British Journal of Pharmacology (2005) 144, 875–884
www.nature.com/bjp

Synergistic and additive interactions of the cannabinoid agonist CP55,940 with μ opioid receptor and α_2-adrenoceptor agonists in acute pain models in mice

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1 Cannabinoid receptor agonists elicit analgesic effects in acute and chronic pain states *via* spinal and supraspinal pathways. We investigated whether the combination of a cannabinoid agonist with other classes of antinociceptive drugs exerted supra-additive (synergistic) or additive effects in acute pain models in mice.

2 The interactions between the cannabinoid agonist CP55,940, α_2-adrenoceptor agonist dexmedetomidine and μ-opioid receptor agonist morphine were evaluated by isobolographic analysis of antinociception in hot plate (55°C) and tail flick assays in conscious male Swiss mice. Drug interactions were examined by administering fixed-ratio combinations of agonists (s.c.) in 1 : 1, 3 : 1 and 1 : 3 ratios of their respective ED_{50} fractions.

3 CP55,940, dexmedetomidine and morphine all caused dose-dependent antinociception. In the hot plate and tail flick assays, ED_{50} values (mg kg^{-1}) were CP55,940 1.13 and 0.51, dexmedetomidine 0.066 and 0.023, and morphine 29.4 and 11.3, respectively. Synergistic interactions existed between CP55,940 and dexmedetomidine in the hot plate assay, and CP55,940 and morphine in both assays. Additive interactions were found for CP55,940 and dexmedetomidine in the tail flick assay, and dexmedetomidine and morphine in both assays.

4 Thus, an α_2-adrenoceptor agonist or μ opioid receptor agonist when combined with a cannabinoid receptor agonist showed significant synergy in antinociception in the hot plate test. However, for the tail flick nociceptive response to heat, only cannabinoid and μ opioid receptor antinociceptive synergy was demonstrated. If these results translate to humans, then prudent selection of dose and receptor-specific agonists may allow an improved therapeutic separation from unwanted side effects.
British Journal of Pharmacology (2005) **144,** 875–884. doi:10.1038/sj.bjp.0706045
Published online 13 December 2004

Keywords: α_2-Adrenoceptor; antinociception; cannabinoid receptor; CP55,940; dexmedetomidine; hot plate; isobolographic analysis; morphine; synergy; tail flick

Abbreviations: Δ^9-THC, Δ^9-tetrahydrocannabinol; MPE, maximum possible effect; Z_{add}, theoretical doses of a purely additive interaction for an ED_{50} effect; Z_{mix}, experimental doses of drug combination for an ED_{50} effect

Introduction

Marijuana (*Cannabis sativa*) has been used for medicinal purposes through history. Its main psychoactive compound Δ^9-tetrahydrocannabinol (Δ^9-THC) causes a variety of effects in different animal species, such as antinociception, hypoactivity, catalepsy, hypothermia and cardiovascular changes (Chaperon & Thiebot, 1999; Randall *et al*., 2004). There has been much interest in Δ^9-THC and its synthetic derivatives (e.g. CP55,940) due to their antinociceptive effects and ability to increase the potency of other analgesic drugs, such as μ opioid and α_2-adrenoceptor agonists. The identification and cloning of the two cannabinoid receptors, CB_1 and CB_2, in the 1990s and the subsequent synthesis of selective receptor ligands have greatly elucidated many aspects of cannabinoid pharmacology and the endocannabinoid system (Pertwee, 2000; 2001). CB_1 receptors are present only in low levels in the hypothalamus and almost absent in the respiratory region of the brainstem (Howlett *et al*., 2002). This correlates with the absence of respiratory depression and low mortality rates associated with cannabis overdose. Thus, the antinociceptive effects of cannabinoid receptor agonists may offer clinical therapeutic advantages. CP55,940, a bicyclic nonclassical cannabinoid, is a full agonist at both CB_1 and CB_2 receptors and 10–50 times more potent than Δ^9-THC (Pertwee, 2000; Howlett *et al*., 2002).

The CB_1, α_2-adrenoceptor and μ opioid receptors are all seven-transmembrane $G_{i/o}$ protein-coupled receptors sharing signal transduction pathways, such as inhibiting adenyl cyclase and modulating K^+ and Ca^{2+} channel activity (Welch *et al*., 1995a; Khan *et al*., 1999; Manzanares *et al*., 1999; Morisset *et al*., 2001; Przewlocki & Przewlocka, 2001; Howlett *et al*., 2002). These receptors are also similarly distributed in the periaqueductal gray and substantia gelatinosa, areas in the CNS highly implicated in antinociception (Yaksh, 1985; Behbehani, 1995; Lichtman *et al*., 1996; Martin & Lichtman, 1998; Furst, 1999; Khan *et al*., 1999; Pertwee, 2001). Both supraspinal and spinal pathways are involved in α_2-adrenoceptor-mediated analgesia. The spinal antinociceptive actions

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Published online 13 December 2004

of α_2-adrenoceptors are mediated mainly by the α_{2A}-subtype (Jones *et al*., 1982; Khan *et al*., 1999; Kamibayashi & Maze, 2000; Malmberg *et al*., 2001), although α_{2C}-adrenoceptors also contribute (Fairbanks *et al*., 2002). Dexmedetomidine is a selective α_2-adrenoceptor agonist currently approved for clinical use under intensive care conditions (Khan *et al*., 1999; Coursin & Maccioli, 2001).

Due to these similarities between the cannabinoid, opioid and adrenergic systems, it is likely that one or more combinations of selective agonists for each system will interact and possibly elicit supra-additive (or synergistic) antinociceptive effects. The aim of this work was to investigate the antinociceptive activity of combinations of doses (based on equieffective ED_{50} fractions) of CP55,940 with morphine or with dexmedetomidine using acute pain models in conscious mice. In addition, the combination of dexmedetomidine and morphine was tested. The effects of the combined drug treatments were tested by isobolographic analysis to determine if interactions were synergistic or merely additive, as described by Tallarida (2001). An abstract of some of this work has been published (Tham *et al*., 2002).

Methods

Animals

This study was approved by the University of Melbourne Animal Ethics Experimentation Committee in accordance with the guidelines of the National Health and Medical Research Council of Australia. Swiss male mice weighing 22–25 g at the start of the study were housed in groups of four with *ad libitum* access to food and water, a 12 h light–dark cycle and temperature of $21 \pm 1^{\circ}C$. Prior to the start of each experiment, the animals were given at least 15 min to acclimatise to the laboratory conditions (temperature $21 \pm 1^{\circ}C$). Each animal was used for a maximum of four experiments, with each experiment using a different dosing regimen. A recovery period of at least 7 days was allowed between experiments. The animals were euthanised upon the completion of the last experiment with an overdose of pentobarbitone (approx. 160 mg kg^{-1} i.p.).

Agonists and protocols

CP55,940 ((−)-*cis* 3-(2-hydroxy-4-(1,1-dimethylheptyl)phenyl)-*trans*-4-(3-hydroxypropyl) cyclohexanol; Tocris Cookson Ltd, Bristol, U.K.) dissolved in 1 part ethanol, 1 part cremaphor and 18 parts 0.9% saline (vehicle 1 : 1 : 18) was administered s.c. with doses ranging from 0.3 to 3.0 mg kg^{-1}. The α_2-adrenoceptor agonist dexmedetomidine hydrochloride (IDC Abbott Laboratories Ltd, Queenborough, Kent, U.K.) and the μ opioid agonist morphine sulphate (David Bull Laboratories, Melbourne, Australia) dissolved in saline were administered s.c. with doses in the range of 3–100 μg kg^{-1} and 3–30 mg kg^{-1}, respectively. In separate groups of mice, either saline or 1 : 1 : 18 vehicle was administered (2.5 ml kg^{-1} s.c.).

Each drug was administered separately generating dose–response curves with their respective ED_{50}. Combinations of CP55,940 with dexmedetomidine, CP55,940 with morphine and dexmedetomidine with morphine were then simultaneously administered s.c. in a fixed 1 : 1 ED_{50} ratio of fractions (1.0, 0.5, 0.25, 0.125 and 0.0625) of their respective agonist ED_{50} values. The combination of CP55,940 and dexmedetomidine was analysed further by administering the drugs in 1 : 3 and 3 : 1 ED_{50} ratios, and doses were calculated such that they added to the same effect level as expected above. The resulting data were used to construct combination dose–response curves, which were then subject to 'fixed ratio design' isobolographic analysis. This was to determine whether an interaction was additive or otherwise, assuming the agonists worked through 'similar independent action' (Tallarida, 2000; 2001). Theoretical values for a purely additive interaction were compared to the actual experimental dose-pairs required for the same effect. A simple additive interaction occurred when a known combination of agonist doses caused a mathematically predictable effect, with the knowledge of the individual agonist potencies. Synergism or supra-additivity described the result when the same agonist combinations caused an exaggerated effect, that is, doses less than those predicted by additivity were needed to cause a normal effect (Tallarida, 2000; 2001).

Nociceptive testing

Hot plate assay Each mouse was placed unrestrained on a hot plate (Ugo Basile model 7280, Comerio, Italy; $55 \pm 0.5^{\circ}C$) for the baseline measurement just prior to drug administration. Measurements were then taken 15, 30, 60, 90 and 120 min after drug administration. Two tests were performed 2 min apart and averaged for a measurement at each time. The end point for each test occurred when the animal displayed the characteristic physical reactions to noxious thermal stimuli, that is, paw-licking or jumping (Le Bars *et al*., 2001), upon which the mouse was instantly removed and returned to its home cage. Baseline latencies were usually within the range of 7–10 s. A maximum cutoff time of 50 s was set to prevent tissue damage.

Tail flick assay A modified version of the rat tail flick assay (D'amour & Smith, 1941) was used in mice to elicit a spinal tail flick response to noxious thermal stimuli. Each mouse was restrained in a 50 ml plastic tube generously ventilated with large holes. This unit was placed on a plantar test apparatus (Ugo Basile model 7371; infrared setting 90) modified for tail flick experiments. Measurements were taken as for the hot plate assay. Baseline latencies were between 4 and 6 s, and a 12 s cutoff time was set to prevent tissue damage (Le Bars *et al*., 2001). The movable heat source was placed directly under the tail and when activated, it caused a distinctive tail flick reflex, which stopped an automated timer (based on infrared reflection). Different points on the tail were used to prevent localised tissue damage, and results were averaged. Each mouse was acclimatised to the restraining tube for approximately 30 min on the day prior to its first experiment. Subsequently, it was restrained for approximately 10 min for acclimatisation prior to each measurement, and for no more than 30 min at a time.

Statistical analyses

For each test time, the percentage maximum possible effect (%MPE) was calculated as (test latency−baseline latency) $\times$ (cutoff time−baseline latency)$^{-1} \times 100\%$ (Harris & Pierson, 1964). %MPE values at the time point at which the greatest antinociceptive responses were observed for each respective

agonist (60 min for CP55,940 and morphine; 30 min for dexmedetomidine; see Figure 1) were used to construct dose–response curves, which included 4–5 doses. The ED_{50} (i.e. the dose that caused 50% of maximum antinociception) and associated 95% confidence intervals were generated from standard nonlinear regression analysis of the log dose–response curve (Prism 4.0, Graphpad Software, San Diego, CA, U.S.A.).

Agonist combinations were analysed for additive interactions using 'fixed ratio design' isobolograms whereby combinations of two drugs in known ratios were administered as fractions of their respective ED_{50}, as outlined above (Horvath *et al*., 1992; Pöch, 1993; Tallarida, 2000; 2001; Miranda *et al*., 2002). The isobologram consists of an additivity line that connects $ED_{50,\ Drug\ A}$ on the vertical axis to $ED_{50,\ Drug\ B}$ on the horizontal axis. The theoretical dose required for a purely additive interaction ($Z_{add} = (f)ED_{50,\ Drug\ A} + (1-f)ED_{50,\ Drug\ B}$, where f is the fraction of drug A used) was calculated and compared *via* a modified version of the Student's t-test to the actual dose (Z_{mix}, determined from the ED_{50} of the combination dose–response curve) required to achieve the same effect experimentally (Tallarida, 2000). The variance for Z_{add} was calculated as $Var(Z_{add}) = (f)^2Var(ED_{50,\ Drug\ A}) + (1-f)^2Var(ED_{50,\ Drug\ B})$ and 95% confidence intervals were derived from these variances based on the proportions of the individual drugs. Confidence intervals for Z_{mix} were generated with Prism.

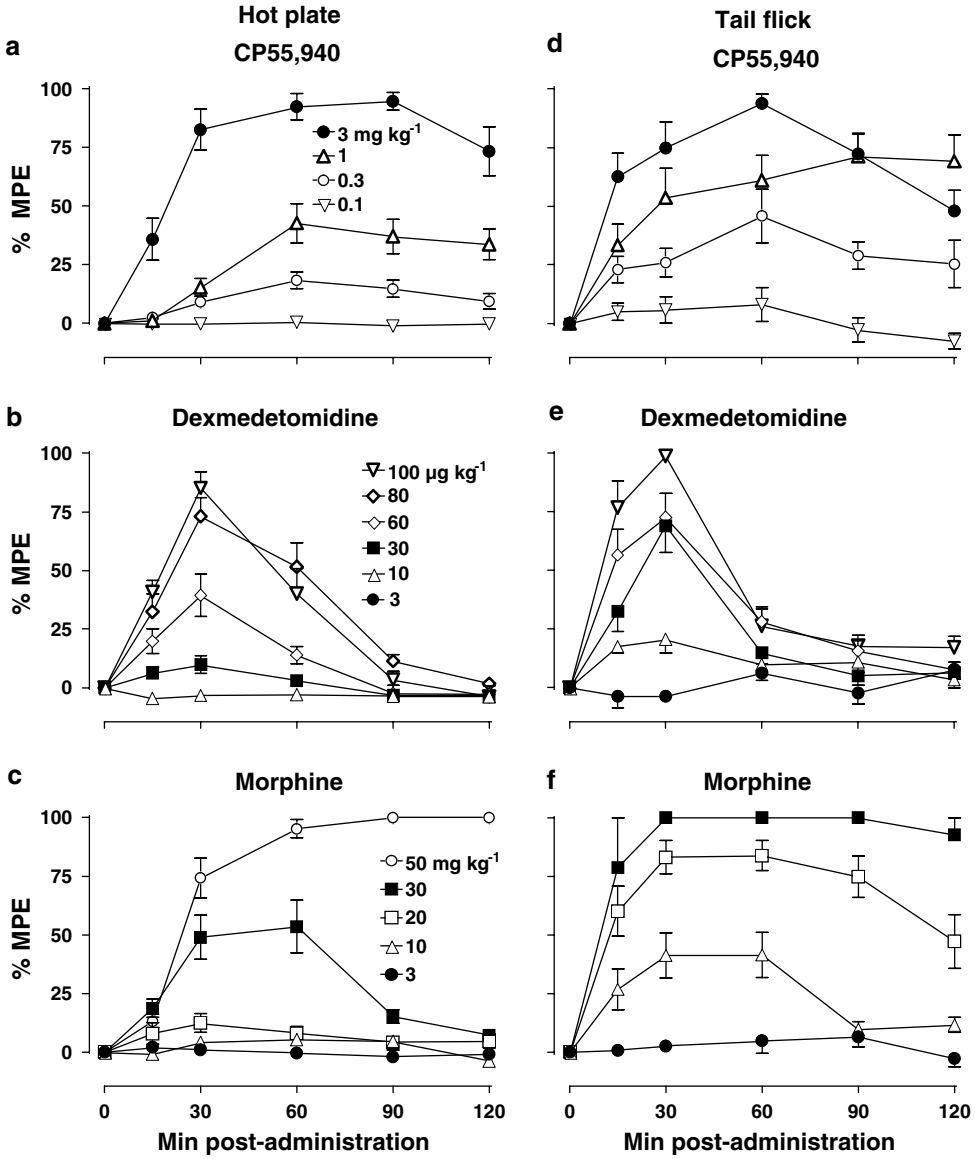

Figure 1 Dose- and time-dependent antinociceptive effects of single s.c. bolus administration of CP55,940 (0.1–3 mg kg^{-1}; top panels), dexmedetomidine (3–100 μg kg^{-1}; middle panels) and morphine (3–50 mg kg^{-1}; bottom panels) in the hot plate (panels a–c) and tail flick (d–f) assays in conscious mice. Responses are expressed as %MPE (see Methods) from 0 min (bolus injection) to 120 min postadministration; symbols are mean ± s.e.m. For each agonist dose, n values in the hot plate and tail flick assays, respectively, were as follows: CP55,940 $n = 8$–16 and 7–11; dexmedetomidine $n = 10$–12 and 6–12; and morphine $n = 6$–8 and 6–12. %MPE values at the time point at which the greatest antinociceptive responses were observed for each respective agonist (60 min for CP55,940 and morphine; 30 min for dexmedetomidine) were used to plot the dose–response curves shown in Figure 2.

Simple additivity was termed when the combination of drugs led to a mathematically predictable effect (i.e. Z_{mix} not significantly different from Z_{add}) with the knowledge of the individual drug potencies. Synergism or supra-additivity described observations in which combinations elicited an enhanced effect, which suggested that drug quantities less than those predicted by additivity (i.e. Z_{mix} significantly less than Z_{add}) were needed for the same effect (Tallarida, 2000; 2001). Values of $P<0.05$ were considered statistically significant.

Results

Antinociceptive activity of CP55,940, dexmedetomidine and morphine

Single drug administration of CP55,940, dexmedetomidine and morphine (s.c.) to conscious male Swiss mice caused dose- and time-dependent antinociceptive effects in the hot plate and tail flick assays, with different individual potencies. The greatest antinociceptive responses for doses of CP55,940 or morphine occurred 60 min after drug administration, and at 30 min after doses of dexmedetomidine, in the hot plate (Figure 1a–c) and tail flick (Figure 1d–f) assays. The agonist log dose–response curves are displayed in Figure 2. The corresponding ED_{50} values with their associated 95% confidence intervals derived from the dose–response curves are shown in Table 1. In separate groups of mice treated with vehicle (saline or 1 : 1 : 18 s.c.), hot plate or tail flick response latencies were consistent over the 0–120 min postadministration test period, as well as between four experimental days (data not shown).

Interactions between CP55,940, dexmedetomidine and morphine

Dose- and time-dependent antinociceptive effects of the drug combinations (s.c.; 1 : 1 fixed ratio of ED_{50} of agonist A : ED_{50} of agonist B) in the hot plate and tail flick assays are shown in Figure 3. The time points at which the greatest antinociceptive effects were observed after coadministration of each combination were 60 min for CP55,940 with either dexmedetomidine or morphine (Figure 3a, b, d and e) and 30 min for dexmedetomidine with morphine (Figure 3c and f). Dose–response curves for drug combinations were then determined for the hot plate (Figure 4a) and tail flick (Figure 4b) assays. Dose fraction (an arbitrary value) ED_{50} values were determined and converted to absolute dose values for isobolographic analysis. The theoretical and experimental values of Z_{add} and Z_{mix}, respectively, in the hot plate and tail flick assays (Table 2) were tested for significance with a modified Student's *t*-test. These values and their corresponding 95% confidence intervals are displayed as isobolograms in Figure 5.

Using 1 : 1 fixed ED_{50} ratios, the combination of CP55,940 and dexmedetomidine (s.c.) resulted in a significant synergistic or supra-additive interaction ($Z_{mix}<Z_{add}$; $P<0.05$) in the hot plate assay (Figure 5a), but not in the tail flick assay (Figure 5d). CP55,940 and morphine caused significantly synergistic interactions in both the hot plate (Figure 5b) and tail flick (Figure 5e) assays. However, the dexmedetomidine and morphine combination only showed simple additive interactions in both hot plate (Figure 5c) and tail flick (Figure 5f) assays, with Z_{add} and Z_{mix} not significantly different (Table 2).

The combination of CP55,940 and dexmedetomidine administered in a 1 : 3 and 3 : 1 ED_{50} ratio gave similar results as for the 1 : 1 ratio combination. Z_{add} and Z_{mix} values were tested for significance (Table 3). Significantly synergistic interactions were observed for the hot plate assay (Figure 6a),

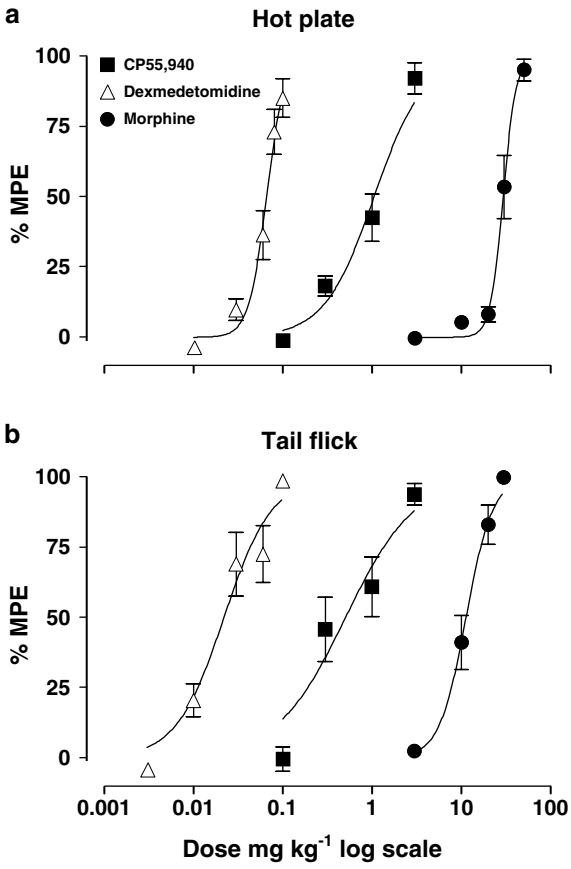

Figure 2 Agonist dose–antinociceptive response curves (single agonist administration s.c.) in the (a) hot plate and (b) tail flick assays in conscious mice. Responses are expressed as %MPE (see Methods). %MPE values at the time point at which peak antinociceptive responses were observed for each respective agonist (see Figure 1) were used to plot the curves. The total *n* values for each agonist curve in the hot plate and tail flick assays, respectively, were as follows: CP55,940 $n=50$ and 33; dexmedetomidine $n=57$ and 46; and morphine $n=34$ and 40. For each agonist dose, *n* values in the hot plate and tail flick assays, respectively, were as follows: CP55,940 $n=8$–16 and 7–11; dexmedetomidine $n=10$–12 and 6–12; and morphine $n=6$–8 and 6–12. Symbols are mean ± s.e.m.

Table 1 Potency of CP55,940, dexmedetomidine and morphine as single s.c. drug treatments in the hot plate and tail flick assays

	Hot plate assay		*Tail flick assay*	
Agonist	ED_{50} (mg kg^{-1})	n	ED_{50} (mg kg^{-1})	n
CP55,940	1.13 (0.83–1.33)	50	0.51 (0.31–0.83)	33
Dexmedetomidine	0.066 (0.060–0.072)	57	0.023 (0.016–0.030)	46
Morphine	29.4 (27.3–31.6)	34	11.3 (9.6–13.4)	40

Values represent ED_{50} (mg kg^{-1}) (with 95% confidence intervals). *n*: number of mice.

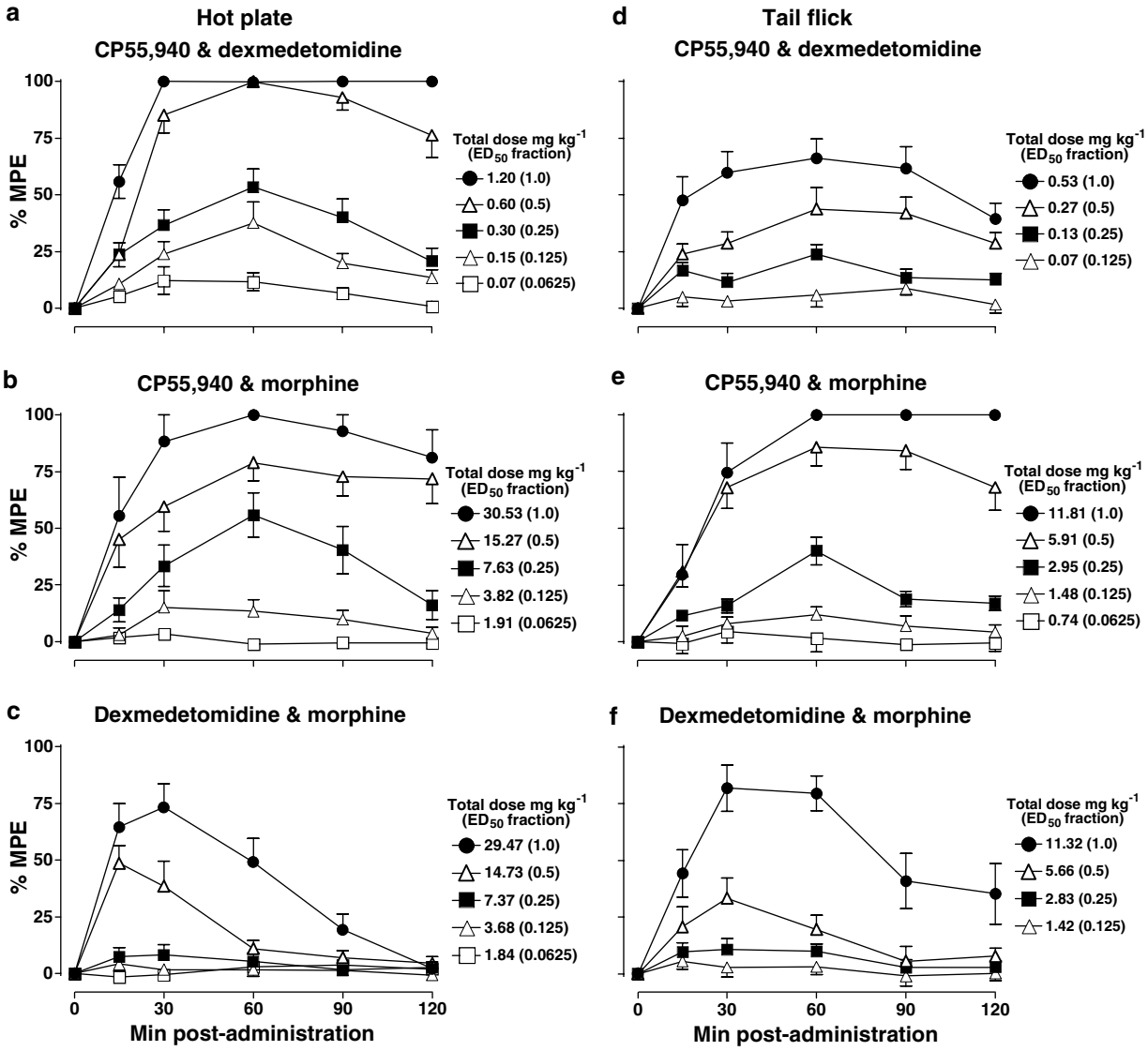

Figure 3 Dose- and time-dependent antinociceptive effects of paired combinations of CP55,940, dexmedetomidine and morphine in the hot plate (panels a–c) and tail flick (d–f) assays in conscious mice. Responses are expressed as %MPE (see Methods) from 0 min (bolus coadministration) to 120 min postadministration; symbols are mean ± s.e.m. The total dose indicated is the sum of the doses of the two drugs being used in combination (mg kg^{-1}). The combinations were of equal fractions (1.0, 0.5, 0.25, 0.125 or 0.0625) of each paired agonist's respective ED_{50} value (see Table 1) coadministered in a fixed 1 : 1 ratio of the ED_{50} of agonist A : ED_{50} of agonist B. For each curve, total n values in the hot plate and tail flick assays, respectively, were as follows: CP55,940 and dexmedetomidine $n = 52$ and 38; CP55,940 and morphine $n = 57$ and 45; and dexmedetomidine and morphine $n = 65$ and 41. For each agonist dose combination, n values in the hot plate and tail flick assays, respectively, were as follows: CP55,940 and dexmedetomidine $n = 7$–13 and 7–11; CP55,940 and morphine $n = 8$–13 and 3–12; and dexmedetomidine and morphine $n = 12$–16 and 10–11. %MPE values at the time point at which the greatest antinociceptive responses were observed for each respective combination (a, b, d, e: 60 min; c, f: 30 min) were used to plot the combination dose–response curves shown in Figure 4.

whereas additive results were obtained with the tail flick assay (Figure 6b). In Figure 7, isobolograms for both the hot plate and tail flick experiments are shown plotted on the same scale for each drug combination to allow a direct comparison of assay sensitivity.

Discussion

We found that single s.c. administration of a cannabinoid, α_2-adrenoceptor or μ opioid receptor agonist was antinociceptive when tested in either the hot plate or tail flick assays in mice. Of great interest were the findings from combinations of two drugs to test for synergy or simple additivity. Our results showed firstly that a cannabinoid agonist combined with a μ opioid agonist displayed synergy in the tail flick and hot plate assays, and secondly that a cannabinoid agonist combined with an α_2-adrenoceptor agonist showed simple additivity in the tail flick assay, but synergy in the hot plate assay. In both assays, combined α_2-adrenoceptor and μ opioid receptor activation was simply additive. These findings indicate that only some combined receptor activations are capable of synergy and that they may be specific to the spinal and supraspinal pathways involved in each type of acute pain stimulus.

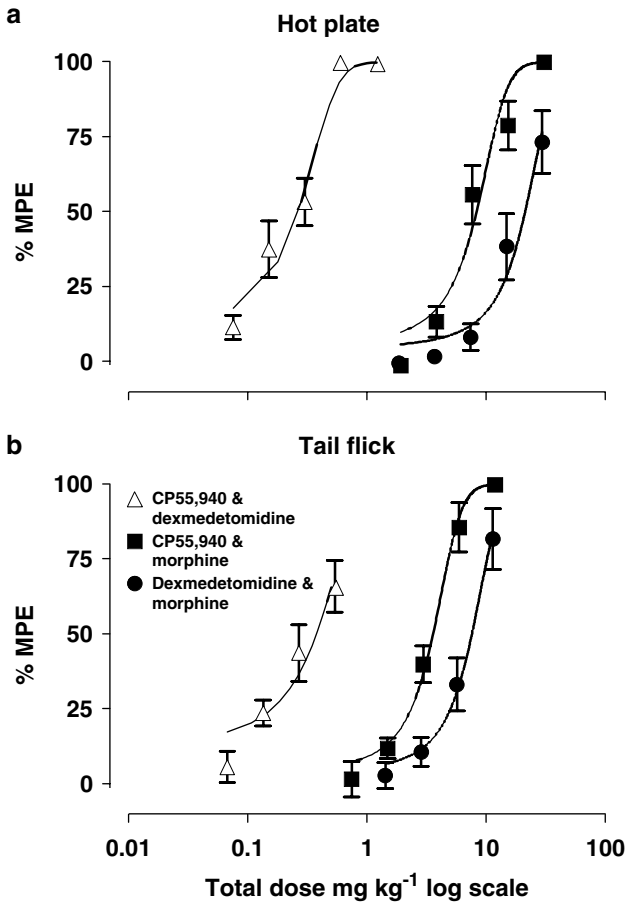

Figure 4 Agonist dose–antinociceptive response curves for paired combinations of CP55,940, dexmedetomidine and morphine (s.c.) in the (a) hot plate and (b) tail flick assays in conscious mice. Responses are expressed as %MPE (see Methods). %MPE values at the time point at which peak antinociceptive responses were observed for each respective agonist (see Figure 3) were used to plot the curves. The total dose for each agonist combination is shown on the *x*-axis. Combinations were of equal fractions (1.0, 0.5, 0.25, 0.125 or 0.0625) of each paired agonist's respective ED_{50} value (see Table 1) coadministered in a fixed 1 : 1 ratio of the ED_{50} of agonist A : ED_{50} of agonist B. For each curve, total *n* values in the hot plate and tail flick assays, respectively, were as follows: CP55,940 and dexmedetomidine $n = 52$ and 38; CP55,940 and morphine $n = 57$ and 45; and dexmedetomidine and morphine $n = 65$ and 41. For each agonist dose, *n* values in the hot plate and tail flick assays, respectively, were as follows: CP55,940 and dexmedetomidine $n = 7$–13 and 7–11; CP55,940 and morphine $n = 8$–13 and 3–12; and dexmedetomidine and morphine $n = 12$–16 and 10–11. Symbols are mean ± s.e.m.

The isobolographic analysis we used is a relatively robust method for testing drug combination *in vivo*. This technique is vulnerable to agonists with different slopes in their individual dose–response curves (Pöch, 1993). To test this possibility, we applied 1 : 1, 3 : 1 and 1 : 3 drug ratios and found consistent results of synergy for α_2-adrenoceptor and cannabinoid receptor activation in hot plate, and additivity for tail flick, assays (Figure 6). Our results therefore support the conclusion of synergy for α_2-adrenoceptor and cannabinoid agonists.

This work shows that the three drugs were consistently more potent (with lower ED_{50} values) as antinociceptive agents in the tail flick assay by three-fold for dexmedetomidine and morphine and two-fold for CP55,940. This apparent increase in sensitivity may be explained by stress-induced analgesia (Lewis *et al*., 1980) caused by the brief restraint of the conscious mice for this test. It is well documented that even brief restraint, or simply frequent handling, of rats and mice elicits a stress response typified by increased levels of endogenous opioid agonists, as well as catecholamines (Yamada & Nabeshima, 1995; Dunn, 1999; D'Arbe *et al*., 2002) and endocannabinoids (Hohmann, 2002). Further, both supraspinal and spinal mechanisms have been shown to be involved in restraint stress-induced enhancement of opioid analgesia (reviewed in Yamada & Nabeshima, 1995). Another possibility is that the choice of a 12 s cutoff time for the tail flick assay, compared to the 50 s of the hot plate test, may have led to an 'apparent' increased sensitivity due to this 'arbitrary' maximum, designed to avoid tissue injury (Le Bars *et al*., 2001). It is most unlikely, however, that the increased sensitivity to the agonists in the tail flick assay (compared with the hot plate test) contributed to the type of interactions found in this study as the cannabinoid and μ opioid agonist combination displayed synergy in both tail flick and hot plate assays, while α_2-adrenoceptor and μ opioid agonist combination consistently displayed additivity (Figure 7).

Table 2 Theoretical Z_{add} and experimental Z_{mix} ED_{50} antinociceptive values obtained from the three drug combinations (s.c.) in the hot plate and tail flick assays

	Hot plate assay			*Tail flick assay*		
Drug combination	Z_{add}	Z_{mix}	n	Z_{add}	Z_{mix}	n
CP55,940 + dexmedetomidine	0.598	0.223*	52	0.266	0.324	38
CP55,940 + morphine	15.3	7.54*	57	5.91	3.31*	45
Dexmedetomidine + morphine	14.7	16.7	65	5.66	6.97	41

n: number of mice. Each combination was a 1 : 1 fixed ratio of the respective ED_{50} of drug A : ED_{50} of drug B. Z_{add}: theoretical dose required for a purely additive interaction; Z_{mix}: experimental value determined from the combination dose–response curve (see Methods). *Statistically significant synergistic or supra-additive interaction ($P < 0.05$).

The mechanism(s) of the synergistic interactions cannot be established by this study. The possible mechanisms include (i) receptor colocation with amplified signal transduction; (ii) pre- and postjunctional stimulation or inhibition; or (iii) receptor stimulation by endogenous opioid, α_2-adrenoceptor or cannabinoid ligands. The synergistic interaction between opioid and cannabinoid agonists has not been completely elucidated although two possible mechanisms have been postulated (Welch *et al*., 1995b; Manzanares *et al*., 1999). The first proposed mechanism is that of signal transduction interactions. Opioid and cannabinoid receptors are both $G_{i/o}$ protein-coupled receptors, with similar intracellular signalling mechanisms (Welch *et al*., 1995b; Manzanares *et al*., 1999; Pertwee, 2001; Przewlocki & Przewlocka, 2001). The assumption is that both opioid and cannabinoid receptors coexist on neurones and therefore share a common pool of G proteins. The coupling of these receptors to a common G protein family may lead to inter-receptor signalling whereby activation of one receptor causes redistribution of its G proteins, which increase the sensitivity of the other receptor (Djellas *et al*., 2000). Another possibility is that cannabinoid agonists induce synthesis and release of endogenous opioid agonists

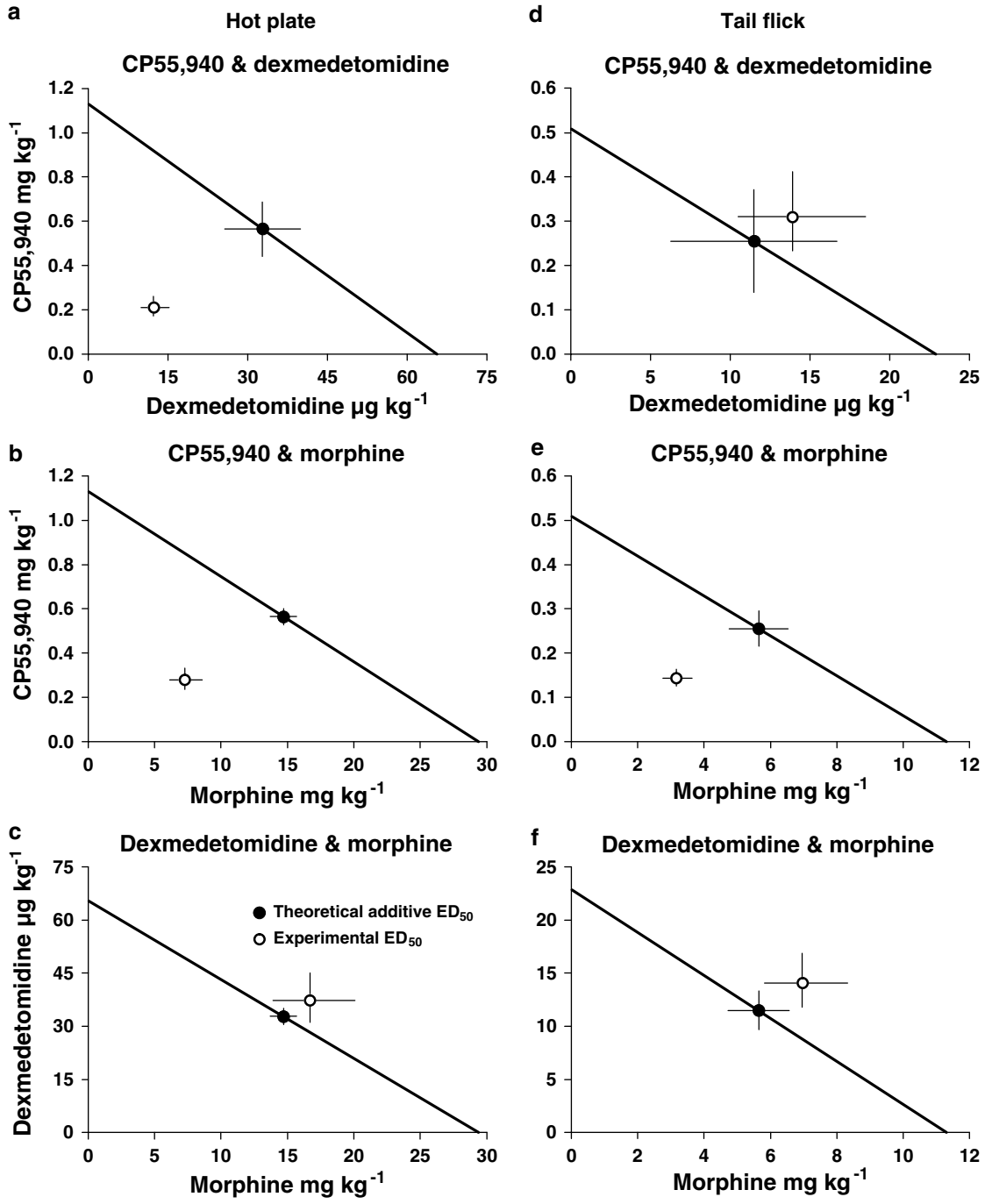

Figure 5 Isobolograms for the effects of simultaneous s.c. administration of drug combinations (in 1 : 1 fixed ratio of ED_{50} of drug A : ED_{50} of drug B) in the hot plate (panels a–c) and tail flick (d–f) assays in conscious mice. The line in each panel connects the ED_{50} of drug A on the *x*-axis and the ED_{50} of drug B on the *y*-axis and represents the locus of points of dose combinations for purely additive interactions. Symbols represent theoretical additive and experimental ED_{50} values, with their associated 95% confidence intervals. The drug combinations (and interactions) were as follows: (a, d) CP55,940 and dexmedetomidine (synergy – hot plate; additive – tail flick); (b, e) CP55,940 and morphine (synergy – both assays); and (c, f) dexmedetomidine and morphine (additive – both assays).

leading to potentiation of opioid antinociception (Cichewicz & McCarthy, 2003); the spinal dynorphin system and other opioid peptides have been implicated (Manzanares *et al*., 1999; Houser *et al*., 2000). In the rat, CP55,940 (i.t.) has been shown to cause release of dynorphin B, which contributes to its antinociceptive actions (Pugh *et al*., 1997). However, the same study found that neither CP55,940 nor dynorphin B itself potentiated the effects of coadministered morphine in the spinal cord of mice (Pugh *et al*., 1997).

CP55,940 and dexmedetomidine interact in a synergistic fashion in the more complex (compared with the tail flick response) supraspinally controlled behavioural response to the hot plate noxious stimulus. Such an interaction may be due to the similarities between the two systems. For instance, both

Table 3 Theoretical Z_{add} and experimental Z_{mix} ED_{50} antinociceptive values for CP55,940 and dexmedetomidine in 1 : 1, 3 : 1 and 1 : 3 ratios (s.c.) in the hot plate and tail flick assays

CP55,940 : dexmedetomidine Fixed ED_{50} ratio	*Hot plate assay*			*Tail flick assay*		
	Z_{add}	Z_{mix}	n	Z_{add}	Z_{mix}	n
1 : 1	0.598	0.223*	52	0.266	0.324	38
3 : 1	0.86	0.33*	47	0.39	0.48	48
1 : 3	0.33	0.11*	48	0.15	0.19	53

n: number of mice. Each combination was a fixed ratio (1 : 1, 3 : 1 or 1 : 3) of the ED_{50} of CP55,940:ED_{50} of dexmedetomidine. Z_{add}: theoretical dose required for a purely additive interaction; Z_{mix}: experimental value determined from the combination dose–response curve (see Methods). *Statistically significant synergistic or supra-additive interaction ($P<0.05$).

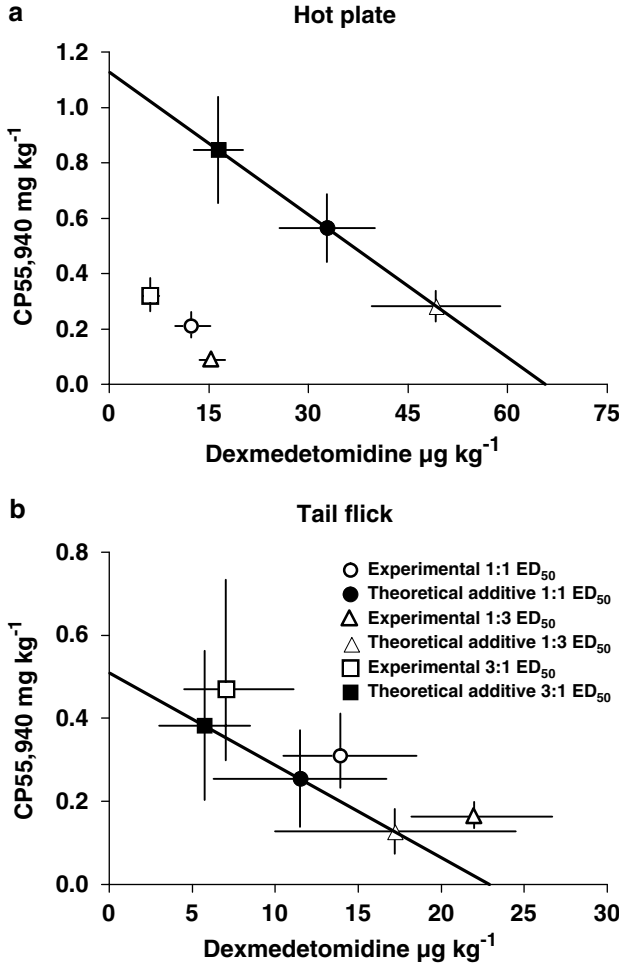

Figure 6 Isobolograms for the effects of simultaneous s.c. administration of CP55,940 with dexmedetomidine in 1 : 1, 3 : 1 and 1 : 3 fixed ratios of ED_{50} of drug A (dexmedetomidine) : ED_{50} drug of B (CP55,940) in the (a) hot plate and (b) tail flick assays in conscious mice. The line in each panel connects the ED_{50} of drug A on the *x*-axis and the ED_{50} of drug B on the *y*-axis and represents the locus of points of dose combinations for purely additive interactions. Symbols represent theoretical additive and experimental ED_{50} values, with their associated 95% confidence intervals. Synergistic interactions were found for all dose ratios in the hot plate assay, while additive interactions occurred in the tail flick assay.

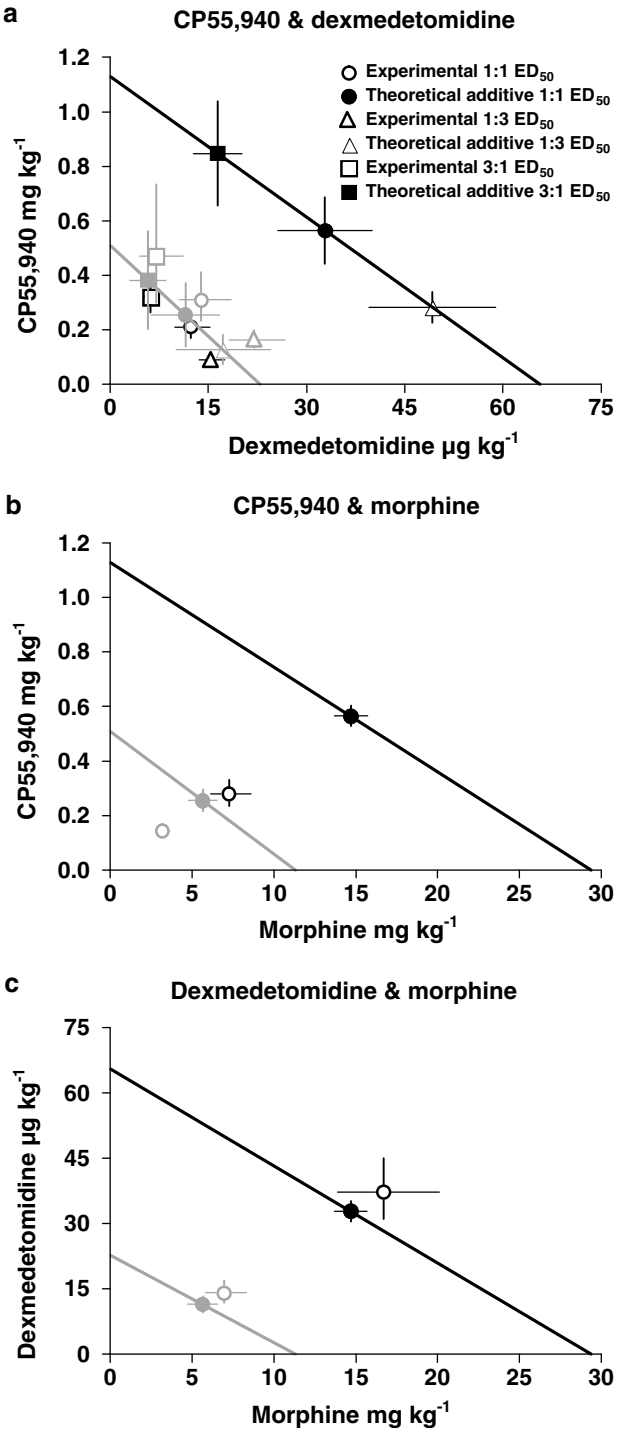

Figure 7 Isobolograms for the effects of simultaneous s.c. administration of drug combinations in hot plate and tail flick assays in conscious mice. The lines (black: hot plate; grey: tail flick) in each panel connect the ED_{50} of drug A on the *x*-axis and the ED_{50} of drug B on the *y*-axis and represent the locus of points of dose combinations for purely additive interactions. Symbols represent theoretical additive and experimental ED_{50} values, with their associated 95% confidence intervals. The drug combinations were (a) CP55,940 and dexmedetomidine; (b) CP55,940 and morphine; and (c) dexmedetomidine and morphine. Fixed ratios of ED_{50} of drug A : ED_{50} of drug B were 1 : 1 (a–c), 3 : 1 (a) and 1 : 3 (a) for the hot plate (black) and tail flick (grey) assays, respectively. Note that for each agonist, the ED_{50} values for antinociception in the hot plate assay were generally three-fold greater than in the tail flick assay.

CB_1 receptors and α_2-adrenoceptors are primarily found on presynaptic membranes and have similar signal transduction, including presynaptic modulation of primary afferent neurones, inhibition of postsynaptic membrane excitability and sensory neurone Ca^{2+} conductance and related neurotransmitter release; other interactions may also exist (Yaksh, 1985; Furst, 1999; Howlett *et al*., 2002). Both receptors are found in areas of the CNS that process and modulate nociceptive information, involving both spinal and supraspinal components (Behbehani, 1995; Lichtman *et al*., 1996; Martin & Lichtman, 1998). Cannabinoid receptors are found in the substantia gelatinosa of the spinal cord, suggesting modulation of nociceptive input from primary afferent neurones, and both cannabinoid receptors and α_2-adrenoceptors are found in the peripheral nervous system on primary afferent neurones (Khan *et al*., 1999; Pertwee, 2001; Howlett *et al*., 2002). However, some studies suggest that the antinociceptive actions of α_2-adrenoceptors are purely mediated at the spinal level, mainly *via* the α_{2A}-subtype present in the substantia gelatinosa (Jones *et al*., 1982; Takano & Yaksh, 1992; Hamalainen & Pertovaara, 1995; Stone *et al*., 1998; Khan *et al*., 1999; Kamibayashi & Maze, 2000; Malmberg *et al*., 2001). Stimulation of α_2-adrenoceptors in the spinal cord causes inhibition of afferent nociceptive impulses from Aδ and C fibres, as well as modulation of substance P and calcitonin gene-related peptide release in the dorsal horn (Yaksh, 1985; Takano *et al*., 1993; Furst, 1999; Khan *et al*., 1999).

Results for the combination of CP55,940 and dexmedetomidine in the mouse tail flick assay were simply additive, implying that cannabinoid receptors and α_2-adrenoceptors cause antinociception in this spinal reflex by independent mechanisms or sites of action. However, in conflict with the latter, Lichtman & Martin (1991) found that Δ^9-THC (i.v.) activated descending noradrenergic pathways in the rat spinal cord, which caused antinociception (tail flick assay) *via* activation of spinal α_2-adrenoceptors. It may be that Δ^9-THC activates or modulates different receptors or mechanisms compared to CP55,940 given the complexity of the descending control pathways (Millan, 2002). Further, in the rodent spinal cord, i.t. Δ^9-THC elicits release of dynorphin A, whereas CP55,940 causes release of dynorphin B (Pugh *et al*., 1997; Houser *et al*., 2000). Opioid agonists may also act supraspinally on spinal function through the activation of descending noradrenergic inhibitory pathways. So, considering the similarities in CNS locations and cell signalling mechanisms of α_2-adrenoceptors and opioid receptors, the two systems may be anticipated to elicit synergistic antinociceptive actions. In rats, Ossipov *et al*. (1990) found that the racemate medetomidine (dexmedetomidine is the dextro-isomer) had a synergistic interaction with morphine in the tail flick, but not hot plate, assay when the drugs were administered to the spinal cord. However, the interaction was only additive in both assays when systemic administration was used (Ossipov *et al*., 1990), which supports our results in mice. Thus, both the route of drug administration and the particular (spinal vs supraspinal) nociceptive pathways involved in responses to types of acute pain appear to determine whether drug combinations will have additive or synergistic effects.

The fact that very low doses of antinociceptive agonists may be used in combination to cause effective pain relief has great potential clinical benefit. Single administration of dexmedetomidine is associated with side effects such as hypotension, dry mouth, impairment of memory and psychomotor performance, as well as sedation (Hall *et al*., 2000). Opioid use may cause constipation, respiratory depression and the development of tolerance and dependence, while cannabinoid drugs elicit central side effects including psychomotor impairment and sedation (Ashton, 2001). Synergistic drug combinations may improve effective pharmacotherapy of pain as the lower clinical dose requirements for each agent will minimise drug-specific adverse effects (Raffa *et al*., 2003).

In conclusion, this study highlights the potential advantage of combining two antinociceptive drugs acting through specific receptor systems to lower the dose of each required for antinociception. That the dose of each can be decreased to less than that for a simple additive effect suggests that a major theoretical advantage can be ascribed to some but not all combinations. Thus, this study in mice demonstrated that an α_2-adrenoceptor agonist or μ opioid receptor agonist when combined with a cannabinoid receptor agonist showed significant potentiation or synergy in hot plate antinociception. For tail flick antinociception, only cannabinoid and μ opioid receptor synergy was demonstrated. If these findings translate to the human clinical situation, then judicial selection of dose and receptor-specific agonists may allow a better therapeutic window from unwanted side effects.

We thank Mrs Kirsten A. Messick for expert research assistance and Dr Arthur Christopoulos for helpful advice on isobolographic and statistical analyses.

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(Received May 21, 2004
Revised August 20, 2004
Accepted October 8, 2004)