Inhibition of calmodulin-dependent myosin light-chain kinase by growth-hormone-releasing factor and vasoactive intestinal peptide

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In view of the ability of calmodulin to bind vasoactive intestinal peptide (VIP) and growth-hormone-releasing factor (GRF) with high affinity [Stallwood, Brugger, Baggenstoss, Stemmer, Shiraga, Landers and Paul (1992) *J. Biol. Chem.* **267**, 19617–19621], the effects of these neuropeptides on a model calmodulin-dependent enzyme, myosin light-chain kinase (MLCK), were studied. Both peptides were potent inhibitors of MLCK activity. The inhibition

of enzyme activity by VIP and GRF was progressively overcome with increasing calmodulin concentrations, with no inhibition observed at a saturating calmodulin concentration. Nanomolar concentrations of MLCK blocked the formation of calmodulin– $[^{125}\text{I-Tyr}^{10}]$ VIP complexes. These data provide support for a functional role of VIP and GRF binding by calmodulin.

INTRODUCTION

The neuropeptide vasoactive intestinal peptide (VIP) displays a broad profile of biological actions [1]. A transmembrane receptor for VIP coupled with adenylate cyclase has been identified [2]. Since GTP potentiates VIP-stimulated adenylate cyclase activity and inhibits VIP binding by membrane preparations [3], a stimulatory G-protein may mediate the receptor–adenylate cyclase coupling. This signal-transduction system, however, does not adequately explain several important aspects of the biological activity of VIP (reviewed in [4]). Some of the observations suggesting that an additional receptor(s) may participate in the actions of this peptide are: (a) VIP-binding membrane proteins ranging in mass from 17 to 80 kDa have been identified [5–7]; (b) VIP stimulates several second-messenger pathways (cyclic AMP [2,3] and cyclic GMP [8] synthesis; NO synthesis [9]; transmembrane Ca^{2+} flux [10]; phosphoinositide hydrolysis [11]; and K^{+} -channel activation [12]); (c) VIP-stimulated hepatic glycogenolysis is Ca^{2+} -dependent and cyclic AMP-independent [13]; (d) contrary to ligand-enhanced GTP-binding noted in simple receptor–adenylate cyclase systems [14], VIP inhibits the binding of a GTP analogue by pituitary cells [15]; (e) apamin, a bee venom peptide, competes with VIP for binding to receptors in some, but not all, tissues, suggesting receptor multiplicity [16]; and (f) in addition to extracellular receptors, VIP-binding proteins are found within cells [17] and the peptide stimulates protein kinase C activity in purified nuclei [18].

We purified a protein from detergent-solubilized lung membranes that displays a high affinity for VIP [7]. N-terminal sequencing of tryptic peptides from this protein revealed it to be calmodulin [19], an intracellular regulator of several key metabolic enzymes [20]. The purified calmodulin also displayed high affinity for growth-hormone-releasing factor (GRF) [19], a hypothalamic peptide that stimulates growth-hormone release [21] and bears a high degree of sequence similarity to VIP (43% identity between VIP and the N-terminal 28 residues of rat GRF). Segments of both neuropeptides can be modelled as a basic amphiphilic helix [22,23], a structural motif known to be recognized by calmodulin [24]. Consideration of a neuropeptide-

receptor function for calmodulin is warranted if two conditions are met: (a) VIP–calmodulin and GRF–calmodulin binding must be productive, in that quantitative or qualitative alterations in the regulatory functions of calmodulin must occur (e.g. enzyme activation by calmodulin); and (b) the neuropeptides must make contact with calmodulin (i.e. the cell-membrane barrier must be transcended by internalization of the neuropeptides or by neuropeptide–calmodulin complex-formation within the membrane).

Internalization of VIP [25,26] and GRF [27] by target cells and formation of complexes of GRF with a protein in intact pituitary cells with mass in the same range as calmodulin have been reported [28], and we have observed rapid accumulation of VIP–calmodulin and GRF–calmodulin adducts by treatment of cultured bronchial epithelial cells and anterior pituitary cells, respectively, with the radiolabelled neuropeptides [29]. The aim of the present study was to evaluate the relationship between neuropeptide–calmodulin binding and the activity of myosin light-chain kinase (MLCK), a model calmodulin-dependent enzyme. Potent inhibition of MLCK activity of VIP and GRF attributable to neuropeptide–calmodulin binding was observed, fulfilling the requirement, in principle, of a signal-transduction event following the binding of calmodulin by the neuropeptides. Although several details of the physiological mechanism, pharmacology and anatomical sites of peptide–calmodulin and peptide–calmodulin–enzyme contact remain to be deciphered, our observations suggest that calmodulin may be considered a potential intracellular receptor for VIP and GRF.

METHODS

MLCK assay

MLCK activity was measured as described in [30] with modifications. Salt-free calmodulin from pig brain (Sigma, St. Louis, MO, U.S.A.) was incubated with MLCK purified from chicken gizzard smooth muscle (provided by Dr. M. Cable, Schering-Plough, Kenilworth, NJ, U.S.A.), Kemptamide (KKRPQRAT-SNVFS-NH₂; Peninsula), ATP (Sigma) and $[^{32}\text{P}]$ ATP (2 μCi ; NEN) for 45 min at 23 °C in a final volume of 0.1 ml of 30 mM

Abbreviations used: VIP, vasoactive intestinal peptide; rGRF, rat growth-hormone-releasing factor; MLCK, myosin light-chain kinase; Kemptamide, KKRPQRATSNVFS-NH₂; DTSP, dithiobis(succinimidyl propionate).

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Mops/NaOH buffer, pH 7.2, containing 0.3 mM MgCl_2 , 0.01 mM CaCl_2 , 0.1 mM dithiothreitol, 1 mM CHAPS and 0.5% BSA. Samples (0.05 ml) of the reaction mixture were plated on Whatman P-81 paper discs; the discs were washed first with 30% acetic acid and then with acetone, and dried; scintillation fluid was added and the radioactivity was determined in a Packard 1600 TR β -counter. Calmodulin and MLCK preparations used were electrophoretically homogeneous. The calmodulin was preincubated in buffer without MLCK in the absence or presence of VIP (Bachem) and rat GRF (rGRF; Peninsula) (60 min). Values for non-specific binding to filters, measured in reaction mixtures that did not contain MLCK, were < 1% of available radioactivity and were essentially identical in the absence or presence of VIP or rGRF (10 μM).

Phosphorylation of Kemptamide by MLCK in the absence of calmodulin and in the absence and presence of neuropeptides was undetectable. The kinetic characteristics of MLCK activity were similar to those described previously [30]. Initial rates at various Kemptamide concentrations and fixed ATP (10 μM), calmodulin (10 nM) and MLCK (20 nM) concentrations were consistent with Michaelis-Menten kinetics (K_m 14 μM ; $V_{\max}$ 94 pmol/min per mg) (results not shown).

Peptide-calmodulin binding

Iodination of synthetic VIP (Bachem) and purification and identification of [^{125}I -Tyr 10]VIP were performed as described previously [31]. Purified calmodulin (0.7 nM) was incubated with [^{125}I -Tyr 10]VIP (1.5 nM) for 60 min (23 °C) in the absence or presence of MLCK or of Kemptamide in 0.04 ml of 30 mM Mops/NaOH, pH 7.2 containing 0.3 mM MgCl_2 , 0.01 mM CaCl_2 , and 1 mM CHAPS. Covalent cross-linking of VIP-calmodulin complexes was by treatment (3 min) with 1 mM dithiobis(succinimidyl propionate) (DTSP), excess cross-linker was quenched by addition of glycine to 0.2 M, and the reaction products were analysed by non-reducing SDS/PAGE, autoradiography and densitometry [7,19]. The MLCK and peptide-calmodulin-binding assays were performed under identical conditions, except for the absence of ATP and dithiothreitol in the latter type of assay. Dithiothreitol was omitted from the binding assays because of the presence of a reducible disulphide bond in DTSP, the cross-linker used to detect calmodulin-neuropeptide complexes by electrophoresis. The absence of dithiothreitol is unlikely to influence the binding reaction, since VIP, GRF and calmodulin do not contain disulphide bonds. In a control binding assay, essentially identical levels of VIP-calmodulin complex-formation were observed in the absence and presence of ATP at 10 μM or 100 μM .

RESULTS AND DISCUSSION

Kemptamide, a 13-residue synthetic peptide (Figure 1) identical with the phosphorylation site in myosin light chains (residues 11–23] at all but one residue (Ser instead of Ala at the C-terminus of the synthetic peptide) [32], was used as substrate for MLCK. At progressively increasing calmodulin concentrations, the rate of incorporation of ^{32}P was saturable, with no increase in rate observed between 1 μM and 5 μM calmodulin. Preincubation of calmodulin with 300 nM rGRF or 5 μM VIP resulted in inhibition of MLCK activity (Figure 2). The inhibition diminished progressively with increasing calmodulin concentration, and the activity was restored to control levels of 5 μM calmodulin. Similar diminutions of the inhibition were observed in experiments conducted at 30 nM or 1 μM rGRF or at 1 μM VIP. We

have previously observed that treatment of [^{125}I -Tyr 10]VIP with calmodulin, followed by covalent cross-linking with DTSP, a bifunctional NH_2 -directed reagent, permits detection of 22–26 kDa radiolabelled VIP-calmodulin complexes [19]. Formation of these complexes is inhibited competitively by VIP and rGRF (IC_{50} 8.1 nM and 6.4 nM respectively) but not by excess BSA, elodeisin, bombesin and neurotensin. In the present study, potent inhibition of [^{125}I -Tyr 10]VIP-calmodulin complex-formation by MLCK was observed (IC_{50} 5.5 nM; Figure 3a). Dissociation of MLCK from MLCK-calmodulin complexes by VIP has been observed previously by indirect fluorimetric methods [33]. These observations suggest that the inhibition of MLCK activity is mediated by neuropeptide-calmodulin binding.

A strong case has been made for the presence of a calmodulin-like site in several calmodulin-dependent enzymes, including MLCK [34]. However, direct inhibitory interaction of the neuropeptides with MLCK is unlikely, for the following reasons: (i) covalently linked radiolabelled bands with mass corresponding to the sum of the molecular masses of VIP (3.3 kDa) and MLCK (133 kDa) were undetectable, arguing against high-affinity VIP-MLCK binding; (ii) the high-affinity calmodulin antagonist melittin binds calmodulin-dependent enzymes with low affinity, compared with potent inhibition of MLCK by VIP and rGRF (see below); (iii) melittin binding to the calmodulin-like site results in weak stimulation, rather than inhibition of enzyme

VIP	<u>H</u> S <u>P</u> <u>D</u> <u>A</u> <u>V</u> <u>E</u> <u>T</u> <u>D</u> <u>N</u> <u>Y</u> <u>T</u> <u>R</u> <u>L</u> <u>R</u> <u>K</u> <u>M</u> <u>A</u> <u>V</u> <u>K</u> <u>K</u> <u>Y</u> <u>L</u> <u>N</u> <u>S</u> <u>I</u> <u>L</u> <u>N</u>
GRF	<u>H</u> <u>A</u> <u>D</u> <u>A</u> <u>I</u> <u>E</u> <u>T</u> <u>S</u> <u>S</u> <u>Y</u> <u>R</u> <u>I</u> <u>L</u> <u>G</u> <u>L</u> <u>Q</u> <u>L</u> <u>Y</u> <u>A</u> <u>R</u> <u>K</u> <u>Y</u> <u>L</u> <u>L</u> <u>H</u> <u>E</u> <u>I</u> <u>M</u> <u>N</u> <u>R</u> <u>Q</u> <u>G</u> <u>E</u> <u>R</u> <u>N</u> <u>Q</u> <u>E</u> <u>R</u> <u>S</u> <u>R</u> <u>F</u> <u>N</u>
MLCK	<u>↓</u> 480 A <u>K</u> K <u>L</u> S <u>K</u> D <u>R</u> M <u>K</u> K <u>Y</u> M <u>A</u> R <u>R</u> K <u>K</u> W <u>Q</u> K <u>T</u> G <u>H</u> A <u>V</u> R <u>A</u> I <u>G</u> R <u>L</u> S <u>S</u>
Kemptamide	K K R <u>P</u> Q R <u>A</u> T <u>S</u> N <u>V</u> F <u>S</u>

Figure 1 Sequences of VIP, rGRF, the calmodulin-binding site in MLCK (beginning with residue 480, from [16]) and the MLCK substrate, Kemptamide

Conserved residues in VIP and GRF are underlined, and similarities in Kemptamide and MLCK (four basic residues and two conserved hydrophobic residues) are shown in bold.

Figure 2 Inhibition of MLCK by rat GRF (300 nM; ▲) and VIP (5 μM ; ●): effect of increasing calmodulin concentration

Data are means $\pm$ S.D. of three replicates, corrected for non-specific association of ^{32}P to filters and expressed relative to ^{32}P incorporation observed in the absence of neuropeptides: (○) shows incorporation in the absence of neuropeptides. Reactant concentrations: 0.1 mM ATP, 2.2 nM MLCK, 20 μM Kemptamide.

Figure 3 Inhibition of [^{125}I -Tyr 10]VIP-calmodulin complex-formation by MLCK and Kemptamide

(a) Representative autoradiogram of SDS/polyacrylamide gels showing [^{125}I -Tyr 10]VIP-calmodulin complexes (22–26 kDa) formed in the absence of inhibitor (lane 12) and in the presence of MLCK (1 μM , 0.1 μM , 10 nM, 1 nM or 0.1 nM: lanes 1–5 respectively), unlabelled VIP (1 μM , lane 6) or Kemptamide (20 μM , 6 μM , 2 μM , 0.2 μM and 0.02 μM : lanes 7–11 respectively). The fast-migrating radioactivity represents uncomplexed [^{125}I -Tyr 10]VIP. (b) Relative optical density (in arbitrary units) of the 22–26 kDa complexes plotted as a function of MLCK ($\blacktriangle$) and Kemptamide ($\bullet$) concentrations. Data are expressed relative to the intensity observed in the absence of inhibitors

activity [34]. We note that these arguments do not rule out low-affinity stimulatory neuropeptide interaction with the putative calmodulin-like site.

Dose-dependent MLCK inhibition was evident at increasing rGRF or VIP concentrations. With substrate (Kemptamide) at 20 μM , IC_{50} values ($\pm$ S.D.) of GRF and VIP were respectively 145 ± 10 nM and 3.9 ± 0.3 μM (Figure 4a). Eleodoin and neurotensin (10 μM), peptides with no sequence similarity to rGRF and VIP, had no effect on the MLCK activity under identical conditions.

Unlabelled VIP and rGRF are potent inhibitors of [^{125}I -Tyr 10]VIP-calmodulin complex-formation ($\text{IC}_{50} < 10$ nM) [7,19], and the K_d of VIP-calmodulin binding estimated by fluorimetric assays is 50 nM [33]. Interference with neuropeptide-calmodulin binding by Kemptamide appears to be responsible, at least in part, for the discrepancy between the calmodulin-binding and MLCK-inhibitory potencies of rGRF and VIP. Kemptamide resembles a segment of the calmodulin-binding sequence in MLCK at 6 of its 13 residues (Figure 1). Peptides that serve as substrates [35] or possess sequence similarity to substrates [36] of calmodulin-dependent enzymes are known to bind calmodulin weakly. In the present study, Kemptamide inhibited the formation of [^{125}I -Tyr 10]VIP-calmodulin complexes (Figure 3) with low potency (IC_{50} 1.0 ± 0.2 μM) compared with unlabelled VIP, GRF and MLCK ($\text{IC}_{50} < 10$ nM). Nevertheless, at the Kemptamide concentration employed in the MLCK assay in Figure 2 (20 μM), $> 85\%$ decrease in VIP-calmodulin

Figure 4 Potencies of rGRF ($\blacktriangle$) and VIP ($\bullet$)-mediated inhibition of MLCK activity at 20 μM (a) and 0.6 μM Kemptamide (b)

Results shown are representative data (means $\pm$ S.D. of three replicates), expressed as percentage inhibition relative to ^{32}P incorporation in the absence of the neuropeptides (94.9 ± 3.9 and 3.7 ± 0.3 pmol/45 min in a and b respectively). Reactant concentrations in (a) and (b) respectively: 2 nM and 10 nM calmodulin, 2.2 nM and 20 nM MLCK, 0.1 mM and 0.01 mM ATP.

Figure 5 Inhibition of MLCK activity by rGRF (300 nM; $\blacktriangle$) and VIP (5 μM ; $\bullet$): effect of substrate (Kemptamide) concentration

Representative data are shown, expressed as percentage inhibition relative to ^{32}P incorporation in the absence of neuropeptide (6.7–456.7 pmol/45 min at substrate concentrations shown on the abscissa). Reactant concentrations: 10 nM calmodulin, 20 nM MLCK, 0.1 mM ATP.

complex-formation can be expected. This is illustrated in Figure 5 by a progressive attenuation of VIP- and rGRF-mediated inhibition of MLCK activity at increasing Kemptamide concentrations. In order to minimize its interfering effect, the con-

centration of Kemptamide was decreased to 0.6 μ M. To permit detection of MLCK activity at this concentration of Kemptamide, the specific radioactivity of [32 P]ATP was increased (by 10-fold; total ATP concn. 10 μ M), and the calmodulin and MLCK concentrations were increased by 5-fold and 9-fold respectively. Under these conditions the IC_{50} for rGRF was 25.9 ± 5.1 nM (Figure 4b). Apparent neuropeptide potencies in the MLCK assay can be expected to be lower, due to use of calmodulin at 10 nM, compared with 1 nM in [125 I-Tyr 10]VIP-binding assay {by 2.6-fold for GRF and 2.3-fold for VIP, assuming absence of interference by other reactants and K_d 8 nM (VIP) or 6 nM (GRF) [29]}. The potencies with which rGRF inhibited MLCK activity and [125 I-Tyr 10]VIP-calmodulin binding are comparable, suggesting that the MLCK inhibition can be explained entirely on the basis of calmodulin-GRF binding. On the other hand, unidentified factors that may limit the MLCK inhibitory potency of VIP should be considered, since the IC_{50} of this peptide (386 ± 64 nM) at 0.6 mM Kemptamide remained about one order of magnitude greater than expected. For example, VIP could stimulate MLCK activity in concert with calmodulin by interaction at the calmodulin-like site in the enzyme, analogous to melittin-mediated stimulation of calmodulin-dependent phosphodiesterase [34]. Detailed study of potential VIP interactions with MLCK and other reactants was beyond the scope of this study. We note that such interactions may underlie the diminished potency of VIP-mediated MLCK inhibition, but cannot be the cause of the inhibition. Since the inhibitory effect was lost at saturating calmodulin concentrations, it remains that neuropeptide-calmodulin binding must mediate MLCK inhibition.

Taken together, the inhibition of a calmodulin-dependent enzyme by VIP and GRF, internalization of VIP and GRF by target cells [24–27], high-affinity binding of the neuropeptides by soluble and membrane-bound calmodulin [7,19] and formation of neuropeptide-calmodulin complexes in unbroken cell preparations [29] suggest that calmodulin is a potential intracellular receptor for VIP and GRF. This conclusion is also consistent with the following indirect lines of evidence: (i) the intracellular concentrations of Ca^{2+} and Mg^{2+} are permissive for neuropeptide-calmodulin binding [37,38]; (ii) at the concentrations of calmodulin found in cells (2–20 μ M) [20], complete complexation of small quantities of internalized neuropeptides can be expected; and (iii) previous reports suggest that the biological effects of VIP [39] and GRF [40,41] in intact cells are Ca^{2+} - and calmodulin-dependent. However, studies conducted using intact cell preparations leave open the possibility of a calmodulin requirement at an unidentified post-receptor signal-transduction step, rather than the neuropeptide-binding step. Unambiguous evaluation of the physiological role of calmodulin in neuropeptide effects will be aided by the development of new reagents like specific antagonists of neuropeptide-calmodulin binding that do not interfere with calmodulin-enzyme interactions, and by identification of the subcellular site(s) of neuropeptide-calmodulin binding in relation to the location of various calmodulin-dependent enzymes. VIP and GRF elicit biological effects in target cells at nanomolar concentrations. Clearly, the internalized neuropeptides cannot be expected to bring about generalized changes in calmodulin function. On the other hand, further investigation of signalling events at the immediate site of neuropeptide-calmodulin binding are warranted, since local modulation of calmodulin function may be an important pathway governing phenotypic effects elicited by VIP and GRF.

Calmodulin is a multifunctional protein responsible for regulation of many different signalling enzymes, e.g. NO synthase, phosphodiesterase, and at least one form of adenylate cyclase

[20,42]. A receptor-like role for calmodulin may explain the broad range of biological effects of VIP and the diverse metabolic and transport systems affected by this peptide [4]. Since the mechanisms of activation of different enzymes by calmodulin are not identical [43], the behaviour of MLCK, the model enzyme examined in the present study, does not permit prediction of the direction (inhibition/stimulation) or potency of potential neuropeptide effects on other calmodulin-dependent pathways.

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