ELSEVIER

European Journal of Pharmacology 337 (1997) 11–17

ejp

Isobolographic analysis of interaction between intrathecal morphine and clonidine in the formalin test in rats

Krzysztof Przesmycki [a,*], Jacek A. Dzieciuch [b], Stanisław J. Czuczwar [b,c], Zdzisław Kleinrok [b]

[a] *Department of Anaesthesiology and Intensive Therapy, Medical University School, Jaczewskiego 8, 20-090 Lublin, Poland*
[b] *Department of Pharmacology and Toxicology, Medical University School, Jaczewskiego 8, 20-090 Lublin, Poland*
[c] *Department of Clinical Toxicology, Institute of Rural Medicine, Jaczewskiego 2, 20-090 Lublin, Poland*

Received 1 May 1997; revised 12 August 1997; accepted 15 August 1997

Abstract

The formalin test, an experimental model of injury-induced central sensitisation, was used. The antinociceptive interaction between intrathecal morphine and clonidine was evaluated based on the inhibition of the phase 1 and 2 of the formalin response, induced by both drugs, given alone or in combinations with fixed dose ratios. Morphine and clonidine, at doses not affecting motor performance, produced dose-dependent inhibition in the formalin test, with similar ID_{50} values in phase 1 and 2; 0.66 and 0.45 nmol and 4.1 and 3.5 nmol, respectively. Isobolographic analysis revealed a significant synergy. The combination ID_{50} was found to be significantly lower than the respective theoretical additive ID_{50} for both fixed dose ratios (1:3 and 1:10) in both phases of the formalin test. The similar total dose fraction of the additive ID_{50} in phase 1 and 2 indicates the same magnitude of synergy and may suggest that the mechanisms of the spinal clonidine-morphine synergy do not differ significantly between both phases of the formalin test. © 1997 Elsevier Science B.V.

Keywords: Morphine; Clonidine; Nociception; Formalin test

1. Introduction

The formalin test is different from most nociceptive tests with phasic mechanical or thermal stimuli in that it allows the assessment of animal responses to continuous pain generated by tissue injury. The mechanisms of nociception and spinal processing in the early and late phase of the formalin response are considered to be different. Phase 1 of the formalin test reflects the acute pain response, while phase 2 reflects injury-induced spinal sensitisation and hyperalgesia (Coderre et al., 1990; Yamamoto and Yaksh, 1992; Coderre and Van Empel, 1994). The formalin test, in the present study, was used as a behavioural model of injury-induced central sensitization.

Recently, it was shown that spinal morphine and an α_2-adrenoceptor agonist, 2-(2,6-diethyl-phenylamino)-2-imidazoline (ST-91), produce a dose-dependent suppression of both phases of the formalin response (Malmberg and Yaksh, 1993a). Both drugs also showed a significant spinal synergy with ketorolac in the formalin test. This potent synergistic interaction supports the use of this class of agents in the treatment of pain states secondary to an acute pain response. These results suggest that clonidine may also possess an antinociceptive effect in the formalin test, despite the difference in the subclass of α_2-adrenoceptors in the spinal cord on which ST-91 and clonidine act (Takano and Yaksh, 1992). Morphine and clonidine coadministered intrathecally possess significant spinal synergy in the tail flick test (Ossipov et al., 1990), however the character of the spinal interaction between both drugs in the formalin test is unknown. Therefore, we decided to study the antinociceptive effect of morphine and clonidine, drugs used clinically, in the formalin test.

The present study was carried out to examine the antinociceptive effect of clonidine and morphine on phase 1 and phase 2 of the formalin test, and to characterise the spinal interaction between both drugs, using isobolographic analysis.

* Corresponding author. Tel.: (48-81) 742-5256; Fax: (48-81) 747-5710.

2. Materials and methods

2.1. Animals

Male Wistar rats (260-350 g), purchased from a licensed dealer, were used. The rats were housed in groups of 8 per cage on a natural light/dark cycle, with free access to water and food. All testing was performed between 10.00 a.m. and 3.00 p.m.

2.2. Drugs and treatment

The following drugs were used in this study: clonidine (clonidine HCl, MW: 266.6, Sigma, St Louis, MO, USA) and morphine (morphine HCl, MW: 375.8, Polfa, Warsaw, Poland). Both drugs were dissolved in 0.9% saline. As determined in preliminary studies, intrathecal (i.t.) morphine and clonidine were injected 15 min prior to formalin testing so that the peak effect of each drug coincided. Before i.t. administration the drugs were mixed so that all doses were delivered in a total volume of 20 μl. All i.t. injections were performed manually over 30 s.

2.3. Intrathecal injections

Drugs or saline were given as i.t. injections by lumbar puncture between the L4 and L5 vertebrae while the rats were under brief halothane anaesthesia. This involved a modification of the method used for mice by Hylden and Wilcox (1980), as applied for rats by Papir-Kricheli et al. (1987). Confirmation that the needle had penetrated the i.t. space was provided by the reflex flick of the rat tail produced by mechanical stimulation of the sacral spinal cord (Coderre and Van Empel, 1994). During the procedure, the rats were lightly anaesthetised for only a very brief period, with the lumbar puncture taking at most 2–3 min. Consequently, the rats were conscious and walking normally within 5 min after lumbar punctures. Formalin injections were performed after 15 min, to allow the agents to penetrate the spinal cord and to ensure that there were no lasting effects of anaesthesia. No rats showed any signs of sedation or any difficulties during locomotion at the time of formalin injection.

2.4. Formalin test

The formalin test was assessed according to Malmberg and Yaksh (1992). Briefly, 50 μl of a 5% formalin solution was injected subcutaneously (s.c.) into the plantar surface of the right hind paw with a 30 gauge needle. The rats were then placed individually in an open plexiglas chamber with a mirror positioned on the opposite side to allow unhindered observation of the formalin-injected paw. Pain behaviour was quantified by periodically counting the incidents of spontaneous flinching/shaking of the injected paw. The animals were observed individually and the flinches counted for 1 min periods at 1 to 2 min, 5 to 6 min and 5 min intervals during the interval from 10 to 60 min. Two phases of spontaneous flinching behaviour were observed as previously described (Wheeler-Aceto et al., 1990): phase 1 started immediately after formalin injection and lasted through the second observation interval (5–6 min), followed by phase 2, which began after 10 min and lasted up to 60 min after formalin injection. After a 1 h observation period, the animals were immediately killed by an overdose with chloroform inhalation anaesthesia.

2.5. Motor performance

The accelerating rotarod test (Jones and Roberts, 1968) was used to determine whether any of the drug severely impaired the animals' motor performance. The rotarod test consisted of placing the rat on a rod with 7 cm diameter, rotating at 2.5 rpm. After 10 s practice run, a timer was started and the speed of rotation was increased from 2.5 to 25 rpm over 5 min. The length of time the rat stayed on the rod was recorded. In each test, the mean of the 2 or 3 pre-drug values was taken as the baseline value for that rat. The accelerating rotarod test was carried out 15 and 30 min after i.t. drug administration.

2.6. Data analysis

The time–response data are presented as the mean number of flinches per min ± S.E.M. for the period of 1 to 2 min, 5 to 6 min and at 5 min intervals thereafter up to 60 min after formalin injection. Dose–response curves are presented as the sum of flinches for phase 1 (0–9 min) and phase 2 (10–60 min), respectively. The cumulative flinching response was calculated for each rat and the dose–response curve represents the mean of these values ± S.E.M. The effective dose resulting in a 50% reduction of the control formalin response was defined as the inhibitory dose 50 (ID_{50}) (Malmberg and Yaksh, 1992). Dose–response curves were derived separately for phase 1 and phase 2 for morphine and clonidine. The log dose–response lines were fitted using least square linear regression, the ID_{50} and 95% confidence interval (CI) for each drug being calculated according to Tallarida and Murray (1987).

The isobolographic analysis for drug–drug interaction was conducted according to Tallarida (1992). The isobolographic method is based on comparison of doses that are determined to be equieffective. To perform the isobolographic analysis, morphine and clonidine were coadministered in two combinations such that the doses were a fixed fraction of dose ratio 1:3 or 1:10 of morphine/clonidine. The dose–response curve of the combined drugs was used to calculate the actual (experimental) ID_{50} value and 95% CI (Tallarida and Murray, 1987). The isobols were drawn by plotting the experimentally determined ID_{50} value of clonidine on the *x*-axis and that of morphine on the *y*-axis,

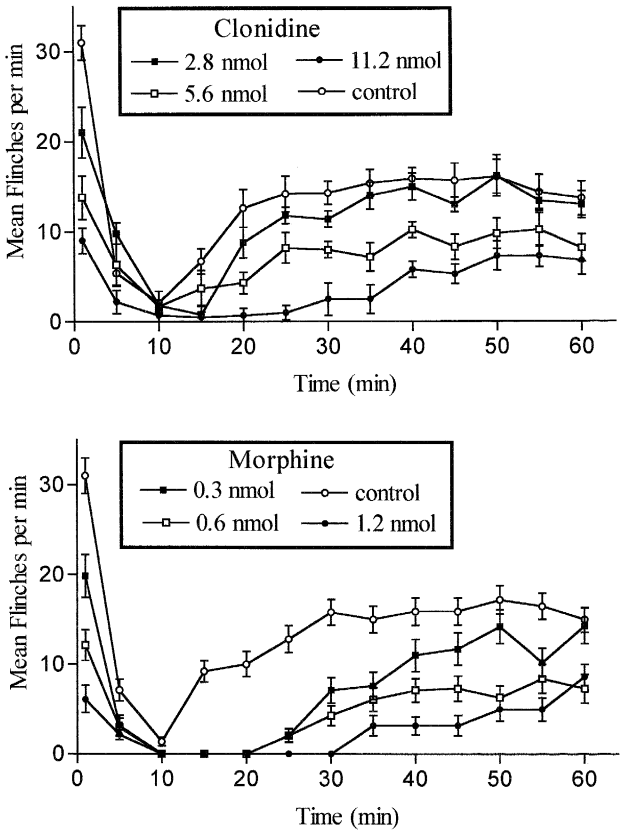

Fig. 1. Effect of intrathecal (i.t.) clonidine and morphine on formalin-induced flinching behaviour. The data are presented as the means ± S.E.M. (6–8 rats per line) of the number of flinches per minute versus the time after formalin injection. Control animals received i.t. 0.9% saline.

delivered alone and in combination. The theoretically additive ID_{50} dose, assuming simple additivity and 95% CI, were calculated according to Tallarida (1992).

To describe the magnitude of the interaction, a total dose fraction value (see formula below) was calculated according to Malmberg and Yaksh (1993a). The ID_{50} values were normalised, so that the ID_{50} value of an agent alone (the value on the axis) was given number 1. The fractional value describes the actual ID_{50} value as a fraction of the additive ID_{50}. Values near 1 indicate an additive interaction and values less than 1 indicate a synergistic interaction.

$$\text{The value of total dose fraction} = \frac{ID_{50}\text{ dose in combination of drug 1}}{ID_{50}\text{ value for drug 1 given alone}} + \frac{ID_{50}\text{ dose in combination of drug 2}}{ID_{50}\text{ value for drug 2 given alone}}$$

2.7. Statistical analysis

The data were evaluated by one-way analysis of variance (ANOVA) followed by the Newman–Keuls test for comparisons between individual treatment groups, provided that the F ratio gave $P < 0.05$.

The value of the experimental ID_{50} for a mixture of drugs was compared to the respective theoretical additive ID_{50}. If the actual ID_{50} is not different from the theoretical additive ID_{50}, the effect of drug administration is additive; otherwise if the mixture ID_{50} is significantly less than the theoretical additive ID_{50}, a synergistic interaction between the drugs occurs. Testing for a significant difference by Student's t-test was not possible since the CIs were not symmetrical around ID_{50}. Therefore, ID_{50} values were considered to differ significantly ($P < 0.05$) from each other, if each ID_{50} value was outside the 95% CI of the other (Tallarida, 1992).

3. Results

Formalin s.c. injection resulted in a reliable flinching response with two distinct phases. The biphasic effect is indicated in the typical results presented in Fig. 1. The timing or magnitude of the behavioural response did not differ in the control groups (i.t. saline) over the time

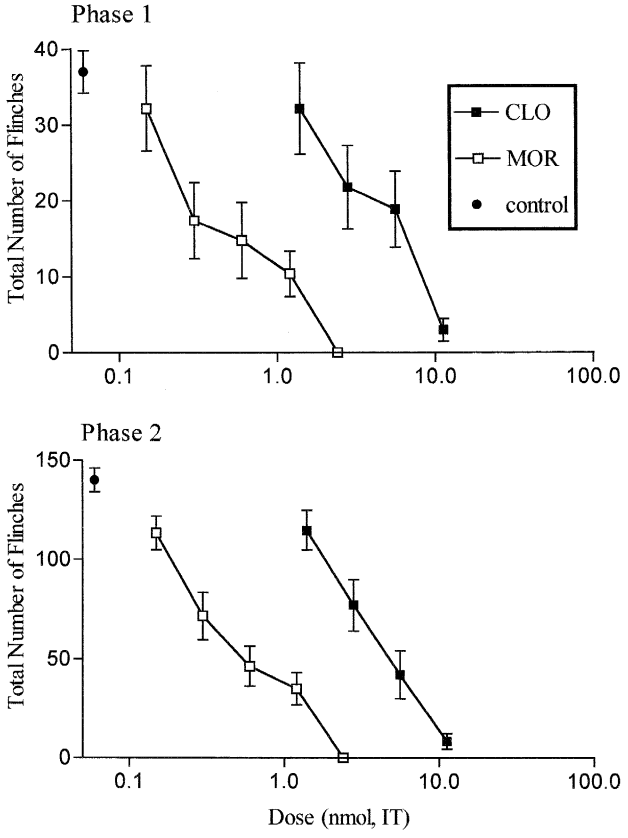

Fig. 2. Dose–effect curves for intrathecal (i.t.) clonidine (CLO) and morphine (MOR), administered 15 min before formalin. Data are presented as the total number of formalin evoked flinches during phase 1 (top) and phase 2 (bottom). Each dose point on the graph represents means ± S.E.M. for 6–8 rats. Control animals received i.t. 0.9% saline.

Table 1
Inhibitory effects of intrathecal morphine and clonidine alone or in combination in the formalin test phase 1 and phase 2

	ID_{50} [a] 95% CI	Add./ Exp. [b]	Total fraction [c]	*R*	*n*
Phase 1					
Morphine	0.66 (0.37–1.18)	—	—	−0.755	23
Clonidine	4.1 (2.8–6.2)	—	—	−0.798	18
Mor:clo 1:3 [d]	1.8 (1.3–2.4)	add.	1	—	—
Mor:clo 1:3	0.66 (0.37–1.18) [e]	exp.	0.39	−0.742	18
Mor:clo 1:10	2.8 (2.0–3.6)	add.	1	—	—
Mor:clo 1:10	1.5 (1.1–1.9) [e]	exp.	0.52	−0.826	18
Phase 2					
Morphine	0.45 (0.28–0.72)	—	—	−0.806	22
Clonidine	3.5 (2.9–3.9)	—	—	−0.944	18
Mor:clo 1:3	1.3 (1.0–1.7)	add.	1	—	—
Mor:clo 1:3	0.51 (0.26–0.99) [e]	exp.	0.40	−0.752	18
Mor:clo 1:10	2.2 (1.8–2.7)	add.	1	—	—
Mor:clo 1:10	1.0 (0.7–1.4) [e]	exp.	0.48	−0.874	18

[a] The ID_{50} value represents the total dose (nmol) resulting in 50% inhibition of the formalin control response.
[b] The theoretical additive (add.) or experimental (exp.) mixture ID_{50} value (as described in the text).
[c] The total dose fraction describes the experimental ID_{50} value as a fraction of the additive ID_{50}.
R: The regression coefficient of the log dose–response curve.
n: Total number of animals per dose–response curve, minimum of five per dose.
[d] The drug ratios are identified as morphine:clonidine.
[e] Significantly different from theoretical additive ID_{50} value ($P < 0.05$).

covered by the study (one-way ANOVA, $P > 0.05$). Results from all control experiments were therefore pooled and treated as a common control group.

The i.t. administration of morphine (0.3–2.4 nmol) and clonidine (2.8–11.2 nmol) did not produce any detectable effect on motor functions. The mean pre-drug rotarod performance time ± S.E.M. in this study was 149 ± 6 s. The i.t. administration of morphine (2.4 nmol) or clonidine (11.2 nmol), either alone or in combination, did not modify motor performance. The mean times for the experimental groups ranged between 87.4 ± 7.7% and 97.4 ± 2.6% of the control group.

Morphine and clonidine, given i.t. alone, resulted in a complete dose-dependent suppression of both phases of the formalin response (Figs. 1 and 2). A typical time course of this effect, with different doses of morphine and clonidine, given 15 min before the injection of formalin, is presented in Fig. 1. Fig. 2 presents the i.t. dose–response curves for morphine and clonidine determined on phases 1 (0–9 min) and 2 (10–60 min) of the formalin test. The ID_{50} doses and 95% CI are summarised in Table 1. The potency of clonidine to inhibit the formalin response, defined by its

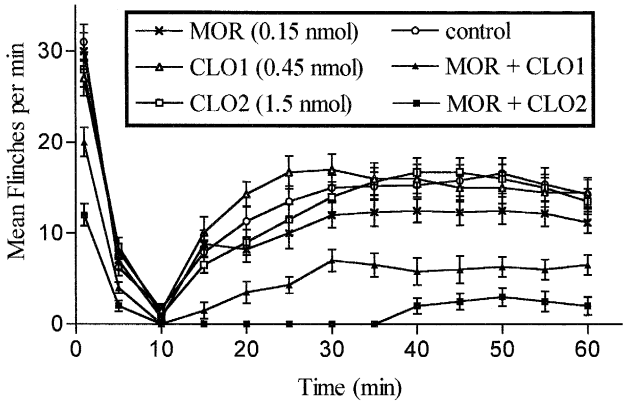

Fig. 3. Effect of clonidine (CLO) and morphine (MOR), coadministered intrathecally (i.t.), on formalin-induced flinching behaviour. The data are presented as the means ± S.E.M. (6–8 rats per line) of the number of flinches per minute versus the time after formalin injection. Control animals received i.t. 0.9% saline. Mean phase 1 flinches/min response: control = MOR = CLO1 = CLO2 > MOR + CLO1 > MOR + CLO2; one-way ANOVA, $P < 0.05$. Mean phase 2 flinches/min response: control = MOR = CLO1 = CLO2 > MOR + CLO1 > MOR + CLO2; one-way ANOVA, $P < 0.05$.

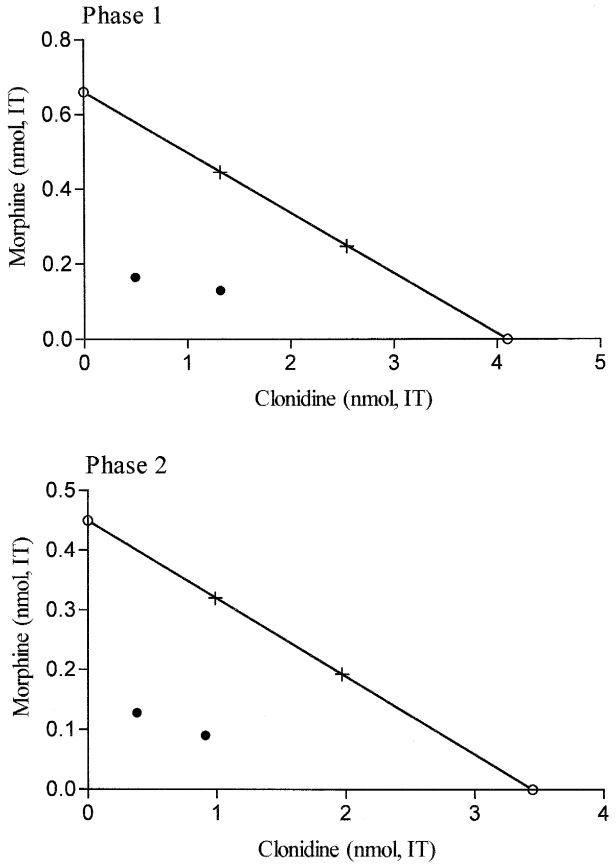

Fig. 4. Isobolograms showing the interaction between intrathecal clonidine and morphine in phase 1 (top) and phase 2 (bottom) of the formalin test. The ID_{50} values of each drug (○) are plotted on the *x*- and *y*-axis, respectively. The line connecting both points is the theoretical line of additivity. In both isobolograms, the experimental ID_{50} values of the fixed dose ratios 1:3 and 1:10 of morphine/clonidine combinations (●) were significantly ($P < 0.05$) below the respective additive points (+); the confidence intervals do not overlap, indicating a supra-additive interaction. The theoretical additive ID_{50} point and its 95% confidence interval (CI) is calculated from the ID_{50} values and CI's of each drug. Each point on the graph represents ID_{50} values from dose–response curves including 18–23 rats.

ID_{50} (nmol) and 95% CI, was of the same magnitude for both phases: 4.1 (2.8–6.2) and 3.4 (2.9–3.9), respectively. Morphine was more potent to inhibit the formalin response than was clonidine. The value of the morphine ID_{50} (nmol) and 95% CI, for phases 1 and 2 of the formalin test was: 0.66 (0.37–1.18) and 0.45 (0.28–0.72), respectively, and did not differ significantly between the two phases.

Morphine and clonidine, given i.t. together, at doses that, alone, had no effect on the formalin response, significantly inhibited both phases of the formalin test (Fig. 3).

The potency ratio of morphine alone versus clonidine alone was 1: 6.2. Because the character of a positive interaction, expected between morphine and clonidine, may be affected by the dose ratio of these drugs, we studied the interaction with the dose ratio (morphine/clonidine) shifted in favour of morphine (1:3) or of clonidine (1:10).

The data for the combinations of morphine and clonidine are presented as isobolograms in Fig. 4. The ID_{50} doses of morphine and clonidine were plotted on the *x*- and *y*-axis, respectively. These points are connected by solid theoretical lines of additivity for phases 1 and 2 of the formalin test separately. The points for experimental ID_{50} values of the fixed dose ratios: 1:3 and 1:10 of morphine/clonidine combinations, were plotted on the isobologram for phase 1 and phase 2, respectively. Isobolographic analysis, using a fixed dose ratio revealed a significant interaction between clonidine and morphine on phase 1 and phase 2 of the formalin response. The experimental values of ID_{50} and 95% CI are significantly ($P < 0.05$) lower than the calculated additive doses, for both fixed dose ratios of drugs used in the study and for both phases of the formalin test (Table 1). Similar total dose fractions and dose ratios for phase 1 and phase 2, indicate that the magnitude of the synergy between clonidine and morphine was the same in both phases of the formalin test (Table 1).

4. Discussion

This study has shown the following: (1) i.t. morphine and clonidine, at doses not affecting motor performance, exert a dose-dependent antinociceptive effect and (2) morphine and clonidine, coadministered i.t., produce greater antinociception than predicted on the basis of simple addition of their effects on both phases of the formalin response in rats.

Clonidine is not considered to be a strong 'analgesic' in acute pain. In a double-blind study, Gordh (1988) demonstrated that epidural clonidine did not significantly decrease the need for supplementary intravenous opioid analgesia in patients with severe postoperative pain after thoracotomy. However, clinical studies (Coombs et al., 1987; Motsch and Graber, 1990; Vercauteren et al., 1990) demonstrated improved analgesia and reduced side effects of epidural opioids when coadministered with epidural clonidine. Most clinical studies compare a fixed dose of opioid with and without clonidine. The fixed opioid dose is often therapeutic itself, thus limiting the ability to observe a synergistic interaction. To evaluate the analgesic interaction and the frequency of side-effects, clinical studies should compare subanalgesic dose combinations of clonidine and opioids with analgesic doses of these agents alone. Thus, while a considerable number of clinical studies have examined the clonidine–morphine combination for pain management, few have clearly identified synergistic drug interactions.

Earlier experimental results suggested a synergistic antinociceptive interaction between α_2-adrenoceptor and opioid agonists in the nociceptive tests reflecting an acute pain response (Hylden and Wilcox, 1983). Isobolographic analysis had not been used in this study. A number of studies have also shown, in the absence of the isobolographic analysis, that combination of morphine and α_2-adrenoceptor agonists (ST-91, norepinephrine, or clonidine) produce enhancement of antinociception (Yaksh and Reddy, 1981; Wilcox et al., 1987; Drasner and Fields, 1988; Ossipov et al., 1989; Monasky et al., 1990; Plummer et al., 1992). In these studies, the character of the positive interaction between morphine and α_2-adrenoceptor agonists (i.e., additive or supra-additive) was not analysed in detail. Either a single dose of each drug was administered and the effect was greater than the sum of effects of each dose alone, or a fixed dose of one drug caused a significant shift to the left in the dose–response curve of the other drug. When a fixed dose of one drug is combined with varying doses of another, the ratio between the two drugs changes at each point of the dose–response curve. This change in ratio may be important, as Ossipov et al. (1990) showed that the interaction between two drugs may be either synergistic or additive, depending on the dose ratio.

Tallarida et al. (1989) provided a model of the statistical interpretation of isobolographic data. The method involves comparison of the effects of doses of each drug alone with those of the drugs in a fixed dose–ratio combination to produce a given level of effect on the dose–response functions of the drugs given alone or in combination. The advantages of this method include statistically derived values independent of graphic analysis and statistical validity without assumptions about mechanism of drug action, shape of the dose–response function, or the need to establish parallelism of the dose–response curves.

Isobolographic analysis at the ED_{50} dose–effect levels of fixed dose–ratio combinations of clonidine with various opioids was used to examine the synergistic inhibition of the tail-flick response in mice (Roerig and Fujimoto, 1989). A synergistic antinociceptive interaction was found between clonidine and morphine or a δ-opioid agonist, whereas the combination of clonidine with μ-opioid or κ-opioid receptor agonists produced only additive antinociceptive effects. Whether the δ-opioid or μ-opioid (Omote et al., 1991; Sullivan et al., 1992) receptor subtype is

involved in this interaction remains a matter of dispute. However, there is a wealth of evidence for morphine interactions with α_2-adrenoceptor agonists in antinociception of the acute pain response.

Synergistic interaction can occur when both drugs affect different critical points along a common pathway (Berenbaum, 1989). Clonidine and morphine share a common second-messenger mechanism: both activate a G-protein coupled receptor which enhances K^+ conductance and leads to hyperpolarization of neurones which may inhibit neuronal discharge and/or decrease neurotransmitter release (North et al., 1987; Ossipov et al., 1990). The action of both agonists may independently alter intracellular mechanisms (second messenger systems within the same cell) coupled to G-protein activation and mediate a synergistic interaction (Malmberg and Yaksh, 1993a).

Another proposed mechanism of synergy involves functional interactions. Synergy may result from distinct drug effects at separate anatomic sites (e.g., pre- and postsynaptic) that may act independently as well as together to inhibit spinal nociceptive processing (Roerig and Fujimoto, 1989). Both pre- and postsynaptic sites of action have been identified for α_2-adrenoceptor and opioid agonists at the spinal level. Thus, simultaneous engagement of pre- and postsynaptic mechanisms may magnify the antinociceptive effects produced by either drug acting at one site independently (Solomon and Gebhart, 1994).

Finally, the pharmacokinetic parameters of one drug may be altered by coadministration of a second drug. Although redistribution of the i.t. administered drugs was not studied, it seems unlikely that the synergistic interaction between clonidine and morphine depends on altered clearance of either drug. In the present study, 15 min after i.t. administration of the drug combination, during phase 1 of the formalin test, the behaviour evoked by the formalin injection was significantly decreased by the combinations at doses that given alone had no effect. It was also shown that spinal α_2-adrenoceptor agonists do not alter the spinal clearance of other spinal agents such as morphine (Monasky et al., 1990).

Most of the above studies, demonstrating the antinociceptive synergy between spinal α_2-adrenoceptor and opioid agonists, were performed with nociceptive tests that reflect only the acute pain response (Yaksh and Reddy, 1981; Wilcox et al., 1987; Monasky et al., 1990; Ossipov et al., 1990; Plummer et al., 1992). In the present study, i.t. morphine and clonidine produced a dose-dependent inhibition of phase 1 and phase 2 of the formalin response, with very similar ID_{50} values. The similar effect of morphine and ST-91 in the formalin test was observed by others (Wheeler-Aceto and Cowan, 1991; Malmberg and Yaksh, 1993a). Malmberg and Yaksh (1993a) demonstrated that both drugs exert a significant synergistic antinociceptive effect with ketorolac for phase 1 and phase 2 of the formalin test. This potent synergy appears to involve the specific μ opioid and α_2 adrenergic receptors, respectively, since the synergy was completely antagonized by naloxone or yohimbine.

The pharmacology of the acute pain response in phase 1 of the formalin test is different from that of phase 2, the subsequent hyperalgesic component. There is considerable evidence to implicate NMDA receptor activation and nitric oxide (NO) formation in the mechanisms that underlie spinal sensitisation in phase 2 of the formalin test (Coderre and Melzack, 1992; Malmberg and Yaksh, 1993b; Meller and Gebhart, 1993).

Morphine-induced antinociception at the spinal level also seems to be dependent on NO formation. Enhancement of morphine antinociception by competitive inhibitors of NO synthase (Przewłocki et al., 1993) and reduction of opioid antinociceptive efficiency by increased NO production were observed in the nociceptive tests reflecting the acute pain response (Brignola et al., 1994). We expected to find a different potency of morphine, as well as a different magnitude of synergy between morphine and clonidine in both phases of the formalin test. However, the potency of the observed antinociceptive effect of morphine and the magnitude of the synergy between clonidine and morphine were very similar in both phases of the formalin response. The synergy was also not affected by changing the dose ratios of both drugs. Based on these results, we cannot propose any explanation for the observed synergism. The above results may only suggest that the mechanisms of spinal clonidine-morphine synergy do not differ significantly between phase 1 and phase 2 of the formalin test.

The potent synergy between spinal clonidine and morphine in the formalin test may prompt further studies of this drug combination in other tests. Tests associated with spinal sensitisation, e.g., an experimental model of a peripheral nerve injury-induced augmented pain processing (hyperalgesia in mononeuropathy), would be more characteristic of neuropathic pain, as was shown for the combination of clonidine and MK-801, an NMDA receptor antagonist (Lee and Yaksh, 1995).

Acknowledgements

This study was supported by a grant from the Medical University School in Lublin No. 90/96. The study design was approved by the ethical committee for animal research at the Medical University School in Lublin.

References

Berenbaum, M.C., 1989. What is synergy?. Pharmacol. Rev. 41, 93–141.
Brignola, G., Calignano, A., Di Rosa, M., 1994. Modulation of morphine antinociception in the mouse by endogenous nitric oxide. Br. J. Pharmacol. 113, 1372–1376.
Coderre, T.J., Melzack, R., 1992. The role of NMDA receptor operated

calcium channels in persistent nociception after formalin-induced tissue injury. J. Neurosci. 12, 3671–3675.

Coderre, T.J., Van Empel, I., 1994. The utility of excitatory amino acid (EAA) antagonists as analgesic agents. I. Comparison of the antinociceptive activity of various classes of EAA antagonists in mechanical, thermal and chemical nociceptive tests. Pain 59, 345–352.

Coderre, T.J., Vaccarino, A.L., Melzack, R., 1990. Central nervous system plasticity in the tonic pain response to subcutaneous formalin injection. Brain Res. 535, 155–158.

Coombs, D.W., Jensen, L.E., Murphy, C.A., 1987. Microdose intrathecal clonidine and morphine for postoperative analgesia. Anesthesiology 67, A238.

Drasner, K., Fields, H.L., 1988. Synergy between the antinociceptive effect of intrathecal clonidine and systemic morphine in the rat. Pain 32, 309–312.

Gordh Jr., T., 1988. Epidural clonidine for treatment of postoperative pain after thoracotomy. A double-blind placebo-controlled study. Acta Anaesthesiol. Scand. 32, 702–709.

Hylden, J.L.K., Wilcox, G.L., 1980. Intrathecal morphine in mice: A new technique. Eur. J. Pharmacol. 67, 313–316.

Hylden, J.L.K., Wilcox, G.L., 1983. Pharmacological characterization of substance P-induced nociception in mice: Modulation by opioid and noradrenergic agonists at spinal level. J. Pharmacol. Exp. Ther. 226, 398–404.

Jones, B.J., Roberts, D.J., 1968. The quantitative measurement of motor incoordination in naive mice using an accelerating rotarod. J. Pharm. Pharmacol. 20, 302–304.

Lee, Y-W., Yaksh, T.L., 1995. Analysis of drug interaction between intrathecal clonidine and MK-801 in peripheral neuropathic pain rat model. Anesthesiology 82, 741–748.

Malmberg, A.B., Yaksh, T.L., 1992. Antinociceptive actions of spinal nonsteroidal anti-inflammatory agents on the formalin test in the rat. J. Pharm. Exp. Ther. 263, 136–146.

Malmberg, A.B., Yaksh, T.L., 1993a. Pharmacology of the spinal action of ketorolac, morphine, ST-91, U50488H and L-PIA on the formalin test and an isobolographic analysis of the NSAID interaction. Anesthesiology 79, 270–281.

Malmberg, A.B., Yaksh, T.L., 1993b. Spinal nitric oxide inhibition blocks NMDA-induced thermal hyperalgesia and produces antinociception in the formalin test in rats. Pain 54, 291–300.

Meller, S.T., Gebhart, G.F., 1993. Nitric oxide (NO) and nociceptive processing in the spinal cord. Pain 52, 127–136.

Monasky, M.S., Zinsmeister, A.R., Stevens, C.W., Yaksh, T.L., 1990. Interaction of intrathecal morphine and ST-91 on antinociception in the rat: Dose–response analysis, antagonism and clearance. J. Pharmacol. Exp. Ther. 254, 383–392.

Motsch, J., Graber, E., 1990. Addition of clonidine enhances postoperative analgesia from epidural morphine: A double-blind study. Anesthesiology 73, 1067–1073.

North, R.A., Williams, J.T., Superenant, A., Christie, M.J., 1987. Mu and delta opioid receptors belonging to a family of receptors that are coupled to potassium channels. Proc. Natl. Acad. Sci. USA 84, 5487–5491.

Omote, K., Kitahata, L.M., Collins, J.G., Nakatani, K., Nakagawa, I., 1991. Interaction between opiate subtype and alpha-2 adrenergic agonists in suppression of noxiously evoked activity of WDR neurones in the spinal dorsal horn. Anesthesiology 74, 737–743.

Ossipov, M.H., Suarez, L.J., Spaulding, T.C., 1989. Antinociceptive interactions between alpha-2 adrenergic and opiate agonists at the spinal level in rodents. Anesth. Analg. 68, 194–200.

Ossipov, M.H., Harris, S., Lloyd, P., Messineo, E., 1990. An isobolographic analysis of the antinociceptive effect of systemically and intrathecally administered combinations of clonidine and opiates. J. Pharmacol. Exp. Ther. 255, 1107–1116.

Papir-Kricheli, D., Frey, J., Laufer, R., Gilon, C., Chorev, M., Selinger, Z., Devor, M., 1987. Behavioural effects of receptor specific substance P agonists. Pain 31, 263–276.

Plummer, J.L., Cmielewski, P.L., Gourlay, G.K., Owen, H., Cousins, M.J., 1992. Antinociceptive and motor effects of intrathecal morphine combined with intrathecal clonidine, noradrenaline, carbachol or midazolam in rats. Pain 49, 145–152.

Przewłocki, R., Machelska, H., Przewłocka, B., 1993. Inhibition of nitric oxide synthase enhances morphine antinociception in the rat spinal cord. Life Sci. 53, 1–5.

Roerig, S.C., Fujimoto, J.M., 1989. Multiplicative interaction between intrathecally and intracerebroventricularly administered mu opioid agonists but limited interactions between delta and kappa agonists for antinociception in mice. J. Pharmacol. Exp. Ther. 249, 762–768.

Solomon, R.E., Gebhart, G.F., 1994. Synergistic antinociceptive interactions among drugs administered to the spinal cord. Anesth. Analg. 78, 1164–1172.

Sullivan, A.F., Kalso, E.A., McQuay, H.J., Dickenson, A.H., 1992. Evidence for the involvement of μ but not δ opioid receptor subtype in the synergistic interaction between opioid and $alpha_2$ adrenergic antinociception in the rat spinal cord. Neurosci. Lett. 139, 65–68.

Takano, Y., Yaksh, T.L., 1992. Characterization of the pharmacology of intrathecally administered alpha-2 agonists and antagonists in rats. J. Pharmacol. Exp. Ther. 261, 764–772.

Tallarida, R.J., 1992. Statistical analysis of drug combinations for synergism. Pain 49, 93–97.

Tallarida, R.J., Murray, R.B., 1987. Manual of Pharmacologic Calculations with Computer Programs, 2nd ed. Springer, New York, NY, pp. 16–17.

Tallarida, R.J., Porreca, F., Cowan, A., 1989. Statistical analysis of drug–drug and site–site interaction with isobologram. Life Sci. 45, 947–961.

Vercauteren, M., Lauwers, E., Meert, T., De Hert, S., Adriaensen, H., 1990. Comparison of epidural sulfentanil plus clonidine with sufentanil alone for postoperative pain relief. Anesthesia 45, 531–534.

Wheeler-Aceto, H., Cowan, A., 1991. Standarization of the rat paw formalin test for the evaluation of analgesics. Psychopharmacology 104, 35–44.

Wheeler-Aceto, H., Porreca, F., Cowan, A., 1990. The rat paw formalin test: Comparison of noxious agents. Pain 40, 229–238.

Wilcox, G.L., Carlsson, K.H., Jochim, A., Jurna, I., 1987. Mutual potentiation of antinociceptive effects of morphine and clonidine on motor and sensory responses in rat spinal cord. Brain Res. 405, 84–93.

Yaksh, T.L., Reddy, S.V.R., 1981. Studies in primate on the analgesic effects associated with intrathecal actions of opiates, alpha-adrenergic agonists and baclofen. Anesthesiology 54, 451–467.

Yamamoto, T., Yaksh, T.L., 1992. Comparison of the antinociceptive effects of pre- and posttreatment with intrathecal morphine and MK801, an NMDA antagonist on the formalin test in the rat. Anesthesiology 77, 757–763.