Synthetic Peptides Corresponding to the Calmodulin-Binding Domains of Skeletal Muscle Myosin Light Chain Kinase and Human Erythrocyte Ca^{2+} Pump Interact with and Permeabilize Liposomes and Cell Membranes†

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ABSTRACT: Synthetic calmodulin-binding (CaM-binding) peptides (CBPs) representing CaM-binding domains of Ca^{2+}/CaM-dependent enzymes have been reported to interfere with the activity of the melanocyte-stimulating hormone (MSH) receptor function in melanoma cells [Gerst, J. E., & Salomon, Y. (1988) *J. Biol. Chem. 263*, 7073–7078]. We postulated that membrane lipids may play an important role in the mode of action of CBPs on cells. We therefore tested the ability of CBPs to interact with membrane bilayers. Using artificial phospholipid vesicles, or M2R melanoma cells and cell membranes derived therefrom, as models, we report here that synthetic peptides representing the CaM-binding domains of skeletal muscle myosin light chain kinase (M5) and the human erythrocyte calcium pump (C28W), as well as other CBPs, interact with lipid bilayers and cell membranes. Significant interactions of CBPs with the lipid bilayer were detected in both model systems. M5 and C28W were found to partition into the lipid bilayer of melanoma cell membranes and soybean lecithin vesicles, and surface partition constants obtained (for the liposome model) were in the range 10^3–10^4 M^{-1}. In addition, C28W and its N-modified NBD derivative were found to inhibit [^{125}I]iodo-[Nle^4,D-Phe^7]αMSH binding to cultured M2R melanoma cells. These and other CBPs were also found to induce the release of cations and calcein from liposomes, suggesting that the interaction of CBPs with the lipid bilayer increases membrane permeability. Nonrelevant peptides used as controls were found ineffective. Melittin, a bee venom derived CBP, and pardaxin, a shark-repellent neurotoxin, both membrane-permeating peptides, were in comparison more potent than the enzyme-derived CBPs that were not lytic when applied to cells. It is proposed that the tested CBPs act as permeators that partition into the lipid bilayer of the cell membrane, thereby also promoting their interaction with hydrophobic domains of membrane proteins such as the MSH receptor, consequently eliciting the observed cellular responses.

Calmodulin (CaM),[1] an acidic highly conserved protein composed of 148 amino acid residues, is a ubiquitous calcium receptor which regulates a host of calcium-mediated cellular and physiological phenomena in eukaryotic cells (Manalan & Klee, 1984). In the last decade, it was discovered that CaM binds to a large number of short natural peptides in a highly calcium-dependent manner, with moderate to high affinities (Anderson & Malencik, 1986; O'Neil & DeGrado, 1990). These peptides can be categorized as cytotoxic venom peptides, such as melittin (honeybee) (Comte et al., 1983) or mastoparan (wasp) (Malencik & Anderson, 1983a), and hormonal peptides, such as adrenocorticotropic hormone (ACTH), vasoactive intestinal peptide (VIP), and β-endorphin (Malencik & Anderson, 1983b). A third group that recently attracted attention relates to synthetic peptides that represent the CaM-binding domain of Ca^{2+}/CaM-regulated enzymes. This group includes the synthetic analogues of the CaM-binding domains of skeletal muscle myosin light chain kinase (skMLCK), M5 (Kennelly et al., 1987), and the human erythrocyte plasma membrane calcium ATPase, C28W (Vorherr et al., 1990), used in this study, and others. Although the primary sequence and chain length of these peptides vary considerably, their affinities for CaM appear to correlate with their secondary structure. Namely, they all contain clusters of highly basic and hydrophobic residues that tend to form, in a hydrophobic environment, an α-helical structure which fits to the hydrophobic core ("bridge") of CaM (Cox et al., 1985). Synthetic fragments corresponding to the CaM-binding domain of MLCK were shown to inhibit CaM-stimulated enzymes due to competition with free CaM (Blumenthal et al., 1988).

We have previously shown that CaM-binding peptides (CBPs), such as melittin (Gerst & Salomon, 1987) and M5 (Gerst & Salomon, 1988), inhibit melanocyte-stimulating hormone (MSH) binding to its membranal receptor and con-

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[1] Abbreviations: AC, adenylate cyclase; ACTH, adrenocorticotropic hormone; CaM, calmodulin; CBPs, CaM-binding peptides; [diS-C_2-(5)], 3,3′-diethylthiadicarbocyanine iodide; DMF, dimethyl formamide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MSH, melanocyte-stimulating hormone; NBD-F, (4-fluoro-7-nitrobenz-2-oxa-1,3-diazole); PC, phosphatidylcholine; PS, phosphatidylserine; RP-HPLC, reversed-phase high-performance liquid chromatography; skMLCK, skeletal muscle myosin light chain kinase; SUV, small unilamellar vesicles.

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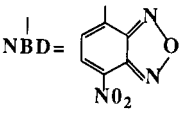
Table I: Amino Acid Sequences of Peptides and Peptide Derivatives Used in This Study



peptide	peptide designation	sequence
1	$ACTH_{1-17}$	β-AYSMEHFRWGKPVGKKKNH$(CH_2)_4$-NH_2
2	NBD-M5	NBD | KRRWKKNFIAVSAANRFG-NH_2
3	NBD-C28W	NBD | LRRGQILWFRGLNRIQTQIKVVNAFSSS
4	melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2
5	pardaxin	GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE
6	A18	EEIPEEELAEDVEEIDHA

sequent accumulation of cyclic AMP, in M2R mouse melanoma cells and cell membrane preparations. The mechanism by which these peptides affected the MSH receptor system remained elusive. The possibility that these CBPs may partition into the lipid bilayer and interact directly with membrane lipids and hydrophobic domains of lipid-inserted proteins was considered.

In the last decade, it was established that natural CBPs can interact with phospholipids, although the exact molecular mechanism involved is as yet unclear. In this regard, peptide toxins (e.g., melittin and mastoparan) were found to change membrane permeability in a nonspecific manner and even induce cell lysis as part of their toxic effect (Dempsey, 1990; Raynor et al., 1991). On the other hand, the interaction of peptide hormones such as glucagon (Epand, 1983), $ACTH_{1-24}$ (Gremlich et al., 1984), $dynorphin_{1-13}$ (Erne et al., 1985), and β-endorphin (Taylor & Kaiser, 1986) with membrane phospholipids was proposed to be beneficial for their function, yet their specific effects were strictly receptor-dependent.

There is much less evidence concerning the interaction of synthetic CBPs with membrane lipids although, as deduced from their structural properties, they should have the necessary capabilities (O'Neil & DeGrado, 1990). In contrast to a random-coil conformation of the free peptides, M5 (Garone & Steiner, 1990) and C28W (Vorherr et al., 1990) were assumed to gain a predominantly α-helical conformation (>50%) in complex with CaM. In an equal manner, as measured by nuclear magnetic resonance spectroscopy, similar conclusions concerning the interaction of M13 (the parental synthetic CaM-binding domain of MLCK) with CaM were recently reported (Ikura et al., 1992). In addition, $ACTH_{1-19}$ was shown to acquire an α-helical structure when dissolved in 2,2,2-trifluoroethanol (Toma et al., 1981). A recent study has shown that C20W, a truncated derivative of C28W, binds acidic phospholipids with high affinity (Brodin et al., 1992). This finding led to the proposal that the CaM-binding domain plays a role in the regulation of pump activity by acidic phospholipids.

In this study, we examined the ability of several CBPs (Table I) to interact with membrane phospholipids using small unilamellar vesicles (SUV) and M2R mouse melanoma cells and cell membrane preparations as model systems. M5 and C28W, as well as $ACTH_{1-17}$, were tested for their effects on phospholipid bilayers and cell membranes by four independent methods: (i) dissipation of valinomycin-induced diffusion potential (Loew et al., 1985), (ii) release of small-size fluorescent molecules entrapped in lipid vesicles (calcein, M_r = 623) (Allen & Cleland, 1980), (iii) interaction of 7-nitrobenz-2-oxa-1,3-diazol-4-yl-labeled (NBD-labeled) M5 and C28W with vesicles (Rapaport & Shai, 1991) or with M2R cell membranes, and (iv) inhibition of [^{125}I]iodo[Nle^4,D-Phe^7]αMSH binding to cultured M2R mouse melanoma cells.

The results demonstrate the ability of the tested CBPs to interact with and permeate artificial and native cell membranes. These properties probably relate to their aforementioned structural features and may be important in their capacity to interact with and affect M2R melanoma cells.

EXPERIMENTAL PROCEDURES

Materials

Chemicals. Calcein was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). 3,3'-Diethylthiadicarbocyanine iodide [diS-C_2-(5)] was obtained from Molecular Probes (Junction City, OR). Phosphatidylcholine (PC) and phosphatidylserine (PS) from bovine spinal cord (grade I), monosodium salts, and egg lecithin (grade I) were purchased from Lipid Products Ltd. (South Nutfield, NJ). Valinomycin and (4-fluoro-7-nitrobenz-2-oxa-1,3-diazole) (NBD-F) was obtained from Sigma Chemical Co. (St. Louis, MO). Cholesterol (extra pure) was purchased from E. Merck (Darmstadt, FRG) and recrystallized twice from ethanol. All other reagents were of analytical grade. Buffers were prepared in double-glass-distilled water.

Peptides. Melittin and [Nle^4,D-Phe^7]αMSH were obtained from Sigma. $ACTH_{1-17}$ (Synchrodyn 1–17) was kindly provided by Dr. Wolfrad von Rechenberg (Hoechst AG, Frankfurt, FRG). $ACTH_{1-24}$ was kindly provided by Dr. J. A. D. M. Tonnaer (Organon, Oss, Holland). C28W and A18 peptides were synthesized by the Fmoc/*tert*-butyl strategy and purified as previously described (Vorherr et al., 1990). Pardaxin was synthesized and purified by RP-HPLC as described previously (Shai et al., 1990). M5 was synthesized by the solid-phase method on (phenylacetamido)methyl-amino acid resin using *tert*-butyloxycarbonyl-amino acids (430A peptide synthesizer, Applied Biosystems) in the core facility of the chemical services at the Weizmann Institute of Science.

The crude peptide in one major peak was found to be 50–60% pure by weight. The peptide was further purified by RP-HPLC on a C_4 reversed-phase Vydac column (300-Å pore size). Elution conditions: linear gradient from 10% acetonitrile (HPLC grade) in 0.1% trifluoroacetic acid at time 0 to 60% acetonitrile in 0.1% trifluoroacetic acid at 40 min. The flow rate was 0.9 mL/min. The pure peptide was shown to be homogeneous (95–99%) by analytical HPLC and amino acid analysis. Peptide identity was further confirmed by amino acid sequence analysis performed at the core facility of the Biological Services of the Weizmann Institute.

Methods

NBD Labeling of CaM-Binding Peptides. Selective labeling of purified peptides with NBD, at their N-terminal amino acid, was achieved under conditions in which the ε-amino group of the lysine residues is not modified, as previously described (Rapaport & Shai, 1991): HPLC-purified M5 and C28W (~1–2 mg, trifluoroacetic acid salt) were reacted with fluoro-NBD (2 equivalents) in dry DMF for 24–48 h. Under these conditions, one product was almost solely obtained possessing an NBD moiety attached to the N-terminal amino group of the peptide. Alternatively, NBD modification of C28W was performed in the resin-bound state prior to HF cleavage, yielding the same peptide derivative (Rapaport & Shai, 1991). NBD-labeled peptides were purified by RP-HPLC on a C_4 reversed-phase Vydac analytical column (300-Å pore size). At a flow rate of 0.9 mL/min, the peptide eluted at 40 min using a linear gradient of 15–60% acetonitrile in water in the presence of 0.1% trifluoroacetic acid (v/v).

Culture of M2R Mouse Melanoma Cells. M2R mouse melanoma cells were cultured as described previously (Gerst et al., 1986).

MSH Binding Studies. Binding of [^{125}I]iodo-[Nle^4,D-Phe^7]αMSH to M2R cells was performed as described previously for [^{125}I]βMSH (Gerst et al., 1986). ^{125}I iodination of [Nle^4,D-Phe^7]αMSH was performed by the chloramine-T method, as previously described (Leiba et al., 1990).

Preparation of a Plasma Membrane-Enriched Fraction from M2R Mouse Melanoma Cells. Plasma membranes from cultured M2R cells were prepared by differential centrifugation as previously described (Gerst et al., 1986). Cell membrane fractions, essentially melanosome-free, in 1 mM bicarbonate buffer (pH 7.0), were divided into aliquots (0.5 mL/tube) and kept frozen in liquid nitrogen until used.

Preparation of Liposomes. Small unilamellar vesicles (SUV) were prepared as described earlier (Shai et al., 1990). Briefly, phospholipids and cholesterol (10% w/w) were dissolved in chloroform/methanol (2:1) in appropriate volumes, to give a concentration of 7.5 mg/mL soybean lecithin (w/v); 7 mg/mL (w/v), 1:1 mixture of PS and PC; or 7 mg/mL (w/v) PC. The solvents were evaporated with a stream of nitrogen, and the lipids were suspended in appropriate buffer by vortex mixing. The lipid dispersion was sonicated 3 × 10 min in a bath-type (16–18 °C) sonicator (J. P. Selecta, Spain). Lipid concentration was determined by phosphorus analysis (Bartlett, 1959).

Membrane Permeability Studies

I. Valinomycin-Mediated Diffusion Potential Dissipation Assay. Pore-mediated diffusion potential collapse was detected fluorimetrically (Loew et al., 1985), as described previously (Shai et al., 1990). Briefly, 4 μL of a liposome suspension prepared in K^+ buffer (50 mM K_2SO_4 and 10 mM HEPES, pH 6.8) was added to 1 mL of isotonic K^+-free buffer (50 mM Na_2SO_4 and 10 mM HEPES, pH 6.8) in a glass tube, and the membrane potential sensitive dye diS-C_2-(5) was added (1 μM final concentration). Under these conditions, the gain of the fluorimeter (Perkin-Elmer LS-5B) was adjusted to 100%. Valinomycin, 1 μL of 10^{-7} M solution, was then added to slowly create a negative diffusion potential inside the vesicles by selectively carrying K^+ outside, which caused an additional 60–80% quenching of the dye's fluorescence. After the fluorescence was stable (10–20 min), the different test peptides were added, causing a relative permeation of the other ions, namely, an influx of Na^+ and an efflux of SO_4^{2-}, resulting in dissipation of the diffusion potential as monitored by the increase of the fluorescence. Fluorescence recovery was monitored continuously for 15 min, until stable (excitation was set at 620 nm, and emission was monitored at 670 nm; slit widths were 10 and 5 nm, respectively).

II. Calcein Release from Vesicles Induced by CBPs. Calcein-containing vesicles [formed from soybean lecithin, PC, or PS/PC (1:1, w/w), all containing 10% (w/w) cholesterol; calcein M_r = 623] were prepared with a self-quenching concentration of 60 mM calcein (Allen & Cleland, 1980) in 10 mM HEPES at pH 7.4. Calcein release by the test peptides was assayed as described previously (Shai et al., 1990; Pouny et al., 1992). Briefly, the nonencapsulated calcein was removed from the liposome suspension by gel filtration, using a Sephadex G-50 (Pharmacia) column connected to a low-pressure LC system (Pharmacia). In a typical run, 20 μL of the liposome suspension was injected onto the column and eluted in 10 mM HEPES, SO_4^{-2}, and 150 mM NaCl, pH 7.4. The eluent was monitored by UV spectroscopy (λ = 280 nm), and the vesicles peak was collected and diluted to a volume of 2 mL, in the same buffer. CBPs, at the indicated concentrations, were added to 1-mL gently stirred vesicle suspensions (containing 1.3 μM liposomes). The resulting peptide-induced calcein leakage, reflected as an increase in fluorescence (Allen & Cleland, 1980), was monitored at room temperature on a Perkin-Elmer LS-5B spectrofluorimeter, at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Complete dye release (set as 100% leakage) was obtained after disrupting the vesicles with Triton X-100 (0.1% final concentration). In the absence of peptide, the leakage rate was less than 1% in 1 h.

Intrinsic Fluorescence Measurements of NBD-Labeled Peptides. Changes in the intrinsic fluorescence of NBD-labeled peptides were measured upon binding to SUVs or M2R cell membranes, as described previously (Rajarathnam et al., 1989; Rapaport & Shai, 1991). The emission spectra of the free NBD-labeled peptides in 2 mL of 10 mM HEPES buffer, pH 7.4, containing 0.2 μM NBD-labeled C28W or 0.2 μM NBD-labeled M5, were recorded as reference. SUVs or M2R cell membranes (~200 μg/20 μL) were added subsequently, to establish a lipid/peptide ratio in which essentially most of the peptide becomes lipid bound. Following a 2-min incubation, maximal emission spectra (λ_{max}) were recorded (3–6 separate experiments) using an SLM-8000 spectrofluorimeter (excitation at 468 nm; 8-nm slit).

NBD-Peptide Binding Experiments. The degree of peptide association with lipid vesicles was measured by adding SUVs (prepared as described above) to a fixed amount of peptide, 0.2 μM NBD-C28W or 0.5 μM NBD-M5, at room temperature. The fluorescence intensity was measured as a function of the lipid/peptide molar ratio (3–7 separate experiments) on an SLM-8000 spectrofluorimeter (excitation was at 468 nm, and emission was at 540 nm; 8- and 4-nm slit, respectively). In order to determine the extent of the contribution of the lipid to any given signal, the readings observed when

unmodified M5 or C28W were titrated with lipid vesicles were subtracted as background from the recorded fluorescence intensity.

The partition coefficient describing the interaction of the NBD-peptides with the liposomes was analyzed from the binding isotherms as a partition equilibrium (Beschiaschvili & Seelig, 1990, 1991; Rizzo et al., 1987; Schwarz et al., 1986), as previously described (Rapaport & Shai, 1991), using the following formula:

$$X_b = K_p \cdot C_f \quad (1)$$

where X_b is defined as the molar ratio of bound peptide per total lipid, K_p corresponds to the partition coefficient, and C_f represents the equilibrium concentration of free peptide in the solution. In order to calculate X_b, the fraction of membrane-bound peptide, f_b, was calculated according to the following formula:

$$f_b = (F - F_0)/(F_\infty - F_0) \quad (2)$$

where F_0 represents the fluorescence of unbound peptide; F, the fluorescence of the peptide after addition of SUVs; and F_∞, the fluorescence obtained when all the peptide is bound to the lipid, an estimate obtained at the maximal plateau level of the lipid-to-protein ratio. The free peptide concentration, C_f, as well as X_b, could now be calculated. As suggested by Beschiaschvili and Seelig (1990), the peptides were assumed to bind only to the outer half-layer of the SUV. Therefore, values of X_b were corrected as follows:

$$X_b^* = X_b/0.6 \quad (3)$$

K_p was calculated from the initial slopes obtained in plots of X_b *versus* C_f. SUVs were used in the assay in order to minimize light-scattering effects (Mao & Wallace, 1984).

RESULTS

We examined whether M5 and C28W can interact with phospholipids by probing the ability of their NBD derivatives to immerse in SUVs and in melanoma cell membranes. We also tested if the interaction of native CBPs induces changes in the membrane permeability by measuring the release of entrapped ions and fluorescent molecules. In addition, the inhibitory effect of C28W and its NBD derivative on MSH binding to M2R mouse melanoma cells was investigated.

I. The Interaction of NBD-CBPs With Liposomes and Cell Membranes. NBD fluorescence reflects the environment in which the NBD group is located, displaying higher quantum yield and blue shift of maximal emission wavelength in more hydrophobic environments. The spectral behavior of NBD-modified peptides can, therefore, provide information about the partition of the peptide between hydrophilic and hydrophobic milieus.

The maximum emission wavelengths of the free NBD-M5 and NBD-C28W are given in Table II. Upon addition of soybean lecithin vesicles or M2R cell membranes to the peptide solutions, fluorescence emission maxima of both peptides exhibited a blue shift and a marked increase in fluorescence intensities. These changes indicate a relocation of the NBD group into a more hydrophobic environment of the liposome. The extent of the blue shift (from 544 ± 1 to 525 ± 1 nm) observed with NBD-C28W was slightly larger, in liposomes and in M2R cell membranes, when compared to the blue shift (from 544 ± 1 to 529 ± 1 nm) exhibited by NBD-M5. In addition, there were differences between the relative fluorescence increases, 4–5-fold for NBD-C28W as compared to 2–3-fold for NBD-M5. However, the extent of the blue shift and the increase in fluorescence intensity induced by each of the NBD peptides in liposomes or M2R cell membranes was very similar (Table II). In these experiments, the lipid/peptide molar ratio was ≥300:1 and the spectral contributions of the free peptide could, therefore, be considered negligible, as further shown below.

Table II: Fluorescence Emission Maxima of NBD-Labeled CBPs, in the Presence of Soybean Lecithin SUVs or M2R Cell Membranes[a]

solution	NBD-derivative[b]	λ_{max} (nm)[c]	rel fluorescence intensity
buffer	NBD-C28W/M5	545 ± 1	1.0
liposomes	NBD-M5	530 ± 1	2.7
liposomes	NBD-C28W	525 ± 1	4.6
buffer	NBD-C28W/M5	543 ± 1	1.0
cell membranes	NBD-M5	529 ● 1	2.0
cell membranes	NBD-C28W	526 ± 1	4.8

[a] Changes in the intrinsic fluorescence of NBD-M5 (0.2 μM) and NBD-C28W (0.2 μM) were measured upon mixing with soybean lecithin SUVs (200 μM) or with M2R cell membranes (200 μg) in 2 mL of 10 mM HEPES, pH 7.4, and were further monitored as described under Experimental Procedures. [b] Peptide designations are as in Table I. [c] The excitation wavelength was 468 nm.

In order to assess the possible involvement of electrostatic forces in the CBPs/lipids interaction, we conducted similar experiments in the presence of up to 1 M NaCl. We found that the blue shift and the increase in fluorescence intensity induced by NBD-M5 and NBD-C28W were unaffected under these conditions. The truncated pardaxin NBD-*C*-helix that interacts strongly with SUVs and the NBD-*S*-protein, which is inert in this regard (Rapaport & Shai, 1991), served as positive and negative controls, respectively, in this study. Independent assessments for the interaction of the NBD-peptides (represented by NBD-C28W) with liposomes (Figure 3A) and melanoma cells (Figure 4) are further described below.

II. Characterization of Binding Isotherms of NBD-C28W and NBD-M5. We next determined the partition coefficients of both NBD-peptides in liposomes (Figures 1 and 2). NBD-C28W (0.2 μM) or NBD-M5 (0.5 μM) was titrated with increasing concentrations of soybean lecithin SUVs. The increases in the fluorescence intensities of NBD-M5 (Figure 1A) and NBD-C28W (Figure 2A) were plotted as a function of the lipid/peptide molar ratios. The concentrations of the peptides used were kept low enough to minimize aggregation in the aqueous phase. Control experiments were performed by titrating unlabeled C28W or M5 with soybean vesicles up to the maximal concentration used in the assay. The fluorescence intensities of these solutions (≤10% of the experimental value) were subtracted. In order to compare the binding characteristics of the NBD-labeled CBPs in the soybean vesicles system, the binding isotherms were further analyzed to calculate the partition coefficients, K_p (Figures 1B and 2B). The partition coefficients ($K_{pNBD\text{-}M5} = 7 \times 10^3$ M^{-1}; $K_{pNBD\text{-}C28W} = 2.8 \times 10^4$ M^{-1}) were estimated from the initial slopes of the curves (Figures 1B and 2B, insets).

Valinomycin-Mediated Diffusion Potential Assay. The ability of the CBPs to permeate the liposome membrane was assessed in the dissipation of diffusion potential experiments, by monitoring the fluorescence recovery until a plateau level was attained. CBPs, at increasing concentrations up to 10 μM, were mixed with SUVs (at constant concentration) composed of soybean lecithin (Figure 3A) or PS/PC (Figure 3B), pretreated with the fluorescent dye [diS-C_2-(5)] and valinomycin. The peptide/lipid ratios ranged from 0.009:1 to 0.2:1 for the tested CBPs. Recovery of the fluorescence (initially quenched by valinomycin) was measured at equi-

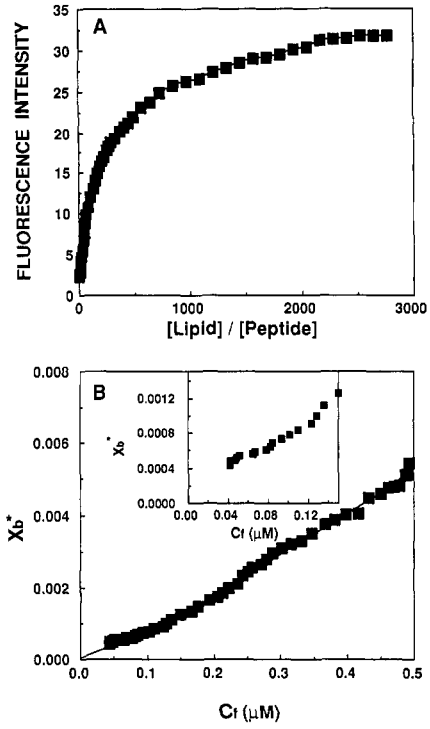


FIGURE 1: (A) Changes in fluorescence of NBD-M5 (0.5 μM final concentration) upon titration with soybean lecithin vesicles. Excitation was set at 468 nm, and emission was monitored at 530 nm. The experiment was performed at room temperature in 10 mM HEPES buffer, pH 7.4. (B) Binding isotherms derived from panel A by plotting X^*_b (extent of binding) *versus* C_f (free peptide). Calculations of X^*_b and C_f were performed according to eqs 1, 2, and 3 under Experimental Procedures. The partition coefficient was estimated from the initial slope of the curve as shown in the inset.

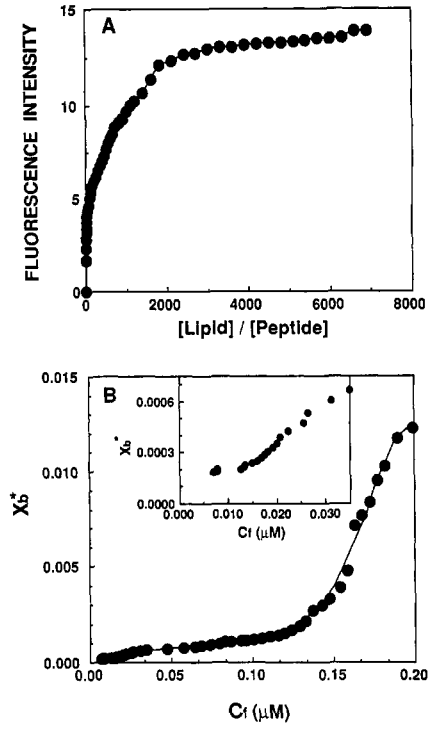


FIGURE 2: (A) Changes in fluorescence of NBD-C28W (0.2 μM final concentration) upon titration with soybean lecithin vesicles, under the same conditions as described in Figure 1A. (B) Binding isotherms derived from panel A by application of the procedure described in Figure 1B. The partition coefficient was estimated from the initial slope of the curve as shown in the inset.

librium (usually within 15–20 min) for each peptide concentration. This level was taken as the maximal activity attainable at each concentration of the peptide tested. In general, the profile of the fluorescence recovery, induced by the CBPs in both soybean and PS/PC liposomes, was found to be similar. One can clearly see that M5 and $ACTH_{1-17}$ have a lower potency than C28W in inducing the fluorescence recovery. Compared to M5, $ACTH_{1-17}$ is slightly more potent in soybean vesicles and slightly less potent in PS/PC vesicles. In preliminary experiments, we found $ACTH_{1-24}$ to be less efficient than $ACTH_{1-17}$, while α-MSH, which is equivalent to $ACTH_{1-13}$, was found to be totally inactive in this assay. The shark-repellent peptide toxin pardaxin, used as a reference in this study, was much more potent, irrespective of the phospholipid composition of the liposomes. On the other hand, A18, a nonrelevant, negatively charged, 18 amino acid synthetic peptide derived from the C-terminus of the human erythrocyte Ca^{2+} ATPase (Vorherr et al., 1990), was totally ineffective in this regard. In similar experiments conducted with PC vesicles, no effect of the tested CBPs was observed (data not shown).

In addition, we tested in this assay the NBD-modified C28W. This was performed in order to examine whether the presence of the NBD group altered the activity of the NBD-peptide, compared to the parent peptide. It can be seen that the activities of both peptides are essentially identical, indicating that the introduction of the NBD group at the N-terminus of C28W had no effect on the peptide membrane-permeating activity (Figure 3A). Each experiment was repeated three to five times with freshly prepared SUVs.

Inhibition of [^{125}I]Iodo-[Nle^4,D-Phe^7]αMSH Binding to M2R Mouse Melanoma Cells by C28W and NBD-C28W.

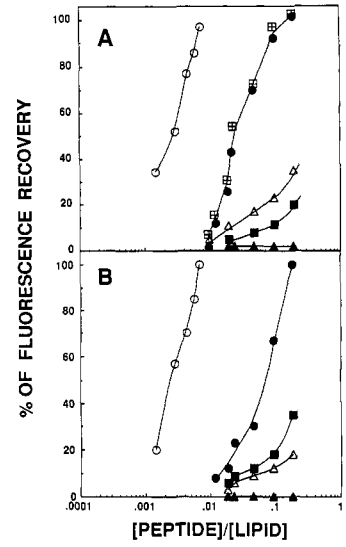


FIGURE 3: Dissipation of diffusion potential induced by CBPs. CBPs and/or control peptides were added to 1 mL of buffer containing a constant concentration of soybean lecithin vesicles (A) or PS/PC vesicles (B) (phospholipid concentration = 38 μM), preequilibrated with the fluorescent dye diS-C_2-(5) and valinomycin. Fluorescence recovery was measured at equilibrium (after 10–15 min) and is plotted as a function of the peptide/lipid molar ratio. ○, pardaxin; △, $ACTH_{1-17}$; ■, M5; ●, C28W; ⊞, NBD-C28W; ▲, A18.

We performed another independent assay in order to examine whether the NBD moiety, attached to the N-terminus of C28W, affects the biological activity of the native peptide. When M2R mouse melanoma cells were incubated with increasing concentrations of C28W or NBD-C28W (0.3–20 μM) in the presence of [^{125}I]iodo-[Nle^4,D-Phe^7]αMSH (1 nM), similar dose-dependent inhibition profiles of hormone binding were observed (Figure 4). In good agreement with the

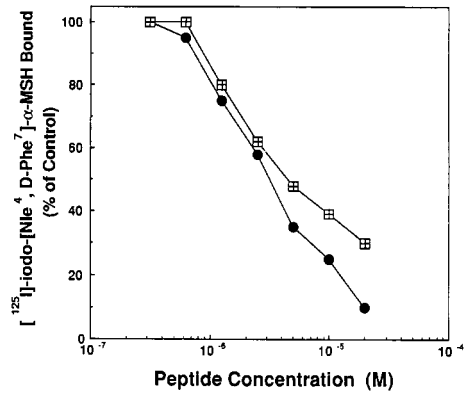


FIGURE 4: Dose-dependent inhibition of [^{125}I]iodo-[Nle^4,D-Phe^7]-αMSH binding to M2R mouse melanoma cells by C28W and NBD-C28W. M2R cells, cultured in 24-well plates, were incubated for 45 min with [^{125}I]iodo-[Nle^4,D-Phe^7]αMSH (1 nM) and increasing concentrations of C28W and NBD-C28W (0.3–20 μM), under standard binding assay conditions (see Experimental Procedures). ●, C28W; ⊞, NBD-C28W. Binding in the absence of CBP amounted to 14 000 cpm; nonspecific binding determined in the presence of 1 μM unlabeled [Nle^4,D-Phe^7]αMSH was 90 cpm. Specific binding, taken as 100%, was calculated as the difference between these two values and served as a control.

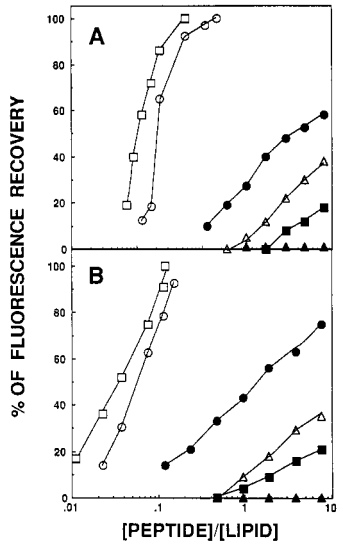


FIGURE 5: Calcein release induced by CBPs. Increasing amounts of CBPs were added to 2.5 μM soybean lecithin vesicles (A) or PS/PC vesicles (B) containing entrapped calcein at a self-quenching concentration in 1 mL of buffer (10 mM HEPES and 150 mM NaCl, pH 7.4). Fluorescence recovery was measured after 10 min and is plotted as a function of the peptide/lipid molar ratio. Designations are as in Figure 3; □, melittin.

experiments described earlier (Figure 3A), these results also show that the NBD group does not appear to affect the interaction of C28W with cell membranes or liposomes. This representative experiment was repeated three times with similar results.

Calcein Release Assay. Leakage of small fluorescent molecules entrapped in liposomes, such as calcein, could either result from the formation of large pores in the liposome membrane or be due to destabilization of the lipid bilayer/membrane structure. The CBPs were tested for their capability to evoke calcein release from SUVs. The dose–response behavior of the calcein release induced by the CBPs as a function of the peptide/lipid molar ratios is shown for soybean lecithin (Figure 5A) and for PS/PC (Figure 5B). It can be seen that the potency of the CBPs in this measurement was similar to those observed in the diffusion potential assay. As above, among the nontoxic CBPs, C28W was the most efficient candidate for permeating the membrane. ACTH$_{1-17}$ had a slightly higher potency than M5 in both liposome preparations. The toxin peptides pardaxin and melittin were much more potent in their ability to release calcein relative to the CBPs. The peptide/lipid molar ratios in this assay for the CBPs varied from 0.1:1 to 8:1, which is about 10-fold higher than required for inducing the dissipation of the diffusion potential by these peptides. As above, A18 appeared to be totally ineffective. As in the diffusion potential assay, the CBPs were found to be inactive when tested with PC liposomes.

DISCUSSION

Using four independent experimental methods, we demonstrated in this study that the enzyme-derived CBPs M5, C28W, and NBD-C28W interact with artificial phospholipid bilayers and with native M2R melanoma cells and cell membranes. While the NBD-CBP derivatives directly and quantitatively probed the partition of CBPs into the lipid phase of the membrane, the dissipation of the membrane potential and the dye release assay served as measures for changes in membrane permeability induced by CBPs. It is proposed that this property of CBPs forms the basis for the observed effects of M5, C28W, and other CBPs on the cellular response to MSH, reported here (Figure 4) and described previously (Gerst & Salomon, 1987, 1988; Eshel et al., 1991).

Fluorescence measurements with NBD-M5 and NBD-C28W were first employed to assess the interaction with soybean lecithin SUVs and M2R cell membranes. The blue shift in the spectra of the NBD-peptides, as well as the increase in the fluorescence intensity induced upon addition of the SUVs or M2R cell membranes, clearly showed that NBD-CBPs partition into membrane phospholipid vesicles and native biological membranes (Table II). Hydrophobic, rather than electrostatic, forces seem to be cardinal in the interactions of NBD-CBPs with the lipid bilayer, since the inclusion of 1 M NaCl had no effect on the spectrum profile. Yet, when tested on PC vesicles, CBPs were ineffective (data not shown). Since the tested peptides are positively charged, the difference in their activities with PC or PS/PC liposomes could be explained by the fact that negative and positive charges counteract each other in the PS/PC–peptide system but not in the PC–peptide system. In the latter case, electrostatic repulsion between peptide monomers would eliminate their aggregation within the membrane, thus preventing the permeation effects. Further support that electrostatic interactions may be involved in the binding of the CaM-binding domain of the plasma membrane calcium pump with acidic phospholipids was given recently, using the truncated C20W peptide (Brodin et al., 1992).

The greater blue shift and larger increase in fluorescence intensity seen with NBD-C28W as compared to NBD-M5 indicate that the NBD moiety is in a more hydrophobic environment. Using either soybean liposomes or M2R cell membranes resulted in the same phenomenon, suggesting that the nature of interaction of NBD-CBPs in both model systems is likely to be similar. The fact that the cell membranes contain proteins appears to be of minor importance in the initial engagement of NBD-CBPs with the bilayer. However, our results suggest that if interactions of CBPs with specific membrane proteins (e.g., the MSH receptor, G-proteins, or others) indeed occur, they may also take place within lipid-embedded domains of these proteins and affect their activity. Such effects may be reflected by the inhibitory activity of CBPs on MSH binding and activation of AC (Gerst &

Salomon, 1987, 1988; Leiba et al., 1990), as also demonstrated for C28W and its NBD derivative in this work (Figure 4). As mentioned, the ability of C20W to interact with acidic phospholipids and compete with their activation of the Ca^{2+} ATPase has been shown recently (Brodin et al., 1992).

The interaction of the CBPs with phospholipid vesicles appears to destabilize the vesicles' structure and consequently permeabilize them, as revealed by the diffusion potential experiments (Figure 3) and the calcein release assay (Figure 5). In these assays, C28W was more potent (~10-fold) than M5. The higher potency of C28W compared to M5 might be explained by the fact that, at similar peptide/lipid molar ratios, more C28W is bound to the membrane than M5 (Figures 1 and 2). This is further demonstrated by the upper curvature of the binding isotherm of C28W (Figure 2B) compared to that of M5 (Figure 1B). Although C28W binds to phospholipid membranes in approximately the same way as pardaxin (Shai et al., 1990) or melittin (Schwarz & Beschiaschvilli, 1989), its permeabilizing activity is much lower. This might explain the higher toxicity of pardaxin and melittin, compared to C28W.

While the binding isotherm for NBD-M5 was linear over the tested range of concentrations (Figure 1B), the binding isotherm describing the partition of NBD-C28W bent upward at $Cf \sim 0.15\ \mu M$ (Figure 2B). This change in slope for C28W argues in favor of a biphasic process, whereby the peptide first incorporates into the bilayer and then self-associates. Such a behavior around a threshold concentration of the peptide was previously described for alamethicin (Rizzo et al., 1987) and pardaxin (Rapaport & Shai, 1991) and was related to their pore-forming activity. Thus, the steep slope in the curves obtained with NBD-C28W (Figures 2 and 3) may be compatible with pore-forming activity of this peptide, observed at relatively higher concentrations compared to pardaxin and its analogues (Shai et al., 1990; Rapaport & Shai, 1991). However, a simple aggregation process, which leads to defects in the membrane's structure, cannot be excluded. In contrast, the linearity of the binding isotherm of NBD-M5 suggests that such a critical concentration point, if exists for this peptide, was not reached.

$ACTH_{1-17}$, which is a potent ligand of the MSH receptor in melanoma cells (Eberle, 1988; Gerst et al., 1986), also falls into the same category as M5, within the weaker group of the tested peptides (Figures 3 and 5). Therefore, at the range of concentrations tested, it is quite obvious that M5 and probably $ACTH_{1-17}$ cannot be designated as pore-forming peptides. However, they can affect cell membrane permeability by interacting with membrane lipids, without inducing cell lysis. Moreover, these peptides, including C28W, may have a better chance to interact with membrane proteins, including hormone receptors, and in this way induce cellular effects that are milder and more selective. Unfortunately, the partition coefficients for NBD-M5 and NBD-C28W in M2R cell membranes could not be determined due to the fact that increasing concentrations of membranes in the cuvette caused light scattering levels that interfered with the fluorescence measurements. However, using an independent measure, we confirmed the interaction of C28W and NBD-C28W with cultured melanoma cells. In this method, we determined the inhibition of [^{125}I]iodo-[Nle^4,D-Phe^7]αMSH binding to the cells by increasing concentrations of the CBPs, obtaining a similar IC_{50} around 5 μM (Figure 4). This observation, together with the results shown in Figure 3A, provided independent evidence that the modification of CBPs with NBD does not change the interaction properties of these peptides with liposomes and cell membranes to any large extent.

For ACTH specifically, Schwyzer and his colleagues suggested that the lipid milieu of the membrane actually serves as a catalyst that facilitates the binding of the hormone to its receptor (Sargent & Schwyzer, 1986). By using different biophysical methods, these investigators demonstrated that ACTH fragments interact strongly with artificial membranes (Gremlich et al., 1984, 1981; Gysin & Schwyzer, 1983, 1984; Schoch et al., 1979). Herein, we found that $ACTH_{1-17}$, which was not tested by these investigators, also interacts with artificial liposomes. $ACTH_{1-17}$ had a slightly higher potency than $ACTH_{1-24}$ in inducing the dissipation of the diffusion potential in soybean vesicles. On the other hand, αMSH, which corresponds to $ACTH_{1-13}$, was totally ineffective in this regard (data not shown). Our results are in good agreement with a recent report concerning the interaction of $ACTH_{1-24}$, and a few other bioactive peptides, with PC- and cardiolipin-containing vesicles (De Kroon et al., 1991).

We showed here (Figure 4) and previously that melittin (Gerst & Salomon, 1987), M5 (Gerst & Salomon, 1988), and M5 and C28W (Eshel et al., 1991) inhibit MSH binding and stimulation of AC in melanoma cells and in lacrimal tissue (Leiba et al., 1990). These effects were dose-dependent in a similar concentration range (1–10 μM) to those seen in this study on liposome permeability (Figures 3 and 5). Yet another indication for the interaction of these CBPs with the cell membrane became recently evident, when we found that these and other synthetic CBPs, on their own, are weak stimulators of AC activity in M2R cells, under certain assay conditions (Y. Eshel and Y. Salomon, unpublished results). Cumulatively, these observations all suggest that the CBPs first penetrate the lipid bilayer of the cell membrane and may subsequently interact with protein domains embedded therein. These proteins may include components of the hormone-sensitive AC system, not excluding intracellular proteins such as G-protein subunits that associate with the cytoplasmic face of the plasma membrane.

The possibility that enzyme-derived CBPs, represented in this study by M5 and C28W, may also be involved in certain discrete interactions of their respective enzymes with membrane lipids can be postulated, on the basis of the results presented above and in the previously mentioned work on the stimulation of the plasma membrane Ca^{2+} pump with acidic phospholipids (Brodin et al., 1992). Moreover, these peptides have the capacity to assume an α-helical conformation (Garone & Steiner, 1990; Toma et al., 1981; Vorherr et al., 1990) which appears to be mandatory for establishing the hydrophobic interaction with CaM (Bagchi et al., 1992) and with the endogenous autoinhibitory pseudosubstrate sites in the respective CaM-dependent enzymes (Kennelly et al., 1987; Vorherr et al., 1990). The occurrence of amphipathic α-helix-forming peptides in amphitropic enzymes is recently being recognized as potentially important in their interaction with and translocation to membranes. These include diacylglycerol kinase (DGK) (Sakane et al., 1991), phosphocholine cytidylyltransferase (Kalmar et al., 1990), carboxypeptidase E (Fricker et al., 1990), and apolipoprotein A-1 (Fukushima et al., 1979; Segrest et al., 1974). However, the isolated peptides containing these amino acid sequences were not tested experimentally (except for apolipoprotein A-1; Fukushima et al., 1979) for their ability to interact with lipid bilayers.

Although the exact mechanism by which CBPs affect cells remains elusive, the results herein correlate with the biological effects of these peptides, which are attributed to their presence

within the cell membrane. The possibility that moderate membrane permeators of this class can act upon cells may have biomedical significance extending beyond the scope of this study. More experiments are needed to determine whether changes in cell membrane permeability alone, or possibly also interaction with putative membrane-bound enzymes, are important in the mode of action of CBPs upon cells.

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