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Determinants of Potency and Temperature-Dependent Function in the *Aplysia* Bag Cell Peptides

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BERRY, R. W., R. HANU, R. S. REDMAN AND J. J. KIM. *Determinants of potency and temperature-dependent function in the Aplysia bag cell peptides.* PEPTIDES **15**(5) 855–860, 1994.—Structure–activity relationships were determined for the natural bag cell peptides (BCPs) and for a series of synthetic analogues in terms of their ability to stimulate (at 30°C) and to inhibit (at 15°C) bag cell adenyl cyclase. We found that the core RLRF motif shared by all these peptides is active in this assay, and is stimulatory. The histidine residue *C*-terminal to this motif in β-BCP is superfluous in this respect. An electronegative residue *C*-terminal to RLRF is sufficient to induce temperature-dependent function.The Ala-Pro pair that is *N*-terminal to this motif in α-BCP increases potency, but does not alter function.

Bag cell peptides Adenyl cyclase *Aplysia* Egg laying Temperature G-proteins Structure–activity

BIOACTIVE peptides smaller than about 10 residues in length are particularly useful for the study of peptide ligand–receptor interactions. They are large enough to interact with multiple receptors, and being peptides, their interactions are amenable to understanding at the level of the linear sequence of their residues. They are small enough that the influence of this linear sequence on secondary and tertiary structure is not likely to be a major factor in ligand–receptor interactions. Moreover, peptides of this size are not apt to be much larger than the binding pocket of the receptor. Thus, experimental manipulations of their structures can be used to gain insights into the nature of their receptors. This approach has been notably successful in the case of the enkephalins (5), and FMRFamide (11), among others, but has yet to be fully exploited in the case of the neuroactive bag cell peptides (BCPs) of *Aplysia*.

The bag cell peptides (α-, β-, and γ-BCP) are small (five–nine residues) secreted products of the neuroendocrine bag cell neurons of the mollusk, *Aplysia* (16,17). The bag cells produce and secrete a peptide egg-laying hormone (ELH) that triggers secretion of the egg string and egg-laying behavior [reviewed in (2)]. The ELH precursor, proELH, also gives rise to the BCPs (16), and is thus a polyhormone, generating multiple nonidentical bioactive peptides in a fashion similar to vertebrate proopiomelanocortin (9). Again by analogy to proopiomelanocortin, which contains multiple peptides that can mediate physiological responses to stress, it has been suggested that the multiple products of proELH—ELH and the BCPs—mediate coordinated behaviors appropriate to egg laying (16); although ELH itself is responsible for initiating behavior directly concerned with deposition of the egg mass on the substrate, a variety of evidence suggests that the BCPs act on central neurons to inhibit the animal's attentiveness to external stimuli during egg laying (4,17).

In addition, the BCPs exert feedback effects on the bag cells themselves that may underlie the temperature dependence of egg laying in this animal (13). *Aplysia californica* lay eggs only rarely at 15°C, but virtually daily at 20°C (12). Both α- and γ-BCP stimulate bag cells and can induce discharges at 20°C and above, but inhibit them at 15°C. β-Bag cell peptide does not share this temperature dependence, being always stimulatory (13) . There is convincing evidence that bag cell excitability is positively correlated with bag cell cAMP levels (7), and BCP effects are consistent with this (8,13,14). Recently, we have reproduced this temperature-dependent effect on bag cell cAMP levels in an in vitro assay of adenyl cyclase enzyme activity using bag cell membranes (15). With this preparation, it has been possible to demonstrate that the stimulatory and inhibitory effects of α-BCP are mediated by G_s- and G_i-type GTP binding proteins, respectively.

Finally, the BCPs have another point in common with the products of proopiomelanocortin and similar precursors, such as proFMRFamide and proenkephalin, in which a common sequence fragment is repeated in several of the products. The BCPs share a common internal motif, RLRF, both among themselves and with peptides A and B of the atrial gland (an exocrine reproductive structure) of this animal (6). This motif is extended in the *C*-terminal direction by one additional residue in β- and

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γ-BCP and by three residues in α-BCP and the atrial peptides (structures are given in Table 1). It is extended in the *N*-terminal direction by two residues in α-BCP, by 26 residues in the atrial peptides, and not at all in β- and γ-BCP.

Thus, the BCPs offer an advantageous model system with which to explore the relationship between the structure of small peptides and the dual constraints of receptor binding and receptor activation. First and foremost, they—collectively and individually—have diverse actions in the physiological system of which they are a part; to a certain extent, nature has already provided some structure–function information. Second, the basic structure of these peptides is small enough to allow experimental manipulations to further elucidate structure–function correlations. Finally, these peptides are active in an in vitro effector assay that accurately reflects physiological effects, and that allows the generation of meaningful dose–response curves.

In the present study, we have constructed a set of peptides in which certain residues of the naturally occurring BCPs have been deleted or replaced and have assessed the effect of these alterations on potency and function in the adenyl cyclase assay. The results of this study have disclosed a consistent picture of the roles of these residues and provide insights into the structure of the receptor or receptors responsible for the multiple actions of the BCPs.

METHOD

Reagents

The naturally occurring BCPs [α(1–7), α(1–9), β, and γ) were obtained from Penninsula Laboratories (Belmont, CA) and Bachem (Torrance, CA). Calculations in this article are based on the peptide content stated by the supplier. FY was obtained from Sigma (St. Louis, MO). The remainder of the peptides were synthesized by manual solid-state methods, using Wang resins and Fmoc (9-fluorenylmethoxycarbonyl) chemistry (3). Resins and Fmoc amino acids were from Novabiochem (San Diego, CA). The trityl derivative of Fmoc-histidine and the Pmc (2,2,5,7,8-pentamethylchroman-6-sulphonyl) derivative of Fmoc-arginine were used. Coupling was performed in reaction vessels supplied by SafeLab (Santee, CA), agitated by bubbling anhydrous N_2. After cleavage from the resin in 95% trifluoroacetic acid/5% phenol, and (for peptides larger than four residues) ether extraction, the peptides were purified by reversed-phase HPLC on C18 columns, using gradients of 0.1% trifluoroacetic acid/acetonitrile. Purity assessment and quantitation were by TLC analysis following dansylation and by HPLC after conversion to phenylthiohydantoins of the amino acids generated by acid hydrolysis of the purified peptides (1). In all cases, purity exceeded 95%.

Assays

Peptides were tested for activity in an adenyl cyclase assay that we have described previously in detail (15). Briefly, membranes prepared from bag cells of animals obtained between March and December 1992 and June and September 1993 were incubated for 15 min at pH 8.0 at 15° or 30°C in the presence of GTP, ATP, 200 m*M* NaCl, and varying concentrations of peptide. The cAMP was then measured by radioimmune assay (Biomedical Technologies, Stoughton, MA).

Data Analysis

Several experiments were required to construct each dose–response curve. Each experiment included three or four controls without added peptide. The effect of the peptide was then calculated as a percentage of the mean cAMP production of the controls in that experiment. Control membranes synthesized cAMP at 7–15 pmol/mg/min, which is within the range of our previous studies, as are the magnitudes of maximal stimulation and inhibition by the naturally occurring BCPs (15). Curves were generated from the data using Sigmaplot (Jandel Scientific, Madera, CA). Error bars in the figures represent the SEM of three–nine points (average = 5). Thus, 95% confidence limits were, on average, 2.78 times the standard errors. For points on the asymptotic parts of the curves, these limits did not overlap zero.

Molecular Modeling

The structures of the peptides used in this study were investigated using Hyperchem (Autodesk, Sausalito, CA), release 2, running on a 50 MHz 486 MS-DOS computer. Each structure was constructed and energy-minimized in vacuo using the AMBER force field and the Polak–Ribiere conjugate gradient. In the case of β-BCP, this procedure was also carried out in solution and the results were compared by eye. Because of the length of this computation, we did not solvate all peptides.

RESULTS

We generated dose–response curves for four of the naturally occurring BCPs and eight synthetic BCP-related peptides. The results are summarized in Table 1. The dose–response curve for α-BCP(1–7) has been presented previously (15). It proved to be the most potent of the peptides tested, and exhibited temperature-dependent bifunctionality. This peptide is generated by extracellular proteolysis from α-BCP(1–9) (17), which we found to be about twofold less potent in this assay (Table 1). This may indicate that Tyr^7 is important for potency, and is somewhat hindered by the Ser-Leu extension in α-BCP(1–9).

γ-Bag cell peptide is an order of magnitude less potent than α-BCP in this assay , but also exhibits temperature-dependent bifunctionality (Fig. 1, Table 1). This peptide differs from α-BCP(1–7) by lacking the *N*-terminal Ala-Pro pair and by replacement of the electronegative *C*-terminal tyrosine with aspartate. The amino-terminal region of α-BCP cannot be responsible for temperature dependence, because γ-BCP lacks these residues. To investigate the relative roles of the *N*- and *C*-

TABLE 1

PEPTIDE EFFECTS ON ADENYL CYCLASE

Peptide	Sequence	EC_{50} (μ*M*)	Maximum Effect 15°	Maximum Effect 30°
α-BCP (1–7)	APRLRFY	0.50	−20 ± 4	+25 ± 3
α-BCP (1–9)	APRLRFYSL	1.3	ND	+36 ± 5
γ-BCP	RLRFD	8.3	−15 ± 3	+31 ± 3
	RLRFY	4.5	−8 ± 5	+32 ± 9
β-BCP	RLRFH	21	+24 ± 3*	+34 ± 5
	RLRF	29	+24 ± 3*	+28 ± 4
	PRLRF	12	+23 ± 2*	+34 ± 3
	APRLRF	12	+27 ± 3*	+34 ± 2
	APRLRFH	10	+17 ± 4*	+26 ± 3

EC_{50} is the concentration for half-maximal stimulation at 30°C. Maximum effect is the maximum stimulation of adenyl cyclase (percent above or below control ± SEM) observed at saturating peptide concentration, in those cases for which complete dose–response curves were obtained.

* Indicates cases where a complete dose–response curve was not obtained at 15°; value is for 100 μ*M* peptide.

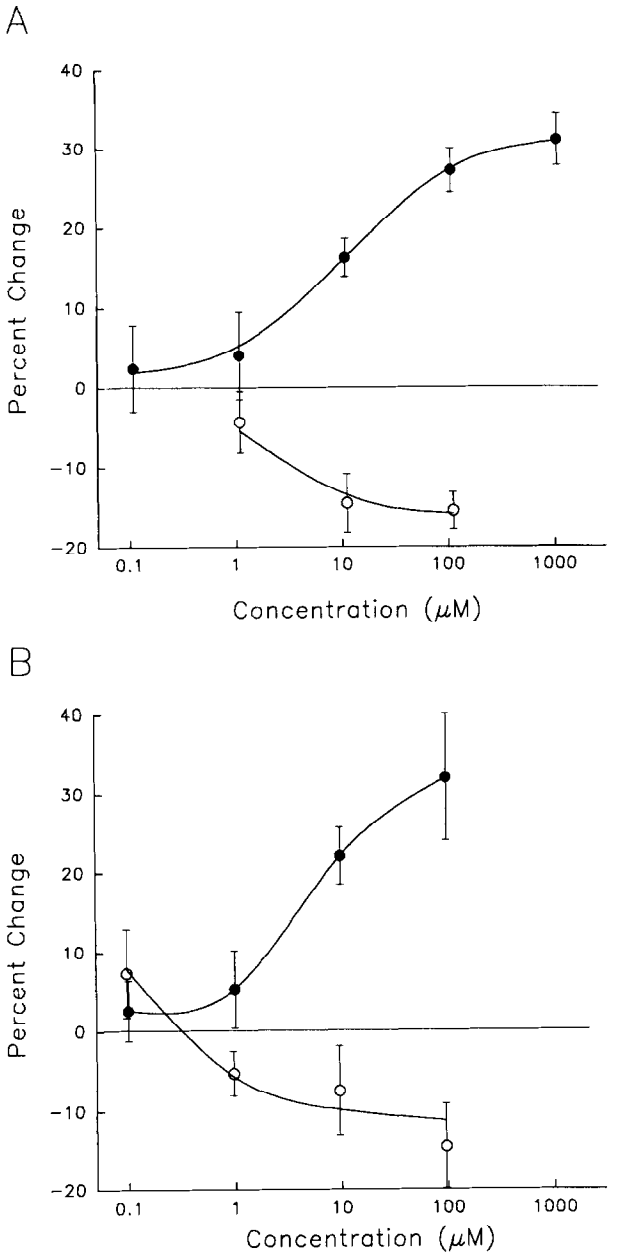

FIG. 1. Dose-response curves for γ-BCP (A) and RLRFY (B) at 30°C (●) and 15°C (○). The ordinate is the percentage stimulation or inhibition of adenyl cyclase, calculated according to the Method section. Error bars represent SEM.

termini in determining potency, we synthesized and tested RLRFY, which can be regarded as [des-Ala^1,Pro^2]α-BCP(1–7) or as [Tyr^5]γ-BCP. As expected, this peptide exhibited temperature dependence (Fig. 1, Table 1). It was also approximately twofold more potent than γ-BCP, but 10-fold less potent than α-BCP(1–7). This result indicates that, although *C*-terminal aspartate and tyrosine are roughly equivalent in terms of potency and functionality, the *N*-terminal Ala-Pro pair is an important determinant of potency.

The other naturally occurring bag cell peptide, β-BCP, is less potent than γ-BCP in this assay, and in accord with results on intact bag cells (14) is stimulatory at both temperatures (Fig. 2, Table 1). This supports the inference from the previous result that a *C*-terminal electronegative residue is both important for potency and necessary for temperature-dependent functionality. In turn, this would imply that the stimulatory effect of β-BCP is due not to its *C*-terminal histidine, but rather to the RLRF motif that is shared by all of the BCPs. We tested this by synthesizing and testing RLRF. As shown in Fig. 2 and summarized in Table 1, the tetrapeptide closely approximates β-BCPs potency and stimulatory effect. Thus, the RLRF motif itself is sufficient for activating the receptor or receptors that mediate the stimulatory effect of the BCPs on bag cell adenyl cyclase. Moreover, histidine in the *C*-terminal position has, at most, a modest effect on potency.

We have not attempted to further dissect the RLRF motif to determine the minimal requirement for activity in this system. However, we were unable to detect any activity in this assay of the dipeptides Phe-Tyr, Phe-His, or Phe-Asp at 15° or 30°C at concentrations of 1 m*M*. These fragments represent the *C*-termini of α-, β-, and γ-BCP, respectively, and this result confirms that the RLRF motif, or possibly some subset of it, is necessary for activity in this system.

The foregoing results lead to the prediction that extension of RLRF by the *N*-terminal Ala-Pro pair that is present in α-BCP would result in a peptide of greater potency, but which is stimulatory at both temperatures. Accordingly, we synthesized and tested PRLRF and APRLRF. Both were more potent than RLRF or β-BCP, and both were stimulatory at 15° and 30°C (Table 1). Interestingly, the data from these two peptides were superimposable. Thus, the entire increase in potency was achieved with the addition of *N*-terminal proline, and the further addition of alanine had no discernable effect.

The increase in potency that results from replacing the *C*-terminal histidine of β-BCP with tyrosine (RLRFY) or aspartate (γ-BCP) is as large or larger than that produced by extending the *N*-terminus. Because the *C*-terminal replacements also induce temperature dependence, it could be argued that the latter effect is the result of the former. To test this hypothesis, we synthesized and tested APRLRFH, that is, β-BCP extended at the *N*-terminus. This produced the expected increase in potency (two- to threefold) but did not induce temperature dependence (Table 1).

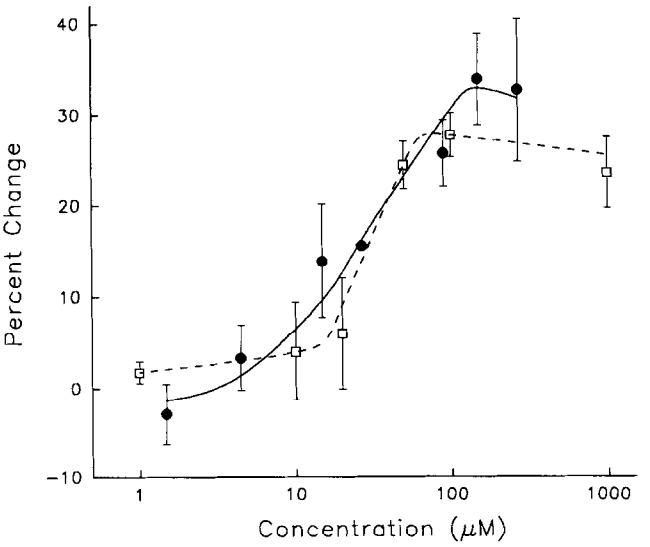

FIG. 2. Dose-response curves for β-BCP (solid line, ●) and RLRF (broken line, □) at 30°C. Details as for Fig. 1.

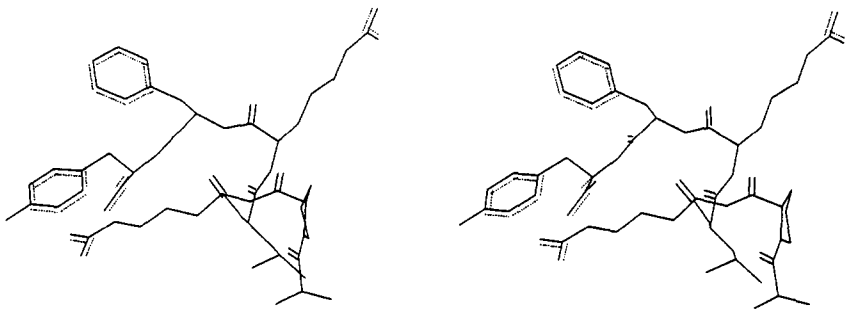

FIG. 3. Stereo pair depicting α-BCP, energy-minimized as described in the Method section. In this orientation, the amino-terminus is to the left rear, and the *C*-terminal tyrosine points toward the viewer. Hydrogen atoms have been omitted for clarity.

Finally, all of the active peptides had roughly the same maximal effect at 30°C, but this was not the case at 15°C. As can be seen from Table 1, the inhibition produced by RLRFY and γ-BCP is somewhat less than that produced by α-BCP(1–7). Although this difference was not statistically significant, it was consistent. Thus, it may be that these peptides are partial agonists. This may also be responsible for the fact that the apparent EC_{50} for these peptides is lower at 15° than at 30°C (Fig. 1). Although we did not obtain complete dose–response curves at 15°C for the peptides that remain stimulatory at this temperature, all but RLRF were tested at two concentrations, and these points were not significantly different at the two temperatures. Thus, as for α-BCP, it is unlikely that the potency of these compounds is affected appreciably by temperature.

DISCUSSION

Peptide Residues Responsible for Activity, Potency, and Function

These results indicate that the RLRF motif that is common to all of the naturally occurring BCPs, or some subset of this motif, is sufficient to stimulate bag cell adenyl cyclase at both 15° and 30°C. The histidine *C*-terminal to this motif in β-BCP appears to be entirely neutral with respect to both potency and function. However, the addition of an electronegative residue (tyrosine in α-BCP, aspartate in γ-BCP) to the carboxy-terminus of RLRF confers the property of temperature-dependent adenyl cyclase inhibition. Conversely, the amino-terminal Ala-Pro pair that is present in α-BCP is not in itself responsible for temperature-dependent enzyme inhibition.

Although RLRF, or some subset of it, can be regarded as necessary for interacting with the bag cell receptor or receptors mediating regulation of adenyl cyclase, appropriate extensions at both ends of the tetrapeptide can increase potency. Proline at the amino-terminus of RLRF or RLRFH leads to a two- to threefold increase in potency. This cannot be interpreted unambiguously as an effect on receptor binding, because cleavage of the Pro^2-Arg^3 bond is the initial event in a major pathway of α-BCP degradation in the extracellular space (10). In view of the unique structure of this imino acid, we did not explore the effect of other residues in this position. We do note, however, that a proline *N*-terminal to RLRF is present in atrial gland peptides A and B (6). The PRLRF sequence is located near the *C*-terminus of these larger peptides, and although little information exists with respect to their potency relative to the BCPs, it is clear that receptor interaction is not seriously compromised by the *N*-terminal extension of 26 residues in these molecules.

In addition to its role in temperature-dependent inhibition, the residue immediately *C*-terminal to RLRF also is an important determinant of potency, at least when this residue is electronegative. γ-Bag cell peptide (RLRFD) is somewhat more potent than RLRF, and RLRFY is more potent still. These modifications to the carboxy-terminus would not be expected to influence degradation in the extracellular space (10,18), and thus may be influencing receptor binding. Moreover, appropriate additions to the *N*- and *C*-termini have a synergistic effect on potency: amino-terminal AP increases potency two- to threefold with histidine or no extension at the *C*-terminus, but 10-fold with a tyrosine in this position. This is more easily explained by an effect on receptor binding of proline in the *N*-terminal region than by an effect of this residue on extracellular degradation.

The increase in potency effected by a tyrosine at the *C*-terminus is compromised by extending the chain further, as is evident from the loss in potency in going from α-BCP(1–7) to α-BCP(1–9). Interestingly, certain *C*-terminal extensions may also interfere with temperature-dependent function: the carboxy-terminal region of atrial peptide A is an analogue of α-BCP (. . . TPRLRFYPI), yet this peptide resembles β-BCP in increasing bag cell cAMP at both low and high temperature (14). This extension is probably sufficiently bulky to sterically hinder the interaction of Tyr^7 with its binding site, further emphasizing the importance of the carboxy-terminal residue in temperature-dependent function.

Physiological Significance

The relative roles of the amino- and carboxy-terminal regions of the BCPs in potency and function have an interesting consequence: a major pathway in the degradation of α-BCP by extracellular proteases proceeds in the amino to carboxy direction (10,18), being initiated by cleavage of the amino-terminal AP dipeptide. Although this would reduce the potency of α-BCP about 10-fold, it would not affect this peptide's temperature-dependent function. Also, the concentration of α-BCP in the vicinity of bag cell neurons has been estimated to exceed 100 μ*M* during a discharge (18). Because RLRFY is active at 10 μ*M*, an initial cleavage at Pro^2-Arg^3 would not be expected to render α-BCP ineffective on bag cells. The other major degradative path is initiated by cleavage between residues 5 and 6, producing [from α-BCP(1–7)] Phe-Tyr, which is ineffective in this assay. Thus, extracellular proteolysis could inactivate α-BCP or not, depending on which pathway was more active in a particular physiological situation, but neither pathway would influence the temperature dependency of α-BCP's function.

The Nature of the BCP Receptor(s)

The observed structure–activity relationships allow some inferences to be made concerning the receptor or receptors mediating BCP actions on bag cells. This process can be facilitated by reference to Fig. 3, which portrays a configuration of α-

BCP(1–7). This structure was obtained by energy minimization computations in vacuo, so three caveats are necessary. First, this structure does not necessarily represent a global energy minimum. Nonetheless, all of the peptides used in this study (with the exception of the dipeptides, which were not modeled) assume broadly similar configurations when minimized in the same way. Second, solvent effects have been neglected. Interestingly, solvation was not found to alter the major features of β-BCP's overall configuration. Finally, and most importantly, it is to be expected that interaction with a receptor may alter the conformation of the peptide ligand significantly. Nevertheless, the conformation depicted serves as a convenient starting point for discussion.

Beginning at the amino-terminus, the first general feature is a "kinked tail" induced by Pro^2. Because the amino-terminal extension that is present in the atrial gland peptides apparently does not interfere with activity, we presume that this extension points away from the receptor. We emphasize that the peptide configuration depicted in the figure may not accurately reflect the actual torsion angle of the Pro-Arg bond in the receptor-bound configuration. A second prominent feature is that the two positively charged arginine side chains are disposed on opposite sides of the molecule and are approximately coplanar. This places the hydrophobic Leu^4 and Phe^6 residues far away from the charged arginines. This conformation does minimize charge interactions between the arginines, but these may be neutralized by pairing with acidic residues in the receptor. Nonetheless, the alternation of hydrophobic and charged residues in the basic binding motif is likely to reflect a similar spatial alternation of residues in the binding pocket. The remainder of the peptide as modeled, namely the orientation of Phe^6 and Tyr^7, is likely to be due to the modeling conditions. In this structure, the electronegative Tyr^7 and the terminal carboxyl are attracted to Arg^3. If, as seems likely, tyrosine interacts with a receptor residue, this orientation easily could be altered. The near equivalence between the potencies of RLRF and RLRFH argues that the terminal carboxylic acid of RLRF has little effect on potency, and probably on binding as well. Finally, peptides lacking the Ala-Pro pair at the amino-terminus, but possessing an electronegative *C*-terminal residue (i.e., RLFY and γ-BCP), may be only partially effective at 15°C. If so, this would suggest that binding of both the *N*- and *C*-termini is required to fully activate the inhibitory form or conformation of the receptor.

The foregoing considerations apply equally to a single, multifunctional receptor and to a series of single- or multifunction receptors that possess various components of the binding pocket described above. For example, the study by Owens et al. (10) indicates that the Phe-Tyr dipeptide is both necessary and sufficient to mimic the electrophysiological inhibition of the LUQ cells of the *Aplysia* abdominal ganglion by α-BCP. In addition, there was some indication in that study that α-BCP(1–6) and -(1–5) could mediate excitation in these cells. Thus, it is conceivable that the LUQ cells contain multiple BCP receptors that respond to different regions of the APRLRFY sequence. This could be true of the bag cells as well, although our failure to observe an effect of the FY and FD dipeptides argues that at least the presumptive FY receptor is not present in the bag cells.

The issue of multiple receptors is crucial to understanding the temperature dependence of modulation of adenyl cyclase in the bag cells by α- and γ-BCP. We have suggested previously that this is due to a single temperature-dependent multifunctional receptor because the EC_{50} of α-BCP(1–7) is identical for stimulation at 30°C and inhibition at 15°C, and because abolition of inhibition by treatment with pertussis toxin does not add to the stimulatory effect of this peptide at the higher temperature (15). The present results add support to this notion: all of the peptides we tested are full agonists for stimulation at 30°, and the potency series we observed at this temperature strongly implies that the receptor responsible for stimulation must include the binding site for the *C*-terminal tyrosine of α-BCP(1–7). Yet this tyrosine (or aspartic acid) is required for inhibition, and indeed, the effect on potency of this residue, judging from the similar potencies of α-BCP(1–7) at 15° and 30°C, would be the same for stimulation and inhibition of the enzyme. It is therefore unlikely that the effect of temperature on this process is mediated by differential binding of α-BCP, or of Tyr^7. Considerations that we have addressed previously lead us to suggest that the effect of temperature is not the result of unique G-proteins or of the adenyl cyclase, but resides at the level of the receptor (15). The present results further narrow the issue to a point subsequent to binding of the peptide to the receptor, but prior to the release of a stimulatory or inhibitory G-protein.

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