative preview (including background), indicating that there is less than 3% contamination by deletion-containing error peptides (Tregear et al., 1977).

Functional Characterization of Synthetic Melittin. The purified, synthetic melittin (free N-terminus) was further characterized in its ability to induce a time- and voltage-dependent conductance in lipid bilayers.

Figure 3 shows the time course of the current flowing across a bilayer exposed to melittin in response to a constant-voltage pulse. As previously found (Tosteson et al., 1985a) when asolectin membranes were exposed to unfractionated melittin, the current response exhibits three regions in which $dI/dt = 0$: an early (e) region reached with a half-time of 1–2 ms followed by a sigmoidal rise to a peak (p) (half-time of 1–6 s); this region is then followed by a decline in the current to the third region in which $dI/dt = 0$, labeled "i". The conductance of regions e and p has been found to be dependent on voltage and melittin concentration, as illustrated in Figure 3. This dependence can be expressed as follows:

$$G = [\text{melittin}]^m \exp(neV/kT)$$

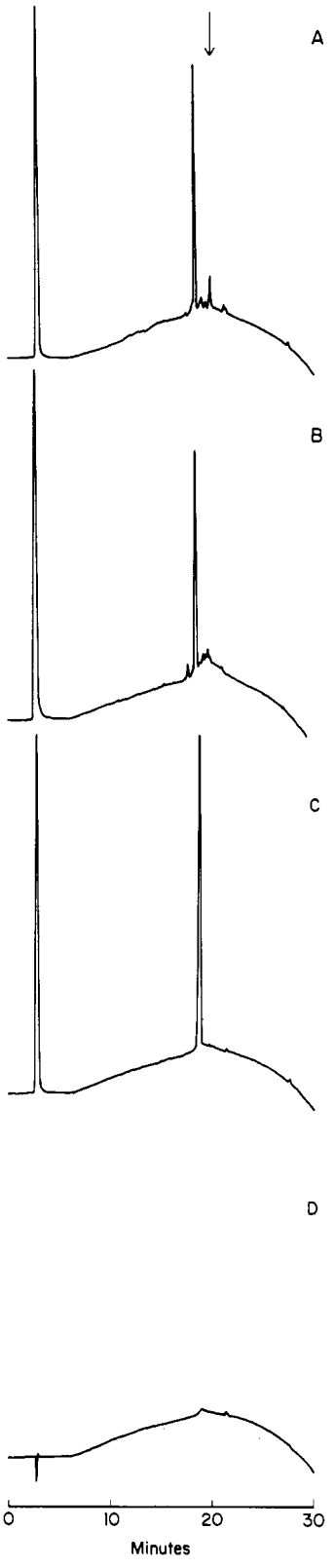

FIGURE 1: HPLC separation of melittin preparations. (A and B) Two different preparations of melittin obtained commercially (M7391 and M2272, respectively). No efforts were made to identify the contaminating peaks. (C) Chemically synthesized melittin, after purification by HPLC, as indicated under Experimental Procedures. Some of the impurities might correspond to aggregates of melittin. (D) Blank injection. Conditions: 30-min linear gradient of 9.75–97.5% CH_2CN in 0.1% TFA at a flow rate of 1.5 mL/min through a stainless-steel column packed with Vydac C_4-bonded silica (4.6 × 15 cm, 5-μm particle size, 300-Å pore size).

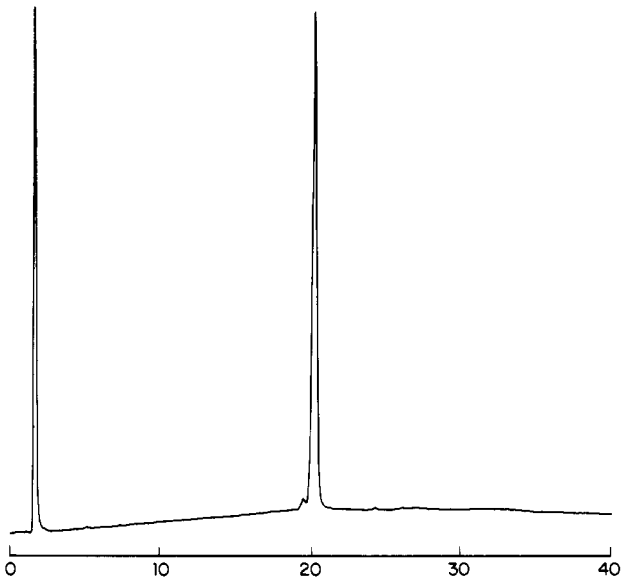

FIGURE 2: HPLC separation of synthetic melittin. Conditions: 40-min linear gradient of 24–43% CH_3CN in 0.1% TFA at a flow rate of 1.5 mL/min through a stainless-steel column packed with Vydac C_4-bonded silica (4.6 × 15 cm, 5-μm particle size, 300-Å pore size).

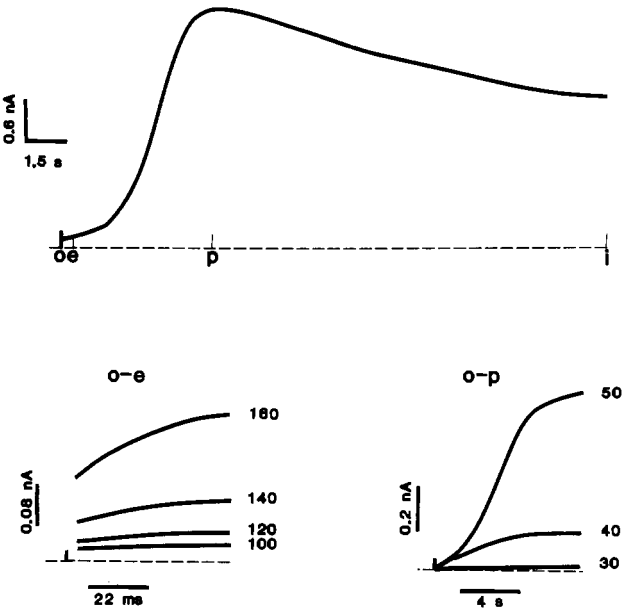

FIGURE 3: Time course of the current response to a constant-voltage pulse. (Top) Retrace of the current response to a step in voltage. The +60-mV pulse was started at "o" and maintained through "i". (Bottom) "o–e", retrace of the current response to steps in voltage of short duration. 25 pulses of 60-ms duration were applied to the bilayer with a frequency of 1 Hz, 25 min after the curve shown in the top trace. The individual current responses were added in a signal averager. "o–p", retrace of the current response to a step in voltage of 11-s duration, taken after "o–e". Dashed lines indicate the current level at 0 mV. Melittin concentration (cis side only) was 8×10^{-8} M.

where e is the electronic charge, k is the Boltzmann constant, T is the temperature, and m and n are constants describing the dependence of the conductance on the concentration of melittin in the aqueous phases and on the applied voltage (V), respectively. The values found for these constants (shown in Figure 4) are very similar to those previously reported. Thus, the free N-terminal melittin seems to aggregate to form tetramers ($m = 4$) when it forms ion-permeable pathways through bilayers. The opening of these conductive pathways seems to be associated with the movement of the equivalent of one electronic charge during the early phase of the activation

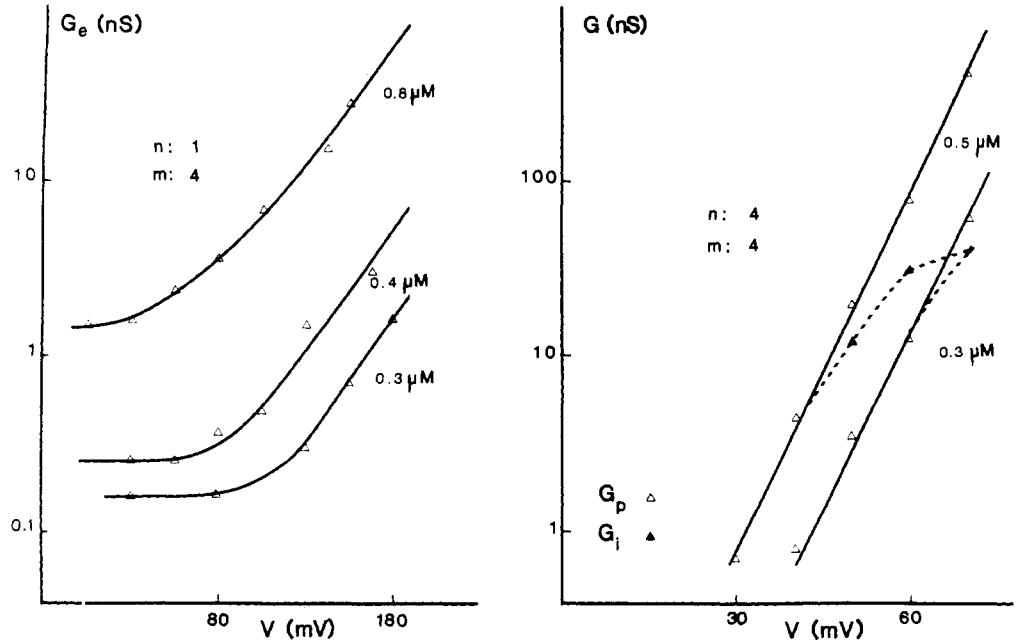

FIGURE 4: Voltage dependence of the steady-state conductance of regions "e" (G_e) and "p" and "i" (G_p and G_i). The current at each potential was determined as detailed in Figure 3 and the conductance calculated by dividing the steady-state current by the applied potential. After each addition of an aliquot of a concentrated solution of melittin, the current response to trains of pulses of short duration (60 ms), +100-mV amplitude, was tested every 10–20 min until it reached a constant, reproducible value (generally after 40 min of addition). At each melittin concentration, the current vs voltage (I–V) curve of region "e" was determined first, followed by the I–V curve of regions "p" and "i".

(n = 1). At the peak of the current activation, the ion-permeable pathways are also tetramers (m = 4), but the opening of these pathways is associated with the movement of the equivalent of four electronic charges (n = 4) (i.e., Figure 4).

The next step in the functional characterization of the synthetic melittin was to determine if the conductance induced by the peptide arises from the formation of many discrete channels. Figure 5 shows a family of averaged currents obtained at three different voltages (A) together with two of the individual current records obtained at +130 mV (B). The results shown in this figure indicate that the probability of a channel being open (averaged current) is both time and voltage dependent, just like the conductance (current) of a multichannel membrane (cf. Figure 3). However, the steady-state conductance calculated from these averages increases e-fold per 10–12-mV increase in the voltage, in contrast to an increase of e-fold per 6-mV increase in the voltage for the macroscopic conductance, G_p (cf. Figure 4). Moreover, no inactivation of the current is apparent in any of the curves shown in Figure 5. We propose that both these results are consistent with a population of channels which is heterogeneous both in amplitude and in kinetic characteristics and that the probability of opening and closing of channels in membranes containing relatively few channels (Figure 5) does not reflect fully the process by which the conductance of membranes containing many channels arises (Figure 4).

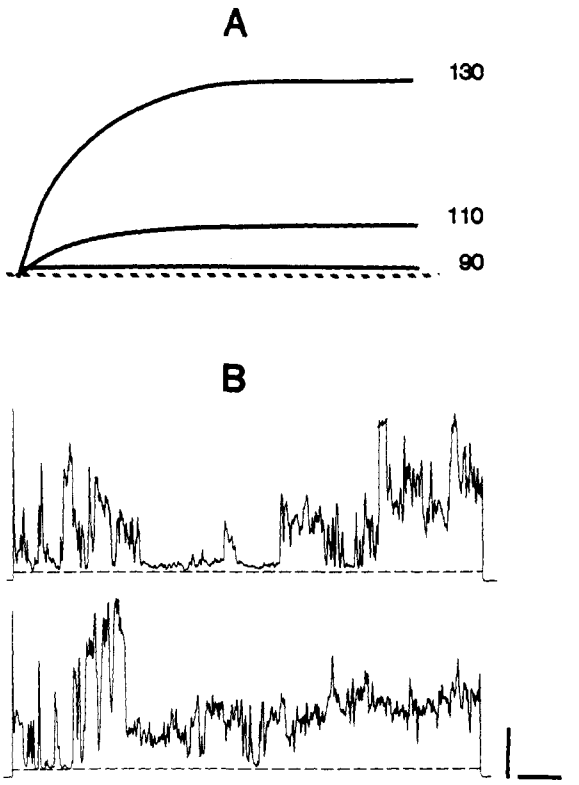

FIGURE 5: Melittin-induced current in bilayers exposed to low melittin concentrations. (A) Retrace of the averaged current response to voltage pulses of 10-s duration applied every 2 min. At each applied potential, the individual current responses were added in a signal averager. (B) Individual current responses to voltage pulses of +130-mV amplitude. The dashed lines indicate the current level in the absence of an open channel. Number of traces analyzed at each voltage was 30. Melittin concentration (cis side only) was 6 nM. Filter, 1 kHz; current scale, 75 pA; time, 1.1 s.

DISCUSSION

The synthesis of hydrophobic peptides composed of more than 20 amino acids is still a challenging task. Although in the past years melittin fragments and melittin analogues have been synthesized (Dorman & Markley, 1971; DeGrado et al., 1981), melittin itself has been obtained only by purification from natural sources. This material is heterogeneous (cf. Figure 1A,B), the main contaminants consisting of phospholipase A_2 and an N-terminally formylated form of melittin (Habermann, 1972; Kreil & Kreil-Kiss, 1967; Maulet et al., 1982).

We have described in this paper the synthesis of one of the chemical forms of melittin, using the stepwise, solid-phase method. Our efforts were concentrated exclusively on the synthesis and purification of the predominant form of the bee

venom toxin, without regard for the yield of synthetic product. We have succeeded in obtaining better than 99% pure free N-terminus melittin, totally devoid of phospholipase A_2 and *N*-formylmelittin, as described under Results (cf. Figure 1).

When tested for its ability to promote a time- and voltage-dependent conductance in asolectin bilayers, it was found that the synthetic product induced a macroscopic conductance with characteristics similar to those which had been previously described using commercially available preparations [cf. Tosteson and Tosteson (1981), Tosteson et al. (1985a), and Figure 4]. Furthermore, when the concentration of melittin in the aqueous phase was low enough to ensure that the probability of forming anion-permeable pathways was low, the characteristics of the single-channel conductance were found to be qualitatively similar to those reported previously (Hanke et al., 1983; Tosteson et al., 1985a; Figure 5B). These results indicate either that both forms of melittin present in the native mixture induce comparable time- and voltage-dependent conductance in bilayers or that only melittin with a free N-terminus induces this type of conductance. The nature of the multisized channels remains undetermined. The results presented in this paper rule out the possibility that heterogeneity in the chemical forms of melittin could lead to the heterogeneity observed in the single channels. We cannot rule out at present that these various conductive pathways could arise from channels composed of a different number of melittin molecules. Neither can we exclude the possibility that channels containing the same number of melittin monomers (i.e., four) could differ in the spatial relations between them (e.g., head-to-tail or head-to-head arrangements).

We believe that the successful synthesis and purification of full-length melittin is a step formward in the elucidation of the nature of the active form(s) of melittin which we plan to pursue through the synthesis of formylmelittin, as well as the synthesis of melittin analogues. Moreover, we will be able to address directly the question of the molecular determinants of the melittin-induced conductance through studies of the relation between the primary structure of melittin analogues and their ability to induce anion-selective, voltage-dependent channels in lipid bilayers.

ACKNOWLEDGMENTS

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Registry No. Boc-Gly-OH, 4530-20-5; Boc-Ile-OH, 13139-16-7; Boc-Ala-OH, 15761-38-3; Boc-Val-OH, 13734-41-3; Boc-Leu-OH, 13139-15-6; Boc-Lys(2-ClZ)-OH, 54613-99-9; Boc-Thr(CH_2Ph)-OH, 15260-10-3; Boc-Pro-OH, 15761-39-4; Boc-Ser(CH_2Ph)-OH, 23680-31-1; Boc-Trp(CHO)-OH, 47355-10-2; Boc-Arg(Tos)-OH, 13836-37-8; Boc-Gln-OH, 13726-85-7; melittin, 20449-79-0.

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