Synthesis and characterization of bivalent peptide ligands targeted to G-protein-coupled receptors

Michael D Carrithers[1] and Michael R Lerner[2,3†]

Background: Through the effects of avidity, multivalency can increase the apparent affinity of a ligand for its binding site. Low molecular weight, high affinity, multivalent ligands theoretically could be used to deliver a variety of agents to specific cell subtypes. In order to target specific G-protein-coupled receptors, a series of monospecific peptide dimers were synthesized that are designed to bind to two adjacent receptor sites.

Results: Three dimers, consisting of a ligand region, a short, flexible, uncharged spacer, a longer, polylysine spacer and a single cysteine residue to permit dimerization, and the corresponding monomers were synthesized by solid-phase peptide synthesis. The ligand domain was either α-melanocyte stimulating hormone (α-MSH), an α-MSH receptor antagonist (α-MSH-ANT), or bombesin. These ligands were characterized in a functional melanocyte dispersion assay. In wild-type melanophores, the α-MSH dimer stimulated dispersion with an EC_{50} approximately seven-fold lower than that of the corresponding monomer. Similarly, in cells transfected with bombesin receptor cDNA, the bombesin dimer was approximately five-fold more potent than the monomer. The α-MSH-ANT monomer specifically inhibited α-MSH-mediated dispersion with no significant agonist activity, but the dimer acted predominantly as an agonist.

Conclusions: Peptide dimers can be synthesized easily and have enhanced functional activity; monospecific dimers have greater avidity and bispecific dimers are likely to have greater selectivity. They may therefore have practical potential as specific cell-targeting agents.

Addresses: Departments of [1]Neurology, [2]Pharmacology, and [3]Internal Medicine, Yale University School of Medicine, New Haven, CT 06520-8024, USA.

Present address: [†]Department of Dermatology, University of Texas Southwestern Medical Center, F4.100, 5323 Harry Hines Boulevard, Dallas, TX 75235-9069, USA.

Correspondence: Michael Lerner
e-mail: MRLERNER@aol.com

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Introduction

The high affinity of immunoglobulins for their binding sites is at least partially attributable to their multivalency. Based on the entropic effects of avidity, multivalency can theoretically increase apparent binding affinity by several orders of magnitude [1]. Although the observed affinity gain is usually more modest, multivalent molecules have potentially powerful applications in clinical pharmacology. For example, synthetic bivalent ligands can be used to target toxins, drugs and, potentially, plasmid DNA to specific cell subtypes.

Thermodynamically, the binding of a multivalent antibody to adjacent epitopes on the cell surface is similar to the chelate effect [2,3]. Although this effect was described originally to explain the enhanced stability of chelate rings, it is also relevant in rate accelerations of enzymic reactions and in base-pair formation by polynucleotides [4,5]. The common feature of these reactions is that following the initial reaction (e.g., binding of one antibody 'arm' to its antigen), each succeeding reaction (e.g., binding of the second antibody 'arm' to an adjacent epitope) is more favorable because the entropy loss is decreased.

Specific examples of bivalent molecules include small bivalent antibodies composed of either antibody fragments (F_{ab}) or single chain antibodies (F_v) [6–8]. In addition, numerous other multivalent molecules have been designed, including bivalent carbohydrates and a variety of synthetic drug delivery systems [9,10].

Bivalent peptides, such as receptor-adhesive modular proteins (RAMPs), represent an alternative approach to cell targeting [11,12]. These large synthetic peptides, which contain two ligand sites separated by a spacer region and a dimerization domain, are designed to bind to two membrane receptors simultaneously. In the original design, the dimerization domain consisted of a two-stranded parallel α-helical coiled coil, and the ligand region was composed of two identical integrin receptor binding peptides. The two ligand domains were separated by at least 50 Å, but the authors were unable to detect increased affinity of their dimeric constructs. These results suggested that these dimers could not bind to two receptors at the same time.

We here propose a simplified approach for the synthesis of peptide dimers. The design consists of monomeric

ligands with a ligand domain, a short, uncharged spacer region, a longer, charged polylysine region and a sulfhydryl dimerization domain. The monomers can be synthesized rapidly by solid-phase peptide synthesis and dimerized by oxidation. This design provides both proper spacing between the ligands and appropriate flexibility. In addition, the polylysine region permits the conjugation of small molecular weight drugs to the dimer and could also allow its incorporation into a synthetic gene-therapy vector [13].

We report the design and synthesis of three monospecific peptide dimers to target two specific G-protein-coupled receptors. Prior work in another laboratory demonstrated that antibody-mediated dimerization of an agonist of the gonadotropin-releasing hormone receptor resulted in a modest increase in functional activity and that antagonist dimerization caused conversion of the ligand to an agonist [14,15]. Here we have used ligand-binding domains consisting of α-melanocyte stimulating hormone (α-MSH), an α-MSH receptor antagonist (α-MSH-ANT) [16], or the neuropeptide bombesin. The dimeric and monomeric forms of these constructs were characterized functionally in a melanocyte dispersion assay [17–20].

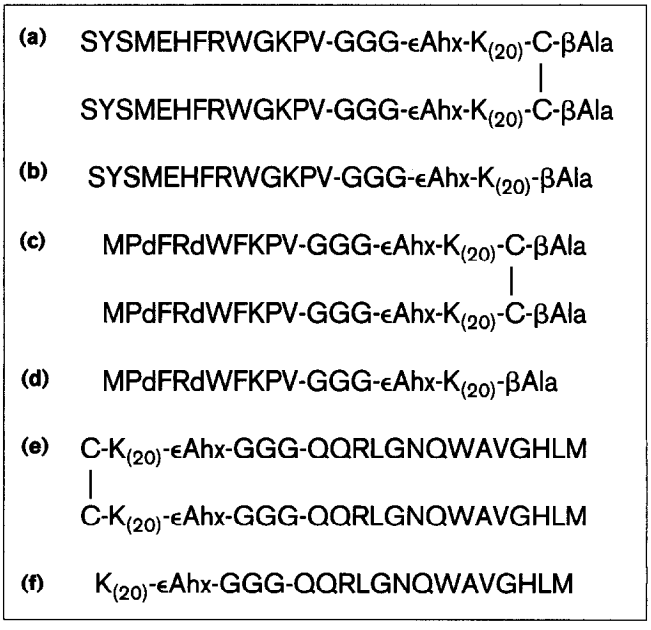
Results

Peptide design and synthesis

Our goal was to design a dimer that had the potential to bind to two receptor sites simultaneously. We reasoned that the spacer region needed to be long enough to span two receptors but flexible enough to permit binding to the second site. Prior work in other laboratories suggested that the minimal distance between two G-protein-coupled receptors is 40 Å [21]. In addition, structural studies of immunoglobulins have demonstrated that the distance between antigen binding sites is 100–150 Å [1]. The spacer region, therefore, needed to consist of residues that could assume an extended secondary structure. Initial attempts to use a polyglycine or a polyproline spacer proved unsuccessful because of solubility and flexibility limitations, respectively.

To achieve the goals of length, flexibility, solubility and maintenance of specific ligand binding, the final design consisted of a polylysine backbone coupled to the ligand region by an uncharged spacer. Representative constructs are shown in Figure 1. For the α-MSH and α-MSH-ANT dimers, the amino-terminal region consisted of the ligand-binding domain. This was followed by a short, uncharged region consisting of three glycine residues and an amino-hexanoic acid spacer, a longer, charged spacer region of 20 lysine residues, a cysteine residue (absent in the monomeric peptides) and, finally, a β-alanine residue at the carboxyl terminus. The bombesin ligand was synthesized in the opposite direction with the ligand-binding domain at the carboxyl terminus because, unlike the MSH peptides, the carboxyl terminus is crucial for bombesin binding.

Figure 1



Three pairs of monomers and dimers were synthesized for the present study. For the **(a)** α-MSH dimer, **(b)** α-MSH monomer, **(c)** α-MSH-ANT dimer and **(d)** α-MSH-ANT monomer constructs, the ligand-binding domain was located at the amino terminus and was followed by a short, flexible spacer region (three glycines and an amino-hexanoic acid (εAhx)), a longer, polylysine spacer, a cysteine residue (present in dimers only) and a β-alanine residue. The **(e)** bombesin dimer and **(f)** bombesin monomer peptides were synthesized in the opposite direction with the ligand domain at the carboxyl terminus.

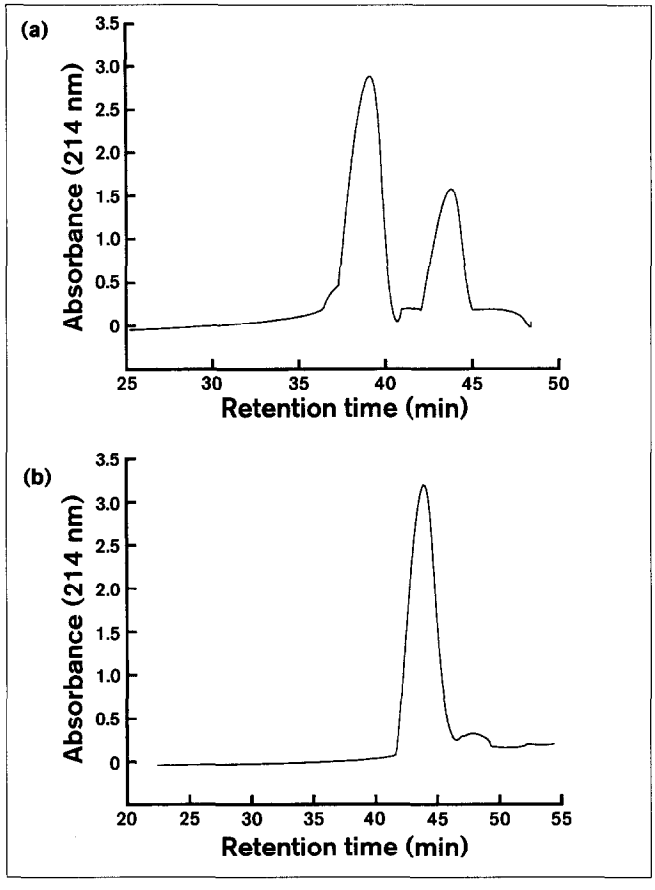
Following high-pressure liquid chromatography (HPLC) purification, both cysteine- and non-cysteine-containing peptides were oxidized by treatment with 20 % DMSO for 4 h [22]. Peptides were then repurified by size-exclusion HPLC (Fig. 2).

Melanocyte dispersion assay

We have developed a rapid, functional assay for G-protein-coupled receptors in frog-melanophore cells [16–20]. Ligands that mediate increases in cellular cyclic AMP levels in these cells, such as α-MSH, or that stimulate phosphatidylinositol (PI) metabolism, such as endothelin 3 [19], cause pigment dispersion and cell darkening in these cells, whereas inhibitors of cyclic AMP synthesis like melatonin cause pigment aggregation and cell lightening.

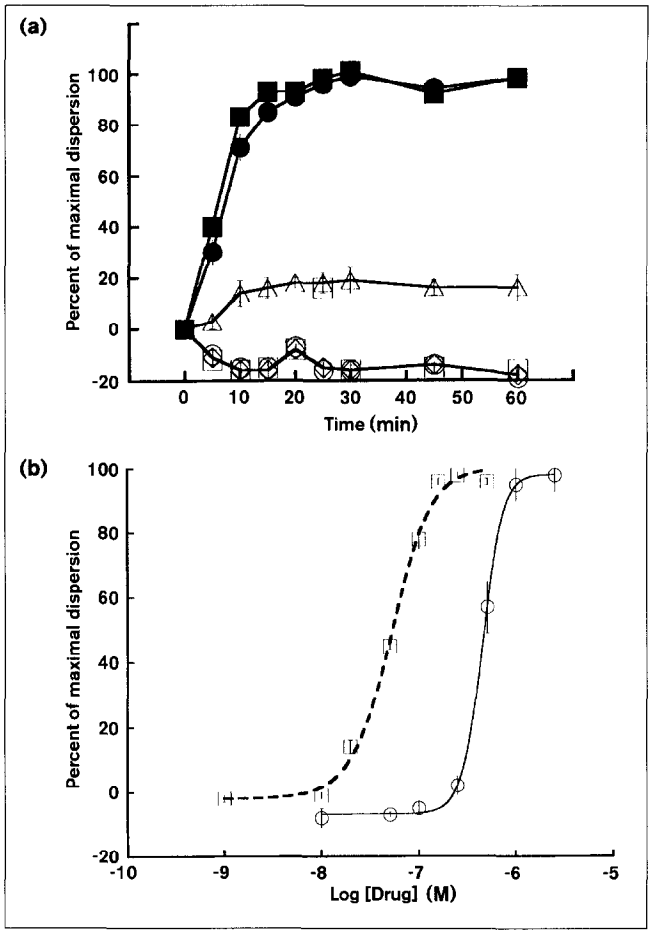
In this study we determined the effect of monomeric and dimeric peptides on pigment dispersion. In wild-type melanophores, the monomeric and dimeric α-MSH peptides stimulated dispersion in a time- and dose-dependent manner (Fig. 3). The α-MSH dimer stimulated

Figure 2



Purification of peptides. Following oxidation in 20 % DMSO, monomer and dimer peptides were repurified by HPLC size-exclusion chromatography (Pharmacia Superose 6 column). Representative chromatograms are shown to demonstrate purification of **(a)** dimer and **(b)** monomer peptide. The dimer required two gel filtration purification steps. The retention times (R_t) were ~39.0 and ~44.0 min for the dimer and the monomer, respectively. Molecular weights were confirmed by mass spectroscopy, and concentrations were determined by amino-acid analysis (data not shown).

Figure 3



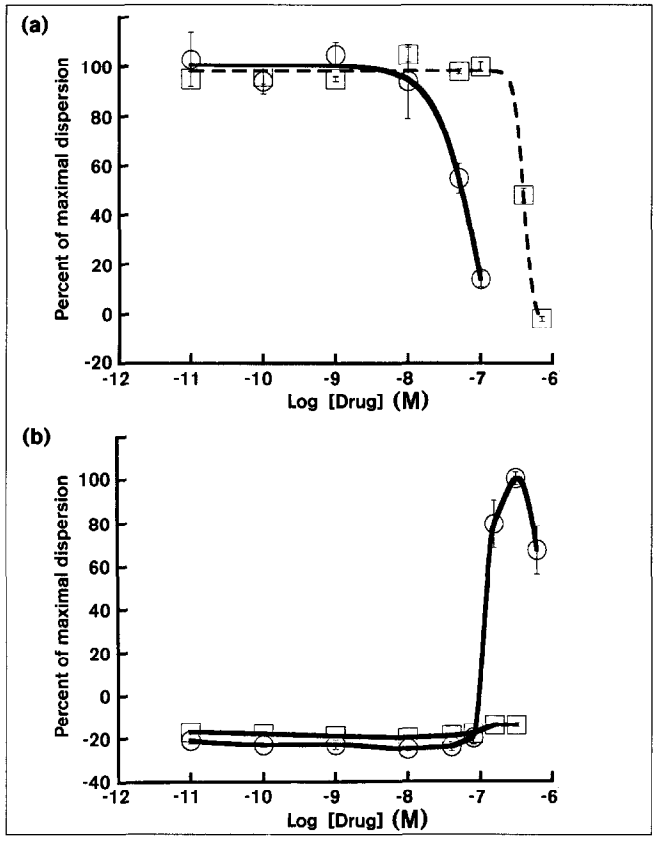
The stimulation of melanocyte dispersion by α-MSH dimer is dependent on both time and dose. **(a)** The dimer was added to a final concentration of ○ 1 nM, □ 10 nM, ◇ 25 nM, △ 50 nM, ● 100 nM or ■ 500nM. **(b)** Values for both the monomer (○) and dimer (□) are shown. Calculated EC_{50} (t = 30 min) values were 51.8 ± 5.4 nM and 372 ± 18 nM for the monomer and dimer, respectively. Each point in the graph represents the mean from triplicate samples.

dispersion with an EC_{50} (at t = 30 min) that was approximately seven-fold lower than the monomer value (Fig. 3b). Calculated EC_{50} values were 51.8 ± 5.4 nM and 372 ± 18 nM for the dimer and monomer, respectively.

The effects of a dimeric α-MSH-ANT on pigment dispersion were also examined. As expected, the monomer inhibited α-MSH-mediated dispersion in a dose dependent manner with an IC_{50} of 120 ± 6 nM (Fig. 4a). The dimer also inhibits α-MSH-mediated dispersion (Fig. 4a) but with a two-fold lower IC_{50} (57 ± 74 nM), although it was not possible to calculate this value accurately because at higher concentrations (>100 nM) the dimeric form predominantly stimulated dispersion (Fig. 4b). In the absence of α-MSH, the calculated EC_{50} for the dimer was 138 ± 4 nM; however, above a concentration of ~350 nM, dimer agonism diminished (Fig. 4b). The monomer did not stimulate dispersion below a concentration of 1 μM. Above 1 μM, all peptides stimulated a small but significant degree of nonspecific pigment dispersion.

To assess whether similar effects could be observed for other ligands, wild-type cells were transfected with a plasmid encoding the human bombesin receptor. This receptor is linked functionally to PI hydrolysis and has been demonstrated to mediate pigment dispersion following transfection [15]. Similar to the results obtained with the α-MSH peptides, the bombesin dimer stimulated pigment dispersion in a dose-dependent manner with an EC_{50} approximately five-fold lower than the corresponding monomer (Fig. 5). The EC_{50} was 23.4 ± 5.6 nM for the dimer and 110 ± 9 nM for the monomer. The bombesin peptides did not stimulate

Figure 4



Monomeric α-MSH-ANT acts only as an antagonist, but at high concentrations the dimer is also an agonist. **(a)** Dispersion mediated by α-MSH (5 nM) is inhibited by monomeric α-MSH-ANT (□; $IC_{50} = 120 \pm 6$ nM) and dimeric α-MSH-ANT (○; $IC_{50} = 57 \pm 74$ nM) in a dose-dependent manner. **(b)** At higher concentrations, and in the absence of α-MSH, the dimeric α-MSH-ANT (○) acts as an agonist with an $EC_{50} = 138 \pm 4$ nM. No significant dispersion was observed in the presence of the monomer (□) alone. Each point in the graph represents the mean from triplicate samples.

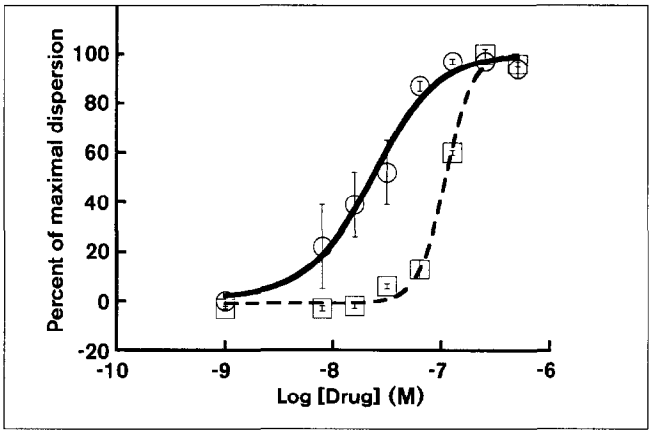
dispersion in wild-type (untransfected) melanophores within the concentration range used (data not shown).

Discussion

The present study demonstrated that dimeric peptides targeted to the α-MSH and bombesin receptors have an affinity approximately five to seven-fold higher than the corresponding monomers. As the dimers contain twice the number of potential ligands, the avidity effect was slightly more than two-fold. Although this effect is modest, it is the first demonstration to our knowledge that an entirely synthetic agonist peptide dimer can enhance G-protein-mediated signaling.

Our results for the two agonist peptides α-MSH and bombesin were similar to those previously reported for gonadotropin-releasing hormone (GnRH) peptides [14]. In that study, short, crosslinked GnRH peptide dimers

Figure 5



Dimeric bombesin is a more potent agonist than the monomer. In melanophores transfected with a plasmid encoding the bombesin receptor, the bombesin monomer (□) and dimer (○) stimulated dispersion in a dose-dependent manner. The EC_{50} (t = 30 min) was 23.4 ± 5.6 nM for the dimer and 110 ± 9 nM for the monomer. Each point in the graph represents the mean from triplicate samples.

were slightly less potent than native GnRH in a functional luteinizing hormone release assay, but generation of more widely spaced ligand sites by incubation of the dimers with anti-GnRH antibodies resulted in an increase in activity of approximately four-fold.

It remains unclear why, in both our study and the prior one, the effect of bivalent binding to G-protein-coupled receptors is relatively modest. One possible explanation is that the βγ subunits of the G-protein complex may prevent excessive signal amplification. Furthermore, models that predict an affinity increase of an order of magnitude or more assume a very high concentration of antigen or receptor on the target surface [1]. In the case of G-protein-coupled receptors, the local concentration of receptor is probably a limiting factor in affinity enhancement by multivalency.

It is also possible that the design of our dimer is suboptimal and that only a small percentage of ligands crosslink to two receptors simultaneously. The length of either the flexible spacer or the longer polylysine region may be too short or too long to obtain optimal binding, the distance between binding sites (>40 Å) may be large enough to limit the entropic benefits of a chelate-like effect [1,4], or the polylysine backbone may have such a high background of non-specific binding that bivalent binding is a rare event. Future designs must attempt both to optimize the length of the spacer regions, and to decrease the total amount of charge without decreasing solubility significantly or eliminating the potential carrier function of polylysine.

Perhaps the increased potency of the dimeric peptides is not due to a chelate-like effect, but rather to enhanced

signaling through G proteins. In the prior GnRH-receptor studies, Conn hypothesized that microaggregation of the receptor proteins may facilitate second messenger events [14,15]. More recent molecular studies of G-protein-coupled receptors suggested that these proteins can interact and form dimers that involve the exchange of amino- and carboxy-terminal domains [23]. These dimers may activate G proteins more efficiently than monomeric receptors. Further studies are needed to resolve these issues.

Perhaps the most interesting and clinically useful observation in this study was that the dimeric α-MSH-ANT acted as an agonist. This result is also reminiscent of a prior finding by Conn and coworkers [15], who demonstrated that antibody-mediated dimerization of a GnRH antagonist could convert that ligand to an agonist. In our study, at low concentrations the α-MSH-ANT dimer acted as an antagonist, but at higher concentrations it had agonist activity. We hypothesize that the dimers may mediate a cooperative effect when the two 'arms' of the ligand are bound to adjacent receptors. In this scenario, receptor microaggregation, as Conn *et al.* suggested, or dimerization can occur and is sufficient to stimulate a second messenger response [14,15]. The agonism of dimeric antagonists diminished at higher concentrations in both studies. This result suggests that the concentration of the receptor may become rate limiting at very high ligand concentrations [24], with monomeric binding of ligand saturating receptor-binding sites, and resulting in inhibition of receptor dimerization.

These results with antagonist dimers suggest a possible mechanism for enhanced cell targeting by dimeric bispecific antagonists [25]. In the present study, we synthesized only monospecific dimers. In other words, the ligand domains were the same in both 'arms' of the molecule. Using a similar synthesis strategy, it is possible to synthesize bispecific dimers. For example, one ligand region could contain an α-MSH-ANT while the other domain could consist of a bombesin antagonist. Hypothetically, bispecific dimeric antagonists would have agonist activity only against cells expressing both receptor types, and these target cells would specifically endocytose the dimer. Cells only expressing one or none of the receptors would endocytose the dimer far less efficiently. Combined with the benefits of enhanced avidity, bispecific dimeric antagonists potentially could enhance uptake by targeted cells by an order of magnitude. Future studies are needed to test this hypothesis as well as optimize the design and synthesis of these molecules.

Significance

The design of agents that can deliver low molecular weight drugs and DNA to specific cell types or across the blood brain barrier has become increasingly important in clinical pharmacology. The goal of the present study was to design, synthesize and characterize monospecific peptide dimers that had the potential to bind to two adjacent G-protein-coupled receptors. Using a functional assay in frog melanophore cells, we demonstrated a modest increase in potency with dimeric agonists and conversion of antagonist peptides to agonists following ligand dimerization. The results with the dimeric antagonist suggests a potentially powerful way to target drugs to specific cell types. We propose that bispecific dimeric antagonists (each ligand 'arm' contains antagonists to two different receptors) would only act as agonists on cells expressing both receptor types, and therefore would only be endocytosed efficiently by those cells. These constructs could potentially increase delivery to specific cell populations by an order of magnitude.

Materials and methods

Peptide synthesis

Peptide synthesis, analytical HPLC, laser-desorption mass spectroscopy and amino-acid analysis were performed at the W.M. Keck Foundation Resource Laboratory of Yale University. Peptides were synthesized by solid phase on a Rainin Symphony multiple peptide synthesizer using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry. Following cleavage from the rink amide methylbenzhydrylamine (MBHA) resin by trifluoroacetic acid (TFA), peptides were analyzed by reverse phase HPLC (Vydec C18 column) and mass spectroscopy.

HPLC

HPLC purification of peptides was performed on a Pharmacia SMART system using Superdex peptide and Superose-6 size-exclusion columns. Following synthesis, peptides were purified on a Superdex Peptide column in phosphate-buffered saline (PBS) at a flow rate of 50 μl min^{-1} and used immediately for oxidation reactions. Following oxidation, monomers and dimers were repurified on a Superose-6 column in PBS at a flow rate of 50 μl min^{-1}. Dimers usually required two successive gel filtration steps to achieve suitable purity. Peptides were stored in PBS at −20 °C. The concentration of the peptides was determined by amino-acid analysis, and molecular weights were confirmed by mass spectroscopy.

Oxidation

After peptides were purified on a Superdex peptide column, they were oxidized at room temperature for 4 h in PBS (pH = 7.0) containing 20 % DMSO [15]. The average yield for the dimeric peptides was ~20 %.

Functional bioassay

Xenopus laevis melanophores were maintained in culture as described previously [11–13]. Transient expression of human bombesin-receptor plasmid DNA (pJG3.6BR) in melanophores was achieved by electroporation [12]. Melanophores were plated (15 000 per well) in 96-well tissue culture plates (Falcon), and time- and dose-response curves were obtained by microtiter-plate assays [10–14]. Prior to addition of monomeric or dimeric peptides, cells were washed and then incubated for 1 h with 0.7 x Leibovitz 15 medium supplemented with 1 nM melatonin. This preincubation causes the cells to aggregate their pigment and lighten. Drugs were added to the microtiter wells in 20 μl aliquots at 10x their final concentration. For IC_{50} curves, the media was supplemented with 5 nM α-MSH.

Phototransmission was measured at 620 nm using a 340 ATTC microtiter-plate reader (SLT Lab Instruments). Transmission readings were taken 1 h following the addition of melatonin (T_i), and drugs were added immediately. Additional readings (T_f) were made at various time points (5–60 min). Data was curve fitted by nonlinear regression with

$y = 1 - (T_f/T_i)$. The final data were normalized and expressed as a percentage of maximal pigment dispersion (y_{max}).

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