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European Journal of Pharmacology 412 (2001) 239–245

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Caffeine increases paragigantocellularis neuronal firing rate and induces withdrawal signs in morphine-dependent rats

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Received 8 June 2000; received in revised form 20 December 2000; accepted 22 December 2000

Abstract

Using single unit recording in nucleus paragigantocellularis neurons located in the rostral ventrolateral medulla, and measuring the precipitated withdrawal syndrome, we investigated whether chronic morphine administration would produce adaptive changes in the adenosine system. Caffeine (50 mg/kg, i.p.) induced withdrawal signs (head shakes, tooth chattering, ejaculation, chewing, and irritability) in morphine-dependent rats 10–18 min after the injection. Only the tooth chattering and diarrhea were expressed following a direct paragigantocellularis injection of caffeine (200 μM, 0.5 μl). The spontaneous activity of paragigantocellularis neurons was significantly decreased by microinjection of both adenosine (10 nM) and an adenosine A_1 receptor-selective agonist, cyclohexyladenosine (200 μM), into the paragigantocellularis nucleus of both control and morphine-dependent rats, but the decrease in firing rate of paragigantocellularis neurons of morphine-dependent rats was greater than that of control ones. There was also a significant enhancement of spontaneous activity of paragigantocellularis neurons 8–15 min after caffeine administration (50 mg/kg, i.p.) and 10–18 min after the injection of an adenosine A_1 receptor-selective antagonist 8-phenyltheophylline (10 mg/kg, i.p.) in both control and morphine-dependent rats. However, the effect of the antagonists was greater in morphine-dependent rats than in control ones. These data suggest that there is an increase in the sensitivity of nucleus paragigantocellularis neurons to adenosine receptor ligands in morphine-dependent rats that may be associated with the ability of caffeine to produce withdrawal signs.

Keywords: Morphine tolerance; Morphine dependence; Nucleus paragigantocellularis; Single unit recording; Adenosine receptor; Withdrawal sign

1. Introduction

The important role of the opiate system in the relief of pain has been well proven. Therefore, ordinarily, exogenous morphine is used to alleviate nociception. Unfortunately, morphine consumption has several side-effects including tolerance, dependence and withdrawal syndrome (Rasmussen et al., 1990). In spite of the fact that there are many reports on the subject of appearance of tolerance, dependence and withdrawal syndrome (Rasmussen and Aghajanian, 1989; Haghparast et al., 1998), the exact mechanism of these phenomena, especially the withdrawal syndrome, has not yet been elucidated.

More recent work has indicated that there are a large number of projections from the nucleus paragigantocellularis to the nucleus locus coeruleus (Cedarbaum and Aghajanian, 1978; Luppi et al., 1993). While paragigantocellularis predominantly activates the locus coeruleus via an excitatory amino acid pathway (Ennis and Aston-Jones, 1988), its activity plays an important role in the opiate withdrawal-induced activation of locus coeruleus (Rasmussen and Aghajanian, 1989). In the opiate-dependent rats, there is a marked increase in the firing rate of locus coeruleus neurons during opiate antagonist-precipitated withdrawal (Akaoka and Aston-Jones, 1991; Rasmussen et al., 1990). This increase in the activity of locus coeruleus neurons has been hypothesized to mediate the opiate withdrawal syndrome (Maldonado and Koob, 1993). However, it should be mentioned that the occurrence of morphine tolerance and dependence results from low paragigantocellularis neuronal activity (Haghparast et al., 1998). During

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)00718-X

morphine withdrawal, there is an increase in unit activity of paragigantocellularis neurons that, in turn, increases the unit activity of locus coeruleus neurons (Guyenet and Young, 1987; Punch et al., 1997).

A wealth of experimental data suggests a convergent relationship between adenosine receptors and opiate system (Dionyssopoulos et al., 1992; Michalska and Male, 1993; Zarrindast et al., 1997). Initially, it was shown that following chronic morphine administration, there is an increase in brain cAMP content (Bernstein and Welch, 1997; Salles et al., 1978), and up-regulation or hypersensitivity of adenosine A_1 receptors (Ahlijanian and Takemori, 1986; Kaplan et al., 1994). Another work has shown that adenosine analogues can be used to treat opiate addiction (Dionyssopoulos et al., 1992). Since adenosine agents can alleviate the morphine withdrawal syndrome (escape attempts, body shakes, jumping and diarrhea), and since caffeine as an adenosine receptor antagonist enhances the morphine withdrawal syndrome (Zarrindast et al., 1997; Michalska and Male, 1993), the adaptive changes in the adenosine system of paragigantocellularis nucleus were investigated in morphine-dependent rats. Both single unit recordings and behavioral measurements were used.

2. Materials and methods

2.1. Animals

National Medical Research Institute (NMRI) male rats (300–380 g) were divided into two groups. The first group, the control, received 3% sucrose in tap water and the second group, the dependent group, received morphine sulfate and 3% sucrose in tap water. There were four subgroups in each group: (1) adenosine, (2) N^6-cyclohexyladenosine, (3) caffeine and (4) 8-phenytheophylline subgroups. Adenosine (10 nM, 0.5 μl) and N^6-cyclohexyladenosine (200 μM, 0.5 μl) were microinjected into the paragigantocellularis nucleus, while caffeine (10, 20 and 50 mg/kg) and 8-phenyltheophylline (10 mg/kg) were administered intraperitoneally. Dependence was assessed behaviorally based on evaluation of a naloxone (2 mg/kg, s.c.)-precipitated abstinence syndrome.

2.2. Drugs

Morphine sulfate (Temad, Iran) was dissolved in tap water. Caffeine (Fluka) was dissolved in distilled water for i.p. injection and 8-phenyltheophylline initially was solubilized in ethylenediamine and then was diluted in distilled water for i.p. injection. Adenosine (Sigma), caffeine and N^6-cyclohexyladenosine were dissolved in artificial cerebrospinal fluid (ACSF) for microinjection. The composition of ACSF consisted of (in mM): NaCl 124, KCl 5, $MgCl_2$ 2, $NaHCO_3$ 26, $CaCl_2$ 2, KH_2PO_4 1.25, D-glucose 10.

2.3. Chronic morphine administration

Animals were kept four per cage with controlled temperature and 12-h light–dark cycle. Rats were fed ad libitum (food pellets) and given morphine sulfate, in solution, as sole source of fluid. Sucrose 3% w/v was added to the morphine solution (tap water with morphine) to mask the bitter taste of morphine sulfate. Animals were made dependent by chronic administration of morphine 0.1, 0.2 and 0.3 mg/ml each for 48 h and 0.4 mg/ml during the following 15 days (Badavy and Evans, 1982). In our pilot study, with measurement of the animal's morphine sulfate intake, the morphine received was about 48 mg/kg/day. In control rats, there was only sucrose in the tap water. The withdrawal syndrome precipitated by naloxone (2 mg/kg, s.c.) was used as an indicator of the development of dependence on morphine. The behavioral parameters of the withdrawal syndrome are wet-dog shake, diarrhea, ptosis, tooth chattering, writhing, paw tremor, irritability to touch and handling.

2.4. Experimental procedures

2.4.1. Behavioral experiments

We examined the behavioral effect of systemic and local caffeine in morphine-dependent rats. The effects of systemic caffeine on occurrence of withdrawal signs were investigated using caffeine (50 mg/kg, i.p.) administration in control and dependent rats. For illustration of the effect of local caffeine on the occurrence of behavioral signs, initially the animals (control and dependent rats) were anesthetized with ketamine (125 mg/kg, i.p.) and then were fixed in a stereotaxic instrument. A 2-mm diameter hole was made in the skull above of paragigantocellularis nucleus (11.65–11.96 mm caudal to bregma and 1.6–1.7 mm lateral to midline) according to the atlas of Paxinos and Watson (1986). Then, a guide cannula was conducted vertically toward the paragigantocellularis nucleus with the microdriver of the stereotaxic instrument (9.6–10.1 mm ventral to the skull surface). Finally, the guide cannula was fixed to the skull with dental cement. After 1-day recovery, animals were given 0.5 μl caffeine (200 μM) microinjected into the paragigantocellularis nucleus. Microinjection was carried out via a guide cannula with a microinjector needle connected to a Hamilton syringe. Following drug administration, behavioral signs were observed in control and dependent rats.

2.4.2. Assessment of withdrawal signs

At the end of the 21-day treatment period, the rats were weighed and injected i.p. (50 mg/kg) or intranuclearly (200 μM, 0.5 μl) with caffeine and placed into clear Perspex boxes ($20 \times 20 \times 30$ cm). The presence or absence of withdrawal signs was noted for each animal over a 20-min observation period immediately following caffeine administration. Assessment was performed on two

rats simultaneously. Quantal assessment was used, i.e. data were calculated as the number of animals per group displaying each specific sign. At the end of the observation period rats were removed from the observation boxes, reweighed and weight loss during withdrawal was calculated.

2.4.3. Paragigantocellularis recording and data collection

NMRI rats were anesthetized with urethan (1.2 g/kg, i.p.), and maintained in a deep anesthesia with additional doses (0.15 g/kg) every 1 h as needed. Then, after insertion of a tracheal cannula (ventilation by spontaneous respiration), the animals were placed in a stereotaxic instrument. Body temperature was maintained at 35.5–36.8°C with a thermistor-controlled heating pad. For single unit recording, a 2-mm diameter hole was drilled in the skull above the paragigantocellularis nucleus, 11.65–11.96 mm caudal to bregma, 1.6–1.7 mm lateral to the midline according to the atlas of Paxinos and Watson (1986), and the dura was reflected.

For microinjection of drugs in the paragigantocellularis nucleus, a guide cannula was implanted in paragigantocellularis with a 30° angle from coronal plane, 5.7 mm caudal to the drilled hole and 10.3 mm ventral to skull surface (5.7 and 10.3 mm calculated according to 30° angle tangent). Then, the set-up for drug microinjection which comprised a microinjector needle connected to a Hamilton syringe, was guided toward the paragigantocellularis nucleus via a guide cannula.

For extracellular recording from individual neurons, a glass micropipette (2–12 MΩ impedance) filled with 2% pontamine sky blue dye in 0.5 M sodium acetate was used.

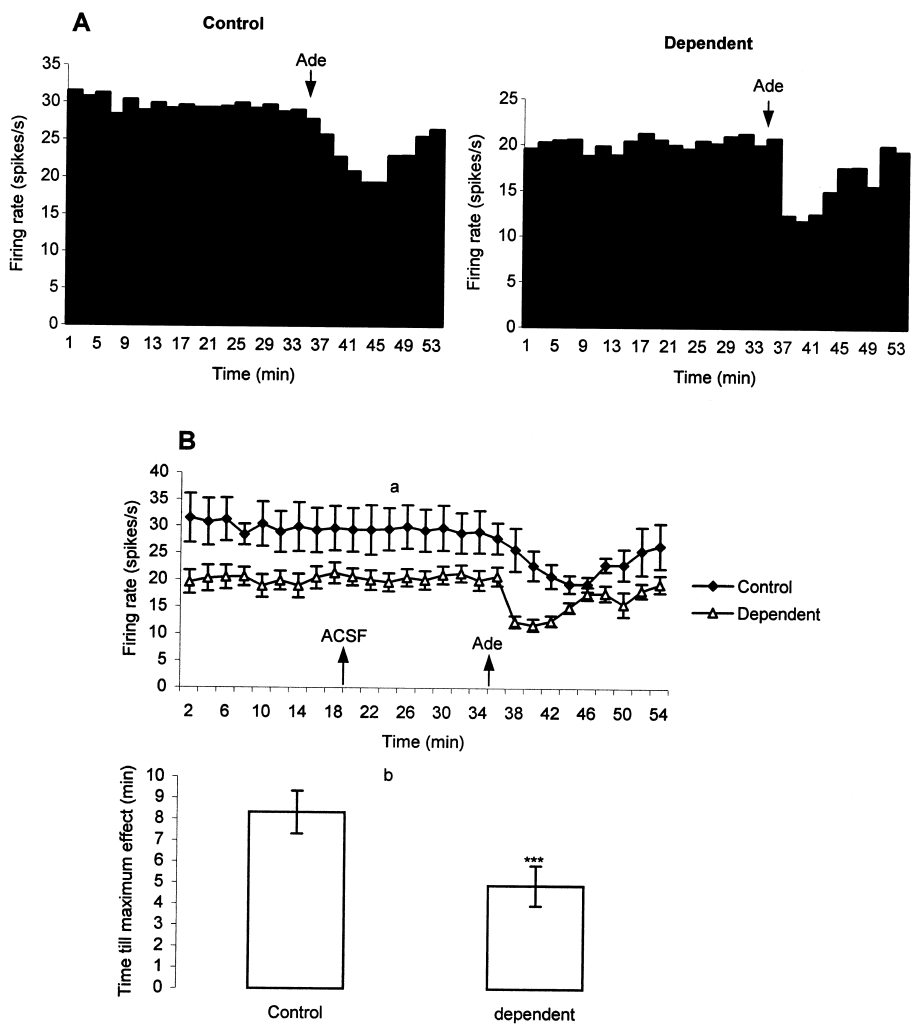

Fig. 1. Two typical PSTH unit activities of paragigantocellularis neurons in control and dependent rats during adenosine (10 nM, 0.5 μl) microinjection into the paragigantocellularis nucleus (A). Adenosine (Ade) significantly decreased unit activity in control (22.4%) and dependent (40.7%) rats. Paragigantocellularis neuronal firing rates (spikes/s) in control and dependent rats (B_a). Data expressed as mean values of paragigantocellularis neuronal firing rate ± S.E.M. during baseline recording, vehicle (ACSF) and adenosine application. Statistical analysis revealed a significant difference within 2–6 min after adenosine administration between control and dependent groups. These results are representative of seven animals. The time till maximum effect of adenosine in control and dependent rats (B_b). Data (means ± S.E.M.) were analyzed by two-factor ANOVA and multiple comparison test (Tukey) that showed a significant difference between control and dependent rats. $^{***}P < 0.001$.

The stereotaxic instrument was used to lead the micropipette into paragigantocellularis (approximately 9.6–10.1 mm ventral to skull surface), for finding single unit spikes. Following this, unit activity was amplified with a microelectrode amplifier (Nihon Kohden), and displayed continuously on a storage oscilloscope (Tektronix) as unfiltered and filtered (100–10 kHz) signals. At the same time, an audio monitor monitored the signals. A window discriminator (WPI) isolated action potentials from background activity, and led to a computer for on-line data collection. Our data, as the number of spikes in a time unit (time unit was set manually between 100 ms and 3600 s), were saved in the computer in the Peri Stimulus Time Histogram (PSTH) program. In these experiments, the time setting for data collection was 180 s with 500-ms bin size as a file which was saved continuously on hard disk during the experiment and unit activity was calculated by computer as an average frequency (spikes/s). For data presentation, unit activity is shown at 15-min intervals.

2.4.4. Data processing

With recording from a single unit and at a stable firing rate (20–30 min), pre- and post-drug injection, spontaneous firing rate was defined so as to yield comparable data. Adenosine (10 nM) and cyclohexyladenosine (200 μM) were microinjected into the paragigantocellularis nucleus (Thomas and Spyer, 1996), caffeine (10, 20 and 50 mg/kg) and 8-phenyltheophylline were administered intraperitoneally. Following drug administration, unit activity recording was continued till recovery of neuronal firing rates. A change in paragigantocellularis neuronal activity was defined as drug effect (Figs. 1A and 2A) which showed a decrease or an increase from mean baseline activity ± two standard deviations (2 SD), respectively.

The results were expressed as means ± standard error of mean (S.E.M.). Firing rates before and after drug administration were compared by Student's *t*-test and percent change was calculated [mean post-drug injection − mean pre-drug injection/mean pre-drug injection]. The data were subjected to one-way and two-way analysis of variance (ANOVA) followed by a protected Tukey's test for multiple comparisons, as needed. A $P < 0.05$ was considered to represent a significant difference.

2.4.5. Histological verification

After the end of each experiment, pontamine sky blue dye marked the recording site and then 0.9% saline followed by 10% formalin phosphate buffer solution was perfused through the heart. The brain was removed, paraffin-sectioned (10 μm thick) and stained (H and E) for histological examination. The recording sites were checked against the atlas of Paxinos and Watson (1986). Our data were confirmed for paragigantocellularis recording sites.

3. Results

3.1. Effects of adenosine on the baseline firing rate of paragigantocellularis neurons

In our experiments, after the stabilization period (20–30 min) and baseline recording (20 min) in each group (control and dependent rats), adenosine was administered and unit activity recording was continued till abolishment of

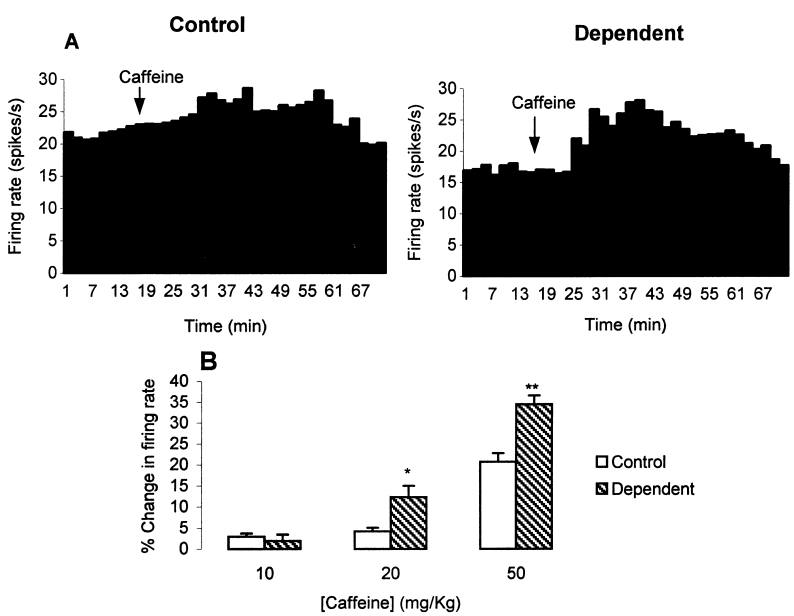

Fig. 2. Two typical PSTH unit activities of paragigantocellularis neurons in control and dependent rats during caffeine (50 mg/kg, i.p.) administration (A). As indicated, the maximum effect of caffeine occurred 8–15 min after the injection. Data analysis showed a significant difference between control (21.5%) and dependent (33.3%) groups. Effects of caffeine (10, 20 and 50 mg/kg, i.p.) on paragigantocellularis neuronal firing rates in control and dependent rats (B). These results are representative of six to seven animals. Bars show percentage change in firing rate due to caffeine administration (mean ± S.E.M.). $^{*}P < 0.05$, $^{**}P < 0.01$, Two-factor ANOVA followed by Tukey's test.

the drug effect and recovery of neuronal firing rate. Fig. 1A shows typical neuronal activity in control and dependent rats. As indicated, adenosine (3–6 min after the administration) decreased paragigantocellularis neuronal firing rates 22.4% and 40.7% in control and dependent rats, respectively. Student's *t*-test revealed that the difference between control and dependent rats was significant ($P < 0.001$). In dependent rats, adenosine thus affected paragigantocellularis neuronal activity more rapidly and more strongly than in control rats (Fig. 1B). The onset of suppression occurred within 1–3 min in control and 20–90 s in the dependent rats, and the maximum effect was observed 4–12 min and 3–6 min in control and dependent rats, respectively. Thus, the time till the maximum effect in dependent rats was shorter than in the control rats (Fig. 1B).

3.2. Effects of caffeine on the baseline activity of paragigantocellularis neurons

Fig. 2 shows that increasing the dose of caffeine significantly increased the spontaneous activity of paragigantocellularis neurons by 2.9–21.5% in control rats. The caffeine effect in dependent rats was also dose-dependent and caffeine significantly increased the spontaneous activity of paragigantocellularis neurons by 1.89–33.3%. After the data were subjected to two-way ANOVA, an overall significant difference was shown between control and dependent groups [$F(1,28) = 13.8643$, $P < 0.001$]. Post hoc analysis revealed that the difference between control and dependent rats was significant at 20 and 50 mg/kg ($P < 0.01$, Fig. 2B).

3.3. Effect of N^6-cyclohexyladenosine and 8-phenyltheophylline on baseline activity of paragigantocellularis neurons

Fig. 3 shows the effects of N^6-cyclohexyladenosine (200 μM, 0.5 μl) as an adenosine A_1 receptor agonist and 8-phenyltheophylline (10 mg/kg, i.p.) as an adenosine A_1 receptor antagonist on paragigantocellularis neuronal activity in control and dependent rats. As indicated (Fig. 3B), cyclohexyladenosine decreased paragigantocellularis neu-

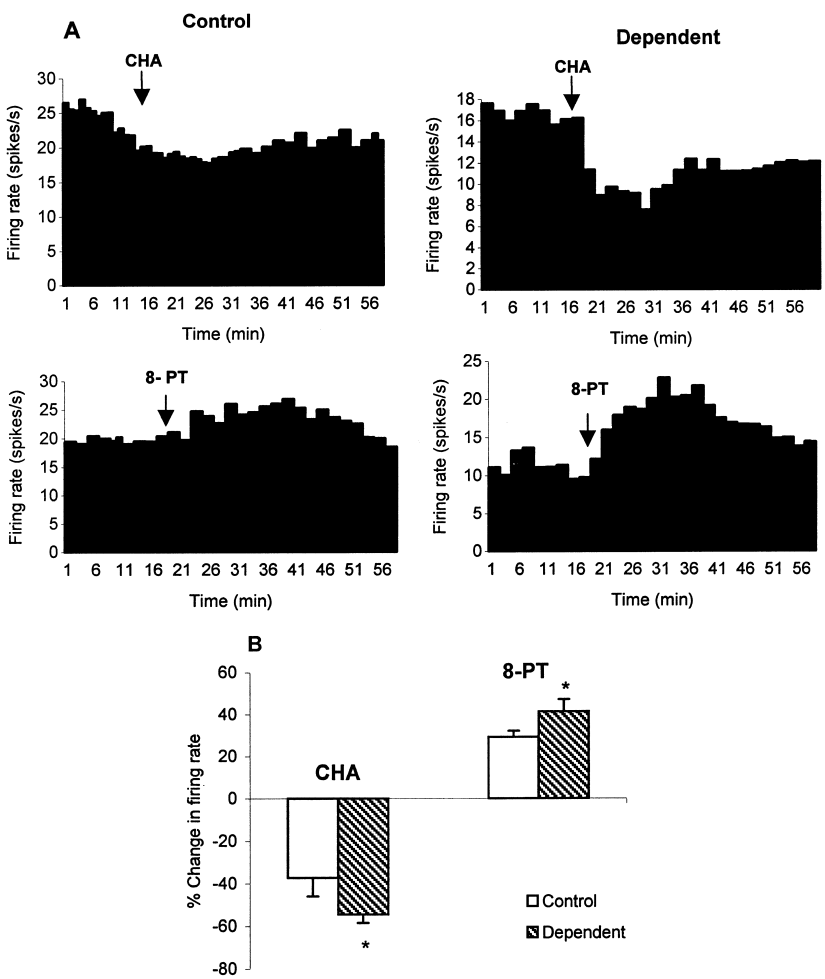

Fig. 3. Four typical PSTH unit activities of paragigantocellularis neurons in control and dependent rats during N^6-cyclohexyladenosine (200 μM, intranuclear, CHA) and 8-phenyltheophylline (10 mg/kg, i.p., 8-PT) administration (A). The effects of N^6-cyclohexyladenosine and 8-phenyltheophylline in control and dependent groups. These results are representative of six animals in each group. * $P < 0.05$, unpaired *t*-test.

ronal activity in both dependent (54.5%) and control (37.2%) rats. The difference between the two groups was significant ($P < 0.05$, unpaired *t*-test). The 8-phenyltheophylline increased paragigantocellularis neuronal activity in both dependent (41.3%) and control (29.2%) rats. The absolute effect of 8-phenyltheophylline in dependent rats was greater than that in control rats ($P < 0.05$, unpaired *t*-test).

3.4. Systemic and intranuclear caffeine induced withdrawal signs

To find the behavioral correlate of caffeine effects on paragigantocellularis neurons, the precipitation of withdrawal signs was evaluated following systemic and local caffeine injection. Surprisingly, 10–18 min after i.p. caffeine administration, five withdrawal signs (head shakes, tooth chattering, ejaculation, chewing, and irritability) were expressed in morphine-dependent rats (Fig. 4A). The latency of expressed withdrawal signs was the same as that of caffeine effects on paragigantocellularis neurons. When caffeine (200 μM) was microinjected into the paragigantocellularis nucleus only the tooth chattering and diarrhea were expressed (Fig. 4B).

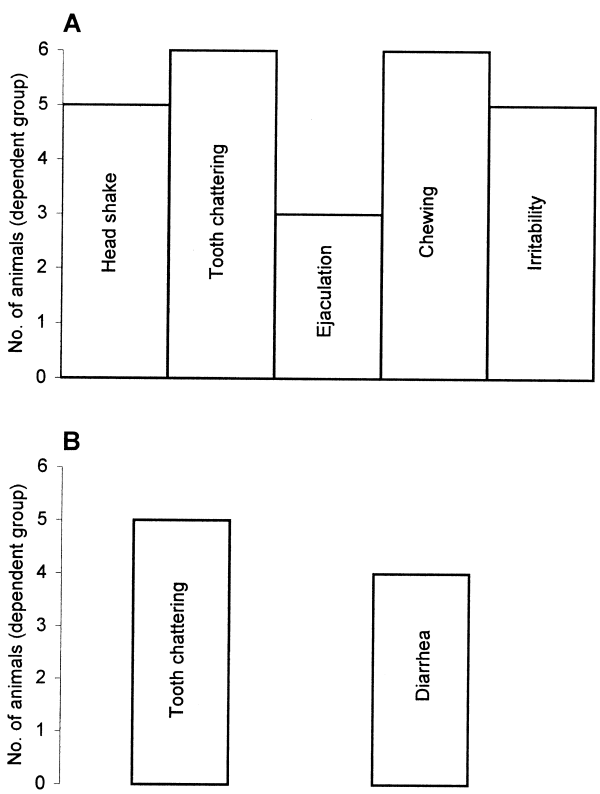

Fig. 4. Withdrawal signs precipitated by caffeine administration (50 mg/kg, i.p.) in morphine-dependent rats (A). Withdrawal signs precipitated by caffeine microinjection (200 μM, 0.5 μl) into paragigantocellularis nucleus in morphine-dependent rats (B). These results are representative of six animals.

4. Discussion

There are different protocols to induce tolerance to, and dependence on morphine. In our experiments, morphine was administered in the drinking water to avoid stress of handling and injection. The development of dependence with this protocol was checked in our pilot study. This mode of dependence is more similar to human dependence and addiction, because the animals adjust the amount of drug received during the development of dependence (Haghparast et al., 1998).

In our experiments, the waveforms, amplitude and spontaneous activity of paragigantocellularis neurons were similar, as had been reported by Ennis and Aston-Jones (1987). In addition, it has been indicated that the paragigantocellularis nucleus and its projections to the locus coeruleus have an important role in morphine tolerance, dependence and withdrawal syndrome. A low and high paragigantocellularis neuronal activity during dependence and withdrawal syndrome, respectively, have been shown (Rasmussen and Aghajanian, 1989; Haghparast et al., 1998).

We found that while both adenosine and cyclohexyladenosine (an adenosine A_1 receptor-selective agonist and metabolically resistant analogue of adenosine) can markedly decrease the spontaneous activity of paragigantocellularis neurons in morphine-dependent rats, both caffeine and an adenosine A_1 receptor-selective antagonist (8-phenyltheophylline) tended to increase it. These results probably indicate adaptive changes in the paragigantocellularis adenosine system, in particular, in adenosine A_1 receptors due to chronic morphine consumption. Phenomena of adaptation to chronic morphine treatment have been described in various experimental models of tolerance to, and dependence on morphine. Hypersensitivity and up-regulation of adenosine A_1 receptors have been reported for cortical, striatal and hypothalamic sites (Kaplan et al., 1994). Michalska and Male (1993), showed that adenosine analogues could alleviate the morphine withdrawal syndrome and that an adenosine receptor antagonist, caffeine, could enhance withdrawal signs, (Zarrindast et al., 1997). Aley et al. (1995) have found that an opiate receptor antagonist, naloxone, could produce withdrawal signs in rats tolerant to adenosine receptor agonists, in particularly, adenosine A_1 receptor agonists. In contrast, an adenosine A_1 receptor antagonist, 1,3-dipropyl-8-(2-amino-4-chlorophenyl)-xanthine (PAPCX), could induce withdrawal signs in morphine tolerant rats.

We showed that caffeine increased the spontaneous activity of paragigantocellularis neurons and that the increase was greater in morphine-dependent rats. The result is in accordance with the work of Kaplan et al. (1994), who report that the central up-regulation of adenosine A_1

receptors induced by tolerance to and dependence on morphine, coincides with a significant alleviative effect of adenosine on the expression of morphine withdrawal signs.

To find the behavioral correlate of the caffeine effect on the activity of paragigantocellularis neurons, precipitation of morphine withdrawal signs following caffeine microinjection into the paragigantocellularis nucleus or i.p. caffeine administration was considered. While most of the withdrawal signs appeared 10–18 min after caffeine administration (50 mg/kg, i.p.), only two withdrawal signs (diarrhea and tooth chattering) were observed in response to caffeine microinjection into the paragigantocellularis nucleus. Therefore, the difference in behavioral repertoire could indicate a meaningful role for the paragigantocellularis nucleus in physical dependence. Our behavioral data are consistent with the reports of Zarrindast et al. (1997) and Michalska and Male (1993) that adenosine analogues can alleviate the morphine withdrawal syndrome (escape attempts, body shakes, jumping, and diarrhea), and that caffeine or theophylline as adenosine receptor antagonists enhance morphine withdrawal signs.

In conclusion, the results show that there is an increase in sensitivity to chemicals which interact with adenosine receptors in morphine-dependent rats, and that adaptive changes in the paragigantocellularis adenosine system due to chronic morphine play a role in the expression of physical dependence on opioids. Further studies are needed to dissect out the molecular mechanisms by which opiate tolerance and dependence can affect the sensitivity and number of adenosine receptors in the paragigantocellularis nucleus and to assess the contribution of this effect to phenomena of adaptation to opiate tolerance and dependence. This approach might help us to develop new strategies in treatment of opiate tolerance and dependence.

Acknowledgements

We wish to thank the Vice-chancellorship office for Research Affairs of Tarbiat Modarres University for the grant supporting this work.

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