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ANALGESIC ACTION OF CLONIXIN, NIFEDIPINE AND MORPHINE USING THE FORMALIN TEST

D. BUSTAMANTE, H. F. MIRANDA, T. PELISSIER and C. PAEILE
Department of Pharmacology, Faculty of Medicine, Universidad de Chile, P.O. Box 70,000, Santiago 7, Chile

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Abstract—1. The analgesic effect of ClX, nifedipine, metamizol, indoprofen and morphine in the pain induced by formalin injection (formalin test) was studied.
2. Attempts to demonstrate tolerance to ClX were unsuccessful.
3. In the analgesic test nifedipine and morphine are approximately 10 times more potent than ClX.
4. The present results suggest that the analgesic action of ClX is not mediated by μ_1, δ or κ-opioid receptors and the anti-nociceptive effect of nifedipine may be associated with the blockade of the transmembrane inward movements of calcium.

INTRODUCTION

It has been recently reported that clonixin (ClX), a nonsteroidal analgesic produces a significant reduction in the neurogenic muscular twitch of the isolated rat vas deferens. Additionally, it produces changes in the M_1-ACh and α_2-NE responses with no modifications in the M_2-ACh and α_1-NE effects in the same preparation being obtained (Bustamante *et al.*, 1988). The above findings suggest that ClX besides its ability to block the release of NE, has some common pharmacological properties, closely allied with those exhibiting the "calcium antagonist", "calcium-channels blockers", "calcium modulators", "calcium influx blockers" or "slow-channels inhibitors".

The important role of calcium ions in the regulation of many cellular functions is widely recognized and so consequently the calcium-channel blockers can modify some of the biological processes by inhibiting the entry of Ca^{2+} into cells. This agent acts not only on the cardiovascular system but also on smooth muscle and on a variety of other tissues, including chromafin tissue, pancreatic beta and other secretory cells, as well as mast cells (Braunwald, 1982). Numerous reports suggest that calcium antagonists inhibit neuromuscular transmission through postsynaptic mechanisms (Bondi, 1978; Ribeiro *et al.*, 1979; Publicover and Duncan, 1979; Bikhazi *et al.*, 1982; Lawson *et al.*, 1983; Williams *et al.*, 1983; Durant *et al.*, 1984; Del Pozo and Baeyens, 1986; Wali, 1987).

In addition, administration of calcium or calcium channel antagonists can regulate the analgesic effects of morphine, β-endorphin, fentanyl and others more specific μ and δ opioids (Kakunaga *et al.*, 1966; Guerrero-Muñoz *et al.*, 1981; Chapman and Way, 1982; Harris *et al.*, 1975; Belger *et al.*, 1985; Hoffmeister and Tettenborn, 1986; Kavaliers and Ossenkopp, 1986, 1987; Kavaliers, 1987).

Since calcium antagonists have proved to be extremely useful in the treatment of exertional angina, vasospastic angina, and essential hypertension and their efficacy in these conditions is related primarily to their greater vascular selectivity (Freedman and Waters, 1987), they probably act by blocking the transmembrane inward movement of Ca^{2+} and possess many important therapeutic properties useful in the management of certain cardiovascular and related disorders in man (Sorkin *et al.*, 1985). A direct and precise relationship between the relief of pain in these conditions and the effects of calcium antagonists has not been explained. The present study was undertaken to investigate the hypothesis that the calcium antagonists might possess anti-nociceptive properties and correlate this action with the analgesic activity of ClX. In addition the purpose of this study was to investigate whether ClX produces dependence when administered to experimental animals and if it does occur whether it is like that rendered by morphine.

MATERIAL AND METHODS

Adult male and female Wistar rats and Swiss AsW mice were used throughout this study.

Study of tolerance

Tolerance to morphine or ClX was induced by daily subcutaneous administration of the drugs, according to the method of Cox *et al.* (1975) as modified by Miranda *et al.* (1987). Control rats and mice received daily subcutaneous injections of saline, in the case of morphine, and the solvent, in the case of ClX, which itself produced no effect on the animals. At the end of 8 days of treatment, 1 hr after the last one injection of morphine or ClX, rats and mice received naloxone (1 mg/kg i.p.) or the rats received the LD_{50} of ClX.

Assay of the LD_{50} of ClX

The animals were injected with ClX i.p. and then the behavior was observed for 1 hr. The LD_{50} was calculated by interpolation from log dose–response plots.

Study of analgesia in rats

Analgesia was assessed by the formalin test according to Dubuisson and Dennis (1977). Briefly, each rat was placed in a special chamber for observation 45 min prior to the formalin injection and during the last 15 min of this period its rated-behavior constituted the pain-free baseline. An injection of 50 μl of sterile 5% formalin was then made under the skin of the dorsal surface of the forepaw and the pain response rated for 30 min.

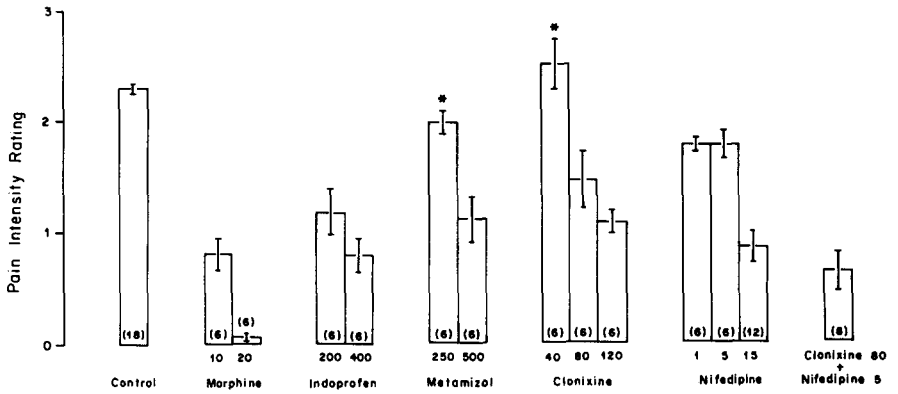

Fig. 1. Analgesic effects of various drugs measured by the formalin test. The numbers above the name of the analgesic are the doses administered (mg/kg). Each bar represents the mean of the number of experiments indicated in parentheses, error bars are the SEM. All the values are significantly different from the control ($P < 0.05$) except these denoted by (*).

The following groups were assayed: (1) Control groups received 1 ml/kg saline solution or 0.8 mg/kg ethanol (nifedipine solvent), (2) the morphine group received 10 or 20 mg/kg morphine; (3) the clonixin group, received 40, 80 or 120 mg/kg ClX; (4) the nifedipine group, received 1, 5 or 15 mg/kg nifedipine; (5) the metamizol (dipyrone) group, received 250 or 500 mg/kg metamizol; (6) the indoprofen group, received 200 or 400 mg/kg indoprofen; (7) the clonixin–nifedipine group, received 80 mg/kg ClX plus 5 mg/kg nifedipine. In all groups the administration of saline, solvent or drugs were made via i.p. 30 min prior to the formalin injection.

The pain response in the different groups of rats was recorded on a Grass Polygraph for 30 min and numerical values assigned according to the rating scale for rats of Dubuisson and Dennis (1977).

The data obtained are expressed as mean ± SEM. Significances of the differences were analyzed according to Student's *t*-test. The 0.05 level of probability was accepted as significant.

Drugs

The following drugs were used. Formalin, ethanol and morphine hydrochloride were obtained from Merck Chemical Co., Darmstadt, West Germany; nifedipine, dipyrone, indoprofen and naloxone hydrochloride were purchased from Sigma Chemical Co., St Louis, Missouri, U.S.A. and clonixin was generously supplied by Pharma Investi Labs, Santiago, Chile.

RESULTS

Study of LD_{50} and tolerance to ClX

The administration of ClX produced a different LD_{50} to the drug in mice and rats, corresponding to 266.4 ± 4.1 mg/kg ($n = 28$) and 308.0 ± 2.6 mg/kg ($n = 12$) respectively. However, in rats pretreated by daily administration of ClX for 8 days (from 20–100 mg/kg) the LD_{50} to ClX was not changed.

Attempts to demonstrate tolerance were unsuccessful. First, the administration of naloxone (1 mg/kg i.p.) to rats and mice chronically treated with ClX (Cox *et al.*, 1975 and Miranda *et al.*, 1987) was unnoticed, since naloxone failed to elicit the characteristic typical withdrawal symptoms. Another attempt to demonstrate tolerance was made in which the criterion for tolerance was the LD_{50} value of ClX. In this case the administration of the LD_{50} of ClX (308.0 mg/kg i.p.) to rats, chronically treated with ClX, induced the same lethality as in untreated rats.

Assessment of analgesia in rats

The results of the pain intensity rating for the different groups of rats used are shown in Figs 1 and 2. The average rating given to these animals prior to the formalin injection was 0.41 ± 0.06 and due to the whole activity, including rearing, burnishing, grooming and other movements of the animals. This value is the pain-free baseline and was omitted for the sake of neatness in Fig. 2.

Figure 1 indicates that the formalin injection induced a marked nociceptive response. There were no significant differences between the pain intensity induced by formalin in the group of saline and ethanol injected rats. The temporal course displayed by the nociceptive response in control groups was characterized by a constant and consistent pain formalin-induced during the 30 min of observation, as can be seen in Fig. 2.

Morphine-treated rats (doses of 10 or 20 mg/kg) were able to display a dose-dependent analgesic effect. These responses by the morphine-treated rats were significantly greater than those of the control animals (Fig. 1) although they were similar in fashion in the temporal course. However, increased analgesia in the morphine group compared with the control rats, was observed (Fig. 2). The administration of indoprofen 200 or 400 mg/kg significantly reduced the pain responses, although at a dose of 250 mg/kg metamizol had a slight analgesic effect and at the dose of 400 mg/kg, this drug induced a significant decrease in the formalin-induced pain, see Fig. 1. The non-steroidal analgesic, ClX, 40 mg/kg, had no effect on the nociceptive response, whereas the calcium channel antagonist, nifedipine, 1 or 5 mg/kg, had a significantly greater action on the pain response, using the formalin test. Otherwise increasing the dose of ClX, 80 and 20 mg/kg, and nifedipine, 15 mg/kg, both drugs induced a dose-dependency and significantly decreased the pain rating, as can be seen in Fig. 1. However, the temporal course of the analgesia

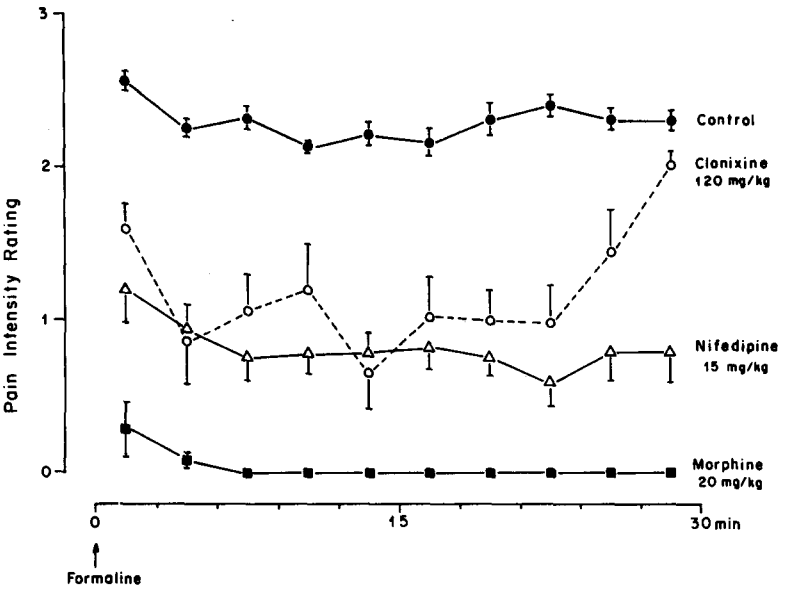

Fig. 2. Pain intensity rating curves of control, clonixine 120 mg/kg, nifedipine 15 mg/kg and morphine 20 mg/kg using the formalin test. Each point represents the mean of 6–12 experiments ± SEM.

was different for both drugs; while nifedipine developed an analgesic curve of similar fashion to that developed by morphine, clonixin developed a non-straight line temporal course characterized by a return of the pain rating to the values of the control rats at the end of the observation period (Fig. 2). The mixture, ClX 80 mg/kg and nifedipine 5 mg/kg, had a significant effect in the reduction of the pain intensity induced by the formalin-injection, as can be seen in Fig. 1.

DISCUSSION

The main finding obtained in the present study indicates that clonixin and nifedipine have antinociceptive properties that can be evaluated using the formalin test, which is a technique that has the advantage over other pain tests. The pain induced by means of the formalin test is continuous and because of this resembles the most clinical pain. Consequently little or no restraint is necessary, permitting unhindered observation of the complete range of behavioral responses (Dubuisson and Dennis, 1977).

The results of the present experiments allow a breakdown of the mechanism by which ClX produces analgesia and in the mechanism of induction of antinociceptive action by nifedipine and morphine, using the same test. Firstly, the relative potencies of ClX, nifedipine and morphine in the experimental model used were markedly different. If ClX and nifedipine were producing the analgesic effect at the same type of receptor site, it would be expected that the relative potencies of these two drugs to be approximately the same. They would be equal to morphine potency, if they were acting on a similar type of receptor-site which is opioid in nature. However, in the analgesic test, nifedipine (15 mg/kg) and morphine (10 mg/kg) are approx 10 times more potent than ClX (120 mg/kg), whereas nifedipine (15 mg/kg) is about equipotent to morphine (10 mg/kg). Secondly the temporal course of analgesia for nifedipine, ClX and morphine indicates a segregation for mechanisms for the antinociceptive effect. As can be seen in Fig. 2, nifedipine shares with morphine the development of the time-course of analgesia, whereas ClX shows a different profile.

In addition, the unsuccessful attempts to produce tolerance, by the repeated administration of ClX, which was tested by administration of the LD_{50} of the drug or by injection of naloxone, reinforced the present suggestion that ClX is acting at a different type of receptor-site that it is not closely correlated with those activated by opioid-drugs. However this result is concordant with that reported by Ling *et al.* (1984) which suggests the possibility of different receptor mechanisms mediating morphine analgesia and many of the withdrawal signs associated with morphine dependence. Furthermore, it has been suggested by Pasternak and Wood (1986) that the analgesia induced by opioids implies the participation of the subtype opioids receptors μ_1 and δ or κ, while most of the commonly observed signs of precipitated withdrawal are not mediated through μ_1.

Furthermore, the role of the opioid receptor in the analgesic action of morphine is widely recognized since the prototypic opioid antagonists, naloxone and naloxonazine, are able to antagonize morphine-induced analgesia (Martin, 1984; Ling *et al.*, 1984; Kavaliers and Ossenkopp, 1987). Unpublished results from this laboratory indicate that naloxone was ineffective in producing a blockade of the antinociceptive action of ClX in the rat, using the formalin test.

Taking into account the findings described above, the action of the drugs at molecular levels, specifically their interaction with the binding receptor-sites, with the different calcium-channels described, the differences in the tissue regulation of calcium chan-

nels, the tissue distribution of calcium-channels and the pharmacokinetics properties of nifedipine, morphine and ClX and also the different receptor associated with pain, it is conceivable that the results reported here suggest that the analgesic action of ClX is not mediated by μ_1, δ or κ-opioid receptors. Moreover these opioid receptor subtypes are implicated in the analgesic action of morphine and the analgesic action of nifedipine is probably associated with the blockade of the transmembrane inward movement of calcium.

Based on these theories, the results obtained on the association between nifedipine and ClX may be explained as a synergistic action rather than a simple dose combination of both drugs. Further investigations are necessary to determine the precise mechanisms by which nifedipine interact with ClX.

The analgesic potency of nifedipine which we found suggests that it is a significant component that could help to explain the relief of the pain associated with the vasospastic disorders obtained by the wide therapeutic use of nifedipine in the treatment of cardiovascular disease.

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