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Antinociceptive effect of α-trinositol, a novel D-*myo*-inositol phosphate derivative, in the formalin test in rats

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Abstract

The antinociceptive effect of α-trinositol was examined in rats using the formalin test following systemic, spinal and local subcutaneous administration. Injection of formalin into the paw evoked two phases (phase 1: 0–9 min; phase 2: 10–60 min) of flinching behavior of the injected paw. Intrathecal administration of α-trinositol resulted in a dose-dependent suppression of the first (ED_{50}: 8 μg) and second (ED_{50}: 9 μg) phase of formalin-evoked behavioral response. Similarly, intraperitoneal delivery showed a dose-dependent reduction of the first (ED_{50}: 83 mg/kg) and second (ED_{50}: 56 mg/kg) phase of the formalin test. Subcutaneous injection of 100 μg, but not 10 μg, α-trinositol into the rat paw together with the formalin solution, had no effect on the first phase, but reduced by 20% the second phase of behavior. These data show that α-trinositol produces a suppression of acute and prolonged nociceptive behaviors with a central mechanism of action, although some peripheral component may contribute to the reduction of the late phase following systemic administration.

Keywords: α-Trinositol; Antinociception; Intrathecal; Formalin test; Behavior; (Rat)

1. Introduction

The inositol phosphates constitute a large family of compounds incorporating both L and D forms of the mono-, bi-, tris-, tetra-, penta- and hexa-phosphates. Endogenous inositol phosphates include inositol 1,4,5-trisphosphate (Ins[1,4,5]P_3), which is an important intracellular second messenger (Berridge and Irvine, 1989; Berridge, 1993) and inositol 1,3,4,5-tetrakisphosphate (Ins[1,3,4,5]P_4) which may be produced upon hormonal stimulation of cells (Houslay et al., 1987). Ins[1,4,5]P_3 binds to specific receptors in the endoplasmatic reticulum, thereby inducing the release of intracellularly stored Ca^{2+} via a tetrameric Ca^{2+} ion channel (Mikoshiba, 1993). Ins[1,3,4,5]P_4 has been suggested to regulate the influx of extracellular Ca^{2+} (Lückhoff and Clapham, 1992), although an extracellular receptor has not yet been described for this inositol phosphate.

There is little current information available on the potential pharmacological effects of the various inositol phosphate analogs, but some interesting effects of α-trinositol (D-*myo*-inositol 1,2,6-trisphosphate, also known as PP56) have been found in different animal models (see Sirén et al., 1991). α-Trinositol is an inositol trisphophate isomer which, like Ins[1,4,5]P_3, is produced by the partial degradation of phytic acid by phytase (Goldschmidt, 1990). However, the intracellular properties of α-trinositol are different from those of Ins[1,4,5]P_3. There is no evidence for an interaction of α-trinositol with Ins[1,4,5]P_3 receptors (Authi et al., 1989). Instead, recent studies suggest that α-trinositol has binding properties similar to Ins[1,3,4,5]P_4 (Yoo et al., 1994).

A number of studies have suggested that α-trinositol acts as a selective antagonist of neuropeptide Y-induced vasoconstriction, both in vitro and in vivo (Edvinsson et al., 1990; Adamsson and Edvinsson, 1991; Adamsson et al., 1992; Potter et al., 1992; Sun et al.,

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1991, 1992), although this finding has been debated (Pernow et al., 1992; Feth et al., 1993). α-Trinositol has also been shown to normalize motor nerve conduction velocity in diabetic rats (Carrington et al., 1993) and possess anti-inflammatory properties in several models of inflammation (Claxson et al., 1990; Nakazawa et al., 1994; Lund and Reed, 1993).

Since peripheral inflammation activates and sensitizes nociceptive fibers and α-trinositol exhibits anti-inflammatory actions, we were interested in the potential antinociceptive properties of α-trinositol. In a preliminary study, we found that systemic administration of α-trinositol produced a potent suppression of the behavioral response to the formalin pain test and reduced formalin-evoked changes in the spinal levels of excitatory amino acids (A.B. Malmberg et al., unpublished observations). The present study was performed to locate the site of action of this antinociceptive effect by comparing spinally delivered α-trinositol with systemic and local administration. We used the formalin test to study antinociception because it provides information about modulation of both acute and prolonged nociceptive behavior. The first phase of behavior induced by formalin injection into the paw results from activation of primary afferent fibers while the second phase is believed to contain both inflammation-evoked afferent activity together with facilitatory processes at the spinal cord level (Dickenson and Sullivan, 1987; Puig and Sorkin, 1994).

2. Material and methods

The experiments were carried out according to protocols approved by the Committee of Ethics for Animal Experiments at the University of Göteborg, Sweden.

2.1. Animal preparation

Male Sprague-Dawley rats (280–320 g, ALAB, Sollentuna, Sweden) were used in the studies. For intrathecal (i.t.) injection studies, the rats were anesthetized by an intraperitoneal (i.p.) injection of a mixture of xylazine chloride (13.2 mg/kg, i.p., Rompun vet., Bayer AG, Germany) and ketamine hydrochloride (66.5 mg/kg, i.p., Ketalar, Parke-Davis/Warner-Lambert, USA), and implanted with a polyethylene catheters (PE-10) extending from the cisterna to the rostral edge of the lumbar enlargement (Yaksh and Rudy, 1976). Intrathecal injection studies were started 3–5 days after implantation. Only animals with normal motor function were used.

2.2. Assessment of general behavior and motor function

General behavior was carefully monitored throughout the study. Motor function was examined by assessing the placing/stepping reflex, where normal behavior is a stepping reflex when the hind paws are drawn across the edge of a table. Righting and ambulation were assessed by placing the rat horizontally with its back on the table which normally gives rise to an immediate coordinated twisting of the body to an upright position. Irritability in the form of touch-evoked agitation or vocalization was assessed by gently stroking the flank of the rat with a pencil.

2.3. The formalin test

Rats were lightly anesthetized with 3% halothane before injecting 50 μl of 5% formalin solution subcutaneously (s.c.) into the dorsal surface of the right hind paw. The rat was then placed in an open plexiglas chamber and the formalin-injected paw observed for 60 min. Because the flinching and shaking evoked by the formalin injection is the most consistent behavior (see also Wheeler-Aceto et al., 1990; Malmberg and Yaksh, 1992), pain-related behavior was quantified by counting the incidence of spontaneous flinching of the injected paw. The number of flinches were counted every min for the first 6 min after formalin injection. Thereafter, starting 10 min after the formalin injection, the incidence of paw flinching was counted at 5 min intervals in periods of 1 min. After the observation period, the animals were killed with an overdose of pentobarbital (180 mg/kg, i.p., Apoteksbolaget, Sweden).

2.4. Drugs and injections

α-Trinositol (Perstorp Pharma, Lund, Sweden) was dissolved in physiological saline (0.9% w/v NaCl). For i.t. injection studies, the agents were mixed such that the doses were delivered in a total volume of 10 μl followed by 10 μl saline to flush the catheter. Intrathecal saline injections were carried out as controls. For i.p. injections, the agent was mixed at a concentration such that a 300 g rat would receive an i.p. injection of 300 μl of the drug solution or 300 μl saline as control. The i.t. and i.p. injections were performed 5 min before the formalin injection. Local injection of the drug or saline into the paw, was as 10 μl added to 50 μl of formalin.

2.5. Data presentation and statistics

Time-response data from the formalin test are presented as the means ± S.E.M. number of flinches per min. Comparisons of the different treatments were carried out using the sum of flinches during phase 1 (0–9 min) or phase 2 (10–60 min). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Student Newman-Keuls test provided that the F ratio gave $P < 0.05$. ED_{50} (effec-

tive dose resulting in a 50% reduction of the control formalin response) and 95% confidence intervals were calculated using a least square linear regression method according to the formulae given by Tallarida and Murray (1987).

3. Results

3.1. General behavior and motor function

There was no detectable prolonged effect on general behavior or motor function by α-trinositol administered i.p. at doses of 30–300 mg/kg, i.t. at doses of 1–10 μg or s.c. at doses of 10–100 μg. However, in some of the rats (approximately 50%) injected with the highest i.p. (300 mg/kg) or i.t. (10 μg) dose of α-trinositol we noted a short lasting increase in motor activity, including circling behavior and extension of the hind limbs. This behavior lasted for about 1–2 min and was absent by the time the formalin injection was performed. In pilot studies, higher intrathecal doses of α-trinositol (100 μg, $n = 2$) again produced spontaneous agitation, tremor, serpentine-like tail movements, circling behavior and motor dysfunction. This behavior was reversible and the rats showed normal motor function and behavioral responses 60–90 min after the injection. All of the rats used in the i.t. injection studies showed normal behavior and motor function after the implantation of the i.t. catheter.

3.2. Effect of spinal α-trinositol

Formalin injection into the paw of rats which had received i.t. saline produced two distinct phases of paw flinching of the injected paw (Fig. 1). Intrathecal delivery of α-trinositol produced a dose-dependent suppression of both phase 1 and phase 2 of the formalin test (Fig. 1 and Table 1). The ED_{50} (95% confidence intervals) for i.t. delivery of α-trinositol was 8 (4–16) μg for phase 1 and 9 (6–14) μg for phase 2.

Table 1
Effect of systemic, spinal or local α-trinositol injection on formalin-evoked flinching behavior

Treatment	N	Sum of flinches ± S.E.M. and statistical significance [a]	
		Phase 1	Phase 2
I.t. injection			
Saline	5	17 ± 1	166 ± 8
1 μg	5	19 ± 2 ns	151 ± 8 ns
3 μg	5	12 ± 2 $P < 0.05$	120 ± 12 $P < 0.01$
10 μg	5	9 ± 1 $P < 0.01$	101 ± 8 $P < 0.001$
I.p. injection			
Saline	6	16 ± 2	170 ± 6
30 mg/kg	5	17 ± 2 ns	152 ± 7 ns
100 mg/kg	5	9 ± 2 $P < 0.01$	118 ± 17 $P < 0.05$
300 mg/kg	5	1 ± 1 $P < 0.001$	55 ± 12 $P < 0.001$
Local s.c. injection			
Saline	6	17 ± 2	171 ± 5
10 μg	6	17 ± 3 ns	168 ± 11 ns
100 μg	6	16 ± 2 ns	144 ± 7 $P < 0.05$

[a] Statistical analysis was carried out by one-way analysis of variance followed by Student-Newman-Keuls test comparing the α-trinositol group with the appropriate saline treatment. ns: $P > 0.05$.

3.3. Effect of systemic α-trinositol

Rats given an i.p. injection of saline before the formalin test showed a similar biphasic flinching behavior in response to formalin compared to rats receiving i.t. saline. As with spinal administration, α-trinositol administered i.p. produced a dose-dependent suppression of both phase 1 and phase 2 of the formalin test (Fig. 2 and Table 1). ED_{50} and 95% confidence intervals were 83 (64–108) mg/kg for phase 1 and 56

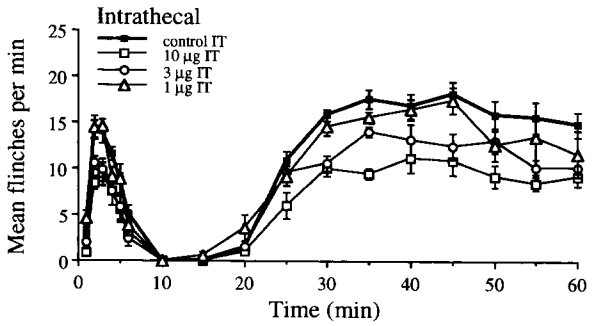

Fig. 1. Effect of i.t. injection of α-trinositol on formalin-evoked behavior. Data are presented as the mean number of flinches per min ± S.E.M. versus the time after formalin injection. The i.t. injection of α-trinositol or saline was performed 5 min before the formalin injection.

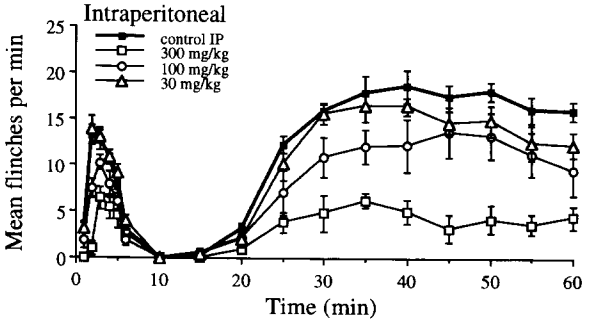

Fig. 2. Effect of i.p. injection of α-trinositol on formalin-evoked flinching behavior. Data are presented as the mean number of flinches per min ± S.E.M. versus the time after the formalin injection. The i.p. injection of α-trinositol or saline was performed 5 min before the formalin injection.

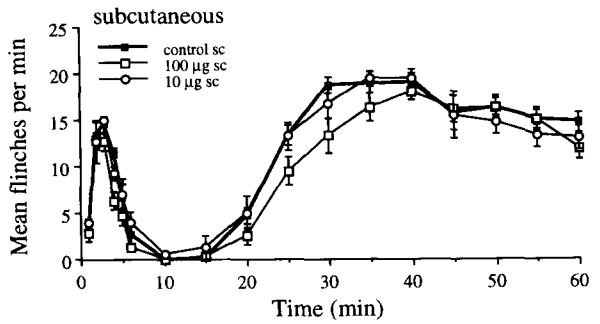

Fig. 3. Effect of local s.c. injection of α-trinositol on formalin-evoked behavior. Data are presented as the mean number of flinches per min ± S.E.M. versus the time after the formalin injection. Local s.c. delivery was performed together with the formalin injection into the rat paw.

(36–88) mg/kg for phase 2 after i.p. injection of α-trinositol.

3.4. Effect of local subcutaneous α-trinositol

In contrast to the effects of spinal and systemic α-trinositol, local s.c. injection of 100 µg α-trinositol together with formalin had no effect on the first phase behavior, but reduced the second phase response by approximately 20% (Fig. 3 and Table 1). Local administration of 10 µg α-trinositol had no effect on the formalin-evoked behavior.

4. Discussion

This study demonstrated that spinally and systemically delivered α-trinositol produced a dose-dependent suppression of both the acute and prolonged behavioral response evoked by paw formalin injection, while local injection of α-trinositol produced only a mild suppression of the second phase. This suggests that α-trinositol reduces the acute pain-related response by a central mechanism, while inhibition of the second phase may be the result of both peripheral and central effects. A central effect of α-trinositol is further supported by the observation that spinally delivered α-trinositol was effective on phase 1 at a dose 30 times lower than local injection. Intrathecal administration of α-trinositol showed similar potency in reducing phase 1 and 2 (similar ED_{50}), while i.p. α-trinositol was more effective in suppressing the phase 2 response (phase 1 ED_{50} > phase 2 ED_{50}). This may indicate that systemic α-trinositol acts at a central site to suppress phase 1 and at both central and peripheral sites to reduce phase 2 of the formalin test.

Injection of formalin into the paw produces an acute activation of Aβ-, Aδ- and C-fibers at the site of injection (Puig and Sorkin, 1994). C-fibers located at the injection site are also involved in the second phase, together with Aδ- and C-fibers from adjacent sites that are activated by the spread of inflammation (Puig and Sorkin, 1994). Recordings from spinal cord neurons show two phases of activity that correspond to the periods of flinching behavior (Dickenson and Sullivan, 1987). The second phase is sensitive to blockade of spinal glutamate receptors of the *N*-methyl-D-aspartate (NMDA) subtype (Haley et al., 1990; Coderre and Melzack, 1992; Yamamoto and Yaksh, 1992). and there is evidence to suggest that the NMDA receptor is essential for initiation of central sensitization (see for example Woolf and Thompson, 1991). The second phase response has also been shown to be dependent on activity during the first phase of the formalin test by studies showing that, if the first phase is suppressed by morphine administration, naloxone administration after the second phase has no effect on the morphine-induced antinociception (Dickenson and Sullivan, 1987; Yamamoto and Yaksh, 1992). The mechanism that underlies this effect may be related to the ability of morphine to suppress primary afferent neurotransmitter release thereby also reducing secondary events in the spinal cord. Because α-trinositol effectively reduced the first phase of the formalin test it is possible that a similar mechanism is involved. In recent preliminary studies we have found that α-trinositol attenuates formalin-evoked increases in spinal glutamate concentrations (Malmberg et al., unpublished observations). Alternatively, α-trinositol may act through a suppression of prostaglandin formation, although this effect appeared unrelated to a direct inhibition of cyclooxygenase inhibition (Perstrop Pharma, unpublished observations). Involvement of spinal prostaglandins in the behavioral response of the formalin test has been suggested by several studies including antinociceptive effect of spinally delivered cyclooxygenase inhibitors (Malmberg and Yaksh, 1992), prostaglandin E receptor antagonists (Malmberg et al., 1994) and also detection of two phases of increased spinal prostaglandin E_2 concentrations by intrathecal microdialysis (Malmberg and Yaksh, 1995). The α-trinositol-mediated reduction of the first phase of the formalin test is, however, greater than observed with cyclooxygenase inhibitors or prostaglandin E receptor antagonists suggesting that an additional mechanism is involved.

The antinociceptive effect of spinal α-trinositol in our study initially appears inconsistent with a recent finding showing that i.t. administration of α-trinositol facilitates the flexion reflex in decerebrated, spinalized rats (Xu and Wiesenfeld-Hallin, 1995). In this study, α-trinositol was also reported to cause obvious motor excitation, as indicated by muscle contractions and spontaneous tail movements (Xu and Wiesenfeld-Hal-

lin, 1995). We also observed increased motor activity, circling behavior, serpentine-like movements of the tail and tremor at our highest i.t. dose (10–100 μg). More surprising in the work by Xu and Wiesenfeld-Hallin (1995) was that systemic administration of α-trinositol had no consistent effect on the flexion reflex, including both reduction and facilitation. It is possible that the difference is related to the use of spinalized and anesthetized rats in these studies. Alternatively, the antinociceptive effects of α-trinositol may be mediated by supraspinal mechanisms. However, α-trinositol was delivered i.t. only 5 min before the formalin injection and this produced a significant suppression of the first phase. It is very unlikely that supraspinal redistribution occurs within that short time interval. It is thus more likely that i.t. α-trinositol produced a local spinal effect that is dependent on an intact nervous system.

Several studies have shown that α-trinositol potently inhibits the actions of the neuropeptide Y, including neuropeptide Y-induced blood vessel contraction (Edvinsson et al., 1990; Potter et al., 1992; Sun et al., 1991), and neuropeptide Y-enhanced hypertension and sympathomimetic vasoconstrictor responses (Adamsson et al., 1992; Sun et al., 1992). The antagonism seems to be at a functional level, because α-trinositol does not interfere with neuropeptide Y receptor binding, while neuropeptide Y-induced Ca^{2+} increases in vascular smooth muscle cells are reduced (Wahlestedt et al., 1992). In contrast to systemic effects, central neuropeptide Y-induced effects are only mildly suppressed by central injections of α-trinositol (Heilig et al., 1991) and spinal delivery of α-trinositol had no effect on neuropeptide Y-induced suppression of the nociceptive flexion reflex (Xu and Wiesenfeld-Hallin, 1995). Further, spinal administration of neuropeptide Y has been shown to produce antinociception in the rat (Hua et al., 1991). It appears therefore unlikely that the central antinociceptive properties of α-trinositol in the present study are related to modulation of neuropeptide Y-evoked effects.

Phase 2 of the formalin test is accompanied by paw swelling and inflammation, which appears to trigger continued activation of C- and Aδ-fibers both in and around the injection site (Puig and Sorkin, 1994). Despite the relatively minor peripheral effect of α-trinositol, the reduction of the second phase is consistent with studies showing an anti-inflammatory activity of α-trinositol. It has been shown that α-trinositol effectively reduced carrageenan-induced foot pad edema (Claxson et al., 1990), adjuvant arthritis inflammation (Claxson et al., 1990), edema generation and albumin extravasation in thermally injured skin (Lund and Reed, 1994), neurogenic inflammation of the trachea (Woie and Reed, 1994), and smoke-induced lung edema (Nakazawa et al., 1994). The anti-inflammatory effect has been suggested to be related to metal chelating properties of α-trinositol, thereby influencing inflammation-evoked free radical formation (Claxson et al., 1990). Edema and extravasation after injury may also be related to endothelial cell Ca^{2+} permeability (Nakazawa et al., 1994). Because α-trinositol reduced Ca^{2+} influx in smooth muscle cells (Wahlestedt et al., 1992), it is possible that suppression of injury-evoked changes by α-trinositol is related to suppression of Ca^{2+} influx (Nakazawa et al., 1994). It has also been reported that α-trinositol exerts edema-preventing effects through modulation of β-1 integrin function (Rodt et al., 1994). Thus it appears that several mechanisms may be involved in the anti-inflammatory and anti-edema effect of α-trinositol and the relation between these mechanisms and antinociception is not clear. However, the anti-inflammatory effect may be enough to explain the local peripheral antinociceptive effect on phase 2.

Recently, the existence of specific binding sites for α-trinositol was demonstrated in rat tissue, including the brain (Yoo et al., 1994). Competition binding studies indicated that Ins(1,3,4,5)P_4 and InsP_6, but not Ins(1,4,5)P_3, potently inhibited [^{3}H]α-trinositol binding. Analyses of binding at different pH and ionic conditions showed clear differences between α-trinositol and the other inositol phosphates other than Ins(1,3,4,5)P_4, which shared several similarities with α-trinositol (Yoo et al., 1994). However, to date there is no evidence to support that a Ins(1,3,4,5)P_4 receptor is located on the cell surface. In the studies by Yoo and colleagues (1994), it was shown that binding sites for α-trinositol were membrane-associated. While the exact transduction mechanism for α-trinositol receptors is unknown, studies indicate that α-trinositol interferes with influx of extracellular Ca^{2+}, as shown in vascular smooth muscle (Wahlestedt et al., 1992). This provides a potential antinociceptive mechanism, as increases in intracellular Ca^{2+} are believed to be essential for neurotransmitter release and blockade of Ca^{2+} channels of N- and P-type produces antinociception in the formalin test (Malmberg and Yaksh, 1994). Further studies are clearly required to identify the exact mechanism of antinociceptive action and to further characterize the receptor system involved.

In summary, α-trinositol suppresses both phases of nociceptive behavior in response to formalin injection into the rat paw. The reduction of the first phase appears to be related to a central effect of α-trinositol. Becuase peripheral injection of α-trinositol only produced a mild reduction of the second phase behaviors, it is likely that a central mode of action is also more important also in the suppression of the second phase following systemic administration. These studies are consistent with preliminary clinical observations (J. Cassuto and T. Hedner, personal communication) reporting analgesic effects of α-trinositol and support

further clinical trials of α-trinositol for the treatment of pain.

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