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Inhibition by immunophilin ligands of morphine-induced tolerance and dependence in guinea pig ileum

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Abstract

Immunophilin ligands, cyclosporine A and FK506 (tacrolimus), besides their immunosuppressive action, have several effects on different neural functions, such as modulation of the release of many neurotransmitters, the reduction of nitric oxide (NO) production by the inhibition of dephosphorylation of neuronal nitric oxide synthase (nNOS) and the alteration of the expression of certain genes. Many of these actions apparently occur through the inhibition of calcineurin, a calcium-calmodulin-dependent phosphatase. On the other hand, several studies have shown that NO has a critical role in opioid-induced tolerance and dependence in both in vivo and in vitro models. In the present study, the effect of cyclosporine A and FK506 on the development of tolerance to and dependence on morphine in the guinea pig ileum was assessed. Morphine inhibited electrically stimulated twitch of ileum in a concentration-dependent manner ($pD_2 = 7.45 \pm 0.07$). Tolerance to this effect was induced by incubation of ileum with $2 \times IC_{50}$ or $4 \times IC_{50}$ of morphine for 2 h that induced a degree of tolerance of 6.81 and 18.10, respectively. The co-incubation of ileum with morphine along with either cyclosporine A or FK506 reduced the degree of tolerance significantly ($P < 0.05$) and restored the sensitivity of ileum to the morphine inhibitory effect. Dependence was induced by incubation with $4 \times IC_{50}$ of morphine for 2 h and was assessed based on naloxone-induced contractions (10^{-5} M). Cyclosporine A (10^{-9} M) and FK506 (10^{-9} M) can attenuate the development of dependence to morphine as shown by the significant decrease in naloxone-induced contractions ($P < 0.05$). These results suggest that immunophilin ligands at very low concentrations (nanomolar) can reduce the induction of acute tolerance to and dependence on morphine in the myenteric plexus of guinea pig ileum.

Keywords: Cyclosporine A; FK506; Ileum, guinea pig; Tolerance; Dependence; Morphine

1. Introduction

The chronic use of opioids is often accompanied by the development of tolerance to and/or dependence upon these agents. The mechanisms involved with these phenomena are not entirely clear and there may be more than one. While all the mechanisms by which tolerance to and dependence on morphine have yet to be determined, a growing body of evidence suggests the participation of a nitric oxide (NO)-cGMP pathway (Kolesnikov et al., 1992, 1993; Adams et al., 1993; Elliot et al., 1994; Kimes et al., 1993; Przewlocki et al., 1993; Babey et al., 1994; Bhargava, 1994, 1995; Bhargava and Bian, 1998). In addition, we have reported the involvement of the nitrergic system in the naloxone-precipitated withdrawal signs in animal models of acute cholestasis, a condition that is associated with increased activity of the endogenous opioid system (Ghafourifar et al., 1997; Dehpour et al., 1998). The first study that evaluated the effect of NO in an in vitro model of dependence on morphine was done by Capasso et al. (1998). They confirmed the involvement of NO in the expression of opioid dependence in a guinea pig ileum model.

Cyclosporine A, a lipophilic undecapeptide, and FK506 (tacrolimus), a macrolide antibiotic, which are used clinically as potent immunosuppressor agents (Borel et al., 1996), have many effects in the nervous system such as modulation of the release of certain neurotransmitters, neurotrophic influences and protection against glutamate-induced neurotoxicity (Steiner et al., 1996; Kikuchi et al., 1998; Ruiz et al., 2000; Lyons et al., 1994). These agents reduce the catalytic activity of neuronal nitric oxide synthase (nNOS) and subsequently

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cause the inhibition of NO release (Dawson et al., 1993; Sharkey and Butcher, 1994; Rao et al., 1996; Snyder et al., 1998). This latter mechanism has been implicated in some of the important functions of cyclosporine A in the nervous system (Sabatini et al., 1997; Ruiz et al., 2000; Sanchez-Lozada et al., 2000; Homayoun et al., 2002a,b).

Some early reports have shown that cyclosporine A modulates opioid-induced dependence (Dafny et al., 1985; Dougherty et al., 1986a, 1987, Dougherty and Dafny, 1988; Berthold et al., 1989; McVaugh et al., 1989) and alters the acute antinociceptive effects of morphine (Thompson et al., 2000). The mechanisms of these effects have not been sufficiently explained. Early studies have tried to relate such apparently neural effects of cyclosporine A to its modulatory properties on the immune system (Dougherty et al., 1986a,b, 1987; McVaugh et al., 1989). However, it has been recently revealed that protein receptors of cyclosporine A and FK506, immunophilins, are abundant in the brain in discrete neural structures where they are co-localized with the Ca^{2+}-activated phosphatase-2B (calcineurin) (Steiner et al., 1992; Dawson et al., 1994; Sabatini et al., 1997) and in the peripheral nervous system (Lukyanetz, 1997; Seaitz et al., 2002). Recently, we have reported that cyclosporine A can attenuate the development and expression of tolerance to and dependence on morphine in mice by decreasing NO production (Homayoun et al., 2002c). Since all these studies have been done in in vivo animal models where neural and immunological actions of these ligands cannot be separated, the aim of the present study was to investigate the effect of cyclosporine A and FK506 on acute morphine-induced tolerance and dependence in an in vitro model of isolated guinea pig ileum which has been extensively used for the assessment of these effects of opioids (Goldstein and Schulz, 1973; Schulz and Herz, 1976; Collier et al., 1981; Dehpour et al., 2000). The opioid receptors of the myenteric plexus in guinea pig ileum show characteristics similar to those in the central nervous system (Lujan and Rodriguez, 1981), so this method let us specifically assess the neural actions of immunophilin ligands on the tolerance and dependence induced by morphine.

2. Materials and methods

2.1. Animals

Adult male guinea pigs (300–350 g) purchased from Institute of Razi (Tehran, Iran) were used in the experiments. Animal care and uses were in accordance with institutional guidelines for laboratory animals. The animals were housed in colony cages (four guinea pigs each) with free access to food and water. They were maintained in a climate- and light-controlled room (12:12 h dark/light cycle) for at least 7 days before testing. Each experiment was performed with at least six to nine isolated preparations from different animals.

2.2. The preparation of excised tissues

The method used has been described previously (Collier et al., 1981; Rezvani et al., 1983). Animals that had been fasted overnight were killed by a blow on the head. The terminal portion of the ileum, with the 10 cm nearest the caecum discarded, was kept in Tyrode solution (mM: NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2.6H_2O$ 0.5, $NaH_2PO_4.H_2O$ 0.4, $NaHCO_3$ 11.9, glucose 5) for 30 min and then washed free of faecal matter. Segments of 2–3 cm length from the same animals were placed between platinum electrodes and were fixed at a resting tension of 0.5 g in a 20-ml organ bath and, before the administration of any drug, were equilibrated for 60 min with washing out every 15 min. Electrical stimulation was applied through a parallel platinum electrode on either side of the tissue, using supramaximal rectangular pulses for all preparations (150 V of 1 ms duration at a frequency of 0.1 Hz). Twitches were recorded isometrically with a Narco Grass force transducer connected to a Narco Grass polygraph. The bath solution was maintained at 37 °C and bubbled continuously with O_2.

2.3. Assessment of the degree of tolerance

The method used to elicit morphine tolerance and to determine of the degree of tolerance was the same as was described by Collier et al. (1981) and Rezvani et al. (1983). After 60-min equilibration, the tissues were stimulated until steady state amplitude was obtained. Morphine was added to the bath cumulatively. The half-maximal concentration of morphine that inhibited electrically induced contractions (IC_{50}) was determined.

The tissues were made tolerant by adding morphine to the Tyrode solution in different concentrations representing $2\times$ and $4\times IC_{50}$ of morphine. The tissues were washed every 15 min over a period of 2 h with Tyrode solution containing the same concentration of morphine. At the end of the incubation, the tissues were stimulated as previously until a steady amplitude comparable to that observed before preincubation with drug was obtained. The IC_{50} of morphine was then redetermined, while the concentration of morphine in the media was maintained. The degree of tolerance induced was expressed as a ratio, IC_{50} tolerant/IC_{50} non-tolerant. In order to assess the effect of immunophilin ligands on the degree of tolerance, the tissues were incubated with Tyrode solution containing cyclosporine A (10^{-9} M) or FK506 (10^{-9} M) (Snyder et al., 1998; Steiner et al., 1992) and $2\times$ and $4\times IC_{50}$ of morphine for 2 h and the degree of tolerance in this preparation was determined.

2.4. Assessment of morphine dependence

The methods used to elicit morphine dependence and typical contracture response of ileum to repeated challenge with morphine and naloxone were the same, as reported previously (Collier et al., 1981; Capasso et al., 1998). The

ilea were allowed to equilibrate for 40–60 min without washing and the response to acetylcholine (10^{-6} M) was determined three times so that the response could be expressed as a percentage of the acetylcholine maximum response. The preparation was electrically stimulated for 10–20 min (0.5 ms duration at a frequency of 0.1 Hz at supramaximal voltage). Before the addition of morphine to the bath, the electrical stimulation was switched off. Morphine ($4 \times IC_{50}$) was added to the bath and after 2 h, the exposure to naloxone (10^{-5} M) induced a strong contracture. After washout, another acetylcholine response was elicited (to verify whether ileum responsiveness was modified after the withdrawal contracture). The amplitude of contracture produced by naloxone challenge is expressed as a fraction of the maximum contraction obtained with the subsequent addition of acetylcholine to the same piece of tissue, according to a modification of the method of Collier et al. (1981):

$$\frac{\text{Response to naloxone}}{\text{Maximum response to acetylcholine}} \times 100 = \text{Tension ratio}$$

2.5. Drugs

Drugs used were morphine sulphate (Temad Pharmaceutical, Tehran, Iran), cyclosporine A (Zakaria Pharmaceutical, Tabriz, Iran), FK506 (Fujisawa Pharmaceutical, Osaka, Japan), naloxone (Tolid Daru, Tehran, Iran) and acetylcholine (Sigma, St. Luis, MO, USA). All drugs were dissolved in deionized water with the exception of cyclosporine A and FK506, which were dissolved in dimethyl sulfoxide (DMSO).

2.6. Statistical analysis

Data are expressed as means ± S.E.M. One-way analysis of variance (ANOVA) was used to compare mean pD_2 values, negative logarithm of IC_{50} and tension ratios in various experiments. $P < 0.05$ was considered as the significant level for differences between groups.

3. Results

Morphine inhibited the electrically stimulated contraction of guinea pig ileum in a concentration-dependent manner

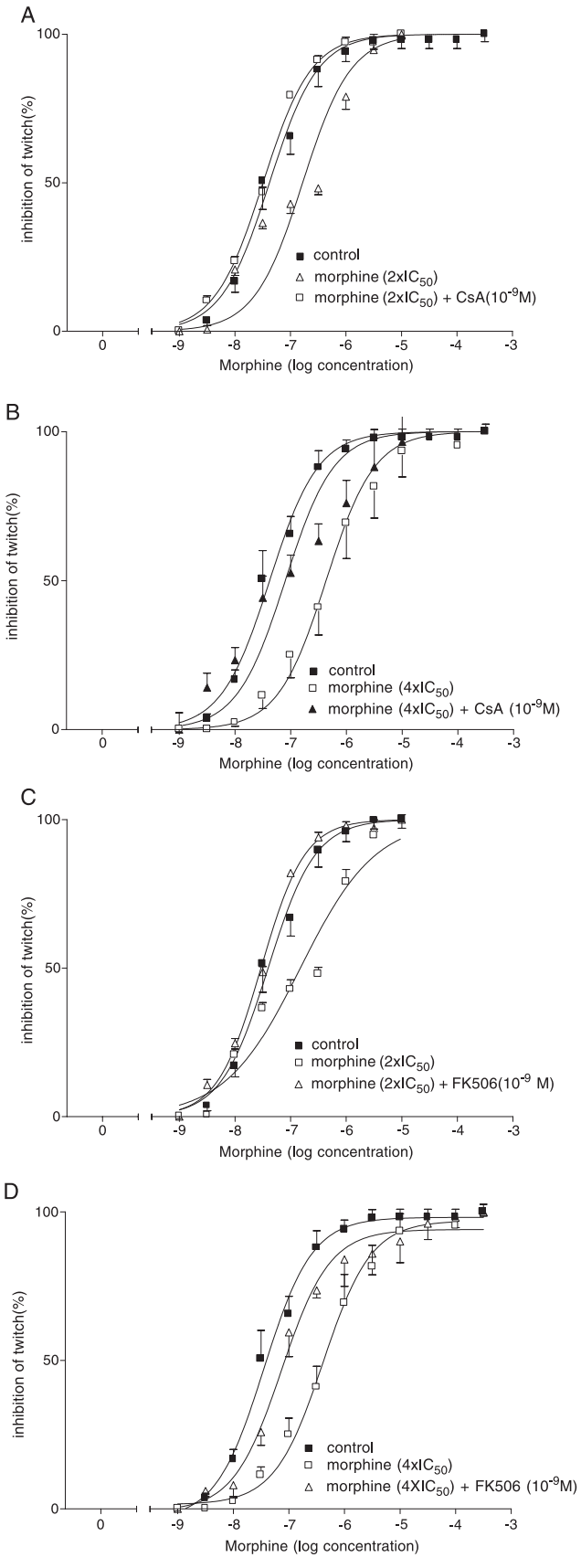

Fig. 1. Concentration–response curve for morphine inhibition of electrically induced contractions in isolated guinea pig ileum. (A) 2-hour incubation with: morphine ($2 \times IC_{50}$)[a] (□), morphine ($2 \times IC_{50}$) + CsA (10^{-9} M)[b] (▲), (B) 2-hour incubation with: morphine ($4 \times IC_{50}$)[a] (□), morphine ($4 \times IC_{50}$) + CsA (10^{-9} M)[b] (▲), (C) 2-hour incubation with: morphine ($2 \times IC_{50}$)[a] (□), morphine ($2 \times IC_{50}$) + FK506 (10^{-9} M)[b] (▲), (D) 2-hour incubation with: morphine ($4 \times IC_{50}$)[a] (□), morphine ($4 \times IC_{50}$) + FK506 (10^{-9} M)[b] (▲). Cyclosporine A (CsA). [a]$P < 0.05$ compared to control (non-tolerant) (■) group. [b]$P < 0.05$ compared to corresponding tolerant group.

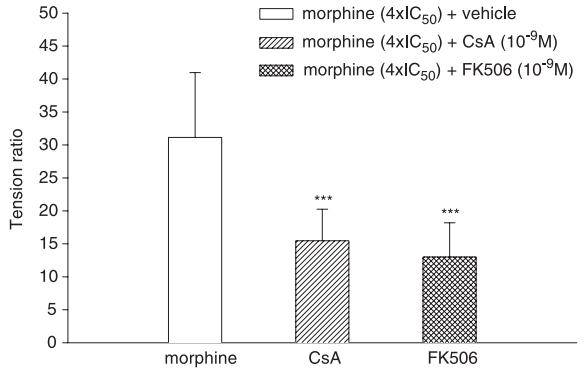

Fig. 2. The effect of CsA (10^{-9} M) and FK506 (10^{-9} M) on morphine induced dependence in isolated guinea pig ileum. Cyclosporine A (CsA). ***$P<0.05$ compared to corresponding group incubated with morphine ($4 \times IC_{50}$).

($pD_2 = 7.45 \pm 0.07$). After incubation of tissues with $2 \times IC_{50}$ or $4 \times IC_{50}$ of morphine for 2 h, the concentration–response curve for the inhibitory effect of morphine was shifted significantly to right (pD_2 of each treatment is 6.62 ± 0.07 and 6.19 ± 0.31, respectively) ($P<0.05$) and the degree of tolerance for each treatment was 6.81 and 18.10, respectively.

Cyclosporine A (10^{-9} M) co-incubated with $2 \times IC_{50}$ or $4 \times IC_{50}$ of morphine decreased the degree of tolerance significantly to 0.90 and 2.36, respectively and restored the sensitivity of guinea pig ileum to the morphine inhibitory effect (pD_2 of each treatment, 7.53 ± 0.02, 7.07 ± 0.10, respectively) ($P<0.05$) (Fig. 1A and B). FK506 (10^{-9} M) had a similar effect. The incubation with FK506 along with $2 \times IC_{50}$ or $4 \times IC_{50}$ of morphine decreased the degree of tolerance to 0.82 and 1.46, respectively (pD_2 of each treatment, 7.53 ± 0.02 and 7.28 ± 0.09, respectively)($P<0.05$) (Fig. 1C and D). The incubation of tissues with cyclosporine A (10^{-9} M) or FK506 (10^{-9} M) alone or their vehicle without morphine for 2 h did not affect the IC_{50} of inhibitory effect of morphine (pD_2 of each treatment is 7.97 ± 0.07,7.83 ± 0.06, 7.95 ± 0.06, respectively) ($P>0.05$).

Regarding the induction of morphine dependence in guinea pig ileum, we incubated the tissues with $4 \times IC_{50}$ of morphine for 2 h and thereafter added naloxone (10^{-5} M), which induced a large contraction (tension ratio = 31.14 ± 9.83). Incubation of tissues with cyclosporine A (10^{-9} M) or FK506 (10^{-9} M) along with morphine ($4 \times IC_{50}$) significantly reduced the tension ratio to 15.47 ± 4.78 and 13.00 ± 5.19, respectively ($P<0.05$) (Fig. 2). Naloxone (10^{-5} M) could not induce any contracture after a 2-h incubation with cyclosporine A (10^{-9} M) or FK506 (10^{-9} M) alone or their vehicles without morphine.

4. Discussion

There has been no previous demonstration of the inhibitory effect of immunophilin ligands on the induction of tolerance to and dependence on morphine in isolated tissues in vitro. The purpose of this study was to find out whether these ligands have inhibitory effects on the acute induction and development of these phenomena in the guinea pig ileum model.

Dafny et al. (1985) first reported that cyclosporine A considerably modified the behavioral signs of naloxone-induced opioid withdrawal in morphine-dependent rats. The exact mechanism of action of cyclosporine A was not clear at that time, so they concluded that the immune system may have a role in the chronic effects of opioids. Dougherty et al. (1987) showed that following intracerebroventricular (icv) administration, cyclosporine A can attenuate the opiate withdrawal syndrome precipitated by naloxone and suggested that cyclosporine A had direct action on the central nervous system. Berthold et al. (1989) also reported that an intact immune system is not a necessary prerequisite for cyclosporine A to attenuate withdrawal. But several early studies had tried to relate such apparently neural effects of cyclosporine A to its modulatory properties on the immune system (Dougherty et al., 1986a,b, 1987; McVaugh et al., 1989). Our results show that cyclosporine A and FK506, at very low concentrations, can inhibit morphine-induced tolerance and dependence in isolated guinea pig ileum. Since the neural elements of the myenteric plexus anatomically and neurochemically closely resemble those of the central nervous system, and since its opiate receptors show characteristics similar to those of these receptors in the central nervous system (Lujan and Rodriguez, 1981), these results suggest that the neural actions of immunophilin ligands, and not their immunologic actions, are important for the inhibition of morphine-induced tolerance and dependence. The results are consistent with the surprising finding that the levels of immunophilins in the nervous tissues are much higher than those in tissues of the immune system, implying a neural role for cyclosporine A and other immunophilin-binding ligands (Steiner et al., 1992). Further research revealed that, in the nervous system, the complex of cyclosporine A and FK506 with their protein receptors, cyclophilin A and FKBP-12, respectively, bind to and inhibit the activity of calcineurin phosphates, which in turn leads to an increase in phosphorylated levels of several important proteins including some transcriptional factors and neuronal nNOS (Dowson et al., 1994). Thus, immunophilin ligands can reduce the catalytic activity of nNOS. Meanwhile, although the mechanisms involved in the tolerance to and dependence on opioids are still not entirely clear, a growing body of evidence suggests participation of the NO-cGMP pathway. The involvement of NO in these phenomena has been suggested by reports showing that the nitric oxide synthase inhibitors abolish some aspects of the naloxone-precipitated withdrawal and attenuate the expression of both tolerance and physical dependence in in vivo rodent models (Adams et al., 1993; Cappendijk et al., 1993; Dambisya and Lee, 1996). Capasso et al. (1998) first reported that N^G-nitro-L-arginine methyl ester (L-NAME)

could prevent morphine-induced dependence in the guinea pig ileum model. According to these results, FK506 and cyclosporine A may exert their effects through inhibition of calcineurin phosphatase activity and the subsequent decrease of NO release, leading to inhibition of morphine-induced tolerance and physical dependence in guinea pig ileum. This possibility is consistent with our recent finding that cyclosporine A can prevent morphine-induced tolerance and dependence, possibly by reducing NO production in the in vivo mouse model (Homayoun et al., 2002c).

The latter study used a 2-day escalating-dose regimen for the induction of tolerance and dependence and showed the inhibitory effect of cyclosporine A on both development and expression of tolerance, but only on the expression, and not the induction, of dependence in an in vivo model of mice. This raises the possibility that the observed effects of immunophilin ligands on morphine dependence in the guinea pig ileum model may be due to mechanisms directly affecting the expression of the naloxone-precipitated response. Alternatively, it is possible that different mechanisms are involved in the induction of dependence in the in vitro rapid-exposure guinea pig ileum model and in vivo chronic treatment model. Accordingly, it is known that, at the cellular level, exposure for minutes or hours to the high concentrations of opioid agonists activates rapid opioid-specific changes in receptor-G protein coupling and adenylyl cyclase cascade, while exposure for days activates distinct mechanisms involving down-regulation of the sodium pump and altered membrane potential (Johnson and Fleming, 1989; Nestler and Aghajanian, 1997; Taylor and Fleming, 2001).

In conclusion, FK506 and cyclosporine A at low concentrations, which can interact with calcineurin-dependent pathways, can inhibit the induction of morphine-induced tolerance and dependence in the guinea pig ileum model. These effects may be due to decreased activity of nNOS, to alterations in neurotransmitter release, or may involve changes in nNOS gene expression, the underlying mechanism(s) warrant further investigation.

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