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Interactions of intracerebroventricular pertussis toxin treatment with the ataxic and hypothermic effects of ethanol

Michael J. Durcan, Richard G. Lister, Philip F. Morgan, and Markku Linnoila

Laboratory of Clinical Studies, National Institute on Alcohol Abuse & Alcoholism, DICBR, Bldg. 10 3C102, Bethesda, MD 20892, USA

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Summary. Pretreatment with pertussis toxin (0.5 and 1.0 μg/animal, i.c.v., seven days prior to testing) reversed the reduction in locomotor activity in the holeboard test caused by administration of the alpha$_2$-adrenoceptor agonist, medetomidine (0.1 mg/kg, i.p.). Intrinsic behavioral effects of pertussis toxin treatment were also observed, these included a reduction in exploratory head-dipping and an increase in locomotor activity. These doses of pertussis toxin also reduced the ataxia induced by a 2.4 g/kg dose of ethanol. Pertussis toxin treated animals also exhibited a diminished hypothermic response to ethanol (2 g/kg), although the pertussis toxin treated animals had lower body temperatures prior to ethanol administration compared to sham treated animals. Neither the behavioral effect of pertussis holotoxin in the holeboard nor its effects on reversing medetomidine hypolocomotion or ethanol-induced ataxia were seen following administration of the binding oligomer of pertussis toxin which binds to the cell membrane but does not possess the enzymatically active subunit. These findings implicate mechanisms involving pertussis toxin sensitive G-proteins in modulating some behavioral and physiological effects of ethanol.

Key words: Pertussis toxin – Pertussis toxin B-oligomer – G-proteins – Ethanol – Ataxia – Hypothermia

Introduction

Many neurotransmitter receptors are linked to their effector mechanisms via guanine nucleotide-binding proteins (G-proteins) (Gilman 1987; Worley et al. 1987). This linkage can be via second messenger systems such as production of cyclic adenosine monophosphate (cAMP) or inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, or may involve actions on ions channels (Schramm and Selinger 1984; Cockcroft and Gomperts 1985; Holz et al. 1986). G-proteins can be inhibitory (G_i) or stimulatory (G_s), although other G-proteins (G_o) whose exact role is not fully understood also exist. Pertussis toxin inactivates G_i and G_o proteins by ADP-ribosylation and therefore uncouple receptors from their effector mechanisms (Ui et al. 1988).

Receptors linked to pertussis toxin-sensitive G-proteins include alpha$_2$ adrenoceptors (Bloom 1975), adenosine A_1 (Wojcik et al. 1985), 5-HT_{1A} (Harrington et al. 1988), muscarinic M_2 (Olianas et al. 1983), dopamine D_2 (Cooper et al. 1986), $GABA_B$ (Wojcik and Neff 1983), and, mu (Cooper et al. 1986) and sigma (Schoffelmeer et al. 1986) opiate receptors. Some of these receptors, such as alpha$_2$ adrenoceptors (Durcan et al. 1989a, b, c, d; Lister et al. 1989), adenosine A_1 receptors (Clark and Dar 1988; Dar et al. 1987) and $GABA_B$ receptors (Allan and Harris 1989), have been implicated in the mediation of the behavioral and physiological effects of ethanol.

The aim of the present study was to assess the effect of pretreatment with pertussis toxin on the ataxic and hypothermic effects of ethanol. Additionally, the effects of the binding (B) oligomer of pertussis toxin, which binds to, but does not penetrate, cell membranes were also investigated in order to assess if this oligomer had actions similar to the enzymatically active holotoxin. The effect of pertussis toxin treatment on the motility reducing properties of medetomidine, which are thought to be mediated via G_i linked alpha$_2$-adrenoceptors, was used to validate and probe the effectiveness of the pertussis toxin pretreatments. The interaction of pertussis toxin treatment with the ataxic and hypothermic effects of ethanol was investigated using a toxin regimen derived from the medetomidine studies. Ethanol was administered as in previous investigations (Durcan et al. 1989b, c, d; Lister et al. 1989).

Methods

Animals. Naive NIH Swiss male mice were housed in groups of 10 on a 12:12 hour light:dark cycle with food and water available ad

Send offprint requests to M. J. Durcan, Bldg. 10 Rm 3C102, 9000 Rockville Pike, Bethesda, MD 20892, USA

lib. The mice weighed between 25 and 28 g at the time of pertussis toxin or sham treatment. All mice were housed individually after pertussis toxin or sham treatment.

Pertussis toxin treatment. Mice were treated with pertussis holotoxin or pertussis toxin binding oligomer (List Biological Labs., Campbell, CA) injected in a volume of 5 µl directly into the lateral ventricle of the brain. Animals were anesthetized using chloral hydrate, a small incision made in the scalp, and the lateral ventricle accessed (co-ordinates from Bregma: 1 mm lateral, 1 mm rostral, −3 mm vertical). Pertussis toxin was injected at a rate of 5 µl/min for 1 min, the injection needle remained in place for a further 2 min before being slowly retracted and the wound closed using a wound clip. Sham animals were injected with the vehicle buffer in which the toxin was dissolved (0.01 M sodium phosphate, 0.05 M sodium chloride, pH 7.0).

Holeboard testing. The holeboard consisted of a Plexiglas box (40 × 40 × 30 cm), the floor of wich had four equally spaced holes, 3 cm in diameter. In two opposite walls, 2 cm above the floor, were four equally spaced infra-red photobeams to measure movement in the box. There were also photobeams beneath each hole to measure the number and duration of head-dips. The apparatus was interfaced with a PDP-11 computer running SKED-11 software (State Systems Inc., Kalamazoo, MI).

The holeboard testing, which took place in a dimly lit room, involved placing a mouse in the center of the holeboard floor and tracking its movements using the photobeams for 8 min.

Ataxia testing. Mice were injected i.p. with 2.4 g/kg ethanol (at a volume of 20 ml/kg) and after 5 min rated for ataxia using the following scale, modified from Majchrowicz (1975): 0 = no observable effect; 1 = mild ataxia; 2 = moderate ataxia; 3 = severe ataxia; 4 = very severe ataxia, only just able to recover righting reflex; 5 = loss of righting reflex.

Temperature recording. Core body temperatures were measured using a rectal probe and a digital thermometer (Sensortek Inc.). The probe was inserted 2.5 cm into the colon of each mouse.

Interactions with medetomidine-induced hypomotility. Groups of animals ($N = 14-22$) were treated with either pertussis toxin (1.0 µg/animal, i.c.v.) or with buffer. Two, four or seven days following pertussis toxin or sham treatment the animals were divided into approximately equal groups and injected with either 0.1 mg/kg medetomidine or distilled water vehicle 30 min prior to testing in the holeboard. This dose of medetomidine was chosen on the basis of previous studies (Durcan et al. 1989b) demonstrating a marked reduction in locomotor activity in the holeboard test. Separate sets of animals were used for each time point.

In a subsequent experiment groups of animals ($N = 18-22$) were injected i.c.v. with either pertussis holotoxin (1.0 µg/animal), pertussis toxin B-oligomer (1.0 µg/animal) or with buffer. Seven days following pertussis holotoxin, B-oligomer or sham treatment the animals were divided into approximately injected with either 0.1 mg/kg medetomidine or distilled water vehicle 30 min prior to testing in the holeboard.

In a different set of experiments, groups of animals were injected i.c.v. with two lower doses of pertussis toxin 7 days prior to testing in the holeboard. Mice ($N = 18-20$) were treated with 0.50 µg/animal pertussis toxin or with buffer. Seven days later half of each group were treated with either 0.1 mg/kg medetomidine (i.p.) or distilled water vehicle 30 min prior to testing in the holeboard. Separate groups of animals ($N = 18-22$) were treated with 0.25 µg/animal pertussis toxin or sham injected with buffer. Seven days later half of each group received either 0.1 mg/kg medetomidine (i.p.) or distilled water vehicle 30 min prior to testing in the holeboard.

Interactions with ethanol-induced ataxia. Groups of animals ($N = 8-20$) were treated with either 0.125, 0.250, 0.500 or 1.00 µg/animal or sham injected as described above. Seven days later all animals were injected i.p. with 2.4 g/kg ethanol dissolved in a distilled water vehicle. Every 5 min up to 25 min after the ethanol injection the animals were "blindly" rated for ataxia using the scale described above.

In a subsequent experiment, groups of animals ($N = 10$) were injected i.c.v. with either pertussis holotoxin (1.0 µg/animal), pertussis toxin B-oligomer (1.0 µg/animal) or with buffer. Seven days following pertussis holotoxin, B-oligomer or sham treatment all animals were injected i.p. with 2.4 g/kg ethanol dissolved in a distilled water vehicle. Every 5 min up to 25 min after the ethanol injection the animals were "blindly" rated for ataxia using the scale described above.

Interactions with ethanol-induced hypothermia. Groups of animals ($N = 15$) were pretreated with either 1.0 µg/animal pertussis toxin or sham treated as described above. Seven days later all animals were administered 2 g/kg ethanol (i.p.) immediately following a core body temperature recording. Twenty minutes later the body temperature of each animals was again measured.

Blood ethanol determinations. Groups of animals ($N = 15$) were pretreated with either 1.0 µg/animal pertussis toxin or sham treatment as described above. Seven days later these animals were administered ethanol (2 g/kg, i.p.) 20 min prior to decapitation. Trunk blood was collected in heparinized tubes. Blood ethanol concentrations were determined using a modification of the Sigma diagnostics alcohol procedure No. 332-UV (Sigma Chemical Co., St Louis, MO) which involves the conversion of ethanol to acetaldehyde and nicotinamide adenine dinucleotide (NAD) to NADH catalyzed by the enzyme alcohol dehydrogenase (Bucher and Redetzki 1951). The levels of NADH were measured photometrically.

Statistics. Data were analyzed using analysis of variance (with repeated measures when appropriate). When significant differences between groups were found from the ANOVA, post hoc comparisons between individual means were made using Fisher's least significant difference test.

Results

Interactions with medetomidine-induced hypomotility

In all experiments medetomidine (0.1 mg/kg) significantly reduced both locomotor activity and exploratory head-dipping in sham treated animals.

Two days after i.c.v. administration of pertussis toxin no effect on locomotor activity was seen as compared to vehicle treated sham controls, although a significant ($p < 0.05$) reduction in head-dipping was detected (Fig. 1A). Four days following pertussis toxin or sham treatment, vehicle treated pertussis toxin animals showed significantly ($p < 0.05$) elevated locomotor activity and significantly ($p < 0.01$) reduced exploratory head-dipping compared to vehicle treated sham controls (Fig. 1B). However, the pertussis toxin pretreated animals were significantly ($p < 0.05$) less affected by the medetomidine than were the sham operated controls. Seven days after pertussis toxin or sham treatment medetomidine significantly ($p < 0.001$) reduced exploratory head-dipping in both groups but only reduced locomotor activity in the sham operated animals (Fig. 1C). Pertussis toxin treatment significantly ($p < 0.05$) elevated locomotor activity and significantly ($p < 0.001$) reduced exploratory as compared to sham-vehicle controls. However, a highly signifi-

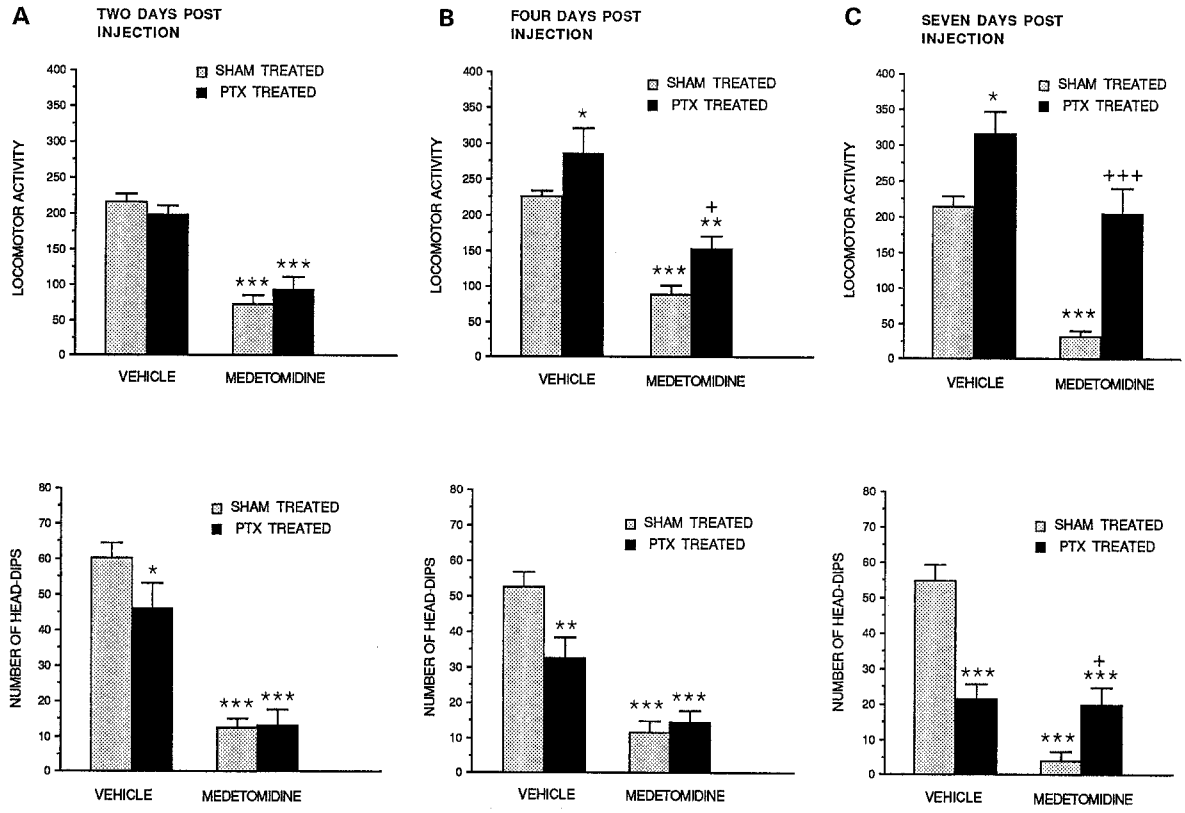

Fig. 1. Effect of pretreatment with pertussis toxin (1.0 μg/animal, i.c.v.) administered 2, 4 or 7 days prior to treatment with medetomidine and testing in the holeboard. * $p < 0.05$, *** $p < 0.001$ vs. sham treated groups; $^{+}$ $p < 0.05$, $^{+++}$ 0.001 vs. sham treated medetomidine groups

cant difference ($p < 0.001$) was seen between the pertussis toxin treated and sham treated animals for locomotor activity following medetomidine administration; locomotor activity in the medetomidine-pertussis toxin treated animals was almost the same as for vehicle-sham treatment. A significant difference ($p < 0.05$) was also seen between the pertussis toxin treated and sham treated animals for exploratory head-dipping following medetomidine administration.

Pretreatment with the B-oligomer of pertussis toxin (1.0 μg/animal, 7 days prior to testing) had no significant effect on either locomotor activity or exploratory head-dipping (Fig. 2). Pertussis holotoxin (1.0 μg/animal, 7 days prior to treatment) elevated motor activity. In this experiment this effect was not statistically significant, but it reduced exploratory head-dipping significantly ($p < 0.001$) as seen in the previous experiments. Medetomidine (0.1 mg/kg) significantly reduced locomotor activity in the sham treated and B-oligomer treated groups but showed no effect in the pertussis toxin treated animals, which did not differ from vehicle treated sham controls. Neither pertussis holotoxin nor B-oligomer pretreatment had any effect on the medetomidine induced reduction in exploratory head-dipping.

Pretreatment with 0.25 μg/animal pertussis toxin did not significantly alter locomotor activity but did significantly ($p < 0.001$) reduce exploratory head-dipping in vehicle treated animals compared to vehicle treated sham controls 7 days after treatment (Fig. 3A). Medetomidine administration significantly ($p < 0.01$) reduced both locomotor activity and exploratory head-dipping in both pertussis toxin and sham treated animals. No significant differences were detected between the pertussis toxin and sham treated animals following medetomidine administration. Seven days after 0.5 μg/animal pertussis toxin or sham treatment there was a significant reduction in exploratory head-dipping in the vehicle treated pertussis toxin animals compared to the vehicle-sham group ($p < 0.05$) but no difference between the two groups for locomotor activity (Fig. 3B). There was, however, a highly significant ($p < 0.001$) difference between the two groups following medetomidine treatment for locomotor activity; the pertussis toxin group showing hardly any hypomotility in response to medetomitine administration. There was no attenuation of medetomidine-induced reduction in exploratory head-dipping.

Effect of pertussis toxin on blood ethanol concentration

Pretreatment with pertussis toxin, 1.0 μg/animal, administered 7 days prior to ethanol, had no effect on blood ethanol concentration compared to sham treated controls 20 min after administration of 2 g/kg ethanol. The mean blood ethanol concentration in the 1.0 μg pertussis toxin pretreated animals was 240.5 ± 5.4 mg/100 ml and in the sham treated animals it was 251.2 ± 11.3 mg/100 ml.

Interaction of pertussis toxin with ethanol-induced ataxia

Five minutes after ethanol (2.4 g/kg) administration animals pretreated with pertussis toxin did not show signifi-

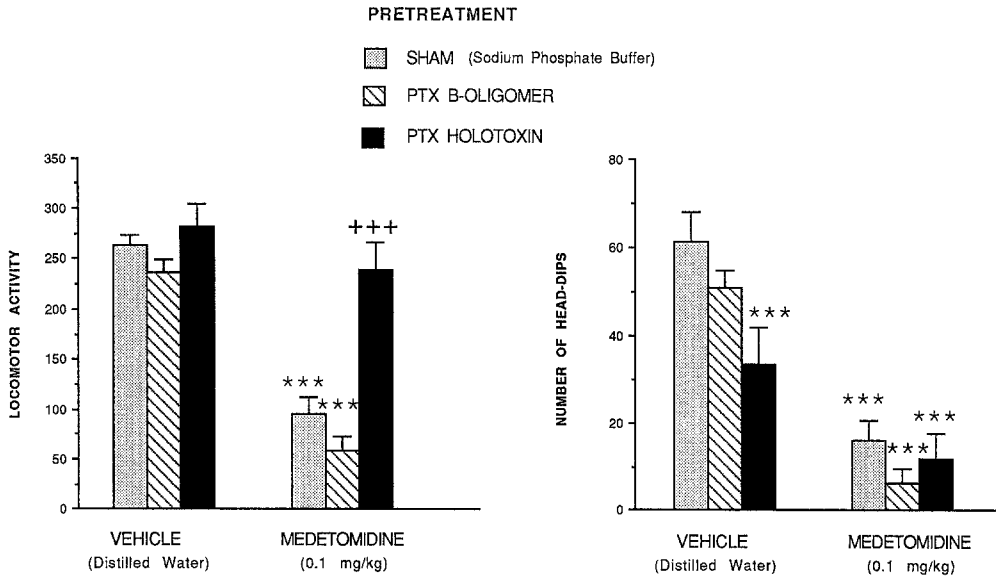

Fig. 2. Effect of pretreatment with pertussis holotoxin (1.0 µg/animal, i.c.v.), pertussis toxin B-oligomer (1.0 µg/animal, i.c.v.) or sham treatment administered 7 days prior to treatment with medetomidine and testing in the holeboard. *** $p < 0.001$ vs. sham treated vehicle groups; $^{+++}$ $p < 0.001$ vs. sham treated medetomidine groups

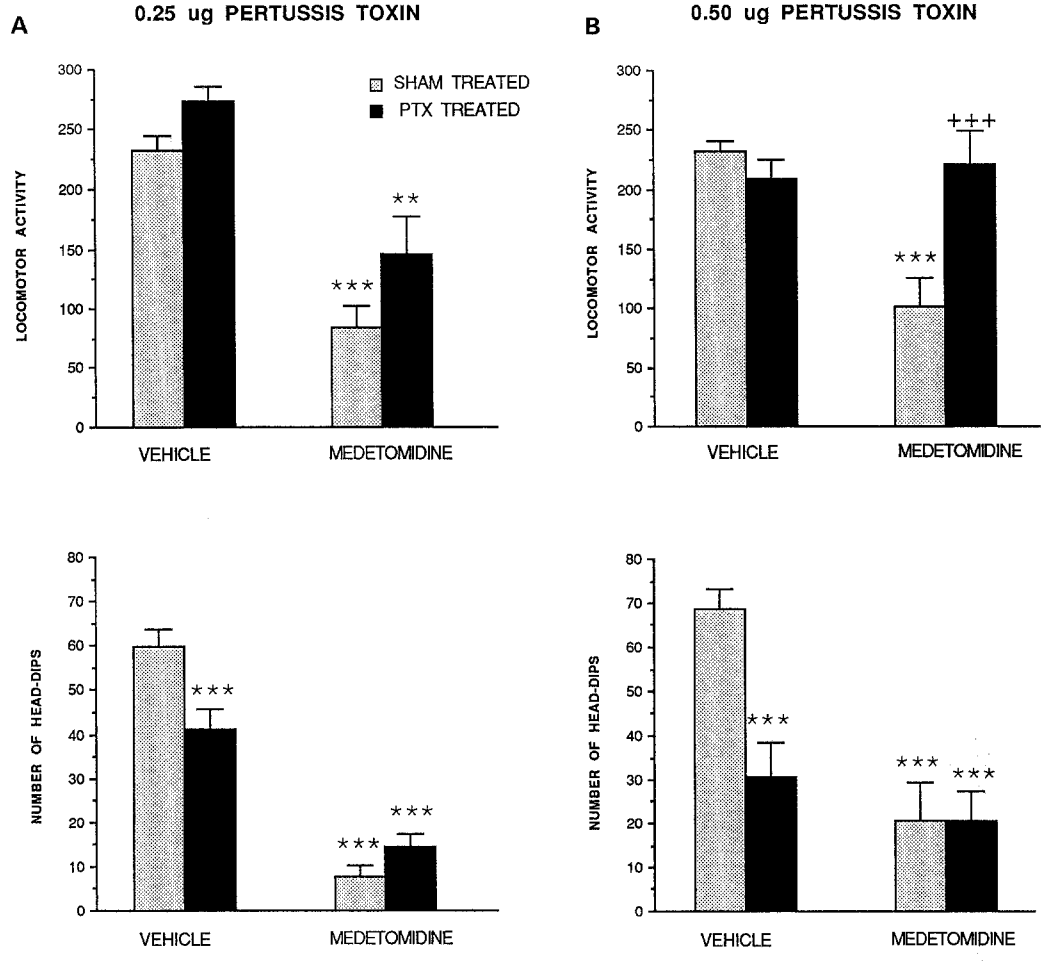

Fig. 3. Effect of pretreatment with either 0.25 or 0.50 µg/animal pertussis toxin (i.c.v.) 7 days prior to treatment with medetomidine and testing in the holeboard. ** $p < 0.02$, *** $p < 0.001$ vs. sham treated vehicle groups; $^{+++}$ $p < 0.001$ vs. sham treated medetomidine groups

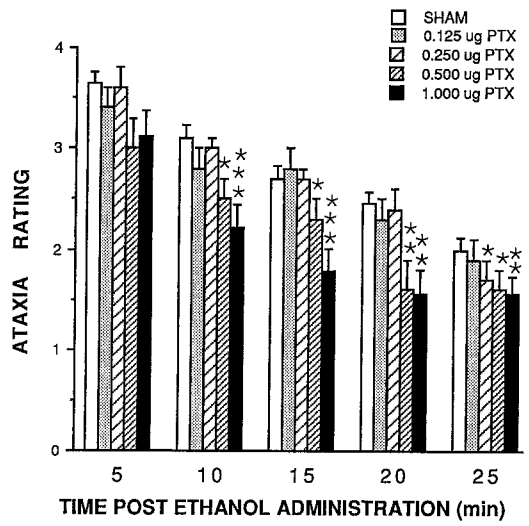

Fig. 4. Effect of pretreatment with either 0.125, 0.25, 0.50 or 1.0 μg/animal pertussis toxin (i.c.v.) 7 days prior to investigating the ataxic effect of 2.4 g/kg ethanol (i.p.) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. sham treated group

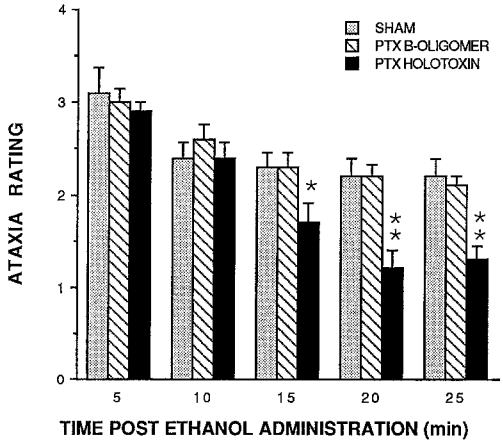

Fig. 5. Effect of pretreatment with pertussis holotoxin (1.0 μg/animal, i.c.v.), B-oligomer of pertussis toxin (1.0 μg/animal, i.c.v.) or sham treatment 7 days prior to investigating the ataxic effect of 2.4 g/kg ethanol (i.p.). * $p < 0.05$, ** $p < 0.01$, vs. sham treated group

cantly less ataxia than sham treated controls at the same time point (Fig. 4). At later time points animals pretreated with either 0.5 or 1.0 μg pertussis toxin showed a significantly ($p < 0.05$) lesser degree of ataxia than the sham treated controls at the same time points. Of the lower dose treatments of pertussis toxin only the 0.25 μg pretreatment showed any significant ($p < 0.05$) reduction in ataxia compared to sham treated controls. This effect was only seen at the 25 min time point.

Mice pretreated with B-oligomer showed no difference from sham treated controls at any time point (Fig. 5) whereas animals pretreated with pertussis holotoxin showed significantly ($p < 0.05$) lesser ataxia than sham pretreated animals at the 15, 20 and 25 min time points.

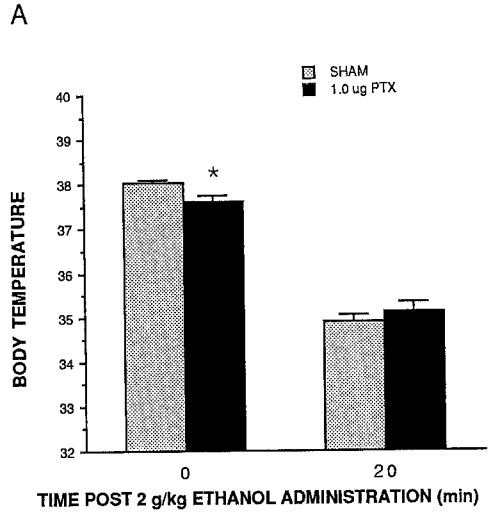

Fig. 6. Effect of pretreatment with 1.0 μg/animal pertussis toxin (i.c.v.) 7 days prior to investigating the hypothermic effect of 2.0 g/kg ethanol (i.p.). (**A**) Body temperatures immediately prior to (T_0) and 20 min after (T_{20}) administration of 2 g/kg ethanol. (**B**) Change in body temperature between T_0 and T_{20} for both pertussis toxin and sham pretreated animals. * $p < 0.05$ vs. sham treated group

Interaction of pertussis toxin with ethanol-induced hypothermia

A significant interaction between pertussis toxin treatment and ethanol administration was found for body temperature ($p < 0.05$), however, a significant difference in initial (pre-ethanol) body temperature was detected as well (Fig. 6A), although no differences between the groups were seen 20 min after ethanol administration. When the change in body temperature was investigated (Fig. 6B) a significantly ($p < 0.05$) greater fall in body temperature was seen in the sham treated animals as compared to those pretreated with pertussis toxin.

Discussion

The interactions of pertussis toxin pretreatment with the alpha$_2$-adrenoceptor agonist medetomidine, revealed

that the hypomotility induced by medetomidine was significantly attenuated in the pertussis toxin pretreated animals. These findings are consistent with observations by other investigators who have used clonidine as the alpha$_2$-adrenoceptor agonist (Nomura et al. 1987; De Sarro et al. 1989). Pretreatments of 0.5 μg pertussis toxin/animal or greater were required to achieve a reversal of the effect of medetomidine on locomotor activity in the holeboard test and this effect was the most marked 7 days following the treatment with pertussis toxin. This time course is also similar to that found for the effects of pertussis toxin treatment on other measurements (De Sarro et al. 1989). The pertussis toxin treatment was not, however, without intrinsic behavioral effects, a significant elevation in locomotor activity was seen in vehicle treated animals four and seven days after pertussis toxin treatment. This intrinsic effect complicates the interpretation of the reversal of the medetomidine-induced reduction in activity. This effect of pertussis toxin is not, however, always detected, for example, no significant elevation in motor activity was detected following pretreatment with 0.5 μg/animal pertussis toxin in the dose response experiment or following 1.0 μg/animal pertussis holotoxin in the B-oligomer experiment but in both cases a reversal of medetomidine hypomotility was seen. A similar behavioral excitation has been reported previously in rats pretreated with pertussis toxin (Nomura et al. 1987) although the exact mechanism of this effect is at present unclear. The reversal of medetomidine-induced reduction in locomotor activity was not seen following pretreatment with the binding oligomer of pertussis toxin and thus the enzymatically active protomotor of the holotoxin appears to be required for this reversal to take place.

Pertussis toxin treatment also significantly reduced exploratory head-dipping in vehicle injected animals, an effect which became progressively more marked with increasing the interval between the pertussis toxin treatment and testing and there was no consistant reversal of the effects of medetomidine on this behavior. No significant reduction in exploratory head-dipping was seen following pretreatment the B-oligomer, although these animals did head-dip somewhat less than the sham pretreated animals. Whilst the exact nature of the intrinsic effects of pertussis toxin remains to be fully elucidated it would appear that the holotoxin is required for their expression since no intrinsic action of the binding oligomer could be detected. The pertussis toxin treatment, at the appropriate time and dose, attenuated the behavioral effects of medetomidine, the actions of which are thought to be mediated via G_i protein linked alpha$_2$-adrenoceptors (Sheinin et al. 1989; Durcan et al. 1989b). This attenuation of medetomidine hypolocomotion required pretreatment with the enzymatically active holotoxin and was not seen following pretreatment with the B-oligomer of pertussis toxin.

Seven days after pretreatment with 0.5 or 1.0 μg/animal pertussis toxin a significant reduction in the degree of ataxia induced by 2.4 μg/kg ethanol was observed. A subsequent experiment revealed that this effect was only seen following pretreatment with pertussis holotoxin and that the B-oligomer of pertussis toxin was ineffective in reducing ethanol-induced ataxia.

The effects of pertussis toxin pretreatment on ethanol induced hypothermia were less clear. The pertussis toxin treated animals appeared to be less affected by the ethanol than did the sham treated animals, although the interpretation of this must be tempered by the fact that there was a significant difference in the initial body temperatures of the two groups. It was the pertussis toxin group which had lower body temperatures immediately prior to the ethanol administration, and the fall in temperature in this group was not as great as in the sham treated animals. The lack of a convincing effect of pertussis toxin would not necessarily rule out G_i and G_o linked receptors in the hypothermic effects of ethanol. Alpha$_2$-adrenoceptors, for example, have been implicated in the modulation of the hypothermic effect of ethanol (Durcan et al. 1989d; 1990) and pertussis toxin treatment has also been demonstrated to attenuate the hypothermia induced by other compounds such as dynorphin in rats (Cavicchini et al. 1990). Pertussis toxin is a large protein and its penetration in to different brain areas may vary (Van der Ploeg et al. 1990), therefore it may not reach all sensitive sites in an adequate concentration to inactive them in sufficient numbers.

It is important to note however, that mechanisms other than the inactivation of G-proteins may be responsible for the observed effects of pertussis toxin. Pertussis toxin is made up of six protein subunits (consisting of five different proteins with one repeat), the largest of these is the enzymatically active A-protomer, whilst the remaining pentamer forms the binding B-oligomer (Tamura et al. 1982). The A-protomer is responsible for catalyzing the ADP-ribosylation of susceptible G-proteins. The B-oligomer binds to the cell membrane and is required to deliver the catalytic subunit to the target cell. Whilst the B-oligomer has no effect on G-proteins it is not completely without biological effect (Burns 1988) and therefore activity of this binding oligomer may possibly contribute to the effects of pertussis toxin treatment. In these experiments however the enzymatically active A-protomer would appear to be required since the effects of pertussis holotoxin in the holeboard experiments or in the ataxia studies could not be seen following pretreatment with the B-oligomer alone.

The pertussis toxin A-protomer inactivates both G_i or G_o proteins (Gilman 1987) and therefore the uncoupling of receptors linked via these G-proteins to their second messenger systems may be contributing to the effects reported here. It is, however, important to point out that without direct measurement of ADP-ribosylation it is not possible to assess if the effects seen here are directly related to modulation of G-protein action and pertussis toxin can have activity at a number of transmembrane signalling systems (Burns 1988). Notwithstanding this caveat, a number of antagonists of receptors thought to be linked via G_i proteins to adenylyl cyclase have been implicated in reducing the ataxic effects of ethanol as discussed earlier. The effects of pertussis toxin treatment on the G-proteins linked to some or all of these receptors

(or other pertussis toxin sensitive receptors) may be responsible for the reductions in ethanol-induced ataxia).

It remains to be clarified as to whether the interactions of pertussis toxin with ethanol are due to the inactivation of a particular set of pertussis toxin sensitive receptors or due a functional loss of all sensitive receptors, i.e. to a general loss of the inhibitory effects of these receptors on effector mechanisms. It is interesting to note in this context that pertussis toxin treatment also reduces the seizure threshold to the excitatory amino acid receptors agonist, NMDA (Durcan and Morgan 1991), but it remains unclear as to whether the G_i or G_o proteins (or both) are primarily responsible for these effects.

In conclusion, pertussis toxin pretreatment, at a time point and with a dose that reverses the effects of the $alpha_2$-adrenoceptor agonist medetomidine, antagonizes the ataxic effects of ethanol and may influence its hypothermic effects. The effects on medetomidine-induced hypolocomotion and ethanol induced ataxia require the enzymatically active holotoxin and no significant effects are found following treatment with the toxin binding subunit. These results provide evidence which may further implicate receptors linked to pertussis toxin sensitive G-proteins (G_i and G_o) in the modulation of some of the behavioral and physiological effects of ethanol.

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