0022-3565/89/2492-0544$02.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 249, No. 2
Printed in U.S.A.

Novel Peptidic *Mu* Opioid Antagonists: Pharmacologic Characterization *in Vitro* and *in Vivo*[1]

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Accepted for publication February 15, 1989

ABSTRACT

A series of six synthetic octapeptides, structurally related to somatostatin, demonstrate high affinity and selectivity for *mu* opioid receptors in radioligand binding assays. The compounds, D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (CTP), D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP), D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP), D-tetrahydroisoquinoline carboxylic acid (D-Tic)-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (D-Tic-CTP), D-Tic-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (D-Tic-CTOP) and D-Tic-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (D-Tic-CTAP), were tested *in vitro* and *in vivo* for agonist and antagonist potency and selectivity. *In vitro* bioassays included the guinea pig ileum, mouse vas deferens and rabbit vas deferens. *In vivo* tests included hotplate antinociception and gastrointestinal transit inhibition, performed in mice. *In vitro*, all six derivatives were competitive, highly selective *mu* antagonists (pA_2 values from 6.4–7.9). The compounds demonstrated varying degrees of intrinsic agonist activity especially in the mouse vas deferens, the least active being CTAP and D-Tic-CTAP, which showed no *mu* or *kappa* agonist actions, and *delta* activity only at very high (>3 μM) concentrations. *In vivo*, none of these compounds showed antinociceptive actions when administered i.c.v. in mice. All were competitive *mu* antagonists in the hotplate antinociception test. Antagonist potency for all the derivatives was similar and approximately 10-fold greater than naloxone in this *in vivo* test (pA^2 values from 11.2–11.9) CTAP and CTOP gave comparable results (competitive *mu* antagonism, lack of agonist activity) when administered intraspinally in the gastrointestinal transit inhibition test, with apparent antagonist potency also approximately 10-fold greater than naloxone. (pA_2 values of 11.4 and 11.3, respectively). At present, these compounds represent the most selective and potent *mu* opioid antagonists yet synthesized.

Research in opioid pharmacology has presented considerable challenges to investigators since the concept of multiple types of opioid receptors has become widely accepted. The discovery of multiple receptor types was not closely paralleled by the development of ligands selective for each site. Instead, new, more specific agents emerged years after these receptors were first postulated and described (Martin *et al.*, 1976). Also, although both agonist and antagonist drugs are necessary for thorough pharmacologic investigations, researchers currently lack a full complement of highly selective agonist and antagonist ligands for each of the accepted opioid receptor types. Whereas highly selective agonists are now available for *mu* (Chang *et al.*, 1983), *delta* (Mosberg *et al.*, 1983) and *kappa* (Lahti *et al.*, 1985) receptors, selective antagonist drugs have proven elusive. An antagonist for the *delta* receptor, ICI 174,864 (Cotton *et al.*, 1984), is now available and is becoming generally accepted. Several agents have been proposed as selective *kappa* antagonists over the last few years, perhaps the most promising being norbinaltorphimine (Portoghese *et al.*, 1987; Takemori *et al.*, 1988), but they remain unproven on a widespread basis. A particular disappointment has been the lack of highly selective competitive antagonist ligands for the *mu* receptor.

It is generally accepted that while alkaloidal antagonists such as naloxone and naltrexone have high affinity for the *mu* receptor, their selectivity is marginal at best. Several groups of investigators have turned to peptide drugs in an effort to achieve the superior selectivity possessed by peptidic opioid agonists. As early as 1977 an attempt was made to modify the endogenous peptide Met-enkephalin to produce an antagonist. The resulting compounds displayed mixed agonist and antagonist activity (Pert *et al.*, 1977). More recent attempts based on Met-enkephalin have shed some light on the structure/activity relationships of opioids, but again produced compounds

Received for publication November 7, 1988.
[1] This work was supported by U.S. Public Health Service Grants DA02163, DA04248, DK36289, DK33547 and NS19972.

ABBREVIATIONS: CTP, D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2; GPI, guinea pig ileum longitudinal muscle/myenteric plexus; MVD, mouse vas deferens; DPDPE, [D-Pen^2-D-Pen^5]enkephalin; D-Tic, D-tetrahydroisoquinoline carboxylic acid; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2; LVD, rabbit vas deferens; HP, hotplate antinociception; GIT, gastrointestinal transit; i.t., intrathecal.

with mixed agonist and antagonist activity, poor potency compared with naloxone and very short durations of action (Judd *et al.*, 1987).

The tetradecapeptide somatostatin, in addition to its functions in controlling growth hormone release and gastrointestinal absorption/secretion, has demonstrable, although relatively weak, opioid receptor affinity (Terenius *et al.*, 1976). Its cyclic structure provides reduced conformational flexibility and may also provide some additional degree of biological stability. A novel opioid peptide with structural similarity to somatostatin was characterized recently by members of our group. This compound, a cyclic octapeptide, CTP, demonstrated high affinity for *mu* opioid receptors, and low affinity for *delta* and somatostatin receptors in radioligand binding studies (Pelton *et al.*, 1986). *In vitro* bioassays, including the GPI and MVD, showed CTP to be a potent and selective *mu* opioid antagonist (Shook *et al.*, 1987b). However, CTP also possessed significant agonist actions in the MVD preparation, which were shown to be the result of both *delta* opioid and somatostatin receptor interactions. *In vivo* testing revealed highly potent and long-lasting opioid antagonist properties for CTP (Shook *et al.*, 1987a), but selectivity was less than optimum. Thus, whereas the antinociceptive effects of *mu* agonists in mice were antagonized competitively by CTP, the effects of the *delta* agonist DPDPE were also antagonized, in a noncompetitive fashion.

In an effort to improve upon the *in vitro* and *in vivo* selectivity profile of CTP, a series of new CTP derivatives was synthesized recently and tested in radioligand binding assays (Kazmierski *et al.*, 1988). CTP was modified at two positions to produce five additional derivatives. Ornithine or arginine was substituted for lysine in position 5, and the constrained D-phenylalanine analog D-Tic was substituted for D-phenylalanine in position 1 (fig. 1). The results of these modifications were increased *mu* receptor affinity, and decreased affinity for the *delta* opioid and somatostatin receptors.

CTP

D–Phe–Cys–Tyr–D–Trp–Lys–Thr–Pen–Thr–NH_2

CTOP

D–Phe–Cys–Tyr–D–Trp–Orn–Thr–Pen–Thr–NH_2

CTAP

D–Phe–Cys–Tyr–D–Trp–Arg–Thr–Pen–Thr–NH_2

D–Tic–CTP

D–Tic–Cys–Tyr–D–Trp–Lys–Thr–Pen–Thr–NH_2

D–Tic–CTOP

D–Tic–Cys–Tyr–D–Trp–Orn–Thr–Pen–Thr–NH_2

D–Tic–CTAP

D–Tic–Cys–Tyr–D–Trp–Arg–Thr–Pen–Thr–NH_2

Fig. 1. Amino acid sequences for CTP, CTOP, CTAP, D-Tic-CTP, D-Tic-CTOP and D-Tic-CTAP. Lines indicate cyclization points.

The objectives of the present work were 3-fold. We wished to examine the *in vitro* and *in vivo* opioid activity profiles of the six derivatives (including CTP) to classify them as agonists or antagonists. We chose multiple *in vitro* bioassays for their different populations of opioid receptors and thus their differential responsiveness to opioids of differing selectivities; two *in vivo* bioassays were used to confirm the effectiveness of these novel drugs on multiple, independent opioid systems. We then assessed *mu* receptor potency and selectivity, and any residual intrinsic activity. Finally, we have attempted to select the most appropriate derivative for further study and general use as a pharmacologic tool.

Methods

The experimental protocols described herein were reviewed and approved by the University of Arizona Animal Care and Use Committee at the Arizona Health Sciences Center. Housing and care of the animal subjects, as well as experimentation, conformed with the guidelines for care and use of laboratory animals as adopted and promulgated by the National Institutes of Health.

Animals. All animals used were experimentally naive and were tested only once. All were kept on a 12 hr/12 hr light/dark cycle at 72–74°F before experimentation. Male Hartley guinea pigs, 250 to 500 g (Harlan, Indianapolis, IN), were housed six per cage and maintained on tap water and guinea pig chow (Eagle Milling, Tucson, AZ) *ad libitum*. Sacrifice was achieved by stunning and exsanguination. Male ICR mice, 20 to 30 g (Harlan), were housed five per cage and maintained on Lab Blocks (Ralston Purina) and tap water *ad libitum*. Sacrifice was by cervical dislocation. Male New Zealand White rabbits, 2.5 to 3.5 kg (Bell Rabbits, Clovis, NM), were housed one per cage and maintained on rabbit pellets (Eagle Milling, Tucson, AZ) and tap water *ad libitum*. Sacrifice was performed with a high velocity pellet gun, a single shot being delivered to the back of the skull between the ears.

Drugs. PL017 [N-Me-Phe^3,D-Pro^4]morphiceptin, DPDPE and somatostatin were obtained from Peninsula Laboratories (Belmont, CA). Naloxone HCl was purchased from Endo Laboratories (Garden City, NY). ICI 174,864 [N,N-diallyl-Tyr^1,Aib^{2,3}]Leu-enkephalin was obtained from Cambridge Research Biochemicals (Cambridge, England). CTP, CTOP, CTAP, D-Tic-CTP, D-Tic-CTOP and D-Tic-CTAP were synthesized as described previously (Pelton *et al.*, 1986; Kazmierski *et al.*, 1988). For *in vitro* use, drugs were dissolved in distilled water. For *in vivo* administration, drugs were dissolved in sterile 0.9% saline. Dry peptides were stored refrigerated in a desiccator; after reconstitution, drugs were kept refrigerated and used within 1 week.

***In vitro* bioassays.** The GPI preparation was used as described previously (Kosterlitz *et al.*, 1970). The tissues were suspended under a final tension of 1 g in organ baths, bathed with Krebs' buffer (millimolar): NaCl 118; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.19; $MgSO_4$, 1.18; $NaHCO_3$, 25; and glucose, 11.48, maintained at 37°C and aerated with 95% O_2-5% CO_2. Electrical stimuli were 0.4 msec pulses of supramaximal voltage, at a rate of 6/min. Isometric contractions were measured *via* strain gauge force transducers on chart recorders. The MVD preparation was also performed as described (Hughes *et al.*, 1975). The Krebs' buffer was made as above, but without magnesium. The tissues were suspended under a final tension of 500 mg. Pulse duration was 2 msec. The LVD preparation (Oka *et al.*, 1980) was modified as follows: only the prostatic and midde portions of the vasa deferentia were utilized and the tissues were allowed to equilibrate for a period of 4 hr without stimulation after being stretched in the organ bath (500 mg tension), before testing. Stimulation parameters and buffer were identical to those for the MVD. Dose-response testing in all preparations was carried out in noncumulative fashion (*i.e.*, buffer was changed between doses), unless otherwise noted.

Intrinsic activity. All six somatostatin analogs were tested for intrinsic agonist activity in the three *in vitro* bioassays. The agonist activity measured was inhibition of electrically stimulated contraction

of the smooth muscle strips. Concentrations studied ranged from 1 nM to 30 μM. Where agonist activity was observed, selective antagonism was attempted. Thus, concentrations showing intrinsic activity for each analog were tested in the presence of naloxone, to define opioid activity and, in the MVD, the *delta*-selective antagonist ICI 174,864 (Cotton *et al.*, 1984), in an attempt to further define the opioid selectivity of the agonist effect. In both of the vasa deferentia preparations, intrinsic activity was also tested after the induction of somatostatin tolerance, as somatostatin also has significant inhibitory actions in these preparations. Somatostatin tolerance was induced by adding somatostatin to the organ bath in 1-μM increments until inhibitory responses were no longer observed in response to further additions, and the contraction strength of the tissue in response to the electrical stimulation had returned to at least 90% of base line.

Antagonist potency and selectivity. The somatostatin analogs were tested in the GPI as opioid antagonists. The *mu* agonist compound in all cases was PL017. Antagonist potency was determined by the pA_2 method as described originally (Schild, 1947). Concentrations of antagonist tested were 100, 300 and 1000 nM. PL017 concentrations ranged from a low of 30 nM to as much as 30 μM when required to achieve maximal effect in the presence of antagonist. The antagonists were also tested as antagonists of the highly *delta*-selective peptide DPDPE (Mosberg *et al.*, 1983) in the MVD. The antagonists were tested only at a concentration of 1 μM. DPDPE concentrations ranged from 0.3 to 1000 nM.

***In vivo* bioassays.** The HP test was performed as described previously (Shook *et al.*, 1987a). A 55°C hotplate was used; cutoff time was 60 sec. Antinociception was quantitated from the test and control latencies to hindpaw licking by the relationship:

$$\% \text{ Antinociception} = (\text{Test-Control})/(60\text{-Control}) \times 100$$

Both agonist and antagonist were administered i.c.v. Mice were anesthetized lightly with ether and the skin of the scalp split with a scalpel to reveal the skull. A Hamilton microliter syringe, equipped with a fixed 25-gauge needle was used freehand to deliver the injection. The needle was fitted with a plastic sleeve to prevent more than 2.5 mm penetration beyond the skull surface. The injections were placed 2 mm caudal to bregma and 1.5 mm lateral to the midline in order to reach the lateral ventricle. Doses of the test compounds alone ranging from 10 ng to 1 μg were studied for intrinsic analgesic or hyperalgesic activity as well as for gross behavioral effects. Similar doses were examined for their ability to antagonize the analgesic effects of the *mu* agonist PL017. PL017 doses ranged from 30 ng to as much as 30 μg i.c.v., when necessary to reach maximal analgesia in the presence of antagonist. pA_2 analyses were performed on the data from these experiments to estimate the *in vivo mu* antagonist potency of the somatostatin derivatives. The derivative CTAP was tested additionally in doses of 1 to 1000 ng as an antagonist of the *delta*-selective agonist DPDPE. DPDPE doses ranged from 0.3 to 30 μg i.c.v.

The GIT inhibition test was performed as described previously (Shook *et al.*, 1987a). Intestinal transit was measured by the geometric center method (Miller *et al.*, 1981). A solution of $Na_2{}^{51}CrO_4$ in water (diluted to approximately 400,000 dpm/ml) was administered in a volume of 0.2 ml to mice by p.o. gavage. The radiochromium marker was administered immediately after the dose of the test compound(s). The animals were sacrificed and the stomach and intestines excised for gamma counting 35 min after dosing. The agonist and antagonist drugs were mixed in the same syringe and administered as a single i.t. injection, by a modification of a previously described method (Hylden *et al.*, 1980). The mice were not anesthetized but were held firmly with the head and forelimbs covered with a cloth. The spinal column was not exposed before injection; the needle was placed freehand through the skin, with a reproducible tail twitch indicating penetration of the cord. Injection volumes were 3 rather 5 μl. Only the analogs CTOP and CTAP were tested. Doses of the two derivatives ranging from 1 ng to 10 μg were examined for intrinsic inhibitory activity on gastric emptying and intestinal transit and for gross behavioral effects. Doses from 30 to 1000 ng were tested for antagonist potency against the *mu* agonist PL017. A pA_2 analysis was performed with the data to estimate the *in vivo* antagonist potency. Naloxone was tested in identical fashion to provide a comparison value.

Calculations. Dose-response data from *in vitro* experiments were analyzed to determine IC_{50} (50% inhibitory concentration) values and their associated S.E.s and 95% CL by means of a computerized pharmacologic data analysis system (Tallarida and Murray, 1987). All Schild analyses (*in vitro* and *in vivo*) were also performed using this system. When slopes of the Schild regressions did not differ significantly ($P < .05$) from −1, the analysis was repeated with the slope constrained to −1. For *in vivo* experiments, data were normalized to a percentage of maximum effect achieved in each experiment and fitted to the Hill equation:

$$E/E_{max} = \text{Dose}^n/(\text{Dose}^n + ED_{50}{}^n)$$

where E_{max} = maximum effect achieved, E = observed effect, ED_{50} = dose producing 50% of the maximal effect and n = sigmoidicity constant (*i.e.*, Hill coefficient). Fitting was achieved with the computerized nonlinear least-squares system, PCNONLIN (Statistical Consultants, Inc., 1986).

Results

In vitro. In the GPI preparation, none of the six somatostatin analogs, in concentrations up to 10 μM, produced notable inhibition of contraction (fig. 2). In the vasa deferentia preparations, more intrinsic activity was observed. In figures 3 and 4, dose-response data for each somatostatin derivative are shown for the MVD (left) and LVD (right). Data for CTP in the MVD (fig. 3, upper left) are provided for comparative purposes. More complete data for CTP have been published (Shook *et al.*, 1987b). Where antagonism by ICI 174,864 is indicated, the reader should be aware that comparable results were obtained with naloxone. Naloxone antagonism data were omitted to limit redundancy. Naloxone and ICI 174,864 were always used in concentrations of 1 μM. For derivatives which demonstrated agonist activity sensitive to somatostatin tolerance, dose-response data obtained after induction of somatostatin tolerance are shown. The compounds showing least agonist activity (either opioid or somatostatin-like) in the two vas deferens preparations were CTAP and D-Tic-CTAP.

Table 1 contains the results of Schild analyses performed with each antagonist and the *mu* agonist PL017. None of the Schild slopes differed significantly from −1, and all experiments indicated competitive antagonism, as maximal inhibition achieved in the control state could be reproduced during antagonism (see fig. 5, for example). All six analogs displayed similar potency, with substantial overlap of the 95% confidence intervals for the pA_2 values. Data from these experiments were reported previously, but the Schild analysis used did not constrain the slope of the regression to −1 (Kazmierski *et al.*, 1988). Only CTOP was significantly less potent than the remaining five compounds. The D-Tic-substituted analogs appeared marginally more potent than the non-D-Tic (D-Phe) analogs, but these differences are not statistically significant. Figure 5 shows the effect of increasing concentrations of CTAP on the dose-response relationship of PL017 in the GPI.

Table 2 summarizes the effect of the six derivatives on the dose-response relationship for DPDPE in the MVD. IC_{50} values and 95% confidence intervals are shown for DPDPE alone (left) and in the presence of 1 μM of each antagonist (right). No antagonist effect, indicated by no significant increase in the DPDPE IC_{50}, was evident for any of the compounds. A very slight leftward shift, statistically significant, was noted for

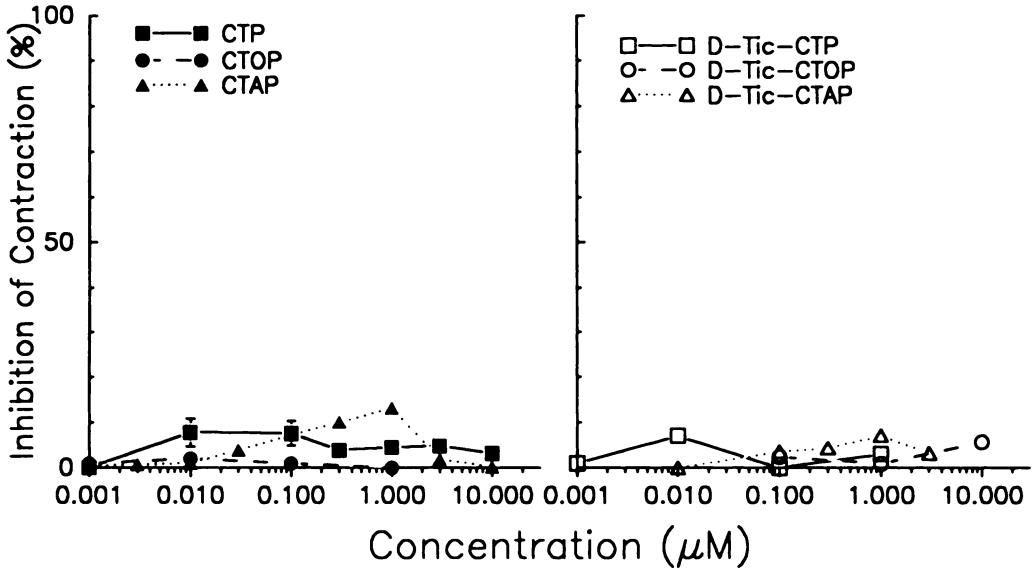

Fig. 2. Intrinsic activity of somatostatin analogs in the GPI. Inhibition of electrically stimulated twitch in the GPI is represented on the *Y*-axis, concentration on the *X*-axis. Each point represents the mean ± S.E. of not less than four determinations.

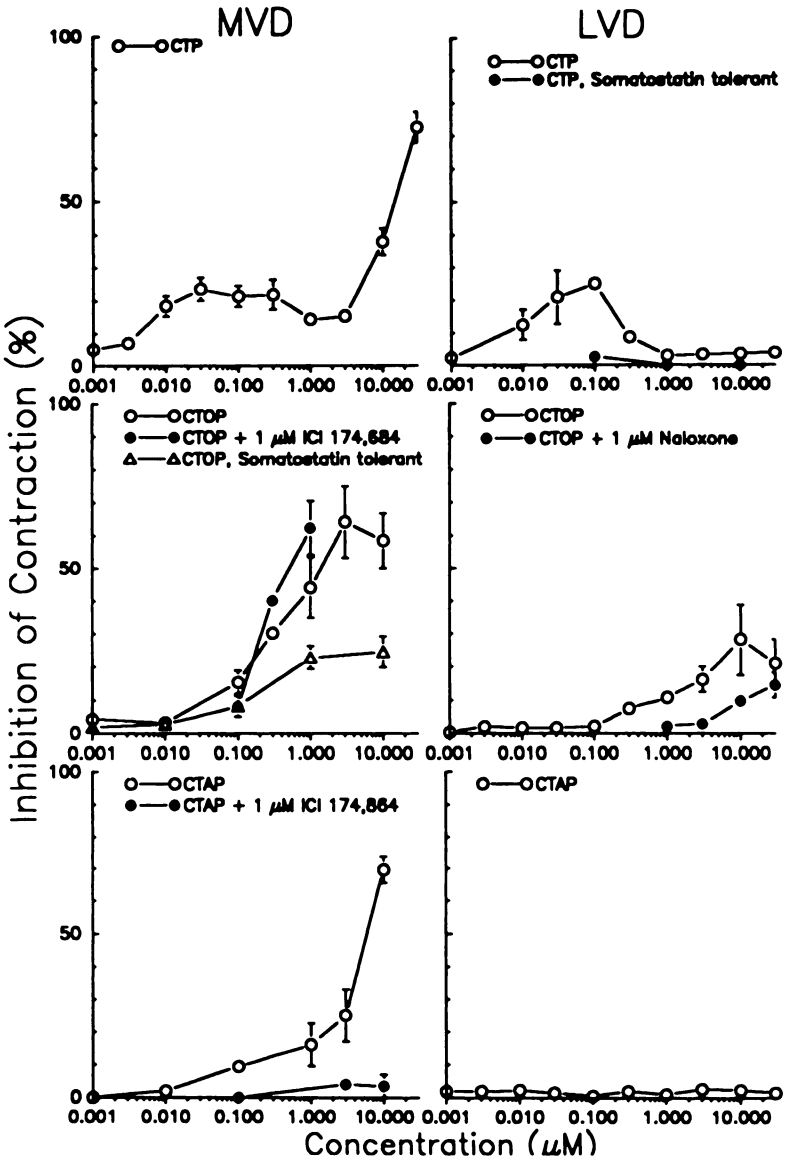

Fig. 3. Intrinsic activity of CTP, CTOP and CTAP in the MVD (left) and LVD (right) preparations. Complete data for CTP in the MVD have been published previously (Shook *et al.*, 1987b). Each point represents the mean ± S.E. of not less than four determinations.

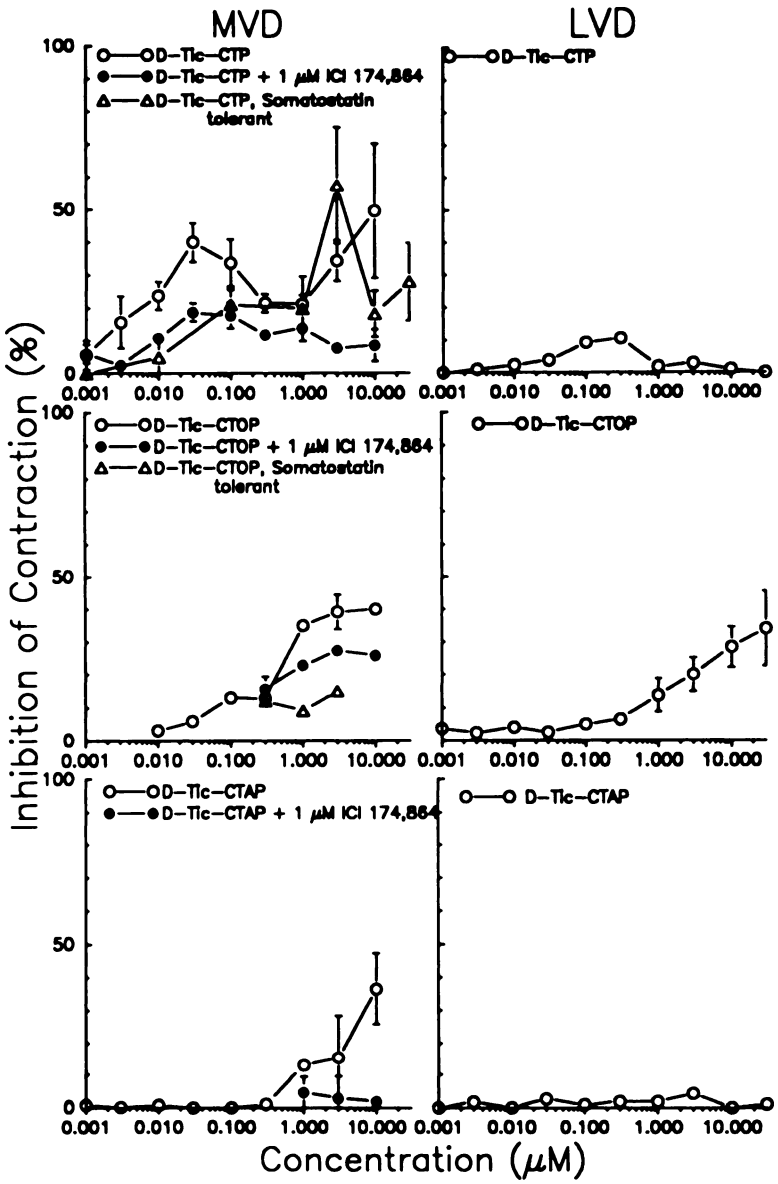

Fig. 4. Intrinsic activity of D-Tic-CTP, D-Tic-CTOP and D-Tic-CTAP in the MVD (left) and LVD (right) preparations. Each point represents the mean ± S.E. of not less than four determinations.

TABLE 1

pA_2 values determined in GPI

Antagonist*	pA_2	95% Confidence Interval
CTP	7.17	6.99–7.34
CTOP	6.38	6.14–6.63
CTAP	7.36	6.93–7.78
D-Tic-CTP	7.82	7.28–8.35
D-Tic-CTOP	7.64	7.37–7.90
D-Tic-CTAP	7.93	7.77–8.08

* Agonist is PL017 in all cases.

DPDPE in the presence of 1 μM CTOP and D-Tic-CTP; this is most likely a result of the intrinsic activity of these two analogs on the MVD.

In vivo. No analgesic or hyperalgesic effect was evident after doses of 1 ng to 1 μg of the test compounds, administered i.c.v. to mice. After the larger doses (300 ng and 1 μg), a transient period of increased grooming activity was often observed. This effect, which occurred with all the analogs, was not quantitated. Somatostatin administered in similar doses did not produce this grooming effect (data not shown). No other acute effects were observed after administration of the test compounds. All

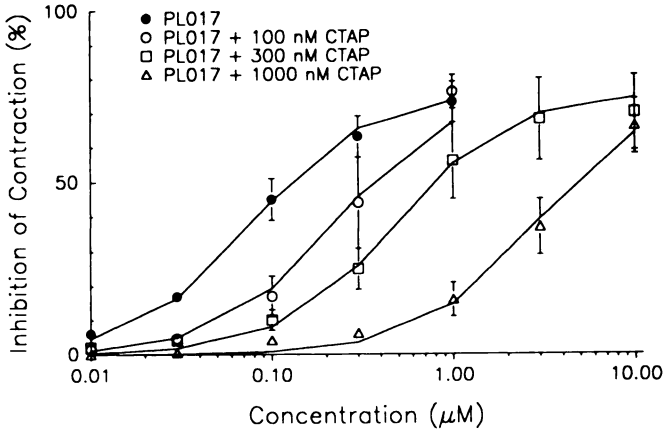

Fig. 5. Antagonism of the *mu* opioid PL017 by CTAP in the GPI. The parallel, progressively rightward shift in dose-response demonstrates competitive antagonism. Each point represents the mean ± S.E. of responses determined in four separate tissues.

TABLE 2

Effects on DPDPE IC_{50}[a] values determined in MVD

Antagonist[b]	IC_{50} Control	95% C.L.	IC_{50} Antagonist	95% C.L.
CTP	4.07	3.7–4.4	4.17	3.6–4.8
CTOP	2.30	2.1–2.5	1.1	1.0–1.2
CTAP	9.10	7.1–11.8	6.2	5.1–7.4
D-Tic-CTP	9.10	7.9–10.5	6.6	5.9–7.6
D-Tic-CTOP	3.20	3.0–3.5	3.1	2.9–3.3
D-Tic-CTAP	3.16	2.9–3.4	2.9	2.5–3.4

[a] Expressed in nanomoles.
[b] All antagonists at 1 μM concentration.

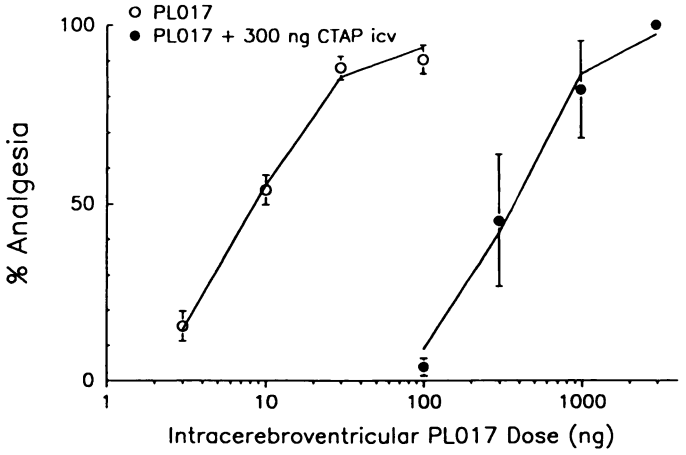

Fig. 6. Antagonism of the analgesic effect of PL017, administered i.c.v., by CTAP in the mouse. Each point represents the mean ± S.E. of responses obtained from 5 to 10 mice.

TABLE 3

pA_2 values determined *in vivo* (HP test)

Agonist and antagonist administered i.c.v.

Antagonist[a]	pA_2	95% Confidence Interval
CTP	11.86	11.40–12.31
CTOP	11.25	10.74–11.76
CTAP	11.37	10.98–11.76
D-Tic-CTP	11.81	11.34–12.27
D-Tic-CTOP	11.48	10.69–12.27
D-Tic-CTAP	11.58	11.23–11.93

[a] Agonist is PL017 in all cases.

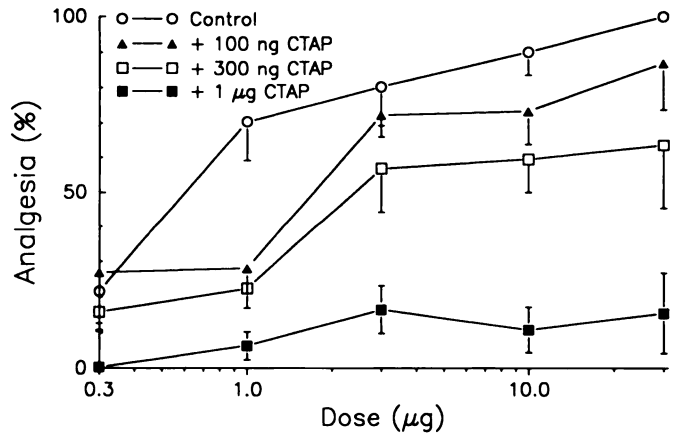

Fig. 7. Inhibition of DPDPE analgesia by CTAP. DPDPE dose response is shifted to the right and flattened by increasing doses of CTAP. Both compounds were administered i.c.v. Each point represents the mean response, ± S.E., determined in at least 5 and in some cases 10 mice.

TABLE 4

pA_2 values determined *in vivo* (intestinal transit)

Agonist and antagonist administered i.t.

Antagonist[a]	pA_2	95% Confidence Interval
CTOP	11.32	10.95–11.69
CTAP	11.41	10.69–12.12
Naloxone	10.08	9 .69–10.46

[a] Agonist is PL017 in all cases.

acted as competitive antagonists of the analgesic effect of PL017 administered i.c.v. Figure 6 shows the rightward shift in the PL017 dose-response produced by coadministration of CTAP. Table 3 shows the results of Schild analyses of the analgesia data from mice receiving PL017 and antagonist. The potency estimates (pA_2 values) were virtually identical for all the compounds; all of the 95% confidence intervals overlap. None of the Schild slopes was significantly different from −1, thus the results shown were obtained from analyses performed with the regression slope constrained to −1. CTAP caused antagonism of the analgesic effects of DPDPE; this interaction did not appear competitive (fig. 7), and potency could not be estimated.

CTOP or CTAP (alone) did not stimulate or inhibit small intestinal transit in the mouse when administered i.t. in doses as high as 10 μg (data not shown). No excess grooming or other unusual behaviors were noted. Both i.t. CTOP and CTAP antagonized the inhibitory effect of i.t. PL017 on intestinal transit, as did naloxone. Results of Schild analyses of these experiments are summarized in table 4; slopes were not significantly different from −1, and were therefore constrained to that value. The potencies of CTOP and CTAP are approximately equivalent, and were as much as 10-fold greater than that of naloxone in this test.

Discussion

The contractions of the GPI in response to electrical stimulation are mediated *via* cholinergic neurons. Acetylcholine release in these neurons is regulated by presynaptic opioid receptors of both the *mu* and *kappa* types. Thus, the lack of intrinsic inhibitory (agonist) activity in the GPI indicates that none of the derivatives possess *mu* or *kappa* opioid agonist activity at concentrations up to 10 μM. In the MVD, significant agonist activity was noted for CTP, D-Tic-CTP and CTOP at low (<300 nM) concentrations. In the case of CTP and D-Tic-CTP, this intrinsic activity was attenuated after somatostatin tolerance was induced in the preparation, whereas for CTOP, naloxone or ICI 174,864 partially blocked intrinsic activity. CTAP and D-Tic-CTAP were least active in the concentration range 1 to 300 nM and appear the most desirable in this respect. All six derivatives produced agonist effects in the MVD with varying potency in concentrations greater than 300 nM. In the case of CTOP and D-Tic-CTOP, these effects were only marginally affected by naloxone, ICI 174,864 or somatostatin tolerance. For CTAP and D-Tic-CTAP, agonist actions above 300 nM were antagonized completely by the antagonist ICI 174,864, indicating weak *delta* agonist activity. CTAP and D-Tic-CTAP were least potent in this respect. In the LVD, CTP and D-Tic-CTP displayed somatostatin like actions below 300 nM, CTOP and D-Tic-CTOP showed naloxone-sensitive actions above 300 nM and CTAP and D-Tic-CTAP were completely devoid of inhibitory activity. On the basis of least intrinsic activity,

CTAP and D-Tic-CTAP were found to be the most successful compounds.

In the GPI, all six derivatives acted as competitive *mu* antagonists as evidenced by Schild plot slopes not significantly different from −1. The overlapping 95% confidence intervals make it impossible to select the most potent compound with certainty; CTOP is the only derivative with significantly lower potency than the remaining five (table 1). It should be noted that the highest concentration of antagonist required to perform the Schild analyses was 1 μM. In the MVD, none of the derivatives (at 1 μM concentration) caused a significant shift in the concentration/response relationship for DPDPE, indicating high selectivity for *mu* over *delta* receptors. DPDPE has subsequently been tested against CTAP in concentrations up to 10 μM (Kramer *et al.*, 1988a) without evidence of antagonism. *Delta* receptor affinity for these compounds was thus impossible to estimate in this preparation, and selection of the optimum derivative on the basis of *mu* to *delta* selectivity *in vitro* is difficult, as all are remarkably specific.

When administered acutely into the brain or spinal cord in mice, these compounds did not induce observable toxic effects. The induction of grooming behavior by the higher doses administered i.c.v. appears unrelated to the somatostatin-like actions in the vas deferens preparations, as somatostatin administered i.c.v. did not produce excess grooming. Additionally, by subjective observation, all these compounds produced an increase in grooming, whereas the CTAP derivatives did not have somatostatin-like actions *in vitro*. The cause of the grooming behavior is therefore unknown. CTP has been reported to cause convulsive behaviors in doses of 10 μg i.c.v., but only during the stimulation of hotplate testing (Shook *et al.*, 1987a). Also, 10 μg of CTP given i.t. caused increased grooming, but no other obvious effects (Shook *et al.*, 1987a). As these agents produce strong *mu* antagonism at considerably lower doses, these behavioral effects may not be important.

None of the derivatives produced analgesia in doses up to 1 μg i.c.v., or did they stimulate or inhibit small intestinal transit rates when administered i.t. in the same doses. This lack of opioid agonist activity generally parallels the results of the *in vitro* bioassays. Whereas *delta* agonist actions were noted in the MVD (figs. 3 and 4), these occurred at concentrations much higher than those required for *mu* antagonist effects. It is probable that extremely high doses would be required *in vivo* to elicit *delta* opioid agonist effects, *e.g.*, analgesia, after i.c.v. administration, or inhibition of GIT after i.t. dosing (Porreca *et al.*, 1984).

Mu antagonism *in vivo* was competitive in both tests (GIT inhibition and HP analgesia). Potency was extremely high; all doses required to perform the Schild analyses were less than 1 nmol. In the HP test, the pA_2 values are all greater than that reported for naloxone in the same test (10.14 ± 0.4, 95% confidence interval) (Shook *et al.*, 1987a), indicating approximately 10-fold greater potency when the compounds are considered as a group. The compounds did not differ significantly in potency, as indicated by the overlapping 95% confidence intervals. In the GIT inhibition test, the potencies of CTOP and CTAP were virtually identical, and again approximately 10-fold greater than that of naloxone.

The apparent antagonism of DPDPE-induced analgesia by CTAP (fig. 7) is somewhat perplexing. This antagonist effect cannot be characterized as competitive, as the dose-response relationship for DPDPE in combination with antagonist does not reach a maximal effect, *i.e.*, the shift in dose response was nonparallel. Noncompetitive antagonism was also noted when CTP was coadministered with DPDPE (Shook *et al.*, 1987a). The concept of *delta*-mediated supraspinal analgesia is becoming well established (Porreca *et al.*, 1984, 1987; Heyman *et al.*, 1988; Kramer *et al.*, 1988b), and it seems unlikely that CTAP is blocking the effect of DPDPE at a *mu* receptor. It is possible to speculate that the *delta* receptors in the MVD are dissimilar from those mediating analgesia in mouse brain, and that CTAP has greater affinity for the latter. Perhaps the more likely explanation lies in the potential allosteric coupling of certain opioid receptor types in brain, by which *mu* and *delta* selective ligands may influence each other's binding (Rothman *et al.*, 1984).

Pharmacokinetic factors and the route of administration almost certainly influence the estimates of *in vivo* potency. Because the somatostatin analogs are hydrophilic peptides, they should remain localized at the site of administration in brain or spinal cord, rather than diffusing away rapidly as the highly lipophilic alkaloid naloxone likely does. CTP has been shown to have a much greater duration of antagonist action than naloxone and naltrexone when administered i.c.v. (Shook *et al.*, 1987a). Conversely, these agents would not be expected to show high antagonist potency in the central nervous system if administered peripherally, due to poor penetration of the blood/brain barrier. Peripheral routes have not been investigated in our laboratories to date.

In summary, a program of synthesis and pharmacologic testing has resulted in the development of several highly potent and selective *mu* opioid receptor antagonist peptides, unrelated in structure to the endogenous opioids. The selectivity of these drugs *in vitro* appears unparalleled by any other available agent at this time. Due to their low intrinsic activity *in vitro*, high antagonist potency *in vitro* and *in vivo* and high degree of selectivity for *mu* opioid receptors, we feel the analogs CTAP and D-Tic-CTAP are the best candidates for further study and exploitation as pharmacologic tools. These selective *mu* antagonist peptides may facilitate significant new advances in the understanding of opioid neurobiology.

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