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Effects of U-50,488H withdrawal on catecholaminergic neurones of the rat ventricle

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1 In the present study the changes in noradrenaline (NA) and dopamine (DA) content and turnover during naloxone-induced withdrawal were analysed in the right ventricle of rats chronically treated with the κ-agonist U-50,488H.

2 Rats were rendered tolerant by administration of U-50,488H twice a day for 4 days. On the day of death the animals were injected with saline or naloxone (3 mg kg^{-1}, s.c.) to precipitate a withdrawal syndrome.

3 After naloxone administration to U-50,488H- treated rats we found neither behaviour signs of physical dependence nor changes in the tissue content of noradrenaline (NA). However, naloxone induced a decrease in both cardiac normetanephrine (NM) levels and NA turnover.

4 Similarly, naloxone enhanced the dopamine content and decreased the 3,4-dihydroxyphenylacetic acid (DOPAC) concentration and dopamine turnover.

5 Importantly and in contrast to μ-agonists, the present results demonstrate that U-50,488H withdrawal produced a decrease in the NA and dopamine turnover, without behavioural signs of physical dependence.

Introduction

The endogenous opioid peptides have multiple physiological actions at both central and peripheral level which are mediated by three main classes of opioid receptors μ, δ and κ. The opioid receptors and the opioid peptides were found to be widely expressed in several peripheral tissues including the heart (Weihe *et al*., 1983; Wegener & Kummer, 1994; Jin *et al*., 1995; Steele *et al*., 1996; Witter *et al*., 1996). Moreover, the co-existence of prodynorphin-derived peptide immunoreactivity with tyrosine-hydroxylase immunoreactivity, a known marker of noradrenergic nerves in the heart, has been reported (Wegener & Kummer, 1994), and it has been proposed that opioid peptides are co-released with catecholamines from nerve terminals in the heart during sympathetic stimulation (Holaday, 1983).

It is known that the catecholaminergic system plays an important role in the development and maintenance of opioid dependence and in the effects upon drug withdrawal (for review see Nestler, 1992; Self & Nestler, 1995). Despite an abundance of substantial evidence that catecholaminergic neurones in the central nervous system are involved in opioid dependence and withdrawal (for review see Self & Nestler, 1995) only a few studies have been performed on the cardiovascular system during opioid withdrawal. Thus, it has been demonstrated that an injection of naloxone in rats pretreated with morphine precipitated a withdrawal response, including an increase in mean arterial blood pressure, biphasic heart rate response and an increase in plasma noradrenaline (NA) and adrenaline (A) levels (Dixon & Chandra, 1987; Chang & Dixon, 1990; Crúz & Villareal, 1993). Moreover, previous studies performed in our laboratory have demonstrated that the hearts of rats that had received chronic morphine-treatment exhibit excitatory reactions to naloxone-precipitated withdrawal characterized by an increase in the turnover of catecholamines (Rabadan *et al*., 1997; 1998). These studies on opioid dependence have focused on morphine which acts mainly on the μ-opioid receptor. The role of the κ-receptors during tolerance/dependence has not been well studied, and there are no conclusive results.

Therefore, the purpose of this study was to elucidate if, like morphine, U-50,488H (a κ-receptor agonist) withdrawal modifies the neurochemical activity of heart catecholaminergic neurones. To accomplish this, we examined the changes in the content of NA, its metabolite normetanephrine (NMN), dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the right ventricle of the rat. We also evaluated the body weight loss (a reliable index of the magnitude of physical dependence). All these measurements were performed after antagonist-precipitated withdrawal from U-50,488H.

Methods

Male Sprague-Dawley rats (weighing 200–220 g at the beginning of experiments) were housed four to five per cage under a 12 h light/dark cycle in a room with controlled temperature (22$\pm$1°C), humidity (50$\pm$10%) with food and water available *ad libitum*.

Experimental procedure

Rats were rendered tolerant/dependent on U-50,488H by injecting the drug twice daily (15 mg kg^{-1}, i.p.) for 4 days. This procedure has repeatedly been shown to produce a high degree of tolerance to the different effects of the drug (Bhargava *et al*., 1989; Milanés *et al*., 1991; Garaulet *et al*., 1994; 1995). Control animals received saline, i.p., on the same time schedule. On day 5, groups of rats were injected with saline, s.c., or naloxone (3 mg kg^{-1}, s.c.) since a previous study demonstrated that the administration of high doses of naloxone (3 mg kg^{-1}) block the effects of U-50,488H in a similar way to the selective κ-antagonist nor-

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binaltorphimine (Milanés *et al.*, 1991; 1994). The rats were killed 30 min after saline or naloxone administration. The possible withdrawal signs were observed before and for 30 min after administration of the opioid antagonist or saline. In addition, weight loss was calculated as the difference between the weight determined immediately before the naloxone or saline injection and a second determination made 30 min after injection.

Analytical procedure for estimation of ventricular catecholamines

After decapitation, the chest was opened with a midsternal incision and the right ventricle was dissected and stored at −80°C until assayed for catecholamines NA and its metabolite NMN; dopamine and its metabolite DOPAC were determined by high-performance liquid chromatography with electrochemical detection (h.p.l.c.–e.d.). Each tissue was weighed, placed in a dry-cooled polypropylene vial and homogenized with a Plytron-type homogenizer (setting 5 for 40 s) in 1.5 ml perchloric acid (0.1 M). The homogenates were centrifuged (20,000 r.p.m.; 4°C 15 min) the supernatant layer was removed into a 1 ml syringe and filtered through a 0.45 μm filter (Millipore) and centrifuged (15,000 r.p.m.; 4°C 20 min) again through Ultrafree MC 0.2 (Millipore). Ten microlitres of each sample were injected into a 5 μm C_{18} reverse-phase column (Waters). Electrochemical detection was accomplished with a glassy carbon electrode set at a potential of +0.65 V *vs* the Ag/AgCl reference electrode (Waters). The mobile phase consisted of a 95:5 (v/v) mixture of water and methanol with sodium acetate (50 mM), citric acid (20 mM), l-octyl-sodium sulphonate (3.75 mM), di-n-butylamine (1 mM) and EDTA (0.135 mM), adjusted to pH 4.3. The flow rate was 0.9 ml min^{-1} and chromatographic data were analysed with a Millennium 2010 Chromatography Manager (Millipore) equipment. Quantification of catecholamines and their metabolites was done by reference to a calibration curve run at the beginning and the end of each series of assays. The ventricular NA, NMM, dopamine and DOPAC concentration are expressed as ng g^{-1} weight of tissue.

Drugs and chemicals

NA bitartrate, NMN, dopamine HCl, DOPAC (used as h.p.l.c. standards) and naloxone HCl, were purchased from Sigma Chemical Co. (St. Louis, MO). Drugs were prepared fresh every day. Naloxone HCl was dissolved in sterile 0.9% NaCl (saline) and given in volumes of 0.1 ml 100 g^{-1} body weight. U-50,488H methane sulphonate (*trans* (±)-3,4 dichloro-N-methyl-N-[2-(1-pyrrolidynyl)ciclohexyl]-benzeneacetamide; a gift from Upjohn) was dissolved in saline and injected in volumes of 0.15 ml 100 g^{-1} body weight. Other reagents were of h.p.l.c. grade.

Statistical analysis

The data are expressed as means ± s.e.mean. The significance of differences in the concentrations of NA, NMN, dopamine, DOPAC and in the NMN/NA and DOPAC/dopamine ratios were determined by analysis of variance followed by the Newman-Keuls test, using a computer programme. Two tailed Student's *t* test was used when comparing the means of body weight change. Differences with a *P* value less than 0.05 were considered significant.

Results

Ventricular concentration of NA and turnover were estimated 30 min after injection of saline (control) or naloxone (U-50,488H withdrawal) to rats treated chronically with saline (control) or U-50,488H (tolerant).

Administration of naloxone to control rats treated for 4 days with saline produced a significant increase in the NMN levels and in the ratio NMN/NA without any changes in the ventricular NA content, when compared with the corresponding control treated with saline s.c. on day 5 (Figure 1). There was no significant change in the ventricular NA content when naloxone was injected to the U-50,488H-tolerant rats, as

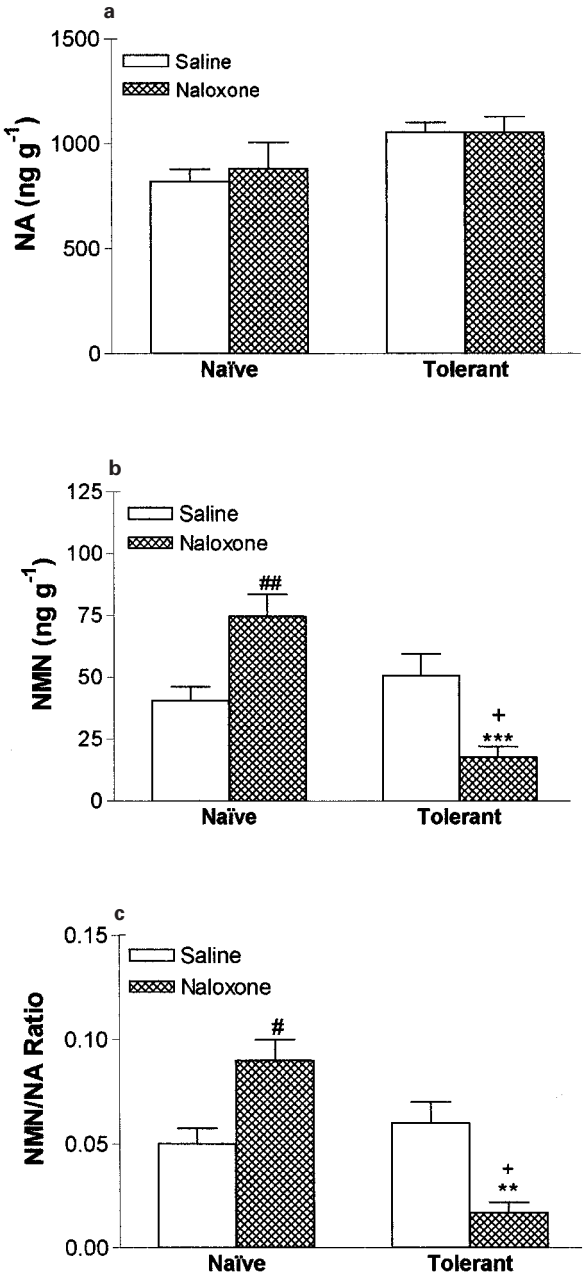

Figure 1 Ventricular (a) noradrenaline (NA), (b) normetanephrine (NMN) and (c) NMN/NA ratio in saline (naïve) and U-50,488H-pretreated (tolerant) rats after 30 min acutely injected saline s.c. or naloxone (3 mg kg^{-1}). Each column represents the mean ± s.e.mean of 5–6 experiments. $^{**}P<0.01$, $^{***}P<0.001$ *versus* naïve + naloxone; $+P<0.05$ *versus* tolerant + saline; $\#P<0.05$, $\#\#P<0.01$ *versus* naïve + saline.

compared to the tolerant group injected with saline (s.c.). However, the ventricular concentration of NMN, and the NMN/NA ratio decreased significantly in the tolerant group injected with naloxone *versus* the tolerant group injected with saline and *versus* the naïve group injected with naloxone (Figure 1).

Figure 2 depicts the ventricular dopamine content and turnover for naïve or tolerant rats. Injection of naloxone to naïve rats did not modify the ventricular concentration of dopamine, DOPAC levels or the DOPAC/dopamine ratio, compared with the naïve group injected with saline. However, the administration of naloxone to rats rendered tolerant to U-50,488H increased the ventricular concentration of dopamine when compared to the tolerant group injected with saline or the naïve group injected with naloxone. In addition, the DOPAC content and the DOPAC/dopamine ratio were significantly lower in the tolerant group injected with naloxone *versus* the tolerant group injected with saline or the naïve group injected with naloxone (Figure 2).

Rats rendered tolerant to U-50,488H receiving naloxone on day 5 did not show any of the behavioural withdrawal signs that are seen during opioid physical dependence (teeth chattering, wet-dog shakes, ptosis, lacrimation) over a 30 min period after naloxone injection. In addition, no significant change in the body weight loss was observed 30 min after naloxone administration to U-50,488-treated rats (−6.1 g), as compared to tolerant rats receiving saline (−6.2 g).

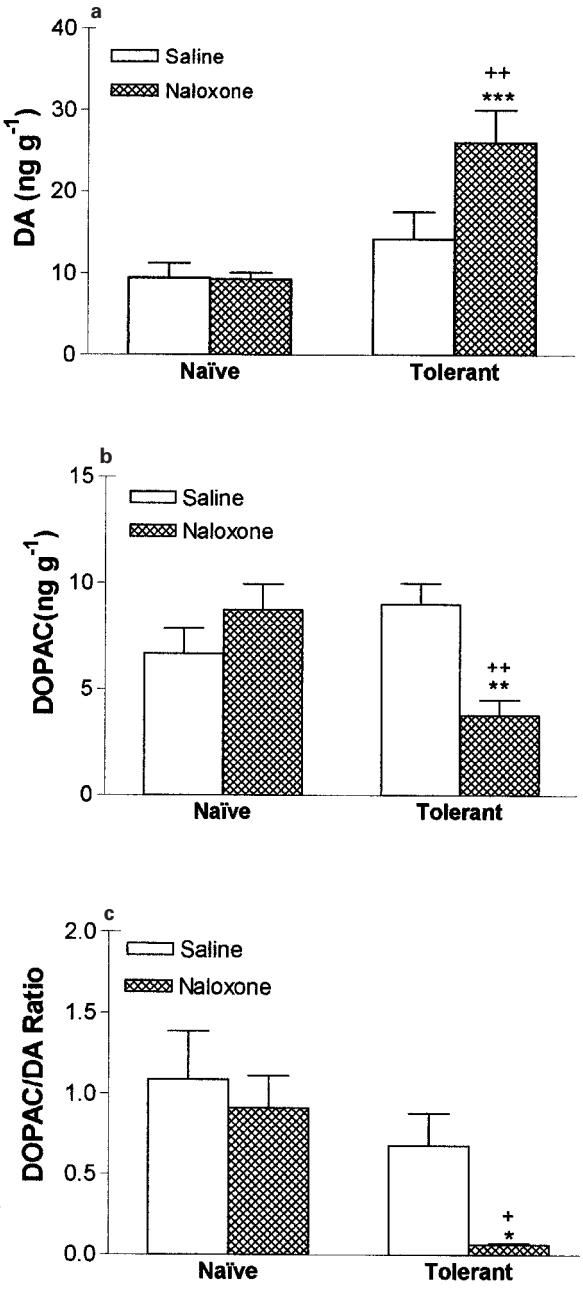

Figure 2 Ventricular (a) dopamine (DA), (b) 3,4-dihydroxy acetic acid (DOPAC) and (c) DOPAC/DA ratio in saline (naïve) and U-50,488H (tolerant)-pretreated rats after 30 min acutely injected saline s.c. or naloxone (3 mg kg^{-1}). Each column represents the mean ± s.e.mean of 5–6 experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ *versus* naïve + naloxone; +$P<0.05$, ++$P<0.01$ *versus* tolerant + saline.

Discussion

Different methods have been used in several studies to induce opioid tolerance. In the present study, the method of chronic U-50,488H administration and schedule used were similar to that previously described (Bhargava *et al*., 1989; Milanés *et al*., 1991; Garaulet *et al*., 1994; 1995) which produces a high degree of tolerance to the effects of U-50,488H in the central and peripheral nervous system. Thus, chronic administration of U-50,488H results in the developments of tolerance to its analgesic and hypothermic action. In addition, rats given a repeated injection of U-50,488H develop tolerance to the stimulant effects of κ-opioid on corticosterone release (Ignar & Kuhn, 1990; Milanés *et al*., 1991; Pechnick, 1993; Alcaráz *et al*., 1993). Previous studies performed in our laboratory have also demonstrated that chronic treatment with U-50,488H induced the development of tolerance to the inhibitory effects of U-50,488H in the guinea pig ileum myenteric plexus-longitudinal muscle (Garaulet *et al*., 1994; 1995).

The studies of opioid dependence in the cardiovascular system have focused on μ-agonists, such as morphine. However, at present the possible changes in the activity of some neurotransmitters, such as catecholamines, have not been established in the heart during U-50,488-withdrawal.

In accordance with previous studies (Ignar & Kuhn, 1990; Milanés *et al*., 1991; Alcaráz *et al*., 1993) we found that the effects of U-50,488H were abolished by the selective κ-opioid receptor antagonist nor-binaltorphimine (Alcaráz *et al*., 1993; Milanés *et al*., 1994) as well as by high concentrations of naloxone (Buckingham & Cooper, 1986; Milanés *et al*., 1991) which also binds to κ-receptors (Blake *et al*., 1996), although much higher doses are required (Magnan *et al*., 1982; Von Voightlander *et al*., 1983). So, the doses of naloxone used in the present study to induce withdrawal were three times the dose required to block the effects of morphine administration, and that previously demonstrated to block the effects of U-50,488H on the hypothalamus-pituitary-adrenal axis in a similar way to the selective κ-antagonist nor-binaltorphimine (Milanés *et al*., 1991; 1994).

Physical dependence associated with chronic morphine treatment is characterized by a withdrawal syndrome comprising various specific behavioural signs, which occur after abrupt cessation of treatment or after administration of an opioid antagonist. Importantly, the present results show that, in contrast to morphine, chronic U-50,488H administration did not produce withdrawal behavioural signs. Body weight loss is a more prominent withdrawal sign in morphine-dependent animals (Tang & Collins, 1985), indicating the involvement of μ-opioid receptors. Our results showing no body weight loss during U-50,488H withdrawal suggest that κ-

receptors are not involved in the development of behavioural signs of opioid physical dependence. The present results agree with previous findings indicating that dependence upon κ-opioid agonists was different from morphine-dependence in rhesus monkeys or in rodents (Gmerek *et al*., 1987; Cowan *et al*., 1988).

In the present study, the increased NMN concentration and NA turnover induced by naloxone in saline treated rats was not accompanied by a decrease in NA levels, suggesting a compensatory increase in the NA synthesis. These results agree with those from a previous study performed in the central nervous system (Lookingland *et al*., 1991) showing that stress-induced activation of noradrenergic neurones that project to the paraventricular nucleus is compensated for by an equivalent increase in the rate of NA synthesis, and this maintains the concentration of NA in this brain region. In addition, the present data suggest that κ-receptors are involved in the endogenous control of catecholaminergic activity, since naloxone increased NMN content and NMN/NA ratio. In contrast, the acute activation of κ-opioid receptors induced an inhibition of neuronal activity as has been demonstrated previously (Ledda *et al*., 1984; Starke *et al*., 1985; Musha *et al*., 1989; Ledda & Mantelli, 1992; Micol & Laorden, 1994; Steele *et al*., 1996).

On the other hand, previous studies have demonstrated that an injection of naloxone into rats treated with morphine precipitated a withdrawal response including an increase in mean arterial blood pressure, biphasic heart rate response and increase in plasma NA and dopamine (Dixon & Chandra, 1987; Chang & Dixon, 1990; Crúz & Villareal, 1993). In addition, studies in our laboratory have demonstrated that naloxone-precipitated withdrawal is characterized by a decrease in the auricular content of NA, A and dopamine, whereas the ratio DOPAC/dopamine was increased, suggesting that the abstinence syndrome is characterized by activation of catecholaminergic neurones in the heart (Rabadan *et al*., 1997; 1998). Importantly, the present results show that, in contrast to morphine, U-50,488H withdrawal was not accompanied by an increase in the heart catecholaminergic activity that was seen after morphine withdrawal. By contrast a greater decrease in the ventricular NMN content and in the NA turnover was observed after naloxone administration to U-50,488H-tolerant rats. The decrease in the NMN levels could be due to inhibition of the activity of catecholaminergic neurones during U-50,488H withdrawal. Similarly, the U-50,488H withdrawal was characterized by a decrease in the DOPAC content and in the turnover of dopamine, suggesting that U-50,488H withdrawal is characterized by a decrease in the activity of catecholaminergic neurones in the heart.

In conclusion, our results show that U-50,488H withdrawal did not induce either behavioural signs of physical dependence or the body weight loss that is seen after μ-agonists withdrawal. In addition the activity of the heart catecholaminergic neurones were decreased, in contrast to the activation of catecholaminergic neurones observed during morphine withdrawal (Rabadan *et al*., 1997; 1998), which might indicate that dependence on κ-agonists is characterized by the opposite changes in NA and dopamine activity compared to those occurring during μ-opioid dependence.

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