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Cortistatin increase of a potassium conductance in rat locus coeruleus *in vitro*

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1 In this study we examined the effects of cortistatin, a putative endogenous ligand for somatostatin (SRIF) receptors, on the membrane properties of rat locus coeruleus (LC) neurones *in vitro*, by use of intracellular and whole cell patch clamp recording. We have compared the actions of cortistatin with those of SRIF and the SRIF analogue D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP).

2 When LC neurones were voltage clamped to −60 mV, application of cortistatin caused an outward current in all cells examined ($n=44$), with a pEC_{50} of 6.62. SRIF also caused an outward current in all cells examined ($n=43$), with a pEC_{50} of 6.93.

3 The outward currents caused by cortistatin in 2.5 mM extracellular K^+ reversed polarity at −106 mV, very close to the predicted K^+ reversal potential of −105 mV. Increasing extracellular K^+ to 10.5 mM resulted in a shift of the reversal potential of +38 mV, a shift consistent with a K^+ conductance. The conductance activated by cortistatin showed mild inward rectification.

4 Continuous application of a high concentration of SRIF (1 μM) resulted in a decrease of the outward current to a steady level of 49% of the maximum response, with a $t_{1/2}$ of 131 s. Application of a high concentration of cortistatin (3 μM) during the desensitized portion of the SRIF response did not result in any further outward current. Continuous application of a high concentration of cortistatin (10 μM) resulted in a decrease of the outward current to a steady level of 42% of the maximum response with a $t_{1/2}$ of 114 s. Application of a high concentration of SRIF (3 μM) during the desensitized portion of the cortistatin response produced only a small outward current.

5 Continuous application of cortistatin (3 μM) also resulted in a decrease of the outward current (by 43%, $t_{1/2}$ of 136 s) and application of a high concentration of CTOP (10 μM) during the desensitized portion of the cortistatin response did not produce any outward current. Continuous application of a high concentration of CTOP (10 μM) resulted in a decrease of the outward current to a steady level of 70% of the maximum response with a $t_{1/2}$ of 143 s. Application of a high concentration of cortistatin (3 μM) during the desensitized portion of the CTOP response did not result in any further outward current.

6 The actions of cortistatin (300 nM – 10 μM) were not affected by the opioid antagonist naloxone (10 μM). Application of met-enkephalin during the desensitized portion of the response to a high concentration of cortistatin (3 μM) produced an outward current similar to that produced by met-enkephalin application alone.

7 Thus cortistatin efficaciously activates an inwardly rectifying K^+ conductance in LC neurones. These actions appear to be mediated by a population of SRIF receptors, at which CTOP is also an agonist. Cortistatin does not appear to be a ligand for μ-opioid receptors in rat LC neurons.

Keywords: Cortistatin; somatostatin; potassium channels; somatostatin receptors; locus coeruleus; CTOP

Introduction

Cortistatin is a novel somatostatin (SRIF)-like neuropeptide that appears to be involved in the modulation of sleep in rats (de Lecea *et al*., 1996). Despite being the product of a separate gene to SRIF, cortistatin is identical to SRIF at 11 of its 14 amino acids, including the Phe-Trp-Lys-Thr sequence required for SRIF binding to its receptors (Veber *et al*., 1979; de Lecea *et al*., 1996). Cortistatin binds with high affinity to SRIF receptors in GH_4 pituitary cells and it mimics the SRIF-induced inhibition of adenylyl cyclase in these cells (de Lecea *et al*., 1996). Cortistatin also mimics SRIF-induced activation of the M-type K^+ current in hippocampal neurones, but it has a markedly different effect from SRIF on field potentials elicited by paired pulse stimulation in the CA1 region of the hippocampus *in vivo*. Additionally, cortistatin has been shown to modulate sleep in rats in a manner different from SRIF (de Lecea *et al*., 1996). These results suggest that cortistatin can act as an agonist at some types of SRIF receptor, but indicate that it may not be an agonist at all types of SRIF receptor identified to date. It has also been suggested that cortistatin may act at other, non-SRIF type receptors; either 'cortistatin-specific' receptors or receptors such as the μ-opioid receptor (de Lecea *et al*., 1996). Very recently, a human analogue of cortistatin was identified, which differs from rat cortistatin by a single amino acid, but which appears to have similar *in vivo* activity (Fukusumi *et al*., 1997).

The locus coeruleus (LC) is a group of noradrenergic neurones in the brainstem that have been implicated in the modulation of diverse neuronal functions, including sleep. Neurones of the LC are hyperpolarized by SRIF acting at SST2-like receptors (Chiu *et al*., 1994; Chessel *et al*., 1996) to open an inwardly rectifying K^+ channel (Inoue *et al*., 1988). We have recently found that the SRIF analogue and μ-opioid antagonist, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP), also opens an inwardly rectifying K^+ channel in LC neurones (Chieng *et al*., 1996). Despite the apparently very low affinity of CTOP for SRIF receptors (Pelton *et al*., 1986), the agonist effects of CTOP may be mediated via a population of SRIF receptors in the LC. In this study we have sought to determine the actions of cortistatin on LC neurones and to compare these effects with those of SRIF, CTOP and com-

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pounds that act at μ-opioid receptors. A preliminary account of this work has been presented to the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (Connor *et al*., 1996a).

Methods

Electrophysiological recordings

Sprague-Dawley rats (150–250 g) of either sex were anaesthetized with halothane and killed by cervical dislocation. Horizontal slices (between 300–350 μm thick) containing the LC were cut and maintained at 35°C in physiological saline of composition (mM): NaCl 126, KCl 2.5, $MgCl_2$ 1.2, $CaCl_2$ 2.4, $NaHCO_3$, 24 and glucose 11; gassed with 95% O_2/5% CO_2. As noted, for a few experiments slices containing LC were cut in the coronal plane. For recording, slices were hemisected, fully submerged and perfused at a rate of 1.5 ml min^{-1}. Drugs were applied to the slice by changing the perfusion buffer to one that differed only in its content of drug.

Intracellular recordings were made with microelectrodes of resistance 35–55 MΩ, filled with 2 M KCl and dipped in 'Sigmacote' (Sigma, Australia). Membrane current and voltage were measured with an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA); the recordings were plotted directly onto chart paper and also digitized for later analyses (PCLAMP and AXOTAPE software, Axon Instruments). Membrane potential was clamped at −60 mV by use of discontinuous voltage-clamp with switching frequencies of 3.5–4.5 kHz. The potential at the headstage was continuously monitored on a separate oscilloscope. Some recordings were made by utilizing the whole cell patch clamp technique, in order to obtain optimal voltage control for the determination of steady state current-voltage relationships in the cells. For these experiments, the electrodes (resistance 3–6 MΩ) contained (mM): K-gluconate 125, NaCl 15, $MgCl_2$, HEPES 10, EGTA 11, MgATP 2, NaGTP 0.25, pH 7.3 with KOH. Recordings were made as described elsewhere (Osborne *et al*., 1996). Cells were clamped at −60 mV and the membrane potential was stepped between −50 and −140 mV for 250 ms, every 2 s. All command potentials were corrected for the junction potential between the intracellular and extracellular solutions. All data are expressed as mean ± s.e.mean, the pEC_{50} values are the geometric mean ± s.e.mean.

Drugs and chemicals

Cortistatin(1–14) (Pro-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Ser-Cys-Lys) was synthesized and purified by Chiron Mimotopes (Clayton, Victoria, Australia). Somatostatin(1–14) and met-enkephalin were purchased from both Auspep (Melbourne, Australia) and Sigma Australia. CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2) was purchased from both Peninsula Laboratories (Belmont CA) and Auspep (Melbourne, Australia) and a further sample, synthesized by Multiple Peptide Systems (San Diego, CA, U.S.A.), was generously donated by the National Institute on Drug Abuse (Bethesda, MD, U.S.A.). Naloxone hydrochloride was purchased from Research Biochemicals International (Natick, MA).

Results

Cortistatin produced a concentration-dependent outward current when applied to LC neurones (Figure 1). All LC neurones responded to cortistatin applied at a concentration of 3 nM or more ($n=44$). The effects of cortistatin reversed on washout. Maximally effective concentrations of cortistatin (10 μM) caused an outward current of 441 ± 13 pA ($n=4$), which is not different from that produced by a high concentration of the opioid agonist met-enkephalin (10 μM) (424 ± 14 pA) in the same cells. A concentration-response relationship for cortistatin activation of the outward current was determined by application of one or more concentrations of cortistatin to single neurones and expressing the resulting currents as a percentage of the current caused by 10 μM met-enkephalin in the same cells (Figure 1b). There appeared to be little desensitization of the cortistatin responses at concentrations of up to 300 nM; in 3 cells where 300 nM cortistatin was applied for between 10 and 20 min, the response declined by only 10%. Similarly, desensitization of responses to SRIF at low SRIF concentrations was not observed (1–100 nM, see Figure 2b in Chieng *et al*., 1996). However, to avoid possible desensitization of agonist responses (see below), low concentrations of agonist (3–100 nM) were not tested on a cell previously exposed to a high concentration (1–10 μM) of cortistatin, SRIF or CTOP. The concentration-response relationship for cortistatin was fitted with a logistic function to produce a pEC_{50} for cortistatin activation of the outward current of 6.62 ± 0.07. The Hill slope of the curve was 0.8 ± 0.01. SRIF also produced an outward current in all LC neurones when applied at concentrations greater than 3 nM ($n=43$). The effects of the application of concentrations of SRIF up to 10 μM reversed on washout. A concentration-response relationship for SRIF was determined in the same way

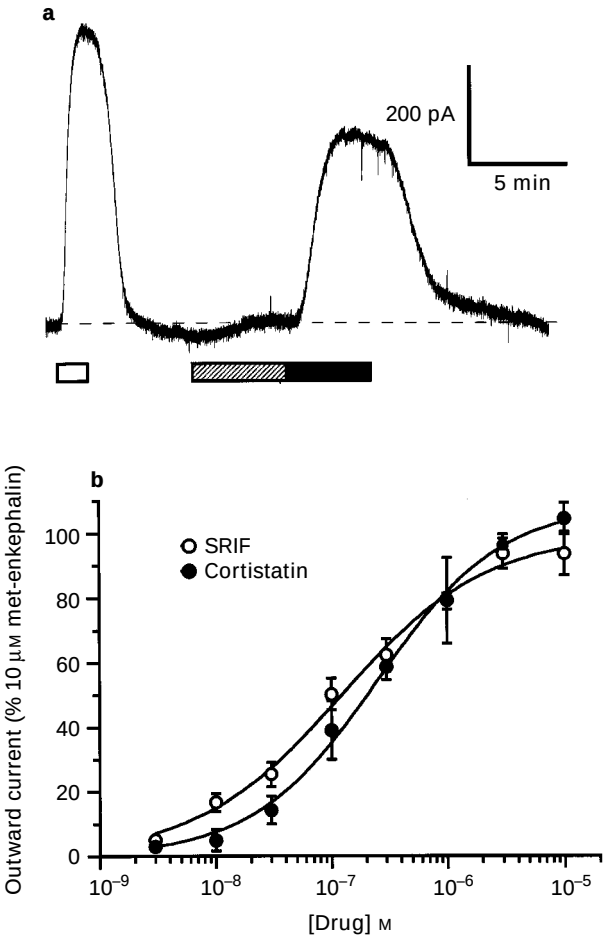

Figure 1 The effect of cortistatin on membrane currents of LC neurones. (a) Membrane currents induced by met-enkephalin (10 μM, open bar) and two concentrations of cortistatin (3 nM, hatched bar, and 300 nM, solid bar) in a single neurone voltage clamped with a microelectrode at −60 mV. Drugs were superfused for the duration shown by the bars. (b) Concentration-response relationship of outward currents induced by cortistatin and SRIF in LC neurones. Each point represents between 3 and 12 individual cells tested at each concentration of agonist. The points were fitted with a logistic function yielding an EC_{50} of 120 nM for SRIF and an EC_{50} of 240 nM for cortistatin. Vertical lines show s.e.mean.

as for cortistatin, the pEC_{50} for SRIF activation of the outward current was 6.93 ± 0.08, the Hill slope of the curve was 0.7 ± 0.1.

Somatostatin increases an inwardly rectifying K^+ conductance in cultured LC neurones (Inoue *et al*., 1988). The conductance activated by cortistatin was examined by determining the steady state current-voltage relationships for LC neurones in the presence and absence of cortistatin. These experiments were performed by use of the whole cell patch clamp technique, in slices of LC cut in the coronal plane, in order to optimize voltage control of the neurones (Travagli *et al*., 1995). In 8 out of 9 cells a clear reversal of the cortistatin-induced current was obtained (e.g. Figure 2). In these 8 cells the reversal potential for the cortistatin-induced current was -106 ± 4 mV, which is similar to the calculated reversal potential for a pure K^+ conductance with 2.5 mM extracellular K^+ (-105 mV). The cortistatin-induced currents were re-examined in 6.5 mM and 10.5 mM external K^+, and the reversal potentials were plotted as a function of the log of increasing K^+ concentration (Figure 2c). The slope of the resulting line was 60 mV per decade increase of $[K^+]$, which is very close to the slope of 61 mV per decade predicted by the Nernst equation. The current activated by cortistatin also showed mild inward rectification. When measured in 2.5 mM extracellular K^+, the slope conductance of the cortistatin-activated current at potentials between -60 and -90 mV was 1.2 ± 0.4 nS, and was 2.2 ± 0.8 nS at potentials between -110 and -130 mV.

Continuous application of SRIF or efficacious agonists at μ-opioid or α_2 receptors to LC neurones results in a homologous, transient desensitization to the agonist (see Discussion). We examined whether cortistatin was acting through SRIF receptors in the LC by performing cross-desensitization experiments. Continuous application of a high concentration of SRIF (1 μM) to LC neurones resulted in a decline in the outward current from maximum of 297 ± 28 pA to steady level of 155 ± 32 pA, a decline of $51 \pm 2\%$, with a $t_{1/2}$ of 131 ± 20 s ($n = 4$) (Figure 3a). When the SRIF response had reached the new steady level, a high concentration of cortistatin (3 μM) was applied to the neurone in the continued presence of SRIF. Cortistatin produced no change in the membrane current of the LC neurones (-10 ± 13 pA, $n = 4$) when applied in the continued presence of SRIF. Continuous application of the highest concentration of cortistatin tested (10 μM) to LC neurones resulted in a decline in the outward current from a maximum of 441 ± 13 pA to a steady level of 185 ± 22 pA, a decline of $58 \pm 6\%$ ($n = 4$) (Figure 3b). The $t_{1/2}$ for this desensitization was 114 ± 13 s. A high concentration of SRIF (3 μM) produced only a small change in the membrane current of LC neurones (33 ± 10 pA, $n = 4$) when applied during the desensitized portion of the cortistatin response.

The cross-desensitization between CTOP and cortistatin was also examined. Continuous application of a high concentration of CTOP (10 μM) to LC neurones resulted in a decline in the outward current from a maximum of 438 ± 18 pA to a steady level of 183 ± 51 pA, a decline of $58 \pm 2\%$, with a $t_{1/2}$ of 143 ± 3 s ($n = 4$). Application of a high concentration of cortistatin (3 μM) during the desensitized portion of the CTOP response produced only a small increase in the membrane current of LC neurones (38 ± 14 pA, $n = 4$). Continuous application of a concentration of cortistatin (3 μM) less than the maximum concentration tested (10 μM) also produced an outward current that desensitized; from 324 ± 35 pA to 179 ± 18 pA, a desensitization of $43 \pm 4\%$ ($n = 9$). The $t_{1/2}$ for this desensitization was 136 ± 15 s. A high concentration of CTOP (10 μM) applied when the cortistatin current had declined to a new steady level produced no outward current (Figure 4a, $n = 4$).

The possible interaction between cortistatin and the μ-opioid receptors present on LC neurones was also examined. The outward currents produced by a submaximally effective concentration of cortistatin (300 nM) were not affected by subsequent co-application of a high concentration of the non-selective opioid antagonist naloxone (10 μM). Similarly, when a high concentration of cortistatin (10 μM) was applied in the continuous presence of naloxone (10 μM), the outward current produced by cortistatin was $104 \pm 1\%$ ($n = 3$) of the current caused by an earlier application of met-enkephalin to the same cells, which is not different from the current caused by cortistatin in cells not treated with naloxone ($104 \pm 2\%$ of the met-

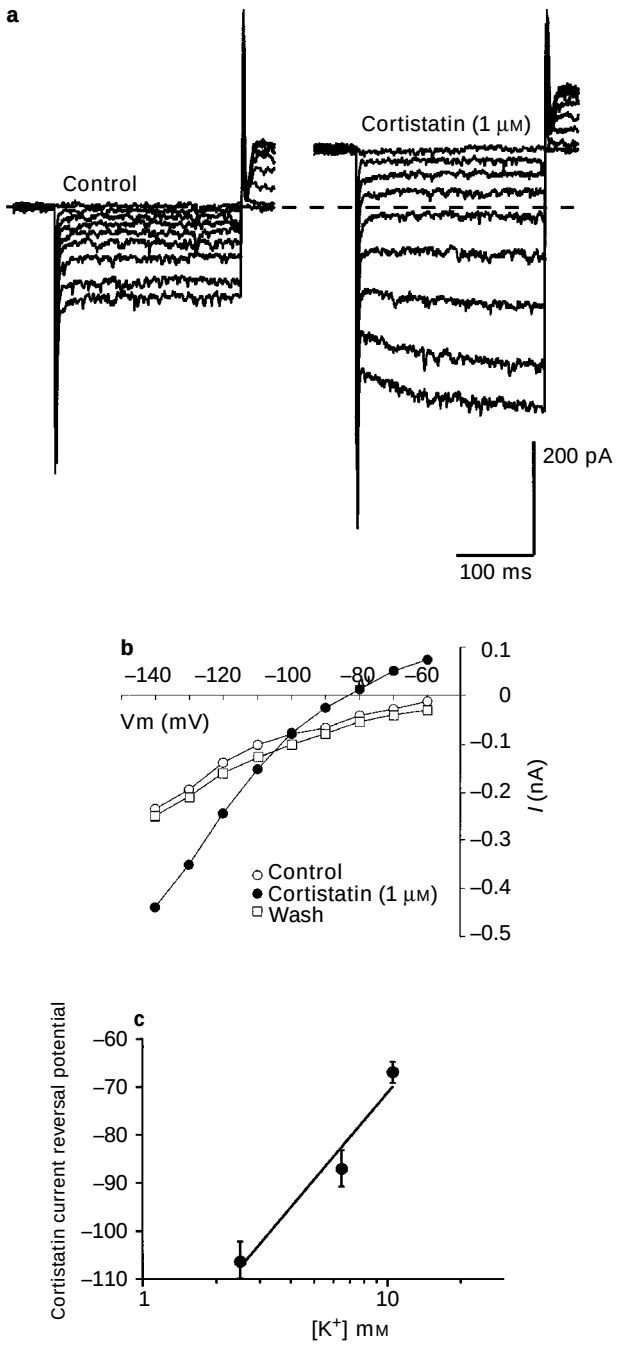

Figure 2 Cortistatin increased inwardly rectifying K^+ conductance of LC neurones. These recordings were made by means of the whole cell patch clamp technique, as outlined in the methods. (a) Voltage command steps 250 ms in duration were made in 10 mV increments from -50 to -140 mV from a holding potential of -60 mV. The resulting currents in the absence (left) and presence (right) of cortistatin (1 μM) in a single neurone are shown. The step to -50 mV has been omitted for clarity. (b) The current-voltage relationships were plotted from the amplitude of evoked currents shown in (a). (c) The reversal potential of the cortistatin-induced outward currents were determined in 2.5 mM, 6.5 mM and 10 mM extracellular K^+ ($n = 6-7$ for each). The slope of the line indicates a 60 mV change in reveral potential for every 10 fold change in extracellular K^+.

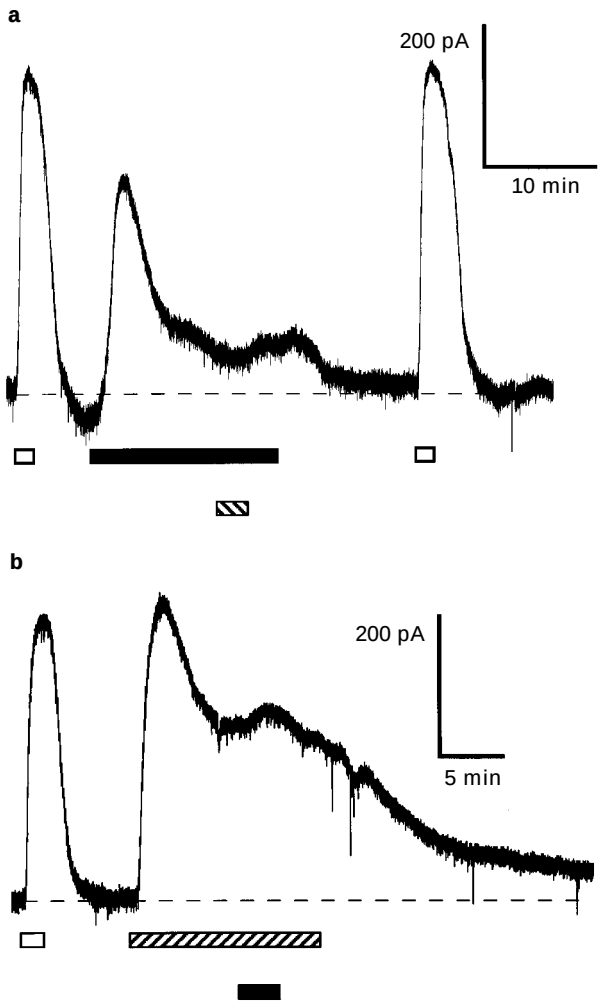

Figure 3 Cortistatin activation of the K^+ conductance cross-desensitized with SRIF in LC neurones. These neurones were voltage clamped at -60 mV with a microelectrode. (a) Applications of a high concentration of SRIF (1 μM, solid bar) following met-enkephalin (10 μM, open bar) application resulted in a large outward current that declined to a plateau over the duration of the SRIF application. Application of a high concentration of cortistatin (3 μM, hatched bar) during the period of SRIF desensitization did not result in any further outward current. (b) Application of a high concentration of cortistatin (10 μM, hatched bar) following met-enkephalin (10 μM, open bar) application also resulted in a large outward current that declined to a plateau over the duration of the cortistatin application. Application of a high concentration of SRIF (3 μM, solid bar) during the period of cortistatin desensitization evoked only a small outward current.

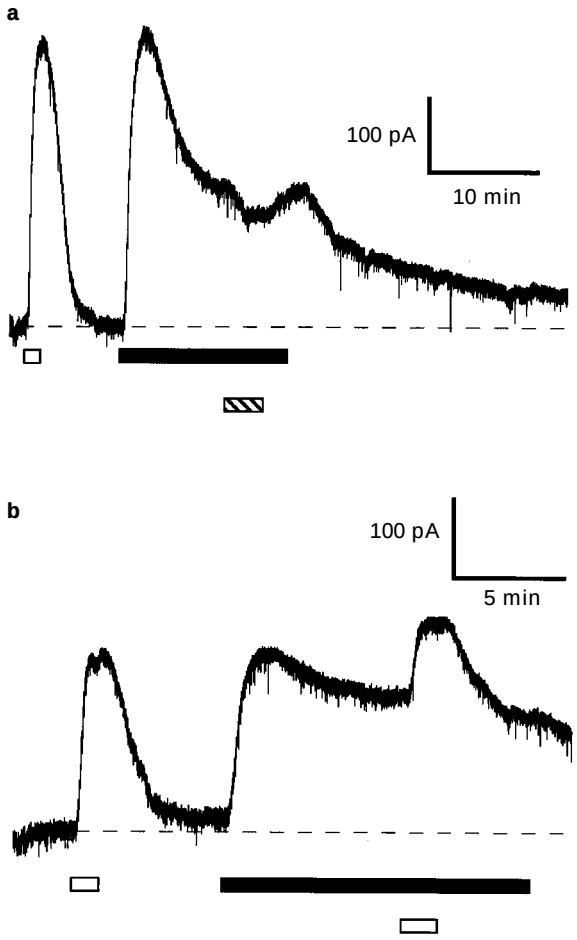

Figure 4 Cortistatin activation of the K^+ conductance cross-desensitized with CTOP, but not with met-enkephalin in LC neurones. These neurones were voltage clamped at -60 mV with a microelectrode. (a) Application of a high concentration of cortistatin (3 μM, solid bar) following met-enkephalin (10 μM, open bar) application resulted in an outward current that declined to a plateau over the duration of the cortistatin application. Applications of a high concentration of CTOP (10 μM, hatched bar) during the period of cortistatin desensitization did not result in any further outward current. (b) Application of a high concentration of cortistatin (3 μM, solid bar) following met-enkephalin (10 μM, open bar) application resulted in a large outward current that declined to a plateau. Application of a met-enkephalin (10 μM, open bar) during the period of cortistatin desensitization still produced a robust outward current.

enkephalin current, $n=4$). Conversely, application of the opioid agonist met-enkephalin (10 μM), during the continuous application of a high concentration of cortistatin (3 μM), produced an outward current not different from that produced by an earlier application of met-enkephalin alone. The met-enkephalin current in cortistatin was $102\pm4\%$ (Figure 4b, $n=5$) of the control met-enkephalin current.

Discussion

This study demonstrates that cortistatin, a recently identified somatostatin-like neuropeptide (de Lecea *et al*., 1996), potently and efficaciously increases an inwardly rectifying K^+ conductance in rat LC neurones. It is likely that cortistatin acted via SRIF receptors in the LC to increase the K^+ current, although this cannot be demonstrated directly in the absence of selective antagonists for SRIF receptors, which were unavailable for this study. However, there was essentially complete, reciprocal cross-desensitization between high concentrations of cortistatin and SRIF. This cross-desensitization between SRIF and cortistatin was likely to reflect actions of these peptides at the same receptor population rather than a heterologous desensitization of the K^+ channels. Previous studies have demonstrated that there is little cross-desensitization between high concentrations of agonists acting at a number of different receptors that mediate activation of the same population of inward rectifier K^+ channels in LC neurones. This homologous desensitization has been shown for μ-opioid receptors, α_2-adrenoceptors and receptors for somatostatin and nociceptin (Harris & Williams, 1991; Fiorello & Williams, 1996; Connor *et al*., 1996b).

There was also virtually complete cross-desensitization between cortistatin and CTOP, which suggests that the two agonists act through a similar population of receptors in the LC. This finding is in contrast to our previous observation that the desensitization between SRIF and CTOP did not operate reciprocally; i.e. SRIF completely cross-desensitized CTOP but

CTOP did not cross-desensitize SRIF (Chieng *et al*., 1996). We interpreted this previous finding as resulting from either SRIF acting on an additional population of receptors to CTOP, or from CTOP being a partial agonist at the same population of receptors as SRIF. In the current series of experiments, cortistatin completely cross-desensitized CTOP, but cortistatin induced a very small outward current when applied during a desensitized CTOP response. Similarly, SRIF completely cross-desensitized cortistatin, but SRIF induced a very small outward current when applied during a desensitized cortistatin response. The residual responses to SRIF in the presence of cortistatin and cortistatin in the presence of CTOP could indicate the presence of different receptor populations, but are most likely due to the use of slightly submaximal concentrations of CTOP and cortistatin. Unfortunately it was not practical to perfuse higher concentrations than those used here.

The somatostatin receptor mediating inhibition of LC neurones *in vitro* has previously been characterized as SST_2-like (Chessel *et al*., 1996), thus our results demonstrate that cortistatin is an effective agonist at rat SST_2-like receptors. It should be noted that the EC_{50} for SRIF activation of the K^+ conductance (120 nM) in this study was much greater than the EC_{50} for SRIF inhibition of spontaneous firing of LC neurones *in vitro* (12 nM, Chessell *et al*., 1996). This difference is probably due to the fact that the maximum effect measured in this study was the activation of all the available K^+ channels in each neurone, while Chessell and colleagues indirectly measured the amount of K^+ conductance activation required to hyperpolarize the LC neurones below the threshold for action potential generation, which is much less than the maximum possible hyperpolarization caused by agonists in the LC (e.g. Harris & Williams, 1991). Another difference between the study of Chessell and colleagues and the experiments described here, as well as by Fiorello & Williams (1996), is the observation of profound desensitization of the SRIF-induced activation of the K^+ conductance, such desensitization was not observed for SRIF inhibition of the firing of LC neurones. The acute desensitization of the SRIF activation of the K^+ current was between 50 and 60% (present study, Fiorillo & Williams, 1996). However, when the firing rate was measured during exposure to high concentrations of SRIF, presumably even a residual 40–50% activation of the K^+ channels was sufficient to maintain the hyperpolarization below threshold. Thus it is clear that SRIF receptors in the LC do desensitize, but apparently only at higher concentrations of agonist.

The EC_{50}s for the SRIF and cortistatin increase of the K^+ conductance in the LC slice are greater than those typically found for agonist activation of recombinant somatostatin receptors (de Lecea *et al*., 1996; Castro *et al*., 1996; Fukusumi *et al*., 1997), native somatostatin receptors found in cultured cells (e.g. Connor *et al*., 1997) or in isolated peripheral tissue (e.g. Mihara *et al*., 1987; Feniuk *et al*., 1993). The reason(s) for the comparatively low potencies of SRIF and cortistatin in this study are not known, but may reflect degradation of the agonists in the slice or a relatively inefficient coupling of the SRIF receptor to the inwardly rectifying K^+ conductance.

SRIF has a low affinity for low opioid receptors (Terenius, 1976; Maurer *et al*., 1982), but SRIF analogues such as SMS 201-995 (Sandostatin) and CTOP bind to opioid receptors with affinities in the nM range (Maurer *et al*., 1982; Pelton *et al*., 1986). Because cortistatin did not exactly mimic the actions of SRIF *in vivo*, the suggestion was made that cortistatin may have also been acting at μ-opioid receptors to produce effects distinct from those of SRIF (de Lecea *et al*., 1996). Our results suggest that this is unlikely. Firstly, application of a high concentration of the non-selective opioid antagonist naloxone failed to affect the outward current caused by either submaximally effective (300 nM) or maximally effective (10 μM) concentrations of cortistatin, indicating that the agonist actions of cortistatin were not mediated via μ-, κ- or δ-opioid receptors. Cortistatin was also unlikely to be a potent antagonist at μ-opioid receptors because a high concentration of cortistatin failed to affect the actions of a high, but not maximally effective, concentration of met-enkephalin. It remains to be established whether cortistatin is an agonist at SRIF receptors other than SST_2 or whether it has actions at non-SRIF receptors other than the μ-opioid receptor.

A human analogue of cortistatin was recently identified which differs by only one amino acid from the 14 amino acid rat peptide. However, it is not clear that cleavage sites in the rat and human precursor peptides are exactly conserved, and the identity of the peptides which may be produced *in vivo* is unclear (Fukusumi *et al*., 1997). The human cortistatin-like peptides bound to all 5 types of human SST receptor and were shown to be agonists at hSST receptors 2–5, although they were generally about 10 fold less potent than SRIF itself.

We have shown that cortistatin is an efficacious agonist at rat SST_2 receptors, and inhibits the activity of LC neurones by opening inwardly rectifying K^+ channels. Cortistatin has previously been shown to activate the M-type K^+ current in hippocampal neurones and to modulate cortical rhythms *in vivo* (de Lecea *et al*., 1996), thus modulatory actions of cortistatin acting at SRIF (and possibly other) receptors are likely to be functionally important in diverse regions of the nervous system.

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