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Pertussis Toxin Inhibits Morphine Analgesia and Prevents Opiate Dependence

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PAROLARO, D., G. PATRINI, G. GIAGNONI, P. MASSI, A. GROPPETTI AND M. PARENTI. *Pertussis toxin inhibits morphine analgesia and prevents opiate dependence.* PHARMACOL BIOCHEM BEHAV **35**(1) 137–141, 1990.—Six days after intracerebroventricular pretreatment of rats with pertussis toxin (PTX 0.5 μg/rat) there was a marked decrease in the antinociceptive effect of morphine, regardless of the route of opioid administration (into the periaqueductal gray matter, intrathecally or intraperitoneally) or the analgesic test used (tail flick and jaw opening reflex). PTX pretreatment also partially attenuated the naloxone-precipitated withdrawal syndrome in morphine-dependent rats, significantly reducing teeth chattering, rearing and grooming. These in vivo findings indicate that G-protein-dependent mechanisms are involved in morphine analgesia and dependence. The biochemical mechanism could be related to ADP ribosylation of Gi coupled to the adenylate cyclase system, but an interaction of PTX with other G-proteins linked to different second messengers or directly to ionic channels cannot be excluded.

Pertussis toxin Morphine Analgesia Dependence

EVALUATION of the physiological responses induced by opiates is extremely complex because of the involvement of different neurotransmitter systems and integrated neuronal circuitries and the existence of heterogeneous opioid receptor subtypes and multiple transduction systems. In the last few years several studies on isolated cells and tissue systems have resulted in better knowledge of the molecular events mediating the actions triggered as a result of opioid receptor binding. The second messenger system most commonly associated with opioid receptors involves inhibition of adenylate cyclase, first described in brain membranes and subsequently in NG 108-15 neuroblastoma × glioma hybrid cells (3, 15, 25, 26). The opening of potassium channels in neurons of the central nervous system and inhibition of voltage-dependent calcium channels in primary cultures of dorsal root ganglia have been observed after opioid receptor activation (17). Recent findings suggest that G-proteins are involved in the inhibition of adenylate cyclase and in the control of the ion channel activity (8, 13, 14, 21, 32).

In a previous paper (18) we reported that injection of pertussis toxin (PTX) into rat cerebral ventricles greatly reduced the antinociceptive action of intracerebroventricular (ICV) morphine. The ability of PTX to reduce the opioid-induced analgesia was independent of the type of opioid receptor involved, although the blockade produced by the toxin was overcome by high doses of (D-Ala^2-N-Me-Phe^4 Gly^5-ol-)enkephalin, but not by comparable high doses of (D-Ala^2, D-Leu^5)enkephalin (22). In this study we tested the effectiveness of PTX in antagonizing the analgesia induced by morphine given by different routes and its effects on the development of physical dependence induced by subcutaneous implantation of morphine pellets.

METHOD

Animals

Male Sprague-Dawley rats, supplied by Charles River, Calco, Italy, weighing 180–200 or 280–300 g depending on experiments, were used and fed a pellet diet with water ad lib. Environmental conditions were standardized (22 ± 2°C, 60% humidity and 12 hr artificial lighting per day).

Microinjections Into the Central Nervous System

Rats were anesthetized with pentobarbital sodium (35 mg/kg IP) and two polyethylene cannulae were inserted into the brain lateral ventricles for ICV injections, according to the Altaffer procedure (1).

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Microinjections into the periaqueductal gray matter (PAG) were made in anesthetized rats as previously described (19). Briefly, 33-gauge stainless steel cannulae were inserted into permanent guides and pushed 1 mm beyond the guide tips into the intended PAG injection site. The coordinates for PAG injection sites were chosen according to the atlas of Paxinos and Watson (20) taking the bregma as a reference point (AP −7.8; L 0.5; V 5.5). The rats were used seven days after surgery. The injection volume was limited to 1 μl to minimize diffusion and tissue damage. At the end of each experiment the brains were removed and fixed in 10% formalin to verify cannula placement. All the brain blocks were frozen 24 hr later, cut to a thickness of 40 μm and alternate sections were mounted and stained with buffered safranin according to Wolf and Yen (30).

For intrathecal injections, a cannula made of polyethylene tubing was inserted into anesthetized rats 7.5–8.5 cm caudally from a transverse slit opened in the cisternal membrane according to the implantation procedure described by Yaksh and Rudy (31). The animals were used after at least one week of recovery.

Analgesic Assays

Six days after PTX pretreatment (0.5 μg/rat dissolved in 0.05 M sodium phosphate buffer pH 7 with 0.15 M NaCl) equiactive doses of morphine were injected IP, ICV into the PAG, or intrathecally, and their analgesic effects were assessed using the tail flick method or tooth pulp stimulation.

The tail flick test was performed according to D'Amour and Smith (6). A maximum cut-off time of 15 sec was used and the rat was removed from the apparatus if it failed to respond within this interval. The results for each time were expressed as the percentage increase (Δ%) over the threshold value measured before morphine, and the total area under the time-response curve (A.U.C.) was calculated.

For tooth pulp stimulation two holes were drilled in the sides of the incisors and filled with silver amalgam; 24 hours later, the experiment was run by adapting hairpins with golden pins to the silver-filled cavities of restrained rats and delivering trains of 10 Hz (8 msec duration) rectangular pulses of constant intensity (6.5 volts) at the time points shown in the figure. Only the rats that showed the characteristic jaw opening reflex (J.O.R.) in response to the electrical stimulation were used in the experiment. The degree of analgesia was determined from the percentage of animals in which the J.O.R. was suppressed by treatment at each stimulation time.

Assessment of Physical Dependence

After an interval of 72 hr after ICV PTX pretreatment (1 μg/rat), the rats were implanted with two 75 mg morphine or placebo pellets. The abstinence syndrome was precipitated 72 hr after pellet implantation by injecting naloxone (2 mg/kg IP). The presence of classical withdrawal signs was evaluated for 30 min after naloxone injection according to Grant and Redmond (12). We considered six counted signs: escape attempts, rearing, teeth chattering, chewing, wet dog shakes, grooming; and six checked signs: ptosis, piloerection, diarrhea, irritability to touch, irritability to handling, dyspnea.

Drugs

The following drugs were used: pertussis toxin (List Biological Labs., Campbell, CA), morphine hydrochloride (S.I.F.A.C., Milan, Italy), naloxone hydrochloride (Endo Laboratories, Garden City, NY).

Statistical Analysis

One-way analysis of variance (ANOVA) was done by collapsing data across all groups. This analysis was followed by individual group comparisons using Student's *t*-test (11). The checked signs were analyzed by Fisher's exact probability test (11).

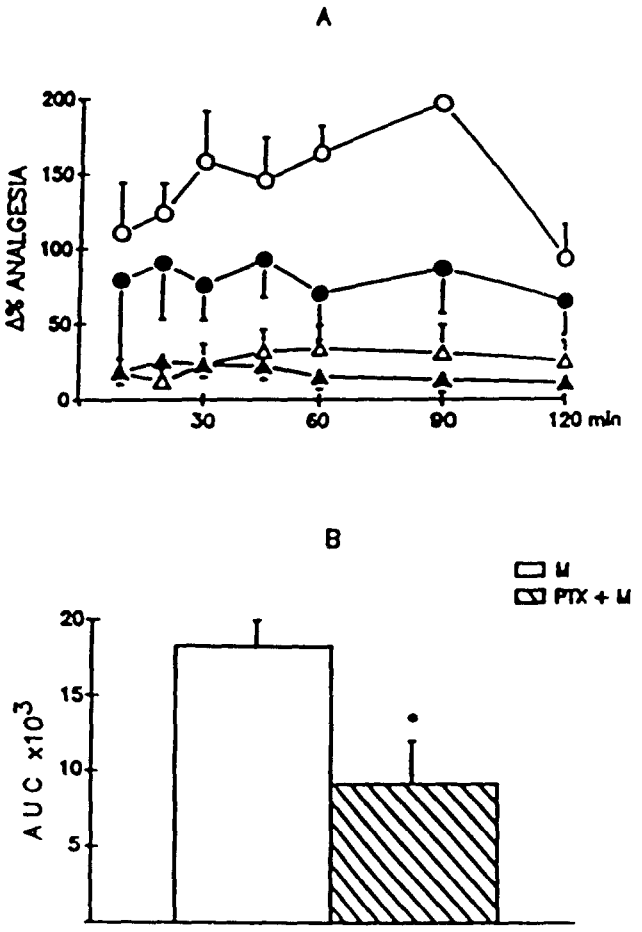


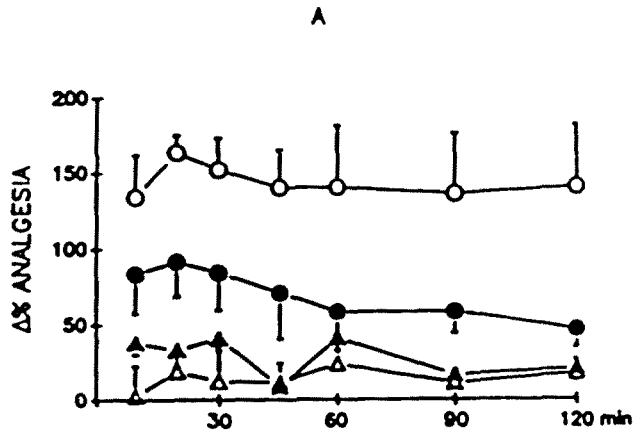
FIG. 1. (A) Effect of PTX pretreatment into the PAG (0.5 μg/rat) on analgesia (tested by tail flick) induced by morphine (3 μg/rat into the same area). △: vehicle + saline; ▲: PTX + saline; ○: vehicle + morphine; ●: PTX + morphine. (B) Effect of PTX pretreatment expressed as area under time-response curve (AUC) shown in panel A. *$p<0.05$ vs. morphine alone.

RESULTS

Figure 1 shows the time course of the analgesic effect of a microinjection of 3 μg of morphine into the ventral PAG in rats treated with PTX in the same area six days before. Basal nociceptive thresholds were the same in the pretreated animals as in controls, but morphine had significantly less antinociceptive effect in the PTX-treated rats (Fig. 1), the time-response curve being reduced to about half the control value.

Pretreatment with intrathecal (IT) PTX also reduced the antinociception produced by IT morphine (2.5 μg/rat) (Fig. 2). The area under the time-response curve for PTX-pretreated rats was reduced 60% compared to that for morphine alone (Fig. 2, panel B).

Pretreatment with ICV PTX also antagonized the analgesia induced by IP morphine (6 mg/kg) tested in rats using the J.O.R. (Fig. 3). In this test, morphine alone abolished the J.O.R. in 90% of the animals, whereas after PTX treatment morphine had no



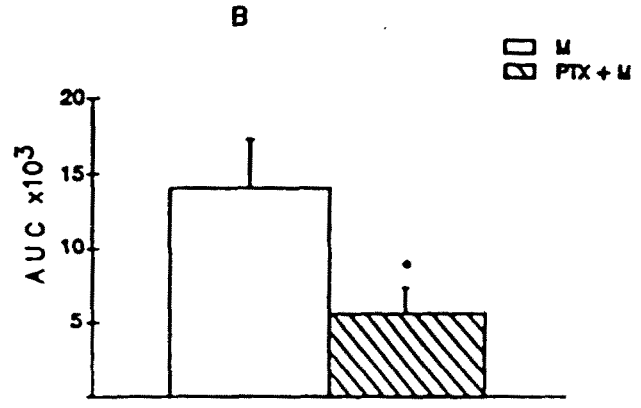


FIG. 2. (A) Effect of PTX pretreatment (0.5 μg/rat IT) on analgesia (tested by tail flick) induced by intrathecal (IT) morphine (3 μg/rat). △: Vehicle + saline; ▲: PTX + saline; ○: vehicle + morphine; ●: PTX + morphine. (B) Effect of PTX pretreatment expressed as area under time-response curve (AUC) shown in panel A. *$p<0.05$ vs. morphine alone.

effect whatsoever on this reflex.

As reported in Fig. 4, animals implanted with morphine pellets showed obvious signs of abstinence 30 minutes after naloxone injection (2 mg/kg). Only a few of these signs occurred occasionally in vehicle/placebo-implanted rats (Fig. 4). In the PTX/placebo group only the incidence of two signs, irritability to handling and dyspnea, was significantly higher than in the vehicle/placebo group. In the PTX/morphine group some of the counted signs such as teeth chattering, grooming and rearing were significantly attenuated, whereas except for dyspnea and irritability to handling whose incidence was enhanced, the checked signs were unaffected (Fig. 4).

DISCUSSION

The present data confirm our previous findings (18) that PTX constantly reduces the antinociceptive effect of morphine regardless of the route of administration of the opioid or the analgesic test

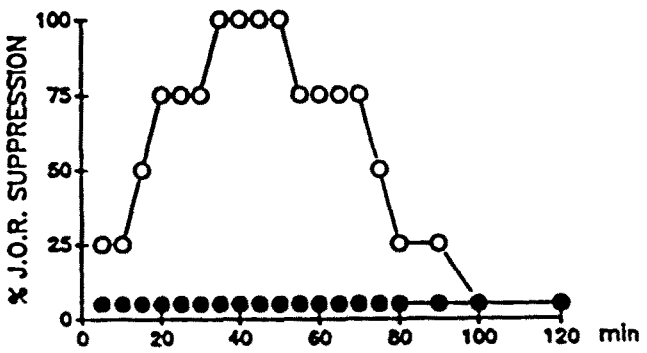


FIG. 3. Effect of PTX pretreatment (0.5 μg/rat ICV) on analgesia (jaw opening reflex, J.O.R.) induced by IP morphine (6 mg/kg). ○: Vehicle + morphine; ●: PTX + morphine.

adopted. Thus, it would appear that both spinal and supraspinal PTX-sensitive G-proteins are a necessary transduction step to start the molecular events leading to an opioid antinociceptive response in the central nervous system. The reduction in antinociception

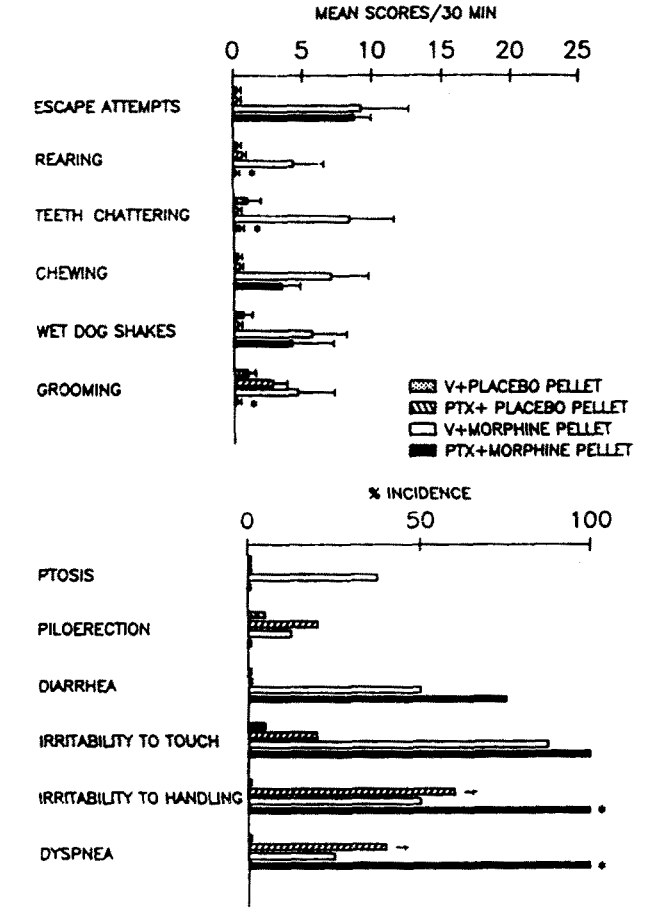


FIG. 4. Effect of PTX pretreatment on naloxone-induced signs of withdrawal in rats implanted with morphine pellets. (A) Counted signs; (B) checked signs. †$p<0.05$ vs. vehicle (V) + placebo pellets. *$p<0.05$ vs. vehicle (V) + morphine pellets.

was more evident with the J.O.R. than the tail flick test. In the J.O.R. test morphine analgesia was completely abolished by PTX pretreatment, whereas the response in the tail flick test was reduced about 60%. The different sensitivity of the two tests to PTX pretreatment could be due to activation of different pathways involved in these reflex responses. According to some authors (7,28) the tail flick test is mainly mediated spinally, while the J.O.R. mainly depends on supraspinal mechanisms (5, 9, 24). Therefore, neuronal circuits involved in the J.O.R. test seem more sensitive to PTX than those mediating tail flick responses.

However, this work provides some evidences that PTX pretreatment interferes with opioid dependence, significantly attenuating only a few signs of withdrawal, namely rearing, teeth chattering and grooming.

In agreement with the concept that withdrawal behaviour involves different neuroanatomical substrates (29), this study suggests that different biochemical events too underlie the development of dependence. Our results indicate that some of these behavioural responses may be linked to a signal transduction mechanism operated by G-proteins. The higher incidence of irritability to handling and of dyspnea observed in morphine-dependent rats pretreated with PTX may be related to the fact that these signs were also increased in rats implanted with placebo pellets and pretreated with PTX. Therefore, the increase of these behaviours could not be related to opiate withdrawal, but probably associated with PTX pretreatment. PTX, by inactivating the influence of some G-protein-dependent mechanisms, may very well cause behavioural excitation. Thus, it appears that G-proteins are only partially responsible for the adaptive mechanisms which are ultimately shown up in vivo as dependence.

Our findings partially agree with the results of Tucker (27) and Lux (15) who found that PTX completely prevented opioid-induced dependence in the guinea pig ileum-myenteric plexus. Though dependence in the guinea pig ileum preparation and the whole animal share many characteristics (2), it must be noted that dependence in the ileum is confined to a single symptom, i.e., the withdrawal contracture (10,23). According to Lux (16), this response is associated with receptors located on nerve somata that may be coupled to adenylate cyclase. So PTX, inactivating Gi through ADP-ribosylation, prevents the opioid inhibition of adenylate cyclase and the expression of dependence.

In contrast, naloxone challenge in whole animals activates several mechanisms, resulting in a variety of withdrawal symptoms, some of which seem dependent on G-proteins. The partial reduction of withdrawal after PTX might be due to some extent to poor diffusion of the toxin in brain tissue, thus not affecting peripheral G-protein-dependent mechanisms involved in the abstinence syndrome. PTX-mediated ADP-ribosylation of tissues from different brain areas of rats injected ICV with 0.5 μg PTX showed a 40–50% reduction in incorporation of ^{32}P-NAD into the protein band corresponding to the 39–41 kDa molecular weight region, indicating that this dose of toxin does not block all G-proteins (data not shown).

The neuronal G-proteins involved in opiate analgesia and in dependence are not well known. Experimental evidence from tissue systems and isolated cells indicates that changes in the activity of cAMP generating systems coupled to G_i may be an intermediate step in production of the acute and chronic effects of opiates (3, 4, 15, 16, 25–27). However, since PTX also interferes with other neuronal G-proteins coupled to different effector systems (e.g., ion channels) (13), our data do not exclude that the toxin may be active on other second messengers or ionic channel-linked G-proteins (8, 13, 14, 21, 32). Moreover, PTX could be working at opiate or nonopiate receptor linked G-proteins or at any G-protein associated with other modulators of the neurons or even other neurons involved in acute and chronic effects of opiates.

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