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Cholera Toxin Effects on Body Temperature Changes Induced by Morphine

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BASILICO, L., M. PARENTI, A. FUMAGALLI, D. PAROLARO, AND G. GIAGNONI. *Cholera toxin effects on body temperature changes induced by morphine*. PHARMACOL BIOCHEM BEHAV **56**(3) 499–505, 1997.— The present study evaluates the influence of cholera toxin and its B-subunit on thermic responses to morphine in the rats. The holotoxin (1 μg/rat) and the B-subunit (5 μg) were administered ICV and three days later rats were challenged ICV with morphine and tested for changes of body temperature. Cholera toxin, but not its B-subunit, modified the time course of the hyperthermic response induced by a low dose of morphine (2.5 μg), converted the hypothermia due to a higher dose of morphine (18 μg) to a consistent hyperthermia and only partially reduced the greater hypothermia induced by 36 μg of morphine. Cholera toxin-induced modifications of thermic responses to morphine were paralleled with a decreased $G_{s\alpha}$ immunoreactivity and a reduced ability for the toxin to catalyse the "in vitro" ADP-ribosylation of $G_{s\alpha}$ in hypothalamic membranes. In contrast, at the same time when morphine-induced effects on body temperature were assessed, no changes in pertussis toxin-mediated ADP-ribosylation of $G_{i\alpha}/G_{o\alpha}$, or basal adenylate cyclase activity, or binding of μ-opioid receptor selective ligand [^{3}H]-DAMGO were observed in hypothalamic areas from rats treated with cholera toxin. These findings suggest that adaptative events secondary to prolonged activation of $G_{s\alpha}$ play a role in the modifications of thermic responses to morphine induced by CTX.

Body temperature Cholera toxin Opioids Morphine Hypothalamus

CHOLERA toxin (CTX) is an exotoxin produced by Vibrio cholerae composed by two protomers, A and B, interacting with one another in a non-covalent manner. The B protomer, which consists of five identical polypeptide chains, binds to GM_1 ganglioside on the surface of mammalian cells and hence promotes the entry of the A protomer into cells (35). The activated A protomer carries an ADP-ribosyltransferase that catalyses the transfer of ADP- ribose from NAD^+ to an arginine residue of α-subunits of heterotrimeric G proteins, including the stimulatory G_s protein of the adenylate cyclase cascade (25). This covalent modification stabilizes the active conformation of $G_{s\alpha}$ by inhibiting the associated GTPase activity (9), and thus induces continual production of cyclic AMP (15), and decreases the sensitivity of the high affinity agonist-receptor complex to the regulatory effects of guanine nucleotides (36).

Recent evidence has shown the coupling of opioid receptors to G_s proteins (13,16,30), other than the generally accepted coupling to the inhibitory pertussis toxin (PTX)-sensitive G_i/G_o-like proteins (17,11,12,38,1). Accordingly, CTX blocks the excitatory electrophysiological responses elicited by low doses of opioid agonists on cultured dorsal root ganglion neurons (30), attenuates the stimulation of basal adenylate cyclase induced by levorphanol in F-11 neuroblastoma × sensory neuron hybrid cells (13), and decreases the ability of low concentrations of opioids to enhance the stimulated release of enkephalin in guinea pig ileum (16). Furthermore, opioid-induced catalepsy, which is PTX-insensitive, is greatly reduced by intracerebroventricular (ICV) CTX pretreatment (22).

More recently it has become apparent that the biochemical consequences of CTX treatment of cells may not be simply due to the ability of catalysing the ADP-ribosylation of G protein α-subunits. The exposure of L6 skeletal myoblasts to the toxin causes the down-regulation of $G_{s\alpha}$ (26) and lowers the density of opioid receptors coupled to PTX-sensitive G proteins in neuroblastoma × glioma hybrid NG108-15 cells (24). The broad spectrum of CTX-induced responses can also account for the ability of CTX to modify some opioid-induced effects sensitive to PTX. "In vitro" CTX treatment of NG108-

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15 cells decreases opioid-mediated inhibition of adenylate cyclase (24), and "in vivo" central administration of CTX to rodents affects opioid analgesia (7,29).

Differently from the "in vitro" studies little is known about the biochemical correlates that underlie the modification of opioid effects induced by CTX "in vivo." Recently, we reported that the hyper—and hypothermic effects elicited by the ICV administration of morphine to rats were sensitive to PTX pretreatment (6). Six days after single ICV injection of PTX hypothermia induced by a high dose of morphine was replaced by a consistent hyperthermia, whereas hyperthermia elicited by low doses of morphine was only partially reduced. The present study evaluates the influence of CTX and its B-subunit (CTX-B) administered ICV to rats on morphine-induced thermic responses. Based on the known biochemical effects of CTX in cultured cells we have measured in hypothalamic membranes from control and CTX-treated rats: (1) the degree of the "in vitro" CTX-catalysed ADP ribosylation of G_{sox}; (2) the tissue levels of G_s; (3) the basal adenylate cyclase activity; and (4) the binding of [^{3}H]-DAMGO to μ-type opioid receptors. Moreover, since CTX treatment might also influence G_i either by decreasing the levels of α_i-subunit and/or by ADP-ribosylation, we have also evaluated the availability of α_i to serve as a substrate for the "in vitro" PTX-catalysed ADP-ribosylation after "in vivo" CTX treatment. The hypothalamic area was chosen since it contains two populations of temperature-sensitive neurons that may account for opioid-induced hyperthermia and hypothermia (3).

METHODS

Animals and Surgery

Female Sprague–Dawley rats (Charles River Italia, Calco, Italy) weighing 150–l80 g were used for all experiments. Food and water were available ad lib. Animals were housed on a 12 h light/12 h dark cycle at constant temperature (22 ± 1°C) and humidity (60%).

For surgery, rats were anesthetized with tribromoethanol (200 mg/kg IP) and placement of ICV microinjection cannulas was performed according to Altaffer's procedure (5). Briefly, anesthetized rats were positioned in a stereotaxic apparatus and the right lateral ventricle located using the stereotaxic atlas of Paxinos and Watson (27). A permanent polyethylene cannula (PE10 type, Becton Dickinson, Parsippany, New Jersey, USA) was implanted 4.5 mm deep from the top of the skull and fixed with dental cement (X-60 type, Hottinger Baldwing Messtechnik, Milan, Italy). After surgery the animals were housed in individual cages and allowed to recover for seven days before experimental testing. Conscious rats were injected with 5 μl volume of each drug solution or vehicle.

Measurement of Body Temperature

All experiments were performed in an acoustically isolated room at temperature of 22 ± 1°C. Starting 48 h after cannula implantation all animals were daily habituated to the testing of rectal temperature as described by Szikszay et al. (37). Following a 1 h acclimatization period in the test room, body temperature was monitored using a rectal thermistor probe (PRA-22002-A, Ellab, Roedovre, Denmark) inserted 6 cm deep into the colon. After a 30 s equilibration period temperature was recorded to the nearest 0.1°C on a CTD-85-M thermometer (Ellab, Roedovre, Denmark). During testing, rats were gently held at the base of their tails. Body temperatures were measured at 0.5, 1, 2, 3, 4, 5, 6, 24, and 72 h after a single ICV injection of CTX (1 μg/rat) or CTX-B (5 μg/rat). Responses to ICV administration of morphine (2.5 or 18 μg/rat) were evaluated three days after injection of CTX, CTX-B, or vehicle, by measuring body temperatures twice before drug administration (baseline), and 15, 30, 45, 60, 90, 120, 180 min after drug injection (longer times, i.e. 240 and 300 min, were also considered when 2.5 μg morphine was employed). In addition the influence of CTX on the hypothermia caused by 36 μg of morphine was assessed. The initial core temperature was similar in all experimental groups (mean ± SEM = 37.27 ± 0.12°C; n = 30). At least five animals in each experimental group were employed and all animals were used only once. All results are expressed as the means ± SEM of centigrade deviation from baseline temperature or area under the time-response curve (AUC).

ADP-Ribosylation Assay

Hypothalamic regions were homogenized with an Ultraturrax (10,000 rpm for 10 s) in 20 volumes of 2 mM Tris-HCl buffer (pH 7.1) containing 1 mM EDTA. Membranes were pelleted by centrifugation at 45,000 × g for 20 min at 4°C, resuspended in the same volume of homogenization buffer and recentrifuged as before. Final pellets were resuspended in 10 mM Hepes-NaOH (pH 7.4) containing NaCl 130 mM, 0.01% (w/v) sodium azide, 0.1 mM phenylmethylsulphonylfluoride and 0.2 u/ml aprotinin and stored at −80°C until use. For ADP-ribosylation CTX was activated by incubation for 10 min at 37°C with 2.5% (w/v) sodium dodecyl sulphate (SDS), 5 mM dithiothreitol (DTT), 130 mM NaCl, and 10 mM Hepes-NaOH buffer (pH 7.4). Hypothalamic membranes (30–40 μg protein/sample) were incubated for 10 min at 25°C with 0.2% (v/v) Triton X-100 and l mM 5′-guanylylimidodiphosphate [Gpp(NH)p]. After addition of equal volumes of a mixture containing activated CTX (0.8 μg/sample), 20 μM [^{32}P]-NAD (30 Ci/mmol), 20 mM isonicotinic acid hydrazide, 1 mM 3-acetylpyridine adenine nucleotide, 10 mM thymidine and 10 mM DTT, incubations continued for 60 min. Pertussis toxin-catalysed ADP-ribosylation was carried out for 45 min at 32°C in a different incubation medium containing 20 mM Tris-HCl buffer (pH 8), 1 mM EDTA, 10 mM thymidine, 0.1% (w/v) Lubrol PX, 1.4 mM DTT, l mM ATP, 0.1 mM GTP, 0.4 μM [^{32}P]-NAD, 0.5 mM NADP, 0.008% (w/v) bovine serum albumin (BSA), 25–50 μg/sample of hypothalamic membranes, and 0.2 μg/sample PTX previously activated by incubation at 37°C for 30 min in the presence of 25 mM DTT.

Reactions were stopped by adding 1 ml ice-cold 10 mM Hepes-NaOH (pH 7.4)/130 mM NaCl and centrifugation for 10 min at 15,000 × g at 4°C. Pellets were resuspended in Laemmli sample buffer (19) and loaded onto a 12.5% (w/v) SDS-polyacrylamide gel electrophoretic system (SDS-PAGE). Dried gels were exposed on Kodak X-Omat AR films and autoradiograms quantitated by optical densitometry.

Adenylate Cyclase Assay

Adenylate cyclase activity was assayed in washed hypothalamic particulate fractions. Briefly, tissues were homogenized by hand with a glass-Teflon Potter in 1 ml of ice-cold 10 mM Hepes-NaOH buffer, 0.32 M sucrose and 1 mM EGTA (pH 7.4) (Buffer "A"). Homogenates were centrifuged at 400 × g for 5 min at 4°C and supernatants spun at 20,000 × g for 10 min. Pellets were resuspended in the same volume of 10 mM Hepes-NaOH buffer (pH 7.4) containing 1 mM EGTA (Buffer "B"), kept 20 min on ice and recentrifuged at 20,000 × g. After resuspension in 30 volumes Buffer "B" (about 40 μg

protein/sample) samples were incubated for 10 min at 30°C in a mixture (0.1 ml total volume) containing 50 mM Hepes-NaOH buffer (pH 7.4), 2 mM $MgCl_2$, 0.5 mM [α-^{32}P]-ATP (30 Ci/mmol), 1 mM cyclic AMP, 0.5 mM isobutylmethylxanthine, 5 mM phosphocreatine, 50 u/ml creatine phosphokinase, 0.01 mM GTP and 0.33 mM EGTA. Incubations were stopped by adding 200 µl of a solution containing 2% (w/v) SDS, 45 mM ATP and 1.3 mM cyclic AMP (pH 7.4). After addition of 15–20,000 c.p.m./sample [2,8-^{3}H]-cyclic AMP (25–40 Ci/mmol), to monitor cyclic AMP recovery, samples were placed in a boiling water bath for 3 min. Cyclic AMP was isolated according to the procedure of Salomon et al. (28).

Western Blotting Analysis

The polyclonal rabbit antiserum CS1 was kindly donated by G. Milligan (Depts. Biochem. & Pharmacol., Univ. Glasgow, Scotland). It was raised against a synthetic peptide, RMHLRQYELL, which corresponds to the C-terminal decapeptide of all forms of α_s (24). For immunoblotting proteins were resolved on a 12.5% (w/v) SDS-PAGE and transferred to nitrocellulose membranes (40). After 3 h blocking in 5% (w/v) dry skimmed milk dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 0.2% (w/v) Tween 20, and 0.02% (w/v) sodium azide (TBS-T) and overnight incubation with a 1,000-fold dilution CS1 antiserum in milk/TBS-T, proteins were detected with alkaline phosphatase-conjugated goat anti-rabbit Ig G using bromochloroindolyl phosphate and nitro blue tetrazolium as substrates.

[^{3}H]-DAMGO Binding

Hypothalamic areas were prepared essentially as described by Klee and Streaty (18). Briefly, tissues were homogenized in ice-cold 0.32 M sucrose (9 ml/g tissue) in a glass Potter with Teflon pestle driven by an electric motor at approximately 1,000 r.p.m. Homogenates were centrifuged at 850 × g for 10 min at 0°C and resulting supernatants spun at 43,500 × g for 20 min. Pellets were resuspended in ice-cold 0.05 M Tris-HCl buffer (pH 7.4) using 1/5 of the original volume. Membranes were stored at −20°C for no more than three weeks before using. All binding assays were carried out in triplicate tubes pretreated with BSA (100 µg/ml). Membrane suspensions were incubated at 37°C for 20 min to remove all endogenous opioids bound to receptors and 100 µl (about 0.3 mg protein) incubated with increasing concentration (0.25–8 nM) of [^{3}H]-DAMGO at 25°C for 60 min in 0.05 M Tris-HCl buffer (pH 7.4) containing bacitracin (50 µg/ml) and reactions terminated by rapid filtration through Whatman GF/B glass fiber filters and rinsing three times with 4 ml ice-cold buffer. Dried filters were counted using Filter-Count as scintillation fluid (Canberra Packard, Pero, Italy). Unspecific binding was defined by 1 µM DAMGO. Scatchard analysis was used to calculate K_d and B_{max} values from saturation isotherms.

Protein Determination

Protein contents of membrane preparations were estimated with the method of Lowry et al. (21) using BSA as a standard.

Drugs and Chemicals

Cholera toxin, cholera toxin B-subunit and pertussis toxin were purchased from Sigma-Aldrich (Milan, Italy) and morphine hydrochloride from S.I.F.A.C. (Milan, Italy). [^{32}P]-NAD, 30 Ci/mmol; [α-^{32}P]-ATP, 30 Ci/mmol; 2.8-[^{3}H]-cyclic AMP, 25–40 Ci/mmol and [^{3}H]-DAMGO 52.5 Ci/mmol were purchased from Dupont NEN (Cologno Monzese, Italy). Chemicals were obtained from Sigma, or Merck (Darmstadt, Germany), unless otherwise stated, and were of the highest purity available.

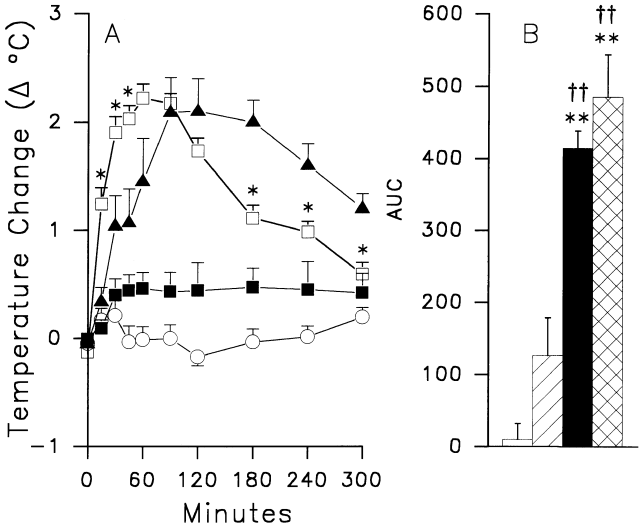

FIG. 1. (A) Effect of CTX pretreatment (1 µg/rat ICV) on hyperthermic response induced by morphine (2.5 µg/rat) administered ICV to rats three days later; (○) vehicle + saline; (■) CTX + saline; (▲) vehicle + morphine; (□) CTX + morphine; *$p < 0.05$ vs vehicle + morphine. Each point is the mean ± SEM of 10 animals. (B) AUC values calculated over the entire time-response curves; (□) vehicle + saline; (▨) CTX + saline; (■) vehicle + morphine; (⊠) CTX + morphine. Histograms represent mean ± SEM, **$p < 0.01$ vs vehicle + saline; ††$p < 0.01$ vs CTX + saline.

Statistical Analysis

All results were expressed as mean ± SEM. Comparisons were made by analysis of variance (ANOVA) followed by post-hoc comparison with Tukey's test in case of multiple means (42).

RESULTS

Effect of CTX on Thermic Responses to Morphine

The ICV administration of CTX to rats (1 µg/animal) neither produced gross behavioral abnormalities nor affected body growth during the three days of observation. However, CTX produced a significant elevation of rectal temperature. Analysis of data with two-way ANOVA revealed significant time effect [$F(9, 90) = 3.493$; $p < 0.001$], drug treatment [$F(1, 90) = 164.550$; $p < 0.0001$] and time vs drug interaction [$F(9, 109) = 8.638$; $p < 0.0001$]. Post-hoc comparisons evidenced a significant difference between CTX- and vehicle-treated groups at 120 to 360 min post injection ($p < 0.001$). The body temperature increment peaked within 120 min, lasted for at least 6 h and disappeared within 24 h (data not shown). The alteration of body temperature induced by CTX alone made it difficult to study the influence of the toxin on thermic response to morphine at short time intervals. Since it has been previously reported (22) that morphine-induced analgesia and catalepsy in rats were antagonized by CTX injected 3 days before, we have chosen this time for the present study. The

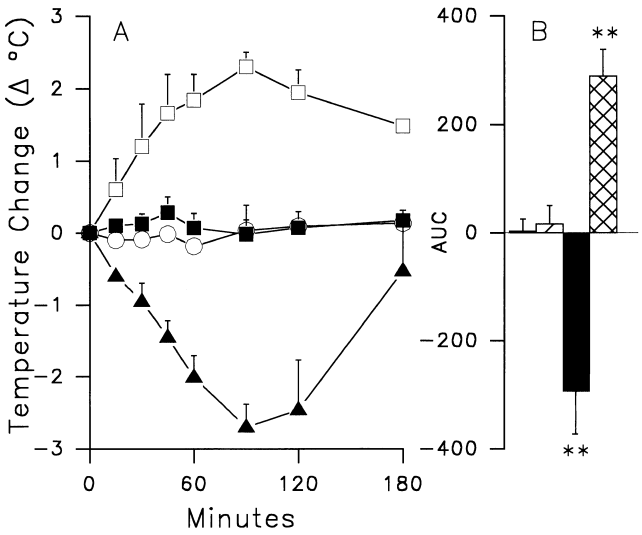

FIG. 2. (A) Effect of CTX pretreatment (1 μg/rat ICV) on hypothermic response induced by morphine (18 μg/rat) administered ICV to rats three days later; (○) vehicle + saline ($n = 7$); (■) CTX + saline ($n = 5$); (▲) vehicle + morphine ($n = 7$); (□) CTX + morphine ($n = 5$). Each point is the mean ± SEM. (B) AUC values over 180 min; (□) vehicle + saline; (▨) CTX + saline; (■) vehicle + morphine; (⊠) CTX + morphine. Histograms represent mean ± SEM. $^{**}p < 0.01$ vs vehicle + saline.

influence of CTX pretreatment on the hyperthermic response elicited by a low dose of morphine (2.5 μg/rat ICV) is shown in Figure 1A and B. The time course of hyperthermia in the group treated with CTX + morphine did not completely overlap with that found in the group treated with morphine alone [$F(9, 180) = 21.659$, $p < 0.0001$ for time; $F(1, 180) = 1.099$, NS for drug treatment; $F(9, 199) = 6.781$, $p < 0.0001$ for drug treatment vs time]. CTX pretreatment shortened the onset of morphine hyperthermic response ($p < 0.05$ at 15, 30, and 45 min) and caused a faster fall of the effect ($p < 0.05$ at 180, 240 and 300 min). However the AUC over 300 min for morphine and CTX+morphine treatments were not significantly different (Figure 1B). The influence of CTX pretreatment on hypothermia elicited by 18 μg/rat ICV of morphine is reported in Figures 2A and B. One-way ANOVA analysis evidenced a significant drug treatment difference [$F(3, 20) = 23.674$; $p < 0.0001$]. Morphine (Figure 2B) caused a significant decrease of body temperature [$p < 0.01$ (post-hoc analysis)] which was converted into hyperthermia by CTX [$p < 0.01$ (post-hoc analysis)]. The AUC over 180 min for rats treated with CTX+morphine was 113-fold higher than for control rats. When the highest dose of morphine tested (36 μg) was administered to rats, the hypothermic response was only partially reduced by CTX, and AUC over 180 min for CTX+morphine was 76% less than morphine alone [$F(1,8) = 5.699$; $p < 0.05$] (data not shown). Purified CTX-B (5 μg/rat ICV) by itself produced a significant rise of rectal temperature that was similar in intensity and time course to that observed with the holotoxin [$F(9, 130) = 8.624$, $p < 0.0001$ for time; $F(1, 130) = 141.098$, $p < 0.0001$ for drug treatment; $F(9, 149) = 4.190$, $p < 0.0001$ for drug treatment vs time] (data not shown). Pretreatment with CTX-B (3 days before morphine) neither affected hyper- nor hypothermia induced by low (2.5 μg) and high (18 μg) doses of the opiate, respectively (data not shown).

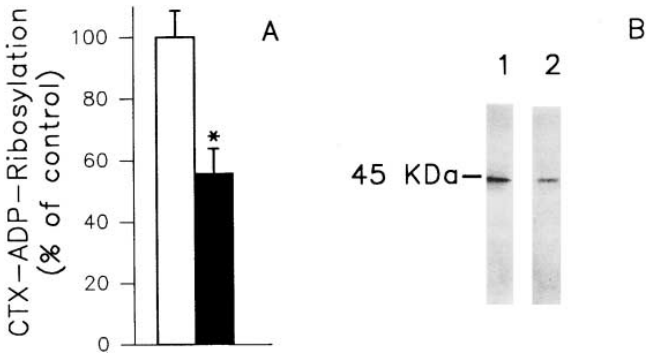

FIG. 3. "In vitro" CTX-catalysed ADP-ribosylation of $G_{\alpha s}$ in hypothalamic membranes from rats injected ICV three days before with CTX or vehicle. (A) Relative quantitation by densitometric analysis of autoradiograms of [^{32}P]ADP-ribose incorporation into 45 kDa band from controls (□) and CTX-pretreated (■) rats. $^{*}p < 0.05$ vs controls. Values are means ± SEM mean of 3 or 4 determinations. (B) Representative autoradiogram. Lane 1 = control; lane 2 = CTX-treated.

Biochemical Studies

All biochemical studies, unless otherwise stated, were performed on hypothalamic membranes prepared from rats treated 3 days before with CTX or its vehicle.

Effect of CTX Treatment on In Vitro CTX- and PTX-Catalysed ADP-Ribosylation of G_{α} Proteins

CTX catalysed the "in vitro" [^{32}P]-NAD-dependent ADP-ribosylation of a major protein band in hypothalamic membranes that migrated on SDS-PAGE in proximity of the 45 kDa protein marker, presumably reflecting the $G_{s\alpha}$-subunit (Figure 3B). The densitometric quantitation of band intensities indicated that radioactivity incorporated into the 45 kDa protein was 51% less in hypothalamic membranes from rats treated with CTX than control animals [$F(1, 4) = 13.98$; $p < 0.05$] (Figure 3A). The "in vitro" ADP-ribosylation of hypothalamic membranes with PTX labelled a single band with apparent molecular mass of about 40 kDa whose intensity was unaffected by "in vivo" CTX treatment (Figures 4A and B).

Effect of CTX on $G_{s\alpha}$-Proteins Levels

The hypothalamic membranes obtained from CTX-treated animals displayed lower $G_{s\alpha}$-immunoreactivity than control membranes by immunoblotting analysis (Figure 5B). About 30% reduction was detected through scanning of the blots by optical densitometry [$F(1, 5) = 8.194$; $p < 0.05$] (Figure 5A).

Effect of CTX on Adenylate Cyclase Activity

The effect of "in vivo" CTX treatment on adenylate cyclase activity in hypothalamic membranes is shown in Figure 6. Statistical analysis of the data revealed significant differences between groups [$F(3,12) = 4.926$; $p < 0.02$]. Post-hoc analysis indicated that one and two days after the "in vivo" injection of CTX the hypothalamic membranes display an increased adenylate cyclase activity (72% and 67%, respectively; $p < 0.05$). At three days the enzyme stimulation was attenuated and the basal adenylate cyclase activity did not differ from that observed in membranes from control or CTX-treated rats.

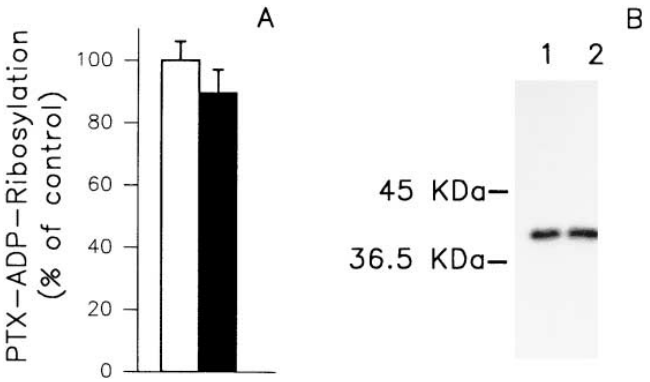

FIG. 4. "In vitro" PTX-catalysed ADP-ribosylation of $G_{\alpha i}/G_{\alpha o}$ in hypothalamic membranes from rats injected ICV three days before with CTX or vehicle. (A) Relative quantitation by densitometric analysis of autoradiograms of [^{32}P]ADP-ribose incorporation into 40 kDa band from controls (□) and CTX-pretreated (■) rats. Values are means ± SEM mean of 4 determinations. (B) Representative autoradiogram showing the positions of two molecular weight markers. Lane 1 = control; lane 2 = CTX-treated.

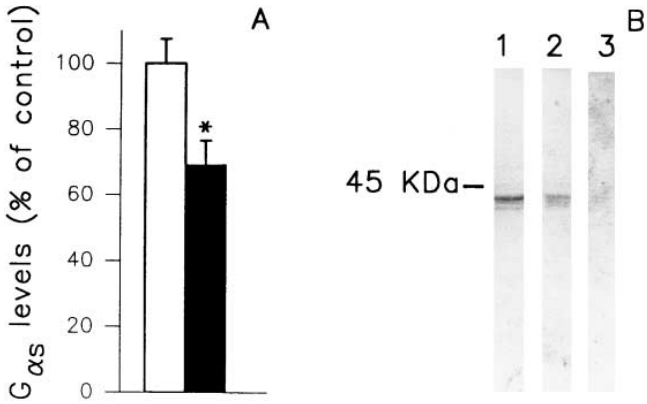

FIG. 5. Immunoblotting analysis of $G_{\alpha s}$ in hypothalamic membranes from rats injected ICV three days before with CTX or vehicle. (A) Relative quantitation by densitometric scanning; vehicle (□) or CTX (■). *$p < 0.05$ vs controls. (B) representative immunoblots of hypothalamic membranes from controls (lane 1) and CTX-treated rats (lane 2); lanes 1 and 2 = $G_{\alpha s}$ antiserum; lane 3 = normal rabbit serum. Values are means ± SEM mean of 4 determinations.

Effect of CTX on μ-Opioid Receptors

The μ-opioid agonist [^{3}H]-DAMGO bound to a single class of sites in hypothalamic membranes from CTX-treated and control animals. The K_d and B_{max} values calculated from equilibrium binding experiments were unaffected by the treatment (control: K_d = 3 0 ± 0.6 nM, B_{max} = 95.1 ±13.1 fmol/mg protein; CTX-treated: K_d = 3.4 ± 0.8 nM, B_{max} = 92.7 ± 16.1 fmol/mg protein) (data not shown).

DISCUSSION

It is well known that administration of opiate and opioid peptides affects body temperature in a manner dependent upon animal species, route of administration, restraint and ambient temperature. Based on the responses induced by selective ligands for μ, k- and δ-opioid receptors it has been suggested that μ- and k-receptors mediate hyper- and hypothermic responses to opioids respectively, whereas the role of δ receptors in temperature regulation is still debated (4). Several authors have reported that morphine given ICV (2,20,39) or into the Preoptic Anterior Hypothalamic Area (POAH) (41,10) to unrestrained rats, caused hyperthermia. In contrast, using a similar experimental setup, we have observed, as previously reported by Ferri et al. (14) a dual response to acutely ICV-injected morphine. The animals responded to low dose (2.5 μg ICV) with hyperthermia and to high doses (9-36 μg ICV) with hypothermia. The reasons for such discrepancies are obscure but we can assume that they may depend on different factors other than doses of morphine. However, in agreement with our findings, Spampinato et al. (34) have recently reported that low dose of DAMGO (0.4 nmol), given ICV to unrestrained rats, increased body temperature, whereas a high dose (2 nmol) caused a significant hypothermia then followed by hyperthermia. Moreover an analog of morphiceptin, PL017, another highly selective μ-agonist caused a dose-related hyperthermic response in rats (0.4–6.3 μg), with an initial hypothermia at the highest dose (8).

Our study shows that soon after administration CTX holotoxin and its B-subunit (CTX-B) produced a significant rise of rectal temperature thus suggesting that they interfere with the thermoregulatory process. While the increase of basal body temperature after CTX treatment could be due to persistent generation of cyclic AMP or be secondary to binding to GM1 ganglioside on the cell surface, that induced by CTX-B might only be dependent upon the latter response. At 72 h CTX had no longer effect on basal temperature but was able to modify the thermic response to morphine. CTX pretreatment shortened the onset of hyperthermia induced by low dose of morphine and attenuated its duration without changing the extent of morphine-induced hyperthermia.

The hypothermia induced by high doses of morphine (18 and 36 μg) was more sensitive to CTX pretreatment (1 μg) than hyperthermia caused by a low dose of the opiate (2.5

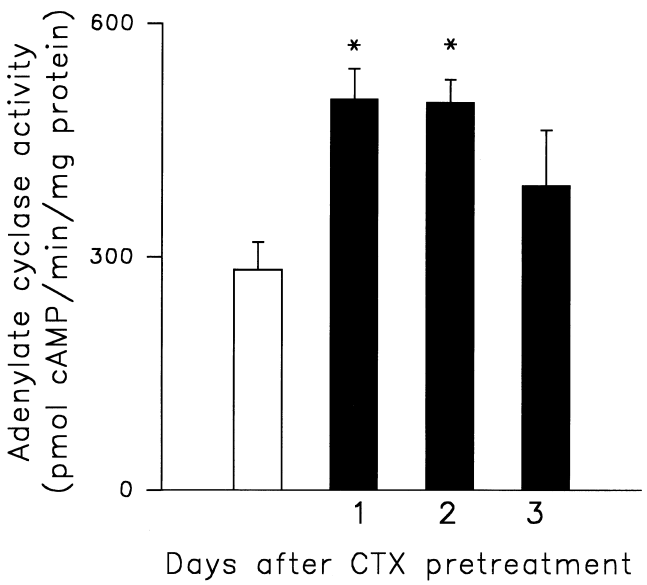

FIG. 6. Adenylate cyclase activity in hypothalamic membranes from rats injected ICV 1, 2, 3 days before with CTX (■) or vehicle (□). *$p <$ 0.05 vs controls. Values are means ± SEM mean of 4 determinations.

μg). The effect of CTX on hypothermia was dependent upon the dose of morphine administered, i.e. the toxin partially blocked the response induced by 36 μg of morphine, whereas it converted the hypothermia caused by 18 μg into hyperthermia. Perhaps the amount of CTX is not enough to completely counteract the response produced by high doses of morphine. However, higher quantities of CTX could not be administered to animals due to its toxicity. An earlier report (33) showed that the hypothermic effect of the two k-opioid receptor agonists U-50488H and PD117302 administered ICV to mice was not affected by CTX given 48 and/or 96 h before. These results suggest that the hypothermic responses to opioid agonists acting on different receptor subtypes are differentially affected by CTX and/or that toxin effectiveness shows species specificity.

The toxin B-subunit, even at higher concentration (5 μg), did not alter the modification in body temperature induced by morphine. However, this finding does not rule out the possibility that CTX-B may exert some short-term modulatory effects on morphine thermic responses. Accordingly, electrophysiological studies on nociceptive neurons in dorsal root ganglion explants in culture showed that CTX-B could selectively block opioid-induced excitatory effects on the action potential duration within a few minutes, presumably by interfering with GM1 gangliosides associated with opioid receptors (31,32). The time at which CTX pretreatment influenced body temperature changes elicited by morphine suggests that such effects were not due to early events caused by the toxin but to later adaptive changes perhaps secondary to a prolonged activation of the adenylate cyclase signalling cascade and subsequent alterations of the cyclic AMP-dependent phosphorylation of some cellular substrates, including receptors and other signalling proteins. Biochemical and electrophysiological evidence suggests that CTX effect on $G_{s\alpha}$ is partially reversible (30). Accordingly, the activation of adenylate cyclase found 24 and 48 h after CTX administration was no longer observed at 72 h when the CTX effect on morphine-induced alterations of body temperature was instead detected. Also the reduced cellular availability of $G_{s\alpha}$ caused by CTX could well be involved in the modifications of thermic responses to morphine induced by the toxin since 72 h after the "in vivo" administration of CTX there was a significant reduction of hypothalamic $G_{s\alpha}$ immunoreactivity and of the ability of this protein to serve as a substrate for "in vitro" CTX-catalysed ADP-ribosylation. In this respect the biochemical effects induced by "in vivo" CTX were similar to those reported in cultured cell lines, such as F-11 cells and L6 skeletal myoblasts, where the exposure to CTX resulted in a down-regulation of $G_{s\alpha}$ but not of $G_{i\alpha}$, levels (6,13). In a previous paper (6) we reported that PTX administered ICV to rats converted morphine hypothermia into hyperthermia thus suggesting an involvement of G_i protein. However, in the present study, the "in vivo" administration of CTX did not seem to affect inhibitory G_i/G_O-like protein in hypothalamus, since the "in vitro" ADP-ribosylation of the α-subunits catalysed by PTX was unaffected. It seems also unlikely that the activity of G_i might be disrupted by CTX injection, since already McKenzie and Milligan (24) demonstrated that G_i was equally active in membranes from untreated and CTX-pretreated NG108-15 cells. In the latter system, Gpp(NH)p, a non-hydrolysable analogue of GTP that displays a greater affinity for G_i than for G_s, was able to produce a maximal inhibition of forskolin-activated adenylate cyclase in membranes from untreated and CTX-treated cells. Thus blockade of morphine hypothermia induced by CTX was not dependent upon an impairment of G_i protein-mediated transduction pathways. This hypothesis is also supported by the observation that morphine hyperthermia was partially reduced by ICV administration of PTX (6), while CTX acted differently, i.e. modifying the time course of hyperthermia without changing its magnitude. Then we considered the possibility that CTX had converted the morphine hypothermia into hyperthermia by affecting hypothalamic μ-opioid binding sites, based on the findings of McKenzie and Milligan (24) that reported a decrease in δ-type opioid receptors in NG108-15 cells exposed to CTX. However, this hypothesis could be excluded since no changes were detected in the affinity or maximal capacity of [^{3}H]-DAMGO binding to membranes from rats treated three days before with the toxin. Nevertheless, possible changes of other opioid receptor subtypes involved in body temperature regulation, particularly κ receptors, must be considered.

This study shows that CTX affects the body temperature changes induced by morphine. The effect of CTX is partially superimposable to that of PTX (6), since both toxins were able to convert morphine hypothermia into hyperthermia. However, CTX changed the time course of morphine hyperthermia whereas PTX reduced the hyperthermic effect. CTX did not seem to modify the thermic response to morphine by interfering with either μ-opioid receptors and/or their "classically" associated transduction mechanisms, operated by inibitory G_i/G_O-like proteins. The effect of CTX, rather than implying a role for G_s in morphine receptor function, could depend upon reduced cellular availability of $G_{s\alpha}$ and/or on altered phosphorylation state of some cellular substrates such as receptor(s) and/or effector(s) caused by prolonged activation of $G_{s\alpha}$.

On the other end the impairment of G_i/G_O-like proteins coupled to opioid or to any other inhibitory receptors mediating the thermic responses to morphine could account for the PTX activity. However, we can not rule out that, in the time between PTX administration and the morphine challenge, intervening adaptive events primed by G_i/G_O inactivation may indeed be responsible of the toxin activity.

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REFERENCES

1. Abood, M. E.; Lee, N. M.; Loh, H. H. Modification of opioid agonist binding by pertussis toxin. Brain Res. 417:70–74; 1987.
2. Adler, M. W.; Hawk, C.; Geller, E. B. Comparison of intraventricular morphine and opioid peptides on body temperature of rats. In: Lomax, P.; Schonbaum, E:, eds. Environment, drugs and thermoregulation. Basel: Karger; 1983;90–93.
3. Adler, M. W; Geller, E. B. The opioid system and temperature regulation. Ann. Rev. Pharmacol. Toxicol. 28:429–449; 1988.
4. Adler, M. W.; Geller, E. B. Physiological functions of opioids: Temperature regulation. In: Herz, A.; Akil, H.; Simon, E. J., eds. Opioids. Berlin: Springer-Verlag; 1991:205–238.
5. Altaffer, F. B.; De Balbian, W.; Hall, P. S.; Long, C. J.; D' Encarnacao, P. A simple and inexpensive cannula technique for chemical stimulation of the brain. Physiol. Behav. 5:119–121; 1970.
6. Basilico, L.; Abbondi, M.; Fumagalli, A.; Parolaro, D.; Gori, E.; Giagnoni, G. Influence of pertussis toxin on thermic responses

to morphine and neurotensin in rats. Eur. J. Pharmacol. 222:241–245; 1992.
7. Bodnar, R. J.; Paul, D.; Rosenblum, M.; Liu, L.; Pasternak, G. W. Blockade of morphine analgesia by both pertussis and cholera toxins in the periacqueductal gray and locus coeruleus. Brain Res. 529:324–328; 1990.
8. Bradley, E. A.; Geller, E. B.; Piliero, T.; Adler, M. W. Actions of μ-selective opioid agonists and antagonist on body temperature in the rat. FASEB J. 5:A861; 1991.
9. Cassel, D.; Selinger, Z. Mechanism of adenylate cyclase activation by cholera toxin: inhibition of GTP hydrolysis at the regulatory site. Proc. Natl. Acad. Sci. USA 74:3307–3311; 1977.
10. Cox, B.; Ary, M.; Chesarek, W.; Lomax, P. Morphine hyperthermia in the rat: an action on the central thermostats. Eur. J. Pharmacol. 36:33–39; 1976.
11. Crain, S. M.; Crain, B.; Makrnan, M. H. Pertussis toxin blocks depressant effects of opioid, monoaminergic and muscarinic agonists on dorsal-horn network responses in spinal cord-ganglion cultures. Brain Res. 400:185–190; 1987.
12. Crain, S. M.; Shen, K. F. Opioids can evoke direct receptor-mediated excitatory effects on sensory neuron. Trends Pharmacol. Sci. 11:77–81; 1990.
13. Cruciani, R A.; Dvorkin, B.; Morris, S. A.; Crain, S. M.; Makman, M. H. Direct coupling of opioid receptors to both stimulatory and inhibitory guanine nucleotide-binding proteins in F-11 neuroblastoma-sensory neuron hybrid cells. Proc. Natl. Acad. Sci. USA 90:3019–3023; 1993.
14. Ferri, S.; Arrigo Reina, R; Santagostino, A.; Scoto, G. M. and Spadaro, C. Effects of met-enkephalin on body temperature of normal and morphine-tolerant rats. Psychopharmacology 58: 277–281; 1978.
15. Gill, D. M.; Meren, R. ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase. Proc. Natl. Acad. Sci. USA 75:3050–3054; 1978.
16. Gintzler, A. R.; Xu, H. Different G proteins mediate the opioid inhibition or enhancement of evoked (5-methionine) enkephalin release. Proc. Natl. Acad. Sci. USA 88:4741–4745; 1991.
17. Hescheler, J.; Rosenthal, W.; Trautwein, W.; Schultz, G. The GTP-binding protein, G_O, regulates neuronal calcium channels. Nature 325:445–447; 1987.
18. Klee, W. A.; Streaty, R. A. Narcotic receptor sites in morphine-dependent rats. Nature 248:61–63; 1974.
19. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685; 1970.
20. Lin, M. T. An adrenergic link in the hypothalamic pathways which mediate morphine- and β-endorphin-induced hyperthermia in the rat. Neuropharmacology 21:613–617; 1982.
21. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265–275; 1951.
22. Massi, P.; Rubino, T.; Parenti, M.; Giagnoni, G.; Groppetti, A.; Parolaro, D. Cholera toxin antagonizes morphine-induced catalepsy through a cyclic AMP-independent mechanism. Eur. J. Pharmacol. 243:65–71; 1993.
23. McKenzie, F. R.; Milligan, G. Prostaglandin E1-mediated, cyclic AMP-independent, down-regulation of $G_{s\alpha}$ in neuroblastoma × glioma hybrid cells. J. Biol. Chem. 265:17084–17093; 1990.
24. McKenzie, F. R.; Milligan, G. Cholera toxin impairment of opioid-mediated inhibition of adenylate cyclase in neuroblastoma × glioma hybrid cells is due to a toxin-induced decrease in opioid receptor levels. Biochem. J. 275:175–181; 1991.
25. Milligan, G. Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding proteins. Biochem. J. 255:1–13; 1988.
26. Milligan, G.; Unson, C. G.; Wakelam, M. J. O. Cholera toxin treatment produces down-regulation of the α-subunit of the stimulatory guanine-nucletide-binding protein (G_s). Biochem. J. 262: 643–649; 1989.
27. Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. Second edition. San Diego: Academic Press Inc.; 1986.
28. Salomon, Y.; Londos, C.; Rodbell, M. A highly sensitive adenylate cyclase assay. Anal. Biochem. 58:541–548; 1974.
29. Sanchez-Blazquez, P.; Garzon, J. Cholera toxin and pertussis toxin on opioid- and α_2-mediated supraspinal analgesia in mice. Life Sci. 48:1721–1727; 1991.
30. Shen, K. F.; Crain, S. M. Cholera toxin-A subunit blocks opioid excitatory effects on sensory neuron action potentials indicating mediation by G_s-linked opioid receptors. Brain Res. 525:225–231; 1990.
31. Shen, K. F. and Crain, S. M. Cholera toxin-B subunit blocks excitatory effects of opioids on sensory neuron action potentials indicating that GM1 ganglioside may regulate G_s-linked opioid receptor functions. Brain Res. 531:1–17; 1990.
32. Shen, K. F.; Crain, S. M.; Ledeen, R. W. Brief treatment of sensory ganglion neurons with GM1 ganglioside enhances the efficacy of opioid excitatory effects on the action potential. Brain Res. 559:130–138; 1991.
33. Shukla, V. K.; Turndorf, H.; Bansinath, M. Pertussis and cholera toxins modulate κ-opioid receptor agonists-induced hypothermia and gut inhibition. Eur. J. Pharmacol. Environ. Toxicol. Pharmacol. Section 292:293–299; 1995.
34. Spampinato, S.; Speroni, E.; Govoni, P.; Pistacchio, E.; Romagnoli, C.; Murari, G.; Ferri, S. Effect of ω-conotoxin and verapamil on antinociceptive, behavioural and thermoregulatory responses to opioids in the rat. Eur. J. Pharmacol. 254:229–238; 1994.
35. Spiegel, S.; Fishman, P. H. Gangliosides as bimodal regulators of cell growth. Proc. Natl. Acad. Sci. USA 84:141–145; 1987.
36. Stadel, J. M.; Leikowitz, R. J. Differential effects of cholera toxin on guanine nucleotide regulation of beta-adrenergic agonist high affinity binding and adenylate cyclase activation in frog erythrocyte membranes. J. Cyc. Nucl. Res. 7:363–374; 1981.
37. Szikszay, M.; Benedek, G.; Obal, Jr. F. Capsaicin pretreatment interferes with thermoregulatory effect of morphine. Pharmacol. Biochem. Behav. 18:373–387; 1983.
38. Taussig, R; Sanchez, S.; Rifo, M.; Gilman, A. G.; Belardetti, F. Inhibition of the omega-conotoxin sensitive calcium current by distinct G proteins. Neuron 8:799–809; 1992.
39. Thornhill, J. A.; Desaultes, M. Is acute morphine hyperthermia of unrestrained rats due to selective activation of brown adipose tissue thermogenesis? J. Pharmacol. Exp. Ther. 231:422–429; 1984.
40. Towbin, H.; Staehelin, T.; Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350–4354; 1979.
41. Trzcinka, G. P.; Lipton, J. M.; Hawkins, M.; Clark, W. G. Effects on temperature of morphine injected rats into the preoptic/anterior hypothalamus, medulla oblongata, and peripherally in unrestrained and restrained rats. Proc. Soc. Exp. Biol. Med. 156:523–526; 1977.
42. Zar, J. H. Biostatistical analysis. New York: Prentice-Hall Inc. Englewood Cliffs; 1974.