0026-895X/96/030650-06$3.00/0

MOLECULAR PHARMACOLOGY, 50:650–655 (1996).

The μ-Opioid Receptor Antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) [but not D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP)] Produces a Nonopioid Receptor-Mediated Increase in K^+ Conductance of Rat Locus Ceruleus Neurons

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Received December 15, 1995; Accepted May 9, 1996

SUMMARY

The somatostatin analogues D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP) have been used widely as selective antagonists of μ-opioid receptors. Actions of CTOP and CTAP on the membrane properties of rat locus ceruleus neurons were studied using intracellular recordings of membrane currents in superfused brain slices. CTOP increased a K^+ conductance with an EC_{50} of 560 nM. The maximal conductance increase produced by CTOP (10 μM) was similar to that produced by high concentrations of the μ-opioid agonists D-Ala-Met-enkephalin-glyol (1 μM) and Met-enkephalin (10 μM), as well as an α_2-adrenoceptor agonist (UK14304, 3 μM) and somatostatin (1 μM). The K^+ current produced by CTOP was not antagonized by naloxone (1 μM), suggesting it was not mediated by μ-opioid receptors. The K^+ currents induced by high concentrations of CTOP desensitized to 42% of the initial maximum after prolonged superfusion ($t_{1/2}$ = 247 sec). In the presence of fully desensitized CTOP responses, somatostatin (1 μM) still produced near-maximal K^+ currents; i.e., there was no cross-desensitization, which suggests that CTOP might act on a receptor distinct from somatostatin receptors. However, the converse did not apply; high concentrations of CTOP (30 μM) did not produce any additional current in the presence of desensitized somatostatin responses. No cross-desensitization was observed between CTOP (10–30 μM) and Met-enkephalin (30 μM) or nociceptin (3 μM) regardless of the order of drug application. Cyclo-(7-aminoheptanoyl-Phe-D-Trp-Lys-Thr[*Bzl*], antagonized both somatostatin- (K_D = 10 μM) and CTOP- (K_D = 8 μM) induced K^+ currents with similar potency. Concentrations of CTOP (100 nM) that produced a small K^+ current partially antagonized the actions of Met-enkephalin (10 μM) on μ-opioid receptors. In contrast to CTOP, CTAP produced no K^+ current at concentrations of 300 nM and 1 μM and little current at 10 μM. CTAP potently antagonized K^+ currents produced by the μ-opioid receptor agonist D-Ala-Met-enkephalin-glyol, with an equilibrium dissociation constant of 4 nM (Schild analysis). CTAP did not antagonize K^+ currents produced by CTOP or somatostatin. These results demonstrate that CTOP is a potent and efficacious agonist at nonopioid receptors, whereas CTAP is a potent μ-opioid receptor antagonist with little nonopioid agonist activity in rat locus ceruleus neurons. The receptor activated by CTOP has yet to be fully resolved but seems to be similar to the somatostatin type 2 receptor or perhaps to a receptor closely related to somatostatin or opioid receptors.

A range of analogues of SST have been developed as selective μ-opioid receptor antagonists (1–3). CTOP and CTAP have been most widely used for this purpose because of their high affinity and selectivity for μ receptors. Equilibrium dissociation constants (K_D) at μ receptors reported for CTOP range from 0.4 to 3 nM in radioligand binding assays (1, 3). K_D values from Schild analysis ranged from 0.7 nM for [^{3}H]norepinephrine release from cortical slices (4) to 400 nM in the guinea pig ileum (2). CTOP has also been reported to have very low affinity for SST (1, 3) (IC_{50} = 24 μM; Ref. 3), δ-opioid (IC_{50} = 14 μM; Ref. 3), and κ-opioid receptors (K_i of U69593 = 5 μM; Ref. 1). CTAP was reported to have similar affinity and selectivity for μ receptors as CTOP (2, 3).

The antagonist actions of CTOP and CTAP at μ receptors have been substantiated by numerous functional studies *in vitro* (2, 4–6) and *in vivo* (7), but weak agonist actions have also been reported. In mouse vas deferens, CTOP produced

This work was supported by National Health and Medical Research Council of Australia.

ABBREVIATIONS: SST, somatostatin; CPP, cyclo(7-aminoheptanoyl-Phe-D-Trp-Lys-Thr[*Bzl*]; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; DAMGO, D-Ala-Met-enkephalin-Gly-ol; LC, locus ceruleus; ORL_1, opioid receptor-like receptor$_1$; SSTR*n*, somatostatin receptor, where *n* is type number (molecular classification).

weak inhibition of electrically evoked twitch, which showed some cross-desensitization with SST (2). Weak δ-opioid receptor agonist actions of CTAP have also been reported (2).

CTOP and CTAP have also been reported to produce functional effects in the absence of opioid agonists. The results of some such studies have differed from those reported using alkaloid antagonists (7), whereas the results of others have been paradoxical (8–11). For example, microinjections of CTOP into the ventral tegmental area have been reported to antagonize the μ receptor agonist-mediated increase in dopamine release in nucleus accumbens (9, 12), but when injected alone, CTOP either decreased dopamine release (12) or increased dopamine metabolite concentrations (9) and increased locomotor activity (13). Functional effects of CTOP and CTAP have generally been explained in terms of antagonism of tonic endogenous opioid release (9–12) or novel properties of μ-opioid receptors (8), but the possibility that CTOP might also have nonopioid actions has generally not been considered.

The current study arose from preliminary experiments[1] in which several SST analogues were found to produce an outward current in rat LC neurons. LC neurons express μ-opioid (14, 15), α_2-adrenoceptor (15), and SST receptors (16), which functionally converge to activate an inwardly rectifying K^+ conductance. We report here that CTOP, but not CTAP, produces an increased K^+ conductance in LC neurons that is not mediated by opioid receptors but resembles that produced in the same neurons by μ-opioid, α_2, nociceptin, and SST agonists.

Experimental Procedures

Preparation of tissue and solutions. Male Sprague-Dawley rats (150–250 g) were anesthetized with halothane and killed by cervical dislocation, and horizontal brain slices containing 300 μm LC were cut and maintained in physiological saline at 35°. Hemisected slices containing LC were submerged and superfused with physiological saline at 35° (1.5 ml/min). The physiological saline solution contained 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 11 mM glucose, and 24 mM $NaHCO_3$ and was gased with 95% O_2/5% CO_2. Drugs were applied to the slice by changing the solution to one that differed only in its content of the drug.

Recording procedure and measurement of K^+ currents. Intracellular recordings of membrane potential and current were made with microelectrodes (30–50 MΩ) filled with 2 M KCl and immersed in Sigmacote (Sigma Chemical, St. Louis, MO) using a single-electrode current- and voltage-clamp amplifier (Axoclamp-2A; Axon Instruments, Burlingame, CA) as described previously (17). Recordings of membrane potential and current were plotted directly on chart recorder paper as well as being digitized for later analyses (pClamp and Axotape software; Axon Instruments). Electrode resistance was monitored and balanced throughout experiments. Only cells that had action potential amplitudes of ≥70 mV were used for data analysis. Membrane potential was clamped at −60 mV, unless otherwise indicated, using discontinuous voltage-clamp at switching frequencies of 3–5 kHz during continuous monitoring of the potential at the headstage with a separate oscilloscope. All data are expressed as mean ± standard error.

Materials. Met-enkephalin was purchased from Sigma Chemical and CPP was purchased from Sigma Chemical and Bachem (Bubendorf, Switzerland). SST_{1-14} was purchased from Auspep (Melbourne, Australia). UK14304, idazoxan hydrochloride, and naloxone hydrochloride were from Research Biochemicals (Natick, MA). Nociceptin (Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln) was synthesized and purified (>95% by high performance liquid chromatographt) by Chiron Mimotopes (Clayton, Victoria, Australia). Three separate samples of CTOP were tested with similar results. Two different lots of CTOP were purchased from Peninsula Laboratories (Belmont, CA), and a third sample, synthesized by Multiple Peptide Systems (San Diego, CA), was generously donated by the National Institute on Drug Abuse (Bethesda, MD). The National Institute on Drug Abuse also generously donated CTAP and DAMGO, which was synthesized by Multiple Peptide Systems.

Results and Discussion

CTOP (but not CTAP) increases K^+ conductance via a naloxone-insensitive mechanism. Fig. 1A demonstrates outward currents produced in a single LC neuron by superfusion of CTOP (100 nM to 1 μM) and Met-enkephalin (10 μM). As shown in Fig. 1B, the maximal outward currents produced by CTOP (Peninsula) and the selective μ receptor agonist DAMGO were similar, demonstrating that CTOP is a full agonist in LC neurons. Similar maximal outward currents were produced by CTOP (97 ± 7% of current produced by Met-enkephalin, 10 μM; four experiments; CTOP from both suppliers), SST (1–3 μM; 96 ± 2%, nine experiments), and UK14304 (3 μM; 95 ± 2%, four experiments; see Fig. 2, C and D). The conductance change responsible for the outward current produced by CTOP did not differ qualitatively from that produced by Met-enkephalin, nor did the actual or extrapolated reversal potentials (E_{rev} = −150 ± 4 mV for CTOP and −152 ± 7 mV for Met-enkephalin; four experiments). These results demonstrate that CTOP, like μ-opioid, α_2, and SST agonists, increase a K^+ conductance in LC neurons. Although the reversal potentials of CTOP- and Met-enkephalin-induced currents seemed to be more negative than E_K, this has previously been demonstrated to be due to poor space-clamp of K^+ currents arising from regions remote from the soma (18).

Concentration-response curves were prepared exclusively using CTOP purchased from Peninsula Laboratories. The concentration-response curves presented in Fig. 1B were fit by a logistic function, yielding an EC_{50} for CTOP of 560 nM and a maximal response that was 99% of that produced by 10 μM Met-enkephalin. The EC_{50} for DAMGO was 43 nM, and maximal response was 107% of that produced by 10 μM Met-enkephalin, which is similar to previously reported values (19, 20). CTAP produced no outward current at concentrations of 300 nM and 1 μM and little outward current at 10 μM. These results demonstrate that CTOP, but not CTAP, has potent and efficacious agonist actions in LC neurons.

Fig. 1C demonstrates that naloxone (1 μM) completely antagonizes the actions of Met-enkephalin (10 μM) on μ receptors but has little or no effect on the action of CTOP (1 μM). Naloxone (1 μM) failed to block CTOP induced currents in 6 neurons, producing 8 ± 9% inhibition [three experiments, paired t test, $p > 0.4$ (NS)]. The μ but not δ or κ receptor agonists have been widely reported to increase K^+ conductance of LC neurons (14, 18, 21), and naloxone antagonizes this action with a K_D value of ~2 nM (14). The failure of 1 μM naloxone to antagonize the action of CTOP therefore demonstrates an agonist action on a receptor distinct from μ receptors.

These results demonstrate that CTOP, but not CTAP, acts

[1] M. J. Christie and J. T. Williams, unpublished observations.

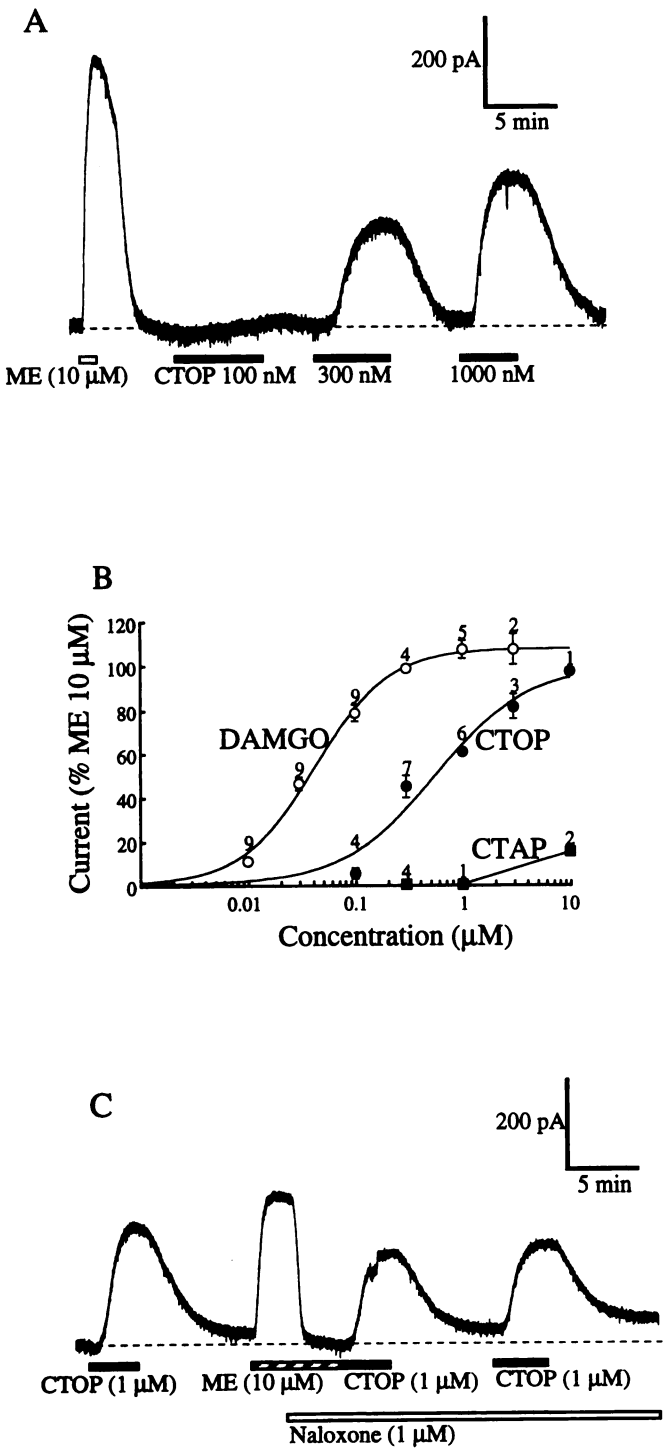

Fig. 1. CTOP increases K^+ conductance of LC neurons by a μ receptor independent mechanism. A, Both Met-enkephalin (*ME*) and CTOP produce outward currents in a single LC neuron. *Bars*, period of drug superfusion (holding potential, −60 mV). B, Concentration-response relationships for DAMGO, CTOP, and CTAP expressed as a percentage of the outward current produced by Met-enkephalin (*ME*) (10 μM). *Numbers above symbols*, number of cells for each data point. EC_{50} values estimated from logistic functions were 43 nM, 560 nM, and $\gg$10 μM for DAMGO, CTOP, and CTAP, respectively. C, Naloxone (1 μM) completely antagonized the outward current produced by Met-enkephalin (*ME*) (10 μM) but had little effect on the outward current produced by CTOP (1 μM) in a single neuron.

as a potent and efficacious nonopioid receptor agonist with actions resembling those of other inhibitory receptor agonists in LC neurons. Functional studies using CTOP should therefore be interpreted with great caution, particularly when effects are observed after application of high concentrations of the peptide (e.g., 3 μM to 3 mM in Ref. 9) in the absence of opioid agonists. Until the receptor mediating the agonist effects of CTOP has been identified, it will not be clear how widespread this action is throughout the nervous system. We have observed[2] but not fully characterized a similar agonist effect of CTOP in single rat periaqueductal gray neurons, suggesting that agonist actions of CTOP are not confined to the LC.

CTOP is an agonist at a receptor similar to, but possibly not identical with SST receptors. SST opens the same population of K^+ channels as μ-opioid and α_2-adrenoceptor agonists on rat LC neurons (15, 16, 21). *In situ* hybridization studies have demonstrated mRNA encoding both SSTR2s and SSTR3s in LC neurons (22), but SSTR2 is predominant (for the molecular classification used here and below, see Ref. 23). Although CTOP was reported to have very low affinity for SST receptors in brain (1, 3), an action on SST receptors was still explored to account for the agonist actions of CTOP in LC neurons, particularly because weak SST-like agonist actions of CTOP have been reported in other tissues (2).

As shown in Fig. 2, A and B, the putative SST antagonist CPP (23) weakly antagonized the effects of both CTOP and SST. The pA_2 of CPP to antagonize CTOP and SST responses was estimated by Schild analysis in single neurons from cumulative dose-response curves in the absence and presence of one to three concentrations of CPP, assuming a competitive interaction. Fully surmountable antagonism was not demonstrated because high concentrations of both CTOP and SST produced profound desensitization. However, the antagonist did not interact nonspecifically with the K^+ conductance or coupling mechanisms because agonist actions of UK14304 (1 μM, three experiments) and nociceptin (100 nM, three experiments) were completely unaffected by CPP (3–10 μM). The estimated pA_2 of CPP was similar for both CTOP (pA_2 = 5.1 ± 0.1, four experiments) and SST (5.0 ± 0.2, four experiments). SST receptor subtypes in rat LC have not previously been functionally characterized, but CPP has been reported to bind to SSTR2s with a K_D of >1 μM compared with 141 nM for SSTR1s and 107 nM for SSTR3s (23). It therefore seems likely that the actions of CTOP were mediated by an agonist action on SSTR2s. However, an agonist action of CTOP on SSTR2s in LC is not consistent with the very low affinity of CTOP reported for SST receptors (IC_{50} = 24,330 nM) labeled in brain by [^{3}H]CGP23,996 (3). CTAP (1 μM) did not antagonize K^+ currents produced by CTOP (300 nM) or SST (30 nM), demonstrating that CTAP does not interact with the receptors activated by CTOP or SST. These results suggest that CTOP, but not CTAP, acts as an agonist at SSTR2s in LC, but they must be considered tentative until the binding of CTOP to SSTR2s is reevaluated.

Cross-desensitization experiments were performed to further explore the involvement of SST receptors in the agonist actions of CTOP. Homologous, transient desensitization of μ-opioid and SST receptors occurs in LC neurons after expo-

[2] B. Chieng and M. J. Christie, unpublished observations.

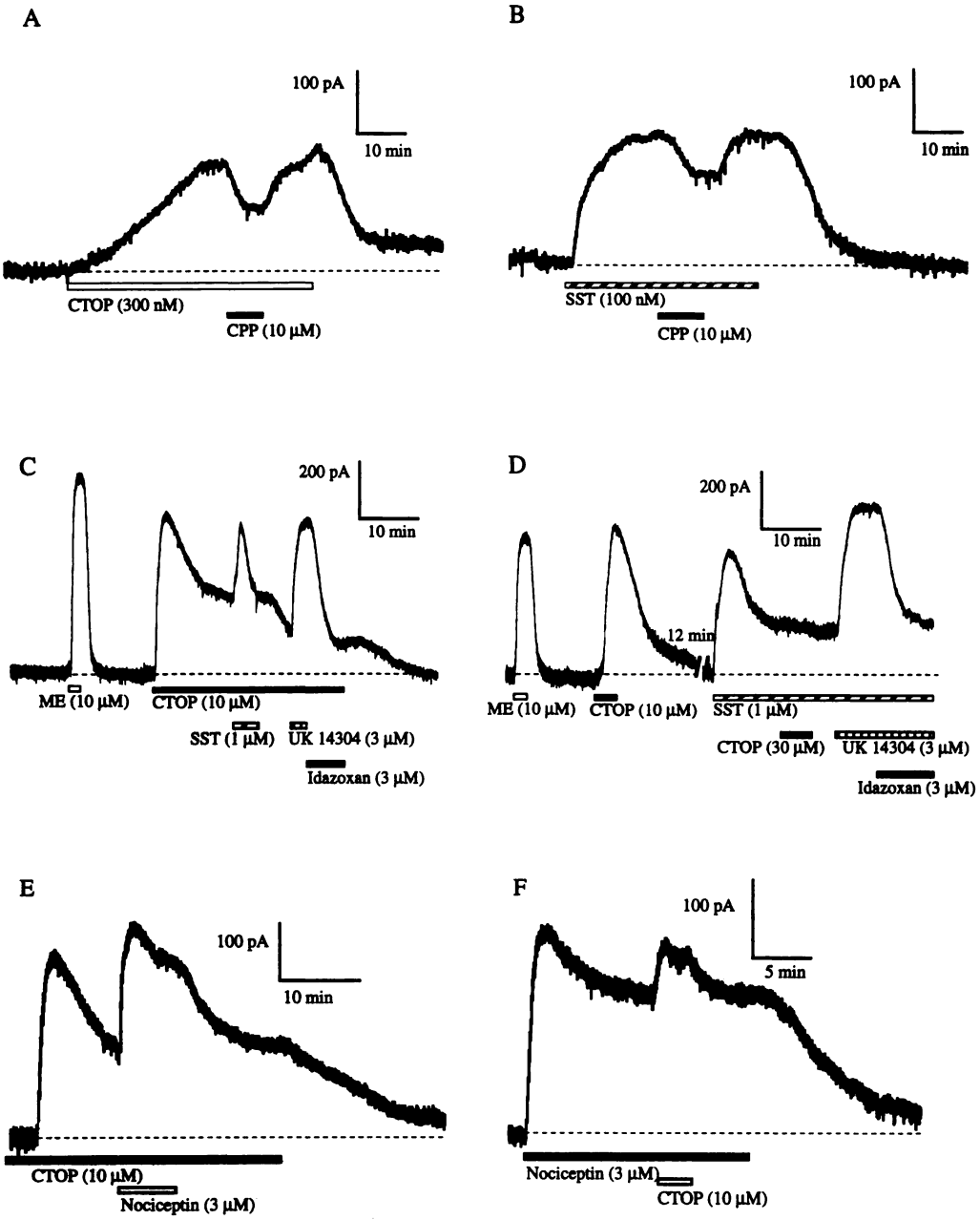

Fig. 2. Possible involvement of SST receptors in the K^+ current produced by CTOP. A, The SST receptor antagonist CPP weakly antagonized the K^+ current produced by a submaximal concentration of CTOP. B, CPP also weakly antagonized the response to a submaximal concentration of SST in the same neuron as that shown in A. C, CTOP (10 μM) did not produce cross-desensitization of maximal responses to SST (1 μM); i.e., SST was still capable of producing a near-maximal response after the response to CTOP was maximally desensitized. UK14304 (3 μM) still produced a maximal response, which was reversed by idazoxan. Also note the second phase of desensitization after washout of the test application of SST. *ME*, Met-enkephalin. D, SST (1 μM; different neuron than that shown in C) produced cross-desensitization of responses to CTOP (30 μM); i.e., CTOP was unable to produce any additional response after the response to SST was fully desensitized. UK14304 (3 μM) still produced a maximal response in the presence of desensitized responses to SST. *ME*, Met-enkephalin. E, The ORL_1 ligand nociceptin (3 μM) did not produce cross-desensitization of responses to CTOP (10 μM). F, CTOP did not produce cross-desensitization of responses to nociceptin (3 μM).

sure to high concentrations of efficacious μ receptor agonists for several minutes (19, 24, 25). If CTOP were acting on SST receptors, then additional actions of high concentrations of SST should be abolished after prolonged superfusion with maximally desensitizing concentrations of CTOP, and *vice versa*. As shown in Fig. 2C, responses to high concentrations of CTOP (10 μM) desensitized to 42 ± 7% of the initial maximum with a $t_{1/2}$ of 247 ± 24 sec (four experiments). SST (1 μM) was still able to produce a near-maximal effect (88 ± 10% of original maximum; four experiments) when superfused during a maximally desensitizing concentration of CTOP (10 μM, three experiments; 30 μM one experiment).

These results suggest that CTOP might act on a receptor distinct from those acted on by SST. If this were the case, then the converse experiment should yield parallel results; i.e., CTOP should still produce maximal responses in the presence of fully desensitized SST responses. As shown in Fig. 2D, this was not the case. Responses to SST (1 μM) desensitized to a similar extent (36 ± 6% of initial maximum; three experiments) but with a greater rate than CTOP ($t_{1/2}$ = 139 ± 22 sec; unpaired *t* test, $p < 0.05$, five degrees of freedom). However, superfusion of CTOP (30 μM) during fully desensitized SST responses produced no additional current (37 ± 6% of initial maximum; three experiments).

There are several possible explanations for this asymmetrical cross-desensitization. One possibility is that SST acts on more than one subtype of SST receptor in LC, but CTOP acts on, and therefore produces, desensitization at only one subtype of SST receptor. If correct, then CTOP would be expected to produce desensitization at only one subtype (e.g., SSTR2), and the other subtype (e.g., SSTR3) would still be available to produce a maximal response to the test bolus of SST. This mechanism might also be expected to shift the dose-response curve for SST to the right after desensitization of CTOP responses, but this

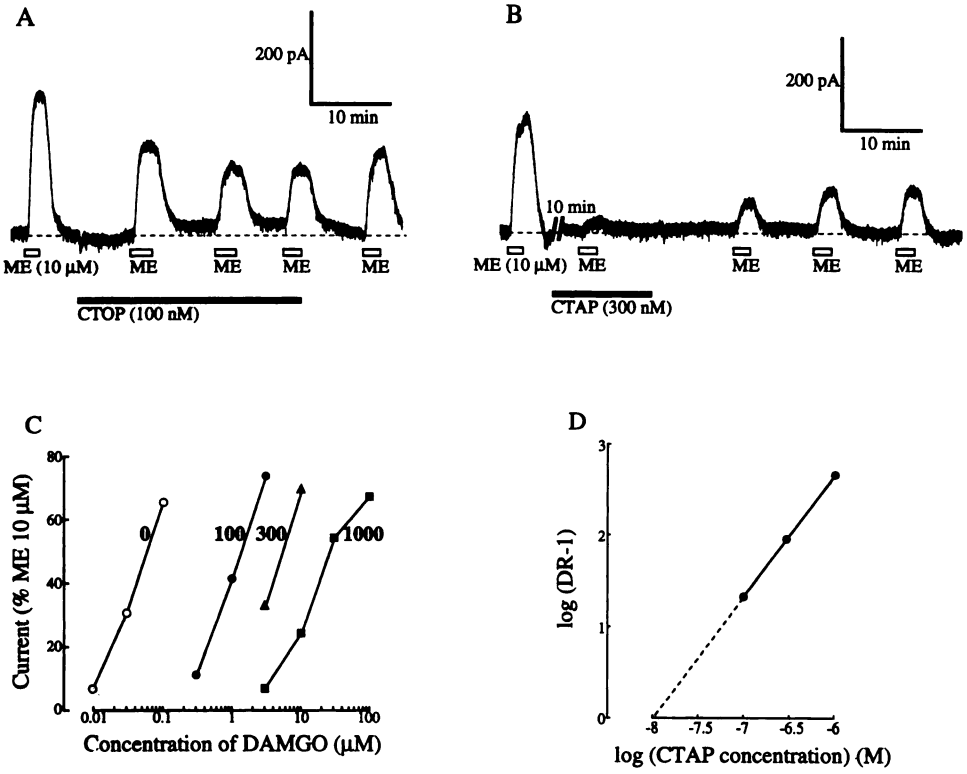

Fig. 3. CTOP and CTAP act as μ receptor antagonists in LC neurons. A, concentration of CTOP (100 nM) that produced little outward current partially antagonized the outward current produced by Met-enkephalin (*ME*) (10 μM throughout). The response to Met-enkephalin partially recovered after washout of CTOP. B, CTAP (300 nM) almost completely antagonized the effect of Met-enkephalin (*ME*) (10 μM throughout). The response to Met-enkephalin recovered slowly after washout of CTAP. C, Competitive antagonism by increasing CTAP concentrations of the outward current produced by DAMGO in a single neuron. Concentration of CTAP (nM) is indicated next to each concentration-response curve. *ME*, Met-enkephalin. D, Schild plot of data presented in C. The pA_2 was 8.0, with a slope of 1.3.

could not be tested due to the slow washout of high concentrations of CTOP. In contrast, prolonged superfusion of SST would be expected to desensitize both SST receptor subtypes, including the type acted on by CTOP. The expression of both SSTR2s and SSTR3s in LC is consistent with this possibility (22), but the weak interaction of CPP with responses to both SST and CTOP suggests that SSTR2s predominantly mediate responses to both agonists.

A second possibility is that CTOP acts on the same receptors as SST but has considerably less intrinsic efficacy. The more efficacious SST might then be expected to produce greater desensitization than that produced by continuous superfusion of CTOP, after which CTOP would no longer be able to produce the same response as before because of its lower intrinsic efficacy (19, 24). The converse might not occur; i.e., prolonged superfusion of CTOP might not produce sufficient desensitization to prevent responses to high concentrations of SST. Low intrinsic efficacy of CTOP at SST receptors might also account for the curious second phase of profound desensitization (Fig. 2C) that occurred in the presence of CTOP (10 μM) after superfusion with a test bolus of SST (1 μM). After superfusion of SST, the response to CTOP desensitized to a new minimum of 27 ± 10% (four experiments) of the original maximum. However, this interpretation is speculative because there is no direct evidence that CTOP has lower intrinsic efficacy than SST at SST receptors. Furthermore, the very low binding affinity of CTOP reported for SST receptors (1, 3) contradicts this interpretation. It also is not supported by the observations that CTOP and SST produced a similar maximal response and a similar extent of desensitization. CTOP also failed to act as a partial agonist when superfused during desensitized responses to SST, but the concentration of CTOP might not have been sufficiently high for such an effect to be observed.

A third possibility is consistent with the reported failure of CTOP to bind to SST receptors and arose from the second phase of profound desensitization (Fig. 2C) produced by SST in the presence of CTOP (10 μM). It is possible that CTOP acts on a non-SST receptor, and SST receptor activation produces cross-desensitization of "CTOP receptors." Prolonged superfusion of SST would then be expected to cross-desensitize CTOP receptors before superfusion of the test bolus of CTOP, and therefore no additional response would be observed. Such cross-desensitization produced by SST receptor activation would be limited to a subset of receptors because desensitization of α_2-adrenoceptors was not observed (see Fig. 2C). We therefore considered the possibility that other receptors (e.g., μ receptors and orphan opioid receptors; see below) might show similar cross-desensitization after prolonged superfusion of SST (1 μM), but we observed none using Met-enkephalin (30 μM) as the agonist (two experiments; data not shown).

We further explored the possible interaction of CTOP with non-SST receptors by examining cross-desensitization with responses to nociceptin. Nociceptin is an endogenous ligand of the opioid-like receptor ORL_1 (26), which is abundant in LC neurons (27). We previously reported that nociceptin, acting on ORL_1, increases K^+ conductance of dorsal raphe nucleus neurons (28). As shown in Fig. 2E, nociceptin also produced a K^+ conductance increase in LC neurons that was naloxone insensitive (10 μM; not shown). The nociceptin-induced K^+ current desensitized during prolonged superfusion of high concentrations (Fig. 3E) but did not show cross-desensitization with responses to CTOP, regardless of the order of application (Fig. 2, E and F; four experiments). These results suggest that CTOP does not interact with ORL_1 in LC neurons.

In summary, our studies of antagonist potency and cross-

desensitization are generally consistent with an agonist interaction of CTOP with SSTR2s on LC neurons. However, we cannot yet rule out an action on other SST receptor subtypes or as-yet-unidentified nonopioid or non-SST receptors, which might explain asymmetrical desensitization between CTOP and SST.

CTOP and CTAP are μ receptor antagonists. The K_D for CTOP at μ receptors could not be fully characterized because of the agonist current it produced. Superfusion of CTOP at a concentration that produced little outward current (100 nM) partially inhibited the K^+ current induced by Met-enkephalin (10 μM; Fig. 3A). With the assumption that the interaction is competitive and that the EC_{50} for Met-enkephalin is ~1 μM[3] (15), the K_D for CTOP is of the order of 10 nM, which is consistent with receptor binding and functional studies (see introduction). These experiments demonstrate that CTOP, when used in concentrations that do not produce substantial agonist actions, does not completely antagonize the actions of Met-enkephalin in the LC. If similar agonist actions of CTOP occur in other cell types, then functional effects of CTOP (see introduction) could be incorrectly attributed to simple antagonism of μ receptor-mediated phenomena.

CTAP was a potent, competitive antagonist at μ receptors. Fig. 3B shows that superfusion with 300 nM CTAP almost completely abolished Met-enkephalin (10 μM) currents. Dose-response curves constructed in a single neuron using DAMGO as the μ receptor agonist demonstrated that CTAP was a competitive antagonist (Fig. 3C), with a K_D determined by Schild analysis of 10 nM (Fig. 3D). The estimated pA_2 from four such experiments was 8.4 ± 0.2. These results demonstrate that CTAP is a potent competitive antagonist in LC neurons at concentrations that produce no agonist actions. It would, however, be premature to assume that the same holds for functional studies of other types of neurons. For example, CTAP was reported to have weak δ receptor agonist actions in mouse vas deferens (2). Furthermore, anomalous actions of CTAP (8) and closely related analogues (29) that are inconsistent with simple μ receptor antagonism have also been reported.

Acknowledgments

The technical assistance of Gina Cleva and the helpful comments of Peregrine B. Osborne are gratefully acknowledged.

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