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Comparison of the Antinociceptive Effects of New [D-Arg^2]-Dermorphin Tetrapeptide Analogs and Morphine in Mice

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CHAKI, K., S. SAKURADA, T. SAKURADA, T. SATO, S. KAWAMURA, K. KISARA, H. WATANABE AND K. SUZUKI. *Comparison of the antinociceptive effects of new [D-Arg^2]-dermorphin tetrapeptide analogs and morphine in mice*. PHARMACOL BIOCHEM BEHAV 31(2) 439–444, 1988.—The antinociceptive effects of synthetic dermorphin tetrapeptide analogs containing D-Arg in position 2, H-Tyr-D-Arg-Phe-Gly-NH_2 and H-Tyr-D-Arg-Phe-β-Ala-OH, were measured in mice by the tail-pressure test. The antinociceptive effect produced by intracerebroventricular (ICV), intrathecal (IT) and subcutaneous (SC) administration of either peptide was greater than that produced by morphine. Oral (PO) administration of the peptides showed approximately the same antinociceptive potency as morphine. In addition, the antinociceptive effect produced by SC and PO administration of either peptide was of longer duration than morphine. Pretreatment with naloxone resulted in nearly complete antagonism of the antinociceptive effects produced by ICV and IT administration of either peptide or morphine. Dose ratios (ICV/IT) of H-Tyr-D-Arg-Phe-Gly-NH_2 and H-Tyr-D-Arg-Phe-β-Ala-OH, which were calculated from the AD_{50} (Antinociceptive Dose=50% MPE) values, were 5.8 and 6.2, respectively, whereas that of morphine was only 1.46. These results suggest that the mechanisms of the antinociceptive effects of [D-Arg^2]-dermorphin tetrapeptide analogs differ from morphine, and that these peptides may possess higher affinities than does morphine for opioid receptors in the spinal cord.

[D-Arg^2]-dermorphin tetrapeptide analogs Morphine Antinociceptive effects Tail-pressure test ICV IT SC PO Mice

DERMORPHIN (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2), a heptapeptide amide, was isolated (4) and identified (14) from the skin of the South American frog, and found to produce a potent and long-lasting antinociceptive effect (1, 2, 6, 8). Though it was also proposed that the N-terminal tetrapeptide amide (H-Tyr-D-Ala-Phe-Gly-NH_2) was the minimal fragment required for full opioid activity (1, 3, 19), this fragment was somewhat less potent than the parent heptapeptide amide (1, 5, 18). In the structure-activity relationships of dermorphin fragments, D-Ala^2 in the peptide chain of dermorphin has been considered to be of crucial importance for opioid activity (1, 3, 19). In fact, [L-Ala^2]-dermorphin is practically inactive (2, 5, 7, 16). The activity of dermorphin was completely antagonized by the opioid antagonist naloxone.

An endogenous opioid peptide, kyotorphin (H-Tyr-Arg-OH), was isolated from bovine brain (24). The substitution of Arg with D-Arg can remarkably enhance its activity (17,23).

Subsequently, it has been reported that the antinociceptive effect of the D-Arg^2 substituted dermorphin tetrapeptide (H-Tyr-D-Arg-Phe-Gly-OH) is greater than the parent tetrapeptide (20). Additionally, in the structure-activity relationships of [D-Arg^2]-dermorphin fragments, N-terminal tetrapeptide amide (H-Tyr-D-Arg-Phe-Gly-NH_2) was the most potent fragment (12).

Based on these reports, we newly synthetized D-Arg^2 and

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TABLE 1

THE AMINO ACID SEQUENCES OF DERMORPHIN, [D-Arg^2]-DERMORPHIN AND [D-Arg^2]-DERMORPHIN TETRAPEPTIDE ANALOGS

	1 2 3 4 5 6 7
Dermorphin	H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2
[D-Arg^2]-dermorphin	H-Tyr-D-Arg-Phe-Gly-Tyr-Pro-Ser-NH_2
[D-Arg^2]-dermorphin (1–4)-NH_2	H-Tyr-D-Arg-Phe-Gly-NH_2
[D-Arg^2,β-Ala^4]-dermorphin (1–4)	H-Tyr-D-Arg-Phe-β-Ala-OH

β-Ala^4 substituted N-terminal tetrapeptide of dermorphin.

In the present study, we have examined the antinociceptive effects of [D-Arg^2]-dermorphin tetrapeptide analogs, H-Tyr-D-Arg-Phe-Gly-NH_2 (TDAPG-amide) and H-Tyr-D-Arg-Phe-β-Ala-OH (TDAPA), when administered intracerebroventricularly (ICV) and intrathecally (IT) in comparison with those of morphine to investigate the sites of action in the central nervous system. To compare with the antinociceptive effects of systemically administered morphine, these peptides were also administered subcutaneously (SC) or orally (PO).

The amino acid sequences of dermorphin, [D-Arg^2]-dermorphin and [D-Arg^2]-dermorphin tetrapeptide analogs (TDAPG-amide, TDAPA) are shown in Table 1.

METHOD

Male Std-ddY mice weighing 20 to 28 g were used in the present study. The animals were housed at 22±2°C, and were supplied food and water ad lib. A standard light-dark cycle was maintained with a timer regulated light period from 9:00 a.m. to 9:00 p.m.

The antinociceptive effect was measured using the tail-pressure method as previously described (17). Briefly, mechanical pressure was applied to the base of the tail at a rate of 10 mmHg/sec and biting or struggling behavior in mice to which pressure was applied mechanically was an indication of response threshold. Only mice responding behaviorally to a tail-pressure of 40 to 50 mmHg were selected for this experiment. The trials were terminated at the level of 100 mmHg to prevent tail tissue damage. For the tail-pressure assay, the mean±s.e.m. of the pressure level was plotted. To obtain the response curve, the dose was plotted against % of maximum possible effect (% of MPE) calculated using the following equation, % of MPE = $(P_2 - P_1/100 - P_1) \times 100$, where P_1 is the response pressure before drug administration (mmHg) and P_2 is the response pressure after drug administration (mmHg).

Compounds used were: [D-Arg^2]-dermorphin tetrapeptide analogs, TDAPG-amide and TDAPA (synthetized by Suzuki *et al.*), morphine hydrochloride (Sankyo) and naloxone hydrochloride (Endo laboratories).

All compounds for ICV and IT administration were dissolved in sterile Ringer's solution. For ICV administration, a modification of the method of Haley and McCormick (10), and for IT administration, the direct lumbar puncture method of Hylden and Wilcox (11) was used with a constant volume of 10 and 5 μl, respectively. All compounds for SC and PO administration were dissolved in physiological saline and distilled water, respectively, each in a volume of 0.1 ml/10 g of body weight. Naloxone hydrochloride was dissolved in physiological saline and was administered intraperitoneally (IP) in a volume of 0.1 ml/10 g body weight at 5 min before either peptide or morphine administration.

Statistical significance of the data was estimated by an analysis of variance (ANOVA) (9) with Dunnett's test. AD_{50} (Antinociceptive Dose=50% MPE) values and their 95% confidence limits were determined by the method of Litchfield and Wilcoxon (13). These values were calculated from the values obtained at the time of peak effect after either peptide or morphine administration.

RESULTS

Antinociceptive Effects Produced by ICV and IT Administration of TDAPG-amide, TDAPA and Morphine

As shown in Fig. 1, ICV administration of TDAPG-amide, TDAPA and morphine produced dose-related and significant antinociceptive effects. Maximally effective doses of TDAPG-amide (20.00 pmol), TDAPA (10.00 pmol) and morphine (2400.00 pmol) produced peak antinociceptive effects 10 min after administration. The AD_{50} (95% confidence limits) values for TDAPG-amide, TDAPA and morphine were 5.80 (3.70–9.10), 3.10 (2.18–4.41) and 1050.00 (551.51–1999.07) pmol/mouse, respectively (Table 2). From the values, the antinociceptive potency of TDAPG-amide was 181.03 times, and TDAPA was 338.71 times that of morphine.

IT administered TDAPG-amide and TDAPA showed exceedingly potent antinociceptive effects. Maximally effective doses of all compounds peaked at 10 min after administration (Fig. 2). The AD_{50} (95% C.L.) values for TDAPG-amide, TDAPA and morphine were 1.00 (0.62–1.61), 0.50 (0.28–0.90) and 720.00 (425.04–1219.65) pmol/mouse, respectively (Table 2). The antinociceptive potency of TDAPG-amide was 720.00 times, and TDAPA was 1440.00 times that of morphine.

The dose ratios (ICV/IT) of TDAPG-amide and TDAPA, which were calculated from the AD_{50} values, were 5.8 and 6.2, respectively, whereas that of morphine was only 1.46 (Table 2).

The antinociceptive effects induced by these peptides and morphine were completely antagonized by the pretreatment with naloxone (0.5 mg/kg IP) (Figs. 1 and 2).

Antinociceptive Effects Produced by SC and PO Administration of TDAPG-amide, TDAPA and Morphine

Figure 3 shows that SC administered TDAPG-amide and TDAPA were both found to possess significant and dose-related antinociceptive effects. Moreover, the antinocicep-

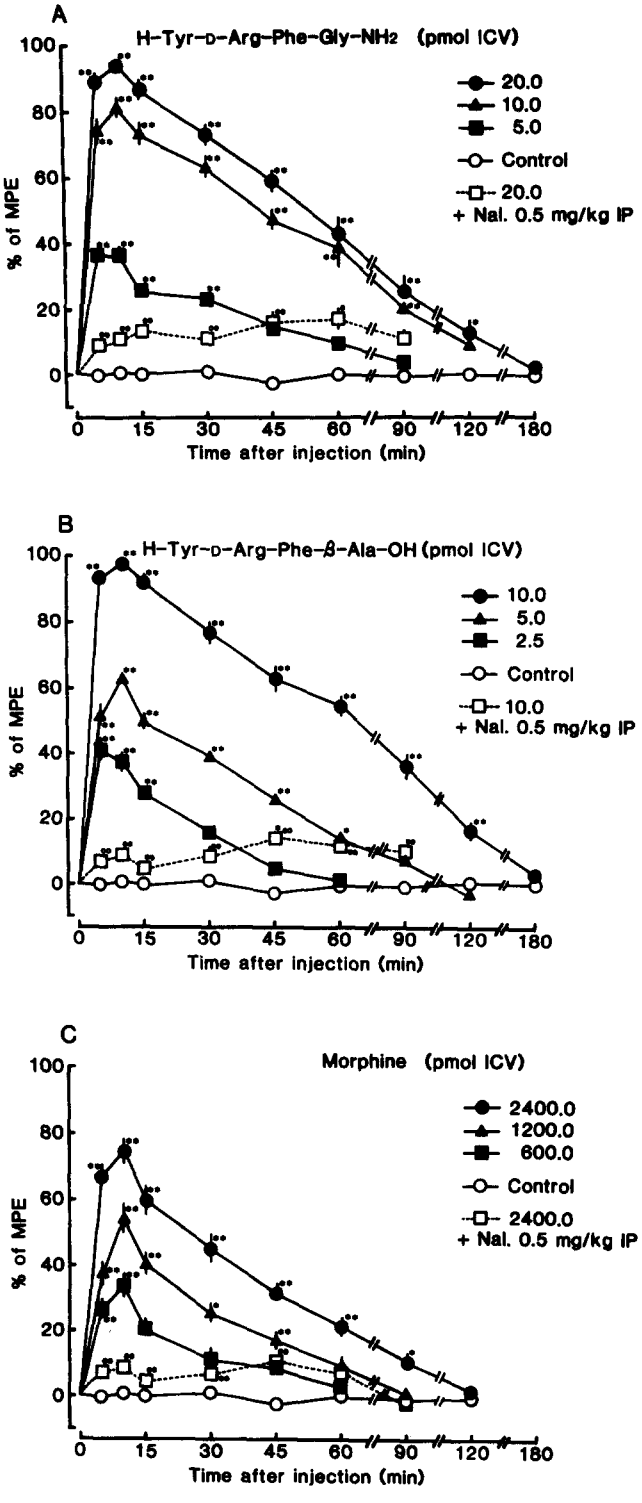

FIG. 1. The time courses of the antinociceptive effects of TDAPG-amide (A), TDAPA (B) and morphine (C) administered intracerebroventricularly (ICV) in mice. Control groups were treated with Ringer solution. Each point represents % of MPE values mean, with vertical lines showing s.e. mean, of ten mice in each group. Significant differences from control groups are indicated with $*p<0.05$, $**p<0.01$: Significant differences between maximum dose (20.00 pmol) of TDAPG-amide and naloxone (0.5 mg/kg) + maximum dose of TDAPG-amide are indicated with $^{o}p<0.05$, $^{oo}p<0.01$.

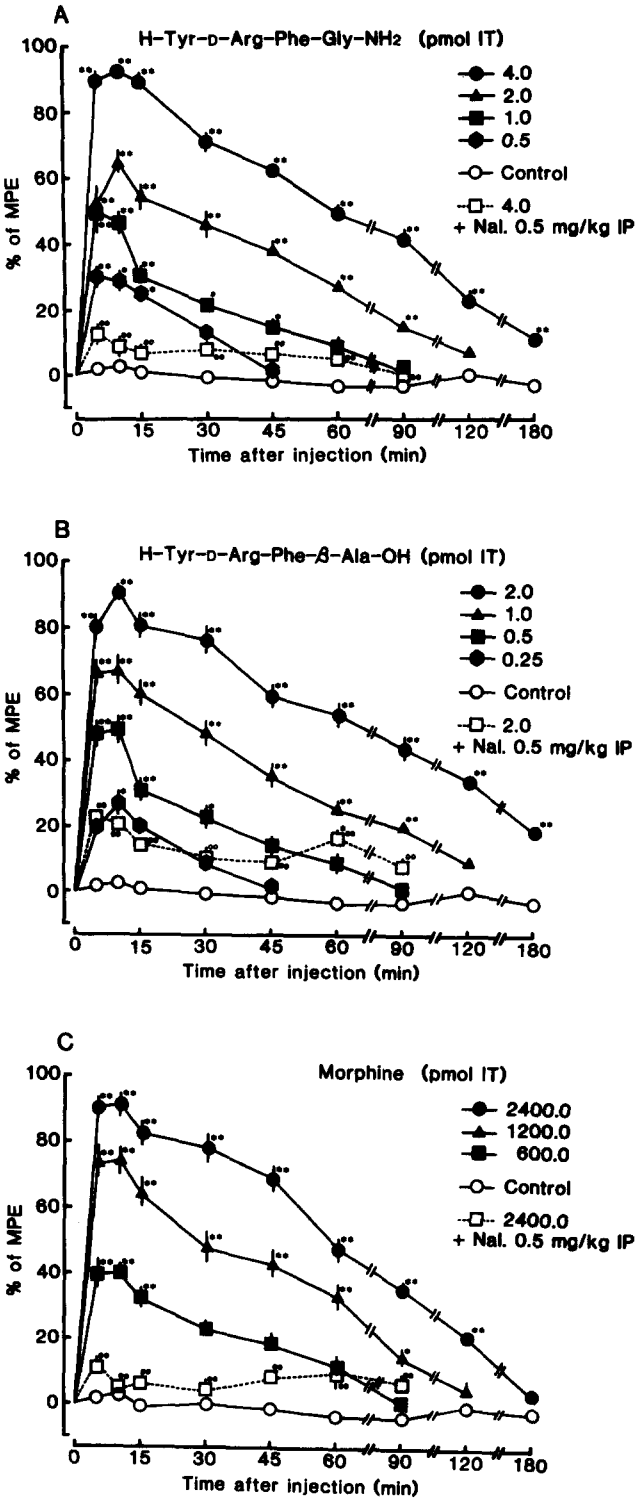

FIG. 2. The time courses of the antinociceptive effects of TDAPG-amide (A), TDAPA (B) and morphine (C) administered intrathecally (IT) in mice. Control groups were treated with Ringer's solution. For other details see Fig. 1.

TABLE 2

ANTINOCICEPTIVE EFFECTS PRODUCED BY INTRACEREBROVENTRICULAR (ICV) AND INTRATHECAL (IT) ADMINISTRATION OF EACH COMPOUND, AS MEASURED BY THE TAIL-PRESSURE TEST IN MICE

Compounds	AD_{50} (pmol ICV)	AD_{50} (pmol IT)	Dose Ratios (ICV/IT)	Antagonism by Naloxone* ICV	IT
H-Tyr-D-Arg-Phe-Gly-NH_2	5.80 (3.70–9.10)	1.00 (0.62–1.61)	5.80	+	+
H-Tyr-D-Arg-Phe-β-Ala-OH	3.10 (2.18–4.41)	0.50 (0.28–0.90)	6.20	+	+
Morphine	1050.00 (551.51–1999.07)	720.00 (425.04–1219.65)	1.46	+	+

AD_{50} values were calculated from the values obtained at the time of peak effect. Ninety-five percent confidence limits are given in parentheses.
*0.5 mg/kg IP.

TABLE 3

ANTINOCICEPTIVE EFFECTS PRODUCED BY SUBCUTANEOUS (SC) AND ORAL (PO) ADMINISTRATION OF EACH COMPOUND, AS MEASURED BY THE TAIL-PRESSURE TEST IN MICE

Compounds	AD_{50} (μmol/kg SC)	Relative Potency	AD_{50} (μmol/kg PO)	Relative Potency
H-Tyr-D-Arg-Phe-Gly-NH_2	1.00 (0.61–1.65)	12.24	102.67 (63.55–165.87)	0.75
H-Tyr-D-Arg-Phe-β-Ala-OH	0.81 (0.53–1.25)	15.11	70.06 (43.52–112.78)	1.10
Morphine	12.24 (7.96–18.82)	1.00	77.16 (51.97–114.55)	1.00

AD_{50} values were calculated from the values obtained at the time of peak effect. Ninety-five percent confidence limits are given in parentheses.

tive effect induced by either peptide was of longer duration than that induced by morphine, with peak effect not seen until 45 min, and the significant effect lasted 240 min (maximum dose) after administration. From the AD_{50} values, the relative potencies of TDAPG-amide and TDAPA were 12.24 and 15.11, respectively (morphine=1).

PO administration of the peptides was also found to possess significant and dose-related antinociceptive effects (Fig. 4). As Table 3 shows, though antinociceptive effect of TDAPA showed approximately equipotent to that of morphine, the effect of TDAPG-amide was less intense than the effect of morphine. However, the antinociceptive effect induced by either peptide was of longer duration than that induced by morphine (Fig. 4).

DISCUSSION

It is well known that the opioid activity of dermorphin is more powerful than that of other naturally occurring opioid peptides or of morphine. As Broccardo *et al.* reported, N-terminal tetrapeptide amide is the minimal fragment required for full opioid activity (1). The substitution of D-Arg for D-Ala in the N-terminal tetrapeptide of dermorphin markedly enhanced the activity (20). Subsequently, Kisara *et al.* studied the structure-activity relationships of [D-Arg^2]-dermorphin fragments and showed that the N-terminal tetrapeptide amide (H-Tyr-D-Arg-Phe-Gly-NH_2) was the most potent fragment (12). From these reports, we have examined the structure-activity relationships of [D-Arg^2]-dermorphin tetrapeptide analogs. We found that the substitution of β-Ala for Gly in the H-Tyr-D-Arg-Phe-Gly-OH peptide chain resulted in greater antinociceptive effect than seen for other [D-Arg^2]-dermorphin tetrapeptide analogs.

In the present tail-pressure assay, the antinociceptive effects produced by ICV, IT and SC administered TDAPG-amide and TDAPA were greater than morphine. Even administered PO, these peptides showed approximately the same antinociceptive potency as morphine. Moreover, the antinociceptive effect induced by either peptide was of longer duration than that induced by morphine in a SC or PO route. Sasaki *et al.* previously reported that the D-Arg^2 substituted N-terminal tetrapeptide of dermorphin (H-Tyr-D-Arg-Phe-Gly-OH) is characterized by a longer duration of action than the parent tetrapeptide (H-Tyr-D-Ala-Phe-Gly-OH). In addition, H-Tyr-D-Arg-Phe-Gly-OH was more stable than H-Tyr-D-Ala-Phe-Gly-OH to the cleavage both by aminopeptidase M and carboxypeptidase Y (20). Therefore, the long duration of its effect appears to be due mainly to resistance to enzymatic degradation on account of the D-amino acid residue. This result suggests that the D-Arg^2

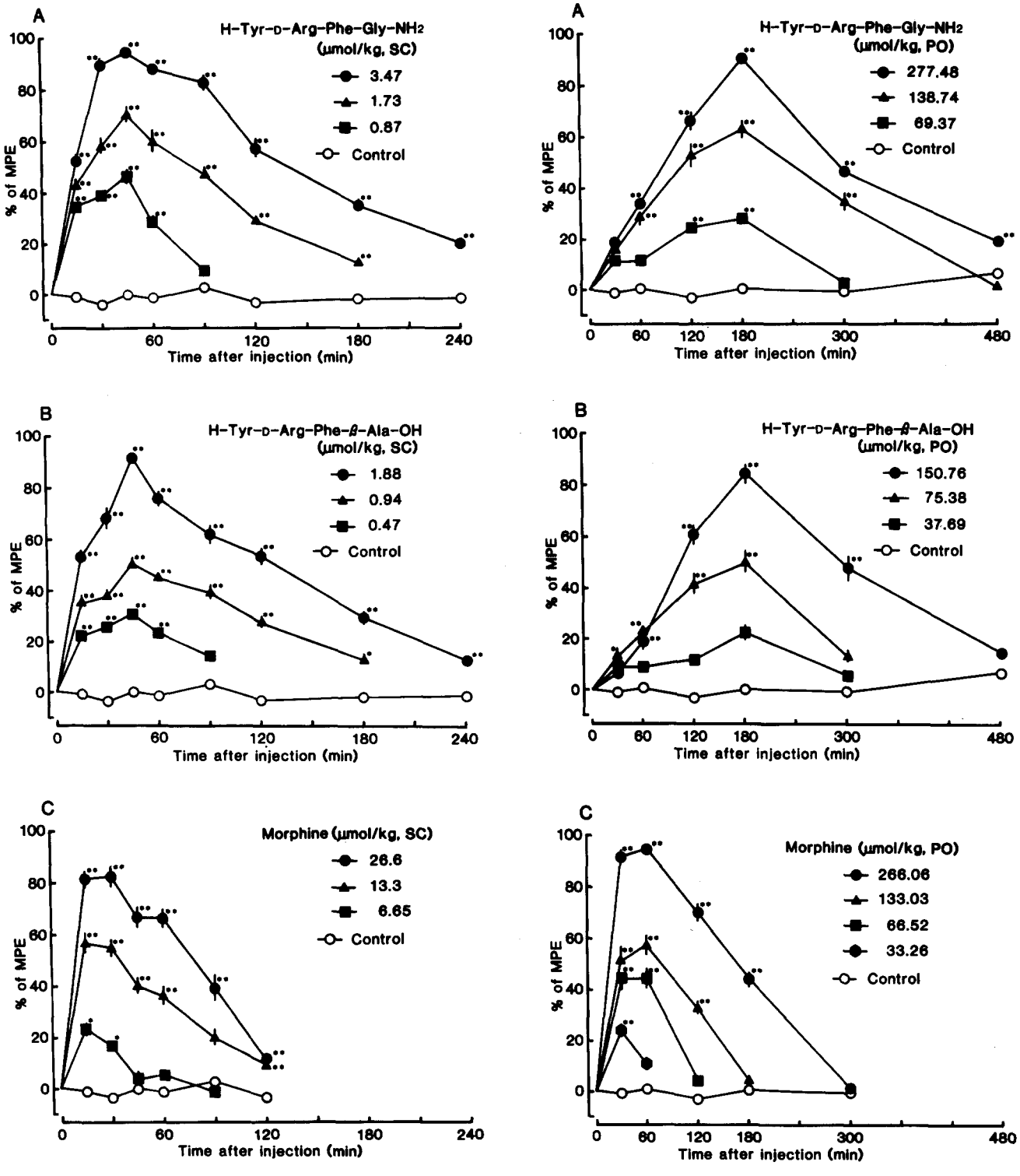

FIG. 3. The time courses of the antinociceptive effects of TDAPG-amide (A), TDAPA (B) and morphine (C) administered subcutaneously (SC) in mice. Control groups were treated with saline solution. For other details see Fig. 1.

FIG. 4. The time courses of the antinociceptive effects of TDAPG-amide (A), TDAPA (B) and morphine (C) administered orally (PO) in mice. Control groups were treated with distilled water. For other details see Fig. 1.

in the peptide chain of [D-Arg^2]-dermorphin tetrapeptide analogs is of crucial importance for opioid activity. In fact, H-Tyr-L-Arg-Phe-Gly-OH was practically inactive in the previous tail-pressure assay (20).

We have recently reported that [D-Arg^2]-dermorphin tetrapeptide analogs, H-Tyr-D-Arg-Phe-Gly-OH and H-Tyr-D-Arg-Phe-Sar-OH, showed high affinities for μ-type opioid receptors in the radioreceptor assay utilizing [^{3}H]-naloxone as the opioid receptor ligand. The affinity ratios of H-Tyr-D-Arg-Phe-Gly-OH was 5.33 times and H-Tyr-D-Arg-Phe-Sar-OH was 25.45 times that of morphine (21). From this report, the antinociceptive effects of these analogs have been considered to be mainly due to the specific interaction with opioid receptors in the central nervous system. As present results show, the antinociceptive effects produced by ICV and IT administration of TDAPG-amide and TDAPA were completely antagonized by the pretreatment with naloxone. Moreover, dose ratios (ICV/IT) of TDAPG-amide and TDAPA, which were calculated from the AD_{50} values, were 5.8 and 6.2, respectively, whereas that of morphine was only 1.46. These results indicate that these peptides may possess higher affinities than does morphine for opioid receptors in the spinal cord.

Nakata *et al.* previously reported that in the physical dependence of [D-Arg^2,Sar^4]-dermorphin (1–4) (H-Tyr-D-Arg-Phe-Sar-OH), abrupt withdrawal after chronic administration of this peptide produced only a slight loss of body weight at 24 hours after withdrawal as compared to the weight on last day of this peptide administration. On the contrary, morphine-dependent rats produced sharp loss of body weight at 72 hours after withdrawal. Naloxone precipitated withdrawal signs after chronic administration of this peptide were significantly less intense than those after chronic administered morphine (15). Thus, [D-Arg^2]-dermorphin tetrapeptide analogs are expected to be clinically useful, and it will not be very long before such peptides are employed as peptidic analgesics.

In summary, these previous and present results suggest that the mechanisms of the antinociceptive effects of [D-Arg^2]-dermorphin tetrapeptide analogs differ from morphine. Furthermore, it is of interest that PO administration of these analogs exhibited not only antinociceptive effects equipotent to morphine, but also a much longer duration of action.

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