

Dermorphin Analog Tyr-D-Arg²-Phe-Sarcosine-Induced Opioid Analgesia and Respiratory Stimulation: The Role of μ_1 -Receptors?¹

P. PAAKKARI, I. PAAKKARI, S. VONHOF, G. FEUERSTEIN and A.-L. SIRÉN

Department of Neurology (P.P., I.P., S.V., A.-L.S.), Uniformed Services University of the Health Sciences, Bethesda, Maryland; the Department of Pharmacology (G.F.), SmithKline Beecham, King of Prussia, Pennsylvania; and the Department of Pharmacology and Toxicology (P.P., I.P.), University of Helsinki, Helsinki, Finland

Accepted for publication March 12, 1993

ABSTRACT

Tyr-D-Arg²-Phe-sarcosine⁴ (TAPS), a μ -selective tetrapeptide analog of dermorphin, induced sustained antinociception and stimulated ventilatory minute volume (MV) at the doses of 3 to 100 pmol i.c.v. The doses of 30 and 100 pmol i.c.v. induced catalepsy. The effect of TAPS on MV was in negative correlation with the dose and the maximal response was achieved by the lowest (3 pmol) dose (+63 ± 23%, $P < .05$). Morphine, an agonist at both μ_1 and μ_2 sites, at a dose of 150 nmol i.c.v. (equianalgetic to 100 pmol of TAPS decreased the MV by 30%, due to a decrease in ventilatory tidal volume. The antinociceptive effect of TAPS was antagonized by naloxone and the μ_1 receptor antagonist, naloxonazine. Naloxonazine also attenuated the catalepsy produced by 100 pmol of TAPS i.c.v. and the respiratory stimulation produced by 3 pmol of TAPS i.c.v.

Pretreatment with 30 pmol of TAPS antagonized the respiratory depression induced by the μ opioid agonist dermorphin (changes in MV after dermorphin alone at 1 or 3 nmol were -22 ± 10% and -60 ± 9% and, after pretreatment with TAPS, +44 ± 11% and -18 ± 5%, respectively). After combined pretreatment with naloxonazine and TAPS, 1 nmol of dermorphin had no significant effect on ventilation. In contrast, pretreatment with a low respiratory stimulant dose (10 pmol i.c.v.) of dermorphin did not modify the effect of 1 nmol of dermorphin. In conclusion, the antinociceptive, cataleptic and respiratory stimulant effects of TAPS appear to be related to its agonist action at the μ_1 opioid receptors. TAPS did not induce respiratory depression (a μ_2 opioid effect) but antagonized the respiratory depressant effect of another μ agonist. Thus, *in vivo* TAPS appears to act as a μ_2 receptor antagonist.

Dermorphin is a heptapeptide (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH₂) derived from amphibian skin. Dermorphin selectively binds to μ -opioid receptors (de Castiglione and Rossi, 1985; Krumins, 1987) and displays potent opioid effects such as analgesia (Broccardo *et al.*, 1981; Stevens and Yaksh, 1986), catalepsy and respiratory depression (Paakkari and Feuerstein, 1988; Paakkari *et al.*, 1990). The N-terminal tetrapeptide of dermorphin is the minimum sequence for opioid activity but the fragment is less potent than the parent heptapeptide (Broccardo *et al.*, 1981; Salvadori *et al.*, 1982). However, substitution of the D-Ala² residue with D-Arg and Gly⁴ with sarcosine markedly enhances the potency of the tetrapeptide. In antinociceptive tests, TAPS was about 6 times as potent as dermorphin (Sasaki *et al.*, 1984; de Castiglione and Rossi, 1985; Sato *et al.*, 1987). The D-Arg²-Sar⁴-substitutions also increased binding of dimeric dermorphin analogs to μ receptors while de-

creasing binding to delta receptors, yielding a very high μ / δ selectivity ratio (Lazarus *et al.*, 1989).

Opioid analgesia has been suggested to be mediated by the μ_1 receptors, whereas μ_2 receptors are thought to be involved in respiratory depression and inhibition of the guinea pig ileum contractions (Ling *et al.*, 1985; Gintzler and Pasternak, 1983; Pasternak and Wood, 1986). Furthermore, signs of opiate withdrawal syndrome seem to be mediated through binding sites other than μ_1 receptors (Ling *et al.*, 1984). Because TAPS was more potent than dermorphin in analgesia tests but less potent in the guinea pig ileum (Sato *et al.*, 1987) and induced a less severe withdrawal syndrome than morphine (Nakata *et al.*, 1986), we proposed that TAPS might stimulate primarily μ_1 receptors and therefore display less respiratory depression than dermorphin. To test this hypothesis, the respiratory effects of TAPS were compared with those of morphine and dermorphin. Pretreatment with the μ_1 receptor antagonist naloxonazine was used to elucidate the role of μ_1 receptors in TAPS- and dermorphin-induced antinociceptive, behavioral and respiratory changes.

Received for publication August 24, 1992.

¹This work was supported by the ADAMHA grant award R01DA07212 for Dr. Sirén.

ABBREVIATIONS: TAPS, Tyr-D-Arg²-Phe-sarcosine; MV, minute volume; TV, tidal volume; VR, ventilatory rate; ANOVA, analysis of variance.

Methods

Surgical procedures. Male Sprague-Dawley rats (300 ± 40 g, Taconic Farms, Germantown, NY) were used in all experiments. The rats were anesthetized with an intramuscular injection of 0.13 ml/100 g ketamine-acepromazine solution (100 mg/ml of ketamine and 1 mg/ml of acepromazine). A stainless steel guide cannula was stereotaxically inserted (David Kopf Instrument, Tujunga, CA) into the right lateral brain ventricle and fixed with instant glue (Eastman 910 adhesive, Eastman Kodak, Rochester, NY). Coordinates for the lateral ventricle were AP = -0.8 mm and L = 1.2 mm. After the operation, the rats were allowed to recover for 1 to 3 days in separate plastic cages, with food and water *ad libitum*, and a 12/12-hr light/dark cycle. On the day of the experiment, a premeasured 30-g cannula was inserted into the ventricular space through the guide cannula. The injection cannula was connected by polyethylene tubing to a Hamilton microliter syringe and the drug solution was injected over a period of 20 sec in a volume of 10 μ l. The proper position of the i.c.v. cannula was ascertained at the end of the experiment by dissection of the brain after an injection of methylene blue (10 μ l).

Evaluation of antinociceptive and cataleptic effects. Analgesia in rats was determined using a tail flick apparatus (Socrel, Milano, Italy). The principle of the radiant heat tail flick technique has been described by D'Amour and Smith (1941). Base-line latencies are determined for each animal and, after drug administration, the reaction times are measured repeatedly. A maximal cutoff time of the heat was 12 sec to prevent tissue damage.

Catalepsy was evaluated by placing the front limbs of the rat over a 10-cm high horizontal bar and measuring the time that the animal maintained that posture. If the rat remained 60 sec on the bar, it was defined as cataleptic. In evaluation of the righting reflex, the animals were designated as having lost their righting reflex if they remained in a supine position for 10 sec.

Recording of respiration. The respiration rate and relative respiration tidal volume were monitored using the OxyMax 85 system (Columbus Instruments, Columbus, OH). This computerized recording system consists of two Plexiglas test chambers and one reference chamber with a constant flow of room air of 2 liters per minute. The respiration rate was determined based on the frequency (respirations per minute) of pressure changes due to the ventilatory movements of the rat's thorax. The TV was recorded as an integral of pulses; this was proportional to the amplitude of the pressure changes and compared with the pulse count in the first sample measurement of the experiment. The TV therefore was expressed as the relative TV in arbitrary units. The relative ventilatory MV was calculated as the arithmetical product of the respiration rate and the relative TV. The accuracy of the respiration rate recording was $\pm 1\%$. The respiratory data were gathered at 2-min intervals.

Initially, the rats were allowed to accommodate for 40 min before the administration of drugs. The average of five recordings during 10 min before the first drug administration was taken as the base-line level of ventilation. To minimize the effect on ventilation caused by handling the animals during injection procedures, the data were evaluated starting 30 min after the drug injection.

Data processing and statistics. The data from the recording instruments were stored and, in part, processed by the computer program Dataquest provided by the manufacturer (Columbus Instruments). In general, averages of five consecutive measurements were calculated and median time points were used. The calculations of the data were carried out in a standard spreadsheet. For statistical analysis the CSS: STATISTICA software package (StatSoft, Tulsa, OK) was used. The results were expressed as means \pm S.E. for five to eight experiments. For analysis of the dose-response relationships, the respiratory effects of TAPS were assessed as areas under the curves of the percent changes in relative MV over time (114 min), using a trapezoidal method. This approximative procedure consists of the summation of the areas of various trapezoids fitted to the curve segment representing the treatment period. For statistical analysis of data with

equal variances, the ANOVA followed by Tukey's multiple comparison test or Student's *t* test were used. The Kruskal-Wallis nonparametric ANOVA and the Mann-Whitney *U* test were used for groups with unequal variances. The dose-response curves were analyzed using linear regression of all data points rather than the mean responses. The doses producing antinociceptive ED₅₀ responses were calculated from the linear portion of the log of the dose-response curve and the ED₅₀ values with their associated 95% confidence intervals have been reported.

Drugs. Dermorphin and TAPS (Peninsula, Belmont, CA), naloxone (DuPont Pharmaceuticals, Wilmington, DE) and naloxonazine (Research Biochemicals, Natick, MA, and a generous gift from Dr. G. W. Pasternak, Memorial Sloan-Kettering Cancer Center, New York, NY) were dissolved in 0.9% saline (naloxonazine with a few drops of glacial acetic acid). The final concentration of acetic acid in the solution was 2%, pH 5. Vehicle (2% acetic acid in saline) or naloxonazine (10 or 20 mg/kg) was administered i.v. 20 to 24 hr before the experiment. Other pretreatments were injected i.c.v. 20 min before the test drug.

Results

Antinociceptive effect of TAPS after i.c.v. administration. Administration of TAPS i.c.v. produced dose-related antinociceptive effects (fig. 1). The dose of 1 pmol (0.5 ng) of TAPS induced a transient increase in the tail-flick latency corresponding to an antinociceptive response of about 20% of the maximal possible effect. The dose of 3 pmol (1.7 ng) of TAPS induced analgesia, which was about 30% of the maximum possible effect and lasted about 1 hr. The dose of 6 pmol (3.2 ng) produced an antinociceptive effect that was 48% of the maximal analgesia. After the dose of 10 pmol (5.3 ng) of TAPS, an antinociceptive effect of 98% of the maximal possible effect was observed 30 min after the injection and was still more than 30% of the maximal analgesia at 2 hrs postinjection. After the dose of 30 pmol (17 ng), maximal antinociception was evident 15 min after the injection and the antinociception was about 50% of the maximal possible effect 2 hr after injection. The highest dose tested, 100 pmol (53 ng), produced maximal analgesia that lasted for about 2 hr and the antinociception was more than 60% of the maximal possible effect 3 hr postinjection (fig. 1). The doses of 1, 3, 6, 10 and 30 pmol of TAPS were plotted into a log dose-response curve (fig. 2, upper panel) and the curve was analyzed with least squares linear regression yielding a positive correlation between dose and effect (slope = 56.802; 95% confidence interval, 40.273-73.331; $r = .805$; $P < .0001$) and an ED₅₀ value of 4.1 pmol (95% confidence interval, 2.1-6.9 pmol).

The antinociceptive effect of 30 pmol of TAPS was blocked by treatment with naloxone (5 mg/kg i.v., fig. 3). Pretreatment with naloxonazine (10 or 20 mg/kg i.v.) also attenuated the antinociception induced by TAPS doses of 30 pmol (fig. 3) and 100 pmol (table 1).

Antinociceptive effect of TAPS after i.v. or p.o. administration. TAPS produced dose-related antinociceptive effects after i.v. and p.o. administration (fig. 1). On i.v. administration, nearly maximal analgesia (97% of the maximal possible effect) was obtained 30 min after the dose of 1.5 mg/kg (2.7 μ mol/kg). Orally, TAPS doses of 10 and 20 mg/kg (18 and 36 μ mol/kg) produced about 60% of maximum analgesia, which subsided gradually during 3 hr (fig. 1). When the i.v. doses of TAPS were plotted into a log dose-response curve (fig. 2, lower panel) and the curve was analyzed with least squares linear regression, a positive correlation between dose and effect (slope = 80.742; 95% confidence interval, 61.530-99.954; $r = .891$; $P < .0001$) was found. A positive correlation between dose and

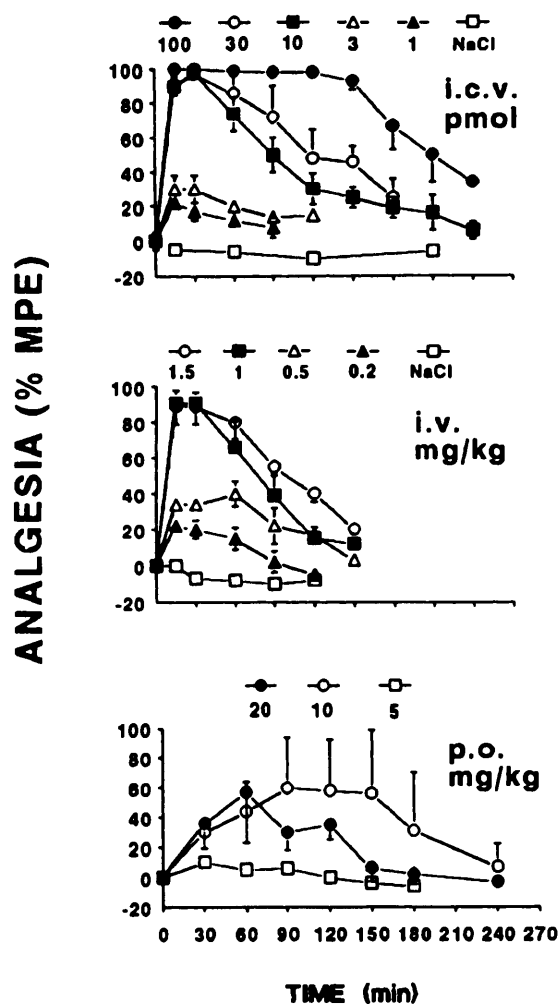


Fig. 1. Analgesic effect of TAPS i.c.v. (1, 3, 10, 30 or 100 pmol/rat, upper panel, $n = 5-6$ in all groups), i.v. (0.5 or 1.5 mg/kg, middle panel, $n = 5-6$ in all groups) and p.o. (5, 10 or 20 mg/kg, lower panel, $n = 3$ in all groups). The results are shown as percents of maximal possible effect (%mpe). Two-way ANOVA with repeated measures was used to analyze the antinociceptive effect of TAPS i.c.v. and i.v., while statistical analysis of the analgesia after TAPS p.o. was not done due to the small number of animals in these groups ($n = 3$). After TAPS i.c.v., a significant interaction between time and dose ($P < .001$) were found at time points 15, 30 and 120 min. The statistical significances for individual doses were analyzed by Tukey's test: 3 pmol of TAPS compared with saline ($P < .01$); 10, 30 or 100 pmol of TAPS compared with saline ($P < .001$). After TAPS i.v., a significant interaction between time and dose ($P < .001$) was found at all time points. The statistical significances for individual doses were analyzed by Tukey's test: 0.5, 1 or 1.5 mg/kg of TAPS compared with saline ($P < .001$).

effect was also found for oral TAPS (slope = 86.350; 95% confidence interval, 42.733-129.968; $r = .871$; $P < .0023$). The ED_{50} values calculated from log dose-response curves were 0.8 $\mu\text{mol/kg}$ (95% confidence interval, 0.4-1.3 $\mu\text{mol/kg}$ or 0.43 mg/kg) for TAPS i.v. and 26 $\mu\text{mol/kg}$ (13.9 mg/kg) for TAPS p.o. (fig. 2, lower panel).

For comparison, the antinociceptive effect of morphine 30 and 150 nmol i.c.v. was evaluated. After the 30-nmol dose of morphine i.c.v., a peak antinociceptive response of $80 \pm 11\%$ was produced ($n = 6$, $P < .05$ compared with that of saline control) of the maximal possible effect, which was reached 60 min after morphine administration. The higher dose (150 nmol) produced nearly maximal analgesia ($90 \pm 10\%$ to $95 \pm 4\%$, $n =$

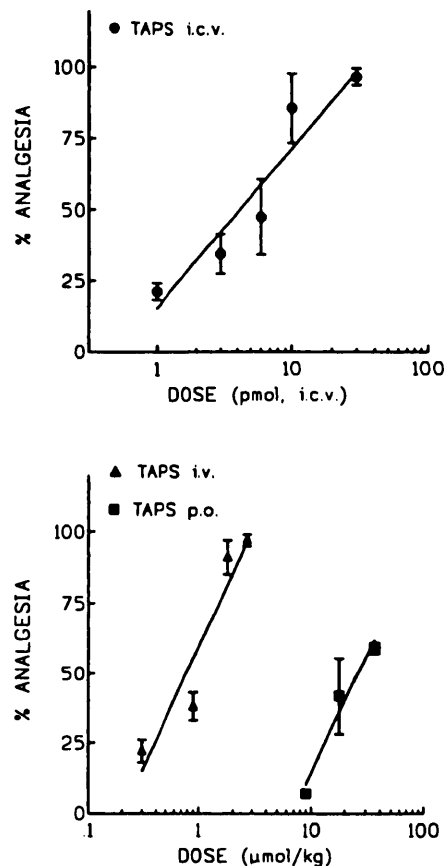


Fig. 2. Dose-response relationship of the TAPS-induced antinociception. The upper panel depicts the effect of TAPS after i.c.v. doses of 1, 3, 6, 10 and 30 pmol; the lower panel depicts the effect of TAPS after i.v. doses of 0.3, 0.9, 1.8 and 2.7 $\mu\text{mol/kg}$ or after the p.o. doses of 9, 18 and 36 $\mu\text{mol/kg}$. The results are shown as percents of the maximal possible effect. Linear regression analysis of the curves revealed that the slope of each curve was significantly different from zero. The slopes with 95% confidence limits, correlation coefficients and P values for each curve were as follows: slope = 56.802, 95% confidence interval, 40.273 to 73.331, $r = .805$, $P < .0001$ (TAPS i.c.v.); slope = 80.742, 95% confidence interval, 61.530 to 99.954, $r = .891$, $P < .0001$ (TAPS i.v.); slope = 86.350, 95% confidence interval, 42.733 to 129.968, $r = .871$, $P < .0023$ (TAPS p.o.).

6, $P < .05$ compared with that of saline control) for more than 3 hr.

Cataleptic effect of TAPS after i.c.v. administration. A dose of TAPS of 100 pmol i.c.v. produced catalepsy, which lasted 60 min in all animals. At 180 min, one of the five rats was still cataleptic (table 1). After the 30 pmol dose of TAPS i.c.v., three of the six rats (50%) were cataleptic for 15 min and two of the six rats (33%) were cataleptic 30 min after injection. None of the animals were cataleptic 60 min after the injection. At none of the doses tested did the rats lose the righting reflex. None of the animals that received TAPS i.c.v. at the doses of 1, 3, 6 or 10 pmol or the animals that received TAPS i.v. or p.o. was cataleptic.

Pretreatment with naloxonazine (20 mg/kg i.v.) attenuated the catalepsy induced by 100 pmol of TAPS (table 1).

Ventilatory effects of TAPS and morphine after i.c.v. administration. Due to the increases in ventilatory TV and VR, TAPS doses up to 100 pmol i.c.v. increased ventilatory MV (figs. 4 and 5, upper panel). The maximum increase in MV after 3 pmol of TAPS was $63 \pm 23\%$ ($P < .05$, compared with base-line values). The corresponding effect after 100 pmol was

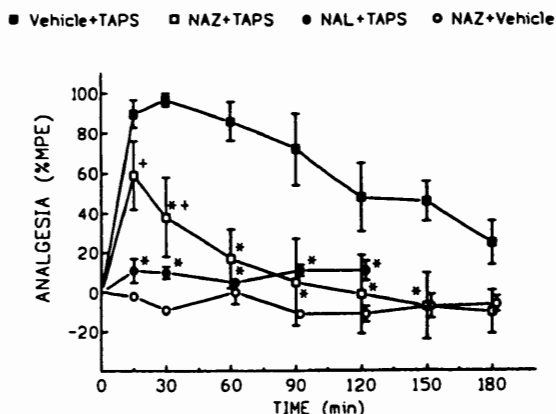


Fig. 3. The analgesic effect of 30 pmol of TAPS i.c.v. in vehicle-treated rats (vehicle + TAPS, $n = 5$), naloxonazine-treated rats (NAZ, 10 mg/kg i.v., $n = 5$) or in naloxone-treated rats (NAL, 5 mg/kg i.v., $n = 4$). The group that received naloxonazine and saline (NAZ + vehicle, $n = 4$) is also shown. * denote statistical significance (Tukey's test) compared with the group that received vehicle and TAPS (* $P < .05$). † indicate statistical significance compared with the group that received NAZ and vehicle († $P < .05$).

TABLE 1
Antagonism by naloxonazine (20 mg/kg i.v.) of analgesia and catalepsy induced by TAPS (100 pmol/rat i.c.v.)

Naloxonazine or vehicle (2% acetic acid in saline) was injected into the right jugular vein 20 hr before the administration of TAPS (100 pmol/rat i.c.v.). The data represent means \pm S.E. of five animals in the control group and seven rats in the naloxonazine-treated group. Analgesia data was analyzed using two-way ANOVA and Tukey's test; Fisher exact probability test was used to evaluate the catalepsy data.

Time min	Vehicle + TAPS		Naloxonazine + TAPS	
	Analgesia %mpe*	%Cataleptic	Analgesia %mpe	%Cataleptic
30	100 \pm 0	100	95 \pm 4	29*
60	99 \pm 1	100	92 \pm 4	29*
90	98 \pm 2	60	57 \pm 11*	0*
120	98 \pm 2	60	44 \pm 11*	0*
150	93 \pm 5	40	35 \pm 9*	0
180	67 \pm 14	20	28 \pm 7*	0
210	50 \pm 16	0	16 \pm 7*	0
240	34 \pm 4	0	14 \pm 7	0

* Percent of the maximal possible effect.

* $P < .05$ between naloxonazine- and vehicle-treated groups.

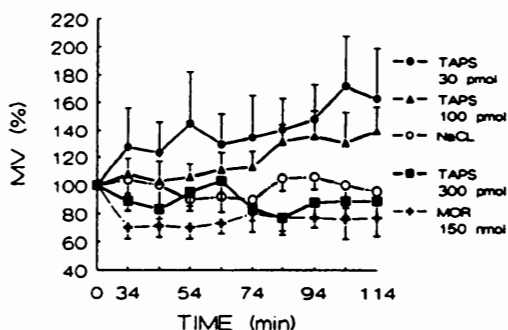


Fig. 4. Effect of i.c.v. treatments of 30 pmol of TAPS ($n = 8$), 100 pmol ($n = 6$) or 300 pmol ($n = 8$), morphine (150 nmol of MOR, $n = 7$) or saline ($n = 6$) on ventilatory MV, shown as a percent change from the base level. One-way ANOVA with repeated measures revealed a significant interaction between treatment and time for all time points for 100 pmol of TAPS and 150 pmol of MOR ($P < .01$).

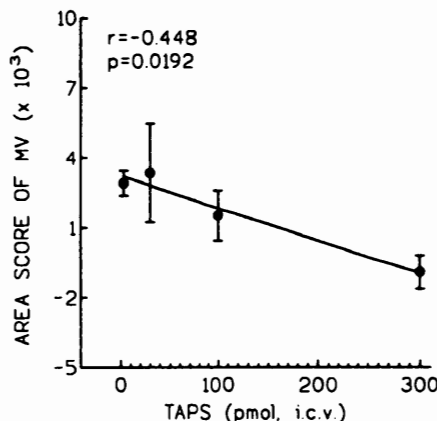
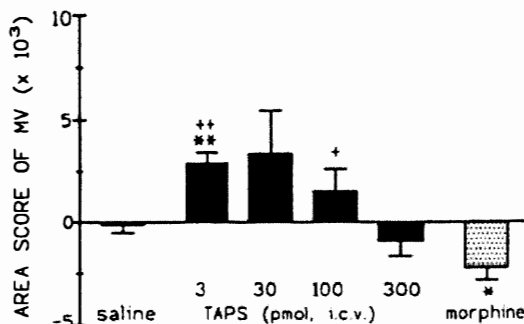


Fig. 5. Dose-response relationship of the TAPS effect on MV. The upper panel demonstrates the areas under the curve of the time-response curves for TAPS and saline as seen in figures 4 and 6. Statistical analysis by Kruskal-Wallis nonparametric ANOVA revealed a significant treatment effect ($P = .03$). The Mann-Whitney U test was used for comparisons of individual groups. * denote statistical significance compared with the saline-treated group (* $P < .05$ and ** $P < .01$). †† indicate statistical significance compared with morphine († $P < .05$ and †† $P < .01$). The lower panel denotes the corresponding values plotted as a dose-response curve. Linear regression analysis of the curve revealed a negative correlation between dose and effect (slope -14.313 ; 95% confidence interval, -26.092 to -2.535 ; $r = -0.448$; $P = .019$). The number of rats at each dose level was $n = 5$ (3 pmol of TAPS and morphine), $n = 6$ (100 pmol of TAPS and saline) and $n = 8$ (30 and 300 pmol of TAPS).

39 \pm 18% ($P < .05$ compared with base-line values). The changes in MV after saline injection were not significant (figs. 4 and 5). The areas under the curve for the TAPS-induced changes in MV were plotted as a dose-response curve (fig. 5, lower panel). Linear regression analysis of the dose-response curve (fig. 5, lower panel) yielded a negative correlation between dose and effect (slope -14.313 ; 95% confidence limit, -26.092 to -2.535 ; $r = .448$; $P = .019$).

The respiratory stimulant effect of TAPS (3 pmol i.c.v.) was attenuated in animals treated with the μ_1 antagonist naloxonazine (fig. 6).

Morphine 150 nmol i.c.v. (equianalgetic to 100 pmol of TAPS i.c.v.) depressed MV (maximal decrease $-32 \pm 8\%$, $P < .05$ compared with saline, fig. 4). The depressant effect of morphine was due to a decrease in TV (maximal decrease $-40 \pm 2\%$, $P < .01$ compared with base-line values) but morphine had no significant effect on VR (fig. 7).

Effect of TAPS, naloxonazine or dermorphin pretreatment on dermorphin-induced ventilatory depression. The interactions of TAPS with the μ_1 -opioid agonist dermorphin are summarized in figure 8. Dermorphin alone (3 nmol i.c.v.) decreased MV by $-60 \pm 9\%$ ($P < .05$ compared with

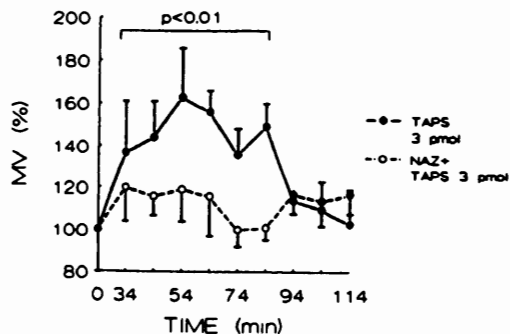


Fig. 6. Effect of 3 pmol of TAPS in the presence and absence of naloxonazine (NAZ) pretreatment (10 mg/kg i.v.) on ventilatory MV. The number of rats was six in both groups. The data are expressed as the percent change from the base line. Two-way ANOVA revealed a significant difference between groups ($P < .01$) during 34 to 84-min postinjection.

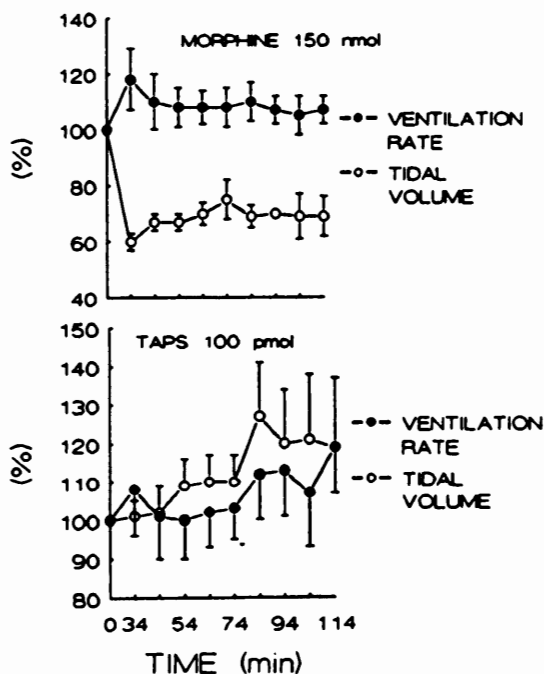


Fig. 7. Effect of 150 nmol of morphine (upper panel, $n = 5$) or 100 pmol of TAPS (lower panel, $n = 6$) on VR and TV. The data are shown as the percent change from the base line. The morphine-induced depression of TV was significant ($P < .001$) when analyzed by one-way ANOVA with repeated measures.

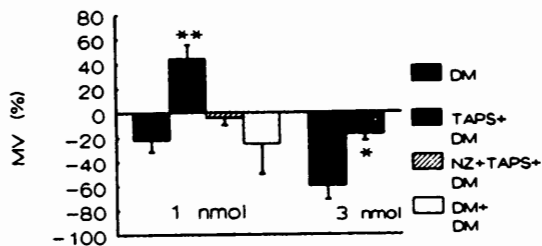


Fig. 8. Percent changes in ventilatory MV after dermorphin (DM) doses of 1 or 3 nmol in the absence or presence of pretreatments with 30 pmol of TAPS, naloxonazine (NAZ 10 mg/kg i.v.) plus 30 pmol of TAPS or DM at a dose of 10 pmol. * indicate statistical significance 1 nmol of DM compared with TAPS + 1 nmol of DM, ** $P < .01$ by Tukey's test; 3 nmol of DM compared with TAPS + 3 nmol of DM, * $P < .05$ by Student's t test. Number of rats was five to six in all groups.

base-line values). After pretreatment with TAPS (30 pmol i.c.v.), the respiratory depressant effect of 3 nmol of dermorphin was not significant ($-18 \pm 5\%$, not significant compared with base-line values). Another dose of dermorphin (1 nmol/rat i.c.v.) tended to decrease MV ($-22 \pm 10\%$, not significant compared with base-line values) when administered after saline pretreatment. After pretreatment with TAPS (30 pmol i.c.v.), this dermorphin dose increased MV maximally by $44 \pm 11\%$ ($P < .05$ compared with base-line values). After combined pretreatment with naloxonazine plus TAPS, dermorphin (1 nmol i.c.v.) had no effect on respiration. Pretreatment with a low dose (10 pmol i.c.v.) of dermorphin did not modify the respiratory effect dermorphin (1 nmol i.c.v.).

Discussion

In the present study, the μ -selective dermorphin tetrapeptide, TAPS produced dose-dependent antinociceptive effects after i.c.v. administration. At low doses of TAPS i.c.v., the TAPS-induced antinociception was not accompanied by any motor deficits. At the two highest i.c.v. doses tested, 30 pmol and 100 pmol, TAPS-induced analgesia was accompanied by catalepsy. However, the cataleptic effect of TAPS 30 pmol was transient (60 min after the injection, none of the animals was cataleptic), whereas a significant antinociceptive effect has been observed for more than 2 hr after administration of this dose of TAPS. A dose of TAPS of 100 pmol i.c.v. produced catalepsy that lasted 1 hr in all animals; nearly maximal analgesia was observed for 3 hr. In a previous study, another μ -agonist dermorphin (90 pmol i.c.v.) produced catalepsy in 85% of rats with the average duration of catalepsy of 37 ± 10 min (Paakkari and Feuerstein, 1988). It is noteworthy that TAPS elicited dose-related antinociceptive effects also after i.v. or even p.o. administration. While maximal analgesia was obtained by picomolar doses of TAPS i.c.v., 80% to 90% of the maximal possible analgesic effect was observed after i.v. injections of micromolar doses of TAPS.

The TAPS-induced antinociception was an opioid receptor-mediated effect because the effect was effectively abolished by naloxone. Moreover, naloxonazine attenuated TAPS-induced analgesia and catalepsy, which suggests that these effects are mediated by μ_1 receptors because naloxonazine antagonizes binding at the μ_1 sites in a long-lasting manner, whereas its binding to other opioid receptors is reversible (Hahn *et al.*, 1982; Ling *et al.*, 1986).

The doses of 3 pmol and 100 pmol of TAPS increased the ventilatory MV by 63% and 35%, respectively. A negative correlation was found between the TAPS dose and the respiratory stimulant effect. However, even at a supramaximal antinociceptive dose (300 pmol i.c.v.), TAPS did not depress respiration. An equianalgetic dose of morphine decreased the MV about 30%, mainly by decreasing the ventilatory TV. Like its analgesic and cataleptic effects, the respiratory stimulation induced by TAPS was antagonized by naloxonazine.

Biochemical (Wolozin and Pasternak, 1981; Clark *et al.*, 1988; Lutz *et al.*, 1985; Blurton *et al.*, 1986) and autoradiographic (Goodman and Pasternak, 1985; Moskowitz and Goodman, 1985) receptor binding studies have suggested the existence of opioid μ receptor subtypes. In pharmacological studies, opioid effects have been classified as naloxonazine-sensitive μ_1 effects and naloxonazine-insensitive μ_2 effects (Pasternak and Wood 1986). Our present data lend further support to the

results of previous studies using naloxonazine or naloxazone that suggest that supraspinal analgesia and catalepsy are μ_1 receptor-mediated opioid effects, whereas respiratory depression (Ling *et al.*, 1985), bradycardia (Holaday *et al.*, 1983; Paakkari *et al.*, 1992) and inhibition of guinea pig ileum contractions or mouse gastrointestinal motility (Gintzler and Pasternak, 1983; Heyman *et al.*, 1988) are mediated by μ_2 receptors. Moreover, our present study further extends the earlier findings by demonstrating that opioids acting on μ_1 receptors produce antinociception with respiratory stimulation.

In a previous study, we demonstrated that dermorphin had a biphasic effect on respiration. Low doses of dermorphin (3–10 pmol i.c.v.) stimulated respiration, whereas doses greater than 300 pmol shared the respiratory depressant effect of μ -opioid agonists (Paakkari *et al.*, 1990). Naloxonazine antagonized the respiratory stimulating effect of very low doses of dermorphin while the respiratory depression produced by high dermorphin doses was potentiated (Paakkari *et al.*, 1990). A possible explanation would be that, at low doses, the μ agonist dermorphin binds to high-affinity μ_1 receptors only and that activation of these receptors induces respiratory stimulation. The suggestion that μ_1 receptors mediate respiratory stimulation (Paakkari *et al.*, 1990) has been supported by studies in fetal lambs, in which naloxonazine increased the number of apneic episodes and pauses and reduced the regularity of the breathing pattern (Cheng *et al.*, 1991). Furthermore, low doses of morphine were also shown to stimulate respiration measured with chronically implanted diaphragmatic electrodes. Morphine induced an increase of diaphragmatic bursts, an increase or no change in breathing rate and a decrease in the number of apneic episodes per hour. All stimulatory effects were completely abolished with naloxonazine (Szeto *et al.*, 1991).

The potent antinociceptive, cataleptic and respiratory stimulating μ_1 effects of TAPS are in concordance with the cardiovascular actions of this tetrapeptide. Low doses of TAPS (3–30 pmol i.c.v.) induced profound naloxonazine-sensitive tachycardia in the rat. Even with relatively high doses, TAPS induced less μ_2 -mediated bradycardia than dermorphin (Paakkari *et al.*, 1992).

Furthermore, TAPS produced, despite intense antinociception, clearly less dependence than morphine. Abrupt withdrawal produced only slight loss of body weight and naloxone-precipitated withdrawal signs were less intense than in morphine-treated rats (Nakata *et al.*, 1986). The authors found it incomprehensible that a large dose of TAPS (64 times the ED₅₀) could not substitute for morphine in preventing withdrawal-induced weight loss, regardless of its high affinity for the μ receptors. This might be explained by the hypothesis that the withdrawal signs are μ_2 mediated, as suggested by Ling *et al.* (1984), and TAPS acts preferentially as a μ_1 agonist. It is also noteworthy that, even at these large doses of TAPS, this effect may be due to cross tolerance with morphine. However, the lack of mortality after TAPS administration could be due to the absence of respiratory depression, a μ_2 receptor-mediated effect.

To study the possibility that TAPS might act as a partial agonist at the μ_2 receptor, we tested whether TAPS would block dermorphin-induced respiratory depression. Pretreatment with TAPS (30 pmol i.c.v.) converted the 22 ± 10% decrease of MV induced by 3 nmol of dermorphin to a 44 ± 11% increase of the MV and the effect of a higher dose of dermorphin was significantly reduced. The possibility that the

antagonistic effect would be due to stimulation of the μ_1 receptor was further studied by blocking the μ_1 sites with naloxonazine. After the combined naloxonazine-TAPS pretreatment, dermorphin neither stimulated nor depressed ventilation, suggesting that both μ receptor subtypes were blocked. A stimulatory dose of 10 pmol of dermorphin did not antagonize the respiratory depressant effect of a higher dose of dermorphin. This implies that the reduced ventilatory depression after TAPS pretreatment was not due to a rapid development of tolerance. Acute tolerance would not be plausible, anyhow, because Ling *et al.* (1989) demonstrated that no significant tolerance developed to μ_2 actions (respiratory depression and gastrointestinal transit) during a 10-hr morphine infusion, whereas μ_1 actions (analgesia and prolactin release) diminished rapidly.

Preliminary binding studies in our laboratory indicated that the K_i values for TAPS at μ_1 and μ_2 opioid binding sites were 0.4 and 1.3 nM, respectively. In the presence of guanosine nucleotides, the affinity of TAPS at the μ_1 site was reduced significantly more than at the μ_2 site, which may indicate a partial agonistic activity at the μ_2 site (S. Vonhof *et al.*, manuscript in preparation).

The present data demonstrated that TAPS produced strong and long-lasting analgesia without respiratory depression in the rat. This would be the profile of an ideal analgesic drug if these effects can be obtained in higher species as well. As a possible μ_2 antagonist, TAPS should induce less side effects, such as inhibition of gastric motility or physical dependence, the latter of which has been shown in the rat (Nakata *et al.*, 1986). TAPS induced antinociception also i.v. and p.o., which would make it even more useful as a possible analgesic.

Acknowledgments

The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or as necessarily reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences. There is no objection to its presentation and/or publication. The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Medicine, National Research Council, DHEW publication no. (NIH) 85-23, 1985. The authors thank Mr. James Millison for his excellent technical assistance and Mrs. Ferne Robinson for skillful editing of the manuscript.

References

- BROCCARDO, M., ERSAMER, V., FALCONIERI ERSAMER, G., IMPROTA, G., LINARI, G., MELCHIORRI, P. AND MONTECUCCHI, P. C.: Pharmacological data on dermorphins, a new class of potent opioid peptides from amphibian skin. *Br. J. Pharmacol.* 73: 625–631, 1981.
- BLURTON, P., BROADHURST, A. M., WOOD, M. D. AND WYLLIE, M. G.: Is there a common, high affinity opioid binding site in rat brain? *J. Recept. Res.* 6: 85–93, 1986.
- CHENG, P. Y., DECANA, J. A., WU, D. L., CHENG, Y. AND SZETO, H. H.: The effects of selective MV: Blockade on breathing patterns in the fetal lamb. *Pediatr. Res.* 30: 202–206, 1991.
- CLARK, J. A., HOUGHTEN, R. AND PASTERNAK, G. W.: Opiate binding in calf thalamic membranes: A selective μ_1 binding assay. *Mol. Pharmacol.* 34: 308–317, 1988.
- D'AMOUR, F. E. AND SMITH, D. L.: A method for determining loss of pain sensation. *J. Pharmacol. Exp. Ther.* 72: 74–79, 1941.
- DE CASTIGLIONE, R. AND ROSSI, A. C.: Structure-activity relationships of dermorphin synthetic analogues. *Peptides (Fayetteville)* 6: 117–125, 1985.
- GINTZLER, A. R. AND PASTERNAK, G. W.: Multiple μ receptors: Evidence for μ_2 sites in the guinea pig ileum. *Neurosci. Lett.* 39: 51–56, 1983.
- GOODMAN, R. R. AND PASTERNAK, G. W.: Visualization of μ_1 opiate receptors in rat brain using a computerized autoradiographic subtraction technique. *Proc. Natl. Acad. Sci. U.S.A.* 82: 6667–6671, 1985.
- HAHN, E. F., CARROLL-BUATTI, M. AND PASTERNAK, G. W.: Irreversible opiate agonists and antagonists: The 14-hydroxydihydromorphinone azines. *J. Neurosci.* 5: 572–576, 1982.
- HEYMAN, J. S., WILLIAMS, C. L., BURKS, T. F., MOSBERG, H. I. AND PORRECA, F.: Dissociation of opioid antinociception and central gastrointestinal propul-

- sion in the mouse: Studies with naloxonazine. *J. Pharmacol. Exp. Ther.* **245**: 238-243, 1988.
- HOLADAY, J. W., PASTERNAK, G. W. AND FADEN, A. I.: Naloxone pretreatment modifies cardiorespiratory, temperature, and behavioral effects of morphine. *Neurosci. Lett.* **37**: 199-204, 1983.
- KRUMINS, S.: Characterization of dermorphin binding to membranes of rat brain and heart. *Neuropeptides* **9**: 93-102, 1987.
- LAZARUS, L. H., GUGLIETTA, A., WILSON, W. E., IRONS, B. J. AND DE CASTIGLIONE, R.: Dimeric dermorphin analogues as mu-receptor probes on rat brain membranes. *J. Biol. Chem.* **264**: 354-362, 1989.
- LING, G. S. F., MACLEOD, J., LEE, S., LOCKHART, S. H. AND PASTERNAK, G. W.: Separation of morphine analgesia from physical dependence. *Science (Wash. DC)* **226**: 462-464, 1984.
- LING, G. S. F., PAUL, D., SIMANTOV, R. AND PASTERNAK, G. W.: Differential development of acute tolerance to analgesia, respiratory depression, gastrointestinal transit and hormone release in a morphine infusion model. *Life Sci.* **45**: 1627-1636, 1989.
- LING, G. S. F., SIMANTOV, R., CLARK, J. A. AND PASTERNAK, G. W.: Naloxonazine actions in vivo. *Eur. J. Pharmacol.* **129**: 33-38, 1986.
- LING, G. S. F., SPIEGEL, K., LOCKHART, S. H. AND PASTERNAK, G. W.: Separation of opioid analgesia from respiratory depression: Evidence for different receptor mechanisms. *J. Pharmacol. Exp. Ther.* **232**: 149-155, 1985.
- LUTZ, R. A., CRUCIANI, R. A., MUNSON, J. P. AND RODBARD, D.: Mu-1: A very high affinity subtype of enkephalin binding sites in rat brain. *Life Sci.* **36**: 2233-2238, 1985.
- MOSKOWITZ, A. S. AND GOODMAN, R. R.: Autoradiographic distribution of μ_1 - and μ_2 -opioid binding in the mouse central nervous system. *Brain Res.* **360**: 117-129, 1985.
- NAKATA, N., SAKURADA, S., SAKURADA, T., KISARA, K., SASAKI, Y. AND SUZUKI, K.: Physical dependence of a dermorphin tetrapeptide analog, [D-Arg², Sar⁴]-dermorphin (1-4) in the rat. *Pharmacol. Biochem. Behav.* **24**: 27-31, 1986.
- PAKKARI, P. AND FEUERSTEIN, G.: Antagonism of dermorphin-induced catalepsy with naloxone, TRH-analog CG 3703 and the benzodiazepine antagonist Ro 15-1788. *Neuropharmacology* **27**: 1007-1012, 1988.
- PAKKARI, P., PAKKARI, I., FEUERSTEIN, G. AND SIRÉN, A.-L.: Evidence for differential opioid μ_1 - and μ_2 -receptor mediated regulation of heart rate in the conscious rat. *Neuropharmacology* **31**: 777-782, 1992.
- PAKKARI, P., PAKKARI, I., SIRÉN, A.-L. AND FEUERSTEIN, G.: Respiratory and locomotor stimulation by low doses of dermorphin, a mu₁ receptor-mediated effect. *J. Pharmacol. Exp. Ther.* **252**: 235-240, 1990.
- PASTERNAK, G. W. AND WOOD, P. J.: Minireview: Multiple mu opiate receptors. *Life Sci.* **38**: 1889-1898, 1986.
- SALVADORI, S., SARTO, G. AND TOMATIS, R.: Synthesis and pharmacological activity of dermorphin and its N-terminal sequence. *Int. J. Pept. Protein Res.* **19**: 536-542, 1982.
- SASAKI, Y., MATSUI, M., TAGUCHI, M., SUZUKI, K., SAKURADA, S., SATO, T., SAKURADA, T. AND KISARA, K.: D-Arg²-dermorphin analog: A potent and long-lasting analgesic activity after subcutaneous administration. *Biochem. Biophys. Res. Comm.* **120**: 214-218, 1984.
- SATO, T., SAKURADA, S., SAKURADA, T., FURUTA, S., CHAKI, K., KISARA, K., SASAKI, Y. AND SUZUKI, K.: Opioid activities of D-Arg²-substituted tetrapeptides. *J. Pharmacol. Exp. Ther.* **242**: 654-659, 1987.
- STEVENS, C. G. AND YAKSH, T. L.: Spinal action of dermorphin, an extremely potent opioid peptide from frog skin. *Brain Res.* **385**: 300-304, 1986.
- SZETO, H. H., CHENG, P. Y., DWYER, G., DECANA, J. A., WU, D. L., CHENG, Y.: Morphine-induced stimulation of fetal breathing: role of mu₁ receptors and central muscarinic pathways. *Am. J. Physiol.* **261**: R344-R350, 1991.
- WOLOZIN, B. L. AND PASTERNAK, G. W.: Classification of multiple morphine and enkephalin binding sites in the central nervous system. *Proc. Natl. Acad. Sci. U.S.A.* **78**: 6181-6185, 1981.

Send reprint requests to: Anna-Leena Sirén, M.D., Ph. D., Department of Neurology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814.
