A strategy has been developed for the identification of inhibitors of toxins or regulatory proteins. This approach is based on blocking the access of such proteins to their biological targets during their solution transport. This approach uses the strength of nonsupport-bound synthetic combinatorial libraries (SCLs) for the study of acceptor-ligand interactions. A non-receptor assisted toxin, melittin, was selected for the present study to illustrate this application of the SCL approach. Hexapeptide SCLs were assayed for their ability to inhibit the cytolytic activity of melittin toward bacterial and erythrocyte cells. Over 20 inhibitory hexapeptides were identified following the screening and deconvolution processes from millions of sequences. The identified inhibitory peptides appeared to interact directly with melittin. These interactions appear to decrease melittin's ability to undergo lipid- and/or polysaccharide-induced conformational changes, and are demonstrated by fluorescence and circular dichroism spectroscopy.

The mechanism of action of the majority of biologically active peptides involves an initial interaction between the peptide and a specific, singular acceptor system (e.g. receptor, antibody, enzyme, etc.). However, a number of toxins have been reported to exert their biological activity directly on cell membranes not by binding to a specific single binding site but through a cascade of different possible mechanisms (reviewed in Ref. 1). Due to the potential difficulty in targeting a specific mechanism of action, an effective means to inhibit the activity of such toxins can be envisioned to involve inhibition of the toxins’ access to cell membranes during the solution transport of the toxins. We have developed an approach involving the use of synthetic combinatorial libraries (SCLs)† to identify short peptide sequences able to bind to the toxins and, in turn, to inhibit their biological effects. The SCLs generated in this laboratory are composed of mixtures of nonsupport-bound compounds that can be used in virtually any bioassay system. Recently, highly specific, short bioactive peptides have been discovered from pools of millions of other related peptides using SCLs based on their binding affinity to specific receptors of interest (i.e. antibodies (2-5), enzymes (6, 7), and opioid receptors (8, 9)). The use of SCLs for the study of nonspecific interactions was only applied to the identification of antimicrobial, antifungal, and antiviral compounds (2, 10-13). Although potent compounds were discovered in this manner, their mechanisms of action have yet to be determined. These successful applications suggest that SCLs may be a useful means for the rapid identification of inhibitors of nonreceptor-assisted, biologically active proteins such as toxins and regulatory proteins.

In a first study of the ability to use SCLs for inhibiting protein activities through peptide-protein interactions, inhibitors of the cytolytic activity of melittin, a potent toxin isolated from bee venom (14), were identified. Besides its implication in the toxic effect of a bee sting, melittin is useful model system to study nonreceptor-assisted toxicity, because it is known to bind spontaneously to biological and synthetic model membranes (reviewed in Ref. 15). Although the mechanism of melittin’s cytolytic activity remains unclear, binding to a specific receptor has not been reported. One of the proposed mechanisms of melittin’s toxicity toward erythrocyte cells involves the accumulation of peptide molecules on the outer leaflet of the bilayer, which causes a perturbation in the arrangement of the membrane structure. This, in turn, results in an increased membrane permeability and, ultimately, cell lysis (15-17).

Using an SCL composed of 52 million hexapeptides, we have developed an inhibitory assay in which each peptide mixture making up an SCL can be tested for its ability to inhibit melittin’s lytic activity on erythrocyte or bacterial cells. Several series of individual hexapeptides were identified that were found to inhibit melittin’s hemolytic and antimicrobial activity. As demonstrated by CD and fluorescence spectroscopy, this inhibition of melittin’s toxicity was found to result from the formation of a complex between melittin and these peptides.

MATERIALS AND METHODS
Preparation of SCLs and Individual Peptides—The peptide mixtures were prepared using the process of divide, couple, and recombine (2, 18, 19) in conjunction with simultaneous multiple peptide synthesis (20-22). All individual peptides were prepared using simultaneous multiple peptide synthesis. Peptide mixtures and individual peptides were solubilized in H2O or 5% dimethylformamide/H2O, aliquote, and stored at -20 °C. Individual peptides were characterized by laser desorption time-of-flight mass spectroscopy analyses (Kompact Maldi-ToF mass spectrometer, Kratos, Ramsey, NJ) and purified by preparative reversed phase-high performance liquid chromatography (Millipore Corp., Waters Division, San Francisco, CA).

Inhibition of Melittin’s Hemolytic Activity—The inhibitory activities of the peptide mixtures and individual peptides were determined using human red blood cells (RBCs) as described elsewhere (23). The assays were carried out in a manner similar to the hemolytic assay (24) but with the addition of melittin (final concentration at 7.5 μg/ml or as mentioned in the text) to each well. In 96-well flat bottom plates (Costar, Pleasanton, CA), an equal volume of a 0.5% RBC suspension in phosphate-buffered saline (35 mM phosphate, 0.15 M NaCl, pH 7.0) was added to the peptide mixtures at concentrations varying from 5000 to 4 μg/ml derived from serial 2-fold dilutions mixed with melittin. Follow-
Inhibitors of Melittin from Combinatorial Libraries

Inhibitors of Melittin from Combinatorial Libraries

RESULTS

Screening of the Dual Defined Position SCLs—The initial SCL tested consisted of 400 separate hexapeptide mixtures having a C-terminal amide and an acetylated N terminus. A length of six amino acids was chosen for this initial library based on a general consensus regarding the appropriate length involved in peptide-receptor interactions (i.e. antigenic determinants, hormone interactions, etc.). The presence of an N-acetyl group is anticipated to prevent electrostatic repulsion between the mixtures and basic melittin. Each separate hexapeptide mixture had the first two positions defined with one of the 20 naturally occurring amino acids (referred to as O) and the remaining four positions as close to equimolar mixtures of 19 of the naturally occurring amino acids (referred to as X - cysteine was omitted). Each peptide mixture can be represented by the formula Ac-OXXX-NH₂, and is comprised of 130,321 (19⁴) individual hexapeptides, for a total of 52,128,400 (400 × 19⁴) hexapeptides.

Taking into account the large number of individual peptides present in a peptide mixture, one must optimize an assay system in order to maximize the signal detection from the background level, permitting the differentiation of the activities between the peptide mixtures. In a competitive assay, the intensity of the signal depends on the concentration of the desired inhibitory peptides in 1 ml of phosphate-buffered saline buffer. The fluorescence intensity was corrected by the contribution of background level, permitting the differentiation of the activity in the same peptide mixture, and therefore, a substantially higher "effective" molar concentration.

As shown in Fig. 1, a small number of the 400 peptide mixtures assayed from the SCL were found to have significant inhibitory activity at 1.25 mg/ml. In addition, at 1.25 mg/ml, none of the peptide mixtures affected cell membranes, as shown by the absence of significant hemolytic activity. In order to determine the relative activities of the most active peptide mixtures found through this initial screening, I₅₀ values were calculated for those mixtures inhibiting more than 50% lysis by melittin at this concentration. The I₅₀ values are based on the percentage of inhibition at 2-fold serial concentrations in peptide mixtures, thereby providing a more accurate relative evaluation of the peptide mixture activities than a single concentration screening. Fifteen out of the 400 peptide mixtures showed I₅₀ values varying from 350 to 1300 μg/ml. Upon screening an equivalent non-N-acetylated hexapeptide SCL, weaker inhibitory activities were observed (I₅₀ > 2000 μg/ml), which indicates the value of having a blocked N-terminal amino group for the desired inhibitory activity. Although several of the 15 active mixtures have been selected for the iterative process, Ac-IVXXXX-NH₂ is used here to illustrate these studies.

Iterative Process—A new series of peptide mixtures were prepared in which the first three residues were specifically defined and the three remaining positions were made up of a close to equimolar mixture of 19 of the naturally occurring amino acids (i.e. Ac-IIVOXXX-NH₂). Each peptide mixture now contains 6,859 individual hexapeptides (19³). At the highest concentration tested, 1 mg/ml, each individual peptide is at 0.2 μM (i.e. molar ratio of peptide to melittin, 1:13). Ac-IIVOXXX-NH₂ and Ac-IVLXXX-NH₂ were the most active peptide mixtures from the series (Table I). Due to the similar chemical character of the third amino acid, only Ac-IIVOXXX-NH₂ was initially pursued in the iterative process and is presented below. It should be noted that isoleucine was found to define the third position of the most active peptide mixtures in the different parallel iterative processes carried out for inhibition studies (data not shown). Isoleucine, or a hydrophobic residue, appears therefore to be crucial at the third position for inhibition to occur.

A 5-fold increase in activity was obtained upon defining the fourth position of Ac-IIVOXXX-NH₂ (i.e. Ac-IIVOXXX-NH₂; Table I). Ten peptide mixtures had inhibitory activity ranging from 20 to 40 μg/ml, indicating a lower specificity for the fourth position. As was found for the third position, the 10 most active mixtures were defined by an hydrophobic residue at the fourth position. Although the iterative process is illustrated in Table I for the peptide mixture Ac-IIVOXXX-NH₂, the mixtures Ac-IIVVX-NH₂, Ac-IIVCXX-NH₂, Ac-IIVWXX-NH₂, and Ac-IIV-IFXX-NH₂ were also selected as leading sequences for parallel iterative processes. Upon defining the fifth position (as illustrated in Table I), increases of up to 2-fold were observed. In the final iterative step, a number of potent defined inhibitors of the hemolytic activity of melittin were identified (I₅₀ values ranging from 5 to 10 μg/ml; Table I). Other inhibitors identified through parallel iterative processes are shown in Table II. All of the inhibitors identified can be described as having a strong hydrophobic character and by the presence of a negatively charged residue or a polar amino acid at the C terminus.

Comparative Study Using a Dual Defined Position SCL and...
Single Defined Position Positional Scanning SCL—Using an N-acetylated hexapeptide SCL in a positional scanning (PS) format (i.e. Ac-XXXXX-NH₂, Ac-XOXXXX-NH₂, Ac-XXOXXX-NH₂, Ac-XXXOXX-NH₂, Ac-XXXXOX-NH₂, and Ac-XXXXXO-NH₂), a series of individual peptides with potent inhibitory activities were identified (Table II and Ref. 23). Although the dual defined position SCL and the single defined position PS-SCL are composed of the same diversity of individual peptides, they differ by the deconvolution process, as well as by the number and concentration of the individual peptides present in a mixture. In contrast to the iterative process, the PS-SCL approach allows the identification of individual active sequences from the direct screening of the mixtures making up the library (23). Thus, each single position SCL provides information on the most active residues at each position, and when used in concert, this information allows the identification of the entire active sequences. Synthesis and screening of such sequences confirm the screening data as well as permit the determination of the relative activity of each of these individual sequences.

The sequences of the inhibitors identified from the PS-SCL approach are very similar to those obtained using the iterative process described above in that they are strongly hydrophobic and contain a glutamic acid at the C terminus. It should be noted...
that whereas the three N-terminal positions were highly specific in the screening of the PS-SCL, the last three positions were found to be redundant with at least 10 of the 20 peptide mixtures showing similar activity (23). These results may explain the differences in the final sequences obtained from the two libraries, which appear primarily in the last three residues of the identified sequences. They are also in agreement with the large number of peptide mixtures having similar activity found upon defining the last three positions. As shown in Table III, the three N-terminal residues identified were among those defining the mixtures having the three greatest activities of each set of the positional SCLs. For instance, isoleucine represented the only peptide mixture having activity from the single position SCL, Ac-XXOXXX-NH₂ and was found to characterize the most active peptide mixtures upon defining the third position during the separate iterative processes. On the other hand, the peptide mixtures corresponding to the two N-terminal residues of the individual peptide inhibitors derived from the screening of the PS-SCL, Ac-FIXXXX-NH₂ and Ac-IIIXXXX-NH₂, were among the 15 most active peptide mixtures of the dual defined position SCL (Table III).

Inhibitory Activity—The importance of the molar ratio between the peptide inhibitors and melittin was studied using Ac-IIXYFE-NH₂ and Ac-FIIFWE-NH₂ by varying the melittin concentration. An average molar ratio of 7 was found for the two inhibitors when melittin was used at a higher concentration, i.e. the lytic activity of melittin is maximal (Table IV). Because the RBC concentration was kept constant during this study, the constant molar ratio observed indicates that the peptide inhibitors found are more likely to be interacting with melittin than with RBCs. In support of this hypothesis, a longer incubation time for melittin and Ac-IIXYFE-NH₂ or Ac-FIIFWE-NH₂ prior to the addition of RBCs resulted in greater inhibition (Fig. 2A). As anticipated from these results, the ability of Ac-IIXYFE-NH₂ or Ac-FIIFWE-NH₂ to inhibit the

| Inhibitor sequence                        | IC₅₀ | Rank | Most active mixture IC₅₀ |
|-------------------------------------------|------|------|-------------------------|
| Ac-IIXXXX-NH₂                             | 2780 | 1    | 2780                    |
| Ac-XXXXX-NH₂                              | 4990 | 3    | 3990                    |
| Ac-XXIXX-NH₂                              | 2810 | 1    | 2810                    |
| Ac-XXLXX-NH₂                              | 4320 | 10   | 2340                    |
| Ac-XXLXX-NH₂                              | 2860 | 7    | 2100                    |
| Ac-XXIXXW-NH₂                             | >5000| 2    | 2010                    |
| Ac-XXIXXNE-NH₂                            | 2050 | 2    | 2010                    |
| Ac-XXXXTX-NH₂                             | >5000| 10   | 2010                    |
| Ac-XXXXXXM-NH₂                            | 4290 | 10   | 2010                    |
| Ac-XXXCCX-NH₂                             | >5000| 19   | 2100                    |
| Ac-XXXVX-NH₂                              | 2600 | 2100 |
| Ac-XXXNE-NH₂                              | 2050 | 2    | 2010                    |
| Ac-XXXQX-NH₂                              | 2470 | 17   | 2100                    |
| Ac-XXXQQ-NH₂                              | 2750 | 7    | 2010                    |
| Ac-XXXFXX-NH₂                             | >5000| 2340 |
| Ac-XXXFX-NH₂                              | 2250 | 2    | 2100                    |
| Ac-XXXAXD-NH₂                             | 2460 | 4    | 2010                    |

Inhibitors derived from dual position SCL

| Inhibitor sequence                        | IC₅₀ | Rank | Most active mixture IC₅₀ |
|-------------------------------------------|------|------|-------------------------|
| Ac-FIIXXXX-NH₂                            | 569  | 7º   | 358                     |
| Ac-IIXXXX-NH₂                             | 461  | 3º   | 358                     |
| Ac-FIXXXX-NH₂                             | >1300| 358  |
| Ac-IXQXXX-NH₂                             | >1300| 358  |

a The rank was determined within the series of 20 peptide mixtures.

b The rank was determined within the 400 peptide mixtures.

| Melittin concentration | Ac-IIXYFE-NH₂ | Ac-IIIFWE-NH₂ |
|------------------------|---------------|---------------|
| µg/ml                  | µM            | µM            | µM            | µM            | µM            |
| 7.5                    | 2.6           | 5             | 5.8           | 2.2           | 5             | 6             |
| 15                     | 5.3           | 32            | 38            | 7.2           | 41            | 45            |
| 18.7                   | 6.6           | 39            | 46            | 7.0           | 46            | 51            |
| 30                     | 10.5          | 60            | 72            | 6.9           | 54            | 60            |

R, molar ratio of peptide to melittin.
hemolytic activity of melittin was found to decrease when melittin was incubated with RBCs prior to the addition of the peptides (Fig. 2B).

Although the lysis mechanism of RBCs relative to that of bacterial cells by melittin may differ, it is logical to assume that it involves the initial accumulation of melittin on the respective cell membranes. If the mechanism of inhibition involves a strong interaction between melittin and the peptide inhibitors rather than an interaction between the cells and the peptide inhibitors, as indicated above, then similar inhibitory activities would be expected with bacterial cells. The four peptides tested were found to inhibit melittin against both Gram-positive (S. aureus) and Gram-negative (E. coli) bacteria (Table V).

**FIG. 2.** Effect of preincubation on the inhibitory activity of Ac-IIIYFE-NH₂ and Ac-FIIWFE-NH₂. A, melittin (7.5 μg/ml) was preincubated with Ac-IIIYFE-NH₂ (14 μg/ml) or Ac-FIIWFE-NH₂ (6 μg/ml) at 37 °C for up to 30 min, prior to the addition of RBCs (0.25%). B, melittin (7.5 μg/ml) was preincubated with RBCs (0.25%) at 22 °C for up to 60 min, prior to the addition of Ac-IIIYFE-NH₂ (14 μg/ml) or Ac-FIIWFE-NH₂ (6 μg/ml). In both cases, the percentage of inhibition is plotted as a function of preincubation time.

**TABLE V**

| Sequences          | IC₅₀  | S. aureus | E. coli |
|--------------------|-------|-----------|---------|
| Ac-IIIYFE-NH₂      | 12    | 13        |         |
| Ac-FIIWFE-NH₂      | 6     | 5         |         |
| Ac-IVILLE-NH₂      | 28    | 27        |         |
| Ac-IVILLW-NH₂      | 14    | 26        |         |

*a* Melittin used at 8 μg/ml.

*b* Melittin used at 30 μg/ml.

**FIG. 3.** Fluorescence studies of Ac-IIIYFE-NH₂-melittin interactions. A, the fluorescence spectra of melittin (7 μM) in the absence (solid line) or presence of Ac-IIIYFE-NH₂ (85 μM, dotted line). B, stoichiometric titration of melittin (7 μM) in the presence of up to 85 μM Ac-IIIYFE-NH₂. The tryptophan fluorescence of melittin at 340 nm after excitation at 294 nm was measured. The increases in tryptophan fluorescence at 340 nm relative to the spectra of melittin in the absence of the inhibitor (Δ λ₃₄₀) are plotted as a function of the inhibitor concentration.

The occurrence of conformational changes in melittin upon adding an increasing concentration of Ac-IIIYFE-NH₂ was studied by CD spectroscopy. Melittin was found to have a
random conformation at 22 μM in phosphate-buffered saline ([θ]_{222} = −5000 degrees cm² dmol⁻¹) with no significant change in ellipticity at 222 nm upon the addition of Ac-IIIYFE-NH₂, even at an Ac-IIIYFE-NH₂ to melittin ratio of 7:1. These results indicate that the binding of the inhibitory peptides to melittin does not induce significant changes in the overall conformation of melittin in buffer. Furthermore, these results support the lack of an inhibitor-induced tetramerization of melittin, which would result in an induced α-helical conformation (30–32), and, in turn, agree with the formation of a melittin/inhibitor complex evidenced earlier by fluorescence spectroscopy. The occurrence of conformational changes of melittin in the presence of the inhibitors was further evaluated when melittin binds to two model biological membrane systems: naturally occurring polymers of N-acetyl neuraminic acid (referred to as polysialic acids), which are present on the surface of cell membranes and lysophosphatidylcholine micelles as representative of the lipid bilayer of the cell membranes. As shown in earlier studies (32), melittin was readily induced into an α-helical conformation upon binding to colaminic acid (poly-2,8-N-acetyl neuraminic acid; Fig. 4). After subtracting the contribution of the inhibitor from the overall spectra, the ellipticity at 222 nm of melittin CD spectra was found to decrease dramatically in the presence of Ac-IIIYFE-NH₂ (Fig. 4). This was found to be independent of the mixing order of the three components (melittin, Ac-IIIYFE-NH₂, and colaminic acid; Fig. 4). In a similar manner, lower ellipticity at 222 nm was observed at several melittin/lysophosphatidylcholine ratios (Rₜ, varying from 0 to 30) in the presence of Ac-IIIYFE-NH₂ (Fig. 5).

**DISCUSSION**

The soluble SCL technology developed in this laboratory has been proven in a wide variety of assays to allow the rapid identification of potent biologically active peptides (reviewed in Ref. 33) and, more recently, peptidomimetics (11). In the present study, the power of the SCL approach was used to develop a new strategy to identify inhibitors of known cytolytic compounds through peptide-protein interactions in solution using melittin as a model system.

Two deconvolution processes have been used for the identification of individual active compounds from an SCL: an iterative process (2) and a positional scanning process (5). The comparison of these two approaches described here leads to the conclusion that very similar information about the overall physico-chemical properties of the final sequences and the relative importance of an amino acid or building block at a given position can be obtained using either deconvolution format. This is true even though relatively minor differences in sequences are likely to be obtained depending on the level of specificity of a given amino acid within the sequence. Thus, in the present case, the results from either SCL indicate that the peptides must have an overall hydrophobic character for inhibition to occur and that the presence of a negatively charged residue at the C terminus (E or D) is important but not an absolute necessity in the inhibition mechanism. The strong similarities observed for the first three residues of the individual peptide sequences identified indicate that highly specific residues can be readily identified through the use of either library format. On the other hand, families of amino acids having slightly different chemical character are most likely to be identified in the case of less specific positions (i.e., the three C-terminal residues).

Similar inhibitory activity was found when using different cell types (Gram-positive and Gram-negative bacteria and erythrocytes), indicating that the mode of inhibitory action occurred through binding to melittin. These results are supported by the greater inhibitory activity observed upon longer incubation times between melittin and inhibitors prior to the addition of the cells. Loss in inhibitory activity was observed when the N terminus of the inhibitory peptides was not blocked with an acetyl group (data not shown). This can also be explained by inhibitor-melittin interactions. Thus, electrostatic repulsions between the positively charged N terminus of this peptide and the basic residues of melittin may lessen or prevent such interactions from occurring and, in turn, eliminate the inhibitory activity. Melittin is known to bind to a number of proteins such as calmodulin (34), troponin C (35), and phosphorylase kinase (36). In particular, it has been reported that melittin binds to calmodulin (34) or troponin C (35) through the exposed hydrophobic surfaces. The hydrophobic character of the inhibitors found in this study suggests the occurrence of hydrophobic-hydrophobic interactions with melittin similar to those reported for proteins.

Using over 50 single substitution analogs of melittin, we have found that the self association of melittin to its transitory tetrameric aggregate is required for penetration of the carbo-
hydrate barrier present in biological membranes and for lysis to occur (32). In addition, the seven residues of the hydrophobic segment (i.e. proline-14 to isoleucine-20) of melittin have been described as a nucleation center in the folding of monomeric melittin into its self-assembled tetramer (37). One can envision the mode of action of the identified inhibitors through hydrophobic interactions with this segment of melittin. This would perturb melittin’s ability to undergo lipid- and/or polysaccharide-induced conformational changes as observed by CD spectroscopy. In agreement with this hypothesis, our fluorescence studies showed that the environment of tryptophan-19 of melittin is more hydrophobic in the presence of the inhibitors. In conclusion, we believe that the present studies show that not only can potent inhibitors of a potentially lytic compound be rapidly identified using the SCL approach but also that one can find specific agents that block the access of a peptide and/or protein to its biological target. The application of combinatorial chemistry may also be useful for blocking or modifying the activity of other toxins, as well as regulatory proteins.

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Sylvie E. Blondelle, Richard A. Houghten and Enrique Pérez-Payá

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