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Activity of mu- and delta-opioid agonists in vas deferens from mice deficient in MOR gene

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1 Mice lacking the mu-opioid receptor have been recently generated. Centrally mediated responses of mu-opioid agonists are suppressed whereas some of the delta-opioid responses are preserved in these mutant mice.

2 The vas deferens bioassay has been used in this study to investigate the functional activity at a peripheral level of mu- and delta-opioid agonists in mice lacking mu-opioid receptors.

3 The different mu-opioid agonists evaluated, morphine, DAMGO, dermorphin and [Lys^7]-dermorphin produced an inhibitory response in vas deferens from wild-type mice but had no relevant activity on vas deferens from mutant mice.

4 The selective delta-opioid agonists DPDPE, BUBU, deltorphin I, deltorphin II and [D-Met^2]-deltorphin induced inhibitory effects in vas deferens from both wild-type and mutant mice. However, the biological activities of these ligands were slightly reduced in preparations from mutant mice. The inhibitory responses of all these delta-opioid agonists were prevented by the administration of the selective delta-opioid antagonist naltrindole.

5 These data indicate that delta-opioid agonists, but not mu-opioid agonists, are biologically active in vas deferens from mice lacking mu-opioid receptors. The decreased response of delta-agonists in mutant mice suggests that some cooperativity may exist between mu- and delta-opioid receptors in these vas deferens preparations.

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Abbreviations: BUBU, Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr(OtBu); DAMGO, Tyr-D-Ala-Gly-N(Me)-Phe-GlyOl; DPDPE, Tyr-D-Pen-Gly-Phe-D-Pen; MOR, mu-opioid receptor

Introduction

Three major types of opioid receptors, mu, delta and kappa, have been pharmacologically and biochemically characterized and recently cloned (Kieffer, 1995). The activation of these three receptors is responsible for the responses induced by exogenous (Kieffer, 1999) and endogenous (Roques *et al*., 1993) opioids. The mu-opioid receptor (MOR) gene has been recently disrupted in mice by homologous recombination (Matthes *et al*., 1996; Sora *et al*., 1997b). Antinociception, behavioural responses and physical dependence produced by morphine were completely abolished in these mice, even when morphine was administered at high concentrations (Matthes *et al*., 1996). The availability of mice lacking mu-opioid receptors also highlight the specific contribution of delta-opioid receptors in the biological activity of prototypic delta-agonists and the possible functional cooperativity between mu- and delta-opioid receptors. Previous studies have shown that antinociceptive responses to the delta-opioid agonist Tyr-D-Pen-Gly-Phe-D-Pen (DPDPE) were slightly decreased (Matthes *et al*., 1998) or abolished (Sora *et al*., 1997a) in MOR-deficient mice, whereas deltorphin II antinociception was maintained in these animals (Matthes *et al*., 1998; Hosohata *et al*., 2000). Besides, the decrease of the respiratory frequency produced by deltorphin II in wild-type mice was not observed in MOR-deficient mice (Matthes *et al*., 1998). All the responses that have been evaluated at the present moment in MOR-deficient mice are related to the activation of opioid receptors in the central nervous system.

Several pharmacological effects of the opioids are due to the stimulation of peripheral opioid receptors. These peripheral opioid responses are important for some of their therapeutic applications, such as analgesia (Stein *et al*., 1989) and constipation (Kromer, 1993). One of the most useful assays to evaluate the functional activity of peripheral opioid receptors in mice is the vas deferens preparation (Hughes *et al*., 1975), which permits an easy and accurate comparison of the potency of analogous compounds (Erspamer & Severini, 1992). The effect of opioid receptor activation in this isolated organ preparation is to reduce smooth muscle contraction *via* inhibition of excitatory neurotransmitter release, revealed by

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measuring the inhibitory action on electrically-stimulated contractions of the vas deferens (Smith & Leslie, 1993). Mouse vas deferens mainly contains delta-opioid receptors that are probably different in functional terms from brain delta-receptors (Vaughn *et al*., 1990). However, these preparations are sensitive to mu-, delta- and kappa-opioid agonists (Smith & Leslie, 1993). Delta-opioid agonists are potent inhibitors of muscle contraction in mouse vas deferens (Lord *et al*., 1977), but the absolute potencies of mu- (Hughes *et al*., 1975) and kappa-opioid agonists (Lord *et al*., 1977) are lower in mouse vas deferens than in other preparations.

In this study, the effects of different opioid agonists have been investigated in vas deferens preparations from wild-type and MOR-deficient mice, as a model of opioid responses on peripheral nervous system. In a first step, classic agonists for the mu-, morphine and Tyr-D-Ala-Gly-N(Me)-Phe-GlyO (DAMGO) (Handa *et al*., 1981), and delta-, DPDPE (Mosberg *et al*., 1983) and Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr(OtBu) (BUBU) (Gacel *et al*., 1988), opioid receptors were used. We have also evaluated the responses induced by the naturally occurring opioid heptapeptides from amphibian skin, dermorphin and [Lys^7]-dermorphin, that are potent and selective mu-opioid receptor agonists (Erspamer, 1992; Negri *et al*., 1992; Melchiorri & Negri, 1996), as well as deltorphin I, deltorphin II and [D-Met^2]-deltorphin (Erspamer *et al*., 1989; Kreil *et al*., 1989), that bind with high affinity and selectivity to delta-opioid receptors. The ability of the delta-opioid selective antagonist naltrindole to reverse the responses induced by the different delta-agonists was also investigated.

Methods

Animals

Male mice and their wild-type littermates were used in this study. All mice were 1 : 1 hybrids from 129/SV and C57Bl/6 mouse strains (Matthes *et al*., 1996). Animals were housed in a controlled environment maintained at $22 \pm 2^{\circ}$C and 50–60% humidity with a natural day/night light cycle until they were killed.

Drugs

DPDPE (Mosberg *et al*., 1983), BUBU (Gacel *et al*., 1988), deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2) (Kreil *et al*., 1989), deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2), [D-Met^2]-deltorphin (Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2) (Erspamer *et al*., 1989), DAMGO (Handa *et al*., 1981), dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) (Rossi *et al*., 1986), and [Lys^7]-dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Lys-NH_2) (Negri *et al*., 1992) were synthesised and purified as previously described. Morphine and naltrindole were purchased from Research Biochemicals Inc., Natick, MA, U.S.A.

Mouse vas deferens preparations

Preparations from mouse vas deferens of wild-type and mutant mice were disposed as previously described (Hughes *et al*., 1975). Vasa were mounted in an organ bath of 10 ml capacity. The tissues were bathed in modified Krebs solution (mM): NaCl 118, KCl 4.75, CaCl2 2.54, KHPO4 0.93, NaHCO3 25, glucose 11, gassed with 95% O_2 and 5% CO_2. No protease inhibitor was added to the Krebs solutions. The bath temperature was 37°C. Longitudinal contractions were recorded isometrically at a constant tension of 0.5 g by a strain gauge transducer (DY 1, Basile, Milan) and displayed on a recording microdynamometer (Unirecord, Basile, Milan). The intramural nerves were stimulated with trains of rectilinear pulses of 1 ms duration. After an equilibration period of 30 min, stimulation trains were given at intervals of 20 s and consisted of six stimuli of 1 ms duration with intervals of 10 ms. Compounds were dissolved in distilled water, added in the 10 ml bath in a volume of 50 μl and washed away after 6 min contact. When the preparation was completely recovered after washing, a higher concentration (double) of the same compound was added to the bath. Extensive concentration-response curves were performed in all the experiments in order to allow calculation of IC_{50} values: DAMGO (from 77 to 384 nM), morphine (from 665 to 6650 nM), dermorphin (from 12.5 to 100 nM), [Lys^7]-dermorphin (from 24 to 189 nM), DPDPE (from 7.7 to 77 nM), BUBU (from 2.6 to 15.6 nM), deltorphin I (from 0.13 to 1 nM), deltorphin II (from 0.65 to 5.2 nM) and [D-Met^2]-deltorphin (from 1.05 to 8.4 nM). At least 10 experiments were performed for each drug in each genotype. For antagonism experiments, naltrindole (45 nM) was added in the bath in a volume of 50 μl after obtaining the maximal response of the delta-opioid agonists. Delta agonists were then added again in the bath after naltrindole administration. At least six antagonism experiments were performed for each delta agonist in each genotype.

Statistical analysis

Dose-response lines were analysed with a linear regression method (Tallarida & Murray, 1986), and IC_{50} were calculated only from the linear portion of the dose-response curves. Two-tailed non-paired Student *t*-test was used to estimate the significance level of the observed differences in IC_{50} values between wild-type and mutant groups. The level of significance was 0.05.

Results

Biological activity of mu-opioid selective agonists

Biological activities of the mu-opioid agonists DAMGO, morphine, dermorphin and [Lys^7]-dermorphin were investigated in mouse vas deferens preparations from wild-type and MOR-deficient mice. The IC_{50} values obtained in preparations from wild-type mice were 176.9 ± 12.32 nM for DAMGO, 43.9 ± 3.81 nM for dermorphin and 47.3 ± 5.68 nM for [Lys^7]-dermorphin. The biological effect of morphine did not reach the 50% of the maximum possible inhibitory response. Significant slopes were obtained for the concentration-response curves of the different mu-opioid agonists in preparations from wild-type mice. These mu-opioid agonists did not induce any biological effect in preparations from MOR-deficient mice when administered

at low concentrations. Only a small trend to inhibit the response was observed after the administration of very high concentrations of morphine, dermorphin and [Lys^7]-dermorphin. Concentration-response curves of the different mu-opioid agonists in MOR-deficient mouse vas deferens show slopes not significantly different from zero (Figure 1 and 4).

Biological activity of delta-opioid selective agonists

Biological activities of the selective delta-opioid agonists DPDPE, BUBU, deltorphin I, deltorphin II and [D-Met^2]-deltorphin were also investigated in the mouse vas deferens preparations. The IC_{50} values obtained in preparations from wild-type mice were 13.9 ± 1.40 nM for DPDPE, 3.6 ± 0.28 nM for BUBU, 0.39 ± 0.04 nM for deltorphin I, 1.92 ± 0.24 nM for deltorphin II and 2.46 ± 0.18 nM for [D-Met^2]-deltorphin. In the case of preparations from MOR-deficient mice, the IC_{50} values for DPDPE (22.5 ± 2.38 nM), BUBU (6.03 ± 0.98 nM), deltorphin I (0.64 ± 0.09 nM), deltorphin II (3.51 ± 0.61 nM) and [D-Met^2]-deltorphin (5.19 ± 0.42 nM) were higher. Significant slopes were obtained for the concentration-response curves of the different delta-opioid agonists in preparations from both wild-type and MOR-deficient mice. The comparison between genotypes revealed significantly different IC_{50} values for [D-Met^2]-deltorphin ($t_{(1,22)} = 4.900$, $P < 0.0001$) and deltorphin II ($t_{(1,22)} = 2.134$, $P < 0.05$). However, the comparison of the effects induced in both genotypes by DPDPE ($t_{(1,22)} = 1.741$, $P = 0.09$), deltorphin I ($t_{(1,22)} = 1.932$, $P = 0.06$) and BUBU ($t_{(1,20)} = 1.754$, $P = 0.09$) did not reach the significance. The rank order of potency of delta-opioid agonists (deltorphin I > deltorphin II > [D-Met^2]-deltorphin > BUBU > DPDPE) was similar in preparations from wild-type and mutant mice (Figures 2 and 4).

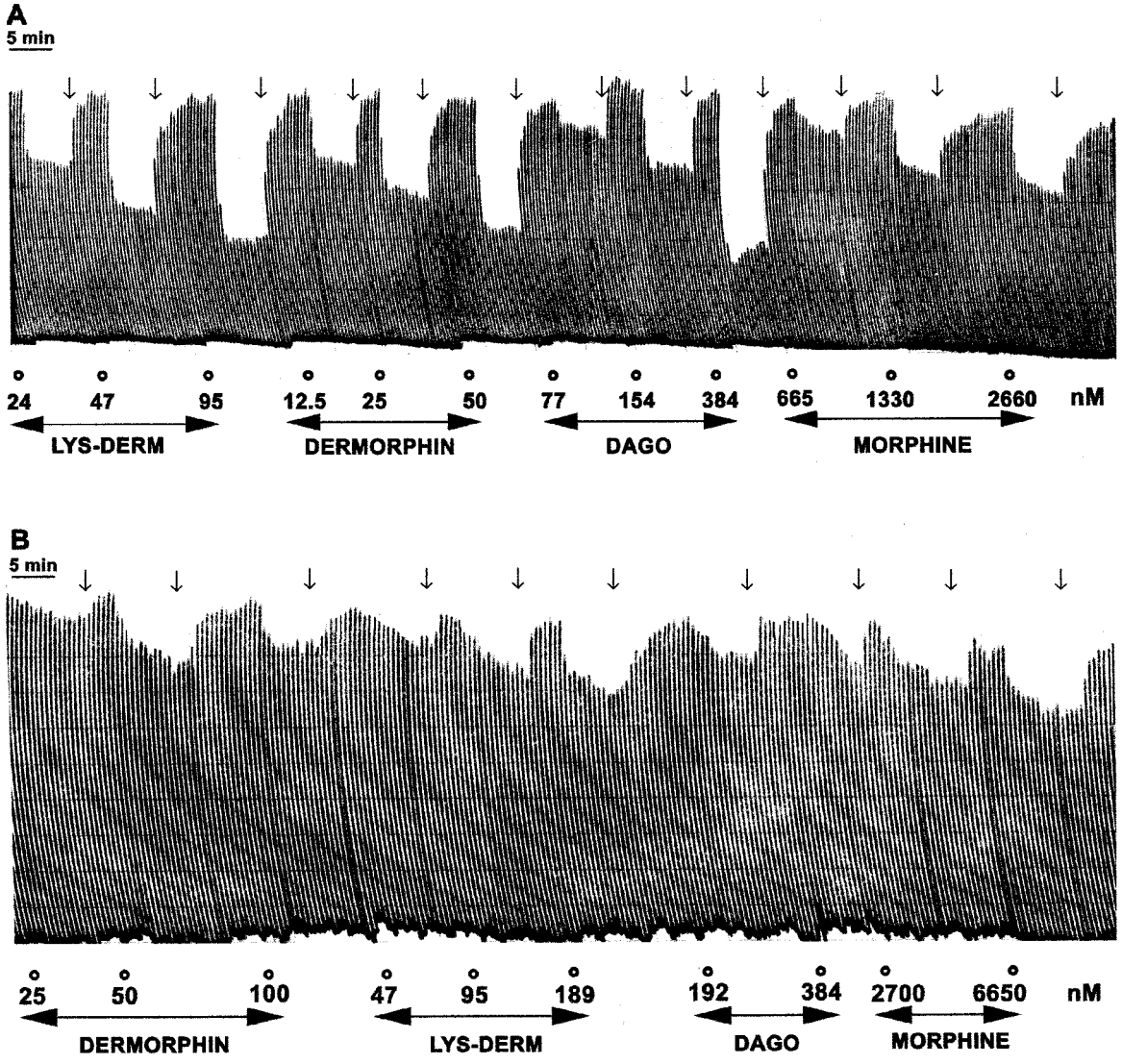

Figure 1 Representative experiment showing depression of twitch contraction induced by graded doses of mu opioid agonists in electrically stimulated vas deferens preparations from wild-type (A) and MOR-deficient mice (B). At least 10 experiments were performed for each drug in each genotype. Extensive concentration-response curves were performed in all the experiments in order to allow calculation of IC_{50} values. Compounds were added to the 10 ml bath in a volume of 50 μl (circles) and washed away after 6 min contact (arrows). Numbers refer to drug concentration in nM.

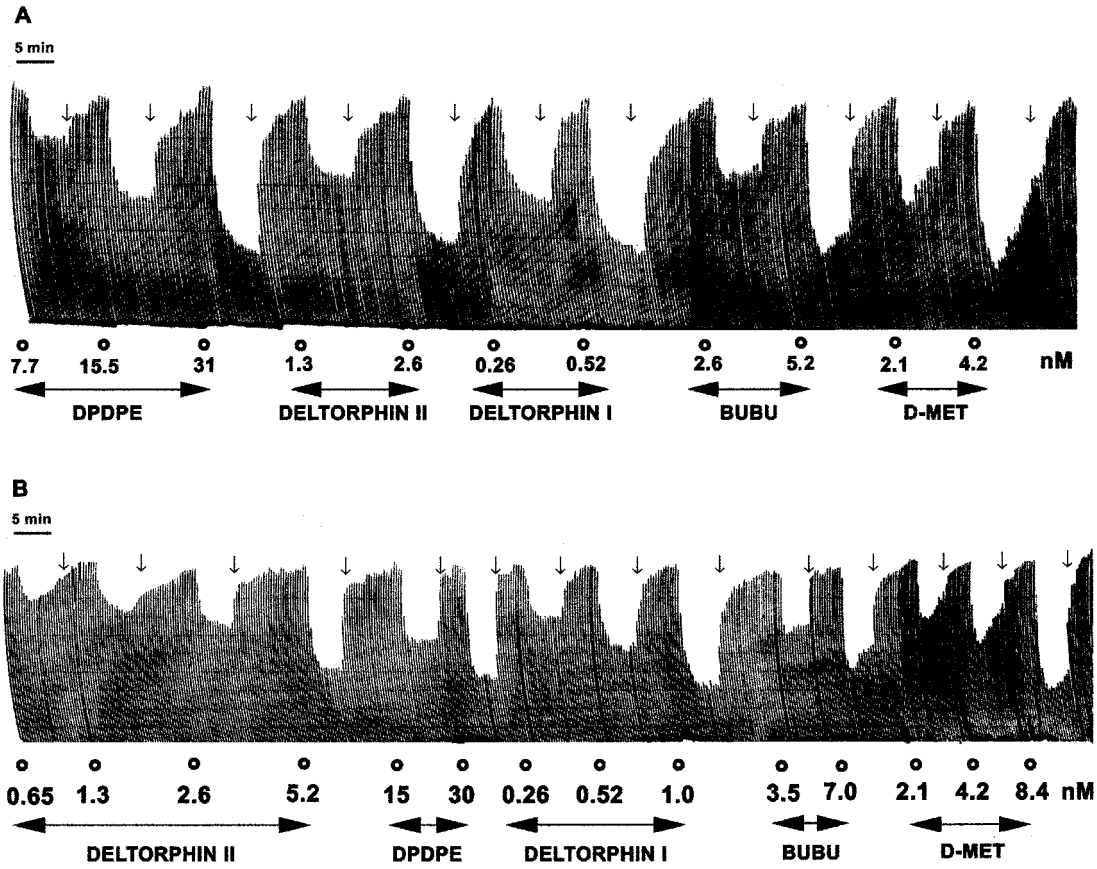

Figure 2 Representative experiment showing effects of delta opioid agonists in electrically stimulated vas deferens preparations from wild-type (A) and MOR-deficient mice (B). At least 10 experiments were performed for each drug in each genotype. Extensive concentration-response curves were performed in all the experiments in order to allow calculation of IC_{50} values. Compounds were added to the 10 ml bath in a volume of 50 μl (circles) and washed away after 6 min contact (arrows). Numbers refer to drug concentration in nM. The shape of the response evoked by the enkephalins, DPDPE and BUBU is different from that evoked by deltorphin I and deltorphin II, i.e., promptness in twitch depression and in recovery upon wash. [D-Met^2]-deltorphin (D-MET) begins to recover before washing probably due to a rapid enzymatic degradation.

Blockade by naltrindole of the effects produced by delta-opioid agonists

The selective involvement of delta-opioid receptors in the responses produced by delta selective agonists was evaluated by using naltrindole, a selective delta-opioid antagonist. Naltrindole administration (45 nM) has no intrinsic effect on muscle contraction in vas deferens preparations from wild-type and mutant mice. The inhibitory effects produced by DPDPE (15 nM), deltorphin I (0.4 nM), deltorphin II (2.5 nM), [D-Met^2]-deltorphin (3.1 nM) and BUBU (3.5 nM) in preparations from wild-type mice were completely prevented after the administration of naltrindole. Indeed, the baseline responses of these vas deferens preparations were not modified when the different delta-opioid agonists were administered after naltrindole exposure. A similar blockade of the inhibitory responses induced by DPDPE (30 nM), deltorphin I (0.7 nM), deltorphin II (6 nM), [D-Met^2]-deltorphin (7 nM) and BUBU (6 nM) was also observed after the administration of naltrindole in preparations from MOR-deficient mice (Figure 3).

Discussion

The biological activities of several agonists of mu- and delta-opioid receptors have been evaluated in mouse vas deferens preparations from wild-type and MOR-deficient mice. As expected, the different mu-opioid agonists, dermorphin, [Lys^7]-dermorphin, DAMGO and morphine produced inhibitory responses in preparations from wild-type mice. Only a negligible response was observed in preparations from MOR-deficient mice when dermorphin, [Lys^7]-dermorphin and morphine were administered at very high concentrations. The different delta-opioid agonists DPDPE, BUBU, deltorphin I, deltorphin II and [D-Met^2]-deltorphin showed biological activities in preparations from both genotypes. However, the IC_{50} values for all the delta agonists were higher in MOR-deficient mice, and [D-Met^2]-deltorphin and deltorphin II showed a significantly lower activity in preparations from MOR-deficient mice. The inhibitory responses of DPDPE, BUBU, deltorphin I, deltorphin II and [D-Met^2]-deltorphin in preparations from both wild-type and MOR-deficient mice were prevented by

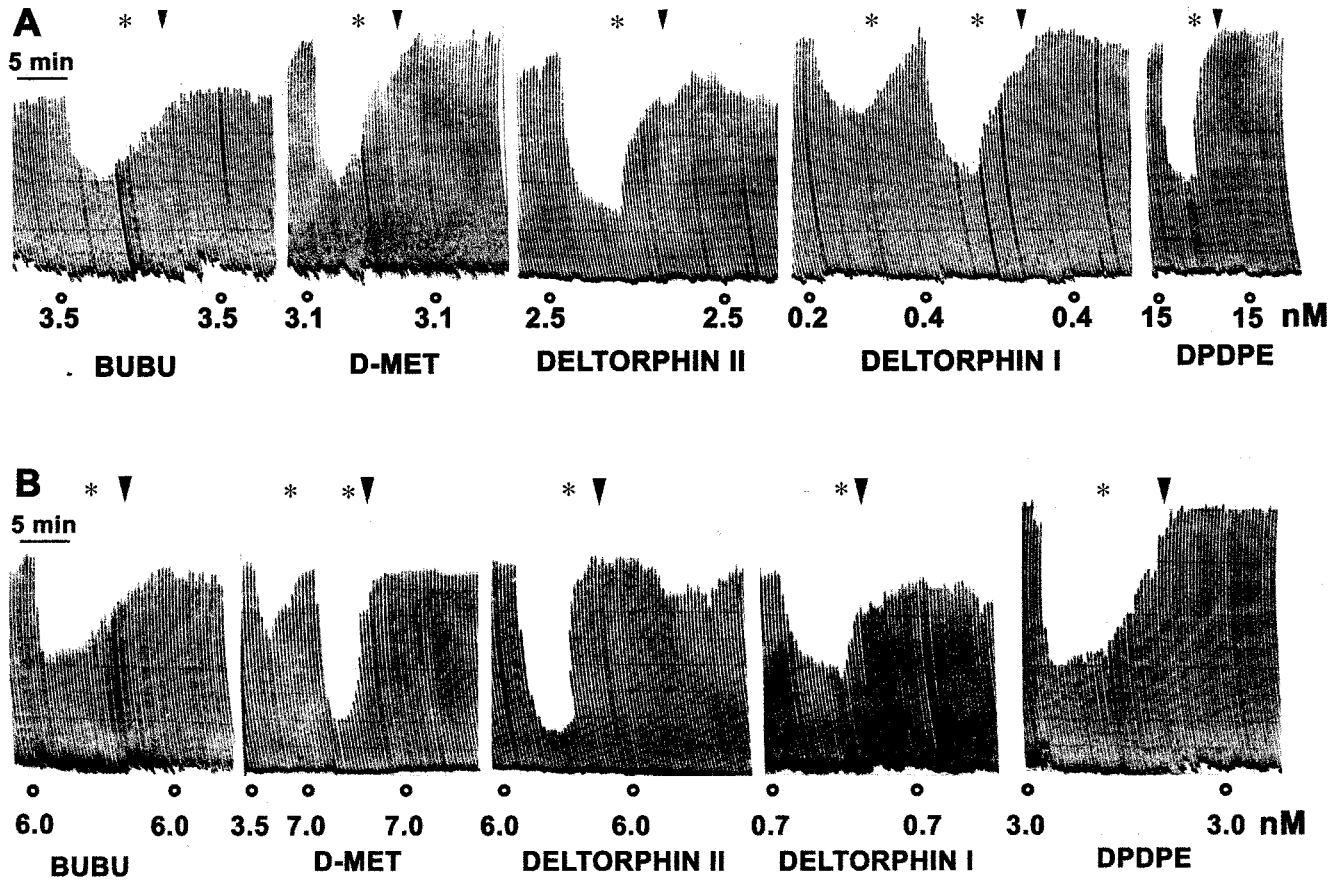

Figure 3 Representative experiment showing antagonism by naltrindole of the effects produced by delta opioid agonists in electrically stimulated vas deferens preparations from wild-type (A) and MOR-deficient mice (B). At least six experiments were performed for each drug in each genotype. Circles and arrows indicate the addition in the bath of delta-opioid agonists and naltrindole (45 nM) respectively. Stars indicate washing. Numbers refer to drug concentration in nM.

the administration of naltrindole. This result reveals a specific involvement of delta-opioid receptor activation in the biological activities of these selective delta-opioid agonists in both genotypes. A similar activity profile to that found with mu- and delta-opioid agonists in preparations from wild-type mice (1.1 hybrids from 129/SV and C57Bl/6) was previously reported in Albino Swiss mice (Melchiorri *et al*., 1991). However, the biological activities of these opioid agonists in preparations from wild-type mice was less intense than those obtained in Albino Swiss mice: the IC_{50} values for the different compounds were from two (most of the delta-opioid agonists) to three times (most of the mu-opioid agonists) higher in this study. In agreement with these results, strain differences in the sensitivity of mouse vas deferens preparations to the activity of opioid agonists have been previously reported. Thus, a reduced response of morphine, but not of enkephalins was reported in vas deferens from C57/BL strain mice (Berti *et al*., 1978) in comparison with Albino Swiss mice.

Mouse vas deferens preparations mainly contain delta-opioid receptors but are sensitive to the biological effects induced by both mu- and delta-opioid agonists (Smith & Leslie, 1993). Binding studies have reported a high selectivity ratio for mu- vs delta-opioid receptors of DAMGO (ratio of 603), dermorphin (ratio of 845) (Negri *et al*., 1998) and [Lys^7]-dermorphin (ratio of 12,277) (Negri *et al*., 1992) in membranes from rat brain homogenates. In agreement with this high selectivity, the present findings indicate that the biological effects of these three mu-opioid agonists in the mouse vas deferens preparations are exclusively due to the activation of mu-opioid receptors. Interestingly, the biological activity of the less selective mu-opioid agonist morphine (ratio of 50 in membranes from guinea-pig brain homogenates) (Corbett *et al*., 1993) was also abolished in preparations from MOR-deficient mice. Therefore, this peripheral response of morphine seems to be exclusively mediated by the activation of mu-opioid receptors. This finding is in agreement with previous reports showing that other centrally mediated effects of morphine are also completely abolished in MOR-deficient mice (Matthes *et al*., 1996; Sora *et al*., 1997b), and provides new evidence for the crucial role of mu-opioid receptors in the pharmacological responses of morphine. When administered at very high doses, the mu-opioid agonists morphine, dermorphin and [Lys^7]-dermorphin produced a negligible and non dose-dependent twitch inhibition in preparations from MOR-deficient mice. Moreover, this small response showed no relationship with the respective selectivity of these agonists for MOR. Therefore, the effects induced by

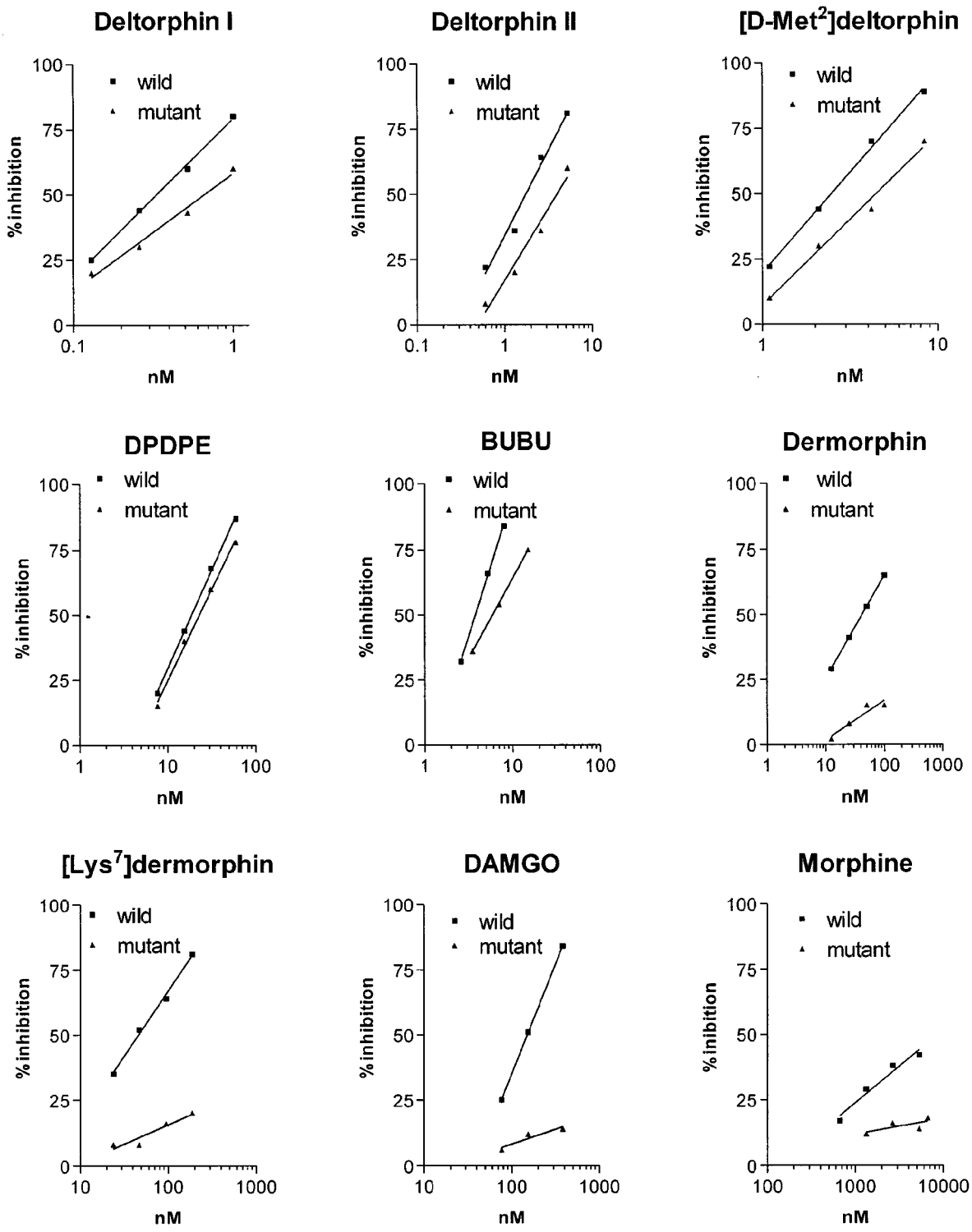

Figure 4 Concentration-response curves for delta- (deltorphin I, deltorphin II, [D-Met^2]-deltorphin, DPDPE and BUBU) and mu- (dermorphin, [Lys^7]-dermorphin, DAMGO and morphine) opioid agonists in electrically stimulated vas deferens preparations from wild-type and mutant mice. At least 10 experiments were performed for each drug in each genotype. Values represent percentage of twitch inhibition. Comparisons of the curves of each delta-opioid agonist in the two genotypes indicate that deltorphin II ($P<0.05$) and [D-Met^2]-deltorphin ($P<0.0001$) are significantly more potent in wild-type than in MOR-deficient mouse preparations.

mu-opioid agonists in MOR-deficient mice seem to be due to a non specific response related to the high concentrations used in these experiments.

High selectivity ratios for delta- vs mu-opioid receptors have been reported by previous binding studies using DPDPE (ratio of 116), deltorphin I (ratio of 14,318), deltorphin II

(ratio of 8448), [D-Met^2]-deltorphin (ratio of 1,358) (Melchiorri *et al*., 1991) and BUBU (ratio of 332) (Gacel *et al*., 1988) in membranes from rat brain homogenates. These delta-opioid selective agonists induced a biological activity in vas deferens from mice deficient in the MOR gene, but a slight decrease in their IC_{50} values was observed in these preparations. The relative potencies of delta-opioid selective agonists in mouse vas deferens preparations have been reported to correlate well with those for inhibition of radioligand binding to delta-sites in the brain (Lord *et al*., 1977; Leslie *et al*., 1980). In agreement, deltorphin I has the most elevated selectivity and affinity for delta-opioid receptors (Melchiorri *et al*., 1991) and showed in this study the highest potency in preparations from both wild-type and MOR-deficient mice. On the contrary, the delta compound displaying the lowest selectivity ratio for delta-opioid receptors, DPDPE also had the lowest potency in vas deferens from both genotypes. The different compounds used in this study are highly selective for delta-opioid receptors and there was no correlation between the specific selectivity of each delta-opioid agonist and the loss of biological activity in MOR-deficient mice. Therefore, the decreased response of delta agonists in vas deferens from mutants lacking mu-opioid receptors does not seem to be due to a cross reactivity with these mu-opioid receptors in preparations from wild-type mice. Accordingly, the biological responses of the highly selective delta-opioid agonists [D-Met^2]-deltorphin and deltorphin II were significantly reduced in preparations from MOR-deficient mice, whereas the responses of other less selective delta agonists, such as DPDPE and BUBU, were similar in vas deferens from both genotypes. Functional interactions between mu- and delta-opioid receptors have been reported for several biochemical and pharmacological responses (Vaught *et al*., 1982; Schoffelmeer *et al*., 1990; Rothman *et al*., 1993; Traynor & Elliot, 1993). These interactions could also occur on vas deferens tissue and would explain the attenuation of the responses of delta-opioid agonists in MOR-deficient mice.

Mouse vas deferens preparations also provide information on the efficacy of proteolytic inactivation, as deduced from the shape of the twitch depression curve (Erspamer & Severini, 1992). The biological effects of [D-Met^2]-deltorphin, but not of other opioid compounds, begins to disappear before washing vas deferens preparations, which was probably due to a rapid enzymatic degradation of the peptide. Some active metabolites of [D-Met^2]-deltorphin, such as penta- and tetra-C terminal fragments, lose their selectivity for delta-opioid receptors (Sagan *et al*., 1989; Melchiorri *et al*., 1991), and may also bind at mu-opioid receptor. Therefore, we cannot completely exclude a possible involvement of mu-opioid receptors in the responses induced by [D-Met^2]-deltorphin in preparations from wild-type mice.

The decreased biological activity of the different delta-opioid agonists in peripheral tissue preparations from MOR-deficient mice is in agreement with previous studies indicating that some centrally mediated effects of delta-opioid agonists are modified in these knockout animals. Thus, the effects of deltorphin II in respiratory frequency were abolished in mice lacking mu-opioid receptors (Matthes *et al*., 1998). The antinociceptive responses produced by DPDPE (Sora *et al*., 1997a) and SNC 80 (Sora *et al*., 1999) were also abolished in MOR-deficient mice. Other studies have reported that the antinociceptive effects of DPDPE (Loh *et al*., 1998) and deltorphin II (Matthes *et al*., 1998) as well as [^{35}S]-GTPγS stimulation elicited by these delta compounds (Matthes *et al*., 1998; Narita *et al*., 1999) were preserved in MOR-knockout mice. Therefore, it has been suggested that under some circumstances a synergistic interaction between mu- and delta-opioid receptors could take place between distant receptors located in separate neurones, or arise from receptor cross-talk at the cellular level. All the present data show that a similar interaction may also occur in the mouse vas deferens preparations.

In summary, the present findings revealed that delta-opioid agonists, but not mu-opioid agonists, are biologically active in vas deferens from mice lacking mu-opioid receptors. The decreased responses of delta-opioid compounds in mutant mice suggest that some cooperativity exists between mu- and delta-opioid receptors in these isolated vas deferens preparations, and support the hypothesis of synergistic interactions between these two opioid receptors. This experimental model represents a useful assay to evaluate the selectivity of opioid agonist compounds for mu-opioid receptors.

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